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THE PREEMPTIVE USE OF INHALED NITRIC OXIDE DURING CARDIOPULMONARY BYPASS IN AN EXPERIMENTAL PIG MODEL

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a été évalué par un jury composé des personnes suivantes

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Abstract

Introduction: Inhaled nitric oxide (INO) is clinically approved by the Food and Drug Administration (FDA) in the treatment of persistent pulmonary hypertension of the newborn. Additionally, it has achieved widespread use in a range of other conditions. Its use is not without risks, particularly rebound pulmonary hypertension following weaning of INO.

Aim: To describe the effects of a controlled weaning protocol of INO on hemodynamic and respiratory parameters.

Methods: 13 pigs were randomly split into two groups; one to undergo cardiopulmonary bypass (CPB) with continuous INO, the other to undergo CPB with INO with weaning. Both groups were subjected to a CPB procedure lasting 90 minutes and maintained anesthetised and mechanically ventilated for a total duration of 24 hours. INO weaning began after CPB. The following parameters were measured or calculated: mean pulmonary arterial pressure (MPAP), mean systemic arterial pressure (MAP), cardiac index (CI), pulmonary vascular resistance (PVR), systemic vascular resistance (SVR), PaO_2:FIO_2, shunt and pulmonary compliance.

Results: A significantly higher CI was detected in the weaned INO group and a significantly greater SVR was detected in the continuous INO group. No other significant differences parameters were detected between groups.

Over time, within each group, MPAP and PVR showed a significant increase. Mean systemic arterial pressure, CI, PaO_2:FIO_2 and pulmonary compliance showed a significant decrease over time. Rebound pulmonary hypertension was not observed in the weaned INO group.

Conclusions: We have demonstrated the safety of a controlled weaning process. With regards to the major advantages observed in previous studies comparing CPB with and without continuous INO, our INO weaning protocol looks promising for future clinical use.

Key words: **cardiopulmonary bypass, nitric oxide, inhaled nitric oxide, porcine model**

Résumé

Introduction : Le monoxyde d'azote inhalé (INO) est reconnu cliniquement par la *US Food and Drug Administration* dans le traitement de l'hypertension pulmonaire persistante du nouveau-né. Son utilisation s'est répandue dans la gestion de diverses pathologies mais n'est pas sans risque. Le sevrage du patient en INO peut ainsi être associé à une hypertension pulmonaire rebond très délétère.

But : Décrire l'effet d'un protocole contrôlé de sevrage sur les paramètres hémodynamiques et respiratoires.

Méthodologie : 13 cochons furent répartis aléatoirement en deux groupes; un soumis à une circulation extracorporelle (CEC) avec du INO en continu, l'autre soumis à une CEC avec INO et sevrage. La CEC a duré 90 minutes, la ventilation mécanique et l'anesthésie furent maintenues durant 24 heures. Le sevrage en INO commença après la CEC. Les paramètres suivants furent mesurés ou calculés : pression artérielle pulmonaire moyenne (PAPm), pression artérielle systémique moyenne (PASm), index cardiaque (IC), résistances vasculaires pulmonaires (RVP), résistances vasculaires systémiques (RVS), ratio PaO_2/FiO_2, gradient alvéolo-artériel en oxygène, admission veineuse / shunt physiologique et compliance pulmonaire.

Résultats : Un IC significativement plus élevé fut observé dans le groupe INO sevré et des RVS significativement plus grandes dans le groupe INO continu. Il n'y eut pas de différences significatives entre les deux groupes pour les autres paramètres hémodynamiques et respiratoires.

Avec le temps, dans chaque groupe, la PAPm et les RVP ont connu une augmentation significative. La PASm, l'IC, le PaO_2:FiO_2 et la compliance pulmonaire ont montré une baisse significative dans le temps. Aucune hypertension pulmonaire rebond ne fut observée dans le groupe INO sevré.

Conclusions : Nous avons démontré la sécurité d'une procédure de sevrage contrôlé de l'INO post-CEC sur des paramètres hémodynamiques et respiratoires. En comparaison de la différence observée lors d'études antérieures en faveur du traitement continu en INO *versus* une CEC en absence d'INO, le protocole de

sevrage élaboré dans la présente étude s'avère extrêmement prometteur pour une future utilisation clinique dans le cadre des chirurgies cardiaques avec CEC.

Mots Clés : **circulation extracorporelle, monoxyde d'azote/ oxyde nitrique, monoxyde d'azote inhalé, modèle porcin**

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ABBREVIATIONS

AA	arachidonic acid
ALI	acute lung injury
BAL	bronchoalveolar lavage fluid
CABG	cardiac artery bypass graft
CI	cardiac index
CK-MB	myocardial fraction of creatinine kinase
COMP	compliance
COP	colloid osmotic pressure
COX	cyclooxygenase
CPB	cardiopulmonary bypass
CVA	cerebrovascular anomaly
CVP	central venous pressure
DNA	deoxyribose nucleic acid
ECG	electrocardiography
EDRF	endothelium - derived relaxing factor
ESR	electron spin resonance
ET	endothelin – 1
FAD	flavin adenine dinucleotide
FDA	Food and Drug Administration
FMN	flavin mononucleotide
FiO_2	inspired oxygen fraction
cGMP	cyclic guanosine monophosphate
Hb	hemoglobin
HCC	heparin coated circuit
ICU	intensive care unit
IL	interleukin
INO	inhaled nitric oxide
IRV	inverse ratio ventilation
LPS	lipopolysaccharide

LT	leukotriene
MAP	mean systemic arterial pressure
MAP	mitogen activated protein
MI	myocardial infarction
MMP	matrix metalloproteinase
MODS	multiorgan dysfunction syndrome
MPAP	mean pulmonary arterial pressure
NADPH	nicotinamide adenine dinucleotide phosphate
NANC	non-adrenergic non-cholinergic
NF-κB	nuclear factor κB
NMA	NG-methyl-L-arginine
NO	nitric oxide
cNOS	constitutive nitric oxide synthase
eNOS	endothelium nitric oxide synthase
iNOS	inducible nitric oxide synthase
nNOS	neuronal nitric oxide synthase
O_2^-	superoxide anion
$ONOO^-$	peroxynitrite
$PaCO_2$	arterial partial pressure of carbon dioxide
PAF	platelet activating factor
PAH	pulmonary arterial hypertension
PaO_2	arterial partial pressure of oxygen
PaO_2:FiO_2	arterial partial pressure of oxygen:inspired fraction of oxygen
PAP	pulmonary arterial pressure
PEEP	positive end-expiratory pressure
PG	prostaglandin
PMN	polymorphonucleocytes
ppm	parts per million
PVR	pulmonary vascular resistance
ra	receptor antagonist

ROS	reactive oxygen species
SIRS	systemic inflammatory response syndrome
sr	soluble receptors
SVR	systemic vascular resistance
TNF	tumor necrosis factor
Tx	thromboxane
VALI	ventilator-associated lung injury
VILI	ventilator-induced lung injury
V/Q	ventilation perfusion

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1. Introduction

At present, the use of INO is clinically approved by the Food and Drug Administration (FDA) for persistent pulmonary hypertension of the newborn, following evidence from large, controlled multicentre trials.[1-4] It has also achieved widespread use in a multitude of other clinical situations, such as acute respiratory distress syndrome (ARDS), acute pulmonary hypertension, and pulmonary hypertensive crises during cardiopulmonary bypass

The primary benefits of INO are a selective pulmonary vasodilatation (both arteries and veins) of ventilated areas of lung resulting in decreased intrapulmonary shunt and improved PaO_2. This, and the absence of effect on systemic vascular tone has been demonstrated in numerous studies.[5-7]

In addition, a reduction in neutrophil adhesion (a crucial step in acute lung injury) to both pulmonary[8-10] and distal[11] systemic vasculature has been demonstrated. It should be noted that the timing of INO administration appears to be key to these beneficial effects, with contrasting results found in studies delivering INO after pulmonary injury was established.[12,13]

The use of INO in clinical practice has not been without problems, primarily due to the occurrence of rebound pulmonary artery hypertension (PAH) associated with an increase in right to left shunting and a resultant decrease in PaO_2 following discontinuation of INO.[14] It has been proposed that downregulation of endogenous cNOS during INO could result in a lack of endogenous NO following weaning of INO,[15-20] resulting in rebound PAH. Also, endothelin (ET) receptor (A) blockade has been shown to prevent rebound PAH indicating a possible interaction between NO, ET, and its receptor.[21,22]

Circumvention of rebound PAH is achievable through careful, stepwise weaning of INO.[23-25]

The **aim of this study** was to evaluate the hemodynamic and respiratory effects of a INO weaning protocol during a cardiopulmonary bypass procedure (CPB) procedure in a porcine model (previously reported by our group[26]), compared with

continuous delivery of INO. Inhaled Nitric oxide was delivered prophylactically *i.e.* on induction of anesthesia, prior to the CPB procedure.

2. Review of the literature I

2.1 Introduction

The aims of this review are twofold:

1. To briefly discuss the physiological similarities between the porcine and human cardiovascular system with reference to suitability for cardiovascular research.
2. To review the current knowledge of the effects of cardiopulmonary bypass (CPB) on the cardiovascular and respiratory systems.

2.2 The validity of pigs as animal models for cardiovascular research; anatomy and physiology

Of the large animal models currently used for cardiovascular research, the pig has particular advantages over other species with respect to its similarities to humans. These include size, digestive physiology, dietary habits, kidney structure and function, pulmonary vasculature bed structure, coronary artery distribution, respiratory rate, many hematological parameters, immunology and cardiovascular anatomy and physiology.[27]

2.2.1 Anatomy

Anatomically, the porcine pulmonary system is similar to that of humans. Grossly, the human left lung is made up of an upper and lower lobe and the right lung of an upper, middle and lower lobe. The porcine pulmonary system is comprised of a right lung made up of cranial, middle and caudal lobes and a left lung made up of a split cranial lobe and caudal lobe. There is also a left accessory lobe. In addition, the anatomy of the bronchial artery and local anastamoses closely resembles that of humans. This is an important feature due to the importance of bronchial artery supply during CPB.[28,29] Continuous pulmonary perfusion with oxygenated blood was associated with lower pulmonary

inflammation (reduction in polymorphonucleocytes (PMNs; neutrophils) and neutrophil elastase concentrations in bronchoalveolar lavage fluid (BAL)), when compared with CPB without continuous pulmonary perfusion in a neonatal pig model of CPB.[29]

2.2.2 Physiology

The cardiovascular and pulmonary systems are similar to that of humans.[27,30] The size of pigs often used in laboratory investigations (25-30 kg) have a heart size to body weight ratio of 0.005, identical to that of humans (70 kg). Other similarities include, a very low incidence of pre-existing collateral coronary vessels (under 2%), a similar end-artery coronary anatomy, distribution and size of coronary vessels,[31] and similar hemodynamic parameters (see Table 1). However, their pattern of venous drainage differs from man in that there is a large hemiazyguous (left azygous) vein.[31]The importance of this during cardiac investigations involving invasive techniques is the risk of hemorrhage due to vessel fragility.

The electrophysiology of the heart is different in the pig compared to man though the intracardiac electrophysiologic parameters resemble man closer than any other non-primate animal.[31] The porcine conduction system has the following differences from humans: different location of AV node, shorter and more proximally branching penetrating bundle, more connective tissue, less elastic tissue and a larger number of nerve fibres, implying an important neurogenic component to conduction.[32] These differences have been demonstrated in immunohistochemical studies.[33,34]

Importantly, with respect to CPB, the pig heart is very susceptible to ventricular fibrillation (which may or may not be preceded by atrial fibrillation), frequently requiring defibrillation and/or pre-treatment with anti-arrhythmic agents such as bretylium tosylate.[30,35] This susceptibility highlights the importance of good CPB technique and myocardial protection when using this animal for CPB.

In addition, the metabolic response of the porcine myocardium to ischemia is similar to that of human myocardium; rendering the porcine model relevant to the

study of post CPB ischemia and reperfusion injury, and acute myocardial infarction.[31]

Parameter	Human	Pig (30 kg)
Cardiac output (l/min)	2.5-3.5	2.0-2.5
Right atrial pressure (mmHg)	0-8	1-9
Right ventricular pressure (mmHg)	15-30	24-30
Pulm. arterial pressure (mmHg)	15-30	24-30
Left ventricular pressure (mmHg)	110-140	116-150
Aortic pressure (mmHg)	70-105	114-126

Table I: Comparison of cardiovascular system parameters between humans and pigs.[27]

Other advantages

In addition to their physiological and physical parameters, their availability (compared to non-human primates) and breeding characteristics make them particularly suitable as large animal models. With regard to breeding characteristics, they have large litters, reach sexual maturity rapidly (4-5 months) and ovulate every 3 weeks. They also adapt well to the laboratory environment and have few disease problems when housed correctly.

2.3 The systemic inflammatory response and cardiopulmonary bypass (CPB)

The systemic inflammatory response syndrome (SIRS) and its possible sequelae were defined at a consensus conference of the American College of Chest Physicians and the Society of Critical Care Medicine.[36] It has been recognised that SIRS may occur as a result of infectious or non-infectious causes (such as CPB) and a clinical progression to sepsis, severe sepsis, septic shock, multiple organ dysfunction syndrome (MODS) and death has been demonstrated.[37] This study[37] also showed that patients with negative bacterial cultures had similar

levels of morbidity and mortality rates, highlighting the importance of non-infectious causes of SIRS such as CPB.

The pathogenesis of SIRS is still unclear due to the many complex interactions involving various facets of the immune system, factors associated with CPB equipment and techniques, patient factors and the generation of a reliable and consistent animal model of CPB.

At present, the progression of SIRS following CPB, towards adverse sequelae is thought to depend on the balance of pro and anti-inflammatory cytokines, within each organ system. Following CPB, the theory of multiple hits states that CPB primes the immune system (PMN priming and pulmonary sequestration), and secondary minor insults result in serious sequelae, deranging attempts of the body at re-establishing homeostasis.[38-41]

SIRS: diagnosis requires presence of 2 or more of the following : Temp > 38°C or < 36°C Heart rate > 90 bpm Resp rate > 20 breaths/min or $PaCO_2$ < 32 mmHg Leukocytes > 12000, < 4000/ mm^3 or > 10% immature (band) forms
Sepsis: SIRS with documented infection
Severe sepsis: sepsis associated with organ dysfunction, hypoperfusion or hypotension
Septic shock: sepsis with hypotension despite adequate resuscitation along with the presence of perfusion abnormalities
MODS: a state of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention

Table II: Criteria for the diagnosis of SIRS, sepsis and MODS.[42]

2.3.1 Pathophysiology of the inflammatory response to CPB.

The inflammatory response to CPB is mediated through contact activation, ischemia- reperfusion induced injury to various organs including brain, heart and blood, kidney, liver and lungs, and, endotoxemia *via* splanchnic hypoperfusion.

2.3.1.1 CPB effects on the cardiovascular system.

The occurrence of major cardiovascular complications (cardiac death, myocardial infarction, heart failure) in cardiac artery bypass graft (CABG) patients is

considerable (10%).[43] The overall incidence of myocardial infarction (MI) in a multicentre study of 566 human patients was up to 25% and the majority of cases occurred within 16 hours after the release of aortic occlusion.[44] Independent predictors of myocardial infarction (MI) were intraoperative ST segment deviation, intraventricular conduction defect, left bundle branch block, duration of hypotension (systolic arterial blood pressure less than 90 mmHg) after CPB and duration of CPB.

Proinflammatory cytokines released as a result of CPB have been shown to play a role in the myocardial dysfunction and ischemic episodes associated with CPB procedures).[45] In a study of 22 human patients,[45] levels of TNF-alpha, interleukin (IL)-6 and IL-8 were determined to exhibit two peaks; the first early in the post operative period and the second approximately 18 hours after the CPB procedure. Left ventricular wall motion abnormalities were associated with raised IL-6 and IL-8 levels and postoperative myocardial ischemic episodes were associated with raised IL-6 levels. Aortic cross clamp time was independently predictive of these postoperative cytokine levels. In addition, Oddis and Finkel showed that TNF-alpha, IL-1beta and IL-6, and NG-methyl-L-arginine (NMA; a NO synthase inhibitor) all completely blocked the positive chronotropic effects of the beta-adrenergic agent isoproterenol on cardiac myocytes.[46] This demonstrates a link between cytokines and NO and myocardial beta-adrenergic desensitisation and may provide a partial explanation for the myocardial depression seen post CPB.

The levels of production of NO by the myocardium may have both beneficial cardioprotective and deleterious effects. A constitutive cardiac nitric oxide synthase (cNOS) has been demonstrated in both humans and animal models and its activity linked to the contractile state of the heart, possibly through beta-adrenergic pathways.[47,48] Nitric oxide produced *via* cNOS has inhibitory roles in myocardial contractility and the degree of platelet adhesion to endothelium.[49,50] During an inflammatory response, NO production is upregulated *via* inducible NOS (iNOS) and this may have deleterious effects for myocardial function.

Increased NO concentrations have been associated with myocardial stunning in humans.[51] There may be several mechanisms underlying the end result such as the inhibition of mitochondrial activity, alterations in platelet-endothelial adhesion, and the formation of radical oxygen species such as peroxynitrite and beta-adrenergic desensitisation.[46,48,50,52,53]

Recent evidence implicates a role for ET (a potent vasoconstrictor produced by endothelial cells) in myocardial depression following CPB as demonstrated by local production of this vasoactive mediator and the beneficial effects of ET receptor antagonism on myocardial function.[54,55]

In addition, myocardial cyclooxygenase (COX)-2 protein and mRNA levels have been shown to increase significantly in an animal model of CPB and cardioplegia. Inhibition of prostaglandin production has been associated with improved systemic arterial blood pressure and urine production during CPB.[56,57]

2.3.1.2 CPB effects on hemostasis

The inflammatory response to CPB affects hemostasis through contact activation of the coagulation and fibrinolytic cascades (Figures 2 and 3), endothelial damage *via* inflammatory mediators, and leukocyte and platelet activation.[58-62]

The duration of CPB, postoperative skin temperature and plasma complement (C3) levels have been positively correlated with postoperative bleeding time and levels of blood loss.[63]

The use of hemofiltration during CPB was associated with a reduction in cytokines (TNF, IL-10), myeloperoxidase, C3a and a reduction in postoperative bleeding.[64]

The involvement of platelets was demonstrated with significantly less postoperative blood loss and improved levels of pulmonary function (post extubation gas exchange, lowered ventilation times) following the post CPB infusion of pre-operatively harvested platelet rich plasma.[65]

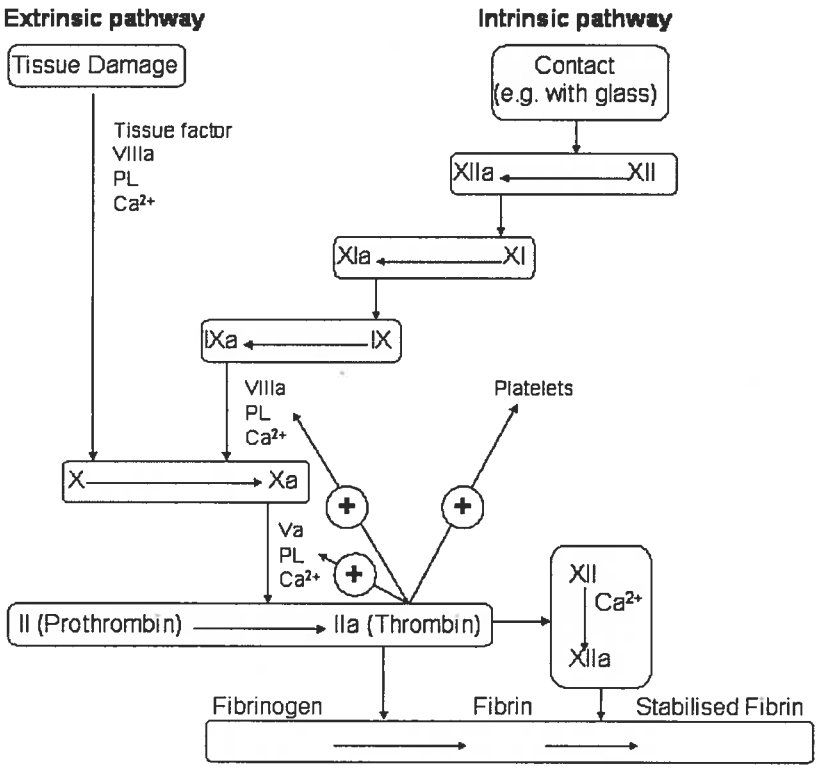

Figure 1: The coagulation cascade. Adapted from Rang *et al.*[66]

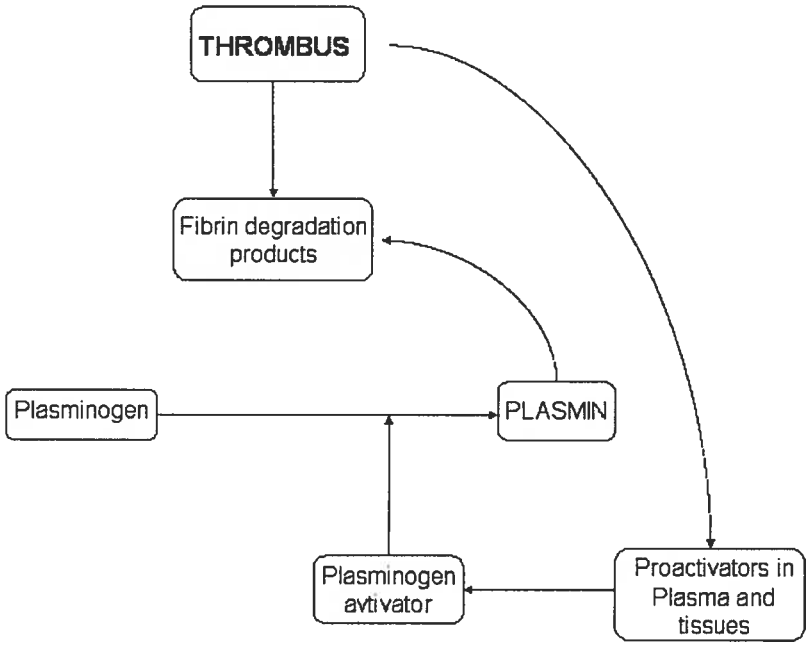

Figure 2: The fibrinolytic cascade. Adapted from Rang *et al.*[66]

2.3.1.3 CPB effects on pulmonary function.

Cardiopulmonary bypass is well known for its effects on the pulmonary system, namely Acute Lung Injury (ALI) and the more extreme form of this syndrome, the Acute Respiratory Distress Syndrome (ARDS). This syndrome, in addition to the effects of CPB on other organ systems puts the patient at risk of developing MODS.[67] The incidence and mortality rates of ARDS vary between surveys with currently accepted figures of an incidence between 1 - 3% with a mortality of 30 - 50%.[42,68,69] Mortality rates have dropped markedly from approximately 90% in the early 1970s despite no major advances in pharmacological therapy in 30 years of research. Improved ventilation strategies have been responsible for the majority of this improvement.[69,70]

The current definition of ALI is hypoxemia (PaO_2:$FIO_2 \leq 300$ mmHg), bilateral pulmonary infiltrates (indicating inflammatory change) on thoracic radiographs and normal pulmonary capillary wedge pressure.[71] The progression of ALI to ARDS has been described as involving a proliferative phase when fibroblasts infiltrate and remodel areas of inflammation followed by fibrosis and consolidation of the lung parenchyma and a PaO_2:$FIO_2 \leq 200$ mmHg.[69]

The risk of development, and severity of ALI, has been positively linked to CPB duration.[72]

Pulmonary dysfunction below the threshold of ALI classification occurs in 12% of patients and includes perfusion/ventilation mismatch, reduced oxygenation index and reduced lung compliance.[73]

In addition, and perhaps relevant to MODS, an association between early pulmonary dysfunction (defined by mechanical ventilation with an PaO_2:$FIO_2 \leq$ 150 mmHg and pulmonary infiltrates) and renal and neurological dysfunction, nosocomial infections, prolonged mechanical ventilation, increased intensive care unit (ICU) and hospital stay and mortality has been identified.[72]

Many studies have demonstrated the role of the inflammatory response in pulmonary dysfunction.[74,75] However the importance of various inflammatory mediators within the inflammatory process is still the subject of investigation.

Histologically, the inflammatory response and pulmonary damage following CPB has been demonstrated.[76] Neutrophils and their associated inflammatory mediators play an important role; 50% of circulating PMNs are sequestered to the lungs following CPB (from the release of the aortic cross-clamp) and are associated with a significant increase in oxidative activity.[75] Granulocyte elastase, IL-6 and IL-8 levels correlate with a reduced respiratory function.[74] Reactive oxygen species (ROS) have also been associated with an increase in pulmonary endothelial permeability.[77]

Sinclair *et al.* studied pulmonary endothelial permeability and found a transient increase in permeability in all patients (20 patients) post-CPB but no association between markers measured (protein accumulation index, and BAL to serum urea ratio) and the development of ARDS. Increased levels of these markers were associated with a longer duration of CPB. They postulated that though PMNs had a role to play in pulmonary dysfunction, cellular activation occurred at a systemic rather than local level supporting the theory of a systemic inflammatory response to CPB.[78]

Recently, ET has been implicated in the pathogenesis of increased pulmonary vascular resistance following CPB. Several animal studies have shown increased plasma ET, ET receptor and ET mRNA concentrations within pulmonary vascular endothelium and ET receptor blockade has been shown to decrease the levels of pulmonary vascular resistance.[79-81]

The enzyme COX 2 and prostaglandin (PG) synthesis may also play a role in increased pulmonary vascular resistance, with increased levels of COX 2 protein and mRNA associated with increased pulmonary resistance.[82]

2.3.2 The effects of extracorporeal perfusion on the inflammatory response

Almost every component of extracorporeal perfusion has been shown to have a potential effect on the inflammatory system. This includes perfusate temperature; cardioplegia temperature; circuit type; oxygenator type; priming solution composition; the heparin-protamine complex; pulsatile *versus* nonpulsatile perfusion; pump types; and shear stress.

2.3.2.1 Perfusate temperature

A study involving 18 patients undergoing elective CABG compared the effects of tepid (34°C) and hypothermic (28°C) perfusate on markers of inflammation, respiratory index, systemic vascular resistance and intubation time.[83] Tepid CPB resulted in a reduction in respiratory dysfunction with a shorter intubation time compared with that of the hypothermic group.

Menasché *et al.* suggested allowing the core temperature of patients to drift between 32-34°C during CPB following the results of a clinical study of 30 patients. This study demonstrated increased levels of cytokines and an increased requirement for vasopressor support in patients undergoing normothermic (37°C) CPB, compared with patients undergoing hypothermic CPB (28-30°C).[84]

An animal study (pigs) found that moderate hypothermia (28°C) was associated with the lowest histological degree of organ damage.[85] This was associated with lower TNF and higher IL-10 levels.

In contrast, a recent study did not show a significant difference in the postoperative levels of any of the inflammatory markers measured (C-reactive protein, IL-6) between normothermic (37°C) and hypothermic (26°C) CPB patients.[86] Different perfusate temperatures (20°C, 32°C, 37°C) were found to have no relationship to neurological dysfunction following CPB.[87]

2.3.2.2 Cardioplegia temperature

Warm blood cardioplegia was found to reduce the levels of TNF, IL-6 and IL-8 post-operatively compared with cold crystalloid cardioplegia.[88] However, a study comparing the effects on complement and PMN activation between warm and cold blood cardioplegia showed that both complement and PMN activation was higher in the warm blood group.[89]

2.3.2.3 Circuit type

The balance of cytokines is pushed in favour of inflammation as a result of the interaction of blood with a CPB circuit, demonstrating the need for improvements in circuit biocompatibility (see section 2.3.1.3).[90]

Such improvements (a conventional circuit *versus* a polymer coated circuit) have been shown to decrease fibrinolysis and thrombin generation and preserve platelets.[91]

The use of heparin coated circuits (HCC) *versus* conventional circuits in high risk patients undergoing CPB found that HCC resulted in a shorter intensive care and post-operative hospital stay, and a lower incidence of lung and renal dysfunction.[92] In a similar study comparing HCC to conventional circuits, a reduction in post-operative morbidity (myocardial infarction, arrhythmias, respiratory insult, neurological dysfunction) and intensive care stay was demonstrated.[93]

With respect to inflammation, a comparison of the concentrations of IL-1, IL-6, IL-8 and TNF by HCC and conventional circuits showed that levels of IL-6 and IL-8 levels were reduced in the serum of patients in the HCC group.[94] In addition, the method of circuit coating and type of heparin employed have effects on the degree of activation of various components of the inflammatory cascade.[95]

2.3.2.4 Oxgenator type

Results from studies comparing the effect of bubble *versus* membrane oxygenators on inflammation and post-operative outcome have been mixed. This may be due, at least in part, to the difference between experimental studies and the clinical situation.

Two studies by the same group found that though there was no difference between the degree of complement activation between two groups of patients undergoing CPB with either a membrane or bubble oxygenator; evidence of PMN activation (plasma lactoferrin and myeloperoxidase plasma concentrations) was significantly lower in the membrane oxygenator group.[96,97] Looking at organ dysfunction in this study, cardiac (post-operative need of inotropic support), renal

(serum creatinine levels) and pulmonary (alveolar-arterial oxygen pressure gradient), but not cerebral function (psychometric tests and adenylate kinases levels), was improved in patients with membrane oxygenators *versus* those with bubble oxygenators.

The effect of bubble oxygenators on respiratory function was associated with an increase in extravascular lung water and atelectasis (on chest radiographs), compared with membrane oxygenators.[98] However, these changes had no effect on the duration of postoperative ventilation, mortality or hospital stay.[98]

A smaller, more recent study looking at complement activation, respiratory function, granulocyte activation and endothelial damage between CPB with membrane *versus* bubble oxygenators showed no significant differences between the groups for all variables studied.[99]

A comparison of flat sheet and hollow fibre membrane oxygenators in terms of shear stress showed a positive correlation between the pressure drop across flat sheet oxygenators (shear stress generated) and the degree of neutrophil elastase released; a marker of PMN activation.[100]

2.3.2.5 Priming solution composition

The effect of crystalloid *versus* colloid priming solutions on colloid osmotic pressure (COP) in 20 patients during CPB found that COP decreased by a significantly lesser amount both during and after CPB in patients receiving a colloid prime.[93] Measures of complement (C3b/c and C4b/c) and PMN activation (neutrophil elastase) and TNF increased similarly in both groups.

Patients with colloid prime showed an improved postoperative fluid balance, shorter duration of intubation, smaller rectal to skin temperature gradient and shorter hospital stay.

In addition, a colloid prime has been shown to improve cardiac index and prevent extravascular lung water accumulation postoperatively when compared with a crystalloid prime.[101]

2.3.2.6 Heparin and protamine complex

Heparin and protamine (antagonises the anticoagulant effect of the heparin-antithrombin III complex; Figures 2 & 3) are given prior to, and at the termination of CPB respectively, in order to minimise the coagulation response to CPB. Together they form a complex which has been shown to activate complement *via* the classical pathway with many ensuing deleterious effects.[102,103] These complexes may cause increased pulmonary arterial pressure, decreased systolic and diastolic arterial blood pressure, myocardial oxygen consumption, cardiac output, heart rate and systemic vascular resistance.

The impaired balance of pro and anti-coagulants in patients following CPB has important roles in postoperative coagulopathies and multiple organ dysfunction.

2.3.2.7 Pulsatile *versus* non - pulsatile perfusion

A clinical study comparing CPB with pulsatile and nonpulsatile flows demonstrated lower levels of endothelial damage (reflected by lower ET levels) and cytokine activation (lower IL-8 concentrations) with pulsatile flows.[104] Levels of endogenous endotoxin are lower with pulsatile CPB whilst cNOS, which plays an important role in the modulation of vascular compliance *via* NO, is activated by pulsatile flow.[105,106]

2.3.2.8 Pump types

A clinical study measuring the levels of inflammatory mediators TNF, IL-1beta, IL-8, IL-6, PMN counts, neutrophil elastase and terminal complement components in patients undergoing CPB with either a centrifugal or roller pump demonstrated that centrifugal pumps generated a greater inflammatory response in terms of a significant increase in IL-6, PMN counts and neutrophil elastase concentrations.[107] However, a recent retrospective study on the effect of pump type on neurological outcomes found that patients undergoing CPB procedures with centrifugal pumps had a lower incidence of permanent neurological deficit and coma compared with roller pumps; there was no difference in mortality rates.[108]

2.3.2.9 Shear stress

Within the vasculature, shear stress acts as a physiologic stimulus contributing to vasoregulation via the endothelium. An animal model (pig) comparing the effects of pulsatile *versus* nonpulsatile flow CPB demonstrated a reduction in endothelial NO production in the nonpulsatile group. It is known that NO contributes to vascular tone and may partially explain the increased vascular resistance seen with nonpulsatile CPB.[109]

However, the shear stress associated with CPB may be excessive and have detrimental effects. It has been demonstrated that hypothermia, plasma dilution and shear stress acted synergistically to decrease erythrocyte deformability and cause immediate and delayed hemolysis which may impair microcirculation and oxygen supply.[110]

An *in vitro* study demonstrated the detrimental effects of shear stress on leukocytes. At levels of shear stress less than that required to cause erythrocyte hemolysis, there was evidence of leukocyte destruction, disruption, aggregation, cytoplasmic granule release and increased adhesiveness.[111] Increased platelet activation has also been linked to shear stress.[112]

Endothelial injury as a result of shear stress may alter the interaction of these cells with the cells of the immune system and underlying smooth muscle.[113]

2.3.3 Inflammatory markers

Due to the cascade nature of enzyme systems and interaction between various aspects of the inflammatory response there are a plethora of inflammatory markers available as indicators of the degree, chronicity, type of inflammatory response and response to treatment.

In terms of CPB, many markers have been studied but only those of particular relevance to CVS and respiratory dysfunction will be discussed.

2.3.3.1 Cytokines

Cytokines are soluble paracrine mesengers that play a key role in the homeostasis of the immunological and physiological systems. The actions of individual cytokines may be loosely described as pro or anti-inflammatory though specific actions are dependent on each situation. For instance, IL-6, described as a pro-inflammatory cytokine has been demonstrated to play a protective role in hyperoxic lung injury in mice through the inhibition of cell death and matrix metalloproteinases (MMP) expression.[114]

Interleukin-1beta and IL-6 plasma concentrations have been shown to be predictive of a poor outcome in ARDS and plasma IL-8 and IL-18 levels were raised in non survivors following cardiac surgery and the subsequent development of SIRS compared with survivors.[115,116]

Bimodal serum peaks for TNF (2 and 18 hours post CPB), IL-6 (immediately and 12-18 hours post CPB) and IL-8 (early and 16-18 hours post CPB) have been documented.[45,117]

Striking a balance with proinflammatory cytokines are antiinflammatory cytokines, cytokine receptors and cytokine receptor antagonists; in particular, IL-10, IL-1 receptor antagonist (ra), TNF soluble receptors 1 and 2 (TNFsr 1 and 2) and transforming growth factor beta (TGFβ).[64,90]

In a similar fashion to pro-inflammatory cytokines, elevations of components of the anti-inflammatory response are staggered in time following CPB. In pediatric CPB, the plasma response began with IL-10 (increased levels during CPB and peaking 24 hours post CPB), followed by IL-lra (increased levels 2 hours post CPB and peaking at 24 hours post CPB) and TNFsr (increased levels 2 hours post CPB and peaking at 24 hours post CPB). From BAL samples, only IL-8 and IL-10 were significantly elevated following CPB.[118]

In adult CPB patients, IL-10, TGF-beta and IL-1ra plasma levels increased early following CPB (levels peaked within 2 hours post CPB and had decreased by 24 hours post CPB). Tumour necrosis factor soluble receptors 1 and 2 levels

increased following CPB and remained elevated at the 24 hour sample time.[90,119,120]

Pulmonary dysfunction following CPB is a frequent complication and believed to be largely mediated through local PMN sequestration and activation, through adherence, degranulation of enzymes and superoxide production.[121-126]

A comparison of BAL and plasma specimens from patients with ARDS, demonstrated that the lungs may be the primary source of IL-8.[127] Furthermore, Donnelly *et al.* provided evidence of a link between IL-8 in BAL and the development of ARDS.[128] No such link was established with plasma IL-8. Increased concentrations of IL-8 and IL-1beta (at levels ten times in excess of IL-1beta ra) are present for prolonged periods in the BAL of patients with ARDS.[129] Interleukin-8 showed a strong correlation with the PMN concentration of the BAL. Elevated IL-1beta levels on day 7 after the onset of ARDS was correlated with increased mortality.

A comparison between two subsets of CPB patients (postoperative cardiovascular dysfunction and postoperative lung injury) found that increased plasma concentrations of IL-8, IL-6 and platelet activating factor (PAF) were associated with cardiovascular dysfunction.[130] Patients with severe lung injury had increased plasma thromboxane (Tx) B2 and decreased plasma PAF, PG E2 and 6-keto PG F1alpha. BAL samples were not taken.

Tumour necrosis factor may play a pivotal role in cardiac insufficiency following CPB through its release following ischemia and reperfusion; this has been demonstrated by the comparison of blood samples from peripheral arterial blood, coronary sinus blood and mixed venous blood in patients undergoing elective CABG.[131] They showed increased levels of TNF and IL-6 in coronary sinus blood following aortic declamping compared with peripheral arterial blood indicating the myocardium as an important source of TNF and IL-6. The effect of the inflammatory process on the heart following CPB is myocardial depression with decreased contractile function and myocyte apoptosis.[132,133] Tumour necrosis factor is believed to create these effects through at least two different

mechanisms; an early depressive phase that is sphingosine mediated and a later component mediated through inducible NOS.[133]

2.3.3.2 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases are involved in the degradation of collagen and the remodelling of the extracellular matrix. They are believed to play an important role in MI, cardiac ischemia - reperfusion injury, ventricular dilation and heart failure, ALI and ARDS.[134-136] Use of an animal model of ALI has demonstrated the importance of MMPs (MMP 2 and MMP 9) in the mediation of ALI.[136] Patients with ARDS have increased levels of MMPs (2 and 9) in BAL samples.[137] Matrix metalloproteinase 9 is released as part of the inflammatory response in humans during CPB.[138] Matrix metalloproteinases 2 and 9 appear to play prominent roles following CPB and the associated inflammatory and ischemia reperfusion injuries.[134,139-142]

Cytokines play an important role in modulating MMP gene expression; in particular TNF and IL-1beta both increase the production of MMP.[143] In addition, they increase the activity and stability of MMPs, and the activation of CPB-primed PMNs by cytokines leads to the release of MMPs.

Measurements of MMP have been undertaken in both the plasma and BAL of patients and animal models with pulmonary dysfunction. Increased BAL concentrations of MMP 2 and MMP 9 in patients with ARDS compared with healthy controls has been demonstrated and MMP 9 levels correlated with BAL PMN concentrations.[137] There have been similar findings in the lungs of rats with hyperoxic lung injury.[144]

The importance of MMPs in different clinical conditions is still under investigation. Though their roles in cardiac and pulmonary dysfunction have been demonstrated (as referenced above), their role in the dysfunction of other organs is not clear. Ziswiler *et al.* (2001) found no evidence for a role of MMP in an animal model of renal ischemia reperfusion injury.[145]

Appendix 1 contains an accepted article from our group entitled, "Increased alveolar and plasma gelatinases activity following porcine cardiopulmonary

bypass: Inhibition by inhaled nitric oxide". This offers further discussion on the relationship between INO and MMPs.

2.3.3.3 Reactive oxygen species (ROS)

Reactive oxygen species may play an important role in the damage and dysfunction associated with the inflammatory response to CPB and the associated ischemia reperfusion injury.[146-148] The sources of ROS and their relative importance have been difficult to identify due to their highly reactive and short lived nature, difficulties in direct measurement and unreliabiltiy of indirect measurements.[146]

It has been demonstrated that ROS are generated by the myocardium as a result of ischemia reperfusion and play a role in myocardial dysfunction.[149]

A study in humans undergoing CPB using a direct method of detecting ROS [electron spin resonance (ESR) spectroscopy] has showed a release of ROS, independent of myocardial ischemia and reperfusion; detection of ROS occurred from the onset of CPB until the end of the CPB part of the procedure.[146] This provides evidence of the role of CPB (and possibly decreased perfusion) in the generation of ROS, as no increases were associated with any preparatory surgery (sternotomy, vascular graft preparation) prior to the onset of CPB.

Reactive oxygen species generated as a result of CPB may play roles in myocardial dysfunction and systemic PMN activation, feeding the inflammatory response. [150,151]

More commonly, indirect measures of oxidation have been used; signs of oxidative protein damage in BAL of ARDS patients (chlorotyrosine, orthotyrosine, nitrotyrosine; Lamb *et al.*), plasma antioxidant levels (ascorbate, alpha-tocopherol, retinol, beta-carotene, selenium, lipid peroxidation products) and the plasma antioxidant enzyme activity of catalase, superoxide dismutase and glutathione-peroxidase in erythrocytes.[152,153]

Reactive oxygen species have been shown to play an important role in ALI and ARDS and it has been suggested that an oxidant-antioxidant imbalance exists.[152]

Reactive oxygen species take part in chain reactions involving cell membranes

resulting in the destruction of membrane integrity, the release of cytotoxic substances and DNA damage with resultant alterations in protein synthesis. Reactive oxygen species affect vascular endothelium through PMN - dependent and - independent mechanisms resulting in dysfunctional vascular tone. In animal models of pulmonary vascular smooth muscle, the effects of ROS have been mixed with reports of both vasodilatation and constriction.[154]

The origin of ROS in ALI and ARDS may be from a range of sources. The stimulation of PMNs and other leukocytes by cytokines (TNF-alpha) and endotoxin leads to ROS production. Complement plays a role in the stimulation of ROS production from primed leukocytes following contact activation associated with extra-corporeal circulation and endotoxin stimulation. A major source of ROS for all organs following CPB is ischemia reperfusion injury. Here, ROS is endogenously produced and it has been demonstrated that 20-40% of the free radicals detected following heart-lung bypass are produced by endothelial cells.[156] Finally, the aggressive oxygen therapy used in the management of ARDS can result in hyperoxic toxicity, caused by ROS.[157] In an animal model of cerebral ischemia reperfusion it was demonstrated that PMN production of ROS (superoxide anion) increased in line with the duration of ischemia, suggesting their role in cerebral ischemia reperfusion injury.[158]

2.3.3.4 Endothelin 1 (ET)

Endothelin 1 is a vasoactive peptide with fibrogenic properties whose production, by endothelial cells, has been investigated widely in various cardiovascular disorders (ischemic heart disease, hypertension, cardiogenic and endotoxic shock).[159] It may exert its effects through stimulation of arachidonic acid (AA) metabolism, and eicosanoids have been used as markers of ET activity.[160] Increased plasma concentrations following CPB have been associated with an increase in length of intensive care stay and prolonged pharmacological management.[161] However, its role in inflammation is not fully understood; whether its presence is a reflection of vascular injury or a cause of

vasoconstriction. In a study of patients with mixed connective tissue disease, plasma ET reflected damage and correlated with an increase in von Willebrand's Factor (a recognised marker of endothelial damage). The plasma concentrations measured were not enough to cause vasoconstriction *in vitro*.[159]

A comparison of ET levels from various sites has demonstrated that plasma ET levels were a systemic product and not of cardiac origin.[162] In a similar study of patients with sepsis, with and without ARDS, the lung did not appear to be a major site of ET production. In one study of CPB patients, no association was demonstrated between increases in plasma ET levels and pulmonary vasoconstriction. On the contrary, plasma ET was associated with pulmonary vasculature vasodilation.[163] However, another study in CPB patients, found an association between pulmonary plasma concentrations of ET and an increase in pulmonary vascular resistance.[164]

ET levels have been positively correlated with an increased organ failure score and oxygen consumption and negatively correlated with the PaO_2:FIO_2 ratio.[165]

More recently, in several animal models (pigs), ET production and rises in systemic plasma levels have been associated with local tissue production (myocardial and pulmonary vascular endothelial cells) and spill over to the systemic circulation. The release of ET was documented to begin immediately following the onset of circulatory arrest and continue throughout this period.[54,81]

In further animal models, ET has also been demonstrated play an important role in myocardial depression and increases in pulmonary vascular resistance following CPB.[55,79-81]

3. Review of the Literature II:

Inhaled Nitric Oxide

3.1 Introduction

Nitric oxide (NO)

Initially described as endothelium - derived relaxing factor (EDRF) before its identification as NO.[166,167] Its discovery resulted in award of the Nobel Prize in Physiology or Medicine in 1998 to RF Furchgott, LJ Ignarro and F Murad. Its potential physiological, pathological and therapeutic roles have received widespread interest over the last 25 years. What follows is a brief summary of the current state of knowledge of NO and its potential and actual therapeutic roles.

3.1.1 Biosynthesis

The role of NO is as an endogenous activator of soluble guanylate cyclase resulting in the formation of cyclic guanosine monophosphate (cGMP), a second messenger of many cells (nerves, smooth muscle, monocytes, platelets).

The biosynthesis of NO is controlled by nitric oxide synthase (NOS) enzymes, catalyzing a reaction between molecular oxygen and L-arginine. There are 3 known isofoms of this enzyme; inducible (iNOS), expressed in macrophages, Kupffer cells, neutrophils, fibroblasts, vascular smooth muscle and endothelial cells, and two constitutive (cNOS) forms, one in the endothelium (eNOS; also found in cardiac myocytes, renal mesangial cells, osteoblasts, osteoclasts and platelets) and the other in neurons (nNOS). Levels of iNOS and hence NO production, increase in response to pathological stimuli as part of the immune system, whereas the primary role of cNOS is in normal physiological states.

The amount of NO produced as a result of catalysis by iNOS, eNOS and nNOS varies; constitutive forms producing picomolar concentrations of the molecule and iNOS capable of producing millimolar concentrations.

3.1.2 Enzyme characteristics

The NOS isoforms exist as dimers, each containing heme, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and tetrahydrobiopterin as prosthetic groups. In addition, they have binding sites for L-arginine, nicotinamide adenine dinucleotide phosphate (NADPH) and calcium-calmodulin. Activation of the dimers is controlled by these prosthetic groups in conjunction with ligand binding.

NOS enzymes are associated with the Golgi apparatus and plasma membranes. The details of the enzymatic process are unclear but it appears that NOS is bimodal in combining oxygenase and reductase activities at distinct structural domains catalyzing the following reaction.

$$O_2 + \text{L-arginine} \rightarrow \text{NO} + \text{citrulline}$$
$$\text{(NOS)}$$

Enzymatic activity is the rate-limiting step as substrates are abundant in the cytoplasm.

3.1.3 Isoform activity

cNOS activity is controlled by intracellular calcium-calmodulin, the production of which is stimulated by a variety of stimuli.

Vascular resistance control under physiological conditions is likely in response to pulsatile flow and shear stress.

iNOS activity is calcium independent (though calcium-calmodulin will stimulate its activation) and induced instead, by lipopolysaccharide (LPS) and/ or cytokines synthesized in response to LPS or inflammation.

3.1.4 Nitric oxide carriage and breakdown

Nitric oxide is freely diffusible across cell membranes thus explaining its local paracrine effects (on smooth muscle, monocytes and platelets adherent to

endothelium). NO carriage in the bloodstream is through a high affinity for heme (as part of Hb). In the absence of oxygen, bound NO is relatively stable. However, in the presence of oxygen, NO is rapidly inactivated by conversion to nitrate and the heme (to which it is attached) oxidized to metHb. Outside Hb, NO can react with oxygen resulting in nitrate and nitrite formation which are excreted in urine. Reversible binding of NO to globin *via* its –SH groups (nitrosothiol formation) is also possible, resulting in S-nitrosylated Hb allowing NO to be effective at sites distant from its production.[11]

3.2. Physiological and pathological roles of NO

The effect of NO may be mediated through either autocrine or paracrine action resulting in effects such as vasodilatation in vascular smooth muscle, decreased platelet adhesion and aggregation and the inhibition of monocyte adhesion and migration. These effects are through its action on cGMP. Cytotoxic actions are mediated through combination with superoxide anion (O_2^-), with which it reacts very rapidly, out competing superoxide dismutase and producing peroxynitrite ($ONOO^-$).

3.2.1 Vascular effects

The physiological control of peripheral (both pulmonary and systemic) vascular resistance through eNOS is constantly present and active. Endothelial NOS expression is increased by vascular shear stress and eNOS activity is increased by mediators which increase intracellular calcium, such as bradykinin and acetylcholine.

At a cellular level, NO produced by endothelial cells (or administered as INO) diffuses into vascular smooth muscle cells. Here, NO activates soluble guanylate cyclase, which catalyses cGMP. Through cGMP-mediated protein kinases, NO exerts its clinical effects of vascular smooth muscle relaxation. In addition, protein kinases inhibit leukocyte adhesion, platelet adhesion and activation, and cell prolifereation.

The action of cGMP is balanced by its hydrolysis to GMP. This is catalysed by phosphodiesterase 1 (brain, heart, lung, testis) and 5 (lung, platelets, vascular smooth muscle and kidney), in humans. Inhibition of phosphodiesterase 5, as a drug target (zaprinast, sildenafil, dipyridamole), increases endothelium-dependant vasodilation.

3.2.2 Neuronal effects

As a non-adrenergic non-cholinergic (NANC) neurotransmitter it has a role in neuronal development and plasticity.

3.2.3 Immune system

iNOS appears to play an important role in the innate immune system, responding to a variety of pathogens. Here its activity is through free radical generating, nitrosylation and heme-binding properties.

3.2.4 Nitric oxide and pathophysiology

The number of pathological conditions in which NO is known to play a part is likely to increase with further research due to the systemic distribution of NOS and the large number of cell types upon which NO has been shown to act. For example, sepsis states involve the overproduction of NO through iNOS recruitment leading to systemic vasodilatation. Whilst inadequate NO production by cNOS is associated with artherogenesis (resultant from hypercholesterolemia and cigarette smoking) and impotence.

3.3 Therapeutics

Inhaled NO (INO) has found use in a range of conditions (acute pulmonary hypertension, right ventricular failure, pulmonary hypertensive crises following CPB and in neonates, heart, lung and liver transplantation), all of which have two factors in common: the desired effect is within the pulmonary system (the effect of INO is limited to this system), leading to a reduction in intra-pulmonary

shunting or, is gained secondary to changes in the pulmonary circulation, such as reduced workload on the right side of the heart.

Pulmonary hypertensive crises following CPB is part of a range of vasomotor dysfunction following CPB.[168] Nitric oxide plays a central role to post-CPB vasomotor function; the activity of eNOS is decreased as a result of several mechanisms, including alterations in cell membrane potential, substrate and cofactor depletion, alterations in intracellular calcium, and injury to cellular components.[168]

The clinical effect of INO is limited to the pulmonary vasculature, with a half life of a few seconds. Inactivation of NO is primarily as a result of binding of NO to O_2^- and to the heme component of hemoglobin, resulting in the release of NO_3^-. Binding of NO to thiols is another source of NO inactivation.

A potentially serious drawback to the delivery of INO is the risk of rebound pulmonary hypertension.[169-171] This complication may result in hypoxemia and right ventricular overloading and failure.

The delivery and monitoring of INO will be discussed later, suffice to say that concentrations of NO less than 50 parts per million (ppm) do not appear to be toxic.[169]

Traditional NO donors, such as nitroglycerin, nitroprusside and S-nitrosoglutathione have systemic effects that may or may not be desirable depending on the condition being treated.

NO inhibition is currently limited to experimental studies though this may have therapeutic potential in cases of NO over production such as sepsis. These compounds are primarily L-arginine analogues and include N^{γ}-monomethyl-L-arginine (L-NMMA) and N^{γ}-nitro-L-arginine methyl ester (L-NAME).

3.4. INO toxicity

There are three potentially toxic products associated with INO; NO_2 resulting from combination with oxygen, $ONOO^-$ from combination with superoxide ion and metHb following the oxidation of nitrosylHb.

NO_2 inhalation studies have found that inhalation of concentrations greater than 10 ppm results in pulmonary edema, alveolar hemorrhage, alteration in surfactant properties, hyperplasia of Type II alveolar epithelial cells, intrapulmonary accumulations of fibrin, neutrophils and macrophages and death.[172] Acute overdose of INO leads to rapid NO_2 accumulation, methemoglobinemia, pulmonary edema and hemorrhage, hypoxemia and death.[173] The Occupational safety and Health Administration of the United States has set 8 hour time weighted average exposure limits in the workplace at 5 ppm. Chronic (9 weeks), low level exposure of 0.5- 1.5 ppm caused focal degeneration of pulmonary interstitial cells and mild emphysematous changes in rats.[174]

Peroxynitrite is readily formed from the reaction of NO and superoxide. Under physiologic conditions this is limited by the superoxide scavengers, primarily superoxide dismutase. However, under pathological conditions when superoxide concentrations may be increasd or scavengers exhausted, peroxynitrite concentrations increase.[175] Endogenous peroxynitrite production is useful through its ability to cause oxidation, peroxidation and nitration of lipids, proteins and DNA.[176,177] This enables the destruction of microorganisms and tumour cells[178,179] but is a double edged sword, resulting in surfactant alteration (decrease in surface tension,[180] macromolecular structural changes and apoptosis induction.[176,177]
MetHb production is limited in the normal individual by reduction back to Hb by cytochrome b_5, methemoglobin reductase within erythrocytes, and by glutathione.

3.4.1 NO and DNA

NO has several effects on DNA; the formation of mutagenic nitrosamines,[176] DNA strand breakage[181] and inhibition of DNA enzyme repair systems.[178]

3.4.2 NO and lipids

The *in vivo* effects of NO on lipids are unclear, with the production of both pro - and anti - artherogenic lipoproteins demonstrated (through oxidation and

peroxynitrite). These effects appear to depend on relative concentrations of the various molecules involved.[182,183]

Evidence from INO clinical trials between 1988-1997 show that significant methemoglobinemia or NO_2 formation was uncommon and a reduction in INO was usually sufficient to decrease levels. Discontinuation of INO was required in only 0.6 % of patients.[174]

The side effects of long term INO usage are complicated by the possible multiple sources of markers of NO related damage.[184,185] However, no changes in surfactant properties[186] or pulmonary function either during or after mechanical ventilation as a result of INO have been observed.

3.4.3 INO and inflammation

There is still conflicting evidence regarding the role of endogenous and INO in inflammation. Much of this appears to depend on the concentration of NO and timing of its delivery.[42,174]

The low concentrations of NO normally produced by cNOS are essential for the maintenance of vascular tone plus effects on platelet and leukocyte function.[8,11,187]

It has been reported that inhibition of cNOS during inflammatory insult worsens the resultant inflammation.[188]

By contrast, iNOS leads to much higher concentrations of NO and it is this which is believed to act in a pro-inflammatory fashion.[189-192]

Inhaled NO is capable of decreasing elevated pulmonary artery pressure (PAP), improving hypoxemia by reducing intra-pulmonary shunt, and optimizing ventilation perfusion (V/Q) matching. Inhaled NO can also inhibit the inflammatory process by reducing cytokine synthesis and inactivating nuclear factor κB (NF-κB), as several cytokines contain a binding site for NF-κB in their promoter regions.[193] Nitric oxide can also decrease the expression of adhesion molecules preventing neutrophil adhesion and migration. Inhaled NO can exert its effect on the lung, on leukocytes trapped in the pulmonary area but as it is

transported by red blood cells to the general circulation, INO could have extrapulmonary effects.

3.4.4 Importance of timing of delivery

Pretreatment with INO or delivery early in the onset of the insult appears to be the key to maximizing the anti-inflammatory effects of NO.[8,171,194,195]

Though the role of NO in inflammation is undoubtedly complex, much of the conflicting data regarding the pro- and anti-inflammatory effects of INO can be explained by the timing of delivery of INO.

Appendix 2 contains an article submitted to *Nitric Oxide-Biology and Chemistry* (Impact factor of 2.545), represents results of a previous series of experiments. It demonstrates the safe use of pre-emptive, continuous INO and its protective effects against lung ischemia/ reperfusion associated with CPB. Such results are very promising for promoting the use of pre-emptive INO therapy during cardiac surgery with CPB. Before expecting such clinical advance, one point remained to be solved: Could INO be reasonably and safely weaned for the cardiac patient after CPB?

4. Project aim and null hypothesis

The aim of this study was to evaluate a INO weaning protocol during a CPB procedure in a porcine model (as validated by our group[26]), and use this model to compare the hemodynamic and respiratory effects of a weaning INO protocol against continuous INO.

Null hypothesis: step-wise weaning of INO does not result in clinically significant rebound pulmonary hypertension. There is no difference between weaned and continuous INO treatment.

5. Methodology

This study was performed at the Notre Dame Hospital (CHUM) animal centre with the approval of the institutional animal care committee (Centre de recherche du CHUM) in compliance with the guidelines of the Canadian Council on Animal Care.

5.1 Animals

Pigs weighing between 30 and 35 kilograms, free of clinical pulmonary disease were purchased from Vadnais Farm Inc. (Drummondville, Québec, Canada). The animals were allowed an acclimatization period of at least 3 days in our housing facilities (Notre-Dame Hospital research laboratories) prior to the procedure. Animals were housed individually with twice daily access to proprietary feed and *ad libitum* access to water.

5.2 Experimental procedure

The following sections on anesthesia, ventilation, INO administration, monitoring, CPB procedure and post-operative protocol were carried out as published by our group.[26] Animals were randomized into 2 groups. One group

(weaned INO; n=6) had INO weaned following the end of the CPB procedure (weaning began at T4, *i.e.* 4 hours after inducing CPB) whilst the remaining group (continuous INO; n=7) had INO continued until termination of the experimental period at T24 (24 hours after induction of CPB). Individuals collecting data were blinded to the INO protocol.

5.2.1 Anesthesia

Animals were fasted 12 hours prior to surgery. Premedication consisted of intramuscular atropine (0.04 mg/kg), azaperone (4 mg/kg) and ketamine (25 mg/kg). Anesthesia was induced thirty minutes later with intravenous fentanyl (5 μg/kg) and thiopental (5 mg/kg) delivered through a 20 gauge catheter placed in an ear vein. Intubation was performed with an 8 mm endotracheal tube (Mallinckrodt Company, Mexico city, DF, Mexico) facilitated with a laryngoscope and surgical preparation (washing, shaving and first disinfection) followed. The animals were attached to the operating room table in a supine position. A single dose of antibiotic (enrofloxacin 5 mg/kg) was given intravenously at this time.

A total intravenous anesthesia technique was used for the maintenance of anesthesia with an infusion of thiopental (5 mg/kg/hr) and fentanyl (20 μg/kg/hr) in the same syringe. Neuromuscular blockade was performed with pancuronium through a loading dose of 0.2 mg/ kg followed by an infusion of 0.2 mg/kg/hr.

Lactated Ringer's solution (Baxter Corporation, Toronto, Ontario, Canada) was given at a basal rate of 10 ml/kg/hr throughout.

5.2.2 Hemodynamic monitoring and support

Monitoring was by continuous ECG, rectal temperature, urine output (Foley catheter placed by cystotomy), and systemic arterial blood pressure [*via* a 20 gauge arterial catheter (Arrow International Inc., Reading, PA, USA) placed in the left carotid artery]. Pulmonary arterial pressure, pulmonary capillary wedge pressure, central venous pressure, cardiac output (through thermodilution) and blood temperature were monitored *via* a Swan-Ganz pulmonary artery catheter (Abbot Laboratories, Chicago, IL, USA) placed in the right internal jugular vein.

Hemodynamic data were collected by an M1166A™ Model 66S cardiovascular monitor (Hewlett Packard Ltd., Palo Alto, CA, USA), which also provided the following calculated variables: systemic vascular resistance, pulmonary vascular resistance and cardiac index.

Fluid and drug administration was *via* an 18-14 gauge double lumen venous catheter (Arrow International Inc., Reading, PA, USA) placed in the left external jugular vein.

5.2.3 Mechanical ventilation and respiratory monitoring

Mechanical ventilation was provided by a 7200E™ Puritan Bennett ventilator (Puritan Bennett, Carlsbad, CA, USA) in the volume-controlled mode with a positive end expiratory pressure (PEEP) of 5 cm H_2O. Tidal volume was 10 ml/kg and respiratory rate adjusted to maintain an end tidal CO_2 pressure between 40 and 50 mm Hg. Inspired oxygen fraction (FiO_2) was 1.0 during the surgical part of the procedure and reduced to 0.5 following chest closure (provided the arterial partial pressure of oxygen; $PaO_2 > 85$ mm Hg).

During CPB, ventilation was continued at a reduced tidal volume of 3 ml/kg and respiratory rate of 8 breaths per minute.

Respiratory data were collected with a Capnomac Ultima monitoring system (Datex Instrumentation Corp., Helsinki, Finland). Data monitored, or calculated by the monitoring system, were: peak, plateau and mean airway pressures, end tidal pressure of carbon dioxide, PEEP, respiratory rate, tidal volume, inspiratory: expiratory ratio and dynamic compliance. Arterial and venous blood gas and electrolyte analysis was carried out with an iSTAT Blood Gas Analyzer™ (I-STAT® Portable Clinical Analyzer, Sensor Devices Inc., Waukesha, WI, USA). Gas analyzers were calibrated in accordance with manufacturers' instructions by a respiratory technician.

5.2.4 Inhaled NO administration

NO gas (1000 ppm NO, balanced N_2 cylinder; VitalAire Santé Ltée., Montréal, Canada) was cyclically injected into the inspiratory line during inspiration by an

NO injector developed by our group and used in both animal and human studies.[23,26] The delivered NO concentration was monitored by a chemiluminescence technique (Polytron NO/NO_2, Drager, AG). During CPB, NO was delivered directly to the membrane oxygenator.

Weaning of INO (for the weaned INO group) was carried out in a stepwise fashion with a 50% reduction in INO every 20 minutes, until 6.3 ppm, then stopped after a further 10 minutes. Weaning procedure took 90 minutes in total and commenced following chest closure (T4). No reduction in INO was performed in the continuous INO group.

5.2.5 Cardiopulmonary bypass procedure

Following instrumentation (T0; venous, arterial and urinary catheters), a median sternotomy was performed and the pericardium opened with a simultaneous injection of intravenous heparin (4 mg/kg) to give an activated clotting time > 400 seconds (measured on site). An aortic cannula (20 French; Chase Medical Inc., Richardson, TX, USA) was placed in the aortic root followed by placement of a multiple hole venous drainage cannula in the inferior vena cava *via* the right auricular appendage. A cardioplegia cannula (9 French; Medtronic Inc., Grand Rapids, MI, USA) was then placed in the aortic root proximal to the aortic valve, enabling cold blood cardioplegia.

The CPB circuit consisted of a membrane oxygenator (Trillium Affinity NT oxygenator, Medtronic Inc., MN, USA), cardiotomy reservoir (Affinity CVR, Medtronic Inc., MN, USA), filter (Affinity 351, Medtronic) and tubing. A Sarns roller pump 7000 (Sarns Inc., Ann Arbor, MI, USA) was used and cardioplegia provided by a Myotherm XP cardioplegia delivery system (Medtronic Inc., MN, USA).

The circuit was primed with a total volume of 1750 ml composed of: Lactated Ringer's solution (1000 ml; Baxter Corporation, Toronto, Ontario, Canada), Pentaspan (500 ml; DuPont, Pharma Inc., Missisauga, Ontario, Canada), sodium bicarbonate (1 mEq/ kg), mannitol (200 ml) and heparin (5000 IU).

Cardiopulmonary bypass was maintained at a flow rate of 70-ml/kg/min and blood temperature decreased to 32°C. The aorta was clamped and hyperkalemic, oxygenated, cold blood cardioplegia instituted at a flow rate of 300 ml/m^2, not exceeding a perfusion pressure of 100 mmHg. This resulted in rapid cardiac arrest. Heart temperature (measured with a temperature probe placed in the myocardium) was maintained between 12 and 15°C.

Partial pressure of arterial oxygen was maintained between 300 and 350 mmHg and partial pressure of venous oxygen between 50 and 60 mmHg, by adjustments to fresh gas flow.

Mean systemic arterial pressure maintained between 50 and 65 mmHg by adjustment to the flow rate plus a bolus of phenylephrine [0.5- 1 ml (0.1 mg/ ml)] if required.

5.2.6 Re-warming and CPB weaning procedure

The aortic clamp time was 75 minutes and the total CPB time 90 minutes. Blood re-warming commenced 10 minutes prior to aortic de-clamping using water at 38°C. The aortic clamp was removed and cardioplegia stopped when the blood temperature attained 35°C. Defibrillation was necessary in all cases, except one, as ventricular fibrillation was observed as the temperature of the heart increased. Internal defibrillation was carried out with 20 joules once myocardial temperature was 32°C. The animals were weaned off CPB by gradual clamping of the venous outflow line. Cardiopulmonary bypass was stopped once the heart was able to maintain a stable arterial pressure. Heparin antagonism with protamine sulphate (1 mg/100 IU heparin) commenced at weaning.

Following removal of the CPB cannulae, a thoracic drain was placed and the chest closed (T3).

Time point (T = x hours)	Procedures
T0	End of instrumentation, sternotomy, CPB (90 minutes)
T4	Start of INO weaning following chest closure

Table III: Experimental time points

5.2.7 Post-operative period

Blood remaining in the CPB circuit was collected and transfused. Aims during the post-operative period were homeostasis of cardiopulmonary and renal function.

To address these aims, protocols were established for the treatment of cardiac insufficiency, ventricular arrythmias, hypovolaemia and tachycardia (Appendix 2).

The duration of follow-up monitoring was until T24.

5.2.8 Statistical analysis

Analysis of continuous dependent variables was performed with a linear mixed model of repeated measures (SAS version 9.0, Cary, NC, USA). *A priori* contrasts were performed for between groups comparisons at different time points. The effect of time for each group was analysed, and *post hoc* analysis was performed with Dunnett's test. Values are presented as mean ± SEM.

Significance was claimed when $P \leq 0.05$.

6. Results of INO weaning *versus* continuous administration

6.1 Losses

In total, the procedure was performed in 17 pigs. Of these, 4 pigs died during the experimental procedure: one due to likely underlying respiratory infection, one to surgical error (lacerated aorta) and two to technical difficulties during CPB (failure of CPB circuit integrity).

6.2 Hemodynamic data

6.2.1 Mean pulmonary arterial pressure (MPAP)

Comparison between groups did not detect significant differences ($P = 0.36$), though there was a tendency to higher values of MPAP in the weaned group compared with the INO group at T7 ($P = 0.06$), T8.5 ($P = 0.09$) and T10 ($P = 0.07$).

There was a significant ($P < 0.0001$) main effect in pooled data of both groups of an increase in MPAP over time. There was a significant difference at all time points compared with T0, except for T5 ($P = 0.07$), T6 ($P = 0.08$) and T6.5 ($P = 0.10$). (Figure 3).

6.2.2 Mean systemic arterial pressure (MAP)

No significant main effect of group was detected ($P = 0.53$). There was no significant interaction of group with time ($P = 0.70$). Over time there was a significant main effect in pooled data for both groups ($P < 0.0001$). Dunnett's *post hoc* test revealed a significantly lower MAP at all time points compared with T0.

6.2.3 Cardiac index (CI)

Between groups comparisons detected a significantly higher CI ($P = 0.01$) in the weaned INO group compared with the continuous INO group at all timepoints. In addition, there was a significantly lower CI at all time points (pooled data from both groups) compared with T0 ($P < 0.0001$). There was no difference in the

effect induced in each group because the interaction term (group x time) was non significant (P = 0.55). (Figure 4)

6.2.4 Pulmonary vascular resistance (PVR)

Between groups comparisons did not detect significant differences (P = 0.57). However, *a priori* comparisons detected a statistically significant difference between at T4 (P = 0.02) and T24 (P = 0.005). This difference reflected a greater PVR in the continuous INO group. There was a significant (P < 0.002) main effect in pooled data of both groups of an increase in PVR over time

There was a significant effect of time (P = 0.002), with an increase in PVR compared with T0 at all timepoints except for T5 (P = 0.15), T6 (P = 0.16) and T6.5 (P = 0.20). (Figure 5)

6.2.5 Systemic vascular resistance (SVR)

Between groups comparisons detected a significantly higher (P = 0.03) SVR in the continuous INO group compared with the weaned INO group. There was a tendency towards a significant effect of time (P = 0.05), although there was no significance detected at any time point compared with T0. The effect observed on SVR was identical in both groups (non significant interaction; time x group, P = 0.92).

6.2.6 Summary

Of the cardiovascular parameters monitored, significant differences were not detected between groups for the following parameters: pulmonary vascular resistance, mean pulmonary arterial pressure and mean systemic arterial pressure. Significant differences were detected between groups for cardiac index (greater in the weaned INO group) and systemic vascular resistance (greater in the continuous INO group).

There was a significant increase in MPAP and PVR for both groups over time.

There was a significant decrease in CI, SVR, and MAP over time in both groups.

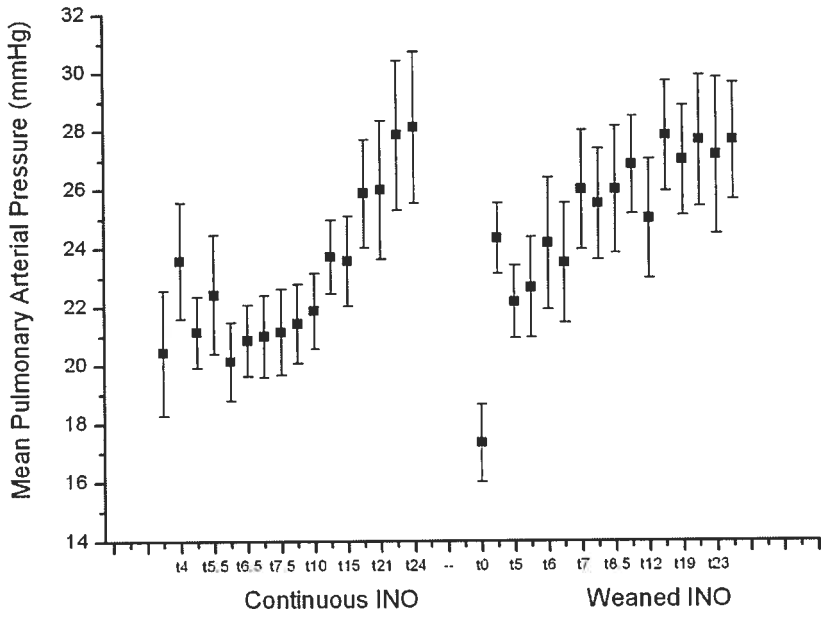

Figure 3: MPAP for continuous *vs* weaned INO groups. Data points represent mean ± SEM. From left to right, time points represent T0, T4, T5, T5.5, T6, T6.5, T7, T7.5, T8.5, T10, T12, T15, T19, T21, T23 and T24 for each group. Weaning of NO occurred between T4 and T5.5.

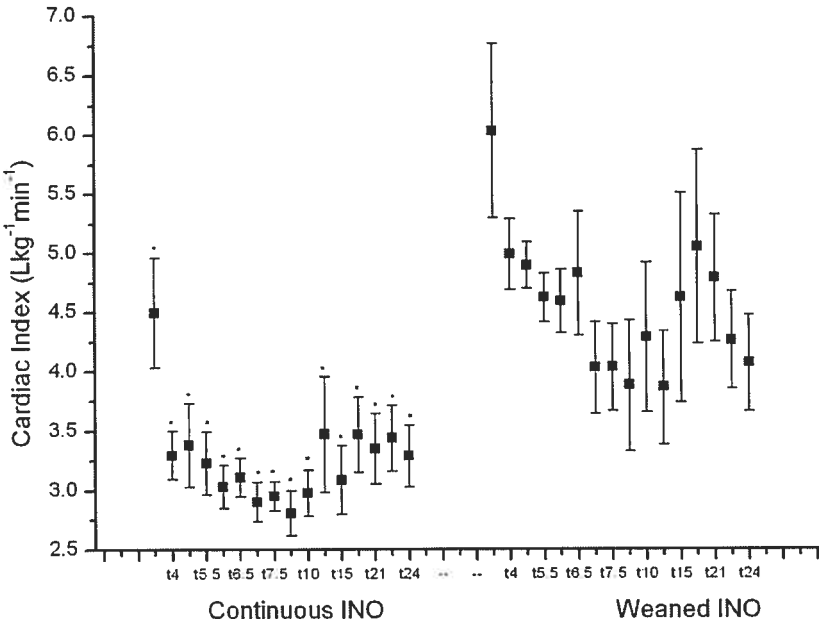

Figure 4: CI for continuous *vs* weaned INO groups. From left to right, time points represent T0, T4, T5, T5.5, T6, T6.5, T7, T7.5, T8.5, T10, T12, T15, T19, T21, T23 and T24 for each group. Weaning of NO occurred between T4 and T5.5. Asterisks represent time points at which significant differences were detected between groups.

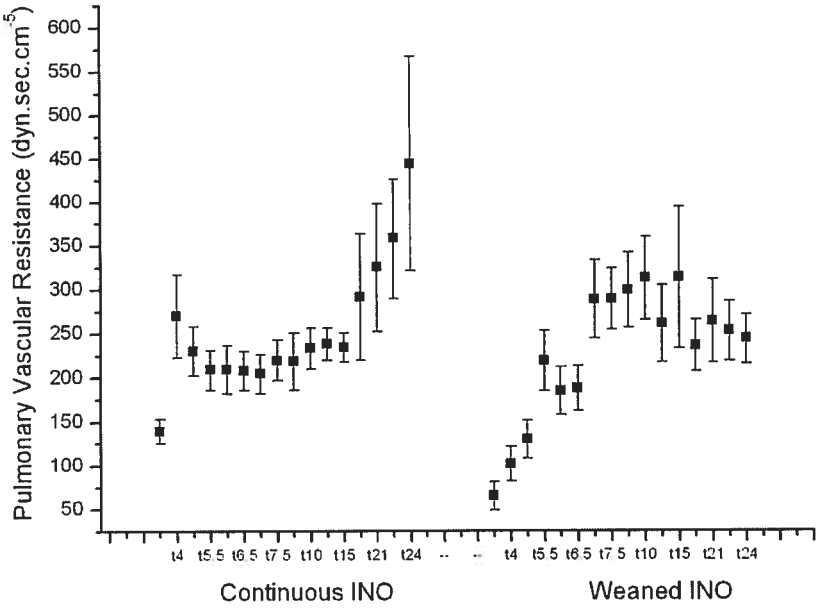

Figure 5: PVR for continuous *and* weaned INO groups. From left to right, time points represent T0, T4, T5, T5.5, T6, T6.5, T7, T7.5, T8.5, T10, T12, T15, T19, T21, T23 and T24 for each group. Weaning of NO occurred between T4 and T5.5.

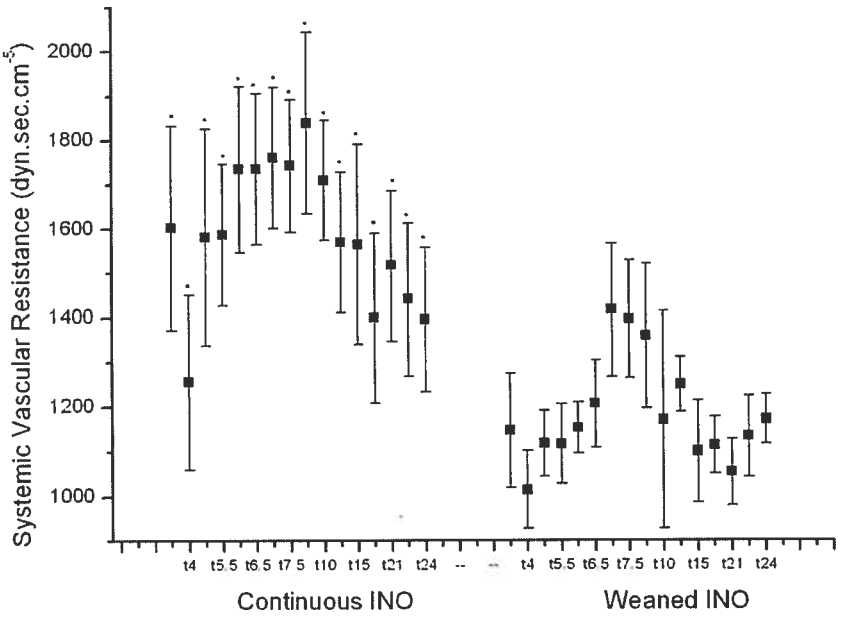

Figure 6: SVR for continuous *and* weaned INO groups. From left to right, time points represent T0, T4, T5, T5.5, T6, T6.5, T7, T7.5, T8.5, T10, T12, T15, T19, T21, T23 and T24 for each group. Weaning of NO occurred between T4 and T5.5. Asterisks represent time points at which significant differences were detected between groups.

6.3 Respiratory data

6.3.1 PaO_2:FIO_2 ratio

No significant differences were detected between groups (P = 0.26) with all time points included in the analysis. However, a significant interaction term ($P = 0.03$) was associated with a significantly greater PaO_2:FIO_2 ratio in the continuous group at T0 ($P = 0.002$). A significant effect of time was present for pooled data ($P < 0.0001$), with significantly decreased PaO_2:FIO_2 ratio at all time points compared with T0 ($P < 0.0001$ for all comparisons).

6.3.3 Physiologic Shunt (Qs/Qt)

There was no significant difference between groups (P = 0.52). Over time, a significant decrease in shunt at T4 was detected compared with T0 for both groups (P = 0.027).

6.3.5 Compliance

Significant differences were not detected in the between groups comparison (P = 0.74).

There was a significant decrease ($P < 0.0001$) over time in pooled data from both groups, at all time points compared with T0, with the exception of T4 (P = 0.21).

6.3.6 Summary

Of the respiratory parameters monitored, there was no overall significant difference between groups. Pooling data from both groups over time, there was a siginificant decrease in the PaO_2:FiO_2 ratio and compliance, and a tendency towards a significant increase in deadspace

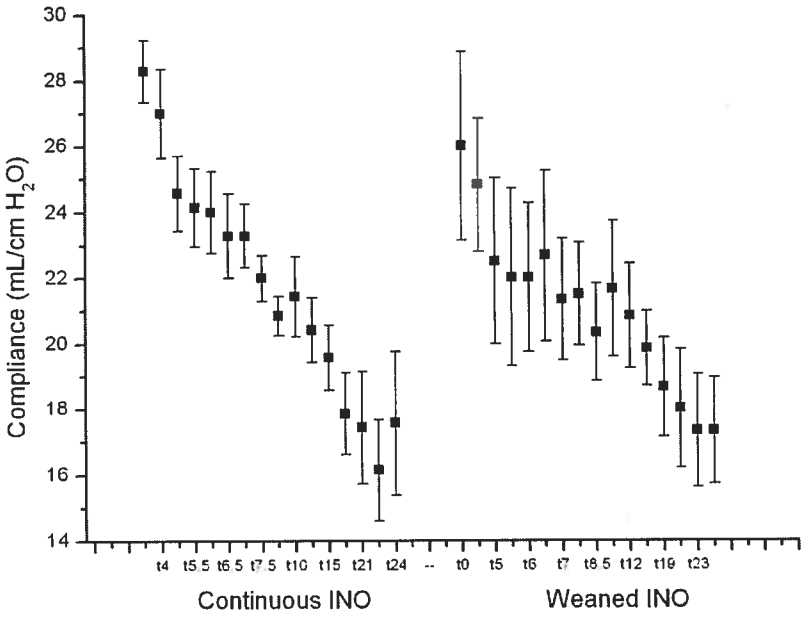

Figure 7: Compliance for continuous *and* weaned INO groups. From left to right, time points represent T0, T4, T5, T5.5, T6, T6.5, T7, T7.5, T8.5, T10, T12, T15, T19, T21, T23 and T24 for each group. Weaning of NO occurred between T4 and T5.5.

7. Discussion

Within our study, every pig was weaned successfully without further intervention. Of the hemodynamic parameters measured, CI and SVR were significantly different between groups.

Therefore, the null hypothesis of absence of difference between weaned and continuous INO therapy is rejected.

The lack of significant differences in PVR between the weaned or continuous INO groups highlights the effectiveness of a carefully managed weaning strategy in avoiding rebound pulmonary hypertension, as was encountered by many early studies.[19,21]

7.1 Inhaled NO and rebound hypertension

Abrupt discontinuation of INO treatment in adults and children results in a transient, though potentially life threatening, rebound increase in pulmonary arterial hypertension (PAH), an increase in intrapulmonary right-to-left shunting and PVR, a decrease in PaO_2[14,196,197] and a decrease in dynamic respiratory system compliance[24] *i.e.* reversal of the benefits gained from INO. This response to the abrupt discontinuation of INO is irrespective of initial response, though occurs only after exposure to INO of several hours.

The underlying mechanisms of such a reaction are unclear, though several candidates have been proposed:

1) Inhibition of eNOS upon exposure to INO (reversible following removal of INO) was the first mechanism proposed.[15,17] The mechanism of action is believed to be through direct action of INO on eNOS[15] or *via* interaction with superoxide[18] leading to a reduction in NOS activity without a reduction in either NOS protein expression[17,19] or alteration in gene expression.[18,20]

2) More recently, ET has been implicated in rebound PAH. Physiologically, there is evidence for regulation between NO and ET through an autocrine feedback loop.[198] The delivery of INO has been associated with an increase in ET plasma

levels (but without concomitant increases in gene expression), suggesting a possible role for ET following INO withdrawal in rebound PAH.[22] Tying in with earlier research implicating a role for superoxide interaction with eNOS,[18] it was recently demonstrated that an ET receptor may be involved in the production of superoxide.[21]

Rebound PAH and hypoxemia can be minimised with careful stepwise weaning of INO, initiated in stable patients (in terms of PaO_2 and required FiO_2). Experience has shown that weaning from a low concentration of INO (1 ppm or less) is likely to have a lesser (rebound and deleterious) effect on PaO_2[23,25] than from a higher concentration (4 or 16 ppm).[199]

7.1.1 Effectiveness of weaning

Sign	**Occurrence**
Pulmonary arterial hypertension	No significant differences between groups, significant increase over time in both groups.
Pulmonary vascular resistance ↑	Increased in both groups over time. No significant difference between groups
Decreased oxygenation	PaO_2:FIO_2 decreased over time. No significant differences between groups.
Decreased compliance	Decreased in both groups over time. No significant difference between groups

Table IV: Signs associated with potential rebound pulmonary hypertension and occurrence during experimentation.

7.1.2 Effects of continuous INO *versus* control groups

Our initial assessment was a comparison of the effects of continuous INO *versus* control groups (see attached article, Appendix II, "Pre-emptive and continuous inhaled NO counteracts the cardiopulmonary consequences of extracorporeal circulation in a pig model"). The conclusion of this study was that inhaled NO had no long-term beneficial effect on lung mechanics and surfactant homeostasis despite improving lung hemodynamics, inflammation and oxygenation, and that

the use of pre-emptive and continuous inhaled NO therapy has protective and safe effects against lung ischemia/ reperfusion associated with CPB.

7.1.3 Effects of continuous *versus* weaned INO groups

Comparisons over time for each variable in both the continuous and weaned INO groups revealed several results of interest.

7.1.3.1 Hemodynamic data

MPAP: both groups showed a significant increase over time (Figure 3). This change is likely due to the increasingly present effects of VILI and the systemic inflammatory response to CPB. It should be noted that continuous INO, when compared with animals not receiving INO, showed a statistically significant beneficial effect (lower value for MPAP; see attached paper, Appendix II). The lack of significant differences between groups highlights the safety of the weaning protocol employed.

PVR: There was no significant difference between groups.

A significant effect of time was detected in both groups. The likely reason behind this change is as explained above for MPAP; VILI has been demonstrated to cause pathological changes similar to ALI/ ARDS[200] and would therefore contribute to the deleterious effects of a systemic inflammatory response to CPB. The two components of MPAP are CI and PVR, Therefore the absence of difference in CI induced in either group explains that the change in MPAP observed is a result of alteration in pulmonary vasoconstriction. Using the ventilation strategy employed in our study, VILI was unavoidable, though it was out with the aims of the study to employ protective ventilation strategies. More work still remains to be done on the protocols of such strategies and with our model a change in ventilation protocol would have been necessary as soon as signs of VILI emerged (difficult to distinguish from inflammation in response to CPB) and introduced variability whilst interfering with the desired controlled delivery of INO. Furthermore, a study examining the effects of INO in a ventilated (using a very similar ventilatory strategy to ours) porcine model

without surgical interference, found stability in both hemodynamic and respiratory parameters over time, suggesting a minor contribution from VILI.[201] It is unclear why PVR was at a lower, albeit non significant level at T0 ($P = 0.29$) and T5 ($P = 0.15$) in the weaned group, compared with the continuous INO group. And also, why there was a significant difference between groups at T4 and T24.

Two surprising results were with CI and SVR.

CI: there was a significantly higher CI in the weaned group compared with the continuous INO group at all timepoints. However, looking at T0, the weaned group was at a higher level of CI. The effect of termination of CPB induced a similar decrease in CI in both groups. As the continuous INO group had a lower CI at T0, the overall effect, despite a similar decrease post-CPB, was a lower CI at all time points compared with the weaned INO group. Indeed, the INO weaning process did not induce a greater degree of CI depression despite the theoretical fact that the initially higher value of CI in the weaned INO group, could magnify any subsequent difference between groups.

SVR: there was a significantly higher SVR in the continuous INO group compared to the weaned group. In both groups there was a pattern to a sharp decrease in SVR immediately following termination of CPB, followed by an increase, then decrease in SVR over the remainder of the experiment. This was likely due to a variable other than the localized effects of INO. A systemic inflammatory response would be associated with vasodilatation and a decrease in SVR. The greater level of SVR in the continuous INO group is in accordance with the lower CI seen in this group. A greater degree of vasoconstriction was required to maintain a stable MAP (no significant difference between groups). A greater degree of vasodilation was not observed in the weaned INO group.

Another possibility is that the time of increase (T6- T7.5) reflected a time of instability following CPB, associated with the use of vasoconstrictors (as standardized in the Methodology section and to be used as required).

MAP: there were no significant differences in MAP between groups. There was a significantly lower MAP in both groups over time compared with T0. The observed decrease following end of CPB was as a result of the systemic effects of

CPB. However, any difference was artificially maintained above an MAP of 60 mm Hg in accordance with experimental protocol.

7.1.3.2 Respiratory data

Of the respiratory data, oxygenation was assessed by the PaO_2:FIO_2 ratio.

The PaO_2:FIO_2 ratio decreased over time in both groups to similar degrees and over a similar time frame. These changes are unlikely to be due to INO weaning. If weaning were to have had an effect, a difference would be expected more acutely at T4. The effect of the ventilatory strategy used and the likely occurrence of VILI, in conjunction with a systemic inflammatory response may explain the changes seen over time in both groups. Though any benefits of INO appear limited over time in our model, it is clear there are benefits with the use of INO compared with controls groups (see attached paper, "Pre-emptive and continuous inhaled NO counteracts the cardiopulmonary consequences of extracorporeal circulation in a pig model." [Appendix II]).

Lung compliance: although there were no significant differences between groups, compliance decreased significantly over time in both groups, again reflecting the combined effects of VILI in conjunction with the systemic inflammatory response. It should be noted that weaning of INO did not result in a further decrease in compliance compared with the continuous INO group. Moreover, as observed previously (Appendix II), INO treatment does not counteract the progressive decrease in compliance following CPB.

Physiologic Shunt: no significant differences were detected between groups. However there was a significant decrease in shunt at T4 compared with T0. This is likely due to the beneficial effect of INO (see Appendix II).

7.2 Ventilator-induced lung injury (VILI)

VILI is a well documented syndrome[200] whereby barotrauma[202-204] and biotrauma[205] can lead to lung injuries in normal lungs functionally and histologically similar in appearance to ALI and ARDS[204] in animal models. Furthermore, damage in already injured lungs is likely to be increased; the range

of compliance found in lungs with ARDS favours further damage as a result of traditional mechanical ventilation.[206] The counterpart of VILI in humans is ventilator-associated lung injury (VALI).[207]

7.2.1 Pathophysiology of VILI

VILI is associated with alveolar hemorrhage, hyaline membrane formation, pulmonary edema and inflammation, atelectasis, hypoxemia and the release of inflammatory mediators.[207,208]

Protective ventilation strategies, as presented below, have a beneficial effect in patients with ALI and ARDS. The main determinants of VILI are excessive tidal volume and/ or end-inspiratory alveolar volume.[207] However, the mechanisms underlying VILI and therefore the means of improvements are yet to be fully understood.

Alveolar epithelial plasma membrane stress failure as a result of mechanical stretch induced injury is an important basic mechanism of VILI.[204] It occurs when the supporting matrix of alveolar epithelial cells undergoes large deformations as may occur with mechanical ventilation. Underlying lung lesions such as atelectasis, as found in ALI/ ARDS patients, may increase susceptibility to such mechanical stress. The precise method of mechanical ventilation is important in that deformation frequency, duration and amplitude play roles of varying importance in causing injury, the inter-relationships are yet to be fully understood[209] though it has been found that reducing the amplitude of deformation through PEEP, decreased lung injury.[203]

Surfactant dysfunction and depletion, as occurs in ARDS and ALI leads to alveolar instability, atelectasis and increased shear stress under ventilation. This has been documented in vivo using video microscopy during ventilation of normal and surfactant deactivated lungs.[210] In normal lungs, the alveoli never collapsed and there was little change in volume. In the surfactant deactivated lungs, alveoli collapsed, reopened and over distended during ventilation. Subsequently, PEEP applied to surface deactivated lungs was found to reduce alveolar size increases to control levels.[211]

Computed tomography demonstrated that the lungs of patients with ARDS is not uniformly damaged, leading to mechanically driven air following a path of least resistance within the lungs and causing alveolar over distension and bullae formation.[205]

At a cellular level, over inflation results in alveolar epithelial[212] and endothelial[213] permeability. This permeability follows from increases in intracellular calcium through stretch-activated ion channels leading to the involvement of tyrosine kinases, activation of the calcium-calmodulin pathway and phosphorylation of the myosin light chain.[200] Such biochemical evidence indicates more than merely physical damage as a cause for increased permeability.

Conventional mechanical ventilation in both intact lungs and those with ALI leads to the recruitment and activation of inflammatory cells (primarily neutrophils and macrophages).[204,214] Such recruitment, *via* pro-inflammatory cytokines, is an important aggravating factor in VILI. A large number of animal studies using cytokine receptor antagonists, antibodies, detection of cytokines in BAL and gene expression have uncovered the involvement of interleukin-1, interleukin-8, transforming growth factor-beta and interleukin-6 in leukocyte recruitment and subsequent VILI.[200]

For some time, there has been interest in the possibility of decompartmentalization, whereby VALI contributes to systemic inflammation and MODS through loss of the compartmentalization/ localization of inflammatory mediators and bacteria to the lungs.[215,216] However, clinical evidence of this hypothesis is sparse. The recent prospective, randomized, multicenter trial investigating the effects of low tidal volume and plateau airway pressure on ARDS patient mortality also demonstrated a reduction in plasma IL-6, highlighting the possibility of a link between improved mortality and levels of inflammatory mediators.[207]

In summary, it remains that the improvement in mortality rates over the last 30 years have resulted solely from alterations in mechanical ventilator strategy, with strong evidence now backing up clinical practice.[207] Undoubtedly, mechanical ventilation has more than a physically injurious effect on the pulmonary system

but a clear link between cytokines, resultant inflammation, and pulmonary injury possibly extending to systemic effects is yet to be clearly demonstrated.[217,218]

7.3 Conclusions

- Comparing weaned and continuous INO groups demonstrated no significant difference between groups in pulmonary vascular resistance, highlighting **the safety of a controlled weaning** process.

- Changes in hemodynamic and respiratory parameters over time may reflect the effects of systemic and localised (pulmonary) inflammation as a result of an inflammatory response to CPB and possibly VILI opposing any beneficial effects of INO.

Our experiments have effectively demonstrated the safety of careful weaning, but were weak in describing contributions by other factors such as VILI, probably as a result of small sample size.

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Appendix 1

Increased alveolar and plasma gelatinases activity during post-pump syndrome: Inhibition by inhaled nitric oxide

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Abstract

Post-pump syndrome is associated with systemic inflammation. Matrix metalloproteinases (MMP)-2 and -9 contribute to pro-inflammatory and platelet-activator reactions. Nitric oxide is involved in regulation of MMPs. The objectives of our study were to investigate the intensity of inflammation induced by three different surgical procedures, its effects on the activity of MMPs and its regulation by inhaled nitric oxide (20 ppm). Inhaled nitric oxide was initiated immediately after tracheal intubation and maintained for total duration of experiments. Thirty pigs were equally randomized into 6 groups [sham; sham + nitric oxide; cardiopulmonary bypass; bypass + nitric oxide; bypass + lipopolysaccharide (1 μg/Kg for 50 min); bypass + lipopolysaccharide + nitric oxide] and animals were subjected to anesthesia and mechanical ventilation up to 24 hours. The levels of MMP-2 and MMP-9 in plasma and bronchoalveolar lavage were measured using zymography. Bypass resulted in a time-dependent rise in MMPs activity, an effect potentiated by lipopolysaccharide. Inhaled nitric oxide attenuated the effects of bypass + lipopolysaccharide. These results confirm that MMP-2 and MMP-9 are associated with the inflammatory process causing the post-pump syndrome. Pre-emptive and continuous administration of inhaled nitric oxide helps to prevent increased MMP-2 and MMP-9 activity.

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Matrix–metalloproteinases (MMPs) are zinc-dependent endopeptidases, known for their ability to cleave one or several constituents of the extracellular matrix. Zymogen forms of the MMPs (pro-MMPs) are secreted in the matrix from a large number of cells types such as epithelial, endothelial or smooth muscle cells [1,2]. Activation of the pro-MMPs in the local environment can result in discrete alterations in tissue architecture and their main role is physiologic tissue remodeling during wound repair and growth development [1]. Several studies have shown that extracellular matrix degradation by MMPs, specifically MMP-9, is involved in the pathogenesis of a wide spectrum of cardiovascular disorders, including atherosclerosis, restenosis, cardiomyopathy, congestive heart failure, myocardial infarction, aortic aneurysm and post-pump syndrome [3-5]. The major physiologic inhibitors of the MMPs *in vivo* are α-2 macroglobulin and the family of specific tissue inhibitor of MMPs (TIMPs), naturally occurring proteins specifically inhibiting these proteases and produced by many cell types. The TIMPs bind with high affinity in a 1:1 molar ratio to the catalytic site of active MMPs, resulting in loss of proteolytic activity, particularly TIMP-1 to 72-kDa (MMP-2) and 92 kDa (MMP-9) gelatinases. Moreover, TIMP-1 and TIMP-2 can form a specific complex with pro-MMP-9 and pro-MMP-2, respectively. This interaction has been suggested to provide an extra level of regulation by potentially preventing activation. The production of gelatinase is controlled by a variety of agents including pro-inflammatory cytokines, such as interleukin 1-β and tumor necrosis factor-α [6].

Since the early days of cardiac surgery, it has been recognized that cardiopulmonary bypass (CPB) is associated with systemic inflammation, occasionally leading to major organ dysfunction. When organ dysfunction cannot be directly attributed to a specific cause, such as infection or ischemia, the concept of the "post-pump syndrome" or "systemic inflammatory response syndrome to CPB" is used as an alternative explanation [7]. Reduced arterial oxygenation is a common complication of the post-pump syndrome [8]. In a study of 400 patients undergoing a variety of cardiac surgeries with CPB, there was a 40% decrease in dynamic lung compliance within the first 4 hours, and in alveolar-arterial oxygen gradient (from 296 at intensive care unit arrival to 152 at 12 hours and 181 mmHg at 24 hours) [9]. Acute respiratory distress syndrome (ARDS), the most severe form of lung dysfunction with a PaO_2/FiO_2 ratio below 200 mmHg, has an incidence rate after CPB of 0.5 to 1.7 % with mortality reported to be between 40 to 60 % or higher [7,10].

Numbers of studies have associated the release of gelatinases with CPB and pulmonary injury [4,11-14]. During and after CPB in humans, these MMPs are synthesized and released in plasma [11-13] and in heart tissue (right atrial biopsy specimens) [13]. In addition, MMP-2 and MMP-9 levels are increased in bronchoalveolar lavage (BAL) of ARDS patients [14]. In experimental conditions, isolated rat hearts submitted to ischemia-reperfusion have highlighted the deleterious role of MMP-2 in the recovery of mechanical function during reperfusion [15]. In an animal model of CPB-induced acute lung injury (ALI) the importance of MMPs (-2 and -9) was demonstrated when all pathological changes typical of ALI after CPB were prevented by using a chemically modified tetracycline, a potent MMP and elastase inhibitor [4].

Usually, nitric oxide (NO) inhalation improves hypoxemia in ALI / ARDS patient [16]. The mediator NO has been suggested to have anti-inflammatory properties. It inhibits the release of cytokines and prevents the expression of adhesion molecules in endothelial cells, smooth muscle cells, leukocytes and platelets [17,18]. The administration of a NO donor (S-nitroso-glutathione) during CPB reduced the Ca^{2+}-independent activity of NO synthase (NOS) [19]. Furthermore, NO donors and prostacyclin inhibited the secretion of gelatinase A (MMP-2) induced by collagen and thrombin in intact platelets, indicating that enzyme release is controlled by both compounds [20]. On the other way, pre-emptive infusion with a NO scavenger decreased gelatinases activities in the lung, left ventricle and atrium in a canine CPB model [21]. With use of a non-selective NOS and soluble guanylate cyclase inhibitors, it was suggested that NO and cyclic GMP are necessary to up-regulate the expression of MMP-9 [22].

Our objective in this study was first to measure the activity of MMP-2 and MMP-9 in sham-operated pigs and in animals subjected to CPB in the presence or absence of lipopolysaccharide (LPS). Acute lung injury / ARDS after CPB can develop after consecutive minor insults, with CPB acting as the initial inflammatory event [23]. A short period of CPB alone is rather innocuous, but when combined with a subsequent, seemingly benign insult (*i.e.*, transient hypoxia, ischemia, endotoxemia with a low dose of LPS), the result is an overwhelming inflammatory response leading to endothelial injury, pulmonary edema, and ALI / ARDS [24]. We also aimed to examine if pre-emptive administration of inhaled NO exerts significant effects on MMPs activity in our anesthetized and mechanically ventilated porcine model of extracorporeal circulation [25].

Experimental procedures

This study was performed with the approval of the institutional animal care committee in compliance with Canadian Council on Animal Care guidelines.

Animals and experimental protocol

Thirty acclimated, 4-month old hybrid (Pietrain x Landrace) castrated male pigs (37.2 ± 2.5 Kg), free from clinically evident pulmonary disease were randomized into six groups of five animals. The first (sham) group was submitted to sternotomy with a 90-minute pericardial opening, followed by closure. The second (CPB) group was subjected to 90-minute CPB with 75-minute aortic clamping. The third (CPB + LPS) group was subjected to the CPB procedure as above, plus receiving an infusion of 1 μg/Kg of *E. coli* LPS O111:B4 (Sigma-Aldrich; Stockholm, Sweden) mixed in 100 ml of isotonic saline and delivered at a flow rate of 120 ml/hour during 50 minutes commencing one hour post-operatively [23]. Fourth (sham + inhaled NO), fifth (CPB + inhaled NO), and sixth (CPB + LPS + inhaled NO) groups were submitted to the same procedure as their respective controls plus the administration of 20 parts per million inhaled NO, initiated immediately after induction of anesthesia and maintained for the surgery and the whole 24 hours follow-up period.

Experimental set-up, ventilation strategy, CPB and inhaled NO administration (20 parts per million) were performed as previously described by our group [25]. We have selected a dose of inhaled NO (20 parts per million), as this dose causes a significant pulmonary vasodilatation [26], whilst ensuring an inspired fraction of NO_2 below 1 part per million with our synchronized intermittent mandatory ventilation system of NO administration [27].

Pigs were premedicated intramuscularly with atropine (0.04 mg/Kg), azaperone (4 mg/Kg) and ketamine (25 mg/Kg), and anesthesia induced with intravenous (auricular vein) fentanyl (5 μg/Kg) and thiopental (5 mg/Kg). After intubation with an 8 mm ID endotracheal tube (Mallinckrodt Company, Mexico City, DF, Mexico), the pigs were placed in a supine position. Anesthesia was maintained by continuous infusion of thiopental (5 mg/Kg/h) and fentanyl (20 μg/Kg/h). Muscle relaxation was induced with 0.2 mg/Kg pancuronium with intermittent re-injection (0.1 mg/Kg) to achieve optimal surgical and ventilatory conditions. After endotracheal intubation, 20 ppm of NO gas was injected cyclically into the inspiratory line during the inspiratory phase by a NO injector for 24 hours. A 1,000 ppm NO balanced N_2 cylinder™ was obtained from VitalAire Santé Ltd. (Montreal, Quebec, Canada). The NO and NO_2 concentrations delivered to the animals were monitored with an electrochemical device (Polytron NO/NO_2™, Drager A.G., Lubeck, Germany). During CPB, NO was added directly to the gas mixture delivered to the oxygenator. Hemodynamic and respiratory monitoring was constant and for the whole (24 hours) duration of the experiment [28].

CPB materials and procedure

A median sternotomy was performed, and at the time of pericardial opening an intravenous injection of heparin (4 mg/Kg) was given to achieve an activated clotting time > 400 seconds. An aortic cannula (20 French; Chase Medical Inc., Richardson, TX, USA) was placed in the aortic root followed by

placement of a multiple hole venous drainage cannula in the caudal vena cava *via* the right auricular appendage. A cardioplegia cannula (9 French; Medtronic Inc., Grand Rapids, MI, USA) was then placed in the aortic root proximal to the aortic valve, enabling cold blood cardioplegia. The CPB circuit consisted of a membrane oxygenator (Trillium Affinity NT oxygenator™, Medtronic Inc., MN, USA), a cardiotomy reservoir (Affinity NT541™, Medtronic Inc., Minneapolis, MN, USA), a filter (Affinity 351™, Medtronic Inc.), tubing and a Sarns™ roller pump (Sarns Inc., Ann Arbor, MI, USA). The circuit was primed with 1,500 mL lactated Ringer's solution™ (Baxter Corporation, Toronto, Ontario, Canada), 500 mL Pentaspan™ colloidal fluid (DuPont Pharma Inc., Mississauga, Ontario, Canada), 1 meq/Kg sodium bicarbonate, 5,000 IU heparin, and 200 mL mannitol. Cardiopulmonary bypass was initiated at a flow rate of 3.0 L/min/m^2 and blood temperature was decreased to 32°C. Following aortic clamping, cardiac arrest was induced by continuous hyperkaliemic cold blood cardioplegia (8°C to 12°C) through the aortic cardioplegia cannula at a flow rate of 500 mL/min, not exceeding a perfusion pressure of 100 mm Hg. Heart temperature measured by a temperature probe placed in the left ventricular myocardium was maintained below 15°C throughout the procedure. Pulmonary capillary wedge pressure was kept less than 15 mm Hg by intermittent interruption of cardioplegia and cardiac decompression through the cardioplegia aortic cannula. Mean systemic arterial blood pressure (mSAP) was maintained between 50 and 65 mm Hg by adjustment to the flow rate, plus a bolus of phenylephrine [0.5-1 mL (0.1 mg/mL)] if required. Arterial partial pressure of carbon dioxide ($PaCO_2$) was maintained between 40 and 50 mm Hg by adjusting fresh gas flow. The aorta was clamped for 75 min. Blood re-warming commenced 10 min prior to aortic de-clamping using water at 38°C. Following aortic clamp removal, the heart was electrically defibrillated (20 joules) as ventricular fibrillation is observed in most pigs after reperfusion [25]. The cardioplegia was stopped and the animals were subsequently weaned from CPB once rectal temperature was above 35°C. The total length of CPB was at least 90 min. Intravenous protamine (1 mg/100 IU heparin) normalized activated clotting time. Hemostasis was performed after removal of the CPB cannulae, thoracic drainage placed under negative pressure, and the chest was closed. Blood from the CPB circuit was subsequently transfused.

At T0 (just before chest opening), T4 (2 hours post-CPB) and T24 (22 hours post CPB), blood and BAL samples were collected for the measurement of MMPs. Fiberoscopy was undertaken for BAL with isotonic sterile saline solution injected as 3 aliquots of 25 ml. Lavage was performed at T0 in the right accessory lobe, T4 in the lower left lobe, and T24 in the lower right lobe. The total recovered BAL samples (kept at 4°C throughout procedure) were filtered, centrifuged for 8 min at 150×*g* to separate the surfactant (supernatant) from cells and cellular debris. Five (5) mL of the supernatant was divided into 500 µL aliquots and frozen at -80°C for further analysis (IL-8 [29,30]; TNF-α [30]; MMPs). Total cell count in BAL was achieved by the hemocytometer method.

MMP-2 and MMP-9 analysis

The release of MMP-2 and MMP-9 during CPB was measured by zymography as described previously [20,31]. This method has been validated for the MMP measurement in humans and in a canine model of CPB [13,21]. Briefly, zymography is performed by subjecting samples (20 μg protein each) to 8% SDS-PAGE with copolymerized gelatin (2 mg/ml; Sigma, St-Louis, MO) as a substrate. After electrophoresis, the gels are washed with 2% Triton X-100, and then incubated in buffer (50 mM Tris-HCl buffer with 0.15 M NaCl, 5 mM $CaCl_2$, and 0.05% NaN_3, pH 7.5) at 37°C until the activities of the enzymes can be determined. After incubation, the gels are stained with 0.05% Coomassie brilliant blue G-250 (Sigma, St-Louis, MO) in a mixture of methanol: acetic acid: water (2.5:1:6.5) and destained in 4% methanol with 8% acetic acid. The gelatinolytic activities are detected as transparent bands against the background of Coomassie brilliant blue-stained gelatin. Enzyme activities are quantified using a gel documentation system (Bio-Rad Laboratories, Mississauga, ON) and expressed as specific activity per milligram of proteins (UI/mg proteins) [20,31].

Statistical analysis

The comparison of MMP-2 and MMP-9 activities between experimental groups was done using Kruskall-Wallis and Mann Whitney tests with Dunn's correction for *post hoc* analysis. Time-courses of this release were compared using Friedman test. Where significance was found in this intra-group comparison, a Wilcoxon test was used for *post hoc* analysis, followed by Dunn's correction. The level of statistical significance was set at 0.05 before correction and data are presented as mean ± standard deviation.

Results

Inter-groups comparison

In plasma, the rise of MMP-2 activity occurred at time T24 in pigs submitted to CPB *versus* sham ($p < 0.05$) (Fig 1A,B) and to CPB + LPS *versus* sham at time T4 ($p < 0.01$) (Fig 1A) and T24 ($p < 0.05$) (Fig 1B). The rise of MMP-9 activity was significantly higher after CPB + LPS *versus* sham and CPB at time T4 ($p < 0.01$) (Fig 2A). The difference was no more present at time T24.
In BAL, MMP-2 activity increased significantly in pigs submitted only to CPB + LPS *versus* sham at time T4 ($p < 0.01$) (Fig 1C) and at time T24 ($p < 0.01$) (Fig 1D). The rise of MMP-9 activity was significantly higher in pigs submitted to CPB + LPS *versus* sham ($p < 0.01$) at time T24 (Fig 2D).

Time- dependent effect of the insult on MMPs for each group

In BAL and plasma, there was no difference in time for MMP-2 and MMP-9 in the sham group without or with inhaled NO.
The MMP-2 activity increased in BAL over time in the CPB group at time T4 compared to time T0 (ns) and at time T24 *versus* time T0 ($p < 0.01$) (Fig 3); this effect was also present in the CPB + LPS group ($p < 0.05$ at time T24 *versus* time T0) (Fig 4). Activity of MMP-9 increased significantly over time in BAL in the CPB group at time T24 *versus* time T0 ($p < 0.05$) and in CPB + LPS group at time T24 *versus* time T4 ($p < 0.05$).
In plasma, MMP-2 also demonstrated an increased activity over time in both CPB ($p < 0.01$ at time T24 *versus* time T0) (Fig 3) and CPB + LPS groups ($p < 0.01$) at time T24 *versus* time T0) (Fig 4). In plasma, the rise of MMP-9 activity occurred in the CPB + LPS group at time T4 *versus* T0 ($p < 0.05$) then MMP-9 activity returned to the initial value at time T24.

Effect of the procedure

The sham procedure did not induce any change in MMPs.
The CPB alone induced an increase of MMP-2 activity in plasma and BAL at T24, and of MMP-9 only in BAL at T24. This was corroborated by the statistically significant comparison between sham and CPB groups at T24 for MMP-2 in plasma.
The CPB + LPS procedure similarly induced an increase of MMP-2 and MMP-9 activity in BAL at T24, and of MMP-2 in plasma at the same time. Moreover, an increase in MMP-9 was observed in plasma for this group at T4, but was no more present at T24. This was corroborated by the statistically significant comparison between sham and CPB + LPS groups at T4 and T24 for MMP-2 in plasma and BAL, and for MMP-9 in plasma at T4 and in BAL at T24.

Effect of inhaled NO (Fig 1, Fig 2)

In sham group, MMP-2 and MMP-9 activities were not changed with inhaled NO both in BAL and in plasma at time T4 and time T24.
In the CPB group, the rise of MMP-2 activity in BAL was significantly higher compared to CPB + inhaled NO group at time T24 ($p < 0.05$) (Fig 1D). In plasma, the rise of MMP-2 activity was also significantly higher in the CPB

group compared to CPB + inhaled NO at time T4 ($p < 0.05$) (Fig 1A) and time T24 ($p < 0.05$) (Fig 1B). These differences were not found for MMP-9 activities both in BAL and in plasma.
In CPB + LPS group, the rise of MMP-2 activity in BAL was significantly higher in CPB + LPS group compared to CPB + LPS + inhaled NO at time T4 ($p < 0.05$) (Fig 1C) and time T24 ($p < 0.05$) (Fig 1D). This effect was significant for MMP-9 activity in BAL only at time T24 ($p < 0.05$) (Fig 2D). In plasma, the rise of MMP-2 activity was significantly higher in the CPB + LPS compared to CPB + LPS + inhaled NO group at time T4 ($p < 0.05$) (Fig 1A) and T24 ($p < 0.05$) (Fig 1B). This effect was not found for MMP-9.

Cell count in BAL and leukocyte count in blood

There was no change in the BAL cell count over time neither in the three inhaled NO-treated groups, or in the sham group. However, at T24, the BAL cell count was statistically higher compared to T0 in both CPB ($p = 0.007$) and CPB + LPS ($p = 0.02$) groups. The increased value at T24 in CPB and CPB + LPS groups was higher than the value in the sham group ($p < 0.03$). The difference between control groups and inhaled NO-treated groups did not reach statistical significance ($p = 0.09$) at T24.
Moreover, the neutrophil differentials in BAL were also greatly augmented ($p < 0.05$) at T24 in the CPB (+206%) and CPB + LPS (+244%) groups, but were lower in the inhaled NO treated animals without reaching statistical significance. No similar difference could be observed for monocytes or lymphocytes in BAL. The CPB + LPS procedure had a significant effect on the blood leukocyte count over time: whereas it decreased with time in the sham and CPB ($p = 0.004$) groups, it increased in the CPB + LPS group. In the sham and CPB groups, leukocyte count decreased over time respectively from $18.9 \pm 5.1\ 10^3$ / mm^3 at time T0 to $13.7 \pm 1.6\ 10^3$ / mm^3 at time T24 and $22.4 \pm 5.5\ 10^3$ / mm^3 at time T0 to $13 \pm 1.6\ 10^3$ / mm^3 at time T24. In the CPB + LPS group, leukocyte count increased from $16.7 \pm 3.5\ 10^3$ / mm^3 at time T0 to $18.9 \pm 7.3\ 10^3$ / mm^3 at time T24. Moreover, inhaled NO had no effect on blood leukocyte count over time.

Discussion

The post-pump syndrome constitutes a model of systemic and local inflammatory reactions with cellular (leukocytes, platelets, endothelial cells) activation and the release of pro-inflammatory cytokines and enzymes mediating acute inflammation and organ injury [7,24]. We have developed a porcine model to study the gelatinase activity after CPB and their potential implication in the associated inflammatory response.
As CPB constitutes only an initial stimulus not systematically associated with consequences, inflammatory reaction was potentiated with subsequent LPS infusion. At 1 μg/Kg for 50 min, LPS in association with CPB increases the inflammatory reaction to induce a real post-pump syndrome and at this dose, it does not induce ALI alone [4,23,24].

Inhaled NO decreases neutrophil and platelet activation and inhibits neutrophil function in ischemia / reperfusion [32] and ARDS [33] conditions. Moreover, combined therapy with NO gas (20 ppm) and iloprost (a stable analog of prostacyclin, 2 ng/Kg/min) reduced the deleterious effects of CPB in human patients, such as thrombocytopenia, platelet activation, platelet-leukocyte aggregate formation, and suppression of platelet aggregative responses [34]. This result was in agreement with previous demonstration of a possible beneficial effect of NO donors to inhibit the Ca^{2+}-independent activity of NOS increased after CPB [19], or the secretion of MMP-2 [20]. In the same time, inhibition of the NO pathway decreased the MMP-2 and –9 activities after CPB [21] and prevented the up-regulation of MMP-9 [22]. As neutrophils and platelets are implicated in the physiopathology of post-pump lung syndrome and release of gelatinases, we have studied the effect of inhaled NO on MMP-2 and MMP-9 activity in plasma and BAL in pig submitted to sham operation, CPB and CPB + LPS. We hypothesized that the post-pump syndrome could be induced by an initial deficiency in endogenous NO in relation with the non-pulsatile blood flow generated by the CPB machine, decreasing the shear stress, source of endothelial NOS stimulation. Deficient NO production affects the inflammatory cascade, allowing the vascular adhesion of inflammatory cells primed by contact with the extracorporeal circuit. Indeed, we have observed with the same model decreased levels of nitrite, nitrate (NOx), stable plasma metabolites of the endogenous NO production in all three groups treated without inhaled NO (sham, CPB, CPB + LPS) [29,30]. This was not the case in comparative groups treated with pre-emptive 20 ppm inhaled NO in swine [29,30] and in human patient [35]. This initial deficit in endothelial NO synthesis is particularly marked in older patients and could be judiciously replaced by a pre-emptive administration of exogenous NO donor [19] or inhaled NO [29,30,35] at low dose. The pre-emptive use of a NO donor suppressed the increased Ca^{2+}-independent activity of iNOS observed after CPB [19] and the inhibition of the NO pathway decreased gelatinases activity after CPB [21]. Therefore, we speculated that NO replacement therapy could blunt the consequences of induction of NOS activity and systemic inflammation [35], leading to a decreased activity of gelatinases.
The release of MMPs during CPB appears to be dependent on the intensity (as reflected by the initial inflammatory insult induced by CPB and amplified by repeated subsequent stimuli such as mechanical ventilation or the LPS

perfusion) of the inflammatory stimuli. Indeed, in BAL and plasma, the levels of MMP-2 and MMP-9 remained stable in the sham group over the 24 hours period. In pigs submitted to CPB, MMP-2 activity increased only in plasma after 24 hours, compared to the sham group. This could reflect the summation of inflammatory stimuli induced initially by CPB and repeated by the ventilator-induced lung injury under general anesthesia. In pigs submitted to CPB + LPS, the increase in MMP-2 activity occurred both in plasma and in BAL immediately after the end of LPS administration (at T4) and for a period of 22 hours and was significantly higher than in the sham group. The rise of MMP-9 activity was significantly higher in plasma after LPS infusion (at T4) compared to CPB group, but only occurred in BAL after 24 hours compared to the sham group. We can conclude that the LPS administration after CPB constitutes a potent stimulus for the rise of the MMPs activities. Our data confirm the results described by Carney *et al.* [4] and Picone *et al.* [23]. Interestingly the CPB + LPS group was the only group that developed severe physiological lung injury typical to ARDS associated to post-pump syndrome [4,23,28]. This was corroborated by the greater release of MMPs in BAL and blood, as well as the increase in leukocyte levels in blood.

Our results may be explained by the different expression patterns of MMP-2 and MMP-9. Whereas MMP-2 is constitutively expressed in various cell types, MMP-9 is strongly induced in epithelial cells by inflammatory cytokines, particularly TNF-α [6,36]. Inflammatory conditions are also associated with increased activation of oxidant-producing enzymes and NO, which could directly affect MMP expression or activation [19–22]. The release of MMP-2 from intracellular reserve in plasma and BAL cells may explain the precocious rise of MMP-2 activity in plasma and in BAL. The same mechanism could explain the rapid rise of MMP-9 activity in plasma. As a matter of fact, infusion of LPS to healthy volunteers resulted in a rapid increase of plasma MMP-9 activity, likely resulting from liberation of this gelatinase from leukocytes, endothelium and vascular cells [37]. However, the rise of MMP-9 activity in BAL appeared only after 24 hours in our experiment. This time allows for induction of expression of MMP-9 [36]. This could also reflect the role of the lung as end-organ to present the induced release of MMP-9 in plasma with a lag time in BAL.

Inhaled NO abolished significantly the rise in MMP-2 activity in plasma after 4 and 24 hours and in BAL after 24 hours in CPB group without effect in sham operation, whereas neither CPB alone or CPB + inhaled NO did affect MMP-9. The effect of inhaled NO was marked in the CPB + LPS group where the rise in gelatinase activity was the highest. In this group, inhaled NO abolished significantly the rise in MMP-2 both in plasma and BAL after 4 and 24 hours. The effect of inhaled NO on MMP-9 was not significant in plasma (whereas MMP-9 increased in plasma at T4 for CPB + LPS) while this effect was present in BAL after 24 hours. As inhaled NO did not (or minimally) influence cells count, cells composition in BAL, leukocytes and platelets count in blood, these results may be explained by the inhibition of the release from intracellular stores or by a decreased induction of MMPs synthesis by cells. Inhaled NO acted as an anti-inflammatory agent by decreasing neutrophil numbers in BAL and its major chemoattractant, IL-8 [29,30], as well as MMPs. Inhaled NO also increased cell apoptosis in the lungs during

inflammatory conditions, which may explain, in part, how it resolves pulmonary inflammation [29,30].
Okamoto, *et al.* [38] demonstrated that the inhibitory effects of *S*-nitrosothiols on MMP-9 expression were associated with diminished nuclear translocation and activation of NF-κB. NF-κB activation is involved in the induction of various pro-inflammatory genes and is critical in inflammatory-immune processes in the lung. Various studies have indicated that NO (or *S*-nitrosothiols) may regulate inflammatory processes by suppressing NF-κB activation (*e.g.*, in neutrophils or alveolar macrophages)[39,40]. Consequently, the inhibitory effects of *S*-nitrosothiols on MMP-9 expression may be caused by inhibition of NF-κB activation, and the effects on MMP-9 are most likely not specific but may be common to other NF-κB–regulated genes [38].
Badly, neither Okamoto, *et al.* [38] or ourselves have measured the TIMP-1 level in plasma and BAL. To have a complete idea of the regulation of MMP activity, it would have been indicated to do it (but technically more difficult), particularly with the recent demonstration that exogenous NO has no modulatory effect on the extracellular TIMP-1 content but strongly amplifies the early increase in cytokine-induced TIMP-1 mRNA and protein levels [39].

We conclude that MMP-2 and MMP-9 may play a role in the pathogenesis of inflammatory lesions encountered during the use of CPB procedure, particularly when associated with a post-pump syndrome. Furthermore, the release of MMPs appears to be related to the severity of inflammatory stimuli. The pre-emptive, continuous administration of inhaled NO attenuates the rise of MMP-2 and MMP-9 activity related to the inflammatory reaction. These results, in association with the beneficial effects of inhaled NO on oxygenation and pulmonary hemodynamics in the same pig CPB model [28], and the reduced postoperative bleeding observed with the combined treatment of inhaled NO and iloprost [34], suggest that the pre-emptive and continuous administration of inhaled NO will have to be considered as a possible new therapeutic option for patients subjected to CPB: before that, the exact mechanisms of action have to be précised, as well as the clinical use including selection of patients, starting and weaning conditions of the NO inhalation, possible toxicity and side effects.

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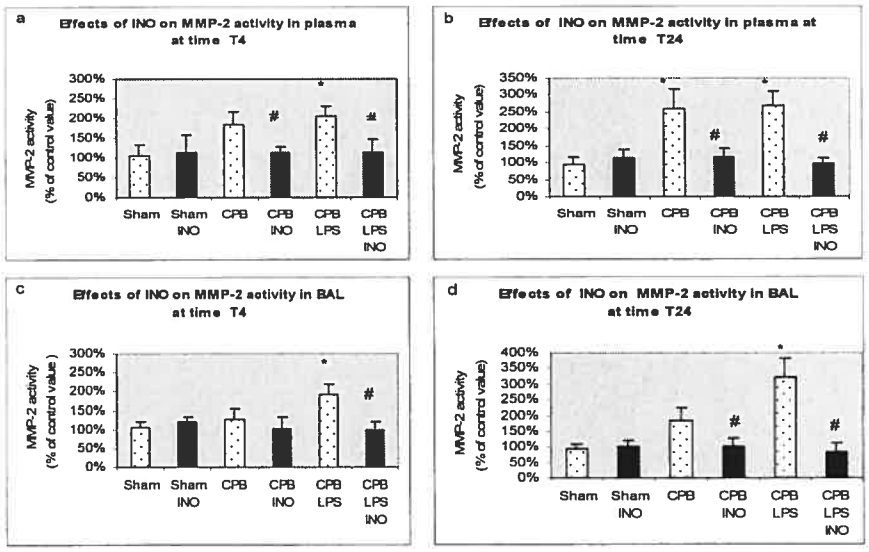
Fig 1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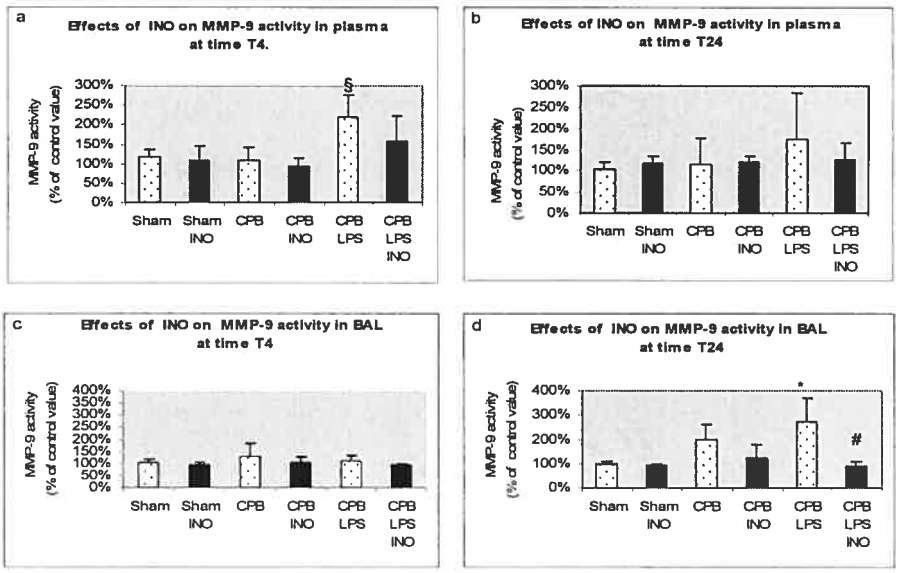
Effects of INO on MMP-2 activity in plasma at time T4
MMP-2 activity (% of control value)
300%
250%
200%
150%
100%
50%
0%
Sham
Sham INO
CPB
CPB INO
CPB LPS
CPB LPS INO
b
Effects of INO on MMP-2 activity in plasma at time T24
MMP-2 activity (% of control value)
350%
300%
250%
200%
150%
100%
50%
0%
Sham
Sham INO
CPB
CPB INO
CPB LPS
CPB LPS INO
c
Effects of INO on MMP-2 activity in BAL at time T4
MMP-2 activity (% of control value)
300%
250%
200%
150%
100%
50%
0%
Sham
Sham INO
CPB
CPB INO
CPB LPS
CPB LPS INO
d
Effects of INO on MMP-2 activity in BAL at time T24
MMP-2 activity (% of control value)
400%
350%
300%
250%
200%
150%
100%
50%
0%
Sham
Sham INO
CPB
CPB INO
CPB LPS
CPB LPS INO

Fig 2


a
Effects of INO on MMP-9 activity in plasma at time T4.
MMP-9 activity (% of control value)
300%
250%
200%
150%
100%
50%
0%
§
Sham
Sham INO
CPB
CPB INO
CPB LPS
CPB LPS INO
b
Effects of INO on MMP-9 activity in plasma at time T24
MMP-9 activity (% of control value)
300%
250%
200%
150%
100%
50%
0%
Sham
Sham INO
CPB
CPB INO
CPB LPS
CPB LPS INO
c
Effects of INO on MMP-9 activity in BAL at time T4
MMP-9 activity (% of control value)
400%
350%
300%
250%
200%
150%
100%
50%
0%
Sham
Sham INO
CPB
CPB INO
CPB LPS
CPB LPS INO
d
Effects of INO on MMP-9 activity in BAL at time T24
MMP-9 activity (% of control value)
400%
300%
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#
Sham
Sham INO
CPB
CPB INO
CPB LPS
CPB LPS INO

Fig 3

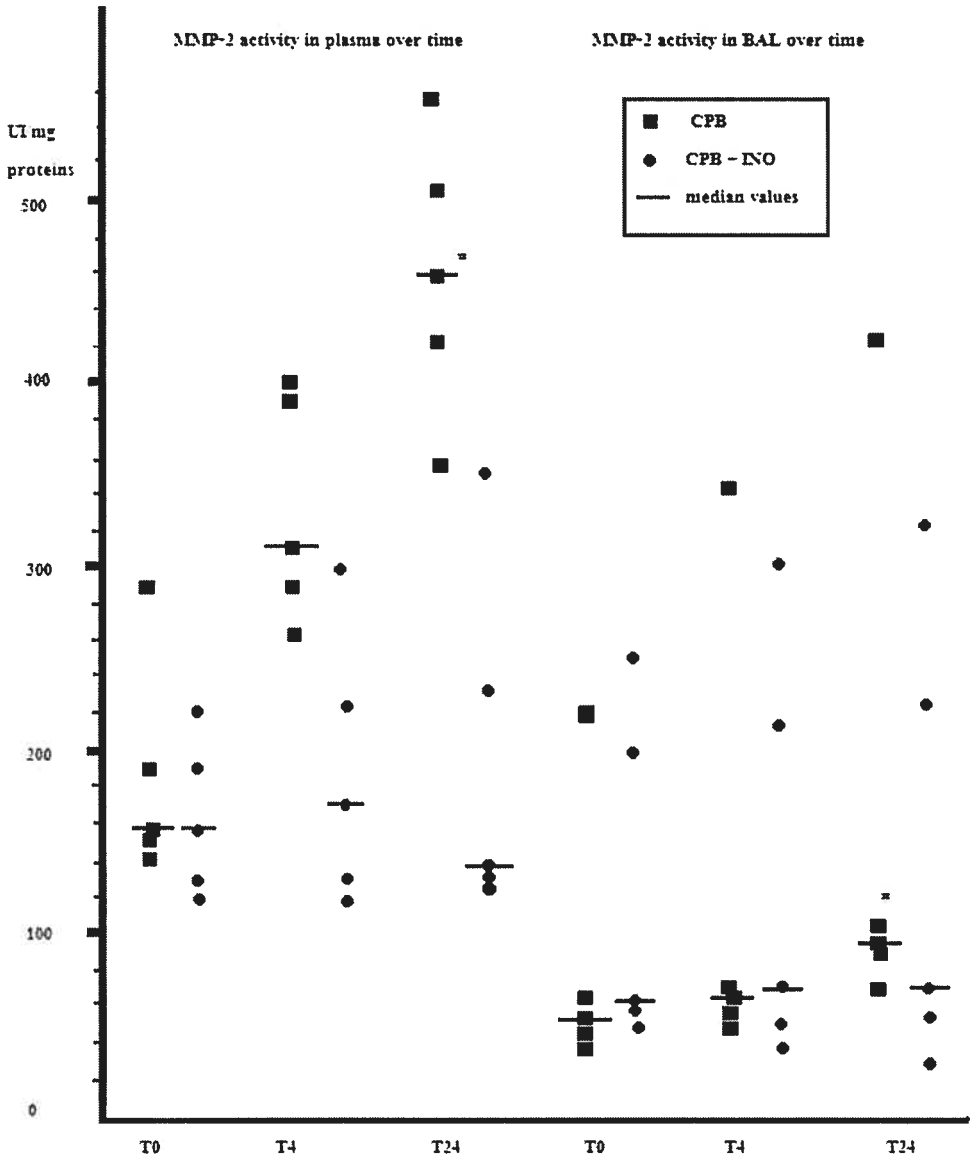
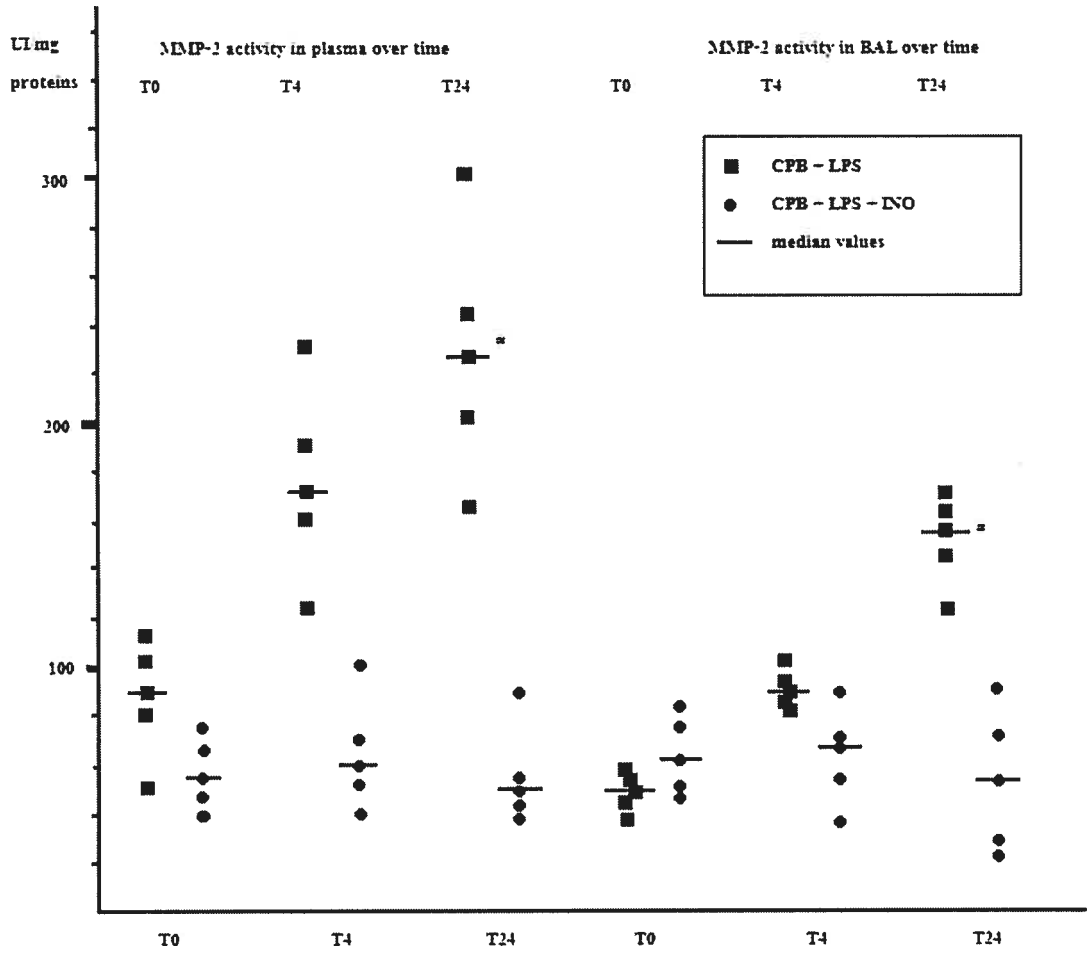

MMP-2 activity in plasma over time
MMP-2 activity in BAL over time
UI mg
proteins
500
400
300
200
100
0
CPB
CPB + INO
median values
T0
T4
T24
T0
T4
T24

Fig 4


UI/mg proteins
MMP-2 activity in plasma over time
MMP-2 activity in BAL over time
T0
T4
T24
T0
T4
T24
300
200
100
CPB – LPS
CPB – LPS – INO
median values
*
*
T0
T4
T24
T0
T4
T24

Legends

Fig. 1. Variation of MMP-2 activity in BAL and plasma at time T4 and T24 versus T0 (%).
Intergroup comparison * $p < 0.05$ *versus* sham, # $p < 0.05$ *versus* group without inhaled NO.

Fig. 2. Variation of MMP-9 activity in BAL and plasma at time T4 and T24 versus T0 (%). Intergroup comparison * $p < 0.05$ *versus* sham, § $p < 0.05$ *versus* CPB, # $p < 0.05$ *versus* group without inhaled NO.

Fig. 3. MMP-2 activity in plasma and BAL in CPB group over time (UI/mg proteins).
# $p < 0.05$ *versus* T0.

Fig. 4. MMP-2 activity in plasma and BAL in CPB + LPS group over time (UI/mg proteins). # $p < 0.05$ *versus* T0.

Appendix 2

Pre-emptive and continuous inhaled NO counteracts the cardiopulmonary consequences of extracorporeal circulation in a pig model.

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Abstract

Cardiopulmonary bypass (CPB) activates a systemic inflammatory response characterized clinically by alterations in cardiovascular and pulmonary function. The aim of this study was to measure the cardiopulmonary consequences in sham-operated pigs, and in animals subjected to CPB in the presence or absence of lipopolysaccharide (LPS). We also investigated, if the perioperative administration of inhaled NO exerts significant cardiopulmonary effects in an anaesthetized and mechanically ventilated pig model of extracorporeal circulation. Thirty pigs were randomized into 6 equal groups (sham; sham + INO; CPB; CPB + INO; CPB + LPS; CPB + LPS + INO) and subjected to anaesthesia with mechanical ventilation for up to 24 hours. We found that CPB + LPS group has the highest degree of lung injury. We also demonstrated that there was a significant difference on the cardiovascular parameters (heart rate, central venous pressure, stroke volume index, and mean systemic arterial blood pressure) between the CPB groups and the sham groups. The deteriorated lung mechanics was associated with a decrease in active subfraction of surfactant (LA) with time during the procedure ($P = 0.0003$), on which inhaled NO had only an initial beneficial effect. In our model, inhaled NO had no long-term beneficial effect on lung mechanics and surfactant homeostasis despite improving lung haemodynamics, inflammation and oxygenation. We conclude from this study that the use of pre-emptive and continuous inhaled NO therapy has protective and safe effects against lung ischemia/reperfusion associated with CPB.

WORD COUNT: 237 words

KEY WORDS: Inflammation, Lung, Cardiopulmonary bypass, Nitric oxide, Inhalation.

Cardiopulmonary bypass (CPB) is the most common means of enabling cardiac surgery. Approximately 750,000 cases of cardiac surgery with a CPB period are performed annually in North America [1].

Postoperative lung dysfunction is a common complication after CPB [2-4]. For up to 75,000 of these patients, the procedure induces an inflammatory process that results in acute pulmonary injury, namely acute lung injury (ALI) and the more extreme form of this syndrome, acute respiratory distress syndrome (ARDS) [5]. The general incidence of ARDS is 1-3% with a mortality of 30-50%, whilst the incidence of ALI after CPB is five times higher [6-8]. The risk of development and severity of ALI have been positively linked to CPB duration [9].

Pulmonary dysfunction below the threshold of ALI classification occurs in 12% of patients and includes ventilation/perfusion (V/Q) mismatch, reduced oxygenation index and reduced lung compliance [10].

Nitric oxide (NO) inhalation improves hypoxaemia in ALI/ARDS patients [11,12]. Inhaled NO decreases elevated pulmonary arterial pressure (PAP). It reduces right ventricle afterload as in cases of pulmonary embolism [13], heart and/or lung transplantation [14] and various high-risk cardiac procedures involving coronary artery bypass grafting [15,16], Fontan-type operation [17], and/or left ventricular assist device placement [18,19]. It improves hypoxaemia by reducing intra-pulmonary shunt, and optimizing V/Q matching. Moreover, NO inhalation is associated with: (i.) a decrease in pulmonary neutrophil and platelet sequestration in animal models with acute pulmonary injury [20-24] and, (ii.) a decreased secretion of oxidative substances by neutrophils during ALI/ARDS, suggesting that the deleterious effects induced by neutrophils could be reduced [25]. Inhaled NO can also inhibit the inflammatory process by reducing cytokine synthesis and inactivating nuclear factor–κB [26-28], and by decreasing the expression of adhesion molecules, preventing neutrophil adhesion and migration. The Main effects of inhaled NO are localised to the lung, *e.g.* on leukocytes trapped in the pulmonary area. However, through carriage by blood borne molecules to the general circulation, inhaled NO also has extrapulmonary effects [29,30].

The relationship between the severity of CPB-induced inflammatory response and the cardiopulmonary complications has not been established yet. Our objective in this study was twofold: Firstly, to measure the cardiopulmonary consequences in sham-operated pigs, and in animals subjected to CPB in the presence or absence of lipopolysaccharide (LPS); Secondly, to examine if the perioperative administration of inhaled NO exerts significant cardiopulmonary effects in an anaesthetized and mechanically ventilated porcine model of extracorporeal circulation [31].

Experimental procedures

This study was performed with the approval of the institutional animal care committee in compliance with Canadian Council on Animal Care guidelines.

Animals and experimental protocol

Thirty acclimated, hybrid male pigs (mean ± SD; 37.15 ± 2.48 Kg), free from clinically evident pulmonary disease were randomized into six groups of five animals. The procedure was similar for all pigs. After induction of anaesthesia and instrumentation, a 15-min period was allowed for stabilizing haemodynamic and blood gas parameters, at which point data was recorded as baseline values (T_{base}). Over the next 2-2.5 hours, the surgical procedure was performed, the end of which was recorded as T_0: the first group (sham) was submitted to sternotomy with a 90-min pericardial opening, followed by surgical site closure; the second group (CPB) was subjected to 90 min CPB with 75-min aortic clamping; the third group (CPB + LPS) was subjected to the CPB procedure as above, plus an infusion of 1 μg/Kg of *E. coli* LPS O111:B4 (Sigma-Aldrich; Stockholm, Sweden) mixed in 100 mL of isotonic saline and delivered at a flow rate of 120 mL/h over a 50 min period [32], which commenced one hour post-operatively, *i.e.* from T_1 to T_{18}. Fourth (sham + INO), fifth (CPB + INO), and sixth (CPB + LPS + INO) groups were submitted to the same procedure as their respective controls plus the administration of 20 parts per million (ppm) inhaled NO, initiated immediately after induction of anaesthesia and maintained for the surgery and the entire post-operative period, which ended at T_{22}, *i.e.* 24-hours after T_{base}.

Experimental set-up, ventilation strategy, CPB and inhaled NO administration (20 ppm) were performed as previously described by our group [31]. The dose of inhaled NO (20 ppm) was selected to achieve significant pulmonary vasodilatation [33], whilst ensuring an inspired fraction of NO_2 below 1 ppm with our system [34]. Pigs were premedicated intramuscularly with atropine (0.04 mg/Kg), azaperone (4 mg/Kg) and ketamine (25 mg/Kg), and anaesthesia induced with intravenous (auricular vein) fentanyl (5 μg/Kg) and thiopental (5 mg/Kg). After intubation with an 8 mm ID endotracheal tube (Mallinckrodt Company, Mexico City, DF, Mexico), the pigs were placed in a supine position. Anaesthesia was maintained by continuous infusion of thiopental (5 mg/Kg/h) and fentanyl (20 μg/Kg/h). Muscle relaxation was induced with 0.2 mg/Kg pancuronium with intermittent re-injection (0.1 mg/Kg) to achieve optimal surgical and ventilatory conditions. After endotracheal intubation, 20 ppm of NO gas was injected cyclically into the inspiratory line during the inspiratory phase by a NO injector for 24 hours. A 1,000 ppm NO balanced N_2 cylinder™ was obtained from VitalAire Santé Ltd. (Montreal, Quebec, Canada). The NO and NO_2 concentrations delivered to the animals were monitored with an electrochemical device (Polytron NO/NO_2™, Drager A.G., Lubeck, Germany). During CPB, NO was added directly to the gas mixture delivered to the oxygenator.

Haemodynamic monitoring and support

Monitoring was by continuous ECG, rectal temperature, urine output (Foley catheter placed by cystotomy), and systemic arterial blood pressure [*via* a 20 gauge arterial catheter (Arrow International Inc., Reading, PA, USA) placed in the left carotid artery]. Mean PAP, pulmonary capillary wedge pressure (PCWP), central venous pressure (CVP), cardiac output (in duplicate at end-expiration) and blood temperature were monitored *via* a Swan-Ganz pulmonary artery catheter (Abbott Laboratories, Chicago, IL, USA) placed in the right internal jugular vein.

A cardiovascular monitor (M1166A™ Model 66S, Hewlett Packard Ltd., Palo Alto, CA, USA) collected haemodynamic data, including the following calculated variables: cardiac index, stroke volume index (SVI), systemic vascular resistance index (SVRI) and pulmonary vascular resistance index (PVRI).
Fluid and drug administration was performed *via* an 18-14 gauge double lumen venous catheter (Arrow International Inc., Reading, PA, USA) placed in the left external jugular vein.

Ventilation strategy and respiratory monitoring

A ventilator (7200AE™, Puritan Bennett, Carlsbad, CA, USA) was used for mechanical ventilation in the intermittent positive pressure mode with positive end-expiratory pressure (PEEP) of 5 cm H_2O. Tidal volume (V_T; 10 mL/Kg) and respiratory frequency were adjusted to maintain end-tidal CO_2 partial pressure between 40 and 50 mm Hg. The F_iO_2 was maintained at 1.0 during the surgical procedure, and was reduced to 0.5 at the end of the procedure, maintaining $PaO_2 > 85$ mm Hg. Ventilation was continued during CPB with a reduced V_T (3mL/Kg) and ventilation frequency (8 breaths/min). All respiratory data was measured with a Capnomac Ultima™ monitoring system (Datex Instrumentation Corp., Helsinki, Finland). Parameters monitored were pulmonary mechanics (peak, plateau, end-expiration and mean airway pressures, PEEP, respiratory rate, V_T, minute volume, end-tidal CO_2 and dynamic thoraco-pulmonary compliance), and gas exchange (arterial and mixed venous blood gas and electrolyte analyses; I-STAT™ Clinical Analyzer, Sensor Devices Inc., Waukesha, WI, USA). The oxygenation index (PaO_2/FiO_2), arterial-to-venous O_2 content difference ($C_aO_2 - C_vO_2$) alveolar-to-arterial O_2 tension difference ($A\text{-}aDO_2$) and physiologic shunt/venous admixture (Q_S/Q_T) were calculated.

All vascular and airway pressures, CO, arterial and mixed venous blood gas samples, and urine output measurements were recorded at T_{base}, hourly from T_0 (end of CPB) to T_6, and at T_{10}, T_{16} and T_{22}.

CPB materials and procedure

A median sternotomy was performed, and at the time of pericardial opening an injection of intravenous heparin (4 mg/Kg) was given to achieve an activated clotting time > 400 seconds. An aortic cannula (20 French; Chase Medical Inc., Richardson, TX, USA) was placed in the aortic root followed by placement of a multiple hole venous drainage cannula in the caudal vena cava *via* the right auricular appendage. A cardioplegia cannula (9 French; Medtronic Inc., Grand Rapids, MI, USA) was then placed in the aortic root proximal to the aortic valve, enabling cold blood cardioplegia. The CPB circuit consisted of a membrane oxygenator (Trillium Affinity NT oxygenator™, Medtronic Inc., MN, USA), cardiotomy reservoir (Affinity NT541™, Medtronic Inc., Minneapolis, MN, USA), filter (Affinity 351™, Medtronic Inc.), tubing and a Sarns™ roller pump (Sarns Inc., Ann Arbor, MI, USA). The circuit was primed with 1,500 mL lactated Ringer's solution™ (Baxter Corporation, Toronto, Ontario, Canada), 500 mL Pentaspan™ colloidal fluid (DuPont Pharma Inc., Mississauga, Ontario, Canada), 1 meq/Kg sodium bicarbonate, 5,000 IU heparin, and 200 mL mannitol. Cardiopulmonary bypass was initiated at a flow rate of 3.0 L/min/m² and blood temperature was decreased to 32°C. Following aortic clamping, cardiac arrest was induced by continuous hyperkaliemic cold blood cardioplegia (8°C to 12°C) through the aortic cardioplegia cannula at a flow rate of 500 mL/min, not exceeding a perfusion pressure of 100 mm Hg. Heart temperature measured by a temperature probe placed in the left ventricular myocardium was maintained below 15°C throughout the procedure. Pulmonary capillary wedge pressure was kept less

than 15 mm Hg by intermittent interruption of cardioplegia and cardiac decompression through the cardioplegia aortic cannula. Mean systemic arterial blood pressure (mSAP) was maintained between 50 and 65 mm Hg by adjustment to the flow rate, plus a bolus of phenylephrine [0.5-1 mL (0.1 mg/mL)] if required. Arterial partial pressure of carbon dioxide ($PaCO_2$) was maintained between 40 and 50 mm Hg by adjusting fresh gas flow. The aorta was clamped for 75 min. Following aortic clamp removal, the heart was electrically defibrillated (20 joules) as ventricular fibrillation is observed in most pigs after reperfusion [31]. The animals were subsequently weaned from CPB once rectal temperature was above 35°C. The total length of CPB was at least 90 min. Intravenous protamine (1 mg/100 IU heparin) normalized activated clotting time. Haemostasis was performed after removal of the CPB cannulae, thoracic drainage placed under negative pressure, and the chest was closed. Blood from the CPB circuit was subsequently transfused.

Bronchoalveolar lavage (BAL) sampling and analyses

At T_{base} (just prior to chest opening), T_2 (2 h post-CPB) and T_{22} (22 h post CPB), blood and BAL samples were collected [35]. All respiratory parameters, even those with continuous follow-up, will be presented for the same time-points to facilitate comparison. The total recovered BAL samples (kept at 4°C throughout procedure) were filtered, centrifuged for 8 min at 150×*g* to separate the surfactant (supernatant) from cells and cellular debris. Five mL of the supernatant was divided into 500 µL aliquots and frozen at -20°C (total surfactant; TS) and -80°C for further analysis (IL-8 [35,36]; TNF-α [36]; MMPs [37]). The remaining supernatant was centrifuged at 40,000×*g* for 15 min to separate the two subfractions of surfactant: large aggregates (LA; the pellet) and small aggregates (SA; the supernatant). Both LA and SA were stored at -20°C for further analysis.

Surfactant analysis

Phospholipid (PL)-phosphorus measurement was used to determine the total quantity of surfactant PLs. Lipids were first extracted from the samples (TS, LA and SA), using the method of Bligh and Dyer [38]. Phospholipid-phosphorus levels were determined using the Duck-Chong phosphorus assay as described previously [39]. Briefly, 100 µL of 10% $MgNO_3$ in CH_3OH were added to the extracted lipids. Samples were then dried and ashen in a fume hood on an electric rack for approximately 1 min. One mL of 1M HCl was added to the samples. Samples were covered and reheated at 95°C for 15 min. After cooling, 66 µl of each sample was added to individual wells of a 96-well plate along with 134 µl of a dye: 4.2% ammonium molybdate in 4.5 M HCl with 3% malachite green (1: 3 vol./vol.). The absorbency of the samples (triplicate) was read at 650 nm, and the phosphorus concentration was calculated using a standard curve of phosphorus ranging from 0.1 to 1.1 µg on the sample plate. The amount of surfactant in each fraction was calculated and expressed as milligrams of PL per kilogram of body weight (mg PL/Kg).

Cell analysis

The pellet of the 150×*g* centrifugation was resuspended in 10 mL RPMI-1640 + 10% foetal calf serum, and viability was determined by trypan blue exclusion. Total cell count was achieved by the haemocytometer method; differential cytospin slides were produced and stained with Wright-Giemsa for cell differentiation. Cells were counted under a microscope at 100× magnification [35,36].

Statistical analysis

Analysis of continuous dependent variables was performed with a linear mixed model for repeated measures (SAS version 9.0, Cary, NC, USA). *A priori* contrasts were performed to compare different levels of the independent variables at different time points. In particular, we analyzed the variation with time for each group taken separately or pooled adequately with regards to the procedure. Variation between groups at different time-points was analyzed by comparison of groups for procedure (sham *vs.* CPB *vs.* CPB + LPS) or treatment with inhaled NO. Values are presented as mean ± SD. The level of statistical significance was set at 0.05.

Results

For all parameters, we will first present the effect of the treatment, inhaled NO, followed by the effect of the procedure (sham *vs.* CPB *vs.* CPB + LPS). There was no statistically significant difference in rectal temperature and urine output between groups throughout the experimental period.

Haemodynamic data

All groups demonstrated a significant variation with time of mPAP (Fig. 1).
-When looking at the overall effect of inhaled NO, higher mPAP values were observed in the groups without inhaled NO from T_1 to T_{22} ($P < 0.05$). This was confirmed by the pair-wise comparisons demonstrating a significant difference between the two sham groups from T_3 to T_4 ($P < 0.03$); between the two CPB groups from T_1 to T_{16} ($P < 0.03$); and between the two CPB + LPS groups from T_3 to T_5 ($P < 0.01$).
-The two CPB + LPS groups, with and without inhaled NO, differed significantly from the remaining groups at T_2 (*i.e.* first time point after the end of LPS administration), and the CPB + LPS group differed significantly from the remaining groups from T_3 to T_5 ($P < 0.04$). There was no significant difference between the sham groups, with and without inhaled NO, and the CPB groups, with and without inhaled NO, respectively, at any time.

-The PVRI followed a similar course, with the exception of the CPB + INO group, which demonstrated no significant variation with time (Fig. 2).
A significant difference between groups, with higher PVRI values in the groups without inhaled NO, was observed from T_1 to T_{22} ($P < 0.01$). The pair-wise comparisons confirmed a significant difference between the two sham groups from T_2 to T_4 and T_{10} to T_{22} ($P < 0.05$), between the two CPB groups from T_1 to T_3 and T_6 to T_{16} ($P < 0.04$), and between the two CPB + LPS groups from T_3 to T_5 and T_{10} to T_{16} ($P < 0.02$).
-The CPB + LPS group PVRI was higher than all other groups at T_4 and T_5 ($P < 0.04$).

-Treatment with inhaled NO had no significant effect on other haemodynamic parameters.
-No significant difference was found between groups for cardiac index, SVRI, and PCWP. The two latter variables (SVRI and PCWP) did not show any statistically significant difference over time. The cardiac index decreased significantly from T_{base} (5.2 ± 1.3 L/min.m^2) to T_1 (3.6 ± 0.9 L/min.m^2) for all groups (n=30). A significantly lower heart rate (Fig. 3) was observed in the sham groups from T_3 to T_{22} ($P < 0.05$) compared to the four CPB groups. Central venous pressure was similarly affected, with a significant difference from T_5 to T_{22} between the two sham groups (7.1 ± 2 mm Hg; n=10) and the four CPB groups (10.2 ± 1.8 mm Hg; n=20). For the four CPB groups, the mSAP decreased significantly from T_{base} (69.5 ± 13.2 mm Hg; n=20) to T_1 (58.7 ± 10 mm Hg), but not in the two sham groups (60.8 ± 11.6 at T_{base} *vs.* 61.2 ± 7 mm Hg at T_1; n=10). From T_{10} to T_{22}, mSAP was significantly higher in the two sham group (77.5 ± 10.7 mm Hg; n=10) compared with the four CPB groups (61.5 ± 9.3 mm Hg; n=20) ($P < 0.05$). Similar in-time differences occurred for SVI (from T_2 to T_{22}; $P < 0.02$) between the two sham groups and the four CPB groups.

Respiratory data

Pulmonary mechanics

-Treatment with inhaled NO had no significant effect on any parameter. There was no significant difference in respiratory rate, V_T, minute volume and end-tidal CO_2 between groups.
-In all groups, compared to baseline, dynamic compliance was significantly decreased at T_{22} ($P < 0.005$), and was significantly decreased at T_2 in the CPB + LPS and CPB + LPS + INO groups (Fig. 4) ($P < 0.002$). Peak airway pressure demonstrated opposite changes, with a significant increase for all groups at T_{22} ($P < 0.02$), and a significant increase occurring at T_2 in the CPB + LPS and CPB + LPS + INO groups ($P < 0.04$).

Gas exchange

-A significant difference between groups, with higher PaO_2/FiO_2 values in the inhaled NO treated groups, was observed (Fig. 5) at T_{22} ($P = 0.001$). The pair-wise comparisons demonstrated a significant difference between the two CPB groups at T_{22} ($P = 0.02$); and between the two CPB + LPS groups at T_{22} ($P = 0.02$).
-The oxygenation index demonstrated a significant variation with time for the three control (without inhaled NO) groups when compared to baseline, but not for the three inhaled NO treated groups (Fig. 5) confirming the inhaled NO effect. The observed decrease in control groups was present at T_2 and T_{22} for the CPB group and at T_{22} for the sham and CPB + LPS groups. There was no significant difference between the sham groups, with and without inhaled NO, at any time.

Moreover, inhaled NO was associated with a significant improvement in $P(A\text{-}a)O_2$, $C_aO_2 - C_vO_2$, and Q_S/Q_T in each treated group ($P < 0.05$) demonstrating the effect of inhaled NO on oxygenation index at T_{22}.

Effect of the procedure on BAL surfactant

There was no significant difference in BAL return volume between groups. There was a non-significant trend of TS and SA to decrease with time with no inter-group difference. The active subfraction of surfactant (LA) decreased significantly with time during the procedure (P = 0.0003) (Fig 6). Particularly, the observed decrease with time was statistically significant in the inhaled NO-treated groups (T_{22} vs. T_0): sham + INO group (P = 0.0007), CPB + INO group (P = 0.05) and CPB + INO + LPS group (P = 0.04).

With the comparison of the pooled groups (inhaled NO treatment *vs.* without inhaled NO), the LA content was significantly higher in the inhaled NO groups at T_{base} ($P = 0.03$), an effect observed after only 75-90 min exposure to inhaled NO. However, this effect disappeared at T_2 and T_{22} (Fig. 6). The pair-wise comparison was not statistically significant at any time.

Cells count in BAL and leukocytes count in blood

There was no change in the BAL cells count over time neither in the three inhaled NO-treated groups, nor in the sham group. However, at T_{22}, the BAL cells count was statistically higher compared to T_{base} in both CPB ($P = 0.007$) and CPB + LPS ($P = 0.02$) groups. The increased value at T_{22} in CPB and CPB + LPS groups was higher than the value in the sham group ($P < 0.03$). The difference between control groups and inhaled NO-treated groups was close to statistical significance ($P = 0.09$) at T_{22}. Moreover, the neutrophil differentials in BAL were also greatly augmented ($P < 0.05$) at T_{22} in the CPB (+206%) and CPB + LPS (+244%) groups, but were lower in the inhaled NO treated animals without reaching statistical significance. No similar difference could be observed for monocytes or lymphocytes in BAL. The CPB procedure had a significant effect on the blood leukocyte count over time: whereas it decreased with time in the sham and CPB ($P = 0.004$) groups, it increased in the CPB

+ LPS group. Moreover, inhaled NO had no effect on leukocyte count in blood over time.

Discussion

Whereas CPB alone was not different from Sham (thoracotomy surgery alone) for its effect on pulmonary haemodynamics, the addition of an LPS perfusion to CPB induced a clear pulmonary vasoconstriction leading to an increased mPAP. At T_2, *i.e.* the first time-point after the end of LPS perfusion, inhaled NO had no effect in counter-acting the increased pulmonary vasoconstriction, but there was an effect at the next time-point, T_3. Compared to CPB alone, the addition of LPS after CPB accelerated the decrease in lung compliance and the increase in peak airway pressure. From this, we suggest that exposure to a benign event (this low dose of LPS is supposed to reproduce a low level exposure to infectious agents) aggravates the lung injury initially induced by the CPB procedure. However, after T_5, the LPS effects are disappearing resulting in no difference between the CPB and CPB + LPS groups.
Without LPS, CPB alone is not different from Sham for pulmonary haemodynamics and slightly different for gas exchange parameters. A large difference for the systemic haemodynamic variables (heart rate, central venous pressure, mSAP and SVI) was present. It should be noted that inhaled NO had no effect on these systemic haemodynamic parameters. And, as expected, by decreasing pulmonary vasoconstriction in all treated groups, inhaled NO improved the oxygenation index by reducing Q_S/Q_T and improving V/Q matching.

Validation of the model

Picone *et al.* [32] demonstrated that the severe lung injury typical of post-pump syndrome could be caused by multiple, sequential insults. Their data established that CPB causes pulmonary neutrophil sequestration without lung injury. In the most widely studied model of sequential insult ARDS, gut ischemia serves as the initial insult and causes pulmonary PMN sequestration without lung injury [40-42]. A subsequent exposure to an otherwise benign insult (LPS 1 μg/Kg over 50 minutes in this case) activates primed and sequestered neutrophils to cause vascular injury, which leads to post-pump syndrome [32]. Moreover, a dose of 100 μg/Kg LPS is necessary to cause ARDS in pigs not subjected to CPB [43]. Our data confirm the observation of Picone *et al.* [32]: in the CPB + LPS group, mPAP, PVRI, and peak airway pressure increased to a higher level and/or more quickly, whereas the lung compliance decreased faster than in the other groups. This was corroborated by the greater release of MMPs in BAL and blood, as well as the increase in leukocyte levels in blood [37]. It must be highlighted that Picone *et al.* stopped their follow-up 3 hours after the end of CPB (when the CPB + LPS group showed the highest degree of failure), corresponding to T_3 to $T_{3.5}$ in our design [32]. It was also at this time that our CPB + LPS showed the highest degree of insult. However, in addition our study demonstrates that the gradation in lung injury between sham, CPB and CPB + LPS is, for the most part, transient. The three groups were similar at T_{22} for lung compliance, peak airway pressure and oxygenation index. However, there was a net difference on the cardiovascular parameters (HR, CVP, SVI and mSAP) and, as we demonstrated in previous studies [35-37], on the BAL values (cell count, neutrophil differential, IL-8 and MMPs concentration) between the CPB groups and the sham group, reflecting the difference in inflammatory reaction.

Effects of pre-emptive and continuous inhaled NO therapy
The L-arginine-NO system has been strongly implicated in the pathophysiology of ischemia/reperfusion [42,44] and sepsis [45]. NO is a multifaceted agent with roles in vascular homeostasis, neurotransmission, and inflammation. It is produced by three isoforms of NOS, of which two are expressed in endothelial cells (eNOS) and

neurons (nNOS), and the third (iNOS) is mainly induced in macrophages, vascular smooth muscle cells, and other cell types only after specific stimuli [46]. Endothelial NO is a potent vasodilator. It inhibits platelet aggregation, attenuates endothelial cells and leukocytes interactions, and may maintain the microvascular permeability barrier [47]. It has been shown that a rapid decrease in eNOS activity follows ischemia/reperfusion promoting leukocyte adherence, activation, and migration across the endothelial barrier, and increases microvascular permeability thus enhancing the tissue inflammatory reaction [48]. Leukocyte-endothelial interaction involves a complex interplay between adhesion molecules and their ligands. Three endothelial adhesion molecules (P-selectin, E-selectin, and ICAM-1) have been linked to the effect of decreased NO on leukocyte adhesion. A strong link has been established between loss of eNO during splanchnic ischemia/reperfusion and increased expression of P-selectin that is responsible for early rolling of leukocytes in systemic postcapillary venules [49]. Indeed, we also observed in our swine model that NOx production is reduced after CPB [36] and CPB + LPS [35], leading to a potential deficit in endogenous NO, which was counteracted by the use of exogenous inhaled NO in the corresponding groups. Inhaled NO acted as an anti-inflammatory agent by decreasing neutrophil numbers in BAL and its major chemoattractant, IL-8, as well as MMPs [37]. Inhaled NO also increased cell apoptosis in the lungs during inflammatory conditions, which may explain, in part, how it resolves pulmonary inflammation [35,36].

All the anti-inflammatory effects of pre-emptive inhaled NO in our model were translated as beneficial effects on cardiopulmonary parameters, mainly on mPAP and oxygenation index. In addition, pre-emptive and continuous inhaled NO was devoid of any adverse systemic cardiovascular effect, nor was it associated with any deleterious (pro-inflammatory) effect. In contrast, inhaled NO did not counteract the CPB-induced alterations on lung mechanics (thoraco-pulmonary compliance and airway pressure), despite an interesting initial effect on surfactant component (LA). Lung surfactant is one of the most important factors contributing to lung stability and maintains lung compliance by reducing surface tension at the air-liquid interface [50]. It is composed of 90% lipid, mostly PLs, and 10% surfactant-specific proteins. Surfactant lipids and proteins are stored in lamellar bodies of type II pneumocytes, and secreted by exocytosis. Under normal conditions, approximately 50% of the surfactant present in the alveolar space is in the form of functionally active LA, and approximately 50% in the form of small surfactant vesicles SA. The LA subtype is primarily responsible for the surface tension-lowering property of surfactant [51,52]. The decreased synthesis, secretion and/or dysfunction of surfactant may cause alveolar collapse (atelectasis), increasing airway pressure and decreasing lung compliance, and favouring ventilation/perfusion mismatch or shunt and subsequent hypoxaemia, as a consequence [50]. To the best of our knowledge, this is the first report indicating that short-term (75-90 min) inhaled NO can increase LA content. Despite the ability of inhaled NO to exert this protective effect, the beneficial impact on surfactant was of short duration, as it disappeared after 4 hours, even in the sham-operated group. This is disappointing, particularly because it has been observed that the surface activity of the LA surfactant was impaired on days 1 to 3 following CPB in infants (< 1 year old) [53]. We propose that prolonged inhaled NO has no long-term effect on the derangement of type II pneumocyte homeostasis caused by hyperoxia, mechanical ventilation and inflammation.
The addition of a 20 ppm NO to the inhaled air during endotoxin shock in pig [54] or hyperoxia-induced pulmonary dysfunction in newborn guinea pigs [55] improved oxygenation index and reduced PAP. However, it did not affect lung mechanics or inflammatory indices (levels of BAL fluid total protein, tumour necrosis factor-alpha, IL-8 and neutrophil counts), despite prophylactic administration. Moreover, the short-term exposure (< 5 days) to NO/O_2 delayed onset of respiratory distress and neither

exacerbated nor attenuated pulmonary dysfunction compared with O_2 exposure alone [55].
We used high FiO_2 (1 and 0.5), mechanical ventilation with low PEEP on paralyzed pigs in supine position to match extreme clinical practice, and the clinically relevant concentration of 20 ppm inhaled NO was delivered. With this combination, inhaled NO had no long-term beneficial effect on lung mechanics and surfactant homeostasis despite improving lung haemodynamics, inflammation and oxygenation. This is encouraging to associate pre-emptive inhaled NO with protective ventilatory strategies (low FiO_2, permissive hypercapnia, prone position, etc.).

Importance of the timing of administration of inhaled NO and of animal model
In the situation of ischemia/reperfusion or sepsis, therapies are directed on one side at inhibition of excessive NO production by nonselective or selective iNOS inhibitors and, on the other side, at L-arginine supplementation or administration of exogenous NO [45]. Compensation of deficient NO production by substrate supplementation or by its exogenous administration may indeed diminish the inflammatory process. This anti-inflammatory effect could be related to the decreased expression of adhesion molecules (*via* a pivotal inhibiting role on nuclear factor–κB [26-28]), the direct effect on neutrophil function, and also the effect on improved blood flow with increased shear rate and hydrodynamic forces that reduce leukocyte adhesion [42,44,49,56]. This latter role is supported by increasing evidence of NO-carrier forms allowing extra-pulmonary effects from inhaled NO [29,30,57].
By its effects (summarized in the introduction), inhaled NO was demonstrated a safe therapy for acutely decreasing lung dysfunction, but without substantial impact on the duration of ventilatory support or survival in infants [58-60], or in adults [60,61]. Therefore, inhaled NO was presented as a therapy in search of an indication. The search continues [62]. This lack of long term clinical benefit of inhaled NO in ALI/ARDS may be attributable to the presence of multiple pathophysiological features, including alveolocapillary leakage and tissue oedema, neutrophil infiltration and activation, and tissue oxidative injury [63,64]. The disparity between its potential anti-inflammatory activity and the lack of significant clinical benefit of inhaled NO may be related to the timing of inhaled NO therapy. Most clinical trials have assessed the effect of inhaled NO therapy in patients with well-established ARDS. In this situation, inhaled NO may be potentially pro-inflammatory [65].
Few studies have initiated inhaled NO prior to, or concurrent with, an inflammatory insult. Under experimental conditions, pre-emptive inhaled NO demonstrated anti-inflammatory effects on isolated rat lungs [20,66]. This has been confirmed in *in vivo* models of ALI, induced by injection of, endotoxin in rats [21], and of endotoxin [67] or platelet-activating factor [68] in pigs. Curiously, similar results were not initially reproduced in rabbits [69,70]. Decreased lung inflammation and injury with pre-emptive inhaled NO was associated with decreased pulmonary iNOS activity in a mouse model of sepsis-induced ALI [71], with delayed release of reactive oxygen species in pigs [44] and rabbit lungs [72] and with decreased activation/migration of leukocytes in rats [42] during ischemia/reperfusion-induced ALI.

As stated above, the mechanisms involved in the variation of inhaled NO effect counteracting ALI are not clear. To the extent of our knowledge, these disparate actions could be related to 1) differences in the timing of administration and/or 2) differences in the concentration of inhaled NO used, or 3) model differences.
It should be emphasized that large amounts of NO may also exert proinflammatory effects [46,73,74]. However, most of the available experimental data support a beneficial net anti-inflammatory effect of NO therapy in diverse acute models of ALI. Particularly, inhaled NO therapy was studied in ALI induced on isolated lungs or by induction of sepsis or ischemia/reperfusion in various species, and it was found that pre-treatment [20,21,42,66-68,71] and "preconditioning" [44,72] of the lung

vasculature with NO administration maintains endothelial integrity and attenuates the increase in microvascular leak. Such studies support initiation of inhaled NO therapy, at reasonable concentrations [54,75] before the inflammatory insult. This approach, which was assessed in our experiments of CPB-induced ALI in pigs, may prove effective in relevant clinical conditions in which NO therapy may be initiated prior to an anticipated proinflammatory stimulus (*e.g.*, before reperfusion of ischemic organs).

Concerning the model chosen (CPB-induced ALI in pigs) and its validity to evaluate an anti-inflammatory effect of inhaled NO, it is extremely important to consider that all the numerous models presented previously do not replicate the clinical situation, with the exception of CPB-induced inflammation in pigs [23]. Firstly, CPB-induced ALI/ARDS is a clinical reality encountered daily in cardiac surgical patients, which could be quite easily reproduced in pigs. Secondly, pigs have a cardiopulmonary physiology, pharmacology and anatomy comparable in many aspects to those of humans [76]. Finally, all the other models are only representative of one, or more, components of the inflammatory process leading to ALI/ARDS or sepsis in humans.

Picone *et al.* speculated, in their conclusion, that if the inflammatory effects of CPB can be reduced, the incidence of post-pump syndrome will be diminished [32]. Firstly, we reproduced the data of Picone *et al.* [32], where sham induces a minor insult compared to CPB, and where the subsequent exposure to an otherwise benign insult (minimal dose of LPS) aggravates significantly the lung injury. This confirms the possible multiple, sequential nature of insults leading to the typical post-pump ALI/ARDS. In contrast to Picone *et al.* [32], we demonstrated that the gradation in lung injury between sham, CPB and CPB + LPS is, for the most part, transient. Secondly, from the series of experiment we have conducted in CPB-induced ALI/ARDS in pigs, the use of pre-emptive and continuous inhaled NO therapy looks promising, with regards to its anti-inflammatory and protective effects on pulmonary haemodynamics and oxygenation, as well as its safety. It still remains to study the weaning strategy before considering a future new clinical indication for inhaled NO.

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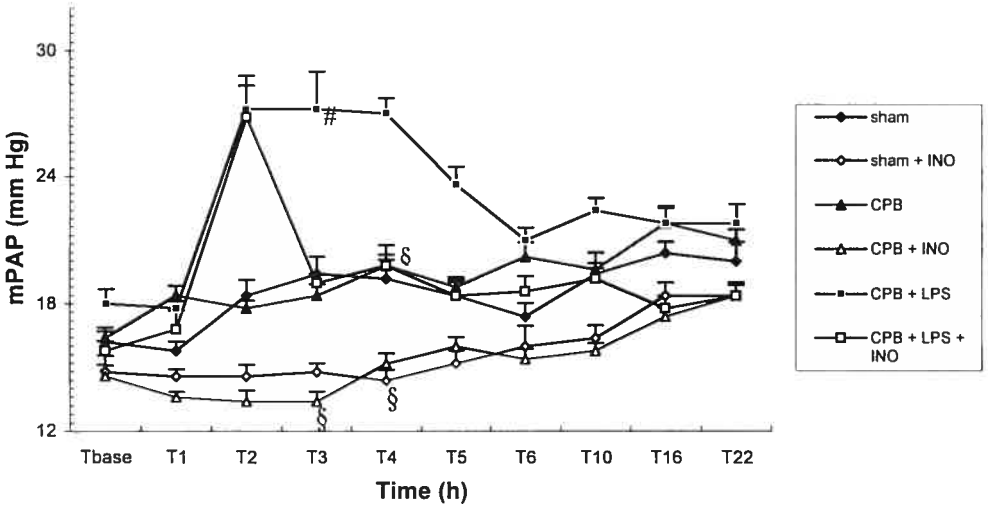
Fig. 1


30
24
18
12
mPAP (mm Hg)
Tbase
T1
T2
T3
T4
T5
T6
T10
T16
T22
Time (h)
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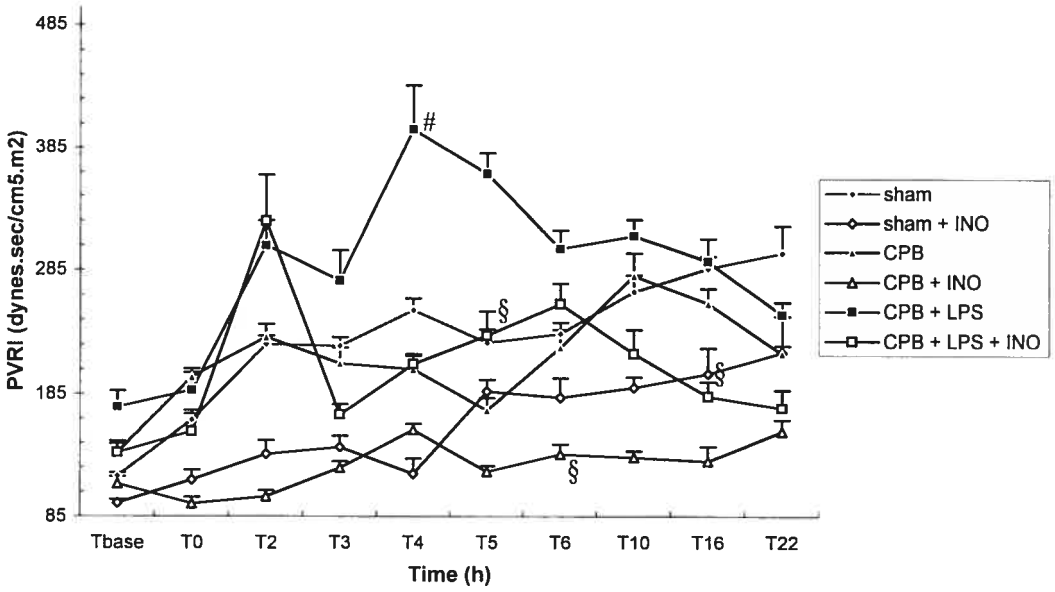
sham
sham + INO
CPB
CPB + INO
CPB + LPS
CPB + LPS + INO

Fig. 2


485
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285
185
85
PVRI (dynes.sec/cm5.m2)
Tbase
T0
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T3
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T6
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T16
T22
Time (h)
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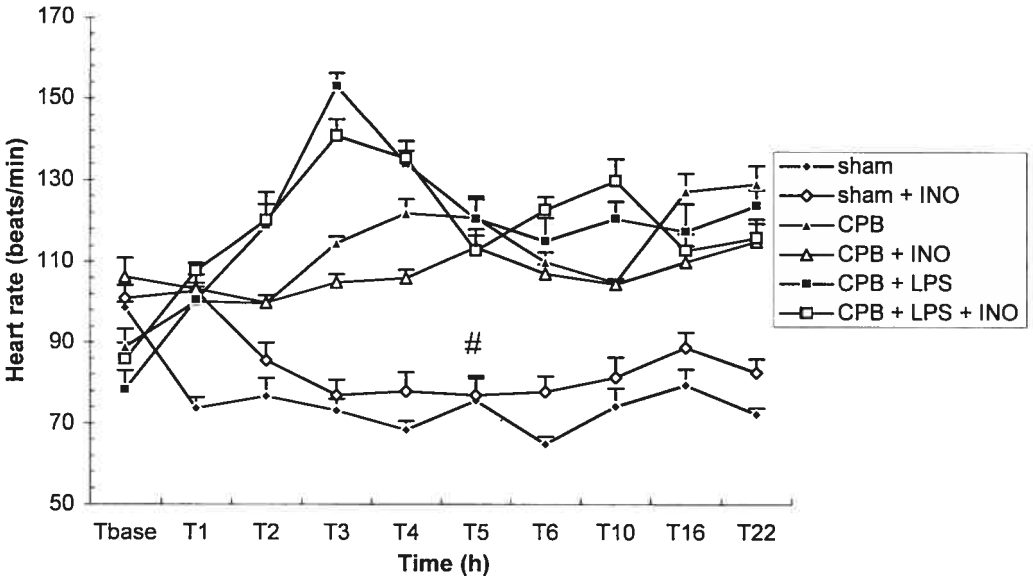
sham
sham + INO
CPB
CPB + INO
CPB + LPS
CPB + LPS + INO

Fig. 3


170
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130
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90
70
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Heart rate (beats/min)
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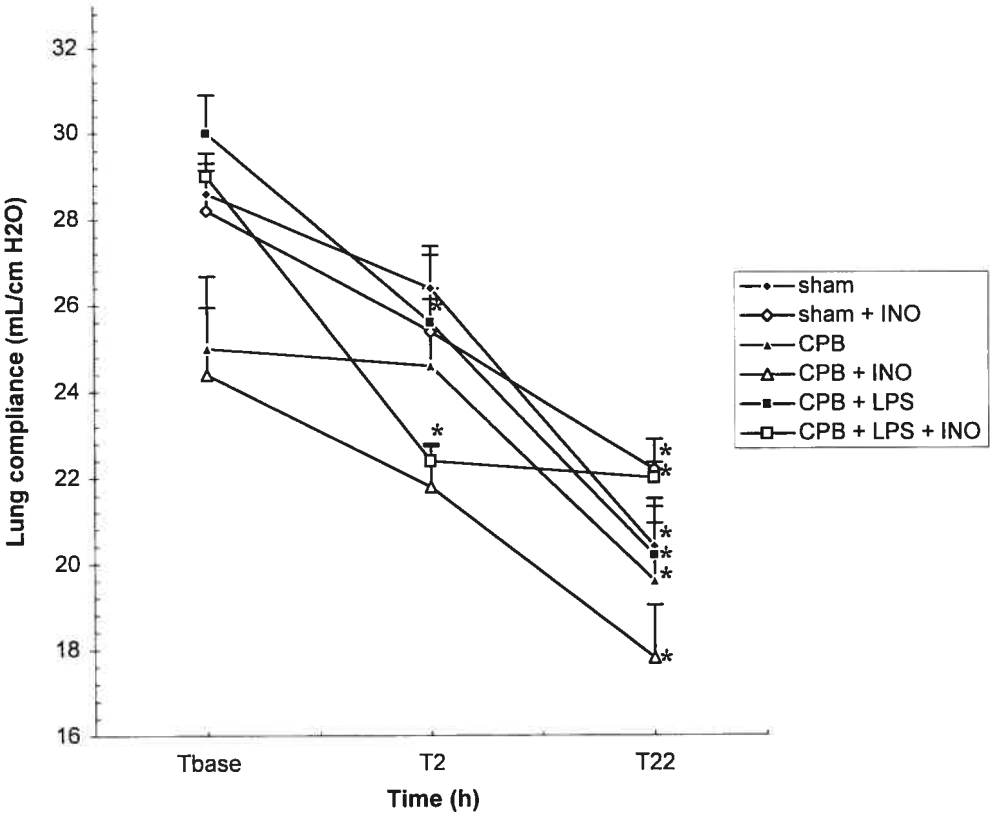
sham + INO
CPB
CPB + INO
CPB + LPS
CPB + LPS + INO

Fig. 4


Lung compliance (mL/cm H2O)
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Tbase
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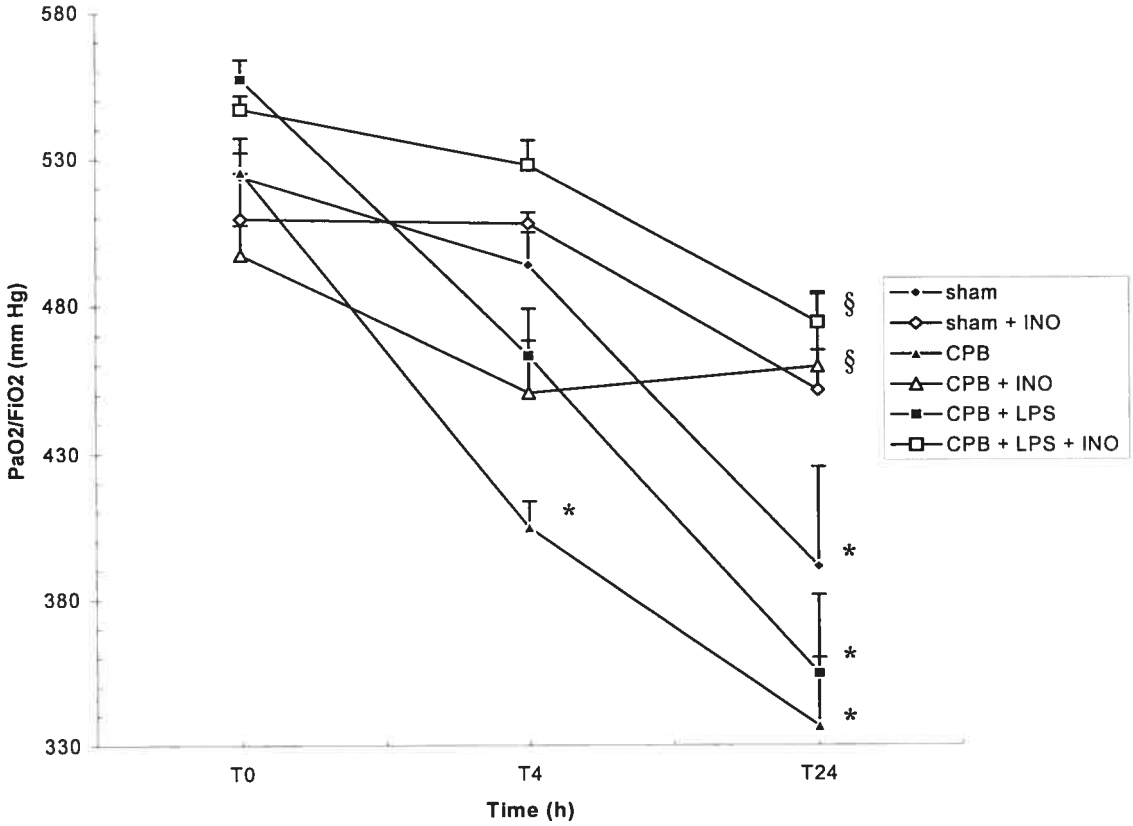
Time (h)
sham
sham + INO
CPB
CPB + INO
CPB + LPS
CPB + LPS + INO


580
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480
430
380
330
PaO2/FiO2 (mm Hg)
T0
T4
T24
Time (h)
sham
sham + INO
CPB
CPB + INO
CPB + LPS
CPB + LPS + INO
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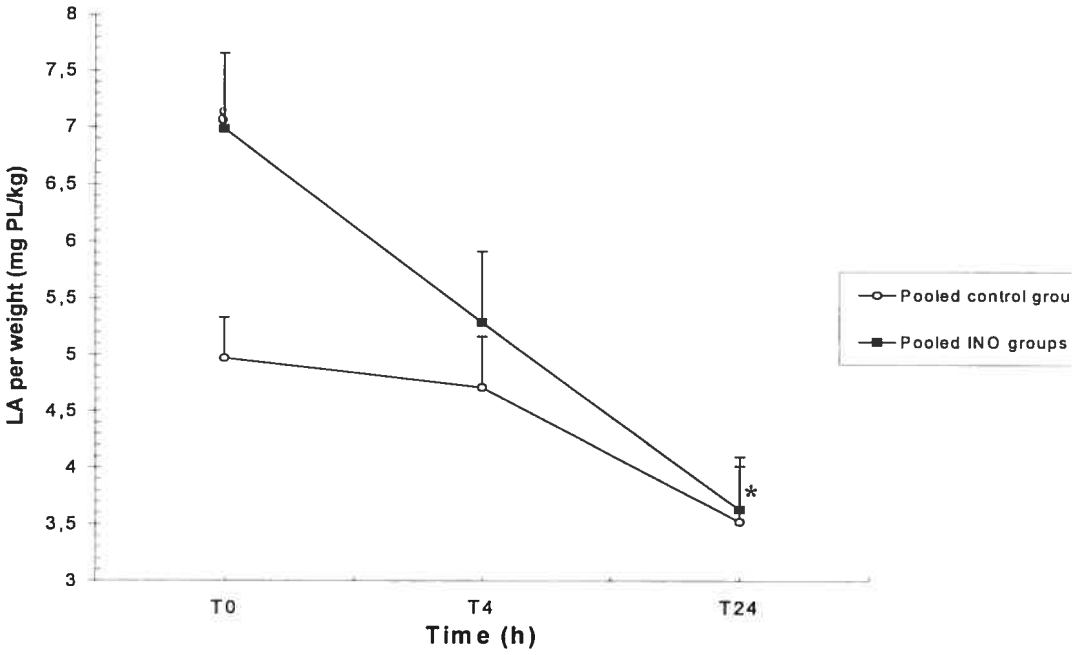
Fig. 5

Fig. 6


8
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7
6,5
6
5,5
5
4,5
4
3,5
3
LA per weight (mg PL/kg)
T0
T4
T24
Time (h)
Pooled control grou
Pooled INO groups
*

Legends

Fig. 1. Mean pulmonary arterial pressure (mPAP) (mm Hg) over time.
Significant effect of time (compared to T_{base}) for sham (T_3, T_{10}, T_{16}, T_{22}), CPB (T_4, T_6, T_{10}, T_{16}, T_{22}), CPB + LPS (T_1 to T_{22}), sham + INO (T_{16}, T_{22}), CPB + INO (T_{22}), CPB + LPS + INO (T_2, T_3, T_4, T_{10}).
Effect of inhaled NO on mPAP over time: § $P < 0.05$ *vs.* same group without inhaled NO (see text for more details).
Effect of the procedure on mPAP over time: CPB + LPS # $P < 0.05$ *vs.* other groups (see text for more details).

Fig. 2. Pulmonary vascular resistance index (PVRI) (dynes.sec/cm^5.m^2) over time.
Significant effect of time (compared to T_{base}) for sham (T_2 to T_{22}), CPB (T_2, T_6, T_{10}, T_{16}, T_{22}), CPB + LPS (T_2 to T_{22}), sham + INO (T_5 to T_{22}), CPB + LPS + INO (T_2, T_5, T_6, T_{10}).
Effect of inhaled NO on PVRI over time: § $P < 0.01$ *vs.* same group without inhaled NO (see text for more details).
Effect of the procedure on PVRI over time: CPB + LPS # $P < 0.05$ *vs.* other groups (see text for more details).

Fig. 3. Heart rate (beats/min) over time.
Significant effect of time (compared to T_{base}) for sham (T_1 to T_{22}), CPB (T_3 to T_6, T_{16}, T_{22}), CPB + LPS (T_1 to T_{22}), sham + INO (T_3 to T_6), CPB + LPS + INO (T_1 to T_{22}).
Effect of the procedure on heart rate over time: the two sham groups # $P < 0.05$ *vs.* the four other CPB groups (see text for more details).

Fig. 4. Lung compliance (mL/cmH_2O) over time.
Effect of the procedure on lung compliance over time: * $P < 0.05$.
At T_{22}, the lung compliance in all groups was significantly decreased. At T_2, only in the CPB + LPS and CPB + LPS + INO groups, this decrease was significant.

Fig. 5. Oxygenation index (PaO_2/FiO_2) (mm Hg) over time.

Significant effect of time (compared to T_{base}) for sham (T_{22}), CPB (T_2, T_{22}), and CPB + LPS (T_{22}) : * $P < 0.05$.

Effect of inhaled NO on PaO_2/FiO_2 over time: § $P < 0.05$ *vs.* same group without inhaled NO (see text for more details).

Fig. 6. Large aggregates (LA) content in BAL per weight (mg PL/kg) over time.

Significant effect of time (compared to T_{base}) for the inhaled NO treated groups (T_{22}): * $P < 0.05$.

Effect of inhaled NO on LA content: § $P < 0.05$ *vs.* same group without inhaled NO (see text for more details).

Appendix 3

Operating Protocols

Problem	Signs	Treatment
Hypovolemia	CVP < 5 mmHg PCWP < 10 mmHg CI < 2.5 L/ min/ m^2 MAP < 50 mmHg Urine prod. < 1 ml/ kg/ hr	Pentaspan bolus: 5 – 10 ml/ kg over 45 mins.
Cardiac insufficiency	CI < 2.5 L/ min/ m^2 CVP > 15 mmHg PCWP > 18 mmHg MAP < 50 mmHg	NE: 8 – 12 mcg/ kg/ hr
Ventricular tachycardia		Mg sulphate 1g IV Lidocaine 1 mg/ kg IV Bretylium tosylate 0.5 mg/ kg IV

Appendix 4

Arterial blood gas analyses

Parameter	Timepoint (hrs)	Range (minimum)	(maximum)	Mean	SD
PH	Baseline (T0)	7.37	7.51	7.46	.05
	CPB (T2)	7.27	7.53	7.44	.09
	Post CPB (T4)	7.37	7.47	7.42	.04
	T7	7.30	7.53	7.46	.08
	T8.5	7.46	7.56	7.50	.04
	T24	7.30	7.53	7.45	.09
$PaCO_2$ (mmHg)	Baseline (T0)	37	50	42.3	4.7
	CPB (T2)	34	61	42.9	9.2
	Post CPB (T4)	35	44	39.3	3.5
	T7	34	52	40.0	6.2
	T8.5	33	44	38.2	4.0
	T24	36	60	41.5	9.8
PaO_2 (mmHg)	Baseline (T0)	459	542	496.1	29.3
	CPB (T2)	93	602	335.9	200.8
	Post CPB (T4)	234	507	316.1	101.0
	T7	134	219	188.9	29.3
	T8.5	149	231	205.2	29.8
	T24	75	210	148.7	48.8
HCO_3^- (mmHg)	Baseline (T0)	28.8	33.9	30.7	1.7
	CPB (T2)	25.5	29.5	27.9	1.5
	Post CPB (T4)	20.9	28.0	25.2	2.5
	T7	24.8	31.6	28.3	2.2
	T8.5	27.9	30.9	29.2	1.2
	T24	23.1	29.9	28.1	2.5
BE	Baseline (T0)	4.7	7.4	6.3	1.0
	CPB (T2)	0.3	6.1	3.6	2.7
	Post CPB (T4)	-3.5	3.8	0.9	2.9
	T7	4.1	5.9	5.2	0.7
	T8.5	4.4	6.5	5.3	1.0
	T24	-2.0	4.8	2.3	3.1

Table of select arterial blood gas analysis results. Weaned INO group.

Parameter	Timepoint (hrs)	Range (minimum)	(maximum)	Mean	SD
PH	Baseline (T0)	7.39	7.54	7.50	0.06
	CPB (T2)	7.28	7.60	7.40	0.11
	Post CPB (T4)	7.30	7.43	7.39	0.05
	T7	7.34	7.47	7.41	0.06
	T8.5	7.41	7.49	7.50	0.03
	T24	7.29	7.51	7.42	0.08
$PaCO_2$ (mmHg)	Baseline (T0)	34	49	41.7	5.4
	CPB (T2)	29	48	43.2	7.1
	Post CPB (T4)	41	53	44.5	4.7
	T7	38	54	45.0	5.8
	T8.5	32	46	41.0	5.2
	T24	37	61	43.6	9.8
PaO_2 (mmHg)	Baseline (T0)	479	641	562.0	56.9
	CPB (T2)	188	612	379.8	160.5
	Post CPB (T4)	207	337	256.5	48.4
	T7	149	249	213.0	40.1
	T8.5	149	247	216.7	35.4

	T24	48	209	128.0	62.4
HCO_3^- (mmHg)	Baseline (T0)	25.5	33.1	28.9	2.5
	CPB (T2)	20.0	28.7	25.9	3.3
	Post CPB (T4)	23.1	27.3	26.0	1.6
	T7	22.6	32.3	28.2	3.6
	T8.5	23.0	30.9	28.3	2.7
	T24	25.5	29.9	27.7	1.9
BE	Baseline (T0)	1.1	9.0	5.2	2.9
	CPB (T2)	-5.5	6.4	1.6	4.5
	Post CPB (T4)	-1.4	2.9	1.4	2.0
	T7	-2.3	8.3	3.9	3.9
	T8.5	0.4	7.3	4.3	2.5
	T24	1.7	6.0	3.7	2.1

Table of select arterial blood gas analysis results. Continuous INO group.