ORIGINAL ARTICLE

Adverse Placental Effect of Formic Acid on hCG Secretion Is Mitigated by Folic Acid

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Abstract — Aims: Formic acid has recently been detected in maternal blood and umbilical cord blood of infants born to alcohol abusing mothers. This toxic metabolite of methanol requires folate for detoxification. We hypothesized that formic acid produced in the maternal circulation will transfer across the placenta and will be toxic to the placenta. Our objectives were, first, to determine whether formic acid transfers across the human placenta and whether it is toxic to the placenta and second, to determine whether folate can decrease transplacental transfer of formic acid and mitigate toxicity. Methods: Dual perfusion of a single placental lobule ex vivo was used to characterize the transfer of formic acid across the placenta. After a 1-h control period, formic acid (2 mM) was introduced into the maternal circulation with (n = 4) or without folate (1 µM) (n = 4) and was allowed to equilibrate for 3 h. Results: Formic acid transferred rapidly from the maternal to the fetal circulation, and transfer was not altered with the addition of folate. Compared with the control period, there was a significant decrease in hCG secretion (P = 0.03) after addition of formic acid. The addition of folic acid to the perfusate mitigated the decrease in hCG. Conclusions: Formic acid rapidly transfers across the placenta and thus has the potential to be toxic to the developing fetus. Formic acid decreases hCG secretion in the placenta, which may alter steroidogenesis and differentiation of the cytotrophoblasts, and this adverse effect can be mitigated by folate.

INTRODUCTION

Alcoholic beverages may contain a small amount of methanol as a congener (Lachenmeier et al., 2011). Furthermore, methanol is produced endogenously in the pituitary from S-adenosylmethionine (Axelrod and Daly, 1965; Sarkola and Eriksson, 2001). In heavy drinkers, methanol may accumulate since ethanol has a higher affinity for alcohol dehydrogenase (ADH) and may reach plasma concentrations above 2 mM (Majchorowicz and Mendelson, 1971; Kapur et al., 2007). Formic acid, the toxic metabolite of methanol, has been detected in both sera and cerebrospinal fluid of alcoholics in concentrations that are neurotoxic (Kapur et al., 2007). Formic acid has also been recently detected in maternal blood and umbilical cord blood of infants born to heavy drinkers (Kapur et al., 2009).

Formic acid requires folate for detoxification and folate status can influence the clearance rate (Johlin et al., 1987; Sokoro et al., 2008). Studies on rat brain hippocampal slices showed that formic acid can cause neuronal cell death, and this toxicity can be mitigated by folate (Kapur et al., 2007). Formic acid has also been shown in animal studies to lead to growth restriction, physical malformations and depletion of glutathione in the embryo (Brown-Woodman et al., 1995; Andrews et al., 1998; Harris et al., 2004; Hansen et al., 2005). Furthermore, human studies have reported fetal alcohol syndrome-like facial features in infants born after solvent abuse (including methanol) by the mother during pregnancy (Scherees and Chudley, 2002).

The placenta separates the maternal and fetal circulations throughout human pregnancy. Heavy alcohol consumption during pregnancy is associated with numerous placental alterations, including placental dysfunction, decreased placental size, gene expression changes and endocrine disruptions (Burd et al., 2007; Rosenberg et al., 2010). Since formic acid can produce oxidative stress (Harris et al., 2004), it may be placentotoxic. Compromised placental function is associated with growth restriction, a deficit associated with the fetal alcohol spectrum disorders (FASDs). Since the placenta is a reservoir for folate (Henderson et al., 1995), there may also be a potential role for detoxification of formic acid within the placenta itself. The objectives of the study were to first determine whether formic acid transfers across the placenta and is toxic to the placenta and, second, determine whether folate can decrease transplacental transfer of formic acid and mitigate potential toxicity.

MATERIALS AND METHODS

Materials

Sodium formate was obtained from Sigma (St. Louis, MO, USA). The water used for all experimental procedures was obtained from a Milli-Q Advantage A10 Ultrapure Water Purification System (Millipore, Billerica, MA, USA).

Placental perfusion

The dual perfusion of a placental lobule was previously described by Miller et al. (1985) and adapted in our laboratory (Derewlany et al., 1991; Pollex et al., 2008). Immediately after elective Cesarean sections, term placentas were obtained from healthy mothers with uncomplicated pregnancies, from St. Michael’s Hospital, Toronto, ON, Canada. Research ethics board approval (REB # 08-024) was obtained and maternal consent was attained prior to the surgery. For each placenta, a vein/artery pair supplying a clearly identifiable cotyledon was chosen for cannulation, and maternal and fetal circulations were established within 30 min of delivery (Derewlany et al., 1991).

The maternal perfusate was equilibrated with 95% O2, 5% CO2 and the fetal with 95% N2, 5% CO2. The perfusate...
consisted of M199 tissue culture medium (Sigma) containing heparin (2000 U/l), glucose (1.0 g/l), kanamycin (100 mg/l) and 40,000 molecular-weight dextran (maternal 7.5 g/l; fetal 30 mg/l). As a marker of passive diffusion, antipyrine (1 mM) was added to the maternal circulation. The fetal and maternal circuit flow rates were maintained at 2–3 and 13–14 ml/min, respectively, and the temperature of both circuits and the perfusion chamber was kept at 37°C. Small volumes of sodium bicarbonate and hydrochloric acid were added to the perfusate to maintain the maternal and fetal circuits at pH 7.4 and 7.35, respectively. This pH mimics the slightly more acidic fetal circulation observed in vivo (Reynolds and Knott, 1989).

Each perfusion consisted of a 1-h pre-experimental control period, followed by a 3-h experimental period, where both were in a recirculating (closed) configuration. The perfusates in both circulations were replaced with fresh media prior to each of the two periods. During the pre-experimental period, samples were taken every 15 min to analyze glucose and oxygen consumption, and human chorionic gonadotropin (hCG) secretion as measures of tissue viability. During the experimental period, these measures were taken every 30 min. Tissue viability measures were calculated as previously described (Pollex et al., 2008). Fetal perfusion pressure and fetal reservoir volume were monitored as an indicator of tissue integrity. The perfusion was terminated at any time if there was a >3 ml/h loss in fetal reservoir volume. pH, pO2 and pCO2 were monitored using a blood gas analyzer (Radiometer ABL 725, Copenhagen, Denmark).

During the experimental period, 2 mM sodium formate was added to the maternal circulation. Two millimolar was chosen since this is a clinically relevant concentration that was previously observed in heavy drinkers (Kapur et al., 2007). For perfusions that included folate, 1 μM was added into both the fetal and maternal circuits at the beginning of both the pre-experimental and experimental periods. Samples were taken for the measurement of formate and folic acid every 10 min for the first half-hour and every half-hour following.

Sample analysis

At the end of each perfusion, the perfused lobule was isolated. Perfused and unperfused tissue from the same placenta was homogenized (1:5 [w/v] in phosphate-buffered saline, pH 7.4) using a Polytron (Brinkmann Instruments, Inc., Westbury, NY) for 2 min. The homogenate was centrifuged at 5000 × g for 15 min at 4°C. The supernatant was removed and analyzed for both hCG and formic acid. hCG levels were measured using enzyme-linked immunosorbent assay (Alpha Diagnostic Intl. Inc., San Antonio, TX) after a 1:80 dilution.

Perfusate samples were stored at −20°C until analysis. Formic acid was detected by gas chromatography-flame ionization detection (GC-FID) using a previously published method (Sokoro et al., 2008). Area under the curve (AUC) was calculated using the trapezoidal rule. Tissue hCG levels of the perfused tissue are expressed as a percentage of the initial tissue concentration (Linnemann et al., 2000). Differences between the pre-experimental control and experimental periods were compared using a paired t-test and differences between perfusion with and without added folate were compared using an independent t-test. Differences were considered significant if $P < 0.05$.

RESULTS

A total of eight lobules from different placentae were perfused with formic acid (four with folate added and four without folate added) and the physical parameters for the perfusions are given in Table 1. The mass of the perfused cotyledons ranged from 8.53 to 17.12 g. Measures of placental viability, integrity and function throughout the experiments remained within normal ranges and were not significantly different between the control and experimental phases (Table 1). Furthermore, the fetal arterial pressures remained similar between the control and experimental periods. The rate of antipyrine disappearance from the maternal circulation was equal to the rate of appearance in the fetal circulation during the experimental period with values of $0.028 ± 0.004$ and $0.029 ± 0.006 \text{ μmol/g} / \text{min}$, respectively.

Formic acid transferred rapidly from the maternal to the fetal circulation (Fig. 1). In the presence or absence of folate to the perfusate, formic acid appeared in the fetal circulation within 10 min in all eight perfusions. The addition of folate into the perfusate did not alter the fetal AUC ($1.30 ± 0.14$ without folate; $1.23 ± 0.48$ with folate; $P = 0.79$). Tissue concentrations of formic acid measured in the perfused lobules at the completion of the experiment were $425.83 ± 57.18$ and $431.18 ± 133.07 \text{ nmol/g}$ for perfusions without and with folate added, respectively.

Compared with the pre-experimental control period, there was a significant decrease in the rate of hCG secretion in the maternal circulation after the addition of formic acid in the experimental period ($P = 0.03$) (Fig. 2). In contrast, there was no significant decrease when folate was present in the

<table>
<thead>
<tr>
<th>Viability parameter</th>
<th>Formic acid without folate (n = 4)</th>
<th>Formic acid with folate (n = 4)</th>
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</thead>
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<tr>
<td>Fetal arterial inflow pressure (mm Hg)</td>
<td>Pre-experimental control</td>
<td>Experiment</td>
</tr>
<tr>
<td>Oxygen (μmol O2/g/min)</td>
<td>43.65 ± 6.02</td>
<td>41.24 ± 2.15</td>
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<tr>
<td>Transfer</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.01</td>
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<tr>
<td>Delivery</td>
<td>0.75 ± 0.33</td>
<td>0.72 ± 0.29</td>
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<tr>
<td>Consumption</td>
<td>0.35 ± 0.16</td>
<td>0.32 ± 0.13</td>
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<tr>
<td>Glucose consumption (μmol/g/min)</td>
<td>0.65 ± 0.58</td>
<td>0.42 ± 0.11</td>
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<tr>
<td>Lactate production (μmol/g/min)</td>
<td>0.38 ± 0.11</td>
<td>0.25 ± 0.06</td>
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<tr>
<td></td>
<td>Pre-experimental control</td>
<td>Experiment</td>
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<tr>
<td></td>
<td>41.50 ± 12.24</td>
<td>38.62 ± 9.05</td>
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<td>0.62 ± 0.09</td>
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<td>0.41 ± 0.09</td>
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<td>0.28 ± 0.17</td>
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perfusate. The percentage of initial placental tissue hCG was decreased in the perfusions without folate compared with perfusions with folate ($P = 0.04$) (Fig. 3).

**DISCUSSION**

The small molecule, formic acid, crosses the placenta rapidly. Since formic acid can reach high concentrations in heavy drinkers (Kapur et al., 2007, 2009), our results suggest that any formic acid produced by the mother is likely to be transferred to the fetus in utero. Although there are a lack of in vivo human studies regarding the developmental toxicity of formic acid (Ema et al., 2010), this molecule has the ability to cause neurotoxicity, growth restriction, physical malformations and depletion of fetal glutathione in animal studies (Brown-Woodman et al., 1995; Andrews et al., 1998; Harris et al., 2004; Hansen et al., 2005; Kapur et al., 2007).

Our current study demonstrates that formic acid is placentotoxic in that placental hCG secretion was decreased. Other toxic compounds have been shown to decrease or inhibit placental hCG secretion including cocaine, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a polychlorinated dibenzo-p-dioxin/polychlorinated dibenzo-p-furan (PCDD/PCDF) mixture, difluoromethylornithine (DFMO), bisphenol A, benzo[a]pyrene and high levels of zidovudine (AZT) (Moore et al., 1988; Zhang et al., 1995; Simone et al., 1996; Boul et al., 1997; Augustowska et al., 2007; Morck et al., 2010). Since formic acid may be present in the maternal circulation at the same time as ethanol, further studies should evaluate how these two agents interact to alter hCG secretion since in vitro studies in human trophoblasts demonstrated that ethanol can increase hCG secretion (Karl and Fisher, 1993; Karl et al., 1994, 1998; Wimalasena et al., 1994). Alterations in hCG secretion by the placenta may have consequential implications for the pregnancy since hCG is vitally important in maintaining the pregnancy, regulating blood flow and delivering nutrients to the conceptus (Boal et al., 1997). In early pregnancy, hCG maintains the corpus luteum for progesterone production. Even in later stages of pregnancy, hCG continues to have numerous functions that have been recently reviewed by Cole (2010). Briefly, hCG promotes cellular differentiation in the placenta to make syncytiotrophoblast cells (Cronier et al., 1994); blocks the maternal immunological response to invading placental cells (Akoum et al., 2005); promotes uterine and umbilical cord

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**Fig. 1.** Maternal-to-fetal transfer of formic acid after dual perfusion of a single placental lobule in the presence ($n=4$) or absence ($n=4$) of folate (1 μM). Formic acid (2 mM) was added to the maternal circulation and transfer was determined for a period of 180 min. Data are shown as mean values ± SEM at each time point.

**Fig. 2.** The rate of hCG secretion into the maternal circulation during the placental perfusion was calculated in the pre-experimental control period (before addition of formic acid) and during the experimental period in the (A) presence ($n=4$) and (B) absence ($n=4$) of folate. (C) The rate of hCG secretion was compared between the experimental period in the presence and absence of folate.
needed to characterize the full dose–perfusion model is very tedious, future
one concentration of formic acid, 2 mM. Since the placental
is present in the blood (Majchrowicz and Mendelson, 1971; Andrews
et al., 2007). Studies evaluating fetal effects of formic acid
using rodent whole-embryo culture have demonstrated
concentration-dependent toxicity (Brown-Woodman et al., 1995; Andrews et al., 1998; Hansen et al., 2005). After microinjection of sodium formate into the amniotic fluid of
pregnant mice and rats, incomplete fetal axial rotation was
observed at concentrations as low as 0.029 mM (Hansen
et al., 2005).
A limitation of our results is that we evaluated toxicity at
one concentration of formic acid, 2 mM. Since the placental
perfusion model is very tedious, future in vitro studies are
needed to characterize the full dose–response relationship for
placental toxicity. Formic acid in heavy drinkers and in preg-
nant women has not been characterized in large populations.
It is possible that higher concentrations of formic acid may
be obtained as methanol may accumulate as long as ethanol
is present in the blood (Majchrowicz and Mendelson, 1971; Kapur
et al., 2007). To properly characterize formic acid
congratulations in pregnant alcohol-consuming women, studies are needed that capture the full time–concentration
curve and these patients will be difficult to recruit. However,
theoretically, we expect formic acid concentrations to reach
similar levels in pregnant women compared with non-
pregnant adults. Preliminary results from our group show
formic acid concentrations as high as 140 and 533 μM in
maternal blood and umbilical cord blood, respectively,
although these samples were not obtained at times where
peak formic acid concentrations are expected. In non-
pregnant adults, a serum formic acid concentration of 2 mM
was observed with a blood alcohol concentration of 150 mM
(Kapur et al., 2007). This would correspond to consuming
over 12 standard drinks on one occasion. Binge drinking at
and exceeding this level has been reported in pregnant
women (Malone et al., 2010; Kuehn et al., 2012), thus it is
likely that similar concentrations of formic acid are achieved
in this population.
We have recently shown that folate transport to the fetus is
compromised in pregnancies affected by heavy and chronic
alcohol exposure (Hutson et al., 2012). Although we did not
focus on FASDs, one could speculate that with decreased
folate transport there will be an increase in formic acid
which may play a significant role in the etiology of FASDs.

CONCLUSIONS
In summary, formic acid at peak concentrations observed in
heavy drinkers can rapidly transfer across the placenta.
Formic acid may be harmful to the developing fetus, and our
results demonstrate that it is also directly placentotoxic.
Formic acid decreases hCG secretion in the placenta, which
may alter steroidogenesis and differentiation of the cytotro-
phoblasts, and this can be mitigated by folate.

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