Hemodynamic Changes During High Frequency Oscillatory Ventilation in Newborn Piglets with Respiratory Distress Syndrome

By

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

Medical Sciences - Pediatrics

University of Alberta

ABSTRACT

Respiratory failure is common in critically ill neonates, with refractory cases often needing high frequency oscillatory ventilation (HFOV). Volume guarantee mode has been added to some HFOV ventilators for theoretical better maintenance of normocapnia. There is little information on the systemic and regional hemodynamic effects of HFOV and this additional modality. We modified an established acute model of moderate-severe respiratory distress syndrome induced by saline lavage over 45-60 minutes in newborn piglets and compared ventilatory modes of HFOV with and without volume guarantee, and conventional mechanical ventilation. Using a randomized controlled approach based on the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments), we compared the systemic and cerebral hemodynamic parameters in newborn piglets with severe respiratory failure (alveolar arterial oxygen gradient > 350 mmHg) using ultrasonic flow probes and near-infrared spectroscopy probes. They were ventilated over a total period of 4 hours with HFOV with and without volume guarantee or conventional mechanical ventilation (n=8 per group), whereas 6 sham-operated piglets were instrumented as normoxic references. We found that HFOV with volume guarantee had no effect on cardiac index (primary outcome), a positive effect on left ventricular contractility, and a negative effect on both cerebral perfusion and oxygenation. The left ventricular cardiac index, P_aCO_2, and pH were also lower, higher, and lower, respectively, over time in the CMV group. Further translational and clinical studies are needed to confirm our findings.

PREFACE

This thesis is an original work by Jagmeet S. Bhogal. He was involved in helping design the study, assisting with animal surgery, and managing protocol timing during the experiment and bloodwork collection. He was the person primarily responsible for collecting and recording raw data, data analysis (including statistical analysis), and writing all of the thesis.

ACKNOWLEDGEMENTS

I would first like to thank Po-Yin Cheung for being the primary supervisor for this thesis. His experience, good humour, feedback, and most of all exceptional patience is what has made this thesis possible. Despite his busy schedule, he always made time to meet with me, and kept in touch to keep me on track with milestones.

I also want to thank Raymond Lee and Min Lu for running the lab and the piglet instrumentation and surgeries. Raymond has such a breadth of experience, patience, and good sense of humour that he made such animal research less stressful, not only for me but also (hopefully) for the piglets. Min, as the surgeon, was also very patient, well humoured and skillful, which greatly increased the consistency and scientific value of the experiments. Raymond and Min also performed the tissue biomarker analysis. The experiments and this thesis could not have happened without them!

I would like to also thank the members of my supervisory committee: Lisa Hornberger, Chlöe Joynt, David Olson, and Georg Schmölzer for their feedback and assistance with the project's progress and final thesis reviews. I also appreciate Greg Funk's feedback for revisions of the thesis.

I would like to thank Megan Schmölzer for her assistance in analyzing the lung tissue, as it would not have been possible without her. The assistance of Anne Lee Solevåg was also appreciated early on in the project regarding study design and performing echocardiography.

I want to recognize the financial and academic support I received from, not only the Neonatal-Perinatal Medicine fellowship program at the University of Alberta, but also the

Clinical Investigator Program and the Deloitte Clinical/Stollery Academic Fellowship. The operating funds were provided by the Neonatal Research Fund of the Northern Alberta Neonatal Program and the Women and Children Health Research Institute (both were awarded to Dr. Cheung).

CONTENTS

CHAPTER 1: Neonatal transition and respiratory physiology……1

Respiratory distress syndrome……..……...4

Bronchopulmonary dysplasia……..5

Conventional and high frequency oscillatory mechanical ventilation modes…….7

History of high frequency ventilation……..9

Comparing conventional and high frequency oscillatory mechanical ventilation…….12

High frequency oscillatory ventilation and volume guarantee……..13

References……..19

CHAPTER 2: Cardiopulmonary interactions……...31

Hemodynamic monitoring…….33

Hemodynamic effects of positive pressure ventilation……..34

Measuring hemodynamic and oxygenation parameters…….38

Monitoring of cerebral perfusion and oxygenation……...44

Biomarkers of inflammation, oxidative ischemia, and ischemia……...45

References……..47

CHAPTER 3: Piglet model of neonatal respiratory distress syndrome…….58

Piglet model of respiratory distress syndrome……...60

References……..63

CHAPTER 4: Experience in developing a piglet model of respiratory distress syndrome……...66

References……..70

CHAPTER 5: Effects of high frequency oscillatory ventilation with volume guarantee on left ventricular function, systemic and regional hemodynamics in a piglet model of respiratory distress syndrome……..71

Abstract…… 72

Introduction……..74

Materials and Methods……..75

Results…..…….86

Discussion…….89

Conclusions……..96

References……97

CHAPTER 6 – Summary of findings and clinical and future relevance..……125

Clinical relevance and future applications……...129

References……131

Common references list…….132

LIST OF FIGURES

1.1 – Cardiovascular shunts and blood flow, from prenatal to postnatal……...27

1.2 – Pulmonary aeration and blood flow transition from prenatal to postnatal……28

1.3 – High-frequency oscillator diagram, with piston to generate oscillations…….29

1.4 – Illustrations showing the 'smoke spike' experiment……30

2.1 – Relationship between right atrial pressure and venous return……..54

2.2 – Example of pressure-volume loops……...55

2.3 – Schematic representation of a conductance catheter in the left ventricle…….56

2.4 – Relative depiction of GSH/GSSG and lactate level trends during hypoxia/ischemia and reoxygenation……..57

3.1 – Schematic representation of piglet model setup with instrumentation/monitors…..59

5.1 – Schematic representation of piglet model setup…….114

5.2 – Pressure-volume loops from sham-operated and experimental groups…….....115

5.3 – Timeline of experimental protocol……..…….…….……..119

5.4 – Line plot of left ventricular cardiac index as function of mode and time……...120

5.5 – Line plot of P_aCO_2 as function of mode and time…….…….……121

5.6 – Bar plot of post-mortem tissue lactate (left ventricle, brain, left kidney)……...122

5.7 – Bar plot of post-mortem tissue GSSG/GSH ratio……...123

5.8 – Bar plot of post-mortem lung tissue IL-8 and TNF-α …….……..124

Appendix 1 – ARRIVE checklist……150

LIST OF TABLES

5.1 – Mean arterial blood pressure, heart rate, mean airway pressure…….101

5.2 – Oxygenation index and alveolar-arterial oxygen diffusion gradient……..103

5.3 – Left ventricle function parameters……..104

5.4 – Systemic oxygen delivery, arterial-venous SO_2 difference, oxygen extraction, lactate……...108

5.5 – Cerebral and renal near-infrared spectroscopy, right common carotid artery flow index…….110

5.6 – pH, HCO_3, base excess, P_aCO_2..…….112

ABBREVIATIONS

$AaDO_2$ – alveolar-arterial oxygen difference

ANOVA – analysis of variance

AVO_2 – arterial-venous oxygen difference

BPD – bronchopulmonary dysplasia

CI – cardiac index

CMV – conventional mechanical ventilation

$CrSO_2$ – near-infrared spectroscopy reading of cerebral oxygen saturation

dP/dt – measure of ventricular contractility

DV – ductus venosus

EF – ejection fraction

ETT – endotracheal tube

F_iO_2 – fraction of inspired oxygen

GSH – reduced glutathione

GSSG – oxidized glutathione

HCO_3 – bicarbonate

Hgb - hemoglobin

HFPPV – high frequency positive pressure ventilation

HFJV – high frequency jet ventilation

HFOV – high frequency oscillatory ventilation

IL-8 – interleukin-8

LV – left ventricle

MAP – mean airway pressure

NIRS – near-infrared spectroscopy

P_aCO_2 – partial pressure of arterial carbon dioxide

P_aO_2 – partial pressure of arterial oxygen

RCAFI – right common carotid artery flow index

RDS – respiratory distress syndrome

S_aO_2 – percent saturated arterial hemoglobin

S_vO_2 – percent saturated venous hemoglobin

SV – stroke volume

TNF-α - tumour necrosis factor alpha

VG – volume guarantee

VT – tidal volume

Chapter 1 - Neonatal Transition and Respiratory Physiology

There is a significant transition that occurs between intrauterine and extrauterine respiratory physiology, and consequently it is no surprise that respiratory illness is the most common challenge in the neonatal intensive care unit (NICU). As a fetus, the lung airways produce and contain fluid which creates an end-expiratory pressure from laryngeal abduction and promotes lung growth **[2]**. However, the fetal lungs do not function in oxygenation or ventilation, as that is the placenta's role. As a result, the majority of cardiac output bypasses the lungs via both the foramen ovale and patent ductus arteriosus to the systemic circulation **[1]** (Figure 1.1).

As shown in Figure 1.1, the placenta is a low resistance vascular system which results in a low afterload for the heart. However, after the umbilical vessels are clamped and the low resistance placental circulation removed, the increase in afterload results in the reversal of direction of shunts (blood flow) from left to right **[1]**. The ductus arteriosus shunt changes to primarily left to right (from aorta to pulmonary artery), and along with an increase in PaO_2 facilitates the closure of the ductus arteriosus. With increased pulmonary circulation due to lung inflation and decreased resistance, as seen in Figure 1.2, there is increased blood flow to the left atrium which results in the foramen ovale shunt changing to primarily left to right (left to right atrium) and the foramen ovale functionally closes **[1]**. With the absence of blood flow from the umbilical vein after birth, the ductus venosus constricts and closes. Regarding lung transition, it is apparent from Figure 1.2 that any complications occurring around the time of birth that delay the transition of fluid removal could result in respiratory distress in the newborn.

With persistence of lung fluid after birth, important aspects of lung dynamics, compliance and resistance, are therefore qualitatively low and high, respectively,

compared to respective postnatal parameters in a well-aerated lung. Compliance is a measure of the pressure required to cause a change in volume. Resistance is a measure of the amount of pressure required to generate flow **[2]**. These parameters can be described mathematically as:

$$C = \Delta V/\Delta P$$

$$R = \Delta P/\nu$$

Where C = compliance, V = volume, P = pressure, R = resistance, and ν = flow.

Consequently, if transitioning from a fluid-filled lung to a well-expanded air-filled lung is affected negatively during birth, a neonate can be very ill from a pulmonary perspective which necessitates mechanical ventilatory support. Conditions which affect transitioning include respiratory distress syndrome (RDS), pneumonia, meconium aspiration syndrome, persistent pulmonary hypertension, and pulmonary hypoplasia. The most common of these is RDS which occurs due to a lack of or inactivation of surfactant in the alveoli, which is a mix of proteins and lipids that serve to reduce alveolar surface tension and thereby improve the compliance of the lungs **[2]**. Surface tension can be understood by the Law of LaPlace relating to the air-fluid interface of a bubble:

$$P = 2T/r$$

Where P = pressure needed to inflate bubble, T = surface tension,

r = radius of bubble

From the formula, it can be seen that the pressure needed to inflate an alveolus is directly proportional to the surface tension and indirectly proportional to the size of the alveolus. Surfactant, by reducing surface tension, reduces the pressure needed for alveolar inflation and facilitates air movement between different sized alveoli and equilibration **[2]**.

Respiratory Distress Syndrome

RDS was a management challenge in NICUs in the era before antenatal steroids and surfactant **[2]**. It is a disease characterized by hypoxemia due to surfactant deficiency resulting in atelectasis with subsequent ventilation-perfusion mismatching **[2]**. In 1929, von Neergaard et al discovered that surface tension contributes to lung recoil **[3]**. Gruenwald showed in 1947 that stillborn infant lungs have a high surface tension **[4]**. Prattle speculated in 1955 that the absence of surfactant material contributed to premature respiratory distress, and in 1957 Clement described surfactant dysfunction in animal experiments confirming its relation to neonatal lung disease **[5]**. Subsequently in 1959 Avery and Mead specifically showed that RDS in human infants is caused by surfactant deficiency **[6]**. Interest and funding was augmented in 1963 after President Kennedy's infant son died at 34 weeks gestation from RDS **[2]**. Management with mechanical ventilators was fraught with complications, notably pneumothoraces which were so frequent that chest tubes were commonplace **[2]**. Ventilation improved following the finding of Gregory et al in 1971 that maintaining continuous positive airway pressure as a distending pressure helped management in certain respects, but air leaks were still

common **[7]**. Natural surfactant was shown to delay progression of RDS in preterm rabbits in 1972, and in 1980 Fujiwara et al demonstrated the first successful use of exogenous surfactant in human infants **[8]**. By the 1990s exogenous surfactant was commonly used in the developed world and significantly improved outcomes of babies with RDS. A meta-analysis in 2009 of 13 RCTs showed it reduces the risk of pneumothorax by 58%, pulmonary interstitial emphysema (PIE) by 55%, mortality by 32%, and combined outcome of bronchopulmonary dysplasia (BPD) or death by 17% **[9]**. During this period, RDS management was also improved by the discovery that antenatal steroids reduce the risk of RDS in 1972 by Liggins and Howie **[10]**. Use of antenatal steroids became widespread after the NIH Consensus Panel in 1995 indicated they should be used for premature fetuses at 24-34 weeks of gestation **[11]**.

Bronchopulmonary Dysplasia

BPD, also known as chronic lung disease of prematurity, is the result of a combination of multiple factors including, but not limited to, arrested lung development from premature birth, prenatal inflammation, and postnatal insults resulting in lung inflammation such as mechanical ventilation, oxygen exposure, and infection **[12]**. This inflammation causes lung injury which is associated with impaired alveolarization and abnormal pulmonary vasculogenesis, Pulmonary function deteriorates with BPD, particularly acutely with decreased compliance, increased resistance, and air trapping at the more severe end of the spectrum **[12]**. It has been reported that about 97% of BPD cases occur in preterm infants with a birthweight less than 1250 grams **[13]**, with a range

of 22-38% of neonates born less than 28 weeks being affected **[14]**. Most commonly, BPD is clinically defined as requiring any respiratory support at or beyond 36 weeks corrected gestational age based on a National Institute of Child Health and Human Development (NICHD) consensus statement in 2001 **[15]**. The spectrum ranges from mild to severe, with the severity classification depending on the respiratory support required. There are multiple lifelong implications of having BPD, including the independent association with neurodevelopmental impairment as well as pulmonary morbidity such as increasing hospitalizations and reduced pulmonary function **[13]**. There have been multiple strategies shown to reduce the risk of developing significant BPD including antenatal corticosteroid administration, postnatal early surfactant therapy, optimizing nutrition and maternal breastmilk use, reduction of infections, minimizing oxygen administration, and one of the most significant which is to reduce the duration of invasive mechanical ventilation **[14]**. As a result, management of neonates born less than 28 weeks has changed from including being intubated soon after birth in order to administer surfactant, to currently avoiding intubation using non-invasive ventilation and only administering surfactant (either by intubating or using less invasive techniques) if the neonate's respiratory status deteriorates to a pre-specified level **[42]**. However, for those neonates that require invasive mechanical ventilation, evidence has been inconsistent regarding whether special ventilatory modes such as high frequency oscillatory ventilation (HFOV) reduce BPD when compared to conventional mechanical ventilation (CMV) **[14]**. There is ongoing research looking at outcomes in neonates on HFOV, especially those placed on it electively when they are first intubated.

Unfortunately, there has been a lack of convincing data to suggest specific ventilatory modes are superior to others. In particular, CMV has been the gold standard as it was the initial mode developed and remains the initial mode started in the vast majority of neonates treated with invasive mechanical ventilation in many centers. However, HFOV was developed in the 1980s for use in human patients, and has been shown to be at least as effective as CMV in a variety of situations, though not clearly superior, in terms of outcomes such as mortality and neurodevelopmental morbidities. Notably, HFOV is still mostly used as a rescue mode of ventilation when CMV is unable to adequately oxygenate or ventilate a patient.

Conventional and High Frequency Oscillatory Mechanical Ventilation Modes

The function of CMV is to ventilate and oxygenate via a constant end-expiratory pressure and intermittent inspiratory pressures, either flow- or time-cycled, which provide inflations at a physiologic rate **[16]**. The main mechanism is via a bulk flow of gas into the lungs, which requires the tidal volume (VT) to be greater than the dead space volume (DV). This mechanism of ventilation can be particularly challenging in lung diseases with significantly reduced compliance and/or air trapping, where there is insufficient inspiratory or expiratory time available to allow the lung to oxygenate and ventilate sufficiently. These pathophysiological conditions of the lung are where HFOV is most commonly used as a rescue mode, since it is able to oxygenate and/or ventilate at lower VTs which are often even lower than DV. HFOV uses a constant mean airway pressure (MAP) around which a diaphragm or piston (depending on the ventilator) oscillates at a

set amplitude resulting in active inspiration and expiration (Figure 1.3) **[16]**. This differs from CMV due to the oscillator actively pushing air in and then drawing air out, at a high frequency typically ranging from 8 to 12 Hz. The oscillation occurs at a set amplitude which is a measure of how far the piston or diaphragm moves back and forth, and which the VT is directly proportional to.

There are multiple theoretical mechanisms behind how HFOV works. Chang elucidated, in 1984, five mechanisms of transport that may be active in HFOV **[17]**:

1) direct alveolar ventilation of lung units near airway opening;

2) bulk convective mixing in conducting airways as a result of recirculation of air between units of inhomogeneous time constants, also known as the Pendelluft effect;

3) convective transport of gases as a result of asymmetry between inspiratory and expiratory velocity profiles;

4) longitudinal dispersion due to interaction between axial velocities and radial transports as a result of turbulence and/or secondary swirling motions, known as Taylor-type dispersion;

5) molecular diffusion near the alveolocapillary membrane.

More recently in the past 2 decades, the importance of adequate recruitment when using HFOV has been found to be even more significant than with CMV given the difference in mechanisms **[18]**. Recruitment refers to the process of opening the lungs, notably alveoli, and achieving optimal inflation by increasing the MAP to a maximal level based on patient context, clinical response, and clinician preference, then titrating it

down based on the clinical response of the patient including oxygenation and ventilation. Its significance is likely partly related to the fact that HFOV doesn't provide inflations at physiologic volume which can open the lung, leaving it more susceptible to atelectasis if the mean airway pressure is insufficient when compared to CMV **[62]**. As a result, patients often require higher MAPs to maintain an optimal inflation, to optimize oxygenation and ventilation, when on HFOV due to the lack of tidal volume breaths as when CMV is used **[62]**. However, providing a high MAP and thus intrathoracic positive pressure may compromise the right ventricular preload which may have a negative impact on hemodynamics **[19]**. This may explain the commonly seen clinical phenomenon that patients who are placed on HFOV subsequently require more cardiac support, either in terms of intravenous fluids and/or inotropes. However, given HFOV is commonly used as a rescue mode of ventilation, it is may be that the cardiac compromise is due to the patient population itself.

History of High Frequency Ventilation

One of the driving forces behind investigating the use of high frequency ventilation (HFV) in neonates was the difficulty in treating RDS and BPD. As noted earlier, neonatal mechanical ventilation was initially associated with significant morbidity and mortality **[20]**; the mortality was up to 80% in the 1960s for infants who were mechanically ventilated. Outcomes began to improve with the introduction of continuous positive airway pressure by Gregory **[46]**. In 1972 Kirby et al trialed the Babybird ventilator, which was a continuous-flow pressure-limited and time-cycled device, and

found less paralysis was needed since babies could take spontaneous breaths in-between cycles. This mode of invasive mechanical ventilation became the standard for newborns and adults **[20]**. With these significant improvements in mechanical ventilation came a focus on decreasing morbidity and mortality in the 1970s, and new approaches were developed to optimize positive end-expiratory pressure, inspiratory time, and positive inspiratory pressure which resulted in decreasing FiO_2 needs. Despite improved oxygenation, the rates of BPD remained unchanged which was a surprise since oxygen was thought to be the main causative factor at the time. As a result, high MAP causing barotrauma was then thought to be a significant factor behind BPD and the focus shifted to reducing MAP which subsequently lead to more research on HFV **[20]**.

The road to developing HFV largely began in the early 1900s. Henderson et al in 1915 wondered how dogs in heat managed to ventilate considering their polypnoea **[21]**. Their experiments with different VTs indicated that during rapid shallow breathing, the dead space is less than when taking larger breaths. Further experiments discovered a 'spike' of inspiratory gas that happens when blowing smoke into the end of a cylindrical tube, and that this thin smoke spike penetrates down the tube with the dead space air moving to the periphery (Figure 1.4). When the puff of smoke was stopped by blocking the proximal end of the tube with the experimenters' tongue, the spike instantly broke apart and the tube was filled with a mixture of smoke and air with a progressively decreasing gradient of smoke from proximal to distal ends **[21]**. They repeated the experiment with a glass bulb connected in the middle of the tube, and found that the smoke spike would shoot through the bulb from one tube to the other without much contamination of surrounding air. However, when the puff was stopped by blocking the

tube there would be almost instantaneous mixture as noted before **[21]**. These results would later help explain some of the basis behind HFV since theoretically a spike should enable ventilation at lower VTs than a square front would **[22]**. Briscoe investigated alveolar ventilation using low VTs in 1954 on five healthy adults using an experimental apparatus **[22]**. Subjects would breathe in a mixture of helium gas at small VTs, down to as little as 60 ml (typical adult tidal volume is 500 ml). The amount of helium in expired gas was then measured as an indication of alveolar ventilation. Their results showed that a VT less than or equal to dead space volume can indeed reach the alveoli and therefore participate in gas exchange, which was supported by Henderson's 'spike' observations previously shown **[22]**.

Research on HFV methods progressed in the 1960s, with high frequency positive pressure ventilation (HFPPV) being studied which is effectively CMV used at high frequencies up to 150 bpm **[23]**. HFPPV appeared to be beneficial in lung diseases that required high pressures, as it enabled ventilation at higher frequencies and lower pressures **[23]**. Further research done on humans, including premature infants, showed the equivalence of HFPPV to CMV but at lower pressures. The results of HFPPV research lead to the development of more efficient high frequency modalities including HFOV and high frequency jet ventilation in the 1970-1980s. Though HFJV is an important and essential ventilator modality in the NICU, I will focus on HFOV in the thesis.

Comparing Conventional and High Frequency Oscillatory Mechanical Ventilation

Multiple studies in the 1980s, both animal and human, showed the efficacy of HFOV in ventilating and oxygenating at tidal volumes less than physiologic dead space **[24][25][26][27]**. Typical tidal volumes in neonates range from 5-6 ml/kg, whereas HFOV volumes are typically 2-3 ml/kg. Since the thought process at the time was that high pressures result in barotrauma, these results encouraged further clinical trials assessing HFOV as compared to CMV. The first major trial was the HIFI trial in 1989 which was a randomized controlled trial comparing CMV to HFOV on premature infants randomized electively for respiratory distress **[28]**. This trial took place before the routine use of surfactant and recruitment techniques to optimize FRC. The results showed a similar incidence of BPD and mortality in both groups, along with an increase in pneumothorax, grade 3 and 4 intraventricular hemorrhage, and periventricular leukomalacia with HFOV. As a result, the study group concluded that HFOV did not offer any advantage over CMV as studied in the trial.

Recent systematic reviews and meta-analyses have looked at the use of HFOV versus CMV in both rescue and elective situations, without any significant advantages of HFOV noted in both preterm and term neonates **[29][30]**. Adequate lung recruitment strategies have been used for just over a decade, so many of the HFOV studies did not use techniques that are now generally considered important for optimal use of HFOV, especially for babies with relatively homogeneous lung disease. As a result, more recent studies using HFOV electively in extremely premature infants showed a potential reduction in BPD, or its risk factors such as duration of intubation, when compared to

CMV **[31][32]**. This is a potentially significant finding, since subgroup analyses in the systematic reviews of studies using recruitment strategies did not consistently show significant differences due to heterogeneity between studies over the past 3 decades **[33]**.

Currently worldwide, HFOV is most commonly used as a rescue mode when there is ventilation or oxygenation failure with CMV. The common respiratory illnesses which benefit from HFOV include congenital diaphragmatic hernia, meconium aspiration syndrome, severe RDS, persistent pulmonary hypertension , pulmonary hypoplasia, and severe BPD **[45]**.

HFOV and Volume Guarantee

Recently, newer ventilators have been developed with HFOV capabilities and a volume guarantee (VG) function **[34]**. In contrast, previous ventilators have the amplitude set and the VT is given based on amplitude depending on lung compliance. Thus, as a result the volume delivered during HFOV would change if lung properties fluctuate. However with VG, the ventilator theoretically maintains a set VT by altering the amplitude which theoretically enables a more consistent ventilation strategy **[34]**. This additional feature may result in more consistent P_aCO_2 levels, which should help reduce the systemic effects of fluctuating CO_2 levels **[47]**. Given that maintaining a consistent volume results in fluctuating amplitude (thus intrathoracic pressures) delivered to the patient, it has not been studied yet how this could potentially affect hemodynamics in comparison to not having VG enabled.

Another aspect of VT consistency relates to the effects of P_aCO_2 on systemic hemodynamics as well as regional organ perfusion. The relationship between P_aCO_2 and cerebral blood flow has been studied in piglets, including by Stiris et al in 1989 **[60]** and Bauer et al in 1999 **[61]** which both found a direct relationship where cerebral blood flow increases with hypercarbia (increased P_aCO_2). Cerebral blood flow in the human neonate has been studied extensively given the significance of intraventricular hemorrhage in preterm infants. Studies have shown that cerebral blood flow is more labile in preterm infants because the brain's vascular autoregulation is immature and less able to compensate for systemic hypotension or hypertension, thereby being at a greater risk for ischemia or hemorrhage, respectively **[35][48][49][50][51]**. Hypercarbia can increase cerebral blood flow secondary to vasodilation, and conversely hypocarbia (decreased P_aCO_2) can decrease cerebral blood flow due to vasoconstriction **[35][52]**. Both hypercarbia and hypocarbia can increase the risk for IVH and brain injury **[35]**. Cardiovascular effects of changes in P_aCO_2 levels are also well known. Studies on adult human volunteers in 1974 showed that hypercarbia causes increased cardiac output along with decreased systemic vascular resistance but overall resulting in increased mean arterial pressure **[36]**. Physiologically it was presumed that elevated $PaCO_2$ stimulates the sympathetic system to increase cardiac output and vascular tone, but that the direct effect of CO_2 on peripheral vasculature resulting in vasodilation must override the indirect effects of vasoconstriction secondary to increased sympathetic tone **[36]**. An early animal study on cardiovascular effects of hypercarbia was done in neonatal lambs by Stahlman et al in 1967, where they induced hypercarbia by providing 8% CO_2 for inhalation **[53]**. Results showed increased cardiac output, decreased pulmonary and

systemic vascular resistance, and a slight decrease in mean aortic blood pressure. Interestingly, a study in preterm infants by Fenton et al in 1992 found that increasing P_aCO_2 resulted in increased blood pressure without a change in heart rate along with a decrease in stroke and cardiac minute volume **[54]**. These results suggested that elevated P_aCO_2 may result in increased resistance in some components of peripheral vasculature, since it was known that it vasodilates cerebral, limb, and splanchnic circulations **[55]**, which the study group hypothesized may relate to varying autonomic innervation of the heart in premature infants.

Furthermore, myocardial blood flow and its relation to P_aCO_2 has also been studied in dogs in 1970 **[37]**. Ledingham et al anesthetized and paralyzed dogs, and assessed changes in myocardial blood flow at different P_aCO_2 concentrations by using radioactive isotopes **[37]**. They noted that at a P_aCO_2 of 100mmHg, there was an increase in myocardial blood flow and right atrial pressure, but there was no consistent changes in mean arterial blood flow, heart rate, or cardiac output **[37]**.

Renal blood flow has also been studied in dogs by Norman et al **[38]**. They found that renal perfusion remained consistent until the P_aCO_2 went above 70mmHg, after which there was a progressive decrease in renal blood flow along with a decrease in glomerular filtration rate and electrolyte excretion **[38]**. Interestingly, this change in renal blood flow was prevented by inducing renal nerve blockade. Therefore they concluded that the effects were likely secondary to the effect of hypercarbia on the sympathetic system **[38]**.

The effects on hepatic and splanchnic circulation has also been studied in beagles by Fujita et al in 1989 **[39]**. Their results showed that hypercarbia with a P_aCO_2 of

60mmHg results in vasodilation of the systemic and splanchnic circulation. Furthermore, there was increased portal venous flow and decreased hepatic arterial flow, and decreased hepatic function which may be secondary to the lower pH **[39]**. On the other hand, hypocapnia with a P_aCO_2 of 22mmHg caused a decrease in hepatic arterial flow **[39]**.

Effects in neonatal lambs were shown by Stahlman et al, with P_aCO_2 being inversely proportional to systemic and pulmonary vascular resistance, and directly proportional to cardiac output **[43]**. There are limited clinical studies in human neonates assessing the effects of P_aCO_2 and blood flow, with the focus being on the brain. Multiple studies have shown that cerebral blood flow is directly proportional to P_aCO_2, with increasing sensitivity with gestational age **[44]**. The effects of P_aCO_2 on cardiac output and other organs in human neonates currently are extrapolated from animal studies as mentioned above.

Given the various systemic and regional blood flow effects of P_aCO2 that have been discovered over the years, it is an important aspect for further research regarding ventilation modes.

Sánchez-Luna et al have conducted several studies on HFOV as well as the VG mode, including animal and human studies **[34][56][57][58][59]**. They have studied aspects including effects of using higher frequencies, different I/E ratios, and using lower tidal volumes. In one of their early studies in 2013, Sánchez-Luna et al studied piglets to compare HFOV with VG in relation to gas exchange **[34]**. Their main goal was to assess gas exchange with VG given changes in lung compliance with bronchoalveolar lavage. They used six healthy 2-day old piglets, with weight range 2.57 +/- 0.26 kg. Piglets were anesthetized and intubated with a seal around the ETT to prevent any leak. HFOV

ventilator parameters included FiO_2 of 0.3, MAP of 10 cmH_2O, baseline VT 2 ml/kg, and frequency 10 Hz with I:E ratio of 1:1. They monitored heart rate, ECG, S_pO_2, blood pressure, and blood gases. Protocol involved increasing the VT from 2 to 2.5 to 3 ml/kg at 15 min intervals and taking measurements at the end of each interval. After the initial series of VT increases, bronchoalveolar lavage was done with three aliquots of 10 ml/kg isotonic saline to induce surfactant depletion and mimic RDS. A recruitment maneuver was then done and the HFOV was set at a VT of 3 ml/kg for 30 min before repeating the measurements. Results showed that HFOV parameters had been kept consistent except for FiO_2 during the bronchoalveolar lavage. As VT was increased, P_aCO_2 decreased and HFOV amplitude increased as expected. Notably, after the bronchoalveolar lavage the VT and P_aCO_2 remained constant despite the amplitude increasing in three piglets, decreasing in two, and remaining unchanged in one. They also calculated the CO_2 diffusion coefficient (DCO_2) which is a marker of CO_2 removal, and found it was inversely proportional to the P_aCO_2 level as expected. Though there was no control group in this study, the group speculated that using HFOV with VG may help maintain more consistent ventilation and P_aCO_2 levels, notably if there is a significant change in lung properties such as compliance **[34]**.

More recently there have been several pilot clinical studies looking at the effects of HFOV with VG on ventilation and oxygenation in preterm infants. Iscan et al. in 2015 looked at the effects of HFOV with VG on preterm infants in a randomized crossover pilot study **[40]**. Their sample size was 20, and included infants less than 32 weeks who required intubation and surfactant in the first 6 hours of life. Each infant was randomized to either HFOV with VG or HFOV without VG, and they were then crossed over to the

other mode after 2 hours with a 'washout' period on CMV in between to achieve a similar baseline oxygenation and ventilations status. Results indicated that HFOV with VG may enable less P_aCO_2 fluctuation and help maintain a more consistent VT. Enomoto et al in 2016 looked at 6 preterm infants born at less than 1000 grams who had a stable respiratory status and were older than 28 days of age **[41]**. This study was done in Japan from 2012-2013, where it is common to invasively ventilate infants for over a week on HFOV until they were extubated. They placed these babies on HFOV with VG for 6 hours and then without VG for 6 hours, with a 'washout' period in between. They found that oxygen saturation and P_aCO_2 appeared to have less variation with VG. A retrospective study in 2019 by Belteki and Morley looked at data collected from 17 infants on HFOV+VG and analyzed the VG effect on parameters of the ventilator and blood gases **[47]**. They found that VG helps maintain the VT close to the target set over the long term, and that the VT needed was rarely higher (defined as > 2.5 ml/kg).

Taken altogether, few studies have assessed the efficacy of HFOV with VG in terms of ventilation and oxygenation parameters, and none have compared differential effects on other physiologic parameters such as regional and systemic hemodynamics. Given the sensitivity of neonatal lungs, particularly in premature infants, and the risk of cardiac compromise, it is important to study new ventilation modes in further detail. The focus of this thesis is to assess the hemodynamic effects at a cardiac, systemic, and regional level of HFOV with and without VG in a piglet model of respiratory distress syndrome.

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Figure 1.1 – Cardiovascular shunts and blood flow, from prenatal (top) to postnatal (bottom). Prenatally, oxygenated blood flows from the umbilical vein to the ductus venosus (DV), into the inferior vena cava (IVC), into the right atrium (RA), and then into either the left atrium (LA, majority of blood flow) or right ventricle (RV, minority of blood flow). It then preferentially bypasses the lungs through the ductus arteriosus (DA) to the aorta (Ao). Postnatally, with increased systemic pressures, the foramen ovale (FO) closes to a smaller patent foramen ovale (PFO) and blood flow to the lungs increases with reduced pulmonary vascular resistance.

PDA = patent ductus arteriosus (P = patent), SVC = superior vena cava, MPA = main pulmonary artery, LV = left ventricle. Arrows and weighting/length indicate direction of blood flow and relative amounts.

(Original illustration. *Reference: Goldsmith JP, Chapter 26: Delivery room resuscitation of the newborn. In: Martin RJ, Fanaroff AA, and Walsh MC, ed. Fanaroff and Martin's Neonatal-Perinatal Medicine, 9th edition, volume 1. St. Louis, Missouri: Elsevier Mosby; 2003: 450.)*

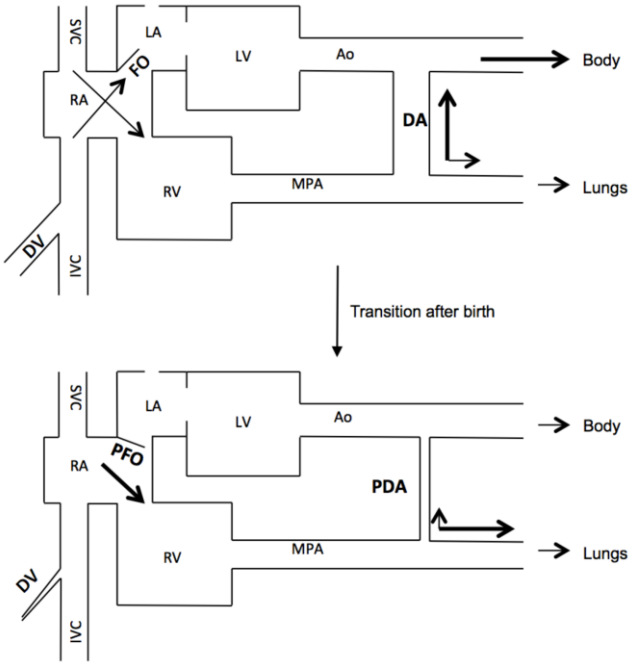


Figure 1.2 – Pulmonary aeration and blood flow transition from prenatal to postnatal states. A) In utero, lungs are less inflated and fluid filled with high vascular resistance, with majority of right ventricular blood flow bypassing lungs through the ductus arteriosus (DA) to aorta. B) After birth transition, lungs are aerated with lower vascular resistance, with the majority of right ventricular blood flow going to the lungs. (Original illustration. *Reference: Goldsmith JP, Chapter 26: Delivery room resuscitation of the newborn. In: Martin RJ, Fanaroff AA, and Walsh MC, ed. Fanaroff and Martin's Neonatal-Perinatal Medicine, 9th edition, volume 1. St. Louis, Missouri: Elsevier Mosby; 2003: 450.)*

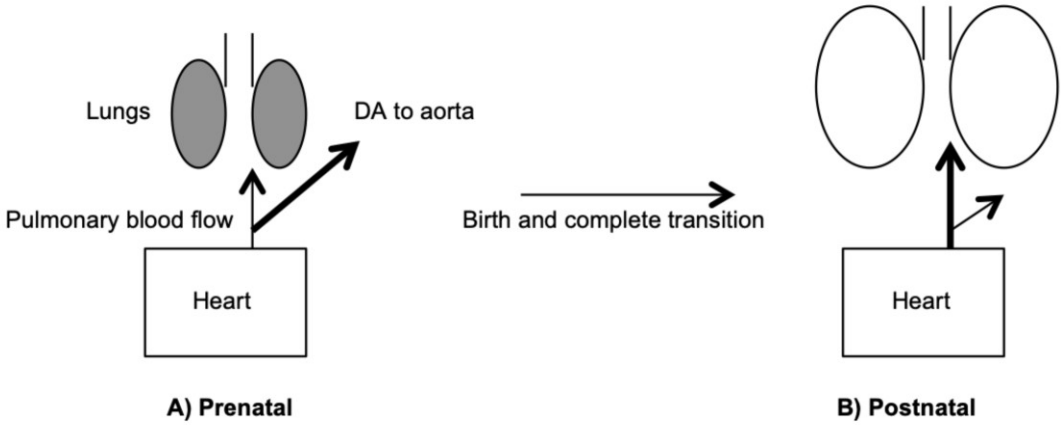


Figure 1.3 – High-frequency oscillator diagram, with piston to generate oscillations. *FGF = fresh gas flow*. (From Thompson WK, et al. High-frequency oscillation compared with standard ventilation in pulmonary injury model. *J Appl Physiol* 1982;52:543)

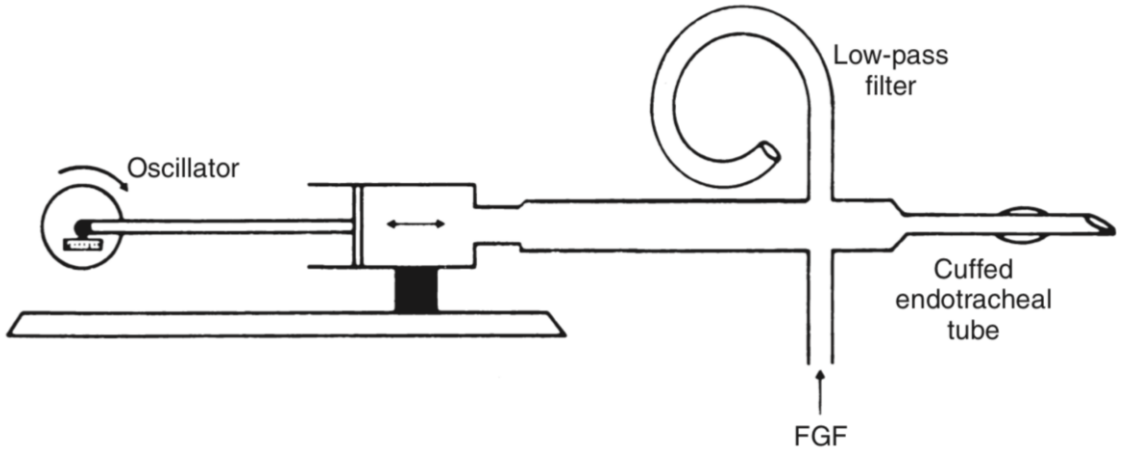
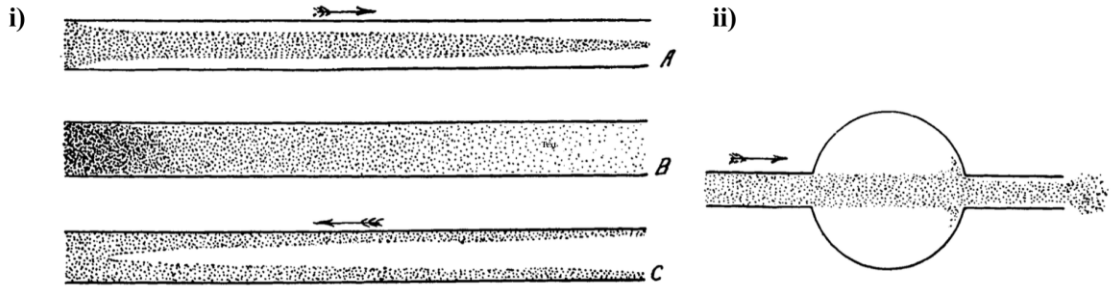


Figure 1.4 – Illustrations showing the 'smoke spike' experiment. i) A shows the spike moving through the tube, B shows when the flow is stopped with instantaneous mixing, and C shows a clear air spike on inspiration. ii) shows the smoke crossing a bulb with little to no mixing. (From Henderson Y, Chillingworth FP, Whitney JL. The respiratory dead space. *Am J Physiol*. 1915; 38(1): 1-19)



Chapter 2 - Cardiopulmonary Interactions

The pulmonary and cardiovascular systems are intrinsically linked throughout life due to their physiological interactions. Common interactions include negative intrathoracic pressure increasing preload to the right ventricle, pulmonary vascular resistance affecting right ventricle (RV) function and output, and pulmonary venous return affecting preload of the left ventricle (LV) and consequently its function **[1]**. In "normal" breathing, the respiratory muscles (primarily diaphragmatic and intercostal muscles) contract to generate negative intrathoracic pressure which draws air into the lungs. This results in an increased venous return to the RV and thus increased RV output. The alveoli are able to inflate relatively easily due to the surfactant coating on their surfaces, which reduces surface tension, reduces areas of atelectasis, and decreases the pressure required for inflation **[2]**. The alveolar inflation further lowers the pulmonary vascular resistance, reduces the RV afterload, and increases pulmonary blood flow.

Transition at birth involves further complex interactions between the pulmonary and cardiovascular systems **[3]**. Important aspects of transition from fetal to neonatal life include: decreasing pulmonary vascular resistance, increasing systemic vascular resistance, increasing lung compliance, and decreasing lung resistance. The postnatal pulmonary changes are due to breathing with subsequent inflation and recruitment of the lungs. These changes initiate increased pulmonary circulation to facilitate increased gas exchange, which occurs concomitantly with increased cardiac or LV output, thereby supplying the increased metabolic requirements in terms of oxygen delivery and carbon dioxide removal as compared to the fetus **[3]**. Multiple changes must occur for this transition to happen, and complications around the time of birth which affect any of these variables may result in pulmonary and/or cardiovascular dysfunction. A common

example is RDS which occurs when surfactant is either quantitatively or qualitatively deficient, the most common reason being prematurity. This can result in atelectasis and decreased compliance, which causes the neonate to use more force to inflate alveoli and generate air flow leading to hypoxemia and hypercarbia **[39][40]**. If not provided respiratory support, the inability to achieve sufficient gas exchange can result in a persistently elevated pulmonary vascular resistance, also commonly known as persistent pulmonary hypertension of the newborn because the pulmonary vascular resistance is normally elevated prenatally compared to postnatally as described earlier in this paper. This is a serious condition which can result in a vicious cycle of worsening hypoxemia, hypercarbia, and metabolic acidosis which further worsens the persistent pulmonary hypertension and may cause significant morbidity or even mortality. Persistent pulmonary hypertension of the newborn is a pathology which exemplifies the interaction between pulmonary and cardiovascular systems, since with worsening persistent pulmonary hypertension, the RV and LV functions are both compromised. This occurs because the RV afterload increases and, with decreased pulmonary blood flow, the LV preload decreases, thereby decreasing the overall cardiac output. Therefore, not only is there hypoxemia and hypercarbia, but there is also decreased cardiac output which compromises the body's ability to maintain the necessary oxygen delivery and carbon dioxide removal **[3]**.

Hemodynamic monitoring

Monitoring the hemodynamic effects of various ventilation methods entails

measuring parameters at the level of ventricular function, systemic and regional blood flow, regional oxygenation, and biomarkers of tissue inflammation, ischemia, and injury. The following is a summary of the main parameters used in this study to assess hemodynamic function ranging from ventricular to tissue level.

The basic physiology of cardiorespiratory interactions relates to changes in pleural pressure secondary to inspiration and expiration. Physiologic breathing occurs via negative pressure inspiration which results in more negative intrapleural pressure, subsequently creating lower right atrial pressure (Pra). Low Pra facilitates blood flow from the vena cavae into the right atrium and increases right-sided preload which improves cardiac output overall **[1]**. There are two main factors: inspiration which decreases intrapleural pressure and subsequently reduces Pra, and diaphragm descent which increases intra-abdominal pressure and thereby increases pressure in the venous capacitance vessels thus increasing flow into the inferior vena cava. The relationship between Pra and venous return is shown in Figure 2.1.

Hemodynamic effects of positive pressure ventilation

It has been known for some time in the scientific literature that positive pressure ventilation, notably when intubated, has negative effects on various parameters of cardiac output. In the 1950s-1960s, animal studies were done showing that mechanical ventilation resulted in cyclic changes in vena cava, pulmonary artery, and aortic blood flow **[4]**. The negative changes were noted to be associated with inspiration, and decreased vena cava flow was related to increased right atrial pressure and compression

of the vena cava secondary to increased pleural pressure, which is the opposite from physiologic negative pressure breathing **[5]**.

The effects of positive pressure ventilation on left ventricular function is different when compared to the right ventricle. The two ventricles are interdependent so that, for example, when the RV output is decreased this simultaneously decreases the LV preload. Nevertheless, there are benefits of positive intrathoracic pressure to the LV, notably with regards to afterload **[6]**. Based on the law of LaPlace, the afterload (or transmural pressure, Ptm) experienced by a ventricle correlates with the pressure inside the ventricle, the thickness of the ventricular wall, and the radius of the ventricle, all of which is illustrated by the formula:

Pressure = (2 x Thickness x Ptm) / Radius,

rearranging for "Ptm":

(1) Ptm = (Pressure x Radius) / 2 x Thickness

The "pressure" is that within the ventricle itself. The Ptm is illustrated by:

(2) Ptm = intraventricular pressure – intrapleural pressure

The ventricular Ptm is directly proportional to afterload, so that the lower the Ptm is the lower the afterload that the ventricle experiences will be. As illustrated in equation (1), Ptm is positively correlated with ventricular pressure and radius, while it is negatively correlated with ventricular wall thickness. Positive intrathoracic pressure

functions to decrease the wall stress and consequently reduce the afterload by increasing intrapleural pressure, as indicated by equation (2). These physiologic effects serve to improve LV output if afterload is the major limiting factor.

Neonatal myocardial function is a topic of active research, with cardiac output relating to four important factors: preload, contractility, afterload, and heart rate **[1]**. The ventricles are interdependent, with the function of one relating to the function of the other. Increased afterload negatively affects ventricular function, with increased LV afterload being most common soon after birth due to increased systemic vascular resistance secondary to loss of the low resistance placental circulation **[1]**. Though pulmonary vascular resistance typically decreases after birth, positive pressure ventilation may serve to increase it if there is hyperinflation of the lungs, which serves to increase RV afterload, and positive pressure ventilation may also decrease RV preload thereby compromising RV function. Elevated pulmonary vascular resistance serves to increase RV afterload, and positive pressure ventilation may also function to increase pulmonary vascular resistance, as well as RV preload, thereby compromising RV function. This subsequently affects LV preload and LV function, due to the interdependency of the ventricles. Given this is a common situation encountered clinically with conditions causing respiratory distress such as respiratory distress syndrome and meconium aspiration syndrome, supporting RV function is an important management consideration notably when mechanically ventilated **[52]**.

There have been several notable studies investigating the relationship between high frequency ventilation and hemodynamics, including both animal and human. Traverse et al in 1989 studied cats and cardiovascular effects of both HFOV and high

frequency jet ventilation **[47]**. They found that both modes were comparable, but that increasing MAP resulted in decreasing cardiac output and increasing pulmonary vascular resistance in both healthy and saline lung-lavaged cats, though the effect of MAP was greater in healthy cats. The Kinsella et al group studied CMV versus HFOV's hemodynamic effects in the premature baboon in a paper published in 1991 **[48]**. They titrated the MAP based on the arterial to alveolar oxygen ratio. Results showed that, since the HFOV MAP was reduced more than the CMV MAP, there were no differences in hemodynamic parameters including LV output, cerebral blood flow, and central venous pressure. Laubscher et al conducted one of the first studies of hemodynamic effects of HFOV in preterm infants published in 1996 **[49]**. They conducted a crossover trial where infants were initially on CMV and then on HFOV, and they found that the LV output decreased on HFOV. However, the MAP in the HFOV group was higher than the CMV group. Another comparative study on neonates was published by Simma et al in 2000 where they measured hemodynamic markers in neonates who were changed from CMV to HFOV for rescue therapy **[50]**. Their findings, though again confounded by a higher MAP in the HFOV group, found that HFOV compromised LV output via decreased LV filling. A 2004 study of mainly term neonates with a variety of respiratory pathologies on HFOV studied the effect on changing MAP on cardiac output compared to a control group on HFOV with a constant MAP **[51]**. This group of Gullberg et al. found that the cardiac output decreased with increasing MAP, with the most significant effect at the highest MAP. These studies emphasize the relationship between MAP and cardiac output that is seen clinically.

Measuring hemodynamic and oxygenation parameters

In order to determine effects on systemic and regional perfusion, validated parameters must be measured. These include markers of ventricular function, blood flow in systemic vessels, and indicators of oxygen delivery to tissues.

Left ventricular function

Assessment of LV function in a piglet animal model, as used in this study, includes a variety of methods that are non-invasive and invasive. Non-invasive methods include heart rate and cuff blood pressures, though these are limited as they relate to LV function indirectly **[7].** Transthoracic echocardiography enables direct assessment of LV function, in particular its cardiac output, and is non-invasive **[8]**. Invasive methods include continuous arterial blood pressure monitoring, as well as flow probes on the aorta and main pulmonary arteries which provide direct and indirect assessments, respectively. Main pulmonary artery flow probes are not used in this thesis' investigation **[7].** A direct way of measuring LV function invasively is via an intraventricular catheter **[8]**, which will now be described in detail.

In 1988 Sagawa and colleagues published studies on the relationship between cardiac contraction and pressure-volume loops (PV loops) **[9]**, which provide the basis behind much of what is now used to assess cardiac function. These PV loops have enabled measurement of various parameters including end-systolic volume and pressure, end-diastolic volume and pressure, stroke volume and work, ejection fraction, cardiac output, dP/dt max (a measure of contractility), and Tau (isovolumic relaxation time

constant, a measure of LV diastolic function). Research on cardiac function has continued over the decades, with the most convenient and accurate way to obtain PV loop data being a conductance catheter inserted into the ventricle along its long axis, which provides real-time volume and pressure readings **[10]**. Please refer to Figure 2.2 for an example of PV loop data.

The earlier work measuring ventricular function included Baan et al in 1981 where they developed a 7 French catheter with 8 equidistant platinum electrodes that was advanced into the left ventricle of dogs **[11]**. The difference in electrical resistance measured at each electrode during the cardiac cycle enabled calculation of cardiac output and stroke volume which correlated well ($r = 0.99$) with an electromagnetic flowmeter, the gold standard at the time. In 1990, Applegate et al used dogs to compare the measure of LV volume by conductance catheter with that of ultrasound using endocardial crystals surgically implanted in the endocardium to facilitate measuring LV dimensions **[12]**. This enabled measurement of parameters including end-systolic volume, end-diastolic volume, dP/dt, and stroke work. They found an excellent correlation of $r = 0.97 +/- 0.04$ between the catheter conductance measurements and LV volume measurements under steady-state conditions. This correlation dropped at lower volumes such as with bicaval occlusion. They also noted that the conductance catheter measured direction and magnitude of the change in contractile status, such as with use of inotropes.

Following this in 1993, Steendijk et al studied the use of a conductance catheter with two more electrodes which utilized a "dual-excitation technique" to generate a more homogeneous intracavitary electric field (Figure 2.3)**[13]**. The dual excitation helped reduce the limitations with better correlation with the electromagnetic flow probes,

though the correlation still decreased with increasing ranges of ventricular volume such as in animals with dilated hearts. Limitations to the conductance catheter are largely related to the fact that the homogeneity of the electric field only holds within a limited range of ventricular volumes, because some of the ventricular blood is located in areas of reduced field strength and therefore affects the resistance differently.

The Millar® catheter (Millar Inc, Houston, TX, USA) is one such conductance catheter which is commonly used at this current time, and is the catheter used in the study. It has been and continues to be used in animals for research purposes, including mice, rats, and piglets **[6][10][53]**. As will be explained in the methods section, calibration to the animal's blood and then, after insertion, to a saline volume bolus is required for optimal measurements given each individual animal is different **[10]**.

Regional perfusion

Assessing regional perfusion grossly requires monitoring the flow of arterial blood in the main vessel supplying an organ. Ultrasonic flow probes and Doppler ultrasound have been used to assess blood flow to organs in various animal models as markers of regional perfusion. Correlation between the two measurement devices, ultrasonic flow probes and Doppler ultrasound, is good as shown by studies such as that by Haaland et al in 1994 assessing cerebral perfusion via carotid artery flow in newborn piglets **[14]**. Pearson et al studied the flow in the superior mesenteric and left renal arteries of newborn piglets in relation to dopamine and fenoldopam infusions to assess dopaminergic receptor-mediated effects **[15]**. Animal models studying the effects of cardiopulmonary bypass and deep hypothermia circulatory arrest also benefit from using

flow probes, such as Tirilomis et al studying carotid artery flow in newborn piglets after cardiopulmonary bypass and deep hypothermic circulatory arrest **[16]**. These and many other studies have shown, particularly in the newborn piglet model, the efficacy of using flow probes to continuously monitor regional perfusion via arterial blood flow. In comparison, fluorescent or radioactive microspheres techniques can only provide intermittent regional perfusion or blood flow measurements in a few time points during the experimental period **[28]**. For the study of this thesis, ultrasonic flow probes were chosen due to ease and familiarity of using them in the lab.

Regional oxygenation

Measurements of cardiac function and regional blood flow assess several aspects of hemodynamics, but cannot predict the distribution of oxygen to the tissues. A common tool used in the past two to three decades to measure regional oxygenation is that of near-infrared spectroscopy (NIRS). Its use was first published in 1977 by Frans Jobsis where his group tested NIRS on shaved areas of the scalp on cats. It was known at that time that scattering and absorption are wavelength-dependent properties of light, and thus infrared light was favoured for assessing properties of tissues **[17]**. Wavelengths below 700nm are scattered by tissues or largely absorbed by hemoglobin, and wavelengths above 1300nm are mostly absorbed by water in normally hydrated tissues. The infrared wavelengths used generally range from 700nm to 1000nm, with the main molecules absorbing at those wavelengths being water, cytochrome c oxidase, oxygenated hemoglobin (HbO_2), and reduced hemoglobin (HbR). HbR's absorption peak is at a different wavelength than HbO_2's, thus allowing their relative concentrations to be

determined. In the study by Jobsis et al, they applied fiberoptic bundles carrying near-infrared light to one temple of the cats and received the transmitted light from the other temple via an IR sensitive photomultiplier tube. By monitoring the difference between the peak absorbance wavelengths for HbO_2 and HbR, they showed that there was increased HbR detected during anoxic periods (ie. interrupting artificial respiration for 3 minutes). The results of this and other experiments indicated the potential for non-invasive monitoring of regional oxygenation based on NIRS. The main limitation at the time was delivering sufficient light energy at the specific wavelengths to enable entering and exiting the tissue and remaining detectable **[17]**.

Studies regarding NIRS were also done with humans, with oxygen delivery to the brain a priority as it is a vital organ. One of the earliest on newborns was by Wyatt et al in 1986 **[18]**. Their goal was to be the first to quantify changes in cerebral oxygenation in sick newborn infants. They conducted NIRS early in life and at ages ranging from 12 hours to 10 weeks of age. They built their own portable NIRS apparatus which involved directing a fiber-optic bundle equidistant between the anterior fontanelle and the external auditory meatus, with the receiving fiber-optic bundle located on the opposite side – a setup similar to that of Jobsis et al. They simultaneously measured S_aO_2 and P_aCO_2. Their results showed that NIRS detected increasing HbO_2 with increasing S_aO_2, and they were also able to show increasing oxidation of cytochrome c oxidase which presumably indicated increased oxygen delivery to mitochondria. Research on a variety of animal models and humans occurred over the following decades. In 2002, Brown et al studied NIRS for monitoring cerebral hemodynamics in piglets by injecting indocyanine green and found it correlated highly with CT scan, and was superior to ultrasonography in

assessing overall cerebral blood flow changes **[19]**. Tichauer in 2006 studied the cerebral metabolic rate of oxygen in piglets by using NIRS and found it to be an effective and non-invasive method of doing so, which could potentially serve as an early marker of cerebral energy dysfunction such as with neonatal hypoxic-ischemic events **[20]**. There has been much research done on NIRS in neonates, infants, and children including from those born with congenital heart defects which require cardiac surgery **[21][22][23][24]**. Studies in neonates often focus on cerebral NIRS including the effect of patent ductus arteriosus **[41][42][43]**, but have also assessed splanchnic NIRS notably in relation to necrotizing enterocolitis **[44]**. Overall, NIRS has been found to be most sensitive regarding cerebral oxygenation. Generally, it has been found that trending the NIRS values at a combination of sites on the body, such as combining cerebral and renal regional oxygenation values, provides even more useful clinical information **[25]**. As a result, NIRS currently remains a clinically valuable tool for monitoring trends of regional oxygenation though it has not yet been determined whether its use is associated with any changes in long-term outcomes **[26]**. A randomized-controlled trial done by the SafeBoosC II team in 2016 showed that using cerebral NIRS to help guide management using a standardized guideline for the first 72 hours of life in infants less than 28 weeks reduced the hypoxia and hyperoxia burden of those infants **[45]**. However, 2 year neurodevelopmental outcomes showed no differences, though the study was not powered for that **[46]**. For the present thesis, the use of NIRS provides a non-invasive and real-time measure of regional oxygenation.

Monitoring of cerebral perfusion and oxygenation

The brain is the most important organ, other than the heart, to maintain perfusion to, and the regulation of its blood flow continues to be a significant topic in animal and human neonatal research. Maintaining perfusion and oxygenation of the brain has been shown to be associated with long-term neurodevelopmental outcomes (cognitive assessments up to around 5 years of age), in populations such as congenital heart surgery neonates and small-for-gestational-age fetuses, where blood and oxygen delivery to the brain is at risk of being compromised **[36][37][38]**. Technologies such as NIRS have shown potential in detecting cerebral ischemia non-invasively, notably when compared to other markers of cerebral blood flow such as anterior cerebral artery Doppler flow and radioactive microsphere technique **[27]**. Common carotid artery blood flow (CCAF) measured via Doppler flow probe has also been shown to correlate with global cerebral blood flow in piglets **[14][28]**. The combination of NIRS and CCAF probes can provide more data regarding cerebral perfusion and oxygen delivery which, for this thesis' investigation, is a particular area of importance to monitor given the association with long-term outcomes in neonates. A study conducted by Gavilanes et al in 2001 looked at piglets with hypotension secondary to hypovolemia, and measured changes in cerebral electrocortical activity, hemodynamics, and oxygenation **[29]**. In particular, they used NIRS and CCAF probes and found that there was a direct correlation between carotid flow and mean arterial blood pressure, but no significant change in NIRS regardless of the blood pressure. In piglets with hypoxia, CCAF was found to initially increase and, with persistent hypoxia, to later decrease when compared to their normoxic counterparts

[30]. CCAF is, of course, not a direct measurement of cerebral blood flow. Interestingly, in exercising adult humans it has previously been shown that with increasing exercise intensity, the left CCAF continued increasing while the left middle cerebral artery initially increased and then decreased, indicating an increased global cerebral blood flow but only in specific areas **[31].** Therefore, changes in CCAF can be considered a surrogate marker of global cerebral blood flow changes.

Biomarkers of inflammation, oxidative injury, and ischemia

Though not available in real time since they are obtained from tissue samples post-mortem, biomarkers help quantify injury, inflammation, and/or ischemia to various organ systems. Lactate helps quantify the amount of ischemia experienced by a tissue because it serves as an end-product of glycolysis during hypoxia to maintain generation of adenosine triphosphate **[32]**. Glutathione functions largely in the body's protective mechanisms related to reactive oxygen species. Reduced glutathione (GSH) and oxidized glutathione (GSSG) function as electron donors and acceptors, respectively. Maintaining their optimal ratio, GSH/GSSG is critical to cell survival and relates to levels of stress being experienced by the cellular environment since a deficiency of GSH results in an increased risk of oxidative damage **[33]**. Figure 2.4 illustrates trends in the aforementioned biomarkers.

Inflammation is indicated by a large variety of biomarkers, and a part of this thesis' research is determining the extent of lung injury and inflammation produced by

the saline lung lavage. This is a method previously described in many animal models, and which is detailed later in this thesis. Common markers associated with the extent of lung injury and inflammation, in conditions such as acute lung injury and acute respiratory distress syndrome, include interleukin-8 and tumour necrosis factor-alpha **[34]**. A more recent development in immunology is that of pattern recognition receptors which are part of the innate immune system and the first line of defense. In relation to acute respiratory distress syndrome, the inflammatory signaling cascades initiated by pattern recognition receptors commonly involve proinflammatory cytokines such as tumour necrosis factor-alpha, interleukin-1-beta, and interleukin-8. Interleukin-8 functions largely as a neutrophil attractant and activator, which are found in various cases of acute lung injury with neutrophilic infiltration into the alveolar space **[34]**.

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Figure 2.1 – Relationship between *Pra* and venous return (*VR*) under normal conditions. Venous return plateaus when *Pra* becomes negative because the vena cavae collapse as they enter the thorax. *Pms* = mean systemic pressure **[1]**

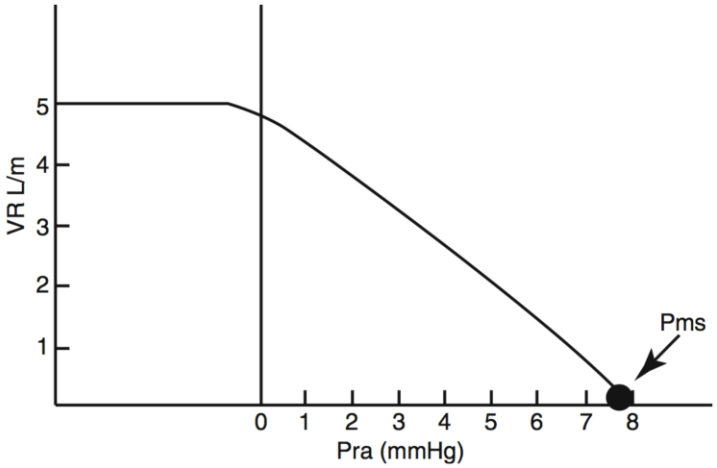


Figure 2.2 – Examples of pressure-volume (PV) loops: variably loaded left ventricular PV loops in dogs with conductance catheter, produced by caval occlusion at rest and during exercise **[34]**

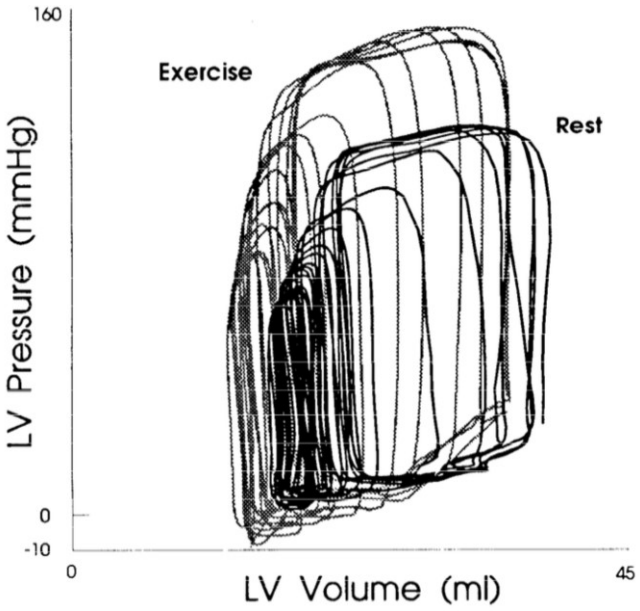


Figure 2.3 – Schematic representation of a conductance catheter positioned along the long axis of LV, similar to the catheter used in this thesis' study. Conventional single-excitation field is generated by passing a current I_s, via electrodes *1* and *10*. Superposition of a second current I_c via electrodes *2* and *9* results in a "dual excitation" field. Dotted lines represent division of ventricle into 5 segments. Voltage over each segment is measured continuously and divided into total current to yield segmental conductances. Note that *1-10* and *2-9* currents are opposite in polarity. (**[13]** – Figure 1, page H2199**)**

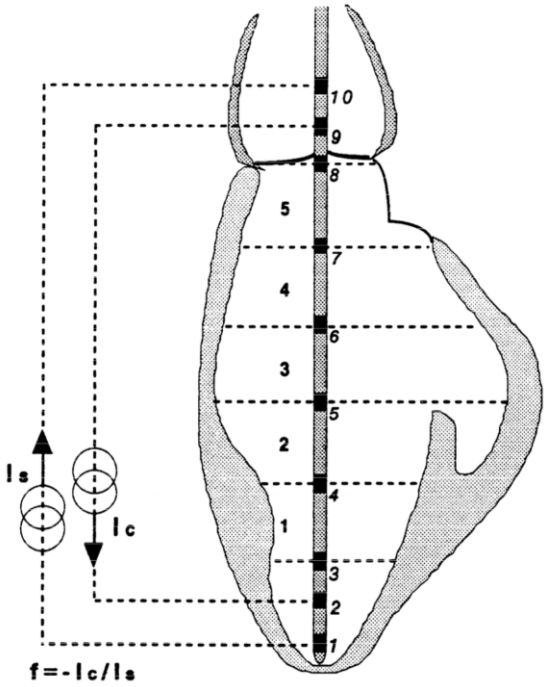
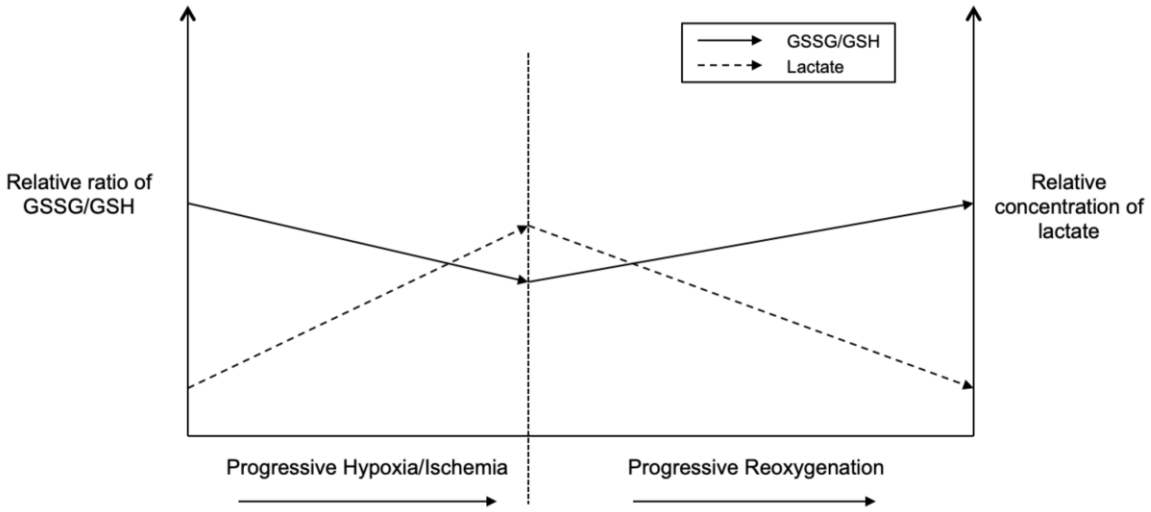


Figure 2.4 – Relative depiction of trends of GSSG/GSH ratio and lactate levels during hypoxia/ischemia and reoxygenation. Not to scale or illustrating specific values. **[32][33]**



Chapter 3 – Piglet model of neonatal respiratory distress syndrome

Basic science research entails a variety of models used to generate knowledge about anatomy and physiology without the risks of experimentation on actual human subjects. Neonatal research has commonly used models such as rat pups, lambs, rabbits, and piglets. Among the large animal models, newborn piglets are often used in hemodynamic patho-physiological studies as they have similar anatomy, genetics, and physiology as humans, and the breeds vary from miniature to large **[1]**. Practically and ethically speaking, they are also more acceptable and cost effective to use compared to primates, which are in turn closer in physiologic and genetic similarity to humans **[3]**. Xenotransplantation of pig organs to primates have apparently been successful, as this is a potential source of organs for humans in the future if incompatibilities can be identified and addressed **[2]**. Of note from such research, the pig renal artery is particularly susceptible to spasm **[2]**, which is relevant to this thesis' study as will be discussed later. The full-term piglet is equivalent in maturity to a 36-38 week gestation human newborn **[3]**. The pulmonary, cerebrovascular, and cardiovascular systems are also similar, with the main differences being higher pulmonary and systemic resistances as well as a less compliant chest wall **[3][17]**. A limitation of the piglet model, however, is that of smaller sample size due to cost and complexities of instrumentation **[3]**. Premature piglets were not used primarily due to the need to deliver them via Cesarean section which would result in euthanization of the sow and the piglets that were unable to be used.

Piglets have been used to study a variety of organ systems, such as the central nervous and cardiovascular systems. The effects of hypoxia on cerebral energy metabolism and encephalopathy, as well as potential therapies, have been studied in piglets. These investigations prompted human trials and the now accepted use of

therapeutic hypothermia for moderate to severe hypoxic-ischemic encephalopathy **[4,5]**. Hemodynamic parameters and their response to interventions have also been studied in a variety of piglet models of shock **[6,7]**. These parameters may include mean arterial pressure, systolic and diastolic pressures, heart rate, cardiac index, central venous pressure, pulmonary artery pressure, and central venous oxygen saturation.

In terms of models of respiratory distress, one of the first newborn animal experiments studying the effects of inhaled nitric oxide on pulmonary hypertension was done in newborn piglets at the same center as this thesis, the University of Alberta. Etches et al, published in 1994, used an acute hypoxic pulmonary hypertension piglet model by exposing piglets to a FiO_2 of 0.1 to 0.14 with a target SpO_2 of 35-45% **[8]**. Inhaled nitric oxide was then administered at varying doses, and multiple parameters including the pulmonary arterial pressure were measured. The results showed that pulmonary pressures decreased at all levels of inhaled nitric oxide, and more quickly and significantly than providing an FiO_2 of 1.0. This study, among others, led to multiple randomized and controlled clinical trials that resulted in inhaled nitric oxide now being the standard of care for near-term and term infants, and some specific populations of more premature infants, with significant pulmonary hypertension **[9]**.

Piglet Model of Respiratory Distress Syndrome

RDS, detailed earlier, is ultimately due to surfactant deficiency. This results in increased surface tension which in turn decreases the compliance of the lung due to generalized or diffuse alveolar collapse. Studying lamb lungs at differing gestational

ages, Brumley et al in 1967 found that there is an inverse relationship between alveolar surface tension and gestational age, and a direct relationship between the concentration of disaturated phosphatidylcholine (a component of surfactant) and gestational age **[10]**. They concluded that changes in the pressure-volume curve characteristics of lungs relate to the amount of surfactant present, and likely explain the challenges they faced with premature neonates and respiratory failure which was consistent with previous studies. Following many studies including surfactant administration to animals, a randomized controlled trial of surfactant instillation in premature neonates was conducted in 1985 by Enhorning et al **[11]**. They intubated infants immediately after birth with subsequent instillation of surfactant, and found significantly improved gas exchange in those infants who received surfactant compared to those who received nothing.

In an effort to study more severe lung pathology in animal models, there was a need to create an animal model of respiratory distress syndrome. Some of the first attempts used asphyxia at birth to induce respiratory distress in lambs, before the specific association between surfactant and respiratory distress syndrome was known **[12]**. This model, however, was not practical to study the lungs in a clinical manner given the whole animal was asphyxiated. The first saline lung lavage model was done in guinea-pigs and published by Lachmann et al in 1980 **[13]**. Prior experiments by the same group in 1978 had shown that lavages resulted in removal of surfactant phospholipids from the lungs. In a 1980 study the investigators used physiological saline at body temperature and a volume of 35 ml/kg per lavage over ten lavages, each of which lasted approximately 20 seconds. The lavages resulted in decreased lung compliance as indicated by pressure-volume loops, and significantly worsened oxygenation and ventilation as indicated by

P_aO_2 and P_aCO_2 trends **[13]**. Lung lavages enabled more control over the severity of lung disease, and histology confirmed the similarity to respiratory distress syndrome. The following decades found many studies using a similar saline lung lavage model in a variety of experimental animals, including piglets **[14,15,16]**. The piglet model notably facilitates the study of ventilation modes and surfactant in a neonatal animal model.

Given the biological similarities between humans and pigs, the economic and ethical advantages, and the history of over three decades of piglet models to study various neonatal conditions; it is a validated model that was chosen to be used in this thesis' study given the focus on a combination of respiratory and cardiovascular parameters in real-time and post-mortem.

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Chapter 4 – Experience in developing a piglet model of respiratory distress syndrome

For the past approximately 2 decades the animal lab used for this study has utilized piglet models for neonatal basic science experiments, notably those of hypoxia-reoxygenation models **[1][2][3][4][5]**. However, this thesis' study was the lab staff's first time using high frequency oscillatory ventilation (HFOV) and saline lung lavage. As a result, it took several trial runs to determine the appropriate ventilator settings needed for adequate oxygenation and ventilation, both for HFOV with and without volume guarantee (VG). It was determined that similar tidal volumes as used in the neonatal unit, starting at 2 ml/kg, were adequate to start on HFOV along with the inspiratory:expiratory ratio, frequency, amplitude, and mean airway pressure. To avoid endotracheal tube leak causing varying pressure delivery between piglets, a tracheotomy was done to insert the endotracheal tube and the trachea was sutured securely around it. This was important due to the inability to measure leak accurately while on HFOV.

A significant challenge was determining the protocol for a saline lung lavage-induced piglet model of respiratory distress syndrome. This included the time over which the saline would be administered, how long before suctioning it out, how frequently to administer it, and what volumes to administer. Though there have been multiple studies of using warmed 0.9% sodium chloride for lung lavage in animals including piglets as discussed earlier, quantification of lung disease severity are limited. One of the challenges in particular entailed determining what severity of lung disease, as determined by the calculated alveolar-arterial oxygen difference or gradient ($AaDO_2$), to achieve to enable a reproducible experiment with clinically relevant results. $AaDO_2$ is a measure of the difference in partial pressure of oxygen between the alveoli and blood, therefore being an indicator of the severity of lung disease. Initially the goal was to achieve an

$AaDO_2$ of around 400-450 mmHg based on literature reviewed of neonates with respiratory distress **[1][2]**. However, it was noticed that several piglets became hypoxic and acidotic in the process to the point that they did not survive the experiment due to the $AaDO_2$ progressing beyond 500 mmHg. As a result, the goal $AaDO_2$ was changed to 300-450 mmHg, and the saline lavages would be stopped once the gradient went over 300 mmHg because the $AaDO_2$ typically increased after the lavages were finished. The volumes used to initially lavage were also decreased from an initial volume of 20 ml/kg to 10 ml/kg so as to reduce the chance of overshooting the desired $AaDO_2$, after noting that some piglets would increase their $AaDO_2$ significantly with a given lavage. There was also careful attention paid to ensuring the piglet was supine and level to optimize equal distribution of saline into both lungs. After achieving the goal $AaDO_2$, a repeat blood gas to calculate the $AaDO_2$ was done 15 minutes later to ensure stability as some piglets had a large decrease in their $AaDO_2$ indicating they needed further lavages. The total lavage time was limited to a maximum of 60 minutes to avoid it being a confounding variable. The clinical applicability has one pertinent limitation with regards to HFOV: recruitment maneuvers are becoming more commonly used in newborns on HFOV to optimize lung inflation, and such a technique was not used in this investigation **[18][19]**. Details of the saline lavage and ventilation protocols are in Chapter 5 in the "Materials and Methods" section.

Other challenges included attempting to incorporate echocardiography into the experiment. However, it was noted that these hypoxic animals were critically ill and unstable and the procedure was not tolerated. In terms of monitoring, the renal artery flow probe often stopped reading presumably due to the displacement of, and the piglet's

position, in this experimental model. There may have also been a component of renal artery spasm at times, as was discussed in the previous chapter. Given the severity of the piglets' cardiopulmonary status during experimentation and their supine position, the renal artery flow probe was unable to be accessed without risking destabilization of the piglet. As a result, insufficient data was obtained for analysis of renal flow. The neonatal S_pO_2 probe occasionally had issues detecting pulsatile flow after about 2-3 hours, often despite moving it to different areas of the piglet, which was presumed secondary to poor peripheral perfusion and it was compensated for by performing blood gases more frequently to monitor S_aO_2. There was consistent correlation between S_aO_2 and pre-ductal S_pO_2 prior to the probes being unable to read, so there was no evidence of pulmonary hypertension sufficient to cause a large right-to-left shunt resulting in deoxygenation in any of the piglets in the early part of the experimental period.

These notable challenges helped refine the protocol used, with more reliable data collection and reduced morbidity and mortality. The specific methods, results, and limitations will be discussed in Chapter 5.

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Chapter 5 - Effects of High Frequency Oscillatory Ventilation with Volume Guarantee on Left Ventricular Function, Systemic and Regional Hemodynamics in a Piglet Model of Respiratory Distress Syndrome

Abstract

Respiratory failure is a common condition faced by neonates in the NICU. High frequency oscillatory ventilation (HFOV) is often used for neonates with refractory respiratory failure. Volume guarantee (VG) mode has been added to some HFOV ventilators. The systemic and cerebral hemodynamic effects of HFOV, and the impact of adding VG mode, is not well understood. This study used a piglet model of respiratory distress syndrome (RDS) induced by saline lavage. Piglets (full term piglets, 1-3 days, weight 1.5-2.4 kg) were randomized to either conventional mechanical ventilation (CMV), HFOV, or HFOV+VG. Hemodynamic and pulmonary parameters were collected over 240 minutes in these newborn piglets with severe RDS treated by CMV, HFOV or HFOV+VG. Experimental groups had similar mean airway pressures and alveolar-arterial oxygen diffusion gradients ($p>0.05$). Left ventricular cardiac index, stroke volume, and ejection fraction decreased significantly in CMV compared to sham-operated groups after 120 minutes of moderate-severe RDS (up to 69%, 52%, and 57% relative differences, respectively; $p<0.05$). At all time points except 240 minutes, HFOV+VG had a higher dP/dt max than sham-operated (up to 73% relative difference; $p<0.05$). Cerebral near-infrared spectroscopy and right common carotid artery flow decreased more in HFOV+VG during the last hour when compared to HFOV and its own baseline value, respectively (30% vs. 43% and 14 ml/kg/min vs. 31 ml/kg/min, respectively; $p<0.05$). The arterial-venous SO_2 difference was greater in HFOV+VG mode compared to CMV at 240 minutes (56% vs. 36%, $p<0.05$). Tissue analysis of

markers of oxidative stress and ischemia indicated no significant differences. This study found that HFOV+VG had no effect on left ventricular cardiac index, and a negative effect on both systemic oxygen delivery and cerebral perfusion and oxygenation.

Introduction

Respiratory failure is the most common medical challenge in the neonatal intensive care unit (NICU). Respiratory distress syndrome (RDS) and bronchopulmonary dysplasia (BPD) in particular are frequent causes of respiratory failure, notably in preterm infants **[1]**. There are multiple modes of invasive mechanical ventilation, including conventional mechanical ventilation (CMV) and high frequency oscillatory ventilation (HFOV). HFOV is commonly used as a 'rescue' mode of ventilation when CMV is insufficient. Elective use of HFOV is an ongoing topic of research with no definitive benefits shown yet when compared to CMV **[2]**. The basic mechanism is that of tidal volumes less than dead space and at higher frequencies, whereas CMV uses tidal volumes above dead space and at physiologic frequencies. HFOV enables ventilation without using the bulk convection of CMV but rather a variety of other theoretical processes as described by Chang **[3]**. A common clinical concern is the effect of HFOV on the hemodynamic stability of a patient, given the difference in positive intrathoracic pressure when compared to CMV **[4]**. This is notable since patients placed on HFOV are often more critically ill and at higher risk of hemodynamic compromise.

Within the past decade, the addition of "volume guarantee" (VG) mode to some HFOV ventilators has occurred, though without research indicating its efficacy or safety **[5]**. VG mode on CMV reduces the damage to lungs, notably those of premature babies, and therefore has become the gold standard **[13]**. Theoretically this is related to the ventilator adjusting the pressures as needed to maintain a

consistent volume, thereby adapting in real time to the compliance of the lung **[11]**. There has been limited research done on HFOV with VG (HFOV+VG) since its implementation, with studies done to date consisting of pilot studies in a clinical setting **[5,6,7]**. There are no prospective trials comparing short and long term outcomes of HFOV and HFOV+VG, nor has there been research looking at hemodynamic effects of HFOV and the possible effects of VG mode. Since there are effects of positive pressure ventilation on cardiac function, and HFOV+VG titrates pressures to maintain a set volume, there is a potential for differing effects on cardiac function.

Given the relative novelty of the HFOV+VG mode, we considered it important to collect detailed hemodynamic and pulmonary data in a piglet model of RDS induced by saline lung lavage. We compared CMV, HFOV, and HFOV+VG along with a sham-operated group. Our hypothesis was that HFOV+VG would have at least a 25% decrease in left ventricular (LV) function compared to HFOV and CMV, with the primary outcome being LV cardiac index (CI), due to the dynamic changes in amplitude to maintain a consistent tidal volume resulting in the heart being exposed to varying pressures at high frequency.

Materials and Methods

The design of our study entailed a newborn piglet model of full-term piglets ranging from 1 to 3 days of age, weighing 1.5 to 2.4 kg, Yorkshire-Landrace, obtained from the Swine Research Technology Centre at the University of Alberta

(Edmonton, AB, Canada). Housing and husbandry of the piglets was conducted at that center, not by the study team, based on policies and procedures that met the Canadian Council on Animal Care and the Animal Care and Use Committees guidelines and approval **[30]**. Premature piglets were not considered due to their lack of availability, and also due to increased fragility and practicality of instrumenting a small animal. Ethics approval was obtained and the study was conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Animal Care and Use Committee for the University of Alberta. ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) were followed in the design, conduction, and reporting of the experiments; please see Appendix 1 for the checklist **[16]**. Piglets have been shown to have similar cerebrovascular, cardiovascular and respiratory systems, with the main difference being higher pulmonary and systemic vascular resistance and a stiffer chest wall **[17]**, with a term piglet being generally equivalent to a 36-38 week gestation human infant.

Instrumentation and anesthesia

The piglets were initially anesthetized with isoflurane and spontaneously breathing. Please refer to Figure 5.1 for a diagram of piglet monitoring and instrumentation. Body temperature was maintained between 38.5 and 39.5 °C by titrating overhead radiant heaters and a heating pad. A percutaneous oxygen saturation sensor was placed to monitor S_pO_2 throughout the experimental period

with the saturation sensor on the right front limb (pre-ductal position). Heart rate was monitored via femoral arterial line. Via a right femoral cut-down, 5-French Argyle catheters (Sherwood Medical Co., St. Louis, Mo) were inserted into the femoral vein and artery to provide intravenous access and venous blood sampling, as well as blood pressure monitoring and arterial blood sampling, respectively. An anterior neck cut-down was then performed and a tracheostomy was created with insertion of a #4.0 Mallinckrodt® endotracheal tube with sutures tied securely around it and the trachea for a secure airway without a leak. Immediately following this, analgesia and anesthesia was initiated with morphine (0.1 mg/kg/hr) and propofol (5-10 mg/kg/hr) infusions, respectively. Maintenance fluids given consisted of 5% dextrose at 10 ml/kg/hr and 0.9% sodium chloride at 2 ml/kg/hr through the venous and arterial catheters, respectively. The piglet was then connected to a Fabian HFO ventilator (Acutronic Medical Systems AG, Hirzel, Switzerland) and started on CMV at baseline settings of: positive end-expiratory pressure (PEEP) 6 cmH_2O, tidal volume (VT) of 10 ml/kg for goal end-tidal CO_2 partial pressure ($ETCO_2$) range 40-60 mmHg, ventilator rate 60 bpm, and inspiratory time 0.3 sec. A urinary catheter was then inserted via cut-down in the midline lower abdomen and inserted transmurally into the bladder and secured with sutures. INVOS near-infrared spectroscopy (NIRS) probes (Covidien, Minneapolis, MN, USA) were placed on the forehead and right flank. A cut-down was done on the left flank to place an ultrasonic flow probe (Transonic Systems Inc., Ithaca, NY, USA) on the left renal artery. The right common carotid artery also had an ultrasonic flow probe placed. After appropriate calibration of a Millar® catheter

(Millar Inc., Houston, TX, USA) in normal saline for a minimum of 30 minutes, and calibration of the software using a fresh arterial blood sample from the piglet, the catheter was inserted into the left common carotid artery and threaded into the LV as indicated by achieving the appropriate pressure-volume loop (PV loop). This was judged by the shape of the loop and systolic and diastolic blood pressures obtained (Figure 5.2). A 1 mL normal saline (0.9% NaCl) bolus was then used to calibrate the Millar® catheter as per manufacturer's instructions.

After the instrumentation phase was complete, the piglet underwent 60 minutes of stabilization with a Ringer's lactate 10 ml/kg bolus halfway through in an effort to facilitate tolerance of the saline lung lavage based on initial experience with bradycardias and hypoxia that could occur during the lavages. Stabilization was defined as a consistent F_iO_2 to maintain S_aO_2 92% or greater, P_aCO_2 between 40-60 mmHg on consistent ventilation settings for 10-15 minutes, and stable HR and blood pressure (fluctuation within 10%). The sham-operated group underwent the same initial instrumentation and anesthesia as well as the subsequent 4-hour monitoring period on CMV, but did not undergo saline lung lavage in order to serve as a control regarding the effects of surgery and anesthesia. The timeline of the protocol is shown in Figure 5.3.

Saline lung lavage

The saline lung lavage model developed by Lachmann et al has been used for several decades to simulate RDS with decreased pulmonary compliance and increased resistance **[12]**. Our approach to the lung lavage was based on the

Lachmann RDS model and modified after the initial pilot experimental runs, due to the lung disease often becoming more severe than intended after the goal alveolar-arterial oxygen difference ($AaDO_2$) was achieved. The goal $AaDO_2$ was determined to be approximately 300 to 450 mmHg, as this indicated moderate to severe RDS based on the piglet responses and literature reviewed **[8]**. The $AaDO_2$ is directly proportional to the severity of lung disease because it's the difference between alveolar and arterial oxygen partial pressure:

$$AaDO_2 = [FiO_2(P_{atm} - P_{H2O}) - (P_aCO_2/0.8)] - P_aO_2$$

After the one hour stabilization period, the piglets in the experimental groups underwent warmed saline lung lavage. This entailed instilling normal saline warmed to approximately 39°C into the endotracheal tube. The tube was then clamped, and it was suctioned after approximately 20 seconds or sooner if the S_pO_2 dropped below 40% or bradycardia occurred less than 90 bpm. This was repeated approximately every 2 minutes with the average total duration of around 45 minutes including a 15 minute stabilization period after the goal $AaDO_2$ was achieved. The initial seven lavages were of 10 ml/kg to avoid overshooting the $AaDO_2$ target range, and the volume was then increased to 20 ml/kg until the $AaDO_2$ gradient reached the desired range due to some piglets requiring more lavages to achieve the desired $AaDO_2$. Blood gases were typically done every two to three lavages, or more frequently, depending on the change in F_iO_2 and S_pO_2 with each lavage. The saline lung lavage was completed within 60 minutes to avoid prolonged

hypoxia that would confound the cardiovascular function and the duration was comparable among groups. Fifteen minutes later, a repeat arterial blood gas was done to confirm the $AaDO_2$ gradient that was achieved.

Ventilation

We further developed a standardized protocol for titrating ventilation based on S_pO_2 and P_aCO_2. The baseline settings for each mode of ventilation after randomization were also part of the protocol. All piglets underwent lavage on CMV at a PEEP of 8 cmH_2O, and otherwise the settings noted previously. After completion of the lavage, the ventilation mode for the subsequent 240 minute experimental period was chosen. HFOV started with a mean airway pressure (MAP) of 2 cmH_2O above the MAP that was required on CMV at the end of the lavages, inspiratory to expiratory ratio 1:2, frequency 10 Hz, and amplitude 30 cmH_2O. HFOV+VG started with a MAP of 2 cmH_2O above the MAP on CMV, inspiratory to expiratory ratio 1:2, frequency 10 Hz, and VT 2 ml/kg (maximum amplitude 45 cmH_2O). For P_aCO_2 out of range (40-60 mmHg), the CMV VT was changed by 1 ml/kg, HFOV amplitude was changed by 5 cmH_2O, and HFOV+VG VT was changed by 0.5 ml/kg. For S_pO_2 out of range (less than 90%), the FiO_2 was titrated and the MAP only increased if desaturating despite a F_iO_2 of 1.0. The goal was to wean the MAP as quickly as possible to a goal of 12 cmH_2O, while maintaining the S_pO_2 above 90% by titrating the FiO_2, to avoid MAP being a confounding variable relating to hemodynamic effects.

Monitoring

The randomization to ventilation mode was done after collecting baseline vitals, hemodynamic and ventilatory parameters, and bloodwork. These included: body temperature, HR, arterial blood pressure, central venous pressure (CVP), urine output, S_pO_2, central venous S_vO_2, S_aO_2, $ETCO_2$, F_iO_2, MAP, dynamic and static compliance, resistance, cerebral and renal NIRS, right carotid and left renal ultrasonic flows, P_aO_2, P_aCO_2, hemoglobin, pH, and lactate. Regional oxygenation and perfusion was assessed by using a combination of NIRS and ultrasonic flow probes, respectively, because of the relative ease of placement, continuous measurements in real-time, and because the combination of the two provides different but related data. To help ensure comparable experimental groups, the $AaDO_2$ and ventilator MAP were monitored. Mean arterial blood pressure and HR were also monitored continuously. A Millar® catheter was chosen to measure parameters of LV function due to its continuous data collection and ability to convert its analog signal to digital data for storage and analysis. LV function parameters included CI, stroke volume, ejection fraction, end-diastolic pressure and volume, end-systolic pressure and volume, contractility (dP/dt max), and Tau (time constant of isovolumetric relaxation time which is a measure of diastolic function). During the first hour of randomization, these blood samples and recordings were done every 15 minutes to ensure stability. During the remaining three hours, it was done every hour unless there was clinical instability in which case it was done sooner. At the end of the 4-hour experimental period, the piglet was euthanized with intravenous pentobarbital (100 mg/kg) and the following organs harvested for

analysis: brain, right lower lobe of lung, left ventricle portion, and left kidney en bloc. The lung was analyzed for tumour necrosis factor alpha (TNF-α) and interleukin-8 (IL-8). The other organs were analyzed for lactate and oxidized glutathione to reduced glutathione ratio (GSSG/GSH), which are the markers of ischemic and oxidative stress, respectively. These biochemical markers of inflammation, ischemia, and oxidative stress were chosen due to their common use and availability, and subsequent importance in interpreting the severity of end-organ injury at the end of the experimentation period.

It should be noted that an attempt was made to assess right ventricular function, pulmonary hypertension, and shunts by echocardiography during the 4-hour monitoring period. However, several piglets became severely hypoxic during the echocardiogram despite increasing ventilatory support and developed severe metabolic lactic acidosis, which resulted in significant morbidity and mortality thus confounding the analysis. As a result, it was decided to not continue with the echocardiogram monitoring, thereby limiting the cardiac function information. These piglets were not included in the final data analysis.

Tissue biochemical analysis

Immediately after euthanasia, the brain was placed in 2-methylbutane (isopentane) and the other tissues were flash frozen in liquid nitrogen, and all were stored at -80°C. Assays of lactate in LV, brain, and renal tissues were done by addition of perchloric acid and EDTA with subsequent homogenization and centrifugation. The supernatant was then isolated, neutralized with potassium

carbonate, and underwent conversion to pyruvate using an assay with lactate dehydrogenase to generate NADH which, via spectroscopy at 340 nm, provided the concentration of lactate in the samples. The lactate concentration was calculated as relative to the amount of protein in the sample, which was determined by using the same supernatant by a protein assay using Bradford Dye Reagent® and bovine serum albumin as a control for spectroscopy. The GSSG/GSH ratio was also determined using homogenized and centrifuged samples, but with phosphate buffered saline and EDTA. The supernatant was again isolated with a subsequent assay using a combination of nitrobenzoic acid and GSH to produce 5-thio-2-nitrobenzoic acid which can be measured using spectroscopy at 405-414 nm. The total GSH was determined, and then GSSG was determined by derivatizing GSH, converting GSSG to GSH, and reacting with nitrobenzoic acid to produce 5-thio-2-nitrobenzoic acid which was subsequently measured. This enabled measurement of the concentrations of GSH and GSSG and therefore determination of the ratio (both lactate and GSSG/GSH assays conducted by ML). Lung tissue preparation was done by placing sample in a lysis buffer (tween-20, phosphate buffer solution, protease inhibitor cocktail) followed by homogenization and centrifugation to isolate the supernatant which was split into samples for lung markers and protein assay. Both lung inflammatory markers IL-8 and TNF-α were quantified by using a sandwich enzyme immunoassay technique which utilized porcine monoclonal antibodies to the marker of interest that were pre-coated onto an enzyme-linked immunosorbent assay (ELISA) plate. The supernatants were added to the wells, along with a control of varying dilutions, and the inflammatory marker was bound to the antibody. The

plate was washed after a 2 hour incubation period, and then a conjugate monoclonal antibody specific for that biomarker was added which has an enzyme on its Fc region. After another 2 hour incubation, a blue substrate solution was added followed by a 30 minute incubation in darkness. The enzyme converted the substrate to a yellow product which can be measured via spectroscopy at 540/570 nm. This enabled quantification of both IL-8 and TNF-α as a ratio of the protein present in each sample (conducted by MS).

Statistical analysis and sample size calculation

The primary outcome of interest in this study was LV output, as indicated by CI, at any time point in the study. Our hypothesis was that HFOV+VG would have a significant negative effect resulting in reduction in LV CI when compared to the other modes of ventilation due to the fluctuation in amplitude to maintain a consistent volume administered. Our power calculation involved finding a difference of 25% in CI between the experimental groups to be considered significant. Based on an α value of 0.05 and β error of 0.2, the sample size was calculated to be 8 piglets per group which we increased to 10 based on the potential for 20% mortality given the lab's prior experience with piglet models, notably hypoxic-ischemic studies **[9]**. Six animals were used in the sham-operated group, due to limited availability of piglets from the Swine Research Technology Centre during the timeframe of the study. Each piglet was randomized into one of the four groups, which was done by picking a card from an envelope. At the start of a block there would be four cards in the envelope, and by the end of the block there would

be one card to choose. Each of the four different groups (CMV, HFOV, HFOV+VG, sham-operated) were done per block of four experiments, in an effort to use as many piglets from the same litter as possible. Since the age range was 1-3 days, it was not possible to use all piglets from the same litter for each block of experiments. The study was not blinded due to the ventilator mode being visible and, to an extent, audible regarding CMV versus HFOV modes. Secondary outcomes included ejection fraction, stroke volume, stroke work, end-diastolic volume and pressure, end-systolic volume and pressure, dP/dt max (measure of contractility), Tau (measure of diastolic function), arterial-venous SO_2 difference, cerebral NIRS, right carotid artery flow index, renal NIRS, P_aCO_2, oxygenation index, pH, lactate, tissue markers including GSSG/GSH ratio and lactate, and lung markers of inflammation including IL-8 and TNF-α. Differences between groups were determined by two-way repeated measures ANOVA, which facilitated investigating interactions between ventilatory modes and time points. *Posthoc* pairwise comparisons between modes and time points were done using the Holm-Sidak method as it was the recommended post-test analysis for the data in question as it was the more conservative method to find differences compared to other tests available in the software. One-way ANOVA was used for the analysis of tissue biochemical markers. Data are presented in mean ± SD. Statistical software used was SigmaPlot (version 11.0, Systat Software, San Jose, CA, USA).

Results

A total of 44 piglets were instrumented with 30 (experimental and sham-operated) included in the study. There were 11 (25%) animals which were euthanized prior to the end of the experiment because of peri-moribund state as related to: excessive lung disease and/or metabolic acidosis (n = 5, prior to initiation of experimental protocol of ventilatory support; piglets #1, #3, #4, #5, #23), intraventricular catheter perforating LV (n = 1, piglet #18), severe illness possibly associated with congenital anomaly (n = 2, piglet #17 and #38), attempting echocardiography resulting in severe illness (n=2, piglet #19 and #26), or excessive bleeding during surgery (n = 1, piglet #32). Of these, 1 piglet was euthanized early at the pilot stage and 10 during the study. Three animals were excluded because of not achieving the goal $AaDO_2$ or prolonged lung lavage (piglets #7, #10, #11). There were no significant differences in mean arterial blood pressure, HR, MAP, CVP, oxygenation index and $AaDO_2$ among groups (Tables 5.1 and 5.2)($p>0.05$). Hemoglobin levels also remained comparable throughout the experiment (data not shown).

Effects on left ventricular function

Representative PV loops of the experimental groups are shown in Figure 5.2. Invasive data acquired using the Millar® catheter indicated that, over the 4-hour experimental period, LV CI of both HFOV modes did not change significantly over time and did not differ significantly from values of CMV or sham-operated groups

(Table 5.3 and Figure 5.4)($p>0.05$). CMV LV CI did decrease significantly over time compared to sham-operated groups (fraction of baseline: 0.63±0.30 vs. 1.32±0.50, $p<0.05$). The LV stroke volume and ejection fraction did not differ over time or between modes including between HFOV modes (Table 5.3)($p>0.05$). A measure of contractility, dP/dt max, was higher in the HFOV+VG mode compared to sham-operated at all time points except 240 minutes (fraction of baseline: up to 1.36±0.53 vs. up to 0.73±0.13, $p<0.05$). Among experimental groups there were no significant differences in stroke work, end-diastolic volume, end-diastolic pressure, end-systolic pressure, and Tau (Table 5.3 continued).

Effects on systemic oxygen metabolism and regional perfusion

The arterial-venous SO_2 difference (AVO_2) was larger in the HFOV+VG mode compared to CMV mode at 240 minutes (56±9% vs. 36±8%, $p<0.05$). This is pertinent given there were no differences between groups in systemic oxygen delivery or oxygen extraction; however, CMV did have a higher P_aCO_2 over time which can affect AVO_2. Serum lactate levels were also similar (Table 5.4).

Cerebral NIRS ($CrSO_2$) at 240 minutes was lower in HFOV+VG when compared to HFOV, but not compared to CMV or sham-operated groups (30±11% vs. 43±10%, $p<0.05$). The right common carotid artery ultrasonic flow probe results, represented by right carotid artery flow index (RCAFI), showed that HFOV+VG decreased more significantly at 180 and 240 minute time points when compared to its starting baseline (10±9 and 14±10 ml/kg/min respectively vs.

31±11 ml/kg/min, $p<0.05$) while other groups did not change significantly (Table 5.5).

Renal blood flow data was limited due to intermittent failure of the renal artery ultrasonic flow probe often by 120 minutes after the commencement of HFOV or CMV, likely related to the supine positioning, possible renal artery spasm, and inability to access the probe due to instability of the piglets. The renal NIRS ($RrSO_2$) results indicate no significant differences between groups over time (Table 5.5).

Oxygenation and CO_2 removal

Both HFOV modes were superior to CMV for removal of CO_2. The P_aCO_2 increased more significantly in CMV over time when compared to its baseline at time 0 minutes (up to 59.1±12.6 mmHg vs. 40.4±4.0 mmHg, $p<0.05$) , while there was no difference in either HFOV mode (Figure 5.5). Furthermore, minute ventilation was consistently higher in both HFOV modes when compared to CMV during the 4-hour experimental period. Oxygenation index was higher in the experimental groups compared to sham-operated, but by the end of the experiment only the oxygenation index of CMV group remained significantly higher than that of the sham-operated piglets (15.1±17.0 vs. 3.9±0.8, $p<0.05$)(Table 5.2). $AaDO_2$ remained higher than sham-operated in all experimental groups as expected, and not different from each other (Table 5.2). Arterial blood gas parameters shown in Table 5.6 show CMV mode had a lower pH at later time points when compared to the sham-operated group (minimum of 7.14±0.19 vs. 7.36±0.07, $p<0.05$). Both

HFOV modes kept P_aCO_2 significantly lower than CMV mode by the end of the experiment. HCO_3 and base excess were not significantly different between groups.

Tissue biochemical assays

Markers of tissue injury were evaluated in the samples of brain, right lower lung lobe, left ventricle, and left kidney (Figures 5.6 and 5.7). There were no significant differences between modes in terms of lactate or GSSG/GSH concentrations. Lung samples also showed no differences between modes in terms of IL-8 or TNF-α concentrations. CMV, HFOV, and HFOV+VG had significantly higher IL-8 in the lung tissue than that of the sham-operated group ($p<0.05$)(Figure 5.8).

Discussion

Invasive positive pressure ventilation is commonly used in sick neonates, with potential adverse effects on cardiac function. Ventilators have become more accurate and more powerful in their ability to ventilate and oxygenate **[26]**. However, as with any tool, the effectiveness of their use depends on the operator's skill and the given patient and lung pathology involved, and every ventilation mode has its benefits and risks – notably the effects on hemodynamics. This study was conducted to assess the effects of HFOV with and without VG on pulmonary and hemodynamic parameters, as well as with comparison to CMV. This information is important to gather given the frequent use worldwide of HFOV in some of the most

critically ill neonates and, in some centers, the use of the largely untested VG mode with HFOV.

Our groups were comparable in terms of MAP and $AaDO_2$, therefore making them comparable in terms of pressure delivered to the proximal endotracheal tube and lung disease severity. It is known the differing MAP between CMV and HFOV may impact hemodynamics . We found that LV CI was negatively affected by CMV over time compared to the sham-operated group (decrease from baseline of 69% more than sham-operated by 240 minutes). There was no significant effect of HFOV with or without VG on LV CI (Figure 5.4). This indicates our hypothesis was incorrect. There was a negative effect over time using CMV in LV EF and SV as well compared to sham-operated, while HFOV+VG had a negative impact on $CrSO_2$ compared to HFOV (13% less at 240 minutes) and in RCAFI over time compared to its baseline (21 and 17 ml/kg/min less at 180 and 240 minutes respectively). Combined with the fact that P_aCO_2 was increasing over time in CMV compared to both HFOV groups, and that CMV had a lower pH which appears to have been related to the hypercarbia, the finding of decreased LV CI may be partially explained by acidosis and elevated P_aCO_2. The decrease in $CrSO_2$ and RCAFI related to HFOV+VG does not have an obvious etiology. As has been previously demonstrated, hypercarbia can result in both systemic and cerebral vasodilation with subsequent increased blood flow **[18][19]**. It is also known that acidosis decreases myocardial function and may increase pulmonary vascular resistance, which may relate to the negative effect seen in CMV over time **[20]**. This is supported by the finding that dP/dt max, a measure of contractility, was significantly higher in HFOV+VG

compared to the sham-operated at all points except at the end of the experiment. Since there was not a negative effect noted on ventricular function with HFOV+VG, and even a positive effect on contractility, then the difference in regional markers of blood and oxygen delivery is likely related to local differences such as vascular resistance. The lack of effect on LV CI in the HFOV+VG group, despite improved contractility, is likely related to other factors such as reduced preload which is related to RV function that we were unable to collect data for. Though CVP may be considered a surrogate marker for RV pressure and function, there was no statistically significant difference between groups, and the average CVP was actually higher in the CMV group by 240 minutes than either HFOV group (5.7 vs. 4.5 and 5.0 mmHg).

These findings are especially interesting because, clinically, it is often seen that markers of regional perfusion, such as urine output, show signs of poor blood flow and/or oxygen delivery before other measures of ventricular function such as blood pressure or pulse assessment on clinical exam **[15]**. This may be due to blood pressure being related to blood flow and systemic vascular resistance, while urine output relates to adequate perfusion of the kidneys which receive a significant amount of blood compared to most other organs **[15]**. Other findings were not consistent with the observation that CMV reduced LV function, such as comparable oxygen delivery and extraction between groups, which may be related to elevated P_aCO_2 given its effects on vasodilation. Regardless, these findings suggest potential differences between ventilation modes that justify further exploration in either

further animal studies or novel human studies with additional non-invasive monitoring such as NIRS and neonatal echocardiography.

To date there have not been any studies looking at the differences in hemodynamic effects of HFOV with and without VG, and comparing HFOV modes to CMV. There have been pilot studies comparing HFOV modes with and without VG use on preterm neonatal patients and the effect on stability of oxygenation and ventilation over a period of hours. Enomoto et al. in 2017 showed that in an open, non-randomized study of 6 preterm neonates with birthweights less than 1000 grams and postnatal age greater than 28 days, there was less significant fluctuation (as measured by standard deviation) in S_pO_2, minute ventilation, and DCO_2 with HFOV+VG when compared to HFOV **[6]**. In an unblinded and randomized pilot study of 20 preterm neonates less than 32 weeks gestation and less than 6 hours of life after surfactant administration for RDS, HFOV+VG treatment had higher mean VT and DCO_2, more consistent VT, and less fluctuation of PCO_2 than those of HFOV only. There was no difference noted in F_iO_2 **[7].** The results in our current study are consistent with that described in these two reports. Though not comparing HFOV with and without VG mode, there have been other neonatal studies of note regarding HFOV+VG. In 2016, a paper by Gonzalez-Pacheco et al found that using HFOV+VG with very high frequencies (up to about 18 Hz) and low VT (down to about 1.2 ml/kg) was able to maintain ventilation **[21]**. A retrospective study was done by Belteki and Morley in 2019 on 17 neonates on HFOV+VG, looking at the VT and its stability **[22]**. They found that VG mode appeared to help maintain the VT very close to the target volume over the long term, though there was no control

group to compare. They also noted the amplitude being automatically titrated with changing compliance, such as sedation and/or muscle relaxation. Both of these studies suggest potential benefits of HFOV with VG, though there is no direct comparison to HFOV without VG.

There are several limitations to the present study. It is an animal study of term piglets aged 1-3 days and the experimental period is limited to four hours of data collection before euthanasia. This results in limited extrapolation to human neonates and to a timeframe over four hours whereas differences between modes may become more obvious over a longer period of experimentation time. The saline lung lavage model, though validated in terms of $AaDO_2$ gradient achieved, had a gradually decreasing $AaDO_2$ gradient over time in all experimental groups up to 150-200 mmHg less by 240 minutes compared to 0 minutes. This suggests a possible acutely resolving mechanism such as pulmonary edema secondary to retained saline from the lavage, in addition to surfactant deficiency, that improves over the short time frame of experimentation. This would need further studies to confirm the pathophysiology involved. The OI decreased less over time and remained higher, though not statistically significant, in the CMV group with the 240 minute OI being 15.1 versus 9.9 in HFOV and 10.1 in HFOV+VG. This may relate to HFOV's ability to oxygenate more effectively at a given pressure, especially when lung compliance is limited, which is extrapolated from the fact that it is typically used as a rescue mode of ventilation **[24][25]**.

Unfortunately, echocardiography was not tolerated by this piglet model, so cardiac function data was limited to the LV. When echocardiography was

attempted, significant refractory hypoxemia along with subsequent lactic metabolic acidosis occurred which often resulted in significant morbidity and mortality. As a result, data on right ventricular function, shunts, valvular regurgitation (notably aortic regurgitation related to the intraventricular catheter), and more precise intraventricular catheter position could not be obtained. This ideally would need to be incorporated in similar future studies given the interdependency of the cardiac ventricles and pulmonary circulation, especially in relation to intrathoracic pressure and mechanical ventilation. CVP was measured via the femoral venous catheter which provides a measure of right atrial pressure and preload **[27][28]**. This can relate to RV function in cases such as increasing RV pressure with subsequently increasing CVP. Flow measurements of different cerebral arteries may have provided more information regarding regional cerebral blood flow when combined with RCAFI. This is notable considering the Millar® catheter may theoretically obstruct blood flow through the left common carotid artery to an extent, though this was not found to have been assessed in any studies reviewed. Adding in Doppler measurements of cerebral blood flow in the left and right hemispheres, such as the anterior cerebral artery, could help determine if it is significant **[29]**.

The S_pO_2 probe was initially placed on the right limb in the pre-ductal position due to the possibility of PPHN and shunting through the patent ductus arteriosus, though it would likely be closed in the majority of piglets after a day of age. The S_pO_2 probe in a minority of the piglets stopped reading reliably typically 1-2 hours into the experimentation period. This required re-siting of the probe, which was not always successful in obtaining a reading, and introduced a potentially

falsely low post-ductal S_pO_2 if there was significant right to left shunting through the PDA. However, the pre-ductal S_pO_2 and post-ductal S_aO_2 correlated relatively well prior to S_pO_2 probe failure, so this was unlikely a significant concern given the peak of right to left shunting would most likely happen when the $AaDO_2$ gradient was highest, if there was even a significant PDA present. Using NIRS to measure regional oxygenation has the strength of continuous non-invasive monitoring, with the weakness of the overall trend mattering more than the absolute number. This may be considered a weakness because absolute numbers may facilitate guiding more standard management. The ultrasonic flow probes also helped provide information on regional blood flow with real-time information, with the main drawback being their sensitivity to their position on the vessel. The right carotid artery flow probe was adjusted when needed, but the renal artery flow probe was unable to be adjusted after placement due to the need to maintain the piglet's position and deep location of the probe on the renal artery. The tissue biomarkers are beneficial in terms of assessing markers of ischemia, injury, and inflammation that are not currently easily measured via other means. They represent the cumulative impact of the experimental period, not the effects at different time points, and therefore provide a broader overview of oxygen delivery and tissue injury.

Ventilation parameters were standardized in terms of weaning, and the MAP was kept consistent between modes given it could be a confounding variable in terms of hemodynamic effects. However, this again makes extrapolation to clinical use in the NICU limited because HFOV modes are typically used at a higher MAP given its mechanics and, often, the higher level of illness in the patient it is used for.

Related to ventilation, we were unable to keep P_aCO_2 levels consistent between CMV and HFOV modes due to the inherent limited ventilatory ability of CMV, thus lower minute ventilation. Though we aimed for and were able to maintain P_aCO_2 at a clinically relevant range between 40-60 mmHg, the levels were significantly higher compared to baseline in CMV mode which did not occur in HFOV modes. Given the association between P_aCO_2 and hemodynamics, notably cerebral perfusion **[14]**, this is a limitation of the study when comparing the hemodynamic results of the CMV mode to those of other modes.

Conclusions

In our newborn term piglet model of moderate to severe RDS, there was no effect of HFOV or HFOV+VG on LV CI; however, there was a negative effect of HFOV+VG on cerebral perfusion and oxygenation. CMV had a negative effect over time on LV CI. Given P_aCO_2 was elevated in CMV compared to the HFOV groups, this may relate to some differences observed. Overall, these results suggest there are differing effects of ventilatory modes on hemodynamics that justify further studies with additional monitoring including that of right ventricular function.

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Table 5.1 Mean arterial blood pressure, heart rate, mean airway pressure (mean ± SD) of saline lung-lavaged piglets treated with CMV, HFOV and HFOV+VG at different time points during the experiment. No significant differences ($p>0.05$).

MBP = mean arterial blood pressure, CVP = central venous pressure, HR = heart rate, MAP = mean airway pressure

	Time point (minutes)				
Parameter and mode	**0**	**60**	**120**	**180**	**240**
MBP (mmHg)					
Sham	63 ± 5	55 ± 10	49 ± 4	46 ± 10	43 ± 7
CMV	62 ± 9	61 ± 10	54 ± 17	54 ± 19	43 ± 17
HFOV	69 ± 14	73 ± 13	63 ± 19	54 ± 12	52 ± 9
HFOV+VG	61 ± 8	56 ± 11	49 ± 10	41 ± 10	35 ± 12
CVP (mmHg)					
Sham	4.0 ± 0.7	4.0 ± 0	3.5 ± 1.2	4.4 ± 0.9	5.3 ± 3.1
CMV	4.4 ± 1.1	4.6 ± 1.1	5.3 ± 2.4	5.4 ± 1.2	5.7 ± 2.3
HFOV	4.8 ± 1.2	3.9 ± 0.6	4.5 ± 1.4	4.5 ± 1.4	4.5 ± 1.3
HFOV+VG	4.8 ± 1.5	4.6 ± 0.7	4.6 ± 0.9	4.9 ± 1.8	5.0 ± 0.6
HR (bpm)					

Sham	208 ± 35	213 ± 30	221 ± 36	225 ± 13	236 ± 29
CMV	230 ± 22	237 ± 37	239 ± 30	248 ± 32	227 ± 24
HFOV	205 ± 30	228 ± 25	216 ± 32	206 ± 34	220 ± 24
HFOV+VG	225 ± 21	222 ± 33	231 ± 21	233 ± 20	215 ± 39
MAP (cmH_2O)					
Sham	9.9 ± 1.0	9.5 ± 0.8	10.2 ± 1.1	9.8 ± 1.4	10.1 ± 1.4
CMV	15.4 ± 2.1	14.0 ± 2.6	13.2 ± 2.9	13.4 ± 3.3	13.8 ± 5.5
HFOV	15.6 ± 1.6	15.4 ± 2.6	13.7 ± 3.5	13.3 ± 2.6	13.3 ± 2.5
HFOV+VG	15.6 ± 2.1	15.6 ± 2.7	14.3 ± 3.0	13.9 ± 2.9	13.0 ± 2.7

Table 5.2 Oxygenation index and $AaDO_2$ (mean ± SD) of sham-operated and saline lung-lavaged piglets treated with CMV, HFOV and HFOV+VG at different time points during the experiment. Formula for oxygenation index is below the table. *OI = oxygenation index*

	Time point (minutes)				
Parameter and mode	**0**	**60**	**120**	**180**	**240**
OI					
Sham	3.8±0.4	3.6±0.8	4.4±0.3	3.8±1.1	3.9±0.8
CMV	17.3±7.0*	14.1±7.5*	12.7±8.0	13.3±11.2	15.1±17.0*
HFOV	16.5±3.5*	13.5±5.0*	13.2±5.9	10.4±6.6	9.9±5.6
HFOV+VG	18.8±7.4*	13.4±4.4*	14.6±6.7	10.9±2.1	10.1±3.3
$AaDO_2$ gradient (mmHg)					
Sham	61±9	62±18	72±19	65±23	71±14
CMV	405±63*	384±181*	345±179*	328±207*	291±183*
HFOV	402±39*	417±130*	366±126*	269±117*	248±157*
HFOV+VG	423±79*	419±86*	395±148*	345±153*	299±126*

*vs. sham-operated ($p < 0.05$)

Oxygenation index = $(F_iO_2*MAP)/P_aO_2$

Table 5.3 LV function parameters (mean ± SD) as fraction of value at baseline (after stabilization) of sham-operated and saline lung-lavaged piglets treated with CMV, HFOV and HFOV+VG at different time points during the experiment. *CI = cardiac index, SV = stroke volume, EF = ejection fraction, dP/dt max = measure of contractility*

	Time point (minutes)				
Parameter and mode	**0**	**60**	**120**	**180**	**240**
CI (fraction of baseline)					
Sham	0.96 ± 0.33	1.02 ± 0.21	1.20 ± 0.40	1.26 ± 0.64	1.32 ± 0.50
CMV	0.92 ± 0.38	0.89 ± 0.41	0.87 ± 0.34	0.78 ± 0.24*	0.63 ± 0.30*
HFOV	0.76 ± 0.29	0.87 ± 0.44	0.80 ± 0.26	1.00 ± 0.37	1.05 ± 0.43
HFOV+VG	0.82 ± 0.30	0.86 ± 0.32	1.10 ± 0.37	1.01 ± 0.59	0.92 ± 0.59
EF (fraction of baseline)					
Sham	0.93 ± 0.24	0.94 ± 0.21	1.01 ± 0.28	1.17 ± 0.45	1.24 ± 0.42
CMV	0.95 ± 0.21	0.81 ± 0.19	0.75 ± 0.18	0.76 ± 0.20*	0.67 ± 0.24*
HFOV	0.87 ± 0.18	0.88 ± 0.26	0.84 ± 0.25	0.97 ± 0.25	1.06 ± 0.24
HFOV+VG	1.00 ± 0.10	1.10 ± 0.29	1.04 ± 0.13	1.14 ± 0.36	0.95 ± 0.21
SV (fraction					

of baseline)					
Sham	0.92 ± 0.27	0.93 ± 0.21	1.00 ± 0.31	1.16 ± 0.43	1.19 ± 0.35
CMV	0.91 ± 0.18	0.77 ± 0.20	0.71 ± 0.20	0.73 ± 0.20*	0.67 ± 0.27*
HFOV	0.81 ± 0.20	0.80 ± 0.28	0.78 ± 0.28	0.93 ± 0.31	1.02 ± 0.30
HFOV+VG	0.85 ± 0.18	0.79 ± 0.23	0.82 ± 0.31	0.89 ± 0.34	0.86 ± 0.35
dP/dt max (fraction of baseline)					
Sham	0.73 ± 0.13	0.63 ± 0.13	0.68 ± 0.19	0.62 ± 0.16	0.62 ± 0.21
CMV	0.79 ± 0.13	0.78 ± 0.17	0.69 ± 0.23	0.55 ± 0.24	0.67 ± 0.51
HFOV	0.81 ± 0.12	0.88 ± 0.28	0.72 ± 0.35	0.69 ± 0.32	0.67 ± 0.28
HFOV+VG	1.18 ± 0.30*	1.36 ± 0.53*	1.29 ± 0.66*	1.11 ± 0.54*	0.88 ± 0.23

*indicates significant difference from Sham-operated group at corresponding time ($p<0.05$)

Table 5.3 (continued) LV function parameters (mean ± SD) as percent of value at baseline (after stabilization) of sham-operated and saline lung-lavaged piglets treated with CMV, HFOV and HFOV+VG at different time points during the experiment. No significant differences ($p < 0.05$). *SW = stroke work, EDV = end-diastolic volume, EDP = end-diastolic pressure, ESP = end-systolic pressure*

	Time point (minutes)				
Parameter and mode	**0**	**60**	**120**	**180**	**240**
Tau (ms)					
Sham	22 ± 7	20 ± 5	21 ± 7	22 ± 6	29 ± 16
CMV	23 ± 8	18 ± 5	24 ± 7	25 ± 10	17 ± 4
HFOV	21 ± 3	18 ± 6	16 ± 4	18 ± 3	18 ± 2
HFOV+VG	21 ± 5	20 ± 9	18 ± 2	18 ± 4	19 ± 6
SW (mmHg*ml)					
Sham	40.8 ± 31.8	36.5 ± 22.9	34.1 ± 21.8	41.1 ± 20.4	38.7 ± 18.0
CMV	34.4 ± 14.7	28.6 ± 14.6	24.8 ± 11.1	19.6 ± 8.6	24.0 ± 8.7
HFOV	34.4 ± 11.4	34.8 ± 19.5	29.7 ± 12.5	37.4 ± 12.0	41.3 ± 18.1
HFOV+VG	36.7 ± 17.6	29.9 ± 15.9	27.9 ± 17.6	27.3 ± 20.4	28.2 ± 21.6
EDV (fraction of					

baseline)					
Sham	0.99 ± 0.08	1.00 ± 0.07	1.00 ± 0.07	1.00 ± 0.07	0.95 ± 0.12
CMV	0.98 ± 0.08	0.91 ± 0.05	0.98 ± 0.06	0.98 ± 0.06	0.99 ± 0.08
HFOV	0.91 ± 0.05	0.90 ± 0.09	0.91 ± 0.09	0.93 ± 0.11	0.94 ± 0.11
HFOV+VG	0.98 ± 0.06	0.95 ± 0.08	0.95 ± 0.09	0.95 ± 0.11	0.96 ± 0.12
EDP (fraction of baseline)					
Sham	0.85 ± 0.16	0.91 ± 0.16	1.26 ± 0.71	1.03 ± 0.17	0.99 ± 0.22
CMV	1.17 ± 0.32	1.38 ± 0.70	1.20 ± 0.32	1.80 ± 1.71	1.56 ± 0.65
HFOV	1.04 ± 0.17	0.91 ± 0.29	0.88 ± 0.22	0.99 ± 0.30	0.96 ± 0.29
HFOV+VG	1.18 ± 0.13	1.39 ± 0.90	1.09 ± 0.26	1.34 ± 0.72	1.20 ± 0.24
ESP (fraction of baseline)					
Sham	0.97 ± 0.04	0.86 ± 0.10	0.85 ± 0.11	0.78 ± 0.09	0.78 ± 0.06
CMV	0.95 ± 0.13	0.92 ± 0.14	0.84 ± 0.18	0.77 ± 0.27	0.80 ± 0.26
HFOV	0.94 ± 0.17	1.00 ± 0.18	0.89 ± 0.20	0.91 ± 0.24	0.82 ± 0.15
HFOV+VG	0.99 ± 0.28	0.98 ± 0.31	0.91 ± 0.28	0.89 ± 0.36	0.74 ± 0.35

Table 5.4 Systemic oxygen delivery, arterial-venous SO_2 difference, oxygen extraction, and lactate levels (mean ± SD) of sham-operated and saline lung-lavaged piglets treated with CMV, HFOV and HFOV+VG at different time points during the experiment. Formulas used for calculations are below table. *DO_2 = systemic oxygen delivery*

	Time point (minutes)				
Parameter and mode	**0**	**60**	**120**	**180**	**240**
DO_2 (ml/kg/min)					
Sham	919.7±677	945.3±625	991.9±507	1098.1±540	1188.2±673
CMV	838.5±221	805.6±236	770.1±220	704.9±160	542.6±346
HFOV	763.7±245	879.5±320	799.4±195	953.8±218	982.0±242
HFOV+VG	711.8±292	777.3±319	852.1±465	865.5±575	711.4±648
AVO_2 difference (%)					
Sham	57±12	53±8	46±5	46±12	47±10
CMV	55±10	57±8	48±12	41±11	36±8
HFOV	52±10	57±11	44±14	46±15	44±14
HFOV+VG	60±10	53±13	55±10	56±7	56±9*

Oxygen extraction (%)					
Sham	61±12	57±10	51±8	49±13	50±10
CMV	61±11	59±9	51±14	48±20	46±23
HFOV	55±11	58±11	46±15	49±15	48±15
HFOV+VG	64±10	55±14	57±10	59±7	61±12
Lactate (mmol/L)					
Sham	4.1 ± 1.2	4.6 ± 2.8	4.6 ± 0.6	3.3 ± 0	3.9 ± 3.4
CMV	4.6 ± 1.2	4.8 ± 1.2	4.6 ± 1.9	7.1 ± 4.9	4.8 ± 5.6
HFOV	4.1 ± 1.6	4.1 ± 2.0	4.4 ± 1.0	4.0 ± 0.6	4.7 ± 3.6
HFOV+VG	5.3 ± 1.5	5.8 ± 2.7	6.1 ± 3.2	4.8 ± 1.2	7.6 ± 5.0

*vs. CMV at corresponding time ($p < 0.05$)

Systemic oxygen delivery = CI x $[(Hb*S_aO_2*1.34)+(P_aO_2*0.003)]$

$AVO_2 = S_aO_2 - S_vO_2$

Oxygen extraction = $(S_aO_2 - S_vO_2)/S_aO_2$ x 100

Table 5.5 Comparison of cerebral NIRS ($CrSO_2$), right common carotid artery flow index (RCAFI), and renal NIRS ($RrSO_2$) (mean ± SD) of sham-operated and saline lung-lavaged piglets treated with CMV, HFOV and HFOV+VG at different time points during the experiment.

	Time point (minutes)				
Parameter and mode	**0**	**60**	**120**	**180**	**240**
$CrSO_2$ (%)					
Sham	39 ± 4	41 ± 5	42 ± 7	40 ± 10	34 ± 11
CMV	35 ± 11	37 ± 10	40 ± 11	38 ± 12	32 ± 12
HFOV	41 ± 7	47 ± 9	42 ± 7	43 ± 9	43 ± 10
HFOV+VG	39 ± 3	42 ± 6	39 ± 8	37 ± 9	30 ± 11*#
RCAFI (ml/kg/min)					
Sham	27 ± 6	25 ± 8	25 ± 10	26 ± 17	22 ± 11
CMV	25 ± 7	20 ± 6	28 ± 12	23 ± 15	21 ± 21
HFOV	30 ± 7	26 ± 7	31 ± 12	25 ± 16	29 ± 22
HFOV+VG	31 ± 11	26 ± 16	20 ± 12#	10 ± 9#	14 ± 10#
$RrSO_2$ (%)					
Sham	51 ± 11	50 ± 12	52 ± 10	55 ± 13	53 ± 12
CMV	49 ± 13	47 ± 10	50 ± 9	50 ± 11	51 ± 11

HFOV	50 ± 8	50 ± 9	51 ± 11	52 ± 15	47 ± 9
HFOV+VG	41 ± 10	41 ± 9	41 ± 8	43 ± 8	40 ± 9

* vs. HFOV at corresponding time ($p<0.05$)

vs. respective baseline ($p<0.05$)

Table 5.6 Arterial blood gas parameters: pH, HCO_3, base excess, and P_aCO_2 (mean ± SD) of sham-operated and saline lung-lavaged piglets treated with CMV, HFOV and HFOV+VG at different time points during the experiment.

	Time point (minutes)				
Parameter and mode	**0**	**60**	**120**	**180**	**240**
pH					
Sham	7.39±0.07	7.40±0.07	7.36±0.05	7.36±0.03	7.36±0.07
CMV	7.33±0.05	7.28±0.08*	7.21±0.12	7.14±0.19*	7.15±0.19*
HFOV	7.35±0.10	7.28±0.10	7.27±0.08	7.29±0.10	7.28±0.11
HFOV+VG	7.30±0.07	7.22±0.13	7.24±0.12	7.32±0.06	7.20±0.18
HCO_3 (mmol/L)					
Sham	23.1±1.4	23.1±2.6	22.2±2.4	22.6±2.3	23.6±2.4
CMV	21.1±1.6	21.1±1.8	21.7±3.3	19.2±5.1	21.5±6.1
HFOV	23.0±2.6	22.2±2.0	21.3±3.6	21.8±4.4	21.0±4.6
HFOV+VG	21.5±2.8	21.2±3.3	20.8±3.7	23.7±2.0	18.9±6.7
Base excess (mmol/L)					

Sham	-1±1	-1±3	-3±2	-3±2	-2±3
CMV	-5±2	-6±2	-6±4	-10±8	-7±9
HFOV	-2±3	-5±3	-6±4	-6±6	-5±6
HFOV+VG	-5±3	-7±5	-6±5	-2±3	-9±9
P_aCO_2 (mmHg)					
Sham	38.4±6.5	35.4±10.2	37.3±13.1	37.3±12.3	40.0±12.0
CMV	40.4±4.0	45.2±8.9	56.5±17.9*	57.2±17.5	59.1±12.6#
HFOV	41.8±8.9	48.4±10.9*	46.2±7.2	44.2±6.5	43.5±4.5
HFOV+VG	43.3±5.8	52.0±9.7	48.2±7.6	46.3±2.6	46.2±11.1

*vs. sham-operated ($p<0.05$)

#vs. sham-operated, HFOV, HFOV+VG ($p<0.05$)

Figure 5.1 – Schematic representation of piglet model setup with instrumentation and monitoring. Ventral view of piglet shown (viewed from top of warmer with piglet laying supine).

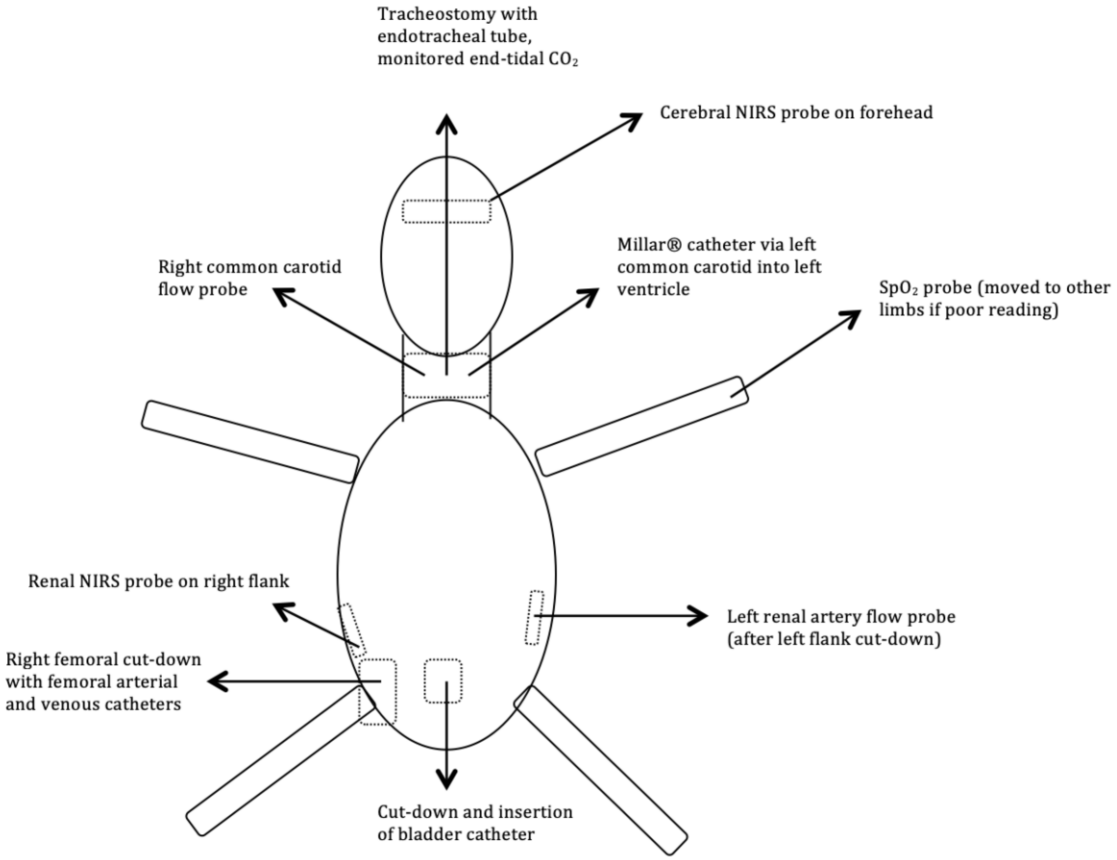
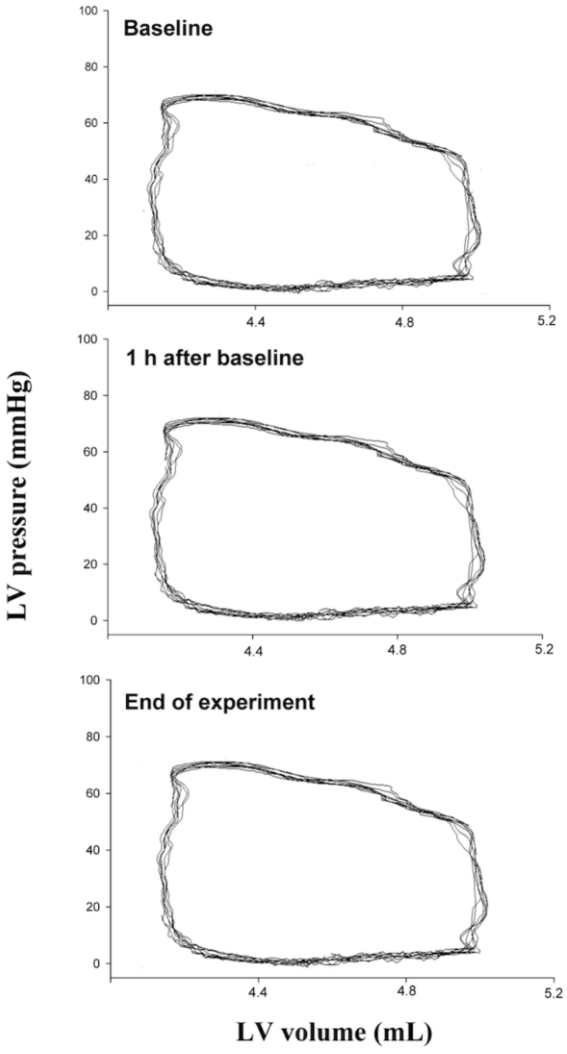
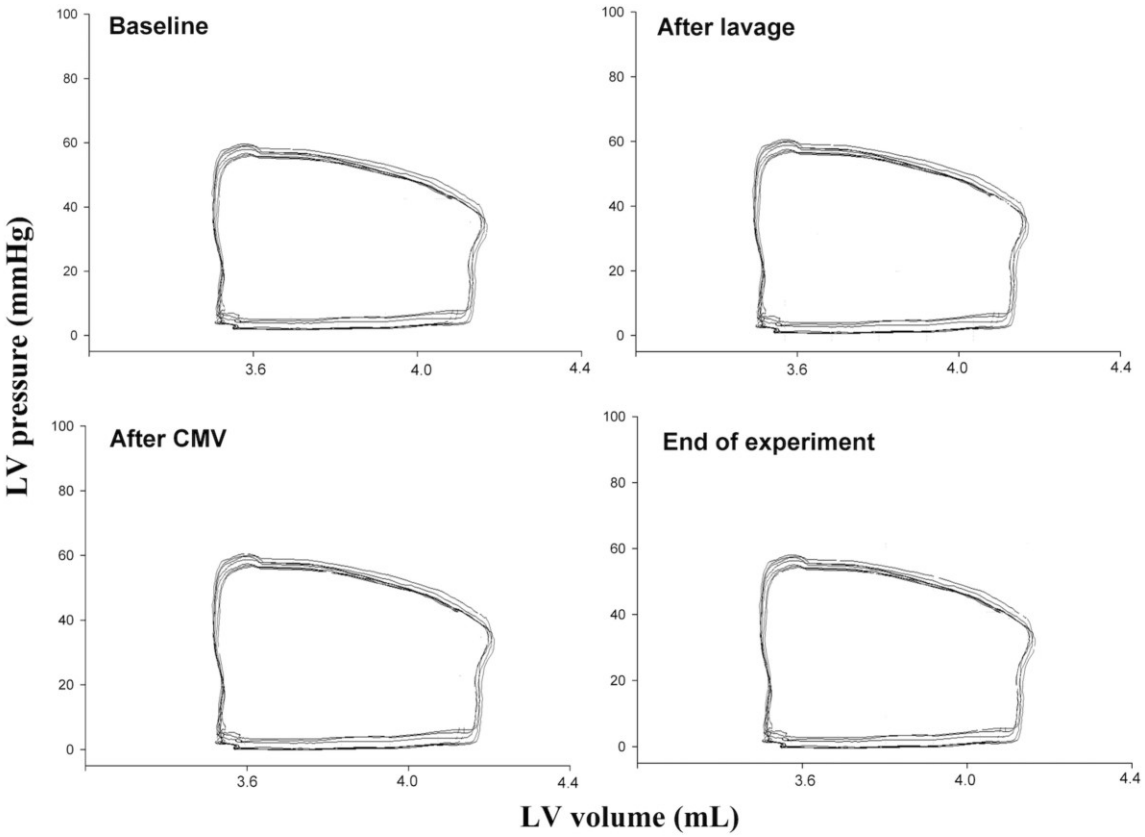


Figure 5.2 Pressure-volume loops obtained from Millar® catheter in left ventricle, examples from sham-operated and each mode at different time points.

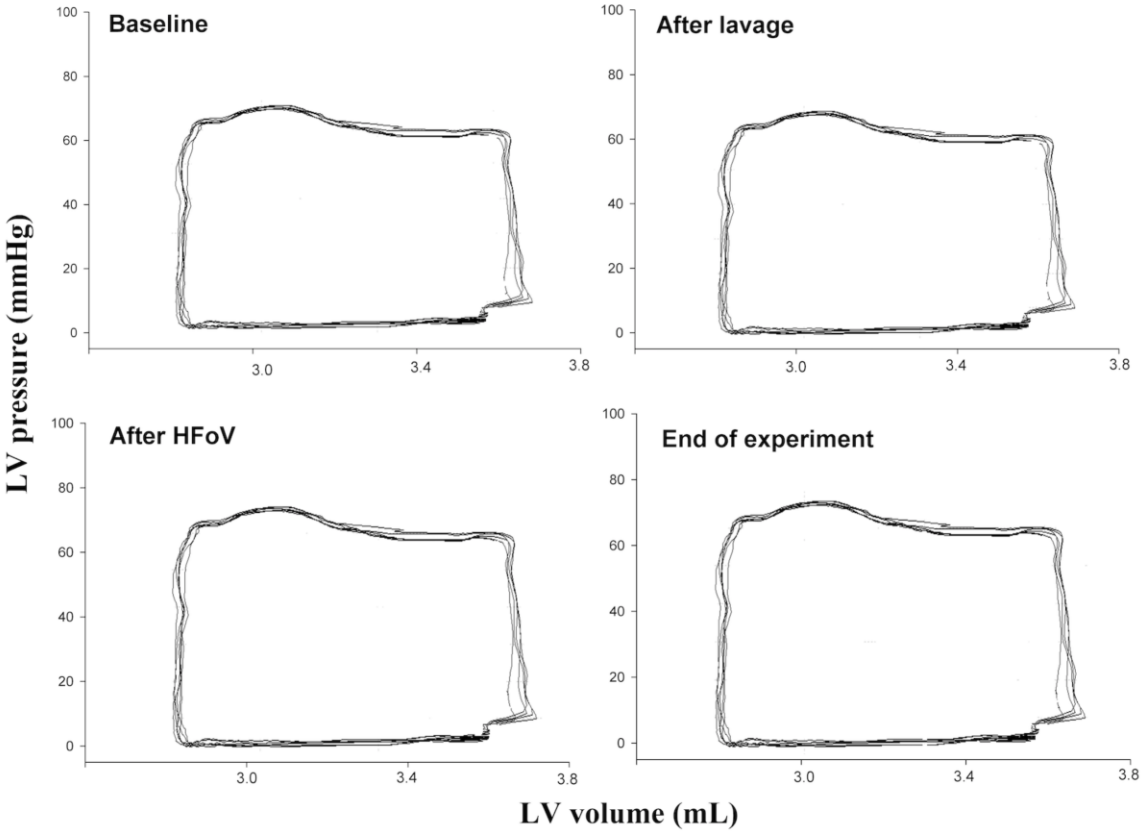
A) Sham-operated



B) CMV


Baseline
After lavage
After CMV
End of experiment
LV pressure (mmHg)
LV volume (mL)
100
80
60
40
20
0
3.6
4.0
4.4

C) HFOV


Baseline
After lavage
After HFoV
End of experiment
LV pressure (mmHg)
LV volume (mL)
100
80
60
40
20
0
3.0
3.4
3.8

D) HFOV+VG

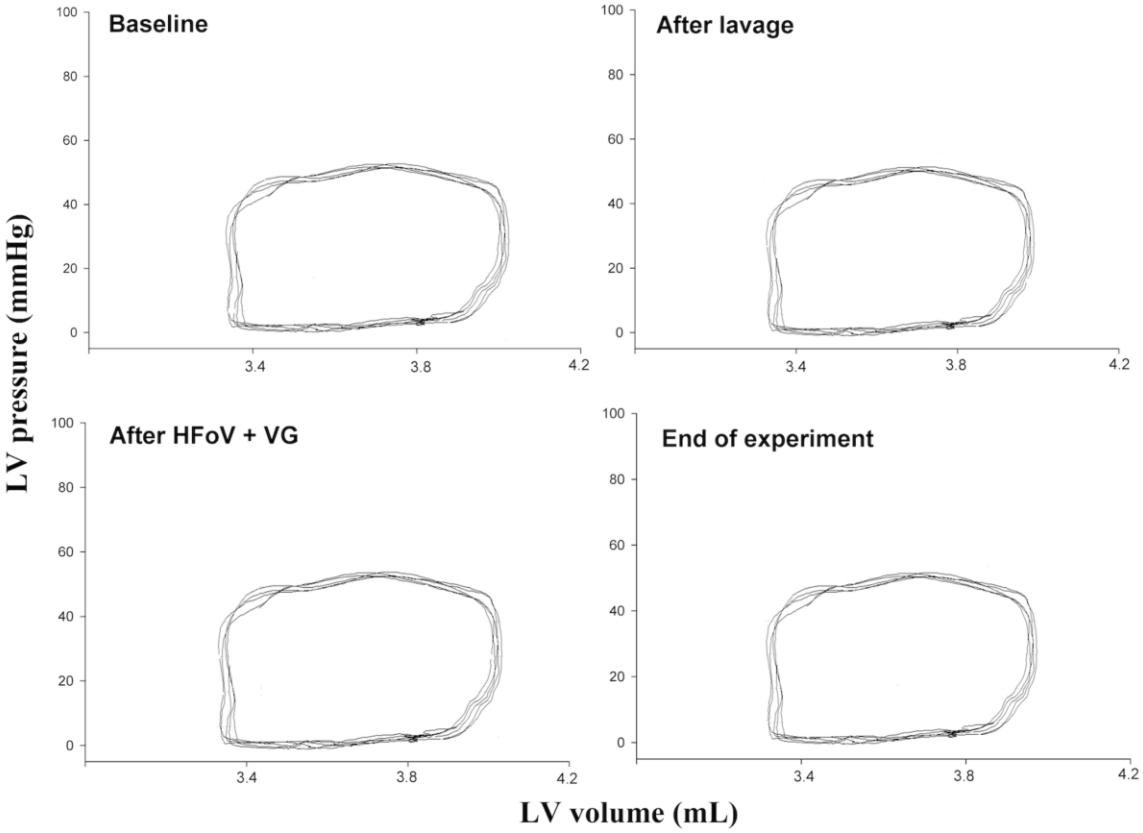

Baseline
After lavage
After HFoV + VG
End of experiment
LV pressure (mmHg)
LV volume (mL)
100
80
60
40
20
0
3.4
3.8
4.2

Figure 5.3 Timeline of experimental protocol. Baseline is at end of stabilization, and time 0 min is at end of saline lung lavage with 60 min intervals afterwards ending at 240 min. Euthanasia occurred after 240 minute collections (RL = Ringer's Lactate, ABG = arterial blood gas)

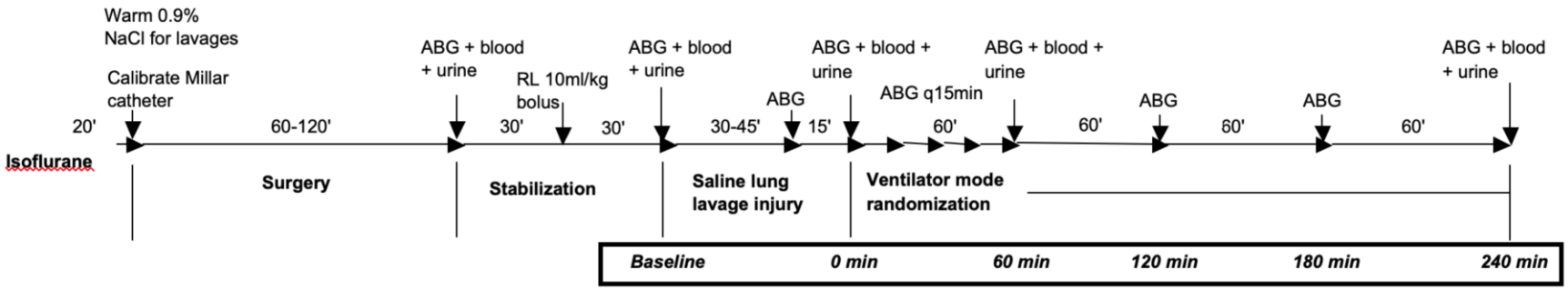


Figure 5.4 Line plot with standard deviations showing fraction change in CI over time of sham-operated and saline lung-lavaged piglets treated with CMV, HFOV and HFOV+VG at different time points during the experiment.

(* indicates $p<0.05$ vs. sham-operated group).

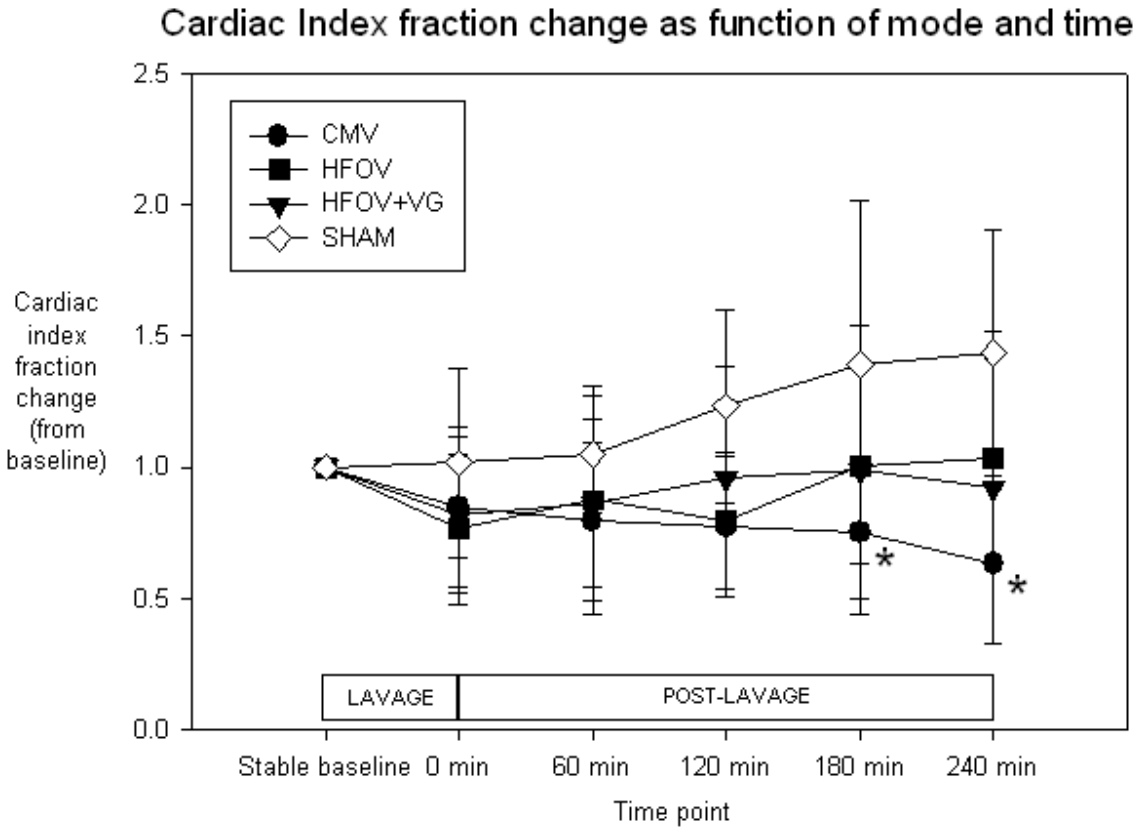


Figure 5.5 Line graphs of P_aCO_2 levels as a function of ventilation mode and time. (*p<0.05 vs. sham-operated, HFOV and HFOV+VG groups)

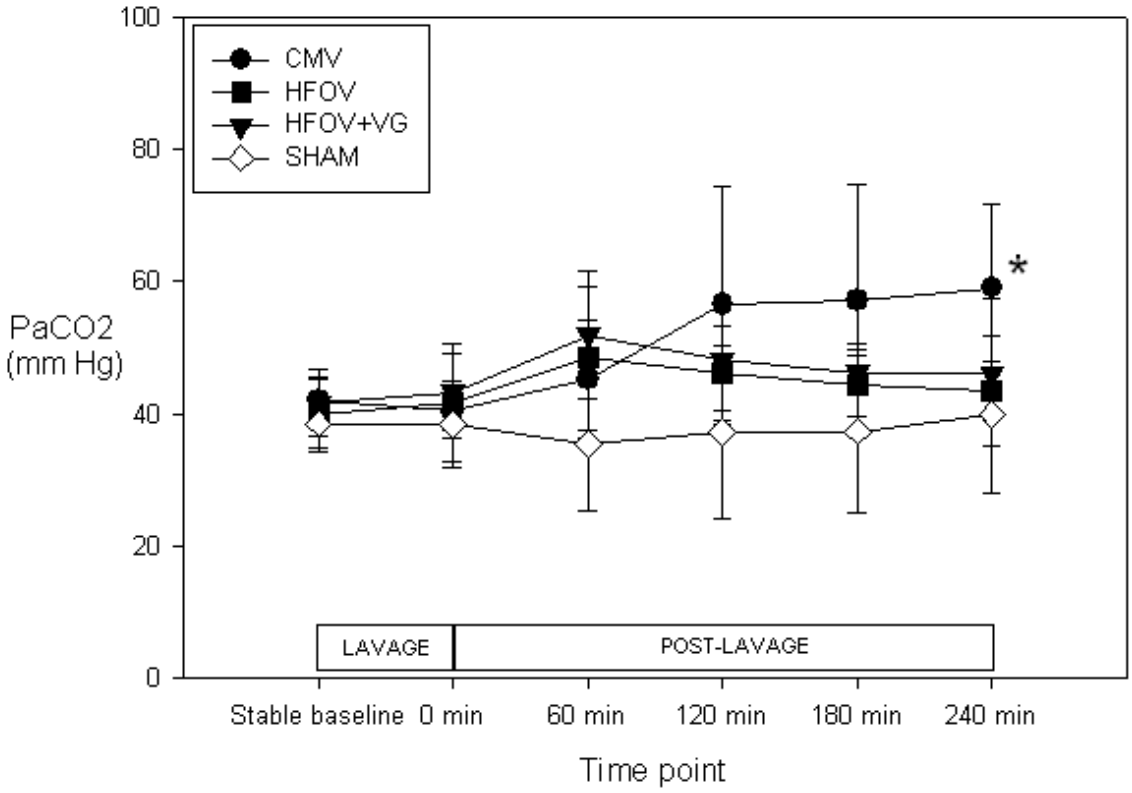


Figure 5.6 – Bar plot with standard deviations showing immediate post-mortem tissue analysis for lactate (mmol/mg) of sham-operated and saline lung-lavaged piglets treated with CMV, HFOV and HFOV+VG. No significant differences between groups.

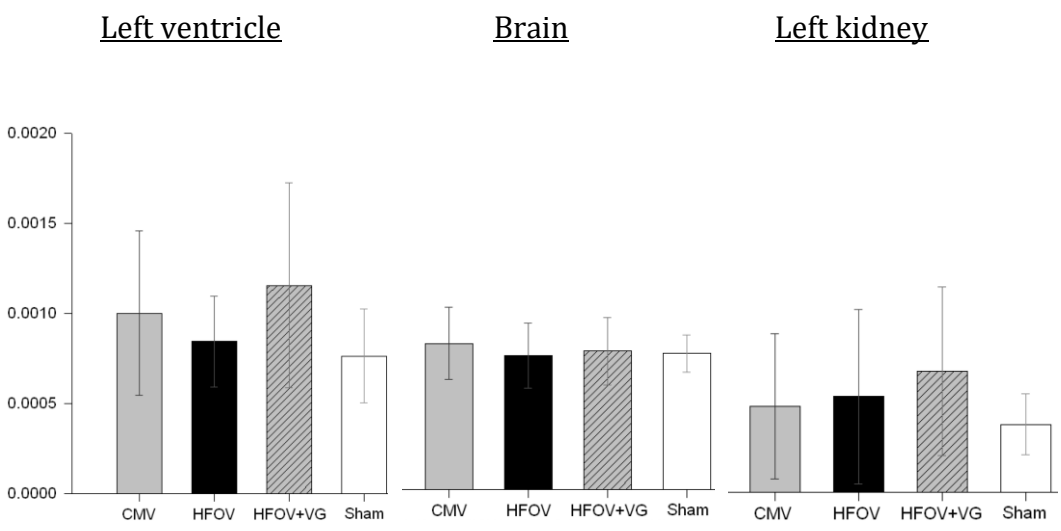


Figure 5.7 Bar plot with standard deviations showing immediate post-mortem tissue analysis for GSSG/GSH ratio of sham-operated and saline lung-lavaged piglets treated with CMV, HFOV and HFOV+VG. No significant differences between groups.

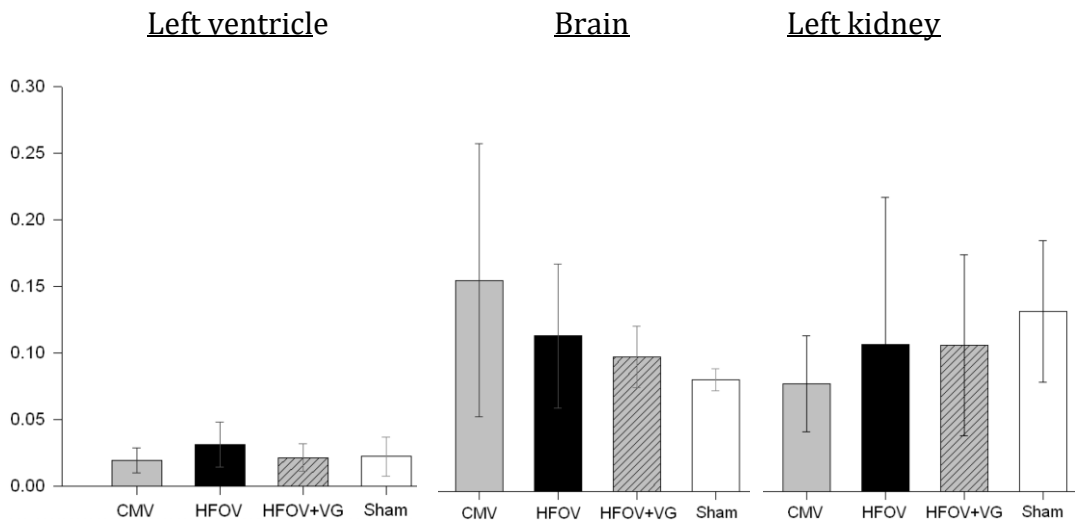
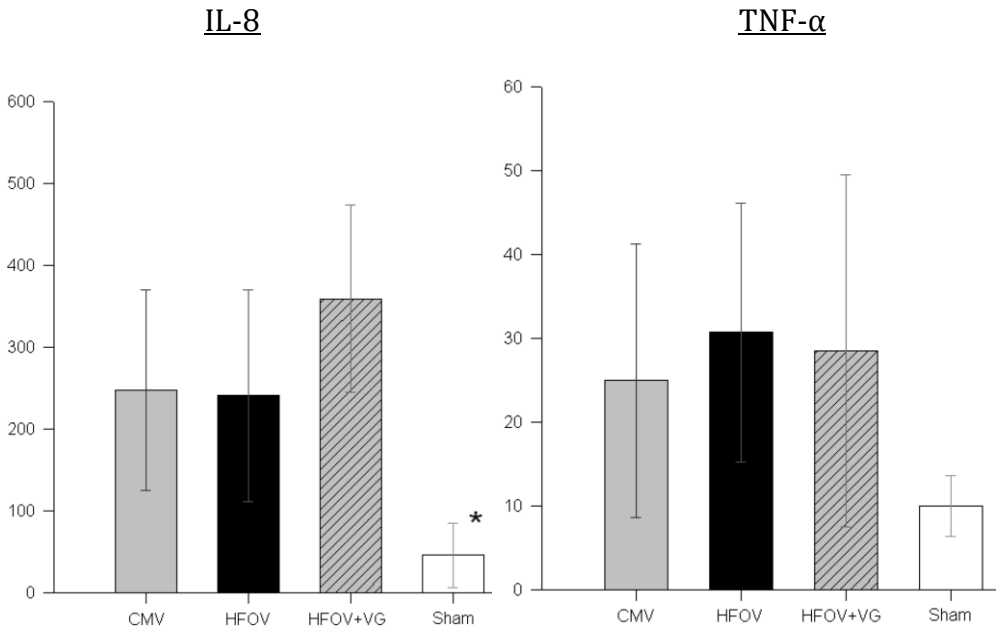


Figure 5.8 Bar plot with standard deviations showing immediate post-mortem lung tissue analysis for IL-8 (pg/mg protein) and TNF-α (pg/mg protein) of sham-operated and saline lung-lavaged piglets treated with CMV, HFOV and HFOV+VG. (*indicates $p<0.05$ vs. all experimental groups)



Chapter 6 – Summary of findings, clinical relevance and future applications

The goal of this study was to assess the hemodynamic effects of HFOV with and without VG, when compared to CMV, in a newborn piglet model of respiratory distress syndrome. This is particularly important in neonates due to the vulnerability of their respiratory and cardiovascular systems, and the fact that respiratory failure requiring positive pressure ventilation assistance is the most common organ failure seen in the NICU. It is a particular challenge that the more compromised a neonate's lungs are often means the higher the mean airway pressures required, which in turn increases the risk for cardiovascular compromise, with HFOV being the common modality. In this study, given our standardization of ventilation weaning, there was no significant difference in either the mean airway pressure or alveolar-arterial S_pO_2 gradient between groups.

The primary outcome of LV CI showed that CMV resulted in a significantly lower LV CI compared to sham-operated over time, though direct comparison to the other experimental groups showed no difference with both HFOV modes showing no significant effect over time. This difference was similarly found with LV SV and EF. Both the cerebral NIRS and the right common carotid doppler flow probe, which are measures of cerebral regional perfusion, showed that HFOV+VG had a significant decrease over time when compared to HFOV and its own starting baseline, respectively. The AVO_2 gradient also showed a significant increase in the HFOV+VG group compared to CMV over time. P_aCO_2 levels increased significantly and pH decreased significantly in CMV compared to HFOV groups over time. These changes in carbon dioxide and pH are important given their effects on vascular resistance and myocardial function, and those effects on regional perfusion and oxygen extraction. The decrease in LV CI and AVO_2 may be related to the decrease in pH and increase in P_aCO_2, respectively.

Tissue analysis of biomarkers for ischemia and oxidation/reperfusion injury showed no differences between GSH/GSSG or lactate concentrations. This lack of a difference in biomarkers, when other markers of regional perfusion indicate a potential difference, may relate to several factors including the relatively short timeframe of the experiment.

Given these findings, our hypothesis that HFOV+VG would have a more negative effect on LV CI was not supported. The combination of parameters measuring LV function and those measuring regional perfusion and oxygen delivery indicates a mixed picture where CMV has negative effects on LV function while HFOV+VG has negative effects on cerebral perfusion and overall oxygen delivery, though these differences may partially be explained by the differences in P_aCO_2 and pH.

Currently, only three previous studies have assessed HFOV+VG to date include those with first authors Sanchez, Iscan, and Enomoto. However, none looked at the effects on hemodynamics. Sanchez et al. in 2013 studied newborn piglets before and after lung lavage with saline, and found that VG appeared to help keep P_aCO_2 stable with changes in compliance; however, there were no control groups **[1]**. The studies by Iscan and Enomoto were both on human neonates, though at differing postnatal ages **[2][3]**. They also found, through a 'crossover' design between HFOV with and without VG, that the VG mode appeared to maintain more stable PaCO2.

There are several limitations to the study of this thesis. As an animal study in piglets its application to humans is limited, and furthermore they are of term gestation so the cardiovascular and pulmonary physiology will differ from preterm neonates to an extent. Full term piglets, in general, are equivalent to approximately 36-38 week

gestation humans, including the pulmonary and cardiovascular systems. The main differences being that piglets have higher pulmonary and systemic vascular resistances, as well as a less compliant chest wall **[5]**. In addition, the sample sizes were calculated to detect a minimum of 25% difference in LV CI, and the sham group resulted in piglets at 6 due to a limitation in availability from the center providing the piglets during the study period. The saline lung lavage model results in pulmonary pathology that is different than the other common conditions encountered including meconium aspiration syndrome and bronchopulmonary dysplasia. Echocardiogram was attempted on the piglets, notably to assess right ventricular function, shunts, and presence of pulmonary hypertension. Unfortunately, due to the severity of the lung disease and consequent fragility of the piglets, progressive hypoxia and lactic acidosis secondary to echocardiography resulted in demise for most piglets prior to the end of the experiment. Echocardiography was therefore eliminated from the study protocol. Another issue was that the renal artery doppler flow probe frequently stopped reading and, due to the stability of the piglets and depth of the probe, it was unable to be repositioned during the experiment thereby making the renal flow data insufficient to make conclusions. Our use of HFOV was also different than that used clinically in many centers due to us not using recruitment maneuvers **[4][6]**. At the time of the study, recruitment ("open-lung") strategies were not standard of care in our center though they are becoming more commonly used **[4]**. Given lung recruitment strategies on HFOV are used in many centers around the world, the lack of this approach in this study could suggest suboptimal use of HFOV when compared to standard of care since such an "open-lung" strategy has been shown to be safer, notably for risk of lung injury and subsequent bronchopulmonary dysplasia, for premature lungs

in CMV and improves oxygenation and ventilation in HFOV **[4]**. Furthermore, HFOV typically operates at a higher mean airway pressure than CMV, but given the need to standardize mean airway pressures to avoid a confounding variable with relation to cardiac function, that was an important and conscious decision. However, as a result, HFOV modes may not have been optimized from a lung volume point of view, and possibly may not have had as much of an impact on cardiac function as HFOV does in a clinical setting with a higher mean airway pressure.

The results of this study show significant negative effects of CMV on LV CI, along with HFOV+VG effects on cerebral blood flow and oxygenation. There was a confounding variable of P_aCO_2 being elevated in the CMV group. These findings indicate differences between ventilator groups in a piglet model of respiratory distress syndrome, which indicate further animal studies and/or human studies with supplementary non-invasive monitoring such as NIRS and echocardiography.

Clinical relevance and future applications

Though the results are not consistent with the hypothesis that HFOV+VG would have more negative effects on LV CI, they do provide some data on parameters that we cannot typically measure in clinical practice. The potential negative effects are a potent reminder of the risks of invasive ventilation, and the possible negative effects of HFOV on regional perfusion and oxygen delivery illustrate the need to ensure adequate

monitoring and cardiac support for those babies receiving HFOV due to respiratory failure.

Given these findings, future animal studies looking at HFOV and hemodynamics would benefit from incorporating echocardiography to assess right ventricular function parameters as well as the presence of shunts and pulmonary hypertension. It may be considered to use an animal model with healthy lung to avoid the risk of respiratory compromise with echocardiography. Future clinical studies could consider assessing markers of regional oxygenation, notably cerebral NIRS, in conjunction with modalities such as echocardiography and biochemical markers of perfusion, to compare hemodynamic effects of CMV and HFOV modes. Though not the focus of this investigation, there could be an additional component of monitoring oxygenation and ventilation stability over time, notably with compliance changes such as giving surfactant, since such stability is one of the proposed yet unproven benefits of adding the VG mode to HFOV. Obtaining such data is important in providing information regarding the potential benefits and drawbacks of a new mode of ventilation and HFOV in general, especially since it is essential and often used on some of the most critically ill neonates.

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Appendix 1: ARRIVE Checklist [Chapter 5, reference 16]

	ITEM	RECOMMENDATION	Section/ Paragraph
Title	1	Provide as accurate and concise a description of the content of the article as possible.	Page 71
Abstract	2	Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.	Page 72
INTRODUCTION			
Background	3	a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale. b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology.	Page 74
Objectives	4	Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.	Page 85
METHODS			
Ethical statement	5	Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.	Page 76
Study design	6	For each experiment, give brief details of the study design including: a. The number of experimental and control groups. b. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when). c. The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.	Page 84, 85 Figure 5.3 (page 123)
Experimental procedures	7	For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day). c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).	Page 75-82
Experimental animals	8	a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.	Page 75-76

Appendix 1 continued (ARRIVE Checklist [Chapter 5, reference 16])

Housing and husbandry	9	Provide details of: a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish). b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment). c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.	Page 75-76
Sample size	10	a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group. b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used. c. Indicate the number of independent replications of each experiment, if relevant.	Page 84-85
Allocating animals to experimental groups	11	a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done. b. Describe the order in which the animals in the different experimental groups were treated and assessed.	Page 84
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).	Page 84-85
Statistical methods	13	a. Provide details of the statistical methods used for each analysis. b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron). c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.	Page 85
RESULTS			
Baseline data	14	For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing. (This information can often be tabulated).	Page 75-76
Numbers analysed	15	a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50%[2]). b. If any animals or data were not included in the analysis, explain why.	Page 84, 85-86
Outcomes and estimation	16	Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).	Tab 5.1-5.6 Fig 5.6-5.8
Adverse events	17	a. Give details of all important adverse events in each experimental group. b. Describe any modifications to the experimental protocols made to reduce adverse events.	Page 85-86
DISCUSSION			
Interpretation/ scientific implications	18	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results[2]. c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research.	Page 89-95
Generalisability/ translation	19	Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.	Page 130-134
Funding	20	List all funding sources (including grant number) and the role of the funder(s) in the study.	Page iv and v