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***In utero* ethanol exposure impairs defenses against experimental *Group B Streptococcus* in the term guinea pig lung**

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Summary

Background—The effects of fetal alcohol exposure on the risks of neonatal lung injury and infection remain under investigation. The resident alveolar macrophage (AM) is the first line of immune defense against pulmonary infections. *In utero* ethanol (ETOH) exposure deranges the function of both premature and term guinea pig AM. We hypothesized that fetal ETOH exposure would increase the risk of pulmonary infection *in vivo*.

Methods—We developed a novel *in vivo* model of *group B streptococcus* (*GBS*) pneumonia using our established guinea pig model of fetal ETOH exposure. Timed-pregnant guinea pigs were pair fed $\pm$ ETOH and some were supplemented with the glutathione (GSH) precursor S-adenosyl-methionine (SAM-e). Term pups were given *GBS* intratracheally while some were pre-treated with inhaled GSH prior to the experimental *GBS*. Neonatal Lung and whole blood were evaluated for *GBS* while isolated AM were evaluated using fluorescent microscopy for *GBS* phagocytosis.

Results—ETOH-exposed pups demonstrated increased lung infection and sepsis while AM phagocytosis of *GBS* was deficient compared to control. When SAM-e was added to the maternal diet containing ETOH, neonatal lung and systemic infection from *GBS* was attenuated and AM phagocytosis was improved. Inhaled GSH therapy prior to *GBS* similarly protected the ETOH-exposed pup from lung and systemic infection.

Conclusions—*In utero* ETOH exposure impaired the neonatal lung's defense against experimental *GBS*, while maintaining GSH availability protected the ETOH-exposed lung. This study suggested that fetal alcohol exposure deranges the neonatal lung's defense against bacterial infection, and support further investigations into the potential therapeutic role for exogenous GSH to augment neonatal AM function.

Keywords

fetal alcohol; *GBS* pneumonia; newborn; alveolar macrophage

Introduction

The use and abuse of alcohol prior to and during pregnancy continues to be a significant problem in our society despite education regarding its dangers, resulting in a significant proportion of both premature and term newborns exposed to alcohol *in utero* (Albertsen et al., 2004; Drews et al., 2003; Ebrahim and Gfroerer, 2003; Lester et al., 2001). Alcohol has recently been associated with an increased risk of extreme premature delivery at an Odds

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Ratio of ~35 (Sokol et al., 2007). However, the majority of alcohol-exposed newborns do not have the recognized phenotypic changes of fetal alcohol syndrome. This results in a clinical under-identification of the alcohol-exposed newborn (Little et al., 1990; Mattson et al., 1997) and an under-estimation of the adverse outcomes in the newborn infant.

We continue to investigate the effects of fetal alcohol exposure on the newborn, with particular interest in the immune function of the developing lung. Chronic exposure to alcohol is well described to increase the risk of pneumonia in adults (Baker and Jerrells, 1993; Szabo, 1999). As a professional phagocyte, the resident immune cell in the lung, the alveolar macrophage (AM), defends the lung by initiating an immune response, participating in the phagocytosis and clearance of infectious particles, and regulating subsequent inflammatory processes within the lung (Fels and Cohn, 1986; Standiford et al., 1995). The adverse effects of chronic alcohol exposure on the adult AM and the risks of pulmonary morbidities continue to be elucidated (Brown et al., 2006; Brown et al., 2007; Joshi et al., 2006; Joshi et al., 2005; Nelson and Kolls, 2002).

However, almost nothing is known about the effects of fetal alcohol exposure on the risks of lung injury and sepsis in the newborn infant. Limited clinical evidence suggests that fetal alcohol exposure increases the risk of neonatal infection in premature (Gauthier et al., 2004) and term newborns (Gauthier et al., 2005a). In previous investigations using a guinea pig model of fetal ethanol exposure, we have demonstrated that, similar to the adult AM exposed to chronic ethanol (ETOH), the neonatal AM exposed to ETOH *in utero* exhibited significant dysfunction at both premature (Gauthier et al., 2005b) and term gestations (Ping et al., 2007). This AM dysfunction was modulated, in part by the availability of the antioxidant glutathione (GSH). Because of this demonstrated severe ETOH-induced dysfunction in the neonatal AM, we *hypothesized* that fetal ETOH exposure would increase the risk of infection in the ETOH-exposed term guinea pig lung *in vivo*. The *purpose* of this study was to examine the effects of chronic *in utero* ETOH exposure on the risk of infection *in vivo* due to experimental group *B streptococcus* pneumonia. Furthermore, we evaluated whether potential therapeutic interventions, including maternal supplementation with S-adenosyl-methione (SAM-e) during ETOH exposure or inhaled GSH (iGSH) post natally to the ETOH-exposed pup would protect against experimental *GBS*.

Materials and Methods

Guinea pig model of fetal ethanol exposure

Our model of fetal ETOH exposure is based on models of ETOH-induced injury in the timed pregnant guinea pig (Abdollah and Brien, 1995; Kimura and Brien, 1998; Kimura et al., 1996) with modifications as previously reported by our laboratory (Gauthier et al., 2005b; Ping et al., 2007). Timed-pregnant pathogen-free guinea pigs (Elm Hill) were shipped on ~day 32 gestation (d32, term ~71 days) and randomly assigned on ~d35 to $\pm$ ETOH in the drinking water with incremental increases up to 4% ETOH (Abdollah and Brien, 1995; Kimura and Brien, 1998; Kimura et al., 1996) (25% calories + 8mg/ 100 ml saccharin) by ~d40 gestation. This experimental mixture was the only access to drinking water. Solid diet was provided *ad libitum* to the ETOH group, while the other groups were pair fed a solid diet to match the ETOH dam. In some experiments, the GSH precursor S-adenosyl-methionine (SAM-e) (10mM Sigma, St. Louis, MO) was added to the drinking water containing the ETOH. The dam's pregnancy was allowed to proceed until spontaneous delivery at term gestation. In our model, the blood alcohol concentration is approximately $0.05 \pm 0.01\%$ in both the ETOH or the ETOH + SAM-e dam and in her pups (Gauthier et al., 2005b). All animals were used in accordance with the NIH Guidelines (Guide for the Care and Use of Laboratory Animals) with protocols reviewed and approved by the Emory University Institutional Animal Care Committee.

Bacterial Culture

Serotype III *group B streptococcus* (*GBS*, ATCC # BAA-22) was aliquoted and stored at -70°C until experimental use. Prior to each experiment, bacteria were grown at 37°C and 5% CO_2 for 24 h in Todd-Hewitt broth and serially washed by centrifugation in 0.9% sterile normal saline at 8500 rpm for 15 min. *GBS* was suspended in normal saline at concentrations of 1×10^7 colony forming units (CFU)/cc (Herting et al., 1998; Juul et al., 1996). The final CFU concentration of each suspension was confirmed using serial dilutions.

In vivo model of Group B *Streptococcus* (*GBS*) Pneumonia

Based on models of *GBS* pneumonia from premature and term rabbits, (Hall and Sherman, 1992; Herting et al., 1999) timed-pregnant guinea pigs were similarly exposed to the experimental diet as described above. The pregnancy was allowed to proceed until spontaneous delivery at term gestation. The pup was sedated with ketamine (20–40 mg/kg) and xylazine (2 mg/kg) intra peritoneally and the trachea surgically identified. Using a tuberculin syringe, the trachea was instilled with 1×10^7 CFU/kg body weight *GBS* in 0.5cc volume within 72 h after delivery (Herting et al., 1998; Juul et al., 1996). The neck incision was closed with a surgical staple and the pups were allowed to recover from anesthesia for four hours.

In vivo inhaled Glutathione (iGSH)

We hypothesized that inhaled GSH would benefit the ETOH-exposed pup since the ETOH diminished GSH availability in the epithelial lining fluid of the lung, and impaired AM function in the GP model (Gauthier et al., 2005b; Ping et al., 2007). After term delivery, a subset of Control pups and ETOH-exposed pups were given intranasal GSH. Capitalizing on the natural sniffing reflex, iGSH (500 μM diluted in normal saline) was instilled in 2 divided doses (50 μl each) into each nares of the unanaesthetized pup (Ping et al., 2007) 6 hrs prior to administration of *GBS* as described above.

Alveolar Macrophage Isolation

Four hrs after intra tracheal *GBS* instillation, the pups were anesthetized with sodium pentobarbital (40 mg/kg) intra peritoneally and the trachea was re-cannulated for bronchoalveolar lavage and collection of AM. The fetal lungs were lavaged with 1.5 cc of sterile phosphate buffered saline (37°C , pH 7.4). The sample was collected and centrifuged at 1200 rpm for 8 min and the cell pellet resuspended in Dulbecco's modified Eagle's medium (DMEM- F-12) media with 2% Fetal Bovine Serum. The supernatant epithelial lining fluid (ELF) was saved for culture (see below). Cells in the lavage fluid were evaluated by Diffquik (Dade Behring, Newark, DE) and cell viability assessed with the calcein/ethidium iodide "live-dead" stain.

Lung Histology

The right upper lung of the term guinea pig lung was isolated and perfusion fixed in 4% paraformaldehyde and embedded in paraffin. Thin sections (5 μ) were obtained and stained with haematoxylin and eosin (H&E) for light microscopic analysis of the lung at 40X magnification (1960; Herting et al., 1998).

GBS growth

To quantify *GBS* in the term pup, the fetal ELF, the blood, and lung homogenate samples were collected and cultured for bacterial growth. ELF samples were collected as described above. Whole blood was collected via sterile cardiac puncture. Fluid samples were plated in 100 μl serial dilutions onto sheep blood agar (ATCC) for 18 h of culture. Left lung tissue was homogenized and serial dilutions of the tissue slurry plated for culture. Lung tissue

protein was determined via the Bradford assay. *GBS* growth is presented in CFU/100 μ l fluid and CFU/mg of protein of lung tissue (Herting et al., 1998).

Confocal Microscopic Analysis of Alveolar Macrophage

The *in vivo* phagocytosis of *GBS* by the guinea pig AM was evaluated as previously described using confocal fluorescent microscopy (Olympus, Melville, NY) (Ping et al., 2007). After cell isolation via bronchoalveolar lavage, the AM were fixed with 4% paraformaldehyde and non-specific binding blocked with bovine serum albumin. The cells were incubated with the primary antibody to *GBS* (Fitzgerald Ind, Concord, MA) and then with a FITC fluorescently tagged secondary antibody (Sigma, St. Louis, MO). Using three dimensional confocal analyses, we evaluated the cell at 50% cell depth in the z plane to localize the internal fluorescence of phagocytosed *GBS* (within the cell and not within the outer 10 μ m plasma membrane). Background fluorescence of macrophages not stained with the FITC-labeled antibody was used to distinguish autofluorescence from engulfed bacteria (Brown et al., 2007; Ping et al., 2007). Fluorescence was determined via quantitative digital analysis via FluoView. Values for phagocytosis (defined as internalization of the *GBS*) are presented the mean percentage of cells with internalized fluorescence $\pm$ SEM as tallied from at least 10 experimental fields/ pup.

Statistical Analyses

Sigma Stat for Windows was used for statistical calculations. Analysis of Variance (ANOVA) was used to detect overall treatment differences. Statistical differences were detected by post-hoc analysis (Holm-Sidak) and a $p < 0.05$ was considered significant. Values presented represent mean $\pm$ SEM. Each litter of pups was considered a separate $n = 1$.

Results

There was no difference in the weight of the pups after the experimental diets (Control- 107 ± 7 grams vs ETOH- 98 ± 15 grams vs ETOH + SAM-e- 99 ± 16 grams, $p=NS$). The pups tolerated the intra tracheal administration of *GBS* without significant respiratory distress during the experimental observation period. There were no deaths due to the instillation of the *GBS*. Four hr after the intra tracheal *GBS* instillation, the pups were sacrificed and the ELF and cell pellet retrieved via bronchoalveolar lavage. There were no significant differences in the retrieved cell count ($p=NS$) among the experimental groups at this time point (Table 1). Approximately 50% of the retrieved cells were AM while the remaining percentage were neutrophils in all the groups ($p=NS$) (Table 1).

In utero ETOH exposure increased lung injury in the exposed pup

Representative H&E sections of the right lung tissue 4 hrs after experimental *GBS* treatment are presented in Figure 1 (40X magnification). In the ETOH-exposed lung, there was an increase in cellular infiltrates and loss of airspaces compared to the Control. With the addition of maternal SAM-e during the ETOH diet, pup lung histology revealed a more normal architecture with open airspace and decreased consolidation (Figure 1). Furthermore, histology from pups exposed to *in utero* ETOH + iGSH prior to *GBS* demonstrated decreased cellular infiltrates and consolidation, suggesting that GSH availability via maternal SAM-e or exogenous GSH to the pup via iGSH attenuated the increased sensitivity of the ETOH-exposed lung to acute lung injury.

GBS in the ELF was similar across experimental groups

ELF obtained from bronchoalveolar lavage was plated for *GBS* on nutrient agar and incubated at 37 °C for 18 h. There was no significant difference in *GBS* in the ELF across experimental groups (Control - 233 ± 83 CFU vs ETOH- 285 ± 60 CFU vs ETOH + SAM-e- 735 ± 277 CFU, $p=NS$, $n=$ at least 6 litters in each group). The addition of iGSH did not alter ELF *GBS* in either the ETOH exposed pups (ETOH + iGSH- 325 ± 140 CFU ($n=6$), or the Control (Control + iGSH- 60 ± 36 CFU, $n=4$).

***In utero* ETOH exposure increased GBS in the neonatal lung**

After sacrifice, the left lung was homogenized and 100 μ l of the tissue slurry plated for bacterial culture as described above. In Control pups, minimal *GBS* was present in the lung (Figure 2). However, the *GBS* in the lung was exponentially increased after *in utero* ETOH exposure (Figure 2 * $p<0.05$ vs Control). Lung *GBS* was significantly attenuated in the ETOH + SAM-e pups compared to ETOH exposure alone (Figure 2 ** $p<0.05$ vs ETOH). The addition of iGSH to the Control pup did not significantly change the minimal *GBS* found in the lung ($p=NS$ vs Control). However, the addition of iGSH prior to *GBS* administration significantly diminished *GBS* in the ETOH-exposed lung to control levels (Figure 2 ** $p<0.05$ vs ETOH).

***In utero* ETOH increased systemic sepsis after experimental GBS pneumonia**

After sacrifice of the pup, whole blood (100 μ l) was obtained via sterile cardiac puncture and was similarly plated for bacterial culture on nutrient agar and incubated at 37 °C for 18 h. Minimal *GBS* was found in the blood of either Control or Control + iGSH pups (Control 60 ± 44 CFU/100 μ l vs 100 ± 54 CFU/100 μ l, $p=NS$). However, *GBS* in the ETOH-exposed pup was exponentially increased compared to Control pups (Figure 3, * $p<0.05$ vs Control). The ETOH-induced increase in *GBS* in the blood was significantly decreased in the ETOH + SAM-e pups (Figure 3, ** $p<0.05$ vs ETOH). Furthermore, the addition of iGSH prior to *GBS* exposure also significantly attenuated the increased systemic sepsis seen in the ETOH-exposed pup (Figure 3, ** $p<0.05$ vs ETOH).

***In utero* ETOH impaired phagocytosis of GBS in vivo**

Bronchoalveolar lavage was performed 4h after intra tracheal instillation of *GBS* and the AM obtained by centrifugation for analysis of phagocytosis. The cells were plated and fixed with 4 % paraformaldehyde. AM were identified by Diffquik staining and morphology. Internalized *GBS* within the isolated AM was localized via fluorescent confocal microscopy. In Control pups, internalized phagocytosis of *GBS* was present in the majority (90 ± 2 %) of the cells. The addition of iGSH to Control prior to bacterial instillation did not change internalization of *GBS*, remaining present in the majority of the cells (85 ± 4 %, $p=NS$). However, *in vivo* phagocytosis of *GBS* in the ETOH-exposed cells was dramatically reduced and internalized bacteria was present in only 63 ± 4 % of the AM (Figure 4, * $p<0.05$ vs Control). The addition of maternal SAM-e during the ETOH ingestion significantly increased phagocytosis of *GBS* in the AM, with 83 ± 1 % of the ETOH + SAM-e cells positive for internalized bacteria (Figure 4, ** $p<0.05$ vs ETOH). Finally, ETOH pups receiving iGSH prior to *GBS* administration demonstrated increased internalization of 75 ± 3.7 % compared to the ETOH alone (** $p<0.05$ vs ETOH, $n=6$).

Discussion

Alcohol abuse is well described to diminish immune defenses in the adult population, particularly by impairing the function of the AM, the resident immune cell in the lung (Brown et al., 2006). ETOH exposure diminishes the adult AM's ability to phagocytose and

clear infectious organisms (Baughman and Roselle, 1987; Greenberg et al., 1999; Nelson and Kolls, 2002; Omidvari et al., 1998; Zhang et al., 1998). Immune paralysis of the AM, with increased apoptosis, deficient phagocytosis and increased oxidant stress with depletion of the critical antioxidant GSH is characteristic of the adult AM exposed to chronic ETOH (Brown et al., 2007).

Alcohol use during pregnancy remains a significantly under-recognized problem in society, particularly when recent data suggests a staggering 35-fold increased risk of extreme premature delivery with maternal alcohol use (Sokol et al., 2007). Despite well recognized alcohol-related risks to adult immune functioning in the lung, the effects of *in utero* alcohol exposure on the immune defenses of the developing baby, particularly the developing AM remain under-recognized and poorly understood. In experimental animal models, *in utero* ETOH impairs the developing immune system resulting in cellular deficits in B cell, T cell and lymphocyte subsets which continues even when those animals are examined as juveniles or adults (Biber et al., 1998; Giberson et al., 1997; Jerrells and Weinberg, 1998; Redei et al., 1989). We hypothesized that the resident AM in the neonatal lung would be similarly impaired by *in utero* ETOH. Our laboratory was the first to demonstrate that *in utero* ETOH exposure increased oxidant stress in the developing lung, causing derangements in the function and viability of the premature (Gauthier et al., 2005b) and term AM (Ping et al., 2007). Hallmarked by decreased GSH availability in the ETOH-exposed fetal lung, impaired AM phagocytosis of inactivated *staph aureus* after *in utero* ETOH exposure was demonstrated both *in vitro* and *in vivo*. Furthermore, maternal ingestion of the GSH donor SAM-e maintained GSH in the ELF of the fetal lung and in the fetal AM, improving the function of the AM both *in vitro* and *in vivo* (Gauthier et al., 2005b; Ping et al., 2007).

The goal of the current study was to extend our investigations of *in utero* ETOH's infection risk for the neonate into a novel guinea pig model of *in vivo* group *B streptococcus* pneumonia. The current study demonstrates that after *in utero* exposure, ETOH pups exhibited increased lung infection, increased systemic sepsis, and impaired phagocytosis of *GBS* by the term ETOH-exposed resident AM compared to Control pups. Because of diminished phagocytosis, AM dysfunction contributed to this increased risk of pulmonary and systemic *GBS in vivo* in the ETOH-exposed pup. Interestingly, as we have previously demonstrated with inactivated *staph aureus* in the term guinea pig (Ping et al., 2007), the ETOH-exposed AM demonstrated an inability to internalize *GBS in vivo*, similar to that demonstrated in the adult AM exposed to chronic ETOH (Brown et al., 2007). The increase in systemic sepsis demonstrated in the ETOH-exposed pups in this 4 hour experimental period also suggests that the ETOH-induced defects in immune response extend beyond the role of the resident AM into the epithelial barrier of the lung, as has been demonstrated in adult studies of the "alcoholic lung" (Bechara et al., 2004; Guidot et al., 2000). Furthermore, investigations into *in utero* ETOH's effects on the peripheral circulating monocyte, the precursor of the alveolar macrophage, are warranted.

Maintaining GSH availability through maternal ingestion of SAM-e protected the ETOH-exposed pup from experimental *GBS* in both the lung and the blood, while significantly attenuating the ETOH-induced AM inability to internalize the *GBS*. Maternal SAM-e to Control litters did not significantly alter *GBS* in the blood or the lungs of the exposed pups (data not shown). The current study suggests that although exogenous iGSH did not alter *GBS* in the Control group, exogenous GSH via iGSH holds promise as a therapy to protect the alcohol-exposed newborn. iGSH, when given to the ETOH pup prior to the *GBS* exposure, dramatically decreased both pulmonary and systemic *GBS* and improved AM phagocytosis. We chose the 500 μ M GSH concentration for the iGSH for the current study since this approximates the GSH concentration observed in the normal adult ELF (Yeh et al., 2007). Aerosolized GSH has been proposed for adult disease states hallmarked by

increased oxidant stress and infectious complications such as cystic fibrosis (Day et al., 2004; Roum et al., 1999). Furthermore, in initial studies of premature newborns, inhaled GSH reduced markers of oxidant stress in the airway (Cooke and Drury, 2005). Since minimal *GBS* was found in the blood or lungs of the Control pups, we can not exclude the possibility that iGSH would be beneficial to Control pups exposed to a higher concentration of infectious organisms. Furthermore, it remains to be determined whether “rescue” therapy with iGSH given after the bacterial exposure would prove beneficial for the neonatal lung. The current study supports further investigations into exogenous GSH as either a protective strategy or a rescue therapy for the at-risk newborn under exaggerated oxidant stress, particularly the premature newborn exposed to alcohol *in utero*.

In the newborn infant, particularly the premature newborn, the baseline function of the resident AM is impaired (Bellanti and Zeligs, 1995; Hall and Sherman, 1992; Sherman et al., 1992). Defenses against organisms in the lung such as *GBS* are compromised due to reduced number of AM, deficient phagocytosis, decreased chemotaxis and impaired bacterial killing (Kurland et al., 1988). Indeed, pneumonia and sepsis continued to cause significant morbidity and mortality, particularly in the premature newborn, (Bizzarro et al., 2005) despite maternal screening and prophylactic antepartum antibiotic administration (Puopolo et al., 2005).

Clinical data addressing the risk of infection in the alcohol-exposed human offspring remain limited (Johnson et al., 1981; Oleson et al., 1998). Retrospective analyses suggest that fetal alcohol exposure increases the risk of infection in both the premature (Gauthier et al., 2004) and term newborns (Gauthier et al., 2005a), but larger investigations are necessary. The current study demonstrates that exposure to alcohol *in utero* could exacerbate the already dysfunctional neonatal AM, increasing the risk of systemic sepsis for the exposed newborn challenged to clear pulmonary bacteria.

Since the resident AM is the conductor of the inflammatory milieu within the newborn airway, it is responsible not only for the initiation of the immune response but also the appropriate termination of this response. With inflammatory states, chronic disease, infection, and lung injury, the AM population in the lung contribute to the severity of the local disease state in the adult (Calhoun and Salisbury, 1989; Hance et al., 1985; Meyer et al., 1993; Rosseau et al., 2000). The neonatal AM's role in this regard remains unknown. Adverse outcomes of prematurity, such as chronic lung disease, have strongly been associated with an inability to terminate the pro-inflammatory environment within the developing lung (Bustani and Kotecha, 2003; De Dooy et al., 2001; Jobe and Ikegami, 1998; Jobe and Ikegami, 2001; Lyon, 2000). Therefore, future investigations evaluating the risk of lung injury and respiratory infection for the alcohol-exposed newborn are warranted to determine clinically whether the results of the current study are translated into the newborn intensive care unit.

In summary, in the current investigation, we have demonstrated that *in utero* ETOH exposure increased the severity of experimental *GBS* infection in the term guinea pig. Deficient AM phagocytosis contributed to an increased *GBS* in the lung and increased systemic sepsis in the ETOH-exposed pup. Maternal supplementation with SAM-e protected the ETOH-exposed pup from these derangements while neonatal treatment with iGSH to the ETOH-exposed pup also protected against increase lung infection and sepsis, suggesting that GSH availability to the pup modulated *in vivo* AM defense against experimental *GBS* pneumonia.

The results of this study note the vital importance for increased surveillance and identification of the alcohol-exposed neonate in order to develop a more comprehensive

understanding of alcohol's effects on the newborn's host immune system. Furthermore, investigations into the mechanisms of ETOH's impact on AM functioning as well as potential clinical interventions, such as GSH replacement to augment immune function remain under investigation. Such clinical interventions to augment the immune function of the ill newborn under exaggerated oxidant stress, such as that seen with *in utero* alcohol exposure will advance the care of our smallest at-risk patients.

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References

- Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. New York: McGraw Hill Publishers; 1960.
- Abdollah S, Brien JF. Effect of chronic maternal ethanol administration on glutamate and N- methyl-D-aspartate binding sites in the hippocampus of the near-term fetal guinea pig [published erratum appears in Alcohol 1996 Jan- Feb;13(1):107]. Alcohol. 1995; 12(4):377–382. [PubMed: 7546336]
- Albertsen K, Andersen AM, Olsen J, Gronbaek M. Alcohol consumption during pregnancy and the risk of preterm delivery. Am J Epidemiol. 2004; 159(2):155–161. [PubMed: 14718217]
- Baker RC, Jerrells TR. Recent developments in alcoholism: immunological aspects. Rec. Dev. Alcohol. 1993; 11:249–271.
- Baughman RP, Roselle GA. Surfactant deficiency with decreased opsonic activity in a guinea pig model of alcoholism. Alcoh. Clin. Exp. Res. 1987; 11(3):261–264.
- Bechara RI, Brown LA, Roman J, Joshi PC, Guidot DM. Transforming growth factor beta1 expression and activation is increased in the alcoholic rat lung. Am J Respir Crit Care Med. 2004; 170(2):188–194. [PubMed: 15105163]
- Bellanti JA, Zeligs BJ. Developmental aspects of pulmonary defenses in children. Pediatr Pulmonol Suppl. 1995; 11:79–80. [PubMed: 7547359]
- Biber KL, Moscatello KM, Dempsey DC, Chervenak R, Wolcott RM. Effects of in utero alcohol exposure on B-cell development in the murine fetal liver. Alcohol Clin Exp Res. 1998; 22(8):1706–1712. [PubMed: 9835284]
- Bizzarro MJ, Raskind C, Baltimore RS, Gallagher PG. Seventy-five years of neonatal sepsis at Yale: 1928–2003. Pediatrics. 2005; 116(3):595–602. [PubMed: 16140698]
- Brown LA, Cook RT, Jerrells TR, Kolls JK, Nagy LE, Szabo G, Wands JR, Kovacs EJ. Acute and chronic alcohol abuse modulate immunity. Alcohol Clin Exp Res. 2006; 30(9):1624–1631. [PubMed: 16930226]
- Brown LA, Ping XD, Harris FL, Gauthier TW. Glutathione availability modulates alveolar macrophage function in the chronic ethanol-fed rat. Am J Physiol Lung Cell Mol Physiol. 2007; 292:L824–L832. [PubMed: 17122355]
- Bustani P, Kotecha S. Role of cytokines in hyperoxia mediated inflammation in the developing lung. Front Biosci. 2003; 8:s694–s704. [PubMed: 12700133]
- Calhoun WJ, Salisbury SM. Heterogeneity in cell recovery and superoxide production in buoyant, density-defined subpopulations of human alveolar macrophages from healthy volunteers and sarcoidosis patients. J Lab Clin Med. 1989; 114(6):682–690. [PubMed: 2556490]
- Cooke RW, Drury JA. Reduction of oxidative stress marker in lung fluid of preterm infants after administration of intra-tracheal liposomal glutathione. Biol Neonate. 2005; 87(3):178–180. [PubMed: 15591818]
- Day BJ, van Heeckeren AM, Min E, Velsor LW. Role for cystic fibrosis transmembrane conductance regulator protein in a glutathione response to bronchopulmonary pseudomonas infection. Infect Immun. 2004; 72(4):2045–2051. [PubMed: 15039325]
- De Dooy JJ, Mahieu LM, Van Bever HP. The role of inflammation in the development of chronic lung disease in neonates. Eur J Pediatr. 2001; 160(8):457–463. [PubMed: 11548181]

- Drews CD, Coles CD, Floyd RL, Falek A. Prevalence of prenatal drinking assessed at an urban public hospital and a suburban private hospital. *J Matern Fetal Neonatal Med.* 2003; 13(2):85–93. [PubMed: 12735408]
- Ebrahim SH, Gfroerer J. Pregnancy-related substance use in the United States during 1996–1998. *Obstet Gynecol.* 2003; 101(2):374–379. [PubMed: 12576263]
- Fels AO, Cohn ZA. The alveolar macrophage. *J Appl Physiol.* 1986; 60(2):353–369. [PubMed: 3005225]
- Gauthier TW, Drews-Botsch C, Falek A, Coles C, Brown LA. Maternal alcohol abuse and neonatal infection. *Alcohol Clin Exp Res.* 2005a; 29(6):1035–1043. [PubMed: 15976530]
- Gauthier TW, Manar MH, Brown LAS. Is maternal alcohol use a risk factor for early-onset sepsis in the premature newborn? *Alcohol.* 2004; 33:139–145. [PubMed: 15528011]
- Gauthier TW, Ping X-D, Harris FL, Wong M, Elbahesh H, Brown LAS. Fetal alcohol exposure impairs fetal alveolar macrophage function via decreased glutathione availability. *Pediatric Research.* 2005b; 57:76–81. [PubMed: 15531743]
- Giberson PK, Kim CK, Hutchison S, Yu W, Junker A, Weinberg J. The effect of cold stress on lymphocyte proliferation in fetal ethanol-exposed rats. *Alcohol Clin Exp Res.* 1997; 21(8):1440–1447. [PubMed: 9394116]
- Greenberg SS, Zhao X, Hua L, Wang JF, Nelson S, Ouyang J. Ethanol inhibits lung clearance of *Pseudomonas aeruginosa* by a neutrophil and nitric oxide-dependent mechanism, in vivo. *Alcohol Clin Exp Res.* 1999; 23(4):735–744. [PubMed: 10235311]
- Guidot DM, Modelska K, Lois M, Jain L, Moss IM, Pittet JF, Brown LA. Ethanol ingestion via glutathione depletion impairs alveolar epithelial barrier function in rats. *Am J Physiol Lung Cell Mol Physiol.* 2000; 279(1):L127–L135. [PubMed: 10893211]
- Hall SL, Sherman MP. Intrapulmonary bacterial clearance of type III group B streptococcus is reduced in preterm compared with term rabbits and occurs independent of antibody. *Am Rev Respir Dis.* 1992; 145(5):1172–1177. [PubMed: 1586063]
- Hance AJ, Douches S, Winchester RJ, Ferrans VJ, Crystal RG. Characterization of mononuclear phagocyte subpopulations in the human lung by using monoclonal antibodies: changes in alveolar macrophage phenotype associated with pulmonary sarcoidosis. *J Immunol.* 1985; 134(1):284–292. [PubMed: 3964815]
- Herting E, Gan X, Rauprich P, Jarstrand C, Robertson B. Combined treatment with surfactant and specific immunoglobulin reduces bacterial proliferation in experimental neonatal group B streptococcal pneumonia. *Am J Respir Crit Care Med.* 1999; 159(6):1862–1867. [PubMed: 10351931]
- Herting E, Strayer DS, Jarstrand C, Sun B, Robertson B. Lung function and bacterial proliferation in experimental neonatal pneumonia in ventilated rabbits exposed to monoclonal antibody to surfactant protein A. *Lung.* 1998; 176(2):123–131. [PubMed: 9500297]
- Jerrells TR, Weinberg J. Influence of ethanol consumption on immune competence of adult animals exposed to ethanol in utero. *Alcohol Clin Exp Res.* 1998; 22(2):391–400. [PubMed: 9581645]
- Jobe AH, Ikegami M. Mechanisms initiating lung injury in the preterm. *Early Hum Dev.* 1998; 53(1):81–94. [PubMed: 10193929]
- Jobe AH, Ikegami M. Prevention of bronchopulmonary dysplasia. *Curr Opin Pediatr.* 2001; 13(2):124–129. [PubMed: 11317052]
- Johnson S, Knight R, Marmer DJ, Steele RW. Immune deficiency in fetal alcohol syndrome. *Pediatr Res.* 1981; 15(6):908–911. [PubMed: 7195540]
- Joshi PC, Applewhite L, Mitchell PO, Fernainy K, Roman J, Eaton DC, Guidot DM. GM-CSF receptor expression and signaling is decreased in lungs of ethanol-fed rats. *Am J Physiol Lung Cell Mol Physiol.* 2006; 291(6):L1150–L1158. [PubMed: 16877635]
- Joshi PC, Applewhite L, Ritzenthaler JD, Roman J, Fernandez AL, Eaton DC, Brown LA, Guidot DM. Chronic ethanol ingestion in rats decreases granulocyte-macrophage colony-stimulating factor receptor expression and downstream signaling in the alveolar macrophage. *J Immunol.* 2005; 175(10):6837–6845. [PubMed: 16272341]

- Juul SE, Kinsella MG, Truog WE, Gibson RL, Redding GJ. Lung hyaluronan decreases during group B streptococcal pneumonia in neonatal piglets. *Am J Respir Crit Care Med*. 1996; 153(5):1567–1570. [PubMed: 8630603]
- Kimura KA, Brien JF. Hippocampal nitric oxide synthase in the fetal guinea pig: effects of chronic prenatal ethanol exposure. *Brain Res Dev Brain Res*. 1998; 106(1–2):39–46.
- Kimura KA, Parr AM, Brien JF. Effect of chronic maternal ethanol administration on nitric oxide synthase activity in the hippocampus of the mature fetal guinea pig. *Alcohol Clin Exp Res*. 1996; 20(5):948–953. [PubMed: 8865973]
- Kurland G, Cheung AT, Miller ME, Ayin SA, Cho MM, Ford EW. The ontogeny of pulmonary defenses: alveolar macrophage function in neonatal and juvenile rhesus monkeys. *Pediatr Res*. 1988; 23(3):293–297. [PubMed: 3353176]
- Lester BM, ElSohly M, Wright LL, Smeriglio VL, Verter J, Bauer CR, Shankaran S, Bada HS, Walls HH, Huestis MA, Finnegan LP, Maza PL. The Maternal Lifestyle Study: drug use by meconium toxicology and maternal self-report. *Pediatrics*. 2001; 107(2):309–317. [PubMed: 11158464]
- Little BB, Snell LM, Rosenfeld CR, Gilstrap LC 3rd, Gant NF. Failure to recognize fetal alcohol syndrome in newborn infants. *Am J Dis Child*. 1990; 144(10):1142–1146. [PubMed: 2403097]
- Lyon A. Chronic lung disease of prematurity. The role of intra-uterine infection. *Eur J Pediatr*. 2000; 159(11):798–802. [PubMed: 11079191]
- Mattson SN, Riley EP, Gramling L, Delis DC, Jones KL. Heavy prenatal alcohol exposure with or without physical features of fetal alcohol syndrome leads to IQ deficits. *J Pediatr*. 1997; 131(5):718–721. [PubMed: 9403652]
- Meyer KC, Powers C, Rosenthal N, Auerbach R. Alveolar macrophage surface carbohydrate expression is altered in interstitial lung disease as determined by lectin-binding profiles. *Am Rev Respir Dis*. 1993; 148(5):1325–1334. [PubMed: 8239171]
- Nelson S, Kolls JK. Alcohol, host defence and society. *Nat Rev Immunol*. 2002; 2(3):205–209. [PubMed: 11913071]
- Oleson DR, Magee RM, Donahoe RM, Falek A, Coles CD. Immunity and prenatal alcohol exposure. A pilot study in human adolescents. *Adv Exp Med Biol*. 1998; 437:255–264. [PubMed: 9666278]
- Omidvari K, Casey R, Nelson S, Olariu R, Shellito JE. Alveolar macrophage release of tumor necrosis factor- α in chronic alcoholics without liver disease. *Alcohol Clin Exp Res*. 1998; 22(3):567–572. [PubMed: 9622433]
- Ping X-D, Harris FL, Brown LAS, Gauthier TW. In vivo dysfunction of the term alveolar macrophage after in utero ethanol exposure. *Alcohol Clin Exp Res*. 2007; 31(2):308–316. [PubMed: 17250624]
- Puopolo KM, Madoff LC, Eichenwald EC. Early-onset group B streptococcal disease in the era of maternal screening. *Pediatrics*. 2005; 115(5):1240–1246. [PubMed: 15867030]
- Redei E, Clark WR, McGivern RF. Alcohol exposure in utero results in diminished T-cell function and alterations in brain corticotropin-releasing factor and ACTH content. *Alcohol Clin Exp Res*. 1989; 13(3):439–443. [PubMed: 2546466]
- Rosseau S, Hammerl P, Maus U, Walmrath HD, Schutte H, Grimminger F, Seeger W, Lohmeyer J. Phenotypic characterization of alveolar monocyte recruitment in acute respiratory distress syndrome. *Am J Physiol Lung Cell Mol Physiol*. 2000; 279(1):L25–L35. [PubMed: 10893199]
- Roum JH, Borok Z, McElvaney NG, Grimes GJ, Bokser AD, Buhl R, Crystal RG. Glutathione aerosol suppresses lung epithelial surface inflammatory cell-derived oxidants in cystic fibrosis. *J Appl Physiol*. 1999; 87(1):438–443. [PubMed: 10409605]
- Sherman MP, Johnson JT, Rothlein R, Hughes BJ, Smith CW, Anderson DC. Role of pulmonary phagocytes in host defense against group B streptococci in preterm versus term rabbit lung. *J Infect Dis*. 1992; 166(4):818–826. [PubMed: 1527417]
- Sokol RJ, Janisse JJ, Louis JM, Bailey BN, Ager J, Jacobson SW, Jacobson JL. Extreme prematurity: an alcohol-related birth effect. *Alcohol Clin Exp Res*. 2007; 31(6):1031–1037. [PubMed: 17403063]
- Standiford TJ, Kunkel SL, Lukacs NW, Greenberger MJ, Danforth JM, Kunkel RG, Strieter RM. Macrophage inflammatory protein-1 α mediates lung leukocyte recruitment, lung capillary leak, and early mortality in murine endotoxemia. *J Immunol*. 1995; 155(3):1515–1524. [PubMed: 7636213]

- Szabo G. Consequences of alcohol consumption on host defence. *Alcohol Alcohol*. 1999; 34(6):830–841. [PubMed: 10659718]
- Yeh MY, Burnham EL, Moss M, Brown LA. Chronic alcoholism alters systemic and pulmonary glutathione redox status. *Am J Respir Crit Care Med*. 2007; 176(3):270–276. [PubMed: 17507544]
- Zhang Y, Crichton RR, Boelaert JR, Jorens PG, Herman AG, Ward RJ, Lallemand F, de Witte P. Decreased release of nitric oxide (NO) by alveolar macrophages after in vivo loading of rats with either iron or ethanol. *Biochem Pharmacol*. 1998; 55(1):21–25. [PubMed: 9413925]

Control**ETOH****ETOH + SAM-e****ETOH + iGSH****Figure 1. *In utero* ETOH increases lung injury from experimental *GBS***

Term guinea pig pups were given with 10^7 CFU/kg body weight of *GBS* intra tracheally. Representative hematoxylin and eosin sections at 40X magnification 4hr after *GBS* instillation are presented. The ETOH-exposed lung is consolidated with loss of airspaces and increased cellular infiltrates compared to the Control lung. After maternal ingestion of ETOH + SAM-e, the pup lung demonstrates diminished cellular infiltrates and less consolidation after experimental *GBS* compared to the lung exposed to ETOH alone. With iGSH, given to the pup after *in utero* ETOH exposure but prior to experimental *GBS*, the lung also demonstrated less infiltrates and decreased airway consolidation.

Figure 2. *GBS* in the neonatal guinea pig lung

Term guinea pig pups were inoculated with 10^7 CFU/kg body weight of *GBS* intra tracheally. After 4hr, lung samples were homogenized and plated (100 μ l) onto sheep blood agar. CFU were determined after 18 hr culture normalized to tissue protein. Bar height represents mean $\pm$ SEM of CFU/mg protein. * $p < 0.05$ vs Control, ** $p < 0.05$ vs ETOH; n=at least 6 separate litters for Control, ETOH, ETOH + SAM-e, or ETOH + iGSH.

Figure 3. *GBS* in the blood of the neonatal guinea pig

Term guinea pig pups were inoculated with 10^7 CFU/kg body weight of *GBS* intra tracheally. After 4hr, blood samples were obtained via sterile cardiac puncture and plated (100 µl) onto sheep blood agar. CFU were determined after 18 hr culture. Bar height represents mean $\pm$ SEM of CFU/100 µl. * $p < 0.05$ vs Control, ** $p < 0.05$ vs ETOH; n=at least 6 separate litters for Control, ETOH, or ETOH + SAM-e or ETOH + iGSH.

Figure 4. *In vivo* phagocytosis of *GBS* by the guinea pig AM

After inoculation with 10^7 CFU/kg body weight of *GBS* intra tracheally, cells were isolated by bronchoalveolar lavage and the location of *GBS* within the AM assessed at 50% cell depth in the z plane by fluorescent confocal microscopy. Bar height represents mean $\pm$ SEM of the percent of cells positive for internalized *GBS*. * $p<0.05$ vs Control, ** $p<0.05$ vs ETOH; n=at least 3 separate litters for Control, ETOH, or ETOH + SAM-e or ETOH + iGSH.

Table 1 Composition of bronchoalveolar lavage fluid after experimental *GBS*

Experimental Group	Cell Count (total)	Alveolar Macrophage (%)	Neutrophils (%)
Control	6.8 ± 2.4 million	61 ± 9	39 ± 9
ETOH	5.2 ± 1.2 million	54 ± 16	46 ± 16
ETOH + SAM-e	3.3 ± 0.3 million	62 ± 13	42 ± 16
ETOH + iGSH	6.6 ± 0.3 million	47 ± 3	50 ± 16