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Cholesterol-Mediated Dysfunction of Surfactant Effects of Surfactant Protein-A and Diet-Induced Hypercholesterolemia

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

CHOLESTEROL-MEDIATED DYSFUNCTION OF SURFACTANT: EFFECTS OF SURFACTANT PROTEIN-A AND DIET-INDUCED HYPERCHOLESTEROLEMIA

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by

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Abstract

This thesis explored the effects of cholesterol and SP-A on surfactant function *in vitro*, and lung function *in vivo*. In the first experiment, we determined whether SP-A could mitigate cholesterol-mediated surfactant dysfunction. We hypothesized that SP-A can mitigate the surfactant inhibition caused by high cholesterol. In the second experiment, we tested the contribution of diet-induced serum hypercholesterolemia to surfactant composition and the development of lung injury in rats. We hypothesized that serum hypercholesterolemia would increase the amount of cholesterol in surfactant and would cause rats to develop more severe lung injury. Our results indicate that SP-A mitigates cholesterol-mediated surfactant inhibition; however, serum levels of cholesterol do not affect surfactant composition or the severity of lung injury. In conclusion, elevated cholesterol within surfactant represents a mediator of surfactant dysfunction which can impede proper lung function, but SP-A can mitigate this form of inhibition.

Keywords

Acute respiratory distress syndrome, captive bubble surfactometer, cholesterol, diet, hypercholesterolemia , mechanical ventilation, surfactant, surfactant protein-A

Co-Authorship Statement

Chapter 2: SP-A mitigates cholesterol-mediated surfactant inhibition and improves the oxygenation in surfactant depleted rats.

The experiments presented in this chapter were performed in the laboratory of Dr. Veldhuizen, Dr. Lewis and Dr. Yamashita. In experiment #1, human alveolar proteinosis surfactant was collected by Dr. Lewis and Dr. Bosma at University Hospital at the University of Western Ontario. Isolation of large aggregate and SP-A from the human proteinosis lavage was conducted by Li-Juan Yao. Furthermore, Li-Juan Yao was heavily involved in teaching me the techniques to extract the human surfactant via organic solvent extraction and the reconstitution of both cholesterol and SP-A into the human surfactant. Additionally, the techniques for use with the captive bubble surfactometer (CBS) were taught by laboratory technician Li-Juan Yao who also provided assistance with the analysis of the data.

In experiment #2 utilizing surfactant depleted rats, Lynda McCaig was instrumental in providing technical assistance to the surgical, ventilator and surfactant depletion procedure associated with the experiment. Jessie Schoenberg provided assistance in the preparation of surfactant samples including organic solvent extraction and reconstitution with cholesterol and SP-A and was also heavily involved in monitoring the animals during experimentation.

Through both experiments, Dr. Lewis and Dr. Yamashita provided insights into the interpretation of the data collected and thesis preparation. Finally, Dr. Veldhuizen had an active involvement into the development of the experiment, interpretation and statistical analysis of the data and preparation of this section of the thesis as well.

Chapter 3: The effect of diet-induced serum hypercholesterolemia on the development of lung injury in three distinct rat models.

The experiments presented in this chapter were performed in the laboratory of Dr. Veldhuizen, Dr. Lewis and Dr. Yamashita. In the following experiments, the technical assistance of Lynda McCaig with regards to the surgical and ventilator procedure was instrumental to the outcomes of the animal models. In experiments #1 and #2, Brandon Banaschewski provided assistance in the physiological monitoring of animals. In experiment #3, Jesse Lewis provided conducted analysis of lavage samples recovered from the animals.

Through all experiments, Dr. Lewis and Dr. Yamashita provided insights into the interpretation of the data collected and thesis preparation. Finally, Dr. Veldhuizen had an active involvement into the development of the experiment, interpretation and statistical analysis of the data and preparation of this section of the thesis as well.

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Secondly, it goes without saying that the lung lab's laboratory technicians, Lynda McCaig and Li-Juan Yao, have been instrumental not only in the completion of my projects, but my development as a scientist. To Yao: thank you for teaching me about all the techniques that were critical to all of my projects. To Lynda, thank you for not only having the patients to teach me the necessary surgeries for my experiments, but also putting-up with me the entire time we spent on the sixth floor. I would have been a wreck without you guys. Thank you for being great at your jobs and even better friends.

Thirdly, I have had the pleasure of working with stellar students during my tenure at the lung lab, but there are a few that require specific mention. To Brandon Banaschewski: I'm sorry for all of the broken clipboards. And blood. But I'm thankful that we got to work together during your fourth year project and I'm even more thankful that you stayed afterwards to pursue your masters. You're a talented scientist and I consider myself extremely lucky to have you as a friend. To Jesse Schoenberg: as smooth as I thought your fourth year project

was going to go, I was wrong. The best plans of rats and men, right? Nevertheless, all my frustrations during that time would have been exponentially more had you not been right there with me. Thank you for not running out of the sixth floor screaming in terror. Thank you for ordering all of that fast-food with me and most of all thank you for your patience with me. We will golf soon. Lastly, to Valeria Puntorieri: words can't describe how grateful I am to have been able to work with you. Your intelligence and work ethic are things that I've tried to emulate but, admittedly, fallen short of. You make being smart seem so easy. If I can be even half as good in my career as you are at being a scientist then I know I'll be successful.

I must also credit both Jim and Cory. I'm not sure how you guys are able to do everything in your lives. Take care of patients, look after us kids in the lung lab and still maintain a home-life. You're both supermen in my books. Your guidance, suggestions and support throughout the years that I've been a member of the lung lab have been instrumental into all of my projects. Thank you for showing me what hard work looks like.

Finally To my mentor and supervisor, Dr. Ruud Veldhuizen: I'm really sorry completing this thesis was like pulling teeth, but thank you for not killing me. I hope you don't regret your decision for hiring me to work in the lung lab as a summer student, then as a fourth year student and then taking me on as a master's student. These years spent under your tutelage were head and shoulders more difficult than any class during my undergrad years, but also far more rewarding and far, far more enjoyable. I've loved the time I've had in the lung lab

and I don’t think it would have been anywhere near the same experience had I not had you as a mentor. Your insight, kindness, generosity and, above all, your passion for science have been a source of inspiration for me. For all of my accomplishments, all the credit goes to you. Officially, I still owe you two beers. Unofficially, I owe you a lot more.

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List of Abbreviations

%LA	Calculated percentage of total surfactant in the large aggregate form
∆P	Change in Pressure
∆V	Change in Volume
γ	Surface tension
AFM	Atomic Force Microscopy
ARDS	Acute Respiratory Distress Syndrome
ARDSnet	Acute Respiratory Distress Syndrome Network
ANOVA	Analysis of Variance
BAL	Broncho-Alveolar Lavage
BL	Baseline
BLES	Bovine Lipid Extract Surfactant
BP	Blood Pressure
BPM	Breaths Per Minute
BW	Bodyweight
CBS	Captive Bubble Surfactometer
DPPC	Dipalmytoylphosphatidylcholine
FiO_2	Fraction of Inspired Oxygen
HCl	Hydrochloric Acid
HR	Heart Rate
IP	Intraperitoneal

LA	Large Aggregate
Lyso-PC	Lyso-Phosphatidylcholine
MBCD	Methyl-Beta-Cyclodextran
N_2	Nitrogen
nRDS	Neonatal respiratory distress syndrome
$PaCO_2$	Partial Pressure of Arterial Carbon Dioxide
PaO_2	Partial Pressure of Arterial Oxygen
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEEP	Peak end-expiratory pressure
PG	Phosphatidylglycerol
PIP	Peak Inspiratory Pressure
PL	Phospholipid
r	Radius
RR	Respiratory Rate
SA	Small Aggregate
SC	Subcutaneous
SM	Sphyngomyelin
SP-A	Surfactant Protein-A
SP-B	Surfactant Protein-B
SP-C	Surfactant Protein-C

SP-D	Surfactant Protein-D
TS	Total Surfactant
ToFS-SIMS	Time of Flight Secondary Ion Mass Spectometry
VILI	Ventilator Induced Lung Injury
wt	Weight

Chapter 1

1 Introduction and general literature review

1.1 Overview

The primary purpose of the lungs is to facilitate gas exchange between the external and internal environment. Specifically, oxygen from the environment diffuses into systemic circulation while carbon dioxide diffuses out of the systemic circulation (West, 2005). Paramount to the proper function of the lungs is a lipoprotein complex called pulmonary surfactant (Possmayer, 2004). Surfactant is produced endogenously and its primary purpose is to reduce surface tensions within the lung to maintain lung compliance and prevent alveolar collapse. These properties help with the lung's ability to facilitate respiration (West 2005). Impairments in surfactant function have been shown to result in lung dysfunction; one such consequence of lung dysfunction is hypoxemia – a potentially life-threatening condition wherein a patient is unable to maintain adequate levels of blood oxygenation. Hypoxemia is one characteristic of severe lung dysfunctions such as the acute respiratory distress syndrome (ARDS) (Brower *et al.,* 2000; Rubenfeld *et al.,* 2012) – one of the main focuses of this thesis.

ARDS is a severe pulmonary disorder characterized by severe hypoxemia, decreased lung compliance and bilateral chest infiltrates with no indication of cardiac failure (Brower *et*

al., 2000; Rubenfeld *et al.,* 2012). These patients ultimately require the use of mechanical ventilation (MV) as a supportive therapy to maintain oxygenation when lung dysfunction is severe (Lee & Slutsky, 2001). Common amongst patients with ARDS are changes to their pulmonary surfactant system. As noted above, surfactant is a lipoprotein complex found within alveoli and its main purpose is to reduce surface tensions within the lungs. Surfactant is predominantly composed of phospholipids (PL), but also contains neutral lipids, of which cholesterol is the major component, and four associated proteins: surfactant protein (SP)-A, SP-B, SP-C and SP-D (Possmayer, 1988; Veldhuizen *et al.,* 1998; Orgeig & Daniels 2001; Hawgood & Shiffer, 1991; Haagsman & Diemel, 2001). Alterations to surfactant's composition, such as decreased SP-A levels, may render surfactant susceptible to biophysical impairment. Recently, elevated cholesterol within surfactant has also been shown to cause surfactant dysfunction (Davidson *et al.,* 2000; Panda *et al.,* 2004; Gunasekara *et al.,* 2005; Vockeroth *et al.,* 2010); however, it is currently unknown how surfactant cholesterol can become elevated.

Given that surfactant cholesterol predominantly originates from the systemic circulation (Guthmann, 1997; Orgeig & Daniels, 2001), it may be possible that serum hypercholesterolemia affects surfactant cholesterol levels. Due to the growing prevalence of serum hypercholesterolemia in humans, determining whether serum cholesterol levels affects surfactant cholesterol levels may identify a potential patient population at risk of surfactant inhibition and, ultimately, developing ARDS. Furthermore, as mentioned above, SP-A may have important properties in maintaining surfactant function despite inhibitory agents; however, it remains to be determined

whether the presence of SP-A can mitigate the inhibitory effects of elevated surfactant cholesterol levels.

The purpose of this thesis is to determine these two unknown factors regarding surfactant cholesterol levels. Specificaly, chapter two will evaluate whether SP-A is able to mitigate the inhibitory effects of high cholesterol in surfactant. Secondly, chapter 3 will explore how systemic levels of cholesterol impact surfactant composition and the development of lung injury. Lastly, chapter 4 will outline specific future experiments that can be undertaken to further explore the role of high cholesterol in surfactant. The remainder of chapter 1 will discuss general concepts in lung physiology, surfactant biophysics and its role in ARDS and will conclude with the rationales and hypothesis for chapters 2 and 3.

1.2 Lung function and structure

The main function of the lungs is to facilitate gas exchange between the environment and systemic circulation. This process allows for carbon dioxide from circulation to diffuse out of circulation while oxygen from the environment can diffuse into the circulation in order to supply oxygen to tissues and vital organs. The first step in the process of breathing is a process called inspiration (West, 2005). Inspiration is facilitated by active contraction of the diaphragm and the external intercostal muscles of the chest (West,

2005). This process of contraction increases the volume of the chest cavity and causes a decrease in the air pressure within the lungs (West, 2005). At this point, the air pressure within the lungs is negative relative to the pressure of the external environment. This negative pressure promotes air to flow from the area with higher pressure (the external environment) to the areas of lower pressure (the alveoli) (West, 2005).

Air travels through the mouth and nostrils which direct the air through the trachea and flows through the branching pathways of the bronchi and enter smaller, further branching bronchioles. Each of the bronchioles terminate at small, air-filled sacs called alveoli which are the sites of gas exchange within the lungs (West, 2005). Alveoli are able to efficiently facilitate gas exchange because of three important characteristics. First, there exists a very thin layer of cells between the air within the lungs and the blood vessels. Alveolar thickness is very small measuring 2 μm - 200 nm which reduces the distance required for gas to diffuse into circulation (West, 2005). Secondly, in humans, there are approximately 300 million alveoli providing 50 – 100 m^2 of surface area where gas exchange can occur (West, 2005). Lastly, each alveolus is surrounded by a network of capillaries which ensures a constant and close blood supply. This association of capillaries to alveoli is termed the blood gas barrier (BGB) and it is where carbon dioxide can diffuse out from systemic circulation and oxygen from respired air can diffuse into circulation (West, 2005). These three properties of alveoli are important to facilitate efficient gas exchange at the BGB. The rate of diffusion across the BGB can be calculated by Fick's law. In general, Fick's law states that the rate of transfer of a gas is proportional to tissue area and inversely proportional to tissue thickness. Therefore, since

the lungs have a very high surface area and the distance between the epithelium and vasculature is very small, the rate of gas exchange within the lungs is very high.

Besides the alveolar properties which ensure efficient gas exchange between the lungs and circulation, the ability of alveoli to maintain patency, thereby optimizing surface area, throughout respiration is of utmost importance for gas exchange (West, 2005). Alveoli are able to remain open, in part due to i) the negative pleural pressure, which exists between the exterior of the lungs and the chest cavity, and ii) the reduction of surface tension at the air-liquid interface due to the presence of pulmonary surfactant. The intricacies of surface tension and the characteristics of surfactant will be discussed in the following sections.

1.2.1 Surface tension

An aqueous layer exists between the alveolar epithelial cells and the air within alveoli (Possmayer, 2004). This forms an air-liquid interface within the lungs and is the site where surface tension occurs. Within the bulk-phase of the liquid, water molecules have an equal attractive force to other water molecules immediately surrounding resulting in a net attractive-force on that molecule of zero **(Figure 1.1A)**. However, water molecules located immediately at the air-liquid interface have a net attraction into the bulk-phase of the liquid due to the lack of water molecules immediately above them **(Figure 1.1A)**. This attraction downwards into the bulk phase causes high surface tensions and promotes the aggregation of water molecules in order to reduce the overall surface area exposed to

the air. As a consequence of this high surface tension, the tendency within the lungs would be for alveoli to collapse thus inhibiting gas exchange. To reduce this surface tension within the lungs, a lipoprotein complex termed pulmonary surfactant is produced **(Figure 1.1B)** (Possmayer, 1988; Goerke, 1998; Possmayer, 2004). The relationship between surface tensions within alveoli and the importance of surfactant can be explained further using LaPlace's Law **(Figure 1.2)**.

LaPlace's law **(Figure 1.2)** states that the pressure difference across a given sphere is equal to twice the surface tension of the sphere divided by its radius. Based on this law, if the radius of a given sphere is reduced, the pressure gradient increases. If surface tension remained constant through this process the pressure differences across such an alveolus would therefore increase causing alveolar collapse **(Figure 1.2)**. In the normal and healthy lung, however, surface tension is significantly reduced as the radius decreases, via the biophysical properties of pulmonary surfactant.

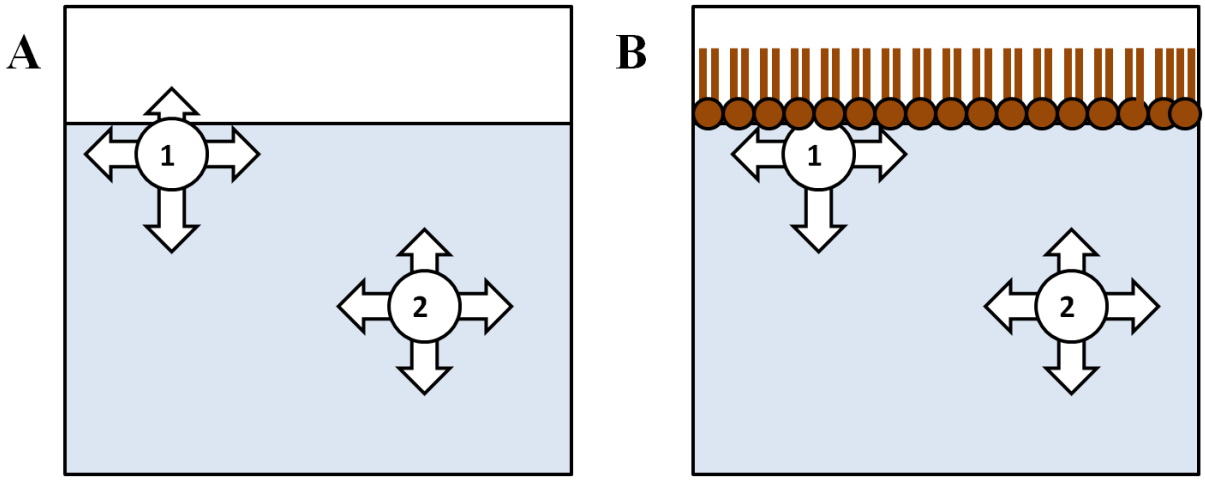

Figure 1.1 Surface tension within the alveoli without and with surfactant present at the air-liquid interface. In (A), a water molecule is shown in bulk of the liquid phase (2) which experiences attractive forces from all directions creating a net-zero force acting on the molecule. Water molecule (1) however has reached the air-liquid interface and experiences a net downwards force active on the molecule into the liquid. In (B), we see how surfactant lipids prevents molecule (1) from reaching the air-liquid interface thereby reducing the net-downwards force and reducing surface tension.

The importance of surface tension and surfactant within the lung is best described in premature neonates afflicted with neonatal respiratory distress syndrome (nRDS). First described by Avery and Mead in 1959, premature infants diagnosed with nRDS presented with hypoxemia and laboured breathing (Avery & Mead, 1959). These symptoms were attributed to the high surface tension present within their lungs due to surfactant deficiency. To treat this condition, clinicians now administer exogenous surfactant to newborns with nRDS before the first breath. As a result of exogenous surfactant therapy, infants with nRDS have significantly improved lung compliance, blood oxygenation and, importantly, decreased overall mortality (Enhorning *et al.,* 1985; Robertson *et al.,* 1989). The effects of surfactant deficiency and subsequent successes of surfactant therapy in this setting emphasize the impact that a functional surfactant system has on proper lung function. In this thesis, conditions that alter the surfactant system within the mature lung will be addressed.

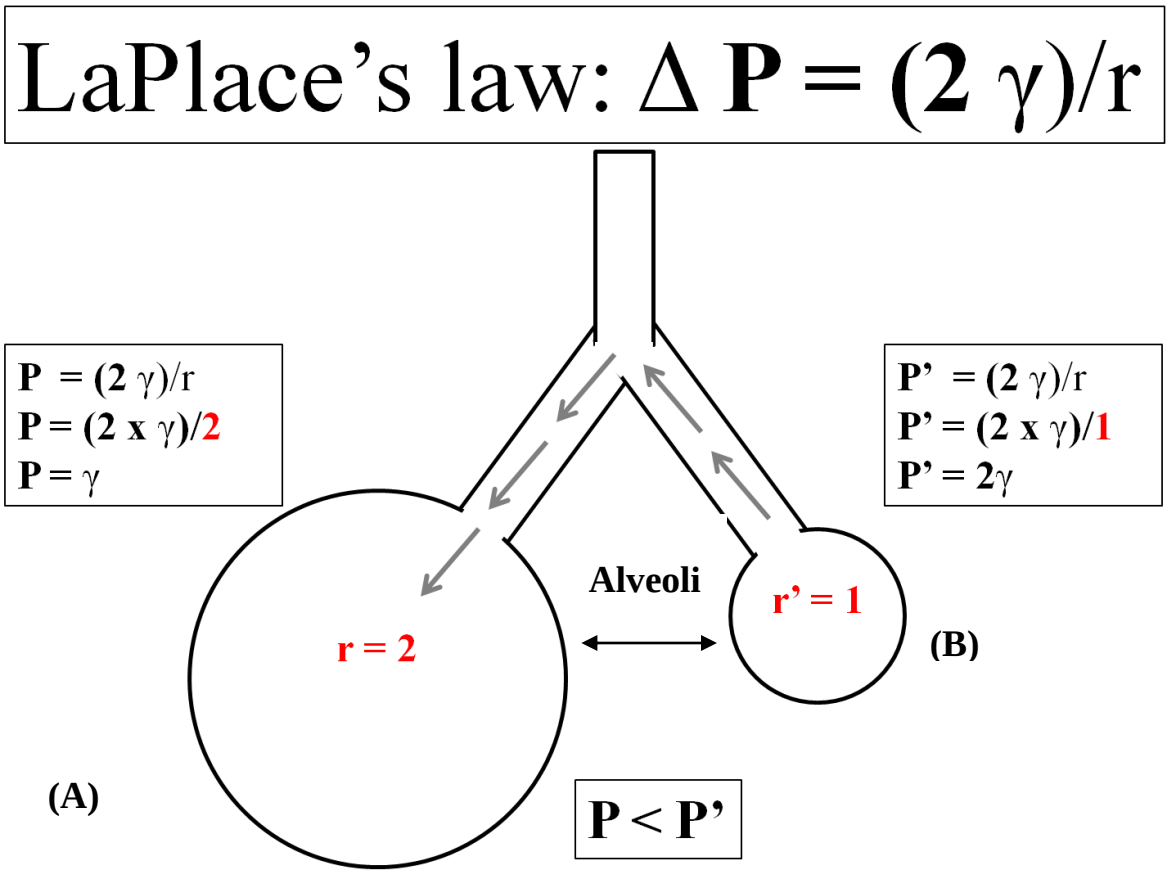

Figure 1.2 Schematic diagram of LaPlace's Law demonstrating alveolar collapse. The difference in pressure across two alveoli can be determined as a relationship between the surface tension present within the alveoli (γ) and the alveoli radius (r). In this scenario the alveoli pictured on the left (A) has a larger radius compared to the smaller alveoli (B). As a result, the pressure within B is greater than in A. The consequence of this pressure gradient is air within alveoli (B) enters alveoli (A) causing alveoli (B) to collapse.

1.2.2 Pulmonary surfactant: composition, metabolism and function

Pulmonary surfactant is an important homeostatic lipoprotein complex formed within alveoli (Possmayer, 2004). Its primary purpose is to reduce the surface tensions within alveoli to near 0 mN/m at the end of exhalation. Proper surfactant composition is crucial to the material's biophysical properties which are necessary for normal lung function. The following sub-sections will address surfactant's composition, metabolism, and biophysical functions. Ramifications of surfactant impairment on lung physiology will then be discussed.

1.2.3 Surfactant composition

Surfactant is a lipoprotein complex primarily composed of phospholipids (PL), neutral lipids and surfactant associated proteins. Surfactant composition is highly conserved amongst homeothermic mammals (Veldhuizen *et al.*, 1998, Orgeig & Daniels, 2001). PL comprises 80-85% of surfactant by weight, of which 80% is phosphatidylcholine (PC), which, in turn is 40-50% in disaturated (dipalmitoylphosphatidylcholine, DPPC) form (Possmayer, 1988; Goerke, 1998; Veldhuizen *et al.*, 1998). DPPC is the key phospholipid responsible for the surface tension reducing capabilities of surfactant. The disaturated nature of DPPC molecules allow them to associate tightly together during periods of surface area compression. This property of DPPC will be further examined in a subsequent section. In addition to DPPC, surfactant also contains phosphtidylglycerol (PG, 10-15% of PL), phosphatiylethanolamine (PE, 3% of PL) and sphingolmyelin (SM) (Veldhuizen *et al.*, 1998). These surfactant PL are thought to enhance the rate of adsorption of surfactant. Lastly, surfactant contains neutral lipids composing

approximately 5-10% wt/wt PL (Orgeig & Daniels, 2001). Within the neutral lipid component of surfactant, free cholesterol represents the predominant species (Guthmann, 1997; Orgeig & Daniels, 2001). The purpose of neutral lipids within surfactant remains uncertain. However, it must be noted that elevated cholesterol levels within surfactant have been shown to cause biophysical dysfunction within the material (Davidson *et al.*, 2000; Panda *et al.*, 2004; Gunasekara *et al.*, 2005; Vockeroth *et al.*, 2010). Thus, cholesterol levels within surfactant may be a relevant consideration in the setting of surfactant alterations in severe lung disorders such as the acute respiratory distress syndrome (ARDS).

In addition to the lipids, there are also four specific surfactant-associated proteins: surfactant protein (SP)-A, -B, -C and –D. These four proteins represent 8-10% of surfactant by weight and can be categorized into two main types: hydrophobic (SP-B and SP-C) and hydrophilic (SP-A and SP-D) (Possmayer, 1988; Goerke, 1998; Haagsman & Diemel, 2001). The smaller hydrophobic SP-B and SP-C molecules are embedded within the acyl-tails of surfactant lipids and have an important role in the biophysical function of surfactant (Weaver & Conkright, 2001). SP-B knockout mouse models have been shown to be embryonic lethal thus emphasizing the protein's important role within the lungs (Clark *et al.*, 1995; Tokieda *et al.*, 1997; Weaver & Conkright, 2001). Furthermore, while SP-C knockout mice are phenotypically normal, *in vitro* assessments of the biophysical function of their surfactant demonstrated surface film instability compared to surfactant from wild-type mice (Glasser *et al.*, 2001). Based on these observations, SP-B

and SP-C are both crucial proteins to the proper biophysical function of surfactant and lung function as well.

With regards to the hydrophilic proteins, SP-A and D are larger, multimeric proteins (Goerke, 1998). SP-A is the most abundant surfactant associated protein and represents approximately 5% of surfactant PL by weight, while SP-D represents approximately 1% of surfactant PL by weight (Goerke, 1998). Both SP-A and SP-D belong to the collectin family of proteins, thus they both have roles in the innate immune defense of the lung (Weaver & Whitsett, 1991). Specifically, SP-A and SP-D have the ability to opsonise bacterial cells for phagocytotic removal by alveolar macrophages (Weaver & Whitsett, 1991). Although this concept of innate immunity provided by SP-A and SP-D is an important feature of the lungs, the topic is beyond the scope of this thesis; however, many excellent reviews on the subject are available (Wright, 1997; McCormack & Whitsett, 2002; Sano & Kuroki, 2005). Biophysically, SP-A has been shown to enhance the rate at which surfactant is able to adsorb at the air-liquid interface (Weaver & Whitsett, 1991). However, Korfhagen and colleagues (1996) showed that SP-A null mice were phenotypically normal with regards to lung physiology. Furthermore, the biophysical function of surfactant from SP-A null mice was not significantly different compared to surfactant containing SP-A. However, at decreased concentrations of DPPC, surfactant from SP-A null mice had impaired ability to reduce surface tensions compared to surfactant with SP-A (Korfhagen *et al.*, 1996). Thus, although SP-A may not be necessary for surfactant function in the normal lung, SP-A may play an important role in maintaining surfactant function when it would otherwise be compromised.

The presence of both surfactant lipids and associated proteins within the surfactant film at the air-liquid interface are crucial for the biophysical function of surfactant. With that said, the surfactant film itself is not a static complex. Surfactant continuously undergoes stages of metabolism in order to maintain a consistent film at the air-liquid interface in order to confer its surface tension reducing capabilities to the lungs.

1.2.4 Surfactant metabolism

Surfactant is synthesized within type-2 alveolar cells **(Figure 1.3)**. After surfactant is synthesized, the material is stored intracellularly within organelles known as lamellar bodies (Mason & Voelker, 1998). A variety of factors can stimulate the release of lamellar bodies from within the alveolar type 2 cell. For instance, adenosine triphosphate may signal the release of surfactant by a protein-kinase A phosphorylation pathway (Chandler & Fisher, 1999; Rooney, 2001). Furthermore, adenosine, itself a neuronaltransmitter, may also stimulate surfactant secretion (Chandler & Fisher, 1999; Rooney, 2001). Lastly, repeated stretching of type-2 cells during respiration also stimulates the fusion of lamellar bodies to the type-2 cellular membrane thereby releasing surfactant into the alveolar space (Mason & Voelker, 1998). Once in the alveolar space, lamellar bodies unwind and form a structure known as tubular myelin (Possmayer, 2004). Tubular myelin contains all of surfactant's lipids and associated proteins with the exception of SP-D. Tubular myelin is able to form a surface-active film at the air-liquid interface which is capable of reducing surface tensions to near 0 mN/m at. This reduction in surface tension is necessary to optimize lung compliance (described via LaPlace's Law) thereby preventing alveolar collapse and decreases the effort required to breathe. It

is believed that tubular myelin also acts as a reservoir from which a surfactant film can be formed. This concept is important during inhalation when the surface area is stretched as surfactant lipids and proteins are able to re-insert themselves into the film in order to maintain functional surfactant film.

Experimentally, extracellular, or alveolar, surfactant can be obtained via whole-lung lavage and can be separated into two distinct surfactant sub-fractions by high speed centrifugation: a large aggregate (LA) pellet (Veldhuizen *et al.,* 1993) and a small aggregate (SA) supernatant (Wright 1990; Brackenbury *et al.,* 2002). The LA pellet, containing tubular myelin, has been shown capable of reducing surface tensions *in vitro* and improve lung physiology when given to surfactant depleted animals *in vivo*. The SA sub-fraction contains small lipid vesicles and SP-D, although, *in vitro* experiments have shown that the SA sub-fraction of surfactant is not as surface active as LA (Yamada *et al*., 1990; Brackenbury et *al*., 2002).

During respiration, repeated changes to alveolar surface area occurs with each breath, causing surfactant to convert from its surface active LA component to the non-surface active SA component. Using intratracheally instilled and radiolabelled LA, Wright (1990) showed that *in vivo*, LA is converted into SA and larger changes in lung surface area corresponded with increased conversion of LA to SA. SA were then taken-up by the type-2 cells within alveoli where it can be recycled back into lamellar bodies or degraded.

Overall, it has been estimated that the process of producing and recycling surfactant takes approximately 7 – 10 hours (Jacobs *et al.,* 1982).

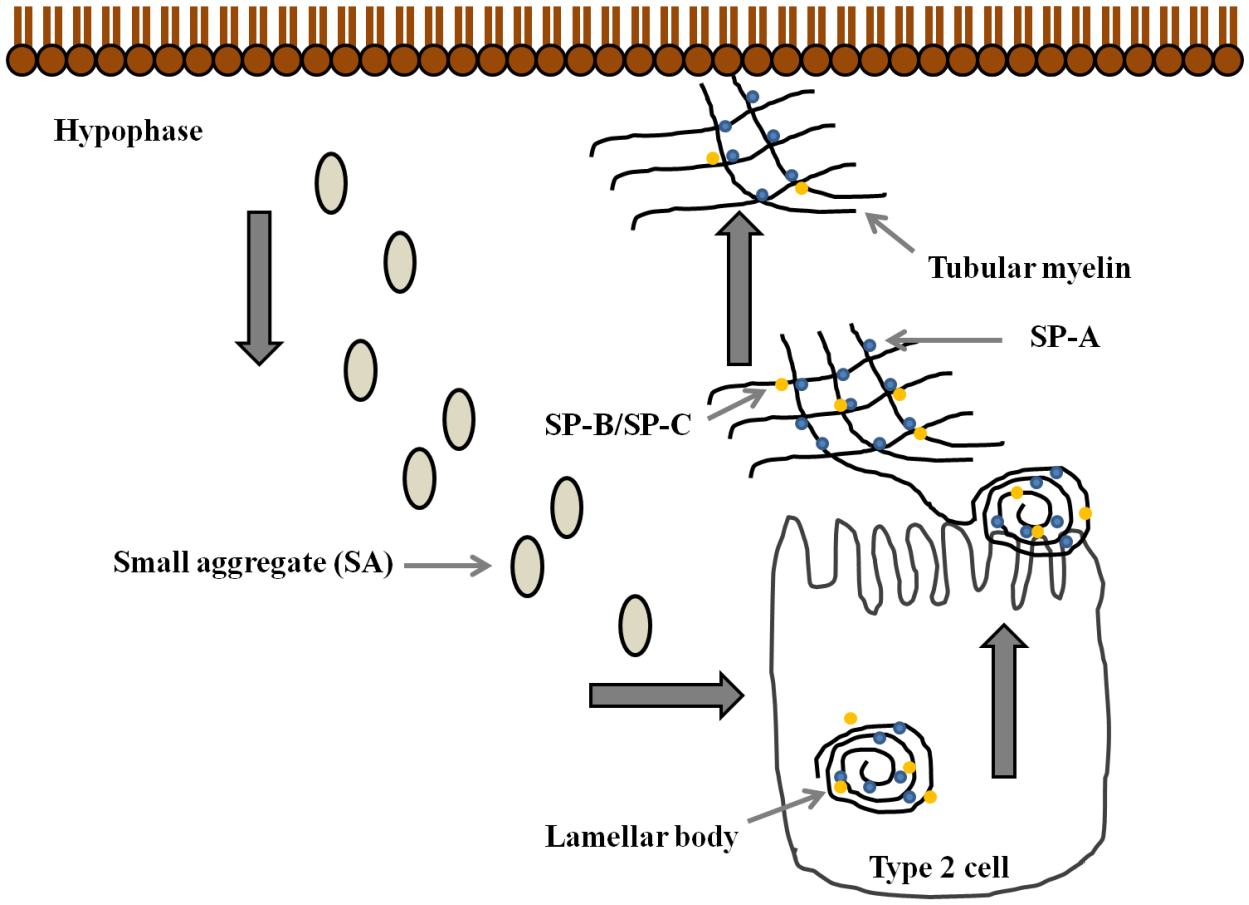

Figure 1.3 Representation of surfactant metabolism. Surfactant is produced within type-2 alveolar cell and stored in an organelle termed lamellar bodies. These organelles are exocytosed where they become unravel to become tubular myelin structures. These act as surfactant reservoirs and during respiration, functional surfactant in the form of large aggregates (LA) adsorb to the air-liquid interface to form a functional surfactant film. Repeated surface area changes during respiration changes LA into the non-functional small aggregate (SA) sub-fraction which can be taken-up by the type-2 cell and recycled back into LA or degraded.

1.2.5 Biophysical function of surfactant

As mentioned previously, the primary purpose of surfactant is to reduce the surface tension within the lungs. The ability of surfactant to reduce the surface tension can be divided into two distinct stages: 1) adsorption and 2) compression and expansion cycles. These two processes describe the ability of surfactant to initially form a film at the air-liquid interface and its ability to further reduce the surface tension when lung volumes are reduced when the film is compressed at the end of exhalation. These two properties will be further discussed in subsequent sections.

1.2.5.1 Adsorption

Adsorption is the process in which surfactant lipids and associated proteins are able to form a surface-active film at the air-liquid interface of alveoli (Walters *et al.*, 2000; Possmayer, 2004). Tubular myelin released from type-2 alveolar cells is able to move through the hypophase towards the air-liquid interface. Since PL are amphipathic, they are able to orient themselves at the air liquid interface with their hydrophobic acyl-tails directed towards the air-space and their hydrophilic polar head-group towards the hypophase. In this orientation, the hydrophilic heads of PL are able to displace water molecules.

Although primarily composed of lipids, this process requires the presence of the surfactant-associated proteins to facilitate rapid adsorption. *In vitro* studies have shown that surfactant films composed of lipids alone cannot rapidly adsorb to the air-liquid

interface without the presence of SP-B and/or SP-C (Possmayer, 2004). Specifically, in 1990, Yu and Possmayer showed that *in vitro*, surfactant films composed of DPPC/PG were able to adsorb more rapidly when SP-B and SP-C were added to surfactant. Additionally, while SP-A is not necessary to facilitate adsorption, its presence in surfactant enhances the overall rate of adsorption of the material in conjunction with SP-B (Hawgood *et al.,* 1987; Rodriguez-Capote *et al.,* 2001). This ability for surfactant to rapidly form a surface active film is crucial to its ability to subsequently reduce surface tensions within the lung during compression.

1.2.5.2 Compression/expansion of surfactant films

As the alveolar surface area decreases during exhalation, the surfactant film undergoes a process termed "compression" wherein the surfactant associated proteins and lipids act in conjunction to reduce the surface tensions within alveoli to near 0 mN/m. With regards to the surfactant associated proteins, SP-B and –C are highly associated with the PL film due to their hydrophibocity (Weaver & Conkright, 2001). They are thought to link surfactant lipids at the air-liquid interface to a surfactant reservoir directly underneath the film in order to facilitate the re-insertion of surfactant lipids to the air-liquid interface (Schurch *et al.,* 1995; Veldhuizen *et al.,* 2001). This is important during inhalation as alveolar surface area expands and the insertion of PL to the air-liquid interface is required in order to maintain a concurrent lipid film. In contrast to SP-B and SP-C, the role of SP-A during compression is less well known.

During compression, PL molecules become tightly packed and it is believed that it is this tight lateral organization of PL which is responsible for reducing the surface tension to near 0 mN/m. It must be noted, however, that not all lipids are capable of forming a stable surface active film upon compression. For instance an *in vitro* study conducted by Hawco and colleagues (1981) utilized surfactant films comprised of only PC. It was observed that this film had an impaired ability to attain low surface tensions upon compression. It was determined that the unsaturated nature of PC molecules limited the film's ability to attain low surface tension values. Specifically, the film composed of PC was able to attain a minimum surface tension of 20 mN/m which is higher than films with DPPC which have been shown capable of attaining minimum surface tensions of 0 mN/m during compression (Ingenito *et al.*, 1999).

1.2.6 Experimental techniques to measure surfactant function

The ability for surfactant to reduce surface tensions to very low values within alveoli during periods of adsorption and compression are essential to the proper function of the lungs. In order to examine the role of specific components of surfactant, including associated proteins such as SP-A, neutral lipids and cholesterol, in the biophysical function of surfactant, we have utilized various *in vitro* and *in vivo* approaches. Relevant to this thesis is a discussion of one such *in vitro* technique, the captive bubble surfactometer (CBS) **(Figure 4)** and an *in vivo* surfactant depleted animal model.

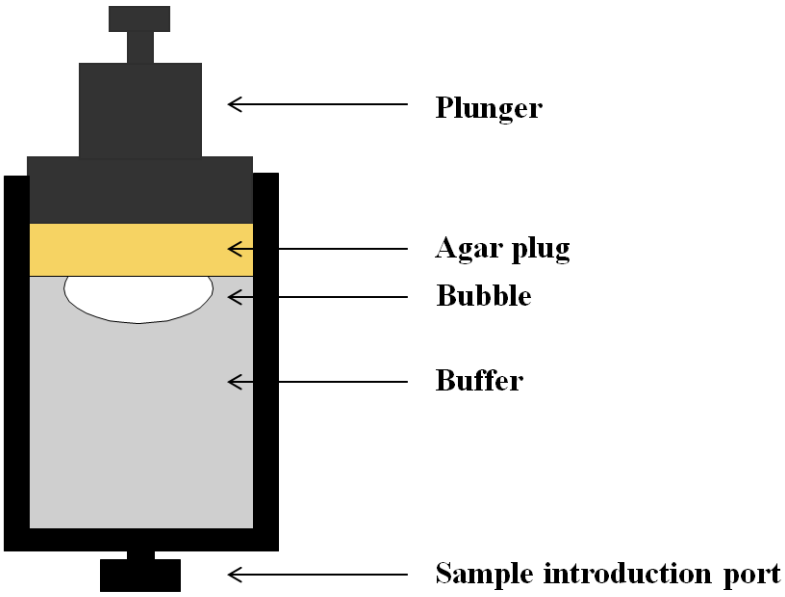

Figure 1.4 Schematic representation of the chamber used in the captive bubble surfactometer (CBS). The surfactant sample of known concentration is introduced with a micropipette via the sample induction port. The surfactant rises to the top of the chamber towards the bubble due to its high buoyancy in the sucrose buffer. The shape of the bubble can be controlled by a motor which moves the plunger up and down. As the bubble changes in volume, changes in surface area can be calculated by determining the bubble's height:width ratio.

1.2.6.1 *In vitro* assessment of surfactant function: captive bubble surfactometer

The CBS, designed by Schurch, is an apparatus which measures the surface tension reducing capability of surfactant preparations through stages of adsorption, compression and expansion cycles (Schurch, 1989). It consists of an air-tight chamber containing a sucrose gradient buffer wherein an air bubble approximately 8 mm in diameter is introduced **(Figure 1.4)**. The bubble rises to the top of the chamber and comes to rest at a hydrophilic agar surface. Surfactant preparations are then introduced into the chamber and adsorbs to the air-liquid interface of the air bubble and surrounding liquid.

To determine the surface tension reducing capabilities of the surfactant, a computer controlled piston decreases or increases the volume of the bubble within the chamber. For example, changes in chamber volume influence changes in chamber pressure causing the bubble to either expand or contract in volume. This change in bubble volume is meant to mimic the changes in alveolar volume during inhalation and exhalation. To determine the surface tension during these dynamic compression-expansion cycles, the shape of the bubble is recorded by a digital camcorder and the height:width ratio of the bubble can be used to calculate the volume and area of the bubble which corresponds to the surface tension of the surfactant film. Overall, the CBS allows for accurate and rapid assessment of a surfactant samples ability to adsorb and attain minimum surface tensions during compression. The assessment of these biophysical properties of surfactant have been important in determining the functionality of patients with severe lung disorders such as the acute respiratory distress syndrome (ARDS).

While the CBS provides experimenters with a rapid and accurate quantitative tool by which to measure surfactant's biophysical properties, the *in vitro* nature of the apparatus cannot assess the physiological consequences of various surfactant preparations. To address how various surfactant preparations affect lung function and physiology, the use of surfactant-depleted animal models can be used.

1.2.6.2 Surfactant depleted animals

Surfactant depleted animals provide a model to study the effects various surfactant preparations on lung physiology. Lachmann and colleagues (1989) used surfactant depleted rabbits to study the pathophysiology ARDS It was shown that adult rabbits made surfactant depleted via repeated saline whole-lung lavages mimicked the physiological signs of ARDS including increased peak inspiratory pressure (PIP) and significantly reduced arterial oxygenation (PaO_2) indicting the development of hypoxemia (Lachmann *et al.,* 1989). With the use of surfactant depleted animals, it became possible to determine the efficacy of exogenous surfactant preparations on lung function. For example, Lewis and colleagues (1993) used surfactant deficient adult sheep to show that the application of exogenous surfactant can improve the PIP and PaO_2 of the surfactant depleted sheep. Importantly, surfactant deficient animals can also be used to determine how surfactants afflicted with different forms of inhibition can affect lung function. For instance, in 2007, Keating and colleagues showed that surfactant deficient rats treated with exogenous surfactant reconstituted with cholesterol had decreased PaO_2 as compared to rats treated with surfactant without cholesterol.

Overall, the use of the CBS allows for the quantification of surfactant's surface tension reduction capabilities. Meanwhile, the use of surfactant depleted animals allows researchers to observe the effects of various surfactant preparations on lung physiology. Both of these investigational tools have been used to ascertain whether changes to surfactant's biophysical function are present in the surfactant collected from patients with various forms of lung injury such as ARDS.

1.3 Acute respiratory distress syndrome

Although the definition and criteria of the acute respiratory distress syndrome (ARDS) has been re-evaluated several times over the years, the hallmarks of the disorder has remained the same: decreases in lung compliance, severe hypoxemia and bilateral chest infiltrates not due to cardiac failure (Bernard *et al.,* 1994; Reubenfeld *et al.,* 2012). The development of ARDS can occur from both indirect and direct insults to the lung (Ware & Matthay, 2000). Indirect insults including sepsis, trauma and multiple blood transfusions can promote systemic inflammation which can also affect the lungs. Direct insults to the lungs include smoke inhalation, gastric acid aspiration and pneumonia and these factors directly impair the lung's epithelium and surfactant system decreasing compliance and oxygenation. Clinically, ARDS presents as severe hypoxemia and the severity of ARDS is reflected in the overall level of oxygenation in arterial circulation (Bernard *et al.,* 1994; Reubenfeld *et al.,* 2012).

1.3.1 Alterations to surfactant composition in ARDS

Studies investigating the biophysical properties of surfactant collected from patients with ARDS have shown that their surfactant has altered composition and impaired surface tension reducing properties (Gunther *et al.,* 1996). Unlike neonates afflicted with nRDS, the onset of ARDS is not predicated on surfactant deficiency. Indeed, surfactant from patients with ARDS has been shown to have a complex array of changes to surfactant's composition and biophysical dysfunction of the material (Gregory *et al.,* 1991; Gunther *et al.,* 1996). Changes to surfactant's PL profile, increased conversion of LA to SA and the presence of serum proteins in surfactant have been observed in lavage collected from ARDS patients (Cockshutt *et al.,* 1991; Veldhuizen *et al.,* 1995; Gunther *et al.,* 1996; Lewis & Veldhuizen, 2002; Lewis & Maruscak, 2006). Recently, it has also been suggested that elevated levels of cholesterol within surfactant contribute to surfactant dysfunction and may play a role in the development of ARDS. These alterations to surfactant may contribute to the overall biophysical impairment of the material and mediate the development of ARDS.

Changes to surfactant's PL profile and associated protein levels have been identified in patients with ARDS when analyzing lavage material collected from their lungs. Specifically, it has been reported that patients with ARDS have a decreased amount of DPPC, PC and PG and an increase in the amount of PE, SM and lyso-phosphatidylcholine (lyso-PC) (Pison *et al.,* 1987; Gunther *et al.,* 1996; Schmidt *et al.,* 2007). Given that DPPC is the PL primarily responsible for the surface tension reducing properties of surfactant, decreases in DPPC's concentrations may affect the overall

biophysical function of surfactant. With regards to the surfactant associated proteins, it has been observed that SP-A, SP-B and SP-C levels are also reduced in surfactant obtained from ARDS patients and this decrease in surfactant associated proteins may be due to alterations in type-2 cell metabolism or, possibly, elevated proteolytic activity (Lee *et al.,* 1981; McGuire *et al.,* 1982). As mentioned previously, Korfhagen and colleagues (1996) were able to show that SP-A null mice were phenotypically normal, but their surfactant was more sensitive to inhibition as compare to surfactant with SP-A. Furthermore, serum-protein mediated surfactant inhibition has also been shown to be mitigated by the presence of SP-A (Cockshutt *et al.,* 1991). Therefore, while SP-A is not necessary for proper lung function under healthy conditions, its role in mitigating surfactant inhibition when the system is under duress highlights the importance of SP-A and the decreases in SP-A levels in ARDS patients may render their surfactant to be more susceptible to inhibition. Taken together, these observations show that changes to surfactant's PL and protein composition affects surfactant's function and may contribute to the development of ARDS.

Additionally, the alterations to the composition of surfactant's sub-fractions have also been observed in the lavage of patients with ARDS. For example, Veldhuizen and colleagues (1995) showed that lavage from ARDS patients showed an increased ratio of SA:LA as compared to lavage from non-ARDS patients (0.48 vs. 0.20 respectively). Additionally, Gunther and colleagues (1996) also showed that in lavage collected from patients with ARDS contains 36% LA (as a percentage of total surfactant) as compared to lavage from healthy lungs which contain between 60-75% LA. Given that *in vitro*

experiments have shown that SA is less biophysically active than LA (Brackenbury *et al.*, 2002), an increased SA/LA ratio suggests that surfactant's surface tension reducing capability may be compromised. Interestingly, the increased conversion of LA into SA may be a consequence of mechanical ventilation (MV) (Veldhuizen *et al*., 1996; Ito *et al*., 1997). The role of MV in increasing the SA/LA ratios will be discussed in more detail in a following section.

Although not natural components of surfactant, the presence of serum proteins within lavage recovered from patients with ARDS due to leakage into the airspace from the serum, also represent agents which can impair surfactant function (Ware & Matthay, 2000). Due to the close and extensive proximity of alveoli to the systemic circulation, insults compromising the barrier between the two regions can cause an influx of serum proteins during pulmonary edema. Studies have shown that serum proteins such as hemoglobin, albumin and fibrinogen can impair the surface-tension reducing capability of surfactant by physically interfering with PL from adsorbing to the air-liquid interface (Seeger *et al.*, 1985; Cockshutt *et al.*, 1990). This interference ultimately results in deficiencies in surfactant's ability to achieve low surface tensions within the lung thus affecting compliance.

Recently, studies have also shown that elevations in the neutral lipid component of surfactant, of which cholesterol is the major component, is also present in surfactant obtained from patients with ARDS. For example, Markat and colleagues (2007) showed

that lavage from patients afflicted with ARDS had twice the amount of neutral lipid as compared to lavage obtained from healthy subjects. Additionally, *in vitro* studies analyzing the biophysical properties of surfactant reconstituted with elevated levels of cholesterol has shown that the material is unable to achieve low surface tensions compared to surfactant with physiological concentrations of cholesterol (Gunasekara *et al.,* 2005; Keating *et al.,* 2007). The contribution of cholesterol to the biophysical impairment of surfactant is an important concept for the purposes of this thesis. To that end, further detail regarding cholesterol mediated surfactant dysfunction will be discussed in subsequent sections as it applies to injuries sustained due to MV and the contribution of serum levels of cholesterol as well.

1.3.2 Mechanical ventilation

MV is a necessary intervention in patients with ARDS. Without the use of MV to maintain oxygenation, it has been estimated that the mortality rate of patients afflicted with ARDS would approach 90% (Reubenfeld, 2003). Despite the necessity of MV, MV itself has been shown to propagate the development of further lung injury (Webb & Tierney, 1974; Dreyfuss & Saumon, 1998; Brower *et al.,* 2000; Veldhuizen *et al.,* 2001). The contribution of MV to the development of further lung injury can be attributed to two main causes: 1) shear forces within alveoli and 2) over-distension of alveoli epithelium (Slutsky, 1999). Shear forces occur during repeated collapse and opening of alveoli during ventilation. Recent advance in ventilation techniques have demonstrated that the use of positive end expiratory pressure (PEEP), or a volume of air left in the lungs after

exhalation, maintains alveolar patency. In effect, PEEP reduces the degree of repeated alveolar collapse and recruitment.

The importance of over-distension of alveoli due to MV in the progression of lung injury was investigated in a large multi-centered clinical trial conducted by the Acute Respiratory Distress Syndrome Network (ARDSnet) in 2001. In this study, it was demonstrated that ARDS patients placed on MV with PEEP and low tidal volumes (6 mL/kg) had significantly lower mortality (31%) than ARDS patients placed on MV with higher tidal volumes (12 mL/kg, 40% mortality). This observation demonstrates how specific ventilator parameters can influence the progression of lung injury and, ultimately, mortality. Given the importance of MV for patients with ARDS and the associated damages that MV can also cause to the lung, research has been undertaken to better understand how MV contributes to the development of lung injury. One method has been to use animal models placed on high-stretch mechanical ventilation to develop lung injury. These animals are considered to be afflicted with ventilator-induced lung injury (VILI).

1.3.3 Animal models of VILI

As discussed previously, MV can progress the severity of lung injury in patients due to repetitive alveolar collapse and over-distension of the alveolar epithelium (Slutsky, 1999). These factors can be mimicked in animal models by utilizing ventilation strategies with high tidal volumes and zero PEEP. The high tidal volume ensures that

alveoli become over-distended, and zero PEEP promotes alveolar collapse. Although use of high tidal volumes and zero PEEP are effective in developing VILI in animal models, one limitation is these settings do not reflect clinical realities. Patients with severe lung injuries are typically ventilated with low tidal volumes with additional PEEP to minimize lung damage (Brower *et al.,* 2001). Furthermore, animal studies of VILI are conducted over the course of a few short hours as compared to patients who may require ventilation for days. Nevertheless, several studies have shown that similar changes are present in rat models of VILI and patients with lung injuries such as ARDS including decreased oxygenation (Veldhuizen *et al.,* 2002; Maruscak *et al.,* 2008), altered surfactant composition (Verbruggae *et al.,* 1998; Maruscak *et al.,* 2008) and diminished biophysical properties (Panda *et al.,* 2004).

For instance, alterations the percentage of LA has been shown in models of VILI (Maruscak *et al.,* 2008). Ito and colleagues (1997) observed that large changes to alveolar surface area due to high tidal volume ventilation resulted in increased conversion of LA to SA *in vivo* (Veldhuizen *et al*., 1996; Ito *et al*., 1997). This observation has been further confirmed with *in vitro* experiments indicating that increased changes to surface area promotes the conversion of LA to SA (Veldhuizen *et al.,* 2000). These observations indicate that the observed elevation in SA/LA ratio in surfactant from ARDS patients may be as a direct result of MV.

1.3.4 Surfactant cholesterol and VILI

One important change in surfactant composition observed in animals with VILI are elevations to the amount of cholesterol within surfactant. Using a VILI model in rats, Maruscak and colleagues (2008) observed that the surfactant of animals with VILI had significantly increased cholesterol levels as compared to rats without lung injury. Importantly, Maruscak and colleagues also observed that the elevations in surfactant cholesterol levels were present in rats placed on high-stretch ventilation prior to the physiological manifestations of VILI such as increased PIP and decreased PaO_2. This observation indicates that changes to surfactant composition, such as elevated cholesterol levels, may occur early and potentially contributes to the development of VILI.

In a follow-up study, Vockeroth and colleagues (2010) showed that, *in vitro*, the biophysical properties of surfactant obtained from rats with VILI was improved following sequestration of cholesterol from the material. Moreover, reconstitution of high levels of cholesterol into surfactant resulted in decreased surface tension reducing properties of the material. Overall, elevated levels of cholesterol within surfactant may represent another mechanism of surfactant inhibition. Unlike serum protein mediated inhibition, however, cholesterol is an inherent component of surfactant and it is currently unknown how cholesterol levels become augmented within surfactant. One consideration into the potential mechanisms which elevate cholesterol levels within surfactant may be related to the levels of cholesterol within the body's circulatory system.

1.4 The role of serum cholesterol

As mentioned previously, surfactant composition includes approximately 4-7% wt/wt PL neutral lipids of which cholesterol is the major component. In an effort to determine the origin of cholesterol within surfactant, a study was conducted in 1995 by Orgeig and Daniels which utilized radio-labeled cholesterol injected into the tail-vain of rats. Upon obtaining the alveolar lavage of these rats, Orgeig and Daniels observed that the overwhelming constituency (~99%) of cholesterol within alveoli was radio-labeled. Furthermore, the presence of both LDL and vLDL receptors on the basolateral membrane of alveolar type-2 cells suggest a mechanism by which facilitate the uptake of cholesterol from serum (Guthmann *et al.,* 1997). These observations indicate that alveolar cholesterol levels predominantly originate from the serum. Since surfactant cholesterol predominantly originates from the serum, it has been postulated that serum levels of cholesterol may affect the amount of cholesterol present within surfactant.

A study conducted by McCrae and colleagues in 2008 was conducted to determine whether high serum cholesterol levels would affect surfactant levels of cholesterol and, ultimately, surfactant function. McCrae and colleagues placed mice on a high cholesterol diet for a period of 16 weeks. At the end of this dietary period, lavage analysis showed that mice fed a high cholesterol diet had a significantly higher proportion of cholesterol as compared to the lavage of mice fed a standard diet (0.26±2.83 mol% vs. 7.84±1.44, respectively). To test the effect of elevated cholesterol within surfactant, McCrae and

colleagues utilized capillary surfactometry. In this test, surfactant was pipetted into a small capillary tube. Pressurized air was then injected through the tube for a period of 120 seconds in order to pierce the meniscus of surfactant and to measure the overall patency of surfactant present. Their results showed that surfactant containing elevated cholesterol was less patent than the surfactant with normal levels of cholesterol. This decreased patency indicated that the surfactant with high cholesterol was less stable than surfactant with normal levels of cholesterol. Therefore, this study indicated that high cholesterol in surfactant may render the material less capable of forming a stable surface-active film. Despite this finding, however, the observations from this study have not been reported in any other animal models. Therefore, it is currently unknown whether the effects of diet-induced serum hypercholesterolemia observed in this particular study will translate to other animals such as rats and, ultimately, humans.

A clinical study conducted by O'Neal and colleagues (2011) attempted to determine the rates of developing ARDS in critically ill patients based on their prior use of cholesterol lowering drugs – HMG-CoA inhibitors or "statins". Their study analyzed the development of ARDS in 575 critically ill patients admitted to a medical or surgical ICU. The results of this study indicated that the patients who had taken statins prior to hospitalization had significantly reduced chance of developing sepsis or ARDS as compared to patients not placed on statins. Despite the findings of this study, it is unclear whether the observed decrease in development of ARDS in patients was due to the cholesterol lowering effects of statins or due to the known anti-inflammatory effects of the drugs themselves.

Recently, a study conducted by Yamashita and colleagues (2012) attempted to determine whether serum hypercholesterolemia affected the severity of lung injury. In their study, Yamashita and colleagues used an apolipoprotein-E (ApoE) null mice as a model of serum hypercholesterolemia. These mice were then treated with hydrochloric acid (HCl) to develop acid-induced lung injury – a model of gastric acid-aspiration induced lung injury. Gastric acid-aspiration induced lung injury represents the second leading cause of ARDS worldwide. In the study conducted by Yamashita and colleagues, the ApoE null mice were observed have increased pulmonary inflammation as compared to wild-type mice. These findings indicate that serum hypercholesterolemia may also play a role in promoting the development of more severe lung injury. However, due to the use of genetically modified mice it remains to be determined whether the increased pulmonary inflammation can be truly attributed to serum hypercholesterolemia, or as a result of ApoE deficiency.

Based on the aforementioned studies, it is evident that elevated cholesterol within surfactant can cause biophysical impairments of the material and may render the surfactant film less stable at the air-liquid interface. Furthermore, serum hypercholesterolemia may render patients more susceptible to the development of more severe ARDS. However, due to the limitations inherent within these studies, there is still a lack of conclusive evidence linking serum hypercholesterolemia in and of itself to the development and ultimate severity of ARDS. Due to the ever-growing prevalence of

serum hypercholesterolemia in humans, determining whether there is a connection between serum hypercholesterolemia and surfactant function is an important consideration. Investigating this potential relationship may reveal a patient population at greater risk of developing lung injuries such as ARDS and this knowledge can be used to prescribe more specific and precise interventions to reduce the risk of developing ARDS.

1.5 Rationale and hypotheses

The lungs are an essential organ responsible for maintaining life by facilitating gas exchange (West, 2005). The biophysical properties of pulmonary surfactant are important to the lung's ability to function and impairments to surfactant's surface tension reducing capabilities lead to the development of severe lung injuries such as ARDS. Surfactant inhibition can occur as a result of a multitude of changes to its composition including an altered lipid profile, changed LA/SA ratios, PL oxidation and the presence of inhibitory agents such as serum proteins. Recent studies have also shown that elevated cholesterol can inhibit surfactant function. Importantly, although SP-A has been shown capable of mitigating the effects of surfactant inhibition due to decreased DPPC concentrations, PL oxidation, and serum protein-mediated inhibition, it is currently unknown whether SP-A can also mitigate the effects of cholesterol-mediated surfactant inhibition. Furthermore, there is evidence supporting that serum hypercholesterolemia may affect surfactant cholesterol levels as well as increase the propensity to develop

more severe ARDS. It is currently unknown, however, whether diet-induced serum hypercholesterolemia will affect the severity of various forms of lung injury.

In the first data chapter, we explored the effects of high cholesterol levels in surfactant and whether SP-A can mitigate its effects. We explored this by using the CBS to determine the biophysical properties of surfactant with high cholesterol with and without SP-A and *in vivo* by administering exogenous surfactant with various levels of cholesterol with and without SP-A to surfactant depleted rats. Given SP-A's ability to mitigate serum-protein mediated surfactant dysfunction and ability to maintain surfactant function even with decreased PL concentrations, SP-A may be able to also mitigate the inhibitory effect of high surfactant cholesterol. The data gathered from this experiment will shed insight into the mitigating effects of SP-A and the effects of high cholesterol in surfactant on physiological data such as blood oxygenation.

In the second data chapter, our objective was to determine whether diet-induced serum hypercholesterolemia will cause changes to surfactant composition and predispose the development of more severe ARDS. Since the majority of surfactant cholesterol is taken-up from systemic circulation, elevations in the degree of cholesterol in the blood may influence surfactant cholesterol levels as well. To explore this, we placed rats on a high cholesterol diet and determined the degree of physiological injury and changes to their surfactant composition in three different forms of lung injury: VILI, acid-aspiration and surfactant depletion.

This study has two hypotheses. First, it was hypothesized that elevated cholesterol in surfactant will cause impairment to the material's biophysical properties and affect lung physiology, but the addition of SP-A will mitigate these results. Secondly, it was hypothesized that diet-induced hypercholesterolemia will affect surfactant composition and result in the development of more severe ARDS.

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Chapter 2

2 SP-A mitigates cholesterol-mediated surfactant inhibition and improves the oxygenation in surfactant depleted rats.

2.1 Introduction

Pulmonary surfactant is a lipoprotein complex formed within alveolar type-2 cells and secreted into the alveolar space which acts to reduce surface tensions at the alveolar surface (Possmayer *et al.*, 1984; Goerke, 1998; Possmayer, 2004). Surfactant is composed of 80-90% phospholipids, of which dipalmitoylphosphatidylcholine (DPPC) is the major component, 5-10% neutral lipids, of which cholesterol is the major constituent, and 5-10% surfactant associated proteins (Veldhuizen *et al.*, 1998; Postle, 2001; Possmayer, 2004). Surfactant released into the alveolar space, termed large aggregate (LA), quickly adsorbs to the air-liquid interface where it forms a surface active film (Possmayer, 2004). As lung surface area decreases during exhalation, the surfactant film undergoes compression which reduces the surface tension to near 0 mN/m (Possmayer, 2004). This ability of surfactant to reduce surface tension prevents alveolar collapse, maintains compliance of the lungs and reduces the effort required to breathe (Possmayer, 2004).

Patients afflicted with lung injuries, such as the acute respiratory distress syndrome (ARDS), have dysfunctional surfactant (Gregory *et al.,* 1991; Gunther *et al.,* 1996; Gunther *et al.,* 2001). The causes for dysfunction of the material include alterations to the lipid composition, reduction in the surfactant associated proteins and the presence of inhibitory agents (Cockshutt *et al.,* 1990; Seeger *et al.,* 2001; Veldhuizen *et al.,* 1995; Gunther *et al.,* 1996). One such agent that has been investigated is the presence of serum protein in the lungs which can occur as a result of pulmonary edema (Ware & Matthay, 2000). Proteins, such as albumin and fibrinogen interfere with surfactant's ability to form a film at the air-liquid interface via competitive inhibition (Cockshutt *et al.,* 1990; Possmayer, 2004; Da Silva *et al.,* 2005). This results in increased surface tension within the lung and contributes to the decreased oxygenation and reduced lung compliance. Recently, the presence of elevated cholesterol has also been implicated as a mechanism for surfactant inhibition (Davidson *et al.,* 2000; Panda *et al.,* 2004; Gunasekara *et al.,* 2005; Vockeroth *et al.,* 2010). Mechanically ventilated rats with lung injury were shown to have an elevated proportion of cholesterol within their surfactant (Maruscak *et al.,* 2008). Further *in vitro* investigation revealed that this surfactant had dysfunctional biophysical properties which could be restored by removal of cholesterol (Vockeroth *et al.,* 2010). Whereas inhibition of surfactant by serum proteins has been studied extensively, cholesterol-mediated inhibition of surfactant's biophysical function is a relatively recent discovery which requires further examination.

One aspect that has been examined in the context of serum protein inhibition is the protective role of surfactant protein-A (SP-A) (Cockshutt *et al.,* 1990). This protein has

been shown to mitigate serum protein-mediated surfactant dysfunction by enhancing surfactant's ability to adsorb to the air-liquid interface. However, unlike serum proteins, cholesterol is an integral component of surfactant therefore its mechanism of inhibition is different than that of serum protein. It is currently unknown if SP-A has the ability to mitigate cholesterol-mediated dysfunction. Based on this information, the purpose of this study was to delineate the effects of elevated cholesterol on the biophysical function of surfactant and the effects of SP-A on this material. To investigate these topics, we tested the biophysical function of human surfactant reconstituted with various levels of cholesterol with and without SP-A *in vitro* in a captive bubble surfactometer (CBS). Additionally, to determine the effects of elevated surfactant cholesterol and SP-A on lung function, we administered exogenous surfactant reconstituted with 5 and 20% cholesterol with and without SP-A to surfactant depleted rats and assessed their physiology. It was hypothesized that elevated surfactant cholesterol levels would impair the biophysical properties of the material leading to decreased lung function, but the presence of SP-A could mitigate these effects.

2.2 Methods

2.2.1 Surfactant and SP-A collection from human proteinosis patient

Alveolar proteinosis surfactant was obtained via a whole lung lavage after obtaining informed consent. Briefly, the patient was anesthetized and intubated using a dual lumen endotracheal tube in order to ventilate each lung independently. Whole lung lavage was

then performed on the left lung and the lavage material was processed immediately to obtain Surfactant LA and SP-A from the lavage material.

Immediately following lung lavage procedures, samples were centrifuged for 10 minutes at 150g to remove cellular debris. (Veldhuizen *et al.,* 1996) The supernatant was centrifuged at 40,000g for 15 minutes to obtain a pellet of the LA sub-fraction of surfactant. The LA pellet was re-suspended in 150mM NaCl and frozen at -20^{o}C until further use. The amount of LA was analyzed after a chloroform extraction via a phospholipid-phosphorus analysis. Briefly, aliquots LA samples were added to a solution containing 1 mL ddH2O, 1 mL methanol and 2 mL chloroform. This mixture was then centrifuged at 150 g for 10 mins to separate the aqueous and organic solvent layers. The organic solvent layer was extracted and dried-down via exposure to O2 gas in a 37°C water bath until the liquid chloroform had evaporated. The remaining solute was ashed on a hot-plate after which 1 mL of 1 N HCl was added. Then, the samples were then placed into a heating block at 100°C for 15 mins. Malachite green dye was added to all surfactant samples and the resulting colour was analyzed on a plate reader.

Human SP-A was purified using a similar method as previously described by Haagsman *et al* (1989). Briefly, to purify SP-A, lavage obtained from human alveolar proteinosis patient was centrifuged at 6500 rpm for 30 mins. The supernatant was discarded and the pellet was suspended in re-suspended butanol. The solution was then centrifuged at 6500 rpm for 30 mins and then dried with nitrogen gas. Following, the pellet was

homogenized in 20 mM n-octyl-B-D-glucopyranoside in 10 mM HEPES (pH 7.4) and then centrifuged at 200,000g for 30 mins. The supernatant was discarded and the remaining pellet was resuspended in n-octyl-B-D-glucopyranoside and was centrifuged once again at 200,000g for 30 mins. The resulting pellet was then re-suspended in 5mM Tris and transferred to a dialysis membrane over night. The next day, the sample was centrifuged at 200,000g for 30 mins. Supernatant was aliquoted and stored at -20^{o}C.

2.2.2 Human proteinosis surfactant cholesterol depletion and reconstitution

Cholesterol depletion was done via lipid extraction of crude LA by the method described by Bligh & Dyer (1959). First, the sample was shell-dried with nitrogen gas then re-suspended with 20 mL of acetone which was then centrifuged at 150g for 15 mins. Following centrifugation, acetone was discarded and replaced with fresh acetone solution and the solution was stored at -20°C overnight. This process was repeated until cholesterol levels were below the detection threshold of a commercially available free cholesterol assay kit (Wako Chemicals, Richmond, VA, USA). The recovered amount of phospholipid was determined by phosphorus assay as described later, the sample was re-suspended in chloroform to make a final concentration of 5 mg/mL phospholipid (PL). This material was utilized to create samples containing either 0, 5, 10 or 20% cholesterol wt/wt PL. Cholesterol was obtained from Sigma (St. Louis, MO) and received as a powder. These samples were dried-down and re-suspended in 0.5 mL of buffer (140 mM NaCl, 10 mM Hepes, and 2.5 mM $CaCl_2$, pH = 6.9) at a final PL concentration of 10

mg/mL. In addition, 4 similar samples were reconstituted with 5% SP-A wt/wt PL. The samples were analyzed for their biophysical function on the CBS as described below.

2.2.3 Assessment of Surface Tension

The surface activity of the LA surfactant samples described above was assessed using a computer-controlled CBS (Schurch, 1989). The chamber of the CBS was filled with a buffer solution (140 mM NaCl, 10 mM Hepes, and 2.5 mM $CaCl_2$, pH = 6.9) containing 10% sucrose (by mass) to increase the buffer density above that of surfactant (Gunasekara *et al.*, 2005). A small bubble (0.035-0.045mL) was introduced into the chamber and allowed to float up and rest against the concave agarose plug at the top of the chamber. Approximately 0.1-0.15 μL of LA sample was deposited just below the air-liquid interface by a transparent capillary attached to a micromanipulator used for the precise approach of the capillary to the bubble. The bubble was imaged using a video camera (Pulnix TM-7CN, S/N: 017505) and recorded for future analysis. Throughout the experiment the chamber was kept at 37°C. Upon injection of surfactant, a 300s adsorption period was allowed, during which time there was no manipulation of the bubble and the change in the shape of the bubble was monitored. The chamber was then sealed and the bubble was rapidly expanded to a volume of 0.13 mL over a 1 second interval. Following this 5 minute period, quasi-static cycling was performed by compressing and then expanding the bubble in a stepwise manner in increments of 20 % of the total volume in order to set the minimum (0.0090 mL) and maximum bubble volume (0.13mL) for subsequent assessment. Dynamic cycling, in which the bubble volume was smoothly changed to the defined volumes for 20 cycles at a rate of 20

cycles/min, was then performed to determine the minimum achievable surface tension during compression (minimum surface tension). The surface tensions of the bubbles were calculated based on the shape of the bubble at various stages throughout the CBS technique. Briefly, during initial adsorption images at precisely 0, 1, 5, 10, 30 and 300 seconds were analyzed for surface tension. During dynamic cycling, every fifth image was analyzed, and the lowest minimum surface tension that was achieved for cycles 1, 2, 5 and 10 were determined.

2.2.4 Exogenous bovine lipid extract (BLES) surfactant cholesterol and SP-A reconstitution

BLES was generously gifted by BLES Biochemicals (London, ON, CA). BLES was extracted using chloroform and stored at -20°C until use. Cholesterol was obtained from Sigma (St. Louis, MO) and received as a powder. Cholesterol was dissolved in chloroform and reconstituted into BLES up to 5 or 20% w/w PL. This solution was shell-dried with gas and re-suspended with saline to a concentration of 15 mg/mL PL. Five percent (w/w PL) human SP-A or an equal volume of saline was added to samples to create surfactant with 5% cholesterol ± SP-A and 20% cholesterol ± SP-A. Subsequently, 1.5 mM $CaCl_2$ was added to all samples and saline was added to make a final concentration of 10 mg PL/mL.

2.2.5 Surgical procedure

All procedures were approved by the animal use subcommittee at the University of Western Ontario in agreement with the guidelines of the Canadian Council of Animal Care. Animals were acclimatized for three days and had free access to water and a standard laboratory diet. The surgical procedure for this experiment was similar to the methodology utilized by Maruscak and colleagues (2008) and Vockeroth and colleagues (2010). Animal were subsequently anesthetized via intraperitoneal injection of 75 mg/mL ketamine and 5 mg/mL xylazine in sterile 0.15 M NaCl. Once a toe-pinch could not be observed, animals were given a subcutaneous injection of an analgesic (0.1 mg/kg buprenorphine) and a 0.2 mL subcutaneous injection of a topical anesthetic (sensorcaine 0.5%) at the site of incision. The left and right jugular veins and the right carotid artery were exposed and catheterized with PE-50 tubing. The left jugular catheter was used to deliver anesthetic/analgesic (0.5 – 2.5 mg/100g/h propofol) and the right jugular catheter was used to deliver fluid (sterile NaCl with 100 IU heparin/L) continuously (0.5 – 1.0 mg/100g/h). The carotid artery catheter was used to measure blood pressure and heart rate, collect blood for blood gas measurements (ABL 500 Radiometer, Copenhagen, DK) and deliver fluids (sterile NaCl with 100 IU heparin/L) continuously (0.5 – 1.0 mg/100g/h). Fluids and anesthetic/analgesic were delivered via infusion pumps (Harvard Apparatus Inc, South Nantucket Massachusetts). Subsequently, the trachea was exposed and a 14 gauge endotracheal tube was inserted and secured with 3-0 silk.

2.2.6 Mechanical ventilation procedure

Following surgical preparation, animals were given a 0.1 mL bolus of a neuromuscular inhibitor (2 mg/mL pancurium bromide) and immediately connected to a volume-cycled rodent ventilator (Harvard Instruments, St. Laurent, PQ, CA) set to deliver 8 mL/kg tidal volume (Vt), respiratory rate (RR) of 54-58 breaths per minute (bpm), 5 cmH_2O positive end-expiratory pressure (PEEP) and a fraction of inspired oxygen (FiO_2) of 1.0. Animals were ventilated for fifteen minutes prior to measuring initial baseline blood-gas measurements. The inclusion criteria for all experiments were a blood gas measurement of $PaO_2 \geq 400$ mmHg.

To deplete endogenous surfactant from the animals, a procedure as similarly used by Bailey and colleagues (2006) and Keating and colleagues (2007) was used. Briefly, rats meeting the inclusion criteria were disconnected from the ventilator and given one total-lung lavage of 10 mL 0.15 N NaCl via syringe attached to the endotracheal tube. The peak inspiratory pressure (PIP) pre/post lavage and lavage volume administered/recovered were recorded. After withdrawal of the lavage, the animal was re-attached to the ventilator. The expiratory line was occluded for one breath to inflate the lungs and recruit any collapsed regions. This process was repeated three additional times with 8 minutes between each lavage. Following the fourth lavage, a blood gas measurement was taken to determine the animal had a $PaO_2 \leq 150$ mmHg. Animals failing to meet the criteria were treated with further lavages ranging in volumes from 2-5 mL until $PaO_2 \leq 150$ mmHg up to a maximum of eight total lavages. Rats meeting the inclusion criteria were then randomized to receive either a 1 mL/kg BW of bolus of air or

a 1 mL/kg BW volume of surfactant (10 mg PL/mL) with 5% cholesterol (n=6), 5% cholesterol + SP-A (n=7), 20% cholesterol (n=6) or 20% cholesterol + SP-A (n=6) intratracheally. Rats were then placed back on ventilation, given one breath hold and ventilated for 120 minutes. Airway pressure, hemodynamics, and blood gas measurements were recorded every 15 minutes.

2.2.7 Rat surfactant lavage collection and processing

Rats were administered 75 mg/kg BW sodium pentobarbital via the left jugular catheter to lower blood pressure. Animals were exsanguinated by severing the descending aorta. A midline sternectomy was performed to open the chest cavity. After, lungs were treated to a whole-lung lavage using five 10 mL aliquots of 0.15 M saline; each aliquot was instilled and removed three times (Veldhuizen *et al.*, 2002).

Collected lavage samples were centrifuged at 150 g for 10 mins. Five mL of supernatant from the lavage containing the surfactant sub-fraction (TS) was aliquoted in 1 mL volumes and stored at -20°C. The remaining surfactant was centrifuged at 40 000g for 15 minutes to separate the supernatant containing the small aggregate (SA) sub-fraction (Yamada *et al.*, 1990) and the pellet containing the large aggregate (LA) was re-suspended in 2 mL of 0.15 M NaCl saline. The SA and LA sub-fractions were stored with the TS sub-fraction at -20°C.

2.2.8 Surfactant analysis

A modified Duck-Chong phosphorous assay was performed on each total surfactant, LA and SA sample to determine the phospholipid content in each (Duck-Chong, 1979). Briefly, aliquots of total surfactant, LA and SA samples were added to a solution containing 1 mL ddH2O, 1 mL methanol and 2 mL chloroform. This mixture was then centrifuged at 150 g for 10 mins to separate the aqueous and organic solvent layers. The organic solvent layer was extracted and dried-down via exposure to O2 gas in a 37°C water bath until the liquid chloroform had evaporated. The remaining solute was ashed on a hot-plate after which 1 mL of 1 N HCl was added. Then, the samples were then placed into a heating block at 100°C for 15 mins. Malachite green dye was added to all surfactant samples and the resulting colour was analyzed on a plate reader.

2.2.9 Statistical Analysis

Statistical analysis of cholesterol and SP-A reconstituted human proteinosis surfactant was performed using a two-way repeated measures analysis of variance (ANOVA) with a Tukey's post-hoc test. Analysis of surface area reduction for human proteinosis surfactant during compression was done by t-test. Results from our study using rats treated with BLES elevated to 5 and 20% cholesterol with and without SP-A were analyzed with a two-way ANOVA followed by Tukey's post-hoc test. Means are reported ± SEM. Values were considered significantly different at a probability value < 0.05.

2.3 Results

2.3.1 *In vitro* experiment

2.3.1.1 Adsorption

We tested the surface tension at the air-liquid interface within the CBS of human surfactant reconstituted with different levels of cholesterol with and without SP-A by allowing the surfactant to adsorb to the air-liquid interface over the course of 300 s. First, **figure 2.1A** shows the effect of increasing concentrations of cholesterol, without SP-A, on the surface tension following adsorption of human surfactant. Surfactant with 0% cholesterol demonstrated rapid adsorption reaching equilibrium surface tension of approximately 23mN/m in 30 seconds. Human surfactant containing 5% cholesterol had adsorption values that were not significantly different from the 0% cholesterol samples at any time during adsorption except for the 5 second time-point. At this time, the 5% cholesterol samples had significantly higher surface tension values compared to the 0% cholesterol sample. Samples with 10 and 20% cholesterol had slower adsorption compared to the 0 and 5% cholesterol sample. This difference was statistically significant from 5 to 60 seconds for 10% cholesterol, and from 5 to 30 seconds for 20% cholesterol, as compared to the 0% cholesterol sample.

The surface tension during 300 s of adsorption at the air liquid interface for human proteinosis surfactant samples reconstituted with various levels of cholesterol and SP-A is shown in **figure 2.1B.** Over the course of 300 s, there were no significant differences

amongst the surfactant samples reconstituted with different levels of cholesterol. Comparisons between surfactant containing similar levels of cholesterol but with or without SP-A showed that there were no significant differences in surface tension between surfactant containing 5% cholesterol regardless of the presence of SP-A. However, it was observed that surfactant containing 10% cholesterol + SP-A had significantly reduced surface tension from 0 – 60 s as compared to surfactant with 10% cholesterol without SP-A. Similarly, surfactant containing 20% cholesterol with SP-A showed significantly lower surface tension from time points 10 – 60 s as compared to surfactant containing 20% cholesterol without SP-A.

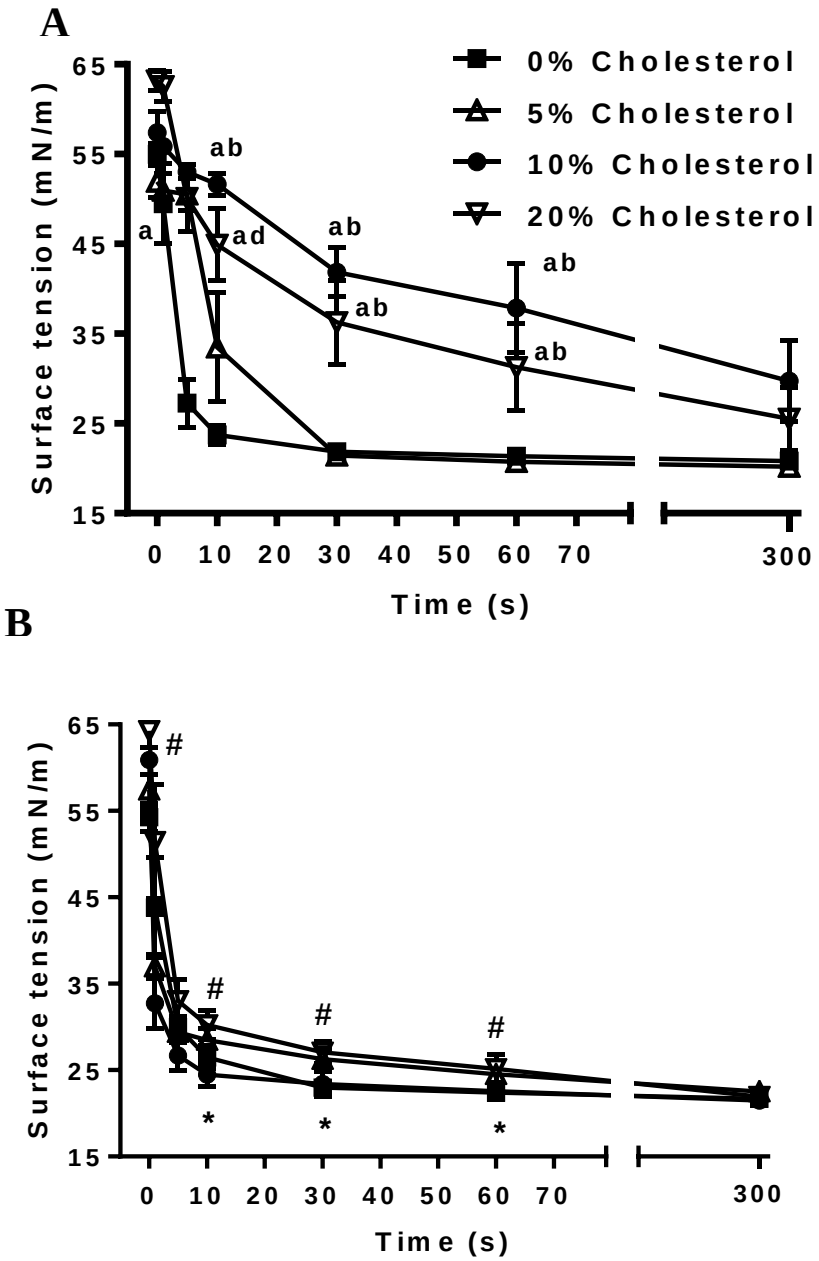

Figure 2.1 Surface tension (mN/m) during 300 s of initial adsorption of human surfactant reconstituted with different levels of cholesterol without (A) and with (B) SP-A, as indicated in the legend. Values are reported as mean ± standard error, a = p <0. 05 versus 0% cholesterol, b = p<0.05 vs. 5% cholesterol, #= p <0.05 versus 10% cholesterol without SP-A, *= p<0.05 vs. 20% cholesterol without SP-A, n = 3/group.

2.3.1.2 Minimum achievable surface tension

The minimum achievable surface tension of human proteinosis surfactant reconstituted with various levels of cholesterol over the course of ten dynamic expansion-compression cycles is shown in **figure 2.2A**. Within each surfactant group, there were no significant differences in minimum surface tension throughout all expansion-compression cycles. Comparison between the surfactant samples with 0% cholesterol and 5% cholesterol revealed only a significantly higher surface tension during 10^{th} dynamic expansion-compression cycle in the 5% cholesterol group as compared to the 0% cholesterol group. In contrast, samples with 10% cholesterol and 20% cholesterol had significantly higher surface tensions than the 0% cholesterol and 5% cholesterol groups throughout all cycles. Additionally, only during cycle #1 did samples containing 20% cholesterol have a significantly higher minimum surface tension than samples containing 10% cholesterol.

The minimum achievable surface tension of human surfactant with various levels of cholesterol and SP-A during expansion-compression cycles 1, 2, 5 and 10 is shown in **figure 2.2B**. No significant differences in minimum surface tension within each surfactant group were found throughout all expansion-compression cycles. Amongst surfactant groups reconstituted with different levels of cholesterol with added SP-A, there were no significant differences in minimum surface tension between groups with 0, 5 or 10% cholesterol throughout all expansion-compression cycles. However, surfactant containing 20% cholesterol + SP-A had significantly higher minimum surface tension than all other surfactant groups reconstituted with SP-A. Additionally, there were significant differences between groups treated with SP-A and those without.

Specifically, surfactant with 5% cholesterol + SP-A had a significantly lower minimum surface tension at the end of cycle 10 as compared to surfactant with 5% cholesterol without SP-A. Similarly, surfactant with 10 and 20% cholesterol + SP-A had significantly lower minimum surface tensions when compared to similar treatments lacking SP-A throughout all expansion-compression cycles.

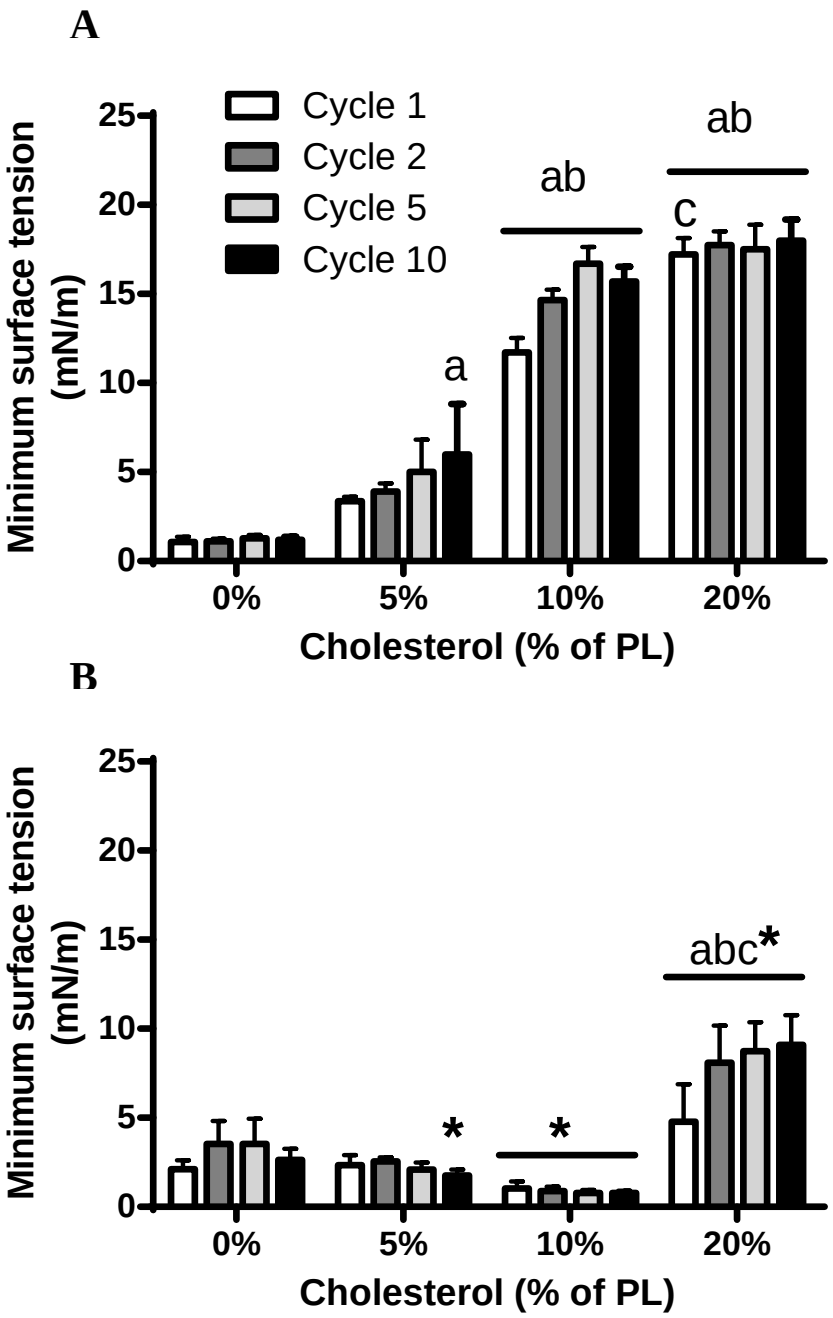

Figure 2.2 Minimum surface tension (mN/m) of human surfactant reconstituted with different levels of cholesterol without (A) and with (B) SP-A during various dynamic compression-expansion cycles. Values are reported as mean ± standard error, a = $p < 0.05$ vs. 0% cholesterol, b = $p<0.05$ vs. 5% cholesterol, c = $p < 0.05$ vs. 10% cholesterol and *= $p<0.05$ versus same sample without SP-A, n = 3/group.

Table 2.1 shows the relative changes in surface area needed to attain minimum surface tension values in samples containing 0 or 20% cholesterol with or without SP-A in order to represent the extreme minimum and maximum cholesterol concentrations in our study. Firstly, there were no differences in the minimum surface tension or relative changes in surface area within each surfactant group between the first and tenth expansion-compression cycle. Secondly, the minimum surface tension achieved in surfactant with 0% cholesterol ± SP-A was lower than the surfactant with 20% cholesterol ± SP-A. However, the % relative area required to attain these values was different between the 0% cholesterol ± SP-A and 20% cholesterol + SP-A. Lastly, surfactant treated with 20% cholesterol alone had increased minimum surface tension and decreased relative surface area than all other samples in compression-expansion cycles 1 and 10.

Table 2.1 Relative surface area (%) required to attain minimum achievable surface tension (mN/m) of human surfactant reconstituted with 0 or 20% cholesterol with and without the addition of SP-A; n = 1/group.

	Expansion-compression cycle #1		**Expansion-compression cycle #10**	
	Surface tension (mN/m)	**% relative area**	**Surface tension (mN/m)**	**% relative area**
0% cholesterol	1.13	51.81	0.91	57.67
0% cholesterol + SP-A	1.95	44.45	2.14	46.56
20% cholesterol	15.70	18.53	15.60	18.45
20% cholesterol + SP-A	10.62	49.69	8.98	47.84

2.3.2 *In vivo* experiment

2.3.2.1 Arterial oxygenation

Surfactant depleted rats were treated with exogenous surfactant of various compositions in order to determine the physiological effects of excess cholesterol and SP-A in surfactant. Arterial oxygenation was measured to determine lung function over the course of 120 minutes of MV at 15 minute intervals. Firstly, **Figure 2.3** shows that the oxygenation of animals receiving 5% cholesterol was significantly increased 15 mins after the last sequential lavage measurement was recorded and remained significantly higher throughout ventilation. Additionally, rats administered surfactant containing 5% cholesterol had significantly higher oxygenation throughout ventilation as compared to rats treated with an air bolus alone. Rats administered surfactant containing 20% cholesterol did have an increase in oxygenation after their last sequential lavage and they had similar oxygenation to animals treated with an air bolus alone throughout ventilation.

We also tested the effects of SP-A on the oxygenation of rats administered surfactant containing 5 or 20% cholesterol. **Figure 2.3** shows that rats administered surfactant containing 5% cholesterol + SP-A had significantly increased oxygenation after the last sequential lavage and significantly higher oxygenation throughout ventilation as compared to rats treated with an air bolus alone. Similarly, rats administered surfactant reconstituted with 20% cholesterol + SP-A had significantly higher oxygenation after their last sequential lavage and also had significantly higher oxygenation than air treated rats from time points 15 – 75.

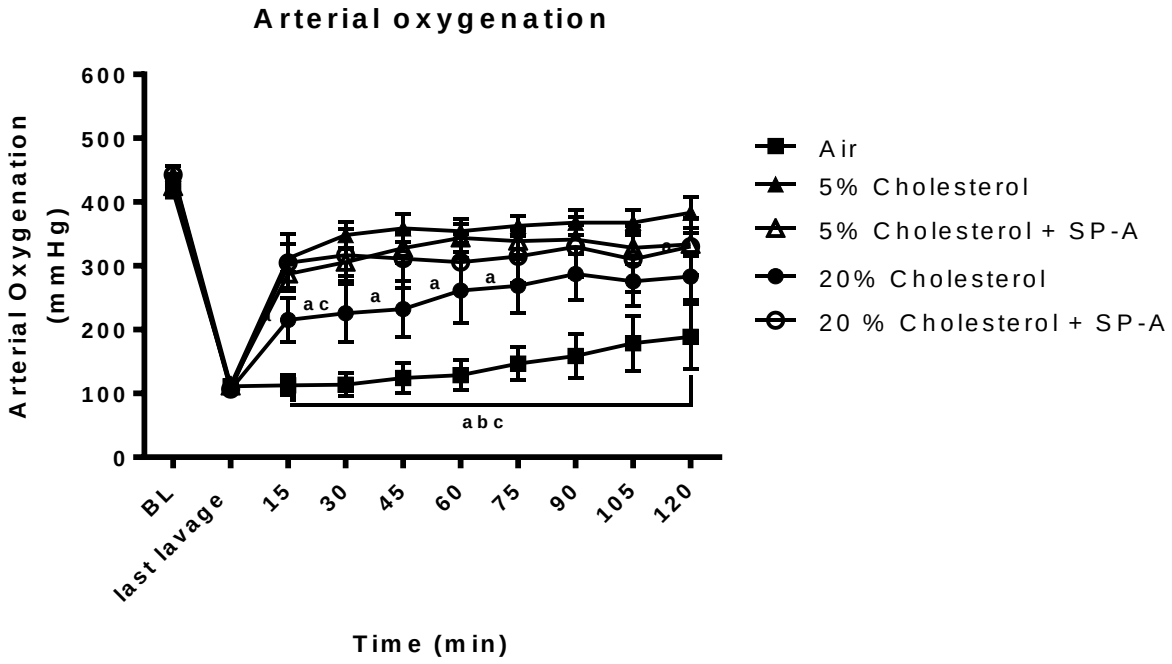

Figure 2.3 Arterial oxygenation content (mmHg) after endogenous surfactant depletion and administration of BLES with various amounts of cholesterol ± SP-A and 120 minutes of mechanical ventilation. Values are means ± SE, n = 6-7/group, a $p<0.05$ vs. 5% cholesterol, b $p<0.05$ vs. 5% cholesterol + SP-A, c $p<0.05$ vs. 20% cholesterol + SP-A.

2.3.3 Surfactant analysis

After 120 minutes of MV, rats were sacrificed and were given a whole lung lavage to recover surfactant. Analysis of collected surfactant from lavage after exogenous surfactant administration and mechanical ventilation showed that within the total surfactant **(2.4A)**, LA **(2.4B)** or SA **(2.4C)** sub-fractions of surfactant, there were no significant differences amongst treatment groups regardless of cholesterol or SP-A content.

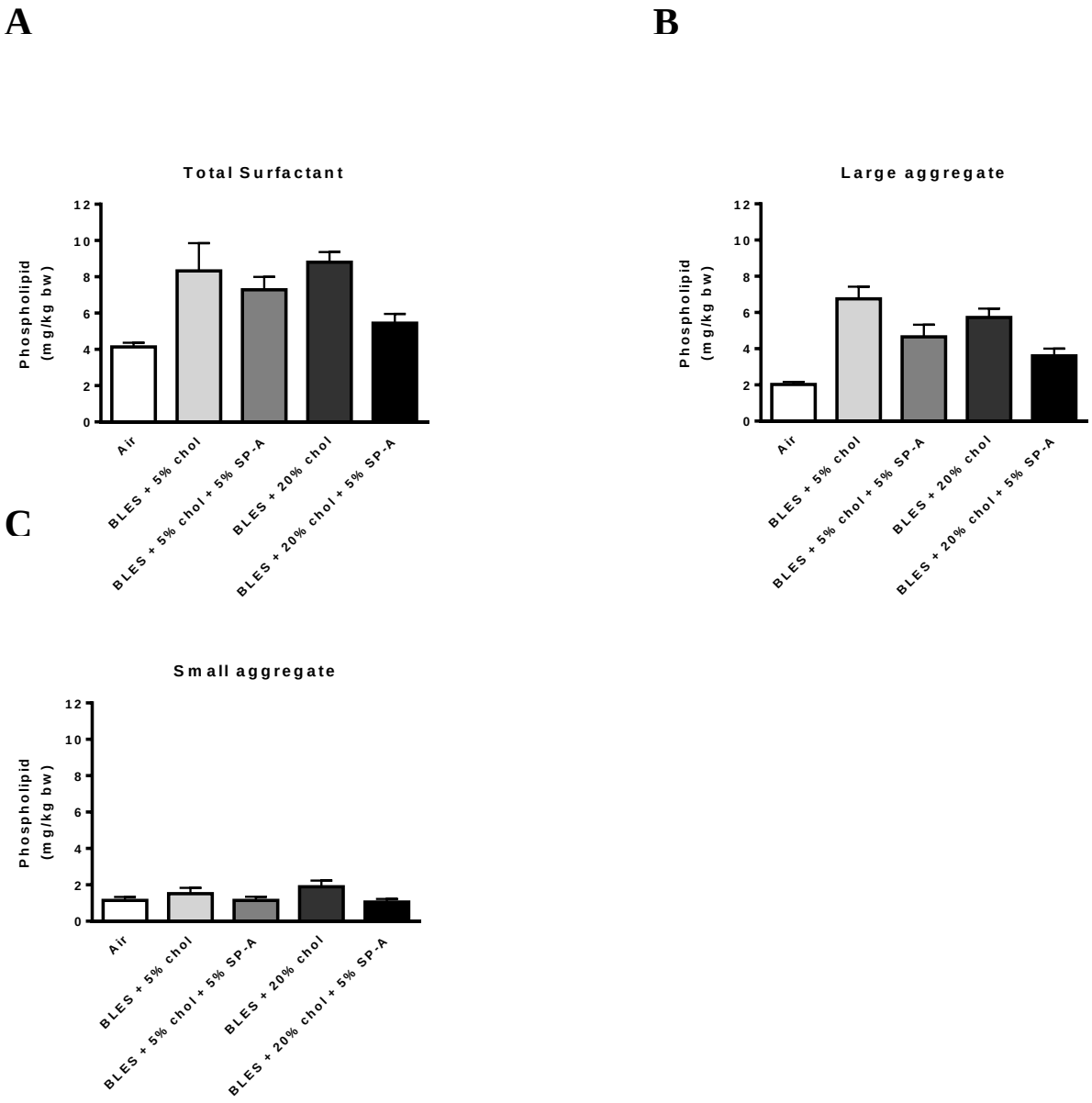

Figure 2.4 Surfactant pool sizes (mg PL/kg BW) between rats depleted of endogenous surfactant administration of BLES with various amounts of cholesterol ± SP-A and 120 minutes of mechanical ventilation. Measurement of (A) total surfactant (B) large and (C) small aggregate pool sizes. Values are means ± SE, n = 6-7/group.

2.4 Discussion

The hypothesis for this study was that elevated cholesterol in surfactant impairs the biophysical properties of the material contributing to decreased lung function, but the presence of SP-A could mitigate these effects. To test this hypothesis we performed *in vitro* and *in vivo* experiments, both of which provided results that indicated that elevated cholesterol was detrimental and that SP-A could mitigate the effect. For the *in vitro* studies outcomes were biophysical in nature, whereas our *in vitro* experiments determined the effects of high cholesterol in surfactant on physiology. Together, these observations support the conclusion that elevated cholesterol in surfactant inhibits the biophysical properties of the material which contributes to lung dysfunction, but SP-A can mitigate these effects and improve physiology.

The first part of our hypothesis examined whether elevated surfactant cholesterol levels would affect the biophysical properties of the material and whether impairments would lead to physiological dysfunction. To investigate this, we tested the biophysical properties of surfactant reconstituted with various levels of cholesterol on the CBS. In a subsequent experiment, we administered surfactant containing 5 or 20% cholesterol to ventilated and surfactant depleted rats. Based on these two experiments, we determined that high cholesterol in surfactant impairs the surface tension reducing capability of the material by observing that surfactant containing 10 and 20% cholesterol had high surface tensions after adsorption and higher minimum achievable surface tensions during

compression. The dysfunction results in impaired oxygenation in rats treated with surfactant containing 20% cholesterol. Previous studies have investigated the physiological effects of surfactant reconstituted with cholesterol (Keating *et al.,* 2007); however, ours is the first to show that cholesterol-mediated surfactant dysfunction results in decreased oxygenation. These findings suggests that biophysical impairment due to high cholesterol in surfactant contributes to dysfunctional lung function and may propagate the development of lung injury.

The second part of our hypothesis was to investigate whether SP-A can mitigate the effects of cholesterol-mediated surfactant dysfunction. Previous *in vitro* studies have determined that reconstitution of SP-A to surfactant inhibited by serum-proteins can mitigate surfactant dysfunction (Cockshutt *et al.,* 1990). Despite this knowledge of SP-A's ability to mitigate this particular form of surfactant inhibition, it was unknown whether it would also be able to restore surfactant function when the material is inhibited by high cholesterol. To determine the effects of SP-A on surfactant inhibited by high cholesterol, we performed two different experiments. Our *in vitro* study utilized surfactant reconstituted with various concentrations of cholesterol with or without 5% SP-A and examined their biophysical properties on the CBS. Our *in vivo* experiment utilized ventilated and surfactant depleted rats and administered exogenous surfactant containing 5 or 20% cholesterol with and without SP-A. These two experiments revealed two important observations regarding SP-A and cholesterol mediated surfactant dysfunction. First, the addition of SP-A improved the biophysical properties of surfactant with high cholesterol by reducing the surface tension after adsorption and minimum

achievable surface tension. Secondly, the presence of SP-A significantly increased the oxygenation in rats treated with surfactant containing 20% cholesterol when compared to baseline (BL) and air treated animals. These observations indicate that SP-A can mitigate cholesterol-mediated surfactant dysfunction which results in significant improvement of the lung's ability to facilitate oxygenation.

While previous studies have shown that SP-A has an important role to enhance the biophysical properties of surfactant, it has also been shown that SP-A, under physiologically normal conditions, is not necessary for lung function (Korfhagen *et al.*, 1996). Specifically, a study conducted by Korfhagen and colleagues (1996) used SP-A null mice and showed that their lung compliance, physiology and surfactant function *in vitro* was not affected as compared to wild-type mice. However, mice lacking SP-A had increased minimum surface tensions when phospholipid concentrations were decreased. This finding indicates that SP-A has an important role in maintaining surfactant function when the system is otherwise compromised. This notion is further supported by work showing that SP-A can mitigate protein-mediated surfactant dysfunction (Cockshutt *et al.*, 1990); oxidized lipid-mediated inhibition (Capote *et al.*, 2003; Bailey *et al.*, 2006) and, as our observations now indicate, cholesterol-mediated dysfunction. Based on these aforementioned studies, while SP-A enhances the biophysical properties of surfactant, SP-A is not wholly necessary for proper lung function under normal and healthy conditions (Korfhagen *et al.*, 1996). However, SP-A has an important role in maintaining lung and surfactant function when surfactant's biophysical properties are compromised by a variety of inhibitory mediators.

The mechanism(s) of cholesterol-mediated surfactant inhibition has been studied, but the observations are open to various, sometimes conflicting, interpretations. For instance, the effect of cholesterol on the biophysical function of surfactant films can vary based on the lipid profile of the film examined. For example, several studies have shown that surfactant films composed of purely DPPC are rendered dysfunctional when physiological amounts of cholesterol are present (Suzuki *et al.,* 1982; Yu & Possmayer, 1998; Yu *et al.,* 1999). In contrast, surfactant films composed of mixed-lipids are not impaired by physiological amounts of cholesterol, but tend to show dysfunction when cholesterol levels are increased (Gunesakara *et al.,* 2005). In general, studies attempting to reconcile the effects of cholesterol on surfactant's biophysical properties suggest that elevated cholesterol affects the fluidity of the lipid film by affecting the organization of surfactant lipids (Keating *et al.,* 2007). Additionally, Panda and colleagues (2004) observed an inability to form organized lipid domains in surfactants with high cholesterol. These lipid structures are not present in dysfunctional surfactant and are thought to be necessary for proper surfactant function. Based on these observations, it is suggested that high cholesterol modifies the lipid organization of surfactant and these changes to the organization of surfactant films are thought to be the principle mechanism of cholesterol-mediated inhibition. Although we have shown that SP-A is able to mitigate the inhibitory effects of high cholesterol on surfactant function, it is currently unclear how SP-A is able to facilitate this. One theory is SP-A is able to act in conjunction with SP-B to enhance the adsorption properties of surfactant which corresponds to improved biophysical function (Venkitaraman *et al.,* 1990). Further

research should be conducted to elucidate the molecular mechanism behind SP-A's ability to mitigate cholesterol-mediated inhibition.

Although not the main focus of this study, we also examined the surfactant subfractions from our ventilated and surfactant depleted rats. This experiment was done in order to better understand the effects of elevated cholesterol and exogenous SP-A on surfactant metabolism in an *in vivo* setting. Previous *in vivo* experiments regarding SP-A and surfactant metabolism have shown that SP-A promotes the re-uptake of surfactant into type-2 cells (Wright & Dobbs, 1991) and may modulate the secretion of surfactant as well (Rice, 1987; Wright & Dobbs, 1991). However, these experiments used concentrations of SP-A that were higher than what would be present physiologically. Based on the surfactant we recovered, it was observed that there were no significant differences in the amounts of total surfactant, SA or LA sub fractions recovered between animals treated with 5 and 20% cholesterol. However, we observed a trend of lower recovered surfactant pool sizes from animals treated with surfactant reconstituted with SP-A as compared to animals treated with surfactant lacking the protein. Thus, our observations suggest that while elevated cholesterol does not affect surfactant metabolism, SP-A may have a regulatory role on surfactant metabolism within the lungs. Lastly, it is important to mention that our *in vitro* determination of surfactant inhibition due to high cholesterol was performed on surfactant obtained from a human patient. Although surfactant composition is highly conserved amongst homeothermic mammals (Veldhuizen *et al.,* 1998), to our knowledge this is the first study to examine the role of

excess cholesterol on human surfactant and, importantly, implicate it as a potential contributor to the development of lung injury in human patients.

2.5 Conclusion

In conclusion, this study demonstrated that elevated levels of cholesterol in surfactant impairs the material's ability to decrease and this has physiological consequences in terms of arterial oxygenation levels in ventilated rats. However, the presence of SP-A can improve the surface tension reducing capability of surfactant with cholesterol mediated impairment which also improves oxygenation *in vivo*.

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Chapter 3

3 The effect of diet-induced serum hypercholesterolemia on the development of lung injury

3.1 Introduction

Acute Respiratory Distress Syndrome (ARDS) is a severe pulmonary disorder caused by both direct, such as acid aspiration and indirect insults to the lungs, such as systemic bacterial infection (Ware & Matthay, 2000). Clinically, ARDS can range from a "mild" form which is defined as having arterial oxygenation between 200 mmHg < PaO2/FiO2 ≤ 300 mmHg to "severe" ARDS with PaO2/FiO2 ≤ 100 mmHg (Rubenfeld *et al.*, 2012). Currently, patients afflicted with ARDS require the use of mechanical ventilation (MV) as a supportive measure (Lee & Slutsky, 2001); however, MV also carries a risk of causing further pulmonary damage (Webb & Tierney, 1974; Dreyfuss & Saumon, 1998; Brower *et al.*, 2000; Veldhuizen *et al.*, 2001). Overall, patients with ARDS have a 30% mortality rate with no known curative therapy (Brower *et al.*, 2000). This high risk of mortality due to ARDS emphasizes the need to better understand the pathophysiology of the disorder.

Although ARDS can arise from both direct and indirect insults to the lungs, one common feature among patients with the condition is impairment to their pulmonary surfactant (Gregory *et al.*, 1991; Veldhuizen *et al.*, 1995; Gunther *et al.*, 1996). Pulmonary

surfactant is a lipoprotein complex found at the air-liquid interface of alveoli (Possmayer *et al.,* 1984; Goerke, 1998; Possmayer, 2004). It is produced by type-two alveolar cells which release surfactant in the form of what are called large aggregates (LA) into the hypophase within alveoli where it can form a surface-active film. This surface active film decreases the effort required to breathe and prevents alveolar collapse at low lung volumes (Possmayer, 2004). Changes to the alveolar surface area during respiration causes LA to convert into a non-surface active component (small aggregates, SA) (Gross, 1995; Lewis *et al.,* 1990). The SA are then taken-up by type-two cells and can be recycled into large aggregates (Wright, 1991) or processed for degradation (Wright, 19901. Surfactant is composed mainly of phospholipids (80-90%) but also contains neutral lipids, such as cholesterol (3-10%), and surfactant associated proteins (5-10%) (Veldhuizen *et al.,* 1998; Possmayer, 2004). The precise balance of surfactant's constituent components is paramount to the material's biophysical properties and changes to the composition can impair surfactant's ability to function.

In the context of ARDS, changes to surfactant's composition and function have been shown to be associated with this condition. Numerous studies have demonstrated alterations to the surfactant phospholipid composition (Gunther *et al.,* 1996; Schmidt *et al.,* 2001), changes to the surfactant proteins levels (Gregory *et al.,* 1991; Gunther *et al.,* 1996; Schmidt *et al.,* 2007) and decreased levels of the LA form of surfactant (Veldhuizen *et al.,* 1995; Gunther *et al.,* 1996). These changes, together with inhibition by serum proteins that have leaked into the lung (Cockshutt, 1991), have traditionally thought to be the main contributors to surfactant dysfunction. Interestingly, recent *in vitro*

studies and experiments with animal models of lung injury have demonstrated that an excess cholesterol within surfactant also represent an important mechanism by which surfactant becomes dysfunctional (Davidson *et al.,* 2000; Panda *et al.,* 2004; Gunasekara *et al.,* 2005; Vockeroth *et al.,* 2010). For example, an experiment using an *in vivo* rat model of ventilator induced lung injury (VILI) showed that removing cholesterol from the surfactant obtained from injured animals restored its ability to reduce surface tension (Vockeroth *et al.,* 2010). Since the majority of surfactant cholesterol originates from the serum with only 1% of surfactant cholesterol endogenously produced within the lungs (Guthmann *et al.,* 1997; Orgeig & Daniels, 2001) this observation of cholesterol-mediated inhibition has raised the question whether serum hypercholesterolemia increases one's susceptibility towards developing more severe lung injury

Therefore, to investigate the role of serum cholesterol in lung injury and surfactant, we used a model of diet-induced hypercholesterolemia in male Wistar rats and randomized them into three distinct injury models: a ventilator-induced lung injury (VILI) model, an acid-aspirated lung injury model and a surfactant depleted lung injury model. In all of these models, outcomes that were assessed were physiological measurements and surfactant analysis. We hypothesized that serum hypercholesterolemia will predispose the rats to developing severe lung injury due to changes to their pulmonary surfactant system.

3.2 Methods

3.2.1 Animals

Eighty-six six-week old male Wistar rats (Charles River, St. Constant, PQ, CA) were used for this experiment. All procedures were approved by the animal use subcommittee at the University of Western Ontario in agreement with the guidelines of the Canadian Council of Animal Care. Animals were acclimatized for three days and had free access to water and a standard laboratory diet. Following this period, animals were randomized into either a standard laboratory diet ("standard diet") or a high cholesterol diet ("high cholesterol diet": 0.5% cholic acid and 1.25% cholesterol by mass; Harlan Teklad, Madison, WI, USA). Rats were fed their respective diet for 17 – 20 days.

3.2.2 Surgical procedure

All animals included in each of the 3 experimental models that underwent MV all underwent the same initial surgical procedure. This surgical procedure was similar to those used by Maruscak and colleagues (2008) and Vockeroth and colleagues (2010). Animals were anesthetized via intraperitoneal injection of 75 mg/mL ketamine and 5 mg/mL xylazine in sterile 0.15 M NaCl. Once a reaction from a toe-pinch could not be observed, animals were given a subcutaneous injection of an analgesic (0.1 mg/kg buprenorphine) and a 0.2 mL subcutaneous injection of a topical anesthetic (sensorcaine 0.5%) at the site of incision. The left and right jugular veins and the right carotid artery were exposed and catheterized with PE-50 tubing. The left jugular catheter was used to

deliver anesthetic/analgesic (0.5 – 2.5 mg/100g/h propofol) and the right jugular catheter was used to deliver fluid (sterile NaCl with 100 IU heparin/L) continuously (0.5 – 1.0 mg/100g/h). The carotid artery catheter was used to measure blood pressure and heart rate, collect blood for blood gas measurements (ABL 500 Radiometer, Copenhagen, DK) and deliver fluids (sterile NaCl with 100 IU heparin/L) continuously (0.5 – 1.0 mg/100g/h). Fluids and anesthetic/analgesic were delivered via infusion pumps (Harvard Apparatus, Model 22). Subsequently, the trachea was exposed and a 14 gauge endotracheal tube was inserted and secured with 3-0 silk.

Following surgical preparation, animals were given a 0.1 mL bolus of a neuromuscular inhibitor (2 mg/mL pancuronium bromide) and immediately connected to a volume-cycled rodent ventilator (Harvard Instruments, St. Laurent, PQ, CA) set to deliver 8 mL/kg tidal volume (Vt), respiratory rate (RR) of 54-58 breaths per minute (bpm), 5 cmH_2O positive end-expiratory pressure (PEEP) and a fraction of inspired oxygen (FiO_2) of 1.0. Animals were ventilated for fifteen minutes prior to measuring initial baseline blood-gas measurements. The baseline (BL) inclusion criteria following this procedure was a blood gas measurement while breathing 100% oxygen of $PaO_2/FiO_2 \geq 400$ mmHg.

3.2.3 High tidal volume mechanical ventilation model of lung injury

In this experiment standard diet and high cholesterol diet animals each were randomized to 3 experimental groups: non-vent, low-stretch and high-stretcth mechanical ventilation for 120 mins. The ventilator procedure used in this part of the study was based on the

procedure used by Maruscak and colleagues (2008). Animals randomized to no mechanical ventilation ("Non-vent", n = 5-6/group) were sacrificed via sodium pentobarbital overdose delivered via intraperitoneal injection. A total of 9 – 10 mL aliquot of blood was collected via the descending aorta. Of this, 3.5 mL was collected in a 6 mL Lithium Heparin Vacutainer tube (BD, Franklin Lakes, NJ, USA) for total serum cholesterol, triglyceride and HDL analysis. 5.5 – 6.5 mL of blood was collected in a 10 mL syringe with 0.1 mL of heparin (1000 USP/mL) for further serum analysis. Animals were then exsanguinated by severing the descending aorta. A midline sternectomy was performed to expose the chest cavity and a pressure-volume curve was determined and animals were treated to a whole-lung lavage as described below.

Animals randomized to one of the ventilation strategies underwent the surgical procedure described above followed by either continued ventilation with "low stretch" (n = 6-7: Vt = 8 mL/kg, RR = 54-58 bmp, PEEP = 5 cmH_2O, FiO_2 = 1.0), or "high stretch" (n=4-6: Vt = 30 mL/kg, RR = 16 – 18 bmp, PEEP = 0 cmH_2O, FiO_2 = 1.0). Animals were detached from the initial ventilator for 10 seconds and re-attached to the either the ventilator with the low stretch or the high stretch settings to begin the 2 hours of ventilation. Airway pressure, hemodynamics, and blood gas measurements were monitored every 15 minutes.

In a separate experiment, animals fed a standard or high cholesterol diet were ventilated with high-stretch ventilation for 180 minutes to ascertain whether the effects of diet

induced serum hypercholesterolemia on lung function would be further exacerbated over time.

3.2.4 Blood collection and lavage collection.

After 120 or 180 mins of ventilation, 3.5 mL of blood was collected from animals via the carotid artery catheter in a 6 mL Lithium Heparin Vacutainer tube (BD Biosciences, Franklin Lakes, NJ, USA) for serum cholesterol analysis. Following, animals were euthanized with an overdose of sodium pentobarbital (110 mg/kg) via the left jugular catheter to lower blood pressure. Animals were then exsanguinated by severing the descending aorta and a midline sternectomy was performed to open the chest cavity. After, lungs were washed with a whole-lung lavage using five 10 mL aliquots of 0.15 M saline. Each aliquot was instilled and removed three times and the total volume of the five aliquots was recorded (Veldhuizen *et al.*, 2002). The lavage material was subsequently processed as described below.

3.2.5 Acid induced lung injury model

The second model of injury was a model of acid aspiration injury. For this part of the study, a similar methodology as described by Folkesson and colleagues (1995) was used to generate an acid-induced lung injury model. Following the surgical procedure described above, rats meeting the inclusion criteria were randomized to receive either 1M HCl (1 mL/kg) or air (1 mL/kg). The rats were disconnected from ventilation and placed on their right side. Half of the treatment volume was administered intratracheally via

pipette after which the animal was reconnected to the ventilator and given two breath holds. The animal was then placed in a supine position for five breaths after which the animal was disconnected from the ventilator and the procedure was repeated on its left side. Rats were then ventilated with Vt = 8 mL/kg, RR = 54-58 bmp, PEEP = 5 cmH_2O and FiO_2 = 1.0 for 120 minutes with airway pressure, hemodynamics, and blood gas measurements monitored and recorded every 15 minutes. Animals were euthanized and lavaged as described above.

3.2.6 Surfactant depletion model

The third model involved surfactant depletion. Our methods were similar to described by Bailey and colleagues (2006) and Keating and colleagues (2007). Animals meeting the inclusion criteria following the surgical procedure described above were disconnected from the ventilator subjected to a whole lung lavage using 10 mL 0.15 N NaCl via syringe attached to the endotracheal tube. The peak inspiratory pressure (PIP) pre/post lavage and lavage volume administered/recovered were recorded. After withdrawal of the lavage, the animal was re-attached to the ventilator and given a single breath hold. This process was repeated four times with 10 minutes between each lavage. Following the fourth lavage, a blood gas measurement was taken to determine if the animal had met the second inclusion criteria of $PaO_2 \leq 250$ mmHg and PIP ≤ 16 cmH_2O. Animals failing to meet the criteria were treated with further lavages ranging in volumes from 2-5 mL up to a maximum of seven total lavages. These repeated lavage samples were collected and analyzed for surfactant composition as described below. Subsequently, animals were ventilated for 120 min, euthanized and lavaged as described above.

3.2.7 Serum total cholesterol analysis

Blood samples collected in Lithium Heparin Vacuatiner tubes were analysed for total cholesterol levels via as performed by the University Hospital Core Lab (University Hospital, London, ON, CA).

3.2.8 Surfactant processing and analysis

Blood samples and post-ventilation and total lavage were centrifuged at 150 g for 10 mins. The resulting blood serum was extracted and stored at -80°C. Five mL of supernatant from the lavage containing the surfactant was aliquoted in 1 mL volumes and stored at -20°C. The remaining surfactant (TS) was centrifuged at 40 000g for 15 minutes to separate the supernatant containing the small aggregate (SA) (Yamada *et al.*, 1990) sub-fraction and the pellet containing the large aggregate (LA) (Veldhuizen *et al.*, 1993); the LA was re-suspended in 2 mL of 0.15 M NaCl saline. Surfactant and surfactant sub-fraction samples were stored with the collected surfactant at -20°C.

A modified Duck-Chong phosphorous assay was performed on each total surfactant, LA and SA sample to determine the phospholipid content in each (Duck-Chong, 1979). Briefly, aliquots of total surfactant, LA and SA samples were added to a solution containing 1 mL ddH2O, 1 mL methanol and 2 mL chloroform. This mixture was then centrifuged at 150 g for 10 mins to separate the aqueous and organic solvent layers. The

organic solvent layer was extracted and dried-down via exposure to O2 gas in a 37°C water bath until the liquid chloroform had evaporated. The remaining solute was ashed on a hot-plate after which 1 mL of 1 N HCl was added. Then, the samples were then placed into a heating block at 100°C for 15 mins. Malachite green dye was added to all surfactant samples and the resulting colour was analyzed on a plate reader.

Total protein in surfactant samples were measured via micro BCA protein assay according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA). Free cholesterol in LA samples was determined by a Free Cholesterol-E kit according to the manufacturer's instructions (Wako Chemicals, Richmond, VA, USA).

3.2.9 Statistical analysis

Data are expressed as means ± standard error (SE). Differences between groups measured over time was done by a two-way repeated measures analysis of variance (ANOVA) with Tukey's post-hoc test. A two-way student's T-test was used to determine significance for comparisons between two groups and in the surfactant depletion study. For all other measures, a two-way ANOVA followed by Tukey's post-hoc test was used. All statistics were performed using statistical analysis program GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Probability (p) values of less than 0.05 were considered statistically significant.

3.3 Results

Figure 3.1 shows the concentration of total serum cholesterol in rats after 14-17 days on either the standard or high cholesterol diet. Rats fed the high cholesterol diet had significantly higher total serum cholesterol compared to rats fed the standard diet ($P<0.0001$). However, there were no significant differences between the weights of rats fed the standard or high cholesterol diet (369 ± 4 g vs. 366 ± 4 g, respectively).

3.3.1 Experiment #1: High tidal volume mechanical ventilation model of lung injury

Figure 3.2 shows the peak inspiratory pressure **(PIP)** of rats placed on either **(A)** low stretch or **(B)** high stretch MV. The PIP of animals placed on low stretch MV did not show significant increases at any time point from BL through to the end of MV. Additionally, there were no significant differences of PIP at any time point during MV between animals fed a control or high cholesterol diet. In contrast, rats placed on high-stretch MV did have significantly increased PIP immediately after BL. However, there were no significant differences in PIP of animals fed either the control or high cholesterol diet throughout the duration of MV.

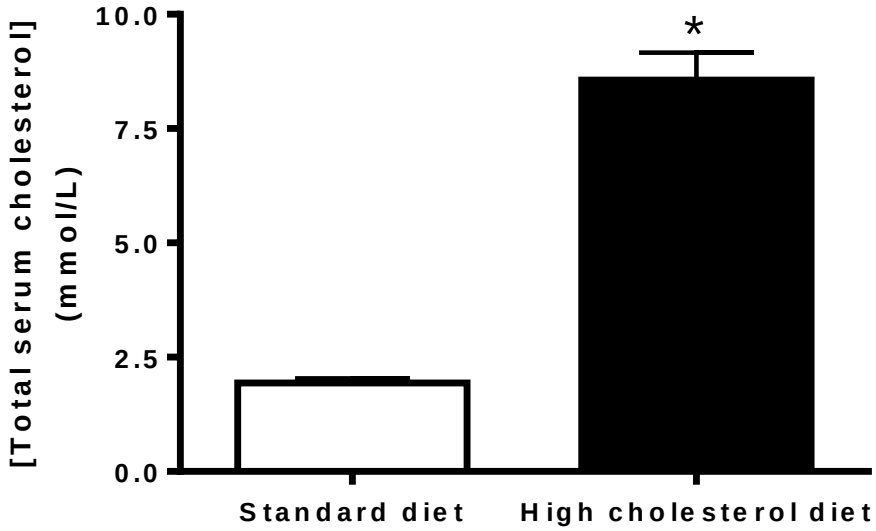

Figure 3.1 Concentration of total serum cholesterol (mmol/L, n = 14 – 19/group) in rats fed either a standard or high cholesterol diet. Values are means ± SE, *P<0.0001 vs. standard diet.

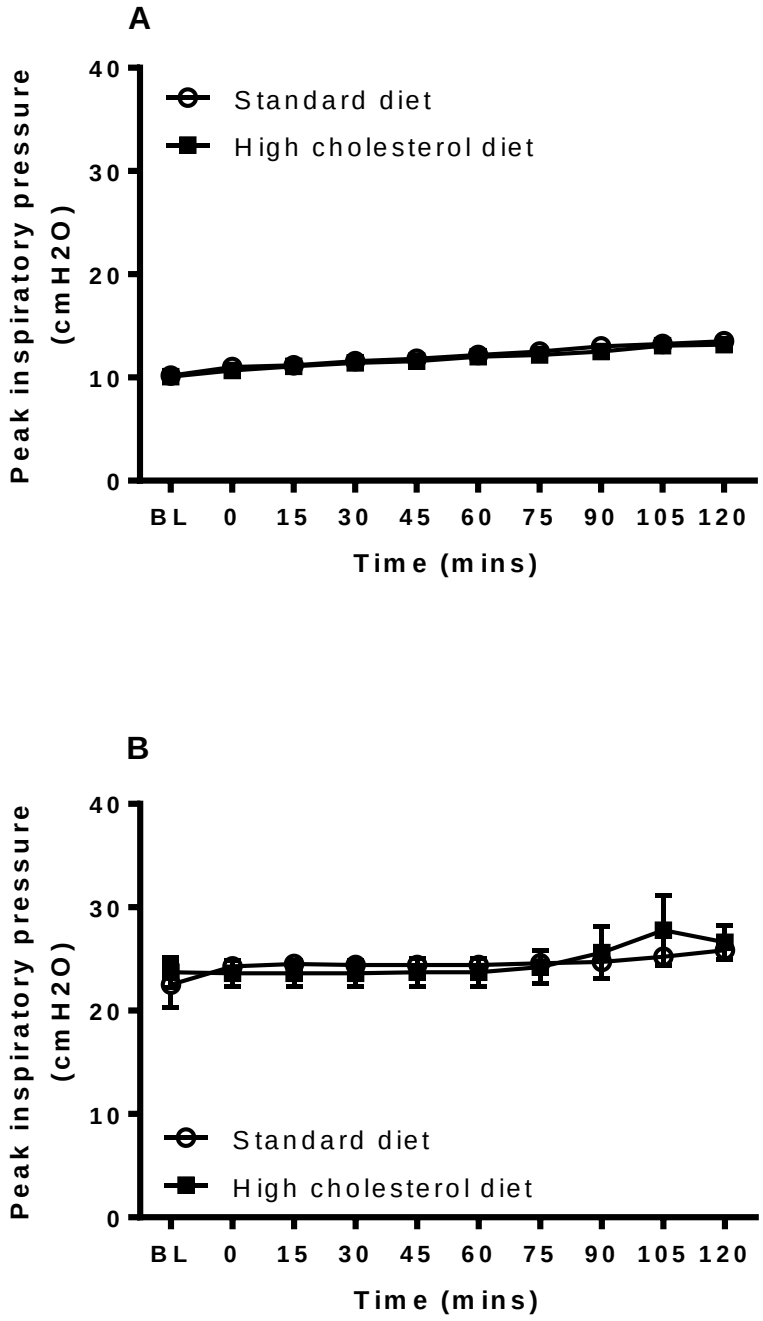

Figure 3.2 Peak inspiratory pressure (cmH2O) throughout 120 minutes of low stretch (A) or high stretch (B) ventilation. Values are means ± SE, n = 5-7/group.

Figure 3.3 shows the arterial oxygenation **(PaO_2)** of rats placed on the standard or high cholesterol diet and randomized to receive either **(A)** low stretch or **(B)** high stretch mechanical ventilation. In rats ventilated with low-stretch ventilation, there were no significant differences between oxygenation values throughout ventilation as compared to BL values in either diet group. Furthermore, there were no significant differences in oxygenation between rats fed a control diet or rats fed a high cholesterol diet at any time point during MV. Similarly, rats placed on high-stretch MV did not show any significant changes in oxygenation after BL and there were no significant differences in oxygenation between rats fed a control diet or rats fed a high cholesterol diet **(figure 3.3B)**.

Surfactant **(A)**, SA **(B)**, LA **(C)** pools and percentage cholesterol within the large aggregate component of surfactant **(D)** are shown in **figure 3.4**. There were significant increases in the amount of surfactant, SA and LA found within the lavage of animals placed on high-stretch MV as compared to non-ventilated animals and a significant increase in the amount of surfactant and SA in animals placed on high-stretch MV as compared to the low-stretch group **(figure 3.4A, B, C)**. There were no significant differences in pool size between animals in either diet strategy in any of the surfactant subfractions measured. Furthermore, there was no significant difference in the percentage of cholesterol found within the surfactant between ventilation strategies or dietary conditions within each ventilation group **(figure 3.4D)**.

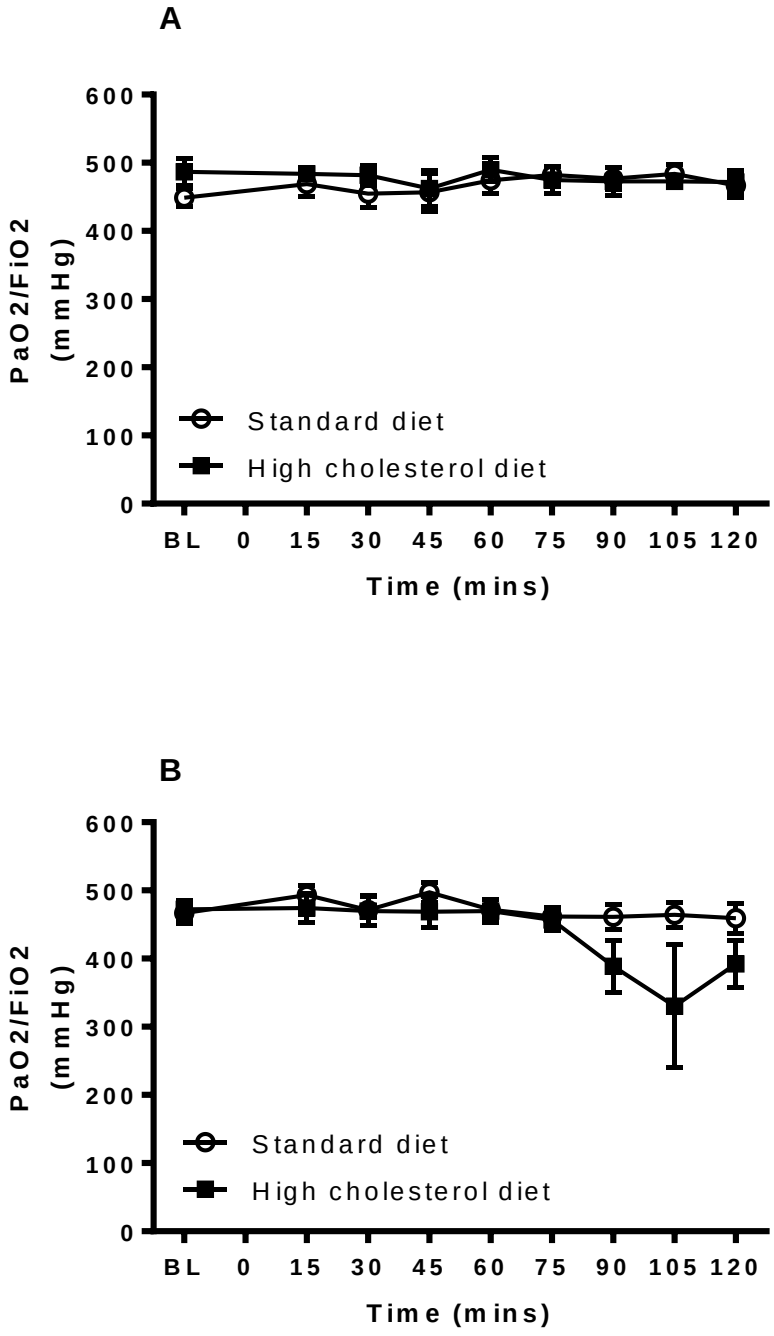

Figure 3.3 Arterial oxygen (mmHg) throughout 120 minutes of low stretch (A) or high stretch (B) ventilation. Values are means ± SE, n = 5-7/group

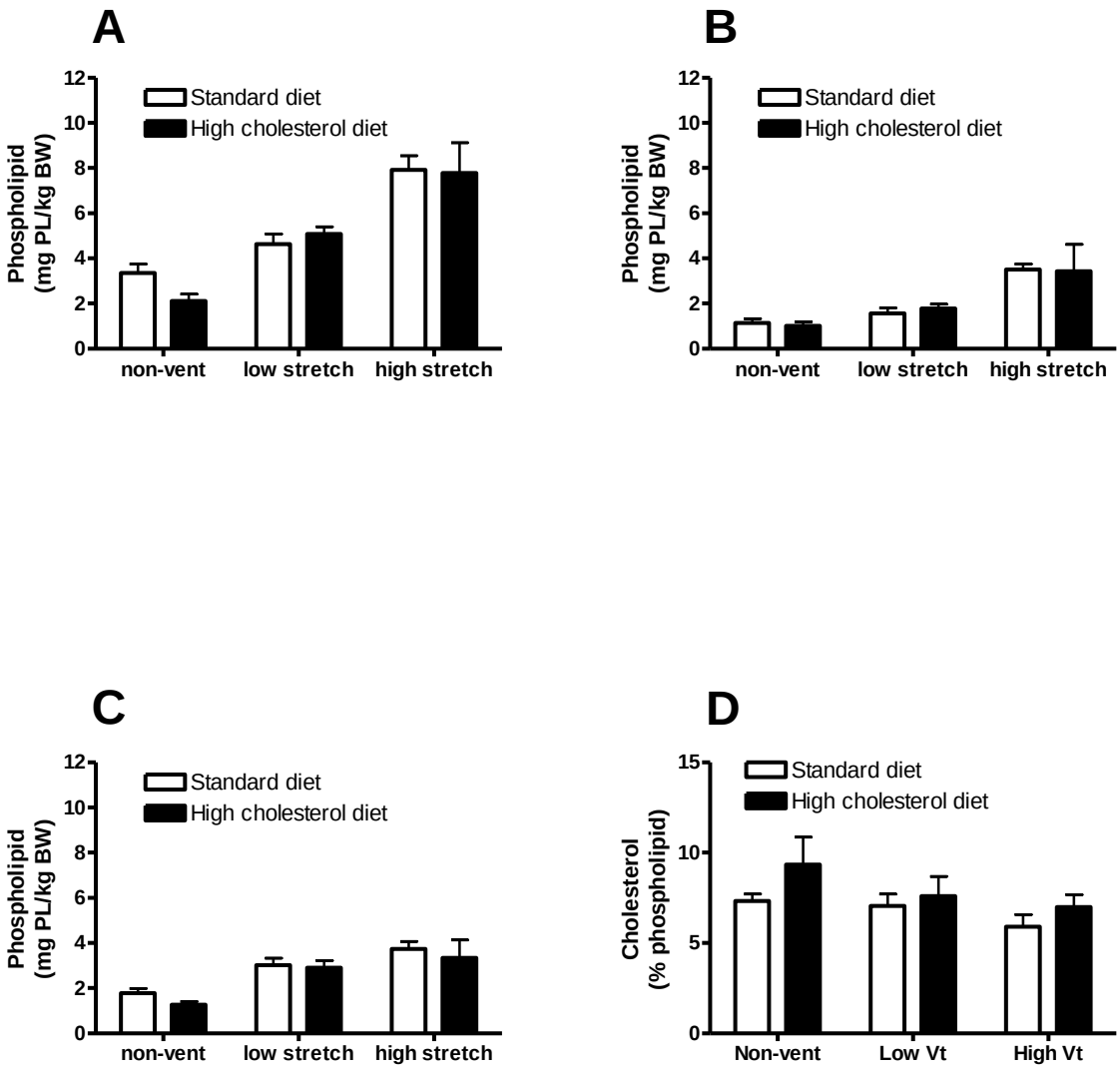

Figure 3.4 Surfactant pool sizes (mg PL/kg BW) between dietary conditions and ventilation strategies. Measurement of (A) total surfactant (B) small aggregate (C) large aggregate pool sizes and (D) percentage of cholesterol LA. Values are means ± SE, n = 5-7/group

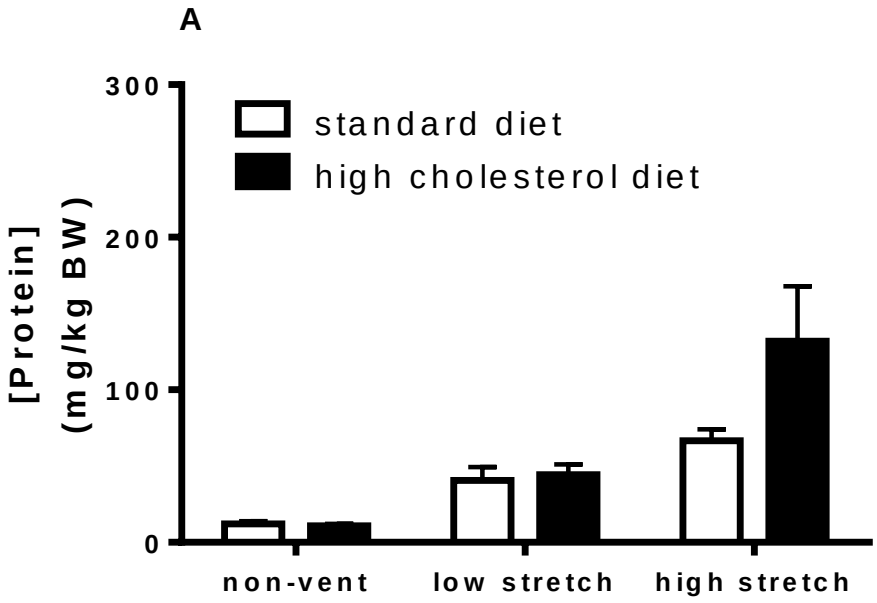

Figure 3.5 Concentration of protein (mg/kg BW) in surfactant among diet and ventilation groups. Values are means ± SE, n = 5-7/group.

Figure 3.5 shows the total protein within the lung lavage from rats in both diet conditions placed in each ventilation strategy. Rats placed on low- or high-stretch MV had a significantly increased amount of total protein within their lavage as compared to non-ventilated rats. However, there were no significant differences in the amount of lavage protein between diet conditions.

In additional experiment, a separate cohort of rats fed either high cholesterol or standard diet were exposed to high-stretch MV for 180 mins. This was done to exacerbate the degree of injury as the PaO_2 of rats placed on high-stretch ventilation after 120 mins did not significantly decrease over time. After 180 minutes of high-strech MV, there was a significant increase in the PIP of the animals from t=15-180 mins **(figure 3.6A)** as compared to BL and there was a significant decrease in the PaO_2 of the animals after 150 mins of ventilation **(figure 3.6B)**. However, there were no significant differences in terms of PIP or PaO_2 between rats fed either diet at any time point during ventilation **(figure 3.6A, B)**.

Table 3.1 shows that after 180 minutes of high-stretch MV, there were no significant differences in phospholipid pool sizes for total surfactant, SA, LA or cholesterol levels in the lavage of rats fed either diet. Also, it was revealed that there was no significant difference between rats fed a standard or high cholesterol diet in terms of the amount of total protein found within the lung lavage of rats placed on either diet after 180 minutes of high stretch mechanical ventilation.

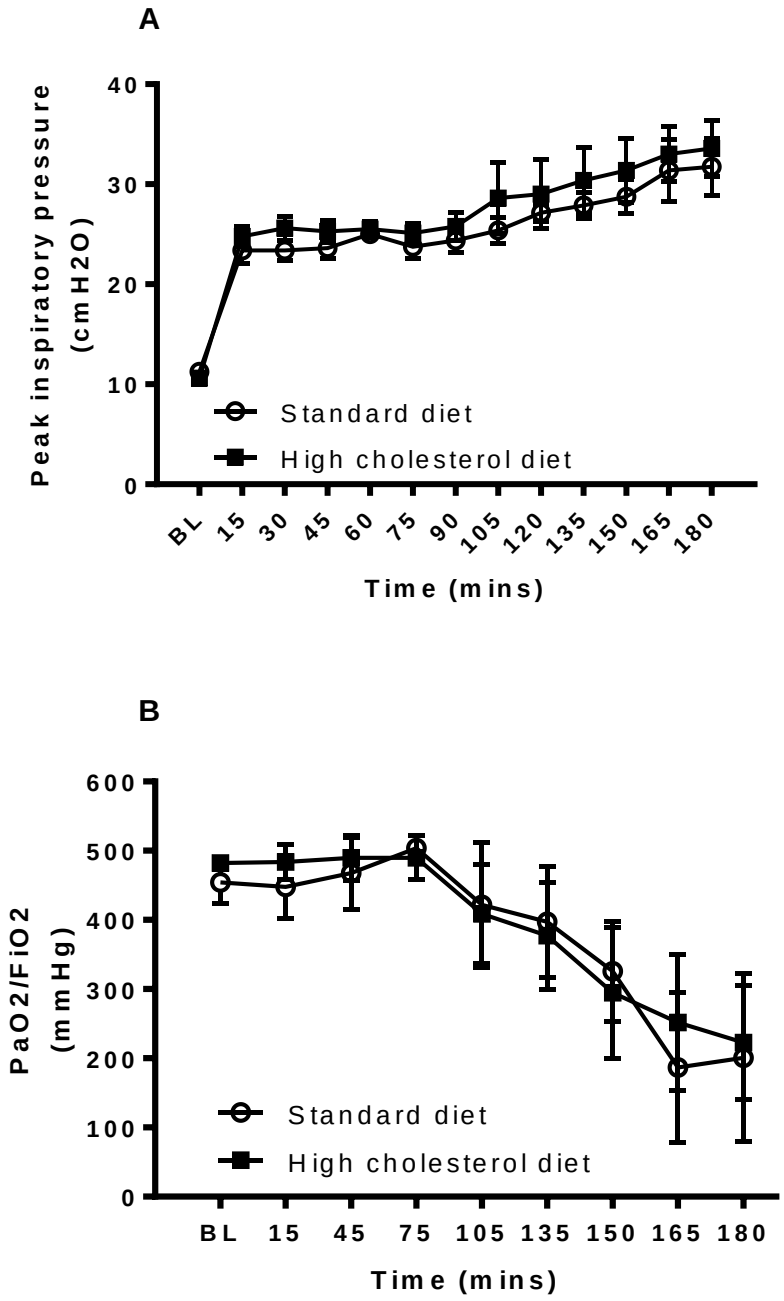

Figure 3.6 Peak inspiratory pressure (cmH2O) (A) and arterial oxygenation content (mmHg) (B) throughout 180 minutes of high stretch mechanical ventilation. Values are means ± SE, n = 4-5/group

Table 3.1 Surfactant pool sizes (mg PL/kg BW), lavage cholesterol levels (% of PL) and lavage protein levels (mg/kg BW) between dietary conditions after 180 minutes of high stretch mechanical ventilation. Values are means ± SE, n = 4-5/group.

Diet	**Total surfactant (mg PL/kg BW)**	**SA (mg PL/kg BW)**	**LA (mg PL/kg BW)**	**Lavage cholesterol (% of PL)**	**Lavage protein (mg/kg BW)**
Standard	6.37±0.92	2.03±0.35	3.27±0.56	10.45±1.26	150.5±41.5
High cholesterol	6.24±0.87	1.89±0.51	2.06±0.15	11.98±0.67	196.6±77.8

3.3.2 Experiment #2: Acid-induced lung injury model

Figure 3.7A shows the PIP of rats fed different diets after administration air or acid followed by 120 mins of MV. Rats given a bolus of acid showed an increase in their PIP throughout the course of MV as compared to BL, however these increases were not significant. There were no observed changes in the PIP of rats given a bolus of air at any time point during MV. With regards to the effect of diet, rats fed either a standard or high cholesterol diet did not have any effect on PIP regardless of air or acid administration. This effect was consistent throughout 120 mins of MV.

Figure 3.7B shows the PaO2 of rats fed different diets after administration of air or acid during 120 mins of MV. Although rats administered a bolus of acid resulted in significantly lower PaO2 as compared to rats administered air from t=15 mins onwards, rats fed either standard or high cholesterol diet did not show any significant differences regardless of air or acid administration any time point during ventilation.

Analysis of collected lavage surfactant after air or acid administration and mechanical ventilation showed that there were no significant differences between the pool sizes of surfactant **(Figure 3.8A)**, SA **(Figure 3.8B)** or LA **(Figure 3.8C)** in rats administered either air or acid and fed the standard or high cholesterol diet. Similarly, there was no significant difference in the amount of cholesterol within the LA **(Figure 3.8D)** regardless of diet or air or acid administration.

With regards to the concentration of total protein **(Figure 3.9)** within the lavage after 120 minutes of mechanical ventilation, there a significant increase in the amount of total protein and in the lavage of animals given acid as compared with air. However, there were no significant differences in the amount of total protein in rats fed a standard or high cholesterol diet regardless of administration of air or acid.

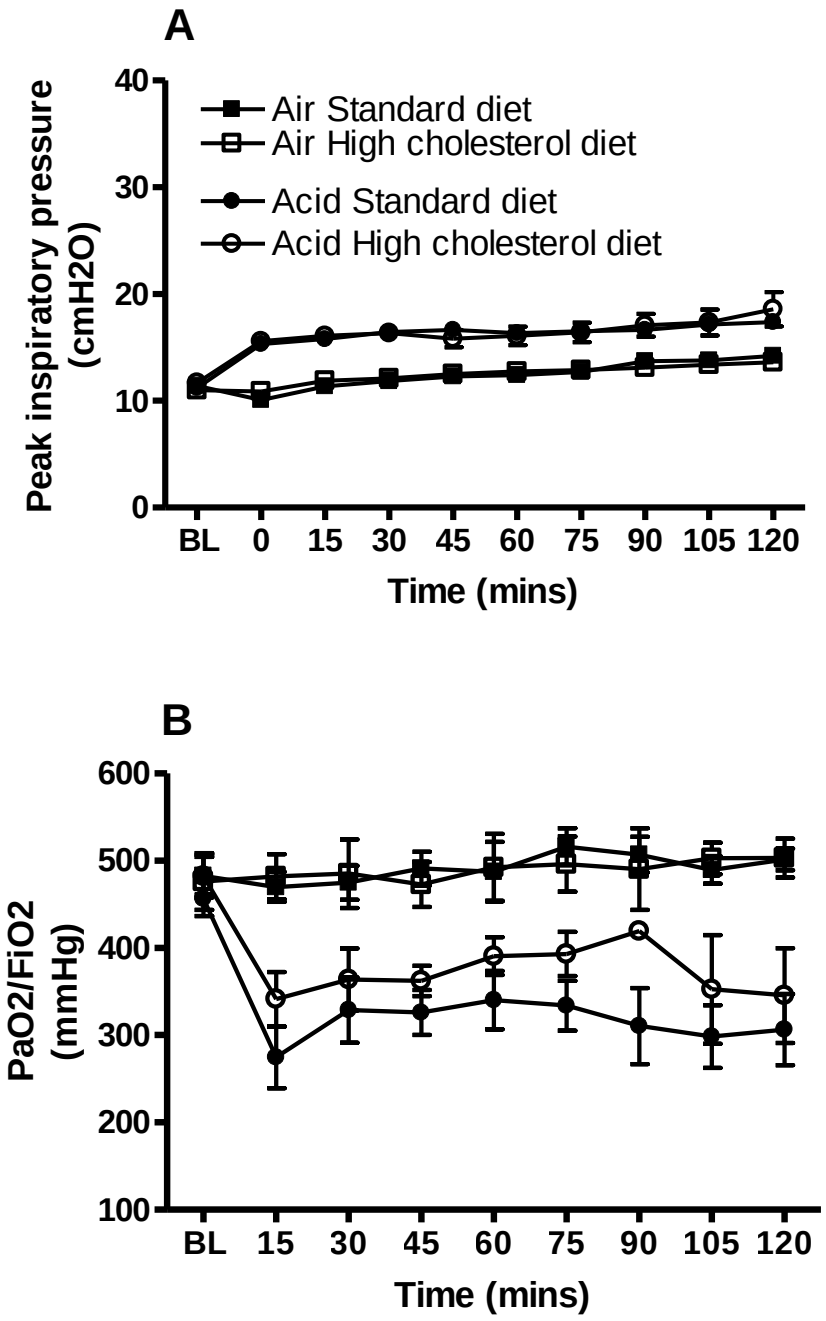

Figure 3.7 Peak inspiratory pressure (cmH2O) (A) and arterial oxygenation content (mmHg) (B) in rats with and without acid induced lung injury and after 120 minutes of mechanical ventilation. Values are means ± SE, n = 4-9/group.

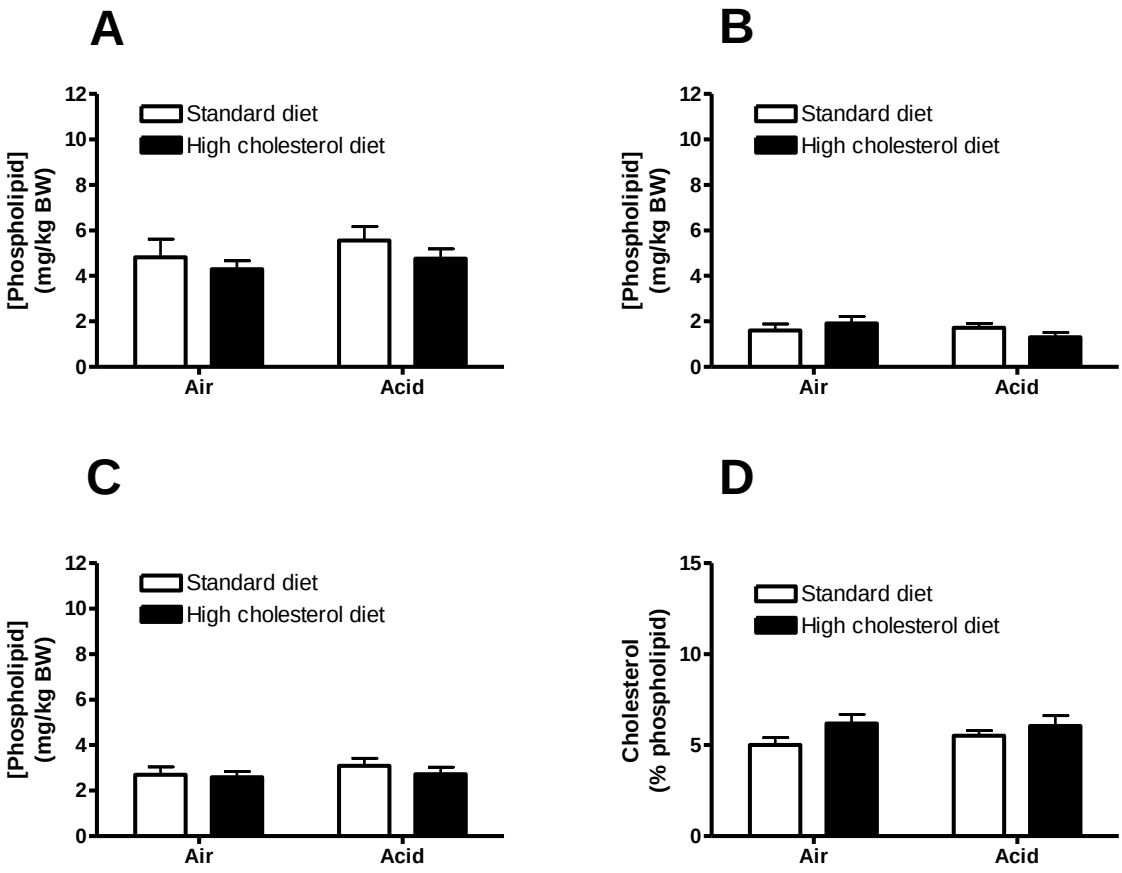

Figure 3.8 Surfactant pool sizes (mg PL/kg BW) between dietary conditions after acid/air treatment and 120 minutes of mechanical ventilation. Measurement of (A) total surfactant (B) small aggregate (C) large aggregate pool sizes and (D) percentage of cholesterol within LA. Values are means ± SE, n = 4-9/group.

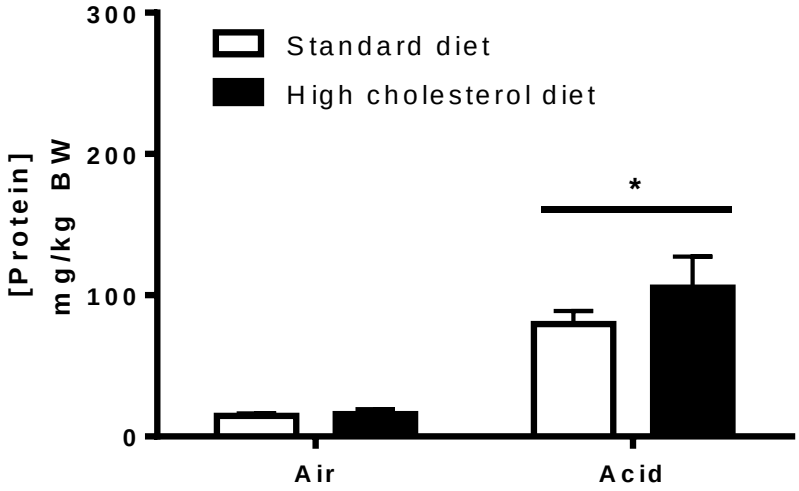

Figure 3.9 Concentration of protein (mg/kg BW) in surfactant in rats fed a standard or high cholesterol diet with and without acid induced lung injury after 120 minutes of mechanical ventilation. *P<0.05 vs. rats fed a similar diet but treated with air. Values are means ± SE, n = 4-9/group.

3.3.3 Experiment #3: Surfactant depletion model

Rats from both diet groups were depleted of surfactant via repeated whole-lung saline lavages and ventilated for 120 mins. There was no significant difference between the number of lavages necessary to deplete the rats of their surfactant between rats fed the standard diet or high cholesterol diet (5.12±0.35 vs. 4.88±0.40 lavages, $p>0.05$). **Table 3.2** compares the surfactant, SA and LA phospholipid amounts in the first and final sequential lavages in rats fed the standard or high cholesterol diet. There were no significant differences in the amount of surfactant, SA or LA found between each diet, but there was a significant decrease in the amount of phospholipid between the first and final sequential lavages across surfactant, SA and LA ($P<0.05$) within each diet group.

Figure 3.10A shows the PIP of rats at BL and after endogenous surfactant depletion fed either a standard or high cholesterol diet. During the course of MV, there was a significant increase in the PIP as compared to BL, however there were no significant differences in the PIP of rats fed either a standard diet or high cholesterol diet. This trend was evident throughout 120 mins of MV.

Figure 3.10B shows the PaO2 of rats at BL and after exogenous surfactant depletion fed either a standard or high cholesterol diet. Although there was a significant decrease in oxygenation after BL throughout the 120 mins of ventilation, there were no significant differences in terms of PaO2 of rats fed either a standard or high cholesterol diet at any time point during MV

Table 3.2 Surfactant phospholipids (PL) measured in the first and final sequential lavages of surfactant depleted rats. *P<0.05 between first and final sequential lavages within each component of surfactant. Values of surfactant, small aggregate (SA) and large aggregate (LA) phospholipids are shown as means ± SEM, n = 8/group.

Diet	**Surfactant (mg PL/kg BW)**		**SA (mg PL/kg BW)**		**LA (mg PL/kg BW)**	
	First sequential lavage	*Final sequential lavage*	*First sequential lavage*	*Final sequential lavage*	*First sequential lavage*	*Final sequential lavage*
Standard	1.94±0.25	0.53±0.05*	0.87±0.19	0.20±0.04*	1.01±0.09	0.25±0.05*
High cholesterol	1.74±0.17	0.67±0.08*	0.78±0.13	0.18±0.03*	0.94±0.08	0.39±0.05*

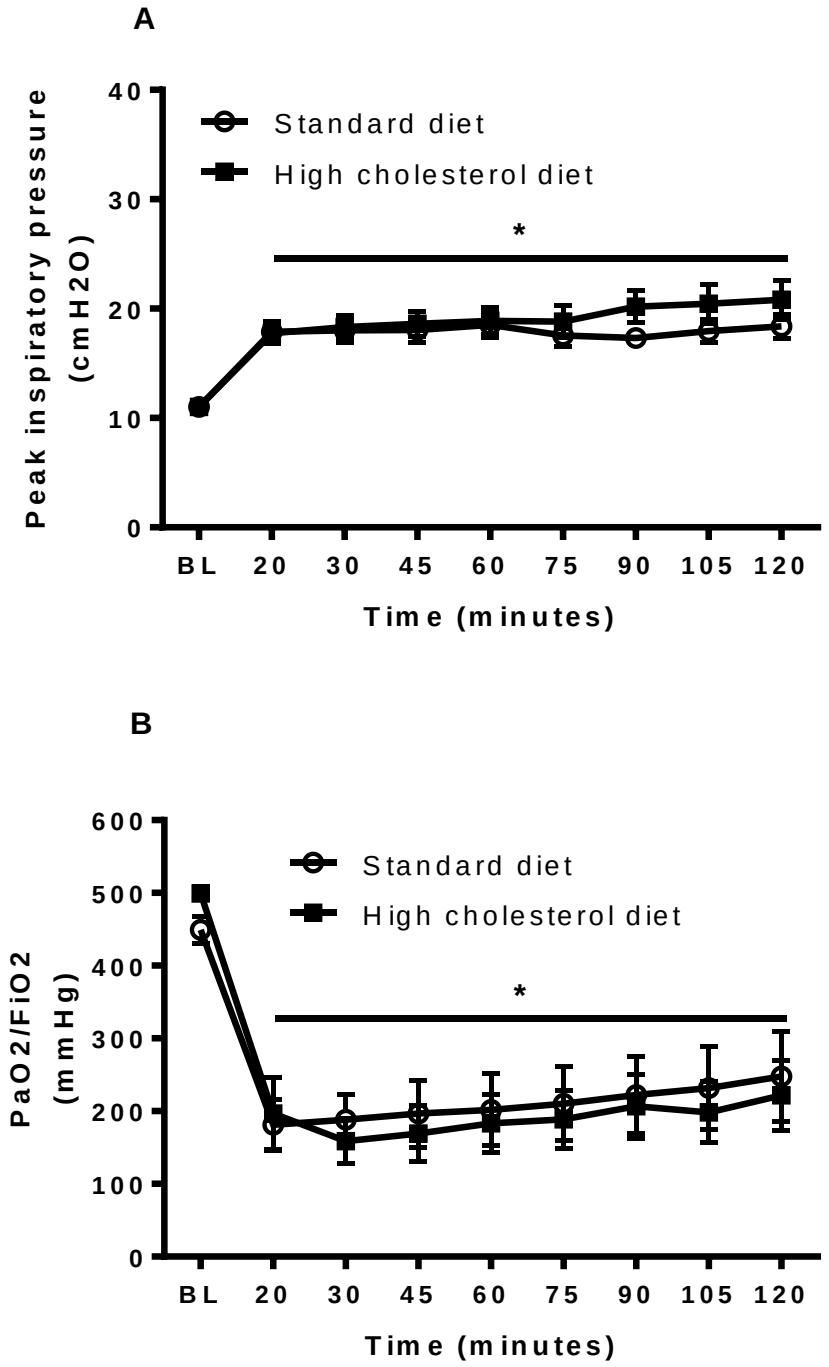

Figure 3.10 Peak inspiratory pressure (cmH2O) (A) and arterial oxygenation content (mmHg) (B) after endogenous surfactant depletion and 120 minutes of mechanical ventilation. *P<0.05 vs. BL. Values are means ± SE, n = 8/group.

Table 3.3 shows the analysis of phospholipid levels of surfactant subfractions, amount of cholesterol and protein in the lavage recovered after depletion and 120 minutes of mechanical ventilation. There was no significant difference between rats fed a standard or high cholesterol diet in terms of the total surfactant, small aggregate or large aggregate phospholipid pool size. Similarly, there was no significant difference in the amount of cholesterol found within the large aggregate component of surfactant and no significant differences were measured in the amount of protein in the lavage between the two diet groups.

Table 3.3 Surfactant pool sizes (mg PL/kg BW), lavage cholesterol levels (% of PL) and lavage protein levels (mg/kg BW) between dietary conditions after endogenous surfactant depletion and 120 mins of MV. Values are means ± SE, n = 5/group.

Diet	**Total surfactant PL (mg/kg BW)**	**SA surfactant PL (mg/kg BW)**	**LA surfactant PL (mg/kg BW)**	**Lavage cholesterol (% of PL)**	**Lavage protein (mg/kg BW)**
Standard	3.06±0.37	1.22±0.15	1.67±0.23	4.49±1.21	37.7±6.6
High cholesterol	3.61±0.43	1.56±0.42	1.41±0.21	6.30±0.67	56.4±16.3

3.4 Discussion

Previous studies have implicated increased cholesterol within surfactant as a contributor to the material's biophysical function (Chapter 2; Maruscak *et al.,* 2008; Vockeroth *et al.,* 2010); however, it was unknown to what extent serum cholesterol levels would affect surfactant cholesterol. It was hoped that by delineating this relationship, a potential patient population predisposed to ablated surfactant function could be identified as candidates for preventative therapies to reduce their risk of developing ARDS. Our original hypothesis was that serum hypercholesterolemia would predispose the rats to developing more severe lung injury due to changes to their pulmonary surfactant system. Based on the data from our studies, serum hypercholesterolemia does not increase levels of surfactant cholesterol. Despite having over a four-fold increase in serum cholesterol, none of the models used in this study had statistically significant differences between hypercholesterolemic and control rats with regards to the severity of lung injury, as measured through PIP, PaO_2 or protein levels in their lavage regardless of the type of lung injury incurred. Furthermore, surfactant composition was not significantly altered in rats fed either diet and analysis of surfactant cholesterol revealed nearly identical proportions of cholesterol between hypercholesterolemic and control rats. Thus, it is concluded that diet-induced hypercholesterolemia does not lead to increased cholesterol content within pulmonary surfactant does not affect the development of lung injury.

We employed three distinct models of lung injury that allowed us to examine the role of serum hypercholesterolemia in different settings. Our first model was VILI in which high tidal volume ventilation cause lung injury. This model was utilized since it was previously shown to result in elevated levels of cholesterol in surfactant (Maruscak *et al.*, 2008). Our second injury was induced by administration of acid to the lung; this is a clinically relevant model reflecting gastric acid aspiration in which a chemical burn initiates lung injury and is further propagated by recruitment of neutrophils into the alveolar space (Folkesson *et al.*, 1995). Additionally, a previous study in our lab used an acid-aspiration model in the hypercholesteremic apolipoprotein-E null mice and demonstrated that these mice had more severe lung injury and higher pulmonary inflammation than wild-type mice (Yamashita *et al.*, 2012). Our third model was a surfactant depletion model in which the repetitive lavage procedure caused depletion of surfactant stores and allowed the evaluation of nascent surfactant produced and secreted during the two hour ventilation period (Lackmann *et al.*, 1989). All three models demonstrated significant lung injury through physiological measurements and elevated proteins in the lavage. By using these models in our study, we were able to investigate the effects of cholesterol across a broad range of injuries rather than focus on a specific sub-set. The fact that all three injury models demonstrated no significant effect of hypercholesteremia on the development of lung injury provides strong support for the conclusion that hypercholesteremia by itself is not a risk factor for the development of ARDS.

In addition to a lack of an effect of serum hypercholesterolemia on lung physiology, we also observed that the cholesterol diet did not affect the surfactant system compared to control rats in any of our models. This finding was somewhat surprising in light of an earlier study conducted by McCrae and colleagues (2008) which examined surfactant's biophysical activities from mice fed a high cholesterol diet. These mice had a significant increase in both their serum cholesterol levels and the amount of cholesterol in their surfactant. Furthermore, when the surfactant was tested, it was found that the surface active film had decreased ability to maintain patency at 37°C as compared to surfactant with endogenous levels of cholesterol (McCrae *et al.,* 2008). While our study did not test the biophysical properties of the surfactant collected, we did analyze the amount of cholesterol within surfactant and found that in all our experiments, in both injured and non-injured rats, there were no significant differences in the cholesterol levels of surfactant recovered from either hypercholesterolemic or control rats. The difference in results between this study and the one conducted by McCrae and colleagues may be due to the different metabolic profiles between the different species used in the studies (rats vs. mice) or the length of diet used (14- 17 days vs. 16 weeks). Because of the possible differences associated with species and/or acute vs. chronic exposure to hypercholesterolemia, future studies looking into the effects of chronic hypercholesterolemia in other species is warranted.

The surfactant collected from our VILI rat model was the only injury model amongst the acid-induced lung injury and surfactant depleted rat models that showed an increase in cholesterol within surfactant. The rats with VILI had ~12% (w/w PL) cholesterol within

their surfactant while our acid-injury model and surfactant depleted models had surfactant cholesterol levels of ~5-7% wt/wt PL. This finding supports previous observations made by Maruscak and colleagues in 2008 when they found that surfactant from Sprague-dawley rats afflicted with VILI had increased surfactant cholesterol as compared to rats that did not have lung injury (Maruscak *et al.,* 2008). It is thought that mechanical stretch of lung epithelium stimulates alveolar type 2 cells to secrete surfactant (Wright *et al.,* 1991; Edwards *et al.,* 2001). The composition of surfactant can also be affected by large tidal volumes used during ventilation (Veldhuizen *et al.,* 1999). For example, adult rabbits ventilated with higher tidal volumes had a higher ratio of SA/LA in their lavage as compared to rabbits ventilated with lower tidal volumes (Veldhuizen *et al.,* 1999). Thus the type of MV protocols used can affect surfactant's composition and overall function. It must be stated that MV alone does not seem to cause changes to surfactant's composition or cholesterol content. In our surfactant depleted model of injury, nascent surfactant collected from rats after 120 mins of non-injurious MV did not have an increased amount of surfactant cholesterol. Based on this observation, we can conclude that mechanical over-distension of the lungs is responsible for increasing the amount of cholesterol within surfactant.

Given the increase in the proportion of cholesterol within surfactant observed in our VILI model discussion regarding the contribution of high-stretch MV to this result is warranted. Based on the data gathered, the elevation of cholesterol seen in this particular model is was not a consequence of increased lung permeability. This is supported by our observation that there was no significant increase in total protein levels in the lungs of

animals fed either the standard or high cholesterol diet in our VILI model. Furthermore, animals with lung injury in our other models showed an increase in the amount of protein in their lavage compared to non-injured animals; however, lavage samples from these animals did not contain elevated cholesterol regardless of diet. These observations indicate that in our VILI model, the increase in cholesterol was not due to cholesterol leaking into the lungs directly from circulation implying that high-stretch ventilation changes the lung's surfactant metabolism.

Although we were successful in the development of hypercholesterolemic rats due to diet the onset of their hypercholesterolemia was acute and does not represent the normal development of the condition which requires chronic ingestion of high-fat and high-cholesterol foods (Steinberg, 2005). To address this, future studies can extend the duration of a hypercholesterolemic diet to better mimic a chronic exposure to high levels of systemic cholesterol. However, the risk of developing other cardiovascular factors, such as hypertension and atherosclerosis (Steinberg, 2005), could have impacted our results. Delineating the specific contribution of serum hypercholesterolemia on the cardiovascular and surfactant systems would have shifted the focus away from our original hypothesis that dealt with only the effects of hypercholesterolemia the development of lung injury due to surfactant. Furthermore, we believe that increasing the duration of the diet would not have led to increases in surfactant cholesterol levels. The synthesis of new surfactant requires 7 – 10 hours and the half-life of phosphatidylcholine, which accounts for 80% of surfactant phospholipids, is approximately 17 hours (Jacbos *et al.,* 1982). Given that our rats fed a high cholesterol diet had a significantly higher

amount of serum cholesterol levels after 14-17 days on the diet, a sufficient period of time had passed wherein newly metabolized surfactant could have incorporated excess cholesterol from the serum.

3.5 Conclusions

In conclusion, diet-induced serum hypercholesterolemia did not predispose rats to the development of more severe ARDS or affect their surfactant composition. However, increases in surfactant cholesterol levels can occur by over-distension of alveoli and this increase is not present in other models of lung injury. Our data argues against either hypercholesteremia or direct leak of cholesterol from the serum as a mechanism by which cholesterol levels within surfactant are affected, other mechanism require further examination.

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Chapter 4

4 Future directions and overall significance

4.1 Introduction

This thesis focused on two aspects of the role of elevated cholesterol in surfactant function and ARDS. In chapter 2, we established that elevated cholesterol in human surfactant affects the material's biophysical properties *in vitro*. We also showed evidence that surfactant with high cholesterol is detrimental to the oxygenation of ventilated and surfactant depleted rats. Importantly, this study also showed that SP-A can mitigate the inhibition caused by elevated cholesterol improving both the biophysical properties of surfactant with high cholesterol and improving the oxygenation of rats treated with surfactant containing high cholesterol. Given the evidence that high cholesterol affects surfactant function, chapter 3's main objective was to determine to whether serum hypercholesterolemia predisposed rats to the development of more severe lung injury and changes to surfactant composition. We fed rats a high cholesterol diet which elevated their serum cholesterol levels to four-times greater than in our control rats. However, our data indicate that regardless of the form of lung injury incurred, there was no difference in the degree of lung injury sustained between diet and control rats and that within each mode of injury there were no changes to the composition of surfactant regardless of the type of diet.

Based on the results from these two chapters, we can conclude that high cholesterol in surfactant affects the material's biophysical properties and oxygenation, but SP-A can mitigate these effects. Furthermore, although cholesterol is an inhibitor to surfactant function, cholesterol levels within surfactant are not affected by diet alone. The purpose of this final chapter is to examine preliminary data determining whether cholesterol-mediated surfactant inhibition affects ventilated human trauma patients and to discuss future experiments. Finally, this chapter will conclude by discussing the overall clinical significance of our findings thus far.

4.2 The role of MV in cholesterol-mediated surfactant dysfunction

Mechanical ventilation (MV) is a supportive therapy necessary in patients that are otherwise unable to maintain proper oxygenation (Lee & Slutsky, 2001); however, studies suggest that the use of MV can also propagate the development of lung injury (Webb & Tierney, 1974; Dreyfuss & Saumon, 1998; Brower *et al.*, 2000; Veldhuizen *et al.*, 2001). Moreover, the contribution of cholesterol in lung injury developed due to MV (i.e. ventilator induced lung injury, "VILI") has shown that there are increases in the amounts of cholesterol within surfactant (Maruscak *et al.*, 2008; Vockeroth *et al.*, 2010). This is further supported by our observations made in chapter 3 which showed that, regardless of diet, rats with VILI had increased cholesterol in their surfactant when compared to rats with other forms of lung injury such as acid aspiration or surfactant

depletion. Despite these findings, limitations with these animal models of VILI create a dissonance between observations in a laboratory setting and clinical realities.

Animal studies of VILI, including ours presently, have predominantly focused on the use of high-tidal volume MV for brief periods of time in animals (Maruscak *et al.,* 2008; Vockeroth *et al.,* 2010). In contrast, critically ill patients require the use of MV for prolonged periods of time extending to days. Furthermore, VILI studies utilize high-stretch MV to develop lung injury within a short time frame. However, clinical MV strategies predicate the use of low-tidal volume strategies which have reduced the mortality rate of patients with ARDS from 40 to 30% according to the large, multi-center trial conducted by ARDSnet (Brower *et al.,* 2000). Given the discrepancy between laboratory models of VILI and clinical realities, it is unknown whether the consequences of MV can be applied to a ventilated, human patient population.

4.2.1 The role of cholesterol in ventilated, human trauma patients.

In order to investigate the role of cholesterol in patients undergoing MV, nine surfactant samples were obtained from eight human trauma patients. These patients were placed on different ventilation strategies as determined by their attending physician, represented a range of ages from 20 – 84 and had different causes of trauma. Common amongst all patients was the requirement for the use of MV for a minimum of 24 hours. We tested the patient's surfactant biophysical properties on the CBS with and without incubation

with methyl-beta cyclodextrinn (MBCD) – a water-soluble molecule capable of sequestering cholesterol from lipid films such as surfactant.

4.2.2 Preliminary results

4.2.2.1 Adsorption

The surfactant large aggregates collected from the trauma patients were tested on the CBS for the material's adsorption properties. Methods as outlined in chapter 2 were used for this experiment. **Figure 4.1** shows the surface tension of human trauma patient surfactant after 300 s of adsorption with and without incubation with MBCD. Overall, surfactant samples collected from the human trauma patients showed significantly reduced surface tension after sample incubation with cholesterol sequestering agent, MBCD after 300 s of adsorption. Other time points during the 300 s of adsorption were measured and at all other points, a similar effect was observed with surfactant samples having significantly lower surface tension after incubation with MBCD than without.

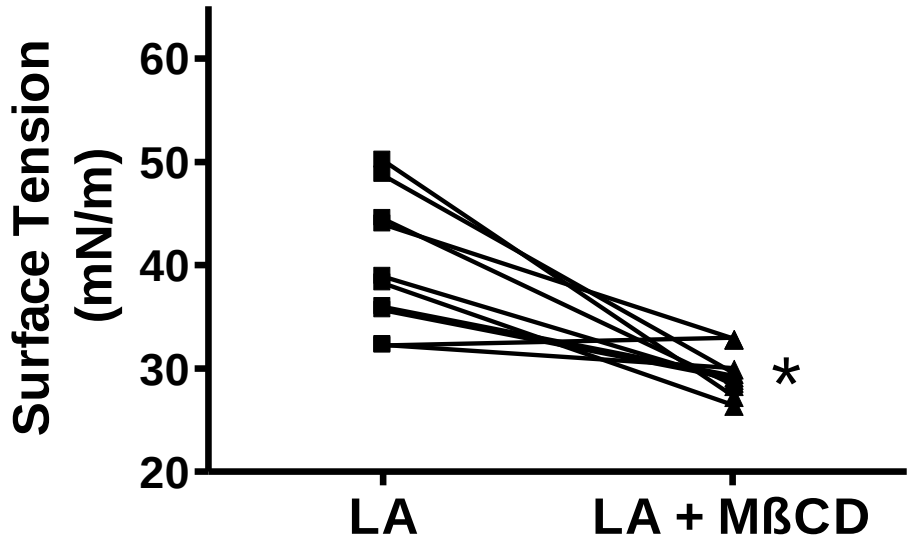

Figure 4.1 Surface tension of human trauma patient surfactant after 300 s of initial adsorption with and without treatment of MBCD. Closed squares represent patient LA without incubation with cholesterol sequestering agent MBCD while closed triangles represent patient LA incubated with MBCD. Lines connecting squares and triangles represent LA from the same patient. Values are reported as mean ± standard error; *$P<0.05$, $n = 8$.

4.2.2.2 Minimum achievable surface tension

Figure 4.2, A, B, C and D shows the minimum achievable surface tension of the surfactant obtained from eight human trauma patients before incubation with MBCD through dynamic expansion-compression cycles #1, 2, 5 and 10. Overall, surfactant samples tested in the presence of MBCD showed a significant reduction in minimum achievable as compared to surfactant not incubated with MBCD. This observation was consistent through cycles 1, 2, 5 and 10 of the dynamic expansion-compression cycles.

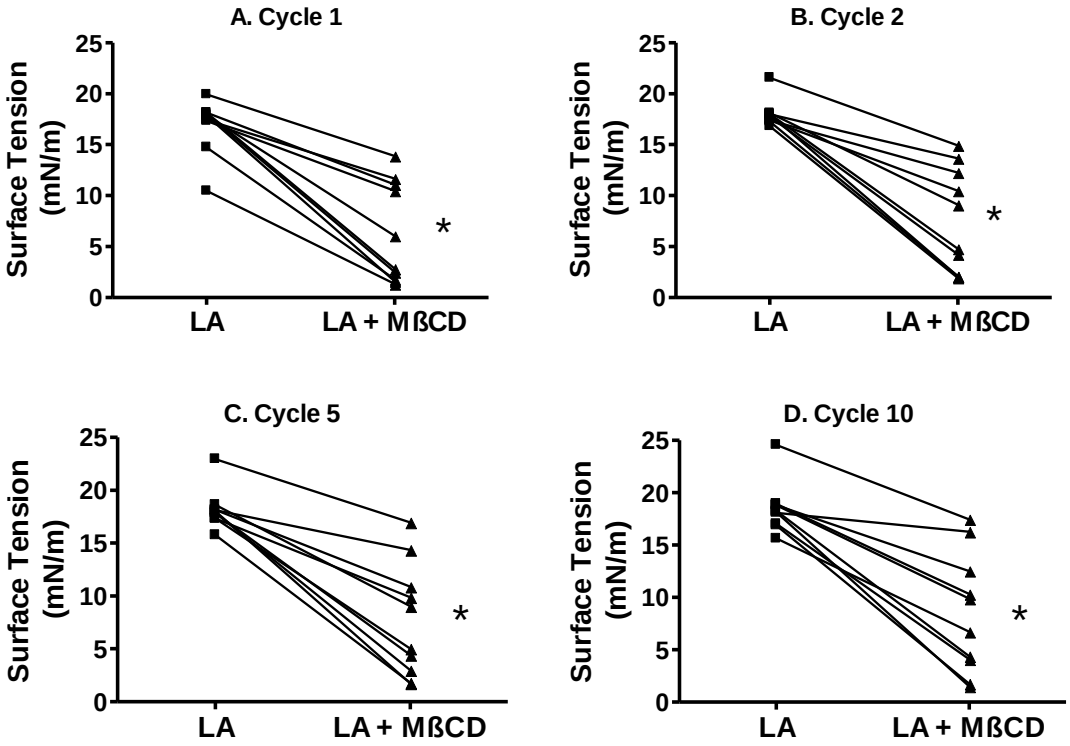

Figure 4.2 Minimum achievable surface tension of human trauma patient surfactant after cycle 1 (A), cycle 2 (B), cycle 5 (C) and cycle 10 (D) of dynamic compression-expansion with and without the addition of MBCD. Closed squares represent patient LA without incubation with cholesterol sequestering agent MBCD while closed triangles represent patient LA incubated with MBCD. Lines connecting squares and triangles represent LA from the same patient. Values are reported as mean ± standard error *P<0.05, n = 8.

4.2.3 Discussion

Based on this preliminary data presented, it is concluded that cholesterol mediated-surfactant inhibition occurs in ventilated human patients. This is further evidence that MV can affect the functional properties of surfactant in part due to affecting cholesterol levels in the material. In contrast with previous studies utilizing animal models of VILI, this preliminary study focused on a non-homogenous population of human patients with non-specific forms of trauma requiring the use of prolonged MV. Therefore, our findings indicate that human patients placed on prolonged MV have surfactant with dysfunctional biophysical properties which can be improved by the sequestration of cholesterol. Future studies investigating the mechanisms of ventilation affecting surfactant cholesterol metabolism is warranted.

4.3 Reconstitution of SP-A into ventilated human patient surfactant

In chapter 2, we determined that the addition of SP-A to surfactant with high levels of cholesterol improves the material's biophysical capabilities which results in improved oxygenation in ventilated, surfactant depleted rats. This observation emphasizes the importance of SP-A in mitigating surfactant dysfunction including inhibition due to elevated cholesterol. Although our preliminary experiment determined that surfactant obtained from ventilated human patients are inhibited by cholesterol, the surfactant

samples underwent organic-solvent extraction prior to testing on the CBS and lacks SP-A. Therefore, it is currently unknown whether the addition of SP-A to human patient surfactant would improve the material's biophysical function.

To investigate this, surfactant samples from ventilated human trauma patients can be reconstituted with 5% SP-A in a similar fashion as in the surfactant samples employed in chapter 2. After, the biophysical function of the human trauma patient surfactant with SP-A can be determined on the CBS as we have described presently in this chapter and previously in chapter 2. Given SP-A's ability to mitigate the inhibitory effects of serum proteins, cholesterol and decreased PL concentrations in surfactant (Cockshutt *et al.,* 1990; Korfhagen *et al.,* 1996; Yu & Possmayer, 1998; Yu *et al.,* 1999; Keating *et al.,* 2007), it is expected that the addition to SP-A to human trauma patient surfactant will result in improved surface tension reducing capabilities. This information would be invaluable to the development of effective exogenous surfactant therapies to treat severe lung disorders such as ARDS.

To date, the application of exogenous surfactant therapy has only proven effective when treating neonatal respiratory distress syndrome (Yee & Scarpelli, 1991; Jobe, 1993) and not ARDS (Lewis & Veldhuizen, 2002; Maruscak & Lewis, 2006). The difficulty in treating ARDS is impaired surfactant function due to serum-protein inhibition, altered PL profiles within the material and, cholesterol inhibition (Veldhuizen *et al.,* 1995; Gunther *et al.,* 1996; Panda *et al.,* 2004) which can also inhibit exogenously applied surfactant.

However, if we can provide evidence that SP-A can mitigate surfactant inhibition in humans, then perhaps exogenous surfactant therapies reconstituted with SP-A may prove to be an effective pharmacological treatment for severe lung maladies such as ARDS.

Limitations in the developmental process of exogenous surfactant preparations make this proposition difficult. Clinically used surfactants undergo a process of organic-solvent extraction which removes inhibitory peptides and also to concentrate the material. However, this process also removes SP-A. Furthermore, reconstitution of SP-A is not currently feasible since the artificial production of human SP-A yields very small quantities and is prohibitively expensive. Nevertheless, the ability of SP-A to prevent various forms of surfactant inhibition is an important consideration into its importance in exogenous surfactant therapies. Surfactants with SP-A may represent an effective pharmacological treatment for patients with lung disorders which may reduce rates of mortality.

4.4 The effect of chronic serum hypercholesterolemia on surfactant function and lung injury

Data in chapter 3 indicated that high of serum cholesterol levels do not affect surfactant composition or the development of lung injury. This is in contrast to findings by McCrae and colleagues (2008) who showed that mice with hypercholesterolemia had elevated surfactant cholesterol levels and their surfactant had increased patency when analyzed *in*

vitro. With that said, species differences (mice vs. rats) and the duration of high cholesterol diet (16 weeks vs. 14-17 days) are apparent between the two studies. Furthermore, the acute development of hypercholesterolemia present in our models of lung injury in chapter 3 do not reflect the development of hypercholesterolemia in humans. Typically, dyslipidemia in humans occurs over a prolonged period of time and chronic ingestion of high fat and high cholesterol food. If it is revealed that surfactant is affected by chronic hypercholesterolemia, this result may signify a specific patient population at risk for the development of lung injuries such as ARDS. Therefore, investigation into the effects of a prolonged high cholesterol diet on surfactant composition and function may be warranted.

To test whether chronic exposure to a high cholesterol diet does change surfactant cholesterol levels, a study can be conducted in which rats are fed the diet used in chapter 3 for 16 weeks as done by McCrae and colleagues (2008). Following, these rats can be randomized into the three models of lung injury as outlined in chapter 3: VILI, acid-induced and surfactant depleted. The results from these experiments can be used to determine whether chronic hypercholesterolemia affects the development of lung injury and would more closely resemble the chronic nature of hypercholesterolemia as present in a human patient population. Furthermore, the biophysical properties of the material can be examined on the CBS as previously described.

This experiment would help to determine whether chronic exposure to high cholesterol levels affects surfactant composition and function. However, due to the chronic nature of this study, however, the potential development of cardiovascular maladies may pose a limitation. Specifically, the development of atherosclerotic plaques due to the high cholesterol diet may cause systemic inflammation which may cause lung inflammation and ultimately lung injury. Thus, the lung injury would not be due to cholesterol directly, but rather due to the development of cardiovascular abnormalities. Secondly, the rate of turnover for surfactant is approximately 7-10 hours (Jacbos *et al.*, 1982). This indicates that in our 14-17 day diet used in chapter 3, we would have expected changes to surfactant's composition to have occurred as a result of the high cholesterol diet. Therefore, it is unclear whether extending the high cholesterol diet to a period of weeks would have any effect on surfactant as well.

4.5 Analyzing the topography of surfactant films with elevated cholesterol and SP-A

The biophysical data obtained in chapter 2 showed that SP-A can mitigate surfactant dysfunction caused by high levels of cholesterol. While the CBS enabled us to accurately measure the surface tension reducing capabilities of our surfactant film samples, it does not provide insight into the molecular mechanisms of cholesterol mediated impairment and how SP-A is able to mitigate this. Two ways to determine the effects of SP-A on surfactant films with high cholesterol on a molecular level is to use atomic force microscopy (AFM) and time-of-flight-secondary ion mass spectrometry (ToF-SIMS).

AFM can be used to measure height-differences in a given surfactant film and provide information about the topographic organization of lipids in the film. ToF-SIMS is a mass-spectrometry technique which allows identification of specific components of surfactant within specific regions of the film.

Studies utilizing AFM imaging and ToF-SIMS on surfactant containing cholesterol has been previously conducted (Panda *et al.,* 2004; Leonenko *et al.,* 2006, Leonenko *et al.,* 2006; 2007; Keating *et al.,* 2007). Using AFM, Panda and colleagues (2004) showed that surfactant from injured lungs lacked organized domains which are thought to be crucial for proper surfactant function. Furthermore, a recent study showed that BLES reconstituted with cholesterol had smaller and more numerous micro-domain as determined by ToF-SIMS (Keating *et al.,* 2007).

Based on these observations, cholesterol affects surfactant function by modifying the lipid organization of surfactant. Since we have shown that SP-A is able to mitigate the biophysical impairments caused by high cholesterol, it would be interesting to examine if SP-A is able to facilitate this by affecting the topographical organization of these lipid films. To do this, we can utilize surfactant samples reconstituted with SP-A and cholesterol to levels similar to those used in the CBS in chapter 2 and then analyze these samples using AFM and ToF-SIMS. The use of AFM and ToF-SIMS may elucidate the mechanisms which enable SP-A to mitigate cholesterol-mediated surfactant dysfunction.

4.6 Overall relevance

The results from this thesis emphasize the importance of cholesterol as a mechanism of surfactant dysfunction, especially within the context of MV, and the role of SP-A in mitigating this form of surfactant dysfunction. Given the evidence which shows that surfactant inhibition occurs in patients with severe lung disorders such as ARDS, it is important to continue investigating the mechanisms behind surfactant dysfunction to better understand the pathophysiology of the disease. Furthermore, although serum hypercholesterolemia did not predispose the development of more severe lung injury or changes to surfactant composition in our models, further research into determining whether specific patient populations are at greater risk of developing lung injuries should be pursued. It is hoped that by identifying a patient population with greater risk of developing lung injuries, therapeutic options, such as exogenous surfactant therapy, can be administered prior to the development of lung injury. This tactic may prove effective in protecting patients from the development of pulmonary maladies such as ARDS and decrease the syndrome's overall rate of mortality.

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