

Markers of Oxidative Stress and Systemic Vasoconstriction in Pregnant Women Drinking ≥ 48 g of Alcohol per Day

Caroline Signore, Sofía Aros, Jason D. Morrow, James Troendle, Mary R. Conley, Elizabeth Y. Flanigan, Fernando Cassorla, and James L. Mills

Background: The precise pathway by which alcohol causes the characteristic features of fetal alcohol spectrum disorders is unknown. Proposed mechanisms for fetal injury from maternal alcohol use include cellular damage from oxidative stress and impaired fetal oxygenation related to maternal systemic vasoconstriction. Our objective was to compare the levels of urinary markers of oxidative stress and systemic vasoconstriction between women consuming large amounts of alcohol during pregnancy and women who did not drink alcohol during pregnancy.

Methods: Pregnant women consuming ≥ 48 g alcohol per day ($n = 29$) on average and pregnant women who abstained from alcohol use ($n = 39$) were identified using detailed interviews and home visits. Random maternal urine specimens were collected. Urinary levels of the oxidative stress marker, 8-isoprostane F2 α , and of the vasoactive prostaglandin metabolites, 2,3-dinor-6-keto-prostaglandin F1 α (a vasodilator) and 11-dehydro-thromboxane B2 (a vasoconstrictor), were measured using mass spectrometric methods. All analyte levels were corrected for urinary creatinine.

Results: In crude analyses, there was no significant difference in 8-isoprostane F2 α between pregnant drinkers and nondrinkers (2.16 vs. 2.08 ng/mg creatinine, respectively, $p = 0.87$). There were no significant differences between the drinking and nondrinking groups in levels of 2, 3-dinor-6-keto-prostaglandin F1 α (1.03 vs. 1.17 ng/mg creatinine, respectively, $p = 0.50$), 11-dehydro-thromboxane B2 (0.72 vs. 0.59 ng/mg creatinine, respectively, $p = 0.21$), or the ratio of vasodilatory metabolite to vasoconstrictive metabolite (1.73 vs. 2.72, respectively, $p = 0.14$). Adjusting for maternal age, marital status, smoking, and gestational age at sampling did not substantially alter the results.

Conclusion: Our results show no difference in levels of urinary eicosanoid markers of oxidative stress and systemic vasoconstriction between pregnant women who drink heavily and pregnant women who abstain. These findings speak against a role for maternal oxidative stress or systemic vasoconstriction in the pathogenesis of alcohol damage to the fetus.

Key Words: Alcohol, Pregnancy, Isoprostanes, Prostacyclin, Thromboxane, Fetal Alcohol Spectrum Disorders.

THE FETAL ALCOHOL spectrum disorders (FASD) comprise a range of developmental abnormalities among children exposed to alcohol in utero. Fetal alcohol syndrome (FAS) is characterized by pre- or postnatal growth restriction, facial dysmorphology, and neurodevelopmental

disability (Centers for Disease Control, 2002a,b; Committee on Substance Abuse and Committee on Children With Disabilities, 2000). While the precise mechanism by which alcohol causes the characteristic features of FAS is unknown, a number of potential pathways have been proposed (West, 1994).

From the Epidemiology Branch, Division of Epidemiology, Statistics, and Prevention Research (CS, MRC, JLM), Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, United States Department of Health and Human Services, Bethesda, Maryland; Department of Pediatrics, School of Medicine (SA), University of Chile, San Borja Arriarán Clinical Hospital, Santiago, Chile; Division of Clinical Pharmacology, Departments of Medicine and Pharmacology (JDM), Vanderbilt University School of Medicine, Nashville, Tennessee; Biometry and Mathematical Statistics Branch, Division of Epidemiology, Statistics, and Prevention Research (JT), Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, United States Department of Health and Human Services, Bethesda, Maryland; Department of Pediatrics (EYF), Uniformed Services University of the Health Sciences and Walter Reed Army Medical Center, Washington, District of Columbia; and Division of Pediatric Endocrinology, Department of Pediatrics (FC), Institute of Maternal and Child Research, University of Chile, Santiago, Chile.

Ethanol consumption promotes systemic oxidative stress in humans, and some authors suggest that alcohol-related fetal brain injury may be a result of cellular damage from oxidative stress and reactive oxygen species (Cohen-Kerem and Koren, 2003; Guerri et al., 1994). Others theorize that abnormal brain development and intrauterine growth restriction may arise from impaired placental oxygen and nutrient delivery due to ethanol-induced vasoconstriction in the umbilical vessels and placental bed (Siler-Khodr et al., 2000; West, 1994).

The F2-isoprostanes are a class of prostaglandin-like compounds that are considered as the best markers of oxidative stress in vivo (Roberts and Morrow, 2000). These compounds are excellent candidates for exploring mechanisms of alcohol-related fetal injury because they both reflect the levels of oxidative stress and have potent intrinsic vasoconstrictive effects (Morrow, 2006). Thus, increased isoprostane levels may reflect oxidative injury to the fetal brain and may directly impair fetal growth via uteroplacental vasoconstriction. Isoprostane levels have not been measured in pregnant drinkers.

Ethanol may affect fetal development by a second eicosanoid pathway as well. It promotes vasoconstriction by reducing prostacyclin, a vasodilator, and by increasing thromboxane A2, a vasoconstrictor (Nanji et al., 1994; Siler-Khodr et al., 2000; Yokoyama et al., 2005). Ylikorkala et al. (1988) measured the urinary levels of prostacyclin and thromboxane metabolites in pregnant women who drank heavily during pregnancy. They reported a relative excess of thromboxane B2, suggesting that a vasoconstricted state is present in pregnant women who consume alcohol. However, the metabolites measured in their study are derived mainly from the kidney, and it is not clear whether they are a good measure of systemic balance between prostacyclin and thromboxane.

We measured the levels of urinary eicosanoids in a group of pregnant women known to be consuming ≥ 48 g alcohol per day and in a group of nondrinking pregnant women. Specifically, we examined levels of 8-isoprostane F2 α , the most widely studied isoprostane, to assess systemic levels of oxidative stress. In addition, we measured urinary metabolites of systemic vasoactive prostaglandins: 2,3-dinor-6-keto-prostaglandin F1 α , a vasodilator, and 11-dehydro-thromboxane B2, a vasoconstrictor. We hypothesized that if oxidative stress and prostanoid-related vasoconstriction were important mediators of alcohol-induced fetal injury, then we would detect increased isoprostanes and a relative excess of vasoconstrictive prostaglandin metabolites among women who drink heavily during pregnancy.

MATERIALS AND METHODS

Study subjects are a subset of a cohort of women enrolled in a study of outcomes in children exposed to large amounts of alcohol in utero. Detailed methods for identifying the full cohort of study participants are described elsewhere (Aros et al., 2006). Briefly, women ($n = 9,628$) presenting for prenatal care at the Maipú Clinic in Santiago, Chile, between August 1995 and July 2000, were questioned about alcohol use. There were 887 women who gave answers

suggestive of risky alcohol use (see Aros et al., 2006 for details), whom we further evaluated with home visits. Using information from the home visits, we identified 101 pregnant women who were consuming ≥ 48 g alcohol per day on average during pregnancy. We used the same method to identify a control group of nondrinking pregnant women ($n = 101$) matched for age (± 2 years) and parity (0 or ≥ 1). None of the women included in this study had a history of chronic hypertension, pregestational or gestational diabetes, gestational hypertension, or preeclampsia.

The larger study described above was underway, when sample collection for the current investigation began. Random urine specimens were collected during pregnancy from 26 of the matched drinking and nondrinking pairs. In addition, unpaired urine specimens were collected from 3 drinking gravidas and 13 nondrinking gravidas. Thus, samples were available for a total of 29 drinkers and 39 nondrinkers. Urine samples were stored at -70°C until analysis.

Subjects were classified as FAS if they demonstrated evidence of growth deficiency, characteristic facial dysmorphism, and neurodevelopmental abnormalities as described by Jones et al. in (1973). Growth deficiency was defined as weight or length ≤ 10 th percentile at any one or more assessments in infancy or childhood. Facial dysmorphism was determined by serial examinations by a geneticist. Specific features required for diagnosis were short palpebral fissure length, thin vermilion border, and flat philtrum. Neurodevelopmental impairment was based on evidence of microcephaly, results of developmental and cognitive testing (Bayley Scales of Infant Development II, Wechsler Preschool and Primary Scale of Intelligence, and Wechsler Intelligence Scale for Children), serial examinations by pediatric neurologists, and evaluation by a pediatric psychologist. We considered a child to have partial FAS if he exhibited abnormalities in 2 of the above 3 domains.

Free 8-isoprostane F2 α (8-iso-P), 2,3-dinor-6-keto-prostaglandin F1 α (2,3-dinor), and 11-dehydro-thromboxane B2 (11-dehydro-TxB2) were quantified as previously described using highly accurate and precise mass spectrometric methods (Daniel et al., 1994; Morrow and Minton, 1993; Morrow and Roberts, 1999). Precision of each of the 3 assays was $\pm 4\%$, $\pm 5\%$, and $\pm 7\%$, respectively. Intra- and inter-day variabilities were $< 10\%$. All results were corrected for intersubject differences in renal function and are expressed in ng/mg urinary creatinine. All analyses were conducted by personnel blinded to the alcohol exposure status of the subjects.

Visual inspection of the data demonstrated an outlying (> 3.2 standard deviations above the mean) 8-iso-P value in a control subject; this value was deleted from our final analyses.

Baseline characteristics of the drinking and nondrinking subjects were compared using the Wilcoxon rank sum test for continuous variables, and Fisher's exact test for categorical variables. Multiple linear regression was used to compare transformed values of eicosanoid levels after adjusting for maternal age, marital status, number of cigarettes smoked per day, and gestational age at sampling. To express the result in more meaningful units, the eicosanoid values were first log-transformed until approximately normally distributed, and then standardized by the control group mean and standard deviation. Regression analyses were conducted on both the set of matched pairs, and on the larger, unmatched study sample. Because the results of the matched and unmatched analyses did not differ substantially, only the unmatched analyses are presented. All data were analyzed using SAS v. 9.0 (SAS System, Cary, NC). A p -value of < 0.05 was considered statistically significant.

Written informed consent was obtained from all study participants. This study was approved by the Institutional Review Boards of both the National Institute of Child Health and Human Development and the San Borja Arriarán Hospital, which is affiliated with the University of Chile School of Medicine.

Table 1. Characteristics of the Study Sample

	Drinkers (≥48 g per day) <i>n</i> = 29	Nondrinkers, <i>n</i> = 39	<i>p</i> -value
Age (years)	23.4 ± 7.5	25.1 ± 8.1	0.37
Parity			0.63
0	16 (55.2)	19 (48.7)	
≥1	13 (44.8)	20 (51.3)	
Education (years)			0.65
<8	3 (10.3)	2 (5.1)	
8	6 (20.7)	4 (10.3)	
9–11	7 (24.1)	13 (33.3)	
12	7 (24.1)	10 (25.6)	
>12	6 (20.7)	10 (25.6)	
Marital status			0.12
Never married	23 (79.3)	23 (59.0)	
Married/divorced/widowed	6 (20.7)	16 (41.0)	
GA at sampling (weeks)	27.5 ± 7.4	26.6 ± 6.5	0.58
Current smoker	17 (58.6)	6 (15.4)	0.0003
Cigarettes per day (among current smokers)	6.3 ± 7.8	5.5 ± 3.7	0.80

GA, gestational age.

Values are expressed as mean ± standard deviation or number (percent).

RESULTS

Characteristics of the study population are presented in Table 1. There were no significant differences in age, parity, educational level, marital status, or gestational age at urine sample collection between drinking and nondrinking subjects. A significantly higher proportion of drinkers smoked during the index pregnancy.

As shown in Table 2, there were no significant differences in 8-iso-P, 2,3-dinor, 11-dehydro-TxB2, or the ratio of

Table 2. Urinary Eicosanoid Concentrations Among Drinking and Nondrinking Gravidas

	Drinkers <i>n</i> = 29	Nondrinkers <i>n</i> = 37	<i>p</i> -value ^a
8-Iso-P			
Mean ± SD	2.16 ± 1.11	2.08 ± 0.87	0.87
Median	1.86	1.88	
IQR	1.35–3.02	1.53–2.35	
Range	0.76–5.84	0.911–4.72	
2,3-Dinor			
Mean ± SD	1.03 ± 0.47	1.17 ± 0.60	0.50
Median	0.92	1.10	
IQR	0.750–1.374	0.73–1.396	
Range	0.150–2.24	0.194–2.988	
11-Dehydro-TxB2			
Mean ± SD	0.72 ± 0.44	0.59 ± 0.31	0.21
Median	0.57	0.54	
IQR	0.463–0.823	0.379–0.714	
Range	0.216–2.030	0.061–1.561	
2,3-Dinor/11-dehydro-TxB2 ratio			
Mean ± SD	1.73 ± 0.92	2.72 ± 3.18	0.14
Median	1.57	1.75	
IQR	1.067–2.438	1.322–2.927	
Range	0.50–3.47	0.64–19.34	

8-Iso-P, 8-isoprostane F2_x; 2,3-dinor, 2,3-dinor-6-keto-prostaglandin F1_x; 11-dehydro-TxB2, 11-dehydro-thromboxane B2.

Values are expressed as ng/mg creatinine.

^aWilcoxon rank sum test.

Table 3. Adjusted^a Mean Urinary Eicosanoid Levels^b in Drinkers Expressed as Standard Deviations (z scores) From the Control Mean

	Parameter estimate	SE	<i>p</i> -value
8-Iso-P	−0.13	0.30	0.67
2,3-Dinor	−0.37	0.24	0.13
11-Dehydro-TxB2	0.21	0.22	0.35
2,3-Dinor/11-dehydro-TxB2 Ratio	−0.43	0.22	0.06

8-Iso-P, 8-isoprostane F2_x; 2,3-dinor, 2,3-dinor-6-keto-prostaglandin F1_x; 11-dehydro-TxB2, 11-dehydro-thromboxane B2.

^aMultiple linear regression on z-score transformations, adjusted for maternal age, marital status, number of cigarettes smoked per day, and gestational age at sampling.

^bValues log-transformed as needed.

2,3-dinor/11-dehydro-TxB2 between pregnant women drinking ≥48 g alcohol per day and nondrinking pregnant women. Results of multiple linear regression analyses adjusted for maternal age, marital status, number of cigarettes smoked per day, and gestational age at sampling indicated no significant differences in urinary eicosanoids between drinking and nondrinking gravidas in either the matched or unmatched analyses (unmatched analyses, Table 3).

To investigate whether eicosanoid levels differed between women drinking most heavily at the time of sampling and nondrinkers, we conducted a subgroup analysis limiting the drinking group to those who reported heavy alcohol use through the second and third trimesters (*n* = 19/29, 66%). Of these 19 women, a substantial majority were binge drinkers; only 2 of 19 drank daily. On average, these women reported drinking 3.2 ± 1.8 days per week, and consumed 147 ± 142 g of ethanol per drinking day. There were no significant differences in 8-iso-P, 2,3-dinor, 11-dehydro-TxB2, or the ratio of 2,3-dinor/11-dehydro-TxB2 between this subgroup of highest quantity and frequency drinkers and nondrinkers (data not shown).

We compared maternal urinary eicosanoid concentrations between the subset of drinking pregnant women whose children were later diagnosed with FASDs (FAS, *n* = 1; partial FAS, *n* = 7) and concentrations in nondrinking pregnant women (*n* = 39), and found no significant differences (data not shown). Though statistically significant differences would not be expected with only 1 case, we hypothesized that isoprostane and vasoactive prostanoid levels would be most altered in the mother of the child with the most severe phenotype, i.e., FAS. Results for maternal 8-Iso-P (1.19 ng/mg creatinine), 2,3-dinor (1.14 ng/mg creatinine), 11-dehydro-TxB2 (0.95 ng/mg creatinine), and the 2,3-dinor/11-dehydro-TxB2 ratio (1.19) were not markedly different in the mother of the FAS case from the mean or median levels of these analytes in either the drinking or nondrinking mothers (Table 2).

DISCUSSION

Exposure to alcohol in utero is a leading cause of disability among children. Several mechanisms by which alcohol may exert its teratogenic effect have been proposed, including lipid

peroxidation resulting from oxidative stress and impaired placental function resulting from alterations in vasoactive prostaglandins. We assessed each of these processes in women who drank heavily during pregnancy and women who abstained from alcohol during pregnancy by testing maternal urine for isoprostanes and stable prostaglandin metabolites. We found no difference in urine levels of 8-isoprostane F_{2α}, 2,3-dinor-6-keto-prostaglandin F_{1α}, or 11-dehydro-thromboxane B₂ between pregnant women drinking ≥48 g alcohol per day and nondrinking pregnant controls.

Isoprostanes, a class of prostaglandin-like compounds that are generated from free-radical peroxidation of arachidonic acid (Basu, 2004), are widely held to be the most accurate markers for oxidative stress *in vivo* (Griffiths et al., 2002; Kadiiska et al., 2005; Montuschi et al., 2007; Roberts and Morrow, 2000; Tsimikas, 2006). Plasma isoprostanes have a short half-life, making urine the specimen of choice for measuring effects of nonacute exposures (Griffiths et al., 2002; Milne et al., 2007). These compounds are very stable in urine (Cracowski et al., 2002), and day-to-day fluctuations of urinary isoprostane concentrations in both health and disease are limited (Bachi et al., 1996; Meagher et al., 1999).

Isoprostanes not only reflect oxidative injury, but also have potent inherent biological activity, and may themselves mediate oxidative injury (Morrow, 2006; Morrow and Roberts, 1997; Morrow et al., 1999). 8-isoprostane F_{2α} is a potent vasoconstrictor (Morrow, 2006). Evidence suggests that 8-isoprostane F_{2α} may cause vasoconstriction in the placenta and thereby promote uteroplacental insufficiency and growth restriction. Exposure to 8-isoprostane F_{2α} reduces trophoblast invasion *in vitro* (Staff et al., 2000), and elevated isoprostane levels have been demonstrated in placentas of women with preeclampsia (Staff et al., 1999; Walsh et al., 2000), a condition known to be associated with impaired uteroplacental circulation and fetal growth restriction (Papageorgiou et al., 2004; Sibai et al., 2005). Alcohol exposure has been shown to increase markers of oxidative stress in placental villi *in vitro* (Kay et al., 2006). Ours is the first investigation of isoprostane levels in drinking and nondrinking pregnant women.

The vasodilator, prostacyclin, and the vasoconstrictor, thromboxane A₂, are rapidly metabolized *in vivo*. Levels of these metabolites may be indicators of general uteroplacental dysfunction. A number of authors (Chavarria et al., 2003; de Jong et al., 2000; Malatyalioglu et al., 2000; Trudinger et al., 1989; Walsh et al., 1993) have reported that alterations in levels of renally derived prostacyclin and thromboxane A₂ metabolites, 6-keto-prostaglandin F_{1α} (PGF_{1α}), and thromboxane B₂ (TxB₂), are associated with vasoconstriction and impaired fetoplacental blood flow, though others have found no such association (Sorem and Siler-Khodr, 1995; Ylikorkala et al., 1983, 1984), and none of these studies specifically assessed the effects of alcohol exposure on prostanoid concentrations. The compounds tested in the current study, 2,3-dinor-6-keto-prostaglandin F_{1α} and 11-dehydro-thromboxane B₂, are stable metabolites of PGF_{1α} and TxB₂,

respectively, and are produced systemically. Interestingly, alterations in urinary levels of these metabolites are associated with placental insufficiency in the form of preeclampsia (Mills et al., 1999).

There are fewer data on the effect of ethanol on umbilical vessel and placental tissue prostanoid production, and results are conflicting. In *in vitro* studies, ethanol exposure has been associated with increased (Siler-Khodr et al., 2000), decreased (Ylikorkala et al., 1987), or unchanged (Randall and Saulnier, 1995) levels of vasoconstrictive prostanoids in human umbilical vessels and placenta. Very few measurements of prostanoid metabolites in pregnant drinkers have been reported. One previous study of heavily drinking (140 g to 840 g ethanol per week during the first half of pregnancy or beyond) pregnant women, showed that urinary levels of renally derived prostanoid metabolites were altered favoring vasoconstriction (Ylikorkala et al., 1988). However, it is unlikely that urinary concentrations of these compounds (6-keto-prostaglandin F_{1α} and thromboxane B₂) reflect systemic effects capable of affecting placental blood flow (Daniel et al., 1994; Morrow and Minton, 1993). The vasodilator (2,3-dinor) and vasoconstrictor (11-dehydro-TxB₂) tested in the current study more accurately represent systemic effects; we found no difference between drinkers and nondrinkers in urinary levels of these compounds, or in their ratio.

Because isoprostanes and other vasoactive prostanoids could not be measured directly in the placenta, we cannot rule out the possibility that ethanol induces oxidative stress or prostanoid alterations localized to the uteroplacental circulation that are not reflected in maternal urine levels. A potential limitation of our study is that our sample size might have been too small to allow us to detect significant differences between the alcohol exposed and unexposed pregnancies. However, assuming a 2-sided analysis at the 5% level, our study had 80% power to detect median differences between drinkers and nondrinkers of 25 to 36% (relative to the control median) in each of our analytes. Thus, we have confidence that there are not major changes in these particular eicosanoids associated with heavy drinking in pregnancy. We are not concerned about an unmeasured confounding influence of antioxidant vitamin intake in our study because prenatal or other vitamins are not commonly used by pregnant women in Chile, and because it is not clear from the current literature whether antioxidant supplements affect urinary F₂-isoprostane levels (DeCaterina et al., 2002; Montuschi et al., 2007; Reilly et al., 1996). Another strength of our study was the rigorous assessment of maternal alcohol use and nonuse through home visitation and interviews.

In summary, our results show no difference in levels of urinary eicosanoid markers of oxidative stress and systemic vasoconstriction between women who drink heavily during pregnancy and women who abstain from alcohol during pregnancy. These findings speak against a role for maternal oxidative stress or systemic vasoconstriction in the pathogenesis of alcohol damage to the fetus.

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DISCLOSURE STATEMENT

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