Université de Montréal

# EVALUATION OF OXYTOCIN PHARMACOKINETIC / PHARMACODYNAMIC PROFILE AND ESTABLISHMENT OF ITS CARDIOMYOGENIC POTENTIAL IN SWINE.

par

NORMA THELMA YBARRA NAVARRO

Département de biomédecine vétérinaire

Faculté de médecine vétérinaire

Thèse présentée à la Faculté de médecine vétérinaire

en vue de l'obtention du grade de

*Philosophiae doctor* (*Ph.D.*)

en sciences vétérinaires

option pharmacologie

Août, 2010

©Norma Thelma Ybarra Navarro, 2010

Université de Montréal

Faculté de médecine vétérinaire

Cette thèse intitulée

**EVALUATION OF OXYTOCIN PHARMACOKINETIC / PHARMACODYNAMIC PROFILE AND ESTABLISHMENT OF ITS CARDIOMYOGENIC POTENTIAL IN SWINE.**

présentée par

Norma Thelma Ybarra Navarro

a été évaluée par un jury composé des personnes suivantes

Bruce D. Murphy, président-rapporteur

Éric Troncy, directeur de recherche

Jérôme R.E. del Castillo, codirecteur

Jean-François Tanguay, membre du jury

Joanne Paquin, examinateur externe

Christopher A. Price, représentant du doyen

# RÉSUMÉ

La thérapie cellulaire est une avenue pleine de promesses pour la régénération myocardique, par le remplacement du tissu nécrosé, ou en prévenant l'apoptose du myocarde survivant, ou encore par l'amélioration de la néovascularisation. Les cellules souches de la moelle osseuse (CSMO) expriment des marqueurs cardiaques *in vitro* quand elles sont exposées à des inducteurs. Pour cette raison, elles ont été utilisées dans la thérapie cellulaire de l'infarctus au myocarde dans des études pre-cliniques et cliniques. Récemment, il a été soulevé de possibles effets bénéfiques de l'ocytocine (OT) lors d'infarctus. Ainsi, l'OT est un inducteur de différenciation cardiaque des cellules souches embryonnaires, et cette différenciation est véhiculée par la voie de signalisation du monoxyde d'azote (NO)-guanylyl cyclase soluble. Toutefois, des données pharmacocinétiques de l'OT lui attribue un profil non linéaire et celui-ci pourrait expliquer les effets pharmacodynamiques controversés, rapportés dans la lttérature.

Les objectifs de ce programme doctoral étaient les suivants :

1) Caractériser le profil pharmacocinétique de différents schémas posologiques d'OT chez le porc, en développant une modélisation pharmacocinétique / pharmacodynamique plus adaptée à intégrer les effets biologiques (rénaux, cardiovasculaires) observés.

2) Isoler, différencier et trouver le temps optimal d'induction de la différenciation pour les CSMO porcines (CSMOp), sur la base de l'expression des facteurs de transcription et des protéines structurales cardiaques retrouvées aux différents passages.

3) Induire et quantifier la différenciation cardiaque par l'OT sur les CSMOp.

4) Vérifier le rôle du NO dans cette différenciation cardiaque sur les CSMOp.

Nous avons constaté que le profil pharmacocinétique de l'OT est mieux expliqué par le modèle connu comme *target-mediated drug disposition* (TMDD), parce que la durée du séjour de l'OT dans l'organisme dépend de sa capacité de liaison à son récepteur, ainsi que de son élimination (métabolisme).

D'ailleurs, nous avons constaté que la différenciation cardiomyogénique des CSMOp médiée par l'OT devrait être induite pendant les premiers passages, parce que le nombre de passages modifie le profile phénotypique des CSMOp, ainsi que leur potentiel de différenciation. Nous avons observé que l'OT est un inducteur de la différenciation cardiomyogénique des CSMOp, parce que les cellules induites par l'OT expriment des marqueurs cardiaques, et l'expression de protéines cardiaques spécifiques a été plus abondante dans les cellules traitées à l'OT en comparaison aux cellules traitées avec la 5-azacytidine, qui a été largement utilisée comme inducteur de différenciation cardiaque des cellules souches adultes. Aussi, l'OT a causé la prolifération des CMSOp. Finalement, nous avons observé que l'inhibition de la voie de signalisation du NO affecte de manière significative l'expression des protéines cardiaques spécifiques.

En conclusion, ces études précisent un potentiel certain de l'OT dans le cadre de la thérapie cellulaire cardiomyogénique à base de cellules souches adultes, mais soulignent que son utilisation requerra de la prudence et un approfondissement des connaissances.

Mots-clés: Ocytocine, cellules souches adultes, différenciation cardiomyogénique, monoxyde d'azote, porc, pharmacokinetic, pharmacodynamic

## ABSTRACT

Cell therapy has been suggested as a promising treatment for myocardial regeneration through cardiomyocyte replacement or by preventing apoptosis of surviving myocardium and/or improving neovascularisation. Bone marrow stem cells (BMSCs) express cardiac markers *in vitro* upon stimulation with different inducers. The BMSCs have been used as cell therapy after myocardial infarction (MI) in pre-clinical and clinical studies. Recent reports have uncovered the potential beneficial effects of oxytocin (OT) after MI. Particularly, OT is an inducer of cardiomyogenic differentiation of embryonic stem cells and this differentiation is mediated by the nitric oxide (NO)-soluble guanylyl cyclase pathway. However, some studies have shown that OT exhibits nonlinear pharmacokinetics and that this could explain the previously described controversial hemodynamic alterations.

Therefore the objectives of the present work were to:

1) Characterize the pharmacokinetic profile of different dosing regimens of OT in swine, by using a more suitable pharmacokinetic / pharmacodynamic modelization that could explain the time-course of cardiovascular and renal effects observed following OT administration.

2) To isolate, differentiate and find the optimum time of porcine BMSC (pBMSC) differentiation based on the expression of cardiac related transcription factors and structural proteins expressed at different passages.

3) To induce and quantify the OT-mediated cardiomyogenic differentiation of pBMSCs.

4) To document the role of the NO pathway in the OT-mediated cardiomyogenic differentiation of pBMSCs.

We found that OT pharmacokinetics are better explained by target-mediated drug disposition (TMDD) kinetics, because the time-course of plasma OT concentration depends on the binding capacity to its receptor, as well as OT elimination (metabolism).

Also, we found that OT-mediated cardiomyogenic differentiation of pBMSCs should be induced during the first passages, because passaging affects the phenotypic profile of pBMSCs, as well as the differentiation potential of pBMSCs.

We observed that OT induces cardiomyogenic differentiation of pBMSCs, because OT-induced cells expressed cardiac markers, and the expression of cardiac specific proteins was more abundant in OT-treated cells *vs.* 5-azacytidine-treated cells, which has been used widely as a cardiomyogenic differentiation inducer of adult stem cells. Moreover, OT improved proliferation of pBMSCs. Finally, we observed that the inhibition of the NO pathway significantly affects the expression of cardiac specific proteins.

To conclude, these studies demonstrate some interesting potential in cardiomyogenic differentiation of adult stem cells for OT, but its precise role in cell therapy will need prudence and further investigations.

Keywords: Oxytocin, adult stem cells, cardiomyogenic differentiation, nitric oxide, swine, pharmacokinetics, pharmacodynamics.

# TABLE OF CONTENTS

# LIST OF TABLES

## LIST OF FIGURES

### CHAPTER 2. LITERATURE REVIEW



### CHAPTER 3. FIRST ARTICLE



### CHAPTER 4. SECOND ARTICLE

## LIST OF ABBREVIATIONS

| | |
|---|---|
| 5-Aza | 5-azacytidine |
| µg | Micrograms |
| µmol/min | Micromolar per minute |
| µU | Microunits |
| 3-D | Three-dimensional |
| ABC | ATP-binding cassette transporters |
| Abcg2 | ATP-binding cassette, sub-family G member 2 |
| ANOVA | Analysis of variance |
| ANP | Atrial natriuretic peptide |
| ATP | Adenosine triphosphate |
| ATPase | ATP hydrolytic enzyme |
| AVP | Arginine vasopressin |
| B220 | Surface marker of human hematopoietic stem cells, equivalent to murine CD45 |
| BM | Bone marrow |
| BM MNC | Mononuclear population cells of the bone marrow |
| Bmax | Maximum receptor density |
| BMP-2 | Bone morphogenic protein-2 |
| *Bmpr1a* | Bone morphogenetic protein receptor, type IA |
| BMSCs | Bone marrow stem cells |
| BNP | Brain natriuretic peptide |
| bp | Base pairs |
| bpm | Beats per minute |

| | |
|---|---|
| $C_a$ | Blood concentration (arterial) |
| $Ca^{2+}$ | Calcium |
| CAD | Coronary artery disease |
| cAMP | Cyclic adenosine monophosphate |
| CD4 | Cluster of differentiation 4 |
| CD8 | Cluster of differentiation 8 |
| CCD camera | Charge-coupled device |
| CD20 | Cluster of differentiation 20 |
| CD31 | Cluster of differentiation 31 |
| CD34 | Cluster of differentiation 34 |
| CD45 | Cluster of differentiation 45 |
| CD45RO | Cluster of differentiation 45 RO isoform |
| CD90 | Cluster of differentiation 90 |
| cDNA | Complementary DNA |
| cGMP | Cyclic guanosine monophosphate |
| c-Kit | Also known as CD117 cytokine receptor |
| Cl | Clearance |
| $Cl^-$ | Chloride ion |
| CLd | Distribution clearance |
| CLt | Elimination clearance |
| $cm^2$ | Squared centimetre |
| cMHC | Cardiac myosin heavy chain |
| CNS | Central nervous system |
| CO | Cardiac output |
| $CO_2$ | Carbon dioxide, capnography |

| | |
|---|---|
| COS-1 | Cell line obtained by immortalizing a CV-1 cell line derived from kidney cells of the African green monkey. |
| CRI | Constant rate infusion |
| CSCs | Cardiac stem cells |
| CSF | Cerebrospinal fluid |
| CSMO | *Cellules souches de la moelle osseuse* |
| CSP | Cardiomyocyte structural proteins |
| cTNI3 | Cardiac troponin I-3 |
| cTnT or TNNT2 | Cardiac troponin T-2 |
| ctrl | Control |
| $C_u$ | Urine concentration |
| $C_v$ | Blood concentration (venous) |
| CVP | Central venous pressure |
| Cys-1 | Amino acid cysteine, position 1 |
| DAG | Diacylglycerol |
| DEA/NO | 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide |
| DMEM-LG | Dulbecco's Modified Eagle Medium-low glucose |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| EBs, EB | Embryoid bodies, embryoid body |
| ECG | Electrocardiogram tracings |
| ECMs | Embryonic cardiomyocytes |
| EDTA | Ethylenediaminetetraacetic acid |
| eNOS | Endothelial nitric oxide synthase |
| $E_{PAH}$ | Para-amino-hippuric acid extraction coefficient |

| | |
|---|---|
| Erk1/2 | Extracellular signal-regulated kinases 1 and 2 |
| ERPF | Effective renal plasma flow |
| ES | Embryonic stem |
| ESCs | Embryonic stem cells |
| F | Forward |
| FBS | Fetal bovine serum |
| $FiO_2$ | Inspired fraction of oxygen |
| FITC | Fluorescein isothiocyanate isomer 1 |
| Flk-1 | Kinase insert domain receptor |
| Flt-1 | Fms-related tyrosine kinase 1 |
| fmol | Fentomoles |
| GATA-4 | GATA binding protein 4, family of zinc-finger transcription factors |
| GFP | Green fluorescent protein |
| GPCR | G protein-coupled receptors |
| Gr-1 | Surface marker myeloid cells |
| h | Hour |
| H+ | Hydrogen cation |
| HR | Heart rate |
| HSC | Hematopoietic stem cell |
| HUVEC | Human umbilical vein endothelial cells |
| I/R | Ischemia/reperfusion |
| ICC | Immunocytochemistry |
| IgG | Immunoglobulin |
| IL-1β | Interleukin one beta |

| | |
|---|---|
| IL-6 | Interleukin 6 |
| iNOS | Inducible nitric oxide synthase |
| IP3 | Inositol 1,4,5-trisphosphate |
| $K^+$ | Potassium |
| $K_{ATP}$ channels | ATP-sensitive potassium channels |
| $K_d$ | Constant of dissociation |
| kg | Kilograms |
| L/min | Liter per minute |
| $Lin^-$ | Lineage negative |
| L-NAME | $N_\omega$-Nitro-L-arginine methyl ester hydrochloride |
| M | Molar |
| Mac-1 | Macrophage-1 antigen |
| MAP | Mean arterial pressure |
| MAP kinase | Mitogen-activated protein kinase |
| MDRs | Multidrug resistances |
| Mef2 | Myocyte enhancer factor |
| MEF-2C | Myocyte enhancer factor-2C |
| $Mg^{2+}$ | Magnesium |
| MHC | Myosin heavy chain |
| MI | Myocardial infarction |
| min | Minutes |
| mL | Millilitres |
| mL/beat | Milliliter per beat |
| mL/min | Milliliter per minute |
| MLC-2a | Myosin light chain-atrial |

| | |
|---|---|
| MLC-2v | Myosin light chain-ventricular |
| mmHg | Millimeter of mercury |
| MNCs | Mononuclear cells |
| mRNA | Messenger ribonucleic acid |
| MSCs | Mesenchymal stem cells |
| mU | Milliunits |
| Na+ | Sodium ion |
| ng | Nanograms |
| ng/kg or ng $kg^{-1}$ | Nanograms per kilogram |
| ng/kg/h | Nanograms per kilogram per hour |
| ng/mL or ng $mL^{-1}$ | Nanograms per millilitre |
| Nkx2.5 | NK2 transcription factor related, locus 5 |
| nmol | Nanomoles |
| nNOS | Neuronal nitric oxide synthase |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| $O_2$ | Oxygen |
| ºC | Degrees Celsius |
| ODQ | 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one |
| OT | Oxytocin |
| OTase | Oxytocinase |
| OTR | Oxytocin receptor |
| P | Probability |
| P1 | Passage one |
| PAH | Para-amino-hippuric acid |

| | |
|---|---|
| PAP | Pulmonary artery pressure |
| pBMSCs | Porcine bone marrow stem cells |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PCV | Packed cell volume |
| PCWP | Pulmonary capillary wedge pressure |
| PD | Pharmacodynamics |
| PDE | Phosphodiesterase |
| pg/mL or pg $mL^{-1}$ | Picograms per millilitre |
| pH | Potential for hydrogen ion concentration |
| PIP2 | Phosphatidylinositol 4,5-bisphosphate |
| PK | Pharmacokinetics |
| p$K_a$ | Potential of acid dissociation constant |
| PKC | Protein kinase C |
| PKG | Protein kinase G |
| PLB | Phospholamban |
| PLC | Phospholipase C |
| pM | Picomolar |
| pBMSC | Porcine bone marrow stem cells |
| PVDF | Polyvinylidene difluoride |
| PVN | Paraventriculear nucleus |
| R | Reverse |
| RNA | Ribonucleic acid |
| RPE | R-Phycoerythrin |
| RPF | Renal plasma flow |

| | |
|---|---|
| RPFc | Corrected renal plasma flow |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| SAS | Statistical analysis software |
| Sca-1 | Stem cell antigen-1 |
| SD | Standard deviation |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SE | Standard error |
| SEM | Standard error of the mean |
| SERCA-2 | Sarco/Endoplasmic Reticulum $Ca^{2+}$-ATPase type 2 |
| sGC | Soluble guanylyl cyclise |
| SNAP | S-nitroso-N-acetyl-d,l-penicillamine |
| SON | Supraoptic nucleus |
| SP | Side population |
| $SpO_2$ | Pulse oximetry |
| SV | Stroke volume |
| SVR | Systemic vascular resistance |
| TBS | Tris buffered saline |
| TBX5 | T-box 5 |
| Tef-1 | Transcriptional enhancer factor-1 |
| TER119 | Marker of erythroid lineage |
| Thy1 or CD90 | Thymocyte differentiation antigen |
| TMDD | Target-mediated drug disposition |
| Tyr-2 | Aminoacid tyrosine, position 2 |
| U | Units |

| | |
|---|---|
| UF | Urine flow |
| uNOS | Universal nitric oxide synthase (antibody detecting three NOS isoforms) |
| USMC | Human uterine smooth muscle cells |
| UV | Ultraviolet |
| V | Volts |
| V1a-type | Arginine vasopressin receptor type 1 (vascular) |
| V2 | Arginine vasopressin receptor (renal) |
| V3 (also known as V1b) | Arginine vasopressin receptor (pituitary) |
| Vc | Central volume of distribution |
| VEGF | Vascular endothelial growth factor |
| *vs*. | Versus |
| Vss | Steady-state volume of distribution |
| Wnt | coined as a combination of Wg (wingless) and Int |
| α1 | Alpha one |
| α-MHC | Alpha myosin heavy chain |
| β1 | Beta one |
| β-MHC | Beta myosin heavy chain |
| μM | Micromolar |

*A mi madre y familia*

## ACKNOWLEDGMENTS

First, I would like to thank Dr. Éric Troncy for being my director during my MSc and PhD programs. I want to thank you for your support and guidance during the last almost 6 years, thanks for giving me the opportunity to work with you. Thanks for your trust and support during this time; I really appreciate your kindness and patience. Thanks for all the good advices and for also being a friend. Thanks for playing an important role in my professional and personal development.

I would also like to thank Dr Jerome del Castillo, for being my co-director and for his enthusiasm for explaining pharmacokinetics and statistics, aunque ambos sabemos no compartimos la misma pasión. Thanks for sharing your knowledge with your students, also thanks for your patience and for always having a good disposition to offer your help, also I enjoyed the good jokes in Spanish. Muchas gracias.

I want to thank Valerie Morin, who studied her master degree under the supervision of Dr Troncy, thanks a lot for being my first friend in Quebec, thanks for all your support and friendship especially in those moments of confusion, and for all the fun times we spent together (sometimes dangerous), I will never forget the times when we worked until very late during the night and we were so exhausted, but you still had energy to make jokes and make me laugh.

I want to thank Dr Simon Authier for all his invaluable help and support during all the experiments performed during my master's degree program, your enthusiasm is always inspirational. I wish I could have your energy!

I want to thank my laboratory collegues, Dominique Gauvin for her help and support during these years, and for sharing her knowledge in laboratory techniques and protocols.

Thanks to Colombe Otis for her help in data analysis and protocols preparations.

Thanks to Pascale Rialland for all the nice times and her friendship also thanks for the good jokes and the time we laughed together.

I want to especially thank Dr Bruce Murphy for very precious help and support during my PhD program, thanks a lot for always have been willing to share your knowledge and to always have open doors of your lab to other students that sometimes are not from the CRRA. Thanks a lot for always having the will of help the others; I also appreciate your jokes.

I want to thank Mira Dobias, for her invaluable help and for always been willing to share her experiences and knowledge, also thanks a lot for always saying hello with a smile in your face, and always make me feel welcome to your lab.

I want to thank Dr. Lawrence Smith and his research associates, Patrick Vincent, France, Jacinthe, for being so open and willing to share knowledge, without mentioning your ever present smiles and kindness. Thanks Patrick for your enthusiasm in sharing your knowledge and for always being willing to help.

Mercie beaucoup Mme St Germain pour toutes les fois que vous m'avais auxilié avec toute sorte des questions et aussi des problèmes, merci beaucoup pout tout votre aide depuis le debut. Thanks a lot to all the staff working in the secretariat. Thanks to Mme Rodier for her help and advice.

In fact, thanks to all the CRRA staff and students!

I also want to thank my dear friends at the CRRA: Adriana Verduzco, Joelle Demarais, Simon Demers not only for your precious friendship but also for sharing with me your knowledge and experience in the lab.

Also thanks a lot to all my friends in the faculty for sharing your time with me, thanks for the fun times you made me have during my post-graduate studies: Adriana, Joelle, Simon, Ricardito, Aron, Lan, Virginie, Pavine, Johanna.

But of course, I want to thank the most important person in my life, my mother for always being there for me. Simplemente a ti te debo todo lo que soy, no tengo palabras para expresarte mi gratitud y admiración. I want to thank my whole family, my brothers, my uncles, thanks for your love and support. Gracias a mis hermanos. Especialmente gracias tío Miguel, por siempre estar con nosotros apoyándonos, gracias porque siempre podemos contar contigo y por darnos tu cariño incondicional. A mi madrina, porque a pesar de la distancia siempre está al pendiente de mi, gracias por tus palabras de aliento.

A mis mejores amigas Karla, Mickey y Lucia, estaremos lejos físicamente pero siempre están ahí para apoyarme y quererme, gracias por siempre escucharme y hacerme sentir mejor cuando más las necesito. Gracias a todos mis amigos.

# CHAPTER 1. INTRODUCTION

Cardiovascular diseases remain the leading cause of mortality and morbidity worldwide. Despite substantial improvements in acute management, survivors of myocardial infarction often progress to heart failure (Siu, Moore et al. 2007). Heart failure is a condition that can result from any structural or functional cardiac disorder. It is defined as the inability of the left ventricle to fill with and/or eject sufficient blood to meet the metabolic requirements of the body. It is undoubtedly an increasing common condition (Harris 1994).

Despite major advances in treatment, the prognosis after a diagnosis of heart failure is poor and comparable to that of several forms of cancer. Even though, the rate of fatalities per case associated with heart failure has declined, the crude number of deaths attributed to the condition has increased, primarily because of increasing prevalence of the condition. Several epidemiological investigations have identified the main risk factors for heart failure, which are: increasing age, hypertension, coronary artery disease (CAD), diabetes, obesity, valvular heart disease, and the metabolic syndrome (Kenchaiah, Narula et al. 2004).

The relative contribution of risk factors to the occurrence of heart failure in the community may be changing over time. The aging of the population, the better treatment of CAD, and the improved survival and ‘‘salvage’’ of patients with myocardial infarction (MI) with subsequent progression to pump failure are believed to be some factors contributing to the growing burden of heart failure. Heart failure is also predicted to increase in line with increased atrial fibrillation and diabetes among the elderly population. Nowadays, hypertension and CAD are the major modifiable risk factors for heart failure. Globally, CAD and arterial hypertension alone or in combination account for more than 90% of cases of heart failure (Lloyd-Jones, Evans et al. 2002; Ezekowitz and Kaul 2010).

Although human cardiomyocytes are reported to proliferate and contribute to the increase in muscle mass of the myocardium after myocardial infarction (Beltrami, Urbanek et al. 2001), their capacity for regeneration, mitigation of the adverse effects of ventricular remodelling,

and contribution to cardiac function is limited. Among other factors, cardiac wall thinning and cardiac remodelling lead to a diminished capacity of the heart to pump blood effectively leading to eventual heart failure and ultimately death of the patient. Heart transplantation is currently the last resort for end stage heart failure, but is hampered by rejection and a severe shortage of donor organs. The main purpose of cell-based therapies as applied for the treatment of myocardial infarction is to prevent heart failure by either rescuing the host myocardium or regenerating cardiac cells (Christoforou and Gearhart 2007; Tao and Li 2007; Dimmeler and Zeiher 2009).

In the past, a variety of cell types have been used experimentally, obtaining promising results, mainly by improving neovascularization, reducing infarct size, limiting wall thinning, and providing proangiogenic and antiapoptotic factors promoting tissue repair in a paracrine manner. By all these mechanisms cell therapy may curb the degeneration to heart failure (Tao and Li 2007).

The cell types used previously include skeletal myoblasts, as a non-cardiac contracting cell, and foetal cardiomyocytes, but these approaches are also limited by cell availability or side effects, mainly due to a non-cardiac identity, as observed in the case of skeletal myoblasts. There is a special interest in the use of stem cells in cell-based therapies, because of their capacity of cell renewal, and differentiation potential. *In vitro* studies examined the ability of stem cells to differentiate into cardiomyocytes and then proceeded to investigate the functional characteristics of these cells. *In vivo* studies examined the capacity of stem cells to graft into the host myocardium and then assayed the functional recovery of the diseased heart.

During the latest years, pre-clinical and clinical studies exploiting stem cells from different sources for transplantation in animal models and patients have reported favourable outcomes (Christoforou and Gearhart 2007; Tao and Li 2007; Dimmeler and Zeiher 2009).

Unfortunately, full recovery has not been achieved yet, due to many variables that need to be considered. For instance, the optimal cell type, which in turn should generate contractile cells that integrate both functionally and structurally into the surrounding viable myocardium. These cells have to beat in a synchronized manner to avoid alterations in the electrical conduction and syncytial contraction of the heart, these cells should also be able to survive in the hostile environment into which they will be grafted (Tao and Li 2007; Dimmeler and Zeiher 2009).

# CHAPTER 2. LITERATURE REVIEW

## 2.1 Stem cells

### 2.1.1 Definition and properties

Stem cells are unspecialized cells, with the long-term capacity of symmetrical self-renewal. Differentiation is a process that involves undifferentiated or unspecialized cells progressing into specialized cells with restricted developmental potential. They can differentiate into specialized cells, including cardiomyocytes (Blau, Brazelton et al. 2001; Bishop, Buttery et al. 2002). The differentiation capacity of these specialized cells *in vivo* to form mature cell types ultimately depends on the state of commitment of the cell and both intrinsic factors and the extra-cellular environment (niche) (Perino, Yamanaka et al. 2008).

The genetic and cellular mechanisms that initiate stem cell differentiation are poorly understood. Transplanted stem cells also undergo a "homing" process in which they are attracted to the site of injury (Hardy 1995). The exact homing mechanism and organ-specific differentiation signals for stem cells are not clearly understood but may be related to microenvironmental factors that are favourable to stem-cell growth and function, integrin and other adhesion molecules, homing receptors, ischemia, and increased expression of different paracrine factors and cytokines (Lee, Wolf et al. 2000; Caplice and Gersh 2003).

### 2.1.2 Classification

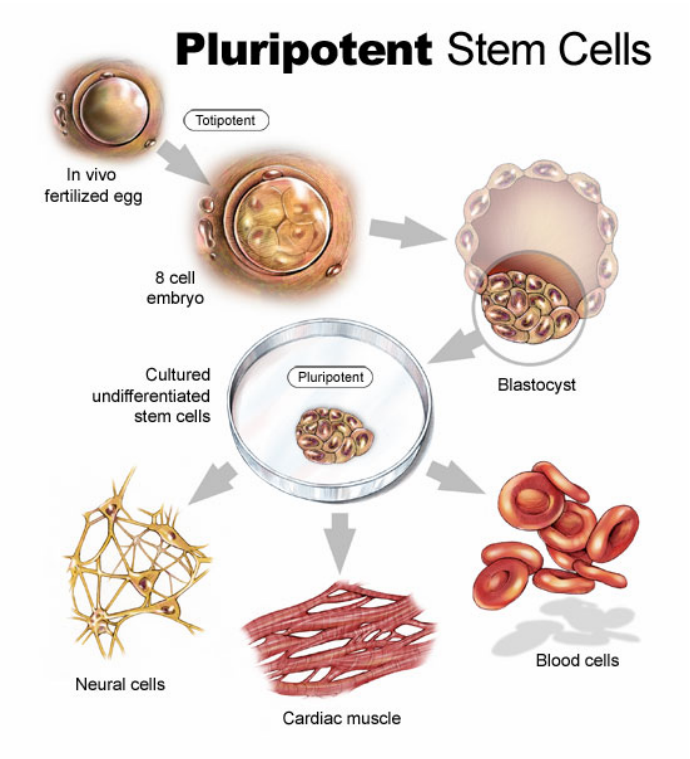
Stem cells have been classified based on their source of isolation, and by their differentiation potential into embryonic stem cells, adult stem cells and induced pluripotent stem cells.

### 2.1.3 Embryonic stem cells (ESCs)

Embryonic stem cells can be derived from the embryo at *morulae* stage, based on their differentiation potential, they are known as totipotent cells because they can give rise to

extraembryonic and embryonic tissues. Stem cells derived from the inner cell mass of the embryo at blastocyst stage are known as pluripotent cells. These *pluripotent* cells can differentiate into the three primary germ layers, endoderm, mesoderm, and ectoderm, meaning they have the potential to differentiate into all tissue specific cells of an embryo, and if the embryo is implanted *in utero* following tetraploid aggregation, an entire ES cell-derived embryo, excluding some extra-embryonic tissues, can be formed (Nagy, Gocza et al. 1990).

**Figure 1. Embryonic stem cells derived from embryos at morulae and blastocyst stage**



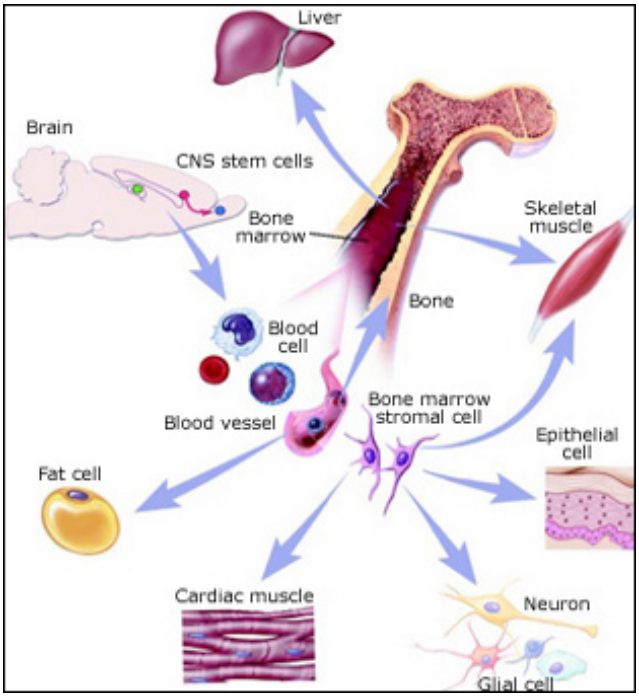

Source: http://www.stemcellresearchfoundation.org

Embryonic stem cells can also be used for the generation of chimeric animals, in which the ES cell genotype can be passed through the germline (Illmensee and Mintz 1976). Another pluripotent cell type includes germ cells, progenitor cells of the germline, these cells are found in a specific part of the foetus called the gonadal ridge, germ cells can also give rise to cells from the three germ layers (Moreno-Ortiz, Esteban-Perez et al. 2009).

### 2.1.4 Adult stem cells

Adult stem cells possess a more restricted developmental potential, giving rise to a subset of cells belonging to the same germ layer, they are generally considered to be *multipotent* to *unipotent*. They typically produce only cells of a closely related family (cells from the tissue or organ in which they reside), providing "new" cells in order to replenish damaged specialized cells in the adult (Wobus and Boheler 2005; Perino, Yamanaka et al. 2008).

**Figure 2. Adult stem cells derived from adult tissues**




Source: National institutes of health. www.nih.gov

Both embryonic and adult stem cells are capable of cell division in the undifferentiated state (self-renewal). Embryonic stem cells readily form tumours when implanted outside the blastocyst, adult stem cells (*e.g.*, hematopoietic, mesenchymal, neuronal) may or may not form tumours, but both ES and adult stem cells are different to cancer stem cells (Perino, Yamanaka et al. 2008).

Noteworthy, cancer stem cells are oncogenic, have lost the ability to prevent uncontrolled proliferation or differentiation, and are therapeutically unviable, properties that make them different from normal stem cells (Maenhaut, Dumont et al. 2010).

### 2.1.5 Cardiomyogenic potential of embryonic stem cells

Embryonic stem cells are conducive to cell implantation therapy, mainly because they are pluripotent. These unique pluripotent cell lines can be propagated in the undifferentiated state in culture and coaxed to differentiate into cell derivatives of the three germ layers, including cardiomyocytes (Capi and Gepstein 2006; Tao and Li 2007).

Doetschman and colleagues (Doetschman, Eistetter et al. 1985) made the initial observation that when ESCs are grown in suspension culture and under conditions favourable for differentiation (without feeder layer and cytokines that maintain an undifferentiated state, *i.e.* leukaemia inhibitory factor –LIF–), ESCs aggregated to form spherical structures called embryoid bodies (EBs). Stochastic differentiation within EBs resulted in the juxtaposition of different developmental fields, thereby mimicking induction cues that occurred during normal embryogenesis. As a consequence, cell lineages of endoderm, ectoderm, and mesoderm origin were observed to appear in differentiating EBs. Prominent cardiomyogenesis occurred during EB differentiation, as evidenced by the presence of well-formed myofibers, as well as spontaneous contractile activity. Thus, differentiating ESCs might provide a surrogate source of donor cardiomyocytes for therapeutic cell transplantation (Rubart and Field 2006; Perino, Yamanaka et al. 2008).

Studies with ESCs derived cardiomyocytes revealed that cardiomyogenic differentiation in EBs closely paralleled that observed for early stages of heart development *in vitro* (Doevendans, Kubalak et al. 2000), and that cardiomyocytes with the typical characteristics of the primitive heart tube and early chamber myocardium could be easily identified (Fijnvandraat, van Ginneken et al. 2003; Fijnvandraat, van Ginneken et al. 2003). The

temporal phenotypic changes in myofiber structure in ES-derived cardiomyocytes closely paralleled those observed in cardiomyocytes *in vivo* (Guan, Rohwedel et al. 1999). Similarly, ES-derived cardiomyocytes exhibited a temporal pattern of cell cycle withdrawal and multinucleation similar to that observed *in vivo* (Klug, Soonpaa et al. 1995). Electrophysiological studies revealed that cells with characteristics of atrial, ventricular, and sinus-nodal foetal cardiomyocytes were present following terminal differentiation of ESCs (Maltsev, Rohwedel et al. 1993), a result that was confirmed *via* molecular analyses (Miller-Hance and Chien 1993). The developmental changes in the electrophysiological properties of ESCs derived cardiomyocytes from initial cardiomyoblast commitment (Kolossov, Fleischmann et al. 1998), through formation of three-dimensional, spontaneously contracting structures (Maltsev, Rohwedel et al. 1993; Banach, Halbach et al. 2003) have been well characterized.

The reproducible differentiation observed in cultured EBs has been exploited not only to identify factors that could be able to enhance cardiomyogenic induction, but also it has been useful to identify transcription factors or signalling pathways important for cardiomyogenic differentiation. Examples of these transcription factors are: Nkx2.5, GATA-4 and Mef2, which also have been used as markers of differentiation. Some studies have implicated the signalling pathways of bone morphogenic proteins and Wnt family members in cardiomyogenic differentiation of ESCs (Winnier, Blessing et al. 1995; Czyz and Wobus 2001; Behfar, Zingman et al. 2002; Kawai, Takahashi et al. 2004; Terami, Hidaka et al. 2004). In addition, signalling through the fibroblast growth factor (Dell'Era, Ronca et al. 2003; Kawai, Takahashi et al. 2004), insulin growth factor-II (Morali, Jouneau et al. 2000), Cripto (Parisi, D'Andrea et al. 2003), cardiotrophin (Sauer, Neukirchen et al. 2004), transforming growth factor beta (Behfar, Zingman et al. 2002), and dynorphin B (Ventura, Zinellu et al. 2003) pathways enhanced cardiomyogenesis during EB differentiation. Cardiomyogenic differentiation has being promoted also by indirect mechanisms, such as

LIF-regulated cardiomyogenesis in ESCs cultures *via* its effect on parietal endoderm differentiation (Bader, Al-Dubai et al. 2000). Thus, altering the relative content of other cell lineages subsequently provided cardiomyogenic inducing factors, that enhances the yield of cardiomyocytes.

In addition to these defined factors, co-culture experiments revealed that precardiac endoderm contained factors capable of enhancing ESC cardiomyogenic differentiation (Rudy-Reil and Lough 2004). Other inducers used in enhancing ESC cardiomyogenic differentiation are some organic compounds such as dimethysulfoxide (DMSO) (Rudnicki, Jackowski et al. 1990), a novel butyric and retinoic acid linked ester of hyaluronan (Ventura, Maioli et al. 2004), and inorganic compounds including lithium (Schmidt, Guan et al. 2001), reactive oxygen species (Sauer, Neukirchen et al. 2004), and nitric oxide (NO) and its signalling components which apparently play a pivotal role during cardiomyogenesis (Bloch, Fleischmann et al. 1999), and enhance the differentiation of ESCs into cardiomyocytes (Kanno, Kim et al. 2004; Mujoo, Krumenacker et al. 2006). Also the neurohypophyseal hormones arginine vasopressin and oxytocin (OT) enhanced the ESC cardiomyogenic differentiation (Paquin, Danalache et al. 2002; Gassanov, Jankowski et al. 2007).

Gene transfer experiments identified additional pathways that enhanced cardiomyogenic differentiation in ESCs. For example, overexpression of GATA-4 (Arceci, King et al. 1993; Grepin, Nemer et al. 1997), alpha-1,3-Fucosyltransferase (Sudou, Muramatsu et al. 1997), and TBX5 (Fijnvandraat, Lekanne Deprez et al. 2003) resulted in increased yields of cardiomyocytes in differentiating EB cultures.

In summary, the reports described above identified many factors that could increase cardiomyocyte content in differentiating ESCs cultures. The information obtained from such studies can in turn be exploited to enhance the production of ESCs derived cardiomyocytes for clinical applications, or to discover signalling pathways that could be used to improve the differentiation of adult stem cells.

In spite of all the interesting qualities of ESCs, there are some ethical concerns about their use in cell therapy. The use of human ESCs in research requires the generation of human ESC lines. The lines currently used were mostly produced from fertilized oocytes that had undergone about 7-8 divisions. The major objection to the use of ESCs is that their generation is purported to involve an "act of killing". The resulting controversy has delayed or stopped human ESCs research in some countries, and could affect the extent to which human ESCs derived therapies are developed and used (Robertson 2001).

Many obstacles remain to be overcome before ESCs can be expected to undergo evaluation in clinical trials. Further, the multiple regulatory issues that surround the use of ESCs and their derivatives, including the difficulties this potentially raises with respect to federal funding, continue to heavily influence the research that is conducted with these cells. Despite this, steady progress continues to be made toward understanding the mechanisms/pathways that control ESCs. A particularly important issue is the potential of ESCs to induce teratoma formation when transplanted. This may be dependent on many factors, including the experimental model and the exact nature of the ESCs population that is administered (Gonzales and Pedrazzini 2009). Currently, the consensus view is that the administration of ESCs that are at least partially committed to a certain fate may considerably reduce the risk of teratoma formation (Kovacic, Harvey et al. 2007).

Given the possible risk of graft rejection, the ethical and other concerns surrounding the use of human ESCs, a recent possible alternative for the possible graft rejection, could be the ESCs like induced pluripotent stem (iPS) cells initially derived from murine fibroblasts. The iPS cells have been created by the retroviral introduction of Oct3/4, Sox2, c-Myc, and Klf4, these factors have been related to pluripotency in ESCs (Kovacic, Harvey et al. 2007). The iPS could be considered a substitute in the evolution of the stem cell field and for cell-based therapies. Unfortunately, the high risk of teratoma formation remains. Furthermore, various safety concerns exist that must be resolved before iPS cell therapy becomes a reality.

Potential risks are related to the delivery of the endogenous factors, alterations in target cells, the cellular effects of the expression and reactivation of the factors that induce pluripotency, and safety issues related to the incorrect characterization and incomplete differentiation of the reprogrammed cells (Jalving and Schepers 2009).

An interesting alternative to the controversial destruction of a human embryo and the possible teratoma formation, is the use of stem cells obtained from adult tissues (Dickens and Cook 2007).

Cell therapy with autologous stem cells from adults, *i.e.* bone marrow cells is completely justified ethically, except for the small numbers of patients with direct or indirect bone marrow disease (e.g. myeloma, leukaemic infiltration) in whom there would be lesions of mononuclear cells (Strauer, Brehm et al. 2008).

The use of human autologous adult (bone marrow) stem cells is clinically justified and ethically unquestionable because no side-effects have been reported, especially with regard to teratocarcinoma. Moreover, in contrast to differentiation of embryonic stem cells immunosuppressive therapy is unnecessary. Thus, the therapeutical advantage clearly prevails, and clinical use has already been realized, at least in cell therapy for MI, obtaining promising results and no arrhythmogenic potential has been reported (Strauer, Brehm et al. 2008).

Further and intensified research using autologous human bone marrow stem cells is needed and is essential in order to promote stem cell therapy for the numerous cardiac diseases.

Clinical use of autologous bone marrow mononuclear cells has no ethical problems. Therapy is performed with usual cardiac catheterization techniques (Strauer, Brehm et al. 2008).

### 2.1.6 Adult stem cells with cardiomyogenic potential

Whereas ESCs clearly have the capacity to differentiate into a variety of cell types and give rise to all tissues, adult stem cells are more specified (more lineage committed) and the use of adult stem cells for cell therapy appears to be rather limited.

The cardiomyogenic potential of adult stem cells is a continuous source of controversy, due to the diversity of experimental protocols, cell sources, the limited *in vitro* functional tests, and the difficulties in the follow up of transplanted cells *in vivo*. The follow up of the *in vivo* differentiation of adult stem cells represents a special challenge because of the presence of two types of artefacts, the transfer of cell labels used to track implanted cells to neighbouring cells (Burns, Ortiz-Gonzalez et al. 2006), and heterotypic cell fusion, which is a process whereby donor and host cells merge, resulting in a fused cell with the genetic information of both cells, including any genetic marker (Nygren, Jovinge et al. 2004; Reinecke, Minami et al. 2008). Both artefacts have been observed *in vitro* and *in vivo*, and complicate the demonstration of adult stem cell *in vivo* differentiation potential (Reinecke, Minami et al. 2008).

Demonstration of *in vitro* differentiation potential of adult stem cells also represents some challenges, for instance the demonstration of an unambiguous cardiac phenotype. The most common approach is to show the expression of one or more cardiac specific markers by immunocytochemistry (ICC), reverse transcriptase-polymerase chain reaction (RT-PCR) (Antonitsis, Ioannidou-Papagiannaki et al. 2007), and western blot (Genovese, Spadaccio et al. 2009). In reality, there are very few specific cardiac markers, and so this approach requires the judicious selection of multiple markers and appropriate controls. Ideally, such phenotyping by cardiac markers is accompanied by functional assays (Rubart and Field 2006), such as action potential recordings or fluorescent calcium ($Ca^{2+}$) imaging (Yoon, Choi et al. 2008).

Even with all the difficulties listed above there are several reports showing the existence of adult stem cells with cardiomyogenic potential which reside mainly in the heart itself or in the bone marrow.

#### 2.1.6.1 Resident cardiac stem cells (CSCs)

Several independent laboratories have reported the existence of CSCs, based on the presence of the receptor tyrosine kinase c-Kit (Beltrami, Barlucchi et al. 2003), stem cell antigen-1 (Sca-1) (Oh, Bradfute et al. 2003), or the presence of side population (SP) cells that express multidrug resistance transporter genes and exclude Hoechst dye (Liang, Tan et al. 2010).

Proponents of the stem cell theory of cardiac self-renewal postulate that these cells, isolated from cardiac tissues, easily differentiate into cardiomyocytes. Some reports have described these observations as experimental artefacts or cell fusion. Perhaps more importantly, opponents have suggested that some of the putative stem cell derivatives may arise from de-differentiated adult cardiac myocytes, trans-determination or even trans-differentiation events and therefore these cells are not authentic or resident stem cells. The origin of cardiomyogenic stem cells is also open to debate, partly because hematopoietic stem cell markers such as c-Kit and Sca-1 are most prominent outside the heart, suggesting that they may originate elsewhere (Braun and Martire 2007). Although SP cells have been associated at least partially with hematopoietic stem cell (HSC) populations, others have shown that the HSCs were equally distributed in the non-SP population, suggesting that precaution should be taken with respect to the claim that SP cells represent a stem cell population (Goodell, Brose et al. 1996; Morita, Ema et al. 2006).

#### 2.1.6.2 The c-Kit positive cells

Beltrami *et al.* first reported the discovery of a c-Kit$^{+}$ population of resident stem cells that could be isolated from adult rat heart and expanded *in vitro* under limiting dilution conditions

(Beltrami, Urbanek et al. 2001). These relatively small cells were calculated to be present at only about 1 per 100 cardiomyocytes and were lineage negative ($Lin^-$) for blood lineage surface markers [cluster differentiation (CD) CD34, CD45, CD20, CD45RO and CD8]. The c-$Kit^+$ cells were also very heterogeneous, with less than 10% of the cells expressing Nkx2.5, GATA-4 and Mef2 cardiac transcription factors, and less than 0.5% of the cells expressing sarcomeric proteins. Under appropriate conditions, these cells could differentiate into cardiomyocytes (Beltrami, Urbanek et al. 2001). Based on the proof of cell division, using Ki-67 protein as a recognized marker strictly associated to cell proliferation, the authors also indicated that repair occurred independently of cell fusion. However, most of the derived cardiomyocytes appeared immature with either limited sarcomeric structures or the presence of stress fibers that are typical of fibroblasts or myofibroblasts (Urbanek, Quaini et al. 2003).

**2.1.6.3 The Sca-1 positive cells**

Sca-1 is one of the most recognized HSCs markers in mice, and an anti-Sca-1 antibody is routinely used to identify and isolate murine HSC from bone marrow. Oh *et al.* identified a small number of Sca-$1^+$ cardiac cells that overlapped with a SP of cells from heart. The cardiac Sca-$1^+$ cells lacked blood lineage surface markers (CD4, CD8, B220, Gr-1, Mac-1, and TER119), c-Kit, Flt-1, Flk-1, vascular endothelial-cadherin, von Willebrand factor, and HSC markers CD45 and CD34. Moreover, these cells expressed GATA-4, Mef2 transcription factors and Tef-1, but not Nkx2.5 or genes that encoded cardiac sarcomeric proteins. The Sca-$1^+$ stem/progenitor cells did not spontaneously differentiate into cardiomyocytes, but following induction with 5-azacytidine, a DNA demethylating agent that causes pronounced epigenetic modifications, genes for Nkx2.5, α-myosin heavy chain (MHC), β-MHC, and the type 1*A* receptor for bone morphogenetic proteins (*Bmpr1a*) were induced, and contraction was observed. The cells were however mononucleated and fibroblast-like in structure (Oh, Bradfute et al. 2003).

On the other hand, Matsuura *et al.* reported that OT, but not 5-azacytidine, induces Sca-$1^+$ cells from the adult murine heart to differentiate into functional, spontaneously beating, immature cardiomyocytes with $Ca^{2+}$ transients typical to those found in heart cells (Matsuura, Nagai et al. 2004). The 5-azacytidine-treated cells however developed a fibroblast-like morphology and never spontaneously contracted. In this study, both treatments up-regulated cardiac transcription factors Nkx2.5, GATA-4, and MEF-2C and structural proteins for α- and β-MHC, myosin light chain (MLC)-2a, MLC-2v, and cardiac α-actin. The OT-treated cells that formed cardiomyocytes were few in number, but these cells had positive inotropic responses to isoproterenol *via* $\beta_1$-adrenergic receptor signalling (Matsuura, Nagai et al. 2004). Given the apparently small number of cardiomyocytes generated *in vitro* from this induction, it seems that cardiomyogenesis is not a default pathway for these cells, and the potential to differentiate into true cardiac progenitors and cardiomyocytes requires further investigation (Matsuura, Nagai et al. 2004).

#### 2.1.6.4 Side population cells

The ATP-binding cassette (ABC) transporter family contains 50 members that use the hydrolysis of ATP to pump toxins from cells. The ABC transporters encoding multidrug-resistance genes (MDRs) also efflux the DNA binding dye Hoechst 33342, which allows for the easy identification and sorting of ABC transporter-positive and transporter-negative cells by flow cytometry. In most cases, cells positively expressing ABC transporters comprise a very small percentage of freshly isolated cells, which appear as a "side population" (SP) of cells relative to the majority of non-SP cells as observed using flow cytometry. The SP cells and especially the Abcg2-dependent SP cell populations, have been associated with stem/progenitor cells and with long-term self-renewal (Goodell, Brose et al. 1996). The ATP-binding cassette, sub-family G, member 2, also known as Abcg2 gene is robustly expressed in the developing heart (Martin, Meeson et al. 2004). At later developmental stages and in the

adult heart, this transporter could also be used to identify a rare cell population, this SP phenotype comprising around 1% of all cells in the mouse heart and these cells did not express hematopoietic surface markers that was not enriched for the hematopoietic markers CD34, c-Kit, Sca-1, Flk-2, and Thy1 (Hierlihy, Seale et al. 2002). Further experiments, in which cardiac SP cells from adult green fluorescence protein (GFP) transgenic mice were isolated and co-cultured with cardiac main population cells from wild-type mice, showed that after 14 days, an unspecified fraction of the GFP positive SP-derived cells immunostained for the striated muscle marker α-actinin. The purified myocardial SP cells from GFP transgenic mice were capable of forming cardiomyocytes, but similarly to the c-Kit$^+$ cells previously described, only did so under co-culture conditions with primary cardiomyocytes.

#### 2.1.6.5 Non-resident cardiomyogenic stem cells

Many non-resident stem cell populations with cardiomyogenic potential have been described. Many clinical trials have shown the safety and therapeutic potential of bone marrow mononuclear cells after myocardial infarction (Strauer, Brehm et al. 2002; Lipinski, Biondi-Zoccai et al. 2007; Arminan, Gandia et al. 2008). However, this population is very heterogeneous and is not enriched with stem or progenitor cells, therefore recent clinical trials using cellular cardiomyoplasty have focused on the potential of specific subpopulations, such as mesenchymal stem cells (MSCs), or HSCs.

#### 2.1.6.6 Hematopoietic stem cells

Therapeutic applications of HSCs in patients with infarcted myocardium have resulted in very different results. Probably, this was due to differences in purification protocols, influencing the outcome of the therapies. Transplanted cells may have contained different contents of stem cells or maybe the selected cells were a heterogeneous population containing other cells with regenerative potential (paracrine effects). The latter is quite possible because

different CD surface antigens were used to purify the definitive stem cell populations. The HSCs are the most studied and well characterized population of stem cells. These cells display a unique set of markers (Murine: c-Kit$^{+}$, Sca-1$^{+}$, Lin$^{-}$; Human: CD34$^{+}$, Thy-1$^{+}$, Lin$^{-}$). Cells expressing these markers correspond to a resident stem cell population suitable for long-term replacement therapy. Some reports have shown that HSCs have the ability to promote myocardial regeneration; Orlic *et al.* mobilized HSCs from bone marrow to show that the "trans-differentiated cells" could repair the infarcted heart (Orlic, Kajstura et al. 2001; Perino, Yamanaka et al. 2008). In a complementary study, they isolated HSCs from mice that constitutively expressed the GFP and used these cells to look for cardiac integration and repair (Orlic, Kajstura et al. 2001). After two weeks of myocardial infarction induction, the mice transplanted with the GFP-labelled HSCs presented improved function of the myocardium, and the newly formed myocardium contained GFP-labelled cells that occupied up to 68% of the infarcted portion of the ventricle. These labelled cells also expressed sarcomeric actins and myosins, troponin and several cardiac-associated transcription factors; however, the sarcomeric structures appeared disorganized. These results were further supported with a report by Rota *et al.* (2007) who showed that bone marrow cells engraft, survive, and grow within the spared myocardium after infarction (Rota, Kajstura et al. 2007). More specifically, they reported that locally delivered bone marrow cells generate new myocardium composed of integrated cardiomyocytes and coronary vessels, and that the cardiomyogenesis was independent of cell fusion, but dependent on close contact with resident cardiomyocytes (Rota, Kajstura et al. 2007).

On the other hand, several reports have contradicted these findings. Murry *et al.* reported that the HSCs did not express cardiac specific genes, and that they could not detect any increase in cardiomyocytes in a murine model of MI region of the heart (Murry, Soonpaa et al. 2004). Also, Kawada *et al.* showed that mobilized murine HSCs expressing GFP do not differentiate into cardiomyocytes after myocardial infarction (Kawada, Fujita et al. 2004).

### 2.1.6.7 Mesenchymal stem cells

Mesenchymal stem cells possess multipotent capabilities; they can proliferate *in vitro* and *in vivo*. These cells are able to induce angiogenesis, and to differentiate into cell types belonging to the mesodermal lineage, such as osteogenic, chondrogenic, adipogenic and myogenic cells. The MSCs have been first isolated from bone marrow (Pittenger, Mackay et al. 1999), which is the main source of MSCs. But they have also been isolated from other sources, like dental pulp (Pierdomenico, Bonsi et al. 2005), adipose tissue (Izadpanah, Trygg et al. 2006), umbilical cord blood (Wang, Seshareddy et al. 2009), chorionic *villi* of the placenta (Igura, Zhang et al. 2004), amniotic fluid (Tsai, Lee et al. 2004), and others.

Although, MSCs obtained from different sources share the expression of certain genes, differences in self-renewal, proliferation, and differentiation have been reported to exist among these various sources of MSCs (Arminan, Gandia et al. 2008).

Animal studies and early clinical studies suggest that therapeutically delivered MSCs can improve heart function after acute MI. More specifically, MSCs seemed to improve contractility, wall thickness and decrease necrosis (Nagaya, Fujii et al. 2004). It has been proposed that the observed therapeutic benefits are probably mediated through the release of a variety of signalling molecules, which may be anti-inflammatory, anti-apoptotic and angiogenic.

The paracrine factors secreted by bone marrow mononuclear cells and endothelial progenitor cells are well characterized (de Macedo Braga, Lacchini et al. 2008). More recently, the gene expression profiles of cultured MSCs were determined by microarray. In particular, transcripts of IL-6, leukemia inhibitory factor (LIF) and vascular endothelial growth factor (VEGF) family members were found to be expressed in human MSCs. Also expressed are several mRNAs for matrix-mediating factors such as matrix metalloproteinase (MMP)-2, inhibitors such as tissue inhibitor of metalloproteinase-1, 2, and matricellular proteins

(thrombospondin-1 and tenascin C) were also highly expressed in human MSCs. The results of the microarray analysis demonstrated that cultured human MSCs expressed mRNAs for a variety of secreted factors that may be cardioprotective and reparative (Iso, Spees et al. 2007). The anti-apoptotic role and broader spectrum of all the paracrine molecules produced by MSCs suggest that the release of these mediators could augment angiogenesis, reduce apoptosis and promote the recruitment of circulating progenitor stem cells into the injured tissue, leading to the favourable remodelling of the damaged heart (Kinnaird, Stabile et al. 2004).

Studies by Tang *et al.* have reported that the expression of basic fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and stromal cell-derived factor (SCDF-1) is increased after MSCs in a rat model of MI. The FGF, is a mostly mitogenic protein, is produced by MSCs and stimulates sustained quiescence and proliferation in uncommitted and committed MSCs (Benavente, Sierralta et al. 2003), and it has been suggested that SCDF-1 may have tissue protective and regenerative roles in solid tissues, this by activating the cell-survival pathway mediated by protein kinase B (Tang, Zhao et al. 2005; Saxena, Fish et al. 2008).

These data suggest that paracrine effects are responsible for the cardioprotection seen with MSC therapy cells in the bone marrow.

Some authors claim that there is only limited data suggesting cardiomyogenic differentiation of MSCs (Li, Guo et al. 2009). But apparently, MSCs have a wider differentiation potential than what it has been previously thought. Some reports have shown that MSCs can differentiate into vascular endothelial cells, and smooth muscle cells (Song, Lee et al. 2007), but more importantly, it has been shown that MSCs are able to differentiate into cardiomyocytes *in vivo* and *in vitro.* For instance, *in vitro* studies have shown that MSCs express some cardiac transcription factors and structural proteins when exposed to 5-azacytidine, a DNA demethylating agent, which induces pronounced epigenetic changes. The

reputed formation of cardiomyocytes after 5-azacytidine exposure was based on the spontaneous contraction of cells in culture, action potentials similar to those found in foetal cardiomyocytes, the expression of sarcomeric proteins, and the expression of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). These peptides are expressed in cardiac muscle; but the sarcomeric proteins used as markers, skeletal α-actin and β-MHC, are associated with both cardiac and skeletal muscle cells. In addition, published images clearly showed multinucleated cells, which are most typical of skeletal muscle (Makino, Fukuda et al. 1999). These results suggest that the MSCs exposed to 5-azacytidine were not authentic cardiomyocytes, but probably a mixed population of muscle cells, due to the hypomethylation of regulatory genes caused by 5-azacytidine treatment. Therefore, it is very possible that 5-azacytidine differentiation induction is not cardiac specific (Bittira, Kuang *et al.* 2002).

Differentiation of MSCs has also been achieved by co-culture with cardiomyocytes (Rangappa, Entwistle et al. 2003); these cells after co-culture with cardiomyocytes were positive for β-myosin heavy chain, cardiac troponin T, cardiac troponin I, cardiac α-actin, and desmin. They also exhibit $Ca^{2+}$ transients, and several types of action potentials (Li, Yu et al. 2008). Furthermore, it has been reported that even without stimulation, MSCs express certain cardiac markers, such as connexin-43, α-actinin, and GATA-4, but fail to express cardiac specific structural proteins (Bayes-Genis, Roura et al. 2005). The ability of MSCs to form cardiomyocytes is still in doubt and in almost all the cases in which cardiomyogenic differentiation has been reported, the cells had to go through a cultivation period that may have altered some basic properties. *In vivo* differentiation has also been reported, there are some studies stating that MSCs can differentiate into cardiomyocytes when injected into healthy murine heart (Toma, Pittenger et al. 2002). The MSCs however remain attractive as a vehicle for cell transplantation or for tissue engineering, because they can be obtained in relatively large numbers and are easily expanded in culture, in addition they can be obtained

from the bone marrow of the patient, eliminating the problems related with graft rejection. Therefore, MSCs remain a promising source for cell therapy in cardiac diseases.

There are many factors influencing the capabilities of MSC differentiation, such as: culture medium, presence and concentration of undetermined growing factors in the foetal bovine serum (FBS), number of passages, as well as differentiation inducer(s) added to the medium. In fact, some reports have shown that MSCs have certain predisposition to differentiate into cardiomyocytes (Bayes-Genis, Roura *et al.* 2005) or have passage-restricted differentiation potential into cardiomyocyte-like cells depending on the time they have been in culture, with a very possible variation among species (Zhang, Li *et al.* 2005). Phenotype and gene expression are affected during culture (Vacanti, Kong *et al.* 2005). For these reasons, it is possible that full and effective cardiomyogenic differentiation of MSCs has not been achieved yet.

## 2.2. Oxytocin (OT)

### 2.2.1 Definition and structure

The peptide hormone OT has many functions in the human body, including facilitating milk expulsion during lactation (Nishimori, Young et al. 1996), and mediating maternal behaviour (Young, Wang et al. 1998). However, OT is best known as a powerful uterotonic agent that stimulates uterine contractions during labour, for which it is frequently used to augment labor and to prevent postpartum haemorrhage (Wise and Clark 2008). Oxytocin was the first hormone to have its structure elucidated and the first to be chemically synthesized in a biologically active form (Du Vigneaud, Ressler et al. 1953). Oxytocin is a cyclic nonapeptide (single linear chain of nine amino acids), derived from large precursor proteins called pro-peptides, which in turn are derived from even larger molecules known as pre-propeptides. The molecular formula of OT is $C_{43}H_{66}N_{12}0_{12}S_2$ and it has a molecular weight of 1,007 Da. It possesses a cyclical disulphide configuration being the ring shape essential to its biological

effect. There is a close homology between OT and vasopressin, the difference consisting only in two amino acids. The similarity in chemical structure explains several common properties which can become evident when OT is administered in high doses (Lippert, Mueck et al. 2003).

### 2.2.2 Oxytocin synthesis

The synthesis of OT occurs in the neural cell bodies of the *supraopticus nucleus* (SON) and *paraventricularis nucleus* (PVN) of the hypothalamus. From this nuclei, it is transported into the neurohypophysis in the form of a precursor molecule, oxytocin-neurophysin, this complex passes down the axons of hypothalamic nuclei to be stored as a dimeric in dilated nerve terminals in the posterior lobe of the pituitary gland (neurohypophysis), from which it is released into the blood stream in a pulsatile form (al-Eknah and Homeida 1991; Lippert, Mueck et al. 2003).

The inactive precursor protein is progressively hydrolyzed into smaller fragments (one of which is neurophysin I) via a series of enzymes. The last hydrolysis which releases the active oxytocin nonapeptide is catalyzed by peptidylglycine alpha-amidating monooxygenase (Sheldrick and Flint 1989).

The release of the neurosecretory product from the terminals occurs mainly as a consequence of invasion of the terminal membranes by action potentials. The electrical activity of the neuron is thus the immediate determinant of secretory output (Bicknell 1985).

### 2.2.3 Other sites of OT synthesis

Recent studies have shown that OT is a hormone that can be synthesized at many sites and several physiological activities have been attributed to this peptide.

It was believed that both OT and arginine vasopressin were exclusively released from the neurohypophysis and synthesized only by hypothalamus, but it has been reported that OT can be founded in other tissues, such as *corpus luteum*, heart, and vasculature as described next.

#### 2.2.3.1 Heart

Oxytocin may be involved in the regulation of blood volume because of its natriuretic properties and its relationship with the modulation of blood pressure secondary to the release of ANP. Based on this hypothesis, Jankowski *et al.* (Jankowski, Hajjar et al. 1998) carried out a study to determine whether OT was synthesized locally in the heart. They reported that, in fact, the heart is a site of OT synthesis and also of release. The presence of OT was detected by radioimmunoassay in the four chambers of the rat heart, and the transcription of OT gene was demonstrated by the amplification of rat heart cDNA using PCR technique. They have shown the presence of OT in the effluent of isolated rat heart perfusates and in the medium of cultured atrial myocytes, which confirms that OT is synthesized and released from the heart. It was shown that cardiac OT is structurally identical to, and therefore derived from the same gene as, the OT found primarily in the hypothalamus.

The localization of OT in the heart was also determined by immunostaining, showing its presence in the atria and ventricles, the signal being stronger in the atria, the right atrium with the strongest signal. The immnunolocalization revealed the presence of OT in fibroblasts and atrial myocytes. These results suggested that OT present in the heart is rather constitutively secreted than stored because immunostaining did not reveal abundant OT tissue deposits, but faint staining was localized in small groups or single cells randomly distributed throughout the atrium; OT was found in the heart perfusate and indicating it is secreted from the heart, and the OT concentration was relatively high in the incubation medium of cultured atrial

myocytes and low within these cells, which suggests immediate secretion of OT by the cells after its synthesis and posttranslational processing (Jankowski, Hajjar et al. 1998).

#### 2.2.3.2 Vasculature

Oxytocin has been identified in rat, sheep, and dog vessels by radioimmunoassay technique, but also OT transcripts containing the coding sequence of the OT gene using the RT-PCR have been found (Jankowski, Wang et al. 2000).

In rats, the superior vena cava and the thoracic aorta were collected to determine the presence of OT in the homogenate of these vessels by radioimmunoassay with an antibody that recognizes specifically biologic active OT (Jankowski, Wang et al. 2000).

The vessels of the rat and other species contain significant amounts of OT immunologically equivalent with synthetic OT. Levels of vascular OT (2–4 ng/mg protein) are comparable to those obtained in other peripheral tissues, such as the ovary (Ivell and Richter 1984) and thymus (Geenen, Legros et al. 1986), and are only 2- to 4-fold lower than the levels found in the hypothalamus (Jankowski, Wang et al. 2000).

#### 2.2.3.3 Ovary and uterus

The synthesis of OT by the ovary of the human and sheep was demonstrated by radioimmunoassay and chromatography (Wathes and Swann 1982). Some years later it was found that OT is synthesized in the ovary of other species like cow (Wathes, Swann et al. 1983), cynomolgus monkey (Khan-Dawood, Marut et al. 1984), goat (Homeida 1986), baboon (Khan-Dawood, Huang et al. 1988) and sow (Jarry, Einspanier et al. 1990). It has been shown that the *corpus luteum* of the sow only stores OT, because the synthesis of OT is carried out by the uterus (Hu, Ludwig et al. 2001), which is the major site of production of reproductive tract origin OT. This was also observed in the rat (Zingg, Rozen et al. 1995) and the mare (Behrendt-Adam, Adams et al. 1999).

### 2.2.4 Regulation of oxytocin production and secretion

The human gene for OT-neurophysin I encoding the OT pre-propeptide is mapped to chromosome 20p13, and consists of three exons: the first exon encodes a translocator signal, the nonapeptide hormone, the tripeptide processing signal, and the first nine residues of neurophysin; the second exon encodes the central part of neurophysin (residues 10-76); and the third exon encodes the COOH-terminal region of neurophysin (residues 77-93/95)(Rao, Loffler et al. 1992).

The OT pre-propeptide is subject to cleavage and other modifications as it is transported down the axon to terminals located in the posterior pituitary (Brownstein, Russell et al. 1980). The mature peptide products, OT and its carrier molecule neurophysin, are stored in the axon terminals until neural inputs elicit their release (Renaud and Bourque 1991). The main role of neurophysin, a small (93-95 residues) disulfide-rich protein, is related to the proper targeting, packaging, and storage of OT within the granules before release into the bloodstream. Oxytocin is found in high concentrations (>0.1 M) in the neurosecretory granules of the posterior pituitary complex in a 1:1 ratio with neurophysin. In such complexes, OT-neurophysin dimers are the basic functional units as suggested by the crystal structure of the neurophysin-OT complex (Rose, Wu et al. 1996). The Cys-1 and Tyr-2 in the OT molecule are the principal neurophysin binding residues. In particular, the protonated α-amino group (Cys-1) in OT forms an essential contact site to neurophysin *via* electrostatic and multiple hydrogen bonding interactions. Due to its dependence on amino group protonation ($pK_a$ ~6.4), the binding strength between OT and neurophysin is much higher in an acidic compartment like the neurosecretory granules (pH ~5.5). Conversely, the dissociation of the complex is facilitated as the complex is released from the neurosecretory granules and enters the plasma (pH 7.4) (Gimpl and Fahrenholz 2001).

### 2.2.5 Central regulation of oxytocin production and secretion

In the central nervous system, the OT gene is primarily expressed in magnocellular neurons in the hypothalamic PVN and SON. Action potentials in these neurosecretory cells trigger the release of OT from their axon terminals in the neurohypophysis (Poulain and Wakerley 1982).

In the PVN, two populations of OT-staining neurons have been identified: magnocellular neurons which end in the neurohypophysis and parvocellular neurons which end elsewhere in the central nervous system (CNS). The strength and frequency of electric stimulation received at the PVN and SON will determine the rate of OT secretion in the blood stream (Poulain and Wakerley 1982; Bicknell, Chapman et al. 1985).

The OT concentrations present in the extracellular fluid of the SON have been reported to be >100- to 1,000-fold higher than the basal OT concentration in plasma, meaning more than 1-10 nM. For example, milk ejection reflex, might release even higher local OT concentrations (Landgraf, Neumann et al. 1992).

Plasma OT does not readily cross the blood-brain barrier, and there is no relationship between the release of OT into the blood by the neurohypophysis and the variations in OT concentrations in the cerebrospinal fluid (CSF). Peripheral stimulations such as suckling or vaginal dilation that elicit large increases in plasma OT may or may not change the concentration of OT in the CSF. As shown in rats, electrical stimulation of the neurohypophysis only evokes the release of OT into the blood, whereas stimulation of the PVN elicits a release of OT into the blood and into the CSF (Jones, Robinson et al. 1983). After hypophysectomy, OT disappears from the blood, whereas its concentration increases in

the CSF (Dogterom, Van Wimersma Greidanus et al. 1977). The OT in the CSF is probably derived from neurons that extend to the third ventricle, the limbic system, the brain stem, and the spinal cord. In the CSF, OT is normally present at concentrations of 10-50 pM, and its half-life is much longer (28 min) than in the blood (1-2 min) (Jones and Robinson 1982). In humans and in monkeys, a circadian rhythm in the OT concentrations in the CSF has been found with peak values at midday. Also it has been described that environmental lighting and circadian rhythms regulate levels of OT in the CSF in mice and rats (Forsling 2000; Devarajan and Rusak 2004; Devarajan, Marchant et al. 2005). Circadian rhythms have also been observed in plasma OT concentrations (Lindow, Newham et al. 1996; Morawska-Barszczewska, Guzek et al. 1996; Devarajan and Rusak 2004).

Therefore, at the OT plasma concentration level, OT secretion is produced in an intermittent manner without being ruled by a biologic rhythm, consequently OT secretion is dictated by peripheral stimuli (Brimble and Dyball 1977; Brimble, Dyball et al. 1978).

It was thought that contrary to other hormones, OT secretion was not controlled by a feedback loop that allows the modulation of its production. And that because of this reason, it is possible to observe high plasma concentrations during certain physiologic situations; OT secretion occurs in a pulsatile manner during birth and lactation (Brimble, Dyball et al. 1978; Higuchi, Honda et al. 1985; Fuchs, Romero et al. 1991; Uvnas-Moberg and Eriksson 1996). More recently, an *in vitro* study reported that progressive increases from extremely low OT concentrations (0.1–10 fM) to high concentrations (0.1–10 nM) induced excitation and subsequent spike frequency reduction (*i.e.* a gradual **reduction** of the firing **frequency**) in OT neurons. These results suggest that the specific autoregulatory effects of OT, and perhaps other neuropeptides as well, are time and concentration dependent (Wang, Ponzio et al. 2006).

On the other hand, it has been reported that during parturition, a receptor-mediated positive feedback action of OT on its own release within the SON, which seems to be involved in the progress of parturition without significantly affecting circulating OT levels. Oxytocin released within the SON might be important for the coordinated activation of OT neurons and for the synergistic central and peripheral OT effects involved in the regulation of parturition-related events necessary for the survival of the newborn, including the onset of lactation (Neumann, Douglas et al. 1996).

Pulsation frequencies reach their maximum during the last phase of birth (Fuchs, Romero et al. 1991). This type of secretion represents an advantage during a long labour procedure, by assuring an effective and rapid contractile activity from the uterus, that responds to OT stimuli (Crall and Mattison 1991).

### 2.2.6 Other factors controlling oxytocin secretion

Oxytocin is very similar in structure to vasopressin (AVP). Vasopressin is also synthesized and realized by mangnocellular neurosecretory neurons when action potentials arrive from the cell bodies in the SON and PVN (Leng, Brown et al. 1999). The major stimuli that excite these AVP neurons are an increase in extracellular osmolarity, generally a result of increased [Na+] (with Cl-), and decreased system blood volume and pressure (Leng, Brown et al. 2001; Bourque 2008).

The AVP and OT amino acid sequences differ only in the 3 and 8 positions. Oxytocin secretion could also be stimulated by hyperosmolarity of extracellular fluid, at least in the rat (Leng, Brown et al. 2001)and it has peripheral natriuretic actions. These natriuretic actions involve direct effects in the kidney (Antunes-Rodrigues, de Castro et al. 2004) and actions in the right atrium to stimulate atrial natriuretic peptide (ANP) secretion (Haanwinckel, Elias et al. 1995). Release of OT is also stimulated by induced hypovolemia in a rat model (Stricker, Hosutt et al. 1987).

Moreover, OT is secreted postprandially (Haanwinckel, Elias et al. 1995), and may contribute to post-prandial ANP secretion (McCann, Gutkowska et al. 2003), and hence post-prandial natriuresis.

Oxytocin is a more potent natriuretic hormone than AVP. These effects can be explained by a direct action of both peptides on specific receptors already shown to be present in the tubular cells of the kidney (Samson and Schell 1995). The different potencies of these hormones can be attributed to a relative affinity of OT for its own receptor or to its lower affinity for $V_2$ and $V_1$ AVP receptors.

Also, some studies have suggested a synergistic effect of AVP and OT in the inner medullary collecting duct, where both peptides induce an increase in cAMP accumulation and natriuresis (Baek, Kwon et al. 1996; Evans 1996). Oxytocin binds to the AVP subtype $V_2$ receptor because of its structural similarity to AVP. The urinary sodium excretion induced by OT is completely blocked by pre-treatment with an OT receptor antagonist, but not affected by an AVP subtype $V_1$ receptor antagonist (Stock, Granstrom et al. 1989).

The central administration of OT decreases salt intake (Blackburn, Samson et al. 1993). The inhibitory role of OT in the control of sodium appetite has been supported by studies in OT knock-out mice that display an enhanced salt appetite compared with OT +/+ mice after water deprivation (Amico, Morris et al. 2001).

In women, during pregnancy OT is involved in the maintenance of hypervolaemia. Salt appetite is increased in pregnancy (Atherton, Dark et al. 1982) and total circulating sodium is increased, although water retention reduces plasma [Na+] while increasing volume. In these circumstances a natriuretic response can be expected, through stimulation of ANP secretion by right atrial distension (Haanwinckel, Elias et al. 1995), but this kind of response is either

ineffective or suppressed in pregnancy. Indeed, circulating ANP levels decrease in late pregnancy (Nadel, Ballermann et al. 1988; Jansakul, King et al. 1989; McCann, Antunes-Rodrigues et al. 2002). Reduced OT content in the atria in pregnancy, and reduced OTR mRNA expression near term (McCann, Antunes-Rodrigues et al. 2002) may contribute to this reduced ANP secretion.

### 2.2.7 Oxytocin receptor: Classification and activation

The physiological effects of OT are primarily mediated through a specific G protein-coupled receptor (GPCR), the OTR. The OTR preferentially couples to Gαq/11 proteins to activate phospholipase C (PLC) which hydrolyzes the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) to form inositol 1,4,5-trisphosphate (IP3), which mobilizes $Ca^{2+}$ from the sarcoplasmic reticulum, and diacylglycerol (DAG), which activates protein kinase C, resulting in the phosphorylation of several target proteins (Ku, Qian et al. 1995; Holda, Oberti et al. 1996; Willets, Brighton et al. 2009).

Oxytocin causes a rapid increase in intracellular free $Ca^{2+}$, activates mitogen-activated protein (MAP) kinase, and stimulates prostaglandin $E_2$ synthesis by activating cyclooxygenase. In addition, an increase in $Ca^{2+}$ bound calmodulin leads to the activation of kinase primarily responsible for the phosphorylation of MLC, regulating myometrial contractility (Nakamura, Itakuara et al. 2000; Gimpl and Fahrenholz 2001).

The high-affinity receptor state requires both $Mg^{2+}$ and cholesterol, which probably act as allosteric modulators (Gimpl and Fahrenholz 2001).

The presence of OTR in several tissues and organs demonstrates that OT effects are not only restricted to the reproductive system (Nakamura, Itakuara et al. 2000). The OTR has been cloned from human (Nakamura, Itakuara et al. 2000), rat (Bale and Dorsa 1997) and other species. It is highly conserved across the species and has been found in a variety of tissues, particularly in the uterus (Kimura, Tanizawa et al. 1992), but also in the mammary and

pituitary glands, brain (Adan, Van Leeuwen et al. 1995), kidney (Stoeckel and Freund-Mercier 1989), thymus (Elands, Resink et al. 1988), ovary (Sernia, Gemmell et al. 1989), testis (Bathgate and Sernia 1994), heart (Jankowski, Hajjar et al. 1998) and blood vessels (Jankowski, Wang et al. 2000).

The function and physiological regulation of the OT/OTR system is strongly steroid dependent, but the OTR modulators are not only present among hormones, they also include neurotransmitters and cytokines, like IL-1β and IL-6 which have inhibiting effects on OTR expression in myometrial cells. This observation was supported by the report of Rauk and Friebe-Hoffmann who found down regulation of OTR in cultured uterine myocytes treated with IL-1 for 24 hours (Rauk and Friebe-Hoffmann 2000; Bussolati and Cassoni 2001; Schmid, Wong et al. 2001).

The regulation by gonadal and adrenal steroids is one of the most remarkable features of the OT system, but unfortunately this has not been clarified (Gimpl and Fahrenholz 2001). On the other hand, it has been demonstrated that the estrogens cause an improvement in the OT and OTR gene expression (Gimpl and Fahrenholz 2001; Richter, Kubler et al. 2004; Hong, Park et al. 2006).

### 2.2.8 Oxytocin receptor

#### 2.2.8.1 Brain

There is a high diversity of OTR distribution among the species (Gimpl and Fahrenholz 2001). For example, the *ventral subiculum* in the *hippocampus* contains high densities of OT binding sites in the rat (Shapiro and Insel 1989), whereas in the guinea pig (Tribollet, Barberis et al. 1992), the hamster (Dubois-Dauphin, Pevet et al. 1992), and the marmorset (Schorscher-Petcu, Dupre et al. 2009), no OT binding sites were detected in this area. In monogamous *vs.* polygamous voles, OTR distribution was shown to reflect social

organization (Insel and Shapiro 1992). In human brains, dense OTR were visualized in the *pars compacta* of the *substantia nigra* unlike in several other species examined so far. Thus, in humans, nigrostriatal dopamine neurons could be a target for OT, and the OT system may be involved in motor and other basal ganglia-related functions. Strong OT binding intensity was also observed in the basal nucleus of Meynert, but OT binding sites were lacking in the *hippocampus*, *amygdala*, *entorhinal cortex*, and olfactory bulb of human brains (Loup, Tribollet et al. 1991).

**2.2.8.2 Kidney**

Studies performed in rats showed by autoradiographical analysis the existence and precise localization of OTR in the kidney. The distribution of OT binding sites experience some changes during postnatal development and this event was also observed in the rat brain during maturation (Schmidt, Jard et al. 1990).

Specific OT binding sites have been first detected at embryonic day 17 in the renal cortex, while in the medulla OT binding sites were detected at embryonic day 19 when this region is experiencing its developmental phase. In the adult rat, OT binding sites were exclusively localized in the cortex, mainly concentrated in the *macula densa*. In the inner medulla, the OT binding sites were found on the loops of Henle of the juxtamedullary nephrons (Arpin-Bott, Waltisperger et al. 1997).

It is important to underline that renal cortical OT-binding sites had a higher selectivity for OT *vs.* AVP compared with the medullar sites of binding for both hormones. It was therefore suggested that OT binding sites of the *macula densa* and Henle's loop could represent two subtypes of OTR. The specific profile of localization of the receptors suggests a role performed by OT in the regulation of tubuloglomerular feedback and solute transport (Arpin-Bott, Waltisperger et al. 1997).

Gene expression of OT and OTR in the kidney is regulated by other hormones, such as steroids. It was shown that estrogens induced an increase of OTR gene expression in the outer medulla region and also in OTR at the *macula densa* and proximal tubule cells of ovariectomized female and adrenalectomized male rats. Since, it was probable that OTR may mediate estrogen-induced alterations in renal fluid dynamics, Ostrowski *et al.* (1995) realized a study in which they measured the OTR mRNA levels in kidneys of late-pregnant, peri-parturient, and lactating rats. They found that OTR transcripts could not be detected in renal tissues of peri-parturient females, because, at this time, OTR mRNA levels were highest in uterus but also levels of estrogens were low, there is a stronger influence of progesterone instead (Ostrowski and Lolait 1995).

However, OTR gene expression in *macula densa* cells gradually reappeared and again achieved control levels by *day 20* post partum. It was observed that both the OTR mRNA levels, as well as, the OT binding capacity of rat renal extracts were significantly lower at term. Although in the rat kidney there was no indication for another than the uterine-type OTR gene expression, the mechanisms controlling the expression of OTR genes in uterus, pituitary gland, and kidney are obviously different (Breton, Pechoux et al. 1995).

**2.2.8.3 Heart and cardiovascular system**

The OTR transcripts and OT binding sites were shown to be present on atrial and ventricular sections as detected by *in situ* hybridization and autoradiograpy. Furthermore, the OTR gene is expressed in all chambers of the rat heart, and the analysis of the RT-PCR products indicated the presence of the uterine-type OTR in the heart. Although the OTR mRNA levels in the atria were found to be higher than in the ventricles, overall the OTR mRNA levels were calculated to be at least 10 times lower than the OTR mRNA level present in the uterus of a non-pregnant rat (Gutkowska, Jankowski et al. 1997). The OTR mRNA has been also found in vascular tissue. This was demonstrated by RT-PCR amplification (Jankowski, Wang et al.

2000). Additionally, it has been demonstrated that human vascular endothelial cells express OTR that are structurally identical to the uterine and mammary OTR (Thibonnier, Conarty et al. 1999).

### 2.2.9 Oxytocin effects extern to the reproductive system

The OT and OTR effects in the reproductive system are well known. But, the proofs of other sites of OT synthesis, out of the reproductive system, and the wide distribution of its receptors have shown that the OT system is much more complex. Therefore, OT is related with more physiological activities than previously thought. Recently, it has been shown that OT/OTR also participate in the regulation of different behavioural, neuro-mediated functions, including sexual and maternal behaviour, memory, food and drink intake, and modulation of anorexia, this activities have shown marked differences among the species (Gimpl and Fahrenholz 2001; Rotzinger, Lovejoy et al. 2009; Stein 2009; Andari, Duhamel et al. 2010; Gouin, Carter et al. 2010).

### 2.2.10 Oxytocin renal effects

One of the main targets of the neurohypophyseal hormones is the control of fluid and electrolytes excretion is the kidney. It has been documented that OT is involved in the management of water and electrolytes excretion. Renal effects of OT have been reported in rats, dogs and more recently in men (Andersen, Engstrom et al. 1992; Conrad, Gellai et al. 1993; Forsling, Judah et al. 1994; Rasmussen, Simonsen et al. 2004). Though, these effects are variable depending on the target species, dose and administration regimen (bolus, infusion, pulsatile).

The stimuli that promote OT release are the hypovolemia or hyperosmolarity. Oxytocin is a non-hypertensive natriuretic agent and it is involved in normal osmolar regulation, which is

presumably different from the volume regulatory components of $Na^+$ homeostasis (Gimpl and Fahrenholz 2001; Mahia, Bernal et al. 2009).

Some studies have been developed to elucidate the complex effects of OT in renal function. For example, the acute administration of physiological doses of synthetic OT in conscious rats produced a modest increase in glomerular filtration rate (GFR) and effective filtration fraction (Conrad, Gellai et al. 1993).

It has been reported that OT has natriuretic effects in rats. Haanwinckel *et al.* (1995) showed that OT produce a release of ANP, which is a peptide hormone synthesized in the atria of the heart that also regulates $Na^+$ and water excretion. The release of ANP was mediated by the interaction of OT with its receptors found in the right atrium, ANP release caused in consequence natriuresis and diuresis suggesting that the natriuresis and diuresis effects of OT are mediated by ANP release (Haanwinckel, Elias et al. 1995).

In addition, it has been shown that OT has natriuretic effects by itself. Soares *et al.* (1999) found that the natriuretic effect of OT is caused by the activation of OTR and further production of NO in proximal tubule and *macula densa* cells, resulting in reduced fractional $Na^+$ reabsorption (Soares, Coimbra et al. 1999). It was suggested that OT binding with its receptors on these nitrergic cells in the proximal tubules and *macula densa* leads to an increase of intracellular $Ca^{2+}$ concentration, followed by $Ca^{2+}$-calmodulin-induced stimulation of NO synthase (NOS) and subsequent activation of the soluble guanylate cyclase (sGC) by released NO. The consequent release of cyclic guanosine monophosphate (cGMP) could then mediate natriuresis and kaliuresis *via* closure of $Na^+$ and $K^+$ channels. In cortical collecting duct cells, it has been shown that the NO-inhibited $Na^+$ transport is associated with increased cGMP content. Overall in rats, OT, as well as ANP, induces a concomitant reduction of both extracellular and intracellular fluid volume in states of increased body fluid volume (Soares, Coimbra et al. 1999).

Verbalis *et al.* (1991) found that OT produces natriuresis in rats at physiological plasma levels and that elevated plasma OT levels correlate with increased $Na^+$ excretion (Verbalis, Mangione et al. 1991).

In rats, the natriuresis is not only dose dependent. Sjoquist *et al.* (1999) found that the pulsatile increase in plasma OT did not produce natriuresis. When OT was administrated in bolus with 5 to 10 minutes intervals simulating the OT release in physiologic states, like during suckling, OT did not produce natriuresis. Although, when the same dose of OT was administrated by constant rate infusion (CRI), it produced natriuresis. So, it is possible that the receptors in mammary glands and uterus are sensitive to pulsatile secretion of OT and then the OTR mediating natriuresis require more continuous exposure (Sjoquist, Huang et al. 1999).

Similar effects have been described in the dog, in which OT had a natriuretic but not diuretic effect when administrated also as a one hour infusion (Andersen, Engstrom et al. 1992). In opposition with the effects reported in rats and dogs, Rasmussen *et al.* (2004) reported an anti-diuretic and anti-natriuretic effect in men and this effect has been also reported in non-human primates (Wesley and Gilmore 1985; Rasmussen, Simonsen et al. 2004).

Earlier reports mention that the natriuretic effect of OT might be species-specific or that it is only observed in lower animal species (Conrad, Gellai et al. 1993; Rasmussen, Simonsen et al. 2004), however, it is quite possible that the discrepancies found in the articles reporting the renal effects of OT are related with the differences in animal preparation methodology, doses and administration regimen.

### 2.2.11 Oxytocin cardiovascular effects

Oxytocin may modify blood pressure and heart rate (HR) by affecting CNS controls, or other organs such as the heart, blood vessels, and kidney, or by acting on other mediators like ANP and NO (Petersson 2002).

The cardiovascular effects of OT have been studied mainly in rats; *in vivo* studies reported that daily central administration of OT can cause different effects; intracerebroventricular daily injection during five days caused a long term decrease in mean arterial pressure (MAP) without affecting HR. This effect was present even at eight days after the last injection (Petersson, Alster et al. 1996); in another experiment, OT infusion into the dorsal vagal complex induced a sustained hypertension (Tian and Ingram 1997). Conversely, other authors have not found any change in MAP and HR when OT was administered by microinjection into the *nucleus tractus solitarius* (Vallejo, Carter et al. 1984). Oxytocinergic projections from the hypothalamus to the brain stem appear to be important for the baroreflex control of the HR (Higa, Mori et al. 2002).

Peripheral administration of OT resulted in short lived increase (Costa, Pereira-Junior et al. 2005; Bakos, Bobryshev et al. 2008) followed by a prolonged decrease (Petty, Lang et al. 1985; Petersson, Lundeberg et al. 1999) in MAP. Oxytocin treatment also produced a reduction in HR and contractility (Costa, Pereira-Junior et al. 2005). In human studies, intravenous bolus injection of synthetic OT was found to be associated with hypotension (Thomas, Koh et al. 2007).

The effects of OT, in the absence of central control mechanisms, have been also described in isolated heart of rats and dogs, finding that OT causes negative inotropic and chronotropic effects (Mukaddam-Daher, Yin et al. 2001; Costa, Pereira-Junior et al. 2005). Even though the exact mechanism of these effects have not been fully studied, apparently they are mediated by local release of ANP (Favaretto, Ballejo et al. 1997).

As was mentioned before, the OT/OTR system is present in the heart and it seems that because OT was found stored only in small amounts, it is immediately secreted, which means that it may act locally producing ANP release. After blood volume expansion, ANP also plays a physiological role by causing a negative chronotropic and inotropic effect, and in consequence a decrease in cardiac output resulting in a reduction in the circulating blood

volume (Gutkowska, Jankowski et al. 2000). So, the physiologic mechanism that OT plays in the heart has been explained as follows, the blood volume expansion as a result of increased venous return to the heart would stretch the cardiomyocytes activating OT release. The OT locally released would promote the release of ANP that in turn acts on its receptors to activate particulate guanylyl cyclase and reduce intracellular $Ca^{2+}$ release. The decrease in intracellular $Ca^{2+}$ causes the negative inotropic and chronotropic effects, which in turn reduce cardiac output and in consequence the effective circulating blood volume (McCann, Antunes-Rodrigues et al. 2002).

Gutkowska *et al.* proposed that OT and ANP act in concert in the control of body fluid and in cardiovascular homeostasis (Gutkowska, Jankowski et al. 1997).

In addition, OT was found to modulate the intrathoracic sympathetic ganglionic neurons regulating the canine heart (Armour and Klassen 1990). These authors showed that when OT was injected into right atrial ganglionated plexi, HR and atrial forces were reduced in 5 of 10 dogs studied. However, no cardiac changes occurred when OT was injected into left atrial or ventricular ganglionated plexi. In this study, the connectivity with the CNS was found to be necessary to elicit consistent OT-induced responses. Although OT elicited neuronal and concomitant cardiovascular responses in most dogs when administered adjacent to spontaneously active intrinsic cardiac neurons, OT injection into the superior vena cava only evoked a slight systemic hypotension in two of seven dogs.

In the vasculature, particularly in the endothelial cells (Thibonnier, Conarty et al. 1999), OTR activation leads to an increase in intracellular $Ca^{2+}$, which is related with cell proliferation. It has been proposed that the OTR trophic properties may participate in the maintenance of the integrity of the vascular endothelial lining. This mitogenic action of OT involves several mediator including $Ca^{2+}$ calmodulin kinase II and PKC.

The presence of the OT/OTR system in the vasculature suggests that OT might also play a role in the regulation of vascular tone and therefore in blood pressure. Effects of OT on

vasculature are controversial. *In vitro* experimentations have shown that OT can cause both vasorelaxation and vasoconstriction (Katusic, Shepherd et al. 1986). Oxytocin vasodilatory effect was demonstrated using isolated canine basilar arteries; OT caused endothelium-dependent vasorelaxation, which means that when endothelium was mechanically removed, the vasodilatory effect disappeared (Katusic, Shepherd et al. 1986). On the other hand, OT presented a potent vasoconstrictor effect in uterine arteries, when administrated at great doses; this vasoconstrictor effect was partially counteracted with the administration of an OT antagonist, suggesting the possible binding of OT with arginine vasopressin receptor V1a-type (Chen, Shepherd et al. 1999; Loichot, Krieger et al. 2001).

The controversial effects of OT on vasculature may be related with OTR expression in endothelial cells and vascular smooth muscle cells. Activation of OTR in endothelial cells produces a $Ca^{2+}$-dependent vasodilatory response *via* stimulation of the NO/sGC pathway, consequently producing vasorelaxation (Thibonnier, Conarty et al. 1999). Whereas OTR activation in smooth muscle cells produces an increase in IP3 production and intracellular $Ca^{2+}$ concentration, thus causing vasoconstriction (Yazawa, Hirasawa et al. 1996). Whether, the vasodilatory or vasoconstrictor effects of OT are mediated through its receptor is not clear because OTR and vasopressinergic V1 receptor are co-localized in endothelial and smooth muscle cells along the vasculature. It has been demonstrated that if OT is administrated at high doses, OT can bind to V1a receptors (Chen, Shepherd et al. 1999; Loichot, Krieger et al. 2001; Miller, Davidge et al. 2002).

### 2.2.12 Oxytocin cardio-protective effects

It has been recently reported that perfusion with OT before ischemia can attenuate the extent of irreversible myocardial damage in an induced ischemia/reperfusion (I/R) *in vitro* model of MI in rats; this was demonstrated as a reduced size of infarction. Oxytocin also decreased or mitigated the post-ischemic cardiac stunning, which is a condition characterized by

prolonged mechanical dysfunction that persists after brief episodes of ischemia and reperfusion, without no histological signs of irreversible injury to cardiomyocytes (Pomblum, Korbmacher et al. 2010). Apparently the negative chronotropic but not inotropic action of OT appeared to be involved in the mediation of its protective effects (Ondrejcakova, Ravingerova et al. 2009).

In a I/R rabbit model, postinfarct treatment with OT reduced MI size and improved left ventricle function and remodeling by activating OTR and prosurvival signals, and by exerting anti-fibrotic and angiogenic effects through activation of matrix metalloproteinase-1 (MMP-1), endothelial NO synthase, and vascular endothelial growth factor (Kobayashi, Yasuda et al. 2009).

Using a swine MI model, it was demonstrated that endogenous pre-MI OT levels and timing of administration post-MI are important criteria, if OT is intended to be used as a therapeutic strategy for ischemic heart disease. Pigs with low endogenous OT level receiving OT subcutaneous infusion (10 ng·kg−1·hr−1) starting 8 days post-MI, as well as pigs with high endogenous OT level receiving saline presented lower MI area measured by planimetry, and cardiac function was also less affected. Moreover, high endogenous OT level pigs receiving OT infusion had highly-affected cardiac function associated to bigger MI area, as well as down-regulation of myocardial OTR (Authier, Tanguay et al. 2010).

Recently, Jankowski *et al.* (2010) examined the effect of OT infusion on cell apoptosis, expression of proliferating cell nuclear antigen (PCNA) and inflammation in the acute and subacute phases of rat MI model. Oxytocin infusion increased the number of cells expressing PCNA in the infarct zone, diminished cell apoptosis and fibrotic deposits in the remote myocardium. In OT treated rats, inflammatory response was reduced, since there was less infiltration of neutrophils, macrophages and T lymphocytes. Moreover, expression of proinflammatory cytokines, tumour necrosis factor-alpha and interleukin-6 was also reduced in OT-infused rats with promotion of transforming growth factor-beta. Oxytocin treatment

reduced expression of atrial and brain natriuretic peptides in the infarcted ventricle, as well as the concentration of both peptides in the circulation. These results indicated that continuous OT delivery reduces inflammation and apoptosis in infarcted and remote myocardium, thus improving function in the injured heart (Jankowski, Bissonauth et al. 2010).

### 2.2.13 Oxytocin in cardiomyogenesis

As mentioned before, heart is a site of OT synthesis and release, and OTR is also expressed in all the chambers of the heart. There are supporting data showing a potential role of OT/OTR system during cardiomyogenesis. Protein levels of OT and OTR are up-regulated in the developing rat heart at day 21 of gestation and postnatal days 1-4, when cardiomyocytes are at a stage of intense hyperplasia. Between postnatal days 1 and 66, OT decreased linearly in all the chambers of heart (Jankowski, Hajjar et al. 1998). The expression of OTR, which is eminent in postnatal cardiomyocytes, declined with age to low levels in adults.

### 2.2.14 Oxytocin in cardiomyogenic differentiation of stem cells

When cultures of embryonal carcinoma P19 cell line, often used for studying *in vitro* embryonic development, were supplemented with OT, there was an increased production of beating cell colonies, this increased production of beating cell colonies was completely inhibited when OT supplemented cells were exposed to an OT antagonist (Paquin, Danalache et al. 2002).

Oxytocin also improved cardiomyogenic differentiation of embryonic and adult stem cells. Matsuura *et al.* (2004) reported the presence of Sca-$1^+$ cells in adult hearts which presented some stem cell features. When treated with OT, Sca-1+ cells expressed genes of cardiac transcription factors and contractile proteins and showed sarcomeric structure and spontaneous beating. Isoproterenol treatment increased the beating rate, which was accompanied by intracellular $Ca^{2+}$ transients. The cardiac Sca-$1^+$ cells also expressed OTR

transcripts, and this expression was up-regulated upon OT treatment (Matsuura, Nagai et al. 2004). Oxytocin appeared to be a more potent differentiation inducer of adult Sca-$1^+$ cells than the widely used cardiomyogenic agent 5-azacytidine.

The involvement of the OT/OTR system in cardiomyocyte differentiation was also confirmed by using murine ES cells, as an experimental model. Hatami *et al.* (2007) showed that murine ESCs treated or not with OT could effectively differentiate into functional cardiomyocytes. This conclusion was based on the contractility of the differentiated cultures, the response of these differentiated cells to cardioactive drugs, the typical ultrastructure of cardiomyocytes, and by the specific expression of multiple cardiac-associated molecular markers by the differentiated cells. In addition, this report revealed that OT led to an increase in the differentiation of ESCs into cardiomyocytes and, perhaps, the acceleration in the maturation of ESCs-derived cardiomyocytes. Also, the expression of OTR was detected in the undifferentiated ESCs and OT-induced cells had an up-regulated expression of OTR (Hatami, Valojerdi et al. 2007).

Oyama *et al.* (2007) reported the presence of SP cells in the neonatal rat heart, these cells when treated with OT, expressed cardiac specific transcripts (GATA-4, Nkx2.5, MLC-2v) and proteins (cTnT, MLC-2v, ANP), and they also showed spontaneous beating (Oyama, Nagai et al. 2007).

### 2.2.15 Oxytocin pharmacokinetics and therapeutic use

Even though the wide therapeutic use of OT, in animals and humans obstetrics, the pharmacokinetics of OT have been reported only in a few studies (Homeida and Cooke 1984; Seitchik, Amico et al. 1984; Thornton, Davison et al. 1990; Morin, Del Castillo et al. 2008). The time course of OT plasma concentrations following an intravenous bolus administration in humans is usually described with an open two-compartment model. The pharmacokinetic profile described for animals is similar to that of humans. It has been reported that in goats

after receiving a single intravenous injection of OT (2000 ng/kg), the plasma concentrations of this hormone also followed a bi-exponential decay, with an initial (distribution phase) mean (SD) 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 of OT in goats is 0.46 (0.02) L/kg (Homeida and Cooke 1984), a three times higher value than in men (De Groot, Vree et al. 1995). 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 (Lundin, Broeders et al. 1993).

Recently, a study performed in anesthetized rats provided evidence for non-linear pharmacokinetics, concluding that The pharmacokinetic properties and persistence of exogenous OT are not proportional to dose (Morin, Del Castillo et al. 2008).

In humans, the recommended method of OT administration for labour induction is the CRI at an initial rate of 0.5 – 1 mU/min (0.85 – 1.71 ng/min), followed by subsequent increases of 1 mU/min (1.71 ng/min) for 40 to 60 minutes. These recommendations are based on a study performed in 11 patients in the early 80's. It was found that this rate of perfusion produces a linear increase in the plasma concentrations of OT, and that a perfusion of around 40 minutes is needed to reach the steady state. This finding suggested that the increase of the doses before this 40 minutes period will carry out an inutile exposure of the patient to higher doses than those actually needed (Seitchik, Amico et al. 1984).

It is important to highlight that OT plasma concentrations do not reflect the actual response of the uterus or labour progression. Moreover, no correlation has been found between the increases in plasma concentrations of administered OT and the increases in uterine contractions (Perry, Satin et al. 1996).

Pulsatile OT administration during birth has been used in order to better approximate normal physiology; this fact reduces the total quantity of OT required in comparison to the CRI

(Cummiskey, Gall et al. 1989). Therefore, it is still controversial, which is the best method for labour induction, even if OT has been used for several years.

Oxytocin is well absorbed after oral and nasal administration (Seitchik, Amico et al. 1984). After oral administration, OT is metabolized by enzymatic destruction in the gastrointestinal tract. Once, absorbed OT is distributed in the extracellular volume and it does not bind to plasma proteins (Arias 2000).

The plasma half-life of OT is of only a few minutes, the reported half-life values of OT go from 10 - 12 minutes, this was reported in women but it was not specified if women were pregnant or not (Arias 2000). In other studies, a plasma half-life of 5 minutes has been described in pregnant women (Seitchik, Amico et al. 1984).

Baseline plasma concentrations of OT have been described in humans. Leake *et al.* (1981) measured the baseline plasma OT in 25 healthy men, 102 non-pregnant women, and 59 pregnant women from 15–42 weeks gestation. In addition, plasma OT levels were measured at the onset, peak, and immediately after a single uterine contraction in 6 women in the latent phase and 14 women in the active phases of labour, as well as in 19 women at initial presentation of the foetal head on the perineum, and 11 women at the time of delivery of the head during a normal vaginal delivery. Baseline plasma OT concentrations (mean SE) did not vary significantly among men, (1.5 ± 0.2 μU/ml), non-pregnant women (1.4 ± 0.2 μU/ml), or pregnant women before labour (1.3 ± 0.1 μU/ml) and did not differ in an additional subgroup of 20 women receiving oral contraceptive medication (1.8 ± 0.7 μU/ml) (Leake, Weitzman et al. 1981).

In studies conducted during labour, plasma OT concentrations did not correlate with uterine pressure measurements and did not increase significantly over baseline pregnancy concentrations during the latent (1.3 ± 0.2 μU/ml) or active (1.6 ± 0.2 μU/ml) phases of labour. But there was a significant increase in plasma OT levels from the time of initial

visualization of the foetal head to the time of delivery of the head (1.1 ± 0.1 to 4.2 ± 1.1 μU/ml, respectively; $P < 0.05$) (Leake, Weitzman et al. 1981).

In men, after the intravenous administration of 1 IU or (1.71 μg) of OT, the pharmacokinetics of the OT can be described using a two compartment model, the obtained parameters were (mean SE): distribution half-life 2.94 ± 0.96 min, elimination half-life 19.8 ± 13.8 min, total clearance 67.1 ± 13.4 L/h, volume of distribution 33,2 ± 28,1 L, steady state volume of distribution 12.2 ± 5,6 L (De Groot, Vree et al. 1995).

#### 2.2.15.1 Oxytocin elimination

The OT is metabolised mainly in the liver, in the plasma (by oxytocinase) and the kidney, and only a small fraction of unchanged OT is excreted in urine (Seitchik, Amico et al. 1984).

*Metabolic clearance of oxytocin*

The proteolytic enzymes metabolically degrade the peptides by hydrolyzing peptide bonds. Depending on the site of activity the enzymes are classified either as exopeptidases (hydrolyze peptides from either the C- or N- terminal region) or endopeptidases (cleave internal bonds of the peptide). In addition to whole body clearance, there is evidence that OT released at synaptic sites in the brain is also subject of degradation by peptidase enzymes (Claybaugh and Uyehara 1993; McEwen 2004).

The enzymes that participate in the metabolism of OT and other peptides by causing a nonspecific cleavage can give origin to the formation of metabolites (Claybaugh and Uyehara 1993).

These enzymes are:

**Table 1. Enzymes participating in oxytocin metabolism**

| Enzyme | Type | Metabolites |
|---|---|---|
| Post-Proline cleaving enzyme | Serine protease | Leu-Gly-$NH_2$ |
| Chymotrypsin-like enzyme | Carbosypeptidase | Gly- $NH_2$ |
| Disulfide bond cleaving enzyme | Thiol: protein disulfide oxydoreductase | |

(Claybaugh and Uyehara 1993)

The OT is removed from the circulation to be principally metabolized by the oxytocinase (OTase), this enzyme has been studied a few years ago by Nakamura *et al.* (2000) who studied the tissue distribution of the enzyme by locating the mRNA, finding that the enzyme has a broad distribution and it is not limited to the placenta. The sites of location of this aminopeptidase include vascular endothelium, gastrointestinal mucosa, pancreas, bile duct, bronchial epithelium, renal tubules (where OT is metabolized and eliminated), sweat glands, adipocytes and skeletal muscle (Nakamura, Itakuara et al. 2000).

To elucidate the mechanism of action of OTase and its relationship with OT/OTR system Nakamura *et al.* (2000) developed a study in which they used a cell line of human umbilical vascular endothelial cells (HUVEC), It is recognized that these cells express OTR (Nakamura, Itakuara et al. 2000).

It was surprising to find the effects of OT on (OTase) activity, they reported that there is an increase in the OTase activity on the cell surfaces of HUVEC probably caused by the

interaction with OTR, suggesting that the receptor stimulation results in a translocation of intracellular OTase to the plasma membrane *via* signal transduction pathway, so the mechanism of translocation of intracellular OTase in response to OT give as a consequence an increase in the degradation of OT at the cell membrane level. This interesting finding suggests a mechanism of negative feedback of peptide hormones at the cellular level; bioactive peptides stimulate degrading protease activity on cell membranes (Nakamura, Itakuara et al. 2000).

*Renal clearance of oxytocin*

As previously mentioned OT is mainly inactivated by the enzyme OTase, which is present in the plasma and different tissues, but as mentioned above OT is also eliminated in an unchanged state in the urine.

Normally a drug that is filtered, not secreted or reabsorbed by the kidney will have a renal clearance equal to the glomerular filtration rate (LeBlanc PP 1997). In the specific case of OT, since it affects the renal and cardiovascular functions depending on many factors previously discussed, OT could also influence its own elimination by reducing renal clearance.

It has been suggested that OT clearance is increased during gestation. Most of the studies have been focused on humans. Initial investigations in women failed to find differences in metabolic clearance rate of OT during gestation (Amico, Seitchik et al. 1984). However, more recently, Thornton *et al.* (1990) reported a four- to fivefold increase in OT clearance in pregnant compared with nonpregnant women (Thornton, Davison et al. 1990).

While it is generally agreed that OT is rapidly inactivated in plasma from pregnant women (50 % of OT disappears in 5 min at 38$^{o}$ C from plasma obtained at term (Mendez-Bauer CJ 1961), reports of its stability in plasma from non-pregnant subjects vary. Ultrafiltration of human plasma containing exogenous OT and AVP at concentrations ranging from 50-400

μU/ml showed that OT was completely unbound to plasma proteins, while AVP binding was 30% independently on the concentration (Fabian, Forsling et al. 1969).

#### 2.2.15.2 Evidences of non-linear oxytocin pharmacokinetics

While no evidence could be found for the binding of OT, its apparent volume of distribution was not very different from that of AVP that is about two thirds of the extracellular volume (interstitial and intravascular compartments). Fabian *et al.* (1969) reported that after an OT bolus of 2 U, volume of distribution was 7.4 L, and for an OT CRI of 500 mU/min the volume of distribution was 10.5 L. The authors reported that both OT and AVP volumes of distribution exceeded the plasma volume and that diffusion presents an additional complicating factor (Fabian, Forsling et al. 1969).

As the distribution volumes of OT might include the interstitial volume, the determination of their half-life in the blood, following an infusion, may be influenced by diffusion across the capillary membrane (Fabian, Forsling et al. 1969).

Some studies have described OT binding to cloned human OTR in COS-1 transfected cells, in this study the affinity of the cloned OTR to different ligands was analyzed. The authors reported that OTR had a single high-affinity binding site with a dissociation constant ($K_d$) of 6 ± 1.1 nmol/l, this value is similar to previous reports of binding assays in human myometrium (Fuchs, Fuchs et al. 1984) and rat mammary gland (Muller, Soloff et al. 1989). The maximum density of the OT binding sites in the COS-1 transfected cells was $8.9 \times 10^5$ sites/cell (Kimura, Makino et al. 1994).

More recently, Tahara *et al.* (2000) studied the binding of radioactive labelled OT to receptor found in human uterine smooth muscle cells (USMC), finding that specific binding of OT to USMC plasma membranes was dependent upon time, temperature and membrane protein concentration. Scatchard plot analysis of equilibrium binding data revealed the existence of a

single class of high-affinity binding sites with an apparent equilibrium dissociation constant ($K_d$) of 0.76 nM and a maximum receptor density (Bmax) of 153 fmol $mg^{-1}$ protein.

Competitive inhibition of radioactive labelled OT binding showed that OT and AVP receptor agonists and antagonists displaced radioactive OT in a concentration-dependent manner. The order of potencies for peptide agonists and antagonists was: oxytocin> [$Asu^{1,6}$]-oxytocin>AVP=atosiban>d$(CH_2)_5$Tyr(Me)AVP>[$Thr^4$,$Gly^7$]-oxytocin>dDAVP (Tahara, Tsukada et al. 2000).

As previously discussed, OT is closely related to AVP, that produces opposite vascular effects to those described for OT (vasorelaxatory effects). Vasopressin acts through its receptors classified as V1a vascular, V2 renal, and V3 pituitary (also known as V1b). The V1a vascular receptor has been located in endothelial cells and vascular smooth muscle cells. The V1a receptor is found at high densities in vascular smooth muscles causing vasoconstriction by an increase in intracellular calcium *via* the phosphatidyl–inositol-bisphosphonate cascade (Holmes, Landry et al. 2003). However, it has been demonstrated that AVP binding to V1a receptor produces vasoconstriction only in non-vital circulations, whereas in vital circulations like coronary and basilar arteries, as well as renal circulation, AVP causes endothelium-dependent relaxation mediated by NO signalling pathway (Katusic, Shepherd et al. 1986; Hirata, Hayakawa et al. 1997; Evora, Pearson et al. 2003).

The selectivity of OT to OTR and AVP's subtype receptors has been studied, showing that AVP receptors are very selective to AVP. However, *in vitro* experimentations demonstrated that high concentrations of OT (>$10^{-8}$ M) caused vasoconstriction that was eliminated by a specific vasopressin V1a receptor antagonist, demonstrating that high concentrations of OT can cause an erratic binding (Miller, Davidge et al. 2002). This fact could explain the different cardiovascular and renal effects observed in the different pharmacokinetic studies reported until today, not only in humans but also in animal models. It is possible that all the

observed discrepancies are related to OT dosing regimens, and subject preparation (species, gender, and physiological state). These were the general explanations proposed to explain the observed differences among the reports.

On the other hand, most of OT pharmacokinetic studies have used an open two-compartment model to describe the disposition of OT after intravascular administration, but in a recent study from our group (Morin, Del Castillo *et al.* 2008), in which the pharmacokinetic profile after the bolus administration of several doses of OT was analyzed, the authors reported that 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. The administered dose significantly affected the distribution and elimination clearances (CLd and CLt), as well as the central volume of distribution (Vc; hypothetical volume into which a drug initially distributes upon administration), but marginally affected the steady-state volume of distribution (Vss; the volume of distribution observed after the drug has distributed into the tissues). Overall, systemic OT exposure did not increase following the principle of superposition (Wagner 1973) as the time-course of plasma OT concentrations became biexponential at increasing doses (non-linear pharmacokinetics). The main problem of this study was the use of sparse blood sampling; this fact implies limitations for elaborating a more realistic pharmacokinetic model. However, the results obtained in the study performed by Morin *et al.* (2008) allowed revealing that other types of modelling could better explain OT disposition after intravascular administration.

Levy introduced the term target-mediated drug disposition (TMDD). The TMDD model captures the capacity limited binding and saturation of a drug–receptor complex, where a significant portion of the drug binds to its target such that this interaction impacts the disposition of the drug per se (Levy G., 1994). Whereas plasma concentrations of most drugs greatly exceed receptor or target concentrations, agents exhibiting TMDD are bound with

high affinity and to a significant degree (relative to dose), such that this interaction influences the temporal profile of plasma drug concentrations. Although originally posed to describe the effects of extensive drug–target binding in tissues, TMDD has received considerable interest owing in part to its role in saturable clearance mechanisms for specific peptide and protein pharmaceuticals (*e.g.*, receptor-mediated endocytosis) (Lobo, Hansen et al. 2004; Tang, Persky et al. 2004).

It is possible that the pharmacokinetics of exogenous OT depend on its binding kinetics to OTR, and that the time-course of cardiovascular and renal OT effects described in the several studies previously mentioned are linked to the rate of OT-OTR complex formation.

Due to the possible therapeutic potential of pharmacological doses of OT in cardiac regeneration, related with its properties of cardiomyogenic differentiation in adult and ES cells, and its role during cardiomyogenesis, in addition to the recent report of cardio-protective effects related to a reduction in the extent of myocardial damage after induced myocardial infarction, it will be important to better characterize the pharmacokinetic profile of OT in order to minimize the possible side effects related to the use of OT as a therapy.

## 2.3 Nitric oxide

### 2.3.1 Definition and synthesis

Nitric oxide is an uncharged free radical gas produced in biological tissues. It exerts a large number of critical regulatory functions and also plays an important role in pathogenesis of cellular injury (Mujoo, Krumenacker et al. 2006; Li, Cui et al. 2008). Indeed, NO possesses multiple biological actions that contribute to the maintenance of cardiovascular homeostasis (Moncada, Palmer et al. 1991). It is synthesized from its precursor L-arginine by the enzyme NOS, which utilize oxygen ($O_2$) and reduced nicotinamide adenine dinucleotide phosphate as co-substrates. L-citrulline is generated as a co-product (Palmer, Ashton et al. 1988; Wang-Rosenke, Neumayer et al. 2008). The NOS system consists of 3 distinct NOS isoforms,

encoded by 3 distinct NOS genes, including neuronal (nNOS), also known as NOS-1, inducible (iNOS) or NOS-2, and endothelial NOS (eNOS) or NOS-3 (Ignarro 1990; Tsutsui, Shimokawa et al. 2009).

Initial studies indicated that nNOS and eNOS are constitutively expressed mainly in the nervous system and the vascular endothelium, respectively, synthesizing a small amount of NO in a $Ca^{2+}$-dependent manner, both under basal conditions and upon stimulation (Tanaka, Hassall et al. 1993; Ursell and Mayes 1995). The localization of eNOS in the vascular endothelium is of particular importance for cardiovascular physiology, as eNOS maintains basal vascular tone through its release of low levels of NO (Loscalzo and Welch 1995). Initial studies also indicated that iNOS is induced only when stimulated by microbial endotoxins or certain pro-inflammatory cytokines, producing a greater amount of NO in a $Ca^{2+}$-independent manner (Stuehr, Cho et al. 1991). However, recent studies have revealed that both nNOS and eNOS are subject to expressional regulation, meaning that these constitutive isoforms are subjected to up-regulation or down-regulation depending on the stimuli to which NOS expressing cells are subjected (Forstermann, Boissel et al. 1998). Also, iNOS is constitutively expressed at low levels under physiological conditions in various cells and not only in cells involved with immune response (Park, Park et al. 1996). It has also become apparent that in addition to eNOS and iNOS, nNOS plays important roles in the cardiovascular system. Apparently, nNOS plays a role in suppressing arteriosclerotic/atherosclerotic vascular lesion formation. Upregulation of nNOS may play a compensatory role in the presence of reduced eNOS activity (*e.g.*, inflammation and arteriosclerosis) to maintain vascular homeostasis (Wilcox, Subramanian *et al.* 1997).

Thus, NO is involved in more physiological activities than previously anticipated (Tsutsui, Shimokawa et al. 2009).

Nitric oxide exerts its effect through either cGMP-dependent or -independent mechanisms (Mujoo, Krumenacker et al. 2006). The enzyme sGC converts guanosine triphosphate to cGMP (Wang-Rosenke, Neumayer et al. 2008). Soluble guanylyl cyclase is a heme-containing, heterodimeric NO receptor. It consists of two subunits α and β, which make up the active enzyme, this sGC enzyme becomes active when NO diffuses into the cell and binds to the heme domain of sGC (Mujoo, Krumenacker et al. 2006). The cGMP is an intracellular second messenger which regulates various effector systems, such as cGMP-depending protein kinases (PKG), phosphodiesterases (PDE) and ion channels, thus modifying many physiological processes (Wang-Rosenke, Neumayer et al. 2008).

### 2.3.2 Nitric oxide cardio-protective effects

Nitric oxide has been extensively studied in animal models of MI using I/R injury as a model. Some studies demonstrated that the deficiency of eNOS exacerbates myocardial I/R injury (Jones, Girod et al. 1999; Sharp, Jones et al. 2002), while the over-expression of eNOS in transgenic mice (Jones, Greer et al. 2004; Elrod, Greer et al. 2006), the administration of NO donors (Pabla and Curtis 1996), and inhaled NO gas therapy (Hataishi, Rodrigues et al. 2006), conferred protection after MI induction. Some of NO physiological properties make it a potent cardio-protective signalling molecule (Calvert and Lefer 2009). Nitric oxide is a potent vasodilator, which could allow for an improved reperfusion of the injured tissue. Moreover, NO reversibly inhibits mitochondrial $O_2$ consumption, therefore the inhibition or modulation of mitochondrial $O_2$ consumption during early reperfusion leads to a decrease in mitochondrial-driven injury by extending the zone of adequate tissue cellular oxygenation away from vessels (Loke, Laycock et al. 1999). Also, NO is a potent inhibitor of neutrophil adherence to vascular endothelium (Ma, Weyrich et al. 1993). Neutrophil adherence is an important event initiating further leukocyte activation and superoxide radical generation, which in turn leads to injury to the endothelium and perivascular myocardium (Palazzo, Jones

et al. 1998). Besides inhibiting neutrophil adherence to vascular endothelium, NO prevents platelet aggregation, both actions attenuate clot formation (Walter and Gambaryan 2004). Finally, it has been reported that NO has anti-apoptotic properties in hepatocytes, either directly or indirectly by inhibiting caspase-3-like activation *via* a cGMP-dependent mechanism and by direct inhibition of caspase-3-like activity through protein S-nitrosylation (Kim, de Vera et al. 1997; Li and Billiar 1999). Even though, most of the experimental studies investigating the effects of NO after MI induction have reported that NO protects the ischemic myocardium, there are some studies reporting negative effects of NO. An important consideration when evaluating the nature of NO effects in cardiovascular disease models is the concentration or dose of NO that is used. It is now well known that physiological levels (nanomolar) of NO promote cytoprotection and higher levels (high micromolar and millimolar) mediate cellular necrosis and apoptosis (Hibbs, Taintor et al. 1988; Beckman, Beckman et al. 1990; Beckman 1991; Gaillard, Mulsch et al. 1991; Radi, Beckman et al. 1991; Siegfried, Carey et al. 1992; Calvert and Lefer 2009).

A number of studies have evaluated the cytoprotective signalling pathways activated by NO in various experimental systems including isolated cardiac cells and intact hearts. Nitric oxide has been shown to activate a number of cellular targets that are linked to cardio-protection, including components of the reperfusion injury salvage kinase pathway, such as PKC and Erk1/2 (Xuan, Guo et al. 2007). Nitric oxide has also been shown to directly protect cultured rat neonatal cardiomyocyte mitochondria by modulating mitochondrial $Ca^{2+}$ handling, which in turn diminishes reoxygenation-associated $Ca^{2+}$ overload. Mitochondrial $Ca^{2+}$ overload is critical in the pathogenesis of irreversible ischemic cell death. During ischemia, mitochondrial $Ca^{2+}$ uptake limits the increase in cytosolic $Ca^{2+}$ concentration. Cell studies suggest that the extent of rise in mitochondrial $Ca^{2+}$ concentration during ischemia determines the likelihood of reoxygenation-induced hypercontracture and cell death (Rakhit, Mojet et al. 2001). Nitric oxide inhibits the activity of L-type $Ca^{2+}$ channels through S-nitrosylation modifications (Hu,

Chiamvimonvat et al. 1997), leading to less sarcoplasmic reticulum $Ca^{2+}$ loading and less $Ca^{2+}$ induced ischemic injury (Sun, Steenbergen et al. 2006). Furthermore, an increase in NO has been shown to be involved in cardio-protection *via* activation of PKG, which leads to activation of mitochondrial pathways including activation of an ATP-regulated mitochondrial channel that allows transport of $K^+$ into the mitochondria $K_{ATP}$ channel, mitochondrial depolarization secondary to opening $K_{ATP}$ channels protects the heart by reducing mitochondrial $Ca^{2+}$ uptake (Garlid, Paucek et al. 1996; Costa, Pereira-Junior et al. 2005).

### 2.3.3 Nitric oxide in cardiomyogenesis

Preliminary studies have implicated the importance of NO signalling during early cardiomyogenesis in embryonic development. Bloch *et al.* (1999) investigated the expression pattern of NOS isoforms and of the target signalling components of NO pathway, sGC and cGMP, during rat and mouse embryonic cardiomyogenesis. They demonstrated that iNOS and eNOS isoforms are expressed early during mouse and rat embryonic heart development, and that these isoforms were down-regulated starting from embryonic day 14.5 (E14.5). The authors also reported drastic changes in sGC expression and cGMP. To further confirm the role of NO pathway components during cardiomyogenesis, the authors used ESC system as *in vitro* development model or cardiomyogenesis. They confirmed *in vivo* observations, reporting an identical NOS-expression pattern at different stages of cardiomyogenesis (Bloch, Fleischmann et al. 1999).

Krumenacker *et al.* (2006) had studied the role of the NO signalling pathway components (eNOS, iNOS, nNOS), sGC (sGC $\alpha_1$ and $\beta_1$) and PKG genes and sGC activity in murine ESCs subjected to differentiation by EB formation, again ESCs were used as model of *in vitro* embryonic development. The authors reported that un-differentiated ESCs expressed nNOS, eNOS, and sGC$\beta_1$. When cells were subjected to differentiation (EB formation), nNOS decreased and iNOS became detectable, also eNOS expression was up-regulated. In

addition, expression levels of both subunits of sGC, and PKG increased gradually over the time of differentiation in EB outgrowths. Purification of ESC-derived cardiomyocytes revealed that transcript expression on NOS isoforms was very low while both sGC subunits transcripts were abundant and cGMP production mediated by sGC was apparent in this population of cells (Krumenacker, Katsuki et al. 2006). In addition, it has been reported the expression of NO signalling pathway components in human ESCs. The expression of these components followed a similar pattern of that observed in murine ESCs, besides the expression of NO signalling components, when cells were subjected to EB differentiation, cardiac specific markers were detected along with NO components, suggesting the role of NO pathway in the differentiation events of human ESC-derived cardiomyocytes (Mujoo, Krumenacker et al. 2006).

### 2.3.4 Nitric oxide in cardiomyogenic differentiation of stem cells

It has been proposed that NO is a potential differentiation signal. Nitric oxide and NOS isoforms have been shown to be involved in the differentiation of several cell types, including nerve cells (Peunova and Enikolopov 1995), some tumour cell types (Magrinat, Mason et al. 1992), and the heart (Bloch, Fleischmann et al. 1999). For instance, *in vitro* studies performed using murine ESCs have shown that NOS inhibitors arrest differentiation toward a cardiac phenotype and that this effect can be rescued by NO donors (Bloch, Fleischmann et al. 1999).

Kanno *et al.* (2004) reported that NO facilitates cardiomyogenesis in murine ESCs. They reported that exposure to NO, either using NO donors or iNOS transfection, increased both the number and the size of beating foci in EB outgrowths. Cardiac specific genes and protein expression were significantly increased in NO-treated ESCs. These data showed that murine ESC cardiomyogenic differentiation is improved by NO treatment. The authors also suggested that NO may influence cardiac differentiation by either inducing a switch toward a

cardiac phenotype or by inducing apoptosis in cells not committed to cardiac differentiation (Kanno, Kim et al. 2004).

It has also been reported that treatment with NO donors increases the expression of cardiac specific genes and proteins in adult stem cells. Rebelatto *et al.* (2009) reported the effects of two NO donors on two different sources of adult stem cells. Bone marrow and adipose tissue derived MSC were exposed to NO donors. Both *S*-nitroso-*N*-acetyl-d,l-penicillamine (SNAP) and 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEA/NO) are able to activate cGMP-dependent and -independent pathways. The authors found that untreated (control) adipose tissue and bone marrow-derived MSC expressed some muscle markers, and that after treatment with NO donors, the cells presented an up-regulation of some cardiac markers. Moreover, NO donors considerably increased the pro-angiogenic potential mostly of bone marrow-derived MSCs as determined by an increased expression of vascular endothelial growth factor (VEGF) transcripts (Rebelatto, Aguiar et al. 2009).

**2.4 Problematics, hypothesis and objectives**

Due to the beneficial effects related to OT administration after MI, we attempted to characterize the hemodynamic effects of pharmacological doses of OT, using swine as an animal model. Based on the literature review, OT hemodynamic effects are controversial, and have been described to be dependent on the dosing administration or even species-specific. We hypothesize that these controversial effects among the species are related with the complex pharmacokinetics of exogenous OT administration. Therefore, the objectives of the first article, here included were:

- To develop a pharmacokinetic model for OT that will include both endogenous and exogenous OT inputs.
- To evaluate the fitting of the data to the selected pharmacokinetic model.
- To evaluate the time-course relationship of the hemodynamic effects with the time-course of the selected pharmacokinetic model.

In addition, OT has been described as a potent cardiomyogenic differentiation inducer of ESCs, and it has been shown that OT cardiomyogenic differention of ESCs is mediated by the NO pathway, which is also implicated in cardiomyogenic differentiation.

Our hypothesis for the second article was that OT induces cardiomyogenic differentiation of bone marrow derived stem cells, which can be easily obtained and their use does not represent any ethical concerns. Therefore the objectives of this second article were:

- To isolate and characterize porcine bone marrow stem cells (pBMSC).
- To induce OT-mediated cardiomyogenic differentiation of pBMSC.
- To compare the effects of OT differentiation induction with 5-azacytidine, which has been widely described as a cardiomyogenic inducer of adult stem cells.

The hypothesis of the third article was that OT-cardiomyogenic differentiation of pBMSC is also mediated by the NO-sGC pathway. The objectives were:

- To reproduce OT-induced pBMSC cardiomyogenic differentiation
- To elucidate the involvement of the NO-sGC pathway in the OT-mediated differentiation by using pharmacological manipulation.
- To evaluate the effects of the pharmacological manipulation (inhibitors of NO pathway) on the OT-mediated cardiomyogenic differentiation process.

# CHAPTER 3. FIRST ARTICLE

**Oxytocin receptor-mediated pharmacokinetics and its hemodynamic implications in pigs.**

Norma Ybarra, MSc, Eric Troncy, *PhD*, Valerie Morin, MSc, Simon Authier, *PhD*, Sylvain Bichot, MSc, Colombe Otis, MSc, Dominique Gauvin, BSc, Jérôme RE del Castillo**,** *PhD*

GREPAQ, Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, Quebec, J2S 7C6, Canada.

[*] **Corresponding author**:

Jérôme RE del Castillo

GREPAQ – Department of veterinary biomedicine, Faculté de médecine vétérinaire, Université de Montréal, P.O. box 5000, St-Hyacinthe, Quebec, J2S 7C6, Canada

Phone: 450-773-8521 ext. 8288, Fax: 450-778-8102

**Short title:** *OXYTOCIN PHARMACOKINETICS AND PHARMACODYNAMICS*

Experimental work was undertaken at: **GREPAQ**

**Source of support**: This work was funded by the Canadian Institutes of Health Research / Heart and Stroke Foundation of Canada New Emerging Team (#108291) program (E.T.) for operating grant, and the On-going New Opportunities (#9483) fund from Canada Foundation for Innovation (E.T.) for infrastructure support.

## 3.1 ABSTRACT

INTRODUCTION: Due to oxytocin therapeutic potential not only in obstetrics, *e.g.* myocardial regeneration, oxytocin (OT) use represents big challenge not only related to its previously demonstrated pharmacodynamical (PD) effects, but also to its recently observed nonlinear pharmacokinetics (PK). Using conscious and anæsthetized pigs, the aims of the study were to *1)* confirm its nonlinear PK; *2)* develop a physiologically-based PK (PBPK) modeling to describe its fate after administration, with *i)* a target-mediated drug disposition (TMDD) PK model, and *ii)* PK/PD modelization on associated hæmodynamic effects. METHODS: Single *i.v.* bolus of OT was given in conscious male pigs (n=5) 20-25 kg, at doses of 298, 993, 2979, 4964 pmol $kg^{-1}$, and a single *i.v.* bolus of OT at doses of 298 (n=3), or 4964 pmol $kg^{-1}$ (n=9), or a constant rate infusion (CRI) at doses of 6.6 (n=3) or 49.6 pmol $kg^{-1}$ $min^{-1}$ (n=9), were given to anæsthetised male pigs. Hæmodynamic effects (systemic mean arterial pressure and heart rate) of OT administered as a bolus or a CRI were collected. The individual time-course of plasma OT concentration was subject to moment PK analysis. As a null-hypothesis model, an open two-compartment model was tested, as well as the alternative-hypothesis TMDD model. RESULTS: The PK profiles of OT showed a triphasic pattern, with a short-lived distribution phase, a convex transition segment that evolves into an elimination phase. The assessment of goodness of fit revealed that the null-hypothesis model failed to accurately predict plasma concentrations and underestimated the observed values. The goodness of fit of the TMDD model better described OT PK and the saturable binding kinetics of OT with its receptor. Moreover, TMDD model revealed the linear relationships between the observed increases in mean arterial pressure and predicted concentrations. CONCLUSION: The diversity and persistence of OT hæmodynamic effects were strongly connected with the complex PK profile of OT. The CRI input rate offered advantage to a progressive exposure of OT receptors to low plasma OT concentrations that would allow taking most advantage of the TMDD fate of OT in living organism.

## 3.2 INTRODUCTION

Oxytocin (OT) is a versatile neuropeptide that accomplishes many physiological activities besides parturition and milk ejection, both in males and females. Indeed, OT receptor (OTR) expression is not limited to uterus and mammary gland (Gutkowska, Jankowski *et al.* 2000; Gimpl and Fahrenholz 2001), but also in several peripheral tissues such as the thymus, fat cells, pancreas, adrenal glands, kidney, heart and large vessels; suggesting that OT exerts effects over these organs (Gimpl and Fahrenholz 2001; Petersson 2002).

Recent reports have shown that OT reduced infarct size in an *in vitro* Langendorff-perfused rat myocardial infarction model, decreasing post-ischemic cardiac stunning. Apparently the negative chronotropic, but not inotropic action of OT appeared to be involved in the mediation of its protective effects (Ondrejcakova, Ravingerova *et al.* 2009). Also, OT improved cardiac function and remodeling in an *in vivo* rabbit MI model (Kobayashi, Yasuda *et al.* 2009).

But the therapeutic use of OT may cause untoward side effects. Cardiovascular effects of OT have been studied mainly in rats. For instance, subcutaneous or intracerebroventricular OT daily injections caused a decrease in mean arterial pressure (MAP) with no heart rate (HR) repercussions, that outlasted the duration of the dosing regimen (Petersson, Alster *et al.* 1996). In contrast, other authors reported a short-lived increase in MAP accompanied by a simultaneous decrease of HR after intravenous OT administration (Costa, Pereira-Junior *et al.* 2005). In *ex vivo* heart preparations, OT caused negative inotropic and chronotropic effects through mechanisms that remain to be elucidated (Mukaddam-Daher, Yin *et al.* 2001; Costa, Pereira-Junior *et al.* 2005).

In the vasculature, OT effects are controversial, as *in vitro* experimentations have shown that OT can cause both vasorelaxation and vasoconstriction. Vasodilatative effect of OT was

demonstrated using isolated canine basilar arteries; OT caused vasorelaxation only if the endothelium was preserved (Katusic, Shepherd *et al.* 1986). On the other hand, high OT doses caused a potent vasoconstriction of uterine arteries, an effect that was only partially counteracted with the administration of an OT antagonist. This finding suggests the interaction of OT with arginine vasopressin (AVP) receptor V1a-type located on vascular smooth muscle cells (Chen, Shepherd *et al.* 1999).

In a previous study in rats from our group (Morin, Del Castillo *et al.* 2008), we found evidence that OT has nonlinear pharmacokinetics (PK). The administered dose significantly affected the distribution and elimination clearances (CLd and CLt) of OT, as well as its central volume of distribution (Vc), but marginally affected its steady-state volume of distribution (Vss). Overall, systemic OT exposure did not increase following the principle of superposition (Wagner 1973) as the time-course of plasma OT concentrations became biexponential at increasing doses. Because this study used sparse blood sampling designs, the structure of a physiologically realistic PK model could not be elucidated. However, our results revealed the hallmarks of target-mediated drug disposition (TMDD) kinetics (Levy 1994; Mager and Jusko 2001), where the time-course of plasma drug concentrations depends on the binding capacity of its receptor. Versatile PK models of TMDD have been developed in the last decade (Mager and Jusko 2001; Mager 2006), and the influence of its parameters on the shape of the PK profile has been tested through partial derivatives sensitivity analyses (Abraham, Krzyzanski *et al.* 2007).

We hypothesize that the PK of exogenous OT depends on its binding kinetics to OTR, and that the time-course of cardiovascular OT effects are linked to the rate of OT-OTR complex formation. Hence, our study objectives are to develop a TMDD PK model for OT that includes both endogenous and exogenous inputs, evaluate the fitting of the data to the TMDD

model, and to evaluate the relationship of the time-courses of cardiovascular effects with the time-course of the OT-OTR complex pool.

## 3.3 MATERIALS AND METHODS

*Animals, housing and dosing regimens*

All experiments described in this report were approved by the Université de Montréal animal care and use committee (09-Rech-1234). The animals were handled according to Canadian Council on Animal Care guidelines (Olfert 1993).

To elucidate the structure of the PK system, a pilot study was performed with conscious male pigs (n=5), and a main study using anæsthetized male pigs (n=24) (Table 1). All conscious pigs (20-30 kg BW) were dosed a single *i.v.* OT (Bachem Americas, Inc., Torrance, CA, USA) bolus (at least one-week interval) at two doses of 993 and 4964 pmol $kg^{-1}$ BW, and one pig was given all four doses (298, 993, 2979 and 4964 pmol $kg^{-1}$ BW) at 1-wk intervals (see Table 1), giving a total of n=12 bolus experiments. In the main PK study, anaesthetized pigs were dosed a single *i.v.* bolus of OT at doses of 298 or 4964 pmol $kg^{-1}$ BW, or a constant rate infusion (CRI) at doses of 6.6 or 49.6 pmol $kg^{-1}$ $min^{-1}$ (n=3 pigs/dose/dosing), giving a total of n=6 bolus experiments and n=6 CRI experiments. Moreover, one bolus (4964 pmol $kg^{-1}$ BW) and one CRI (49.6 pmol $kg^{-1}$ $min^{-1}$) were each administered to n=3 pigs with limited sampling time-points.

The OT cardiovascular effects were investigated in separate experiments using 23-31 kg BW male pigs (n=6). For each experiment, the pigs were randomized across two OT dosing groups consisting in a 4964 pmol $kg^{-1}$ BW *i.v.* bolus or a 49.6 pmol $kg^{1}$ $min^{-1}$ CRI of 90 minutes duration (n=3 pigs/dose/experiment).

Therefore, in total, 12 bolus and 12 CRI experiments were completed under anæsthesia, and 12 bolus administrations in conscious pigs.

*General anæsthesia protocol*

Anesthetized pigs were premedicated with azaperone 2 mg $kg^{-1}$ *i.m.* and ketamine 15 mg $kg^{-1}$ *i.m.* After induction of anæsthesia with propofol 4 mg $kg^{-1}$ *i.v.*, and tracheal intubation for dispensing a 1.5 - 2% isofluorane/$O_2$ mixture, a Lactated Ringer's solution fortified with 0.43 mg/mL lidocaine was infused intravenously at a rate of 7 ml $kg^{-1}$ $h^{-1}$ to meet fluid maintenance and analgesia needs during the surgery. Ceftiofur sodium 3 mg $kg^{-1}$ *i.m.* was administrated for prevention of infection during surgery. A two-lumen indwelling catheter (Arrow International, CS-16702) was inserted in the external jugular vein of all pigs to enable drug administration and blood sampling.

In all anæsthetized pigs, electrocardiogram tracings (ECG), pulse oximetry ($SpO_2$), capnography ($CO_2$), temperature, inspired fraction of oxygen ($FiO_2$) and inspired-expired anæsthetic gas were monitored continuously during the procedure with the LifeWindow™6000 system (Digicare™ Biomedical Technology, Inc., Boynton Beach, FA, USA). At the end of surgical preparations, a period of at least 30 minutes was allowed for stabilization before starting the evaluation of OT effects on cardiovascular and renal systems.

*Cardiovascular instrumentation*

For this experiment, the carotid artery was fitted with a Micro-Renathane® catheter (MRE-065®, Braintree Scientific, Inc., Braintree, MA, USA) connected to a pressure transducer (Medex LogiCal®, MedExSupply Medical Supplies, Inc., Monsey, NY, USA) for invasive MAP monitoring with the LifeWindow™6000 system. A Swan-Ganz catheter (Medex

LogiCal®) was advanced through the left femoral vein up to a capillary of the pulmonary artery, and connected to identical pressure transducer and monitor for simultaneous recording of pulmonary artery pressure (PAP), pulmonary capillary wedge pressure (PCWP) by wedging a segment of the pulmonary artery, central venous pressure (CVP) and cardiac output (CO).

*Cardiovascular measurements*

Before OT administration, at least three baseline values were recorded every five minutes for HR, MAP, PAP, PCWP, CVP, and CO. After OT administration, the above-listed parameters were continuously recorded for 150 minutes (*i.v.* bolus) or 200 minutes (CRI). Other hemodynamic parameters such as systemic vascular resistance (SVR) and pulmonary vascular resistance (PVR) were calculated additionally using standard equations (Troncy, Francoeur, *et al.* 1997).

*Plasma OT concentration measurements*

The table 1 lists the timing of serial blood sampling (2 mL) used in each experiment.

Blood samples were transferred to chilled EDTA vacuum tubes (Vacutainer, BD, Franklin Lakes, NJ, USA), fortified with 50 nmol pepstatin A, and 100 nmol phenylmethanesulfonyl fluoride (PMSF). Upon blood collection, blood samples were processed and stored as described above. Plasma OT concentration was determined by radioimmunoassay, as previously described by our group (Morin, Del Castillo, *et al.* 2008). Plasma samples were serially diluted to obtain a concentration in the linear analytical range, Standard curves generated with $^{125}$I-OT demonstrated a sensitivity of 0.1 pg and linearity in the range of 0.25-

199 pmol. The cross-reactivity of the antibody was <1% with AVP and vasotocin (Jankowski, Hajjar *et al.* 1998).

*Pharmacokinetic/pharmacodynamic modeling*

The individual time-course of plasma OT concentration was subject to moment PK analysis (Yamaoka, Nakagawa *et al.* 1978) in order to derive the initial estimates of CLt, Vc after bolus *i.v*. administration, and Vss to be used in the compartmental modeling. The general TMDD PK model proposed by Mager and Jusko (Mager and Jusko 2001) was modified to account for endogenous OT production. Briefly, the PK system is composed of two subsystems: the first predicts the fate of plasma and tissue free OT, and consists in an open two-compartment model with exogenous (CRI) and endogenous rate inputs into the central, exogenous *i.v.* bolus input and steady-state clearance output (*i.e.* endogenous input rate, divided by CLt) from the central compartment. The second subsystem predicts the turnover of OTR, with production and degradation of free OTR, saturable binding kinetics of the OT-OTR complex using the apparent dissociation constant (Kd) reported by Kimura *et al.* (1994) (Kimura, Makino *et al.* 1994), and metabolism of the OT-OTR complex, according to Nakamura *et al.* (2000) (Nakamura, Itakuara *et al.* 2000). As a null-hypothesis model, an open two-compartment model with time and dose-invariant parameters was used. Data from all pigs was fitted simultaneously with the ADAPT software, version 5 using the nonparametric expectation minimization algorithm (Wang, Schumitzky *et al.* 2009). The relative goodness of fit of each model was assessed by comparison of their respective Akaike information criterion values (Yamaoka, Nakagawa *et al.* 1978).

In order to further verify the proportionality of plasma OT concentration with respect to administered dose (*i.e.* the null hypothesis), plasma OT concentrations were normalized with respect to dose by using the following transformation:

$$Cp(t)' = Cp(t) \cdot \frac{Dose_{min}}{Dose}$$

Where Cp(t)' and Cp(t) are respectively the predicted and raw plasma OT concentration at time *t*, $Dose_{min}$ is the lowest dose used in a given experiment, and *Dose* is the actual dose administered to the pig.

The changes on cardiovascular parameters over the total time of monitoring (before and after OT administration) were examined separately for each dosing regimen with an ANOVA repeated-measures linear model. A Dunnett's post hoc test was used to compare each interval after OT administration with baseline values. All tests were performed at a 0.05 alpha level.

## 3.4 RESULTS

*Dose-proportionality in pharmacokinetic response*

The individual time-courses of plasma OT concentration associated with *i.v.* bolus or CRI OT administration are depicted in Figure 1. In all experiments using *i.v.* bolus dosing (Fig. 1 A to C), the OT PK profiles showed a triphasic pattern, with a short-lived distribution phase, a convex transition segment that evolves into an elimination phase. The latter resumes back to baseline plasma OT concentrations. The PK profiles of the two highest OT bolus doses (2979 and 4964 pmol $kg^{-1}$) were not superimposable to those of the lower doses, especially during the distribution phase and the transitional phase between elimination and back to baseline (Fig. 1-A). In addition, the dose-normalized PK profiles for the 4964 pmol $kg^{-1}$ OT bolus dose were shifted upwards with respect to those for the 2979 pmol $kg^{-1}$ (Fig. 1-B). We observed that the elimination of the highest dose seems to be more abrupt. A similar lack of superposition in dose-normalized plasma OT concentration was recorded for the two tested

levels of CRI (6.6 or 49.6 pmol $kg^{-1}$ $min^{-1}$): steady state plasma OT concentration was reached faster in pigs dosed with the highest rate of CRI (Fig. 1-D).

*Modeling*

The assessment of goodness of fit with both models revealed that the plasma concentration data could not be predicted accurately with the null-hypothesis model (Figure 2 A to E). The lack of fit of this model was greater for the CRI data, as it could not discriminate between baseline concentration values and plasma concentrations associated with the exogenous OT input. In contrast, the TMDD model correctly predicted the accumulation of OT during the CRI. The standardized regression residuals of each model are plotted against measured OT concentration (Fig. 2-A and 2-B) and time (Fig. 2-C and 2-D). The range of dispersion is largely in favour of the alternative-hypothesis.

These graphs reveal that the lack of fit is greater for the null-hypothesis model, especially during the distribution phase, and leverages the regression curve to a point that trough concentrations are systematically underestimated. As a result, the scattering of predicted concentrations generated by the null-hypothesis model (Fig. 2-E) was considerably larger than the one of the TMDD model. The predictive values from the H0 model underestimated the observed values.

The Table 2 presents the average moment PK parameters and variables of OT as a function of administered dose. Interestingly, the estimates of lambda Z (terminal elimination rate), mean residual time (MRT; average amount of time that a drug spends in the body), Vss and Vc are affected both by dose and OT input rate. In contrast, CLt (total body clearance, rate at which a drug is removed from the body, considered as a single unit, the sum of renal clearance and metabolic (hepatic) clearance, expressed as volume per unit time), R (endo) (endogenous

secretion) and IC(1) (constant of proportionality between stationary plasma concentration and constant of drug elimination) are only affected by the highest dose.

The estimates of clearance ($CL_T$) and IC(1) were similar between bolus and CRI administrations Interestingly, the estimates of steady state (Vss), and peripheral ($V_p$) volumes were significantly affected by OT input rate, with higher values with CRI compared to bolus. In contrast, central volume ($V_c$) was lower after CRI compared to bolus.

*Cardiovascular effects*

Statistical analysis showed (Table 3) that the *i.v.* bolus administration of OT caused pronounced increases in vascular pressure, with significant differences being recorded for MAP ($p<0.0001$), PCWP ($p=0.004$) and CVP ($p=0.04$). In contrast, the effects of the OT CRI administration were only significant for MAP ($p=0.009$). The time-courses of MAP and HR associated with *i.v.* bolus administration of OT (plasma OT concentration superposed) are depicted in Figure 3-A. The increase in MAP following OT administration is immediate, and parallels the decay in plasma OT concentration during its distribution phase. In contrast, the decrease in HR lags for some minutes the time of OT administration.

The results of Dunnett's post-hoc tests performed to compare each time of measurement of cardiovascular parameters with the baseline values of each parameter are reported in Table 3. This type of test allowed obtaining the duration of statistically significant changes in the parameters over time. Changes were considered significant when $p<0.05$.

The time-courses of MAP and HR associated with predicted OT concentrations after *i.v.* bolus administration (n=3) using the alternative-hypothesis model are depicted in Figure 3. The increase in MAP following OT administration is immediate, and parallels the decay in plasma OT concentration during its distribution phase (*data not shown*). In consequence, a linear relationship is observed between MAP and predicted OT concentration (Fig. 3-A). In

contrast, the decrease in HR lags for some minutes the time of OT administration and looks more as reactive to changes induced in MAP. When looking for the same correlation with CRI administration (n=3), the result was less evident (Fig. 3-B) and must be related to the lower plasma OT concentration observed with this mode of administration and dosage.

The plasma concentrations modelized with the hæmodynamics changes observed on n=3 pigs receiving 4964 pmol $kg^{-1}$ BW *i.v.* bolus or 49.6 pmol $kg^{-1}$ $min^{-1}$ CRI were predicted to the time-points of hæmodynamic measurements using the alternative-hypothesis (TMDD) model.

## 3.5 DISCUSSION

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, we demonstrated a non-stationarity of OT PK parameters with respect to dose in anæsthetized rats (Morin, Del Castillo *et al.* 2008). Studies reporting the PK of OT after an *i.v.* bolus all used a two compartment model to describe the plasma concentration-time curves (Seitchik, Amico *et al.* 1984; Conrad, Gellai *et al.* 1993; Lundin, Broeders *et al.* 1993; De Groot, Vree *et al.* 1995). In our previous study, a one compartment model best described the PK parameters at doses of 200, 300 and 500 $ng \cdot kg^{-1}$ OT. However, an open two compartment model best described profiles observed at doses of 1000, 3000, 5000 and 10000 $ng \cdot kg^{-1}$ OT in anæsthetized rats (Morin, Del Castillo *et al.* 2008). At this time, to explain why the peptide did not follow a dose-independent relationship in body, we proposed that, at least partially, this would result from the physiological effects of OT that modulate its own PK.

Indeed, the results obtained on the $CL_T$ supported the hypothesis of a possible dose-dependent saturation of the enzyme oxytocinase with a plateau reached at the dose of 1000

$ng \cdot kg^{-1}$ OT, but also, may suggest a modification in the renal functions induced by OT at that dose. This point of saturation is found again in the present study, and is important to be kept in mind. Secondly, the changes observed on the volumes of distribution, $V_c$ and $V_p$, strongly suggested that $V_p$ would represent the population of OTR found on the vascular bed. The receptors, present in limited number, appear to have saturated at the plateau dose of 1000 $ng \cdot kg^{-1}$ OT. The presence of OTR in the vascular endothelium was demonstrated and is well characterized (Thibonnier, Conarty *et al.* 1999). Because no distribution process was observed for doses lower than 1000 $ng \cdot kg^{-1}$ OT, as shown with the compartment model, we hypothesized that the number of OTRs exceeded the OT molecules and no saturation was observed at these doses. Then, OT binds rapidly to the receptors without saturation and the homogenization is free of impairment. As the OT dose increases, metabolism and elimination processes, *via* the OTR/oxytocinase couple, influence the equilibrium in the homogenization between the two compartment ($V_c$ and $V_p$). A rapid decline in that homogenization is observed with doses of 1000 $ng \cdot kg^{-1}$ OT and above.

*Pharmacokinetic and pharmacodynamic modeling*

Though using a narrower range of dosing regimens, the results of this study confirm our previously published observations in a rat model (Morin, Del Castillo *et al.* 2008). Noteworthy, the two highest bolus doses (2979 and 4964 pmol $kg^{-1}$) were not superimposable to the lower 993 pmol $kg^{-1}$ dose (Figure 1), and the principle of superposition was also not applicable between the two highest bolus doses, as well as between the two CRI doses (6.6 and 49.6 pmol $kg^{-1}$ $min^{-1}$). So, once again we could propose the hypothesis of a saturation of OTR pool to explain such results. This OTR pool in close relationship with the central (blood) volume could be located on the endothelium and would represent $V_p$.

The goodness of fit of the alternative-hypothesis (TMDD) model in comparison to the null-hypothesis of an open two-compartment model (Figure 2) highlights new physiologically-based PK explanations. It is very interesting to note that the CRI administration, compared to bolus administration, led to increased Vss, and mostly $V_p$. Indeed, with *i.v.* bolus administration (*data not shown*), Vss and mean residence time (MRT) decreased with the dose, as well as $CL_T$. Our PK TMDD system was composed of two subsystems: the first predicts the fate of plasma and tissue free OT, and consists in an open two-compartment model with exogenous and endogenous rate inputs into the central compartment ($V_c$). The second subsystem predicts the turnover of OTR, with production and degradation of free OTR, saturable binding kinetics of the OT-OTR complex using the apparent dissociation constant (Kd) reported by Kimura *et al.* (2000) (Kimura, Makino *et al.* 1994), and metabolism of the OT-OTR complex, according to Nakamura *et al.* (Nakamura, Itakuara *et al.* 2000).

Finally, the time-course of MAP associated with predicted OT concentrations after *i.v.* bolus administration using the alternative-hypothesis TMDD model presented linear relationships between the measured increase in MAP and the increase in predicted concentrations.

To link together these apparently independent discoveries related to OT, we could propose the following scenario:

> The binding kinetics of OT-OTR is saturable, but it is also the signal of membrane translocation of oxytocinase. The latter activity is in consequence saturable and would mainly explain the dose-dependency in $CL_T$, and PK non-linearity observed in our studies in rats and pigs. But once OT is degraded and metabolized by OTase, this leads to an immediate availability of OTR. Remaining OT in the central compartment, which did not yet have access to OTR, is therefore able to bind to OTR

and induce its physiological action. And the higher is the plasma OT concentration, the higher is the increase in MAP. There is close relatedness of OT with the neuropeptide AVP, another vasoactive peptide. The latter acts through specific receptors classified as V1a vascular, V2 renal, and V3 pituitary (also known as V1b). The V1a vascular receptor has been located in endothelial cells and vascular smooth muscle cells. The V1a receptor is found at high densities in vascular smooth muscles causing vasoconstriction by an increase in intracellular calcium *via* the phosphatidyl–inositol-bisphosphonate cascade (Holmes, Landry *et al.* 2004). However, it has been demonstrated that AVP binding to V1a receptor produces vasoconstriction only in non-vital circulation, whereas in vital circulation like coronary and basilar arteries, as well as renal circulation, AVP causes endothelium-dependent relaxation mediated by NO signalling pathway (Katusic, Shepherd *et al.* 1986; Hirata, Hayakawa *et al.* 1997; Evora, Pearson *et al.* 2003).

The selectivity of OT to OTR and AVP's subtype receptors has been reported, showing that AVP receptors are very selective to AVP. However, *in vitro* experimentations demonstrated that high concentrations of OT ($>10^{-8}$ M) caused vasoconstriction that was eliminated by a specific vasopressin V1a receptor antagonist (Miller, Davidge *et al.* 2002), demonstrating that high concentrations of OT may cause an erratic binding. Plasma concentrations reached after OT bolus and CRI administration were superior to $10^{-8}$ M, therefore it is very possible that these high plasma concentrations of OT caused OT binding not only to its receptors but also to vasopressinergic V1a receptors, resulting in an additive effect and a very striking and relatively sustained vasoconstriction on peripheral vasculature.

On the other side, because the oxytocinase metabolic pathway is saturable, the initial plasma OT "in excess" in the circulating central compartment will be exposed to a

renal elimination presenting a lower rate of elimination, explaining the decrease in $CL_T$ observed with increasing doses.

When administering OT by CRI, the Vss, and mostly $V_p$, increase as the progressive exposure of $V_c$ to OT allows to not reach saturation of OTR/oxytocinase. But as demonstrated on Fig. 3-B, the challenge would be to administer dosing rate high enough to induce significant PD effects. Moreover, too low CRI dosing rates would lead to a potential lag in the expected PD effects. Because OT has potential usefulness in the regenerative therapy of post-ischæmic myocardial disease, finding an appropriate dosing regimen for this new therapeutic use has crucial importance.

Finally, it would remain to explore the potential consequences of the physiologically-induced changes on cardiovascular and renal functions on OT own PK. Indeed, we observed a ceiling diuretic effect of OT bolus administration in rats, with a peak around 3000 $ng \cdot kg^{-1}$ OT. Higher doses led to a decrease in glomerular filtration rate that would reduce the elimination of OT (Morin, Del Castillo *et al.* 2008).

*Clinical perspective*

When comparing the PK/PD with *i.v.* bolus *versus* CRI administration, it is evident that the intensity of PD effects was lower with CRI, or more exactly with the lower plasma concentrations predicted by the TMDD model with CRI administration. This has its importance with regards to the objective of determining an appropriate dosing regimen in post-ischæmic myocardial disease. The present discovery of a PK TMDD model describing adequately the PK of OT opens promising avenues to detect this appropriate dosing regimen in the near.

## 3.7 REFERENCES

Abraham, A. K., W. Krzyzanski, *et al.* (2007). "Partial derivative-based sensitivity analysis of models describing target-mediated drug disposition." AAPS J **9**(2): E181-189.

Chen, Y. L., C. Shepherd, *et al.* (1999). "Oxytocin and vasopressin constrict rat isolated uterine resistance arteries by activating vasopressin V1A receptors." Eur J Pharmacol **376**(1-2): 45-51.

Conrad, K. P., M. Gellai, *et al.* (1993). "Influence of oxytocin on renal hemodynamics and sodium excretion." Ann N Y Acad Sci **689**: 346-362.

Costa, E. S. R. H., P. P. Pereira-Junior, *et al.* (2005). "Cardiac effects of oxytocin: is there a role for this peptide in cardiovascular homeostasis?" Regul Pept **132**(1-3): 107-112.

De Groot, A. N., T. B. Vree, *et al.* (1995). "Bioavailability and pharmacokinetics of sublingual oxytocin in male volunteers." J Pharm Pharmacol **47**(7): 571-575.

Evora, P. R., P. J. Pearson, *et al.* (2003). "Effect of arginine vasopressin on the canine epicardial coronary artery: experiments on V1-receptor-mediated production of nitric oxide." Arq Bras Cardiol **80**(5): 483-494.

Gimpl, G. and F. Fahrenholz (2001). "The oxytocin receptor system: structure, function, and regulation." Physiol Rev **81**(2): 629-683.

Gutkowska, J., M. Jankowski, *et al.* (2000). "Oxytocin is a cardiovascular hormone." Braz J Med Biol Res **33**(6): 625-633.

Hirata, Y., H. Hayakawa, *et al.* (1997). "Receptor subtype for vasopressin-induced release of nitric oxide from rat kidney." Hypertension **29**(1 Pt 1): 58-64.

Holmes, C. L., D. W. Landry, *et al.* (2004). "Science Review: Vasopressin and the cardiovascular system part 2 - clinical physiology." Crit Care **8**(1): 15-23.

Jankowski, M., F. Hajjar, *et al.* (1998). "Rat heart: a site of oxytocin production and action." Proc Natl Acad Sci U S A **95**(24): 14558-14563.

Katusic, Z. S., J. T. Shepherd, *et al.* (1986). "Oxytocin causes endothelium-dependent relaxations of canine basilar arteries by activating V1-vasopressinergic receptors." J Pharmacol Exp Ther **236**(1): 166-170.

Kimura, T., Y. Makino, *et al.* (1994). "Molecular characterization of a cloned human oxytocin receptor." Eur J Endocrinol **131**(4): 385-390.

Kobayashi, H., S. Yasuda, *et al.* (2009). "Postinfarct treatment with oxytocin improves cardiac function and remodeling via activating cell-survival signals and angiogenesis." J Cardiovasc Pharmacol **54**(6): 510-519.

Levy, G. (1994). "Pharmacologic target-mediated drug disposition." Clin Pharmacol Ther **56**(3): 248-252.

Lundin, S., A. Broeders, *et al.* (1993). "Pharmacokinetic and pharmacologic properties of antiuterotonic oxytocin analogs in the rat." J Pharmacol Exp Ther **264**(2): 783-788.

Mager, D. E. (2006). "Target-mediated drug disposition and dynamics." Biochem Pharmacol **72**(1): 1-10.

Mager, D. E. and W. J. Jusko (2001). "General pharmacokinetic model for drugs exhibiting target-mediated drug disposition." J Pharmacokinet Pharmacodyn **28**(6): 507-532.

Miller, M. E., S. T. Davidge, *et al.* (2002). "Oxytocin does not directly affect vascular tone in vessels from nonpregnant and pregnant rats." Am J Physiol Heart Circ Physiol **282**(4): H1223-1228.

Morin, V., J. R. Del Castillo, *et al.* (2008). "Evidence for non-linear pharmacokinetics of oxytocin in anesthetizetized rat." J Pharm Pharm Sci **11**(4): 12-24.

Mukaddam-Daher, S., Y. L. Yin, *et al.* (2001). "Negative inotropic and chronotropic effects of oxytocin." Hypertension **38**(2): 292-296.

Nakamura, H., A. Itakuara, *et al.* (2000). "Oxytocin stimulates the translocation of oxytocinase of human vascular endothelial cells via activation of oxytocin receptors." Endocrinology **141**(12): 4481-4485.

Olfert ED, C. B., McWilliam AA (1993). Manuel sur le soin et l'utilisation des animaux d'expérimentation. Ottawa, Canada, Conseil Canadien de Protection des Animaux.

Ondrejcakova, M., T. Ravingerova, *et al.* (2009). "Oxytocin exerts protective effects on in vitro myocardial injury induced by ischemia and reperfusion." Can J Physiol Pharmacol **87**(2): 137-142.

Petersson, M. (2002). "Cardiovascular effects of oxytocin." Prog Brain Res **139**: 281-288.

Petersson, M., P. Alster, *et al.* (1996). "Oxytocin causes a long-term decrease of blood pressure in female and male rats." Physiol Behav **60**(5): 1311-1315.

Seitchik, J., J. Amico, *et al.* (1984). "Oxytocin augmentation of dysfunctional labor. IV. Oxytocin pharmacokinetics." Am J Obstet Gynecol **150**(3): 225-228.

Thibonnier, M., D. M. Conarty, *et al.* (1999). "Human vascular endothelial cells express oxytocin receptors." Endocrinology **140**(3): 1301-1309.

Troncy, E., M. Francoeur, *et al.* (1997). "Extra-pulmonary effects of inhaled nitric oxide in swine with and without phenylephrine." Br J Anaesth **79**(5): 631-640.

Wagner, J. G. (1973). "A modern view of pharmacokinetics." J Pharmacokinet Biopharm **1**(5): 363-401.

Wang, X., A. Schumitzky, *et al.* (2009). "Population Pharmacokinetic/Pharmacodyanamic Mixture Models via Maximum a Posteriori Estimation." Comput Stat Data Anal **53**(12): 3907-3915.

Yamaoka, K., T. Nakagawa, *et al.* (1978). "Application of Akaike's information criterion (AIC) in the evaluation of linear pharmacokinetic equations." J Pharmacokinet Biopharm **6**(2): 165-175.

Yamaoka, K., T. Nakagawa, *et al.* (1978). "Statistical moments in pharmacokinetics." J Pharmacokinet Biopharm **6**(6): 547-558.

## 3.8 TABLES AND FIGURES

**Table 1. Time-points of serial blood sampling for determining plasma oxytocin concentrations**

| Experiment | Anæsthesia | Dose amount (pmol $kg^{-1}$) | Dosing rate | Sampling time (min) Pre-OT | Post-OT |
|---|---|---|---|---|---|
| PK | No (n=1) | 298 | Bolus | -60, -50, -40, -30, -20, -10 | 2, 3, 4, 5, 6, 10, 30, 60, 90, 120, 150, 180, 190, 200, 230, 250. |
| | | 993 | | | |
| | | 2979 | | | |
| | | 4964 | | | |
| | No (n=4) | 993 | Bolus | -10, -5, -1 | 2, 4, 6, 8, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90 |
| | | 4964 | | | |
| | Yes (n=3 for each dose) | 298 | Bolus | -60, -50, -40, -30, -20, -10 | 2, 3, 4, 5, 6, 7, 10, 20, 30, 45, 60, 75, 90, 120, 150, 180, 200, 220 |
| | | 4964 | | | |
| | | 596 | CRI 90 min | -60, -50, -40, -30, -20, -10 | 15, 30, 60, 75, 90, 95, 100, 120, 150, 180, 210 |
| | | 4468 | | | |
| | | 4964 | Bolus CRI 90 min | -60, -30 -60, -30 | 2, 16, 60, 110, 130 4, 60, 105, 133, 231 |
| | | 4468 | | | |
| PK/PD Cardio-vascular | Yes (n=3 for each dose) | 4964 | Bolus | -50, -40, -30, -20, -10 | 1.3, 1.4, 2.6, 16.3, 21, 60, 61, 62, 131, 132, 150 |
| | | 4468 | CRI 90 min | -50, -40, -30, -20, -10 | 1.46, 1.47, 1.52, 62, 105, 133.6, 135.8, 229.8, 230.1, 231 |

Legend: Summary of the experimental groups with the administered doses and number of animals in each group, the time-points for blood sampling are alse explained. Abbreviations: CRI, constant rate infusion; PK, pharmacokinetics; PK/PD, pharmacokinetic/ pharmacodynamic; Post-OT, sampling times following the onset of OT administration; Pre-OT, baseline sampling times.

**Table 2. Average moment pharmacokinetic parameters and variables as a function of oxytocin administered dose**

| PK variable | Unit | Estimates for Bolus Administration | | Estimates for CRI | | Population estimates | |
|---|---|---|---|---|---|---|---|
| | | Mean | SE | Mean | SE | Mean | SE |
| CLt | $L \cdot min^{-1} \cdot kg^{-1}$ | $1.89 \times 10^{-2}$ | 0.0013 | $1.73 \times 10^{-2}$ | 0.0014 | | |
| R(endo) | $pmol \cdot min^{-1} \cdot kg^{-1}$ | 0.3485 | 0.0163 | 0.4783 | 0.0451 | 0.376 | 0.020 |
| IC(1) | $pmol \cdot kg^{-1}$ | 3.6672 | 0.2905 | 3.8446 | 0.4182 | 3.42 | 0.353 |
| Vss | $L \cdot kg^{-1}$ | 0.4194 | 0.0152 | 0.9856 | 0.2141 | 0.398 | .045 |
| Vc | $L \cdot kg^{-1}$ | 0.3257 | 0.0206 | 0.2127 | 0.0214 | 0.270 | 0.028 |
| Vp | $L \cdot kg^{-1}$ | 0.0936 | 0.0135 | 0.7729 | 0.2295 | 0.128 | 0.017 |

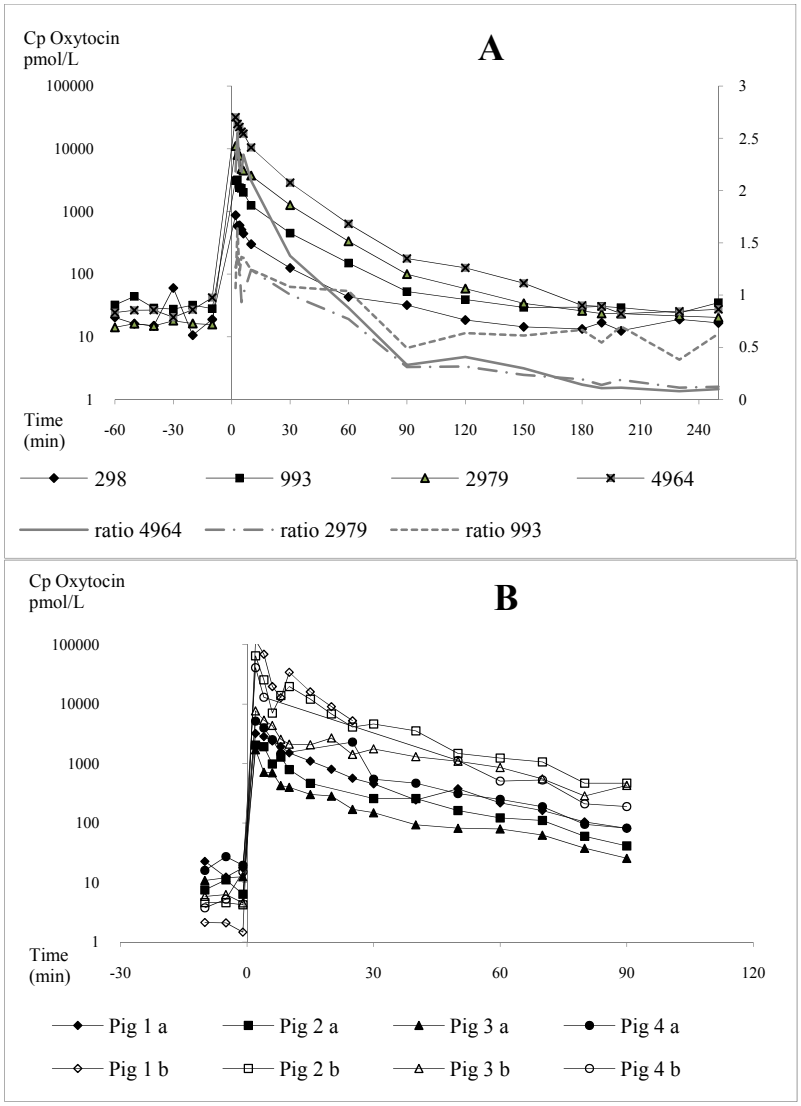
Legend: PK, pharmacokinetic; CRI, constant rate infusion; Clt, total clearance; R(endo), endogenous secretion; IC(1), constant of proportionality between stationary plasma concentration and constant of drug elimination; Vss, steady-state volume of distribution; Vc, central volume of distribution; Vp, peripheral volume of distribution.

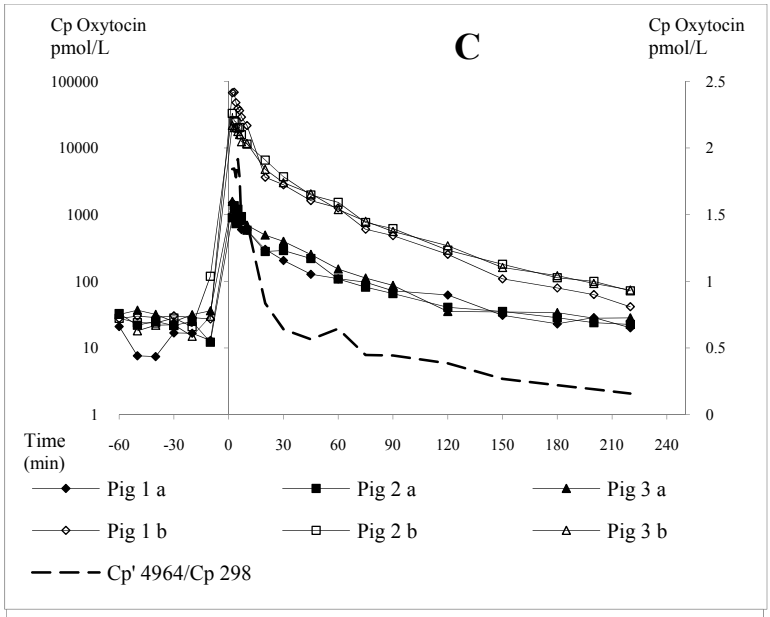
**Table 3. Summary of mean overall changes in cardiovascular parameters after bolus or constant rate infusion of oxytocin in pigs.**

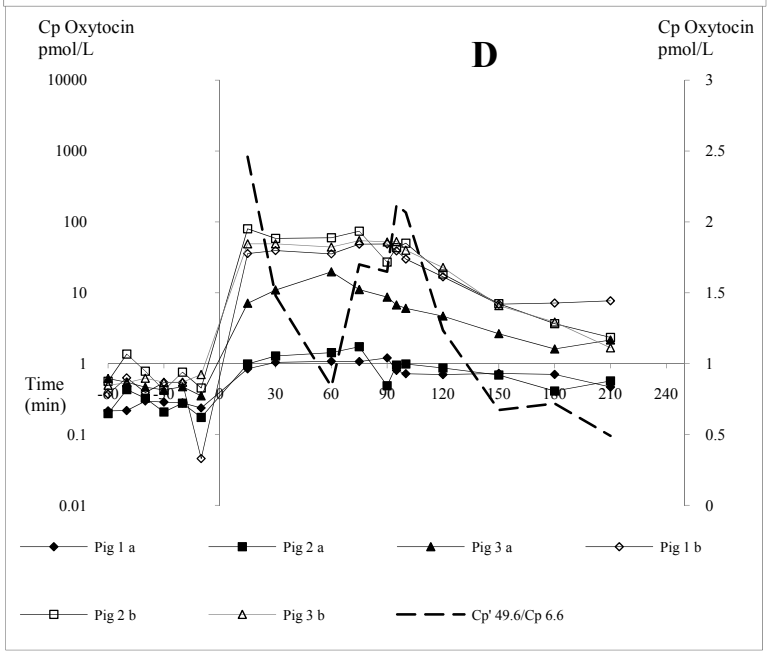
| Variable | (units) | Bolus 4964 pmol $kg^{-1}$ | | CRI 49.6 pmol $kg^{1}$ $min^{-1}$ | |
|---|---|---|---|---|---|
| | | Trend, peak, % | Duration (min) | Trend, peak, % | Duration (min) |
| MAP | mmHg | ↗ 28.06±3 +42% | 0-50 | ↗ 10.45±3 16% | 10-90 |
| HR | bpm | ↘ 5.01±2 -5% | 0-10 | none | 0 |
| CO | L $min^{-1}$ | ↘ | 0-5 | none | 0 |
| SV | mL $beat^{-1}$ | ↘ | 0-5 | none | 0 |
| PCWP | mmHg | ↗ 3.56±1 +47% | 0-20 | ↗ 3.13±1 +35% | 20-60 |
| CVP | mmHg | ↗ 0.99±0.3 +15% | 0-5 | ↘ 1.4±0.4 -18% | 0-5 |
| PAP | mmHg | None | 0 | none | 0 |
| SVR | dynes $s^{-1}$ $cm^{5}$ | ↗ 756.2±292 +52% | 0-20 | ↗ 512.71±207 +24% | 20-100 |
| PVR | dynes $s^{-1}$ $cm^{5}$ | None | 0 | none | 0 |

Legend. Trend (↘decreased or ↗increased compared to baseline value and duration (min) after oxytocin bolus or constant rate infusion administration. Peak are statistically significant differences and these changes were considered significant $p<0.05$. Abbreviations: mean arterial pressure (MAP), heart rate (HR), cardiac output (CO), stroke volume (SV), pulmonary capillary wedge pressure (PCWP), central venous pressure (CVP), pulmonary artery pressure (PAP), systemic vascular resistance (SVR), pulmonary vascular resistance (PVR).

**Figure 1. Plasma oxytocin concentrations associated with intravenous administration of exogenous OT in pigs.**




C
Cp Oxytocin pmol/L
Cp Oxytocin pmol/L
100000
10000
1000
100
10
1
2.5
2
1.5
1
0.5
0
Time (min)
-60 -30 0 30 60 90 120 150 180 210 240
Pig 1 a
Pig 2 a
Pig 3 a
Pig 1 b
Pig 2 b
Pig 3 b
Cp' 4964/Cp 298


D
Cp Oxytocin pmol/L
Cp Oxytocin pmol/L
10000
1000
100
10
1
0.1
0.01
3
2.5
2
1.5
1
0.5
0
Time (min)
30 60 90 120 150 180 210 240
Pig 1 a
Pig 2 a
Pig 3 a
Pig 1 b
Pig 2 b
Pig 3 b
Cp' 49.6/Cp 6.6

Legend. Panel A: Pilot experiment conducted on a conscious pig dosed with *i.v.* bolus administration of oxytocin (OT) at the doses described in the chart, discontinuous grey lines represent the ratios of Cp'(plasma concentration reached after each dose multiplied by the ratio of the lower dose/each given dose). Panel B: Pilot experiment on conscious pigs dosed with *i.v.* bolus administrations at doses of 993 pmol $kg^{-1}$ (black lines with closed symbols), and 4964 pmol $kg^{-1}$ (lines with open symbols). Panel C: Main experiment in anæsthetized pigs dosed with *i.v.* bolus administration at 298 pmol $kg^{-1}$ (black lines with closed symbols), or 4964 pmol $kg^{-1}$ (lines with open symbols). Panel D: Main experiment in anæsthetized pigs dosed with *i.v.* constant rate infusions at 6.6 pmol $kg^{-1}$ $min^{-1}$ (black lines with closed symbols), or 49.6 pmol $kg^{-1}$ $min^{-1}$ (lines with open symbols). Pigs a (low dose), pigs b (high dose). In all panels, discontinuous lines represent the ratio of dose-normalized plasma OT concentration (Cp') of higher dose, divided by the concurrent raw plasma OT concentration (Cp) of the lowest dose used in the experiment. Under the null hypothesis, the ratio should not differ significantly from 1.

**Figure 2. Comparison of null-hypothesis and alternative-hypothesis models adequacy.**

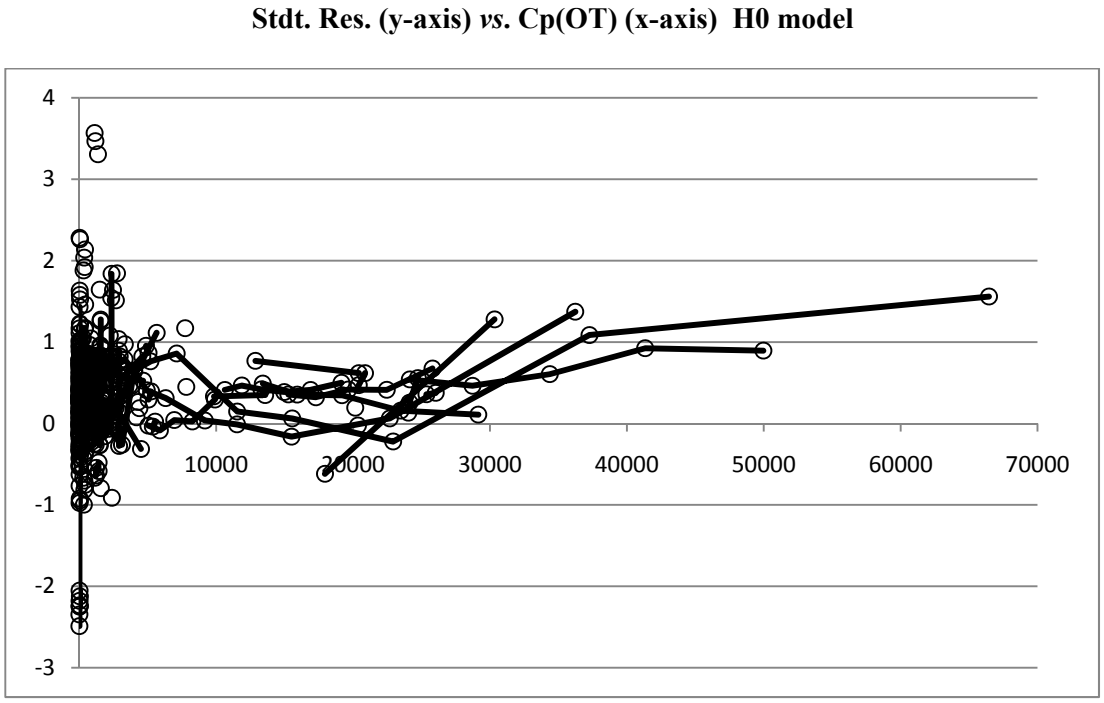



Fig. 2-A. Standardized regression residuals *versus* the measured plasma OT (pmol/L) concentration for the null-hypothesis model, which attempted to verify the proportionality of plasma OT concentration with respect to administered dose using the two-compartment pharmacokinetic model.

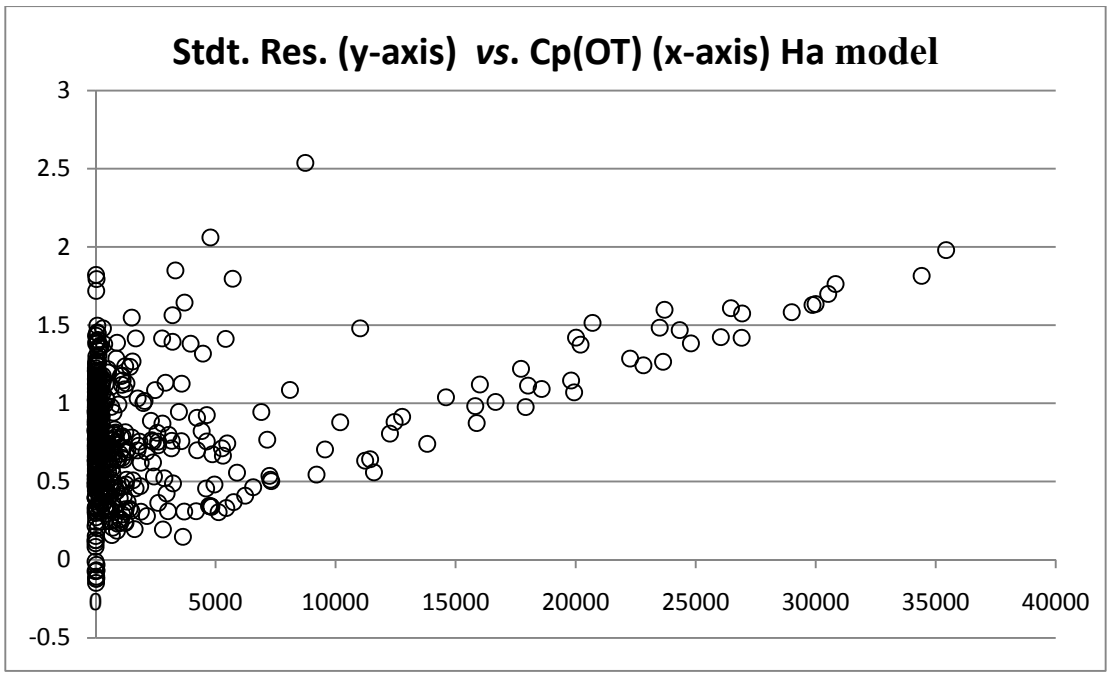



Fig. 2-B. Standardized regression residuals *versus* the measured plasma OT (pmol/L) concentration for the alternative-hypothesis model, which attempted to verify the proportionality of plasma OT concentration with respect to administered dose using the target-mediated drug disposition (TMDD) model.

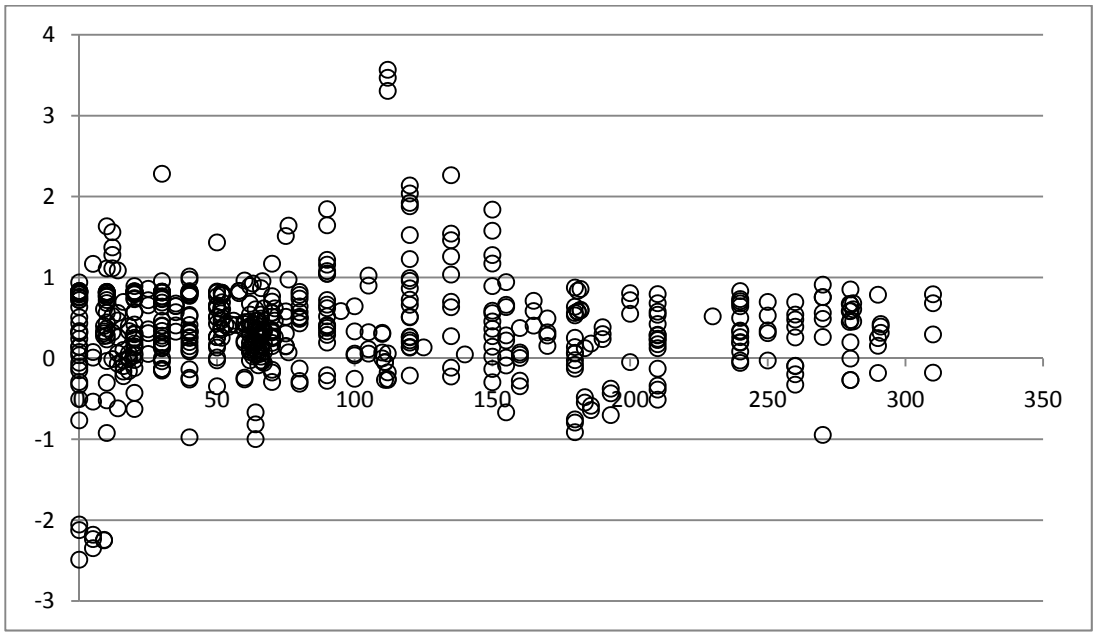



Fig. 2-C. Standardized regression residuals (y-axis) *versus* time in minutes (x-axis) for the null-hypothesis model, which attempted to verify the proportionality of plasma OT concentration with respect to administered dose using the two-compartment pharmacokinetic model.

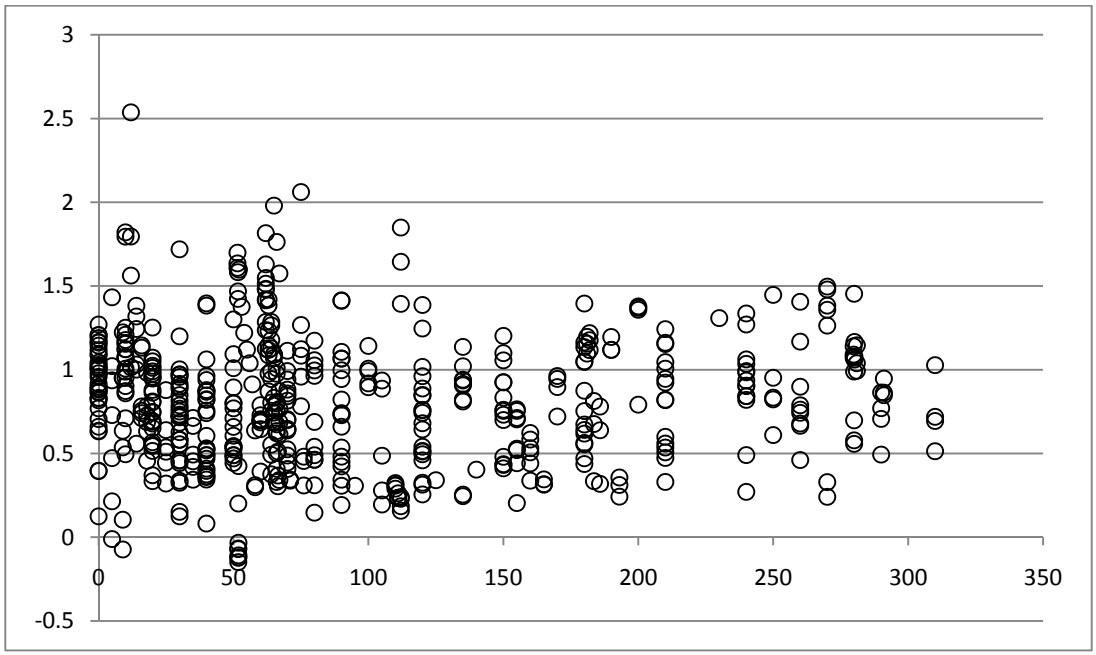



Fig. 2-D. Standardized regression residuals (y-axis) *versus* time in minutes (x-axis) for the alternative-hypothesis model, which attempted to verify the proportionality of plasma OT concentration with respect to administered dose using the target-mediated drug disposition (TMDD) model.

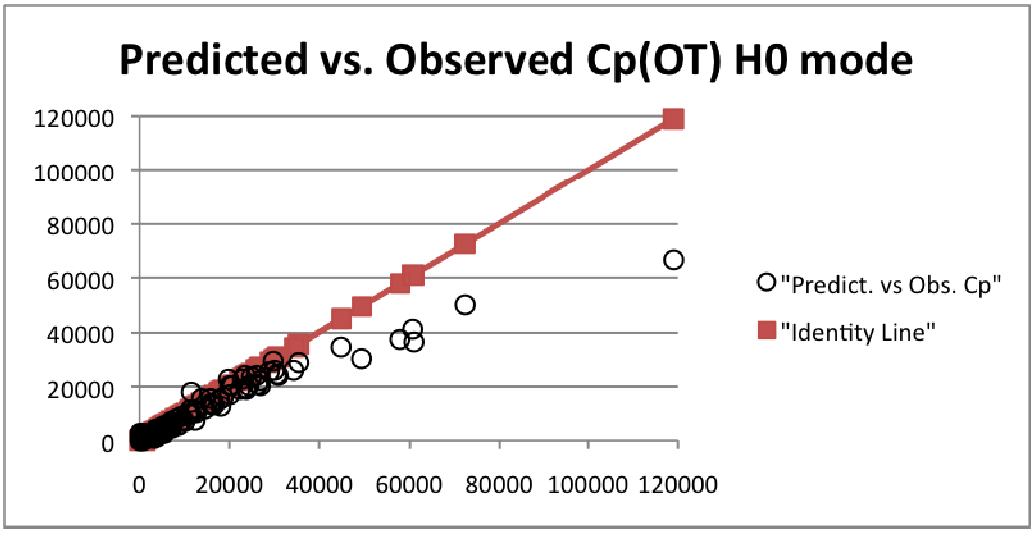



Fig. 2-E. Predicted concentrations from the null-hypothesis model, which attempted to verify the proportionality of plasma OT concentration with respect to administered dose using the two-compartment pharmacokinetic model, compared to the observed plasma OT concentrations.

**Figure 3. Oxytocin pharmacokinetic / pharmacodynamic (cardiovascular) correlation.**

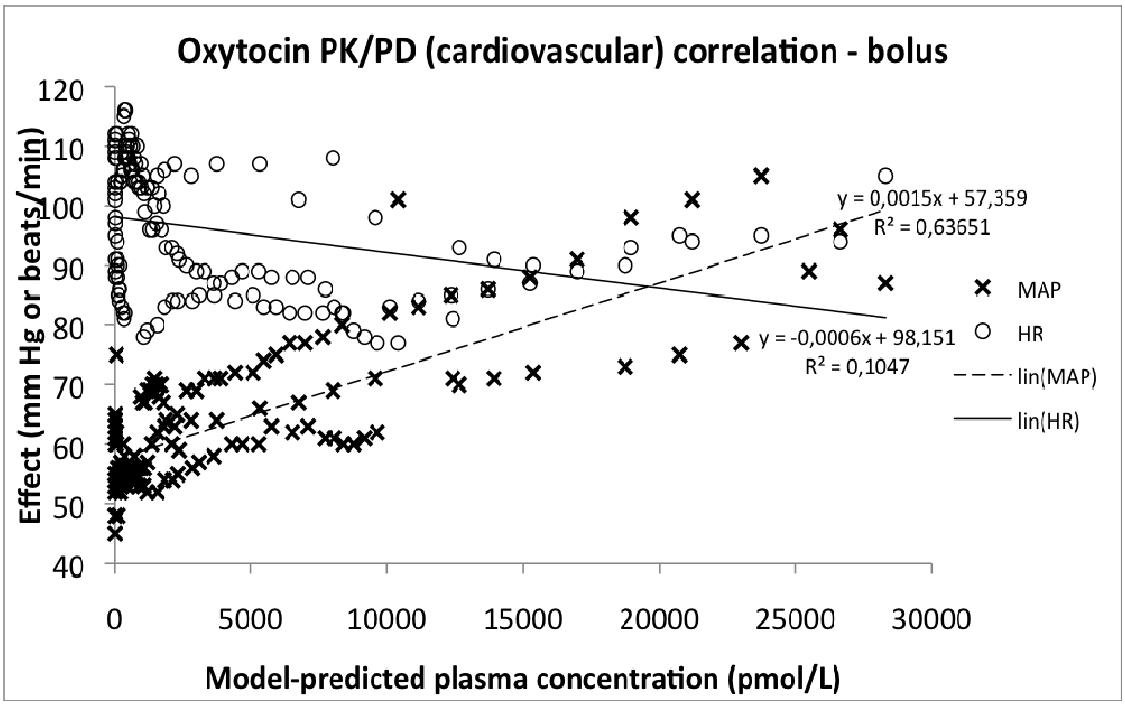



Fig. 3-A. Oxytocin pharmacokinetic (PK)/pharmacodynamic (PD) (cardiovascular) correlation with bolus administration on anæsthetized pigs (n=3). Correlation of measured parameters with oxytocin predicted plasma concentrations. Abbreviations: MAP (mean arterial pressure), HR (heart rate).

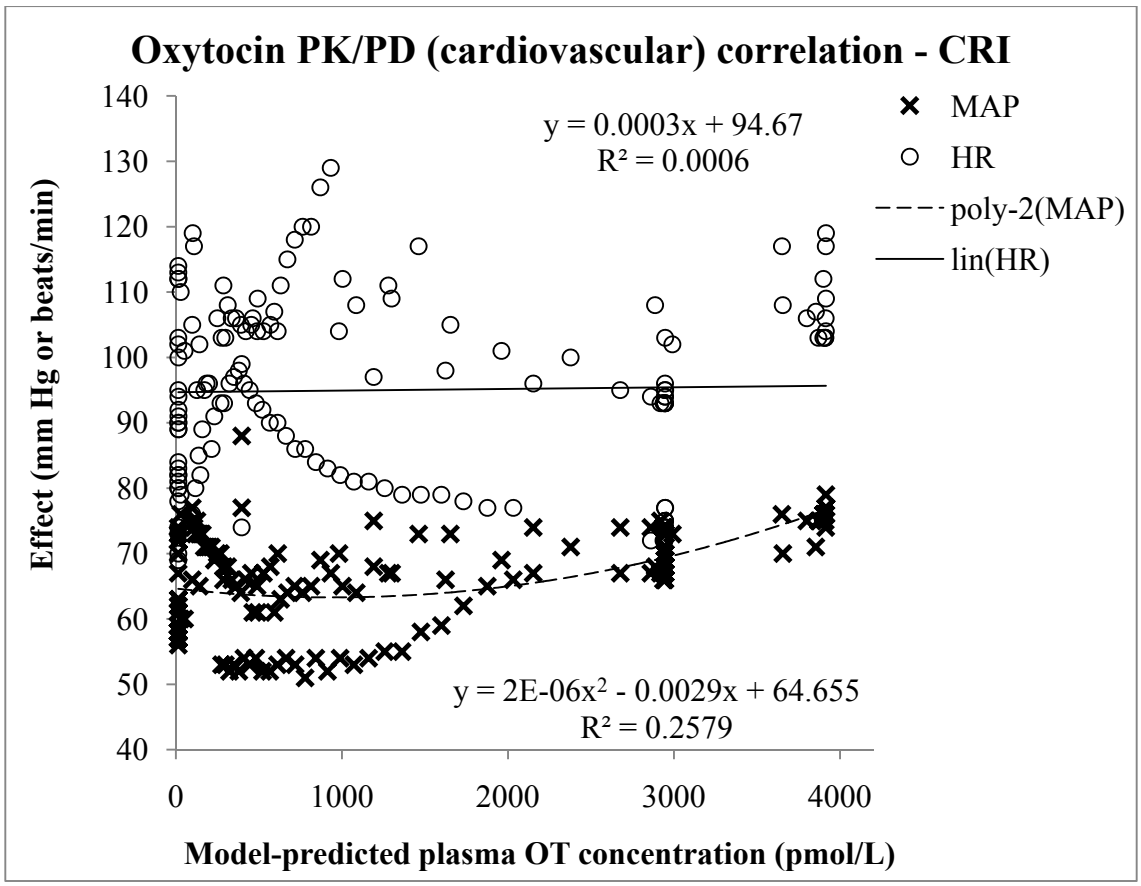



Fig. 3-B. Oxytocin pharmacokinetic (PK)/pharmacodynamic (PD) (cardiovascular) correlation with CRI administration on anæsthetized pigs (n=3). Correlation of measured parameters with oxytocin predicted plasma concentrations. Abbreviations: MAP (mean arterial pressure), HR (heart rate).

# CHAPTER 4. SECOND ARTICLE

# Oxytocin induces *in vitro* cardiomyogenic differentiation of porcine bone marrow stem cells

Norma Ybarra, MSc [a]; Patrick Vincent, *PhD* [b];

Lawrence C Smith, *PhD* [b]; Eric Troncy, DV, *PhD* [a,*]

[a] GREPAQ, [b] CRRA – Department of veterinary biomedicine, Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, Quebec, Canada.

[*] **Corresponding author**:

Eric Troncy

GREPAQ – Department of veterinary biomedicine, Faculté de médecine vétérinaire, Université de Montréal, P.O. box 5000, St-Hyacinthe, Quebec, J2S 7C6, Canada

Phone: 450-773-8521 ext. 8399, Fax: 450-778-8102

**Short title:** *OXYTOCIN-INDUCED DIFFERENTIATION*

Experimental work was undertaken at: **GREPAQ**

**Source of support**: This work was funded by the Canadian Institutes of Health Research / Heart and Stroke Foundation of Canada New Emerging Team (#108291) program (E.T.) for operating grant, and the On-going New Opportunities (#9483) fund from Canada Foundation for Innovation (E.T.) for infrastructure support.

## 4.1 ABSTRACT

The bone marrow stem cells (BMSCs), when 5-azacytidine induced, are a source of stem cells with myogenic differentiation potential. Oxytocin (OT) induces cardiomyogenic differentiation of murine embryonic and cardiac stem cells. The role of OT as a cardiomyogenic differentiation inducer of BMSCs has never been studied. Here, we attempted to isolate, characterize, and induce OT-mediated cardiomyogenic differentiation of porcine BMSCs (pBMSCs). The pBMSCs were characterized by surface marker expression using fluorescent-activated cell sorting. Expression of Oct4, GATA4, and cardiomyocyte structural protein (CSP) transcripts were assessed by RT-PCR from passage 0 to 4. First passage pBMSCs were divided into 3 treatment groups: control, OT, and 5-azacytidine (as positive control of myogenic differentiation). After induction, gene and protein expression of GATA4 and CSP were assessed by RT-PCR, immunocytochemistry and western blot, respectively. The CD29+, CD90+, and CD45- pBMSCs expressed the transcripts of Oct4, GATA4, OT receptor, and phospholamban during early passages. After induction, RT-PCR showed upregulation of GATA4 in OT- and 5-azacytidine-induced groups. By immunocytochemistry, expression of cardiac troponin T and myosin heavy chain was significantly more abundant in OT than in 5-azacytidine-induced groups ($p=0.01$). Western blot analysis showed significant upregulation of cardiac troponin I in OT-induced pBMSCs ($p=0.01$). The pBMSCs should be induced during early passages when they expressed transcription factors related to pluripotency and cardiomyogenesis, as well as OT receptor. These results show that expression of cardiac specific proteins was more abundant in OT-treated pBMSCs than in 5-azacytidine treated cells, OT could be a more potent cardiomyogenic inducer of pBMSC.

**Keywords:** Bone marrow stem cells; Cardiomyogenic differentiation; Oxytocin.

## 4.2 INTRODUCTION

The possibility of reconstituting the damaged heart has introduced a new paradigm in cardiovascular biology and created the potential for a new therapeutic approach in the cardiovascular medicine.[1] Restoration of cardiovascular function is the ultimate goal of stem-cell-based therapy. In principle, cardiovascular stem cells can improve cardiac function via *de novo* cardiomyogenesis, enhanced myocardial neovascularization, and prevention of post-infarct remodeling. Stem cell transplantation to improve cardiac function has generated mixed results in animal models and human clinical trials.[1-4]

Although no major adverse events have been reported in the small studies completed thus far (100–300 patients), cases of patients developing intractable ventricular tachycardia,[5] aggravation of in-stent restenosis,[6] or luminar loss of the infarct-related artery after cell infusion have been described.[7] As with any experimental strategy, the likelihood of benefit must outweigh the risk of harm. Beyond safety, the next most important issue is the mechanism for the observed clinical benefit.

Clinical studies using bone-marrow-derived adult stem cells (BMSC) infused by intracoronary injection after a recent myocardial infarction suggest the possibility of a functional benefit.[1] In 1999, Tomita *et al.* showed that bone marrow cells had little effect when injected directly into cryoinjury induced cardiac scar tissue, but that bone marrow cells differentiated with 5-azacytidine (5-Aza) into cardiomyocyte-like cells were of benefit to heart function.[8] Later studies showed that the infusion of purified (expanded and labeled) BMSCs into the ascending aorta led to their subsequent accumulation in both the infarct-induced scar tissue (as fibroblasts) and normal myocardium (as cardiomyocytes).[9] Even later, it was suggested that direct cell-to-cell contact between cardiac myocytes and mesenchymal stem cells (MSC, bone marrow subpopulation of stem cells), was necessary for the

differentiation of stem cells into cardiac myocytes.[10] Regardless of the mechanism responsible, fusion *vs*. transdifferentiation, it is generally agreed that the number of reported cardiomyocytes derived from exogenously delivered BMSCs remains relatively low and cannot physically account for the observed functional improvements. As such, one alternative proposed mechanism is stem cell–mediated paracrine effects, stem cells produce cytokines that improve neovascularization or reduce inflammation.[11, 12]

Rather than using stem cells directly as therapy, strategies that target the mobilization, expansion, activation or differentiation of endogenous stem cells by the introduction of drugs or biological molecules may be more promising. Recent evidences suggest that oxytocin (OT), a hormone mostly known for its role during childbirth and lactation, could exert protective effects after induced myocardial infarction (MI). Oxytocin reduced infarct size in an *in vitro* Langendorff-perfused rat MI model,[13] and improved cardiac function and remodeling in an *in vivo* rabbit MI model.[14] A potential role of OT during cardiomyogenesis has been suggested. Oxytocin and OT receptors (OTR) protein expression are strongly upregulated in the developing rat heart. Moreover, OT improves cardiomyogenic differentiation of P19 murine embryonic carcinoma (EC) cells and murine embryonic and cardiac stem cells (CSCs).[15-18] Furthermore, embryonic stem cells (ESCs), P19 EC and CSCs express OTR and they are up-regulated when OT, dimethyl sulfoxide (DMSO) or retinoic acid are added to the culture medium for differentiation induction purposes.[15-18] Recently, in a pig model of MI, our group added information about the potential role of OT in cardiac regenerative therapy.[19] In particular, pre-treatment endogenous OT levels, dose of OT, and timing of OT administration post-MI appear to impact outcome in this porcine MI model.

Porcine BMSCs (pBMSC) have been poorly studied yet, with a few studies reporting that they express some cardiac transcription factors and structural proteins after *in vitro* induction

with 5-Aza, without reaching full differentiation.[20, 21] Moreover, to the best of our knowledge, the role of OT/OTR system in BMSCs cardiomyogenic differentiation has not been considered. The experiments reporting OT as a cardiomyogenic inducer have been performed using stem cells mostly from murine origin, which represents certain clinic translational limitations, due to species differences. Therefore, it would be interesting to test the potential use of OT, as a cardiomyogenic inducer of BMSCs obtained from a more suitable animal model, based on the future perspective of testing these OT-induced pBMSCs in a porcine MI.

Our hypothesis is that OT is a more potent *in vitro* cardiomyogenic inducer of BMSCs than 5-Aza. Therefore, we attempted to isolate, characterize, find the best time for cardiomyogenic differentiation induction of pBMSCs, and finally estimate OT-induced differentiation of pBMSCs into cardiomyocytes by quantifying the number of cells expressing cardiac specific proteins and by semi-quantifying the total protein expression of cardiac troponin I.

## 4.3 MATERIALS AND METHODS

Experiments were performed according to the guidelines from the Canadian Council on Animal Care with the approval of the institutional animal care and use committees before initiation of the study (#09-Rech-1234).

### 4.3.1 Isolation and culture of pBMSCs

All the reagents used in this study, unless stated, were purchased from GIBCO Invitrogen, Burlington, ON, Canada.

Bone marrow (BM) was aspirated from the humeral head of ten different juvenile pigs under general anesthesia. The animals, weighing 25 to 30 kg, were anesthetized with a combination of ketamine (Vetalar, Bioniche Animal Health Canada Inc., Belleville, ON, Canada) 15 mg/kg body weight i.m., and xylazine (Anased, Novopharm, Toronto, ON, Canada) 2 mg/kg

body weight i.m. BM aspirates, approximately 10 ml, were obtained using an 11 gauge biopsy aspiration needle (Tyco Healthcare Kendall, Mansfield MA, USA) attached to a syringe containing 10,000 I.U. of heparin (Leo Pharma, Inc., Thornill, ON, Canada). BM samples were immediately transported to the laboratory for further processing. For isolation of pBMSCs, mononuclear cells (MNCs) were separated by density gradient centrifugation using a commercially available solution (Histopaque-1077 Hybri-Max, Sigma, MO, USA), following the manufacturer's instructions. Briefly, 5 ml of phosphate buffered saline (PBS) were added to 3 ml of BM/blood mix and gently mixed in a 15 ml centrifuge tube. The cell suspension was deposited over 3 ml of Histopaque, and centrifuged at 400 x *g* for 30 min at room temperature. MNCs were recovered from the opaque interface with a Pasteur pipette and PBS washed thrice by centrifugation (300 x *g* for 10 min). After washing, MNCs were re-suspended in Dulbecco's Modified Eagle Medium-low glucose (DMEM-LG) supplemented with 15% of fetal bovine serum (FBS), and antibiotic-antimycotic, plated on T-75 plastic flasks (BD Falcon, Bedford, MA, USA) at a density of approximately 500,000 cells/$cm^2$ and incubated in a humidified 5% $CO_2$ atmosphere at 37$^oC$. Non-adherent cells were washed away by changing medium 24 h after first plating. Adherent fibroblast-like cells pBMSCs colonies were observed at 5 to 7 days after plating. Cells were allowed to grow, medium was changed every third day. When cells reached 80 to 90% of confluence, they were passaged by trypsinization (0.25% Trypsin with EDTA) and reseeded at a density of 5000 to 6000 cells/$cm^2$ in T-75 plastic flasks.

### 4.3.2 Characterization

Confluent pBMSCs collected at passage 1 were analyzed for surface epitopes by Fluorescent-activated cell sorting (FACS). Cells were trypsinized, washed in PBS and re-suspended in 1 ml of blocking buffer (20% FBS/PBS) and incubated on ice for 30 min, to avoid non-specific binding. Cells (about 2.5 x $10^5$), were re-suspended in staining buffer (2% FBS/PBS)

containing an appropriate concentration of conjugated antibodies: R-phycoerythrin (RPE) conjugated CD90 (Clone 5E10, BD Pharmingen, San Diego, CA, USA) and CD31 (Clone TLD-3A12, BD Pharmingen, San Diego, CA, USA), fluorescein isothiocyanate (FITC) conjugated CD29 (Clone MEM-101A, Abcam, Cambridge, MA, USA) and CD45 (Clone K252.1E4, AbD Serotec, Kidlington, Oxford, UK). The adequate concentration of antibody was determined by previous titration. Negative controls used for the analysis were isotype match control immunoglobulins conjugated to a fluorochrome, either IgG1 FITC-conjugated (CloneW3/25, AbD Serotec, Kidlington, Oxford, UK) or IgG1κ RPE-conjugated (Clone MOPC-21; BD Pharmingen, San Diego, CA, USA). Cells were incubated on ice for at least 45 min. pBMSCs were washed and re-suspended in 500 µl of staining buffer. Analysis of stained cells was performed on FACSvantage SE flow cytometry system (Becton Dickinson, Mississauga, ON, Canada) and data analyzed by Cellquest software (Becton Dickinson, Mississauga, ON, Canada).

The pBMSCs collected from passage 0 to 4 were analyzed qualitatively by RT-PCR for gene expression of transcription factors and cardiac structural proteins, in an attempt to determine the best time for OT differentiation induction. Three different isolates were analyzed. Total RNA was extracted from pBMSCs collected by trypsinization from passage 0 to 4, using an RNeasy Mini Kit (Qiagen, Gaithersburg MD, USA) following manufacturer's instructions. Briefly, pBMSCs pellet was exposed to a lysis buffer and transferred to the QIAshredder spin column (Qiagen), for cell homogenization. Ethanol 70% was added to the homogenized cell lysate and transferred to RNeasy spin column. After appropriate washing steps, using buffers provided with the kit, RNA was eluted from the column by adding 30 µl of RNase-free water. Total RNA was measured by loading 1 µl of undiluted RNA directly onto the lower measurement pedestal of a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Two micrograms of total RNA were treated with one unit of DNase

I (amplification grade) to avoid genomic DNA contamination. cDNA was synthesized in a final volume of 20 µl, containing 4 µl of first-strand buffer, 2 µg of RNA, 1 µl of Oligo(dT)20 Primer 50 µM, 1 µl of 10 mM dNTP Mix, 1 µl of RNAseOUT Recombinant Ribonuclease Inhibitor 40 U/µl, 1 µl of 0.1 M Dithiothreitol and 1 µl SuperScript™ III Reverse Transcriptase (200 units/µl). cDNA (2 µl) was then used for PCR amplification in a 25 µl reaction mixture following the protocol for Platinum *Taq* DNA Polymerase, using specific primers sequences for Oct4 (POU5F1), GATA4, phospholamban (PLB), desmin, cardiac troponin I (cTnI), OTR and β-actin genes.

Primer sequences, specific annealing temperatures, number of cycles and amplicon size, are summarized in Table 1. Negative controls for PCR amplification were run at the same time as samples, either water or non-reverse transcribed RNA were used instead of cDNA to discard genomic DNA contamination.

The PCR products were size-fractioned by 1-1.5% agarose gel electrophoresis and photographed under UV light (Chemi genius 2, Bioimaging system, Syngene, Frederick, MD, USA.).

**4.3.3 Cardiomyogenic differentiation**

For cardiomyogenic differentiation, pBMSCs collected at passage 1 were subcultured at a density of 20,000 cells/cm$^2$ in DMEM-LG medium containing 15% FBS and antibiotic-antimycotic mix. After one day of subculture, cells were divided into three treatment groups: control group (no treatment), OT treated group, and 5-Aza treated group (as a positive control of differentiation). For the control group, the medium was replaced by the same medium described before without adding any differentiation inducer (OT or 5-Aza). In OT group, medium was replaced with medium containing OT (Bachem, King of Prussia, PA, USA) at a concentration of $10^{-5}$ M. In the 5-Aza group, differentiation was induced by adding medium

containing 5-Aza (Sigma, St. Louis, MO USA) at a 10 μM concentration, as previously described.[20, 22] Cells were exposed (day 0) to the medium containing or not the differentiation inducers. Medium was changed twice a week for three weeks until termination of the experiment. The optimal time for differentiation induction and the optimal concentration of OT, in OT-mediated differentiation induction, were determined from previous assays (data not shown). Cardiomyogenic differentiation was assessed qualitatively by RT-PCR as described above (Table 1), cardiac specific proteins expression was assessed by immunocytochemistry (ICC) and by western blot. Also, the ultrastructure of induced (OT and 5-Aza groups) and non-induced (control group) cells was analysed using transmission electron microscopy (TEM).

**4.3.4 Immunocytochemistry**

Induced (OT and 5-Aza groups) and non-induced (control group) cells, from three different isolates, grown on glass coverslips were collected three weeks after differentiation induction. Cells were washed twice in PBS (Roche, Indianapolis IN, USA) and fixed in ice-cold methanol for 10 min and stored at -20$^{o}$C, pending analysis. Fixed cells were washed thrice for 5 min in PBS. To avoid non-specific binding, cells were incubated overnight at 4$^{o}$C in a blocking solution consisting in 10% of normal goat serum in PBS. Then, cells were again incubated overnight at 4$^{o}$C with primary antibodies directed against: PLB 1:200 (ABR Affinity BioReagents, Golden, CO, USA),cardiac isoform troponin-T (cTnT) 1:100 (Lab Vision, Fremont, CA, USA), and cardiac myosin heavy chain (cMHC) 1:100 (ABR Affinity BioReagents). After 3 x 5 min washing steps in PBS, cells were incubated with Alexa fluor conjugated goat anti-mouse IgG secondary antibody 1:1000 for 1 h at room temperature. After 3 x 5 min washing steps in PBS, nucleus were stained by a 3 min incubation with Hoechst 33342 solution 1:1500, washed twice and mounted using Vectashield mounting medium for fluorescence (Vector laboratories Inc., Burlingame, CA, USA). For ICC negative

controls, cells were incubated only with secondary antibody. Slides were observed under a fluorescence microscope (Leica ASLMD microscope; Leica Cambridge, Cambridge, UK) equipped with a mercury lamp (HBO 103 W/2; LEJ, Jena, Germany). Micrographs were acquired with a Leica DC500 camera and a DFC Twain Software (Leica).

To determine the degree of cardiomyogenic differentiation induced by OT and 5-Aza, cells expressing cMHC and cTnT, were counted from three different micrographs taken from three different microscopic fields (200x magnification). Cell counts are reported as a percentage of cells expressing these cardiac specific proteins, the percentage being calculated by counting the total number of cells (nucleus counted) in each microscopic field and from these cells the number of cells expressing the cardiac specific proteins mentioned above. Cell counts were performed from three different isolates.

**4.3.5 Western Blot analysis**

Total protein expression of cTnI was determined by semi-quantitative analysis. Two weeks after differentiation, induced and non-induced cells were washed once with Dulbecco's PBS. Cells were collected and lysed with cell lysis/extraction buffer (CelLytic™ M, Sigma Aldrich, Oakville, ON, Canada), supplemented with protease inhibitor cocktail (Sigma Aldrich), according to the supplier's recommendations. Briefly, cell lysates were centrifuged at 17,000 x *g* for 15 min; supernatants were stored at -80° C pending analysis. Protein concentration was determined using Bio-Rad Protein Assay (Bio-rad Laboratories, Hercules, CA, USA), based on Bradford method.[23] Protein samples (80 μg) from collected cells obtained form three different isolates, and protein from porcine heart (40 μg) were subjected to electrophoresis on a 12% sodium dodecyl sulfate-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 primary antibody directed against cTnI (AbD Serotec, Cat. No. MCA1208, Cedarlane Laboratories Ltd., Burlington, ON, Canada), at a 1:500 concentration. Membranes were washed thrice with Tween 20-TBS and incubated for 30 min at room temperature, with the horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (Calbiochem, VWR CANLAB Mississauga, 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, Hercules, CA, USA), 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). Semi-quantitavive analysis was performed by densitometry using GeneSnap from Syngene (Perkin Elmer). Protein expression was represented as a value relative to ß-actin expression. Triplicate gels and immunoblots were run for all samples, from three different isolates.

**4.3.6 Transmission electron microscopy**

Ultrastructural analysis was performed by TEM. Induced and non-induced obtained from three different isolates cells were grown in plastic coverslips (Nalge Nunc International, Rochester, NY, USA). Three weeks after treatment cells were washed in PBS and fixed overnight at 4$^{o}$C, in cacodylate buffer (0.05 M pH 7.4) containing 2.5% glutaraldehyde. Cells were then rinced thrice in cacodylate buffer, and post-fixed in 2% osmium tetroxide in cacodylate buffer, dehydrated and stained in uranyl acetate 0.5% in maleate buffer 0.05 M pH 5.2 for 1 h at 4$^{o}$C. Cells were then embedded in epoxy resin. Ultrathin sections, cut transversal to the growing surface were viewed under a TEM Philips 300 (Royal Philips Electronics, Eindhoven, the Netherlands).

### 4.3.7 Statistical analysis

The analysis for cell counts and western blot was performed from three different cell isolates. Data from cell counts percentages and western blot ratios were ranked within each individual across treatments and an ANOVA was performed on the ranked values with treatment as a factor. This is the equivalent of the Friedman test for repeated measures. Tukey's post-hoc tests were used to compare mean values between pairs of treatments. The significance level was set at 0.05 and SAS version 9.1 (Cary, N.C.) was used for the analysis.

## 4.4 RESULTS

### 4.4.1 Isolation and characterization of pBMSCs

The pBMSCs were successfully isolated from BM aspirates. Most of non-adherent cells were removed with medium changes at 24 and 72 h after the first plating. Small colonies of adherent fibroblast-like cells attached to the plastic were evident after 5 days in culture. The number of cells and the size of colonies increased progressively. Cells reached 80-90% of confluence at 10 to 15 days after first seeding.

The pBMSCs from passage 1 analyzed for surface epitopes were positive for CD29 (β1-integrin), and CD90 (Thy-1), and were partially negative for CD31 (PECAM-1), as shown in Figure 1. As expected, these cells were negative for CD45, a leukocyte marker.

Before differentiation induction, pBMSCs expressed Oct4, GATA4, OTR and PLB, as shown in Figure 2. These markers were found only during passages 0 and 1, and disappeared or were down-regulated in subsequent passages. Porcine BMSCs did not express other cardiomyocyte structural proteins, such as cTnI and desmin (data not shown).

### 4.4.2 Cardiomyogenic differentiation induction

Both induced and non-induced cells expressed genes involved in cardiomyogenesis. RNA was extracted for cDNA synthesis at days 4, 6, 10, and 14 after differentiation induction, showing a pattern of early and late gene expression. Four days after differentiation induction, none of the genes listed in table 1 were expressed (data not shown). Six days after treatment, induced and non-induced cells expressed only GATA4, with a clear up-regulation in treated cells (Fig. 3). GATA4 expression was down-regulated or not apparent 10 days after treatment (data not shown). Conversely, gene expression of cardiomyocyte structural proteins: PLB, desmin and cTnI were observed only 10 days after treatment, in induced and non-induced cells, without visible differences in transcripts expression among the groups, as shown in Figure 3.

By ICC analysis, it was observed that, two weeks after differentiation induction, PLB protein was expressed in all groups. However, the fluorescence signal was weaker in the control group or non-induced cells, without significant differences. On the other hand, expression of cTnT (13.6±2.7 SD %) and cMHC (12.8±6.9 SD %), was lower in the control group and 5-Aza-treated group, cTnT (12.5±5.2 SD %) cMHC (11.5±4 SD %), compared to the expression observed in OT-treated, which was significantly stronger cTnT p=0.01 (26.2±7.1 SD %), cMHC p=0.01 (26.3±7.7 SD %). Moreover, there was no significant difference on cTnT and cMHC expression, between non-induced cells and 5-Aza-induced cells (Fig. 4). The percentages mentioned above refer to the immunopositive cells found in each microscopic field, as described in the methods section.

On semi-quantitative protein analysis, it was observed that protein expression of cTnI was more abundant in OT-treated cells compared with control cells and 5-Aza-treated cells (Fig.

5). There was a significant effect of OT treatment in the up-regulation of cTnI protein expression ($p<0.01$). Post-hoc Tukey's analysis indicated that the mean of the ranks was significantly more elevated in the OT-treated cells when compared with control cells and 5-Aza-treated cells, but there was no significant difference when control cells and 5-Aza-treated cells were compared.

The ultrastructural analysis of the transversal sections of induced pBMSCs showed that, three weeks after induction with OT and 5-Aza, the cells presented centrally positioned nucleus, numerous mitochondria and myofilaments in the cytoplasm, aligned in a parallel fashion but without forming typical striated sarcomeres (Fig. 6).

## 4.5 DISCUSSION

Stem cells, especially BMSCs, are a promising source for MI cell-based therapy. The BMSCs can be obtained from patient's bone marrow by performing a minor procedure, or they can be obtained from a donor. Since they apparently are not immunogenic, there are no complications related to allogenic rejection.[24] They can be easily expanded *in vitro*. Moreover, BMSCs are able to express cardiac markers after induction with 5-Aza, growth factors cocktails, and difluoromethylornithine.[21, 25, 26] Some reports have shown that BMSCs delivered into the infarct area have the ability to promote angiogenesis and prevent apoptosis of surviving cardiomyocytes. Moreover, these effects were mediated by paracrine factors secreted by BMSCs.[12, 27] By using BMSCs, ethical concerns and the possible teratoma formation related to the use of ESCs are avoided.[28]

In this study, we were able to isolate and characterize pBMSCs. There are many factors influencing the capabilities of BMSCs differentiation, such as: culture medium, presence and concentration of undetermined growing factors in the FBS, number of passages, as well as

differentiation inducer(s) added to the medium. In fact, some reports have shown that BMSCs, particularly MSC subpopulation, have certain predisposition to differentiate into cardiomyocytes or have passage-restricted differentiation potential into cardiomyocyte-like cells, depending on the time they have been in culture with a very possible variation among species.[29] Specifically, porcine MSCs phenotype and differentiation potential are affected during culture.[30]

Here, we report that pBMSCs are affected by the time in culture and the number of passages, not only in size and shape, but also gene expression was altered through the passages, in the culture conditions previously described for this study. Based on the results obtained during pBMSC characterization, it seemed appropriate to induce differentiation during early passages, specifically during passage 1, when pBMSCs correspond to the phenotypic profile of surface epitopes described for MSCs.[21, 31, 32]

At passage 1, they also expressed important levels of OTR transcripts, which could be an indication of responsiveness to OT stimulation. As mentioned before, OT/OTR system is actively expressed during early cardiogenesis, and OTR are upregulated in P19 EC cells, ESCs and CSCs upon cardiomyogenic differentiation induction with OT, DMSO and retinoic acid.[15-17, 33] In addition, pBMSCs also expressed transcription factors, Oct4 previously related to pluripotency,[34] and GATA4 implicated in cardiac differentiation and regulation of expression of most cardiac genes. Indeed, GATA4 binds and transactivates the promoters or enhancers of cMHC[35] and cardiac troponins, and it is also involved in heart tube formation.[36] Finally, pBMSCs expressed PLB, integral membrane protein that regulates the $Ca^{2+}$ pump in cardiac and skeletal muscle cells. It is likely that pBMSCs expressed PLB transcripts, because as previously reported,[37] MSCs experience a certain degree of myogenic differentiation once they are expanded *in vitro,* even without differentiation induction.

However, if not induced they fail to express cardiac specific markers present in fully differentiated cells. For example, they failed to express cardiac troponin I and SERCA-2,[37] possibly because they cannot fully differentiate, or because they only have the potential to differentiate in myogenic phenotypes, without appropriate stimulation.

After differentiation induction of pBMSCs, the expression of GATA4, desmin, PLB, and cTnI transcripts was more abundant in the OT- and 5-Aza-induced cells than in non-induced cells. It is noteworthy to mention that when pBMSCs were passaged at 80-90% of confluency for characterization purposes, desmin and cTnI transcripts were not observed. However, when cells were subcultured for differentiation purposes, desmin and cTnI transcripts were even expressed in pBMSCs control group cells, meaning pBMSCs experienced a certain degree of differentiation, even without induction. This could be related to the role that cellular density and/or aggregation have over differentiation. It is known that maintenance of undifferentiated nature is closely related to cell density.[38-40]

Conversely, protein expression of cardiomyocyte specific proteins was more abundant in the induced groups. Even though there was no clear difference in gene transcripts expression in the induced groups, protein expression of cardiac specific proteins was more abundant in OT-induced cells. Expression of cardiac MHC and cTnT was more abundant in OT- *vs.* 5-Aza-induced cells, and this difference was statistically significant, the percentage of cells expressing these cardiac specific proteins was higher in OT-treated *vs.*5-Aza-treated cells. Nevertheless, PLB expression was similarly up-regulated in all groups, but PLB is expressed not only in cardiomyocytes but also in skeletal and smooth muscle cells. In addition, semi-quantitative protein analysis revealed that OT- induced cells have more abundant expression of cTnI compared to control group and 5-Aza-induced cells, with no significant difference between control and 5-Aza groups cells.

The 5-Aza being a cytidine analog and demethylating agent, inhibitis DNA methyltransferase, improving BMSC transdifferentiation potential by hypomethylating regulatory genes. Therefore, 5-Aza is not a cardiac specific differentiation inducer.[41] It also activates the expression of genes and proteins not involved in cardiomyogenic differentiation. Some have been characterized,[42] but others remain unknown. In addition, 5-Aza inhibits the synthesis of total cellular proteins, while RNA synthesis overall rate remains nearly unchanged[43, 44] or even up-regulated, as observed here for GATA4, PLB, and cTnI transcripts expression. If 5-Aza inhibits protein synthesis by affecting the function of several types of RNA that are essential for the process of protein synthesis, it is a highly toxic agent and produces cell death.[45] In consequence, in our study 5-Aza caused a lower expression of cardiac structural proteins: cardiac MHC and troponin T. This might also interfere with the cardiomyogenic differentiation, without mentioning the cellular toxic effects caused by this agent. In accordance with the results observed in this study, 5-Aza induction of CSCs, another source of adult stem cells with cardiomyogenic potential, also induced expression of cardiac genes but did not induce protein expression of cardiac troponin T.[18] These results suggest that treatment with 5-Aza induced incomplete differentiation of CSCs, as observed here for pBMSCs.

Ultrastructural analysis of pBMSCs revealed that both non-induced and induced cells presented an inner cytoplasmic zone rich in organelles, especially mitochondria and Golgi apparatus, as observed in human MSCs.[22] On the other hand, OT-induced cells presented numerous myofilaments, but the formation of visible sarcomeres was not observed neither in the OT-, nor in 5-Aza-induced cells. This is in accordance with the ultrastructure observations of human MSCs, 5-Aza-induced, but in contrast with the observations reported in murine MSCs, which presented typical striation and sarcomere formation eight weeks after treatment with 3 μM 5-Aza.[46]

These variations in the ultrastructural analysis of BMSCs could be related with the difference in stem cell biology among the species, as previously described for the specific case of ESCs [For review see ref.[47]]. The differences in stem cells biology could be related with the great disparity in life-span between murine models and humans. Fukuda found sarcomeres formation only 8 weeks after 5-Aza differentiation induction.[46] But, Xu *et al.* found no sarcomere formation in human MSCs two weeks after treatment with 10 μM 5-Aza.[48] It is likely that the time for observing sarcomere formation in human and porcine BMSCs is longer since life-span is notoriously longer in porcine and humans compared to mice.

The signaling pathway by which OT induces cardiomyogenic differentiation of P19 EC cells, ESCs and CSCs has not been elucidated yet. In the case of pBMSCs, we observed an up-regulation of GATA4, which is closely related to cardiogenesis.[49] But further investigation needs to be carried out, in order to understand and clarify the possible mechanisms related with OT-induced cardiomyogenic differentiation. A better understanding of the signaling pathways stimulated after OTR activation could improve the potential of OT as cardiomyogenic inducer.

In this study, we observed that OT is able to increase the expression of cardiac specific structural proteins in pBMSCs, as previously described for murine P19 EC cells, ESCs and CSCs.[16-18] Oxytocin was a more potent inducer of cardiac differentiation of pBMSCs than 5-Aza, because cardiac structural proteins were more abundant in the OT-induced cells compared with 5-Aza cells. These results on OT-induced pBMSCs look promising for testing their cardioregenerative potential on a porcine MI model. The MI murine models have important differences related to their size and life-span compared to those of humans. The porcine model is more suitable, considering its similarities with human anatomy, physiology, and life-span.[50] Moreover, the *in-vivo* porcine model of reperfused MI is well-established and

largely used,[51, 52] it offers the possibility of actively choosing the location of the occlusion of the coronary artery, and control over the duration of the ischemia. Also, there is low additional morbidity related with the experimental model itself, it is possible to use a minimal invasive approach, therefore avoiding any possible additional influence on experiments, and collect repeated blood and tissue samples. Finally the porcine model has been demonstrated as a well-suited model for acute and chronic myocardial infarctions.[51] Post-infarct OT treatment with 10 mg/kg SC (once a day for 5 consecutive days) reduced MI size and improved left ventricle function and remodeling by activating OTR and prosurvival signals and by exerting antifibrotic and angiogenic effects.[14] Our group, using the swine MI model, recently demonstrated that endogenous pre-MI OT levels and timing of administration post-MI are important criteria, if OT is intended to be used as a therapeutic strategy for ischemic heart disease.[19] Pigs with low endogenous OT level receiving OT subcutaneous infusion (10 $ng \cdot kg^{-1} \cdot hr^{-1}$) starting 8 days post-MI, as well as pigs with high endogenous OT level receiving saline presented lower MI area measured by planimetry, and cardiac function was also less affected. Moreover, high endogenous OT level pigs receiving OT infusion had highly-affected cardiac function associated to bigger MI area, as well as down-regulation of myocardial OTR. Such results highlighted the major interest of OT-based therapeutics in ischemic heart disease, but also the apparent narrow therapeutic window for direct administration of OT endogenous peptide.[19]

Considering the importance of the microenvironment in determining the differentiation of BMSCs,[53] it is probable that pBMSCs require *in vitro* pre-programming before the implantation. Therefore, OT-predifferentiated pBMSCs with concomitant OT treatment could improve the recovery in a porcine MI model, by supporting a better engraftment and survival of the cells in the MI area, improving angiogenesis, reducing apoptosis and promoting recruitment of circulating progenitor stem cells in the injured tissue, while minimizing side

effects occurrence.[54] A major discrepancy between animal studies and clinical trials is the age of the cells delivered. Animal models have tested BMSCs harvested from young healthy animals, which are then injected into young animal models with a single myocardial injury. Proliferative and transdifferentiation potential of those cells is high. By contrast, the mobilised BMSCs from middle-aged or elderly patients with heart failure[55] or coronary disease[56] are dysfunctional, and the likelihood that they have significant capacity to transdifferentiate into cardiomyocytes is low. It is important to better stimulate grafting, transdifferentiation of purified and pre-differentiated BMSCs, and/or mobilization and homing of endogenous BMSCs,[57] it will be interesting to test OT potential to improve these conditions. Finally, OT use could be considered in the avenue of induced pluripotent stem cells while testing if OT could facilitate cardiac differentiation of reprogrammed somatic cells,[58] as this has been the case with 5-Aza, ascorbic acid, bone morphogenetic proteins and vascular endothelial growth factor.[59, 60]

## 4.6 ACKNOWLEDGEMENTS

Special thanks to Dr Guy Beauchamp for performing the statistical analysis in this report. Also very special thanks to Dr. Bruce D. Murphy and the entire lab staff of CRRA, Faculté de médecine vétérinaire, Université de Montréal, for their technical assistance in conducting this project. Many thanks to: Dominique Gauvin for her help and technical assistance, Dr André Blouin and Céline Viens for their assistance in interpretation and sample preparation for transmission electron microscopy.N.Y. is a *Ph.D.* student funded by the Canadian Institutes of Health Research – Strategic Training Initiative GREAT program, and the CONACyT (*Consejo Nacional de Ciencia y Tecnología*), Mexico.

Conflict of interest: The authors state no conflict of interest.

## 4.7 REFERENCES

1. Partovian C, Simons M. Stem cell therapies in cardiovascular disease A "realistic" appraisal. Drug Discov Today Ther Strateg 2008;5(1):73-8

2. Tao ZW, Li LG. Cell therapy in congestive heart failure. J Zhejiang Univ Sci B 2007 Sep;8(9):647-60

3. Dimmeler S, Burchfield J, Zeiher AM. Cell-based therapy of myocardial infarction. Arterioscler Thromb Vasc Biol 2008 Feb;28(2):208-16

4. Liu J, Sluijter JP, Goumans MJ, Smits AM, van der Spoel T, Nathoe H, et al. Cell therapy for myocardial regeneration. Curr Mol Med 2009 Apr;9(3):287-98

5. Villa A, Sanchez PL, Fernandez-Aviles F. Ventricular arrhythmias following intracoronary bone marrow stem cell transplantation. Europace 2007 Dec;9(12):1222-3

6. Kang HJ, Kim HS, Zhang SY, et al. Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: the MAGIC cell randomised clinical trial. Lancet 2004 Mar;363(9411):751-6

7. Mansour S, Vanderheyden M, De Bruyne B, et al. Intracoronary delivery of hematopoietic bone marrow stem cells and luminal loss of the infarct-related artery in patients with recent myocardial infarction. J Am Coll Cardiol 2006 Apr;47(8):1727-30

8. Tomita S, Li RK, Weisel RD, et al. Autologous transplantation of bone marrow cells improves damaged heart function. Circulation 1999 Nov;100(19 Suppl):II247-56

9. Wang JS, Shum-Tim D, Chedrawy E, Chiu RC. The coronary delivery of marrow stromal cells for myocardial regeneration: pathophysiologic and therapeutic implications. J Thorac Cardiovasc Surg 2001 Oct;122(4):699-705

10. Wang T, Xu Z, Jiang W, Ma A. Cell-to-cell contact induces mesenchymal stem cell to differentiate into cardiomyocyte and smooth muscle cell. Int J Cardiol 2006 Apr;109(1):74-81

11. Liao R, Pfister O, Jain M, Mouquet F. The bone marrow--cardiac axis of myocardial regeneration. Prog Cardiovasc Dis 2007 Jul-Aug;50(1):18-30

12. Xu S, Xu X, Guo Y, Gao W. Inflammatory responses after intracoronary mononuclear BM cell therapy in swine. Bone Marrow Transplant 2009 Oct;44(7):427-31

13. Ondrejcakova M, Ravingerova T, Bakos J, Pancza D, Jezova D. Oxytocin exerts protective effects on in vitro myocardial injury induced by ischemia and reperfusion. Can J Physiol Pharmacol 2009 Feb;87(2):137-42

14. Kobayashi H, Yasuda S, Bao N, et al. Postinfarct treatment with oxytocin improves cardiac function and remodeling via activating cell-survival signals and angiogenesis. J Cardiovasc Pharmacol 2009 Dec;54(6):510-9

15. Paquin J, Danalache BA, Jankowski M, McCann SM, Gutkowska J. Oxytocin induces differentiation of P19 embryonic stem cells to cardiomyocytes. Proc Natl Acad Sci USA 2002 Jul;99(14):9550-5

16. Fathi F, Murasawa S, Hasegawa S, Asahara T, Kermani AJ, Mowla SJ. Cardiac differentiation of P19CL6 cells by oxytocin. Int J Cardiol 2009 May;134(1):75-81

17. Hatami L, Valojerdi MR, Mowla SJ. Effects of oxytocin on cardiomyocyte differentiation from mouse embryonic stem cells. Int J Cardiol 2007 Apr;117(1):80-9

18. Matsuura K, Nagai T, Nishigaki N, et al. Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes. J Biol Chem 2004 Mar;279(12):11384-91

19. Authier S, Tanguay JF, Geoffroy P, et al. Cardiovascular effects of oxytocin infusion in a porcine model of myocardial infarct. J Cardiovasc Pharmacol 2010 Jan;55(1):74-82

20. Liu J, Hu Q, Wang Z, et al. Autologous stem cell transplantation for myocardial repair. Am J Physiol Heart Circ Physiol 2004 Aug;287(2):H501-11

21. Moscoso I, Centeno A, Lopez E, et al. Differentiation in vitro of primary and immortalized porcine mesenchymal stem cells into cardiomyocytes for cell transplantation. Transplant Proc 2005 Jan-Feb;37(1):481-2

22. Antonitsis P, Ioannidou-Papagiannaki E, Kaidoglou A, Papakonstantinou C. In vitro cardiomyogenic differentiation of adult human bone marrow mesenchymal stem cells. The role of 5-azacytidine. Interact Cardiovasc Thorac Surg 2007 Oct;6(5):593-7

23. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976 May;72:248-54

24. Mishra PK. Bone marrow-derived mesenchymal stem cells for treatment of heart failure: is it all paracrine actions and immunomodulation? J Cardiovasc Med (Hagerstown) 2008 Feb;9(2):122-8

25. Bartunek J, Croissant JD, Wijns W, et al. Pretreatment of adult bone marrow mesenchymal stem cells with cardiomyogenic growth factors and repair of the chronically infarcted myocardium. Am J Physiol Heart Circ Physiol 2007 Feb;292(2):H1095-104

26. Muscari C, Bonafe F, Carboni M, et al. Difluoromethylornithine stimulates early cardiac commitment of mesenchymal stem cells in a model of mixed culture with cardiomyocytes. J Cell Biochem 2008 Mar;103(4):1046-52

27. Uemura R, Xu M, Ahmad N, Ashraf M. Bone marrow stem cells prevent left ventricular remodeling of ischemic heart through paracrine signaling. Circ Res 2006 Jun;98(11):1414-21

28. He Q, Trindade PT, Stumm M, et al. Fate of undifferentiated mouse embryonic stem cells within the rat heart: role of myocardial infarction and immune suppression. J Cell Mol Med 2009 Jan;13(1):188-201

29. Zhang FB, Li L, Fang B, Zhu DL, Yang HT, Gao PJ. Passage-restricted differentiation potential of mesenchymal stem cells into cardiomyocyte-like cells. Biochem Biophys Res Commun 2005 Oct;336(3):784-92

30. Vacanti V, Kong E, Suzuki G, Sato K, Canty JM, Lee T. Phenotypic changes of adult porcine mesenchymal stem cells induced by prolonged passaging in culture. J Cell Physiol 2005 Nov;205(2):194-201

31. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999 Apr;284(5411):143-7

32. Guo KT, SchAfer R, Paul A, Gerber A, Ziemer G, Wendel HP. A new technique for the isolation and surface immobilization of mesenchymal stem cells from whole bone marrow using high-specific DNA aptamers. Stem Cells 2006 Oct;24(10):2220-31

33. Jankowski M, Danalache B, Wang D, et al. Oxytocin in cardiac ontogeny. Proc Natl Acad Sci USA 2004 Aug;101(35):13074-9

34. Lengner CJ, Camargo FD, Hochedlinger K, et al. Oct4 expression is not required for mouse somatic stem cell self-renewal. Cell Stem Cell 2007 Oct;1(4):403-15

35. Molkentin JD, Kalvakolanu DV, Markham BE. Transcription factor GATA-4 regulates cardiac muscle-specific expression of the alpha-myosin heavy-chain gene. Mol Cell Biol 1994 Jul;14(7):4947-57

36. Narita N, Bielinska M, Wilson DB. Cardiomyocyte differentiation by GATA-4-deficient embryonic stem cells. Development 1997 Oct;124(19):3755-64

37. Bayes-Genis A, Roura S, Soler-Botija C, et al. Identification of cardiomyogenic lineage markers in untreated human bone marrow-derived mesenchymal stem cells. Transplant Proc 2005 Nov;37(9):4077-9

38. Arai F, Ohneda O, Miyamoto T, Zhang XQ, Suda T. Mesenchymal stem cells in perichondrium express activated leukocyte cell adhesion molecule and participate in bone marrow formation. J Exp Med 2002 Jun 17;195(12):1549-63

39. Reyes M, Lund T, Lenvik T, Aguiar D, Koodie L, Verfaillie CM. Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood 2001 Nov;98(9):2615-25

40. Sekiya I, Larson BL, Smith JR, Pochampally R, Cui JG, Prockop DJ. Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. Stem Cells 2002;20(6):530-41

41. Bittira B, Kuang JQ, Al-Khaldi A, Shum-Tim D, Chiu RC. In vitro preprogramming of marrow stromal cells for myocardial regeneration. Ann Thorac Surg 2002 Oct;74(4):1154-9

42. Ye NS, Chen J, Luo GA, Zhang RL, Zhao YF, Wang YM. Proteomic profiling of rat bone marrow mesenchymal stem cells induced by 5-azacytidine. Stem Cells Dev 2006 Oct;15(5):665-76

43. Cihak A. Biological effects of 5-azacytidine in eukaryotes. Oncology 1974;30(5):405-22

44. Reichman M, Penman S. The mechanism of inhibition of protein synthesis by 5-azacytidine in HeLa cells. Biochim Biophys Acta 1973 Oct;324(2):282-9

45. Vesely J. Mode of action and effects of 5-azacytidine and of its derivatives in eukaryotic cells. Pharmacol Ther 1985;28(2):227-35

46. Fukuda K. Development of regenerative cardiomyocytes from mesenchymal stem cells for cardiovascular tissue engineering. Artif Organs 2001 Mar;25(3):187-93

47. Van Hoof D, Passier R, Ward-Van Oostwaard D, et al. A quest for human and mouse embryonic stem cell-specific proteins. Mol Cell Proteomics 2006 Jul;5(7):1261-73

48. Xu W, Zhang X, Qian H, et al. Mesenchymal stem cells from adult human bone marrow differentiate into a cardiomyocyte phenotype in vitro. Exp Biol Med (Maywood) 2004 Jul;229(7):623-31

49. Auda-Boucher G, Bernard B, Fontaine-Perus J, Rouaud T, Mericksay M, Gardahaut MF. Staging of the commitment of murine cardiac cell progenitors. Dev Biol 2000 Sep;225(1):214-25

50. Swindle MM. Swine as replacements for dogs in the surgical teaching and research laboratory. Lab Anim Sci 1984 Aug;34(4):383-5

51. Krombach GA, Kinzel S, Mahnken AH, Gunther RW, Buecker A. Minimally invasive close-chest method for creating reperfused or occlusive myocardial infarction in swine. Invest Radiol 2005 Jan;40(1):14-8

52. Suzuki Y, Lyons JK, Yeung AC, Ikeno F. In vivo porcine model of reperfused myocardial infarction: in situ double staining to measure precise infarct area/area at risk. Catheter Cardiovasc Interv 2008 Jan;71(1):100-7

53. Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation 2002 Jan;105(1):93-8

54. de Macedo Braga LM, Lacchini S, Schaan BD, et al. In situ delivery of bone marrow cells and mesenchymal stem cells improves cardiovascular function in hypertensive rats submitted to myocardial infarction. J Biomed Sci 2008 May;15(3):365-74

55. Heeschen C, Lehmann R, Honold J, et al. Profoundly reduced neovascularization capacity of bone marrow mononuclear cells derived from patients with chronic ischemic heart disease. Circulation 2004 Apr;109(13):1615-22

56. Walter DH, Haendeler J, Reinhold J, et al. Impaired CXCR4 signaling contributes to the reduced neovascularization capacity of endothelial progenitor cells from patients with coronary artery disease. Circ Res 2005 Nov;97(11):1142-51

57. Ballard VL, Edelberg JM. Stem cells for cardiovascular repair - the challenges of the aging heart. J Mol Cell Cardiol 2008 Oct;45(4):582-92

58. Boer B, Cox JL, Claassen D, Mallanna SK, Desler M, Rizzino A. Regulation of the Nanog gene by both positive and negative cis-regulatory elements in embryonal carcinoma cells and embryonic stem cells. Mol Reprod Dev 2009 Feb;76(2):173-82

59. Gai H, Leung EL, Costantino PD, et al. Generation and characterization of functional cardiomyocytes using induced pluripotent stem cells derived from human fibroblasts. Cell Biol Int 2009 Nov;33(11):1184-93

60. Moretti A, Bellin M, Jung CB, et al. Mouse and human induced pluripotent stem cells as a source for multipotent Isl1+ cardiovascular progenitors. FASEB J 2010 Mar;24(3):700-11

## 4.8 TABLES AND FIGURES

**Table 1. PCR primers and reaction conditions.**

| Gene | GenBank Accession No. | Primer sequence | Annealing Temp, $^{o}$C | PCR cycles | Product size, bp |
|---|---|---|---|---|---|
| Oct4 | **AJ251914** | F: AGGTGTTCAGCCAAACGACC | 60 | 35 | 341 |
| | | R: TGATCGTTTGCCCTTCTGGC | | | |
| GATA4 | **NM_214293** | F: GCCTCTACCACAAGATGAAT | 53.3 | 35 | 598 |
| | | R: AGTGATTATGTCCCCATGAC | | | |
| PLB | **NM_214213** | F: CTTTTTCAGCTTTCTCTTG | 51 | 35 | 530 |
| | | R: ACCCCTAGTTCATCCTCA | | | |
| Desmin | **NM_001001535** | F: CCAGCGGCTACCAGGACAACAT | 62 | 33 | 587 |
| | | R: CCAAGGGCCAGGCTCACTCACT | | | |
| cTnI | **NM_001098599** | F: CCCACCTCAAGCAGGTGAAGA | 65 | 35 | 128 |
| | | R: GCCAGCTCAGCCCTCAAACTT | | | |
| OTR | **NM_214027** | F: GGCCGAGCGGCTCTGGCCCGCGTCA | 61 | 35 | 408 |
| | | R:CGTGGATGGCTACGAGCAGCTCTTCTG | | | |
| β-Actin | **U07786** | F: GGACTTCGAGCAAGGAGATGG | 54 | 30 | 233 |
| | | R: GCACCGTGTTGGCGTAGAGG | | | |

F= Forward and R= reverse primer sequences for semi-quantitative RT-PCR. Abbreviations: Octamer 4 = POU class 5 homeobox 1 (Oct4 = POU5F1); GATA4 (GATA binding protein 4); phospholamban (PLB), cardiac troponin I (cTnI), oxytocin receptor (OTR).

**Figure 1. Fluorescent-activated cell sorting analysis of porcine bone marrow stem cells (pBMSCs) surface markers.**

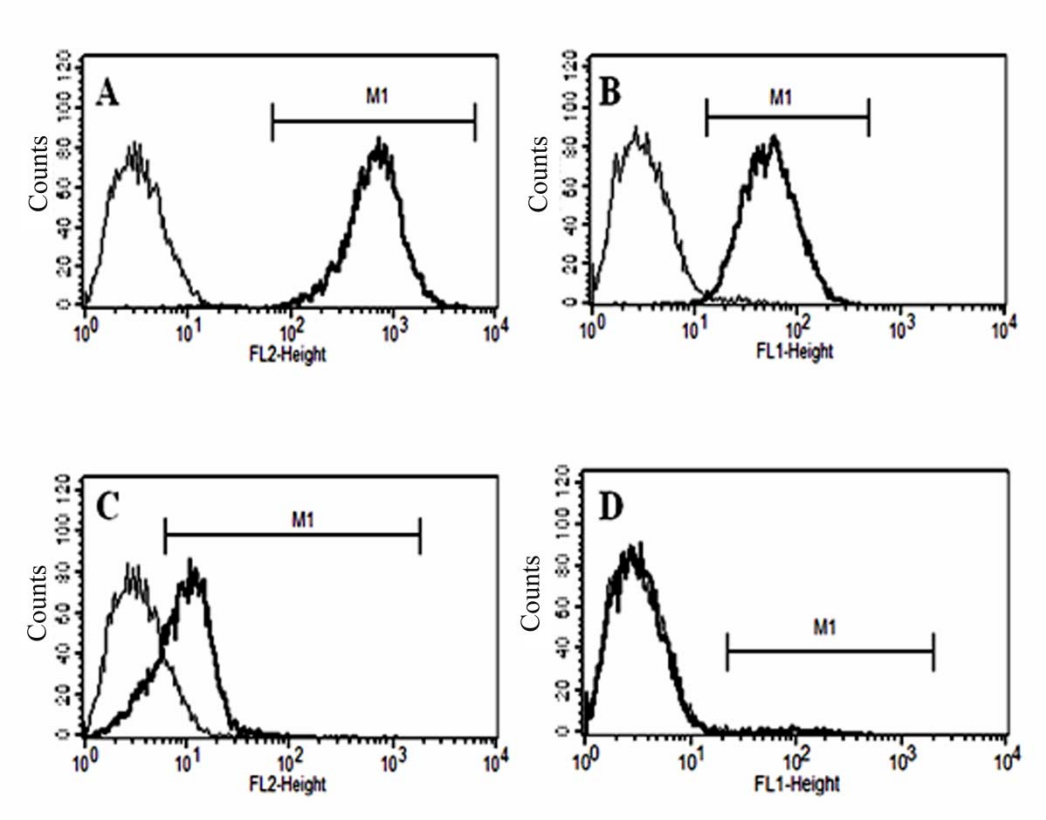



Fig. 1. Antibodies used were conjugated CD90-RPE (A), CD29-FITC (B), CD31-RPE (C), CD45-FITC (D). Normal line curves represent isotype controls used for calibration. Bold line curves represent cells incubated with the conjugated antibody. The pBMSCs used here were at passage 1. Abbreviations: FITC (Fluorescein isothiocyanate isomer 1), RPE (R-phycoerythrin).

**Figure 2. Semi-quantitative RT-PCR analysis of gene expression profile in porcine bone marrow stem cells (pBMSCs).**

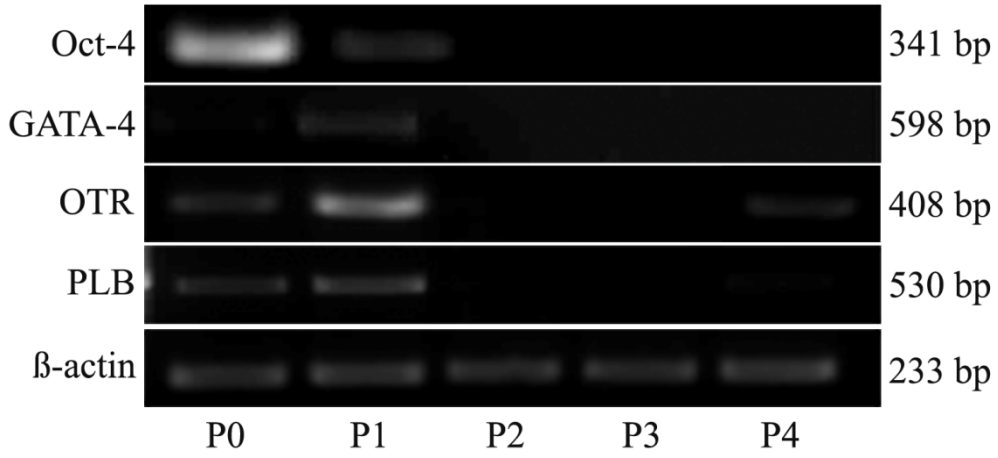



Fig. 2. Transcripts expressed by pBMSCs (porcine bone marrow stem cells) collected from passage (P) 0 to 4. Oct4, GATA4, oxytocin receptor (OTR), phospholamban (PLB), and β-actin used as an internal control. Representative images of gene expression profile observed in three different cell isolates.

**Figure 3. Semi-quantitative RT-PCR analysis of gene expression profile after differentiation induction.**

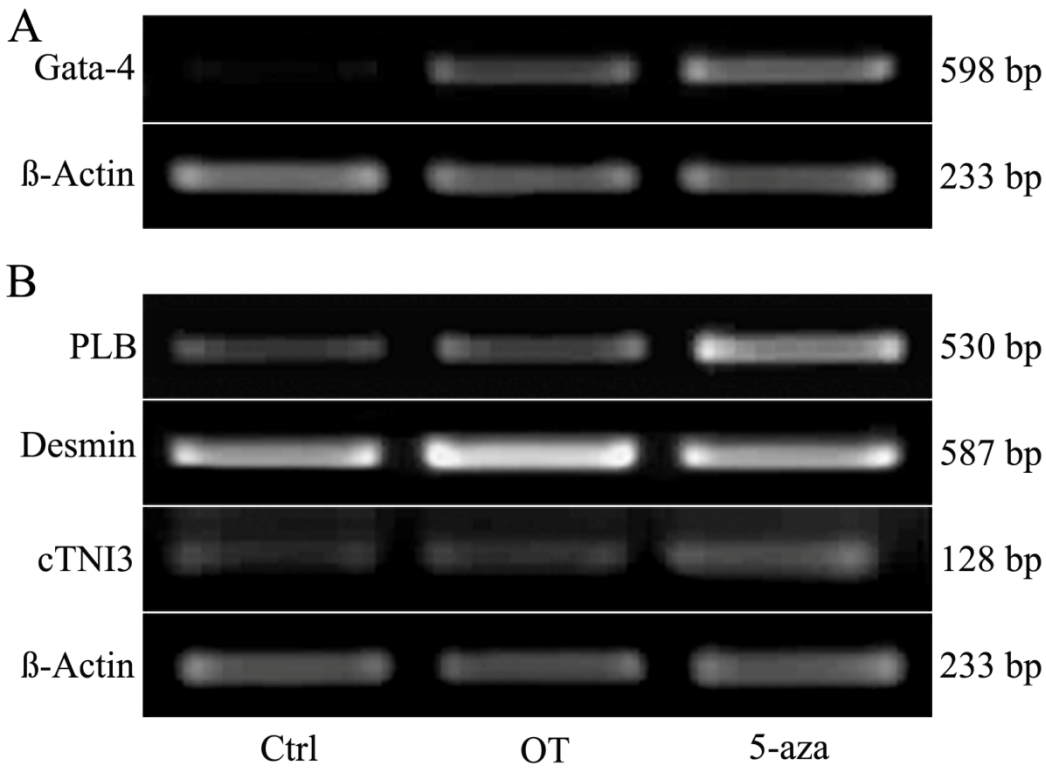



Fig. 3. Gene expression profile of GATA4 after 6 days of differentiation induction *(A)*. Phospholamban (PLB), desmin, and cardiac troponin I (cTnI) after 10 days of differentiation induction *(B)*. Non-induced (NI), oxytocin (OT)-induced, 5-azacytidine (5-Aza)-induced cells. ß-actin was used as an internal control. Representative images of gene expression profile observed in three different cell isolates after differentiation induction.

**Figure 4. Cardiac protein expression on pBMSCs.**

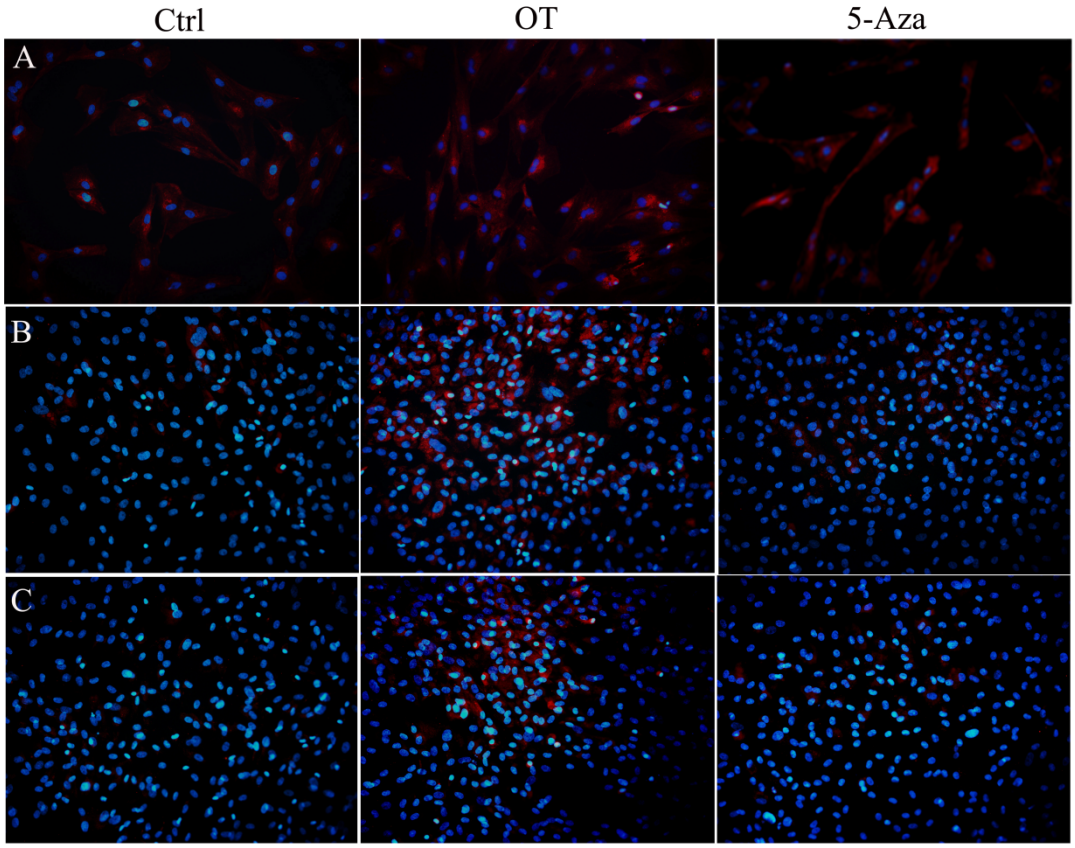



Fig. 4. Protein expression of phospholamban (PLB) *(A)*; cardiac troponin-T (cTnT) *(B)*; and cardiac myosin heavy chain (cMHC) *(C)* in non-induced (Ctrl), oxytocin (OT)-induced, and 5-azacytidine (5-Aza)-induced porcine bone marrow stem cells (pBMSCs). Magnification x 400. The pBMSCs were collected and fixed three weeks after differentiation induction. Representative images of protein expression observed in three different cell isolates.

**Figure 5. Western blot analysis of cardiac troponin I**

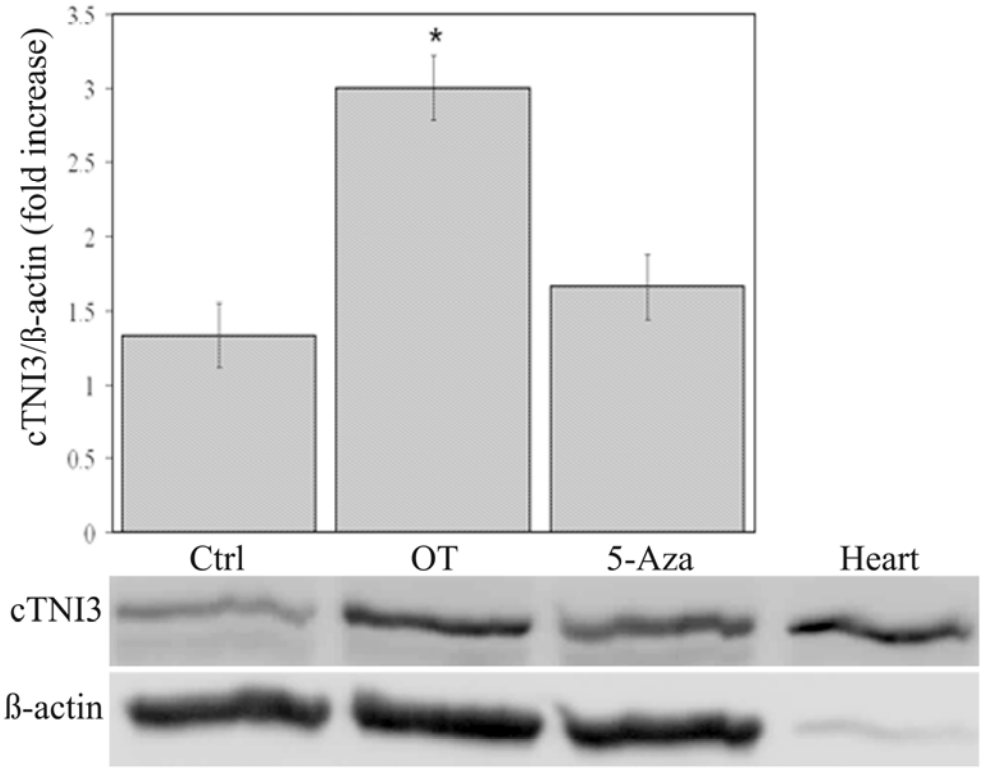



Fig. 5. Effects on cardiac troponin I (cTnI) expression in control (non-induced cells), oxytocin (OT)-, and 5-azacytidine (5-Aza)-induced cells, protein extracted from swine heart was used as positive control. Representative image of three different cell isolates *(A)*. Protein was extracted from three different isolates and western blot analysis was performed in triplicates. Error bars represent ± SEM; * indicates significant increase in OT-induced cells ($P < 0.05$), compared with control and 5-Aza-induced cells *(B)*.

**Figure 6. Transmission electron microscope images of pBMSCs**

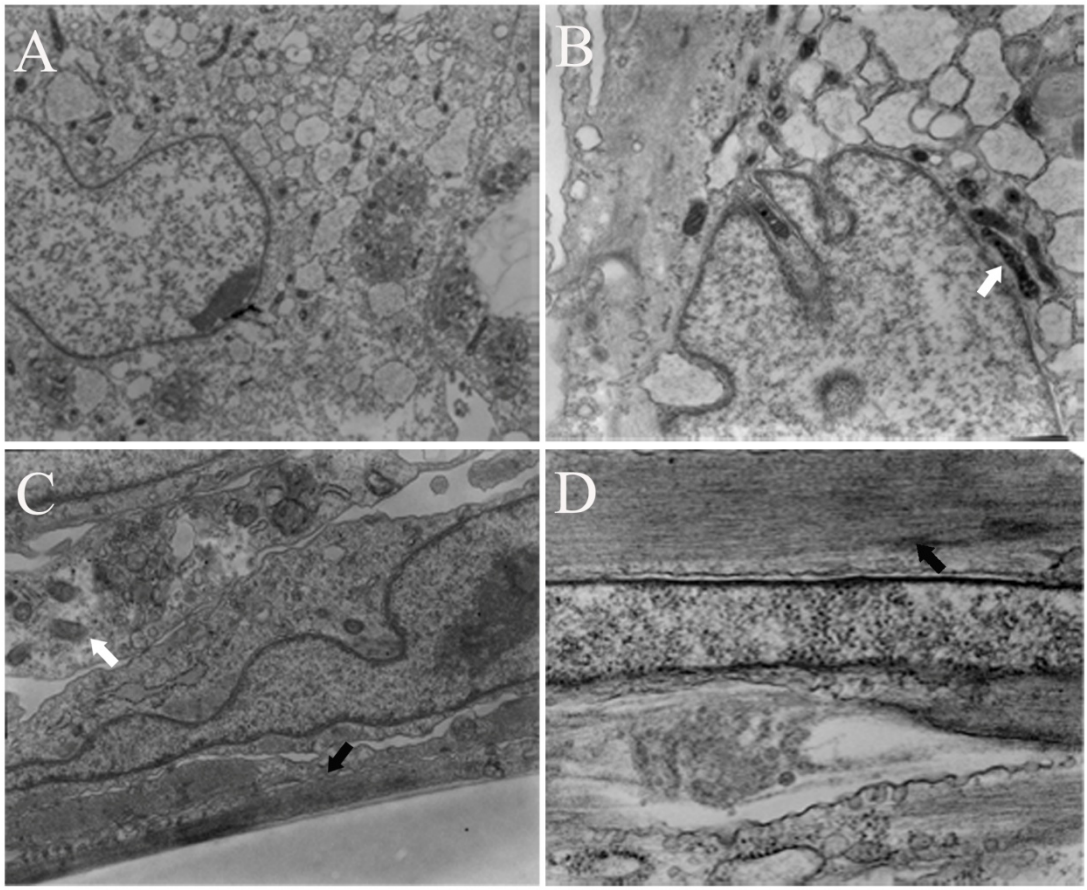



Fig. 6. Transmission electron microscope images of pBMSCs non-induced cells 7,000x (A), cells induced with 5-azacytidine 15,000x (B), and cells induced with oxytocin 15,000x (C) with closer view of myofilaments found in oxytocin treated cells 30,000x (D), after 4 weeks of differentiation. Induced cells show a certain degree of transdifferentiation, with cardiomyocyte-like structures: multiple mitochondria (white arrows, B and C) and myofilaments aligned in a paralleled manner (black arrows C and D). Representative images of similar structures observed in three different isolates.

# CHAPTER 5. THIRD ARTICLE

**Involvement of the nitric oxide – soluble guanylyl cyclase pathway in the oxytocin-mediated differentiation of porcine bone marrow stem cells into cardiomyocytes**

Norma Ybarra, Jérôme RE del Castillo, Eric Troncy

GREPAQ, Department of veterinary biomedicine, Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, Quebec, Canada.

**Corresponding author**:

Norma Ybarra

GREPAQ – Department of veterinary biomedicine, Faculté de médecine vétérinaire, Université de Montréal, P.O. box 5000, St-Hyacinthe, Québec, J2S 7C6, Canada

Phone: 450-773-8521 ext. 8660, Fax: 450-778-8102

**Short title:** *OXYTOCIN-NITRIC OXIDE INDUCED DIFFERENTIATION*

**Experimental work was undertaken at**: GREPAQ

**Source of support**: This work was funded by the Canadian Institutes of Health Research / Heart and Stroke Foundation of Canada (E.T.) New Emerging Team (#108291) program, and the On-going New Opportunities fund (#9483) from Canada Foundation for Innovation (E.T.) for infrastructure support.

## 5.1 ABSTRACT

Bone marrow stem cells (BMSCs) express cardiac markers *in vitro* and *in vivo* upon induction. Cardiomyogenic differentiation of embryonic stem cells induced by oxytocin (OT) involves the nitric oxide (NO)-soluble guanylyl cyclase (sGC) pathway. Also, OT improved cardiomyogenic differentiation of porcine BMSCs (pBMSCs). Here, we document the role of NO pathway in OT-mediated cardiomyogenic differentiation of pBMSCs obtained from bone marrow aspirates of juvenile pigs. Cells were exposed (OT cells) or not (control cells) to OT, in presence or absence of a NO synthase inhibitor (L-NAME) and a sGC inhibitor (ODQ). Gene (RT-PCR) and protein expression (immunocytochemistry) of NOS was up-regulated after OT induction. Exposure of OT cells to L-NAME, ODQ, or both, leaded to a significant reduction in cardiac troponin I transcripts, and protein (Western Blot) expression. For the latter, ODQ looked more performing in inhibition than L-NAME. Expression of cardiac troponin T and myosin heavy chain (immunocytochemistry) was less abundant in OT cells exposed to inhibitors without apparent synergic effect between L-NAME and ODQ. In control cells, protein expression remained low. Moreover, OT induced cell proliferation, and this effect was counteracted by NOS/sGC inhibitors. Inhibiting NO production and NO effector, sGC, affected the OT-mediated differentiation of pBMSCs, because abundance of cardiac proteins was reduced to levels similar to those observed in control cells. We propose that following treatment with OT, activation of NO pathway directs pBMSCs to a preferential cardiomyogenic phenotype and stimulates cell proliferation.

Keywords: Bone marrow stem cells; Cardiomyogenic differentiation; Oxytocin

## 5.2 INTRODUCTION

Myocardial infarction (MI), characterized by a massive necrosis of heart tissue, is a leading cause of death in developed countries. Cell therapy has been identified as a promising treatment for myocardial regeneration through cardiomyocyte replacement or by preventing apoptosis of surviving myocardium and improving neovascularization (for review see [1]). There is a special interest in using stem cells, due to their differentiation potential and capacity of symmetrical self-renewal (for review see [2]). Embryonic stem (ES) cells are able to differentiate into any cell type, and are useful tools for the study of functional genomics, developmental processes, and the elucidation of signalling pathways involved in cell differentiation [3]. It has been reported that oxytocin (OT), a hormone mostly known for its role during childbirth and lactation, is involved in cardiac differentiation during embryonic development [4] and has acted as a potent and specific inducer of the differentiation of P19 murine embryonic carcinoma cells [5], ES cells [6,7], and cardiac stem cells [8] into beating cardiomyocytes. Moreover, the OT receptors (OTR) expressed in ES cells have been shown to up-regulate when OT and dimethyl sulfoxide are added to the culture medium for inducing cell differentiation [5].

Nitric oxide (NO) has been shown to promote ES cell differentiation [9], including cardiomyogenic differentiation. Uncharged free radical gas, NO is synthesized from L-arginine by three isoforms of nitric oxide synthase (NOS): the constitutively active neuronal (nNOS) and endothelial (eNOS) isoforms, and the inducible isoform (iNOS) that is expressed in response to stress and inflammatory conditions (for review see [10]). Both eNOS and iNOS isoforms are prominently expressed during early stages of cardiomyogenesis. The synthesis of NO is required for cardiomyogenesis because NOS inhibitors are reported to prevent the maturation of ES cells in terminally differentiated cardiomyocytes [11]. In addition, exposure to the NO donor SNAP (S-nitroso-N-acetylpenicillamine), and iNOS gene

transfection improved cardiomyogenic differentiation of ES cells by increasing the number and the size of beating foci in embryoid body outgrowths [12].

In addition to NO, it has been shown that its main effector, soluble guanylyl cyclase (sGC), is important in the OT-mediated cardiomyogenic differentiation of P19 EC cells. Indeed, the NOS inhibitor N (G)-nitro-L-arginine methyl ester (L-NAME), and the sGC inhibitor 1H-(1,2,4) oxadiazolo [4,3-a] quinoxalin-1-one (ODQ), both reduced the OT-evoked cardiomyogenic differentiation of ES cells [13]. The exact mechanism by which OT induces or improves cardiomyogenic differentiation of ES cells has not been elucidated y. It has been reported that OT could induce cell proliferation, depending on the subcellular localization of its receptor [14]; therefore, it is likely that OT additionally induces proliferation of cells committed to cardiac differentiation.

The use of human ES cells for cell therapy is still a subject of debate, due to ethical concerns related to the destruction of a viable embryo. Therefore, an interesting alternative of stem cells with cardiomyogenic potential are bone marrow-derived stem cells (BMSCs), which are obtained from adult tissues. Indeed, these cells have been used for cell therapy after myocardial infarction in preclinical [15] and clinical studies (for review see ref. [16]) obtaining promising results.

*In vitro* studies have shown that BMSCs express cardiac specific proteins that can be up-regulated in the presence of 5-azacytidine [15], of a cardiomyogenic differentiation medium containing insulin, dexamethasone, linoleic acid, and ascorbic acid [17], and when BMSCs are co-cultured with cardiomyocytes [18]. We have previously demonstrated that the OT/OTR system is present in porcine BMSCs (pBMSCs), which we consider a more suitable animal model, based on the future perspective of testing OT-induced pBMSCs in a porcine MI animal model. Moreover, OT seemed to be a more potent and specific *in vitro*

cardiomyogenic inducer of pBMSCs than 5-azacytidine, as based on the expression of cardiac specific structural proteins [19].

We hypothesized that NO-sGC pathway is involved in the OT-mediated cardiomyogenic differentiation of pBMSCs, and the proliferation of pBMSCs expressing cardiac markers while OT-induced. Therefore, in the present study we aimed to elucidate the role of the NO-sGC pathway in the OT-mediated cardiomyogenic differentiation of pBMSCs.

## 5.3 MATERIAL AND METHODS

Experiments were performed according to the guidelines from the Canadian Council on Animal Care with the approval of the institutional animal care and use committee before initiation of the study (#09-Rech-1234).

### 5.3.1 Isolation and culture of pBMSCs

All the reagents used in this study, unless stated, were purchased from GIBCO Invitrogen, Burlington, ON, Canada.

The BM was aspirated from the humeral head of three juvenile pigs, weighing 25 to 30 kg, that were anesthetized with a i.m. combined injection of ketamine (Vetalar, Bioniche Animal Health Canada Inc., Belleville, ON, Canada) at 15 mg/kg body weight, and xylazine (Anased, Novopharm, Toronto, ON, Canada) at 2 mg/kg body weight. Approximately 10 mL BM aspirates were obtained using an 11-gauge biopsy aspiration needle (Tyco Healthcare Kendall, Mansfield, MA, USA) attached to a syringe containing 10 000 I.U. of heparin (Leo Pharma, Inc., Thornill, ON, Canada). The BM samples were immediately transported to the laboratory for pBMSCs isolation. To this end, mononuclear cells (MNCs) were separated by

density gradient centrifugation using a commercially available solution (Histopaque®-1077 Hybri-Max, Sigma Aldrich, Oakville, ON, Canada), following the manufacturer directions. Briefly, 5 mL of phosphate buffered saline (PBS) were added to 3 mL of BM/blood mix and gently mixed in a 15 mL centrifuge tube. The cell suspension was poured over 3 mL of Histopaque®, and centrifuged at 400 × *g* for 30 min at room temperature. The MNCs were recovered from the opaque interface with a Pasteur pipette, being washed thrice with PBS and centrifugation (300 × *g* for 10 min). Then, MNCs were resuspended in Dulbecco's Modified Eagle Medium, low glucose (DMEM-LG) supplemented with 15% of fetal bovine serum (FBS), and Antibiotic-Antimycotic mix, plated on T-75 plastic flasks (BD Falcon, Bedford, MA, USA) at an approximate density of 500 000 cells/cm$^2$ and incubated at 37$^o$C in a humidified atmosphere with 5% $CO_2$. Non-adherent cells were discarded by changing the medium 24 h after first plating. Adherent fibroblast-like cell colonies were observed 5 to 7 days after plating. Cells were allowed to grow, and medium changed every third day. When cells reached 80 to 90% of confluence, they were harvested by trypsinization (0.25% trypsin with EDTA) and distributed evenly in 7 treatment groups.

### 5.3.2 Cell treatment

To explore the role of NO-sGC pathway in the OT-mediated cardiomyogenic differentiation of pBMSCs, first-passage cells were subcultured at a density of 20 000 cells/cm$^2$ in DMEM-LG containing 15% FBS and Antibiotic-Antimycotic mix. After one day of being subcultured (day 0), the cells were assigned to one of the following conditions. For differentiation induction, cells were exposed for one day either to (induced cells) $10^{-5}$ M OT (Bachem, King of Prussia, PA, USA), or culture medium alone (control cells), and then were exposed (or not) to NOS inhibitor, $10^{-4}$ M L-NAME (Sigma Aldrich, Oakville, ON, Canada), sGC inhibitor, 10 µM ODQ (Sigma Aldrich), or to the combination of both inhibitors at identical concentrations. The concentrations of the inhibitors used were selected from previous

publications [13]. Exposure to the inhibitors was maintained for six days, with daily replacement of the fortified (containing inhibitors in the above mentioned concentrations) culture media. After this period, the fortified media were changed twice a week for the remaining period of experiment, up to day 15.

### 5.3.3 Effect of the treatments on OT-mediated cardiomyogenic differentiation

*Semi-quantitative RT-PCR*

First, the expression of eNOS and iNOS isoforms was assessed in control and OT-induced cells. Second, the effect of the inhibitors on gene expression of cardiomyogenic (cardiac troponin I, cTNI3) and myogenic (desmin and phospholamban, PLB) markers was assessed. The pBMSCs were collected by trypsinization at days 1, 5, and 10. Total RNA was extracted from collected pBMSCs using an RNeasy Mini Kit (Qiagen, Gaithersburg, MD, USA). Briefly, the pBMSC pellet was treated with lysis buffer and homogenized in a QIAshredder spin column (Qiagen, Gaithersburg, MD, USA). Ethanol (70%) was added to the homogenized cell lysates and transferred to RNeasy spin column. After the appropriate washing steps using the buffers provided with the kit, RNA was eluted from the column by adding 30 μL of RNase-free water.

Total RNA was measured by loading 1 μL of undiluted RNA directly onto the lower measurement pedestal of a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Two micrograms of total RNA were treated with one unit of DNase I, amplification grade to avoid genomic DNA contamination. The cDNA was synthesized in a final volume of 20 μL, containing 4 μL of first-strand buffer, 2 μg of RNA, 1 μL of Oligo(dT)20 Primer 50 μM, 1 μL of 10 mM dNTP Mix, 1 μL of RNAseOUT Recombinant Ribonuclease Inhibitor 40 U/μl, 1 μL of 0.1 M Dithiothreitol, and 1 μL SuperScript™ III Reverse Transcriptase (200 units/μL). Then, cDNA (2 μL) was then used for PCR

amplification in a 25 μL reaction mixture following the protocol described for Platinum *Taq* DNA Polymerase (Invitrogen), using specific primers sequences for eNOS, iNOS, cTNI3, desmin, PLB and β-actin genes.

Primer sequences, specific annealing temperatures, number of cycles and amplicon product size are summarized in Table 1. Negative controls for PCR amplification were run at the same time as samples, either water or non-reverse transcribed RNA were used instead of cDNA to discard genomic DNA contamination. The PCR products were size-fractioned by 1-1.5% agarose gel electrophoresis, visualized by ethidium bromide staining and photographed under UV light (Chemi genius 2, Bioimaging system, Syngene, Frederick, MD, USA.). Ratios of band density of specific products to β-actin were evaluated.

*Immunocytochemistry*

The inducing effect of OT on NOS expression and the effect of the inhibitors on cardiac specific proteins expression were verified qualitatively. For this purpose, pBMSCs grown on glass coverslips were collected two weeks after initial treatment, washed twice in PBS (Roche, Indianapolis, IN, USA), fixed in ice-cold methanol for 10 min, and stored at -20$^{o}$C pending analysis. Fixed cells were then washed thrice for 5 min in PBS. To avoid non-specific binding, cells were incubated overnight at 4$^{o}$C in a blocking solution consisting in 10% of normal goat serum in PBS. Then, cells were incubated again overnight at 4$^{o}$C with a solution of primary antibodies directed against universal NOS at 1:100 (v/v) (uNOS, detects three NOS isoforms; ABR Afffinity BioReagents, Golden, CO, USA), 1/200 cardiac isoform troponin-T (TNNT2; Lab Vision, Fremont, CA, USA), or 1/200 cardiac myosin heavy chain (cMHC; ABR Afffinity BioReagents, Golden, CO, USA). After three 5-min washing steps in PBS, cells were incubated for 1 h at room temperature in a 1:100 solution of an FITC conjugated goat anti-rabbit IgG secondary antibody (uNOS; Zymed, Invitrogen), and a 1:1

000 solution of Alexa fluor 594 conjugated goat anti-mouse IgG secondary antibody (TNNT2 and cMHC; Molecular Probes, Invitrogen). After three other 5 min washing steps in PBS, nuclei were stained by a 3-min incubation in a 1:1 500 solution of Hoechst 33342, washed twice and mounted using Vectashield mounting medium for fluorescence (Vector laboratories, Inc., Burlingame, CA, USA). For the negative controls, cover slips were incubated only with the secondary antibody. The signal was visualized by fluorescence microscopy (Leica ASLMD microscope; Leica Cambridge, Cambridge, UK) with a mercury lamp (HBO 103 W/2; LEJ, Jena, Germany). Micrographs were acquired with a Leica DC500 camera and a DFC Twain Software (Leica, Cambridge, UK).

*Western Blot Analysis*

Protein expression of cTNI3 in pBMSCs was semi-quantitatively assessed after exposure of the cells to OT and NO-sGC inhibitors. Two weeks after initial treatment, cells were washed once with Dulbecco's PBS, collected and lysed with cell lysis/extraction buffer (CelLytic™, Sigma Aldrich, Oakville, ON, Canada) supplemented with the Protease Inhibitor Cocktail (Sigma Aldrich, Oakville, ON, Canada), according to supplier's guidelines. Afterwards, cell lysates were centrifuged at 17 000 × *g* for 15 min, and supernatants stored at -80° C pending analysis. Protein concentration was determined using the Bio-Rad Protein Assay (Bio-rad Laboratories, Hercules, CA, USA), based on Bradford's method [20]. Briefly, protein samples (60 µg) were separated electrophoretically on 12% SDS-polyacrylamide gel, and then blotted onto polyvinylidene difluoride (PVDF) membranes at 90 V for 90 min (Hybond-P; Amersham GE Healthcare, Buckinghamshire, UK). Membranes were incubated for 2 h at room temperature in a blocking buffer made of 5% nonfat dry milk in Tris buffered saline (TBS) with Tween 20, at a pH value of 8 (Sigma Aldrich, Oakville, ON, Canada). After an overnight (12–16 h) incubation at 4°C with the primary antibody directed against cTNI3 1:500 (AbD Serotec, Cedarlane Laboratories Ltd., Burlington, ON, Canada), membranes

were washed thrice with TBS-Tween 20 and incubated for 30 min at room temperature with 1:15 000 horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (Calbiochem, VWR CANLAB Mississauga, ON, Canada). The antigen-antibody complexes were visualized by chemiluminescence system (Immun-Star$^{TM}$ WesternC$^{TM}$, Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions. The signal was visualized with a cooled CCD camera (Chemi genius$^2$, Syngene, Perkin Elmer, Woodbridge, ON, Canada). Protein bands were quantified by densitometry using the GeneSnap image acquisition software (Syngene, Frederick, MD, USA). Protein expression was represented as a value relative to ß-actin expression. Triplicate gels and immunoblots were run for all samples.

*Cell proliferation*

To determine whether OT treatment induces a proliferation of pBMSCs that depends on NO synthesis, pBMSCs seeded on glass coverslips at a density of 20 000 cells/cm$^2$ were collected two weeks after being exposed to the different treatments. Cells were fixed and stained with Hoechst 33342 nuclear staining and counted using the ImageJ freeware (National Institutes of Health). For each treatment, cells were counted from three different micrographs taken from three different microscopic fields. Cell counts were performed on all cell isolates.

### 5.3.4 Statistical analyses

All statistical tests were performed at the $\alpha$=0.05 significance level with SAS software version 9.1 (SAS Institute Inc., Cary, NC, U.S.A.). First, a generalized linear model [21] was built to estimate the effects of various OT, L-NAME, and ODQ treatment combinations on net cell proliferation. Cell counts were modeled with the Poisson distribution and the log link function. The value of the Pearson Q statistic, divided by the model degrees of freedom, was used as scaling factor to adjust for overdispersion (*i.e.* to comply with the equivalence of

mean and variance assumption of the Poisson distribution). The generalized estimating equations method was used to account for the correlated counts of replicated experiments with each cell isolate [22]. The within-cell correlation was modeled with a structure-free covariance matrix. *A priori* contrasts were computed to examine the effect of OT on cell counts, to compare cell counts in cells exposed to OT alone or combined with L-NAME and/or ODQ, and to examine a possible synergistic inhibitory effect of exposing OT-treated cells to both L-NAME and ODQ, as compared to either inhibitor alone.

Following this analysis, a repeated-measures linear mixed model was used to estimate the effects of treatment combinations on the expression of cardiac markers transcripts (cTNI3, desmin, and PLB), as well as, on the expression of cTNI3 protein [23]. Treatment was a fixed-effect factor, and random factors were cell line and replicate within cell. Because cTNI3 transcript expression was shown to be heteroscedastic, its statistical analysis was performed on log-transformed data. A second model where cell count was added as a covariate was used to examine whether the expression of the protein of cTNI3 resulted from direct OT stimulation or was secondary to OT-induced cell proliferation. Selection of the covariance model was made following a strategy proposed by Littell et al [24]. Differences between treatment groups were assessed with least-square means.

## 5.4 RESULTS

The population of cells selected in our culture conditions was positive for CD29 (β1-integrin), and CD90 (Thy-1), and was partially negative for CD31 (PECAM-1), and negative for CD45 (a leukocyte marker). These pBMSCs expressed transcription factors related to pluripotency and cardiomyogenesis only during passage 0 and 1 [19].

### 5.4.1 Expression of NOS in pBMSCs cardiomyogenic differentiation

Gene expression in pBMSCs during the process of OT-differentiation was up-regulated for both NOS transcript products, especially for eNOS (Fig. 1A). In addition, protein expression of NOS was abundantly detected in the cytoplasm of OT-treated cells (Fig. 1B).

### 5.4.2 Effect of the inhibitors on OT-mediated cardiomyogenic differentiation of pBMSCs

The statistical analysis revealed significant effects of treatments on the gene expression of cTNI3, desmin and PLB genes (Fig. 2). Treatment of pBMSCs with $10^{-5}$ M OT (induced cells) produced a significant up-regulation ($p=0.0003$) in the gene expression of cardiac specific marker cTNI3 with no change for desmin and PLB, compared to control cells. The inhibitors had no statistically significant effect on control cells, but in OT-induced cells, both L-NAME and ODQ, alone or in combination, induced a down-regulation of the cTNI3 transcript, as shown in OT *vs*. OT+L-NAME ($p=0.0001$), OT *vs*. OT+ODQ ($p=0.0001$), and OT *vs*. OT+L-NAME+ODQ ($p=0.0001$). A similar down-regulation was noted for desmin in OT-induced cells, but only in groups exposed to ODQ either alone ($p=0.0048$) or in combination ($p=0.0167$). For PLB, there was no significant effect when groups were compared.

Immunocytochemistry analysis showed that both control and OT-induced pBMSCs expressed cardiac specific proteins TNNT2 and cMHC, but their expression appeared more abundant in OT-induced cells. Conversely, when control and OT-induced cells were exposed to the NOS and sGC inhibitors, the expression of these cardiac specific proteins was significantly lower (B, C, E, G, in Fig. 3 and Fig. 4) without additional effect when mixing both inhibitors on OT-induced cells (F in Fig. 3 and Fig. 4).

The Western Blot analysis of cTNI3 protein expression in cells exposed to OT-induction and NO-sGC inhibitors paralleled the results recorded for cTNI3 transcript expression (Fig. 5). The cTNI3 protein expression increased significantly in OT-induced cells compared to control cells (Ctrl *vs.* OT; p=0.0002). In addition, cTNI3 protein expression was significantly reduced when OT-induced cells were exposed to both inhibitors alone or in combination (OT *vs.* OT+inhibitors; p<0.0001), but there was no significant effect in protein expression when control cells were exposed to the inhibitors. The effect of ODQ on the reduction of cTNI3 expression was stronger than the one of L-NAME in OT-induced cells.

### 5.4.3 Effect of treatments on cell count

Statistical analysis showed that the only treatment that significantly increased cell proliferation compared to control was the use of OT alone (p<0.0001). The statistical a priori contrast used to verify the effect of inhibitors on OT-induced cell proliferation revealed that cell counts of OT-induced cells exposed to inhibitors used alone or in combination were significantly lower than the cell counts of OT-induced cells (p=0.0052). There was no significant potentiating effect with the combined use of the inhibitors L-NAME and ODQ (p=0.9074).

### 5.4.4 Cell count as potential confounding factor of cTNI3 induction by OT?

A statistical model devised for testing whether the expression of cTNI3 protein resulted from direct OT-induction or if it was secondary to OT-induced cell proliferation revealed that cTNI3 protein expression was not affected by cell proliferation (p=0.1111), but only by direct OT induction (p=0.0011). Therefore cTNI3 protein expression is primarily mediated by OT-induced cardiomyogenic differentiation and not by the OT stimulation of cell proliferation.

The effects of cell treatment, presented as mean ± SE, on cell counts and the expression of transcripts and proteins expressed as ratios are summarized in Table 2.

## 5.5 DISCUSSION

Previous studies have shown that OT improves cardiomyogenic differentiation of P19 EC [5], ES [6,7], cardiac [8], and pBMSCs [19] cells. It has been previously reported that NO pathway components, such as NOS isoforms and sGC subunits α1 and β1, are expressed during cardiomyogenic differentiation of murine [25], and human [26] ES cells. Specifically, it has been reported that nNOS is expressed in undifferentiated ES cells and that this expression is down-regulated upon differentiation; together with this, up-regulation of iNOS and eNOS isoforms are observed in ES cells [25]. This led us to hypothesize that the NO pathway should be involved as well in the cardiomyogenic differentiation of adult stem cells.

In the present study, nNOS was detected neither in undifferentiated nor in differentiated pBMSCs. However, eNOS and iNOS isoform transcripts were expressed in both non-induced and OT-induced pBMSCs, and OT induction up-regulated the expression of both isoforms, especially eNOS. It is possible that the nNOS isoform could not be detected because pBMSCs possess a more limited differentiation potential than ES cells. Noteworthy, nNOS has been recorded only in undifferentiated ES cells, and upon differentiation by embryoid body formation the expression of this isoform was down-regulated and even undetectable [25]. Bloch *et al.* [11], have observed that eNOS and iNOS are prominently expressed during early stages of cardiomyogenesis (differentiation phase), and that eNOS presents a sustained expression up to the adult stage. The results obtained in the present study with adult stem cells support similar findings.

Danalache *et al*. have reported the functional involvement of iNOS/eNOS/sGC in OT-mediated cardiomyogenic differentiation of murine P19 embryonic carcinoma cells [13]. Additionally, OT exhibited a more efficient cardiomyogenic action than did NO donor SNAP, as assessed by the size and number of beating cell cultures and by the expression of cardiac marker MLC-2v associated to the expression of reporter gene GFP. The authors suggested that this could be related to a fine regulation of NO synthesis mediated by OT/OTR action, or to the contribution of additional NO-independent transduction pathways triggered by OT. In addition, upon OT induction of P19 cells, eNOS gene expression was upregulated, together with cardiac markers while the iNOS transcript was undetectable [13]. However, they found that when blocking with 1400W, a specific inhibitor of iNOS, P19 cell cardiomyogenic differentiation was greatly reduced [13]. In our study, we were able to detect eNOS and iNOS transcripts in pBMSCs, which expression was up-regulated after OT-induction. Particularly, the expression of eNOS looked upregulated after pBMSC OT-induction. It has been reported that neonatal administration of OT increases the expression of eNOS transcripts in the rat heart [27]. OT additionally enhanced iNOS transcripts and protein expression in fetal membranes, though the mechanism by which OT increases iNOS expression in this tissue has not been yet clarified [28].

Because most structural proteins expressed in cardiomyocytes are expressed both in skeletal and smooth muscle cells as well, there are few cardiac-specific proteins that could be used as markers of cardiomyogenic differentiation of pBMSCs. For this reason, in the present study, to semi-quantify the effect of NOS and sGC inhibitors on the OT-mediated cardiomyogenic differentiation of pBMSCs, we used cTNI3, a protein widely used as a cardiac specific marker for the detection of myocardial necrosis [29]. We found that NO, and its effector sGC, contribute to the OT-mediated cardiomyogenic differentiation of pBMSCs: when OT-induced cells were exposed to L-NAME, and/or ODQ, the transcript and protein expressions

of cardiac specific cTNI3 were significantly down-regulated. The combination of both inhibitors did not cause a greater reduction of cTNI3 transcripts, but ODQ looked more performing in inhibiting cTNI3 protein expression. This would need further investigation. In addition, the signal of TNNT2 and cMHC protein expression, as observed by immunocytochemistry analysis, was weaker in cells exposed to the inhibitors used alone or in combination. It has been previously reported that BMSCs express cardiac or myogenic markers even without induction. Apparently, to expose the cells to inhibitors in the absence of OT did not affect the differentiation process that BMSCs experiment under *in vitro* conditions [30], since cTNI3 and skeletal muscle markers (PLB and desmin) expression was also found in non-induced and induced cells exposed to the inhibitors at similar levels to those observed in the control cells. On the other hand, the expression of cTNI3 protein was significantly higher in OT-induced cells compared to non-induced cells, suggesting that OT improves the expression of this cardiac marker and possibly the cardiac specific differentiation of pBMSCs. The increased cTNI3 expression elicited by OT was significantly down-regulated in OT-induced cells exposed to inhibitors, and the expression levels of cTNI3 in OT-induced cells exposed to inhibitors was similar to those observed for control group. These results suggest that the increased expression of cardiac specific markers in OT-induced cells is strictly dependent to the NO-sGC signalling pathway. The transcripts of non-cardiac specific markers, desmin and PLB, were also affected in some cases by the exposure to the inhibitors. For example desmin was importantly down-regulated in OT-induced cells exposed to inhibitors (particularly ODQ), but it was slightly but not significantly up-regulated in non-induced cells exposed to L-NAME (Fig. 2). In the case of PLB, transcript expression was more constant in all analyzed groups. It has been mentioned above that BMSCs have myogenic potential, and taking together the observations of transcripts expression in different treatment groups, it seems that myogenic differentiation is also, though slightly, affected by treatment with NO/sGC pathway inhibitors. These results are in accordance with the results

previously reported by Danalache *et al*., in which they observed that, when OT-induced P19 cells were exposed to L-NAME, the inhibitor predominantly reduced the production of ventricular cardiomyocytes and had a minimal effect on simultaneously generated populations of skeletal myocytes [13].

It seems clear that cardiac specific differentiation of pBMSCs is elicited by OT treatment, because cardiac specific markers are importantly up-regulated. Moreover, this up-regulation is mediated by the NO/sGC pathway, while expression of cardiac specific markers is significantly down-regulated (coming back to non-induced cells levels) when induced cells are exposed to the inhibitors. Apparently, OT has no effect in the myogenic differentiation of pBMSCs, because myogenic markers (desmin and PLB) were not up-regulated in OT-induced cells.

Some reports have shown that OT possesses proliferative properties depending on the cell type and the subcellular localization of OTR. Recent data on the membrane localization of OTR indicate that, when the vast majority of OTR are excluded from lipid rafts enriched in caveolin-1, OT inhibits cell proliferation; but when OTR are targeted to lipid rafts, OT has a strong mitogenic effect [14]. The same anti-proliferative [31], and proliferative effects have been described for NO [32], especially in endothelial cells. Thibonnier *et al*., reported that human vascular endothelial cells expressed OTR and that when these cells were stimulated with OT, the nonapeptide induced proliferation of endothelial cells, and that this proliferation was mediated by NO release [33]. BMSCs and even MSCs are a heterogenous population of cells, in which probably a reduced number have cardiomyogenic potential, and therefore it could be possible that OT promotes the proliferation only of cells with cardiomyogenic potential. Here, we observed that OT-induced cardiomyogenic differentiation of pBMSCs is mediated by NO, and it has been reported that NO may influence cardiac differentiation by

both inducing a switch toward a cardiac phenotype and inducing apoptosis in cells not committed to cardiac differentiation [12]. In addition, by analyzing cell counts of pBMSCs exposed to the different treatments, we observed that OT increased the number of cells, but when OT-induced cells were exposed to the inhibitors, the number of cells was similar to that found in control conditions. Moreover, control cells exposed to inhibitors did not present any proliferation. Therefore, it seems that OT has an effect on cell proliferation and that this effect is also mediated by NO. Further studies need to be carried out in order to determine the mechanism by which OT improves cardiomyogenic differentiation of stem cells. We suspected that OT, by inducing cell proliferation, will indirectly increase the expression of cTNI3, since it has been documented that cell aggregation (more abundant in presence of cell proliferation) plays an important role on stem cell differentiation [34]. Using a statistical model specifically devised to explore this possibility, we rejected this hypothesis: The increased expression of cTNI3 most likely results from direct OT-induced cardiomyogenic differentiation rather than OT-mediated cell proliferation. It will also be important to determine if the derived cells expressing the cardiac markers could perform as functional cardiomyocytes.

In the present report we describe that the inhibition of NO production and the inhibition of NO effector, sGC, affect the OT-mediated differentiation of pBMSCs. Abundance of cardiac specific proteins was reduced to levels similar to those of control group. Endogenously produced NO and the activation of sGC-dependent pathway(s) seem to be important in OT-mediated specific cardiomyogenic differentiation of pBMSCs, since only cardiac specific proteins expression was affected. These results suggest that treatment with OT and NO pathway activation direct the pBMSCs to a preferential cardiomyogenic phenotype, and opens attractive therapeutic avenues for MI based on OT-mediated cardioregeneration.

## 5.6 ACKNOWLEDGEMENTS

Special thanks to Dr. Bruce D. Murphy and Dr. Lawrence C. Smith and the entire lab staff of CRRA, Faculté de médecine vétérinaire, Université de Montréal, for their technical assistance in conducting this project. Many thanks to: Dominique Gauvin for her help and technical assistance. N.Y. is a *Ph.D.* student funded by the Canadian Institutes of Health Research – Strategic Training Initiative GREAT (Gasotransmitter REsearch and Training) program, and the CONACyT (*Consejo Nacional de Ciencia y Tecnología*), Mexico.

## 5.7 REFERENCES

[1] S. Itescu, M.D. Schuster, and A.A. Kocher, New directions in strategies using cell therapy for heart disease. J Mol Med 81 (2003) 288-96.

[2] K. Tateishi, N. Takehara, H. Matsubara, and H. Oh, Stemming heart failure with cardiac- or reprogrammed-stem cells. J Cell Mol Med 12 (2008) 2217-32.

[3] S. Sudheer, and J. Adjaye, Functional genomics of human pre-implantation development. Brief Funct Genomic Proteomic 6 (2007) 120-32.

[4] M. Jankowski, B. Danalache, D. Wang, P. Bhat, F. Hajjar, M. Marcinkiewicz, J. Paquin, S.M. McCann, and J. Gutkowska, Oxytocin in cardiac ontogeny. Proc Natl Acad Sci U S A 101 (2004) 13074-9.

[5] J. Paquin, B.A. Danalache, M. Jankowski, S.M. McCann, and J. Gutkowska, Oxytocin induces differentiation of P19 embryonic stem cells to cardiomyocytes. Proc Natl Acad Sci U S A 99 (2002) 9550-5.

[6] F. Fathi, S. Murasawa, S. Hasegawa, T. Asahara, A.J. Kermani, and S.J. Mowla, Cardiac differentiation of P19CL6 cells by oxytocin. Int J Cardiol 134 (2009) 75-81.

[7] L. Hatami, M.R. Valojerdi, and S.J. Mowla, Effects of oxytocin on cardiomyocyte differentiation from mouse embryonic stem cells. Int J Cardiol 117 (2007) 80-9.

[8] K. Matsuura, T. Nagai, N. Nishigaki, T. Oyama, J. Nishi, H. Wada, M. Sano, H. Toko, H. Akazawa, T. Sato, H. Nakaya, H. Kasanuki, and I. Komuro, Adult cardiac Sca-1-

positive cells differentiate into beating cardiomyocytes. J Biol Chem 279 (2004) 11384-91.

[9] A. Cheng, S. Wang, J. Cai, M.S. Rao, and M.P. Mattson, Nitric oxide acts in a positive feedback loop with BDNF to regulate neural progenitor cell proliferation and differentiation in the mammalian brain. Dev Biol 258 (2003) 319-33.

[10] K.S. Madhusoodanan, and F. Murad, NO-cGMP signaling and regenerative medicine involving stem cells. Neurochem Res 32 (2007) 681-94.

[11] W. Bloch, B.K. Fleischmann, D.E. Lorke, C. Andressen, B. Hops, J. Hescheler, and K. Addicks, Nitric oxide synthase expression and role during cardiomyogenesis. Cardiovasc Res 43 (1999) 675-84.

[12] S. Kanno, P.K. Kim, K. Sallam, J. Lei, T.R. Billiar, and L.L. Shears, 2nd, Nitric oxide facilitates cardiomyogenesis in mouse embryonic stem cells. Proc Natl Acad Sci U S A 101 (2004) 12277-81.

[13] B.A. Danalache, J. Paquin, W. Donghao, R. Grygorczyk, J.C. Moore, C.L. Mummery, J. Gutkowska, and M. Jankowski, Nitric oxide signaling in oxytocin-mediated cardiomyogenesis. Stem Cells 25 (2007) 679-88.

[14] F. Guzzi, D. Zanchetta, P. Cassoni, V. Guzzi, M. Francolini, M. Parenti, and B. Chini, Localization of the human oxytocin receptor in caveolin-1 enriched domains turns the receptor-mediated inhibition of cell growth into a proliferative response. Oncogene 21 (2002) 1658-67.

[15] J. Liu, Q. Hu, Z. Wang, C. Xu, X. Wang, G. Gong, A. Mansoor, J. Lee, M. Hou, L. Zeng, J.R. Zhang, M. Jerosch-Herold, T. Guo, R.J. Bache, and J. Zhang, Autologous stem cell transplantation for myocardial repair. Am J Physiol Heart Circ Physiol 287 (2004) H501-11.

[16] S. Singh, R. Arora, K. Handa, A. Khraisat, N. Nagajothi, J. Molnar, and S. Khosla, Stem cells improve left ventricular function in acute myocardial infarction. Clin Cardiol 32 (2009) 176-80.

[17] W.S. Shim, S. Jiang, P. Wong, J. Tan, Y.L. Chua, Y.S. Tan, Y.K. Sin, C.H. Lim, T. Chua, M. Teh, T.C. Liu, and E. Sim, Ex vivo differentiation of human adult bone marrow stem cells into cardiomyocyte-like cells. Biochem Biophys Res Commun 324 (2004) 481-8.

[18] T. Wang, Z. Xu, W. Jiang, and A. Ma, Cell-to-cell contact induces mesenchymal stem cell to differentiate into cardiomyocyte and smooth muscle cell. Int J Cardiol 109 (2006) 74-81.

[19] N. Ybarra, P. Vincent, Smith LC, Troncy E, Cardiomyogenic differentiation of porcine bone marrow stem cells induced by oxytocin. Transl Res (2010; submitted).

[20] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72 (1976) 248-54.

[21] A. Alan, Categorical data analysis, John Wiley & Sons, Inc, Hoboken NJ, USA, 2002.

[22] H.J. Hardin JW, Generalized estimating equations, Chapman & Hall/CRC, Boca Raton, FL, USA, 2003.

[23] M.G. Littell RC, Stroup WW, Wolfinger RD, Schabenberger O *SAS for Mixed Models*, SAS Publishing, Cary NC, USA, 2006.

[24] R.C. Littell, J. Pendergast, and R. Natarajan, Modelling covariance structure in the analysis of repeated measures data. Stat Med 19 (2000) 1793-819.

[25] J.S. Krumenacker, S. Katsuki, A. Kots, and F. Murad, Differential expression of genes involved in cGMP-dependent nitric oxide signaling in murine embryonic stem (ES) cells and ES cell-derived cardiomyocytes. Nitric Oxide 14 (2006) 1-11.

[26] K. Mujoo, J.S. Krumenacker, Y. Wada, and F. Murad, Differential expression of nitric oxide signaling components in undifferentiated and differentiated human embryonic stem cells. Stem Cells Dev 15 (2006) 779-87.

[27] H. Pournajafi-Nazarloo, A. Perry, L. Partoo, E. Papademeteriou, F. Azizi, C.S. Carter, and B.S. Cushing, Neonatal oxytocin treatment modulates oxytocin receptor, atrial natriuretic peptide, nitric oxide synthase and estrogen receptor mRNAs expression in rat heart. Peptides 28 (2007) 1170-7.

[28] C. Ticconi, A. Zicari, M. Realacci, M. Di Vito, P. Denora, M. Narcisi, M.A. Russo, and E. Piccione, Oxytocin modulates nitric oxide generation by human fetal membranes at term pregnancy. Am J Reprod Immunol 52 (2004) 185-91.

[29] E.M. Antman, M.J. Tanasijevic, B. Thompson, M. Schactman, C.H. McCabe, C.P. Cannon, G.A. Fischer, A.Y. Fung, C. Thompson, D. Wybenga, and E. Braunwald,

Cardiac-specific troponin I levels to predict the risk of mortality in patients with acute coronary syndromes. N Engl J Med 335 (1996) 1342-9.

[30] A. Bayes-Genis, S. Roura, C. Soler-Botija, J. Farre, L. Hove-Madsen, A. Llach, and J. Cinca, Identification of cardiomyogenic lineage markers in untreated human bone marrow-derived mesenchymal stem cells. Transplant Proc 37 (2005) 4077-9.

[31] W. Yang, J. Ando, R. Korenaga, T. Toyo-oka, and A. Kamiya, Exogenous nitric oxide inhibits proliferation of cultured vascular endothelial cells. Biochem Biophys Res Commun 203 (1994) 1160-7.

[32] M. Ziche, L. Morbidelli, E. Masini, H. Granger, P. Geppetti, and F. Ledda, Nitric oxide promotes DNA synthesis and cyclic GMP formation in endothelial cells from postcapillary venules. Biochem Biophys Res Commun 192 (1993) 1198-203.

[33] M. Thibonnier, D.M. Conarty, J.A. Preston, C.L. Plesnicher, R.A. Dweik, and S.C. Erzurum, Human vascular endothelial cells express oxytocin receptors. Endocrinology 140 (1999) 1301-9.

[34] B.A. Pallante, I. Duignan, D. Okin, A. Chin, M.C. Bressan, T. Mikawa, and J.M. Edelberg, Bone marrow Oct3/4+ cells differentiate into cardiac myocytes via age-dependent paracrine mechanisms. Circ Res 100 (2007) e1-11.

## 5.8 TABLES AND FIGURES

**Table 1. PCR primers and reaction conditions.**

| Gene | GenBank Accession No. | Primer sequence* | Annealing Temp, $^{o}$C | PCR cycles | Product size, bp |
|---|---|---|---|---|---|
| eNOS | **NM_214295** | F: TCTGCAGAGACTGGCCTTATTCCT | 62.5 | 35 | 116 |
| | | R: TGGCTCTAGTCTCCGGAATACCAC | | | |
| iNOS | **NM_001143690** | F: CCATGGAACACCCCAAATACGAGT | 60.6 | 35 | 224 |
| | | R: GTGTGTTTCCAGGCCCATCCTTCT | | | |
| cTNI3 | **NM_001098599** | F: CCCACCTCAAGCAGGTGAAGA | 65 | 35 | 128 |
| | | R: GCCAGCTCAGCCCTCAAACTT | | | |
| Desmin | **NM_001001535** | F: CCAGCGGCTACCAGGACAACAT | 62 | 33 | 587 |
| | | R: CCAAGGGCCAGGCTCACTCACT | | | |
| PLB | **NM_214213** | F: CTTTTTCAGCTTTCTCTTG | 51 | 35 | 530 |
| | | R: ACCCCTAGTTCATCCTCA | | | |
| β-Actin | **U07786** | F: GGACTTCGAGCAAGGAGATGG | 54 | 30 | 233 |
| | | R: GCACCGTGTTGGCGTAGAGG | | | |

Legend: *F=Forward primer sequence; *R= Reverse primer sequence. Abbreviations: endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), cardiac troponin I (cTNI3), phospholamban (PLB).

**Table 2. Effect of cell treatment expressed as mean ± SE of transcripts and protein ratios, as well as total cell counts.**

| | Ratio Mean ± SE | | | | |
|---|---|---|---|---|---|
| | Transcripts | | | Protein | |
| Treatment groups | cTNI3 | Desmin | PLB | CTNI3 | Cell counts (No.) |
| Ctrl | 0.19 ± 0.02 | 1.59 ± 0.21 | 0.39 ± 0.12 | 4.0 ± 1.05 | 311 ± 21.77 |
| OT | 0.26 ± 0.04 | 1.65 ± 0.04 | 0.33 ± 0.11 | 6.1 ± 1.08 | 564 ± 32.36 |
| Ctrl+L-NAME | 0.20 ± 0.03 | 1.92 ± 0.14 | 0.31 ± 0.08 | 4.4 ± 1.18 | 301 ± 50.17 |
| OT+L-NAME | 0.15 ± 0.01 | 1.66 ± 0.04 | 0.32 ± 0.07 | 5.3 ± 1.12 | 323 ± 16.34 |
| OT+L-NAME+ODQ | 0.15 ± 0.02 | 1.39 ± 0.11 | 0.31 ± 0.05 | 3.9 ± 0.49 | 333 ± 22.71 |
| Ctrl+ODQ | 0.19 ± 0.04 | 1.64 ± 0.13 | 0.31 ± 0.03 | 3.9 ± 0.68 | 329 ± 35.64 |
| OT+ODQ | 0.17 ± 0.02 | 1.34 ± 0.10 | 0.26 ± 0.07 | 3.5 ± 0.75 | 347 ± 32.18 |

Effect of cell treatment groups on cell counts, and transcripts and protein ratios of cardiac troponin I (cTNI3), desmin, and phospholamban (PLB). Abbreviations: L-NAME, *N*,G-nitro-L-arginine-methylester; ODQ, 1H-(1,2,4)oxadiazolo[4,3-a]quinoxalin-1-one; Ctrl, control; OT, oxytocin. Ratios were normalized using β-actin as a housekeeping.

**Figure 1. Effects of oxytocin (OT) on endothelial (eNOS) and inducible (iNOS) nitric oxide synthase transcripts and proteins expression of both enzymes.**

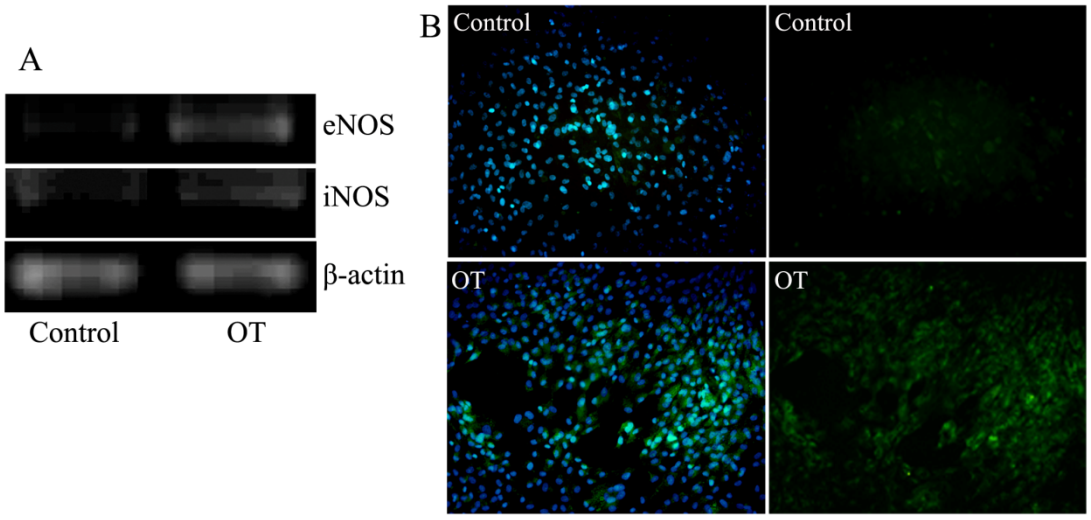



Fig. 1 Effects of oxytocin (OT) on endothelial (eNOS) and inducible (iNOS) nitric oxide synthase transcripts (A) and proteins (B) expression of both enzymes (uNOS). An antibody which detects both isoforms was used. Non-induced pBMSCs were used as negative controls. β-actin was used as an internal control (A).

**Figure 2. Representative image of semi-quantitative RT-PCR analysis of cardiac markers gene expression 10 days after initial treatment**

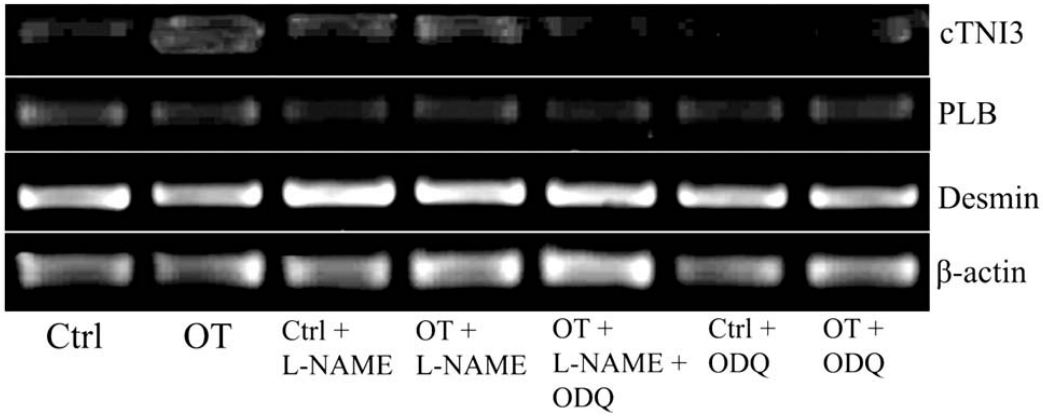



Fig. 2. Representative image of semi-quantitative RT-PCR analysis of gene expression of cardiac markers 10 days after initial treatment in control cells, OT-induced cells, and both control and OT-induced cells exposed to inhibitors L-NAME and ODQ, or both. mRNA levels were normalized to β-actin. Abbreviations: L-NAME, *N*,G-nitro-L-arginine-methylester; ODQ, 1H-(1,2,4)oxadiazolo[4,3-a]quinoxalin-1-one; Ctrl, control cells; OT, oxytocin.

**Figure 3. Expression of cardiac troponin-T protein on treated cells in the presence and absence of nitric oxide pathway inhibitors.**

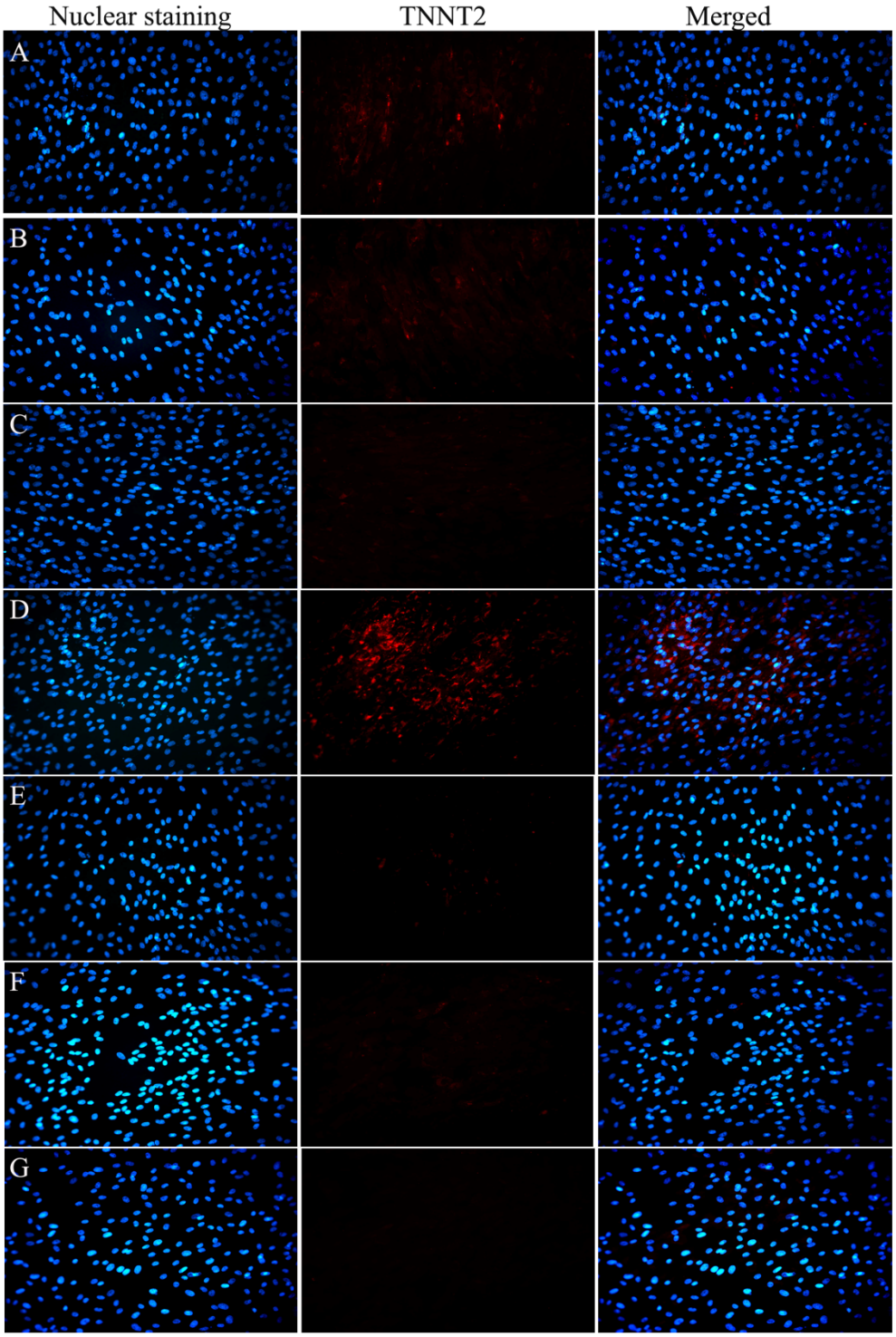


Fig. 3. Expression of cardiac troponin-T (TNNT2) protein in Ctrl cells (A), Ctrl+L-NAME cells (B), Ctrl+ODQ cells (C), OT-induced cells (D), OT+L-NAME cells (E), OT+L-NAME+ODQ cells (F), OT+ODQ cells (G). Abbreviations: Ctrl, control; OT, oxytocin; L-NAME, *N*,G-nitro-L-arginine-methylester; ODQ, 1H-(1,2,4)oxadiazolo[4,3-a]quinoxalin-1-one. Magnification 200x.

**Figure 4. Expression of cardiac myosin heavy chain protein on treated cells in the presence and absence of nitric oxide pathway inhibitors.**

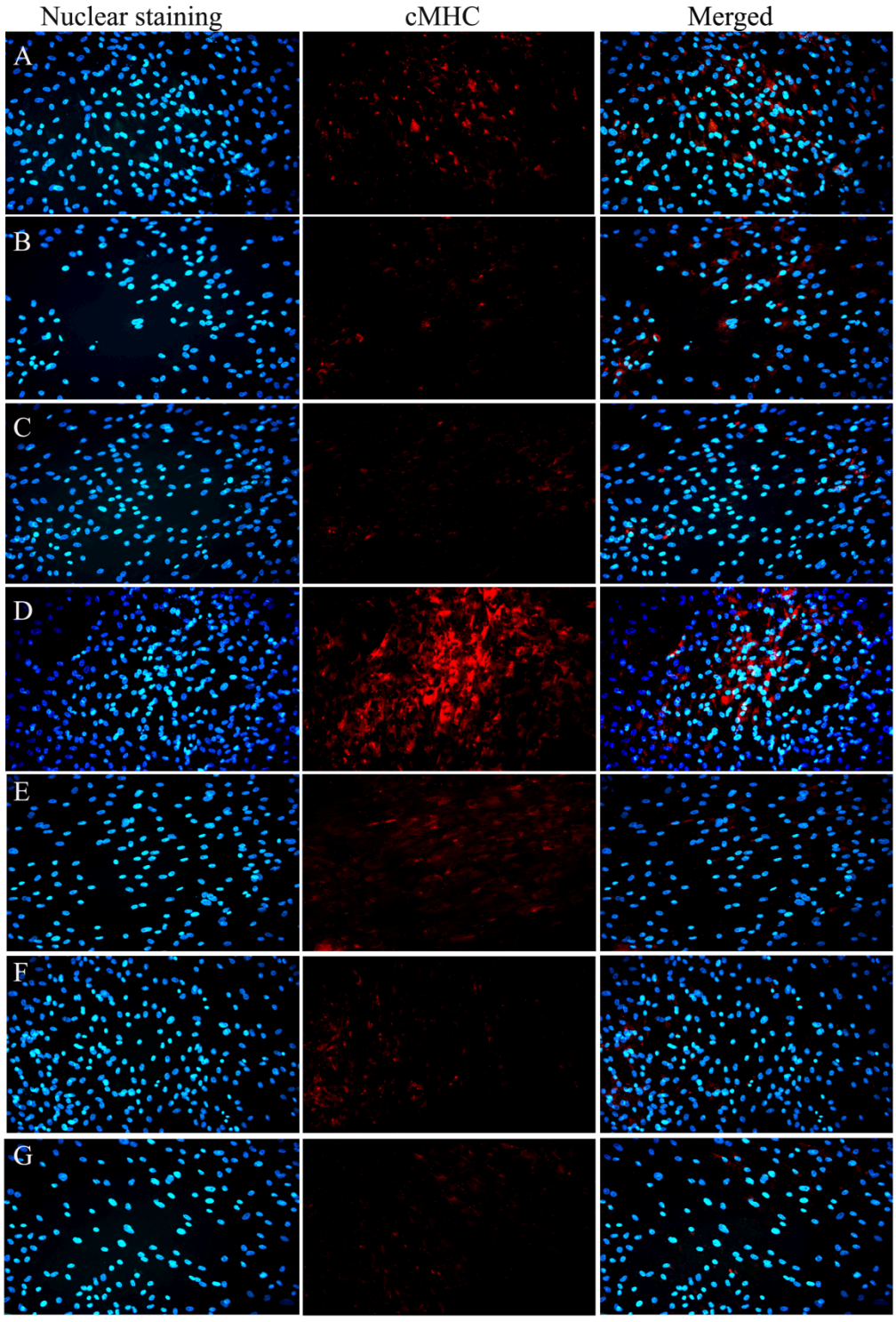


Fig. 4. Expression of cardiac myosin heavy chain (cMHC) protein in Ctrl cells (A), Ctrl+L-NAME cells (B), Ctrl+ODQ cells (C), OT-induced cells (D), OT+L-NAME cells (E), OT+L-NAME+ODQ cells (F), OT+ODQ cells (G). Abbreviations: Ctrl, control; OT, oxytocin; L-NAME, *N*,G-nitro-L-arginine-methylester; ODQ, 1H-(1,2,4)oxadiazolo[4,3-a]quinoxalin-1-one. Magnification 200x.

**Figure 5. Semi-quantitative western blot analysis of protein expression of the cardiac specific marker cardiac troponin I, effects of inhibitors of nitric oxide pathway**

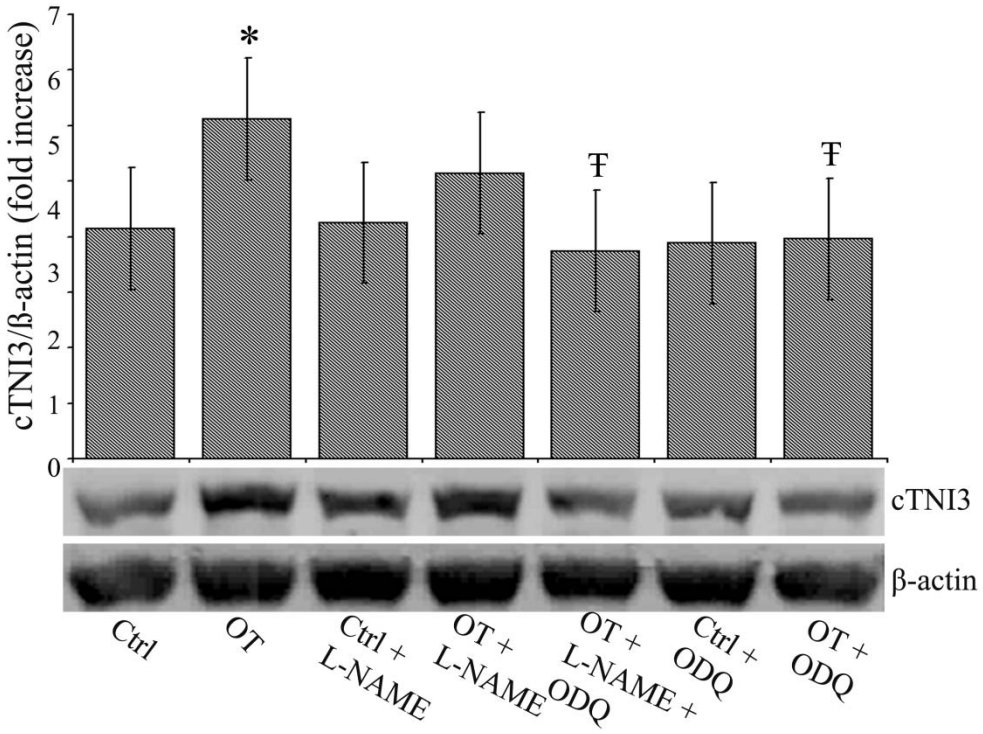



Fig. 5. Semi-quantitative western blot analysis of protein expression of the cardiac specific marker cardiac troponin I (cTNI3) two weeks after initial treatment in: control cells, OT-induced cells, and both control and induced cells exposed to inhibitors L-NAME and ODQ, or both. Protein levels were normalized to β-actin. Protein was extracted from three different isolates and western blot analyses were performed in triplicates. Error bars represent ± SEM; * indicates significant increase when control cells were compared with OT-induced cells ($p=0.0002$); Ŧ significant decrease in cTNI3 expression of OT-induced cells in the presence of the inhibitors, OT *vs.* OT+L-NAME+ODQ ($p=0.0002$); OT *vs.* OT+ODQ ($p<0.0001$). Abbreviations: L-NAME, *N*,G-nitro-L-arginine-methylester; ODQ, 1H-(1,2,4)oxadiazolo[4,3-a]quinoxalin-1-one; Ctrl, control cells; OT, oxytocin.

# CHAPTER 6. GENERAL DISCUSSION

### 6.1 Oxytocin pharmacokinetics and pharmacodynamics

Due to the potential beneficial effects of OT in regenerative medicine in post-ischemic myocardial disease, it was considered that finding an appropriate dosing regimen for this new therapeutic use of OT requires knowledge of its dose-concentration relationship. Therefore, the objectives of the article entitled "Oxytocin receptor-mediated pharmacokinetics and its hemodynamic implications in pigs" together with the article "Evidence for non-linear pharmacokinetics of oxytocin in anesthetizetized rat", written by a member of our group Valerie Morin, were to characterize the pharmacokinetic profile of different doses and dosing regimens of OT (Morin, Del Castillo et al. 2008). We also considered important to characterize the renal and cardiovascular effects of this hormone, using the rat and the pig as animal models. We observed that the intravenous administration of OT, at the doses and administration regimens used, caused changes in cardiovascular and renal parameters in some cases similar to previous reports using lower doses of OT. Regarding OT pharmacokinetics, the results of this study performed in pigs confirmed the previously published observations in the rat model. In particular, the volume of distribution of OT decreased significantly with the bolus dose amount, which generated strong evidence of nonlinear pharmacokinetics. Oxytocin pharmacokinetics have been previously described using the open two-compartment model, but the long lasting cardiovascular effects found in the previously mentioned articles of our groups could be better explained with the type of modelization presented in the above mentioned article. The TMDD pharmacokinetic model takes in account the binding of drugs to their cellular receptors which additionally are characterized by low dissociation constants, therefore increasing the drug effects duration, as observed here with the dosing regimens of OT used in the first article here included. Moreover, dose-concentration and concentration effect relationship will be dependent of the expression drug receptors, as well as, the affinity of the drug to its receptor.

The significant and in some cases long lasting changes in renal and cardiovascular parameters highlight the importance of choosing an appropriate dosing regimen which could provide the "cardio-protective" effects of OT reported recently.

Our group, using the swine MI model, recently demonstrated that endogenous pre-MI OT levels and timing of administration post-MI are important criteria, if OT is intended to be used as a therapeutic strategy for ischemic heart disease (Authier, Tanguay et al. 2010). Pigs with low endogenous OT level receiving OT subcutaneous infusion (10 $ng \cdot kg^{-1} \cdot hr^{-1}$) beginning 8 days post-MI, as well as pigs with high endogenous OT level receiving saline, presented lower MI area measured by planimetry, and cardiac function was also less affected. However, high endogenous OT level pigs receiving OT infusion had highly-affected cardiac function associated to bigger MI area, as well as down-regulation of myocardial OTR (Authier, Tanguay et al. 2010). These results highlight the importance of an adequate dosing regimen and the knowledge of basal endogenous OT since these two factors importantly affect the outcome after MI. Indeed, non-linear pharmacokinetics (Morin, Del Castillo et al. 2008) 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. In our previous study, treatment of pigs (n=4) with OT ($10\ ng \cdot kg^{-1} \cdot hr^{-1}$) for 7 days commencing immediately after MI presented significantly decreased fraction shortening. 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, as 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 *vs.* 58.3 ± 12.2% with placebo-saline infusion, n=3) (Authier, Tanguay *et al.* 2010). The outcome was completely different when the same treatment was initiated one-week after MI, after

inflammation had mostly resolved, hypothesizing that early treatment with OT after MI could indeed worsen the pathophysiological ischemia/reperfusion situation, and that the cardiovascular and renal side effects related to different OTR presence could be highly deleterious.

The disturbances in cardiovascular hemodynamics vary with the severity of the MI. In mild cases there are no significant abnormalities. But in cases of cardiogenic shock, the most marked disturbances are the serious reduction of the SV of the heart and the marked increase in total SVR. The moderately severe and severe cases form an intermediate group between the mild and the cardiogenic shock cases, in hemodynamic abnormalities. Thus, the severity of the MI appears to be correlated directly with the degree of depression of SV. If a MI is extensive or if it involves a previously damaged myocardium, there is diminished contractility and hence a failure of stroke output. The consequent reduction of arterial pressure immediately results in the activation of neurogenic reflexes and possibly other compensatory mechanisms which produce an increase in total peripheral resistance (SVR) and tachycardia (Freis, Schnaper *et al.* 1952). Thus, the hemodynamic response is not unlike the compensated phase of hypovolemic shock, except that the disturbance is due to ineffectual contraction of the myocardium rather than to blood loss (Freis, Schnaper *et al.* 1952).

Based on the results obtained in this report of a MI swine model, it seems appropriate to conclude that the endogenous basal levels of OT are variable and that they affect the outcome after a MI. In the first article here included, we observed that high plasma OT concentrations cause significant alterations on cardiovascular hemodynamics. For instance, we observed increases in MAP and SVR, and these two variables are seriously affected after a MI. Therefore, the plasma concentrations reached after OT administration in pigs with high endogenous levels will be high enough to additionally alter cardiovascular hemodynamics. And the situation could even be worse with a negative feedback of this high OT expression

on OTR expression, a result observed in our study of OT therapy efficacy on the pig MI model (Authier, Tanguay *et al.* 2010). Moreover, we did not report in the first article that OT CRI also causes diuresis, kaliuresis and natriuresis, which will alter the volemia and the electrolyte homeostasis, compromising even more the outcome after a MI. This was corroborated by the fact that in pharmacodynamical evaluation of OT on anaesthetized rats (*data not shown*), where we observed induction of arrhythmia (premature ventricular contractions observed in two different rats) with high dose of OT, and the suspicion that one OT-treated pig immediately after MI died from arrhythmia.

Nevertheless, OT beneficial effects after MI have been recently reported, based on the observations of the studies performed by our group. It seems important to consider the basal levels of OT and the best moment of OT administration, as well as the complex pharmacokinetic profile of this neuropeptide.

Considering the other report on OT cardio-protective effects, in one of the reports the authors (Ondrejcakova, Ravingerova et al. 2009) used an *ex vivo* model therefore the alterations in hemodynamics are not taken in account, for the other two reports (Kobayashi, Yasuda et al. 2009; Jankowski, Bissonauth et al. 2010), it will be interesting to monitor if the species used in these also have subpopulations with low and high basal endogenous OT concentrations, of if there is a mechanism altering the basal concentrations in the pig model.

A very interesting feature of OT is that it has improved the cardiomyogenic differentiation potential of embryonic and cardiac stem cells, and cell therapy is thus an interesting alterative in patients affected by MI and congestive heart failure. We therefore decided to test the hypothesis of OT-mediated pBMSCs cardiomyogenic differentiation potential, as mentioned before these cells offer the possibility of being obtained from the patient's bone marrow, therefore there is no possibility of rejection. Also these cells are obtained from adults tissue, avoiding the ethics problematic related to the use of embryos.

Unfortunately, full differentiation of BMSCs into cardiomyocytes has not been achieved completely. There is still a lack of understanding of the differentiation process of stem cells, and in order to achieve full differentiation (functional) other possibilities need to be explored. It is important to take in account the complex process of differentiation of stem cells within their niche. The studies reported in the present thesis showed that pBMSCs have a potential to express cardiac markers when induced with OT, and that this process is mediated by the NO pathway. As mentioned in the discussion enclosed in the second article, it is important to test the functionality of these cells, but also it will be interesting to try different culture conditions that could improve the differentiation attained with OT alone. Once the differentiation and functionality of these cells are achieved, it will be very interesting to test their potential to improve cardiac function after MI. Eventhough these cells have been already used in preclinical and clinical trials obtaining promising results, these results have been more related to the possible anti-inflammatory and pro-angiogenic effects that they provide when transplanted after MI. Some propositions of future experiments that could be performed in order to improve the potential use of BMSCs for MI cell therapy are discussed more in detail in the subsequent sections.

### 6.2 Cell therapy in cardiovascular disease: why promote adult stem cells and particularly bone marrow derived stem cells?

During the last decades stem cell research has revealed many aspects related to embryonic and adult stem cell biology, which in turn has led to potential uses of stem cells in regenerative medicine. Even though general knowledge about stem cells has greatly increased during the past years, there are still many unknown aspects of stem cell biology that need to be elucidated in order to exploit the full potential of these cells.

The use of stem cells for myocardial regeneration has received a great deal of attention, and there are many reports of pre-clinical and clinical studies showing in general beneficial

effects. Stem cell therapy reflects a new form of therapy for heart disease that is otherwise refractory to conventional pharmacological therapy (Kovacic, Harvey et al. 2007).

As mentioned before, ESCs have the potential to differentiate into cardiomyocytes *in vivo* and *in vitro* even without induction, but ESCs and ESC derived cardiomyocytes have only been used in pre-clinical trials after MI induction. Most of the pre-clinical studies have used ESCs in the undifferentiated state, which implies the possible risk of teratoma formation (Boyd, Higashi et al. 2005), which works against the possible therapeutic benefits of ESCs. Alternatively, when using ESC derived cardiomyocytes some beneficial effects have been observed, for example: ventricular function, regional blood flow and arteriolar density were improved, and moreover there was a reduction in the myocardial remodelling process (Hodgson, Behfar et al. 2004; Singla, Hacker et al. 2006). Using ESC derived cardiomyocytes the possibility of teratoma formation is reduced. Unfortunately, even when ESC derived cardiomyocytes have many of the phenotypic properties of authentic cardiomyocytes (Abdul Kadir, Ali et al. 2009), recent reports have shown that $Ca^{2+}$ handling, crucial to excitation-contraction coupling of ES-derived cardiomyocytes is functional but immature (Liu, Lieu et al. 2009). Therefore full and efficient differentiation of ESC derived cardiomyocytes has not been achieved yet. In addition, it has been previously reported that ESC derived cardiomyocytes are potentially arrhythmogenic, which means that they might not be an optimal cell source for cardiac cell therapy (Zhang, Hartzell et al. 2002). There is also the possibility of immune rejection (Nussbaum, Minami et al. 2007), as well as the ethical concerns related to the use of ESCs, previously discussed (2009).

On the other hand, pre-clinical and clinical studies using adult stem cells for myocardial regeneration after MI have reported similar beneficial effects to those reported in pre-clinical studies using ESCs and ESCs derived cardiomyocytes. By using stem cells from adult origin

the problems related to ethical concerns, teratoma formation, and the possible rejection of the grafted cells are negligible, as previously discussed. Recently, the discovery of iPS has generated expectations, but the manipulation of the somatic cell genome could generate insertional mutagenesis [for review see ref.(Rolletschek and Wobus 2009)], as previously observed in certain cases of gene therapy (Herzog, Cao et al. 2010). Adult stem cells used in pre-clinical and clinical studies are mainly derived from the mononuclear population cells of the bone marrow (BM MNC). Recent clinical studies have reported how BMSCs proliferate, mobilize and incorporate into the myocardium (Wollert, Meyer et al. 2004; Engelmann, Theiss et al. 2006; Zohlnhofer, Ott et al. 2006; Tse and Lau 2007; Meyer, Wollert et al. 2009; Overgaard, Ripa et al. 2009).

The BMSCs exhibit a high degree of plasticity and participate in the repair process after MI. These cells, attracted to the site of ischemic injury, improve vascularization and reduce cardiac remodelling process (Wang, Haider et al. 2006; Wang, Johnsen et al. 2006), but the potential of differentiation of these cells into real cardiomyocytes is a continuous source of debate, as mentioned before. On the other hand, there are no reports of arrhythmogenic potential of adult stem cells in the clinical assays performed using these cells.

Recent studies have focused on a local, intramyocardial deposit of BMSCs, selected for their immature nature such as the IMPACT-CABG project (*Implantation of autologous CD133+ stem cells in patients undergoing coronary artery bypass grafting*) (UdeMNouvelles, 13 avril 2010).

Although both embryonic (pre-clinical studies) and adult stem cells (pre-clinical and clinical studies) have shown to be beneficial as an alternative therapy after MI, there are still some aspects that need to be improved. These aspects are mainly related to the efficient cardiomyogenic differentiation, the long-term survival of both cell sources, together with the incorporation of the grafted cells to the host myocardium. Also, in the special case of BM

MNC the low percentage of stem cells contained in the BM aspirates, together with the low delivery efficiency, and finally, poor survival, engraftment and integration of the implanted cells.

The beneficial effects observed after treatment with BMSCs have been described as transient or marginal. As previously described, the effects of BMSCs are mainly related to paracrine molecules expressed and secreted by the cells.

### 6.3 How to optimize BMSCs for cell therapy

In the future, it will be of great importance to understand the direct mechanisms of the transplanted cells on the heart as a whole. Basically, it is crucial to track the fate of implanted cells *in vivo* and their interactions with the host myocardium after transplantation. Also, it is important to better characterize and understand the biology of BMSCs and their subpopulations, in order to find which of these subpopulations accounts for major beneficial effects, ideally these cells should be able to give rise to functional cardiomyocytes together with the known properties of conferring anti-apoptotic, and anti-inflammatory effects and neoangiogenesis.

In most cases, acquiring a sufficient number of a specific cell type from the BM may require either extremely large quantities of BM from patients, or *ex vivo* expansion of cells by culture, which takes time and decreases the possibility of employing the cells when they could exert their beneficial effects, so it is important to find the way of increasing the clonogenicity of such cells in a shorter period, with a safe methodology. In addition, improving the survival of these cells which will be exposed to a hostile environment by up-regulating some pathways related with cell survival should be beneficial in increasing the clinical effects on long-term.

Another important subject that needs to be optimized when using BMSCs for treatment is the delivery method. Local delivery of BMSCs avoids diffusion of the cells to other tissue and/or

organs. Therefore cell delivery procedures also need to be improved. At this moment the most prevalent methods for cell delivery are the catheter-based stem cell delivery modalities, such as intracoronary infusion and transendocardial injection, which are commonly adopted in pre-clinical studies and human trials. Intracoronary infusion is the most common cell delivery route in clinical studies. Similar to percutaneous coronary intervention, cells can be delivered *via* a balloon catheter placed in the affected coronary artery with the inflated balloon causing temporary occlusion of the proximal section of the coronary artery to prevent back flow of the cells. Although considered a safe procedure, cell infusion represents some risks. It has been reported that cell infusion by this method is related with an increased incidence of coronary events, such as coronary artery restenosis after coronary angioplasty (Kang, Kim et al. 2004) and a higher incidence of decreased coronary blood flow (Freyman, Polin et al. 2006).

Using this method of cell delivery it has been observed that a significant quantity of the implanted cells could home in to non-targeted organs, which will potentially reduce the observation of beneficial effects in the area of interest (Freyman, Polin et al. 2006; Kurpisz, Czepczynski et al. 2007). An alternative to this method of delivery is the transendocardial injection, which delivers cells to small areas of abnormal heart tissue intramyocardially by using an injection catheter guided by a cardiac mapping system. This technique has been described as safe, and it has been tested on animal models and on human subjects (Amado, Saliaris et al. 2005; Dohmann, Perin et al. 2005). Transendocardial injections may improve the efficacy of cell therapy with fewer cells required by preventing the homing of the cells to other tissues and organs.

### 6.4 Potential of oxytocin as a therapy for myocardial infarction: effects on embryonic and adult stem cells, on isolated *ex vivo* organs, and preclinical models in rats and pigs.

Oxytocin-mediated cardiomyogenic differentiation has already been reported in embryonic carcinoma cells (Paquin, Danalache et al. 2002). Embryonic carcinoma cells have been a good model for developmental studies, but they might not share all the differentiation characteristics of ESCs. Also, ESCs OT-mediated cardiomyogenic differentiation has been reported previously (Hatami, Valojerdi et al. 2007), ESCs could cause teratoma formation, and could be rejected by the host immune system since they are not obtained from the patient's own tissues. As previously discussed, these cells are not good candidates for clinical trials. Therefore, it was interesting for us, to verify if OT was a more efficient differentiation inducer of stem cells from adult tissues. In this specific case, our objective was to test if OT is a better inducer of porcine BMSCs (pBMSCs), than other inducers previously used, such as 5-azacytidine. Swine are more suitable animal models than murine models because the latter have important differences related to their size and life-span compared to those of humans. The porcine model is more adequate, considering its similarities with human anatomy, physiology, and life-span (Swindle 1984).

Recently, it has been suggested that OT could be potentially useful as an alternative therapy after MI. Moreover, it has also been reported that OT induces the proliferation of endothelial cells (Cattaneo, Chini et al. 2008), inducing neovascularisation and in consequence an improved myocardial regeneration after infarction.

In addition to the beneficial effects of OT on cardiomyogenic differentiation and endothelial cell proliferation, a recent report has shown that OT protects the kidney tissue against I/R-induced oxidative damage (Tugtepe, Sener et al. 2007). Consequently, Ondrejcakova *et al.* reported that OT can attenuate the extent of irreversible myocardial damage in an induced I/R

model of MI in rats; this was demonstrated as a reduced size of infarction. Oxytocin also decreased or mitigated the post-ischemic cardiac stunning. Apparently the negative chronotropic, but not inotropic action of OT appears to be involved in the mediation of its protective effects (Ondrejcakova, Ravingerova et al. 2009).

The beneficial effects of OT administered after MI have been reported in *ex vivo* preparations of isolated Langendorff-perfused rat hearts (Ondrejcakova, Ravingerova et al. 2009), and animal models of MI as previously mentioned in the review of literature. In a recent report of our group, using the porcine MI model, we observed that endogenous pre-MI OT levels, OT dosing and timing of administration post-MI are important criteria in the outcome of the infarcted animals treated with OT. These results highlight the importance of better characterizing the dosing regimen and the potential signaling pathways activated by OT administration.

On the other hand, *in vitro* cardiomyogenic differentiation of adult stem cells is another very interesting feature of OT. To optimize grafting and transdifferentiation of adult stem cells, particularly taking account the difference in environment of the target population (old people with lower potential of regeneration), the strategy to add factor promoting pre-differentiation to BMSCs culture has been proposed (Khan, Mohsin et al. 2009). Moreover, because our group has demonstrated that the strategy to use OT administration to increase mobilization, the homing and differentiation of endogenous progenitor stem cells in the MI target area has some interest in rat (Jankowski, Bissonauth et al. 2010) and swine (Authier, Tanguay et al. 2010) MI models, it became evident that we must learn more about the potential of OT to act as adult stem cell differentiation inducer.

### 6.5 Mechanisms of action of OT-induced cardiomyocyte differentiation (role of NO, future implications, propositions of future tests)

Due to the possible additive effects of OT and stem cells for myocardial regeneration after infarction, the experiments presented in this thesis had the objective of exploring the OT-mediated cardiomyogenic differentiation of pBMSCs, and the role of the NO-cGMP pathway in the differentiation process elicited by OT treatment. It is important to understand and to explore the possible mechanisms that induce the differentiation of stem cells to a certain cell type and, it has been previously reported that some of the OT effects in different systems are mediated through the NO pathway (Thibonnier, Conarty et al. 1999; Danalache, Paquin et al. 2007).

An interesting finding was that OT is a better cardiomyogenic differentiation inducer of pBMSCs than widely used 5-azacytidine. This was demonstrated by increased cardiac specific protein expression. Similar results have been reported with adult stem cells from the heart (Matsuura, Nagai et al. 2004; Oyama, Nagai et al. 2007). In the present study, it was possible to demonstrate an improvement in cardiomyogenic differentiation of pBMSCs, based on an increased cardiac protein expression observed in OT-treated cells, compared with control cells and 5-azacytidine-treated cells. It would be interesting to develop culture systems that could allow us to test the physiological changes that these cells experience after OT differentiation induction, since the objective of cell therapy for myocardial infarction is not only to use cells that express cardiac markers, but to use cells that are able to couple and act in synchrony with the host cardiomyocytes.

Moreover, we also found that not only OTR is expressed in pBMSCs, but also NOS (endothelial and inducible) are expressed. Both OT and NO systems are expressed and functional during embryonic development of the heart and are important for cardiogenesis. This has been reported in experiments using ESCs as developmental models (Bloch,

Fleischmann et al. 1999; Paquin, Danalache et al. 2002; Krumenacker, Katsuki et al. 2006; Mujoo, Krumenacker et al. 2006; Hatami, Valojerdi et al. 2007). The fact that pBMSCs also express both systems could be indicative of the native cardiomyogenic potential of these cells, meaning that it is possible that pBMSCs have a cardiomyogenic differentiation potential similar to the one of ESCs during early cardiomyogenesis.

Furthermore, Danalache *et al.* (2007) already demonstrated that OT cardiomyogenic differentiation of embryonic carcinoma cells is mediated by the NO pathway, because they observed an up-regulation of eNOS transcripts, and demonstrated that inhibition of NO pathway using competitive inhibitors of L-arginine drastically reduced the number of beating cell colonies. They also reported that OT was a better differentiation inducer than NO donor SNAP (Danalache, Paquin et al. 2007).

It is important to understand the signalling pathways that could be involved during OT differentiation, because these pathways could be also exploited in order to improve myocardial function after I/R injury. In the present study, we found that induction of pBMSCs with OT, elicits up-regulation of NOS, therefore if these cells are used for cell therapy in a MI animal model, these cells could be a source of NO production, and it has been proved that NO itself promotes the migration and proliferation of endothelial cells, which in turns will lead to angiogenesis, improving $O_2$ availability and, in consequence, myocardium long-term survival. In fact, as mentioned before, it has been reported that OT promotes angiogenesis and this angiogenesis is in part mediated by NO pathway (Cattaneo, Chini et al. 2008).

Furthermore, it has been reported that nitrite anion represents an important storage form of NO in blood and tissues. Nitrite is an oxidative breakdown product of NO. Previously, nitrite has only been considered as an acute marker of NO flux/formation in biological systems, and

an indirect determinant of NOS activity (Gladwin, Raat et al. 2006). At physiological ranges of pH and $O_2$ tension, nitrite has been considered to be a highly stable and inert metabolic end-product of NO oxidation with limited intrinsic biological activity (Kelm 1999).

However, nitrite anion can be reduced to NO under certain pathological conditions. Recently, it has been found that nitrite anion is cytopretective in a number of animal models of disease including myocardial I/R injury. Moreover, during ischaemia, the ability of eNOS to generate NO, through the conventional L-arginine pathway is severely reduced because of an inadequate delivery of $O_2$ and co-factors (Becker, Kupatt et al. 2000). Recent reports have shown that eNOS can reduce nitrite under anoxia, and the magnitude and duration of this reduction is relevant for the fast delivery of NO in hypoxic vascular tissues (Vanin, Bevers et al. 2007).

In the results presented in this thesis we observed that OT up-regulates eNOS expression, a finding of significance if OT-treated BMSC are transplanted in an MI animal (porcine) model, transplanted cells could increase the local production of NO from nitrite contained in the blood and tissues, even under low $O_2$ tension conditions, and therefore improve angiogenesis and subsequent $O_2$ delivery.

Even though, OT apparently improves the cardiomyogenic differentiation of embryonic and adult stem cells, the mechanism by which OT is improving this differentiation has not been clarified yet. We know from the results presented here that a blockage in NO pathway decreases the expression of cardiac specific proteins coming from OT-mediated cardiomyogenic differentiation. In addition, these results are in accordance with the results presented by Danalache *et al.* (2007) using embryonic carcinoma cells, where they described that OT treatment increased the number of beating cell colonies (Danalache, Paquin et al. 2007).

Furthermore, we also observed that there was a significant increase in the total cell count of pBMSCs treated with OT, when compared to the cell count of pBMSCs not induced with OT. In the case of embryonic carcinoma cells, the authors reported an increased number of beating cell colonies (Paquin, Danalache et al. 2002). It has been previously reported that OT could induce cell proliferation depending on the subcellular localization of OTR (Guzzi, Zanchetta et al. 2002). Therefore, it could be possible that OT induces proliferation of cells already committed with cardiac lineage, which will result in an apparently increased cardiomyogenic differentiation. The OT-induced cell proliferation could also increase the number of cells that could be used for cell therapy, reducing the time of *ex vivo* expansion previously discussed in order to improve the outcomes of BMSCs therapy for MI. On the other hand, OT differentiation-induction of embryonic and adult stem cells activates the NO pathway, and it has been proposed that NO could be acting as an apoptotic agent of cells not committed with cardiac lineage, therefore causing an apparent increased number of ESCs derived cardiomyocytes (Kanno, Kim et al. 2004).

### 6.6 Potential roles of oxytocin in the future therapies: with BMSCs, hypotheses of research, as well as limitations

As mentioned before one of the main problems when inducing cardiomyogenic differentiation of embryonic or adult stem cells is to prove that the differentiated cell has not only the phenotypic and structural characteristics of a cardiomyocyte, but also that the derived cell is functionally like an adult cardiomyocyte. An interesting feature of ESCs is that once they are allowed to differentiate by embryoid body aggregation, these cells exhibit spontaneous cell beating, this fact allows us to observe a certain degree of functionality. Unfortunately, there are reports showing that functionally ESCs, derived cardiomyocytes do

not act like adult cardiomyocytes, thus full differentiation into adult cardiomyocytes has not been achieved (Dolnikov, Shilkrut et al. 2006; Binah, Dolnikov et al. 2007).

It will be interesting to verify the functional properties of either OT-induced embryonic or adult stem cells, to see if OT is able not only to improve the phenotypic cardiomyogenic differentiation of stem cells but also the functional characteristics of the OT-treated derived cells.

Unfortunately, we did not observe spontaneous beating in the OT induced pBMSCs, although there are some publications that describe spontaneous beating in BMSCs *in vitro* (Dolnikov, Shilkrut et al. 2006). This could be related to differences in culture conditions and differences among species, since spontaneous beating has been reported in murine BMSC (Fukuda 2002).

In the future, it will be interesting to test the functional properties of OT induced pBMSCs grown in different culture conditions. For example, in order to observe spontaneous beating, pBMSCs need to be cultured under conditions that allow them to aggregate and form three-dimensional structures, because under the conditions described here, adherence to a flat and rigid surface and growing in monolayers, it is not possible to observe spontaneous beating. Also, it was observed that when pBMSCs are suspended in a solution, on low adherent surfaces, they tend to agglomerate; this could compromise the viability of these cells, because the cells that are in the inner part of the agglomerate won't be in contact with the culture medium nutrients and $O_2$. Furthermore, another less well-known but essential requirement for nearly all cells is the need for a specific physical substrate. Most cell types, including cardiaomyocytes (Curtis and Russell 2009) and pBMSCs (personal observation) proliferate only when attached to a suitable supporting surface.

A possible interesting approach for improving cardiomyogenic differentiation and survival of pBMSCs is the use of biomaterials and scaffolds for tissue engineering. Tissue engineering is intended to mimic the natural tissue structure, by offering the cells a surface to adhere and

proliferate in a more "natural" manner. The purpose of using tissue engineering will be to recreate a viable cellular environment through the use of biologically acceptable materials. It has been shown that slight changes on the surface provided to the cells to attach affect cardiomyocyte viability. This surface should support cardiomyocyte adhesion and at the same time allow the cells to exhibit spontaneous beating (Curtis and Russell 2009). Same principle could be applied to stem cells subjected to *in vitro* differentiation. Most of the current research has focused on inducing stem cells to become cardiomyocytes by using *in vitro* treatment or *in vivo* differentiation when transplanted to the host myocardium, but until today full differentiation has not been achieved and the survival of the cells is not optimal, therefore it could be possible that both technologies, tissue engineering and *in vitro* inducers, improve the cardiomyogenic differentiation potential of stem cells.

Specifically, it would be interesting to combine tissue engineering and OT treatment for inducing the differentiation of BMSCs. In a very recent report, Valarmathi *et al.* (2010) propose a very interesting approach to improve what until now they described as inadequate and inconsistent differentiation of BMSCs into cardiomyocytes, as well as the poor survival and integration of the BMSCs-derived cardiomyocytes, after implantation into ischemic myocardium. The authors developed an *in vitro* model of three-dimensional (3-D) cardiac muscle using a co-culture of rat ventricular embryonic cardiomyocytes (ECMs) and BMSCs. They found that when ECMs and BMSCs were seeded sequentially onto a 3-D tubular scaffold engineered from topographically aligned type I collagen-fibers and cultured in basal medium for 7, 14, 21, or 28 days, the maturation and co-differentiation into a cardiomyocyte lineage was observed. They observed expression of transcripts coding for cardiomyocyte phenotypic markers and the immunolocalization of cardiomyogenic lineage-associated proteins revealed typical expression patterns of cardiomyogenesis. They concluded that the 3-D co-culture system sustains the ECMs *in vitro* continuum of differentiation process and simultaneously induces the maturation and differentiation of BMSCs into cardiomyocyte-like

cells (Valarmathi, Goodwin et al. 2010). The main inconvenient of this study was the use of ECMs, because as previously discussed, it will be difficult to know if the BMSCs fused to the ECMs, since they did not test this possibility, more over if we think in the possibility of using this construct for a clinical application, the use of a cell source that does not belong to the own patient adds the possibility of future tissue rejection. Therefore, it would be interesting to test if this type of 3-D scaffold could be used in the OT-mediated cardiomyogenic differentiation of BMSCs, this scaffold which will provide a structural matrix to which BMSCs will attach, to grow and differentiate, and since there is evidence supporting that OT improves the number of BMSCs expressing cardiac markers, there is no need to co-culture the stem cells with differentiated cells to provide needed microenvironment, *e.g* ECMs. For this purpose it will be necessary to test different concentrations of OT and exposure timings, since the 3-D configuration of the cells could affect the concentration at which the cells will be exposed, also it will be important to verify the effect of the collagen scaffold on OT-treated BMSCs. It could be possible that the cells will need repeated exposure to low concentrations of OT in an attempt to mimic what it happens during embryogenesis. The progressive cell aggregation to which the BMSCs will be exposed together with OT exposure could definitively improve the cardiomyogenic differentiation of BMSCs.

The very encouraging results obtained to date in clinical studies using BMSCs, together with the promising results obtained in some pre-clinical studies using OT after MI, could lead to the future combination of both technologies. Even though, cautions should be taken if OT is planned to be administered after MI, this based on the observations found by our group (Authier, Tanguay et al. 2010).

Furthermore, it is important to monitor the progression of the differentiation process as well as the detection of spontenous beating, in a recent report Ohn *et al.* (2009) described a very interesting method of imaging and analysis procedure that yields a dynamic depiction of heart development that included, at each developmental stage studied, the shape of the heart in any

of its contraction states. This method combines automated high-speed fluorescence and brightfield microscopy with high-dimensional image sequence synchronization to reveal the dynamics of cardiac morphogenesis with cellular resolution and without perturbing cardiac beat. The resulting images are highly suitable for automatic image analysis, specifically, to extract quantitative velocity fields and track cell motions both as the heart beats and over the course of cardiac development (Ohn, Tsai et al. 2009). This method of imaging and analysis could provide a better follow up of the differentiation process of OT-treated BMSCs. The ultimate goal of combining these technologies will be to engineer new heart tissue that can be implanted into damaged areas. By using tissue engineering and *in vitro* stem cells differentiation technologies, some of the problems related to direct cell transplantation could be reduced, for example: when cells are injected directly to the myocardium or delivered intravascularly, there is cell migration to other organs (Barbash, Chouraqui et al. 2003); in consequence there are a reduced number of cells acting locally in the heart. In contrast, if cells are implanted within a matrix or a support material like collagen, it will be possible to limit cell migration to the infarcted area rather than to other organs. In addition, it will be possible to have a better follow up of the implanted cells. Moreover, it will also be possible to have an improved survival of these cells, since the implanted cells will not be in direct contact with the hostile environment (inflammatory cytokines) found after MI, without mentioning the possible enhanced differentiation achieved.

Currently, there are many studies documenting the use of different materials to build scaffolds and cell sheets for tissue engineering after MI (Ifkovits, Devlin et al. 2009; Nakamuta, Danoviz et al. 2009; Singelyn, DeQuach et al. 2009; Seif-Naraghi, Salvatore et al. 2010; Zakharova, Mastroeni et al. 2010). Extensive research needs to be done in this area and stem cells cardiomyogenic differentiation before both technologies could be used together.

Finally, it is possible that these scaffolds with engineered tissue could be made with biodegradable materials that could be delivered to the MI area by endoscopic surgery, similarly to the endoscopic coronary artery bypass.

# CHAPTER 7. GENERAL CONCLUSIONS

**7.1 Oxytocin pharmacokinetics and pharmacodynamics**

Oxytocin hemodynamic effects are strongly connected with the pharmacokinetic TMDD behavior of this hormone. This emphasizes the need for reassessing the therapeutic index of OT in a context of patients affected with cardiovascular pathologies. The TMDD pharmacokinetic-pharmacodynamic model proposed here may become a tool for understanding the complex balance between OT desired effects and untoward outcomes.

**7. 2 Isolation and characterization of pBMSCs**

The pBMSCs differentiation potential was affected by the time in culture and the number of passages: cells were affected not only in size and shape, but also their gene expression was altered.

It seemed appropriate to induce cardiomyogenic differentiation during early passages, specifically during passage 1, because at this time pBMSCss expressed:

- Phenotypic profile of surface epitopes described for MSC.
- Important levels of OTR transcripts.
- Transcription factors, Oct-4 previously related to pluripotency, and GATA-4 implicated in cardiac differentiation.

**7.3 Cardiomyogenic differentiation induction of pBMSCs**

- Expression of GATA-4, desmin, PLB and cTNI3 transcripts was up-regulated in OT- and 5-azacytidine-induced cells when compared to non-induced cells.

- Protein expression of cardiomyocyte specific proteins MHC, TNNT2 and cTNI3 was up-regulated in OT-induced cells; however PLB was also expressed, although weaker in non-induced cells.
- Expression of cardiac specific proteins was more abundant in OT- *vs.* 5-azacytidine-treated cells, and PLB expression was up-regulated similarly in both induced groups. Although, gene transcripts seemed to be more abundant in 5-azacytidine-treated cells. Semi-quantitative analysis demonstrated that cTNI3 protein expression was significantly up-regulated in OT induced cells compared to control and 5-azacytidine treated cells.

In this study, OT induced expression of cardiac specific structural proteins in pBMSCss, as previously described for murine ES cells and cardiac stem cells. Oxytocin was a more potent inducer of cardiac differentiation of pBMSCs than 5-azacytidine, based on cardiac protein expression.

**7.4 Involvement of the NO-sGC pathway in the OT mediated cardiomyogenic differentiation of pBMSCs**

- pBMSCs express endothelial and inducible nitric oxide synthase isoforms.

OT induction:

- Up-regulates transcript expression of eNOS and iNOS, it also increases expression on NOS protein.
- Increases abundance of cardiac specific proteins cMHC, TNNT2 and cTNI3.

Specifically in OT-induced pBMSCs, inhibiting NO production with L-NAME, a competitive inhibitor of NOS, and inhibiting sGC using ODQ, significantly affected the expression of cardiac specific markers transcripts and proteins.

- The exposure of pBMSCs to NOS and/or sGC inhibitors reduced the abundance of cardiac specific markers to levels similar to those found in control cells.
- Endogenously produced NO under OT stimulation, with the consequent activation of sGC-dependent pathway(s) seem to be important in OT-mediated specific cardiomyogenic differentiation of pBMSCs, since only cardiac specific protein expression was affected.

These results suggest that OT induction with the subsequent activation of NO pathway directs pBMSCs to a preferential cardiomyogenic phenotype.

- OT increases total cell count of pBMSCs and this increase seem to be also dependent of the NO-sGC pathway, since inhibition of NO pathway with L-NAME and ODQ gave the similar cell counts of those registered in control cells.
- Apparently, OT, through NO-sGC pathway, increases proliferation of pBMSCs.

# CHAPTER 8. REFERENCES

(2009). "Embryonic stem cells, Francis Collins, and the NIH." Lancet **374**(9685): 175.

Abdul Kadir, S. H., N. N. Ali, et al. (2009). "Embryonic stem cell-derived cardiomyocytes as a model to study fetal arrhythmia related to maternal disease." J Cell Mol Med.

Adan, R. A., F. W. Van Leeuwen, et al. (1995). "Rat oxytocin receptor in brain, pituitary, mammary gland, and uterus: partial sequence and immunocytochemical localization." Endocrinology **136**(9): 4022-4028.

al-Eknah, M. M. and A. M. Homeida (1991). "A review of some aspects of the pharmacology of oxytocin in domestic animals." Vet Res Commun **15**(1): 45-55.

Amado, L. C., A. P. Saliaris, et al. (2005). "Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction." Proc Natl Acad Sci U S A **102**(32): 11474-11479.

Amico, J. A., M. Morris, et al. (2001). "Mice deficient in oxytocin manifest increased saline consumption following overnight fluid deprivation." Am J Physiol Regul Integr Comp Physiol **281**(5): R1368-1373.

Amico, J. A., J. Seitchik, et al. (1984). "Studies of oxytocin in plasma of women during hypocontractile labor." J Clin Endocrinol Metab **58**(2): 274-279.

Andari, E., J. R. Duhamel, et al. (2010). "Promoting social behavior with oxytocin in high-functioning autism spectrum disorders." Proc Natl Acad Sci U S A **107**(9): 4389-4394.

Andersen, S. E., T. Engstrom, et al. (1992). "Effects on renal sodium and potassium excretion of vasopressin and oxytocin in conscious dogs." Acta Physiol Scand **145**(3): 267-274.

Antonitsis, P., E. Ioannidou-Papagiannaki, et al. (2007). "In vitro cardiomyogenic differentiation of adult human bone marrow mesenchymal stem cells. The role of 5-azacytidine." Interact Cardiovasc Thorac Surg **6**(5): 593-597.

Antunes-Rodrigues, J., M. de Castro, et al. (2004). "Neuroendocrine control of body fluid metabolism." Physiol Rev **84**(1): 169-208.

Arceci, R. J., A. A. King, et al. (1993). "Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart." Mol Cell Biol **13**(4): 2235-2246.

Arias, F. (2000). "Pharmacology of oxytocin and prostaglandins." Clin Obstet Gynecol **43**(3): 455-468.

Arminan, A., C. Gandia, et al. (2008). "Cardiac Differentiation Is Driven by NKX2.5 and GATA4 Nuclear Translocation in Tissue-Specific Mesenchymal Stem Cells." Stem Cells Dev.

Armour, J. A. and G. A. Klassen (1990). "Oxytocin modulation of intrathoracic sympathetic ganglionic neurons regulating the canine heart." Peptides **11**(3): 533-537.

Arpin-Bott, M. P., E. Waltisperger, et al. (1997). "Two oxytocin-binding site subtypes in rat kidney: pharmacological characterization, ontogeny and localization by in vitro and in vivo autoradiography." J Endocrinol **153**(1): 49-59.

Atherton, J. C., J. M. Dark, et al. (1982). "Changes in water and electrolyte balance, plasma volume and composition during pregnancy in the rat." J Physiol **330**: 81-93.

Authier, S., J. F. Tanguay, et al. (2010). "Cardiovascular effects of oxytocin infusion in a porcine model of myocardial infarct." J Cardiovasc Pharmacol **55**(1): 74-82.

Bader, A., H. Al-Dubai, et al. (2000). "Leukemia inhibitory factor modulates cardiogenesis in embryoid bodies in opposite fashions." Circ Res **86**(7): 787-794.

Baek, K. J., N. S. Kwon, et al. (1996). "Oxytocin receptor couples to the 80 kDa Gh alpha family protein in human myometrium." Biochem J **315 ( Pt 3)**: 739-744.

Bakos, J., P. Bobryshev, et al. (2008). "Phenylethanolamine N-methyltransferase gene expression in the heart and blood pressure response to oxytocin treatment in rats exposed to voluntary wheel running." Ann N Y Acad Sci **1148**: 302-307.

Bale, T. L. and D. M. Dorsa (1997). "Cloning, novel promoter sequence, and estrogen regulation of a rat oxytocin receptor gene." Endocrinology **138**(3): 1151-1158.

Banach, K., M. D. Halbach, et al. (2003). "Development of electrical activity in cardiac myocyte aggregates derived from mouse embryonic stem cells." Am J Physiol Heart Circ Physiol **284**(6): H2114-2123.

Barbash, I. M., P. Chouraqui, et al. (2003). "Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium: feasibility, cell migration, and body distribution." Circulation **108**(7): 863-868.

Bathgate, R. A. and C. Sernia (1994). "Characterization and localization of oxytocin receptors in the rat testis." J Endocrinol **141**(2): 343-352.

Bayes-Genis, A., S. Roura, et al. (2005). "Identification of cardiomyogenic lineage markers in untreated human bone marrow-derived mesenchymal stem cells." Transplant Proc **37**(9): 4077-4079.

Becker, B. F., C. Kupatt, et al. (2000). "Reactive oxygen species and nitric oxide in myocardial ischemia and reperfusion." Z Kardiol **89 Suppl 9**: IX/88-91.

Beckman, J. S. (1991). "The double-edged role of nitric oxide in brain function and superoxide-mediated injury." J Dev Physiol **15**(1): 53-59.

Beckman, J. S., T. W. Beckman, et al. (1990). "Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide." Proc Natl Acad Sci U S A **87**(4): 1620-1624.

Behfar, A., L. V. Zingman, et al. (2002). "Stem cell differentiation requires a paracrine pathway in the heart." FASEB J **16**(12): 1558-1566.

Behrendt-Adam, C. Y., M. H. Adams, et al. (1999). "Oxytocin-neurophysin I mRNA abundance in equine uterine endometrium." Domest Anim Endocrinol **16**(3): 183-192.

Beltrami, A. P., L. Barlucchi, et al. (2003). "Adult cardiac stem cells are multipotent and support myocardial regeneration." Cell **114**(6): 763-776.

Beltrami, A. P., K. Urbanek, et al. (2001). "Evidence that human cardiac myocytes divide after myocardial infarction." N Engl J Med **344**(23): 1750-1757.

Benavente, C. A., W. D. Sierralta, et al. (2003). "Subcellular distribution and mitogenic effect of basic fibroblast growth factor in mesenchymal uncommitted stem cells." Growth Factors **21**(2): 87-94.

Bicknell, R. J. (1985). "Endogenous opioid peptides and hypothalamic neuroendocrine neurones." J Endocrinol **107**(3): 437-446.

Bicknell, R. J., C. Chapman, et al. (1985). "Neurohypophysial opioids and oxytocin secretion: source of inhibitory opioids." Exp Brain Res **60**(1): 192-196.

Binah, O., K. Dolnikov, et al. (2007). "Functional and developmental properties of human embryonic stem cells-derived cardiomyocytes." J Electrocardiol **40**(6 Suppl): S192-196.

Bishop, A. E., L. D. Buttery, et al. (2002). "Embryonic stem cells." J Pathol **197**(4): 424-429.

Bittira, B., J. Q. Kuang, et al. (2002). "In vitro preprogramming of marrow stromal cells for myocardial regeneration." Ann Thorac Surg **74**(4): 1154-1159; discussion 1159-1160.

Blackburn, R. E., W. K. Samson, et al. (1993). "Central oxytocin inhibition of salt appetite in rats: evidence for differential sensing of plasma sodium and osmolality." Proc Natl Acad Sci U S A **90**(21): 10380-10384.

Blau, H. M., T. R. Brazelton, et al. (2001). "The evolving concept of a stem cell: entity or function?" Cell **105**(7): 829-841.

Bloch, W., B. K. Fleischmann, et al. (1999). "Nitric oxide synthase expression and role during cardiomyogenesis." Cardiovasc Res **43**(3): 675-684.

Bourque, C. W. (2008). "Central mechanisms of osmosensation and systemic osmoregulation." Nat Rev Neurosci **9**(7): 519-531.

Boyd, A. S., Y. Higashi, et al. (2005). "Transplanting stem cells: potential targets for immune attack. Modulating the immune response against embryonic stem cell transplantation." Adv Drug Deliv Rev **57**(13): 1944-1969.

Braun, T. and A. Martire (2007). "Cardiac stem cells: paradigm shift or broken promise? A view from developmental biology." Trends Biotechnol **25**(10): 441-447.

Breton, C., C. Pechoux, et al. (1995). "Oxytocin receptor messenger ribonucleic acid: characterization, regulation, and cellular localization in the rat pituitary gland." Endocrinology **136**(7): 2928-2936.

Brimble, M. J. and R. E. Dyball (1977). "Characterization of the responses of oxytocin- and vasopressin-secreting neurones in the supraoptic nucleus to osmotic stimulation." J Physiol **271**(1): 253-271.

Brimble, M. J., R. E. Dyball, et al. (1978). "Oxytocin release following osmotic activation of oxytocin neurones in the paraventricular and supraoptic nuclei." J Physiol **278**: 69-78.

Brownstein, M. J., J. T. Russell, et al. (1980). "Synthesis, transport, and release of posterior pituitary hormones." Science **207**(4429): 373-378.

Burns, T. C., X. R. Ortiz-Gonzalez, et al. (2006). "Thymidine analogs are transferred from prelabeled donor to host cells in the central nervous system after transplantation: a word of caution." Stem Cells **24**(4): 1121-1127.

Bussolati, G. and P. Cassoni (2001). "Editorial: the oxytocin/oxytocin receptor system-expect the unexpected." Endocrinology **142**(4): 1377-1379.

Calvert, J. W. and D. J. Lefer (2009). "Myocardial protection by nitrite." Cardiovasc Res.

Capi, O. and L. Gepstein (2006). "Myocardial regeneration strategies using human embryonic stem cell-derived cardiomyocytes." J Control Release **116**(2): 211-218.

Caplice, N. M. and B. J. Gersh (2003). "Stem cells to repair the heart: a clinical perspective." Circ Res **92**(1): 6-8.

Cattaneo, M. G., B. Chini, et al. (2008). "Oxytocin stimulates migration and invasion in human endothelial cells." Br J Pharmacol **153**(4): 728-736.

Chen, Y. L., C. Shepherd, et al. (1999). "Oxytocin and vasopressin constrict rat isolated uterine resistance arteries by activating vasopressin V1A receptors." Eur J Pharmacol **376**(1-2): 45-51.

Christoforou, N. and J. D. Gearhart (2007). "Stem cells and their potential in cell-based cardiac therapies." Prog Cardiovasc Dis **49**(6): 396-413.

Claybaugh, J. R. and C. F. Uyehara (1993). "Metabolism of neurohypophysial hormones." Ann N Y Acad Sci **689**: 250-268.

Conrad, K. P., M. Gellai, et al. (1993). "Influence of oxytocin on renal hemodynamics and sodium excretion." Ann N Y Acad Sci **689**: 346-362.

Costa, E. S. R. H., P. P. Pereira-Junior, et al. (2005). "Cardiac effects of oxytocin: is there a role for this peptide in cardiovascular homeostasis?" Regul Pept **132**(1-3): 107-112.

Crall, H. D. and D. R. Mattison (1991). "Oxytocin pharmacodynamics: effect of long infusions on uterine activity." Gynecol Obstet Invest **31**(1): 17-22.

Cummiskey, K. C., S. A. Gall, et al. (1989). "Pulsatile administration of oxytocin for augmentation of labor." Obstet Gynecol **74**(6): 869-872.

Curtis, M. W. and B. Russell (2009). "Cardiac tissue engineering." J Cardiovasc Nurs **24**(2): 87-92.

Czyz, J. and A. Wobus (2001). "Embryonic stem cell differentiation: the role of extracellular factors." Differentiation **68**(4-5): 167-174.

Danalache, B. A., J. Paquin, et al. (2007). "Nitric oxide signaling in oxytocin-mediated cardiomyogenesis." Stem Cells **25**(3): 679-688.

De Groot, A. N., T. B. Vree, et al. (1995). "Bioavailability and pharmacokinetics of sublingual oxytocin in male volunteers." J Pharm Pharmacol **47**(7): 571-575.

de Macedo Braga, L. M., S. Lacchini, et al. (2008). "In situ delivery of bone marrow cells and mesenchymal stem cells improves cardiovascular function in hypertensive rats submitted to myocardial infarction." J Biomed Sci **15**(3): 365-374.

Dell'Era, P., R. Ronca, et al. (2003). "Fibroblast growth factor receptor-1 is essential for in vitro cardiomyocyte development." Circ Res **93**(5): 414-420.

Devarajan, K., E. G. Marchant, et al. (2005). "Circadian and light regulation of oxytocin and parvalbumin protein levels in the ciliated ependymal layer of the third ventricle in the C57 mouse." Neuroscience **134**(2): 539-547.

Devarajan, K. and B. Rusak (2004). "Oxytocin levels in the plasma and cerebrospinal fluid of male rats: effects of circadian phase, light and stress." Neurosci Lett **367**(2): 144-147.

Dickens, B. M. and R. J. Cook (2007). "Acquiring human embryos for stem-cell research." Int J Gynaecol Obstet **96**(1): 67-71.

Dimmeler, S. and A. M. Zeiher (2009). "Cell Therapy of Acute Myocardial Infarction: Open Questions." Cardiology **113**(3): 155-160.

Doetschman, T. C., H. Eistetter, et al. (1985). "The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium." J Embryol Exp Morphol **87**: 27-45.

Doevendans, P. A., S. W. Kubalak, et al. (2000). "Differentiation of cardiomyocytes in floating embryoid bodies is comparable to fetal cardiomyocytes." J Mol Cell Cardiol **32**(5): 839-851.

Dogterom, J., T. B. Van Wimersma Greidanus, et al. (1977). "Evidence for the release of vasopressin and oxytocin into cerebrospinal fluid: measurements in plasma and CSF of intact and hypophysectomized rats." Neuroendocrinology **24**(2): 108-118.

Dohmann, H. F., E. C. Perin, et al. (2005). "Transendocardial autologous bone marrow mononuclear cell injection in ischemic heart failure: postmortem anatomicopathologic and immunohistochemical findings." Circulation **112**(4): 521-526.

Dolnikov, K., M. Shilkrut, et al. (2006). "Functional properties of human embryonic stem cell-derived cardiomyocytes: intracellular Ca2+ handling and the role of sarcoplasmic reticulum in the contraction." Stem Cells **24**(2): 236-245.

Du Vigneaud, V., C. Ressler, et al. (1953). "The sequence of amino acids in oxytocin, with a proposal for the structure of oxytocin." J Biol Chem **205**(2): 949-957.

Dubois-Dauphin, M., P. Pevet, et al. (1992). "Localization of binding sites for oxytocin in the brain of the golden hamster." Neuroreport **3**(9): 797-800.

Elands, J., A. Resink, et al. (1988). "Oxytocin receptors in the rat thymic gland." Eur J Pharmacol **151**(2): 345-346.

Elrod, J. W., J. J. Greer, et al. (2006). "Cardiomyocyte-specific overexpression of NO synthase-3 protects against myocardial ischemia-reperfusion injury." Arterioscler Thromb Vasc Biol **26**(7): 1517-1523.

Engelmann, M. G., H. D. Theiss, et al. (2006). "Autologous bone marrow stem cell mobilization induced by granulocyte colony-stimulating factor after subacute ST-segment elevation myocardial infarction undergoing late revascularization: final results from the G-CSF-STEMI (Granulocyte Colony-Stimulating Factor ST-Segment Elevation Myocardial Infarction) trial." J Am Coll Cardiol **48**(8): 1712-1721.

Evans, J. J. (1996). "Oxytocin and the control of LH." J Endocrinol **151**(2): 169-174.

Evora, P. R., P. J. Pearson, et al. (2003). "Effect of arginine vasopressin on the canine epicardial coronary artery: experiments on V1-receptor-mediated production of nitric oxide." Arq Bras Cardiol **80**(5): 483-494.

Ezekowitz, J. A. and P. Kaul (2010). "The epidemiology and management of elderly patients with myocardial infarction or heart failure." Heart Fail Rev.

Fabian, M., M. L. Forsling, et al. (1969). "The clearance and antidiuretic potency of neurohypophysial hormones in man, and their plasma binding and stability." J Physiol **204**(3): 653-668.

Favaretto, A. L., G. O. Ballejo, et al. (1997). "Oxytocin releases atrial natriuretic peptide from rat atria in vitro that exerts negative inotropic and chronotropic action." Peptides **18**(9): 1377-1381.

Fijnvandraat, A. C., R. H. Lekanne Deprez, et al. (2003). "TBX5 overexpression stimulates differentiation of chamber myocardium in P19C16 embryonic carcinoma cells." J Muscle Res Cell Motil **24**(2-3): 211-218.

Fijnvandraat, A. C., A. C. van Ginneken, et al. (2003). "Cardiomyocytes derived from embryonic stem cells resemble cardiomyocytes of the embryonic heart tube." Cardiovasc Res **58**(2): 399-409.

Fijnvandraat, A. C., A. C. van Ginneken, et al. (2003). "Cardiomyocytes purified from differentiated embryonic stem cells exhibit characteristics of early chamber myocardium." J Mol Cell Cardiol **35**(12): 1461-1472.

Forsling, M. L. (2000). "Diurnal rhythms in neurohypophysial function." Exp Physiol **85 Spec No**: 179S-186S.

Forsling, M. L., J. M. Judah, et al. (1994). "The effect of vasopressin and oxytocin on glomerular filtration rate in the conscious rat: contribution to the natriuretic response." J Endocrinol **141**(1): 59-67.

Forstermann, U., J. P. Boissel, et al. (1998). "Expressional control of the 'constitutive' isoforms of nitric oxide synthase (NOS I and NOS III)." FASEB J **12**(10): 773-790.

Freis, E. D., H. W. Schnaper, et al. (1952). "Hemodynamic alterations in acute myocardial infarction. I. Cardiac output, mean arterial pressure, total peripheral resistance, central and total blood volumes, venous pressure and average circulation time." J Clin Invest **31**(2): 131-140.

Freyman, T., G. Polin, et al. (2006). "A quantitative, randomized study evaluating three methods of mesenchymal stem cell delivery following myocardial infarction." Eur Heart J **27**(9): 1114-1122.

Fuchs, A. R., F. Fuchs, et al. (1984). "Oxytocin receptors in the human uterus during pregnancy and parturition." Am J Obstet Gynecol **150**(6): 734-741.

Fuchs, A. R., R. Romero, et al. (1991). "Oxytocin secretion and human parturition: pulse frequency and duration increase during spontaneous labor in women." Am J Obstet Gynecol **165**(5 Pt 1): 1515-1523.

Fukuda, K. (2002). "Reprogramming of bone marrow mesenchymal stem cells into cardiomyocytes." C R Biol **325**(10): 1027-1038.

Gaillard, T., A. Mulsch, et al. (1991). "Regulation of nitric oxide production by stimulated rat Kupffer cells." Pathobiology **59**(4): 280-283.

Garlid, K. D., P. Paucek, et al. (1996). "The mitochondrial KATP channel as a receptor for potassium channel openers." J Biol Chem **271**(15): 8796-8799.

Gassanov, N., M. Jankowski, et al. (2007). "Arginine vasopressin-mediated cardiac differentiation: insights into the role of its receptors and nitric oxide signaling." J Biol Chem **282**(15): 11255-11265.

Geenen, V., J. J. Legros, et al. (1986). "The neuroendocrine thymus: coexistence of oxytocin and neurophysin in the human thymus." Science **232**(4749): 508-511.

Genovese, J. A., C. Spadaccio, et al. (2009). "Electrostimulated bone marrow human mesenchymal stem cells produce follistatin." Cytotherapy **11**(4): 448-456.

Gimpl, G. and F. Fahrenholz (2001). "The oxytocin receptor system: structure, function, and regulation." Physiol Rev **81**(2): 629-683.

Gladwin, M. T., N. J. Raat, et al. (2006). "Nitrite as a vascular endocrine nitric oxide reservoir that contributes to hypoxic signaling, cytoprotection, and vasodilation." Am J Physiol Heart Circ Physiol **291**(5): H2026-2035.

Gonzales, C. and T. Pedrazzini (2009). "Progenitor cell therapy for heart disease." Exp Cell Res **315**(18): 3077-3085.

Goodell, M. A., K. Brose, et al. (1996). "Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo." J Exp Med **183**(4): 1797-1806.

Gouin, J. P., C. S. Carter, et al. (2010). "Marital behavior, oxytocin, vasopressin, and wound healing." Psychoneuroendocrinology.

Grepin, C., G. Nemer, et al. (1997). "Enhanced cardiogenesis in embryonic stem cells overexpressing the GATA-4 transcription factor." Development **124**(12): 2387-2395.

Guan, K., J. Rohwedel, et al. (1999). "Embryonic stem cell differentiation models: cardiogenesis, myogenesis, neurogenesis, epithelial and vascular smooth muscle cell differentiation in vitro." Cytotechnology **30**(1-3): 211-226.

Gutkowska, J., M. Jankowski, et al. (1997). "Oxytocin releases atrial natriuretic peptide by combining with oxytocin receptors in the heart." Proc Natl Acad Sci U S A **94**(21): 11704-11709.

Gutkowska, J., M. Jankowski, et al. (2000). "Oxytocin is a cardiovascular hormone." Braz J Med Biol Res **33**(6): 625-633.

Guzzi, F., D. Zanchetta, et al. (2002). "Localization of the human oxytocin receptor in caveolin-1 enriched domains turns the receptor-mediated inhibition of cell growth into a proliferative response." Oncogene **21**(11): 1658-1667.

Haanwinckel, M. A., L. K. Elias, et al. (1995). "Oxytocin mediates atrial natriuretic peptide release and natriuresis after volume expansion in the rat." Proc Natl Acad Sci U S A **92**(17): 7902-7906.

Hardy, C. L. (1995). "The homing of hematopoietic stem cells to the bone marrow." Am J Med Sci **309**(5): 260-266.

Harris, P. (1994). "The problem of defining heart failure." Cardiovasc Drugs Ther **8**(3): 447-452.

Hataishi, R., A. C. Rodrigues, et al. (2006). "Inhaled nitric oxide decreases infarction size and improves left ventricular function in a murine model of myocardial ischemia-reperfusion injury." Am J Physiol Heart Circ Physiol **291**(1): H379-384.

Hatami, L., M. R. Valojerdi, et al. (2007). "Effects of oxytocin on cardiomyocyte differentiation from mouse embryonic stem cells." Int J Cardiol **117**(1): 80-89.

Herzog, R. W., O. Cao, et al. (2010). "Two decades of clinical gene therapy--success is finally mounting." Discov Med **9**(45): 105-111.

Hibbs, J. B., Jr., R. R. Taintor, et al. (1988). "Nitric oxide: a cytotoxic activated macrophage effector molecule." Biochem Biophys Res Commun **157**(1): 87-94.

Hierlihy, A. M., P. Seale, et al. (2002). "The post-natal heart contains a myocardial stem cell population." FEBS Lett **530**(1-3): 239-243.

Higa, K. T., E. Mori, et al. (2002). "Baroreflex control of heart rate by oxytocin in the solitary-vagal complex." Am J Physiol Regul Integr Comp Physiol **282**(2): R537-545.

Higuchi, T., K. Honda, et al. (1985). "Release of oxytocin during suckling and parturition in the rat." J Endocrinol **105**(3): 339-346.

Hirata, Y., H. Hayakawa, et al. (1997). "Receptor subtype for vasopressin-induced release of nitric oxide from rat kidney." Hypertension **29**(1 Pt 1): 58-64.

Hodgson, D. M., A. Behfar, et al. (2004). "Stable benefit of embryonic stem cell therapy in myocardial infarction." Am J Physiol Heart Circ Physiol **287**(2): H471-479.

Holda, J. R., C. Oberti, et al. (1996). "Characterization of an oxytocin-induced rise in [Ca2+]i in single human myometrium smooth muscle cells." Cell Calcium **20**(1): 43-51.

Holmes, C. L., D. W. Landry, et al. (2003). "Science review: Vasopressin and the cardiovascular system part 1--receptor physiology." Crit Care **7**(6): 427-434.

Homeida, A. M. (1986). "Evidence for the presence of oxytocin in the corpus luteum of the goat." Br J Pharmacol **87**(4): 673-676.

Homeida, A. M. and R. G. Cooke (1984). "Biological half-life of oxytocin in the goat." Res Vet Sci **37**(3): 364-365.

Hong, E. J., S. H. Park, et al. (2006). "Identification of estrogen-regulated genes by microarray analysis of the uterus of immature rats exposed to endocrine disrupting chemicals." Reprod Biol Endocrinol **4**: 49.

Hu, H., N. Chiamvimonvat, et al. (1997). "Direct inhibition of expressed cardiac L-type Ca2+ channels by S-nitrosothiol nitric oxide donors." Circ Res **81**(5): 742-752.

Hu, J., T. E. Ludwig, et al. (2001). "Autocrine/paracrine action of oxytocin in pig endometrium." Biol Reprod **64**(6): 1682-1688.

Ifkovits, J. L., J. J. Devlin, et al. (2009). "Biodegradable fibrous scaffolds with tunable properties formed from photo-cross-linkable poly(glycerol sebacate)." ACS Appl Mater Interfaces **1**(9): 1878-1886.

Ignarro, L. J. (1990). "Biosynthesis and metabolism of endothelium-derived nitric oxide." Annu Rev Pharmacol Toxicol **30**: 535-560.

Igura, K., X. Zhang, et al. (2004). "Isolation and characterization of mesenchymal progenitor cells from chorionic villi of human placenta." Cytotherapy **6**(6): 543-553.

Illmensee, K. and B. Mintz (1976). "Totipotency and normal differentiation of single teratocarcinoma cells cloned by injection into blastocysts." Proc Natl Acad Sci U S A **73**(2): 549-553.

Insel, T. R. and L. E. Shapiro (1992). "Oxytocin receptor distribution reflects social organization in monogamous and polygamous voles." Proc Natl Acad Sci U S A **89**(13): 5981-5985.

Iso, Y., J. L. Spees, et al. (2007). "Multipotent human stromal cells improve cardiac function after myocardial infarction in mice without long-term engraftment." Biochem Biophys Res Commun **354**(3): 700-706.

Ivell, R. and D. Richter (1984). "The gene for the hypothalamic peptide hormone oxytocin is highly expressed in the bovine corpus luteum: biosynthesis, structure and sequence analysis." EMBO J **3**(10): 2351-2354.

Izadpanah, R., C. Trygg, et al. (2006). "Biologic properties of mesenchymal stem cells derived from bone marrow and adipose tissue." J Cell Biochem **99**(5): 1285-1297.

Jalving, M. and H. Schepers (2009). "Induced pluripotent stem cells: will they be safe?" Curr Opin Mol Ther **11**(4): 383-393.

Jankowski, M., V. Bissonauth, et al. (2010). "Anti-inflammatory effect of oxytocin in rat myocardial infarction." Basic Res Cardiol **105**(2): 205-218.

Jankowski, M., F. Hajjar, et al. (1998). "Rat heart: a site of oxytocin production and action." Proc Natl Acad Sci U S A **95**(24): 14558-14563.

Jankowski, M., D. Wang, et al. (2000). "Oxytocin and its receptors are synthesized in the rat vasculature." Proc Natl Acad Sci U S A **97**(11): 6207-6211.

Jansakul, C., R. G. King, et al. (1989). "Plasma levels of atrial natriuretic peptides during pregnancy and post partum in the rat." J Endocrinol **120**(1): 113-117.

Jarry, H., A. Einspanier, et al. (1990). "Release and effects of oxytocin on estradiol and progesterone secretion in porcine corpora lutea as measured by an in vivo microdialysis system." Endocrinology **126**(5): 2350-2358.

Jones, P. M. and I. C. Robinson (1982). "Differential clearance of neurophysin and neurohypophysial peptides from the cerebrospinal fluid in conscious guinea pigs." Neuroendocrinology **34**(4): 297-302.

Jones, P. M., I. C. Robinson, et al. (1983). "Release of oxytocin into blood and cerebrospinal fluid by electrical stimulation of the hypothalamus or neural lobe in the rat." Neuroendocrinology **37**(6): 454-458.

Jones, S. P., W. G. Girod, et al. (1999). "Myocardial ischemia-reperfusion injury is exacerbated in absence of endothelial cell nitric oxide synthase." Am J Physiol **276**(5 Pt 2): H1567-1573.

Jones, S. P., J. J. Greer, et al. (2004). "Endothelial nitric oxide synthase overexpression attenuates myocardial reperfusion injury." Am J Physiol Heart Circ Physiol **286**(1): H276-282.

Kang, H. J., H. S. Kim, et al. (2004). "Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: the MAGIC cell randomised clinical trial." Lancet **363**(9411): 751-756.

Kanno, S., P. K. Kim, et al. (2004). "Nitric oxide facilitates cardiomyogenesis in mouse embryonic stem cells." Proc Natl Acad Sci U S A **101**(33): 12277-12281.

Katusic, Z. S., J. T. Shepherd, et al. (1986). "Oxytocin causes endothelium-dependent relaxations of canine basilar arteries by activating V1-vasopressinergic receptors." J Pharmacol Exp Ther **236**(1): 166-170.

Kawada, H., J. Fujita, et al. (2004). "Nonhematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction." Blood **104**(12): 3581-3587.

Kawai, T., T. Takahashi, et al. (2004). "Efficient cardiomyogenic differentiation of embryonic stem cell by fibroblast growth factor 2 and bone morphogenetic protein 2." Circ J **68**(7): 691-702.

Kelm, M. (1999). "Nitric oxide metabolism and breakdown." Biochim Biophys Acta **1411**(2-3): 273-289.

Kenchaiah, S., J. Narula, et al. (2004). "Risk factors for heart failure." Med Clin North Am **88**(5): 1145-1172.

Khan-Dawood, F. S., J. C. Huang, et al. (1988). "Baboon corpus luteum oxytocin: an intragonadal peptide modulator of luteal function." Am J Obstet Gynecol **158**(4): 882-891.

Khan-Dawood, F. S., E. L. Marut, et al. (1984). "Oxytocin in the corpus luteum of the cynomolgus monkey (Macaca fascicularis)." Endocrinology **115**(2): 570-574.

Khan, M., S. Mohsin, et al. (2009). "Repair of senescent myocardium by mesenchymal stem cells is dependent on the age of donor mice." J Cell Mol Med.

Kim, Y. M., M. E. de Vera, et al. (1997). "Nitric oxide protects cultured rat hepatocytes from tumor necrosis factor-alpha-induced apoptosis by inducing heat shock protein 70 expression." J Biol Chem **272**(2): 1402-1411.

Kimura, T., Y. Makino, et al. (1994). "Molecular characterization of a cloned human oxytocin receptor." Eur J Endocrinol **131**(4): 385-390.

Kimura, T., O. Tanizawa, et al. (1992). "Structure and expression of a human oxytocin receptor." Nature **356**(6369): 526-529.

Kinnaird, T., E. Stabile, et al. (2004). "Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms." Circ Res **94**(5): 678-685.

Klug, M. G., M. H. Soonpaa, et al. (1995). "DNA synthesis and multinucleation in embryonic stem cell-derived cardiomyocytes." Am J Physiol **269**(6 Pt 2): H1913-1921.

Kobayashi, H., S. Yasuda, et al. (2009). "Postinfarct treatment with oxytocin improves cardiac function and remodeling via activating cell-survival signals and angiogenesis." J Cardiovasc Pharmacol **54**(6): 510-519.

Kolossov, E., B. K. Fleischmann, et al. (1998). "Functional characteristics of ES cell-derived cardiac precursor cells identified by tissue-specific expression of the green fluorescent protein." J Cell Biol **143**(7): 2045-2056.

Kovacic, J. C., R. P. Harvey, et al. (2007). "Cardiovascular regenerative medicine: digging in for the long haul." Cell Stem Cell **1**(6): 628-633.

Krumenacker, J. S., S. Katsuki, et al. (2006). "Differential expression of genes involved in cGMP-dependent nitric oxide signaling in murine embryonic stem (ES) cells and ES cell-derived cardiomyocytes." Nitric Oxide **14**(1): 1-11.

Ku, C. Y., A. Qian, et al. (1995). "Oxytocin stimulates myometrial guanosine triphosphatase and phospholipase-C activities via coupling to G alpha q/11." Endocrinology **136**(4): 1509-1515.

Kurpisz, M., R. Czepczynski, et al. (2007). "Bone marrow stem cell imaging after intracoronary administration." Int J Cardiol **121**(2): 194-195.

Landgraf, R., I. Neumann, et al. (1992). "Push-pull perfusion and microdialysis studies of central oxytocin and vasopressin release in freely moving rats during pregnancy, parturition, and lactation." Ann N Y Acad Sci **652**: 326-339.

Leake, R. D., R. E. Weitzman, et al. (1981). "Plasma oxytocin concentrations in men, nonpregnant women, and pregnant women before and during spontaneous labor." J Clin Endocrinol Metab **53**(4): 730-733.

LeBlanc PP, A. J., Besner JG, Buri P, Lesne M. (1997). Traité de Biopharmacie et Pharmacocinétique. Paris, France.

Lee, S. H., P. L. Wolf, et al. (2000). "Early expression of angiogenesis factors in acute myocardial ischemia and infarction." N Engl J Med **342**(9): 626-633.

Leng, G., C. H. Brown, et al. (2001). "Responses of magnocellular neurons to osmotic stimulation involves coactivation of excitatory and inhibitory input: an experimental and theoretical analysis." J Neurosci **21**(17): 6967-6977.

Leng, G., C. H. Brown, et al. (1999). "Physiological pathways regulating the activity of magnocellular neurosecretory cells." Prog Neurobiol **57**(6): 625-655.

Li, H., H. Cui, et al. (2008). "Nitric oxide production from nitrite occurs primarily in tissues not in the blood: critical role of xanthine oxidase and aldehyde oxidase." J Biol Chem **283**(26): 17855-17863.

Li, J. and T. R. Billiar (1999). "The anti-apoptotic actions of nitric oxide in hepatocytes." Cell Death Differ **6**(10): 952-955.

Li, X. H., X. Y. Yu, et al. (2008). "[Human bone marrow mesenchymal stem cells cocultured with semi-permeable membrane separated neonatal rat ventricular myocytes differentiated into cardiomyocyte phenotype]." Zhonghua Xin Xue Guan Bing Za Zhi **36**(7): 651-654.

Li, Z., J. Guo, et al. (2009). "Paracrine role for mesenchymal stem cells in acute myocardial infarction." Biol Pharm Bull **32**(8): 1343-1346.

Liang, S. X., T. Y. Tan, et al. (2010). "Differentiation and migration of Sca1+/CD31- cardiac side population cells in a murine myocardial ischemic model." Int J Cardiol **138**(1): 40-49.

Lindow, S. W., A. Newham, et al. (1996). "The 24-hour rhythm of oxytocin and beta-endorphin secretion in human pregnancy." Clin Endocrinol (Oxf) **45**(4): 443-446.

Lipinski, M. J., G. G. Biondi-Zoccai, et al. (2007). "Impact of intracoronary cell therapy on left ventricular function in the setting of acute myocardial infarction: a collaborative systematic review and meta-analysis of controlled clinical trials." J Am Coll Cardiol **50**(18): 1761-1767.

Lippert, T. H., A. O. Mueck, et al. (2003). "Effects of oxytocin outside pregnancy." Horm Res **60**(6): 262-271.

Liu, J., D. K. Lieu, et al. (2009). "Facilitated maturation of Ca2+ handling properties of human embryonic stem cell-derived cardiomyocytes by calsequestrin expression." Am J Physiol Cell Physiol **297**(1): C152-159.

Lloyd-Jones, D. M., J. C. Evans, et al. (2002). "Treatment and control of hypertension in the community: a prospective analysis." Hypertension **40**(5): 640-646.

Lobo, E. D., R. J. Hansen, et al. (2004). "Antibody pharmacokinetics and pharmacodynamics." J Pharm Sci **93**(11): 2645-2668.

Loichot, C., J. P. Krieger, et al. (2001). "High concentrations of oxytocin cause vasoconstriction by activating vasopressin V1A receptors in the isolated perfused rat kidney." Naunyn Schmiedebergs Arch Pharmacol **363**(4): 369-375.

Loke, K. E., S. K. Laycock, et al. (1999). "Nitric oxide modulates mitochondrial respiration in failing human heart." Circulation **100**(12): 1291-1297.

Loscalzo, J. and G. Welch (1995). "Nitric oxide and its role in the cardiovascular system." Prog Cardiovasc Dis **38**(2): 87-104.

Loup, F., E. Tribollet, et al. (1991). "Localization of high-affinity binding sites for oxytocin and vasopressin in the human brain. An autoradiographic study." Brain Res **555**(2): 220-232.

Lundin, S., A. Broeders, et al. (1993). "Pharmacokinetic and pharmacologic properties of antiuterotonic oxytocin analogs in the rat." J Pharmacol Exp Ther **264**(2): 783-788.

Ma, X. L., A. S. Weyrich, et al. (1993). "Diminished basal nitric oxide release after myocardial ischemia and reperfusion promotes neutrophil adherence to coronary endothelium." Circ Res **72**(2): 403-412.

Maenhaut, C., J. E. Dumont, et al. (2010). "Cancer stem cells: a reality, a myth, a fuzzy concept or a misnomer? An analysis." Carcinogenesis **31**(2): 149-158.

Magrinat, G., S. N. Mason, et al. (1992). "Nitric oxide modulation of human leukemia cell differentiation and gene expression." Blood **80**(8): 1880-1884.

Mahia, J., A. Bernal, et al. (2009). "The natriuretic effect of oxytocin blocks medial tuberomammillary polydipsia and polyuria in male rats." Eur J Neurosci **29**(7): 1440-1446.

Makino, S., K. Fukuda, et al. (1999). "Cardiomyocytes can be generated from marrow stromal cells in vitro." J Clin Invest **103**(5): 697-705.

Maltsev, V. A., J. Rohwedel, et al. (1993). "Embryonic stem cells differentiate in vitro into cardiomyocytes representing sinusnodal, atrial and ventricular cell types." Mech Dev **44**(1): 41-50.

Martin, C. M., A. P. Meeson, et al. (2004). "Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart." Dev Biol **265**(1): 262-275.

Matsuura, K., T. Nagai, et al. (2004). "Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes." J Biol Chem **279**(12): 11384-11391.

McCann, S. M., J. Antunes-Rodrigues, et al. (2002). "Oxytocin, vasopressin and atrial natriuretic peptide control body fluid homeostasis by action on their receptors in brain, cardiovascular system and kidney." Prog Brain Res **139**: 309-328.

McCann, S. M., J. Gutkowska, et al. (2003). "Neuroendocrine control of body fluid homeostasis." Braz J Med Biol Res **36**(2): 165-181.

McEwen, B. B. (2004). "General introduction to vasopressin and oxytocin: structure/metabolism, evolutionary aspects, neural pathway/receptor distribution, and functional aspects relevant to memory processing." Adv Pharmacol **50**: 1-50, 655-708.

Mendez-Bauer CJ, C. M., Cabot HM, Negreiros de Paiva CE, Gonzalez-Panizza VH. (1961). Studies on plasma oxytocinase. In Oxytocin. London, Editor CALDEYRO-BARCIA, R. & HELLER, H.,.

Meyer, G. P., K. C. Wollert, et al. (2009). "Intracoronary bone marrow cell transfer after myocardial infarction: 5-year follow-up from the randomized-controlled BOOST trial." Eur Heart J **30**(24): 2978-2984.

Miller-Hance, W. C. and K. R. Chien (1993). "Of mouse and man: molecular switches that control cardiac growth and development." Heart Dis Stroke **2**(2): 157-165.

Miller, M. E., S. T. Davidge, et al. (2002). "Oxytocin does not directly affect vascular tone in vessels from nonpregnant and pregnant rats." Am J Physiol Heart Circ Physiol **282**(4): H1223-1228.

Moncada, S., R. M. Palmer, et al. (1991). "Nitric oxide: physiology, pathophysiology, and pharmacology." Pharmacol Rev **43**(2): 109-142.

Morali, O. G., A. Jouneau, et al. (2000). "IGF-II promotes mesoderm formation." Dev Biol **227**(1): 133-145.

Morawska-Barszczewska, J., J. W. Guzek, et al. (1996). "Cholecystokinin octapeptide and the daily rhythm of vasopressin and oxytocin release." Exp Clin Endocrinol Diabetes **104**(2): 164-171.

Moreno-Ortiz, H., C. Esteban-Perez, et al. (2009). "Isolation and derivation of mouse embryonic germinal cells." J Vis Exp(32).

Morin, V., J. R. Del Castillo, et al. (2008). "Evidence for non-linear pharmacokinetics of oxytocin in anesthetizetized rat." J Pharm Pharm Sci **11**(4): 12-24.

Morita, Y., H. Ema, et al. (2006). "Non-side-population hematopoietic stem cells in mouse bone marrow." Blood **108**(8): 2850-2856.

Mujoo, K., J. S. Krumenacker, et al. (2006). "Differential expression of nitric oxide signaling components in undifferentiated and differentiated human embryonic stem cells." Stem Cells Dev **15**(6): 779-787.

Mukaddam-Daher, S., Y. L. Yin, et al. (2001). "Negative inotropic and chronotropic effects of oxytocin." Hypertension **38**(2): 292-296.

Muller, M., M. S. Soloff, et al. (1989). "Photoaffinity labelling of the oxytocin receptor in plasma membranes from rat mammary gland." FEBS Lett **242**(2): 333-336.

Murry, C. E., M. H. Soonpaa, et al. (2004). "Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts." Nature **428**(6983): 664-668.

Nadel, A. S., B. J. Ballermann, et al. (1988). "Interrelationships among atrial peptides, renin, and blood volume in pregnant rats." Am J Physiol **254**(5 Pt 2): R793-800.

Nagaya, N., T. Fujii, et al. (2004). "Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis." Am J Physiol Heart Circ Physiol **287**(6): H2670-2676.

Nagy, A., E. Gocza, et al. (1990). "Embryonic stem cells alone are able to support fetal development in the mouse." Development **110**(3): 815-821.

Nakamura, H., A. Itakuara, et al. (2000). "Oxytocin stimulates the translocation of oxytocinase of human vascular endothelial cells via activation of oxytocin receptors." Endocrinology **141**(12): 4481-4485.

Nakamuta, J. S., M. E. Danoviz, et al. (2009). "Cell therapy attenuates cardiac dysfunction post myocardial infarction: effect of timing, routes of injection and a fibrin scaffold." PLoS One **4**(6): e6005.

Neumann, I., A. J. Douglas, et al. (1996). "Oxytocin released within the supraoptic nucleus of the rat brain by positive feedback action is involved in parturition-related events." J Neuroendocrinol **8**(3): 227-233.

Nishimori, K., L. J. Young, et al. (1996). "Oxytocin is required for nursing but is not essential for parturition or reproductive behavior." Proc Natl Acad Sci U S A **93**(21): 11699-11704.

Nussbaum, J., E. Minami, et al. (2007). "Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response." FASEB J **21**(7): 1345-1357.

Nygren, J. M., S. Jovinge, et al. (2004). "Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation." Nat Med **10**(5): 494-501.

Oh, H., S. B. Bradfute, et al. (2003). "Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction." Proc Natl Acad Sci U S A **100**(21): 12313-12318.

Ohn, J., H. J. Tsai, et al. (2009). "Joint dynamic imaging of morphogenesis and function in the developing heart." Organogenesis **5**(4): 166-173.

Ondrejcakova, M., T. Ravingerova, et al. (2009). "Oxytocin exerts protective effects on in vitro myocardial injury induced by ischemia and reperfusion." Can J Physiol Pharmacol **87**(2): 137-142.

Orlic, D., J. Kajstura, et al. (2001). "Transplanted adult bone marrow cells repair myocardial infarcts in mice." Ann N Y Acad Sci **938**: 221-229; discussion 229-230.

Orlic, D., J. Kajstura, et al. (2001). "Mobilized bone marrow cells repair the infarcted heart, improving function and survival." Proc Natl Acad Sci U S A **98**(18): 10344-10349.

Ostrowski, N. L. and S. J. Lolait (1995). "Oxytocin receptor gene expression in female rat kidney. The effect of estrogen." Adv Exp Med Biol **395**: 329-340.

Overgaard, M., R. S. Ripa, et al. (2009). "Timing of granulocyte-colony stimulating factor treatment after acute myocardial infarction and recovery of left ventricular function: Results from the STEMMI trial." Int J Cardiol.

Oyama, T., T. Nagai, et al. (2007). "Cardiac side population cells have a potential to migrate and differentiate into cardiomyocytes in vitro and in vivo." J Cell Biol **176**(3): 329-341.

Pabla, R. and M. J. Curtis (1996). "Effect of endogenous nitric oxide on cardiac systolic and diastolic function during ischemia and reperfusion in the rat isolated perfused heart." J Mol Cell Cardiol **28**(10): 2111-2121.

Palazzo, A. J., S. P. Jones, et al. (1998). "Coronary endothelial P-selectin in pathogenesis of myocardial ischemia-reperfusion injury." Am J Physiol **275**(5 Pt 2): H1865-1872.

Palmer, R. M., D. S. Ashton, et al. (1988). "Vascular endothelial cells synthesize nitric oxide from L-arginine." Nature **333**(6174): 664-666.

Paquin, J., B. A. Danalache, et al. (2002). "Oxytocin induces differentiation of P19 embryonic stem cells to cardiomyocytes." Proc Natl Acad Sci U S A **99**(14): 9550-9555.

Parisi, S., D. D'Andrea, et al. (2003). "Nodal-dependent Cripto signaling promotes cardiomyogenesis and redirects the neural fate of embryonic stem cells." J Cell Biol **163**(2): 303-314.

Park, C. S., R. Park, et al. (1996). "Constitutive expression and structural diversity of inducible isoform of nitric oxide synthase in human tissues." Life Sci **59**(3): 219-225.

Perino, M. G., S. Yamanaka, et al. (2008). "Cardiomyogenic stem and progenitor cell plasticity and the dissection of cardiopoiesis." J Mol Cell Cardiol **45**(4): 475-494.

Perry, R. L., A. J. Satin, et al. (1996). "The pharmacokinetics of oxytocin as they apply to labor induction." Am J Obstet Gynecol **174**(5): 1590-1593.

Petersson, M. (2002). "Cardiovascular effects of oxytocin." Prog Brain Res **139**: 281-288.

Petersson, M., P. Alster, et al. (1996). "Oxytocin causes a long-term decrease of blood pressure in female and male rats." Physiol Behav **60**(5): 1311-1315.

Petersson, M., T. Lundeberg, et al. (1999). "Short-term increase and long-term decrease of blood pressure in response to oxytocin-potentiating effect of female steroid hormones." J Cardiovasc Pharmacol **33**(1): 102-108.

Petty, M. A., R. E. Lang, et al. (1985). "The cardiovascular effects of oxytocin in conscious male rats." Eur J Pharmacol **112**(2): 203-210.

Peunova, N. and G. Enikolopov (1995). "Nitric oxide triggers a switch to growth arrest during differentiation of neuronal cells." Nature **375**(6526): 68-73.

Pierdomenico, L., L. Bonsi, et al. (2005). "Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp." Transplantation **80**(6): 836-842.

Pittenger, M. F., A. M. Mackay, et al. (1999). "Multilineage potential of adult human mesenchymal stem cells." Science **284**(5411): 143-147.

Pomblum, V. J., B. Korbmacher, et al. (2010). "Cardiac stunning in the clinic: the full picture." Interact Cardiovasc Thorac Surg **10**(1): 86-91.

Poulain, D. A. and J. B. Wakerley (1982). "Electrophysiology of hypothalamic magnocellular neurones secreting oxytocin and vasopressin." Neuroscience **7**(4): 773-808.

Radi, R., J. S. Beckman, et al. (1991). "Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide." J Biol Chem **266**(7): 4244-4250.

Rakhit, R. D., M. H. Mojet, et al. (2001). "Mitochondria as targets for nitric oxide-induced protection during simulated ischemia and reoxygenation in isolated neonatal cardiomyocytes." Circulation **103**(21): 2617-2623.

Rangappa, S., J. W. Entwistle, et al. (2003). "Cardiomyocyte-mediated contact programs human mesenchymal stem cells to express cardiogenic phenotype." J Thorac Cardiovasc Surg **126**(1): 124-132.

Rao, V. V., C. Loffler, et al. (1992). "The human gene for oxytocin-neurophysin I (OXT) is physically mapped to chromosome 20p13 by in situ hybridization." Cytogenet Cell Genet **61**(4): 271-273.

Rasmussen, M. S., J. A. Simonsen, et al. (2004). "Effects of oxytocin in normal man during low and high sodium diets." Acta Physiol Scand **181**(2): 247-257.

Rauk, P. N. and U. Friebe-Hoffmann (2000). "Interleukin-1 beta down-regulates the oxytocin receptor in cultured uterine smooth muscle cells." Am J Reprod Immunol **43**(2): 85-91.

Rebelatto, C. K., A. M. Aguiar, et al. (2009). "Expression of cardiac function genes in adult stem cells is increased by treatment with nitric oxide agents." Biochem Biophys Res Commun **378**(3): 456-461.

Reinecke, H., E. Minami, et al. (2008). "Cardiogenic differentiation and transdifferentiation of progenitor cells." Circ Res **103**(10): 1058-1071.

Renaud, L. P. and C. W. Bourque (1991). "Neurophysiology and neuropharmacology of hypothalamic magnocellular neurons secreting vasopressin and oxytocin." Prog Neurobiol **36**(2): 131-169.

Richter, O. N., K. Kubler, et al. (2004). "Oxytocin receptor gene expression of estrogen-stimulated human myometrium in extracorporeally perfused non-pregnant uteri." Mol Hum Reprod **10**(5): 339-346.

Robertson, J. A. (2001). "Human embryonic stem cell research: ethical and legal issues." Nat Rev Genet **2**(1): 74-78.

Rolletschek, A. and A. M. Wobus (2009). "Induced human pluripotent stem cells: promises and open questions." Biol Chem **390**(9): 845-849.

Rose, J. P., C. K. Wu, et al. (1996). "Crystal structure of the neurophysin-oxytocin complex." Nat Struct Biol **3**(2): 163-169.

Rota, M., J. Kajstura, et al. (2007). "Bone marrow cells adopt the cardiomyogenic fate in vivo." Proc Natl Acad Sci U S A **104**(45): 17783-17788.

Rotzinger, S., D. A. Lovejoy, et al. (2009). "Behavioral effects of neuropeptides in rodent models of depression and anxiety." Peptides.

Rubart, M. and L. J. Field (2006). "Cell-based approaches for cardiac repair." Ann N Y Acad Sci **1080**: 34-48.

Rudnicki, M. A., G. Jackowski, et al. (1990). "Actin and myosin expression during development of cardiac muscle from cultured embryonal carcinoma cells." Dev Biol **138**(2): 348-358.

Rudy-Reil, D. and J. Lough (2004). "Avian precardiac endoderm/mesoderm induces cardiac myocyte differentiation in murine embryonic stem cells." Circ Res **94**(12): e107-116.

Samson, W. K. and D. A. Schell (1995). "Oxytocin and the anterior pituitary gland." Adv Exp Med Biol **395**: 355-364.

Sauer, H., W. Neukirchen, et al. (2004). "Involvement of reactive oxygen species in cardiotrophin-1-induced proliferation of cardiomyocytes differentiated from murine embryonic stem cells." Exp Cell Res **294**(2): 313-324.

Saxena, A., J. E. Fish, et al. (2008). "Stromal cell-derived factor-1alpha is cardioprotective after myocardial infarction." Circulation **117**(17): 2224-2231.

Schmid, B., S. Wong, et al. (2001). "Transcriptional regulation of oxytocin receptor by interleukin-1beta and interleukin-6." Endocrinology **142**(4): 1380-1385.

Schmidt, A., S. Jard, et al. (1990). "Oxytocin receptors in rat kidney during development." Am J Physiol **259**(6 Pt 2): F872-881.

Schmidt, M. M., K. Guan, et al. (2001). "Lithium influences differentiation and tissue-specific gene expression of mouse embryonic stem (ES) cells in vitro." Int J Dev Biol **45**(2): 421-429.

Schorscher-Petcu, A., A. Dupre, et al. (2009). "Distribution of vasopressin and oxytocin binding sites in the brain and upper spinal cord of the common marmoset." Neurosci Lett **461**(3): 217-222.

Seif-Naraghi, S. B., M. A. Salvatore, et al. (2010). "Design and Characterization of an Injectable Pericardial Matrix Gel: A Potentially Autologous Scaffold for Cardiac Tissue Engineering." Tissue Eng Part A.

Seitchik, J., J. Amico, et al. (1984). "Oxytocin augmentation of dysfunctional labor. IV. Oxytocin pharmacokinetics." Am J Obstet Gynecol **150**(3): 225-228.

Sernia, C., R. T. Gemmell, et al. (1989). "Oxytocin receptors in the ovine corpus luteum." J Endocrinol **121**(1): 117-123.

Shapiro, L. E. and T. R. Insel (1989). "Ontogeny of oxytocin receptors in rat forebrain: a quantitative study." Synapse **4**(3): 259-266.

Sharp, B. R., S. P. Jones, et al. (2002). "Differential response to myocardial reperfusion injury in eNOS-deficient mice." Am J Physiol Heart Circ Physiol **282**(6): H2422-2426.

Sheldrick, E. L. and A. P. Flint (1989). "Post-translational processing of oxytocin-neurophysin prohormone in the ovine corpus luteum: activity of peptidyl

glycine alpha-amidating mono-oxygenase and concentrations of its cofactor, ascorbic acid." J Endocrinol **122**(1): 313-322.

Siegfried, M. R., C. Carey, et al. (1992). "Beneficial effects of SPM-5185, a cysteine-containing NO donor in myocardial ischemia-reperfusion." Am J Physiol **263**(3 Pt 2): H771-777.

Singelyn, J. M., J. A. DeQuach, et al. (2009). "Naturally derived myocardial matrix as an injectable scaffold for cardiac tissue engineering." Biomaterials **30**(29): 5409-5416.

Singla, D. K., T. A. Hacker, et al. (2006). "Transplantation of embryonic stem cells into the infarcted mouse heart: formation of multiple cell types." J Mol Cell Cardiol **40**(1): 195-200.

Siu, C. W., J. C. Moore, et al. (2007). "Human embryonic stem cell-derived cardiomyocytes for heart therapies." Cardiovasc Hematol Disord Drug Targets **7**(2): 145-152.

Sjoquist, M., W. Huang, et al. (1999). "Sodium excretion and renin secretion after continuous versus pulsatile infusion of oxytocin in rats." Endocrinology **140**(6): 2814-2818.

Soares, T. J., T. M. Coimbra, et al. (1999). "Atrial natriuretic peptide and oxytocin induce natriuresis by release of cGMP." Proc Natl Acad Sci U S A **96**(1): 278-283.

Song, Y. S., H. J. Lee, et al. (2007). "Potential differentiation of human mesenchymal stem cell transplanted in rat corpus cavernosum toward endothelial or smooth muscle cells." Int J Impot Res **19**(4): 378-385.

Stein, D. J. (2009). "Oxytocin and vasopressin: social neuropeptides." CNS Spectr **14**(11): 602-606.

Stock, S., L. Granstrom, et al. (1989). "Elevated plasma levels of oxytocin in obese subjects before and after gastric banding." Int J Obes **13**(2): 213-222.

Stoeckel, M. E. and M. J. Freund-Mercier (1989). "Autoradiographic demonstration of oxytocin-binding sites in the macula densa." Am J Physiol **257**(2 Pt 2): F310-314.

Strauer, B. E., M. Brehm, et al. (2008). "The therapeutic potential of stem cells in heart disease." Cell Prolif **41 Suppl 1**: 126-145.

Strauer, B. E., M. Brehm, et al. (2002). "Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans." Circulation **106**(15): 1913-1918.

Stricker, E. M., J. A. Hosutt, et al. (1987). "Neurohypophyseal secretion in hypovolemic rats: inverse relation to sodium appetite." Am J Physiol **252**(5 Pt 2): R889-896.

Stuehr, D. J., H. J. Cho, et al. (1991). "Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein." Proc Natl Acad Sci U S A **88**(17): 7773-7777.

Sudou, A., H. Muramatsu, et al. (1997). "Le(X) structure enhances myocardial differentiation from embryonic stem cells." Cell Struct Funct **22**(2): 247-251.

Sun, J., C. Steenbergen, et al. (2006). "S-nitrosylation: NO-related redox signaling to protect against oxidative stress." Antioxid Redox Signal **8**(9-10): 1693-1705.

Swindle, M. M. (1984). "Swine as replacements for dogs in the surgical teaching and research laboratory." Lab Anim Sci **34**(4): 383-385.

Tahara, A., J. Tsukada, et al. (2000). "Pharmacologic characterization of the oxytocin receptor in human uterine smooth muscle cells." Br J Pharmacol **129**(1): 131-139.

Tanaka, K., C. J. Hassall, et al. (1993). "Distribution of intracardiac neurones and nerve terminals that contain a marker for nitric oxide, NADPH-diaphorase, in the guinea-pig heart." Cell Tissue Res **273**(2): 293-300.

Tang, L., A. M. Persky, et al. (2004). "Pharmacokinetic aspects of biotechnology products." J Pharm Sci **93**(9): 2184-2204.

Tang, Y. L., Q. Zhao, et al. (2005). "Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction." Ann Thorac Surg **80**(1): 229-236; discussion 236-227.

Tao, Z. W. and L. G. Li (2007). "Cell therapy in congestive heart failure." J Zhejiang Univ Sci B **8**(9): 647-660.

Terami, H., K. Hidaka, et al. (2004). "Wnt11 facilitates embryonic stem cell differentiation to Nkx2.5-positive cardiomyocytes." Biochem Biophys Res Commun **325**(3): 968-975.

Thibonnier, M., D. M. Conarty, et al. (1999). "Human vascular endothelial cells express oxytocin receptors." Endocrinology **140**(3): 1301-1309.

Thomas, J. S., S. H. Koh, et al. (2007). "Haemodynamic effects of oxytocin given as i.v. bolus or infusion on women undergoing Caesarean section." Br J Anaesth **98**(1): 116-119.

Thornton, S., J. M. Davison, et al. (1990). "Effect of human pregnancy on metabolic clearance rate of oxytocin." Am J Physiol **259**(1 Pt 2): R21-24.

Tian, P. S. and C. D. Ingram (1997). "Evidence for independent hypertensive effects of oxytocin and vasopressin in the rat dorsal vagal complex." Neurosci Res **27**(3): 285-288.

Toma, C., M. F. Pittenger, et al. (2002). "Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart." Circulation **105**(1): 93-98.

Tribollet, E., C. Barberis, et al. (1992). "Localization and characterization of binding sites for vasopressin and oxytocin in the brain of the guinea pig." Brain Res **589**(1): 15-23.

Tsai, M. S., J. L. Lee, et al. (2004). "Isolation of human multipotent mesenchymal stem cells from second-trimester amniotic fluid using a novel two-stage culture protocol." Hum Reprod **19**(6): 1450-1456.

Tse, H. F. and C. P. Lau (2007). "Therapeutic angiogenesis with bone marrow--derived stem cells." J Cardiovasc Pharmacol Ther **12**(2): 89-97.

Tsutsui, M., H. Shimokawa, et al. (2009). "Nitric oxide synthases and cardiovascular diseases." Circ J **73**(6): 986-993.

Tugtepe, H., G. Sener, et al. (2007). "The protective effect of oxytocin on renal ischemia/reperfusion injury in rats." Regul Pept **140**(3): 101-108.

Urbanek, K., F. Quaini, et al. (2003). "Intense myocyte formation from cardiac stem cells in human cardiac hypertrophy." Proc Natl Acad Sci U S A **100**(18): 10440-10445.

Ursell, P. C. and M. Mayes (1995). "Anatomic distribution of nitric oxide synthase in the heart." Int J Cardiol **50**(3): 217-223.

Uvnas-Moberg, K. and M. Eriksson (1996). "Breastfeeding: physiological, endocrine and behavioural adaptations caused by oxytocin and local neurogenic activity in the nipple and mammary gland." Acta Paediatr **85**(5): 525-530.

Vacanti, V., E. Kong, et al. (2005). "Phenotypic changes of adult porcine mesenchymal stem cells induced by prolonged passaging in culture." J Cell Physiol **205**(2): 194-201.

Valarmathi, M. T., R. L. Goodwin, et al. (2010). "A 3-D cardiac muscle construct for exploring adult marrow stem cell based myocardial regeneration." Biomaterials **31**(12): 3185-3200.

Vallejo, M., D. A. Carter, et al. (1984). "Haemodynamic effects of arginine-vasopressin microinjections into the nucleus tractus solitarius: a comparative study of vasopressin, a selective vasopressin receptor agonist and antagonist, and oxytocin." Neurosci Lett **52**(3): 247-252.

Vanin, A. F., L. M. Bevers, et al. (2007). "Nitric oxide synthase reduces nitrite to NO under anoxia." Cell Mol Life Sci **64**(1): 96-103.

Ventura, C., M. Maioli, et al. (2004). "Butyric and retinoic mixed ester of hyaluronan. A novel differentiating glycoconjugate affording a high throughput of cardiogenesis in embryonic stem cells." J Biol Chem **279**(22): 23574-23579.

Ventura, C., E. Zinellu, et al. (2003). "Dynorphin B is an agonist of nuclear opioid receptors coupling nuclear protein kinase C activation to the transcription of cardiogenic genes in GTR1 embryonic stem cells." Circ Res **92**(6): 623-629.

Verbalis, J. G., M. P. Mangione, et al. (1991). "Oxytocin produces natriuresis in rats at physiological plasma concentrations." Endocrinology **128**(3): 1317-1322.

Wagner, J. G. (1973). "A modern view of pharmacokinetics." J Pharmacokinet Biopharm **1**(5): 363-401.

Walter, U. and S. Gambaryan (2004). "Roles of cGMP/cGMP-dependent protein kinase in platelet activation." Blood **104**(8): 2609.

Wang-Rosenke, Y., H. H. Neumayer, et al. (2008). "NO signaling through cGMP in renal tissue fibrosis and beyond: key pathway and novel therapeutic target." Curr Med Chem **15**(14): 1396-1406.

Wang, L., K. Seshareddy, et al. (2009). "Effect of initial seeding density on human umbilical cord mesenchymal stromal cells for fibrocartilage tissue engineering." Tissue Eng Part A **15**(5): 1009-1017.

Wang, Y., H. Haider, et al. (2006). "Evidence for ischemia induced host-derived bone marrow cell mobilization into cardiac allografts." J Mol Cell Cardiol **41**(3): 478-487.

Wang, Y., H. E. Johnsen, et al. (2006). "Changes in circulating mesenchymal stem cells, stem cell homing factor, and vascular growth factors in patients with acute ST elevation myocardial infarction treated with primary percutaneous coronary intervention." Heart **92**(6): 768-774.

Wang, Y. F., T. A. Ponzio, et al. (2006). "Autofeedback effects of progressively rising oxytocin concentrations on supraoptic oxytocin neuronal activity in slices from lactating rats." Am J Physiol Regul Integr Comp Physiol **290**(5): R1191-1198.

Wathes, D. C. and R. W. Swann (1982). "Is oxytocin an ovarian hormone?" Nature **297**(5863): 225-227.

Wathes, D. C., R. W. Swann, et al. (1983). "Characterization of oxytocin, vasopressin, and neurophysin from the bovine corpus luteum." Endocrinology **113**(2): 693-698.

Wesley, C. R. and J. P. Gilmore (1985). "Effects of oxytocin, vasotocin and alpha-MSH on renal function in the nonhuman primate." Horm Res **21**(1): 55-59.

Wilcox, J. N., R. R. Subramanian, et al. (1997). "Expression of multiple isoforms of nitric oxide synthase in normal and atherosclerotic vessels." Arterioscler Thromb Vasc Biol **17**(11): 2479-2488.

Willets, J. M., P. J. Brighton, et al. (2009). "Regulation of oxytocin receptor responsiveness by G protein-coupled receptor kinase 6 in human myometrial smooth muscle." Mol Endocrinol.

Winnier, G., M. Blessing, et al. (1995). "Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse." Genes Dev **9**(17): 2105-2116.

Wise, A. and V. Clark (2008). "Strategies to manage major obstetric haemorrhage." Curr Opin Anaesthesiol **21**(3): 281-287.

Wobus, A. M. and K. R. Boheler (2005). "Embryonic stem cells: prospects for developmental biology and cell therapy." Physiol Rev **85**(2): 635-678.

Wollert, K. C., G. P. Meyer, et al. (2004). "Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial." Lancet **364**(9429): 141-148.

Xuan, Y. T., Y. Guo, et al. (2007). "Endothelial nitric oxide synthase plays an obligatory role in the late phase of ischemic preconditioning by activating the protein kinase C epsilon p44/42 mitogen-activated protein kinase pSer-signal

transducers and activators of transcription1/3 pathway." Circulation **116**(5): 535-544.

Yazawa, H., A. Hirasawa, et al. (1996). "Oxytocin receptors expressed and coupled to Ca2+ signalling in a human vascular smooth muscle cell line." Br J Pharmacol **117**(5): 799-804.

Yoon, J., S. C. Choi, et al. (2008). "Bone marrow-derived side population cells are capable of functional cardiomyogenic differentiation." Mol Cells **25**(2): 216-223.

Young, L. J., Z. Wang, et al. (1998). "Estrogen receptor alpha is essential for induction of oxytocin receptor by estrogen." Neuroreport **9**(5): 933-936.

Zakharova, L., D. Mastroeni, et al. (2010). "Transplantation of cardiac progenitor cell sheet onto infarcted heart promotes cardiogenesis and improves function." Cardiovasc Res.

Zhang, F. B., L. Li, et al. (2005). "Passage-restricted differentiation potential of mesenchymal stem cells into cardiomyocyte-like cells." Biochem Biophys Res Commun **336**(3): 784-792.

Zhang, Y. M., C. Hartzell, et al. (2002). "Stem cell-derived cardiomyocytes demonstrate arrhythmic potential." Circulation **106**(10): 1294-1299.

Zingg, H. H., F. Rozen, et al. (1995). "Oxytocin and oxytocin receptor gene expression in the uterus." Recent Prog Horm Res **50**: 255-273.

Zohlnhofer, D., I. Ott, et al. (2006). "Stem cell mobilization by granulocyte colony-stimulating factor in patients with acute myocardial infarction: a randomized controlled trial." JAMA **295**(9): 1003-1010.