Published in final edited form as:

Alcohol Clin Exp Res. 2018 June ; 42(6): 1022–1033. doi:10.1111/acer.13754.

Dietary Iron Fortification Normalizes Fetal Hematology, Hpcidin, and Iron Distribution in a Rat Model of Prenatal Alcohol Exposure

Shane M. Huebner, Ph.D.¹, Kaylee K. Helfrich, B.S.³, Nipun Saini, Ph.D.³, Sharon E. Blohowiak, M.S.², Adrienne A. Cheng, B.A.¹, Pamela J. Kling, M.D.², and Susan M. Smith, Ph.D.^{1,3}

¹Dept. Nutritional Sciences, University of Wisconsin-Madison, Madison WI 53706

²Dept. Pediatrics, University of Wisconsin-Madison, Madison WI 53706

³Nutrition Research Institute, University of North Carolina at Chapel Hill, Kannapolis NC 28081

Abstract

Background—Prenatal alcohol exposure (PAE) causes neurodevelopmental disability. Clinical and animal studies show gestational iron deficiency exacerbates PAE's behavioral and growth deficits. In rat, PAE manifests an inability to establish iron homeostasis, increasing hepcidin (maternal and fetal) and fetal liver iron while decreasing brain iron and promoting anemia. Here, we hypothesize dietary iron fortification during pregnancy may mitigate alcohol's disruption of fetal iron homeostasis.

Methods—Pregnant Long-Evans rats, fed iron-sufficient (IS; 100ppm iron) or iron-fortified diets (IF; 500ppm iron), received either 5g/kg alcohol (PAE) or isocaloric maltodextrin (M) daily on gestation days (GD) 13.5 through 19.5. Maternal and fetal outcomes were evaluated on GD20.5.

Results—PAE reduced mean fetal weight ($p<0.001$) regardless of maternal iron status, suggesting iron fortification did not improve fetal growth. Both PAE ($p<0.01$) and IF ($p=0.035$) increased fetal liver iron. In fetal brain, PAE ($p=0.015$) affected total ($p<0.001$) and non-heme iron ($p<0.001$) such that iron fortification normalized ($p=0.99$) the alcohol-mediated reductions in brain iron and non-heme iron. Iron fortification also improved fetal hematological indices in PAE including hemoglobin, hematocrit, and mean cell volume ($p's<0.001$). Iron fortification also normalized hepcidin expression in alcohol-exposed maternal and fetal liver. Neither diet nor PAE affected transferrin and ferritin content in fetal liver, nor transferrin or transferrin receptor in fetal brain. However, IF-PAE fetal brains trended to less ferritin content ($p=0.074$), suggesting greater availability of non-storage iron. In PAE, hepcidin levels were linearly related to increased liver iron stores and decreased RBC and brain iron.

Corresponding Author: Susan Smith, Ph.D., Professor, Department of Nutrition, UNC-Nutrition Research Institute, Rm 3104, University of North Carolina at Chapel Hill, 500 Laureate Way, Kannapolis, NC 28081, Susan_Smith@unc.edu, Tel: (704) 250-5065, Fax: (704) 250-5001.

PROF. SUSAN M. SMITH (Orcid ID : 0000-0003-4782-6857)

The authors have no conflict of interest to declare.

Conclusions—Maternal oral iron fortification mitigated PAE's disruption of fetal iron homeostasis and improved brain iron content, hematological indices, and hepcidin production in this rat PAE model. Clinical studies show maternal iron deficiency substantially enhances fetal vulnerability to PAE, and our work supports increased maternal dietary iron intake may improve fetal iron status in alcohol-exposed pregnancies.

Keywords

fetal alcohol spectrum disorder; iron deficiency; hepcidin; anemia; iron homeostasis

INTRODUCTION

Prenatal alcohol exposure (PAE) is a leading cause of neurodevelopmental disability in the United States (NIAAA, 2015). Even with increased public understanding of the negative effects of PAE, 1 in 10 pregnant women in the US use alcohol and 1 in 33 report binge drinking in the previous month (CDC, 2015). In the US, 24 to 48 per 1000 children are estimated to exhibit fetal alcohol spectrum disorder (FASD; May, et al., 2018). PAE can cause life-long physical, cognitive, and behavioral abnormalities (NIAAA, 2015). It is estimated that the US alone spends \$4 billion annually on FASD related disabilities (Lupton, et al., 2004). Because of its severity and prevalence in both developed and developing countries, identification and implementation of nutritional interventions to mitigate the negative consequences of PAE are a priority.

Iron deficiency (ID) is the most common micronutrient deficiency in the world and affects approximately 30% of the global population and 50% of women of reproductive age (Asobayire, et al., 2001; Beard, 2003). Iron is essential for cellular energy generation and red cell formation, and in neurodevelopment is required for neurotransmitter formation as well as neuronal myelination (Beard, 2003; Lozoff, et al., 2006). Clinically, maternal ID strongly correlates with worsened outcomes in FASD including body growth, birth weight and size, and head circumference (Carter et al. 2010, 2012). Our previous work in a rat model of PAE affirmed that the combination of maternal prenatal ID and PAE worked synergistically to impair associative learning and white matter formation (Rufer, et al., 2012; Huebner et al. 2015). Molecular studies in those animals suggest the interaction between PAE and ID is due, in part, because alcohol disrupts maternal and fetal iron homeostasis, producing fetal anemia and brain iron-inadequacy while the liver accumulates iron stores due to alcohol's induction of hepcidin, which serves to limit iron absorption and transport (Huebner et al. 2016).

Because PAE dysregulates fetal iron utilization, and because ID exacerbates alcohol's damage, dietary iron supplementation under PAE conditions may improve fetal outcomes. Iron metabolism is tightly regulated, as free iron is highly toxic and causes oxidative damage (Tussing-Humphreys, et al., 2012). Therefore, optimization of dietary iron intake according to individual and population needs is paramount. Multiple factors must be considered when determining the amount of iron supplementation to provide, such as an individual's diet and the duration of public health intervention (Beard, 2000). A recent study indicated that oral iron and folate supplementation was able to significantly improve maternal iron biomarkers

in ID Tanzanian women as well as decrease perinatal mortality (Abioye, et al., 2016). However, whether pregnant women should receive iron supplementation in the absence of iron deficiency anemia remains under debate (Siu and USPSTF, 2015).

The *primary goal* of this study was to test the hypothesis that maternal consumption of an iron-fortified diet prior to and during pregnancy would restore iron homeostasis in both the dam and fetus in the presence of PAE. Multiple studies have shown that regulation of hepcidin, the primary stimulus for iron trafficking, is disrupted in the presence of alcohol exposure, and alcohol significantly decreases hepcidin levels in non-pregnant individuals, although much of that work is in the context of alcoholic liver disease (Ohtake, et al., 2007; Harrison-Findik, et al., 2009). In contrast, iron fortification alone could increase hepcidin levels (Pantopoulos, et al., 2012). Therefore, our *secondary goal* was to examine whether iron supplementation had detrimental effects upon iron homeostasis, divalent metal content, and oxidative stress.

MATERIALS AND METHODS

Animals and diets

We used our established model of dietary iron intervention (Rufer, et al., 2012; Huebner et al., 2015, 2016). Nulliparous, 8-week old Long Evans female rats (Envigo, Richmond IN) were randomly assigned to consume *ad libitum* either an iron-sufficient (IS, 100ppm Fe; TD. 06016) or iron-fortified (IF, 500ppm; TD.110880) diet at least 2 wks prior to and throughout pregnancy. The diet composition is based on AIN-93G (Supplemental Table 1), and food intake did not differ by treatment. Gestational day (GD) 0.5 was defined as the morning a vaginal plug was discovered. Litters containing < 9 or >15 pups were excluded from all analyses to minimize differences in iron distribution across litters within a treatment group. Fetuses were analyzed on GD20.5, which is the day prior to birth and is when placental iron transport is largely complete; it is the equivalent of human fetus at 39-40 weeks with respect to maternal-fetal iron transfer (Rao and Georgieff, 2002). On GD20.5, following isoflurane overdose and maternal perfusion, maternal and fetal blood and tissues were collected, flash frozen in liquid nitrogen, and stored at -80°C until further analysis. A subset of liver specimens was formalin-fixed and paraffin-blocked for histological analysis. All protocols were approved by the Institutional Animal Care and Use Committee.

Alcohol exposure

We used our established model of alcohol exposure (Huebner, et al., 2016). On GD 0.5, IS and IF dams were randomly assigned to receive either 5.0 g/kg body weight alcohol (PAE; 200 proof ethanol, USP grade; Decon Labs) given as 40% alcohol in water, or isocaloric maltodextrin (M) in water. Both alcohol and maltodextrin were administered by gastric gavage and were given daily as two half-doses 2 hrs apart on GD13.5 through G19.5.

Tissue iron analysis

Iron, copper, and zinc content was quantified at G20.5 in maternal liver, fetal brain, and fetal liver using inductively coupled plasma atomic emission spectroscopy (UW Soil Science Analysis Laboratory, Madison, WI) as described (Huebner et al., 2016). Fetal tissue could

not be perfused; therefore, non-heme iron content was quantified using the method of Rebouche, et al., (2004) and expressed as $\mu\text{g/g}$ wet weight and total μg of iron. Total RBC iron was calculated from hemoglobin values in Table 3 and using the formula: $\text{mg Fe in circulation} = \text{blood volume (90 ml/kg rat weight)} \times \text{hemoglobin (g/ml)} \times 3.47 \text{ mg Fe/g hemoglobin}$ (Foman et al. 1988). To assess how iron status and alcohol treatment affected iron distribution across fetal compartments, we calculated the ratios of liver non-heme iron (from Figure 4A) to total RBC iron (Table 3), brain non-heme iron (Figure 4B), and relative hepcidin expression (Figure 1B); values for ID fetuses were from Huebner et al. (2016).

Hematological Measures

On GD 20.5, dam and fetal blood samples were collected by cardiac puncture and placed immediately into a lithium heparin microtainer. Hematologic measures were determined using a pocH-100i™ hematology analyzer (Sysmex, Mundelein, IL). In addition to erythrocyte counts (RBC), hemoglobin, hematocrit, and the RBC indices mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC) were obtained. Unbound and total iron binding capacity was determined by TIBC kit (Pointe Scientific, Canton MI). Plasma ferritin (FTN) was quantified by ELISA (Immunology Consultants, Newberg, OR). Wright-Giemsa Smears were used to assess cellular morphology and count nucleated RBCs. A total of 100 nucleated cells were counted to distinguish between nucleated RBC and WBCs because the hematology analyzer is unable to do so. The WBC count is corrected by subtracting nucleated RBC.

To quantify RBC pockets within fetal liver, formalin-fixed, paraffin-embedded fetal liver specimens were sectioned at $10 \mu\text{m}$ and stained with either H&E or with enhanced Prussian Blue, which is a sensitive indicator of Fe^{2+} and Fe^{3+} deposits (Schroeter et al., 2004). Two central veins were centered and photographed at 20x using SPOT Advanced software (Diagnostic Instruments, Sterling MI). Using NIH ImageJ, thresholding for dark purple of nuclei of early RBC or the rust of ePBR staining was defined by changing the image type to an RGB Stack. Percent threshold staining area compared to the whole area of the slide was quantified by defining minimum and maximum threshold for each stack.

Western blot analysis

This was performed as previously described (Huebner et al. 2016). Briefly, $10 \mu\text{g}$ protein per sample was analyzed. Primary antibodies for iron homeostatic proteins were used in a 1:2000 dilution against FTN heavy chain (sc-25617, Santa Cruz Biotechnology, Dallas TX), transferrin (TF sc-30159, Santa Cruz Biotechnology), transferrin receptor-1 (TFRc 13-6890, Life Technologies, Rockville MD), and GAPDH (1:5000, G8795; Sigma-Aldrich, St Louis MO). Immunoreactive bands were visualized utilizing isotype-specific IR-dye conjugated secondary antibodies (A-21109, Life Technologies; #926-68022, 1:15,000, LI-COR, Lincoln, NB; #610-132-007, 1:30,000, Rockland Immunochemicals, Gilbertville, PA). Band IR intensities were quantified using the Odyssey infrared imaging system and Image Studio software (both from LI-COR). Molecular weights for bands of interest were calculated against color-tagged protein standards (Precision Plus Protein, BioRad, Hercules CA). Protein band intensity was normalized to GAPDH expression within the same sample and protein abundance was compared between samples; PAE does not affect GAPDH abundance

in this model, as determined by comparison to total protein loaded per lane (Huebner et al. 2016). Samples were assayed in triplicate.

Real-time Quantitative PCR

Methodology conforms to MIQE (Minimal Information for Publication of Quantitative Real-Time Experiments) guidelines for quantitative PCR analysis. RNA extraction, cDNA synthesis, and qPCR protocol were performed as described (Flentke, et al., 2004). Hepcidin (Hamp, NM_053469.1) primer sequences were: forward, 5'-AGACACCAACTTCCCCATATGC-3', reverse 5'-ACAGAGACCACAGGAGGAATTCTT-3'. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh, NM_017008.4) primer sequences were: forward, 5'-TGACAAAGTGGACATTGTTGC-3', reverse 5'-CTTGCCGTGGGTAGAGTCAT-3'. Samples were run in triplicate, and hepcidin expression was normalized to GAPDH (Gapdh, NM_017008.4) content. The mean relative expression change was calculated using the 2^{-CT} method (Livak and Schmittgen, 2001). Neither alcohol nor iron status affected GAPDH transcript abundance in this model.

DNPH Assay for Protein Carbonylation

Total protein carbonyl content in maternal rat liver was determined using 2, 4-dinitrophenylhydrazine (DNPH) as described (Reznick and Packer, 1994). Liver tissue was homogenized in 50mM phosphate buffer containing 0.1% digitonin, 1mM EDTA, and an antiprotease cocktail. Following centrifugation to remove solids, nucleic acids were removed from the supernatant using a 1% streptomycin sulfate precipitation, and protein was quantified using the BCA assay. Each protein sample at 5mg/mL was combined with 4 volumes of 20mM DNPH in 2.5M HCl and incubated for 1 hour in the dark with occasional vortex mixing. A sample omitting DNPH was run in parallel. The protein was precipitated twice in a final concentration of 10% trichloroacetic acid and then washed 3 times in ethyl acetate/ethanol (1:1, v/v). The final precipitate was dissolved at 37°C in 400µL of 6M guanidine hydrochloride. The carbonyl content was calculated from the absorbance at 375nm of the DNPH-treated proteins and using a molar extinction coefficient ϵ of 22,000 M⁻¹ cm⁻¹. For each sample, a parallel sample that omitted DNPH was run as a background control. Total protein concentration was determined by absorption at 280nm. Carbonyl content is expressed as mol carbonyl per mg total protein.

Data analysis

Animals in the IS and IF cohorts were generated contemporaneously with the iron-deficient (ID) cohort described in Huebner et al. (2016). Comparison of the IS and ID cohorts is presented in Huebner et al. (2016), and comparison of IS and IF cohorts is described herein. Select data for the IS-M and IS-PAE litters described in Huebner et al. (2016) are reproduced herein for comparative purposes against IF-M and IF-PAE, and those data are clearly identified within each table. The measures reproduced from Huebner et al. (2016) are: IS-M and IS-PAE maternal body weight (Table 1), fetal body and organ weights (Table 1), tissue mineral content (Table 2, Figure 4, Supplemental Table 2), and CBCs (Tables 2 and 3). All IS-M and IS-PAE data for the mRNA and protein quantitation (Figures 1–3), liver hematology (text), and the protein carbonylation (Supplemental Figure 1) are new data

generated from samples not previously analyzed. All IF-M and IF-PAE data are from animals not previously analyzed or published.

In the data analysis, for all fetal measurements excluding CBCs, the litter was classified as the experimental unit with 2 fetuses sampled per litter per endpoint and comparing consistent uterine position, because previous work shows intra-litter variation for experimental endpoints is lower than inter-litter variation within a treatment cohort. For CBCs, fetal blood was pooled and analyzed across an entire litter. A two-way ANOVA was employed using SAS Procedure MIXED (SAS Institute, Cary, NC) to determine differences due to dietary iron status and PAE. Fixed effects were iron status, PAE, and the interaction of iron status and PAE. The analysis included the random effect of litter. Each dependent variable was tested with two models utilizing equal or unequal variance among the four treatment groups. A likelihood ratio test was employed to determine which model was statistically appropriate. When the effect of iron status, PAE, and/or their interaction was significant ($P < 0.05$), the ANOVA was followed by *a priori* comparisons of the four treatments to one another in a planned, pairwise fashion (*e.g.* IS-M vs. IS-PAE, etc.) utilizing Tukey's HSD test to identify treatment differences while limiting overall comparison-wide error rate. Differences were considered significant at $P < 0.05$. Values are presented as Mean $\pm$ SEM unless otherwise noted.

RESULTS

Maternal-fetal weight parameters

Neither iron status nor PAE affected litter size (Table 1) and final maternal weight did not differ between treatments. Iron status had no significant effect upon pregnancy weight gain, but a non-significant trend was seen for increased gain in IF versus IS counterparts ($P = 0.068$). PAE dams has significantly lower gestational weight gain compared with their maltodextrin-exposed controls ($P = 0.035$). Maternal brain, heart, and liver weights did not significantly differ by treatment (not shown).

PAE reduced total fetal weight ($P < 0.001$) and reduced absolute brain ($P = 0.030$), liver ($P < 0.001$), and placental ($P = 0.034$) weights regardless of maternal diet (Table 1). When expressed as a percentage of body weight, PAE fetuses had higher brain and heart sizes irrespective of diet ($P < 0.001$), suggestive of organ sparing at the expense of body growth. The placental-to-body-weight ratio was significantly higher in PAE pregnancies ($P < 0.001$), and iron fortification did not mitigate this. Multiple comparisons test revealed that the IS-PAE group had a significantly higher placental-to-body-weight ratio compared with IS-M ($P = 0.032$) and IF-M ($P = 0.038$), whereas the IF-PAE values did not significantly differ from control values.

Maternal-fetal hepcidin mRNA expression

We have previously shown that PAE alters the expression of signals crucial for the regulation of iron fate in both the maternal and fetal compartments (Huebner et al. 2016). One such signal is hepcidin, a key regulator of iron absorption and transport, which is increased by PAE in both IS and ID dams and fetuses. We tested if dietary iron fortification would

normalize its expression. IS-PAE dams had significantly elevated liver hepcidin mRNA expression when compared to IS-M controls ($P = 0.024$). Iron fortification normalized maternal hepcidin expression in the presence of PAE (Figure 1A), and did not further affect hepcidin in controls. With respect to the fetus, a trend was present showing elevated hepcidin in IS-PAE fetuses and levels increased 66%, similar to that reported previously, while levels in IF-M and IF-PAE fetuses were essentially equivalent to IS-M controls (Figure 1B). The lack of excessive stimulation of hepcidin expression by the IF diet suggested that the five-fold increase in dietary iron intake was not excessive.

Maternal-fetal ferritin and transferrin

The hepcidin expression results suggested that providing dams with an iron-fortified diet prior to and during pregnancy may have helped to lessen the impact of PAE on iron sensing and/or utilization. To explore this further, we evaluated the maternal and fetal expression of ferritin (FTN), transferrin (Tf), and transferrin receptor (TfRc), which help regulate iron flux between liver and extrahepatic compartments. We reported previously that PAE blunted the response of these proteins to iron deficiency (Huebner et al. 2016). In maternal liver, alcohol itself did not affect the content of these proteins in the IS animals (Figure 2). Iron fortification elevated hepatic FTN and reduced TfRc, a response consistent with the dams' elevated iron intake (Pantopoulos et al., 2012). Under the IF-PAE combination, these effects of IF were attenuated and FTN and TfRc content did not differ from that of IS animals, whereas Tf was significantly reduced ($P = 0.048$).

Within the fetus, both diet ($P = 0.020$) and PAE ($P = 0.003$) increased liver FTN in the IS and IF fetuses (Figure 3A), although the additional increase was only a trend for the IF fetuses ($P = 0.13$). PAE and maternal diet did not affect fetal liver TfRc or Tf (Figure 3B, 3C). In fetal brain, diet did not affect the content of these three proteins (Figure 3E-G). However, within the developing brain, PAE significantly reduced the content of both FTN ($P < 0.001$) and Tf ($P = 0.007$), and iron fortification did not counter these effects of alcohol. IF-PAE fetal brain had significantly lower FTN content when compared to IS-M brain ($P = 0.0071$).

Maternal and Fetal Hematological Parameters

We previously reported that, even though their dams consumed an IS diet, PAE-exposed fetuses exhibited a normocytic anemia that became hypochromic anemia under ID-PAE (Huebner et al. 2016). The normalized hepcidin and iron regulatory proteins in the IF-PAE fetuses suggested iron fortification may have normalized their iron distribution. To test this, we compared the complete red cell counts in IF-M and IF-PAE dams and fetuses against those from IS-M and IS-PAE. In dams, independent of PAE status, iron fortification elevated RBC ($P < 0.001$), hemoglobin ($P < 0.001$), and hematocrit ($P = 0.001$) values compared to IS (Table 2); however, these elevated values were still within normal ranges for the rat (Quesenberry and Carpenter, 2004). Iron status and PAE also interacted to affect RBC numbers ($P = 0.02$) and hematocrit ($P = 0.045$), and post-hoc analysis revealed that IF reversed the reductions in RBC and hematocrit that were observed in the IS-PAE dams (Table 2, Huebner et al., 2016). Neither maternal diet nor PAE significantly affected maternal erythrocyte indices including MCV, MCH, MCHC, and RDW.

With respect to the fetus, we previously reported that PAE significantly reduced RBC numbers ($P = 0.025$), hemoglobin ($P = 0.034$), and hematocrit ($P = 0.034$), even when their dams consumed an IS diet (Table 3). Fetuses from iron-supplemented pregnancies had higher RBC ($P = 0.0002$), hemoglobin ($P = 0.001$), and hematocrit values ($P < 0.001$) regardless of their alcohol exposure status, and these values were significantly improved in the IF-PAE fetuses compared with their IS-PAE counterparts. These elevated red cell indices suggested that the extra iron may have stimulated erythropoiesis and, in support of this, the RBCs in the IF fetuses had a larger MCV ($P = 0.002$) and the appropriate hemoglobin content for their increased cell size as shown by higher MCH ($P = 0.012$) and normal MCHC, and this appearance was consistent with a normochromic phenotype. Although IF diet increased the nucleated RBC count ($P < 0.001$), review of Wright-Giemsa blood smears failed to reveal signs of maternal or fetal megaloblastic RBC or hypersegmented neutrophils, as seen with folate or B12 deficiency. Quantitation of RBC pockets along the sinusoids of fetal liver showed that immature RBC numbers were elevated in the IS-PAE liver, and these values were normalized by the IF diet in the presence of PAE. Taken together, these data show that iron fortification reversed the fetal red cell anemia caused by PAE.

Maternal and fetal iron content

We investigated whether iron fortification also normalized fetal iron distribution, as this is significantly disrupted by PAE (Huebner et al. 2016). Dams consuming the IF diet had significantly increased liver iron content ($P < 0.001$), independent of their alcohol exposure and consistent with their increased iron intake (Table 2); PAE did not affect hepatic iron content in those dams. In the fetus, PAE significantly increased hepatic iron content ($P < 0.001$) and this represented both total and non-heme iron (Figure 4A). Although hepatic iron was also increased in IF-PAE, this likely represented an effect of dietary iron and not PAE, as liver non-heme iron content did not differ in IF-M and IF-PAE. In fetal brain, alcohol also affected both total ($P = 0.015$) and non-heme brain iron content ($P < 0.001$), as reflected in sharply reduced brain iron content in the IS fetus (Figure 4B). Importantly, dietary iron fortification to the mother reversed that decline and total iron and non-heme iron content was normalized in the IF-PAE fetal brain.

To gain insight into the relationship between iron storage and hepcidin under PAE, we compared the distribution of iron between liver stores (non-heme iron) and the erythron (total RBC iron) and brain (non-heme iron) compartments (Figure 4C, D). In the non-alcohol-exposed fetuses, iron status significantly affected iron distribution to the erythron ($P = 0.0043$) and brain ($P = 1.53 \times 10^{-7}$), such that the ratio of liver-to-tissue iron increased commensurate with maternal iron uptake. This is expected and reflects that, under normal conditions, iron is prioritized to the erythron and developing brain; the non-heme liver iron/tissue iron is an order of magnitude lower for the erythron compared with brain under ID, consistent with the higher priority assigned to erythropoiesis. In contrast, PAE significantly skewed the relationship between liver storage iron and erythron iron ($P = 0.0017$) and brain iron ($P = 2.2 \times 10^{-16}$). In the erythron, iron was disproportionately allocated to liver stores under ID, and the liver stores/RBC iron ratio was independent of iron intake during PAE (Figure 4C). In brain, an interaction between iron status and PAE ($P = 2.06 \times 10^{-11}$) was associated with significantly elevated liver/brain iron ratios under ID and IS, and this ratio

declined as iron intake increased (Figure 4D). These data reinforce the hypothesis that, under PAE, iron is directed to liver instead of to the RBCs and brain, even when iron is limiting.

We then compared the relationship between fetal iron distribution and hepcidin. This comparison was made at the treatment level because technical limitations precluded assay of liver iron content and hepcidin expression in the same fetus. We observed a positive relationship between hepcidin and iron storage, such that the hepcidin increases were linearly related to the increased fraction of hepatic iron storage relative to the iron content of the erythron (Figure 4E) and brain (Figure 4F).

Markers of Iron Overload and Oxidative Stress

Excessive iron intake in pregnancy has been linked to poor perinatal outcomes (Casanueva and Viteri, 2003). With respect to the dam, PAE did not affect maternal plasma TIBC and TSAT, and the iron-fortified diet did not further influence those values (Table 2). Iron fortification can reduce the bioavailability of other divalent metals due to direct competition for transport and export. The iron-fortified diet modestly elevated the zinc content of maternal liver ($P=0.004$) and fetal liver ($P<0.001$), and trended to increase it in fetal brain ($P=0.07$) (Supplemental Table 2). The IF diet also increased the copper content of maternal liver ($P<0.001$), fetal liver ($P=0.051$), and fetal brain ($P=0.051$). PAE ($P=0.003$) increased zinc in fetal liver, and no iron-PAE interaction affecting the mineral content of these tissues was found. Overall, the iron-fortified diet did not negatively impact zinc and copper availability for the dam and fetus. Excessive tissue iron also can promote oxidant stress through Fenton chemistry. Thus, we quantified levels of protein carbonylation in maternal liver. We found no differences in protein carbonyl content with respect to alcohol exposure or iron intake (Supplemental Figure 1).

DISCUSSION

The most important finding from this study is the demonstration that maternal dietary iron fortification significantly improved iron distribution and utilization within the fetus. Infants born to binge-drinking mothers are more likely to have iron-deficient anemia, and those infants have greater growth deficits and head circumference reductions than those who were iron-adequate (Carter et al. 2007, 2012). In animal models, ID and PAE interact to worsen growth and behavioral outcomes (Rufer et al. 2012; Huebner et al. 2015, 2016). The basis for this interaction may be, in part, because PAE alters iron distribution within the fetal compartment and thus hinders the ability of tissues to control their iron status (Miller et al. 1995; Huebner et al. 2016). Our demonstration here that dietary iron fortification normalizes hepcidin expression, brain iron content, and erythropoiesis within the fetal compartment endorses that dietary approaches including iron fortification should be further explored as potential interventions to improve outcomes in alcohol-exposed human pregnancies. These findings have additional clinical relevance as both human and rat fetuses experience a rapid growth spurt at the end of pregnancy and must accumulate significant iron stores prenatally, because their postnatal growth outstrips the iron quantity delivered by milk. Thus, prenatal

iron deposition is necessary in both rat and human to offset the risk for postnatal iron deficiency during lactation.

The dietary iron fortification improved maternal and fetal hematological parameters, which impose a substantial burden on iron stores due to the rapid expansion of maternal and fetal circulation during pregnancy. Although the IS dams were not anemic, they had lower RBC numbers and the iron-fortified diet increased the hematologic values of IF-PAE dams such that these were now indistinguishable from those of IF-M dams. This suggested that iron stores were limiting for erythropoiesis in the IS-PAE dams and, potentially, that the IF dams achieved a more effective delivery of oxygen to the placenta. This hematologic improvement was even more dramatic in the PAE fetus. Whereas IS-PAE fetuses were overtly anemic, the dietary intervention reversed this anemia and the IF-PAE fetuses exhibited hematologic indices that were indistinguishable from those of IF-M fetuses. This indicated that the iron-fortified diet improved iron delivery and/or utilization by the fetal erythron, needs that were unmet under conditions of IS-PAE. Under most physiological circumstances, including anemia, iron delivery to the fetal erythrocytes is prioritized over other iron-dependent tissues such as brain (Zamora, et al., 2016). However, under limited conditions including inflammation (Fisher and Nemeth 2017) and (now) PAE (Huebner et al., 2016), iron delivery is not prioritized to fetal erythrocytes and is instead routed to storage. The inability of IS-PAE, but not IF-PAE, fetuses to maintain RBC production suggests that PAE disturbed the ability to fulfill that priority, and our calculations of tissue iron partitioning support this interpretation. Although iron fortification was associated with larger MCVs, further investigation revealed this did not represent megaloblastic anemia, but rather the increased presence of nucleated RBCs, suggesting IF promoted erythropoiesis in the PAE fetus. Iron supplementation is known to stimulate erythropoiesis and nucleated RBC release (Kling et al., 2008). The vulnerability of PAE fetuses to anemia gains significance because maternal iron inadequacy is present in populations at high risk for FASD (May et al., 2014, 2016; Streissguth et al., 1983). An improved fetal erythron would benefit fetal development particularly with respect to brain, and in other organ systems as well.

Improvement of the erythron in IF-PAE fetuses was accompanied by improved iron status in IF-PAE brain. The developing brain is a major target of iron-PAE interactions, and ID synergizes with alcohol to reduce associative learning, myelination, and head circumference in the offspring (Carter et al., 2007, 2013; Rufer et al. 2012). Even when the mother is iron-adequate, PAE causes lasting reductions in brain iron content (Miller et al., 1995). Iron is essential for healthy brain development, and thus it is noteworthy that dietary iron fortification normalized fetal brain iron status such that total brain iron was equivalent between IF-PAE and IS-M and IF-M controls. Non-heme iron, which better reflects the non-vascular iron content in these unperfused brains, had a greater content in the IF brains regardless of alcohol exposure, indicating an improved iron status. A greater proportion of this non-heme iron likely represents non-storage forms because the PAE brains also had reduced ferritin content. The iron-adequacy of the IF-PAE brain is further supported by its normal Tf and TfRc content, which otherwise increases in response to iron-insufficiency. Although iron still accumulated in the liver of PAE fetuses, the accrual of non-heme iron in IF-PAE brain combined with normal Tf and TfRc suggests the iron-fortified diet potentially promoted the more effective use of iron within the fetus.

Although this study was not designed to identify the mechanism by which iron fortification normalized iron distribution and iron status, an important clue emerged from the demonstration that the IF diet normalized fetal and maternal hepcidin expression in alcohol's presence, such that hepcidin levels in IF-PAE were indistinguishable from those in IS-M and IF-M. Hepcidin is a peptide hormone synthesized by liver and a 'master regulator' of iron homeostasis. Under iron sufficiency or iron excess, hepatic hepcidin output increases to reduce intestinal iron absorption and direct iron into storage, thereby limiting iron circulation. Conversely, under times of iron deficiency, hepcidin production decreases, signaling the liver to release iron from storage and into the bloodstream (Pantopoulos, et al., 2012). Within the fetal compartment, hepcidin similarly acts to regulate placental transfer of maternal iron and its distribution into storage or target tissues. Our studies (Huebner et al., 2016 and herein) reveal that alcohol disrupts this relationship between iron status and hepcidin, such that alcohol increased the fractional storage of iron relative to that in erythron and brain under conditions when iron was limiting. This shift was linearly related to the increased hepcidin production and suggests that the attenuated hepcidin in IF-PAE favored the redirection of fetal iron toward brain and erythron. Previous studies have shown that alcohol decreases hepcidin in adult humans with alcoholic liver disease, and in animal models of both acute and chronic exposure (Harrison-Findik, 2009; Ohtake, et al., 2007; Varghese, et al., 2016). In contrast, gestational alcohol exposure increases hepcidin mRNA in both dam and fetus (Huebner et al., 2016). Although these latter results are seemingly incongruous with prior work, one contribution to this elevated hepcidin may be alcohol's proinflammatory properties. Cytokines including IL-6 and IL1 β stimulate hepcidin transcription (Lee et al., 2005), and our preliminary findings observe dysregulated cytokine production in response to alcohol and iron status in dams and fetuses (Huebner et al., in preparation). An additional contribution to the elevated hepcidin levels in PAE may be the incomplete erythropoiesis observed in the IS-PAE and ID-PAE fetuses (Huebner, et al., 2016). Although we did not measure erythropoiesis directly, the reduced hematocrits in the IS and ID fetuses are consistent with such a mechanism and the IF diet may have ameliorated this by enhancing the pool of available (non-storage) iron.

PAE has complex effects upon growth and can result in delayed prenatal and/or postnatal growth, catch-up growth, or no growth delays at all (Carter et al. 2016). Iron-deficiency at infancy worsens these post-natal growth delays at 6 months (trend) and 12 months of age, but not in childhood (Carter et al. 2007, 2012). Rat models of PAE support those clinical findings and show that ID interacts with alcohol to worsen fetal and postnatal growth, compared with IS-PAE controls (Rufer et al., 2012; Huebner et al. 2016). PAE exerts multiple influences upon offspring growth by affecting nutrient availability (Sawant et al. 2015), placental function (Lo et al. 2017), and perhaps metabolic programming (Lunde et al. 2016), among others. Collectively, these clinical and rodent model findings affirm that iron status is an important modulator of growth in FASD, and worsened growth outcomes occur, in part, in those pregnancies in which iron was limiting.

In summary, this study shows that maximizing maternal iron stores prior to and during pregnancy can mitigate the deleterious effects of high-dose alcohol exposure on fetal erythrocyte-iron delivery. A limitation is that we do not know if the benefit was derived from the iron preload prior to pregnancy, from the gestational supplementation, or both, and this

must be ascertained because elevated hepcidin in heavy-drinking pregnancies would reduce dietary iron absorption. The increased non-storage iron content in IF-PAE brain suggests greater iron availability for neurodevelopmental processes such as monoamine neurotransmitter synthesis and myelination (Georgieff, 2007), and studies to test the impact of iron fortification on neurobehavioral outcomes in PAE are underway. However, the decision to fortify with iron during pregnancy is complex. Diagnosing iron deficiency is not always straightforward, as maternal anemia during the third trimester can be attributed to normal physiology during pregnancy (Scholl, 2005). Supplementation during this period can be detrimental, because high doses promote Fenton peroxidation and may increase susceptibility to developing gestational diabetes (Lachili, et al., 2001; Chen, et al., 2006). Promotion of iron supplements during pregnancy is consistent with public health policy, and results here show that oral iron delivery can improve maternofetal iron status under conditions of PAE. However, we used an iron-fortified diet rather than a concentrated oral iron supplement, and we note the latter can be associated with symptoms such as gastrointestinal stress, abdominal cramping, nausea, and constipation, which may deter many pregnant women (Pereira, et al., 2014; Smith, et al., 2014). Iron deficiency is common in both developed and developing countries (Scholl, 2005), including areas where alcohol abuse is high in pregnant women (May et al., 2014, 2016). Although fetal outcomes would be most improved by limiting maternal alcohol use, this is difficult in practice and alcohol abuse remains a leading public health problem including for pregnant women (CDC, 2015). Recently, micronutrient supplementation concurrent with prenatal alcohol exposure has been shown to improve neurodevelopmental outcomes in the offspring (Coles et al., 2015; Kable et al., 2015), and our rat model supports this approach to help reduce the infant's vulnerability to PAE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by NIH award #R01 AA22999, MERIT award R37 AA11085, and Hatch Act formula funds from UW-Madison to SMS, and F32 AA21311 to SMH.

References

- Abioye AI, Aboud S, Premji Z, Etheredget AJ, Gunaratna NS, Sudfeld CR, Mongi R, Meloney L, Darling AM, Noor RA, Spiegelman D, Duggan C, Fawzi W. 2016; Iron Supplementation Affects Hematologic Biomarker Concentrations and Pregnancy Outcomes among Iron-Deficient Tanzanian Women. *J Nutr.* 146:1162–1171. [PubMed: 27121530]
- Asobayire FS, Adou P, Davidsson L, Cook JD, Hurrell RF. 2001; Prevalence of iron deficiency with and without concurrent anemia in population groups with high prevalences of malaria and other infections: a study in Cote d'Ivoire. *Am J Clin Nutr.* 74:776–82. [PubMed: 11722959]
- Beard JL. 2003; Iron deficiency alters brain development and functioning. *J Nutr.* 133:1468S–1472S. [PubMed: 12730445]
- Beard JL. 2000; Effectiveness and strategies of iron supplementation during pregnancy. *Am J Clin Nutr.* 71:1288S–94S. [PubMed: 10799404]
- Beard JL, Connor JR. 2003; Iron status and neural functioning. *Annu Rev Nutr.* 23:41–58. [PubMed: 12704220]

- Carter RC, Jacobson JL, Molteno CD, Dodge NC, Meintjes EM, Jacobson SW. 2016; Fetal Alcohol Growth Restriction and Cognitive Impairment. *Pediatrics*. 138:e20160775. [PubMed: 27401098]
- Carter RC, Jacobson JL, Molteno CD, Jiang H, Meintjes EM, Jacobson SW, Duggan C. 2012; Effects of heavy prenatal alcohol exposure and iron deficiency anemia on child growth and body composition through age 9 years. *Alcohol Clin Exp Res*. 36:1973–1982. [PubMed: 22897691]
- Carter RC, Jacobson SW, Molteno CD, Jacobson JL. 2007; Fetal alcohol exposure, iron-deficiency anemia, and infant growth. *Pediatrics*. 120:559–67. [PubMed: 17766529]
- Carter RC, Jacobson JL, Sokol RJ, Avison MJ, Jacobson SW. 2013; Fetal alcohol-related growth restriction from birth through young adulthood and moderating effects of maternal prepregnancy weight. *Alcohol Clin Exp Res*. 37:452–62. [PubMed: 23013325]
- Casanueva E, Viteri FE. 2003; Iron and oxidative stress in pregnancy. *J Nutr*. 133:1700S–1708S. [PubMed: 12730487]
- CDC. Fetal Alcohol Spectrum Disorders (FASDs)—Alcohol Consumption Rates [Online]. 2015. Available: <http://www.cdc.gov/ncbddd/fasd/data.html> [Accessed June 22 2016]
- Chen X, Scholl TO, Stein TP. 2006; Association of elevated serum ferritin levels and the risk of gestational diabetes mellitus in pregnant women: The Camden study. *Diabetes Care*. 29:1077–82. [PubMed: 16644640]
- Cohen E, Wong FY, Horne RS, Yiallourou SR. 2016; Intrauterine growth restriction: impact on cardiovascular development and function throughout infancy. *Pediatr Res*. 79:821–30. [PubMed: 26866903]
- Coles CD, Kable JA, Keen CL, Jones KL, Wiertelcki W, Granovska IV, Pashtepa AO, Chambers CD, CIFASD. 2015; Dose and Timing of Prenatal Alcohol Exposure and Maternal Nutritional Supplements: Developmental Effects on 6-Month-Old Infants. *Matern Child Health J*. 19:2605–2614. [PubMed: 26164422]
- Costa Matos L, Batista P, Monteiro N, Ribeiro J, Cipriano MA, Henriques P, Girao F, Carvalho A. 2013; Iron stores assessment in alcoholic liver disease. *Scand J Gastroenterol*. 48:712–718. [PubMed: 23534461]
- Fisher AL, Nemeth E. 2017; Iron homeostasis during pregnancy. *Am J Clin Nutr*. 106(Suppl 6): 1567S–1574S. [PubMed: 29070542]
- Flentke GR, Baker MW, Docterman KE, Power S, Lough J, Smith SM. 2004; Microarray analysis of retinoid-dependent gene activity during rat embryogenesis: increased collagen fibril production in a model of retinoid insufficiency. *Dev Dyn*. 229:886–98. [PubMed: 15042712]
- Fomon SJ, Janghorbani M, Ting BTG, Ziegler EE, Rogers RR, Nelson SE, Ostedgaard LS, Edwards BB. 1988; Erythrocyte Incorporation of Ingested 58-Iron by Infants. *Pediatric Res*. 24:20–24.
- Gambling L, Czopek A, Andersen HS, Holtrop G, Srai SKS, Krejpcio Z, McArdle HJ. 2009; Fetal iron status regulates maternal iron metabolism during pregnancy in the rat. *Am J Physiol Regul Integr Comp Physiol*. 296:R1063–1070. [PubMed: 19176888]
- Georgieff MK. 2007; Nutrition and the developing brain: nutrient priorities and measurement. *Am J Clin Nutr*. 85:614S–620S. [PubMed: 17284765]
- Harrison-Findik DD. 2009; Is the iron regulatory hormone hepcidin a risk factor for alcoholic liver disease? *World J Gastroenterol*. 15:1186–1193. [PubMed: 19291818]
- Huebner SM, Blohowiak SE, Kling PJ, Smith SM. 2016; Prenatal Alcohol Exposure Alters Fetal Iron Distribution and Elevates Hepatic Hepcidin in a Rat Model of Fetal Alcohol Spectrum Disorders. *J Nutr*. 146:1180–1188. [PubMed: 27146918]
- Huebner SM, Tran TD, Rufer ES, Crump PM, Smith SM. 2015; Maternal iron deficiency worsens the associative learning deficits and hippocampal and cerebellar losses in a rat model of fetal alcohol spectrum disorders. *Alcohol Clin Exp Res*. 39:2097–2107. [PubMed: 26399568]
- Kable JA, Coles CD, Keen CL, Uriu-Adams JY, Jones KL, Yevtushok L, Kulikovskiy Y, Wiertelcki W, Pedersen TL, Chambers CD, CIFASD. 2015; The impact of micronutrient supplementation in alcohol-exposed pregnancies on information processing skills in Ukrainian infants. *Alcohol*. 49:647–656. [PubMed: 26493109]
- Kling PJ, Willeitner A, Dvorak B, Blohowiak SE. 2008; Enteral erythropoietin and iron stimulate erythropoiesis in suckling rats. *J Ped Gastroent Nutr*. 46:202–207.

- Lachili B, Hininger I, Faure H, Arnaud J, Richard MJ, Favier A, Roussel AM. 2001; Increased lipid peroxidation in pregnant women after iron and vitamin C supplementation. *Biol Trace Elem Res*. 83:103–10. [PubMed: 11762527]
- Lee P, Peng H, Gelbart T, Wang L, Beutler E. 2005; Regulation of hepcidin transcription by interleukin-1 and interleukin-6. *Proc Natl Acad Sci USA*. 102:1906–1910. [PubMed: 15684062]
- Livak JJ, Schmittgen TD. 2001; Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 25:402–8. [PubMed: 11846609]
- Lo JO, Schabel MC, Roberts VH, Wang X, Lewandowski KS, Grant KA, Frias AE, Kroenke CD. 2017; First trimester alcohol exposure alters placental perfusion and fetal oxygen availability affecting fetal growth and development in a non-human primate model. *Am J Obstet Gynecol*. 216:302.e1–302.e8. [PubMed: 28153658]
- Lozoff B, Beard J, Connor J, Barbara F, Georgieff M, Schallert T. 2006; Long-lasting neural and behavioral effects of iron deficiency in infancy. *Nutr Rev*. 64:S34–43. [PubMed: 16770951]
- Lunde ER, Washburn SE, Golding MC, Bake S, Miranda RC, Ramadoss J. 2016; Alcohol-induced developmental origins of adult-onset diseases. *Alcohol Clin Exp Res*. 40:1403–1414. [PubMed: 27254466]
- Lupton C, Burd L, Harwood R. 2004; Cost of fetal alcohol spectrum disorders. *Am J Med Genet C Semin Med Genet*. 127C:42–50. [PubMed: 15095471]
- May PA, Chambers CD, Kalberg WO, Zellner J, Feldman H, Buckley D, Kopald D, Hasken JM, Xu R, Honerkamp-Smith G, Taras H, Manning MA, Robinson LK, Adam MP, Abdul-Rahman O, Vaux K, Jewett T, Elliott AJ, Kable JA, Akshoomoff N, Falk D, Arroyo JA, Hereld D, Riley EP, Charness ME, Coles CD, Warren KR, Jones KL, Hoyme HE. 2018; Prevalence of Fetal Alcohol Spectrum Disorders in 4 US Communities. *JAMA*. 319:474–482. [PubMed: 29411031]
- May PA, Hamrick KJ, Corbin KD, Hasken JM, Marais AS, Blankenship J, Hoyme HE, Gossage JP. 2016; Maternal nutritional status as a contributing factor for the risk of fetal alcohol spectrum disorders. *Reprod Toxicol*. 59:101–108. [PubMed: 26656914]
- May PA, Hamrick KJ, Corbin KD, Hasken JM, Marais AS, Brooke LE, Blankenship J, Hoyme HE, Gossage JP. 2014; Dietary intake, nutrition, and fetal alcohol spectrum disorders in the Western Cape Province of South Africa. *Reprod Toxicol*. 46:31–39. [PubMed: 24568797]
- Miller MW, Roskams AJI, Connor JR. 1995; Iron regulation in the developing rat brain: effect of in utero ethanol exposure. *J Neurochem*. 65:373–380. [PubMed: 7790882]
- NIAAA. Fetal Alcohol Exposure [Online]. 2015. Available: <http://pubs.niaaa.nih.gov/publications/FASDFactsheet/FASDfact.htm> [Accessed 2016]
- Ohtake T, Saito H, Hosoki Y, Inoue M, Miyoshi S, Suzuki Y, Fujimoto Y, Kohgo Y. 2007; Hepcidin is down-regulated in alcohol loading. *Alcohol Clin Exp Res*. 31:S2–8. [PubMed: 17331161]
- Pantopoulos K, Porwal SK, Tartakoff A, Devireddy L. 2012; Mechanisms of mammalian iron homeostasis. *Biochemistry*. 51:5705–5724. [PubMed: 22703180]
- Pereira DI, Couto Irving SS, Lomer MC, Powell JJ. 2014; A rapid, simple questionnaire to assess gastrointestinal symptoms after oral ferrous sulphate supplementation. *BMC Gastroenterol*. 14:103. [PubMed: 24899360]
- Quesenberry, KE, Carpenter, JW. *Ferrets, Rabbits, and Rodents Clinical Medicine and Surgery*. 2nd. Saunders; St. Louis MO: 2004.
- Rao R, Georgieff MK. 2002; Perinatal aspects of iron metabolism. *Acta Paediatr Suppl*. 438:124–129.
- Rebouche CJ, Wilcox CL, Widness JA. 2004; Microanalysis of non-heme iron in animal tissues. *J Biochem Biophys Methods*. 58:239–51. [PubMed: 15026210]
- Reznick AZ, Packer L. 1994; Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Meth Enzymol*. 233:357–363. [PubMed: 8015470]
- Rufer ES, Tran TD, Attridge MM, Andrzejewski ME, Flentke GR, Smith SM. 2012; Adequacy of maternal iron status protects against behavioral, neuroanatomical, and growth deficits in fetal alcohol spectrum disorders. *PLoS One*. 7:e47499. [PubMed: 23094056]
- Sanchez J, Casas M, Rama R. 1988; Effect of chronic ethanol administration on iron metabolism in the rat. *Eur J Haematol*. 41:321–325. [PubMed: 3197820]

- Sawant OB, Wu G, Washburn SE. 2015; Maternal L-glutamine supplementation prevents prenatal alcohol exposure-induced fetal growth restriction in an ovine model. *Amino Acids*. 47:1183–1192. [PubMed: 25740606]
- Scholl TO. 2005; Iron status during pregnancy: setting the stage for mother and infant. *Am J Clin Nutr*. 81:1218S–1222S. [PubMed: 15883455]
- Schroeter M, Saleh A, Wiedermann D, Hoehn M, Jander S. 2004; Histochemical detection of ultrasmall superparamagnetic iron oxide (USPIO) contrast medium uptake in experimental brain ischemia. *Magn Reson Med*. 52:403–406. [PubMed: 15282824]
- Siu AL, U.S. Preventive Services Task Force. 2015; Screening for Iron Deficiency Anemia and Iron Supplementation in Pregnant Women to Improve Maternal Health and Birth Outcomes: U.S. Preventive Services Task Force Recommendation Statement. *Ann Intern Med*. 163:529–536. [PubMed: 26344176]
- Smith GA, Fisher SA, Doree C, Di Angelantonio E, Roberts DJ. 2014 Oral or parenteral iron supplementation to reduce deferral, iron deficiency and/or anaemia in blood donors. *Cochrane Database Syst Rev*. :CD009532. [PubMed: 24990381]
- Streissguth AP, Barr HM, Labbe RF, Smith JR, Darby BL, Smith NJ, Martin DC, Doan RN. 1983; Alcohol use and iron status in pregnant women. *Alcohol Clin Exp Res*. 7:227–230. [PubMed: 6346927]
- Tussing-Humphreys L, Pusatcioglu C, Nemeth E, Braunschweig C. 2012; Rethinking iron regulation and assessment in iron deficiency, anemia of chronic disease, and obesity: introducing hepcidin. *J Acad Nutr Diet*. 112:391–400. [PubMed: 22717199]
- Varghese J, James JV, Sagi S, Chakraborty S, Sukumaran A, Ramakrishna B, Jacob M. 2016; Decreased hepatic iron in response to alcohol may contribute to alcohol-induced suppression of hepcidin. *Br J Nutr*. 115:1978–1986. [PubMed: 27080262]
- Vokurka M, Krijt J, Sulc K, Necas E. 2006; Hepcidin mRNA levels in mouse liver respond to inhibition of erythropoiesis. *Physiol Res*. 55:667–674. [PubMed: 16497104]
- Zamore TG, Guiang SF 3rd, Widness JA, Georgieff MK. 2016; Iron is prioritized to red blood cells over the brain in phlebotomized anemic newborn lambs. *Pediatr Res*. 79:922–928. [PubMed: 26866907]

Figure 1. Maternal and fetal hepcidin production in response to PAE and dietary iron fortification

(A) Feeding an iron-fortified (IF) diet to dams significantly reduced the PAE-induced expression of hepcidin within maternal liver at GD20.5 to levels not different from IF and IS controls. (B) In fetal liver at GD20.5, PAE was associated with a trend to elevated hepcidin expression in IS fetuses, whereas feeding the IF diet to dams attenuated hepcidin expression in IF-PAE fetuses to levels not different from IS-M controls. Values are means $\pm$ SEMs of hepcidin mRNA content, normalized to GAPDH mRNA content and expressed relative to IS-M, representing $n=6$ litters and sampling 2 fetuses/litter. Figure panels present overall P

values as determined by 2-factor ANOVA, followed by post-hoc analysis in which means without a common letter are significantly different, $P<0.05$. IS, iron-sufficient; IF, iron-fortified; M, maltodextrin; PAE, prenatal alcohol exposure. The IS-M and IS-PAE dams studied here were previously studied in Huebner et al. (2016), but the presented values were generated from separate aliquots of frozen liver that has not been previously analyzed. The IS-M and IS-PAE fetal values presented here were all generated from fetuses that have not been previously analyzed. The IF values have not been previously reported.

Figure 2. Western blot quantification of ferritin, transferrin receptor, and transferrin proteins in maternal liver at GD20.5 in response to alcohol and dietary iron fortification

(A) Dietary IF but not alcohol significantly increased the ferritin (21 kDa) protein content in maternal liver, consistent with their elevated iron intake. (B) Dietary IF but not alcohol significantly reduced TfRc (95 kDa) protein content in maternal liver. (C) Transferrin (78 kDa) protein content was significantly reduced in maternal livers that experienced IF-PAE. (D) Representative Western blots for FTN, TfRc, Tf, and GAPDH protein in maternal livers at GD20.5. Values in (A-C) are means $\pm$ SEMs for FTN, TfRc, or Tf protein content normalized to GAPDH protein content, and expressed relative to IS-M, with $n=6$ dams/treatment group. Figure panels present overall P values as determined by 2-factor ANOVA, followed by post-hoc analysis in which means without a common letter are significantly different, $P<0.05$. IS, iron-sufficient; IF, iron-fortified; M, maltodextrin; PAE, prenatal alcohol exposure; FTN, ferritin; TfRc, transferrin receptor; Tf, transferrin. The IS-M and IS-PAE dams studied here were previously studied in Huebner et al. (2016), but the presented values were generated from a separate aliquot of frozen liver that has not been previously analyzed. The IF values have not been previously reported.

Fetal Liver & Brain FTN, TfRc, Tf

Figure 3. Western blot quantification of ferritin, transferrin receptor, and transferrin proteins in fetal liver (A-D) and fetal brain (E-H) at GD20.5 in response to alcohol and dietary iron fortification

(A) Dietary IF and alcohol significantly increased the ferritin (21 kDa) protein content of fetal liver. (B) Neither dietary IF nor alcohol affected TfRc (95 kDa) protein content of fetal liver. (C) Dietary IF and alcohol did not appreciably affect Transferrin (78 kDa) protein content in fetal liver. (D) Representative western blots for FTN, TfRc, Tf, and GAPDH protein in fetal livers at GD20.5. (E) Alcohol significantly reduced the ferritin (21 kDa) protein content of fetal brain, and this was not reversed by dietary IF. (F) Neither dietary IF nor alcohol affected the TfRc (95 kDa) protein content of fetal brain. (G) Alcohol reduced Transferrin (78 kDa) protein content of fetal brain relative to dietary IF but not relative to dietary IS. (H) Representative western blot for FTN, TfRc, Tf, and GAPDH protein in fetal brains at GD20.5. Values are means $\pm$ SEMs of FTN, TfRc, and Tf protein content normalized to GAPDH protein content, and expressed relative to IS-M, with $n=6$ litters per treatment and sampling 2 fetuses/litter. Figure panels present overall P values as determined by 2-factor ANOVA, followed by post-hoc analysis in which means without a common letter are significantly different, $P<0.05$. IS, iron-sufficient; IF, iron-fortified; M, maltodextrin; PAE, prenatal alcohol exposure; FTN, ferritin; TfRc, transferrin receptor; Tf, transferrin. The IS-M and IS-PAE fetal values presented here were all generated from fetuses that have not been previously analyzed. The IF values have not been previously reported.

Figure 4. Iron status and PAE affect total and non-heme iron content in GD20.5 fetal liver and brain

(A) Mean iron content in fetal liver, normalized to liver wet weight. Open bar represents total iron and shaded bar represents non-heme iron. (B) Mean iron content in fetal brain, normalized to brain wet weight. Open bar represents total iron and shaded bar represents non-heme iron. (C) Ratio of non-heme liver iron (from 4A) to total RBC iron, plotted as a function of iron status and alcohol treatment. Total RBC iron is calculated using the formula: mg Fe in circulation = blood volume (90 ml/kg rat weight) × hemoglobin (g/ml) × 3.47 mg

Fe/g hemoglobin (Foman et al. 1988). **(D)** Ratio of non-heme liver iron to non-heme brain iron (from 4B), plotted as a function of iron status and alcohol treatment. **(E)** Comparison of liver hepcidin expression (from Figure 1B) versus the ratio of non-heme liver iron to total RBC iron (from 4C), expressed as the mean fold-change in the ID (■), IS (▲) and IF (●) PAE fetuses relative to their respective dietary controls. **(F)** Comparison of liver hepcidin expression versus the ratio of non-heme liver iron to non-heme brain iron (from 4D), expressed as the mean fold-change in the ID (■), IS (▲) and IF (●) PAE fetuses relative to their respective dietary controls. For **(A-D)**, values are means $\pm$ SEMs, with n=6 litters per treatment for brain and liver, and n=3-4 litters for RBCs, and sampling 2 fetuses/litter. Figure panels present overall P values as determined by 2-factor ANOVA, followed by post-hoc analysis in which means without a common letter are significantly different, $P<0.05$. IS, iron-sufficient; IF, iron-fortified; M, maltodextrin; PAE, prenatal alcohol exposure. The brain and liver iron content and hemoglobin values of IS and ID fetuses were previously presented in Huebner et al. (2016) and are reproduced here for comparative purposes against the IF-M and IF-PAE values, which have not been previously reported.

Table 1

Effect of dietary iron status and PAE on maternal growth, litter size, and fetal rat tissue weights on gestational day 20.5¹

	IS-M ²	IS-PAE ²	IF-M	IF-PAE	P		
					Iron	PAE	Iron × PAE
<u>Maternal Weight, g</u>							
Final, total	337.6 ± 12.0	331.0 ± 4.8	326.0 ± 10.5	307.9 ± 10.5	0.13	0.28	0.61
Pregnancy gain	75.6 ± 3.8 ^{ab}	69.6 ± 5.5 ^b	94.5 ± 5.4 ^a	73.8 ± 7.0 ^{ab}	0.068	0.035	0.24
% of total gain	22.5 ± 1.1 ^b	21.0 ± 1.9 ^b	29.0 ± 1.4 ^a	23.6 ± 1.6 ^{ab}	0.007	0.035	0.20
<u>Litter size, <i>n</i></u>	12.0 ± 0.3	11.1 ± 0.3	11.6 ± 0.6	12.8 ± 0.5	0.22	0.70	0.042
<u>Fetal Weight, g</u>							
Body	3.21 ± 0.13 ^a	2.60 ± 0.13 ^b	3.20 ± 0.06 ^a	2.64 ± 0.11 ^b	0.86	<0.001	0.82
Liver	0.23 ± 0.01 ^{ab}	0.19 ± 0.01 ^b	0.24 ± 0.01 ^a	0.19 ± 0.01 ^b	0.41	<0.001	0.55
Brain	0.16 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.15 ± 0.01	0.56	0.030	0.73
Heart	0.018 ± 0.001	0.016 ± 0.001	0.016 ± 0.001	0.015 ± 0.001	0.053	0.17	0.66
Placenta	0.38 ± 0.01 ^a	0.37 ± 0.02 ^{ab}	0.39 ± 0.01 ^a	0.35 ± 0.01 ^b	0.63	0.034	0.17
<u>% (w/w) of total body weight</u>							
Liver	7.1 ± 0.2	7.3 ± 0.2	7.6 ± 0.1	7.3 ± 0.1	0.13	0.89	0.09
Heart	0.55 ± 0.03 ^b	0.65 ± 0.02 ^a	0.50 ± 0.01 ^b	0.58 ± 0.02 ^a	0.003	<0.001	0.73
Brain	5.1 ± 0.1 ^b	5.9 ± 0.2 ^a	4.9 ± 0.1 ^b	5.8 ± 0.2 ^a	0.27	<0.001	0.97
Placenta	11.9 ± 0.4 ^a	14.0 ± 0.6 ^b	12.2 ± 0.5 ^a	13.2 ± 0.4 ^{ab}	0.83	<0.001	0.22

¹ Values are means ± SEM, *n* = 6 litters. Labeled means within a row without a common letter differ in the post-hoc comparison at *P* < 0.05; means without letters did not significantly differ. IS, iron sufficient; IF, iron fortified; M, maltodextrin; PAE, prenatal alcohol exposure.

² IS-M and IS-PAE data are reproduced from Huebner et al. (2016) and are presented here for comparative purposes against IF values, which have not been previously reported.

Table 2

Effect of iron status and PAE on liver iron content and clinical blood counts of rat dams on gestational day 20.5¹

		IS-M ²	IS-PAE ²	IF-M	IF-PAE	P		
						Iron	PAE	Iron × PAE
Liver iron, $\mu\text{g/g}$		233 ± 19 ^c	249 ± 23 ^{bc}	346 ± 31 ^a	330 ± 22 ^{ab}	<0.001	0.99	0.50
RBC, $\times 10^6 \text{ cells}/\mu\text{L}$		6.0 ± 0.3 ^a	5.4 ± 0.1 ^c	6.5 ± 0.2 ^{ab}	6.8 ± 0.1 ^b	<0.001	0.31	0.02
Hemoglobin, g/dL		10.9 ± 0.4 ^a	10.0 ± 0.2 ^a	12.0 ± 0.4 ^b	12.1 ± 0.8 ^b	0.001	0.29	0.13
Hematocrit, %		34.3 ± 1.8 ^a	30.4 ± 0.6 ^b	37.3 ± 0.7 ^c	38.3 ± 0.2 ^c	<0.001	0.20	0.045
MCV, fL/cell		57.0 ± 1.2	56.6 ± 1.7	57.3 ± 0.9	56.4 ± 0.8	0.97	0.58	0.83
MCH, pg/cell		18.2 ± 0.2	18.5 ± 0.5	18.4 ± 0.1	17.9 ± 0.3	0.49	0.82	0.18
MCHC, pg/dL		31.4 ± 0.7	30.5 ± 0.1	32.1 ± 0.2	31.7 ± 0.5	0.054	0.21	0.56
RDW, %		12.7 ± 0.4	12.0 ± 0.1	12.3 ± 0.3	12.6 ± 0.2	0.70	0.48	0.10
TIBC, $\mu\text{g}/\text{dL}$		550 ± 32	429 ± 59	478 ± 60	471 ± 15	0.73	0.14	0.19
TSAT, %		42.1 ± 7.5	47.4 ± 3.7	40.0 ± 11.1	57.4 ± 10.8	0.70	0.29	0.57

¹Values are means ± SEM, $n = 3-4$ except liver iron, $n = 6$. Labeled means within a row without a common letter differ in the post-hoc comparison at $P < 0.05$; means without letters did not significantly differ. IS, iron sufficient; IF, iron fortified; M, maltodextrin; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; MCV, mean cell volume; PAE, prenatal alcohol exposure; RBC, red blood cell count; RDW, red blood cell distribution width; TIBC, total iron binding capacity; TSAT, transferrin saturation.

²IS-M and IS-PAE data are reproduced from Huebner et al. (2016) and are presented here for comparative purposes against IF values, which have not been previously reported.

Effect of iron status and PAE on hepatic and clinical blood counts of fetal rats on gestational day 20.5¹

Table 3

	IS-M ²	IS-PAE ²	IF-M	IF-PAE	P		
					Iron	PAE	Iron × PAE
RBC, $\times 10^6$ cells/ μ L	1.2 $\pm$ 0.1 ^{ab}	0.9 $\pm$ 0.1 ^b	1.6 $\pm$ 0.1 ^a	1.5 $\pm$ 0.1 ^a	<0.001	0.025	0.09
Hemoglobin, g/dL	6.9 $\pm$ 0.6 ^b	4.8 $\pm$ 0.6 ^c	9.9 $\pm$ 0.3 ^a	9.6 $\pm$ 0.1 ^a	<0.001	0.034	0.10
Hematocrit, %	20.9 $\pm$ 1.8 ^b	13.8 $\pm$ 2.0 ^c	28.3 $\pm$ 0.7 ^a	27.4 $\pm$ 1.1 ^a	<0.001	0.034	0.83
MCV, fL/cell	166 $\pm$ 5 ^a	163 $\pm$ 3 ^a	180 $\pm$ 2 ^b	184 $\pm$ 4 ^b	0.002	0.93	0.34
MCH, pg/cell	54.9 $\pm$ 1.5 ^a	57.2 $\pm$ 2.2 ^a	65.0 $\pm$ 2.2 ^a	64.2 $\pm$ 4.5 ^a	0.012	0.73	0.53
MCHC, pg/dL	32.9 $\pm$ 0.5	35.0 $\pm$ 1.2	35.8 $\pm$ 1.2	35.1 $\pm$ 1.9	0.29	0.50	0.23
cWBC, $\times 10^3$ cells/ μ L	1.16 $\pm$ 0.13 ^b	1.38 $\pm$ 0.14 ^b	2.75 $\pm$ 0.47 ^a	1.81 $\pm$ 0.27 ^b	<0.001	0.19	0.032
nRBC $\times 10^3$ cells/ μ L	1.79 $\pm$ 0.14 ^c	1.91 $\pm$ 0.10 ^c	9.52 $\pm$ 1.44 ^a	6.17 $\pm$ 0.39 ^b	<0.001	0.022	0.014
Liver nRBC %	3.67 $\pm$ 0.85 ^b	6.48 $\pm$ 0.84 ^a	3.86 $\pm$ 0.29 ^b	3.05 $\pm$ 0.31 ^b	0.025	0.014	0.14

¹Values are means $\pm$ SEM, $n = 3-4$. Labeled means within a row without a common letter differ in the post-hoc comparison at $P < 0.05$; means without letters did not significantly differ. IF, iron fortified; IS, iron sufficient; M, maltodextrin; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; MCV, mean cell volume; nRBC, nucleated RBC in peripheral circulation; PAE, prenatal alcohol exposure; RBC, red blood cell count. cWBC, white blood cell count corrected for nucleated RBC; Liver nRBC %, % of hepatic surface area containing nucleated RBC.

²IS-M and IS-PAE blood hematology (RBC, hemoglobin, hematocrit, MCV, MCH, MCHC) is reproduced from Huebner et al. (2016) and are presented here for comparative purposes against IF values, which have not been previously reported.