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The Biophysical Alterations to Pulmonary Surfactant during Lung Injury

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Abstract:
Pulmonary surfactant is a lipoprotein mixture responsible for reducing surface tension of the lung. In this thesis, we investigate the impact of lung injury on surfactant composition and function, with a specific focus on cholesterol content. First, the impact of a high cholesterol diet on surfactant function was assessed in three models of lung injury. It was hypothesized that serum hypercholesterolemia would increase host susceptibility to surfactant functional impairments. Secondly, the impact of injurious ventilation on intracellular surfactant within the lung was investigated. It was hypothesized injurious ventilation would produce alterations to lamellar body surfactant. Overall, the data suggest that a high cholesterol diet contributes to altered surfactant function during lung injury. Additionally, injurious ventilation promoted functional impairments to intracellular surfactant prior to secretion into the airspaces. In conclusion, we provide evidence of diet induced alterations to surfactant function and novel insight into intra-alveolar alterations to surfactant during mechanical ventilation.

Keywords: Pulmonary surfactant, mechanical ventilation, ventilation induced lung injury, lamellar body, minimum surface tension, cholesterol, acute respiratory distress syndrome.
Co-Authorship Statement:

All the experiments presented in this thesis were performed in the lab of Dr. Ruud Veldhuizen and Dr. Cory Yamashita. Throughout these studies Dr. Yamashita and Dr. Veldhuizen were actively involved in the development of experimental design, the interpretation and analysis of data, and the presentation of this thesis.

In the first experiment presented in this thesis, lavage surfactant samples from rats fed a standard and high cholesterol diet were acquired from a previous experiment conducted under the supervision of Dr. Veldhuizen performed by a former student, Josh Qua Hiansen (Qua Hiansen Thesis, 2013). During this previous experiment Josh, Brandon Banaschewski, Li-Juan Yao, and Lynda McCaig were heavily involved in animal experimentation, recovery and processing of the lavage surfactant samples, and interpretation of the experimental data.

In the second experiment involving the isolation of lamellar bodies, several other individuals contributed to the work presented in this thesis. Li-Juan Yao provided general guidance on techniques used during extractions and phosphorus assays. The assistance of Lynda McCaig was instrumental in regards to surgical and ventilator preparation of all rats. She also provided assistance in the physiological monitoring of animals while on the ventilator.

All transmission electron microscopy images of isolated lamellar body samples from non-ventilated rat lungs were obtained by Reza Khazaee at Biotron at The University of Western Ontario. Reza additionally provided supplementary data and images of lamellar bodies in situ within lung tissue follow ventilation, which contributed toward the discussion of this thesis.
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This thesis is dedicated to my grandparents Frank and Rene Milos
   Thank you both for everything you have done for me
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Abbreviations:

AIIE – Alveolar Type II Cell
ADSA – Axisymmetric Drop Shape Analysis
ANOVA – Analysis of Variance
ARDS – Acute Respiratory Distress Syndrome
ARDSnet – National Institute of Health, Acute Respiratory Distress Syndrome Network
BLES – Bovine Lipid Extract Surfactant
BPM – Beats per Minute
CSD – Constrained Sessile Drop Surfactometer
CV – Control Ventilation
DPPC – Dipalmitoylphosphatidylcholine
ER – Endoplasmic Reticulum
FiO₂ – Fraction of Inspired Oxygen
FPS – Frames per Second
MβCD – Methyl-Beta-Cyclodextrin
NV – Non-ventilated
PaO₂ – Partial Pressure of Oxygen in arterial blood
PaCO₂ – Partial Pressure of Carbon Dioxide in arterial blood
PC – Phosphatidylcholine
PG - Phosphatidylglycerol
PEEP – Positive End-Expiratory Pressure

PIP – Peak Inspiratory Pressure

RDS – Neonatal Respiratory Distress Syndrome

RR – Respiratory Rate

SPA – Surfactant Protein A

SPB – Surfactant Protein B

SPC – Surfactant Protein C

SPD – Surfactant Protein D

TEM – Transmission Electron Microscopy

VILI – Ventilation Induced Lung Injury

VT – Tidal Volume

ZEEP – 0 cmH₂O Positive End Expiratory Pressure

**Symbols:**

ΔP – Change in Pressure (LaPlace’s Law)

r – Radius (LaPlace’s Law)

γ - Surface Tension (LaPlace’s Law)

γ adsorption – Surface tension following adsorption

γ min – Minimum Surface Tension

γ max – Maximum Surface Tension
Chapter 1 - General Introduction and Literature Review

1.1 Overview

Pulmonary surfactant is a significant contributor toward normal respiratory mechanics and arterial blood oxygenation. Surfactant is a complex mixture composed primarily of phospholipids, surfactant-associated proteins, and neutral lipids such as cholesterol. Secreted into lung airspaces, surfactant’s function is to reduce surface tensions, which helps to facilitate breathing and prevent alveolar collapse at low lung volumes (West, 1995). The specific constituents of surfactant are important for its ability to reduce surface tension while alterations to surfactant composition may contribute to its dysfunction and impaired arterial oxygenation.

Acute respiratory distress syndrome (ARDS) is a severe respiratory disorder with a high mortality. Patients with ARDS present with a variety of pathological changes in the lung (Ware and Matthay, 2000), however across all causes of ARDS, dysfunctional surfactant forms contribute to impairments in surface tension reduction and gas exchange (Günther et al., 2001). In order to accurately reverse low arterial oxygenation in these patients, mechanical ventilation is employed as a supportive therapy to promote gas exchange in the lungs. Unfortunately, the use of mechanical ventilation in pre-injured lungs or through the use of inappropriate ventilation strategies may promote lung injury via localized over-distension of the healthy lung regions and repeated alveolar collapse. The generation of these damaging forces may induce or exacerbate alterations to the surfactant system, further contributing to poor lung function and patient survival (Tremblay and Slutsky, 2006).

The purpose of this thesis was to investigate the role of diet induced serum hypercholesterolemia and intracellular surfactant alterations within the context of surfactant function following exposure to injurious mechanical ventilation of the lung. This introductory chapter focuses on basic concepts such as structure and function of surfactant, mechanical ventilation of the lung, and finally pathological alterations to surfactant typically associated with mechanical ventilation.
1.2 Structure and Function of Lungs

The primary function of the lung is to facilitate the exchange of O\textsubscript{2} and CO\textsubscript{2} between the external environment and the blood. Gas exchange is facilitated by the respiratory cycle which consists of both inspiration and expiration. Inspiration begins with contraction of the diaphragm and intercostal muscles, increasing the volume of the thoracic cavity and creating a negative intrathoracic pressure relative to the external environment. Due to this pressure gradient between the external environment and intrathoracic cavity, approximately 0.5L of air from the external environment flows into the lungs causing them to inflate (West, 1995). This volume of inspired air at each breath is called the tidal volume (VT) and is ~6-8mL/kg in humans. Expansion of the lungs during inspiration helps to promote the formation of a large surface area at which O\textsubscript{2} from the external environment and CO\textsubscript{2} from the blood may be exchanged. Following inspiration, the diaphragm and intercostal muscles relax and the chest cavity decreases in volume, expelling air and CO\textsubscript{2} in a process called expiration.

The structure of the lungs makes this organ well suited for gas exchange as the lungs form a branching network of airways that get increasingly smaller in diameter toward the distal regions. At the ends of these airways are millions of small air-sacs called alveoli. The alveolar tissue is well perfused with blood from nearby capillaries, which provide a short diffusion distance (~0.3\textmu m) between the external environment and the systemic circulation allowing for efficient gas exchange. The high number and small size of these alveoli contribute to a large surface area of up to 50-100m\textsuperscript{2} at which gas exchange can occur (West, 1995). Because of these factors, the alveoli allow for efficient gas exchange, producing high partial pressure of arterial blood oxygen (PaO\textsubscript{2} = 85-100mmHg, PaO\textsubscript{2}:FiO\textsubscript{2} = 400-500mmHg) and relative low partial pressure of carbon dioxide (PaCO\textsubscript{2}) in the blood (37-43mmHg), satisfying the metabolic demands of systemic tissues (West, 2003).

The surface of the alveolus is composed of two types of cells; alveolar type I, and a smaller proportion of alveolar type II epithelial cells (AIIE). Directly between the alveolar surface and the nearby airspace is a thin lining of aqueous fluid, termed the alveolar hypophase, which extends from the interior of alveolar sacs where it is secreted, into the lung’s airways. This aqueous fluid exerts a large force (or surface tension) upon
the lung, causing a high energy requirement for recruiting the alveolar sacs. Fortunately, AIIIE cells near the airspaces secrete a surface active phospholipid-protein mixture, called surfactant, into the alveolar hypophase in order to counteract these forces which ultimately facilitate the ease of respiration. Alveolar type I cells, although not the focus of this thesis, have an important role as their elongated structure creates the large, thin alveolar surface that allows for efficient gas exchange.

The remarkable ability of the lungs to efficiently exchange gases during respiration is highly dependent on pulmonary surfactant which is responsible for promoting high pulmonary compliance (i.e. the change in lung volume associated with a given distending pressure) by reducing these high surface tensions of the alveolar hypophase. High compliance allows the lungs to easily inflate upon relatively small changes in intrathoracic pressure helping to facilitate respiration. Furthermore, this phospholipid-protein mixture prevents the collapse of alveolar sacs by maintaining stability at low lung volumes (i.e. during exhalation). The importance of surfactant for reducing surface tension in the lung and its subsequent impact on respiratory function will be discussed in the following sections.

1.2.1 – Surface Tension

The primary function of surfactant within the lung is to reduce the high surface tensions at the air-liquid interface of the alveolar hypophase. In order to sufficiently understand the actions of surfactant in the lung, a thorough understanding of the concept of surface tension is required. Presented in Figure 1.1 is the basic concept of how surface tension arises at an interface. Water molecules within the bulk of the aqueous solution are surrounded by other molecules in each direction, resulting in a molecule at equilibrium with no net force in any direction. However, molecules within 1nm of the air-liquid interface do not possess equal attractive forces between air vapour molecules above compared to those within the aqueous hypophase (Zinke-Allmang, 2009). The result of these unbalanced electrostatic forces is a net force driving the interfacial molecule into the bulk phase (Figure 1.1A). The electrostatic imbalance at the interface produces a high
energy requirement for the formation of a surface, as molecules must be moved to the interface against these net forces. This force required for a surface film to resist expansion is called surface tension, which is measured as force/unit area (Possmayer, 1992). This phenomenon produces a tendency for materials with high surface tension such as water (surface tension of 70mN/m at 37°C), to minimize surface area by forming shapes such as spheres or droplets, while materials with low surface tensions form puddles.

![Diagram of surface tension reduction](image)

Figure 1.1 – The generation of high surface tension at an aqueous air-liquid interface (A). Phospholipids lower surface tension by forming an interfacial film and reducing the magnitude of the net downward force on interfacial aqueous molecules beneath the surface. (B). PL-phospholipid (Electrostatic forces are indicated by arrows, the magnitude of the force is indicated by size of the arrow).

1.2.2 – How Surfactant Reduces Surface Tension

Surfactant aids in surface tension reduction by adsorbing to the air-liquid interface and orienting in such a way that the polar head groups are contained within the bulk phase forming a monolayer, while hydrophobic acyl tails protrude into the airspaces above. The molecular orientation of the phospholipid molecules creates electrostatically favourable interactions between the charged phospholipid head and the aqueous molecules near the
lining of the air-liquid interface, reducing the net downward force on the interfacial water molecules (Figure 1.1B). When this happens, the intermolecular attractive forces responsible for generating high surface tensions are reduced (West, 1995).

The physiological impact of surfactant on pulmonary surface tension within the alveolus is best illustrated using LaPlace’s law [1], applied to a bubble.

\[
\Delta P = \frac{2\gamma}{r}
\]  

[1]

Where \(\Delta P\) is the pressure difference across the drop or alveolus, \(\gamma\) is the surface tension within the alveolus, and \(r\) is the radius of the alveolus. How surfactant stabilizes the alveolus is shown in Figure 1.2. According to LaPlace’s law, as the radius of the alveolus decreases during exhalation, the pressure across the alveolus increases if surface tension remains the same. Surfactant acts to reduce the surface tension of the alveolar hypophase, therefore reducing the pressure across alveoli at a given radius. Because alveolar size in the lung is not uniform, higher surface tensions in the lung would produce a larger pressure gradient across smaller alveoli, promoting the tendency for these alveoli to collapse inward, shunting air toward the larger, more stable alveoli. Furthermore, a key feature of surfactant is that surface tension is further reduced to near 0mN/m values during instances of lateral compression due to an enrichment of tightly packed saturated phospholipids at the interfacial film. Therefore surfactant at the air-liquid interface reduces the tendency of alveolar collapse by increasing alveolar stability through surface tension reduction at low lung volumes (Clements et al., 1961).

The reduction of surface tension within the alveolus is highly dependent on surfactant’s composition, as biochemical alterations have been shown to contribute to impairments in surface tension reduction. The interactions between surfactant constituents in the alveolar hypophase create a large multi-layered scaffold capable of reducing surface tension to low values.
LaPlace’s Law (Drop): $\Delta P = 2\gamma/r$

Figure 1.2 – LaPlace’s Law applied to a bubble (inverse configuration), modelling the alveolus. Surfactant reduces surface tensions at the air-liquid interface, thereby reducing pressure across the alveolus, contributing to alveolar stability and preventing collapse at low lung volumes. $\gamma$ -surface tension.
1.3 –Surfactant Composition, Lifecycle, and Biophysical Function

Surfactant within the alveolar airspaces is crucial for reducing surface tension in the lung. Several factors are responsible for surfactant’s ability to reduce surface tension once secreted into the airspaces, including 1) proper composition and 2) maintenance and metabolism of the interfacial surface film.

1.3.1- Surfactant Composition

The composition of surfactant constituents has been widely studied and is generally well conserved across most mammalian species (Veldhuizen et al., 1998; Orgeig and Daniels, 2001). Surfactant consists of a complex mixture of a variety of phospholipid species (80-90% by weight), surfactant proteins A through D (5-10%), and neutral lipids - the majority of which are cholesterol (4-10%) (Pérez-Gil, 2008; Zuo et al., 2008).

The single most prevalent surfactant component is phosphatidylcholine (PC) composing 70 - 85% of all lipids. Of the PC found in surfactant, 30-60% is composed of saturated dipalmitoylphosphatidylcholine (DPPC), consisting of two palmitic acids (16:0) attached to a glycerol backbone (Kyriakides et al., 1976; Burnell et al., 1978; Zuo et al., 2008). The remaining portion of PC is predominately composed of a variety of unsaturated phospholipids (Zuo et al., 2008). Beyond PC, other phospholipid species, most of which are unsaturated, make up the remaining portion of surfactant phospholipids including: positively charged phosphatidylethanolamine (3-10%), negatively charged phospholipids such as, phosphatidylinositol (2-4%) and phosphatidylglycerol (PG) (7-10%), and sphingomyelin (2-3%) (Rooney et al., 1975; Post et al., 1982; Veldhuizen et al., 1998; Zuo et al., 2008). In addition to phospholipids, neutral lipids make up the other lipid portion of surfactant. The majority of neutral lipids in surfactant consist of free and esterified cholesterol, while other tri-, di-, and mono-glycerides contribute to the smaller remaining lipid pool (Post et al., 1982; Orgeig and Daniels, 2001). Although the phospholipid and cholesterol constituents in surfactant are generally fairly consistent within a species, it has been reported that intake of certain diets may impact its lipid composition (Davidson et al.,
1997; Murray et al., 2000; Wolfe et al., 2002; McCrae et al., 2008). However the functional consequences of these changes in surfactant composition remain poorly characterized.

Finally, surfactant contains four surfactant proteins including large hydrophilic proteins, surfactant protein A (SPA) and surfactant protein D (SPD), and two smaller hydrophobic proteins, surfactant protein B (SPB) and surfactant protein C (SPC) (Weaver and Conkright, 2001; Haagsman and Diemel, 2001; Perez-Gil, 2008). SPA, a member of the collectin family of proteins, makes up the majority of surfactant proteins by mass (~5% total), while SPB, SPC, and SPD each compose a lesser proportion. These surfactant proteins tend to associate with surfactant phospholipids with the exception of SPD. For this reason, discussion of SPD will largely be excluded from this thesis (for detailed review, see Hartl and Griese, 2006). The hydrophobic proteins SPB and SPC associate with phospholipids acyl tails within surfactant and are vital for endogenous surfactant biogenesis and function (Serrano and Pérez-Gil, 2006). Furthermore, SPB is positively charged allowing for interactions with negatively charged phospholipids such as PG (Weaver and Conkright, 2001).

The presence and proper ratio of these above surfactant constituents are paramount for the proper function of surfactant films (Zuo et al., 2008). Furthermore, the processes involved in the formation of surfactant at the air-liquid interface to mediate surface tension reduction are highly dynamic. Surfactant is continuously produced, secreted and metabolized within the lung in order to promote the formation of a film capable of achieving optimal biophysical function during respiration.

1.3.2 - Alveolar Surfactant

The AIIE cells at the alveolar surface are constantly producing, secreting, re-internalizing, and recycling the surfactant mixture that covers alveolar epithelial surfaces (Perez-Gil and Weaver, 2010). Within the healthy lung at any given time there are two broad localizations of surfactant: 1) intracellular surfactant, such as lamellar bodies, acting as surfactant storage vesicles within AIIEs, and 2) extracellular surfactant within the alveolar hypophase of the airspaces forming a structurally dense 3D multilayer network of
surfactant bilayers at the air-liquid interface, directly responsible for surface tension reduction. There exists a dynamic turnover between these two localizations of surfactant; intracellular lamellar bodies are secreted into the airspace where internal contents are unravelled to form multilayers and a surface active film. In response to changes in surface area that occur during respiration, surfactant constituents no longer contributing to surface tension reduction, disassociate from the multi-layer network to form unilamellar vesicles which are eventually internalized and recycled by the AIIE into new surfactant constituents or targeted for degradation (Figure 1.3).
Figure 1.3 – The endogenous pulmonary surfactant system. 1. Surfactant components are first produced within AIIE cells and packaged into lamellar bodies. 2. Upon cellular stimuli, lamellar bodies fuse with the apical plasma membrane and expel and unravel internal surfactant bilayers into the airspaces, where it may associate with SPA to form multilayer networks. 3. Multi-layer large aggregate structures form a monolayer and surface membrane phase acting as surfactant associated surface reservoir (enlarged portion). 4. During alveolar surface area cycling, inactive unilamellar vesicles are formed and targeted for recycling within the AIIE. Note: not shown are surfactant proteins or the graphical representation of cholesterol within the phospholipid bilayer phases.

1.3.2.1 - Intracellular Surfactant Production and Secretion

Surfactant production begins in the AIIE where phospholipid and surfactant protein constituents are intracellularly synthesized and packaged together with cholesterol from
the serum into bilayers along the cell exocytic pathway. The primary intracellular storage vesicle for surfactant prior to its secretion into the airspace is the lamellar body. Lamellar bodies are composed of dense, tightly packed stacks of lipid bilayers with proteinaceous cores surrounded by an external limiting membrane (Gil and Reiss, 1973; Ochs, 2010). Although other less abundant intermediate surfactant storage vesicles exist within the AIIE, such as multivesicular bodies, the lamellar body represents final intracellular ‘precursor’ before secretion into the airspaces (Schmitz and Müller, 1991).

In the AIIE, the majority of surfactant phospholipids, including PC and PG, are synthesized and remodelled in the endoplasmic reticulum (ER) (Batenburg, 1992; Schmitz and Müller, 1991). Following synthesis, phospholipids are packaged into multivesicular vesicles and continue along the exocytic pathway. During multivesicular body maturation into lamellar bodies, additional phospholipids from the ER may transverse the lamellar body limiting membrane into the lumen via lipid transporters such as ABCA3, promoting the accumulation of lipids in the lamellar body cores (Perez-Gil and Weaver, 2010).

In addition to the surfactant phospholipids, SPB and SPC are synthesized within the ER prior to concentration into secretory vesicles which mature into lamellar bodies (Weaver, 1998; Haagsman and Diemel, 2001). These surfactant proteins are vital for the phospholipid bilayer accumulation and organization into dense lamellae within lamellar bodies; lack of SPB production results in respiratory deficiency and impaired maturation of lamellar bodies from multivesicular structures (Clark et al., 1995; Melton et al., 2003). The production and trafficking of SPA to surfactant membranes is less clear. Although studies have previously localized SPA within lamellar bodies of AIIEs (Oosterlaken-Dijkstra et al., 1991a), it has become widely accepted that the majority of SPA is secreted independently of lamellar bodies from the ER into the air-spaces (Ochs et al., 2002; Pérez-Gil, 2008).

Incorporation of cholesterol into surfactant phospholipid membranes normally occurs during lamellar body formation. Hass and Longmore have previously demonstrated that the majority of surfactant cholesterol (99%) is derived from serum lipoproteins rather than endogenous synthesis (Hass and Longmore, 1979). However, less is known concerning intra-alveolar trafficking of cholesterol from the serum into these surfactant
storage organelles once inside the AIIE (Orgeig and Daniels, 2001; Gowdy and Fessler; 2013).

Once all surfactants constituents have been assembled into mature lamellar bodies, cellular stimuli such as alveolar stretch (Dietl et al., 2010), triggers intracellular Ca\(^{2+}\) accumulation (Wirtz and Dobbs, 1990) promoting lamellar body exocytosis at the apical membrane of the AIIE. Fusion of the limiting membrane at the apical plasma membrane ultimately unravels the dense phospholipid bilayers and protein cores into the aqueous hypophase.

1.3.2.2 – Extracellular Surfactant and Metabolism

Upon lamellar body secretion, the dense folds of phospholipid membranes from within lamellar body cores form a large multi-layer network of bilayers in the alveolar hypophase (Fig 1.3). At the alveolar air-liquid interface is a surface film which is connected with the multilayer surfactant reservoir below. The formation of this multilayer structure is thought to be aided through lipid-protein interactions mediated by SPA (Schürch et al., 1992; Perez-Gil, 2008), SPB (Nag et al., 1999; Serrano and Perez-Gil, 2006) and SPC (Leonenko et al., 2007). It is these extracellular surfactant structures that are directly responsible for reducing the surface tension in the lung. Although extracellular surfactant was previously conceptualized as a simple monolayer, visualizations of the air-liquid interface have demonstrated surfactant solutions form a series of dense phospholipid multilayers just below the surface monolayer (Schürch et al., 1998; Leonenko et al., 2007; Follows et al., 2007; Ochs, 2010). To this end, surfactant should be conceptualized as a complete structure developed in the aqueous lining as a whole termed the ‘membrane surface phase’, rather than a simple interfacial monolayer (Perez-Gil, 2008). The membrane surface phase complexes are experimentally described as large aggregates and may be isolated from the airspaces of the lung by washing the lungs with fluid, a technique known as broncho-alveolar lavage. Previous estimates have suggested extracellular surfactant concentration within the alveolar hypophase is quite high (Zuo et al., 2008), with
phospholipid concentration estimations of 30 – 120 mg/mL found in adult small mammal lungs (Ohashi et al., 1994; Putz et al., 1994).

The surfactant lining the alveolar surface is constantly exposed to compression and expansion during respiration, resulting in changes in alveolar surface area. During interfacial surface area cycling, spent or inactivated surfactant components from the surface membrane phase disassociate to form small unilamellar vesicles, experimentally referred to as small aggregates (Veldhuizen et al., 1996; Ito et al., 1997; Günther et al., 1999). Previous studies have demonstrated these unilamellar vesicles do not possess surface tension reducing capabilities (Yamada et al., 1990; Veldhuizen et al., 1993). Although it has been demonstrated these small aggregates are composed of similar lipid species as large aggregates, it has been suggested that small aggregates may be devoid of SPB protein (Veldhuizen et al., 1993; Günther et al., 1999). Unilamellar vesicles that dissociate into the hypophase may subsequently be internalized into the AIIE where the constituents may be recycled into new surfactant membranes or degraded (Batenburg, 1992; Perez-Gil and Weaver, 2010).

Extracellular surfactant forms a structurally dense 3D network within the alveolar hypophase that is directly responsible for surface tension reduction, helping to stabilize the lung during respiration. The surface active properties of these extracellular films are discussed in the following section.

1.3.3 Surfactant Biophysical Properties

For surfactant within the airspaces to be truly effective during respiration, it must possess several important properties which will be referred to as surfactants ‘biophysical properties’. Biophysical properties of a competent surfactant are I) rapid adsorption to the air-liquid interface upon initial introduction to the hypophase (in vitro), II) achievement of very low surface tension (~0mN/m) upon lateral compression, and III) efficient re-spraying to the air-liquid interface upon re-expansion (Zuo et al., 2008; Possmayer et al., 2010). Although surfactant is important for in vivo lung stability and function, the current model of how surfactant functions to promote these biophysical properties is primarily
based off evidence from *in vitro* studies using devices to visualize surfactant and measure surface tension (see section 1.3.4). Importantly, no single component of surfactant is efficient at achieving all three biophysical properties on its own (*in vitro*), indicating the importance of cumulative effects of the various surfactant constituents.

The dominant phospholipid within surfactant is DPPC. Pure DPPC bilayers have a relatively high melting temperature (42°C) promoting a gel-like membrane phase at physiological temperatures (Possmayer et al., 2001). However, the addition of other bulkier lipid components, such as unsaturated phospholipids and large amounts of cholesterol, to DPPC membranes *in vitro* tends to lower the melting temperature of these membranes closer to physiological temperatures (37°C) (Egberts et al., 1989). In turn, this promotes an increase in the lateral mobility of phospholipids within these membranes, a factor referred to as fluidity (Zuo et al., 2008). *In vitro* biophysical studies have generally shown that the saturated components of surfactant are important for reducing surface tensions during lateral compression, while the more fluidizing phospholipid species are thought to contribute to adsorption, re-spreading, and monolayer to multilayer transition.

1.3.3.1- Rapid Adsorption

Adsorption is the process by which surfactant proteolipid complexes move from the sub-phase to an interface, forming a surface film (Lopez-Rodriguez and Perez-Gil, 2014). As a result of surfactant movement to the interface, aqueous molecules at the surface of the air-liquid interface are replaced with a film of phospholipids, reducing high surface tensions of the aqueous molecules. If the adsorbing surfactant phospholipid is sufficiently concentrated, the surface tension of pure water will be reduced from 70mN/m toward an equilibrium tension of ~22-25mN/m (at 37°C), at which point phospholipids at the monolayer are in equilibrium with molecules in the bulk of the hypophase. *In vivo*, this process only occurs in the lungs at birth, after which the film is ever-present and dynamically maintained. Thus, to study the adsorptive capacity of surfactant, *in vitro* techniques using model surfactant preparations have been developed.
**In vitro**, effective surfactant preparations are expected to adsorb to the air-liquid interface within several seconds (Zuo et al., 2008; Lopez-Rodriguez and Perez-Gil, 2014). Interestingly, biphasic phospholipid mixtures modelling the lipid components of surfactant (DPPC and unsaturated PC or PG) do not possess the ability to rapidly reduce surface tension at the air-liquid interface upon deposition within the sub-phase *in vitro*. However, the addition of hydrophobic surfactant proteins, SPB and SPC to these biphasic phospholipid mixtures, immediately improves the rate of adsorption (Oosterlaken-Dijksterhuis et al., 1991b; Nag et al., 1999; Rodriguez-Capote et al., 2001). Furthermore, in the presence of SPB, SPA has been shown to enhance phospholipid adsorption *in vitro* (Schürch et al. 1992; Yu and Possmayer, 1996; Rodriguez-Capote et al., 2001). It should be noted that SPA is not absolutely required for surfactant biophysical function as indicated by SPA knockout animals which have phenotypically normal lung physiology (Korfhagen et al., 1998). Based on these *in vitro* findings, it is thought that these hydrophobic surfactant proteins help to facilitate the transfer of surfactant from the multilayer surfactant reservoir into the interfacial film (Perez-Gil and Weaver, 2010). Finally, it should be noted that the addition of fluidizing lipids to DPPC mixtures may also promote the rate at which surfactant may adsorb to the interface compared to pure DPPC *in vitro* (Walters et al., 2001). However, the role of these fluid lipid components in this process appears to be relatively minor compared to the role of surfactant proteins (Holm et al., 1996; Diemel et al., 2002).

### 1.3.3.2 - Low Surface Tension upon Lateral Compression

The most physiologically important biophysical property of surfactant *in vivo* is the ability to reduce surface tensions to near 0mN/m values upon expiration, allowing the stabilization of the alveolus (see LaPlace’s Law). Achievement of low surface tension upon compression *in vivo* is a continuous process that occurs upon every exhalation of the respiration cycle. In a competent surfactant, compressions of about 20-30 % surface area reduction should be enough to produce a stable, a near zero minimum surface tensions (Schürch et al., 1976), corresponding to the changes in alveolar surface area that occur during resting ventilation (Bachofen and Schürch, 2001). Although very low surface
tension have been measured within the lung, the models explaining how low surface
tension is achieved has predominately been based on in vitro experiments involving
compression and expansion of phospholipid bilayer and monolayers.

In vitro studies compressing monolayers of surfactant phospholipids have
concluded that surfactant surface films composed of DPPC and other saturated
phospholipids are predominately responsible for the near 0 mN/m surface tensions
achieved in the lung (Notter and Morrow, 1975; Hawco et al., 1981). Compression of other
bulkier unsaturated surfactant PC films are generally unable to achieve low surface
tensions of the same magnitude (Notter and Morrow, 1975; Possmayer et al., 2001). This
phenomenon is explained by the ability for DPPC molecules to be tightly compressed
together with little interference from neighbouring saturated acyl tails, forming highly
packed membranes at the interface. Alternatively, phospholipid membranes composed of
unsaturated lipids or the addition of high levels of cholesterol to a phospholipid membrane
prevents as close of an association to neighbouring phospholipids resulting in more fluid
membranes, which cannot pack tightly to reduce surface tension to the same extent when
compressed as a monolayer.

The alveolar surface is constantly exposed to compression and expansion. To
stabilize the alveolar surface during low lung volumes, low surface tensions must be
achieved. Given that surfactant phospholipid films are typically composed of 30-60%
DPPC and considerable quantities of other fluidizing unsaturated phospholipids and
cholesterol, in order to reach near zero surface tensions, it is believed the surface film must
be remodelled during compression (Figure 1.4). It is currently theorized that during
interfacial compression as would occur during respiration, there is a progressive refinement
of the interfacial surface, such that more fluid lipid components such as unsaturated
phospholipids and cholesterol are squeezed out from the interfacial surface (Perez-Gil and
Weaver, 2010; Lopez-Rodriguez and Perez-Gil, 2014; Baoukina et al., 2014). This type of
lipid refinement would favour DPPC enrichment at the interface, resulting in a tightly
packed surface membrane capable of achieving surface tensions near 0 mN/m (Figure
1.4A). Several other studies have suggested SPB and SPC help to refine the surface film
during instances of lateral compression (Diemel et al., 2002; Nag et al., 1999; Leonenko et
al., 2007; Lee, 2008; Lopez-Rodriguez and Perez-Gil, 2014). Despite refinement of surfactant at the air-liquid interface during compression, highly fluidized membranes may also impair the ability of the surfactant to achieve low surface tensions during compression. For example, *in vitro* studies by Hawco et al., demonstrated that addition of unsaturated phospholipids in high proportions to DPPC monolayers impaired their ability to reduce to low surface tension upon compression with a Langmuir balance. Furthermore, as the proportion of these unsaturated lipids increased within the surfactant membrane, so did the minimum surface tensions achieved of the lipid monolayers (Hawco et al., 1981). Similar studies have demonstrated similar findings with the addition of ~10-20% cholesterol (Gunasekara et al., 2005; Notter et al., 1980). It has been suggested that these bulky fluidizing molecules impair the ability to achieve an enriched DPPC membrane upon compression.
Figure 1.4 – Surfactant interfacial films during compression and expansion of the interface. (A) Compression of the interfacial film is believed to promote exclusion of more fluid components of surfactant (unsaturated phospholipids and cholesterol) into the bilayer phases below the interfacial film. This produces an enrichment of DPPC molecules at the interface, resulting in low surface tension (~0mN/m). (B) During expansion of the interfacial film, material from the surfactant reservoir in the multilayer is re-adsorbed into the interfacial film. Note: the hydrophobic proteins SPB/C play a major role in these processes, however, are not shown in this figure for simplicity.

1.3.3.3 - Efficient Re-spreading

Low surface tensions reflect the ability of surfactant to stabilize the lung during exhalation. Conversely, during lung re-expansion or inhalation, the surface area of the air-liquid interface increases and surfactant from the multilayer reservoir below must be re-inserted at the interfacial film to prevent high surface tensions of the expanding aqueous hypophase (Figure 1.4B). In a healthy lung in vivo, it is expected that surface tensions achieved during re-expansion would not exceed equilibrium surface tension of ~22-
25mN/m (Schurch et al., 1978; Zuo et al., 2008) reflecting efficient re-insertion of surface active surfactant back into the surface. Although *in vitro* maximum surface tensions beyond equilibrium values may be sustained due to differences in the environment at which surfactant is exposed, maximum surface tension is a good indicator of the efficiency at which surfactant is re-incorporated into the expanding interface in experimental samples (Diemel et al., 2002; Baumgart et al., 2010). In regards to re-spreading, it is the unsaturated phospholipids and cholesterol within surfactant lipids that are thought to be important for re-incorporation into the surface film (Notter et al., 1980; Taneva and Keough, 1997). For example, compressing binary mixtures of DPPC with physiological levels of unsaturated PC (1:1) or cholesterol (9:1), promoted significantly enhanced re-spreading of material to the interface as compared to DPPC alone (Notter et al., 1980). SPB and SPC additionally play an important role in facilitating the re-spreading of phospholipids upon changes in alveolar surface area (Baumgart et al., 2010), likely by aiding and stabilizing the transfer of surfactant from the reservoir to the interface (Lopez-Rodriguez and Perez-Gil, 2014).

1.3.4 - Experimental Assessment of Surfactant Biophysical Function

Surfactant is able to rapidly adsorb to the air-liquid interface, reach very low surface tension upon film compression, and re-insert back into the surface layer at the air-liquid interface during film expansion, completing one dynamic compression-expansion cycle. *In vivo*, it is difficult to study the surface tension reducing properties of surfactants produced endogenously within the host. The majority of methods used to study surfactant biophysical function *in vivo* involve removal or administration of surfactant from the lungs and observing the subsequent impact on gas exchange or pulmonary compliance rather than precise measurements of surface tension achieved in the lung (Zuo et al., 2008). Although certain techniques (microdroplet technique) have been used to directly study surface tensions *in situ* within lung (Schürch et al., 1976), these methods are fairly inconvenient. As such, methods to study surfactant function, and measure surface tension reduction have been made simpler and more direct using *in vitro* techniques, by means of instruments such as Langmuir balances and surfactometers. These devices can replicate the dynamic surface area behaviour of the alveolar hypophase during respiration, allowing for accurate
assessments of surface tension in isolated surfactant films outside of the lung during instances of lateral compression and relaxation.

1.3.4.1 - The Constrained Sessile Drop Surfactometer (CSD)

*In vitro* analysis of surfactant biophysical function may be assessed through the use of a variety of surfactometers, each with their own specific set of advantages and disadvantages (Zuo et al., 2008). To assess isolated surfactant biophysical function in this thesis, a constrained sessile drop surfactometer (CSD) was used (Figure 1.4). The CSD was first developed by Wulf and colleagues to measure the surface tension of polymer melts, and was later modified by Yu and associates to allow for the application of isolated surfactant surface tension assessment (Wulf et al. 1999; Yu et al., 2004). The CSD is an ideal surfactometer for assessment of surfactant biophysical function because of i) the environmentally controlled chamber allowing the experimenter to mimic lung temperature and humidity, ii) the requirement for low surfactant sample volumes (~10µL per replicate), and iii) a razor shape edge on the pedestal preventing surfactant sample leakage, which was common in other surfactometers (Yu et al., 2004). Furthermore, the CSD presents with very few disadvantages within the context of surfactant biophysical assessment (Zuo et al., 2008).

Surfactant adsorption, surface tension reduction upon compression, and re-spreading capabilities may all be assessed on the CSD. The CSD consists of a metal pedestal within an environmentally controlled chamber set at 37°C, connected to a syringe filled with water (representing the alveolar hypophase) via tubing (Fig 1.4A). To begin biophysical assessment, a small volume (~9-10µL) of surfactant sample is deposited on top of the CSD pedestal. The surfactant sample is allowed to equilibrate over several minutes to determine the surface tension during adsorption. To model the respiratory cycle and assess change to surface tension during changes in surface area, the surfactant sample is exposed to dynamic compression-expansion cycles (Fig 1.4B). This is done by exchanging fluid from the surfactant sample drop and the loaded CSD syringe. Within the centre of the pedestal, there is a 1mm pinhole directly in contact with the water filled syringe. The piston
within the syringe may be withdrawn or advanced using a custom designed software (‘The Dropper’) and a computer controlled stepper motor to influence the volume and surface area of the surfactant sample on the pedestal, causing surfactant to compress and expand, respectively. Along the horizontal plane of the pedestal is a monochromatic light source and a microscopic camera, used for capturing images of the illuminated CSD pedestal and sample. During biophysical assessment, images are recorded and later analyzed for surfactant surface tension measurements in conjunction with software Axisymmetric Drop Shape Analysis (ADSA) (Kalantarian et al., 2011). Thus, the CSD allows for in vitro assessment surface tension during adsorption and dynamic cycling.
Figure 1.4 – A schematic of the constrained sessile drop surfactometer (A). Surfactant samples are placed on the surfactant pedestal forming a sessile drop. The volume of the drop is increased and decreased, laterally expanding and compressing the drop respectively (B). Images are rapidly recorded with a microscopic camera for later analysis with ADSA – axisymmetric drop shape analysis. \( \gamma \) = surface tension.
1.3.3 Clinical Significance of Surfactant

The importance of surfactant membrane secretion, maintenance, and biophysical function toward normal respiratory mechanics and aeration of the lung is best illustrated by clinical examples of patients afflicted by neonatal respiratory distress syndrome (RDS), or acute respiratory distress syndrome (ARDS).

RDS is characterized by underdevelopment of the lungs; newborns affected exhibit increased work of breathing, lower pulmonary compliance, collapsed alveoli, and impaired gas exchange. In 1959, Avery and Mead discovered that lung extracts isolated from infants that died of RDS had impaired surfactant surface tension reduction, compared to the lung extracts of infants who died of non-pulmonary related disorders (Avery and Mead, 1959). These findings and subsequent studies would later establish that surfactant deficiency and associated dysfunction was responsible for the poor respiratory function of those afflicted by RDS (Farrell and Avery, 1975; Reynolds et al., 1968). Today, RDS is less of a clinical concern due to advances in surfactant replacement therapy used to treat the lungs of premature infants. Instillation of a single dose of exogenous surfactant preparations in the trachea of an RDS neonate counteracts surfactant insufficiency and increases arterial oxygenation and overall survival (Fugiwara et al., 1980; Smyth et al., 1983; Enhorning et al., 1985). The impact of surfactant deficiency on lung function and subsequent reversal using adequate instillation of exogenous surfactant formulations clearly demonstrates the importance of surfactant within alveolar airspaces toward healthy respiratory function.

ARDS, a disease of the mature lung, is defined by acute lung inflammation and increased pulmonary vascular permeability, preventing aeration of lung tissue. Similar to RDS, surfactants isolated from ARDS patients have impaired function; however it is qualitative biochemical and biophysical surfactant abnormalities which contribute to respiratory failure, rather than sheer surfactant deficiency (Günther et al., 2001). Unfortunately, despite improving patient oxygenation, surfactant replacement therapy has largely been unsuccessful regarding the improvement of patient mortality within the context of ARDS (Günther et al., 2001; Dushianthan et al., 2012). As will become apparent, surfactant alterations during ARDS clearly demonstrate that the precise balance of surfactant constituents is crucial for normal surfactant biophysical function within the lung.
1.4 Acute Respiratory Distress Syndrome and Mechanical Ventilation

1.4.1 – Acute Respiratory Distress Syndrome

ARDS is a pulmonary disorder caused by direct (e.g. trauma or acid aspiration) or indirect (e.g. sepsis or inflammation) pulmonary insults promoting impaired aeration of alveolar tissue. These insults lead to the hallmark characteristic of reduced partial pressure of arterial blood oxygen (Ware and Matthay, 2000). In addition to hypoxemia, ARDS patients also present with bilateral lung infiltrates, and decreased pulmonary compliance. ARDS is sub-classified according to the severity of hypoxemia and ranges from mild ARDS (PaO2: FiO2<300) to severe ARDS (PaO2: FiO2<100) (Ranieri et al., 2012).

The potential causes of ARDS include many noxious insults to the lung, each of which may lead to different intracellular alterations in the lung, making this disorder particularly difficult to treat. Unfortunately, no effective pharmacotherapy currently exists which benefits all patients afflicted by ARDS (Bosma et al., 2010; Del Sorbo et al., 2011). This lapse in effective ARDS patient therapy is reflected by a relatively unchanged mortality of 30-40% (Ware and Matthay, 2000; Phua et al., 2009). Due to the inadequate oxygenation of patients diagnosed with ARDS, supportive mechanical ventilation is used as an essential therapeutic intervention. Mechanical ventilation is the artificial inflation and deflation of the lung to facilitate gas exchange and represents the standard of care for patients with ARDS. However, mechanical ventilation of the pre-injured lung may exacerbate pulmonary injury through several mechanisms.

1.4.2 – Injurious Mechanical Forces on the Lung

Although mechanical ventilation is the primary supportive therapy to ARDS patients, it represents a significant risk toward the development of more severe lung injury. In general, two factors influence the progression of lung injury during mechanical ventilation: 1) alveolar over-distension, and 2) repeated alveolar recruitment and de-recruitment (Tremblay and Slutsky, 2006). Over-distension occurs during inhalation when
excessive VTs flow into the lung, causing deformation of alveolar tissue (Dreyfuss and Saumon, 1998; Dreyfuss et al., 1988). Conversely, alveolar de-recruitment occurs when pressure remaining in the lung at the end of expiration (also called positive end-expiratory pressure, or PEEP) is inadequate to prevent collapse of the alveolus, and subsequent re-opening of the collapsed alveolus during the next inhalation cycle produces shear stress on the alveolar tissue (Del Sorbo et al., 2011).

1.4.3 – Mechanical Ventilation of ARDS Patients.

Mechanical ventilation represents a large risk to ARDS patients; however certain levels of VT and PEEP have been directly related to the generation of excessive forces on alveolar tissue during mechanical ventilation. Traditionally, patients afflicted with ARDS were ventilated with large VTs (10-15mL/kg) exceeding normal resting VTs (~6-7mL/kg) in efforts to promote increased arterial oxygenation and prevent high arterial PaCO₂. Very little change in the way ARDS patients are ventilated was established until the results of a large multi-centered trial by the ARDS Network were published in 2000. This seminal study demonstrated that ARDS patients ventilated with low tidal volumes of 6mL/kg and plateau pressure restriction had significantly lower mortality (22%) and increased number of ventilator-free days as compared to patients ventilated with traditional tidal volumes of 10-12ml/kg (ARDSnet, 2000). Thus, it was recommended that clinicians provide lung protective ventilation strategies using low VT in ARDS patients to improve overall patient survivability and minimize the risk of injury incurred on the ventilator.

In addition to reducing VT during ventilation, there is a multitude of evidence from animal models to suggest maintaining small amounts of pressure in the lung at the end of expiration (PEEP) during mechanical ventilation is protective against alveolar collapse and the development of lung injury, edema, and surfactant alterations that may occur when PEEP is absent (Dreyfuss et al., 1988; Webb and Tierney, 1974; Verbrugge et al., 1998; Tremblay and Slutsky, 2006). Although these models have inferred that the higher levels of PEEP may protect from the development of lung injury, trials in human ARDS populations on the mechanical ventilator have led to inconsistent results (Dasenbrook et al. 2011). For example, in a follow-up study to the ARDSnet study in 2000, it was found that
mechanical ventilation of ARDS patients with lower levels of PEEP (8cmH2O) compared to higher levels (13cmH2O) had no beneficial impact on mortality when patients were ventilated with a given tidal volume (ARDSnet, 2004). It is now suggested that extent of PEEP applied to the lung during mechanical ventilation of ARDS patients should be tailored according to the trans-pulmonary pressure and the individual’s lung mechanics (Fan et al., 2013).

Although these large trials have demonstrated that lung protective ventilation strategies should be applied to ARDS patients, there is still a risk for the development of lung injury. Ventilation of the pre-injured lung with physiologically relevant VTs (6-7mL/kg) may result in regional areas of alveolar over-distension (Tremblay and Slutsky, 2006). For example, in localized areas of injury where the alveoli have collapsed or been flooded with proteinaceous edema (i.e. in ARDS patients), physiological VTs are unevenly distributed to healthy regions of the lung, exposing some alveoli to relatively large VTs (Gattinoni et al., 1986). Furthermore, pre-existing surfactant impairments that may have occurred during ARDS may result in increased susceptibility to alveolar collapse (Taskar et al., 1997), and may promote exertion of a large force on lung tissue during inhalation. Thus within clinical practice, lung injury may be incurred during protective mechanical ventilation of the injured lung.

1.4.4 – Ventilation Induced Lung Injury

It is difficult to discern between injury induced by the ventilator and that of the underlying disease for which mechanical ventilation was initiated due to the similar pathological manifestations of the respiratory system (Fan et al., 2013; Tremblay and Slutsky, 2006). Thus, in order to study the damaging effects of mechanical ventilation in isolation, animal models employing high VTs and 0 cmH2O PEEP (ZEEP) have been developed in which the injury is called ventilation induced lung injury (VILI) (Dreyfuss and Saumon, 1998). In these models, the use of large VTs (>20mL/Kg) promotes excessive alveolar distension, while ZEEP ensures an inadequate pressure upon the end of expiration, promoting alveolar de-recruitment and collapse. These models have promoted similar
physiological findings to patients diagnosed with ARDS and exposed to mechanical ventilation, producing changes to the lung such as protein edema, loss of arterial oxygenation, and altered surfactant composition and function (Webb and Tierney, 1974; Dreyfuss et al., 1988; Dreyfuss and Saumon, 1998; Veldhuizen et al., 2000; Veldhuizen et al., 2002; Tremblay and Slutsky, 2006).

The above observations indicate how parameters set by the respirator (VT, PEEP) during mechanical ventilation may impact the development of injury and subsequent mortality. Considering the requirement for mechanical ventilation in ARDS patients, and the deleterious physiological effects that may occur as a result of ventilation of the pre-injured lung, it is important to understand how mechanical ventilation may contribute to the propagation of lung injury.

1.4.5 – Surfactant Alterations during VILI

It has been shown mechanical ventilation further contributes to alterations and impairments of the endogenous surfactant system resulting in worsening of lung mechanics. Preliminary studies conducted by Greenfield and co-workers initially reported alterations to surfactant function following mechanical ventilation of dog lungs. In this study, lung extracts isolated from dogs ventilated for 2 hours with large inspiratory pressures and tidal volumes, demonstrated significant impairment in surface tension reduction compared to dogs ventilated with physiological tidal volumes when surfactants were compressed on a Wilhelmy balance (Greenfield et al., 1964). Later, in 1974 Webb and Tierney postulated that surfactant dysfunction associated with certain ventilation strategies likely contributed to the progression of lung injury due to alveolar instability (Webb and Tierney, 1974; Tremblay and Slutsky, 2006). Studies have since shed light on the role of surfactant dysfunction in the progression of VILI (Verbrugge et al., 1998; Veldhuizen et al., 1996; Veldhuizen et al., 2000; Veldhuizen et al., 2002; Panda et al., 2004; Maruscak et al., 2008; Vockeroth et al., 2010). While these studies have demonstrated the adverse alterations to surfactant that occur upon secretion into the alveolar hypophase, there have been very few investigations into the impact of ARDS or mechanical ventilation on intracellular surfactants, such as lamellar bodies. As such, the
intracellular impact of alveolar over distension and repeated collapse induced by VILI on surfactant prior to secretion into the airspace remains unknown and will represent a primary focus of a study in this thesis.

Changes to extracellular surfactant have been well-characterized in the lungs of animals exposed to VILI induced through ventilation with high VT and lack of PEEP. These alterations are I) increased conversion of surfactant large aggregates to biophysically inactive small aggregates (Veldhuizen et al., 1996; Ito et al., 1997; Verbrugge et al., 1998; Malloy et al., 2000) II) reduced expression of SPA, SPB, and SPC (Veldhuizen et al., 2000) and III) serum protein leakage into the airspaces interfering with surfactant formation at the air – liquid interface (Veldhuizen et al., 1996; Verbrugge et al., 1998; Malloy et al., 2000; Gunther et al., 2001).

For example, in a rabbit model of VILI using high VT (13-15mL/kg), increased large aggregate surfactant conversion to its biophysically inactive small aggregates was observed compared to rabbits ventilated with low VTs (Veldhuizen et al., 1996). The increased conversion has been demonstrated to be dependent on large changes in alveolar surface area associated with the ventilation strategy (Ito et al., 1997; Gunther et al., 1999). Furthermore, it has been demonstrated that increased conversion of the large aggregate fraction to small aggregates is associated with impairments in surfactant surface tension reduction in vitro (Gunther et al., 1999).

More recently it has been demonstrated that high levels of cholesterol in surfactant films impair its overall biophysical function. Furthermore, increases in cholesterol recovered in surfactant has been described within the context of lung injury induced by VILI (Panda et al., 2004; Maruscak et al., 2008) suggesting that this type of surfactant modification may represent an important mediator of surfactant dysfunction.

1.4.6. Elevated Surfactant Cholesterol

Within the past ten years, there has been a growing amount of evidence to suggest that elevated levels of cholesterol beyond normal physiological levels found in surfactant
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films (>4-10% phospholipid) are detrimental to its biophysical function in vitro. These in vitro studies using exogenous surfactant preparations, such as bovine lipid extract surfactant (BLES) composed of phospholipids and hydrophobic surfactant proteins, have demonstrated adding cholesterol to levels of 10-20% phospholipids (20-40 mol%) alters surfactant such that adsorption, reversible monolayer to multilayer transition, and surface tension reduction during compression are all significantly impaired (Saad et al., 2009; Gunasekara et al., 2008; Gunasekara et al., 2005; Leonenko et al., 2007; Keating et al., 2007).

Elevated levels of cholesterol in surfactant may also represent a mechanism by which surfactant may become impaired in vivo. For example, a previous study conducted in our lab has demonstrated surfactant isolated via lung lavage of VILI exposed rats injured with high VTs (30mL/kg) and ZEEP, had significantly elevated levels of cholesterol associated with surfactant (~13% phospholipid) compared to non-injurious ventilation (~8% phospholipid) (Maruscak et al., 2008). In a follow-up study using the same model of lung injury, it was demonstrated these high quantities of surfactant cholesterol contribute, in part, to the impairments in surfactant surface tension reduction isolated from VILI injured animals (Vockeroth et al., 2010). When surfactant isolated from rats exposed to VILI had free cholesterol removed via chemical sequestration with methyl-beta-cyclodextrin (MβCD), a significant restoration toward the achievement of low surface tension was observed. Furthermore, re-adding cholesterol back to original quantities measured restored the naive biophysical impairment of crude surfactant films (Vockeroth et al., 2010). Together, these studies concluded that this type of surfactant alteration is relevant toward the progression of lung injury in VILI exposed animals.

These experiments were performed in experimental rat models of VILI and as such, it is unknown whether supra-physiological levels of cholesterol represent a broad marker of surfactant inactivation in ARDS or whether these changes are unique to the pathological consequences of VILI. However, several studies have indicated high levels of cholesterol in surfactant may play a role in lung injury in human patients on the mechanical ventilator. For example, ARDS patients receiving supportive mechanical ventilation demonstrated higher levels of cholesterol and other neutral lipids within surfactant large aggregates.
recovered by lavage compared to healthy control subjects (Markart et al., 2007). Furthermore, in a separate study by Qua Hiansen and colleagues, surfactant isolated by lavage from patients undergoing long-term mechanical ventilation for the treatment of major trauma had significantly improved surface tension reduction when surfactant was co-administered with cholesterol sequestering agent MβCD (Qua Hiansen et al., 2015). The results of these studies in humans have suggested that high levels of cholesterol in surfactant may be relevant to mechanical ventilation of patients with ARDS. However, future studies are required to confirm such speculations.

Cholesterol is a natural component of surfactant, which may become elevated following exposure to injurious mechanical ventilation. However, at this time it remains unknown precisely how surfactant cholesterol levels may become augmented in response to VILI or mechanical ventilation. Because the majority of surfactant cholesterol is incorporated from the serum, one possibility is that increased cholesterol substrate may favour increased incorporation into surfactant membranes (McCrae et al., 2008). Furthermore, since the majority of surfactant cholesterol is first incorporated into surfactant during lamellar body production (Hass and Longmore, 1979), it is worthwhile to see how VILI impacts intracellular incorporation of cholesterol into surfactant.

1.5 – Rationale and Hypotheses

The ability of the lungs to successfully facilitate gas exchange is highly dependent on the proper biophysical properties of surfactant. During ARDS, alveolar gas exchange becomes highly impaired and patients require mechanical ventilation. Unfortunately, mechanical ventilation may promote lung injury, causing broad alterations to the pulmonary surfactant system which exacerbate pre-existing lung injury. Recent investigations regarding cholesterol content in extracellular surfactant has led to the conclusions that high surfactant cholesterol represents a distinct mechanism by which surfactant becomes biophysically inactivated in animal models of VILI.

The objective of this thesis is to provide insight into the biophysical impairments associated with surfactant in the lung following exposure to ARDS and mechanical
ventilation. To complete this objective, two related, but independent studies were performed in rats. Firstly, the impact of serum hypercholesterolemia and its downstream consequences on alveolar surfactant cholesterol levels and their contribution toward the development of experimental ARDS and VILI was assessed. Secondly, a novel investigation into the impact of VILI on lamellar body surfactant was undertaken to determine if any surfactant alterations to composition and function may be mediated intracellularly, prior to secretion into the airspaces.

1.5.1 – Objective 1 - Impact of Diet-Induced Serum Hypercholesterolemia

The first objective of this thesis aims at determining if serum hypercholesterolemia is a major risk factor toward the dysfunction of surfactant biophysical function after exposure to lung injury. Despite what is known regarding normal alveolar cholesterol regulation and incorporation into surfactant, the physiological mechanism behind these surfactant cholesterol increases induced over the course of injurious ventilation has yet to be elucidated. Because ~99% of surfactant cholesterol originates from serum lipoproteins (Hass and Longmore, 1979), it was investigated whether serum hypercholesterolemia predisposed to increased cholesterol incorporation into surfactant, due to increased availability. Serum cholesterol overload may potentially contribute toward impairments in AIIE cholesterol handling by promoting the incorporation of cholesterol into surfactant membranes, marring surface tension reduction, and contributing to more severe lung injury.

Samples used in this biophysical study were obtained from a cohort of rats used in a previously completed experiment investigating the physiological consequences and basic compositional changes to surfactant induced by a high cholesterol diet following exposure to various lung insults (Milos et al., 2016). It was hypothesized that rats fed a high cholesterol diet would have impaired surfactant biophysical function compared to rats fed a standard diet. Because this thesis investigates the biophysical function of previously obtained samples, a brief discussion of this initial study and its results are presented below.
Rats were exposed to a standard or acute high cholesterol diet for several weeks and were randomized to receive one of three experimental models of ARDS and associated controls: VILI, acid aspiration, and surfactant depletion. All animals were ventilated following experimental treatment or injury, and lung physiology (arterial oxygenation and peak inspiratory pressure [PIP] - Table 1.1) was assessed to determine whether the diet produced an increased susceptibility to a more severity lung injury. Following ventilation, surfactant was collected by lavage for basic compositional and pool-size analysis (Table 1.2) to determine any impact diet-induced serum hypercholesterolemia may have had.

The physiological impact of a high cholesterol diet on the severity of lung injury is shown in Table 1.1. Within all three separate injury models, there were significant changes in arterial oxygenation and PIP following the initiation of mechanical ventilation as compared to baseline (with the exception of the VILI – 2-hour groups). However, there were no significant differences in any measured physiological outcomes between rats fed either diet. These data conclude that although these experimental animal models induced injury to the host, serum hypercholesterolemia was not a risk factor for the development of more severe lung injury outcomes.
Table 1.1 – Lung physiology of standard and high cholesterol diet fed rats exposed to 3 experimental models of ARDS and associated controls.

<table>
<thead>
<tr>
<th>Time</th>
<th>PaO₂:FiO₂ (mmHg)</th>
<th>PIP (cmH2O)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard</td>
<td>HCD</td>
</tr>
<tr>
<td><strong>Control Ventilation – 2 hours</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>448.8±13.0</td>
<td>486.7±20.1</td>
</tr>
<tr>
<td>120 minutes</td>
<td>466.7±17.1</td>
<td>471.8±16.9</td>
</tr>
<tr>
<td><strong>VILI – 2 hours</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>466.7±15.7</td>
<td>472.4±13.2</td>
</tr>
<tr>
<td>120 minutes</td>
<td>459.0±22.3</td>
<td>392.0±35.4</td>
</tr>
<tr>
<td><strong>VILI – 3 hours</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>454.3±30.5</td>
<td>482.0±11.3</td>
</tr>
<tr>
<td>180 minutes</td>
<td>200.5±121.6$</td>
<td>222.5±82.7$</td>
</tr>
<tr>
<td><strong>Air Aspiration + 2 hours MV</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>482.7±21.7</td>
<td>476.0±32.4</td>
</tr>
<tr>
<td>120 minutes</td>
<td>501.3±12.6</td>
<td>503.0±22.2</td>
</tr>
<tr>
<td><strong>Acid Aspiration + 2 hours MV</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>456.6±20.0</td>
<td>482.3±24.7</td>
</tr>
<tr>
<td>120 minutes</td>
<td>306.4±11.6$</td>
<td>345.4±54.3$</td>
</tr>
<tr>
<td><strong>Surfactant Depletion + 2 hours MV</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>449.4±18.6</td>
<td>499.0±9.5</td>
</tr>
<tr>
<td>120 minutes</td>
<td>247.7±61.6$</td>
<td>221.6±48.7$</td>
</tr>
</tbody>
</table>

# indicates P<0.05 - acid vs air aspiration within diet. § indicates P<0.05 - baseline vs end of ventilation within diet. n=4-9/treatment group. MV – mechanical ventilation.
Analysis of extracellular surfactant phospholipid pools and cholesterol content isolated from rats via lung lavage exposed to the three models of injury are shown in Table 1.2. Overall within each of the three models of ARDS, there were no significant differences in the amount of recovered total surfactant or surfactant large aggregates between rats fed the two diets, concluding that this type of diet did not affect surfactant pool sizes. Furthermore, there was no difference in the amount of cholesterol measured within large aggregates between the two diets within any experimental model. Only rats exposed to VILI for 3 hours had a statistically significant increase (P<0.05) in surfactant cholesterol levels beyond those of naïve animals (data not shown).
**Table 1.2** – Surfactant composition isolated from rats fed a standard and high cholesterol diet and exposed to 3 experimental models of ARDS and associated controls

<table>
<thead>
<tr>
<th>Diet</th>
<th>Total Surfactant (mg/kg BW)</th>
<th>Large Aggregates (mg/kg BW)</th>
<th>Cholesterol (%Large Aggregate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Ventilation – 2 Hours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td>4.63 ± 0.45</td>
<td>3.03 ± 0.30</td>
<td>7.06 ± 0.40</td>
</tr>
<tr>
<td>High Cholesterol</td>
<td>5.09 ± 0.31</td>
<td>2.90 ± 0.32</td>
<td>7.60 ± 1.09</td>
</tr>
<tr>
<td></td>
<td>VILI– 2 Hours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td>7.92 ± 0.63</td>
<td>3.73 ± 0.35</td>
<td>5.90 ± 0.69</td>
</tr>
<tr>
<td>High Cholesterol</td>
<td>7.78 ± 1.74</td>
<td>3.08 ± 0.97</td>
<td>6.99 ± 0.69</td>
</tr>
<tr>
<td></td>
<td>VILI– 3 Hours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td>6.37 ± 0.92</td>
<td>3.27 ± 0.56</td>
<td>10.45 ± 1.26*</td>
</tr>
<tr>
<td>High Cholesterol</td>
<td>6.24 ± 0.87</td>
<td>2.06 ± 0.15</td>
<td>11.98 ± 0.67*</td>
</tr>
<tr>
<td></td>
<td>Air Aspiration + 2 Hours MV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td>4.81 ± 0.81</td>
<td>2.69 ± 0.35</td>
<td>5.00 ± 0.40</td>
</tr>
<tr>
<td>High Cholesterol</td>
<td>4.29 ± 0.38</td>
<td>2.58 ± 0.25</td>
<td>6.18 ± 0.52</td>
</tr>
<tr>
<td></td>
<td>Acid Aspiration + 2 Hours MV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td>5.56 ± 0.61</td>
<td>3.10 ± 0.33</td>
<td>5.52 ± 0.28</td>
</tr>
<tr>
<td>High Cholesterol</td>
<td>4.77 ± 0.42</td>
<td>2.73 ± 0.31</td>
<td>6.06 ± 0.56</td>
</tr>
<tr>
<td></td>
<td>Surfactant Depletion + 2 Hours MV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td>3.06 ± 0.37</td>
<td>1.67 ± 0.23</td>
<td>4.49 ± 1.21</td>
</tr>
<tr>
<td>High Cholesterol</td>
<td>3.61 ± 0.43</td>
<td>1.41 ± 0.21</td>
<td>6.30 ± 0.67</td>
</tr>
</tbody>
</table>

* indicates P<0.05, VILI- 3 hours vs. naïve, non-ventilated rat (data not shown), n=4-9/group
Despite the absence of differences between diets in regards to rat lung physiology, surfactant pool size, and surfactant cholesterol content, analysis comparing surfactant biophysical function in these samples had not yet been assessed. Therefore in this thesis, the general *in vivo* contribution of a high cholesterol diet toward surfactant biophysical function and surface tension reduction *in vitro* was investigated.

*Note: these studies involving pulmonary physiology, surfactant composition, and surfactant biophysical function discussed in the coming chapters, have been consolidated and published as Milos et al., 2016.*

1.5.2 –Objective 2 - Impact of VILI on Lamellar Body Surfactant

The second objective of this thesis focused on biophysical alterations to intracellular lamellar body surfactant following exposure to VILI induced by high VT and ZEEP. There have been extensive investigations into biophysical impairments of extracellular surfactant isolated from lavage following VILI, however it is unknown to what extent biophysical function of surfactant is impaired during intracellular lamellar body synthesis and production within AIIE in response to this type of injury. The understanding into how surfactant impairments are mediated in response to VILI may help to address the negative impact of ventilation in ARDS patients. It was hypothesized that VILI would promote significant alterations to lamellar body phospholipid recovery, composition, and biophysical function compared to those isolated from ventilation controls.
Chapter 2 – Methods

To assess the role of VILI on isolated surfactant biophysical function in this thesis, two independent experiments were performed. In the first experiment, extracellular surfactant was isolated from injured rats fed either a standard or high cholesterol diet to determine the impact of a serum hypercholesterolemia on surfactant biophysical function \textit{in vitro}. Since these samples were obtained from a previous study (Milos et al., 2016), the initially obtained data that is relevant to this thesis is shown in Tables 1.1 and 1.2. Furthermore, a brief description of the methodology used to isolate the samples is presented. In the second experiment, lamellar bodies were isolated from mechanically ventilated rats to determine the impact of VILI on intracellular surfactant’s basic composition and biophysical function \textit{in vitro}.

2.1 Experimental Designs

2.1.1 – Impact of Diet-Induced Serum Hypercholesterolemia

Samples used to assess the impact of a high cholesterol diet on surfactant biophysical function were obtained from a previous experiment. A schematic of the experimental design used in this study is shown in Figure 2.1. In this study, six week old male Sprague-Dawley rats (Charles River, St. Constant, PQ, CAN) were randomized to receive an acute diet of standard chow (~0% cholesterol and cholic acid) or a high cholesterol diet (1.25% cholesterol and 0.5% cholic acid, by mass). Rats were fed these diets for the next 17-20 days, after which serum cholesterol levels were measured. Upon diet completion, it was observed that rats fed a high cholesterol diet had a significant elevation in serum cholesterol levels (8.57±0.6mM) compared to rats fed a standard diet (1.94±0.1mM), P<0.05, n=8-13/diet. Rats (weighing 340-425g) were subsequently grouped into one of three experimental models of ARDS and their respective controls described below: VILI or control ventilation (CV), acid or air –aspiration control, and surfactant depletion (n=4-9/group). Once assigned to a group, each rat was prepared for mechanical ventilation.
Figure 2.1 – Experimental design of the first study investigating the impact of a high cholesterol diet on extracellular surfactant biophysical function following exposure to three independent models of lung injury. Surfactant was assessed in vitro on the constrained sessile drop surfactometer (CSD). * indicates the additional use of 180 minutes of mechanical ventilation.

2.1.2 – Impact of VILI on Lamellar Body Surfactant

An overview of the experimental design investigating the role of VILI on lamellar body composition and biophysical function is presented in Figure 2.2. Male Sprague-Dawley rats age 6 to 10 weeks old and weighing 300-550g were used for this experiment. Rats were acclimatized to the housing facility for at least 72 hours prior to experimentation and were allowed food and water ad libitum. Rats were then randomized to one of three groups (n=6-8) within the experimental model of VILI: non-ventilated controls (NV), control ventilation (CV), and ventilation induced lung injury (VILI). CV and VILI animals were prepared for mechanical ventilation, while NV animals were not ventilated and were immediately prepared for isolation of lamellar bodies. A separate cohort of NV rats (n=3) was used for the image analysis of isolated lamellar body via transmission electron microscopy (TEM).
Figure 2.2 – Experimental design of the second experiment investigating the impact of VILI on isolated lamellar body composition and biophysical function assessed with the constrained sessile drop surfactometer (CSD). A subset of non-ventilated animals had lamellar bodies isolated for visualization via transmission electron microscopy (TEM).

2.2 – Surgical Procedures

All procedures were approved by the Animal Care Committee at the University of Western Ontario in agreement with the Canadian Council of Animal Care.

2.2.1 – Ventilated Rats

For all experiments involving mechanical ventilation, rats were surgically prepared for connection to a rodent ventilator as previously described (Aspros et al., 2010; Vockeroth et al., 2010). Rats receiving mechanical ventilation were first anesthetized using intraperitoneal injections of a ketamine (70-100 mg/kg) and dexmetatomidine or xylazine (1 mg/kg, or 5 mg/kg respectively) mixed in sterile saline. Once successful anesthesia had been achieved as indicated by a lack of toe-pinchester response, rats were given subcutaneous injections of analgesic buprenorphine (0.05-0.1 mg/kg) and local anesthetic bupivacaine.
(0.1 - 0.25mL – 2.5%) at the intended site of incision. The ventral neck area was exposed to allow for catheterization prior to placement on the ventilator. The left and right jugular veins were exposed and catheterized to continuously deliver anesthetic (propofol - 0.5-2.0 mg/h), neuromuscular blocker (cisatracurium), and sterile fluids (heparinized saline). The carotid artery was exposed and catheterized to allow for blood pressure, heart rate, and blood gas measurements, and to allow continuous delivery of fluids (heparinized saline). All fluids were delivered using infusion pumps (Model 22, Harvard Apparatus, St. Laurent, PQ, CAN). Heparinized saline distributed to the veins and arteries was adjusted such that total fluid received by the rat at any given time was 1 mL/100g/hr.

Following catheterization, the trachea of the rat was exposed and a 14-gauge endotracheal tube was inserted and tightly secured. Rats were given a dose of neuromuscular blocker, 100µg cisatracurium, and were simultaneously connected to a volume cycled rodent ventilator (Model 683, Harvard Apparatus). Once animals were placed on the rodent ventilator, they were ventilated with baseline settings: tidal volume (VT) = 8mL/kg, respiratory rate (RR) = 54-58 beats/minute, positive end-expiratory pressure (PEEP) = 5cmH₂O, and fraction of inspired oxygen (FiO₂) = 1.0. Animals were given two breath holds (or sighs) to promote alveolar recruitment and were ventilated at these settings for 15 minutes prior to sampling of baseline arterial blood gas. Rats were further dosed with 100µg cisatracurium, administered every 15 minutes. The temperature of rats over the course of mechanical ventilation was measured with a rectal thermometer and temperature was maintained at 37 °C using heating pads. Blood gas measurements of PaO₂ and PaCO₂ were assessed using a blood-gas analyzer (ABL 500, Radiometer, Copenhagen, DK). Peak inspiratory pressure (PIP) was measured with a pressure monitor (Model Criterion 40, Caradyne, Galway, IRE) connected to the rodent ventilator. To ensure animals did not have pre-existing lung injury, inclusion criteria of arterial oxygenation PaO₂:FiO₂ > 400mmHg was established.
2.2.2 – Non-Ventilated Rats

NV animals were used within the lamellar body experiment only. Animals randomized to NV did not undergo any of the above surgical preparations required for mechanical ventilation and were instead euthanized immediately with an overdose of sodium pentobarbital (110 mg/kg). Whole lungs were collected for isolation of lamellar bodies (see section 2.4.2).

2.3 – Rats Models of Lung Injury

2.3.1 – Ventilation Induced Lung Injury

Both of the experiments described in this thesis utilized the same VILI model to induce lung injury. Rats within each study were exposed to VILI or non-injurious CV. Following baseline measurements, rats randomized to CV were ventilated without alteration of the baseline ventilator settings (VT = 8mL/kg, RR=54-58bpm, PEEP = 5cmH2O, FiO2 = 1.0) for 120 or 180 minutes during which PIP, vital signs, and blood oxygenation measurements were recorded every 15 to 30 minutes. Rats randomized to receive VILI were transferred to a second rodent ventilator with a larger piston (Model 683, Harvard Apparatus) set to deliver an injurious ventilation strategy. The injurious ventilator settings were as follows: VT = 25-30mL/kg, RR=15-18 bpm (beats per minute), PEEP = 0cmH2O, FiO2 = 1.0. These rats were ventilated for 120 or 180 minutes and airway pressure (PIP), vital signs, and blood oxygenation measurements were taken every 15 to 30 minutes.

2.3.2 – Acid Aspiration

Rats fed standard or high cholesterol diets assigned to the acid aspiration model of lung injury followed a protocol similar to that of Aspros and colleagues (Aspros et al., 2010). Following baseline measurements, animals were disconnected from the ventilator and received an intratracheal dose of either 1M HCl (1 mL/kg) or a control air bolus (1
mL/kg). Rats were ventilated for 120 minutes at baseline settings (VT=8mL/kg, RR=54-58 bpm, PEEP= 5 cmH₂O, FiO₂ = 1) during which PIP, vital signs, and blood oxygenation measurements were taken every 15 to 30 minutes.

2.3.3 – Surfactant Depletion

Rats fed a standard or high cholesterol diet randomized to the surfactant depletion model of lung injury underwent procedures similar to those described previously (Bailey et al., 2004). Briefly, rat lungs were repeatedly lavaged with aliquots of 10mL sterile saline to remove the endogenous extracellular surfactant. After meeting the inclusion criteria of PaO₂:FiO₂ < 250, animals were re-attached to the ventilator and ventilated at baseline settings (VT=8mL/kg, RR=54-58 bpm, PEEP= 5 cmH₂O, FiO₂ = 1) for 120 minutes. Airway pressure, vital signs, and blood oxygenation measurements were taken every 15 to 30 minutes.

2.4 - Surfactant Sample Collection

2.4.1 - Lavage Collection and Extracellular Surfactant Processing

Following the duration of ventilation (120 or 180 minutes), standard or high cholesterol diet fed rats were euthanized with pentobarbital (110 mg/kg) via the left jugular catheter and disconnected from the rodent ventilator. Whole lungs were lavaged five times with 10mL of saline via a syringe connected to the endotracheal tube to isolate extracellular surfactant. Each aliquot of 10mL of saline flushed the lung three times. Recovered lavage fluid was pooled and spun at 150 x g to pellet cells and debris, separating them from surfactant. Surfactant was spun at 40,000 x g to produce a pellet containing the large aggregate sub-fraction (Veldhuizen et al., 1993) separate from small aggregates in the supernatant. The large aggregate pellet was resuspended in saline and frozen at -4°C for later analysis.
2.4.2 Isolation of Lamellar Bodies

Following completion of 180 minutes of mechanical ventilation, rats were euthanized with an overdose of sodium pentobarbital (110 mg/kg) via the left jugular catheter and disconnected from the ventilator. Ventilated rats and NV rats had a midline sternotomy performed, and isolation of lamellar bodies from whole lung tissue was performed as described by Sanders and colleagues, with a few minor practical modifications reported in similar studies (Gil and Reiss, 1973; Sanders et al., 1980; Chander et al., 1986). The pulmonary circulation was perfused by flowing 10mL of ice-cold 0.32M sucrose solution from the inferior vena cava out a hole cut in the left ventricle of the heart. All solutions used during experimentation were pre-cooled to 4°C, saline free, contained 0.01M HEPES, and had a pH of 7.4. Lungs were excised out of the chest cavity and briefly rinsed in 1.0M sucrose. Visible large airway tissue was removed from lungs prior to weighing and tissue homogenization.

Lung tissue homogenization was carried out in steps. First, lungs were cut into smaller pieces and chopped lengthwise, widthwise, and diagonally using a tissue chopper (McIlwain Tissue Chopper, Mickle Laboratory Engineering Co. LTD, ENG) set to cut at 0.25 mm slice intervals. Next, tissue was added to 1.0M sucrose solution producing a 10-20% (weight/volume) lung homogenate suspension. Homogenization was carried out using five passes of a serrated, 0.15-0.23 mm clearance pestle in a Potter-Elvehjem homogenizer to promote the release of intracellular contents from lung tissue. The collected homogenate was filtered through 4-ply gauze to remove large cellular debris. Smaller debris was pelleted and removed from the homogenate solution via centrifugation at 500 x g for 15 minutes at 2°C.

Lamellar bodies were isolated and purified from homogenate through the use of discontinuous sucrose gradients. Briefly, 1.0M sucrose tissue homogenate was layered below volumes of 0.32M sucrose and 0.68M sucrose before centrifugation in a SW28 rotor at 64,000 x g for 2 hours at 4°C with no brake in a Beckman Couture Optima L-80 XP ultra-centrifuge. The lamellar body containing interfacial band between 0.32M and 0.68M sucrose was collected. With the aid of a refractometer (ABBE Refractometer, model A300A, Fisher Scientific Co, Waltham, MA, USA), concentrated 1.0M sucrose was added
to the collected interfacial materials to produce a sucrose solution of 0.58M. To account for the lamellar body contribution to refractive index the solution was over-estimated at 0.61M sucrose (Sanders et al., 1980). The lamellar body containing 0.58M sucrose solution was then layered atop of 0.32M sucrose and 0.45M sucrose before being centrifuged in a SW28 rotor at 64,000 x g, for 2 hours at 4°C with no brake in the same ultracentrifuge. Purified lamellar bodies were collected at the 0.45-0.58M sucrose interfacial band (Gil and Reiss, 1973; Sanders et al., 1980). Next, the isolated lamellar body solution was diluted to 0.2M sucrose with the aid of the refractometer, and lamellar bodies were pelleted by centrifuging the collected materials at 40,000 x g for 15 mins at 4°C. The resulting lamellar body pellet was resuspended in 300µL of saline and frozen at -4°C for later analysis.

In the case of lamellar bodies isolated from NV rats for the purposes of TEM visualization, lamellar bodies were collected in 0.2M sucrose and 0.1M sodium cacodylate buffer (pH=7.35) with primary fixatives 1.2% glutaraldehyde and 1.2% formaldehyde (Schmiedl et al., 2005) and were centrifuged at 40,000 x g for 15 minutes at 4°C to produce a pellet.

2.5 - Transmission Electron Microscopy

Recovered lamellar body pellets from NV animals were immediately fixed in sodium cacodylate buffer for 2 hours. Following fixation, samples were washed in buffer, and stained and fixed with 1% osmium tetroxide for 1 hour. Lamellar body phospholipids were stained en-bloc overnight with half saturated uranyl acetate and were then dehydrated using serially increasing acetone to water ratios (Acetone: 20%, 30%, 50%, 70%, 90%, 95%, and 3 x 100%) (Post et al., 1982). Dehydrated samples were embedded in epon-araldite resin and polymerized at 60°C for 3-5 days. After heating, plastic blocks containing sample were trimmed and ultra-thin (70nm) sections were cut using an Ultramicrotome (Reichert-Jung Ultracut E, Leica Microsystems, Wetzlar, DE) and mesh nickel grids. Lastly, samples were post-stained with 2% uranyl acetate for 15 minutes and 2% lead citrate for 2 minutes. Isolated samples were washed and dried before imaging with a Philips CM10 transmission electron microscope at a high voltage between 80-100kV.
2.6 - Surfactant Biochemical Analyses

2.6.1 – Phosphorus Assay

Aliquots of extracellular surfactant large aggregates and lamellar bodies were extracted via the method of Bligh and Dyer, and a modified Duck-Chong Phosphorus assay was used to quantify the phosphorus content (Bligh and Dyer, 1959; Duck-Chong, 1979). Briefly, samples in saline were extracted by adding chloroform and methanol in a ratio of 1:2:1 (sample: chloroform: methanol) after which the samples were vortexed and centrifuged at 500 x g for 15 minutes. The chloroform fraction was recovered and 100 µL of 0.39M Mg(NO₃)₂ was immediately added to the sample before drying with N₂ or O₂ gas over a heated water bath. Dry samples were heated using an electric rack element, and the resultant residue was resuspended in 1M HCl and heated at 90-100 °C for 15 minutes. The resulting inorganic phosphate was added to dye consisting of a 3:1 ratio of 0.3% malachite green and 42% ammonium molybdate (in 3.5M HCl), and 1% Triton-X, to produce an absorbance product. Five minutes were allowed for completion of the reaction, and absorbance was read at 655nm and compared to known standards to calculate phosphorus in each sample. Phospholipid content in each sample was quantified based on phosphorus measurements.

2.6.2 – Cholesterol Assays

The amount of free cholesterol in extracellular large aggregate samples as a percentage of total phospholipids was quantified using an enzymatic colorimetric Free Cholesterol-E assay kit as per the manufacturer’s instructions (Wako Chemicals, Richmond, VA, USA).

Total cholesterol levels (free and esterified cholesterol) in isolated lamellar bodies were quantified using an enzymatic colorimetric Amplex Red cholesterol assay kit, as per manufactures instructions (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, lamellar body sample, cholesterol esterase, cholesterol oxidase, hydrogen peroxidase, and
Amplex red reagent were combined and reacted to produce a fluorescent resorufin product. An incubation period of 1 hour was allowed for the complete liberation of cholesterol esters (Amundson and Zhou, 1999), after which fluorescence was detected using an absorbance spectrum of 570 nm and an emission spectrum of 590 nm.

2.7 Surfactant Biophysical Analysis

2.7.1 – Constrained Sessile Drop Surfactometer (CSD)

Surfactant biophysical function was assessed using a constrained sessile drop surfactometer (CSD) as previously described (Yu et al., 2004). A graphic displaying the overall set up of the CSD surfactometer is presented in Figure 1.3. Distilled water was loaded into a 2.5 mL syringe (Hamilton Syringe, Reno, NV, USA) and connected to a stepping motor (LTA-HS actuator, Newport Corporation, Irvine, CA, USA) connected to a personal computer. The syringe was connected with tubing to a small, hollowed metal platform within an environmentally controlled chamber set at 37°C. In the middle of the platform, a 3mm diameter pedestal with a razor sharp edge and a 1mm pinhole was inserted, in direct contact with the tubing attached to the water within the syringe. Water was then flushed through the tubing from the loaded syringe up out the top of the pedestal pinhole via control of the stepping motor. The use of custom designed software ‘The Dropper’ (developed by Yi Zuo, The University of Hawaii at Manoa, 2014) allowed for experimental control of the stepping motor to promote movement of the syringe piston forward or backwards within the syringe. Aligned within the horizontal plane of this pedestal was a red LED spotlight (Telecentric Backlight Illuminator, Edmund Optics, Barrington, NJ, USA) positioned to illuminate samples atop the pedestal. Planar with the light source and pedestal was a camera (CMOS monochrome camera, Edmund Optics) which functioned to record images of samples. Images of samples were rapidly captured at a rate of 10 frames per second (FPS) and were later analyzed to obtain an accurate measurement of surface tension and surface area using a software program, ADSA (Kalantarian et al., 2013).
2.7.2 – Dynamic Compression-Expansion Cycles

Biophysical activity was assessed by exposing samples to repeated compression-expansion cycles on the CSD. Aliquots of isolated large aggregate and lamellar body samples were centrifuged at 21,000 x g for 15 minutes at 2°C to concentrate samples at 2mg/mL phospholipid in buffer (140mM NaCl, 2.5mM HEPES, and 1.5mM CaCl₂, pH=7.4). Samples were heated at 37°C for at least one hour prior to functional assessment on the CSD. Small glass beads were added to samples to promote mixing of the sample prior to analysis. A drop (9-10uL) of a vortexed sample was deposited on the pedestal within the 37°C chamber at atmospheric humidity. This drop was allowed to adsorb for 120-180 seconds on the pedestal without manipulation. Following adsorption, 20 dynamic compression-expansion cycles were completed at a frequency of 30 cycles per minute to an intended compression of 25-30% original area. One second before the initiation of dynamic cycling, images were recorded at a rate of 10 FPS, during which captured images had surface tension to measure adsorption. Images of the sample were acquired at this rate for the duration of the dynamic cycling. Dynamic compression-expansion cycling occurred as follows: the sample was first laterally compressed via withdrawal of surfactant subphase (distilled water) into the 1mm pinhole by movement of the motor-driven syringe, reducing volume and surface area of the sample drop. Following drop compression, the same volume of hypophase that was removed during compression was inserted back into the sample through the pedestal pinhole allowing the surfactant sample to expand, and the cycle was repeated. Following dynamic compression-expansion, recorded images were analyzed with ADSA to assess surface tension reduction and surface area during the dynamic cycling. One to four technical replicates of each sample were exposed to dynamic compression-expansion cycles, depending on sample availability.

An example of a surfactant surface tension versus surface area isotherm and the associated biophysical parameters relevant to this thesis are shown in Figure 2.3. This figure illustrates the first compression-expansion cycle of clinical surfactant, BLES, at 2mg/mL phospholipid. During the first compression cycle, a surfactant sample at equilibrium is compressed, reducing the surface tension as the area of the sample is compressed. During expansion, the surfactant sample area increases, along with surface
tension. The primary outcome of isolated sample biophysical analysis was minimum surface tension ($\gamma_{\text{min}}$). Of secondary importance were surface tension after 120-180 seconds of adsorption ($\gamma_{\text{adsorption}}$), maximum surface tension at each cycle ($\gamma_{\text{max}}$), overall compression, and compression required to reach minimum surface tension. In all compression-expansion isotherms, the lower part of the curve represents the surface tension achieved during surfactant compression, while the upper portion represents the surface tension upon expansion.

Figure 2.3 – A representative surface tension ($\gamma$) versus surface area (relative area) isotherm of surfactant (BLES) at the first dynamic compression-expansion cycle assessed on the constrained sessile drop surfactometer (CSD). Samples are compressed (---) and expanded (●). Analysis of isotherms yield information on surface tension following adsorption ($\gamma_{\text{adsorption}}$), minimum surface tension ($\gamma_{\text{min}}$), maximum surface tension ($\gamma_{\text{max}}$) as well as maximum compression and compression required to achieve minimum surface tension.
2.8 – Statistical Analysis

Technical replicates of surfactant function assessed on CSD are averaged as an individual data point (except in the presentation of surface tension versus surface area isotherms). Data is expressed as mean ± standard error (SE). All statistics were performed using statistical analysis software GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Probability (p) values of less than 0.05 were considered statistically significant.

Statistical comparisons between surfactant isolated from control and high cholesterol diet rats within the three experimental models of ARDS measured over the course of dynamic compression expansion cycling, were assessed by a two-way repeated measures analysis of variance (ANOVA) with Bonferroni post hoc test. For comparisons between air instilled and acid instilled rats or between CV-2h and VILI-2h rats with respect to diet, a two-way ANOVA with a Bonferroni post hoc test was used. An unpaired, two-way student’s T-test was used to determine significance for comparisons between the two diets within VILI – 3h rats and surfactant depleted rats.

Statistical comparisons between lamellar bodies isolated from VILI and controls over the course of ventilation or dynamic cycling on the CSD were assessed using a two-way repeated measures analysis of variance (ANOVA) with a Bonferroni post hoc test. Comparisons between ventilation strategies with regards to biochemical and individual time point assessments on the CSD were made using a one-way ANOVA with a Tukey post hoc test.
Chapter 3 – Results

3.1 – Impact of Diet-Induced Serum Hypercholesterolemia on Surfactant Function

3.1.1 Ventilation Induced Lung Injury – 2 hours

The biophysical analysis of surfactant isolated from rats fed either a normal or high cholesterol diet and exposed to VILI and CV for 2 hours are shown in Figure 3.1, 3.2, and Table 3.1. Representative surface tension versus surface area isotherms (at cycle 1 and 10) during compression-expansion cycles are shown in Figure 3.1. The isotherms of surfactant obtained from the CV group displayed reduction to low surface tensions upon surfactant compression, achieving minimum surface tensions of near 5-7mN/m (Fig 3.1A). Isotherms of surfactant isolated from rats exposed to 2 hours of VILI and fed a high cholesterol diet (Figure 3.1B), had minimum surface tensions plateau around 12-15mN/m, which was not observed in normal diet group. In general, the amount of compression required to reach minimum surface tensions appeared larger in the high cholesterol diet groups at each ventilation strategy.

Examination of the minimum surface tensions at cycles 1-20 of these samples is shown in Figure 3.2. There were no differences between diets regarding minimum surface tension achieved at any cycle measured within the CV or VILI ventilation strategy (Figure 3.2). Furthermore, there was no statistical difference within each diet comparing minimum surface tension achieved between the two ventilation strategies or between dynamic cycle numbers (Figure 3.2).
Figure 3.1 – Representative surface tension (mN/m) versus surface area (fraction of the initial area) isotherms at dynamic compression-expansion cycles 1 and 10 of surfactant isolated from rats exposed to two hours of CV (A) and VILI (B) fed a standard or high cholesterol diet. Compressions are shown in light points (white-standard diet, grey-high cholesterol diet), while expansions are represented by black points. One technical replicate is shown.
Figure 3.2- Minimum surface tension (mN/m) of isolated surfactant during 20 dynamic compression-expansion cycles from rats fed a standard or high cholesterol diet and subsequently exposed to two hours of either control ventilation (CV) or VILI. No statistical significance, P>0.05, n=5-7/treatment groups.

Further quantification and statistical analyses of the data from all isolated surfactant isotherms is shown in Table 3.1. There was a significant decrease in surface tension achieved following 120-180 seconds of adsorption on the CSD pedestal in surfactant isolated from rats fed a high cholesterol diet, compared with rats fed a standard diet (P<0.05). This was observed in both the CV and VILI ventilation groups. Secondly, all surfactant isolated from rats fed a high cholesterol diet underwent a significantly larger maximum compression (expressed as a percentage of initial area) as compared to the samples isolated from rats fed a standard diet when assessed on the CSD (P<0.05). Thirdly, during sample re-expansion, there was no difference in absolute maximum surface tension achieved between diets during dynamic cycling of surfactant obtained from both
ventilation strategies. However, samples obtained from rats fed a high cholesterol diet exposed to VILI, tended to achieve maximum surface tensions earlier upon re-expansion, producing flatter isotherms compared to standard diet fed rats exposed to VILI (Figure 3.1B). Lastly, comparisons between ventilation strategies within each diet generated no significant differences in any of these measured surfactant biophysical outcomes.

Table 3.1 – Area compression, adsorption, and maximum surface tension of isolated rat surfactant following 2 hours of mechanical ventilation.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Control Ventilation (CV) - 2 Hours</th>
<th>Ventilation Induced Lung Injury (VILI) - 2 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Surface Tension Following Adsorption (mN/m)</td>
<td>Standard</td>
</tr>
<tr>
<td></td>
<td>Surface Tension Following Adsorption (mN/m)</td>
<td>24.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Maximum Compression (Percent original area)</td>
<td>75.68 ± 0.5</td>
</tr>
<tr>
<td>Cycle #1</td>
<td>Area at MST (Percent of original area)</td>
<td>84.0 ± 1.4</td>
</tr>
<tr>
<td>Cycle #10</td>
<td>Maximum Surface Tension (mN/m)</td>
<td>37.65 ± 2.2</td>
</tr>
<tr>
<td>Cycle #10</td>
<td>Area at MST (Percent of original area)</td>
<td>79.6 ± 0.7</td>
</tr>
<tr>
<td>Cycle #10</td>
<td>Maximum Surface Tension (mN/m)</td>
<td>40.93 ± 1.8</td>
</tr>
</tbody>
</table>

* - indicates P<0.05 standard vs. high cholesterol diet. $ - indicates P<0.05 cycles 1 vs cycle 10. n=5-7/treatment group. MST-minimum surface tension.
3.1.2 Ventilation Induced Lung Injury – 3 hours

A subset of rats was ventilated with a prolonged injurious ventilation protocol, extending the duration of VILI to three hours. This extended duration was not assessed in rats exposed to CV. Representative surface tension versus surface area isotherms of surfactant isolated from rats exposed to VILI for 3 hours at dynamic compression-expansion cycles 1 and 10 measured in are shown in Figure 3.3, with quantification of minimum surface tension and other surfactant biophysical parameters shown in Figure 3.4 and Table 3.2, respectively.

All isotherms displayed flattened shapes, exhibiting impaired surface tension reduction beyond 10 mN/m during instances of compression, particularly at the later dynamic cycles (Figure 3.3). Generally, isotherms obtained from surfactant isolated from animals fed either diet displayed the most change in surface tension during the initial compression (first 5-10% area reduction) as indicated by the steepest slope on the compression isotherm, while increasing compression beyond these area reductions caused the slope of the isotherm to become shallower, and low surface tensions were not achieved. When statistically comparing the minimum surface tension achieved by these surfactant samples, it was found that there were no significant differences between surfactant isolated from rats fed either diet (Figure 3.4). Additionally, there was no difference in minimum surface tension achieved over the course of continuous dynamic cycling (Figure 3.4).
Figure 3.3 – Representative surface tension (mN/m) versus surface area (fraction of the initial area) isotherms at dynamic compression-expansion cycles 1 and 10 of surfactant isolated from rats fed a standard or high cholesterol diet and exposed to VILI for 3 hours. Compressions are shown in light points (white-standard diet, grey-high cholesterol diet), while expansions are represented by black points. One technical replicate is shown.
Figure 3.4 - Minimum surface tension achieved (mN/m) by surfactant during 20 dynamic compression-expansion cycles, isolated from rats exposed to a standard or high cholesterol diet and three hours of VILI. No statistical significance, P>0.05, n=6-7/diet.

The surface tension following adsorption, maximum surface tensions, and compressions (as a fraction of the initial area) measured during the biophysical assessment of surfactant obtained from rats fed a standard and high cholesterol diet exposed to VILI for three hours are shown in Table 3.2. Surfactant samples isolated from high cholesterol diet rats had a significantly lower surface tension measured following 120-180 seconds of adsorption upon the CSD pedestal compared to surfactants isolated from rats fed a standard diet (P<0.001). Secondly, much like the 2 hour CV and VILI groups (Table 3.1), samples obtained from rats fed a high cholesterol diet were exposed to increased compression (as a percentage of initial area) during analysis on the CSD as compared to rats fed a standard diet (P<0.001). Additionally, surfactant obtained from rats fed a high cholesterol diet had their minimum surface tensions achieved at larger compression distances for both cycles investigated. Lastly, surfactant samples isolated from rats fed a high cholesterol diet had
significantly reduced maximum surface tensions upon sample expansion as compared to surfactant isolated from rats fed a standard diet. Furthermore, there were no significant differences between any parameter comparing dynamic cycle 1 and 10.

**Table 3.2 – Area compression, adsorption, and maximum surface tension of surfactant isolated from rats exposed to three hours of VILI**

<table>
<thead>
<tr>
<th></th>
<th>Ventilation Induced Lung Injury (VILI) - 3 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diet</td>
</tr>
<tr>
<td></td>
<td>Standard</td>
</tr>
<tr>
<td>Surface Tension Following Adsorption (mN/m)</td>
<td>25.3 ± 0.6</td>
</tr>
<tr>
<td>Maximum Compression (Percent of original area)</td>
<td>76.6 ± 0.4</td>
</tr>
<tr>
<td>Cycle #1</td>
<td>Area at MST (Percent of original area)</td>
</tr>
<tr>
<td></td>
<td>Maximum Surface Tension (mN/m)</td>
</tr>
<tr>
<td>Cycle #10</td>
<td>Area at MST (Percent of original area)</td>
</tr>
<tr>
<td></td>
<td>Maximum Surface Tension (mN/m)</td>
</tr>
</tbody>
</table>

*-indicates P<0.05 - standard versus high cholesterol diet. n=6-7/diet group. MST-minimum surface tension
3.1.3 Acid Aspiration

The second injury model examined in our diet study was acid aspiration. Representative surface tension versus surface area isotherms during dynamic compression-expansion cycles 1 and 10 of surfactant isolated from rats exposed to air and acid aspiration are shown in Figure 3.5, with quantification of minimum surface tension and other surfactant biophysical parameters from all samples assessed in these groups shown in Figure 3.6 and Table 3.3, respectively.

Isotherms of extracellular surfactant isolated from rats exposed to the air control group were similar between the two diets at each respective cycle number (Figure 3.5A). Additionally, isotherms of surfactant isolated from acid exposed rats demonstrated similar isotherms between the two diets (Figure 3.5B).

Minimum surface tensions were not different between rats fed a standard or high cholesterol diet exposed to air aspiration (Figure 3.6). There were several differences however, as acid administered rats exposed to a high cholesterol diet exhibited higher minimum surface tensions during compression at cycle’s #9-13, and 16, as compared to standard diet counterpart rats (Figure 3.6). There was no difference in surfactant minimum surface tension achieved within each diet, regardless of material instilled (Figure 3.6). Furthermore, there was no difference in minimum surface tension achieved over the course of dynamic cycling as compared to cycle #1 in any of the four surfactant samples (Figure 3.6).
Figure 3.5 - Representative surface tension (mN/m) versus surface area (fraction of the initial area) isotherms at dynamic compression-expansion cycles 1 and 10 of surfactant isolated from rats fed a standard or high cholesterol diet, exposed to air aspiration (A) and acid aspiration (B) followed by 120 minutes of non-injurious mechanical ventilation. Compression is shown in light points (white-standard diet, grey-high cholesterol diet), while expansion is represented by black points. One technical replicate is shown.
Figure 3.6- Minimum surface tension (mN/m) of surfactant during 20 dynamic compression-expansion cycles, isolated from rats fed a standard or high cholesterol diet and exposed to air and acid aspiration followed by 2 hours of baseline mechanical ventilation. * indicates P<0.05, high cholesterol diet versus standard diet exposed to acid, n=4-9/treatment group.

The additional outcomes of surfactant biophysical assessment, obtained from rats fed a standard and high cholesterol diet exposed to air or acid aspiration, are shown in Table 3.3. Surfactant isolated from rats fed either diet and exposed to either air or acid aspiration, did not have any significant difference between them regarding surface tension achieved following 120-180 seconds of adsorption on the CSD pedestal. During sample compression on the CSD, all four sample groups achieved very comparable maximum compressions (~76% original area), although there was a significantly reduced compression as percentage of initial area required to achieve minimum surface tension at the first dynamic cycle for surfactant isolated from air instilled hypercholesterolemic rats.
compared to normcholesterolemic counterparts (P<0.05). Overall, there were no differences in surfactant sample compression between acid exposed animals. There was no difference in maximum surface tension achieved between diets regarding surfactant collected from the air instilled animals. However, in animals exposed to acid aspiration, maximum surface tension of surfactant isolated from rats fed a high cholesterol diet was significantly reduced compared to those fed a standard diet at both cycles investigated. Lastly, it should be noted that there were no significant differences between any surfactant biophysical parameters comparing the material instilled within each diet.

Table 3.3 – Area compression ratios, adsorption, and maximum surface tension of surfactant isolated from rats exposed air or acid aspiration.

<table>
<thead>
<tr>
<th></th>
<th>Air Aspiration</th>
<th>Acid Aspiration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diet</strong></td>
<td>Standard</td>
<td>High Cholesterol</td>
</tr>
<tr>
<td><strong>Surface Tension</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Following Adsorption</td>
<td>23.5 ± 0.2</td>
<td>23.0 ± 0.3</td>
</tr>
<tr>
<td><strong>Maximum Compression</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Percent of original area)</td>
<td>75.9 ± 0.4</td>
<td>76.2 ± 0.4</td>
</tr>
<tr>
<td><strong>Area at MST</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Percent of original area)</td>
<td>78.2 ± 0.5</td>
<td><strong>80.2 ± 0.5</strong> *</td>
</tr>
<tr>
<td><strong>Maximum Surface Tension</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mN/m)</td>
<td>36.3 ± 0.6</td>
<td>35.1 ± 1.0</td>
</tr>
<tr>
<td><strong>Area at MST</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Percent of original area)</td>
<td>77.8 ± 0.3</td>
<td>78.8 ± 0.3</td>
</tr>
<tr>
<td><strong>Maximum Surface Tension</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mN/m)</td>
<td>36.5 ± 0.5</td>
<td>34.9 ± 1.2</td>
</tr>
</tbody>
</table>

*-indicates P<0.05 – standard diet versus high cholesterol diet. n=4-9/treatment group.
MST-minimum surface tension
3.1.4 – Surfactant Depletion

The third model for which the biophysical function of isolated extracellular surfactant was measured was that of surfactant depletion. Similar to the previous two injury models, representative surface tension versus surface area isotherms during dynamic cycles 1 and 10 of extracellular surfactant obtained from rats exposed to surfactant depletion and 2 hours of mechanical ventilation are displayed in Figure 3.7, and quantified data is presented in Figure 3.8 and Table 3.4.

Comparing isotherms between diets at each cycle, surfactant isolated from rats fed a standard diet exhibited the tendency to reach very low surface tensions (~5mN/m), while surfactant from rats fed a high cholesterol diet had impaired minimum surface tension, only reaching surface tension of 10-15mN/m at maximal of area compression (Figure 3.7). When this was quantitatively compared, surfactant isolated from rats fed a high cholesterol diet had significantly higher minimum surface tensions achieved at cycles # 4, 8-15, and 17-20, compared to surfactants isolated from rats fed a standard diet (Figure 3.8). There was no statistical difference in surface tension reduction over the course of dynamic cycling as compared to cycle 1 in surfactants obtained from both diets (Figure 3.8).
Figure 3.7 – Representative surface tension (mN/m) versus surface area (fraction of the initial area) isotherms at dynamic compression-expansion cycles 1 and 10 of surfactant isolated from rats fed a standard or high cholesterol diet, exposed to surfactant depletion followed by 120 minutes of mechanical ventilation. Compression is shown in light points (white-standard diet, grey-high cholesterol diet), while expansion is represented by black points. One technical replicate is shown.
Figure 3.8 - Minimum surface tension (mN/m) of isolated surfactant during 20 dynamic compression-expansion cycles from rats exposed to a standard or high cholesterol diet followed by surfactant depletion and mechanical ventilation for 2 hours. *- indicates P<0.05 – standard versus high cholesterol diet, n=8/treatment group.

Within surfactant depleted sample biophysical analysis, it was also found that there was no difference between diets in regards to surfactant surface tension achieved following 120-180 seconds of adsorption on the CSD platform (Table 3.4). Assessment of compression percentage of the original sample area revealed that during dynamic cycling of surfactant from both diets, samples were maximally compressed to the same extent (~74.5% of initial area), and there was no difference in compression required to achieve minimum surface tension. However, at cycle 10, surfactant isolated from rats fed a high cholesterol diet was able to reach minimum surface tensions after less compression compared to those of the standard diet. Maximum surface tensions achieved upon sample re-expansion were not significantly different between diets at any cycle measured.
Table 3.4—Area compression, adsorption, and maximum surface tension of surfactant isolated from rats exposed to surfactant depletion.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Surfactant Depletion</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard</td>
<td>High Cholesterol</td>
</tr>
<tr>
<td><strong>Surface Tension Following Adsorption</strong> (mN/m)</td>
<td>24.15 ± 0.3</td>
<td>24.09 ± 0.2</td>
</tr>
<tr>
<td><strong>Maximum Compression</strong> (Percent of original area)</td>
<td>74.3 ± 0.2</td>
<td>74.7 ± 0.3</td>
</tr>
<tr>
<td>Cycle #1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Area at MST</strong> (Percent of original area)</td>
<td>76.8 ± 0.5</td>
<td>78.2 ± 0.7</td>
</tr>
<tr>
<td><strong>Maximum Surface Tension</strong> (mN/m)</td>
<td>37.56 ± 1.1</td>
<td>35.72 ± 0.8</td>
</tr>
<tr>
<td>Cycle #10</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Area at MST</strong> (Percent of original area)</td>
<td>76.8 ± 0.2</td>
<td><strong>80.5 ± 1.0</strong>*</td>
</tr>
<tr>
<td><strong>Maximum Surface Tension</strong> (mN/m)</td>
<td>37.95 ± 1.1</td>
<td>36.51 ± 0.8</td>
</tr>
</tbody>
</table>

* = indicates P<0.05 standard vs high cholesterol diet, n=8/treatment group MST minimum surface tension
3.2 - Impact of VILI on Lamellar Body Surfactant

The second set of experiments focused specifically on the lamellar body fraction of surfactant in a three-hour model of VILI.

3.2.1 - Physiology during Ventilation

The arterial oxygenation measured throughout the course of three hours of CV and VILI are shown in Figure 3.9. All rats maintained arterial oxygenation at baseline values up until 150 minutes into mechanical ventilation. At this time point, significant reductions in arterial oxygenation were observed in rats exposed to VILI compared to CV. Furthermore, at 150 minutes of ventilation and through to the end of 180 minutes of ventilation, rats exposed to VILI had significantly lower arterial blood PaO₂: FiO₂ as compared to CV rats. Rats exposed to CV did not achieve differences in arterial oxygenation as compared to baseline at any point during ventilation.

Figure 3.9 – Arterial oxygenation (mmHg) measured in rats throughout the course of three hours of control ventilation (CV) or VILI. # - P<0.05 CV vs. VILI, § - P<0.05 baseline vs. time point of VILI rats. n=6/treatment group.
Additional measurements of physiological parameters recorded in rats at baseline and end of mechanical ventilation are shown in Table 3.5. Firstly, none of the physiological parameters measured between CV and VILI rats at baseline were significantly different. Secondly, after three hours of CV or VILI mechanical ventilation, significant increases in peak inspiratory pressure (PIP) and reductions in blood pressure were measured at the end of ventilation as compared to baseline measurements. Comparing the two ventilation strategies, VILI promoted significant increases in PIP as compared to those measured in rats exposed to CV, however no other differences in measured physiology were observed between the ventilation strategies. Lastly, three hours of CV promoted significant increases in heart rate as compared to baseline; a phenomenon not observed in rats exposed to VILI.

Table 3.5 – Physiological measurements of ventilated rats.

<table>
<thead>
<tr>
<th>Physiological Parameter</th>
<th>Control Ventilation (CV)</th>
<th>VILI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>180 minutes</td>
</tr>
<tr>
<td>PIP (cmH\textsubscript{2}O)</td>
<td>10.4 ± 0.4</td>
<td>14.6 ± 0.2\textsuperscript{§}</td>
</tr>
<tr>
<td>PaCO\textsubscript{2} (mmHg)</td>
<td>33.0 ± 1.4</td>
<td>35.8 ± 1.6</td>
</tr>
<tr>
<td>Blood Pressure (cmH\textsubscript{2}O)</td>
<td>173.0 ± 8.6</td>
<td>79.2 ± 5.1\textsuperscript{§}</td>
</tr>
<tr>
<td>Heart Rate (bpm)</td>
<td>243.8 ± 5.0</td>
<td>266.8 ± 23.2\textsuperscript{§}</td>
</tr>
</tbody>
</table>

\textsuperscript{§} - P<0.05 – baseline vs. 180 mins, \textsuperscript{#} - P<0.05 – control ventilation vs. VILI. n=6/treatment group. Bpm – beats per minute,

3.2.2 – Verification of Lamellar Body Isolation

Lamellar bodies were isolated from NV rats to confirm the isolation technique. Representative TEM images of lamellar body samples are shown in Figure 3.10. Images showed isolation of lamellar body structures without the presence of other identifiable cellular structures, such as mitochondria. Lamellar bodies displayed characteristic features of dense osmiophilic folds of phospholipids surrounded by an intact limiting membrane.
3.2.3 – Biochemical Analysis and Quantification

Phospholipid and relative cholesterol (% of phospholipid) measured in isolated lamellar bodies are shown in Figure 3.11. CV rats ventilated for 180 minutes resulted in significant increases in total phospholipid levels recovered in the lamellar bodies (3.11A) following the isolation process as compared to both NV rats and VILI rats. However, there was no difference in recovered lamellar body phospholipid between NV animals and those exposed to VILI. Quantification of total cholesterol (free cholesterol + esterified cholesterol) as a percentage of phospholipid obtained in isolated lamellar bodies (3.11B)
yielded no statistically significant differences in the relative amount of cholesterol composing lamellar body surfactant material following exposure to different ventilation strategies.

Figure 3.11 – Phospholipid (mg/Kg BW) (A) and total cholesterol as a percentage of phospholipids (B) recovered in isolated lamellar bodies from non-ventilated rats, or rats exposed to three hours of CV and VILI. # - indicates $P<0.05$ CV and NV vs. VILI, n=6-8/treatment group.
3.2.4 – Biophysical Analysis

Following isolation and analysis of lamellar body phospholipid and cholesterol content, isolated lamellar body samples had their biophysical properties assessed on the CSD. Representative surface tension versus surface area isotherms of isolated lamellar bodies are shown in Figure 3.12. Whereas these representative isotherms illustrate the overall surfactant surface tension changes during cyclical compression and expansion of the representative samples, the quantification and statistical analysis of minimum surface tension and other biophysical parameters from all lamellar body samples are shown in Figure 3.13 and Table 3.6, respectively.

Lamellar bodies isolated from NV and CV rats displayed low surface tensions upon the first compression (<5mN/m), while rats exposed to VILI had an impaired ability to reduce surface tension at cycle #1, reaching surface tension of 15mN/m (Figure 3.12). Repeated dynamic cycling lowered the minimum surface tension achieved in all lamellar body samples compared to the first dynamic cycle. Statistically comparing the surface tension reduction of lamellar body samples obtained from rats exposed to various ventilation patterns, it was demonstrated that rats exposed to VILI had significantly higher minimum surface tensions achieved compared to associated controls. The impairment associated with VILI lamellar body samples was statistically different at cycles 1-10, and cycles 1-14 and 18 compared to the minimum surface tension of surfactant obtained from CV and NV rats, respectively (Figure 3.13). There were no significant differences in surface tension reduction upon compression of isolated lamellar bodies between NV and CV samples. Comparing minimum surface tensions achieved over the course of dynamic cycling, it was demonstrated that significant reductions in minimum surface tension were achieved as compared to cycle 1 for both NV (cycle 2 and onward) and VILI (cycle 3 and onwards) exposed animals.
Figure 3.12 - Representative surface tension (mN/m) versus surface area (fraction of the initial area) isotherms at dynamic compression-expansion cycles 1 and 10 of lamellar bodies isolated from NV rats or rats exposed to three hours of CV or VILI. Compression is shown in white points, while expansion is represented by black points. One technical replicate is shown.
Figure 3.13 - Minimum surface tension (mN/m) of isolated lamellar bodies during 20 dynamic compression-expansion cycles from NV rats, or rats exposed to 3 hours of control ventilation (CV) or VILI. * indicates $P<0.05$ – NV vs VILI, # indicates $P < 0.05$ - CV vs VILI, $n=6-8$/treatment group.

The additional outcomes of isolated lamellar body biophysical assessment are shown in Table 3.6. Firstly, there were no significant differences between VILI and controls with respect to the surface tension achieved following 120-180 seconds lamellar body sample adsorption on the CSD pedestal. Secondly, all lamellar body samples regardless of origins were compressed the same extent during the biophysical assessment, as indicated by maximum compression at cycle 1. Although there were no differences in area compression required to achieve minimum surface tension between the different ventilation strategies, lamellar bodies isolated from NV rats required significantly less compression to achieve minimum surface tension at cycle 10 as compared to cycle 1.
Thirdly, there were no differences in maximum surface tension between lamellar bodies isolated from rats exposed to VILI or associated controls achieved during dynamic cycles 1 or 10.

Table 3.6– Area compression, adsorption, and maximum surface tension of lamellar bodies isolated from rats exposed to ventilation

<table>
<thead>
<tr>
<th></th>
<th>NV</th>
<th>CV</th>
<th>VILI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Surface Tension Following Adsorption</strong> (mN/m)</td>
<td>23.2 ± 0.3</td>
<td>22.9 ± 0.2</td>
<td>24.1 ± 0.4</td>
</tr>
<tr>
<td><strong>Maximum Compression</strong> (Percent of original area)</td>
<td>73.6 ± 0.6</td>
<td>71.7 ± 0.8</td>
<td>73.0 ± 0.7</td>
</tr>
<tr>
<td><strong>Cycle #1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Area at MST</strong> (Percent of original area)</td>
<td>77.5 ± 0.8</td>
<td>76.5 ± 0.9</td>
<td>76.6 ± 0.8</td>
</tr>
<tr>
<td><strong>Maximum Surface Tension</strong> (mN/m)</td>
<td>34.0 ± 0.8</td>
<td>32.4 ± 0.6</td>
<td>32.3 ± 0.6</td>
</tr>
<tr>
<td><strong>Cycle #10</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Area at MST</strong> (Percent of original area)</td>
<td>81.0 ± 0.6‡</td>
<td>78.7 ± 1.4</td>
<td>78.6 ± 1.4</td>
</tr>
<tr>
<td><strong>Maximum Surface Tension</strong> (mN/m)</td>
<td>34.3 ± 0.4</td>
<td>33.6 ± 0.6</td>
<td>33.8 ± 1.0</td>
</tr>
</tbody>
</table>

‡ indicates P<0.05 – cycle 1 versus cycle 10, n=6-8/treatment group. MST-minimum surface tension
Chapter 4: Discussion and Conclusions

4.1 Overview

During ARDS and associated mechanical ventilation, pathophysiological changes occur in the lung, including alterations to the endogenous pulmonary surfactant system. These surfactant alterations contribute to impairments in biophysical function, most importantly impaired surface tension reduction, contributing toward the progression of lung injury within ARDS patients. The objective of this thesis was to investigate the alterations and impairments of surfactant biophysical function that occur in the context of lung injury, with a specific focus on VILI.

The biophysical analysis of extracellular surfactant isolated from rats fed a standard and high cholesterol diet presented in this thesis were obtained from a previously performed study within our lab (Qua Hainsen, 2013). This study focused on changes in lung physiology and surfactant composition induced by a high cholesterol diet following exposure to three distinct models of lung injury. The results from the initial study and the biophysical analysis of the samples presented in this thesis were combined and recently published (Milos et al., 2016). Therefore, the initial part of this discussion will focus on the combined observations of these studies, leading to the first conclusion that serum hypercholesterolemia does not lead to increased severity of lung injury. Secondly, this discussion will focus in-depth on the analysis of surfactant biophysical function assessed using the CSD. The biophysical assessment of surfactant allows for a comprehensive evaluation of surfactant function in the context of exposure to a high cholesterol diet and lung injury. This leads to the second conclusion that despite the absence of changes in the severity of lung injury, a high cholesterol diet promotes significant alterations to surfactant biophysical function during injury. Finally, a study involving the investigation of lamellar bodies isolated from rat lungs exposed to VILI provides a unique characterization of intracellular surfactant biophysical function following injury. This analysis provides the basis for the third conclusion that VILI induces intracellular changes which impair the biophysical activity of the lamellar body derived surfactant.
4.2 – Diet-Induced Serum Hypercholesterolemia– Combined Studies

It has been demonstrated that elevated levels of cholesterol within surfactant impair its function (Gunasekara et al., 2005) and that this type of biophysical inhibition occurs in VILI (Panda et al., 2004; Vockeroth et al., 2010). However, it was unknown whether high serum cholesterol impacts surfactant cholesterol alterations during lung injury. Therefore, the effect of diet-induced serum hypercholesterolemia on the severity of lung injury was investigated in three independent, clinically relevant rat models of ARDS. Based on the observation that the majority of surfactant cholesterol is derived from the serum, it was hypothesized that a high cholesterol diet would lead to increased severity of lung injury outcomes due to increased incorporation of cholesterol into surfactant membranes, impairing surfactant biophysical function.

The overall findings of these studies demonstrated that despite achieving significantly increased serum cholesterol levels, a high cholesterol diet did not increase the susceptibility to lung injury, as indicated by typical markers of pulmonary function, such as PaO₂:FiO₂ and PIP (see Table 1.1), across all three models lung injury. Furthermore, serum hypercholesterolemia induced by the diet did not affect surfactant pool sizes or cholesterol content recovered in surfactant large aggregates within any model of injury or their associated controls (see Table 1.2). Interestingly considering the lack of differences in physiology and surfactant composition, a high cholesterol diet promoted significant impairments in surface tension reduction within acid aspiration and surfactant depletion models of lung injury compared to rats fed a standard diet. Therefore, in these two models, it appeared a high cholesterol diet had an effect on surfactant surface tension reducing capabilities as assessed on the CSD. However, these impairments in surface tension reduction measured in vitro were not of sufficient magnitude to affect lung function. Therefore, it is concluded that serum hypercholesterolemia is not a significant risk factor for increased severity or susceptibility towards lung injury.
The reported observation of elevated cholesterol content recovered in lavage surfactant of experimental animal models has been largely restricted to studies investigating VILI (Panda et al., 2004; Maruscak et al., 2008; Vockeroth et al., 2010). Thus, it was unknown whether significant increases in surfactant cholesterol occurred in the context of other initiating insults of lung injury. In order to thoroughly investigate the contribution of a high cholesterol diet on surfactant cholesterol content and its subsequent impact on its biophysical function, three distinct animal models of ARDS were utilized. The use of high VT and 0 cmH₂O PEEP was employed to study the effects of alveolar over-distension and repeated collapse (VILI) on surfactant cholesterol content since increases in surfactant associated cholesterol had previously been described in the lavage of animals exposed to this type of injury. Acid aspiration, modelled gastric acid inhalation, allowed for a clinically relevant assessment as to whether high serum cholesterol predisposes a risk toward impairments in surfactant biophysical function. This type of injury occurs due to a chemical burn in the lung and promotes significant inflammation and leakage of contents from the serum into the alveolar airspaces (Aspros et al., 2010; Reiss et al., 2012). Lastly, a model of surfactant depletion combined with mechanical ventilation was utilized to induce lung dysfunction and to allow de novo surfactant secretion (Bailey et al., 2004). Despite the various lung pathologies associated with each model injury, the lack of differences regarding lung physiology and surfactant cholesterol content induced by the diet across all models provides strong evidence to for us to suggest that serum hypercholesterolemia does not influence the development or severity of lung injury.

Upon comparison of cholesterol content recovered in surfactant obtained from lavage of all rat models of ARDS (Table 1.2), it was observed that only rats exposed to three hours of VILI had significantly elevated levels of cholesterol in surfactant compared to naive rats (Milos et al., 2016). Considering other reports also demonstrating this observation, we speculate that high surfactant cholesterol may be a specific biomarker indicative of exposure to VILI. Although investigation into other models of lung injury is required to determine if high surfactant cholesterol is truly specific to the damaging effects of mechanical ventilation, evidence limiting surfactant cholesterol increases to VILI suggest that the changes are likely mediated by altered mechanotransduction signalling.
within the alveolar epithelial cells upon exposure to forces such as over-distension or shear stress during alveolar collapse (Vlahakis and Hubmayr, 2003). Furthermore, a recent preliminary clinical study has suggested that cholesterol mediated surfactant impairment may be relevant in human patients receiving mechanical ventilation (Qua Hiansen et al., 2015). When surfactant isolated from the lavage of severely injured trauma patients undergoing mechanical ventilation had surfactant cholesterol removed using chemical sequestration with MβCD, surfactant’s biophysical function was greatly improved (Qua Hiansen et al., 2015). Although it has not been proven that these cholesterol increases are due to alveolar over-distention and repeated collapse, this study provides evidence to suggest that increased cholesterol in surfactant may play a role in the surfactant dysfunction of these ventilated patients. Thus it is speculated that increases in surfactant cholesterol are sequela of lung injury incurred exclusively during the process of injurious mechanical ventilation.

Finally, it is concluded that elevated cholesterol recovered in lavage surfactant was unlikely to be caused by excessive leakage from the serum. In injury models of acid aspiration and VILI, there was diffuse endothelial-epithelial barrier damage as indicated by high protein content recovered in the lavage of rats fed both a standard and a high cholesterol diet with no significant difference in protein recovered between diets (data not shown). Thus, if cholesterol levels were increasing in lavage due leak from the serum, it would be expected that there would be higher levels of cholesterol recovered in the lavage of both the acid and VILI exposed rats fed a high cholesterol diet compared to the standard diet, which was not observed. Thus, our results indicate to us that elevated cholesterol in surfactant is a consequence of the damaging effects of mechanical ventilation, regardless of diet.

When interpreting the results of this study investigating the alterations to surfactant and lung physiology upon exposure to acute serum hypercholesterolemia, it is important to consider limitations. The acute duration of a high cholesterol diet (17-20 days) used in this study is not reflective of the human condition. Typically in humans, serum hypercholesterolemia is induced through intake of high fat and high cholesterol foods over the span of several years (Civin, 1972). In this study, our short duration high cholesterol
diet was used to avoid induction of confounding cardiovascular and disease co-morbidities. Additionally, the injury models used were of relatively short duration, limited three hours of mechanical ventilation. In reality, ARDS patients require ventilation for several days to weeks before lung injury may be resolved, pending severity of disease (Ranieri et al., 2012). It is possible that a longer duration of exposure to serum hypercholesterolemia or increased duration of mechanical ventilation (with clinically relevant protective ventilation strategies) may have impacted cholesterol incorporation into surfactant differently, however this remains to be investigated.

In summary, our results demonstrate a high cholesterol diet does not impact the progression or severity of lung injury through alterations in surfactant cholesterol content. However, observations from functional biophysical analysis led us to speculate that high serum cholesterol levels may predispose to cholesterol-independent impairments in surfactant biophysical function following exposure to lung injury. Furthermore, increases in surfactant-associated cholesterol were only observed in the VILI model of lung injury following short injury times periods, suggesting increases in surfactant cholesterol may be related to the damaging effects of alveolar over distension and repeated collapse and reopening.

4.3 – Diet-Induced Serum Hypercholesterolemia – Biophysical Studies

Although there were no differences with respect to physiology or surfactant content measured between the two diets, the second component of this investigation was to provide an in-depth biophysical evaluation of surfactants isolated from rats fed these diets. Assessing surfactant biophysical alterations after exposure to a high cholesterol diet would allow us to further assess our hypothesis that serum hypercholesterolemia is a risk factor for increased susceptibility toward the development of more severe lung injury.

The major findings of surfactant biophysical assessment on the CSD were that there are alterations to all three biophysical parameters measured between the two diets in our various injury models. It is important to note that during biophysical assessment, surfactant
samples were generally compressed to the same relative area between the two diets allowing for accurate comparisons between the other biophysical parameters. The exceptions to this were due to technical issues, and in these cases, samples between the two diets were compressed slightly differently. Nevertheless, within each model of lung injury, minimum surface tension achieved during surfactant film compression was impaired when isolated from hosts fed a high cholesterol diet compared to standard diet counterparts. A second general trend regarding surfactant biophysical function from hypercholesterolemic rats compared to standard diet rats, was lower maximum surface tension achieved upon dynamic expansion. Lastly, although only observed in the surfactant isolated from rats exposed to the VILI model of injury, surfactant isolated from rats fed a high cholesterol diet had improved ability to adsorb to the air-liquid interface. These results collectively implicate a high cholesterol diet in modulating surfactant function. It should be appreciated that these alterations in function were observed independently of increased cholesterol incorporation into surfactant membranes across all models of experimental lung injury and diet, inferring a role of unknown contributors toward these surfactant alterations.

The overall finding that serum hypercholesterolemia did not promote increases in surfactant cholesterol is in contrast to a previously published report (McCrae et al., 2008). This study performed by McCrae and colleagues demonstrated one month of feeding adolescent female mice a high cholesterol diet successfully induced increases in surfactant associated cholesterol in the alveolar lavage (12 mol%) compared to a standard diet fed mice (7 mol%). Alternatively, several other studies have investigated the effect of pharmacologically lowering serum cholesterol on surfactant function. When rat serum cholesterol levels were successfully lowered with treatment of an intraperitoneal injection of 1 4-aminopyrazolo[3,4-d] pyrimidine, there was no difference in cholesterol quantities recovered in lamellar body or extracellular surfactant fractions compared to untreated rats with normal serum cholesterol levels (Suzuki and Tabata, 1980; Davidson et al., 1997). Although these two latter studies suggest a role of the AIIE in equilibrating surfactant cholesterol when availability is low, it is possible that differences in experimental design such as method used to alter serum cholesterol, organismal species, and host species age and sex, may have contributed to the observed differences in surfactant cholesterol content induced by altered serum cholesterol levels.
Although a discrepancy exists between various experimental studies in regards to changes to surfactant cholesterol induced by altering dietary cholesterol, our study and studies by McCrae and Davidson collectively suggest altered serum cholesterol content detrimentally impacts surfactant function in rodent animal models. When surfactant function was assessed with a capillary surfactometer designed to assess unrestricted air flow through a capillary tube, surfactants isolated from the lavage of high cholesterol diet mice had impaired patency or an inability to maintain airway flow, compared to surfactant isolated from those fed a standard diet (McCrae et al., 2008). Conversely, surfactant biophysical function from serum hypo-cholesterolemic rats was assessed at 3mg/mL phospholipid using a pulsating bubble surfactometer, and demonstrated impaired reduction to low minimum surface tensions during compression compared to rats with normal serum cholesterol (Davidson et al. 1997). Taken together, the results from these studies suggest an important role of normal serum cholesterol levels in vivo towards the biophysical function of surfactant in vitro, acting independently of actual cholesterol incorporation into surfactant films. Because there are only a limited number of studies investigating surfactant function associated with altered serum cholesterol, additional studies are required to confirm this relationship and determine such a mechanism by which physiologically normal serum cholesterol levels may help maintain proper surfactant biophysical function.

Although a diet high in cholesterol appeared to impact surfactant’s biophysical behaviour, there were no differences measured in regards to known mediators of surfactant dysfunction between the two diets. Therefore, we speculate that intake of a high cholesterol diet may have caused alterations in surfactant phospholipid composition. Indeed, previous studies investigating the impact of higher levels of various dietary lipids on pulmonary surfactant have produced findings demonstrating alterations to phospholipid species recovered in surfactant lavage (Kyriakides et al., 1976; Burnell et al., 1978; Alam and Alam 1984; Wolfe et al., 2002; Murray et al., 2000). For example, rats fed a diet free of essential fatty acids or a diet high in saturated fat, had significantly reduced recovery of DPPC recovered in lavage (Kyriakides et al., 1976; Burnell et al., 1978; Alam and Alam 1984). Furthermore, surfactant from the lungs of these animals fed a diet free of essential fatty acids were associated with significant impairments in minimum surface tension reduction, likely related to reduced DPPC content (Burnell et al., 1978). Other studies investigating
the role of a diet high in fatty acids such as linoleate or unsaturated fish oils, have demonstrated AIIE cells may favour the incorporation of fatty acids that are abundant in the blood serum into surfactant phospholipid films (Murray et al., 2000; Wolfe et al., 2002). The tendency to favour incorporation of unsaturated fatty acids into surfactant phospholipids may result in reduced saturated surfactant composition, altering fluidity and diminishing the capacity for surfactant to reduce surface tension to very low values upon compression. In our study, it is speculated that serum hypercholesterolemia may have had an alternate effect beyond simply raising cholesterol in serum, impacting the serum fatty acid profile and altering the relative composition of surfactant phospholipids.

The collective alterations to surfactant biophysical function measured in rats exposed to serum hypercholesterolemia and lung injury provide evidence for us to suggest the diet has induced alterations to surfactant film fluidity. Evidence from previous in vitro studies have demonstrated that increasing amounts of fluidizing components in surfactant phospholipid mixtures (such as unsaturated phospholipids), promotes impaired surface tension reduction upon compression and enhanced adsorption and re-spreading upon film formation (Taneva and Keough, 1997; Lee, 2008; Hawco et al., 1981; Notter et al., 1980). Based on our findings that a high cholesterol diet induced similar biophysical changes across all surfactant samples obtained from three models of lung injury, we suspect this diet may have increased the amounts of unsaturated phospholipids and reduced the amount of DPPC present in surfactant. To help confirm such alterations to fluidity induced by the diet, the phospholipid species present in surfactant obtained from both diets needs to be assessed.

Considering the importance of surface tension reduction for alveolar stability during exhalation, it was notable that impaired surface tension was not correlated with increased severity of lung injury in hypercholesterolemic animals. We provide several possible explanations for these findings. First, the discrepancy may have arisen due to the environment in which surfactant function was assessed in vitro, and the environment in vivo at which surfactant acts to stabilize the lung. In our study, assessment of surfactant biophysical function occurred at relatively low concentrations of 2mg/ml phospholipid. This concentration is much lower than what has been estimated to be found lining the lung
of small mammals, ~30mg/mL in adult rats, and ~120 mg/mL in rabbits (Ohashi et al., 1994; Putz et al., 1994). Moreover, low concentrations of surfactant phospholipid are more susceptible to biophysical impairments by serum proteins or high levels of cholesterol (Zasadzinski et al., 2005; Gunasekara et al., 2005); increasing the phospholipid concentrations improves its interfacial biophysical properties. Second, the impaired minimum surface tension upon compression noticed in the high cholesterol diet groups compared to the control diet group was counterbalanced by the differences in re-spread capabilities as indicated by lower maximum surface tension during sample re-expansion between the two diets. Therefore, it is possible that despite high cholesterol diet surfactant samples not being able to stabilize the lung at low volumes as well as the standard diet group, the surfactant from this group was able to adsorb and re-form a surface active membrane upon expansion more efficiently helping to reduce surface tension during inspiration, and reducing the work of breathing. Finally, it is possible that although the sensitivity of the CSD allowed us to observe statistically significant differences between samples, the magnitude of changes between the two different diets was relatively small and likely not sufficient to be reflected in physiological differences. For example, the differences in minimum surface tension detected in our study were ~5mN/m, whereas in previous studies by Veldhuizen et al., and Vockeroth and colleagues have demonstrated differences on the order of 15-20mN/m to be associated with altered lung physiology (Veldhuizen et al., 1997; Vockeroth et al., 2010).

In summary, our results demonstrate that upon initiation of lung injury and exposure to a high cholesterol diet, there are measurable alterations to surfactant biophysical function at relatively low surfactant concentrations in vitro compared to surfactant isolated from standard diet fed rats. These collective biophysical alterations imply that the diet may have had an effect on surfactant film fluidity. These biophysical alterations were measured independently of amounts of cholesterol recovered in surfactant and therefore it remains unknown which specific biochemical factors may be responsible for the biophysical impairments. These biophysical observations support the findings that a high cholesterol diet leads to significant alterations in surfactant function, however future studies are required to equate these in vitro results to the in vivo setting.
4.4 – Impact of VILI on Lamellar Body Surfactant

The impact of VILI on surfactant’s biophysical function has been extensively examined in studies on the extracellular surfactant fraction obtained via lung lavage (Greenfield et al., 1964; Verbrugge, 1999; Malloy et al., 2000; Veldhuizen et al., 2000; Veldhuizen et al., 2002; Panda et al., 2004; Keating et al., 2007; Bailey et al., 2008; Maruscak et al., 2008; Vockeroth et al., 2010). The role of VILI on the intracellular surfactant (i.e. the lamellar body) has not been previously investigated. It is therefore unknown as to whether intracellular production and packaging of surfactant into lamellar bodies is altered during VILI which could contribute to the observed biophysical impairments in extracellular surfactant. It was hypothesized that there would be alterations to lamellar body surfactant recovery, composition, and biophysical function following exposure to VILI. To test this hypothesis, lamellar bodies were isolated from rat lung tissue homogenate after exposure to three experimental ventilation strategies. The results demonstrated that minimum surface tension reduction of unravelled lamellar bodies isolated from rats exposed to VILI was significantly impaired during early dynamic compression-expansion cycles compared to non-injured controls. Secondly, the amount of lamellar body associated phospholipids recovered from lung tissue was reduced following exposure to VILI. Taken together, it is concluded that VILI causes significant alterations to lamellar bodies. We speculate that these observed changes contribute to impairments in surface tension reduction within the alveolar hypophase, thereby exacerbating lung injury.

To investigate the impact of VILI on intracellular surfactant within AlIEs, lamellar bodies were isolated from rat lung tissue homogenate (Sanders et al., 1980) following exposure to injurious ventilation with high VT and ZEEP based on previously described experiments (Maruscak et al., 2008; Dreyfuss and Saumon, 1998). The model of VILI utilized in this experiment was verified by indicators of pulmonary function, such as reduced $\text{PaO}_2:\text{FiO}_2$ and increased PIP compared to control ventilation strategies, demonstrating detrimental alterations in gas exchange and normal lung mechanics following the ventilation period. Verification of lamellar body isolation technique was confirmed by TEM visualization of lamellar body pellets isolated from non-ventilated rat lungs. Within these images, lamellar bodies displayed dense lamellae structures surrounded
by an intact limiting membrane and were without the presence of contaminating cellular structures such as microsomes or mitochondria, suggesting a relatively pure fraction of lamellar bodies had been isolated (Gil and Reiss, 1973; Post et al. 1981).

One of the objectives of our study, based on previous results of increased cholesterol within endogenous surfactant obtained via lavage was to measure the relative amount of cholesterol in the isolated lamellar bodies (see section 4.2.1; Panda et al., 2004; Maruscam et al., 2008; Vockeroth et al., 2010). Our quantification of isolated rat lamellar body cholesterol content (% phospholipids) was similar to that of previously published studies in rats (Longmore and Hass, 1979; Orgieg and Daniels, 2001) and our analysis did not indicate any difference in isolated lamellar body cholesterol content following exposure to VILI. Given the discord in relative cholesterol levels measured in extracellular and intracellular surfactant following exposure to our rat model of VILI, it is proposed that increases in extracellular surfactant cholesterol following alveolar over-distension and collapse are not mediated through increased trafficking of cholesterol into lamellar bodies prior to secretion into the airspaces.

The most likely explanation for the differences in cholesterol recovered between intracellular and extracellular surfactant within our rat model of VILI arises due to dysregulated exocytosis during large changes in alveolar surface area. Although lamellar body luminal contents are unravelled into the air spaces to form the membrane surface phase, dense phospholipid cores of lamellar bodies may not represent the primary storage site for surfactant cholesterol within the AlIE (Orgeig et al., 2003). For example, injection of radiolabelled cholesterol into the rat tail vein showed selective cholesterol accumulation (3:1) in the lamellar body limiting membrane compared to the dense cores (Orgeig and Daniels, 2001). Upon normal lamellar body exocytosis, the limiting membrane fuses with AlIE plasma membrane and internal bilayers are expelled into the alveolar hypophase, leaving the lamellar body limiting membrane its associated cholesterol at the cell membrane (Ryan et al., 1975; Perez-Gil, 2008). Although cholesterol content in lamellar body limiting membranes and luminal cores were not distinguished between experimental groups in this study, it is speculated that high alveolar stretch may promote dysregulated
exocytosis of lamellar bodies from lung tissue, potentially expelling cholesterol-rich limiting membranes into the alveolar spaces along with other surfactant constituents.

Our assessment of lamellar body biophysical function on the CSD following VILI is novel in two ways. Firstly, this experiment represents the primary investigation of isolated lamellar bodies’ surfactant biophysical function on the CSD, providing an in-depth assessment of adsorption and surface tension reduction during surface area cycling. Although Haller’s group has successfully investigated lamellar body adsorptive capacity and unravelling at the air-liquid interface using immunofluorescent labelled lamellar body surfactant isolated from cell culture (Hobi et al., 2014; Hobi et al., 2016), our study builds on these findings by subjecting lamellar bodies isolated in vivo to dynamic compression-expansion cycles. Secondly, this study is the first assessment of intracellular surfactant composition and biophysical function following exposure to VILI. Several studies have investigated alterations to lamellar body composition following induction of lung injury (Kirkland and Bray, 1988; Kirkland and Bray, 1989), however this is the first to do so from a biophysical perspective. Intact lamellar bodies isolated in vivo are unravelled in vitro by adding Ca²⁺ to surfactant buffer, allowing lamellar body surfactant to form multi-layer structures in the presence of surfactant proteins, as would occur lining the lung extracellular space (Engle et al., 1976). Our results indicate this method of functional assessment is not only feasible for healthy lung lamellar bodies, as indicated by achievement of very low surface tension (in some cases <1mN/m), but also has the sensitivity to detect biophysical alterations in surfactant function that may occur as a result of injury as observed in our samples obtained from rats exposed to VILI.

In our experiments, impairments in surface tension reduction were observed in VILI samples during the early compression-expansion cycles, however continuous cycling improved minimum surface tensions achieved over time. Therefore, lamellar bodies isolated from VILI exposed rats were able to overcome their initial impairment by means of several repeated compressions and expansions, as would occur during continuous respiration. The phenomenon of improved surfactant surface tension reduction during repeated lateral compression is likely explained by interfacial film remodelling, involving
the continuous removal (or ‘squeeze out’) of fluidizing, unsaturated phospholipid and cholesterol from the monolayer into the surfactant multi-layers below at each compression (Zuo and Possmayer, 2007). This process promotes the formation of a DPPC enriched membrane and is aided by surfactant proteins, presumably playing a role in the organization of lipid domains within the surface membrane phase (Leonenko et al., 2007; Lee, 2008; Perez-Gil, 2008). Our data lead us to suggest that upon film formation at air-liquid interface, lamellar body surfactant isolated from NV or CV rats were able to immediately form a DPPC enriched membrane upon the first compression, while lamellar bodies from VILI exposed rats required several compressions to result in a more purified surface membrane.

Given the myriad of studies investigating the biochemical and biophysical impact of VILI on extracellular surfactant isolated from the lung via lavage, potential culprits mediating impaired surface tension reduction of intracellular lamellar bodies may be speculated. Previously, alterations to extracellular surfactant during VILI have been described as: increased conversion of functional surfactant large aggregates to inactive small aggregates (Veldhuizen et al. 2002; Bailey et al., 2008); altered expression of surfactant proteins (Maruscak et al., 2008); serum protein leakage into the airspaces competing with surfactant at the air-liquid interface (Veldhuizen et al., 2000; Maruscak et al., 2008); and increased cholesterol incorporation into surfactant membranes (Panda et al. 2004; Vockeroth et al. 2010), all of which have been associated with altered surfactant function to some degree. However, focusing our assessment on intracellular surfactant allows for the exclusion of several of these factors that may impact extracellular surfactant during VILI. For example, during alveolar over-distension, the pulmonary endothelial-epithelial barrier permeability increases, promoting leakage of serum contents into the lung airspaces (Dreyfuss and Saumon, 1998). Presumably, proteins that have leaked from the serum bypass the lamellar body synthesizing ALIEs, moving directly into the airspace and thus are likely not responsible for the interference of lamellar body surfactant function. Furthermore, lamellar body surfactant material is believed to be composed strictly of large aggregates, and thus conversion to small aggregates that occurs during surface area cycling would not have occurred prior to assessment (Veldhuizen et al., 1996). Finally, our investigation into levels of cholesterol in lamellar bodies yielded no difference between
ventilation groups. Given the absence of differences between known mediators of extracellular surfactant dysfunction, we suspect that intracellular alterations in the production of phospholipids and surfactant proteins likely contribute to these biophysical observed between VILI exposed samples and controls. Evidence for these types of changes has been provided by Veldhuizen et al., who previously demonstrated the mRNA levels of SPB and SPC in the lung tissue are reduced following exposure to VILI, suggesting alterations in the transcription and production of these proteins intracellularly (2000). Investigation into phospholipids species and proteins expression between samples is required to confirm such speculation.

Although the primary finding of this study was altered biophysical quality of surfactant isolated from lamellar bodies, it is suspected that quantitative changes to lamellar bodies produced within the AIIEs also plays a role in the pathogenesis of VILI. Lamellar bodies isolated from VILI exposed lungs had reduced recovery of surfactant phospholipids suggesting reduced lamellar body phospholipid incorporation, increased lamellar body secretion into alveolar airspaces, or both during instances of high VT and repeated alveolar collapse. To further investigate the impact of VILI on lamellar bodies, a parallel study of lamellar bodies in situ within lung tissue was performed. Using a similar experimental design, lungs were fixed and TEM images of AIIE cells were obtained. These images were analyzed for lamellar body morphological characteristics, numbers, and percent area occupied within the AIIE. Results of this study are shown in Figure 4.1 and Figure 4.2, respectively (personal communication with Reza Khazaee, data from unpublished undergraduate thesis).

Representative TEM images of lamellar bodies visualized in situ within AIIE cells are shown in Figure 4.1A. Generally there were no differences between ventilation groups regarding lamellar body morphological characteristics such roundness, diameter, or individual size, as assessed by Image Pro software (data not shown). However, lamellar body staining intensity was significantly reduced in images of AIIEs exposed to VILI (Figure 4.1B), suggesting to us that there was perhaps less surfactant phospholipid incorporation into these lamellar bodies during ventilation with injurious strategies.
Figure 4.1 – Representative TEM images of alveolar type II cells (AIIE) at 10,000X magnification (scale bar represents 2µm), and in situ lamellar bodies at 46,000X magnification (scale bar represents 100nm) imaged within rat lungs exposed to various ventilation strategies (A). The intensity of stained materials (intensity/pixel) visualized in lamellar bodies in situ within the AIIE (B). # indicates P<0.05, CV vs. VILI, n=3 (Adapted from work by Reza Khazaee, unpublished).
Quantification of *in situ* lamellar body number and % occupancy of the AIIE using TEM image analysis is shown in Figure 4.2. Results were largely in accordance with what was observed regarding phospholipid recovery of isolated lamellar bodies from lung tissue (Figure 3.10A), namely, rats exposed to VILI had a significantly reduced number of lamellar bodies visualized within the unit area of AIIEs images compared to CV and NV animals (Figure 4.2A). AIIEs of NV and CV rats had similar absolute numbers of lamellar bodies visualized *in situ*, however there was considerably less recovery of surfactant phospholipid in lamellar bodies isolated from NV rat lung tissue homogenate compared to CV. This discrepancy was resolved by investigation of lamellar body occupancy within the AIIE (Figure 4.2B). Significant differences in lamellar body occupancy of AIIE were observed between all three groups from TEM images obtained *in situ*, matching the trend in recovered lamellar body phospholipid, such that lungs exposed to VILI had significantly less lamellar bodies present in AIIE images compared to both control groups. Thus, overall ventilation with high VT and ZEEP showed reduced lamellar body number, % area of lamellar bodies occupying the AIIE, phospholipid recovery, and phospholipid staining intensity, strongly indicating that this type of ventilation affects surfactant production and assembly within the AIIE.
Figure 4.2 – Number of lamellar bodies (LBs) (A) and relative area of ALII occupied by LBs (B) imaged in lungs isolated from rats following various ventilation strategies. Results are presented as means ± SEM. * indicates P<0.05 – NV versus VILI, # indicates P<0.05 – CV versus VILI. Φ indicates P<0.05 – CV vs NV, n=3/treatment group (Personal communication, Reza Khazaee).
The proposition that ventilation strategy impacts the amount of intracellular lamellar bodies present within the AIIE is supported by additional reports claiming higher levels of stretch promote increased surfactant secretion from the AIIE (Wyszogrodski et al. 1975; Vlahakis and Hubmayr, 2003; Wirtz and Dobbs, 1990). Similar to the supplementary *in situ* data presented, Massaro and Massaro showed diminished lamellar body volume density within AIIE of rat lungs following just one hour of mechanical ventilation with intermittent high VTs (Massaro and Massaro, 1983). The mechanism behind these increases in lamellar body surfactant secretion during high VT ventilation was proposed by Wirtz and Dobbs who demonstrated increasing magnitudes of stretch on rat AIIE cells in culture produced a dose-dependent, transient increase in intracellular calcium, correlated with higher levels of PC exocytosis (Wirtz and Dobbs, 1990). Overstretching of the lung with higher tidal volumes favours lamellar body exocytosis, due to direct stimulation of the AIIE calcium ion channels in response to changes in surface area (Dietl et al., 2010). Given our observations regarding the alterations to lamellar body surfactant recovered from lung homogenate in response to VILI and the fact high VT ventilation promotes lamellar body exocytosis, we speculate lamellar bodies may be secreted into the airspaces before complete phospholipid accumulation or maturation. These immature lamellar bodies may not possess the complete set of surfactant constituents required for biophysical function of maturated lamellar bodies. It is speculated that stimulated secretion of pre-mature lamellar bodies may contribute to the biophysical impairments of extracellular surfactant, worsening lung injury.

Despite the successful isolation of surface-active lamellar bodies from lung tissue homogenate of VILI exposed rats, this study has several limitations. A limitation exists that all previously described methods of lamellar body isolation were from healthy rat lungs and thus it is unknown how or if the ventilation regimes would affect the surfactant recovery during the isolation procedure. Our method for lamellar body isolation was modelled after that of Sanders et al., 1980 and is dependent on the density of intact lamellar body structures. Alterations in lamellar body density may have reduced the fraction of material recovered at the 0.45-0.58M sucrose and thus we cannot be certain this technique
was successful in isolating the complete set of lamellar bodies present in the lung. Counteracting this argument is the morphological data which showed reduced lamellar body content without major morphological differences, correlating well with our phospholipid recovery data. Nevertheless, a more broad characterization of how VILI affects surfactant density and recovery during isolation procedure requires investigation.

It should be noted that there are inherent limitations associated with our model of VILI as it relates to the changes that may occur in humans undergoing mechanical ventilation. Within the clinical setting, human patients that may be subject to mechanical ventilation have pre-existing lung injury or ARDS responsible for impaired arterial oxygenation and necessitating mechanical ventilation in the first place (Del Sorbo et al., 2011; Ranieri et al., 2012). In this study, lung injury is initiated through alveolar over-distension and collapse associated with high tidal volumes (>25mL/kg) and 0 cmH₂O PEEP and as such, represents an unrealistic initiating insult as seen in the clinic setting. Nonetheless, in cases where the lung may be heterogeneously injured or has regions of collapse, physiologically low tidal volumes of 6mL/kg may pose risk toward subjecting healthy lung regions to relatively large localized volumes causing alveolar over-distension (Gattinoni et al., 1986). Although not directly applicable toward the initiation of ventilation in human patients, this model of high tidal volume provides insight into effects of localized over-distension on alveolar tissue responsible for assembling fresh surfactant into lamellar bodies.

In summary, this study has extended knowledge regarding surfactant biophysical impairments associated with VILI through demonstrating alterations to surfactant function prior to secretion in the airspaces. These alterations suggest to us that overstretched and collapse due to high tidal volumes and lack of PEEP, promote alterations to lamellar body surfactant quality and quantity. In addition, we provide evidence that these impairments are independent of cholesterol content, indicating elevated cholesterol in surfactant may be
a relevant inhibitor of surfactant function only in the context of extracellular surfactant and VILI.

### 4.5 – Novelty of the Constrained Sessile Drop Surfactometer

The use of the CSD to assess surfactant biophysical function of both extracellular and lamellar body surfactant was one of the strengths of this thesis. For reasons discussed in section 1.3.4.1 the CSD was the ideal surfactometer for assessing isolated rat surfactant. By exposing surfactant to repeated dynamic compression-expansion cycles on the CSD, changes in lung hypophase surface area during respiration are mimicked, allowing *in vitro* assessment of isolated surfactant biophysical function such as adsorption, minimum surface tension, and re-spreading capabilities. To date, only a handful of studies have published their biophysical findings regarding surfactant obtained from experimental animal models when assessed on the CSD (Ge et al., 2016; Otsubo et al., 2015; Yamashita et al., 2016). The results obtained in this study indicating functional differences between rats fed a standard and high cholesterol diet exposed to three models of ARDS, provide supporting evidence to suggest the CSD as a particularly sensitive instrument capable of detecting alterations to surfactant function assessed *in vitro*.

Intracellular surfactant biophysical function is largely ignored in experimental studies of lung injury, as lavage surfactant has predominately been assessed to gain an understanding of surfactant function at the air-liquid interface. Although extracellular surfactant is directly responsible for the reduction of surface tension across the alveolus, investigation into surfactant alterations as they occur intracellularly can provide valuable insight into the pathology behind surfactant dysfunction, contributing to our understanding of lung diseases. Our study represents the first investigation into the complete biophysical function of isolated lamellar body surfactant. The ability to perform a detailed assessment of adsorption and surface tensions during dynamic cycling on lamellar bodies is largely due to the low sample requirement and delicate control of the compression area of the CSD.
4.6 – Future Directions and Conclusions

In summary, we have characterized biophysical alterations to extracellular surfactant following exposure to serum hypercholesterolemia, lung injury, and mechanical ventilation, as well as impairments in surface tension reduction of lamellar body surfactant isolated following exposure to VILI. Each of the two independent experiments offers several frontiers of which future studies may be explored. Furthermore, the overall conclusions of these experiments have provided a foundation upon which future experiments may be based.

4.5.1 – Impact of Diet-Induced Serum Hypercholesterolemia

Considering our results demonstrating biophysical alterations to surfactant following exposure to a high cholesterol diet, there are several future directions that may be undertaken which may help determine a biochemical culprit responsible for altered surfactant function. An immediate future direction would be to quantify the various phospholipids present in the isolated surfactant samples using liquid chromatography-mass spectrometry. This would provide the relative quantities of PC, PG and other minor phospholipids, as well as the relative fatty acid profiles of each, present in surfactant. Based on biophysical differences measured in our study, it is suspected a high cholesterol diet may have reduced DPPC or increased unsaturated phospholipid content, in some unknown manner altering membrane fluidity. It is also worthwhile to determine if there are any alterations to SPA, SPB, and SPC expression recovered in lavage using western blot and densitometry as these proteins may also impact dynamic surfactant function (Perez-Gil, 2008), and may be altered during various patterns of stretch (Veldhuizen et al., 2002; Vlahakis and Hubmayr, 2003; Maruscak et al., 2008). Assessment of these experimental outcomes would provide a complete quantitative assessment of surfactant film constituents isolated from rat lavage exposed to these diets. Determining the precise composition of these surfactants may help to resolve the functional differences observed between lavage isolates from rats fed a standard and high cholesterol diet and may further infer a risk factor
of serum hypercholesterolemia toward the development of dysfunctional surfactant and potential lung injury.

Although this thesis has presented findings regarding diet-induced changes to surfactant function, the diet used was limited in its relatively short duration. To this end, the role of chronically elevated serum cholesterol towards alterations of surfactant function and lung physiology within animal models and human populations should be assessed. Extending the duration of diet and applying it to the experimental design used in the initial diet study, the impact of long-term serum hypercholesterolemia may be assessed to give a better indication as to whether serum hypercholesterolemia is truly a relevant risk factor for the development of ARDS in humans. In addition, investigating other causes of serum hypercholesterolemia, such as genetic polymorphisms (Bender et al., 2012), may further provide evidence of a risk factor associated with the development of lung injury.

Using three independent models of lung injury, we have demonstrated cholesterol may be a specific biomarker indicative of exposure to injurious ventilation or VILI. Although there is preliminary evidence to suggest high levels of cholesterol may play a role in surfactant alterations and dysfunction in human patients receiving mechanical ventilation (Markart et al., 2007; Qua Hiansen et al., 2015), this remains to be tested. A future study may be directed at determining whether ARDS patients on the ventilator may have alterations to surfactant cholesterol content in vivo over the course of mechanical ventilation. This could be investigated by recruiting newly diagnosed ARDS patients and obtaining a lavage sample around the time supportive mechanical ventilation is initiation. These patients may then be monitored closely over the duration of mechanical ventilation and a second lavage sample may be obtained at a time point following significant exposure to mechanical ventilation. Once lavage samples pre and post mechanical ventilation have been obtained, comparisons may be made to determine if the amount of cholesterol in surfactant isolated by lavage increased over the course of ventilation.
4.5.2 – Impact of VILI on Lamellar Body Surfactant

In this thesis, we provide a novel investigation into lamellar body surfactant biophysical activity isolated from lungs exposed to various ventilation strategies. It was found that crude lamellar bodies isolated from rat lungs after exposure to VILI produced significant impairments in surface tension reduction at early cycles. Although we speculate these alterations occurred due to altered surfactant protein expression or phospholipid species present in lamellar body samples, this has not been investigated. Therefore, an immediate future direction is to measure isolated lamellar body phospholipid species and surfactant protein expression using mass spectroscopy and western blots, respectively. Complete characterization and quantification of surfactant constituents may help determine any pathological alterations to AIIE intracellular signalling during surfactant production and processing in response to overstretched, helping to characterize the detrimental changes that occur to human patients’ lungs during VILI.

Furthermore, because the assessment of lamellar body’s biophysical properties by CSD had not been completed before this study, it is worthwhile to assess the lamellar body biophysical function on the CSD following lipid extraction. Using chloroform and methanol extraction (Bligh and Dyer, 1959), surfactant phospholipids and hydrophobic proteins SPB and SPC may be separated from other hydrophilic proteins or possible contaminants that may have accumulated with the lamellar body samples during the isolation procedure. If similar trends regarding the surface tension reduction of extracted lamellar bodies isolated from VILI exposed animals are observed compared to crude samples, this assessment will further solidify the findings that there are alterations to the biophysical properties of lamellar bodies, removing the possibility of interfering contaminants. In addition, because the majority of SPA is secreted independently of lamellar bodies (Ochs et al., 2002) and removed during the extraction process, the impact of adding SPA (5%w/w PL) to extracted lamellar body samples should be examined. The addition of SPA enhances surfactant biophysical properties in the presence of SPB, contributing to the enrichment of DPPC membranes during compression and improving interfacial surface active properties (Schürch et al. 1992). Thus, it is possible that adding SPA to lamellar bodies isolated from VILI exposed animals may improve minimum
surface tension achieved upon early compressions. This type of differential assessment of surfactant biophysical properties may lead to an understanding of how surfactant impairments during VILI are mediated and may further provide insight into how these impairments may be mitigated.

Alterations to intracellular surfactant within the context of ARDS have largely not been studied. Given the excellent surface tension reducing capabilities of lamellar bodies isolated from naive rat lung tissue homogenate and the measurable impairments following exposure to VILI in vitro on the CSD, we propose future animal studies investigating the impact of ARDS on surfactant function should not neglect to assess lamellar body surfactant biophysical function. Assessment of intracellular surfactant function within lamellar bodies remains a potentially fruitful area of investigation for surfactant research in order to indicate alterations to the way AIIE cells handle surfactant synthesis, packaging, and secretion into the airspaces. This sort of biophysical and compositional assessment may be applied to other causes of ARDS (such as gastric acid aspiration) to determine the various intracellular effects on AIIE surfactant production induced by various models of lung injury. Distinguishing intracellular alterations to surfactant during various insults of injury may help to provide beneficial therapy for patients targeted to their specific cause of the injury.

4.5.3 – General Future Directions

In past experiments using rat models of VILI, supra-physiological levels of cholesterol have been observed in lavage surfactant (Panda et al., 2004; Maruscak et al., 2008; Vockeroth et al., 2010). In this study, we have provided evidence that these changes are likely a consequence of the damaging effects of injurious ventilation on the lung. Despite the consistency of this observation, the physiological and molecular mechanisms behind these pathological changes to cholesterol regulation remain unknown. Specifically, it remains to be determined which forces acting upon the alveoli contribute toward cholesterol dysregulation in the AIIE in our model of VILI. By studying rats grouped to different ventilation strategies with varying degree of alveolar over-distension (dependent
on the level of VT and PEEP applied) and shear forces at each breath (PEEP vs. ZEEP), the mechanism of injury in which cholesterol is increased in surfactant may be elucidated. Determination of the ventilator setting that contributes to these cholesterol mediated surfactant biophysical impairments may help in developing superior protective ventilation strategies to help prevent these adverse consequences and improve the patient mortality associated with mechanical ventilation of ARDS patients.

In addition to the ventilation parameters responsible for elevated surfactant cholesterol, the molecular mechanisms behind the increased incorporation of free cholesterol into surfactant extracellular films within the AIIE are unknown. In this thesis, we have provided evidence to suggest that during VILI 1) excessive cholesterol does not become increased in surfactant due to leakage from the serum, and 2) surfactant cholesterol is not increased intracellularly within lamellar bodies as a whole prior to secretion. Thus to gain a complete understanding of cholesterol regulation and metabolism within the AIIE in response to VILI, other storage localizations for alveolar cholesterol within the AIIE such as the cytoplasm, lamellar body limiting membrane, and lipofibroblasts should be investigated (Orgeig and Daniels, 2001) to gain a broader understanding of how this type of injury impacts the surfactant synthesizing cells. Cholesterol metabolism within the AIIE involves a variety of differentially expressed proteins responsible for cholesterol internalization and export and from the serum. Specific focus for future studies may be determining changes in AIIE expression to proteins like ABCA1, and ABCA3 during various levels of mechanical stress, as these proteins regulate the movement of cholesterol from the serum into the AIIE and further regulate cholesterol during lamellar body formation (Perez-Gil and Weaver, 2010; Bates et al., 2005). The overall lack of understanding of AIIE intracellular cholesterol transport highlights the need to understand the storage and liberation of alveolar intracellular cholesterol. Determining the expression patterns of protein in response to various levels of alveolar stretch may help to provide a molecular basis for the increased trafficking of cholesterol to surfactant during VILI. Once these molecular targets have been established, pharmacological measures may be undertaken to attempt to control the amounts of surfactant cholesterol during mechanical ventilation, helping to reduce surfactant dysfunction.
4.5.4 – Overall Conclusions

In conclusion, the two major findings of this thesis are 1) diet-induced serum hypercholesterolemia during experimental lung injury promotes biophysical alterations to surfactant isolated from rat lavage compared to rats fed standard diet, and 2) VILI promotes significant alterations to lamellar body surfactant biophysical function prior to secretion into the airspaces. These results emphasize the detrimental changes that occur to surfactant function during injurious ventilation and provide novel insight into the functionality of intracellular surfactant before secretion into the airspaces. Furthermore, these experiments attempt to resolve the mechanism by which surfactant cholesterol content is increased following VILI. While they unfortunately do not provide a direct answer, they provide valuable insight into the regulation of cholesterol within the AIIE. Given the effects of altered surfactant composition and function toward impaired respiratory mechanics, it is important to understand how these biophysical changes are mediated within the lung. Ultimately, understanding these changes may help to develop therapeutics which may prevent or mitigate these adverse consequences, and improve overall patient survival.
Reference List:


Animal Ethics Approval

Western

**AUP Number:** 2012-017  
**PI Name:** Veldhuizen, Ruud  
**AUP Title:** The Effect Of Mechanical Ventilation On Surfactant

**Approval Date:** 06/28/2012

**Official Notice of Animal Use Subcommittee (AUS) Approval:** Your new Animal Use Protocol (AUP) entitled "The Effect Of Mechanical Ventilation On Surfactant" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2012-017::1

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura  
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University Council on Animal Care
2012-017::1:

AUP Number: 2012-017
AUP Title: The Effect of Mechanical Ventilation on Surfactant

Yearly Renewal Date: 07/01/2013

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2012-017 has been approved, and will be approved for one year following the above review date.

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REQUIREMENTS/COMMENTS

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AUP Number: 2012-017
AUP Title: The Effect of Mechanical Ventilation on Surfactant

Yearly Renewal Date: 07/01/2014

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AUP Number: 2012-017

AUP TITLE: The Effect of Mechanical Ventilation on Surfactant

Yearly Renewal Dates: 07/01/2015

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2012-017 has been approved, and will be approved for one year following the above review date.

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Health certificates will be required.

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**Abstracts:**


• **Milos S**, Khazaee R, McCaig LA, Nygard K, Yamashita CM, Veldhuizen RAW. (2016). The Effects of Ventilation-Induced Lung Injury on the Intracellular Surfactant System. *Accepted for Poster Presentation at Department of Medicine Research Day 2016*