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The role of neuronal apoptosis in Valproic Acid brain-related teratogenesis: a histochemical and immunohistochemical study in BALB/c mice

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Abstract

Objectives: The purpose of this study was to examine the teratogenic effects of Valproic Acid (VPA) and to investigate the role of apoptosis in neural tissue development. Although an apoptotic activity due to VPA has been reported, a direct connection of VPA-induced apoptosis with embryonic brain and/or spine malformations and teratogenesis has not yet been established. *Materials and Methods*: VPA was administered to BALB/c mice, from the 7th to the 10th gestational days. Macroscopical congenital anomalies were registered under a stereomicroscope and were further histologically studied. Immunohistochemistry was performed with terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) staining. *Results*: Birth defects were described and an increase of the apoptotic activity in the brain was immunohistochemically identified. *Conclusions*: Considering the increased and very intense TUNEL expression of the neural cells of treated animals' fetuses, it is suggested that VPA triggers a pathological increase of apoptosis resulting in an imbalance between cell proliferation and cell death, the final result of which is malformation.

Keywords: Valproic Acid, teratogenesis, apoptosis, TUNEL staining, immunohistochemistry, fetal brain.

Introduction

Valproic Acid (VPA) is widely used as a first-line antiepileptic drug, but it can also be used to treat migraine headaches and bipolar disorder [1]. Despite its efficacy, an elevated incidence of major congenital malformations, associated with its use, has been a consistent finding in studies of patient registries and in several large case series [2] and it is now considered to be a dose-related teratogen [3]. According to extended studies, maternal exposure to VPA during the first trimester of pregnancy significantly increases the risk of major fetal malformations including neural tube defects, such as spina bifida [4, 5]. It is recognized that the risk of such defects in the developing embryo may reach 1–2% of pregnancies [6]. However, these findings have often been limited by a relatively small population size of exposed women and lack of retrospective study design [7]. In an effort to circumvent these pitfalls animal studies are often performed.

VPA affects cell growth, differentiation, and apoptosis of cancer cells *in vivo* and *in vitro* [8]. Its property of inhibiting histone deacetylase places it to an emerging new class of antitumoral agents that can deactivate gene expression, inhibit growth of tumor cells, and induce apoptosis in a wide range of tumor-derived cell lines [9]. VPA has been reported to inhibit the growth of prostate cancer cells [10, 11]. It also increases apoptosis in human melanoma cells [12, 13] and is considered a promising agent for targeted therapy [14]. It can induce apoptosis and differentiation in acute myeloid leukemia [15, 16] and in medulloblastomas [17]. It is documented that it induces apoptosis, and it inhibits growth of gastric cancer cells [18], of colon adenocarcinoma and hepatocellular carcinoma [9]. All the recent research on VPA suggests a potential use in therapeutic protocols and novel cancer treatments, partly based on its ability to induce apoptosis [9, 19]. It has recently been suggested that teratogenicity after VPA administration could also be attributed to apoptosis [19]. VPA has induced cell apoptosis in brain microglia of BALB/c mice [20] and apoptotic neuro-degeneration in regions of the brain, such as hippocampus, thalamus, frontal and parietal cortex of neonatal rats [21, 22]. VPA treatment enhanced apoptotic cell death in the cerebellum and hippocampus of treated mice [23]. On the other hand, there are recent studies that report attenuation of apoptosis [24] or reduction of the rate of neuronal apoptosis with VPA [25] and reduced death of neural progenitor cells by VPA at developmentally critical periods [26]. The fact is that the exact action mechanisms of VPA-induced teratogenicity are still unclear [19, 27].

Aim

Considering the above, the aim of this study was to evaluate the macroscopic and microscopic defects in the central nervous system (CNS) of BALB/c mouse fetuses, after VPA administration and to investigate apoptotic activity, if any, as a possible mechanism of teratogenesis.

Materials and Methods

Experimental animals

Twenty BALB/c mice, 12 weeks old, were used for this experimental study. The animals were fed *ad libitum* under a controlled 12-hours light:12-hours dark cycle. Virgin female mice weighing 30–35 g were mated overnight. The first 24 hours after conception were considered gestational day (GD) 0. Pregnant animals were randomly divided into four groups, containing five mice each. Each pregnant mouse was housed individually.

Drug and doses

Pregnant mice were injected intraperitoneally 500 mg/kg VPA/day on GD 7 and 8 (group A) or on GD 8 and 9 (group B) or on GD 7, 8 and 9 (group C). The time-period between GD 7 and 10 is considered very important because it is the time organogenesis commences and specific areas of the CNS begin to form with neurogenesis and migration processes [28]. Any alterations in these processes can result in severe congenital abnormalities. This is also the time that anterior and posterior neural tube closure occurs in the mouse model [29]. The 7^{th} to 9^{th} days of gestation in the mouse embryo correspond to the 3^{rd} to 4^{th} weeks of human gestation [30]. Group D formed the control group, in which animals were injected with saline on GD 8 and 9. VPA (Sodium Valproate) was obtained from Sanofi Winthrop Industrie (France).

Early in the morning of GD 18, a day that the external brain morphology has been completed, pregnant mice were weighed and then euthanized with a lethal dose of CO_2. The uteri were removed, and the implantation sites were identified. The fetuses were collected, and the malformations were observed with a stereomicroscope and were recorded.

The protocol of this study was approved by the Local Ethics Committee for Animal Research and the experiments complied with current laws of the country.

Stereomicroscope

All the collected material was carefully examined with a stereomicroscope so the details could be observed in a three-dimensional way. Macroscopic anomalies were recorded, and photographs were taken for documentation.

Light microscopy

The fetal heads of the collected material were fixed in 10% neutral buffered formalin, dehydrated, and then embedded in paraffin wax. Using a microtome, a series of frontal or sagittal sections of 7 μm thickness were cut. The first of each eight serial sections was histochemically stained with Heidenhain's Azocarmine–Aniline Blue (AZAN). This trichrome staining renders the condensation of the nucleus (deep red color) and the shrinkage of the cytoplasm better recognizable, in comparison to other histochemical stainings and, thus, is suitable for the detection of cellular apoptosis [31].

Sections chosen to be immunohistochemically stained were outlined with DAKO pen that formed a waterproof ring around them allowing the confinement of the reagents. The procedure was performed at room temperature. Deoxyribonucleic acid (DNA) fragmentation was detected by the modified terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) assay [32]. The method is based on the specific binding of TdT to 3'-OH ends of DNA. The kit used was provided by Promega Corporation.

The sections were counterstained with Hematoxylin for one minute, mounted with DPX Mountant and viewed with a light microscope.

In an attempt to compare the results from all the groups, TUNEL-positive cells were counted. Tissue slides from two fetuses were randomly chosen from each group. From each fetus, seven areas coming from different depths were selected for the measurements. From a population of 100 cells in each area, the cells with a positive staining (both mild and intense) and the unreactive cells were counted.

Statistical analysis

Standard statistical methods were used for descriptive statistics. *Chi*-squared test was used for the statistical analysis. The null hypothesis was discarded when the probability (p) of a type I error was <5%. Analyses were performed using the IBM Statistical Package for the Social Sciences (SPSS) Statistics for Windows, version 23.0 (IBM Corp., Armonk, NY, USA).

Results

Gross anomalies

All the fetuses were collected and counted. Fetuses from the control group appeared as normal. Some of the fetuses from the treated groups were malformed to such a large extent that they were visually unreminiscent of a normal fetus. They were counted separately and are referred to as "malformed". The data are given in Table 1.

Table 1 – ***VPA administration in different GD and the registration of fetuses: experimental groups***

Groups	VPA administration	Weight GD 18 [g]	Fetuses	Additional "malformed" fetuses
A	GD 7 and 8	26.7±3.8	11	7
B	GD 8 and 9	25.5±3.6	9	6
C	GD 7, 8 and 9	25.7±2.2	16	9
D	Control	28.1±1.9	31	0
Total			67	22

GD: Gestational days; VPA: Valproic Acid.

Encephaloceles, anencephaly and meroanencephaly, myeloceles, spina bifida, cranioschisis, microcephaly, exophthalmos, dilation of the ventricular system and narrowness of the skull were some of the most common congenital anomalies observed in the fetuses that were treated with VPA, as demonstrated in Table 2. In Figure 1, a fetus from group C is shown, presenting meroanencephaly, exophthalmos and external ear defects (Figure 1).

Histochemical study

The histochemical study of the control group fetuses revealed no visible malformations. Most of their nuclear population had no obvious abnormalities. Namely, apart from the pyknotic, hyperchromatic nuclei, there were no other evident morphological changes on the light microscope, with Heidenhain's AZAN staining.

Table 2 – ***The most common congenital anomalies documented in the fetuses of the experimental groups***

Groups / Congenital anomalies	A (*n*=11)	B (*n*=9)	C (*n*=16)	D (*n*=31)
Encephaloceles	5	3	7	0
Myelomeningoceles	2	0	5	0
Anencephaly	1	1	3	0
Meroanencephaly	2	1	3	0
Cranioschisis	3	4	6	0
Microcephaly	3	2	5	0
Scaphocephaly	0	2	3	0
Exophthalmos	6	2	8	0
Spina bifida	4	5	9	0
Dilation of the ventricular system	7	6	11	2

n: No. of fetuses.

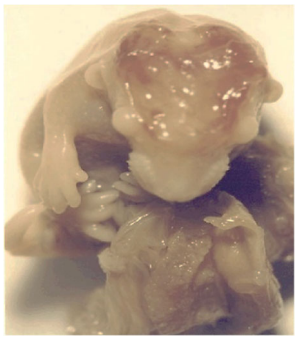

Figure 1 – ***Mouse fetus from group C with cranioschisis and meroanencephaly. A bilateral exophthalmos is also observed.***

Many fetuses from the treated groups exhibited neural tube defects. Encephaloceles were noted, usually located on the back side of the skull, in the occipital region, sometimes very extensive (Figure 2). In a few cases, the encephaloceles were extended towards the forehead and nose. Myelomeningoceles were also noticed in several cases. In a few animals, a major portion of the brain or skull was absent. In addition to anencephaly, many fetuses, mainly from group C, also appeared to have cranioschisis. The open skull was observed mostly at the occipital region. The sites with defective skull bone formation either appeared uncovered or displayed an amorphous tissue covering, resembling a meningeal or a thin skin cover.

Some of the fetuses exhibited an obvious microcephaly. Occasionally, their heads appeared longer and narrower, indicating scaphocephaly. A common finding was the presence of bilateral exophthalmos, sometimes very prominent.

One of the most common histological findings, using the Heidenhain's AZAN dye, was the dilation of the ventricular system of the fetal brain. The study of serial sections revealed that the dilation could be local or extended to the entire ventricular system, varying in degree, depending on the treatment. The dilation was mostly apparent in the parietal lobe but was, quite often, also distinct in the occipital lobe (Figure 3).

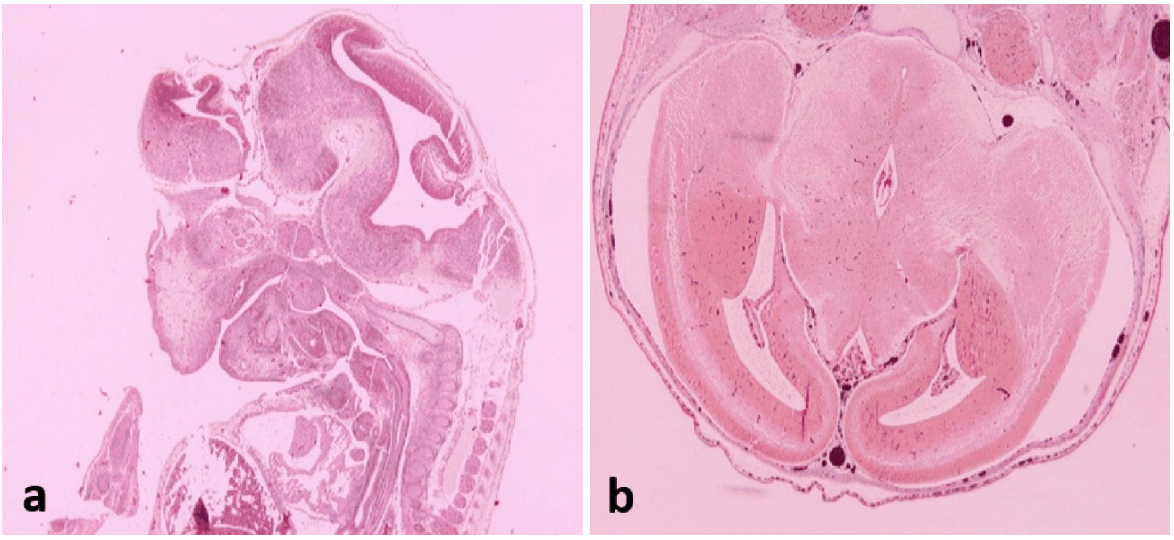

Figure 2 – ***Tissue sections with Heidenhain's AZAN staining (×200), mouse fetuses from group C: (a) Sagittal section that demonstrates an encephalocele anteriorly and a dilation of the ventricular system in the occipital lobe – spina bifida is also recognized; (b) Sagittal section from the control group.***

In the affected fetuses, neural cells seemed to be loosely arranged. In some areas, the cells formed loose aggregates of variable size. The cells in some aggregates and in fewer areas appeared to be more closely arranged. Near the ventricular lumen the aggregates seemed to be larger. A great number of especially condensed-pyknotic were detected which had an intense, uniform eosinophilic color. A high number of them exhibited evident and easily distinguishable morphological deviations, such as a particularly high density and an irregular shape with an irregular outline of the nucleus. These nuclei were usually easily distinguished from the rest of the nuclear population which exhibited a less intense eosinophilic color and had no obvious abnormalities.

Immunohistochemical study – TUNEL assay

Most of the neural cells from the control group exhibited a mild reaction to the antibody. Different areas of the control group exhibited a great variation in numbers of positive TUNEL cells, a variation which seemed to be greater than in the other groups. Most of the apoptotic cells were located near the ventricular lumen, in the midbrain, and their nuclei exhibited no or only minor obvious on the microscope morphological abnormalities, which were usually not easily recognized in the Heidenhain's AZAN staining group (Figure 4a).

The expression of the antibody was clearly higher in the VPA-treated groups (Figure 4, b–d). Most of the

positive cells were large and irregular in shape and, amongst them, many appeared to be vacuolated. Most of the nuclei were pyknotic and sometimes gigantic. The positively stained cells often formed loose aggregates of variable sizes (Figure 4, c and d).

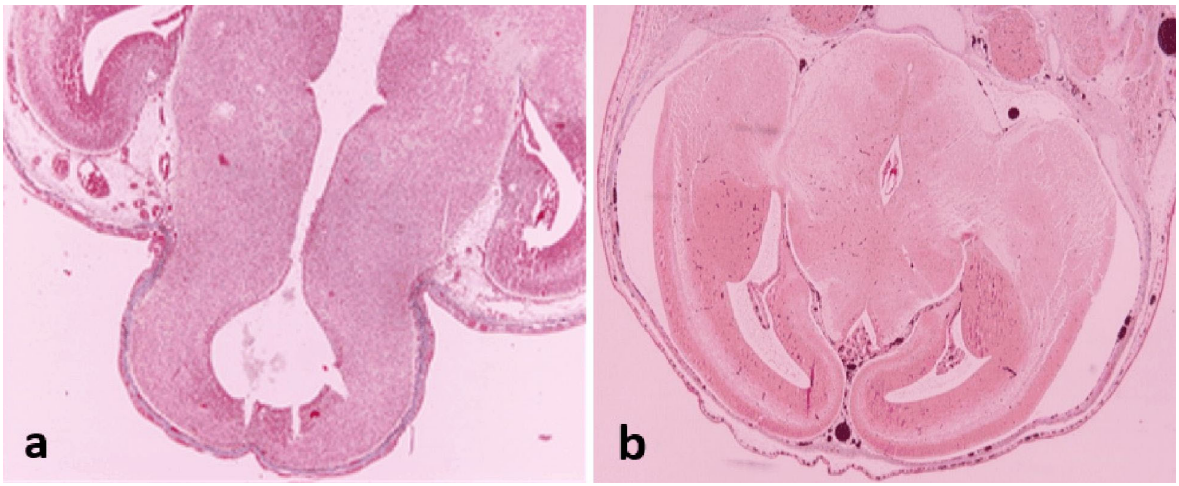

Figure 3 – ***Tissue sections with Heidenhain's AZAN staining (×200), mouse fetuses from group C: (a) Frontal section with a marked ventricular dilation in the occipital lobe – the protrusion in the developing cranium corresponds to the affected area; (b) Frontal section, corresponding area, from the control group.***

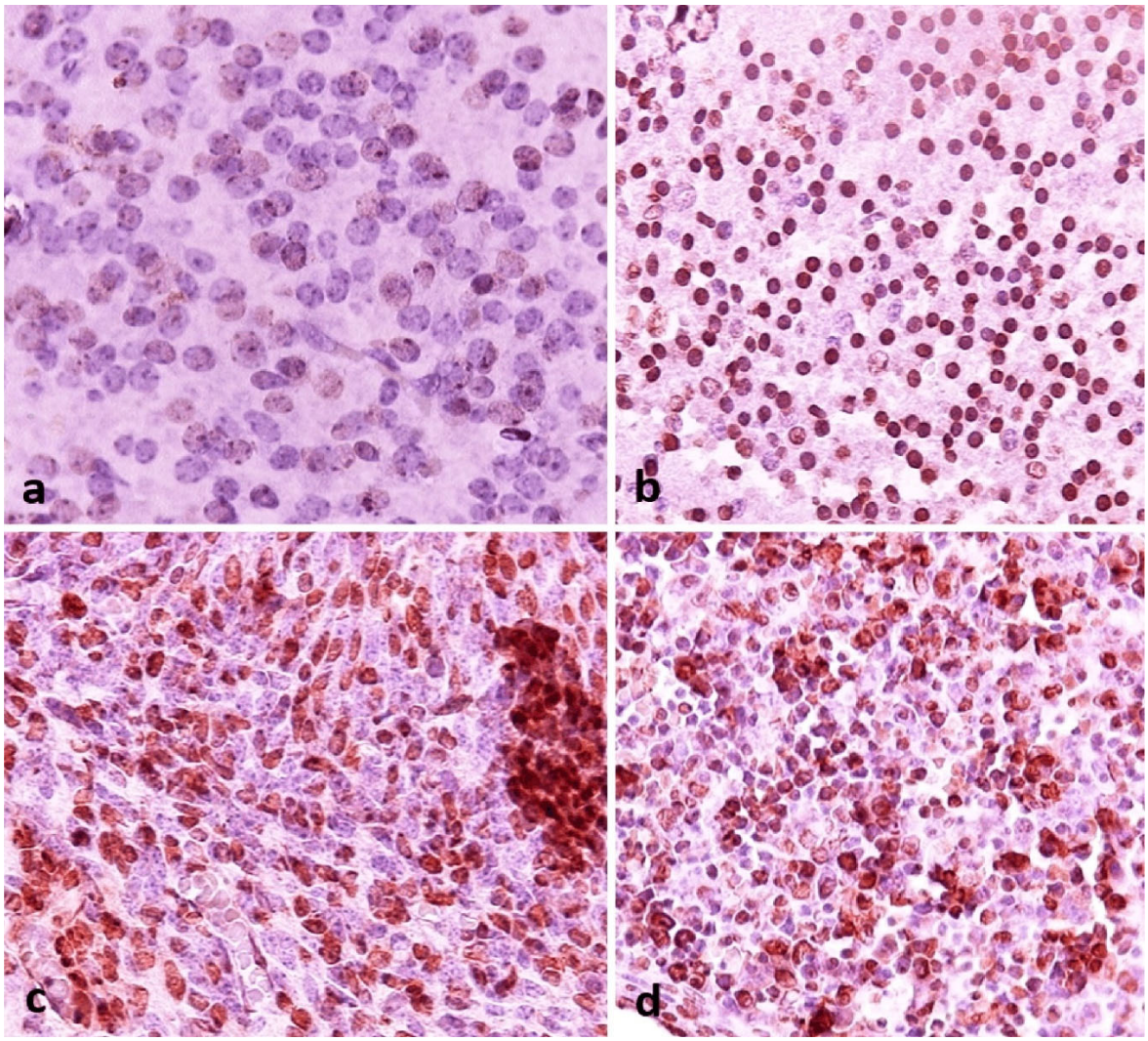

Figure 4 – ***Light microscopy photos of TUNEL-stained histological brain sections (×400): (a) Control group fetus – rare positively stained nuclei; (b) Group A fetus – small, positively stained nuclei; (c) Group C fetus – many dark, larger, positively stained nuclei, presence of an aggregate of apoptotic cells; (d) Group C fetus – many larger, positively stained nuclei, with irregular shapes; small, loose aggregates of apoptotic cells. dUTP: Deoxyuridine triphosphate; TdT: Terminal deoxynucleotidyl transferase; TUNEL: TdT-mediated dUTP nick-end labeling.***

TUNEL-positive cells were counted. Tissue slides from two fetuses were randomly chosen from each group. From each fetus, seven areas coming from different depths were selected for the measurements. From a population of 100 cells in each area, the cells with a positive staining (both mild and intense) and the unreactive cells were counted. The total number of apoptotic cells in groups A, B and C were 62%, 67% and 72%, respectively ($p<0.05$). The higher numbers of positively stained apoptotic cells were measured near the ventricular system and this was a constant finding in all fetuses of treated groups.

In the control group, the percentage of apoptotic cells was 49% and differed significantly from the VPA groups ($p<0.001$). Only a very small number from the total population of apoptotic cells had a high expression of the antibody with mild morphological alterations. The measurements with the higher numbers were usually in the midbrain.

Moreover, further grading was performed separating the morphological features of the apoptotic cells in intense and mild. In the control group, intense apoptotic morphological alterations were observed in 2.4% of cells, whereas the corresponding percentages in the VPA groups were 58.7% in group A, 60.2% in group B, and 68.2 in group C ($p<0.001$).

Discussions

VPA and teratogenicity

VPA is one of the four most widely prescribed anti-epileptic drugs and is effective and regularly approved in migraines, bipolar disorders and recently in cancer patients for clinical trials [33]. Many national institutes of health classify VPA as one of the drugs contraindicated during pregnancy, because it may cause birth defects, but there are undoubtedly benefits from its use [2]. Its teratogenic potential has been demonstrated in both experimental models and human clinical studies. Its use during the first trimester of pregnancy is known to have a higher risk of developing spina bifida, cleft palate and craniosynostosis. It may also be associated with neuro-developmental delay and autistic spectrum disorders in children of pregnant women exposed to the drug [34]. Recently, it was suggested that a patient's predisposition to VPA toxicity may be influenced by environmental and genetic factors [2], the latter being both maternal and fetal [1].

VPA may elicit teratogenicity in various ways, namely by promoting folic acid deficiency, inducing oxidative stress, leading to the ω- and β-oxidation, inhibiting histone deacetylase, counteracting angiogenesis, and damaging DNA [27]. There is also a report suggesting that maternal exposure to VPA in mice can cause epigenetic alterations in the embryo and this contributes to its mechanism of teratogenesis [35]. The fact is that the exact mechanisms leading to VPA-induced teratogenicity are still unclear [19, 27].

This study demonstrates the teratogenic effect of VPA and describes newly recorded malformations, such as encephaloceles, myelomeningoceles, anencephaly, cranioschisis, microcephaly, exophthalmos, dilation of the ventricular system and possible scaphocephaly in mice fetuses.

Apoptosis, natural and drug-induced

Studies performed on a variety of drugs, including antiepileptic drugs and in particular VPA, strongly indicate that they trigger neuronal apoptosis throughout the developing brain when used by pregnant women. A reduction of brain volume and a permanent deletion of neurons are some of the features observed when studying the effect of such drugs, but there can be several different patterns of degeneration [30].

For the normal growth and development of all embryonic and fetal organs and systems, a balance between cell production, differentiation and apoptosis is essential. Apoptosis is a natural phenomenon during development, an active process under genetic control that is crucial for embryogenesis and tissue homeostasis. It is well regulated, and its main role is to remove abnormal, misplaced, nonfunctional or harmful cells from the tissue without damaging it. Apoptosis also eliminates unwanted structures, controls cell numbers, and contributes to the formation of vesicles and tubes [36]. A high percentage of neural precursors normally dies during development and most of differentiated neurons becomes integrated during the period of synaptogenesis. The developing neurons, which fail to make appropriate synaptic connections and to integrate with the growing network, eventually commits suicide through apoptosis [30]. It is documented that apoptosis within a specific subdomain of the anterior neural tube is required within a limited developmental time window to ensure proper forebrain development [37]. This indicates that the time during which apoptotic cell death occurs, is also very important for normal development.

Apoptosis also includes pathological cell death due to internal and external factors. The questions under investigation are whether the drug administered to pregnant females causes an increase of the normal apoptosis, whether this additional apoptosis can overall cause a pathologically increased rate of cell death and if this situation can cause or can explain the teratogenicity of the drug.

Apoptosis and teratogenicity

The immunohistochemical assay revealed that the injection of VPA in pregnant mice increases the TUNEL-positive cells in fetuses, compared to the control group. Moreover, the highest number of apoptotic cells was observed in group C, where three doses were administered. The findings of our study are consistent with the recent studies supporting that VPA administration leads to an increase of apoptosis in the developing brain, which seems to be dose-dependent [30]. Initiation of VPA administration on the 8th day seems to increase apoptosis compared to the 7th day of gestation. Furthermore, the increase in VPA doses from two to three in group C is related to both an increase in the total number of apoptotic cells, and to more intense morphological alterations of the apoptotic cells.

Different areas of the control group exhibited a greater variation in numbers of positive TUNEL cells, compared

to the other groups. This suggests that the arrangement of apoptotic cells in the brain tissue is not uniform and the number of apoptotic cells varies considerably depending on the area. It seems like most apoptotic cells are located in the midbrain, so this is the area where cell death normally mostly occurs. In the affected fetuses, the number of TUNEL-positive cells was more consistent and higher. The increase of the total number of apoptotic cells also created an imbalance between apoptotic and non-apoptotic cells.

One of the most characteristic findings was the marked intensity of the TUNEL positivity of the cells in the affected fetal brain. Most of these cells also had very obvious apoptotic morphological characteristics, easily recognizable histochemically, probably suggesting that cell death was about to occur very soon. On the other hand, the positivity of the cells in the control group was usually very mild without any evident morphological apoptotic features. This could mean that apoptotic death will need here a longer time to be completed. Considering the above, it could be speculated that VPA not only creates an imbalance in the number of apoptotic cells but also accelerates the actual time of death of apoptotic cells, aggravating an already impaired situation.

Conclusions

The neural cells of a developing brain are regulated by balanced biological mechanisms that promote both cell survival and apoptosis. VPA upsets this balance by increasing cell apoptosis to a much higher pathological rate. This is accomplished either by increasing the number of cells that are programmed to die or by reaching them prematurely to the beginning steps of apoptosis or even maybe by reducing the time required for the process of apoptotic death to occur. When apoptosis occurs massively and briefly a major part of the cell population, which in this proliferative stage consists of progenitor cells, will vanish in short time. Obviously, the massive reduction of the cell number, which is necessary to create the mature neural cells and glia, cannot be compensated. Therefore, structural and functional organization of the brain tissue is seriously disrupted affecting the final morphology of the structures and resulting in teratogenesis.

Conflict of interests

The authors declare to have no conflict of interests.

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