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Neurobehavioral effects of maternal triphenyl phosphite exposure: A comparative experimental study using the valproic acid model

Maternal trifenil fosfit maruziyetinin nörodavranışsal etkileri: valproik asit modeli ile karşılaştırmalı deneysel bir çalışma

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ABSTRACT

Aim: Neurodevelopmental disorders are characterized by impairments in one or more developmental domains originating in the embryonic period and persisting throughout life. Autism, experimentally induced by valproic acid (VPA) in animal models, is a neurodevelopmental disorder. This study aimed to compare the outcomes of maternal exposure to triphenyl phosphite (TPP), a neurotoxic organophosphorus compound, and VPA in terms of neurobehavioral alterations.

Materials and Methods: Pregnant rats were randomly assigned to three groups: VPA (n=3), TPP (n=3), and control (n=3). Each group received an intraperitoneal injection of either VPA, TPP, or saline (for the control group) on embryonic day 12.5. Male offspring (n=10 per group) were evaluated on postnatal days 33–34 using the Marble Burying Test and the Three-Chambered Social Interaction Test. Animals were perfused on postnatal day 35 for immunohistochemical (IHC) analysis.

Results: TPP group exhibited a significant increase in marble burying behavior ($p<0.05$). Also, Three-Chamber Social Interaction Test revealed significant impairments in social interaction in the VPA and TPP groups compared to the control ($p<0.05$). Histologic evaluation revealed higher nestin-positive cell density in the subgranular zone of the control group compared to VPA and TPP groups ($p<0.05$). Synaptophysin expression in the CA3 region was higher in the control group than in the VPA and TPP groups ($p<0.05$). Connexin-43 expression increased in the CA1 region of the TPP group compared to controls ($p<0.05$), while no significant change was observed in the VPA group.

Conclusion: Maternal TPP exposure may be regarded as a risk factor for autism-related neurodevelopmental disorders.

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ÖZ

Amaç: *Nörogelişimsel bozukluklar, embriyonik dönemde ortaya çıkan ve yaşam boyunca devam eden bir veya daha fazla gelişimsel alandaki bozukluklarla karakterize edilmektedir. Nörogelişimsel bozukluklardan biri olan otizm, hayvan modellerinde valproik asit (VPA) ile indüklenebilmektedir. Bu çalışma, nörotoksik bir organofosfor bileşiği olan trifenil fosfit (TPP) ve VPA'ya maternal maruziyetin sonuçlarını nörodavranışsal değişiklikler açısından karşılaştırmak amacıyla planlanmıştır.*

Gereç ve Yöntem: *Gebe sıçanlar randomize şekilde üç gruba ayrılmıştır: VPA (n=3), TPP (n=3) ve kontrol (n=3). Her gruba embriyonik 12.5. günde intraperitoneal VPA, TPP veya salin (kontrol grubu için) enjeksiyonu yapılmıştır. Erkek yavrular (grup başına n=10) doğum sonrası 33-34. günlerde Üç Odacıklı Sosyal Etkileşim Testi ve Mermer Gömme Testi kullanılarak değerlendirilmiştir. Hayvanlar immünohistokimyasal (IHC) analiz için postnatal 35. günde perfüze edilmiştir.*

Bulgular: *Üç Odacıklı Sosyal Etkileşim Testi sonuçları; VPA ve TPP gruplarında kontrol grubuna kıyasla sosyal etkileşimde anlamlı bozulmalar olduğunu göstermiştir ($p<0.05$). Ayrıca, TPP grubunda bilye gömme davranışında anlamlı bir artış saptanmıştır ($p<0.05$). Histolojik analizler ile kontrol grubunun subgranüler zonunda (SGZ) VPA ve TPP gruplarına göre daha yüksek nestin-pozitif hücre yoğunluğu tespit edilmiştir ($p<0.05$). Connexin-43 ekspresyonunda, TPP grubunun CA1 bölgesinde kontrol grubuna kıyasla artış bulunmuş ($p<0.05$) ancak VPA grubunda ise anlamlı bir değişiklik olmamıştır. Sinaptofizin ise CA3 bölgesinde kontrol grubunda, VPA ve TPP gruplarına kıyasla daha yüksek eksprese edilmiştir ($p<0.05$).*

Sonuç: *Araştırmanın bulguları, TPP'nin maternal maruziyetinde yavruların nörogelişimsel bozukluklar açısından özellikle otizm için riskli olarak değerlendirilebileceğini ortaya koymuştur.*

Anahtar Sözcükler: *Nörogelişimsel Bozukluklar; Otizm Spektrum Bozukluğu; Maternal Maruziyet; Organofosfor Bileşikleri; Hipokampus.*

INTRODUCTION

Neurodevelopmental disorders (NDs) are lifelong conditions characterized by impairments in social communication, dysfunctions in motor skills, and deficits in cognitive and sensory processes, commonly emerging in early childhood (1). The occurrence and severity of symptoms may differ among individuals (2). While these disorders are thought to have a genetic basis, non-genetic factors that are considered potential triggers have yet to be fully elucidated. Exposure to environmental factors during the embryonic period and early postnatal stages is recognized as a substantial risk factor for the onset of NDs. Such factors include socioeconomic status, malnutrition, advanced maternal age, stress, inflammation, exposure to high levels of air pollution, and contact with chemicals such as heavy metals and organophosphate pesticides (3–5).

Triphenyl phosphite (TPP) is an acetylcholinesterase inhibitor and an organophosphorus-derived neurotoxic agent classified as Type II. It is commonly used in the agriculture sector as an insecticide and fungicide, in the plastic and rubber industry as a stabilizer, anti-wear additive, and epoxy resin. It causes delayed ataxia, paralysis, and changes in the nervous system prospectively (6). As a result of the administration of TPP at a dose of 1184 mg/kg, axonal and terminal degeneration was observed in the vestibular complex, cerebellum, superior and inferior colliculi, and cochlear nuclei. Degenerative axons and terminals were also identified in the cerebral cortex, hippocampus, thalamus, hypothalamus, septal region, substantia nigra, and subthalamic nucleus. Prominent degeneration was evident in the sensorimotor cortex, magnocellular preoptic and medial mammillary nuclei of the hypothalamus, as well as in the mediodorsal, ventromedial, and medial geniculate nuclei of the thalamus, while degeneration within the fasciculus gracilis and its associated nuclei appeared limited (7). Furthermore, TPP exposure at 250 mg/kg impaired learning abilities in male offspring (8). However, its effects on brain

development and behavioral outcomes have not been clarified yet.

Autism, one of the most common ND, is associated with an increased risk of occurrence subsequent to maternal exposure to detrimental environmental factors during critical developmental periods (9–12). Valproic Acid (VPA) is a histone deacetylase inhibitor and modulates neurotransmission and gene expression. It is an antiepileptic drug and is used in mood disorders (13). VPA exposure in the early embryonic period causes deterioration in the structure of the nervous system and behavior in rats (14). Rodier et al. proposed VPA as a model of autism in 1996, and the VPA model has become one of the most frequently studied models today (15,16).

This study sought to compare the neurobehavioral outcomes identified in juvenile offspring following maternal TPP exposure and the VPA-induced autism model.

MATERIALS AND METHODS

Approval of the experimental protocol was granted by the Local Ethics Committee for Animal Experiments affiliated with Ege University (Approval No: 2017-081).

Animals

Adult female Wistar albino rats (250–300 g, n=9) were first assessed for their estrous cycle stage using vaginal smears. Females at the appropriate stage were housed overnight with males, and the presence of vaginal plugs detected the following morning was used to indicate the onset of pregnancy, marking the first day of gestation. After delivery, only male offspring were included in all experimental procedures to minimize potential variability caused by hormonal fluctuations. Throughout the study, animals were maintained under controlled laboratory conditions (22 ± 2 °C, 50 ± 10% humidity) with a 12-hour light/dark cycle and had ad libitum access to food and water.

Chemicals

VPA was used in the form of sodium valproate (DEPAKIN®, Sanofi-Aventis), which was diluted with 0.9% sodium chloride solution and administered at a dose of 500 mg/kg (13). TPP (Sigma-Aldrich, T84654) was initially administered at a non-toxic dose of 250 mg/kg, as reported by Levin et al. (8); however, due to mortality observed on the fourth day following administration, the dose was reduced to 100 mg/kg and subsequently administered intraperitoneally in propylene glycol (1 ml/kg) in the main study.

Experimental design

Following confirmation of pregnancy by the detection of vaginal plugs, nine rats were randomly divided into three groups: TPP, VPA, and control. TPP and VPA were administered intraperitoneally on embryonic day 12.5 (E12.5), while the control group received an equivalent volume of saline. However, two of the pregnant rats receiving TPP at 250 mg/kg did not survive until the fourth day post-injection, and the remaining rat was excluded from the study. Subsequently, three pregnant rats, not previously subjected to experimental procedures, were randomly selected and administered TPP at a reduced dose of 100 mg/kg in propylene glycol (1 ml/kg) via intraperitoneal injection on E12.5. The revised dose did not significantly affect maternal weight gain during gestation, and offspring delivery occurred on gestational days 21 and 22. Offspring remained with their dams until weaning. Behavioral testing was conducted on P33–P34 (n=10 per group), and animals were perfused for immunohistochemical (IHC) analysis on P35 (n=5 per group).

Behavioral Tests

The male offspring (n=10/each group) were handled for 5 days before behavioral experiments which were conducted between 09:00 a.m. and 4:00 p.m in a room with dim light.

Marble Burying Test

Repetitive behaviors in rodent models of autism are commonly evaluated using the Marble Burying Test. Twenty regular glass marbles were placed on the odorless and unused sawdust with a depth of 5 cm in the same order for each cage. The rat was released from the corner of the cage not to disturb the placement of the marbles and the sawdust bed. The cages were covered after releasing and expected them to bury the marbles in 30 minutes. The animals were not fed or given water during the experiment and the marbles were cleaned with 70% alcohol between trials. After the test, animals were transferred to their home cages without disturbing the sawdust and marbles. If 2/3 of each marble is covered with sawdust, it was recorded as buried. The scores of three different researchers were averaged and the final score was determined (17).

Three-Chamber Social Interaction Test

Crawley's Three-Chamber Social Interaction Test was utilized to evaluate the social preference of rodents (18). In this study, the test was conducted on P33–P34 using a three-chambered rectangular apparatus, in which the central compartment provided two openings allowing access to the side chambers. In the habituation stage, the subject was released in the middle compartment with the blocked entrances for 5 minutes. In session I, a stranger animal (stranger 1) with similar characteristics (same sex, similar weight, and age) of the subject and had not encountered with the subject before was placed in one of the right or left chambers (changed systematically). The entrances were opened to allow the subject to explore other chambers for 10 minutes. In session II, another stranger animal (stranger 2) was placed in the other empty compartment and allowed the subject to explore for 10 minutes. A basket with holes was placed to prevent stranger rats from leaving the chamber and contact with the subject physically. Test materials were cleaned with 70% alcohol for each trial.

Histopathologic Evaluation

Perfusion procedures for histopathological analysis were initiated on postnatal day 35 (P35). Anesthetic agents Ketamine (80–100 mg/kg) and Xylazine (10–12.5 mg/kg) were administered to the animals. Following anesthesia, brains were removed and fixed in 4% formaldehyde for three days, then processed in Phosphate Buffer Solution (PBS) on the fourth day. The hippocampus was carefully removed following the stereotaxic coordinates described by Paxinos and Watson (19). Subsequently, the removed tissue was incubated for 24 hours in a solution containing 0.1 M cacodylate buffer and formalin. Hippocampal tissues were embedded in paraffin, sectioned at 5 µm thickness, and stained with routine Haematoxylin and Eosin (H&E).

Immunohistochemical Procedures

Based on the previously described protocol, sections were first incubated with 10% hydrogen peroxide (H_2O_2; Sigma-Aldrich, Inc., Saint Louis, MO, USA) for 30 minutes to block endogenous peroxidase activity, and subsequently treated with Super Block (ScyTek Laboratories, Inc., Logan, UT, USA) for 1 hour at room temperature to minimize non-specific antibody-antigen binding. Primary antibodies specific for nestin (Bioss Antibodies, Woburn, MA, USA), synaptophysin (Sigma-Aldrich, Inc., Saint Louis, MO, USA), and connexin43 (Abcam, Cambridge, UK) were used at a dilution of 1:100. Tissue sections were incubated with these antibodies at +4 °C for 24 hours. Following incubation, a biotinylated secondary antibody (ScyTek Laboratories, Inc., Logan, UT, USA) was applied, followed by horseradish peroxidase (HRP)-conjugated streptavidin (ScyTek Laboratories, Inc., Logan, UT, USA). Finally, sections were washed and incubated with diaminobenzidine (DAB) and Mayer's hematoxylin (Merck KGaA, Darmstadt, Germany) for visualization under a light microscope (20).

Image Analysis

SYN and Cx-43 expression in CA1 and CA3 regions, along with the density of nestin-positive cells in the Subgranular zone (SGZ) (cells/mm²), were evaluated under a light microscope (Olympus BX-51) and imaged with a digital camera (Olympus C-5050). ImageJ (https://imagej.nih.gov/ij) and cellSens (Olympus Corporation, Japan) software were used for analysis. IHC evaluations were independently conducted by three blinded researchers, each assessing a minimum of 100 randomly selected neurons per rat. Immunoexpression levels were quantified using the H-score method, and the mean values of these scores were used for statistical analysis (21).

Data analysis

Statistical evaluations were conducted using IBM SPSS Statistics version 22. The Shapiro–Wilk test was applied to assess data normality and homogeneity. Datasets meeting these assumptions were analyzed through one-way ANOVA followed by Bonferroni post hoc comparisons, while non-normally distributed data were assessed using the Kruskal–Wallis test, with pairwise comparisons performed by the Mann–Whitney U test. Results are presented as mean ± standard error of the mean (SEM), and a threshold of $p<0.05$ was set for statistical significance.

RESULTS

Behavioral Tests Results

Marble Burying Test

Mann Whitney test revealed that there was a significant difference ($p=0.029$). Control group buried 10-15% of the marbles while TPP

(p=0.037) and VPA (p=0.012) groups buried 30-35% regarding the repetitive behavior (Figure- 1).

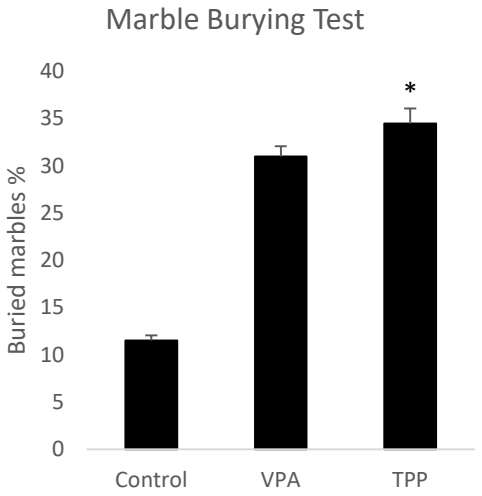

Figure-1. The percent of buried marbles was higher in TPP group than controls significantly (n=10/each group).
*p<0.05 vs VPA and control

Three-Chamber Social Interaction Test

Kruskal-Wallis test demonstrated a significant difference between groups in session I (p=0.009). The duration in the empty chamber of VPA and TPP groups was longer than controls which were prone to explore the chamber of the stranger rat 1 significantly (p=0.009) (Figure-2A).

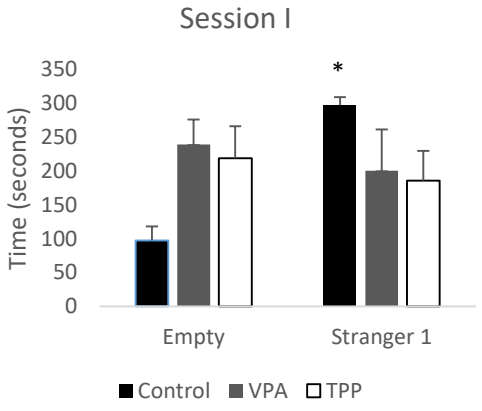

Figure-2. (A) Control rats preferred the chamber of the stranger rat 1 while they spent more time in the chamber of stranger rat 2 in session II.

In session II, control group spent time in the chamber of the stranger rat 2 more than the TPP group significantly (p=0.005). Moreover, the findings showed that the TPP group preferred the chamber of the stranger rat 1 more than the VPA group (p=0.048) (Figure-2B).

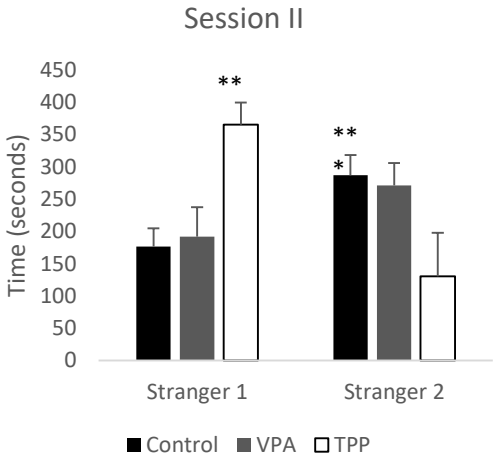

Figure-2. (B) Further, TPP group preferred the chamber of the stranger rat 1 rather than stranger rat 2 as a result of impaired social novelty.
p*<0.05 vs VPA and TPP, p**<0.05 vs TPP, p***<0.05 vs TPP

Histopathological Results

Histological analysis of hippocampal tissue sections stained with H&E revealed notable histological differences between the experimental groups (Figure-3). The control group exhibited a typical hippocampal architecture, characterized by well-preserved pyramidal cell layers and normal neuronal morphology. In contrast, hippocampal sections from the VPA and TPP groups demonstrated neuronal abnormalities and evidence of apoptosis. These findings suggest that both VPA and TPP administration led to observable histopathological alterations in hippocampal structure compared to the control group.

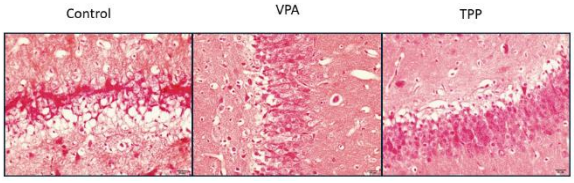

Figure-3. H&E-stained hippocampal tissue samples obtained from each experimental group (scale bar = 20 µm). Typical histological architecture was observed in the hippocampal tissue of the control group, in contrast to the neuronal abnormalities and apoptotic changes detected in the VPA and TPP groups.

Immunohistochemical Results

Nestin expressions in SGZ

SGZ of hippocampus was examined by anti-nestin immunostaining. Nestin-positive cells were denser and more compact in control group as seen by arrows in Figure-4B while large cavity and disorganized cells were observed in the TPP group regarding neurodevelopmental delay. One-way ANOVA results showed a significant difference between groups ($F_{(2,15)}$=18.536, p=0.000). Post hoc analyses demonstrated that nestin expression was significantly higher in the control group (26.12±3.07) compared to both the VPA (10.75±1.05, p=0.001) and TPP (9.74±1.75, p=0.000) groups (Figure-4A).

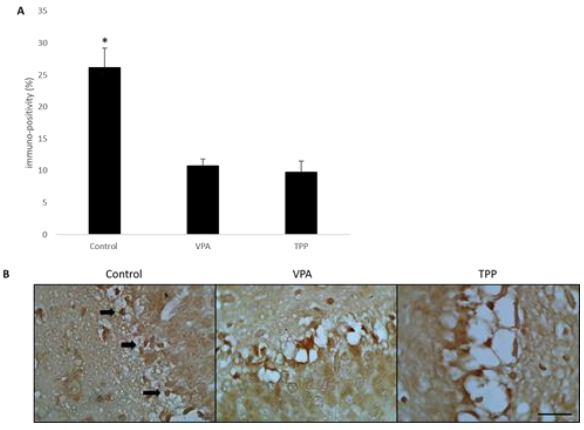

Figure-4. (A) Nestin expression in SGZ of control group was higher than other groups significantly.
(B) Representing microphotographs of nestin expressions in the SGZ of hippocampus. The arrows indicate intense nestin-positive cells in SGZ (n=6/each group, scale bar = 10 μm, magnification x40).
p*=0.001 vs VPA, p*=0.000 vs TPP

Synaptophysin and Connexin-43 expressions in CA1 and CA3

CA1 and CA3 areas of the hippocampus were examined to compare SYN and Cx-43 expressions within groups. Cx-43 expression of the TPP group (38.79± 2.46) was higher than control (p=0.05, 30.49±3.07) in CA1 (Figure-5A). Furthermore, the scattered and impaired pyramidal neuron layer and Cx-43 immuno-expression are quite evident in the CA1 of the TPP group (Figure-5B).

Post hoc Bonferonni test following one-way ANOVA ($F_{(2,15)}$=5.193, p=0.02) revealed that SYN expression of the control group (37.75±2.34) was significantly higher than VPA (28.93±2.07, p=0.04) and TPP (28.21±2.55, p=0.03) groups in CA3 as seen in (Figure-5C). The impairment is prominent with dispersed neurons in CA3 of the TPP group and structural neuronal changes in the VPA group (Figure-5D).

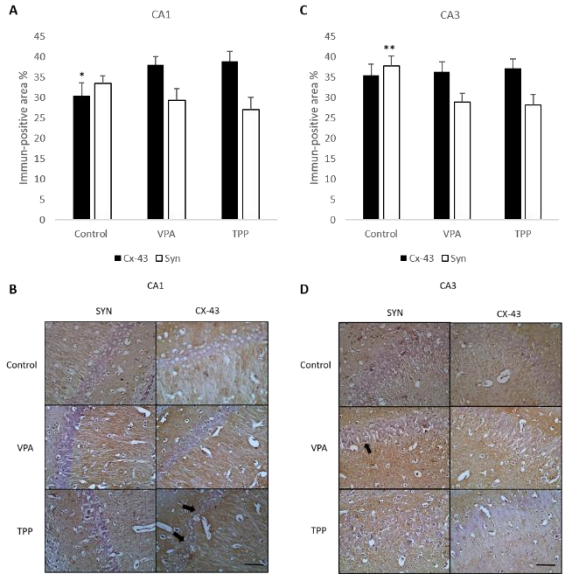

Figure-5. Cx-43 expression was higher in the TPP group than control significantly (A). The arrows indicate the impaired homogeneity of the CA1 pyramidal layer (B). SYN expression was higher in the control group more than VPA and TPP (C) and apoptotic-like neurons were seen in the CA3 of the VPA group as indicated by the arrow (D). p*= 0.05 vs TPP group, p**=0.02 vs VPA and TPP groups (scale bar = 20 μm)

DISCUSSION

Autism is a ND characterized by various neurological and behavioral impairments, showing significant differences in etiology, onset, and severity (22) It has been suggested that the behavioral changes seen in autism cannot be associated with a single region of the brain, but is related to many brain regions such as the limbic system, thalamus and cerebellum (23,24). One of the regions where structural changes seen in autism is the hippocampus (25). The embryonic maturation process of the hippocampus begins at day 12.5 and maternal VPA administration on that day causes autism-like behaviors in rats (26). The problems seen in the behaviors accompanying the developmental disorders of people with autism are caused by these abnormalities in hippocampus (27). Neuron loss was evident in the hippocampus

area in autism models of animals, and accordingly, impairments in learning skills were observed (28).

In this study, VPA model was compared with TPP, which is a environmental neurotoxic substance and behavioral changes and IHC differences in hippocampus were evaluated in juvenile rats.

Levin et.al. (8) indicated that TPP (250 mg/kg) causes learning impairments in male offspring However, in the present study, administration of TPP at a dose of 250 mg/kg resulted in maternal mortality, requiring a modification of the dose to 100 mg/kg for ensuing experiments. The Marble Burying Test was used to evaluate repetitive behaviors seen in autism. Wu et al. (29) showed that VPA-treated group showed more repetitive behavior by burying marbles compared to the control group. In a different model of autism studied by Degroote et al. (30) repetitive behaviors were measured with the Marble Burying Test, and a higher marble burying score was obtained in the experiment group than the controls. The number of marble burying increases in rodent models is associated with repetitive behaviors created by genetic manipulations (17) In present study, repetitive behaviors were observed in both VPA and TPP groups proving that TPP causes autism-like repetitive behavior.

The Three-Chamber Social Interaction Test was used to measure the deterioration in social communication seen in autism. The results in the VPA model of Dai et al. (31) demonstrated that the social interaction of the VPA group was impaired. A significant difference was found and social interaction impairment was also observed in the VPA group in the first session and second session scores of the three-chamber social interaction test performed by Kim et al. (32) and Wu et al. (33) In this study, VPA group was similarly dissociated and deteriorated and there was a decrease in social innovation preferences of the experimental group as compared to the control group. The effects of maternal TPP exposure on the social behavior were also found to be similar to VPA group. Maternal TPP exposure led to increased repetitive behaviors in the Marble Burying Test and impaired sociability and social novelty preferences in the Three-Chamber Social Interaction Test, which may reflect autism-like features.

The VPA model causes a delay in neurogenesis by substantially affecting interneurons, cell proliferation in SGZ and neuroplasticity of hippocampus granular cells (17). In this study, nestin staining was used to show the differences of proliferation in SGZ. Cai et al. (34) showed a decrease in nestin-positive cells in SGZ in a mouse model of autism, and stated that this decrease was associated with a delay in migration towards the dentate gyrus. Similarly, the present study showed that maternal TPP exposure impaired nestin expression in the SGZ, suggesting a neurodevelopmental delay during the juvenile period, which may not only reflect reduced proliferation but also involve impairments in neuronal progenitor cells and neurogenesis processes.

Synaptic dysfunction is known to occur in animal models of autism induced by VPA (Iijima et al., 2016). Codagnone et al. (35) showed reduced SYN immunoreactivity in the hippocampal regions of VPA exposed rats. The present findings demonstrated scattered neurons and decreased SYN immunoreactivity in the CA3 region of TPP group refers to impaired synaptic transmission. Furthermore, studies have provided evidence of neuroglial responses and neuroinflammation in autism (36). The expression level of Cx-43, a major component protein in the astrocytic gap junction, is increased in the frontal cortex of post-mortem brain tissue from patients with autism (Mony et al., 2016). Chávez et al. (37) reported an increase in the activity of Cx-43 in the hippocampal regions in lipopolysaccharide model of autism associated with hippocampal neurotoxicity. In this study, increased Cx-43 expression, a marker of neuroinflammation and neurotoxicity implicated in the pathogenesis of NDs, particularly autism, was noticeably observed in the TPP group.

CONCLUSION

This study highlights the potential neurodevelopmental effects of maternal exposure to environmental toxicants. Maternal TPP administration may induce autism-like behaviors and IHC alterations in the hippocampus of juvenile rats, resembling changes observed in the VPA model. Although initial, these findings suggest that TPP exposure could be considered as a complementary approach in future ND models, particularly in studies focusing on autism. Further research is required to explore the effects of TPP at varying doses, on brain regions potentially associated with neurodevelopmental alterations,

additional behavioral outcomes, and across different developmental stages.

LIMITATIONS

The limitations of this study include the absence of early postnatal growth and maturation data, the unavailability of a separate propylene glycol vehicle control group, the lack of direct apoptosis measurement, and the possibility of an insufficient sample size.

Conflicts of interest: Authors declared no conflict of interest.

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Data Availability Statement

The data used to support the findings of study are included within the article.

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Important Note

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