Université de Montréal

VALIDATION DES MODÈLES DE PHARMACOLOGIE DE SÉCURITÉ ET ÉVALUATION DE LA VALEUR THÉRAPEUTIQUE DE L'OXYTOCINE DANS LE TRAITEMENT DE L'INFARCTUS DU MYOCARDE

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En février, 2009 un rapport de PHRMA (*Pharmaceutical Research and Manufacturers of America*) confirmait que plus de 300 médicaments pour le traitement des maladies cardiaques étaient en phase d'essais cliniques ou en révision par les agences règlementaires. Malgré cette abondance de nouvelles thérapies cardiovasculaires, le nombre de nouveaux médicaments approuvés chaque année (toutes indications confondues) est en déclin avec seulement 17 et 24 nouveaux médicaments approuvés en 2007 et 2008, respectivement. Seulement 1 médicament sur 5000 sera approuvé après 10 à 15 ans de développement au coût moyen de 800 millions $. De nombreuses initiatives ont été lancées par les agences règlementaires afin d'augmenter le taux de succès lors du développement des nouveaux médicaments mais les résultats tardent. Cette stagnation est attribuée au manque d'efficacité du nouveau médicament dans bien des cas mais les évaluations d'innocuité remportent la palme des causes d'arrêt de développement. *Primum non nocere*, la maxime d'Hippocrate, père de la médecine, demeure d'actualité en développement préclinique et clinique des médicaments. Environ 3% des médicaments approuvés au cours des 20 dernières années ont, par la suite, été retirés du marché suite à l'identification d'effets adverses. Les effets adverses cardiovasculaires représentent la plus fréquente cause d'arrêt de développement ou de retrait de médicament (27%) suivi par les effets sur le système nerveux. Après avoir défini le contexte des évaluations de pharmacologie de sécurité et l'utilisation des bio-marqueurs, nous avons validé des modèles d'évaluation de l'innocuité des nouveaux médicaments sur les systèmes cardiovasculaires, respiratoires et nerveux.

Évoluant parmi les contraintes et les défis des programmes de développements des médicaments, nous avons évalué l'efficacité et l'innocuité de l'oxytocine (OT), un peptide endogène à des fins thérapeutiques. L'OT, une hormone historiquement associée à la reproduction, a démontré la capacité d'induire la différentiation *in vitro* de lignées cellulaires (P19) mais aussi de cellules souches embryonnaires en cardiomyocytes battants. Ces observations nous

ont amené à considérer l'utilisation de l'OT dans le traitement de l'infarctus du myocarde. Afin d'arriver à cet objectif ultime, nous avons d'abord évalué la pharmacocinétique de l'OT dans un modèle de rat anesthésié. Ces études ont mis en évidence des caractéristiques uniques de l'OT dont une courte demi-vie et un profil pharmacocinétique non-linéaire en relation avec la dose administrée.

Ensuite, nous avons évalué les effets cardiovasculaires de l'OT sur des animaux sains de différentes espèces. En recherche préclinique, l'utilisation de plusieurs espèces ainsi que de différents états (conscients et anesthésiés) est reconnue comme étant une des meilleures approches afin d'accroître la valeur prédictive des résultats obtenus chez les animaux à la réponse chez l'humain. Des modèles de rats anesthésiés et éveillés, de chiens anesthésiés et éveillés et de singes éveillés avec suivi cardiovasculaire par télémétrie ont été utilisés. L'OT s'est avéré être un agent ayant d'importants effets hémodynamiques présentant une réponse variable selon l'état (anesthésié ou éveillé), la dose, le mode d'administration (bolus ou infusion) et l'espèce utilisée. Ces études nous ont permis d'établir les doses et régimes de traitement n'ayant pas d'effets cardiovasculaires adverses et pouvant être utilisées dans le cadre des études d'efficacité subséquentes.

Un modèle porcin d'infarctus du myocarde avec reperfusion a été utilisé afin d'évaluer les effets de l'OT dans le traitement de l'infarctus du myocarde. Dans le cadre d'un projet pilote, l'infusion continue d'OT initiée immédiatement au moment de la reperfusion coronarienne a induit des effets cardiovasculaires adverses chez tous les animaux traités incluant une réduction de la fraction de raccourcissement ventriculaire gauche et une aggravation de la cardiomyopathie dilatée suite à l'infarctus. Considérant ces observations, l'approche thérapeutique fût révisée afin d'éviter le traitement pendant la période d'inflammation aigüe considérée maximale autour du 3^{ième} jour suite à l'ischémie. Lorsqu'initié 8 jours après l'ischémie myocardique, l'infusion d'OT a engendré des effets adverses chez les animaux ayant des niveaux endogènes d'OT élevés. Par ailleurs, aucun effet adverse (amélioration non-significative) ne fût observé chez les animaux ayant un faible niveau endogène d'OT. Chez les animaux du groupe placebo, une tendance à observer une meilleure récupération chez ceux ayant des niveaux endogènes

initiaux élevés fût notée. Bien que la taille de la zone ischémique à risque soit comparable à celle rencontrée chez les patients atteints d'infarctus, l'utilisation d'animaux juvéniles et l'absence de maladies coronariennes sont des limitations importantes du modèle porcin utilisé.

Le potentiel de l'OT pour le traitement de l'infarctus du myocarde demeure mais nos résultats suggèrent qu'une administration systémique à titre de thérapie de remplacement de l'OT devrait être considérée en fonction du niveau endogène. De plus amples évaluations de la sécurité du traitement avec l'OT dans des modèles animaux d'infarctus du myocarde seront nécessaires avant de considérer l'utilisation d'OT dans une population de patients atteint d'un infarctus du myocarde. En contre partie, les niveaux endogènes d'OT pourraient posséder une valeur pronostique et des études cliniques à cet égard pourraient être d'intérêt.

MOTS CLEFS :

Oxytocine ; Pharmacologie de Sécurité; Effets Cardiovasculaires Adverses; Pharmacologie; Physiologie; Toxicologie; Étude préclinique, Infarctus du Myocarde; Fraction d'Éjection; Planimétrie; Porc; Utilisation Thérapeutique; Infusion Continue; Pharmacocinétique.

SUMMARY

In february 2009, a report from PHRMA (Pharmaceutical Research and Manufacturers of America) confirmed that more than 300 drugs for treatment of cardiovascular diseases were in clinical trials or under review by regulatory agencies. Despite the abundance of new cardiovascular therapies, the number of new drugs approved each year (all indications combined) is declining steadily with only 17 and 24 new drugs approved in 2007 and 2008, respectively. Only 1 drug out of 5000 candidates will be approved after 10 to 15 years of development with an average cost of $800 millions. Several initiatives have been launched by regulatory agencies to increase the success rate in drug development but results are still awaited. This stagnation is attributed to the lack of efficacy of several drug candidates but safety assessments are the leading cause of drug development discontinuation. *Primum non nocere*, the maxim from Hippocrate, father of medicine, remains of major relevance in preclinical and clinical drug development. Over the past 20 years, approximately 3% of approved drugs were subsequently withdrawn from the market due to adverse effects. Cardiovascular adverse effects represent the most frequent cause of drug development discontinuation or withdrawal (27%) followed by effects on the nervous system. After defining the context of safety pharmacology evaluations and the use of biomarkers in drug development, we validated safety pharmacology models to investigate drug-induced cardiovascular, respiratory and neurological effects.

As we progressed within constraints and challenges of drug development, we evaluated the efficacy and safety of oxytocin, an endogenous peptide with therapeutic potential. Oxytocin (OT), a hormone historically associated with reproduction, demonstrated the ability to induce *in vitro* differentiation of cell lines (P19) but also embryonic stem cells into beating cardiomyocytes. These observations lead us to consider the use of OT as a treatment for myocardial infarct. To achieve this ultimate goal, we first evaluated the pharmacokinetic of OT in an anesthetized rat model. These investigations highlighted the unique characteristics

of OT including a very short half-life and a non-linear pharmacokinetic profile in response to the dose.

Cardiovascular effects of OT in healthy animals were then evaluated in various species. In preclinical research, the use of various animal species and state of consciousness (conscious or anesthetized) is recognized as one of the best strategies to increase the predictive value of results obtained in animals to the human response. Our initial investigations of OT treatment regimens used various animal models including conscious and anesthetized rats, anesthetized pigs, conscious dogs with indirect blood pressure monitoring and diuresis and conscious monkeys with cardiovascular telemetry monitoring. These studies confirmed OT to have significant hemodynamic effects with variable responses depending on the state of consciousness (conscious or anesthetized), the dose, the administration protocol (bolus or infusion) and the species that were used. These screening studies enabled selection of a treatment regimen and dose without adverse effects that could subsequently be tested in efficacy studies.

A porcine myocardial infarct (MI) model with reperfusion was used to evaluate the effects of OT following myocardial ischemia. In a pilot project, continuous infusion of OT initiated immediately at coronary reperfusion induced cardiovascular adverse effects in all treated animals including a reduction of left ventricular fraction shortening and worsening of dilated cardiomyopathy which is typical following MI. Considering these observations, the therapeutic strategy was revised to avoid OT treatment during the inflammatory phase which was considered maximal around day 3 post-ischemia. When initiated 8 days after MI, OT infusion induced adverse effects in animals with elevated endogenous levels of OT. In contrast, no significant effects (not statistically significant improvement) were observed in animals with low endogenous OT baseline. In placebo treated animals, a trend to observe a better recovery was noted in animals with high endogenous OT baseline. While the size of the ischemic zone was comparable to human patients with MI, the use of juvenile animals and the absence of coronary disease are important limitations of the porcine model.

The potential of OT for treatment of MI remains but our results suggest that systemic administration of OT by continuous infusion as part of a replacement therapy should be investigated further in relation to endogenous levels. Further investigations on safety of the treatment with OT on animal MI models are warranted before the use of OT can be considered in the patient population after myocardial infarct. On the other hand, endogenous levels of OT may have a prognostic value and clinical trials to investigate this hypothesis may be of interest.

KEY WORDS :

Oxytocin; Safety Pharmacology; Adverse Cardiovascular Effects; Pharmacology; Physiology; Toxicology; Preclinical Study; Myocardial infarct; Ejection Fraction; Planimetry; Pig; Continuous Infusion; Pharmacokinetic.

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LISTE DES SIGLES ET ABBRÉVIATIONS

AAALAC : *Association for Assessment and Accreditation of Laboratory Animal Care International*, Association Internationale pour l'Évaluation et l'Accréditation Des Soins aux Animaux

ADME : *Absorption distribution metabolism elimination,* absorption, distribution, métabolisme et élimination

ADP : Adénosine diphosphate

ADR : *Adverse drug reaction reports*, rapport d'effet adverse d'un médicament

AE : *Adverse effect*, effet adverse

AIDS : *Acquired immunodeficiency syndrome*, syndrome de l'immunodéficience acquise

AIP : *Apoptosis-inducing factor*, facteur induisant l'apoptose

AIT : *Association of inhalation toxicologists*, association des toxicologues de l'inhalation

ALS : *Amyotrophic lateral sclerosis*, sclérose latérale amiotrophique

ANOVA : *Analysis of Variance*, analyse de variance

ANP : *Atrial natriuretic peptide*, peptide natriurétique atrial

ATP : Adénosine triphosphate

ARNm : Acide ribonucléique messager

Beecf : *Base excess in extra-cellular fluids*, excès de base dans le fluide extra-cellulaire

BLA : *Biologic license application*, application de licence pour un produit biologique

bpm : *beat per minute*, battements par minute

BSA : *Body surface area*, surface corporelle

BW : *Body weight*, poid corporel

Ca^{2+} : ion calcique

cAMP : 3',5'-adénosine monophophate cyclique

CAST :	*Cardiac Arrhythmia Suppression Trial*, essai clinique de suppression des arythmies cardiaques
CCK-4 :	*Cholecystokinine tetrapeptide*, tétrapeptide cholécystokinine
CDER :	*Center for Drug Evaluation and Research*, centre pour l'évaluation et la recherche pour les médicaments
CFR :	*Code of Federal Regulations*, code de règlementations fédérales
cGMP:	*Cyclic guanylate cyclase*, guanylate cyclase cyclique
CHMP:	*Committee for Medicinal Products for Human Use*, comité pour les produits médicinaux pour utilisation chez l'humain
cm :	Centimètre
CNS :	*Central nervous system*, système nerveux central
CO_2 :	Dioxyde de carbone, gaz carbonique
COD :	*Coefficient of determination*, coefficient de détermination
CREB:	*cAMP-responsive element-binding protein*, protéine liante répondant à l'AMPc
CRO :	*Contract research organization*, organisation de recherche contractuelle
CSA :	*Controlled Substances Act (of the US)*, l'Acte pour les substances contrôlées (des États-Unis)
CSS :	*Controlled Substances Staff (of the US)*, personnel des substances contrôlées (des États-Unis)
CVS :	*Cardiovascular*, cardiovasculaire
CYPs :	Cytochromes
DII :	Dérivation électrocardiographique II
DAC :	*Data acquisition controller*, contrôleur d'acquisition de données
DEA :	*Drug Enforcement Agency (of the US)*, agence de surveillance des substances contrôlées
dL :	Déciitre
DMARDs :	*Disease Modifying Antirheumatic drugs*, médicaments modifiant la maladie contre l'arthrite rhumatoïde

DMOADs : *Disease Modifying Osteoarthritis Drugs*, medicaments modifiant la maladie contre l'arthrose

DNA : *Deoxyribonucleic acid*, acide déoxiribonucléique

DPE : *Division of Pharmacovigilance and Epidemiology*, division de pharmacovigilance et épidémiologie

DRF : *Dose range finding*, évaluation du spectre de dose

EBM : *Evidence based medicine*, médecine factuelle, basée sur les évidences

ECG : Électrocardiographie

EDRF : *Endothelium-derived relaxing factor*, facteur de relaxation dérivée de l'endothélium

EEG : Électroencéphalographie

EF : *Ejection fraction*, fraction d'éjection

e.g. : *exempli gratia*, par exemple

EGAPP : *Evaluation of Genomic Applications in Practice and Prevention Working Group (of the US)*, groupe de travail sur l'évaluation des applications de la génomique en pratique et en prévention (des États-Unis)

EKG : Électrocardiographie

EMEA : *European Medicines Agency*, agence européennes des médecines

eNOS : *Endothelial nitric oxide synthase*, synthase de monoxyde d'azote endothélial

ELISA : *Enzyme-Linked ImmunoSorbent Assay,* épreuve immunoenzymatique par colorimétrie

etc. : *et cetera,* et autres

EU : *European Union*, Union européenne

Fab : *Fragment antigen binding*, fragment liant un antigène

FADD: *Fas associated death domain*, domaine de Fas associé à la mort cellulaire

FIH : *First in man*, première administration chez l'humain

FDA :	*Food and Drug Administration*, l'administration pour les aliments et les médications
FFT	*Fast Fourier Transform*, transformation de Fourier
FOB :	*Functional Observation Battery of safety tests*, batteries d'observations fonctionnelles pour évaluation de la sécurité
FS:	*Fraction shortening*, fraction de raccourcissement
hERG :	*Human ether-a-go-go channel,* canal éther à go-go humain
HSP-70 :	*Heat shock protein 70*, protéine de choc 70
HTS :	*High throughput screening*, dépistage à rendement élevé
Hz :	Hertz
g :	Gramme
GAPDH :	Glyceraldehyde-3-phosphate dehydrogenase, déhydrogénase glycéraldéhyde-3-phosphate
GLP :	*Good Laboratory Practices*, bonnes pratiques de laboratoire
GMP :	Guanylate monophosphate
GTN:	Glycerol trinitrate
GTP :	Guanylate triphosphate
h :	Heure
H_2O_2 :	Peroxyde d'hydrogène
Hb :	Hémoglobine
HCO_3^-:	Bicarbonates
HDL :	*High density lipoprotein*, lipoprotéine de haute densité
HIV :	*Human Immunodeficiency virus*, virus d'immunodéficience humaine
HNO_2 :	Acide nitreux
HR :	*Heart Rate*, fréquence cardiaque
IACUC :	*Institutional Animal Care and Use Committee*, comité institutionnel de soins et d'utilisation des animaux
IAP :	*Inhibitor of apoptosis protein*, protéine inhibitrice de l'apoptose
ICH :	*International Conference on Harmonisation,* Conférence internationale pour l'harmonisation
I_{Ca}:	*Calcium current*, courant calcique

ICE : *Interleukin-1B-converting enzyme*, enzyme de conversion de l'interleukine-1B

i.e. : *id est*, c'est-à-dire

IFN-γ : Interféron gamma

I_{K1} : *Inward rectifier potassium current*, courant de correction entrant potassique

I_{KAch} : *Acetylcholine-activated potassium current*, courant potassique activé par l'acétylcholine

I_{KNa} : *Sodium-activated potassium current*, courant potassique activé par le sodium

I_{Kr} : *Rapid delayed rectifying potassium current*, courant potassique rapide de correction retardée

I_{Ks} : *Slowly activating delayed rectifying potassium current*, courant retardé de correction potassique d'activation lente

IL-1: Interleukine-1

IL-1 beta : Interleukine-1 bêta

IL-6 : Interleukine-6

IL-17 : interleukine-17

IL-18 : Interleukine-18

ILLUMINATE : *Investigation of lipid level management to understand its impact in atherosclerotic events*, essai clinique pour comprendre les impacts de la gestion des niveaux lipidiques dans les événements athérosclérosiques

IM : Intramusculaire

I_{Na} : *Sodium current*, courant sodique

IND : *Investigational New Drug*, nouveau médicaments sous investigation

iNOS : *Inducible nitric oxide synthase*, synthase inductible de monoxyde d'azote

IPRG :	*Interdisciplinary Pharmacogenomic Review Group (of the US)*, groupe interdisciplinaire de révision en pharmacogénomique (des États-Unis)
IQ :	*Installation Qualification*, qualification d'installation
I_{to} :	*Outward potassium current*, courant potassique sortant
IV :	Intraveineux
JPMA :	*Japanese Pharmaceutical Manufacturers Association*, association japonaise des manufacturiers pharmaceutiques
kg :	kilogramme
kPa :	kilo-Pascal
L :	litre
LAD :	*Left anterior descending coronary artery*, artère coronarienne antérieure descendante gauche
LDH :	*Lactate dehydrogenase*, lactate déhydrogénase
LDL :	*Low density lipoprotein*, lipoprotéine de faible densité
LVESD :	*Left ventricular end systolic diameter*, diamètre ventriculaire gauche en fin de systole
mAb :	*monoclonal antibody*, anticorps monoclonal
mEq :	Milliéquivalent
mg:	Milligramme
Mg^{2+} :	Magnésium ionique
MHLW :	*Ministry of Health, Labor and Welfare (of Japan)*, Ministère de la santé, du travail et du bien-être (du Japon)
MI :	*Myocardial infarct*, infarctus du myocarde
min :	Minute
mL :	Millilitre
mm :	Millimètre
mmHg :	Millimètre de mercure
MPT :	*Mitochondrial permeability transition*, transition de perméabilité mitochondrial
MRI :	*Magnetic resonance imaging*, imagerie par résonnance magnétique

mRNA : *messenger ribonucleic acid*, acide ribonucléique messager

ms : Milliseconde

mt-NOS : *Mitochondrial nitric oxide synthase*, synthase mitochondriale de monoxyde d'azote

MV : *Minute ventilation*, ventilation minute

n = : Taille d'échantillon

Na^+ : Ion sodique

NADH : *Reduced nicotinamide adenosine dinucleotide*, nicotinamide adénosine dinucléotide réduit

NADPH : *Reduced nicotinamide adenosine dinucleotide phosphate*, nicotinamide adénosine dinucléotide phosphate réduit

NCE : *New Chemical Entity*, nouvelle entité chimique

NCI : *National Cancer Institute (of the US)*, institut national du cancer (des États-Unis)

nhp : *Non human primate*, primates non-humains

NIDA : *National Institute on Drug Abuse (of the US)*, institut national sur la dépendance aux médicaments (des États-Unis)

NIH : *National Institute of Health*, institut national de santé

NMDA : N-méthyl-D-aspartate

NME : *New Molecular entity*, nouvelle entité moléculaire

nNOS : *Neural nitric oxide synthetase*, synthase neuronale de monoxyde d'azote

NO : *Nitric oxide*, monoxyde d'azote

NO^-: Anion nitroxyl

NO_2 : Dioxyde d'azote

NOAEL : *No observed adverse effect level*, dose sans effet secondaire adverse

NOEL: *No observed effect level*, dose sans effet pharmacologique

NOS : *Nitric oxide synthase*, enzyme synthase de monoxyde d'azote

NO_x : Oxydes d'azote

NSAID : *Non steroidal anti-inflammatory drug*, anti-inflammatoire non-stéroïdien

NTG : *Nitroglycerine*, nitrogycérine

O_2 : Oxygène

O_2^- : Superoxide

OA : *Osteoarthritis*, arthrose

$ONOO^-$: Peroxynitrite

OQ : *Operation Qualification*, qualification de fonctionnement

OT : Oxytocine

OTBL : Plasma oxytocin baseline, niveau plasmatique de base de l'oxytocine

OTR : *Oxytocin receptor*, récepteur d'oxytocine

$PaCO_2$: Pression partielle artérielle en dioxyde de carbone

PaO_2 : Pression partielle artérielle en oxygène

PCI : *Percutaneous coronary intervention*, intervention coronarienne percutanée

PCV : *Packed cell volume*, volume de cellules centrifugées, hématocrite

PD : *Pharmacodynamic*, pharmacodynamique

PES : *Programmed electrical stimulation,* stimulation électrique programmée

pH : Potential Hydrogen, logarithme de la concentration d'ion hydrogène

Ph.D. : *Philosophiae Doctor*, Doctorat de 3^{ième} cycle universitaire

PK : *Pharmacokinetic*, pharmacocinétique

PMS : *Post-Marketing Surveillance*, surveillance post-approbation de commercialisation

PNF : Procédures normalisées de fonctionnement

PQ : *Performance Qualification*, qualification de performance

PSA : *Prostate specific antigen*, antigène spécifique à la prostate

PTZ : *Pentylenetetrazole*, pentylènetétrazole

PVC : *Premature ventricular contraction*, contraction ventriculaire prématurée

qMRI : *Quantitative magnetic resonance imaging*, imagerie par résonnance magnétique quantitative

QTc : *Corrected QT interval*, intervalle QT corrigé

QTcB : *QT interval corrected with Bazett's formulae*, intervalle QT corrigé avec la formule de Bazett

QTcF : *QT interval corrected with Fredericia's formulae*, intervalle QT corrigé avec la formule de Fridericia

QTcV : *QT interval corrected with Van De Water's formulae*, intervalle QT corrigé avec la formule de Van De Water

QTd : *QT distribution*, distribution de l'intervalle QT

RLE : *Rare lethal event*, événements fatal rare

RNOS: *Reactive NO species*, formes réactives de monoxyde d'azote

RNS : *Reactive nitrogen species*, formes réactive d'azote

ROS : *Reactive oxygen species*, radicaux libres

RR : *Respiratory rate*, fréquence respiratoire

s : Seconde

SaO_2: pour-centage de saturation artérielle en oxygène de l'hémoglobine

SAP : *Systemic arterial pressure*, pression systémique artérielle

SC : Sous-cutané

SD : *Standard deviation*, variance

SEM : *Standard error of the mean*, erreur type

sGC : *Soluble guanylyl cyclase*, guanylyl cyclase soluble

SNPs : *Single-nucleotide polymorphisms*, polymorphismes sur un seul nucléotide

SOD : Superoxide dismutase

SOP : *Standard Operating Procedure*, procédures normalisées de fonctionnement

SpO_2: *Pulsatile saturation of hemoglobin*, saturation pulsatile de l'hémoglobine

SRS: Spontaneous Reporting System, système de rapport spontané

SSRI: Selective serotonin reuptake inhibitors, inhibiteur sélectif de la ré-entrée de la sérotonine

TBS : *Tris buffered saline*, saline tamponnée tris

TDP: Torsade de pointes

TNF-α:	*Tumor necrosis factor alpha,* facteur de nécrose tumorale alpha
TNFR1:	*Tumor necrosis factor receptor-1,* récepteur du facteur de nécrose tumorale alpha
TQTS:	Thorough QT Study, étude approfondie de l'intervalle QT
TRADD :	*TNFR 1-associated death domain*, domaine de mort cellulaire associé à TNFR1
TRIaD :	*Triangulation reverse use dependence and instability*, triangulation de dépendance inverse et instabilité
TV :	*Tidal volume*, volume courant
µg :	microgramme
VF :	*Ventricular fibrillation*, fibrillation ventriculaire
VPB:	*Ventricular premature beat*, battement ventriculaire prématuré
VT:	*Ventricular tachycardia*, tachycardie ventriculaire
WHO :	World Health Organization, organisation mondiale de la santé
w/w :	*weight/weight*, poids/poids
yrs:	Years, années
® :	Marque déposée
°C:	Degrés Celsius
% :	Pour-cent

DÉDICACE

« *It is a miracle that curiosity survives formal education.* C'est un miracle que la curiosité survive à l'éducation formelle. »

Albert EINSTEIN (1879-1955)

À Dominique ;
Mon alter ego sur tous les horizons, une source d'inspiration vive et constante. En reconnaissant la valeur de mes efforts, elle a rendu possible mon épanouissement personnel et professionnel.

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INTRODUCTION

Les maladies cardiovasculaires sont responsables de la majorité des décès en Amérique du Nord depuis près d'un siècle (Braunwald, 1997). Selon l'Organisation Mondiale de la Santé, les maladies coronariennes deviendront la cause la plus fréquente de décès dans les pays en voie de développement à compter de 2020 (Aboderin *et al.*, 2002). On projette que le nombre de décès liés à l'infarctus du myocarde, en valeur absolue, augmentera de près de 100% entre 2000 et 2035 (Elkins & Johnston, 2003). Basée sur ces tendances sociétales, l'importance du développement de nouvelles thérapies pour le traitement des maladies cardiovasculaires est sans équivoque.

Les modalités de traitements de l'infarctus du myocarde évoluent constamment suite aux avancées pharmacologiques et technologiques. Dans la phase aiguë de l'infarctus du myocarde, l'acronyme MONA résume les traitements traditionnellement utilisés incluant **m**orphine, **o**xygène, **n**itroglycérine et **a**spirine. Au-delà des traitements d'urgence, les discussions abondent quant aux traitements à administrer suite à la reperfusion de la zone infarctée. Très peu de thérapies font l'unanimité ou même connaissent une approbation définitive. Pendant qu'on discute de nouvelles thérapies innovatrices, l'efficacité de médicaments largement utilisés en clinique comme l'ézétimibe reste à confirmer de façon définitive par des essais cliniques post-approbation de longue durée (Bays *et al.*, 2008). L'ézétimide, approuvé en 2002 sur la base de la diminution des niveaux de LDL dans deux études cliniques (Dujovne *et al.*, 2002; Knopp *et al.*, 2003), n'a pas démontré d'efficacité dans une étude de 2 ans impliquant 720 patients en dépit de vente de plus de 5 milliards en 2007 (Berenson *et al.*, 2008; Psaty & Lumley, 2008). Cette étude a résulté en une progression plus rapide mais non-significative (p=0.29) de l'épaississement coronarien chez les patients recevant l'ézétimide et la simvastatine (0.1111 mm) lorsque comparé aux patients recevant la simvastatine seule (0.0058 mm) (Anonyme, 2008). L'utilisation des statines dont l'usage est largement répandu à titre préventif demeure un sujet des plus discutés (Arca *et al.*, 2007 ; Santos *et al.* 2008; Steinberg 2008 ; Holmes *et*

al., 2009). Alors que les traitements dans la classe des *petites molécules* subissent des vagues de remise en question, les essais cliniques utilisant des cellules souches mésenchymateuses dans le traitement de l'infarctus du myocarde sont partagés (Singh *et al*. 2009) et une plus grande compréhension des mécanismes moléculaires serait nécessaire afin d'arriver à des bénéfices cliniques (Liu *et al.*, 2009). De nombreuses avenues thérapeutiques prometteuses sont ouvertes. La recherche sur les thérapies de l'infarctus de myocarde est un sujet d'actualité et le restera probablement pour les décennies à venir. Cette thèse vise à présenter les étapes d'évaluation d'une nouvelle thérapie potentielle ainsi que le contexte d'évaluation des nouveaux médicaments.

Ce programme de doctorat avait pour objectif de *(1)* comprendre les étapes et les enjeux du développement des médicaments, *(2)* valider des modèles précliniques d'évaluation de l'innocuité et de l'efficacité des nouveaux médicaments *(3)* évaluer le potentiel thérapeutique de l'oxytocine (OT) dans le traitement de l'infarctus du myocarde à l'aide d'un modèle préclinique. Ultimement, le programme d'étude visait à répondre à la question suivante : «Est-ce que l'OT pourrait avoir un rôle à jouer comme thérapie systémique de l'infarctus du myocarde ?».

La thèse s'articule donc en trois parties commençant par la présentation de la pharmacologie de l'innocuité (*British Journal of Pharmacology* 2008,154:1382-1399) suivie de l'utilisation des bio-marqueurs dans le développement de nouveaux médicaments (*Cardiovascular biomarkers as examples of success and failure in predicting safety in humans*, Chapitre soumis à l'auteur du livre). Cette première partie établit le cadre dans lequel le développement de nouveaux médicaments s'effectue ainsi que les enjeux réglementaires. Après avoir établi les assises du développement des nouveaux médicaments, la deuxième partie de la thèse traite plus spécifiquement des modèles précliniques d'évaluation de l'innocuité et d'efficacité. Dans un premier temps, des travaux expérimentaux réalisés par notre groupe ont permis de caractériser et de valider des modèles précliniques d'évaluation de l'innocuité de

nouvelles thérapies sur la fonction cardiovasculaire. La validation du modèle a d'abord été abordée sous un angle non-pharmacologique (*Journal of Pharmacological and Toxicological Methods* 2007, 56 : 115-121) suivi d'une validation pharmacologique (*Journal of Pharmacological and Toxicological Methods* 2007, 56 : 122-130) pour finalement présenter les avantages et applications du modèle anesthésié d'évaluation de l'innocuité cardiovasculaire des nouveaux médicaments (*Journal of Pharmacological and Toxicological Methods* 2008, 58 : 94-98). Ensuite, des travaux expérimentaux de validation d'un modèle d'évaluation de l'innocuité sur la fonction respiratoire (*Journal of Pharmacological and Toxicological Methods* 2008, 57 : 52-60) sont présentés et discutés. Afin d'approfondir les discussions sur l'évaluation de l'innocuité respiratoire, des travaux originaux effectués par notre groupe comparent les modèles précliniques utilisant différentes espèces dont le chien, le rat et le singe (*Regulatory Toxicology and Pharmacology* 2009, 55(2):229-235). Afin de compléter cette revue de la pharmacologie de l'innocuité et d'efficacité, les modèles couramment utilisés lors des évaluations règlementaires sont présentés et discutés.

La troisième partie présente notre évaluation de l'OT comme nouvelle thérapie cardiovasculaire. Dans un premier temps, une revue de littérature présente les connaissances actuelles sur les effets cardiomyogéniques de l'OT et les hypothèses sur lesquelles nos travaux étaient fondés. Ensuite, les résultats de travaux expérimentaux d'évaluation de l'innocuité de l'OT dans divers modèles précliniques sont présentés. Les effets cardiovasculaires de l'OT sur des modèles de rats anesthésiés et conscients, de chiens conscients et anesthésiés ainsi que de singes conscients dressent un tableau complet des résultats obtenus avec ces modèles précliniques d'innocuité. Les effets pharmacologiques de l'OT sont évalués afin d'orienter le choix de la dose et du protocole de traitement pour les évaluations subséquentes dans un modèle d'efficacité du traitement de l'infarctus du myocarde porcin (*Journal of Cardiovascular Pharmacology*, manuscript soumis). Après avoir présenté les résultats expérimentaux obtenus avec les

différents modèles, cette troisième partie discute des implications des résultats sur le développement de cette protéine thérapeutique pour le traitement de l'infarctus, des implications cliniques et des perspectives futures d'évaluation de ce traitement. Cette dernière partie permet d'évaluer les effets bénéfiques et délétères de la thérapie systémique d'OT.

À titre de conclusion générale, nous présentons nos recommandations liées au rôle potentiel de l'OT dans le traitement de l'infarctus du myocarde ainsi qu'un résumé des grandes lignes directrices en matière de développement des médicaments.

"If we doctors threw all our medicines into the sea, it would be that much better for our patients and that much worse for the fishes."

"Si nous, médecins jetions toutes nos médications à la mer, ce serait tellement mieux pour nos patients et tellement pire pour les poissons."

Oliver Wendel Holmes, M.D. (1841-1935)

Première partie

MÉTHODOLOGIES D'ÉVALUATION DES NOUVEAUX MÉDICAMENTS

Chapitre 1

REVUE DE LITTÉRATURE

1. PRINCIPES DE BASE EN PHARMACOLOGIE DE SÉCURITÉ

Ce premier article (*British Journal of Pharmacology* 2008,154:1382-1399) présente la pharmacologie de sécurité, une discipline relativement nouvelle dans le domaine du développement des médicaments. Cet article couvre certains aspects du premier objectif de cette thèse soit, la compréhension des étapes et des enjeux du développement des médicaments. Après avoir présenté l'historique et les définitions propres à la discipline, l'agenda de la pharmacologie de sécurité présente au lecteur une vue d'ensemble des champs d'activités et des grands axes d'activité incluant l'évaluation de la fonction cardiovasculaire, respiratoire, neurologique ainsi que les études supplémentaires courantes. Ensuite, le développement, la validation et l'accréditation des modèles de pharmacologie de sécurité sont présentés. Une description plus approfondie des méthodes fait suite à la présentation générale de la discipline. La pharmacologie de sécurité répond à des considérations scientifiques plutôt que règlementaires et cette tendance est discutée. En terminant, l'article présente les principes de pharmacologie de sécurité et les besoins non-comblés.

Cet article présente la démarche d'évaluation s'appliquant à l'ensemble des nouveaux médicaments d'où sa pertinence dans le développement de l'OT comme traitement de l'infarctus du myocarde. Les concepts et considérations qui y sont discutés servent à guider les études animales qui ont été effectuées par notre groupe afin d'évaluer l'OT comme traitement potentiel de l'infarctus du myocarde.

Le Docteur Simon Authier a participé de façon active à la rédaction de cet article publié (*British Journal of Pharmacology* 2008; 154 : 1382-1399) avec comme responsabilité l'écriture des sections décrivant l'agenda de la pharmacologie de sécurité, le développement, la validation et l'accréditation des modèles ainsi qu'une contribution importante à la présentation détaillées des modèles utilisés.

Principles of Safety Pharmacology

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Abstract

Safety Pharmacology is a rapidly developing discipline that uses the basic principles of pharmacology in a legal-driven process to generate data to inform risk:benefit assessment. The aim of Safety Pharmacology is to characterize the pharmacodynamic/pharmacokinetic (PK/PD) relationship of a drug's adverse effects using continuously evolving methodology. Unlike toxicology, Safety Pharmacology includes within its remit a requirement to predict the risk of rare lethal events. This gives Safety Pharmacology its unique character. The key issues for Safety Pharmacology are detection of an adverse effect liability, projection of the data into safety margin calculation, and finally clinical safety monitoring. This article sets out to explain the drivers for Safety Pharmacology in order that the wider pharmacology community is better placed to understand the discipline. It concludes with a summary of principles that may help inform future resolution of unmet needs (especially establishing model validation for accurate risk assessment). Subsequent articles in this issue of the journal address specific aspects of Safety Pharmacology in order to explore the issues of model choice, the burden of proof and to highlight areas of intensive activity (such as testing for drug-induced torsades de points liability).

Abbreviations

AE, Adverse effect; ADR, Adverse drug reaction reports; ADME, Absorption distribution metabolism elimination; ADR, Annual number of adverse drug reaction reports; CERT, Arizona Center for Education and Research on Therapeutics; CDER, Center for Drug Evaluation and Research; CHMP, Committee for Medicinal Products for Human Use; CRO, Contract Research Organization; CSA, Controlled Substances Act (of the US); CSS, Controlled Substance Staff (of the FDA); DPE, Division of Pharmacovigilance and Epidemiology; DRF, Dose range finding; DEA, Drug Enforcement Agency; ECG, Electrocardiogram; EMEA, European Medicines Agency; EU, European Union; FIH, First in human (first time a new class of treatment is administered to humans); FDA, Food and Drug Administration of the United States; FOB, Functional observational battery of safety tests; GLP, Good Laboratory Practice; HTS, High throughput screening; hERG, Human ether-a-go-go; ICH, International Conference on Harmonization; I_{Kr}, Rapid delayed rectifying potassium current; IND, Investigational New Drug; I_{to}, Transient outward potassium current; JPMA, Japanese Pharmaceutical Manufacturers Association; NCI, National Cancer Institute; NIDA, National Institute on Drug Abuse; NIH, National Institute of Health; NCE, New chemical entity; PD, Pharmacodynamic; PK, Pharmacokinetic; PMS, Post-Marketing Surveillance; PES, Programmed electrical stimulation; RLE, Rare (but potentially) lethal event; SRS, Spontaneous Reporting System; Liability, Tendency to cause an adverse effect (universal jargon term in Industry); TQTS, Thorough QT Study (jargon term for QT prolongation liability testing in humans); TRIaD; triangulation; reverse use dependence and instability, TdP, Torsades de pointes; WHO, World Health Organisation

1. A definition and history of Safety Pharmacology

Safety Pharmacology is the discipline that seeks to predict whether a drug, if administered to human (or animal) populations, is likely to be found unsafe, and its professional mandate is to prevent such an occurrence. Prior to 1990, pharmaceutical companies conducted toxicological testing of lead compounds as part of preclinical drug discovery. However, it has become increasingly clear over several decades that drugs may progress as far as Phase 3 clinical trials (i.e., the intended patient population) before rare and potentially lethal adverse effects become apparent. The vigilant Post-Marketing Surveillance (PMS) efforts by regulatory authorities necessary to confirm the existence of a rare adverse event occurs after approval for human use. The Food and Drug Administration of the United States/Center for Drug Evaluation and Research (FDA/CDER) uses tools such as drug experience reports (MEDWatch), medical literature (clinical trial data) and multiple Federal agency data sources (Drug Enforcement Agency: DEA, National Institute of Health: NIH, National Institute on Drug Abuse: NIDA) in conjunction with the Division of Pharmacovigilance and Epidemiology (DPE) which utilizes the Spontaneous Reporting System (SRS) to monitor adverse drug effect patterns potentially indicative of a public health concern (a potential "signal"). The SRS receives adverse drug reaction reports derived from health care providers and hospitals. When an adverse effect is very rare, it may require millions of prescriptions before an awareness of its existence emerges. There are numerous examples of this in the literature (e.g., Kemp 1992); one of the best is terfenadine.

In the mid 1990s the antihistamine, terfenadine (Seldane®, Marion Merrell Dow), was withdrawn following a growing awareness that the drug could evoke the potentially life threatening cardiac syndrome, torsades de pointes (TdP), in otherwise healthy patients (Monahan *et al.*, 1990; June *et al.*, 1997). Prior to this, only cardiac/cardiovascular compounds were considered to possess such a tendency (liability). The problem here was that terfenadine, a non-cardiovascular

drug, had low efficacy to evoke TdP making it so rare an event that it required several million prescriptions before its liability became suspected. The other important consideration here is that the indication for which terfenadine was used (hayfever) is itself far from life threatening. Therefore risk (death) clearly outweighs benefit.

This episode was of great importance to what we now call Safety Pharmacology (a discipline that did not exist at the time). This is because predicting terfenadine's TdP risk was not possible by the conventional *preclinical toxicity testing* methods conducted at the time. Preclinical toxicology testing involved determining the high dose adverse event profile of a compound given at chronic, toxic doses, but would not have detected a rare lethal event liability. Indeed, screening for TdP liability risk in animals or in Phase 1 and 2 clinical investigations (whether by evaluating QT prolongation or by exploring other putative biomarkers) was not recognized as relevant, let alone necessary, in the late 1980s and early 1990s. Moreover the magnitude of the effect of terfenadine on QT interval is small and peak effects may exhibit a delayed onset (Ollerstam *et al.* 2007), and so the effect is hard to detect even if one is looking. This problem could have been avoided if, instead of routine toxicology, a programme of specific high throughput screening (HTS) for TdP liability had been utilized in early drug discovery at the time, but consideration of biomarkers for rare adverse event liability was not part of the toxicology agenda in the early 1990s.

Even though it was known that repolarization delay in the ventricles of the heart was associated with TdP occurrence, it was not until 1996 that Brown's group identified the likely mechanism of terfenadine's 'cardiotoxic' actions (Roy et al., 1996) and Rosen, by bring together for consumption in the mainstream literature (Rosen 1996) all of the threads, helped to inform a growing awareness in Industry that Toxicology alone, as practiced at the time, was insufficient for detecting rare but lethal adverse effect liability. In response to this, within 4 years, Safety Pharmacology had evolved into an Industry department-based discipline

designed to bridge the gap between preclinical toxicology and (preclinical and clinical) drug development (Bass et al., 2004a).

The creation of Safety Pharmacology has not resolved all challenges, especially with respect to detection of rare and lethal adverse effect liability. One of the most difficult problems in Safety Pharmacology is how to conduct early HTS for adverse effect liability with precision and accuracy and in a manner that the data set for a drug deemed "safe" can be presented in a convincing way to regulators. This is a particular problem for rare but potentially lethal adverse drug effects. Furthermore, to interject into this discourse, we noted earlier that Safety Pharmacology (as exists today) is tasked with identifying drugs as *unsafe* (within the therapeutic window) so, in effect, the data set the company presents to regulators is a failure to disprove that the drug is likely to be unsafe, rather than positive indication of likely safety. The extent of this difficulty becomes clear when we consider that even though more than 10 years has passed since the terfenadine episode, it is still not possible to *quantify*, with high certainty, a risk:benefit assessment for TdP liability for a drug about to enter Phase 1 clinical studies based on preclinical (or even clinical QT test) data sets (Shah 2008). Thus, we really remain years away from being able to take a drug's range of IC_{50} values for different molecular targets (i.e., its selectivity profile) and generate a number that reflects its risk (i.e., liability to evoke TdP) that can then be balanced against a number that reflects its likely therapeutic benefit. This model applies to all and any rare, but potentially lethal, adverse effect issues.

So, how has this impacted on the unfolding (and evolving) history of Safety Pharmacology? In the absence of quantification of the predictive value of tests and programmes, industry and the regulators have attempted to accommodate one another through a series of industry- and regulatory-led initiatives. Of the latter, the most important is the International Conference on Harmonization (ICH). The ICH is a project started in 1990 that utilizes the regulatory authorities of the United States, Europe and Japan in conjunction with experts from the pharmaceutical industry (from the three regulatory regions) to

discuss scientific and technical aspects of therapeutic drug registration (Bass et al., 2004a). What has this to do with pharmacology? The answer is that Safety Pharmacology has been shaped in structure and function by this ongoing accommodation between pharmacologists and regulatory authorities.

2. The agenda of Safety Pharmacology

Regulatory authorities [the FDA (US); Health Canada (Canada); European Medicines Agency (EMEA) and Japanese Pharmaceutical Manufacturers Association (JPMA) for example] give approval for drug use in humans. Therefore convincing the regulators that a drug is safe and efficacious is a key part of the drug discovery/development process. Thus, it is important to consider who the regulators are and what they want to know. The structure of a Safety Pharmacology "core battery" programme (Fig 1) is to determine the potential undesirable pharmacodynamic effects of a drug on the central nervous, cardiovascular and respiratory systems, as well as to implement supplementary tests to evaluate other organ systems (Pugsley, 2004; Bass et al., 2004b). Thus it is primarily designed to take account of regulatory requirements; scientific issues are secondary. Follow-up studies may be triggered if there is a need to characterize specific adverse effects found in initial Safety Pharmacology studies. Although follow-up may appear more scientifically driven than the core programme, the design of follow-up studies is nevertheless based on what is perceived by the pharmaceutical company to be the data required by the regulators. This gives a rather special flavour to Safety Pharmacology – it serves the needs of regulatory authorities primarily, and scientific proof is a secondary issue. But pharmacology is a science; this article therefore sets out to interrogate the Safety Pharmacology agenda and explore how far its mores digress form the rubric of science.

The practical agenda of Safety Pharmacology (to determine if a drug is "unsafe", and, if this is not the case, to inform drug discovery that the drug is

likely to be “safe”) is the flip side of drug discovery itself (to determine whether a drug is “effective”). If these semantics are kept in mind, the process of Safety Pharmacology is exactly the same as that for discovery; for a drug to progress to patients, Safety Pharmacology must conclude that a drug has a sufficiently low potential to evoke adverse effects to be trialed in patients, while discovery must conclude that a drug has a sufficiently high potential for benefit to be trialed in patients. Clearly, therefore, Safety and discovery pharmacology are interconnected, since a greater potential benefit may offset a greater potentially adverse effect liability.

Both Safety Pharmacology and discovery rely on preclinical (animal) research prior to Phase 1 human testing. Thus both are subject to the same issues, namely concerns over whether the animal models will allow accurate and complete detection of “hits” without false positives or negatives. Thus, both seek to identify and use full-scale clinically relevant endpoints (e.g., detection of disease generation in Safety Pharmacology, and protection against generated disease in discovery). At the same time, for reasons of practicality, both tend to use biomarkers (surrogate endpoints) such as kinase inhibition as a biomarker for cancer suppression in drug discovery (Garber 2006), and hERG block for TdP in safety (Sanguinetti and Mitchelson, 2005) to reduce the need for experimental complexity. Expert use of biomarkers is the most challenging aspect of Safety Pharmacology, and is a topic to which we will return on more than one occasion in this article, especially in the context of HTS for rare but serious adverse drug effect liability (e.g., TdP liability).

One of the key roles of Safety Pharmacology is to help inform the decision to begin testing in humans. Pharmacology alone does not define the fate of a new drug. The issues determining the point at which it is ethical/legal to proceed with clinical trials informs a risk:benefit assessment that is weighed against clinical development costs and the potential market. Risk:benefit assessment may appear to be a rather simple process: it is not. Determining the risk:benefit ratio is especially difficult when rare, but potentially lethal, events are a concern for a

drug which is intended for use against a non-life-threatening condition. The key point to emphasize again in this regard is that just as preclinical discovery studies never *prove* that a drug will be effective in patients, preclinical Safety Pharmacology studies never *prove* that a drug will be safe in patients. Thus the point at which preclinical data is sufficient to inform a decision on whether or not to proceed with a drug into clinical investigation is subjective and a matter of judgment (both for the company and for the regulators who scrutinize the application).

How is the decision to begin human testing made? In the absence of precise guidance from regulators (in some areas the ICH recommendations are vague) the decision-making process is difficult to understand, especially if a drug is found in preclinical tests to have a *possible* liability to evoke a *serious* adverse effect. Ultimately the regulators will decide whether to allow the drug to proceed to humans . . . or not (there is no halfway house).

It is important to note that both discovery and safety ought to take account of dosage in order to inform a likely safety margin for the drug. If animal models can be used to reliably predict the necessary dosage for benefit and the maximum tolerated dosage it may be possible to calculate a projected safety margin. Of course this begins to become a challenge if one attempts to equate *in vitro* data (using drug concentrations) with *in vivo* data (and dosage) Moreover, if biomarkers are used to substitute for real benefit or real risk in discovery or Safety Pharmacology this may lead to over- or under-estimation of projected safety margins. This is a highly problematic area. Unless one proposes that both discovery efficacy and Safety Pharmacology studies be conducted entirely in human volunteers (which effectively means abandoning all scientific research in medicine), the solution is to minimize the use of surrogate biomarkers when estimating dose response relationships in preclinical discovery and in preclinical Safety Pharmacology.

As an aside, it is worthwhile at this point to define the concept of Good Laboratory Practice (GLP). In discovery and Safety Pharmacology there comes a

stage when it is necessary to prepare investigational new drug (IND) documentation for submission for regulatory approval. Regulators take most notice of GLP studies, which use models that are formally validated (in as much as all procedures are defined, monitored and documented according to a recognized procedure, consideration of the details of which is beyond the scope of this article). GLP ensures the generation of verifiable quality data for the drug in development and as such defines the framework in which preclinical studies for regulatory submission must be conducted. GLP regulations encompass all components of regulated preclinical studies including the scientists involved (Study Director/Monitor), the test facility, the test system and the test article (test drug). The FDA regulates the conduct of preclinical laboratory studies under Part 58 (Good Laboratory Practice for Non-clinical Laboratory Studies) of Title 21 of the Code of Federal Regulations (US FDA, 2005a).

Once human testing has begun, risk:benefit assessment continues, this time in the patient population. This means taking into account the seriousness of the disease as well as the seriousness of any adverse effects. This is primarily relevant to Pharmaceutical company choice-making about investment (spending). Thus, a very promising cure for a rapidly progressing disease with a poor prognosis, such as pancreatic cancer, will likely be allowed to enter Phase 1 clinical trials in pancreatic cancer patients with minimal preclinical Safety Pharmacology testing. In which case, the extent of Safety Pharmacology investment will be minimized. Thus, the oncology division at the FDA may not fully enforce ICH S7A (the regulatory guidance document that provides general principles and recommendations for safety pharmacology studies) depending on the seriousness of the disease and current therapy or the absence of current therapy in this population, in which case the company will be able to minimize their spending (by carrying out more focused, and hence fewer Safety Pharmacology tests).

An IND is a request, under FDA's jurisdiction, to allow initiation of clinical trials. A successful IND may be filled with an abbreviated version of the

core battery investigation if the regulator deems it is worth providing this drug to patients quickly. Of course, preclinical scientists who are dealing with, for example, the oncology division of the FDA, know the requirements of this division because they will be aware of a number of IND packages that do not fully adhere to the S7A guidance, and yet were approved to allow clinical trials to proceed. This informs investment choices (spending). Subsequently, the regulatory authorities will judge if the Safety Pharmacology data is *sufficient* to establish that the drug does not expose patients to an unreasonable risk; with a rapidly lethal disease and a new type of treatment, time is of the essence and minor adverse effects may not be a critical concern. Other considerations for regulators (when deciding whether to allow a drug to progress to patients) include manufacturing information documenting consistency of the drug, the proposed clinical protocol and the qualifications of the clinical investigators charged with managing the proposed studies. The flexibility of requirements associated with variations in disease severity, variations in the needs for a new drug and variations in the anticipated adverse effects of the new drug should not be regarded as a charter for corner cutting as even abbreviated submissions are subjected to rigorous scrutiny.

Thus, industrial Safety Pharmacology departments seek to fulfill the requirements of the S7A core battery using different combinations of tests based on scientific judgment and the particularities of each drug candidate (i.e., on a case-by-case basis). Experience is a major component in this process from both the scientist and regulator perspectives. The process is not dissimilar to jurisprudence (in the legal milieu). Once a company (or a scientist) has submitted a successful package (submission to the regulator), this constitutes a positive signal from the regulator that the approach that informed generation of the package is acceptable. As an example (from personal experience) a single study with telemetry recording of cardiovascular parameters, CNS neurological examination and a respiratory profile using a pneumotachometer in only n=4 dogs may be sufficient to fulfill the core battery requirement for a drug indicated for a

life-threatening disease. On the other hand, if the condition to be treated is not life-threatening it may be necessary to implement the full functional observational battery (FOB). The FOB is a formalized systematic evaluation of nervous system function in the rat, comprising more than 30 parameters across autonomic, neuromuscular, sensorimotor and behavioural domains in rats (Redfern et al., 2005), respiratory function in a second study in rats (Murphy and Joran, 1992) and haemodynamic telemetry in dogs (Ollerstam et al., 2007). Drugs for diseases for which treatments are already available (even life-threatening diseases such as Hodgkin's lymphoma) will usually require a complete Safety Pharmacology investigation programme and a relatively favorable safety profile. Thus, there exists a risk:benefit *continuum*; many currently available anticancer drugs are not in any way "safe" for healthy humans but they are considered "safe" for cancer patients given their debilitating condition. Likewise, given the anticipated adverse effects of some anticancer drugs, no testing is needed in healthy human volunteers (see Fig 2 for details on the continuum).

The nature of the drug is also an important factor that will modulate the requirements for Safety Pharmacology testing. As an example, a monoclonal antibody will be allowed to progress to first in human (FIH) studies with minimal investigation of, for example, TdP liability. In contrast, a small molecule first in a new drug class will require a complete Safety Pharmacology assessment before it can progress to Phase 1 assessment in healthy volunteers, owing to the probability that an entirely new class of drug will have the greatest scope for unforeseen adverse effect.

Because, the decision from the regulators for a given product is a risk:benefit continuum that goes beyond the ICH guidelines (U.S. FDA, 2001), the company must choose which Safety Pharmacology tests to perform based on a subjective judgment that takes into account the need to test for "safety preclinical signals" in the context of the potential benefit for the patient population by considering currently available drugs for the indication and their adverse effects (severity and reversibility of the adverse effects). After all, if, for example, current

best therapy for a lethal indication is highly unsafe then to test a new drug for this indication for possible trivial adverse effect liability is needless, and constitutes development procrastination. Given that peoples' lives are at stake, the drug developer is under extreme time pressure to assemble a Safety Pharmacology portfolio that will provide the regulator with data in which the risk:benefit ratio favours use of the drug in such patients. The pressure exerted by patent laws (time-limited protection) on bringing a drug as quickly as possible to market must also be acknowledged and set against the existence of regulatory guidance on what is regarded as "reasonable promise" of expected effectiveness as well as "reasonable expectation of safety" if a drug is allowed to pass from preclinical to clinical investigation. It is therefore clearly difficult for regulators and drug developers to know where to set the threshold for determining a judgment of "reasonable promise", and how to weigh this against the scope for ambiguity regarding "reasonable expectation of safety" in many areas (e.g., TdP liability).

In the future we can anticipate Safety Pharmacology studies (especially non-GLP screening) being completed earlier in the process of drug discovery and development, with preclinical Safety Pharmacology data used to inform decision-making.

3. The development, validation and accreditation of preclinical Safety Pharmacology methods.

As we have explained, the primary agenda of Safety Pharmacology is to provide companies with data to discontinue development of (kill) unsafe drugs early in the preclinical development phase. The sooner a decision is made to kill a drug the sooner the company can begin to strategize on development pathways, i.e., either develop another drug backup using a similar chemical scaffold or consider a dissimilar drug class or programme. Since real (human) safety can usually only be decided after conduct of a Meta analysis of clinical trials (a statistical approach that evaluates the combined results of several independent clinical investigation

studies, each of which has addressed a related hypothesis) which takes place *after* drug approval and extensive human exposure, preclinical Safety Pharmacology does not seek to ordain a drug as "safe". Indeed it cannot (especially for very rare but potentially lethal adverse effect liabilities, such as TdP). The best it can do is to attempt to identify a drug as *potentially* safe. This means that, in Safety Pharmacology, expense (of time, human resource and money) is spent in pursuit, not of bringing drugs to the market, but in stopping drugs going to market.

Understandably, therefore, it may be that companies with limited resources to develop drugs could work on the principle of "as little Safety Pharmacology as necessary" or "only what is required". This may result in *cutting corners to terminate* a drug project, not cutting corners to ordain it as safe. On the other hand, large pharmaceutical companies will commit to spend more in order to achieve an integrated (in this context we mean comprehensive) Safety Pharmacology programme that provides the best prediction of human response in the shortest time. Essentially, a larger, resource-rich, company may be *less ruthless in terminating* a drug candidate early than a smaller company because a more extensive safety profile can be afforded to be developed. This ensures a reduced probability of inappropriately terminating development of what may eventually become a therapeutically useful drug. There are two ways of proceeding. First, in order to ensure appropriate decisions are made, a more comprehensive (and expensive) Safety Pharmacology programme may be judged to be required. To make such a comprehensive programme work, the focus is then placed upon integrating the use of time, resources and decision-making procedures. On the other hand, the company may focus on avoiding drug failures owing to adverse effects and attempt to ruthlesslessly weed out potential failures using approaches that may have fewer false negatives in the hope of achieving close to zero false positives. Both approaches are subjective: one seeks to avoid throwing out the baby with the bathwater while the other seeks to avoid leaving a piranha in the bath with the baby. The guiding principle, once again, is to optimize the overall preclinical Safety Pharmacology programme to minimize

testing time and most importantly, to identify quickly and accurately, any “show stopper” adverse effect liability as soon as possible. If drug development can be terminated during the preclinical testing phase instead of Phase 3, this is a major resource and financial advantage.

The guiding principle that informs the selection of what safety tests to conduct, in accordance with those outlined in guidance documents (S7A and S7B), may therefore be “as little as is necessary and no more than is sufficient”. Safety Pharmacology is not, after all, about establishing likely therapeutic benefit, but rather is primarily about preventing further cost with uncertain benefit.

What is necessary to achieve in the laboratory becomes increasingly clear the closer one gets to a lead candidate. Thus, with a new chemical entity (NCE) no safety tests are conducted until there is reason for considering the NCE to be a potential lead compound (i.e. until there is some discovery data available). In contrast, at the other end of the discovery process, just prior to Phase 1 (FIH) clinical investigations, there will have evolved a detailed portfolio of Safety Pharmacology data for the “nominated” compound that will most typically include GLP study findings. The interesting challenge therefore is to know when to do what and how to interpret the findings, since the onus on Safety Pharmacology is to inform the risk:benefit assessment at all stages of drug discovery and development (see Fig 3)..

As is inevitable, a nominated compound will have a specific preclinical safety portfolio. However, an NCE that fails in preclinical safety assessment could have an incomplete portfolio (the final entry of which will be the outcome that indicated that potential risk outweighed potential benefit). For any NCE that fails it is the goal of safety assessment to inform the decision as early as possible in the discovery process for reasons of cost (animal and monetary) and time. Therefore choice of test and timing of testing are critical.

There is a core battery of CNS, respiratory and cardiovascular tests that will need to be completed if an NCE is to become a drug (see above), constructed for purposes of legal compliance as well as reason of good scientific practice.

However, as safety assessment progresses from no data to completion, the timing of the deployment of different safety tests (core battery and other studies conducted either in house or at a contract research organization: CRO) is a matter of subjective judgment (Table 1). This means that different companies likely conduct studies at different times (Friedrichs et al., 2005; Lindgren et al., 2008), choosing different non-core-battery tests from among those available according to in-house judgment and expertise. While a majority of these tests may have been validated by blinded experimentation (e.g., Hamlin et al., 2004; Lawrence et al., 2006) others may not. For these reasons it is beyond the scope of this article to provide a logical explanation for the process of deployment of each test based on an appraisal of model validation and cost effectiveness, so we offer here a mere brief description of typical practice.

In some large drug development platforms (usually large pharmaceutical companies), Safety Pharmacology may be divided into complementary phases. The initial phase is part of the process that informs lead candidate selection and optimization, and is usually not conducted under GLP compliance. For most small molecule drug candidates, this initial phase includes cardiovascular screening. This initial phase typically includes ion channel inhibition (e.g. I_{kr} also known as the hERG potassium channel assay; Murphy et al., 2006) and may include an isolated organ preparation (wedge preparation and/or isolated Langendorff heart; Wang et al., 2008; Hondeghem et al., 2003; Hamlin et al., 2004), an anesthetized animal model (dogs or monkeys) using continuous step infusion (n=2/compound) and a non-GLP conscious telemetry study (Shah 2008). Drug class and early findings such as hERG assay results effects help define the next step.

The first phase of Safety Pharmacology that is usually done prior to the core battery is subjective, based on needs, findings and experience. Drug development teams will rely on the experience of their safety pharmacologist team through meetings to decide on the successive steps – the process is dynamic. It is important to remember that drug discovery research (tests in disease models) occurs in parallel to safety assessment, and the outcomes of each process inform

the decision making in each process (Figure 2). This is the type of decision-making practice for how drugs are developed. A simple analogy is the decision making one may make about taking a swim in the sea in the UK (for non UK residents, we note that the sea in the UK can range from balmy and rewarding to chilly, turbulent and treacherous, and the typical UK swimmer can range in competence from Olympic to dyslipedemic). First we decide how strongly we desire to swim. Then we consider the weather. Then ultimately, having travelled to the coast, we dip our toes into the sea and decide (factoring in our general health and fitness) whether or not to take the plunge. This is how drugs go to market: is there a market? What conditions prevail? Do we have a launchable product?

To provide a broader assessment of the safety profile, the animal species selected for an anesthetized animal study may often be different from that used for conscious animal telemetry. If the drug is bioavailable and has adequate PK in different species, it will be tested in a range of species. If only monkeys or dog are suitable (i.e., owing to the unique expression in these species of the drug's primary molecular target for benefit and/or possible anticipated adverse effects), a single species can be used. For some drug classes, additional screening models may be added. This early phase of Safety Pharmacology may appear somewhat random but this is because it is the most difficult to design. Should every NCE be administered to monkeys? Obviously not, especially given that the goal is to find a read-out that justifies terminating drug development. It would be most ideal for a number of reasons if this assessment could be achieved using a test tube assay. However we know that this is not achievable, nor realistic despite attempts to suggest otherwise – humans are complex, integrated physiological systems so similarly complex systems are needed to evaluate the safety profile of the compound. Thus, for very novel NCEs, model and test choice may be impossible to prejudge, and decision-making processes are likely to be frequently reviewed.

For the core battery of safety tests, there are regulatory guidelines that test for potential undesirable pharmacodynamic effects on physiological functions in

relation to the nature of the drug exposure (US FDA, 2001). For the early phase Safety Pharmacology investigations, decision-making is conducted on an "as needed basis". As an example, cardiovascular adverse effects (e.g., heart failure liability) of multi-targeted receptor tyrosine kinase (RTK) inhibitors (e.g. sunitinib; Chu et al., 2007; Kerkala et al., 2006) prompt consideration of the use of repeated dose Safety Pharmacology screening methods in conscious animals using telemetry with a focus on systemic arterial pressure and chronotropic effects for drug in the same class (Khakoo et al., 2008). For other drugs, e.g., a topical acne treatment, this would be fatuous. Of course these choices must be reviewed. Overall, previous failures of the chosen Safety Pharmacology screening programme to predict human adverse effects will dictate how the programme should be revised. In drug development; companies may focus on specific therapeutic targets (e.g. a specific enzyme) whereby their chemists will produce a number of iterations (backup compounds) of parent drug. This may reduce the required extent of safety assessment.

This initial phase of Safety Pharmacology testing is a luxury that most smaller drug development platforms (e.g. small biotechnology and pharmaceutical companies) cannot afford. So why do the bigger companies do this if (apparently) they do not need to do so? The answer may be perhaps (but not all of the time) that the small companies do not mind if their drug ultimately is found to lack success in Phase 3 trials as a possible larger agenda could involve sale of their chemistry, technology and drug development programs to a bigger company after the drug has achieved FIH status (i.e., has been entered into Phase 1 clinical investigation). For this reason, small companies with a single drug or small portfolio are more than likely to follow the tenet "as little testing as needed", as described earlier. Thus, resources may be invested to achieve FIH with possible disregard for determining safety liabilities before FIH (Fig 4)

For drug candidates in the large molecule category, this initial screening step may not be required on the basis of the drug development team experience and the safety profile of other drugs in the same class. In these cases, the

toxicology and Safety Pharmacology assessment programs may share the same first step, known as dose range finding (DRF) studies (sometimes called toleration studies), which are initially conducted in rodents (mice or rats) and followed by studies in a large animal species (e.g. dogs, non human primates or minipigs). The DRF/toleration study design utilizes a dose escalation paradigm to determine the dose at which adverse effects are first seen in a single or limited number of animals (somewhat rather a crude test since statistical proof cannot be part of the process with such an approach). Regardless, such studies characterize the toxicological dose-response profile (usually for the first time for a drug in development) and include cage side observations (for physical and behavioral effects), drug exposure analysis, blood chemistry, hematology, pathology and histopathology. A repeated dose administration toxicology study will often be completed in selected animal species to confirm the dose levels that will then be used in subsequent GLP toxicology and Safety Pharmacology studies.

The second part of the Safety Pharmacology programme is normally conducted in accordance with GLP guidelines for regulatory submission and includes the Safety Pharmacology core battery as defined in the ICH guideline S7A (U.S. Food and Drug Administration, 2001) as well as a GLP hERG assay. Here decisions may be made that defer from “killing-the-drug” mode to “presenting-the-drug-as-likely-safe” mode. Thus there is a changed in development status since the audience is no longer the company’s strategic planners, it is now the regulatory authorities who decide whether the drug is fit to be entered for human consumption.

The Safety Pharmacology core battery is typically conducted with a single administration of drug using the same administration route in conventional toxicology studies (similar to that which will be used clinically) with evaluations usually up to 24 hrs. Cardiovascular safety is assessed in a conscious telemetry study (e.g. n=4) usually in a Latin square or dose-escalation design with sufficient drug “wash out” times between dosing. These studies usually use the same species as in the large animal toxicology studies. Respiratory Safety

Pharmacology is typically evaluated in conscious rats (e.g. n=8 given the greater variability of respiratory parameters) but large animals such as dogs and monkeys may also be used when rodents are not suitable (e.g., if target is absent in rodents or absorption distribution metabolism elimination (ADME) profile is not adequate). Neurological safety is usually evaluated using a modified Irwin test in rats (Irwin, 1968; Mattsson *et al.*, 1996) where qualitative evaluations are conducted by an evaluator blinded to study treatments (e.g. n=10 per group). Neurological evaluations may also be performed in other species (e.g. mice, dogs, minipigs or non human primates; Moscardo *et al.*, 2007; Tontodonati et al., 2007) as for respiratory Safety Pharmacology. Beyond routine CNS Safety Pharmacology evaluations, some models are developed to characterize specific neurological adverse effects with the use of EEG monitoring by telemetry (Durmuller et al., 2007). A trend to integrate some components of the Safety Pharmacology evaluations such as respiratory, CNS and ECG study end-points into toxicology studies is currently noted (Luft & Bode, 2002). Development of non-invasive methodologies such as ECG monitoring (along with respiration, temperature, and animal activity) using jacketed external telemetry (JET^{TM}) systems has significantly contributed to this emerging practice (Morton *et al.*, 2003). Among the advantages of Safety Pharmacology assessments in toxicology studies, we have an increased sensitivity (e.g. increased statistical power) based on the relatively large number of animals in toxicology studies, reduction of the number of animals required for overall safety evaluations, an integration of Safety Pharmacology endpoints with histopathological and hematological/clinical chemistry data and potential cost reduction (e.g. when including FOB and respiratory assessments in toxicology studies).

The key question about the core battery tests (as far as the regulators are concerned) is: are they validated? In other words, does the chosen model accurately identify the safety liability of the drug candidate? Validation of Safety Pharmacology test systems for GLP compliance is achieved at each test site using positive control drugs with currently accepted models (Hauser *et al.*, 2005;

Chaves *et al.*, 2006; Chaves *et al.,* 2007; Authier *et al.*, 2007; Authier *et al.* 2008). At a higher level, some initiatives such as the QT-PRODACT project have helped characterize the sensitivity of the methodologies and inter-facility variability (Ando *et al.*, 2005; Miyazaki *et al.*, 2005; Omata *et al.*, 2005; Tashibu *et al.*, 2005; Toyoshima *et al.*, 2005; Sasaki *et al.*, 2005). These results have contributed to the increasing harmonization of industry practices, making it easier for regulators to make judgments based on retrospective comparison considerations (precedents).

While test system validation for regulatory purposes appears to evolve within an accepted reference frame, does this mean that regulatory authorities will accept as "validated" a method that has not actually been *scientifically* validated? From experience with regulatory audits and IND package submissions, regulatory authorities will accept models that have been demonstrated as reasonably valid in the public domain (i.e., used, and the data published). Accuracy, reliability, use of standard agents as reference, and security of the systems are major elements in GLP validations.

True pharmacological validation remains a vexing issue in Safety Pharmacology in exact mirror image of the issue of validation of disease models in drug discovery. It is important to emphasize that models and biomarkers are "valid" only when they detect all and only those drugs that have the same effectiveness and safety in the human. There is a major paradox inherent in this requirement, one that is not well recognized, and one that is a fundamental problem for the newest most potentially revolutionary drugs. Thus, because new drugs are new by definition (FIH for an untreated condition, NCE, new mechanism of action), the disease for which the drug is intended may have *no presently available treatment*. Clearly without a positive control to provide a template response profile, this means there can be no validated preclinical model for discovery. Thus the models used to identify the new drug are not validated, and will not be validated until the identified drug is shown to be effective in humans. Likewise, in Safety Pharmacology, no model is validated until a range of

positive and negative controls have been shown to produce the same outcome in the model as occurs in humans. This sounds simple; however, it is a huge problem for certain types of adverse effects. Thus to validate a model that is to be used for detecting a liability for a drug to evoke a very rare (but potentially) lethal event (RLE) requires precise and accurate *human* data on the liability of a range of drugs to evoke the RLE (the "gold standard").

Again, drug induced TdP liability testing provides a good example of the problems here. One of the most well known TdP-causing drugs is terfenadine. One would imagine (on the basis of foregoing considerations) that terfenadine would be one of the first drugs to be chosen to be used in validating any new TdP liability-testing model. However, one would need to know the exact rate of occurrence in humans of TdP with terfenadine to make use of terfenadine to validate a model. The rate of occurrence could then be ranked against a range of other drugs to generate a clinical ranking order. This would then serve as the template to validate the model (by generating a comparator ranking order for the model). There are several problems with this, unfortunately. For TdP there exists no reliable clinical ranking order for drugs. This is because when events (such as TdP) are rare the calculation of their preponderance cannot be made with acceptable precision. Thus, for terfenadine, the rate of occurrence of adverse cardiovascular events was 83 cases reported from time of approval in Europe (1981) until 1992 after use in millions of patients (Schiefe and Cramer, 1996; Kemp, 1992; Yap and Camm, 1999). The drug manufacturer reported the "events" (cases presenting as TdP, QT prolongation, ventricular tachycardia, flutter and fibrillation, cardiac arrest, and sudden death) to the FDA which issued a "black box" label warning of cardiovascular risk for the drug alone as well as when prescribed in combination with macrolide antibiotics and azole antifungal drugs (Morris and Carlson, 1998). Based upon these findings the FDA issued a proposal to withdraw terfenadine from the market in 1998 and the manufacturer complied. Interestingly, in a cohort study comparing terfenidine to clemastine, non-prescription antihistamines and ibuprofen, life-threatening ventricular

arrhythmias (used in lieu of categorical confirmed TdP incidence) occurred in less than 0.063% (or 317 out of >500,000) of patients in a Medicaid recipient database (Pratt *et al.*, 1994). The authors concluded that terfenadine users were no more likely to develop arrhythmias than those on ibuprofen or clemastine (Tavist) (Pratt *et al.*, 1994). Darpo (2001) reviewed the annual number of adverse drug reaction (ADR) reports of TdP submitted to the World Health Organization (WHO) drug monitoring center over a 16 year period (1983-1999). Of the 761 cases described 34 (or 4%) were fatal. Of the 20 most commonly reported drugs only ~46% were cardiovascular drugs (Class I, III and IV), the remainder were non-cardiovascular (antibiotics, antihistamines, antipsychotics etc.; Darpo, 2001). Interestingly, over this time period 41 cases (1 fatal) of terfenadine TdP reports were named from 10,047 (or 0.41%) reported ADR for this drug. So, there *is no* reliable "gold standard".

As an alternative, when events are rare, some investigators have attempted to classify large numbers of drugs into a small number (5-7) of distinct classes, reflecting a subjective ranking of perceived risk. Already one can appreciate this process is fraught with uncertainty since it lacks precision and (probably also) accuracy. Indeed, the literature carries examples of variable approaches used to rank the relative risk of different drugs. The Arizona Center for Education and Research on Therapeutics (CERT) website (www.qtdrugs.org or www.torsades.org) provides a regularly updated list of drugs (~132 as of April 2008) that can prolong QT (a subjective biomarker for TdP liability). Risk is categorized using integration of an international medical registry of drug induced arrhythmias, case reports, FDA drug labels and data from pre-clinical, clinical and epidemiological studies by an expert committee of advisors for agents known to cause TdP. Drugs are then assigned as those with a possible risk, those to be avoided in congenital long QT syndrome patients and drugs unlikely to cause TdP unless other risk factors are present. This is not precise. Thus, to use even this approach in order to attempt to validate a model is hazardous.

In summary, when an adverse event is a concern (because it is potentially lethal or debilitating), even if its occurrence is rare, and when the drugs known to have a liability for the event have only a low liability, then it is almost impossible to validate any preclinical test (owing to a lack of precision and accuracy concerning the rank order of liability of the template drugs).

To make matters worse, if a potentially lethal adverse event is actually rather rare, even among the drugs with a known liability, then the putative test model will either have a similar low rate of adverse event making it impossible to use (if the event rate in humans is 1 in 1000 it means that many thousands of tests would be required to detect the adverse event liability in the model), or it will need to me "modified" to exaggerate the drug's adverse even rate. The obvious drawback here is that if the model exaggerates adverse event liability then how reliable (precise and accurate) would be the rank order of liabilities of the range of positive and negative controls (the template drugs) in a validation test? Moreover, in an exaggerated liability model there are likely to be false positives.

Clearly there is no ideal approach to safety testing for rare but serious events. This area remains the most problematic in Safety Pharmacology and has generated numerous publications in recent years (Yamaguchi *et al.* 2003; Hamlin *et al.*, 2004; Thomsen *et al.*, 2004; Valentin *et al.*, 2004; Lawrence *et al.*, 2006; Liu *et al.*, 2006; Kagstrom *et al.*, 2007). In this context, validation of the predictive value of Safety Pharmacology models is an evolving understanding of the relationship between clinical adverse effects and our integrated pre-clinical screening tools.

How might we move forward? Let us again consider drug discovery. The best models in discovery are validated retrospectively when they can be shown to have had a key role in advancing a drug into clinical use. In discovery a model can be perfectly valid even if it provides only partial information (i.e. accurately detects one class of effective drug but not another – e.g., rat hearts are effective for detecting class 1 antiarrhythmic activity (Farkas & Curtis 2002) but not class III antiarrhythmic activity (Rees & Curtis 1996)). Thus, in safety we cannot

necessarily expect validation of the core battery tests. However, safety and discovery differ with respect to the perceived need for validation of models, and in terms of the influence of badly validated models on drug development. Thus, in discovery, if a model gives regular false positives it is quickly abandoned once the first products of the model fail in man due to lack of effectiveness. If the model regularly gives false negatives then there will be no products to test in man, and the model will eventually be abandoned and superseded by another. *Model competition* is inherent in academia and industry, e.g., in antiarrhythmic drug discovery researchers have had species issues (dog vs. cat vs. rat vs. pig); conscious vs. anesthetized vs. isolated heart preparations; myocardial ischemia vs. infarction vs. reperfusion vs. programmed electrical stimulation (PES), with continuous re-appraisal of models (Bellemin-Baurreau et al., 1994; Billman, 2006; Botting et al., 1985; Chung et al 1993; Curtis, 1998; Hamlin, 2007; Johnston et al., 1983). As yet there has been little equivalent assessment in Safety Pharmacology. It is critical that the core battery tests be properly validated by showing they are the best among the possible options in terms of avoidance of false positives and false negatives.

What are the processes that drive new Safety Pharmacology model development and the processes used to "accept" a model? The answer to this question is not satisfactory in terms of science, since the overriding guiding principle is pragmatism. This is exemplified (once again) by reference to TdP liability testing. HTS screening for drug block of the potassium current I_{Kr} (so-called "hERG screening" since the gene, hERG, encodes the channel mediating I_{Kr}) is commonplace in Industry even though it is known to generate either false positives, false negatives or highly variable potency values (IC_{50}) compared to voltage-clamp methods for channel inhibition (Zheng et al., 2004; Sorota et al., 2005; Murphy et al., 2006; Slack et al., 2006). It is used because it is quick and relatively inexpensive, once established in-house. Its use is justified because the frequency of false negatives compared to compound throughput in this crude HTS is considered to be as low as inconsequential. Moreover, on the other hand, the

possibility of a false positive in an inexpensive crude HTS screen is a trivial concern in comparison with not using the screen and ending up with a candidate that has a real adverse effect liability which is detected only much later in drug development when, for example (TdP again), *in vivo* dog telemetry studies are (now routinely) undertaken. Indeed, companies today are likely to avoid what they regard as an I_{Kr} binding pharmacophore in early stage synthesis of new chemical entities. This pragmatic approach is understandable, but it should be remembered that this is a gamble and is not validated in that we do not know how many potentially useful drugs are lost by this process. It has been argued by Hondeghem that crude screens for I_{Kr} block may result in truly valuable agents being lost to medicine (Shah and Hondeghem, 2005). Certainly if a drug is intended for life extension in aggressive carcinoma then it would seem ludicrously inappropriate to discard a potentially useful drug just because it blocks I_{Kr}.

The decision on whether a drug candidate should progress to the next level in the discovery process is one of the most important in Industry. Progression is driven by discovery outcomes (i.e., outcomes in studies focused on potential therapeutic effectiveness) and is halted either because there is a loss of signal (lack of benefit in a disease model, for example, meaning that progression stops owing to efficacy issues) or because of the emergence of an adverse effect signal (meaning that progression may stop owing to safety issues) (see Figure 2). An adverse effect signal may not necessarily end progression, but it will certainly slow it. For a drug that is late in the candidate selection process there is reluctance for an adverse effect signal to be used to terminate progression without a proper scientific interrogation of the signal. This means a safety signal for a “mature” drug in preclinical development is likely to trigger new mechanistic-based studies. These are likely to involve a step-up in perceived clinical relevance (e.g., if the signal was in anaesthetized acutely prepared rodents the follow up studies may be conducted in conscious canines or primates with telemetry). This means a step-up in cost and time. The integrated risk assessment (or evaluation of all non-clinical

study results from the core battery studies including findings obtained from follow-up studies as well as other relevant information including pharmacodynamics, tissue distribution and drug interaction studies) will therefore take into account money already spent and the likely return if the NCE becomes a drug (i.e., whether or not the follow up studies fail to reiterate the adverse effect signal).

If a surrogate endpoint is used as the decision-making safety signal, false positive and false negative results may inform false decision-making. For example, using the QT interval in the ECG as a surrogate biomarker gives a false positive for ranolazine and a false negative for disopyramide (Shah, 2008). There is no absolute threshold for decision making with any of the available surrogate biomarkers. The burden is on the development team (discovery and safety pharmacologists working together) to establish criteria for decision making (see Figure 2). However, if the emphasis is placed on safety signals over effectiveness signals (as is inevitable), errors are unavoidable. For example, while a drug candidate in the small molecule category with IC_{50} for blocking I_{Kr} above 1 mM can usually be considered safe to pursue in early stage pre-clinical development, application of this pragmatic threshold would have lead to development discontinuation of valuable candidates such as amiodarone (Lin *et al.*, 2005). The overall cost of pre-clinical development for a single drug (US$2 to US$10 millions) is trivial compared with clinical trial costs (US$100 to US$800 million; NCI 2007) but a large number of candidates that enter pre-clinical screening programs will be "killed" prior to reaching the FIH milestone. This situation is increasing the weight on resource allocation at an early stage to maximize the output of the drug development pipeline.

Molecule size is an important determinant of the type of safety signal likely to be detected. Thus, drug-induced acute QT prolongation is much more common among small molecules owing to the QT-prolonging mechanism, which commonly requires drug access to discrete molecular targets, specific amino acid residues that form the drug binding site located within the central cavity of the

hERG channel (Sanguinetti & Mitcheson, 2005; Kamiya et al., 2006). In contrast, large molecules such as proteins, peptides or monoclonal antibodies (biologics) that may be too large to affect ion channels (Vargas et al., 2008) may evoke adverse hemodynamic effects owing to actions on more readily accessible targets in the vascular space. A trend for the "pipeline" to contain an increasing number of large molecules has been seen in the past few years (Marafino & Pugsley, 2003) leading to an adjustment of drug screening paradigms and Safety Pharmacology studies based on the type of molecule. Thus, while a respiratory Safety Pharmacology "hit" is rarely the signal for small molecule drug discontinuation, it is increasingly recognized as the signal for large molecules intended for repeated administration, owing to a propensity for sensitization and allergic reactions (Murphy & Joran, 1992).

Species sensitivity should also be considered in the interpretation of Safety Pharmacology study findings. Drug or vehicle administration in dogs occasionally leads to histamine release with associated cardiovascular changes (Eschalier *et al.,* 1988; Masini et al., 1985). This phenomenon is known to be species-specific and has relatively limited clinical relevance. Pretreatment with antihistamine drugs such as diphenhydramine and cimetidine (Kien *et al.*, 1992) or measurement of histamine plasma concentration are determined in non-GLP Safety Pharmacology or mechanistic toxicology studies to confirm histamine mediated effects.

Getting the balance right, therefore, is the big challenge, and different companies take different stances on this. Again, the decision making is informed by an awareness of a paradox. Thus, in preclinical drug discovery the possibility (from studies using disease models or biomarker models of possibly dubious validity) that a drug may be of benefit informs the regulatory process to take an optimistic stance. In safety, the possibility (from preclinical studies of dubious validity) that a drug may be unsafe, informs the regulatory process to take a pessimistic stance. While the latter might mean the delay or preclusion of useful drugs entering clinical use, it might also mean the prevention of harmful drugs doing likewise. The task for scientific Safety Pharmacology is to provide better

evidence to direct the decision making. By this means the present guidelines might be expected to evolve to become less vague.

4. The detail of Safety Pharmacology studies

From the most recent survey of industry practices (conducted by the Safety Pharmacology Society from late 2007-early 2008; Lindgren et al., 2008), we can provide the following summary of the survey regarding issues related to the "frontloading" of Safety Pharmacology studies. "Frontloading" defines a safety study that is conducted with a compound *prior* to its selection as a drug candidate for continued development. According to the survey, ~78% of safety pharmacologists responding conduct such frontloaded studies. Both Discovery (~51%) and Drug Development (~49%) research centers share the responsibility for conducting these studies.

When such safety studies are partitioned and examined (Lindgren et al., 2008) as to whether they are frontloaded or not, all survey responders reported frontloading cardiovascular safety studies (~69% during lead optimization prior to candidate selection). CNS studies are also almost always frontloaded (by ~63% of responders prior to candidate selection). Interestingly, but not surprisingly, frontloading of respiratory studies is low (only 28%) while ancillary organs (such as the gastrointestinal (GI) tract and kidneys) were generally not frontloaded (only ~21% of responders frontload such safety studies). Note that such studies are not usually conducted according to GLP standards.

Of all the studies that can be conducted during this phase of drug development, the hERG assay appears to be frontloaded by all the survey responders. Approximately 60% of respondents require mandatory hERG testing in order to proceed with a development candidate (Lindgren et al., 2008). Of the plethora of available methodologies that can be used to determine drug effect on hERG channels the majority use the following test systems: automated HTS patch clamp (~84%); ligand binding studies (~38%); non-automated patch clamp

(~34%) and Rubidium efflux studies (~9%). Rodent CV and ECG studies along with many methods used to evaluate drug effects on ventricular repolarization such as the action potential duration are frontloaded.

The FDA has recently made abuse liability assessment a mandatory part of the development phases of the submission process for all new CNS-active drug products (see comprehensive reviews by Ator & Griffiths, 2003; Balser & Bigelow, 2003). Abuse dependence liability studies are required under the USCA Title 21, Chapter 13, Controlled Substances Act, as amended February 15, 1996, §811(c), and (f) and is maintained in full accordance with the National Institutes of Mental Health's Methods and Welfare Considerations in Behavioral Research with Animals (Morrison et al., 2002). A pre-clinical abuse liability testing guidance document was recently approved for use by the EMEA (EMEA/CHMP/SWP, 2006); however, such an equivalent guideline is only in draft stage in the US, under the auspices of the Controlled Substance Staff (CSS). The CSS provides expertise to the FDA and CDER divisions in assessing drugs for abuse liability and fulfills this unique role within the FDA under the authority of the Controlled Substances Act (CSA) of 1970. In Canada a clinical testing abuse liability guidance document is also in review (Health Canada, 2006). Many in industry may not be fully aware of the new regulations requiring abuse liability assessments [as originally established according to the FDA Food Drug & Cosmetic Act (FD & C, 1938) and the Control Substances Act (CSA, 1970) which determines, labels and schedules abuse potential]. However, since approval by the EMEA in 2006 numerous scientific and procedural challenges have yet to be fully resolved. Sponsors will need to select appropriate pre-clinical *in vitro* (binding, functional) and *in vivo* (neuropharmacological, behavioral) models based upon the pharmacological nature of the test compound and the onus will be placed upon pharmaceutical companies to "build a case" for appropriate assessment, in consultation with regional regulatory authorities. In the US, numerous agencies are involved in drug scheduling (FDA, CSS, NIDA, DEA), but as with safety studies, the FDA will require *more* testing rather than less and

will, if directed by clinical adverse effect concerns, rely upon pre-clinical models for clarification of mechanisms responsible for the adverse effect. The best characterized, validated and predictable pre-clinical models are used for Schedule I drugs (high abuse potential drugs with no accepted medical use such as lysergic acid diethylamide) and II (high abuse potential drugs with an accepted medical use such as morphine); however, their applicability to "weaker" compounds may not be appropriate such as drugs with Schedule 4 (low abuse potential drugs such as diazepam) or no Schedule effects. One important issue that was introduced resulting in much debate and controversy was that the EMEA regulatory authorities consider that "behavioural pharmacology studies for investigating dependence potential…should be conducted under in compliance to GLP to the greatest extent possible" (EMEA/CHMP/SWP, 2006). Thus, GLP conditions are required in Japan, expected by the EU (according to guidance) and preferred by the FDA. However scientists in Safety/Toxicology areas tend to have limited experience with these models since pre-clinical testing has historically been assessed using behavioral pharmacology models (Ator & Griffiths, 2003). Since only a limited number of CROs conduct pre-clinical abuse liability studies there is an additional concern about the potential for delay in drug package submissions; most studies are conducted at academic institutions who do not comply fully with GLP creating potential discord. Numerous other issues of concern include debate regarding choice of species (rats or non-human primates) – the FDA position is unclear (but more reliance is placed upon primate data) while Japanese regulators prefers non-human primates and the EU recommends avoidance of primates and advocates use of the rat. The FDA, in accordance with the EMEA, will likely suggest that abuse liability potential be characterized over a dose range, specifically up to doses that occur to several fold above the expected clinical exposure (therapeutic) range. The clinical route of drug administration is preferred as with safety/toxicology studies; however, most self administration behavioral study methods require the use of intravenous formulation necessitating development of toxicology and pharmacokinetic information before conduct of

abuse liability studies. Therefore, a better integration process is needed between pre-clinical and clinical studies to provide an adequate "integrated risk assessment" regarding abuse liability potential; pre-clinical data should be used to focus clinical investigations and aid in identification of clinical comparator compounds.

The EMEA guidance document recommends a two-tiered strategy regarding abuse liability. The first tier pharmacology studies involve an assessment of the nature of the compound. Information regarding chemical similarity to known drugs of abuse, whether the mechanism of action is similar to compounds known to have abuse liability potential and data from receptor binding studies, are all early signals for such a potential liability requiring subsequent evaluation. *In vitro* binding and functional cellular studies that are conducted as a part of early development can provide signals for possible dependence potential. Additional functional assays measuring neurotransmitter release and second messenger activity may also be conducted. In vivo neuropharmacological models including microdialysis, neurotransmitter turnover, antinociception and locomotor activity may be used (Johanson, 1990). Combined, these first tier pharmacology studies should aid in elucidation of the compound profile and mechanism of action and establish the degree of elaboration of assessment needed to establish the dependence potential.

A second tier behavioral pharmacology assessment is necessary if these initial signals suggest dependence potential and insufficient information is available to define dependence potential. Numerous animal models have been developed to assess the potential for development of drug abuse liability. Specific selection of an appropriate animal model should be based upon the pharmacological profile constructed (see above). A complete dose-response profile using multiple study endpoints (including motor and cognitive function) should be conducted and parent and metabolites considered. Clinical route of administration and appropriate animal species must be used in animal models (EMEA/CHMP/SWP, 2006; Weerts et al., 2007; Feltenstein & See, 2008) that

include physical dependence (drug withdrawal), reinforcing properties (self-administration), discriminative effects (drug discrimination) and tolerance.

5. The emergence of scientific Safety Pharmacology

Although the practice of Safety Pharmacology is dictated principally by regulatory need, its development as a scientific discipline is informed by the same issues as any other biological science that requires the use of animals, namely the issues that inform appraisal of the extent to which the data sets are relevant to humans. By this we mean everything from whether the human molecular target is expressed in the chosen animal and whether the animal's basic physiology and biochemistry is sufficiently similar to that of man, through to the bioassay characteristics of the animal disease model and its cost effectiveness. However, there are distinct differences between safety and discovery pharmacology in the way these issues are treated, partly alluded to earlier. Safety Pharmacology is a discipline whose external role is simply to provide an integrated assessment of data that addresses risk and determines whether a drug will not likely be unsafe in man. In science one can never prove a negative and yet proving a negative is the bottom line in Safety Pharmacology. This has affected the evolution of the discipline.

The key issue to consider in this regard, given that the best test bed for human safety is a human test bed (Phase 1 testing) is: How much *preclinical* Safety Pharmacology is necessary? Presently the guidance (ICH S7A; US FDA, 2001) is moderately explicit, but the suggestions for ancillary studies are almost open-ended. Thus, individuals conducting Safety Pharmacology studies are actually shaping the guidance in an ongoing manner by virtue of the nature of the data they generate and the nature of its relationship with the eventual clinical outcome. Thus, if the preclinical novel-type ancillary data on a new drug is accepted by regulators, its validity will presumably be assessed later by consideration of how the drug fared in man from a safety perspective. However,

this process of ongoing validation will proceed only if there is *scrutiny* and *publication* of findings. This means that there is an onus on Industry and CROs to publish their Safety Pharmacology data. Indeed we hope that this will become mandatory.

In discovery, historically there has been a very meager documentation in the literature of exactly what preclinical tests and preclinical thinking was involved in the generation (from the first idea through the preclinical screening and testing for potential effectiveness) of a commercially successful drug (note that there is an obvious reluctance to divulge thinking/serendipity because of the potential for competitors developing similar drugs). If the same holds sway with regard to Safety Pharmacology in terms of the ideas behind and the development and validation of whatever methods a company has successfully used to selectively extract potentially unsafe drugs (as is likely) then it is difficult to imagine how the process of validation will proceed in any sort of systematic fashion. Safety pharmacologists need to publish on their emerging battery of HTS safety screens, not only to reveal their validity but also to publicly display the company's safety screening prowess. This will add to credibility when presenting a drug for consideration that is claimed to be safe.

Another key issue is the relationship between preclinical Safety Pharmacology and Phase 1 clinical studies. Here it is important to acknowledge the regrettable clinician concept that preclinical (animal) Safety Pharmacology studies are minimally useful or predictive. Once again, the issue of drug induced TdP provides a good basis for elaborating this important point. For an example of this presumption, in a recent authoritative book on cardiac safety of non-cardiac drugs edited by respected clinicians (Morganroth and Gussack, 2005) only 4 out of 18 chapters focused on relevant preclinical aspects (and of those, one chapter discussed molecular aspects of ion channels and another was concerned with pharmacogenomics). The vast majority of chapters were concerned with the minutiae of recording and identifying subtle indicators of TdP liability in human subjects (from phase 1 assessment of small QT interval and QT shape changes to

"thorough QT" evaluation). This focus on determining whether a horse has bolted from the barn or is about to bolt would surely be better dealt with by ensuring the barn door had been locked in the first place (i.e., by ensuring that *preclinical* methods are sufficient to block any progression of an unsafe drug to the clinical setting).

Nevertheless, it is undeniable (and quite proper) that once reliable human data has been generated this renders all preclinical data redundant. Pre-clinical Safety Pharmacology models are constantly evolving and improving under the pressure of clinical trial findings. This is illustrated by the recent findings with sunitinib (the first multi-targeted receptor tyrosine kinase inhibitor approved simultaneously for the treatment of renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumor; Demetri et al., 2006) in which a cardiovascular adverse effect liability (heart failure) was not detected in initial Safety Pharmacology studies (Khakoo *et al.*, 2008).

Interestingly, pre-clinical model validation that is not tailored to clinical results has only a minor impact on industry practices. As an example, the lack of the transient outward potassium current (I_{to}) in minipigs (Mow *et al.*, 2008) has not triggered major concerns for the use of this species for *in vivo* QT evaluations. The physiological and Safety Pharmacology role of I_{to} has been extensively characterized in various species including humans (Patel *et al.*, 2005) and, even though I_{to} contributes to ventricular repolarization, its block has not been associated with significant arrhythmogenic potential in humans. In other words, the fact that pigs have no I_{to} has not stopped this species from being used for QT testing, because there is no positive data to show that selective I_{to} blockers have no TdP liability. Thus although the pig will become a pariah if it fails to pick up QT widening by I_{to} block for a drug that is later found to have a TdP liability in humans, the absence of a "hit" in this case means the pig is presently acceptable. This is pragmatism, and this is an important reality of Safety Pharmacology where the inability to predict a human response is usually needed before the industry and regulators correct the tools that are accepted. Again this is similar to jurisprudence

where similar logical disposition is granted in relation to historical precedent, experience and judgment, all of which have a role to play in decision making. The problem with this is the consequence it has for preclinical Safety Pharmacology.

When a decision is to be made whether to take a new drug into clinical studies, the decision makers and clinical trial designers are required to know that the drug they hope they can show to be effective will also be safe. Thus, the clinical development attitude to Safety Pharmacology tends to reduce to a request for a simple yes/no answer. Thus, the bottom line in preclinical Safety Pharmacology is to generate a provisional integrated risk assessment that may be contemplated by individuals in charge of clinical development, and also to provide advice concerning whether the drug is likely to be sufficiently safe to warrant the start of clinical investigation. In other words: "will it be safe?" Indeed, often the question asked by the next level of management is even more demanding: "is it safe?" Thus, preclinical Safety Pharmacology involves an integrated risk assessment but the need for a risk:benefit calculation requires an unequivocal assessment of risk. This is very challenging.

Given these considerations it is understandable that those in charge of clinical development require clear guidance (yes or no) and even then will wish to rely on clinical data when managing development of the drug. Indeed, (back to TdP liability once more) the concept of "Thorough QT" assessment (TQT) in humans has emerged in recent years (U.S. FDA, 2005). "Thorough QT" assessment derives from the ICH E14 guidance document (Darpo et al., 2006) that provides recommendations concerning the design, conduct, analysis and interpretation of *clinical* studies to assess the potential of a drug to delay cardiac repolarization (Shah, 2005; 2008). Such a clinical trial is applicable to both new drugs with systemic bioavailability but also to approved drugs where there may be a change in dose or route of administration (resulting in an increase in exposure) or a change in patient population (US FDA, 2005b). Similarly, such a study may be triggered in response to the pharmacological class to which the drug being

developed belongs where there may be an association with QT/QTc interval prolongation or TdP during post marketing surveillance (US FDA, 2005b). A "thorough QT" study is extremely expensive to conduct (US$3-5 million), and, since it measures only QT (a putative surrogate biomarker for TdP risk) and not TdP itself, is not necessarily predictive of TdP liability. This (the cost and the uncertainty of human biomarker data sets) is one further reason why preclinical safety assessment should be inclined to take a safety-first attitude to "hits" in safety screens.

As noted earlier, this is no different in qualitative terms from the drug discovery position, whereby clinical development will proceed on the basis of a yes/no judgment about likely effectiveness. However, once again, the stakes are different between discovery and Safety Pharmacology. If a drug fails due to lack of effectiveness, the apparent preclinical false positive will not kill off further preclinical discovery efforts. On the other hand, when a drug fails due to adverse effects in man the consequences for preclinical development are catastrophic. In the cardiac arrhythmia suppression trial (CAST) and survival with oral D-sotalol trial (SWORD) trials, two drugs intended to treat ventricular arrhythmias were found to *evoke* ventricular arrhythmias and kill patients (Weiss et al., 1999; Pratt et al., 1998). In the preclinical studies conducted at the time, although the types of proarrhythmia studies that may be used today were not undertaken, there was nevertheless an apparent failure in adequate safety testing since the proarrhythmic liability of these compounds went undetected. The consequences were that, not only did the preclinical disease models used to detect potential ventricular arrhythmia effectiveness fall into disrepute, but also the world's pharmaceutical drug development programs for treatment of ventricular arrhythmias were abandoned owing to lack of faith by the pharmaceutical industry in preclinical models in this area. This is catastrophic because 30-40% of adults today will die from ventricular arrhythmias for which there are no adequate prophylaxis (one of the largest untapped markets in the drug world). So the stakes for inadequate Safety Pharmacology are much higher than the stakes for flawed discovery

(effectiveness) pharmacology. The upshot is that the scope and extent of preclinical Safety Pharmacology data sets necessary to support a claim that a drug is safe is ever growing. Moreover if, among a large set of preclinical Safety Pharmacology data that shows a lack of safety risk there is one subset that can be interpreted as hinting at the possibility that the drug may be unsafe, a great deal of notice is taken by the regulatory authorities. The upshot is that better confidence is required for preclinical Safety Pharmacology method validity. To achieve this will require a better and more generally accepted methodology for *validation* of Safety Pharmacology approaches.

6. The principles of Safety Pharmacology and the unmet needs

When there are a large number of drugs that have precise and known relative liabilities for producing common and frequent minor adverse effects it is a simple matter to validate preclinical models using the human template of responses to positive and negative controls. The challenge in Safety Pharmacology is dealing with rare events of a life threatening nature, especially for drugs aimed at treating non life-threatening diseases. Here follows a simple guide. It is not intended to be prescriptive and we invite the community to interrogate it, modify it and challenge it.

- Preclinical Safety Pharmacology models require better validation
- Validation requires a quantitative and accurate human template of liabilities of positive and negative controls with which to compare model data sets
- Validation is not possible for models screening for liabilities that are rare or imprecise with current drugs in humans
- Validation is also not possible for methods for evaluating human-specific biologics (that are antigenic in animals)

- When validation is not possible, especially when the liability in humans is rare but life threatening, the use of surrogate biomarkers is unavoidable
- It must be understood that interpretation of surrogate biomarker data sets is unavoidably subjective
- Preclinical safety testing in a non-validated setting must therefore be regarded as non-scientific whereby yes:no judgments will remain subjective in the absence of true validation of the models available
- Scientific validation of safety testing methods remains the goal, however elusive this may seem
- Scientific validation requires blinded randomized testing of drugs known to have and known to not have a liability for the specific adverse effect in humans
- A rank order of liable drugs in humans ("gold standard") is the best template
- It must be acknowledged that a gold standard does not exist for most adverse effect liabilities. This poses a problem
- In the absence of validation it is better to live with false positives than risk the chance of false negatives

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Figure Legends:

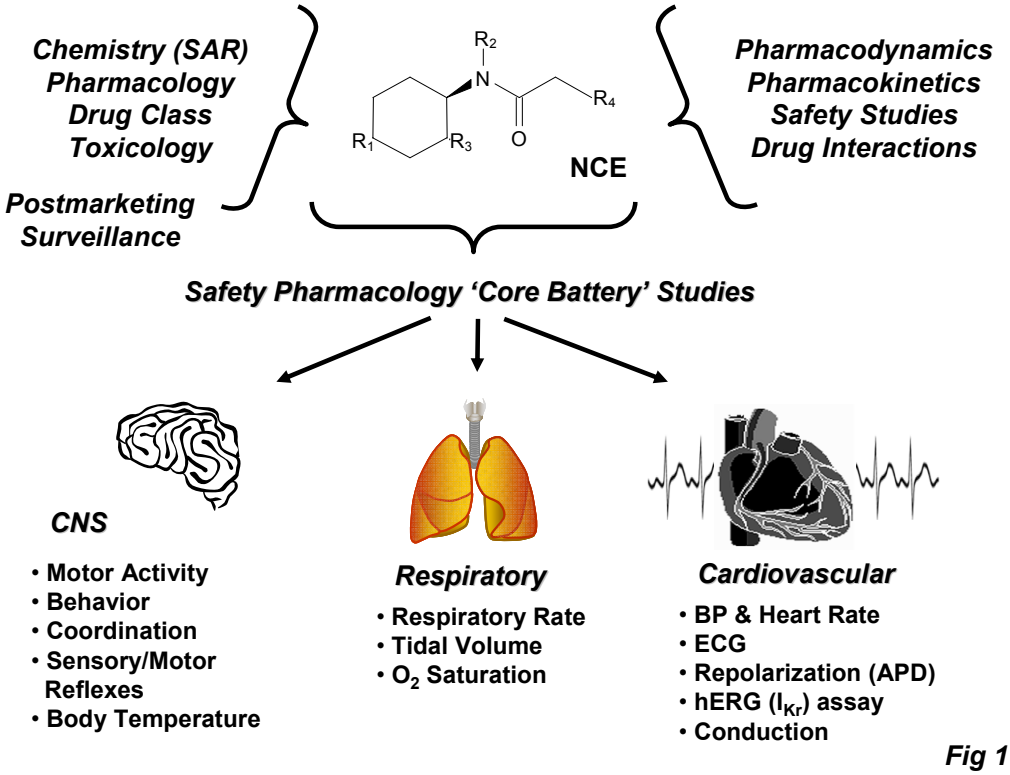

Figure 1

An overview of the multidisciplinary integration required to evaluate the safety profile of a new chemical entity (NCE) in Safety Pharmacology. Consideration is required of the physicochemical and pharmacological nature of the compound, along with Toxicological and associated ADME and pharmacokinetic findings. The lower panel of the figure depicts some of the possible non-clinical methods/parameters recommended for assessment in the safety pharmacology core battery of tests by ICH Guidelines S7A and S7B.

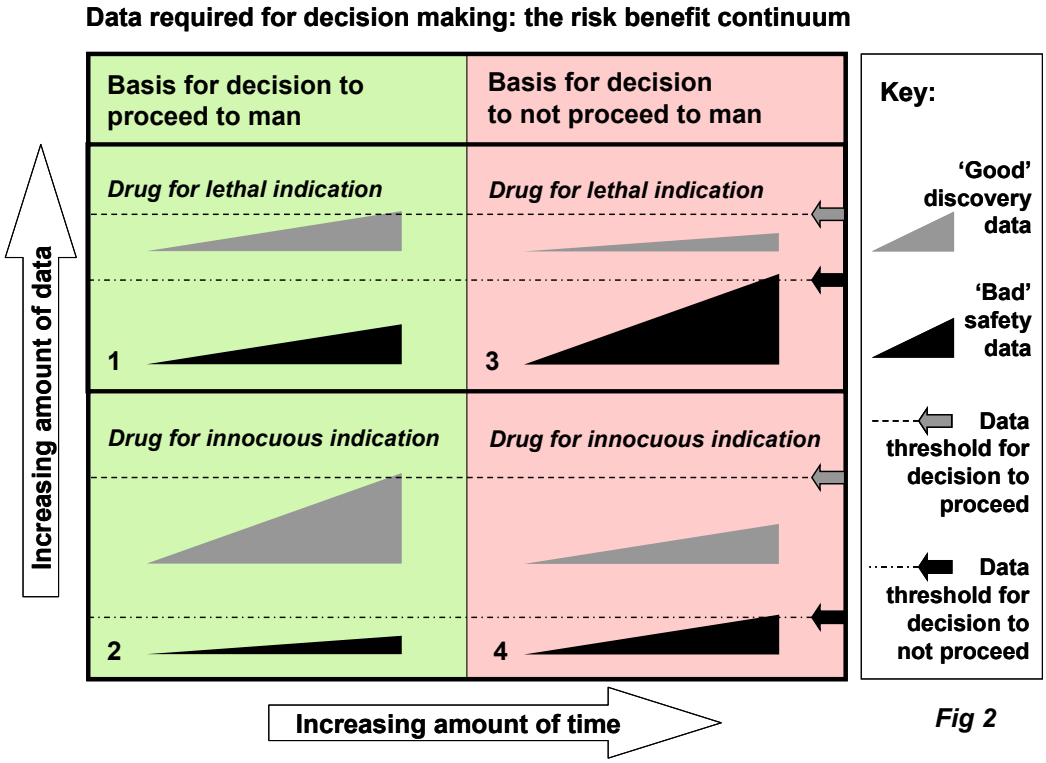

Figure 2

Risk:Benefit Continuum. In this figure, the wedge symbols represent the accumulation of data for positive discovery outcomes and negative safety outcomes. At some point a decision needs to be made to proceed to human studies. This decision is taken when a subjective threshold is met (indicated by arrows and dotted lines). The decision is an integrated risk assessment. The amount of time required to reach the decision is arbitrary since it is the amount of information accumulated that is paramount. The extent data (discovery and safety) necessary and sufficient for a decision is a trade off. Thus, for a drug for a lethal indication, only a moderate amount of positive discovery data is necessary for a decision to proceed provided that a sufficient amount of worrisome ('bad') safety pharmacology data has not accumulated (quadrant labeled '1'). If a threshold level of bad safety data has accumulated before the threshold amount of 'good' discovery data is reached the drug will be killed (quadrant labeled '2'). The same rules apply for a drug for an innocuous indication, except that the threshold amount of necessary positive discovery data is greater (quadrant labeled

‘3’), while the threshold amount of bad safety data sufficient to kill the drug is much less (quadrant 4). This figure emphasizes the role of subjective judgment in decision making, and the influence of disease severity on the risk:benefit calculation.

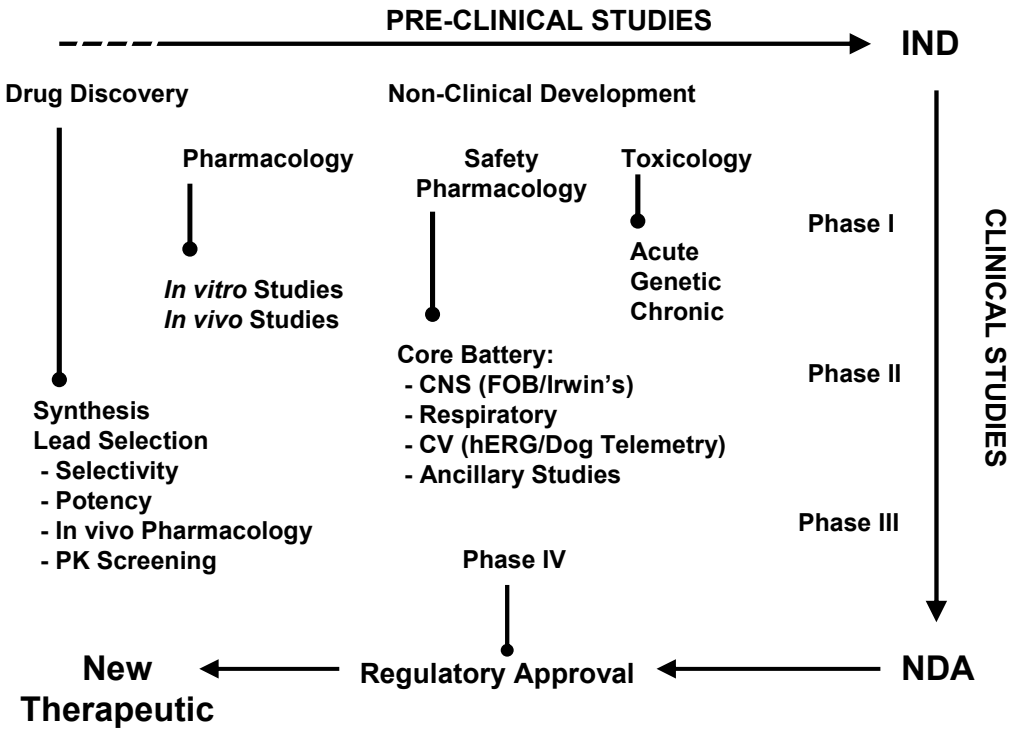

Figure 4

Schematic depicting the complex interaction of pre-clinical scientific disciplines and study models used to characterize the safety profile of a new chemical entity. A non-clinical development program includes data from drug discovery models up through Safety Pharmacology and Toxicology where an investigational new drug application (IND) is filed for a candidate drug. The IND is the means by which a pharmaceutical company obtains regulatory permission (from the FDA) to provide drug to clinical investigators for use in Phase I clinical trials. The FDA reviews the IND application for safety to assure that clinical research subjects will not be subjected to unreasonable risk. The candidate drug then proceeds through multiple clinical trials (Phase I-III) after which an NDA or New

Drug Appliation is made to regulatory authories. In this document drug sponsors propose that the FDA approve a new pharmaceutical for sale and marketing. The goals of the NDA are to provide enough information to permit FDA reviewers to establish whether the drug is safe and effective for its proposed indication

Table 1 - Non-clinical methods recommended for use in the safety pharmacology core battery of tests by ICH Guidelines S7A and S7B.

SAFETY PHARMACOLOGY CORE BATTERY	MEASURED VARIABLES
Central Nervous System (Modified) Irwin's Test Functional Observation Battery (FOB)	Coordination, Body Temperature, Behavior, Neuromuscular, Sensorimotor, Convulsions.
Respiratory System Plethysmography	Respiratory Rate, Tidal Volume, Airway Resistance/Compliance, pO_2
Cardiovascular System QT Interval (Telemetrized Dog) hERG Isolated Purkinje Fibers (Langendorff Isolated Hearts) (Proarrhythmia Models)	Blood Pressure, Heart Rate, ECG, Cardiac Output, Left-Ventricular Pressure, Contractility, TRIaD, hERG IC_{50}
Supplemental Systems* Gastrointestinal Renal/Genitourinary Blood Inflammation Immunological	Intestinal Transit Time, Gastric Emptying and Secretion, Urine Volume, Total Protein, Electrolytes, BUN, Platelet Aggregation, Bleeding Time

TRIaD (triangulation, reverse use dependence and instability) refers to the integrated risk assessment of Luc Hondeghem (Hondeghem et al., 2003). Note that there are a number of additional supplemental systems that could be interrogated, such as the immune system. This Table is not meant to be comprehensive list. Refer to S7A and S7B guidance documents for additional study details.

Un concept essentiel extrait de cet article sur la pharmacologie de sécurité est la nécessité de choisir les modèles d'un plan de développement d'un médicament en fonction de considérations scientifiques (sensibilité, pertinence de l'espèce, etc.) mais aussi en fonction des particularités de la population de patients ciblés. Il est important d'identifier les effets adverses potentiels auxquels cette population est particulièrement susceptible et de choisir des modèles animaux permettant de détecter des changements associés à ceux-ci. Dans le cas de l'infarctus du myocarde, la population de patients est particulièrement susceptible aux effets adverses cardiovasculaires. Cette susceptibilité peut être attribuée à une réduction de la réserve fonctionnelle chez ces patients. Cette population pouvant développer une insuffisance cardiaque chronique (Meris *et al.*, 2009) ou aigüe (Felker *et al.,* 2003) est particulièrement sensible aux effets hémodynamiques hypertenseurs. Les effets délétères d'une hausse de pression artérielle systémique, même minime, ont été démontrés dans une méta-analyse (Lewington *et al.*, 2002) ayant révélé une hausse considérable de la morbidité et de la mortalité suite à un accroissement de la pression artérielle. Le choix des modèles animaux utilisés dans le développement d'un médicament est donc le résultat d'une analyse des biomarqueurs ayant la plus grande valeur prédictive de la sécurité de celui-ci. Il importe de faire une adaptation de la planification des études précliniques selon les études cliniques devant être accomplies pour un traitement donné. Pour un grand nombre de médicaments n'ayant jamais été utilisés chez l'humain, le plan de développement devra faire une démonstration de la sécurité chez des individus sains par l'intermédiaire d'une étude clinique de Phase 1. Cette situation est différente pour les médicaments déjà en utilisation clinique et qui sont évalués pour une indication différente comme c'est le cas pour l'OT. Celle-ci, couramment utilisée en obstétrique, est évaluée pour le traitement de l'infarctus du myocarde dans le cadre de cette thèse. Dans ce cas spécifique, les études précliniques sont axées sur les caractéristiques de la population cible car la sécurité de l'OT chez des individus sains a été démontrée par son utilisation en obstétrique.

Le cheminement rationnel menant au choix des études précliniques souligne l'importance d'une connaissance approfondie des biomarqueurs tel que présenté dans l'article qui suit. La démarche traditionnelle de planification des études précliniques est basée sur une approche par étape et combine les biomarqueurs des différentes disciplines précliniques incluant la pharmacologie de sécurité mais aussi la toxicologie qui s'effectue tôt dans le processus de développement comme l'indique le schéma présenté (Figure 4) dans l'article précédent. Parmi les objectifs de la toxicologie, on retrouve l'identification des organes cibles de toxicité, la détermination de la dose sans effets secondaires adverses (objectif commun avec la pharmacologie de sécurité) (Baldrick, 2008). Tout comme la pharmacologie de sécurité, la toxicologie utilise deux espèces animales incluant un rongeur et un non-rongeur. Les études de toxicologie permettent habituellement de choisir les doses à utiliser pour les études de pharmacologie de sécurité. Dans le cas du développement de l'OT comme thérapie cardiovasculaire, la pharmacologie de sécurité occupe une place prépondérante étant donné que les études de toxicologie ont été complétées et que tel que mentionné plus haut, la sécurité de ce nanopeptide a été largement démontrée chez l'humain. Le choix des biomarqueurs dans nos travaux suit donc une voie non-traditionnelle dans le développement de ce médicament en donnant une place prépondérante aux études de pharmacologie de sécurité qui permettent de sélectionner les doses pour les expérimentations animales suivantes visant à évaluer l'efficacité de ce traitement de l'infarctus du myocarde.

2. LES BIOMARQUEURS CARDIOVASCULAIRES COMME EXEMPLES DE SUCCÈS ET D'ÉCHEC AFIN DE PRÉDIRE LA SÉCURITÉ CHEZ L'HUMAIN

Ce deuxième document de revue de littérature présente la notion de biomarqueur dans le cadre du développement des médicaments. Ce chapitre de livre donne suite à une invitation d'un éditeur pour un livre sur le développement des médicaments (Wiley Interscience, Hoboken, NJ, USA, 2009) et fût écrit par le Docteur Simon Authier avec l'assistance de ces mentors. Le chapitre présente la complexité associée à l'utilisation de modèles visant à prévoir les réponses chez les patients. En plus de donner une image plus approfondie du contexte réglementaire de développement des médicaments, ce chapitre présente l'interdépendance entre les études cliniques et précliniques et les conséquences importantes qui découlent de l'association ou du manque d'association entre les biomarqueurs et les résultats cliniques incluant la mortalité et la morbidité.

Ce chapitre s'inscrit dans la démarche générale de la thèse visant à définir les enjeux et les limitations possibles dans le développement d'un plan d'évaluation de la sécurité d'un médicament. On introduit un sujet épineux pour la communauté pharmaceutique et médicale, celui de la démonstration de l'efficacité des nouveaux médicaments.

En effet, tel que présenté dans le chapitre, l'industrie du développement pharmaceutique connait une stagnation en matière de développement des médicaments. Celle-ci est attribuable, au moins en partie, aux difficultés à démontrer l'efficacité des nouvelles thérapies. La présente thèse, ayant pour objectif l'évaluation de la sécurité mais aussi l'efficacité de l'OT dans le traitement de l'infarctus du myocarde, s'articule autour de cette inextricable utilisation des biomarqueurs comme outils principaux d'évaluation des deux considérations essentielles : la sécurité et de l'efficacité. Comme le suggère Zhao *et al*. (2009), les échecs en développement de médicament doivent forcer un changement de stratégie. Le nouveau paradigme utiliserait les bio-marqueurs

comme assise d'une médecine axée sur l'identification de cibles thérapeutiques pour ensuite mettre en place des nouvelles thérapies pour celles-ci.

Cardiovascular biomarkers as examples of success and failure in predicting safety in humans

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"*Biomarker: A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.*" (National Institute of Health Biomarker Definitions Working Group, 2001).

"*A surrogate endpoint or marker is a laboratory measurement or physical sign that is used in therapeutic trials as a substitute for a clinically meaningful endpoint that is a direct measure of how a patient feels, functions or survives and is expected to predict the effect of the therapy*" (Temple, 1999)

Introduction

Drug discovery begins with a hypothesis. This hypothesis leads to development of a new chemical entity (NCE) that is designed to alter a particular target. However, before clinical efficacy can be tested, proof of concept requires identification of a likelihood of efficacy. This is interrogated using animal models of disease and usually involves use of biomarkers. Biomarkers are also used as surrogate indicators of the state of a disease (for diagnosis and prognosis). Biomarkers (those used for safety assessment purposes as well as efficacy) are therefore cornerstones of medical research. In their central role, biomarkers reflect the presumed link between conceptual understanding of pathophysiological processes and their modulation for therapeutic purposes. In this unique position, biomarkers serve a role in translational medicine where research advances are converted into practical decision algorithms for clinicians or potential new therapies for development by the pharmaceutical industry. While this concept may appear simple, it depends on the predictivity of the biomarker. Given the close relatedness of biomarkers used in drug discovery (non-clinical) and clinical applications, both perspectives will be presented and discussed.

For clinicians, biomarkers are useful for diagnosis, prognosis or to help select and guide treatment for a given patient. In drug development, biomarkers are the scientific endpoints that orient study design and decision-making. In clinical trials, a biomarker may be elevated to the status of 'surrogate endpoint' where it serves as a quantitative measure of the effectiveness or the potential safety of a treatment. A surrogate endpoint is therefore a biomarker which (to be of value) has been validated for a given application as a predictive measure of the true clinical outcome. The controversial process by which a biomarker is validated to become a surrogate marker will be discussed throughout the chapter.

Biomarkers are used in all areas of medicine and many well-known examples include the use of bone density as a surrogate of fractures in osteoporosis (Marshall *et al.* 1996; Cranney *et al*., 2007), CD4 lymphocyte count and quantitative measurement of viral load and proviral DNA in human immunodeficiency virus (HIV) (Antiretroviral Therapy Cohort Collaboration 2008; Torti *et al.*, 2008), or albuminemia in chronic renal disease (Honda *et al.*, 2008).

The interdependency of non-clinical research, clinical trials and therapeutics

To set the basis for discussion, the context of drug development will be outlined. Drug development can be divided into clinical and non-clinical areas of investigation. Clinical investigation includes research activities commensurate with study in healthy volunteers or patients while non-clinical investigation encompasses research activities for drug development that include a diverse spectrum of *in vitro* and *in silico* assays and animal models. Non-clinical research is initiated prior to first in human (FIH) administration (preclinical) but can continue during clinical trials. Non-clinical testing requirements increase as the process proceeds and the NCE advances in development; however, note that data received in clinical trials may also prompt additional non-clinical testing. The different requirements of non-clinical testing for drug development in relation to clinical trials and drug approval will be presented later. Drug development

research and clinical diagnosis share the same final interest - the patient. As a result, a significant proportion of biomarkers used in non-clinical research and early clinical trials are also used in the clinic during the conduct of Phase-3 (i.e., randomized controlled multicenter trials on large patient populations) and 4 (post-marketing safety surveillance) studies.

There is a growing consensus among regulatory authorities and the pharmaceutical industry regarding the need for biomarkers that are common to non-clinical research and clinical trials, and which can eventually be used by clinicians. This is important because non-clinical data can be used to inform clinical decision making when issues of efficacy and (more commonly) safety arise. For example, a biomarker used in non-clinical research to quantify liver toxicity could later be used to monitor signs of possible hepatic toxicity in clinical trials. Thus, common clinical and non-clinical biomarkers offer the promise of greater coherence and ease of decision making. However, for this to work, a question about the process of biomarker development must be addressed.

Biomarkers are developed by integrating the outcomes of clinical trials, clinical research (independent of ongoing evaluation of therapeutic interventions), and non-clinical research. Drugs with known clinical effects are most useful for development and validation of biomarkers in non-clinical models, where non-clinical biomarkers are assessed for their ability to predict a confirmed clinical outcome. Clinical research excluding therapeutic interventions may identify biomarkers for diagnostic or prognostic purposes. However, establishing causality between change to the biomarker and improvement of clinical outcome due to the treatment (drug) is often no more than a ‘leap of faith’ as will be explained later using high-density lipoproteins (HDL) for cardiovascular disease as an example.

Evolution of biomarker development

The development of biomarkers and their role in drug development has evolved rapidly leveraged primarily by advances in life science technologies. At a time when biomarkers carry great hope for medical advances, the history of biomarkers

may have a lesson to teach the modern medical world. Considerable efforts have been invested to characterize the predictive value of each current biomarker as a surrogate endpoint. The iconic Framingham heart study (started in 1947) was amongst the pioneer initiatives of the era of prospective epidemiological clinical studies to undertake systematic investigation of causes of cardiovascular disease and risk factors. Findings from the Framingham study allowed for an assessment of biomarker validation for cardiovascular disease resulting in the utilization and subsequent adoption of serum cholesterol (Oppenheimer 2005) as a primary biomarker for cardiovascular health status. As described in the initial study outline by Meadors (1947): "*this project is designed to study the expression of coronary artery disease in a normal or unselected population and to determine the factors predisposing to the development of the disease through clinical and laboratory examination and long term follow-up of such a group*". While serum cholesterol can be used as a biomarker to establish the general health status of the cardiovascular system, the troponins (T and I), recognized as highly sensitive and specific markers of myocardial damage, illustrate biomarkers that have been developed to provide direct evidence of disease (The Joint European Society of Cardiology/American College of Cardiology Committee, 2000). The use of biomarkers to assess disease risk factors is common in clinical diagnosis (*e.g.* for identification of signs of malignancy by histology of tumor biopsy) but also in clinical trials (*e.g.* from assessment of QT prolongation) whereas the effect of a treatment on a biomarker may be used to predict efficacy or safety (*e.g.* troponin T and I, serum level of low density lipoprotein (LDL) or glomerular filtration rate).

However the use of unvalidated biomarkers (i.e., characteristics that have not yet been determined to be reliable) is potentially hazardous. First, it is recognized that a treatment effect on a surrogate endpoint does not necessarily guarantee correct inference of the treatment effect on the relevant clinical endpoint (Baker & Kramer, 2003; Berger 2004; Prentice, 1989). The concept of biomarkers as risk factors is intimately related to validation of surrogate

endpoints. Surrogate endpoints may include biomarkers which represent direct evidence of disease as illustrated previously with troponins or biomarkers validated as predictive of clinical outcome exemplified by the QT interval that is widely used to assess the risk of the syndrome *torsades de pointes* (TdP). The QT interval, which represents the interval between the start of ventricular depolarization and the end of repolarization, is recognized by the scientific community (Lawrence *et al.*, 2006; Wallis 2007) and regulatory agencies (Anon, 2005) as the most convenient biomarker to assess the risk of developing TdP. Consequently, most of the attention from both the scientific community and regulatory agencies (FDA, EMEA and MHLW) has been directed toward QT prolongation as a risk factor for drug-induced TdP. Sensitivity and specificity limitations of QT prolongation have been reported by several groups (Eckardt *et al.*, 2002; Redfern *et al.*, 2003) and there has been criticism of over-reliance on the use of the QT interval (Hondeghem 2008). Some drugs such as amiodarone and pentobarbital induce QT prolongation but have no reported ability to cause TdP. The use of QT prolongation as a surrogate for TdP in drug development may lead to discontinuation of valuable treatments. On the other hand, while increasing evidence is emerging that QT *shortening* predisposes to ventricular fibrillation (Lu *et al.,* 2008), regulatory guidelines on QT interval have yet to address this possible concern. As a result, a widely accepted and validated biomarker used as risk factor for a potentially fatal condition relies on questionable and evolving foundations. Despite the limitations of QT prolongation, ethical and economical considerations prevent use of the true clinical endpoint (TdP in patients) to assess the safety of new treatments. Considerable efforts have been made to refine and validate the use of QT as a surrogate marker for TdP (Fossa, 2008; Nolan *et al.*, 2006; Ollerstam *et al.* 2007; Pugsley *et al.*, 2008) but this has tended to serve only to emphasize its limitations. Increasingly QT is seen as just one part of an integrated risk assessment (Gintant 2008).

Composite endpoints

As one might expect, given the complex nature of most diseases, if there is no single definitive biomarker for a given condition, a combination of biomarkers is normally used to forecast potential clinical outcome (mortality, morbidity and quality of life). Medicine has always aimed at improving the predictive value of biomarkers. Selection and validation processes have evolved into an organized framework where evidence based medicine (EBM) benefits from meta-analyses of the medical literature, risk-benefit assessment and randomized controlled trials to weigh the predictive value of biomarker combinations. The use of an integrated approach combining more than one biomarker to increase the predictive value is noted in the clinic where prognostic indexes using multiple biomarkers have been developed in major areas of medicine including, but not limited to, cardiology (Meuwissen *et al.*, 2008, Lev 2008), oncology (Rees *et al.*, 2008; Mitry *et al.*, 2004) and neurology (Hansson *et al.*, 2006). Similar approaches have been developed and are now utilized in non-clinical drug development where an integrated risk assessment is used to estimate the sensitivity and specificity of a combination of non-clinical models. Pollard *et al.* (2008) recently assessed the predictive value of a combination of non-clinical assays to quantify TdP risk potentials. When combining *in vitro* (hERG) and *in vivo* QT data, the predictive value to man was reported to be >80%. This may seem high, but in fact it implies a 20% failure rate to predict a potentially life threatening condition. Initiatives to assess the integrated predictive value of drug development screening platforms may have a long-term impact on development of new therapies where selection and timing of the various assays is traditionally based on experience of the research groups rather than on calculated and proven predictive value. With calculated predictive value, one could reassess the construct of a drug development program and optimize timeline and resource allocation.

Among the disciplines using multiple factor analysis, genomic and proteomic approaches offer potentially one of the best hopes for rapid medical progress. With the increasing availability of microarray technologies, genomics

and proteomics give rise to a new paradigm in biomarker development. The quest to establish a relationship between biomarkers and clinical outcome has challenged medical research for the past century. The modern medical world is now faced with a unique challenge: determination of whether the correlations identified have genuine predictive value. Genomics and proteomics are particularly affected by this since although they allow extensive characterization of chromosome and protein expression, so much data is generated by these powerful screening technologies that correlation of one or more biomarker with an experimental variable is inevitable. It then becomes necessary to interrogate the relevance of the correlation. This validation of biomarker candidates is an area of intensive activity. The imperative to confirm the scientific value of "discoveries" from high output microarray technologies requires novel approaches to data analysis supported by bioinformatics (Gormley *et al.*, 2007; Hwang *et al.*, 2008). At bedside, patient genome screening is now commercially available and can be used to evaluate multiple single-nucleotide polymorphisms (SNPs) for disease susceptibility. Genome profiling is a start point for personalized preventive medicine and targeted therapies (Sawyers, 2008). In spite of recognized potential for improved diagnosis, the clinical utility of SNPs remains limited given the lack of controlled clinical trials to evaluate the clinical value of genetic biomarker screening (Hunter *et al.*, 2008).

Considerations for the use of biomarkers: Is validation achievable?

Validation requires value as well as validity. A biomarker is useful only if it is sufficiently accurate *and* a therapeutically useful drug with a good risk/benefit ratio is available (*i.e.,* the biomarker can be used to usefully inform therapeutic decision making). Prostate specific antigen (PSA; also known as kallikrein III or P30 antigen) is a prostate-specific protein that is usually present in minute quantities in the serum of normal men but which is elevated in prostate cancer (Thompson *et al.*, 2004). The measurement of PSA for use in prostate cancer assessment began commercially in1982 – yet more than twenty-five years later,

its value as a routine screening diagnostic tool is still debated (Lin *et al.*, 2008) partly due to a relatively high rate of false negatives (reported to be as high as 27%; Carter, 2004). The psychological consequences of a false positive in the case of a cancer biomarker may outweigh the biomarker's diagnostic value as a routine screening tool.

This emphasizes the key driver in validation: the patient is the primary focus. New generations of biomarkers succeed older generations on the basis of improved sensitivity, specificity or other considerations such as economical and psychological impacts. Thus, lactate dehydrogenase (LDH), a marker of cardiac ischemia (Randall & Jones, 1997) has been largely replaced by troponin T, and troponin T is now challenged by a more sensitive marker (H-FABP) for early detection of myocardial ischemia (Ishii *et al.*, 2005; McCann *et al.*, 2008).

Biomarkers used as surrogate endpoints evolve in a regulated environment where generic validation for a clinical condition takes priority over validation for a given drug or treatment (Katz, 2004). In other words, an ideal validation would demonstrate the predictive value of a surrogate endpoint across different drug classes to treat a given clinical indication (Hughes, 2002). However, even widely accepted biomarkers struggle to comply with such stringent validation requirements, as will be discussed below, but first the regulatory context of biomarker validation will be presented.

Regulatory considerations

In the pharmaceutical industry, guidelines that have been provided by regulatory authorities serve as a start point for non-clinical and clinical study designs. Regulatory approval is usually based on the manifest effects of the treatment on survival or on the symptoms of the disease (Katz, 2004). Approval is based "*...upon a determination that the product has an effect on a clinical endpoint or on a surrogate endpoint that is reasonably likely to predict clinical benefit".* Examples of surrogate endpoints that were accepted by the US Food and Drug Administration (FDA, www.fda.gov) and the European Medicines Agency

(EMEA, http://www.emea.europa.eu) include blood pressure and cholesterol for heart attacks, stroke and death.

Validation of biomarkers is recognized as a process that needs to be independent from drug submission review (Goodsaid & Frueh, 2007). Several initiatives by regulatory authorities have provided for a better understanding of biomarkers and their use in the regulatory approval of investigational drugs. A pilot group structure was developed by the FDA around the Interdisciplinary Pharmacogenomic Review Group (IPRG). Although the primary mission of the IPRG was to establish a scientific and regulatory framework for reviewing genomic data, it was also logical to allow the contributors from this group to aid in the qualification of new biomarkers for the evaluation of new drugs. This subsequent initiative comprised of FDA experts from the Center for Drug Evaluation and Research (CDER), Center for Biologicals Evaluation and Research (CBER), Center for Devices and Radiological Health and National Center for Toxicological Research, and is known as the Biomarker Qualification Review Team. This team is mandated to coordinate the evaluation of data submitted as related to the qualification of novel biomarkers of drug safety using clinical, non-clinical and statistical methodology (Goodseid and Frueh, 2007). Coordination initially involves a review of the intended context of use of the biomarker utilizing data submitted from the applicant. The context of use is a critical component of qualification, since a biomarker may be relevant in more than one particular clinical setting. Thus, once the context of use has been reviewed, the biomarker qualification study strategy is devised and, in an iterative process, a consensus can be sought between the regulatory authority and the sponsor. After completion of the qualification study, the Biomarker Qualification Review Team will decide on approval or rejection of the new biomarker based upon the study results.

While the urge to develop biomarkers is recognized by regulatory authorities, motives to prioritize the task differ from one clinical area to another. In some cases, new technologies provide unmatched opportunities for biomarker

development such as imaging for lung tumors (Petrick *et al.*, 2008). In other cases, such as cardiovascular disease, the use of clinical outcome as a primary endpoint may not be feasible and the use of biomarkers such as LDL-cholesterol level (a biomarker for atherosclerosis) provides a reasonably validated (albeit not wholly definitive) surrogate endpoint.

Other initiatives to facilitate biomarker development include scientific forums such as the *Cardiovascular Biomarkers and Surrogate Endpoints Symposium* held annually since 2003. The symposium includes international experts and FDA representatives in a collaboration designed to address issues surrounding biomarker and surrogate endpoint application for the assessment of cardiovascular disease along with evaluation of the development of novel diagnostics. The symposium has been marked by controversies regarding the value of biomarkers in cardiovascular (and diabetes) drug approvals. In spite of significant efforts to increase the use of biomarkers, the debate on the validity of surrogate endpoints remains unresolved. Rosiglitazone (Avandia®), a thiazolidinedione agonist of the nuclear peroxisome proliferation-activation receptor gamma (PPAR-γ) was fast-tracked and received regulatory approval in 2000 for the treatment of type II diabetes mellitus based on its ability to improve glycemic control by improving insulin sensitivity. However, a meta-analysis revealed that rosiglitazone increases the risk of myocardial ischemic events (Nissen & Wolski, 2007).

Problems arising from the use of unvalidated biomarkers

The use of biomarkers as surrogate endpoints may lead to grossly inaccurate predictions as illustrated by the Cardiac Arrhythmia Suppression Trial (CAST). Prior to conduct of this clinical trial an increased risk for cardiovascular death was believed to correlate with the incidence of ventricular premature beats (VPB) (Bigger, 1984). Encainide and flecainide, class I sodium channel blocking antiarrhythmic drugs, effectively suppressed VPB incidence and were consequently approved by the FDA for life-threatening and symptomatic

ventricular arrhythmias. The CAST trial confirmed a reduction in the incidence of VPBs in survivors of myocardial infarction treated with encainide and flecainide. However, encainide and flecainide also caused a higher rate of lethal ventricular arrhythmia incidence and total mortality (The CAST Investigators, 1989). Paradoxically it was accepted at the time that VPBs were only hypothetically a surrogate for lethal arrhythmias, and the CAST study was in fact billed as an attempt to test the 'cardiac arrhythmia suppression hypothesis' – the notion that if a drug suppresses VPBs acutely in hospital it will improve long term survival (Pratt and Moyé 1995). This illustrates the danger of using an unvalidated surrogate to direct drug discovery.

Aside from the risk of lack of causality with clinical outcome, using a surrogate endpoint in a clinical trial is unlikely to identify off target adverse effects or beneficial effects mediated through biomarker unrelated mechanisms. The presence of unexpected toxicity or lack of efficacy sometime requires large clinical trials to be confirmed. The use of biomarkers to assess efficacy and safety of drugs warrants a conservative position both for regulators and the pharmaceutical (and biopharmaceutical?) industry. In spite of biomarker drawbacks, they remain a key component in drug development and present definitive advantages in clinical trials allowing trials completion in much shorter time (Ledford, 2008).

Biomarkers and drug development stagnation

In March 2004, the US FDA launched the Critical Path Initiative in response to a marked decrease in the number of innovative medical products submitted for regulatory approval. In its report, the FDA highlighted the difficulties of medical product development and called for joint efforts (by both industry and regulators) to improve the use of scientific tools including validated biomarkers. In 2006, the FDA released the Critical Path Opportunities List which provided a list of greatest opportunities in which genomics, proteomics, imaging and bioinformatics were recognized as valuable components of medical/medicinal product development. In

2007, three years after the Critical Path Initiative was launched, only a total of 17 new molecular entities (NMEs) and 2 biologic license applications (BLAs) that were submitted were approved by the US FDA - a record low since 1983 (Hughes, 2008). While the urge to accelerate drug development is keenly felt by all, appropriate solutions still await to enhance approval yet provide a greater degree of drug safety. One explanation for the drug development stagnation resides in the approval of drugs in newer therapeutic areas (i.e., 'first in class' drugs) where regulators require more comprehensive clinical (and pre-clinical) data due to the limited experience with the therapeutic target and developed drugs for that target. Thus, the risk-benefit assessments by regulators become obscured by the increased volume of data on the NCE.

Another possible explanation for drug development stagnation is the absence of biomarkers for predicting short-term efficacy in some degenerative diseases such as osteoarthritis. Osteoarthritis is one of the most prevalent disease in North America with a patient population of 27 million in the United States alone (Zhang & Jordan, 2008), representing 3.5% of home care patients (National Home and Hospice Care Survey, 2002, NCHS, CDC). While disease-modifying drugs such as IL-6 (Sebba, 2008) and TNF-a (Mancarella *et al.*, 2008) blockers are developed for the treatment of rheumatoid arthritis (DMARDs), OA remains a condition with scarce drug candidate development potential (DMOAD). Yet approved treatments are mainly limited to drugs that alleviate pain and symptoms (Sun *et al.*, 2007) but have limited to no impact on actual disease progression.

Drug development in this field suffers from the general perception of limited predictive value of small animal research models (hence low research output). In recent studies, the Pond-Nuki dog model has, however, been proven to be predictive as positive results with diacerhein (Brandt, 2006), doxycycline (Yu *et al*., 1992), and licofelone (Moreau *et al*., 2006) were all reproduced in clinical trials (Dugados *et al*., 2001; Brandt *et al*., 2005; Raynauld *et al*., 2008). When large animal models present the only real predictive *in vivo* model, financial and ethical considerations become a significant burden to high throughput screening

of drug candidates.

The reduction in the progression of structural changes in OA induced by drugs does not always translate into clinical benefits. Absence of symptom improvement with DMOADs could be related to a number of factors such as the selection of patient population (*e.g.* inadequate stage of the disease, limited alteration in biomechanics), slow disease progression and chronic disability rendering the evaluation of the efficacy of treatment difficult, or simply due to the absence of a validated surrogate endpoint. Osteoarthritis involves all synovial joint tissue components and the emphasis on the loss of cartilage in the evaluation of efficacy of new treatments was likely misleading. Osteoarthritis is a painful disorder. Since articular cartilage is not innervated, the link between cartilage and pain severity may be due to other aspects of OA disease pathology, involving a potential role for cartilage. Hence, there is no reason to expect that pain or discomfort would accompany cartilage loss. In contrast, longitudinal magnetic resonance imaging (MRI) studies correlating pain with cartilage loss found no association between the severity of pain and the severity of cartilage morphology alterations (Hunter *et al*., 2006; Torres *et al*., 2006). The above-indicated points have all been barriers to drug development and are consequences of technical and measurement limitations that new approaches will have to overcome. Indeed, a recent study of Raynauld *et al.* (2008) using quantitative MRI (qMRI – Figure 1) allowed to link structural joint benefits and pain relief of a DMOAD (licofelone) in a multi-centre trial of knee OA patients. These results suggest that technology advances are likely to hold part of the solution to the drug development stagnation through refinement of biomarkers. Further expanding on technology driven opportunities, the use of microarray analysis allowed characterization of the early response of adult human articular cartilage to injury. Dell'Accio *et al*. (2008) described the molecular signaling pathways altering chondrocyte biology after injury. Therapeutic targeting of such pathways may improve current protocols of joint surface defect repair and/or prevent the evolution of such lesions into post-traumatic OA. Drug development based on disease targets has become the new

standard in the twenty-first century. Once again, technology advances will be central to achieve high throughout and accelerate drug development.

The future of biomarkers: development of drugs for personalized medicine

Oncology biomarkers have been correlated with disease risk factors (Ankerst *et al.*, 2008), prognosis (Xie & Minna, 2008) and biological properties of tumors (Weigelt *et al.*, 2008). However, they also provide information regarding the susceptibility of tumors to treatment, thus providing a potential additional step toward personalized medicine (van't Veer *et al.*, 2008). Recent developments in pharmacogenetics and pharmacogenomics currently support a shift from generic treatment schemes (*i.e.*, the same treatment for all patients) to therapeutic approaches adapted to individuals. It is now recognized that not only genetics and gender, but also many other factors, influence drug treatment responses. Personalized medicine is developing rapidly with the use of gene profiling to tailor medical care (*e.g.* drug and dose level selection) for patients. The latter is emerging with contributions from the pharmaceutical industry under the auspice of the various regulatory authorities. The CDER created a table of valid pharmacogenomic biomarkers (Anon., 2008), which solidifies translational medicine efforts into a formal reference for clinicians. The table provides comprehensive information including clinical response, risk identification, dose selection guidance, susceptibility, resistance and polymorphic drug targets. The table illustrates the usefulness of biomarkers as practical tools in the clinic. In the pharmaceutical industry, specific disease targets are now the starting point of research teams that will use various biomarkers to measure the effects of drug candidates. Tyrosine kinase inhibitors such as imatinib (Gleevec®) have occupied the top of the "medical billboard" for the past few years where this targeted drug development strategy seems to have yielded remarkable public benefit.

An increase in drug labels containing pharmacogenomic information was noted over the past years (Frueh *et al.*, 2008) but the use of biomarkers in personalized medicine has not achieved its potential (Lesko, 2007; Davies, 2006).

The benefits of pharmacogenomic biomarkers include efficacy screening such as if found with tumor over-expression of human epidermal growth factor receptor 2 (HER2/neu) required for treatment with trastuzumab (Herceptin®). Improved pharmacokinetic analysis is another important advance of pharmacogenomic biomarkers as illustrated by the impact of the cytochrome P450 (CYP) mixed-function oxidase variants on metabolism. Cytochrome P450 2D6 shows the largest phenotypical variability among the CYPs (largely due to genetic polymorphism). Variants have been identified in patients experiencing reduced as well as increased drug metabolism which may undermine efficacy or lead to unexpected toxicity in the absence of appropriate phenotype testing.

An important question is whether available pharmacogenomic tools will become widely used in clinical practice. An evaluation group recently concluded that insufficient information was available to recommend genetic evaluation of CYP isoenzymes in the use of selective serotonin reuptake inhibitors (SSRI) (Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Working Group, 2007). CYP biomarkers included in the FDA table of valid genomic biomarkers are either for informational purposes only or recommended for application; however, none of the current CYP biomarkers are required (*i.e.*, no mandatory testing).

Drug development faces an increase in use of biomarkers that identify subpopulations of patients with specific disease characteristics indicative of either therapeutic efficacy or development of adverse event susceptibility. However the introduction of these biomarkers as routine clinical tools has progressed very slowly. New differences in drug responses between subpopulations of patients are continually being uncovered. Thus, population dynamics (gender, age, and genetic profile) is playing an increasingly important role in drug development. For a number of anesthetic drugs, gender has been shown to significantly influence pharmacodynamic response, potentially altering the depth of anesthesia. Gender is not the only important genetically determined marker of safety or efficacy. Genomic variations influencing response to pharmacotherapy of pain are under

investigation (Stamer & Stüber, 2007). Candidate genes such as (opioid)-receptors, transporters and drug metabolizing enzymes represent major targets of ongoing research aimed to identify associations between genetic profiles and individual drug response (pharmacogenetics). Polymorphisms of cytochrome P450 enzymes (CYP2D6) also influence analgesic properties of codeine, tramadol and tricyclic antidepressants. Blood levels of some NSAIDs are dependent on CYP2C9 activity, whereas opioid-receptor polymorphisms could support differences observed in opioid mediated analgesia and side effects (Chou *et al*., 2006).

What if we could predict preoperatively how patients might respond to common pain medications, anticoagulants and anti-emetics? In the coming decades, opportunities to develop medications that are specifically designed for patients with unique metabolic characteristics, receptor affinity or gene expression will arise. Although far from reality at this time, it is possible that someday we will have a preoperative analysis of buccal cells that will guide with opioid selection to achieve appropriate perioperative pain relief while avoiding side effects such as respiratory depression, nausea, and pruritus. Similarly, tailored interventions based on patient-specific genetic analyses may be possible for hemodynamic management, treatment of perioperative sepsis, and other perioperative issues. For these new biomarkers of safety or efficacy to emerge there will need to be a structured characterization of patient populations including correlation with clinical endpoints. The process will entail several challenges, since obtaining patient information with subsequent storage and analysis of personal medical files raises several serious issues such as confidentiality, discrimination, accuracy and clinical practicality.

Long-term initiatives including prospective epidemiological clinical studies will take decades to yield results but in the mean time, biomarkers have already entered the clinic for specific fields of applications such as oncology. How should the drug development industry react to this situation? The cost of genetic or enzymatic profiling is currently prohibitive and pharmaceutical

companies generally seek a traditional 'one-size-fits-all' dosing. The cost of genetic and metabolic profiling is high currently but should decrease over the next years which will make development of "personalized drugs" potentially more attractive. Efforts to identify drugs targeted for specific patient/disease subpopulation may provide safer or confirmed efficacy. Steering drug development with efficacy or safety biomarkers is an appealing approach to increase chances of success in an industry where very few drug candidates reach bed side. Only the future will tell if research and development efforts based on biomarkers will translate into expected public health benefits.

Biomarkers in regulatory research

Biomarkers are embedded in regulatory approval of new drugs. The passage from non-clinical testing to FIH is vastly supported by a panel of biomarkers aimed to demonstrate safety of the drug candidates (Table 1). Regulatory guidelines for non-clinical testing are generally comparable between the European Union, Japan and United States as a result of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (www.ich.org). The organization provides recommendations (ICH guidelines), which reduce or obviate the need to duplicate testing done for development and approval of new drugs amongst different countries. The ICH guidelines for *in vitro* and *in vivo* preclinical studies allow greater harmonization between countries and a more economical use of human, animal and material resources and the elimination of unnecessary delay in the global development and availability of new medicines. The selection of biomarkers to include in preclinical studies is influenced by primary goals of these studies which include identification of 1) an initial safe dose and subsequent dose escalation schemes in humans, 2) potential target organs for toxicity and reversibility and 3) safety parameters for clinical monitoring (ICH S6, 1997). The process is under strict scrutiny of regulatory agencies as illustrated by U.S. FDA procedures.

To initiate clinical research in the United States an Investigational New Drug Application (IND) must be filed with the FDA (Anon. 2, 2008). The design of animal studies for an IND application is a science-based process where drug characteristics (indication, drug class, administration route, toxicokinetic, mechanism of action, etc.) and regulatory requirements are evaluated to generate a study plan to inform on drug candidate safety. For products intended to treat life-threatening or severely debilitating illnesses the pharmaceutical company may request an early consultation meeting with the FDA. The Pre-IND meeting is intended to reach an agreement on the design of animal studies (including evaluated biomarkers) needed to initiate clinical trials.

As previously mentioned, the study plan endeavors to identify non-clinical biomarkers that could later be used to monitor clinical trials. Animal studies usually include two relevant mammalian species although a single species may be acceptable in some cases (*e.g.* mAb for which the target is only expressed in non human primates). The animal species selection is based on the predictive value of biomarkers in animals to the human response. As an example, the rat is not suitable for assessment of the risk of TdP as the ventricular myocardium in this species lacks the slow inward rectifying potassium (I_{Kr}) current (encoded by the hERG gene) which is the major current responsible for ventricular repolarization in humans.

The relevance of biomarkers in animal safety studies also depends on the nature of the drug candidate as illustrated with biologics. Also referred to as 'biotechnology-derived' pharmaceuticals, biologics are products that originate from characterized cells using various expression systems (mammalian, insect, bacterial or yeast) including cytokines, plasminogen activators, recombinant plasma factors, growth factors, fusion proteins, enzymes, receptors, hormones, and monoclonal antibodies (mAbs) (ICH S6,1997). Biologics are usually larger molecules thought to be less prone to untoward adverse effects than small (NCE) molecules (Zhou, 2007). As a result, *in vitro* hERG assays recommended by the

ICH guideline S7B, are not considered required for large molecules such as monoclonal antibodies (>140,000 Da) (Vargas *et al.*, 2008).

Concerted efforts for development of biomarkers

Significant financial and human resources are invested in research conducted by the pharmaceutical industry for discovery, lead optimization and regulatory submission. Research funded by granting agencies such as the National Institute of Health (NIH) will usually lead to publication of results. However, there are comparably fewer publications from the pharmaceutical industry on the development of biomarkers. More importantly, results from clinical trials that may be used to validate biomarkers are often not published. The US Food and Drug Administration approved 90 new drugs between 1998 and 2000. More than half (515/909, 57%) of the 909 drug trials supporting these approvals remained unpublished five years later (Lee *et al.*, 2008). In 2007, the Food and Drug Administration ruled that the key results from all drug trials must be made publicly available within one year of trial completion or of the drug's approval.

Concerted efforts are required to ensure effective development of biomarkers in drug development. Aligned with this global perspective, The Biomarkers Consortium (www.biomarkersconsortium.org) was created to search for and validate new biomarkers which may then be applied in research for important indications such as diabetes, cancer and heart diseases. The Biomarkers Consortium is a public-private biomedical partnership that includes major stakeholders such as FDA, NIH and The Pharmaceutical Research and Manufacturers of America (www.phrma.org).

Conclusion

Biomarkers will occupy a central role in the development of medicine and will constitute a strategic component of the drug development industry faced with a poor success rate at carrying new therapies from discovery to bed side.

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Table 1

Biomarkers in non-clinical toxicology and safety pharmacology

	Biomarkers	Common Methodology	Comments
General Toxicology	Body weight	Usually evaluated once weekly or more often when adverse effects are noted.	Used as a non specific indicator of toxicity. The growth curve is well characterized in animal species used in toxicology studies especially in rodents. Comparable age and body weight ranges between control and treated groups is essential to ensure validity of statistical analysis on body weight. Correlated with food consumption and possibly dehydration.
	Food consumption	Usually evaluated once daily or weekly. May be evaluated per animal or per cage (all animals from a cage in the same group).	Used as a non specific indicator of toxicity. Decreased food consumption is a frequent sign of drug toxicity. Higher sensitivity in rodents due to the larger number of animals in each group.
	Electrocardiography (PR, PQ, QRS, QT, RR)	External ECG leads (erivation II). Continuous monitoring of unrestrained animals using jacket in large animals such as dogs and non human primates Evaluated once before start of dosing and at study completion (e.g. last week of treatment).	Toxicology studies include higher number of animals than safety pharmacology offering the potential for increased statistical power. Toxicology studies also offer the advantage of repeated dose administration compared with safety pharmacology which is often single dose. Technology advances including continuous non-invasive ECG monitoring using jackets increase the sensitivity of ECG evaluations in toxicology studies.
	Hematology (CBC)	Analysis of plasma: EDTA or Heparin as anti-coagulant. Evaluated once during pre-treatment and at study completion.	Evaluates toxicity of drug candidates on platelets, erythroid and myeloid lineages. Interpretation is often correlated with bone marrow histopathological evaluations. Hematology is a standard component of immunotoxicity testing including lymphocytic, monocytic and polymophonuclear cells.
	Clinical Chemistry	Analysis of serum: Serum separator tubes (SST). Evaluated once during pretreatment and at study completion.	Biomarkers of hepatic (*e.g.* alanine aminotransferase for hepatocellular injury), renal, muscular (or gastrointestinal toxicity. Globulin level is also part of the FDA (but not EMEA) first tier immunotoxicity test.
	Coagulation	Analysis of plasma: Citrate as anti-coagulant. Evaluated once during pretreatment and at study completion.	Activated partial thromboplastin time (APTT) to assess intrinsic coagulation and Prothrombin time (PT) to evaluate extrinsic coagulation.
	Urinalysis Physicochemical (volume, density, color and appearance, pH, glucose, ketones, etc.) and microscopic evaluation (erythrocytes, protein casts, leucocytes, bacteria, etc.).	Urine collection cage to prevent water supply contamination. Evaluated once during pretreatment and at study completion.	Used to detect signs of renal toxicity and often correlated with clinical chemistry results. Casts (cylindruria) is an important component of microscopic evaluations. Hyaline, granular or red cell or epithelial cell casts be indicative of renal parenchyma toxicity.

Table 1 (cont'd)

Biomarkers in non-clinical toxicology and safety pharmacology

	Biomarkers	Common Methodology	Comments
General Toxicology (cont'd)	Systemic arterial pressure (systolic, mean and diastolic pressures, rate)	Indirect sphygmomanometry. Evaluated once during pretreatment and at study completion.	Occasionally included in toxicology studies although telemetry is used as a definitive evaluation of systemic arterial pressure. Recognized limitations including restraining which induces tachycardia and increased arterial blood pressure. Accuracy of diastolic and mean arterial pressures measured with indirect sphygmomanometry have limited value. Indirect systolic arterial pressure measurement considered valid.
	Organ weights	Performed after organs have been trimmed free of fat at necropsy.	Used to identify drug induced atrophy, hypertrophy, edema or other pathophysiological process that alter organ weight. Organ weight results are correlated with gross necropsy and histopathology observation for interpretation of toxicological findings.
Safety Pharmacology	Systemic arterial pressure (systolic, mean and diastolic pressures)	Direct arterial pressure measurement (fluid filled or digital catheter). Implantable radiotelemetry transmitters or anesthetized models instrumented with arterial catheters.	Investigation of arterial pressure effects (hyper or hypotension). A recent interest for chronobiology (*e.g.* lost of circadian rhythm with some hypertensive drug candidates). Drug candidate with hypertensive effects have high risk of adverse effects in patients given the prevalence of hypertension in the human population. Dog is the most frequent model for cardiovascular safety pharmacology. Rat models often used for screening of arterial pressure after repeated dose administration but unsuitable for evaluation of QT prolongation due to lack of HERG channel. Monkeys required based on pharmacological considerations (*e.g.* biologics where the target is only expressed in this species or when the pharmacokinetic in the monkey is the most relevant to humans). Pig cardiomyocytes lack the Ito potassium channel (present in humans) but are still used for QT assessments. Pig, with rapid coagulation time compared to other species, is prone to pressure catheter occlusion when using telemetry implant and chronic monitoring.
	Electrocardiography (ECG)	External, subcutaneous, intracardiac or pericardiac ECG leads. Implantable radiotelemetry transmitters often in derivation II. Precordial derivations (*e.g.* V3) also used to improve T-end detection.	Subcutaneous ECG leads are usually considered adequate in dogs, pigs and rats. Monkeys often present important skeletal muscle artifacts when using subcutaneous ECG leads which favors the use of pericardiac leads.

Table 1 (cont'd)

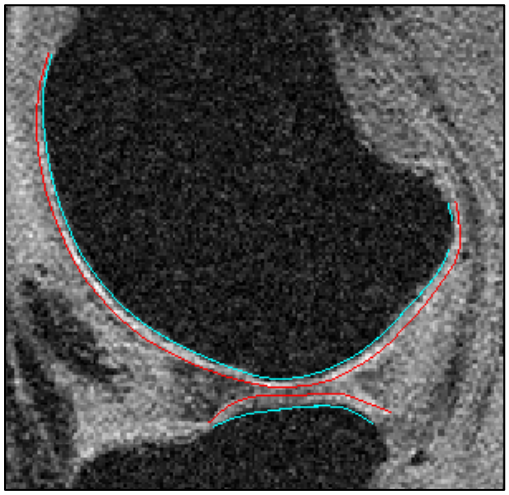
Biomarkers in non-clinical toxicology and safety pharmacology

	Biomarkers	Common Methodology	Comments
Safety Pharmacology (cont'd)	Respiratory function	Head-out plethysmography. Whole body plethysmography. Head chamber with bias flow for monkeys. Mask with pneumotachometer or thoracic bands in dogs.	Rats are often the preferred species for respiratory safety pharmacology for ethical and economical (less testing material required) reasons. Large animal species (such as dogs and monkeys considered for pharmacological reasons (*e.g.* Presence of target).
	Functional Observation Battery	Functional Observation Battery (FOB) to evaluate the nervous system. Includes a number of physiological parameters such as physical activity, body temperature, grip strength, mobility, etc.	Functional observation battery is most often performed in rats but may occasionally require the use of dogs or monkeys based on pharmacological considerations. General toxicity will often translate into decreased activity level which is usually interpreted as a non-specific sign of toxicity.
Supplemental safety pharmacology studies	Left ventricular function	Anesthetized animal models or conscious unrestrained telemetered animals with left ventricular catheter.	dP/dT+, a measure of isovolumetric contraction, is used to assess cardiac contractility. Positive and negative inotropic properties of drugs evaluated.
	Gastrointestinal (gastric secretion, intestinal transport rate, gastric emptying, etc.)	Gastric cannula for secretion volume, phenol red for gastric emptying, charcoal propulsion for intestinal transport, gastric samples for pH.	Gastrointestinal adverse effects often result from pharmacological activity of the test drug but also from chemical or physical properties (*e.g* elevated viscosity potentially leading to paralytic ileus in rats).
	Electroretinography (a-wave, b-wave, oscillatory potentials)	Anesthetized ERG recording using corneal and subpalpebral electrodes and ground. Includes scotopic (dark) and photopic (background light) evaluations with single response and repeated light flashes (flicker response). Advanced protocols include luminance curve response for calculation of retinal sensitivity (Log K)	Used to evaluate functional signs of retinal toxicity as well as reversibility of the effects (*e.g.* amplitude and latency of a-wave and b-wave or attenuation of oscillatory potentials). ERG baseline results in animals are often less variable than humans due to homogeneity of laboratory animal population.
	Electroencephalography (spectral analysis, spike trains, sleep scoring, etc.)	External, subcutaneous or dural EEG leads. Emerging technologies enable computerized EEG monitoring and analysis.	EEG investigations required most of the time as follow-up to adverse clinical signs from toxicology studies. Identification of paroxysmal EEG activity which represents an increased risk for seizure. Useful to detect presence of sedation.

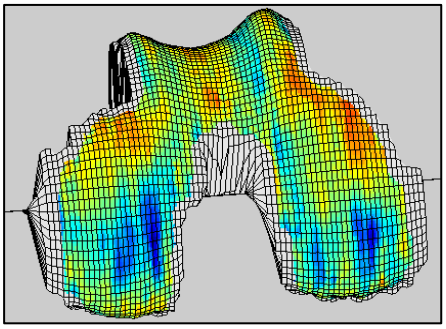
Figure 1. Cartilage quantitative evaluation of knee joint using magnetic resonance imaging (MRI). Pictures are courtesy of ArthroLab / ArthroVision, Montreal (QC), Canada.

From the MRI picture (A), the cartilage is mapped and reconstructed in 3D (B, C). The cartilage mapping upon MRI is useful to track cartilage defects (D), and validate them to macroscopic defects on this dog model (E). Quantitative MRI could also allow computerized measurement of cartilage volumetry (F).

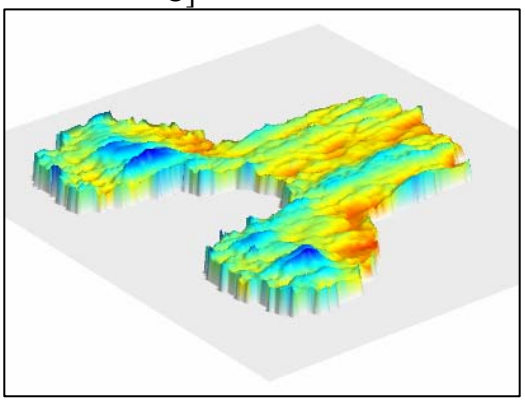
A]



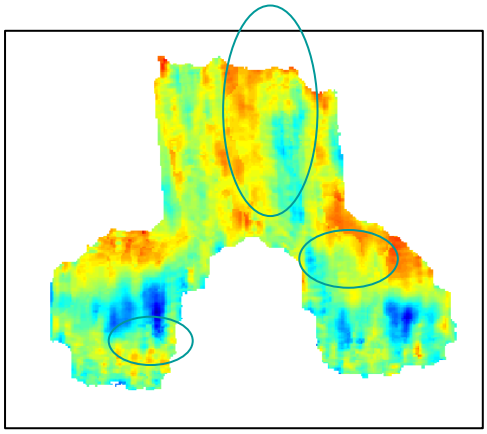
B]



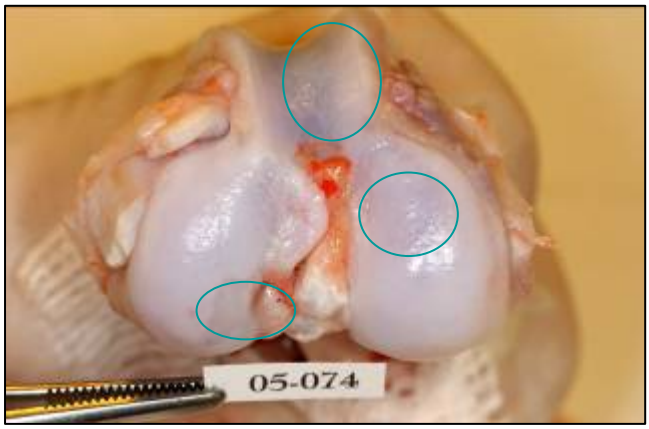
C]



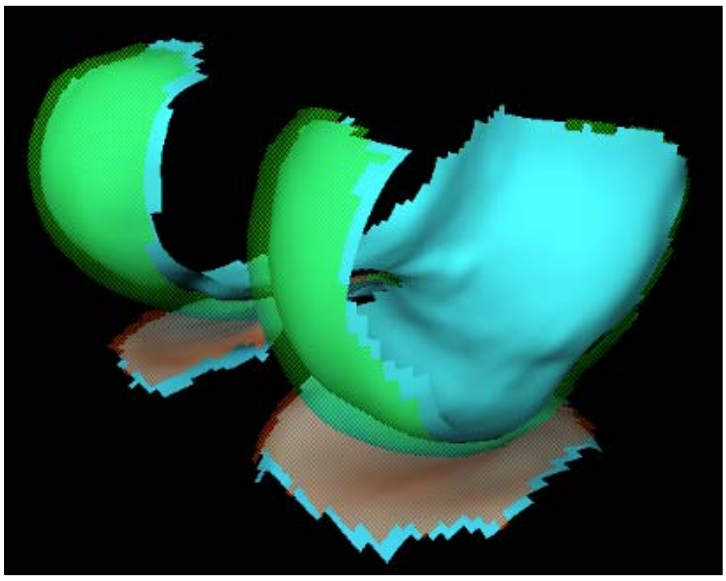
D]



E]



F] Green = femoral cartilage interface; Red = Tibial cartilage interface



Ce chapitre sur les biomarqueurs soulève plusieurs concepts d'importance liés au premier objectif de cette thèse et pave la voie à la deuxième partie de la thèse sur la validation des modèles précliniques. Tel que souligné dans ce chapitre, le biomarqueur idéal doit avoir une valeur prédictive du dénouement clinique. Pour les études cliniques, le lien entre le biomarqueur chez l'humain et le dénouement clinique comporte une étape de substitution de l'élément qu'on cherche à prédire. Tel que décrit dans le chapitre, ce lien entre le biomarqueur chez le patient et le dénouement clinique est loin d'être facile à démontrer. Pour les études précliniques, cette valeur prédictive revêt un caractère particulier en impliquant une étape additionnelle de substitution entre le paramètre mesuré chez l'animal et le dénouement qu'on cherche à prédire chez l'humain. Cette étape supplémentaire réduit la valeur prédictive des études animales (Valentin *et al.,* 2009 ; Valentin & Hammond, 2008) qui demeurent tout de même essentielles au développement des médicaments.

En plus de sa valeur prédictive, le biomarqueur idéal en recherche préclinique devrait pouvoir être transféré chez l'humain lors des études cliniques. Ce transfert des études précliniques aux études cliniques est recherchée à la fois par les agences règlementaires mais aussi par les clinicien(ne)s soucieux(ses) d'assurer un suivi étroit des premières étapes cliniques de développement d'un nouveau médicament. Ce concept de transfert des études précliniques aux études cliniques s'applique à plusieurs biomarqueurs utilisés dans l'évaluation de l'efficacité de l'OT pour le traitement de l'infarctus du myocarde et qui seront présentés dans la troisième et dernière partie de la thèse. Parmi les biomarqueurs du modèle porcin d'infarctus du myocarde pouvant être transférés aux études cliniques, on compte les paramètres d'échocardiographie et le marqueur sérologique de dommage myocardique, la troponine T. Au-delà des avantages de continuité, cette particularité de pouvoir transférer le biomarqueur en clinique ne confirme pas sa valeur prédictive en comparaison des autres biomarqueurs utilisés.

Ceci nous amène à l'essence de la prochaine partie de la thèse, soit la validation des modèles précliniques. Cette prochaine partie visant à mieux définir

les outils du développement des médicaments s'articule entre deux pôles complémentaires que sont les validations scientifique et règlementaire des modèles précliniques.

Chapitre 2 : PHARMACOLOGIE DE SÉCURITÉ CARDIOVASCULAIRE

ÉTUDES EXPÉRIMENTALES

3. PARTIE 1 : VALIDATION NON-PHARMACOLOGIQUE D'UN SYSTÈME D'ENREGISTREMENT DE LA FONCTION CARDIOVASCULAIRE CHEZ LE SINGE CYNOMOLGUS

Avant d'amorcer les évaluations pharmacologiques des effets de l'OT, plusieurs étapes nous ont permis de caractériser les modèles utilisés dans l'évaluation de ce peptide thérapeutique.

Première étape de ce plan de validation des modèles, cet article (*Journal of Pharmacological and Toxicological Methods* 2007, 56 : 115-121) présente la validation non-pharmacologique d'un modèle d'évaluation de l'innocuité des nouveaux médicaments sur la fonction cardiovasculaire. Ces travaux ont permis d'isoler les activités de validation souvent laissés en arrière-plan dans le développement de nouveau médicament. L'article fourni un exemple de mise en application des procédures de validation permettant de confirmer la précision et reproductibilité des mesures pour ensuite mettre en place des procédures normalisées de fonctionnement (PNFs) assurant la bonne utilisation du système lors des essais pharmacologiques précliniques.

Cet article présentera une étape essentielle dans le développement des médicaments, soit la validation non-pharmacologique des modèles. Ce faisant, cet article permet de mieux comprendre les composantes de la validation des modèles de développement des médicaments, le deuxième objectif principal de cette thèse.

Les résultats et discussions inclus dans cet article ont été présentés par le Docteur Simon Authier au Congrès Annuel de la Société Canadienne de l'Assurance Qualité à Montréal en 2007.

A CARDIOVASCULAR MONITORING SYSTEM IN CONSCIOUS CYNOMOLGUS MONKEYS FOR REGULATORY SAFETY PHARMACOLOGY:

PART 1: NON-PHARMACOLOGICAL VALIDATION

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Short title: Cardiovascular telemetry validation in cynomolgus monkey: Part 1

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Suggested section: Original article

ABSTRACT

INTRODUCTION: This project addresses validation study design of test system using a telemetered non-human primate model for cardiovascular safety pharmacology evaluations. METHODS: The validation provided by the supplier evaluated installation (IQ) and operation (OQ) qualifications. This protocol was completed with tests evaluating electronic data management and accuracy and precision of transmitter (n=4) measurements for temperature and pressure criteria with a series of tested values. As part of performance qualification, physical activity (for 24 hours) as well as cardiovascular, ECG (20 complexes for each animal) and systemic arterial blood pressure (SAP, 10 different measures), data were recorded simultaneously from the same animals (n=4) using certified equipment and the telemetry system. Reliability was evaluated over 60 days. RESULTS: The IQ and OQ were completed successfully. The electronic data management was performed successfully. The *ex-vivo* evaluation for temperature and pressure showed high correlation ($R^2 > 0.99$) but with slight pressure shift as expected with this transmitter model. For physical activity, the correlation coefficients were good to excellent with high activity counts but the comparison demonstrated a limited sensitivity of the telemetry system with animal presenting low activity levels. ECG interval measurement using the telemetry software was considered at least equivalent to manual measurement, but with some limitations in the reading of ECG. The comparison between both methods of SAP measurement showed adequate precision ($R^2 = 0.969$) but no accuracy. DISCUSSION: Reference monitoring methods are important to ensure proper test system validation. Monitoring with a reference methodology and the telemetry system is important to evaluate precision and accuracy of the test system. Computerized analysis may lack the capability to analyze ECG complexes with abnormal morphologies. This reinforces the need to have ECG evaluation prior to telemetry implantation along with visual evaluation of ECG tracing at standard speed (e.g. 50 mm/sec) at all time points.

Keywords: Cardiovascular, methods, non-human primates, pharmacology, preclinical, safety

1. Introduction

The importance of telemetric monitoring in biomedical research involving laboratory animals has grown significantly over the past years. This technology is an important tool for collection of a considerable number of physiological parameters including electrocardiograms, electroencephalograms, electromyograms, arterial blood pressures, ventricular blood pressures, locomotor activity, core body temperature and pleural pressures (Brockway *et al.*, 1998). For researchers, especially those in the fields of pharmacology and toxicology, telemetry provides a valuable tool to define the physiological and pathophysiological consequences derived from advanced molecular, cellular, and tissue biology and to predict new compounds effectiveness and safety in humans. Particularly, systemic arterial pressure (SAP), heart rate (HR) and electrocardiography (ECG) should be evaluated as part of preclinical safety pharmacology (US FDA – ICH S7A, 2001, US FDA – ICH S7B, 2005).

If continuous measurement of cardiovascular parameters in experimental animals is essential for cardiovascular research, species selection for safety pharmacology and drug toxicity testing is important to develop new clinically useful pharmaceuticals. Historically, canine models were most frequently used for cardiovascular safety pharmacology studies when large laboratory animals were required. Some considerations for selection of relevant preclinical models have justified sensitivity evaluation and validation of telemetered non-human primate models (Chaves *et al.*, 2006). New methods for correcting QT interval for HR have been recently evaluated in cynomolgus monkey (Holzgrefe *et al.*, 2006). Data obtained in conscious cynomolgus monkeys have also been published recently (Ando *et al.*, 2005; Gauvin *et al.*, 2006). The pharmacokinetics of xenobiotics in humans is closer to non-human primates than to dogs for a number of drugs (Ward and Smith, 2004). *In-vivo* metabolism also supports the use of non-human primate models for various drugs as metabolites may be responsible for adverse cardiovascular effects (Fermini and Fossa, 2003). Metabolism in non-

human primates is closer to human than dog for some drugs (Zuber *et al.*, 2002). Reducing the use of nonhuman primates in research is an important overall objective from both an ethical and resource perspective. However, the high degree of relevance of monkeys for some drugs makes it a judicious model for cardiovascular safety pharmacology evaluation when justified.

In light of studies described in the literature (Schlatter and Zbinden, 1982; Kramer *et al*., 2000; Schierok *et al*., 2000; Harkin *et al*., 2002; Akita *et al*., 2004), it was concluded that the use of radiotelemetry to measure SAP, ECG, HR, body temperature and locomotor activity in rodents has been sufficiently validated (Kramer *et al.*, 2001; Kramer and Remie, 2005; Shiotani *et al.* 2007). Data on circadian rhythms of SAP (Schnell and Wood, 1993; Gerber *et al*., 2000; Gauvin *et al.*, 2006), HR (Schnell and Wood, 1993; Gerber *et al*., 2000, Gauvin *et al.*, 2006), and body temperature (Cilia *et al*., 1998; Palkova *et al*., 1999; Gauvin *et al.*, 2006) in marmoset and cynomolgus monkeys have been reported in the literature. For the latter, most of the data has only been reported in abstracts of annual meetings (Kamenosomo *et al*., 1999; Kito *et al*., 1999), or were using a simple Holter monitor (Macallum and Houston, 1993). Even if non-human primates are used routinely for regulatory cardiovascular safety pharmacology, validation study results are rarely reported in the scientific literature and data available is often limited (Omata *et al.*, 2005; Ando *et al.*, 2005). Validation of electronic technologies to generate electronic records and electronic signatures has been the subject of significant discussions among interested parties following issuance of the final Code of Federal Regulation 21 Part 11 (US FDA – CFR 21 Part 11, 1997). As a result of these concerns, FDA issued a guidance document providing insights on FDA interpretation of part 11 requirements (US FDA – CFR 21 Part 11, 2003). This guidance emphasizes the importance of using a documented risk assessment to determine the extent of system validation.

The aim of the current project is to evaluate, similarly to what was done with rodents, the installation, operation and performance qualification of a telemetry system using a telemetered non-human primate model.

2. Methods

2.1. Hardware and Software

This study evaluated the following components of the Data Science International (DSI, St-Paul, MN, USA) telemetric system:

- Temperature and physical activity transmitters (Model TA10TAD70)
- Pressure, biopotential, temperature and physical activity monitoring transmitters (Model D70-PCT)
- Telemetry receivers (Model RMC-1)
- Telemetry Data Exchange Matrix (Data Exchange Matrix™)
- Ambient Pressure Reference (Model APR1)
- Data acquisition and analysis software (Dataquest A.R.T. ™ Gold Version 3.01)
- Electrocardiogram analysis software (Physiostat™ ECG Analysis 4.01)

The system was installed by the DSI technical staff on a desktop computer (Optiplex GX270™, Dell, North York, ON, Canada). The study was conducted in accordance with the Good Laboratory Practice (GLP) regulations of the United States Food and Drug Administration (21 CFR Part 58 and subsequent amendments). The test plan consisted of the four phases presented below.

2.2. DSI Validation Protocol

First, the supplier (DSI) performed a series of tests (GLP Large Animal Validation Protocol) developed for the validation of the system based on 21 CFR Part 11 and Part 58. The validation protocol performed on-site by the supplier included installation and operation qualifications. Tests performed by the supplier evaluated all recording and analysis functions of the software in the absence of animals. Security checks and audit trails were also tested. Lastly, a transmitter simulator (TSS-1™, DSI) producing a signal with known characteristics was used to validate accuracy of radiowave signal capture.

2.3. Electronic Data Management

The ability to generate accurate and complete copies of electronic records is critical to allow proper interpretation of experimental results and is a requirement of 21 CFR Part 11. Samples of the electronic raw data (6080 values) were printed directly from the telemetry system software. The same electronic data was imported using Microsoft Excel software (Microsoft Canada Co., Mississauga, ON, Canada). Then, the Excel documents were converted into a PDF document (Adobe Acrobat Professional 5.0, San Jose, CA, USA) and printed. The printed raw data was compared to the printed Adobe Acrobat data for quality control of data transfer. A sample of the electronic study data was also transferred to archives (DVD) and integrity was evaluated after 1 week and 6 months.

2.4. Ex-Vivo Evaluation

Ex-vivo tests evaluated transmitter precision and accuracy in controlled conditions without animal. These tests are equivalent to standard curves used with most measurement systems.

2.4.1. Temperature

Four transmitters (TA10TAD70™, DSI) were placed consecutively in a beaker containing water at temperatures *within the physiologically possible range (34ºC to 40ºC)*. The temperature of the water was recorded simultaneously every min for at last 20 min using a calibrated digital thermometer (*016-605™, AMG Medical Inc., Montreal, QC, Canada*) and the telemetry system.

2.4.2. Pressure

Four transmitters (TL11M2-D70-PCT™, DSI) were placed consecutively in a pressure chamber (DSI, St-Paul, MN, USA). The pressure in the chamber was monitored simultaneously using a calibrated manometer (DPM-1b, Biotek®, *Winooski, VT, USA*) and the telemetry system. Pressure increments of 25 mmHg

from 0 to 250 mmHg were applied. For each pressure increment, the values obtained from the manometer and telemetry system were recorded every min for 10 min.

2.5. In-Vivo Evaluation

During the study, the care and use of animals were conducted in accordance with the principles outlined in the current Guide to the Care and Use of Experimental Animals as published by the Canadian Council on Animal Care and the Guide for the Care and Use of Laboratory Animals, a National Research Council publication. LAB Research Inc.'s facility is AAALAC accredited.

Four cynomolgus monkeys (*Macaca fascicularis*), 2 males and 2 females, were used for ECG, SAP and locomotor activity evaluations. At study initiation, monkeys were 2.7 to 5.8 years old and weighed between 2.5 and 4.4 kg. The animal room environment was controlled (temperature 21 ± 3°C, humidity 30-70%, 12 h light, 12 h dark, 10-15 air changes per h) and temperature and relative humidity were monitored continuously. A standard certified commercial primate chow (Certified Primate Diet 2055C™, Harlan Teklad, Madison, WI, USA) was available to each monkey twice daily, except on the day prior to surgery at which time animals were fasted overnight.

2.5.1. Anesthesia

Animals were anesthetized with an intramuscular (IM) injection of acepromazine (Atravet®, 10 mg/ml, 0.14 mg/kg, Ayerst, Guelph, ON, Canada) and ketamine (Ketaset™, 100 mg/ml, 13.6 mg/kg, Ayerst, Guelph, ON, Canada). Lidocaine spray (Lidodan™, 10% w/w, Odan Laboratories Ltd., Pointe-Claire, QC, Canada) was administered onto the arytenoids prior to endotracheal intubation using laryngoscopy. A sterile ophthalmic ointment (Duratears®, Alcon Canada Inc., Mississauga, ON, Canada) was applied to both eyes to prevent drying of the cornea. Animals were then placed on a heating pad and inhaled a mixture of oxygen (O_2) and isoflurane (AErrane™, Baxter Corporation, Mississauga, ON, Canada) with the O_2 flow meter and the vaporizer set approximately at 1.0 L/min,

and 2.5%, respectively. Respiratory rate was maintained between 10 and 12 breaths/min with an inspiratory airway pressure between 18 and 20 cm H_2O using a mechanical ventilator (2002, Hallowell EMC, Pittsfield, Massachusetts, USA). Monitoring during anesthesia included HR and pulsatile hemoglobin saturation in O_2 (VetOx 4404™ pulse oximeter, Heska™, Fribourg, Switzerland). Prophylatic antibiotic therapy (Cefazolin injectable, 25 mg/kg, Novopharm®, Toronto, ON, Canada) was administered by IM injection at least 1 h prior to surgery, at the end of surgery and every 8 h for 24 h post-surgery. Analgesic (buprenorphine, Temgesic™, 0.05 mg/kg, Schering-Plough, Welwyn Garden City, Hertfordshire, United Kingdom) was administered by IM injection upon completion of the surgery and every 8 h for 24 h post-surgery. Fluid therapy was given intravenously (IV) throughout anesthesia using sterile Lactated Ringer's solution at a rate of 10 ml/kg/hr. The surgical site was shaved and aseptically prepared using chlorhexidine gluconate 4% and isopropyl alcohol.

2.5.2. Transmitter implantation

An incision parallel to the *linea alba* was made in the abdominal wall to allow D70-PCT™ (DSI) transmitter placement. The latter was inserted between the abdominal internal oblique and the abdominal transverse muscles through a longitudinal incision in the middle of rectus abdominis muscle. A surgical approach to the right inguinal region gave access to the femoral artery. The monitoring catheter was tunneled subcutaneously to the inguinal incision using a trocar. An arteriotomy was performed and the monitoring catheter was inserted into the femoral artery. After securing the monitoring line with non-absorbable sutures (Polybutester 4-0, Novafil™, Tyco Healthcare Group LP, Norwalk, CT, USA) the surgical sites were flushed with warm sterile saline. Then, skin incisions were made on the left lateral aspect of the thorax just cranial to the last rib and on the right side of the thorax in the area of the thoracic inlet, to allow ECG lead placement (DII). The skin incisions were closed with interrupted intradermal buried sutures using absorbable suture material (Polyglactin 3-0, Vicryl™, Ethicon

Inc., Somerville, New Jersey, USA). This suture pattern ensured that no monkey could easily remove any suture.

An incision was made in the left inguinal region for femoral vein infusion catheter placement. A second incision was made in the interscapular region for catheter exteriorization. The catheter was secured using non-absorbable suture material (Polypropylene 3-0, Prolene™, Ethicon Inc., Somerville, New Jersey, USA). The surgical sites were irrigated with sterile warm saline. A loop of catheter was secured into a s.c. skin pocket made in the inguinal region. The incision was closed with interrupted buried sutures using absorbable material (Polyglactin 3-0, Vicryl™). Following surgery, each animal was equipped with a jacket and tether system. Rectal body temperature was monitored in the post-operative period until animals reached at least 37.0 °C, where they were returned to their cage.

2.5.3. Procedures

2.5.3.1. Physical activity monitoring

Locomotor activity was monitored simultaneously using the telemetry system and an external physical activity monitoring device (Actical™, Mini Mitter®, Bend, OR, USA) attached to the jacket of each cynomolgus monkey for a period of at least 24 h. Upon completion of the monitoring period, the data from the external device was uploaded and compared to the DSI telemetry data. To allow comparison, the sum of activity intensity for each animal, for each hour, obtained from DSI and Actical™ systems were used for statistical analysis.

2.5.3.2. Cardiovascular monitoring

2.5.3.2.1. Electrocardiography

Electrocardiograms from 4 conscious restrained cynomolgus monkeys were recorded simultaneously with the telemetry system and with a medical electrocardiograph (MAC 1200™, GE Medical System IT Inc., Milwaukee, WI,

USA) for a period of at least 1 min on 2 different occasions. The following parameters were evaluated: PQ, PR, QRS, QT, RT, RR, RTp (R to T peak) and Tpe (peak to end) intervals, ST segment, QTd (QT dispersion), and HR (in beats per min, bpm). Parameters from 10 complexes recorded with the MAC 1200™ electrocardiograph, distributed in each one-min period, were manually calculated by a veterinarian. The electrocardiogram ruler used for manual interval measurement had a precision of 0.02 s at 50 mm/s. Parameter averages (2 occasions x 10 complexes x 4 monkeys = 80 values) manually calculated were compared with intervals measured by the software for the same period. All ECG mark placed by the software for each one-min period were reviewed prior to interval computation to ensure adequate complex processing.

2.5.3.2.2. Systemic arterial blood pressures

Blood pressures (systolic, mean and diastolic SAP) were measured using a non-invasive electronic oscillometric sphygmomanometer (Minipack 911™, Pacetech Inc., Clearwater, FL, USA) on 10 different occasions in 2 cynomolgus monkeys. The oscillometric sphygmomanometer pediatric cuff was placed on a thoracic limb (middle third of the humerus) while direct arterial pressure was measured by the telemetry system through the catheter in the femoral artery.

2.6. Statistical Methods

Simple linear regression (SPSS, Chicago, IL, USA) was performed on both *ex-vivo* (temperature and pressure) and *in-vivo* (physical activity, SAP) data. Pearson's correlation coefficient (R^2), slope and origin ordinate were used to evaluate accuracy and detect measurement shifts between measuring methods. Statistical tests were performed at the 0.05 threshold of significance. Mean ± SD data are presented.

2.7. Acceptance Criteria

Complete integrity of electronic data must be preserved to consider electronic data management acceptable. Given the experimental conditions for *ex-vivo* temperature and pressure evaluations (beaker containing water and pressure chamber) and the precision of reference measurement methods (0.1°C and 1 mmHg), a maximal difference of 1°C and 2 mmHg were considered acceptable for temperature and pressure evaluations, respectively. Electrocardiographic analysis performed using the Physiostat™ ECG Analysis version 4.01 software was considered accurate if the maximal difference between computed and manual ECG analysis was less than 0.03 s, which is 1.5 times the precision of the electrocardiographic ruler (0.02 s) used for manual interval measurements. For SAP, correlation was a more important criterion than accuracy between both used methods of measurement. Reliability and consistent intended performance as required by 21 CFR Part 11 were evaluated throughout the study conducted over a 60-day period.

3. Results

3.1 DSI Validation Protocol

Tests performed by the supplier included installation and operation qualifications. Verification that all software recording and analysis functions were correctly operating, as per specifications in the absence of living animals, was done. Security checks and audit trails were also successfully tested. Lastly, the average of 10 cycles of the signal simulator was confirmed to be within the range specified in the acceptance criteria for temperature, HR (pressure chanel), systolic, mean and diastolic pressure, HR (ECG), pulse pressure and activity.

3.2. Electronic Data Management

All numerical values were accurately processed into Adobe Acrobat tables from the electronic raw data. The integrity of the archived electronic raw data was preserved and copies could be generated. The electronic raw data could not be altered by users or the system administrator. The electronic raw data could be deleted from the computer by the administrator but could not be deleted from the archived media (DVD). When the internal clock of the computer was adjusted by the administrator to a previous time where telemetry data was recorded, no overwriting was possible. If the original electronic raw data files had been deleted, new electronic raw data could be retrospectively generated at the exact same time as the study was conducted but this fraud could be detected throught the audit trail, which could not be altered by the administrator.

3.3. Ex-Vivo Evaluation

3.3.1. Temperature

The correlation coefficients of simple linear regressions were between 0.999 and 1 for the 4 transmitters evaluated. Differences between temperatures from the digital thermometer and the telemetry system ranged from -0.3 to 0.3°C. The slopes obtained from the 4 transmitters were between 1.002 and 1.008. Therefore, increments measured by the calibrated digital thermometer were considered accurately quantified by the telemetry system. The values of origin ordinates ranged between -0.200 and -0.456 °C.

3.3.2. Pressure

Correlation coefficients for pressures measured with a calibrated manometer and the telemetry system were maximal ($R^2 = 1.00$) for all transmitters evaluated. The origin ordinate of linear regression functions ranged between -3.648 and -4.342

mmHg, indicating a negative pressure shift (Figure 1). The linear regression slopes ranged between 0.996 and 1.005.

3.4. In-Vivo Evaluation

3.4.1.1. Physical activity monitoring

The correlation coefficients of linear regression ranged from 0.51 to 0.96. Better correlation ($R^2 > 0.9$) was noted for animals with high activity counts. The absence of activity count was frequent with the telemetry system, particularly when the monkeys were less active.

3.4.1.3. Cardiovascular monitoring

3.4.1.3.1. Electrocardiography

The ECGs of 3 of the 4 animals could be analyzed with the computerized telemetered system. Standard and telemetry ECG confirmed that 1 animal had reduced R-wave amplitude with a deep S-wave in derivation II (Figure 2). The Physiostat™ ECG Analysis software was unable to measure intervals and amplitudes of complexes with reduced R-wave amplitude. A fifth cynomolgus monkey with normal QRS complexes (normal R-wave amplitude) was surgically prepared with a telemetry transmitter and was used to complete the validation protocol.

Manual (ECG ruler) and computerized interval measurement for one-min periods revealed a difference of -14.1 to 25.8 ms. Comparison for QTd was not considered relevant given that the precision of the ECG ruler was less than the expected QTd (<0.02s). Mean HR were 181 ± 14 and 178 ± 15 bpm for manual and telemetry measurements, respectively.

3.4.1.3.2. Systemic arterial blood pressures

A correlation coefficient of 0.969 was present between systolic SAP values evaluated with the automated sphygmomanometer and the telemetry system. All values obtained with the sphygmomanometer were lower than those obtained with the telemetry system. The average indirect systolic SAP was 139 mmHg compared to 150 mmHg for the telemetry system.

4. Discussion

This study demonstrated that the installation and operation qualifications of the DSI telemetered cardiovascular monitoring system were successful. Particularly, electronic data transfer, security checks, audit trials, signal tests, electronic signature, archive and measurement precision and accuracy were verified with *ex-vivo* tests that were designed to be representative of standard operating procedures with normal and abnormal (intentional or not) use of the system. The *ex-vivo* evaluation tested, in reference to calibrated monitors, the temperature and the pressure measurement precision and accuracy. Precision is how repeatable the measurements are. Accuracy is how close a value is to the true value. An inaccurate, but precise monitor can be re-calibrated, but an imprecise monitor cannot be improved (Szocik *et al.*, 2005). For both variables, the correlation coefficients of simple linear regressions were excellent. The differences observed for absolute temperature values (from -0.3 to 0.3°C) and for the origin ordinate (between -0.200 and -0.456 °C) were not considered clinically significant. Both reference and tested thermometers were thermistor probes and their comparison showed them to be precise. The pressure increments, measured by the telemetry system and the reference manometer, were considered equivalent but the presence of a possible pressure shift (Figure 1) should be evaluated for telemetry transmitters prior to implantation and once the transmitter is removed at necropsy.

During the *in-vivo* evaluation, the procedures completed the comparative validation of accuracy and precision for physical activity monitoring, ECG and SAP measurements. Briefly, the DSI telemetry device was found to be less efficient than the Actical™ external monitor for locomotor activity monitoring. In contrast, evaluation of ECG and SAP with the comparative methods (manual ECG ruler, and non-invasive oscillometric sphygmomanometry of SAP) showed evidences of limitations compared to the telemetry system. From a surgical perspective, the use of buried suture for skin incision eliminated post-operative complications due to removal of sutures by non-human primates. The suture pattern used in the current study was used in 118 telemetry implantation surgeries in non human primates over the past 2 years in our laboratory with no case of wound dehiscence or infection (Authier *et al*., 2006, unpublished observation).

For the locomotor activity counting, the correlation coefficients were good to excellent with high activity counts. When the monkeys were less active, inducing less signals, activity count absence was more frequent with the DSI telemetry system, which lead to poor correlation coefficient value. With lower activity, the locomotor activity analysis is more limited. This highlights a limited sensitivity for the DSI device, when compared to the Actical™ monitor. Based on these results, interpretation of locomotor activity data from this telemetry system should be done with care and activity should only be used as a complement to interpretation of the data of interest (cardiovascular or core body temperature).

Only monkeys presenting normal ECG complexes including the presence of normal amplitude R-waves should be used for ECG evaluation when using the Physiostat™ ECG Analysis software. An ECG evaluation is required prior to surgical preparation of the animals and all time points evaluated following drug administration should include a sample ECG at standard speed (e.g. 50 mm/sec). Differences observed with intervals measurement are considered mainly attributable to the measurement precision of the ECG ruler (20 ms). Differences could also result from the limited number of complexes evaluated manually (10 complexes) in each one-minute period compared to evaluation of all complexes

with the telemetry system. Given these results, electrocardiographic interval measurement using the Physiostat™ ECG Analysis software is considered at least equivalent to manual measurement when all complexes included in the analysis are reviewed for accuracy of computerized interval mark placement.

Common sources of error during non-invasive oscillometric sphygmomanometry include selection of an inappropriate cuff size. The width of the SAP cuff should be 20% greater than arm diameter or 40% of the limb circumference, and it should be applied snugly after any residual air has been squeezed out. Although a too large cuff will generally work well and produce little underestimating error, the use of cuffs that are too narrow will result in an overestimation of SAP. The American Association for the Advancement of Medical Instrumentation standards require that a monitor records SAP within a 5 ± 8 mm Hg (mean ± SD) prediction error with respect to the reference method (Weiss and Pasch, 1997). Even though automated non-invasive SAP measurement techniques are considered non-invasive and relatively safe, complications have been reported. These morbid events include pain, petechiae and ecchymoses, limb edema, venous stasis and thrombophlebitis, peripheral neuropathy, and even compartment syndrome (Sutin *et al.*, 1996; Weiss and Pasch, 1997). They occur more often after prolonged periods of excessively frequent cuff inflations/ deflations cycling, are visible in critically ill patients, particularly in humans, and are due to trauma or impaired distal limb perfusion. In animals, these complications are rare, but cuff displacement may occur in conscious monkeys. Our results reinforce the need to compare values recorded with the same measuring method to obtain valid conclusions. The widespread application of invasive SAP monitoring in anesthesia and intensive care is also related to the good safety record of the technique. Large clinical investigations confirm the low incidence of long-term complications after distal artery cannulation, in particular, the small risk of distal ischemia, which is estimated at less than 0.1% (Mandel and Dauchot, 1977). Other complications of direct SAP monitoring include hemorrhage, arterial embolization, infection, peripheral neuropathy and most commonly,

misinterpretation of data or misuse of equipment (Mandel and Dauchot, 1977; Cockings *et al.*, 1993).

Interpretation of regulatory guidelines in the preclinical industry was the basis for selection of our validation study design. In response to regulatory guidelines, preclinical laboratories working under GLP regulations will elaborate validation protocols, which will differ to a certain extent from one laboratory to another. Comparison of study design and results among laboratories is often not possible due to the legal environment in which contractual research organizations and pharmaceutical companies evolve. In addition to the design and results that are presented in the current study, several elements were considered important and are presented below. The use of proper reference measurement methods (thermometer, manometer, external physical activity counter, oscillometric sphygmomanometer and certified external ECG) was important to evaluate the accuracy and precision of the system.

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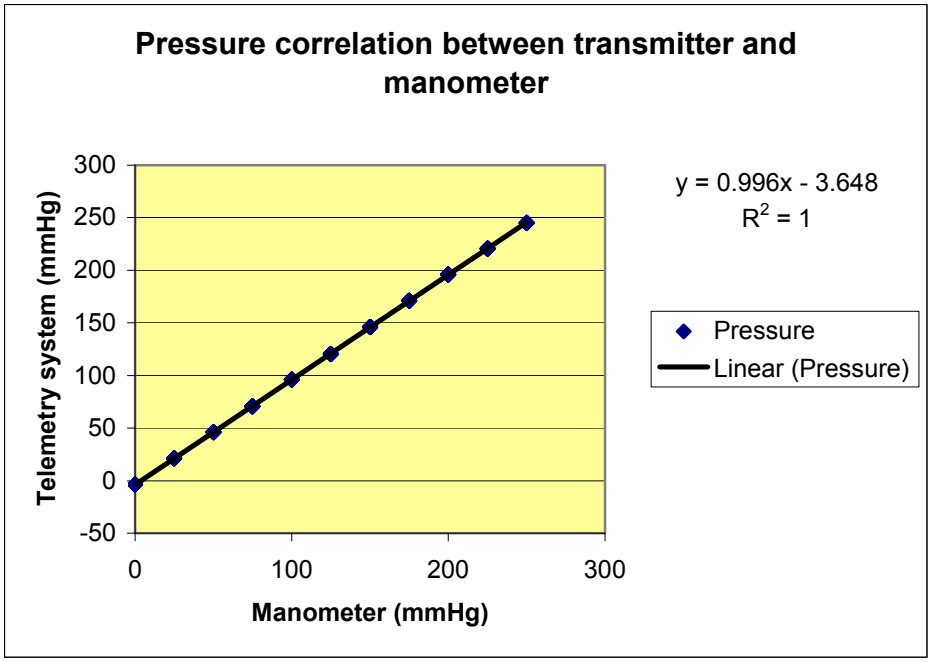
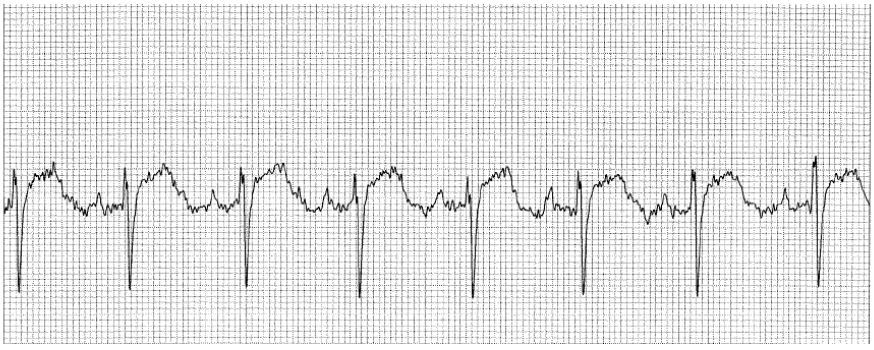
Figures

Fig. 1.



Pressure correlation curve comparing the telemetry system and the reference manometer (*ex-vivo* evaluation).

Fig. 2.



Electrocardiographic tracing of a cynomolgus monkey with reduced R-wave and deep S-wave in derivation II.

L'article précédent introduit la première étape non-pharmacologique de validation des modèles précliniques. La tolérance aux risques d'effets secondaires adverses associés à l'administration de médicaments est fonction 1) du bénéfice, 2) de la sévérité de la condition traitée et 3) des alternatives thérapeutiques disponibles. Dans le cas des patients sains des études cliniques de Phase 1, cette tolérance est limitée étant donné que la santé et ultimement la vie de ceux-ci est en jeu et qu'il n'y a pas de bénéfice sur le plan de la santé pour l'individu. De façon comparable, les traitements développés des conditions bénignes ou pour lesquelles des thérapies efficaces et sécuritaires existent déjà sont assujettis à des exigeances strictes de sécurité. Le cas particulier du traitement de l'infarctus du myocarde exige que les traitements développés présentent un profil de sécurité favorable puisque de nombreux traitements sécuritaires sont disponibles. Cette situation de tolérance limitée aux risques oblige l'industrie pharmaceutique à prendre toutes les précautions nécessaires pour s'assurer de la validité des essais de sécurité des nouveaux traitements. La fiabilité, la sécurité et la précision sont donc des notions primordiales dans la validation des modèles précliniques et mènent à l'adoption d'une approche structurée, voire même contrôlée, permettant de confirmer que le système rencontre les attentes techniques et règlementaires applicables. Tel que souligné dans l'article, la validation des systèmes informatisés précliniques doit se faire en accord avec la règlementation incluse dans le *Code of Federal Regulation 21 Part 11* (U.S. FDA – CFR 21 Part 11, 1997). Cette règlementation nécessite la mise en place de plusieurs fonctionalités et caractéristiques visant à assurer l'authenticité, la sécurité, le suivi des demandes d'accès pour ne nommer que les principales exigeances.

Une fois cette première partie complétée, la validation pharmacologique permet d'évaluer le système en conditions réelles d'utilisation. Suite logique de la validation non-pharmacologique, cette prochaine étape permet aux pharmacologistes d'évaluer la performance du système par l'intermédiaire d'agents pharmacologiques reconnus pour induire des effets secondaires communs.

4. PARTIE 2 : VALIDATION PHARMACOLOGIQUE D'UN SYSTÈME D'ENREGISTREMENT DE LA FONCTION CARDIOVASCULAIRE CHEZ LE SINGE CYNOMOLGUS

Cet article (*Journal of Pharmacological and Toxicological Methods* 2007, 56 : 122-130) présente la validation pharmacologique d'un modèle de pharmacologie de sécurité comportant des analyses hémodynamiques et ECG. Cette deuxième partie de la validation communément appelée validation de la performance (en anglais : *performance qualification* ou *PQ*) permet de confirmer la capacité du modèle à identifier les effets secondaires adverses. Les validations de modèles de pharmacologie de sécurité publiées chez le chien (Gauvin *et al.*, 2006; Ollerstam *et al.,* 2007) mettent en évidence les avantages des suivis pharmacologiques chez des animaux conscients. En effet, le suivi cardiovasculaire continu avec télémétrie permet d'identifier les effets pharmacologiques lorsqu'un délai est présent entre l'administration du composé et la réponse.

L'article qui suit met en relief l'importance de choisir des contrôles positifs altérant tous les paramètres mesurés par le système. Cet article permet d'illustrer la dernière étape de la validation des modèles de développement des médicaments avant leur utilisation pour l'évaluation des médicaments.

Les résultats de cet article ont été présentés par Docteur Simon Authier au 44^{ième} congrès annuel de la *Society of Toxicology* à la Nouvelle-Orléans en Louisiane en 2005 ainsi qu'à la rencontre sur la prolongation de l'intervalle QT de la British Society of Cardiovascular Research Society à Londres en 2007.

CARDIOVASCULAR MONITORING SYSTEM IN CONSCIOUS CYNOMOLGUS MONKEYS FOR REGULATORY SAFETY PHARMACOLOGY:

PART 2: PHARMACOLOGICAL VALIDATION

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Short title: Cardiovascular telemetry validation in cynomolgus monkey: Part 2

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ABSTRACT

INTRODUCTION: This project addresses validation study design of a test system using a telemetered non-human primate model for cardiovascular safety pharmacology evaluations. METHODS: In addition to non-pharmacological validation including installation and operation qualifications, performance qualification (locomotor activity and cardiovascular evaluations) was completed on free-moving cynomolgus monkeys by quantifying the degree of cardiovascular response measured by the telemetric device to various positive control drugs following their intravenous administration. Remifentanil (0.0005, 0.001, 0.002, 0.004, 0.008 and 0.016 mg/kg) was given to induce bradycardia and hypotension. Medetomidine (0.04 mg/kg) was used to induce an initial phase of hypertension followed by hypotension and bradycardia. Esmolol (0.5, 1.0 and 2.0 mg/kg) was used to induce bradycardia. Dopamine (0.002, 0.008, 0.01, 0.02, 0.03 and 0.05 mg/kg/min) was infused over 30 min to induce an increase in arterial and pulse pressures and tachycardia. Amiodarone (0.4, 0.8 and 1.6 mg/kg/min) was infused over 10 min to induce QT interval prolongation. Potassium chloride (0.08 mEq/kg/min) was infused for periods of less than 30 min to induce electrocardiographic changes characteristic of hyperkalemia. Reliability was evaluated over 60 days. RESULTS: Monitoring with a reference methodology and the telemetry system was important to evaluate precision and accuracy of the test system. Positive control drugs induced a wide range of cardiovascular effects with different amplitudes, which were useful to identify the limits of the test system. DISCUSSION: Reference monitoring methods and selection of a battery of positive control drugs are important to ensure proper test system validation. Drugs inducing not only QT prolongation but also positive and negative chronotropic effects, positive and negative systemic arterial pressure changes and ECG morphology alterations were useful to identify test system limitations during performance qualification. ECG data processing at significantly elevated heart rates revealed that all cardiac cycles evaluated by computer should be reviewed by a trained observer.

Keywords: Cardiovascular, methods, non-human primates, pharmacology, preclinical, safety

1. Introduction

Many studies using cynomolgus monkey (*Macaca fascicularis*) are drug trials involving safety profiling, as promulgated by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (US FDA – ICH S7A, 2001, US FDA – ICH S7B, 2004), and particularly specific safety pharmacology evaluations. Studies conducted under the guidance of the ICH are designed to discover and characterize the potential adverse cardiovascular effects of biologically active new chemical entities (NCE) that may present as an unintended consequence of NCE administrations (ICH S7A, ICH S7B). Monkeys are one of the most commonly used preclinical safety species after dogs because of their genetic, cardiovascular and metabolic similarities to humans, but only a few published reports illustrates the usefulness of telemetry in conscious monkeys (Benardeau et al., 2000, Gauvin et al., 2006, Hassimoto & Harada, 2003 and Kaufman & Detweiler, 1999). A recent study (Gauvin et al., 2006) has published a significant dataset on normal values for core body temperature, hemodynamic, and ECG parameters for well-acclimated, freely moving laboratory-housed cynomolgus monkeys using radiotelemetry recording methodologies. This study also highlighted the numerous advantages of using telemetered cardiovascular safety pharmacology devices, such as obtaining physiological measurements from awake and freely moving laboratory animals without introducing either physically or chemically mediated stress or restraint artefacts in the data. Moreover, there has been explicit acknowledgement in ICH and other regional regulatory drug safety testing guidelines that conditions present during routine telemetric monitoring may most closely approximate the normal physiological state of the animal, and therefore safety endpoints evaluated under such conditions may consequently demonstrate the greatest predictive validity to

outcomes of similar testing in humans. Indeed, the authors of this recent publication presented a convincing list of advantages for using remote radio-monitoring in preclinical safety pharmacology. This list of advantages includes a humane, affordable, accurate, readable (without human contact, and as such eliminating a major source of interfering stress), reliable and, for long-term use, without special animal care or maintenance method (Gauvin et al., 2006). Moreover, another recent study tested the rhesus monkey telemetry model as a preclinical predictor of pro-arrhythmic potential (specifically QT interval prolongation) of human pharmaceuticals (Chaves et al., 2006). Limited information is available on precise analysis of ECG (Ohmura et al., 1999; Horii et al., 2002; Hassimoto and Harada, 2003). Particularly, the measurement of corrected QT interval (QTc by Bazett (1920) and/or Fridericia's (1920) correction formula) in cynomolgus monkey appeared to be a useful approach to evaluate the potential cardiotoxicity of histamine H_1 receptor antagonists (Ohmura et al., 1999; Horii et al., 2002). Nicardipine (Ca^{2+} channel blocker) at 30 mg/kg (po) caused sustained hypotension and tachycardia (Horii et al., 2002). Results obtained with various agents have also confirmed the sensitivity of the non-human primate model for non-clinical assessments of the potential for drug-induced QT prolongation (Ando et al., 2005). These recent studies provided convincing arguments showing that these procedures represent the contemporary industry's preferred practices for measuring such cardiovascular parameters under the ICH guidelines, and are amenable to routine use in a variety of other relevant safety/efficacy studies. They also confirmed, in our opinion, the necessity to validate the telemetric device with *ex vivo* and *in vivo* non-pharmacological and pharmacological evaluation of precision, accuracy and repeatability. Even if non-human primates are routinely used for regulatory cardiovascular safety pharmacology, validation study results are rarely reported in scientific literature, and available data is limited. The aim of the current project is to evaluate, similarly to what was done with rodents, the installation, operation and

performance qualification of a telemetry system using a telemetered non-human primate model.

2. Methods

2.1. Hardware and Software

This study evaluated the following components of the Data Science International (DSI, St-Paul, MN, USA) telemetric system:

- Temperature and physical activity transmitters (Model TA10TAD70)
- Pressure, biopotential, temperature and physical activity monitoring transmitters (Model D70-PCT)
- Telemetry receivers (Model RMC-1)
- Telemetry Data Exchange Matrix (Data Exchange Matrix™)
- Ambient Pressure Reference (Model APR1)
- Data acquisition and analysis software (Dataquest A.R.T. ™ Gold Version 3.01)
- Electrocardiogram analysis software (Physiostat™ ECG Analysis 4.01)

The system was installed by the DSI technical staff on a desktop computer (Optiplex GX270™, Dell, North York, ON, Canada). The study was conducted in accordance with the Good Laboratory Practice (GLP) regulations of the United States Food and Drug Administration (21 CFR Part 58 and subsequent amendments). The test plan consisted of the four phases presented below. The serial number of each system components was recorded, verified at each step of validation, and included in the study report.

2.3. In-Vivo Evaluation

During the study, the care and use of animals were conducted in accordance with the principles outlined in the current Guide for the Care and Use of Laboratory Animals, a National Research Council publication (Anon., 1996). LAB Research Inc.'s facility is AAALAC accredited.

Four cynomolgus monkeys (*Macaca fascicularis*), 2 males and 2 females, were used for ECG, systemic arterial blood pressure (SAP) and locomotor activity evaluations. Surgical implantation and anesthesia were performed as described in Part 1 of the current article (Authier et al., 2007). At study initiation, monkeys were 2.7 to 5.8 years old and weighed between 2.5 and 4.4 kg. The animal room environment was controlled (temperature 21 ± 3°C, humidity 30-70%, 12 h light, 12 h dark, 10-15 air changes per h) and temperature and relative humidity were monitored continuously. A standard certified commercial primate chow (Certified Primate Diet 2055C™, Harlan Teklad, Madison, WI, USA) was available to each monkey twice daily, except on the day prior to surgery at which time animals were fasted overnight.

2.3.3. Procedures with drug-induced cardiovascular effects

Six drugs with known cardiovascular effects were administered to 4 telemetered cynomolgus monkeys. This included drugs mainly affecting HR (either decreasing it, remifentanil; medetomidine; and esmolol; or increasing it, dopamine), SAP (either decreasing it, remifentanil; and esmolol; or increasing it, dopamine; or with a biphasic effect, medetomidine) or ECG [QT, QTcV (van de Water), QTcF (Fridericia), QTcB (Bazett), amiodarone; and multiple effects on ECG, potassium chloride]. Most drugs including remifentanil (Ultiva®, Abbott Laboratories Ltd., Vaughan, ON, Canada), naloxone (Sabex, Boucherville, QC, Canada), dopamine (Inotropin™, Bristol-Myers Squibb, Montreal, QC, Canada), esmolol (Brevibloc®, Baxter Corporation, Mississauga, ON, Canada) and amiodarone (Sabex, Boucherville, QC, Canada) were purchased from a local pharmacist (Pierre Dannel, Laval, QC, Canada). Potassium chloride (Hospira Healthcare Corporation, St-Laurent, QC, Canada) and medetomidine (Domitor®, Novartis, Missisauga, ON, Canada) were purchased from a veterinary product distributor (CDMV Inc., St-Hyacinthe, QC, Canada). To minimize artifacts following IV administration, drugs available as commercial solution forms except

for remifentanil that needed to be reconstituted, were given to unrestrained animals equipped with a jacket and tether connected to a continuous infusion line and pump (Table 1). These drugs were mainly selected with regards to their very short elimination half-life (esmolol, remifentanil), and/or the possibility to effectively antagonize their effects (remifentanil, medetomidine, potassium chloride).

2.6. Statistical Methods

Drug-induced effects were evaluated using a two-way ANOVA for repeated measures (SAS, Cary, North Carolina, USA). *A posteriori* contrasts were conducted using Dunnett's test. To facilitate analysis, a period of data collection was selected with regards to observed peak effect following drug administration and compared to a similar data collection period prior to drug administration. For example, short acting cardiovascular effects were observed immediately after remifentanil injection. As a result, statistical analysis was conducted on a 1 min period average starting 1 min after administration, which was compared with the average from 1 min prior to remifentanil administration. Statistical tests were performed at the 0.05 threshold of significance. Mean ± SD data are presented.

2.7. Acceptance Criteria

Identification of hemodynamic and ECG changes following administration of selected drugs with known effects was considered to be an important component of performance qualification of the system. A validation study should be performed over a period of time permitting assessment of the system reliability and consistent intended performance. As required by 21 CFR Part 11, reliability and consistent intended performance were evaluated over a 60-day period, which is considered representative of normal conditions of use for the system.

3. Results

3.1. In-Vivo Evaluation

3.1.1. Drug-induced cardiovascular effects

3.1.1.1. Remifentanil

The telemetry system identified HR and diastolic SAP modifications ($p<0.05$) with the greatest effects observed at 0.016 mg/kg ($p<0.05$) (Table 2). An increased QT interval was identified after the administration of 0.016 mg/kg ($p<0.05$), but QTcV, QTcF and QTcB were not statistically different.

A dose of 0.016 mg/kg produced evidence of electromechanical dissociation in 1 animal. This observation was preceded by sinoatrial node blockade. Naloxone was given as an IV bolus (0.1 mg/kg) to reverse the effect of remifentanil. Upon return to sinusal rhythm, increased T-wave amplitude (Figure 1) and ventricular premature contractions (Figure 2) were noted for this animal.

3.1.1.2. Medetomidine

A biphasic effect of medetomidine on SAP was identified by the telemetry system on all dosing occasions (Table 3). As expected, systolic, diastolic and mean SAPs increased followed by an hypotensive phase associated with a decrease in HR. A statistically significant increase in QT, QTcV and QTcF intervals was noted at 30 min compared to baseline, with some episodic occurrence (noted two times, in one monkey) of sinus arrhythmia (second degree atrioventricular block – Mobitz II) during the first 30 min after injection

3.1.1.3. Esmolol

A significant decrease in systolic, mean and diastolic SAPs combined with a decrease in pulse pressure and HR was identified with the telemetry system. The highest dose (2.0 mg/kg) induced the greatest changes (Table 4), particularly on systolic and mean SAPs.

3.1.1.4. Dopamine

At lower doses (0.002, 0.008 and 0.01 mg/kg/min), dopamine infusion had no apparent effect on cardiovascular monitoring parameters. With a 0.02 mg/kg/min infusion rate, the diastolic SAP showed a decrease without reaching statistical significance but translated in a significant increase in arterial pulse pressure (Table 5). This effect on arterial pulse pressure was also present with an infusion rate of 0.03 mg/kg/min, but it was associated with a return to baseline value for diastolic SAP and an increase in systolic SAP that did not reach statistical significance. With an infusion rate of 0.05 mg/kg/min, the increase in systolic SAP was statistically significant as was the increase in arterial pulse pressure, but the latter showed a statistically lower increase compared to the one observed with the two previous infusion dose rates.

3.1.1.5. Amiodarone

Following administration of 4, 8 and 16 mg/kg (total dose), prolongation of the QT ($p<0.0001$), QTcV ($p<0.0005$), QTcF ($p<0.005$) and QTcB ($p<0.05$) intervals were noted for all animals. QT prolongation observed at 4 (Figure 3) and 8 mg/kg were equally significant, with a maximal 21% increase in QT duration compared to saline. Administration of the highest dose (16 mg/kg) induced an increase in QRS duration (+32%), with an increase in QT (+26.1%) and QTcB (+11%).

3.1.1.6. Potassium chloride

In the first monkey (infusion over a 2 h and 6 min) the ECG showed T-wave peak, widening and slurring of the QRS complex, flattening and loss of P-wave and atrial fibrillation. Early signs of hyperkalemia are presented in Figure 4. Emergency treatments were provided to the animal. Subsequent infusions were limited to 30 min, before all these ECG changes occurred for the other animals to minimize the risk of serious deleterious effects. They were indeed stopped at the first occurrence of ECG signs of hyperkalemia: an increase in T-wave amplitude

($p<0.05$) combined with a decrease in R wave amplitude ($p<0.05$) were noted for all animals.

4. Discussion

Procedures with pharmacological manipulations are critical to the validation protocol most importantly for performance qualification. Arterial pressure can be divided into steady (mean SAP) and pulsatile components (pulse pressure) (Berne & Levy, 1992). Mean SAP is determined by cardiac output and vascular resistance. The pulse pressure component, representing the variation in pressure around the mean, is influenced by left ventricular ejection, large artery stiffness, early pulse wave reflection, and HR. Cardiac output, peripheral vascular resistance and artery stiffness influence systolic SAP. In contrast, diastolic SAP is determined mainly by peripheral vascular resistance and cardiac output.

The predominant and usual effect of opioids on HR is to produce bradycardia resulting from stimulation of the central vagal nucleus. Remifentanil is an ultra short-acting (μ)-opioid agonist that induces bradycardia (Elliott et al., 2000) and hypotension in humans (Schuttler et al., 1997). Conversely, remifentanil IV bolus (0.002 mg/kg) administered to human volunteers resulted in a transient increase in SAP and HR (Glass et al., 1993). Similar dual biphasic changes (increase followed by decrease in tension) with increasing concentrations of fentanyl were reported in basal canine epicardial coronary artery rings (Introna et al. 1995). Although increases in SAP and HR were not significant at similar doses in monkeys (0.002 mg/kg), hypotension was accurately identified at higher doses (Table 2). In dogs, remifentanil produces hemodynamic effects, which include decreases in contractility, cardiac output, HR and SAP (James *et al*., 1992). In our study, a more pronounced effect was observed on HR and SAP at the highest dose tested (*i.e.* 0.016 mg/kg). The fact that the effect was more pronounced on diastolic SAP than on systolic and mean SAPs suggests a possible decrease in peripheral vascular resistance associated to a negative inotropic and chronotropic effect. Indeed, pharmacological studies evaluating alfentanil, fentanyl, and

sufentanil in the dog demonstrated direct peripheral vessel smooth muscle relaxation (White et al., 1990). Direct effects of remifentanil on smooth muscles were also shown on isolated rat tissues (Unlugenc et al., 2003). Based on our data, the same response may be present in cynomolgus monkey. A decrease in HR induces QT interval (Bazett, 1920) physiological prolongation, which was identified by the system after administration of remifentanil at 0.016 mg/kg. As expected, no statistically significant differences were present for QTcV, QTcF, and QTcB. Indeed, opioids may depress cardiac conduction (Fattorini et al., 2003). Other studies have reported that fentanyl slowed atrioventricular node conduction, and prolonged RR interval, atrioventricular node refractory period and Purkinje fiber action potential duration (Royster et al., 1988; Blair et al., 1989). Opioids can also prolong the QT interval (Blair et al., 1987), but the overall effect of opioid anesthesia is anti-arrhythmic (Atlee & Bosnjak, 1990). We observed cardiac brady-arrhythmia and ECG signs of myocardial ischemic suffering, with ventricular premature contractions and increased T-wave amplitude (Somers et al., 2002), in one animal after remifentanil injection at the dose of 0.016 mg/kg.

Medetomidine is a selective alpha$_2$-adrenergic agonist which, following IV injection, produces an initial phase of hypertension followed by hypotension and bradycardia (Pypendop & Verstegen, 1998; Capuano et al. 1999). This biphasic effect of medetomidine on SAP was also observed in our study. However, the degree of bradycardia (about 14% in this study) was 4.5 times lower than the one observed in dogs (63% of decrease) for the same dose (Vainio & Palmu, 1989), and 3 times lower than for rhesus macaques with 0.05 mg/kg IV (40% of decrease) (Capuano et al., 1999). This could be explained by the lower baseline value of HR in our monkeys. Also, we did not observe similar occurrence of sinus arrhythmias (complete sino-atrial block/sinus arrest with junctional escape beats, and infrequent ventricular premature contractions in 10 of 15 tested animals) (Capuano *et al*., 1999). Similarly to dogs, occasional second degree atrioventricular blocks were observed in the first 20 (Vainio & Palmu, 1989) to 30

min after injection in one monkey. In comparison, SAP was initially increased by 11 mmHg on average after IV administration of dexmedetomidine (0.002 mg/kg) to humans (Penttila, J. et al., 2004) compared with an increase of 29 mmHg in cynomolgus monkeys in the current study. Dexmedetomidine being the active molecule in the racemic mixture of medetomidine, we could extrapolate that the potential dose of dexmedetomidine in our study was 0.02 mg/kg. The 10 times higher dose administered to monkeys explains a greater, although not proportional, response magnitude. The effects on SAP were identical to those observed by Capuano et al. (1999) with a lower magnitude but a higher sensitivity related to the direct monitoring of SAP in our monkeys. Medetomidine was not selected as a positive control drug to evaluate QT interval prolongation but its ECG effect has been reported in other species (Kinjavdekar *et al.*, 1999). In our study, we observed significant QTcV and QTcF prolongation but no significant increase in QTcB.

Esmolol is a beta-adrenoceptor blocker reported to be cardioselective ($beta_1$-blocker), with no intrinsic sympathetic activity and low lipophilicity (Harrold et al., 1998). It produces dose-dependant bradycardia (Murthy *et al.*, 1983; Gorczynski *et al.*, 1984) associated to a decrease in velocity of atrioventricular conduction and a decrease in contractility. With larger doses, the relative selectivity to $beta_1$-adrenergic receptors is lost, and $beta_2$-receptors are also blocked, with potential bronchoconstriction, peripheral vasoconstriction, and decreased glycogenolysis (Opie *et al*., 1995). Its extremely short half-life results from its hydrolysis by red blood cell esterase. A significant decrease in systolic, mean and diastolic SAPs combined with a decrease in pulse pressure and HR were identified with the telemetry system but the extent of the effect was limited to 10-15% and only observed at the highest tested dose (2 mg/kg). This is indicative of a relatively good sensitivity of the telemetry system to detect slight but clinically relevant changes as observed with similar doses in humans (Kindler et al., 1996; Sintetos et al., 1987). Moreover, the more important effects observed on systolic and mean SAPs are suggestive of a prominent effect of esmolol on

cardiac output (negative chronotropic and inotropic effects) (Berne & Levy, 1992).

Experimental hemodynamic studies in anesthetized dogs indicate that an IV injection of dopamine (0.001-0.009 mg/kg) induces a slight depressor response associated with a decrease in peripheral vascular resistance, a decrease in renal vascular resistance, an increase in renal blood flow, and an increase in cardiac output (McNay & Goldberg, 1966; Setler *et al*., 1975). The dopaminergic receptors in vascular smooth muscle (DA_1) subserves vasodilatory responses in the renal, visceral, coronary and cerebral beds (Chapman *et al*., 1980; Goldberg & Rajfer, 1985). High dose levels (0.009-0.018 mg/kg) produce pressor responses and a more pronounced increase in myocardial contractile force ($beta_1$–mediated chronotropic and inotropic positive effects) leading to increased systolic, mean and diastolic SAPs (Setler *et al*., 1975; Abdul-Rasool *et al.*, 1987). Widening of pulse pressure is also reported in dogs (Robie & Goldberg, 1975). Considering that the highest dose tested (0.05 mg/kg/min) only induced a statistically significant increase in systolic SAP, it could be hypothesized that this effect was related to a $beta_1$ stimulation. Effects observed at lower doses most likely resulted from a $beta_2$ stimulation, leading to vasodilation, decreased total peripheral vascular resistance and diastolic SAP, as well as increased pulse pressure. In the dog, doses higher than 0.01 mg/kg/min present a risk of alpha-vasoconstrictor effect due to the loss of dose-dependent dopamine receptor selectivity. Based on our results, the vasculature of cynomolgus monkeys appears to be less sensitive than the dog to the pressor effects of dopamine, since the significant increase in systolic SAP (only observed at a dose of 0.05 mg/kg/min) is limited (approximately 10%). In humans, chronotropic and pressor effects of dopamine were significant in larger cohorts (n=16) of healthy volunteer at lower doses (0.003 mg/kg) (Marinac et al., 2001). These effects may also be significant in cynomolgus monkeys when using corresponding group sizes.

As a Class III antiarrhythmic agent, amiodarone selectively prolongs the action potential and refractory period without effect on the resting membrane potential

of the cell. This is clinically expressed as a prolongation of atrioventricular nodal conduction time and an increase in atrial and ventricular refractory periods (Novotny & Adams, 1986). Also, amiodarone induces QT interval prolongation after IV administration (Bertholet *et al.*, 1983) as well as a slight prolongation of the QRS complex (Cascio *et al*., 1988). The telemetered ECG monitoring reported all these effects. This wide range of ECG effects induced by amiodarone is a consequence of interactions with several cellular targets. Amiodarone blocks, the rapid delayed rectifying potassium current (I_{Kr}), which prolongs the action potential and translates into QT interval prolongation. In addition, amiodarone blocks *β*-adrenoreceptors (Charlier, 1970) and several ion channels including calcium current (I_{Ca}) (Nishimura et al., 1989), sodium current (I_{Na}) (Follmer et al., 1987) and several potassium current such as I_{Ks}, I_{to}, I_{K1}, I_{KACh} and I_{KNa} (Yoshida et al., 2002). Despite very interesting pharmacodynamic properties and its popularity as a cardiovascular drug (Siddoway, 2003), amiodarone presents a highly variable pharmacokinetic profile with an elimination half-life averaging 58 days in humans. These characteristics may be considered prohibitive for some validation studies where animals need to be reused in subsequent studies after an appropriate wash-out period. Given these considerations, specific I_{Kr} blockers with shorter half-life such as sotalol (Poirier et al., 1990) or dofetilide (Ollerstam et al., 2006) may be preferred as positive controls for QT prolongation in validation studies.

The infusion of potassium chloride was used to verify the ability of the system to detect ECG morphology changes associated with hyperkalemia. Hyperkalemia lowers the resting membrane potential of excitable cardiac cells and decreases the duration of the myocardial action potential and upstroke velocity. This decreased rate of ventricular depolarization, plus the beginning of repolarization in some areas of the myocardium while other areas are still undergoing depolarization, produces characteristic ECG changes: peaked T- waves, widening of the QRS complex that merges with the T-wave into a sine wave appearing at severely elevated levels, and loss of P-waves (Mattu *et al.*, 2000). The earliest

manifestations of hyperkalemia are narrowing and peaking of the T-wave. Though not diagnostic of hyperkalemia (Somers *et al.*, 2002), T-waves are almost invariably peaked and narrowed when serum potassium levels range between 7 to 9 mEq/L. The telemetered ECG system detected this early change in all monkeys. When serum potassium levels exceed 7 mEq/L, atrial conduction disturbances appear as a decrease in P-wave amplitude and an increase in PR interval. Supraventricular tachycardia, atrial fibrillation, premature ventricular complexes, ventricular fibrillation, or sinus arrest may occur. We did observe in the first cynomolgus monkey (longer infused with the potassium solution) losses of P-waves and atrial conduction disturbances. This is also a beneficial element with regards to the system sensitivity to detect ECG arrhythmias.

Interpretation of regulatory guidelines in the preclinical industry was the basis for selection of our validation study design. In response to regulatory guidelines, preclinical laboratories working under GLP regulations will elaborate validation protocols, which will differ to a certain extent from one laboratory to another. Comparison of study design and results among laboratories is often not possible due to the legal environment in which contractual research organizations and pharmaceutical companies evolve. Based on our results, several elements were considered important for validation of this test system. Validation of both numerical values and ECG tracing in normal and positive control drug treated animals was central to confirm accuracy of this study endpoint. Results from the validation allowed definition of precise experimental conditions in which this test system could operate. As an example, computerized ECG analysis provided accurate interval measurement in saline-treated cynomolgus monkeys. In presence of severely increased HR following drug administration, analysis with the software revealed that, with standard settings, the software was unable to identify individual complexes. This observation prompted the need to have all complexes used for computerized ECG analysis reviewed by trained personnel. In conclusion, this validation study was pivotal to the preparation of standard

operating procedures to ensure that the test system would be used within conditions that ensure accurate and reliable safety pharmacology evaluations. The design of the current validation is considered adequate as an initial validation. More drug responses may be useful to better characterize the sensitivity of the model and more animals would be needed to further assess drug reproducibility.

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Tables

Table 1

Cardiovascular positive control drugs

IV bolus	**Dose level (mg/kg)**
Remifentanil	0.0005
	0.001
	0.002
	0.004
	0.008
	0.016
Medetomidine	0.04
Esmolol	0.5
	1.0
	2.0
IV infusions (duration)	**Dose rate**
Dopamine mg/kg/min (30 min step-infusion)	0.002
	0.008
	0.01
	0.02
	0.03
	0.05
Amiodarone mg/kg/min (10 min)	0.4
	0.8
	1.6
Potassium chloride mEq/kg/min (up to 30 min)	0.08

Table 2
Cardiovascular effects of remifentanil in conscious cynomolgus monkeys (n=4)

Dose level (mg/kg)	Systolic SAP (mmHg)	Mean SAP (mmHg)	Diastolic SAP* (mmHg)	Arterial Pulse Pressure (mmHg)	HR* (beats/min)
0	130 ± 18	111 ± 16	90 ± 13	40 ± 5	159 ± 25
0.0005	131 ± 15	111 ± 14	89 ± 11	43 ± 5	149 ± 39
0.001	133 ± 10	113 ± 8	93 ± 6	40 ± 5	160 ± 31
0.002	137 ± 9	116 ± 9	94 ± 8	44 ± 3	191 ± 20
0.004	135 ± 9	112 ± 8	89 ± 9	46 ± 6	192 ± 20
0.008	130 ± 21	102 ± 16	78 ± 16	52 ± 13	151 ± 56
0.016	110 ± 50	80 ± 41	63 ± 32†	47 ± 19	106 ± 62†

* $p<0.05$ ANOVA; † $p<0.05$ Dunnett's test compared to baseline, analysis of data recorded over 1 min starting 1 min after administration.

Table 3
Cardiovascular effects of medetomidine in conscious cynomolgus monkeys (n=4)

	Time post-Rx (min)		
SAP (mmHg)	Baseline	1	30
Systolic*	118 ± 13	153 ± 12†	96 ± 8
Mean*	100 ± 12	129 ± 12†	83 ± 6†
Diastolic*	84 ± 15	109 ± 28†	69 ± 28
HR (bpm)*	143 ± 39	125 ± 33	95 ± 17†
ECG intervals (s)			
PR	0.103 ± 0.008	0.099 ± 0.018	0.109 ± 0.014
QRS	0.306 ± 0.008	0.305 ± 0.008	0.320 ± 0.010
QT*	0.206 ± 0.048	0.211 ± 0.031	0.275 ± 0.031‡
QTcV (van de Water)*	0.249 ± 0.039	0.253 ± 0.022	0.302 ± 0.021†
QTcF (Fridericia)*	0.269 ± 0.033	0.265 ± 0.024	0.317 ± 0.025†
QTcB (Bazett)	0.308 ± 0.026	0.297 ± 0.026	0.341 ± 0.028
RR*	0.445 ± 0.161	0.517 ± 0.131	0.684 ± 0.206‡

* $p<0.05$ ANOVA; † $p<0.05$ Dunnett's test compared to baseline; ‡ $p<0.01$ Dunnett's test compared to baseline, analysis of data recorded over 1 min at specified time.

Table 4
Cardiovascular effects of esmolol in conscious cynomolgus monkeys (n=4)

Dose level (mg/kg)	Systolic SAP* (mmHg)	Mean SAP* (mmHg)	Diastolic SAP* (mmHg)	Arterial Pulse Pressure* (mmHg)	HR* (beats/min)
0	139 ± 11	121 ± 10	98 ± 9	43 ± 4	194 ± 36
0.5	131 ± 9†	112 ± 8†	92 ± 6	40 ± 4†	172 ± 34
1.0	132 ± 9	113 ± 7	94 ± 5	37 ± 5	168 ± 31
2.0	124 ± 7‡	107 ± 5‡	87 ± 5†	37 ± 4†	167 ± 17†

* p<0.05 ANOVA; † p<0.05 Dunnett's test compared to baseline; ‡ p<0.01 Dunnett's test compared to baseline, analysis of data recorded over 1 min immediately following administration.

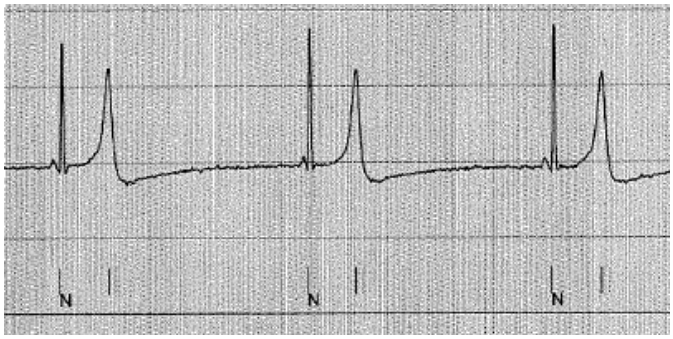
Table 5
Cardiovascular effects of dopamine infusion in conscious cynomolgus monkeys (n=4)

Dose level (mg/kg/min)	Systolic SAP* (mmHg)	Mean SAP (mmHg)	Diastolic SAP (mmHg)	Arterial Pulse Pressure (mmHg)	HR (beats/min)
0	137 ± 18	118 ± 15	98 ± 12	39 ± 8	173 ± 28
0.002	137 ± 15	118 ± 13	98 ± 11	39 ± 6	185 ± 31
0.008	133 ± 18	117 ± 16	93 ± 13	39 ± 5	198 ± 33
0.01	134 ± 16	113 ± 14	92 ± 12	42 ± 5	182 ± 24
0.02	138 ± 19	113 ± 16	89 ± 13	45 ± 6‡	168 ± 26
0.03	146 ± 19	121 ± 16	96 ± 12	51± 7‡	176 ± 27
0.05	154 ± 20†	129 ± 16	105 ± 13	49 ± 7†	196 ± 28

* p<0.05 ANOVA; † p<0.05 Dunnett's test compared to baseline; ‡ p<0.01 Dunnett's test compared to baseline, analysis of data recorded during the last minute (1 min) of infusion for each dose level.

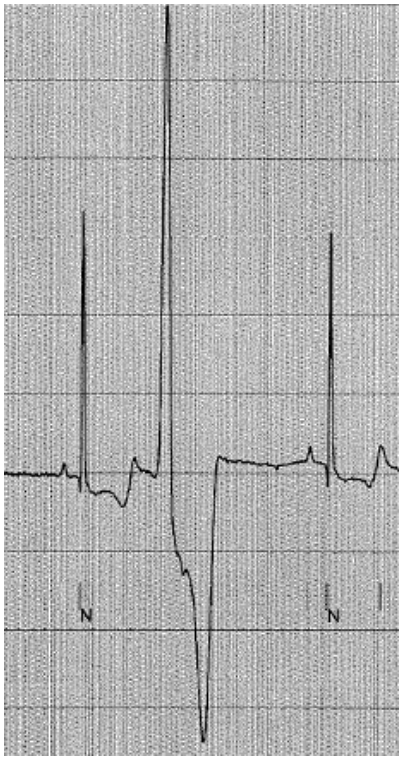
Figures

Fig. 1.



Increased T wave amplitude after myocardial ischemia in a male cynomolgus monkey. The ECG was obtained shortly after electromechanical dissociation caused by remifentanil at high dose (0.016 mg/kg) and reversed by naloxone (Derivation II). The T wave amplitude was increased for a period approximately 2 min starting 30 sec after return to sinusal rhythm with ventricular contractions.

Fig. 2.



Premature ventricular contraction (PVC) observed in a male cynomolgus monkey after electromechanical dissociation subsequent to remifentanil at high dose (0.016 mg/kg). A total of ten (10) PVC were observed during a period of 1 min shortly after reversal of remifentanil with naloxone (Derivation II).

Fig. 3.

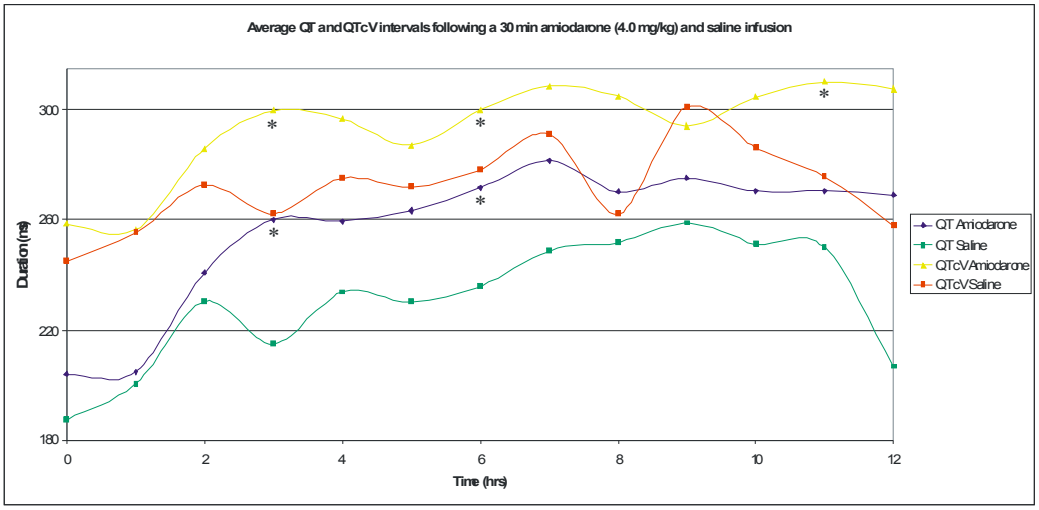
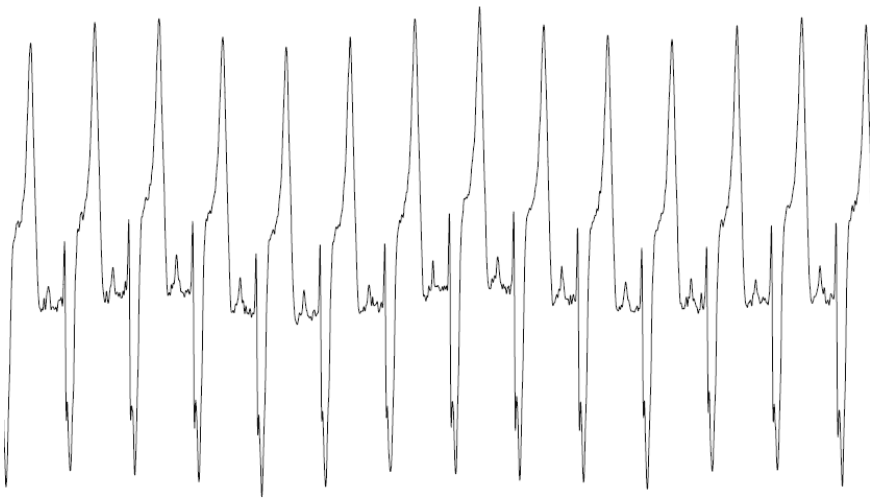


Illustration of the effect of amiodarone infusion (0.4 mg/kg/min for 10 min) on QT and QTcV intervals in conscious telemetered cynomolgus monkeys (n=4). Amiodarone infusion was performed over a 30 min period ending at 1h on the abscise axis of the above graph. ECG values obtained from the same monkeys at the same time of the day (6 pm to 6 am) prior to amiodarone administration were used as control for statistical analysis (* $p<0.05$ for paired comparison).

Fig. 4



Characteristic ECG changes induced by potassium chloride including T-wave peak, widening and slurring of the QRS complex, flattening of P-wave. This animal also presented a right bundle branch block.

Tel qu'illustré dans cet article, la validation pharmacologique, étape ultime avant la mise en production d'un modèle préclinique, nécessite la démonstration d'une réponse dose-dépendante pour les biomarqueurs principaux mesurés par le système évalué par la validation. La courbe de réponse à la dopamine présentée dans l'article précédent illustre bien cette stratégie qui permet de confirmer les caractéristiques du système préclinique testé. De façon générale, les courbes dose-réponse permettent d'évaluer la sensibilité du système en comparaison des autres systèmes utilisés par l'industrie pharmaceutique. Un problème de fond se pose toutefois dans cette démarche de comparaison avec les modèles couramment utilisés. Dans le domaine de la recherche académique subventionnée par des agences nationales, les incitatifs à publier les résultats expérimentaux sont nombreux. En effet, l'attribution et/ou le renouvellement des subventions sont basés en partie sur la prolificité des groupes de recherche telle qu'évaluée par le nombre et la qualité des articles. En contraste, il y a peu d'incitatifs à publier les résultats des études de validation dans le domaine pharmaceutique privé où des considérations de confidentialité et les avantages concurrentiels règnent. Des efforts nationaux ont mené à la publication de validations des modèles précliniques de pharmacologie de sécurité au Japon (Hashimoto, 2008; Omata *et al.*, 2005; Miyazaki *et al.*, 2005 ; Sasaki *et al.* 2005 ; Kano *et al.*, 2005 ; Ando *et al.*, 2005 ; Tashibu *et al.* 2005 ; Toyoshima *et al.*, 2005 ; Ki *et al.*, 2005 ; Yamazaki *et al.*, 2005 : Hayashi *et al.*, 2005). Malgré ces résultats largement discutés par la communauté scientifique, force est de constater que la littérature en matière de validation des modèles précliniques demeure très limitée en dépit des ressources colossales investies en recherche pharmaceutique à l'échelle planétaire. En 2008 seulement, l'industrie pharmaceutique a investi 65.2 milliards dans le développement des médicaments (Pharmaceutical Research and Manufacturers of America, 2009) et moins de vingt articles ont été publiés au cours de la même année sur la validation des modèles précliniques. L'article qui suit présente une comparaison entre deux modèles précliniques d'évaluation de la sécurité cardiovasculaire des médicaments et permet de mieux caractériser les

outils qui seront utilisés pour les évaluations des effets de l'OT. La connaissance des modèles précliniques permettra de sélectionner des modèles complémentaires sur le plan de la sensibilité et de la valeur prédictive de la réponse chez l'humain. Ce faisant, le programme de développement peut utiliser le nombre minimal d'animaux nécessaire et atteindre un des grands objectifs en médecine des animaux de laboratoire, soit la réduction (Russell & Burch, 1959).

5. COMPARAISON DE LA SENSIBILITÉ HÉMODYNAMIQUE DES MODÈLES DE PRIMATES NON-HUMAIN CONSCIENTS ET ANESTHÉSIÉS

Cet article (*Journal of Pharmacological and Toxicological Methods* 2008, 58 : 94-98) introduit le modèle anesthésié d'évaluation de l'innocuité des nouveaux médicaments sur la fonction cardiaque. Le but de cette étude était de caractériser les avantages et inconvénients du modèle anesthésié ainsi que la corrélation avec la réponse chez les patients. La littérature traitant de l'anesthésie chez le singe est peu abondante et notre groupe a confirmé l'utilisation des protocoles courant chez le cynomolgus (Authier *et al.*, Journal of the American Association of Laboratory Animal Science 2006, article en annexe). Notre hypothèse était que l'anesthésie réduirait les artéfacts liés aux stimulations externes augmentant ainsi la sensibilité du modèle. En contrepartie, l'anesthésie pourrait réduire la sensibilité aux médicaments à l'essai ayant des effets cardiovasculaires par l'intermédiaire d'un mécanisme d'action agissant sur le système nerveux central.

Les résultats et la discussion de cet article ont été présentés par Docteur Simon Authier au 45^{ième} congrès annuel de la *Society of Toxicology* à San Diego en Californie en 2006.

CONSCIOUS AND ANESTHETIZED NON HUMAN PRIMATE SAFETY PHARMACOLOGY MODELS: HEMODYNAMIC SENSITIVITY COMPARISON

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SUMMARY

Background and purpose. Drug-induced cardiovascular effects identified in conscious cynomolgus monkeys equipped with tethers and prepared for radiotelemetry were compared with results from anesthetized non-human primate (cynomolgus and rhesus) models.

Experimental approach. Remifentanil (4.0 μg/kg, bolus), esmolol (2.0 mg/kg, bolus) and dopamine (0.05 mg/kg/min, 30 min infusion) were given intravenously to all models.

Key results. Remifentanil decreased heart rate (HR), systolic, mean and diastolic systemic arterial pressures (SAP) in anesthetized animals while conscious monkeys presented an increase in HR, systolic, mean and diastolic SAP, as seen in humans for the respective state of consciousness (conscious and anesthetized). Esmolol decreased HR, systolic, mean and diastolic SAP in anesthetized monkeys while only HR, systolic and mean SAP achieved a statistically significant decrease in the conscious model. The amplitude of SAP reduction was greater in anesthetized models, while the amplitude of HR reduction was greater in the conscious and anesthetized cynomolgus models than in the anesthetized rhesus model. Dopamine induced a significant increase in HR, systolic, mean and diastolic SAP in anesthetized models without any statistically significant effect on HR and SAP in the conscious model.

Conclusions and Implications. The amplitude of hemodynamic and chronotropic alterations induced by positive control drugs was generally greater in anesthetized

than in conscious models and statistical significance was achieved more often with the anesthetized models. These results suggest that an anesthetized model may be valuable as part of a drug screening program for cardiovascular safety evaluations in addition to a conscious model.

Keywords: Cardiovascular, blood pressure, sensitivity, monkey, pharmacology, safety, conscious, anesthetized

INTRODUCTION

Repolarization-associated ventricular tachyarrhythmia has received considerable attention over the past years from regulatory agencies as highlighted in ICHS7A and ICHS7B guidelines (U.S. Food and Drug Administration, 2001; U.S. Food and Drug Administration, 2005) and from the scientific community (Detweiler, 1985; Redfern *et al*., 2003). A variety of methodologies to assess QT interval prolongation and analyze torsadogenic potential have been developed (Matsunaga *et al*., 1997; Spence *et al*., 1998; Fossa *et al.* 2006; Tattersall *et al*., 2006). On the other hand, systemic arterial pressure is recognized as a significant risk factor for mortality at all ages in humans (Kannel, 2000). A meta-analysis in human patients revealed that a 2 mmHg change in blood pressure translates into a 10% change in stroke and a 7% change in death from ischaemic heart disease (Lewington *et al*., 2002). These observations highlight the importance of sensitive safety evaluation methodologies in drug development. Non-clinical models used for drug safety assessment have been characterized using several approaches. Recently, statistical power simulations were calculated based on historical hemodynamic, inotropic and chronotropic data from a Beagle dog model (Chiang *et al*., 2004). Conscious and anesthetized animal models for the identification of torsadogenic effect have been discussed both for small and large laboratory animals (Hamlin *et al*., 2003; Vormberge *et al*., 2006). On the other hand, there is a paucity of data comparing conscious and anesthetized models for the identification of hemodynamic alterations using positive control drugs. The current study compares hemodynamic and chronotropic responses of conscious

and anesthetized non-human primate (NHP) models to positive control drugs. Importantly, the goal was not to confirm the effects of control drugs which have been extensively characterized in animals and humans but to compare the sensitivity of non-clinical screening models, including their limitations when used in the drug development industry.

MATERIALS AND METHODS

Statement on use and care of animals. During the study, care and use of animals were conducted in accordance with principles outlined in the current Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). LAB Research Inc.'s facility is AAALAC accredited. All procedures were conducted as per Standard Operating Procedures (SOPs) in place, and according to Good Laboratory Practices (GLP).

Animal housing and preparation. For conscious evaluations, 4 cynomolgus (*Macaca fascicularis*) monkeys (2 males and 2 females) were surgically prepared with telemetry transmitters *(TL11M2-D70-PCT™, DSI, St-Paul, MN, USA)* and femoral vein infusion catheters and were equipped with jackets and tethers (Lomir, Notre-Dame-de-l'Île Perrot, QC, CAN) connected to continuous infusion pump (AS50, Baxter, Mississauga, ON, CAN). Analgesic (buprenorphine, Temgesic™, 0.05 mg/kg, Schering-Plough, Welwyn Garden City, Hertfordshire, UK) was administered by intramuscular (IM) injection upon completion of the surgery and every 8 h for at least 24 h post-surgery. Four (4) cynomolgus

(*Macaca fascicularis*) and 4 rhesus (*Macaca mulatta*) monkeys (2 males and 2 females each) were used for anesthetized cardiovascular evaluations. At study initiation, monkeys were 2.7 to 5.8 years old and weighed between 2.5 and 5.9 kg. The animal room environment was controlled (temperature 21 ± 3°C, humidity 30-70%, 12 h light, 12 h dark, 10-15 air changes per h) and temperature and relative humidity were monitored continuously. A standard certified commercial primate chow (Certified Primate Diet 2055C™, Harlan Teklad, Madison, WI, USA) was available to each monkey twice daily.

Experimental methods. For unconscious NHP evaluations, anesthesia was induced with isoflurane using a mask, followed by intubation. Animals were placed on a heating pad and inhaled a mixture of oxygen (O_2) and isoflurane (AErrane™, Baxter Corporation, Mississauga, ON, CAN) with the O_2 flow meter and the vaporizer set at 1.0 L/min, and 1.5 to 2.0%, respectively. Respiratory rate was maintained between 10 and 13 breaths/min with an inspiratory airway pressure between 15 and 20 cm H_2O using a mechanical ventilator (2002, Hallowell EMC, Pittsfield, MA, USA). During anesthesia, monitoring included heart rate and pulsatile hemoglobin saturation in O_2 (VetOx 4404™ pulse oximeter, Heska, Fribourg, Switzerland). Anesthetized monkeys were instrumented with a systemic arterial catheter placed in the aorta approximately at the level of the renal artery, a left ventricular catheter inserted through the left carotid, and a Swan-Ganz catheter (Edwards LifeSciences, Irvine, CA, USA) placed in the pulmonary artery under fluoroscopy imaging. Hemodynamic parameters were continuously recorded in anesthetized monkeys, using a

computerized data acquisition system (Modular Instruments Inc., Malvern, PA, USA).

Positive control drugs included remifentanil (Ultiva®, Abbott Laboratories Ltd., Vaughan, ON, CAN), esmolol (Brevibloc®, Baxter Corporation, Mississauga, ON, CAN) and dopamine (Inotropin®, Bristol-Myers Squibb, Montreal, QC, CAN). Dose levels and administration protocol were the same for conscious and unconscious NHP models. Dose levels above human clinical doses were selected to induce moderate hemodynamic changes and assess the sensitivity of both models. Conscious animals were freely moving during administration and received control drugs through a permanent femoral catheter. Remifentanil and esmolol were given as an intravenous (IV) bolus at 4.0 μg/kg and 2.0 mg/kg, respectively. Dopamine was administered as a 0.05 mg/kg/min continuous infusion over 30 min.

Statistical methods. Statistical analysis was carried out using SAS version 9.0 (Cary, NC, USA). The analysis was performed using an analysis of variance (ANOVA) with the treatment and the NHP model for each parameter and the significance of inter-group delta differences was analyzed by Dunnett's t-test. Delta effect was calculated as the difference between baseline and peak drug effect post-treatment. The amplitude of drug-induced effects was calculated as percentage of baseline.

RESULTS

Cardiovascular effects induced by remifentanil. Remifentanil induced a statistically significant decrease in systolic, mean and diastolic systemic arterial pressures associated with a reduction in heart rate in anesthetized cynomolgus and rhesus monkeys (as shown in Fig. 1). In contrast, the same drug administered to conscious cynomolgus monkeys induced a slight increase in systolic, mean and diastolic systemic arterial pressures. Changes in heart rate were not statistically significant in the conscious cynomolgus NHP model. Individual data review revealed that all animals presented an increase in heart rate after remifentanil with increases above 40% of baseline in some cases. The elevated response variability most likely contributed to the lack of statistical significance but in the context of drug safety evaluation, these changes would be considered of pharmacological importance. Effects on systolic, mean, and diastolic arterial pressures were significantly more important in anesthetized cynomolgus (between group comparison; $p=0.003$, $p=0.006$, $p=0.005$) and rhesus (between group comparison; $p=0.02$, $p=0.02$, $p=0.008$) monkeys than the changes observed in the conscious cynomolgus NHP model. The effect on heart rate was also more important in anesthetized cynomolgus ($p=0.03$) and rhesus ($p=0.02$) NHP models than in the conscious cynomolgus NHP model. There was no statistically significant difference between the two monkey strains for the anesthetized NHP models for any of the parameter evaluated.

Cardiovascular effects induced by esmolol. In all anesthetized and conscious NHP models, esmolol induced a reduction in heart rate with a concomitant

decrease in systolic, mean and diastolic systemic arterial pressures (Fig. 2). If the decrease observed in the 4 parameters was statistically significant for both anesthetized NHP models, in the conscious cynomolgus model, the decrease was not statistically significant for the diastolic systemic arterial pressure. Pulse pressure was significantly reduced in both anesthetized models, and also in the conscious NHP model. There was significant difference in the amplitude of decrease observed with each model. Again, the effect was significantly more important in anesthetized cynomolgus monkeys for systolic ($p=0.0001$), mean ($p=0.0003$) and diastolic ($p=0.0003$) arterial pressures, when compared to conscious cynomolgus monkeys. The anesthetized rhesus NHP model also presented more important reduction of systolic ($p=0.049$) and mean ($p=0.01$) arterial pressures in comparison to the conscious cynomolgus model. There was no significant difference between the two anesthetized NHP models for hemodynamic effects. The effect on heart rate was significantly more important in conscious ($p=0.04$) and anesthetized ($p=0.02$) cynomolgus monkeys than in anesthetized rhesus monkeys. There was no significant difference between conscious and anesthetized cynomolgus monkeys for heart rate effects.

Cardiovascular effects induced by dopamine infusion. Dopamine in anesthetized cynomolgus and rhesus models resulted in significant increases of systolic, mean and diastolic arterial pressures (Fig. 3). In contrast, no statistically significant increase in arterial pressures was noted in the conscious cynomolgus model. Again, individual data review showed that all animals presented an increase in systemic arterial pressure following dopamine reaching up to +23%, which was

considered pharmacologically significant. The amplitude of diastolic (p=0.03) arterial pressure alterations was greater in anesthetized cynomolgus monkeys than in conscious animals. The effect on systolic (p=0.006) and mean (p=0.009) systemic arterial pressures was more important in the anesthetized rhesus model when compared with the conscious cynomolgus model. No statistically significant chronotropic effect was observed in the conscious cynomolgus model with the power used (n=4), while a significantly increased heart rate was noted in both cynomolgus and rhesus anesthetized NHP models (Fig. 3). No difference in amplitude of response was observed for any parameter between both anesthetized NHP models.

DISCUSSION

While the number of new drugs and biological applications submitted to the FDA is declining, most investigational products entering clinical trials are unsuccessful, increasing the overall costs of drug development (U.S. Food and Drug Administration, 2004). The need to perform more applied scientific work to develop tools to evaluate safety and effectiveness of new products is essential to improve the critical path from laboratory concept to commercial product.

The current study assessed sensitivity of conscious and anesthetized NHP models for the detection of hemodynamic and chronotropic effects. Cynomolgus monkeys have been widely used for regulatory cardiovascular safety assessments (Gauvin *et al.,* 2006). The extensive use of cynomolgus over rhesus monkeys in conscious cardiovascular models may result from the smaller size of cynomolgus monkeys, reducing costs of test article manufacturing; abundant historical data

available at each testing facilities for this strain; milder aggressive behaviours in cynomolgus monkeys (Zumpe *et al.*, 2005); as well as sporadic limitations of rhesus monkeys supplies. On the other hand, rhesus monkey offers some advantages for unconscious NHP models due to its larger size, which facilitates heart chamber instrumentation and decreases hemodynamic impacts of intracardiac catheters on heart function (*e.g.* Swan Ganz).

Remifentanil is an ultra short-acting (μ)-opioid agonist that has been used in conscious and anesthetized humans. The monkey dose of 4.0 μg/kg is equivalent to 1.3 μg/kg in humans after conversion with the human equivalent dose factor based on body surface area (U.S. Food and Drug Administration, 2002). A dose of remifentanil comparable to the one used in monkeys is reported to induce a transient increase in systolic blood pressure and heart rate when given as a bolus to conscious humans (Glass *et al.*, 1993). In contrast, remifentanil was reported to induce hypotension and bradycardia when administered to anesthetized humans (Elliott *et al.*, 2000). Drug effects obtained with monkeys were representative of the human response for the respective state of consciousness (conscious and anesthetized). These observations reinforce the need to include conscious animal data when using anesthetized animal data to extrapolate to the conscious human response.

Cardiovascular changes following esmolol administration were characteristic of $beta_1$ receptor blocker in both conscious and anesthetized NHP models, with negative chronotropic effects and decreased systemic arterial pressures (Gorczynski *et al.*, 1984; Kindler *et al.*, 1996; Cuneo *et al.*, 1994). The

amplitude of hemodynamic and chronotropic effects following esmolol administration was suggestive of model specific sensitivity. Once again decrease in systemic arterial pressures was more important in both anesthetized NHP models than in the conscious NHP model, suggesting a greater sensitivity of the anesthetized NHP model. Bradycardia induced by esmolol was more important in cynomologus (both conscious and anesthetized) when compared with rhesus monkeys, while baseline heart rate was similar in both strains for anesthetized animals (cynomolgus 119 bpm ± 4; rhesus 120 bpm ± 16). It remains that expected pharmacological effects were observed with both models and illustrate the variability of different animal strains. Similarly, different animal species such as dogs and monkeys are expected to present pharmacological responses that will differ to a given extent depending on the test article. As a result, using different animal species (e.g. dogs and monkeys) and different states (conscious and anesthetized) in the safety screening program could increase the overall sensitivity and most importantly increase the predictive value to the human response.

Results following dopamine infusion revealed that hemodynamic pressor effects (Abdul-Rasool *et al.*, 1987; Setler *et al.,* 1975) were more important in anesthetized monkeys. In addition, statistical significance for positive chronotropic effects was only achieved in anesthetized NHP models. The amplitude of systemic arterial pressure response was also greater in anesthetized monkeys when compared to the conscious NHP model. As previously reported, anesthesia with isoflurane is expected to result in lower mean arterial pressure baseline, when compared with the conscious state (Hom *et al.,* 1999).

Consequently, we could hypothesize that a lower arterial pressure baseline may enable greater pressor effects following dopamine infusion. As observed with remifentanil, lower systemic arterial pressure baseline in the anesthetized NHP model did not impair the sensitivity of this model for identification of hypotensive effects. Our results suggest that anesthetized cardiovascular models (NHP or other species as appropriate) may be valuable as part of the screening program in drug development. This screening step is typically conducted at an early stage of drug development in non-GLP studies using a very limited number of animals. In addition, the lack of statistical significance for some expected effects in the conscious model illustrate limitations of statistical analysis which may not completely capture pharmacologically significant changes when using relatively small group size for non-clinical drug screening. Statistical analysis should be combined with individual data evaluation to confirm that all relevant pharmacological effects have been identified.

Evaluation of drug potential for QT prolongation is an important factor in the choice of the most appropriate *in-vivo* safety model(s). Volatile anesthetics have been shown to prolong QT and QTc intervals in animals and humans (Riley *et al*., 1988; Schmeling *et al*., 1991). As a result, the conscious model is normally preferred for electrocardiographic safety evaluations. It would be interesting to compare the sensitivity of conscious and anesthetized NHP models for electrocardiographic alterations, including use of different anesthesia protocol (isoflurane, sevoflurane, etomidate, alpha-chloralose, propofol, etc.).

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Figure legends

* $p < 0.05$ for comparison to baseline

** $p < 0.01$ for comparison to baseline

*** $p < 0.001$ for comparison to baseline

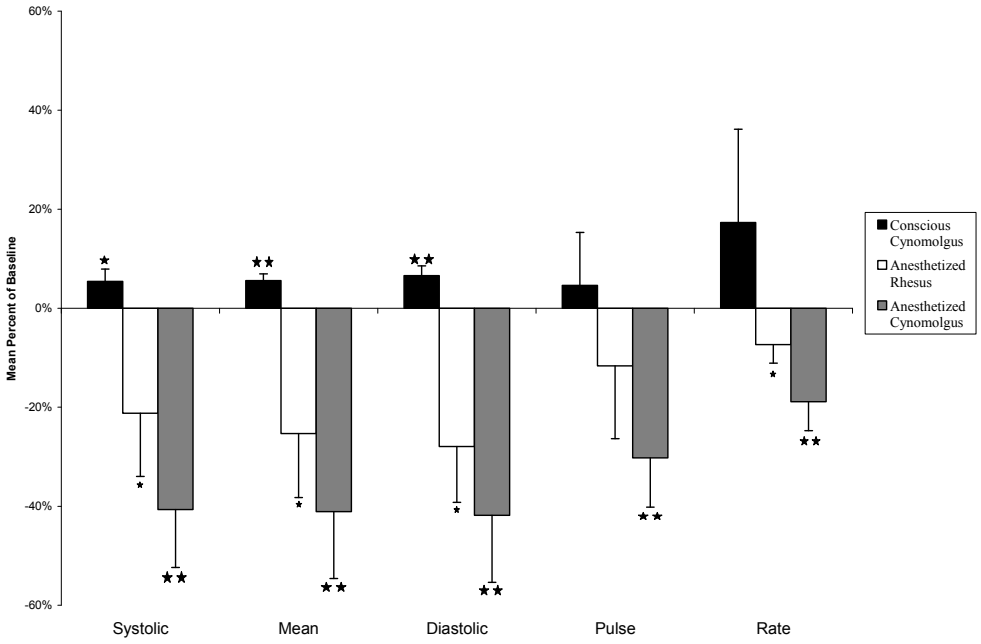

Fig. 1. Maximal cardiovascular effects (systolic, mean, diastolic systemic arterial pressures, arterial pulse pressure and heart rate) of a remifentanil (4.0 μg/kg) intravenous bolus in conscious freely-moving cynomolgus monkeys (n=4), anesthetized rhesus monkeys (n=4) and anesthetized cynomolgus monkeys (n=4).

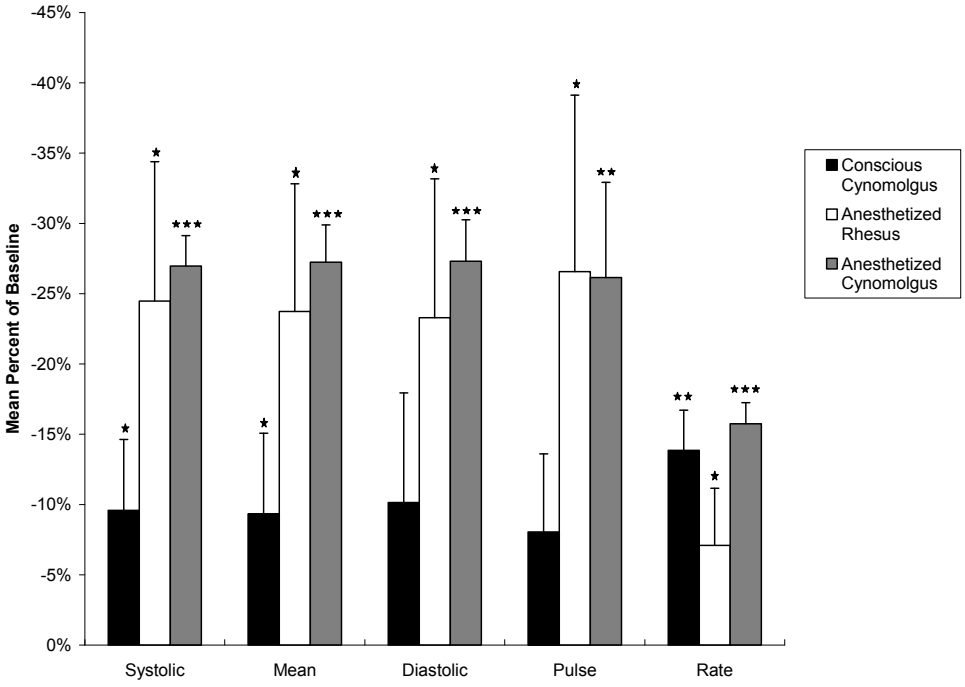

Fig. 2. Maximal cardiovascular effects (systolic, mean, diastolic arterial pressures, arterial pulse pressure and heart rate) of an esmolol (2.0 mg/kg) intravenous bolus in conscious freely-moving cynomolgus monkeys (n=4), anesthetized rhesus monkeys (n=4) and anesthetized cynomolgus monkeys (n=4).

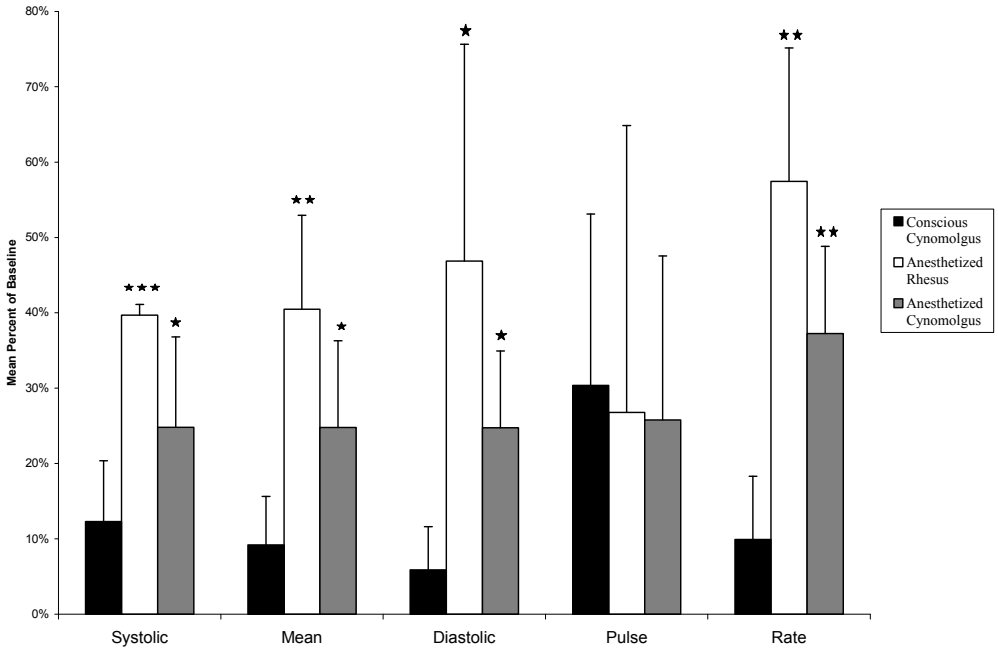

Fig. 3. Maximal cardiovascular effects (systolic, mean, diastolic arterial pressures, arterial pulse pressure and heart rate) of a dopamine (0.05 mg/kg/min) intravenous infusion in conscious freely moving cynomolgus monkeys (n=4) and anesthetized rhesus monkeys (n=4) and anesthetized cynomolgus monkeys (n=4).

Tel que présenté dans la revue de littérature en première partie de cette thèse, l'évaluation des effets sur la fonction cardiovasculaire revêt une importance particulière dans nos travaux d'évaluation des effets de l'OT comme peptide thérapeutique étant donnée la population cible souffrant d'infarctus du myocarde. Ce premier chapitre des études expérimentales a permis de définir les étapes de validation des modèles précliniques de pharmacologie de sécurité pour le système cardiovasculaire. Bien que les effets adverses cardiovasculaires constituent la majorité des causes d'arrêt de développement des médicaments (Valentin & Hammond, 2008), les agences règlementaires demandent l'évaluation de l'innocuité des médicaments sur les systèmes physiologiques essentiels que sont le système cardiovasculaire, respiratoire et nerveux. Le troisième chapitre élargit les horizons du deuxième objectif principal de la thèse en présentant la validation des modèles d'évaluation de la pharmacologie de sécurité du système respiratoire.

Chapitre 3 : PHARMACOLOGIE DE SÉCURITÉ RESPIRATOIRE

ÉTUDES EXPÉRIMENTALES

6. VALIDATION PHARMACOLOGIQUE D'UN SYSTÈME D'ENREGISTREMENT DE LA FONCTION RESPIRATOIRE CHEZ LE CHIEN BEAGLE CONSCIENT ET ANESTHÉSIÉ

Cet article (*Journal of Pharmacological and Toxicological Methods* 2008, 57 : 52-60) présente la validation pharmacologique d'un modèle de pharmacologie de sécurité chez le chien conscient et anesthésié. À l'instar du modèle cardiovasculaire, notre hypothèse pour ce projet était que la réponse chez le chien anesthésié bénéficierait de l'absence d'artéfacts liés aux stimulations externes. D'autres parts, un effet d'atténuation des effets pharmacologiques était attendu avec le modèle anesthésié principalement pour les effets médiés par le système nerveux central. Finalement, le modèle anesthésié, avec l'utilisation d'une sonde œsophagienne, permettait l'enregistrement de la résistance et l'élastance pulmonaire, deux paramètres importants dans l'évaluation de la pharmacologie de l'innocuité respiratoire. Lors de la conception d'un programme d'études précliniques, les biomarqueurs minimaux devant être inclus sont dictés par les lignes directrices S7A de International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). Ce premier article présente donc la validation du modèle d'évaluation de la fonction respiratoire chez le chien conscient rencontrant les conditions minimales d'évaluation fixées par le S7A alors que le modèle d'évaluation chez le chien anesthésié est classifié comme une étude de suivi plus approfondi. Une hypothèse notable de nos travaux expérimentaux était la faible sensibilité des gaz sanguins à identifier des effets pharmacologiques chez des chiens en santé. En effet, les lignes directrices du S7A de l'ICH recommandent l'utilisation des gaz sanguins comme biomarqueur de première ligne dans l'évaluation de la fonction respiratoire.

Les résultats et discussions de cet article ont été présentés par Docteur Simon Authier au 27^{ième} congrès annuel de l'*American College of Toxicology* à Palm Spring en Californie ainsi qu'au 6^{ième} congrès annuel de la *Society of Safety Pharmacology* à San Diego en Californie en 2006.

VALIDATION OF RESPIRATORY SAFETY PHARMACOLOGY MODELS: CONSCIOUS AND ANESTHETIZED BEAGLE DOGS

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Abstract

Installation, operation and performance qualifications were performed on a test system for respiratory monitoring. For performance qualification, conscious dogs received saline (0.2 mL/kg, iv, n=12), albuterol (100 µg/kg, inhalation, n=5), methacholine (2.0 and 8.0 µg/kg, iv, n=8) and remifentanil (4.0 µg/kg, iv, n=7). Following anesthesia with propofol infusion, dogs received saline (iv, n=15), albuterol (100 µg/kg, inhalation, n=8), methacholine (8.0 µg/kg, iv, n=8), remifentanil (4.0 µg/kg, iv, n=7), cholecystokinine tetrapeptide (CCK-4) (10 µg/kg, iv, n=7) and were exposed to hypoxic gas mixture (10% oxygen) (n=12). Saline had no significant respiratory effect. Albuterol increased tidal volume (TV) (+28%, $p<0.05$) and minute ventilation (MV) (+96%, $p<0.01$) in conscious dogs. In anesthetized dogs, MV was significantly increased (+23%, $p<0.05$) but the difference was not statistically significant for TV and respiratory rate (RR). Methacholine at 2.0 µg/kg increased MV (+45%, $p<0.01$) in conscious animals while 8.0 µg/kg increased RR (+66%, $p<0.01$), TV (+24%, $p<0.05$) and MV (+88%, $p<0.05$). In anesthetized dogs, methacholine increased RR (+51%, $p<0.05$), MV (+34%, $p<0.05$), lung elastance (+36.9%, $p<0.01$), and resistance (+45.8%, $p<0.01$). Remifentanil decreased MV in conscious dogs (-68%, $p<0.01$) while transient apnea was observed in all anesthetized dogs. CCK-4 increased RR (+328%, $p<0.01$) and MV (+127%, $p<0.05$) and decreased TV (-58%, $p<0.01$). Exposure to hypoxic gas mixture increased MV and RR ($p<0.01$). Baseline MV was lower ($p<0.05$) in anesthetized than in conscious dogs. Arterial blood gas values, particularly SaO_2, presented a limited sensitivity to detect any ventilation disturbance, but allowed confirmation of both ventilatory compensatory phenomenon (when present) and initial pharmacologic drug effect. These results also highlight the greater sensitivity of the conscious model when compared to anesthetized dogs.

Keywords: Respiratory, methods, dogs, pharmacology, preclinical, safety, validation

1. Introduction

Non-clinical safety pharmacology testing paradigms are in constant evolution with rapidly improving technologies and guidelines supported by regulatory authorities. Results obtained with a number of non-clinical respiratory safety pharmacology models have been published in different species including rat (Heaton et al. 2007; Kim et al. 2005; Schierok et al. 2000), mouse (Flandre et al. 2003), guinea pig (Kim & Shin 2005; Kim et al. 2003; Pennock et al. 1979), monkey (Malis et al. 1975; Murphy et al. 2001) and dog (Mauderly 1974; 1989; Joseph et al. 1997; Baldrick et al. 2002; Chanda et al. 2005). The International Conference on Harmonization (ICH), which represents regulatory agencies from U.S.A., Europe, and Japan, has established guidelines for the safety testing of pharmaceuticals on organ functions. References provided in the ICH guideline S7A (US FDA – S7A, 2001) include a publication from Murphy 1994, which discusses the study design in respiratory safety pharmacology and reports use of both conscious and anesthetized models. Results from respiratory safety pharmacology studies in conscious (Murphy et al. 1998; Murphy et al. 2001; Flandre et al. 2003; Kim & Shin 2005; Kim et al. 2005) and anesthetized (Malis et al. 1975; Joseph et al. 1997; Schierok et al. 2000; Baldrick et al. 2002; Chanda et al. 2005; Heaton et al. 2007) animals are frequently reported but rarely compared. Recently, the sensitivity of conscious (non-invasive) and anesthetized (invasive) models was discussed in a variety of efficacy and safety pharmacology applications in rodents (Glaab 2005; Hoymann 2007). In addition, literature on respiratory safety pharmacology frequently presents data obtained with experimental drugs that may not be commercially available. Data obtained with commercially available drugs are most useful for validation of study design, more specifically for performance qualification (PQ). As part of the "core battery" of safety pharmacology studies, the ICH guideline requires that the effect of drugs on respiratory function be evaluated prior to first human administration (Murphy 2005). To fulfill regulatory requirements, validation of the sensitivity, specificity and reproducibility of respiratory function evaluation is critical.

The aim of the present study was to validate a respiratory safety pharmacology model in dogs, to test different strategies and techniques considered to be most appropriate for the detection and characterization of drug-induced respiratory disorders in non-clinical safety studies, and to compare the conscious and anesthetized states using inert and positive control drugs.

2. Methods

2.1 Supplier Qualification and Justification for CFR 21 Part 11 Compliance

Respiratory system vendor qualification was initially performed using a standard questionnaire. User requirements were provided to several suppliers before selection of a respiratory monitoring system. Then, a risk assessment was documented in a prospective validation protocol to justify the requirement for part 11 compliance as suggested in the FDA *Guidance for the Industry* (US FDA – CFR 21 Part 11, 2003).

2.2 Test System Description

2.2.1 Animal Management

All animals used during this study were maintained in accordance with the *Guide for Care and Use of Laboratory Animals* at LAB Research Inc. (Laval, Quebec, Canada), a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) before study initiation. Study population comprised eighteen (18) beagle dogs (6 males and 12 females) with an age range of 1 to 4 yrs old and a body weight ranging from 8 to 18 kg. The study was conducted in accordance with the Good Laboratory Practice regulations of the United States Food and Drug Administration (21 CFR Part 58 and subsequent amendments).

The animal room environment was controlled (targeted ranges: temperature 21 ± 3°C, humidity 30-70%, 12 hours light, 12 hours dark, 10-15 air changes per hour).

Temperature and relative humidity were monitored continuously. A standard certified commercial dog chow (400 g of Harlan Teklad Certified 25% Lab Dog Diet #8727C) was provided to the animals once daily during a 2-hour feeding period. Clinical signs were evaluated at cage side at least once daily, and a detailed clinical examination was performed at transfer and once weekly throughout the study.

2.2.2 Respiratory Monitoring System

Respiratory monitoring was performed using a computerized system composed of a data acquisition controller (DAC 8, Scientific Respiratory Equipment Quebec Inc. (SCIREQ), Montreal, QC, Canada) connected to a computer (OptiPlex GX280 Workstation, DELL, Dallas, TX, U.S.A.) where a respiratory monitoring software (flexiWare 5.1, SCIREQ Inc., Montreal, QC, Canada) was installed. The respiratory hardware connected to the flexiWare 5.1® software included a pneumotachometer (Model 3719 with a 100L/min capacity, HANS RUDOLPH, MO, U.S.A.) with individual heater controllers (HANS RUDOLPH, MO, U.S.A.). Precision differential pressure transducers connected to the data acquisition matrix were connected with the pneumotachometer (Model UT-PDP-02, 0.2 kPa nominal, SCIREQ Inc., Montreal, QC, Canada) and the esophageal pressure probe (Model UT-PDP-75, 7.5 kPa nominal, SCIREQ Inc., Montreal, QC, Canada). The latter could only be used in anesthetized animals.

2.2.3 Conscious and Anesthetized Dog Models

For conscious evaluations, all dogs were acclimated to the respiratory monitoring procedures for 2 weeks prior to study initiation. Animals were placed on a mobile restraint unit (LOMIR BIOMEDICAL, Notre-Dame-de-l'Île-Perrot, QC, Canada) and equipped with custom made transparent masks (LNR Inc., Ville St-Laurent, QC, Canada). No supplemental specific procedure was used in conscious animals. For unconscious monitoring, anesthesia was induced with propofol (PropoFlo™, Abbott Laboratories Ltd., Montreal, QC, Canada) given intravenously (IV) into

the cephalic vein to effect (6 to 15 mg/kg, 10 mg/mL) followed by continuous infusion (24 mg/kg/h to 42 mg/kg/h). Animals were carefully oro-tracheally intubated. Lidocaine spray (10% w/w) was administered onto the glottis prior to intubation, when necessary. An ophthalmic ointment was applied to both eyes to prevent drying of the cornea. A thoracic esophageal pressure probe, confirmed under fluoroscopy when required, was positioned at the level of the heart (caudal portion) to measure pleural pressure. Using a pulse oximeter and a capnometer, continuous monitoring (only during anesthesia) included: oxygen saturation of hemoglobin (SpO_2), inspiratory and end-tidal CO_2 levels, heart and respiratory rates. Lactated Ringer's solution was administered iv throughout the procedure at a rate of 10 mL/kg/hr.

Respiratory monitoring included respiratory rate (RR), tidal volume (TV) and minute ventilation (MV) for both conscious and anesthetized dogs. In addition, the pleural pressure probe, in anesthetized dogs, allowed measurement of resistance, elastance and compliance. The flexiWare 5.1® software calculates dynamic lung resistance and compliance using the linear first-order single compartment model in anesthetized animals. Dynamic compliance captures the ease with which the lungs can be extended, and is useful as a measure of the comparative stiffness of the lung, including resistance of small airways, presence of edema or accumulation of fibrous tissue in the lung. Elastance (the inverse of compliance) represents elastic rigidity of the lungs and resistance to deformation. Dynamic lung resistance includes contributions from central airways, peripheral airways, as well as friction in the tissues. The coefficient of determination (COD) is a quality control parameter evaluating the data fit to a single compartment model. The COD was used as an exclusion factor for values of respiratory mechanics in unconscious animals. All values of respiratory mechanics associated with COD values below 0.9 were excluded from the analysis. Values of respiratory mechanics with a COD above 0.9 were reviewed for quality by a trained veterinarian, before inclusion in the analysis. The revision of the data

included an evaluation of pleural pressure and air flow tracings to confirm the quality of the breaths (amplitude, correlation between pleural pressure and flow and absence of artifacts). Data used in statistical analysis for these parameters were collected at maximal effect for each tested drug, which was globally 1 to 2 min after administration. Arterial blood samples were taken 5 min after dosing, in all dogs. The collection site was the dorsal pedal artery in conscious dogs, and the femoral artery using an indwelling catheter in anesthetized dogs. Parameters evaluated were pH, $PaCO_2$, PaO_2, HCO_3^-, TCO_2, base excess in extracellular fluids (Beecf) and SaO_2, as well as electrolytes (Na^+, K^+, and ionized Ca^{2+}), packed cell volume (PCV), hemoglobin and glucose concentrations (i-STAT®, Heska, ON, Canada).

2.2.4 Installation and Operation Qualifications

Installation (IQ) and operation (OQ) qualifications were performed on-site (at LAB Research Inc.) by SCIREQ Inc. Additional OQ tests were performed by LAB Research Inc. staff to fulfill validation. Tests included in the OQ were designed to reflect actual conditions of utilization including worst-case scenarios (pressures and volumes outside of the hardware specifications). System precision was assessed using reference modalities such as a calibrated reference manometer (DPM-1b®, BIOTEK, Winooski, VT, U.S.A.) and a calibrated volume calibration syringe (HANS RUDOLPH, Kansas City, MO, U.S.A.). The ability of the computerized system to record accidental or intentional alterations of the computer internal clock through the audit trail was tested.

2.2.5 Performance Qualification

For PQ, conscious dogs received saline at 0.2 mL/kg, iv (BAXTER, ON, Canada), albuterol at 100 µg/kg by inhalation (RATIOPHARM Inc, QC, Canada), methacholine at 2.0 and 8.0 µg/kg, 0.2 mL/kg, iv (SIGMA-ALDRICH, ON, Canada) and remifentanil at 4.0 µg/kg, 0.2 mL/kg, iv (Ultiva®, ABBOTT LABORATORIES Ltd., ON, Canada). The same drugs with the addition of

cholecystokinine tetrapeptide (CCK-4) at 10 μg/kg, 0.2 mL/kg, iv (BACHEM CALIFORNIA, CA, U.S.A.) were administered to anesthetized dogs. Respiratory effects of a hypoxic mixture of oxygen (10%) and nitrous oxide (90%) was also evaluated in anesthetized animals. Anesthetized dogs were exposed to the hypoxic gas mixture until SpO_2 reached 50%. Oxygen was then returned to 100% until SpO_2 returned to 90%.

2.2.6 Statistical Analysis

Data was analyzed using SAS/STAT software, Version 9.1 of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA). Trend analysis was performed to assess the presence of respiratory changes following PQ inert control (saline) iv injection to conscious or unconscious animals. Values exported every 10 seconds, from -300 sec to 1200 seconds after saline injection, were used for statistical trend analysis to detect any effect of the inert control drug over this period. Parameters analysis included RR, TV and MV for both conscious and anesthetized dogs. Analysis for each drug including saline was conducted using a paired t-test based on paired values obtained 5 min prior to dosing, and after each test agent administration. Analyses were done for each test agent, each parameter and for unconscious and conscious models, independently, and maximal effects induced by tested drug compared to pre-treatment values are presented in the results section. Comparison of conscious and unconscious models was first done with results obtained following saline administration. A t-test compared the difference of results obtained from conscious and unconscious models for each time-point, independently. Results obtained in conscious and unconscious models following albuterol, methacholine (8.0 μg/kg) and remifentanil treatments were compared using a t-test, for each group independently. First, the percentage of change induced by each test agent on each parameter was calculated. The difference was then submitted to a t-test to evaluate differences between the effect noted in conscious and unconscious models. In presence of unequal variance, the Satthertwaite correction was used. Time delay to obtain maximal effect for each

parameter (RR, TV and MV) with these three treatments was compared between conscious and anesthetized animals. The exception to the above-presented analysis was the response to hypoxic mixture in anesthetized dogs. Statistical analysis of respiratory parameters with this positive control was done by comparing the values recorded 2 min before treatment, with the average obtained when SpO_2 was between 60 and 75%, corresponding to the initial phase of hypoxemia. For arterial blood gases and pH, only effects of positive control drugs were compared to inert control saline for both conscious and unconscious states. Moreover, the effect of anesthesia on arterial blood gases and pH was evaluated by comparing values obtained after saline administration in conscious and unconscious dogs. Comparison was done using a t-test for each parameter. Correlation coefficient calculation was used to evaluate data obtained during IQ and OQ. The threshold for statistical significance was set at 0.05; results are presented as mean ± S.D.

3. Results

3.1 Installation, Operation and Performance Qualifications

IQ and OQ confirmed accuracy of the system within normal conditions of use (Fig. 1).

Results following inert and positive control drugs administration to conscious dogs are presented in Table 1 while results from anesthetized dogs are reported in Tables 2 and 3.

3.1.1 Saline

Trend analysis and comparison of baseline with post-saline administration values indicated no statistically significant respiratory effect following saline administration in either conscious or anesthetized dogs.

3.1.2 Albuterol

Increases in MV ($p<0.01$) and TV ($p<0.05$) were noted following albuterol administration to conscious dogs. No statistically significant RR modification

was identified. Following albuterol administration, no biochemical parameter was significantly different from values recorded following saline administration in conscious dogs (Table 4). As with conscious animals, albuterol induced a significant increase in MV in anesthetized animals ($p<0.05$). Average MV increased from 2.472 L/min to 3.051 L/min, which represents a 23.4% increase compared to a 96.4% increase in conscious dogs. Lung resistance decreased by 17.3% in average following albuterol administration, but the difference was not significant ($p=0.11$). Compared to the saline unconscious group, albuterol arterial blood gases and pH values in anesthetized dogs indicated a slight ($p=0.04$) increase in pH associated with an improvement in oxygenation ($p=0.02$ for PaO_2).

3.1.3 Methacholine

In conscious animals, methacholine at 2.0 µg/kg induced a significant increase in MV ($p<0.01$). At high dose, methacholine (8.0 µg/kg) created a significant increase in all respiratory parameters. No biochemical parameter in both methacholine groups was statistically different from saline group. In anesthetized dogs, methacholine at 8.0 µg/kg resulted in a significant increase in pulmonary elastance (+36.9%, $p<0.01$) and resistance (+45.8%, $p<0.01$) with significant increases in RR ($p<0.05$) and MV ($p<0.05$). Compared to unconscious dogs treated with saline, respiratory acidosis was exacerbated, with deteriorated pH ($p<0.01$) and $PaCO_2$ ($p=0.02$).

3.1.4 Remifentanil

After remifentanil administration, a significant decrease in MV was noted in conscious dogs ($p<0.01$). Biochemical evaluation confirmed hypoventilation with decreased pH ($p=0.0005$) and increased $PaCO_2$ ($p=0.0002$). Respiratory acidosis was also associated with a significant ($p=0.008$) increase in plasma glucose (112.3±16.3 *vs*. 91.8±9.1 mg/dL in the saline conscious group) and a marginally significant ($p=0.05$) increase in PCV (39.8±11 *vs*. 30.8±5.4%) and

hemoglobin concentration (13.6±3.7 *vs.* 10.5±1.8 g/dL). All anesthetized dogs presented a period of apnea with variable breathing patterns during post-apneic recovery. Some animals presented an abrupt return to elevated MV while others progressively increased their MV. No significant modification to respiratory mechanical parameters was noted in the anesthetized dog model. However, the arterial blood gases and pH values indicated a major respiratory acidosis ($p<0.001$ for pH and $PaCO_2$) associated with deteriorated oxygenation ($p<0.000001$ for PaO_2 and SaO_2).

3.1.5 CCK-4

In anesthetized dogs, CCK-4 induced a significant increase in RR (+328%, $p<0.01$) and MV (+127%, $p<0.05$) associated with a decrease in TV (-58%, $p<0.01$). No significant change was noted for pulmonary resistance, elastance and compliance. When compared to the anesthetized group treated with saline, the respiratory acidosis ($p<0.01$ for pH and $PaCO_2$) was associated with deteriorated oxygenation ($p<0.0001$ for PaO_2 and SaO_2) and increased ($p=0.002$) glucose concentration (114.9±13 *vs*. 96.9±11 mg/dL).

3.1.6 Hypoxic mixture

Following exposure of anesthetized dogs to a hypoxic gas mixture, a progressive decrease in SpO_2 was recorded for all animals. MV and RR were increased ($p<0.01$) while TV remained relatively stable. SpO_2 values between 60 and 75% were correlated with increased MV and RR. Hypoxia was associated with major hypoxemia ($p<0.0000001$ for PaO_2 and SaO_2), and triggered a hyperventilating response. The latter led to restored ($p<0.05$) pH and $PaCO_2$ compared to respiratory acidosis values recorded in saline anesthetized dogs.

3.2 Statistical comparison of conscious and anesthetized models

Baseline MV was significantly lower ($p<0.05$) in anesthetized animals. Following albuterol, methacholine (8.0 µg/kg) and remifentanil treatments, the time delay to

get maximal effects on RR, TV and MV was always shorter in unconscious animals. With the three treatment groups pooled together, time delay in conscious *vs.* unconscious model was 01:42±00.25 *vs.* 01:25±00:21 min for RR (p=0.03), 01:33±00.21 *vs.* 01:19±00:19 min for TV (p=0.03) and 01:48±00.28 min *vs.* 01:28±00:22 for MV (p=0.01). When comparing each treatment, only MV time delay in the methacholine group showed statistical significance (p=0.04). Comparison of arterial blood gases and pH between conscious and unconscious dogs receiving saline demonstrated that propofol anesthesia induced a significant (p<0.01) respiratory acidosis characterized by decreased pH and increased $PaCO_2$, TCO_2 and HCO_3^- (Table 4). The effect of albuterol on MV was significantly more important (p<0.01) in the conscious model. Similarly, the effects of methacholine (8.0 µg/kg) (p<0.01) and remifentanil (p<0.05) on TV were significantly more important in the conscious model. As illustrated in Fig. 2, transient hypoventilation following remifentanil administration to conscious animals was followed by a hyperventilatory response.

4. Discussion

The purpose of this study was to validate a respiratory safety pharmacology model in conscious and anesthetized beagle dogs, and to compare both models. Because the respiratory system consists of two functional units, the pumping apparatus and the gas exchange unit, a study designed to evaluate the potential for new drugs to produce adverse effects on respiratory function must evaluate both of these components (Murphy 2005). Evaluating dose-response relationships, determining the degree of change in specific respiratory parameters, and characterizing changes in arterial blood gases and pH are used to establish the liability associated with the functional changes. Because the safety profile defined by this type of study can have a significant impact on the successful development of new therapeutic agents, it is important that the techniques and assays used minimize the occurrence of false negative and false positive results. For this reason, techniques that provide direct measures of respiratory parameters should

be used (Murphy 2004; 2005). In this study, the tested respiratory device is based on facemask or intubated pneumotachometry and esophageal pleural pressure determination (in anesthetized dogs only). A complete validation process of this respiratory model is presented, including IQ, OQ and PQ. Installation qualification and OQ allowed system functionalities characterization and constitute important milestones for preparation of standard operating procedures.

It is widely accepted in respiratory safety pharmacology that, because most drugs are intended for use in conscious patients and because most anesthetics, analgesics, and sedatives can alter ventilatory reflexes, respiratory drive, and airway reactivity, non-clinical studies evaluating the effects of drugs on respiratory function should utilize conscious animal models (Murphy 2005). To the authors' knowledge, a quantification of the possible interfering effect of anesthesia on respiratory safety pharmacology parameters has not previously been reported. Inert control (saline) did not produce any statistically significant change in respiratory parameters in both conscious and anesthetized models. However, comparison of arterial blood gases and pH values recorded 5 minutes after saline administration in all dogs clearly reported the centrally mediated respiratory depression induced by propofol anesthesia. Respiratory acidosis results from a build-up of carbon dioxide in blood, usually due to hypoventilation. Moreover, the intravenous agent propofol depresses the hypercapnic-induced ventilatory response at the level of central chemoreceptors (Nieuwenhuijs et al. 2001). Consequently, ventilatory parameters (TV, RR, MV, lung compliance and resistance) were not altered by anesthesia. However, to keep similar ventilatory response pattern in comparison to conscious animals, animals under propofol anesthesia requires higher level of $PaCO_2$, which resulted in decreased plasma pH. This is by definition respiratory acidosis. Arterial blood gases classically reveal a pH below 7.35 with elevated $PaCO_2$ (>45 mmHg / 6 kPa). Bicarbonate could be either normal or increased (chronic compensation with renal HCO_3^- retention or acute shift of the Henderson-Hasselbalch equation as observed in our study). It was also reported that propofol could depress ventilatory parameters

(TV, MV) in humans, as the ventilatory response to carbon dioxide was 58% of baseline awake control (Goodman et al. 1987). Such an effect could be a major interfering factor when evaluating respiratory safety pharmacology, and the results obtained with positive control drugs confirmed this possibility. Apnea is common following profofol bolus administration, and increasing dose or injection rate will increase incidence and duration of apnea (Morgan & Legge 1989 ; Muir & Gadawski 1998). Propofol induction doses > 9 mg/kg resulted in transient cyanosis in healthy dogs whereas doses > 14 mg/kg translated into apnea with dose-dependent increase in duration (Muir & Gadawski 1998). Despite slow injection, cyanosis is often observed; mask oxygenation during induction is recommended. Maintenance with propofol infusion appears to be less depressant than induction. In humans, a maintenance dose of 6 mg/kg/h resulted in decreased TV, increased RR and variable changes in MV. Doubling the infusion rate caused further decrease in TV but no change in RR (Goodman et al. 1987). All injectable anesthetic agents are reported to depress ventilatory response to carbon dioxide, particularly propofol and thiopental.

Similarly to previously reported effects in conscious humans, in conscious dogs albuterol increased TV and MV without significant effect on RR (Sorbini et al. 1984). These effects were considered expected effects of a beta-$_2$ selective bronchodilator in normal animals, which translated into an increase in MV. This increase in ventilation had no consequence on arterial blood gases and pH values. However, in anesthetized dogs presenting respiratory depression, the increase in MV, mainly related to an increase in TV and a bronchodilating effect of albuterol induced a significant increase in pH and oxygenation.

Increased lung resistance and elastance was observed with methacholine as previously reported in anesthetized dogs (Ramsdell & Georghiou 1979). Methacholine-induced bronchoconstriction increases the resistive load of ventilation, and the narrowing of airways can reduce the efficiency of gas exchange by decreasing airflow. However, in this study, the obstructive and restrictive disorder was counter-acted by a stimulation of respiratory function,

particularly evident in conscious animals. A drug-induced dose response was also noted in the conscious model, with more important respiratory alterations at higher doses. This dose-response was related to a full compensation by hyperventilation, preventing alterations to arterial blood gases and pH values in conscious dogs. However, hyperventilatory response to increased resistance was not fully demonstrated under anesthesia, leading to partial compensation, which translated into respiratory acidosis.

The short acting opioid remifentanil induced typical central depression of the pumping apparatus (Smith et al. 1997; Moerman et al. 2003), which translated into decreased MV and respiratory acidosis in conscious dogs, and transient apnea and respiratory acidosis in anesthetized dogs. In conscious dogs, respiratory depression, apnea and associated hypoxia appear to have induced a stress response marked by increased plasma glucose, and blood viscosity. This effect was unexpected and should be further documented before drawing final conclusions. One possible explanation for increased PCV is a splenic contraction mediated by the release of catecholamines during apnea and/or possible hypoxia. The function of the spleen as a reservoir for the formed elements of the blood is well-recognized: the circulating hemoglobin concentration of Weddel seals increases by 60% during the first 10-12 min of a dive (Qvist et al. 1986). Similarly to the observed contraction in exercising horses (Persson et al. 1973) and other terrestrial mammals (Miller & Rhoads 1933; Anderson & Rogers 1957; Hannon et al. 1985), including dog (Sato et al. 1995), it has been confirmed that the spleen of the Weddell seal contracts and supplies red blood cells into the peripheral circulation. Depending on conditions and species, the splenic contraction leads to a 15-60% increase in circulating red blood cells and this increased O_2-carrying capacity has been related to catecholamine release (Sato et al. 1995; Hurford et al. 1996). In this experiment, after remifentanil administration in conscious dogs, PCV and hemoglobin concentration increased by about 30%. Moreover, in absence of any compensatory response, remifentanil added to the respiratory depression induced by propofol with apparition of a

deteriorated oxygenation. Apnea periods observed after remifentanil administration in anesthetized dogs explain the lack of statistically significant results on mechanical respiratory parameters upon return to tidal respiration. Nevertheless, remifentanil's short half-life makes it a useful positive control in validation studies given its short wash-out period.

As previously reported, CCK-4 induced in anesthetized dog a MV increase of more than 100%, without any respiratory mechanical parameters modification (Bates & Bradwejn 1995). In comparison to other hyperventilating pharmacological stimulation induced by albuterol, methacholine, or hypoxia, CCK-4 induced hyperventilation was only due to a stimulation of RR and was associated with a statistically significant decrease in TV. Indeed, it could be hypothesized that the rapid shallow breathing did not allow sufficient gas flow to reach an adequate TV. This resulted in respiratory acidosis associated with oxygenation deterioration. Even under general anesthesia, there were stress response evidences with increased plasma glucose.

In the anesthetized dogs, the hypoxic mixture oxygen (10%) and nitrous oxide (90%) induced hypoxemia, which progressively increased MV. This ventilatory response to hypoxia corrected the acidosis induced by propofol anesthesia. Given major hypoxemia induced by our procedure, the increase in ventilation was not sufficient to return to normal oxygenation values. Noteworthy, the use of nitrous oxide instead of an inert gas could have negatively influenced the amplitude of response to hypoxia. Nitrous oxide is reported to have no effect on bronchomotor tone (Rooke et al. 1997), and minimal interference with spirometry parameters (TV, MV) and subsequently $PaCO_2$ in dogs (Warner et al. 1998). Nitrous oxide affects breathing by changing the distribution and timing of neural drive to the respiratory muscles in a species-dependent manner, rather than by causing a global depression (Warner et al. 1998). Also, nitrous oxide impaired mucociliary function (Lichtiger et al. 1975) and the hypoxic pulmonary vasoconstriction (Bindslev et al. 1986) worsening oxygenation. Finally the central depression induced by nitrous oxide attenuates hypercarbia-induced increase in ventilatory

drive (Royston et al. 1983) and the ventilatory response to hypoxia (Yacoub et al. 1976) in a dose-dependent manner.

Validation studies should be performed in experimental conditions similar to those that will prevail during regulatory testing. Results from validation studies confirm the ability of the test system to identify a series of pharmacodynamic effects, which may be observed in human trials. Positive control agents' choice was based on their mechanisms of action. Test article respiratory effects may be elicited by various physiological pathways including bronchodilation, as seen with albuterol (Goubault et al. 2001), bronchoconstriction, as with methacholine (Crimi et al. 2002), central nervous system depression, as observed with mu opioid agonist remifentanil (Bouillon et al. 2003, Egan et al. 2004), or psychoneurological responses such as panic attacks, induced by CCK-4 (Bradwejn et al. 1998). Hypoxia is also a well-characterized phenomenon, which triggers increased ventilatory response (Sjogren et al. 1999). The hypoxic mixture allowed SpO_2 validation as a systemic oxygenation measure, and confirmed the test system's ventilatory response to hypoxia. It is also important to note that all procedures tested in this experiment induced different ventilatory responses (either hyperventilation or hypoventilation), based on different parameters (RR, TV and MV), and, each time, associated with different effects on gas exchange as characterized by acid-base and oxygenation status. We consider that this experimental design combining respiratory mechanics, arterial blood gases and pH evaluations, is an important part of a test system validation in large laboratory animals.

The S7A guideline recommends the use of unanesthetized models for safety pharmacology evaluations. When evaluating tidal midexpiratory flow (EF_{50}) as a respiratory endpoint, in comparison to the anesthetized model, lower sensitivity and greater variability were reported with the conscious rat model, (Hoymann 2007). In addition, anesthetized models often permit respiratory mechanics evaluation, which may not be available with non-invasive monitoring. These factors may justify the use of anesthetized models for some applications. In the

current study, we compared the sensitivity of a conscious and an anesthetized respiratory model in dogs. Administration of the same drugs at the same dose levels to both models resulted in greater respiratory effects in conscious dogs. This could be attributed to an interfering effect of the anesthetic drug either by direct depression of central nervous system, or indirectly, by affecting the animal's baseline respiratory status. This was particularly obvious in anesthetized dogs with partial hyperventilatory compensation to methacholine or albuterol, and aggravation of respiratory depression induced by remifentanil. The respiratory response to remifentanil of conscious dogs was an initial phase of hypoventilation, followed by compensatory hyperventilation, as shown in Fig. 2. The anesthetized model lacks this second phase of the respiratory response to remifentanil. These results were expected given the well-known respiratory depressive effects of anesthetic agents such as propofol (Nieuwenhuijs et al. 2001). These results suggest that the conscious model is more sensitive than the anesthetized model for respiratory effect identification. It concurs with the recommendation of S7A. Moreover, the evaluation of peak effects in this study demonstrated that the time to maximal effect of tested drugs was longer in conscious animals, suggesting the intervention of some compensatory phenomenon.

Like other safety pharmacology studies, respiratory monitoring should be based on the pharmacokinetic of the test article and should include monitoring at peak plasma concentration (Murphy 1994). In conscious models, respiratory monitoring on day(s) following treatment (*e.g.* 24 hrs post-dosing or as needed) can be useful to assess the reversibility of acute respiratory effects or to evaluate the presence of delayed respiratory effects. The most sensitive parameter of total respiratory system evaluation was MV, which was significantly altered with all positive control drugs at all doses, and in both models, except for remifentanil in anesthetized dogs, where apnea was present for all animals. Apnea is the most extreme expression of hypoventilation and confirms the suitability of the system for pharmacodynamic evaluations. As illustrated by standard deviations reported

in the current study, intrinsic inter-individual variability of respiratory baseline values is greater than cardiovascular safety pharmacology models observed variability (Authier et al. 2007). In this context, the authors consider that individual data should be reviewed by a respiratory pharmacologist in addition to statistical analysis of respiratory data, arterial blood gases and pH evaluation, when applicable, to ensure adequate conclusions. As previously reported, nearly all facilities use statistical analysis in safety pharmacology studies (Kinter et al. 1994). Statistical analysis is an important tool in safety pharmacology but the absence of statistically significant pharmacodynamic changes does not preclude that some animals may present drug induced adverse effects which may be relevant in human population and need to be reported.

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Conflict of Interest Statement

The authors declare that no conflict of interest that could influence the work submitted is present.

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Tables

Table 1
Effect of inert and positive control drugs on respiratory parameters in conscious beagle dogs

Test Agents		Respiratory Rate (b/min) Pre-Rx	Respiratory Rate (b/min) Post-Rx	Tidal Volume (mL) Pre-Rx	Tidal Volume (mL) Post-Rx	Minute Ventilation (mL/min) Pre-Rx	Minute Ventilation (mL/min) Post-Rx
Saline (0.2 mL/kg)	(n=12)	17.1 ± 4.5	16.4 ± 3.3	208 ± 59	217 ± 75	3409 ± 709	3443 ± 1201
Albuterol (100 µg/kg)	(n=5)	17.8 ± 7.0	21.7 ± 7.6	187 ± 80	239* ± 108	2933 ± 682	5759 ** ± 1364
Methacholine (2.0 µg/kg)	(n=8)	15.4 ± 5.1	17.1 ± 5.6	255 ± 39	308 ± 77	3776 ± 871	5490** ± 772
Methacholine (8.0 µg/kg)	(n=8)	16.0 ± 2.8	26.6** ± 7.4	236 ± 70	292* ± 55	3712 ± 1096	6994* ± 3025
Remifentanil (4.0 µg/kg)	(n=7)	16.8 ± 7.5	11.9 ± 4.8	289 ± 94	205 ± 154	4871 ± 1352	1576** ± 553

*p< 0.05, **p<0.01

Table 2

Effect of inert and positive control drugs on respiratory parameters in anesthetized beagle dogs

Test Agents		Respiratory Rate (b/min) Pre-Rx	Respiratory Rate (b/min) Post-Rx	Tidal Volume (mL) Pre-Rx	Tidal Volume (mL) Post-Rx	Minute Ventilation (mL/min) Pre-Rx	Minute Ventilation (mL/min) Post-Rx
Saline (0.2 mL/kg)	(n=15)	18.5 ± 10.9	21.0 ± 14.7	163 ± 7	170 ± 76	2490 ± 1060	2640 ± 1032
Albuterol (100 µg/kg)	(n=8)	18.7 ± 7.4	20.7 ± 7.1	154 ± 9	168 ± 80	2472 ± 514	3051* ± 425
Methacholine (8.0 µg/kg)	(n=8)	23.5 ± 15.8	35.6* ± 26.4	162 ± 9	157 ± 67	3299 ± 1542	4407* ± 2604
Remifentanil (4.0 µg/kg)†	(n=7)	18.7 ± 10.5	11.9 ± 4.2	168 ± 6	131 ± 61	2869 ± 1162	1877 ± 1235
CCK-4 (10 µg/kg)	(n=7)	14.5 ± 8.2	62.1** ± 37.4	179 ± 9	76** ± 22	2216 ± 720	5027* ± 3185
Hypoxic mixture (10% O_2)	(n=12)	25.7 ± 14.3	41.9** ± 27.0	177 ± 9	167 ± 59	3241 ± 1593	5384** ± 2414

*p< 0.05, **p<0.01, † transient apnea was present for all dogs following remifentanil injection.

Table 3

Effect of inert and positive control drugs on respiratory mechanics in anesthetized beagle dogs

Test Agents		Resistance (cm H_2O/L/s) Pre-Rx	Resistance (cm H_2O/L/s) Post-Rx	Elastance (cm H_2O/L) Pre-Rx	Elastance (cm H_2O/L) Post-Rx
Saline (0.2 mL/kg)	(n=15)	0.98 ± 0.48	0.93 ± 0.41	17.4 ± 7.6	16.7 ± 9.3
Albuterol (100 µg/kg)	(n=8)	1.13 ± 0.65	0.93 ± 0.66	18.5 ± 5.6	15.9 ± 3.5
Methacholine (8.0 µg/kg)	(n=8)	0.88 ± 0.22	1.28 ± 0.35**	22.9 ± 7.0	31.4 ± 4.7**
Remifentanil (4.0 µg/kg)†	(n=7)	0.78 ± 0.34	1.40 ± 1.05	23.3 ± 9.5	34.9 ± 14.7
CCK-4 (10 µg/kg)	(n=7)	0.99 ± 0.32	0.74 ± 0.21	19.4 ± 9.6	20.2 ± 9.1
Hypoxic mixture (10% O_2)	(n=12)	1.04 ± 0.75	1.37 ± 0.71	23.1 ± 13.4	20.0 ± 13.2

**p< 0.01, † transient apnea was present for all dogs following remifentanil injection.

Table 4
Effect of inert and positive control drugs on arterial blood gases and pH parameters in conscious and anesthetized beagle dogs

Blood gas analysis in conscious dogs	Glucose (mg/dl)	PCV (%)	Hb (g/dL)	pH	$PaCO_2$ (mmHg)	PaO_2 (mmHg)	HCO_3^- (mmol/L)	Beecf (mmol/L)	SaO_2 (%)
Saline (0.2 mL/kg)	91.8 ± 9.1	30.8 ± 5.4	10.5 ± 1.8	7.33 ± 0.04	34.9 ± 2.5	78.8 ± 14.5	18.7 ± 1.8	-7.2 ± 2.1	88.8 ± 13.7
Albuterol (100 µg/kg)	105.5 ± 17.7	31.5 ± 6.4	10.7 ± 2.1	7.32 ± 0.06	35.1 ± 5.4	70.5 ± 31.8	17.9 ± 0.1	-8.5 ± 0.7	87.5 ± 13.4
Methacholine (2.0 µg/kg)	86.8 ± 16.3	26.0 ± 7.1	8.8 ± 2.4	7.32 ± 0.03	37.1 ± 2.8	71.0 ± 19.2	19.1 ± 1.4	-7.0 ± 1.8	87.7 ± 10.2
Methacholine (8.0 µg/kg)	82.7 ± 20.5	28.3 ± 4.5	9.6 ± 1.5	7.31 ± 0.08	35.1 ± 4.9	65.3 ± 21.6	17.7 ± 2.9	-8.3 ± 4.0	87.7 ± 8.1
Remifentanil (4.0 µg/kg)†	112.3 ± 16.3**	39.8 ± 11.0	13.6 ± 3.7	7.25 ± 0.02**	42.2 ± 2.8**	67.5 ± 11.6	18.6 ± 1.6	-8.5 ± 1.87	88.5 ± 6.5
Blood gas analysis in unconscious dogs									
Saline (0.2 mL/kg)	96.9 ± 11.1	30.3 ± 3.8	10.3 ± 1.3	7.26 ± 0.05	48.0 ± 6.1	70.9 ± 9.7	21.3 ± 1.9	-5.8 ± 2.0	89.9 ± 5.5
Albuterol (100 µg/kg)	102.0 ± 9.2	31.6 ± 4.5	10.7 ± 1.6	7.30 ± 0.03*	44.5 ± 1.3	80.7 ± 6.2*	21.7 ± 1.1	-4.9 ± 1.5	94.0 ± 1.6
Methacholine (8.0 µg/kg)	102.8 ± 16.3	32.4 ± 9.4	11.0 ± 3.2	7.21 ± 0.02**	54.8 ± 7.1*	63.6 ± 24.3	21.6 ± 2.0	-6.1 ± 1.8	77.6 ± 28.4
Remifentanil (4.0 µg/kg)†	97.3 ± 10.4	29.5 ± 5.0	10.0 ± 1.7	7.18 ± 0.03**	58.7 ± 4.2**	39.7 ± 7.4**	21.9 ± 1.5	-6.5 ± 1.9	59.2 ± 5.2**
Cholecystokinin-4 (10 µg/kg)	114.9 ± 13.0**	32.4 ± 2.8	11.0 ± 1.0	7.20 ± 0.03**	54.9 ± 3.9**	46.9 ± 9.8**	21.5 ± 1.9	-6.6 ± 2.2	70.1 ± 11.9**
Hypoxic mixture (10% O2)	103.2 ± 15.5	32.8 ± 8.9	11.2 ± 2.7	7.30 ± 0.06*	42.3 ± 8.8*	36.1 ± 3.4**	20.6 ± 2.4	-5.8 ± 2.4	63.4 ± 4.9**

*p< 0.05, **p<0.01 intergroups comparison to the saline group of either conscious or unconscious status

Beecf: Base excess in extracellular fluids

Figures

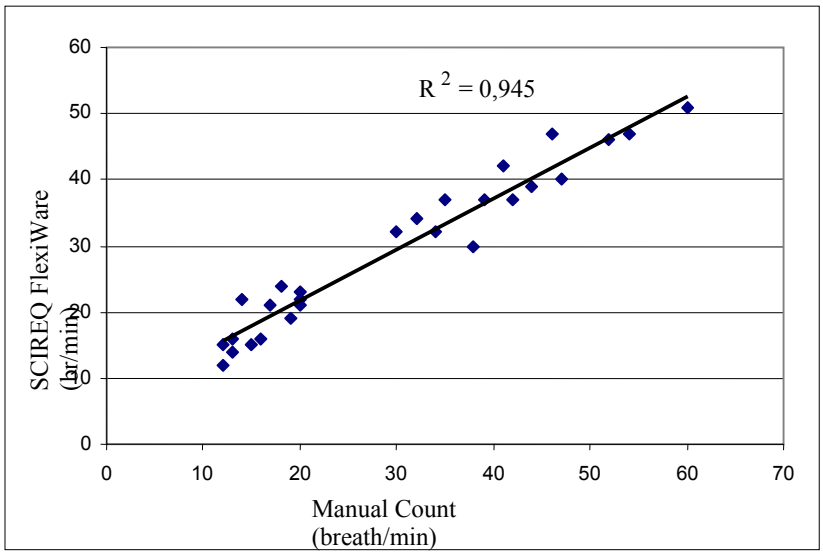

Fig. 1. Correlation between computerized and manual respiratory rate monitoring in conscious beagle dogs.

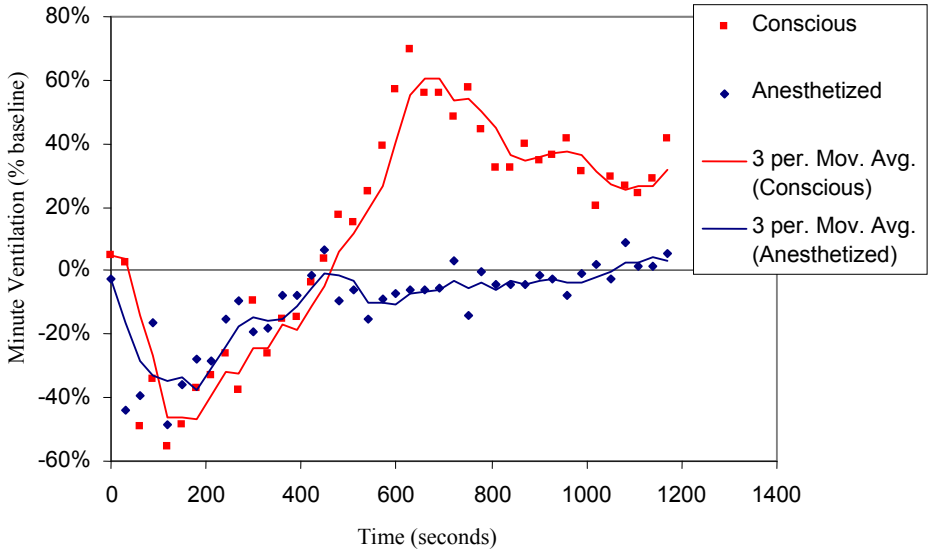

Fig. 2. Effect of remifentanil on minute ventilation in conscious and anesthetized beagle dogs. Data recorded at 200Hz and exported every 10 seconds with a 3 period moving average trend line (p<0.01).

Le point marquant de cet article est la démonstration de la faible sensibilité des gaz sanguins comme biomarqueurs pour évaluer les changements pharmacodynamiques du système respiratoire. Faisant contraste avec les recommandations internationales du S7A de l'ICH présentées précédemment, cet article démontre l'importance de la validation pharmacologique des modèles. De plus, la conclusion de l'article illustre l'importance de fonder la sélection des modèles précliniques sur des considérations scientifiques supportées par des données expérimentales. Des recommandations comparables ont été formulés pour le choix des formules de corrections de l'intervalle QT devant être choisies en fonction des données historiques de chaque laboratoire (Tattersall *et al.*, 2006). Un nombre importants de médicaments ont été évalués par l'utilisation des gaz sanguins comme biomarqueurs principaux de l'innocuité respiratoire sans que les fondements des recommandations des agences règlementaires soient remis en question. Depuis la publication de cet article, des changements dans le choix des biomarqueurs de pharmacologie de sécurité respiratoire ont été discutés par Dennis Murphy, expert international en pharmacologie de sécurité respiratoire, au 7^{ième} congrès annuel de la *Society of Safety Pharmacology* à Édinbourg en Écosse ainsi qu'au 11^{ième} congrès de la *International Congress of Toxicology* à Montréal en 2007.

Le choix des biomarqueurs discutés ci-haut, bien qu'important dans la conception d'un programme d'évaluation d'un médicament, n'est qu'un des facteurs déterminant du succès du plan de développement d'un médicament. Le fil conducteur dans ce processus de conception du plan d'évaluation préclinique demeure la pertinence des modèles utilisés en relation avec les objectifs des études précliniques. À cet effet, le choix des espèces animales se fait sur la base de considérations scientifiques, éthiques et souvent aussi économiques. La valeur prédictive de la réponse clinique est critique pour un effet secondaire adverse potentiellement fatal comme la torsade de pointe (Puglsey *et al.*, 2009). Par opposition, la valeur prédictive n'est pas aussi importante pour une modification mineure à modérée de la fonction respiratoire qui soit réversible. L'article qui suit présente des données comparatives de validation de différents modèles d'évaluation de la fonction

respiratoire. Notre démarche scientifique et les résultats permettent d'illustrer ce concept reconnu en développement pharmaceutique selon lequel les modèles choisis doivent être : "*Fit for use*".

7. PHARMACOLOGIE DE SÉCURITÉ RESPIRATOIRE CHEZ LE RAT SPRAGUE-DAWLEY, LE CHIEN BEAGLE ET LE SINGE CYNOMOLGUS: EST-CE QUE TOUTES LES ESPÈCES PRÉDISENT LA RÉPONSE CHEZ L'HUMAIN DE FAÇON ÉGALE ?

Cet article (publié dans *Regulatory Toxicology and Pharmacology*) est l'aboutissement de travaux de notre groupe afin de caractériser la réponse respiratoire (ventilation) chez le rat, le chien et le singe. Ces trois espèces couramment utilisées en pharmacologie de sécurité présentent des caractéristiques physiologiques et anatomiques menant à des différences de réponses respiratoires pour un même contrôle positif. En plus des différences pharmacodynamiques, ces espèces de niveau évolutif tout aussi différent suscitent des considérations éthiques dans le choix de l'espèce utilisée. Ce projet présente donc, grâce à l'utilisation de contrôles positifs dont la dose a été ajustée en fonction de la surface corporelle (*body surface area*, BSA), des résultats permettant d'évaluer la sensibilité relative des différentes espèces mais aussi la valeur prédictive de la réponse chez les patients.

Les résultats et discussions de cet article ont été présentés par Docteur Simon Authier au 7^{ième} congrès annuel de la *Society of Safety Pharmacology* à Édinbourg en Écosse ainsi qu'au 11^{ième} congrès de la *International Congress of Toxicology* à Montréal en 2007.

Respiratory Safety Pharmacology: Positive Control Drug Responses in Sprague-Dawley Rats, Beagle Dogs and Cynomolgus Monkeys

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Suggested section: **Safety Evaluation**

Short title: RESPIRATORY SAFETY IN THREE SPECIES

Experimental work was undertaken at: LAB Research Inc., 445 Armand Frappier, Laval, Québec, H7V 4B3, Canada

ABSTRACT

Rats are most frequently used to fulfill ICH S7A requirements for respiratory safety pharmacology. We hypothesized that the models used to assess respiratory safety pharmacology present different ventilatory responses to bronchoconstriction, bronchodilation and respiratory depression. Respiratory monitoring was performed with head-out plethysmographs for rats, masks for dogs and bias airflow helmets for monkeys. Respiratory rate (RR), tidal volume (TV) and minute volume (MV) were recorded. Forty rats, 18 dogs and 8 monkeys were acclimated to the respiratory monitoring equipment. Animals received saline (IV), albuterol (inhalation), methacholine (IV) and remifentanil (IV). Albuterol increased TV in all species. Methacholine decreased TV and MV in monkeys. In dogs, methacholine increased TV, RR and MV. In rats, methacholine increased TV and decreased RR. Remifentanil induced central respiratory depression in all species with decreased MV, except in rats. Dogs presented a biphasic response to remifentanil with hypoventilation followed by delayed hyperventilation. The monkeys presented similar responses to humans which may be due to biologic similarities. Dogs and rats presented clinically significant ventilatory alterations following positive control drugs. Although, the response to bronchoconstriction in dogs and rats was different from humans, the two species presented ventilatory changes that highlight the potential adverse effect of test articles.

Keywords: Respiratory safety, ICH S7A, rat, monkey, dog, conscious, tidal volume, respiratory rate, minute volume

1. INTRODUCTION

The ICH S7A Guideline defines the safety pharmacology core battery including cardiovascular (CVS), central nervous system (CNS) and respiratory (U.S. Food and Drug Administration, 2001). The guideline states that "*respiratory rate and other measures of respiratory function (e.g. tidal volume or hemoglobin oxygen saturation) should be evaluated*". A recent industry survey revealed that respiratory rate, tidal volume and minute volume are included in 98 to 100% of core battery and supplemental respiratory safety pharmacology studies (Lindgren et al. 2008). Study design in safety pharmacology is constantly evolving prodded by emerging technologies (Hoymann, 2007; Murphy *et al.*, 1998; Murphy *et al.*, 2001) and integrated drug development where *in vitro*, preclinical and clinical safety testings share a common goal, drug approval. Sensitivity of the model should be commensurate with the risk in our drug development industry where resources are scarce and time is felt by drug companies with aging drug patents. Blood gases, reported to have limited sensitivity (due to complex compensatory mechanisms) to detect drug-induced adverse effects (Authier *et al.,* 2008), and RR are occasionally used as sole *in-vivo* markers of preclinical respiratory safety. With the addition of histopathological assessments, these two *in-vivo* biomarkers (blood gases and RR) are, in some cases, considered acceptable for respiratory safety testing (*e.g.* some oncology indications).

When non-clinical and clinical reasons for drug development discontinuation are combined, respiratory adverse effects are less frequent than CVS or CNS (Valentin & Hammond, 2008). Moreover, respiratory safety pharmacology is less frequently frontloaded (performed earlier in the drug

development process) than CVS and CNS which may suggest a perceived lower risk of respiratory liability in comparison to the other two systems included in the safety pharmacology core battery.

The rat is used as the preferred species for respiratory safety in most investigational new drug (IND) programs (Lindgren et al. 2008). Advantages to the use of rats are numerous including high genetic homogeneity which reduces variability, lower test material requirements, abundant historical data and ethical considerations which favour the use of a phylogenically lower laboratory animal species. On the flip side, the use of rats may present disadvantages including limited genetic diversity which may not be representative of the human patient population. Respiratory anatomy, physiology and pathology in rats are relatively different from humans, an adaptive response to their respective normal habitat. Large laboratory animals such as dogs and monkeys present respiratory system characteristics which could be considered closer to humans.

IND submissions often require that a small and a large animal species be selected for toxicology studies. CVS safety pharmacology assessments are most frequently performed in large animals (Lindgren *et al.*, 2008) using dogs (Gauvin *et al.*, 2006) or monkeys (Authier *et al.*, 2007). Respiratory assessments to fulfil S7A can be performed using minimally invasive methodologies in toxicology studies or in large animals used for CVS safety pharmacology without the need to use additional animals. As large animals present biologic similarities with human patients, inclusion of respiratory measures in large animal studies may increase the clinical relevance of these investigations. The current project presents the ventilatory responses of

common respiratory safety pharmacology models to bronchodilation, bronchoconstriction and respiratory depression using well characterized pharmaceutical agents.

2. MATERIALS AND METHODS

2.1. Statement on use and care of animals and regulatory compliance. During the study, care and use of animals were conducted in accordance with principles outlined in the current Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care and the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). LAB Research Inc.'s facility is AAALAC accredited. Data were obtained under studies conducted in accordance with the Good Laboratory Practice (GLP) regulations of the United States Food and Drug Administration (21 CFR Part 58 and subsequent amendments).

2.2. Animal housing and preparation. The experimental population comprised forty (40) male Sprague-Dawley rats (242 to 315g, 59 to 65 days), eighteen (18) Beagle dogs (6 males and 12 females, 8 to 18 kg, 1 to 4 yrs) and eight (8) cynomolgus (*Macaca fascicularis*) monkeys (4 males and 4 females, 2.5 to 4.6 kg, 3 to 5 yrs old). The animal room environment was controlled and monitored continuously (targeted ranges: temperature 21 ± 3°C, humidity 30-70%, 12 hours light, 12 hours dark, 10-15 air changes per hour). Rats were fed a standard rodent chow *ad libitum* (Teklad Certified 18% Rodent Diet #2018C). Dogs received a standard certified commercial dog chow (400 g of Harlan Teklad Certified 25% Lab Dog Diet #8727C) over a 24-hour feeding period. A standard certified commercial primate chow (Teklad Certified

Global 25% Primate Diet # 2055C) was made available to each monkey twice daily. Clinical signs were evaluated at cage side at least once daily, and a detailed clinical examination was performed at transfer and once weekly throughout the studies.

2.3. Respiratory Monitoring System. Respiratory rate (RR), tidal volume (TV) and minute volume (MV) were recorded continuously at a sampling frequency of 200 Hz in all species. Respiratory function was monitored in rats using head-out plethysmographs (Model PC-H2, SCIREQ Inc., Montreal, QC, Canada) with opaque headrest connected to a pneumotachometer (Model 8420 with a 5 L/min capacity, HANS RUDOLPH, MO, U.S.A.). The headrests were such that the nose of the rats was open to ambient air but visual and auditory stimulations were attenuated providing a more comfortable environment for the animals. Airflow signals from rat pneumotachometers were monitored using precision differential pressure transducers (Model UT-PDP-02, 0.2 kPa nominal, SCIREQ Inc., Montreal, QC, Canada) connected to the data acquisition matrix (In expose-08, Data Acquisition Controller 8 channels, SCIREQ Inc., Montreal, QC, Canada) and a real-time respiratory analyzer (flexiWare 5.3.1, SCIREQ Inc., Montreal, QC, Canada).

Respiratory monitoring was performed in conscious Beagle dogs as previously described (Authier *et al.*, 2008). In brief, canine respiratory function was monitored using a computerized system composed of a data acquisition controller (DAC 8, Scientific Respiratory Equipment Quebec Inc. (SCIREQ), Montreal, QC, Canada) using a real-time respiratory analyzer (flexiWare 5.1, SCIREQ Inc., Montreal, QC, Canada) connected to pneumotachometer (Model 3719 with a 100L/min capacity, HANS

RUDOLPH, MO, U.S.A.) with individual heater controllers (HANS RUDOLPH, MO, U.S.A.) connected to a face mask.

Respiratory function was monitored in conscious cynomolgus monkeys using a computerized system composed of a data acquisition controller (In expose-08, Scientific Respiratory Equipment Quebec Inc. (SCIREQ), Montreal, QC, Canada) connected to a computer (OptiPlex GX280 Workstation, DELL, Dallas, TX, U.S.A.) with a real-time respiratory analyzer (flexiWare 5.1, SCIREQ Inc., Montreal, QC, Canada). The respiratory hardware included pneumotachometer (Model 3500 with a 35L/min capacity, HANS RUDOLPH, MO, U.S.A.) with individual heater controller (HANS RUDOLPH, MO, U.S.A.). Monkeys were acclimated to a restraining chair and a transparent plexiglass helmet (LOMIR BIOMEDICAL, Notre-Dame-de-l'Île-Perrot, QC, Canada) with a biais airflow (inExpose pump module 2.5 lpm, SCIREQ Inc., Montreal, QC, Canada) and continuous helmet pressure monitoring (Model UT-PDP-25, 2.5 kPa nominal, SCIREQ Inc., Montreal, QC, Canada). Helmet pressure was maintained neutral to ambient pressure.

Rats, dogs and monkeys were acclimated to respiratory monitoring on three (3) different occasions before initiation of treatment. Rats and dogs that did not tolerate the respiratory monitoring were replaced by animals kept in the same experimental conditions. Respiratory monitoring was well tolerated by all monkeys assigned to the study and no replacement was required.

2.4. Positive and negative control drugs. Saline (BAXTER, ON, Canada), albuterol (RATIOPHARM Inc, QC, Canada), methacholine (SIGMA-ALDRICH, ON, Canada) and remifentanil (Ultiva®, ABBOTT LABORATORIES Ltd., ON, Canada) were administered to conscious rats,

dogs and monkeys at doses selected based on historical data obtained in previous studies conducted at LAB Research and adjusted for body surface area (BSA) to induce slight and moderate to severe respiratory effects as presented in Table 1. The dose volume was 2.0 mL/kg for rats, and 0.2 mL/kg for dogs and monkeys (all intravenous agents). Albuterol was administered using a pressurized metered-dose inhaler with a holding chamber and a mask. Monitoring was initiated at least 15 min before dosing in all species. Respiratory data during the first 20 min after administration was used for comparison between species given the rapid onset of pharmacological effects with selected agents.

2.5. Data analysis. Data were averaged every 5 min and results following positive control drugs compared with saline administration. For rats, one (1) min averages at peak effect were also calculated. Normality of distribution was evaluated using the Shapiro-Wilk test. The Levene test was used to examine the homogeneity of group variances. When both of these tests were found to be non-significant, analysis of variance (ANOVA) was considered appropriate. Whenever the overall group differences were shown significant (F-Test for ANOVA), then pair-wise comparisons were conducted using Dunnett's test for ANOVA. Comparisons among dose levels at each timepoint were done using a T-test and including the Satthertwaite method in presence of heterogenous group variances. Results are presented as mean values ± standard error of the mean (SEM).

3. RESULTS

Average RR after saline treatment was 175 ± 15 b/min, 16.4 ± 3.3 b/min and 50.5± 3.7 b/min in rats, dogs and monkeys, respectively. Average TV after saline treatment was 1.30 ± 0.07 mL, 217 ± 75 mL and 42 ± 17 mL in rats, dogs and monkeys, respectively. Mean MV following saline administration were 0.257 ± 0.004 L/min, 4.050 ± 0.407 L/min and 2.054 ± 0.315 L/min in rats, dogs and monkeys, respectively.

Albuterol administered by inhalation induced an increase in TV (Fig. 1) in rats, dogs and monkeys when using an analysis with 5 min averages for a 20 min monitoring period compared with saline treatment. Changes to RR were not statistically significant (Fig. 2) in any species during the 20 min monitoring period following treatment, while MV was significantly increased in dogs and monkeys (Fig. 3).

Monkeys presented a decrease in TV ($p<0.01$) and MV ($p<0.01$) following administration of methacholine with a trend to a compensatory increase in RR at higher doses (Fig. 4). In contrast, respiratory monitoring in dogs differed with the dose of methacholine: at low dose (2 μg/kg), only MV presented a statistically significant change associated with a decrease in TV and an increase in RR at T10 min; at high dose (8 μg/kg) dogs presented an initial increase in RR ($p<0.01$) followed by an increase in TV ($p<0.05$) associated to a decrease in RR and a sustained increase in MV ($p<0.05$) (Fig. 5). In rats, systemic administration of methacholine resulted in dose-dependent effects: at low dose (28 μg/kg), no statistically significant effect was observed (Fig. 6), but at high dose (136 μg/kg), methacholine induced a significant increase in TV ($p<0.01$) with a decrease in RR ($p<0.05$) (Fig. 6).

As expected for a potent mu agonist opioid, remifentanil induced a significant respiratory depression in all three species (Table 2). Rats presented an initial and transient (at T5) decrease in RR, immediately compensated by a transient (at T10 and T15) increase in TV, without significant change in MV. When using one (1) min averages starting at pharmacological onset, RR (-37.4% ± 4.2%) and MV (-32.6% ± 5.3%) were significantly decreased (p<0.01) in rats following 14 μg/kg remifentanil administration. Dogs presented a biphasic response with hypoventilation followed by a delayed phase of hyperventilation with increased RR. Panting was noted in some dogs when adverse respiratory effects were seen which complicated respiratory analysis with this species. In monkeys, apnea was observed in 3 out of 7 monkeys at high dose (6.4 μg/kg), which prompted reversal of the opioid effects with naloxone (0.4 mg/mL, IV).

4. DISCUSSION

Species differences are recognized by regulators and dictate selection of maximum safe starting dose during initial clinical trials in healthy volunteers (U.S. Food and Drug Administration, 2005). Allometric correlations have been reported between species for metabolic rates but also for physiological parameters such as heart rate (West *et al.*, 2002), cardiac output, respiratory rate and ventilation (Lindstedt & Schaeffer, 2002). Allometric correlation between body weight and MV has been recognized for several decades (Guyton, 1947; Stahl, 1967). Inter-species correlations are recognized *de facto* in toxicology which uses models from multiples species to predict the human response. Allometric scaling of respiratory parameters is also a central exercise in inhalation toxicology studies for dose estimation. A

formula ($V(m)=0.608\ BW^{0.852}$) to estimate respiratory MV adapted to laboratory animals (mouse, rat, dog and monkey) was recently published by the Association of Inhalation Toxicologists (AIT) (Alexander *et al.*, 2008). When using the recent AIT formula, the predicted MV (rats 0.191 ± 0.003 L/min; dogs 5.227 ± 0.225 L/min; monkeys 1.759 ± 0.100 L/min) were closer to actual respiratory measurements following saline administration than the predicted MV calculated (rats 0.166 ± 0.002 L/min; dogs 3.846 ± 0.196 L/min; monkeys 1.368 ± 0.074 L/min) with the Bide formula ($V(m)=0.499\ BW^{0.809}$) which is commonly used for dose calculation in inhalation toxicology studies (Bide *et al.,* 2000). These results support the superior predictive value of the recent AIT formula to estimate MV when compared with the Bide formula. This closer correlation between predicted and measured MV using the AIT formula supports the use of the later in our experimental conditions. The importance to evaluate standard formulas with in-house data was demonstrated for QT correction formulas (Tattersall *et al.*, 2006). Similarly, allometric formulas used in dose calculation for inhalation toxicology studies benefit from qualification using actual MV values recorded in experimental conditions that prevail in each laboratory.

In humans, systemic administration of albuterol induces a significant increase in TV and MV (Sorbini *et al.*, 1984) which is thought to result from an increased metabolic rate and serum lactate (Tobin *et al.*, 2006). Similar to humans, monkeys and dogs presented a significant increase in TV and MV, while only increase in TV reached statistical significance in rats when compared with Saline. The duration of effects was longer in monkeys when compared to dogs at equivalent doses (0.1 mg/kg in dogs and 0.2 mg/kg in

monkeys). This could be related to a greater respiratory reserve in dogs which resulted in a rapid compensation. Albuterol by inhalation induces tachycardia with a decrease in systolic pressure in beagle dogs (Petruska *et al.*, 1997). Similarly, rats presented tachycardia following inhaled albuterol at doses of 84 μg/kg (4.8 times lower than the dose used in the current study). Doses up to 7.5 times higher than the high dose (0.2 mg/kg) used in this study did not produce significant cardiovascular effects in cynomolgus monkeys. Cardiovascular effects of albuterol, also reported in healthy volunteers (Corea *et al.*, 1984), may contribute to respiratory changes when using this positive control drug in respiratory safety models and in humans. Differences in ventilatory responses observed between the three models may be due to species specific sensitivity to cardiovascular and/or respiratory effects. Cardiopulmonary dependency is an emerging concept in safety pharmacology that often requires monitoring of the respiratory and cardiovascular systems simultaneously in the same animals to identify correlation between the two systems.

Methacholine at the high dose (8 μg/kg in dogs and 13.5 μg/kg in monkeys) induced diametrically opposite effects with a significant increase of MV in dogs while monkeys presented a decreased MV. Whereas TV was consistently decreased in monkeys at high dose methacholine, in dogs, TV was initially slightly reduced as previously reported (Savoy *et al.*, 1982) and associated with an abrupt increase in RR, followed by a second phase of respiratory response including increase in TV and return toward baseline values for RR. Such results suggest that the dog is highly responsive to the bronchoconstrictive effects of methacholine resulting in an abrupt increase in

RR. Healthy humans are reported to present a decreased MV and TV (Fujimori K *et al.*, 1996) similar to the response observed in monkeys. Similar to dogs, the rats presented a response different from humans and monkeys with an increase in TV and a decrease in RR at the doses used in the current study. It remains that all three (3) species presented significant ventilatory changes following bronchoconstriction with methacholine and are considered suitable models to detect the presence of respiratory liability. Core battery safety pharmacology studies should identify potential adverse effects in human patients. The response to bronchoconstriction, although different between species, will yield the same conclusion; identification of a potential respiratory (adverse) effect in humans which will trigger appropriate monitoring during early clinical trials.

Remifentanil, a potent mu-agonist opioid, leads to respiratory depression in humans (Smith *et al.*, 1997), which translates into decreased MV. Expected effects were observed in all species, but in rats the depression was more evident with the analysis at one (1) minute interval. Indeed for rats, analysis at onset of pharmacological effects with one (1) min averages revealed higher sensitivity to detect changes after remifentanil administration. The difference in statistical results between one (1) and five (5) min averages highlights the importance of post-acquisition data analysis which needs to be tailored to each test article in order to capture pharmacological effects. On the other hand, adapting statistical analysis *at posteriori* to capture an unknown pharmacodynamic response is a challenging issue in regulatory toxicology where any modification to the analysis plan would raise concerns on potential bias.

Results from the current study suggest that the ventilatory response to bronchoconstriction in the monkey is closer to humans. These pharmacodynamic similarities correlate with anatomic, physiologic and histologic characteristics of monkeys that resemble humans while dogs and rats present lower level of homology with humans. Monkeys (Dungworth *et al.*, 1975), dogs (Takenaka *et al.*, 1998) and humans (Saetta *et al.*, 1994) have several generations of respiratory bronchioles while rats have either no respiratory bronchioles of a single generation (Tyler & Julian, 1991; Saetta *et al.*, 1994). The number of alveolar pores, which facilitates collateral ventilation, is similar in dog, monkey and human alveolus but lower in rats (Port *et al.*, 1977). Submucosal glands are observed throughout the bronchial tree of dog (Takenaka *et al.*, 1996), monkeys (El-Bermani & Grant, 1975) and humans (Scott, 1973) while rats do not have bronchial glands (Jeffery, 1983). Respiratory neural organization in monkeys, dogs and rats is comparable to humans (Kastner & Gauthier, 2008) with respiratory centers (inspiratory, expiratory, pneumotaxic and apneustic) located in the medulla oblongata and multiple nervous effectors controlling ventilation such as the phrenic and intercostal nerves (King, 2005). Despite central similarities the pulmonary innervation presents differences between species. The rat lung innervation presents significant differences when compared with monkeys (El-Bermani, 1978) while lung innervation in dogs is comparable to monkeys (Knight *et al.*, 1981). Will anatomical and physiological similarities between monkeys and humans translate into increased predictive value with this species? If so, will increased predictive value alter the decision making process during drug development? The answer resides in an integrated risk assessment of the

toxicology testing plan. Despite differences between humans and rats, the rat model is widely accepted in pharmaceutical research (Tschernig *et al.*, 2008) and this species remains predictive of the patient response. Clinically significant alterations to respiratory parameters were noted in rats with all positive control drugs used in this study supporting the use of the three species for respiratory safety investigations.

The current study holds some limitations and complete dose-response curves would be needed to compare species sensitivity. It remains that dose levels that were used reliably induce bronchoconstriction, bronchodilation and respiratory depression given the well-characterized positive control drugs that were selected. As observed in the current study, dogs occasionally present panting when stressed or in response to drug induced adverse effects. Panting during respiratory monitoring acclimation in dogs triggers exclusion of individuals. When present, panting decreases accuracy of the ventilatory measures and increases artefacts due to excessively rapid ventilation of the respiratory dead space. Occasional panting is an inconvenient of the canine model and the rat can be preferred for respiratory safety testing when these two species are selected for toxicology studies. In contrast, the cynomolgus monkey maintains a tidal breathing pattern and the inclusion of non-invasive respiratory (ventilatory) investigations in toxicology studies may present some advantages over ventilatory assessments in rats.

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Table 1

Dose levels of positive control drugs

	Route	Rats	Dogs	Monkeys
Saline	IV	--	--	--
Albuterol (µg/kg)	Inhalation	400	100	100
				200
Methacholine (µg/kg)	IV	28	2	3.4
		136	8	13.5
				68.0
Remifentanil (µg/kg)	IV	14	4	3.4
				6.8

Table 2 – Respiratory parameters following remifentanil (5 min averages starting at injection)

		Dose	Respiratory Rate (b/min)		Tidal Volume (mL)		Minute Volume (mL/min)	
Control Articles		µg/kg	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
Sprague-Dawley Rats	(n=8)	14	188.7 ± 10.4	164.4* ± 7.3	1.517 ± 0.069	1.683* ± 0.089	271.6 ± 13.5	262.4 ± 12.0
Beagle Dogs	(n=7)	4	15.8 ± 2.3	11.6* ± 0.8	270.7 ± 19.4	243.2 ± 24.3	3913 ± 440	2339** ± 193
Cynomolgus Monkeys	(n=8)	3.4	49.2 ± 3.2	39.6 ± 2.5	39.4 ± 3.5	23.0** ± 3.2	1864 ± 163	911** ± 165
	(n=7)[a]	6.8	50.4 ± 4.4	41.7 ± 3.4	41.6 ± 3.9	28.4* ± 4.5	1945 ± 143	1297* ± 287

* $p<0.05$, ** $p<0.01$

[a] Effects of remifentanil was reversed with naloxone IV for 3 out 7 animals due to severe apnea.

Figures

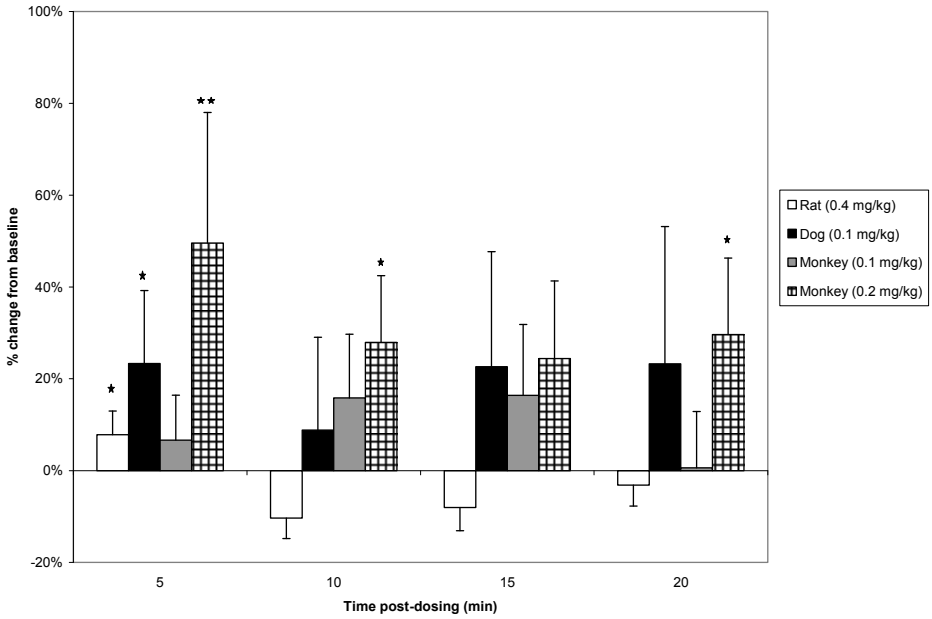


FIG. 1. Tidal volume (TV) after albuterol administered by inhalation to Sprague-Dawley rats (n=8), Beagle dogs (n=5) and cynomolgus monkeys (n=8). Overall group difference when compared with saline was significant for rats (p<0.05), dogs (p<0.05) and monkeys at 0.2 mg/kg (p<0.01).

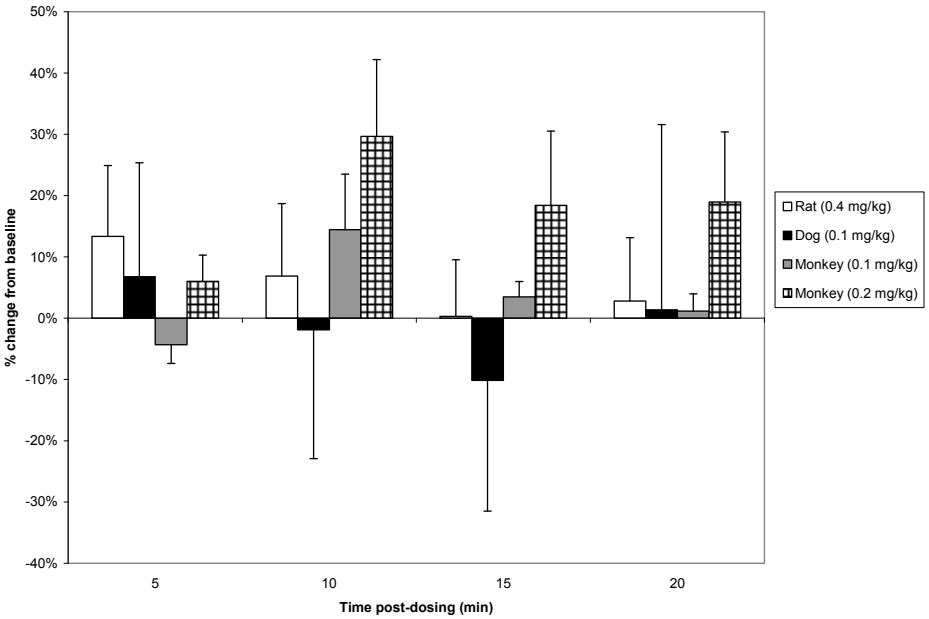


FIG. 2. Respiratory rate (RR) following albuterol administered by inhalation to Sprague-Dawley rats (n=8), beagle dogs (n=5) and cynomolgus monkeys (n=8). Overall group difference when compared with saline using ANOVA was not significant for any of the 3 species during the 20 min monitoring period with 5 min averages.

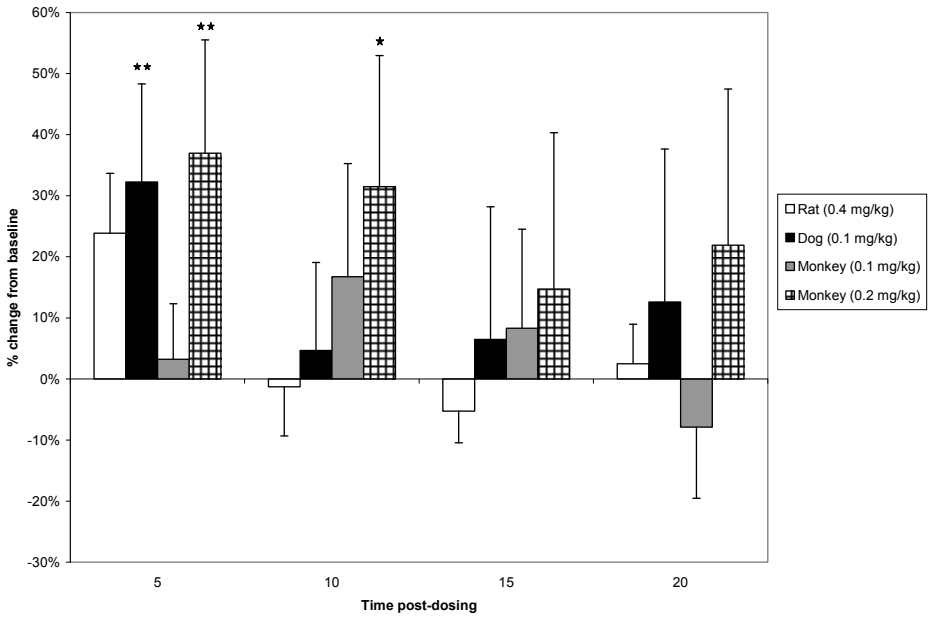


FIG. 3. Minute volume (MV) following albuterol administered by inhalation to Sprague-Dawley rats (n=8), beagle dogs (n=5) and cynomolgus monkeys (n=8). The overall group difference was statistically significant when compared with saline for dogs (p<0.01) and monkey at 0.2 mg/kg (p<0.01), while overall group difference did not reach statistical significance in rats when compared with saline.

** = $p < 0.01$.

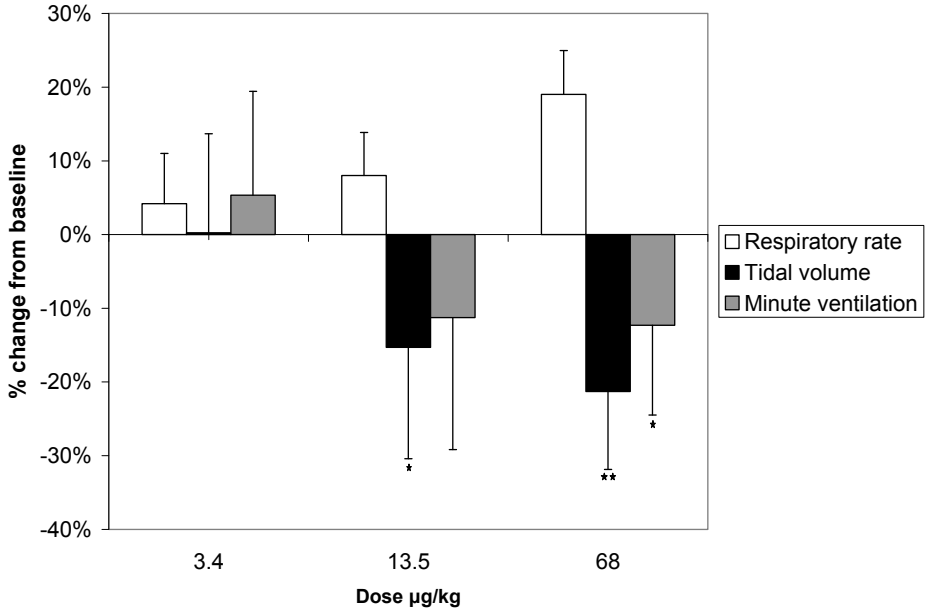
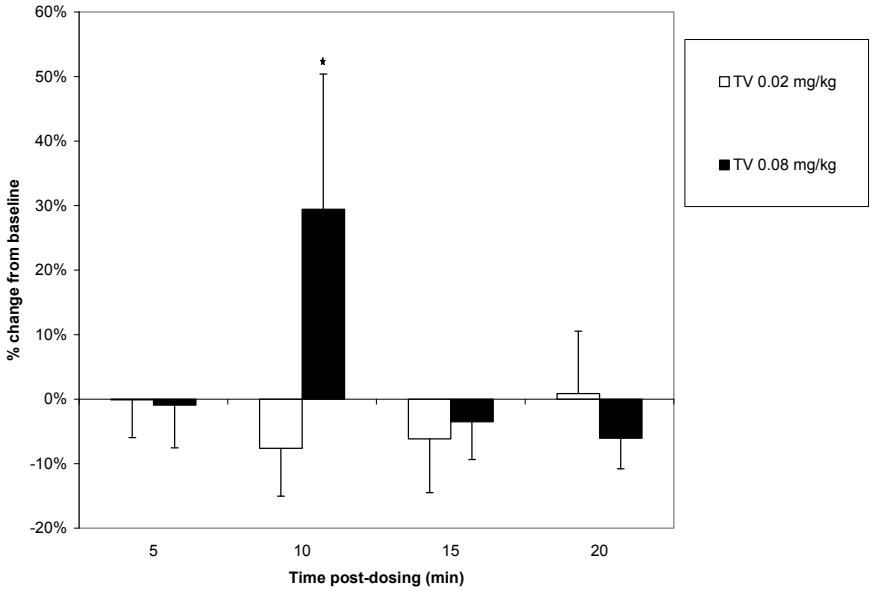


FIG. 4. Respiratory monitoring following methacholine bolus administration (IV) to cynomolgus monkeys (n=8). Overall difference was significant for tidal volume (p<0.01) and minute volume (p<0.01) and significance at each dose is presented above. A trend to compensatory increase in respiratory rate was observed following methacholine administration at 13.5 and 68 µg/kg.

* = $p < 0.05$, ** = $p < 0.01$.

A


60%
50%
40%
30%
20%
10%
0%
-10%
-20%
% change from baseline
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5
10
15
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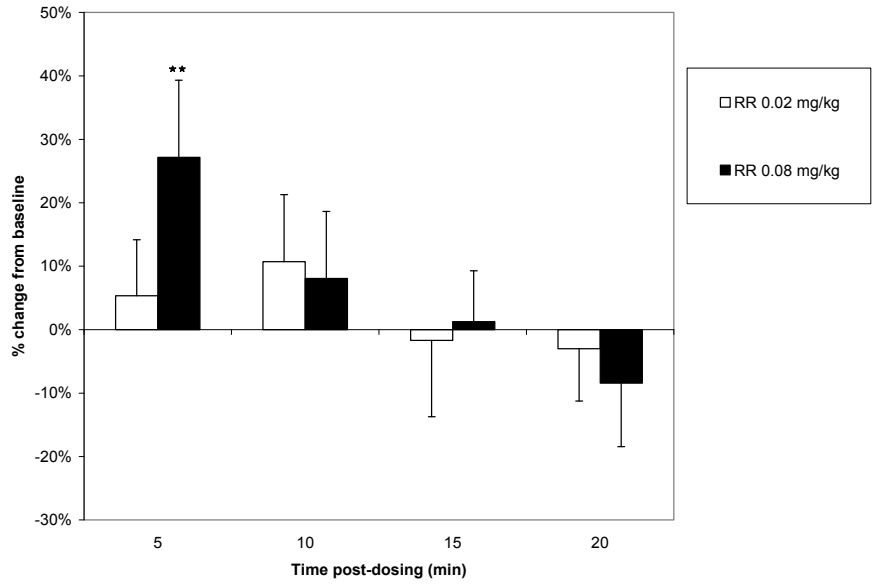
Time post-dosing (min)
TV 0.02 mg/kg
TV 0.08 mg/kg

B


50%
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Time post-dosing (min)
RR 0.02 mg/kg
RR 0.08 mg/kg

C

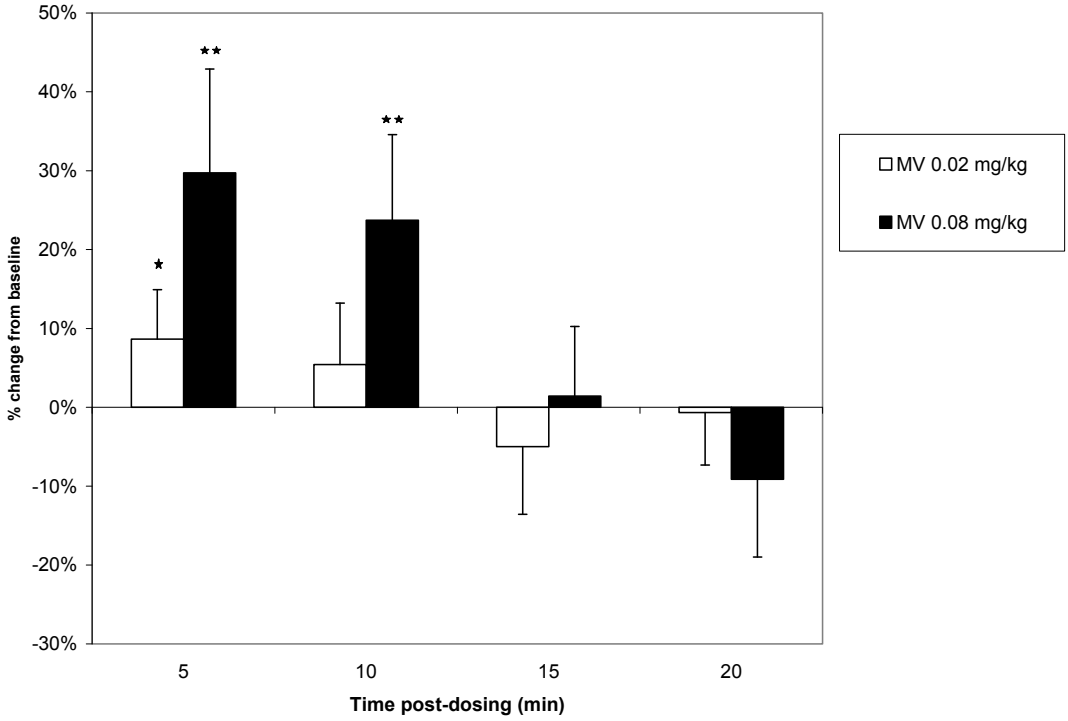

FIG. 5. Tidal volume (5A), respiratory rate (5B) and minute volume (5C) following methacholine bolus administration (IV) to beagle dogs (n=8). The overall difference was significant for MV at 2 μg/kg (p<0.01) and for all 3 parameters at 8 μg/kg (RR, p<0.01; TV, p<0.05; MV, p<0.05). At 8 μg/kg, a sustained increase in MV is explained by a biphasic response, with an initial increase in RR, followed by an increase in TV while RR is returned toward baseline.

* p<0.05; ** p<0.01

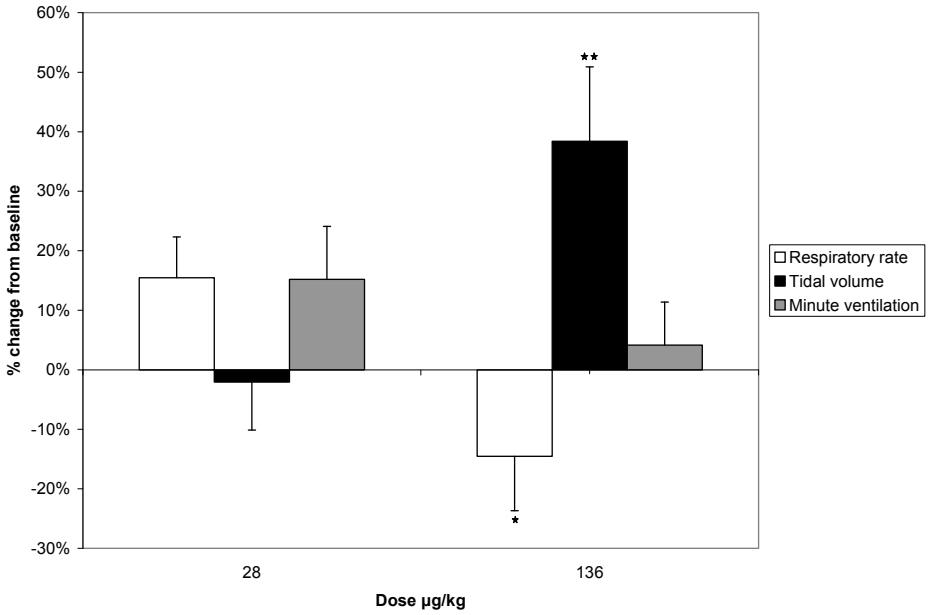

FIG. 6. Respiratory monitoring (5 min average at pharmacological onset) following methacholine bolus administration (IV) to Sprague-Dawley rats (n=8). When compared with saline, RR was decreased (p<0.05) and TV was increased (p<0.01) after methacholine at 136 µg/kg. The changes in RR (p=0.07) and MV (p=0.11) at 28 µg/kg did not reach statistical significance.

* = $p < 0.05$, ** = $p < 0.01$.

Cet article suggère une réponse respiratoire chez les primates plus près de la réponse chez les patients en comparaison des réponses chez le rat et le chien. Malgré cette similarité entre le primate et l'humain, le rat demeure l'espèce la plus communément utilisée en pharmacologie de sécurité respiratoire. Tel que mentionné précédemment, le modèle choisi doit être approprié pour les objectifs à atteindre ("*fit for use*"). En raison de considérations éthiques, le choix des singes comme espèces précliniques n'est justifié que lorsque le rat est jugé comme une espèce inadéquate. La sensibilité moindre des rats reste acceptable considérant la tolérance des patients aux effets pharmacologiques respiratoires. En plus des évaluations de sécurité cardiovasculaire et respiratoire, le système nerveux occupe une position clé dans le processus de confirmation de la sécurité d'un médicament faisant partie des études de base incluses dans les lignes directrices S7A de l'ICH. Les évaluations neurologiques de base largement acceptées par la communauté scientifique en pharmacologie de l'innocuité demeurent pratiquement inchangées depuis plus d'un demi-siècle (Irwin, 1968) comportant des évaluations subjectives et quantitatives permettant d'évaluer la présence d'effets potentiels sur le système nerveux central et périphérique.

En résumé, la conception des études d'évaluation de base de pharmacologie de sécurité est influencée par une multitude de facteurs scientifiques (ex. valeur prédictive, sensibilité), médicaux (ex. possibilité de transfert des biomarqueurs aux études cliniques, bénéfice pour les patients, présence de thérapies alternatives) et règlementaires (ex. fiabilité, conformité avec les lignes directrices, validation des modèles). Au-delà des modèles couramment utilisés, les effets pharmacologiques des médicaments découverts lors des études de bases font éclore de nouvelles préoccupations quant à la sécurité des médicaments à l'essai. Dans une telle situation, les pharmacologistes doivent mettre en place des modèles de pharmacologie de sécurité appropriés pour évaluer en profondeur les effets secondaires observés. À titre d'exemple, l'évaluation des risques épileptogènes des médicaments constitue un champ de recherche soutenu en pharmacologie de sécurité depuis les dernières années. (Dürmüller *et al.*, 2007; Winter *et al.*, 2008 ; Easter *et al.*, 2009). Notre groupe de recherche a ainsi complété la validation d'un modèle

d'évaluation du potentiel épileptogène par mesure de l'electroencéphalographie par radiotélémétrie chez le singe conscient et sans contention (Authier S, Paquette D, Gauvin D, Sammut V, Fournier S, Chaurand F, Troncy E. Video-electroencephalography in conscious non human primate using radiotelemetry and computerized analysis: refinement of a safety pharmacology model. *J Pharmacol Toxicol Methods* 2009;60(1):88-93 article inclus en annexe).

Après avoir présenté le contexte et les enjeux du développement pharmaceutique dans la revue de littérature, cette première partie des résultats expérimentaux a permis de définir les étapes et le processus de validation des modèles précliniques de pharmacologie de sécurité tout en présentant les grandes sphères d'évaluation de la pharmacologie de sécurité. En mettant en évidence la nécessité de justifier le choix des modèles précliniques par des considérations scientifiques, médicaux et éthiques cette première partie de la thèse prépare la scène pour la deuxième partie de la thèse qui évalue la sécurité de l'OT dans différents modèles d'animaux sains suivie de l'évaluation de l'efficacité d'un plan de traitement dans un modèle d'infarctus du myocarde.

Deuxième partie

ÉVALUATION DE LA SÉCURITÉ ET DE L'EFFICACITÉ DE L'OXYTOCINE COMME THÉRAPIE DE L'INFARCTUS DU MYOCARDE

Chapitre 4: LA DIFFÉRENTIATION CELLULAIRE EN CARDIOMYOCYTES INDUITE PAR L'OXYTOCINE

REVUE DE LITTÉRATURE

8. LE RÔLE POTENTIEL DE L'OXYTOCINE COMME TRAITEMENT DE L'INFARCTUS DU MYOCARDE

Au cours des dernières années, le rôle de l'OT dans la différentiation des cardiomyocytes au cours de l'embryogénèse s'est confirmé. Des études récentes chez la souris suggèrent que l'OT joue un rôle très tôt dans le développement embryonnaire. En effet, on observe l'expression de récepteurs d'oxytocine (OTR) chez un nombre élevé de cellules embryonnaires murines à des stades précoces du développement (Stefanidis *et al.,* 2009). De plus, on note une augmentation de l'expression d'OTR au jour 7 de la gestation chez la souris, soit au moment où la différentiation cardiaque survient (Mukaddam-Daher *et al.*, 2002).

Des travaux avec une lignée cellulaire embryonique ont permis de mieux définir le rôle de l'OT dans la différentiation myocardique. L'exposition des cellules de carcinome embryonnaire P19 à l'OT *in vitro* induit la différentiation en cardiomyocytes (Paquin *et al.*, 2002; Bouchard & Paquin, 2008). Une lignée de cellules souches embryonnaires murines (Royan B1, C57BL/6 strain) a également démontré la capacité de se différentier en cardiomyocytes suite à l'exposition à l'OT ce qui fournit une évidence additionnelle du potentiel cardiomyogénique de l'OT (Hatami *et al.*, 2007).

Bien qu'on considère qu'OTR soit impliqué dans la différentiation des cardiomyocytes, les voies de signalisation menant à cette différentiation en cardiomyocytes suite à l'activation du récepteur ne sont pas complètement élucidées. Il y a plus de quinze ans, on démontrait que le dimethylsulfoxide (DMSO) avait la capacité d'induire la différentiation de cellules de carcinome embryonnaire P19 en myocytes ayant des propriétés propres aux cardiomyocytes (Rudnicki *et al.*, 1990). On sait maintenant que le DMSO agit par l'intermédiaire des OTR (Paquin *et al.*, 2002) car la présence d'un antagoniste de l'OT bloque complètement la cardiomyogénèse des cellules P19 exposées au DMSO. Assez peu d'études rapportent l'évaluation des voies cellulaires impliquées en aval de l'activation d'OTR dans ce phénomène de différentiation des cellules embryonnaires en cardiomyocytes. Les travaux de Danalache *et al.* rapportent l'utilisation de l'inhibiteur de la monoxide d'azote

synthétase (NOS), le N,G-nitro-L-arginine-methyl-ester (L-NAME) qui bloque la différentiation des cellules P19 en cardiomyocytes (Danalache *et al.*, 2007) suggérant que ce gazotransmetteur intervient dans la cascade menant à cette différentiation en cardiomyocytes. De plus, l'utilisation du S-nitroso-N-acetylpenicillamine, un générateur de NO, renverse l'inhibition par le L-NAME. D'autres recherches seront nécessaires afin de mieux comprendre les médiateurs impliqués dans ce phénomène complexe et fascinant.

Qu'en est-il des implications cliniques de ces résultats obtenus en grande partie avec des lignées cellulaires ? On a vu accroître le nombre de parents qui demandent le prélèvement du sang du cordon ombilicale contenant des cellules souches embryonnaires au moment de la naissance de leur enfant (Thornley *et al.*, 2009). On peut ainsi envisager que ces cellules pourrait être utilisées afin de développer des thérapies de régénération tissulaires (Buchheiser *et al.*, 2009) utilisant un protocole de traitement de cellules embryonnaires *in vitro* lorsque cette nouvelle génération aura atteint l'âge d'être susceptible à l'infarctus du myocarde. Certaines difficultés pourraient toutefois se dresser dans la mise en place de ces thérapies innovatrices de remplacement tissulaire. Alors que la différentiation de la lignée cellulaire de carcinome embryonnaire P19 fût notée après 8 jours (Paquin *et al.*, 2002), la différentiation des cellules souches embryonnaires de souris en cardiomyocytes fût principalement observée beaucoup plus tard soit après 17 jours de culture en présence d'OT (Hatami *et al.*, 2007). Il est difficile de prédire si l'évolution de la guérison dans la zone infarcie permettra d'obtenir un bénéfice clinique plus de 2 semaines après le dommage myocardique et si les délais de différentiation présenteront des limitations à l'utilisation de l'OT comme thérapie cardiovasculaire. Les études à venir utilisant ces nouvelles thérapies cellulaires permettront de répondre à ces questions.

Le dogme selon lequel la division cellulaire des cardiomyocytes n'est pas possible au stade adulte a été ébranlé il y a une dizaine d'années suite à la démonstration de la présence de cellules en mitose dans le myocarde d'humains sains (Kajstura *et al.*, 1998). Les mêmes études démontraient une augmentation significative du nombre de cellules en mitose dans le myocarde de patients en insuffisance cardiaque incluant les maladies cardiovasculaires

ischémiques terminales et la cardiomyopathie dilatée idiopathique (Kajstura *et al.*, 1998) suggérant une réponse proliférative cellulaire endogène dans le tissu myocardique. Le durée de la mitose était d'environ une heure ce qui laisse entrevoir la possibilité de générer des millions de cardiomyocytes en situation non-pathologique ou pathologique. Cette découverte d'envergure a mené à la mise en évidence de populations de cellules souches résidentes du myocarde exprimant plusieurs marqueurs cellulaires dont Kit, Sca-1 et Isl-1 (Barile *et al.*, 2007). Un groupe de recherche a ensuite démontré la différentiation de cellules souches somatiques résidentes du myocarde chez la souris en cellules présentant des caractéristiques de cardiomyocytes (sarcomère et contractions spontanées) suite à l'exposition à l'OT *in vitro* (Matsuura *et al.*, 2004). L'identification et l'isolation de cellules souches endogènes chez des individus adultes est une découverte majeure qui a amené plusieurs publications (Li *et al.*, 2009; Pallante et al., 2006; Stamm *et al.*, 2009; Tateishi et al., 2007). Ces découvertes ouvrent la voie à l'utilisation d'un traitement systémique d'OT suite à l'infarctus du myocarde pour favoriser la différentiation de cellules souches présentes dans le myocarde en cardiomyocytes tel que proposé dans notre hypothèse de traitement présenté au chapitre 6. D'autre part, il est possible que les bénéfices des cellules souches myocardiques ne soient pas entièrement attribuables à la différentiation des cellules souches en cardiomyocytes. En effet, une étude chez le rat indique que des cellules identifiées comme étant potentiellement des cellules souches somatiques, après avoir été mises en culture *in vitro* avec l'OT, ont la capacité de migrer dans le myocarde endommagé et de se différentier en cardiomyocytes mais aussi en cellules endothéliales et en muscle lisse (Oyama *et al.*, 2007). Des résultats dans un modèle porcin d'infarctus du myocarde suggèrent également que les bénéfices liés à la différentiation des cellules souches mésenchymateuses sont associés à une différentiation en cardiomyocytes, en cellules endothéliales et en cellules de muscle lisse (Quevedos *et al.*, 2009).

Les connaissances actuelles indiquent que l'OT occupe une place prépondérante dans la différentiation en cardiomyocytes. La découverte de cellules souches endogènes dans le myocarde mais aussi la migration de

cellules souches au site de dommages myocardiques suggèrent également un processus actif de régénération du myocarde suite à l'infarctus du myocarde. Ces données supportent notre hypothèse selon laquelle l'administration d'OT à des patients suite à un infarctus du myocarde pourrait avoir des effets bénéfiques.

Chapitre 5 : EFFETS DE L'OXYTOCINE CHEZ LE RAT SPRAGUE-DAWLEY CONSCIENT ET ANESTHÉSIÉ, CHEZ LE SINGE CONSCIENT ET CHEZ LE CHIEN CONSCIENT ET ANESTHÉSIÉ

ÉTUDES EXPÉRIMENTALES

9. LES EFFETS DE L'OXYTOCINE CHEZ DES MODÈLES D'ANIMAUX SAINS

Les modèles précliniques sont couramment utilisés afin de prédire les effets pharmacologiques et toxicologiques de nouveaux médicaments destinés à l'humain. À cet effet, les agences règlementaires publient des références permettant d'extrapoler les doses animales chez l'humain (Food and Drug Administration, 2005). L'utilisation de plusieurs espèces animales est reconnue dans l'industrie du développement des médicaments comme une stratégie permettant d'accroître la valeur prédictive des résultats précliniques. Employant différents plans de traitement, les étapes initiales du développement d'un médicament permettent d'accroître les connaissances sur le composé à l'essai et de choisir une stratégie de traitement pouvant être utilisée dans la population cible de patients. L'administration d'OT en bolus chez l'humain est associée à une chute de pression artérielle accompagnée d'une tachycardie compensatoire (Weis *et al.,* 1975). Lorsqu'administrée en perfusion chez des femmes avant une chirurgie de césarienne, les effets hémodynamiques de l'OT sont significativement atténués (Cyranowski *et al.*, 2008). Malgré cette diminution des effets adverses suite à l'administration par perfusion, le patron de libération pulsatile endogène de l'OT supportait la nécessité d'évaluation de traitements en bolus. Des protocoles de traitement par perfusion ainsi que par injection rapide (bolus) furent donc évalués.

Suite à la caractérisation pharmacocinétique de l'OT (Morin *et al., Journal of Pharmacy and Pharmaceutical Sciences,* 2008;11(4):12-24. Article inclut en annexe), les effets pharmacodynamiques de l'OT ont été évalués chez le rat anesthésié et conscient (télémétrie), chez le singe conscient (télémétrie), chez le porc anesthésié ainsi que chez le chien conscient et anesthésié. Les évaluations des effets de l'OT chez le porc anesthésié ont été effectués dans le cadre des travaux de la Docteure Norma YBarra, étudiante au doctorat avec le Docteur Eric Troncy, et les résultats seront présentés et discutés par celle-ci séparément. Il importe de souligner que les évaluations des effets de l'OT présentées dans ce chapitre ont été effectuées dans le cadre d'une évaluation préliminaire des effets de l'OT. Par conséquent, les

évaluations n'étaient ni exhaustives ou comparables d'une espèce à l'autre. L'objectif principal de ces expériences était de caractériser de façon exploratoire la réponse de différents modèles précliniques à des traitements d'OT dans nos conditions expérimentales afin de déterminer le protocole et la dose pouvant être utilisée dans un modèle d'efficacité du traitement de l'infarctus du myocarde. Toutes les expérimentations présentées dans ce chapitre ont été préalablement soumises au comité institutionnel d'éthique animale pour révision et approbation. Tous les travaux présentés dans ce chapitre à l'exception des évaluations chez le rat par télémétrie ont été effectués à LAB Recherche, un centre de recherche accrédité par le Conseil Canadien de Protection des Animaux et par l'*Assessment and Accreditation of Laboratory Animal Care* (AAALAC). Les études de télémétrie chez le rat ont été effectuées à l'Hôpital Hotel-Dieu de Montréal, une institution membre du Centre de recherche du Centre Hospitalier Universitaire de Montréal (CHUM) aussi accrédité par le Conseil Canadien de Protection des Animaux. Les animaux hébergés à LAB Recherche ainsi qu'à l'Hôpital Hotel-Dieu étaient sous surveillance vétérinaire dans le cadre du programme de soins aux animaux des deux institutions.

9.1 EFFETS DE L'OXYTOCINE DANS UN MODÈLE DE RAT ANESTHÉSIÉ

Tel que présenté au chapitre 2 par la comparaison des résultats chez le singe conscient et anesthésié, les évaluations cardiovasculaires sur des modèles anesthésiés permettent d'obtenir une sensibilité élevée en diminuant la présence d'artéfacts. Chez le rat comme chez la plupart des espèces utilisées en médecine des animaux de laboratoire, les manipulations requises pour des injections parentérales (intraveineuse ou sous-cutanée) occasionnent des changements dans les paramètres cardiovasculaires mesurés. D'autre part, les effets cardiovasculaires de l'OT tel que présenté dans le présent chapitre sont de relativement courte durée. Dans un premier temps, notre objectif était de déterminer une dose d'OT n'ayant pas d'effets cardiovasculaires suite à une administration par bolus intraveineux. Le choix d'un modèle de rat anesthésié permettait donc d'accroître la sensibilité des évaluations cardiovasculaires dans la période immédiatement suite à l'administration. Dans ce contexte, les résultats démontrant la sensibilité plus élevé des évaluations chez le singe anesthésié furent considérés applicables aux modèles chez le rat. Les expériences antérieures avec différents agents pharmacologiques évalués (données non-publiées) supportaient également cette analogie entre les modèles chez le singe et le rat. Les effets de l'administration d'OT en injection et en perfusion continue ont donc été évalués dans un modèle de rat anesthésié.

9.1.1 Matériel et méthodes

Des rats Sprague-Dawley mâles âgés de 4 à 12 mois hébergés en groupe de deux à quatre dans conditions de laboratoire conventionnelles (cycle de lumière de 12 heures, température de 21±3°C) furent utilisés. Afin de minimiser le nombre d'animaux requis et pour éviter les variations possiblement associées aux cycles hormonaux chez la femelle des mâles furent choisis pour cette étape préliminaire d'évaluation de la sécurité cardiovasculaire. Une anesthésie à l'isoflurane (AErrane™, Baxter Corporation, Mississauga, ON, Canada) et oxygène (approximativement 2

L/min) utilisant une chambre d'induction à une concentration de 3 à 5% fût initialement utilisée. Une fois l'atteinte d'un niveau chirurgical confirmé (absence de réflex de retrait) l'anesthésie fut maintenue à un débit d'oxygène de 1 L/min avec une concentration d'isoflurane de 1.5-2 % administré à l'aide d'un masque anesthésique. Les animaux furent maintenus sur un tapis chauffant en plus d'une fluidothérapie de remplacement intraveineuse (saline iotonique) à un débit de 10 mL/kg/heure. L'artère fémorale fût isolée chirurgicalement et canulée avec un cathéter d'enregistrement de pression (Modular Instruments 2, West Chester PA, USA) relié à un manomètre préalablement calibré. Après une période de stabilisation des paramètres cardiovasculaires d'au moins quinze minutes, un traitement d'OT (Peninsula, Laboratories, Belmont, CA, États-Unis) dissoute dans la saline fut administré par injection intraveineuse suivi d'une période d'enregistrement de la fonction cardiovasculaire de 3 heures. À la fin de la période d'évaluation cardiovasculaire, les animaux furent euthanasiés humainement en conformité avec les recommendations du Conseil Canadien de Protection des Animaux.

9.1.2 Résultats

Une augmentation dose-dépendante de la pression artérielle accompagnée d'une diminution de la fréquence cardiaque furent observés chez le rat anesthésié à des doses de 6 000 jusqu'à 50 000 ng/kg (Figures 1 et 2). À la dose la plus élevé de 300 000 ng/kg, une atténuation de l'amplitude des effets hypertenseurs de l'OT fut observée avec des signes cliniques de toxicité incluant la détresse respiratoire. Puisque ces signes cliniques ont été observés chez des rats sous anesthésie à un niveau chirurgical, nous considérons que ces animaux n'ont pas souffert suite au traitement d'OT à dose élevée. La diminution de la fréquence cardiaque à la dose la plus haute malgré la diminution de l'amplitude de l'effet hypertenseur indique que les effets chronotropiques négatifs n'étaient pas uniquement liés à une bradycardie réflexe.

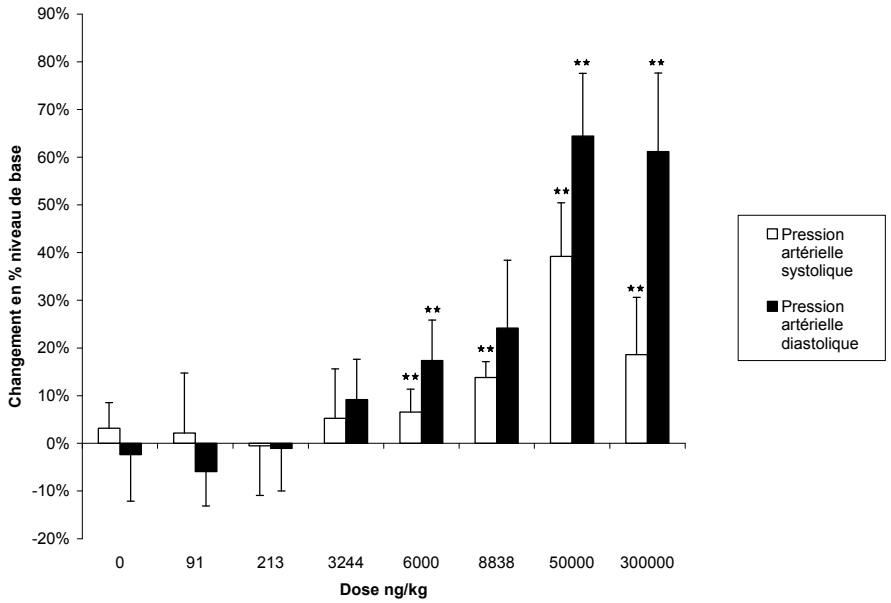

Figure 1. Pression artérielle immédiatement après le traitement de rats Sprague-Dawley mâles anesthésiés. Augmentation dose-dépendante de la pression systolique suite à l'administration d'oxytocine en bolus IV fût notée. n=4 à 6 par dose. ** $p<0.01$. La dose de 3244 ng/kg constitue la dose la plus haute sans effet pharmacodynamique (*no observed effect level*, NOEL).

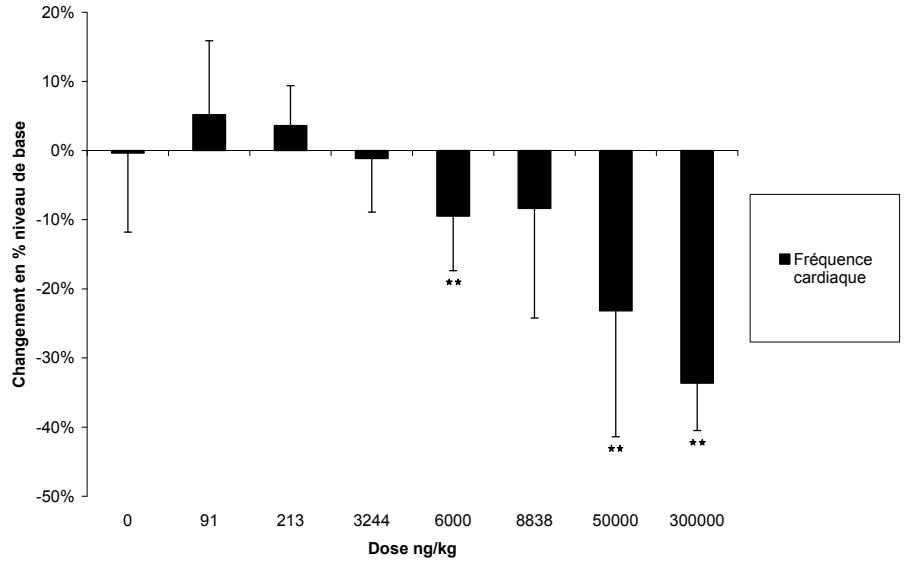

Figure 2. Fréquence cardiaque immédiatement après le traitement de rats Sprague-Dawley mâles anesthésiés. On note une diminution dose-dépendante de la fréquence cardiaque chez le rat Sprague-Dawley mâle anesthésié suite à

l'administration d'oxytocine en bolus IV avec ce modèle anesthésié. La dose de 3244 ng/kg constitue la dose la plus haute sans effet pharmacodynamique (*no observed effect level*, NOEL).

9.1.3 Discussion

Les études chez le rat anesthésié constituaient la première étape d'un processus visant à déterminer la dose pouvant être administrée dans des modèles d'efficacité et ultimement chez des patients sans engendrer d'effets secondaires cardiovasculaires adverses. Les courbes dose-réponse des effets cardiovasculaires de l'OT illustrées par les figures 1 et 2 permettaient d'identifier un phénomène d'effet plateau à des doses supérieures à 50 000 ng/kg. La présence de cette effet plateau pourrait résulter d'une saturation des OTR et possiblement aussi des récepteurs de vasopressine étant donnée que la liaison de l'OT pour le récepteur de la vasopressine (Thibonnier *et al.*, 1998). Dans le cadre du développement d'un protocole de traitement de l'infarctus du myocarde, notre intérêt était principalement du côté des doses n'ayant aucun effet cardiovasculaire adverse. Basé sur ces résultats chez le rat anesthésié, des doses présentées plus loin furent choisies pour les études chez le rat sain et conscient afin de confirmer les observations obtenues dans ce premier modèle.

9.2 EFFETS DE L'OXYTOCINE DANS UN MODÈLE DE RAT CONSCIENT

9.2.1 Matériel et méthodes

Des rats Sprague-Dawley mâles (n=19) âgés de 3 à 6 mois hébergés individuellement dans conditions de laboratoire conventionnelles (cycle de lumière de 12 heures, température de 21±3°C) furent utilisés. Les animaux furent instrumentés chirurgicalement avec des transmetteurs de télémétrie (PA-C40, Data Science International, St-Paul, MN, É.-U.) pour l'enregistrement de la pression artérielle systémique à partir d'un cathéter implanté dans l'artère fémorale. Une antibiothérapie prophylactique (penicilline procaine, 20 000 IU/kg, SC) et une analgésie post-opératoire (buprénorphine, 0.01 mg/kg, SC, BID) furent administrées pour tous les animaux. Les animaux furent répartis entre les différents groupes expérimentaux tel qu'indiqué dans les Tableau I, II et III ci-bas.

Un premier traitement de perfusion sous-cutanée continue utilisant une dose d'OT (Peninsula, Laboratories, Belmont, CA, États-Unis) sélectionnée suite aux résultats des évaluations cardiovasculaires obtenus chez le rat anesthésié visait à définir une dose de perfusion sans effet cardiovasculaire et pouvant ensuite être utilisée dans des études d'efficacité préclinique. La durée de l'infusion par pompe osmotique (Alzet®, DURECT, Cupertino, CA, USA) fût choisie afin de couvrir la période de remodelage tissulaire en phase aigüe suite à l'infarctus du myocarde (Frangogiannis, 2008; Ono *et al.*, 1998).

Tableau I

Groupes	**Dose (ng/kg/hr)**	**Débit de la perfusion (mL/hr)**	**Durée de la perfusion (jours)**	**Nombre d'animaux (Mâles)**
1	0	0.5uL/hr	15	3
2	250	0.5uL/hr	15	3

Un deuxième traitement par d'injection sous-cutanée quotidienne visait à confirmer les effets presseurs de l'OT chez le rat conscient. La dose d'OT choisi dans ce groupe expérimental visait à comparer nos résultats avec ceux précédemment publiés avec l'utilisation de méthodologie de mesure non-invasive de la fonction cardiovasculaire chez le rat conscient avec contention (Pertersson *et al.*, 1996; Pertersson *et al.*, 1997).

Tableau II

Groupes	**Dose (mg/kg)**	**Volume (mL/kg)**	**Durée du traitement (jours)**	**Nombre d'animaux (Mâles)**
3	0	1	15	3
4	1	1	15	4

Un troisième traitement utilisant un protocole de perfusion intraveineuse continue visait à 1) évaluer les effets possibles de la sensibilisation ou désensibilisation suite à une exposition prolongée par perfusion d'OT et à 2) définir le profil de réponse cardiovasculaire en fonction de la dose de perfusion. Un groupe d'animaux fût assigné à un protocole de traitement crescendo alors qu'un deuxième groupe fût assigné à un protocole de traitement decrescendo. Notre hypothèse était que le degré de désensibilisation serait proportionnel à la durée d'exposition à l'OT. Ainsi, notre hypothèse postulait que le groupe crescendo allait présenter une réponse cardiovasculaire moins importante aux doses élevées que le groupe descrescendo suite à l'exposition prolongée aux doses plus basse qui entraînerait une désensibilisation. Ce phénomène d'atténuation de la réponse suite à l'augmentation progressive de la dose d'infusion d'OT est étudié en obstétrique (Dencker *et al.*, 2009). Ce phénomène de désensibilisation présentait un attrait comme plan de traitement de l'infarctus du myocarde. En effet, cette diminition des effets pharmacologiques suite à une augmentation

progressive du débit d'infusion permettrait d'accroître l'exposition à l'OT tout en minimisant les effets secondaires.

Tableau III

Groupes	Traitement	Dose* ng/kg/hr	Concentration ng/mL	Durée de la perfusion (jours)	Nombre d'Animaux (Mâles)
5 crescendo	Saline	0	0	2	3
	Oxytocine	125	312.5	1	
		250	625	1	
		1 000	2 500	1	
		10 000	25 000	1	
		27 300	68 250	1	
		100 000	250 000	1	
		200 000	500 000	7	
6 decrescendo	Saline	0	0	2	3
	Oxytocine	200 000	500 000	1	
		75 000	187 500	1	
		30 000	75 000	1	
		7 500	18 750	1	
		1 000	2 500	1	
		0	0	1	

* Tous les animaux ont reçu 0.4 mL/kg/hr afin d'éviter les effets cardiovasculaires volumétriques.

9.2.2 Résultats

Groupes 1 & 2

L'administration d'OT par perfusion sous-cutanée continue à une dose de 250 ng/kg/heure pour une durée de 15 jours n'a pas engendré d'effet cardiovasculaire significatif lorsque comparé avec le groupe traité avec la saline confirmant ainsi notre hypothèse d'une absence d'effet à cette dose établie par les études chez le rat anesthésié.

Groupes 3 & 4

L'administration sous-cutanée d'OT (1 mg/kg) provoqua une augmentation des pressions artérielles systémiques (systolique, moyenne et diastolique) suivi d'un retour à des valeurs de base. Les pressions artérielles systolique (Figure 3), moyenne et diastolique demeurèrent significativement

élevées jusqu'à 120 minutes suite à l'injection (p<0.01). L'effet de l'OT demeura le même d'un jour à l'autre (p=0.15). Parallèlement, une diminution de la fréquence cardiaque fut observée (Figure 4) jusqu'à 60 minutes après l'injection (p<0.01) suivie d'une augmentation de la fréquence cardiaque lorsque comparée au groupe saline à 200 minutes après le traitement (p<0.05).

Groupes 5 & 6

La perfusion intraveineuse continue à des doses allant jusqu'à 200 000 ng/kg/heure provoqua une augmentation de la pression systémique (systolique, moyenne et diastolique). Le modèle de covariance, avec la concentration comme facteur répété, indiqua que la relation entre la pression artérielle et la dose n'était pas la même dans les groupes crescendo et decrescendo ($p < 0.01$) (Figure 5). Le modèle indiqua également une composante quadratique pour la relation entre la dose et la pression ($p < 0.05$). Les corrélations (quadratique) entre la dose (transformée en log base 10) et les pressions artérielles étaient les suivantes :

<u>Pression artérielle systolique</u>

Doses crescendo : Pression = 171.3 – 38. 4*dose + 6.4*dose^2

Doses decrescendo : Pression = 112.6 + 1.8*dose + 0.6*dose^2

<u>Pression artérielle moyenne</u>

Doses crescendo : Pression = 146.7 – 32. 6*dose + 5.4*dose^2

Doses decrescendo : Pression = 93.6 + 1.4*dose + 0.7*dose^2

<u>Pression artérielle diastolique</u>

Doses crescendo : Pression_artérielle diastolique = 128.0 – 28. 9*dose + 4.8*dose^2

Doses decrescendo : Pression artérielle diastolique = 78.0 + 1.2*dose + 0.7*dose^2

Parallèlement, la fréquence cardiaque fût réduite et le modèle de covariance, avec la dose comme facteur répété, indiqua que la relation entre la fréquence cardiaque et la dose n'était pas la même dans les groupes crescendo et decrescendo ($p < 0.01$). La corrélation (linéaire) était la suivante:

Crescendo : Fréquence Cardiaque = 458.9 – 23.6*dose

Decrescendo : Fréquence Cardiaque = 395.4 – 7.3*dose

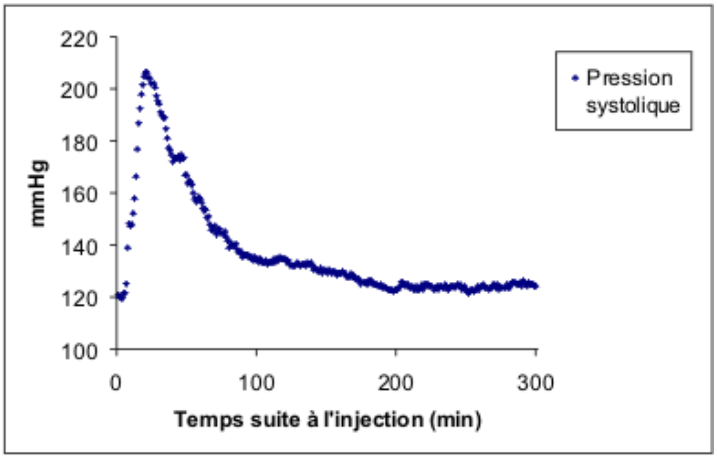

Figure 3. Pression systolique illustrant les effets hypertenseurs de l'OT (1 mg/kg) chez le rat Sprague-Dawley mâle suite à l'injection SC à (n=4).

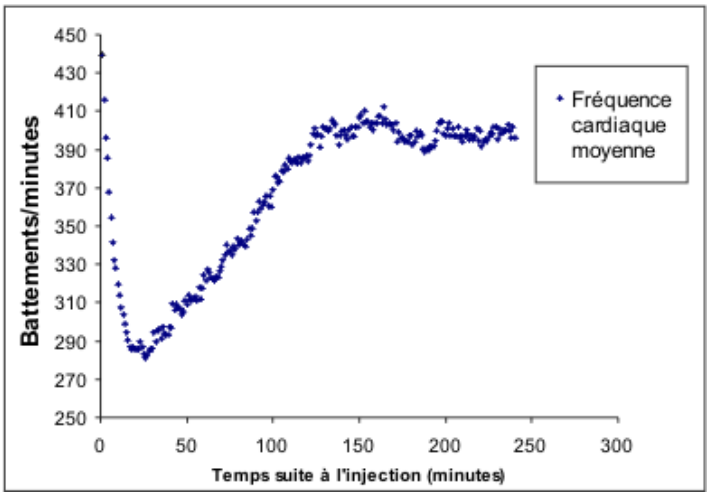

Figure 4. Fréquence cardiaque suite à l'administration SC d'OT (1 mg/kg) chez le rat Sprague-Dawley mâle (n=4) illustrant la bradycardie réflexe.

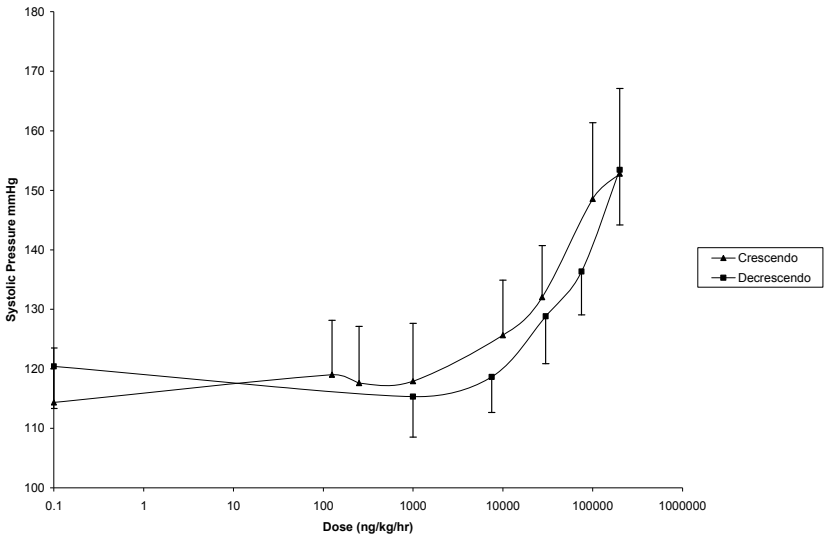

Figure 5. Effets hypertenseurs d'un protocole crescendo et decrescendo d'administration d'OT chez le rat Sprague-Dawley mâle par perfusion continue IV (n=4). Un effet de désensibilisation fût observé avec le protocole d'administration decrescendo dans lequel la corrélation dose/pression systolique était inférieure à l'administration crescendo (p<0.01). Les doses utilisées pour les protocoles crescendo et decrescendo étaient différentes et donc l'analyse statistique s'est effectuée en comparant les corrélations dose-pression.

9.2.3 Discussion

Les études chez le rat conscient s'inscrivaient dans notre démarche visant à établir un protocole de traitement dépourvu d'effet secondaire adverse et pouvant ainsi être utilisés dans le modèle d'infarctus du myocarde. L'absence d'effets cardiovasculaires à la dose de 250 ng/kg/hr confirma notre hypothèse basée sur les études chez le rat anesthésié. Une distinction importante entre notre modèle de rat conscient et la population cible est la présence d'un infarctus du myocarde qui modifie les mécanismes compensatoires. Plusieurs articles publiés à ce jour discutent de la pertinence d'utiliser des modèles animaux présentant une pathologie cardiovasculaire afin d'accroître la sensibilité du modèle aux effets adverses cardiovasculaires (Hamlin *et al.*,

2008; Joshi *et al.*, 2004; Kijtawornrat *et al.*, 2006; Lawrence *et al.*, 2005; Sugiyama, 2008; Pugsley *et al.*, 2008). Cette évaluation dans un modèle représentatif sera effectuée dans la dernière partie de cette thèse avec un modèle d'infarctus du myocarde porcin.

9.3 EFFETS DE L'OXYTOCINE DANS UN MODÈLE DE SINGE CONSCIENT AVEC RADIOTÉLÉMÉTRIE

9.3.1 Matériel et méthodes

Deux singes mâles âgés de 2 à 3 ans hébergés individuellement dans les conditions de laboratoire conventionnelles (cycle de lumière de 12 heures, température de 21±3°C) furent utilisés. Les animaux furent préparés chirurgicalement pour l'enregistrement de la fonction cardiovasculaire tel que décrit au deuxième chapitre de cette thèse. Une solution d'OT (Peninsula, Laboratories, Belmont, CA, États-Unis) diluée dans de la saline stérile fut administrée par injection intraveineuse (bolus) à des doses de 0, 50, 91, 250, 500, 750 et 1000 ng/kg. Il est courant d'utiliser un nombre limité d'animaux (n=2) dans les études de pharmacologie de sécurité préliminaires qui visent seulement à établir la présence ou l'absence d'effet pharmacologique aux différentes doses évaluées. L'analyse est donc fondée sur l'interprétation des réponses individuelles afin de déterminer la dose maximale sans effet et aucun test statistique ne fût utilisé pour ces expérimentations.

9.3.2 Résultats

Une diminution de la pression artérielle fut observée chez les singes conscients recevant une dose de 500 ng/kg ou supérieure (Figure 5). Aucun effet significatif sur la fréquence cardiaque ne fut observé dans ce modèle de primate non humain aux doses évaluées.

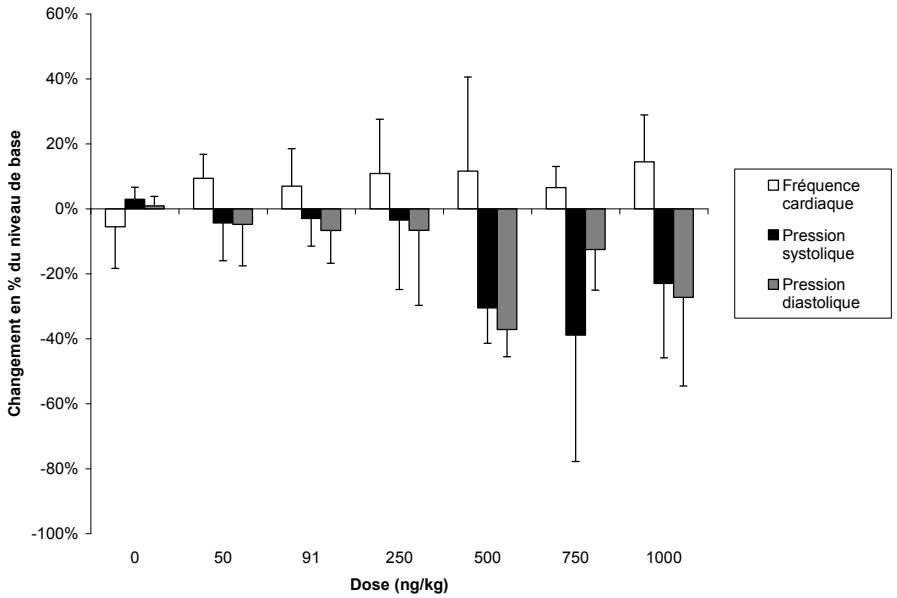

Figure 6. Effets cardiovasculaires de l'OT chez le singe cynomolgus conscient suite à l'administration intraveineuse sans contention (cathéter permanent et dose administrée par un port d'injection à extérieur de la cage). Considérant le nombre limité d'animaux utilisé dans cet essai pharmacologique préliminaire (n=2), les analyses ont été limitées à des statistiques descriptives.

9.3.3 Discussion

Cette première étape d'évaluation des effets cardiovasculaires de l'OT dans une espèce de grands animaux de laboratoire permit d'étendre les connaissances sur les effets de l'OT administrée en bolus intraveineux et de mieux définir les marges thérapeutiques de cet agent. Tel que présenté dans la Figure 6, la réponse du singe fût opposée à celle obtenue chez le rat anesthésié chez qui des effets hypertenseurs avaient été observés. La présence d'effets contraires suite à l'administration de la même drogue dans des modèles différents est reconnue telle qu'illustrée par la réponse au remifentanil dans le deuxième chapitre de cette thèse (Authier *et al.*, 2008). L'interprétation de résultats contradictoires ne pose pas problème car l'objectif principal des études précliniques tel que discuté plus loin dans ce chapitre est d'estimer une dose sécuritaire pouvant être utilisé chez les patients. Tel que discuté dans le deuxième article du troisième chapitre de cette thèse, il importe peu que le

modèle préclinique présente une réponse identique à l'humain pour autant qu'il permette d'identifier la présence d'effets pharmacologiques du composé à l'essai aux doses pertinentes pour l'humain. Cet élément de discussion soulève la question de la pertinence des réponses d'un modèle à la réponse chez l'humain. Celle-ci ne peut être confirmée tant que les études chez l'humain n'ont pas été complétées (Redfern *et al.*, 2002). L'approche couramment utilisée pour maximiser la valeur prédictive des évaluations précliniques est donc de faire l'évaluation de la sécurité d'un composé à l'aide de plusieurs modèles précliniques et de faire une intégration des réponses de ceux-ci dans une évaluation intégrée du risque pour les patients (Valentin *et al.*, 2009).

9.4 EFFETS DE L'OXYTOCINE DANS UN MODÈLE DE CHIEN CONSCIENT

9.4.1 Matériel et méthodes

Quatre Beagles (2 mâles et 2 femelles) âgés de 1 à 3 ans hébergés individuellement dans les conditions de laboratoire conventionnelles (cycle de lumière de 12 heures, température de 21±3°C) furent utilisés pour les évaluations cardiovasculaires chez le chien conscient. Les animaux furent préparés chirurgicalement avec un cathéter veineux fémoral permanent permettant la perfusion continue d'OT sans contention. En bref, une antiothérapie prophylactique (cephazolin, 25 mg/kg, IM) fût administrée au moins 30 min avant l'induction de l'anesthésie. L'induction de l'anesthésie fût effectuée avec une injection de propofol (6 mg/kg, IV) suivi de l'intubation et du maintien de l'anesthésie par isoflurane (2.0-3.5%). Les sites chirurgicaux (interscapulaire et abdominal) furent préparés de façon aseptique avec de la chlorexidine (4% pour 5 min). Les soins post-opératoires comportaient l'administration d'analgésie (buprénorphine, 0.01 mg/kg, IM, BID pour un minimum de 2 jours) et le suivi de la température corporelle jusqu'à ce que les chiens atteignent une température de 37°C.

Une solution d'OT (Peninsula, Laboratories, Belmont, CA, États-Unis) diluée dans de la saline stérile fut administrée par perfusion IV continue à des

doses de 0, 50, 250, 1 000 et 10 000 ng/kg/heure pour une période de 24 heures chacune en utilisant un protocole de traitement crescendo (doses croissantes). La pression artérielle systémique fût évaluée par sphygmomanométrie (pression artérielle indirecte mesurée en triplicat à chaque occasion) avant le début du traitement et à 2, 6 et 23 heures après le début de la perfusion. Le volume d'urine et la natriurèse furent mesurés par récolte complète pour chaque période de 24 heures à chacune des doses.

9.4.2 Résultats

Aucun effet cardiovasculaire ne fut observé aux doses évaluées avec ce modèle (Figure 6). Une diminution de la diurèse fut observée suite au traitement toutes doses confondues (Figure 7). La natriurèse demeura inchangée suite au traitement avec une augmentation proportionnelle de la concentration de sodium suite à la diminution de la diurèse.

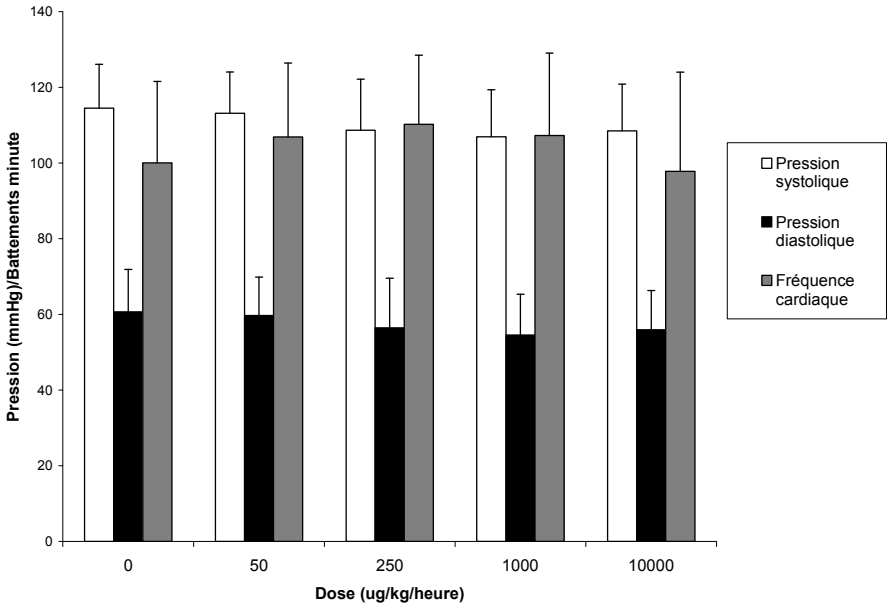

Figure 7. Effets cardiovasculaires de l'OT chez le chien Beagle conscient suite à l'administration intraveineuse par perfusion continue (24 heures) sans contention (cathéter permanent). Aucun effet statistiquement significatif n'a

été observé à aucune des doses évaluées (n=4 par dose). Les valeurs constituent la moyenne de 3 mesures prises à 2, 6 et 24 heures suite à l'initiation de la perfusion continue. L'analyse des différentes occasions n'a révélé aucune différence significative entre les différents temps de mesure.

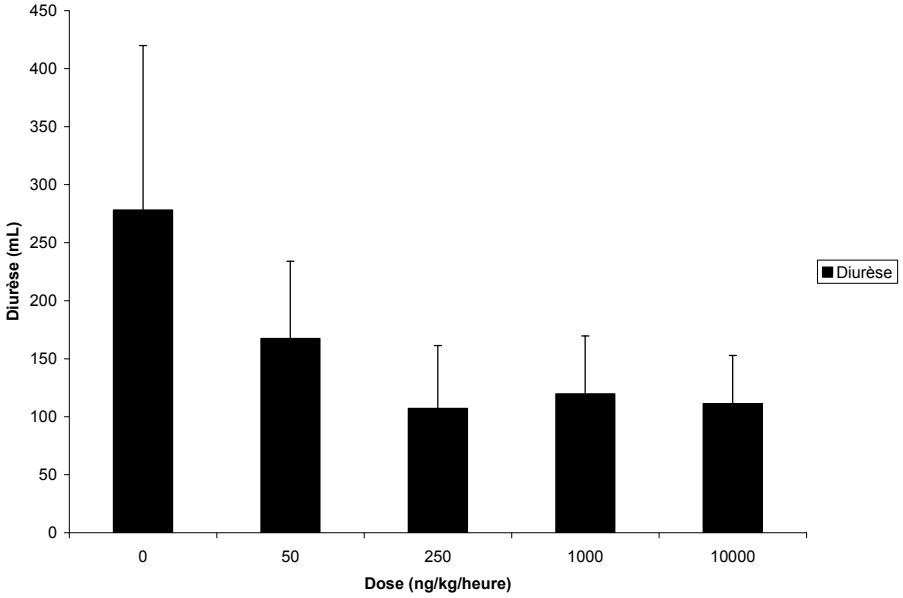

Figure 8. Effets anti-diurétiques de l'OT chez le chien Beagle conscient suite à l'administration intraveineuse par perfusion continue (24 heures) sans contention (cathéter permanent). Une diminution statistiquement significative de la diurèse fût observée suite au traitement (p<0.01).

9.4.3 Discussion

Tel que discuté dans l'article de validation non-pharmacologique de la télémétrie chez le singe du deuxième chapitre de cette thèse, la précision des mesures de fonction cardiovasculaire obtenues par des méthodes indirectes (ex. sphygmomanométrie) présente des limitations et requiert la contention des animaux qui peut introduire la présence d'artéfacts. Ces mesures sont ainsi considérées moins sensibles que celle obtenues par des mesures directes telle que la télémétrie. Pour cette raison, les recommandations des agences règlementaires favorisent l'utilisation des méthodes de mesure sans contention

(Food and Drug Administration, 2001). Ainsi l'absence d'effet cardiovasculaire noté avec ce modèle canin pourrait être liée à cette réduction de la sensibilité des évaluations. Il demeure que l'impact de cette sensibilité limitée est minime puisque les estimations de doses sont toujours basées sur le modèle présentant la sensibilité la plus élevée. Le modèle canin révèle toutefois des résultats importants en mettant à jour les effets d'une infusion d'OT sur la diurèse. Des effets anti-diurétiques similaires sont rapportés chez l'humain (Li *et al.*, 2007). L'utilisation fréquente des agents diurétiques dans la gestion clinique des patients atteints d'insuffisance cardiaque suite à un infarctus du myocarde (Li *et al.*, 2008) laisse entrevoir les effets adverses qu'un traitement ayant des effets anti-diurétiques pourrait avoir sur ces patients. En accroissant le volume circulant, les effets anti-diurétiques de l'infusion d'OT pourraient mener à une augmentation de pression artérielle et de la post-charge ventriculaire précipitant ainsi la progression vers une insuffisance cardiaque. Considérant la sensibilité limitée des mesures de fonction cardiaque indirecte obtenue dans cette étude, l'utilisation d'un modèle canin anesthésié d'évaluation de la fonction cardiaque était la prochaine étape logique visant à raffiner les évaluations de sécurité de l'OT chez cette espèce.

9.5 EFFETS DE L'OXYTOCINE DANS UN MODÈLE DE CHIEN ANESTHÉSIÉ

9.5.1 Matériel et méthodes

Pour les évaluations chez le chien anesthésié, quatre Beagles (2 mâles et 2 femelles) âgés de 1 à 3 ans hébergés individuellement dans les conditions de laboratoire conventionnelles (cycle de lumière de 12 heures, température de 21±3°C) furent utilisés. Les animaux furent anesthésiés par injection IV de propofol à 6 mg/kg (Propoflo, PropoFlo™, Abbott Laboratories Ltd., Montreal, QC, Canada). Suite à l'intubation endotrachéale, l'anesthésie fût maintenue avec l'isoflurane (2%) (AErrane™, Baxter Corporation, Mississauga, ON, Canada) administrée par inhalation à l'aide de système de Bain avec un débit d'oxygène de 200 mL/kg/min. L'homéothermie fût maintenue avec un tapis chauffant tout au long de la procédure. Un cathéter Swann Ganz (Edward LifeScience, Irvine, CA, États-Unis) fut placé dans l'artère pulmonaire afin de mesurer le débit cardiaque. Un cathéter au niveau de l'artère fémorale et du ventricule gauche furent placés afin d'enregistrer la pression artérielle systémique et ventriculaire. Une solution d'OT (Peninsula, Laboratories, Belmont, CA, États-Unis) diluée dans de la saline stérile fut administrée par perfusion intraveineuse en bolus à des doses de 0, 300, 5 000 et 50 000 ng/kg. Aucune analyse statistique ne fût effectuée étant donné le nombre de chiens à chaque dose (n=2) avec ce protocole standard d'évaluation de la réponse cardiovasculaire à des doses croissantes. Les interprétations furent basées sur l'analyse de la réponse individuelle des animaux aux différentes doses.

9.5.2 Résultats

Dans le modèle canin anesthésié, l'OT ne causa aucun effet sur la fréquence cardiaque et sur la pression artérielle aux doses évaluées. Une diminution de la contractilité mesurée par une réduction de dP/dt+ fut notée aux doses de 5 000 ng/kg (-34.0% ± 8.8%) et 50 000 ng/kg (-32.6%). Une diminution cliniquement significative du débit cardiaque fut observée aux doses de 5 000

et 50 000 ng/kg (Figure 9). La dose de 50 000 ng/kg causa une augmentation de pouls de +18.1%.

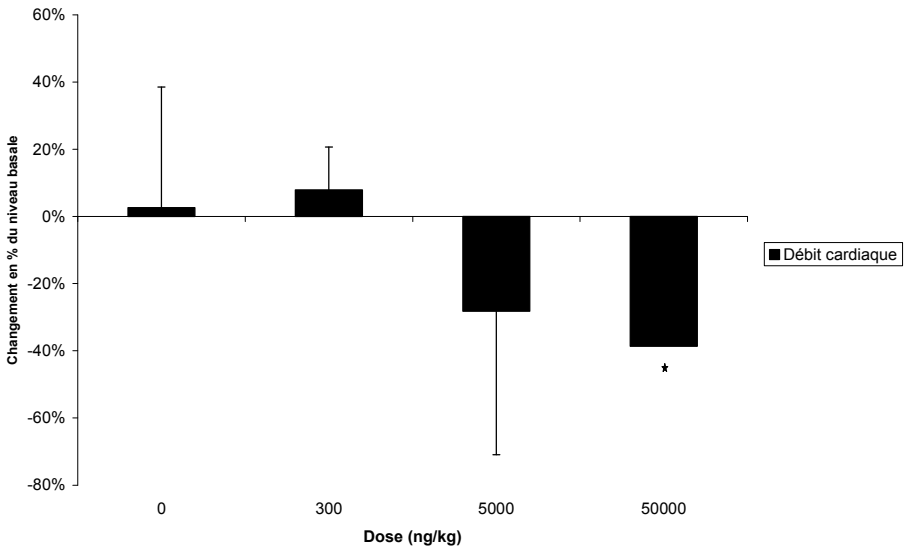

Figure 9. Effets de l'OT sur le débit cardiaque chez le chien Beagle anesthésié suite à l'administration intraveineuse en bolus. Une diminution cliniquement significative fut observée aux doses de 5 000 et 50 000 ng/kg. Suite à la sévérité de l'effet hypotenseur avec la dose la plus élevée (50 000 ng/kg) la présence d'un effet pharmacologique fut confirmée et un seul animal fut utilisé par souci de réduction du nombre d'animaux utilisés.

9.5.3 Discussion

Il importe de souligner que la sensibilité de ce modèle canin anesthésié est limitée considérant le nombre réduit (n=2) de chiens qui fût utilisé. La présence d'un effet inotrope négatif suite à l'administration d'OT dans ce modèle canin d'évaluation de la fonction cardiaque constitue un facteur de risque important pour les patients dans le traitement de l'infarctus du myocarde. En effet, une diminution de la contractilité ventriculaire est présente suite à l'ischémie-reperfusion (Chandrashekhar *et al.*, 1999). De plus, une proportion des patients atteints d'un infarctus du myocarde présenteront éventuellement une insuffisance cardiaque (Ezekowitz *et al.*, 2009). Les effets

négatifs d'un traitement sur la contractilité pourraient précipiter cette progression vers l'insuffisance cardiaque. Il importe toutefois de souligner un point favorable dans l'interprétation des résultats obtenus avec ce modèle canin anesthésié. L'applicabilité des effets secondaires observés dans le modèle préclinique à la réponse chez le patient dépend de la dose à laquelle ces effets sont observés. La marge entre la dose thérapeutique et la dose à laquelle les effets adverses sont observés détermine la pertinence de cette observation pour la population de patients (Hayes, 2007). Ainsi, une marge thérapeutique de 4 est jugée étroite alors qu'une marge thérapeutique de 100 ou plus est considérée élevée. Tel que mentionné plus haut, les effets sur la contractilité chez le chien ont été observés à une dose de 5000 ng/kg ou plus. Puisque la dose thérapeutique n'est pas connue, il n'est pas possible de calculer de marge thérapeutique à ce stade. Une difficulté dans l'établissement de la marge thérapeutique réside dans le manque de connaissances de la dose thérapeutique chez l'humain qui ne peut être établie que par les études cliniques.

9.6 DISCUSSION DES EFFETS DE L'OXYTOCINE CHEZ DES MODÈLES D'ANIMAUX SAINS

Depuis plusieurs décennies, l'OT est reconnue comme une hormone neurohypophysaire ayant des effets cardiovasculaires chez plusieurs espèces de mammifères tel que le rat, le chien (Nakano & Tanem, 1963) le mouton (Roberts *et al.*, 1992) et l'humain (Secher *et al.*, 1978) pour n'en nommer que quelques-unes. Les effets cardiovasculaires observés suite à l'administration d'OT diffèrent en fonction des sites anatomiques d'administration et de l'espèce évaluée.

Au niveau du système nerveux central, l'OT joue un rôle complexe de messager neurohormonal (Leng et Ludwig, 2008; Skuse et Gallagher, 2009). Chez le rat, l'administration d'OT dans les ventricules cérébraux ne produit aucun effet cardiovasculaire (Feuerstein *et al.*, 1984) mais réduit la tachycardie suite à l'exercice (Braga *et al.*, 2000). L'administration d'OT au niveau intrathécal dans la région thoracique chez le rat résulte en une augmentation de la fréquence cardiaque sans effet sur la pression artérielle (Yashpal *et al.*, 1987). L'administration intrathécale d'OT au niveau de la citerne cérébellaire chez le chien anesthésié et le rat conscient n'induit pas d'effet cardiovasculaire notable (Neath *et al.*, 2000; Petty *et al.*, 1985). Les mécanismes par lesquels l'OT exerce ses effets au niveau du système nerveux central demeurent peu connus et les recherches futures permettront de mieux comprendre les rôles de ce messager neurohormonal.

À l'extérieur du système nerveux central, des effets cardiovasculaires sont rapportés par nombre d'études chez plusieurs espèces. Le cœur est connu comme un site de synthèse endogène et d'action de l'OT chez le rat (Coulson *et al.*, 1997 ; Jankowski *et al.*, 1998) et l'humain (Cicutti *et al.*, 1999). Des effets cardiaques directs de l'OT ont été rapportés et constituent une considération pharmacologique importante dans l'utilisation de l'OT comme thérapie potentielle pour l'infarctus du myocarde. Sur des cœurs de rat isolés, l'OT exerce des effets chronotropes négatifs (Coulson *et al*., 1997). Par contre, les résultats publiés sur les effets inotropes de l'OT sur cœur de rat isolé sont contradictoires puisque que certains groupes rapportent une augmentation des

mesures d'inotropie (Coulson *et al*., 1997) et d'autres une diminution (Costa-E-Sousa *et al.*, 2005). Ces résultats opposés pourraient s'expliquer par des conditions expérimentales différentes pouvant avoir des effets importants sur la réponse des modèles de cœurs isolés aux agents pharmacologiques. L'activation de récepteurs fonctionnels d'OT (OTR) a été associée avec la libération du peptide natriurétique atriale (ANP) (Gutkowska *et al.*, 1997) expliquant en partie les effets chronotropes et inotropes négatifs dans des modèles de rats *ex-vivo* (Favaretto *et al.*, 1997).

L'OT est associée à des effets inotropes et chronotropes négatifs (Costa-E-Sousa *et al.*, 2005) chez le rat conscient. Ces résultats obtenus chez le rat conscient concordent avec nos observations où des effets chronotropes négatifs ont été observés en réponse au traitement d'OT en bolus SC et en perfusion IV continue. Les effets chronotropes négatifs de l'OT ont également été rapportés chez le lapin conscient (Uzun *et al.*, 2007) et le chien (Mukaddam-Daher *et al.*, 2001) alors qu'une augmentation de la fréquence cardiaque est observée chez l'humain tel que discuté précédemment. Des études avec des cœurs canins indiquent que les effets chronotropes et inotropes négatifs de l'OT chez cette espèce font intervenir le NO et l'innervation cholinergique cardiaque. Nos résultats chez les chiens anesthésiés confirment cette diminution de contractilité ventriculaire suite à l'administration d'OT aux doses de 5 000 et 50 000 ng/kg en plus d'une diminution du débit cardiaque. Ces considérations importantes orientent la planification de l'étude d'évaluation de la valeur thérapeutique de l'OT dans un modèle d'infarctus du myocarde porcin présenté plus loin. Afin d'évaluer les effets secondaires adverse possibles du traitement sur la contractilité ventriculaire, l'étude avec le modèle porcin comporte l'utilisation de biomarqueurs fonctionnels cardiaques quantifiés à l'aide d'échocardiographie.

Outre les effets de l'OT sur le cœur, des effets de ce nanopeptide sur l'ensemble du système cardiovasculaire sont rapportés. L'administration intraveineuse d'OT chez le rat conscient est associée à une phase initiale d'hypertension possiblement associée à l'activation des récepteurs de la vasopressine suivie d'une phase prolongée d'hypotension relative (Petty *et al.*, 1985; Petersson *et al.*, 1996). L'hypertension suite à l'administration d'OT

chez le rat fût également observée dans nos conditions expérimentales chez le rat anesthésié et conscient mais la phase d'hypotension relative ne fût pas statistiquement significative. Les résultats publiés chez le chien anesthésié (Nakano & Fisher, 1963) concordent avec nos observations pour le débit cardiaque diminué suite au traitement. En contrepartie, l'augmentation de la contractilité ventriculaire, la diminution de pression artérielle et l'augmentation de fréquence cardiaque notées par Nakano & Fisher n'ont pas été observés dans nos conditions expérimentales. Les doses évaluées par Nakano & Fisher (100 à 3 200 ng/kg) étaient comparables à celles que nous avons utilisées tout comme le protocole de traitement. Il est donc possible que le nombre limité d'animaux utilisé dans nos expériences expliquent la moindre sensibilité de nos études.

En obstétrique, l'utilisation d'une perfusion d'OT à dose croissante est une pratique courante afin d'induire l'accouchement (Dencker *et al.*, 2009). Cette stratégie de traitement présente un intérêt sur le plan thérapeutique en permettant d'accroître la dose administrée et possiblement les chances de succès en assumant que les effets soient proportionnelles au temps d'exposition et à la dose. La comparaison de nos résultats de perfusion d'OT à des doses croissantes et décroissantes indique que les effets hypertenseurs et chronotropes négatifs étaient moins importants à compter de la deuxième dose avec le protocole decrescendo. Cette diminution des effets à partir de la deuxième dose pourrait être causée par une diminution de l'expression des OTR suite à l'exposition à des doses élevées d'OT de façon prolongée. On rapporte une diminution de l'expression d'OTR et d'ARNm myométrial suite à une augmentation endogène et exogène d'OT lors de l'accouchement (Phaneuf *et al.*, 2000). Nos résultats de mesure d'OTR chez le porc suite à une infusion d'OT qui sont présentés dans le prochain chapitre concordent également avec cette hypothèse d'une diminution d'expression des récepteurs suite à une exposition prolongée à l'OT.

Les données sur les effets de l'OT sur la contractilité ventriculaire chez les patients humains sont limitées. Des évaluations sur trabécules atriales humaines suggèrent l'absence d'effet inotrope de l'OT sur le myocarde humain (Rosaeg *et al.*, 1998). Considération importante, les effets

inotropiques négatifs, lorsque présents, peuvent avoir des effets très significatifs chez les patients. La sévérité de la réduction de contractilité ventriculaire suite à l'infarctus du myocarde est un indicateur de pronostic (Kelly *et al.*, 1985) et nécessite fréquemment une intervention thérapeutique (Russ *et al.*, 2007). Chez des femmes enceintes, l'administration d'OT résulte en une diminution significative de la pression artérielle systémique accompagnée d'une augmentation de la fréquence cardiaque (Secher *et al.*, 1978; Pinder *et al.*, 2002; Charbit *et al.*, 2004; Thomas *et al.*, 2007). Ces résultats chez l'humain concordent avec nos mesures de fonction cardiaque présentées précédemment chez le singe. Ces effets hémodynamiques pourraient avoir d'importantes conséquences dans la population de patients. Chez les patients atteints de maladies cardiovasculaires, une augmentation, même minime, de la pression artérielle systémique occasionne des effets secondaires importants et une augmentation des mortalités cardiovasculaires (Lewington *et al*., 2003). Les nouvelles drogues ayant des effets hypertenseurs font donc face à des risques d'échec très importants. Les données disponibles suggèrent que l'OT aurait potentiellement des effets hypotenseurs chez les patients, ce qui présente un risque moins grand qu'un effet hypertenseur mais demeure une considération importante dans l'utilisation de ce peptide comme thérapie cardiovasculaire.

Un des objectifs importants de nos études était de déterminer une dose d'OT sans effet pharmacologique adverse (*no observed adverse effect level*, NOAEL) afin de choisir une dose thérapeutique pour les modèles d'efficacité de traitement de l'infarctus du myocarde. Il importe de spécifier qu'une distinction importante existe entre la dose n'ayant aucun effet pharmacologique (*no observed effect level*, NOEL) et la NOAEL. Les bénéfices d'un traitement constituent un effet pharmacologique et donc la dose choisie doit être suffisante pour atteindre ce seuil d'efficacité. La dose ayant des effets adverses est établie à la lumière des résultats obtenus avec les différents modèles précliniques de pharmacologie de sécurité. Les effets observés sont ensuite interprétés en fonction de l'indication clinique prévue. Par exemple, une légère augmentation de pression artérielle ne serait pas considérée comme un effet secondaire adverse limitant dans le cas du

traitement d'un cancer avec un prognostic sombre (ex. cancer du pancréas) mais serait un effet secondaire adverse très important pour le traitement de l'insuffisance cardiaque congestive. Une autre considération importante dans l'interprétation de la nature « adverse » d'un effet pharmacologique est la population dans laquelle les essais cliniques de phase 1 seront effectués. Un effet secondaire rare mais fatal (ex. arythmie) sera jugé adverse et inadéquat pour une étude clinique effectuée chez des volontaires en santé alors que le même effet secondaire dans le cas d'études cliniques de Phase 1 effectué chez des patients en phase terminale d'une maladie ayant un pronostic sombre pour laquelle aucun traitement n'est disponible pourrait être jugé acceptable. Dans le cas du traitement de l'infarctus du myocarde, la population cible présente une fonction cardiaque altérée et une réserve fonctionnelle souvent limitée. La présence d'effets secondaires cardiovasculaires est donc susceptible d'avoir un impact important sur la morbidité et la mortalité. Ces considérations ont dicté une approche conservatrice dans notre choix de dose pour notre étude d'efficacité dans le modèle porcin d'infarctus du myocarde.

9.7 CONCLUSION SUR LES EFFETS DE L'OXYTOCINE CHEZ DES MODÈLES D'ANIMAUX SAINS

De façon courante en pharmacologie de sécurité et en toxicologie, les doses retenues chez chacune des espèces doivent être converties en dose équivalente chez l'humain. Cette conversion allométrique s'effectue habituellement à l'aide des lignes directrices des agences règlementaires (U.S. FDA, 2005). De plus, il est nécessaire de choisir la dose qui sera initialement utilisée chez l'humain en fonction de la dose ayant des n'atant pas d'effets adverses chez l'espèce animale la plus sensible (l'espèce animale présentant des effets secondaires adverses avec la dose humaine équivalente la plus basse). Ce concept est essentiel afin d'assurer la sécurité des patients inclus dans les essais cliniques initiaux. En résumé, les doses n'ayant pas d'effet secondaire adverse (NOAEL) suite à l'administration d'OT dans des modèles précliniques étaient les suivantes:

Tableau IV

Modèle	NOAEL	Effet adverse observé	Route d'administration	Facteur pour convertir la dose animal en dose humaine	Dose humain équivalente
Rat anesthésié	213 ng/kg	Hypertension	Bolus IV	6.2	34.4 ng/kg
Rat conscient	250 ng/kg/hr	Hypertension	Perfusion IV	6.2	40.3 ng/kg/hr
Singe conscient	250 ng/kg	Hypotension	Bolus IV	3.1	80.6 ng/kg
Chien conscient	< 50 ng/kg/hr	Effet anti-diurétique	Perfusion IV	1.8	< 27.8 ng/kg/hr
Chien anesthésié	300 ng/kg	Diminution de la contractilité	Bolus IV	1.8	166.7 ng/kg

Les effets anti-diurétiques chez le chien dictent donc la NOAEL la plus basse. La formule pour convertir les doses animales en dose humaine (ou en dose d'une autre espèce) est la suivante (U.S. FDA, 2005):

Dose humaine équivalente = dose animale en mg/kg x (poids animal en kg/70[a] kg)$^{0.33}$

[a] Poid moyen d'un humain estimé à 70 kg.

La dose de 50 ng/kg/hr chez le chien correspond donc à une dose de 30.6 ng/kg/hr chez le porc et représente la dose sans effet secondaire adverse. Les doses choisies dans nos travaux avec le modèle porcin d'infarctus du myocarde sont basées sur les résultats présentés dans le présent chapitre ainsi que sur les évaluations des effets cardiovasculaires et rénaux suite à l'administration d'OT chez le porc sain conscient et anesthésié. Ces évaluations ont été effectués dans le cadre du projet de thèse de la Docteur Norma Ybarra et seront présentés et discutés par celle-ci. La dose retenue pour les évaluations dans le modèle porcin d'infarctus du myocarde fût de 10 ng/kg, soit une dose 3X moindre que la dose sans effet secondaire adverse présentée ci-haut. La justification de ce choix est fondée sur 1) la nécessité d'inclure une marge thérapeutique et 2) la plus grande susceptibilité aux effets secondaires adverses dans un modèle impliquant une pathologie cardiaque.

Chapitre 6: MODÈLE PORCIN D'INFARCTUS DU MYOCARDE

REVUE DE LITTÉRATURE

10. HISTORIQUE ET PRÉSENTATION DES COMPOSANTES EXPÉRIMENTALES DU MODÈLE PORCIN D'ÉVALUATION DE L'EFFICACITÉ DU TRAITEMENT LORS D'INFARCTUS DU MYOCARDE

La décision de poursuivre le développement d'un médicament dépend de la sécurité de celui-ci mais aussi de son efficacité. Le présent chapitre présente les résultats d'évaluation de l'efficacité de l'OT. Les études expérimentales présentées dans le chapitre précédent avaient pour objectif d'évaluer la sécurité de l'OT comme thérapie cardiovasculaire. Plus spécifiquement, ces études nous ont permis 1) d'identifier les effets secondaires pouvant être associés avec l'administration d'OT, 2) d'identifier des biomarqueurs fonctionnels pertinents pouvant être inclus dans l'étude avec le modèle porcin d'infarctus du myocarde et 3) de déterminer une dose dépourvue d'effets secondaires adverses et donc susceptible d'être adéquate comme estimation de la dose thérapeutique dans un modèle d'évaluation de l'efficacité de ce nouveau traitement.

Le modèle porcin d'infarctus du myocarde fût initialement décrit il y a plus de cinquante ans (Garamella *et al.*, 1957). Les méthodologies d'induction de la pathologie (occlusion coronarienne) et d'évaluation de la condition ont progressé suite aux avancées technologiques et de nombreuses modalités d'évaluation sont aujourd'hui disponibles. L'occlusion coronarienne peut être effectuée chirurgicalement par ligature de l'artère à thorax ouvert (Lie *et al.*, 2008) ou par cathétérisation interventionnelle à thorax fermé (Pérez de Prado *et al*., 2009). Aux cours de la dernière décennie, cette dernière approche minimalement invasive a été utilisée dans la vaste majorité des études publiées avec le modèle porcin d'infarctus du myocarde. Le choix de la durée de l'ischémie est un paramètre important dans le choix du modèle d'infarctus du myocarde avec des répercussions sur le taux de survie ainsi que sur la sévérité du dommage myocardique. La littérature récente rapporte l'utilisation de période d'ischémie variant de 30 min à 180 min (Angeli *et al.*, 2009; Choi *et al.*, 2009; Kaneda *et al.*, 2009; Larsen *et al.*, 2008 ; Liu *et al.*, 2007). Malgré ces variations du modèle, l'utilisation d'une période d'ischémie de 60 minutes

constitue la procédure la plus fréquemment utilisée pour engendrer un infarctus du myocarde dans le modèle porcin (Baumert *et al.*, 2007; Boyle *et al.*, 2008; Grøgaard *et al.*, 2007; Osipov *et al.*, 2009; Otake et al., 2007; Sodha *et al.*, 2009) et résulte, comme nos résultats présentés plus loin le suggèrent, en une taille d'infarctus comparable à la situation clinique. Au-delà des considérations techniques du modèle, des articles récents suggèrent la comparaison des nouvelles thérapies avec les thérapies de référence utilisées cliniquement afin de démontrer dans le modèle préclinique la supériorité du nouveau traitement (Angeli *et al.*, 2009). Cette stratégie est couramment mise en oeuvre lors des essais cliniques (Schwarzwälder *et al.*, 2009). Basé sur ces considérations, nous avons choisi une période d'occlusion de 60 min combinée à l'utilisation prophylactique de traitements pharmacologiques de base incluant la lidocaïne, la nifédipine, l'aspirine et l'héparine.

Une fois le modèle établi, le choix des modalités d'évaluation cardiovasculaires suite aux traitements est tout aussi important et constitue l'étape logique subséquente dans la planification des évaluations d'efficacité. Dans ce modèle, une combinaison de biomarqueurs cardiovasculaires fonctionnels ainsi que de biomarqueurs visant à quantifier le dommage tissulaire myocardique est la plus susceptible de fournir une évaluation complète des effets du traitement à l'essai. Parmi les modalités d'évaluations fonctionnelles disponibles, on compte l'échocardiographie (Angeli *et al.*, 2009), la résonnance magnétique (Mather *et al.*, 2009 ; Ishida *et al.*, 2009), le CT-Scan (Mahnken *et al.*, 2009) et la médecine nucléaire (Morooka *et al.*, 2009) pour ne nommer que les principaux. Nous avons retenu l'échocardiographie comme modalité d'évaluation cardiaque fonctionnelle afin de rencontrer notre objectif de combiner une modalité fonctionnelle avec une évaluation du dommage tissulaire. L'utilisation de la double coloration pour quantifier la zone à risque (zone d'ischémie) avec le bleu d'Evans en proportion de la zone infarcie mesurée avec la coloration au tétrazolium est une méthode largement reconnue avec le modèle porcin. Cette modalité permet de quantifier le dommage tissulaire tout en tenant compte de la sévérité de la zone ischémique qui varie en fonction de l'anatomie coronarienne de chaque animal (Suzuki *et al.*, 2008).

Parmi les marqueurs sériques de dommages myocardiques suite à l'infarctus, la CKMB (Zaninotto *et al.*, 2009) et la troponine T figurent parmi les plus souvent utilisés (Hedström *et al.*). La troponine T est généralement reconnue comme un marqueur plus sensible que la CKMB (Harris *et al.*, 2000 ; Nageh et al., 2003) dans l'évaluation de dommages myocardiques justifiant notre choix d'utiliser cette dernière comme marqueur sérique.

L'annexine-V est présente dans de nombreux tissus et exerce un effet anti-coagulant important *in vitro* (Rand *et al.*, 1998). Certaines études ont associé un niveaux circulant d'annexine-V faible à un état d'hypercoagulabilité chez des individus admis pour un infarctus du myocarde lorsque comparés à une population d'individus sains (Shojaie *et al.*, 2009). D'autres études suggèrent une augmentation de la concentration plasmatique d'annexine-V chez les patients atteints d'un infarctus du myocarde (Kaneko *et al.*, 1996). En recherche, l'annexine-V est reconnue comme un marqueur d'apoptose (Kempf *et al.*) des cardiomyocytes (Han *et al.*, 2003). Ce marqueur constitue une composante de nos évaluations des effets de l'OT sur la zone d'infarctus.

Une hypothèse sur laquelle reposaient nos travaux d'évaluation de la sécurité et de l'efficacité de l'oxytocine est présentée comme préambule à l'interprétation subséquente des résultats. Selon ce concept simple, un médiateur cellulaire doit être maintenu à l'intérieur d'une fenêtre physiologique optimale afin d'assurer ses fonctions. À l'extérieur de cette fenêtre optimale les effets du médiateur deviennent délétères. Ce concept est bien illustré dans un article de revue publié par notre groupe et qui présente l'exemple du monoxide d'azote (NO) (Blaise GA, Gauvin D, Gangal M, Authier S. Nitric oxide, cell signaling and cell death. Toxicology. 2005 Mar 15;208(2):177-192). L'article définit le rôle physiologique du NO ainsi que ses effets toxiques au niveau cellulaire dans le cadre du traitement au monoxyde d'azote par inhalation. Lorsque les niveaux de NO cellulaire passent sous un certain seuil, la cellule prend la voie de l'apoptose. En contre partie, une augmentation des niveaux de NO au-delà des niveaux physiologiques entraîne l'apoptose ou encore la nécrose cellulaire. De façon comparable, nous proposons comme hypothèse que les niveaux d'OT doivent être maintenus à l'intérieur d'une fenêtre physiologique optimale. Le

traitement avec l'OT chez des individus ayant des niveaux endogènes élevés résulterait en des effets délétères du traitement alors que l'administration d'OT à des individus ayant des niveaux endogènes bas résulterait potentiellement en des effets bénéfiques.

Quels sont les mécanismes possibles de l'OT dans l'amélioration de la guérison suite à l'infarctus du myocarde ? Des articles récents suggèrent que les mammifères peuvent répondre à un dommage tissulaire par la migration de cellules mésenchymateuses au site de la lésion. Ce phénomène a été démontré à l'aide de modèle d'infarctus du myocarde chez le rat (Jiang *et al.*, 2006). Les effets potentiels de l'OT sur la guérison suite à l'infarctus du myocarde pourraient être le résultat de la différentiation de cellules souches en cardiomyocytes au site d'infarctus mais ce mécanisme ne représente pas la seule possibilité. Ces cellules mésenchymateuses pourrait également agir par la sécrétion de facteurs de croissance et de cytokines favorisant la guérison par des fonctions paracrines tel que suggéré dans des travaux récents avec des modèles précliniques d'infarctus du myocarde (Korf-Klingebiel *et al.,* 2008; Li *et al.*(2), 2009).

Chapitre 7: EFFICACITÉ ET SÉCURITÉ DE L'OXYTOCINE DANS UN MODÈLE PORCIN D'INFARCTUS DU MYOCARDE

ÉTUDES EXPÉRIMENTALES

11. EFFETS CARDIOVASCULAIRES DE L'OXYTOCINE DANS UN MODÈLE PORCIN D'INFARCTUS DU MYOCARDE

L'article qui suit présente les résultats expérimentaux d'évaluation de l'OT comme traitement de l'infarctus du myocarde. Faisant suite aux évaluations pharmacocinétiques et pharmacodynamiques de l'OT chez les animaux sains, ce projet utilise un modèle d'efficacité préclinique afin d'évaluer les effets bénéfiques ou adverses d'un traitement systémique par perfusion continue d'OT sur les paramètres de la fonction cardiaque. Développé à l'Institut de Cardiologie de Montréal, le modèle d'infarctus du myocarde porcin présente des mesures fonctionnelles incluant l'échocardiographie et l'angiographie ainsi que des évaluations post-mortem telles que la planimétrie, l'analyse d'expression protéique (western blot) et l'immunohistochimie. Ce projet central au programme de thèse a déployé d'importantes ressources, à la fois financières et humaines, et a contribué significativement à la formation du Docteur Simon Authier. Le manuscrit qui suit présente les résultats et interprétations de ces travaux et a été rédigé principalement par le Docteur Simon Authier avec l'assistance de ses mentors et les apports des autres étudiants et personnel impliqués dans le projet. Le manuscrit est actuellement en révision pour publication dans le *Journal of Cardiovascular Pharmacology*.

Cardiovascular Effects of Oxytocin Infusion in a Porcine Model of Myocardial Infarct

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Short title: *OXYTOCIN EFFECTS IN A MYOCARDIAL INFARCT MODEL*

Experimental work was undertaken at: Montreal Heart Institute

ABSTRACT

Summary: The effects of OT on cardiovascular endpoints were assessed in a myocardial infarct (MI) model. OT (10 $ng \cdot kg^{-1} \cdot hr^{-1}$) or saline infusion was initiated at reperfusion (D0) or 8 days (D8) after MI. Our hypothesis was that OT administration to individuals with a low pre-treatment OT levels (PTOT) may be beneficial, whereas individuals with an elevated PTOT would be prone to adverse effects. Starting OT on D0 reduced left ventricular fraction shortening evaluated 8 days post-MI, and had no effect on infarct size. OT initiated on D8 in animals with high PTOT decreased ejection fraction (EF) and increased left ventricular end systolic diameter (LVESD) at 28 days post-MI, but had no significant effects on EF and LVESD in low PTOT animals. OT infusion reduced OT receptor (OTR) protein expression in high PTOT animals but not in low PTOT animals. Amongst placebo-treated individuals, low PTOT presented a trend towards reduced EF and larger infarct size compared with high PTOT. MI areas of fibrosis presented lower Annexin-V expression compared with MI with cardiomyocyte predominance. Pre-treatment endogenous OT levels and timing of OT administration post-MI appear to impact outcome in this porcine model and further investigations are warranted to define potential role of OT in cardiac regenerative therapy.

Key Words: Oxytocin - myocardial infarct – cardiovascular - continuous infusion

1. INTRODUCTION

Oxytocin (OT) was discovered a century ago (1) and has been used in obstetrics for decades (2). This hormone, usually associated with reproduction, has a potential role in clinical management of myocardial infarct (MI). OT was reported to induce *in vitro* differentiation of P19 embryonic stem cells into beating cardiomyocytes (3, 4), an *in vitro* differentiation facilitated by formation of embryoid bodies (cell aggregation) (5). OT-differentiated cardiomyocytes respond to cardioactive drugs (6). OT has also been shown to induce *in vitro* and *in vivo* differentiation of somatic stem cells into cardiomyocytes, with evidences of homing to injured heart area (7). Oxytocin receptor (OTR) expression has been found to correlate with cardiomyocytes differentiation. Elevated cardiac OTR expression was associated with intense cardiomyocyte hyperplasia in neonatal rats, whereas a progressive decline in OTR was noted from infancy to adulthood (8). Recently, up-regulation of OTR was suggested as an important factor to allow differentiation of P19 cells into cardiomyocytes (9). Neonatal exposure to OT resulted in a marked increase of cardiac OTR mRNA (10). Up-regulation of OTR expression during pregnancy is a well-known phenomenon in various species including human (11). During pregnancy, OTR expression is not only modified in the reproductive system but also in other organs, as evidenced by increased cardiac OTR expression during pregnancy in rats (12). Various hormonal combinations were reported to up-regulate OTR expression even in non-pregnant animals (13). Recently, OT treatment was reported to reduce infarct size by 66% in an *in vitro* model of myocardial ischemia in rats (14). The inducible OTR expression in adult non-pregnant animals combined with the

role of OTR in cardiac differentiation suggests that OT could be considered as a systemic treatment for cardiac cell regeneration in adults.

In contrast, non-linear pharmacokinetics (15) associated with potentially dramatic pharmacodynamics responses of OT on the electrolytes / renal homeostasis or cardiovascular function are potential serious adverse effects of this peptide, when administered as therapeutic agent for cardiac regeneration to patient with cardiovascular disease. We expected the fine balance between beneficial and adverse effects of OT to obey similar pathophysiological principles applying to other endogenous mediators. We postulated that OT levels must be maintained within an optimal physiological range to provide beneficial effects on recovery from MI. The concept of optimal dose was seen in a variety of endogenous mediators like gasotransmitters: nitric oxide (NO), hydrogen sulfoxide or carbon monoxide. These gasotransmitters lead to cell death when present in excessive concentration; while NO in insufficient concentration will lead to apoptosis (16). We hypothesized that OT administration to individuals with a low baseline endogenous OT level (low OTBL) may be beneficial for treatment of MI, whereas individuals with an elevated baseline endogenous OT level (high OTBL) would be prone to present adverse effects. The aim of the current study was to evaluate the cardiovascular effects of long-term subcutaneous OT infusion in a porcine model of MI.

2. METHODS

2.1. Statement on use and care of animals. During the study, care and use of animals were conducted in accordance with principles outlined in the

current Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care. Experimental procedures, performed at the Montreal Heart Institute, were reviewed and approved by Institutional Animal Care and Use Committees prior to initiation.

2.2. Animal housing and preparation.

A total of thirty-two (32 domestic Yorkshire-Landrace castrated male pigs were obtained for the experiment. Animals weighted 21 to 29 kg at initiation of the experiment. Animals received acetylsalicylic acid (Aspirin®; 25 mg/kg) and nifedipine (1 mg/kg) orally at least 12 hrs before each percutaneous coronary intervention (PCI). Premedication (Telazol®, 6 mg/kg, IM and atropine 0.03 mg/kg IM) was used ~15 min before anesthesia induction with isoflurane (3-5%), followed by endotracheal intubation. Anesthesia was maintained with isoflurane (1.5-2%) in a 2:1 mixture of oxygen and air with assisted ventilation. Continuous monitoring consisted in heart rate, ECG, and systemic arterial blood pressures.

2.3. Experimental methods.

2.3.1 Animal Management

Animals were allocated into four experimental treatments including OT (10 $ng \cdot kg^{-1} \cdot hr^{-1}$) or saline infusion, initiated immediately at reperfusion (D0; n=3 saline and n=4 OT) or 8 days (D8; n=8 saline and n=14 OT) after MI (Table 1). Echocardiography (Phased Array of 5 MHz, Advance Technology Laboratories, Bothell, WA, U.S.) evaluations were performed by a veterinarian blinded to experimental treatments. Following percutaneous

puncture of the right or left femoral artery, a sheath was placed and heparin (200 UI/kg, Leo Pharma, Thornhill, Canada) and lidocaine (100mg, AstraZeneca, Mississauga, Canada) were administered as intra-arterial boluses. Left coronary angiography (Visipaque, Amersham Health Inc, Oakville, Canada) was performed and the coronary anatomy defined. A site in the left anterior descending (LAD) coronary artery distal to the first septal branch was selected for occlusion (Figure 1A). The selected site of the LAD was occluded for 60 min (confirmed by contrast media injection after occlusion; Figure 1B) to induce an acute anterior wall MI. After balloon occlusion, animals remained anesthetized for a reperfusion period of at least 30 min and were then returned to their cage once complete recovery from anesthesia was achieved. Animals presenting ventricular fibrillation (VF) were defibrillated at 360 J (CodeMaster XL+, Hewlett Packard, Palo Alto, CA) and additional doses of lidocaine were given for refractory cases. Troponin T was measured in the first cohort of seven (7) animals treated immediately after MI but was not measured in subsequent animals to minimize stress from blood collection during the first 24 hrs after MI in animals susceptible to potentially fatal ventricular arrhythmia. OT (Peninsula, Laboratories, Belmont, CA) was dissolved in saline to the required concentration and was administered by continuous subcutaneous infusion using osmotic pumps (Alzet® Model 2ML4, Durect, Cupertino, CA). At study termination, animals were anesthetized using the same procedure. The LAD was reoccluded at the same anatomical location as the initial MI, using playback of fluoroscopic images as reference. Evan's Blue was infused through a pigtail ventricular catheter delineating the risk area by negative coloration (Figure 2). The heart was

excised and sectioned into 7 slices from apex to base and then incubated in triphenyl tetrazolium chloride (TTC, 1%) at 37^0C for 10 min. TTC produces a brick-red formazan pigment in viable myocardium while necrotic myocardium does not stain and appears whitish-tan. The area at risk (AAR) and the infarct area (IA) were determined by computerized planimetry from digital images of the transverse sections. The risk and infarct areas were expressed as a proportion of the total left ventricle (Σ risk or infarct area in all slices / Σ left ventricle area in all slices). Infarct size was calculated as ratio of IA to AAR (17).

2.3.2 Plasma OT and Western blot analysis

Blood samples (baseline and at steady-state) were centrifuged at 4°C in tubes with inhibitors of plasma oxytocinase, pepstatin A and phenylmethanesulfonyl fluoride purchased from Sigma-Aldrich Chemical (Oakville, ON, Canada) and stored at -80°C pending analysis. Plasma OT concentrations were determined using commercially available ELISA detection kits (Assay design, Ann Arbor, MI, U.S.A.). The lower limit of detection was 16 pg/ml, with a sensitivity of 11.7 pg/ml. Animals in which treatment was initiated at D8 post-MI were classified based as low (<115 pg/mL) and high (≥115 pg/mL) OTBL. For Western blot analysis, protein samples (50 µg) obtained from the myocardium of the left ventricle outside of the infarct area for each animal were subjected to electrophoresis on a 10% SDS-polyacrylamide gel and the separated proteins were blotted onto polyvinylidene difluoride membranes at 90 V for 90 min (Hybond-P; Amersham GE Healthcare, Buckinghamshire, UK). Membranes were

incubated with blocking buffer, 5% nonfat dry milk in Tris buffered saline (TBS) with Tween 20 pH 8 (Sigma, St. Louis, MO, USA), for 2 h at room temperature, followed by an overnight (12–16 h) incubation at 4°C with anti-oxytocin receptor antibody (Santa Cruz Biotechnology Inc., sc-8103, Santa Cruz, CA, USA) at a 1:1000 concentration. Membranes were washed trice with TBS-Tween 20 and incubated for 30 min at room temperature with the horseradish peroxidase-labeled anti-goat secondary antibody (R&D Systems, HAF017, Cedarlane Laboratories Ltd., Burlington, ON, Canada) at a dilution of 1:15 000. The antigen-antibody complex was visualized using a highly sensitive chemiluminescence system (Immun-Star^{TM} WesternC^{TM}, Bio-Rad Laboratories), according to the supplier's recommendations. Signal was visualized with an imaging system equipped with a cooled CCD camera (Chemi genius^2, Syngene, Perkin Elmer, Woodbridge, ON, Canada). The quantification of protein bands was performed by densitometry using GeneSnap from Syngene (Perkin Elmer, Woodbridge, ON, Canada). Protein expression was represented as a value relative to ß-actin expression. Triplicate gels and immunoblots were run for all samples.

2.3.3 Annexin-V Cell Expression

Myocardial tissue samples from the infarct and infarct border areas were stained for Annexin-V, a marker of apoptosis commonly used *in vitro* (18) and *in-vivo* (19). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. Non-specific binding was blocked by incubating in PBS containing 10% normal serum for 10 min. Tissues were incubated with commercially available goat polyclonal Annexin-V (Santa Cruz

Biotechnology, Santa Cruz, CA) and normal goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was used as negative control. Antibodies were diluted in PBS containing 1% normal serum and incubated overnight at 4°C in a humidified chamber. Horse anti-goat monoclonal biotinylated antibodies (VectorLabs, Burlingame, CA) were added as secondary antibodies. Sections were then washed, incubated with Streptavidin-HRP (VectorLabs, Burlingame, CA) for 30 min and visualized using DAB-peroxidase substrate solution (VectorLabs, Burlingame,CA). Finally, the sections were counter stained with hematoxylin QS (VectorLabs, Burlingame,CA) and mounted with permount. Semi-quantitative evaluation of Annexin-V expression was performed on histologic cross-sections by a veterinarian blinded to study treatments. Infarct border and infarct areas were quantified separately. Within the infarct areas, subareas with predominance of cardiomyocytes or fibroblasts (fibrosis) were identified and the cell type (cardiomyocytes or fibroblasts) was recorded for each evaluated field.

2.3.4 Statistical Analysis

Statistical analysis was done using a Student T-Test or analysis of variance (ANOVA) as appropriate. When ANOVA was required, normality of distribution was evaluated using the Shapiro-Wilk test. The Levene test was used to examine the homogeneity of group variances. When both of these tests were found to be non-significant, ANOVA was considered appropriate. Whenever the overall group differences were shown significant (F-Test for ANOVA), pairwise comparisons were conducted using Dunnett's test for

ANOVA. $p < 0.05$ was considered significant. Results were expressed as mean ± SEM.

3. Results

All animals presented ventricular arrhythmia at least once during MI or reperfusion. Premature ventricular complexes (PVC), ventricular tachycardia (VT) and VF were the most frequently observed arrhythmia, often present in sequential order (PVC → VT → VF). Emergency procedures, including 360J defibrillation and arterial lidocaine boluses, restored sinusal rhythm except for three (3) pigs that died of VF during MI or reperfusion. These 3 animals were excluded from the analysis. At ventricular planimetry, the AAR of all animals (all groups combined) was 25.7% ± 2.7% of the left ventricle. The IA of placebo-treated animals was 13.9% ± 1.7% of the left ventricle. The infarct size (Σ IA/Σ AAR) for placebo-treated animals was 58.3% ± 12.2% and 57.1% ± 8.9% at 8 and 28 days post-MI, respectively.

OT infusion initiated immediately after MI

Animals treated with OT for 7 days commencing immediately after MI presented significantly decreased fraction shortening (Figure 3). One animal treated with OT immediately after MI died on Day 2. Necropsy of this animal did not reveal any gross pathology lesion and a fatal ventricular arrhythmia was suspected. Planimetry with double staining in situ at D8 post-MI did not reveal any effect of treatment with OT on the infarct size (58.4% ± 8.3% with OT). An increase in Troponin T was noted following MI but no treatment-related effect was observed (Figure 4). No other statistically or clinically

significant difference was noted between saline- and OT-treated animals when treatment was initiated immediately after MI. Baseline plasma OT levels were 83.7 ± 14.5 pg/ml. Plasma OT levels at steady-state (D8) were 119.8 ± 31.3 pg/ml in OT-treated animals.

OT infusion initiated 8 days after MI

All animals treated with OT or saline commencing on Day 8 post-MI survived until scheduled termination (Day 28 post-MI). A significant difference between mean change of ejection fraction (EF, $p < 0.05$) (Figure 5) was noted when comparing groups (Low OTBL + saline, Low OTBL+ OT, High OTBL + saline and High OTBL+ OT). Statistical contrasts indicated a less important decrease of EF in animals with high OTBL treated with saline than animals with high OTBL treated with OT ($p<0.01$). Left ventricular end-systolic diameter (LVESD) was more importantly increased in animals with high OTBL treated with OT than in animals with high OTBL receiving saline ($p<0.05$) (Figure 6), indicative of more severe early ventricular dilation following OT treatment. OTR expression at 28 days post-MI was significantly lower ($p<0.05$) in animals with high OTBL treated with OT than in animals with high OTBL receiving placebo (Figure 7). Placebo-treated animals with low OTBL presented a trend ($p=0.059$) towards larger infarct size (Figure 8). Plasma OT levels are presented in Table 2. At immunohistochemistry, infarct border areas evaluated at 28 days post-MI did not reveal significant expression of Annexin-V in any of the animals. In the infarct area, expression of Annexin-V was not significantly different in all groups (Low OTBL + saline 57.5% ± 9.2%; Low OTBL + OT 30.0% ± 9.7%; High OTBL + saline 40.0%

± 5.9%; High OTBL + OT 45.0% ± 4.6%). As illustrated in Figures 9 and 10, Annexin-V positive cell counts were significantly higher in infarct areas with predominance of cardiomyocytes when compared with areas of fibrosis (fibroblast predominance).

4. Discussion

Infarct size in placebo-treated animals and mortality (8%) prior to treatment were comparable to previously reported values in a porcine MI model with 60 min LAD occlusion (17, 20). The average IA in this porcine model was comparable to IA observed in the human patient population (21, 22, 23). However, the age (juvenile animals) and lack of coronary disease (*e.g.* absence of atheromatous plaques) in the porcine model differ from the human patient population and present potential limitations of the model. Unequal group sizes and the low number of animals in each group also present limitations of the current study.

Treatment of MI and associated risk factors have been vastly dominated by chemical entities for the past decades. However, biologics have emerged as a valuable addition to the medical arsenal with candidates at several stages of development. In 1994, Abciximab, a Fragment antigen binding (Fab), the platelet glycoprotein IIb/IIIa receptor used to prevent ischemic cardiac complications in high risk patients was the second monoclonal antibody (mAb) approved for clinical use in the U.S. More recently, IL-33 was shown to reduce atherosclerosis development in rodent models (24).

The effects of OT on cellular differentiation triggered an interest for regenerative therapies with this endogenous nanopeptide. OT was shown to

stimulate differentiation of human mesenchymal stem cells into osteoblasts over adipocytes, providing a potential application for osteoporosis treatment (25). OT was reported to have protective properties against skin burn and gastric ulcers (26). In addition to *in vitro* cardiomyogenic activity, OT induces differentiation of stem cells present in the adult mouse heart into cardiomyocytes (27). These observations increased the relevance of therapeutic investigations of OT in a preclinical MI model.

When initiated immediately after acute MI in a porcine model, long-term OT infusion (7 days) was associated with worsening of fraction shortening (Figure 3). The mortality of an OT-treated animal on Day 2 could not be excluded as a potential treatment-related effect.

Early ventricular dilatation after acute MI is a well-recognized adverse event in humans (28). Ventricular remodeling, caused by end-diastolic wall stress, leads to a progressive dilation of the infarct zone but also of the healthy myocardium, which puts patients at risk of developing congestive heart failure, ventricular arrhythmias, and free wall rupture. Despite its therapeutic potential, OT holds significant clinical risks as observed in the cohort of animals treated immediately after MI.

In healthy volunteers, OT injection (10 IU) induced chest pain, transient severe tachycardia, hypotension, and concomitant signs of myocardial ischaemia (29). A case of MI was recently correlated with OT treatment in a pregnant woman with obstructive cardiomyopathy (30). Based on our results and clinical reports, continuous infusion of OT starting immediately after MI will require further investigations to understand potential association with adverse effects.

A complex inflammatory response clears the infarct from cells and matrix debris in the first days after an acute MI (31). Gene expression of inflammatory cytokines such as TNF-α, IL-1ß, and IL-6 returns toward baseline at 1-week post-MI (32). The acute inflammatory response present on Day 3 post-MI is progressively replaced by granulation tissue proliferation in the infarct area at 1 week post-MI (33). Based on these considerations, OT treatment was initiated 8 days post-MI to avoid the acute inflammatory phase.

Plasma OT levels remained relatively stable after MI in saline-treated animals (Table 2). Continuous OT infusion increased plasma levels in low PTOT animals but only a marginal increase was observed in high PTOT animals. Exogenous OT in high PTOT animals could decrease endogenous release and/or higher oxytocinase concentrations could be present in animals with high PTOT. Additional investigations would be needed to evaluate these hypotheses.

Treatment with OT starting 8 days after MI in animals with high PTOT resulted in deterioration of the ventricular function at 28 days after MI. The increase in left ventricular end-systolic volume, a primary predictor of long-term survival (34), following treatment with OT was a significant adverse effect observed in our study. In MI patients subjected to coronary artery bypass, EF recovery and long-term survival are inversely correlated with left ventricular end-systolic volume (35), a potentially valuable biomarker of cardiac outcome. Treatment with OT starting 8 days after MI in animals with low PTOT did not significantly alter EF and LVESD (Figures 5 & 6). Interestingly, comparison between placebo-treated animals with low and high PTOT suggested a trend to observe more important deterioration in EF with

low PTOT (Figure 5) when compared with high PTOT. Planimetry, an evaluation modality independent from echocardiography, also suggested a larger infarct area in placebo animals with low PTOT when compared with placebo animals with high PTOT (Figure 8). OT is recognized as a hormone positively correlated with stress (36). It is possible that animal presenting high PTOT perceived more stress which could influence outcome.

The effect of OT on cardiomyogenesis appears to involve NO (37). It is recognized that cellular levels of NO need to be maintained within a physiological range to ensure cell survival. The increased susceptibility to adverse cardiovascular effects of OT in animals with high PTOT suggests that OT levels also need to be maintained within a physiological range to prevent adverse cardiovascular response following MI.

A decrease in cardiac OTR total protein was observed in animals with elevated PTOT receiving OT infusion for 21 continuous days starting 8 days post-MI. Down-regulation of OTR mRNA was reported following OT treatment in cultured myometrial cells but total OTR protein was not modified *in vitro* after OT exposure for up to 48 hrs (38). A 300-fold decrease in OTR mRNA was observed in women receiving OT infusion during labor (39). Results following continuous OT infusion in humans contrast with changes following a single OT injection, in a neonatal rodent model where a transient increase in OTR mRNA expression was observed (10). A limited amount of data is available on regulation of cardiac OTR expression. The current study reports for the first time a decrease in cardiac OTR total protein *in vivo* following long-term OT infusion.

Immunohistochemistry shows higher expression of Annexin-V in infarct areas with predominance of cardiomyocytes when compared with areas with fibroblast predominance (fibrosis). Scar formation is a proliferative process in response to the myocardial ischemic injury that includes newly differentiated fibroblasts which explains the lower expression of a recognized marker of apoptosis, Annexin-V. These results suggest that analysis of Annexin-V expression in MI areas benefits from cell type differentials, as the abundance of fibroblasts, although associated with lower Annexin-V expression, are not expected to yield a favorable outcome in ischemic areas.

In conclusion, OT was shown to induce *in vitro* differentiation of cardiomyocytes. Based on results from the current study, the effects of OT infusion after MI appear to be influenced by the timing of the treatment and endogenous OT levels. Additional investigations would be needed to understand the roles of OT and interactions with other hormonal mediators in response to MI. Conversely, the prognostic value of OT levels post-MI may warrant further investigations and comparison of OT levels with clinical outcome may help to understand the potential role of OT in the MI pathophysiology.

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Table 1. Experimental groups

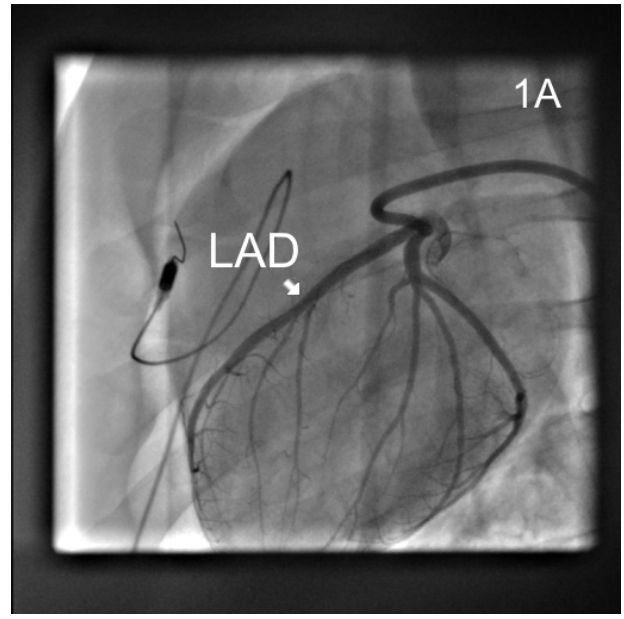
OT Dose rate (ng/kg/hr)	Start of treatment (post-MI)	Oxytocin plasma baseline level	Treatment end and termination day (post-MI)	Number of animals (males)
0	Immediately post-MI	N/A	8	3
10	Immediately post-MI	N/A	8	4
0	Day 8	Low	29	5
0	Day 8	High	29	3
10	Day 8	Low	29	6
10	Day 8	High	29	8

Table 2. Plasma Oxytocin Levels for Animals with Treatment Initiated on Day 8 post-MI

OT Dose rate (ng/kg/hr)	Baseline Oxytocin plasma level at (pg/mL)	Oxytocin plasma level at steady-state# (pg/mL)
0	91.8 ± 5.7	119.3 ± 9.8
0	136.4 ± 10.8	134.4 ± 22.6
10	91.1 ± 8.5	174.9 ± 31.3*
10	217.0 ± 28.9	239.3 ± 52.3

Plasma level at 21 days after initiation of continuous infusion (28 days post-MI).

* $p<0.05$ Significantly different from baseline using a Student T-test for paired samples



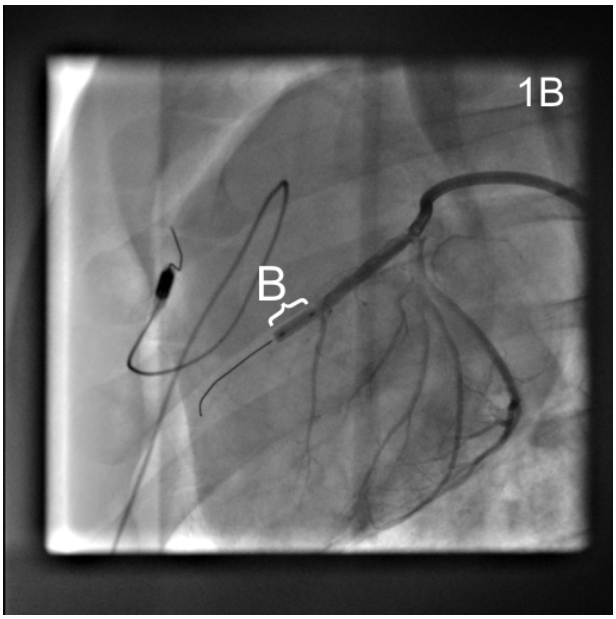


Figure 1A LAD: Left anterior descending coronary artery during angiography in a pig to define coronary anatomy and select the occlusion site. **Figure 1B** B: Balloon catheter occlusion (60 min) of the left anterior descending coronary artery distal to the first septal collateral branch.

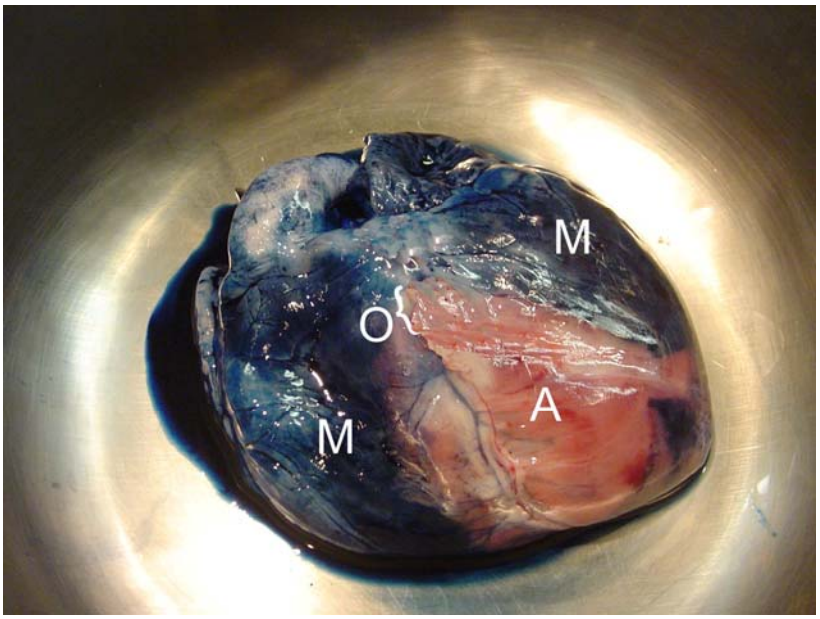


Figure 2 Negative coloration of area at risk with Evan's blue. O: Left anterior descending coronary occlusion site; A: Area at risk, M: Myocardium outside of the ischemic zone.

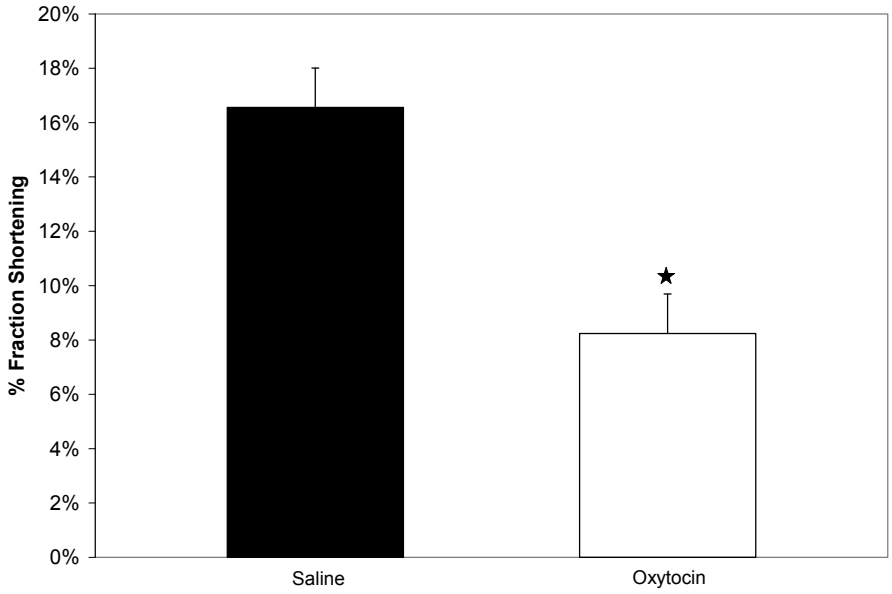


Figure 3 Left ventricular fraction shortening expressed as percent change from baseline evaluated by echocardiography on Day 8 post-MI with treatments initiated immediately after MI. Results indicate adverse effects of the OT with decreased left ventricular fraction shortening.

* $p < 0.05$.

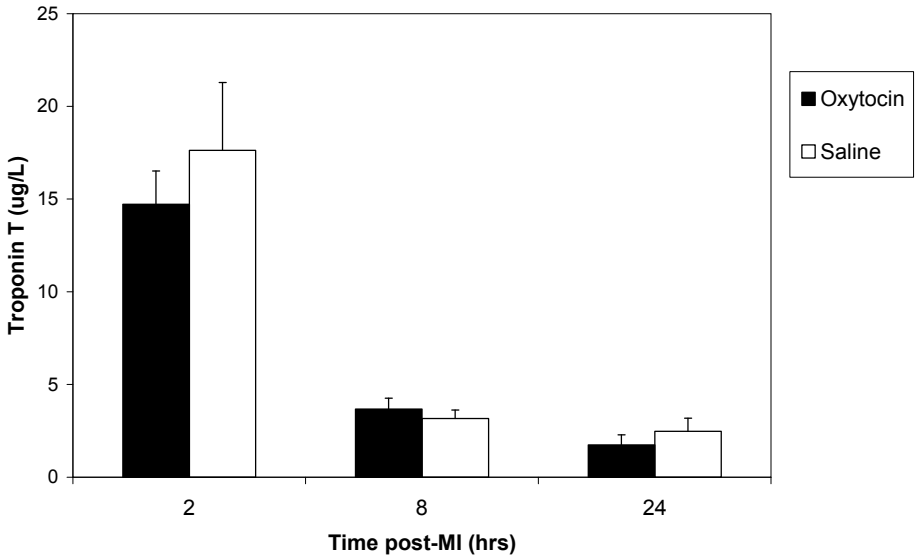


Figure 4 Plasma concentration of Troponin T over time in a porcine model of MI. No significant difference was observed between Saline and OT treated animals (infusion initiated immediately after MI).

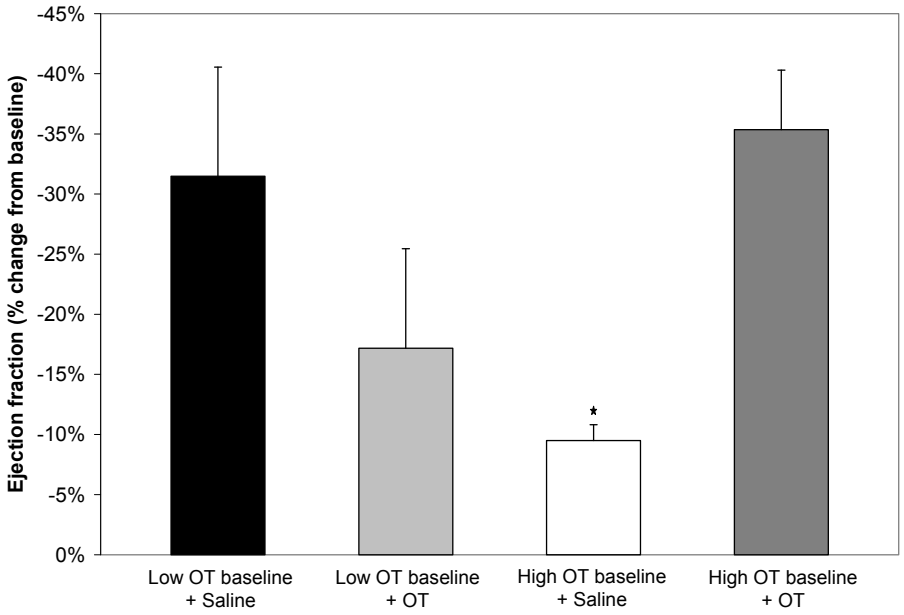


Figure 5 Ejection fraction (EF) evaluated at echocardiography 28 days after MI with treatment initiated 8 days after MI. Placebo-treated animals with high OTBL presented a less severe reduction in EF than animals with high OTBL treated with OT (p<0.01).

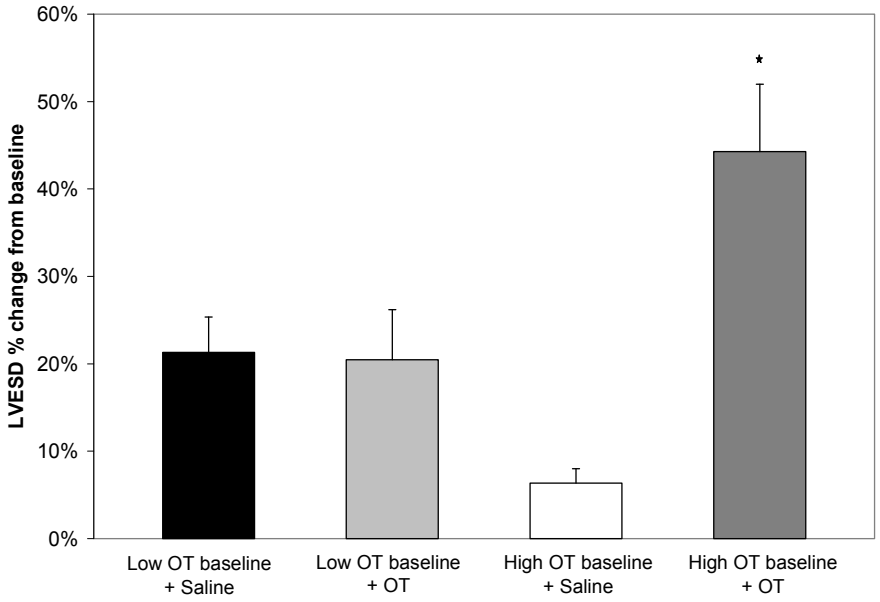


Figure 6 Left ventricular end systolic diameter (LVESD) 28 days after MI. Animals with high OTBL receiving OT starting on Day 8 post- MI presented a more important increase in LVESD than animals with high OTBL receiving Saline (*, $p<0.05$).

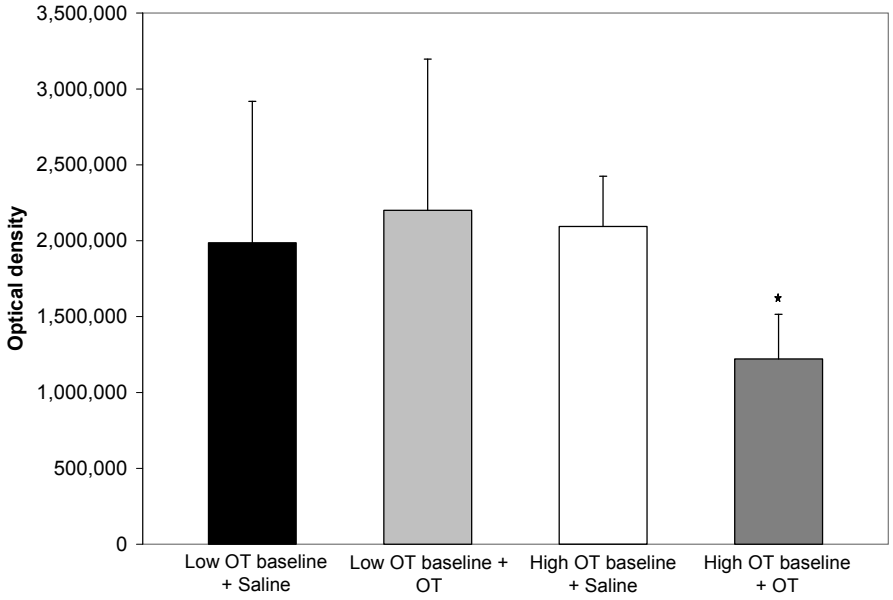


Figure 7 Oxytocin receptor (OTR) expression in healthy myocardium outside of infarct at 28 days post-MI. OTR expression was significantly lower (p<0.05) in animals with high OTBL treated with OT infusion than animals with high OTBL treated with Saline.

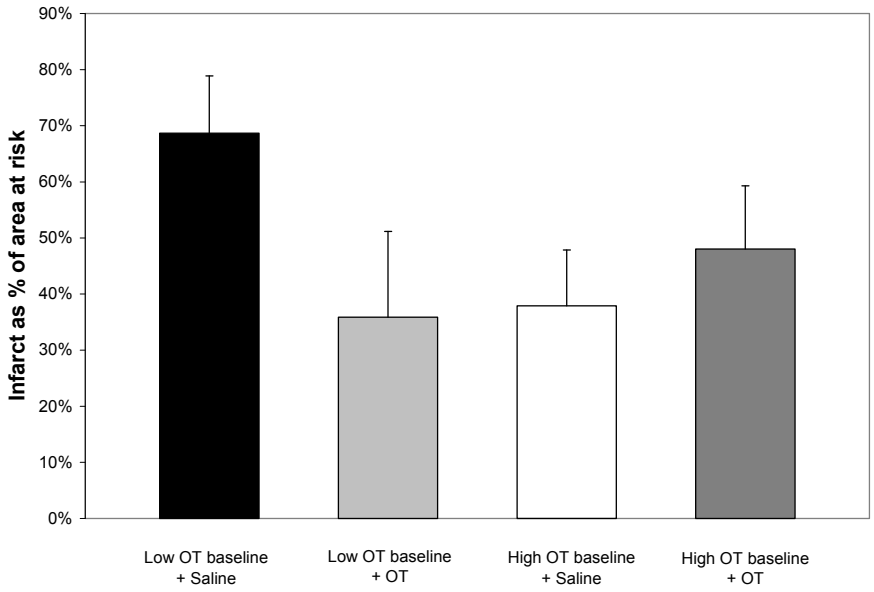


Figure 8 Planimetry 28 days after MI with onset of treatment on Day 8 post-MI. Myocardial infarct area presented as a percent of the area at risk. Placebo-treated animals with low OTBL presented a trend towards larger infarct size (p=0.059).

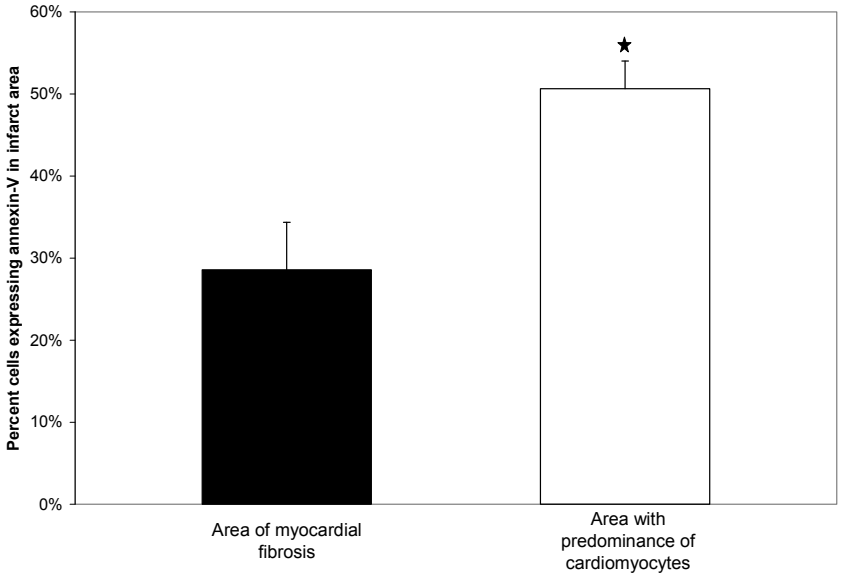


Figure 9 Percent of Annexin-V positive cells 28 days after MI. Higher counts of Annexin-V positive cells were noted in the infarct areas with predominance of cardiomyocytes when compared with areas with fibroblast predominance ($p<0.01$).

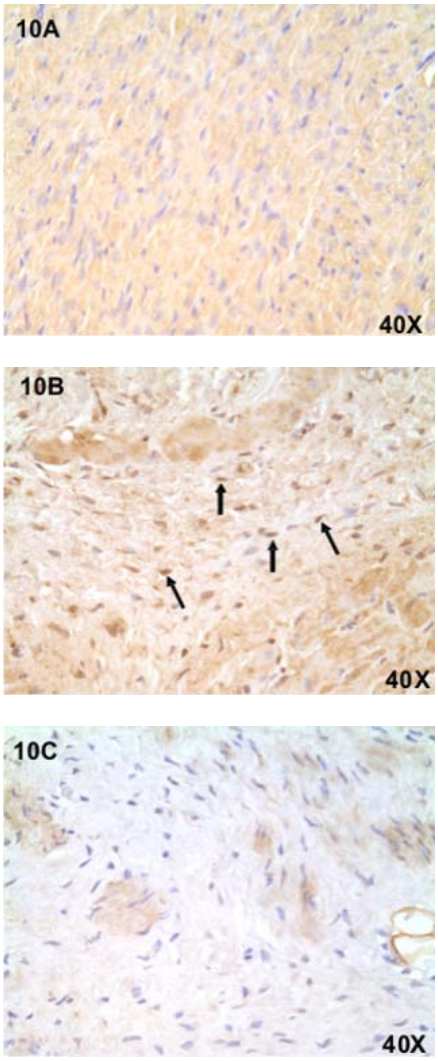


Figure 10 Annexin-V staining in control myocardium (outside infarct area) (10A) compared with myocardium in the infarct area (10B) presenting Annexin-V positive cells (arrows) and area of fibrosis negative for Annexin-V (10C).

Chapitre 8: INTERPRÉTATION DES DONNÉES DE SÉCURITÉ ET D'EFFICACITÉ DANS LE CONTEXTE DU DÉVELOPPEMENT DE L'OXYTOCINE COMME NOUVELLE THÉRAPIE CARDIOVASCULAIRE

12. LE PROFIL DE SÉCURITÉ DE L'OXYTOCINE DANS LE CONTEXTE ACTUEL DU DÉVELOPPEMENT DES MÉDICAMENTS

Notre hypothèse initiale était que l'OT, ayant démontré la capacité d'induire la différentiation de divers types cellulaires en cardiomyocytes *in vitro*, pourrait avoir des effets bénéfiques comme thérapie systémique suite à l'infarctus du myocarde. Ce concept de nouvelle thérapie devait toutefois passer avec succès les étapes du processus de développement des médicaments. Ces étapes entre l'identification d'une indication thérapeutique potentielle et l'approbation d'un nouveau médicament sont nombreuses.

La revue de littérature présentée dans le premier chapitre a permis de dresser un tableau détaillé de ce domaine du développement des médicaments dont les assises dans un environnement scientifique et médical sont sous une forte influence règlementaire. Tel que souligné tout au long de la thèse, très peu de composés candidats à devenir un médicament réussissent à compléter le parcours menant à l'approbation. Les causes d'échec sont discutées par une industrie perplexe (Redfern *et al.*, 2002) sans qu'on puisse vraiment identifier de coupable. On peut toutefois résumer le contexte de la façon suivante. Afin d'obtenir l'approbation règlementaire, un nouveau médicament doit démontrer son efficacité et sa sécurité. Cette adéquation combinant l'absence d'effets secondaires adverses aux doses thérapeutiques et la confirmation d'efficacité est rarement présente. Afin de s'assurer de rencontrer les exigences modernes pour le développement de l'oxytocine comme nouvelle thérapie de l'infarctus, nous avons d'abord complété la validation des modèles requis pour assurer la validité des essais avec l'OT. Après avoir analysé les caractéristiques de la population cible pour ce nouveau traitement, il devint évident que les évaluations de la sécurité cardiovasculaire de l'OT seraient prépondérantes dans l'évaluation de la sécurité de ce traitement pour la nouvelle indication thérapeutique proposée.

La pharmacologie de sécurité, cette nouvelle discipline évaluant les effets pharmacodynamiques des nouveaux médicaments, est en pleine effervescence poussée par les avancées technologiques qui permettent de

raffiner les modalités d'évaluation de la sécurité. La validation des modèles de pharmacologie de sécurité des médicaments est un item majeur à l'agenda de cette nouvelle discipline étant donnés les enjeux médicaux et économiques qui y sont rattachés. D'abord, la fiabilité et l'intégrité de ces modèles d'évaluation de la sécurité des médicaments est essentielle étant donnée que la santé des patients enrôlés dans les études cliniques est en jeu. Ensuite, les modèles de pharmacologie de sécurité ont la responsabilité notable d'identifier les risques d'effets secondaires rares mais pouvant avoir des conséquences graves pour les patients. L'exemple largement discuté de la torsade de pointe, cette arythmie potentiellement fatale pouvant survenir chez une infime proportion de la population traitée rappelle l'importance de la validité des modèles utilisés afin de protéger les patients. Autre exemple, cette fois-ci applicable au programme de développement de l'OT comme thérapie cardiovasculaire, les impacts majeurs des effets hémodynamiques sur la morbidité et la mortalité (Lewington *et al.*, 2002). Deux exemples récents de médicaments en développement démontrent les conséquences des effets hémodynamiques. Le premier connu sous le nom de sunitinib, un anticorps monoclonal inhibiteur de la tyrosine kinase, a été développé par la firme Pfizer pour le traitement du cancer. Alors que le traitement avec une seule dose intraveineuse de sunitinib n'a pas d'effet sur la pression artérielle, l'administration répétée de celui-ci occasionne des effets hypertenseurs (Rivera *et al.*, 2009). Les effets hypertenseurs du sunitinib n'ont été dévoilés pour la première fois que lors d'études cliniques de Phase 3. Il importe de souligner l'ampleur de ressources déployées pour la conduite d'études cliniques de Phase 3 telle que présentée dans le chapitre sur les biomarqueurs inclus dans la première partie de cette thèse. Le constat qui ressort de ce premier exemple est que l'identification tardive des effets hypertenseurs suggère une lacune dans la planification des études de pharmacologie de sécurité préclinique. Cette situation a incité la compagnie (Pfizer) à inclure une évaluation des effets hémodynamiques chez le rat par télémétrie suite à l'administration répétée des composés très tôt dans le développement de ses nouveaux composés (Blasi *et al.*, 2007). Le deuxième exemple probant d'effets secondaires hémodynamiques apparaissant encore une fois suite à l'administration répétée est celui du torcetrapib développé

pour ses effets sur l'accroissement des niveaux de cholestérol associé aux lipo-protéines de haute densité (HDL). Cet exemple présenté dans la revue de littérature du premier chapitre a également contribué à généraliser l'utilisation du modèle d'évaluation des effets cardiovasculaires chez le rat éveillé avec l'utilisation de la télémétrie. Les grands thèmes de la revue de littérature ainsi que la validation des modèles précliniques de pharmacologie de sécurité cardiovasculaire ont ainsi permis de définir la sensibilité de ces modèles et de mieux choisir les modèles que nous avons utilisés pour l'évaluation des effets secondaires potentielles de l'OT comme nouvelle thérapie cardiovasculaire.

Les modèles d'évaluation de la fonction cardiovasculaire chez le rat anesthésié, chez le rat éveillé et chez le singe éveillé ont permis d'identifier les effets hémodynamiques et chronotropiques de l'OT. À l'instar des effets hypertenseurs du sunitinib et du torcetrapib, l'utilisation d'un modèle d'évaluation de la fonction cardiovasculaire chez le rat éveillé par télémétrie nous a permis d'évaluer les effets cardiovasculaires d'une exposition prolongée à l'OT. Contrairement à ces deux exemples pour lesquelles des effets cardiovasculaires progressivement plus importants ont été observés, la réponse cardiovasculaire à l'administration d'OT a présenté un profil de désensibilisation avec une diminution des effets suite à une exposition prolongée. Il demeure qu'en présence d'une population de patients avec une atteinte de la fonction cardiaque, la présence de tels effets présente un risque notable.

En plus des effets cardiovasculaires d'importance, nos travaux ont confirmé la présence d'effets antidiurétiques suite à une infusion prolongée d'OT. Le maintien de l'homéostasie cardiovasculaire fait intervenir un contrôle étroit du volume plasmatique circulant et l'utilisation de médicaments diurétiques constitue une composante majeure de l'arsenal des clinicien(ne)s dans le traitement de l'insuffisance cardiaque. La présence de ces effets antidiurétiques ajoute donc une part de risque. Il reste difficile d'évaluer la pertinence clinique de ces effets car les conséquences délétères d'une augmentation du volume plasmatique peuvent présenter un profil insidieux et difficile à discerner sans des mesures de pression artérielle précises et continues qui n'était pas incluses dans le modèle canin que nous avons utilisé

pour les évaluations sur la diurèse. Cette limitation pourrait être résolue par l'utilisation de télémétrie dans un modèle canin avec infusion continue.

La mise en évidence d'effet inotropique négatif suite au traitement d'OT chez le modèle canin anesthésié est la dernière considération soulevée par nos travaux présentés au cinquième chapitre. Encore une fois, la possibilité d'interférer avec une fonction cardiaque précaire chez le patient suite à l'infarctus présente un risque substantiel qui pourrait être incompatible avec le succès de cette nouvelle thérapie. Tel que présenté dans le cinquième chapitre, la pertinence de cette observation dans le modèle canin est fonction des marges thérapeutiques. Les effets hémodynamiques, chronotropiques et antidiurétiques présentés ci-haut ont tous été observés initialement avec des doses comparables (<10X) aux doses utilisées en obstétrique ce qui augmente le niveau de risque. La situation est différente pour les effets négatifs sur la contractilité ventriculaire alors que les effets sont observés à des doses de 5000 ng/kg ou supérieures ce qui laisse entrevoir des marges thérapeutiques plus élevées une fois la dose efficace confirmée.

Une question centrale demeure : Quelle approche utiliser pour déterminer la dose efficace ? La stratégie que nous avons utilisée dans le développement de l'OT pour cette nouvelle indication cardiovasculaire est de définir la dose sécuritaire la plus élevée (No Observed Adverse Effect Level *NOAEL*) telle que définie au cinquième chapitre et d'évaluer l'efficacité d'une dose 3X moindre que cette dose sécuritaire chez l'animal sain. Il demeure que cette approche empirique d'exposition à un facteur de la dose maximale sécuritaire présente des lacunes et peut ne pas refléter la dose optimale pour atteindre le bénéfice clinique recherché. Pour pallier à cette lacune, l'utilisation de modèles *in vitro* peut, dans certains cas, fournir des données additionnelles menant à un choix éclairé de la dose thérapeutique à utiliser *in vivo*. Plusieurs études avec des modèles *in vitro* ont démontré la capacité de l'OT d'induire la différentiation de cellules pluripotentes en cardiomyocytes (Fathi *et al.,* 2009; Bouchard & Paquin 2008; Danalache *et al.,* 2007; Jankowski *et al.* 2004; Paquin *et al.*, 2002). Les concentrations d'OT utilisées dans ces études avoisinent les 10^{-7} M (moles/litre). La masse moléculaire de l'OT étant de 1007.19 g/mol, la concentration utilisée dans les études *in vitro*

représente ainsi un milieu de culture de 100.719 ng/mL soit des niveaux largement supérieurs aux niveaux plasmatiques obtenus dans les modèles précliniques utilisés. Les doses pouvant être utilisées dans les études animales sont donc beaucoup moindres que celles appliquées dans les protocoles de différentiation *in vitro*. Advenant que les effets bénéfiques de l'OT sur le traitement de l'infarctus du myocarde impliquent la différentiation de cellules en cardiomyocytes et que les concentrations requises *in vitro* ne puissent être réduites, une stratégie de traitement alternative doit être envisagée car ces concentrations utilisées *in vitro* sont associées à des effets adverses *in vivo* importants. Nos résultats soulèvent des préoccupations mais laissent également entrevoir des opportunités de recherches futures. Plusieurs articles récents (Ly et *al.*, 2009 ; Quevedo *et al.*, 2009 ; Schuleri *et al.*, 2009 ; Wang *et al.*, 2009 ; Alt *et al.*, 2009 ; Sheu *et al.*, 2009) rapportent des effets bénéfiques dans le traitement de l'infarctus du myocarde suite à l'utilisation de thérapies cellulaires. La capacité démontrée de l'OT de différentier des lignées cellulaires *in vitro* en cardiomyocytes ouvre la voie à la l'évaluation des effets de l'OT en combinaison avec ces nouvelles thérapie cellulaires. On rapporte que l'infusion de cellules souches embryonnaires produit des effets bénéfiques dans un modèle d'infarctus du myocarde chez le rat (Min *et al.*, 2006). Similairement, des cellules souches embryonnaires humaines administrées chez des rats immunodéficients suite à un infarctus du myocarde se différentient en cardiomyocytes dispersés parmi les cardiomyocytes endogènes (Dai *et al.*, 2007) ce qui suggère une intégration fonctionnelle. Des études indiquent que l'injection de cellules souches mésenchymateuses au site d'infarctus contribue à réduire la taille de l'infarctus (Yamada *et al.*, 2006; Katritsis *et al.*, 2005). Des protocoles de culture de cellules mésenchymateuses *in vitro* avec prétraitement d'OT sont donc envisageables avant une transplantation des cellules dans des modèles précliniques d'infarctus du myocarde. Cette stratégie de traitement bien que possible dans des modèles animaux où les prélèvements peuvent être effectués avant l'infarctus sont plus difficilement envisageables dans un contexte clinique où les prélèvements de cellules autologues ne peuvent être effectués qu'après l'infarctus du myocarde chez le patients. De nombreux défis sont donc aux rendez-vous dans le

développement de nouvelles thérapies cardiovasculaires mais les avancées dans le domaine des thérapies cellulaires laissent présager que des thérapies novatrices encore au stade embryonnaire de développement pourraient rejoindre les rangs des traitements approuvés dans un avenir relativement rapproché.

En accord avec les résultats de pharmacologie de sécurité présentés précédemment, le modèle porcin d'infarctus du myocarde indique que l'administration d'OT présente un risque potentiellement important dans le cadre du traitement de l'infarctus du myocarde. Par contre, ce risque fût principalement identifié pour des individus ayant un niveau d'OT endogène élevé. Les maladies cardiovasculaires et plus particulièrement les maladies coronariennes sont influencées par plusieurs facteurs de risque ayant une composante héréditaire. Il est possible, voire probable, que les niveaux endogènes d'OT soient aussi influencés par des déterminants génétiques comme le sont les niveaux de cholestérol (Vincent *et al.*, 2002; van Aalst-Cohen *et al.*, 2004) ou le développement du diabète (Butty *et al.*, 2008). Ce dernier est parfois causé par une diminution de la sécrétion endogène d'insuline suite à la perte des ilôts de Langherhans pancréatiques. On pourrait ainsi proposer comme hypothèse basée sur nos résultats qu'une diminution de dans la production endogène d'OT serait requise afin de bénéficier d'une thérapie de remplacement avec cette hormone.

Le choix du moment d'initiation du traitement s'est avéré avoir un impact majeur sur les résultats dans ce modèle porcin. La guérison de la zone infarcie a été comparée à la guérison d'une plaie (Frantz *et al.*, 2009) avec une première phase inflammatoire pendant laquelle la matrice extracellulaire est dégradée en plus de la libération de médiateurs inflammatoires et d'une inhibition de la prolifération tissulaire. Nos résultats suite à l'administration d'OT immédiatement après l'infarctus confirment la présence d'effets délétères pendant cette période. Puisque les effets de l'OT sont attendus dans la période de prolifération tissulaire, cette réponse adverse au traitement pendant la phase initiale où la prolifération tissulaire est inhibée paraît logique. La zone infarcie passe ensuite à une phase de réparation avec la prolifération de fibroblastes et de cellules inflammatoires qui entraîne la libération de

cytokines favorisant la fibrose et donc l'augmentation de la synthèse de la matrice extracellulaire pour mener à la formation d'un tissu cicatriciel. Suite à la phase inflammatoire, la guérison optimale requiert des mécanismes qui éliminent l'infiltration de cellules inflammatoires et initient la production de collagène pour aboutir à une cicatrice solide. On peut entrevoir que les effets de l'OT pendant cette période de prolifération pourraient être favorables en favorisant la différentiation en cardiomyocytes. Rétrospectivement, l'administration d'OT après la phase inflammatoire est donc une stratégie de traitement avec un potentiel de succès plus élevé.

En tenant compte des résultats obtenus et des considérations mentionnées précédemment, qu'en est-il du profil de pharmacologie de sécurité de l'OT comme nouvelle thérapie systémique cardiovasculaire ? Globalement, les résultats de pharmacologie de sécurité positionnent l'OT comme un peptide ayant un potentiel de succès mais dont plusieurs effets pourraient nuire à une population de patients ayant une fonction cardiaque compromise. Les évaluations des effets du traitement avec l'OT dans un modèle porcin d'infarctus du myocarde présentent un pas de plus vers la situation clinique et par le fait même pourraient présenter une valeur prédictive supérieure autant sur le plan des évaluations de sécurité que d'efficacité. Basés sur nos résultats, les niveaux endogènes d'OT sont un facteur déterminant dans la réponse au traitement. On pourrait donc postuler que l'administration d'OT doit être considérée comme une thérapie de remplacement hormonale. En comparaison, l'administration d'insuline à un individu sain et normoglycémique aurait des effets secondaires potentiellement adverses en induisant un état d'hypoglycémie pouvant mener à des conséquences sérieuses tel le coma. En contre-partie, un patient atteint de diabète insulino-dépendant présenterait des effets secondaires sérieux si on cessait l'administration d'insuline exogène pour maintenir sa glycémie. De la même façon, l'administration d'OT serait bénéfique seulement chez des patients présentant une déficience de cette hormone.

Afin de confirmer cette hypothèse, des évaluations des niveaux d'OT au sein de la population de patients seraient nécessaires. Existe-t-il une distinction entre les niveaux d'OT des patients atteints d'insuffisance

cardiaque suite à une maladie ischémique coronarienne et ceux ayant présenté une évolution clinique favorable ? Cette hypothèse ne semble pas avoir été vérifiée jusqu'à maintenant. L'évaluation de la concentration d'OT au niveau de l'hypophyse postérieure entre les patients humains normaux et ceux décédés d'insuffisance cardiaque n'a pas révélé de différence entre les deux groupes (Sivukhina *et al.*, 2009). Il reste à savoir si la concentration d'OT hypophysaire présente une corrélation avec la concentration plasmatique d'OT ou même avec la concentration d'OTR des cellules souches endogènes. Certaines conditions dont l'exercice physique modifient la concentration d'OT hypophysaire sans toutefois modifier la concentration plasmatique de celle-ci (Bakos *et al.*, 2007). Similairement, il est possible que des individus ayant des niveaux d'OT hypophysaire comparables, tels que rapportés par Sivukhina *et al.,* présentent des niveaux plasmatiques de cette même hormone qui soient différents. Plusieurs questions restent encore à être évaluées mais le rôle de l'OT comme hormone ayant une fonction cardiovasculaire est un constat qui restera probablement le sujet de travaux de recherche pour les décennies à venir.

13. LE PROFIL DE SÉCURITÉ DE L'OXYTOCINE : IDENTIFICATION D'UN RISQUE NON-ÉVALUÉ

Nos travaux ont tenté de définir le potentiel de l'OT comme traitement cardiovasculaire en évaluant la sécurité et l'efficacité de ce nanopeptide. Bien que nos études soient le fruit d'un processus de planification des études requises sur la base des pratiques actuelles d'évaluation des médicaments, un aspect majeur du profil de sécurité des médicaments n'a pas été abordé dans nos travaux, celui du potentiel arythmogène. L'arythmie revêt une importance particulière dans les évaluations de sécurité des médicaments étant donné ses conséquences potentiellement fatales. Tel que souligné dans le premier chapitre de la thèse, le potentiel arythmogène peut être difficile à quantifier dans des cas où la prévalence des arythmies est très basse comme avec la terfenadine, cet anti-histaminique qui a mené à l'implantation de lignes directrices internationales en matière d'évaluation des effets électrocardiographiques.

Les risques d'arythmies sont supérieurs suite à l'infarctus du myocarde et peuvent prendre la forme de fibrillation ventriculaire (Pride *et al.*, 2009; Sasano *et al.*, 2009), de fibrillation auriculaire (Saczynski *et al.*, 2009) ou autre. Afin de discuter les effets électrocardiographiques de l'OT, il importe de discuter brièvement des changements ECG associés à l'administration d'OT chez les patients humains et des risques potentiellement associés à ces changements. L'administration d'OT a été associée à une prolongation de l'intervalle QTc (Charbit *et al.*, 2004) avec un effet maximal sur le QTc noté 1 minute après l'injection chez des femmes anesthésiées.

Une étude chez des patients subissant une angioplastie par cathéter ballonnet a révélé une augmentation de l'intervalle QTc dans 100% des cas suite à une ischémie transmurale du myocarde (Kenigsberg *et al.,* 2007). Lorsque comparée avec les marqueurs reconnus d'ischémie myocardique (élévation du segment ST et dépression du segment ST), la prolongation de l'intervalle QT était l'anomalie ECG apparaissant le plus rapidement. La prolongation de l'intervalle QTc résulte habituellement en une réduction de l'intervalle TQ (entre la fin de la repolarisation et la dépolarisation

ventriculaire du prochain complexe). Une réduction de l'intervalle TQ a été notée dans 99% des cas dans la période précédant une torsade de pointe, une arythmie associée à une prolongation de l'intervalle QTc (Fossa *et al.*, 2007). Une hypothèse avancée pour expliquer cette association entre la réduction de l'intervalle TQ et la torsade de pointe est que la réduction de l'intervalle TQ augmente les risques de ré-entrée électrique menant à l'arythmie. En diminuant la période de relaxation, l'augmentation de l'intervalle QTc augmenterait le risque d'arythmies.

L'utilité des mesures de l'intervalle QTc chez les modèles précliniques afin de prédire les risques de torsade de pointe chez l'humain est un sujet en continuelle évolution (Redfern *et al.*, 2003 ; Webster *et al.*, 2002 ; Lawrence *et al.*, 2006 ; Hanson *et al.*, 2006) et bien que l'association entre la prolongation QTc et la présence d'arythmie soit variable d'un composé à l'autre, tout agent ayant la capacité d'augmenter l'intervalle QTc doit être évalué pour son potentiel arythmogène. La prolongation QT suite à l'administration d'OT pourrait accroître les risques d'arythmie chez des patients présentant déjà une prolongation QTc suite à l'ischémie myocardique. Nos études avec le modèle d'infarctus du myocarde chez le porc n'ont toutefois pas permis d'évaluer le potentiel arythmogène de l'OT qui nécessite une taille d'échantillon beaucoup plus élevée pour être évalué. Il demeure que l'utilisation d'OT chez des patients présentant des facteurs de risque élevés pour développer des arythmies malignes comme c'est le cas dans la population de patients atteints d'infarctus du myocarde est actuellement contre-indiqué.

Tel que mentionné précédemment, l'utilisation de modèles précliniques de pathologies cardiaques a récemment été recommandée afin d'évaluer le potentiel arythmogène des nouveaux composés (Hamlin & Kijtawornrat, 2008). La présence d'une pathologie augmente la sensibilité du modèle aux arythmies et la pertinence clinique des évaluations. La présence d'une zone infarcie dans un modèle d'infarctus du myocarde a été associée au phénomène de ré-entrée ventriculaire et de fragmentation de l'onde de dépolarisation pouvant mener à la fibrillation ventriculaire (Chow *et al.*, 2004). En modifiant les propriétés de repolarisation ventriculaire, l'OT pourrait être associée à des risques plus élevés d'arythmie. Des études

supplémentaires seront nécessaires afin d'évaluer la sécurité sur le plan électrocardiographique de l'OT dans des modèles d'infarctus du myocarde.

Chapitre 9

CONCLUSIONS GÉNÉRALES

14. LE FUTUR DE L'OXYTOCINE DANS LA GESTION CLINIQUE DES CAS D'INFARCTUS DU MYOCARDE

Très peu de nouvelles thérapies connaissent un parcours favorable et obtiennent l'approbation règlementaire (Hughes, 2008) pour utilisation chez les patients et des efforts considérables sont déployés afin de réduire le taux élevé d'attrition des médicaments en développement (Kola & Landis, 2004). Malgré cette volonté de réduire le taux d'attrition, les stratégies actuellement utilisées par l'industrie du développement des médicaments visent à éliminer les candidats ayant des effets secondaires adverses le plus tôt possible (Valentin *et al.*, 2008). En effet, en cessant le développement d'un traitement infructueux rapidement, les efforts et ressources peuvent être concentrés sur d'autres candidats qui pourront rejoindre les rangs des traitements approuvés.

La perfusion d'OT est largement utilisée pour l'induction de l'accouchement chez la femme (McLeod *et al.*, 2003; Prager *et al.*, 2008) ainsi que chez plusieurs espèces animales incluant le chien, le chat, (Pretzer, 2008), le cheval (Villani & Romano, 2008) et le porc (Yang *et al.*, 1996). L'OT est également utilisée pour contrôler les hémorragies utérines post-partum (Lovold *et al.*, 2008; Hinshaw *et al*, 2008) et dans le cadre des protocoles d'avortement (Nuthalapaty *et al.*, 2005; Kelekci *et al.* 2006). Malgré des rapports de cas présentant des risques d'infarctus du myocarde chez des patients ayant une fonction cardiaque compromise (Schmitto *et al.*, 2008), l'utilisation d'OT en obstétrique est considérée sécuritaire et efficace.

Des résultats récents (Ondrejcakova *et al.*, 2009) suggèrent que l'OT réduit significativement (-66%) la taille de l'infarctus du myocarde dans des modèles *ex vivo* chez le rat. Ces résultats démontrent également que les effets bénéfiques de l'OT *in vitro* s'expliquent en partie par ses effets chronotropes négatifs. En utilisant un modèle porcin d'ischémie-reperfusion myocardique nous avons démontré la présence d'effets secondaires adverses lorsqu'une perfusion continue d'OT est initiée immédiatement après l'ischémie myocardique. L'administration d'OT 8 jours après l'ischémie chez des individus ayant des niveaux d'OT endogènes élevés a également causé des

effets secondaires adverses chez les individus ayant des niveaux endogènes d'OT élevés. Nos résultats suggèrent que l'utilisation de l'OT comme thérapie systémique présente des risques importants incluant les risques d'aggravation de la dilatation ventriculaire gauche suite à l'ischémie myocardique transmurale. En contre partie, l'administration d'OT par perfusion continue IV débutant 8 jours après l'ischémie myocardique chez des individus ayant des niveaux endogènes faibles résulte en une amélioration (certes non-significative) des paramètres d'évaluation de la fonction cardiaque. De plus, la comparaison des animaux des groupes placebo suggère que les animaux ayant un niveau endogène d'OT élevé pourraient avoir une meilleure récupération dans ce modèle porcin d'infarctus du myocarde. Ceci ouvre la voie à un rôle pour l'OT comme biomarqueur pronostic potentiel suite à l'infarctus du myocarde. Plusieurs biomarqueurs sérologiques sont couramment utilisés pour établir un pronostic suite à l'infarctus du myocarde chez les patients humains. Parmi les plus fréquemment utilisés, on compte la troponine I et la créatine kinase MB (Jaffery *et al.*, 2008). En plus de ces biomarqueurs traditionnels, un nombre croissant de biomarqueurs sont évalués comme facteur de pronostic suite à l'infarctus du myocarde. Une augmentation des niveaux sériques d'IL-17 et d'IL-18 (Jafarzadeh *et al.,* 2009), d'albumine (Dominguez-Rodriguez *et al., 2009*), d'acide urique (Nadkar & Jain, 2008*)*, de myéloperoxidase (Chang *et al.*, 2009) et d'érythropoiétine (Belonje *et al.*, 2009) ont tous été récemment associés avec un pronostic défavorable suite à l'infarctus du myocarde. Ces biomarqueurs suggèrent, par leur augmentation, un dommage ou une condition clinique plus sévère et donc des probabilités plus élevées de morbidité ou de mortalité. Bien que le développement de biomarquers suggestifs de la sévérité des dommages soit utile, l'identification de biomarqueurs dont la diminution est associée à un pronostic sombre revêt un intérêt particulier. En effet, les biomarqueurs dont la diminution est associée à un pronostic plus sombre pourraient, dans certains cas, être indicatifs d'une capacité de guérison ou de régénération moindre. Par exemple, une diminution des niveaux sériques de TRAIL a été associée à un pronostic défavorable (Secchiero *et al.*, 2009). Les auteurs soulignent les effets anti-inflammatoires et anti-athérosclérotique de TRAIL qui pourrait expliquer la corrélation entre la diminution des niveaux

sériques de TRAIL et l'augmentation des risques de mortalité de cause cardiaque et d'insuffisance cardiaque. Comme pour TRAIL chez des patients humains, nos résultats suggèrent qu'un niveau endogène d'OT élevé est associé avec un meilleur pronostic dans ce modèle porcin d'infartus du myocarde. Le développement de nouveaux biomarqueurs de pronostic suite à l'infartus du myocarde soulève un intérêt marqué (Howie-Esquivel *et al.* 2008) en permettant une meilleure compréhension des facteurs pathophysiologiques influançant l'évolution clinique. Ces nouveaux biomarqueurs associés au pronostic comme pourrait l'être l'OT laissent entrevoir la possibilité de développer une médecine personalisée où le choix des traitements serait basé sur les profils individuels des patients.

"All drugs are poisons the benefit depends on the dosage."

"Tous les médicaments sont poisons, le bénéfice depend de la dose "

Theophrastus Philippus Aureolus Bombastus von Hohenheim (1493-1541)

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ANNEXE

15. COMPARAISON DE 3 PROTOCOLES ANESTHÉSIQUES POUR L'ADMINISTRATION PAR ENDOSCOPIE CHEZ LE SINGE CYNOMOLGUS

L'article qui suit (*Journal of the American Association of Laboratory Animal* 2006; 47: 774-778) présente les effets cardiovasculaires, le temps de réveil ainsi que le profil d'absorption du glucose chez le singe cynomolgus suite à l'utilisation de différents protocoles anesthésiques. Cet article écrit par le Docteur Authier sous la supervision du Docteur Éric Troncy fût un préembule aux évaluations plus approfondies de l'évaluation de la fonction cardiovasculaire chez des animaux anesthésiés.

Les résultats et discussions inclus dans cet article ont été présentés sous forme d'affiche par le Docteur Authier au 13^{ième} Congrès Annuel de l'International Society for the Study of Xenobiotics à Maui en 2005.

Comparison of Three Anesthetic Protocols for Intraduodenal Drug Administration Using Endoscopy in Rhesus Monkeys (*Macaca mulatta*)

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Short Title: Anesthetic protocols for endoscopy in rhesus monkeys

Author Contributions: SA and ET conceived and designed the experiments. SA, FC, and ML performed the experiments. SA, CB and ET analyzed the data. SA and ET wrote the paper.

Summary: The purpose of this study was to evaluate 3 anesthetic protocols for intraduodenal drug administration by endoscopy in rhesus monkeys (*Macaca mulatta*). Anesthesia was induced using intramuscular ketamine and midazolam, isoflurane (inhalant gas), or intravenous propofol in male and female rhesus monkeys. A noninvasive dosing line was placed in the duodenum by use of endoscopy, and 50% dextrose (3 ml/kg) was administered. Blood pressure, heart rate, body temperature, and reflexes (corneal, palpebral, pharyngeal) and myorelaxation (mandibular reflex and reaction to limb manipulation) were evaluated every 5 min. To estimate intestinal absorption, glycemia was evaluated prior to dextrose administration and at 2, 5, 10, 15, 20, 30, 45, and 60 min after dosing. All 3 protocols resulted in successful induction of anesthesia. Recovery from isoflurane and propofol was significantly faster than from ketamine–midazolam. Duration of the recovery period after isoflurane was less variable than with propofol, but isoflurane produced greater hypothermia. Isoflurane and propofol resulted in predictable glucose absorption after intraduodenal dextrose administration, whereas ketamine–midazolam led to an inconsistent increase in glycemia.

Introduction

Nonhuman primates typically are considered an acceptable model for preclinical bioavailability studies.[6] Preclinical pharmacokinetic and metabolism evaluations of drugs intended for oral administration in humans are often affected by gastric secretions, which may affect oral drug absorption and bioavailability.[17] The gastric pH of fasted cynomolgus monkeys was similar to that in fasted humans, whereas the pH profiles of these species differed after a solid meal.[8] Measurement of gastric acid secretions in fasted rhesus monkeys reveals that gastric pH in this species also is similar to that of humans.[8,15] Because development of an enteric coating for preclinical screening of new drugs is lengthy and expensive, endoscopic administration directly into the duodenum can be used to bypass the effects of gastric secretions. When solid formulations are evaluated, the size of the tablet or capsule modifies average transit time in the stomach,[4,6] thus complicating synchronization of blood collection for pharmacokinetic analysis with absorption of the test substance in the duodenum. In the absence of an enteric coating and to ensure an optimal pharmacokinetic blood collection schedule, administering solid formulations directly into the duodenum may provide an acceptable alternative. In addition, scientific data describing anesthetic recovery in rhesus monkeys are rare. The aim of this study was to compare recovery from anesthesia and intestinal absorption of a test substance after the use of 3 anesthetic protocols for intraduodenal drug administration using endoscopy.

Materials and Methods

Study subjects. All nonhuman primates used during this study were maintained in accordance with the *Guide for Care and Use of Laboratory Animals*[13] at LAB Research Inc. (Laval, Quebec, Canada), which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International-accredited facility. All procedures were reviewed and approved by LAB Research's Institutional Animal Care and Use Committee before study initiation. The study population comprised 3- to 6-y-old male and female rhesus monkeys (*Macaca mulatta*; Covance Research

Products, Alice, TX). Body weights ranged from 3.4 to 4.6 kg for female monkeys and from 3.1 to 3.4 kg for males. Health status evaluation before study initiation confirmed that the monkeys were negative for *Salmonella* spp., *Shigella* spp., *Yersinia* spp., *Cercopithecine herpesvirus 1* (B virus), and tuberculosis. Animals were pair-housed in stainless-steel squeeze-back cages (floor space, 1320 in^2, height, 34 in.), and had been housed in a laboratory environment for 5 mo before initiation of the study. Monkeys received 7 cookies of a standard certified commercial primate chow (Teklad Certified Global 25% Primate Diet 2055C, Harlan Teklad Animal Diet and Bedding, Madison, WI) twice daily and received enrichment including foraging opportunities, television, music, and organic fruits daily. Animals were fasted overnight (food only) prior to anesthesia for endoscopic administration. Municipal tap water purified by reverse osmosis was provided to the animals ad libitum. The environmental conditions in the animal room were controlled (100% fresh conditioned air; temperature, 21 ± 3 °C; humidity, 30% to 70%; 10 to 15 air changes hourly, 12:12-h light:dark cycle).

Study design. We randomly assigned each of 6 rhesus monkeys (3 male and 3 female) to 2 of the 3 anesthetic protocols, giving a sample size of 4 for each protocol. Randomization of male and female monkeys was done separately to maintain an equal-gender ratio. Premedication or drug antagonists were not included in the 3 protocols evaluated to minimize the potential for drug interactions in eventual pharmacokinetic studies. Animals reacclimated for 48 h between the 2 protocols. Because of the nature of the procedure (endoscopy) to be performed, deep surgical anesthesia was not required, and the presence of neurologic reflexes (usually absent under surgical anesthesia) was considered acceptable. Anesthetic dosages were selected in light of experience with endoscopic administration in monkeys (a common procedure at our research facility), the results of pilot experiments (which also were used to estimate the smallest sample size needed to show significant difference), and supervision by a veterinary anesthetist (ET).

For blood glucose evaluation, an indwelling catheter was placed in the cephalic vein of each animal before induction of anesthesia. Another indwelling catheter was placed in the cephalic vein of the opposite arm for

intravenous propofol administration, when applicable. Monkeys were placed on restraining apparatus similar to a hammock (Mobile restraint unit, Lomir Biomedical, Notre-Dame-de-l'Île-Perrot, Quebec, Canada) for most of the experiment. On each of at least 3 d prior to the study, animals had been acclimated to the restraining apparatus, which was used for isoflurane induction, intramuscular and intravenous injections, and blood collection.

The following protocols were used to induce light anesthesia for endoscopy. One protocol comprised midazolam (0.2 mg/kg) and ketamine (8 mg/kg) given intramuscularly in the thigh. In the second of the protocols, isoflurane at 3.0% to 4.0% with 2.0 l/min oxygen was administered through a Bain coaxial system (Moduflex, Dispomed, Joliette, Quebec, Canada) connected to a mask for induction, followed by orotracheal intubation and maintenance with 2% isoflurane with 1.0 l/min oxygen until dextrose administration. The anesthetic system was connected to an exhaust system (Dispomed) to avoid personnel exposure to isoflurane. Monkeys were exposed to isoflurane through a mask for 5 to 13 min before insertion of endotracheal tubes (rubber, cuffed Magill-type; internal diameter, 4 mm [male monkeys] or 3 mm [female monkeys]; Centre de Distribution Médecine Vétérinaire, St-Hyacinthe, Quebec, Canada). Isoflurane was stopped immediately after endoscopy, and animals were extubated as soon as the animal was able to swallow. For the third protocol, propofol (6 mg/kg) given intravenously by slow (30 s) bolus. Half of the dose was given initially, with the remainder titrated to effect, and the level of anesthesia was evaluated visually and through neurologic reflexes. Throughout propofol administration, animals were closely monitored to ensure that spontaneous breathing was preserved.

Intraduodenal endoscopic administration. Monkeys were fasted overnight (approximately 16 h) prior to induction of anesthesia. Immediately after induction, a sterile eye lubricant was applied to prevent ocular desiccation. Monkeys were placed on a heating pad immediately after induction or intubation and remained there until their ambulatory status returned to normal. A protective mouth gag was used to keep the mouth open for endoscopy.

An endoscope (Olympus CV-100 processor, Olympus CLV-U20 light source, and Olympus GIF-100 gastroscope, Carsen Group, Markham, Ontario, Canada) with an insertion tube of 9.5 mm was used; sterile lubricant was used to facilitate introduction of the endoscope into the esophagus. The endoscopic procedure was started immediately after sufficient anesthesia was achieved, to minimize the duration of anesthesia and to ensure comparability of recovery periods. Once the lower esophageal sphincter was passed, the stomach was slightly inflated to allow visualization of the antrum. The tip of the endoscope was slid along the greater curvature and advanced into the antrum until it was immediately in front of the pylorus. The pylorus was kept in the center of the field of vision (Figure 1 A). A dosing line (polyvinyl chloride; dead volume, 0.4 ml) was introduced into the duodenum (Figure 1 B), and the endoscope was inserted into the proximal part of the duodenum (Figure 1 C). A bolus of 50% dextrose (3 ml/kg) was administered, followed by a saline flush of 0.8 ml (that is, twice the dead volume of the dosing line). The dosing line was visualized throughout the dosing procedure to ensure that it was properly placed and that no duodenal reflux occurred. Once the dosing was completed, the dosing line was removed and the stomach was gently deflated using a suction pump (Schuco-Vac model 5711-130, Centre de Distribution Médecine Vétérinaire) connected to the endoscope.

Glycemia. Glycemia was measured by use of a drop of blood collected through an indwelling catheter placed in a cephalic vein. Blood samples were taken after induction or intubation (before endoscopy) and at 2, 5, 10, 15, 20, 30, 45, and 60 min after dextrose administration. Blood samples were processed immediately for glucose concentration analysis (Accusoft Advantage glucometer, Roche Diagnostics Canada, Laval, QC, Canada).

Anesthesia monitoring. The following parameters were evaluated every 5 min after induction to quantify recovery associated with the various anesthesia protocols. Monitoring was started immediately at the onset of induction for all protocols. Systemic arterial pressures and heart rate were measured noninvasively by use of an oscillometric sphygmomanometer (Minipack 911, Pacetech, Tampa Bay, FL). A neonatal cuff (size 3) was placed over the proximal part of the arm between the shoulder and the elbow.

Monkeys were placed in mobile restraint units throughout anesthetic recovery to minimize movements during blood pressure monitoring. Rectal temperature was monitored until it reached 38 °C. Palpebral reflex (medial and lateral), pharyngeal reflex, corneal reflex, masseter tone, and activity level were evaluated sequentially. The corneal reflex was evaluated with a drop of sterile saline to minimize the impact of repeated evaluation on the cornea. We also evaluated the reaction of the monkey to manipulation of a hindlimb or forelimb; we preferred this method of evaluating reaction to limb manipulation to others in an attempt to minimize stress for the monkeys. To assess this parameter, the evaluator lifted one of the monkey's limbs with one hand and let the limb fall into his/her other hand. The reaction of the monkey was given a score from 0 (no reaction) to 3 (monkey withdrew the limb promptly when manipulated). The same animal health technologist, who was blinded regarding anesthetic protocol, evaluated reaction to limb manipulations for all monkeys. The activity level was classified as normal, slightly decreased (monkey was able to stand but had slow reactions), moderately decreased (monkey was able to sit but unable to stand), or severely decreased (unconscious or recumbent).

Statistical analysis

The motivation for anesthetizing each animal with 2 different protocols was to reduce interindividual variability and facilitate comparisons of the results obtained for each protocol. Differences between anesthetic protocols were analyzed by use of 2-way analysis of variance for repeated measures. Time was treated as a within-subject factor and anesthetic protocol as a between-subjects factor. For cardiovascular parameters and rectal temperature, we obtained the baseline (control) value at the end of follow-up, because these parameters could not be followed during the pretreatment period. A posteriori comparisons were done with Dunnett tests. For categorical parameters (for example, presence or absence of neurologic reflexes or masseter tone), Fisher exact tests were used to compare rate of occurrence in each group at induction of anesthesia (time 0) and 10 min thereafter. The threshold for statistical significance was set at 0.05; results are presented as mean.

Results

Induction of anesthesia. The induction mask, when used, was well tolerated by all monkeys. All protocols resulted in uneventful and successful induction of anesthesia. No dysphoria or hyperactivity was noted during induction with the 3 protocols evaluated. Spontaneous respiration was maintained throughout the procedure for all monkeys. All monkeys required the complete dose of propofol to achieve an appropriate anesthetic level before initiation of the endoscopic administration. No additional dose of propofol was required to complete the endoscopic administration of dextrose for any animal in this group.

Endoscopy procedure. All 3 protocols allowed intraduodenal administration of 50% dextrose without complication. The average duration of the complete endoscopic dosing procedure (from first insertion into the mouth to complete withdrawal) was 5 min for isoflurane, 4 min for propofol, and 5 min for ketamine–midazolam.

Glycemia. Blood glucose reached maximum at an average of 37.5 min after dextrose administration for isoflurane compared with 37.7 min for propofol. Of the 4 monkeys anesthetized with ketamine–midazolam, 2 had no distinct increase in glycemia after dextrose administration, and the average increase in glucose concentration in these animals was not statistically significant. In the 2 monkeys showing an increase in blood glucose, maximum glycemia was reached at 10 and 15 min after dextrose administration. An average increase of 103% compared with the baseline (control) level was noted for isoflurane and of 134% for propofol.

Cardiovascular parameters. Monitoring was initiated immediately after induction of anesthesia. Values recorded at the end of the monitoring period were considered to represent baseline because animals had recovered from anesthesia. Compared with the control value measured at 60 min postinduction, heart rate after induction with propofol was globally decreased ($P = 0.0004$), and a posteriori contrast was positive at 0 and 5 min after induction. Heart rate rapidly (approximately 20 min) returned to baseline levels and was stable thereafter. Because of increased variability the apparent initial decrease in heart rate was not statistically significant in the 2 other

anesthesia groups. There was no statistical difference in heart rate among the 3 groups. A statistically significant ($P = 0.02$) decrease in diastolic pressure was noted for isoflurane compared with ketamine–midazolam and propofol.

Rectal temperature. For monkeys receiving isoflurane, rectal temperature could be recorded only after intubation, which explains the lower body temperature values recorded initially. Isoflurane created most prolonged decrease in body temperature, returning to an average of 38 °C at 70 min after isoflurane was stopped compared with 15 min after induction with propofol and 55 min with ketamine–diazepam. The observed difference in the delay before going back to a normal temperature was statistically significant ($P = 0.02$) between the propofol and isoflurane protocols. Among the 3 protocols, propofol created the least decrease in body temperature with a minimum of 37.95 °C at 40 min after induction (Figure 2) and was significantly different ($P < 0.04$ for all comparisons) from the 2 other protocols at 15 and 30 min after induction.

Neurologic reflexes. Palpebral, corneal, and pharyngeal reflexes were preserved at all times for monkeys receiving ketamine–midazolam. Palpebral and corneal reflexes were lost just prior to intubation until 10 min after isoflurane was stopped for the 4 monkeys in this group. For all monkeys anesthetized with isoflurane, pharyngeal reflex was absent from intubation until 15 min after isoflurane was stopped. Monkeys receiving propofol lost palpebral and corneal reflexes for variable periods (0 to 10 min); 1 monkey receiving propofol never lost the pharyngeal reflex. The other 3 monkeys in this group lost the pharyngeal reflex for 5 (1 monkey) and 10 min (2 monkeys) after induction. The loss of neurologic reflexes just after induction and at 10 min later was statistically significantly different ($P = 0.01$ for all comparisons) between the ketamine–midazolam and isoflurane groups; the propofol group did not differ from the 2 other groups. No difference in medial and lateral palpebral reflexes could be identified in any group.

Masseter tone. Masseter tone was lost for a period of 10 min in 1 of the 4 monkeys receiving ketamine–midazolam. All 4 monkeys in this group had decreased masseter tone for 5 to 30 min after injection. Masseter tone was absent throughout the anesthesia maintained with isoflurane and was absent or

severely decreased for 10 (1 monkey) to 15 min (3 monkeys) after isoflurane was stopped. The masseter tone was absent or decreased from 5 (1 monkey) to 10 min (3 monkeys) after propofol induction. The loss of masseter tone just after induction and at 10 min later was statistically significantly different (P = 0.045 for all comparisons) between the ketamine–midazolam and isoflurane groups. The propofol group did not differ from the 2 other groups.

Reaction to limb manipulation, recovery, and ambulatory status. Reaction to limb manipulation was decreased or absent for 15 to 45 min after injection of ketamine–midazolam. Only 2 of the 4 monkeys receiving ketamine–midazolam completely lost reaction to limb manipulation. Monkeys were recumbent for 15 (2 monkeys), 20 (1 monkey), and 30 min (1 monkey) after injection of ketamine–midazolam. Monkeys in this group had normal ambulatory status at 25 (1 monkey), 40 (1 monkey), and 50 min (2 monkeys) after induction. No reaction to limb manipulation was noted from intubation to termination of isoflurane anesthesia; monkeys had normal reaction to limb manipulation and ambulatory status at 15 (1 monkey) and 20 min (3 monkeys) after isoflurane was stopped. Monkeys were recumbent from 10 (2 monkeys) to 15 min (2 monkeys) after induction with propofol and regained normal ambulatory status at 15, 20, 25, and 30 min, respectively, after induction. The comparative analysis showed that duration of recumbency was marginally different (P = 0.05) between the ketamine–midazolam (20 ± 3.53 min) and propofol (12.5 ± 1.44 min) groups. The delay for ambulatory recovery was significantly different (P = 0.01 for both comparisons) between the ketamine–midazolam (41.25 ± 5.9 min) and both propofol (22.5 ± 3.23 min) and isoflurane (18.75 ± 1.25 min), with no difference between the last 2 groups. Consciousness returned faster with propofol, but full recovery appears to be faster after isoflurane. Only 1 monkey vomited during the recovery period; this animal had received ketamine–midazolam.

Discussion

Comparison of different anesthetic protocols in rhesus monkeys is useful not only for selection of an appropriate procedure for endoscopy but for a wide variety of noninvasive procedures. Anesthetic protocols that were evaluated in the current study are similar to protocols used with other species, including

humans; therefore the findings may also be applicable to other species. All 3 anesthetic protocols evaluated were considered acceptable for induction of anesthesia of short duration. Administration routes selected for the different anesthetic protocols included three widely used routes of induction (inhalant gas, intravenous, and intramuscular).

Intramuscular administration of ketamine and midazolam resulted in safe but relatively long-lasting sedation that lasts longer than the recovery time after most general anesthesia protocol. As expected with its mechanism of action, ketamine did not abolish any neurologic reflex. Ketamine has no cardiovascular depressant effect, and in combination with midazolam resulted in relatively stable arterial pressure with variable and slightly reduced heart rate. These variations in heart rate could be attributable to the imperfect loss of consciousness as well as to the retained reaction to limb manipulation that we found in 2 of the 4 monkeys. The hypothermic effect noted with this protocol was compatible with the peripheral vasoconstriction that occurs in humans after induction with ketamine.[5] The relatively high baseline glycemia values seen with this protocol could potentially result from increased plasmatic catecholamine. Interestingly, a combination of ketamine and midazolam increased plasmatic epinephrine and norepinephrine 10 min after intravenous bolus injection in healthy human volunteers.[12] Due to the prolonged sedation it induced, we do not consider this protocol appropriate for intraduodenal endoscopic administration in rhesus monkeys. Nevertheless, this practical intramuscular induction protocol in combination with premedication, including an antiemetic agent, may be useful for other minimally invasive procedures. As complementary information, the preferred anesthetic protocol for minimally invasive procedures in our laboratory is intramuscular injection of ketamine and acepromazine without premedication. A cocktail of 1 ml acepromazine (10 mg/ml) mixed with 10 ml ketamine (100 mg/ml) is given at 0.05 ml/kg for manipulation and detailed physical examinations of aggressive monkeys; at 0.1 ml/kg for intradermal tuberculin injection, dental prophylaxis, and tattoo identification; and at 0.15 ml/kg for minor surgeries (for example, biopsies) and when intubation is needed. Midazolam was preferred to

acepromazine in the current study to minimize the risk of increased pyloric tone, which interferes with duodenoscopy.[11]

The pharmacokinetics of propofol involves a 3-compartment linear model with rapid tissue redistribution, thus explaining its short duration of action.[9] Relatively wide interindividual pharmacokinetic and pharmacodynamic variability is reported to occur in humans.[1,10] The inconsistent retention of reflexes we noted in the current study suggests that a similar variability could be present in rhesus monkeys, but evaluation of a greater number of subjects and assessment of propofol pharmacokinetics in rhesus monkeys would be needed to confirm this hypothesis. Induction with propofol resulted in moderate and transient cardiovascular depression that mostly affected heart rate. Reported cardiovascular effects of propofol in other species include bradycardia, hypotension, and negative inotropism.[14] The presence of minimal cardiovascular effects is likely to be associated with stable renal function. These 2 factors are important to ensure validity of pharmacokinetic studies. Very short duration with minimal effects on body temperature and complete loss of consciousness (as reflected by the loss of neurological reflexes for 10 to 15 min and the absence of reaction to limb manipulation) are also favorable features of the use of this protocol for endoscopic administration. Propofol is metabolized by the liver via glucoronide conjugation mediated by cytochrome P450.[1] Drug interactions between propofol and test substances that are metabolized by this important metabolic pathway can be expected, increasing elimination time for both drugs. This interaction is a possible limitation to the use of this protocol for endoscopic administration of test substances.

With complete recovery of neurologic reflexes and ambulatory status within 20 min after isoflurane was stopped for all monkeys, this protocol had the shortest and most reproducible recovery. The decrease in arterial pressure associated with reflex tachycardia represents an important and well-known cardiovascular effect of isoflurane in other species.[3] Decreasing the level of anesthetic might minimize this effect. Depression of body temperature regulation centers combined with heat lost through airways via the Bain coaxial system likely explain the significant temperature decreases that we

noted. In light of our results, the prolonged and more pronounced decrease in body temperature associated with isoflurane without premedication does not seem to prolong recovery time compared with that of the other protocols evaluated. A study of recovery after anesthesia with isoflurane that compares constant and variable body temperatures would be useful to assess the effect of hypothermia on recovery duration. Isoflurane is metabolized at 0.2% to 1.0% by the liver, mostly by cytochrome P450 CYP2E1.[2,7] We do not consider this slight metabolism of isoflurane to represent a disqualifying condition to using this protocol for endoscopic administration of test substances.

As mentioned previously, the monkeys used in the current study were acclimated to the laboratory environment for 5 mo and to the restraining apparatus (Mobile restraint unit, Lomir Biomedical) for the 3 d preceding the procedure. The specific design of this sling allowed us to place the monkeys in horizontal position, leading to more quiet and comfortable animals. These monkeys were well conditioned to work with technicians, a characteristic that may have facilitated mask induction. We consider that induction with isoflurane without premedication would be insufficient for monkeys restrained by hand or in a chair; aggressive or excited monkeys would not be good candidates for mask induction.

Potential occupational exposure of staff to halogenated anesthetic gases should be considered when using the isoflurane protocol. Conflicting evidence exists in the scientific literature about the effects of trace levels of anesthetic gases on the health and performance of operating room personnel. Genetic mutations, cancer, complications during pregnancy (for example, spontaneous abortion), hepatic and renal disease, immunologic effects, and psychomotor changes have been linked to exposure to trace gases. In most instances, definitive proof is lacking[16]. Any veterinary or laboratory facility using inhalant anesthetics (that is, halogenated hydrocarbons and nitrous oxide) should institute and maintain a control program for waste anesthetic gases, in light of the possibility that trace gases may adversely affect human health. Briefly, this control program includes establishing standard operating procedures for appropriate checkout of materials and equipment and routine maintenance for volatile anesthesia equipment (particularly regarding filling of

vaporizers and verifying scavenging system efficacy), limiting the opening (rate) of flowmeters and vaporizers to their time of use on animals (to the level sufficient for the procedure, such as evaluated in the current study), using cuffed endotracheal tubes, eliminating (in so far as possible) residual gases through the scavenging system before disconnecting the patient, and using mask induction in well-ventilated rooms with nonrecirculating ventilation systems (such as the Bain coaxial system) or under a fume hood.

In conclusion, all 3 anesthesia protocols we tested are appropriate for induction of anesthesia for diagnostic endoscopic examinations. However, in our opinion, intramuscular ketamine–midazolam is contraindicated for endoscopic dosing because of its prolonged recovery time, which subsequently increases the postanesthetic care required by the monkeys. The choice between intravenous propofol and inhalant isoflurane will depend on available technology, the experience of personnel with each method, and potential drug–drug interactions. The quality of the induction, anesthesia, and recovery achieved with these 2 induction agents was comparable. Both agents appear to have a similar effect on duodenal absorption, but isoflurane causes more cardiovascular depression and body temperature alterations despite a trend to provide faster recovery.

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Legends

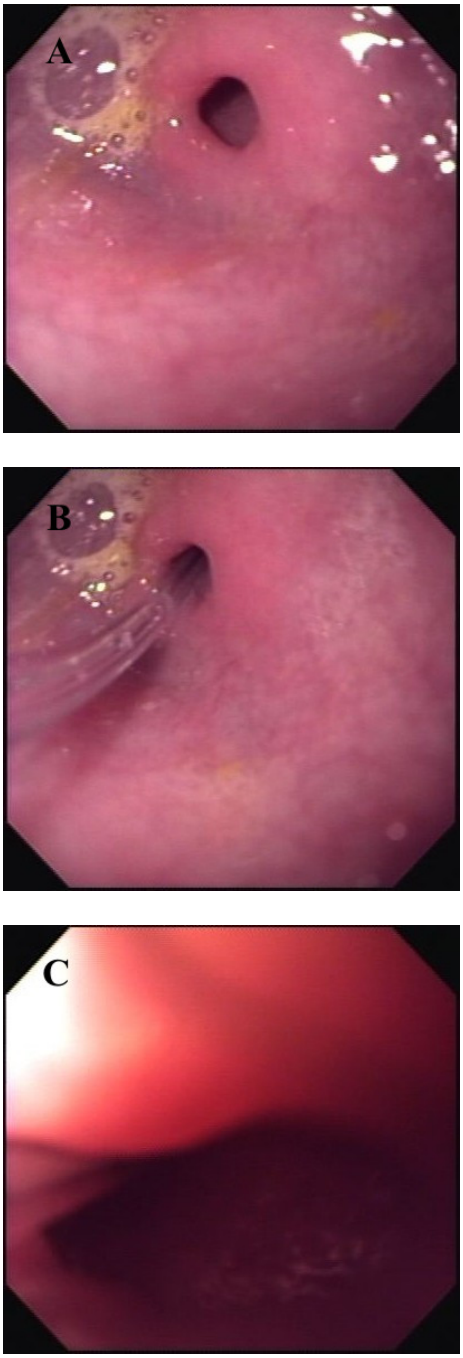

Figure 1. (A) View of the pylorus from the antrum. (B) Dosing line inserted in the pylorus. (C) Proximal duodenal mucosa with characteristic granular surface.

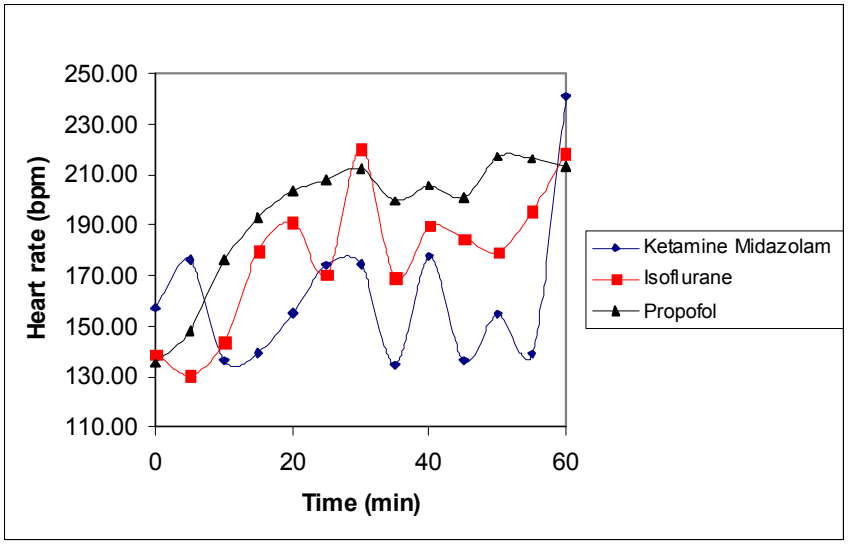

Figure 2. Average heart rate (bpm, beats/min) in rhesus monkeys during recovery from anesthesia induced with ketamine–midazolam, isoflurane, and propofol. Heart rate was decreased globally when anesthesia was induced with propofol ($P = 0.0004$).

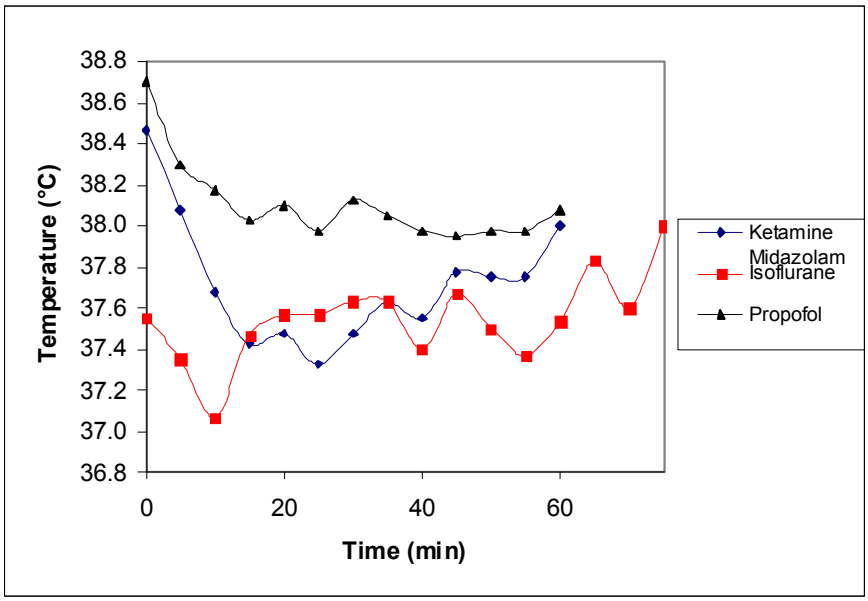

Figure 3. Average rectal temperature (°C) in rhesus monkeys during recovery from anesthesia induced with ketamine–midazolam, isoflurane and propofol. Among the 3 protocols, propofol caused the smallest decrease in body temperature; this protocol differed significantly from the other 2 protocols at 15 and 30 min after induction ($P < 0.04$ for all comparisons).

Table 1. Glycemia of rhesus monkeys

	Glucose (mmol/l)					
	Ketamine–midazolam		Isoflurane		Propofol	
	Baseline	Maximum	Baseline	Maximum	Baseline	Maximum
Male 1	5.4	5.1	na	na	4.1	7.4
Male 2	4.3	4.8	3.4	7.6	na	na
Male 3	na	na	3.0	5.4	3.0	5.6
Female 1	5.8	4.6	na	na	1.9	8.1
Female 2	5.4	7.6	3.5	4.6	na	na
Female 3	na	na	4.1	10.1	3.5	11.7
Average	5.2	5.5	3.2	6.5	3.0	7.0

na, not applicable

Monkeys were randomized to 2 of the 3 protocols, thus yielding 4 animals in each group

Table 2. Systemic arterial pressures

Time (min)	Average systemic arterial pressure (mm Hg)					
	Isoflurane		Propofol		Ketamine–midazolam	
	Systolic	Diastolic	Systolic	Diastolic	Systolic	Diastolic
0	90.25	44.75	98.50	75.25	98.25	61.75
5	90.75	45.00	107.50	60.50	140.75	80.25
10	107.50	69.25	123.25	57.00	116.00	77.75
15	100.00	65.00	112.25	80.00	110.00	65.50
20	106.75	72.75	118.75	76.00	102.50	74.75
25	107.25	70.00	131.00	94.50	128.00	74.00
30	115.00	69.25	122.50	85.50	111.25	78.50
35	97.25	67.75	108.25	69.00	128.75	98.25
40	113.50	78.50	115.00	73.50	127.75	93.25
45	118.50	71.00	118.75	71.50	110.00	69.25
50	104.25	61.50	109.25	58.75	119.75	82.50
55	99.71	46.63	119.96	86.63	102.87	66.35
60	126.50	76.00	129.50	81.75	114.75	83.00

At all time points, the diastolic pressure with isoflurane was significantly lower than that for the two other protocols ($P < 0.05$ for all comparisons

16. VIDÉO-ELETROENCÉPHALOGRAPHIE CHEZ LE SINGE CONSCIENT UTILISANT LA RADIOTÉLÉMÉTRIE ET L'ANALYSE PAR ORDINATEUR

Cet article explore une nouvelle modalité d'évaluation de pharmacologie de sécurité du système nerveux exploitant des technologies de pointe. Cet article présente une solution efficace à des questions de pharmacodynamie du système nerveux dans le développement de nouvelles thérapies. Dans un premier temps, l'enregistrement de l'électroencéphalographie (EEG) par des implants de radio-télémétrie fût développé. Comme pour les systèmes d'évaluations chez les patients humains, ce modèle est combiné au suivi vidéo afin de supporter le diagnostique électroencéphalographique lors de segment EEG anormaux. L'analyse EEG anciennement basé sur l'évaluation visuelle des tracés par un neurophysiologiste passe à l'ère moderne avec des outils informatiques avancés permettant de développer des algorithmes de détection en fonctions des caractéristiques des EEGs de chaque dérivation. Par ailleurs, ce modèle a permis de lever un arrêt d'étude clinique de Phase 2 pour un composé (nom du composé à l'essai et application clinique confidentiels) évalué par le FDA en 2008.

Les résultats et discussions de cet article ont été présentés par Docteur Simon Authier au 8^{ième} congrès annuel de la *Society of Safety Pharmacology* à Madison au Wisconsin en 2008 et seront présentés de nouveau dans le cadre d'un cours de formation continue au 9^{ième} congrès annuel de la *Safety Pharmacology Society* en Septembre 2009 à Strasbourg en France.

VIDEO-ELECTROENCEPHALOGRAPHY IN CONSCIOUS NON HUMAN PRIMATE USING RADIOTELEMETRY AND COMPUTERIZED ANALYSIS: REFINEMENT OF A SAFETY PHARMACOLOGY MODEL

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Short title: VIDEO-EEG FOR SAFETY PHARMACOLOGY IN NHP USING RADIOTELEMETRY

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SUMMARY

INTRODUCTION: Electroencephalography (EEG) investigations are occasionally required as follow-up studies for safety pharmacology core battery (S7A). Video-EEG monitoring is a standard diagnostic tool in humans but limited data is available on its use in telemetered freely moving macaque monkeys for safety pharmacology investigations. While proconvulsant risk evaluations are routinely conducted in rodents, pharmacological or pharmacokinetic considerations lead to the use of non human primates in toxicology and safety pharmacology in some cases. **METHODS:** Cynomolgus monkeys were instrumented with telemetry implants. Placement of EEG electrode was based on the *10-20 system* using three derivations (C3-O1, Cz-Oz and C4-O2). EEG trace analysis was carried out using NeuroScore software. After 24 hrs of continuous video-EEG monitoring, animals received pentylenetetrazole (PTZ, 10 mg/kg/15 min) until convulsions were noted. Convulsions were immediately treated with diazepam (1.0 mg/kg). A seizure detection protocol with a dynamic spike train threshold was used for the entire EEG monitoring period (total of 44 hrs) including periods when PTZ was administered. Spectral analysis was done to quantify the absolute and relative amplitude of EEG frequency bands (delta, theta, alpha, sigma and beta waves). Sleep stages were quantified and EEGs during seizures were analyzed using fast Fourier transformation (FFT) to assess dominant frequencies. **RESULTS:** Spike trains were detected by computerized analysis in all animals presenting PTZ-induced seizures while paroxysmal activities were systematically predictive (at least 4-min prior to generalized seizures). Beta activity increased

with visual stimulation using monkey treats. Characteristics of EEG for all sleep stages (I, II, III and IV) were present in all animals. Delta activity was predominant in normal awake EEG as well as in all sleep stages. Seizure peak frequency was 3-6 Hz on FFT, corresponding to the discharge of the underlying generator. **DISCUSSION:** EEG-video monitoring can be useful when using non human primates to characterize neurological adverse effects with unpredictable onset. Computerized video-EEG analysis was a valuable tool for safety pharmacology investigations including proconvulsant risk assessment, spectral analysis of frequency bands and sleep stage determination.

Keywords: Electroencephalography, monkey, pharmacology, safety, conscious, seizure, pentylenetetrazole

1. INTRODUCTION

Neurological and neuromuscular adverse effects are seen with a number of new drug candidates and characterization of the underlying pathophysiological mechanisms is critical for the future of the drug candidate. A drug that induces life-threatening adverse effects such as generalized seizures or *status epilepticus* may lead to drug development discontinuation. It is critical to differentiate CNS adverse effects from peripheral toxicity. Uncontrolled muscular activities can result from centrally mediated toxicity (*e.g.* seizures) or from neuromuscular transmitter disorder as seen with physostigmine, a cholinesterase inhibitor (Ambrani & Van Woert, 1972). While cholinesterase activity level can be investigated *in vivo* (Thomsen *et al.*, 1988), it may be difficult to differentiate involuntary skeletal muscle contractions caused by other neuromuscular disorders from simple partial seizures when using neurological examination in safety pharmacology studies. Drug-induced muscular contractions can originate from central or peripheral neurological alterations and classification of adverse events can be a complex and challenging task (Lanhunta *et al.*, 2006). The challenge is even greater considering that the timing of adverse effects is often unpredictable. Electroencephalography (EEG) has been used in safety pharmacology to assess the proconvulsant risk in various species (Danielsson *et al.*, 2006; Dürmüller *et al.*, 2007). While proconvulsant risk evaluations are routinely conducted in rodents, pharmacological or pharmacokinetic considerations lead to the use of non human primates in some cases. The use of monkeys is often justified by pharmacokinetic and metabolic profiles that are comparable to humans when rodents are not a suitable model (Oldham *et al.*, 1990). The use

of radiotelemetry with single (Pearce *et al.*, 1998) and dual (Almirall *et al.*, 1999) EEG channel has been reported in non human primates for neurobehavioral studies. Video-EEG monitoring is a standard diagnostic tool in humans (Asano *et al.*, 2005), but limited data is available on its use in telemetered freely moving macaque monkeys for safety pharmacology investigations. Electrophysiology examinations are occasionally required as follow-up studies for safety pharmacology core battery (U.S. FDA, 2001). The aim of the current study was to qualify a telemetered non human primate model of continuous video-EEG monitoring and computerized EEG analysis including seizure detection. Pentylenetetrazole (PTZ) is a well characterized agent inducing seizures in humans (Danielsen & Ellebjerg, 1966) and a variety of species including macaque monkeys (David & Grewal, 1977) that allowed qualification of this safety pharmacology model.

2. MATERIALS AND METHODS

2.1. Statement on use and care of animals. During the study, care and use of animals were conducted in accordance with principles outlined in the current Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). LAB Research Inc.'s facility is AAALAC accredited. All procedures were conducted as per Standard Operating Procedures (SOPs) in place.

2.2. Animal housing and preparation. Three (3) cynomolgus (*Macaca fascicularis*) monkeys were surgically prepared with telemetry transmitters *(TL11M2-D70-EEE™, DSI, St-Paul, MN, USA)*. Monkeys were 3 to 4 years old and weighed between 3.7 and 4.3 kg. The animal room environment was controlled (temperature 21 ± 3°C, humidity 30-70%, 12 h light, 12 h dark, 10-15 air changes per h) and temperature and relative humidity were monitored continuously. A standard certified commercial primate chow (Certified Primate Diet 2055C™, Harlan Teklad, Madison, WI, USA) was available to each monkey twice daily. Prophylactic antibiotics (cefazolin 25 mg/kg) were administered by intramuscular (IM) injection at least 30 minutes prior to surgery and every 4-8 hours post-injection for at least 48-hours post surgery. Preemptive analgesia (buprenorphine, Temgesic™, 0.05 mg/kg, Schering-Plough, Welwyn Garden City, Hertfordshire, UK) was administered by IM injection before surgery and every 6 to 12 h for at least 48 h post-surgery. Animals were placed on a heating pad and inhaled a mixture of oxygen (O_2) and isoflurane (AErrane™, Baxter Corporation, Mississauga, ON, CAN) with the O_2 flow meter and the vaporizer set at 1.0 L/min, and 2.0%, respectively. Respiratory rate was maintained between 10 and 13 breaths/min with an inspiratory airway pressure between 15 and 20 cm H_2O using a mechanical ventilator (2002, Hallowell EMC, Pittsfield, MA, USA). During anesthesia, monitoring included heart rate and pulsatile hemoglobin saturation in O_2 (VetOx 4404™ pulse oximeter, Heska, Fribourg, Switzerland). Bipolar centro-occipital and temporal-occipital EEG derivations were selected to minimize electromyographic (EMG) artefacts as previously described with needle electrodes in macaque monkeys (Danielsson *et al.* 2006). An abdominal

midline skin incision was initially done cranial to the umbilicus and a longitudinal incision was done in the middle of the *rectus abdominis* muscle. The telemetry transmitter was placed between the *internal abdominal oblique* muscle and the aponeurosis of the *transversus abdominis* muscle. The *rectus abdominis* was sutured with a simple continuous suture and EEG electrodes were tunnelled subcutaneously to a small skin incision in the neck. The abdominal skin incision was closed with interrupted buried sutures and the animal was placed in sternal recumbency to expose the cranium for the remainder of the surgery. Electrode placement was based on the internationally standardized *10-20 system* using three derivations (C3-O1, Cz-Oz and C4-O2) (Sharbrough *et al.*, 1991). Incision and electrode placement sites on the cranium were measured and marked with a surgical skin marker before the surgery to ensure precision. A sagittal incision was done in the occipital region over Oz and allowed visualization of O1 and O2. Then, a transverse incision was done over C3, Cz and C4. The temporal muscle at the level of C3 and C4 was visualised and incised parallel to muscle fibers on each side to expose the cranium. A small groove-shaped hole (approximately 3 mm) was drilled through the skull at the level of C3, Cz, C4, O1, Oz and O2 and the electrodes were inserted in the holes and secured with surgical glue (Vetbond™, 3M, St-Paul, MN, USA). The holes were subsequently filled with polymethyl methacrylate. The temporal muscles and the skin were sutured. Immediately at completion of surgery, a local analgesic (Marcaine® E, 2.5 mg/mL, Hospira, Montreal, QC, CAN) was injected in 6-10 sites (0.2 mL/site) around surgical areas on the skull.

2.3. Experimental methods. The EEG-video monitoring included digital color camera with daylight and infrared night vision connected to a computerized system (IBM Intellistation Z pro, Xeon 3.8 Ghz, 3.5 TB hard drive). The video recording was used for observational evaluation of behavioral convulsions both in real-time for diazepam administration and *a posteriori* when EEG traces were interpreted. To maximize telemetry signal quality, two receivers (DSI model RMC-1) were placed in each monkey cage (top and bottom). Acquisition of the telemetry signal was done at sampling rate of 500 Hz with the DSI software (Dataquest A.R.T. 3.01 Gold^{TM}) while the frequency range of the telemetry transmitters (D70-EEE, DSI) was 1-100 Hz. Video and EEG were monitored continuously for 24 hrs to establish baseline prior to subcutaneous injection of PTZ (10 mg/kg, 0.1 mL/kg) approximately every 15 min until seizures were noted. As soon as clonic convulsions were noted on video, a single diazepam injection was administered IV (1.0 mg/kg) and was sufficient to terminate seizure and paroxysmal EEG activity in all animals. Video and EEG were recorded for an additional 20 hrs after diazepam administration. Food treats were presented to induce a visual stimulation during baseline EEG monitoring to induce CNS stimulation.

2.4. EEG analysis methods. Trace analysis of EEG was carried out using NeuroScore software Version 1.1-2242 (*DSI, St-Paul, MN, USA*). A seizure detection protocol was created for cynomolgus monkeys with a dynamic spike train threshold which revealed to be the more sensitive and more specific than absolute amplitude threshold for seizure detection in our experimental conditions. The protocol was applied to the entire EEG monitoring period

including periods when PTZ was administered. All EEG segments classified as spike trains by the software were evaluated by a trained reviewer and correlated with video for interpretation. All EEG traces after initiation of PTZ administration but before seizure onset were also reviewed to evaluate the presence of any undetected EEG spike train. Spectral analysis was also done at different intervals to quantify the absolute and relative amplitude of EEG frequency bands (delta, theta, alpha, sigma and beta waves). EEG characteristics of various sleep stages were identified (I, II, III or IV) and combined with behavior evaluations (video) and spectral analysis for sleep stage classification. Sleep stages III and IV also known as slow-wave sleep (SWS) Finally, EEG during seizures was analyzed using fast Fourier transformation (FFT) and auto-regression spectrum to assess dominant frequencies. When applicable, data is presented as mean ± standard deviation.

3. RESULTS

Telemetry implants and EEG electrodes were well tolerated in all animals and the quality of EEG traces was adequate throughout the monitoring period (Fig. 1). The most common artefact noted during behavioral evaluations from video analysis was EMG activity. Telemetry signal strength was above 25 MKUs throughout the monitoring period, which was considered optimal to minimize artifacts. When present, EMG activity was most important for derivations C3-O1 and C4-O2 while Cz-Oz (Fig. 1) presented significantly less EMG activity. Paroxysmal activity (Fig. 2) was noted at least 4 minutes before general seizure in all animals. Behavioral clonic convulsions were observed in all animals (average cumulative PTZ dose of 70 mg/kg ± 17 mg/kg) and

correlated with EEG spike trains. Animals presented jerky movements before onset of generalized clonic convulsions corresponding to paroxysmal activity. The NeuroScore software seizure detection module identified spike trains during seizure in all animals (Fig. 3). *A posteriori*, video was used to differentiate jerky movements and clonic convulsion from artefacts such as EMG which may present similitude with EEG paroxysmal activity. Software sensitivity to detect spike trains was high (EEG review confirmed that all spike trains had been identified) while specificity was low (large number of EEG segments identified as spike trains by the software that were excluded following video and EEG review). Computer analysis significantly reduced the amount of EEG traces to review for spike trains (less than 5 min per period of 24 hours). Transient background attenuation characteristic of the post-ictal EEG was noted (Fig. 4) in all animals and resulted in increased delta wave amplitude and attenuation of higher EEG frequencies (Fig. 5C). Spectral analysis of EEG throughout the monitoring period revealed that delta activity was predominant in all periods in the cynomolgus monkey (Figs. 5A to 5E). Visual stimulation resulted in a transient increase in beta activity (Fig. 5B). All sleep stages (I, II, III and IV) were present in all animals. A relative increase in beta activity with decreased alpha activity was noted during stage I sleep (Fig 5D) while the presence of sleep spindles and K complexes (Fig. 6) were characteristic of stage II sleep. Increased delta activity (slow wave sleep) was present in stages III (>20% of epoch) and IV (>50% of epoch) sleep stages (Fig. 5E and Fig. 7). During seizure, FFT revealed peak amplitude of 3-6 Hz corresponding to the discharge frequency of the underlying generator. Several

peaks at integer multiples were also present but the higher harmonics could be distinguished from the fundamental frequency (Fig. 8).

4. DISCUSSION

A trend to develop an increasing number of biologic drug candidates (Hughes, 2008) may justify the use of non-human primate for safety investigations for various reasons including the presence of drug target in this species not found in rodents or other animal species. Some drugs administered orally have a relatively slow absorption rate and several hours may elapse between drug administration and the onset of adverse effects. In these cases, it may not be possible or ethical to restrain monkeys for EEG monitoring until an adverse event is noted. Similarly to clinical diagnostic, EEG-video monitoring can be useful when using non-human primates to characterize neurological clinical signs with unpredictable onset. Continuous EEG monitoring using telemetry generates an important amount of tracings which may render manual EEG review impractical. The use of computerized EEG analysis is essential to optimize data processing and facilitate evaluation.

In the current study, PTZ was administered at 15 min interval until clonic convulsions were noted to confirm the method of computerized seizure detection. The software accurately identified spike trains in all animals from 44 hrs of EEG tracings which facilitated data processing. It remains that correlation of spike trains with behavioral activity recorded on video by a trained reviewer was required for interpretation of EEG traces. Dürmüller *et al.* presented paroxysmal activity as an adequate endpoint to assess the proconvulsant risk of drug candidates in telemetered dogs with PTZ infusion

(Dürmüller *et al.*, 2007). Dürmüller reported that the onset of paroxysmal EEG activity was during the 60 s preceding convulsions. The delay between paroxysmal activity and clonic convulsion is critical to use this early EEG sign for evaluation of proconvulsant risk during real-time monitoring. Occasional EMG artefacts may require careful EEG tracing assessment to confirm the presence of paroxysmal activity. The current study allowed identification of paroxysmal activity at least 4 min before generalized seizures. The delay between paroxysmal activity onset and generalized convulsions when using SC injection at 15 min intervals may facilitate the use of this early sign as an indication of the proconvulsant risk while minimizing the overall duration of physical restraining. In contrast, the repeated PTZ administration at fixed doses SC decreases the precision and sensitivity of the proconvulsant model when compared with continuous IV infusion where the PTZ administration can be terminated and the total dose calculated. The current model could be further refined with continuous IV infusion in unrestrained non-human primates. As seen in humans (Tilz *et al.*, 2006) and other species (Cherubini *et al.*, 1981), post-ictal EEG was characterized by background attenuation. A single diazepam IV administration (1.0 mg/kg) was sufficient to control and terminate seizure and paroxysmal EEG activity in all animals. Based on immediate resolution of paroxysmal activity, it is unlikely that brain trauma had occurred.

Alpha activity is predominant in normal awake EEG in adult humans while lower frequencies are predominant during rapid eye movement (REM) sleep (Morisson *et al.*, 1998). Delta activity was predominant in awake as well as all sleep stages in our experimental conditions. While earlier report

suggested predominant sigma activity in awake cynomolgus monkey (Jurko & Andy, 1967), recent spectral analysis in normal awake cynomolgus monkeys supports our observation that delta activity is predominant in this species (Lallement *et al.,* 1998). In human, an increase in beta EEG activity during performance of a mental task is well described (Poupard *et al.*, 2001). The same observation was present in the monkeys of our study with visual stimulation (food treats). Several marketed drugs including benzodiazepines (Bastien *et al.*, 2003), anti-convulsants (Wu & Xiao, 1996), anti-depressants (Feige *et al.*, 2002) and Alzheimer's disease treatments (Gianotti *et al.*, 2008) have known effects on EEG spectral analysis. Video-EEG monitoring can be a useful tool to further characterize drug candidates with central effects. The peak EEG frequencies (3-6 Hz) observed during PTZ-induced seizures were similar to reported values in dogs (4-5 Hz) (Dürmüller *et al.*, 2007) and rats (1-3 Hz) (Mirski *et al.*, 2003) and humans (3 Hz) (Rowan & Tolunsky).

In conclusion, video-EEG with computerized analysis was a valuable tool for safety pharmacology investigations including proconvulsant risk assessment, spectral analysis of frequency bands and sleep stage determination. In spite of considerable increase in efficiency for data processing, EEG review by a trained observer remains essential for definitive interpretations of traces. When evaluating drug candidate proconvulsivant risks, species characteristics must be carefully considered. While most studies have used rats and mice, pharmacological and pharmacokinetic considerations may support the use of non human primates in some cases and this pilot study establishes standards of video-EEG with computerized analysis in cynomolgus monkeys.

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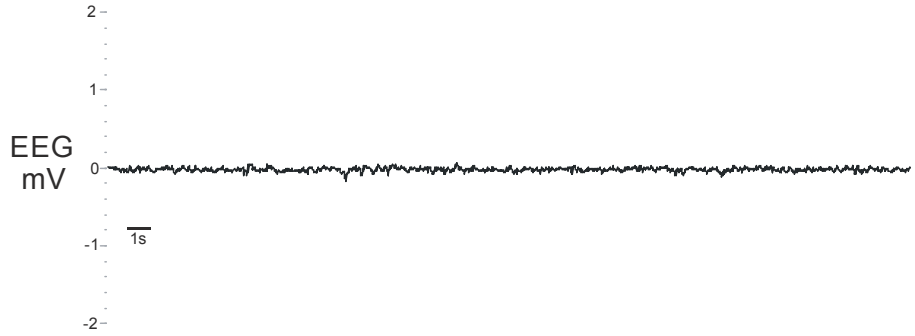

Fig. 1. Electroencephalogram in Cz-Oz derivation from a normal awake freely moving cynomolgus monkey.

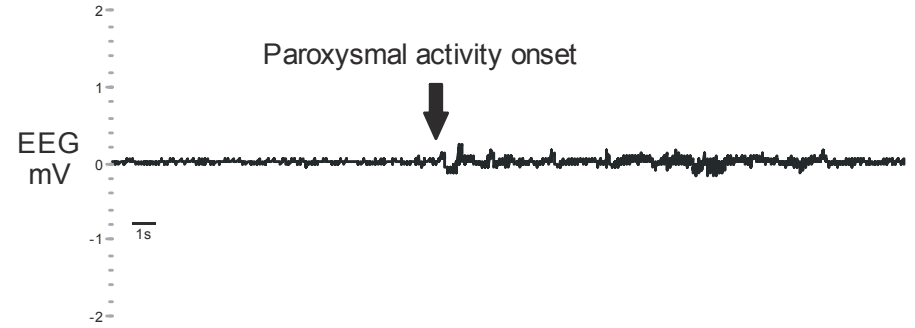

Fig. 2. Illustration of paroxysmal EEG activity in telemetered cynomolgus monkey.

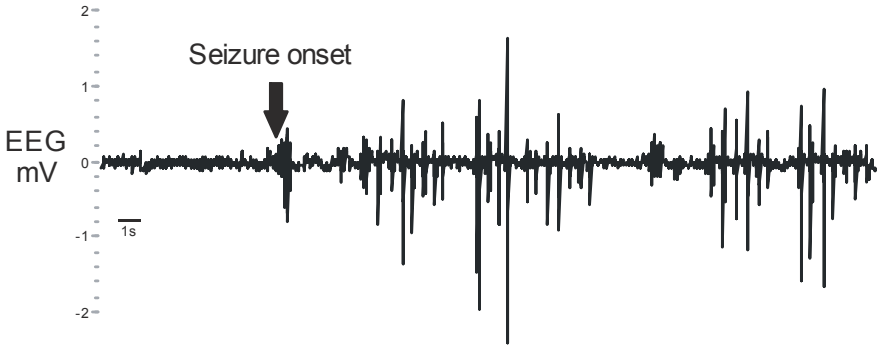

Fig. 3. Spike train detected using seizure detection module of the NeuroScore software in freely moving telemetered cynomolgus monkeys obtained from EEG Cz-Oz derivation.

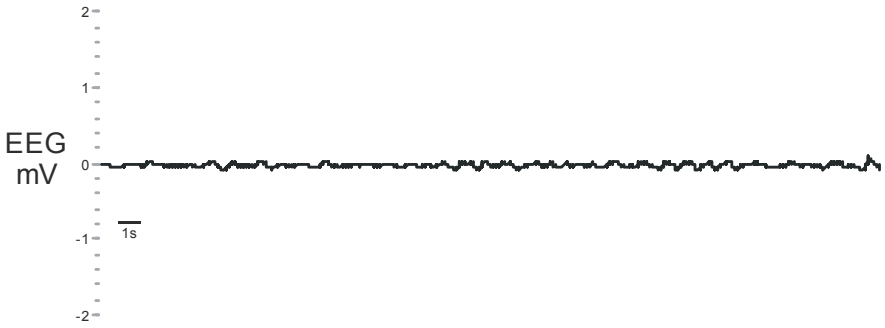

Fig. 4. Post-ictal EEG with background attenuation in Cz-Oz derivation following diazepam injection at onset of seizures in cynomolgus monkey.

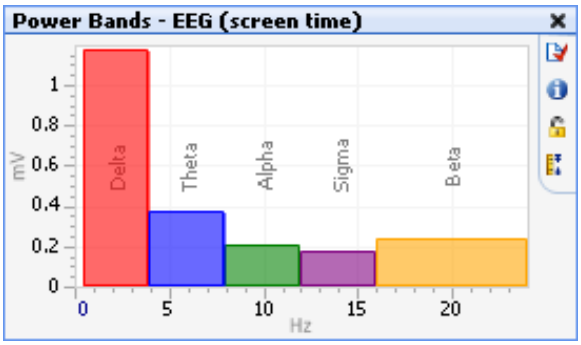

Fig. 5.A

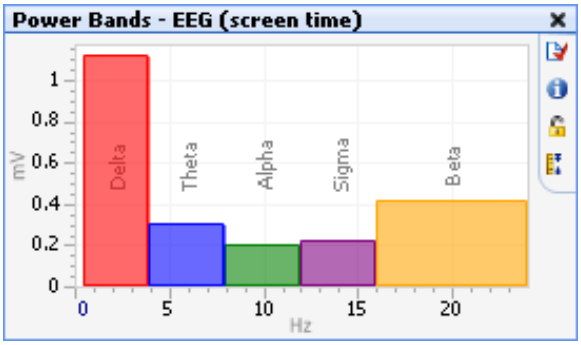

Fig. 5B

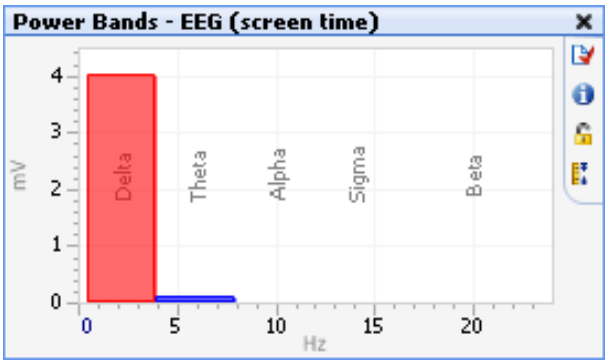

Fig. 5C

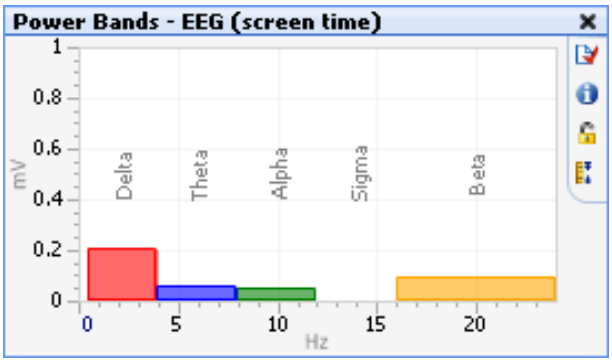

Fig. 5D

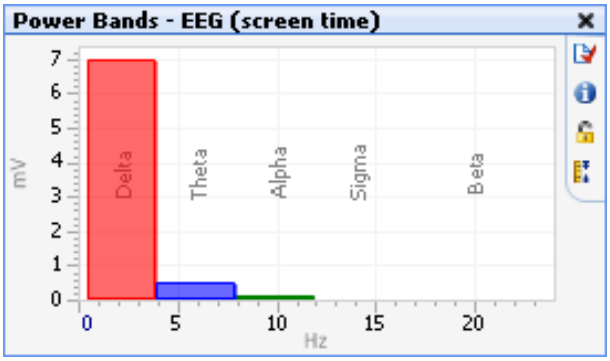

Fig. 5E

Fig. 5. Spectral analysis of EEG in cynomolgus monkey in different states: A) normal awake, B) increased beta activity during visual stimulation C) post-ictal EEG background attenuation with increased delta activity, and attenuation of higher EEG frequencies, D) stage I sleep with enhanced beta activity, E) stages III and IV sleep with increased delta activity and attenuation of higher EEG frequencies.

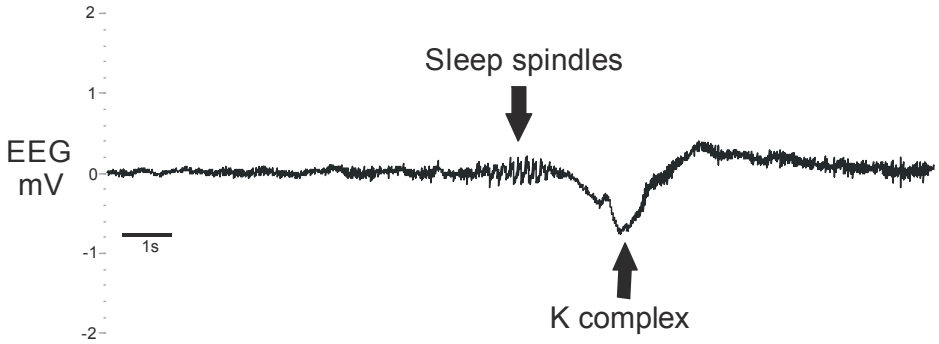

Fig. 6. Electroencephalogram from Cz-Oz derivation during stage II sleep in a cynomolgus monkey.

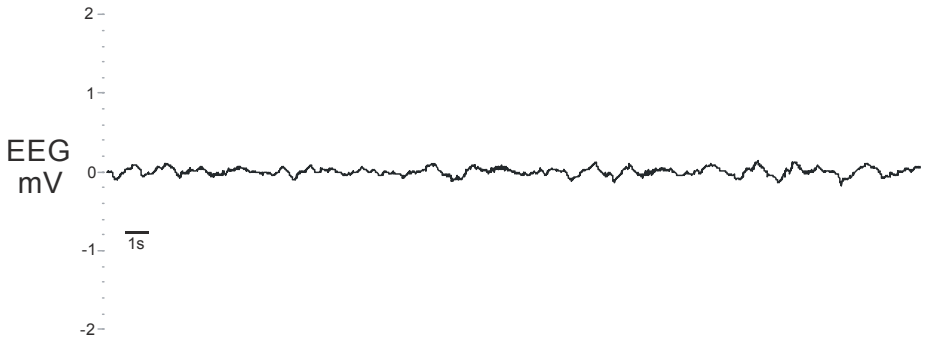

Fig. 7. Electroencephalogram from Cz-Oz derivation during deep sleep (Stage IV) presenting increased delta wave (>75 µV) activity in a cynomolgus monkey.

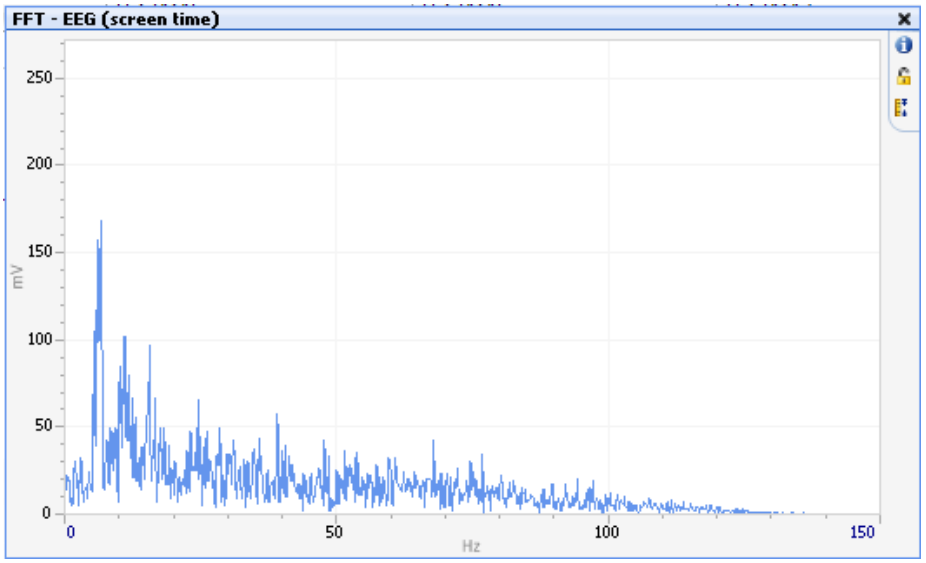

Fig. 8. Fast Fourier Transform of EEG during seizure with peak amplitude at a frequency of 6 Hz.

17. MONOXYDE D'AZOTE, SIGNALISATION CELLULAIRE ET MORT CELLULAIRE

Cet article (*Toxicology* 2005, 208:177-192) présente le rôle physiologique du monoxyde d'azote ainsi que ses effets toxiques au niveau cellulaire ainsi que dans le cadre du traitement au monoxyde d'azote par inhalation. Les implications de ce gaso-transmetteur dans l'apoptose et la nécrose cellulaires sont discutées avec une approche combinant à la fois la perspective clinique et la biologie moléculaire. Cet article introduit un concept fondamental à l'analyse des effets de l'OT comme peptide thérapeutique, soit la nécessité de maintenir un niveau optimal physiologique pour assurer les bienfaits du traitement. Sous la supervision du Docteur Gilbert Blaise, le Docteur Simon Authier a contribué à la rédaction de cet article par la recherche sur le rôle du monoxyde d'azote dans la différentiation, l'apoptose et la nécrose des cardiomyocytes.

Nitric oxide, cell signaling and cell death

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Abstract

Nitric oxide (NO) is an important bioregulatory molecule in the nervous, immune and cardiovascular systems. NO participates in the regulation of the daily activities of cells as well as in cytotoxic events. It possesses a controversial effect on cell viability by acting both as a protection against apoptogenic stimuli, or by inducing apoptosis when produced at elevated concentrations. The mechanisms of NO in regulating these biological functions can be either through cyclic guanylate cyclase (cGMP)-dependent or cGMP-independent pathways. The purpose of this review is to highlight the implication of NO in cell signaling, synaptic transmission, and cell death. We focus also on the protective role as well as the toxicity of NO. Finally, the adverse effects of inhaled nitric oxide are also depicted in this review.

Keywords: Apoptosis; Toxicity; Mechanism; Nitroglycerine tolerance

1. Introduction

In 1980, Furchgott and Zawadzki discovered that endothelial cells stimulated with different agonists liberated a vasodilator that was called endothelium derived relaxing factor (EDRF). Six years later, its nature was identified and it is now well accepted that EDRF is nitric oxide (NO) or a NO-containing substance. NO is produced by a family of enzymes, the nitric oxide synthetases (NOS), through enzymatic oxidation of the guanidine group of l-arginine (Marletta et al., 1998). This occurs in two sequential monooxygenase reactions utilizing NADPH as co-substrate and involving molecular oxygen; citrulline and NO are the terminal products.

The constitutive expression of two NOS isoforms is responsible for a low basal level of NO synthesis in neural cells (nNOS or NOS1) and endothelial cells (eNOS or NOS3). These constitutive NOS isoforms require

Ca^{2+} and several cofactors for their enzymatic activity. Induction of the inducible isoform (iNOS or NOS2) by cytokines and/or bacterial products (endotoxin/lipopolysaccharides) have been observed in virtually all cell types tested (including macrophages, dendritic cells, fibroblasts, chondrocytes, osteoclasts, astrocytes, epithelial cells), and results in the production of large amounts of NO(Nathan, 1997). The mitochondria also contain a unique NO-producing enzyme (mt- NOS).

NO is a small, hydrophobic molecule that can easily pass through membranes. It persists in vivo in the presence of free hemoglobin for a few seconds, and can diffuse only several cell diameters away from its site of synthesis (Taha et al., 1992; Malinski et al., 1993; Lancaster, 1994; Huang et al., 2002). Several molecules containing thiol groups, such as acethylcysteine, albumin and red blood cells, can be not only NO scavengers, but can also protect it from metabolism, and are NO carriers that release active NO or a NO-containing small molecule acting like NO at a distance from the NO site of production. Overwhelming evidence points to a new regulatory role of NO in the human circulation in which red blood cells undergo *S*-nitrosylation during passage through the lungs and subsequently release part of their bound NO during arterial–venous transit, enhancing blood flow and aiding O_2 delivery in the microcirculation (Stamler et al., 1997; Gow et al., 1999; McMahon et al., 2002).

2. NO actions

NO exerts most of its physiological effects by binding to its guanylyl cyclase (GC)-coupled receptors in a specialized heme group, the occupation of which results in conformational changes that trigger GC activity and so the generation of cyclic GMP from GTP then engages various downstream targets including protein kinases, phosphodiesterases and ion channels bringing modifications in cell functions such as smooth muscle relaxation, platelet disaggregation, and synaptic plasticity.

NO regulates a wide range of biological functions via post-translational modification of proteins (Mannick and Schonhoff, 2004). The biological activities of NO can be divided into cyclic guanylate cyclase

(cGMP)-dependent and cGMP-independent pathways (Chung et al., 2001). cGMP formation is considered to be the main physiological signaling NO pathway (Chung et al., 2001).

cGMP production leads to the activation of cGMP dependent protein kinases and the suppression of caspase activity (Dash et al., 2003). High doses of NO may inhibit apoptosis through both cGMP-dependent and -independent mechanisms (Dash et al., 2003).

The three principal targets of cGMP are protein kinase G, cyclic-nucleotide-gated channels, and cyclic nucleotide phosphodiesterase (Ahern et al., 2002). When NO is present in concentrations sufficient to activate soluble guanylyl cyclase (sGC), apoptosis is initiated by protein kinase G-dependent activation of the P13K/Akt pathway, resulting in Bad phosphorylation, and subsequent suppression of cytochrome *c* release and caspase-3 activation (Ha et al., 2003).

NO can also modify proteins through direct chemical reactions, without the need for enzymes.

S-Nitrosylation is the attachment of NO to the thiol group of a cysteine residue. This process requires larger amounts of NO than does activation of sGC. The range of ion channels that is *S*-nitrosylated is nearly as broad as that controlled by cGMP (Ahern et al., 2002).

NO and related species can oxidize, nitrate or nitrosylate proteins (Mannick and Schonhoff, 2002). Tyrosine *nitration* is a covalent protein modification resulting from the addition of a nitro (NO_2) group onto one of two equivalent ortho carbons of the aromatic ring of tyrosine residues (Ischiropoulos, 2003). The addition of the nitro group implies the formation of nitrating species with NO providing the nitrogen source (Ischiropoulos, 2003).

Nitrite (NO_2^-) is a well-known substrate for heme proteins, and its conversion to NO_2 by myeloperoxidase, eosinophil peroxidase as well as other heme proteins (myoglobin, cytochrome P450) could account for the nitration of tyrosine residues (Ischiropoulos, 2003).

The attachment of a NO group to a thiol (R-SH) or a transition metal is called *nitrosylation*, a reversible mechanism involved in cell signaling, which regulates the function of many proteins (Mannick and Schonhoff, 2004). *S*-

Nitrosylation is an important form of posttranslational modification of ion channels, and provides a route by which NO can regulate electrical activity without stimulating cGMP production.

3. Apoptosis versus necrosis

Apoptosis, derived from Greek, refers to the dropping of leaves from the tree (Kiechle and Zhang, 2002). The morphological features of apoptosis can be observed in fixed and stained tissue and they include cytoplasmic blebbing, chromatin condensation, nuclear fragmentation, cell rounding (loss of adhesion) and cell shrinkage (Kiechle and Zhang, 2002). Apoptosis is a genetically controlled process that often requires new protein synthesis. In contrast, necrosis has none of the cellular morphological changes characteristic of apoptosis, and occurs rapidly after the initial insult (Kiechle and Zhang, 1998; Geske and Gerschenson, 2001). Necrosis is also called passive cell death, and apoptosis, active or programmed cell death. As a simple distinction, apoptosis requires activation of cell signaling whereas necrosis does not involve cell signaling.

Apoptosis is an important cellular process in both physiological and pathological conditions. To introduce the role of NO in apoptosis, a brief overview of the major elements of cellular signaling during apoptosis will be presented. Intracellular ATP concentration may be critical in selection of the cell death pathway. A high ATP concentration favors apoptosis, while a low concentration shifts cell death toward necrosis (Los et al., 2002). The activity of poly (ADP-ribose) polymerase (PARP)-1 may be the pivotal point in this cell death decision. If PARP-1 is activated, NAD^+ is used as a substrate for ADP-ribosylation, and ATP depletion may occur to synthesize NAD^+, leading to necrosis (Kiechle and Zhang, 2002). If PARP-1 is inactivated by proteolytic cleavage into two sub-units by caspase-3, ATP concentration will not be depleted, favoring apoptosis (Los et al., 2002).

Apoptosis can be induced by extrinsic and intrinsic pathways. The receptor-linked pathway is known as the extrinsic pathways, and requires binding of a ligand to a death receptor on the cell surface (Kiechle and Zhang, 2002). Tumor necrosis factor (TNF) and Fas ligand (FasL) bind to their cell surface death receptor, TNF receptor type 1 (TNFR 1) and Fas receptor,

respectively. Once activated, these receptors form an homotrimeric complex that recruits signal-producing molecules, the TNFR 1-associated death domain (TRADD) and Fas associated death domain (FADD) (Kiechle and Zhang, 2002). This complex then activates caspase-8, which initiates the protease cascade, leading to apoptosis (Reed, 2000; Mullauer et al., 2001; Zimmermann et al., 2001). (It has been shown that NF-κB can prevent apoptosis induced by TNF-α through transcriptional regulation of factors, such as TNFR-associated factor (TRAF) and inhibitor of apoptosis protein (IAP)).

The intrinsic pathway is mediated by the mitochondria, which integrates multiple stress signals, such as hypoxia, calcium and loss of growth factors. In response to these stimuli, the mitochondria release proapoptotic factors, such as apoptotic-inducing factor (AIF), Smac/Diablo, endonuclease G (endo G) and cytochrome *c*. Smac is pro-apoptotic by inhibiting IAP (Du et al., 2000). Cytochrome *c* is a cornerstone of apoptosis. Once released from the mitochondria, cytosolic cytochrome *c* binds to Apaf-1 in the presence of dATP. This complex recruits pro-caspase-9 to form the apoptosome that finally activates caspase-3 and the apoptotic cascade. An important element of the intrinsic pathway is the activation of transcription factor p53, in response to DNA damage (Fig. 1).

p53 is a DNA-binding protein and transcriptional activator that may have a role in DNA repair, because it appears to accumulate after DNA damage (Donehower and Bradley, 1993). In turn, p53 increases the expression of the pro-apoptotic Bax, and decreases the expression of the anti-apoptotic bcl-2.

When nitrosative and/or oxidative stresses are coupled with a combination of different factors, such as decreased antioxidants, the by-products of homeostatic cell metabolism can cause deleterious effects that can signal apoptotic cell death (Nelson et al., 2003). Apoptosis pathways either involve death receptors (a), or the mitochondria (b) (Brune, 2003).

The Fas ligand, a cell surface type II protein that is found predominantly on activated T cells, is a member of the TNF gene family (Nagata and Golstein, 1995). This ligand triggers apoptosis in a variety of cells bearing Fas on their cell surface (Nagata and Golstein, 1995). Recent work on

the mechanism of Fas signaling suggests that ceramide production via activation of ASM may be important. The addition of synthetic C2-ceramide alone to culture medium can mimic Fas activation and trigger apoptosis (Cifone et al., 1994).

The Fas molecule is a type I membrane protein surface receptor that induces apoptotic cell death. Fas induced apoptosis is regulated by protein nitrosylation. Fas ligation leads to the activation of a family of cysteine proteases called caspases. Caspases are expressed as relatively inactive zymogens that are cleaved to form fully active enzymes. In resting cells, caspase- 3 zymogens in mitochondria are kept inactive via *S*-nitrosylation of their catalytic site cysteine. Caspase-3 may be stably *S*-nitrosylated in the mitochondria due to an association between *S*-nitrosylated caspase-3 and NOS. Moreover, *S*-nitrosylated but not denitrosylated caspase-3 associates with ASM in mitochondria. This association provides another level of apoptosis regulation by inhibiting caspase-3 cleavage and activation by initiator caspases. When cells are stimulated by the Fas ligand, caspase-3 becomes denitrosylated (Mannick and Schonhoff, 2004).

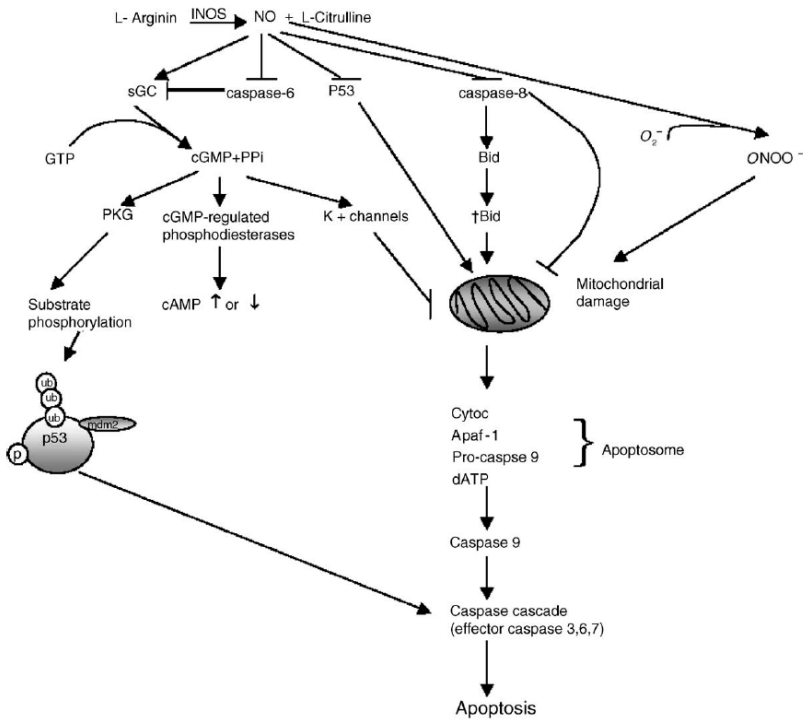

Fig. 1. Implication of nitric oxide in the apoptosis pathways. Nitric oxide can either induce or inhibit apoptosis depending on its site of action. PKG: protein kinase G; iNOS: inducible nitric oxide synthase; sGC: soluble guanylyl cyclase; ub: ubiquinone; p: phosphor; cytoc: cytochrome *c*; $ONOO^-$: peroxinitrite.

4. Mechanism

Fas, which binds the Fas ligand, oligomerizes in the membrane; its cytoplasmic domain contains a 70-residue sequence called the death domain, required for the stimulation of apoptosis (Ashkenazi and Dixit, 1998). Oligomerization then incorporates an intracellular death domain-containing protein called FADD (Fas associated death domain) (Ashkenazi and Dixit, 1998).

The N-terminal region of FADD contains another domain, the death effect or domain. It associates with a death effector domain at the N-terminus of procaspase- 8. Recruitment into the complex brings about pro-caspase-8 dimerization, releasing active caspase- 8. Caspase-8 then initiates the whole

effect or protease cascade by acting on pro-caspase-3 (Ashkenazi and Dixit, 1998).

5. NO and apoptosis

The role of NO in apoptosis and/or necrosis is controversial. Indeed, it has been shown that NO can have both pro- and anti-apoptotic properties. NO can prevent apoptosis in some cell lines, such as endothelial cells, lymphoma cells, ovarian follicles, cardiac myocytes and hepatocytes. To illustrate the need to have a certain level of NO for cell survival, NOS inhibitors increased the incidence of apoptosis (Youn et al., 2001; Ciani et al., 2002). On the other hand, exposure to NO donors also augmented the incidence of apoptosis (Shimojo et al., 1999; Uchiyama et al., 2002) (Fig. 1).

Inhibition of apoptosis by NO may be associated with the induction of heat shock protein 70 (Hsp 70) response, suppression of Bax expression, or GC activation, induction of protective pathways through the induction of heme oxygenase and cyclo-oxygenase; may involve up-regulation of intracellular antioxidant systems, especially glutathione; may inhibit caspase 3-like enzymes via *S*-nitrosylation or through a cGMP dependent mechanism, both leading to inactivation of caspases. It has been shown that the signaling pathway by which NO prevents apoptosis in cardiomyocytes includes the up-regulation of p21cip/waf1, which inhibits the kinase activity of an important cyclin A/cdk2 (Maejima et al., 2003). Inhibitor of NOS down-regulated serine/threonine kinase (Akt) survival pathway, and inhibited phosphorylation of cAMP-responsive element-binding protein (CREB) via cGMP, which was associated with decreased Bcl-2 expression in cerebellar neurons (Ciani et al., 2002). Exposure to NO donors increases cGMP concentration in cardiomyocytes (Shimojo et al., 1999), neural cell lines, and pancreatic beta cell lines (Loweth et al., 1997). NO can augment the concentration of proapoptotic ceramides by decreasing ceramidase activity and inducing a neutral sphingomyelinase (Huwiler et al., 1999; Takeda et al., 1999). Ceramides mediate a pro-apoptotic effect through diminished Bcl-2 expression (Di Nardo et al., 2000). NO has a protective role against these pro-apoptotic effects of ceramides. First, it has been shown that NO inhibits acid

sphingomyelinase via cGMP in the early stage after exposure to TNF-α in a monocytic cell line (Barsacchi et al., 2002). This inhibition could be physiological protection against NO-induced apoptosis in the early stage. NO also acts as an antioxidant that shields against the pro-apoptotic effects of ceramides. Second, NO counteracts the reactive oxygen species (ROS) generated by pro-apoptotic ceramides (Matsunaga et al., 2004). It inhibits the assembly of Apaf-1 and pro-caspase-9, which form the apoptosome that is essential in both stress- and receptor-induced death (Zech et al., 2003) (Fig. 1). Exposure to NO induces Smac release from the mitochondria of lymphoblastoid cell lines (Li et al., 2004). The major effects of NO on the cellular pathways leading to apoptosis are preserved among different cell types. Nevertheless, the variability that subsists among different cell types is probably crucial for proper cell responses.

Stimulation of different cell lines with lipopolysaccharides and several cytokines, including interleukin (IL)-1, interferon (INF)-γ and TNF-α, induces activation of iNOS and cell death (Kitajima et al., 1994; Pinsky et al., 1995; Geng et al., 1996; Lu et al., 1996; Zhang et al., 1997), which can be prevented by NOS inhibitors (Estevez et al., 1998). NO donors can induce apoptosis in many cell types (Kaneto et al., 1995; Pinsky et al., 1995; Geng et al., 1996; Tabuchi et al., 1996; Taylor et al., 2001; Li et al., 2002). Injury-induced cell death results in up-regulation of NOS gene expression and is inhibited by NOS inhibitors. NO toxicity may result from interaction with protein thiol groups or iron sulfur groups (Stamler, 1994; Brune et al., 1996; Kiechle and Malinski, 1996; Christopherson and Bredt, 1997; Heales and Bolanos, 2002). NO attenuates oxidative phosphorylation by inhibiting iron–sulfur cluster enzymes, including NADH ubiquinone/oxidoreductase and NADH succinate/oxidoreductase. NO suppresses glycolysis by reacting with *cis*-aconitase and by competing with oxygen at cytochrome oxidase. It can react rapidly with superoxide (O_2^-) to produce peroxynitrite ($ONOO^-$), which is a powerful oxidant sufficiently stable to diffuse through cells to react with targets. Elevated NO concentrations increase the formation of $ONOO^-$ which creates DNA strand damage. DNA strand breakage leads to up-regulation of p53, which activates the DNA repair enzyme PARP. The energy required for

DNA repair may force the cell to initiate apoptosis. $ONOO^-$ is particularly efficient at oxidizing iron–sulfur clusters, zinc fingers and protein thiols; these reactions also contribute to cellular energy depletion. $ONOO^-$ reacts with superoxide dismutase (SOD), and this combination catalyses the 3- nitration of protein tyrosine residues, particularly those in cytoskeletal proteins. Glutathione plays an important role in protecting cells from oxidative stress and NO-mediated apoptosis (Ho et al., 1997; Kim et al., 1997; Li et al., 1997; Zhao et al., 1997). NO-induced apoptosis is associated with a significant decrease in intracellular glutathione in vascular smooth muscle cells. Glutathione might prevent apoptosis through the maintenance of oxidation-sensitive enzymes in a reduced activity state (Zhao et al., 1997). NO oxidative products (NO_2, $ONOO^-$ ions, HNO_2, 'NOx') may deaminate, crosslink and oxidize DNA bases. In NO induced apoptosis, NO donors evoke the up-regulation of CD95 (APO-1/Fas) ligand, TRAIL/APO-2 ligand and p53 gene expression as well as caspase activation that cleaves nuclear proteins such as PARP (Messmer et al., 1996; Zhao et al., 1997; Chlichlia et al., 1998; Brune et al., 2001). NO-induced apoptosis can be blocked by a broad-spectrum ICE (interleukin-1B-converting enzyme)-protease/caspase inhibitor and by Bcl-2 over expression from Bcl-2 transfectants (Messmer et al., 1996; Chlichlia et al., 1998). NO also regulates the concentration of mRNA coding for a variety of transcription factors and other gene products. For example, the regulation of central signal-responsive transcription factors, such as NF-kB, c-myb and AP-1 by NO, may be associated with NO-induced apoptosis (Tabuchi et al., 1996; DelaTorre et al., 1997; Park et al., 1997; Brendeford et al., 1998; Bogdan, 2001; Pfeilschifter et al., 2001).

The mitochondria comprises a target for NO and there is accumulating evidence that inhibition of respiration may contribute to the pro-apoptotic effect of NO by membrane potential reduction, transition pore opening, and cytochrome *c* release (Brune, 2003).

In the mitochondria, decreased SOD activity can result in the kinetically favorable side-reaction of triplet NO^- with O_2^-. This reaction generates ONOO radical that is damaging to cells. $ONOO^-$ will oxidize free

thiols in the cytosol and form disulfide linkages, potentially affecting protein functions within cells (Nelson et al., 2003).

In the case of decreased SOD, cellular damage causes the immune system to signal iNOS release, which reacts with O_2^- to produce more $ONOO^-$. If glutathione peroxidase or catalase activity is decreased, the resulting OH^- and lipid peroxidation will also signal more iNOS until antioxidants are depleted (Nelson et al., 2003).

Although NO alone does not interact with proteins or nucleic acids, it can combine directly with local electron-accepting species to form reactive NO species (RNOS). RNOS are formed when NO reacts with O_2 or superoxide radicals. RNOS are known to cause injury by further oxidative reactions with proteins in tissues. In many situations, the reaction of RNOS can induce cell death (Kim et al., 2001a, b).

$ONOO^-$ is in dynamic equilibrium with its conjugated acid ONOOH under physiological conditions (Mikkelsen and Wardman, 2003). Under physiological conditions, the relatively low $ONOO^-$ yields are detoxified by direct or radical-mediated reactions with mitochondrial components such as ubiquinol or NADH (Carreras et al., 2004). NO at μM concentrations for long periods inhibits respiration and mitochondrial complex I activity (Carreras et al., 2004).

6. NO and synaptic transmission

NO is implicated in numerous physiological and pathophysiological neurodegenerative diseases and injuries, such as stroke, epileptic seizures, Alzheimer's disease, Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), traumatic brain injury, aging and AIDS dementia. NO is a key modulator in physiological processes such as memory, long-term potentiation, and long-term depression (Tabuchi et al., 1996; Fitzsimonds and Poo, 1998; Hawkins et al., 1998; Tao and Poo, 2001).

In the central nervous system, NO can be produced by neural cells, endothelial cells, and glial cells. It is produced by three isoforms of NOS: (a) nNOS, which is constitutively expressed in a proportion of brain neurons, and is activated by calcium/ calmodulin, particularly after stimulation of *N*-methyl-

d-aspartate (NMDA)-type glutamate receptors; (b) eNOS, which is constitutively expressed in brain endothelial cells and some astrocytes, and is regulated by calcium/calmodulin and phosphorylation/ dephosphorylation; (c) iNOS, which is not normally expressed in the "healthy" brain, but is induced in glial and endothelial cells by pro-inflammatory cytokines and once expressed continuously, produces high levels of NO (Brown and Bal-Price, 2003). iNOS expression is one of the characteristic changes of the "activated" state of microglia and astrocytes induced by inflammation (Brown and Bal-Price, 2003).

Excitatory amino acids, glutamate in particular, are the main neurotransmitters, as it is estimated that over half of the brain's 100 billion neurons secretes glutamate (Tabuchi et al., 1996; Holden, 2003); however, at high extracellular levels, glutamate can kill neurons; a process known as excitotoxicity (Brown and Bal-Price, 2003). It has also been proposed that excitatory amino acid neurotoxicity contributes to the pathogenesis of various neurodegenerative diseases (Meldrum and Garthwaite, 1990). Glutamate-induced death of neurons can be mediated by: (a) activation of the NMDA and AMP A subtype of glutamate ionotropic receptors, resulting in Ca^{2+} and/Na^{+} overload of the neurons, (b) activation of AMP A receptors, resulting in Ca^{2+} and/or Na^{+} overload of neuron; or (c) glutamate inhibition of cysteine uptake, ending in the oxidative stress/death of neurons (Novelli et al., 1988; Dawson et al., 1991; Schinder et al., 1996). With NMDA receptors as a model, Lipton et al. demonstrated that the redox versatility of NO allows its conversion from a neuroprotective molecule to a neurotoxin; neurotoxicity is mediated by $ONOO^{-}$ but neuroprotection is achieved by down-regulation of NMDA receptor activity by *S*-nitrosation (addition of NO^{+}) of the receptor redox modulatory site (Ledo et al., 2004).

Other observations including the potentiation of NO synthesis by ascorbate and the down-regulation of NMDA receptors by NO^{-}, the one-electron reduced form of NO, underscore the notion that a change in the redox milieu, modulated in part by the balance between reactive species (such as superoxide anion and H_2O_2) and neuroprotective compounds (most notably,

ascorbate and glutathione), may determine the biological fate of NO (Ledo et al., 2004).

Reactive free radicals, including ROS and reactive nitrogen species (RNS), are implicated in central nervous system pathogenesis. These highly reactive radicals and oxidants may indiscriminately attack proteins, lipids and DNA, causing oxidative modifications and strand breakage. DNA strands will activate the constitutive nuclear enzyme PARP-1 which is implicated in physiological processes such as DNA repair, genomic stability and apoptosis. PARP-1 catalyses the addition of a long branched chain of poly (ADP-ribose) from its substrate NAD to a set of nuclear proteins. An excess of active PARP-1 is a crucial oxidative factor as it leads to NAD depletion, resulting in a loss of ATP used to synthesize new NAD. Depletion of intracellular NAD slows the glycolysis rate, electron transport and ATP formation leading to cell necrosis due to energy failure.

Glutamate neurotoxicity is mediated in part by NO production by nNOS (Dawson et al., 1991; Strijbos et al., 1996) and NO-induced mitochondrial inhibition or depolarization (Jenner et al., 1992; Bolanos et al., 1996; Almeida et al., 1998). There is a synergy between NO and high Ca^{2+} probably mediated by permeability transition. NMDA receptor-activation causes partial depolarisation, which opens Na+ channels evoking glutamate release, which, in turn, complete a vicious circle by activating the NMDA receptor, with the output of this circle continuing nNOS stimulation that causes neuronal death.

A distinction could be made between chronic and acute excitotoxicity, the former being mediated by stimulation of nNOS, while the latter is mediated by Ca^{2+} uptake into the mitochondria, resulting in massive mitochondrial ROS production (Urushitani et al., 2001).

NO, at respiration-inhibiting concentrations, causes acute glutamate release from synaptosomes and neurons (Meffert et al., 1994), which has been attributed either to inhibition of mitochondrial respiration, followed by reversal of glutamate uptake (McNaught and Brown, 1998; Trabace and Kendrick, 2000; Bal-Price and Brown, 2001), or to direct (Ca^{2+}-independent) activation of vesicular exocytosis. NO also elicits acute glutamate release from

astrocytes (Bal-Price et al., 2002). Astrocytes are known to possess a vesicular pool of glutamate that is rapidly exocytosed in response to agonists that raise intracellular Ca^{2+}. NO can induce glutamate release from astrocytes by Ca^{2+}-induced exocytosis of vesicular glutamate.

Activated astrocytes maintain considerably high levels of extracellular glutamate that are lowered by an iNOS inhibitor, without affecting glutamate uptake. This suggests that iNOS-synthesized NO causes continuous glutamate release (Bal-Price et al., 2002). NO from activated glia might kill neurons by three excitotoxic-related means: (1) NO-induced glutamate release from astrocytes, (2) NO-induced glutamate release from neurons, (3) potentiation of NMDA receptor activation via respiration inhibition-induced neuronal depolarization. In cultured neurons, oxidative phosphorylation or sodium pump inhibitors allow low levels of glutamate to become toxic (Novelli et al., 1988). This phenomenon is due to ATP depletion causing failure of the Na^{+} pump and resulting in plasma membrane depolarization, and release of Mg^{2+} block of the NMDA channel. However, ATP depletion-induced changes in Ca^{2+} transport may also contribute to this synergic effect (Novelli et al., 1988; Siesjo and Bengtsson, 1989). Inhibitors of the mitochondrial respiratory chain or Na+ pump cause rapid glutamate release from neurons and isolated nerve terminals (synaptosomes) apparently due to ATP depletion inducing ionic pump failure, prompting plasma membrane depolarization and resulting in rapid, transient exocytosis of vesicular glutamate, followed by a sustained reversal of the glutamate uptake carrier (Pocock and Nicholls, 1998; Nicholls and Budd, 2000; Bal-Price and Brown, 2001). The synergy between NO and glutamate in excitotoxicity has been demonstrated and could be explained by the following mechanisms: NMDA receptor-activation induces superoxide production which reacts with NO to cause neuronal death. Inhibition of mitochondrial respiration by NO depolarizes the plasma membrane and removes the Mg^{2+} block; glutamate and NO are synergistic in inducing apoptosis through activation of calcineurin and dephosphorylation of the pro-apoptotic Bcl-2 homologous protein Bad, which translocates to the mitochondria and causes cytochrome *c* release (White et al., 2000; Capano et al., 2002).

There are several ways in which NO can induce neuronal cell death: energy depletion-induced necrosis, oxidative apoptosis, and excitotoxicity.

The level of cellular ATP directs cell death pathways either to necrosis or apoptosis. DNA damage leads to PARP activation and NAD and ATP depletion because NAD^+ is required for glycolysis and because it is synthesized from ATP. Both NO-induced respiratory inhibition and mitochondrial permeability transition (MPT) can cause cellular ATP depletion if the cellular glycolytic capacity is a key determinant of cell survival or the mode of cell death in response to NO (Bal-Price and Brown, 2000; Almeida et al., 2001; Brown and Borutaite, 2002). $ONOO^-$ and *S*-nitrosothiols can directly inhibit or uncouple glycolysis at glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) in the condition of glutathione depletion, potentially causing necrosis (Vedia et al., 1992; Albina et al., 1999), even if GAPDH is not normally rate-limiting for glycolysis. NO-induced oxidation of GAPDH can result in its uncoupling from ATP production, potentiating ATP depletion (Albina et al., 1999). PARP-induced cell death could also be mediated by AIF released from the mitochondria, which translocates to the nucleus to induce DNA fragmentation. NO can elicit apoptosis in non-neuronal cells by a variety of means (Bolanos et al., 1994), including (a) MPT activation (probably by $ONOO^-$ or *S*-nitrosothiols); (b) activation of the endoplasmic reticulum stress response (possibly due to ryanodine receptor-mediated Ca^{2+} release); (c) activation of p53 (probably due to DNA damage); and (d) activation of the p38 nitrogen activated kinase MAPK pathway (possibly due to oxidants).

Apoptosis in neuronal cells may be secondary to excitotoxicity. Excitotoxicity may also cause necrosis if high sustained levels of glutamate or aspartate are involved, or when sensitizers such as glycine or respiratory inhibitors are present (Nicholls and Budd, 2000). Such necrosis can result from acute swelling from Na+ influx or delayed mitochondrial dysfunction due to Ca^{2+} influx. Whether a cell dies by apoptosis or necrosis depends on whether the ATP level is maintained by glycolysis (Leist et al., 1999; Bal-Price and Brown, 2000). ATP is required in the apoptotic process. ATP depletion itself causes necrosis as the Na^+ and Ca^{2+} pumps require energy for their function. NO inhibits ATP production and can induce apoptosis by itself.

In some cases, NO induced neuronal cell death involves p38 MAPK activation, followed by Bax translocation to the mitochondria, which causes the release of cytochrome *c* and other apoptogenic proteins (Ghatan et al., 2000).

NO can also protect cells and neurons. *S*-Nitrosating species, particularly *S*-nitrosothiols, can nitrosate and inhibit caspases in non-neuronal cells (Thippeswamy et al., 2001; Torok et al., 2002). In neurons, *S*-nitrosating species can inhibit exitotoxicity by *S*-nitrosating and inhibiting the NMDA receptor (Lipton and Stamler, 1994; Lipton, 1999). In astrocytes, cGMP (from NO-activated sGC) can protect against cell death by inhibiting MPT (via protein kinase G-mediated phosphorylation) (Takuma et al., 2001). Several factors can modulate the effect of NO on cells survival: NO concentration, time course of exposure to NO, type of NO derivative present, cell type, thiol status of the cell, glycolytic capacity, and NO and NO-independent protective mechanisms. In vitro, activated glia stimulated by cytokines can produce enough NO to kill neurons in culture. There is some controversy in vivo about whether inflammation alone is capable of inducing neuronal death (Allan and Rothwell, 2001; Morimoto et al., 2002) or if it has to synergize with excitotoxins to induce neuronal death. In several chronic cerebral pathologies, inflammatory neurodegeneration is one of the mechanisms of disease. Among these pathologies, worth mentioning are Alzheimer's disease, Parkinson's disease, motor neuron diseases, multiple sclerosis, brain ischemia and stroke, traumatic brain injury, epilepsy, aging, as well as viral and bacterial infections.

7. Nitroglycerine (NTG): mechanisms of action and tolerance

Since its discovery more than 150 years ago, the action of NTG on chest pain due to angina has been recognized, and NTG is actually still one of the most often used drugs in cardiology/cardiac surgery (Marsh and Marsh, 2000). It can be administered orally, by nasal or oral spray, through transdermal patches, and intravenously. It is commonly recognized that NTG can release three molecules of NO, and that NO is the active moiety of these molecules. The metabolic pathways leading to NO release are not completely established and are a matter of discussion between experts. One of the

problems of nitrate use is the tolerance that rapidly develops after treatment beginnings. Tolerance can be dissociated into three categories: de novo impairment of tissue responsiveness to nitrates and NO; pseudotolerance, summed attenuation of nitrates by the secretion of substances exerting biologically opposing actions (catecholamines, angiotensin, endothelin, etc.) and true nitrate tolerance; progressive desensitization of blood vessels and platelets to the effects of nitrates.

As it was accepted until recently that the mechanism of action of NTG was through the release of NO, the redox status of thiol groups has been implicated in nitrate tolerance. Thiol deficiency was thought to be the core mechanism behind the development of nitrate tolerance (Needleman and Johnson, 1973). Later studies have failed to demonstrate a beneficial effect of *N*-acetylcysteine supplementation in nitrate tolerance (Parker et al., 1987) or found decreases in plasma or tissue thiols in nitrate-tolerant rats (Boesgaard et al., 1994). Vasoconstrictors release can not only oppose the vasodilatory effect of NTG, but can also induce ROS production which interferes with NTG effect and the endogenous NO pathway. Another mechanism of tolerance could be enhancement of phosphodiesterase activity which degrades cGMP (Kim et al., 2001a,b). More recently, it was shown that nitrate tolerance is accompanied by superoxide anion production and that O_2^- production inhibition or high concentrations of antioxidants, such as Vitamin E and Vitamin C, can protect from nitrate tolerance.

Despite the fact that NTG can stimulate eNOS, randomized controlled clinical trials have demonstrated that continuous glycerol trinitrate (GTN) worsens endothelium dependent relaxation in ischemic and healthy volunteers (Caramori et al., 1998; Gori et al., 2001).

B-nicotinamide adenine dinucleotide phosphate (ADP)-dependent membrane-associated oxidase (NADPH oxidase) may be the main source of O_2^- production responsible for nitrate tolerance. Angiotensin II (Ang II) may represent a link between nitrate tolerance and O_2^- formation, as it is known that Ang II stimulates NADH/NADPH oxidase in smooth muscle cells (Griendling et al., 1994), making ACE inhibitors and A II blockers antioxidants themselves.

GTN is associated with modulation of the eNOS response. The production of an endogenous inhibitor of NOS, asymmetric dimethylarginine, can be enhanced and not only compete with arginine transport through the Y_+ cell transporter, but also inhibit NO synthesis and increase O_2^- production. L-Arginine as well as folic acid supplementation (which is involved in tetrabiopterine (BH4) regeneration (Stroes et al., 2000)) can prevent nitrate tolerance. BH4 is involved as a co-factor that enables the transfer of electrons to L-arginine, and thus forms NO (Stroes et al., 2000). Long-term NTG therapy is known to activate PKC which stimulatesO_2^- formation; furthermore, eNOS is a phosphorylation target for PKC. Thus, uncoupling of eNOS necessary for O_2^- production could be dependent on PKC phosphorylation.

Finally, more recent data suggests that at physiological concentrations NO is not the only intermediate or mechanism of NTG-induced vasodilatation. Indeed, NTG-induced CGRP release from capsaicin-sensitive nerves could explain part of the vasodilatation and tolerance mechanism (Zhou et al., 2001).

At low nanomolar GTN concentrations, the sGC/cGMP/cGMP-dependant protein kinase (cGK-I) signaling pathway is activated without any detectable NO release (Kleschyov et al., 2003). In clinical settings, the GTN/NO pathway is probably limited to cases where GTN is infused at high doses (micromolar concentrations) to induce local vasodilatation (Marczin et al., 1997). There is an apparent link between GTN biotransformation into 1,2-GDN/nitrite and GTN vasoactivity (Bennett et al., 1994). Even if a mechanistic explanation is still forthcoming, GTN can be metabolized by several enzymes. Mitochondrial aldehyde reductase (mtALDH) is able to bioconvert GTN (Chen et al., 2002; Fayers et al., 2003) to a vasodilator as its blockade inhibits vasodilatation induced by nanomolar concentrations of GTN. It has also been shown that angiotensin can evoke GTN tolerance by a mechanism downstream of cGMP. In vascular systems, cGMP activates cGK-I, which is composed of two isoforms (cGK-Iα and cGK-Iβ); the α and β splice variants of the same gene are functionally different (Knot, 2003). The β isoform requires greater cGMP concentration than the α isoform for comparable activation. Angiotensin treatment in rat elicits alternative splicing

with a large increase in mRNA for the β isoform. This happens early after infusion, before any increase in blood pressure. CGK-I phosphorylates and activates the large conductance, Ca^{2+}-activated K^{+} channel (Robertson et al., 1993; Archer et al., 1994) and the sarcoplasmic reticulum Ca^{2+} pump via phosphorylation of phospholambam to reduce intracellular Ca^{2+}.

8. Toxicity and toxicological data

NO is delivered in gas cylinders (approximately 6000–8000 l with a factory concentration of 800–1000 ppm nitrogen). The Occupational and Safety Health Administration (OSHA) (Electronic reference 1, 2004) classified NO as a class A dangerous gas (might be inhaled without any warning signs of exposure).

The published lethal dose (LD50) for mice is 797 ppm for 1-h exposure. The lowest observed lethal dose is 320 ppm. A dose of 315 ppm is considered immediately dangerous to life and health (Electronic reference 2, 2004).

Inhaled NO (INO) might have a mutagenic effect: at 30 ppm for micro-organisms (*Salmonella Typhimurium*), at 27 ppm for 3 h for mammalian somatic cells (rat; continuous inhalation). At recommended therapeutic doses (10–20 ppm), there is no known mutagenic activity.

NO and its derivatives have a direct toxic effect on blood, lungs, nervous system, pancreas and skin.

Skin: Gaseous NO acts as a mild irritant. A more potent irritant is the conversion product NO2, which might produce direct chemical burns. Gaseous NO can be absorbed through the skin, causing symptoms as in inhalation.

Eyes: Severe eye irritation can result from gaseous NO at high (but therapeutic) doses. A direct corneal reaction was observed at a dose of 50 ppm. At 90 ppm, cornea opacity was seen in rabbits after experimental exposure for over 8 h, mainly induced by NO2. *Cardiovascular system*: Gaseous NO, administered by inhalation, has specific, local (pulmonary), short-term vasodilatation activity (Barnes, 1993). Although the general NO

vasodilatory action has been known since 1900, when NTG was first used in the treatment of pectoral angina and nitroprussiate was started for high blood pressure emergencies, only 91 years later, in 1991, was it observed that gaseous NO reduces pulmonary artery pressure without influencing systemic pressure (Taylor et al., 2004). In the same period of time, it was demonstrated that endogenously produced NO is able to relax corpora cavernosa muscles and induce penile erection (Burnett et al., 1992).

Brain and nervous system: INO has a controversial impact on the central nervous system. Endogenously produced NO may act as a neurotransmitter or tie neuronal activity with local blood flow, facilitating classical neurotransmitter synaptic release. Low dose NO protects neurons from degeneration, but high NO concentrations are toxic, inducing neuronal destruction (Castillo, 1999).

Pancreas: Data regarding the toxic effect of INO on pancreatic beta-cells are indirect and controversial. *N*-nitro-l-arginine methyl esther (l-NAME), an inhibitor of endothelial and macrophagic NOS partially suppresses the development of diabetes mellitus in a low-dose streptozotocin-induced diabetes model. At low concentrations, NO acts as an insulin secretion stimulator (McDaniel et al., 1996).

A very interesting clinical application of INO is in stabilizing the blood cell components, after cardiopulmonary bypass or in the sickle cell anemia (Weiner et al., 2003).

9. Symptoms of intoxication in correlation with concentration range (Electronic reference 1, 2004; Electronic reference 2, 2004)

Even if the individual response is not dose dependent, INO's acute toxic action is mainly related to its air concentration. Very few data are available, most of them being extrapolated from other gases that act on blood.

• An 8000-ppm concentration (0.8%) will generate sudden unconsciousness, followed by death in 1 min from acute asphyxia (due to INO methemoglobin (MetHb) production and NO2-induced pulmonary edema).

• At 3000 ppm (0.3%), dizziness or drowsiness is observed in minutes, quickly followed by unconsciousness and death in 5 min.
• A 1600-ppm (0.16%) concentration will generate neuromuscular dysfunction, loss of coordination, tachypnea, tachycardia, and drowsiness in less than 5 min, with unconsciousness in 10 min, and death in 15 min.
• An 800-ppm concentration will mainly induce hematological changes (MetHb production) that are lethal at 70–90% MetHb after 1-h exposure.
• 797 ppm (1068 mg/m3): LC50/1 h inhalation-rat (lethal to 50% of rats in 1 h inhalation).
• 632 ppm: NO2 production will be predominant (as a visible brown cloud) which irritates the eyes and mucous membranes.
• 400 ppm: the first symptoms, similar to those at 1600 ppm, appear within 2 h when MetHb concentration reaches 30–40%. Unconsciousness may occur within 3 h.
• 320 ppm: LCLO/1 h inhalation in mice (the lowest lethal concentration observed).
• 315 ppm: LC50/15 min inhalation in rabbits.
• 100 ppm: immediately dangerous to life or health (IDLH).
• 25 ppm: may be symptom-free for 8 h until 5–48 h after exposure, when air hunger, tightness or burning in the chest, coughing, choking and sleeplessness may develop.
• 1–20 ppm: therapeutic doses. Local, selective pulmonary vasorelaxant. Anti-inflammatory action (anti-apoptotic).
• 0.3–0.9 ppm: pungent odor.

A delayed immune pulmonary response has been described after an acute exposure. After 10 days to 6 weeks of the primary exposure, an immune pulmonary fibrosis reaction (bronchiolitis fibrosa obliterans) may be observed. Classical symptoms include cough, cyanosis, fever, and hypoxemia. Reduced pulmonary function, and a fine, scattered pulmonary Rx pattern are also observed (Harrison, 2000).

Chronic exposure to INO is not described in the medical literature. The pathology will probably include chronic bronchitis, asthmatic attacks, pulmonary function changes and rarely fibrosis or emphysema. Rats exposed

to 4 ppm NO–NO_2 daily for 6 months did not develop any pathology, but those exposed from 9.8 to 25 ppm for up to 18 months showed variable forms of emphysema.

The rapid lethal effect of NO_2 is caused by pulmonary edema. Short-term exposure to more than 150 ppm of NO_2 is usually fatal (Hamilton and Hardy, 1983). In the usual NO doses, such as 0.5–4%, methemoglobinemia is not usually a problem, but this exposure should be monitored closely. The OSHA (Electronic reference 1, 2004) safety limitation for NO is 25 ppm, and for NO_2, 5 ppm, but parenchymal lung injury and airway reactivity have occurred with inhalation of as little as 2 ppm of both gases.

10. Adverse effects of INO

The adverse effects of INO are mainly related to platelet inhibition, left ventricular failure and rebound pulmonary hypertension.

INO studies in patients with ARDS showed that platelet aggregation and agglutination were significantly modified in a non-dose-dependent manner, with maximal platelet inhibition occurring at 3 ppm (not associated with any changes in bleeding time).

High therapeutic doses (40–80 ppm) of INO administered to patients with left ventricular dysfunction decrease pulmonary resistance and elevate pulmonary capillary wedge pressure. An acute increase in left ventricular filling pressure could produce pulmonary edema in patients with pre-existing left ventricular dysfunction.

Withdrawal of INO could present a problem in some patients (decreased arterial oxygenation and pulmonary artery pressure elevation). A transient (4–8 h) deterioration of oxygenation occurs with withdrawal of INO, and potentially life-threatening pulmonary hypertension develops in certain patients.

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18. ÉVIDENCE D'UNE PHARMACOCINÉTIQUE NON-LINÉAIRE DE L'OXYTOCINE CHEZ LE RAT ANESTHÉSIÉ

L'article qui suit (*Journal of Pharmacy and Pharmaceutical Sciences* 2008; 11: 12-24) présente la pharmacocinétique de l'OT dans un modèle de rat Sprague-Dawley anesthésié. Ces travaux ont été effectués par notre équipe au stade préliminaire du développement d'un protocole de traitement d'OT. Ces études nous ont permis de mieux caractériser les effets pharmacodynamiques de l'OT lorsqu'administré à des doses thérapeutiques. Le Docteur Simon Authier a contribué de façon significative au développement des modèles animaux qui ont été utilisés dans ce projet ainsi qu'à la réalisation des expérimentations animales.

EVIDENCE FOR NON-LINEAR PHARMACOKINETICS OF OXYTOCIN IN ANESTHETIZED RAT

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ABSTRACT

Purpose: Because oxytocin (OT) is potentially useful in cardiovascular therapy but has hormonal roles on the cardiovascular and renal systems, we characterized its pharmacokinetic (PK) properties as a function of dose. **Methods**: A single intravenous bolus of OT was given at doses of 200, 300, 500, 1000, 3000, 5000 and 10000 ng/kg to anesthetized male rats ($n \geq 4$ per dose). Blood samples (6) were taken over 72 min to 150 min, depending on dose. The individual time-courses of plasma OT concentrations were analyzed with a one- or an open two-compartment PK model. Kruskal-Wallis tests (alpha=0.05) were used to compare the PK parameters among groups. **Results**: At doses up to 500 ng/kg, OT showed a higher median systemic clearance (CL_T = 0.0624 L/(min·kg); 0.0622 ± 0.0228 as mean ± SD value), a higher median central compartment volume of distribution (V_C = 0.7906 L/kg; 0.6961 ± 0.1754), and a lower median elimination half life ($t½(\lambda z)$ 7.94 min; 9.08 ± 4.3) with respect to the higher doses (CL_T = 0.0266 L/(min·kg); 0.0284 ± 0.0098, V_C = 0.2213 L/kg; 0.2227 ± 0.1142, and $t½(\lambda z)$ 21.09 min; 28.36 ±

21.8), all differences being significant ($p \leq 0.0008$). Minimal differences were found for the estimates of these PK parameters among the 4 higher OT doses. **Conclusion**: The PK properties and persistence of exogenous OT are not proportional to dose, therefore this must be accounted for in dosing regimen design for potential cardiovascular therapy.

Keywords: Pharmacokinetics, non-linear; Peptide, endogenous, oxytocin; rats.
Short running title: Non-linear PK of oxytocin.

INTRODUCTION

Oxytocin (OT) is widely used in gynecology and obstetrics to induce labor in women (1) and mare, to combat uterine inertia during parturition in all species, and to stimulate milk letdown in animals presenting agalactia (2). This hormone is present at equal concentrations in the plasma and neurohypophysis of both men and women (3-4). The absence of gender difference suggests other than the traditionally accepted roles for this nonapeptide. Recently, OT has demonstrated the capacity to differentiate embryonic murine stem cells into beating colonies of cardiomyocytes (5), to induce cardiogenic differentiation of adult stem cells (6-7), and to induce mitotic activity in the vascular endothelium (8) as well as cardiomyogenesis (9- 10).

Despite its widespread therapeutic use in human beings and animals, the pharmacokinetics (PK) of OT has been examined only in a limited number of studies (4,11-15). The time-course of its plasma concentrations in humans receiving a single i.v. bolus usually is described with an open two-compartment model. The PK data reported up to now for animals resembles that in humans. In goats receiving a single i.v. injection of OT (2000 ng/kg), the plasma concentrations of this hormone also followed a biexponential decay, with an initial (distribution phase) half-life of 1.94 ± 0.21 min, a terminal (elimination phase) half-life of 22.3 ± 0.3 min, and a total body clearance of 0.85 ± 0.02 L/(h·kg). However, the apparent volume of distribution (V_{area}) of OT in goats is 0.46 ± 0.02 L/kg (12), a three times higher value than in men (15). A study on anesthetized rats receiving a constant infusion of 6040 ng/(min·kg) reported a systemic clearance of 1.46 ± 0.22 L/(h·kg) and an elimination half-life of 20.70 ± 1.55 min**,** as estimated from steady-state plasma concentrations (14).

From a pharmacodynamic perspective, important effects have been characterized on the cardiovascular and renal functions. Indeed, OT receptors are found in all chambers of the heart and in vascular endothelial cells (8,16), and OT participates in the regulation of cardiovascular functions and volume homeostasis by acting on cardiac receptors to decrease cardiac rate and force of contraction (17-20). These results in isolated rat heart could not be

reproduced exactly either in live rats (21) or in other species such as dog (22-24) and human beings (25). Historically, OT showed a natriuretic effect in rat (26-27) but, there is apparent discrepancy in the literature regarding the saliuretic and diuretic properties of OT: some publications report a clear positive effect in rats (28-31), dogs (32-33), and human beings (34), but others report an absent or negative effect in rats (31,35-36), dogs (37-38) and humans (25). Such differences could be associated with the experimental design, in particular with choices regarding the dose, and administration route and method, as well as the degree of hydration/ volemia, gender or species. We hypothesized that at some OT dose level, the magnitude of its associated negative inotropic and chronotropic effects combined with its effects on renal water and electrolyte balance will result in a decrease of its glomerular filtration rate, hence inducing nonlinearity of its urinary clearance and PK response.

Considering the therapeutic potential of OT for cardiac regeneration, precise characterization of the PK profile for each species targeted as an evaluation model is required. PK characterization is needed to optimize dosage regimen, as well as for recognition of species-specific pharmacodynamic effects. If the PK of OT is truly dose-dependent, prediction of pharmacodynamic effects in a therapeutic dosage regimen is critical, especially in the context of cardiac failure treatment where adverse pharmacodynamic effects could affect clinical outcome. The objective of the study was to document the dose-linearity of the PK response to i.v.-administered OT bolus, by examining the relationship between dose and the area under the plasma concentration curve over time (*AUC*), as well as the stationarity of its compartmental PK parameters with respect to dose.

METHODS

Chemicals

Synthetic OT was purchased from Peninsula Laboratories (San Carlos, CA, USA). A sterile solution of OT in 0.9% NaCl saline was prepared and sterilized by filtration through a 0.45 μm polyethersulfone syringe filter (Whatman Inc., Clifton, NJ, USA) before injection. The concentration of OT was adjusted in order to obtain a constant final dose volume of 1 mL. The inhibitors of plasma oxytocinase, pepstatin A and phenylmethanesulfonyl fluoride were purchased from Sigma-Aldrich Chemical (Oakville, ON, Canada). Specific antibodies against OT nonapeptide were a generous gift from Mariana Morris (Wright State University, Dayton, OH, USA). Chemicals for radioimmunoassay (RIA) analyses were at least of RIA grade. All other reagents and solvents were obtained from commercial source.

Animals

Experiments were performed according to guidelines from the Canadian Council on Animal Care with the approval of the institutional animal care and use committees before initiation of the study. Animal experiments were performed at LAB Research Inc. (Laval, QC, Canada), an AAALAC accredited facility. Adult Sprague-Dawley male rats (400-700 g; 13-21 weeks old) were obtained from Charles River Laboratories (St-Constant, QC, Canada). To avoid any gender effect, we selected only males. Animals were housed in groups of 2 or 3 in a controlled environment (temperature 21 ± 3°C, humidity 30-70%, 12 hr light, 12 hr dark, 10-15 air changes per hr). Laboratory rat chow (Rodent Diet #2018C, Certified 18% crude protein, Teklad, Madison, WI, USA) and tap water were given *ad libitum*.

Animal Preparation

Animals were anesthetized with isoflurane/oxygen mixture with the vaporizer set at 2%. The animals were kept on heating pads (37ºC). A butterfly needle (25 G) was placed in a tail vein for OT administration. Continuous infusion of Ringer's solution using a syringe pump (AS50, One Baxter Parkway,

Deerfield, IL, USA) set at 10 mL/(hr·kg) rate was provided to compensate for fluid loss during the experiment. The femoral vein was catheterized with an MRE-040 (Braintree Scientific Inc, Braintree, MA, USA) for blood sampling.

Experimental Procedures

Treatment was assigned randomly to 7 groups of rats ($n \geq 4$ each) according to a parallel design. In a first pilot study on isoflurane-anesthetized rats ($n = 8$), several doses of OT were tested for their intensity of cardiovascular effects. The highest OT dose without cardiovascular effect was 300 ng/kg. Therefore, we selected doses of 200, 300, 500, 1000, 3000, 5000, 10000 ng/kg given as an i.v. bolus. A second pilot study ($n = 4$) was performed to estimate the baseline plasma OT concentration in rats dosed with a volume of saline equal to that to be used for the administration of OT, and to optimize the sampling timepoints (39) to be used in the main study. Blood samples (1 mL) were taken at the times listed in Table 1, and transferred to chilled EDTA vacuum tubes (Vacutainer, BD, Franklin Lakes, NJ, USA), fortified with 50 nmol pepstatin A, and 100 nmol phenylmethanesulfonyl fluoride. Upon blood collection, tubes were centrifuged for 10 minutes at 1500×*g* and 4ºC. Plasma was immediately harvested and stored at -70°C pending analysis.

Table 1. Dosing groups, number of rats and timing of post-dosing blood samples used in the study.

Dose (ng/kg)	Number of rats	Post-dosing sampling number and time (min)					
		1	**2**	**3**	**4**	**5**	**6**
200	4	2	6	12	24	-	-
300	5	2	4	6	36	60	-
500	4	2	6	12	24		-
1000	4	2	4	6	36	60	-
3000	4	2	4	6	36	60	-
5000	8	4	12	30	90	120	150
10000	4	4	12	30	80	110	140

Analytical Techniques

Plasma OT concentration was determined with a RIA technique developed in our laboratory, using specific antibodies against OT nonapeptide. Briefly, synthetic OT was labeled with ^{125}I-Na using the lactoperoxidase method (16). The iodinated tracer was purified by high performance liquid chromatography (LC-600, Shimadzu, Rochester, NY, USA) on a C_{18} Bondpack column (uBondpack 4.6×250mm, WAT052860, San Jose, CA, USA). The iodinated OT was eluted with 35% acetonitrile in 0.1% trifluoroacetic acid. The RIA was performed in RIA buffer (50 mM $NaPO_4$, pH 7.6; 0.1% Bovine serum albumine; 0.01% sodium azide; 0.01% sodium chloride). All the tubes containing 200 µL of sample or standard (0-200 pg·mL^{-1}) were supplemented with 200 µL of antibody (1:150 000) solution, and 100 µL of ^{125}I-OT at a concentration providing 3 000 counts per minute. Following a 48h-long incubation at 4°C, the antibody-bond radioactivity was separated from free radioactivity by supplementing each tube with 1 mL of dextran-coated charcoal suspension and centrifuging for 25 min at 1500×*g* at 4ºC. The radioactivity in the supernatant was then measured with a gamma counter (E5005 Cobra II Auto Gamma, Perkin-Elmer Packard, Montreal, QC, Canada). Log-transformed data of the standard curve showed linearity within the range of 0.25-200 ng/L, sensitivity of 0.1 ng/L, limit of quantification of

0.1 ng/L, and inter- and intra-assay coefficients of variation of 17.7% and 6.8%, respectively.

Pharmacokinetic analysis

Data from the 2nd pilot study was analysed with WinNonlin, version 1.5 (Pharsight corp., Mountain View, CA, USA) to find the optimal sampling times for the main study by graphic inspection of the partial derivatives of the transfer rate micro-constants k_{10}, k_{12} and k_{21} over time (39). The resulting timepoints allowed a minimum of 4 measurable OT plasma concentrations per animal and tested dose. The mean baseline plasma OT concentration measured with the saline control rats was subtracted from the plasma OT concentrations obtained in the main experiment. Then, the individual sets of OT plasma concentrations were examined first with statistical moment PK analysis (40). The systemic clearance (CL_T), apparent volume of distribution (V_C) and steady-state volume of distribution (V_{ss}) were respectively estimated as:

$$CL_T = \text{Dose}/AUC \quad (1)$$

Where AUC is the area under the curve of plasma OT concentration as a function of time.

$$V_C = \text{Dose}/C_0 \quad (2)$$

Where C_0 is the initial plasma concentration of OT, as estimated from back-extrapolation of the time-concentration curve after the i.v. bolus dosing.

$$V_{ss} = CL_T/MRT = Dose \cdot AUMC/AUC^2 \quad (3)$$

Where MRT is the mean residence time of OT in the PK system (*i.e.*, the average time the administered OT molecules reside in the body before being eliminated), and $AUMC$ is the area under the momentum curve of the product of plasma OT concentration and time, as a function of time (*i.e.*, the first statistical moment).

These statistical moment estimates of PK parameters were used to derive the initial parameter values for a compartmental PK analysis with the ADAPT II software (40). To this end, a set of two ordinary differential equations corresponding to an open two-compartment mammilary model with drug output from the central compartment and first-order drug transfer rates was devised (41). These equations were parameterized with V_C and the transfer rate

micro-constants k_{10}, k_{12}, and k_{21} (see below), and solved with maximum likelihood estimation or generalized least square estimation. Goodness of fit to the data was assessed with the Akaike criterion and graphical examination of the residuals (39). After obtaining the best possible fit, systemic clearance (CL_T) was calculated as:

$$CL_T = V_C \cdot k_{10} \quad (4)$$

Where k_{10} is the elimination rate micro-constant from the central compartment. Distribution clearance (CL_D) was:

$$CL_D = V_C \cdot k_{12} \quad (5)$$

Where k_{12} is the transfer rate micro-constant from the central compartment to the peripheral compartment. The apparent volume of the peripheral compartment V_P and V_{SS} were calculated as:

$$V_P = V_C \cdot k_{12}/k_{21} \quad (6)$$

$$V_{SS} = V_C + V_P = V_C \cdot (1 + k_{12}/k_{21}) \quad (7)$$

Where k_{21} is the peripheral-to-central compartment transfer rate micro-constant.

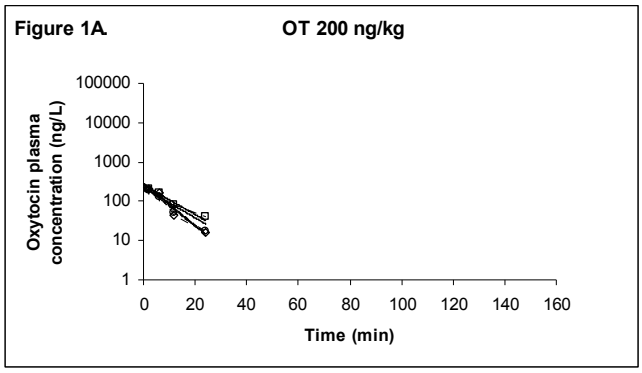
Statistical analysis

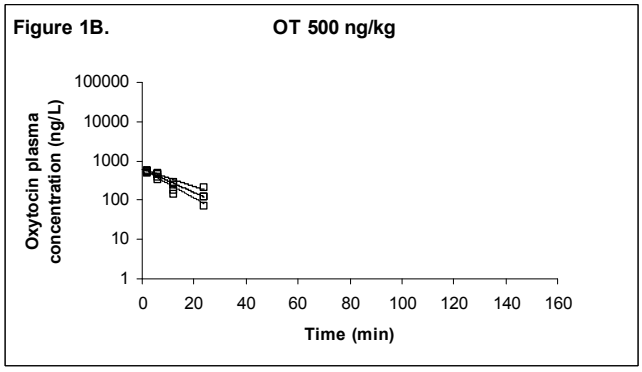
The homogeneity of animal weights across treatment groups and the differences between the estimates of CL_T, CL_D, V_C, V_{SS}, and terminal half life ($t½(\lambda_z)$) were checked with a Kruskal-Wallis rank test analysis at a 0.05 alpha level, because this non-parametric test is deemed more powerful in cases a small sample (NCSS, Kaysville, UT, USA). In case of an overall significant difference in a given PK parameter, the Dunn's *post hoc* test was used to determine which dose groups were different from the others. The relationship between *AUC* and dose was examined with the use of a 3rd order polynomial linear regression model with ordered inclusion of the linear, quadratic, and cubic terms (42), where the statistical significance of the intercept was used to detect departure from dose proportionality, and the statistical significance of the quadratic and cubic terms were used to detect departure from dose linearity (43). Polynomial terms with $p<0.10$ were considered significant.

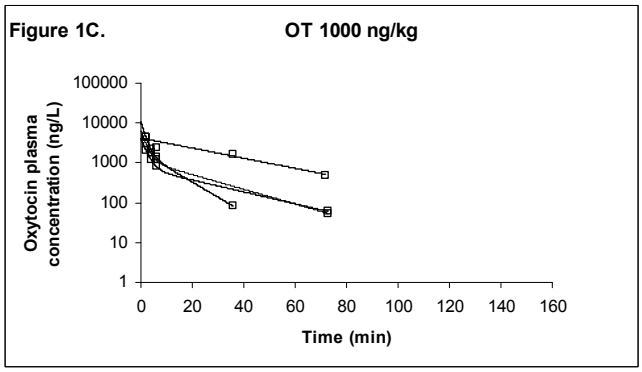
RESULTS

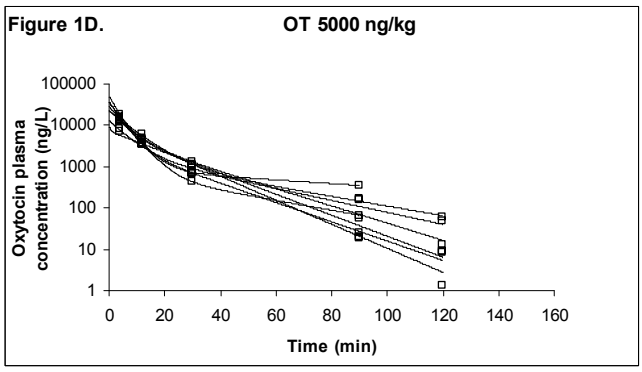
No significant difference in body weight was found for the dose groups. The baseline plasma OT concentration in the saline control group was 22.92 ± 10.89 ng/L (mean ± SD). Figure 1 shows the individual time-courses of plasma OT concentration in anesthetized rats receiving a single i.v. bolus of 200, 500, 1000, 5000 or 100000 ng/kg OT. The decay in plasma OT concentration was clearly monophasic (*i.e.*, a single slope) for the 200 and 500 ng/kg dose, but was biphasic for all doses greater or equal to 1000 ng/kg OT. The time-concentration curves in rats dosed with 300 ng/kg OT were as for the 200 and 500 ng/kg dose groups, therefore the open two-compartment PK model was reduced to an one-compartment model for their respective analysis. The average coefficients of variation (CV%) of their estimated V and k_{10} respectively were 9.1% and 7.5%, with 95% confidence intervals (CI) of [5.8%, 12.4%] and [4.3%, 10.7%]. In contrast, the decay in the time-concentration curves of the 1000, 3000 and 10000 ng/kg dose groups were biphasic as that of the 5000 ng/kg dose, which enables their analysis with the original two-compartment PK model. The average CV% of their estimated V_c, k_{10}, k_{12}, and k_{21} respectively were 13.1%, 12.6% 20.0% and 25.7%, with 95% CI of [3.0%, 23.3%], [3.4%, 21.7%], [7.3%, 32.7%], and [5.4%, 46.0%], respectively. Two of the 8 rats in the 5000 ng/kg dose group behaved like the ones of the 10000 ng/kg OT group, which terminal slope (λ_z) was not as steep as those of all the other rats that received a dose of 1000, 3000 or 5000 ng/kg of OT. A bimodal trend occurs from this dosage.

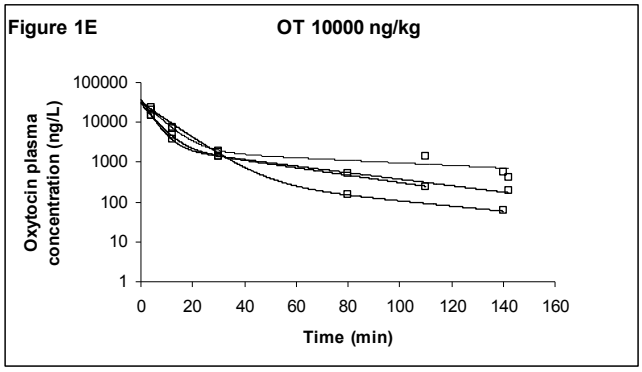
Figure 1. Individual time-courses of dose-normalized, baseline-corrected oxytocin plasma concentrations in rats receiving a single i.v. bolus at a dose of 200, 500, 1000, 5000, and 10000 ng/kg.











The estimates of $t½(\lambda_z)$ were significantly different across treatment groups (Kruskal-Wallis test; $p = 0.0008$). The Dunn's *post-hoc* test allowed distinguishing the 200, 300 and 500 ng/kg OT groups with significantly lower $t½(\lambda_z)$ values than the ones of the 1000, 3000 and 5000 ng/kg OT groups (Table 2). With respect to the $t½(\lambda_z)$ in rats dosed 10000 ng/kg OT, their values were significantly higher than those for all other treatment groups. In addition, the estimated $t½(\lambda_1)$ of the two-compartment model differed from the $t½(\lambda_z)$ estimates of the one-compartment model, a difference that was significantly affected by dose (Kruskal-Wallis test; $p = 0.0004$). The Dunn's *post-hoc* test revealed significant differences between the 200 – 500 ng/kg dose groups on one side, and the 1000 – 5000 ng/kg dose groups on the other. The $t½(\lambda_1)$ estimates of rats dosed with 10000 ng/kg OT were not significantly different from either group, but differences with respect to the 300 and 500 ng/kg dose groups were closest to significance threshold (*i.e.*, *p*-value between 0.10 and 0.05).

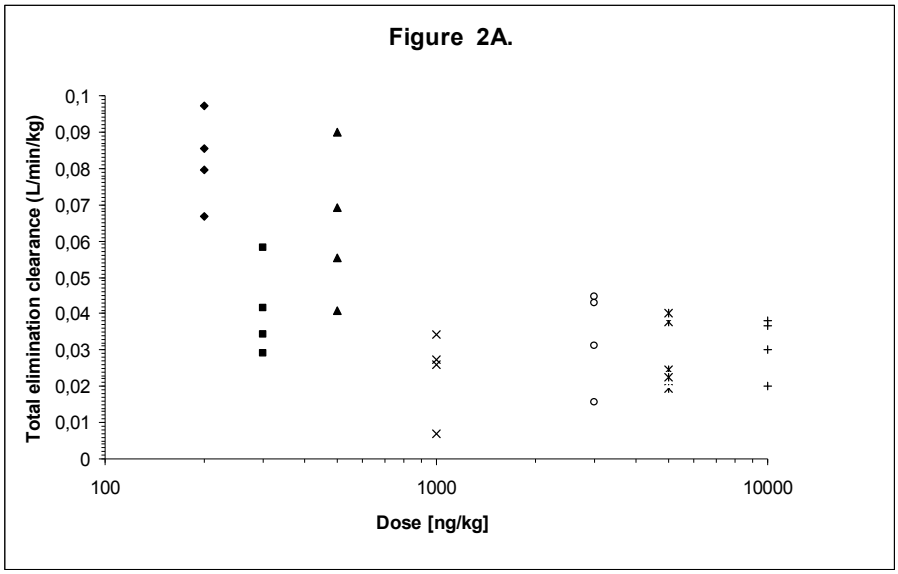
Table 2. Median estimates of selected compartmental pharmacokinetic parameters and variables of oxytocin after bolus intravenous administration in rats, as a function of dose.

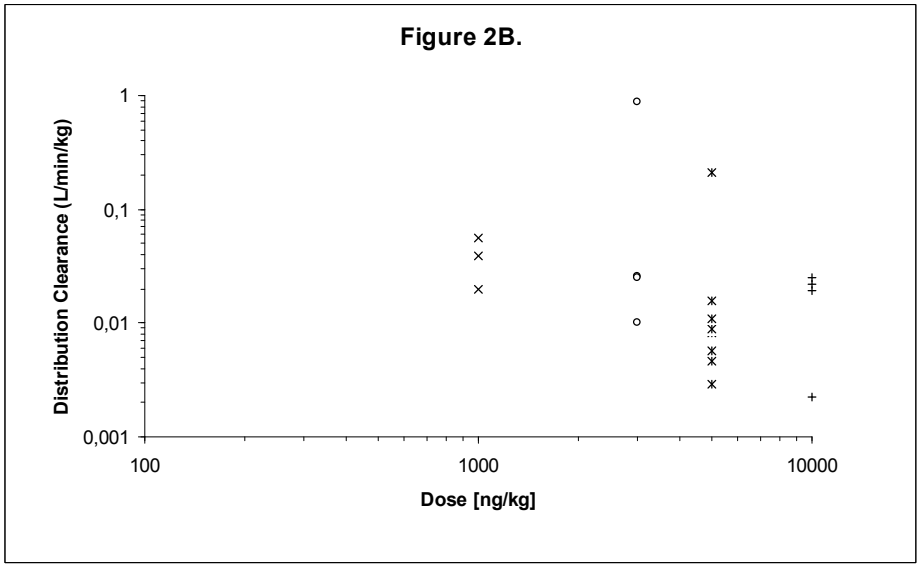
Parameters (units)	Dose (ng/kg)						
	200	300	500	1000	3000	5000	10000
Number of compartments	1	1	1	2*	2	2	2
CL_T (L/(min·kg))	0.0825[a]	0.0417[a]	0.0624[a]	0.0266[b]	0.0370[a]	0.0245[b]	0.0333[b]
CL_D (L/(min·kg))	N/D	N/D	N/D	0.03860[a]	0.02561[a]	0.00858[b]	0.02077[a]
V_C (L/kg)	0.8091[a]	0.4878[a]	0.8076[a]	0.1346[b]	0.0989[b]	0.1384[b]	0.2748[b]
V_{SS} (L/kg)	0.8091[a]	0.4878[a]	0.8123[a]	0.3459[b]	0.4515[a]	0.3455[b]	0.9760[a]
AUC (h·ng/L)	2219[a]	8709[b]	6742[b]	36529[c]	80087[d]	200792[e]	296439[e]
$t½(\lambda_1)$ (min)	N/D	N/D	N/D	1.34[y]	1.84[y]	3.51[y]	4.06[x, y]
$t½(\lambda_z)$ (min)	6.92[a, x]	9.38[a, x]	9.27[a, x]	18.22[b]	20.14[b]	17.63[b]	42.01[c]

Legend: [a, b, c] Results of dose groups with different superscripts statistically differ ($p < 0.05$); [x, y] Results of dose groups with different superscripts significantly differ ($p < 0.05$); N/D not determined; * One subject was best fit with one-compartment PK model.

The estimates of CL_T (Figure 2A) were significantly different among treatment groups ($p = 0.0008$). The *post hoc* test revealed that the lower dose groups (from 200 to 500 ng/kg OT) had significantly higher CL_T estimates than the higher dose groups: 1000 ng/kg ($p = 0.002$), 5000 ng/kg ($p = 0.0003$) and 10000 ng/kg ($p = 0.03$). Marked variations in CL_D were also observed across the range of doses studied: those of the 200 to 500 ng/kg groups were immeasurable, but the estimates for the 1000 and 5000 ng/kg OT were significantly different ($p = 0.04$) (Figure 2B).

Figure 2. Elimination (A) and distribution (B) clearances of oxytocin in rats individually dosed with a single i.v. bolus, as a function of the administered dose.

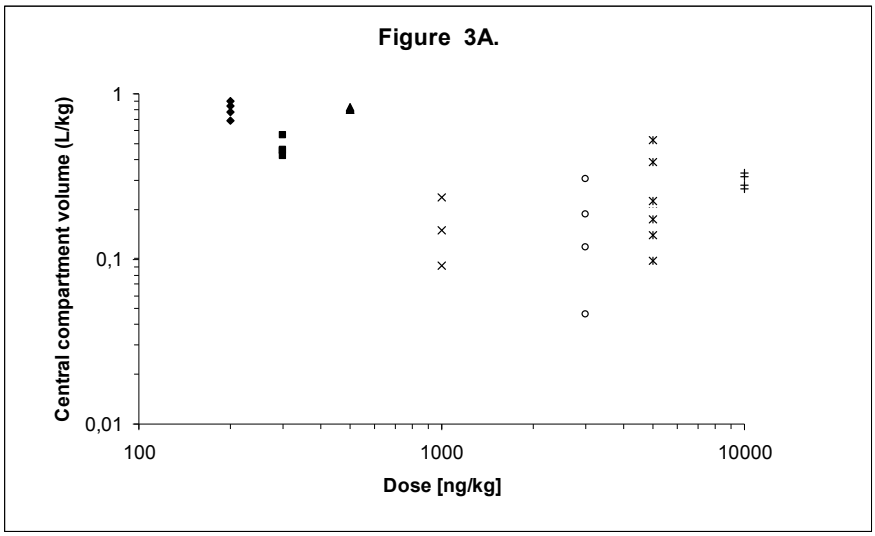


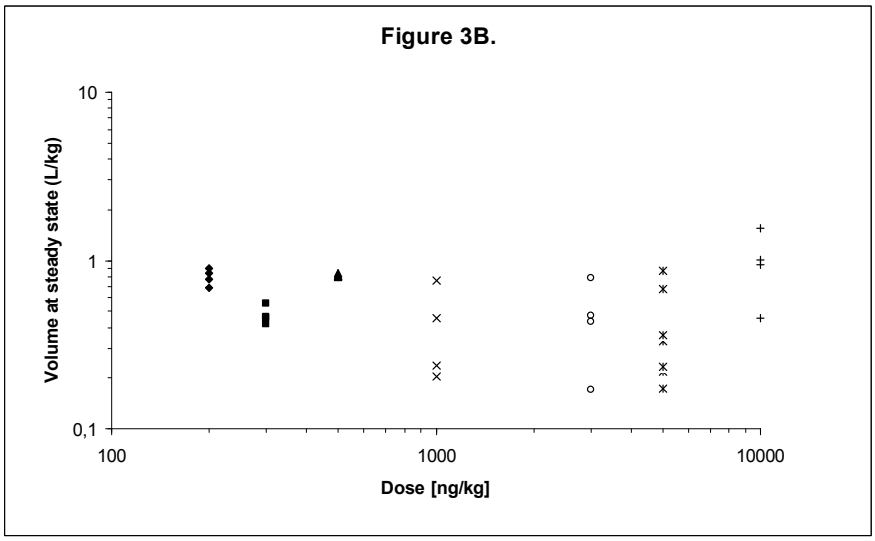


The estimates of V_C (Figure 3A) were significantly different across OT dose groups ($p < 0.0001$). This parameter was significantly higher in the rats dosed 200 to 500 ng/kg OT, as compared to the higher doses. Differences in V_{SS} were not significant for the doses of 200 to 500 ng/kg OT as compared to

the 3000 and 10000 ng/kg (p = 0.15; Figure 3B), but were significant between the 3 lower doses and the 1000 ng/kg (p = 0.04) and 5000 ng/kg (p = 0.02) OT groups.

Figure 3. Apparent volumes of the central compartment (A) and at steady state (B) for oxytocin in rats individually dosed with a single i.v. bolus, as a function of the administered dose.





The estimates of *AUC* (Table 2) were significantly different among treatment groups, as shown with the polynomial regression analysis (p <

0.0001). Significant regression coefficients were recorded for the linear ($p < 0.0001$) and quadratic ($p = 0.03$) terms of dose. It was noteworthy that the quadratic dose term had a negative sign, indicating that the highest OT doses yield AUC values lower than expected from a strict linear relationship.

DISCUSSION

To our knowledge, the stationarity of OT PK parameters with respect to dose has never been examined when this hormone is given at pharmacologic doses. Because OT has potential usefulness in the regenerative therapy of post-ischemic myocardial disease, finding an appropriate dosing regimen for this new therapeutic use requires prior knowledge of its dose-concentration relationship. Most drug possess PK parameters that are independent of the administered dose and of the duration of the dosing regimen, which implies that systemic exposure to the drug increases linearly with the administered dose. In the case of OT, cardiovascular and renal functions may be modified by the hormone to a point that its own plasma concentration is a covariate for its disposition PK parameters. Our study allowed confirmation of this hypothesis, as the distribution and clearance PK parameters of OT significantly depended on dose, and the time-course of dose-normalized plasma OT concentration failed to satisfy the superposition principle (40).

Studies reporting the PK of OT after an i.v. bolus all used an open two-compartment model to describe the time-course of its plasma concentration (12,14-15,44). Another study where rats were infused at a constant rate also reported a two-phase decay after the infusion was stopped (45). In our study, a two-compartment open model best described the time-course of plasma OT concentration at doses of 1000, 3000, 5000 and 10000 ng/kg, but that of the 200, 300 and 500 ng/kg OT doses was best described with the one-compartment model (Figure 1). It could be argued that numerical identification of the peripheral compartment was not possible for the lower doses because an inadequate sampling schedule was used. If this were true, implying that the recorded slope corresponds to the distribution process and a terminal slope with $t½(\lambda_Z)$ of at least 18 min (Table 2) would be found by extending our sampling schedule, plasma OT concentration in rats dosed with

300 µg/kg drug should fall within the analytical range of our RIA assay (0.25 – 200 ng/L) for at least 60 minutes, and a similar finding would be recorded for the 200 and 500 ng/kg dose groups. But all rats in the 300 ng/kg dose group had plasma OT concentrations below the limit of detection at 60 min. In addition, the one-compartment PK model fitted closely to plasma OT concentrations measured in the three lowest dose groups: the choice of this model over the other one was unambiguous, its r^2 values averaged 0.95 and its PK parameters were accurately estimated (*i.e.*, with CV% < 10.5%). These results are inconsistent with the presence of a second, shallower slope in the PK profiles of the 200 – 500 ng/kg OT dose groups, as the plasma concentrations associated with this additional slope would considerably affect the goodness of fit, r^2 value and accuracy of estimated PK parameters. In conclusion, our strategy of devising an optimal sampling schedule (*i.e.*, a schedule that would minimize the error at estimating the parameters of the PK model) based on the results of the pilot experiment allowed minimizing the loss of information associated with the use of restricted sampling schedule.

As the adequacy of the sampling schedule has been assessed, the changes in the time-course of plasma OT concentration may be interpreted as hindrance of the PK processes of distribution and elimination as a consequence of the combined effects of OT on the cardiovascular and renal systems. Noteworthy, the increase in AUC as a function of dose was slightly but significantly curvilinear, and the time-course of the dose-normalized plasma OT concentrations failed to satisfy the superposition principle. Indeed, the graphical assessment of the time-course of dose-normalized plasma OT concentrations revealed three items strongly suggestive of nonlinear PK response. First, the PK profiles were markedly affected by the administered dose (Figure 1), and the estimated $t½(\lambda_Z)$ of the three lowest OT doses significantly differed from the half-lives of the initial and terminal slopes of the four highest OT doses (Table 2). Second, the duration of the distribution phase in rats dosed with ≥ 1000 ng/kg was visibly shorter than the duration of measurable plasma concentrations in rats dosed with the three lowest OT doses (Figure 1). Third, as compared to rats receiving the three lowest OT doses, the dose-normalized plasma OT concentrations in rats dosed ≥ 1000

ng/kg were higher during the whole sampling period (data not shown). Yet, we must point out that the aim of this study was to document dose-related changes in the structure of the PK system of OT, not to build a suitable mechanistic PK model for this hormone. This task would have required a different study design, *e.g.*, with a more intensive sampling schedule and the simultaneous monitoring of the renal and cardiovascular functions. Therefore, the compartmental PK models used here are models of data, which are adequate to fulfill our study objectives and generate specific hypotheses about the PK system of OT, but cannot be used to test these hypotheses because the results would be biased by model misspecification. Nevertheless, the dose-related changes in compartmental PK parameters reported here should provide insight to the cardiovascular and renal effects of OT in rats.

The compartmental PK analysis first revealed that the systemic clearance of OT significantly decreased when administered at doses of 1000, 5000, and 10000 ng/kg, as depicted in Figure 2. Those results indicate that the elimination process of the drug is faster (between 1.5 and 3 times) at doses under 500 ng/kg OT and is considerably reduced at 1000 ng/kg and above, but tends to stabilize when that plasma level corresponding to the 1000 ng/kg dose is reached. In the case of a drug with linear kinetics, CL_T is the proportionality constant that relates plasma drug concentration to its rate of elimination. The OT is eliminated both by renal filtration (46) and metabolism by oxytocinase (47-48). Prior to hydrolysis by this enzyme (EC 3.4.11.3) OT molecule must bind to its receptor on the external surface of the endothelial cell membrane, which afterwards is internalized *via* the classic clathrin-mediated pathway (49) and eventually recycled onto the cell surface (46). In addition, OT binding sites have been found in the *macula densa* and thin Henle's loop of the rat kidney (50), which could mediate dual effects on renal filtration and reabsorption (see below). The dose-related decrease in CL_T suggest a saturation of the pool of OT receptors and/or oxytocinase molecules, with a plateau reached at the dose of 1000 ng/kg OT, or a decrease in renal excretion induced by OT at that dose. We will report in a separate communication the results of a study that examined the effects of OT on renal function. The results on CL_D are also suggestive of such saturation phenomenon with

estimates being not measurable on 200 to 500 ng/kg OT groups, while being quite homogenous between groups with higher doses: Only a marginally significant (p = 0.04) difference was noted between the 1000 and 5000 ng/kg OT groups for this parameter.

Second, it was noteworthy to find that the median V_C decreased abruptly at exogenous OT doses exceeding 500 ng/kg, and its inter-individual variability considerably increased (Figure 3). Similar changes were recorded for V_{SS}, although median values did not decrease with the OT dose as much as that of V_C. Because V_{SS} is the sum of the volumes of V_C and V_P, these combined findings imply a concurrent increase in V_P. This change in the structure of the PK system strongly suggests that V_P represents the population of receptors/enzymes where OT could bind on the vascular bed. The OT receptors of the external surface of the endothelium cell membrane are structurally identical to the uterine and mammary OT receptors (8). These receptors, present in limited number, appear to have saturated at the plateau dose of 1000 ng/kg OT. At doses below 1000 ng/kg OT, as supported by our PK analysis, the pool of unoccupied OT receptors/enzymes would exceed the number of OT molecules available for binding, and the peripheral compartment may act predominantly as an elimination site. That is, the activity of oxytocinase in the peripheral compartment greatly exceeds the rate of transfer of OT back to the central compartment; therefore, the body behaves as a kinetically homogeneous system that could be described with a one-compartment open model. At higher doses, the peripheral metabolic (oxytocinase) pathway saturates, which would restrict the availability of unoccupied OT receptors, hence forcing the peripheral compartment to behave more as a distribution site. Since OT binding to its receptor activates the translocation of oxytocinase and leads to peptide degradation, it would be interesting to quantify OT degradation metabolites. This measurement would represent a direct assessment of the apparent V_p. Unfortunately, no study evaluating OT metabolites is reported yet.

Third, as the OT dose increases and the oxytocinase pathway progressively saturated, it is likely that the rate of recycling of OT receptors decreased, which restricted the pool of unoccupied receptors sufficiently to

delay the equilibrium between the two compartments (V_c and V_p) of the PK system. This delay in homogenization resulted in the development of a distribution phase in the time-course of plasma OT concentration, which slope is λ_1 and PK parameter is CL_D.

Fourth, our estimated CL_T and $t½(\lambda_z)$ recorded for the doses of 1000 ng/kg OT or higher are compatible with those previously reported in the literature for human (4,11,15), goat (12) and rat (14), with CL_T around 1-1.5 L/(h·kg) and $t½(\lambda_z)$ around 20 min. Interestingly, the terminal slope λ_z in the 10000 ng/kg OT group and in 2 individuals receiving 5000 ng/kg (Figure 1B and Table 2) were more than twofold slower (42 min) than the other groups (Table 2). This could be associated, at least in part, with the onset of changes in the renal function following exposure to OT. Indeed, a study in conscious male rats found that 1000 ng i.v. injection of OT induced a +22% temporary (5 min duration) increase in mean systemic arterial blood pressure associated to a decrease in heart rate (-20%) and contractility (-15%) (51). Due to the short duration of cardiovascular effects, their implication on PK parameters dose-dependency is probably limited, if any. Moreover, saliuretic and diuretic/antidiuretic effects of OT have been demonstrated in rats (29-31,36). If the natriuretic effect of OT is relatively constant between rat studies, it was hypothesized that low doses of OT induce diuresis *via* binding on OT receptor (or a non-V_{1a} non-V_2 vasopressin receptor) stimulating an increase in intracellular calcium (52), and high doses of OT induce antidiuresis *via* binding on V_2 vasopressin receptor stimulating a G_s protein, cyclic AMP release and aquaporine-2 opening (53,54). As stated above, these renal effects are highly variable, not only with the OT dose and way of administration, but also with the model: i.v. constant rate infusion of OT induced natriuresis and antidiuresis in dogs (38), and anti-natriuresis and antidiuresis in humans (25). Consequently, we could propose a dose-dependent interference of these pharmacodynamic effects on the PK of OT. Also, a tubuloglomerular reflex in response to increased natriuresis could lead to a reduction in the glomerular filtration rate. As a result, the elimination rate for this 1007 Dalton nonapeptide through the glomerular capillaries would be directly reduced and affect the PK elimination parameters. Our data suggests that the saturation of

the endothelial receptors, of the oxytocinase activity added with a direct anti-diuretic effect and the possible negative feedback of blood perfusion and kidney glomerular filtration rate for fluid homeostasis, explain the diminution of CL_T and the increased elimination half-life observed in animals receiving up to 1000 ng/kg OT.

CONCLUSION

This study showed the non-linearity of PK of OT in rats and proposed several hypotheses to explain this unique PK profile. A deeper understanding of the PK behavior, particularly in relation with the metabolism of the peptide, is needed to confirm underlying mechanisms. Investigations of PK linearity in other species and studies of OT pharmacodynamics on volume homeostasis are also justified. The proof of non-linearity warrants important precautions in the therapeutic use of OT.

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