African Journal of Biotechnology Vol. 9(34), pp. 5616-5622, 23 August, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 ©2010 Academic Journals

Full Length Research Paper

The effect of valproic acid on rat ovarium and the protective role of vitamin E and folic acid: An ultrastructural study

Ayfer Aktas*, Yusuf Nergiz and Murat Akkuş

Departments of Histology and Embryology, Faculty of Medicine, Dicle University, Turkey.

Accepted 2 August, 2010

This study was undertaken to investigate the effect of valproic acid and protective effects of vitamin E and folic acid on rat ovary ultrastructural changes. Twenty-four Wistar rats were used. Animals were divided into four groups. The first group of rats was used as control. The second group was injected valproic acid. The third group was injected valproic acid + folic acid and the fourth group was given valproic acid + vitamin E. At the end of the study, ovarium tissues were taken under anesthesia. Tissues were prepared and examined by transmission electron microscopy. Microscopically, the groups are cited as follows: Control group (in which the ovarium tissue was normal), valproic acid group (which showed increase in lipid content plus mitochondrial crystalysis seen in folliculer and theca interna cells of rat ovarium), valproic acid + folic acid group (in which the theca interna and granulosa cells of rat ovarium had normal appearance) and valproic acid + vitamin E group (where all the organelles of theca interna and granulosa cells of rat ovarium were observed to be normal). Vitamin E and folic acid have protective effects against valproic acid-induced tissue damage in rat ovaries.

Key words: Valproic acid, vitamin E, folic acid, ovarium, rat.

INTRODUCTION

Valproic acid (VPA) caused a teratogenic picture distinct from that of other anticonvulsants. The first warning of the possibility of its danger came from experimentally based calculations that indicated VPA to be a far more potent teratogen than phenytoin (Kalter, 2003). Despite its effectiveness and widespread use, valproic acid is teratogenic in both animals and humans. Of particular concern is the 1 to 2% risk of neural tube defects, most of which are spina bifida, with the use of valproic acid during the first trimester of pregnancy.

Epilepsy will also affect various aspects of sexual and reproductive functioning with reproductive endocrine dysfunction reported during childhood, adolescence and adulthood in females with epilepsy. The most common symptoms are hyperandrogenism, menstrual disorders, ovarian failure, polycystic ovary syndrome (PCOS) with or without divalproex (VPA) therapy, hyperinsulinemia and weight gain especially with VPA therapy (Opaleke and Helmers, 2007). There has been a controversy surrounding PCOS and certain anti-epileptic drugs (AEDs), particularly VPA. Isojärvi et al. (1996) suggested an increase in PCOS related to VPA and associated with weight gain. Others have not shown an increase in PCOS with VPA use (Luef et al., 2002; De Vries et al., 2007). Additionally, when anti-epileptic drugs such as VPA are discontinued, there is still an increased incidence of PCOS in females with epilepsy, suggesting that epilepsy contributes to an associated risk of PCOS (Morrell et al., 2002). Isojarvi et al. (1998, 2001) reported that obese VPA-treated women with polycystic ovaries or hyperandrogenism, or women with both, had hyperinsulinemia and associated unfavorable changes in serum lipid levels that is consistent with insulin resistance. Therefore, they concluded that the use of VPA for cardiovascular disease in obese women was associated with risk factors. They

*Corresponding author. E-mail: aaktas@dicle.edu.tr or fuldic@gmail.com. Fax: +90 412 2488440.

Abbreviations: VPA, Valproic acid; **mEH,** microsomal epoxide hydrolase; **CIF,** caspase inhibitory factor; **ROS,** reactive oxygen species; **PCOS,** polycystic ovary syndrome; **AEDs,** anti-epileptic drugs.

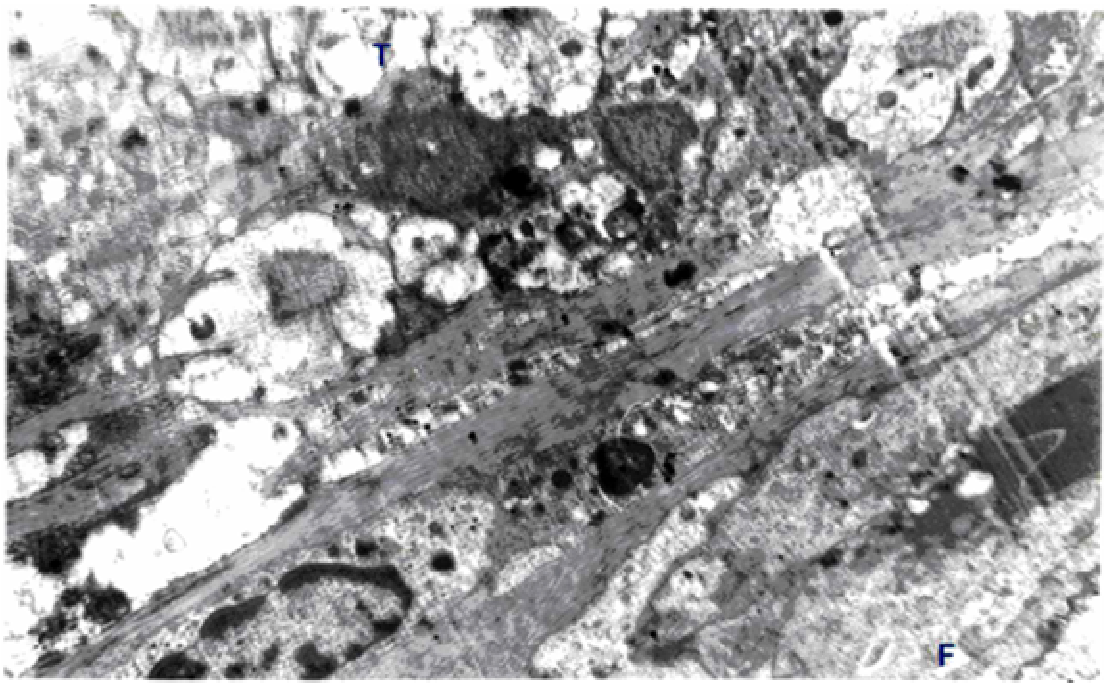

Figure 1. Control group: The control group folliculer cells (F) and theca interna cells of rat ovarium (T) were observed to be normal (uranyl acetate-lead citrate X3.000).

have noted that these VPA-related risks can be reduced by substituting lamotrigine for VPA.

Several studies indirectly suggest that valproic acid-initiated teratogenicity may be caused by oxidative stress. For example, previous research has shown that catalase, which detoxifies hydrogen peroxide, prevented valproic acid-induced lymphocyte toxicity and that 1.10 phenanthroline (an iron chelator) also decreased valproic acid-mediated cytotoxicity *in vitro*. These results suggest that the production of hydrogen peroxide and the succeeding iron-catalyzed formation of hydroxyl radicals may be the specific ROS that mediates valproic acid-induced toxicity. In addition, valproic acid has been shown to inhibit cardiomyocyte differentiation of embryoid bodies derived from murine pluripotent embryonic stem cells through an increase in ROS (Defoort et al., 2006).

In the present study, an investigation was done on the effect of valproic acid that was used widely in the treatment of epilepsy on rat ovarium and the protective role of vitamin E and folic acid.

MATERIALS AND METHODS

The study was conducted in accordance with the National Institutes of Health guidelines for the use of experimental animals. Twenty-four adult female Wistar-albino rats weighing 200 to 250 g, obtained from Experimental Research Institute of Dicle University (DÜSAM), were used. Rats were maintained on a 12 h light/dark cycle at 21 ± 1°C and 50 ± 10% humidity. After mating, pregnant rats were separated into four equal groups, while untreated rats served as controls. The group of valproic acid (sodium valproate and Sigma P 4543) were injected 400 mg/kg valproic acid subcutaneously on the 8th, 9th and 10th day of pregnancy, respectively. The group of valproic acid + folic acid was given 400 µg folic acid (Pteryoglutamic acid vit M, Sigma F 8798). This was added to the drinking water of rats during the period of pregnancy. Supplementarily, 400 mg/kg valproic acid was administered on the 8th, 9th and 10th day of pregnancy, respectively. Valproic acid + vitamin E group with 250 mg/kg vitamin E (α-tocopherol, Sigma T-3251) solved in olive oil was given with gastric intubation. One hour later, after administration of vitamin E, 400 mg/kg valproic acid was injected subcutaneously.

On the 20th day of gestation, all pregnant rats were sacrificed and the fetuses were removed. The ovarium was removed from each group for electron microscopic examination, while the pieces of ovarium tissues were fixed in 2.5% gluteraldehyde in 0.1 M sodium phosphate buffer. Following this, the tissue was fixed for 2 h in the same fixative at 4°C. The tissue was then thoroughly washed three times in a 0.1 M sodium phosphate buffer and post-fixed in 1% osmium tetroxide in a 0.1 M sodium phosphate buffer at 4°C. After repeated washings, the tissue was dehydrated in a graded series of alcohol and embedded in araldite CY212. The blocks were cut on microtome (LKB-8800 ultratome). Semihtin sections (1 µm thick) were routinely stained with toluidin blue for light microscopy. Ultrathin sections (60 - 80 nm thick) were contrasted with uranyl acetate and lead citrate and were examined with a transmission electron microscope (JEOL 1010) operating at 80 kV.

RESULTS

No pathology was observed in the control group of rats' ovaries (Figure 1). In the ovaries of valproic acid's group, lipid accumulation and mitochondrial crystalysis was seen in the cells of theca interna and granulose (Figures 2 and 3).

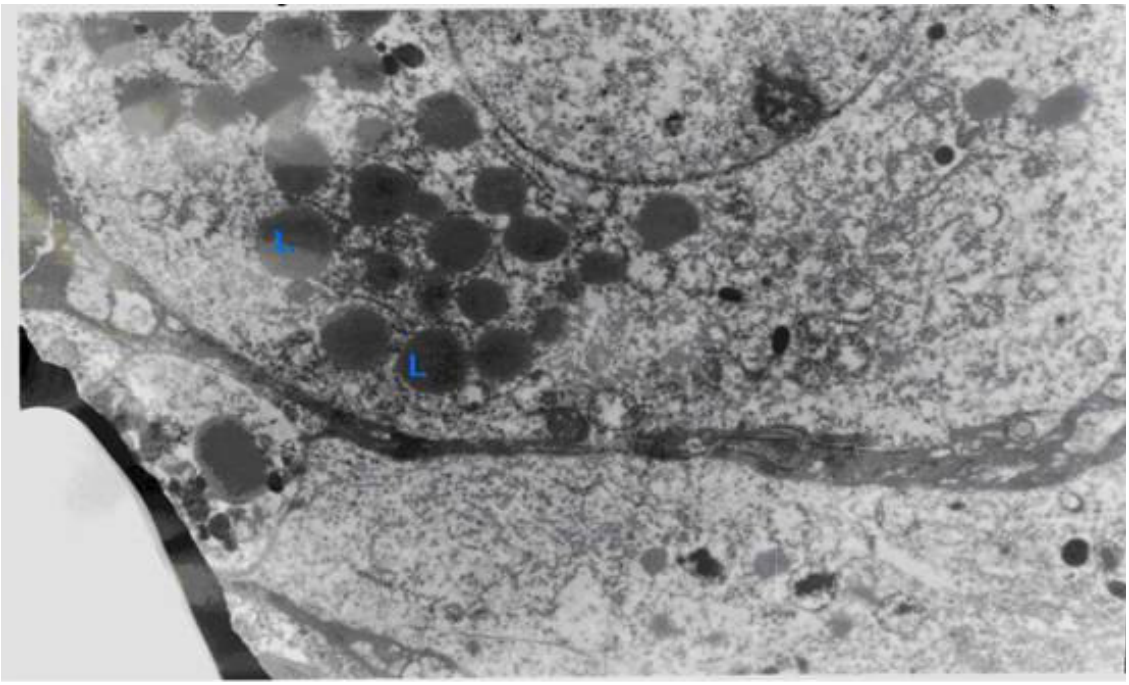

Figure 2. Valproic acid group: Lipid (L) increase was seen in the valproic acid group follicular and theca interna cells of rat ovarium (uranyl acetate-lead citrate X4.400).

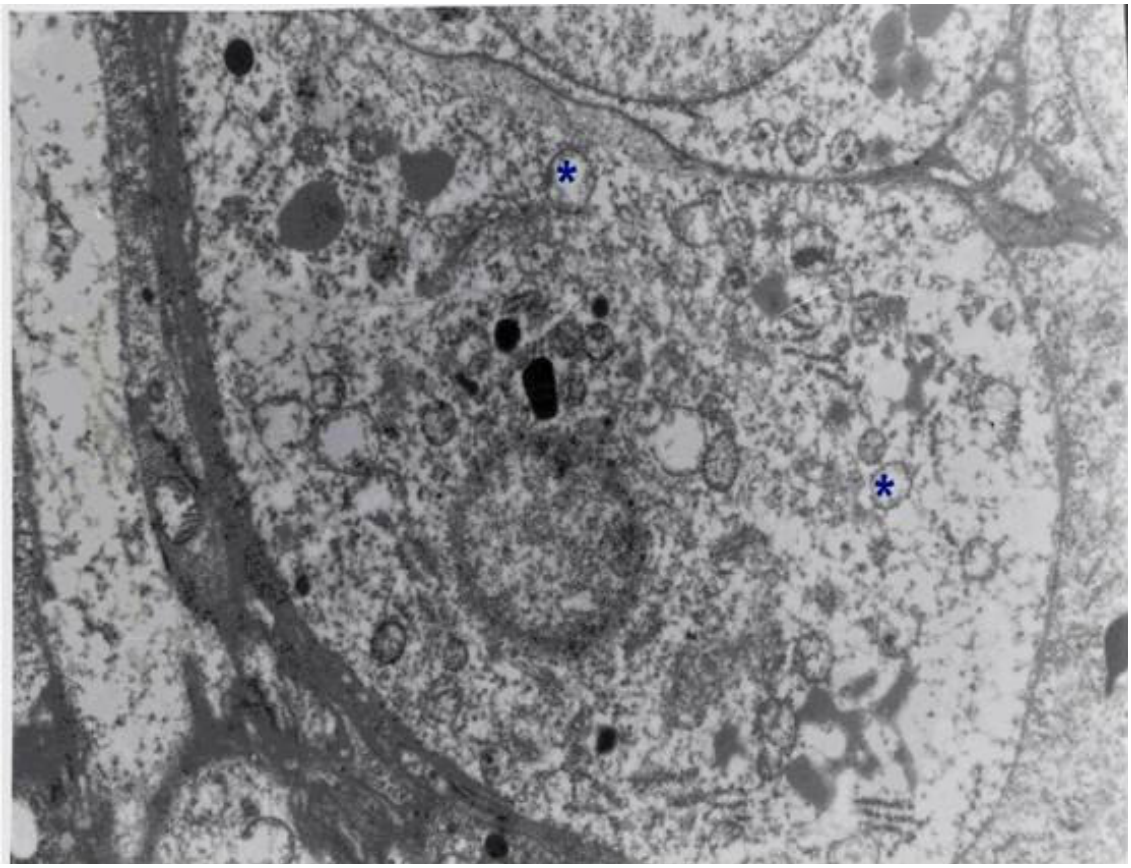

Figure 3. Valproic acid group: The mitochondrial crystalysis (*) was observed in the valproic acid group follicular cells and theca interna cells of rat ovarium (uranyl acetate-lead citrate X4.400).

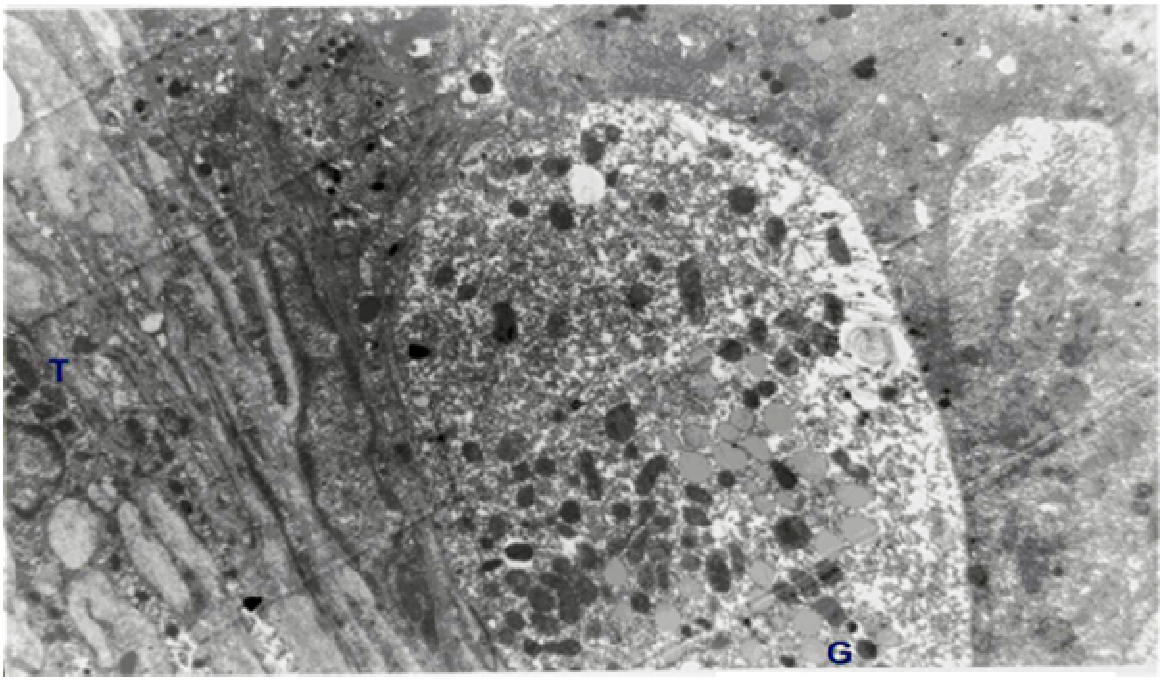

Figure 4. Valproic acid + folic acid group: The valproic acid + folic acid group theca interna (T) and granulosa cells (G) of rat ovarium had normal appearance (uranyl acetate-lead citrate X3.000).

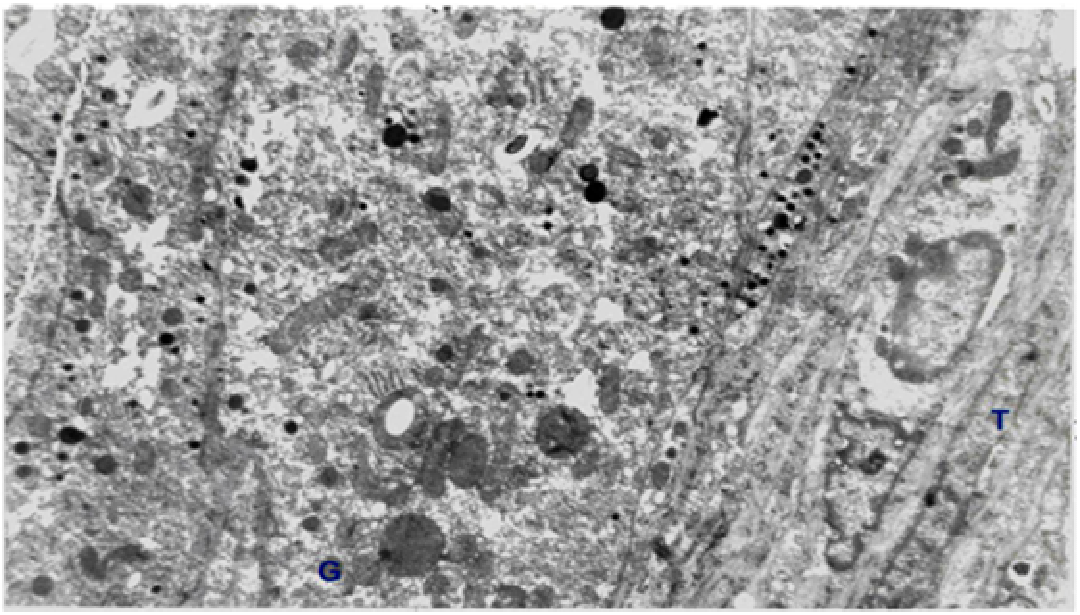

Figure 5. Valproic acid + vitamin E Group: All the organelles of the valproic acid + vitamin E group theca interna (T) and granulosa cells (G) of rat ovarium were observed to be normal (uranyl acetate-lead citrate X3.000).

Normal appearance was shown in the cells of theca interna and granulosa in the group of valproic acid + folic acid (Figure 4). In the group of valproic acid + vitamin E, there was an appearance close to the control group of theca interna and granulosa cells (Figure 5).

DISCUSSION

Since various central nervous systems acting on therapeutic agents have well-defined effects on the neuroendocrine control of reproductive function, it is not

surprising that they have been employed as pharmacological tools to elucidate the functional roles of central nervous system transmitter and peptidergic pathways controlling the pituitary-ovarian axis (Stoker et al., 2001).

The central nervous system plays a critical role in modulating the physiological and behavioral events associated with normal reproductive function in both male and female mammal. In females, the hypothalamus regulates pituitary function in a variety of ways to assure the maintenance of ovarian cycles, ovulation, the initiation of pregnancy, implantation, pregnancy maintenance, after birth and lactation. As a result of its importance to normal pituitary-gonadal function, the hypothalamus is also a significant target site for reproductive toxicants. In rodents, this endocrine event must be precisely timed during the estrous cycle. This occurs in response to circadian factors (that is, light and dark schedule) and fluctuating levels of gonadal steroids in the blood. The estrous cycle in the female rat can be characterized by observing the estrogen progesterone dependent changes in vaginal cytology (Stoker et al., 2001).

In recent years, the polycystic ovarian syndrome (PCOS) has been extensively discussed in relation to the use of valproate as an anti-epileptic drug in humans. Several studies have demonstrated development of menstrual disorders, hyperandrogenism and polycystic ovaries in women on valproate (Isojärvi et al., 1993).

In this study, no pathology was observed in the control group of rats' ovaries (Figure 1). In the ovaries of valproic acid group, lipid accumulation and mitochondrial crystalysis were observed in the cells of theca interna and granulose (Figures 2 and 3). Acquired abnormalities in mitochondrial respiration are caused by several drugs and toxins. The anticonvulsant valproate is metabolized into a 4-envalproic acid, a mitochondrial toxin, which causes liver failure in those with underlying complex I or cytochrome c oxidase deficiency (Chabrol et al., 1994). Valproate alters reproductive endocrine function in non-epileptic rats. The results suggest a peripherally directed effect of the drug in both sexes. In female rats, such an effect is supported by the markedly reduced estradiol concentrations combined with unaltered testosterone levels and also by the minor changes seen in the gonadotropin levels. In addition, the morphologic findings, with increased number of follicular cysts, support a direct effect on the gonadal level (Roste et al., 2001).

Graf et al. (1985) reported that a number of organs from adult female mice were investigated after continuous application of the anticonvulsant drug valproic acid (VPA) by enzyme cytochemistry, light and electron microscopy, pharmacokinetics and clinical chemistry. Microscopic electron, number and length of hepatocytes' microvilli were increased and many of them showed fat inclusions, mitochondrial swellings and autophagic vacuoles. In some of the proximal convoluted tubules of the kidney, the reaction product originating from microvillous and lysosomal hydrolases was diffusely distributed and its amount was lowered. This was parallel to that of the tubular cells with an increased number of fat droplets and swollen mitochondria or destroyed tubular cells, as demonstrated by electron microscopy. Their data indicated that, in addition to the liver, the kidney, thymus and spleen are also target organs of VPA-induced toxicity in the mouse (Graf et al., 1985). Hattori et al. (2000) found that valproate-induced inhibition of the microsomal epoxide hydrolase (mEH) enzyme suppressed the conversion of testosterone to estradiol by granulosa cells cultured *in vitro*. They argued that if VPA inhibits the mEH activity and then suppresses aromatase activity in granulosa cells, testosterone produced by theca interna cells would not be converted to estradiol in the follicles. This effect would lead to an androgen dominant micro-environment in the ovary with low estrogen levels and possibly to the development of polycystic changes in the ovary (Hattori et al., 2000).

Valproate has a significant influence on reproductive endocrine hormones in non-epileptic animals of both genders. The main finding in the study was a significant increase in the testosterone/estradiol ratio, mainly because of the marked reduction in serum estradiol levels. They found that valproate reduced the secretion of testosterone, estradiol and progesterone from follicular cells isolated from small- and medium-sized ovarian follicles (Roste et al., 2002). Tauboll et al. (2003) investigated the mechanisms by which VPA affects ovarian steroidogenesis in female subjects. By use of *in vitro* cell cultures of porcine follicular cells, the study investigated the effect of VPA on both basal and gonadotropin-stimulated steroidogenesis and the conversion of testosterone to estradiol. Secondly, the effects of VPA on cell proliferation and apoptosis were studied (Tauboll et al., 2003). The results in this study showed that when VPA was added to the culture medium, it caused a significant effect on steroidogenesis in both unstimulated and gonadotropin-stimulated porcine ovarian follicular cells. These findings showed a direct effect of VPA on steroidogenesis, independent of epileptic activity, and indicated at least some similarities between the effects of VPA treatment and mechanisms at the ovarian level, responsible for cyst formation. In the ovary, estrogens are of prime importance for the survival and development of pre-ovulatory follicles, whereas androgens are apoptotic factors. Evidence suggests a direct interference of estrogens with apoptotic processes. Recently, estradiol was found to prevent caspase-6–mediated neuronal cell death, probably by inducing a caspase inhibitory factor (CIF) through a receptor-mediated non-genomic pathway. Another example is the stimulation of anti-apoptotic proteins' expression, like Bcl-2 or Bcl-XL, which prevents cytochrome c efflux from mitochondria to cytoplasm and finally, results in the inhibition of caspase-dependent apoptotic cell death. It is also possible that estrogens stabilize mitochondrial function either by scavenging free radicals or affecting adenosine triphosphatase (ATPase)

FOF1 (Kipp and Ramirez, 2001).

Neumana et al. (2001) determined the cytotoxicity of valproic acid (VPA), its metabolite and 4-ene-valproic acid (4-ene-VPA) in human hepatoblastoma cells (Hep G2). Also, they studied the modulatory effect of cytochrome P450 2E1 induction in this model. In this study, a number of morphological changes were observed by TEM in cells exposed to VPA. Noteworthy changes included: a two-fold increase in diameter of mitochondria, vesiculation and dilatation of smooth endoplasmic reticulum, a decrease in diameter of lipid droplets and an increase in the number of lipid droplets per cell. These *in vitro* results support previous findings that VPA causes hepatotoxicity, although, its P450-generated metabolite (4-ene-VPA) is more potent. Using TEM, altered mitochondrial structure was observed in VPA-treated cells compared to untreated cells (Neumana et al., 2001). This damage may be as a result of the VPA inhibition of β-oxidation, which has been demonstrated in rats and their liver homogenates (Kesterson et al., 1984; Bjorge and Baillie, 1991). The other study's findings indicated that acute treatment of freshly isolated rat hepatocytes, with valproic acid, resulted in oxidative stress, which occurred in the absence of cytotoxicity. Also, it indicated that glutathione confers protection of hepatocytes against mitochondrial damage by valproic acid (Tong et al., 2005).

The finding of the present study showed that there was normal appearance in the cells of theca interna and granulosa in the group of valproic acid + folic acid (Figure 4). Women with an increased risk for pregnancy, complicated by neural tube defects, often have a history of epilepsy, that is, the ingestion of valproic acid or carbamazepine for seizure control (Cadrin, 2003; Chodirker et al., 2001; Van Allen et al., 2002). Fetal valproate (teratogen) recognized the condition associated with neural tube defects (MOT Jones, 2006).

Folic acid studies were done in one class of animals namely anti-metabolites, that is, those of pteroyglutamic acid or folic acid as it is most commonly called, which led to the interest of this vitamin's role in human abnormal development (an interest that has extended over 50 years). Firstly, in such experimental work, the anti-metabolites, when administered to female mice and rats earlier or at the time of uterine implantation, caused early prenatal death, but not malformation, thereby suggesting an all-or-none action (Kalter, 2003).

Vitamin E is a beneficial antioxidant in the pharmacological treatment of mitochondrial disorders (Bandyopadhyay and Dutta, 2005). The study's findings in valproic acid + vitamin E group showed that there was an appearance close to the control group of theca interna and granulosa cells (Figure 5). The antioxidant vitamin E has been shown to decrease the frequency of valproic acid-induced neural tube defects in mice, suggesting that reactive oxygen species (ROS) may play a role in the failure of the neural tube to develop properly. In addition, vitamin E has provided significant protection against hydrophobic bile acid toxicity in an *in vivo* rat model (Sokol et al., 1998).

It was concluded that the use of anti-convulsion drug has to be taken into consideration during pregnancy, while folic acid and vitamin E should be increased in the course of medical treatment. Further studies are needed to investigate the protective role of fatty acid (FA) and vitamin-E against toxic effects of valproic acid on fetus ovarium development.

REFERENCES

Bandyopadhyay SK, Dutta A (2005). Update Article: Mitochondrial Hepatopathies. JAPI, 53(10): 973-978.

Bjorge SM, Baillie TA (1991). Studies on the beta-oxidation of valproic acid in rat liver mitochondrial preparations. Drug Metabol. Dispos. 19(4): 823-829.

Cadrin C, Chodirker BN, Davies GAL, Johnson J, Reid GJ, Shaw D, Wilson RD, Young DC (2003). SOGC Genetics Committee: The use of folic acid for the prevention of neural tube defects and other congenital anomalies. SOGC Clinical Practice Guidelines No. 138, Novembe. J. Obstet. Gynaecol. Can. 25(11): 960-965.

Chabrol B, Mancini J, Chretien D (1994). Valproate induced hepatic failure in a case of cytochrome c oxidase deficiency. Eur. J. Pediatr. 153: 133-135.

Chodirker BN, Cadrin C, Davies GAL, Summers AM, Wilson RD, Winsor EJT (2001). SOGC Genetics Committee Members, CCMG Prenatal Diagnosis Committee Members. Canadian guidelines for prenatal diagnosis. Genetic indications for prenatal diagnosis. SOGC Clinical Practice Guidelines, No 105, June 2001. J. Soc. Obstet. Gynaecol. Can. 23(6): 525-531.

De Vries L, Karasik A, Landau Z, Phillip M, Kiviti S, Goldberg-Stern H (2007). Endocrine effects of valproate in adolescent girls with epilepsy. Epilepsia, 48: 470-477.

Defoort EN, Kim PM, Winn LM (2006). Valproic Acid Increases Conservative Homologous Recombination Frequency and Reactive Oxygen Species Formation: A Potential Mechanism for Valproic Acid-Induced Neural Tube Defects. Mol. Pharmacol. 69: 1304-1310.

Graf R, Gossrau R, Merker HJ, Schwabe R, Stahlmann R, Nau H (1985). Enzyme cytochemistry combined with electron microscopy, pharmacokinetics, and clinical chemistry for the evaluation of the effects of steady-state valproic acid concentrations on the Mouse. Histochemistry, 83: 347-358.

Hattori N, Fujiwara H, Maeda M, Fujii S, Ueda M (2000). Epoxide hydrolase affects estrogen production in the human ovary. Endocrinology, 141: 3353-3365.

Isojärvi JIT, Laatikainen TJ, Knip M (1996). Obesity and endocrine disorders in women taking valproate for epilepsy. Ann. Neurol. 39: 579-584.

Isojärvi JIT, Laatikainen TJ, Pakarinen AJ (1993). Polycystic ovaries and hyperandrogenism in women taking valproate for epilepsy. N. Engl. J. Med. 329: 1383-1388.

Kalter H (2003). Teratology in the 20th century Environmental causes of congenital malformations in humans and how they were established. Neurotoxicol. Teratol. 25: 131-282.

Kesterson JW, Granneman GR, Machinist JM (1984). The hepatotoxicity of valproic acid and its metabolites in rats. I. Toxicologic, biochemical and histopathologic studies. Hepatology, 4: 1143-1152.

Kipp JL, Ramirez VD (2001). Effect of estradiol, diethylstilbestrol and resveratrol on F0F1-ATPase activity from mitochondrial preparations of rat heart, liver and brain. Endocrine, 15: 165-175.

Luef G, Abraham I, Haslinger M (2002). Polycystic ovaries, obesity and insulin resistance in women with epilepsy: A comparative study of carbamazepine and valproic acid in 105 women. J. Neurol. 249: 835-841.

Morrell MJ, Giudice L, Flynn KL (2002). Predictors of ovulatory failure in women with epilepsy. Ann. Neurol. 52: 704-711.

MOT Jones KL (2006) Smith's recognizable patterns of human malformation. 6th ed. Philadelphia WB Saunders. pp. 704-705.

Neumana MG, Sheara NH, Jacobson-Browna PM, Katza GG, Neilsona HK, Malkiewicza IM, Camerona RG, Abbott F (2001) CYP2E1-mediated modulation of valproic acid-induced hepatocytotoxicity. Clin. Biochem. 34: 211-218.

Opaleke A, Helmers SL (2007) Hormonal Consequences of Epilepsy. Semin. Pediatr. Neurol. 14: 189-195.

Roste LS, Taubøll E, Berner AA, Isojärvi JIT, Gjerstad L (2001). Valproate, but not lamotrigine, induces ovarian morphological changes in Wistar rats. Exp. Toxicol. Pathol. 52: 545-552.

Roste LS, Taubøll E, Isojärvi JIT, Pakarinen AJ, Huhtaniemi IT, Knip M, Gjerstad L (2002). Effects of chronic valproate treatment on reproductive endocrine hormones in female and male Wistar rats. Reprod. Toxicol. 16: 767-773.

Sokol RJ, McKim JM JR, Goff MC (1998). Vitamin E reduces oxidant injury to mitochondria and the hepatotoxicity of taurochenodeoxycholic acid in the rat. Gastroenterology, 114: 164-174.

Stoker TE, Goldman JM, Cooper RL (2001). Delayed ovulation and pregnancy outcome: effect of environmental toxicants on the neuroendocrine control of the ovary. Environ. Toxicol. Pharmacol. 9: 17-129.

Tauboll E, Gregoraszczuk EL, Kolodziej A, Kajta M, Ropstad E (2003). Valproate Inhibits the Conversion of Testosterone to Estradiol and Acts as an Apoptotic Agent in Growing Porcine Ovarian Follicular Cells. Epilepsia, 44(8): 1014-1021.

Tong V, Teng XW, Chang TKH, Frank Abbott S (2005). Valproic Acid II: Effects on Oxidative Stress, Mitochondrial Membrane Potential, and Cytotoxicity in Glutathione-Depleted Rat Hepatocytes. Toxicol. Sci. 86(2): 436-443.

Van Allen MI, McCourt C, Lee NS (2002). Preconception health: folic acid for the primary prevention of neural tube defects. A resource document for health professionals. Ottawa, Ontario Minister of Public Works and Government Services Canada.