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Neurotoxicity, Hepatotoxicity and Behavioral Effects of Valproic Acid in Autism-like Rats

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Abstract

AUTISM is a neurodevelopmental disorder with serious psychological consequences manifested by social reciprocity and communication disorders. The study explored the influence of valproic acid (VAP) administration on behavioral and histobiochemical markers in autism-like rats. Male Wistar rats were distributed into two groups: The control (T) received saline solution (NaCl 9%), and the treated group (VAP): received valproic acid at 500 mg/kg. Behavioral impairment was measured by different paradigms to evaluate social interaction, memory, and anxiety. Then, the brain and liver were removed for biochemical and histological examinations. Prenatal exposure to VPA at day 12.5 causes long-term effects on behavior in rats, notably a significant reduction of social interactions and memory and an increase in anxiety. The oxidative markers (MDA and CAT) were altered, expressed by an increase of MDA levels in the prefrontal cortex and hippocampus. However, the enzymatic antioxidant activity of CAT in the same areas was depleted. Widespread abnormalities in the brain and liver structure were observed at viable cell levels (pyramidal neurons and Purkinje cells, hepatocytes). In conclusion, in utero VPA exposure causes abnormalities in the brain and liver structures (Purkinje cells and pyramidal neurons) that might be related to oxidative stress. Furthermore, understanding the altered brain architecture involved in neurogenesis and the neurotransmission and its related behavior induced by VPA exposure is needed.

Keywords: Valproic acid-induced autism, Oxidative stress, Behavioral impairment, Rats.

Introduction

Autism spectrum disorder (ASD) is characterized by sociability and alterations in communication, restricted and repetitive behavior patterns, and interests [1–3]. In most cases, ASD is diagnosed with no defined etiology.

It is a neurodevelopmental disorder with serious psychological consequences. Maternal or paternal behavior is not responsible for a child's autism; its origin is strongly linked to genetic factors as well as other environmental risk factors [4,5]. The etiology of autism currently remains extremely complex and poorly understood. In 5 to 10% of cases, there is a comorbidity associated with another disorder, like the syndromes of Fragile X or Down. This is referred to as syndromic autism. However, for the vast majority of cases, it is not possible to clearly identify the origins, and this is referred to as non-syndromic autism [6].

The involvement of purely genetic factors has been demonstrated by comparing the transmission rate of ASD in homozygous and dizygotic twins. The currently identified genes encode, among others, proteins directly and indirectly involved in synaptic transmission and neuronal development [4]. Although these genetic causes alone seem difficult to explain the occurrence of ASD in children, it would appear that it is due to a genetic predisposition associated with environmental factors affecting the mother during her pregnancy. These environmental factors include heavy metals, pesticides, and volatile organic compounds. Other factors, such as maternal stress and certain pharmacological substances, such as sodium valproate (VPA), prescribed during pregnancy, are also risk factors [5].

Autism is linked to abnormal brain development that leads to a mosaic of maladaptive behavioral manifestations. It is thought that either the entire brain development is disrupted from fetal life to

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adolescence or that only certain areas of the brain are affected. These disruptions will subsequently lead, depending on the case, to a cascading alteration in the development and function of other interconnected structures.

A change in brain growth has been reported in autistic patients, with a period of greater growth in patients between the ages of 2 and 4, followed by normal or reduced growth, leading, in adulthood, to a brain of normal or smaller volume [7]. Among the structures that show atrophy are, among others, the amygdala (related to anxiety) and the striatum (related to stereotypies) [8,9].

Post-mortem studies of the brains of autistic patients suggest that there is a dysregulation of cortical development leading, for example, to a reduction in the size and number of pyramidal neurons as well as irregular lamination. These observations may account for deficits in social interactions, problems in the genesis and integration of emotions, and abnormal sensory experience observed in patients with ASD [10]. Abnormal development of the cerebellum, often associated with a loss of Purkinje cells3, is also frequently described. Since the cerebellum is involved in motor learning and motor coordination, this may account for some of the motor deficits observed in some autistic patients. Furthermore, since the cerebellum is also involved in environmental perception, its dysfunction could be at the origin of certain autistic behaviors. Some authors also propose that the cerebellum could guide the maturation of certain brain structures and influence cognitive development [11]. From a functional perspective, imaging studies show both hyper-connected and hypo-connected brain areas [12].

All of this information from human studies is both complex and difficult to interpret. They were conducted on a limited number of individuals whose age is a criterion that must be taken into account. Therefore, in an attempt to better understand the neurodevelopmental abnormalities responsible for autistic behaviors, animal models have been developed that reproduce some of these abnormalities.

To understand the complexity of ASD, several animal models were investigated. Valproic acid (VPA) is an anti-epileptic drug with teratogenic effects. The administration of VPA to pregnant women increases the probability of developing a neurodevelopmental disorder such as ASD [13]. Some studies showed that intraperitoneal (500 mg/kg) and subcutaneous (400 mg/kg) VPA administration induced several behavioral modifications, including increased anxiety-like behavior, social interaction deficits, reductions in sensory processing and attention, [14–16][15][16]. VPA administration in female rats during the early stages of pregnancy manifests autism-like behaviors in the offspring marked by alterations in brain structures and biomarker levels similar to those of patients with ASD [17,18]. Some mechanisms have been proposed, including hepatotoxicity and neurotoxicity caused by VPA; most of them are related to oxidative stress which has been implicated in the development of clinical manifestation of autism [19–21] VPA is implicated in ROS production and enhances the formation of lipid peroxidation [22], induces DNA damage, and decreases the viability of cells in hippocampal neurons [23].

Although ASD has been associated with genetic and environmental etiological factors, it remains a clinical and broad-spectrum diagnosis. This study aims to investigate the VPA-induced oxidative stress levels in brain tissue, liver tissue, and behavioral abnormalities of Wistar rats prenatally exposed to valproic acid by measuring oxidative stress markers and behavioral and structural abnormalities in an autism-like rat model.

Material and Methods

Animals, diets, and drug treatment

Wistar rats (20 females and 10 males) were kept on a 12 h light/dark cycle at 18–22°C, with relative humidity at 50–60%, and received a standard diet and water. Female rats aged 12 weeks old (250–300 g) were mated overnight. The first gestation day (GD1) is estimated by spermatozoa detected. Randomly, female rats were divided into two groups: the control and valproic acid (VAP) group. However, the females of the VPA rats were intraperitoneally injected with 500 mg/kg VPA (in saline pH 8.3, 250 mg/ml), and control rats received saline on GD 12.5. The female rats were housed individually, and their pups were weaned on postnatal day (PND21) and separated by sex. Altered social interaction and behavioral deficits were only observed in all male pups after VPA exposure; then only males were used in biochemical and behavioral tests in the current study from PND 43 to 50. The Ibn Tofail University Kenitra, Morocco, doctoral studies center monitored and approved the procedures.

Tissue isolation and homogenate preparation

The rats were extremely anesthetized with hydrate of chloral (100 mg/kg), and their brains were obtained using bone forceps. However, the prefrontal cortex, hippocampi, and cerebellum were isolated and homogenized in a cold lysis buffer using a Dounce homogenizer. Then centrifuged at 14,000 g for 15 min and stored at -80 °C. Protein levels were later analyzed using the Bradford reagent.

Biochemical assays

Lipid peroxidation assay

Malondialdehyde (MDA) is known as an oxidative stress marker. Lipid peroxide formation was assessed by assessing thiobarbituric-acid-reacting substances (TBARS) in tissues [24]. Samples were added to 1 ml of 10% trichloroacetic acid and 1 ml of 0.67% thiobarbituric acid and then heated in boiling water for 15 min, and butanol (2:1 v/v), and centrifugated at 800 g/5 min, the TBARS were measured at 535 nm [25,26].

Catalase

Using a UV/visible spectrophotometer, catalase (CAT) activity is measured at 240 nm, by determining the optical density of hydrogen peroxide (H_2O_2). For assessing enzymatic reaction, a volume of 20 µl of supernatant was mixed with to 780 ml of phosphate buffered saline (PBS) (0.1 M, pH 7.4) and 200 µl of H2O2 (0.5 M) [27].

Histological examination

Samples of liver and brain structures (hippocampus, cerebellum, and prefrontal cortex) were fixed in 10% buffered formalin and embedded in paraffin. 5 µm sections were stained using hematoxylin and eosin and observed under optical microscopy. The tissue integrity was evaluated by assessing degeneration, necrosis, apoptosis, and leukocyte infiltration [28].

Behavioral tests:

Open field:

A wooden apparatus (100 cm × 100cm) with 40 cm-high walls exposed to high illumination (100 watts, 2 m above the apparatus). The device contains 25 squares (20 cm × 20 cm), including nine in the center and sixteen squares in the periphery. During the first 10-min test, the rat was placed in the center of the device, and its behavior was videotaped. After each rat session, the apparatus was cleaned. The total number of squares visited was assessed [29,30].

Sociability test

The sociability test was performed at PND31. The apparatus is a box of acrylic plastic measuring 120 cm/40 cm/50 cm formed by three chambers; the central one is 60 cm in length, while each side is 30 cm [31]. The subject rat was allowed to explore the chambers freely. The test was conducted in an environment unfamiliar to the test rat, encompassing three communicating compartments [32]. The time spent in a compartment and the number of crosses between compartments were measured for 5 min. Time duration and the entry number into the empty cage and space with the stranger rat were assessed [33,34].

Novel Object Recognition (NOR) Task

Task was conducted in a 40 × 50 cm² open field, surrounded by 50 cm high walls, covered with a thin layer of black plastic. For 5 minutes, and in the absence of any objects, the animals were habituated to freely explore the open field. 24 hours later, NOR training was performed by placing individual rats for 5 minutes in the field containing two similar objects placed in two adjacent corners. Long-term retention was tested 24 hours after training [30].

The objects used had similar size and color but distinct shapes. The ratio between the number of explorations of object B and the sum of the numbers of explorations of objects A and B defined the recognition memory index.

Elevated Plus Maze

The EPM test measures the anxiety degree by assisting increased exploration with open arms, which indicates reduced anxiety-like behaviors. The EPM is a 70 cm high wooden plus-shaped device. It is formed by two opposite arms (50 cm × 10 cm) closed by 40 cm high side and end walls, with an open roof. To prevent falling, the other open arms are surrounded by a 0.5 cm high rim. The intersection of the four arms forms a central platform (10 cm × 10 cm). The device is exposed to a 100 W lamp. Each rat is placed facing the open arm, and its behaviors are recorded for 5 minutes. The ratio of time spent on the open arm to the total time and the ratio of the number of entries to the open arm to the total number of entries define the level of anxiety [29,35].

Statistical analysis

Parameters measured are expressed as the means ± SEM. Data analysis was performed using Student's t-test to assess differences between two groups and one-way ANOVA followed by Tukey's post hoc for multiple comparisons using GraphPad Pad Prism 9.0. The threshold statistical significance was set at $P < 0.05$.

Results

Biochemical assessment in brain tissue homogenates

The prenatal VPA exposure significantly changed the oxidative stress markers (catalase activity and MDA) in the prefrontal cortex ($p<0.001$), cerebellum ($p<0.001$), and hippocampal ($p<0.05$) homogenates compared to the control group (Fig. 1). Moreover, in utero exposure to VPA in rats causes a decrease in catalase activity successively by 1.23-fold in the prefrontal cortex ($p<0.01$), 1.55 in the hippocampus ($p<0.05$), and 3.74 in the cerebellum ($p<0.01$) (Figures A, B, C) compared to a control group. Compared to control results, the post hoc analysis showed that MDA levels after the prenatal VPA exposure increased by 1.42 in the prefrontal cortex ($p<0.001$), 1.38 in the hippocampus ($p<0.001$) and 1.42 in the cerebellum ($p<0.5$) (Fig. D, E, F). (Table 3).

Sociability novelty preference test and social novelty index

Impairment in social interaction is observed to be high in the VPA group (Fig. 2). The post-hoc analysis showed that the VPA group spent more time with the familiar rat chamber and less time with the novel rat chamber ($P<0.001$), indicating a social memory deficit.

Spontaneous locomotor activity seems to be affected within the group exposed to in utero VPA (Fig. 2). Which was significantly reduced compared with normal control. The prenatal VPA exposures caused anxiety-like behavior manifested by a significant decrease in the total number of crossed squares ($p<0.05$) and time spent in the central zone ($p<0.001$), unlike the control ones. The induced anxiety-like behavior was evident in the EPM test, where it was observed that a significant decrease in time spent in open arms was considered an anxiogenic indicator.

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Histopathological analysis

Brain areas

Normal controls have a normal histological architecture, unlike the rats with prenatal exposure (Fig. 3). The VPA group exhibited neurodegenerative alterations in H&E-stained sections of hippocampus areas CA1, CA3, and cerebellar cortex, indicating a significant decrease in viable cell number (Purkinje and pyramidal cells). The VPA group showed perineural spaces with degenerated neurons, whereas it didn't show any variation in dentate gyrus between the two studied groups ($p > 0.05$ VPA versus control).

Liver tissue

The control rats showed normal liver cells with normal central veins and intervening regular sinusoids and portal tracts instead of the VPA groups. The VPA group liver presented a lost architecture showing dysplastic cirrhotic nodules, an enlarged and congested central vein of the liver, and sinusoidal spaces that appear narrowed and filled with blood (Fig. 4).

Discussion

The developmental and cognitive effects of valproic acid on the brain are highly presented in several studies [36,37], in which many characteristics of behavioral alterations caused by VPA administration to rats, like repetitive-like activity and social interaction troubles, have been associated with autism. Following these studies, we investigated the histological alterations both in brain and liver tissues, the oxidative marker modification, and behavioral impairments in the VPA-induced autism-like model in male Wistar rats.

Results from our research showed that the VPA group presented inappropriate social behavior, decreased preference for social novelty, and lack of sociability to a strange rat evaluated in the sociability test. Both an over effect, like impairments of memory, were evaluated in the novel object recognition test, and an anxiogenic effect was noted too. indicating similarities between behavioral alterations in VPA rats and behavioral deficits in patients with autism [15,38].

However, widespread abnormalities in the brain structure and function are related to dysregulation in neurodevelopmental processes. These processes have been demonstrated to cause adverse effects in autistic patients and animal models of autism. Besides other experiments, behavioral alteration increase is accompanied by GABA-ergic signaling alterations attributed to GABA system dysregulation, and excitation-inhibition imbalance in the hippocampal and prefrontal function causes abnormal synaptic plasticity and neural network formation, which could disturb the social deficit, anxiety, learning, and memory processing in VPA-treated groups [39,40]. Recent studies also showed that there are profound modifications in the expression of GABA receptors in the amygdala of the VPA-induced rats. It suggests that targeting the GABAergic system may contribute to correcting the generational pathophysiology of ASD [41,42].

The rat's brain could be used as a good model for understanding the in vivo brain neurotoxicity mechanisms in different VPA-induced toxicity models, which have been considered as a free radical source either causing a decreasing antioxidant capacity of cells [43]. Simultaneously, our work confirmed the similarities of our results with the previous ones, notably, the variation in catalase activity and lipid peroxidation in different brain areas.

In histological brain structures, a diminution of pyramidal neurons and Purkinje cells was observed in various areas, unlike the normal histological architecture of control rats. In most cases, in utero VPA administration in rats causes degenerative changes in the cerebellum layers, in particular affected Purkinje cells, and impairs the mitochondrial morphology in Purkinje cell perikarya [44]. In addition, changes in the number of hippocampus neurons were also found, especially in CA1 and CA3, indicating toxic effects of VPA on neuronal development and inducing apoptotic neurodegeneration in the hippocampus and prefrontal cortex [39]. Furthermore, neurodegenerative abnormalities may arise when ROS is triggered and neuronal damage occurs [45]. Our study is consistent with the previous ones and showed that VPA causes oxidative stress, resulting in increases in the prefrontal cortex, hippocampal, and cerebellum MDA levels and decreases in catalase activity. These side effects of VPA administration on neuronal degeneration might be associated with the inhibition of oxidative phosphorylation, antioxidant enzymes, including catalase, an intracellular metabolic product, and an increase in free radical contents in the brain [44, 46]. Moreover, over sufficient quantities of ROS production could lead to oxidative stress, which is significantly related to neurodegeneration, including cognitive impairment [47]. The histological examination of the liver showed hepatotoxicity aspects with dysplastic cirrhotic nodules, and enlarged sinusoidal spaces filled with blood.

Hepatotoxicity related to AVP administration can lead to irreversible liver failure. Although its mechanism is poorly understood, oxidative stress is considered as a critical cause of hepatotoxicity [46].

In conclusion, our results demonstrate that the VPA-induced autism model in which many aspects of behavioral deficits found in ASD patients, like repetitive-like activity and social interaction problems, profound abnormalities in brain structures, and decreasing antioxidant capacity of the cell induced by oxidative stress markers. Furthermore, understanding the altered brain architecture involved in neurogenesis and the neurotransmission and its related behavior induced by VPA exposure is needed.

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Funding statement

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Declaration of Conflict of Interests

No conflict.

Ethical of approval

The Ibn Tofail University Kenitra, Morocco, doctoral studies center monitored and approved the procedures (ethics number 10/2022).

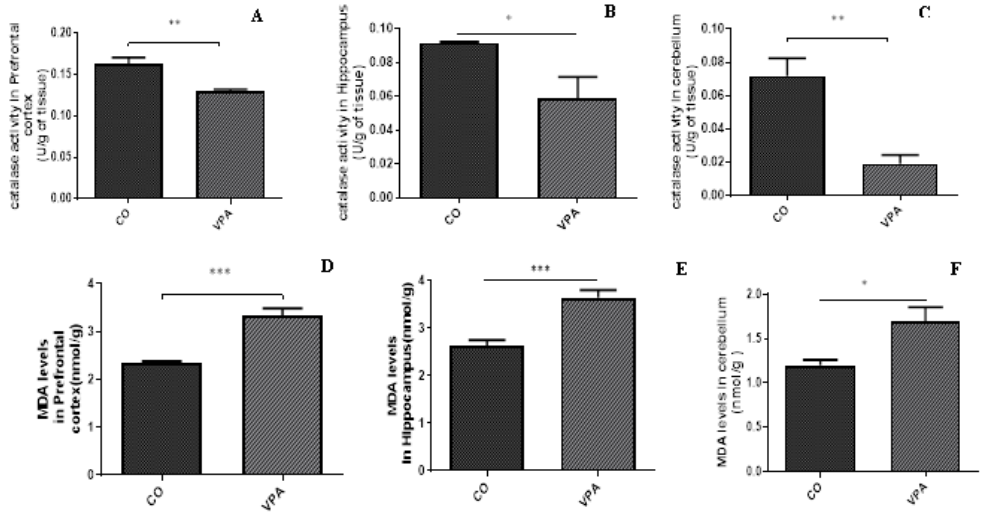

Fig. 1. Effect of prenatal exposure to valproic acid in male Wistar rats on catalase activity in different brain areas. The prefrontal cortex (A), hippocampus (B), the cerebellum (C), and on Oxidative damage in brain regions of VPA group. the prefrontal cortex (D), The hippocampus (E) and cerebellum (F). (Control, n=8). Results are represented as mean ± SEM and expressed in nmol/g of protein. The significance level is *0.05. ** p< 0.01, *** p < 0.001.

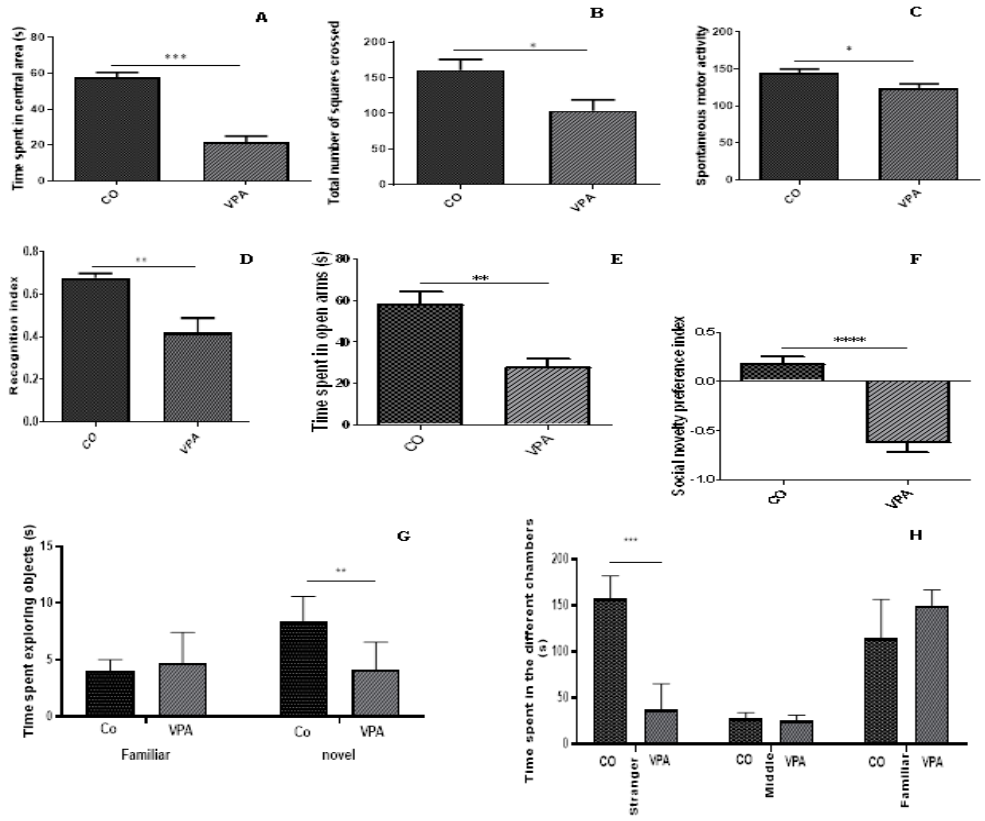

Fig. 2. Autism-related behaviors in valproic acid (VPA), controls. (Co) The time spent in the central area and, (B) Total number of squares crossed; (C) spontaneous motor activity in the open field test; (D) The object recognition index for long-term memory in the object recognition apparatus; (E) Time spent in the open arm in elevated plus maze; (F) Social novelty preference index; (H) Social preference between familiar rats and strangers. Results are represented as mean ± SEM, the significance level is 0.05. * p< 0.05, p<0.01, *** p < 0.001..**

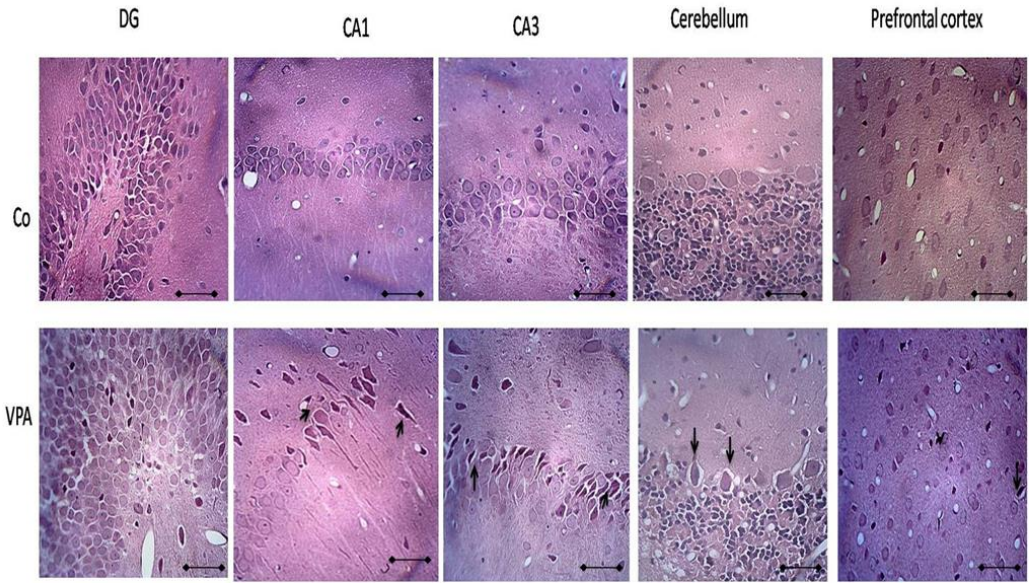

Fig. 3. Hematoxylin and Eosin-stained sections (X400, scale bar = 50 µm) of hippocampus, prefrontal cortex, and cerebellum in the VPA-treated group and the control one. CA: ammon's horn, GD: dentate gyrus, Co: control, VPA: valproic acid. Degenerated neurons.

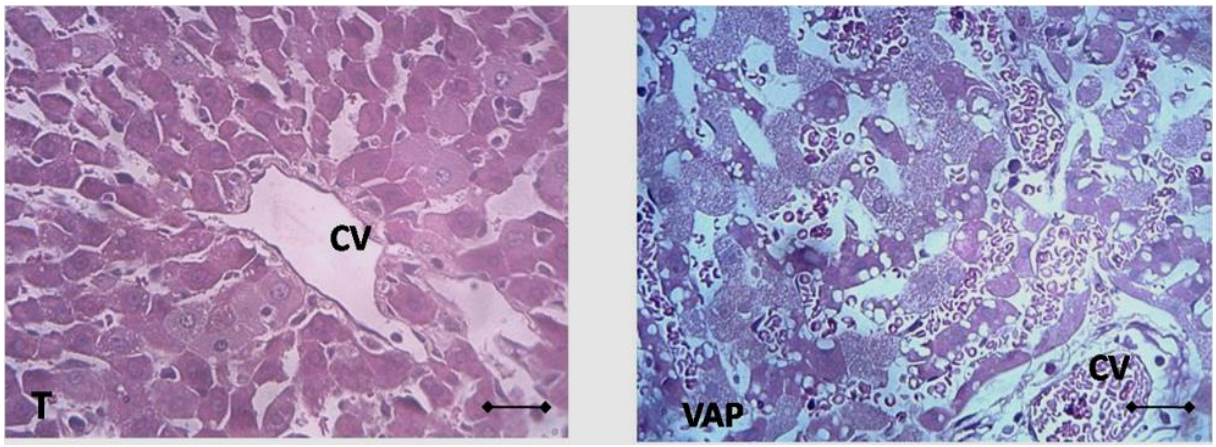

Fig. 4. Histopathological Examination of Rat Liver Tissues (X 400 scale bar=50 µm). (T) Control group; (VAP): valproic acid groups; CV: central vein.

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السمية العصبية والسمية الكبدية والتأثيرات السلوكية لحمض الفالبرويك في الجرذان المصابة بالتوحد

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الملخص

التوحد اضطرابٌ عصبيٌّ نمائيٌّ ذو عواقب نفسية خطيرة، يتجلى في التبادل الاجتماعي واضطرابات التواصل. استكشفت الدراسة تأثير إعطاء حمض الفالبرويك (VAP) على المؤشرات السلوكية والنسيجية والكيميائية الحيوية لدى فئرانٍ شبيهةٍ بالتوحد. قُسِّمت ذكور فئران ويستار إلى مجموعتين: المجموعة الضابطة (T): تلقت محلول ملحي IP (كلوريد الصوديوم 9%)، والمجموعة المُعالَجة (VAP): تلقت حمض الفالبرويك Ip بجرعة 500 ملغم/كغم. تم قياس الاختلال السلوكي باستخدام نماذج مختلفة لتقييم التفاعل الاجتماعي والذاكرة والقلق. بعد ذلك، أُزيل الدماغ والكبد لإجراء فحوصات بيوكيميائية ونسيجية. يُسبب التعرض لحمض الفالبرويك قبل الولادة في اليوم 12.5 آثارًا طويلة المدى على سلوك الفئران بعد الولادة، لا سيما انخفاضًا كبيرً في التفاعلات الاجتماعية والذاكرة وزيادة في القلق. تغيرت مؤشرات الأكسدة (MDA وCAT)، وتجلى ذلك في زيادة مستويات MDA في أنسجة دماغية مختلفة، بما في ذلك القشرة الجبهية الأمامية والحُصين. ومع ذلك، انخفض النشاط الأنزيمي المضاد للأكسدة لـ CAT في نفس المناطق. ولوحظت تشوهات واسعة النطاق في بنية الدماغ والكبد على مستويات الخلايا الحية (الخلايا العصبية الهرمية وخلايا بوركنجي وخلايا الكبد). وختامًا، يُسبب التعرض لـ VPA داخل الرحم تشوهات في بنية الدماغ والكبد (خلايا بوركنجي والخلايا العصبية الهرمية) قد تكون مرتبطة بالإجهاد التأكسدي. علاوة على ذلك، هناك حاجة إلى فهم بنية الدماغ المتغيرة المشاركة في تكوين الخلايا العصبية والانتقال العصبي وسلوكه المرتبط به الناتج عن التعرض لـ VPA.

الكلمات الدالة: التوحد الناجم عن حمض الفالبرويك، الإجهاد التأكسدي، ضعف السلوك، الجرذان.