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VPA-INDUCED NEUROTOXICITY, HEPATOTOXICITY AND BEHAVIORAL ABNORMALITIES IN WISTAR VPA- RAT MODEL OF AUTISM

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Abstract

Autism is a part of the group of pervasive developmental disorders (PDDs) characterized by disorders of social reciprocity; language and communication disorders with a restricted and stereotyped repertoire of interests and activities (ICD-10).To explore the effect of valproic acid (VAP) on various behavioral impairments and histo-biochemical markers in VPA-induced autism-like, the male rats were divided into two groups: Control group (T): received Ip saline solution (NaCl 9%) and treated group (VAP): received Ip valproic acid at 500mg/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. Our results had shown that, prenatal exposure to VPA at day 12.5 had long-term effects on postnatal behavior in rats, notably, a significant reduction of social interactions, memory, and an increase of in anxiety. The oxidative markers (MDA & CAT) were altered, expressed by an increase of MDA level in various brain tissues including the prefrontal cortex, and hippocampus whereas; the enzymatic antioxidants 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, the results suggest that in-utero VPA exposure causes abnormalities in the brain and liver structures (Purkinje cells and pyramidal neurons) that might be linked 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.

1. INTRODUCTION

Autism spectrum disorder (ASD) is characterized by persistent deficits in sociability and communication, as well as restricted and repetitive behavior patterns and interests [1]–[3].In most cases, ASD is diagnosed without any defined etiology. The lack of knowledge about underlying causes has limited the ability to develop and mobilize effective treatments.

To understand the complexity of ASD, serval animal models were investigated. Valproic acid (VPA) is a common anti-epileptic drug that displays teratogenic effects. The administration of VPA to pregnant women increases the probability that their offspring develop a neurodeveplemntal disorder such as ASD [4]. Several studies reported that intraperitoneal (500 mg/kg) and subcutaneous (400 mg/kg) VPA injections induced many behavioral changes including, increased anxiety-like behavior, social interaction deficits, reductions in sensory processing and attention, [5]–[7][6][7]. The intraperitoneal injection of VPA into female rats during the early stages of pregnancy manifest autism-like behaviors in the offspring marked by alterations in brain structures and biomarker levels similar to those of patients with ASD[8], [9]. Some mechanisms have been suggested that hepatotoxicity and

neurotoxicity found in VPA; most of them are associated with oxidative stress which has been implicated in the pathogenesis and development of clinical manifestation of autism [10]–[12] VPA has been shown to induce ROS production and enhance the formation of lipid peroxidation [13], induce DNA damage and decrease cell viability in hippocampal neurons [14].

Although ASD has a plethora of heterogeneous manifestations, and has been associated with several possible etiological factors (both genetic and environmental), it remains a clinical and broad-spectrum diagnosis. The current 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 models.

2. MATERIALS AND METHODS

2.1 Animals, Diets, and Drug Treatment

Wistar rats (10 males and 20 females) from the animal house of Ibn Tofail University, Morocco) were kept on a standard diet and water ad libitum after weaning and maintained on a 12 h light/dark cycle at 18–22°C, with relative humidity at 50–60%. 12 week-old female rats (250–300 g), with controlled fertility cycles, were mated overnight. The vaginal secretion was collected the next morning, and the day on which spermatozoa were detected was considered the first day of gestation (GD1). Randomly, female rats were divided into two groups: the control and valproic acid (VAP) group. However, the females of the VPA group received a single intraperitoneal injection of 500 mg/kg VPA (250 mg/ml in saline, pH 8.3. Sigma, Casablanca, Morocco), and the control ones were injected with saline on GD 12.5. The female rats were housed individually, and were allowed to raise their littles. The pups were weaned on postnatal day (PND21) and separated by sex. Because of the higher male incidence of autism, impaired social interaction and repetitive behaviors were; only observed in all male pups after VPA exposure; only male offspring were used in developmental, biochemical and behavioral tests in the current study from PND 43 to 50.

The Ibn Tofail University Kenitra, Morocco, doctoral studies centre monitored and approved the procedures. Ethics Committee of Moroccan Society of Ethics and Animal Research (MoSEAR) gave its approval. The procedures were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. A minimal number of animals was used to achieve statistical significance and all efforts were made to minimize their suffering. The rats were fed the standard laboratory diet containing all the nutrients and enriched with an additional supplement of vitamin A (15 IU/g), vitamin D (3 IU/g) and vitamin E(10−5 IU/g)..

2.2 Tissue Isolation and Homogenate Preparation

The animals were extremely anesthetized with chloral hydrate (100 mg/kg), and their brains were removed from the skull using appropriate bone forceps. Thereafter, the hippocampi, cerebellum and prefrontal cortex were dissected out and homogenized in ice-cold lysis buffer (RIPA lysis buffer + 1 mM PMSF) using a Dounce homogenizer. The homogenates were centrifuged at 14,000g for 15 min and stored at -80°C. Total protein levels were later analyzed using the Bradford reagent.

2.3 Biochemical Assay

2.3.1 Lipid Peroxidation Assay

Malondialdehyde (MDA) is widely known as a good marker of oxidative stress. In this study, we examined the formation of lipid peroxides by measuring thiobarbituric-acid-reacting substances (TBARS) in cells, as previously described by [15]. Briefly, the samples were mixed with 1 ml of trichloroacetic acid 10% and 1 ml thiobarbituric acid 0.67%, and then heated in boiling water for 15 min, and butanol (2:1 v/v) was later added to the solution. After centrifugation (800 g/5 min), the TBARS were determined by measuring the absorbance at 535 nm [16].

2.3.2 Catalase

The catalase activity (CAT) is measured at 240 nm using a UV/visible spectrophotometer by the variation of optical density consecutive to the disproportion of hydrogen peroxide (H2O2). For the enzyme reaction, 20 µl of supernatant was added to 780 of phosphate buffer saline (PBS) (0.1 M, pH 7.4) and 200 µl of H2O2 (0.5 M) [17].

2.4 Histological Examination

Samples of liver and brains structures (hippocampus, cerebellum and prefrontal cortex) were fixed in 10% buffered formalin embedded in paraffin, sectioned at 5 µm, stained with hematoxylin, and eosin and examined under light microscopy. The histological analysis aimed to evaluate the tissue integrity of organs. The parameters examined were: degeneration, necrosis, apoptosis, and leukocyte infiltration.

2.5 Behavioral Tests

The behavioral test, including open field, Elevated plus Maze, Novel Object Recognition (NOR) Task and three chamber tests were conducted in offspring on PND 45. The eye-opening, ear-unfolding, and development were inspected for each animal. For the righting reflex (at PND7), the rats were put on their backs and the time taken to turn over to all four paws was recorded. The ears were gently touched with a cotton swab, the score being 0 if there was no response, 1 if there was ear movement and 2 if there was avoidance.

2.5.1 Open-field

The investigatory behavior was tested in a wooden apparatus (100 cm × 100cm). As previously reported it was enclosed by 40 cm high walls and placed under strong illumination (100 watts, 2 m above the apparatus). The area was divided into 25 squares (20 cm × 20 cm), defined as nine central and sixteen peripheral squares. At the beginning of the 10-min test, the animal was placed in the center of the apparatus and its behavior was videotaped for subsequent analysis. The device was cleaned after each rat session. The quantified parameters used in this experiment were the total number of squares visited [18, 19].

2.5.2 The Sociability Test

The sociability test involving stranger and a familiar rat, was performed at PND31 [20]. The apparatus is an acrylic plastic box with dimensions 120/40/50 (length/width/height in cm). The box is formed by three chambers; the central chamber is 60 cm in length while each side is 30 cm.

The subject rat was allowed to explore the chambers freely. The test took place in an environment unknown to the rat being tested, in the form of a cage with three communicating compartments. The right compartment contained an unfamiliar rat from another control litter of the same strain, and the left compartment contained a familiar rat from the same litter as the rat being tested.

Unfamiliar and familiar rats were placed in wire cages. At the beginning of the test, the subject rat was placed in the empty central compartment. The time spent in each compartment and the number of crosses from one compartment to another were measured for 5 min. Time duration and the number of entrances into the chamber with the empty cage, as well as into the chamber with the stranger rat, were noted and analyzed [21].

2.5.3 Novel Object Recognition (NOR) Task

The apparatus and procedures for NOR training have been described in many studies [18,19] The task took place in a 40 × 50 cm2 open field surrounded by 50 cm high walls, made of plywood covered by a black fine plastic layer. All animals were given a habituation session where they were left to freely explore the open field for 5 min. No objects were placed in the box during the habituation trial.

Twenty-four hours later, NOR training was conducted by placing individual rats for 5 min in the field, in which two identical objects(objects A1 and A2) were placed in two adjacent corners; 10 cm from the walls. Long-term retention was tested 24 h after training. The same rats explored the field for 5 min in the presence of a familiar object (A) and a novel object (B). A single set of three objects was used for all the animals. All the objects presented similar textures: colors and sizes, but distinctive shapes. The index of recognition memory was defined as the ratio of object B exploration number and the sum of object A's and B's exploration numbers.

2.5.4 Elevated plus Maze

The EPM test measures the degree of anxiety-related, increased open-arm exploration indicates reduced anxiety-like-related behavior. The EPM is a plus-shaped platform elevated 70 cm above the floor; the apparatus is made of wood. On sited to two of the opposing arms (50 cm × 10 cm) are closed by 40 cm-high sides and end walls, having an open roof. To avoid falling, the other two arms (open arms) were surrounded by a 0.5 cm high edge.

The four arms had, at their intersection, a central platform (10 cm × 10 cm). A 100-W lamp was placed exactly on the central platform. Each animal is placed on a platform facing the open arm and the following behaviors are recorded during 5 min. The anxiety level of the rat is assessed by the time spent on the open arm divided by total time, and the number of open-arm entries divided by the total number of arm entries [18], [22].

2.6 Statistical Analysis

All data are expressed as the means ± SEM. Statistical analysis was performed using Student's t-test to analyze the data between two groups, and one-way ANOVA followed by Tukey's post hoc multiple comparison tests using Graph Pad Prism 8.0software (Graph Pad, San Diego, CA, USA). The criterion for statistical significance was set at $P < 0.05$.

3. RESULTS

3.1. Biochemical Assessment in the Prefrontal Cortex, Hippocampus and Cerebellum Homogenates

The prenatal VPA exposure significantly changed the oxidative stress markers (catalase activity & MDA) in the prefrontal cortex ($p<0.001$), cerebellum ($p<0.001$) and hippocampal ($p<0.05$) homogenates compared to the control group (Figure 1). Moreover, utero-exposure VPA rats causes a decrease in catalase activity successively by 1.23 folds in the prefrontal cortex ($p<0.01$), 1.55 in the hippocampus ($p<0.05$) and 3.74 in the cerebellum ($p<0.01$) (Figure 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 1.42in the prefrontal cortex ($p<0.001$), 1.38 in the hippocampus ($p<0.001$) and 1.42 in the cerebellum ($p<0.5$) (Figure D, E, F).

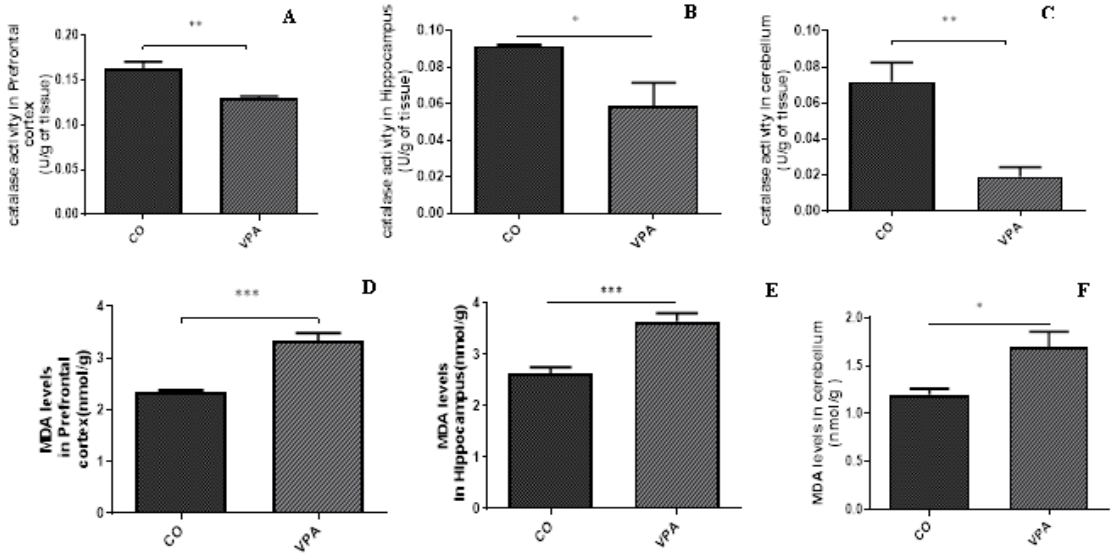

Figure 1: Effect of Prenatal Exposure to Valproic Acid in Male Wistar Rats on Catalase Activity in different Brain Areas. The Prefrontal Cortex (A), Hippocampus (B), the Cerebellum (C), and on Oxidative Damage in Brain Regions of VPA Group. The Prefrontal Cortex (D), the Hippocampus (E) and Cerebellum (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.

3.2. Sociability Novelty Preference Test and Social Novelty Index

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

Spontaneous locomotor activity seems to be affected within the group exposed toin utero VPA (figure 2). Which was significantly reduced compared with normal control. The prenatal VPA exposures induced anxiety –like behavior manifested by a significant decrease in a total number of crossed squares ($p<0.05$) and time spent in central zone ($p<0.001$) unlike 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.

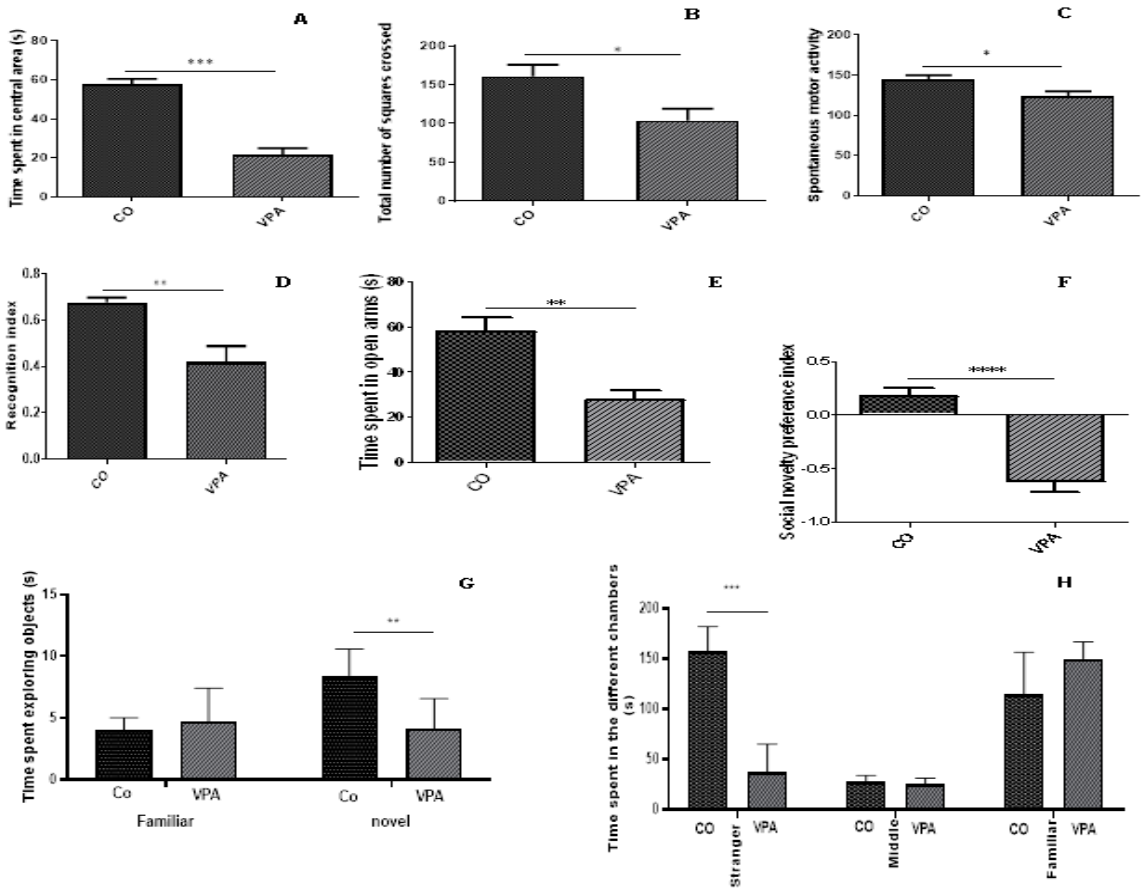

Figure 2: Autism related behaviors in valproic acid (VPA), controls. (Co) The time spent in the central area and, (B) Total number of squares crossed (C) spontaneous motor activity in the open field test. (D) The object recognition index for long-term memory in object recognition apparatus (E) Time spent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

3.3. Histopathological Analysis

areas CA1, CA3 and cerebellar cortex evidenced by a significant decrease in the number of viable cells (pyramidal cells and Purkinje cells respectively), The VPA group showed shrunken degenerated neurons with perineural spaces, whereas it didn't show any variation in dentate gyrus between the two studied groups (p>0.05 VPA versus control).

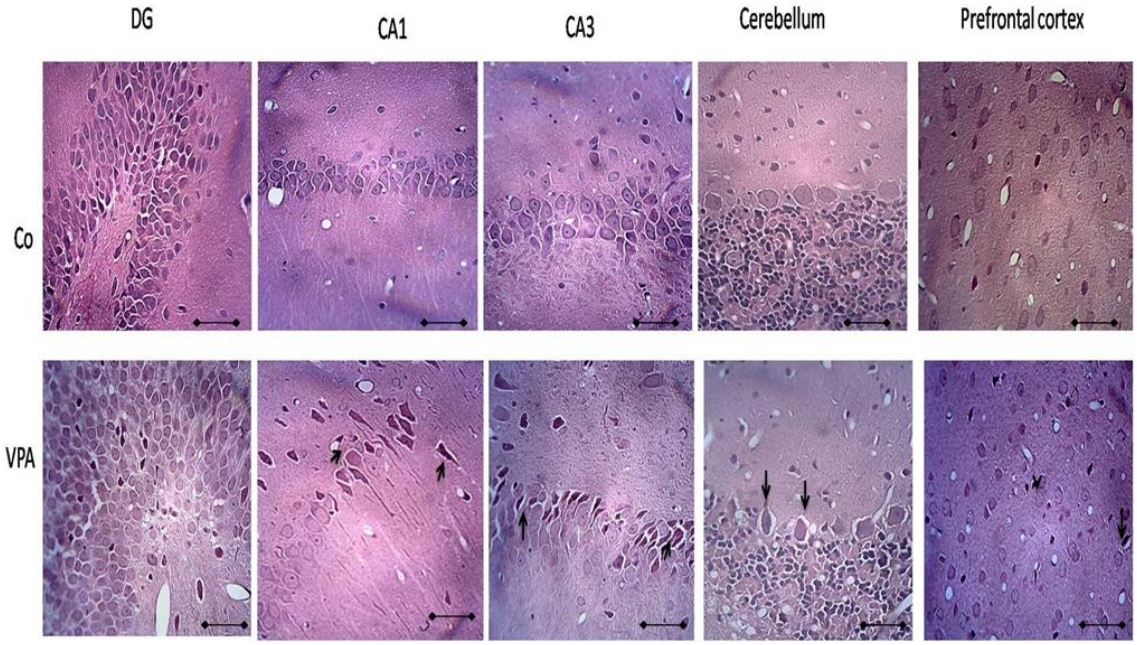

Figure 3: Figure3.Hematoxylin&Eosin-stained sections (X400, scale bar=50 µm) of hippocampus, prefrontal cortex and cerebellum in VPA-treated group and control one.CA: ammon's horn, GD: dentate gyrus, Co: control, VPA: valproic acid. Degenerated neurons.

3.3.2 Liver Tissue

The Control group showed normal liver cell plates with intervening regular sinusoids and normal central veins and portal tracts, instead of (VPA) groups. The examination of the liver from the VPA group presented lost liver architecture with dysplastic cirrhotic nodules, Enlarged and congested central vein of the liver Sinusoidal that spaces appear narrowed and filled with blood.

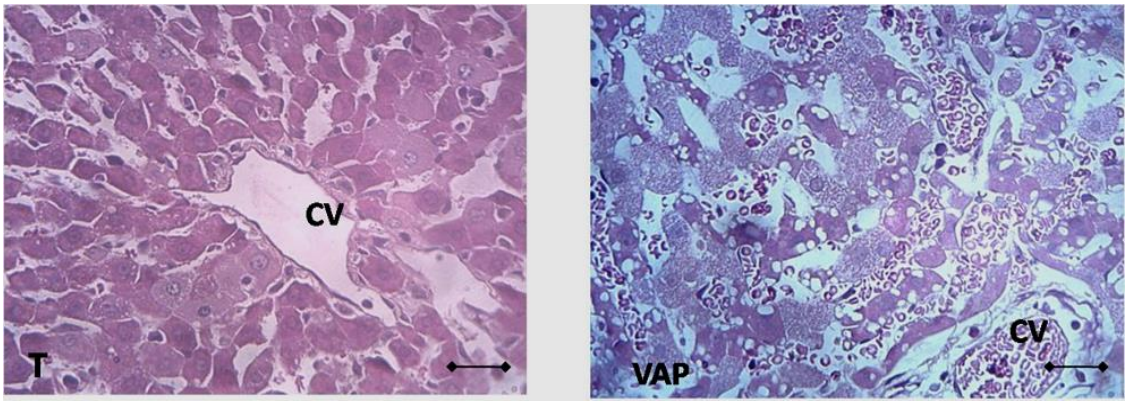

Figure 4: Histopathological Examination of Rat Liver Tissues (X 400 scale bar=50 µm). (T) Control Group, (VAP): Valproic Acid Groups, CV: Central Vein.

4. DISCUSSION

The developmental and cognitive effects of valproic acid on the brain are highly presented in several studies [23], [24], in which many characteristics of behavioral abnormalities found in the VPA – rats such as repetitive/stereotypic-like activity and deficit in social interaction have been correlated with autism.

Following these studies, we investigated the histological alterations both in brain and liver tissues, the oxidative markers modification and behavioral impairments in VPA-induced autism-like a 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 evaluated in novel object recognition test, and an anxiogenic effect was noted too. Suggesting similarities between the observed pattern of behavioral alterations in VPA rats and features of disturbed behavior in autistic patients [6], [25].

However, widespread abnormalities in the brain structure and function caused by dysregulation of neurodevelopmental processes. These processes have been shown to exert adverse effect in autistic individuals and animal models of ASD. Although, many studies have shown that 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[26], [27]. Recent studies also showed that there are profound alterations in the expression and function of GABA receptors (GABA Rs) in the amygdala across generations of the VPA-induced animal models of ASD; It suggests that targeting the GABAergic system may contribute to correcting the generational pathophysiology of ASD[28], [29].

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 has been reported as a "free radical source" either causing a decreasing antioxidant capacity of cells [30], [31].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 impaired the mitochondrial morphology in Purkinje cell perikarya [32].In addition, the changes in a total number of neurons in the hippocampus ware also found especially in CA1 and CA3; This suggests that VPA has toxic effects on neuronal development and induce apoptotic neurodegeneration not only in the hippocampus but also in the prefrontal cortex [27,33]. Furthermore, neurodegenerative abnormalities may arise when ROS triggered and neuronal damage occur [33, 35]. Our study is consistent with the previous ones and showed that VPA causes oxidative stress resulting in an 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 reduction including catalase, an intracellular metabolic product and increase of free radical contents in the brain [32, 36-39]. Moreover, over sufficient quantities of ROS production could lead to oxidative stress which is significantly associated with neurodegeneration including cognitive impairment [40-48]. The histological examination of the liver showed hepatotoxicity aspects with dysplastic cirrhotic nodules, and enlarged sinusoidal spaces filled with blood [49-52].

Hepatotoxicity is frequent after chronic VPA administration and, in certain cases, can lead to fatal irreversible liver failure, although its mechanism is not known, oxidative stress has been widely speculated as a critical mechanism for hepatotoxicity [53-60].

5. CONCLUSION

In conclusion our results demonstrate that the VPA-induced autism model in which many characteristics of behavioral abnormalities found in ASD patients such as repetitive/stereotypic-like activity and deficit in social interaction, profound abnormalities in brain structures 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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