Amelioration of sodium valproate-induced neural tube defects in mouse fetuses by maternal folic acid supplementation during gestation

R. Padmanabhan¹ and M. Mohamed Shafiullah²
¹Department of Anatomy and ²Pharmacology, Faculty of Medicine and Health Sciences, UAE University, Al Ain, United Arab Emirates

ABSTRACT  Infants of epileptic women treated with valproic acid (VPA) during pregnancy have a higher risk of developing spina bifida than those of the general population. VPA induces exencephaly in experimental animal embryos. But the pathogenetic mechanism remains rather elusive. Antiepileptic drugs (AED) in general accentuate pregnancy-imposed fall in maternal folate levels. Periconceptional folic acid supplementation is reported to protect embryos from developing neural tube defects (NTD). Conflicting results have been reported by experimental studies that attempted to alleviate VPA-induced NTD by folic acid. Our objectives were to determine the critical developmental stages and an effective dose of folic acid for the prevention of VPA-induced exencephaly in mouse fetuses. A single teratogenic dose of 400 mg/kg of VPA was administered to TO mice on gestation day (GD) 7 or 8. It was followed by (1) a single dose of 12 mg/kg of FA (folic acid) or (2) 3 doses of FA 4 mg/kg each. In experiment (3), FA (4 mg/kg) was administered thrice daily starting on GD 5 and continued through GD 10. These animals received VPA on GD 7 or 8. VPA and B12 concentrations were determined by radioimmunoassay. The single heavy dose of FA had no rescue effect on NTD. Three divided doses of FA on GD 7 and continuous dosing of FA from GD 5 through GD 10 substantially reduced the VPA-induced exencephaly in the fetuses. In the later experiments, the neural folds elevated faster than the non-supplemented group. VPA considerably reduced maternal plasma folate and B12 concentrations. The heavy dose of FA only moderately improved vitamin levels. Three divided doses of FA elevated the vitamin levels slightly better but it was the prolonged dosing of FA that was associated with sustained elevation of plasma levels higher than the control levels and acceleration of neural tube closure thus accounting for the pronounced protection against VPA-induced NTD development. These data suggest that plasma levels of FA and B12 have to be kept substantially elevated and maintained high throughout organogenesis period to protect embryos against VPA-induced NTD in this mouse model.

Key Words: valproic acid, pregnant mice, neural tube defects, folic acid, vitamin B12, congenital anomalies prevention

INTRODUCTION

Valproic acid (VPA) is one of the most commonly used first generation antiepileptic drugs (AED). It has two major adverse effects: teratogenicity and hepatotoxicity. Since Dalens et al. (1980) first published the teratogenic effects of VPA, several reports on fetal valproate syndrome have appeared in the literature. Birth defects attributed to maternal VPA therapy include prominent forehead, flat nasal bridge, low set unusually shaped-ears, hypertelorism, epicanthal folds, down slanting palpebral fissures, micrognathia, microcephaly, cardiovascular defects, duodenal atresia, renal hypoplasia, congenital hip dislocation, shortened forearm, preaxial reduction deformity of digits, nail hypoplasia, and vertebral malformations (Tein and MacGregor, 1985; Sharony et al., 1993; Kaneko et al., 1988; Nakano and Kaneko, 1992). Three case reports published in early 1980s also described isolated spina bifida in infants prenatally exposed to VPA (Gomez, 1981; Stanley and Chambers, 1982; Blaw and Woody, 1983). Subsequent studies have established that there is an absolute risk of 1-2% for spina bifida in the offspring of VPA-treated epileptic women compared with the risk of 0.06% for the general population (Robert, 1988; Mastroiacovo et al., 1983; Lindhout et
al., 1992). Although myeloschisis or spina bifida aperta is
commonly attributed to VPA therapy, myelomeningoceles
with skin covering have also been reported (Robert, 1988).
The fact that sacral or lumbosacral positions are the preferred
sites of these defects in VPA exposed infants (Ardinger et al.,
1988) suggests that VPA affects the secondary neurulation
involving the neural tube caudal to the posterior neuropore.
In sharp contrast, exencephaly is reported to be the most con-
sistent type of neural tube defects (NTD) in VPA-treated
mouse embryos (Nau et al., 1991; Padmanabhan and Hameed,
bifida in experimental animals has been only rarely reported
(Ehlers et al., 1992; Padmanabhan and Vaidhya, 1990). While
some investigators believe that anencephaly and spina bifida
are distinctly different anomalies (Torielli and Higgins, 1985),
others assume that these are causally related abnormalities
(Seller, 1990). The reason why VPA induces spina bifida in
humans and exencephaly in mouse embryos is not clear. It is
possible that there are some common events in the mecha-
nism of closure of the cranial and caudal neuropores, which
are uniquely susceptible to VPA treatment (Padmanabhan and

That VPA is a neuroteratogen has now been established
unequivocally, but the pathogenetic mechanism remains rather
evasive. VPA-induced alterations in gene expression
(Wlodarczyk et al., 1996), intracellular pH, modifications in
zinc, retinoid and lipid metabolism and changes in folic acid,
homeocysteine and glutathione status have been attributed to
underlie the pathogenesis of these anomalies. These postu-
lates are considered largely speculative and credible evidence
is yet to come (see for references Scott et al., 1997).

NTD are of multifactorial origin with genetic susceptibil-
ity being impacted by environmental factors in a majority of
cases (Seller, 1994, 1995; Sadler and Sulik, 1993). Among
environmental factors, poor diet is one of the most likely can-
didates (Rose and Mennuti, 1994). Recent reports claim that
50-70% of NTD in humans can be prevented by periconce-
ptional consumption of folic acid (Green, 2002; Persad et al.,
2002; Bower et al., 2002). Largely influenced by the re-
sults of two multicenter trials on the beneficial effects of
periconceptional folic acid supplementation in reducing the
risk of NTD (MRC Vitamin Study Research Group, 1991;
Czeizel and Dudas, 1992), food fortification with vitamins
was recommended in many countries. Contrasting results have
been reported (Rosano et al., 1999). Epileptic patients with
malformed infants have a particularly low plasma folate con-
centration (Dancy et al., 1987; Ogawa et al., 1991). The
link between VPA therapy and alterations in folate metabo-
lism comes from the fact that antiepileptic agents (including
VPA) were shown to interfere with folate metabolism in the
embryo (Netzloff et al., 1979; Hanson and Billings, 1985).
Folate levels are known to be lowered during pregnancy and
AEDs including VPA intensify this effect (Hendel et al., 1984;
Lewis et al., 1998). VPA reduces folic acid and B12 levels in
pregnant mice (Padmanabhan, 1997). Exogenous folic acid
has been reported to have ameliorative, augmentative or no
fetal effects in experimental animals (Trotz et al., 1987; Nau
et al., 1991; Hansen et al., 1995; Hansen and Grafton, 1991;
Seller, 1994; Padmanabhan, 1997). Both species and strain
variations, and differences in dose and timing of folic acid
treatment might have contributed to the contrasting re-
results. Studies aimed at investigating pathogenetic mechanisms
of folic acid-related rescue effects on NTD require consist-
ency and reproducibility of the beneficial effects. Trotz et al.
(1987) administered on gestation day (GD) 8 three doses of
4 mg/kg of folic acid (FA) to their VPA-treated mice and
found a reduction in exencephaly incidence in the fetuses. We
had recently exposed our VPA-treated mice to a single dose
of 12 mg/kg of FA on GD 7 or 8 and observed no reduction in
exencephaly incidence (Padmanabhan, 1997). The supple-
mental FA did not show a specific pattern of amelioration on
VPA-induced axial skeletal malformations.

The objective of the present study was to determine the criti-
cal timing, dose and duration of FA supplementation in order to
alleviate VPA-induced exencephaly in a mouse model of
NTD.

MATERIALS AND METHODS

The TO mice used in this study were originally obtained from
Harlan Olac (England) and raised in our local facility. They
were maintained on a commercial laboratory chow and tap
water provided ad libitum and housed in light (12:12 hr light:
dark cycle) and temperature (21 ± 1°C) controlled rooms. Vir-
gin females, about six weeks of age and approximately 30 g
in weight were mated with males of the same stock in the
evening and presence of a vaginal plug observed on the fol-
lowing morning indicated day 0 of gestation. At stage I, groups
of mice were injected intraperitoneally (ip) with a single dose
of 400 mg/kg of VPA (sodium valproate Laboratoires LABAS,
Paris) on gestation day (GD) 7 or 8. The controls were either
injected with a proportionate volume of saline (SAL) or non-
treated. Their food and water consumption were recorded.
They were then killed on GD 18 by cervical dislocation and
fetuses were collected. At stage II, the mice were divided
into three major groups. Group I was treated (ip) with a single
dose of 400 mg/kg of sodium valproate on GD 7 or 8 fol-
lowing a single dose of 12 mg/kg of folic acid (FA) (Fo-
linic acid, Sigma, MO). Folic acid is metabolically the most
active form of the B vitamin folic acid (Trotz et al., 1987).
The controls were injected with FA and saline. Group II
received three doses (ip) of 4 mg/kg of FA at 3 hr intervals with
a single dose of 400 mg/kg of VPA given at the time of mid
dose of FA on GD 7 or 8. The controls received three doses
of FA and a mid dose of saline, equivalent to the volume of
VPA solution. Group III received three doses of 4 mg/kg of
Table 1 Susceptible stages for the induction of NTD by maternal treatment with VPA

<table>
<thead>
<tr>
<th></th>
<th>Untreated Control (GD 5 &amp; GD 6)</th>
<th>Saline Control (GD 7 &amp; GD 8)</th>
<th>VPA-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of litters</td>
<td>10</td>
<td>24</td>
<td>11</td>
</tr>
<tr>
<td>Implantations</td>
<td>121</td>
<td>279</td>
<td>125</td>
</tr>
<tr>
<td>Resorptions (%)</td>
<td>7 (6.0)</td>
<td>16 (5.7)</td>
<td>17 (12.9)*</td>
</tr>
<tr>
<td>Live fetuses</td>
<td>114</td>
<td>263</td>
<td>105</td>
</tr>
<tr>
<td>Fetal weight (g)</td>
<td>1.41 ± 0.09</td>
<td>1.41 ± 0.07</td>
<td>1.20 ± 0.09*</td>
</tr>
<tr>
<td>Exencephaly (%)</td>
<td>4 (3.6)</td>
<td>9 (3.4)</td>
<td>25 (23.2)*</td>
</tr>
</tbody>
</table>

GD 7 and GD 8 Saline control data have been pooled; *p < 0.05 when VPA (GD 7 or GD 8) is compared with the corresponding untreated or saline control.

FA daily at 3 hr intervals starting on GD 5 and continued through GD 10. They also received (ip) a single dose of VPA (400 mg/kg) on GD 7 or 8. The corresponding controls were similarly treated with FA but given a single dose of saline instead of VPA on GD 7 or 8. Both VPA and FA were dissolved in physiological saline. The total volume of fluid injected corresponded to the body weight and did not exceed 0.45 ml. The food and water consumption of all groups was recorded throughout the study. All animals were killed on GD 18 by cervical dislocation and the fetuses were collected. All implantation and resorption sites were counted. The fetuses were cleared of their membranes, blotted dry, weighed and fixed in 95% ethanol and subsequently examined for malformations by a modified method of Sterz and Lehmann (1985). Fetal weight data were analysed by Student t-test and malformation frequency was analysed by X² test with Yates correction (Elzey, 1987). Significance was assumed when p was < 0.05.

In another set of experiments, following daily injection of three doses of 4 mg/kg FA from GD 5 through 10 and VPA (or saline) on GD 8, embryos were collected at 6 hr, 12 hr and then at 24 hr intervals after VPA until GD 10. At each time point, at least three mice were killed and their embryos delivered into phosphate buffered saline. Their development was scored under a stereomicroscope. They were then fixed in Bouin's solution, paraffin embedded, cut serially at 7 μm thickness, stained with haematoxylin and eosin and examined with a Carl Zeiss microscope.

Estimation of plasma folic acid levels

Three and a half hrs after the last dose of FA, as described above, both the control and experimental animals were decerated by cervical dislocation and quickly 0.5 ml of blood was taken from the inferior vena cava into EDTA-coated evacuated tubes. The samples were immediately placed on ice and centrifuged within 60 min at 3,000 × g for 10 min at 4°C. Plasma was then separated and stored at -70°C. Folic acid and vitamin B₁₂ concentrations were measured simultaneously with Dual count solid phase no boil radioassay (Diagnostic Products Corporation, Los Angeles, CA). The three steps (heat denaturation at 100°C, competition for crude intrinsic factor and b-lactoglobulin under acidic condition and separation by coated charcoal are replaced in DPC assay by alkaline denaturation of endogenous proteins, competition for purified binder at pH 9.3 and solid phase separation.

RESULTS

Maternal Effects

There was a slight but insignificant reduction in food and water consumption of the VPA-treated animals. But soon they recovered and consumed food and water as well as the controls and gained a comparable range of body weight. Administration of different doses of FA did not cause any maternal toxicity. A single dose of 400 mg/kg of VPA was found to induce a highly significant rate of resorption, intrauterine growth retardation (IUGR) and exencephaly in both GD 7 and 8 groups (Table 1). Exencephaly was characterised by open cranium and everted rostral neural folds. The unclosed neural tube was haemorrhagic and had undergone varying degrees of degeneration. Their amniotic fluid was blood stained. Maxillary-mandibular hypoplasia, low set microtia and a significant reduction in body weight were other features of these embryos. Administration of a single dose of 12 mg/kg of FA substantially reduced the VPA-induced resorption but did not reduce VPA treatment related IUGR or exencephaly in both GD 7 and 8 groups (Table 2). In order to maintain a steady state of folic acid concentration in maternal plasma, instead of a single dose of 12 mg/kg, we administered three doses of 4mg/kg of FA each on GD 7 or 8. VPA was injected at mid dose of FA. There was no preventive effect of FA on VPA-induced resorption in the GD 7 group; in fact resorption was enhanced by co-treatment with FA. On the contrary there was a considerable protective effect in the GD 8 group (Table 3). VPA-induced IUGR however remained unfluenced by exogenous FA in the GD 8 treatment group. FA almost halved
### Table 2 Effect of a single dose of folic acid (12 mg/kg) treatment on GD 7 and 8 on VPA-induced exencephaly in the mouse embryos

<table>
<thead>
<tr>
<th>VPA-treatment on GD 7</th>
<th>VPA-treatment on GD 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL + VPA</td>
<td>FA + SAL</td>
</tr>
<tr>
<td>No of litters</td>
<td>25</td>
</tr>
<tr>
<td>Implantations</td>
<td>305</td>
</tr>
<tr>
<td>Resorptions (%)</td>
<td>50 (16.4)*</td>
</tr>
<tr>
<td>Live fetuses</td>
<td>255</td>
</tr>
<tr>
<td>Fetal weight (g)</td>
<td>1.27 ± 0.07*</td>
</tr>
<tr>
<td>(Mean+SD)</td>
<td></td>
</tr>
<tr>
<td>Exencephaly (%)</td>
<td>65 (25.8)*</td>
</tr>
</tbody>
</table>

*P < 0.05 when compared with corresponding control: 1) FA + SAL vs SAL + VPA *; 2) SAL + VPA vs FA + VPA **; 3) FA + SAL vs FA + VPA all are significant.

### Table 3 Effect of a triple dose administration of folic acid (4 mg/kg each dose) on GD 7 and 8 on VPA-induced exencephaly in the mouse embryos

<table>
<thead>
<tr>
<th>VPA-treatment on GD 7</th>
<th>VPA-treatment on GD 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL + VPA</td>
<td>FA + SAL</td>
</tr>
<tr>
<td>No of litters</td>
<td>12</td>
</tr>
<tr>
<td>Implantations</td>
<td>149</td>
</tr>
<tr>
<td>Resorptions (%)</td>
<td>20 (13.4)*</td>
</tr>
<tr>
<td>Live fetuses</td>
<td>124</td>
</tr>
<tr>
<td>Fetal weight (g)</td>
<td>1.22 ± 0.14*</td>
</tr>
<tr>
<td>(Mean+SD)</td>
<td></td>
</tr>
<tr>
<td>Exencephaly (%)</td>
<td>32 (25.8)*</td>
</tr>
</tbody>
</table>

*P < 0.05 when compared with the corresponding control: 1) FA + SAL vs SAL + VPA *; 2) SAL + VPA vs FA + VPA **; 3) FA + SAL vs FA + VPA

### Table 4 Effect of a daily triple dose administration of folic acid (4 mg/kg) from GD-5 through GD-10 on VPA-induced exencephaly in the mouse embryos

<table>
<thead>
<tr>
<th>VPA-treatment on GD 7</th>
<th>VPA-treatment on GD 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL + VPA</td>
<td>FA + SAL</td>
</tr>
<tr>
<td>No of litters</td>
<td>16</td>
</tr>
<tr>
<td>Implantations</td>
<td>201</td>
</tr>
<tr>
<td>Resorptions (%)</td>
<td>19 (9.5)</td>
</tr>
<tr>
<td>Live fetuses</td>
<td>182</td>
</tr>
<tr>
<td>Fetal weight (g)</td>
<td>1.29 ± 0.14*</td>
</tr>
<tr>
<td>(Mean+SD)</td>
<td></td>
</tr>
<tr>
<td>Exencephaly (%)</td>
<td>46 (25.2)*</td>
</tr>
</tbody>
</table>

*p < 0.05 when compared with corresponding control: 1) FA + SAL vs SAL + VPA *; 2) SAL + VPA vs FA + VPA **; 3) FA + SAL vs FA + VPA all are significant.
Fig. 1 Mouse embryos of GD 9. A: Control (FA + SAL). This embryo has closed neural tube and well developed pharyngeal arches and frontonasal process (arrowhead). The FA + VPA (GD8 treatment) embryos have closed neural tube but many are very much growth retarded (B) or have partially elevated neural folds and unclosed rostral neuropore (not illustrated). The SAL + VPA (GD8 treatment) embryos (C-F) have widely open mesencephalic neural folds (C, D) or open prosen- mesen- and rostral part of the rhombencephalon (E). In E and F both the mesencephalon (thick arrow) and rhombencephalon (thin arrow) are open dorsally. Also observe the hypoplasia of the frontonasal and maxillary processes (C, D, F). FA = folic acid; SAL = saline; VPA = valproic acid; A-E: 18 ×. 

Fig. 2 Mouse embryos of GD 10. The control (FA + SAL) embryo has well developed brain vesicles (A). The FA + VPA (GD8 treatment) embryo has the neural tube closed, normal frontonasal process as in control (arrowhead) but the lower face and trunk appear poorly developed (D). The SAL + VPA (GD8 treatment) embryos (B, C, E, F) are stunted. Also note that the mesencephalic neural folds are apposed and unclosed (thick arrows in B, C) or open, everted and overgrown (thick arrows in E, F). The maxillary processes are hypoplastic (asterisks in E, F). Embryo C presents median facial cleft due to failure of closure Sites 3 and 2. A-E: 18 ×.
the VPA-induced exencephaly in the GD 7 group but not in the GD 8 group (Table 3). In order to maintain a high plasma folate level of FA over a longer period of time during neurulation, FA was administered (4 mg/kg) thrice daily starting on GD 5 and continued through GD 10. VPA was administered on GD 7 or 8. This dosing regimen of FA also did not rescue the embryos of GD 7 group from being resorbed. On the contrary supplementation was found to result in enhancement of resorption (Table 4). It is likely that the young embryos of GD 7 are particularly susceptible to deleterious effects of VPA plus FA as manifested by a high incidence of embryo lethality (Tables 2 and 3). The preventive effect of successive doses of FA on embryonic resorption in the GD 8 group was about 25% (Table 4). Prolonged supplementation of FA reduced the incidence of exencephaly from 25% to 18% in the GD 7 VPA group, and from 35% to 18% in the GD 8 group (Table 4).

In order to determine what was happening to the neural folds and neural tube of embryos of the FA supplemented groups, FA + VPA group embryos together with age matched controls were harvested at 6, 12, 24 and 48 hrs post-treatment with VPA on GD 8, and examined under a dissecting microscope. The VPA-treated embryos always appeared growth retarded compared to age matched untreated/saline controls. FA supplementation did not appear to have any effect on growth. The neural folds of the VPA group embryos appeared to elevate more slowly in comparison to the controls. The growth of the craniofacial region appeared to be inhibited. The neural folds were smaller than those of the controls and the failure of contact between neural folds of either side appeared to involve different regions in different embryos although most commonly closure site 2 appeared to have been missed (Fig. 1). Closure site 4 was missed next in frequency either partly or completely. Occasionally closure sites 2, 3, and 4 had failed to close completely. These cases of total closure failure would possibly become acrania fetuses similar to the ones with median facial clefts seen in our previous study (Padmanabhan and Ahmed, 1996). The unclosed mesencephalic folds of other embryos became subsequently everted and assumed various shapes (Figs. 2E and 2F) and overgrowth of the unclosed neural tubes were also discernible. Supplementation with FA substantially activated the processes of elevation, apposition and fusion of neural folds in a good number of embryos at each stage of observation (Fig. 3).

**Histological Examination**

Frontal sections of control embryos at 12 hr on GD 8 had the neural folds elevated so that the folds could appose each other (Figs. 4A-D). A few had their anterior neuropore closed though extensive contacts between neural fold were yet to be established in others. The VPA group embryos had everted neural folds or no elevation at all (Figs. 4 and 5). The head ME was sparse and poorly organized. There were numerous apoptotic figures. The pharyngeal arches were hypoplastic. These craniofacial alterations were such that even if the embryos were allowed sufficient time in utero, there might not be much repair and restoration of growth or elevation and fusion of the neural folds (Fig. 5D).

**Plasma folic acid and B12 status**

Maternal plasma folic acid and vitamin B12 levels were significantly reduced in the unsupplemented animals of both GD 7 and 8 VPA groups. Administration of a single but heavy dose (12 mg/kg) of FA to VPA-treated animals improved the plasma folic acid levels substantially but in absolute terms,
Fig. 4 Frontal (A, B, C, F) and sagittal (D) sections of mouse embryos of GD 8-12 hr stained with haematoxylin and eosin (H & E). The controls (FA + SAL) have closed neural tube (NT) and well formed cranial mesenchyme (ME) appropriate for this stage of development (A, D) some of which still have the prosencephalon, rhombencephalon (E) and mesencephalon (F) open. The FA+VPA (GD8 treatment) embryos (B, C) have the prosencephalon and rhombencephalon completely open. P = prosencephalon; M = mesencephalon; ME = mesenchyme; R = rhombencephalon. A-D: 10 x; E: 16 x; F: 5 x.

Table 5 Effect of VPA-treatment on maternal plasma levels (Mean ± SD) of folic acid and vitamin B12 in the TO mouse

<table>
<thead>
<tr>
<th>Folic acid level (ng/ml)</th>
<th>GD 7</th>
<th>GD 8</th>
<th>B12 level (pg/ml)</th>
<th>GD 7</th>
<th>GD 8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SAL</td>
<td>VPA</td>
<td>SAL</td>
<td>VPA</td>
<td>SAL</td>
</tr>
<tr>
<td>Unsupplemented</td>
<td>97.3 ± 7.7</td>
<td>16.9 ± 4.8</td>
<td>84.1 ± 20.2</td>
<td>13.1 ± 4.1</td>
<td>6953 ± 1017</td>
</tr>
<tr>
<td>FA 12 mg/kg</td>
<td>106.5 ± 9.2</td>
<td>65.7 ± 10.1</td>
<td>111.5 ± 20.5</td>
<td>65.2 ± 10.6</td>
<td>11050 ± 71</td>
</tr>
<tr>
<td>FA 4 mg/kg × 3</td>
<td>117.0 ± 9.7</td>
<td>107.8 ± 20.0</td>
<td>120.6 ± 7.4</td>
<td>106.4 ± 8.9</td>
<td>12066 ± 403</td>
</tr>
<tr>
<td>FA 4 mg/kg × 3, GD 5 through 10</td>
<td>149.0 ± 27.1</td>
<td>133.9 ± 57.8</td>
<td>136.9 ± 29.0</td>
<td>130.7 ± 40.7</td>
<td>11113 ± 975</td>
</tr>
<tr>
<td>FA 12 mg/kg × 3, GD 5 through 10</td>
<td>149.0 ± 27.1</td>
<td>133.9 ± 57.8</td>
<td>136.9 ± 29.0</td>
<td>130.7 ± 40.7</td>
<td>11113 ± 975</td>
</tr>
</tbody>
</table>

Mean ± SD derived from 5-7 mice per group.

P < 0.05 when all experimental values are compared with corresponding controls.

The Japanese Teratology Society

DISCUSSION

Valproic acid easily crosses the placental barrier (Barzago et al., 1996). Its teratogenic effects have been unequivocally demonstrated in humans and experimental animals (see for reference Holmes et al., 2001; Padmanabhan et al., 2000). About 1-2% of all infants exposed prenatally to VPA are born with spina bifida whereas about 25-30% of VPA-treated mouse embryos develop exencephaly depending on the strain. The reason why the sites of VPA-induced lesion in the two species differ is not known. Both in human and mouse embryos, the contact between the neural folds is initiated at several sites (see for references Nakatsu et al., 2000) but there are obvious species differences with regards to the onset and direction of progressive closure (Nakatsu et al., 2000). The
intermittent pattern of neural tube closure has been studied in some detail in the mouse (Theiler 1972; Waterman, 1976; Kaufman 1979; Geelan and Langman, 1977; Cole and Trasler, 1980; Juriloff et al., 1991; Golden and Chernoff, 1993). The dorsal edges of the neural folds of mouse embryos appose each other and first make contact in the region of the 2nd to 6th pair of somites (Site 1). This closure progresses bidirectionally. The closure Site 2 begins next at the prosencephalon-mesencephalon junction and progresses bidirectionally. Closure Site 3 begins at the rostral end adjacent to the stomadeum and extends caudally. Closure Site 4, also bidirectional covers the rhombencephalon and meets rostrally the caudal end of Site 2 and caudally the rostral extension of closure Site 1. Closure Site 5 is described to begin at the caudal end of the neural folds and meet with the caudal extension of closure Site 1. The process of initiation of contact and progressive fusion of neural folds is rather rapid and that may be one reason why this phenomenon has not been studied well in human embryos. The study by Nakatsu et al. (Nakatsu et al., 2000), in which the authors had access to a large number of normal and and normal human embryos is possibly the only one exception. Bush et al. (1990) suggest that each major segment of the developing neural tube may require a unique combination of mechanisms to provide the force for elevation of the neural folds. It is possible that there are certain developmental mechanisms common to the closure of the rostral end of the mouse and caudal end of the human neural tube that are uniquely susceptible to the deleterious action of VPA. The beneficial effects of periconceptional folic acid on the outcome of NTD-prone pregnancy has been reported by some studies and contested by others (Mills et al., 1989; Kalter, 2002). Based on the knowledge that most AED depress folic acid levels (see for references Lewis et al., 1998) and that folic acid supplementation can prevent NTD occurrence, experiments were conducted on the effects of exogenous folic acid on VPA-induced embryotoxicity with both positive (Trotz et al., 1987) and negative (Hanson et al., 1995) results. In a preliminary study we observed that FA could

Fig. 5 Paraffin sections of GD 8-12 hr embryos stained with H & E. In the control embryos (A, B), only the diencephalic segment of the NT is partly open (arrow in A, B) but in the SAL + VPA (GD8 treatment) embryos of the corresponding stage the neural tube is widely open (C) and neural folds are everted (D) while the craniophal mesenchyme (ME) remains hypoplastic. A, B, D: 10 ×; C: 16 ×.
substantially reduce VPA-induced exencephaly in NTD-prone TO mice (Padmanabhan, 1997). The results of the present study have confirmed our earlier findings and added morphological evidence for the rescue effects of FA in the mouse model of exencephaly. This study has also demonstrated that neither VPA nor FA administered in the present experiments is overtly toxic to pregnant mice. There was a high incidence of resorption, IUGR and exencephaly in both GD 7 and 8 treatment groups similar to those of our previous study in which we administered different doses of VPA during organogenesis in mice (Padmanabhan and Ahmed, 1996). Supplementation of VPA-treated mothers with a single dose of 12 mg/kg of FA substantially reduced embryonic resorption but not intrauterine growth retardation (IUGR). This dose also did not rescue the embryos from developing NTD. When given in three divided doses, the same quantity of FA almost halved the incidence of NTD in the GD 7 group but not in the GD 8 group. Since the neural tube closure is completed by GD 9, it would seem that the GD 8 VPA + FA group embryos did not have sufficient time for FA to act effectively.

The persistence of a high rate of resorption in the GD 7 group is suggestive of the possibility that more severely malformed embryos were possibly the ones to be resorbed (Wilson, 1973). The resorption rate, reduction in fetal body weight and incidence of NTD in the single (12 mg/kg) and triple FA dose (4 mg/kg) groups of GD 8 were found to be similar. This indicates that mouse embryonic response to FA is developmental stage dependent. That the divided dose regimen of FA in the GD 7 group was more effective in reducing the incidence of exencephaly by about 50% is suggestive that a moderate plasma concentration of FA initiated on GD 7 well before closure is more important than a short (applied on GD 8) but higher level in promoting the neural fold elevation, fusion and subsequent prevention of exencephaly. Divided doses of FA administered daily starting on GD 5 and continued through GD 10 resulted in a greater exencephaly prevention than single doses as well as three doses given only either on GD 7 or 8 (Table 4). Since more embryos were resorbed in the GD 7 group than in the GD 8 group, it would seem that the higher reduction in exencephaly incidence in the GD 8 group was possibly due to the higher survival rate of embryos. In other words, more severely malformed embryos of GD 7 VPA group were possibly the ones to be resorbed.

Plasma folic acid and vitamin B12 determination also revealed that successive dosing starting on GD 5 led to better plasma concentrations than the other two regimens. This observation shows that a plasma concentration of folic acid higher than the control levels initiated early and maintained over a long period during critical stages of embryogenesis allows a greater opportunity for the neural folds to elevate and fuse. This inference is further strengthened by the results of the time-lapse study of the neural tube closure in the control and FA supplementation groups of embryos in which the neural tube closure events appeared to be accelerated in the supplemented group of embryos.

There is a view that NTD do not result from a maternal deficiency of folic acid because mothers of fetuses with NTD have either normal red cell and serum folate levels or are only mildly deficient (see for references Scott et al., 1994). Only some studies have noted significantly lower folate concentrations in women having NTD babies than those with normal fetuses (Smithels et al., 1981). Intestinal absorption of folate is not disturbed in women with a history of carrying NTD affected fetuses (Bower et al., 1993). Whereas this may be the case in the general population of NTD, the situations in epileptic pregnancy in general and in those on AED therapy in particular are different. Phenytoin and VPA have been reported to interfere with folate metabolism in the embryos (Netzlöff et al., 1979; Hansen and Billings, 1985; Lewis et al., 1998). Folate concentrations decrease during pregnancy and AED including VPA intensify this effect (Hendel et al., 1984). Epileptic patients with malformed infants have particularly low levels of plasma folate (Ogawa et al., 1991; Lewis et al., 1998). Our mice treated with VPA exhibited a significant reduction in plasma folic acid and a single dose of 12 mg/kg of FA improved the concentrations but the three divided doses given on one day had a better alleviation effect. Successive doses of FA starting on GD 5 and continued through later stages not only improved the plasma FA levels but also enhanced the concentrations to much higher levels than that of the control and other dosing regimens of FA. It is important to point out here that this higher level sustained over a longer period during neural plate differentiation, neural fold elevation and neural tube closure had a significant bearing on the fetal outcome in terms of a profound reduction in the incidence of exencephaly. This view is further strengthened by Shin and Shiotani’s (Shin and Shiotani, 1999) study, which shows that folic acid-supplementation over a similarly prolonged period during gestation (GD 0.5 through GD 9.5) produces a 30% reduction in maternal hyperthermia-induced NTD in mouse fetuses.

Embryonic development is characterised by an intense mitotic activity in different tissues; hence there is an enhanced need for folic acid for DNA methylation. Low folic acid levels cause chromosomal breaks, hypomethylation of DNA and micronucleus formation (Fenech, 2001). Expression of folate binding protein 1 that mediates cellular uptake of folate has been observed to occur in the dorsal edges of the fusing neural folds of mouse embryos (Saitsu et al., 2003). However, the exact mechanism by which folic acid plays its rescue operation on the neural tube is not clear. In the absence of a clear folic acid deficiency in pregnancies associated with NTD fetuses, attention appears to be directed to genetically determined folate metabolism. Low levels of methionine, elevated levels of homocysteine and alterations in B12 and inositol levels have been implicated in NTD (Copp, 1998). 5,10 Methyl-
ene tetrahydrofolate, homocysteine, methionine, cystathionine, cysteine, vitamin B12 etc are key elements in folate metabolism. Homocysteine appears to be a biomarker of folate status (Locock et al., 1999). Moderately elevated levels of homocysteine have been observed in maternal blood and amniotic fluid of pregnancies with NTD (Steegers-Theunissen et al., 1991; Kirke et al., 1997). Homocysteine has been shown to induce malformations in chick and rat embryos (Rosenquist et al., 1996; Vanaerts et al., 1994). Kirke et al. (1993) observed that folic acid and vitamin B12 are independent risk factors for NTD. The enzyme 5,10-methylene tetrahydrofolate reductase (MTHFR) catalyses the production of 5-methyltetrahydrofolate, a substrate for methionine synthase in folic acid cycle. Reduced activity of MTHFR results in elevated levels of homocysteine and thus diminishes the supply of methyl groups to macromolecules via folate-homocysteine cycle. A thermolabile variant of MTHFR is reported to occur frequently among NTD cases and their families than among controls (Al-Gazali et al., 2001; Ashfield-Watt et al., 2002). The fact that folic acid cannot prevent all NTD points to the heterogenous nature of NTD that do not follow a Mendelian pattern of inheritance. This view is further supported by experimental studies in which folic acid prevents cranial NTD in the homozygous Cartl knockout mouse embryos (Zhao et al., 1996) but the NTD in Axial defects mutants do not respond to folic acid or vitamin B12 but only to methionine (Esseen and Wannberg, 1993). We have recently observed that methionine does not prevent exencephaly but accentuates maternal alcohol-induced axial skeletal defects in the TO mouse embryos (Padmanabhan et al., 2002). But then we only administered exogenous methionine whereas it was not just methionine that was depressed but a host of other amino acids whose plasma concentrations were lowered by ethanol. Neither FA nor B12 was co-administered in that study. Also noteworthy was the fact that a prolonged exposure to a steady concentration of methionine might be more effective than a bolus dose in alleviating teratogen-induced fetal malformations and growth retardation. Further studies are required to clarify certain basic issues in the nutritional requirements of embryos during normal and abnormal morphogenesis of the neural tube.

ACKNOWLEDGEMENT

Mr. Ashok Prasad (Media Unit) assisted us in photography. This work was supported by generous grants (NP/2000/25) from the Faculty of Medicine and Health Sciences, UAE University, Al Ain.

REFERENCES


Padmanabhan R (1997) Effect of cotreatment of valproic acid (VPA) and folinic acid (FA) on axial skeletal morphogenesis in the TO mouse. Cong Anom, 34: 314A.


Dev Biol, 14: 192-205.


