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In-utero exposure to alcohol and nicotine alters the fetal lung innate immunity and development

Tatjana Lazic
Iowa State University

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In-utero exposure to alcohol and nicotine alters the fetal lung innate immunity and development

by

Tatjana Lazic

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Veterinary Pathology

Program of Study Committee:
Mark. R Ackermann, Major Professor
David K. Meyerholz
Jesse M. Hostetter
Christine A. Petersen
Ricardo F. Rosenbusch
Todd A. Wyatt

Iowa State University
Ames, Iowa
2009

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DEDICATION

THIS DISSERTATION IS DEDICATED TO MY FAMILY
# TABLE OF CONTENTS

| LIST OF FIGURES | v |
| LIST OF TABLES | vii |
| ABSTRACT | viii |

## CHAPTER 1. GENERAL INTRODUCTION 1

- Dissertation Organization 1
- Overview 2
- Alcohol (Ethanol) Consumption During Pregnancy 4
- Smoking During Pregnancy 9
- Innate Immunity of the Respiratory Tract 12
- Mucociliary Apparatus 13
- Collectins 21
- *In-utero* Lung Development 26
- Vascular Endothelial Growth Factor (VEGF) 33
- References 41

## CHAPTER 2. MATERNAL ALCOHOL INGESTION REDUCES SP-A EXPRESSION BY PRE-TERM FETAL LUNG EPITHELIA 78

- Abstract 78
- 1. Introduction 80
- 2. Materials and Methods 82
- 3. Results 88
- 4. Discussion 89
- Acknowledgements 96
- References 96
- Tables 103
- Figures 104

## CHAPTER 3. EFFECTS OF NICOTINE ON PULMONARY SURFACTANT PROTEINS A AND D IN OVINE LUNG EPITHELIA 109

- Summary 109
- 1. Introduction 110
- 2. Materials and Methods 111
- 3. Results 116
- 4. Discussion 117
- Acknowledgements 123
- References 124
- Tables 129
- Figures 130

---

*iii*

*TABLE OF CONTENTS*
LIST OF FIGURES

CHAPTER 2

Figure 1. Intra-abomasal cannula for alcohol delivery. 104

Figure 2. Ewes were exposed to alcohol as described. 105

Figure 3. The relative SP-A mRNA expression in pre-term control (PTC), and full-term control (FTC) lambs as well as pre-term alcohol-exposed (in utero) (PTA) and full-term alcohol-exposed (in utero) lambs (FTA). 106

Figure 4. The relative SP-D mRNA expression in pre-term and full-term lambs with or without (control) maternal alcohol administration during gestation. 107

Figure 5. Representative immunohistochemical detection of SP-A protein in bronchial/bronchiolar epithelium of the full-term/alcohol exposed lambs (A), full-term control lambs (B), pre-term/alcohol exposed lambs (C), and pre-term control lambs (D). High distribution and intensity of staining is seen in pre-term lambs receiving alcohol, suggesting that alcohol may affect SP-A protein release from epithelial cells. Arrows depict epithelial cells with SP-A protein expression. 108

CHAPTER 3

Figure 1. The relative SP-A mRNA and SP-D mRNA expression in PTC and FTC lambs as well as PTN and FTN lambs. 130

Figure 2. Representative immunohistochemical detection of SP-A protein in bronchial/bronchiolar epithelium of FTC-(A), FTN-(B), PTC-(C) and PTN-(D) lambs. Arrows depict epithelial cells with SP-A protein expression. 131

Figure 3. Representative Western blot detection of the SP-A and SP-D proteins in the lung tissue of the PTC and PTN lambs. 132

Figure 4. Representative Western blot detection of the SP-A and SP-D proteins in the lung tissue of the FTC and FTN lambs. 133
CHAPTER 4

Figure 1. Relative mRNA expression of hypoxia inducible factor-1α, 2α and 3α (HIF-1α, HIF-2α and HIF-3α respectively) in the neonatal ovine lung.

Figure 2. Relative mRNA expression of vascular endothelial growth factor (panVEGF) in the neonatal ovine lung.

Figure 3. Relative mRNA expression of vascular endothelial growth factor receptor 1 and 2 (VEGFR-1 and VEGFR-2 respectively) in the neonatal ovine lung.

Figure 4. Relative mRNA expression of chemokines and cytokines, in the neonatal ovine lung. Exposure to ethanol in utero reduces mRNA expression of; tumor necrosis factor (TNF)-α; (A), interleukin (IL)-10; (B), chemokine (C-C motif) ligand 5 (CCL5; E) and monocyte chemotactic protein (MCP-1; D) in the lungs of pre-term (PT) lambs when compared to the control lambs of the same age group.

Figure 5. Vascular endothelial growth factor (panVEGF) protein levels in the lung of pre-term lambs.

Figure 6. Periodic acid-Schiff (PAS) stain for glycogen granules in the type II pneumocytes (ATII).

Figure 7. Gomori’s trichrome stain for collagen deposition in the alveolar walls. Collagen fibers are more abundant in the alveolar walls of full-term lambs exposed to ethanol in utero (D) when compared to the control lambs of the same age group (C).
LIST OF TABLES

CHAPTER 2

Table 1. Serum alcohol concentrations in mg/dl, in acclimated (receiving daily ethanol for five days for three weeks) pregnant ewes at six, 12 and 24 hours after a single infusion (40% at 1 mg/kg, V/V).

103

CHAPTER 3

Table 1. The cotinine plasma levels (CPL) measured in the maternal plasma 6, 12 and 24 hr after the patch placement in 6 ewes.

129
ABSTRACT

Despite the evidence that alcohol and cigarette smoke have harmful effects on the developing fetus, alcohol consumption and smoking during pregnancy remains a common behavior among women. Fetal exposure to alcohol can result in numerous developmental abnormalities referred to as fetal alcohol spectrum disorder (FASD). Fetal exposure to cigarette smoke is related to premature birth and low birth weight for gestational age. Recent evidence suggests, however, that both alcohol and cigarette smoke may alter the fetal lung development and immunity and therefore predispose infants to respiratory infections. The purpose of this study was to establish new, advantageous animal models for fetal exposure to alcohol and nicotine; to determine if \textit{in-utero} exposure to alcohol and nicotine alters fetal lung innate immune components, primarily surfactant proteins A and D (SP-A and SP-D, respectively); and finally to determine the possible mechanism by which alcohol alters the fetal lung development and immunity.

In this study, we established new sheep animal models for fetal exposure to alcohol and nicotine. We demonstrated that exposure to low levels of alcohol during the last trimester of pregnancy decreases SP-A gene expression in lambs born prematurely and SP-A protein expression in full-term lambs. Exposure to alcohol did not alter SP-D protein or gene expression in pre-term or full-term lambs. In addition, exposure to low levels of nicotine during the last trimester of pregnancy decreases SP-A gene and protein expression in lambs born prematurely. Exposure to nicotine did not alter SP-D protein or gene expression in pre-term or full-term lambs. Finally, we demonstrated that alteration of SP-A expression in premature lambs exposed to
alcohol in-utero could be attributed to the alcohol-related decreased expression of vascular endothelial growth factor (VEGF) in the fetal lung.

In conclusion, our findings indicate that fetal exposure to alcohol and nicotine alters the fetal lung innate immunity and development which in part may explain alcohol and nicotine-related increased incidence of respiratory infections in neonates.
CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation describes the effects of prenatal exposure to alcohol (ethanol) and nicotine on the innate immunity factors of the developing fetal lung using the *in vivo* sheep animal model. The dissertation is divided into five chapters that include a general introduction, three manuscripts that have been published or submitted for publication, and a general conclusion. Chapter 1 includes the dissertation overview, describes the dissertation organization, and includes: (a) a review of the impact of alcohol and nicotine on immunity with an emphasis on neonatal lung immunity and maturation; (b) a review of lung innate immunity with an emphasis on mucociliary clearance in the respiratory tract and pulmonary collectins; pulmonary surfactant protein A (SP-A), pulmonary surfactant protein D (SP-D); (c) a review of fetal lung development with an emphasis on lung development in sheep in comparison to lung development in humans and rodents; and (d) a review of vascular endothelial growth factor (VEGF) and its role in lung development. Chapter 2 describes a novel sheep model for prenatal alcohol exposure that demonstrates that moderate alcohol exposure during the third trimester of pregnancy is associated with significant reduction of SP-A mRNA expression and cilia beat frequency in lambs born prematurely. This finding may partially explain the increased incidence of pulmonary infections in neonates exposed to alcohol *in utero*. Chapter 3 describes a novel sheep model for prenatal nicotine exposure that demonstrates that low-grade nicotine exposure during the third trimester of pregnancy significantly decreases SP-A mRNA and protein expression in lambs born prematurely. This finding
suggests a possible mechanism by which smoking during pregnancy alters fetal lung maturation and predisposes infants to neonatal pulmonary infections. Chapter 4 describes the possible mechanism underlining the effect of alcohol previously described in Chapter 2. It also describes the influence of prenatal alcohol exposure on the expression of crucial pulmonary immune genes in the neonatal lung. Chapter 5 includes the general conclusions and future plans based on findings in this dissertation.

**Overview**

Alcohol (ethanol) consumption and smoking remain common behavior among women during pregnancy despite the fact that harmful health effects to the fetus are widely recognized. The fetal alcohol spectrum disorder (FASD), which includes multiple birth defects and neurodevelopmental disorders, has been well described in the literature. Little is known, however, about the effects of fetal alcohol exposure on fetal lung development and immunity. It has been recently suggested that fetal alcohol exposure alters the immunity of newborn children and predisposes them to bacterial infections and sepsis. Furthermore, experimental studies done in animal models indicate that alcohol may have a detrimental effect on neonatal lung immunity by impairing the function of alveolar macrophages. Smoking during pregnancy is the leading cause of neonatal morbidity and mortality, which are primarily attributed to the premature birth and low birth weight for gestational age. In addition, infants born to mothers who smoked during pregnancy have impaired pulmonary immunity that results in increased risk of respiratory infections such as respiratory syncytial virus (RSV) infection. These data suggest that both fetal
alcohol and nicotine exposure may alter the lung innate immunity of neonates and, therefore, impair the pulmonary defense against respiratory pathogens. The respiratory tract innate immune system plays a crucial role in preventing the colonization and proliferation of inhaled microorganisms in the airways, thus maintaining the efficient physiological function of the lung: gas exchange. Although the innate immunity of the respiratory tract is a complex system that includes an anatomic barrier, many cellular and secretory components, mucociliary clearance, surfactant protein-A (SP-A), and surfactant protein-D (SP-D) represent important elements of pulmonary first-line defense. Once inhaled, microorganisms are trapped in a thin mucous layer lining the lower respiratory tract, propelled to the oropharynx by synchronized movement of the cilia, and expectorated. If this defense mechanism fails and microorganisms remain in the airways or alveoli, the cellular and secretory mechanisms are triggered. SP-A and SP-D are secreted by type II alveolar epithelial (ATII) cells and Clara cells. These are powerful antimicrobial proteins that can fight a broad spectrum of microorganisms such as bacteria, viruses, and fungal organisms⁸. Impaired function of mucociliary clearance and antimicrobial surfactant proteins opens a window of opportunity for microorganisms to reach and colonize the airways and alveoli. Finally, although research related to the effects of alcohol and nicotine on the developing fetus is broad, there is a lack of animal models that closely parallel the fetal development and the habits of drinking and smoking in humans. The purpose of the work in this dissertation is to:

1. characterize the sheep as an animal model for maternal alcohol administration;
2. test the hypothesis that prenatal alcohol exposure in moderate levels during the last trimester of pregnancy alters the cilia function in the trachea and pulmonary SP-A and SP-D expression in neonatal lambs;

3. characterize an ovine animal model for maternal nicotine administration;

4. test the hypothesis that prenatal, low-grade nicotine exposure during the last trimester of pregnancy alters the expression of pulmonary SP-A and SP-D in neonatal lambs; and

5. determine the possible mechanism by which intrauterine alcohol exposure alters the expression of the key innate immunity components.

**Alcohol (Ethanol) Consumption During Pregnancy**

Alcohol is the most commonly consumed chemical during pregnancy that has harmful effect on the developing fetus. The most recent survey, published in 2009, found that in the United States up to 30% of pregnant women consume alcohol at some point during pregnancy\(^9\). Among these women, 8.3% report binge drinking, 2.7% report drinking during all trimesters of pregnancy, and 7.9% report drinking during the third trimester. Other surveys that analyzed the amount of alcohol consumed during pregnancy found that 5 to 10 out of 1,000 women consume seven or more drinks per week\(^{10-12}\). These alarming numbers clearly indicate that a significant number of neonates have been exposed to alcohol during fetal development.

**Effects of *in-utero* exposure to alcohol (ethanol) on the developing fetus**

It is well established that *in-utero* exposure to alcohol results in a number of physical, behavioral, and cognitive abnormalities in neonates, together referred to as
fetal alcohol spectrum disorders (FASD)\textsuperscript{1}. Incidence of FASD in United States ranges from 7–10 cases per 1,000 live births every year\textsuperscript{13}. FASD includes fetal alcohol syndrome (FAS), alcohol-related birth defects (ARBD), and alcohol-related neurodevelopmental disorders (ARND). FAS encompasses a spectrum of disorders including pre- and post-natal growth retardation, facial defects, and neurological, structural, and functional abnormalities\textsuperscript{14}. In addition to the defects found in FAS, ARBD includes defects in other organ systems such as cardiovascular, skeletal, and renal\textsuperscript{14}. ARND encompasses a range of cognitive, behavioral, and emotional abnormalities in children who were exposed to alcohol during \textit{in-utero} development but do not have abnormalities typical of FAS\textsuperscript{14}. In addition to FASD, alcohol consumption during pregnancy is a risk factor for premature birth and small for gestational age birth\textsuperscript{15,16}.

\textbf{Alcohol (ethanol) and the immune system}

It is established that alcohol impairs the immune system in adults. Alcohol abuse in chronic alcoholics has been linked to increased incidence and severity of infectious diseases such as bacterial pneumonia and hepatitis C\textsuperscript{17,18}. Studies done on humans and experimental animal models have demonstrated that both acute and chronic alcohol exposure results in defects of cellular components of innate and acquired immunity. Acute exposure to alcohol reduces expression of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-\textgreek{a} and interleukin (IL)-1\textgreek{b} in human blood monocytes\textsuperscript{19,20}. It also suppresses adhesion, migration, phagocytosis, and oxidative burst in polymorphonuclear leukocytes (PMN's)\textsuperscript{21,22}. In mice, chronic exposure to alcohol reduces lymphocyte numbers in the spleen,
thymus, and gut-associated lymphoid tissues, most likely through stimulation of apoptosis\textsuperscript{23,24}. In murine natural killer (NK) cells, exposure to alcohol reduces expression of perforin, granzyme A, and granzyme B\textsuperscript{25}. In addition, chronic exposure to alcohol increases production of reactive oxygen species (ROS) by Kupffer cells and reduces antigen presentation by antigen-presenting cells\textsuperscript{26}.

**Alcohol (ethanol) and respiratory tract**

Studies of the effects of alcohol on lung immunity are limited. Epidemiological studies of chronic alcoholics, however, show that these individuals are at increased risk of developing pneumonia caused by Gram-negative bacteria, specifically *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*\textsuperscript{17,18}. This is in part due to suppressed swallowing reflex which can result in aspiration of oral contents; many studies have demonstrated, however, that alcohol directly inhibits immune cell responses in the lung. For example, in humans, alcohol suppress expression of IL-17 by T cells which stimulates recruitment of neutrophils in the lung in response to *K. pneumoniae*\textsuperscript{27,28}. Intraperitoneal injection of alcohol induces suppression of lung chemokine production and defense against *Streptococcus pneumoniae* in mice\textsuperscript{29}. In addition to PMNs, alcohol alters the function of alveolar macrophages by reducing expression of TNF-\textgreek{a}, IL-1\textgreek{b}, and CXC chemokines\textsuperscript{19}. Chronic alcohol ingestion potentiates TNF-alpha-mediated oxidative stress and apoptosis in rat type II cells which are the source of SP-A and SP-D, the major components of pulmonary innate immunity\textsuperscript{30}. In addition to SP-A and SP-D, alcohol ingestion alters airway mucociliary clearance, another major defense mechanism in the lung. The effect of alcohol mucociliary clearance depends on the length of the exposure. If acutely exposed to
alcohol, the ciliary beat frequency in bovine bronchial epithelial cells increases\textsuperscript{31}. In contrast, chronic exposure to alcohol results in decreased ciliary beat frequency\textsuperscript{32}. This same effect of chronic alcohol exposure was demonstrated \textit{in vivo} in rats and mice\textsuperscript{33,34}.

\textbf{Alcohol (ethanol) and neonatal immunity}

Although the data on the effect of \textit{in-utero} exposure to alcohol on neonates are limited, a few recent reports suggest that alcohol may also impair fetal immunity\textsuperscript{2,35}. Children diagnosed with FAS commonly have increased incidence of infection such as meningitis, soft tissue infection, pneumonia, and sepsis\textsuperscript{36}. The incidence of respiratory infections appears to be particularly frequent\textsuperscript{36,37}. The most recent studies done on a guinea pig model of \textit{in-utero} alcohol exposure has demonstrated that alcohol impairs the alveolar macrophages in neonates\textsuperscript{3}. This impairment was attributed to the glutathione depletion in the neonatal lung by alcohol and subsequent increase in oxidative stress and apoptosis of alveolar macrophages\textsuperscript{2,38,39}. In addition to alveolar macrophages, alcohol exposure reduces mitochondrial glutathione levels in alveolar type (ATII) cells to make ATII cells more susceptible to apoptosis and necrosis\textsuperscript{40}. In addition, the majority of children exposed to alcohol \textit{in-utero} do not exhibit typical changes associated with FASD, and consequences of alcohol impact on fetal development and immunity remain undiagnosed.

\textbf{Animal (ethanol) models used in alcohol research}

Rodents are the most common animal model used in alcohol research. Rodents are small, easy to handle, and inexpensive. Their metabolic and endocrine
events during pregnancy share many similarities to humans. Selective breeding has
developed a line of rats which prefer 10% alcohol to water (Alko, Alcohol) and a line
which chooses water over alcohol (Alko, Non-Alcohol)\textsuperscript{41}. Rats can be fed with a
special liquid diet containing alcohol (Lieber and De Carli diet) which provides 36% of energy from alcohol or isocaloric amounts of carbohydrate\textsuperscript{42}. To completely avoid the reduction of weight gain due to decreased dietary intake in alcohol-fed animals, alcohol can be administered via an intragastric tube or intravenously\textsuperscript{43,44}. The guinea pig is another animal model used in alcohol research. As rodents, they are easy to handle, inexpensive, and can receive alcohol orally\textsuperscript{4}. Sheep are a commonly used animal model in alcohol research, especially for experiments addressing \textit{in-utero} exposure to alcohol\textsuperscript{45-47}. Although sheep are an expensive animal model, the fetus is large and robust, which allows easier experimental instrumental manipulation and handling when compared to rodents. Furthermore, the major advantage of the sheep model is lung development that closely corresponds to lung development in the human fetus, which is not the case with rodent species\textsuperscript{48,49}. Sheep, however, cannot be administered alcohol orally due to ruminal microflora which largely degrade ingested alcohol before it reaches systemic circulation\textsuperscript{50}. Therefore, intravenous administration of alcohol is the only technique that has been used to achieve chronic systemic alcohol exposure\textsuperscript{46,47}. This approach has significant experimental deficiencies because intravenously administered alcohol bypasses the first pass hepatic metabolism, which does not resemble the physiological processing of orally consumed alcohol\textsuperscript{51}. 
Smoking During Pregnancy

According to the 2006 survey conducted by the National Center for Chronic Disease Prevention and Health Promotion (CDC), 20.8% of adults in the United States are cigarette smokers, out of which 18% are women\textsuperscript{52}. According to Merzel et al., women tend to quit smoking before or during pregnancy, and up to 85% of female cigarette smokers maintain cessation throughout pregnancy\textsuperscript{53}. The result of the latest CDC survey conducted from 2001 to 2005, however, revealed that 13.8% of women in United States smoke during pregnancy\textsuperscript{54}. These numbers indicate that a large group of neonates is exposed to the cigarette smoke during gestation. In the United States alone, 776 infants die annually as a result of maternal smoking during pregnancy\textsuperscript{55}.

Effects of in-utero exposure to cigarette smoke/nicotine on the developing fetus

Cigarette smoking prior to pregnancy is a risk factor for secondary infertility\textsuperscript{56}. Smoking during pregnancy has a wide range of negative health effects on the developing fetus which include obstetric complications, intra-uterine growth retardation, and postnatal complications\textsuperscript{57}. Premature birth is the most common obstetric complication; it is estimated that smoking during pregnancy is responsible for 15% of all premature births\textsuperscript{58}. Other less common obstetric complications include ectopic pregnancy, spontaneous abortion, and placenta previa\textsuperscript{59,60}. Intra-uterine growth retardation is a consequence of reduced surface area and volume of the placenta which further results in decreased oxygen and nutrient supply to the fetus\textsuperscript{61}. Post-natal complications include increased risk of sudden infant death syndrome.
(SIDS), lower respiratory infections, middle ear infections, compromised lung function, wheezing, and asthma.

**Cigarette smoke/nicotine and the immune system of the respiratory tract**

Cigarette smoke contains thousands of chemicals some of which are proven to have immunomodulatory effect. Nicotine is an important chemical in the cigarette smoke due to its addictive properties and systemic effects on different organs. Recent evidence indicates that nicotine modulates immunity in similar manner as whole cigarette smoke. Exposure to cigarette smoke results in increased permeability and loss of cellular integrity of the airway epithelium. In addition, cigarette smoke stimulates secretion of pro-inflammatory cytokines such as IL-8, IL-6, and TNF-α by the bronchial epithelial cells. The increased mucus production in smokers is attributed to increased expression of the epidermal growth factor receptor (EGFR) on epithelial cells, which is the major regulator of mucus production in the airways. In smokers, broncho-alveolar lavage (BAL) fluid contains reduced levels of SP-A compared to non-smokers, indicating that smoking affects the function of ATII cells. Smoking increases the numbers of alveolar macrophages; these cells, however, are less mature and have reduced capability to phagocyte bacteria. Smoking increases the influx of PMNs in the airways which are more active and release higher levels of proteolytic enzymes. Smoking also increases the numbers of cytotoxic T cells and decreases the ratio of CD4+/CD8+ ratio. In addition, smoking elevates the number of eosinophils in the respiratory tract.
Cigarette smoke/nicotine and neonatal immunity

Children born to mothers who smoked during pregnancy have increased risk of respiratory and middle ear canal infections and increased incidence of asthma and wheezing\textsuperscript{77}. These reports indicate that maternal smoking may alter the immune system in neonates. It has been reported that maternal smoking disrupts the antioxidant system in the placenta which further damages placental trophoblasts which are the source of cytokines important in regulation of fetal immunity\textsuperscript{78}. Furthermore, maternal smoking results in decreased numbers of segmented neutrophils, lymphocytes, and myeloid precursor dendritic cells as well as immunoglobulin (Ig)E in the cord blood\textsuperscript{79,80}. Neonates exposed to cigarette smoke \textit{in-utero} have lower cytokine response upon TLR-2, TLR-3, and TLR-9 activation\textsuperscript{81}. A study conducted in neonatal rats reported that intrauterine exposure to nicotine accelerates ATII cell proliferation; these cells, however, have decreased capability to produce pulmonary surfactant, including SP-A and SP-D\textsuperscript{82}. Overall, maternal smoking suppresses the development of fetal immunity; few studies, however, have adequately addressed this particular problem.

Animal models in cigarette smoke/nicotine research

Rodents are the most commonly used animal models in the cigarette smoke/nicotine research field due to the easy exposure of caged animals to cigarette smoke (cigarette smoke chamber)\textsuperscript{83}. Currently described sheep models in nicotine research utilize intravenous application and placement of subcutaneous infusion pumps for the long-term nicotine delivery\textsuperscript{84,85}. While intravenous administration is less invasive, it can be associated with dosing problems due to the
acute bolus administration of nicotine in circulation. Subcutaneous pumps provide much more controlled long-term nicotine delivery; their placement, however, requires general anesthesia and surgery which significantly increases the cost and complexity of the model.85

**Innate Immunity of the Respiratory Tract**

The respiratory tract represents the largest epithelial surface of the body that is constantly exposed to potentially pathogenic microorganisms, toxins, aerosols, and particulates present in the inspired air. The innate immunity of the respiratory tract is the first line of defense against inhaled microorganisms and consists of three general components:

1. a mechanical defense component, which includes an anatomic barrier and a mucociliary apparatus;

2. a secretory component which includes a broad spectrum of anti-microbial agents; and

3. a cellular component, which includes phagocytic and inflammatory cells.

**Anatomic barrier**

The respiratory epithelium lining the airways together with airway surface liquid (ASL) represents the physical barrier of the respiratory tract that prevents entry and colonization of microorganism in the deeper mucosal and submucosal tissues. The airway is anatomically divided into the upper respiratory and lower respiratory tracts. The upper respiratory tract includes nasal passages, nasal sinuses, and the nasopharynx. The lower respiratory tract consists of the trachea which branches into the bronchi and bronchioles and terminates with alveoli.86 The proximal airways
of the lower respiratory tract such as the trachea and bronchi are lined by pseudostratified columnar ciliated epithelial cells, mucus-secreting goblet cells, and basal cells. The smaller airway branches are lined with fewer basal cells, and epithelial cells are more cuboidal. The bronchoalveolar junction is lined by numerous non-ciliated (Clara) cells which release innate immune product such as surfactant proteins and metabolize toxins. Ninety-five percent of the alveolar surface is lined by thin, elongate epithelial cells (type I pneumocytes, ATI cells), and the rest of the surface is covered by cuboidal to rounded cells (type II pneumocytes, ATII cells). Individual epithelial cells are tightly joined to each other by specialized complexes that include gap junctions, desmosomes, adherence junctions, and zonula occludens or tight junctions. These junctions form a mechanical barrier between the airway lumen and underlying tissue. Desmosomes mediate mechanical adhesion between the neighboring cells, and tight junctions encircle the cells at the apical membrane and completely obliterate the space between the cells. This epithelial organization allows communication between neighboring cells, as well as selective passage of molecules such as antioxidants and components of ASL. In addition to tightly joined epithelial cells, mucous and serous epithelial cells of submucosal glands contribute to epithelial mechanical barrier and produce ASL.

**Mucociliary Apparatus**

The mucociliary clearance is a defense mechanism by which the inhaled particles, including microorganisms, are expelled from the respiratory tract by retrograde expulsion of particulates. It involves ciliated epithelial cells and overlying airway surface liquid (ASL). The inhaled particles are trapped onto a superficial,
sticky, mucus layer of ASL, then propelled by cilia movement into the oropharynx and swallowed or expectorated. Ciliated epithelial cells line the upper respiratory tract from the trachea to the respiratory bronchioles. They account for 56% of the total epithelial cells lining the trachea and about 15% in the lower respiratory bronchioles. Each cell contains approximately 200 cilia that project from the cell surface into the overlying ASF. The length of cilia varies from 6 µm in the trachea to 2 µm in the seventh-generation airways. Each cilium is anchored to the cell by the basal body, which projects its striated rootlet named “basal foot” into the cell cytoplasm. The ciliary body contains a centrally located axoneme composed of two microtubules enclosed within a central sheath. The axoneme is further surrounded by nine microtubule doublets, thus forming the classical “9+2” microtubular arrangement. The outer doublets are connected to axoneme by radial spokes (RS) and to each other by nexin links. Each doublet projects inner and outer dynein arms toward the adjacent doublet. Cilia move (beat) in coordinated fashion through interaction between the dynein arms, adjacent microtubules, and axoneme powered by hydrolysis of adenosine triphosphate (ATP). This interaction results in sliding of the microtubules and restricted by radial spokes, cilia bend. In order to remove mucus efficiently, there is a phase difference between beating cilia which creates a wave, often referred to as “metachronal wave” or “metachronism.” The beat pattern consists of two main strokes, the effective stroke and recovery stroke. The effective stroke is fast, and cilia are in an upright position which enables ciliary tips to embed into the mucus layer. The recovery stroke is slower, and cilia are slightly bent, thus avoiding the contact with mucus. The mechanism by which the pattern of
ciliary beating is regulated is not fully understood. In the human trachea, the ciliary beat frequency (CBF) is approximately 12–15 hertz (Hz)\(^9\), and by beating, cilia propel particles adhered to the mucus at a speed of 0.5mm/s\(^1\). There is a constant, slow rate of CBF (basal level) that requires only ATP.

**Regulation of ciliary beat frequency**

The cilia can respond to insults by increasing the basal level of CBF which is primarily regulated by calcium (Ca\(^{2+}\)) flux and cyclic adenosine monophosphate (cAMP)\(^9\). CBF correlates with intracellular Ca\(^{2+}\) ion [Ca\(^{2+}\)]\(_i\) concentration. Reduction in intracellular [Ca\(^{2+}\)]\(_i\) results in decreased CBF; in contrast, rise in intracellular [Ca\(^{2+}\)]\(_i\) increases CBF\(^9\). The intracellular [Ca\(^{2+}\)]\(_i\) levels are regulated by activation of P2-purinergic receptors by ATP and muscarinic receptors by acetylcholine (ACh) which results in the activation of phospholipase C (PLC)\(^1\). Activated PLC hydrolyzes phosphatidylinositol-4,5-biphosphate to inositol-1,4,5-triposphosphate (IP3) which stimulates the release of Ca\(^{2+}\) from the intracellular stores\(^2\). [Ca\(^{2+}\)]\(_i\) and diacylglycerol activate the protein kinase C (PKC) pathway\(^3\). After the release of [Ca\(^{2+}\)]\(_i\) from the intracellular stores, the [Ca\(^{2+}\)]\(_i\) levels are sustained by a continuous influx of Ca\(^{2+}\) from the extracellular space, which allows CBF to remain at high levels throughout exposure to an agonist\(^4\). It has been demonstrated that ciliated cells have specialized receptor channels designated as P2X\(_{cilia}\), which have relatively high permeability to Ca\(^{2+}\)\(^5\). In addition to the Ca\(^{2+}\), cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) play an important role in CBF regulation\(^6-9\). It was initially thought that Ca\(^{2+}\), cAMP, and cGMP were independent; newer evidence, however, indicates that all three pathways are closely
interconnected. Ciliated cells express Ca^{2+}-dependent adenylate cyclase (AC) and in response to elevated intracellular Ca^{2+}, AC increases cAMP through a calcium-calmodulin complex (Ca-CAM). Ca-CAM further activates nitric oxide synthase (NOS) which leads to activation of cAMP-dependant protein kinase (PKA) and cGMP-dependent protein kinase (PKG). PKA phosphorylates axoneme, which results in strong, short-term CBF enhancement. Axoneme phosphorylation by PKG results in moderate, prolonged CBF enhancement. In addition, the ciliated epithelial cells express mechanoreceptors which, in response to mechanical stimulation, enhance CBF. Ciliated cells stimulated through mechanoreceptors release IP3, which stimulates an adjacent ciliated cell through intercellular gap junctions.

**Airway surface liquid (ASL)**

ASL is composed of three layers: (a) a superficial gel (mucus); (b) the periciliary fluid that covers the apical surface of the epithelial cells; and (c) a thin layer of surfactant which separates the other two layers. The periciliary fluid is secreted by surface epithelial cells. It hydrates the respiratory epithelium and provides a watery environment to enable ciliary movement. In addition to water, glycoproteins, and lipids, it also contains peptides and proteins with antimicrobial activity such as Immunoglobulin A, lysosome, lactoferrin, peroxidase, and antimicrobial peptides including defensins. The depth of periciliary fluid is approximately 5-10 μm, which is crucial for effective ciliary function. If the periciliary fluid is too deep, cilia do not reach the superficial mucus layer and fail to clear it from the airways. If the periciliary fluid is too shallow, cilia get stuck in the mucus which
results in the same ineffective clearance of the airways\textsuperscript{93}. These depths can be altered by dehydration and conditions in which there is impaired periciliary fluid such as cystic fibrosis\textsuperscript{115}. Mucus is the superficial layer of ASL. Mucus is a nonhomogeneous, adhesive, viscoelastic gel that has three main functions in the respiratory tract: (a) it traps the inhaled particles, including microorganisms, which are further expelled from the respiratory tract via mucociliary clearance\textsuperscript{91,116}; (b) it forms the mechanical barrier that protects the underlying respiratory epithelium; and (c) it prevents dehydration of respiratory epithelial cells\textsuperscript{117}. In addition, airway mucus contains embedded neutrophils and macrophages, as well as antimicrobial proteins such as lysozyme, antiproteases, and immunoglobulins which, as indicated, have antimicrobial activity\textsuperscript{117}. Mucus is predominantly produced by goblet cells and the submucosal glands\textsuperscript{95}. It is composed of salts (1%), protein, glycoproteins (mucins) (2–3%), and water (95%). Mucins are composed of carbohydrates (70–80%), protein (20%), and sulfate bound to oligosaccharide side chains (1–2%)\textsuperscript{116}. There are two principal forms of airway mucins: the secreted, gel-forming mucins that form the superficial mucus layer of ASF; and membrane-bound mucins, which may act as cell surface receptors\textsuperscript{91,116}. The mucin genes (MUC genes) encode the protein backbone\textsuperscript{118}. Epithelial cells of the human respiratory tract express at least 13 MUC genes that encode for membrane-associated mucins and 5 MUC genes that encode for gel-forming mucins\textsuperscript{119}. The most important mucins that form airway mucus gel, however, are MUC5AC and MUC5B\textsuperscript{117}. The thin surfactant layer is composed of phospholipids (phosphatidylcholine being the most abundant) (80%), neutral lipids (cholesterol being the most abundant) (12%), and surfactant proteins (8%). The
main function of surfactant in the airways is to preserve periciliary fluid, spread the mucin, and prevent cilia from entangling\textsuperscript{120}.

**Consequences of compromised mucociliary clearance**

Mucociliary clearance is essential for normal lung function and pulmonary defense. The disrupted balance between cilia function, periciliary fluid, and mucus production leads to lung inflammation, damage, and infection. Mucociliary clearance is impaired by dehydration, and in some genetic disorders such as primary ciliary dyskinesia (PCD)\textsuperscript{121,122} and cystic fibrosis (CF)\textsuperscript{115,123}, often results in reoccurring or chronic pulmonary infections\textsuperscript{124-126}. Asthma is another example of disrupted mucociliary apparatus in the respiratory tract\textsuperscript{127}. In asthmatic patients, mucus hypersecretion results in obstruction of airway passages and increased incidence of pulmonary infections\textsuperscript{128}. Other factors such as exposure to pollutants, cigarette smoking, and alcohol consumption may have negative effects on cilia function and mucus production\textsuperscript{34,95,129-131}.

**Mucociliary clearance and alcohol (ethanol)**

Depending on the length of exposure, alcohol may increase or decrease CBF. In bovine bronchial epithelial cells (BBECs), short-term exposure to alcohol stimulates CBF; this stimulation is mediated through activation of both PKA via cAMP and PKG via NO\textsuperscript{31}. When BBECs are preincubated with alcohol for 24hr, the stimulation of PKA with isoproterenol is abrogated\textsuperscript{32}. This indicates that chronic exposure to alcohol down regulates CBF in ciliated epithelial cells; the effect was also demonstrated \textit{in vivo} in mouse and rat animal models\textsuperscript{33,34}. 
Secretory component; antimicrobial factors

The airway epithelium produces a wide range of antimicrobial factors to fight inhaled microorganisms and prevent respiratory tract infection\textsuperscript{8,132}. The list of specific peptides/proteins, the cells of origin, and function is adopted and modified from the paper published by Grubor et al., “Collectins and cationic antimicrobial peptides,” Veterinary Pathology, 2006 September: 43(5):595–612.

Lysozyme is secreted by epithelial cells of the trachea, bronchi, and tracheobronchial submucosal glands, as well as neutrophils, alveolar macrophages, and monocytes. Lysozyme is muramidase that cleaves the glycosidic bonds of the bacterial membrane peptidoglycans or kills the bacteria in nonenzymatic mechanism fashion\textsuperscript{133,134}. Lactoferrin is secreted by epithelial cells of the trachea, serous cells of tracheobronchial submucosal glands, and neutrophils. Lactoferrin is an iron-binding protein that inhibits the growth of iron-requiring bacteria, and can be directly microbicidal through its N-terminal cationic fragment\textsuperscript{135,136}. Surfactant proteins SP-A and SP-D are secreted by ATII and Clara cells. In addition to their antimicrobial activity against bacteria, viruses, and fungal organisms, they are also involved in immunomodulation, inflammation, and regulation of surfactant metabolism\textsuperscript{8}. Anionic peptides (APs) are secreted by epithelial cells of the trachea, bronchi, and bronchioles. APs are bactericidal against Gram-positive and Gram-negative, and they require zinc as a cofactor for bactericidal activity\textsuperscript{137}. Cationic antimicrobial peptides (AMPs) include α-, β-, and θ-defensins, and cathelicidins. α- and β-defensins are secreted by airway epithelia, neutrophils, and possibly alveolar epithelial cells. θ-defensins are secreted by neutrophils, and cathelicidins are
secreted by airway respiratory epithelia, serous, and mucous cells of tracheobronchial submucosal glands and neutrophils. AMPs may potentially fight Gram-negative and Gram-positive bacteria, fungi, viruses, and parasites\textsuperscript{138,139}. Clara cell secretory 10-kD protein (CC10) which plays an anti-inflammatory role in lung diseases is secreted by Clara cells\textsuperscript{140}. Secretory peroxidases include lactoperoxidase (secreted by airway epithelia) and myeloperoxidase (secreted by neutrophils). They act on thiocyanate ions or produce oxygen radicals, which may have a bacteriostatic or bactericidal effect\textsuperscript{88}. Secretory Immunoglobulin (Ig) A is secreted by respiratory epithelia. The IgA play an important protective role against respiratory viruses\textsuperscript{141,142}. Elafin is secreted by tracheal epithelial cells, Clara cells, ATII cells, and macrophages. Elafin is an antiproteinase that inhibits neutrophil elastase and other neutrophil-derived proteinases such as proteinase-3. It can inhibit neutrophil-mediated proteolytic tissue damage but may also act against microbial proteinases\textsuperscript{143}. The secretory leukoprotease inhibitor (SLPI) is secreted by serous cells of tracheobronchial submucosal glands and Clara cells in vivo. In vitro, SLPI is produced by neutrophils, macrophages, monocytes, and epithelial cells of the lower respiratory tract, including alveolar epithelial cells. SLPI is a proteinase inhibitor and inhibits neutrophil elastase via the C-terminal domain\textsuperscript{144}. The respiratory epithelial cells and neutrophils secrete Phospholipase A2 (PLA2). PLA2 is involved in cytokine-mediated inflammation, surfactant degradation, and anti-bactericidal activity, especially against Gram-positive bacteria\textsuperscript{145,146}. There are many other less studied antimicrobial factors in the respiratory tract such as uric acid, aminopeptidases, proline-rich proteins, statherin, cystatin, and PLUNK\textsuperscript{8}. 
Collectins

Collectins belong to the superfamily of Ca\(^{2+}\)-dependant lectins (C-type lectins); so far, nine different members have been identified. They are mannan-binding lectin (MBL), mannose-binding protein (MBP), collectin liver 1 (CL-L1), collectin placenta 1 (CL-P1), collectin of 43 kD (CL-43), collectin of 46 kD (CL-46), SP-A, SP-D, conglutinin; and newly identified collectin kidney (CL-K1\(^{147,148}\)). These proteins share a common structure organized into four regions: Cysteine (cys)-rich N-terminus; followed by a collagen-like sequence; an \(\alpha\)-helical coiled-coil neck; and C-terminus, which is C-type lectin region\(^{149}\). The C-type lectin region contains a carbohydrate recognition domain (CRD), which recognizes and binds glycoconjugates and lipids on microorganisms. This leads to agglutination or neutralization of the microorganism, opsonization for phagocytosis, and recruitment of inflammatory cells\(^{149}\).

SP-A and SP-D protein structure

As mentioned above, SP-A and SP-D share the common collectin structure. They are organized as oligomers assembled of monomers, which contain four structural domains. The first domain (N-terminus) is a short amino- and disulfide-rich domain of 7 (SP-A) to 25 (SP-D) amino acids. N-terminus is involved in the interchain interactions. The second domain (collagen-like domain) consists of Gly-X-Y repeats and forms fibrillar triple helix 20 (SP-A) to 46 (SP-D) nm long. The third domain (neck region) forms a short trimeric coiled-coil domain and links the collagen-like sequence to the fourth domain. The fourth domain is a C-type lectin region that contains a globular CRD. SP-A is the most abundant surfactant protein in
the lung and comprises 5.3% of the total pulmonary surfactant weight. SP-A is assembled as octadecamers that consist of six trimeric subunits that form a “bunch-of-tulips” shape. Each SP-A monomer has molecular weight of 28–36 kDa. SP-D is assembled as dodecamers that contain four homotrimeric subunits arranged in a cruciform shape. Each SP-D monomer has molecular weight of 43 kDa\textsuperscript{150}.

**SP-A and SP-D receptors**

Little is known about collectin receptors. Several SP-A receptors have been identified, including the C1q receptor (also known as CD93), the calreticulin-CD91 complex, the signal-inhibitory regulatory protein-α (SIRP-α), the 210-kDa cell surface protein (SP-R210) and gp-340. SP-D binds the calreticulin-CD91 complex, SIRP-α, and SP-R210 and gp-340\textsuperscript{150}. Binding of SP-A to a certain receptor is regulated by the presence or absence of pathogens. For example, in the absence of potential pathogens, SP-A binds to SIRP-α receptor, which leads to inhibition of inflammatory mediators. In contrast, in the presence of pathogens SP-A binds to the calreticulin-CD91 complex through a free collagen-like region, thus activates immune cells and enhances production of inflammatory mediators such as TNF-α\textsuperscript{151}. It has only recently been demonstrated that SP-A and SP-D suppress alveolar macrophage phagocytosis via interaction with SIRP alpha receptor\textsuperscript{152}. The SP-R210 receptor is localized on the cell surface of ATII cells and macrophages. Binding of SP-A to SP-R210 results in SP-A-mediated uptake and killing of *Mycobacterium bovis*, and inhibition of phospholipid secretion\textsuperscript{153,154}. Both SP-A and SP-D bind to the gp-340 receptor, which has a soluble form and macrophage membrane-bound form. Activation of gp-340 receptor leads to binding and aggregation of some bacteria
such as *Streptococcus mutans*\textsuperscript{155}. Other recent studies have demonstrated that SP-A and SP-D bind TLRs such as TLR-2 and TLR-4\textsuperscript{156,157}.

**Biological activities of SP-A and SP-D**

SP-A and SP-D have several functional roles in the lung such as defense against invading microorganisms, regulation of inflammation, regulation of pulmonary surfactant homeostasis, and immunomodulation\textsuperscript{8}. The focus of this section will be the antimicrobial role of SP-A. These collectins bind a wide range of potentially pathogenic viruses, Gram-negative and Gram-positive bacteria, and fungi through CRD, which leads to agglutination, enhancement of phagocytosis, and respiratory burst, or direct bacteriostatic and fungistatic effect\textsuperscript{150}. Collectins have the ability to bind to enveloped viruses such as influenza A virus (IAV), respiratory syncytial virus (RSV), human immunodeficiency virus (HIV), and herpes simplex virus\textsuperscript{154,158,159}. Both SP-A and SP-D suppress IAV infectivity through agglutination\textsuperscript{160}. SP-A neutralizes IAV by binding and blocking hemagglutinin (HA) cell attachment site expressed on viral surface\textsuperscript{161}. SP-A can also opsonize IAV and enhance phagocytosis by macrophages\textsuperscript{162}. In addition, preincubation of IAV with SP-A enhances the respiratory burst of neutrophils, which phagocytes the virus\textsuperscript{160}. SP-D causes massive agglutination of IAV particles and thus represents more potent collectin in suppression of this virus. SP-D binds to high-mannose oligosaccharides of IAV which are present on the coat HA\textsuperscript{163}. In addition, SP-D enhances the uptake of IAV by neutrophils, which results in protection of the neutrophils from virus-induced suppression and enhances respiratory burst\textsuperscript{163}. SP-A and SP-D opsonize RSV by binding to the F (fusion) and G (adherence) protein in
Ca^{++}-dependent manner\textsuperscript{159,164}. Binding of SP-A to RSV enhances viral uptake by peripheral blood mononuclear cells and macrophages\textsuperscript{159}. Both SP-A and SP-D bind to a broad spectrum of Gram-negative and Gram-positive bacteria, which may results in agglutination, permeabilization of bacterial walls, enhancement of respiratory burst by neutrophils, and macrophages and enhancement of opsonization\textsuperscript{158,165}. Lipopolysaccharide (LPS) represents the most common ligand for binding the Gram-negative bacteria while lipoteichoic acid (LTA) and peptidoglycans represent the most common ligand for binding G-positive bacteria. SP-A binds the following bacterial organisms: \textit{Staphylococcus aureus}\textsuperscript{166}, \textit{Pseudomonas aeruginosa}, \textit{Escherichia coli} J5 (containing O-antigen deficient rough LPS)\textsuperscript{165}, \textit{Mycobacterium tuberculosis}\textsuperscript{167}, \textit{Streptococcus pneumonia}, Group A and Group B Streptococci, \textit{Hemophilus influenzae}\textsuperscript{168}, and \textit{Klebsiella pneumoniae}\textsuperscript{169}. SP-D binds \textit{Mycobacterium tuberculosis}, \textit{Pseudomonas aeruginosa}, \textit{E.coli}, and \textit{acapsular Klebsiella pneumoniae}\textsuperscript{147}. Binding of SP-A and SP-D to the same pathogen may have a different effect; upon binding to \textit{Mycobacterium tuberculosis}, SP-D decreases the bacterial uptake by macrophages. In contrast, SP-A binding to the same pathogen enhances the bacterial uptake by macrophages\textsuperscript{167,170}. Both SP-A and SP-D represent the major defense mechanism against inhaled fungal spores and yeast. The polysaccharides and highly glycosylated proteins on the fungal spore represent the ligands for SP-A and SP-D binding. Both SP-A and SP-D bind the following fungal and yeast organisms: \textit{Saccharomyces cerevisiae}, \textit{Aspergillus fumigatus}, \textit{acapsular Cryptococcus neoformans}, and \textit{Pneumocystis carinii}\textsuperscript{147}. SP-A and SP-D are capable of regulating inflammatory response by stimulating an
anti-inflammatory and/or pro-inflammatory effect\textsuperscript{8}. For example, SP-A interacts with macrophages through binding the CD14 receptor, inhibits the binding of smooth LPS to CD14, and reduces TNF\textgreek{a}-expression induced by smooth LPS. In contrast, SP-A increases the inflammatory response of macrophages induced by rough LPS\textsuperscript{171}. SP-D upregulates expression of pro-inflammatory cytokines such as IL-1, IL-6, and TNF\textgreek{a} by epithelial cells upon stimulation by bacteria\textsuperscript{172}. Lung collectins play a significant role in immunomodulation. For example, SP-D inhibits production of IL-2 by monocytes and consequently suppresses lymphocyte proliferation\textsuperscript{172}. SP-A can also inhibit lymphocyte proliferation through modulation of IL-2 production\textsuperscript{155}. SP-D modulates the inflammatory response to inhaled allergens by inhibition binding of IgE to those allergens\textsuperscript{173}. Primarily SP-A and, to a lesser extent, SP-D regulate surfactant homeostasis by controlling the secretion and uptake of surfactant by ATII. In addition, SP-A and SP-D protect surfactant from functional inhibitors such as phospholipase A1, and they are required for the formation of tubular myelin\textsuperscript{153}.

**SP-A and SP-D metabolism**

SP-A and SP-D are synthesized by ATII and Clara cells\textsuperscript{174}. After synthesis in endoplasmic reticulum (ER), lung collectins are transported into the Golgi apparatus (GA) and stored in the cytoplasmic structures named lamellar bodies (LBs)\textsuperscript{175}. Upon stimulation, LBs are secreted via exocytosis into the alveolar space. Once they reach the alveolar space, LBs swell and unravel, forming a cross-hatched structure named tubular myelin (TM). In addition to surfactant proteins, TM contains surfactant lipids that create a thin surface film which reduces surface tension in the alveoli during the breathing cycle\textsuperscript{175}. Recently, some studies have demonstrated that SP-A
and SP-D trafficking bypasses the LBs and that these two collectins are directly excreted from the GA into the alveolar space\textsuperscript{176,177}. In addition, ATII cells use intracellular glycogen during the synthesis of surfactant phospholipids (28). Therefore, intracellular glycogen stores can be an indicator of ATII cell function and maturation\textsuperscript{178}. The half-life of lung collectins is short. In rabbits, the half-life of soluble SP-A after the intratracheal installation into the lung is only 4.5–6.5 hours\textsuperscript{179,180}. Collectins secreted into the alveolar space are partially internalized and degraded by macrophages and partially reuptaken by ATII cells and recycled\textsuperscript{181,182}.

\textit{In-utero} lung development and regulation of SP-A and SP-D expression

In the neonatal lung, the expression of SP-A and SP-D is developmentally regulated and primarily depends on the level of differentiation and maturation of ATII cells\textsuperscript{177}.

\textit{In-utero} Lung Development

Stages of fetal lung development include the pseudoglandular, canalicular, saccular, and alveolar phase\textsuperscript{183}. In humans, the pseudoglandular phase of lung development occurs from the 6\textsuperscript{th} to the 16\textsuperscript{th} week of gestation. This phase is characterized by extensive branching of the airways. The epithelial cells of the distal airways, including alveolar epithelial cells, are largely undifferentiated, however\textsuperscript{183}. The canalicular phase occurs from the 16\textsuperscript{th} to the 24\textsuperscript{th} week of gestation. This phase is characterized by the remodeling of the distal portion of the branching airways and differentiation of alveolar epithelium. Low numbers of ATII cells are present; they are characterized by small, intracellular glycogen pools and few lamellar bodies. The saccular phase occurs from the 24\textsuperscript{th} to the 36\textsuperscript{th} week of gestation. During this stage,
the terminal airway branches develop into the terminal sacks and, to some extent, alveoli. These alveoli form a thin air-blood barrier, and surfactant production starts. The alveolar phase occurs at the 36th week of gestation and ends at approximately two years of post-natal age. This phase is characterized by extensive alveolar development that includes three stages: secondary septation, remodeling of the alveolar walls, and alveolar growth. During the alveolar phase, the alveoli continue to develop from the terminal sacks, the alveolar walls become thinner, and the numbers of ATII cells increase and mature. The lung development in other mammalian species parallels the lung development in humans; some substantial differences, however, are present. At birth, the lungs of mice are at the saccular phase, and alveolarization does not start until post-natal day 548. Similarly, the alveolar phase in the rat lungs starts at post-natal day 4 and continues until day 14184. Unlike humans, guinea pigs and rabbits develop the majority of their alveoli prior to birth185. The development of lungs in sheep closely parallels the development of human lungs with the exception of the secondary septation stage of alveolarization, which occurs earlier in gestation. SP-A and SP-D in the fetal lung parallels the fetal development and maturation of ATII cells. In the human lung, SP-A is first detectable at the 30th week of gestation (term~40 weeks), while SP-D is first detectable in the second trimester of gestation186,187. In sheep, the SP-A mRNA levels are low at the 100th day of gestation (term~148) and progressively increase starting at about 120 days of gestation to term and into the postnatal period177.
Regulation of SP-A and SP-D expression

In addition to gestational development, SP-A and SP-D expression is also regulated by a number of growth factors, hormones, and regulatory substances, including glucocorticoids, insulin, and vascular endothelial growth factor (VEGF)\(^8\). Glucocorticoids are increasingly produced by the fetal adrenal cortex during the late gestation period which parallels maturation of the fetal lung. In general, glucocorticoids increase production of surfactant phospholipids and surfactant proteins and thus prevent neonates from developing respiratory distress syndrome (RDS)\(^{188}\). Interestingly, if given for prolonged time and in high concentrations, glucocorticoids suppress surfactant protein expression in the lung\(^{189}\). Insulin decreases the expression of SP-D in the human fetal lung, and neonates from diabetic mothers are at increased risk to develop RDS due to insufficient pulmonary surfactant\(^{190}\). The growth factors involved in fetal lung maturation include VEGF, platelet-derived growth factor (PDGF), keratinocyte growth factor (KGF), and transforming growth factor-beta1 (TGF-\(\beta1\))\(^{183}\). The effects of the VEGF on the ATII cell maturation will be described in detail later. The PDGF-A family represents an important regulator of lung maturation, and mice in which the PDGF-A gene is deleted die soon after birth due to respiratory distress\(^{191}\). In addition, PDGF-A regulates post-natal lung development as well, and PDGF-A\(^{-/-}\) mice fail to form alveoli\(^{192}\). KGF is a stimulator of epithelial cell proliferation and surfactant protein gene expression. When adult rats were inoculated intrabronchially with 5 mg/kg KGF, SP-A and SP-D mRNA levels were increased in the lung\(^{193}\). This was attributed mainly to the ATII cell hyperplasia while expression of SP-A and SP-D per
cell remained at the same level. *In vitro* studies done on rat ATII cells treated with KGF demonstrated increased expression of SP-A but did not influence expression of SP-D mRNA\(^{194}\). TGF-β1 reduces SP-A mRNA expression and impairs the pseudoglandular stage of fetal lung development in mice. Other factors involved in lung maturation and surfactant protein expression include cAMP, IFN-γ, TNF-α, IL-4, O₂, NO, *all-trans* retinoic acid (RA), and phorbol myristyl acetate (PMA)\(^{8}\).

**SP-A and SP-D deficiency**

Respiratory distress syndrome (RDS), characterized by a severe deficiency of pulmonary surfactant, is the most common complication of neonates born prematurely\(^{195}\). Pulmonary surfactant primarily functions to regulate the size of alveolar spaces and allows efficient respiration. SP-A and SP-D contribute to the lung stability to a minor extent; they, however, play a major role in pulmonary defense against potentially pathogenic microorganisms. The increased incidence of RSV infection in prematurely born neonates is in part attributed to the decreased levels of SP-A and SP-D in immature lungs\(^{196,197}\). In fact, a study done on the lamb model of RSV infection has demonstrated reduced viral clearance from the lungs of lambs born prematurely\(^{198}\). Furthermore, several studies done on mice deficient in SP-A (SP-A\(^{-/-}\)) or SP-D (SP-D\(^{-/-}\)) have clearly demonstrated the crucial immunological role of these two collectins in the lung. Mice deficient in SP-A and SP-D had normal pulmonary function; the pulmonary defense against different viruses and bacteria, however, was significantly impaired. The SP-A\(^{-/-}\) mice had an increased load of bacterial organisms in the lung following the intratracheal installation of *B streptococcus* (GBS)\(^{199}\). The alveolar macrophages of SP-A\(^{-/-}\) mice
had a reduced rate of phagocytosis of intratracheally administered *Pseudomonas aeruginosa* bacterial organisms\(^{200}\). Similarly, the alveolar macrophages of mice deficient in SP-A and SP-D demonstrated a reduced rate of phagocytosis of *Hemophilus influenza* and GBS\(^{201}\). SP-A\(^{-/-}\) mice have demonstrated an increased susceptibility to RSV, and an intratracheal installation of SP-A enhanced the viral clearance from the lung\(^{202}\). The following study demonstrates that administration of SP-D to the mice infected with RSV enhanced the viral clearance from the lung\(^{203}\). Finally, SP-D\(^{-/-}\) mice have demonstrated reduced clearance of IAV and, as in a previous study, intratracheal installation of SP-D enhanced the viral clearance from the lung\(^{204}\). In conclusion, the major role of SP-A and SP-D in the lung is defense against pathogenic microorganisms; deficiency of these two lung collectins enhances susceptibility of the lung to pathogenic bacterial and viral agents. In addition, Individuals with polymorphisms in SP-A and SP-D genes have increased incidence and severity of RSV infections\(^{205,206}\).

**Lung surfactant replacement therapies**

Therapies used to improve pulmonary function in infants deficient in pulmonary surfactant due to immaturity include oxygen administration, mechanical ventilation, and administration of exogenous glucocorticoids and pulmonary surfactants. Exogenous administration of glucocorticoids to the mother accelerates fetal lung maturation primarily through stimulation of ATII cell differentiation and surfactant production\(^{207,208}\). Therefore, administration of glucocorticoids to pregnant women who are at increased risk of pre-term delivery is a common medical practice\(^{209}\). Fetal exposure to exogenously delivered glucocorticoids is associated
with many undesirable effects. Short-term side effects include hypertension, hyperglycemia, intestinal perforation and bleeding, inhibition of stomach growth, and hypertrophic cardiomyopathy\textsuperscript{210,211}. Long-term side effects include inhibition of lung growth and neurodevelopmental abnormalities\textsuperscript{212}. In addition, surfactant protein expression in the lung is suppressed by prolonged glucocorticoid treatment\textsuperscript{189}.

Exogenous surfactant replacement is another treatment therapy for prematurity-related surfactant deficiency\textsuperscript{213}. Although exogenously administered lung surfactant does not accelerate the process of lung maturation, it does reduce morbidity and mortality rates in prematurely born neonates. Most of the replacement surfactants used today, however, are synthetic and contain lipids, surfactant protein-B (SP-B), and surfactant protein-C (SP-C)\textsuperscript{214}. It has only recently been suggested that natural surfactants, which also contain SP-A and SP-D, may be beneficial in treating RDS but also non-RSD respiratory disorders such as pneumonia and bronchiolitis\textsuperscript{214}. In addition, exogenous surfactant therapy is a short-term therapy, and the procedure needs to be frequently repeated\textsuperscript{215}. In summary, despite the beneficial effects of exogenously administered glucocorticoids and pulmonary surfactants in neonates with inadequate respiratory function, both of these treatments have undesirable effects or questionable efficacy, which indicates a need for new, more efficient therapies.

**Cellular component; phagocytic and inflammatory cells**

Under normal conditions, pulmonary macrophages are the largest population of the phagocytic cells in the lung\textsuperscript{88}. Most of the pulmonary macrophages reside in the alveoli, and thus are designated as pulmonary alveolar macrophages (PAMs).
The main function of PAMs is to phagocytose particulate matter or bacteria that have reached the alveolar space by evading the mucociliary escalator and antimicrobial factors in the airways\textsuperscript{216}. They express several recognition receptors including pattern recognition receptors (PRRs) such as Dectin-1, scavenger receptors, Toll-like receptors (TLRs), and NOD-like receptors (NLRs). Other macrophage-surface receptors include Fc-\(\gamma\) receptor, complement receptors (CR-1, CR-3, and CR-4), and CD40 receptors\textsuperscript{217-221}. Once the particulate matter or microorganism is engulfed, the phagosome fuses with lysosomes which eliminate the phagocytized particles from the alveolar space. PAMs regulate the inflammatory response in the lung by secreting cytokines such as interleukin (IL)-1\(\beta\), IL-6, IL-10, IL-12, and tumor necrosis factor (TNF-\(\alpha\)); chemokines such as IL-8; monocyte chemotactic protein (MCP)-1, and MCP-2; macrophage inflammatory protein (MIP-1\(\alpha\)); and MIP-1\(\beta\); as well as most of the components of the complement pathway\textsuperscript{88}. Compared to other tissue macrophages, PAMs induce minimal inflammatory response in order to protect pulmonary tissue from the destructive consequences of fulminant inflammation. This anti-inflammatory state is displayed through altered cytokine response such as increased expression of anti-inflammatory cytokine, IL-10, and reduced oxidant production\textsuperscript{222,223}. In addition, PAMs secrete antimicrobial factors such as lysozyme and participate in humoral innate immunity of the lung\textsuperscript{224}. If the humoral responses and macrophages are unable to control infectious agents, a massive flux of neutrophils occurs. In these circumstances, neutrophils can constitute up to 80\% of the cells retrieved from the alveolar lavage fluid\textsuperscript{225}. Chemotaxis is stimulated primarily by IL-8 secreted by...
epithelial cells or complement activation. Neutrophils are powerful phagocytic cells; in addition to phagocytosis, they secrete cytokines such as IL-1β, IL-6, and TNF-α as well as defensins. Under normal conditions, eosinophils are present in the low numbers in the lung. Upon the stimulation of T-cell derived cytokines such as IL-3, IL-5, as well as GM-CSF, however, they proliferate. They are involved in inflammatory responses against parasitic agents and allergens. Pathologic conditions such as asthma and chronic eosinophilic pneumonia are characterized by eosinophilic inflammation. Natural killer (NK) cells respond to viruses by lysing the infected cells, releasing interferon (IFN)-γ, and recruiting other inflammatory cells.

Other innate immunity mechanisms and factors

The innate immunity of the lungs involves many other mechanisms and factors that are beyond the scope of this dissertation and therefore will be outlined only briefly. These include complement cascade; antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, and oxidant free radical scavengers; modified epithelial cells (M cells); bronchial associated lymphoid tissue (BALT); antiproteinases; and neurokinins.

Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF) refers to a large superfamily of growth factors primarily involved in growth of blood vessels and lymphatic vessels. The VEGF superfamily includes five subfamilies of polypeptides named VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PLGF). The VEGF-A and VEGF-B family members regulate formation and maintenance of blood vessels while VEGF-C and VEGF-D family members are involved in formation and
maintenance of lymphatic vessels\textsuperscript{229}. The VEGF-A family members are the most intensively studied VEGF growth factors in the past decade and hereafter will be referred to as VEGF.

**Biological activities of VEGF**

The VEGF polypeptides have a fundamental role in physiological vasculogenesis (\textit{de novo} formation of blood vessels during embryogenesis), physiological angiogenesis (formation of blood vessels from pre-existing vasculature)\textsuperscript{230}, and growth of blood vessels in pathological processes such as retinal neovascularization\textsuperscript{231}, age-related macular degeneration\textsuperscript{232}, rheumatoid arthritis\textsuperscript{233}, and neoplasia\textsuperscript{234-236}. The VEGF stimulates blood vessel development by two major mechanisms. First, it simulates proliferation of endothelial cells derived from both arterial and venous blood vessels\textsuperscript{227}. Second, it prevents apoptosis of vascular endothelial cells by activating phosphatidylinositol (PI)-3-kinase-Akt pathway\textsuperscript{237} and anti-apoptotic proteins Bcl-2 and A\textsubscript{1}\textsuperscript{238}. In addition to vascular endothelial cells, VEGF exerts its biological effect on many other non-endothelial cells, stimulates proliferation of airway epithelial cells in the human fetal lung\textsuperscript{30}, promotes monocyte chemotaxis\textsuperscript{239}, induces granulocyte-macrophage progenitor cell colony formation\textsuperscript{240}, inhibits dendritic cell development\textsuperscript{241}, and stimulates production of myeloid cells and B cells\textsuperscript{242}. VEGF also regulates inflammation by inducing vascular leakage\textsuperscript{243,244} and synthesis of nitric oxide (NO) and prostacyclin (PGI\textsubscript{2}) by endothelial cells\textsuperscript{245}. 
VEGF isoforms

The VEGF gene is mapped to chromosome 6p21.3 in humans and organized as eight exons separated by seven introns. Due to alternative exon splicing, this single gene encodes for several isoforms. In humans, there are seven known VEGF isoforms composed of 121, 145, 148, 165, 183, 189, and 206 amino acid residues. There are four analog murine isoforms composed of 120, 144, 164, and 188 amino acid residues, and three analog ovine isoforms composed of 120, 164, and 187 amino acid residues. The VEGF isoforms are named as VEGF<sub>xxx</sub> in which the xxx subscript refers to the number of amino acid residues present in the molecule. These isoforms are glycosylated, dimeric polypeptides which may or may not bind heparin sulphate proteoglycans. VEGF<sub>121</sub> is an acidic, diffusible polypeptide that does not bind heparin. In contrast, VEGF<sub>189</sub> and VEGF<sub>206</sub> are highly basic polypeptides which bind to heparin with high affinity and remain sequestered on the cell surface upon secretion. VEGF<sub>165</sub> is an intermediate isoform and upon secretion partially remains bound to the cell surface and partially diffuses into the extracellular space. Although the biological significance of different VEGF isoforms is not clear, some studies suggest that VEGF<sub>165</sub> has maximal availability and bioactivity.

Regulation of VEGF expression

Oxygen tension is the key regulator of VEGF expression. The tissue oxygenation regulates the VEGF expression through the actions of the transcription factors: hypoxia-inducible factor 1α, 2α, and 3α (HIF-1α, HIF-2α, and HIF-3α, respectively). HIF-1α and HIF-2α upregulate the expression of VEGF; in contrast,
HIF-3α is considered to be a negative regulator of VEGF expression\(^{250}\). HIFs are heterodimers composed of constitutively expressed beta subunit (the aryl hydrocarbon receptor nuclear translocator, Arnt) and one out of three oxygen-sensitive subunits alpha (HIF-1α, HIF-2α, or HIF-3α)\(^{250}\). Under normoxic conditions, the alpha subunit is rapidly hydrolyzed by prolyl hydroxylase, further associated with von Hippel-Lindau (VHL) protein, and thereafter degraded in ubiquitin pathway. In hypoxic conditions, the prolyl hydroxylase is inactive, and an alpha subunit dimerizes with a beta subunit to form the active HIF transcription factor. The active forms of HIF-1α and HIF-2α bind to the promoter on the VEGF gene and upregulates the VEGF mRNA transcription\(^{230}\). Although little is known about HIF-3α, some studies suggest that there is a splice variant of HIF-3α gene known as Per/Arnt/Sim (IPAS). This HIF transcription factor does not have transactivation function and is reportedly a negative regulator of VEGF expression\(^{250}\). In addition to oxygen tension and oxygen-sensitive transcription factors, several growth factors including epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), transforming growth factor beta (TGF-β), fibroblast growth factor (FGF), and platelet growth factor (PGF) upregulate transcription of VEGF mRNA\(^{228,251}\). There is evidence that inflammatory cytokines such as interleukin 1alpha (IL-1α) and interleukin 6 (IL-6) upregulate transcription of VEGF mRNA in several cell types, thus demonstrating that VEGF is a mediator of angiogenesis in inflammatory conditions\(^{251}\). In addition, several studies indicate that oncogenes induce angiogenesis and progressive tumor growth mediated by VEGF\(^{252,253}\).
**VEGF receptors**

VEGF binds three related tyrosine kinase receptors: vascular endothelial growth factor receptor 1, 2, and 3 (VEGFR-1, VEGFR-2, and VEGFR-3, respectively). VEGF-A family members bind to VEGFR-1 and VEGFR-2 to promote development of blood vessels, whereas VEGF-C and VEGF-D members bind to VEGFR-3 to promote development of lymphatic vessels. VEGFR-2 plays a key role in VEGF-mediated effects on endothelial cells such as proliferation, NO synthesis, and vascular permeability. VEGFR-2 has an extracellular region composed of seven immunoglobulin-like extracellular domains, a single transmembrane spanning region, and an intracellular tyrosine kinase region interrupted by tyrosine kinase-insert domain. Upon ligand binding, the tyrosine residues of the tyrosine kinase-insert domain undergo autophosphorylation, and phosphotyrosines further bind proteins containing the Src homology-2 domain. This ultimately leads to endothelial cell proliferation mediated by Erk pathway, endothelial cell survival mediated by 3-kinase/Akt pathway and caspase 9, and Bad inactivation. NO production mediated by NO synthase activation, endothelial cell migration mediated by p38MAPK and paxillin, and blood vessel permeability mediated by Yes tyrosine kinase. VEGFR-1 is structurally similar to the VEGFR-2. Binding of VEGF to VEGFR-1, however, leads to weak autophosphorylation of tyrosine residues on tyrosine kinase-insert domain resulting in weak downstream signaling. In addition, recent studies suggest that there is a soluble form of VEGFR-1, named the VEGF “decoy” receptor. The soluble form of
VEGFR-1 binds VEGF with high affinity and prevents VEGF from binding and activation of VEGFR-2\(^\text{266}\).

**The roles of VEGF, HIF and VEGFR in the lung development**

In the developing lung, VEGF primarily stimulates blood vessel growth and branching\(^\text{258}\). Recently it has been suggested that in addition to vasculature development, VEGF stimulates proliferation of alveolar type II cells (ATII cells) as well as production of lung surfactant proteins by ATII cells, thus accelerating lung maturation\(^\text{30,258,267-269}\). In the human lung, ATII cells are the major source of VEGF\(^\text{270}\), and VEGF levels are higher in broncho-alveolar fluid compared to the blood, suggesting that ATII cells themselves represent the target for VEGF\(^\text{271}\). Thus, addition of exogenous VEGF to the human fetal lung explants resulted in increased proliferation of distal airway epithelial cells\(^\text{30}\). When added to the cell culture, VEGF stimulated expression of SP-A and SP-C in human fetal ATII cells\(^\text{267}\). Furthermore, inhibition of VEGF resulted in respiratory distress syndrome (RDS) in neonatal mice due to insufficient production of pulmonary surfactant by ATII cells\(^\text{268}\). The exact mechanism by which VEGF stimulates pulmonary surfactant production is not clear; in the study done by Comprenolle et al., however, mice deficient in pulmonary surfactant had increased glycogen stores in ATII cells, indicating that VEGF mediates conversion of glycogen into pulmonary surfactant\(^\text{268}\). While the roles of specific VEGF isoforms in the lung are not fully explored, some studies suggest that VEGF\(_{120}\), VEGF\(_{164}\), and, in part, VEGF\(_{188}\), play the most significant roles during intrauterine lung development\(^\text{272}\). As previously mentioned, VEGF expression is tightly regulated by three hypoxia-sensitive transcription factors (HIF-1\(\alpha\), HIF-2\(\alpha\), and
HIF-3α\(^{227}\), and all three transcription factors are expressed in the developing human lung\(^{250}\). Several studies have demonstrated that HIF-1α and HIF-2α are crucial for intrauterine lung development and play a significant role in proliferation and maturation of ATII cells through VEGF action; the role of HIF-3α in lung development, however, is less clarified. For example, HIF-2α gene deletion in neonatal mice resulted in respiratory failure and death due to impaired differentiation of ATII cells and lack of pulmonary surfactant production\(^{268}\). In the same study, intrauterine delivery or postnatal intratracheal delivery of VEGF stimulated conversion of glycogen to surfactant and rescued HIF-2α\(^{-/-}\) from respiratory failure. In another study done in mice, HIF-1α gene deletion resulted in impaired alveolar epithelial differentiation and complete loss of surfactant production, indicating similar roles of HIF-1α and HIF-2α in neonatal lung development\(^{273}\). Furthermore, RDS in pre-term lambs characterized by impaired pulmonary surfactant production in immature lungs was associated with decreased expression of both HIF-1α and HIF-2α\(^{274}\). VEGF binds two tyrosine kinase receptors: VEGFR-1 and VEGFR-2. As stated above, VEGFR-2 is considered the main signaling receptor for VEGF\(^{230}\). Several studies demonstrated that ATII cells express VEGFR-2\(^{30,268,275}\), and intrauterine delivery of anti-VEGFR-2 antibodies in fetal mice resulted in respiratory failure compatible with VEGF deficiency\(^{268}\). In rats, chronic inhibition of VEGFR-2 with SU5146 VEGFR-2 blocker resulted in alveolar epithelial cell apoptosis and pulmonary emphysema\(^{276}\). The data on VEGFR-1 are conflicting. The recent studies suggest that VEGFR-1 may serve as a “decoy” receptor for VEGF and a negative
regulator of VEGF signaling; VEGFR inhibition with a combined VEGFR-1 and VEGFR-2 inhibitor, however, reduced alveolarization in the neonatal rats\textsuperscript{277}.

**VEGF and alcohol (ethanol)**

The data analyzing the influence of alcohol on VEGF are limited. I have not been able to identify publications that report the influence of alcohol on VEGF in either the adult lung or the fetal lung. Exposure to alcohol, however, has been associated with increased morbidity and mortality in trauma patients due to delayed process of wound healing. Radek et al. have demonstrated that angiogenesis, a crucial step in wound healing, was reduced by 61\% in rats acutely exposed to alcohol\textsuperscript{278}. The following study reveals that the mechanism by which alcohol alters wound healing involves an HIF-VEGF-VEGFR pathway as protein levels of HIF-1\(\alpha\) and VEGFR-2 were decreased in alcohol-treated endothelial cells and VEGFR-2 was less responsive to VEGF treatment\textsuperscript{279}. There are studies that describe the opposite effect of alcohol on angiogenesis and VEGF in neoplastic tissues. For example, exposure to alcohol stimulated expression of VEGF and angiogenesis in mice implanted with melanoma cells, which stimulated proliferation and metastasis of neoplastic cells\textsuperscript{280}. In another study, exposure to alcohol stimulated VEGF expression and angiogenesis of human fibrosarcoma cells implanted in chick chorioallantoic membrane\textsuperscript{281}. Although there is evidence that alcohol may stimulate expression of VEGF, these studies are conducted in neoplastic tissues, which may not compare to the physiologically healthy tissues.
The specific goals of this dissertation were to:

1. characterize an ovine model of fetal exposure to alcohol (ethanol) and nicotine,
2. determine the effect of in utero exposure of alcohol (ethanol) and nicotine on pulmonary SP-A and SP-D in lambs born prematurely and lambs that have reached full gestation, and
3. determine the mechanism by which in utero alcohol (ethanol) exposure alters the expression of pulmonary SP-A and SP-D in neonatal lambs.

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CHAPTER 2. MATERNAL ALCOHOL INGESTION REDUCES SP-A EXPRESSION BY PRE-TERM FETAL LUNG EPITHELIA

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Tatjana Lazic, Todd A Wyatt, Milan Matic, David K Meyerholz, Branka Grubor, Jack M Gallup, Karl W Kersting, Paula M Imerman, Marcia Almeida-De-Macedo, Mark R Ackermann

Abstract

In addition to neurodevelopmental effects, alcohol consumption during pregnancy is associated with immunomodulation and premature birth. Premature birth, in turn, is associated with increased susceptibility to various infectious agents such as Respiratory Syncytial Virus (RSV). The initial line of pulmonary innate defense includes the mucociliary apparatus, which expels microorganisms trapped within the airway secretions. Surfactant proteins A and D (SP-A and SP-D, respectively) are additional components of pulmonary innate immunity and have an important role in pulmonary defense against inhaled pathogens. The purpose of this study was to determine if chronic alcohol consumption during the third trimester of pregnancy alters the function of the mucociliary apparatus and expression of SP-A and/or SP-D of fetal lung epithelia. Sixteen date-mated ewes were assigned to two different groups: an ethanol exposed group in which ewes received ethanol through surgically implanted intra-abomasal cannula during the third trimester of pregnancy, and a control group in which ewes received the equivalent amount of water instead
of ethanol. Within these two groups, ewes were further randomly assigned to a full-term group in which the lambs were naturally delivered, and a pre-term group in which the lambs were delivered prematurely via an abdominal incision and uterotomy. Ethanol was administered 5 times a week as a 40% solution at 1gr/kg of body weight. The mean maternal serum alcohol concentration (SAC) measured 6 hr post administration was 16.3 +/- 4.36 mg/dL. Tracheas from 6 full-term lambs were collected to assess ciliary beat frequency (CBF). The lung tissue from all (24) lambs was collected for immunohistochemistry (IHC) analysis of SP-A and SP-D protein production and fluorogenic real-time quantitative polymerase chain reaction analysis (qPCR) of SP-A and SP-D mRNA levels. Exposure to ethanol during pregnancy significantly blocked stimulated increase in CBF though ethanol-mediated desensitization of cAMP–dependant protein kinase (PKA). In addition, prematurely-born/ethanol-exposed lambs showed significantly decreased SP-A m-RNA expression when compared to the prematurely born/control group (p=0.004); no significant changes were seen with SP-D. The full-term/ethanol exposed lambs had no significant alterations in mRNA levels, but had significantly less detectable SP-A protein when compared to the full-term/control lambs (p=0.02). These findings suggest that chronic maternal ethanol consumption during the third trimester of pregnancy alters innate immune gene expression in fetal lung. These alterations may underlie increased susceptibility of premature infants exposed to ethanol in utero to RSV and other microbial agents.
1. Introduction

The innate immune response of lung epithelia represents the first line of defense against potentially pathogenic microorganisms present in inspired air. These responses can immediately prevent colonization and proliferation of microbes, thus minimizing the involvement of the adaptive immune response and allowing efficient gaseous exchange. The respiratory tract innate immune system prevents initial colonization of airborne microorganisms through at least three distinct mechanisms. First, microorganisms trapped in the respiratory mucus can be removed mechanically by mucociliary activity. Second, when invading microbes reach the deeper respiratory compartments, such as the alveolar lumen, cellular and secretory mechanisms are triggered. These include alveolar macrophages, intravascular macrophages, and other cells with phagocytic and pro-inflammatory properties such as eosinophils (Aderem and Underhill 1999; Underhill and Ozinsky 2002). The third mechanism involves antimicrobial peptides, proteins and other molecules secreted by respiratory epithelia (Brogden, Ackermann et al. 2003; Bals and Hiemstra 2004; Hiemstra and Bals 2004). Ethanol exposure significantly alters at least some of these lung innate responses (Happel and Nelson 2005) in adults. While each of these innate immunity mechanisms are important in the initial respiratory host defense, their activity in individuals born pre-term with or without the influence of intrauterine ethanol exposure is poorly understood.

Alveolar type II cells (ATII) as well as non-ciliated bronchial and bronchiolar epithelial cells (Clara cells) secrete lung surfactant. Surfactant is a mixture of phospholipids which primarily functions to prevent alveolar collapse during
end-expiration (Ochs 2006). However, in addition to this physiological function, surfactant has been found to have potent antimicrobial properties which are largely attributed to the hydrophilic, collectin-like surfactant protein A and D (Crouch, Hartshorn et al. 2000; LeVine and Whitsett 2001; Sano and Kuroki 2005; Grubor, Meyerholz et al. 2006; Kingma and Whitsett 2006; Kishore, Greenhough et al. 2006). The secretion of SP-A and SP-D is regulated by developmental stage (King, Ruch et al. 1975; Mescher, Platzker et al. 1975; Kawashima, Meyerholz et al. 2006), hormones and potentially, by environmental factors such as alcohol consumption.

Infants born prematurely exhibit increased susceptibility to and severity of infections with RSV (Rossi, Medici et al. 2007). Risk factors associated with premature birth include maternal alcohol consumption, smoking, drug abuse and poor nutrition (Hack, Flannery et al. 2002; Parazzini, Chatenoud et al. 2003; Albertsen, Andersen et al. 2004; Kyrklund-Blomberg, Granath et al. 2005). Alcohol consumption during pregnancy has been associated with many harmful effects in the developing fetus. Most studies have focused on neurodevelopmental (Riley and McGee 2005; West and Blake 2005) and behavioral anomalies (Mattson, Schoenfeld et al. 2001; Sood, Delaney-Black et al. 2001) in infants born from mothers who drank during pregnancy. Recently it has been suggested that in addition to neurodevelopmental effects, ethanol may play a significant role in immunomodulation and consequential increase in susceptibility to various infectious in newborn infants (Gauthier, Manar et al. 2004; Gauthier, Drews-Botsch et al. 2005; Gauthier, Ping et al. 2005). It is our hypothesis that ethanol consumption during pregnancy alters mucociliary clearance and expression of SP-A and/or SP-D by fetal
lung epithelia. Moreover, we suspect that intrauterine fetal ethanol exposure may more severely affect those born prematurely than those born full-term since full-term infants have had additional time to allow for lung epithelial cell maturation. To do this we have developed an ovine model. Gestating ewes and their offspring are well-suited for these studies since: 1) alveolar development in lambs occurs pre-natally as in humans unlike rodents; 2) lambs can be derived pre-term and survive unlike many other animal models (Meyerholz, Grubor et al. 2004); 3) ovine respiratory epithelia of airways, distal bronchioles and alveoli are similar to those of human lung and the ovine lung has submucosal glands not present in some animal models (Meyerholz, Grubor et al, 2004; Meyerholz, Kawashima et al, 2006); 4) expression of SP-A and SP-D and other innate immune genes of ovine lung are well-characterize and very similar to human (Grubor, Meyerholz et al, 2006; Meyerholz, Kawashima et al 2006); and 5) pre-term lambs are susceptible to RSV, a known pathogen of premature infants and maternal ethanol consumption is a risk for pre-term birth (Meyerholz, Grubor et al, 2004). Finally, sheep have long been used for respiratory studies related to human disease as well as the effects of ethanol on fetal brain development (Mescher, Platzker et al, 1975).

2. Materials and Methods

2.1. Experimental design:

Animal use and experimental procedures were approved by Iowa State University’s Animal Care and Use Committee according to the requirements of the NIH guide for the Care and Use of Laboratory Animals. Abomasal cannula was surgically implanted in 16 mixed breed (1/2 Rambouillet, 1/4 Dorset, and 1/4
Finsheep), date-mated, pregnant ewes approximately 6 weeks prior to lambing
dates. The ewes were randomly assigned to two groups, an ethanol-exposed and a
control group. Within these groups, ewes were further randomly assigned to a
full-term group in which the lambs were naturally delivered and a pre-term group in
which the lambs were delivered prematurely via abdominal incision and uterotomy
(day 138 of gestation, term=147). The ewes received either ethanol or water five
days a week for three weeks. A total of twenty four lambs were obtained and
assigned to experimental groups. Each group had 6 lambs (n=6 lambs).

2.2 Abomasal cannulation:

The delivery of ethanol into the ovine abomasum allows bypass of the rumen
and degradation by ruminal microflora. It also promotes hepatic bio-metabolism as
the abomasum is the “true” stomach of ruminants and empties directly into
duodenum. Pregnant ewes were deprived of water and food for 12 hr prior to
induction of anesthesia. Anesthesia was induced by administration of Telazol (1-2
mg/kg, I.M; Fort Dodge, IA) and ewes were positioned in the dorsal recumbence.
General anesthesia was maintained by inhalation of 2% isoflurane. The median
abdominal and right paracostal regions were prepared for aseptic surgery. The
initial, 10 cm incision was made from umbilicus to the epigastric region. Abomasum
was identified, exteriorized, and isolated by towels moistened with sterile saline. A
½” polyethylene cannula (Bar Diamond, Parma, ID) was placed in the pyloric antrum
and the abomasal incision was closed with purse string sutures. A second 5 cm long
incision was made in the right paracostal region and the cannula shaft was
exteriorized. To secure the cannula placement, an additional 4 sutures through the
cannula ring were added. Finally, both abdominal incisions were sutured closed (Figure 1).

2.3 Ethanol administration protocol:

In 16 pregnant ewes, 40% ethanol at 1gr/kg, V/V (Sigma, St. Louis, MO) administration began 5 days after cannula insertion and was repeated 5 days a week during the last 5 weeks of gestation. Control ewes received the equivalent amount of water. After 4 weeks of ethanol administration (5 days/week) serum alcohol (ethanol) concentrations (SAC) were determined at 6, 12 and 24 hours post a single infusion by headspace gas chromatography analysis (University of Iowa Diagnostic Laboratories, Iowa City, IA)

2.4 Tissue collection:

Within 24 hr of natural lambing or uterotomy, all lambs were euthanized by intravenous administration of sodium pentobarbital and lung tissue was collected immediately. The samples from the left and right cranial lobes were collected into cryovials, snap frozen in liquid nitrogen and stored at -80°C until RNA extraction and qPCR analysis. The remaining tissue from the same lung lobes were fixed in 10% buffered formalin for IHC. The tracheas were collected into complete Dulbecco’s modified Eagle’s medium (complete DMEM), placed on ice and immediately shipped for mucociliary activity analysis (Department of Internal Medicine, University of Nebraska Medical Center, Pulmonary and Critical Care Medicine Section).

2.5 Ciliary Beat Frequency analysis:

A total of 6 lamb tracheae were obtained, 3 from full-term control lambs (n=3 lambs) and 3 from full-term alcohol exposed lambs (n=3 lambs). Lamb tracheae
were sliced into 2-5 mm rings and placed in M199 media in 35 mm tissue culture dishes (Falcon, Franklin Lakes, NJ). Tissues were maintained at room temperature (25°C ± 0.5°C) during assay procedures using a thermostatically-controlled heated stage. Cilia motion frequency was quantitated using the Sisson-Ammons Video Analysis (SAVA) system. Images of ciliated cells were visualized by inverted phase-contrast microscope, digital video images captured and analyzed for motion by SAVA using a process known as Whole Field Analysis (Sisson, Stoner et al. 2003). The SAVA software analyzed each image containing 19,200 possible motile zones to determine the average frequency and the standard error of the mean for each field captured. For each experimental condition, a minimum of 6 separate fields were captured, analyzed and expressed as a data point.

2.6 Determination of cAMP-dependent Kinase Activity:

PKA activity was determined in crude whole-cell fractions of tracheal epithelial cells. Tracheae were exposed to M199 (media control) or 100 µM isoprotrenol for 1 hour and the tracheal epithelial cells were scraped from the luminal surface of the tracheae using a sterile cell lifter. Cells were flash frozen on liquid nitrogen in a cell lysis buffer consisting of phosphate buffered saline (pH 7.4) containing protease inhibitors (1 g/ml each of leupeptin, aprotinin, PMSF and chymostatin). Cell lysates were sonicated and particulates removed by centrifugation. Protein concentration was determined by the Bradford method (Schleicher and Wieland 1978) with Bio-Rad protein reagent (Bio-Rad, Hercules, CA). This PKA assay has been previously described in detail (Wyatt and Sisson 2001). Kinase activity was expressed in relation to total cellular protein assayed and expressed in terms of
picomoles of phosphate incorporated/min/mg of total protein assayed. All samples were assessed in triplicate and no less than three separate experiments were performed per unique parameter.

2.7 qPCR analysis:

Our approach to this procedure has been previously described in detail (Gallup and Ackermann 2006; Kawashima, Meyerholz et al. 2006). Briefly, total RNA was isolated from whole lung tissue samples and assessed for quantity and purity by spectrophotometry. This procedure was followed by DNase treatment (TURBO DNA-free kit, Ambion, Austin, TX). Prior to qPCR analysis, a test plate was run to determine which RNA dilution ranges gave the best signal (lacked inhibition and exhibited LOG-linear behavior and amplification efficiencies >80%) for SP-A and SP-D targets. The DNase treated RNA isolates were diluted 1:10 with nuclease-free water, then diluted to their ideal ranges on a per-sample, per-target basis (according to what was learned from the test plate analysis), and further placed in duplicate into 96-well qPCR reaction plates (Applied Biosystems Incorporated, Forest City, CA). Each initially-prepared 30 μl reaction contained 7.8 μl of DNase treated, appropriately-diluted RNA isolate, 15 μl of a commercially available Applied Biosystems One-Step Master Mix, 1000 nM primers, 150 nM TaqMan (5’-6FAM, 3’-TAMRA-quenched) probe, and nuclease-free water. The negative, no-template control wells contained nuclease-free water (Ambion) instead of RNA isolate. 27 μl of each reaction was used in-well in the 96-well reaction plates. The plates were run in the GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA) for detection and relative quantification of SP-A and SP-D mRNA targets.
The results were analyzed by using the GeneAmp 5700 software and departmentally available Excel files. In addition, the real-time mRNA data are normalized to S15 mRNA.

2.8 IHC analysis:

This procedure and scoring system has been previously described in detail by our laboratory (Grubor, Gallup et al. 2004). Briefly, sections of lung on glass slides were deparaffinized and rehydrated followed by antigen retrieval, a protein blocking procedure, SP-A specific primary antibody incubation (Mouse IgM anti-human SP-A antibody, Chemicon International, Inc., Temecula, CA), secondary antibody application (biotinylated Rat anti-Mouse Isotype Rat (LOU) IgG2a, B.D. Pharmingen, San Diego, CA), streptavidin-conjugated horseradish peroxidase incubation (BioGenex, San Ramon, CA) and Nova Red substrate (Vector, Burlingame, CA) development. All slides were counter-stained with Shandon’s ¼-strength hematoxylin (Shandon-Lipshaw, Pittsburgh, PA), dehydrated through a series of graded solvents, and cover-slipped using Permount (Fisher, Hanover, IL) according to standard IHC protocol. For IHC scoring system, five fields of each lung section were assessed for intensity and distribution of SP-A stained epithelial cells in the terminal bronchioles and bronchiolar/alveolar junctions. Scoring was based on predetermined scale; 0, no staining of cells; 1, <30% of epithelial cells stained with minimal detectable intracytoplasmic staining; 2, 30-60% of epithelial cells stained with minimal detectable intracytoplasmic staining; 3, 30-60% of epithelial cells stained with >50% of cytoplasm stained; 4, >60% of epithelial cells stained with >50% of cytoplasm stained.
2.9. Statistical analysis:

For CBF data, ANOVA was run on each data point. For determination of cAMP-dependent Kinase Activity data was analyzed for statistical significance using one-way ANOVA. For IHC and qPCR data, summary statistics (mean and standard error of the mean) were calculated for each experimental group. Student-t tests (Graph Pad Sigma software, San Diego, CA) were also performed on normalized qPCR and IHC-score data. The acceptable level of significance was p<.05.

3. Results

3.1 Sheep serum alcohol (ethanol) concentration

The ethanol in the maternal serum was measured 6 hr post-infusion and ranged from undetectable (in one ewe) and 10-32 mg/dl (in other 7 ewes), mean was 16.3 +/- 4.36 mg/dL. Ethanol was undetectable at 12 and 24 hours in these ewes (Table 1).

3.2 CBF results:

Ex vivo tracheal CBF was assayed in full-term lambs delivered to both control and ethanol-exposed pregnant ewes. In ethanol-exposed trachea, baseline CBF did not significantly differ from control trachea (Figure 2A). However, isoproterenol-stimulated CBF increases in control lamb tracheal rings were not observed in tracheal rings obtained from ethanol-exposed lambs. Because isoproterenol-stimulated CBF increases require the activation of PKA (Wyatt, Spurzem et al. 1998) epithelial cell PKA activity was also assayed in this study. Isoproterenol significantly increased PKA activity in control trachea, but failed to activate PKA in ethanol-exposed trachea (Figure 2B).
3.3 qPCR results:

Pregnant ewes that received ethanol during the last 5 weeks of gestation showed significantly reduced SP-A mRNA expression in lungs of lambs delivered prematurely (p=0.004) compared to pre-term/control lambs not receiving ethanol (Figure 3). Although maternal ethanol administration exhibited a tendency toward decreased SP-D mRNA expression in pre-term lambs when compared to pre-term/control lambs, this difference was not statistically significant (p=0.4, Figure 4). In full-term lambs, there was no significant change in both SP-A and SP-D mRNA expression between full-term control and full-term/ethanol exposed lambs.

3.4 IHC analysis results:

SP-A antigen was detected within the cytoplasm of terminal bronchiolar and bronchiolar/alveolar junction epithelial cells in all four groups of lambs. The mean score for all for groups was; PTC, 1.7 +/- 0.15; FTC, 2.88 +/-0.39; FTA, 1.66 +/-0.38 and PTA, 2.00 +/-0.41. The pre-term/control group (Figure 5D) had significantly less detectible SP-A-specific staining when compared to the full-term/control group (p=0.02, Figure 5B). The full-term/alcohol exposed group (Figure 5A) had significantly less detectible SP-A-specific staining (reduced SP-A protein expression) when compared to the full-term/control group (p=0.04, Figure 5B).

4. Discussion

The finding that maternal ethanol exposure reduces the expression of SP-A and CBF in fetal lung demonstrates that ethanol has transplacental effects on key aspects of lung innate immunity. The decreased expression of SP-A mRNA in lungs of lambs born prematurely and exposed to ethanol in utero when compared to
prematurely born, control lambs lacking in utero ethanol exposure suggests that ethanol may even further hinder innate immunity beyond the level of a normal pre-term individual. Our previous studies have shown that innate immune gene expression in pre-term lambs is significantly reduced compared to full-term lambs (Hallman, Glumoff et al. 2001; Kawashima, Meyerholz et al. 2006) and ethanol may exacerbate this alteration. In this study, pre-term/control (without ethanol exposure) SP-A mRNA levels tended to be reduced in comparison to full-term lambs. However, this reduction was not significant (p=0.9). This was expected since the gap in age between pre- and full-term lambs in this current study is less (9 days) than in our previous studies (17-32 days) (Meyerholz, Kawashima et al. 2006).

Although SP-A mRNA levels were reduced in the lungs of pre-term lambs exposed to ethanol in utero, SP-A protein expression was not significantly altered. We suspect that this might be explained by decreased secretion of SP-A protein by the immature lung epithelial cells (Hallman, Glumoff et al. 2001). It is possible that ethanol may suppress SP-A transcriptional activity in immature epithelia and SP-A protein release into the airway. Both of these alterations by ethanol may greatly affect innate defense.

In contrast to pre-term lambs, SP-A mRNA levels were not altered in full-term lambs with or without ethanol exposure in utero. We suspect that the additional gestation time may allow the lung epithelia to further mature and reach a critical point for essential SP-A transcription. The reduced SP-A protein detected in lungs of full-term/ethanol exposed lambs compared to the full-term/control lambs may suggest that while a mature level of SP-A gene transcription may occur at this
developmental stage, there may be a time of “catch up” needed in newborn lambs to release mature protein into the airway lumen. Alternatively, ethanol may have a residual effect on post-translational intracellular mechanisms. Both of these findings suggest that ethanol has a suppressive effect on SP-A depending on the developmental stage of the animal. Age-specific mechanisms are likely different as well.

The decrease in stimulated trachea CBF in lambs exposed to ethanol is consistent with previous studies. Ethanol can have a detrimental effect on this lung protective mechanism depending on the length of exposure. When acutely-exposed to ethanol, the ciliary beat frequency in bovine bronchial epithelial cells was increased (Wyatt, Forget et al. 2003). Interestingly, when chronically exposed to ethanol, the effect was quite opposite and ciliary beating was decreased (Wyatt and Sisson 2001). This chronic desensitization of cilia stimulation by ethanol has also been demonstrated in vivo in rat (Wyatt, Gentry-Nielsen et al. 2004) and mouse (Elliott, Sisson et al. 2006) models. Unique to our current study is the observation that maternal transmission of ethanol-mediated desensitization occurs in the full-term lamb as well. The combination of reduced SP-AD expression and decreased clearance due to desensitized CBF may underlie the increased susceptibility of the pre-term lung (exposed to maternal alcohol consumption) to infectious agents such as RSV.

The CBF and SP-A findings in fetal lung are consistent with studies that show an overall immunosuppressive effect of ethanol. For example, chronic alcoholics have an increased incidence and higher mortality rate related to bacterial pneumonia
Maternal alcohol consumption during pregnancy and its effects on fetal immunity remains a largely unexplored area of clinical significance. Several studies have already demonstrated that newborns exposed to ethanol during prenatal life have a higher incidence of infection (Gauthier, Manar et al. 2004; Gauthier, Ping et al. 2005). The exact mechanism by which this occurs is not fully understood but it is possible that reduction in tracheal CBF and SP-A may increase susceptibility to infections. Ethanol consumption has been shown to have a suppressive effect on cellular components of the innate immune system such as macrophages (Gauthier, Ping et al. 2005) and/or neutrophils (Vinson, Carroll et al. 1998), all of which may occur concurrently with reductions in CBF and SP-A.

Although lung epithelia secrete many antimicrobial peptides, proteins and molecules, SP-A and SP-D are among the most extensively-studied pulmonary host defense/innate proteins. They are constitutively expressed and secreted by alveolar type II cells and Clara cells (Crouch, Parghi et al. 1992) and belong to a subgroup of collectin-containing mammalian C-type lectins, called collectins (Kishore, Greenhough et al. 2006). SP-A and SP-D can bind, aggregate and opsonize many microorganisms including Gram-negative and Gram-positive bacteria, enveloped viruses like *Influenza A Virus* (IAV), RSV, non-enveloped viruses like *Rotavirus* and fungal organisms in order to protect lung from possible infection (Grubor, Meyerholz
et al. 2006). In animal studies, the deficiency of SP-A, SP-D or both were associated with increased susceptibility to pulmonary *Pseudomonas aeruginosa* (LeVine, Kurak et al. 1998) and *Streptococcal* infections (LeVine, Bruno et al. 1997). Also, elevated levels of SP-AD mRNA in the lungs of neonatal lambs are associated with clearance of *Parainfluenza virus*-3 (PIV-3) (Grubor, Gallup et al. 2004). Another study showed that SP-AD enhance mannose receptor mediated phagocytosis of *Mycobacterium avium* by macrophages (Kudo, Sano et al. 2004). Also, the reduced clearance of RSV in lambs is associated with prematurely and possibly inadequate expression in pulmonary collectins (Meyerholz, Grubor et al. 2004) while SP-A enhances RSV clearance in mice (LeVine, Gwozdz et al. 1999).

In addition, when SP-A,D are administered to the lung in the murine model of invasive pulmonary aspergillosis, the mortality rate of infected animals was significantly reduced (Kishor, Madan et al. 2002). In rats, administration of SP-A reduces the oxidative damage of ventilated lungs (Bailey, Maruscak et al. 2006). In ventilated lambs, SP-A did not reduce inflammation indicators caused by inflammation (Ikegami and Jobe 2002). However, there were no negative effects associated with SP-A administration.

In this study, we have shown that a moderate level of alcohol consumption during the third trimester of pregnancy significantly decreases the levels of SP-A gene expression in prematurely born lambs while SP-D remained unaffected. The exact mechanism of this alcohol-related immunosuppressive effect is not clear but we suspect that ethanol may have a direct effect on lung epithelial cells, particularly ATII cells and/or Clara cells. For example, Brown et al. have previously shown that
chronic ethanol ingestion potentiates apoptosis of ATII cells in rats (Brown, Harris et al. 2001). The results of this experiment suggest that chronic ethanol exposure decreases the bioavailability of glutathione (GSH) to mitochondria of ATII cells. As a consequence, ATII repair mechanisms are diminished – resulting in a significant decrease in numbers of these SP-A-producing cells. Also, the same investigators have previously published that chronic ethanol exposure and GSH depletion activates matrix metalloproteinases (MMP’s) which consequentially degrade lung epithelial cells (Lois, Brown et al. 1999). Although this might be a possible mechanism of ethanol-induced lung innate immunity impairment, other researchers have shown that prenatal exposure to ethanol effects maternal-fetal-hormonal interactions which in-turn substantially affects the functional immunity of offspring. Zhang et al. have demonstrated that alcohol consumption during pregnancy increases Hypothalamic-Pituitary-Adrenal (HPA) responsiveness in rat pups in such a way that ethanol-exposed neonates exhibit reduced increase responsiveness in early and pre-weaning life (Zhang, Sliwowska et al. 2005). The exact nature and significance of this disturbed hormonal homeostasis still needs to be determined but it is possible that insufficient neonatal corticosteroid levels might be an underlying indicator for inadequate lung surfactant production. In addition, moderate levels of ethanol consumption are related to an increased incidence of premature birth (Parazzini, Chatenoud et al. 2003; Albertsen, Andersen et al. 2004). As previously demonstrated in our lab, premature birth is significantly correlated to decreased expression of SP-AD (Meyerholz, Kawashima et al. 2006).
In developing this ovine model, a cannula was placed into the abomasum for ethanol delivery. This allowed us to bypass the rumen and access a part of the ovine gastrointestinal tract that more accurately mimics the stomach of humans. The abomasum is the "true" stomach of ruminants out of which there is unimpeded flow of abomasal contents into the duodenum and small intestine – similar to gastric contents emptying into the small intestine of humans. The delivery of ethanol into the ovine abomasums in this manner allows for its absorption and passage into the portal veins draining into the liver thereby promoting hepatic bio-metabolism (Lieber, Gentry et al. 1994), which is similar to the route of ethanol in humans.

As indicated, the levels of ethanol used in this study were similar to those of moderate, not excessive alcohol consumers (Brien, Clarke et al. 1987). There is debate over "safe" levels of alcohol consumption during pregnancy, and many studies recommend complete abstinence from alcohol during gestation altogether (Martinez-Frias, Bermejo et al. 2004).

In conclusion, moderate alcohol consumption during the last trimester of pregnancy in sheep is associated with significant reduction of SP-A and CBF in lambs born prematurely. The deficiency in surfactant proteins, including SP-A, is associated with increased susceptibility to neonatal infections including RSV (Griese, Maderlechner et al. 2002). This finding may have clinical significance and will allow us to further investigate RSV infection as a means to address the hypothesis that ethanol exposure during pregnancy may increase RSV susceptibility in human infants.
Acknowledgements

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References


### Tables

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**Table 1.** Serum alcohol concentrations in mg/dl, in acclimated (receiving daily ethanol for five days for three weeks) pregnant ewes at six, 12 and 24 hours after a single infusion (40% at 1 mg/kg, V/V).
Figure 1. Intra-abomasal cannula for alcohol delivery. The canula is present along the ventral right paralumbar area. To deliver alcohol, the screw-cap is released and a catheter is inserted and alcohol enters by gravity flow.
Figure 2. Ewes were exposed to alcohol as described. 6 lambs were sacrificed and tracheal ring cilia were incubated with control media or 100 µM isoproterenol (ISO) for 30 min. CBF (panel A) and PKA (panel B) were assayed. ISO significantly stimulates increased lamb tracheal epithelial CBF and PKA from control ewes (*p<0.05; n=3 lambs). Maternal alcohol exposure desensitizes the lamb cilia to beta-agonist stimulated increases in CBF and PKA (n=3 lambs).
Figure 3. The relative SP-A mRNA expression in pre-term control (PTC), and full-term control (FTC) lambs as well as pre-term alcohol-exposed (in utero) (PTA) and full-term alcohol-exposed (in utero) lambs (FTA). The FTC and PTC values are equal to one. The PTA and FTA values are presented relative to the corresponding controls. The SP-A mRNA expression is significantly reduced (p=0.004) in PTA lambs (n=6 lambs) when compared to PTC lambs (n=6 lambs). There is no significant change in SP-A mRNA expression between FTA and FTC lambs.
Figure 4. The relative SP-D mRNA expression in pre-term and full-term lambs with or without (control) maternal alcohol administration during gestation. Although maternal alcohol administration had a tendency to decrease the SP-D mRNA expression in the pre-term/alcohol exposed lambs (PTS), (n=6 lambs) when compared to the pre-term/control lambs (PTC), (n=6 lambs), this difference was not statistically significant (p=0.4). There is no significant change in SP-D mRNA expression between full-term/alcohol exposed and full-term/control lambs.
Figure 5. Representative immunohistochemical detection of SP-A protein in bronchial/bronchiolar epithelium of the full-term/alcohol exposed lambs (A), full-term control lambs (B), pre-term/alcohol exposed lambs (C), and pre-term control lambs (D). High distribution and intensity of staining is seen in pre-term lambs receiving alcohol, suggesting that alcohol may affect SP-A protein release from epithelial cells. Arrows depict epithelial cells with SP-A protein expression.
CHAPTER 3. EFFECTS OF NICOTINE ON PULMONARY SURFACTANT PROTEINS A AND D IN OVINE LUNG EPITHELIA

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Tatjana Lazic, DVM, MS, Milan Matic, DVM, Jack M. Gallup, MS,
Albert Van Geelen, PhD, David K. Meyerholz, DVM, PhD, Branka Grubor, DVM,
PhD, Paula M. Imerman, PhD, Marcia M. M. A. de-Macedo, PhD,
and Mark R. Ackermann, DVM, PhD

Summary

Maternal smoking during pregnancy increases the incidence and severity of respiratory infections in neonates. Surfactant proteins A and D (SP-A and SP-D, respectively) are components of pulmonary innate immunity and have an important role in defense against inhaled pathogens. The purpose of this study was to determine if nicotine exposure during the third trimester of pregnancy alters the expression of SP-A and SP-D of fetal lung epithelia. Pregnant ewes were assigned to four groups; a nicotine-exposed full-term and pre-term group, and control full-term and pre-term group. Lung tissue was collected for Western blot and IHC analysis of SP-A level, Western blot analysis of SP-D level and qPCR analysis of SP-A and SP-D mRNA expression. Exposure to nicotine significantly decreased SP-A gene expression (p=0.01) and SP-A protein level in pre-term lambs. This finding suggests that maternal nicotine exposure during the last trimester of pregnancy alters a key component of lung innate immunity in offspring.
1. Introduction

The lungs are constantly exposed to potentially pathogenic microorganisms present in the inspired air. Microbial agents trigger responses of the innate immune system of lung epithelia which reduces microbial colonization and infection. In addition, an effective innate immune response by epithelia minimizes the adaptive immune responses, thereby reducing leukocytic infiltration and allowing efficient pulmonary function and gaseous exchange.

The lung innate immune system includes three major components: physical barrier, cellular responses, and secretory products. The physical barrier includes respiratory tract epithelia and constant ciliary movement in order to propel invading particles out of the respiratory tract. The alveolar macrophages, intravascular macrophages, and other cells with phagocytic and pro-inflammatory properties are cellular components which function to prevent pulmonary infection. The secretory component includes antimicrobial peptides, proteins, and other molecules secreted by respiratory epithelia which line the pulmonary airways. Surfactant proteins A and SP-D are key elements of the secretory lung innate immune defense. They are synthesized and secreted by alveolar type II cells (ATII) and non-ciliated bronchial and bronchiolar epithelial cells (Clara cells). Surfactant protein A and SP-D are involved in host defense against various bacterial and viral pathogens. The secretion of SP-A and SP-D is regulated by developmental stage, hormones and, potentially, by environmental factors such as cigarette smoke.

Previous studies have shown that maternal smoking during pregnancy is associated with many adverse health effects in the developing fetus which may be
manifested during postnatal life. Undoubtedly, maternal smoking during pregnancy is a major cause of premature birth and low birth weight for gestational age. There is a relatively more recent body of work, suggesting that cigarette smoke is immunosuppressive and predisposes infants to respiratory infections. Although cigarette smoke contains thousands of different chemicals, it is suspected that nicotine is a major immunomodulatory component since it readily crosses the placenta and accumulates in the fetal compartment.

It is our hypothesis that in addition to premature birth, cigarette smoking during pregnancy may have a significant regulatory role on lung surfactant protein and consequential increase in susceptibility to various respiratory infections in newborn infants.

2. Materials and Methods

2.1. Experimental design:

Animal use and experimental procedures were approved by Iowa State University’s Animal Care and Use Committee according to the requirements of the NIH guide for the Care and Use of Laboratory Animals. The lateral neck region was clipped and fitted with a transdermal nicotine patch (21 mg, Nicoderm CQ, GlaxoSmithKline, Brentford, Middlesex, U.K) in 16 mixed breed (1/2 Rambouillet, 1/4 Dorset and 1/4 Finnish sheep), date-mated, pregnant ewes approximately 6 weeks prior to lambing dates. The ewes were randomly assigned to two groups, nicotine exposed group and a control group. Within these groups, ewes were further randomly assigned to a full-term group in which the lambs were naturally delivered
and a pre-term group in which the lambs were delivered prematurely via abdominal incision and uterotomy (day 138 of gestation, term=147). Prior to uterotomy, the ewes were anesthetized with tiletamine and zolazepam (Telazol, 1–2 mg/kg, I.M; Fort Dodge, IA) and upon uterotomy; the ewes were euthanized by administration of sodium pentobarbital (Beuthanasia-D, 2 ml/ 10 pounds, Schering-Plough Animal Health Corp., Union, NJ). The ewes received either nicotine or supportive bandage alone. A total of twenty four lambs were obtained and assigned to experimental groups. Full-term control (FTC) and pre-term control (PTC) groups had 6 lambs each (n=6), full-term nicotine exposed group (FTN) had 5 lambs (n=5) and pre-term nicotine exposed group (PTN) had 7 lambs (n=7).

2.2. Nicotine administration protocol:

Approximately 20 cm$^2$ of the lateral neck region was shaved and cleaned. After the neck was dry, a 21 mg nicotine patch was placed on the skin and gentle pressure was applied over the patch for 5 minutes. The patch was secured by additional bandaging. Blood samples (10 ml) were drawn from the jugular vein 6, 12, and 24hr after patch placement and assessed for cotinine plasma levels (CPL) by HPLC analysis. The procedure was repeated on a daily basis during the last six weeks of pregnancy.

2.3. Tissue collection:

Lung tissue was collected within 24 hours of natural lambing or uterotomy. The samples from the most lateral portions of the right cranial lobes were collected into cryovials, snap frozen in liquid nitrogen, and stored at -80°C until protein and
RNA extraction for Western blot and qPCR analysis. The remaining tissue from the same lung lobes were fixed in 10% buffered formalin for immunohistochemistry (IHC).

2.4. qPCR analysis:

Methodology for qPCR analysis of SP-A and SP-D mRNA levels has been described previously in detail\textsuperscript{10,11}. Briefly, total RNA was isolated from the fresh, frozen lung tissue and assessed for quantity and purity by spectrophotometry. This procedure was followed by DNase treatment (TURBO DNA-free kit, Ambion, Austin, TX). Prior to qPCR analysis, a test plate was run to determine which RNA dilution ranges gave the best signal for SP-A and SP-D targets. The DNase treated RNA isolates were diluted 1:10 with nuclease-free water, then diluted to their ideal ranges on a per-sample, per-target basis, and further placed in equal amount, in duplicate into 96-well qPCR reaction plates (Applied Biosystems Incorporated, Forest City, CA). The plates were run in the GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA) for detection and relative quantification of SP-A and SP-D mRNA targets. Results were gathered by the GeneAmp 5700 SDS software and processed using custom Excel files. In addition, the real-time mRNA data was normalized to ovine ribosomal protein S15 mRNA.

2.5. IHC analysis:

Surfactant protein A immunohistochemical staining have been described previously in detail by our laboratory\textsuperscript{12}. Briefly, upon collection, the lung tissue was fixed in 10% buffered formalin for 24 hours. Prior to IHC run, 3 um thick tissue
sections were placed on glass slides. The glass slides were deparaffinized and rehydrated followed by antigen retrieval, a protein blocking procedure, SP-A specific primary antibody incubation (Mouse IgM anti-human SP-A, Chemicon International, Inc., Temecula, CA), secondary antibody application (biotinylated Rat anti-Mouse Isotype Rat (LOU) IgG2a, B.D. Pharmingen, San Diego, CA), streptavidin-conjugated horseradish peroxidase incubation (BioGenex, San Ramon, CA) and Nova Red substrate (Vector, Burlingame, CA) development. All slides were counter-stained with Shandon’s ¼-strength hematoxylin (Shandon-Lipshaw, Pittsburgh, PA), dehydrated through a series of graded solvents, and cover-slipped using Permount (Fisher, Hanover, IL) according to standard IHC procedure. For IHC scoring, five fields of each lung section were assessed for intensity and distribution of SP-A-stained epithelial cells in the terminal bronchioles and bronchiolar/alveolar junctions. Scoring was based on a predetermined scale; 0 = no staining of cells; 1 = <30% of epithelial cells stained with minimal detectable intracytoplasmic staining; 2 = 30-60% of epithelial cells stained with minimal detectable intracytoplasmic staining; 3 = 30-60% of epithelial cells stained with >50% of cytoplasm stained; 4 = >60% of epithelial cells stained with >50% of cytoplasm stained.

2.6. Western blot analysis:

Fresh, frozen lung tissues (0.1 gram) collected from the lateral portion of the right, cranial lung lobe were homogenized in 2 ml T-PER® Tissue Protein Extraction Reagent (Pierce, Rockford, IL) containing Complete TM Protease Inhibitor Cocktail (Santa Cruz Biotechnology, Santa Cruz, CA). The tissue homogenates were centrifuged at 13,000 x RPM for 5 minutes at 4°C and supernatant (protein extract)
was collected. The sodium dodecyl sulfate (SDS) sample buffer was added and samples were boiled for 5 minutes. The protein extracts were loaded onto polyacrylamide gel (PAG, 8-16%, Precise TM Protein gels, Thermo scientific, Rockford, IL) for protein separation and then transferred to polyvinylidene fluoride transfer membrane (PVDF transfer membrane, Millipore, Bedford, MA). The membrane was blocked with 5% nonfat dry milk in 1X TBS, after which the membrane was incubated overnight at 4°C with the primary polyclonal rabbit anti bovine SP-A antibodies (Chemicon International, Inc, Temecula, CA) or primary polyclonal rabbit anti human SP-D antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Both antibodies were diluted in 1XTBS, 0.05% Tween and 5% nonfat dry milk at 1:1000. After rinsing 3 times for 5 minutes with 1XTBS, 0.05% Tween the membranes were further incubated for 90 minutes at room temperature with secondary Alexa Fluor 488 goat anti rabbit normal IgG antibodies (Invitrogen, Carlsbad, CA) diluted in 1XTBS, 0.05% Tween at 1:2000. To demonstrate equal loading of samples, a duplicate membrane was probed with monoclonal mouse anti β-actin antibodies (Sigma, St. Louis, MO) diluted in 1XTBS, 0.05% Tween at 1:5000 and secondary antibody goat anti mouse Alexa fluor 488 at 1:2000. The fluorescence was detected by Typhoon imaging system (GE Healthcare, Baie d'Urfe, Quebec, Canada) using excitation wavelength 488 and 520 BP 40 emission filter.

2.7. Statistical analysis:

For qPCR and IHC data, summary statistics (mean and standard error of the mean) were calculated for each experimental group. Student’s-t tests (Graph Pad
Sigma software, San Diego, CA) were also performed on normalized qPCR and IHC data. The acceptable level of significance was p<0.05.

3. Results

3.1. Sheep cotinine plasma levels (CPL):

The cotinine in the maternal plasma was measured 6, 12, and 24 hr after patch placement. 6 hr after patch placement, CPL ranged from 30 to 79 ug/ml with a mean of 49.8+/−6.7 ug/ml. 12 hr after patch placement, CPL ranged from undetectable (in 1 ewe) to 73 ug/ml with a mean of 45.4+/−7.22 ug/ml. 24 hr after patch placement, CPL ranged from undetectable (in 1 ewe) to 38 ug/ml with a mean of 30.0+/−2.59 ug/ml (Table 1).

3.2. qPCR results:

Pregnant ewes exposed to nicotine during the last six weeks of gestation had significantly reduced (p=0.01) SP-A mRNA expression in lungs of lambs to PTC lambs not exposed to nicotine. Although maternal exposure to nicotine exhibited a tendency toward decreased SP-D mRNA expression in PTN lambs when compared to PTC lambs, this difference was not statistically significant (p=0.09). The results are presented in Figure 1. There were no significant changes in both SP-A and SP-D mRNA expression between FTC and FTN lambs.

3.3. IHC analysis results:

The SP-A antigen was detected within the cytoplasm of terminal bronchiolar and bronchiolar/alveolar junction epithelial cells in all four groups of lambs (Figure 2). This cellular localization of SP-A is similar to previous studies. The mean score
for each group was: PTC, 1.9 +/- 0.15; FTC, 2.88 +/-0.39; PTN, 2.09 +/-0.21 and FTN, 3.32 +/-0.15. There was no statistical significance between FTC and FTN groups (p=0.1) as well as PTC and PTN (p=0.08) groups.

3.4. Western blot results:

The Western blot analysis detected a single ~35 kD band that corresponds to the expected size of SP-A protein and ~43 kD band that corresponds to the expected size of SP-D protein. The SP-A and SP-D proteins were detected in all four groups of lambs; PTC and PTN, FTC and FTN lambs. The SP-A bands were significantly less detectable in the PTN group when compared to PTC group (Fig. 3). This indicates decreased SP-A protein level in nicotine exposed, pre-term lambs when compared to the control group. The SP-A bands were similar in size in FTN group when compared with FTC group (Fig. 3). There was no difference in size of SP-D bands in PTN group when compared to PTC group or FTN group when compared to FTC group (Fig. 4).

4. Discussion

We found that maternal nicotine exposure during pregnancy significantly reduces SP-A gene expression and protein level in pre-term born lambs, while it has no significant effect in naturally delivered, full-term lambs. It is likely that the additional gestational time allows more complete transcriptional activation or enhanced cellular differentiation and proliferation of SP-A producing cells in full-term lambs. Nicotine exposure did not alter SP-D gene expression and protein level in pre-term or full-term born lambs. The qPCR results show wide standard errors of
mean. This may be explained by the fact that sheep used in this experiment are outbred. Individual genetic variability in these animals tends to be wide in contrast to commonly used experimental animals such as mice and rats, which are inbred and have less genetic variation within the same strains. Although the wide errors of mean may question the validity of qPCR data, the protein levels determined by Western blot analysis closely parallel the qPCR findings which confirm biological significance of changes determined at mRNA levels. The IHC analysis did not show any statistically significant difference in SP-A protein level in PTN lambs when compared to PTC group; the significant difference was not present between FTN and FTC groups. This finding does not closely match the Western blot results in which the SP-A protein level was reduced in nicotine exposed lambs born prematurely compared to the controls. This may be explained by the fact that the IHC scoring system includes only proteins located within the intracellular compartment (intracytoplasmic SP-A) and does not take into account proteins located within the extracellular compartment (SP-A secreted into intra alveolar space). In contrast, Western blot analysis measures protein located in both intracellular and extracellular compartments and therefore represents a more sensitive and more reliable method of lung surfactant protein level determination.

Surfactant proteins are considered to be the key aspect of secretory lung innate immunity, and nicotine-induced suppressive effects may explain, in part, the increased susceptibility of pre-term born, nicotine-exposed neonates to lower respiratory infections. Although lung epithelia secrete many antimicrobial peptides, proteins and molecules, SP-A and SP-D are among the most extensively studied
pulmonary host defense/innate proteins. They are constitutively expressed and secreted by ATII cells and Clara cells and belong to a subgroup of collectin-containing mammalian C-type lectins, called *collectins*\(^\text{13}\), SP-A and SP-D can bind, aggregate, and opsonize many microorganisms including Gram-negative and Gram-positive bacteria, enveloped viruses like Influenza A Virus (IAV), RSV, non-enveloped viruses like Rotavirus, and fungal organisms in order to protect lung from potential infection\(^\text{13}\). In animal studies, the deficiency of SP-A, SP-D, or both were associated with increased susceptibility to pulmonary *Pseudomonas aeruginosa*\(^\text{14}\) Streptococcal infections\(^\text{15}\). Elevated levels of SP-A and SP-D mRNA in the lungs of neonatal lambs are associated with clearance of Parainfluenza virus-3 (PIV-3)\(^\text{13}\). Also, reduced clearance of RSV in lambs is associated with prematurity and inadequate expression of pulmonary *collectins*\(^\text{12}\), while SP-A has been shown to enhance RSV clearance in mice\(^\text{16}\). In addition, when SP-A and SP-D were administered to the lung in a murine model of invasive pulmonary aspergillosis, the mortality rate of infected animals was significantly reduced\(^\text{13}\). In rats, administration of SP-A reduces the oxidative damage of ventilated lungs\(^\text{17}\).

Published data on the influence of smoking and nicotine on ATII cells, lung surfactant, and surfactant proteins are conflicting. There are several reports clearly indicating that cigarette smoking or nicotine alone affects alveolar ATII cells and, therefore, possibly lung surfactant metabolism. In a study published by Maritz and Thomas, intrauterine exposure of rat pups to nicotine resulted in increased proliferation of ATII cells\(^\text{18}\). Nicotine-exposed ATII cells exhibited obvious mitochondrial swelling and damage which may have disrupted adequate cellular
metabolism, including metabolism of lung surfactant and surfactant proteins. In the most recent study published by Rehan et al., nicotine exposure accelerated ATII cell proliferation, differentiation, and metabolism in rat embryonic lung and stimulated synthesis of surfactant lipids\textsuperscript{19}. In both of these studies, however, the influence of nicotine on SP-A and SP-D was not investigated. A study conducted in healthy adult smokers showed reduced content of SP-A and SP-D in BAL fluid when compared to non-smokers\textsuperscript{20}. A similar study conducted in rats confirmed this finding since rats chronically exposed to cigarette smoke had significantly reduced SP-A and SP-C levels in BAL fluid\textsuperscript{21}. Several studies conducted in rats showed that maternal nicotine or cigarette smoke exposure alters the expression of surfactant proteins in neonatal pups. In one study, maternal nicotine exposure increased SP-A and SP-D expression in neonatal pups on postnatal day 7 and decreased SP-A and SP-D expression on postnatal day 14\textsuperscript{22}. Yet, in another study conducted in rats, maternal side-stream cigarette smoke exposure showed reduced levels of SP-A in BAL of pups on postnatal day 1 and increased levels of SP-A in BAL on postnatal day 21\textsuperscript{23}. There are reports, however, that suggest that maternal smoking does not influence lung surfactant proteins of exposed neonates. For example, neonates from mothers who smoked during pregnancy did not show reduced levels of SP-A according to amniotic fluid measurements\textsuperscript{24}. There is also a report indicating that smoking in healthy individuals increases levels of SP-A when measured in serum\textsuperscript{25}. In addition, there is scientific evidence that prenatal nicotine exposure has permanent detrimental effect on later postnatal lung development and function. It has been shown that in-utero exposure to nicotine influences the “programmed” lung aging
and maintenance thus accelerates aging and predisposes lung to parenchymal
damage\textsuperscript{18}.

In addition, there is controversy related to the safety of nicotine patch use
during pregnancy. Although the nicotine patch is highly recommended as nicotine
replacement therapy in pregnant women\textsuperscript{25} during the smoking cessation, to our
knowledge there are no studies conducted to evaluate the effect of nicotine patch on
developing fetus. In this study, we have shown that nicotine delivered to the mother
by nicotine patch does have an undesirable effect on fetal lung epithelial
development.

Differences of the above studies with the lamb model are likely related to
differences in animal lung maturation at the various stages of lung development,
duration of nicotine or cigarette smoke exposure, as well as in the techniques used
to determine surfactant protein levels. Although cigarette smoking during pregnancy
remains a significant cause of neonatal mortality and morbidity, previous studies
frequently utilized rodents as animal models which do not adequately parallel fetal
lung development in humans. This is especially relevant in alveolar and pulmonary
surfactant development research area. At birth, the lung of rodents is in saccular
stage and alveoli with lung surfactant develop during first three weeks of post natal
life\textsuperscript{26}. In contrast, gestating ewes and their offspring are well-suited for these studies
since: 1) alveolar development in lambs occurs prenatally as in humans\textsuperscript{26}; 2) lambs
can be derived pre-term and survive, unlike many other animal models\textsuperscript{12}; 3) ovine
respiratory epithelia of airways, distal bronchioles, and alveoli are similar to those of
the human lung including similar numbers of Clara cells (which can reach 50% of the
epithelium of mice), and the ovine lung has submucosal glands not present in some animal models\textsuperscript{10,27}; 4) expression of SP-A and SP-D and other innate immune genes of ovine lung are well-characterized and are very similar to human orthologs\textsuperscript{10}; and 5) pre-term lambs are susceptible to RSV, a known pathogen of premature infants, and maternal cigarette smoking is a risk for pre-term birth. Finally, sheep have long been used for respiratory studies related to human disease as well as for studying the effects of nicotine on pulmonary function.

Nicotine is the major cigarette constituent that crosses the placenta and accumulates in the fetal compartment to influence fetal lung growth and development\textsuperscript{9,28}. The transdermal patch as a nicotine delivery method was used for several reasons. First, when fitted to the prepared skin area, the patch slowly and constantly releases nicotine for at least a 12 hr time period, which closely resembles the smoking habit in humans. In contrast, other nicotine delivery techniques such as intravenous administration and nicotine releasing subcutaneous pumps are invasive and may result in excessive nicotine blood levels which are difficult to control. Second, according to several published studies, nicotine metabolism in pregnant women is significantly accelerated compared to non-pregnant females and males\textsuperscript{29}. Also, pregnant women tend to quit or at least significantly reduce the number of cigarettes smoked per day. According to a study published by Peacock et al., pregnant women who smoked during the last trimester of pregnancy most frequently had cotinine plasma levels ranging from 15-75 ng/ml\textsuperscript{30}. Similar levels were present in maternal blood in this study. In addition, depending on cigarette brand and strength, this plasma cotinine level would be equivalent to 2–6 cigarettes smoked per day.
Lastly, although administering nicotine during the entire length of gestation would more closely mimic human behavior, we have chosen to administer nicotine during the last six weeks of ovine gestation since this time frame corresponds to the time frame of pulmonary surfactant development in fetal lambs\textsuperscript{31}.

In conclusion, nicotine exposure during the last trimester of pregnancy in sheep is associated with a significant reduction in SP-A gene expression and protein level in lambs born pre-term. This finding may have clinical significance and will allow us to further investigate RSV infection as a means to address the hypothesis that nicotine exposure during pregnancy may increase RSV susceptibility in human infants. To our knowledge, there are no publications describing similar animal model in lung development research area. In this work we have described an innovative animal model that may be used by the broad research community focused on neonatal nicotine related pathological processes such as respiratory distress syndrome, impaired intra-uterine lung growth, and other pulmonary abnormalities during postnatal life.

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Table 1. The cotinine plasma levels (CPL) measured in the maternal plasma 6, 12 and 24 hr after the patch placement in 6 ewes. The CPL is expressed in ug/ml.
Figure 1. The relative SP-A mRNA and SP-D mRNA expression in PTC and FTC lambs as well as PTN and FTN lambs. The FTC and PTC values are equal to one. The PTN and FTN values are presented relative to the corresponding controls. The SP-A mRNA expression is significantly reduced (p=0.01) in PTN lambs (n=7 lambs) when compared to PTC lambs (n=6 lambs). There is no significant change in SP-A mRNA expression between FTN and FTC lambs. Although maternal nicotine administration had a tendency to decrease the SP-D mRNA expression in the PTN lambs (n=7 lambs) when compared to the PTC (n=6 lambs), this difference was not statistically significant (p=0.09). There is no significant change in SP-D mRNA expression between FTN and FTC lambs.
Figure 2. Representative immunohistochemical detection of SP-A protein in bronchial/bronchiolar epithelium of FTC-(A), FTN-(B), PTC-(C) and PTN-(D) lambs. Arrows depict epithelial cells with SP-A protein expression. There was no statistically significant difference in SP-A level between PTN and PTC group.
Figure 3. Representative Western blot detection of the SP-A and SP-D proteins in the lung tissue of the PTC and PTN lambs. The SP-A protein bands in PTN group are less detectable when compared to PTC group. There is no difference in SP-D protein bands between PTN and PTC group.
Figure 4. Representative Western blot detection of the SP-A and SP-D proteins in the lung tissue of the FTC and FTN lambs. There are no differences in SP-A and SP-D protein bands between FTN and FTC groups.
ABSTRACT

The effects of fetal exposure to ethanol on lungs remain under investigation. Previously, we demonstrated that lambs exposed to ethanol during gestation had impaired expression of pulmonary surfactant protein-A and respiratory cilia function, the two crucial pulmonary innate immune components. In this study, we investigated the effects of in utero exposure to ethanol on the maturation and immunity of the fetal lung. Pregnant ewes were surgically implanted with an abomasal cannula and administered 1 g ethanol/kg (n=8) or water (n=8) five times a week during the last trimester of pregnancy. Lambs were delivered prematurely via Cesarean section or naturally. Lungs from all animals were assessed for markers of maturation (HIF-1α, HIF-2α, HIF-3α, panVEGF, VEGFR-1, VEGFR-2, glycogen, collagen) and immunity (cytokines and chemokines). Pre-term animals exposed to ethanol had significantly reduced panVEGF mRNA (p=0.066) and protein levels, HIF-1α (p=0.055), HIF-2α (p=0.019), VEGFR-1 (p=0.088) and VEGFR-2 (p=0.067) mRNA levels, but no changes in HIF-3α mRNA. In contrast, such changes were not observed in full-term animals exposed to ethanol. Similarly, glycogen levels were significantly higher in
pre-term animals exposed to ethanol (p<0.01) but not in full-term animals. Pre-term animals exposed to ethanol had significantly reduced TNF-α (p=0.05), IL-10 (p=0.03), CCL5 (p=0.017) and MCP-1 (p=0.0004) mRNA. In full-term animals exposed to ethanol, the immune alterations were either sustained (TNF-α, p=0.009; IL-10, p=0.03) or returned to control levels (CCL5 and MCP-1). The ethanol-mediated alterations in fetal lung maturation and immunity may explain the increased incidence of respiratory infections in neonates exposed to ethanol in utero.

INTRODUCTION
Alcohol (ethanol) consumption during pregnancy has been associated with adverse health effects in the developing fetus including premature birth, low birth weight, multiple birth defects, and neurodevelopmental disorders, together named Fetal Alcohol Spectrum Disorder (FASD) (42). Along these well documented health consequences, there have been a few reports suggesting that fetal alcohol exposure has detrimental effects on the developing fetal lung, such as suppressed pulmonary innate immunity (52), and an increased incidence of neonatal upper respiratory infections (11). Despite the evidence that exposure to a low dose of alcohol may have harmful effects on the developing fetus, alcohol consumption remains a common behavior among pregnant women (46) with one out of 29 pregnant women consuming alcohol during pregnancy (14). Moreover, the well-described risks of alcohol use during pregnancy remain underestimated because many newborn infants exposed to varying levels of alcohol in utero do not display typical clinical
symptoms of FASD (37, 38) suggesting a need to widen the research area of alcoho--related fetal adverse health effects.

Although a few studies have looked at the effects of alcohol on lung growth and maturation (36, 52), more studies are needed to address the consequences of maternal alcohol use on both pre-natal and post-natal lung function, maturation, and immune status. Previously, we developed an ovine model of alcohol exposure \textit{in utero} by administering moderate levels of alcohol to pregnant ewes during the last trimester of pregnancy (36). In that study, the maternal blood alcohol levels achieved in pregnant sheep were similar to those of individuals who consume alcohol in moderation (6, 36). Exposure to alcohol had dramatic impact on the fetal lung, as indicated by the reduction in the expression of pulmonary surfactant-A (SP-A) mRNA in pre-term born lambs, the decreased protein expression of SP-A in full-term lambs, and the reduced ciliary beat frequency in full-term lambs. Recently, another group has used a similar ovine model to demonstrate additional alterations in fetal lung due to alcohol exposure \textit{in utero} (52). Although alcohol has deleterious effects on the fetal lung, the exact mechanisms by which alcohol alters pulmonary SP-A and other immune parameters have yet to be determined.

One mechanism by which alcohol can impede with lung development and function is through alterations in the expression of growth factors which are vital for lung development (15). Fetal lung development is a complex process which is tightly regulated, in part by hormones and growth factors (29) such as vascular endothelial growth factor (VEGF) (3). VEGF is crucial for pre-natal and post-natal lung development as it stimulates angiogenesis and vascular permeability (16, 17).
VEGFR blockade results in inhibition of angiogenesis and alveolar formation in the
developing rat lung (33), and VEGF gene deletion results in less complex alveolar
patterning (22). Furthermore, VEGF is essential in pulmonary epithelial cell
maturation and pulmonary surfactant production, as inhibition of VEGF in fetal mice
results in respiratory distress syndrome (RDS) due to decreased pulmonary
surfactant production (12).

VEGF belongs to a super-family composed of five structurally similar but
functionally different members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental
growth factor (PLGF) (58). The VEGF-A family is primarily involved in vascular
development in various organs including lung, and hereafter will be designated as
VEGF. VEGF is encoded by a single gene composed of 8 exons and 7 introns (58).
Due to alternative splicing, this single gene encodes for several isoforms which differ
in the number of amino acid residues present in the molecule. There are 7 human
isoforms which contain 121, 145, 148, 165, 183, 189, and 206 amino acid residues
(39); 4 analog murine isoforms which contain 120, 144, 164, and 188 amino acid
residues (16); and 3 analog ovine isoforms composed of 120, 164, and 187 amino
acid residues (10). The VEGF molecules are therefore named as VEGF.xxx in which
xxx subscript refers to the number of amino acid present in the molecule. There are
several studies which report that the most significant VEGF isoforms in the lung are
VEGF_{120} and VEGF_{164} (34). The term “pan VEGF” and “VEGF-A” are
interchangeably used since antibodies that can bind and detect multiple VEGF-A
isoforms are named as “pan-VEGF” antibodies (2).
The VEGF gene transcription is up regulated by 3 transcription factors: hypoxia inducible factor-1α, 2α, and 3α (HIF-1α, HIF-2α, HIF-3 α respectively) (17, 45). All of these transcription factors are expressed in the lung, and loss of HIF-1α (49) and HIF-2α (12) in mice is lethal due to impaired alveolar epithelial cell maturation and inadequate surfactant production. VEGF binds two related, tyrosine kinase receptors: VEGFR-1 (also known as fms-tyrosine kinase-1 receptor (Flt-1) in rodents) and VEGFR-2 (also known as kinase insert-domain-containing receptor in humans (KDR) or fetal liver kinase-1 receptor (Flk-1) in rodents). VEGFR-2 has been considered as a main VEGF signaling receptor (56). VEGFR-1 functions as a decoy receptor as it undergoes weak tyrosine autophosphorylation upon VEGF activation (17). Both of these receptors have been detected in the lung; VEGFR-2 is detected in the ATII cells and, when activated, enhances ATII cellular growth and surfactant synthesis (12).

There are very few studies that focus on the direct effect of alcohol on VEGF. It has been reported, however, that chronic alcohol exposure down regulates nitric oxide system and VEGF expression by blood vessel endothelial cells resulting in systemic hypertension in rats (31). In addition, acute alcohol intoxication has been associated with increased morbidity and mortality in traumatic injuries (5). Although the precise mechanism by which alcohol influences the post-traumatic outcomes is not known, there are some speculations that alcohol alters the VEGF signaling and slows down angiogenesis and, therefore, wound healing (44). To the best of our knowledge, this is the first study analyzing the effect of alcohol on VEGF in the developing fetal lung.
As VEGF and the factors HIF-1α, HIF-2α, and HIF-3α are known to have key roles in lung function and maturation, and as there is evidence of the deleterious effects of alcohol on lung function, development, and immunity, we tested four hypotheses: 1) Maternal alcohol ingestion during the third trimester of pregnancy will significantly alter lung maturation through reduced expression of HIF-1α, HIF-2α, HIF-3α and their downstream target genes VEGF, VEGFR-1 and VEGFR-2; 2) Maternal alcohol ingestion during the third trimester of pregnancy will significantly alter lung maturation by affecting lung glycogen and collagen contents; 3) Alcohol-related lung development alterations will be more severe in pre-term animals than full-term animals; and 4) Compared to control animals, those exposed to alcohol in utero will display dysregulated expression of genes involved in host defense and the inflammatory response.

**MATERIALS AND METHODS**

*Experimental procedure*

Animal use and experimental procedures were approved by Iowa State University’s Animal Care and Use committee according to the requirements of the NIH guide for the Care and Use of Laboratory Animals. Sixteen mixed (1/2 Rambouillet, 1/4 Dorset, and 1/4 Finsheep) date-mated, pregnant ewes were assigned in four groups: alcohol exposed pre-term and full-term groups and control pre-term and full-term groups. Alcohol was administered through surgically implanted abomasal cannula. This procedure was previously described in detail (36). Ewes assigned to alcohol groups received 40% ethanol at 1gr/kg, V/V (Sigma, St. Louis, MO). Control ewes
received the equivalent amount of water. The ewes received alcohol or water five
days a week for five weeks. Serum alcohol (ethanol) concentrations (SAC) were
determined at 6, 12 and 24 hours post a single infusion by headspace gas
chromatography analysis (University of Iowa Diagnostic Laboratories, Iowa City, IA).
The lambs in the pre-term group were delivered via Cesarean section at 138 days of
gestation (term is ~147 days). The lambs in the full-term group were allowed to
delivered naturally (~147 days); there were a total of twenty-four lambs, and we
assigned six lambs to each group (n=6). After lambing or uterotomy, all lambs were
euthanized by intravenous administration of sodium pentobarbital. The lung samples
from the lateral portions of the right cranial lobes were collected into cryovials, snap
frozen in liquid nitrogen and stored at -80°C until RNA extraction for qPCR analysis
or protein extraction for Western blot analysis. The remaining tissue from the same
lung lobes were fixed in 10% buffered formalin, processed, and embedded in
paraffin from which 3 micron thick sections were cut for periodic acid-Schiff (PAS) or
Gomori's trichrome special staining procedures.

Gene expression analysis
The fluorogenic real-time quantitative polymerase chain reaction analysis (qPCR)
was used to measure alcohol-mediated changes in gene expression of pulmonary
maturation markers; HIF-1α, HIF-2α, HIF-3α, panVEGF, VEGFR-1 and VEGFR-2,
and gene expression of major groups of immune genes in the lung including the
pro-inflammatory cytokine; tumor necrosis factor (TNF)-α, the anti-inflammatory
cytokine; interleukin (IL)-10, chemokine (C-C motif) ligand 5 (CCL5), and monocyte
chemotactic protein (MCP-1). This procedure has been previously described in detail
Briefly, total RNA was isolated from lung tissues sampled from lateral portions of the right cranial lobe, followed by DNase treatment (TURBO DNA-free kit, Ambion, Austin, TX). A test plate for all targets was run to determine the optimal sample dilution. The DNase treated RNA isolates were diluted 1:10 with nuclease-free water, then diluted to their ideal ranges (according to what was learned from the test plate analysis) and placed in duplicate into 96-well qPCR reaction plates (Applied Biosystems Incorporated, Forest City, CA). Each sample contained 7.8 μl of DNase-treated, appropriately-diluted RNA isolate, 15 μl of a commercially available Applied Biosystems One-Step Master Mix, 1000 nM primers, 150 nM TaqMan (5'-6FAM, 3'-TAMRA-quenched) probe and nuclease-free water. The negative, no-template control wells contained nuclease-free water (Ambion) instead of RNA isolate. Twenty-seven μl of each reaction was used in-well in the 96-well reaction plates. The plates were run in the GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). The results were analyzed by using the GeneAmp 5700 software and departmentally available Excel files.

**Western blot analysis**

The western blot analysis was used to measure panVEGF protein levels. The term “pan VEGF” and “VEGF-A” are interchangeably used since antibodies that can bind and detect multiple VEGF-A isoforms are named as “pan-VEGF” antibodies (2). The sections of the lateral portion of the right cranial lung lobes (0.1 g) were homogenized in 2 ml T-PER® Tissue Protein Extraction Reagent (Pierce, Rockford, IL) and Complete TM Protease Inhibitor Cocktail (Santa Cruz Biotechnology, Santa Cruz, CA). The tissue homogenates were centrifuged at 13,000 x RPM for 5 minutes.
at 4°C, protein extracts were boiled for 5 minutes with sodium dodecyl sulfate (SDS) sample buffer, loaded onto polyacrylamide gel (PAG, 8-16%, Precise TM Protein gels, Thermo scientific, Rockford, IL) and then transferred to polyvinylidene fluoride transfer membrane (PVDF transfer membrane, Millipore, Bedford, MA). The membranes were then blocked with 5% nonfat dry milk and incubated overnight at 4°C with the mouse monoclonal anti recombinant VEGF 189 protein antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The next day, the membranes were incubated for 90 minutes at room temperature with secondary Alexa Fluor 488 goat anti mouse normal IgG antibodies (Invitrogen, Carlsbad, CA). To demonstrate the equal loading of the protein samples, the proteins were stripped and the membranes were probed with monoclonal mouse anti β-actin antibodies (Sigma, St. Louis, MO) and secondary antibody goat anti mouse Alexa fluor 488. The fluorescence was detected by using Typhoon imaging system (GE Healthcare, Baie d’Urfe, Quebec, Canada) using excitation wavelength 488 and 520 BP 40 emission filter.

*Staining and microscopic scoring of cytoplasmic glycogen*

To determine the glycogen content in type II pneumocytes, the slides were subjected to the periodic acid-Schiff stain (PAS) procedure as follows: upon collection, the lung tissue was fixed in 10% buffered formalin for 24 hours and 3 μm-thick tissue sections were cut and placed on glass slides. The slides were then deparaffinized, rehydrated, and placed in 1% periodic acid for 10 minutes (Sigma, St. Louis, MO) followed by a thorough wash. After the wash, slides were placed in Sigma Schiff’s reagent (Rowley Biochemical Institute, Denver, MA) for 15 minutes followed by another wash after which they were counterstained with MCB reagent
(Manufacturing Chemists, Inc, Gibbstown, NJ), rinsed in distilled water, dehydrated and cover-slipped using Permount (Fisher, Hanover, IL). Ten alveoli were scored within one tissue section from each lamb. Alveoli were similar in size which ranged between 100–200 μm in diameter. Scoring was based on predetermined scale: 0, no detectable stain; 1, earliest detectable stain; 2, cytoplasmic glycogen granules present in less the 30% of alveolar epithelial cells; and 4, cytoplasmic glycogen granules present in more than 60% of alveolar epithelial cells.

**Staining and microscopic scoring of alveolar collagen**

To determine the content of collagen within the alveolar walls, the slides were subjected to Gomori’s trichrome stain procedure which stains for type I and type III collagen. The procedure was performed as follows: the lung tissue fixed in 10% buffered formalin for 24 hours was cut in 3 μm-thick sections and placed on glass slides. The slides were then deparaffinized, rehydrated, and placed in Picric Acid solution for 30 minutes followed by a thorough wash in running tap water for 10 minutes after which they were rinsed in distilled water and placed in Gomori’s trichrome stain for 15 minutes. After the staining, the slides were placed in 0.5% glacial acetic acid and then dehydrated and cover-slipped using Permount (Fisher, Hanover, IL). 10 alveoli from different fields (50 total) were counted to determine which had complete circumferential positive staining for collagen, which had staining of >50% of the circumference, and then which had >0 but <50% positive staining of the circumference. Staining around the vessels and airways was not taken into account.
Statistical analysis

The summary statistics (mean and standard error of the mean) was calculated for each experimental group. Student’s t-test was performed using Graph Pad Sigma software (San Diego, CA). One-way ANOVA was performed using SAS version 9.1 (SAS Institute, Cary, NC). Mean values of relative mRNA expression levels of genes from pre-term and full-term animals exposed to ethanol were compared to control animals of the same age group at \( p \leq 0.1 \), \( **p \leq 0.01 \), and \( ***p \leq 0.001 \) and expressed as means ± SEM.

RESULTS

Pulmonary HIF, panVEGF and VEGFR gene expression: Compared to control animals, the pre-term animals exposed to ethanol had significantly decreased expression of HIF-1\( \alpha \) \( (p=0.055) \), HIF-2\( \alpha \) \( (p=0.0187) \) (Fig.1), panVEGF \( (p=0.066) \) (Fig. 2), VEGFR-1 \( (p=0.088) \) and VEGFR-2 mRNA \( (p=0.067) \) (Fig.3); no significant changes were seen with HIF-3\( \alpha \). In full-term animals exposed to ethanol, no significant changes in the expression of HIF-1\( \alpha \), HIF-2\( \alpha \), panVEGF, VEGFR-1 and VEGFR-2 mRNA were seen when compared to animals of the same age group.

Pulmonary cytokines and chemokines gene expression: Pre-term animals exposed to ethanol had significantly reduced levels of TNF-\( \alpha \) mRNA \( (p = 0.05) \), IL-10 mRNA \( (p = 0.03) \), CCL5 mRNA \( (p = 0.017) \) and MCP-1 mRNA \( (p = 0.0004) \) when compared to control animals of the same age group (Fig.4). In full-term animals exposed to ethanol, a significant decrease in the expression of TNF-\( \alpha \) mRNA \( (0.009) \) and IL-10 mRNA \( (0.03) \) was observed. However, no significant changes in the expression of
CCL5 mRNA ($p = 0.12$) and MCP-1 mRNA ($p = 0.33$) occurred when compared to control full-term animals.

*Pulmonary panVEGF protein levels:* The western-blot analysis demonstrated significantly decreased levels of panVEGF protein in the lungs of pre-term animals exposed to ethanol when compared to control animals of the same age group (Fig. 5).

*Pulmonary glycogen content:* We found that pre-term animals expressed more glycogen in their lungs when compared to full-term animals (Fig. 6). In addition, the increased glycogen retention in the lungs of preterm animals was more pronounced and statistically significant when the animals were exposed to ethanol ($p < 0.01$) *in utero* during the last trimester of gestation. In newborn lambs, exposure to ethanol did not lead to statistically significant differences in glycogen levels when compared to control animals of the same age group.

*Pulmonary collagen content:* We found that *in utero* exposure to ethanol led to a statistically significant deposition of collagen in the lung of full-term animals ($p = 0.03$) (Fig. 7). Although some collagen deposition was also observed in the lungs of pre-term animals exposed to ethanol, the changes were not statistically significant.

**DISCUSSION**

Studies on the detrimental effects of maternal ethanol (alcohol) on the developing fetus are of utmost importance. The goals of this study were: 1) to determine a mechanism behind alcohol-mediated alterations in lung maturation, and 2) to investigate the effects of *in utero* alcohol exposure on fetal and neonatal pulmonary immunity.
Lung maturation

The current study demonstrated that a potential mechanism of alcohol (ethanol)-mediated alterations in lung maturation is through a decrease in the expression of panVEGF (includes all VEGF isoforms in the lung), its receptors (VEGFR-1, VEGFR-2), and its regulatory components (HIF-1α, and HIF-2α) and through an increase in glycogen retention in lungs. This study demonstrated that the effects of ethanol on the fetal lung are more pronounced in pre-term animals than animals that have reached full gestation (full-term animals). This may explain our previously reported decrease in SP-A mRNA expression in pre-term lambs exposed to alcohol in utero (36). VEGF mediates conversion of glycogen in type II pneumocytes (ATII) into surfactant proteins (12) and increased glycogen content in the lung of pre-term lambs exposed to alcohol most likely resulted from the suppressing effect of alcohol on panVEGF mRNA and protein. The most recent study done by Sozo et al. demonstrated similar effect of alcohol on the fetal lung. In that study, pre-term lambs chronically exposed to alcohol had SP-A and SP-B mRNA levels decreased to one third of control levels (52). The exact mechanism by which alcohol impairs lung maturation in neonates is not clear; however it has been demonstrated that alcohol impairs fetal growth and maturation in general by multiple mechanisms. First, alcohol reduces fetal oxygenation and nutrient transport by reducing blood supply to the fetus (51). Second, inter-uterine alcohol exposure alters growth factors such as growth hormone and insulin-like growth factors-binding proteins (15) both of which may impair lung development. Third, there is growing evidence that alcohol depletes protective, anti-oxidative enzymes in the fetal lung,
thus increasing oxidative stress and damage of the pulmonary epithelial cells, as seen in ATII cells (8). Together, these studies indicate that the alteration of fetal lung maturation by alcohol is most likely multi-factorial. More recent studies have pointed to the impairing properties of alcohol on the HIF-VEGF-VEGFR pathway in cell lines; on the nitric oxide system and the expression of VEGF by blood vessel endothelial cells (31); and on proliferative wound healing in mice (44). In this current study, we looked at additional factors behind the alcohol-mediated alterations on the fetal lung, namely the roles of VEGF, VEGFR-1, VEGFR-2, HIF-1α, HIF-2α, and HIF-3α on pre-natal and post-natal lung development and maturation in consequence to maternal alcohol use. Our finding that fetal exposure to alcohol during the last trimester of gestation significantly reduced the expression of factors including HIF-1α and VEGFR-2 in pre-term lambs is in agreement with those of Radek et al., who demonstrated that acute exposure of endothelial cell cultures to alcohol decreased HIF-1α protein level as well VEGFR-2 (43). A study conducted on chick embryos demonstrated a dose-dependent decrease of VEGF, Flt-1 and Flk-1 mRNA expression following alcohol exposure in area vasculosa, suggesting a mechanism by which alcohol impairs embryonal development (55). However, other studies have shown the opposite effects of alcohol on VEGF expression. In models of pathological conditions such as chick embryo chorioallantoic membrane that bore human fibrosarcoma, alcohol exposure increased VEGF expression and thus stimulated tumor angiogenesis, growth and metastasis (26). In mice chronically exposed to alcohol in utero and post-natally up to weaning, alcohol increased VEGF protein levels in the liver and had a tendency to decrease VEGF protein levels in
hippocampus (18). In another study done on mice implanted with B16F10 cells (melanoma cells), chronic alcohol exposure resulted in significant increases in the expression of VEGF mRNA and protein as well as Flt-1 protein level in melanoma cells, suggesting progression of melanoma angiogenesis and growth (54). The observed differences could be attributed to the fact that in these studies different animal models were used. Furthermore, VEGF was often evaluated in neoplastic cells in which the gene expression does not parallel the gene expression of physiologically normal cells.

The well documented role of VEGF during fetal lung development involves stimulation of endothelial cell proliferation and suppression of endothelial cell apoptosis and therefore stimulation of the blood vessel branching and growth (58). However, there are studies suggesting that in addition to blood vessel growth, VEGF coordinates the development of other pulmonary tissues and cells such as airways and airway epithelial cells; particularly ATII. The major source of VEGF in the lung is ATII cells (1) and in the adult human lung, VEGF levels are higher in the bronchoalveolar lavage fluid than in the blood (35). These data suggest that ATII cells auto-regulate their function by releasing VEGF into the alveolar lumen. Incubation of fetal human lung explants with VEGF\textsubscript{165} resulted in increased size and epithelialization of the distal airway ducts (7). The same treatment increased ATII cell differentiation, SP-A and SP-C mRNA levels and SP-A protein level in ATII cells. The exact mechanism by which VEGF exerts its effect on ATII cells is not known; however, in one study, isolated fetal ATII cells incubated with recombinant VEGF\textsubscript{165} failed to increase transcript levels of SP-A and SP-C which suggested an indirect
effect of VEGF exerted by reciprocal paracrine interactions of other lung cell types (47). Furthermore, inhibition of VEGF resulted in impaired lung maturation and fatal respiratory distress syndrome (RDS) in mice (12). The same study suggested that VEGF stimulates conversion of glycogen stores in ATII cells into surfactant proteins and treatment of prematurely born mice with VEGF prevented respiratory distress and death. These results are in accordance with our finding that the lungs of pre-term lambs contained more abundant glycogen stores within type II alveolar cells, indicating suppressed conversion of glycogen to surfactant proteins due to reduced maturation/differentiation. In addition to RDS, decreased expression of VEGF is associated with bronchopulmonary dysplasia (PBD) in human infants (3).

The main regulators of VEGF gene expression in the lung are hypoxia-inducible transcription factors; HIF-1α, HIF-2α and HIF-3α (17, 45). In response to hypoxic conditions such as intra-uterine environment during gestation, HIF binds hypoxia response element on the VEGF promoter and up-regulate or down-regulate the VEGF gene transcription. HIF-1α and HIF-2α stimulate expression of VEGF and blood vessel formation; in contrast the splice variant of HIF-3α gene known as Inhibitory Per/Arnt/Sim (IPAS) has no endogenous transactivation function and has been reported to be a negative regulator of VEGF (45). The data on HIF-3α are limited; however, in contrast, there are several reports indicating that HIF-1α and HIF-2α are crucial for normal intrauterine lung development. For example, HIF-1α−/− mice embryos die in utero due to insufficient blood vessel formation (32). In another study done on mice, deletion of HIF-1α gene 8 days prior to parturition resulted in 100% mortality rate of pups. Deletion of the same gene 4 to 6 days prior parturition
resulted in 15% survival rate; however, these pups died within hours of parturition exhibiting decreased expression of VEGF, HIF-2α and absence of pulmonary surfactant proteins (49). HIF-2α knock-out mice can survive to term gestation but succumb to severe respiratory distress due to inhibition of VEGF and consequently inadequate pulmonary surfactant production (12). Furthermore, study done on lambs reported that premature birth and RDS is associated with decreased expression of both, HIF-1α and HIF-2α in the lung (25). Although the biological roles of HIF-1α and HIF-2α in the lung development are compatible, there are some indications that HIF-2α may be more significant in VEGF regulation. For example, in the fetal human lung, HIF-1α protein is expressed in the airway epithelium throughout the gestation (45). In contrast, HIF-2α is primarily detected in the pulmonary interstitium during early gestational stage while during the later gestational stages; its expression was detected in interstitium and pulmonary epithelium, primarily ATII cells. While expression of HIF-1α remained constant regardless of gestational age, HIF-2α mRNA expression increased with gestational age and showed positive correlation with VEGF mRNA expression.

VEGF binds 2 tyrosine kinase receptors, VEGFR-1 and VEGFR-2. Upon activation, VEGFR-2 on endothelial cells mediates endothelial cell survival, proliferation, nitric oxide synthesis and increases vascular permeability (56). In contrast, activation of VEGFR-1 by VEGF ligand triggers weak phosphorylation of the receptor and weak activation of downstream signaling (56). Furthermore, targeted mutation of tyrosine kinase domain of VEGFR-1 in mice did not result in any over defects during embryonal development (30) whereas targeted deletion of
VEGFR-2 gene results in embryonal death due to inadequate vasculogenesis and angiogenesis (50). Therefore VEGFR-2 is considered to be the positive regulator of VEGF biological activity whereas VEGFR-1 is considered as “decoy” receptor which prevents VEGF from binding to VEGFR-2. Hypoxia up-regulates expression of both VEGFR-1 and VEGFR-2 genes. It is reported that up regulation of VEGFR-1 gene is mediated by HIF-1α in human umbilical vein endothelial cells when exposed to hypoxic conditions (21); TNF-α regulates expression of VEGFR-2 gene in human endothelial cells in hypoxic conditions (41). Both receptor genes are expressed in the lung, although in areas along endothelial cells, VEGFR-2 gene is expressed on the surface of ATII cells and, when VEGFR-2 is activated, it enhances ATII cellular growth and surfactant synthesis (12). In our studies, we suspect that fetal alcohol exposure may have a direct down-regulating effect on the expression (transcription or protein stabilization) of HIF-1α and HIF-2α, which consequently leads to the down regulation of VEGF and VEGFR expression. Another possible mechanism involves increased oxidative stress by alcohol in the fetal lung which accelerates apoptosis, especially in the ATII (7) cells which are the main source of VEGF protein in the lung. Additional studies are required to further elucidate the underlying mechanistic basis for these findings.

Pulmonary parenchymal elastic tissue which includes collagen supports lung function during inspiration and expiration. The amount of the deposited collagen fibers represents a crucial structural component of the lung since decreased collagen content results in pulmonary emphysema (57) while increased collagen content results in restricted pulmonary function described in pathological conditions.
such as bronchopulmonary dysplasia (BPD) and asthma (9). Chronic alcohol consumption in adults is associated with increased incidence of lung injury in patients with sepsis or trauma (27) which has been attributed in part to the increased expression of profibrotic growth factors in the lung (28). So far, there has been only one report describing the effect of alcohol exposure on collagen deposition in the developing fetal lung (52). Sozo et al reported an increased collagen deposition in lungs of pre-term lambs exposed to alcohol in utero (52). Although we could not find statistically significant differences in the lung collagen content of the pre-term lambs exposed to alcohol, those of full-term animals exhibited a significant increase in the deposition of collagen. The difference in collagen content in pre-term animals in our study and that of Sozo et al may be attributed to experimental procedures: duration of alcohol administration to the pregnant ewes was longer and alcohol was infused directly into the maternal systemic circulation reaching higher maternal blood concentration. Whether the increased collagen deposition in animals that have reached full gestational age can detrimentally affect lung function and whether such defects will persist with age warrants further investigations.

Lung immunity

This study also demonstrated that: 1) alcohol readily passes through the placenta of the ewe and is able to affect the cytokine and chemokine system of the fetal lamb, 2) the immune system of the fetal lamb is responsive at the time of birth, 3) alcohol significantly altered the expression of genes that are crucial to the host defense, and 4) the dysregulation of immune genes occurred in both, pre-term and full-term animals. Recent research has shown that exposure to alcohol can impair
the development and function of immune cells, including reduced T cell development in the thymus (24, 48), reduced B cell development in the liver (4, 40) and impaired macrophage function (19). Alcohol exposure has other suppressive effects on the immune response, such as decreased surfactant synthesis and pro-inflammatory cytokines (13, 23) and impaired response to a bacterial challenge (20). Our findings that maternal alcohol alters the expression of genes that are crucial to host defense and the regulation of the inflammatory response in pre-term animals are in agreement with those of Sozo et al. (52) who looked at pro-inflammatory cytokines. However, we extended our studies by analyzing the effects of ethanol on lung immunity in lambs that have reached full-term gestation and by comparing their immune response to that of pre-term animals. In pre-term animals exposed to ethanol, some genes displayed significant alterations in expression that either persisted to full-term gestation (as seen with TNF-α and IL-10) or were restored to levels similar to those of control animals at full-term gestation (as seen with CCL5 and MCP-1). As TNF-α and IL-10 are cytokines produced by many cells, including antigen presenting cells (APC), the sustained decrease in expression after in utero ethanol exposure suggests that ethanol can interfere with the function of APC. Our findings that ethanol also decreased the expression of the C-C chemokines CCL5 and MCP-1 are supported by other studies that showed in vitro suppressive effects of ethanol on chemokine production (53). The data presented here clearly suggest that ethanol is an immunomodulatory agent. Further investigations will be required to determine how long the observed alterations in lung immunity will persist in the post-natal period, the mechanisms underlying gene alteration and whether the
effects of alcohol on the immune system contribute to the increase in post-natal susceptibility to respiratory infections.

Conclusions

In conclusion, our study demonstrates a novel mechanism by which alcohol impairs fetal lung maturation and which may explain the previously reported decrease in SP-A expression by prematurely born lambs. Our study also demonstrated that, in addition to effects on developing airways, maternal alcohol has significant immunological effects on fetal lambs, and that some of the alcohol-mediated alterations persist until full-term gestation. To the best of our knowledge, this is the first study to report such findings. Considering the importance of VEGF in fetal lung maturation, these results may open a new venue for therapeutic targeting VEGF pathways in pediatric patients suffering from deleterious effects of in utero alcohol exposure.

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Figure 1. Relative mRNA expression of hypoxia inducible factor-1α, 2α and 3α (HIF-1α, HIF-2α and HIF-3α respectively) in the neonatal ovine lung. Exposure to ethanol in utero reduces mRNA expression of HIF-1α and HIF-2α in the lungs of pre-term (PT) lambs when compared to the control lambs of the same age group. Such changes are not observed in the lungs of full-term (FT) lambs exposed to ethanol in utero. No significant changes are seen with HIF-3α mRNA expression levels in both, PT and FT lambs. (*p<0.1, significance was established at 90% confidence interval).
Figure 2. Relative mRNA expression of vascular endothelial growth factor (panVEGF) in the neonatal ovine lung. Exposure to ethanol \textit{in utero} reduces mRNA expression of panVEGF in the lungs of pre-term (PT) lambs when compared to the control lambs of the same age group. Such changes are not observed in the lungs of full-term (FT) lambs exposed to ethanol \textit{in utero}. (*p<0.1, significance was established at 90% confidence interval).
Figure 3. Relative mRNA expression of vascular endothelial growth factor receptor 1 and 2 (VEGFR-1 and VEGFR-2 respectively) in the neonatal ovine lung. Exposure to ethanol *in utero* reduces mRNA expression of VEGFR-1 and VEGFR-2 in the lungs of pre-term (PT) lambs when compared to the control lambs of the same age group. Such changes are not observed in the lungs of full-term (FT) lambs exposed to ethanol *in utero*. (*p<0.1, significance was established at 90% confidence interval*).
Figure 4. Relative mRNA expression of chemokines and cytokines, in the neonatal ovine lung. Exposure to ethanol in utero reduces mRNA expression of; tumor necrosis factor (TNF-α; (A), interleukin (IL)-10; (B), chemokine (C-C motif) ligand 5 (CCL5; E) and monocyte chemotactic protein (MCP-1; D) in the lungs of pre-term (PT) lambs when compared to the control lambs of the same age group. In the lungs of full term (FT) lambs, ethanol exposure in utero reduces mRNA expression of; TNF-α; (A) and IL-10; (B). (*p<0.1, significance was established at 90% confidence interval).
Figure 5. Vascular endothelial growth factor (panVEGF) protein levels in the lung of pre-term lambs. Exposure to ethanol \textit{in utero} reduces panVEGF protein level in the lungs of pre-term lambs when compared to the control lambs of the same age group.
Figure 6. Periodic acid-Schiff (PAS) stain for glycogen granules in the type II pneumocytes (ATII). Glycogen granules in the ATII are more abundant in the pre-term lambs exposed to ethanol \textit{in utero} (B) when compared to the to the control lambs of the same age group (A). Glycogen content in the ATII is similar in the full-term lambs exposed to ethanol \textit{in utero} (D) and the control lambs of the same age group (C).
Figure 7. Gomori’s trichrome stain for collagen deposition in the alveolar walls.

Collagen fibers are more abundant in the alveolar walls of full-term lambs exposed to ethanol *in utero* (D) when compared to the control lambs of the same age group (C). Collagen deposition is similar in the pre-term lambs exposed to ethanol *in utero* (B) and the control lambs of the same age group (A).
CHAPTER 5. GENERAL CONCLUSIONS

Experiments performed in our study hopefully reached several important milestones, which should have a significant impact on the field of neonatal immunology:

1. We characterized a new animal model for in-utero exposure to alcohol and determined the effects of in-utero exposure to alcohol during the last trimester of gestation on:
   a. fetal tracheal ciliary beat frequency (CBF), and
   b. expression of SP-A and SP-D by the fetal lung epithelia.
2. We characterized a new animal model for in-utero exposure to nicotine and determined the effect of in-utero exposure to nicotine during the last trimester of gestation on the expression of SP-A and SP-D by the fetal lung epithelia.
3. We determined the possible mechanism by which in-utero exposure to alcohol alters the expression of SP-A in the fetal lung.

Characterization of the New Animal Model of Fetal Alcohol Exposure

The sheep represents a commonly utilized animal model in research area related to the effects of alcohol on the developing fetal lung\textsuperscript{1,2}. This is due to the fact that in-utero development of the sheep lung closely parallels the development of the lung in humans\textsuperscript{3}. Administration of alcohol directly into the systemic circulation, however, significantly alters the physiological metabolism of alcohol\textsuperscript{4}. Our study demonstrated that administration of alcohol via abomasal cannula to the pregnant sheep allows alcohol to bypass the rumen and avoid degradation by ruminal
microflora. We managed to achieve very controlled, but chronic, fetal exposure to alcohol in our sheep model, and realistically mimicked clinical situation frequently observed in human subjects. Since pregnant women have a tendency to reduce alcohol consumption and drink in moderation, by administering alcohol at 1gr/kg in the sheep model, the achieved blood alcohol concentration (BAC) was very similar to the BAC of humans with moderate alcohol consumption habits\textsuperscript{5}.

**In-utero Exposure to Moderate Levels of Alcohol Alters Fetal Tracheal CBF and Expression of SP-A and SP-D by Fetal Lung Epithelia**

Recent studies have indicated that fetal exposure to alcohol alters the immune system in the fetal lung and predisposes infants to infections and sepsis\textsuperscript{6,7}. Mucociliary apparatus in the airways and pulmonary SP-A and SP-D represent the first line defense mechanisms against inspired microorganisms\textsuperscript{8,9}. In this study, we demonstrated that *in-utero* exposure to moderate levels of alcohol during the last trimester of pregnancy significantly reduces the tracheal CBF and SP-A gene expression and protein levels in the lambs born pre-maturely. The SP-D gene expression remained unchanged in both pre-term and full-term lambs. This finding in part can explain the increased susceptibility to respiratory infections in neonates exposed to alcohol *in-utero*. Furthermore, our results created a strong basis for enhancing recommendations for absolute abstinence from alcohol consumption in any stage of pregnancy.
**Characterization of the New Animal Model of Fetal Nicotine Exposure**

Cigarette smoke contains thousands of chemicals; different studies have demonstrated, however, that immunomodulatory effects of nicotine parallel immunomodulatory effects of the whole cigarette smoke\textsuperscript{10}. The sheep is a commonly used animal model in research related to the \textit{in-utero} nicotine effects on the developing lung\textsuperscript{11,12}. Previously described nicotine administration techniques, however, are invasive and potentially may result in excessive nicotine levels in the maternal blood\textsuperscript{11}. In this study, we have developed a new technique of nicotine administration by simply applying a nicotine patch on the clipped skin of pregnant ewes. By doing so, we have achieved cotinine levels in the maternal blood which are similar to the cotinine levels of humans who smoke five to six cigarettes per day\textsuperscript{13}. This technique is non-invasive, inexpensive, and avoids the risk of excessive administration of nicotine to the pregnant animals.

**In-utero Exposure to Low Levels of Nicotine Alters the Expression of SP-A and SP-D by Fetal Lung Epithelia**

Smoking during pregnancy has detrimental health effects in the developing fetus\textsuperscript{14}. Neonates exposed to cigarette smoke during fetal development have impaired lung function and increased risk of respiratory infections, especially RSV infection\textsuperscript{14,15}. In this study, we demonstrated that \textit{in-utero} exposure to low nicotine levels during the last trimester of pregnancy significantly reduces SP-A gene and protein expression in prematurely born lambs. The SP-D gene and protein expression was not affected by nicotine in both pre-term and full-term lambs. Similarly to our previous observation in alcohol exposed neonates, the results of this
study may provide a strong evidence against chronic use of nicotine in any form during pregnancy (cigarette, nicotine replacement therapy, etc.). Considering that nicotine addiction is frequently difficult to control, however, results of our study may offer potential guidelines for closer monitoring of neonates born from individuals who consumed nicotine in any form during pregnancy. Considering that alcohol and nicotine exposure seem to have a significantly negative effect on innate immunity protein (SP-A), possible therapeutic strategies targeting regulation and production of this protein may have a unique value for effective medical treatment of these conditions.

**In-utero Exposure to Moderate Levels of Alcohol Reduces VEGF Gene Expression and Protein Levels in the Fetal Lung**

In the developing lung, VEGF plays a crucial role in vasculogenesis and angiogenesis. Recent studies, however, indicate that in addition to blood vessel formation, VEGF participates in alveolar type II cell maturation and lung surfactant production. In this study, we demonstrated that *in-utero* exposure to moderate levels of alcohol during the last trimester of pregnancy significantly reduces VEGF gene and protein expression in prematurely born lambs, while VEGF gene expression and protein levels were not affected by alcohol in the lambs that have reached full gestation. Considering that VEGF is a potent regulator of the lung maturation and innate immunity protein production, it is likely that decreased VEGF expression may be the first step in the cascade responsible for the abnormal neonatal lung function and immunity. Considering that during the last decade, VEGF has become a frequently studied and utilized pharmacological target in many
systemic diseases (neoplasia, macular degeneration), it is likely that utilization of this vast biological and pharmacological knowledge of VEGF biology can be effectively used for development of new therapeutic modalities for neonatal lung diseases.

**Future Research Goals**

Our data are strongly suggestive of deficient function of innate immunity components in fetal lungs exposed to chronic alcohol, which may open novel therapeutic opportunities for prematurely born neonates with respiratory infections. Pharmacological targeting of the ciliary motility and pulmonary surfactant proteins can be a reasonable strategy for early medical interventions in this particular patient population, which could be possibly effectively tested in our animal model in the future. We have identified decreased VEGF expression associated with chronic alcohol exposure, and manipulation of the VEGF pathway may prove to be a potentially successful therapeutic strategy for treatment of respiratory abnormalities in prematurely born neonates. Since we successfully demonstrated in the past the capacity of the human RSV to infect ovine lungs, the future experiments may provide more detailed knowledge about interactions between common infectious agents and different components of the neonatal lung immunity affected by chronic alcohol and nicotine administration. Considering that size and developmental biology of the sheep lung is almost identical to human lungs, it is likely that any effective therapeutic modality in the sheep model may become an effective translational step toward successful treatment of human patients.
References


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