Effect of valproic acid on fetal and maternal organs in the mouse: A morphological study

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Résumé
L’acide valproique (AVP), est une drogue à utiliser, comme médicament antépileptique. À cause de ses effets tératogènes connus, l’acide valproïque n’est pas recommandé aux femmes en âge de procréer. L’étude présente vise à évaluer les effets de ce dernier acide sur le fœtus et les organes maternels.
Des groupes randomisés des souris gravides ont été traités de la façon suivante :
Groupe 1 (n = 10), 500 mg/kg d’AVP/jour, les jours de gestation 8-11. Groupe 2 (n = 10), 600 mg/kg d’AVP/jour, les jours de gestation 8-11. Groupe 3 (n = 4), contrôle avec injection de sérum physiologique.
Le 18e jour de gestation, les souris gravides ont été sacrifiées. Les fœtus ont été collectés et préparés pour la Microscopie Electronique à Balayage. En plus les organes des fœtus et les organes maternels ont été examinés en histologie de routine et en immunohistochimie pour les facteurs de croissance (TGF alpha, beta-1, beta-2 et EGF). Enfin les organes des fœtus et les organes maternels ont été examinés en Microscopie Electronique à Transmission.
Pour les fœtus qui ont été examiné en Microscopie Electronique à Balayage, les groupes traités ont montré des lésions spécifiques provoquées par l’AVP chez le fœtus, notamment la spina bifida occulta, l’exencéphalie, et l’exopthalmie. L’examen histologique de routine, l’immunohistochimie et la Microscopie Electronique à Transmission n’ont pas détectés d’autres lésions chez les fœtus ou au niveau des organes maternels. Ces données suggèrent que les lésions présentes chez le fœtus sont dûes à l’effet direct de AVP sur l’acide rétinoïque, à composant partout présent qui joue un rôle dans le développement normal. Plutôt qu’à une insuffisance de transport de substances nutritives au fœtus à cause d’une insuffisance placentaire qui serait dû à la toxicité induite de AVP.


Summary
Valproic acid (VPA) is an antiepileptic drug used clinically. Because of its known teratogenic properties VPA is not recommended for women of child bearing age. The present study was designed to assess the effects of VPA on both fetal and maternal organs. Randomized groups of pregnant mice were treated as follows: Group 1 (n = 10) 500 mg/kg VPA/day on gestation days 8-11; Group 2 (n = 10) 600 mg/kg VPA/day on gestation days 8-11; and Group 3 (n = 4) saline-injected controls. On gestation day 18, the pregnant mice were euthanized, fetuses collected and prepared for scanning electron microscopy. In addition, fetal and maternal organs were processed for routine histology, immunohistochemistry for growth factors (TGF alpha, beta-1, beta-2 and EGF) and transmission electron microscopy. Scanning microscopy revealed specific lesions induced by VPA in the fetus, namely spina bifida occulta, exencephaly, and exophthalmia. On the other hand, there were no detectable morphological changes in fetal or maternal organs by routine histology, immunohistochemistry or electron microscopy. The data suggest that the lesions present in the fetus are due to a direct effect by VPA on retinoic acid, a ubiquitous compound that has a role in normal development, rather than the lack of transport of sufficient nutrients to the fetus as a result of placental insufficiency due to VPA-induced toxicity.

Key words: Mouse fetus. Valproic acid. Developmental defects.

INTRODUCTION
Excessive exposure to chemicals and therapeutic agents is known to be teratogenic in man and animals. One such compound, valproic acid, used to treat absence seizures and generalized tonic-clonic seizures (1) is not recommended for women of child bearing age (2, 3), since it is associated with a spectrum of malformations termed “fetal valproate syndrome” (4, 5). This clinical entity is characterized by phenotype abnormalities of the face, developmental disabilities and occasional major organ abnormalities involving respiratory, cardiovascular, gastrointestinal, genitourinary and skeletal systems (6, 7).
Valproic acid has also been shown to induce developmental defects in experimental animals. The abnormalities typically described are spina bifida and exencephaly in...
mice and hamsters (8-10) and limb defects in mice and rats (11). The etiology of VPA-induced malformations has not been clearly elucidated. Several hypotheses have been proposed, such as interference by VPA with embryonic metabolism of folates (12), zinc (13, 14) and lipid (15), or alteration of intracellular pH as an important parameter for cellular function (16).

In light of the above, the present study was designed to examine the effect of VPA on the structural and hence functional integrity of both maternal and fetal organs to assess whether toxicity of the various organs contributes to the developmental defects seen in the fetus.

**Materials and Methods**

Virgin female mice (Balb c), 30-35 gms, were mated between 6:00-9:00 am and the following 24 hours were considered day 0 of gestation if sperm were detected in the vaginal smears. Animals were kept under controlled lighting conditions (12 hours of light alternating with 12 hours of darkness) and given commercial laboratory chow and water *ad lib*. Animals were randomized into three groups and injected ip as follows: Group 1 (n = 10) 500 mg/kg VPA/day on gestation days 8-11; Group 2 (n = 10) 600mg/kg VPA/day on gestation days 8-11; Group 3 (n = 4) saline-injection on gestation days 8-11 as controls. Valproic acid, (sodium valproate) was obtained from Sanoﬁ Winthrop Industrie, France. On gestational day 18, the pregnant mice were euthanized with a lethal dose of CO2. The uteri were excised and the number of implantation sites recorded. Fetuses were removed, examined individually under a stereomicroscope for external malformations, and processed for morphological assessment. Control and drug treated fetuses with or without external malformations were fixed in 10% buffered formalin and processed for scanning electron microscopy. Organs from the dams and fetuses, with or without external developmental defects, were also processed for routine histology and transmission electron microscopy. Organs collected were maternal liver, lung, and kidney; fetal liver and lung, as well as the placenta. For scanning electron microscopy, the heads of the fetuses were microdissected under a stereomicroscope, dehydrated in a grades series of ethanol and amyl acetate and dried by the critical point method (Critical Point dryer Balzers CPD 020). The fetal heads were attached onto stubs and coated with a 10-20 nm layer of gold and observed and photographed in a JEOL JSM-840A Scanning Microscope at 10 to 15 kV and 10-6 beam current. For routine histology, organs were processed and embedded in paraffin, 6u sections cut and stained with Hematoxylin and Eosin, viewed and photographed on an Olympus photomicroscope. Immunohistochemistry was also performed on fetal heads, fetal and maternal organs. Sections (6um) were cut and stained for localization of growth factors TGF alpha, TGF beta-1 and beta-2, and EGF. Primary antibodies were obtained from the following sources: TGF alpha, TGF beta-1 and beta-2 (Santa Cruz Biotechnology, Santa Cruz, CA), EGF (ICN Biomedicals Inc., Costa Mesa, CA). Primary antibodies were diluted 1:100 and 1:200 and incubated over sections for one hour (20 hours for TGF alpha). Normal rabbit serum was substituted as control for nonspecific binding of secondary antibody and endogenous peroxidase activity. Localization of bound antibody was achieved using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA). Sections were examined with an Olympus microscope and level of expression of each growth factor was scored semi-quantitatively by assigning a grade of staining intensity from 0 (no stain), + 1 (focal), + 2 (weak), + 3 (moderate), or + 4 (strong). For transmission electron microscopy, organs were fixed in glutaraldehyde, post-fixed in osmium tetroxide, dehydrated in a graded series of ethanol and embedded in araldite. Thin sections were cut and stained with uranyl acetate and lead citrate, viewed and photographed on a Philips EM-201 electron microscope. In all instances, organs were coded and examined without foreknowledge of their source.

**Statistical Analysis**

Immunohistochemical scores for each growth factor and frequency of fetal malformations were analyzed with non-parametric statistical methods.

**Results**

Assessment by scanning microscopy revealed the presence of neural tube defects in a number of the fetuses exposed to either 500 or 600 mg/kg of VPA (table I). The lesions noted were spina bifida occulta (fig. 1), exencephaly and exophthalmia (fig. 2). Lesions were not seen in fetuses from the control group (fig. 3-4). These defects occurred in 35% and 36% of the fetuses exposed to the two dosages, respectively, and the incidence was not dose-dependent ($X^2 = 5.67, p = 0.34$). In contrast, the appearance of the various organs, regardless of whether they were examined at the light or electron microscope level revealed no morphological differences, amongst dams drug-treated or saline-treated, or amongst fetuses with or without external anomalies (fig. 5-6). Furthermore, immunohistochemis-

**Table I. Mouse embryo malformations induced with valproic acid.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Spina Bifida Occulta</th>
<th>Spina Bifida Aperta</th>
<th>Exenceph</th>
<th>Exophth</th>
<th>Underdeveloped</th>
<th>Resorption</th>
<th>Aborted</th>
<th>Normal</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10</td>
<td>3</td>
<td>7</td>
<td>7</td>
<td>1</td>
<td>16</td>
<td>0</td>
<td>35</td>
<td>79</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>0</td>
<td>3</td>
<td>13</td>
<td>0</td>
<td>26</td>
<td>1</td>
<td>12</td>
<td>61</td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>33</td>
</tr>
</tbody>
</table>

GROUPS:

A = 500 mg/kg VPA
B = 600 mg/kg VPA
C = Saline-injected

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try performed on the various regions in the fetal heads (mesenchyme, nasal and oral epithelium, cartilage, mandible), maternal (liver, kidney, lung) and fetal (liver, lung) organs as well as the placenta revealed no statistical differences among controls (dams and fetuses) and drug-treated dams and fetuses with or without anomalies. (Mann-Whitney U test, p > 0.05). An example of immunohistochemical staining is illustrated in figs. 7 and 8 for TGF-beta 2. In both control (fig. 7) and experimental (fig. 8) fetal heads, the staining intensity (+3) was similar.

DISCUSSION

Valproic acid (VPA) is a simple fatty acid largely used as an anti-epileptic agent. Side effects are uncommon, but cases of hepatic failure have been reported in children (17) and adults (18). Valproic acid has a potentially teratogenic effect, as well as the capacity for inducing neural tube defects. VPA treatment of amphibian cultured embryos from blastula stage onward, revealed defective neurulation and closure of the neural folds. Furthermore, the neural epithelium was disorganized (19). In the mouse, multiple dosages of VPA (500 mg/kg) administered on day 9 of gestation induced spina bifida, as well as exencephaly (10). In addition, a single dose (500 mg/kg) of an active metabolite of valproic acid, 4-yn-VPA, has also been shown to be highly teratogenic in both the mouse (20) and rat (21). Moreover, it was noted that administration of the S-isomer and not the R-isomer of 4-yn-VPA resulted in failure of closure of the anterior and posterior neuropores, erratic neural seams and reduced telencephalic spheres (20, 21). Direct exposure of mouse embryos (22) to valproic acid resulted in dysmorphology similar to that observed following in vitro exposure of rat embryos (21). The anomalies primarily observed were failure of closure of the anterior and posterior neuropores, erratic neural seams and reduced telencephalic spheres.

The results of our study are of interest and suggest that VPA might have had a non-specific effect in the induction of the malformations noted. Had structural changes, and hence function, been detected throughout the fetus, one could argue that the abnormalities seen could have resulted from the lack of transport of sufficient nutrients and vitamins, notably folates which are essential for normal development (23), as the result of placental and/or maternal pathology due to VPA-induced toxicity. This notion is supported in part by earlier studies in the rat (24) that exami-
ned the histological changes in the placenta, decidua and fetal anomalies induced by maternal-toxic doses of ethylene glycol and cadmium chloride. Changes noted consisted of extensive necrosis and hemorrhage in the decidua basalis and reduced formation of the villi, concurrent with hydrocephaly, rib defects and fetal body weight reduction. Alternately, had structural damage been detected in the placenta, fetal lung and liver, and not the dam, one could conclude that VPA had a generalized toxic effect upon the fetus and not a localized one as it appears to have had in this instance. Another and more probable cause for the induction of the anomalies seen is via the effect that VPA has on serum levels of retinol, a ubiquitous compound that has a role in normal embryonic development (25, 26). Nau et al. (27) reported increased levels of retinol with VPA use and decreased levels of retinol metabolites with VPA in combination with other anticonvulsants. Thus, VPA may alter the balance of retinoids, leading to impaired regulation by retinoic acid of Hoxa-1 gene expression in mesoderm and ectoderm, resulting in malformations (28, 29). Similar events may have led to the abnormalities seen in our study. Support for this hypothesis is seen from the study by Abbott and Birnbaum (30) who assessed the effect of exogenous retinoic acid on the immunohistochemical localization of growth factors in the regulation of development of the palate in the mouse embryo. Growth factors TGF alpha, beta-1, beta-2 and EGF were shown to have specific temporal and spatial expression in the palatal shelf. Exogenous
retinoic acid altered the expression of TGF alpha, beta-1 and beta-2, but not EGF. Moreover, the effects of retinoic acid on growth factor expression were dependent on the gestational age of treatment. In our study, we did not detect any differences in the staining pattern of the various growth factors, a finding that differs from that reported by Abbott and Birnbaum (30). A key difference in our study was that while we administered VPA on GD 8-11, the fetuses were not collected and analyzed until GD 18. Abbott and Birnbaum (30), in contrast, treated embryos on GD 10 or GD 12 and performed their analysis on GD 14 or GD 16. The time lapse from GD 16 to GD 18 might have been sufficient to allow a return to normal levels of growth factor expression even though the defects seen on GD 18 may have been induced during embryonic exposure to VPA on GD 8-11. Further to this, Ehlers et al. (31) reported differing periods of sensitivity of the embryo to VPA and retinoic acid-induced malformations. Fetuses, examined on GD 18, exposed to VPA on GD 8 revealed exencephaly, but not spina bifida, which was initially induced with treatment on GD 9. In contrast, both malformations were detected in GD18 fetuses exposed to retinoic acid on GD 8. Thus taken together, the presence of specific growth factors and relative levels of growth factors in the embryo, as well as timing are equally important in regulating development.

The precise mechanism of action of most teratogens is not known, and VPA is not an exception in this regard. Further studies are warranted to determine the mechanism of action of VPA as it affects cell proliferation, cell to cell interaction, growth factors and apoptosis in the developing embryo, as well as the interaction between VPA and endogenous retinoids.

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