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REDUCTION OF FATTY ACID ETHYL ESTER ACCUMULATION BY GANGLIOSIDE GM1 IN RAT FETUS EXPOSED TO ETHANOL

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Abstract—The biochemical mechanism of alcohol teratogenicity is not known. We have demonstrated that alcohol administration to pregnant rats during gestation days (GD) 6 and 7 and/or 13 and 14 leads to significant accumulation of ethyl esters of long chain fatty acids (FAEEs) in both maternal and fetal organs. This observation extends the report of Bearer *et al.* (*Pediat Res* **31**: 492–495, 1992) who detected the presence of metabolites in maternal and fetal organs of pregnant C57Bl/6J mice exposed to alcohol on GD 7 and/or GD 14. The ethyl esters of arachidonic, linoleic, oleic, stearic and palmitic acids were major metabolites detected in both maternal and fetal organs. It was also demonstrated that detectable levels of FAEEs remain 14 days (GD 20) after initial exposure to alcohol on GD 7. Ganglioside GM1 treatment 1 and 24 hr prior to alcohol exposure on both GD 7 and/or GD 14 reduced the accumulation of FAEEs. A similar regimen was shown to prevent development of tolerance to alcohol in the adult pups exposed to alcohol *in utero* in our previous studies. Thus, the ganglioside GM1 may have therapeutic value in reducing neurobehavioral effects of alcohol exposure *in utero*.

Key words: gestational days; fetal alcohol effects (FAE); fatty acid ethyl esters (FAEEs); ganglioside GM1

Alcohol abuse during pregnancy is linked to a wide range of birth defects of varying severity [1–3]. Acute exposure to alcohol for 1–2 days at a critical period during gestation is shown to produce birth defects in mice [4–7]. The possible long-term effects of an acute intoxication during GD† 8 on the sensitivity to hypnotic effects of alcohol in adult offspring have been studied [8, 9]. In a rat study, administration of a total alcohol dose of 18 g/kg on GD 14 and GD 15 (a critical period in cerebral cortex development) resulted in a thinner, severely disorganized cerebral cortex [10]. Thus, even a short duration of alcohol exposure as compared with chronic ingestion can lead to permanent neurobehavioral deficits. The mechanisms that underlie CNS pathologies associated with both chronic (FAS) and binge-like alcohol exposure (FAE) are unknown. The primary focus of studies aimed at defining mechanisms that underlie prenatal alcohol exposure are placental dysfunction, nutritional deficiency, acetaldehyde toxicity, fetal hypoxia and the role of prostaglandins [11].

Recently, it was demonstrated that acute exposure to alcohol on GD 7 and/or GD 14 leads to significant accumulation of FAEEs, non-oxidative metabolites of ethanol, in the placenta and fetus [12]. In both humans and animals, these metabolites have been shown to accumulate in certain organs that do not metabolize alcohol oxidatively, and that are frequently damaged by alcohol abuse [13, 14]. These lipids are known to bind to mitochondria and uncouple oxidative phosphorylation in mitochondrial preparations [15]. Thus, the presence of these metabolites during a critical period of fetal growth may be injurious and may interfere with normal development of the CNS. This may eventually lead to behavioral manifestations in adulthood.

Exogenous gangliosides are being used therapeutically to treat a variety of neuronal disorders including peripheral neuropathies, spinal cord injury and stroke [16]. We have reported previously that pretreatment of pregnant rats with ganglioside GM1 at 24 hr and 1 hr, prior to alcohol exposure on GD 7 and 8 prevents the development of acute tolerance to alcohol in the adult pup [17]. We investigated the efficacy of GM1 in reducing the alcohol-induced accumulation of FAEEs in various fetal and maternal organs. The results suggest that GM1 pretreatment reduced the accumulation of FAEEs in both fetus and maternal organs.

MATERIALS AND METHODS

Animal protocol. The procedure for alcohol administration is essentially as described by Bearer *et al.* [12] for their FAEE studies in C57Bl/6J mice. The body weight of mothers on GD 4 ranged from 240 to 260 g. Dams were weighed and housed together with other pregnant females until GD 5. On GD 5, the dams were divided into four groups. Group A received ganglioside GM1 (Fidia Corp., Italy, 10 mg/kg, i.m. in saline) on GD 5 and GD

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† Abbreviations: AAEE, arachidonic acid ethyl ester; FAE, fetal alcohol effects; FAEE, fatty acid ethyl ester; FAS, fetal alcohol syndrome; GD, gestation day; and PLA_2, phospholipase A_2.

12, i.e. 24 hr before alcohol administration. Alcohol (5.8 g/kg) in two doses of 2.9 g/kg, 24% (v/v) in saline, spaced 4 hr apart (10:00 a.m. and 2:00 p.m.) was administered i.p. on GD 6 and GD 7 and/or GD 13 and GD 14, following i.m. injection of GM1 (10 mg/kg) 1 hr prior to alcohol administration. Group B received only alcohol solution following vehicle i.m. injection as in group A instead of ganglioside GM1. Group C received only i.m. vehicle and the same volume saline i.p. on GD 6 and 7 and/or GD 13 and 14. Group D received only i.m. injections of GM1. Two hours after the second dose of alcohol, the animals were killed, and fetuses, fetal placenta, maternal brain, liver and heart were collected and analyzed for FAEEs. Additional animals were maintained *ad lib.* and killed on GD 14 and GD 20 to see how long the FAEEs persisted after the initial dose of alcohol on GD 6.

Extraction and isolation of FAEEs. FAEEs were extracted from the tissue as described earlier [14]. To 1 g of tissue was added 10 μg (33.3 nmol) of heptadecanoic acid ethyl ester (internal standard, Sigma Chemical Co., St. Louis, MO) in 10 mL of cold acetone. The homogenate was centrifuged at 2000 *g* for 10 min. The residue was re-extracted with 10 mL of cold acetone. The acetone extract was chromatographed over a column of 1 g Unisil (silicic acid, Clarkson Chemical Co., Williamsport, PA), and FAEEs were recovered by elution with 10 mL of petroleum ether (40°–60°). The eluate was evaporated to dryness under nitrogen, reconstituted in 50 μL cyclohexane and analyzed by GC with flame ionization detector (FID) or by GC/MS [14].

Identification and quantitation of FAEEs. A Hewlett-Packard 5830 gas chromatograph interfaced with a computer integrator and equipped with a glass column (2 m long and 2 mm i.d.), packed with 10% SP 2330 on Chromosorb 100/120 WAW, was employed. Conditions were: oven temp., 200°; injection temp., 240°; FID temp., 275°; and carrier (helium) flow, 30 mL/min. Ethyl esters were identified by comparison of GC retention times with the standard ethyl esters and were quantitated by comparison of integrated peak area to the areas of known amounts of internal standard (IS) (heptadecanoic acid ethyl ester). For GC/MS identification of the compounds, a Hewlett–Packard model 5985 GC/MS with electron impact ionization, equipped with a column packed with 10% SP2330 on 100/120 Chromosorb, was used. The oven temperature was held at 200°, and carrier (helium) flow was 30 mL/min. The ionization energy was 70 eV [14].

RESULTS

FAEE accumulation in fetal tissue and placenta. FAEEs were identified by comparison of GC retention times with those of standards. Further characterization was accomplished by comparison of the fragmentation pattern with standard FAEEs using GC-electron ionization-MS (GC-EI-MS). The MS fragmentations of the unknown FAEEs were identical when compared with the authentic FAEE standards. A typical gas chromatogram of FAEEs extracted from fetal tissue exposed to alcohol on GD 6 and GD 7 is shown in Fig. 1. Alcohol exposure on GD 6 and 7 (total alcohol dose of 11.8 g/kg) resulted in significant accumulation of AAEE (C20:4) in combined fetal and placental tissue (Fig. 2). Palmitic (C16:0), stearic (C18:0), oleic (C18:1) and linoleic (C18:2) acid ethyl esters were also found to accumulate in measurable quantities. Similarly, alcohol exposure on GD 6, 7, 13, and 14 (total alcohol dose of 23.6 g/kg) led to accumulation of highly significant amounts of these major FAEEs in both fetal tissue and placenta (Fig. 3, A and B). The placenta appeared to have 2 to 4-fold higher level of FAEEs compared with fetal tissue. Although AAEE was a major metabolite accumulating in the placenta, there was also significant accumulation of palmitic, stearic, oleic and linoleic esters. Control animals (normal saline control and GM1 alone control) did not have any detectable levels of FAEEs.

FAEE accumulation in maternal organs. The FAEE profile from maternal liver and heart exposed to alcohol on GD 6 and 7 is shown in Fig. 4. It is interesting to note that at this time FAEEs were not detectable in the maternal brain. One possible explanation would be that the synthesis and accumulation of FAEEs may be influenced by gestational hormonal changes, especially during the late gestational period. The FAEE profile from maternal liver, heart and brain exposed to alcohol on GD 6, 7, 13 and 14 (total alcohol dose of 23.8 g/kg) is shown in Fig. 5. FAEEs were not detected in the organs from control groups. Although AAEE was the major metabolite in all the organs examined, other FAEEs (palmitate, stearate, oleate and linoleate) also accumulated in considerable amounts (liver, heart, brain).

These findings are consistent with the results reported by Bearer *et al.* [12]. The levels of FAEEs in maternal organs found in the current study (brain, 60 nmol; heart, 231 nmol; and liver, 303 nmol) were lower than the values (heart, 300 nmol; and liver, 900 nmol) reported by Bearer *et al.* [12]. On the other hand, fetal (51 nmol) and placental (196 nmol) levels were 10 to 20-fold higher than the values (11–16 nmol) reported by Bearer *et al.* [12]. If one excludes the AAEE from the current studies, the tissue specificity appears to be similar in the two studies. These investigators also reported that fatty acid moieties of FAEEs were different in different tissues, e.g. maternal placenta and fetal tissue had C18:0, whereas maternal heart and liver had C18:1, as predominant fatty acids [12]. Our results suggest that in all the tissues examined C20:4 (AAEE) appears to be the predominant fatty acid. The observed discrepancies may be due to the differences in the species used in these studies (C57Bl/6J mice vs Wistar rats in the current study) and/or the period and dosage of alcohol exposure (GD 7, 5.8 g/kg vs GD 6 and 7, 11.6 g/kg; GD 7 and 14, 11.6 g/kg vs GD 6 and 7 and GD 13 and 14, 23.2 g/kg). In any event, FAEEs do accumulate in substantial quantities in all the tissues examined. The significance of their presence in FAE and FAS remains to be established.

Effect of GM1 treatment on FAEE accumulation. Gas chromatographic analysis of FAEEs (Fig. 1, A and B) from fetal tissue exposed to alcohol on GD 6 and 7 suggests that GM1 treatment prior to alcohol

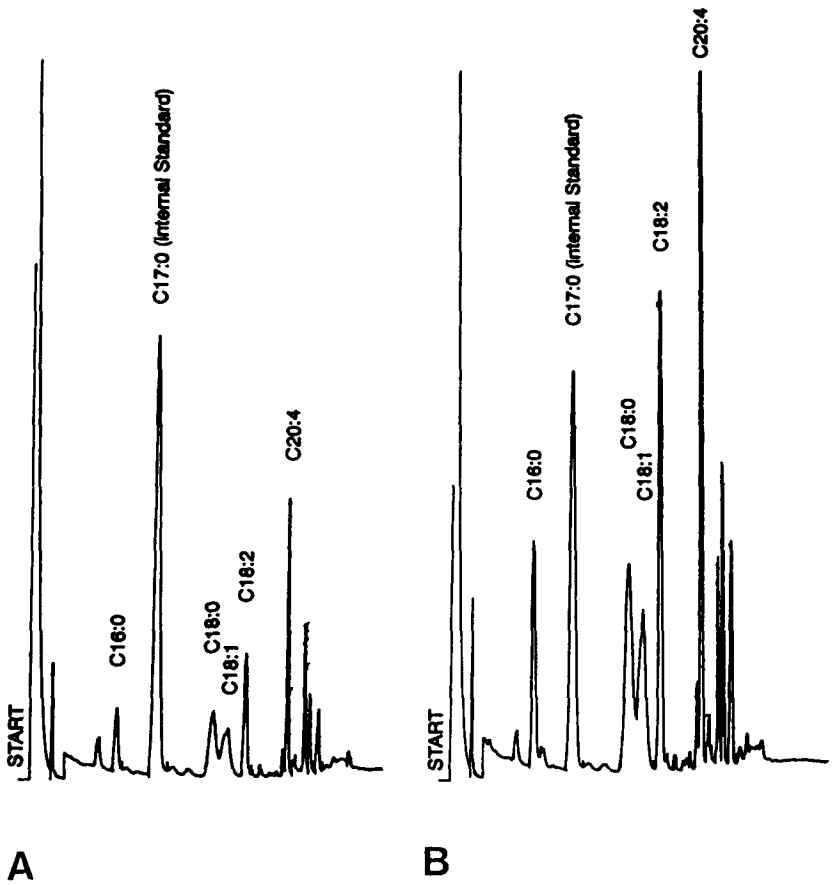

Fig. 1. Gas chromatogram showing analysis of FAEEs from rat fetal tissues exposed to alcohol on GD 6 and 7: (A) alcohol and GM1, (B) alcohol only.

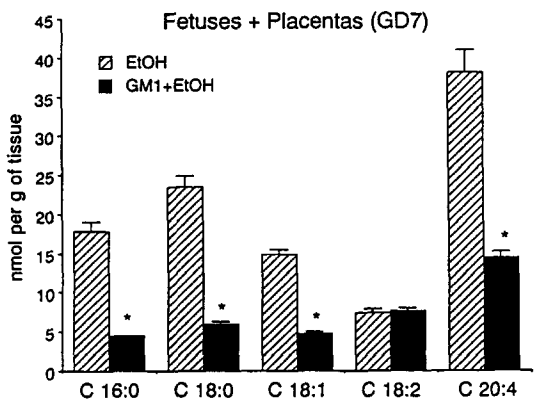

Fig. 2. FAEE analysis on GD 7 of fetal tissues exposed to alcohol on GD 6 and 7. Values are means ± SEM, N = 12 for each group. Key: (*) $P < 0.001$, paired *t*-test.

exposure significantly reduced the accumulation of FAEEs (Fig. 2). A similar effect was observed in fetal tissue and placenta that were exposed to alcohol on GD 6, 7, 13 and 14 (Fig. 3, A and B). Placenta appears to have the maximum accumulation of FAEEs. GM1 treatment also reduced the accumulation of FAEEs in maternal organs (Figs. 4 and 5).

FAEE analysis on GD 14 and GD 20 after initial exposure to alcohol on GD 6 and 7 only suggests that the fetal tissue contained a significant amount of FAEEs on GD 14, similar to that reported by Bearer *et al.* [12] and continued to be present in trace amounts on GD 20 (data not shown). These results suggest that FAEEs once formed remain in the organs for a long period of time. This sustained presence may lead to organ damage and subsequent neurobehavioral abnormality in adulthood.

DISCUSSION

One key question in alcohol research has been, how much alcohol is too much and when is the fetus at greater risk during gestation. While heavy drinking throughout gestation leads to FAS, episodic binge drinking at high levels results in partial expression of a syndrome unique to the gestational period of exposure: FAE. The animal model (binge drinking) employed in this study has been shown to produce teratogenic effects [8, 9]. Studies relating to biochemical mechanisms underlying the FAE and

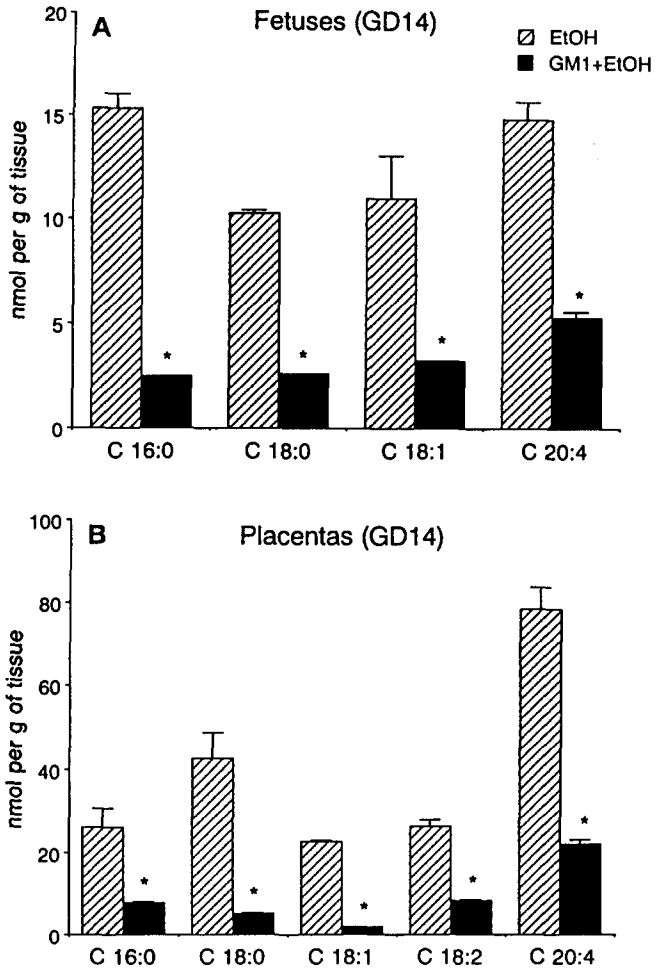

Fig 3. FAEE analysis on GD 14 of (A) fetal and (B) placental tissue exposed to alcohol on GD 6, 7, 13 and 14. Values are means ± SEM, N = 11 for each group. Key: (*) $P < 0.001$, paired *t*-test.

FAS are limited. The recent report by Bearer *et al.* [12], who showed significant accumulations of FAEEs, seems to suggest that FAEEs may play a role in fetotoxicity leading to FAE and FAS.

The findings reported here reflect on a possible biochemical abnormality that correlates with the teratogenic effects of binge drinking during pregnancy. These results confirm the findings of Bearer *et al.* [12] and extend the therapeutic intervention with ganglioside GM1 to preventing/reducing FAE. The persistence of marked levels of FAEEs in the fetal tissue up to 14 days or more after initial alcohol exposure is of significance. Their formation is probably the result of the action of alcohol on the plasma membrane. Alcohol may destabilize the membrane by the activation of PLA_2, thereby releasing the FFAs from membrane phospholipid bilayer. The released FFAs will form FAEEs through fatty acid ester synthase, an enzyme found in most organs especially in extra-hepatic organs that lack alcohol dehydrogenase [13]. FAEEs are known to cause mitochondrial dysfunction, which may, in turn, cause injury to the organs of the alcohol-exposed animals or humans [15]. The presence of these non-oxidative metabolites (FAEEs) may produce injurious effects particularly in the developing fetus. This may lead to abnormal synaptic connections and brain development, resulting in the behavioral manifestations reported in adulthood. These results also suggest that GM1 treatment prior to alcohol exposure reduces FAEE accumulation in both maternal and fetal organs. Following a similar experimental protocol, we have shown previously that *in utero* alcohol exposure on GD 7 and 8 leads to the development of tolerance to alcohol (sleep time) in adult pups (45 days old), and ganglioside GM1 treatment prior to alcohol exposure reduced this effect [17]. Thus, the presence of a large amount of FAEEs during critical periods of brain development may lead to the observed behavioral abnormality.

There are several plausible mechanisms to explain how GM1 reduces the deleterious effects of alcohol. One possible mechanism would be that GM1 inhibits alcohol-induced PLA_2 activity and thereby minimizes the availability of free fatty acid substrate for the

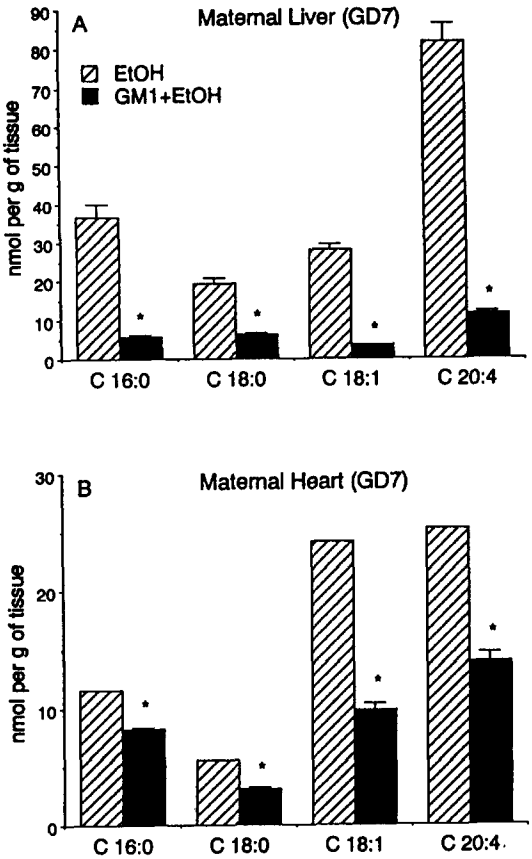

Fig. 4. FAEE analysis on GD 7 of maternal (A) liver and (B) heart exposed to alcohol on GD 6 and 7. Values are means ± SEM, N = 6 for each group. Key: (*) $P < 0.001$, paired *t*-test.

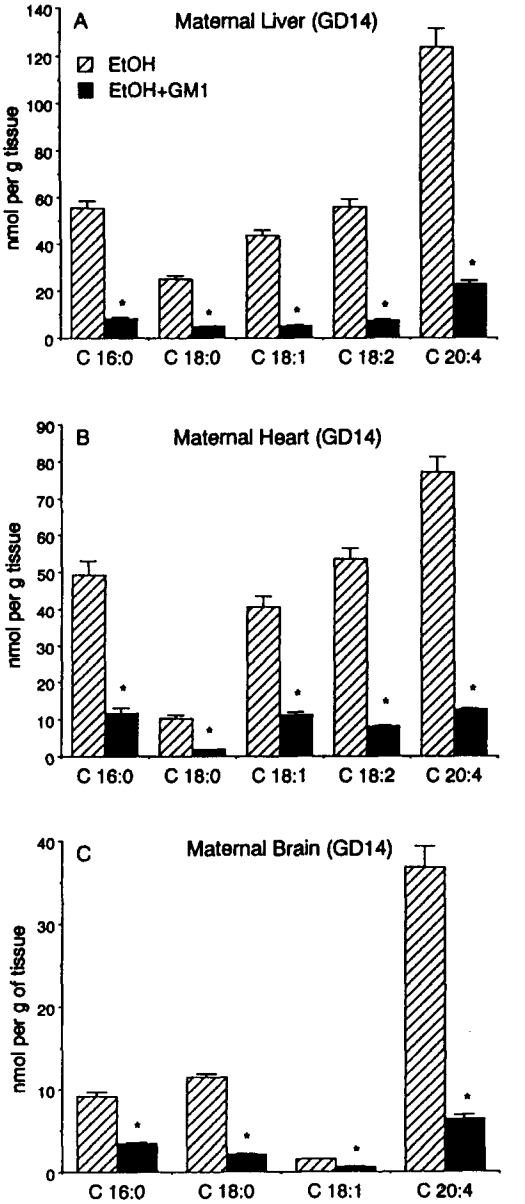

Fig. 5. FAEE analysis on GD 14 of maternal (A) liver, (B) heart and (C) brain exposed to alcohol on GD 6, 7, 13 and 14. Values are means ± SEM, N = 6 for each group. Key: (*) $P < 0.001$, paired *t*-test.

accumulation of FAEEs in the organs. We have demonstrated recently that GM1 treatment reduces the activity of PLA_2 in adult animals exposed chronically to alcohol [18]. A similar effect on PLA_2 has been demonstrated in *in vitro* studies with synthetic lipid bilayers [19]. Alternatively, GM1 may produce its protective effect by inhibiting FAEE synthase, which may lead to reduced FAEE accumulation. This effect may also possibly be brought about by direct insertion of GM1 into the membrane [20]. In this regard, it is noteworthy that [^{3}H]GM1 administered exogenously to pregnant dams passes the placental barrier and remains in fetal organs, including the brain, in a significant amount for up to 48 hr after injection [21]. Such membrane alterations may lead to the rigidization of the membrane, resulting in reduced penetration of ethanol into the membrane [20].

It has been shown that pups treated with GM1 postnatally between the days 5 and 15 exhibit improved learning/memory and increased cholinergic function [22]. It has also been shown that prenatal treatment with ganglioside GM1 enhances brain maturation in the rat [23]. In tissue culture studies, the ganglioside GM1 is shown to enhance neuronal growth and sprouting [24, 25]. These *in vivo* and *in vitro* studies indicate that ganglioside GM1 has the ability to enhance the maturation of the CNS. Therefore, therapeutic intervention by GM1 at a critical gestational time period prior to prenatal alcohol CNS insult may prevent/repair the cellular damage induced by the long-term presence of FAEEs in alcohol-exposed fetal organs, especially fetal brain, and thus prevent neurobehavioral abnormalities [20]. It is also likely that ganglioside GM1 may protect CNS from possible acute ischemia/hypoxia, which is considered to be one of the effects of acute prenatal alcohol exposure, since exogenous

ganglioside GM1 has been shown to prevent CNS ischemic injury in various animal models of ischemia [16].

In conclusion, our studies demonstrated that alcohol exposure *in utero* leads to significant accumulation of FAEEs in various maternal and fetal organs, confirming the findings of Bearer *et al.* [12], and GM1 pretreatment reduced this accumulation. This action of GM1 on alcohol-induced accumulation of FAEEs may be one of the mechanisms involved in the reduction of feto-toxic effects of alcohol. Nevertheless, further morphological and biochemical studies including developmental changes in FAEE-catabolizing and -synthesizing enzymes will provide answers to this phenomenon and will help in understanding the mechanisms of the preventive action of gangliosides in the treatment of alcohol-related birth defects and FAS.

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