The Journal of Nutrition
Nutrition and Disease

Prenatal Alcohol Exposure Alters Fetal Iron Distribution and Elevates Hepatic Hepcidin in a Rat Model of Fetal Alcohol Spectrum Disorders[1–3]

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Abstract

Background: Prenatal alcohol exposure (PAE) causes neurodevelopmental disabilities, and gestational iron deficiency (ID) selectively worsens learning and neuroanatomical and growth impairments in PAE. It is unknown why ID worsens outcomes in alcohol-exposed offspring.

Objective: We hypothesized that PAE alters maternal-fetal iron distribution or its regulation.

Methods: Nulliparous, 10-wk-old, Long-Evans rats were mated and then fed iron-sufficient (100 mg Fe/kg) or iron-deficient (≤4 mg Fe/kg) diets. On gestational days 13.5–19.5, dams received either 5.0 g ethanol/kg body weight (PAE) or isocaloric maltodextrin by oral gavage. On gestational day 20.5, maternal and fetal clinical blood counts, tissue mineral and iron transport protein concentrations, and hepatic hepcidin mRNA expression were determined.

Results: In fetal brain and liver ($P < 0.001$) and in maternal liver ($P < 0.005$), ID decreased iron (total and nonheme) and ferritin content by nearly 200%. PAE reduced fetal bodyweight ($P < 0.001$) and interacted with ID ($P < 0.001$) to reduce it by an additional 20%. Independent of maternal iron status, PAE increased fetal liver iron (30–60%, $P < 0.001$) and decreased brain iron content (total and nonheme, 15–20%, $P \leq 0.050$). ID-PAE brains had lower ferritin, transferrin, and transferrin receptor content ($P \leq 0.002$) than ID-maltodextrin brains. PAE reduced fetal hematocrit, hemoglobin, and red blood cell numbers ($P < 0.003$) independently of iron status. Unexpectedly, and also independent of iron status, PAE increased maternal and fetal hepatic hepcidin mRNA expression >300% ($P < 0.001$).

Conclusions: PAE altered fetal iron distribution independent of maternal iron status in rats. The elevated iron content of fetal liver suggests that PAE may have limited iron availability for fetal erythropoiesis and brain development. Altered fetal iron distribution may partly explain why maternal ID substantially worsens growth and behavioral outcomes in PAE. *J Nutr* 2016;146:1180–8.

Keywords: fetal alcohol spectrum disorders, iron deficiency, hepcidin, neurodevelopment, pregnancy

Introduction

Alcohol abuse is a major public health concern. Among its consequences is fetal alcohol spectrum disorder (FASD)[6], a leading cause of permanent neurodevelopmental disability that affects 2.5–4.6% of school-age children in the United States and has higher rates in alcohol-abusing populations (1). Prenatal alcohol exposure (PAE) causes a distinct behavioral profile that includes impairments in learning, memory attention, executive function, and motor skills (2, 3). Despite increased public awareness regarding FASD, for pregnant women in the United States, 10.2–16.2% report having consumed alcohol in the previous 30 d and 0.7–2.9% report binge drinking (4). Thus, strategies that ameliorate alcohol's damage are a priority. Maternal factors, including low socioeconomic status, poor nutrition, and small stature, substantially worsen FASD outcomes (5, 6). One important nutritional modifier is iron deficiency (ID), the most common micronutrient deficiency during pregnancy (7). Perinatal ID and

[1] Supported by NIH grants F32 AA21311 (SMH, SMS), R01 AA22999 (SMS), and R37 AA11085 (SMS).
[2] Author disclosures: SM Huebner, SE Blohowiak, PJ Kling, and SM Smith, no conflicts of interest.
[3] Supplemental Tables 1–5 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
[6] Abbreviations used: FASD, fetal alcohol spectrum disorder; GD, gestational day; ID, iron deficiency; IS, iron sufficiency; PAE, prenatal alcohol exposure; TFRc, transferrin receptor.
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Manuscript received December 1, 2015. Initial review completed January 15, 2016. Revision accepted March 21, 2016.
First published online May 4, 2016; doi:10.3945/jn.115.227983.

FASD individually affect similar cognitive and behavioral domains (2, 3, 8, 9), and this suggests that PAE and ID may interact mechanistically. Clinical studies endorse this hypothesis. Although alcohol consumption is associated with increased liver iron stores (10, 11), women with the highest binge-drinking rates actually have an elevated risk of ID-anemia (12, 13). In studies of newborns with FASD, those infants who also had ID-anemia had the smallest head circumference and slowest postnatal growth trajectories (13, 14), and these growth delays persisted up to age 9 y (14).

The explanation for this association between alcohol and ID is unknown. One possibility is that PAE may alter maternal-fetal iron requirements or use. Understanding how PAE affects maternal-fetal nutrient use is important because a clinical intervention to improve FASD outcomes through maternal micronutrient supplementation, including iron, is under way (15, 16). Animal models of FASD suggest that alcohol may alter maternal-fetal iron metabolism. In offspring born to otherwise iron-adequate rat dams, PAE alters postnatal brain iron distribution and modifies brain transferrin and ferritin expression (17). We previously showed that gestational ID substantially worsens FASD characteristics, including reduced white matter formation, somatic growth delays, and impaired associative learning (18, 19). Here, this study investigated a potential mechanism underlying this alcohol-ID interaction and tested the hypothesis that PAE disrupts the homeostatic mechanisms that normally ensure normal fetal iron endowment. This experiment modeled maternal nonanemic ID in rats, such that maternal hematologic indicators are normal but liver iron stores are limiting, and targeted PAE to the late gestational period that encompasses when most maternal-fetal iron transfer occurs (20). This revealed that PAE significantly disturbs fetal iron distribution and dysregulates the homeostatic signals governing this process. The fetus's subsequent iron-deficient status may heighten its vulnerability to the damaging consequences of PAE.

Methods

Animals and diets. An established model of alcohol exposure and ID was used (18, 19). Twenty-four nulliparous, 8-wk-old Long-Evans female rats (Harlan) were evenly split across 2 cohorts of 12 and temporally spaced by 1 y and subsequently pooled at study completion. Rats were housed in a temperature-controlled room with a 12-h light/dark cycle. All females were fed an iron-sufficient (IS) diet (TD.06016, Harlan-Teklad; 100-mg/kg diet; **Supplemental Table 1**) until they were mated at 10 wk of age. The detection of a morning vaginal plug was designated gestational day (GD) 0.5, at which time dams were randomly assigned to 1 of 4 dietary iron/ethanol exposure treatments in a manner such that each treatment received a dam assignment before a given treatment received a second assignment. The study concluded with 6 dams/treatment. IS dams continued to be fed the IS diet from GDs 0.5–20.5. Dams assigned to the ID cohort were fed the 100-mg/kg diet from GDs 0.5–5, the 20-mg/kg iron diet (TD.06013; Harlan-Teklad) from GDs 5–13.5, and the 2- to 6-mg/kg iron diet (TD.80396; Harlan-Teklad) from GDs 13.5–20.5; this graded reduction in iron intake limited maternal iron stores while maintaining the dam in a moderate ID state (19). After isofluorane overdose and perfusion of the dam, maternal and fetal tissues were collected on GD 20.5, flash-frozen in liquid nitrogen, and stored at −80°C. Hematologic measures were determined using a pocH-100i hematology analyzer (Sysmex). Protocols were approved by the Institutional Animal Care and Use Committee.

Mineral analysis. Tissue mineral content was quantified using inductively coupled plasma atomic emission spectroscopy (UW Soil Science Analysis Laboratory, Madison, WI). Fetal tissue was not perfused; therefore, the nonheme iron concentration (μg/g wet weight) and total nonheme iron content (μg) of fetal liver and brain tissue were quantified (21).

Ethanol exposure. Starting at GD 13.5 and daily through GD 19.5, the alcohol-exposed IS and ID dams received 5.0 g ethanol/kg body weight (200 proof, USP grade; Decon labs), given as 40% ethanol in water (PAE). Control IS and ID dams instead received isocaloric maltodextrin solutions from GDs 13.5–19.5. Ethanol and maltodextrin were administered daily by gastric gavage and given as 2 half-doses 2 h apart. Blood alcohol content was measured in a separate group of dams 1 h after the second gavage on GD 13.5 and quantified using an Analox GM7 according to the manufacturer's protocol.

Western blot analysis. Fetal and maternal tissue samples were homogenized by a polytron (Brinkman) in a 10-mmol M Tris-HCl buffer/L containing 5 mmol EDTA/L, 1 mmol DTT/L, 1 mmol Na_3VO_4/L, 1% Triton-X100, 2 μmol pepstatin/L, 2 μmol leupeptin/L, 10 kIU aprotinin/mL, 400 μmol PMSF/L. Protein concentration and Western blot analysis were performed as previously described (22). Primary antibodies were directed against ferritin (sc-25617, 1:2000) (23), transferrin (sc-30159, 1:2000) (24), and divalent metal transporter 1 (both isoforms, sc-30120, 1:2000) (25) (all from Santa Cruz Biotechnology); transferrin receptor 1 (13–6890, 1:2000; Life Technologies) (26); ferroportin (MTP-11A, 1:2000; Alpha Diagnostic International) (27); and GAPDH (G8795, 1:10,000; Sigma-Aldrich). Immunoreactive bands were visualized using isotype-specific infrared dye-conjugated secondary antibodies (A-21109, Life Technologies; 926–68022, 1:15,000, LI-COR; 610–132–007, 1:30,000, Rockland Immunochemicals). Band intensities were quantified with the Odyssey infrared imaging system and Image Studio software (both from LI-COR). The band intensity for each protein of interest was normalized to GAPDH expression within the same sample to compare protein content between samples; neither PAE nor iron status affected GAPDH abundance in this model.

qPCR. Methods conformed to the MIQE (Minimum Information for Publication of Quantitative Real-Time Experiments) guidelines. RNA isolation, cDNA synthesis, primer design, and qPCR were performed as described (28) except cDNA was synthesized using random hexamers and ImProm-II reverse transcriptase (Promega) according to the manufacturer. Hepcidin (*Hamp*, NM_053469.1) primer sequences were as follows: forward, 5′-AGACACCAACTTCCCCATATGC-3′; reverse, 5′-ACAGAGACCACAGGAGGAATTCTT-3′. GAPDH (*Gapdh*, NM_017008.4) primer sequences were as follows: forward, 5′-TGACAAAGTGGACATTGTTGC-3′; reverse, 5′- CTTGCCGTGGGTAGAGTCAT-3′. Expression was normalized to GAPDH content. Samples were replicated in triplicate. Mean relative expression change was calculated using the $2^{-\Delta\Delta CT}$ method (29). Neither alcohol nor iron status affected GAPDH transcript abundance in this model.

Data analysis. For all fetal measurements, excluding complete blood counts, the litter was analyzed as the experimental unit, sampling 2 fetuses · litter^{-1} · endpoint^{-1} and comparing consistent uterine position, because intralitter variation for experimental endpoints studied herein was lower than interlitter variation within a treatment cohort. Fetal blood for complete blood counts was analyzed as a pooled sample for the entire litter. A 2-factor ANOVA was conducted with SAS Procedure MIXED (SAS Institute) to determine differences due to iron status and alcohol exposure. The fixed effects were iron status, alcohol exposure, and the interaction of iron status and alcohol exposure, in addition to the random effect of litter. Each dependent variable was tested with 2 models that used either equal or unequal variance among the 4 treatment groups. A likelihood ratio test was employed to determine which model was statistically appropriate. When the effect of iron status, alcohol, and/or their interaction was significant ($P < 0.05$), ANOVA analysis was followed by a priori comparisons of the 4 treatments to one another in a planned, pairwise fashion (e.g., IS-maltodextrin compared with IS-PAE) with Tukey's honestly significant difference test to identify individual treatment differences while also limiting overall comparison-wide error rate. Differences were considered significant at $P < 0.05$. Values are presented as means ± SEMs.

Results

Gestational growth characteristics. Neither PAE nor ID affected the absolute or relative percentage of dam gestational weight gain (**Supplemental Table 2**). Although PAE ($P = 0.012$) influenced the dams' final weight, this was because ID-PAE dams had a lower body weight at conception. Maternal tissue weights (liver, brain, heart, kidney) did not differ between treatments except that alcohol decreased ($P = 0.014$) mean spleen weight regardless of iron status. Although PAE was determined to significantly reduce litter size, planned comparisons revealed that neither IS-PAE nor ID-PAE dams had significantly fewer fetuses than did their respective controls (**Table 1**). Only one resorbed fetus was observed in the study's entirety, supporting a lack of effect of treatment on litter size. Iron status did not influence maternal blood alcohol levels (IS-PAE, 364 ± 24 mg/dL; ID-PAE, 376 ± 19 mg/dL).

PAE led to fetal growth restriction (Table 1), a central diagnostic criterion for FAS (3). PAE reduced absolute fetal body, heart, liver, and brain tissue weights at GD 20.5. The moderate gestational ID itself did not alter fetal body or tissue weight; however, PAE and ID interacted to further reduce total body ($P = 0.025$) and liver ($P = 0.015$) weight compared with the IS-PAE fetus. The effects of PAE on tissue growth were disproportionate, and upon correction for fetal weight, the liver weight as a percentage of body weight did not change, whereas the heart ($P = 0.001$) and brain ($P < 0.001$) weights were increased in relative size. The addition of ID to PAE increased relative brain mass compared with IS-PAE ($P = 0.010$), suggesting a greater impairment of somatic growth. Thus, consistent with aforementioned work (13, 14, 18), PAE and maternal iron status interacted to modify fetal somatic and brain growth.

Maternal and fetal iron status. ID dams were best described as having nonanemic ID, delineated by a 40–50% decrease in liver iron ($P < 0.001$) and normal hematologic values (**Table 2**). PAE did not affect maternal liver iron or hematologic indexes excluding decreased platelet numbers ($P = 0.030$). Neither PAE nor ID altered liver zinc or copper concentration (**Supplemental Table 3**).

In contrast to dams, both PAE and ID had a significant impact on fetal iron distribution. The ID-maltodextrin fetuses exhibited ID-anemia, indicated by their reduced liver iron concentration (total, $P = 0.013$; nonheme, $P < 0.001$), reduced mean corpuscular volume ($P = 0.002$) and mean corpuscular hemoglobin ($P = 0.013$), and increased red blood cell distribution width ($P < 0.001$) compared with IS-maltodextrin controls (**Table 3**). In contrast, PAE had complex effects on indexes of fetal iron status. PAE increased both total and nonheme iron concentration ($P < 0.001$) in fetal liver and in ID-PAE to levels indistinguishable from IS-maltodextrin controls. PAE altered fetal hematologic values independent of ID and decreased red blood cell numbers ($P = 0.001$), hemoglobin concentration ($P = 0.002$), and hematocrit volume ($P = 0.001$). The hematologic profile of ID-PAE fetuses had elements of both PAE and ID and included reduced hemoglobin ($P = 0.021$) and hematocrit ($P = 0.032$) compared with ID-maltodextrin, as well as reduced mean corpuscular volume ($P = 0.035$) and mean corpuscular hemoglobin ($P = 0.017$) compared with IS-PAE. In addition, ID ($P = 0.033$) and PAE ($P < 0.001$) increased the zinc concentration of fetal livers (**Supplemental Table 4**). These data indicate that PAE sequestered iron in the fetal liver and negatively affected fetal blood hematologic indexes, particularly when erythrocyte iron was limiting; thus, PAE led to fetal anemia, independent of maternal iron status.

With respect to the fetal brain, as expected, ID alone reduced brain iron concentration by 31% ($P < 0.001$) compared with IS. PAE decreased brain iron in IS fetuses by 21% ($P = 0.017$ compared with IS-maltodextrin) to levels indistinguishable from ID-maltodextrin and ID-PAE. Neither PAE nor ID altered brain zinc concentration; brain copper was undetectable (**Supplemental Table 5**). PAE exerted opposing effects on fetal brain and liver iron concentration, and this, together with the altered hematologic profiles, strongly suggested PAE disrupted fetal iron distribution.

Expression of proteins regulating iron transport and storage. To investigate factors potentially responsible for changes in fetal iron distribution, the content of proteins that regulate iron transport and storage was evaluated. Fetal iron originates from both maternal diet and maternal liver stores. In maternal liver, dietary iron content influenced both ferritin and transferrin receptor (TFRc) content in the absence of PAE, such that ID reduced ferritin levels 59% ($P = 0.011$) and increased TFRc levels 42% ($P = 0.008$) compared with IS-maltodextrin

TABLE 1 Effect of dietary iron status and prenatal alcohol exposure on litter size and fetal rat tissue weights on gestational day 20.5[1]

					P value		
	IS-M	IS-PAE	ID-M	ID-PAE	Iron	PAE	Iron × PAE
Litter size, *n*	12.2 ± 0.3	11.3 ± 0.4	12.8 ± 0.5	11.3 ± 0.6	0.52	0.031	0.52
Absolute weight, g							
Body	3.19 ± 0.13^{a}	2.59 ± 0.15^{b}	3.31 ± 0.15^{a}	2.10 ± 0.10^{c}	0.17	<0.001	0.025
Liver	0.22 ± 0.01^{a,b}	0.19 ± 0.02^{b}	0.24 ± 0.02^{a}	0.15 ± 0.01^{c}	0.20	<0.001	0.015
Brain	0.16 ± 0.01^{a,b}	0.15 ± 0.01^{b,c}	0.17 ± 0.01^{a}	0.14 ± 0.01^{c}	0.83	<0.001	0.07
Heart	0.018 ± 0.002^{a,b}	0.016 ± 0.001^{a,b}	0.019 ± 0.001^{a}	0.015 ± 0.001^{b}	0.83	0.011	0.19
Placenta	0.38 ± 0.01^{a}	0.36 ± 0.01^{a,b}	0.39 ± 0.01^{a}	0.33 ± 0.02^{b}	0.42	0.006	0.23
Relative weight, g/100 g total body weight							
Liver	7.0 ± 0.1	7.4 ± 0.2	7.2 ± 0.2	7.0 ± 0.3	0.70	0.74	0.15
Heart	0.56 ± 0.03^{b}	0.65 ± 0.02^{a,b}	0.58 ± 0.03^{b}	0.69 ± 0.02^{a}	0.26	0.001	0.70
Brain	5.1 ± 0.1^{c}	6.0 ± 0.2^{b}	4.9 ± 0.2^{c}	6.9 ± 0.2^{a}	0.037	<0.001	0.011

[1] Values are means ± SEMs, $n = 6$ litters (10–15 pups/litter). Labeled means within a row without a common superscript letter differ at $P < 0.05$. ID, iron deficient; IS, iron sufficient; M, maltodextrin; PAE, prenatal alcohol exposure.

TABLE 2 Effect of iron status and prenatal alcohol exposure on liver iron concentration and clinical blood counts of rat dams on gestational day 20.5[1]

					P value		
	IS-M	IS-PAE	ID-M	ID-PAE	Iron	PAE	Iron × PAE
Liver iron, μg/g	233 ± 18[a,b]	249 ± 23[a]	115 ± 9[c]	151 ± 30[b,c]	<0.001	0.26	0.67
RBC, $\times 10^6$ cells/μL	6.0 ± 0.3	5.4 ± 0.1	5.7 ± 0.2	5.7 ± 0.3	0.97	0.18	0.22
Hemoglobin, g/dL	10.9 ± 0.4	10.0 ± 0.2	10.0 ± 0.3	10.6 ± 0.8	0.82	0.69	0.13
Hematocrit, %	34.3 ± 1.8	30.4 ± 0.6	32.0 ± 1.1	35.5 ± 4.7	0.56	0.95	0.15
MCV, fL/cell	57.0 ± 1.2	56.6 ± 1.7	56.1 ± 0.4	58.6 ± 1.6	0.65	0.41	0.25
MCH, pg/cell	18.2 ± 0.2	18.5 ± 0.5	17.7 ± 0.2	18.7 ± 0.5	0.60	0.08	0.37
MCHC, pg/dL	31.4 ± 0.7	30.5 ± 0.1	31.8 ± 0.3	31.5 ± 0.2	0.09	0.16	0.49
RDW, %	12.7 ± 0.4	12.0 ± 0.1	12.7 ± 0.3	13.3 ± 0.8	0.12	0.91	0.14
PLT, $\times 10^3$ cells/μL	689 ± 74	377 ± 95	658 ± 91	488 ± 126	0.69	0.030	0.48
MPV, fL/cell	8.8 ± 0.3	9.2 ± 0.5	8.4 ± 0.1	9.0 ± 0.3	0.41	0.19	0.81
WBC, $\times 10^3$ cells/μL	3.0 ± 0.7	1.7 ± 0.6	2.7 ± 0.2	2.9 ± 0.1	0.41	0.36	0.23

[1] Values are means ± SEMs, n = 3–4 except liver iron, n = 6 litters (10–15 pups/litter). Labeled means within a row without a common superscript letter differ, $P < 0.05$. ID, iron deficient; IS, iron sufficient; M, maltodextrin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume, PAE, prenatal alcohol exposure; PLT, platelets; RBC, red blood cell count; RDW, red blood cell distribution width; WBC, white blood cell count.

(**Figure 1**). PAE did not alter the content of either protein in IS or ID dams, suggesting that PAE did not alter maternal iron storage.

In contrast, PAE produced marked alterations to the fetal iron transport apparatus. In fetal liver, these regulatory proteins responded appropriately to ID in the absence of PAE. Gestational ID alone decreased liver ferritin levels 49% ($P < 0.001$, **Figure 2**) and modestly increased TFRc content (29%, $P = 0.05$), whereas fetal liver transferrin, ferroportin, and divalent metal transporter 1 levels were unchanged compared with IS-maltodextrin controls. PAE did not alter these proteins' abundance under iron adequacy. However, compared with IS-maltodextrin controls, PAE unexpectedly increased liver ferritin by 66% ($P < 0.001$) compared with ID-maltodextrin to levels comparable with IS-maltodextrin. PAE also decreased divalent metal transporter 1 protein content ($P = 0.045$) independent of iron status. Under ID, PAE did not alter TFRc, transferrin, and ferroportin protein abundance in fetal liver.

With respect to the fetal brain, the local expression of iron transport proteins was appropriate for gestational ID. As expected, ID reduced brain ferritin content 59% ($P < 0.001$, **Figure 3**) and increased TFRc levels 39% ($P = 0.003$), reflecting the need for brain iron import (30). In contrast, PAE disrupted the expression of proteins mediating iron import into brain tissue. Under maternal IS, PAE sharply reduced brain ferritin content 54% ($P < 0.001$) to levels well below IS-maltodextrin and no different from ID-maltodextrin brains, reflecting its low iron concentration (Table 3). In addition, PAE and ID interacted ($P = 0.002$) to further reduce brain ferritin content by 52% ($P = 0.002$) compared with ID-maltodextrin and to

TABLE 3 Effect of iron status and prenatal alcohol exposure on liver and brain iron concentration and clinical blood counts of fetal rats on gestational day 20.5[1]

					P value		
	IS-M	IS-PAE	ID-M	ID-PAE	Iron	PAE	Iron × PAE
Liver iron, μg/g							
Total	155 ± 9[a]	182 ± 9[a]	104 ± 15[b]	166 ± 14[a]	0.005	<0.001	0.10
Nonheme	40.5 ± 2.9[b]	54.5 ± 2.1[a]	20.1 ± 2.2[c]	43.2 ± 3.4[b]	<0.001	<0.001	0.11
Brain iron, μg/g							
Total	20.6 ± 1.0[a]	16.2 ± 0.7[b]	14.3 ± 1.2[b]	14.9 ± 0.8[b]	<0.001	0.050	0.016
Nonheme	3.9 ± 0.1[a]	3.3 ± 0.1[b]	2.8 ± 0.1[c]	2.4 ± 0.1[c]	<0.001	<0.001	0.36
RBC, $\times 10^6$ cells/μL	1.2 ± 0.1[a,b]	0.9 ± 0.2[b]	1.6 ± 0.1[a]	1.1 ± 0.1[a,b]	0.047	0.003	0.80
Hemoglobin, g/dL	6.9 ± 0.6[a]	4.8 ± 0.7[b]	6.9 ± 0.6[a]	4.9 ± 0.3[b]	0.94	0.002	0.94
Hematocrit, %	20.9 ± 1.8[a,b]	13.7 ± 2.1[c]	21.8 ± 2.2[a]	15.6 ± 1.3[b,c]	0.40	0.001	0.81
MCV, fL/cell	167 ± 3[a]	163 ± 4[a]	144 ± 5[b]	145 ± 5[b]	<0.001	0.78	0.62
MCH, pg/cell	54.9 ± 1.5[a]	57.2 ± 2.7[a]	45.3 ± 1.3[b]	46.0 ± 0.5[b]	<0.001	0.47	0.70
MCHC, pg/dL	33.0 ± 0.5	35.0 ± 1.2	31.6 ± 0.6	31.8 ± 1.2	0.025	0.26	0.29
RDW, %	23.7 ± 1.3[c]	28.4 ± 0.5[b]	33.8 ± 1.4[a]	27.8 ± 0.8[b]	0.002	0.58	<0.001
PLT, $\times 10^3$ cells/μL	180 ± 57	111 ± 39	102 ± 25	144 ± 3	0.55	0.73	0.17
MPV, fL/cell	10.5 ± 0.2[b]	10.2 ± 0.1[b]	12.0 ± 0.5[a]	10.5 ± 0.6[b]	0.015	0.022	0.07

[1] Values are means ± SEMs, n = 3–4 except liver and brain iron, n = 6 litters (10–15 pups/litter). Labeled means within a row without a common superscript letter differ, $P < 0.05$. ID, iron deficient; IS, iron sufficient; M, maltodextrin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume, PAE, prenatal alcohol exposure; PLT, platelets; RBC, red blood cell count; RDW, red blood cell distribution width.

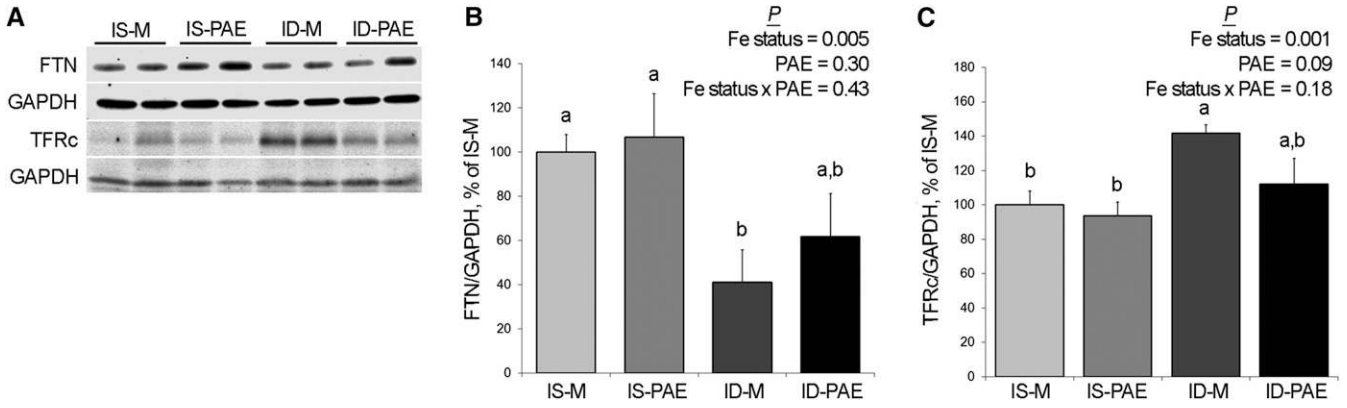

FIGURE 1 Effect of iron status and PAE on FTN and TFRc in hepatic rat dam tissue at gestational day 20.5. (A) Representative Western blot of maternal liver iron homeostatic proteins FTN (21 kDa) (B) and TFRc (90 kDa) (C), normalized to GAPDH (37 kDa). Values are means ± SEMs and expressed relative to IS-M, $n = 6$ litters (2 fetuses/litter). Means without a common letter are significantly different, $P < 0.05$. Figure panels present overall P values as determined by 2-factor ANOVA. See text for detailed statistical analysis. FTN, ferritin; ID, iron deficient; IS, iron sufficient; M, maltodextrin; PAE, prenatal alcohol exposure; TFRc, transferrin receptor.

only 20% ($P < 0.001$) of IS-maltodextrin expression. PAE reduced brain TFRc protein levels such that PAE blunted any ID-associated increase in TFRc ($P < 0.001$). The brain synthesizes its own transferrin for intercellular iron transport (31). Compared with controls, PAE reduced brain transferrin content nearly 50% regardless of iron status ($P < 0.001$). Neither PAE nor ID affected brain ferroportin and divalent metal transporter 1 abundance.

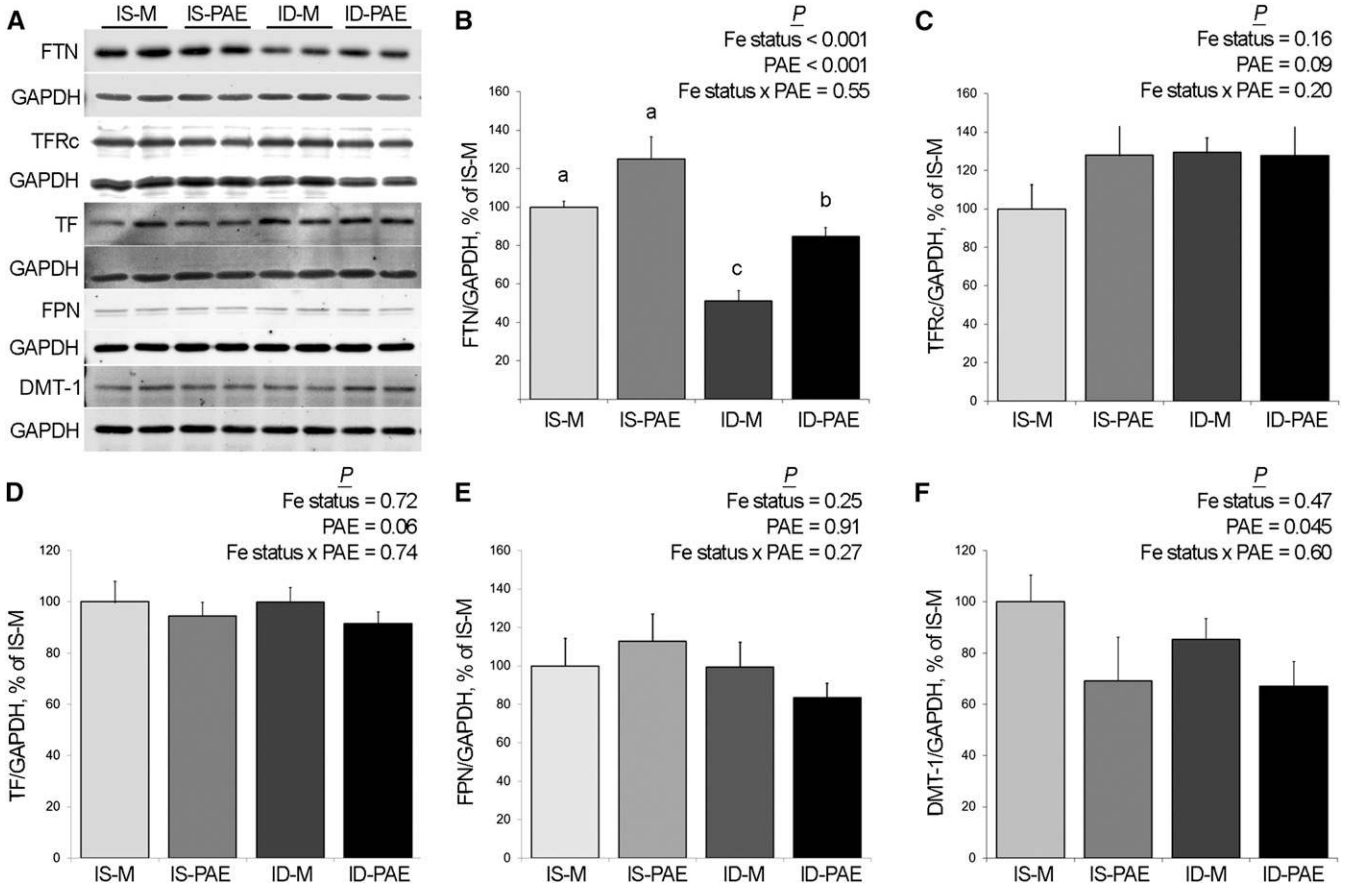

FIGURE 2 Effect of iron status and PAE on the fetal rat hepatic expression of FTN, TFRc, TF, FPN, and DMT-1 at gestational day 20.5. (A) Representative Western blot of fetal liver iron homeostatic proteins FTN (21 kDa) (B), TFRc (95 kDa) (C), TF (78 kDa) (D), FPN (65 kDa) (E), and DMT-1 (68 kDa) (F), normalized to GAPDH. Values are means ± SEMs and expressed relative to IS-M, $n = 6$ litters (2 fetuses/litter). Means without a common letter are significantly different, $P < 0.05$. Figure panels present overall P values as determined by 2-factor ANOVA. See text for detailed statistical analysis. DMT-1, divalent metal transporter 1; FPN, ferroportin; FTN, ferritin; ID, iron deficient; IS, iron sufficient; M, maltodextrin; PAE, prenatal alcohol exposure; TF, transferrin; TFRc, transferrin receptor.

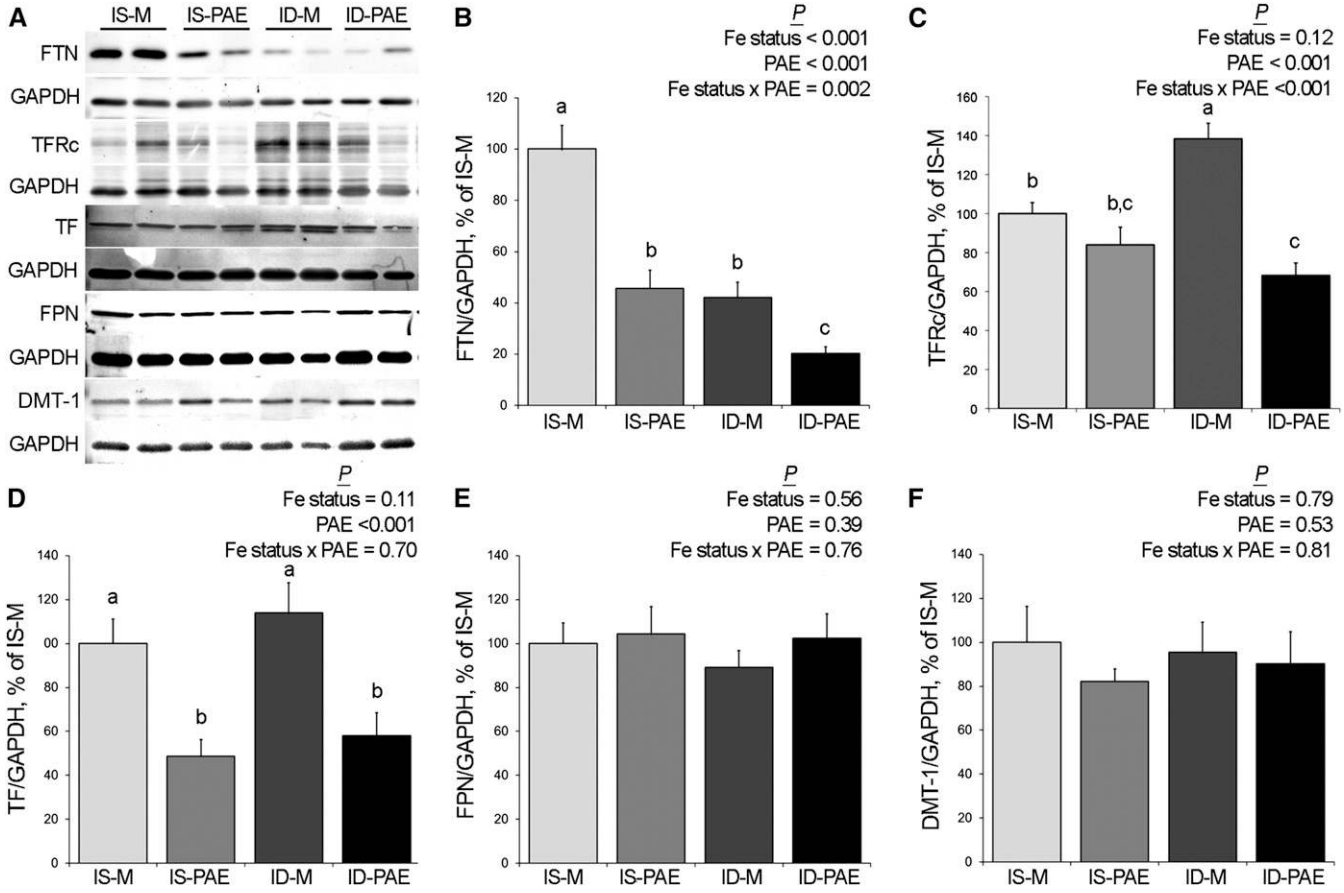

FIGURE 3 Effect of iron status and PAE on the fetal brain expression of FTN, TFRc, TF, FPN, and DMT-1 at gestational day 20.5. (A) Representative Western blot of fetal brain iron homeostatic proteins FTN (21 kDa) (B), TFRc (95 kDa) (C), TF (78 kDa) (D), FPN (65 kDa) (E), and DMT-1 (68 kDa). Values are means ± SEMs and expressed relative to IS-M, $n = 6$ litters (2 fetuses/litter). Means without a common letter are significantly different, $P < 0.05$. Figure panels present overall P values as determined by 2-factor ANOVA. See text for detailed statistical analysis. DMT-1, divalent metal transporter 1; FPN, ferroportin; FTN, ferritin; ID, iron deficient; IS, iron sufficient; M, maltodextrin; PAE, prenatal alcohol exposure; TF, transferrin; TFRc, transferrin receptor.

Maternal-fetal hepcidin gene expression. Given these alterations in iron transport proteins, maternal and fetal livers were analyzed for mRNA expression of hepcidin, the "master" hormone that regulates iron homeostasis (32). In an effort to increase the dams' iron absorption and mobilization, gestational ID alone repressed maternal liver hepcidin mRNA (**Figure 4**) to just 0.2% ($P < 0.001$) of IS-maltodextrin levels. In contrast, PAE unexpectedly increased dam liver hepcidin gene expression ($P < 0.001$) regardless of iron status, a strikingly unexpected outcome given that alcohol suppresses liver hepcidin expression in nonpregnant adults (11). Maternal liver hepcidin mRNA increased 287% ($P = 0.006$) in IS-PAE dams compared with IS-maltodextrin controls and 11,550% ($P < 0.001$) in ID-PAE dams compared with their ID-maltodextrin controls. Similar trends were observed in the fetus; gestational ID alone appropriately decreased fetal liver hepcidin mRNA nearly 70% ($P = 0.005$) compared with IS alone, whereas PAE again increased fetal liver hepcidin mRNA >300% in both IS and ID fetuses compared with their iron-matched control ($P < 0.010$). In addition, PAE increased liver hepcidin mRNA levels in ID fetuses to a level comparable to that of IS-maltodextrin livers.

Discussion

Somatic growth deficits partly typify individuals with FASD (3, 6), and findings herein are in line with previous demonstrations that ID and PAE interact to intensify the alcohol-associated growth restriction in the offspring (13, 14, 18). In young children who are diagnosed with fetal alcohol syndrome/FASD, concurrent ID-anemia is associated with the greatest reductions in head circumference and growth trajectory (13, 14). Those observations were corroborated in this rat model and found that the comorbidity of ID and alcohol acts to decrease the offspring's growth in both the prenatal (herein) and early postnatal periods (18, 19). The unexpected finding that PAE was associated with fetal anemia, irrespective of maternal iron status, offers an insight into a possible mechanism underlying this growth reduction, because anemia and its attendant hypoxia are well known to restrict fetal growth (33, 34). Whether a similar PAE-induced fetal anemia occurs in human pregnancy has not been explored. Such a condition might contribute to fetal alcohol syndrome/FASD growth restrictions by limiting fetal growth capacity, a mechanism that may be similar to other hypoxic states such as maternal smoking (35). Interestingly, maternal smoking also reduces the fetal iron endowment (36, 37), is associated with intrauterine growth restriction (37–39), and is frequently co-morbid with alcohol abuse; thus, pregnancies that are exposed to both PAE and smoking may have an exaggerated risk of intrauterine growth restriction, perinatal iron deficiency, and anemia.

PAE significantly altered fetal iron distribution in this rat model. During PAE, the fetal liver accumulated iron independent of maternal intake, a characteristic also seen in adult alcoholics

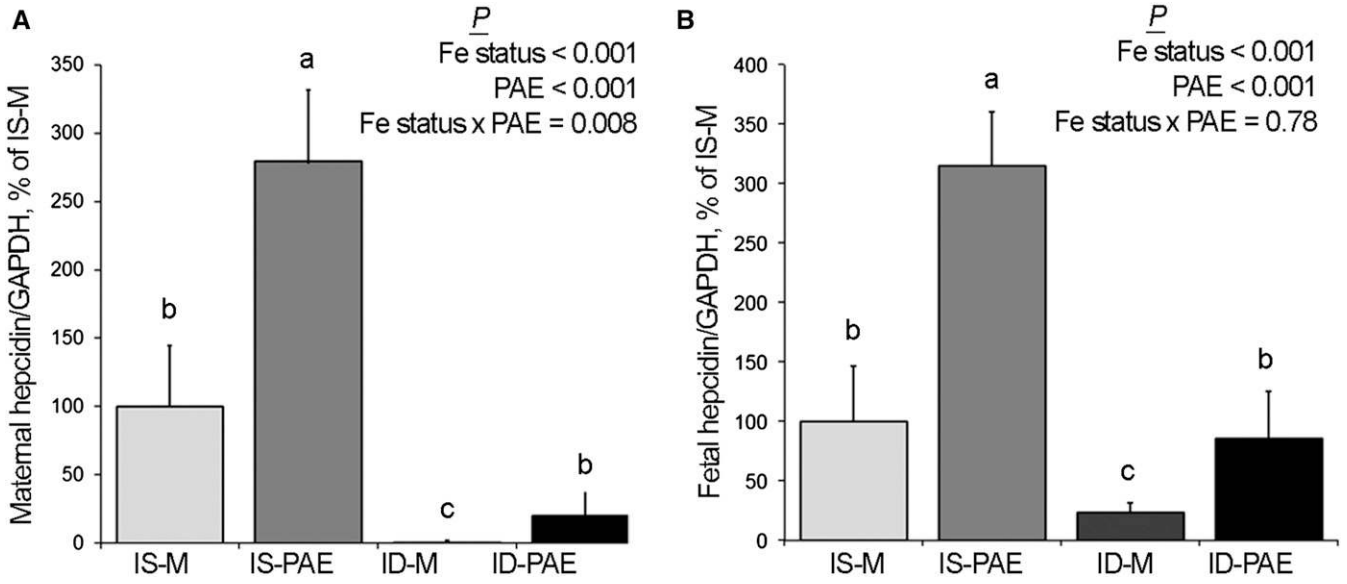

FIGURE 4 Effect of iron status and PAE on hepcidin expression in dam and fetal hepatic tissue at gestational day 20.5: maternal (A) and fetal (B), $n = 6$ litters (2 fetuses/litter). Values are means ± SEMs and expressed relative to IS-M. Means without a common letter are significantly different, $P < 0.05$. Figure panels present overall P values as determined by 2-factor ANOVA. See text for detailed statistical analysis. ID, iron deficient; IS, iron sufficient; M, maltodextrin; PAE, prenatal alcohol exposure.

and in alcohol-exposed neonatal rats (10, 11, 18, 40). Surprisingly, despite their elevated liver iron concentration, the PAE fetuses were anemic, and their anemia worsened when combined with ID. This disconnect between fetal hematopoiesis and liver iron content in PAE is notable because the bulk of fetal erythropoiesis is hepatic (41), and because even under conditions when iron is limiting, such as gestational ID, iron is typically prioritized to the fetal erythron at the expense of other tissues (42). The mechanism responsible for this disconnect between the erythron and iron stores in the PAE fetus is currently unknown. PAE may have reduced iron availability for fetal erythropoiesis, although hepatic iron concentrations were within the adequate range; the elevated fetal hepcidin may have a role here (see below). PAE may have also limited the availability of other nutrients important for red blood cell maturation such as folate (43) and/or may have directly impaired erythrocyte development and maturation, as has been reported for adult alcohol exposure (44). Supporting this latter interpretation, the anemias of the ID and PAE fetuses had distinctive characteristics. The former was microcytic and hypochromic, consistent with ID, whereas that of PAE had fewer red blood cells that were normocytic and normochromic, an appearance suggestive of impaired or incomplete erythropoiesis. Further investigation of this question is under way.

Data herein suggest that a partial explanation for the fetal brain's vulnerability to alcohol is that PAE significantly alters iron distribution between the fetal brain and liver. Although rat dams were iron sufficient, PAE lowered the iron concentration of the fetal brain to levels that did not differ from those of the ID fetus, creating a brain iron-deficiency state. Gestational ID causes lasting impairments in learning, cognition, executive function, and motor function, as shown both in clinical studies and in animal models of ID (8, 9). These impairments persist even after iron status is normalized because ID impedes processes that are fundamental for healthy brain development, including axonogenesis, synaptogenesis, neurotransmitter synthesis and degradation, and white matter formation (9, 45). Although alcohol itself is unquestionably a neurodevelopmental teratogen, PAE's ability to place the developing brain in an ID state may partly account for the overlapping impact of PAE and ID on brain development, because many of the same neurodevelopmental processes disrupted by ID are also impaired by alcohol (2, 3, 8, 9). The PAE-associated anemia may be further contributory. Accompanying the reduced brain iron under PAE was the apparent failure of neuronal populations to make adaptive changes that would otherwise enhance cellular iron uptake, such as increasing their transferrin and TFRc content, and instead the alcohol-exposed neurons had reduced concentrations of these iron transport proteins. Miller et al. (17) described similar maladaptive changes in brain regions in response to PAE, and those changes persisted into early adulthood, although normal, iron-adequate dams raised the neonates. These reports suggest that PAE may have reduced the ability of fetal tissues to sense and adapt to their iron status and that of the mother.

A potential explanation for this altered iron distribution may involve the concurrent dysregulation by PAE of hepcidin, the "master" regulator of iron homeostasis (32). Hepcidin operates as a negative regular of iron bioavailability, and liver hepcidin production increases under iron sufficiency to reduce iron uptake and promote its storage (32). The elevated hepcidin mRNA and altered iron transport protein content in the PAE fetal tissues are consistent with a mechanism that emphasizes liver iron storage rather than distribution to targets such as the brain and erythron. However, these changes in hepcidin are at odds with studies in nonpregnant adults, in whom chronic alcohol abuse represses hepcidin expression and enhances dietary iron absorption (11). Because hepcidin's gestational function is incompletely understood (46, 47), its response to alcohol during pregnancy may be dose dependent and/or distinct from that of the nonpregnancy state. In support of this, moderate alcohol exposure (blood alcohol concentration ~110 mg/dL) in a sheep gestational model did not alter maternal or fetal hepatic hepcidin expression (48). Attempts to resolve this by quantifying rat hepcidin peptide were unsuccessful. A potential explanation for the elevated hepcidin found here may be alcohol's proinflammatory action (49, 50), because inflammatory

signals stimulate hepcidin synthesis and signal the liver to sequester and limit circulating iron (32). Elevated hepcidin mediates the anemia of chronic inflammation by restricting iron availability for erythropoiesis (51), and it reduces brain iron by limiting its transport across the choroid plexus (52), changes that are consistent with findings in these alcohol-exposed fetuses. PAE promotes inflammatory responses in the fetal brain and liver (50), and preliminary work finds elevated IL-1β and TNFα production in these PAE fetuses (SM Huebner, SM Smith, unpublished results, 2016). Although more work is needed, this suggests that strategies to reduce inflammation and normalize hepcidin production may improve iron distribution for the alcohol-exposed pregnancy.

In summary, good data from both clinical studies and from animal models document that maternal iron status has a significant influence on FASD outcome, such that maternal ID enhances fetal vulnerability to alcohol's damage (13, 14, 18, 19). This study provides additional insight to this interaction, as PAE alters iron distribution such that the iron concentration of fetal brain and erythron is reduced, although maternal iron status and fetal liver iron concentration were adequate. In this model, markers of maternal iron status were neither predictive nor representative of PAE's effects on fetal iron metabolism, and this suggests that PAE may make it challenging to identify pregnancies at risk of fetal ID. Both anemic ID and nonanemic ID are common in pregnancy, including those exposed to alcohol (13, 53), and this places alcohol-exposed pregnancies at risk of worsened fetal outcomes due to limited maternal iron stores. However, because prevention of maternal ID clearly mitigates the adverse effects of PAE on growth, learning, myelin formation, and neuronal cellularity (18, 19), dietary iron will likely improve fetal iron status and reduce vulnerability to alcohol-induced injury. This has special relevance for communities where both alcohol abuse and ID are endemic, such as the Western Cape Province of South Africa (13, 14, 53). Increased focus on improving iron status during pregnancy is a likely successful strategy to address the significant public health problem of FASD and fetal alcohol syndrome.

Acknowledgments

We thank Peter Crump for assistance with statistical analysis and Juna Abazi for technical assistance. SMH and SMS designed the research; SMH and SEB conducted the research; SMH conducted the statistical analysis; SMH, SEB, PJK, and SMS wrote the manuscript; and SMS had primary responsibility for final content. All authors read and approved the final manuscript.

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