Journal of Shahrekord University of Medical Sciences

eISSN: 2717-0071 https://j.skums.ac.ir/

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Doi: 10.34172/jsums.1193

Please cite this article as: Forouzandeh Shahrakei Z, Noori A, Amini-Khoei H, Yazdanpanahi N, Fatemi M. Therapeutic Potential of Trigonelline in Alleviating Valproic Acid–Induced Autism-like Symptoms in an Animal Model: Investigating Its Antioxidative Stress Effects. J Shahrekord Univ Med Sci 2026;28(4): x-x. doi: 10.34172/jsums.1193

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Original Article

Submitted: 2026/7/18

Last revision: 2026/8/19

Accepted: 2026/8/22

Therapeutic Potential of Trigonelline in Alleviating Valproic Acid–Induced Autism-like Symptoms in an Animal Model: Investigating Its Antioxidative Stress Effects

Running title: Therapeutic Potential of Trigonelline in Autism

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Abstract

Background and aims: Oxidative stress is a contributing factor in the pathophysiology of autism spectrum disorder (ASD). Given the well-documented antioxidant and neuroprotective properties of trigonelline (TRI), this study investigated whether TRI mitigates valproic acid (VPA)-induced ASD-like behavioral abnormalities in rats via its antioxidant mechanism.

Methods: A single subcutaneous dose of VPA (600 mg/kg) was administered to pregnant Wistar rats (n=14) on gestational day 12 to establish a VPA-induced ASD-like model. Following this, starting from postnatal day 40, the male pups underwent a one-week treatment regimen via intraperitoneal injection of normal saline (10 ml/kg) or TRI at doses of 10, 50, and 100 mg/kg (n=6 animals per group). To evaluate neuropsychological functions, the marble burying test (MBT), Elevated Plus Maze (EPM) , shuttle box test, and three-chamber test were employed to assess repetitive behaviors, anxiety-like symptoms, memory capacity, and social interactions, respectively. Furthermore, oxidative stress markers, specifically malondialdehyde (MDA) levels and total antioxidant capacity (TAC), were quantified in the serum and hippocampus.

Results: The administration of VPA induced a range of behavioral abnormalities, specifically anxiety-like symptoms in the EPM (p<0.01), memory impairments in the shuttle box (p<0.01), repetitive patterns in the MBT (p<0.01), and social dysfunction in the three-chamber test (p<0.001). TRI reversed these behavioral alterations. Moreover, TRI increased hippocampal and serum TAC and reduced MDA concentrations within the hippocampal tissue and serum samples of ASD rats (p<0.001).

Conclusion: These results revealed that TRI, partially by alleviating oxidative stress markers in the hippocampus and serum, mitigated ASD-like behavioral abnormalities in rats.

Keywords: Trigonelline, Autism spectrum disorder, Oxidative stress, Valproic acid, Neuroprotection

Introduction

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ASD is a complex neurodevelopmental condition characterized by a multifaceted clinical presentation. Its core hallmarks include profound deficits in social-communicative functioning encompassing both verbal and nonverbal communication coupled with restricted, repetitive behavioral patterns and highly circumscribed interests (1). Individuals with ASD frequently experience anxiety and depression (2).

A range of teratogenic agents, including VPA, ethanol, and thalidomide, have been implicated in increasing the likelihood of ASD development. VPA is widely prescribed for the management of neurological conditions such as epilepsy and seizure disorders. However, prenatal exposure to VPA can result in valproate syndrome, first described in 1984, which encompasses a spectrum of major and minor abnormalities affecting growth, neurodevelopment, and behavior (3). It has been verified that prenatal exposure of pregnant rodents to VPA leads to the induction of ASD in offspring. This indicates that VPA-induced ASD in rodents is a valid model for the study of autism (4).

The accumulation of reactive oxygen species (ROS) can lead to significant oxidative impairment within biological systems, which serves as a precursor to numerous chronic pathologies. Given its elevated oxygen consumption and high concentration of polyunsaturated fatty acids (PUFAs), the central nervous system exhibits a heightened vulnerability to oxidative-mediated damage. When PUFAs undergo lipid peroxidation, they generate MDA, a widely recognized indicator of oxidative stress. Like other organs, the brain relies on antioxidant defense systems for protection. One key component of this defense is its TAC, which helps counteract oxidative damage. However, when oxidative stress surpasses the brain's antioxidant capacity, the imbalance leads to cellular injury and contributes to neurological dysfunction (5). This suggests that an imbalance between oxidant generation and antioxidant defenses in the brain increases its vulnerability to damage caused by oxidative stress (6-9).

Compelling evidence has recently established a robust nexus between systemic oxidative stress and the progression of neurodevelopmental disorders, specifically ASD (10). Earlier research suggests that ASD is linked to elevated levels of oxidative stress markers, such as MDA, along with a reduction in TAC within the brain (11, 12).

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Recent studies indicate a strong link between oxidative stress and neurological disorders, including ASD (10). Evidence from earlier research shows that ASD is accompanied by elevated oxidative stress markers such as MDA and a reduction in TAC within the brain (11, 12).

In this regard, the literature has shown that agents that may alleviate ASD do so by decreasing MDA and increasing TAC in the brain (12, 13). In recent decades, researchers have increasingly studied natural compounds derived from medicinal plants for various diseases, including neurological disorders (14-16). TRI is an alkaloid identified in numerous plants, particularly in seeds of the *Fabaceae* family, including fenugreek, coffee, barley, melon, maize, onion, chickpeas, and soybeans (17).

TRI has been reported to have multiple pharmacological properties, including antidepressant, anticonvulsant, anti-inflammatory, and antioxidative stress effects (18-20). Given the role of oxidative stress in ASD development and the antioxidant properties of TRI, this study examined whether TRI could alleviate VPA-induced autism-like behaviors, with particular emphasis on its potential to counteract oxidative stress.

Materials and Methods

Animals

Twenty-four Wistar rats (6 males, 18 females; weight: 200–250 g; age: 10–11 weeks) were obtained from the Royan Institute (Isfahan, Iran). The animals were housed at the Shahrekord University of Medical Sciences under controlled environmental conditions, including a 12-h light/dark cycle and a constant temperature of 22±122 \pm 122±1 °C. Standard laboratory chow and water were provided ad libitum. All experimental protocols were conducted in strict accordance with the National Institutes of Health (NIH) guidelines for the ethical treatment of laboratory animals.

Pregnancy and Induction of the Autism Model

Rats were housed in cages containing one male and three females. To monitor estrous cycles, vaginal smears were collected and examined daily; the presence of spermatozoa was utilized to define gestational day (GD) 1. Following

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successful mating, pregnant females were randomly assigned to one of two experimental groups: The **saline control group** (n = 4), which received a subcutaneous injection of normal saline (10 mL/kg) on GD12, and the **VPA-induced ASD group** (n = 14), which received a single subcutaneous dose of (VPA; 600 mg/kg) on GD12. It is important to note that the number of embryo resorptions and neonatal mortality typically varies among individual rats. On average, each dam produces around eight pups, commonly consisting of approximately four males and four females. In this study, 14 pregnant females were selected to ensure an adequate number of offspring exhibiting autism-like characteristics. Following weaning at postnatal day (PND) 21, the male pups were distributed into groups based on the predefined experimental protocols.

Experimental Groups

Treatments began on postnatal day (PND) 40, corresponding to early adulthood, and continued for one week. TRI was obtained from Sigma-Aldrich (USA), CAS No. 6138-41-6, with a purity greater than 98.5% (HPLC). The compound was prepared in saline containing 1% DMSO, following previously published protocols (21, 22).

Group 1 (n = 6): Control animals received saline with 1% DMSO (10 mL/kg, intraperitoneally).

Group 2 (n = 6): VPA-exposed rats were administered the same saline/DMSO vehicle (10 mL/kg, i.p.). Group 3 (n = 6): Rats exposed to VPA received a 100 mg/kg dose of TRI via intraperitoneal (i.p.) injection.

Group 4 (n = 6): Rats exposed to VPA received a 50 mg/kg dose of TRI via intraperitoneal (i.p.) injection. Group 5 (n = 6): Rats exposed to VPA received a 10 mg/kg dose of TRI via intraperitoneal (i.p.) injection

The dosing regimen and treatment duration were selected based on findings from our pilot experiments and prior literature (23). Previous studies report that TRI has a biological half-life of approximately 5 hours (24-27) and exhibits notable neuroprotective properties. TRI is capable of crossing the BBB due to its high lipid solubility, allowing it to reach the brain and exert protective effects even after a single administration (28). TRI has a high distribution and can rapidly enter all tissues (18, 20).

Schedule Behavioral Tests

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Behavioral evaluations were carried out following a seven-day treatment period, covering postnatal days 47 through 50. To maintain consistency across all subjects, each rat completed the entire set of behavioral tasks in a predetermined sequence. The tests were performed in the following order: Three-Chamber Sociability Test, Marble Burying Test, Elevated Plus Maze, and finally the Shuttle Box Test. This organized progression enabled systematic assessment of social interaction, repetitive behaviors, locomotor and anxiety-related responses, as well as passive avoidance learning in a controlled and standardized manner.

Three-Chamber Sociability Test

Social interaction was evaluated using a three-chamber Plexiglas apparatus (120×40×50120×40×50 cm), comprising a middle compartment flanked by two equivalent side chambers. Following a 5-minute habituation period in the center zone, sociability was assessed by introducing a novel, sex- and age-matched conspecific (social stimulus) into one side chamber, while the opposite chamber contained an empty wire enclosure (non-social stimulus). The subject was allowed 10 minutes of exploration, during which time spent in each zone and interaction durations were recorded. The sociability index (SI) was computed as: (social stimulus 1 non-social stimulus)/ (social stimulus 1 + non-social stimulus). To assess social preference, the previously vacant chamber was replaced with a second, unfamiliar, age- and sex-matched rat (social stimulus 2). After another 10-minute exploration period, the social preference index (SPI) was calculated: SPI = (time with social stimulus 2 –time with social stimulus 1) / (time with social stimulus 2 + time with social stimulus 1). These indices (SI and SPI) served as the primary parameters to characterize social behavioral profiles (29-31).

Elevated Plus Maze (EPM)

Anxiety-like tendencies were evaluated using an EPM assembly, comprising a plus-shaped configuration of two open and two closed arms, positioned 50 cm above the ground. During each 5-minute exploration period, rats were positioned on the central platform with their orientation toward a closed arm. Behavioral metrics, including the frequency of arm entries and time intervals spent in both open and closed arms, were manually documented.

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An entry was strictly defined as the moment the animal's four paws crossed into an arm. Higher durations within the closed arms were indicative of increased anxiety. To ensure consistency and mitigate olfactory cues, the maze was sanitized with 70% ethanol between trials, and all procedures were performed in a controlled, low-noise, and dimly lit environment (32).

Marble Burying Test

Animals were subjected to the marble-burying task, a well-established paradigm for quantifying compulsive-like behavior. Twenty marbles were placed on the bedding of a Plexiglas cage in a consistent grid arrangement. Each rat was given 20 minutes to freely explore the cage. A marble was considered buried if at least two-thirds of its surface was obscured by the bedding; subsequently, the total count of such marbles was documented (33).

Shuttle Box Test

The animals' passive avoidance memory was measured in a standard shuttle box, a two-compartment device (light and dark sides) joined by a gate and built with electrified grid floors. The experimental protocol spanned four days. Following an initial 48-hour habituation period where rats explored the device freely for five minutes per session, the acquisition phase commenced on the third day. In this phase, rats were introduced into the illuminated compartment; upon crossing the door into the dark zone, they received a transient 1-second electrical stimulus (1 mA). The latency to traverse into the dark chamber was measured. On the fourth day, during the retention test, the time taken for the rats to enter the dark compartment (capped at 60 seconds) was recorded. To prevent olfactory or sensory interference, the apparatus was sanitized between each trial (34).

Euthanasia and Sample Collection

Once the behavioral testing battery had been completed, deep anesthesia was induced in the rats by administering ketamine (60 mg/kg) in combination with xylazine (15 mg/kg) (35). Once an adequate depth of anesthesia was confirmed, blood was obtained through cardiac puncture. Following blood collection, the brains were excised, and the hippocampal tissue was carefully isolated. The samples were promptly frozen and maintained at −70 °C until further analysis. In

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parallel, blood specimens were centrifuged at 3,200 rpm for 5 min. The resulting plasma fraction was then transferred into designated storage tubes and preserved at −70 °C for subsequent biochemical assays.

Hippocampal isolation

After the skull was opened using appropriate surgical instruments, the brain was gently removed and transferred to a chilled Petri dish placed on ice. The brain was then separated into two hemispheres, and the hippocampi were carefully isolated from their anatomical regions. Each hippocampus was homogenized with a mechanical homogenizer in 0.03 M PBS buffer (pH 7.4) to prepare the tissue for subsequent biochemical analyses (36, 37).

Determination of TAC

Determination of TAC was performed on serum and hippocampal tissue homogenates employing the FRAP procedure. Briefly, the assay exploits the ability of antioxidants to reduce Fe^{3+}-TPTZ complexes to Fe^{2+} under specified conditions (37 °C, pH 3.6), producing a blue chromophore whose intensity is proportional to antioxidant content. Absorbance measurements were taken at 593 nm on an automated plate reader (LQ-300+II-Epson, USA), and concentrations were derived from a standard curve and reported as µg/mL (38).

Measurement of MDA

MDA, the principal end product of polyunsaturated fatty acid peroxidation, was measured in serum and hippocampal homogenates via the TBARS procedure. The reaction was carried out by mixing 100 µL of sample with 900 µL of Tris–KCl buffer, followed by the addition of 500 µL of 30% trichloroacetic acid and 500 µL of 0.75% thiobarbituric acid. After incubation at 80 °C for 45 min in a water bath and centrifugation at 3,000 rpm for 5 min, the absorbance of the clear supernatant was read at 562 nm on an ELISA plate reader. Values, normalized to protein content, were reported as nmol/mg protein (39, 40).

Statistical Analysis

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We utilized GraphPad Prism 8 for all statistical computations. Before conducting parametric analyses, we confirmed data normality and variance homogeneity using the Kolmogorov–Smirnov and Brown–Forsythe tests. One-way ANOVA, followed by Tukey's post hoc analysis, was used to identify significant differences between groups. Values are expressed as mean ± SD, and a p-value of less than 0.05 indicated statistical significance. Furthermore, G*Power software (v3.1.7) was used to calculate the necessary sample size for a power of 0.8 at an alpha level of 0.05

Results

TRI increased the sociability index and social preference index in VPA-exposed rats

Significant variations in the sociability index across the experimental groups were observed via one-way ANOVA ($F(4, 15) = 10.34$, $p = 0.0003$). Post hoc analysis using Tukey's test revealed a significant reduction in the sociability index within the VPA + saline group compared to the control + saline group ($p < 0.001$). Interestingly, administration of TRI (10, 50, and 100 mg/kg) to VPA-treated animals resulted in a substantial recovery of the sociability index when compared to the VPA-only group ($p < 0.05$, $p < 0.001$, and $p < 0.01$, respectively) (Figure 1: A). Social preference index followed a similar pattern, with a one-way ANOVA confirming significant between-group differences ($F(4, 15) = 15.71$, $p < 0.0001$). Tukey's post hoc analysis further indicated that the VPA + saline group had a significantly lower social preference index compared with the control + saline group ($p < 0.001$). Additionally, administration of TRI at 50 and 100 mg/kg significantly elevated the social preference index in VPA-treated animals relative to the untreated VPA group ($p < 0.001$) (Figure 1:B).

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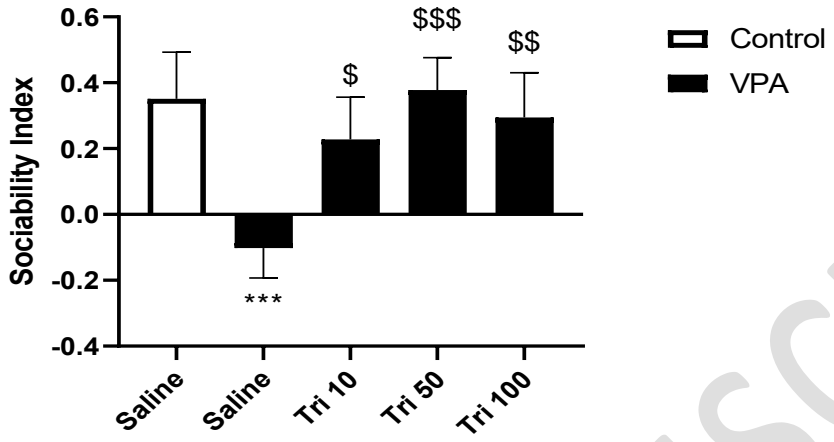

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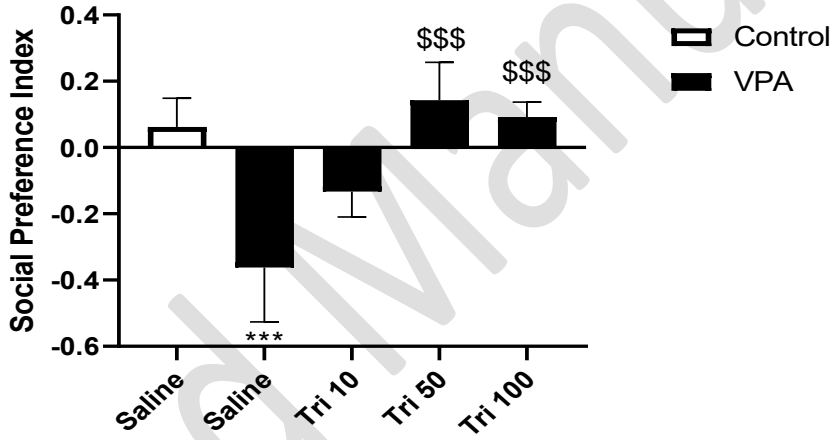

Figure 1. Impact of TRI on social behavior across experimental groups. (**A**) Sociability index and (**B**) social preference index. Values are expressed as mean ± standard deviation (SD). Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. ***$p < 0.001$ versus the saline-treated control group; \$ $p< 0/05$, \$\$ $p<0/01$, \$\$\$ $p < 0.001$ versus the VPA + saline group. TRI, trigonelline; VPA, valproic acid.

TRI reduced repetitive behaviors in the marble burying test

One-way ANOVA revealed significant variations in repetitive behaviors among the various experimental groups ($F(4, 25) = 13.71$, $p < 0.0001$). Tukey's post hoc analysis revealed that the VPA-treated group exhibited a considerable elevation

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in repetitive behaviors compared with the saline-treated controls ($p < 0.001$). Notably, administration of TRI at 50 and 100 mg/kg significantly reduced repetitive behaviors in VPA-exposed rats ($p < 0.01$), whereas the 10 mg/kg dose did not yield a statistically significant change (Figure 2).

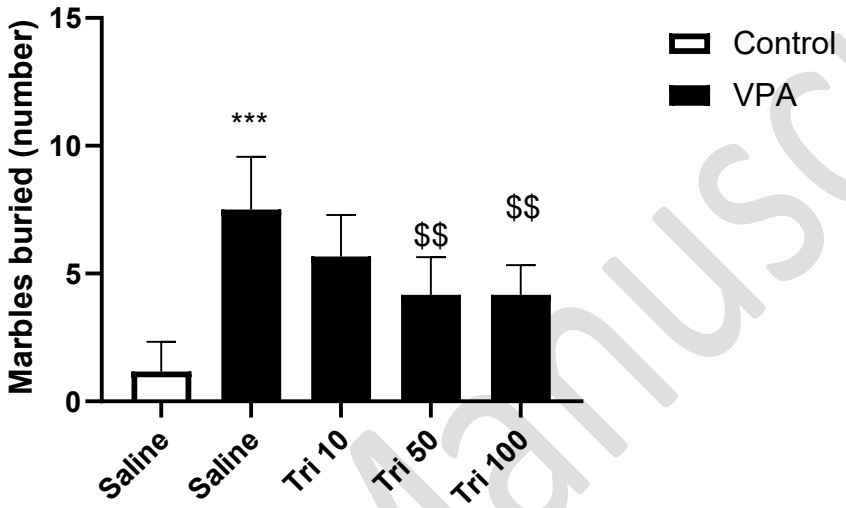

Figure 2. Impact of TRI administration on repetitive behaviors in experimental groups. Results are presented as mean ± SD. Significant differences were identified using one-way ANOVA and Tukey's post hoc analysis, ***$p < 0.001$ compared to the control–received saline group; $$ $p<0.01$ compared to the VPA-treated group. TRI: Trigonelline, VPA: valproic acid.

TRI increased latency to enter the dark compartment in the shuttle box test

One-way ANOVA showed that no significant intergroup variations existed for T1 ($F(4, 18) = 1.862$, $p = 0.1611$) (Figure 3: A). Conversely, T2 values exhibited a significant group effect ($F(4, 15) = 9.708$, $p = 0.0004$). According to Tukey's post hoc analysis, the VPA-treated saline group showed a significantly reduced secondary latency (T2) in comparison to the saline control group ($p<0.01$). Notably, TRI administration at doses of 50 and 100 mg/kg effectively enhanced secondary latency in VPA-exposed rats relative to the VPA-only group ($p<0.05$and $p<0.01$, respectively). In contrast, the 10 mg/kg TRI dose failed to yield a significant improvement in T2 latency (Figure 3: B).

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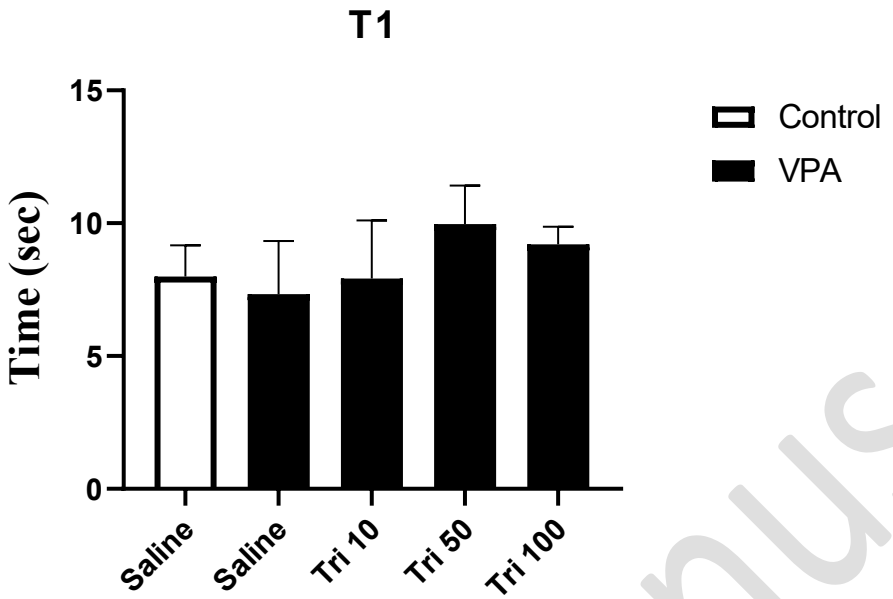

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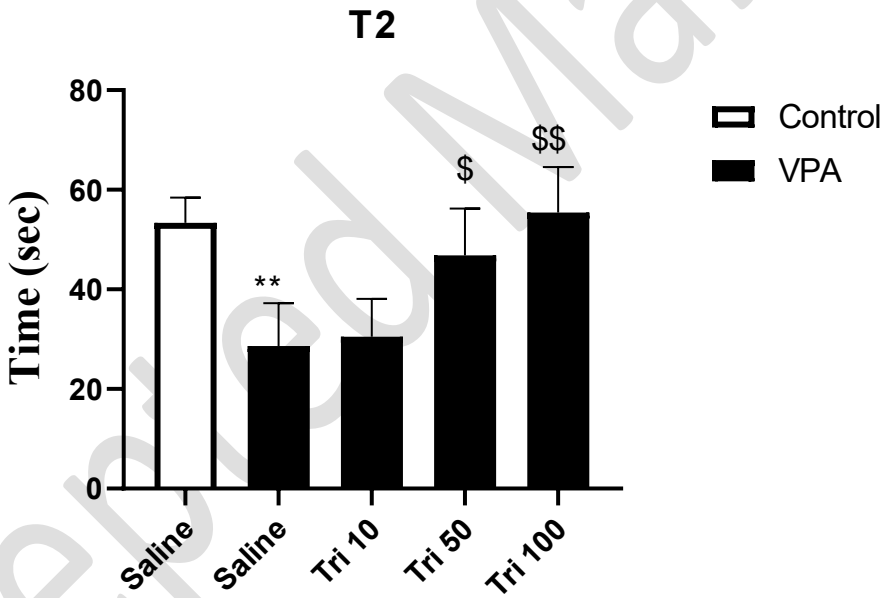

Figure 3. Effects of TRI on latency parameters in the experimental groups. **(A)** Initial latency. **(B)** Step-through (secondary) latency. Values are expressed as mean ± SD. Statistical differences were determined by one-way ANOVA followed by Tukey's post hoc test. ** $p<0.01$ compared to the control–received saline group and $ $p<0.05$ and $$ $p<0.01$ in comparison to the VPA–received saline group. TRI: trigonelline, VPA: valproic acid.

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TRI treatment increased open-arm entries and time spent in the open arms in the elevated plus maze

Significant variations were observed between groups in both open-arm exploration parameters. One-way ANOVA for open-arm entries revealed a significant main effect ($F(4, 15) = 6.940$, $p = 0.0023$), with post hoc analyses indicating that VPA-saline-treated rats had significantly fewer entries than controls ($p<0.01$). While TRI administration at 100 mg/kg significantly rescued this behavior ($p<0.05$), the 10 mg/kg dose failed to elicit a significant response (Figure 4: A). Similarly, a significant group effect was observed for the time spent in the open arms ($F(4, 15) = 17.04$, $p < 0.0001$). Post hoc analysis revealed that the VPA-saline group exhibited **significantly reduced open-arm exploration** compared to the control group ($p<0.001$), a deficit that was markedly attenuated by TRI at doses of 50 and 100 mg/kg ($p<0.01$). Conversely, the 10 mg/kg TRI dose did not significantly impact this parameter (Figure 4: B).

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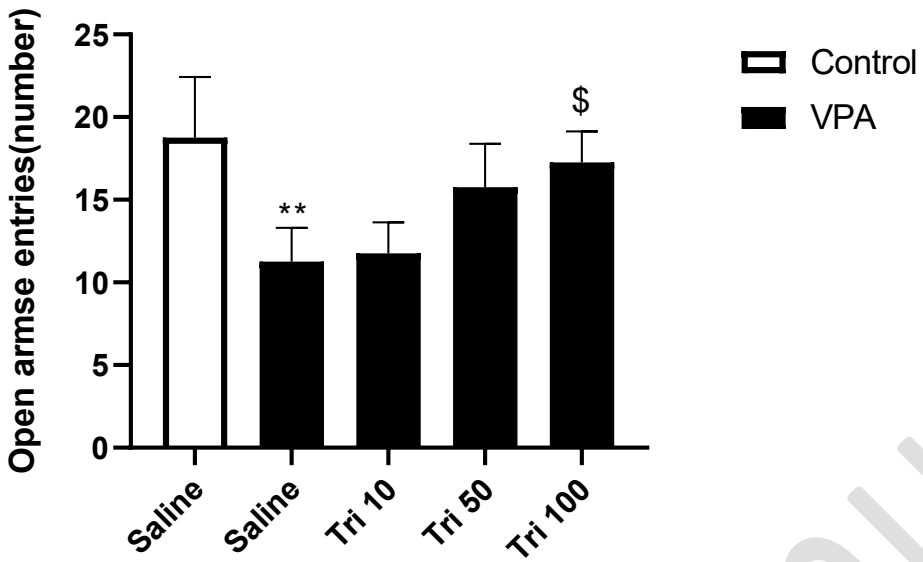

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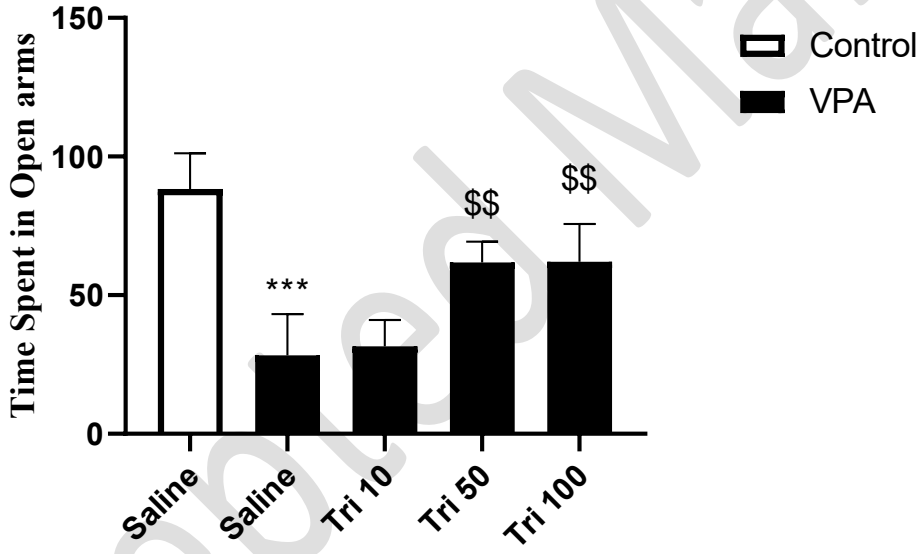

Figure 4. Effects of TRI on open-arm exploration in the experimental groups. Percentage of open-arm entries **(A)** and percentage of time spent in open arms **(B)**. Data are expressed as mean ± SD and were analyzed by one-way ANOVA followed by Tukey's post hoc test. $^{**}p<0.01$ and $^{***}p<0.001$ indicate significant differences compared to the control saline group; \$ $p<0.05$ and \$\$ $p<0.01$ denote significant differences relative to the VPA -saline group. TRI: trigonelline, VPA: valproic acid.

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TRI enhanced hippocampal TAC level

One-way ANOVA confirmed significant variations in hippocampal TAC across the experimental cohorts (F (4, 25) =21.01, p<0.0001). Post hoc comparisons using Tukey's test revealed that VPA administration significantly depleted hippocampal TAC compared to the saline-treated control group (p<0.001). Notably, TRI supplementation at doses of 50 and 100 mg/kg effectively rescued this antioxidant deficit, significantly elevating TAC levels in VPA-exposed rats relative to the VPA-only group (p<0.001). However, no statistically significant amelioration of TAC was observed following administration of 10 mg/kg TRI (Figure 5).

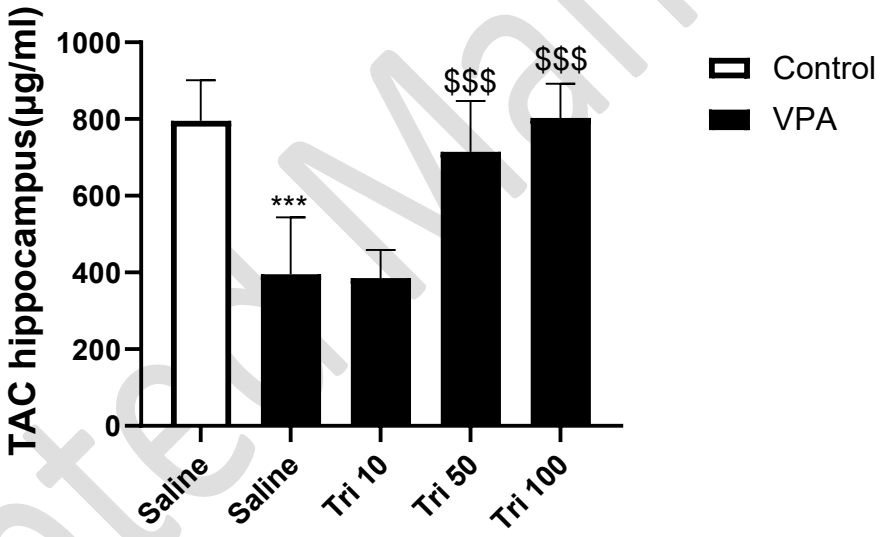

Figure 5. **Effect of TRI on hippocampal TAC across experimental groups.** Data are presented as mean ± SD. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. ***$p < 0.001$ indicates a significant difference versus the control-saline group, whereas \$\$\$ $p< 0.001$ indicates a significant difference versus the VPA-saline group. TRI, trigonelline.VPA: valproic acid.

TRI enhanced serum TAC level

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One-way ANOVA revealed significant differences in serum TAC among the experimental groups (F (4, 25) = 25.93, p < 0.0001) (Figure 6). Post hoc analysis revealed that VPA-saline treatment significantly diminished serum TAC levels in comparison to the control-saline group (p < 0.001). The observed VPA-induced reduction in serum TAC was **significantly attenuated** following treatment with 50 and 100 mg/kg of TRI (p < 0.01). In contrast, TRI at 10 mg/kg did not produce a significant improvement in serum TAC.

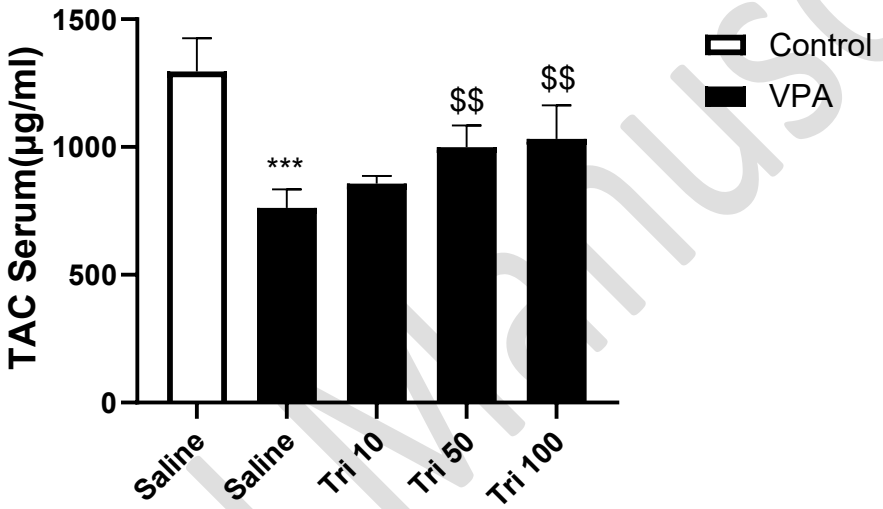

Figure 6. Effects of TRI on serum TAC in experimental groups. Results are shown as mean ±SD and analyzed by one-way ANOVA and Tukey's post hoc test. ***p<0.001 compared to the control–received saline group and $$ p<0.01 in compared to the VPA–received saline group. TRI: trigonelline, VPA: valproic acid.

TRI decreased hippocampal MDA level

One-way ANOVA demonstrated significant differences in hippocampal MDA concentrations among the experimental groups (F (4, 55) =80.97, p<0.0001) (Figure 7). Tukey's post hoc analysis confirmed that VPA exposure **significantly induced** an elevation in hippocampal MDA levels relative to the saline-treated control group (p<0.001). Conversely, TRI treatment significantly attenuated these oxidative stress markers in a dose-dependent manner, with doses of 10, 50, and 100 mg/kg yielding reductions relative to the VPA-only group (p<0.01, p<0.001, respectively).

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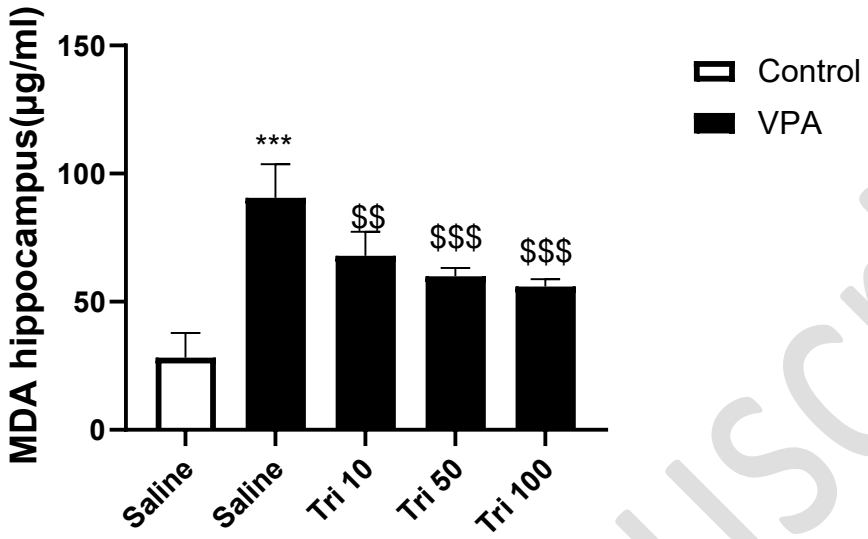

Figure 7. Effect of TRI on hippocampal MDA levels across experimental groups. Data are presented as mean ± SD and were analyzed using one-way ANOVA followed by Tukey's post hoc test. ***p < 0.001 vs. the control-saline group; $$$p$ < 0.01 and $$$ p< 0.001 vs. the VPA-saline group. TRI, trigonelline; VPA: valproic acid.

TRI reduced serum MDA levels

One-way ANOVA revealed significant intergroup disparities in serum MDA concentrations (F (4, 25) =30.99, p<0.0001) (Figure 8). Although VPA exposure markedly escalated lipid peroxidation—manifested by a substantial rise in MDA levels compared to the saline control (p < 0.001)- TRI intervention demonstrated dose-dependent efficacy in mitigating this oxidative insult. Notably, TRI administration at doses of 50 and 100 mg/kg significantly attenuated serum MDA concentrations relative to the VPA-only group (p<0.01and p<0.001, respectively), whereas the 10 mg/kg dose failed to yield a significant therapeutic reduction.

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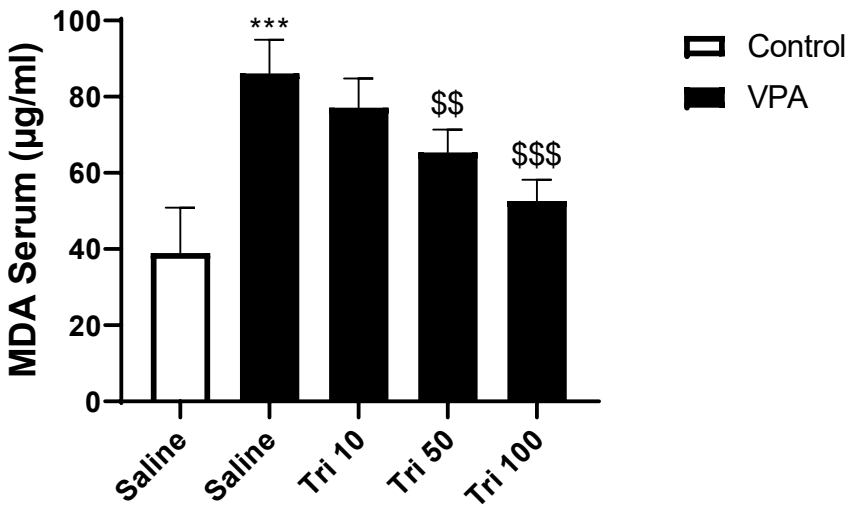

Figure 8. TRI-induced alterations in serum MDA levels. Data are presented as mean ± SD, with statistical comparisons performed using one-way ANOVA and Tukey's post hoc test. ***$p < 0.001$ vs. the control-saline group; $$ $p < 0.01$ and $$$ $p < 0.001$ vs. the VPA-saline group. Abbreviations: TRI:trigonelline, VPA: valproic acid.

Discussion

ASD represents a prevalent neurodevelopmental condition, affecting upwards of 1% of the global population. It is characterized fundamentally by enduring deficits in social communication and the presence of restricted, repetitive patterns of behavior. Despite its relatively high prevalence, no definitive or highly effective treatment has yet been established for ASD (41). A substantial body of research points to a strong association between oxidative stress (OS) and ASD (42, 43). This indicates that reducing oxidative stress may offer therapeutic benefits (10). Supporting this hypothesis, a growing body of evidence indicates that the reduction of oxidative damage serves to mitigate the core symptoms of ASD within various animal paradigms (44).

It has been established that TRI treatment facilitates the upregulation of major antioxidant enzymes, including SOD, CAT, and GPx, thereby augmenting the capacity of the endogenous antioxidant system. Moreover, TRI has been reported to activate the Nrf2–ARE signaling pathway, a central regulatory mechanism that governs cellular defense responses against oxidative stress (45). Despite its potential, the neuroprotective role of TRI in the context of neurodevelopmental

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disorders like ASD remains unexplored. Consequently, this research aimed to investigate whether TRI could mitigate autism-like phenotypes induced by prenatal VPA exposure, with a particular emphasis on its antioxidant properties. Our results indicate that gestational VPA administration effectively modeled ASD-like behavioral abnormalities, characterized by heightened stereotypic and anxiety-like behaviors, alongside deficits in social interaction and cognitive memory. These observations align with established literature, which suggests that prenatal VPA exposure triggers behavioral abnormalities consistent with ASD in rodent models (46, 47). Furthermore, VPA exposure in this study is associated with decreased antioxidant capacity and increased MDA levels in serum and hippocampal samples. These findings indicate that TRI produced notable anti-autistic effects in rats exposed to VPA. TRI administration showed significant therapeutic potential by mitigating repetitive behaviors during the marble burying assay and enhancing social interaction in the three-chamber sociability paradigm. Furthermore, TRI ameliorated cognitive deficits in the shuttle box task and attenuated anxiety-like tendencies as observed in the EPM. These behavioral enhancements are consistent with the findings of Amini et al. (2023), whose research corroborated that VPA exposure precipitates autism-like behavioral deficits in rodent models (4).

The present investigation demonstrated that TRI administration enhances antioxidant capacity while simultaneously lowering MDA concentrations in both hippocampal tissue and serum. These observations align with established literature regarding the neuroprotective efficacy of TRI across various neurological injury models. Notably, Pravalika et al. (2019) observed a dose-dependent reduction in MDA and nitrite levels within the brain parenchyma of mice undergoing cerebral ischemia, an outcome fundamentally driven by TRI's antioxidant properties (48). Qiu et al. (2020) indicated that TRI diminishes present neural damage by modulating the PI3K/Akt pathway and reducing oxidative stress (49). Collectively, these findings support TRI's ability to mitigate oxidative stress-induced brain injury, primarily through its strong antioxidant activity. Overall, the results obtained here demonstrate that TRI can significantly decrease oxidative stress at both serum and neuronal levels. The increase in TAC and the reduction in MDA levels in serum and hippocampal tissue demonstrate this compound's antioxidant ability. Given the high susceptibility of the hippocampus to oxidative stress, these antioxidant properties determine its potential neuroprotective effects. Therefore, TRI may be further

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investigated in future studies as a natural compound with neuroprotective and antioxidative stress properties in ASD models. The lack of significant effects of TRI at a dose of 10 mg in the hippocampus may be due to the relatively short duration of treatment. A prolonged treatment period may be required for this dose to produce therapeutic effects.

The results of this study suggest that TRI can partially counteract the autism-like behavioral abnormalities induced by VPA, likely because it reduces MDA and increases TAC. Nevertheless, additional investigations are required to elucidate the precise mechanisms through which TRI influences autism-related outcomes and to further define its pharmacokinetic profile. Given these observations, TRI may represent a promising natural compound for future research targeting neurodevelopmental disorders and other conditions characterized by oxidative stress. One limitation of the present work is that TRI's effects were not evaluated in female rats, leaving sex-specific behavioral responses unexplored. Future studies should therefore assess whether TRI similarly modulates autism-like behaviors in females. Another limitation is that biochemical analyses were conducted only on the whole hippocampus. Since distinct hippocampal subregions contribute to different cognitive and behavioral functions (50), future research should examine how TRI affects specific hippocampal areas in the VPA model of autism. Additionally, the study did not investigate TRI's effects in non-VPA control rats, which restricts conclusions regarding its broader behavioral impact.

Conclusion

The results indicate that TRI, as a natural compound, partly mitigated the adverse effects of VPA. This compound not only alleviates behavioral symptoms associated with ASD but also exerts protective effects by enhancing antioxidant capacity and reducing hippocampal and serum MDA levels, suggesting its therapeutic potential for improving ASD-like behavioral abnormalities in this animal model.

Conflict of Interest Disclosure

The authors declare that there is no conflict of interest.

Ethical Approval

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All experimental procedures were approved by the Animal Ethics Committee of Islamic Azad University (Ethics code: IR.IAU.NAJAFABAD.REC.14031.155) and were performed according to the NIH Guide for the Care and Use of Laboratory Animals.

Artificial Intelligence (AI) Disclosure Statement

During the preparation of this manuscript, we used ChatGPT only for grammar checking to improve English. All content generated with the assistance of this tool was carefully reviewed, validated, and revised by the authors to ensure accuracy, originality, and scholarly rigor. The authors assume full responsibility for the integrity and final content of the published articles.

Acknowledgment

The authors would like to express their sincere gratitude to Islamic Azad University, Falavarjan Branch, and Shahrekord University of Medical Sciences for their valuable cooperation in this research.

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Funding/Support

This work did not receive funding.

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