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Valproate induced effects on development in the rat are partially prevented by folinic acid and S-adenosylmethionine

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Summary

Valproic acid (VPA) is a teratogenic agent that induces a wide range of tissue alterations, including neural tube defects (NTD) and skull malformations. There is an established dose-dependent effect of VPA and types of malformations in rat. The aim of the present study was to determine morphological, histological and immunohistochemical alterations under an assumedly non teratogenic VPA exposure. Potential prevention by coadministration with folinic acid (FA) and S-adenosylmethionine (SAM) was also assessed.

Wistar rats were treated during gestation with VPA (300 mg/kg/d on days 8, 9, and 10), either alone or in combination with FA (4 mg/kg/d on days 8, 9, and 10) or SAM (10 mg/kg/d from days 1 to 10). Rats were terminated on day 21, and implantation sites were counted.

VPA induced a lower fertility rate due to a higher number of resorptions, which were reduced by coadministration of either FA or SAM. Foetal weight and length were unaffected by VPA treatment. Hepatic injury was observed in foetuses treated with VPA. Studies with specific antibodies against Kupffer cells and T-lymphocytes also showed that Kupffer cells appeared more frequently in the VPA+FA and VPA+SAM groups, whereas folinic acid and exogenous SAM were able to reverse the decrease in VPA-induced foetal liver T-lymphocyte cells. VPA treatment resulted in skeletal modifications in the skull, appendicular bones, vertebrae and ribs. Folate was able to prevent these defects, whereas SAM-coadministration did not show this protective action.

The results are discussed on the basis that VPA may induce other potential alterations different from NTD whose long-term effects are not well understood at present.

Key Words: Valproate - Folinic acid - S-adenosylmethionine - Gestation - Rat

Introduction

Valproate (valproic acid, VPA) is an effective anticonvulsivant drug widely used in the treatment of epilepsy. Many studies have shown that VPA induces neural tube defects (NTDs), and especially spina bifida in humans (Bjerkedal et al., 1982; Robert and Rosa, 1983; Lindhout and Meinardi, 1984) and exencephaly in rodents (Ong et al., 1983; Vorhees, 1987; Ehlers et al., 1992; Padmanabhan et Hameed, 1994). However, VPA also induces other less severe but important alterations, such as foetal skeletal malformations and hepatotoxicity. In fact, Vorhees (1987) has shown a dose-dependent effect for VPA and types of malformations in rat: only when the dose used was above 400 mg/kg/day were NTD induced.

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The mechanism for the drug effect is unknown, but an interaction with folate cycle and methylation cycle has been suggested (Fig. 1). It has been reported that VPA may decrease maternal plasma folate concentrations in rats (Carl, 1986). Many animal models have been developed to study the teratogenic mechanism of VPA, as well as its possible prevention with the vitamin folic acid and the amino acid methionine. In mice, *in vivo* treatment with folinic acid (5-formyltetrahydrofolate) decreases the incidence of VPA induced-NTDs (Trotz et al., 1987), whereas in other similar studies no effects have been observed (Elmazar et al., 1992; Hansen et al., 1995). Rat embryos treated *in vitro* with folinic acid did not show any protective effect (Hansen and Grafton, 1991). In humans, periconcepcional supplementation with folic acid reduces the recurrence (MRC Vitamin study Research Group, 1991) and the occurrence of NTDs (Czeizel and Dudas, 1992).

Coelho et al. (1989) observed that rat embryos cultured in cow serum required methionine for neural tube closure. *In vivo*, methionine decreases the exencephaly rate in mouse embryos (Ehlers et al., 1996). Furthermore, cultured rat embryos revealed that in the absence of methionine the neural tube failed to close (Coelho and Klein, 1990). These authors suggest that methionine may be required for the methylation of the microfilament proteins involved in neural fold apposition. We have previously demonstrated (Alonso-Aperte et al., 1999) that VPA-treated rats had significantly lower plasma methionine levels when compared to untreated rats. However, it remains unclear whether these metabolites participate in VPA-induced hepatotoxicity.

Methionine is transformed in the cell into S-adenosylmethinone (SAM), which serves as the donor of methyl groups in many transmethylation reactions (DNA-, proteins-, lipids, etc). SAM is also important in methionine metabolism since it is a factor that modulates the distribution of homocysteine between the remethylation to methionine and the catabolic transsulfuration pathway (Finkelstein, 1990).

We have previously shown that SAM exerts a protective effect in carbon tetrachloride (CCl_4)-induced hepatic injury since it reverses folate depletion (Varela-Moreiras et al., 1995) and partially corrects the depletion of glutathione (GSH) (Corrales et al., 1992), a molecule involved in the regulation of the antioxidant status. SAM is able to increase GSH bioavailability and this could be the mechanism of its protective effect (Mato, 1994).

A possible factor contributing to VPA-induced hepatotoxicity is the antioxidant status: VPA treatment has been associated with selenium deficiency, which is required for glutathione peroxidase activity (Hurd et al., 1984). It has been proposed that reactive toxic metabolites of VPA could be involved in the hepatic injury (Appleton et al., 1990) and a detoxification role for GSH in the mechanism of toxicity of unsaturated metabolites of VPA has also been suggested (Jurima-Romet et al., 1996).

Since its initial description, several unproved hypotheses have been proposed to clarify the potential folate-VPA interaction. The present study was undertaken to examine the adverse effects of administering an assumedly non-teratogenic dose of VPA to rat foetuses, mainly hepatic injury and skeletal alterations, and the role of folinic acid and SAM as potential protective factors in the incidence of these alterations.

Materials and Methods

Animals

Forty-seven female rats of the Wistar strain (Instituto de Investigaciones Biomédicas, CSIC, Spain) were used in this study. The animals were housed in rooms at 22±2°C on a 12:12 hours light:dark cycle. Virgin females were mated overnight (ratio of one male to five females). The presence of a vaginal plug upon examination the following morning was considered to indicate day one of pregnancy.

Animals were given free access to standard laboratory diet (Panlab, S.A., Spain) and water was also provided *ad libitum*.

All animal experiments were undertaken according to the "Directional Guides Related to Animal Housing and Care", from the European Community Council (1986).

Drugs

Sodium valproate (VPA) and folinic acid (5-formyltetrahydrofolate, calcium salt) were obtained from Sigma Chemical Co (Dorset, UK) and prepared as an aqueous solution. S-adenosylmethionine, in the stable form of sulphate-p-toluensulfonate, produced as Samet® (BioResearch Spa, Milan, Italy), was kindly provided by Europharma (Madrid, Spain).

Treatments

Rats were classified in four groups according to the specific treatment:

*VPA: 16 rats treated with 300 mg/kg body weight of valproic acid, subcutaneously on days 8, 9 and 10 of gestation.

*VPA+FOL: 11 rats treated with 300 mg/kg body weight of valproic acid, subcutaneously on days 8, 9 and 10 of gestation + 4 mg/kg body weight of folinic acid, intraperitoneally, 30 minutes before each VPA injection.

*VPA+SAM: 11 rats treated with 300 mg/kg body weight of valproic acid, subcutaneously on days 8, 9 and 10 of gestation + 10 mg/kg body weight of S-adenosylmethionine, intramuscular, over the first ten days of gestation.

*Control: 9 untreated rats.

Dams were sacrificed on day 21 of gestation. The uteri were removed immediately, and the implantation sites and resorptions were recorded. Each living foetus was weighed and measured individually and inspected for the presence of external malformations. Foetuses were fixed in 10% formalin for morphological study, which consisted of a skeletal analysis and a histological and immunohistochemistry study.

Histological study

Whole fixed foetuses were transferred to 5% trichlororacetic acid, dehydrated through ascending ethanols and butanol and paraffin imbedded. Specimens were cut at 7 µm parasagittal sections on a microtome and then conventionally stained with the hematoxylin-eosin and AZAN (azocarmine-aniline blue) techniques. Sections were visualised under a Nikon Type 115 light microscope, and photographed with a Nikon FX-35 DATA camera.

Immunohistochemistry study

Fixed livers foetuses were removed, rinsed in phosphate buffer saline (PBS), and transferred to 15% sucrose solution. Specimens were cut at 10 µm sections on a Leika cryostat at -20°C and collected on poly-L-lysine glass slides. Sections were rinsed in PBS, transferred to 0.3% hydrogen peroxide and chosen randomly for staining with one of two primary antibodies against Kupffer cells or against T cells and incubated overnight at 4° C. The primary antibody for Kupffer cells (Mouse Anti-Rat Macrophages, Serotec, England) was diluted 1/400 in PBS, and the primary antibody for T cells (Mouse Monoclonal Antibody to Rat Thymocyte and T cells, Serotec, England) was diluted 1/50 in PBS. The following morning sections were rinsed in PBS and the secondary antibody (Affini Pure Rabbit Anti-Mouse Ig G, diluted 1/50, Jackon Immunoresearch Lab Inc., Pennsylvania, USA) was added for two hours at room temperature. Slides were rinsed in PBS and the tertiary antibody (Mouse Peroxidase-Anti-Peroxidase, diluted 1/500, Jackson Immunoresearch Lab Inc., Pennsylvania, USA) was applied for two hours at room temperature. Sections were rinsed in PBS and developed with 0.06% diaminobenzidine (DAB) solution. Sections were observed under a Nikon Type 115 light microscope and photographed with a Nikon FX-35 DATA camera.

Cellular measurement

To measure stained cells, we followed the method of Cope and Dilly (1990) with modifications. One of three sections from each liver was selected randomly. For each section, three fields were chosen and photographed at x20 magnification. Area measurements were made using a milimetric slide, each photography corresponding to 0.2944 mm^2, an area delimited by the viewfinder of the photomicroscope. Counts were made directly on the photographs. Data are expressed as the number of cells per mm^2.

Skeletal study

To analyse the skeletal malformations, half of the foetuses were chosen randomly, skinned and eviscerated. The procedure of cartilage staining with alcian blue and bone staining with alizarin red S as modified by Wassersug (1976) was carried out. Foetuses were examined under a model SMZ-10 Nikon dissecting microscope, following as model a laboratory atlas of the rat skeleton (Walker and Wirtschafter, 1955), and photographed with a Nikon FX-35 DATA camera.

Statistical analysis

Results were compared using one-way ANOVA followed by the Tukey multiple test. Differences were considered significant at $p< 0.05$.

RESULTS

Gestational parameters

VPA treatment on days 8, 9 and 10 of gestation did not especially affect the gestational parameters. The gestation index, expressed as the proportion of animals with live foetuses on gestation day twenty-one from the total of pregnant rats on gestation day one, was lower in the VPA group, although the differences were not statistically significant (p=0.06) (Table 1). Coadminis-

Table 1.- Gestational parameters of Wistar rats treated with VPA alone or in combination with folinic acid or S-adenosylmethionine.

	Control	VPA	VPA+FOL	VPA+SAM
Pregnant females	9	16	11	11
Dams with live foetuses	9	12	10	10
Gestational index (GI, %)	100	75*	91	91
N° resorptions/dam (mean±SEM)	1.11 ± 0.58	1.37 ± 0.55	1.08 ± 0.31	0.90 ± 0.39
Live foetuses/dam (mean±SEM)	12.6 ± 0.92	9.0 ± 1.40*	9.8 ± 1.15	9.1 ± 1.41
Fetal weight (g) (mean±SEM)	3.42 ± 0.06	3.61 ± 0.14	3.54 ± 0.11	3.50 ± 0.30
Fetal length (cm) (mean±SEM)	37.7 ± 0.24	37.4 ± 0.52	37.5 ± 0.35	37.1 ± 0.49

*p = 0.06 with respect to control group

$$GI = \frac{\text{dams with live foetuses}}{\text{Pregnant females}} \times 100$$

tration with FOL or SAM increased the gestation index to values similar to those of the control group. This positive effect was also observed for the number of live foetuses per dam. This parameter was lower in the VPA group than the other cases, but not statistically different (p=0.06). As regards the gestation index, this was slightly elevated when the VPA was administered with FOL or SAM. The number of resorptions, foetal weight and foetal length were not significantly different when compared to the control group values.

Macroscopic analysis

Observation of the external alterations of the foetuses revealed no perceptible anomalies in any of the four groups.

Histological and immunohistochemistry analysis

The use of conventional staining techniques in foetal liver sections revealed the presence of cells other than hepatocytes, with a smaller and denser nucleus (Fig. 8). When a sinusoidal position was analysed, these cells proved to be either Kupffer cells (KC) or T cells (TC), and hence we decided to use two different antibodies to determine the type of cell. KC and TC were easily recognised at light microscopy owing to their brown cytoplasmic staining for DAB.

The values obtained for the cell counts are shown in Table 3. KC were significantly elevated ($p<0.05$) in all three VPA treated groups as compared to the control group. No differences were detected between the VPA group and the supplemented FOL or SAM groups. By contrast, TC levels were significantly reduced ($p<0.01$) in the

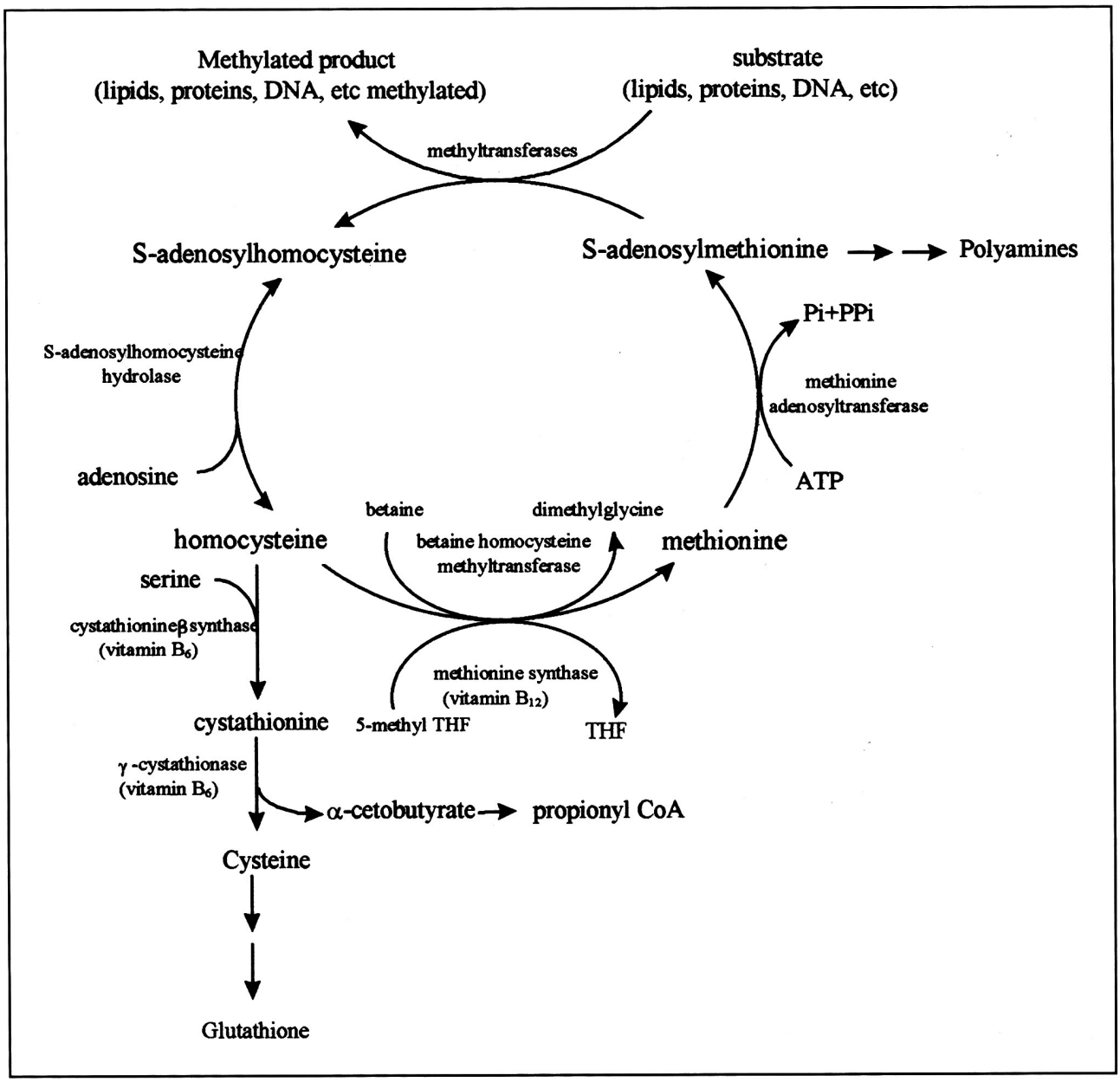
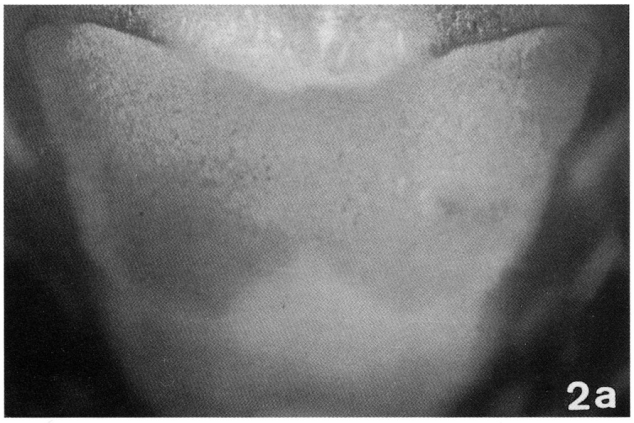


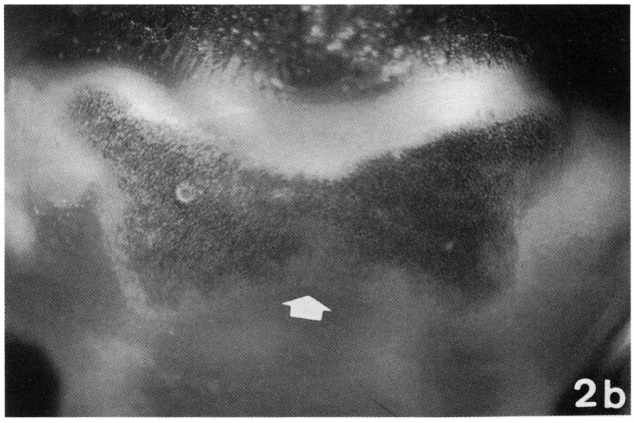
Fig. 1.- Biochemical pathways related to folate and methylation cycles, modified after Scott et al. (1994) and Adams et al. (1995).

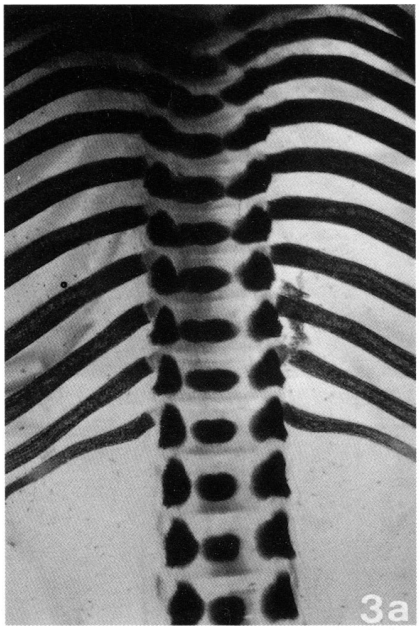
VPA group as compared to the VPA+SAM set, although the decrease in cells was also remarkable with respect to the control and VPA+FOL groups. Thus, supplementation with FOL or SAM reverses the effect of VPA. Figures 9 and 10 show photographs of foetal liver sections, where stained cells are observed and counted.

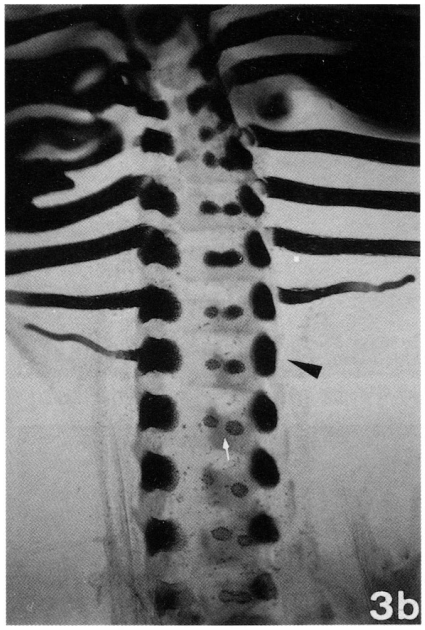
Skeletal analysis

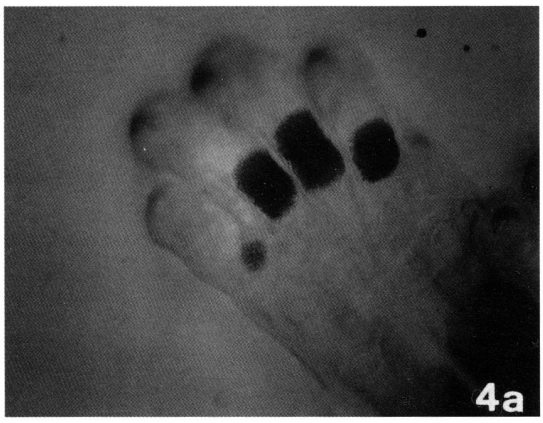
All ossification defects (malformations + development delays) were summarised in three groups depending on the anatomical region where they appeared: skull, trunk or limb. Some of observed skull defects were a delay in supraoccipital development (Fig. 2) and exoci-











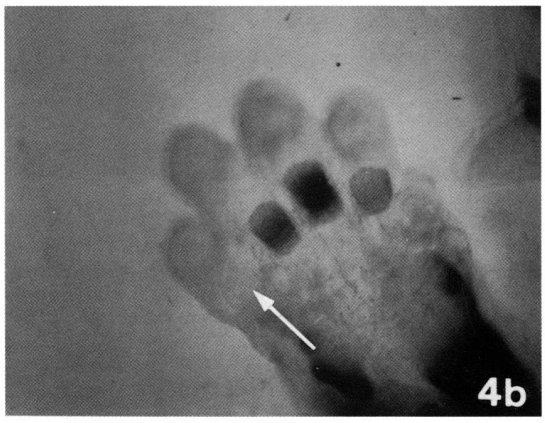
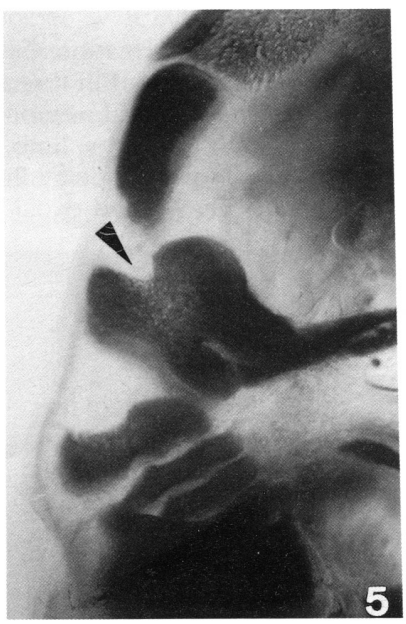
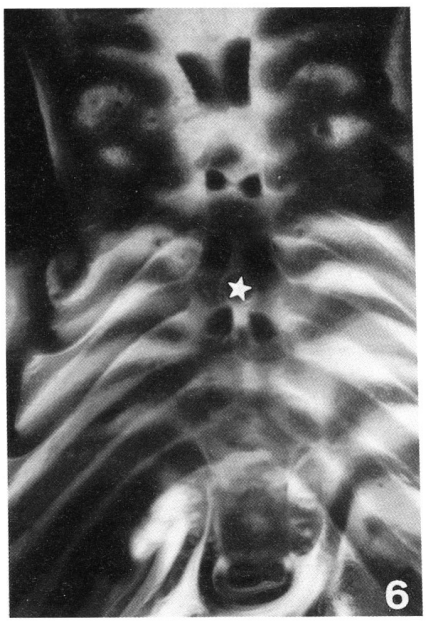


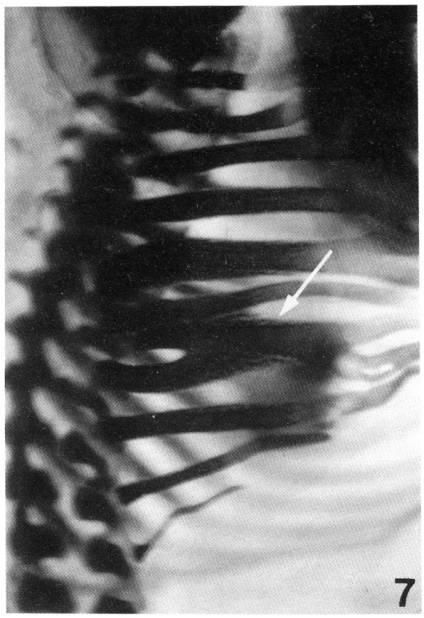
Fig. 2.- **a:** supraoccipital bone in a control rat foetus. **b:** arrow shows smaller and more irregular supraoccipital bone in a VPA treated rat foetus.

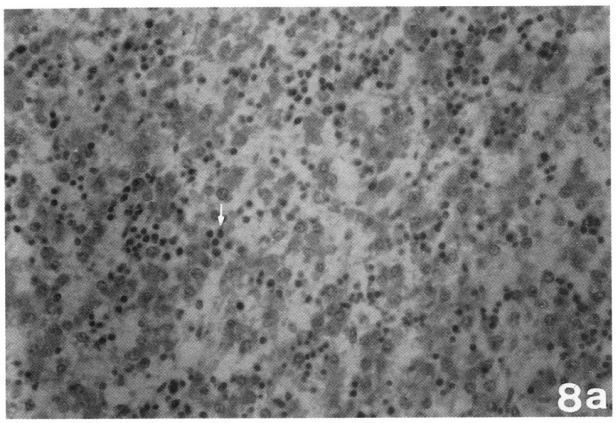
Fig. 3.- **a:** thoracic and lumbar region in a control rat foetus. **b:** arrow shows the delay in vertebral centres, and arrowhead shows lack of 13th rib in a VPA treated rat foetus.

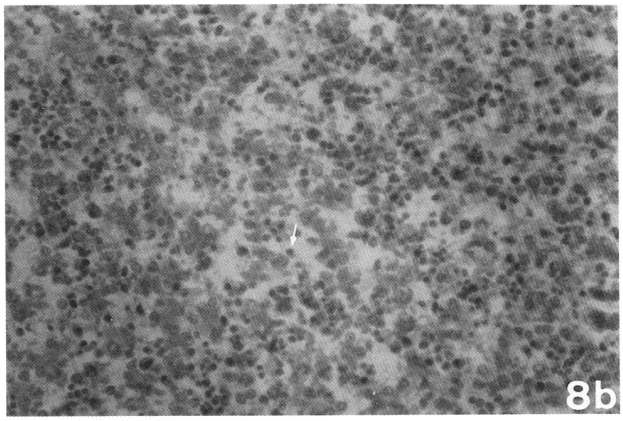
Fig. 4.- **a:** dorsal region of anterior limb with four ossified metacarpal in a control rat foetus. **b:** arrow shows lack of fifth metacarpal in a VPA treated rat foetus.

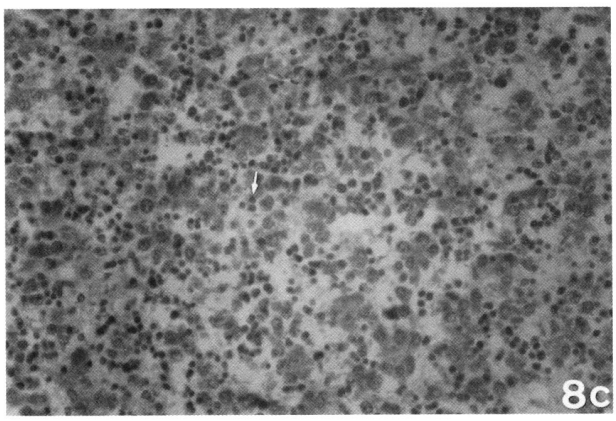












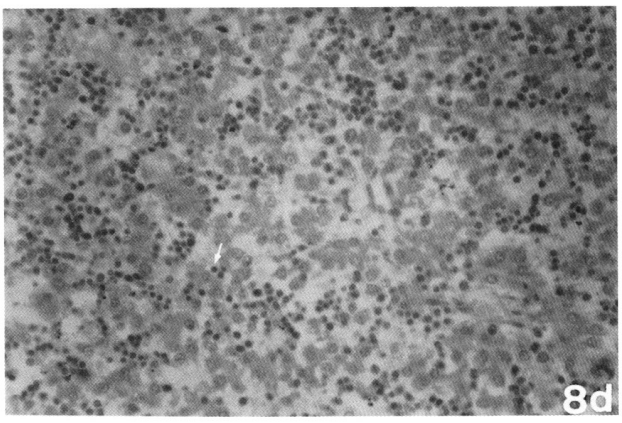


Fig. 5.- Cervical region of a rat foetus treated with VPA: arrowhead shows the exoccipital-atlas fusion.
Fig. 6.- Ventral region of sternum of a rat foetus treated with VPA: star shows lack of sternebras fusion.
Fig. 7.- Lateral thoracic region of a rat foetus treated with VPA: arrow shows fused ribs.
Fig. 8.- Foetal liver sections stained with hematoxilin-eosin: arrows show sinusoidal cells, which are less numerous in the control group than in the three treated groups. x20. **a:** control group. **b:** VPA group. **c:** VPA+FOL group. **d:** VPA+SAM group.

pital-atlas fusion (Fig. 5). Trunk defects were a lack of sternebras fusion (Fig. 6), fused ribs (Fig. 7), a delay in vertebral centre ossification (Fig. 3) and agenesis of the 13th rib. Finally, limb defects were mainly represented by a lack of ossification in the fifth (Fig. 4) or all metacarpal.

The numerical data of the incidence of altered foetuses in each group are shown in Table 2. No significant differences between groups were observed in the skull and limb defects.

In all groups the major defects were the malformations or a delay in ossification appearing in the trunk region.

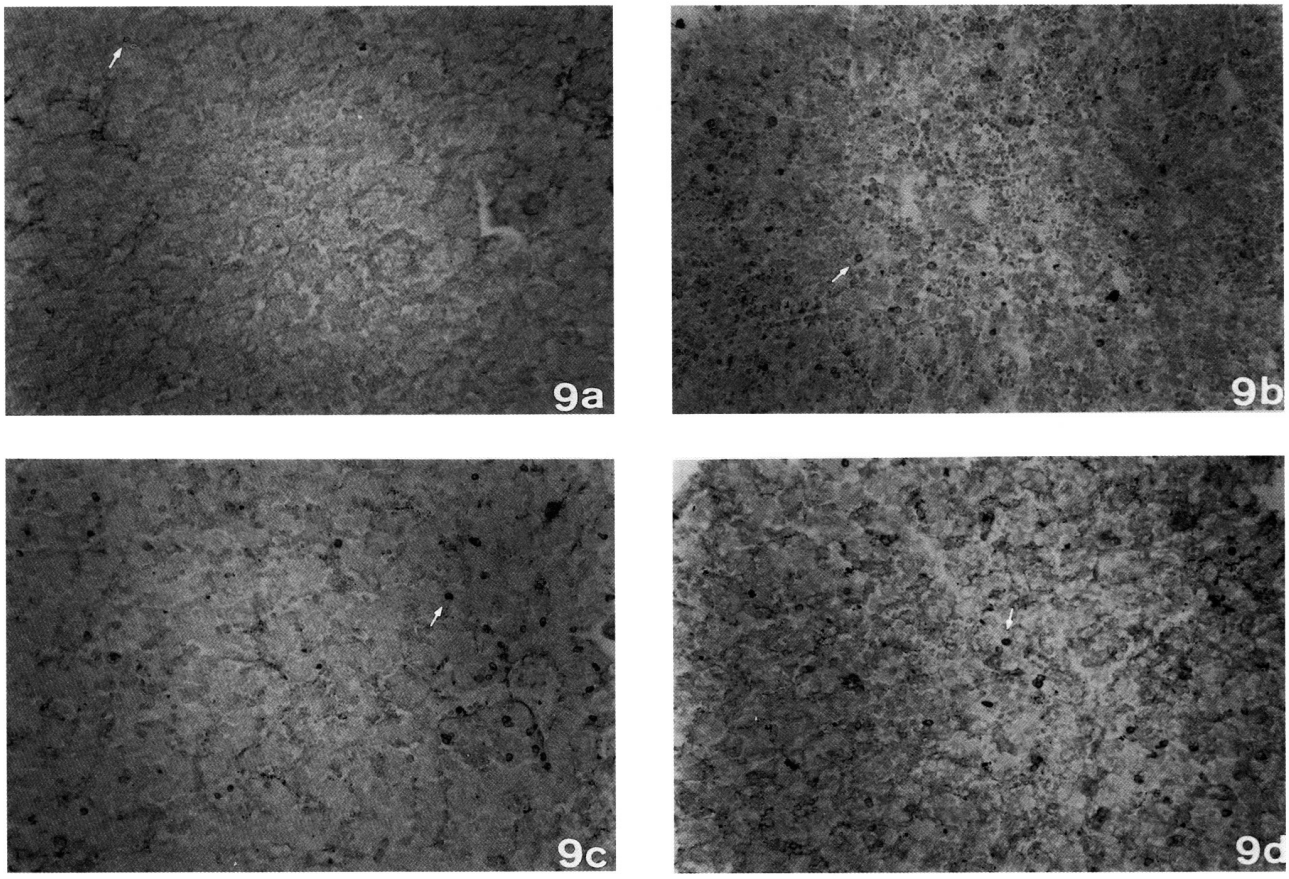


Fig. 9.- Foetal liver sections labelled with Kupffer cell antibody: arrow show labelled cells. x20. **a:** control group. **b:** VPA group. **c:** VPA+FOL group. **d:** VPA+SAM group.

Coadministration of FOL had a protective effect for all defects, since a reduction in the proportion of altered foetuses to nearly control values was observed. However, SAM did not exert a protective effect against the ossification defects.

Discussion

Treatment with VPA caused a marked although not significant reduction in the number of live foetuses per dam and in the gestational index, and an increase in the number of resorptions (Table 1). However, foetal weight and length values were similar to those of the control foetuses. This suggests that drug effect could be mainly related to early development, decreasing embryos implantation or increasing resorptions. However, once this alteration has been overcomed, the development of VPA-affected embryos should be considered normal and extrapolable to control embryos. The embryolethality rate observed in the present study is lower than that reported by Vorhees (1987) in Sprague-Dawley rats and by Nosel and Klein (1992) in CD rats using the same dose. However, in those studies different administration patterns (gavage and intraperitoneally injection) were used. To date, no other comparable studies have been carried out. The administration of FOL or SAM improved the gestational parameters, mainly the gestational index and number of resorptions, which are indicative of foetal viability.

According to Vorhees (1987) and Menegola et al. (1996), administration of 300 mg/kg of VPA

Table 2.- Ossification defects in Wistar rats treated with VPA alone or in combination with folinic acid or S-adenosylmethionine.

	Control	VPA	VPA+FOL	VPA+SAM
Skull defects (%)	8 (4/49)	18 (10/57)	12 (5/42)	18 (7/39)
Trunk defects (%)	29 (14/49)	40 (23/57)	29 (12/42)	62 (24/39)**
Limb defects (%)	12 (6/49)	21 (12/57)	21 (9/42)	38 (15/39)

** $p < 0.01$ with respect to control, VPA and VPA+FOL groups

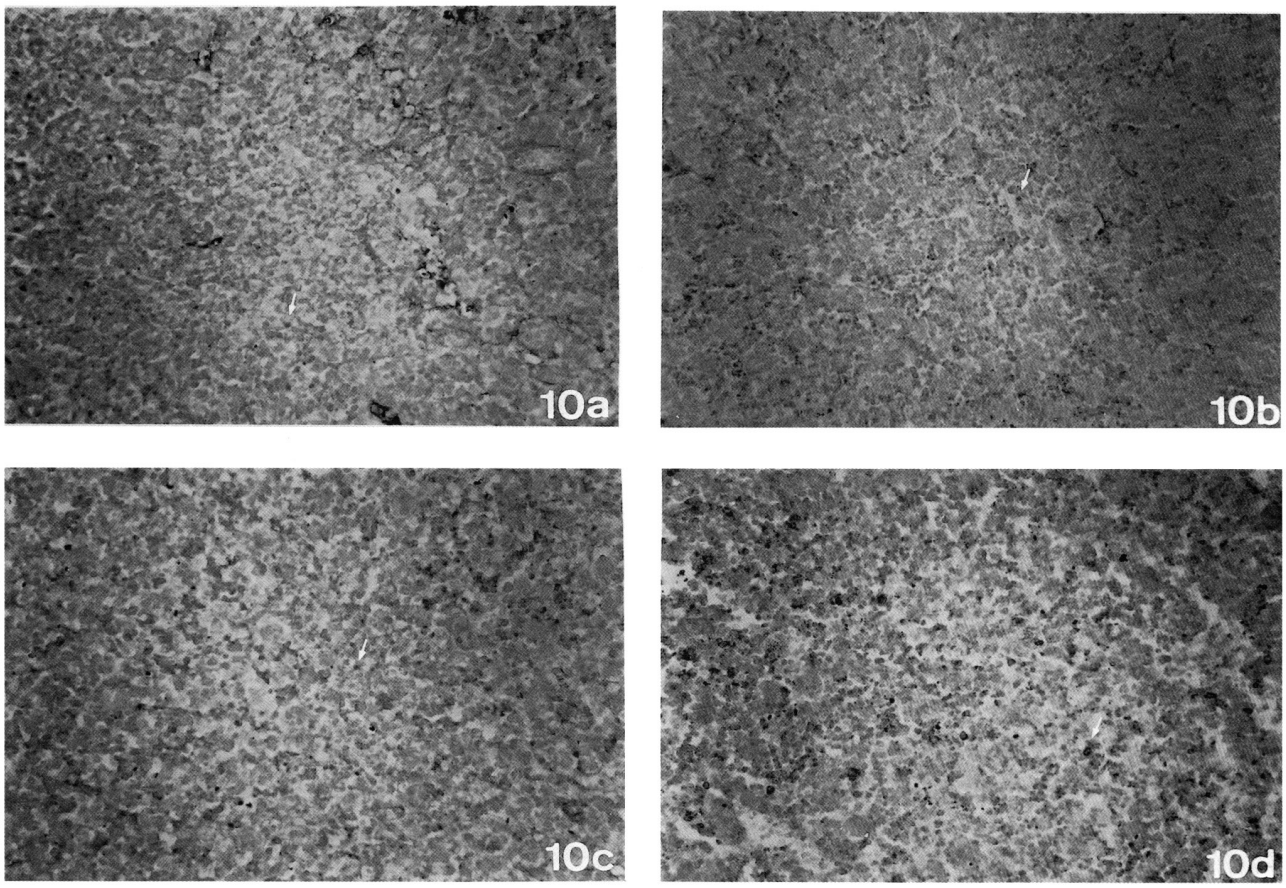


Fig. 10.- Foetal liver sections labelled with T cell antibody: arrow show labelled cells. x20. **a:** control group. **b:** VPA group. **c:** VPA+FOL group. **d:** VPA+SAM group.

does not induce exencephaly or spina bifida at gestation term. However, in our study we observed important alterations in foetal liver and the skeletal system.

When whole foetal sections were conventionally stained with the hematoxylin-eosin or Azan techniques in the sinusoids we observed the presence of cells other than hepatocytes, which were more numerous in the treated groups than in the controls (Fig. 8). The use of specific antibodies against KC or TC allowed us to recognise these cell types.

KC levels were significantly elevated in the groups treated with VPA alone or in combination with folinic acid or S-adenosylmethionine (Table 3; Fig. 9). KC are specific liver macrophages involved in the inflammatory response to xenobiotic-induced liver injury (e.g. carbon tetrachloride, acetaminophen, phenobarbital, alcohol, etc.). The specific location of these cells within the liver varies with the chemical agent used (Laskin and Pendino, 1995). Many reports have shown that when KC are activated by inflammatory stimuli, they release superoxide anion, hydrogen peroxide, nitric oxide, hydrolytic enzymes, and a number of different immunoregulatory and inflammatory cytokines (Decker, 1990; Laskin, 1990). Laskin and Pendino (1995) have suggested that they may act as primary mediators of tissue injury, and/or participate in the inflammatory response by initiating a cascade of additional immunological reactions that result in tissue damage.

Recently, Fox et al. (1997) have reported that N-acetylcysteine and α-tocopherol reverse KC activation *in vitro* and are effective at points beyond the initiation of activation, since these antioxidants suppress cytokine mRNA and TNF-α secretion of KC. However, in our study neither folinic acid nor SAM were able to reverse the KC activation in foetal livers or the consequent inflammation.

On the other hand, folinic acid and SAM did reverse the VPA- induced foetal liver decline in TC levels. This VPA effect was also reported by Almodovar-Cuevas et al. (1985) after chronic exposure to 240 mg/kg in Sprague-Dawley rats. These authors mainly observed a reduction in the numbers of monocyte and lymphocyte cell types, and concluded that the effects of VPA are dependent on drug kinetics. However, there are no data available for the possible effects of prevention through FOL and SAM. The mechanism of this prevention effect is unknown, but it could be related to the process involved in the prevention of other haematological alterations, such as anaemia. More research is needed to confirm or reject this possibility.

Table 3.- Number of Kupffer cells and T cells per mm^2 in foetal livers from Wistar rats treated with VPA alone or in combination with folinic acid or S-adenosylmethionine.

	Control	VPA	VPA+FOL	VPA+SAM
Kupffer cells (mean±SEM)	29.92 ± 12.69	90.77 ± 19.72*	93.2 ± 9.50*	111.11 ± 20.84*
T cells (mean±SEM)	36.92 ± 5.63	14.43 ± 3.45**	30.73 ± 3.36	51.84 ± 12.41

* $p < 0.05$ with respect to control group
** $p < 0.01$ with respect to VPA+SAM group

The axial skeleton is a system which is very sensitive to the effects of VPA. In this study VPA was able to induce a delay in ossification at a higher proportion in the treated animals as compared with the control group. Figure 2 shows the most common VPA-induced developmental delay in the three anatomical regions as compared to the controls: the supraoccipital, thoracic and lumbar centres and the fifth metacarpal. However, the observed differences were not statistically significant (Table 2). These delays in ossification, except for the thoracic and lumbar regions, have not been described in other studies, and are the most characteristic features observed in the present animal model. VPA administration also induced malformations, which were not observed in the control group (Figure 3): atlas-exoccipital fusion, fused ribs and lack of sternebras fusion, also described by Menegola et al. (1996) in mice treated with 150 or 300 mg of VPA/kg b.w. at day 7 of gestation. These VPA-induced malformations in our animal model could affect the foetal viability and were not reversed by supplementation with either folinic acid or S-adenosylmethionine.

Menegola et al. (1998) suggested that there is a characteristic pattern of malformations related to developing times of treatment. This segmentary specificity could be a possible explanation for the predominance of trunk defects, mainly thoracic, in our experiments, although more studies are necessary to confirm such a hypothesis.

Folinic acid reduced the VPA-induced skull and trunk defects, but not significantly. A protective effect of folinic acid on VPA-induced embryotoxicity has been reported by Trotz et al. (1987) and Wegner and Nau (1991) in the NMRI mouse. However, it seems that only when combined with vitamins B_6 and B_{12} is it able to reduce some ossification defects in the same strain (Elmazar et al., 1992). Other experiments demonstrated that there was no protective effect for folinic acid in CD rats *in vitro* (Hansen and Grafton, 1991) or in CD mice or Nctr :SDN rats *in vivo* (Hansen et al., 1995). According to these authors, differences may occur in the absorption/metabolism of folinic acid, the sensitivity to VPA, or in the mechanism of VPA-induced embryotoxicity between strains. As we show in the present study, in Wistar rats folinic acid seems to have no protective effect. The delay in supraoccipital development was also observed by us in the foetuses of rats treated with a FA deficient diet, but not in foetuses chronically treated with a FA supplemented diet (8 mg/kg b.w.) during pregnancy (Alonso-Aperte, 1997).

Coadministration of VPA and SAM increased the delay in ossification, but not the malformations. In fact, trunk defects were significantly elevated as compared to the other groups. However, the defects observed were less severe than those observed in the VPA group. The exogenous administration of SAM seems to enhance the VPA-induced ossification defects. SAM is the universal donor of methyl groups in several transmethylation pathways and is a methylation cycle modulator (Mato et al., 1994). However, the administration of elevated SAM concentrations could cause a metabolic disruption in the methylation cycle that would enhance the effect of VPA resulting in alterations to foetal morphogenesis.

In conclusion, we have shown in Wistar rats that 300 mg/kg b.w. of VPA, an assumedly non-teratogenic dose of drug-results in potentially important foetal alterations, such as skeletal malformations and delays, which are partially prevented by folinic acid supplementation, and hepatotoxicity in liver foetuses. A possible mechanism for the hepatic toxic effect is a relationship between the drug and immunological changes, i.e. macrophages and lymphocytes. Exogenous administration of folinic acid or SAM contributes to reversing the leukopenia observed in foetal livers, but not KC activation, and may be due to a hitherto unknown complicated molecular process. Further studies are urgently needed to evaluate these morphological and biochemical liver alterations, and to establish an adequate dose of VPA to maintain an adequate control of seizures in pregnant women without induce some foetal alterations.

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