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ORIGINAL CONTRIBUTION

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Effect of maternal exposure to homocystine on sodium valproate-induced neural tube defects in the mouse embryos

Summary *Background* Neural tube defects (NTD) are mainly of multifactorial origin. Maternal treatment with valproic acid (VPA) during pregnancy induces NTD in susceptible fetuses. Elevated levels of homocysteine are observed in pregnancies with NTD. The mechanism by which homocysteine might cause NTD is unknown. *Aim of the Study* The aim of this study was to determine if homocystine would augment VPA-induced exencephaly in an experimental model. *Methods* Groups of mice were injected (IP) on gestational day 8 (GD) with a single dose of 75 mg/kg of L-Homocystine (HC) or a proportionate volume of saline, followed by a single dose of 600 mg/kg of VPA or an equal volume of saline. In a second experiment, mice were treated with a daily dose of 75 mg/kg of HC or an equal volume of saline (IP) from GD 5 and continued through GD 10. These animals had a single exposure to 600 mg/kg of VPA or saline (IP) on GD 8. All animals were killed by cervical dislocation on GD 18. Plasma homocysteine, folate and vitamin B12 were determined on GD 8 and GD 10 from single and multiple dose groups of mice, respectively, from additional experiments. *Results* The VPA and HC + VPA induced significantly higher rates of embryonic resorption and intrauterine growth retardation (IUGR) than HC or saline alone. HC + VPA groups had significantly more numerous fetuses with severe IUGR than HC alone or VPA alone groups. Both single and multiple doses of HC augmented VPA-induced reduction in fetal body weight. Successive doses of HC did not augment the rate of IUGR more significantly than a single dose of HC. Incidence of exencephaly was significantly enhanced in the HC + VPA groups compared to that in the HC or VPA alone groups. HC alone was not teratogenic. Plasma homocysteine levels increased several fold both in HC and HC + VPA groups and the increase was not particularly more marked in multiple dose groups than in the single dose groups. VPA did not elevate homocysteine concentration. Both FA and vitamin B12 concentrations were reduced by VPA, HC and HC + VPA, but HC and VPA when combined did not produce an additive effect on vitamin levels. *Conclusion* These data indicate that HC and VPA interact in neurulation stage embryos, affect fundamental processes of closure of the neural tube and lead to enhanced incidence of NTD.

Key words Neural tube defects – valproic acid – folic acid – homocysteine – vitamin B12

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Introduction

Neural tube defects (NTD) such as anencephaly, spina bifida and encephalocele are the most common birth defects, exceeded in frequency only by congenital heart defects [1, 2]. The etiology of NTD is rather complex and imperfectly understood. Most NTD cases are considered to be of multifactorial origin, having a significant genetic component to their etiology that interacts with a number of environmental risk factors [3, 4]. Reported risk factors for NTD include a previous pregnancy with an NTD, insulin-dependent maternal diabetes mellitus, maternal hyperthermia and the use of antiepileptic drugs (AED) during pregnancy [4, 5]. Both clinical and experimental investigations have shown that maternal exposure to valproic acid (VPA) during critical stages of embryonic development can induce NTD in the offspring [6–9]. In addition to a characteristic pattern of craniofacial anomalies, a manifold increase in the incidence of both myeloschisis with spina bifida aperta and myelomeningoceles with skin covering in the lumbosacral or sacral positions have been reported to occur in about 2% of the offspring of epileptic women treated with VPA during pregnancy [9]. With the discovery that periconceptional folate (FA) supplementation could prevent NTD incidence by about 50–70%, several reports have appeared emphasizing a role for micronutrients such as FA, vitamin B12, methionine etc., in the pathophysiology of neural tube development [10–12].

Possibly because of the burden imposed by fetal needs, maternal FA and vitamin B12 concentrations decrease substantially resulting in high levels of homocysteine during pregnancy [13], AED in general, appear to lower the plasma FA concentrations and when epileptic women are treated with these drugs during pregnancy, the situation worsens, thus giving rise to the recommendation that all epileptic women be given additional FA, particularly when pregnancy is contemplated [14, 15]. Since a large number of pregnancies are unintended, there is some consensus that all women of reproductive age group be advised to eat either vitamin fortified food or consume daily 400 μg of synthetic FA [10–12]. However, the mechanism by which FA rescues embryos from being malformed has not been clarified yet [16]. It is also not clear whether FA *per se* or one of its metabolites, which is responsible for the observed responses. Some studies have implicated the deficiency of methionine, an essential amino acid in NTD. There are also reports that suggest that accumulation of homocysteine as a result of either low FA bioavailability or defective metabolism of FA, could affect neural tube closure mechanisms resulting in NTD. Thus increased concentrations of homocysteine have been reported in plasma and amniotic fluid of pregnant women carrying fetuses with NTD [17, 18]. Patients on long-term AED treatment are reported to have low serum vitamin B12 and erythrocyte FA and elevated plasma total homocysteine values [19]. Experiments on chick embryos in ovo and on mouse embryos in vitro have given conflicting results after homocysteine treatment [20–23]. We observed a high incidence of NTD accompanied by reductions in maternal FA and vitamin B12 concentrations when mice were treated with a single dose of VPA during critical stages of neural tube closure [24].

The objective of the present study was to determine if maternal hyperhomocysteinemia in pregnant mice would provide a greater susceptibility to VPA-induced NTD in the offspring. The results of this experiment indicate that hyperhomocysteinemic condition in the pregnant animals can augment VPA-induced NTD in embryos.

Materials and methods

Animals

The TO mice used in this study were obtained from Harlan Olac (England) and raised in our local facility. They were housed in temperature (21 ± 1°C) and humidity (about 65%) controlled rooms and at 12:12 hours of light:dark cycle. All animals had free access to a laboratory chow and tap water provided *ad libitum*. Virgin females weighing about 30 gm were housed overnight with males and vaginal plug observed on the following morning was taken to indicate successful mating. Plug positive day was considered gestation day (GD) 0.

Experiments

All injections were given via intraperitoneal route. Both sodium valproate (VPA) and L-homocystine (L-4, 4′Dithiobis [2-aminobutanoic acid]) were purchased from Sigma (MO, USA) and dissolved in physiological saline. In experiment I, groups of mice were first injected on GD 8 with a single dose of 75 mg/kg of L-Homocystine (HC) or an equal volume of saline. The saline-treated animals were then exposed to a single dose of 600mg/kg of VPA (saline + VPA group) or an equal volume of saline (saline + saline control group). One half of the HC-treated animals then received a single dose of 600 mg/kg of VPA (HC + VPA group), while the other half were injected with a proportionate volume of saline (HC + saline group). In experiment II, mice

were treated with a daily dose of 75 mg/kg of HC or a proportionate volume of saline starting from GD 5 and continued through GD 10. On GD 8, the saline-treated animals were divided into two groups and treated with a single dose of 600 mg/kg of VPA (saline + VPA group) or an equal volume of saline (saline + saline control group), respectively. One half of the HC-treated animals also had a single exposure to 600 mg/kg of VPA (HC + VPA group) or a proportionate volume of saline (HC + saline group) on GD 8. Thus all animals had an equal number of injections via IP route. The total volume of fluid injected corresponded to the body weight and did not exceed 0.45 ml. The 75 mg/kg dose of HC was based on an analogy from our earlier experimental study [25]. The food and water consumption of all groups was recorded throughout the study. The VPA-treated animals on GD 8 did not consume food and water for about 2–3 h. Therefore, food and water were withdrawn from all animals for 3 h on GD 8. All animals were killed on GD 18 by cervical dislocation and fetuses were collected. Implantation and resorption sites were counted. The fetuses were weighed, fixed in 95% ethanol and subsequently examined for malformations according to the methods used previously [25]. Fetuses weighing 1 or 2 Standard Deviations (SD) less than the mean of the control fetuses were considered growth retarded [25].

Experiment III: Estimation of plasma levels of Homocysteine, Folate and Vitamin B12

Additional experiments were performed by following the procedure described above for determination of homocysteine, FA and Vitamin B12 concentrations. Blood samples were obtained on GD 8 from the single dose HC-group and on GD 10 from the multiple dose HC-group, 3 h after the HC administration. Blood samples were also collected from appropriate age-matched controls. The animals were decerebrated by cervical dislocation and quickly 0.5 ml of blood was taken from the inferior vena cava into EDTA-coated evacuated tubes. There were as many as 6–8 samples in each group. The samples were immediately placed on ice and centrifuged at 3000 × *g* for 10 min at 4°C. Plasma was then separated and stored at −80°C until assayed for homocysteine. Homocysteine levels were determined with the IMx Homocysteine assay (Abbott Laboratories, IL, USA), based on Fluorescence Polarization Immunoassay technology in an IMx Analyzer. Total free homocysteine concentrations were measured after conversion of mixed disulfide and protein bound forms by the use of dithiothreitol. A homocysteine standard curve was constructed using homocysteine calibrators ranging from 0 to 50 μmol/l. Four-parameter logistic data reduction was used to generate the calibration curve. The sensitivity of this assay was calculated to be < 0.5 μmol/l.

Dual count solid phase no boil radioassay (Diagnostic Products Corporation, Los Angeles, CA) was used for determining FA and vitamin B_{12} concentrations simultaneously. (Manufacturer's procedure).

Statistical analysis

Data were analyzed using SPSS version 12.0 and SAS version 8.01. We applied logistic regression using PROC GENMOD (SAS 8.01) to test the effect of VPA, HC and HC + VPA on embryonic resorption and exencephaly. To take the cluster effect of "litter" into account, GEE estimates were used with a compound symmetry error structure within litters. The General Linear Model (GLM) procedure was used to test the levels of significance of fetal weights in different treatment groups and to determine if exencephalus malformation had any effect on fetal weights. The homocysteine, vitamin B12 and folate data were graphically displayed using Box-and-whisker plots. To estimate the effects of the four different treatments (Saline, VPA, HC and HC + VPA) and doses (single *vs* multiple), on homocysteine concentrations, Univariate Analysis of Variance and Tukey HSD Post-Hoc tests were used after a logarithmic transformation of their plasma values. Plasma folate and vitamin B12 values were analyzed by Analysis of Variance and Tukey Post-Hoc tests. A *p*-value less than 0.05 was considered significant.

Ethics

The protocol of this study was approved by the Animal Research Ethics Committee of the Faculty of Medicine and Health Sciences, UAE University, Al Ain. All experiments were conducted by following the principles expressed in the Guide for the Care and Use of Laboratory Animals [26].

Results

Maternal effects

For the purpose of simple and convenient description, the saline + saline, saline + VPA, and HC + saline groups are referred to here as the control, VPA and HC groups, respectively. Neither the control nor the HC-group of mice did differ from the non-treated controls in their food and water consumption and body weight gain with advancing gestation. Therefore, the non-treated control data are not presented here. The VPA-treated animals, however, appeared to be

drowsy for 2–3 h. Food and water intake was moderately reduced on the day of treatment but subsequently the drug-treated animals consumed food and water as well as the controls. However, they weighed comparatively less than the controls until GD 18, possibly because of treatment-related increase in embryonic resorption. This data was similar to those of our earlier studies [8].

Fetal effects

Resorption

The rates of embryo implantation, resorption and exencephaly in the different treatment groups of animals are shown in Tables 1 and 2. HC alone caused a modest increase in resorption rate more than the background frequency. All VPA groups (VPA and HC + VPA) had significantly higher rates of embryonic resorption than HC or saline groups. However, both with single and multiple doses of HC, HC + VPA did not induce a statistically significant increase in the rate of resorption in comparison to VPA alone.

Fetal body weight

The reduction in mean fetal body weight of the VPA, HC and HC + VPA groups was significant in comparison to that of the control fetuses. It is a common observation that severely malformed fetuses are stunted. Therefore, we compared the body weights of fetuses both with and without combining exencephaly fetuses. It was observed that both VPA and HC either alone or in combination (HC + VPA) induced significant reductions in mean fetal weight in both dose groups. This was true whether one included or excluded the exencephalic embryos in the comparison. The frequency of IUGR at -2SD level of the HC alone groups appeared to be HC-dosage (single *vs* multiple doses)-dependent (Tables 1 and 2). A 100% incidence of IUGR at -2SD level was observed in the HC + VPA groups of the single and multiple dose groups, significantly more than those observed in VPA or HC

Table 1 Effects of injection of a single dose of homocystine on the reproductive toxic effects of VPA administered on GD 8 in the mouse

	GD-8			
	Saline	VPA	HC	HC + VPA
Numbers of litters	13	12	18	11
No of Implantations	99	113	173	102
Resorption (%)[a, c, d]	7(7.1)	31(27.3)	15(8.7)	28(27.5)
Live fetuses (%)	92(92.9)	82(72.6)	158(91.3)	74(72.6)
Fetuses with exencephaly (%)[a, c, d, e]	1(1.1)	12(14.6)	1(0.6)	32(43.2)
Fetal weight (g) (Mean ± SD)[a, c, d, e]	1.251 ± 0.051	0.992 ± 0.135	1.197 ± 0.129	0.874 ± 0.156
IUGR at −1SD (%)[b, c]	6(6.5)	7(8.5)	28(17.7)	0
IUGR at −2SD (%)[a, b, c, d, e]	0	74(90.2)	41(25.9)	74(100.0)

IUGR = Intrauterine growth retardation; VPA = Valproic acid; HC = Homocystine. Numerals in parentheses are percentages
[a, b, c, d, e] Significant ($p < 0.05$) when comparisons are made as follows:
[a]VPA vs Saline; [b]HC vs Saline; [c]HC vs VPA; [d]HC vs HC + VPA; [e]VPA vs HC + VPA

Table 2 Effect of multiple doses of homocystine on the reproductive toxic effects of VPA injected on GD 8 in the mouse

	GD-5 through GD-10			
	Saline	VPA	HC	HC + VPA
No of litters	13	12	13	16
No implantations	102	107	125	155
Resorptions (%)[a, c, d]	4(3.9)	27(25.2)	8(6.8)	43(38.4)
Live fetuses (%)	98(96.1)	80(74.8)	117(93.6)	112(72.3)
Fetuses with exencephaly (%) [a, c, d, e]	0	20(25.0)	0	48(42.9)
Fetal weight (g) (Mean ± SD) [a, b, c, d, e]	1.260 ± 0.053	1.002 ± 0.156	1.170 ± 0.136	0.887 ± 0.148
IUGR −1SD (%) (b, c)	9(9.2)	9(11.3)	21(18.0)	0
IUGR −2SD (%) [a, b, c, d, e]	0	69(86.3)	45(38.5)	112 (100.0)

IUGR = Intrauterine growth retardation; VPA = Valproic acid; HC = Homocystine. Numerals in parentheses are percentages
[a, b, c, d, e] Significant ($p < 0.05$) when comparisons are made as follows:
[a]VPA vs Saline; [b]HC vs Saline; [c]HC vs VPA; [d]HC vs HC + VPA; [e] VPA vs HC + VPA

alone groups suggesting a strong interaction between these two compounds in restricting fetal growth. There was no additive response in terms of IUGR when HC and VPA were combined.

Neural tube defects

The number of fetuses with exencephaly was remarkably enhanced ($p < 0.001$) in the HC + VPA groups compared to that in the VPA alone groups. When the combined data of both groups were analyzed, an approximately threefold increase in the probability of exencephaly was observed in the HC + VPA group compared to that of the VPA alone group. However, the increase in the multiple dose HC-group was not significantly higher than that of the single dose HC-group (Tables 1 and 2). HC alone did not produce any increase in the incidence of malformations at this dose level. The exencephalic embryos of the VPA alone and HC + VPA groups did not differ in their morphological appearance. The open neural tube had overgrown and was often hemorrhagic. Maxillary and mandibular hypoplasia, low-set microtia, exophthalmia with or without cataract, significant IUGR, blood-stained excess amniotic fluid and a female preponderance in incidence were characteristic of the exencephalic embryos. The associated craniofacial malformations and IUGR in the two groups were also similar in nature. Exencephalic embryos of both groups had similar basicranial and axial skeletal abnormalities (data not presented here).

Homocysteine concentrations

Plasma levels of homocysteine, FA and vitamin B12 were estimated on GD 8 in the single dose group and on GD 10 in the multiple dose group 3 h after the last dose of HC. VPA alone did not alter significantly plasma homocysteine levels. However, there was an overall but highly significant ($p < 0.001$) increase (approximately 20-fold) in the homocysteine values of the other two treatment (HC alone and HC + VPA) groups. The effect of dosage (single *vs* multiple doses) was complex. Single vs multiple dose administration of HC on an average had no effect on homocysteine concentrations of saline control animals (Fig. 1a). However, it interacted with the treatment. Most notably, there was a marked and statistically significant ($p < 0.001$) interaction between dosage and the two treatments (HC and HC + VPA) in terms of a manifold increase in homocysteine concentrations compared to saline control animals. While in HC alone treatment, multiple dose group mice had significantly lower homocysteine values than the single dose group, in HC + VPA treatment group, multiple doses had the opposite effect, viz. it markedly increased the homocysteine values.

Folate concentrations

In the single dose groups, treatment-related reductions in plasma FA levels were significant ($p < 0.001$)

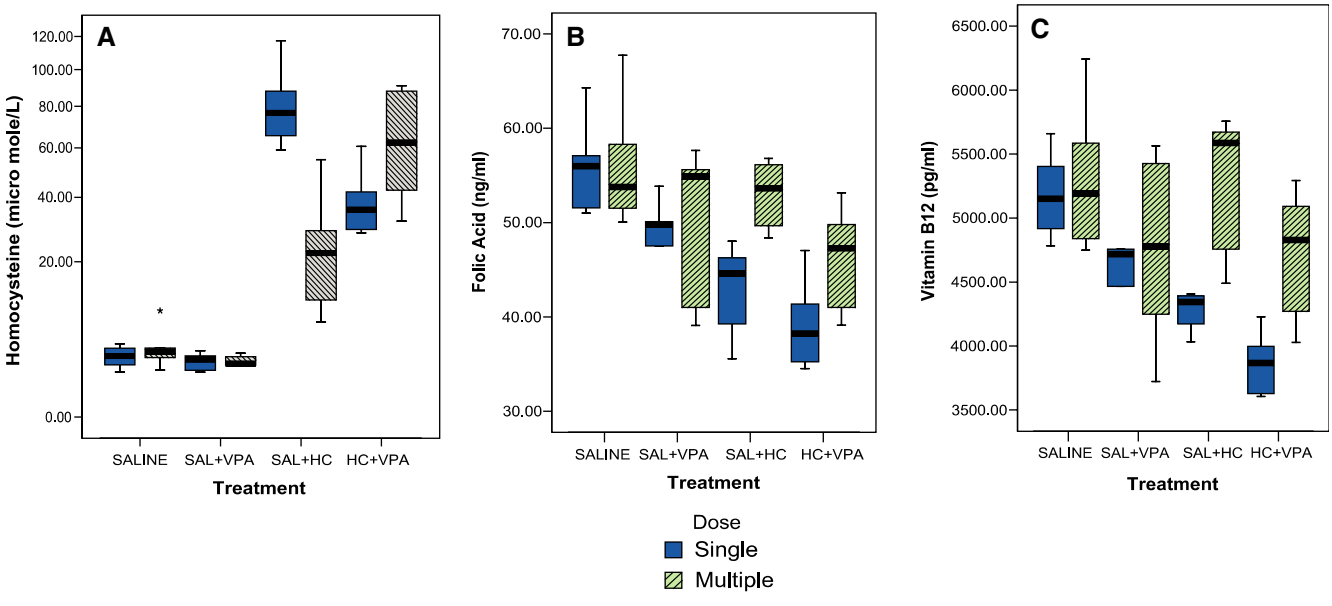

Fig 1 Box plots showing the effect of homocystine treatment on VPA-induced alterations in plasma homocysteine (a), folate (b) and vitamin B12 (c) concentrations in the mouse

except in the case of VPA alone group in which the reduction was only moderate ($p = 0.061$). HC + VPA caused a significantly higher reduction in FA concentration than VPA or HC alone ($p < 0.05$) suggesting a possible interaction between HC and VPA in lowering plasma values of FA. In the multiple dose groups, on the other hand, only HC + VPA lowered FA levels significantly ($p < 0.05$) below the level of the control. When the single and multiple dose groups were combined, all treatment-related reductions in FA concentrations were observed to be statistically significant. In this instance HC + VPA lowered FA level to a greater degree ($p < 0.001$) than HC ($p = 0.003$) or VPA ($p < 0.02$) alone suggesting a strong interaction between HC and VPA in general. Although HC and VPA individually reduced the FA concentrations, it was interesting to note that when combined, they did not produce an additive effect in their FA lowering abilities (Fig. 1b).

Vitamin B12 concentrations

In the single dose groups, all treatment-related reductions in plasma vitamin B12 concentrations were observed to be significant ($p < 0.05$) in comparison to the control values (Fig. 1c). Both HC and VPA individually lowered vitamin B12 levels significantly. HC and HC + VPA produced a greater reduction ($p < 0.001$) than VPA alone ($p < 0.02$) indicating a possible interaction between the nutrient and the drug. When the effect of HC + VPA was compared with that of HC alone, the difference was not found to be significant. Taken together these data would suggest that in the presence of HC, VPA plays a particularly effective role in lowering the vitamin concentration. Comparison of combined data (single and multiple dose groups together) showed that all treatments (HC, VPA, and HC + VPA) induced significant ($p < 0.05$) reductions in vitamin B12 values when contrasted with those of the control. Analysis of the data of multiple dose groups indicated that multiple doses of HC were not particularly more effective than single doses in lowering plasma vitamin levels.

Discussion

It has been suggested that supplemental FA (a) corrects the maternal FA deficiency, (b) compensates for reduced uptake or (3) corrects some defect in FA metabolic pathway and thus prevents NTD. However, mothers with NTD infants are not found to be particularly FA deficient and that their FA absorption is normal [27, 28]. Polymorphisms of genes involved in FA metabolic cycle can account only for a small proportion of all NTD [29]. Folbp1 (−/−) mouse embryos die in utero and only a few of the survivors show NTD. FA supplementation neither restores maternal FA concentration to the level of the wild type, nor does it provide total protection against NTD in their embryos [30]. This situation leads one to believe that the metabolic basis of NTD might involve other members of the FA cycle such as homocysteine, methionine, cysteine etc. Elevated levels of homocysteine concentrations in amniotic fluid appear to correlate more strongly with NTD than with polymorphisms of MTHFR gene [18]. Epileptic patients on AED therapy, particularly those homozygous for MTHFR variant (MTHFR TT) are reported to be associated with elevated homocysteine concentrations [31]. If this is true, then it might at least partly explain why NTD are more common among AED-treated pregnancies than in otherwise normal population. Previous experimental studies that attempted to investigate the reproductive toxicologic effects of homocysteine, exposed chick embryos in ovo [20, 22, 32] or mouse embryos in vitro [21, 23] to variable doses of this amino acid and found both positive and negative responses in terms of the presence or absence of NTD. Direct application of homocysteine to chick embryos in ovo caused several malformations but not NTD, while there was a delay in the closure of the rhombencephalon and transient widening of the rostral neuropore [20]. Mouse embryos cultured in presence of homocysteine were found to develop IUGR, blisters and somite anomalies [23], but not NTD [21, 23]. In low concentrations, homocysteine was reported to promote rat embryo development in a culture medium, which would normally not support embryo development [32]. When exposed to higher concentrations of homocysteine, relatively young embryos were found to develop NTD, which could be attenuated by methionine. Both the in vitro and in ovo studies have an advantage in that they permit direct observation on the homocysteine-treated embryos, but the disadvantage is that they completely eliminate the role of the maternal organism in making any contribution to the fetal outcome. Since the embryos do not continue and complete development in culture, post treatment opportunities for tissue repair and compensatory growth inherent in in vivo do not exist in vitro [33]. In vitro studies also lack the ability to replicate the complex nutrient–nutrient interactions that occur in the maternal, placental and fetal compartments during mammalian embryo development.

The results of the present study show that administration of a single dose of 75 mg/kg of HC on GD 8 or on successive days from GD 5 through GD10 is neither toxic to pregnant mice nor significantly embryolethal. However, it causes a significant reduc-

tion in mean fetal body weight affecting about 26% and 39% of fetuses at -2SD level in the single and successive dose groups, respectively. When HC is co-administered with VPA during the critical phase of neurulation, the embryos respond with a pronounced susceptibility to IUGR and NTD suggesting that there is a strong interaction between these two compounds. Embryotoxicity of VPA in terms of resorption was not enhanced in the single dose HC-group. In the multiple dose experiment, there was a numerical but not statistically significant increase in the frequency of resorption in the HC + VPA group in comparison to that of VPA alone group, possibly because some severely malformed embryos were resorbed due to this combination treatment. Administration of exogenous HC can cause a step-wise increase in plasma homocysteine levels covering the range of clinically observed homocysteine values in the rat [34]. Our results indicate that both single and successive doses HC administered to pregnant mice results in a considerable elevation of their plasma homocysteine concentration. An important observation from this study is the fact that VPA does not induce hyperhomocysteinemia under the conditions of our experiments. This observation is in agreement with that reported clinically [19] in adult patients on VPA-treatment. However, given in an elevated homocysteinemic background, the drug induces substantially enhanced range of embryolethality and malformations including NTD.

In addition to gene–gene and gene–nutrient interactions, drug–nutrient interactions are thought to be important in the pathogenesis of NTD, particularly in situations where chronic therapy is required (e.g. epileptic women on AED treatment) [35]. Anticonvulsants are known to lower plasma FA concentration and increase homocysteine level [19]. Epileptic women are treated with AED even during pregnancy thus allowing a possible elevation of homocysteine levels during critical stages of neural tube development. Thus there is a possibility that AED influences NTD development either directly via cytotoxicity in the developing neuroepithelium or via a mechanism mediated by homocysteine during this period. However of all AED, it is VPA that causes a manifold increase in the incidence of spina bifida in the infants of epileptic women treated with the drug during pregnancy in comparison to the background frequency in the general population and that in non-VPA-treated epileptic pregnancies [6, 7]. This might suggest that VPA-induced elevation in homocysteine concentration is a strong risk factor for NTD. The results of the experiments reported previously [24] and those discussed here indicate that VPA-induces reduction in plasma FA and vitamin B12 concentrations. This study has also shown that the magnitude of reduction in FA and vitamin B12 levels were in the order of HC + VPA > HC > VPA in the single dose HC-group indicating a strong interaction between the drug and HC. However, the accompanying increase in plasma homocysteine levels did not follow the same order of magnitude. VPA alone did not elevate plasma homocysteine levels. Single doses of HC produced a higher plasma homocysteine level than multiple doses. However, HC + VPA interaction produced a significantly greater elevation of homocysteine value in the multiple dose group than in single dose group possibly because with reduced FA and vitamin B12 concentrations, the interaction inhibited either remethylation of homocysteine into methionine or interfered with conversion of homocysteine via transsulfuration pathway into cystathionine and cysteine. This speculation is in conformity with the reported reduction in methionine and elevated homocysteine concentrations in epileptic patients and laboratory animals treated with AED [36–38]. It would mean that a combination of HC and VPA involves other micronutrients [e.g. methionine, cystathionine, cysteine, and betaine) in addition to FA and vitamin B12. One another possible reason why HC + VPA in the multiple dose group resulted in a greater elevation in homocysteine concentration could be the fact that assays were done at different time points in the two groups (i.e. on GD 8 in the single dose group and on GD 10 in the multiple dose group). This might have allowed a prolonged time for exogenous HC to exert its effect in a low plasma FA and vitamin B12 environment. Multiple injections of HC might also have had some direct effect on maternal physiology such as oxidative stress [39], impaired hepatic and renal functions, reduction in glutathione status and or protein binding.

Homocysteine does not occur in the diet. It is derived from methionine. Clinically homocysteine concentrations can rise in a number of situations [40, 41]: The metabolism of FA and vitamin B12 is reported to be altered in families with spina bifida infants [42]. The mechanism of action of homocysteine in abnormal development of the neural tube has not been established. An altered methionine metabolism or an imbalance between methionine and homocysteine levels can lead to an insufficient methyl group donation and consequently hypomethylation of DNA, RNA and proteins and affect the expression of developmental genes during neurulation. Homocysteine may exert some direct toxic effects on developing embryos and cause death or IUGR. There is some evidence that homocysteine-induced birth defects in chick embryos are mediated by competitive inhibition of NMDA receptors [43].

The enhanced incidence of NTD observed in the embryos of HC + VPA group indicates that the interaction between HC and VPA affects fundamental processes of closure of the mouse neural tube and involves micronutrients.

Conclusion

The results of this study indicate that a single or multiple doses of HC administered to mice during organogenesis can aggravate the developmental disturbances caused by a single dose of VPA administered on GD 8. Whereas, VPA lowers significantly plasma FA and vitamin B12 concentrations, it has no direct impact on the homocysteine concentrations. Therefore, it is proposed that high levels of homocysteine disturb the FA, vitamin B12, and possibly methionine metabolism thus providing a favorable situation for VPA to interfere with the development of susceptible embryos.

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