Multiple Point Action Mechanism of Valproic Acid-Teratogenicity Alleviated By Folic Acid, Vitamin C, And N-Acetylcysteine In Chicken Embryo Model

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Running title: VPA damages alleviated by nutraceutics.

Abstract

The teratogenicity of antiepilepsy drug valproic acid (VPA) mostly is found in genetic and somatic levels, causing teratogenesis involving neurotubular defects (NTD), anencephaly, lumbosacral meningomyelocele, and leg dysfunction due to spina bifida aperta. A diversity of nutraceutics have been tried to alleviate the risk of VPA-teratogenicity. The effect was varying. In order to promote the preventive prescription, to find out its action mechanism can be rather crucial. We used chicken embryo model to try the effect of folic acid (FA), ascorbic acid (AA), and N-acetyl cysteine (NAC). VPA at 30 mM showed the higher malformation rate (66.7%) with the least mortality (22.2%). Pathological findings indicated that the cervical muscle was more susceptible to VPA injury than the ankle muscle. VPA downregulated the levels superoxide dismutase (SOD), glutathione (GSH), histone deacetylase (HDAC) and folate; and induced H_2O_2 formation. FA, AA, and NAC significantly upregulated SOD, but only AA alone activated GSH. AA and NAC downregulated H_2O_2, while FA was totally ineffective. All three nutraceutics comparably rescued HDAC with simultaneously suppressed homocysteine accumulation and folate re-elevation, although less effectively by NAC. Based on these data, we conclude VPA possesses “Multiple Point Action Mechanism”. In addition to affecting the cited transcription and translation levels, we hypothesize that VPA competitively antagonize the glutamic

acid to couple with pteroic acid in biosynthesis of dihydrofolic acid (DHFA). H_2O_2 directly destroyed the NADPH reducing system at dihydrofolate reductase (DHFR) and methylene folate reductase (MTHFR) levels, while completely restored by AA, an implication in preservation of intact apoenzymes. In addition, the GSH-GSSG system is sandwiched between the reducing systems NADPH/NADP and DHA-AA, its net balance is highly dependent on in situ *in-vivo* Redox state, hence folic acid transformation is varying. To rescue the VPA-induced teratogenicity, simultaneous multiple prescriptions are suggested.

Keywords: Valproic acid; Folic acid; Ascorbic acid; N-acetylcysteine; Histone deacetylase.

1. Introduction

Comparing to the mouse model (Faiella et al., 2000), the chicken egg and developing embryo are useful models for the study of teratogenecity (Whitsel et al., 2002; Rosenquist et al., 2010; Lie et al. 2010) and embryotoxic potency (Jelinek et al. 1985). The teratogenicity of VPA mostly is found in genetic and somatic levels (Whitsel et al. 2002; Eikel et al., 2006), mostly involving neurotubular defects, (NTD), anencephaly, lumbosacral meningomyelocele (Lindhout et al., 1992; Koch et al., 1996), and spina bifida aperta (Mominoki et al., 2006). The cited mechanism of VPA to elicit teratogenicity involve i) promoting folic acid deficiency (Johannessen, 2000; Chango et al., 2009) and acting as a disrupter of methylenete trahydrofolate reductase (MTHFR) (Karabiber et al., 2003; Roy et al., 2008). ii) inducing oxidative stress (Tabatabaei and Abbott, 1999); iii) leading to the ω- and β-oxidation (Lheureux et al. 2005); iv) inhibiting histone deacetylase (Menegola et al. 2006; Hrzenjak et al., 2006); v) antiangiogenesis (Rosenberg, 2007); and vi) DNA damages (Schulpis et al., 2006). In addition, reduced folate levels may result in hyperhomocysteinemia. Homocysteine has been considered a mediator of the teratogenic potential of VPA (Verrotti et al., 2000; Karabiber et al., 2003). Reduced folate can compromise DNA (Fig. S1, van Gelder et al., 2010). Mild MTHFR deficiency and reduced maternal erythrocyte folate concentration is a particularly strong risk factor for NTD (Amorim et al, 2007)

Therapeutically, a diversity of nutrients and nutraceutics have been tried to prevent or antagonize VPA-induced teratogenicity. Folic acid supplementation in females significantly reduced VPA-induced NTDs in their offspring (Padmanabhan and Shafiullah, 2006; Dawson et al., 2006). The alternate treatments involved methionine (Ehlers et al., 1995), folinic acid, vitamin B_6 plus vitamin B_{12} (Elmarzar et al., 1992), folic acid with tocopherol (Aluclu et al., 2009), and betaine supplement for the MTHFR-defficient (Kelly et al., 2005). Although supplement with folinic acid and vitamins B_6+B_{12} had effectively reduced VPA malformations (Elmazar et al., 1992), the sciatic nerve degeneration in fetal rats caused by VPA was only partially rescued by folic acid or tocopherol, or the combination of these two vitamins (Aluclu et al., 2009). Apparently, these protections were not complete, and obviously some other mechanisms have been embedded behind that need further investigation. This present paper intends to march forward more detail into these unknown action mechanisms, which may provide more reliable evidences in extending the prescription for the VPA therapy and protective strategies.

2. Materials and methods

2.1. Chemicals

N-acetyl-L-cysteine , bovine serum albumin (BSA), sodium dodecyl sulfate (SDS),

Bromophenol Blue, Coomassie Brilliant Blue R (CBR), N,N,N',N'-tetramethyl-ethylenediamine (TEMED), Tween-20, and potassium ferricyanide were purchased from Sigma Co. (St. Louis, MO, USA). Sodium carbonate and acetonitrile (ACN) were supplied by JT-Baker & Co. (Windale, NY, USA).Other undefined products were all manufactured by Wako Pure Chemicals (Tokyo, Japan). All reagents and solution used were prepared following the manufacturer's instruction.

2.2. Source of fertilized eggs and processing

Sixty day-1 fertilized Leghorn eggs supplied by Qing-Dang Chicken Farm (Taichung, Taiwan) were divided into 5 groups, 12 fertilized eggs in each. The fertilized eggs were placed in the incubator (Haw-Yang Agricultural Farm, Taichung, Taiwan) and incubated at 37^{o}C, RH 50-60% for 1.5 days. The fertilized eggs were moved to a laminar flow chamber. A hole having size 2mm×2mm on the egg shell was aseptically drilled through with a pin-driller. The embryos were moved as close as to the hole opening by carefully turning around in between the observer's eyes and a direct strong light source, and injected with a tip injector an amount of 100μL VPA (30mM) alone or with folic acid, ascorbic acid, and N-acetyl cysteine, 10 mM each in PBS, respectively. PBS was used as the negative control. The openings were aseptically sealed with

a 3M tape. The incubation was continued. The sampling points were set at day 5.5 (HH stage-28) (Supplement Fig. S2) and day 21 (Hamburger, and Hamilton, 1951).

2.3. Sample embryo and treatment

The embryo at day 5.5 was carefully removed off their chorioallantoic membranes, blood vessel, yolk and egg white. The embryo was successively rinsed with several times of PBS and deionized water (dw). The weight of embryo was taken and the embryo was stored at -80^{o}C for further use.

2.4. Status of vascularization and vessel density

The day 5.5 fertilized eggs were broken and the embryos were transferred onto a Petri dish. The photos were taken and analyzed with Image-Pro Plus 6.0 software.

2.5. Collection of organs

After hatched out, the chicks were euthanized with CO_2. The brains, hearts, livers, cervical muscle, hind leg, claws with toes, ankle joint, spinal cord, and sciatic nerve were collected, rinsed with PBS, dewatered with tissues and weighed. After the picture of excised organs was taken to examine the pathological changes of outer appearance, the organs were separately dipped into 10% buffered neutral formalin at a ratio 1:30 for 48 h to proceed paraffin

embedding preparation.

2.6. Histopathological Examination

The paraffin-embeded slides were subjected to HE staining, and the slides were observed with Castor Bl-90AMicroscope.

2.7. HPLC analysis for serum folic acid

The nonboiled treatment method of Rodriguez-Bernaldo de Quiros et al. (2004) with slight modification was used for HPLC determination of folic acid. The solutions required for this assay included solutions A, B, C, D, and E. These solutions were prepared prior to HPLC analysis: Solution A was prepared by mixing equal volume of 20mM Tris buffer (pH 7), 20 μM 4-aminoacetophenone, and 5 μM tetrabutylammonium hydroxide. Solution B merely was a PBS solution (0.5%) used to dilute the serum. Solution C merely was a 20 mM Tris buffer (pH 7.0) solution. Solution D was a 20 mM Tris buffer (pH 7) containing 30% methanol. Solution E was a 50 mM phosphate buffer (pH 3) containing 50% methanol.

HPLC Hitachi equipped with L-2130 HTA Pump and L-2485 Fluorescence Detector was a product of Hitachi High Tech (Tokyo，Japan). A separation column Sep-Pak C18 was used for purification of folic acid from serum sample. Before separation, the column was first activated by treating successively with

methanol (2 mL), ddw (2 m), and solution C (3 mL). The blood or serum was centrifuged at 12000g at 4^{o}C, the supernatant serum, separated from the precipitate and diluted with solution B at 1:1 and 1:2 ratio, and stored at -21^{o}C for further use (Serum sample of folic acid, SSFA). To proceed separation, SSFA, each 60 μL, was thoroughly mixed with 180 μL of solution A and then transferred onto the separation column that had been previously activated. After the separation column was flushed with 1 mL of solution C plus 0.25 mL of solution D, solution E (1 mL) was added to conduct the elution. The eluent was filtered through a 0.45 μm Micropore filter and the filtrate was stored at 4^{o}C before HPLC analysis (EFA). To conduct HPLC analysis, a 20 μL aliquot of EFA was injected into the injection port of HPLC held at 30^{o}C. A mobile phase 0.05M KH_2PO_4 (pH 7.0) containing 13.5% acrylonitrile (ACN) was operated at a flow rate 1.0 mL/min for elution. The whole course was held at 30^{o}C. The fluorescence of the eluent excited at λ_{ex} = 250 nm was read at λ_{em} = 350 nm with an ELISA Fluorescence Reader. Similarly, a serial diluted authentic folic acid solutions were prepared by diluting the authentic folic acid solution (10 mM) with appropriate amount of solution B (pH 7.2) to make 6.25 to 200 μM, and the fluorospectrometric determination was conducted to establish the calibration curve.

2.8. HPLC analysis for serum homocysteine (s-Hcy)

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Hitachi HPLC (Hitachi High Tech, Tokyo, Japan) equipped with an L-2130 HTA Pump and an L-2485 Fluorescence Detector was used for assay of homocysteine (Hcy) according to Frick et al. (2003). Briefly, to the serum sample (60µL) and equal volume of authentic homocysteine solution (60 µM) 39 µL Dulbecco's phosphate buffered saline and 10µL TECP were added. The mixture was left to react at ambient temperature for 30 min. To the reaction mixture 90µL trichloroacetic acid solution (100 g/L) containing 1mM EDTA was added and mixed well. The mixture was centrifuged at 4000×g at 4^{o}C for 10 min. To 50 µL aliquot of the supernatant 125 µL of borate buffer (0.125 mM containing EDTA 4 mM), 10 µL NaOH (1.55 M) and 50 µL SBD-F were added and left to react for 1 h at 60^{o}C avoiding direct sunlight. The reaction mixture was filtered through 0.45µM Micropore and an aliquot 20µL of the filtrate was subjected to HPLC analysis. The mobile phase used was the 0.1 M buffered potassium dihydrogen phosphate buffer (pH. 2.7) containing 5% methanol. The flow rate was operated at 1.0mL /min. The fluorescence detector was used to capture the wavelength of emission at 515 nm when excited by wavelength 385 nm. A calibration curve was established using authentic homocysteine solution at concentrations 3.75, 7.5, 15, 30, and 60µM with similarly treated and conducted by the same procedure mentioned in the above.

2.9. Assay for histone deacetylase (HDAC) in tissue

The activity of tissue histone deacetylase was determined according to Kwon et al., (2002). In brief, to tissue (100 mg) 0.5 mL cold lysis buffer (pH 7.5, containing 10 mM Tris-HCl，10 mM NaCl，15 mM $MgCl_2$，250 mM sucrose, 0.5% T riton-X100, and 0.1 mM EGTA) was added, homogenized. After agitated for 10 s the mixture was left to stand at 4^oC for 15 min. To 1.6mL of the mixture cold sucrose cushion (containing 30% sucrose, 10mM of pH 7.5Tris-HCl, 10mM NaCl, and 3mM $MgCl_2$) was added, mixed well and centrifuged at 1300×g at 4^oC for 10 min. The supernatant was discarded. To the residue, 400μL 10 mM Tris-HCl buffer (pH 7.5, containing 10 mM NaCl) was added. The pellets were dispersed and the mixture was centrifuged at 1300×g at 4^oC for 10 min. The supernatant was discarded. To the residue 150μL Extraction Buffer (pH 7.5, containing 50mM HEPES, 420 mM NaCl, 0.5mM EDTA, 0.1mM EGTA, and10% glycerol) was added. The mixture was agitated to resuspend the pellets. After subjected to ultrasonication for 1 min, the mixture was centrifuged at. 10000×g at 4^oC for 10 min. The supernatant was separated and stored at -80^oC for further use. For determination, four supernatant samples, each 10μL, were transferred into a 96-well plate. To two of the four samples, 160μL assay buffer was added, respectively (to serve as sample group A). And to the other two samples, 150μL assay buffer and 10μL Trichostatin A solution was added, respectively (to serve as sample group B). To each of the four samples 10μL HDAC substrate solution was added,

respectively. After agitated at 37^{o}C for 30 min, 40 μL developer agent was added and left to react at ambient temperature for 15min. The fluorescence intensity of the final solution was read with an ELISA Fluorescence Reader at emission wave length 440-465 nm after exited at wavelength 340-360 nm. To establish the calibration curve, the deacetylated histone standard (2.1mM) was first diluted to 10-fold dilution, which was then diluted to 10, 21, 42, 84, and 168μM, respectively. To 10μL of each authentic solution, 160μL assay buffer and 10μL HDAC substrate solution were added. After the mixtures were agitated at 37^{o}C for 30 min, 40 μL developer agent was added and the reaction was allowed to proceed for 15 min. The fluorescence intensity of the final solution was read with an ELISA Fluorescence Reader at emission wave length 440-465 nm after exited at wavelength 340-360 nm. The amount of HDAC was calculated from the calibration curve according to the following equation:

C_{DA} (μM) = [(CSF–intercept at Y axis)/slope]

Where CSF= F_B-F_A;

C_{DA} (μM) = the concentration of deacetylated compound (μM)

F_B: the fluorescence intensity obtained from sample group B.

F_A: the fluorescence intensity obtained from sample group A.

The original HDAC activity was calculated from:

HDAC Activity (nmol/min/mL) = [µM/30 min]× fold dilution

2.10. Assay for superoxide dismutase (SOD) in tissue

Mattiazzi et al. (2002) was followed to determine the tissue superoxide dismutase activity. Briefly, to embryonic tissue (100 mg) 20 mM cold HEPES buffer (containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose) was added, homogenized, and centrifuged at 14000×g at 4^{o}C for 5 min. To 10 µL of supernatant radical detector (200 µL) was added and mixed well with gentle touching on the tube surrounding with fingers. To the mixture, 20 µL of diluted xanthine oxidase solution was added, after gentle shaking, the mixture was left to stand for 20 min. The optical density was read at 450 nm with an ELISA reader.

2.11. Assay for reduced glutathione (GSH) in tissue

The method of Zhang et al. (2010) was followed with slight modification to determine the content of reduced form glutathione. Briefly, to chick embryo (100 mg) 400 µL PBS was added. The mixture was homogenized and centrifuged at 12000×g at 4^{o}C for 30 min. The protein content of the supernatant was determined. The supernatant (100µL) was transferred into 1.5 mL microcentrifuge tube with aid of 100 µL EDTA solution containing 5% TCA. The mixture was thoroughly agitated on the ice to react for 20 min and centrifuged at 12000×g at 4^{o}C for 10 min. To the supernatant (75 µL) imidazole solution (250 mM) 75 µL was added and agitated well. The reaction was

left to proceed for 5 min. DTNB color reagent (100 μL) (1.5 mM in 200 mM potassium dihydrogen phosphate solution) was added and mixed well. An aliquot of the reaction system (200 μL) was transferred into the 96 well plate and read at 410 nm with ELISA reader. A calibration curve was established using the authentic glutathione at concentrations 5, 10, 20, 30, 40, and 50 nM conducted with similar treatment and procedure. The content of reduced glutathione was expressed in nmol/mg protein.

2.12. Assay for hydrogen peroxide (HPO) in tissue

The assay method of tissue hydrogen peroxide level was according to Nourooz –Zadeh et al. (1994). Briefly, to tissue (100mg) PBS (100μL) was added. The tissue was homogenized and centrifuged at 12000×g. To the supernatant (25μL) in a 96 well plate 250μL color reagent was added and left to react for 30 min at ambient temperature. The optical density was read at 620 nm with an ELISA reader. Authentic hydrogen perxoxide solution (Merck, Germany) at concentrations 5, 10, 20 30, 40, 50 and 60 μM were used to establish the calibration curve by similar treatment and procedure.

2.13. Statistical analysis

The data obtained were analyzed with the Statistical Analysis System 9.0 (SAS 9.0) and expressed in mean±SD. The variance between groups was

analyzed using Duncan`s Multiple Range Test. A level of $p<0.05$ was set as the confidence level.

3. Results and Discussion

3.1. The optimum dosages of VPA, folic acid, vitamin C, and NAC to conduct teratogenesis experiment

The percent prevalence of malformation and mortality rate reached 66.7% and 22.2% at VPA 30mM (Table 1). The hatched 1 day chick was totally unable to stand up (Fig. 1, right column), contrasting with the normal (Fig. 1. left column). No mortality was seen at VPA dose ≤ 20 mM (Table 1). Hence the optimum dosage of VPA was found to be 30 mM. This dosage was further combined with the target nutraceutics each at 10 mM to carry out the protective experiments. VPA alone exhibited a malformation rate 62.2% as well as a mortality rate13.7%. VPA (30 mM)+folic acid (10 mM) did not induce any. VPA (30 mM)+vitamin C (10 mM) caused a malformation rate 25.0% without any occurrence of mortality. VPA (30mM)+NAC (10mM) caused 40% malformation rate, also without any mortality (Table 2). To quantify, we established a scoring system to describe malformation grading (Fig. 1B). Based on this system, the extent of malformation was scored and shown in Table 3. As can be seen, the groups VPA, VPA+folic acid, VPA+vitamin C, and VPA+NAC exhibited teratogenic scores:

malformation 為 61.3；mortality 為 16.3%

malformation 為 10%；mortality 為 0%

malformation 為 22.5%；mortality 為 22.5%

malformation 為 30.0%；mortality 為 20%

3.1, 0.4, 3.0, and 2.5 in legs; and 2.1, 0.4, 1.0, and 1.5 in claws, respectively (Table 3). By this scoring system, the optimum teratogenic dosage of VPA was re-confirmed to be at 30 mM.

3.2. Folic acid, vitamin C, and NAC alleviated embryonic vascularization at different degree

VPA inhibited angiogenesis. Less vascularization was seen in VPA group (Fig. 2A). Comparing to the control density (6.6%), VPA group only showed 3.3 % vascularization (Fig. 2B). Folic acid, NAC and vitamin C restored it to 5.75%, 5.7% and 4.3%, respectively (Fig. 2B). The most powerful restoring effect of vascularization implicated the potent angiogenic nature of folic acid. Overtime, the embryonic development was severely inhibited by VPA. The percent embryo wt./egg wt. (= percent $E_m w/E_g w$) attained only 0.23% w/w) comparing to those of nutraceutics-treated, which were comparably retaining at a percent Emw/Egw above 0.47% (Fig. 2C).

3.3. Pathological changes found in cervical muscle and ankle joint

Zenker's necrosis with inflammatory cell infiltration (pathological score 4) as well as fibrosis extensive (pathological score 5) was the major pathological defect frequently found in cervical muscle (Fig. 3), and similarly, synovitis and necrosis with inflammatory cell infiltration (pathological sore 3) were the main pathological

findings in the ankle joint (Fig. 4) when treated with VPA. VPA+folic acid or VPA+vitamin C was shown able to rescue some extent of the cervical muscle defects (Fig. 3, Fig. 4, Table 4). In contrast, no any ankle joint defects could be found in these treated groups, implicating the cervical muscle was more susceptible to VPA toxicity.

3.4. Superoxide dismutase (SOD) was ameliorated by folic acid, vitamin C, and N-acetyl cysteine

VPA suppressed SOD activity from 0.56 to 0.44 U/mg protein. Folic acid, vitamin C, and NAC not only significantly restored but also enhanced its activity to 0.76, 0.88, and 0.88 U/mg protein, respectively (Fig. 5A). N-acetylcysteine increased manganese superoxide dismutase activity in septic rat diaphragms (Barreiro et al., 2005). Superoxide anion ($\cdot O^{2-}$) induced abnormalities of the neural suture in rat embryos (Jenkinson et al., 1986). Glutathione (10 mM) or catalase (50 μg/mL) either partially or completely abolished the effects of X/XO, whereas the addition of SOD (50 μg/mL) or desferrioxamine (1 mM) did not reduce the number of malformed embryos (Jenkinson et al., 1986), suggesting hydrogen peroxide and/or hydroxyl radicals to be responsible for the effects of superoxide anion ($\cdot O^{2-}$) (Jenkinson et al., 1986). Apparently, the elevation of SOD (Fig. 5A) would be effective in reducing the terminal formation of hydrogen peroxide and hydroxyl radicals and simultaneously ameliorating the toxicity originated from SOD.

3.5. Glutathione level could not be restored by folic acid

The glutathione level was reduced from 12 to 6 nmol/mg-protein when treated with VPA. Folic acid and NAC failed to resumed its level. As contrast, vitamin C effectively raised the level to 12.8 nmol/mg-protein (Fig. 5B). Comparable but less effective result was reported by Zhang et al. (2010).

NAC is an acetylated cysteine residue. An optimal thiol redox state has been demonstrated to be of primary importance if attempting to optimize the protective ability of the cell to oxidative stress (Sen, 2001). Relative to glutathione availability, one of the most important considerations has been to properly maintain the availability of cysteine in the blood as that is known to be the rate-limiting substrate for glutathione resynthesis (Sen, 2001). The fact that NAC completely failed to resynthesize GSH while vitamin C successfully resumed the GSH level (Fig. 5B) explains well that VPA merely had attenuated the coenzyme NADPH/$NADP^+$ system of glutathione reductase (GR) at least at this HH28 embryonic stage, and the total GSH-GSSG availability was adequately enough even in the presence of VPA. Under such a circumstance, the need for reactivation would prevail resynthesis of GSH, resulting in the apparent ineffectiveness of NAC (Fig. 5B), and in the meanwhile, implicating both folic acid and NAC not involved in the oxidation-reduction cycle of GSH.

A diversity of physiological roles have been cited for thioredox status, which, in addition to the common well known antioxidant defenses and cell proliferation and apoptosis, otherwise involve xenobiotic metabolism, protein structure and activity, receptor modification, signal transduction, immune regulation, and membrane transport (Kerksick and Willoughb, 2005). Thus suppressing GSH would initiate tremendous biological effects not merely limited to the Redox world.

3.6. Hydrogen peroxide level was effectively suppressed by either vitamin C or NAC

Sha and Winn have shown that VPA exposure leads to both an increase in reactive oxygen species (ROS) production and increased frequency of homologous recombination (HR) (Sha and Winn, 2010).

Valproate inhibition of histone deacetylase 2 involves ROS production, which affects differentiation and decreases proliferation of endometrial stromal sarcoma cells (Hhrzenjak et al., 2006). Level of hydrogen peroxide raised by VPA from 6 nmol/mg-protein to 7.3 nmol/mg-protein was significantly reduced by vitamin C and NAC to the same level 5.0 nmol/mg protein (Fig. 5C), implicating folate does not have any H_2O_2-scavenging capacity, consistent with Huang et al. (2002), although folate exhibited antioxidant behavior toward superoxide anions, hydroxyl radicals and peroxynitrite (Huang et al., 2002).

Endogenous ROS serve as a second messenger in signal transduction (Hansen, 2006) and are thought to be important in ion transport, immunological host defense, transcription and apoptosis of unwanted cells (Dennery, 2007). However, ROS also can be harmful by binding covalently or irreversibly to cellular macromolecules. Oxidative stress, an imbalance between ROS generation and antioxidant defense mechanisms of a cell or tissue, causes irreversible oxidation of DNA, proteins and lipids, leading to inactivation of many enzymes and cell death (Dennery, 2007). In addition to damaging cellular macromolecules, oxidative stress may affect gene expression by interfering with the activity of redox-sensitive transcription factors and signal transduction by oxidizing thiols (Sahambi and Hales, 2006). During the prenatal period, this may result in birth defects and growth retardation, and in severe cases in *in-utero* death (Wells et al., 1997; Hansen, 2006). Gilmour et al. reported hydrogen peroxide inhibited expression of HDAC2 down to 46% of the control level (Gilmour et al., 2003). Recently, van Gelder et al. identified six teratogenic mechanisms associated with medication use: folate antagonism, neural crest cell disruption, endocrine disruption, oxidative stress, vascular disruption and specific receptor- or enzyme-mediated (like HDAC2) teratogenesis. Many medications classified as class X are associated with at least one of these mechanisms (van Gelder et al., 2010).

3.7. Homocysteine accumulation was completely restored by folic acid, vitamin C, and N-acetyl cysteine

Significant homocysteine accumulation was found intensely raised to 21.5μM ($p<0.05$) in the VPA treated (Fig. 6). Complete restoration was achieved by folic acid and NAC to 13.5 and 13.0μM, but not by vitamin C (15.5μM, $p<0.05$) (Fig. 6). Such a discrepancy may be attributed to different antagonistic mechanism of folic acid, NAC, and vitamin C. In vivo, folate is converted through two reduction reactions by dihydrofolate reductase (DHFR) to the naturally bioactive form tetrahydrofolate (THF), which is converted into 5-methyltetrahydrofolate (5-MTHF) monoglutamate. 5-MTHF is the main form of folate in the blood circulation (van der Put and Blom, 2000). Inside the cell, it acts as an essential co-enzyme in many biochemical reactions by being an acceptor or donor of one-carbon units in, for example, purine and pyrimidine synthesis and DNA methylation reactions (Fig. S1, van Gelder et al., 2010). Since rapidly proliferating tissues require DNA synthesis the most, it is obvious that folate-dependent reactions are essential for fetal growth and development and that folate requirements increase during pregnancy. In addition, DNA methylation is known to be involved in the epigenetic control of gene expression during development. Several drugs disturb the folate metabolism and may have a teratogenic effect through inhibition of the folate methylation cycle (van Gelder et al., 2010).

Obviously, the accumultion of homocysteine was directly caused by the defect in methionine synthase (Fig. 6), which in turn will cause brain and nerve damages, increasing the risk of folate sensitive birth defects, such as neural tube defects, orofacial clefts and limb defects (van Gelder et al., 2010). Alternatively, the defect may also points to DHFR, but results are inconsistent (Hermández-Dıáz et al., 2001; Meijer et al., 2005). Our results apparently indicated DHFR was not affected by VPA (Fig. S1). Although it is unclear whether hyperhomocysteinemia is itself teratogenic or whether it is simply a biomarker for disturbances in folate or methionine metabolism, mild maternal hyperhomocysteinemia has been considered to be a risk factor for NTDs (van der Put and Blom, 2000).

3.8. Histone deacetylase activity (HDAC) rescued by folic acid and vitamin C

Histones are covalently modified at the epsilon-amino group of conserved lysines by a class of enzymes called histone acetyltransferases (HATs). HATs come in two flavors, cytoplasmic and nuclear. The cytoplasmic HATs (e.g. Hat1) acetylate histones prior to nuclear localization and chromatin assembly, whereas the nuclear HATs acetylate histones in a manner associated with transcription and other DNA dependent processes. HDAC is a direct target of VPA (Phiel et al., 2001). HDACs deacetylate lysine residues on histone tails and condensate chromatin, resulting in limited access of transcriptional activators to the DNA (Johnstone, 2002). exerting a fundamental

impact on gene expression and therefore possible molecular targets of VPA-induced signaling cascades including neural tube defects (NTDs) (Eikel et al., 2006).

An increase of 6-fold histone H3 acetylation was observed when treated with VPA (20%) comparing to the untreated control (3%) (Chen et al., 2007; Huang et al., 2011). Phiel et al. demonstrated that VPA (5 mM) dowregulated the nuclear HDAC activity by 77% (Phiel et al., 2001). Inhibition of HDACs may elicit VPA-induced teratogenicity (Sha and Winn, 2010), resulting in interruption of cell proliferation, differentiation and apoptosis (Marks et al., 2000; Yoshiura et al., 2005). We found that VPA significantly dowregulated HDACs to 1.15 nmol/min/mg protein comparing to the control (2.10 nmol/min/mg protein), a downregulation of 45%. Folic acid and vitamin C restored it to 2.60 nmol/min/mg protein (+24%) and 2.20 nmol/min/mg protein, respectively. Less effect was found for NAC (1.65 nmol/min/mg protein) (Fig. 7). Although in vivo, only up to 20% of the whole genome is controlled by HDACs, key processes for development, survival, proliferation, and differentiation have been strictly linked to HDAC enzyme functioning (Menegola et al., 2006). To date, all tested HDACi have shown teratogenic effects similar to those described for VPA in many animal models (Menegola et al., 2006).

3.9. Folate concentration was restored by vitamin C, but not by NAC

In the developing chick embryo, the higher polyglutamates are the only folate forms

present and their content increases progressively during the development of the embryo (Marchetti et al., 1982).

The decrease of folate level in chick embryo when exposed to VPA mounted to -44% (from the control 12.5 μM to 7.0 μM in VPA treated chick embryo). Vitamin C (10 mM) effectively restored the folate level to 14.5 μM. NAC (10 mM) was shown to have only partially restored 80% of the folate level (Fig. 8), implication in "Multiple Point Action Mechanisms" of VPA in folate biosynthesis, e.g. direct inhibition on the biosynthesis of the frame structure of folic acid, and the indirect inhibition of reducing coenzyme (like NADPH/$NADP^+$) systems for activation.

Considering the fact that structurally analogous to dihydropteroic acid (DHPA), trimethprim or pterin sulfonamide acts competitively in DHFA synthesis, we hypothesize VPA, or possibly its relevant metabolites, being analogous to glutamic acid, also may exhibit potential to antagonize the DHFA synthesis.

5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase (DHFR) (EC 1.5.1.3) is an enzyme that reduces dihydrofolic acid (7,8-dihydrofolate, DHFA) to tetrahydrofolic acid (5,6,7,8-tetrahydrofolate, THF) using $NADP^+$/NADPH reduction/oxidation system as electron donor within cells (principally in the liver where it is stored) (van Gelder et al., 2010). In addition, polymorphisms in genes associated with the folate metabolism, including methylene tetrahydrofolate reductase (MTHFR) (Botto and Yang, 2000; van

Rooij et al., 2003), methionine synthase reductase (MTSR) (van der Linden et al., 2006) and methylene tetrahydrofolate dehydrogenase (MTHFD) (Parle-McDermott et al., 2006), may lead to differences in the susceptibility of individuals to folate antagonists (Fig. S1, van Gelder et al., 2010).

A summary of the teratogenicity-associated markers as a consequence of exposure to VPA and the different counteracting mechanisms exerted by folic acid, vitamin C, and N-acetyl cysteine is shown in Fig. 9.

We hypothesize that VPA may affect folate methylation cycle by “Multiple-Point Action Mechanism” with varying strength. Briefly, egg white are enriched with folate polyglutamate (Marchetti et al., 1982), which on hydrolysis releases glutamic acid, in addition to the release of folic acid, the released glutamic acid may couple with pteroic acid to produce dihydrofolic acid (Fig. 9). VPA inhibits the endogenous folic acid biosynthesis acting as a structural antagonist for glutamic acid. Moreover, VPA tends to suppress the $NADP^+$/NADPH coenzymes by producing ROS without destroying the apoenzymes DHFR and MTHFR (Fig. 9), hence these steps still retain their reversibility available for transforming folates provided the reducing power is sufficiently retained.

In vivo, the reduced form of GSSG/GSH system can be reactivated by NADPH/$NADP^+$ system, and the reduced GSH may in turn reactivate ascorbic

acid/dehydroascorbic acid (AA/DHA) system, or the vice versa. In mouse embryo, Zhang et al. (2010) demonstrated the re-activation of GSH by AA alone was significant comparing to the VPA treated (p<0.05), but still lower than the control. On the contrary, we indicated that AA alone had highly significantly reassumed level of GSH (12.8 nmol/mg protein) to exceed the control (12 nmol/mg protein). In curling tail mice, zinc (20-150μg/kg), homocysteine (50-400mg/kg), methionine (200-160 mg/kg), and thymidine (25-400 mg/kg) had been tried by Bock and Marsh (1994), none of the medicines tested showed significant difference in the prevalence rate of NTD (Bock and Marsh, 1994). Apparently, the therapeutic effects of medicines on teratogenesis are highly developmental stage- and species-dependant. The embryotoxic potential of a compound depends on factors such as dose, critical window of exposure and sensitivity of the developing morphogenetic system at the time of administration (Joshi, 2011).

Evidenced by these data, we found that VPA possesses “Multiple Point Action Mechanism”. In addition to affecting the cited transcription and translation levels, we hypothesize that VPA competitively antagonize the glutamic acid to couple with pteroic acid in biosynthesis of dihydrofolic acid (DHFA). H_2O_2 directly destroyed the NADPH reducing system at dihydrofolate reductase (DHFR) and methylene folate reductase (MTHFR) levels, while completely restored by AA, an implication in

preservation of intact apoenzymes. In addition, the GSH-GSSG system is sandwiched between the reducing systems NADPH/NADP and DHA-AA, its net balance is highly dependent on in situ *in-vivo* Redox state, hence folic acid transformation is varying. To rescue the VPA-induced teratogenicity, simultaneous multiple prescriptions are suggested.

Conclusion

VPA induces teratogenicity by “Multiple Point Action Mechanism”. The nutraceutics mostly cited to be relatively effective in treatment of VPA teratogenicity all showed only partial alleviation effect. Suggestively, to simultaneously use multiple prescriptions is recommended. Recently, we are undertaking the alternative investigation covering the genomic and proteomic transformations. Expectedly, much more detail novel mechanism(s) will be appeaing in the very near future, which might be helpful in promoting the VPA therapeutics.

Acknowledgements

The authors are grateful for financial support from the National Science Council 99-2313-B-018-002-MY3, 98-2320-B-038-024-MY3, 99-2320-B-038-011-MY3, and 99-2320-B-039-034-MY3. The authors also acknowledge the financial support of

CMU97-234 and CMU99-N1-09 from Chinese Medical University.

Conflict of interest

The authors do not have any conflict of interest in publishing this manuscript.

References

Aluclu M.U., Tuncer M.C., Guzel A., Aluclu M.A., Akkus M. 2009. The effects of valproic acid on sciatic nerve of fetal rats and protective effects of folic acid and vitamin E. Int. J. Mophol. 27, 285-294.

Amorim M.R., Lima M.A., Castilla E.E., Orioli I.M., 2007. Non-Latin European descent could be a requirement for association of NTDs and *MTHFR* variant 677C > T: A meta-analysis. Am J Med Genet. 2007. Am. J. Med. Genetics Part A 143A, 1726–1732.

Barreiro E., Sánchez D., Gáldiz J.B., Hussain S.N.A., Gea J., 2005. N-acetylcysteine increases manganese superoxide dismutase activity in septic rat diaphragms. Clin. Respirat. Physiol. 26, 1032-1039.

Bock G., Marsh J., 1994. (eds): Neural Tube Defects-Congress W3 C161F vol. 181, Ciba Foundation (John-Wiley & Son Ltd. West Sussex, England).

Botto L.D., Yang Q., 2000. 5,10-Methylelenetetrahydrofolate reductase gene variants and congenital anomalies: a HuGE review. Am. J. Epidermal. 151, 862-877.

Chango A., Nour AA., Bousserual S., Eveillard D., Anton P.M., Gueant J.L., 2009.

Time course gene expression in the one-carbon metabolism network using HepG2 cell line grown in folate-deficient medium. J. Nutr. Biochem. 20, 312-320.

Chen C.S., Wang Y.C., Yang H.C., Huang P.H., Kulp S.K., Yang C.C., Lu Y.S. Matsuyama S., Chen C.Y., 2007. Histone deacetylase inhibitors sensitize prostate cancer cells to agents that produce DNA double strand breaks by targeting Ku70 acetylation. Cancer Res. 67, 5318-5327.

Dawson J.E., Raymond A.M., Winn L.M., 2006. Folic acid and pantothenic acid protection against valproic acid-induced neural tube defects in CD-1 mice. Toxicol. Appl. Pharmacol. 211, 124-132.

Dennery P.A., 2007. Effects of oxidative stress on embryonic development. Birth Defects Res. C Embryo Today 81, 155-162.

Ehlers K., Elmaza M.M.A., Nau H., 1995. Methionine reduces the valproic acid –induced spina bifida rate in mice without altering valproic acid kinetics. J Nutri. 126, 67-75.

Eikel D., Lampen A., Nau H., 2006. Teratogenic Effects Mediated by Inhibition of Histone Deacetylases: Evidence from quantitative structure activity relationships of 20 valproic acid derivatives. Chem. Res. Toxicol. 19, 272–278.

Elmarzar M.M.A., Thiel R., Nau H., 1992. Effect of supplementation with folinic acid,

vitamin B_6, and vitamin B_{12} on valproic acid-induced teratogenesis in mice. Fundam. Appl. Toxicol. 18, 389-394.

Faiella A., Werning M., Consalez G.G., Hostick U., Hofmann C., Hustert E., Boncinelli E., Balling R., Nadeau J.H., 2000. A mouse model for valproic acid teratogenicity: parental effects, homeotic transformations, and altered HOX expression. Human Mol. Genet. 9, 227-236.

Frick, B., Schrocksnadel K., Neurauter G., Wirleitner B., E. Artner-Dworzak E., Fuchs D., 2003. Rapid measurement of total plasma homocysteine by HPLC. Clin. Chim. Acta, 331**,** 19-23.

Gilmour P., Rahman I., Donaldson K., MacNee W., 2003. Histone acetylation regulated epithelial IL-8 release mediated by oxidative stress from environmental particles. Am. J. Physiol Lung Cell Mol. Physiol. 284, L533-L540.

Hamburger, V., Hamilton, H.L., 1951. A series of normal stages in the development of the chick embryo. J. Morphol. 88, 49–92.

Hansen J.M., 2006. Oxidative stress as a mechanism of teratogenesis. Birth Defects Res. C Embryo Today, 78, 293-307.

Hermández-Dıáz S., Werler M.M., Walker A.M., Mitchell A.A., 2001. Neural tube defects in relation to use of folic acid antagonists during pregnancy. Am. J. Epidemiol. 153, 961-968.

Hrzenjak A., Moinfar F., Kremser M.-L., Strohmeier B., Staber P.B., Zatloukal K., Denk H., 2006. Valproate inhibition of histone deacetylase 2 affects differentiation and decreases proliferation of endometrial stromal sarcoma cells. Mol. Cancer Ther. 5, 2203–2210.

Huang P.-H., Chen C.-H., Chiu C.-C., Sargeant A.M., Kulp S.K., Teng C.-M., Byrd J.C., Chen, C.-S., 2011. Histone deacetylase inhibitors stimulate histone H3 lysine 4 methylation, in part, via transcriptional repression of histone H3 lysine 4 demethylases. Mol. Pharmacol. 79, 197-206.

Huang R.F., Huang S.M., Lin B.S., Hung C.Y., Lu H.T., 2002. N-Acetylcysteine, vitamin C and vitamin E diminish homocysteine thiolactone-induced apoptosis in human promyeloid HL-60 cells. J. Nutr. 132, 2151-2156.

Jelinek R, Peterka M., 1985. Chick embryotoxicity screening test-130 substances tested. Ind. Exptl. Biol. 23, 588-595.

Jenkinson P.C., Anderson D., Gangolli S.D., 1986. Malformations induced in cultured rat embryos by enzymically generated active oxygen species. Teratogenesis, Carcinogenesis, and Mutagenesis 6, 547-554.

Johannessen, C.U., 2000. Mechanisms of action of valproate: a commentatory. Neurochem. Int. 37, 103–111.

Johnstone RW. 2002. Histone deacetylase inhibitors: novel drugs for the treatment of cancer. Nat. Rev. Drug Discov. 1, 287–299.

Karabiber H, Sommezgoz E, Ozerol E, Yakinci C, Otlu B, Yologlu S., 2003. Effects of valproate and carbamazepine on serum levels of homocysteine, vitamin B12, and folic acid. Brain & Development. 25, 113–115.

Kelly T.L.J. Neaga O.R., Schwahn B.C., Rozen R., Trasler J.M., 2005. Infertility in 5,10-Methylenetetrahydrofolate reductase (MTHFR)-deficient male mice is partially alleviated by lifetime dietary betaine supplementation. Biol. Reprod. 72, 667–677.

Kerksick C., Willoughby D., 2005. The Antioxidant Role of Glutathione and N-Acetyl-Cysteine Supplements and Exercise-Induced Oxidative Stress. 2, 38-44.

Koch S., Jager-Roman E., Losche G., Nau H., 1996. Rating D and Helge H. Antiepileptic drug treatment in pregnancy: drug side effects in the neonate and neurological outcome. Acta Pediatr. 84, 739–746.

Kwon, H.J., Kim M.S., Kim M.J., Nakajima H., Kim K.W., 2002. Histone deacetylase inhibitor FK228 inhibits tumor angiogenesis. Int. J. Cancer, 97, 290-296.

Lheureux P.E.R., Penaloza A., Zahir S., Gris M., 2005. Science review: carnitine in the treatment of valproic acid-induced toxicity – what is the evidence? Crit Care, 9, 431–440.

Lie O.V., Bennett G..D., Rosenquist T.H., 2010. The N-methyl-D-aspartate receptor in heart development: A gene knockdown model using siRNA. Reprod. Toxicol. . 29, 32-41.

Marchetti M., Bovina C., Formiggini G.., Tolomelli B., 1982. Folic acid metabolism in rapidly growing tissues: synthesis of pteroylpolyglutamates. [Article in Italian] Acta Vitaminol. Enzymol. 4,197-205.

Marks P.A., Richon V.M., Rifkind R.A., 2000. Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. J. Natl. Cancer Inst. 92, 1210–1216.

Mattiazzi M., D'Aurelio M., Gajewski C.D., Martushova M., Kiaei M,. Beal F., Manfredi G., 2002. Mutated human SOD1 causes dysfunction of oxidative phosphorylation in mitochondria of transgenic mice. J. Biol. Chem. 277, 29626-29633.

Meijer W.M., de Walle H.E.K., Kerstjens-Frederikse W.S., de Jong-van den Berg L.T.W., 2005. Folic acid sensitive birth defects in association with intrauterine exposure to folic acid antagonists. Reprod. Toxicol. 20, 203–207.

Menegola E.D., Di Renzo F, Broccia M.L. Giavini E., 2006. Inhibition of histone deacetylase as a new mechanism of teratogenesis. Birth Defects Res, 78, 345-353.

Mominoki K., Kinutani M., Wakisaka H., Saito S., 2006. Leg dysfunctions in a hatched chick model of spina bifida aperta. Exptl. Neurol., 2006, 197(1):133-142.

Nourooz-Zadeh, J., Tajaddini-Sarmadi J., Wolff S.P., 1994. Measurement of plasma hydroperoxide concentrations by the ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine. Anal. Biochem. 220, 403-409.

Padmanabhan R., Shafiullah M., Benedict S., Nagelkerke N., 2006. Effect of maternal exposure to homocystine on sodium valproate-induced neural tube defects in the mouse embryos. Eur. J. Nutr. 45, 311–319.

Parle-McDermott A., Kirke P.N., Mills J.L., Molloy A.M., Cox C., O'Leary V.B., Pangilinan F., Conley M., Cleary L., Brody L.C., 2006. Confirmation of the R653Q polymorphism of the trifunctional C1-synthase enzyme as a maternal risk for neural tube defects in the Irish population. Eur. J. Hum. Genet. 14, 768–772.

Phiel C.J., Zhang F, Huang E.Y., Guenther M.G., Lazar M.A., Klein P.S., 2001. Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer and teratogen. J. Biol. Chem. 276, 36734-36741.

Rodríguez-Bernaldo de Quirós A., Castro de Ron C., López-Hernández J., Lage-Yusty M.A., 2004. Determination of folates in seaweeds by high-performance liquid chromatography. J. Chromatogr. A. 1032, 135-139.

Rosenberg G., 2007. The mechanisms of action of valproate in neuropsychiatric disorders: can we see the forest for the trees? Cell. Mol.Life Sci. 64, 2090-2103.

Rosenquist T.H., Chaudoin T., Finnell R.H., Bennett G..D., 2010. Hig receptor in cardiac neural crest migration: A gene knockdown model using siRNA. Dev Dyn. 239, 1136-1144.

Roy M., Leclerc D., Wu Q., Gupta S., Kruger W.D., Rozen R., 2008. Valproic acid increases expression of methylenetetrahydrofolate reductase (MTHFR) and induces lower teratogenicity in MTHFR deficiency. J. Cell Biochem. 105, 467–476.

Sahambi S.K., Hales B.F., 2006. Exposure to 5-bromo-2'-deoxyuridine induces oxidative stress and activator protein-1 DNA binding activity in the embryo. Birth Defects Res. A Clin. Mol. Teratol. 76, 580-591.

Schulpis K.H., Lazaropoulou C., Regoutas S., Karikas G.A., Margeli A., Tsakiris S., Papassotiriou I., 2006. Valproic acid monotherapy induces DNA oxidative damage. Toxicol. 217, 228-232.

Sen C.K., 2001. Antioxidant and redox regulation of cellular signaling: introduction. Med. Sci. Sports Exerc. 33, 368-370.

Sha K., Winn L.M., 2010. Characterization of valproic acid-initiated homologous recombination. Birth Defects Res. B Dev. Reprod. Toxicol. 89,124-132.

Tabatabaei A.R., Abbott F.S.., 1999. Assessing the mechanism of metabolism dependent valproic acid induced in vitro cytotoxicity. Chem. Res. Toxicol. 12, 323–330.

van der Linden I.J.M., den Heijer M., Afman L.A., Gellekink H., Vermeulen S.H.H.M., Kluitmans L.A.J, Blom H.J., 2006. The methionine synthase reductase 66A>G polymorphism is a maternal risk factor for spina bifida. J. Mol. Med. 84, 1047-1054.

van der Put N.M., Blom H.J., 2000. Neural tube defects and a disturbed folate dependent homocysteine metabolism. Eur. J. Obstet. Gynecol. Reprod. Biol. 92, 57–61.

van Gelder M.M.H.J., van Rooij I.A.L.M., Miller R.K., Zielhuis G.A., de Jong-van den Berg L.T.W., Roeleveld N., 2010. Teratogenic mechanisms of medical drugs.

Hum Reprod. Update, 16, 378– 394.

van Rooij I.A.LM., Ocke´ M.C., Straatman H., Zielhuis G.A., Merkus H.M.W.M., Steegers-Theunissen RPM., 2004. Periconceptional folate intake by supplement and food reduces the risk of nonsyndromic cleft lip with or without cleft palate. Prev. Med. 39, 689– 694.

Verrotti A., Pascarella R., Trotta D, Giuva T., Morgese G., Chiarelli F., 2000. Hyperhomocysteinemia in children treated with sodium valproate and carbamazepine. Epilepsy Res. 41, 253–257.

Wells P.G., Kim P.M., Laposa R.R., Nicol C.J., Parman T., Winn L.M., 1997. Oxidative damage in chemical teratogenesis. Mutat. Res, 396, 65-78.

Whitsel A.I., Johnson C.B., Forehand C.J., 2002. An in ovo chicken model to study the systemic and localized teratogenic effects of valproic acid. Teratol 66, 153-163.

Yoshiura K., Nakaoka T., Nishishita T., Sato K., Yamamoto A., Shimada S., Saida T., Kawakami Y., Takahashi, T.A., Fukuda H., Imajoh-Ohmi S., Oyaizu N., Yamashita N. 2005. Carbonic anhydrase II is a tumor vessel endothelium–associated antigen targeted by dendritic cell therapy. Clin. Cancer Res. 11, 8201-8207.

Zhang B., Wang X., Nazarali A.J., 2010. Ascorbic acid reverses valproic acid-induced

inhibition of *Hoxa2* and maintains glutathione homeostasis in mouse embryos in culture. Cell Mol. Neurobiol. 30,137-148.