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Effects of Maternal Protein Restriction on the Pulmonary Surfactant System during the Early Life and Adulthood

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Abstract

Fetal growth restriction (FGR) is defined by low birth weight and contributes to a variety of adult-onset diseases with different severities between males and females. However, the effects of FGR on the pulmonary surfactant are not fully elucidated. In this thesis, first, we investigated the FGR effects on the lung function and the surfactant system at the early postnatal life. It was hypothesized that FGR contributes to alterations of lung mechanics and the surfactant system during the neonatal period. Second, we assessed the FGR effects on the surfactant system in response to sepsis in adulthood. It was hypothesized that FGR contributes to the alteration of the surfactant system in response to sepsis in adulthood. Overall, the data suggest that FGR induced by maternal protein restriction affects lung compliance at the early life and predispose the septic adults to the development of surfactant alterations in a sex-specific manner.

Keywords:

Fetal Growth Restriction, Maternal Low Protein Diet, Pulmonary Surfactant, Lung function, Sepsis, Lung Injury, Acute Respiratory Distress Syndrome, Pulmonary Inflammation

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Co-Authorship Statement:

Chapter 2 and 3 explain the experiments that were performed by Reza Khazaee in the lab of Dr. Ruud Veldhuizen and Dr. Cory Yamashita. Throughout these thesis, Dr. Yamashita and Dr. Veldhuizen actively provided intellectual contributions to all the studies described in this thesis, experimental design, data analysis, and presentation of this thesis.

For experiments presented in Chapter 2, the rat offspring were provided by Dr. Daniel Hardy's lab. Dr. Hardy, also, provided intellectual contribution with experimental design throughout the studies.

For experiments in Chapter 2 and 3, Lynda McCaig was heavily involved in animal experimentation and the process of lavage surfactant, protein and cytokine measurements and the analysis and interpretation of the experimental data.

Shannon Seney performed Multiplex assays and helped with plating blood samples in Chapter 3.

Karen Nygard provided help with histology and microscopy in Chapter 3.

Zhong Huang and Jack Zheng provided assistance with lavage and tissue sample collection, preparation, and storage in Chapter 3.

Wenjia Zhou provided help with designing a software to perform surfactant biophysical analysis in Chapter 2.

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List of Abbreviations and Symbols:

Abbreviations:

ATII – Alveolar Type II Cell

ANOVA – Analysis of Variance

ARDS – Acute Respiratory Distress Syndrome

BAL – Broncho Alveolar Lavage

BW – Body Weight

CSD – Constrained Sessile Drop Surfactometer

DPPC – Dipalmitoylphosphatidylcholine

ECM – Lung Extracellular Matrix

ELISA – Enzyme-Linked Immune-Sorbent Assay

H&E – Hematoxylin & Eosin

ER – Endoplasmic Reticulum

FGR – Fetal Growth Restriction

FIP – Fecal-Induced Peritonitis

G-CSF – Granulocyte Colony Stimulating Factor

ICU – Intensive Care Unit

IFN-γ – Type II Interferon

IL-6 – Interleukin-6

IP – Intraperitoneal

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KC – Keratinocyte Chemoattractant

LA– Large Aggregate

LB – Lamellar Body

LP – Low Protein

MCP-1 – Monocyte Chemotactic Protein-1

NRDS – Neonatal Respiratory Distress Syndrome

PEEP – Positive End-Expiratory Pressure

RR – Respiratory Rate

SA – Small Aggregate

SEM – Standard Error of the Mean

SP-A – Surfactant Protein A

SP-B – Surfactant Protein B

SP-C – Surfactant Protein C

SP-D – Surfactant Protein D

TNF-α – Tumor Necrosis Factor Alpha

TS – Total Surfactant

V_t – Tidal Volume

List of Symbols:

ΔP – Change in Pressure

ΔV– Change in Volume

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v –Velocity Difference

γ – Surface Tension

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Chapter 1-General Introduction and Literature Overview

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1.1. Overview

Over the last few decades, it has become evident that an adverse intrauterine environment during pregnancy leads to fetal growth restriction (FGR) and can have immediate and long-term health impacts in both male and female offspring (Barker et al., 1991; Hoo et al., 2004; Rueda-Clausen, Morton, & Davidge, 2009). FGR is defined as small for gestational age (Mamelle, Cochet, & Claris, 2001; Sohi et al., 2011). FGR contributes to the development of diseases such as diabetes and cardiovascular disorders, often with different outcomes in males and females (Carey et al., 2007; Ozaki et al., 2001; Ozanne et al., 1998; Petry, Ozanne, & Hales, 2001; Rozance et al., 2011). Comparatively, the impact of FGR on the lung development and function in the neonatal period and its contribution to the development of lung diseases later in life, has received far less attention. Several studies have provided preliminary indications that FGR does indeed have impacts on the pulmonary system (Mestan & Steinhorn, 2011; Real et al., 2010; Rozance et al., 2011; Sugahara et al., 1983; Visentin et al., 2014). These effects include alterations in lung mechanics and the pulmonary surfactant; a lipid-protein mixture coats the inside of the lung and eases the lung expansion during respiration (West, 2012). Despite this knowledge, as will be described in more details later in this chapter, a comprehensive analysis is lacking. Therefore, in this thesis, we have utilized a rat model of FGR towards: i) to study lung function and the surfactant system in the neonatal period (chapter 2) and ii) examine the pulmonary response to an inflammatory insult in adult life (chapter 3) in both male and female offspring.

The objective of the first chapter is to provide an overview of the current state of knowledge on Lung development and function, fetal growth restriction effect on the lung development, the pulmonary surfactant, surfactant deficiency, and lung injury. Subsequently, the impact of sepsis, as the most prevalent inflammatory insult, on the lung will be reviewed. Finally, I will discuss FGR focusing on the known information about its effects on the pulmonary system.

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1.2. Lung Development

To ultimately understand the effects of fetal growth restriction on the lung, it is important to first understand normal lung development. Overall, lung development is a series of tightly regulated processes beginning in the embryo and continuing during the postnatal life. Lung development is divided into three different stages; 1) the embryonic period, 2) the fetal period, and 3) postnatal lung development (for detailed review see DiFiore & Wilson, 1994 and Schittny, 2017). Lung organogenesis in humans and rats occurs through the embryonic period between week 4-7 and day 11-13 of embryonic period; this includes formation of the two lung buds which then is followed by the formation of the major airways. During fetal period, there is a repetitive process of formation and branching of the distal airways for both rats and human. This is followed by the first appearance air-blood barrier and the pulmonary surfactant (a lipoprotein complex that facilitates the expanding of the lung) in both species before birth. Beginning of the alveolarization (the process by which the alveoli, the main pulmonary gas-exchange units, are formed) is one of the major differences in the lung development between rats and human. In humans, the first alveolar spaces form in the last trimester of pregnancy, while in rats, it begins at postnatal day 7-21. The process of pulmonary development in both species continues until young adulthood with continuous increasing the alveolar spaces and microvascular maturation (Burri, 1992; Schittny, Mund, & Stampanoni, 2008).

1.3. The Effect of Sexual Dimorphism on the Lung Development

There is evidence from human and animal studies that there are sexual dimorphisms in the regulation of lung development during the fetal and postnatal period (Carey et al., 2007; DiFiore & Wilson, 1994; Hibbert et al.,1995; Joshi & Kotecha, 2007; Milewich et al.,1986; Sekhon et al., 1999; Simard, Provost, & Tremblay, 2006;). During fetal period, one such difference is a higher degree in lung alveolarization and earlier surge in surfactant content in females compared to males in both humans (Fleisher et al., 1985) and animal models (Provost, Simard, & Tremblay, 2004; Torday & Nielsen, 1987). It has been shown that there is higher degree of maturation in surfactant phospholipid profile in the last trimester of a normal pregnancy in healthy females compared to healthy males (Becklake &

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Kauffmann, 1999; Fleisher et al., 1985). Throughout the postnatal life, the most obvious developmental difference between males and females is that lungs of females are smaller in size than males (Thurlbeck, 1982). However, the ratio of alveolar numbers and area to body mass is higher in females compared to males (Carey et al., 2007; Massaro, 2006). Despite all these differences between males and females, there are no significant effects on pulmonary physiological measurements between sexes in healthy individuals throughout life.

1.4. Fetal Growth Restriction Effect on the Lung Development

An important factor that can affect the lung development is alterations in the intra-uterine environment. One such example is fetal growth restriction (FGR), which is defined as small for gestational age (Mamelle, Cochet, & Claris, 2001; Sohi et al., 2011). Common factors results in FGR are oxygen and maternal or fetal nutrient deficiencies which can induce ineffective prenatal and postnatal lung development and maturation mostly in a sex-specific manner (Bose et al., 2009; Harding & Maritz, 2012; Winick & Noble, 1966). Previous animal studies showed that neonate rats with FGR had reduced pulmonary alveolarization and vessel growth and thickening of the distal airways compared to controls (Chen, Wang, & Su, 2004; Rozance et al., 2011). The same results for reduced alveolarization was observed in another study that was done by Maritz et al., in 2001; they showed that placental insufficiency-induced FGR resulted in decreased alveolarization and airspace volume in sheep offspring during infancy and adulthood. These developmental impairments can have consequences on the lung function which is a main focus of this thesis (chapter 2) and will be discussed in following section.

1.5. Lung Function

The primary function of the lung is the process of gas exchange. During breathing, inhaled air enters the lungs and reaches the alveoli at the peripheral site of the lung. Oxygen diffuses rapidly through the blood-gas barrier into the blood in the capillaries while carbon dioxide diffuses out from the blood to the alveoli down its concentration gradient. Eventually, carbon dioxide is expired out of the body during exhalation (Diaz et al., 1997; West, 1977). To allow for efficient gas exchange the lung has several essential anatomical

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and mechanical properties. These anatomical features include repeated branching of the airways and an inner large surface area (~50-100 m^2) available for gas diffusion. Furthermore, proximity of an extended network of blood capillaries wrapped around the alveoli and presence of a thin air-blood barrier at the alveolar level facilitate the process of gas exchange through the alveolar epithelial wall (Ward & Nicholas, 1984).

In addition to the lung structure, the process of gas exchange, also, depends on the mechanical features of the lung and thoracic cavity in which the lungs are located. Inspiration occurs through the contraction of the inspiratory muscles, the diaphragm and external intercostal muscles. The contraction of these muscles causes an increase in the size of the thoracic cavity generating a pressure gradient to a sub-atmospheric value that allows inflow of volumes of air (Grinnan & Truwit, 2005). The expiration process occurs when the inspiratory muscles relax, which produce a positive alveolar pressure compared to the pressure of the external environment helping the movement of air back into the atmosphere (West, 2012).

In addition to respiratory muscles, there are other mechanical aspects inside the lung that affect the breathing process. Two critical aspects of lung mechanics are compliance and airway resistance. Compliance refers to the work required to expand lung surface area during inhalation. There are two main factors can affect lung compliance: the elasticity (stiffness) of the lung tissue and the surface tension arising from the liquid lining the alveolar inner surface area (will be discussed in further details in the following sections). Increased airway resistance in some pulmonary disease (such as asthma) may increase work of breathing. However, in a healthy lung, the effect of airway resistance, relative to tissue elasticity and alveolar surface tension, on work of breathing is negligible (Sidebotham & Le Grice, 2007).

1.5.1. Airway Resistance

Airway resistance is the resistance to the flow of air through the respiratory tract during inhalation and expiration (West, 2012). Total airway resistance is the pressure difference between mouth and the alveoli, divided by airflow. Airway resistance is determined by the diameter of the airways and the velocity of the air flow using the following equation, where

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ΔP is the pressure difference (cmH_2O) driving airflow and v is the velocity difference (ml/s).

$$\textbf{Airway Resistance} = \frac{\Delta P}{v}$$

Clinical analysis has shown that airway resistance can be affected by sex (Becklake & Kauffmann, 1999; Mead, 1980). In humans, new born females have higher ratio of large to smaller airways. This results in having higher conductance and flow rates in female infants compared to males. These differences are also observed until adulthood where larger proximal airways for females during deep breathing results in higher conductance compare to males (Becklake & Kauffmann, 1999). Despite these differences, in healthy individuals, the contribution of airway resistance to the whole respiratory resistance and work of breathing is negligible. However, narrowing of the proximal airways, as a result of some diseases (i.e., obstructive airway disease) can result in airway resistance in a sex-dependent manner (with males more affected), which in turn may impact the work of breathing and the time required for inhalational and deflation (Crouse & Laine-Alava, 1999). Furthermore, experimental and clinical studies have shown that FGR is associated with impaired proximal airway function in neonate and adults (Dezateux et al., 2004; Joss-Moore et al., 2011).

1.5.2. Lung Compliance

Lung compliance is defined as the expandability of the lungs and its ability to inflate during inhalation (Grinnan & Truwit, 2005) and is determined using the following equation, where ΔV is the change in volume (mL) and ΔP is the change in pressure (cmH_2O):

$$\textbf{Compliance} = \frac{\Delta V}{\Delta P}$$

Two key factors impact the lung compliance are resistant force produced by elastic recoil of the lung tissues (tissue stiffness) and the surface tension generated at the alveolar air-liquid interface (Lindahl et al., 1997; Weibel, 2011). Lung elastic recoil is the tendency of lung parenchyma to recoil to the resting volume after expanding which arises from lung extracellular matrix components most importantly collagen (stiff) and elastin (stretchy)

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fibers (Carey et al., 2007; Simard et al., 2006). Changes in the composition and/or orientation of the extracellular matrix components can alter the pulmonary compliance (Andreassen et al., 2010). As mentioned above, surface tension is the other factor that affects lung compliance and it is the result of the attractive forces between water molecules at the alveolar air-liquid interface. In a healthy lung, alveolar surface tension is regulated and maintained at lower values by the presence of surfactant lipid-protein complex secreted into the air-space by alveolar type II (ATII) cells (Ward & Nicholas, 1984).

Opposite to airway resistance, there has not been strong clinical or experimental evidence indicating significant differences in pulmonary compliance between healthy males and females throughout life (Colebatch, Ng, & Nikov, 1979). Although not been studied comprehensively, it has been shown that neonates and adult rat with FGR, induced by maternal calorie restriction, had lower lung compliance compared to controls (males only included) (Rehan et al., 2012). The results of the only study (Albion, 2011) assessed potential sex-based differences of FGR effects on the lung function showed reduced lung compliance in only FGR female mice at early life. Based on these studies, there is evidence indicating a sex-dependent FGR impact on the lung function. As mentioned before, surfactant system directly contributes to regulation of lung compliance. Therefore, reduced lung compliance can be implicated in surfactant deficiency and/or inhibition of its biophysical function.

1.6. The Pulmonary Surfactant System

As mentioned above, pulmonary surfactant has a big impact on maintaining normal lung mechanics by reducing the alveolar surface tension. Impairments of the surfactant system, such as changes to the surfactant composition and biophysical functions, have been known as important contributors to pathogenesis and development of different pulmonary complications (Zuo et al., 2008). The surfactant composition and biophysical properties will be discussed in the next sections.

1.6.1. Extracellular Surfactant Composition

The composition of surfactant has been conserved across different mammalian species;

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(Orgeig & Daniels, 2001;Veldhuizen et al., 1998) surfactant complex is composed of phospholipids ~80-90% by mass (such as phosphatidylcholine), neutral lipids ~3-10% (mainly cholesterol), and surfactant-associated proteins (SP-A, SP-B, SP-C, and SP-D) (5-10%) as well as some other (mostly serum-derived) proteins (Lopez-Rodriguez & Pérez-Gil, 2014).

The most abundant surfactant phospholipid component is dipalmitoyl phosphatidylcholine (DPPC), a saturated lipid that contributes ~40-50% of the total surfactant phospholipid content. DPPC is the main contributor to minimizing surface tension to near zero during exhalation (Yu et al., 2004). Surfactant proteins include the small hydrophobic protein, SP-B and SP-C, that contribute directly to surfactant surface activity, and the hydrophilic proteins, SP-A and SP-D, play significant roles in surfactant metabolism and innate immune defense (for detailed review see Lopez-Rodriguez & Pérez-Gil, 2014). Proper proportion of surfactant lipids and proteins and interactions between these constituents are essential for maintaining the surfactant reducing surface tension ability (Mander et al., 2002).

1.6.2. Extracellular Surfactant Metabolism

Surfactant lipids and associated proteins are produced and exocytosed into the alveolar space by alveolar type II cells (ATII). The majority of intracellular surfactant is assembled into organelles called lamellar bodies (LB) within ATIIs. Upon stimulation, for instance by cyclic stretch, the organelles relocate to the apical membrane of ATIIs, where they release surfactant into the extracellular alveolar space by exocytosis (Schmitz & Müller, 1991).

The extracellular surfactant can be collected by flushing the lungs with saline and collecting the liquid. This process is called bronchoalveolar lavage. In vitro, the isolated alveolar lavage can be separated into two subfractions by centrifugation. The dense structures including surface-active components of surfactant called large aggregates (LA) and lighter surfactant vesicles are called small aggregates (SA). Previous studies showed some differences in the structure of these surfactant subfractions; LA had lipo-protein structures such as LBs, tubular myelin, and surfactant-associated proteins (SP-A, SP-B,

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SP-C, and SP-D) while these organized structures did not present in SA (Veldhuizen et al., 1994).

After being secreted into the alveolar space LBs undergo reorganization into structures called tubular myelin; a network structure adsorbed to the air-liquid interface during inhalation where it forms a surface-active film (Figure 1.1) (Veldhuizen et al., 1994). After being adsorbed to the interface, LA contributes to maintaining alveolar stability by reducing alveolar surface tension arising at the air-liquid interface. During breathing cycles, changes in the surface area is associated with conversion of LA into SA. Ultimately, SA is dissociated from the film and is either uptaken and recycled mostly by ATII cells or cleared and degraded within alveolar macrophages by phagocytosis (Günther et al., 2001).

There has not been clinical or experimental evidence indicating significant sex differences in extracellular surfactant levels between healthy individuals throughout life. However, differences have been observed in the extracellular surfactant levels in human neonates (50 to 100mg/Kg body weight) compared to adults (5-10mg/kg body weight) (Fidanovski et al., 2005; Hamm, Kroegel, & Hohlfeld, 1996; Jobe & Ikegami, 1987; Runge & Patterson, 2007). It is suggested that the large surfactant pool sizes at birth is nescessary to fiacilitate opening of the lungs upon the transition to the air-breathing environment.

There have been previous animal studies which measured levels of specific surfactant lipids or lipo-proteins in lung tissues (Chen et al., 2004; Gortner et al., 2005; Sutherland et al., 2012). Chen et al, in 2004, showed that in a rat model of FGR, induced by 50% maternal calorie restriction, there was a reducton in the level of intracellular saturated surfactant phospholipids in FGR offspring. Another study showed reductions in the expression levels of surfactant lipid-proteins in lung tissues at late gestation in mice fetuses with FGR (induced by hypoxia) (Gortner et al., 2005). These limited studies did not assess whether these changes resulted in the altertation of the extracellular surfactant metabolism. Also, there are no previous studies examining the FGR effect on the surafactant metabolism during the adulthood; this will be discussed in chapter 3.

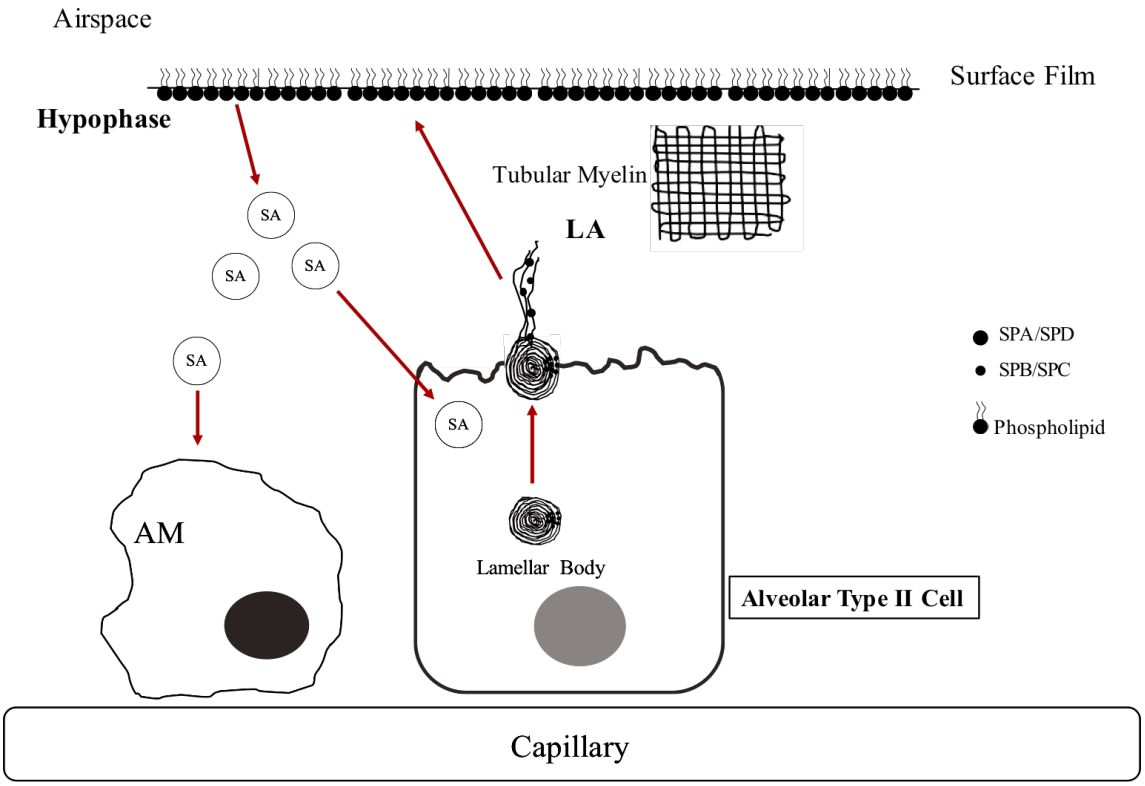

Figure 1. 1. Surfactant Metabolism. Surfactant lipo-protein complex is synthesized within alveolar type II (ATII) cells, and then stored in lamellar bodies (LB). Upon the cellular stimulation, LBs are exocytosed into the liquid hypophase where they unravel and form tubular myelin which then adsorbs into the interface to form a surfactant film. During expansion-compression cycles LA are metabolized into SA which are taken up by ATII cells or cleared by alveolar macrophages (AM).

1.6.3. Surfactant Function

As mentioned in previous section, pulmonary surfactant reduces the alveolar surface tension at the air-liquid interface. In the absence of surfactant, under the surface of a polar liquid, attraction forces of individual molecules pull on each other equally in all directions resulting in a net force of 0. At the surface of an air-liquid interface however, the net attractive forces between molecules is downward letting the water molecules to compress more tightly compared to the molecules inside the aqueous phase. This results in forming stronger bonds at the surface (surface tension) that resists deformation. In the lung, a high surface tension can increase work of breathing during inhalation. Also, this can cause less

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alveolar stability during lung compression which can promote alveolar collapse and, thus, reduction in surface area available for gas exchange (Lopez-Rodriguez & Pérez-Gil, 2014).

In the lung, the presence of surfactant film at the air-liquid interface contributes to reducing the surface tension resistant force and increasing alveolar stability (Figure 1.2). This happens through adsorption and increasing the concentration of the surfactant lipid-protein components into the interface. Upon adsorption, these components displace the water molecules and, ultimately, form the interfacial surface-active film (Goerke, 1998; Possmayer et al., 2010). When the surfactant phospholipid molecules entirely replace the water the surface tension of water would be reduced from ~70mN/m to an equilibrium surface tension of ~23–24mN/m (at 37ºC) (Lopez-Rodriguez & Pérez-Gil, 2014).

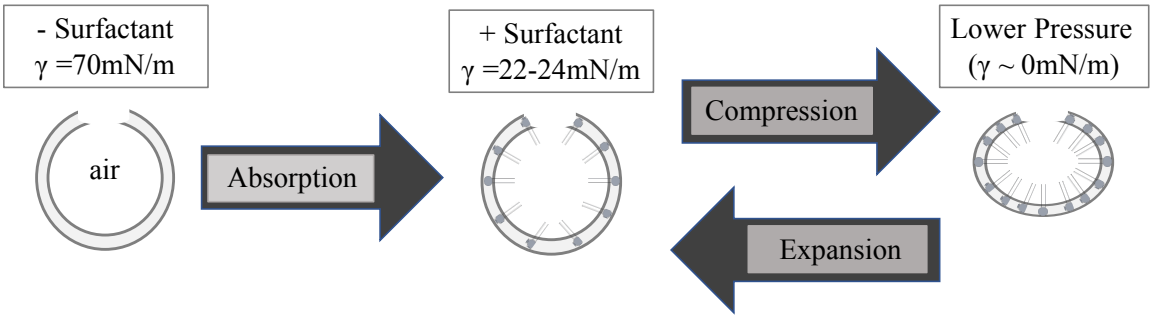

Figure 1. 2. Forming the interfacial surfactant film. Through the adsorption process, surfactant replaces the water molecules and reduced the surface tension from ~70mN/m to an equilibrium surface tension of ~23–24mN/m. At lower volumes, with surfactant present, lateral compression causes surfactant phospholipid molecules move closer together making the surface tension further decrease, thereby, causes more alveolar stabilization and prevention of lung collapse (γ: surface tension).

To stabilize the lung, surfactant should maintain its lowering surface tension properties during breathing cycles. It means concentration of surfactant components should be efficiently regulated at the surfactant film during compression-expansion cycles. During exhalation, lateral compression of surfactant film is a mechanism allows alveolar stabilization by approaching a surface tension near 0mN/m (Lopez-Rodriguez & Pérez-

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Gil, 2014). The lateral compaction of the interfacial film is enhanced through sufficient elevated concentration of saturated DPPC; their saturated acyl chains can get tightly compressed with minimum interactions from neighbor phospholipid acyl chains (Lopez-Rodriguez & Pérez-Gil, 2014; Possmayer et al., 2010). Concentration of saturated DPPC can further achieved by exclusion of more fluid lipids, such as unsaturated phospholipids and cholesterol, from the film. This allows to achieve the minimum surface tension near 0mN/m and therefore more alveolar stability, at the at maximum compression (end of deflation) (Keating et al., 2012; Possmayer et al, 2001)

During inhalation, increases in the interfacial film surface area results in a reduction of phospholipid concentration and therefore surface tension increases. An efficient film re-expansion is achieved by immediate selective re-insertion of the protein-lipid complexes, especially saturated DPPC, from the surfactant reservoir below the interface back into the surfactant film upon initiation of lung expansion (for more details see Baoukina & Tieleman, 2011; Keating et al., 2012; Pérez-Gil & Keough, 1998).

There has been no study indicating sex differences in surfactant biophysical function throughout life. Furthermore, no clinical or experimental study have previously assessed the effect of FGR on surfactant biophysical function during the early life or adulthood. In chapter 2, in addition to lung compliance and surfactant phospholipid measurements, surfactant biophysical analysis was performed on extracellular LA surfactant of FGR offspring throughout the lactation period. The surfactant was collected through the lavage process and its biophysical measurements was performed using a technique called constrained sessile drop surfactometer, which is explained in the section below.

1.7. Analysis of Surfactant Biophysical Function

The *in vivo* assessment of surfactant function is difficult. Therefore, common methods to study the surfactant function include removing of the extracellular surfactant by lavaging the lung and measuring the surfactant functions *in vitro* (Zuo et al., 2008). In this thesis, to perform the *in vitro* surfactant function a constrained sessile drop surfactometer (CSD) was used (L. M. Y. Yu et al., 2004). The CSD's features that make it an ideal tool to measure the surfactant function are: i) for precise assessments, unlike other methods, very low

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sample volumes are needed (9-10µl), ii) the environmentally control chamber allows to replicate lung temperature and humidity, and, finally, iii) dynamic compression-expansion of samples at different frequencies allows to mimic human breathing (L. M. Y. Yu et al., 2004). A schematic diagram of a CSD we used in this thesis (used in chapter 2) is shown in Figure 1.3.

Surfactant surface tension after adsorption and surface tension reducing ability during compression-expansion cycles can be assessed on the CSD. The CSD consists of a stainless-steel pedestal which located in an environmentally controlled chamber at 37°C. To perform the biophysical assessments, a small droplet (9-10µl) of LA surfactant is deposited on top of the pedestal. The droplet is allowed to equilibrate for ~2min during which LA phospholipids absorbed to the air-liquid interface. The droplet is then exposed to compression-expansion cycles by the water pressure that is controlled with a syringe that directs the water to the top of the pedestal below the LA droplet. During the compression cycles, while a light source illuminates the droplet, a microscopic camera takes hundreds of images of the illuminated LA droplet. The images are recorded and later are analyzed for surfactant biophysical measurements by using an Axisymmetric Drop Shape Analysis (ADSA) software (Kalantarian et al., 2011).

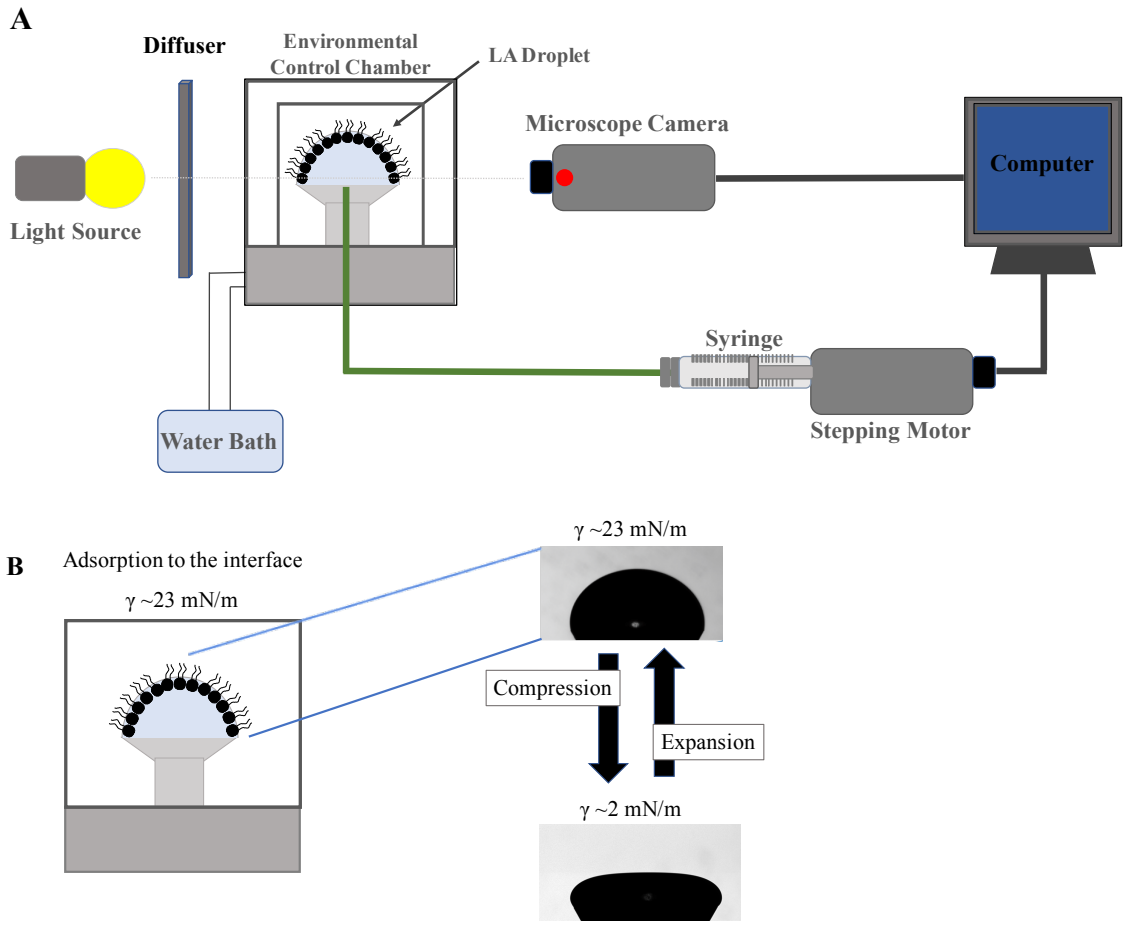

Figure 1. 3. A schematic diagram of the constrained sessile drop surfactometer (CSD). **(A)** This setup allows to precisely assess the surfactant function using a small volume of LA sample (9-10µl) on a pedestal, in an environmentally control chamber. The pedestal is connected to a computer-controlled syringe for dynamic compression and expansion cycling; mimicking breathing cycles. During the cycling, a digital camera takes and records images of the droplet. **(B)** Surfactant function is assessed by image analysis which is based on the shape and surface area of the droplet (Kalantarian, Saad, & Neumann, 2013) (γ: surface tension).

1.8. Clinical Significance of Surfactant

The importance of surfactant metabolism and biophysical function for a normal healthy lung function is best illustrated in patients with respiratory diseases such as neonatal

respiratory distress syndrome and the acute respiratory distress syndrome in adults.

1.8.1. Neonatal Respiratory Distress Syndrome (NRDS)

NRDS is a disease of infants and is characterized by immature lung development and surfactant deficiency. The infants experience reduced lung compliance, increased work of breathing, alveolar collapse, and reduce blood oxygenation (Avery & Mead, 1959; Brumley, Hodson, & Avery, 1967; Davis, Morley, & Manley, 2012). Previous clinical observations showed that there were about 10 times reductions in the total alveolar surfactant pool sizes in premature infants with NRDS compared to healthy offspring (10mg/Kg in NRDS vs 100mg/Kg body weight in control) (Fidanovski et al., 2005). Also, surfactant analysis of infants died from NRDS demonstrated impairments of reducing surface tension property compared to infants died from other diseases (Avery & Mead, 1959). The importance of the pulmonary surfactant has been shown by adverse respiratory outcomes caused by surfactant deficiency in NRDS patients and subsequently alleviating these deleterious outcomes by instillation of exogenous surfactant formulations within the lung lumen of NRDS patients (Enhorning et al., 1985; Hallman et al., 1985; Liechty et al., 1991). In spite of applying surfactant replacement therapy and the implementation of high technology, NRDS is still a common cause of mortality among the neonates with surfactant deficiencies (Kamath et al., 2011).

1.8.2. Acute Respiratory Distress Syndrome (ARDS)

In adults, surfactant alteration has been consistently illustrated in patients with ARDS, which is defined by severe lung dysfunction occurs after initiating direct (i.e., gastric acid aspiration) or indirect (i.e., sepsis) (Akella & Deshpande, 2013; Frerking, Günther, Seeger, & Pison, 2001; R. A. Veldhuizen, McCaig, Akino, & Lewis, 1995) insults to the lung. The pathophysiology associated with ARDS includes acute systemic and pulmonary inflammation and pulmonary edema. These alterations can result in dysregulated surfactant metabolism and inhibition of LA biophysical function (Gregory et al., 1991; Günther et al., 1996; Günther et al., 2001; Schmidt et al., 2007; Veldhuizen et al., 1995; Ware & Matthay, 2000). The ultimate results of surfactant inhibition are reduced lung compliance and hypoxemia, the development of severe lung injury and, ultimately, in critically ill patients,

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lung failure and death. Due to the lack of effective pharmacotherapies the mortality associated with ARDS is high in the ICU (~40% worldwide). One of the common reasons for the failure of therapeutic approaches is that different types of initiating insults can affect different systemic and pulmonary intracellular pathways with different severity. Furthermore, patient-specific characteristics can affect the complexity and severity of the disease. Therefore, one way to interfere with the adverse outcomes of the disease can be identifying the factors that influence the progression of the pulmonary alterations due to common etiologies of ARDS (i.e. sepsis).

1.8.3. Sepsis-Induced ARDS

Sepsis is defined as a dysregulated host immune response to a systemic infection and is the most common etiology of severe lung injury and ARDS in adults (with males more affected) (Fein & Calalang-Colucci, 2000; Hudson et al., 1995; Zellweger et al., 1997). The acute systemic inflammation often causes multi-organ dysfunction in the setting of severe sepsis including lung as one of the first organs adversely affected (Shorr et al., 2006).

Previous clinical and animal studies showed that sepsis-induced lung injury was associated with reductions in the total surfactant content (Huang et al.,2005; Malloy et al., 1997; Pison et al., 1990). Although surfactant replacement therapies have shown significant beneficial impacts in infants with NRDS it has unfortunately failed to show any beneficial clinical effect in adults with sepsis-induced ARDS (Spragg, 2002). Also, no other clinical therapies have been successfully developed to benefit all these patients. One of the other main reasons for high mortality in patients with sepsis-induced ARDS is that the diagnosis of the disease progression is possible when the patient is beyond the time frame for receiving efficient therapies (Shaver et al., 2017). Therefore, as the most common cause of ARDS, identifying risk factors that predispose the septic individuals at higher risks of the development of pulmonary impairments would be beneficial.

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1.8.4. Fetal Growth Effect on the Sepsis-Induced Pulmonary Response

Different patient-specific factors have impacts on the incidence of sepsis and its associated pulmonary pathophysiological outcomes (Danai et al., 2006). There is some evidence that fetal growth restriction (FGR) may be one such potential factor which is defined as small for gestational age (Barker et al., 1991; Hoo et al., 2004; Rueda-Clausen, Morton, & Davidge, 2009).

Clinical and animal studies indicate that FGR is associated with a variety of adult on-set diseases (with males more affected) such as diabetes, cardiovascular diseases, chronic pulmonary hypertension, and chronic systemic inflammation (Barker et al., 1991; Karadag et al., 2009; Murki & Deepak, 2014; Real et al., 2010; Vega et al., 2016; Wong et al., 2008) There are underlying shared mechanism between these morbidities and sepsis-induced ARDS, such as chronic inflammatory responses and ER and oxidative stress (Lv et al., 2013; Maritz et al., 2004; Vega et al., 2016). Despite all this evidence, the impact of FGR on the septic pulmonary response has not been studied in great details.

1.8.5. Analysis of FGR Effect on Sepsis-Induced Pulmonary Response

To study sepsis and FGR there have been some animal models developed with some advantages and disadvantages (Joss-Moore et al., 2011; Maritz et al., 2004; Swanson & David, 2015). For our study, we used rats as experimental species due to some specific features such as: i) rats' surfactant system and lung function have been extensively assessed in many previous studies, including our lab (Huang et al., 2005; Malloy et al., 1997; Orgeig, Barr, & Nicholas, 1995; Wichert et al., 2000) , ii) rats can be used as a clinically relevant animal model in the setting of both FGR and sepsis (Petry, Ozanne, & Hales, 2001) (have been, further, discussed below), iii) it is a, relatively, low cost animal model with high reproduction rates, iv) the relative large size of rats allows for easy handling, breeding, instrumentation, and obtaining enough samples even at the neonatal period. Given that there is a sexual dimorphism in developing sepsis-induced pulmonary dysfunction and FGR-adult onset-diseases, all the treatments and experimental analysis were applied to both male and female rats.

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To induce FGR, we used an isocaloric maternal low protein diet (LP) during pregnancy-lactation period. This model has some features which make it suitable for the purpose of this study, such as i) LP newborn rats display FGR characteristics (Petry, Ozanne, & Hales, 2001; Snoeck et al, 1990), ii) LP offspring manifest various clinical adult-onset morbidities such as hypertension, hypercholesterolemia, and insulin resistance, iii) LP is a relevant animal model of human placental dysfunction-induced FGR (the common etiology in Canada) (Crosby, 1991), and, finally, iv) compared to other FGR models like hypoxia or utero-placental intervention, LP is less technically demanding and accompanied with significantly less maternal mortality and fetal resorption (Hayashi & Dorko, 1988).

Sepsis was induced in rats by fecal-induced peritonitis (FIP). Some features of FIP that make it a proper model to study sepsis are i) consistently mimicking clinical septic-associated pathophysiological outcomes (Vincent et al., 2009), ii) easy controlling of disease severity by adjusting the injecting dose of fecal slurry, iii) no need for surgery and post-surgical recovery procedure, and iv) no need for general anesthesia; it suppress breathing and can affect surfactant metabolism (Petrov & Lyubarskii, 1966; Saraswat, 2015).

The sepsis effect in FGR rats was investigated by: i) measuring levels of systemic and pulmonary inflammatory responses, ii) measuring the extracellular pulmonary surfactant levels, and iii) a preliminary histological assessment.

1.9. Rationale and Hypothesis

The proper lung mechanics and the process of gas exchange are directly dependent on the surfactant function. At the early life, impairments of the lung development may result in significant persisting alterations of the surfactant system with progression of several associated adverse impacts. From previous animal studies, there are some preliminary indications that FGR has potential effects on the lung development and the surfactant system of neonates with life-lasting consequences. Furthermore, clinical and experimental studies demonstrated that FGR adult-onset morbidities may predispose individuals to the development of surfactant impairments and lung injury in response to an insult, such as sepsis, in a sex-dependent manner. Based on this knowledge and lack of effective therapies

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for sepsis-induced pulmonary complications, in-detail analysis the potential FGR pulmonary outcomes may help to identify and, ultimately, interfere with its clinical associated risks.

I had two hypotheses:

Hypothesis 1– FGR induced by maternal protein restriction contributes to the alterations of lung mechanics and of the surfactant system in a sex-dependent manner during the neonatal period (Chapter 2).

Hypothesis 2– In response to sepsis, FGR induced by maternal protein restriction contributes to the alterations of the surfactant system in adults in a sex-dependent manner (Chapter 3).

I had two objectives:

Objective 1– To examine the lung mechanics and amounts and function of the pulmonary surfactant in a rat model of FGR induced by a low maternal protein diet during the neonatal period (Chapter 2).

Objective 2– To examine the effects of FGR induced by maternal protein restriction on the amounts of pulmonary surfactant and development of lung injury in response to sepsis in adults (Chapter 3).

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Chapter 2- Impacts of Fetal Growth Restriction on the Surfactant System and Lung Function during Early Postnatal Life

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2.1. Introduction

During pregnancy, maternal nutrient restriction and/or placental malfunction can result in fetal growth restriction (FGR), where infants have failed to achieve their growth potential in the uterus (Barker et al., 1991; Hoo et al., 2004; Rueda-Clausen, Morton, & Davidge, 2009). FGR is observed in ~8% of newborns in Canada (Canadian Institute for Health Information, 2009). FGR has a variety of health consequences later in life, in a sex-dependent manner. Focusing on the lung, FGR is associated with constrained early postnatal pulmonary development and complications such as decreased lung angiogenesis and alveolarization and chronic pulmonary inflammation (Bose et al., 2009; Carey et al., 2007; Chen et al., 2004; de Onis et al., 1998; Lin & Lechner, 1991; Rozance et al., 2011; Winick & Noble, 1966). These changes can lead to persisting impairments that increase the risk of respiratory morbidities later in life (Lipsett et al., 2006). Therefore, further understanding the pulmonary consequences of FGR is essential to, ultimately anticipate and efficiently interfere with its associated clinical risks.

There is evidence that the developmental changes associated with FGR can affect the lung function in early life. It has been shown that FGR, induced by a 50% maternal calorie restriction, resulted in lower lung compliance in female mice offspring (Albion, 2011). In another study, in the same animal model, it was shown that FGR resulted in changes in lung extracellular matrix composition in neonates which later in life caused a reduction in lung compliance (Rehan et al., 2012). Also, other studies suggested that FGR caused decreases in specific surfactant phospholipids or proteins (Braems et al., 2000;Chen et al., 2004; Gortner et al., 2005; Joss-Moore et al., 2011; Rozance et al., 2011; Sutherland et al., 2012) that potentially can impair surfactant biophysical properties and, ultimately, lung compliance.

Based on the above information, previous studies have provided preliminary indications that in early life FGR affects lung function and the surfactant system. However, a comprehensive analysis is still lacking. It remains unknown whether the impairments observed in lung compliance could be the result of potential alterations in surfactant metabolism and/or surfactant biophysical function. However, the studies that focused on

lung function (Albion, in 2011; Rehan et al., 2012) did not assess whether potential changes in the surfactant system were an underlying mechanism responsible for the impairments of lung compliance in FGR offspring. Furthermore, the studies that investigated the FGR effects on the pulmonary surfactant did not address whether the identified changes in specific surfactant components contributed to inefficient lung mechanics. Ultimately, to our knowledge, whether FGR has impacts on the total surfactant pool sizes that results in alterations in its biophysical properties and the development of lung disease has remained unexamined.

In this study, we hypothesized that FGR induced by maternal protein restriction contributes to the alterations of lung mechanics and of the surfactant system in a sex-dependent manner during the neonatal period. To test this hypothesis, we utilized an animal model of FGR induced by a low maternal protein diet. At different days following birth, lung mechanics and in-depth analysis of the pulmonary surfactant system in both male and female offspring were performed.

2.2. Materials and Methods

2.2.1. Experimental Design and Ethics Statement

All procedures were approved by the Animal Use Subcommittee at the University of Western Ontario (Protocol Number: 2005-009). A total of 10 female and three male Wistar rats at breeding age (250 g) were purchased from Charles River (La Salle, St-Constant, Quebec, Canada). Rats were housed in individual cages and allowed to acclimatize for three weeks on a 12:12 light: dark cycle with free access to water and standard chow. After the acclimatization period, female rats were housed with stud males. The following morning, impregnation was confirmed by the presence of sperm in the vaginal smear. Upon confirmation of impregnation (gestation d1), pregnant rats were housed individually and randomized to one of two dietary conditions: a 20% protein (Control, n=6 litters) or an 8% low protein diet (LP, n=4 litter). The LP diet contained equal fat content and was made isocaloric by the addition of carbohydrates (Bio-Serv, Frenchtown, NJ). At birth, all the litters were culled to 8 and mothers were kept on the same dietary regimes until postnatal

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d21. Food and water were provided *ad libitum*. Weights of the male and female offspring were recorded at d1, d7, and d21 after birth.

2.2.2. Analysis of Lung Mechanics

Rats at postnatal d7 and d21 were euthanized with an intraperitoneal injection (IP) overdose of Sodium pentobarbital (110mg/Kg of body weight [BW]). After performing a tracheostomy and exposing the lungs, animals were connected to the FlexiVent (SCIREQ, Montreal, Quebec, Canada) for ex vivo measurements of lung function. Following connecting to the FlexiVent, rats were immediately exposed to 1min *ex vivo* mechanical ventilation (V_t=10ml/kg, RR=120breath/min, PEEP=0cm). Prior to measurements, to standardize volume history, deep inflation was applied to the lungs from PEEP value to about a pressure of 30cmH_2O. Following deep inflation, we performed two different perturbations: a snapshot perturbation and a prime-8 (Ask et al., 2008; Robichaud et al., 2015). These software-controlled perturbations were performed with 10 seconds intervals of *ex vivo* ventilation. The snapshot perturbation was applied to measure compliance and resistance of the whole respiratory system. Prime-8 perturbation outcomes were values for proximal airway resistance, and tissue elastance.

2.2.3. Bronchoalveolar Lavage collection, Surfactant Isolation and Analysis

Following lung function measurements, whole-lung bronchoalveolar lavages (BAL) were collected through flushing the lungs with saline. For d7 and d21, lungs were lavaged with 4 or 5 aliquots of saline, respectively. For d1 offspring, BAL fluids were collected directly after euthanasia (Sodium pentobarbital IP overdose [110mg/Kg BW]) and tracheostomy; lungs were lavaged with four aliquots of saline. The total volumes of administered and recovered saline were recorded. The lavage samples were centrifuged at 150g for 10min at 4°C to remove all cellular debris from the lavage resulting in a supernatant containing the total surfactant (TS). A volume of TS (~300ul) was stored at -20°C and the remaining volume was centrifuged at 40,000g for 15min to separate the surface-active large aggregate (LA) surfactant subfraction from the supernatant, which contained the inactive small aggregates (SA) surfactant subfraction. The LA pellets were then re-suspended in saline, and both LA and SA subfraction were stored at -20°C for further analysis. The phospholipid

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contents of TS, LA, and SA lipid extracts were chloroform-methanol extracted and measured using a phosphorous assay as previously described (Bligh & Dyer, 1959; Duck-Chong, 1979). Due to significant differences in body weights between control and LP groups, values for surfactant phospholipid levels were calculated as the amount of phospholipid corrected for body weight (mg of the total phospholipid in BAL/gBW). Ultimately, using these corrected values, we calculated the percent LA (LA/(LA+SA)) in each experimental group.

2.2.4. Bronchoalveolar Protein Measurements

Protein concentration in BAL for d21 samples was measured using a Micro BCA protein assay kit (Pierce, Rockford, Ill., USA) according to manufacturer's instructions.

2.2.5. Surfactant Biophysical Analysis

Aliquots of LA were centrifuged at 21,000g for 15 minutes at 4°C, and the resulting pellets were re-suspended at a concentration of 2mg/ml phospholipid in a buffer containing 140mM NaCl, 2.5mM HEPES, and 1.5mM CaCl2, pH=7.4. Samples were incubated at 37°C for about 1hr prior to assessing their surface tension reducing ability using a constrained sessile drop surfactometer (CDS) as previously described (Milos et al., 2017; Valle, Wu, & Zuo, 2015). Briefly, a droplet of LA sample (~10µl) was dispensed onto the CDS drop holder. The droplet was allowed to equilibrate for 2 minutes to allow for surfactant adsorption. Following adsorption, the droplet was exposed to 20 dynamic compression-expansion cycles on the CDS using a computer-controlled stepper motor (LTA-HS actuator, Newport Corporation, Irvine, CA, USA). Compression-expansion cycles were applied at a frequency of 20 cycles per minute and intended compression to ~70-75% of the original area of the droplet. During the dynamic compression-expansion cycles, images of the droplet were taken at a rate of 10 frames per second and the recorded images were analyzed with Axisymmetric Drop Shape Analysis software to assess the sample surface tension and the surface area of each picture.

To better understand the information obtained by biophysical analysis of compression-expansion outcomes, an example of an isotherm (surface tension versus surface area) with

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different surfactant biophysical parameters is shown in Figure. 2.1 The figure displays the first compression-expansion cycle of a surfactant LA sample, at 2mg/mL phospholipid. Before compression, the sample is at its equilibrium surface tension. Over the compression, reducing in surface area directly causes lowering of the surface tension. While during expansion, increases in surface area result in increasing surface tension.

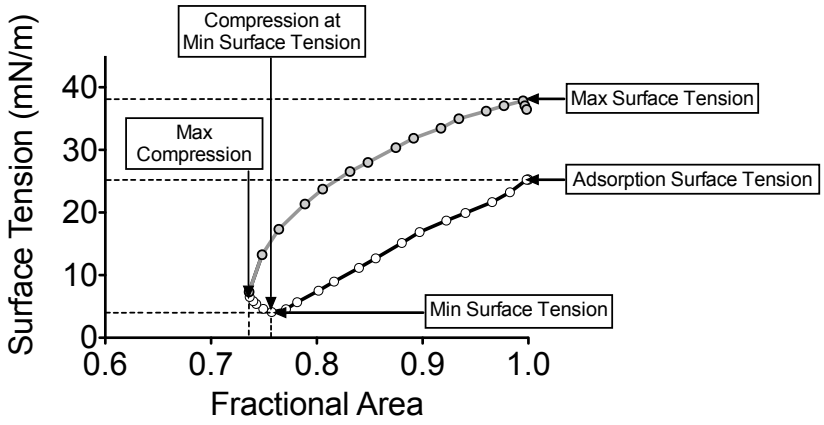

Figure 2. 1. A representative surface tension versus relative surface area (fractional area) isotherm at the first dynamic compression-expansion cycle assessed on the constrained sessile drop surfactometer (CDS). Samples were compressed (-o-) and expanded (-●-). Isotherms give information on surface tension following adsorption (equilibrium surface tension at cycle 1), minimum surface tension, maximum surface tension, and maximum compression needed to achieve minimum surface tension.

2.2.6. Statistical Analysis

All data are expressed as mean ± standard error of the mean (SEM). All statistical analyses were performed using the GraphPad Prism statistical software (GraphPad Software, Inc., La Jolla, CA., USA). Statistical comparisons for weights between control and LP neonatal offspring were performed using an unpaired, two-way student's t-test. Data analysis for lung mechanics parameters and surfactant phospholipid contents, as well as statistical comparisons for large aggregate surfactant function were performed using a two-way repeated measures ANOVA with Bonferroni post hoc test. Technical replicates of surfactant function assessed on CDS were averaged as an individual data point. Probability (p) values of less than 0.05 were considered statistically significant.

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2.3. Results

2.3.1. Effects of LP on Body Weight

Body weights for both male and female LP offspring were significantly lower compared to control males and females at d1 and d21. There was a non-significant trend in body weights of LP male and female offspring compared to controls at d7. Further analysis showed no significant differences in body weights between sexes (males vs. females) in the same dietary groups at each experimental day (Table 2.1).

Table 2. 1. Numbers and body weights of LP and control offspring at d1, d7, and d21

10 Litters n=148	**d1**		**d7**		**d21**	
	Control	LP	Control	LP	Control	LP
Males	7.0 ± 0.2 n=16	6.3* ± 0.3 n=13	21.2 ± 1.2 n=10	17.2 ± 1.5 n=6	73.5 ± 2.5 n=10	50.1* ± 4.0 n=10
Females	6.6 ± 0.2 n=26	5.8* ± 0.1 n=19	18.5 ± 1.6 n=8	15.7 ± 0.7 n=11	71.4 ± 3.0 n=10	45.9* ± 4.7 n=4

Body weights of d1 and d21 LP males and females were significantly lower compared to controls (*= $p < 0.05$ vs. control). There was a non-significant trend in body weights of LP offspring at d7 compared to controls ($p>0.05$). Data are expressed as means ± SEM

2.3.2. Lung Functional Analysis

Lung functional assessments in males and females at d7 and d21 are shown in Figure. 2.1. Lung functional assessments include: i) whole lung compliance (measure of lung expandability), ii) tissue elastance (measure of the lung's parenchymal stiffness), iii) whole respiratory airway resistance (proximal and peripheral airway resistance combined), and

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iv) proximal airway resistance.

2.3.2.1. Lung Function Analysis at d7

Data analysis at d7 showed that LP diet had no effects on different parameters of lung function in males or females ($p>0.05$) (Fig. 2.2). Also, further analysis between males and females at d7 displayed no changes in parameters of lung function between sexes with the same diet: there were no significant changes between control males compared to control females ($p>0.05$) or between LP males compared to LP females ($p>0.05$).

2.3.2.2. Lung Function Analysis at d21

Lung compliance and Tissue Elastance

Data analysis showed that LP resulted in alterations of lung compliance and tissue elastance in d21 females compared to controls ($p<0.01$) (Fig. 2.2. A). However, LP did not result in a significant difference in lung compliance in d21 males compared to controls ($p>0.05$). Furthermore, tissue elastance showed higher elasticity only in LP d21 females compared to control females ($p<0.01$) (Fig. 2.2.B).

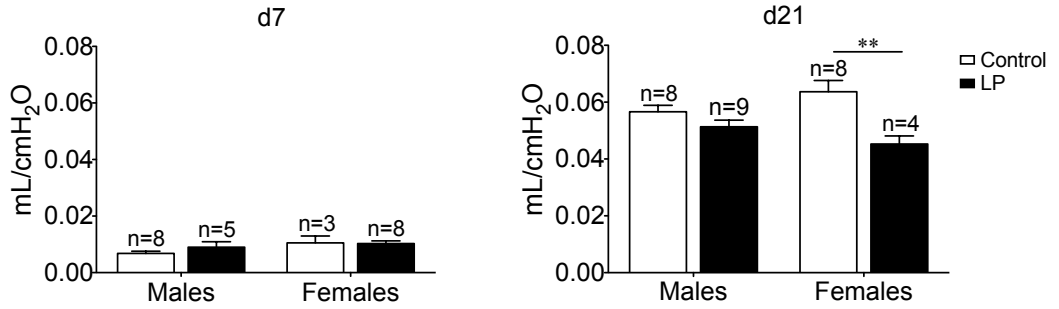
Whole Respiratory and Proximal Airway Resistance

Increase in whole respiratory airway resistance (Fig. 2.2 .C) was observed in response to LP diet in both d21 males ($p<0.001$) and females ($p<0.05$) compared to their respective control groups. However, proximal airway resistance (Fig. 2.2. D) displayed increases in LP males only compared to control males ($p<0.001$).

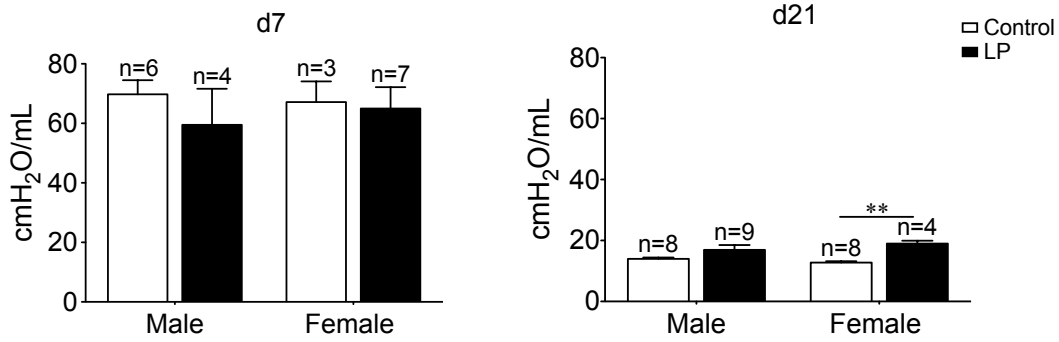
2.3.2.3. Lung Function Analysis: d21 versus d7

Further data analysis demonstrated some significant changes from d7 to d21: i) Lung compliance of d21 males and females were significantly higher compared to the same sex and diet groups at 7 ($p<0.001$), ii) in control groups, from d7 to d21, there were significant decreases for whole respiratory and proximal airway resistance ($p<0.01$ and $p<0.001$), and similar significant differences were observed in LP groups ($p<0.01$ and $p<0.001$).

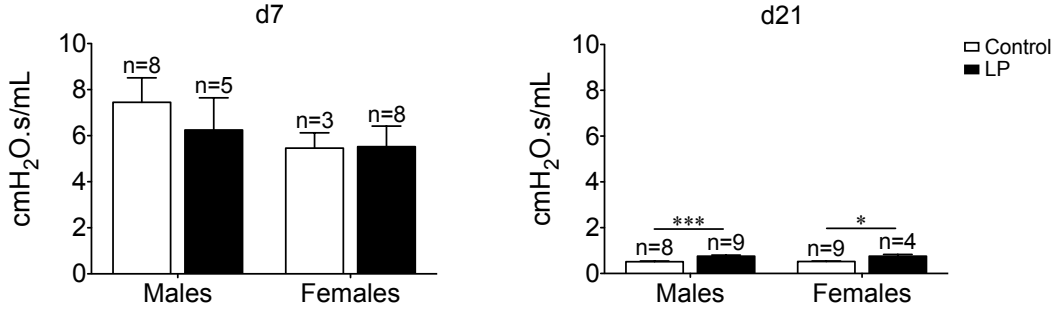
A. Whole Lung Compliance



B. Tissue Elastance



C. Whole Respiratory Airway Resistance



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D.Proximal Airway Resistance

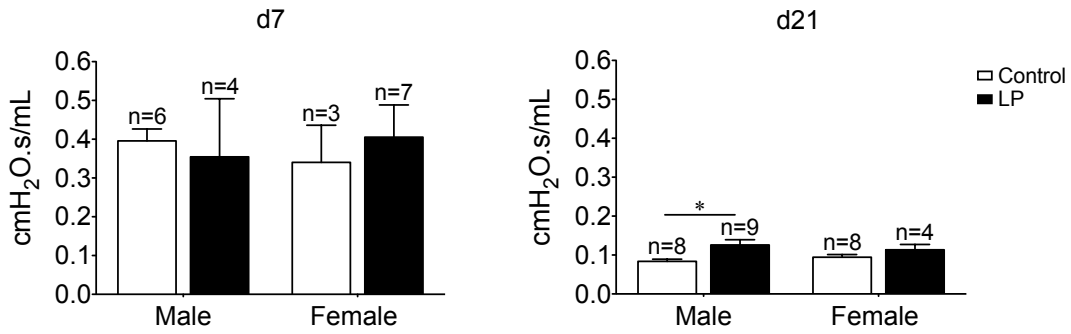

Figure 2. 2. Analysis of respiratory mechanics in Control and LP males and females at postnatal d7 and d21. (A) Whole lung compliance, (B) tissue elastance, (C) whole respiratory airways resistance, and (D) proximal airway resistance. Data are expressed as means ± SEM, *= $p < 0.05$ and **= $p < 0.01$vs. control.

2.3.3. Bronchoalveolar Surfactant Analysis

The total volumes of saline used to lavage the lungs (infused volumes) and the total volumes of collected BAL are displayed in Table 2.2. For d1 and d7, during lavaging, the same volumes of saline could be infused into the lung lumen of LP and control offspring. However, at d21, there were significant differences for the volumes of infused saline ($p<0.05$, $p<0.01$) between LP males and females compared to their respective control groups. Similar results were observed for collected volumes of BAL ($p<0.01$, $p<0.001$).

The results for BAL phospholipid measurements for TS and the surfactant sub-fractions (LA and SA) are shown in Figure. 2.3. Values for surfactant phospholipid levels have been presented as the amount of phospholipid corrected for the body weight (µg/g of body weight).

2.3.3.1. Large and Small Aggregate Phospholipid Levels

Overall, there were no significant differences for LA or SA phospholipid levels between LP offspring compared to the control groups at d1, d7, or d21 ($p>0.05$). Results showed

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that in control d7 and d 21 offspring of both sexes surfactant levels were lower compared to d1 offspring of the same sexes ($p<0.05$). However, there were no further differences in surfactant levels between d7 and d21 offspring ($p>0.05$). Similar findings were observed in LP groups at d7 and d21 compared to d1 offspring.

2.3.3.2. Large Aggregate Surfactant Percent

Further analysis demonstrated that percent LA (LA/(LA+SA)) (Figure. 2.4) did not change in response to LP diet at different ages versus control groups ($p>0.05$). In addition, although from d1 to d7 there were no changes in percent LA ($p>0.05$), however, d21 offspring had significant higher LA percent levels in both sexes compared to d7 and d1 ($p<0.05$). Also, similar results were observed for LP groups.

2.3.3.3. Surfactant Analysis Between Sexes

Finally, comparisons between control males and females displayed no differences in LA and SA phospholipid levels or LA at different days ($p>0.05$) with similar results were observed in LP males and females ($p>0.05$).

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Table 2. 2. Total volumes of saline used to lavage the lungs and volumes of collected BAL.

		Control Diet		LP Diet	
	Volume (ml)	**Males**	**Females**	**Males**	**Females**
d1	**Infused Saline**	1.6 ± 0.1	1.6 ± 0.1	1.5 ± 0.2	1.4 ± 0.1
	Collected BAL	1.4 ± 0.1	1.5 ± 0.1	1.3 ± 0.1	1.2 ± 0.1
d7	**Infused Saline**	3.3 ± 0.3	3.2 ± 0.3	3.1± 0.2	2.8 ± 0.2
	Collected BAL	3.0 ± 0.4	2.6 ± 0.4	2.5 ± 0.3	2.5 ± 0.2
d21	**Infused Saline**	15.8 ± 1.0	14.7 ± 1.5	9.1 ± 1.1##	7.2 ± 0.2#
	Collected BAL	14.2 ± 1.0	12.5 ± 1.0	8.1 ± 1.2***	6.4 ± 0.4**

Except for LP d21 males and females, there was no significant difference between the total volumes of saline infused in and total BAL collected ($p>0.05$) between other groups. **=$p<0.01$ vs d21 control females, ***=$p<0.001$ vs d21 control males, #=$p<0.01$ vs d21 control females, ##=$p<0.001$ vs d21 control males.

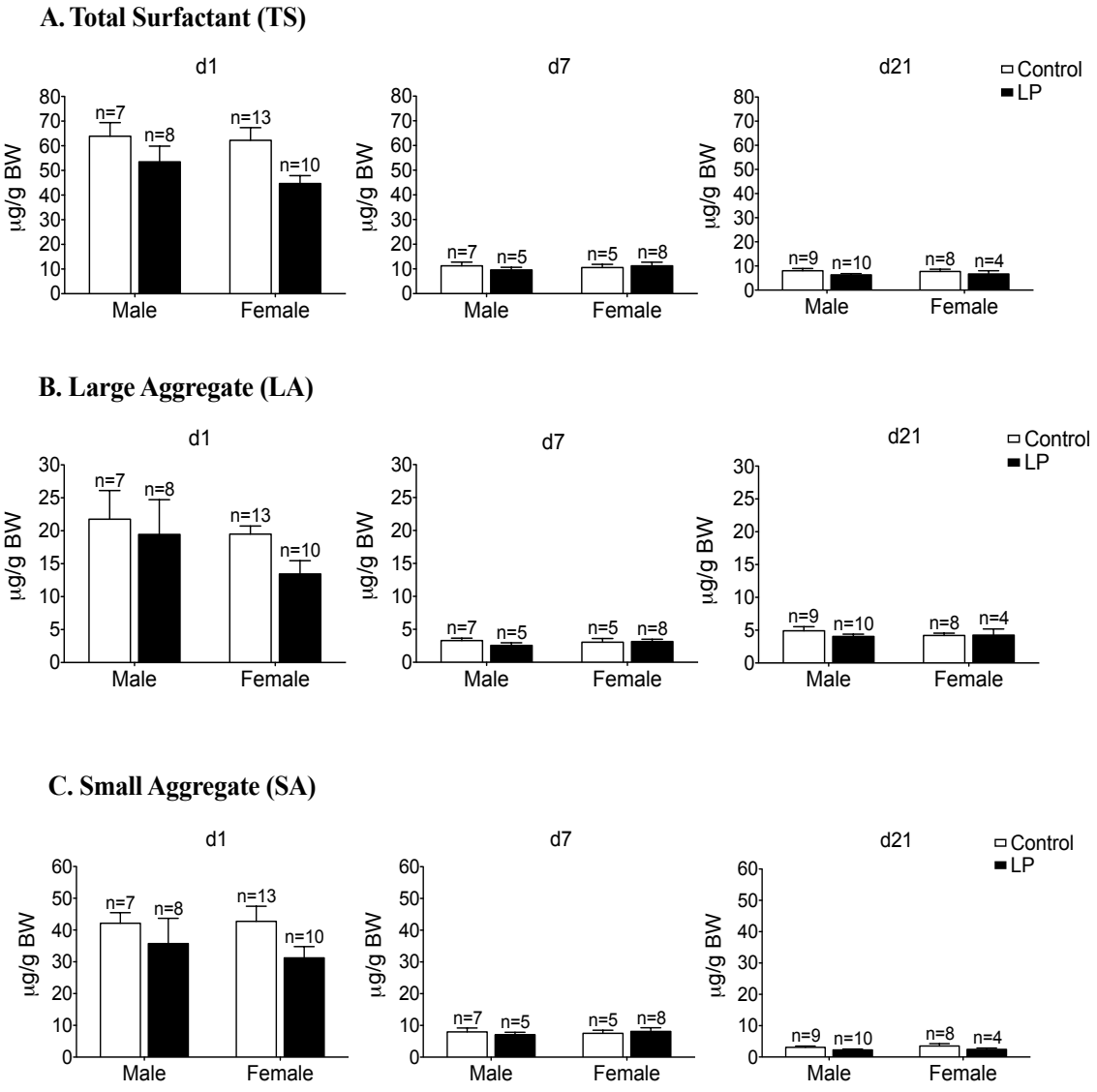

Figure 2. 3. Surfactant pool sizes of BAL fluids recovered from control and LP rat offspring. Surfactant pool sizes of (A) TS, (B) LA, and (C) SA measured by phosphorous assay. Data are expressed as amount of phospholipids/g body weight. Values are expressed as mean ± SEM, within each surfactant subfraction $p>0.05$ vs controls.

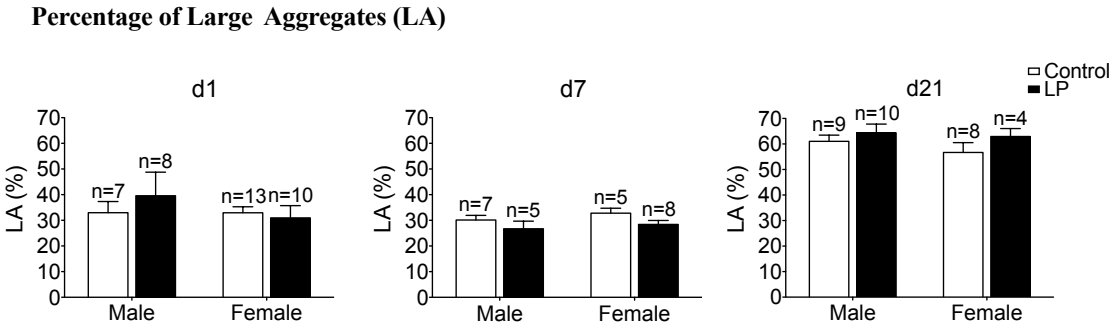

Figure 2. 4. Percent large aggregates measure in BAL at d1, 7 and 21 in LP and Control males and females. Data are expressed as mean ± SEM, $p>0.05$ vs controls.

2.3.4. Surfactant Biophysical Activity

Biophysical properties of isolated LA were assessed on CDS. Results for biophysical measurements have been presented in Figure 2.5, and Table 2.3. In this study, the minimum surface tensions that were achieved during compressions of LA samples were used as the primary indicator for biophysical assessment of surfactant. Surface tension upon adsorption (equilibrium surface tension) was also analyzed. The other measured biophysical parameters were maximum surface tension during expansion and compression required to reach minimum surface tension during each cycle. Figure. 2.5. shows two representative compression-expansion isotherms (at cycle 10) for d21 LP and control males and females. These isotherms demonstrated a reduction in surface tension during compression to ~7-10mN/m (Figure. 2.5. A) for LP and control males and to ~6-7mN/m (Figure. 2.5. B) for LP and control females. Isotherms show that the compressions required to achieve minimum surface tension for LP and control males and females were ~70-74% of the original area of the LA droplets.

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-Isotherms at d21

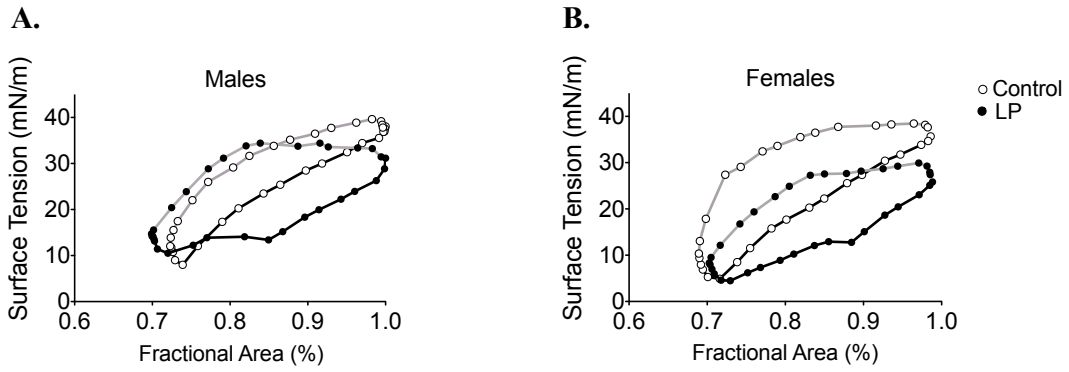
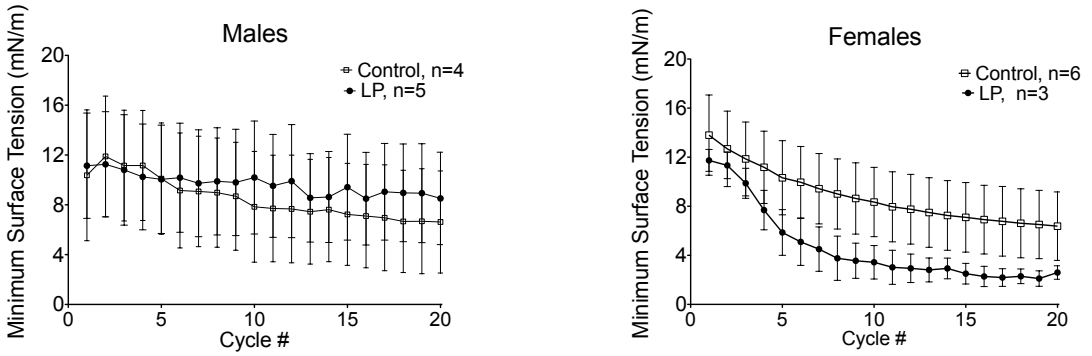


Figure 2. 5. Representative surface tension (mN/m) versus relative surface area (fraction of the initial area) isotherms at dynamic compression-expansion cycle 10 of LA isolated from rats with control and LP diets. Compressions are connected with back (-) and expansions with grey (-) lines.

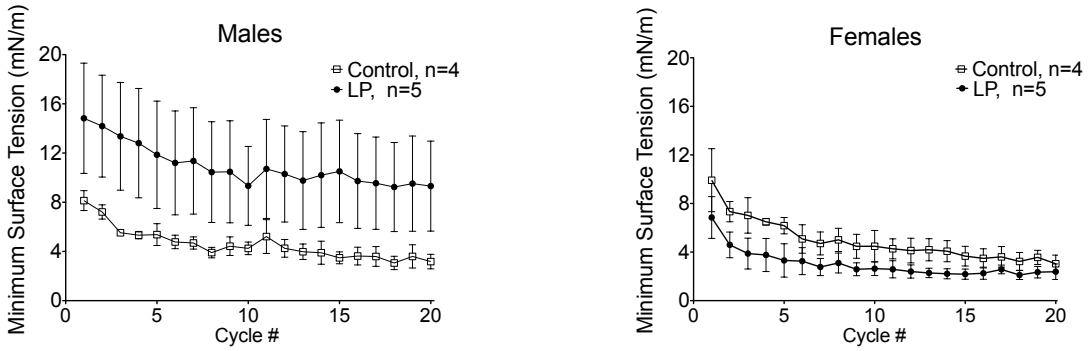
Minimum surface tensions achieved by LA samples at different cycles (cycle 1 to 20) for control and LP offspring at d1, d7, and d21 have been shown in Figure 2.6 A-C. Data analysis revealed that at each postnatal day there were no significant differences in minimum surface tension at any cycle for LP males and females compared to respective control groups ($p>0.05$). Furthermore, at each day no difference was observed in surface tension between control males and females of the same diet ($p>0.05$). The same statistical outcomes were observed for the comparison between LP males and females at different ages ($p>0.05$).

Results for equilibrium surface tension upon adsorption and other surfactant biophysical parameters at cycle 10 have been shown in Table. 2.3. Statistical analysis displayed no significant difference in equilibrium surface tension following 60-120 seconds of adsorption on CDS pedestal in isolated LA surfactant from LP offspring compared to controls for d1, d7, and d21 ($p>0.05$). Furthermore, there was no significant difference observed in maximum surface tension achieved between LP and control groups at different days. Lastly, a comparison between LP and control groups showed no statistical differences for maximum compression at cycle 10 ($p>0.05$).

A. d1



B. d7



C. d21

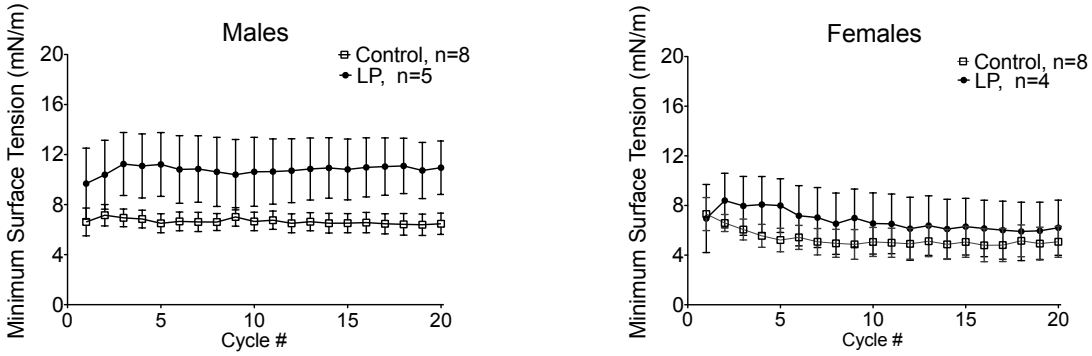


Figure 2. 6. Minimum surface tension during different cycles. Measurements of minimum surface tension (mN/m) during 20 dynamic compression-expansion cycles of LA samples isolated from BAL fluids at (A) d1, (B) d7, and (C) d21.Control vs LP diet. Data are expressed as mean ± SEM, $p>0.05$.

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Table 2. 3. Surfactant equilibrium surface tension upon adsorption, area compression ratios and maximum surface tensions at cycle 10 for control and LP rats at d1, d7, and d21.

	Age	Male		Female	
		Control diet	**LP diet**	**Control diet**	**LP diet**
Surface Tension Following Adsorption (mN/m)	**d1**	25.2 ± 1.3	26.0 ± 0.6	25.2 ± 0.3	26.0 ± 0.6
	d7	24.1 ± 1.0	25.1 ± 2.1	24.8 ± 1.2	24.7 ± 0.5
	d21	23.8 ± 0.4	23.5 ± 0.4	23.7 ± 0.5	23.4 ± 1.1
% of the Original Area at Min Surface Tension (Cycle 10)	**d1**	72.0 ± 2.2	74.4 ± 4.1	71.4 ± 2.2	69.2 ± 1.8
	d7	70.1 ± 1.6	71.8 ± 5.0	74.1 ± 3.3	72.7 ± 2.4
	d21	71.1 ± 0.9	71.5 ± 1.0	70.1 ± 0.6	73.4 ± 1.6
Max Surface Tension (mN/m) (Cycle 10)	**d1**	36.8 ± 2.1	38.2 ± 1.1	41.5 ± 1.5	40.9 ± 0.1
	d7	40.7 ± 0.9	44.4 ± 1.4	36.3 ± 2.2	41.5 ± 1.2
	d21	36.4 ± 1.2	36.2 ± 1.1	37.9 ± 1.8	33.7 ± 2.0

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2.3.5. BAL Protein Content

The analysis of BAL protein contents at d21 for males and females is shown in Figure. 2.7. Overall, there were no significant differences for protein contents in LP offspring compared to the control groups.

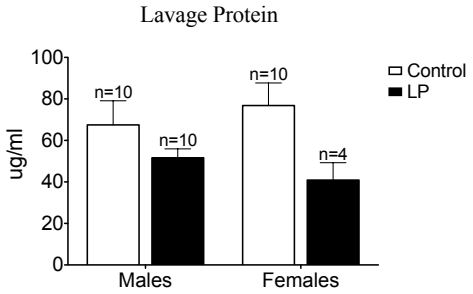

Figure 2. 7. Total protein content in BAL fluids from d21 males and females control and LP groups. Data are expressed as mean ± SEM, $p>0.05$ vs controls.

2.4. Discussion

In this study, we hypothesized that FGR induced by maternal protein restriction contributes to the alterations of lung mechanics and of the surfactant system in a sex-dependent manner during the neonatal period. Results demonstrated altered parameters of lung mechanics at d21 in a sex-specific manner. We showed that lung compliance and tissue elastance were significantly different in LP female offspring at d21. Furthermore, results for surfactant analysis demonstrated that at different postnatal days LP diet has no effect on the surfactant pool sizes or biophysical properties. Also, no alterations were observed in surfactant reducing surface tension ability. It is concluded that, during the neonatal period, FGR causes significant alterations to lung compliance in a sex-specific manner without having any impact on the surfactant system.

An important feature of this study was to analyze the surfactant system and lung function in an established clinically relevant animal model of FGR induced by maternal low protein diet (LP). Similar to previous studies that utilized LP the animal rat model (Petry, Ozanne, & Hales, 2001; Snoeck et al, 1990), we successfully observed lower body weight in LP

male and female newborns (d1) compared to control offspring. Furthermore, another advantage of this animal model was that compared to, relatively, invasive hypoxia or utero-placental interventions, LP is accompanied with significantly less reported maternal mortality and fetal resorption (Hayashi & Dorko, 1988).

Maintaining large surfactant pool sizes at birth, relative to the later ages, is nescessary to ease the opening of the lungs upon the transition to the air-breathing environment. Furthermore, alveolarization process in rats begins and progress during postnatal day 7-day 21 period (Burri, 1992; Schittny, Mund, & Stampanoni, 2008). Therefore, any changes in surfactant contents or lung function at day 1 or between day 7-day 21 would implicate in alterations in the lung development and pulmonary alveolarization process. Based on this knowledge, surfactant pool sizes and lung function were assessed during day 1 to day 21 postnatal life. The most interesting observation made in the current study is the significant decrease in lung compliance at d21 in LP female, but not male, rat offspring. Consistent with this decrease in lung expandability in this experimental group were measurements of increased tissue elasticity (stiffness). A similar observation was made previously using a maternal calorie restriction mouse model of FGR (Albion, 2011). In that study, only reported in a thesis format, lung functional measurements showed that 30-day old FGR female mice had lower lung compliance compared to control female mice (with no effects on males). Together, these studies provide strong support of the novel finding that FGR can lead to altered lung compliance in the neonatal period in a sex-dependent manner.

To further investigate the potential underlying mechanism of decreased lung compliance we investigated the pulmonary surfactant system. In general, lung compliance is affected by lung structure and the function of the pulmonary surfactant system. On the other hand, all the previous studies that investigated the effect of FGR on the pulmonary surfactant only measured the levels of specific surfactant phospholipids or proteins in the total surfactant (mostly intracellular surfactant) (Chen et al., 2004; Gortner et al., 2005; Sutherland et al., 2012). A novelty of my study was, for the first time, I assessed the effects of FGR on the surfactant biophysical activity. Furthermore, I separated and measured the LA surface active and SA inactive surfactant components in a FGR animal model, which

was not in those previous studies. Since the data showed no differences in the amounts of surfactant or the biophysical activity, it appears unlikely that surfactant was a contributor to decreased lung compliance in 21-day old female mice. Thus, although not investigated in the current study, it is possible that structural alterations may be responsible for the observed effect on the lung function. Previous studies showed that the most common cause of increased tissue stiffness is changes in lung structure by altered expression and deposition of lung extracellular matrix (ECM) proteins, most importantly collagen and elastin (Lindahl et al., 1997; Weibel, 2011). Also, Rehan et al., in 2012, showed that FGR induced by maternal calorie restriction resulted in increased levels of collagen subtypes in the lung parenchyma in 21-day old rats (males only examined). Further studies, such as parenchymal morphological and ECM compositional assessments, are required to specifically address the potential structural changes due to FGR and differences among male and female offspring in this process.

Although the main focus of our study was to study the impact of FGR, it is of interest to note that surfactant levels differed among the different age groups in both male and female offspring. Surfactant analysis in different age groups showed relatively higher levels of total surfactant and LA pool sizes at d1 compared to the later ages. Of note, due to differences in body weights between different age groups, the values for surfactant pool sizes were corrected for body-weight to allow for age comparisons. In a previous study, a large surfactant pool size was observed at birth in healthy male offspring (Runge & Patterson, 2007). This study suggested that large surfactant pool sizes at birth is necessary to facilitate opening of the lungs upon the transition to the air-breathing environment. Our results supported this previous observation and expanded upon it by first, showing that the same regulating mechanism to maintain high surfactant pool sizes existed in control female animals, and second, the mechanism was conserved even in LP newborn males and females.

Our findings on lung compliance and tissue stiffness in FGR offspring may have clinical implications. As mentioned above, ECM macromolecules, most importantly collagen and elastin, are responsible for regulating the parenchymal mechanical properties. Also, these proteins, especially collagen, have important roles in regulating homeostasis and cellular

responses to injury. Previous studies showed that chronic dysregulated parenchymal homeostasis co-existing with tissue stiffness may indicate the risk of pulmonary complications such as the progress of fibrosis, alveolar collapse and an increased risk of chronic lung disease (Lambert et al., 1992; Suki & Bates, 2008; Thibeault et al., 2003). In later ages into adulthood, these potential changes can make the pulmonary system more susceptable to different respiratory morbidities with severe clinical outcomes. To explore these potential outcomes in FGR adults, the next chapter of this thesis examined the effects of FGR on the susceptability of adult rats to develop lung injury in response to sepsis (as a common insult) in the same LP model.

It should be mentioned that this study was associated with some limitations. For example, in terms of practical limitations it should be noted that i) the FlexiVent that was used to assess the lung function was not designed for very small d1 offspring, therefore, we could not examine the FGR impacts on the lung function upon the transition to the extra-uterine environment, ii) we could not determine whether the reduced lung compliance in LP at d21 contributed to physiological symptoms, such as altered gas exchange and blood oxygenation levels, iii) since surfactant analysis requires lung lavage procedures which would invalidate structural analyses, there were not enough pups to perform structural assessments in LP neonates in addition to our surfactant analysis; this is considered a potential future direction, and iv) finally, due to the diversity of initiating mechanisms responsible for inducing FGR and, also, genetics and physiologic variations between different animal models and humans, our single animal model does not represent all the pulmonary clinical features associated with FGR.

2.5. Conclusions

Overall, this study showed that, during early postnatal life, maternal protein restriction-induced FGR is associated with changes in pulmonary mechanics in the absence of surfactant alterations. Furthermore, our findings demonstrated a sex-dependent role for the impact of FGR on the development of lung functional impairments. This emphasizes the need for further understanding of the underlying mechanisms of pulmonary complications associated with FGR during the fetal period and their long-term impacts during adulthood.

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Chapter 3- Effects of Fetal Growth Restriction on the Pulmonary Surfactant System in Response to Sepsis in Adult Rats

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3.1. Introduction

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to a systemic infection (Fein & Calalang-Colucci, 2000). The systemic infection and inflammation can have ultimately impacts on different organs with the lungs being one of the first organs adversely affected (Shorr et al., 2006). Unfortunately, despite the improvements in understanding the pathophysiology associated with sepsis, there are no proven specific pharmacotherapies to reverse or even stop sepsis-induced lung injury (Anzueto et al., 1996; Luce et al., 1988; Spragg, 2002; Yu & Tomasa, 1993). As such, pulmonary complications caused by sepsis contributes to high mortality in critically ill patients (Stapleton et al., 2005). Therefore, it is important to identify the factors that influence the development of pulmonary alterations due to sepsis.

There is clinical evidence that patient-specific factors may have impacts on the incidence and development of sepsis and sepsis-induced lung injury. There is experimental and epidemiological evidence indicating that sex, age, and chronic comorbidities (such as diabetes and hypertension) are important factors affecting patient's risk for developing sepsis developing sepsis and sepsis-induced lung injury (Heffernan et al., 2011; Kadioglu et al., 2011; Martin et al., 2003; Oberholzer et al., 2000; Zellweger et al., 1997). Therefore, a septic insult in different individuals may elicit significantly different systemic and pulmonary outcomes (Danai et al., 2006; Martin et al., 2003). There is evidence that fetal growth restriction (FGR) represents a risk factor affecting the pulmonary responses to sepsis. In general, FGR is associated with adult on-set diseases and complications such as diabetes, cardiovascular disorders, and chronic systemic and pulmonary inflammation (Barker et al., 1991; Karadag et al., 2009; Murki & Deepak, 2014; Vega et al., 2016; Wong et al., 2008). There are underlying shared mechanisms between these diseases and sepsis-induced severe lung injury, such as inflammation and elevated levels of ER/oxidative stress (Lv et al., 2013; Maritz et al., 2004; Vega et al., 2016). The impact of FGR on septic responses within the lung have not previously been examined.

Previous animal studies, mostly performed in young healthy male mice and rats, have examined the effects of sepsis on the pulmonary system (Bailey et al.,2002; Huang et al.,

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2005; Luo et al., 2017). These studies showed alterations in the lung surfactant system such as decrease in surfactant levels and inhibition of the surfactant function as well as pulmonary inflammation. On the other hand, there is strong clinical evidence indicating sex-specific differences in the development of sepsis-induced systemic inflammation and lung injury (Kadioglu et al., 2011; Martin et al., 2003; Oberholzer et al., 2000); the incidence of sepsis and sepsis induced-lung injury is higher in males.

In this study, it was hypothesized that in adult rats, FGR affects the sepsis impacts on the surfactant system in a sex-dependent manner. To test our hypothesis, we used an animal rat model of FGR induced by a maternal low protein diet. Sepsis was induced by fecal intraperitoneal injection. This study included both male and female offspring allowing the assessment of sexual dimorphism on pulmonary surfactant and the development of lung injury.

3.2. Materials and Methods

3.2.1. Experimental Design and Ethics Statement

All procedures were approved by the Animal Use Subcommittee at the University of Western Ontario (Protocol Number: 2017-003). A total of 15 female and 5 male Wistar rats at breeding age (250g) were purchased from Charles River (La Salle, St-Constant, Quebec, Canada). Rats were housed in individual cages and allowed to acclimatize for 3 weeks on a 12:12 light:dark cycle with free access to water and standard chow. After the acclimatization period, female rats were housed with stud males. The following morning, impregnation was confirmed by the presence of sperm in the vaginal smear. Upon confirmation of impregnation, pregnant rats were housed individually and randomized to one of two dietary conditions: a 20% protein (Control, n=7 litters) or an 8% low protein diet (LP, n=9 litters). The LP diet contained equal fat content and was made isocaloric by the addition of carbohydrates (Bio-Serv, Frenchtown, NJ). At birth (d1) offspring weights were recorded and the litter size was reduced to ten animals. This ensured a standard litter size for all mothers. After birth, mothers were kept on the same dietary regimes until postnatal d21. Male and female offspring were weighed and separated at postnatal d21. Food and water were provided ad libitum. Since d21, all the offspring were fed with the

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control protein diet until between d130-d150 when the rats were randomly assigned into sham or septic groups. Weights of the offspring were recorded at d1 and d130-150.

3.2.2. Sepsis Model

Sepsis was induced by fecal peritonitis (FIP) by giving each rat an intraperitoneal (IP) injection of a fecal slurry according to the following procedure: equal amount of fresh rat feces was collected from different experimental groups about 12h before the intraperitoneal injection. The collected feces were weighed and mixed with saline solution to give fecal concentrations of 0.5g/ml. This fecal slurry was homogenized by vortexing the fecal solution for 2min. The collected homogenate was pressed through 4-ply gauze to remove particulate matter. Following the filtration, the fecal solution was kept overnight, at 4°C. The next morning, 15 min prior to the IP injection upon randomization, analgesia was provided by a subcutaneous injection of 0.6ml buprenorphine (0.03mg/ml). Once the proper depth of anesthesia was achieved each rat was given an IP injection of the fecal slurry solution (1.0ml/100g of body weight [BW]) using a syringe and 21G needle. Sham rats were received an injection of sterile saline (1.0ml/100gBW).

3.2.3. Monitoring of Rats

We used a standard murine sepsis score (MSS) (Shrum et al., 2014) to monitor and evaluate the health of the animals for 6h; 15 min before and after the IP injection, and every 30min thereafter. To assess the health of the animals, we used the variables that have been described previously (Shrum et al., 2014 and Langford et la., 2010). These variables included respiratory quality (i.e. labored breathing or gasping), appearance (i.e. degree of piloerection or changes in the eye color), responding to auditory stimuli (i.e. fast or slow), and spontaneous activity (i.e. continuous/lack of investigating movements) of the animal. Each of these variables was given a score between 0 to 4. In addition, respiratory rate was measured at each observation. Rats were euthanized if the total MSS at any given time point was greater than 9, or if any of the individual scores increased by more than 3. Following completion of 6h monitoring, animals were euthanized with an overdose of IP injection of sodium pentobarbital (110mg/KgBW).

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3.2.4. Blood Collection and Bacterial Culture

Immediately after euthanasia, blood samples were taken from abdominal aorta for evaluation of systemic infection and inflammatory responses. Colombia blood agar plates (BD Bioscience, Mississauga, ON) inoculated with 0.1ml blood samples were incubated overnight in an aerobic chamber at 37°C. The next morning, the plates were checked to determine the number of bacterial colony-forming units. The remaining volume of blood samples were centrifuged to collect the serum, which was stored at -80°C for cytokine analysis.

3.2.5. Bronchoalveolar Lavage collection, Surfactant Isolation

Whole-lung bronchoalveolar lavage (BAL) were performed after collecting blood by flushing the lungs with saline (~5 x 11ml aliquots of saline). The total volumes of administered and recovered saline were recorded. Total volume of BAL was centrifuged at 150g for 10min at 4°C to separate all the cellular components. A volume of the supernatant (~1.5ml), which contained the total surfactant (TS), was stored at -80°C for cytokine analysis. Also, a 1ml aliquot of TS was stored at -20°C to measure the total surfactant content. The remaining volume was centrifuged at 40,000g for 15min to separate surfactant large aggregate (LA) subfraction from the supernatant which contained the small aggregates (SA) surfactant subfraction. The LA pellets were then re-suspended in 1.0ml saline and both LA and SA subfraction were stored at -20°C for further analysis.

3.2.6. Lavage Cell Analysis

The isolated BAL cellular component was re-suspended in PlasmaLyte (Baxter Healthcare, Dearfeld, IL) (1000 µl to 2000µl depending on cell density). A 25µl aliquot of cell suspension was diluted in an equal volume of 0.4% trypan blue to assess viability through trypan blue exclusion, and subsequently utilized to determine total cell counts with a Bright-Line hemocytometer (Hausser Scientifc, Horsham, PA) and a light microscope (at 10x magnification). For cell differential, aliquots of cell suspension were spun down on cytospin slides at 1000 rpm using a Shandon Cytospin 4 (Termo Scientifc, Cheshire, UK) for 6 minutes at room temperature and stained with Hemacolor® (Harleco, EMD

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Chemicals Inc., Gibbstown, NJ, USA). The stained slides were utilized to perform differential cell counts using light microscopy (at 10x magnification). Leukocytes from five different fields of view were counted and averaged, and the percentage of each inflammatory cell type was calculated. The total number of alveolar macrophages (AM) and other proinflammatory cell types in BAL were then obtained by multiplying their percent by the total cell number previously determined. Ultimately, concentrations of total leukocytes, macrophages, and other proinflammatory cells in BAL were measured.

3.2.7. Bronchoalveolar Surfactant Measurements

The phospholipid contents of TS, LA, and SA lipid extracts were chloroform-methanol extracted and measured using a phosphorous assay as previously described (Bligh & Dyer, 1959; Duck-Chong, 1979). Due to significant differences of body weights between different experimental groups, values for BAL surfactant phospholipid levels were reported as the amount of phospholipid corrected for body weight (mg of the total phospholipid in BAL/KgBW). Ultimately, we calculated the percent LA (LA/(LA+SA)) in in total volumes of BAL fluids from different experimental groups.

3.2.8. Bronchoalveolar Protein Measurements

Total alveolar protein levels were measured in total BAL fluids using a Micro BCA protein assay kit (Pierce, Rockford, Ill., USA) according to manufacturer's instructions. Values for BAL protein levels have been presented as the protein concentration in BAL.

3.2.9. Measurement of Inflammatory Mediators in BAL and Serum

Concentrations of four cytokines (IL-6, G-CSF, TNF-α, and IFN-γ) and two chemokines (KC and MCP-1) were measured in BAL fluids using multiplexed immunoassay kits according to the manufacturers' instructions (R&D Systems, Minneapolis, MN). A Bio-Plex 200 readout system was used (Bio-Rad), which utilizes Luminex® xMAP fluorescent bead-based technology (Luminex Corporation, Austin, TX). Cytokine levels (pg/ml) were automatically calculated from standard curves using Bio-Plex Manager software (v. 4.1.1, Bio-Rad).

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Cytokines: IL-6= interleukin-6, IFN-γ= type II interferon, TNF-α= tumor necrosis factor alpha, and G-CSF= granulocyte colony stimulating factor

Chemokines: MCP-1= monocyte chemotactic protein-1 and Chemokines: KC= keratinocyte chemoattractant

3.2.10. Lung Collection and Tissue Fixation

Following euthanasia, three non-lavaged lungs, from each treatment group, were immediately isolated en-bloc. Briefly, the diaphragm was cut and a midline incision was performed to open the rib cage and expose the lungs. Lungs were fixed immediately with 4% paraformaldehyde solution in 0.1M phosphate buffer (pH=7.4) at a pressure of 22-25 cm water column by the airway instillation method (Weibel, Limacher, & Bachofen, 1982). After filling the airways, the trachea was clamped and the intact lungs were immersed in the same fixation solution for ~24hr. Fixed lungs were rinsed four times (20 minutes each) with 0.1M phosphate buffer (pH=7.4) at 4°C. Each lobe was, consistently, sectioned into slices at 3mm thickness and the sections were transferred into 70% ethanol (v/v%). Once wax embedded, lungs were sectioned into 6um slices and stained with hematoxylin and eosin (H&E) stain for histological assessments. On the H&E stained sections, we did preliminary observational assessments.

3.2.11. Statistical Analysis

All data are expressed as mean ± standard error of the mean (SEM). All statistical analyses were performed using the GraphPad Prism statistical software (GraphPad Software, Inc., La Jolla, CA., USA). Statistical comparisons for weights were performed using an unpaired, two-way student's t-test. Data analysis for total MSS, respiratory rate, surfactant phospholipid contents, and cytokine measurements were performed using a two-way ANOVA followed by a one- way ANOVA with a Tukey's post hoc test. Probability (p) values of less than 0.05 were considered statistically significant.

3.3. Results

3.3.1. Effects of LP on Body Weight

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Data analysis between control and LP groups showed that at d1, body weights for both LP male and female offspring were significantly lower compared to control males ($p<0.01$) and females ($p<0.05$) (Table 3.1. A).

At d130-d150, body weights for LP males were still significantly lower as compared to the control males ($p<0.01$) while LP did not have a similar effect on female body weights ($p>0.05$) (Table 3.1. B).

Comparing body weights between sexes showed that were no significant differences between male and female body weights at d1 ($p>0.05$) (Table 3.1. A). However, at d130-d150, within both diets, females' body weights were significantly lower compared to males' ($p<0.001$).

Table 3. 1. Body weights of (A) newborns and (B) adult rats

A. d1 (16 Litters, N=43)	**Control**	**LP**
Males	7.0 ± 0.3, n=8	6.0**± 0.2, n=8
Females	6.5 ± 0.2, n=14	5.7* ± 0.2, n=13
B. d130-150 (16 Litters, N=102)	**Control**	**LP**
Males	660.6 ± 12.3, n=27	591.1*** ± 11.2, n=29
Females	$379.1^{\#}$ ± 7.2, n=23	$360.7^{\$}$ ± 7.5, n=23

Data expressed as mean ±SE. **(A)** At d1, LP resulted in lower body weight in both males and females (*=$p<0.05$ vs. control females, **=$p<0.01$ vs. control males), n=8-14/group. However, **(B)** in adults, LP resulted in lower body weight only in males with no effect on females (***=$p<0.001$ vs. control males). Also, for both diets, adult females' body weights were significantly lower in compared to males' ($^{\#}$=$p<0.001$ vs. control males and $^{\$}$=$p<0.001$ vs. LP males), n=23-29/group.

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3.3.2. Murine Septic Score (MSS) before Euthanasia

In septic groups, during the experiments, there were 5 animals that were euthanized due to reaching a total MSS score greater than 9. All the euthanized animals were septic and were from both sexes and diets. There were 1 control male, 2 LP male, 1 control female, and 1 LP female septic animals that were euthanized before 6hr with a total MSS score>9.

Before the IP injections, MSS was zero for all the experimental groups. At the end of the experiments, before the euthanasia, comparing septic animals to shams revealed that regardless of diet and sex, the total MSS in all septic rats were significantly higher compared to shams ($p<0.001$) (Table 3.2). Also, results showed that septic LP offspring had higher total MSS than septic controls. Furthermore, data analysis showed no significant differences in septic LP males and females and similar results were observed between septic control males and females.

Table 3. 2. MSS scores at t=6h after the IP injection (before the euthanasia).

Total MSS	Control		LP	
	Sham	Septic	Sham	Septic
Males	1.5 ± 0.3	5.3 ± 0.2***	0.9 ± 0.2	6.3 ± 0.4*** #
Females	0.9 ± 0.2	5.2 ± 0.4***	1.2 ± 0.4	6.3 ± 0.4*** #

Data presented as mean ± SEM. Total MSS was significantly higher in all septic groups compared to shams in both diets. Also, further analysis demonstrated higher MSS score for septic LP males and females compared to septic controls (***=$p<0.001$ vs. LP Sham; #=$p<0.05$ vs Control septics). In addition, no differences were observed between septic LP males and females. n=10-14/group.

3.3.3. Respiratory Rate and Respiratory Score

At the beginning of the experiment, before IP injections, there were no differences for respiratory rate (RR) between different experimental groups (data is not shown; $p>0.05$)

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regardless of diet and sex. Also, results showed no significant changes in RR among different sham groups after 6hr (before euthanasia) (Figure 3.1). However, at the end of the treatments, data analysis displayed decreases in RR for all the septic groups compared to sham groups ($p<0.01$). Regardless of sex and maternal diet, at the end of the experiment, there were no differences between different sham groups ($p>0.05$) and similar results were observed between septic groups.

Before the IP injections, respiratory score was zero for all the experimental groups. At the end of the experiments, before the euthanasia, comparing septic animals to shams revealed that regardless of diet and sex, the respiratory scores in all septic rats were significantly higher compared to sham groups ($p<0.001$) (Table 3.3). Results showed that septic LP offspring had higher respiratory scores than septic controls in both males and females ($p<0.05$) (Table 3.3). Furthermore, data analysis showed no significant differences between septic control males and females and similar results were observed between septic LP males and females ($p>0.05$).

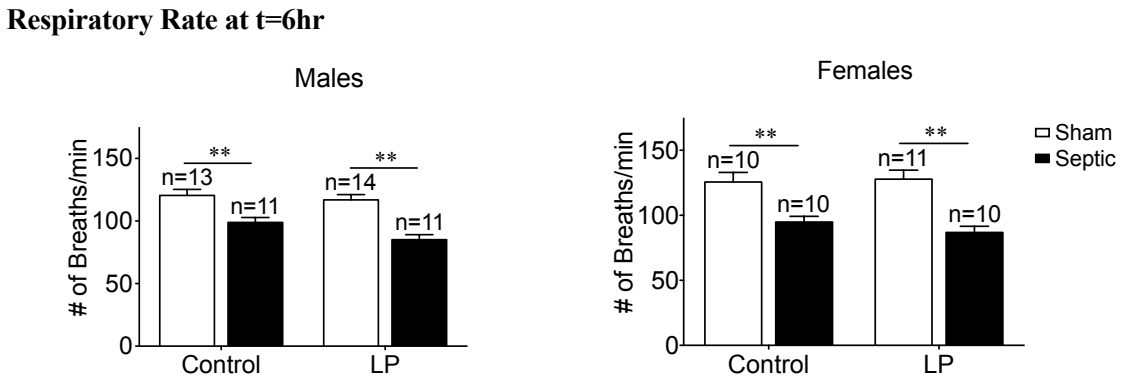

Figure 3. 1. Respiratory rates (RR) before euthanasia. RR of all septic groups were significantly lower than RR of sham offspring 6h after the injection of saline or fecal slurry ($p<0.01$). There were no differences in RR between septic males and females in both diets. Data presented as Mean ± SE, **=$p< 0.01$vs. Shams. n=10-14/group.

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Table 3. 3. Respiratory score. at t=6h after the IP injection (before the euthanasia).

Respiratory Score	Control		LP	
	Sham	Septic	Sham	Septic
Males	0	0.8 ± 0.1***	0.2 ± 0.2	1.7 ± 0.2*** #
Females	0	1.3 ± 0.2***	0	1.8 ± 0.3*** #

Data presented as mean ± SEM. Respiratory score was significantly higher in all septic groups compared to sham in both diets. Also, further analysis demonstrated higher respiratory score for septic LP males and females compared to septic controls (***=$p<0.001$ vs. LP Sham; #=$p<0.05$ vs Control septics). In addition, no differences were observed between septic LP males and females. n=10-14/group.

3.3.4. Blood Culture

Incubated blood cultures consistently showed bacterial growth in aortic blood samples of septic animals (as seen in clinical septic conditions). While blood cultures from sham animals showed negative bacterial growth.

3.3.5. Systemic Inflammatory Mediators

The effect of LP on the systemic inflammation was assessed at the end of the treatments in sham and septic males (Figure 3.2. A-B) and females (Figure 3.3. A-B). The concentrations of four cytokines (IL-6, TNF-α, IFN-γ, and G-CSF) and two chemokines (MCP-1 and KC) were measured at the end of the treatments in blood serum.

In sham groups, except for MCP-1 (Figure 3.2. B and 3.3.B), all the inflammatory mediators were below the detection range ($p>0.05$). Statistical analysis for MCP-1 in females showed significant differences between septic and sham groups ($p<0.05$). However, there were no differences in MCP-1 concentrations between septic and sham groups in both control and LP males ($p>0.05$).

Data analysis between septic male control and LP rats displayed no difference ($p>0.05$) in

concentrations of any inflammatory mediators. Similar results were observed between septic female control and LP rats ($p>0.05$). Also, in each dietary group, no significant differences in concentrations of different inflammatory mediators were observed between septic males compare to septic females ($p>0.05$). e

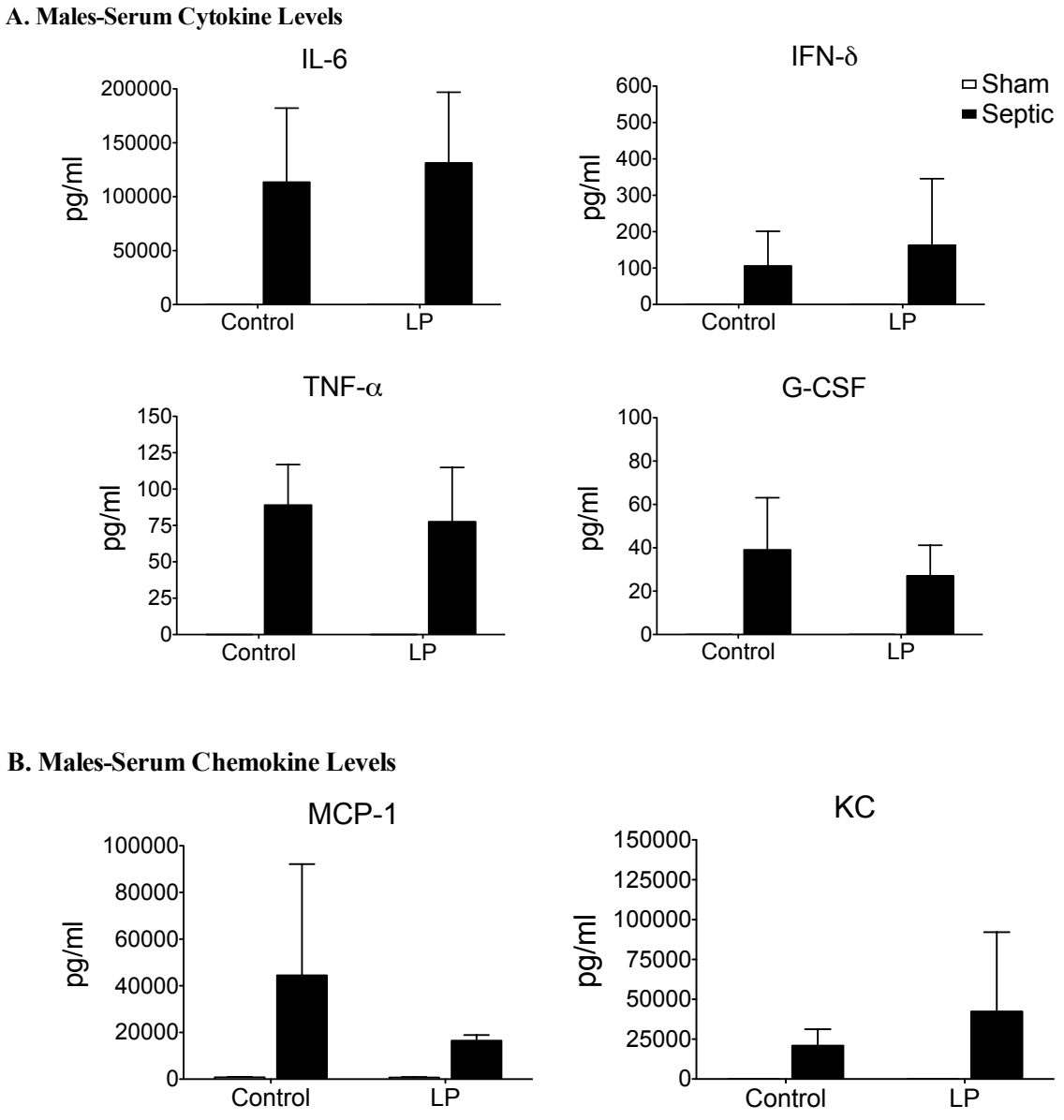
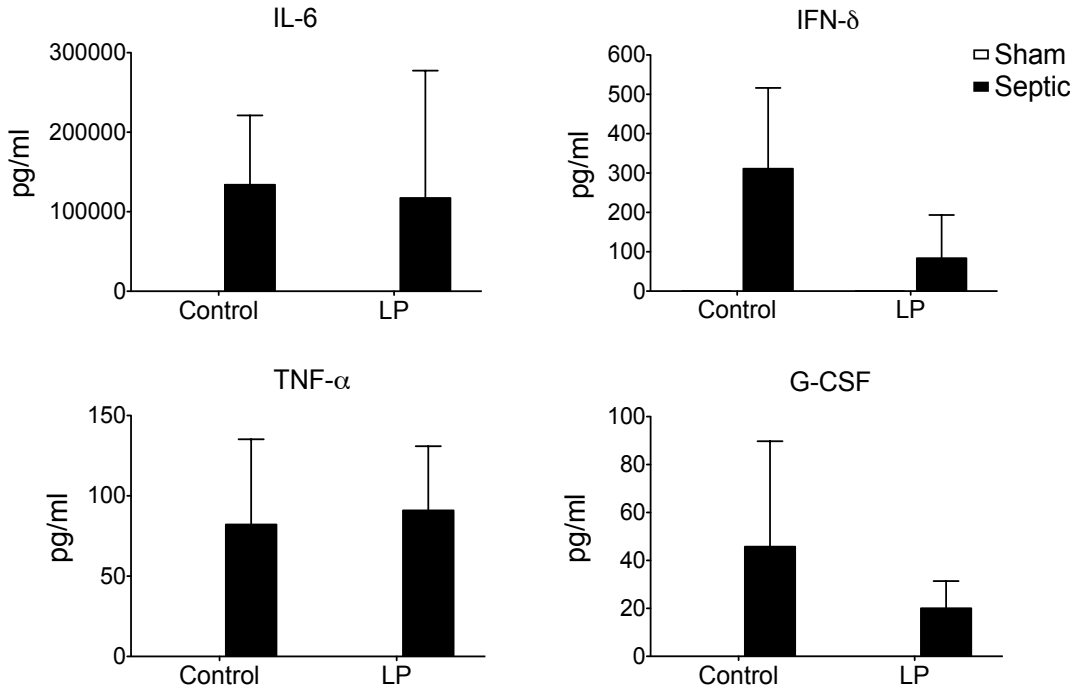


Figure 3. 2. Cytokine and chemokine levels measured in blood serum of males. (A) Analysis showed that LP did not have a significant effect on (A) cytokine and (B) chemokine levels between different septic groups compared to shams. Data presented as mean ± SEM, $p>0.05$. n=4/ sham groups and n=5/septic groups.

A. Females-Serum Cytokine Levels



B. Females-Serum Chemokine Levels

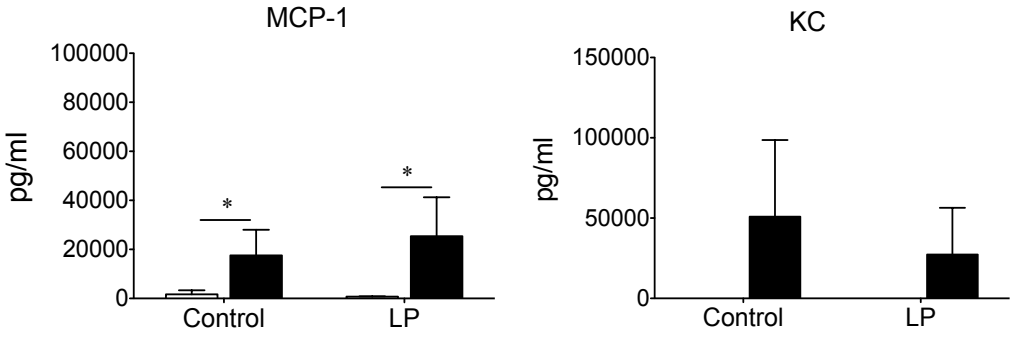


Figure 3. 3. Cytokine and chemokine levels measured in blood serum of females. (A) Analysis showed that LP did not have a significant effect on (A) cytokine and (B) chemokine levels between different septic groups compared to shams. Data presented as mean ± SEM, p >0.05 vs. Shams. Data presented as mean ± SEM, *=p <0.05 vs. Shams. n=4-5/group.

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3.3.6. BAL Pro-Inflammatory Cell Count and Differentiation

There were no differences in the total volumes of BAL fluids collected at end of lavaging for each group ($p>0.05$). This allowed us to compare the cell concentrations between different groups (Figure 3.4). Results showed no significant changes were observed in control septic males and females compared to control shams groups ($p>0.05$) (Figure 3.4 A). However, there were decreases observed in concentrations of total cells in BAL of septic LP males ($p<0.05$) and females compared to LP shams ($p<0.001$). Furthermore, data analysis showed no differences in BAL total cells between septic control males and females, and similar results were observed between septic LP males and females ($p>0.05$).

Differential cell counts demonstrated that decreases in concentrations of total cells in BAL between LP septic and sham animals were due to alterations in concentration of BAL macrophages (Figure 3.4. B) in LP septic rats compared to LP shams. Finally, comparing the concentrations of other types of BAL pro-inflammatory cells, including lymphocytes, monocytes, and basophiles, regardless of sex and diets, showed no changes between different septic and sham animals ($p<0.05$) (Figure 3.4. C).

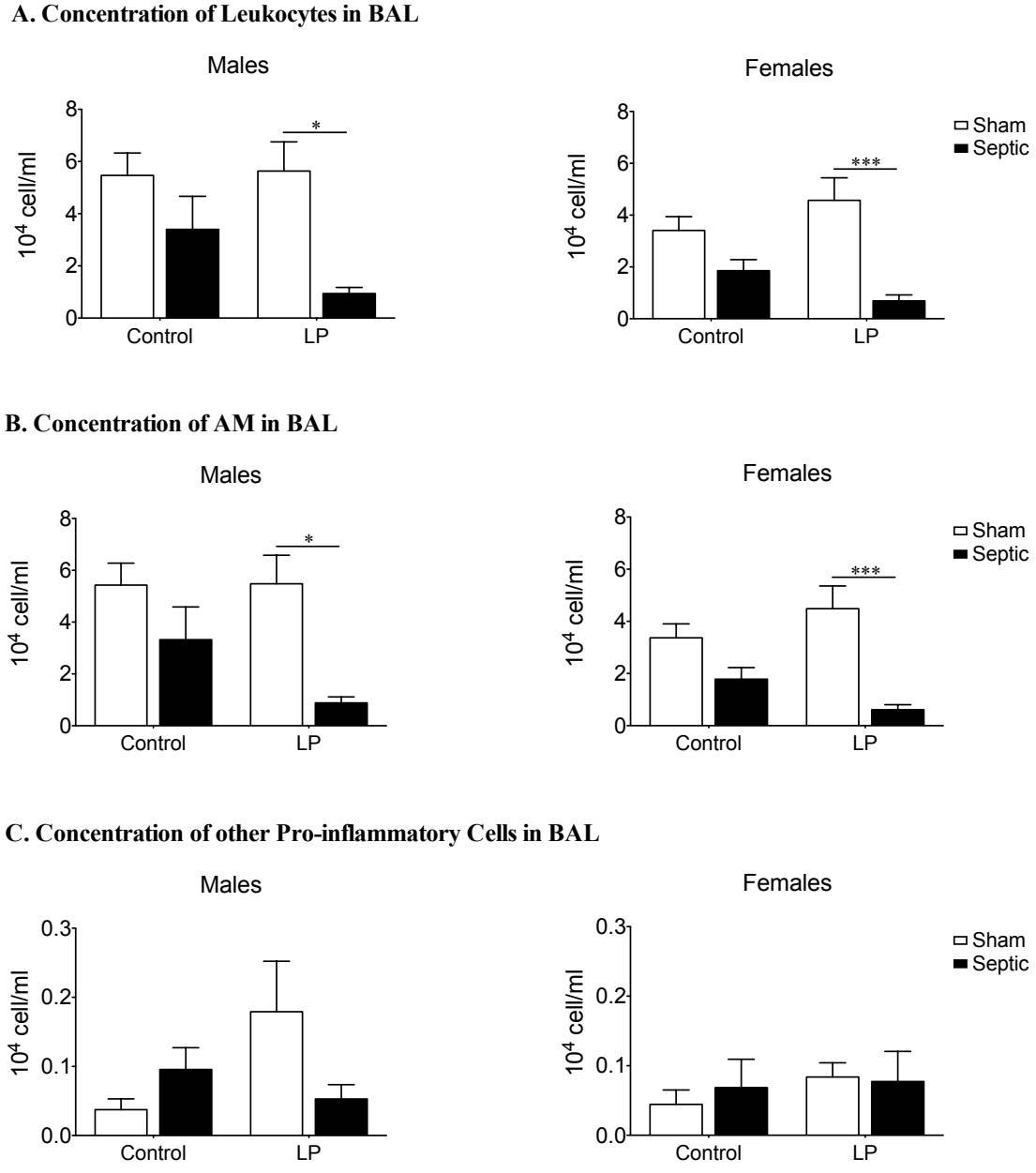

Figure 3. 4. BAL cell counts and cell differentials. (A) Concentration of total cells in BAL in different sham and septic group. Cell differentiation helped to determine the concentration of (B) BAL macrophages and (C) other BAL pro-inflammatory cells. Data presented as mean ± SEM, *=$p<0.05$ and ***=$p<0.001$ vs. Sham LP, n values for different experimental groups are the same as presented in Table 3.3 (n= 7-10/group).

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3.3.7. BAL Surfactant Phospholipid Levels

The results for BAL phospholipid measurements of TS and surfactant sub-fractions (LA and SA) are shown in Figure 3.5. BAL surfactant analysis displayed no changes in LA or SA surfactant subfractions between control septic males compared to sham males ($p>0.05$) (Figure 3.5. A). Similar results were observed between control septic females and shams ($p>0.05$) (Figure 3.5. B).

However, in LP males, there was a reduction in TS phospholipid levels in response to sepsis compared to shams (Fig 3.5. A) ($p<0.05$). Further analysis showed that the difference in TS was the result of a reduction in LA phospholipid levels ($p<0.05$) with no changes in SA ($p>0.05$). In LP females, however, BAL surfactant analysis displayed no changes in LA or SA surfactant subfractions in either control or LP females in response to sepsis compared to shams ($p>0.05$).

In septic and control groups, comparing between sexes showed that LA phospholipid levels in LP males was significantly lower compared to LP females ($p<0.05$). Similar results were not observed between control males and females ($p>0.05$).

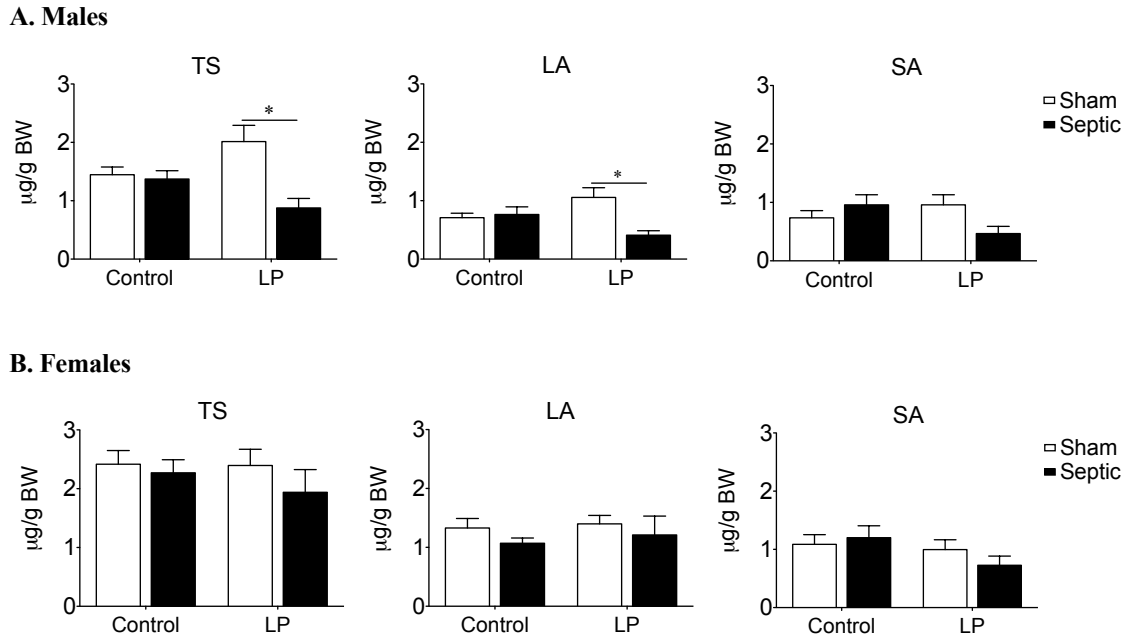

Figure 3. 5. Surfactant pool sizes of BAL fluids recovered from LP and control male and female rats. Data are expressed as mean ± SEM, *=p<0.05 vs. Sham LP (n= 7-10/group).

3.3.8. Percent Large Aggregate in BAL

Statistical analysis demonstrated that sepsis did not induce significant alterations in LA percent whether in LP or control offspring.

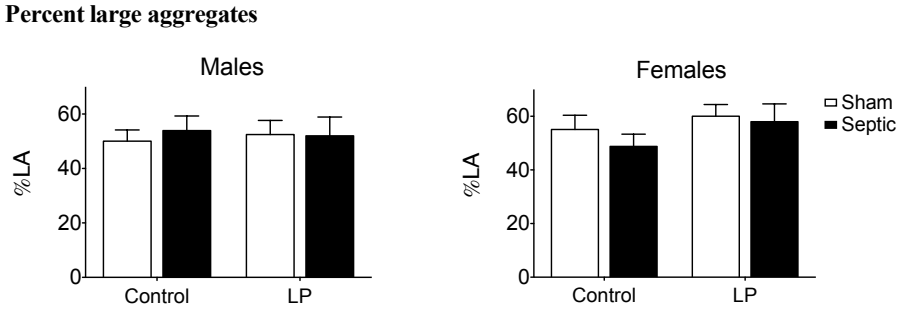

Figure 3. 6. Percent large aggregates (LA) measured in BAL fluids recovered from septic and sham animals. The data display the proportion of LA/(LA+SA) in total BAL volumes. Data have been expressed as mean ± SEM, p>0.05 vs shams (n= 7-10/group).

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3.3.9. BAL Protein Levels

The analysis of BAL protein contents for males and females is shown in Fig. 3.7. Overall, there were no significant differences for protein contents in response to sepsis whether in LP or control groups for both males and females ($p>0.05$).

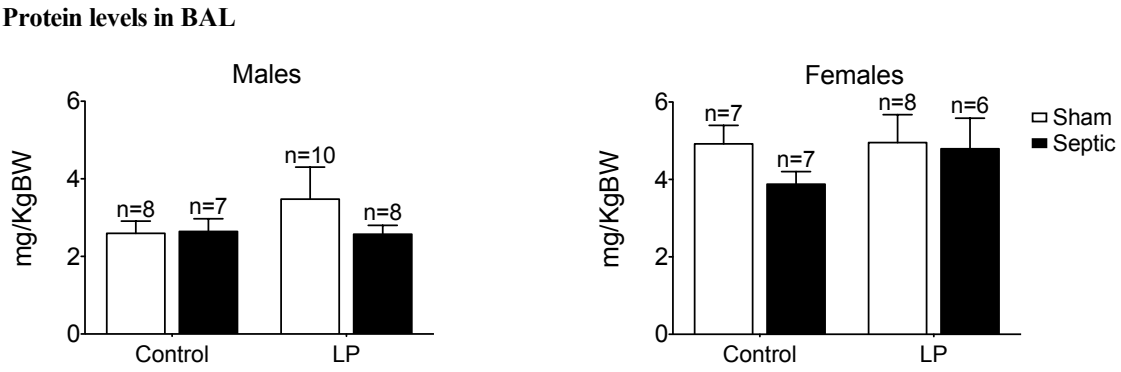

Figure 3. 7. Protein levels in BAL fluids. Results showed no significant changes in BAL protein levels between different treatments. Data presented as mean ± SEM, $p >0.05$ vs. Shams. n=6-10/group.

3.3.10. BAL Inflammatory Mediators

To further analyze the role of LP in intra-alveolar inflammatory response associated with sepsis, the concentrations of four cytokines (IL-6, TNF-α, IFN-γ, and G-CSF) and two chemokines (MCP-1 and KC) were measured at the end of the treatments in BAL fluids. Levels of these cytokines and chemokines were not detectable in any of the experimental groups (data not shown).

3.3.11. Lung Histology

Representative images of septic and sham males and females from both maternal diets have been shown in Figure 3.8 and Figure 3.9. Preliminary qualitative visual assessments illustrated morphological changes in all septic groups compared to shams; it appeared that alveolar edema and hemorrhage and thickening of the alveolar cell wall were presented in all septic groups.

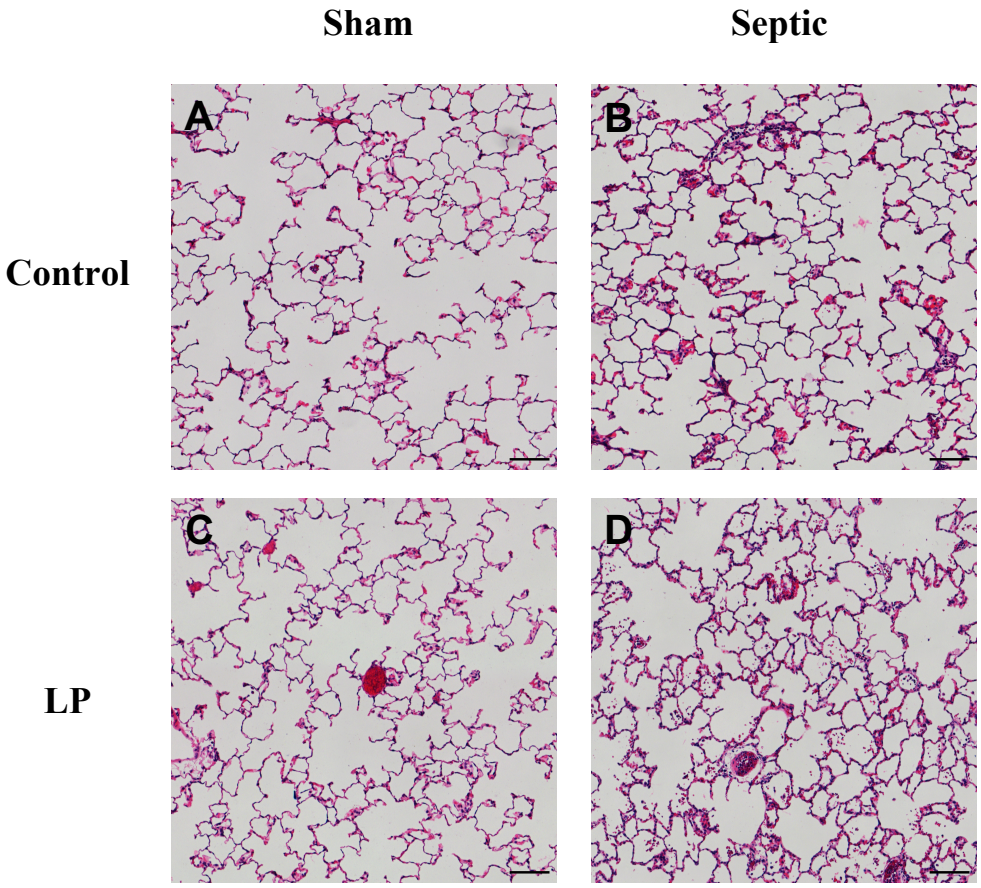

Figure 3. 8. Representative images of hematoxylin-eosin stained lung sections from males. Images of lung sections from sham and septic control (A, B) and LP (C, D) males, 6h after saline or FIP injections. Magnification 10x. Scale bar =100μm shown on the bottom right corner of each image.

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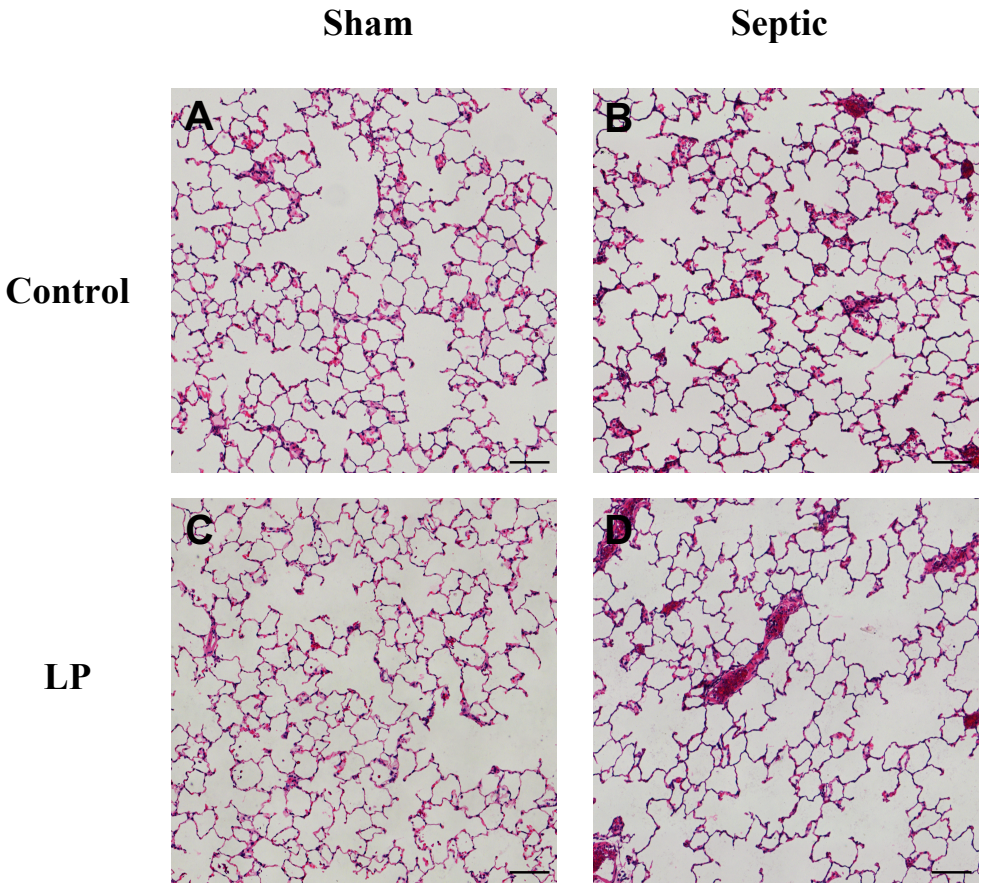

Figure 3. 9. Representative images of hematoxylin-eosin stained lung sections from females. Images of lung sections from sham and septic control (A, B) and LP (C, D) females, 6h after saline or FIP injections. Scale bar=100μm shown on the bottom right corner of each image.

3.4. Discussion:

This study addressed the hypothesis that FGR, induced by maternal protein restriction, is associated with the development of surfactant alteration and lung injury in response to sepsis in a sex-dependent manner during adulthood. The results demonstrated that in response to sepsis surfactant pool sizes decreased in adult LP males. The sepsis-induced systemic inflammation did not lead to augmented pulmonary inflammation or recruitment of pro-inflammatory leukocytes. It is concluded that, in this model of sepsis, FGR resulted in alteration of surfactant system in a sex-dependent manner despite the absence of local pulmonary inflammation.

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To test our hypothesis, we utilized a combination of a LP diet-induced model of FGR, with a FIP model of sepsis. While several previous studies demonstrated a LP rat model resulted in low birth weight in both sexes, as it was shown successfully in this thesis, other studies showed LP is a relevant model to study the fetal origins of adult-onset diseases (Petry, Ozanne, & Hales, 2001; Snoeck et al, 1990). Studies on adult rats with maternal protein restriction demonstrated different clinical FGR adult-onset morbidities associated with this model, in a sex-dependent manner, as it has been shown in clinical observations (Hennington & Alexander, 2013; McMullen, 2004). Furthermore, results from the current study (chapter 2) demonstrated impaired lung function at the end of the lactation period (d21) in LP offspring rats. These features allowed us to use the same LP rat model for studying FGR pulmonary outcomes during adulthood. Furthermore, given that human placental dysfunction can cause protein deficiency in the fetus, LP is a relevant animal model of prevalent human placental dysfunction-induced FGR (the common etiology in Canada) (Crosby, 1991). Finally, an important feature of this LP animal model was that, unlike utero-placental ligation and hypoxia, it was non-invasive and resulted in no moralities among the pregnant rats (Hayashi & Dorko, 1988).

Furthermore, the clinical relevance of this study was based on using an animal model of feces-induced peritonitis (FIP). Sepsis was induced in rats by intraperitoneally injecting a freshly prepared fecal solution. While this FIP model can lead to the development of systemic infection, as we consistently showed by positive bacterial growth, it also mimics the pathophysiological alterations associated with human response to sepsis (Wichterman, Baue, & Chaudry, 1980). Another feature of this sepsis model was that we did not apply general anesthesia; it has been shown that general anesthesia can suppress the surfactant metabolism and lung function (Petrov & Lyubarskii, 1966; Saraswat, 2015). These features allowed us to efficiently examine how LP-induced FGR affect the surfactant system in response to a clinically relevant condition of sepsis.

The lack of alveolar inflammatory responses and neutrophil accumulations in septic groups was unanticipated. One explanation for the absence of inflammatory mediators in BAL fluid was that there was not enough time for the infection to reach the alveolar space and trigger an inflammatory response. To assess this mechanism, in future studies, it would be

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beneficial to extend the treatment for more than 6hr. Also, measuring proinflammatory mediator m-RNA and protein levels (i.e. IL-6 and TNF- α) in BAL and lung tissue at 6hr can give information about the development and on-set of the inflammation process. Furthermore, as previous experimental studies showed that sepsis-induce surfactant changes were accompanied by increased alveolar inflammatory responses (Malloy et al., 1997; Wright et al.,2001), it would be interesting to investigate the underlying mechanism responsible for predisposing FGR males to surfactant alterations even in the absence of inflammation.

An interesting novel observation in this study was related to the sex-difference in how FGR contributes to surfactant alteration in response to sepsis; in our model, significant decrease in alveolar LA surfactant pool sizes was only observed in septic FGR adult males, but not females. Also, results demonstrated significant changes in LA levels in FGR septic males only. This provided strong support for the novel notion that FGR predisposed adults to the development of surfactant deficiency in response to sepsis in a sex-specific manner.

Whether the sex-dependent surfactant deficiency in septic males can potentially increase their susceptibility to develop inhibition of surfactant biophysical function remains to be determined. Surfactant inhibition affects the development of severe lung injury. Preliminary observational assessments indicated degrees of alveolar edema, hemorrhage and thickening of the alveolar cell wall in all septic groups. However, to characterize the stage and severity of the injury a comprehensive image analysis needs to be done. As a future direction, it can include quantitative assessments such as measuring the septal thickening, intra-alveolar infiltration of red blood cells and neutrophils and counting the number of intact alveolar type II cells.

Unanticipated altered LA surfactant content only in male FGR septic animals raised a question about the underlying mechanisms. To our knowledge, this study is the first demonstration of the sex-specific effect of FGR on the surfactant system in a sepsis model, surprisingly, in the absence of a pulmonary inflammatory response. Previous studies on pulmonary responses to sepsis, were only done in healthy male animals. From those studies, alternated conversion of LA to SA, reduced LA biosynthesis, and clearance of LA

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by alveolar macrophages have been reported as the mechanisms responsible for alteration of surfactant levels (Huang et al., 2005; Wichert et al., 2000). However, whether these mechanisms are regulated differently in FGR septic males and females is unknown. Therefore, in our study, further studies are required to address the potential mechanism(s) responsible for sexual dimorphism in surfactant regulations.

Since sepsis-induce severe lung injury is a significant clinical problem, it is important to speculate on the potential clinical implications of our findings. In the current study, we focused on the altertions of the surfactant system as an adverse pulmonary outcome of acute lung injury that is applicable to all septic experimental and clinical situations (Gregory et al., 1991b; Wiener-kronish, Gropper, & Matthay, 1990). One of the reasons for frequent failure of therapies is limited knowledge regrading factors that contribute to progression of septic pulmonary outcomes. Therefore, the diagnosis of the disease progression, commonly, is possible when it has been already byond the time frame for sucesful therapeutic interventions (Walter, Wilson, & Ware, 2014). Our results for surfactant levels in septic animals provided support for considering FGR and sexual dimorphism as contributing factors affecting the disease progression and severity of the outcomes. Thus, based on our experimental animal study, it would be of interest to examine if patients that were born with low birthweight have differential outcomes compared to people with normal birthweights, and if sexual dimorphism affects the pulmonary sepsis outcomes exist in these populations.

It should be mentioned that this study was associated with some limitations. Practical limitations included: i) the animals were not on general anesthesia, therefore, we could not examine the potential contributions of FGR to other pathophysiological outcomes, such as blood gas and systemic inflammation over the course of sepsis progression, ii) in real clinical situations, usually, sepsis-induced surfactant impairments and lung injury develop in a few days, however, this study was relatively time-intensive (limited to 6h), and iii) finally, due to diversity of initiating mechanisms of sepsis, variety of FGR animal models, and variations between animal models and humans, our single experimental model does not represent all the clinical features associated with FGR in response to sepsis.

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3.5. Conclusions

In conclusion, in response to sepsis FGR predisposed adult rats to the development of surfactant alterations even in the absence of pulmonary inflammation. Reductions in surfactant levels were observed only in septic FGR males leading to the suggestion that in response to sepsis sex is a co-founding variable that impacts the mechanism of surfactant alteration in adult rats with FGR. This emphasizes further understanding of the FGR impacts on the mechanism of the disease progression in the search for future personalized therapeutic interventions.

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Chapter 4—Summary and Future Directions

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4.1. Summary

The first objective of the present study was to examine the effects of FGR, induced by maternal protein restriction (LP), on lung mechanics and amounts and function of the pulmonary surfactant system during neonatal life (Chapter 2). Also, we tested the effects of FGR, induced by maternal protein restriction, on the amounts of pulmonary surfactant and development of lung injury in response to a systemic inflammation during adulthood (Chapter 3). Furthermore, we assessed whether there is any sexual dimorphism in the development of potential FGR-associated pulmonary outcomes both during the neonatal period and in adulthood. My hypotheses were I) FGR contributes to alterations of the surfactant system and lung mechanics in a sex-dependent manner during the neonatal period (Chapter 2) and II) In response to a sepsis-induced systemic inflammation, FGR contributes to alteration of the surfactant system in a sex-dependent manner in adults (Chapter 3).

I initially examined the effect of LP on lung function in male and female offspring during the lactation period (day 1-day 21). Large broncho-alveolar surfactant levels in newborns helps them to immediatelty and maximumally open the lungs upon their transition to the air-breathing environment. On ther hands, pulmonary alveolarization process in rat neonates begins and progresses between week one and week three after birth. Therefore, dysregulated lung development during the neonatal period could alter the surfactant production and the lung compliance of the offspring. By investigating different parameters of lung mechanics between day 7 and day 21, we observed that at the end of the lactation period (day 21), FGR induced decreases in lung compliance of female FGR pups only, without affecting FGR males' lung compliance. Furthermore, an increase in the lung stiffness was observed only in FGR females. On the other hand, the study of the effects of FGR on the extracellular surfactant system revealed that LP-induced FGR had no impact on the alveolar surfactant content or its biophysical function compared to control offspring.

We speculated that reduction in the lung expandability properties was due to lung tissue stiffness, which may have been caused through alterations in parenchymal extracellular matrix (ECM) morphology in a sex-dependent manner. ECM proteins such as collagen

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subtypes and elastin contribute the most to lung parenchymal elasticity (Lindahl et al., 1997; Ewald R. Weibel, 2011b). Changes in deposition of these proteins have been previously observed in a FGR sheep model (induced by umbilico-placental embolization), which resulted in thickening of the alveolar cells walls and decreasing alveolarization (Joyce et al., 2001; Maritz et al., 2004; Rozance et al., 2011).

From the beginning, our results raised questions about the potential clinical implications of this alteration. Chronic parenchymal stiffness and remodeling during infancy can contribute to several pulmonary complications and morbidities such as deregulated angiogenesis (Mammoto et al., 2013), immature alveolar formation, developing fibrosis, and increased risk for chronic lung disease (Jakkula et al., 2000; Thibeault, Mabry et al., 2003; Wirtz & Dobbs, 1990). Therefore, although there were no changes in the surfactant levels by the end of the lactation period, a persisting lung stiffness potentially may result in predisposition of FGR offspring to different pulmonary complications in later ages in a sex-dependent manner.

The second hypothesis was that in response to sepsis, FGR contributes to alteration of the surfactant system in a sex-dependent manner in adults. To test this hypothesis, I used a combination of an LP model of FGR with a FIP model of sepsis. While it is well understood that a maternal LP rat model results in low birth weight offspring in both sexes, some compelling studies have shown LP is also a relevant model to study the fetal origins of adult-onset diseases (Petry, Ozanne, & Hales, 2001; Snoeck et al., 1990).

Given that in the first objective it was shown that lung function is affected by FGR during infancy, as the second objective, we investigated whether FGR can predispose adult rats to the development of surfactant alterations and lung injury in response to sepsis. To pursue our objective, we used the same FGR rat model (chapter 2); after d21 all the control and LP offspring were fed with a control protein diet until day 130-day 150. Sepsis was induced by fecal intraperitoneal injection. This study included both male and female offspring, to allow the assessment of sexual dimorphism on pulmonary surfactant and the development of lung injury. Our results displayed that intraperitoneal injection of a rat fecal slurry successfully induced systemic inflammation in all septic groups. However, surprisingly,

there was no intraalveolar inflammation observed in septic animals. Assessment of the BAL fluids showed a different impact on the surfactant pool sizes only in adult septic males with FGR. These observations have led us to some important conclusions. First, FGR predisposed the offspring to develop surfactant impairments in response to sepsis even in the absence of pulmonary inflammation. Second, there is a sexual dimorphism associated with FGR, which affects the pulmonary surfactant response to sepsis.

4.2. Future Directions

Based on our results, which showed an association between FGR and sepsis-induced surfactant deficiency, it would be beneficial to expand this study by assessing the effect of sepsis-induced surfactant reductions on lung compliance and development of lung injury in adult FGR offspring. Also, by assessing lung compliance in adult offspring, it would be possible to test whether the sex-dependent changes in lung compliance observed at the end of the lactation period persist throughout life. Another potential future direction that could complement the current research is the assessment of the biophysical properties of surfactant. Clinical and experimental studies have shown significant changes in the surfactant levels and biophysical function of the surfactant in sepsis-induced acute lung injury/ARDS (Gregory et al., 1991a; Lewis et al., 1994; Wiener-kronish et al., 1990). Therefore, to further elucidate the pathophysiological outcome of FGR in a septic lung, surfactant biophysical assessment is essential. Furthermore, as in FGR males, surfactant depletion occurs before the development of pulmonary inflammation, so the effect of an early exogenous surfactant administration can be investigated as a potential therapy to interfere with the development of surfactant inhibition and lung injury in FGR offspring.

Finally, in the current study, the full extent of (potential) FGR-susceptibility to sepsis and/or ARDS has not been examined. Our model of sepsis had some lung involvements but with no evidence of pulmonary inflammtion. Therefore, in future studies, expanding the assessment of FGR effects on the the development of lung injury could involve direct lung injuries such as using the FGR rat model of ventilation iduced lung injury (Milos et al., 2017) or intratracheal LPS infection. Such studies would further examine the potential harmful effects of FGR associated with lung inflammtion during adulthood.

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4.3. Concluding Remarks

Sepsis-induced severe lung injury is a significant clinical problem which can lead to a life-threatening form of respiratory failure with a mortality rate- higher than the other causes of severe lung injury (~60%). Although overall the mortality induced by severe lung injury has been decreasing in the last few decades, mostly due to improvements in supportive care, there have been no proven effective pharmacological therapies for sepsis-induced pulmonary failure nor for preventing its associated adverse outcomes. Most therapeutic approaches for sepsis-induce lung injury include exogenous surfactant treatment and anti-inflammatory therapies. However, due to the complexity of the disease and the likelihood of late diagnosis, the therapies have not achieved substantial success. Therefore, identifying populations of patients at risk to receive early appropriate therapies could result in improvements of the clinical outcome and reducing the mortality rate associated with lung failure resulting from sepsis. The findings of the present study have contributed to the knowledge of sepsis-associated pulmonary outcomes by i) evaluating FGR as a risk factor for developing lung function at early life, ii) identifying FGR as a risk factor for developing surfactant deficiency in response to sepsis, and iii) identifying sex differences in response to lung function and surfactant deficiency in neonates and adults with FGR, respectively. Further experimental and clinical studies are needed to identify the altered underlying mechanisms associated with FGR impacts on the lung response to sepsis to ultimately provide more effective therapeutic targets and specific treatments to decrease its incidence and mortality.

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Appendix 1: The University of Western Ontario animal use sub-committee protocol approval

Western

AUP Number: 2009-055
PI Name: Veldhuizen, Ruud
AUP Title: The Molecular Mechanisms Underlying How Undernutrition Leads to Long-term Hypercholesterolemia and the Metabolic Syndrome.

Official Notification of AUS Approval: A MODIFICATION to Animal Use Protocol 2009-055 has been approved.
The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.
Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee

Western

AUP Number: 2017-003
PI Name: Veldhuizen, Ruud
AUP Title: The Effect Of Intrauterine Growth Retardation On Surfactant And The Susceptibility Of Lung Injury
Approval Date: 05/23/2017

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "The Effect Of Intrauterine Growth Retardation On Surfactant And The Susceptibility Of Lung Injury" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2017-003::1

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

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

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

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Post-secondary Education and Degrees

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Publications:

Tobias IC, **Khazaee R,** and Betts DH. (2018). Analysis of mitochondrial dimensions and cristae structure in pluripotent stem cells using transmission electron microscopy. *Curr Protoc*. 10:e67. doi: 10.1002/cpsc.67.

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Khazaee R, Milos S, McCaig LA, Nygard K, Yamashita CM, Veldhuizen RAW. (2016). The Effects of Ventilation-Induced Lung Injury on Lamellar Body Morphology Associated with Type-2 Alveolar Cells, *Accepted for poster presentation at London Health Research Day 2016*

Reza Khazaee, Lynda McCaig, Daniel Hardy, Cory Yamashita, Ruud Veldhuizen. (2016). The Effects of Intrauterine Growth Retardation on the

Pulmonary Surfactant System. *Accepted for poster presentation at Physiology and Pharmacology Research Day 2016*

Reza Khazaee, Lynda McCaig, Daniel Hardy, Cory Yamashita, Ruud Veldhuizen. (2017). Maternal Protein Restriction Effects on the Pulmonary Surfactant System and Lung Function During Early Postnatal Life. *Accepted for poster presentation at Physiology and Pharmacology Research Day 2017*

Reza Khazaee, Lynda McCaig, Daniel Hardy, Cory Yamashita, Ruud Veldhuizen. (2017). Intrauterine Growth Restriction Effects on the Pulmonary Surfactant and developing Lung Injury. *Accepted for poster presentation at Department of Medicine Resident Research Day 2017*

Reza Khazaee, Lynda McCaig, Daniel Hardy, Cory Yamashita, Ruud Veldhuizen. (2017). Intrauterine Growth Restriction Effects on the Pulmonary Surfactant and Lung Injury. *Accepted for oral presentation at Western Research Forum 2017*

Reza Khazaee, Lynda McCaig, Daniel Hardy, Cory Yamashita, Ruud Veldhuizen. (2017). The Effects of Intrauterine Growth Restriction on Pulmonary Surfactant and Lung Injury. *Accepted for poster presentation at 15th Annual Paul Harding Research Awards Day 2017*

Reza Khazaee, Lynda McCaig, John Huang, Daniel Hardy, Cory Yamashita, Ruud Veldhuizen. (2018). Maternal Protein Restriction as a Risk Factor for Acute Respiratory Distress Syndrome. *Accepted for poster presentation at Western Research Forum Research Day 2018*

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Reza Khazaee, Lynda McCaig, John Huang, Daniel Hardy, Cory Yamashita, Ruud Veldhuizen. (2018). Effects of Fetal Growth Restriction on the Surfactant System in Response to Sepsis. *Accepted for poster presentation at Department of Medicine Resident Research Day 2018*