Zinc Insufficiency Mediates Ethanol-Induced Alveolar Macrophage Dysfunction in the Pregnant Female Mouse

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Abstract — Aims: (a) Establish the minimum number of weeks of chronic ethanol ingestion needed to perturb zinc homeostasis, (b) Examine intracellular zinc status in the alveolar macrophages (AMs) when ethanol ingestion is combined with pregnancy, (c) Investigate whether in vitro zinc treatment reverses the effects of ethanol ingestion on the AM. Methods: C57BL/6 female mice were fed a liquid diet (±25% ethanol-derived calories) during preconception and pregnancy. The control group was pair-fed to the ethanol group. In the isolated AMs, we measured intracellular AM zinc levels, zinc transporter expression, alternative activation and phagocytic index. Zinc acetate was added to some cells prior to analysis. Results: Intracellular zinc levels in the AM decreased within 3 weeks of ethanol ingestion. After ethanol ingestion prior to and during pregnancy, zinc transporter expression and intracellular zinc levels were decreased in the AMs when compared with controls. Bacterial clearance was decreased because the AMs were alternatively activated. In vitro additions of zinc reversed these effects of ethanol. Conclusion: Ethanol ingestion prior to and during pregnancy perturbed AM zinc balance resulting in impaired bacterial clearance, but these effects were ameliorated by in vitro zinc treatments.

INTRODUCTION

In the United States, one in two women of childbearing age (18–44) consumes alcohol with 15% reporting binge drinking (Centers for Disease Control and Prevention, 2012). Nearly 50% of all pregnancies are unplanned (Finer and Zolna, 2011) and alcohol consumption preconception is strongly associated with drinking during pregnancy (Ethen et al., 2009). Alcohol consumption during pregnancy leads to a range of growth and developmental disabilities classified as fetal alcohol spectrum disorders (Jones and Smith, 1973; Mattson et al., 1997; Hoyme et al., 2005), increases risk for premature delivery (Sokol et al., 2007), and the perinatal morbidity and mortality associated with prematurity (McCormick, 1985; Saigal and Doyle, 2008).

Proper zinc nutrition during pregnancy is essential for fetal development, growth, and immune function (Vallee and Falchuk, 1993) and zinc requirements increase ~30% when compared with the recommended daily allowance for non-pregnant, non-lactating women (Institute of Medicine, 2006). Zinc is an important micronutrient that displays catalytic, structural and regulatory functions (Cousins et al., 2006; King, 2011) and is particularly crucial for the function of highly proliferative systems, such as the immune system. Zinc balance is maintained through tight regulation of absorption and re-absorption, excretion, and distribution to tissues, processes that are carried out primarily by zinc transporters (Krebs, 2000). Zinc transporters are classified into two families that often display opposite roles in regulation of zinc homeostasis. Zip (SLC39) family transporters are responsible in zinc movement into the cytoplasm from the extracellular space or intracellular organelles. In contrast, the ZnT (SLC30) family transporters move zinc from the cytoplasm into cellular compartments or outside the cell (Kambe et al., 2004).

Overt zinc deficiency is rare and mostly observed in conditions such as acrodermatitis enteropathica, which displays, among other manifestations, thymic atrophy and increased bacterial, viral, and fungal infections (Rink and Gabriel, 2001). Mild-to-moderate zinc deficiency is more common and can present itself in diverse ways since zinc is widely involved in the general metabolism. Marginal zinc deficiency results in depression of the immune response, even if clinical manifestations such as skin lesions and alopecia are not observed. Alcohol abuse is also associated with zinc insufficiency, which could be related to a combination of malabsorption (Dinsmore et al., 1985) and poor diet (Lieber, 2000). Animal studies show that zinc levels in the alveolar space decrease even when ethanol ingestion is combined with a well-balanced diet (Joshi et al., 2009). Given that one in two women of childbearing age reports routine alcohol abuse (Centers for Disease Control and Prevention, 2012), zinc insufficiency could be initiated prior to pregnancy. Since nearly 50% of pregnancies are unplanned (Finer and Zolna, 2011), this zinc insufficiency could become exacerbated if alcohol consumption is coupled with pregnancy, leading to further compromised maternal zinc stores and zinc availability to the developing fetus.

In most non-pregnant animal adult models of chronic ethanol ingestion, ethanol comprises 36% of total calories consumed, amount comparable to that of people with alcohol use disorders. Ethanol models include dietary (Joshi et al., 2009; Gauthier et al., 2010), drinking water (An and Zhang, 2013), injection (Wang et al., 2007), inhalation (Karlage et al., 2010) and administration directly into the stomach (Lazic et al., 2007, 2011). These models are mostly rodent models (Gauthier et al., 2005, 2010; Ping et al., 2007) and sheep models (Lazic et al., 2007; Sozo et al., 2009, 2011). Many animal models of fetal alcohol exposure have been established and range from partial (Lazic et al., 2007, 2011; Wang et al., 2007; Sozo et al., 2009) to full (Gauthier et al., 2010) ethanol exposure throughout gestation, with most studies initiating fetal alcohol exposure after conception. In order to maintain pregnancy, ethanol-derived calories are decreased to 25% rather than 36% of total calories consumed, resulting in blood alcohol concentrations of 0.05 ± 0.01% (Gauthier et al., 2005). In our laboratory, this level of ethanol ingestion during gestation does not cause distress or...
loss of pregnancy but results in impaired differentiation and viability of alveolar macrophages (AM) in the newborn pup (Gauthier et al., 2009, 2010). Additionally, phagocytic function of the AM from newborn pups is impaired as shown by decreased bacterial clearance in vitro (Gauthier et al., 2005, 2010; Ping et al., 2007) and with an in vivo Group B streptococcus challenge (Gauthier et al., 2009). The mechanisms by which fetal alcohol exposure alters immune functions continue to be elucidated.

Current models initiating fetal alcohol exposure after conception do not represent the typical societal patterns where alcohol abuse occurs before recognition of the pregnancy. In order to develop a better understanding of the effects of alcohol established prior to conception, we developed a mouse model where chronic ethanol ingestion by the dams started prior to conception and continued for the duration of pregnancy. The goal was to establish the minimum number of weeks of chronic ethanol ingestion needed to perturb zinc homeostasis before initiation of pregnancy. Since chronic ethanol ingestion perturbs zinc homeostasis in the lung, promotes alternative activation of the AMs, and increases the risk of respiratory infections (Joshi et al., 2009), we chose the lung and the AM as the particular site for monitoring the effects on zinc homeostasis. Additionally, we examined whether intracellular AM zinc levels would be further perturbed when alcohol consumption was combined with the demands of pregnancy and whether addition of zinc in vitro would reverse these negative effects of alcohol. In previous studies, we demonstrated that the ethanol-induced impaired AM immune functions (Kamat et al., 2005) were due to the upregulation of TGFβ1, an immunosuppressive cytokine (Brown and Brown, 2012). This immunosuppression was due to alternative activation, the switch from a classically activated/proinflammatory Th1 profile (M1) to the immunosuppressed/regenerative Th2 profile (M2). In the current study, we examined whether zinc reversed the ethanol-induced AM alternative activation.

MATERIALS AND METHODS

Mouse model of ethanol ingestion prior to and during pregnancy

Female C57BL/6 mice shipped from the vendor (Charles River, Burlington, MA, USA) were allowed to acclimate in the Emory Pediatrics facilities for a week. After acclimatization, the mice were introduced to the liquid diet for an additional week and then randomized to receive an isocaloric liquid diet ± 25% ethanol-derived calories. For the ethanol group, the ethanol content of the diet was ramped from 0% (7 days) to 12.5% (3 days) and 25% (2 days) where they were maintained throughout the study. The control group was paired to the ethanol group and 25% of the calories came from maltoose-dextrin. The nutritional content of the liquid diet used is especially designed for experimentation in pregnant rodents, including adequate dietary zinc (Bioserv, Frenchtown, NJ, USA). First, we established the time period of ethanol ingestion at 25% of total calories needed to result in zinc insufficiency in the alveolar space. Non-pregnant mice were assigned to different time points, 2, 3 and 6 weeks. Some mice were fed their assigned diet (±ethanol) and then maintained on the appropriate diet throughout mating and pregnancy. Food consumption was recorded daily and the liquid food was changed daily. The assigned experimental liquid diet was the only access to food and water. Mice were weighed during the diet acclimation period to ensure no weight loss occurred and once a week upon the start of the experiment. Once pregnancy occurred, the mice were only disturbed for food and cage changes. To avoid additional stress, weight measurements during Days 8–20 of pregnancy (mouse equivalent to second and third trimester) were not determined. All animals were used with protocols reviewed and approved by the Emory University Institutional Animal Care Committee (DAR-2002896–090817BA) in accordance with NIH Guidelines (Guide for the Care and Use of Laboratory Animals).

Alveolar macrophage isolation

After anesthesia with intraperitoneal pentobarbital sodium, the trachea was identified and cannulated with a 19-G catheter. The lungs were serially lavaged with 1.5 ml sterile saline (5×) to obtain samples of the alveolar lining fluid. The initial lavage from each mouse was centrifuged (1200 rpm for 8 min) and the supernatant (designated bronchoalveolar lavage fluid) was saved for further analysis. The subsequent lavages from each mouse were pooled and similarly centrifuged. The cell pellet obtained from the initial and subsequent lavages was resuspended in RPMI 1640 1× media containing 10% fetal bovine serum and antibiotics. Cell viability and count were determined with Trypan Blue stain (0.4%; Life Technologies, Grand Island, NY, USA). The cells obtained from the lavage were predominantly AMs (±94%). Each mouse represented an n of 1. Cells were incubated overnight in a 10% CO₂ incubator at 37°C. For some studies, sterile zinc acetate (25 μM; final concentration) was added to the well before the cells were incubated overnight. For each parameter measured, the well contained 125,000 cells.

Zinc levels in AMs

Intracellular zinc levels in the isolated AMs were measured using a membrane permeable zinc-specific fluorescent dye, FluoZin-3AM (Invitrogen, Carlsbad, CA, USA). FluoZin-3AM has a high affinity for zinc (Kd ~ 15 nM) and minimal interfering calcium sensitivity. FluoZin-3AM is suitable for detection of zinc in the 1–100 nM range and is the most sensitive and zinc-specific of the different FluoZin fluorescent dyes. This dye detects small changes in intracellular zinc and has been previously used as an effective strategy to measure zinc in monocytes and macrophages (Haase et al., 2008; Joshi et al., 2009). AMs were incubated with FluoZin-3AM (45 min; 37°C) and then incubated in dye-free medium to allow for complete de-esterification of the intracellular acetoxymethyl esters of the fluorophore. Cells were then washed and fixed with 3.7% paraformaldehyde and fluorescence was quantified using fluorescent microscopy (Olympus Corp, Melville, NY, USA) and ImagePro Plus software for Windows. Data are presented as mean relative fluorescent unit (RFU)/field ± SEM.

Protein expression of zinc transporters and alternative activation

After isolation and fixation with 3.7% paraformaldehyde, immunostaining was used to evaluate protein expression of the zinc transporters Zip1, ZnT1 and ZnT4, as well as TGFβ1 and the M2 marker arginase-1. AMs were incubated with the
primary antibody in a 1:100 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 h, washed (3×; PBS; 5 min), after which the secondary antibody was added (1:200 dilution; 30 min). Zinc transporter expression was quantified using fluorescent microscopy and ImagePro Plus for Windows. Data are presented as mean RFU/field ± SEM. Similarly, protein expression of TGFβ1 and arginase-1 was quantified using fluorescent microscopy and ImagePro Plus for Windows. Data are presented as fold increase relative to cells without zinc acetate addition (CTRL-P) ± SEM.

**AM phagocytic index**

Isolated AMs were incubated overnight, after which pHrodo Staphylococcus aureus bioparticles conjugates (Life Technologies, Grand Island, NY, USA) were added to the wells for an additional 4 h incubation. Cells were then washed with PBS and fixed with 3.7% paraformaldehyde. Fluorescence of phagocytosed TRITC-S. aureus was quantified by fluorescent microscopy (Olympus Corp, Melville, NY, USA) and ImagePro Plus for Windows. Background fluorescence of unstained macrophages was used to account for autofluorescence. The phagocytic index was calculated as previously described (Fitzpatrick et al., 2008) with the percentage of cells positive for internalized fluorescence × mean RFU per field, as tallied from at least 10 experimental fields per set. Values are expressed as the phagocytic index ± SEM.

**Statistical analysis**

GraphPad software (La Jolla, CA, USA) and IBM SPSS Statistics (Armonk, NY, USA) were used for generation of graphs and statistical analysis, respectively. ANOVA was used to detect overall differences between groups and post hoc analysis was conducted (Tukey’s) for group comparisons. When data were not normally distributed, non-parametric tests were done followed by pair-wise comparisons. A P < 0.05 was deemed as statistically significant. Data are presented as mean ± SEM where each n represents one mouse.

**RESULTS**

**Study demographics**

Published data from our laboratory group demonstrated in diverse animal models that lower dietary ethanol (25 vs. 36%) is safely tolerated by pregnant dams with minimal negative pregnancy outcomes. In the current study, the liquid diet was well tolerated by the dams at all exposure periods: 2–6 weeks preconception, during mating and throughout pregnancy, without significant loss of pregnancy or distress. Mice that were not pregnant differed significantly in diet intake when compared with pregnant mice (P = 0.016 vs. pregnant control, P = 0.003 vs. pregnant ethanol) (Table 1). There were no statistically differences between pregnant control and ethanol groups (P = 0.817) and between non-pregnant control and ethanol groups (P = 1.00) in diet consumption. Additionally, there were no statistical differences in initial (P = 0.52) and final weight (P = 0.054) between groups (pregnant and non-pregnant) or within groups (control and ethanol). The period required to establish pregnancy was ~2.5 weeks resulting in female mice fed the appropriate liquid diet for 3 weeks prior to conception, 2.5 weeks for mating and 3 weeks of pregnancy yielding a total of 8.5 weeks. There were no significant differences between control and ethanol-fed groups on diet exposure duration or in the number of successful pregnancies (Table 1). Additionally, there were no significant differences in litter viability or number of abnormal fetuses.

**Macrophase profile**

In the bronchoalveolar lavage fluid, the cells isolated from control and ethanol-fed dams were predominantly AMs (294%). Total cell counts in pregnant groups (control and ethanol) were lower only when compared with non-pregnant ethanol group. However, there were no significant differences in cell viability between AMs from the non-pregnant and pregnant groups or within those groups, control vs. ethanol (Table 2).

**Chronic ethanol ingestion prior to pregnancy by the dam decreased zinc availability in the AMs and bacterial clearance**

Our first goal was to establish a model where dams, fed a diet containing 25% of calories derived from ethanol, entered
pregnancy with AM zinc insufficiency. Non-pregnant adult female mice were given the chronic ethanol diet for 2, 3 and 6 weeks. At the end of each time point, AMs were isolated to measure intracellular zinc and the phagocytic index. For those on the ethanol diet for 2 weeks, no statistical differences in AM intracellular zinc levels or phagocytic index were observed and this group was excluded from further analyses. For control groups at 3 and 6 weeks, no significant differences were observed and these groups were pooled for comparisons with time points of ethanol ingestion. Since the data were not normally distributed, non-parametric tests were performed. After 3 weeks of chronic ethanol ingestion, perturbations in AM intracellular zinc ($P = 0.04$) were observed but these changes were not associated with statistically significant decreases in bacterial clearance ($P > 0.05$) (Figs 1 and 2). In contrast, 6 weeks of ethanol ingestion significantly decreased both AM intracellular zinc ($P = 0.001$) and bacterial clearance ($P = 0.001$) when compared with AMs from control dams (Figs 1 and 2).

Chronic ethanol ingestion decreased AM zinc availability and immune functions in pregnant mice fed ethanol prior to and during pregnancy

We next determined if the zinc insufficiency associated with ethanol prior to pregnancy was exacerbated if the dam was maintained on the ethanol diet throughout pregnancy. AMs isolated from the ethanol + pregnancy group (EtOH-P) had significantly lower intracellular zinc levels when compared with pair-fed control + pregnancy group (CTRL-P) (Fig. 3). AMs isolated from the CTRL-P group had, on average, slightly higher, but not significant, increases in intracellular zinc levels when compared with the control non-pregnant group (CTRL-NP) (Fig. 3). Similarly, bacterial clearance in pregnant ethanol-fed mice (prior to and during pregnancy) was decreased by 40 and 54% when compared with the control non-pregnant and control pregnant groups, respectively (Fig. 4). Similar results were seen in ethanol non-pregnant group (EtOH-NP) when compared with control groups. For AM intracellular zinc levels and phagocytic index, there were no significant differences...
between the control groups, CTRL-P vs. CTRL-NP or between the ethanol groups, EtOH-P vs. EtOH-NP (Figs 3 and 4).

In vitro additions of zinc acetate reversed ethanol-induced effects on intracellular zinc and bacterial clearance in the AMs from the pregnant dams

In a subgroup of pregnant mice, we examined whether changes in AM intracellular zinc and bacterial clearance were related to zinc availability. Chronic ethanol ingestion prior to and during pregnancy (6–8 weeks total) resulted in a 60% decrease in phagocytosis but this was reversed to control levels by *in vitro* additions of zinc acetate (Fig. 5A). Furthermore, *in vitro* addition of zinc acetate restored intracellular zinc in the AMs from ethanol-fed dams when compared with control values without zinc addition, but not to that of the control group with *in vitro* addition of zinc \( (P < 0.05) \) (Fig. 5B). There were no significant differences between control groups (CTRL-P vs. CTRL-P + Zn).

**In vitro addition of zinc acetate reversed ethanol-induced AM upregulation of TGFβ1 and alternative activation**

Perturbations in zinc homeostasis can lead to formation of reactive oxygen species which can ultimately alter immunity. Given that bacterial clearance in the AMs from ethanol-fed dams was reduced 60% when compared with the controls, we determined if this was associated with TGFβ1 upregulation and downstream events like alternative activation. In the respiratory burst, antimicrobial agents nitric oxide and reactive nitrogen species are generated through nitric oxide synthase (Locati *et al.*, 2013). However, availability of the substrate arginine becomes limited when there is also upregulation of arginase-1. Based on our previous results in AMs from adult rats (Brown and Brown, 2012), the ethanol-induced upregulation of protein expression of arginase-1 (Fig. 6A) and TGFβ1 (Fig. 6B) by ∼4× and ∼8×, respectively, were not unexpected. When zinc acetate was added, the ethanol-induced expression of arginase-1 and TGFβ1 were normalized to control values (Fig. 6A and B).
Chronic ethanol ingestion is associated with decreased protein expression of zinc transporters and in vitro addition of zinc reversed ethanol-induced effects on the zinc transporters in the AMs from the pregnant dams

Since AM intracellular zinc was decreased, we determined if this was due to altered expression of the zinc transporters. For Zip1, a transporter that imports zinc into the cell, chronic ethanol ingestion prior to and throughout the pregnancy (6–8 weeks total) significantly decreased AM protein expression ($P < 0.001$) when compared with controls (Fig. 7A). We also examined the ZnT family zinc transporters, which contributes to the cytoplasmic zinc balance by exporting zinc out of the cell. These transporters include ZnT1 and ZnT4.

**Fig. 6.** Chronic ethanol ingestion prior to and during pregnancy increased protein expression of TGF$\beta_1$ and arginase-1 in the AMs but expression was normalized by the addition of zinc acetate. After delivery, the AMs from the dams were isolated and protein expressions of arginase-1 (A) and TGF$\beta_1$ (B) were determined via immunostaining and quantified by fluorescent microscopy and ImagePro Plus analysis. Data were expressed relative to the control AMs and bar heights represent mean RFU ± SEM from at least five different dams. CTRL-P = pregnant control group; CTRL-P + Zn = AMs from pregnant control group with zinc acetate added to the media; EtOH-P = pregnant ethanol group; and EtOH-P + Zn = AMs from pregnant ethanol group with added zinc acetate. For arginase-1 (A), ***denotes $P < 0.001$ for CTRL-P, CTRL-P + Zn vs. EtOH-P + Zn. For TGF$\beta_1$ (B), **denotes $P < 0.01$ for CTRL-P, CTRL-P + Zn vs. EtOH-P + Zn.

**Fig. 7.** Chronic ethanol ingestion prior to and during pregnancy decreased protein expression of zinc transporters in the AMs from the pregnant dams but was restored by addition of zinc acetate. After delivery, the AMs from the dams were isolated and protein expressions of the zinc transporters Zip1 (A), ZnT1 (B) and ZnT4 (C) were determined by computer analysis of fluorescent microscopic images. Data were expressed relative to the control AMs and bar heights represent mean RFU ± SEM from at least five different dams. CTRL-P = pregnant control group; CTRL-P + Zn = AMs from pregnant control group with zinc acetate added to the media; EtOH-P = pregnant ethanol group; and EtOH-P + Zn = AMs from pregnant ethanol group with zinc acetate added to the media. For Zip1 (A), *denotes $P < 0.05$ for CTRL-P, CTRL-P + Zn vs. EtOH-P + Zn, **denotes $P < 0.01$ for EtOH-P + Zn vs. EtOH-P and ***denotes $P < 0.001$ for CTRL-P, CTRL-P + Zn vs. EtOH-P. No significant differences were observed between control groups ($P > 0.05$). For ZnT1 (B), **denotes $P < 0.01$ for CTRL-P, CTRL-P + Zn vs. EtOH-P + Zn and EtOH-P + Zn vs. EtOH-P. No significant differences were observed between CTRL-P, CTRL-P + Zn and EtOH-P + Zn ($P > 0.05$). For ZnT4 (C), ***denotes $P < 0.001$ for CTRL-P, CTRL-P + Zn, and EtOH-P + Zn vs. EtOH-P. No significant differences were observed between CTRL-P, CTRL-P + Zn and EtOH-P + Zn ($P > 0.05$).
cell or by sequestering zinc into intracellular compartments (Kambe et al., 2004). In the current study, AMs isolated from ethanol-fed dams had an ~75% decrease in the protein expression of both ZnT1 (Fig. 7B) and ZnT4 (Fig. 7C) when compared with all other groups. As expected, in vitro addition of zinc to the AMs from ethanol-fed dams improved Zip1 protein expression when compared with the ethanol group without additional zinc acetate (Fig. 7A). Likewise, addition of zinc to the AMs from ethanol-fed dams normalized the expressions of ZnT1 and ZnT4 (Fig. 7B and C). Therefore, ethanol-induced decreases in these transporters were related to decreased zinc availability.

**DISCUSSION**

Most animal models of fetal ethanol exposure rely on initiation of ethanol ingestion after conception but this is unlikely to represent the typical exposure where alcohol abuse occurs before recognition of pregnancy. Therefore, we established a fetal ethanol model where chronic ethanol ingestion by the dams started prior to conception and continued throughout the pregnancy. Our model of fetal ethanol exposure aimed to mimic societal patterns, where women who routinely abuse alcohol are likely to have decreased zinc availability when compared with women who do not drink prior to pregnancy. In this study, we demonstrated that chronic ethanol ingestion prior to pregnancy (≥3 weeks) decreased intracellular AM zinc when compared with the control group; however, the decrease in bacterial clearance did not reach statistical significance. Continuation of chronic ethanol ingestion for 6 weeks was associated with significant reductions in AM zinc as well as decreases in the phagocytic index. The observed decreases in intracellular zinc and phagocytosis were unexpected since higher ethanol doses (36% of calories) and longer periods of ethanol ingestion (12 weeks) are typically used to generate the immunosuppressed phenotype. Decreases in intracellular zinc within three weeks were particularly surprising since the diet contained adequate zinc and other nutrients. Perturbations in zinc homeostasis and phagocytosis were maintained, but not exacerbated, with continued ethanol ingestion during the pregnancy.

Zinc is a cofactor in ~300 enzyme-dependent processes involved in immunity, growth, cell differentiation and metabolism (Cousins, 2006; King, 2011). Zinc homeostasis is tightly regulated by zinc transporters through intestinal absorption, fecal excretion, renal reabsorption and mobilization of zinc pools, primarily carried out via the trans-cellular processes of zinc transporters (Krebs, 2000; Kambe et al., 2004; Cousins et al., 2006). During pregnancy, the recommended daily allowance for many vitamins and minerals increases and suboptimal nutrition can compromise pregnancy outcomes and enhance teratogenicity of other insults such as alcohol (Keen et al., 2010). Proper zinc nutrition during pregnancy is essential for fetal development, growth and immune function (Vallee and Falchuk, 1993) and the recommended daily allowance for zinc in a healthy, well-nourished, pregnant female rises ~27% when compared with her non-pregnant counterpart (Institute of Medicine, 2006). Under normal fortification settings, zinc supplementation should not interfere with the absorption of other micronutrients, such as copper, and vice versa. In normal pregnancies, decreases in zinc result from a combination of hemodilution and decreases in albumin concentration which lead to decreased zinc transport, and possibly due to placental hormonal changes. Copper, on the other hand, steadily increases throughout pregnancy and is twice as much by the third trimester when compared with the non-pregnant counterpart (Tabrizi and Pakdel, 2014).

Animal models of zinc deficiency show that maternal zinc deficiency during pregnancy exerts negative effects on both mother and fetus and may have long-lasting postnatal effects. Prenatal zinc deficiency is associated with depression of the innate immune response characterized by decreased neutrophil chemotaxis and phagocytosis (Wellingham, 2001) and thymic atrophy characterized by reduction in T- and B-lymphocytes that may persist into childhood (Dutz et al., 1976; Ferguson, 1978). Moreover, gestational zinc deficiency in animal models is particularly associated with decreased growth of the newborn lung, spleen and thymus when compared with other organs such as heart, kidney, liver or brain (Beach et al., 1982).

In this study, we focused on zinc insufficiency as opposed to deficiency because the more common marginal zinc deficiency can also result in immune suppression. To date, there is no established marker of zinc status and plasma zinc, the most frequently used biomarker, is affected by diurnal variations, gender, age and state of pregnancy [reviewed in (Gibson, 2005)]. Plasma zinc concentrations rapidly decrease in a dose-dependent manner with severe zinc deficiency (Lowe et al., 2004; King, 2011). However, low-to-moderate zinc deficiency may result in plasma zinc levels that remain in the normal range for weeks due to adjustments in rates of excretion and absorption (Milne et al., 1987; Ruz et al., 1991; Gibson, 2005). Thus, plasma zinc concentration may not reflect cellular zinc status.

Alcohol consumption leads to nutrient deficiency through a combination of a poor diet, inhibition of nutrient absorption and increased excretion (Dinsmore et al., 1985; Lieber, 2000). The increased nutrient demands during pregnancy may exacerbate these negative effects of alcohol on nutrient availability to the fetus. In adult rats, ethanol ingestion at 36% of calories decreases zinc levels in the alveolar space, zinc transporter mRNA expression and phagocytic index in AMs, despite a well-balanced diet (Joshi et al., 2009). However, the current studies demonstrated that the AMs from female mice fed only 25% ethanol-derived calories had perturbations in zinc within 3 weeks, despite a well-balanced diet developed for the pregnant rodent. Continuation of ethanol ingestion at 25% of calories resulted in further decreases in AM zinc and expression of the zinc transporters. Our original hypothesis was that the demands of pregnancy would exacerbate the ethanol-induced zinc insufficiency established prior to pregnancy. However, pregnancy did not exacerbate the decreased zinc status observed in the ethanol groups. The increased dietary intake during pregnancy may have contributed to the maintenance of AM intracellular zinc levels.

In these studies, chronic ethanol ingestion decreased Zip1 expression which would result in decreased zinc import into the cell. Ethanol also decreased ZnT expression which would be protective by decreasing AM zinc export. However, in vitro addition of zinc restored AM expression of Zip1 and ZnT transporters as well as the AM zinc levels suggesting that ethanol-induced decreased zinc availability in the alveolar space was central to AM expression of these zinc transporters and the zinc pool. In previous rat model studies, chronic ethanol
ingestion decreased AM mRNA expression of the major intracellular zinc-binding protein metallothionein-1 which plays an important regulatory role in zinc uptake, distribution, storage and release as well as a scavenger of reactive oxygen species (Hasler et al., 2000; Joshi et al., 2009; Mohammad et al., 2012). Additional studies are needed to determine if less and shorter periods of ethanol intake or pregnancy alter the expression of metallothionein-1. Further studies are also needed to determine if ethanol ingestion increased hepatic metallothionein expression, which could lead to hepatic zinc sequestration and decreased circulating zinc resulting in decreased availability for other organs like the lung (Carey et al., 2000a,b).

In previous studies with adult and fetal models, we have shown that chronic ethanol increases oxidative stress, depletes and oxidizes the antioxidant glutathione, increases inflammatory cytokines, and impairs AM immune functions (D’Souza et al., 1996; Brown et al., 2004, 2007; Gauthier et al., 2005; Kamat et al., 2005). Central to these ethanol-induced events was the upregulation of TGFβ1, a cytokine that promotes immunosuppression through alternative activation, i.e. the switch from a proinflammatory Th1 profile to the immunosuppressed Th2 profile (Brown and Brown, 2012). In those studies, inhibition of TGFβ1 signaling reversed alternative activation and restored phagocytosis. In the current study, we determined if the ethanol-induced TGFβ1 upregulation and alternative activation, a downstream event, were linked to decreased zinc availability. As we observed in the adult rat model (Brown and Brown, 2012), chronic ethanol ingestion increased expression of TGFβ1 and the alternative activation marker arginase-1 in the female mouse model, even though ethanol was 25% of the calories. Furthermore, in vitro addition of zinc reversed ethanol-induced upregulation of TGFβ1 and alternative activation which may account for the restoration of phagocytosis. Further assessment of other markers of ethanol-induced impaired immune function, such as cytokines, IL-6 and TNFα, as well as NfκB, reactive nitrogen species, or reactive oxygen species (D’Souza et al., 1996; Brown et al., 2001, 2004, 2007; Gauthier et al., 2005; Kamat et al., 2005) would helpful in determining the extent to which decreased zinc availability contributes to the immunosuppressed phenotype. However, these observed results suggest that ethanol-induced decreases in zinc availability were etiological in the upregulation of TGFβ1 and the downstream switch to an alternatively activated cell, which is associated with immunosuppression.

In the adult rat model (Joshi et al., 2009), zinc availability was central to the ethanol-induced decreased capacity for microbial clearance and could be reversed by in vitro zinc treatments. In other studies, these effects of chronic ethanol ingestion were secondary to decreased expression of the zinc-dependent transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2), which plays a crucial role in the antioxidant response, including glutathione synthesis (Jensen et al., 2013). With zinc supplementation, Nrf2 expression and signaling is improved, oxidative stress is decreased, and AM microbial clearance improved (Mehta et al., 2011). Whether Nrf2 depletion is an etiological factor in the effects observed with this model with fewer ethanol-derived calories or a shorter period of ethanol ingestion remains to be determined.

In summary, this study focused on zinc insufficiency as opposed to deficiency because even marginal zinc deficiency can depress immune responses. The results of this study suggested that chronic ethanol ingestion decreased protein expression of the zinc transporters Zip1 and ZnT, which in turn, decreased intracellular zinc availability. In vitro addition of zinc reversed the negative effects of chronic ethanol ingestion on protein expression of the zinc transporters and the zinc pool in the AM. Furthermore, addition of zinc reversed ethanol-induced TGFβ1 upregulation and its immunosuppressive downstream events like alternative activation which would contribute to the restoration of phagocytosis. Therefore, ethanol-induced decreases in intracellular zinc were an etiological factor in the immunosuppression of AMs. In this study, we focused on AMs and further studies of the effects on other immune cells are warranted. Additional studies are needed to determine if these negative effects of chronic ethanol ingestion on the maternal zinc levels extend to the developing immune system of the newborn.

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