INDUCTION OF LIVER METALLOTHIONEIN CONTRIBUTE TO THE DEVELOPMENTAL TOXICITY OF VALPROIC ACID IN RAT

[1]FARID ABOLHASSANI, [1]MARYAM AZIZI, [1]MOHAMMAD AKBARI, [2]AHMAD REZA DEHPOUR, [3]MOHAMMAD ANSARI, [1]FARIBA KHOSRAVI

[1]Department of Anatomy, [2]Department of Pharmacology, [3]Department of Biochemistry, Medical school, Tehran University of Medical Sciences. Tehran, Iran.

ABSTRACT

There is an increased risk of neural tube defects and axial skeletal malformations among infants born by mothers who had received Valproic acid...The aim of the present study is, if administration of valproic acid can induce maternal hepatic Metallothionein (MT) synthesis and so secondary decrease of plasma Zn. In the present experimental study, mated rats were divided into four groups of 12 animals each [control, valproic acid (VPA), valproic acid + zinc (VPA+ Zn) and Zinc (Zn) groups]. The VPA group received 300 mg/kg valproic acid; daily. The control group received an equal volume of 0.9% NaCI. The VPA+ Zn group received 300 mg/kg VPA as well as 30 mg/kg zinc sulfate, and the Zn group received 30 mg/kg zinc sulfate, daily. These drugs were administered intraperitoneally from day 6 through day 15 of gestation. Dams were killed on GD 16 or 20. Blood was drawn to determine plasma zinc; furthermore, maternal liver Zn and MT were also determined. The zinc concentration in the plasma of rats treated with valproic acid was significantly lower than those of the other groups on GD 16 (p=0.004), but liver Zn (p=0.016) and MT (p=0.004) were significantly higher than those of the control group. On GD 20 the incidence of skeletal malformations and neural tube defects tended to be higher in VPA group than VPA+ Zn treated group and no anomalies were seen in the control group. The results from the present experiment support hypothesis that one of biochemical lesions causing the teratogenicity of VPA is a drug -induced maternal plasma zinc deficiency secondary to Metallothionein induction in liver.

Keywords: Teratogenicity, Valproic acid, Zinc, Metallothionein.

INTRODUCTION

Epilepsy is a chronic neurological disorder which is characterized by recurrent seizures. It is sudden and unprovoked attacks that is usually associated with altered awareness and involuntary movements (1). Antiepileptic drugs are the mainstay of treatment for most patients with epilepsy. Valproic acid, one of the main antiepileptic drugs is used in both generalized and partial epilepsies (1, 2).

Approved indications of valproic acid are treatment of bipolar disorders, neuropathic pain, and migraine prophylaxis (1-5). Congenital malformations have been reported among infants born whose mother's had received valproate during pregnancy. In these infants, there have been increased risks of neural tube defects such as spina bifida and anencephaly, and a variety of syndromes such as craniofacial and digital abnormalities, cleft lip, cleft palate as well as axial skeletal malformations (3, 5-11).

It has been demonstrated that the teratogenicity of a number of drugs may be linked partly to drug-induced alterations in maternal trace mineral status (12).

There are controversy reports about the relation between valproic acid teratogenicity and the decrease of plasma zinc level (12-16). It is believed that administration of VPA result in an induction of Metallothionein (MT) protein in maternal liver (12, 13, 17), which is the most abundant, nonenzymatic Zinc-containing protein known at present (18). The induction of MT is associated with Zn retention in maternal liver and a reduction in plasma Zn (12, 13).

Zinc (Zn) is an essential element of the nutrition of human beings, animals, and plants. It is a constituent of over 200 metalloenzymes and other proteins involved in immune functions, antioxidant protection, and membrane stabilization. Zinc is required for DNA and RNA synthesis at every step of the cell cycle (3, 8, 18-22) and is essential for normal growth and development. It has been reported that low maternal serum is associated with congenital malformations and fetal dysmaturity. Several groups reported that infants' mothers with congenital anomalies had lower plasma Zn concentrations in comparison with other mothers. Zinc deficiency has been implicated specifically in development of two neural tube defects, anencephaly and spina bifida (21).

Correspondence: Farid Abolhassani, Department of Anatomy, Medical School, Tehran University of Medical sciences, Tehran, Iran, E-mail:abolhasf@sina.tums.ac.ir

It has been hypothesized that the developmental toxicity of certain compounds is, in part, due to maternal toxicity (Induction of maternal hepatic MT synthesis) resulting in alterations in zinc (Zn) metabolism that affects the developing conceptus. In this study the effects of developmentally toxic dose of valproic acid (VPA) on Zn metabolism in the pregnant rat were investigated.

MATERIALS AND METHODS

Virgin female Sprague- Dawley rats (180-200gr) were purchased from Pasteur institute. The rats were housed in cages in a controlled atmosphere room (temperature 23 -25 °C, 12 h light: dark cycle) and were fed with the zinc adequate diet. Diet and water were available throughout the experiment. After 3 weeks of acclimation to the diet and the environment, rats were mated with males of the same strain over night, and successful mating was determined by the presence of vaginal plugs in the next morning, which was designated as gestational day (GD) 0.

The rats were divided into four groups of 12 animals each [control, valproic acid (VPA), valproic acid + zinc (VPA + Zn) and Zinc (Zn) groups].

The valproic acid group received 300-mg/kg valproic acid, daily (Rouz Darou Co.). The Zinc group received 30mg/kg zinc sulphate, daily (Merck Co.). The control group received an equal volume of 0.9% NaCI. The valproic acid + zinc group received 30Omg/kg VPA and 30mg/kg zinc sulfate, daily. Valproic acid, NaCI, and zinc sulfate were administered intraperitoneally (23). To have the same condition for the administration of drugs to all groups, the other drugs were administered by IP injection. These injections were performed from day 6 through day 15 of gestation and on the day of 16, six rats of each group were killed with ether, and the othersix rats were decapitated on GD 20 to evaluate the anomalies among the elder fetuses. Blood was drawn by cardiac puncture into heparinized tubes. Liver was removed quickly, then two sections of it separated and frozen in liquid nitrogen until analyzed for Zn and MT on GD 16. The uterus was removed, and the number of implantation sites, embryos and resorption sites were counted.

Embryos were weighted (by the rate of 0.01 gram, Sartorius balance), and crown-rump lengths were measured (by the rate of 0.1 mm). According to the usual practice in toxicological studies, half of the number of fetuses in each litter were used for evaluation of alteration in soft tissue and the remaining that were used for evaluation of skeletal alterations by clearing and staining of skeletal system (8, 24, and 25); therefore, we performed this method for fetal assessment.

Plasma zinc analysis
Blood samples were centrifuged at 3000 x g at 4°C for 15 minutes and plasma was transferred into separate tubes. Plasma zinc was determined (by the rate of 0.04 ppm) by atomic absorption spectrophotometer (Spectra AA - 220, Varian) in Iranian Atomic Energy Organization.

Liver Zinc analysis
Liver samples were wet-ashed with 16 N HN03, evaporated by heat and were diluted with 0.1 N HN03 (13). Diluted samples were analyzed for Zn concentration (by the rate of 0.04 ppm) by flame atomic absorption spectrophotometer (spectra AA-220 Varian).

Metallothionein analysis
Liver MT concentrations were determined by the Cd saturation method (17). The livers were homogenized in 4 vols. of 0.25 M sucrose in a glass homogenizer which was set in ice. The homogenates were centrifuged at 139500 x g for 30 min at 4°C and 0.2 ml of the supernatant fractions were mixed with 10μg Cd (as CdC12) and the volume was adjusted to 3 ml by addition of a 0.02 M Tris-HN03 buffer (PH 7.4) to total 3 ml. Subsequently, 0.2 ml of rabbit haemoglobin (Sigma Chemical Co.) solution (10mg/ml) was added and the mixture was placed in a boiling water bath for 2 min. After cooling in an ice bath, the mixture was centrifuged at 3000 x g for 20 min at 4°C. This process of haemoglobin-heat treatment was repeated three times. The aliquots of the supernatant fractions were used for determination of Cd by the atomic absorption spectrophotometer. MT concentrations in each experiment were determined by using purified rabbit liver MT (Sigma Chemical Co.), as a standard in each experiment.

Statistical analysis
Kruskal - Wallis test was used for analysis of difference between all of groups. Statistical significance was established at p<0.05. Results are presented as mean ± S.E.M.

Fetal assessment
All fetuses were examined for external abnormalities by stereo-microscope.
Soft tissue evaluation: Half of the numbers of fetuses in each group were evaluated by microscopicand macroscopic sections after fixing in Bouin's solution.
Macroscopic sections were prepared by Wilson's technique (24). Each section was examined with the aid of a stereo-microscope.

Staining of fetal skeletons
Fetal skeletons were stained by modification of a reported method after evisceration of fetuses (24).

After fixation in alcohol for a period of 7 days, each fetus was placed in a dish and when remaining alcohol was drained away, dishes were filled with 1% KOH solution, and fetuses were macerated for approximately 24 h. The KOH solution was then drained and replaced with 1% KOH solution containing the alizarin red- s stain, where fetus were left in the stain for approximately 24 h. Following draining the solution it was replaced with a fresh 1% KOH solution in which the specimens were remained for another 24 h. The KOH solution was again drained, and the fetuses were cleared with progressively higher concentrations of glycerine (20%, 40%, 60%, and 80%) and then the fetuses were stored in 99.5% glycerine with a few crystals of thymol.

RESULTS

The mean of plasma zinc level ± S.E.M among VPA, control, VPA+ Zn and Zn groups was 2.28 ± 0.63, 4.43 ± 1.10, 8.5 ± 1.15, 12.49± 2.88 ppm, respectively (table 1) and differences between groups were significant (p=0.004). The result show that the plasma zinc concentration in rats treated with valproic acid were significantly lower than those of the control rats and the VPA+ Zn treated rats on GD16 (p<0.005).

The differences between plasma zinc in VPA and VPA + Zn group was statistically significant (p=0.006). The 16-day-fetus weight of the Zn group was higher than the fetus weight of the three other groups (p<0.001). The mean of liver zinc concentration ±S.E.M among VPA and control groups was 49.06±13.01 and 22.96±0.93 ppm, respectively (Table 1), which showed that the liver zinc concentrations of rats treated with VPA were significantly higher than those of the control rats (p=0.016). The mean of liver MT concentration ±S.E.M among VPA and control groups was 1.06± 0.13 and 0.47± 0.023 ppm, respectively (table 1), which showed that the liver MT concentrations of rats treated with VPA were significantly higher than those of control groups (p=0.004). Compared to control and Zn groups, VPA+ Zn and VPA treated groups had low fetal weight and crown-rump length on GD 16 (Table 2).

Table 1. The influence of administration of VPA and supplemental zinc (on GD 6-15) on maternal Zn parameters in GD 16 sprague-Dawley rats[a].

Groups	N	Liver MT (ppm)	Liver Zn (ppm)	Plasma Zn (ppm)
Control	6	0.47±0.023	22.96±0.93	4.43±1.10
VPA	6	1.06±0.13[*†]	49.66±13.01[*†]	2.28±0.63[*†]
VPA +Zn	6	48.70±8.86	123.42±6.90	8.57±1.15
Zn	6	76.4±10.1	189±13.88	12.49±2.88

NOTE. Values expressed as mean ± SD. (N= number of dams per group)
* There was significant difference with Control group. † There was significant difference with VPA + Zn group

Table 2. The influence of administration of VPA and supplemental zinc (on GD 6-15) on fetal outcome in GD16 sprague-Dawley rats[a]

Groups	N	Fetal Weight (gr)	Crown-rump Length (cm)
Control	6	0.592 ± 0.03	1.917±0.031
VPA	6	0.474 ± 0.023	1.621±0.025
VPA +Zn	6	0.496±0.023	1.551±0.027
Zn	6	0.66±0.029	1.88±0.065

NOTE. Values expressed as mean ± SD (N= number of dams per group). There was no significant difference with Control group.
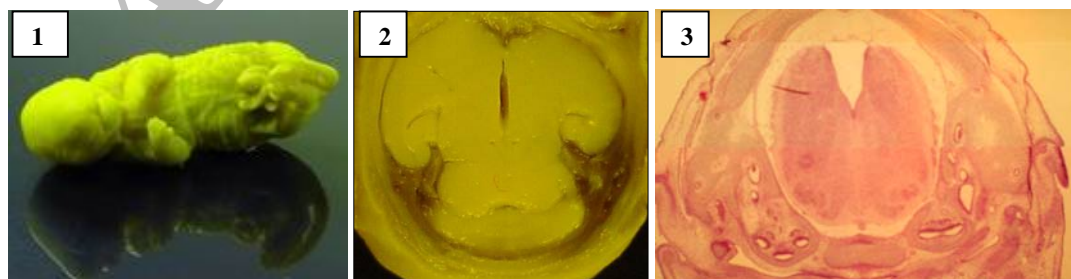

Figure 1. Rat fetus of a valproic acid treated dam in day 20 of gestation.Omphalocele
Figure 2. The sections of rat fetuses of valproic acid group. The transverse section of brain in day 20 of gestation shows dilation of the 3rdventricle (Hydrocephaly). H&E staining 40X.
Figure 3. The sections of rat fetuses of valproic acid group. The transverse section of spinal cord in day 16 of gestation shows spina bifida . H&E staining 40X.

The difference of fetal length between Zn and control groups was not statistically significant (p=0.186). Still birth fetus in VPA and Zn groups were 3.57% and 1.58%, respectively. Control group had low percentage of resorption in comparison to the VPA exposed group while there was not any still birth fetus. No dead or resorbed fetus was observed in the VPA+Zn group.Some anomalies such as omphalocele (Fig. 1), hydrocephaly (Fig. 2), spina bifida (Fig. 3), hemivertebrate, rib malformations and anencephaly were seen in VPA treated group. Compared to VPA group, low percentages of anomalies were observed in the VPA+Zn treated group and there was no anomaly in control group.

DISCUSSION

Results from the present experiment support hypothesis that one of biochemical lesions causing the teratogenicity of VPA is a drug -induced maternal plasma Zn deficiency. The data obtained in this study show that VPA administration to pregnant rats on the days of 6-15 of gestation resulted in induction of maternal liver MT, and this induction was associated with Zn retention in maternal liver and a reduction in plasma Zn, which were probably reflected by reduced placental Zn transfer and embryonic accumulation. It has been reported that administration of VPA induced MT in mouse liver (17). A mechanism common to the developmental toxicity by a number of agents has been Zn deficiency of the conceptus secondary to induction of maternal MT. Induction of MT synthesis can produce hepatic MT concentrations over an order of magnitude higher than normal, leading to substantial sequestration of circulating Zn in the maternal liver, lowered plasma Zn concentrations, and reduced Zn availability to the conceptus. Embryo fetal zinc deficiency secondary to maternal hepatic MT induction has been presented for diverse chemicals including valproic acid (12, 13), 6-mercaptopurine (26, 27), urethane (28), ethanol, and x-hederin (29). In a study of the reported data for some of these compounds, it was found that there are strong positive relationship between maternal hepatic MT induction and

maternal hepatic ^{65}Zn retention and a negative relationship between maternal MT induction and ^{65}Zn distribution to the litter (29, 8).Also it has been shown that administration of valproic acid for one week produced significant depletion of zinc in the plasma of rats (30).

It has been demonstrated while reduction in plasma Zn was in VPA treated rats; there was no evidence of Zn deficiency induced by VPA (15, 16). In another report it has been stated that no zinc deficiency were present after administration of valproic acid (31).

Considering high abnormalities and growth delays in fetuses obtained from VPA treated dams in comparison with fetuses obtained from VPA+ Zn treated dams, it is suggested that the transitory Zn deficiency is induced by VPA which is developmentally toxic.

A double- blind study of 580 African-American women in Alabama provides convincing evidence that poor maternal Zn status may also cause intrauterine growth retardation (21). Meanwhile, it has been reported that administration of zinc during pregnancy was associated with an increase in birth weight, which is the same as our findings in this investigation (32).

Consistent with our findings a different studies show that the neural tube and axial skeleton system are very sensitive to the teratogenicity of valproic acid and results in skeletal and neural tube malformations as well as growth retardation (5, 9, and 10). Our results are similar to these experiments.

It is well established that Zn is essential for normal embryos development, and deficiency of this element may result in congenital defects of multiple organ systems (8, 18, 21, 22, 33, 34).Therefore, the teratogenic effects of VPA may be modulated by supplemental Zinc.

REFERENCES

1. Pedley TA. The epilepsies. In: Goldman L, Bennett X, editors. Cecil Text book of medicine. 21st edition. Philadelphia: W.B. Saunders co; 2000. p. 2151-63.
2. Lowenstein DH. (2001) Seizures and epilepsy. In: Braunwald E,Fauci AS, Kasper DL, et al. editors. Harrison's Principles of Internal Medicine. 15 h edition.USA:Mc Graw Hill; 2001. p. 2354-69.
3. Parfitt K, Sweetman S, Blake P, Parsons A. Martin Dale. The complete drug reference.32nd ed. Pharmaceutical Press; 1999. p. 361-4, 373.
4. Johannessen CU. Mechanisms of action of valproate: a commentatory. Neurochem Int 2000; 37: 103-10.

5. Scott WJ, Schreiner CM,Nau H, Vorhees CV, Beliles RP, Colvin J, McCandless D. Valproate induced limb malformations in mice associated with reduction of intracellular PH. Reprod Toxicol 1997 ; 11:483-9.

6. Decherney AH, Pernol MI. Current Obstetric & Gynecologic Diagnosis & Treatment. 8th edition. Along medical book. 1996.

7. Sadler T W. Langmans Medical Embryology. 9th edition. Lippincotte Williams & Wilkins 2004.

8. Klaassen CD. Casarett & Doull's Toxicology, The basic science of poisons .5th edition International edition 1995.

9. Padmanabhan R, Alimed I. Sodium valproate augments spontaneous neural tube defects and axial skeletal malformations in TO mouse fetuses.Reprod Toxicol 1996; 10: 345-63.

10. Menegola E, Broccia ML,Nau H, M,Ricolfi R,Giavini E. Teratogenic effects of sodium valproate in mice and rats at midgestation and at term.Teratog Carcinog Mutagen 1996; 6: 97-108.

11. Nau H. Valproic acid- induced neural tube defects. Ciba Found Symp 1994; 181: 144 -52.

12. Keen CL, Peters JM, Hurley LS. The effect of valproic acid on 65 Zn distribution in the pregnant rat. J Nutr 1989; 119: 607-11.

13. Bui LM.Taubeneck MW,Commisso JF, Uriu-Hare JY, Faber WD,Keen CL. Altered Zinc metabolism contributes to the developmental toxicity of 2ethylhexanoic acid,2-ethylhexanol and valproic acid. Toxicology 1998; 20; 126: 9-12.

14. Graf WD, Oleinik OE, Glauser TA, Maertens P, Eder DN, Pippenger CE. Altered antioxidant enzyme activities in children with a serious adverse experience related to valproic acid therapy. Neuropediatrics 1998 ; 29: 195-201.

15. Coakley ME, Brown NA. Valproic acid teratogenicity in whole embryo culture is not prevented by Zinc supplementation. Biochem Pharmacol 1986; 35:1052- 55.

16. Daffron JC, Kasarskis Ej. Effect of valproic acid on zinc metabolism in the rat. Toxicol lett 1984; 23: 321-5.

17. Kaji M, Mikawa H. Induction of metallothionein in mouse liver by valproic acid. Toxicology 1991; 69: 143-9.

18. Mahan LK, Stump SE. Krause's Food Nutrition and Diet Therapy. 10th edition. 2000. P. 133-4.

19. Derelanko MJ, Hollinger MA. Hand book of Toxicology. 2nd edition. CRC press. 2001. p. 939.

20. Subramanian P, Sivabalan S, Venugopal PM, Vasudevan K. Influence of chronic zinc supplementation on biochemical variables and circadian rhythms in Wistar rats. Nutrition research 2000; 20: 413-25.

21. King X. Keen CL. Zinc. In: Shils ME, Olson JA, Shike M, Ross AC, editors. Modern Nutrition in Health & Disease. 1999. p. 223-4.

22. Prasad AS. Zinc deficiency in women, Infants and children. J Am coll Nutr 1996; 15: 1133-20.

23. Hill GM, Brewer GJ, Hogikyan ND, Stellini MA.The effect of depot parenteral zinc on copper metabolism in the rat. J Nutr 1984; 114: 2283 -91.

24. Hayes W. Female reproductive and developmental toxicology. Principles and methods of toxicology. 4th edition. Boston Massachusetts. 2001. p.1344 -62.

25. Melby EC, Altman Jr. N.H. Hand book of laboratory animal science. Volume 1. CRC Press.1974.

26. Amemiya K, Keen CL,. Hurley LS. 6-Mercaptopurine-induced alterations in mineral metabolism and teratogenesis in the rat.Teratology. 1986; 34 :321-34.

27. Amemiya K, Hurley LS, Keen CL.Effect of 6-mercaptopurine on 65Zn distribution in the pregnant rat.Teratology. 1989 ;39: 387-93.

28. Daston GP, Overmann GJ, Taubeneck MW, Lehman-McKeeman LD, Rogers JM, Keen CL. The role of metallothionein induction and altered zinc status in maternally mediated developmental toxicity: comparison of the effects of urethane and styrene in rats. Toxicol Appl Pharmacol. 1991 ;110 :450-63

29. Taubeneck MW, Daston GP, Rogers JM, Keen CL.Altered maternal zinc metabolism following exposure to diverse developmental toxicants.Reprod Toxicol. 1994 ;8 :25-40.

30. Hurd RW, Van Rinsvelt HA, Wilder BJ, Karas B, Maenhaut W, DeReu L. Selenium, zinc, and copper changes with valproic acid: possible relation to drug side effects. Neurology 1984; 34: 1393-5.

31. Altunbasak S, Biatmakoui F, Baytok V, Herguner 0, Burgut HR, Kayrin L. Serum and hair zinc levels in epileptic children taking valproic acid.Biol Trace Elem Res 1997; 58: 117-25.

32. Goldenberg RL, Tamura T, Neggers Y, Copper RL, Johnston KE, Dubard MB, Hauth X. The effect of zinc supplementation on pregnancy outcome. JAMA 1995; 274: 463 -8.

33. Sole D, Rieckmann B, Lippelt RM, Lippelt RT, Amancio OM, Queiroz S de S, Naspitz CK. Zinc deficient diet consequences for pregnancy and offsprings of wistar rats. Rev Paul Med 1995; 113: 681-6.

34. Braga Costa TM, De Oliveria LM, Vannucchi H. Effect of zinc deficiency induced before and during pregnancy on the survival of female rats and their pups. Braz J Med Bio Res 1995; 28: 569-74.