The Prophylactic Effects of Folic Acid and Vitamin E against Valproic Acid During Fetal Thymus Development: an Ultrastructural Study

Los Efectos Profilácticos del Ácido Fólico y la Vitamina E contra el Ácido Valproico Durante el Desarrollo Fetal del Timo: un Estudio Ultraestructural

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SUMMARY: To evaluate histopathologic differences in the thymus of Wistar Albino rat fetuses prenatally exposed to valproic acid (VPA), folic acid (FA) and vitamin E (Vit-E). VPA (400 mg/kg), FA (400 mcg/kg) and Vit -E (250 mg/kg) were administered to rats on each of gestation days 8, 9 and 10. The fetuses (n:24) were divided into four groups: control, VPA, VPA+Vit-E and VPA+FA groups. On the 20th day of gestation, all pregnant rats were sacrificed and the fetuses were extracted. Thin sections from thymus of live fetuses were stained with uranyl acetate-lead citrate and were examined under transmission electron microscope. The histopathological findings of control group was normal. In VPA group, it showed extensive degenerative changes by VPA were on all tissue compartments when compared to controls. In VPA-FA group, vacuoles, mitochondrial cristallysis and swelling were decreased in cytoplasm. In VPA-Vit-E group, lipid storage and vacuolization were observed. Mitochondrial cristallysis decreased. Our aim in the present study is to analyze histopathological changes which may occur in a high risk experimental model after giving of VPA. In addition, protective roles of the administration of FA and Vit-E are assessed.

KEY WORDS: Valproic acid; Fetal thymus; Vitamin E; Folic acid.

INTRODUCTION

The human thymus, primary lymphoid organ, is an organ consisting of two lobes located in the anterior mediastinum (Abo, 2001; Lamboez *et al.*, 2002). Thymus organogenesis in vertebrates depends on interactions between cells of all three embryonic germ layer origins: endoderm-derived epithelium, neuroectoderm derived neural crest mesenchyme, and mesoderm derived hematopoietic cells and endothelial cells of blood vessels (Smith, 1965; Moore & Owen, 1967). Its function is critical during embryogenesis and in the early stages of life: during this period, its removal is lethal. However, soon after birth (a few days in mice, a few months in man), the thymus can be taken out with no detrimental effects for the organism. Its role is essential for the development of the T cell repertoire, though some T cells are of extra-thymic origin. T cell maturation is highly complex, and it is thought that the sequence of differentiation of

thymocytes to T cells is controlled by the thymus microenvironment. The thymic microenvironment is made up by epithelial cells and cells of the mononuclear phagocytic system. Thymic epithelial cells show ultrastructural varieties. Electron microscopy enables an easy distinction between four cortical thymic epithelial cells (cTEC) and three medullary thymic epithelial cells (mTEC) in rats (Milic'evic' & Milic'evic', 1997). Precursor T cells originate mainly in the yolk sac at first and then in the liver (during fetal development), subsequently in the bone marrow (after birth), and later in other organs too (in aged individuals). They differentiate from hematopoietic stem cells (HSCs) in the bone marrow, where they undergo several phenotypic transformations to give rise to a number of different cell types, including the lymphoid lineage (Abo; Lamboez *et al.*; Blom *et al.*, 1997; Lamontagne *et al.*, 1998; Poccia *et al.*,

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1998). VPA was first produced in 1882 as an organic solvent (Burton, 1882). The clear, colorless, eight-carbon branched chain fatty acid derived the name 'valproic acid' from the chemical name of 2-propylvaleric acid. The therapeutic potential of VPA was fortuitously discovered in 1962 when Carraz *et al.* used VPA as a solvent for experimental anticonvulsants and recognized that VPA itself had anticonvulsant effects (Meunier *et al.*, 1963). The first clinical trial for VPA in the treatment of epilepsy was reported in 1963 (Carraz *et al.*, 1964). The antiepileptic drug valproic acid (VPA) is a potent inducer of neural tube defects (NTDs) in human and mouse embryos. As with many other developmental toxicants, however, the mechanism of VPA teratogenicity is still unknown (Baran *et al.*, 2006). Thymus is one of the target organs of valproic acid (VPA)-induced toxicity in rat. It causes changes in cortical and medullary morphology of the thymus (Graf *et al.*, 1985). Folic acid (FA), a water-soluble vitamin, has been used since the 1940s to treat some cases of macrocytic anemia without neurologic disease (Swain & St Clair, 1997). Chronic treatment of valproic acid causes FA deficiency (Carl, 1986; Weber & Dib, 2003; Lewis *et al.*, 1998). Vitamin E was discovered over 80 years ago as a lipid-soluble substance in lettuce and wheat necessary for the prevention of fetal death and resorption in rats that had been fed by a rancid lard diet (Evans & Bishop, 1922). Vitamin E is the most important lipid-soluble chain-breaking antioxidant in plasma, red cells and tissues, playing an essential role in maintaining the integrity of biological membranes (Kamal-Eldin & Appelqvist, 1996; Chow, 2001; Tappel, 1972; Lucy, 1972). Vitamin E can react more rapidly with peroxy radicals several orders of magnitude faster than with acyl lipids, and thus prevents free radical-induced peroxidative tissue damage (Kamal-Eldin & Appelqvist; Lucy; Burton & Traber, 1920).

The aim of the present experimental study is to investigate the effects of VPA usage in pregnancy on rat foetal thymus development ultrastructurally and antioxidant effects of folic acid (FA) and vitamin E (Vit-E).

MATERIAL AND METHOD

Animals. In the study, 24 Wistar-rats (weighing approximately 240–280g) obtained from Prof. Dr. Sabahattin Payzin Practice and Research Center of Medical Sciences in Dicle University, Diyarbakir, Turkey were used. The animals were housed in plastic cages, fed a standard laboratory diet and water *ad libitum*, and treated in accordance with the guidelines of the Animal Care Committee of the University of Dicle, Diyarbakir, Turkey. Rats were exposed to a 12 h light:12 h dark cycle, at a room temperature of 18–22° C.

Experimental procedure. Twenty-four Wistar rats were used in this study. They were randomly divided into four groups, each consisting of six rats (n=6): the control (n=6), VPA (n=6), VPA+FA (n=6), and VPA+Vit-E group (n=6). In the control group, normal saline 0.3 ml were injected subcutaneously into rats on the 8-10 days of gestation. In the VPA group, valproic acid (400 mg/kg/day) were given subcutaneously on the 8-10 days of gestation. In the VPA+FA group, folic acid (400 µg/kg/day) were given with the drinking water during the whole period of gestation, and valproic acid (400 mg/kg/day) were given subcutaneously on the 8-10 days of gestation. In the VPA+Vit E group, vitamin E (250 mg/kg/day) were given via orogastric tube on the 8th, 9th and 10th days of gestation, and one hour later valproic acid (400 mg/kg/day) were given subcutaneously on the 8th 9th and 10th days of gestation. All these agents which were subcutaneously injected were given in volume of 1 ml.

Tissue preparation for electron microscope. On the 20th day of gestation, all pregnant rats were sacrificed and the fetuses were removed. Thymus were removed from fetuses. For electron microscopic examination, the pieces of thymic tissues were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, and postfixed in 1% phosphate-buffered osmium tetroxide and then dehydrated in graded alcohol series, and subsequently embedded in araldite CY212. Sections were cut with ultratome. Thin sections were stained with uranyl acetate and lead citrate, and examined using a Carl Zeiss EM 900 transmission electron microscope.

RESULTS

The ultrastructural examination of thymic tissue was carried out by electron microscope (Jeol TEM 1010). In the Control group sections, thymocytes and reticular fibers were observed in their normal appearance. Nucleus was prominent, and small vacuoles were observed. Desmosomal complex were seen in normal appearance (Fig.1).

In the VPA group, electron microscopic observations have shown that the damage produced by VPA were in all tissue compartments. A great deal of swollen mitochondria were observed. The vacuoles that appeared in the cytoplasm of the cells had varying sizes. In thymus parenchyma, mast cells were observed. There is no change in the structure of Mast cells containing granules which were in normal size. Fat droplets were also observed in the cytoplasm. Condensation of chromatin in nuclei was evident. Widened perinuclear cisterna was observed. On the other hand, in some thymocytes, degeneration was seen (Figs. 2, 3).

Fig. 1. Electron micrograph of control group: Normal appearance of thymic parenchyma. 1.Small thymic lymphocytes, 2. Medium-sized thymic lymphocytes, 3. Nucleus of epithelial reticular cell. (Uranyl acetate-lead citrate X3000).

Fig. 2. Electron micrograph of Valproic acid group. Varying size of vacuoles(arrows). (Uranyl acetate-lead citrate X3000).

Fig. 3. Electron micrograph of Valproic acid group. Chromatin condensation(arrow head) and vacuolization(arrows). (Uranyl acetate-lead citrate X3000).

In VPA+FA group, chromatin distribution of nuclei was better than VPA group. Vacuoles, mitochondrial cristallysis and swelling were decreased in the cytoplasm of thymocytes (Fig.4).

In VPA+Vit E group, nuclear chromatin and perinuclear cisterna had normal appearance. Lipid storage and a small amount of vacuolization were observed. Mitochondrial cristallysis decreased (Fig. 5).

DISCUSSION

The thymic stromal compartment consists of several cell types that collectively enable the attraction, survival, expansion, migration, and differentiation of T-cell precursors. The thymic epithelial cells constitute the most abundant cell type of the thymic microenvironment and can be differentiated into morphologically, phenotypically, and functionally separate subpopulations of the postnatal thymus (Holländer *et al.*, 2006). Thymus is divided into two distinct compartments; the outer cortex and the inner medulla. Both regions are densely populated with lymphocytes (or thymocytes while in the thymus). Most of the cortical lymphocytes are immature and unable to carry out immune functions.

Valproic acid formulations are FDA pregnancy category D drugs due to teratogenic effects seen in studies in experimental animals and human case reports. There is reference to the death of a newborn following therapeutic use of valproic acid in the mother during pregnancy, although no specific information is provided (Depakote Product Label Drugs FDA, 2009). In a study carried out by Arudchelvan *et al.* (2005), it was reported that, in thymic tissue samples, thymocyte had a marked nuclei, and there were vacuoles in cytoplasm. In our study, in addition to the points mentioned above, we observed that desmosomal structures were normal. In the same study, they also reported that thymus epithelial cells which were exposed to radiated proliferated 3rd day of recovery period and volume of vacuol within their cytoplasm. Graf *et al.* reported the effects

Fig. 4. Electron micrograph of Valproic acid + Folic acid group. The VPA damage was improved by folic acid. (Uranyl acetate-lead citrate X3000).

Fig. 5. Electron micrograph of Valproic acid + Vit E group. The recovery of thymus with Vit E. 1.Small thymic lymphocytes, 2. Medium-sized thymic lymphocytes, 3.Nucleus of epithelial reticular cell. (Uranyl acetate-lead citrate X3000).

of the steady-state valproic acid concentrations on the mouse thymus. Those showed that cortical and medullary morphology varied considerably in the thymus. In extreme cases, the cortical zone was either reduced in size or the medulla showed a cortex-like structure or vice versa (inverted type of thymus). The thymic cortical reticular cells showed increased aminopeptidase M and alkaline phosphatase reaction (Graf *et al.*). In our findings, in the group valproic acid, we observed that damage occurred in most thymic compartments, there was an increase in the number of vacuole and swelling of mitochondria. In addition, there were fat droplets in some places in cytoplasm, the nuclei was markedly evident, chromatin had a condensated appearance and there were expansions in perinuclear cisternae. However, mast cells had their normal histological structure. Folic acid (the anion form) are forms of a water-soluble B

vitamin. Folic acid is necessary for cell development, for the metabolism of specific biochemical reactions in the body, such as the conversion of homocysteine to methionine; and for the metabolism of specific anticonvulsant drugs (Berg, 1999). Therefore, valproic acid use causes folic acid deficiency. Thus it causes neural tube defects, thymic atrophy, loss of thymic cellularity (Carl; Weber & Dib; Lewis *et al.*). The production of thymic hormones and differentiation of T lymphocytes are reduced. In the present study, chromatin distribution of nuclei was better, and cytoplasmic vacuoles and mitochondrial swelling was reduced in the VPA+FA group. We thought that folic acid decreased the effects of maternal valproic acid on the thymus.

Vitamin E is a fat-soluble vitamin that exists in eight different forms. Each form has its own biological activity, which is the measure of potency or functional use in the body (Traber & Packer, 1995). Alpha-tocopherol (a-tocopherol) is the name of the most active form of vitamin E in humans. It is also a powerful biological antioxidant. Antioxidants such as vitamin E act to protect cells against the effects of free radicals, which are potentially damaging by-products of energy metabolism. Free radicals can damage cells and may contribute to the development of cardiovascular disease and cancer. Studies are underway to determine whether vitamin E, through its ability to limit production of free radicals, might help prevent or delay the development of those chronic diseases. Vitamin E has also been shown to play a role in immune function, in DNA repair, and other metabolic processes. Vitamin E supplementation has various beneficial effects on the immune system. It plays an important role in the differentiation of immature T cells and increases the positive selection by thymic epithelial cells in thymus (Traber, 1999; Farrel & Roberts, 1994). In our study, the decreased cellular immunity with VPA is markedly improved by the intake of vitamin E, and vitamin E also induces the recovery of thymic atrophy. In conclusion, we are of the opinion that valproic acid should not be advised during pregnancy since its use has risks. If its use is a must, it should be given together with folic acid or vitamin E, since those two drugs decrease the harmful effects of VPA on the thymus tissue.

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RESUMEN: Se realizó este estudio para evaluar las diferencias histopatológicas en el timo de fetos de ratas Wistar Albinas expuestas prenatalmente a ácido valproico (VPA), ácido fólico (AF) y vitamina E (Vit-E). VPA (400 mg/kg), FA (400 mcg/kg) y vitamina E (250mg/kg) administradas a ratas en los días 8, 9 y 10 de gestación. Los fetos (n=24) fueron divididos en cuatro grupos: control, APV, APV + vitamina E y VPA + FA. En el día 20 de gestación, todas las ratas preñadas fueron sacrificadas y los fetos fueron extraídos. Se obtuvieron secciones delgadas del timo de los fetos y se tiñeron con citrato de uranilo - acetato de plomo, siendo examinados al microscopio electrónico de transmisión. Los hallazgos histopatológicos del grupo control fueron normales. En el grupo VPA, se observaron cambios degenerativos en todos los compartimentos de tejido en comparación con los controles. En el grupo VPA+FA, las vacuolas, cristalisis mitocondrial e inflamación se redujeron en el citoplasma. En grupo VPA + Vitamina E, se observó el almacenamiento de lípidos y vacuolización. La cristalisis mitocondrial disminuyó. El estudio permitió analizar los cambios histopatológicos que pueden ocurrir en un modelo experimental de alto riesgo después de la administración de VPA, además, las funciones de protección por la administración de AF y vitamina E.

PALABRAS CLAVE: Ácido valproico; Tímo fetal; Vitamina E; Ácido fólico.

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