Turk Neurosurg 34(6):1145-1159, 2024

Original Investigation

DOI: 10.5137/1019-5149.JTN.44980-23.3

Received: 18.07.2023
Accepted: 01.12.2023

Published Online: 23.10.2024

Effects of Vitamin D and Memantine on Repetitive Mild Traumatic Brain Injury via mTOR, TRPM2, and GABA Expression Levels on Juvenile Rats

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ABSTRACT

AIM: To investigate the effects of vitamin D and memantine on the healing process in juvenile rats with repetitive brain injury (rTBI) and to elucidate the mechanisms underlying these potential therapeutic effects.

MATERIAL and METHODS: Juvenile rats were randomly allocated into seven groups, with eight rats per group: sham-operated (Group I), trauma (Group II), memantine supplementation (10 mg/kg) pre-trauma (Group III), vitamin D supplementation (5 μg/kg) pre-trauma (Group IV), vitamin D supplementation post-trauma (Group V), memantine and vitamin D supplementation post-trauma (Group VI), and vitamin D supplementation pre- and post-trauma with post-trauma memantine supplementation (Group VII). A modified repeated weight drop model was employed to induce rTBI. Brain tissues and blood samples were collected for analysis. Expressions of the mammalian target of rapamycin (mTOR), temporary receptor potential (TRPM2), and GABA receptors were assessed via immunohistochemistry. Levels of 8-hydroxy-2-deoxyguanine (8-OHdG) were determined using high-performance liquid chromatography (HPLC). Matrix metalloproteinases -2 and -9, tissue inhibitors of metalloproteinases-1 and-2, and NADPH oxidation-4 levels were determined using commercially available enzyme-linked immunosorbent Test kits. Immunohistochemistry analyses were performed on the brain cortex and hippocampus.

RESULTS: The levels of 8OHdG/106dG, MMP-2, MMP-9, TIMP-1, -TIMP2, and NOX-4 were significantly higher in the trauma group than in the other groups. No difference was found between the control and Pre Vit D+Mem+Post Vit D groups regarding 8OHdG/106dG, MMP-2, -9 and NOX-4 levels. Normalized expressions of mTOR and TRPM2 were observed in Groups VI and VII. Conversely, GABA expression levels decreased in Group II, with the most pronounced therapeutic effects observed in Group VII.

CONCLUSION: Memantine and vitamin D positively affected rTBI when used alone. Their combined use exhibited greater therapeutic outcomes. These effects are mediated by mTOR mRNA, TRPM2 mRNA, and GABA mRNA expressions.

KEYWORDS: Repeated mild traumatic brain injury, Vit D, Memantine, mTOR signaling

INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of death and disability worldwide. In the United States, approximately 70 million individuals experience a TBI annually (11), with approximately 80% of these being mild TBI (mTBI) (28). While a single TBI can induce symptoms like increased stress and mental confusion (24), recurrent mTBI (rmTBI) often results in more severe short-term and

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long-term physiological effects (40). Children are particularly vulnerable to rmTBI due to common incidents, such as falls, bicycle or motorcycle accidents, and sports-related injuries. Such repeated injuries during childhood can lead to persistent behavioral and neurological problems, including conditions such as seizures, sleep disturbances, neurodegenerative diseases, hormonal imbalances, and mental health issues (7).

Vitamin D (Vit D) is essential for cellular health and regular cellular functions (19). Numerous studies have explored the benefits of Vit D, either alone or combined with other compounds, in TBI, observing its influence on survival rates and disease outcomes (2,8). Despite the growing understanding of its mechanisms (23), research on Vit D's therapeutic potential for recurrent brain injuries, especially in children, remains limited.

MMT inhibits the activity of NMDA-5, providing neuroprotection by reducing glutamate-induced toxicity, which can be harmful to neurons, and aiding in cognitive recovery following TBI (20). Although memantine is used in various neurological disorders, its use in TBI, especially rmTBI, is not widespread. A limited number of studies have examined its role in recurrent brain injuries (26). To the best of our knowledge, only one study investigated memantine's role in recurrent brain injuries (32).

Matrix metalloproteinases (MMPs) are multifunctional enzymes that influence various cellular processes in the central nervous system (CNS) (16). MMPs manage the extracellular matrix components in both normal and pathological states of the CNS and peripheral nervous system (43). Elevated MMP activity is observed in various CNS conditions such as Alzheimer's disease, post-injury, or post-stroke brain damage (38,41). MMP-2 and MMP-9 are particularly important for growth, adaptability, and nervous system repair (43). Tissue Inhibitors of Metalloproteinases (TIMPs) are responsible for regulating MMPs by inhibiting MMP-1, MMP-2, and, MMP-9 (10).

Ion channels, composed of proteins, act as cellular gateways that facilitate ion exchange. The transient receptor potential (TRP) protein family is involved in various cellular functions. Usually, TRP channels are considered to be free cation channels and not just select cation channels (39). Among them, TRP-melastatin 2 (TRPM2) has been linked to oxidative stress-induced processes and inflammation. Its function is dependent on the presence of intracellular Ca^{2+}.

The mammalian target of rapamycin (mTOR) pathway is vital for various brain functions, including neuronal protection, cell growth, and learning how to change our minds (21). Neuronal mTOR supports protein synthesis necessary for cellular growth and repair, and mTOR-driven processes are crucial for recovery from CNS damage, including TBI-induced inflammation.

This study aimed to investigate the roles of memantine and Vit D in minimizing post-traumatic neural damage in juvenile rat brains following repeated trauma.

MATERIAL and METHODS

Animals

We obtained 56 male Wistar-albino rats, weighing between 70-85 g and aged three weeks, from the Experimental Medicine Application and Research Center. Ethical permission for the study was granted by the Animal Experiments Local Ethics Committee of Van Yuzuncu Yıl University, Turkey (2020/12-16, Date: 31.12.2020). The animals were randomly assigned to seven groups (eight per group). They were housed in an automatically controlled environment with a temperature of 22 ± 2 °C, 60% humidity, and a 12-hour light/dark cycle. Prior to the experiment, the animals were acclimatized to the environment to ensure that they were not stressed.

The rats had ad libitum access to food and water. A combination of Ketamine (80 mg) and xylazine (10 mg) body weight was administered intraperitoneally before sacrifice. All the procedures were performed in accordance with the principles of the Declaration of Helsinki. All staff members paid attention to minimizing animal suffering during each step of the experiment.

Experimental Protocol

The weight drop method was adapted from Feeney's falling weight (FFW) technique and modified according to the Mychasiuk method in the literature (15). The FFW was utilized for achieving an adequate cranium impact level, while the Mychasiuk method was implemented to reduce animal mortality and improve real-life conditions adaptation (32). The setup involved an aluminum foil-covered sponge placed in a basket. After anesthetization, animals were positioned on the aluminum foil (15,32). A brass cylinder weighing 150 grams was dropped from a height of 0.05 m onto the skull, guided by a vertical glass tube, over four days. The energy (E) of the impact was calculated using the following formula: E = mg x h.

In this formula, E represents energy, mg is mass times gravity, and h denotes height. This system allows for precise and repeatable injuries in different types of animals. This system enabled accurate and repeatable injuries in different types of animals. The animals were immediately placed in their cages following recovery after the weight drop.

Control – Sham-operated: Animals in this group continued their routine lives without any intervention

Trauma group: Following the Feeney model, animals underwent the weight-drop trauma daily for four days (15, 32). No medication or post-traumatic procedures were administered. The animals were sacrificed on the eighth day.

Memantine group with memantine supplementation after trauma: After the weight-drop trauma, the subjects received 10 mg/kg/day of memantine for four days, (44) and were sacrificed on the eighth day.

Pre-Vit D group -with Vit D supplementation before trauma: Animals received 5 µg/kg of Vit D, intraperitoneally, four days before the trauma, followed by the trauma procedure over the next four days. Sacrifice occurred on the eighth day.

Post Vit D group with Vit D supplementation after trauma: Similar to the previous groups, these animals received 5 µg/kg of Vit D, intraperitoneally, for four days post-trauma (42), and were sacrificed on the eighth day.

Post Vit D with memantine supplementation after trauma: Following the weight-drop trauma model, as in the previous groups, animals in this group were administered both memantine (10 mg/kg/day) and Vit D (5 µg/kg, intraperitoneally, for four days during a seven-day period. They were sacrificed on the eighth day.

Pre Vit D + Post Vit D and Memantine group with Vit D supplementation before and after trauma and memantine supplementation after trauma: Animals in this group received Vit D (5 µg/kg), intraperitoneally, starting four days before the weight-drop trauma and continuing until sacrifice. Additionally, they were administered memantine injections (10 mg/kg/day) for the four days following the trauma. Sacrifice occurred on the eighth day.

Histopathological Method

Brain samples were collected at the end of the necropsy. These samples were then fixed in a 10-percent neutral formalin solution for two days. Subsequently, the brain samples underwent standard histological processing. This process began with dehydration in progressively concentrated alcohol solutions. Subsequently, the tissues were treated with xylol using the Leica ASP300S automated tissue processor (Leica Microsystems, Wetzlar, Germany) and then covered with paraffin.

Tissue sections of 5 µm thickness were prepared using the Leica RM2155 rotary microtome (Leica Microsystems, Wetzlar, Germany). These sections were stained with Hematoxylin and Eosin and covered with glass slides, for observation under conventional light microscopy.

Immunohistochemical Method

Brain sections were immunostained using an active TRPM2 (TRPM2 antibody, ab11168), mTOR (mTOR antibody, ab32028; from Abcam, Cambridge, UK), and GABA-receptor antibodies (anti-GABA receptor alpha 5 antibodies, JB34-19, ET7107-08; from Hangzhou HuAn Biotechnology Co. Ltd., USA). In this study, we used Streptavidin and Biotin Peroxidase methods. The procedure began with incubation in the primary antibody for one hour, followed by a secondary immunohistochemical analysis using a biotin-labeled secondary antibody and conjugate from the EXPOSE Mouse and Rabbit IHC Kit (AB80436). Diaminobenzidine was employed as the chromogen. For controls, the primary antibody was replaced with a antigenic dilution medium. All assessments were conducted on anonymized samples.

To quantify positive cells, we counted 100 cells across five random areas of the brain cortex and hippocampus (20 cells in each area). Using commercial analysis software we examined each section in detail. The processing included cropping of images, separation by colour channel, removal of artefacts, and use of an area tool to identify and count neighboring positive cells. Counts were performed blindly, and results were verified by a pathologist from another university The results were then analyzed using a database manual and a morphometric analysis and microphotography system (Olympus Co., Japan).

Biochemical Assays

To evaluate oxidative DNA damage, we assessed the levels of 8-OHdG using HPLC, following the methodology established by Kaur and Halliwell (25). The results are expressed as the ratio of 8-OHdG to 106 dG. This method comprises three stages. Initially, DNA extraction was performed using fresh blood samples, employing the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, CA, USA).

Subsequently, the DNA samples were hydrolyzed using 60% v/v formic acid as detailed by Kaur and Halliwell (25). For the detection of 8-OHdG, an HPLC system equipped with an electrochemical and diode array detector (HPLC-ECD and HPLC-DAD; Waters 2465, The Netherlands) was utilized, as mentioned previously (37). Following hydrolysis, the DNA samples were mixed with HPLC solvent to a final volume of 1 mL. A 20 µL segment of this hydrolyzed mixture was then analyzed using HPLC-ECD and HPLC-DAD. The HPLC analysis employed a reverse phase column (RP-C18, 250 mm x 4.6 mm x 4.0 µm; Phenomenex, CA, USA). While the concentration of dG was monitored via absorbance at 245 nm, 8-OHdG was detected electrochemically at 600 mV. The concentration of 8-OHdG was quantified as the number of 8-OHdG molecules per 106 dG.

The levels of MMP-2, MMP-9, TIMP-1, TIMP-2, and NOX-4 were measured using specific ELISA kits according to the guidelines provided by the manufacturer (YLbiont YI Biotech Co. Ltd., Shanghai, China).

The total antioxidant capacity (TAS) and the total oxidant status (TOS) in the brain tissue were quantified using an automated colorimetric method as described by Erel O (13,14). The results were expressed in mmol Trolox/L for TAS and µmol 2HO Eq/L for TOS.

RESULTS

Immunohistochemistry Findings

Brain trauma caused marked histopathological lesions in the brain cortex, but not in the hippocampus. Immunohistochemical findings revealed increased mTOR and TRPM expression and decreased GABA expression in the cortex and hippocampus. Treatment with either memantine or Vit D resulted in amelioration. The combination of memantine and Vit D led to more effective amelioration, with the most marked improvement observed in Group VII.

Brain Cortex Histopathological Findings

Histopathological examination of the brain cortex showed normal tissue histology in the sham-operated group. In contrast, the trauma group exhibited marked hyperemia, hemorrhage, necrosis, and inflammatory cell infiltrations. A significant decrease in pathological findings was determined in Groups III, IV, V, VI, and VII, with a marked decrease observed in Group VII (Figure 1).

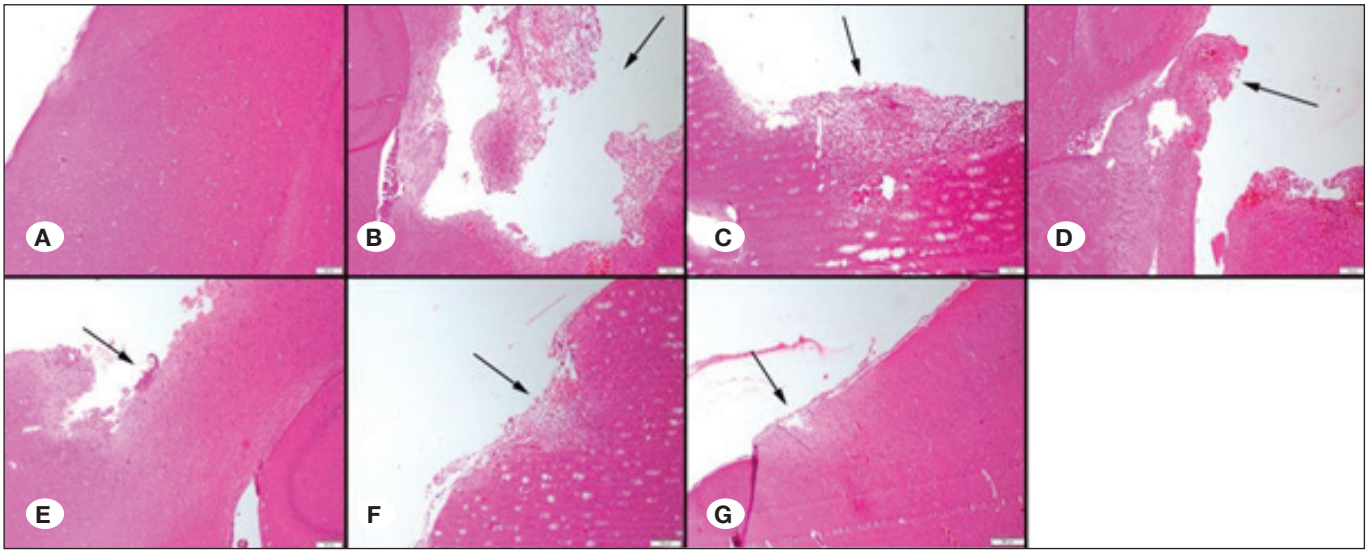

Figure 1: The microscopical appearance of the brain cortex between the groups. **A)** Normal tissue histology in the control group. **B)** Marked necrosis and inflammatory reaction (arrow) in the trauma group. **C)** Decreased pathological reaction (arrow) in the memantine group. **D)** Decreased pathological findings (arrow) in Pre trauma treatment with Vit D. **E)** Moderate decrease in pathological findings (arrow) in Post-trauma treatment with Vit D. **F)** Moderate decrease in pathological findings in (arrow) Memantine + post-Vit D treatment group, **G)** Marked amelioration in pathological findings (arrow) in Pre trauma Vit D + post trauma Vit D and Memantine groups. HE, scale bars=50μm.

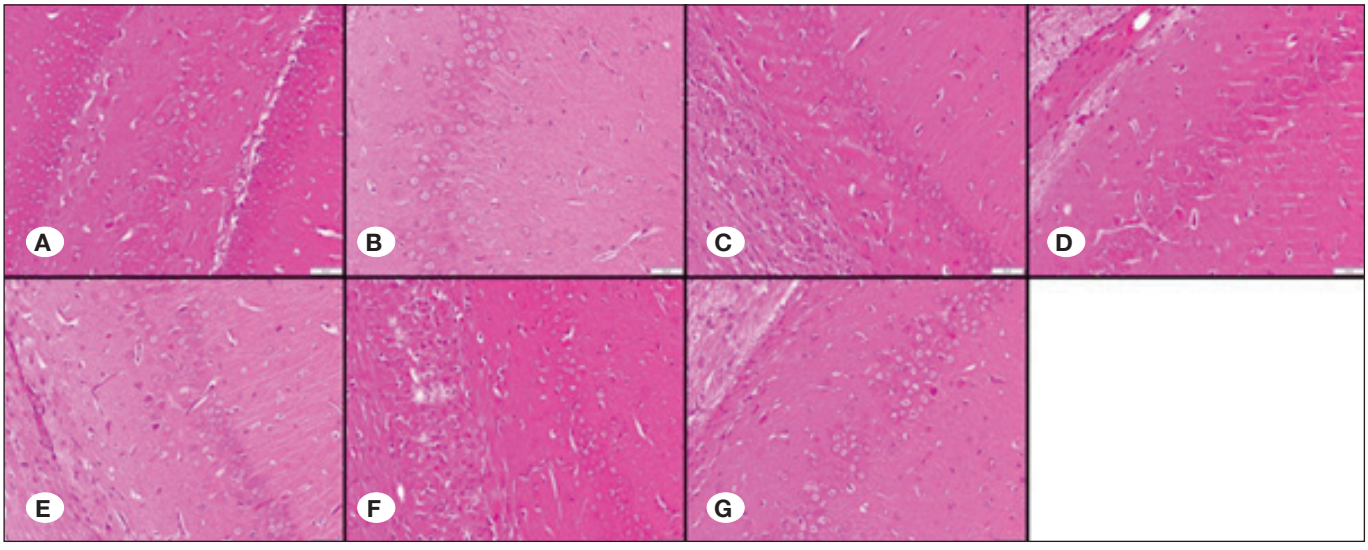

Figure 2: Histopathological appearance of the hippocampus between the groups. Normal tissue histology in the control group **(A)**; in the trauma group **(B)**; in the memantine group **(C)**; in Pre trauma treatment with Vit D group **(D)**; in the Post-trauma treatment with Vit D group **(E)**; in Memantine + post-trauma treatment Vit D group **(F)**; in Pre trauma Vit D + post trauma Vit D and Memantine groups **(G)**. HE, scale bars=50μm.

Hippocampus Histopathological Findings

No pathological findings were observed in any group during the hippocampal histopathological examination (Figure 2).

mTOR Immunohistochemistry Findings in Brain Cortex

mTOR immunohistochemistry findings indicated increased expressions in the trauma group. Decreased expressions were observed in other groups. In Group IV, mTOR expression levels (46.12 ± 2.69) were similar to those in Group III (47.87 ± 2.79). However, Group V exhibited a better therapeutic effect (37.00 ± 1.92). Group VI also showed significantly decreased mTOR expression levels (33.87 ± 3.31). The most marked amelioration was observed in Group VII (20.87 ± 1.12) (Table I, Figure 3).

mTOR Histopathological Findings in the Hippocampus

In the hippocampus, Group II showed significantly increased

mTOR expressions. Slightly decreased expression levels were noted in neurons Groups III (21.12 ± 1.72), IV (15.75 ± 1.03), and V (16.55 ± 1.19). A moderate decrease was observed in Group VI (11.87 ± 1.12), while Group VII exhibited the best therapeutic effect (9.00 ± 0.75), with significant reduction in mTOR staining in neurons (Table I, Figure 4).

TRPM2 Immunohistochemistry Findings in Brain Cortex

TRPM2 immunohistochemistry findings revealed increased expressions in Group II. Decreased expressions were noted in Group III (45.75 ± 1.66). Groups IV and V had TRPM2 expression levels of 41.50 ± 1.41 and 37.62 ± 2.19, respectively. A significant decrease was observed in Group VI (33.75 ± 1.75), with the most effective outcomes in Group VII (27.50 ± 1.85).

TRPM2 is an oxidative stress-sensitive channel, and our results demonstrate that memantine and Vit D play crucial roles in intracellular cascade regulation (Table I, Figure 5).

TRPM2 Immunohistochemistry Findings in the Hippocampus

Similar to the brain cortex, the hippocampal TRPM2 expression levels in the trauma group (Group II) showed a statistically significant increase (51.37 ± 3.33). Memantine treatment (Group III) reduced TRPM2 expression levels (46.50 ± 2.07). Vit D-administered groups (Groups IV and V) showed more reduction in TRPM2 expression levels (44.50 ± 1.51 and 41.62 ± 1.30, respectively) than the memantine-treated group (Group III). However, the reductions in Groups VI and VII were much greater than those in the other groups. The best

Table I: Statistical Analysis Results of Immunohistochemical Scores Between Groups

		Control	Trauma	Memantine	Pre Vit D	Post Vit D	Mem + post Vit D	Pre Vit D + + post Vit D -Mem
mTOR	B	22.75 ± 2.21^{a}	56.37 ± 3.02^{b}	47.87 ± 2.79^{c}	46.12 ± 2.69^{c}	37.00 ± 1.92^{d}	33.87 ± 3.31^{e}	20.87 ± 1.12^{a}
	H	10.00 ± 1.63^{ae}	24.87 ± 3.72^{b}	21.12 ± 1.72^{c}	15.75 ± 1.03^{d}	16.55 ± 1.19^{d}	11.87 ± 1.12^{e}	9.00 ± 0.75^{a}
TRPM2	B	28.00 ± 0.81^{a}	48.87 ± 1.95^{b}	45.75 ± 1.66^{c}	41.50 ± 1.41^{d}	37.62 ± 2.19^{e}	33.75 ± 1.75^{f}	27.50 ± 1.85^{a}
	H	29.00 ± 3.16^{a}	51.37 ± 3.33^{b}	46.50 ± 2.07^{c}	44.50 ± 1.51^{c}	41.62 ± 1.30^{d}	26.50 ± 2.13^{e}	18.25 ± 1.48^{f}
GABA Recept.	B	55.75 ± 0.95^{a}	23.37 ± 1.68^{b}	39.25 ± 4.80^{c}	46.00 ± 1.85^{d}	47.87 ± 1.88^{de}	49.87 ± 1.24^{e}	56.75 ± 2.49^{a}
	H	56.25 ± 0.95^{a}	28.50 ± 1.19^{b}	34.50 ± 1.19^{c}	34.25 ± 1.38^{c}	35.62 ± 1.59^{c}	44.00 ± 2.00^{d}	55.37 ± 4.10^{a}

: Data expressed mean ± standard deviation (SD). One-way ANOVA Duncan test. **: The differences between the groups carrying different letters in same colon are statistically significant, p<0.001. **B: Brain, **H:** Hippocampus.*

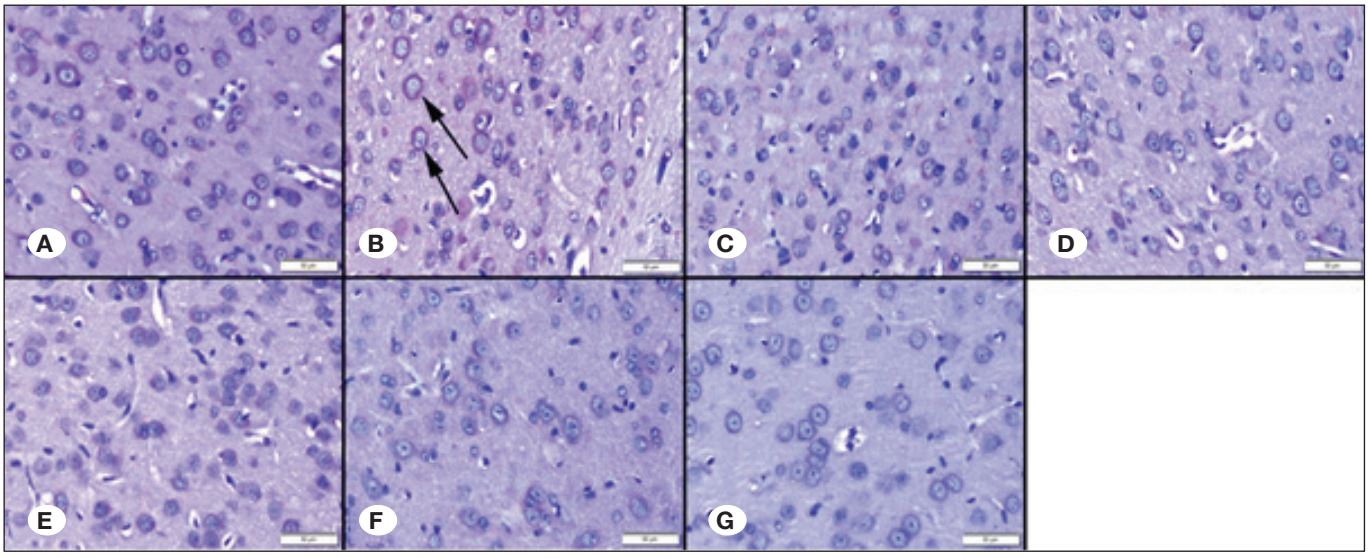

Figure 3: mTOR immunohistochemistry findings in brain cortex between the groups. **A)** Control group animals **B)** Marked increase in expression (arrows) in neurons in the trauma group. **C)** Slightly decreased expressions in neurons in the memantine group. **D)** Slightly decreased expressions in neurons in the Pre trauma treatment with Vit D group. **E)** Slightly decreased expression in neurons in the Post-treatment Vit D group. **F)** Moderate decrease in neurons in the Memantine + post-trauma treatment with Vit D group. **G)** A marked increase in neurons in the Pre trauma Vit D + post-trauma Vit D and Memantine groups. Streptavidin biotin peroxidase method, scale bars=50µm.

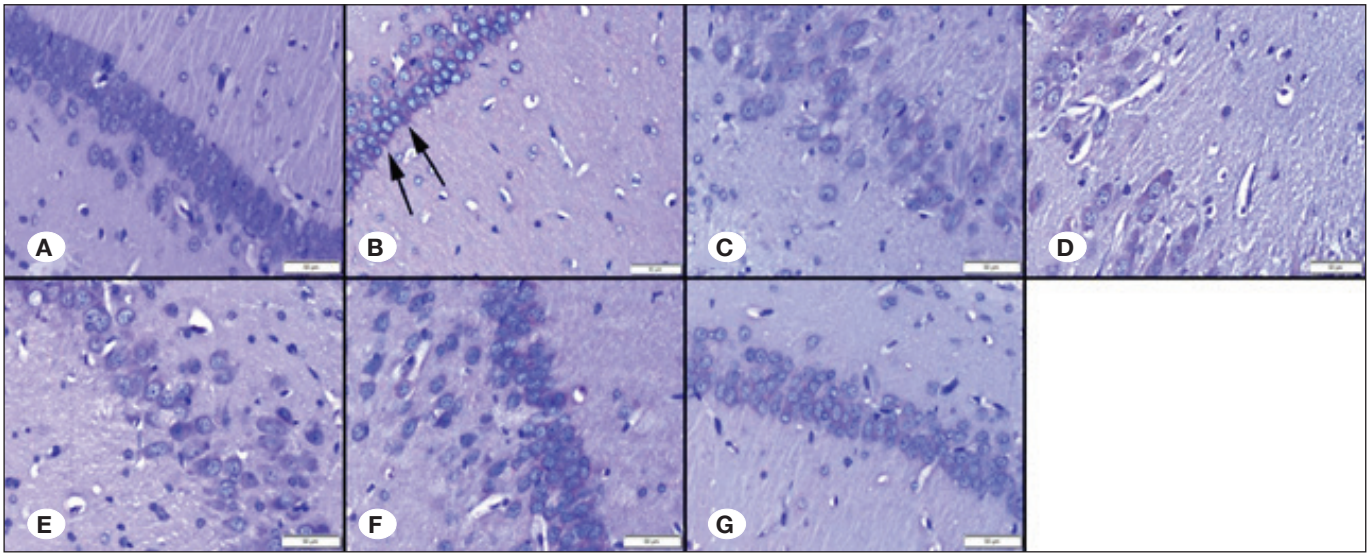

Figure 4: mTOR immunohistochemistry findings in the hippocampus among the groups. **A)** Slight expression in the control group. **B)** A marked increase in neurons (arrows) in the trauma group. **C)** Slightly decreased expression in neurons in the memantine group. **D)** Slightly decreased neuron expression in the Pre trauma treatment with the Vit D group. **E)** Slightly decreased neuron expression in the Post-trauma treatment with the Vit D group. **F)** Moderate decrease in expression in neurons in the Memantine + post-trauma treatment with Vit D group. **G)** A marked increase in neurons in the Pre trauma Vit D + post-trauma Vit D and Memantine groups. Streptavidin biotin peroxidase method, scale bars=50μm.

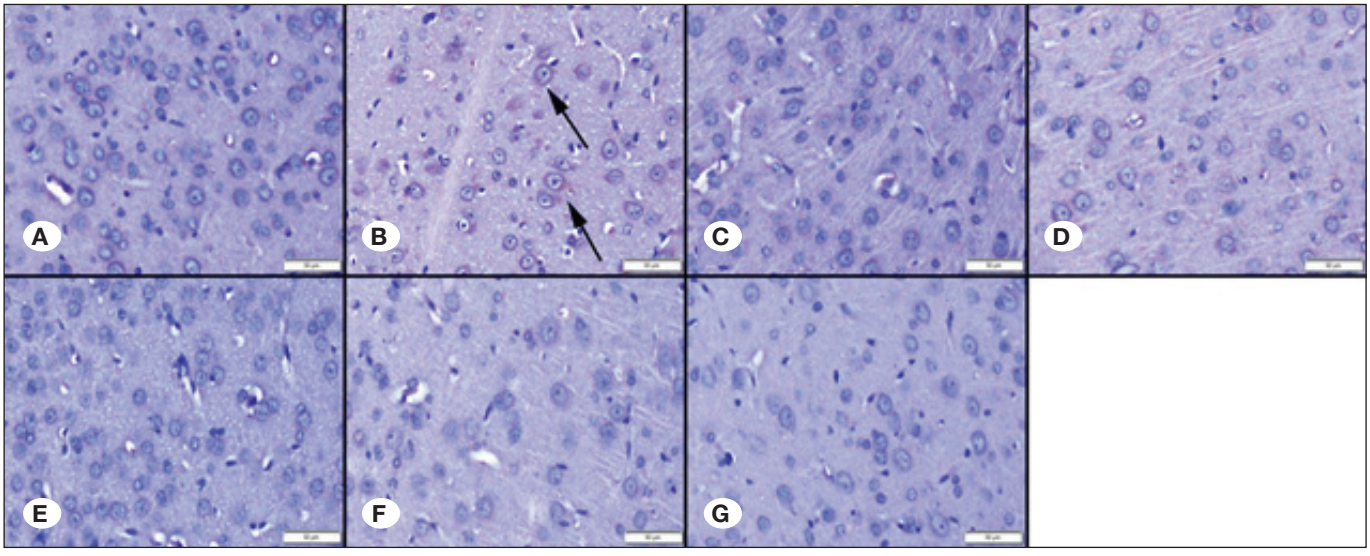

Figure 5: TRPM immunohistochemistry findings in the brain cortex between the groups. **A)** Slight expression in the control group. **B)** A marked increase in neurons (arrows) in the trauma group. **C)** Slightly decreased expression in neurons in the memantine group. **D)** Slightly decreased neuron expression in the Pre trauma treatment with the Vit D group. **E)** Slightly decreased neuron expression in the Post-trauma treatment with the Vit D group. **F)** Moderate decrease in expression in neurons in the Memantine + post-trauma treatment with Vit D group. **G)** A marked increase in neurons in the Pre trauma treatment Pre trauma Vit D + post-trauma Vit D and Memantine groups. Streptavidin biotin peroxidase method, scale bars=50μm.

therapeutic effect was observed in Group VII (18.25 ± 1.48). (Table I, Figure 6).

GABA Receptor Immunohistochemistry Findings in Brain Cortex

GABA receptor immunohistochemistry in the brain cortex showed decreased expression levels in the trauma group (Group II, 23.37 ± 1.68). Following memantine administration (Group III), the expression levels increased (39.20 ± 4.80). Groups IV and V exhibited higher GABA expression levels (46.00 ± 1.85 and 47.87 ± 1.88, respectively). Group VI demonstrated better effects (49.87 ± 1.24), but the most pronounced therapeutic effects were observed in Group VII (56.75 ± 2.49) (Table I, Figure 7).

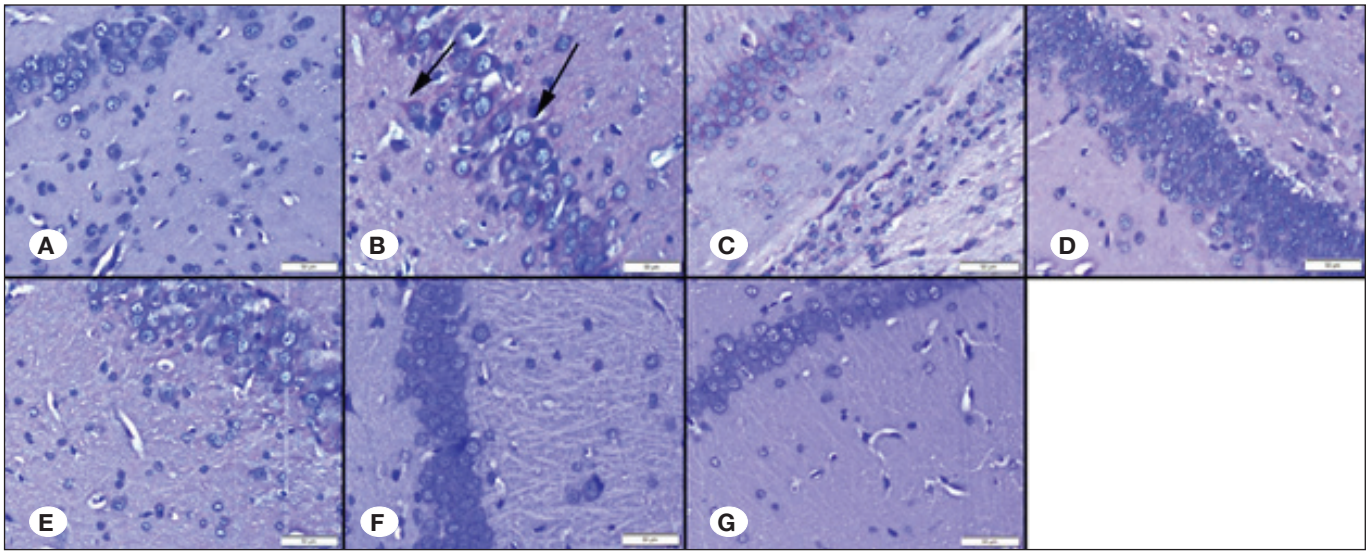

Figure 6: TRPM immunohistochemistry findings in the hippocampus between the groups. **A)** Slight expression in the control group. **B)** A marked increase in neurons (arrows) in the trauma group. **C)** Slightly decreased expression in neurons in the memantine group. **D)** Slightly decreased neuron expression in the Pre trauma treatment with the Vit D group. **E)** Slightly decreased neuron expression in the Post-trauma treatment with the Vit D group. **F)** Moderate decrease in expression in neurons in the Memantine + post-trauma Vit D group. **G)** A marked increase in neurons in the Pre Vit D + Post trauma Mem + Vit D groups. Streptavidin biotin peroxidase method, scale bars=50μm.

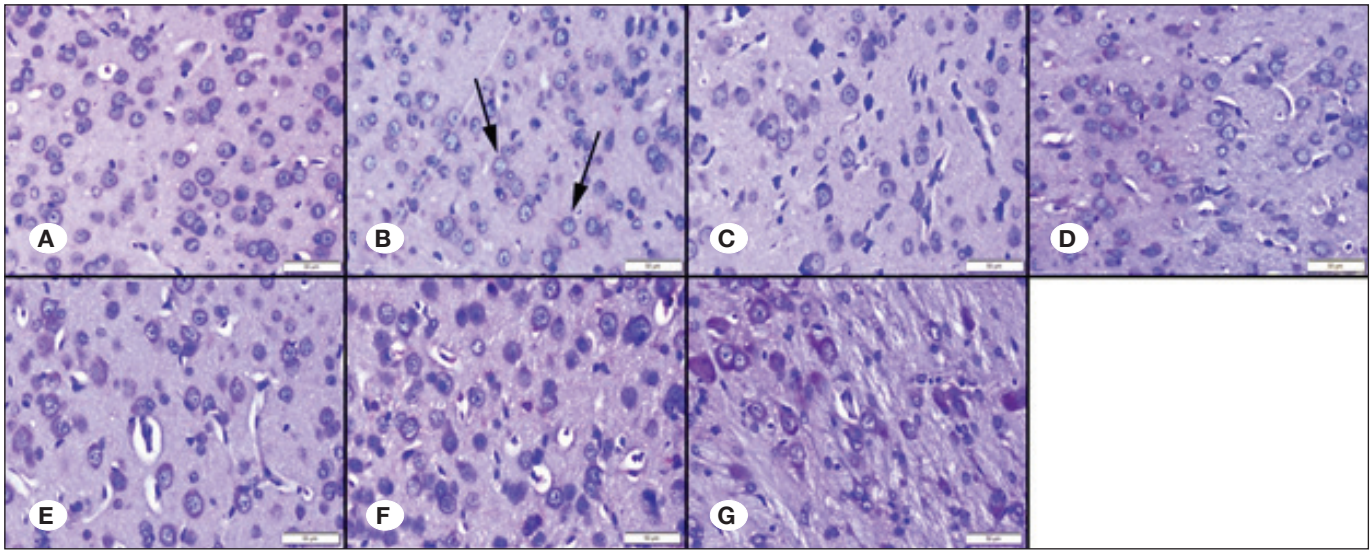

Figure 7: GABA receptor immunohistochemistry findings in the brain cortex between the groups. **A)** Marked expression in neurons in the control group. **B)** A marked decrease in expression in neurons (arrows) in the trauma group. **C)** Slight increase in expression in neurons in the memantine group. **D)** A moderate increase in neuron expression in the Pre trauma treatment with the Vit D group. **E)** A moderate increase in expression in neurons in the Post-trauma treatment with the Vit D group. **F)** A moderate increase in expression in neurons in the Memantine + post-trauma treatment with the Vit D group. **G)** A marked increase in expression in neurons in the Pre Vit D + Post trauma Mem + Vit D groups. Streptavidin biotin peroxidase method, scale bars=50μm.

GABA Receptor Immunohistochemistry Findings in the Hippocampus

In the hippocampus, GABA receptor immunohistochemistry revealed decreased expression levels in the trauma group (Group II, 28.50 ± 1.19). Memantine administration (Group III) led to increased expression levels (34.50 ± 1.19). Group IV and Group V also showed increased GABA expression levels (34.25 ± 1.38 and 35.62 ± 1.59, respectively). Group VI showed improved effects (44.00 ± 2.00). However, Group VII exhibited the most substantial therapeutic effects (55.37 ± 4.10) (Table I, Figure 8).

Biochemical Findings

Examination of the 8-OHdG/106dG ratios revealed that the trauma group had the highest values, notably differing from

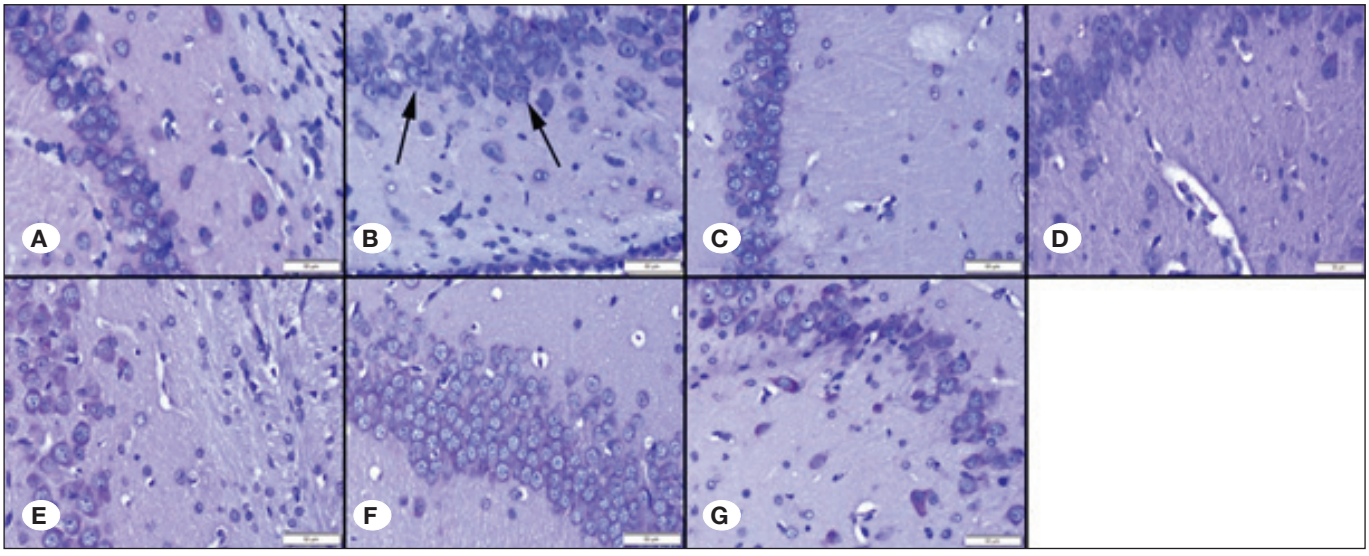

Figure 8: GABA receptor immunohistochemistry findings in the hippocampus among the groups. **A)** Marked expression in the control group. **B)** A marked decrease in neurons (arrows) in the trauma group. **C)** Slight increase in neurons in the memantine group. **D)** Moderately increased neuron expression in the Pre trauma treatment with Vit D group. **E)** A moderate increase in neurons in the Post-trauma treatment with the Vit D group. **F)** A moderate increase in neurons in the Memantine + post-trauma treatment with the Vit D group. **G)** A marked increase in neurons in the Pre Vit D + Post trauma Mem + Vit D groups. Streptavidin biotin peroxidase method, scale bars=50μm.

Table II: The Levels of All Variables in Groups

	Control	Trauma	Memantine	Pre Vit D	Post Vit D	Post Mem + Vit D	Pre Vit D + +Post Vit D+ Mem
8-OHdG / 10^6dG	0.81 ± 0.14^{a}	3.12 ± 0.25^{b}	1.85 ± 0.33^{c}	1.53 ± 0.31^{c,d}	1.26 ± 0.21^{d}	1.25 ± 0.38^{d}	0.82 ± 0.07^{a}
NOX-4 (μmol/L)	8.12 ± 0.83^{a}	18.5 ± 6.56^{c}	14.8 ± 3.25^{b}	13.5 ± 2.21^{b}	14.9 ± 2.31^{b}	8.96 ± 1.46^{a}	9.82 ± 1.07^{a}
MMP-2 (ng/mL)	30.8 ± 3.65^{a}	46.3 ± 4.94^{c}	35.8 ± 3.97^{a}	44.1 ± 6.09^{c}	43.1 ± 5.07^{b,c}	36.6 ± 3.83^{a,b}	31.6 ± 1.92^{a}
MMP-9 (ng/mL)	0.42 ± 0.07^{a}	0.87 ± 0.11^{c}	0.98 ± 0.09^{b}	0.74 ± 0.09^{b}	0.75 ± 0.06^{b}	0.73 ± 0.11^{b}	0.47 ± 0.07^{a}
TIMP-1 (ng/mL)	1.68 ± 0.19^{a}	0.56 ± 0.11^{d}	1.05 ± 0.27^{b,c}	1.26 ± 0.26^{b,c}	0.93 ± 0.07^{c}	1.31 ± 0.32^{b}	1.11 ± 0.06^{b,c}
TIMP-2 (ng/mL)	8.02 ± 0.97^{a}	3.21 ± 0.17^{d}	4.47 ± 0.44^{c}	3.91 ± 0.77^{c,d}	4.22 ± 0.24^{c,d}	3.98 ± 0.28^{c,d}	6.55 ± 0.95^{b}

Different letters in the same row show significant differences. P<0.05. The results were expressed as mean±standart deviation.
^{a}p<0.001 vs Control group, ^{b}p<0.001 vs Trauma group, ^{c}p<0.001 vs Post trauma Memantin group, ^{d}p<0.001 vs Pre trauma Vit D group, ^{e}p< 0.001 vs Post trauma Vit D group, ^{f}p<0.001 vs Pre trauma Vit D +Post trauma Mem + Vit D group.

other groups. The Pre Vit D+Mem+Post Vit D group displayed lower levels, not significantly differing from the control group. Less memantine was used in the trauma group than in any other group, but more memantine was used in all groups except the Pre Vit D group. The Pre Vit D, Post Vit D, and Mem+Post Vit D groups showed no notable differences in 8-OHdG/106dG levels (Table II, Figure 9).

The NOX-4 levels in the trauma group notably surpassed those in the other groups, with the control group having the lowest readings. Although the control group's levels were similar to those of both the Mem+Post Vit D and Pre Vit D+Mem+Post Vit D groups, the memantine group's levels were lower than those of the trauma group. The memantine, Pre-Vit D, and Post-Vit D groups had similar NOX-4 levels, but they were discernibly lower than those in the trauma group and higher than those in the control group, Mem+ Post-Vit D, and Pre-Vit D + Mem + Post-Vit D groups (Table II, Figure 10).

The MMP-2 levels in the Pre Vit D+Mem+Post Vit D group were noticeably lower than those in the other groups, with no significant difference from the control group. The levels in the Mem+Post Vit D group were lower than those in the Pre Vit D and Trauma groups but remained similar to the control, memantine, and Pre Vit D+Mem+Post Vit D groups (Table II, Figure 11).

Regarding MMP-9, the Pre Vit D+Mem+Post Vit D group's readings were distinctly lower than most, except for the control group. The Trauma group showed the highest MMP-9 levels, surpassing all other groups. The memantine, Pre Vit D, Post Vit D, and Mem+Post Vit D groups exhibited similar MMP-9 values (Table II, Figure 12).

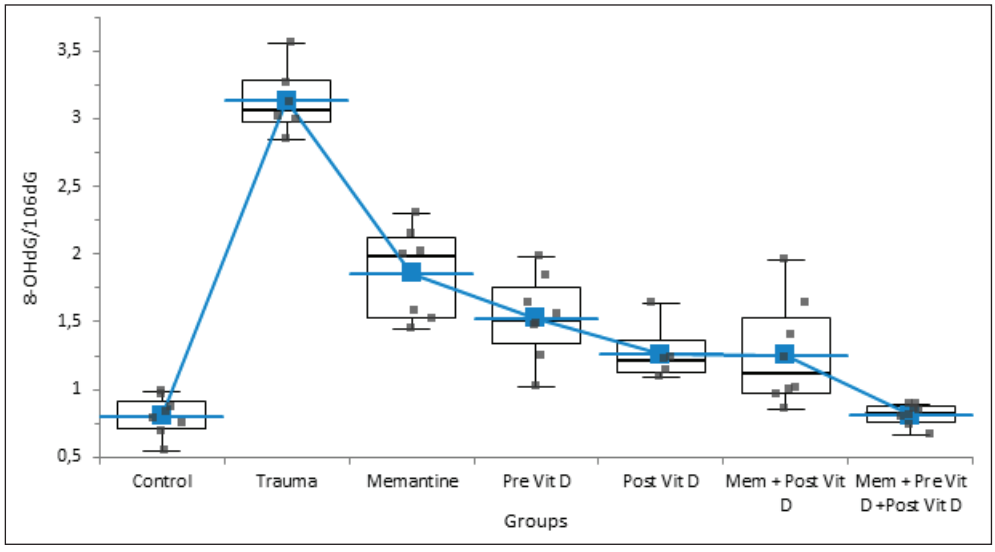

Figure 9: Distribution of 8-OHdG/10^6 dG levels between groups. Box plot presentation.

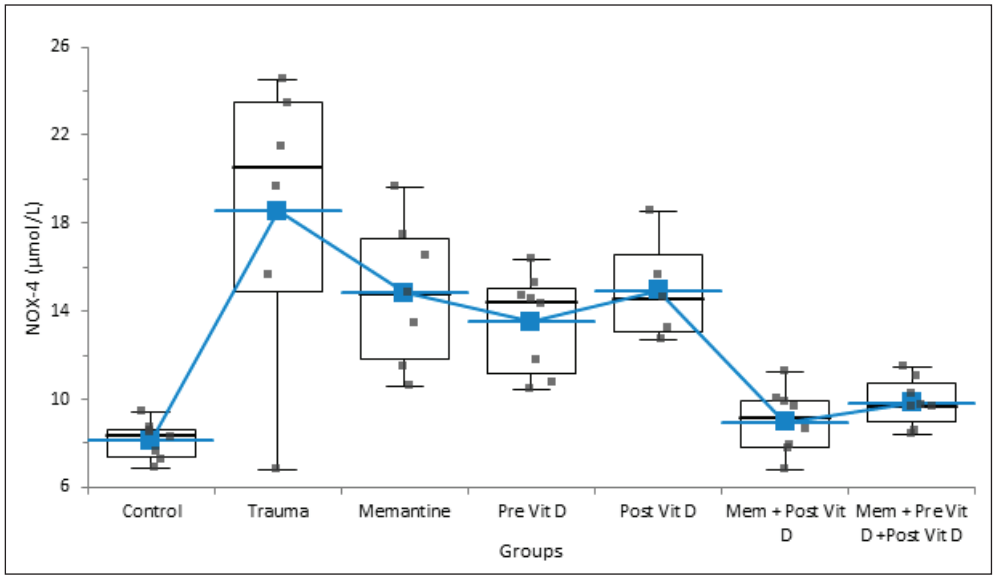

Figure 10: Distribution of NOX-4 (µmol/L) levels between groups. Box plot presentation.

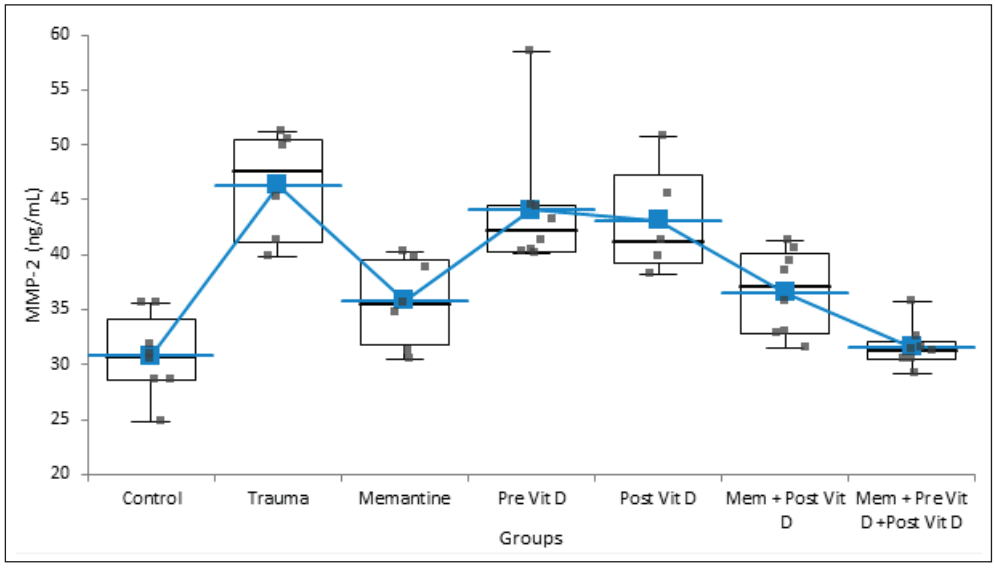

Figure 11: Distribution of MMP-2 ng/mL) levels between groups. Box plot presentation.

TIMP-1 levels were highest in the control group, contrasting with all other groups. The Trauma group had the lowest TIMP-1 levels, while among the treatment groups, those in the Post Vit D group were the lowest. The Mem+Post Vit D group had the highest TIMP-1 level among treatments, yet it was not significantly different from the Memantine, Pre Vit D, and Pre Vit D+Mem+Post Vit D groups (Table II, Figure 13).

The peak TIMP-2 levels were observed in the control group, which contrasted notably with other groups. The Trauma group had the lowest TIMP-2 levels, but among treatments, the Pre Vit D+Mem+Post Vit D group was the highest, differing notably from all groups except the control (Table II, Figure 14).

Correlation analysis revealed a clear positive association between 8-OHdG/106 dG and NOX-4 levels. Additionally, 8-OHdG/106dG levels positively correlated with both MMP-2 and MMP-9 levels. NOX-4 was negatively correlated with TIMP-1 and TIMP-2 but positively correlated with MMP-2 and MMP-9. Clear positive and negative correlations were found between TIMP-1 and TIMP-2 and MMP-2 and MMP-9, respectively. All the correlations are presented in Table III and Figure 14.

DISCUSSION

The results of this study indicate that the use of Vit D and/or memantine, either separately or in combination, promotes healing in repetitive brain injury in juvenile rats. Notably, combined usage provided more substantial healing effects through modulation of mTOR, TRPM2, and GABA expression levels.

The use of Vit D in patients with head trauma has been extensively studied. According to a recent randomized, placebo-controlled study, Vit D administration at different doses had different effects on serum inflammatory factor

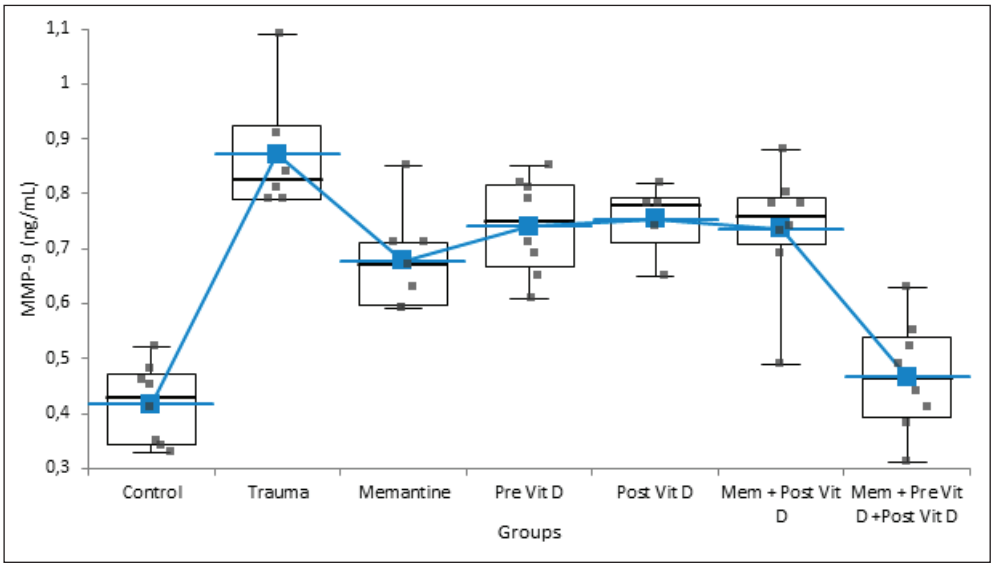

Figure 12: Distribution of MMP-9 ng/mL) levels between groups. Box plot presentation.

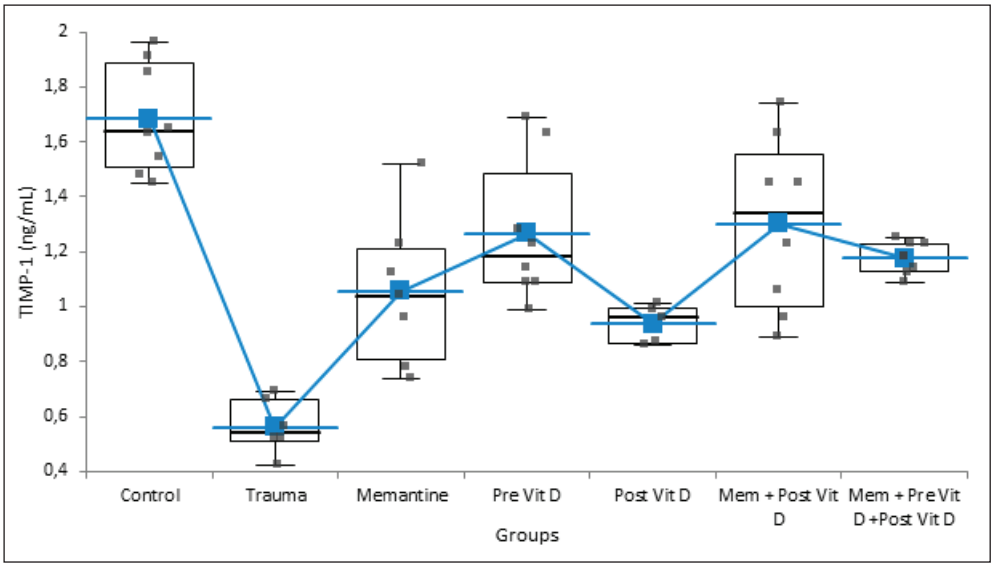

Figure 13: Distribution of TIMP-1 ng/mL) levels between groups. Box plot presentation.

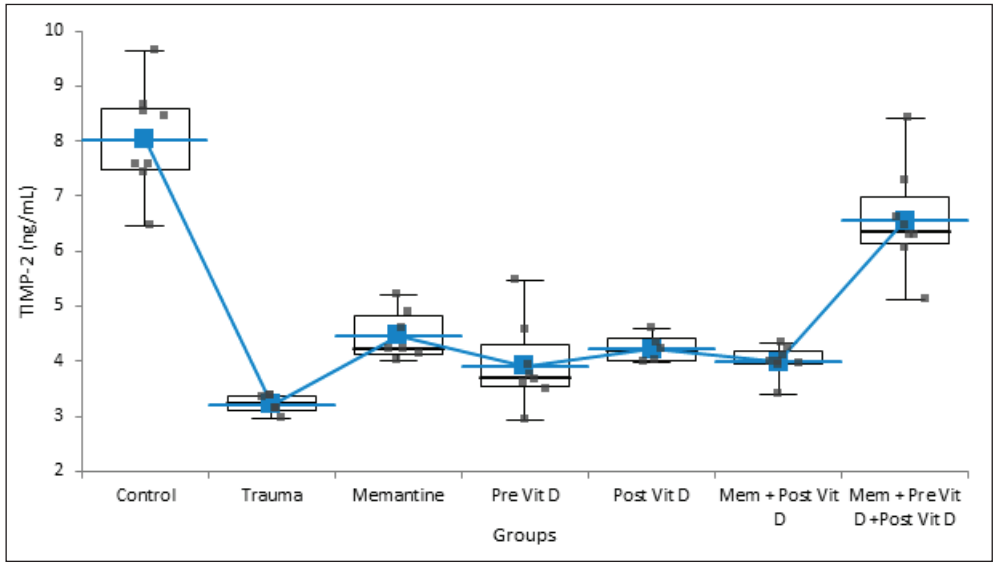

Figure 14: Distribution of TIMP-2 ng/mL) levels between groups. Box plot presentation.

Table III: The Correlation Analysis of Variables

Correlations		8-OHdG/10⁶dG	NOX-4 (µmol/L)	TIMP-1 (ng/mL)	TIMP-2 (ng/mL)	MMP-9 (ng/mL)	MMP-2 (ng/mL)
8-OHdG/10⁶dG	r	1					
	p						
	N	50					
NOX-4 (µmol/L)	r	0.626**	1				
	p	**0.000**					
	N	50	50				
TIMP-1 (ng/mL)	r	-0.661**	-0.612**	1			
	p	**0.000**	**0.000**				
	N	50	50	50			
TIMP-2 (ng/mL)	r	-0.613**	-0.555**	0.564**	1		
	p	**0.000**	**0.000**	**0.000**			
	N	50	50	50	50		
MMP-9 (ng/mL)	r	0.597**	0.526**	-0.527**	-0.789**	1	
	p	**0.000**	**0.000**	**0.000**	**0.000**		
	N	50	50	50	50	50	
MMP-2 (ng/mL)	r	0.530**	0.579**	-0.456**	-.690**	.667**	1
	p	**0.000**	**0.000**	**0.001**	**.000**	**.000**	
	N	50	50	50	50	50	50

***. Correlation is significant at the 0.01 level (2-tailed). **r=** Correlation coefficient.*

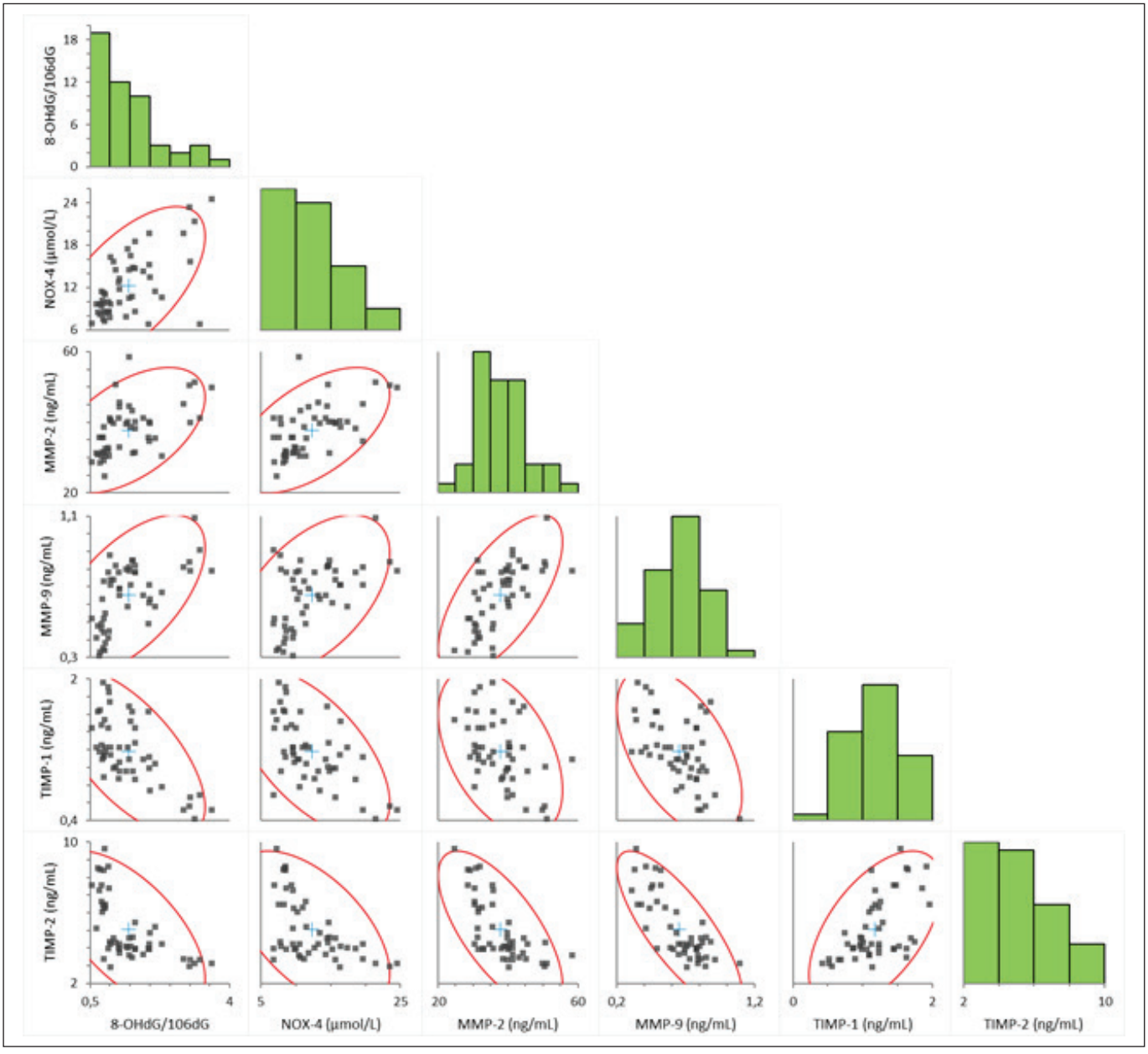

Figure 15: The correlation matrix between variables.

levels and mortality in patients with severe TBI. Based on the findings of this study, Vit D may help patients with TBI achieve better clinical outcomes by lowering the rates of infection, inflammation-related morbidity, and death.

The age group in that study ranged from 18 to 65 years, encompassing young adults, adults, and older patients (19). A separate review focused on the relationship between Vit D and neonatal hypoxic-ischemic brain injury. This review demonstrated that Vit D administration is beneficial in mitigating brain damage (41). Vit D has been identified as a protector against traumatic brain injury through the modulation of microglial polarization and neuroinflammation, dependent on the TLR4/MyD88/NF-κB pathway (23). Another study investigated the synergistic effects of Vit D and progesterone on brain injury, particularly in elderly patients with head trauma, and reported significant results (8).

Our study differs from the existing literature in several ways.

The first distinction lies in the age range of our study group, which aligns with the infancy and play-age childhood group. Children are particularly vulnerable to trauma, and, to our knowledge, no study specifically addresses this age group. The second distinction involves the severity of trauma. Recurrent mild head trauma during infancy and play-age childhood, occurs for various reasons, often due to falling, getting up, or bumping one's head, is common. Our study emphasizes the

importance of addressing these types of injuries. However, the mechanism of action observed in our study aligns with that reported in the literature. Consistent with previous findings, we found that the combined use of Vit D led to more effective healing outcomes.

Memantine is widely used in the clinical treatment of many neurological diseases. There have been various studies have been various studies investigating memantine's mechanism of action, and it has been investigated in several TBI models (6,12,26,36). A study conducted in 2018, explored memantine's effect on repetitive mild brain damage using adult rats. This study demonstrated the effectiveness of memantine in this context (31). Similarly, our study focused on repetitive mild brain injury but differed in the age range of the subjects. Additionally, we revealed another difference by examining the combined effects of Vit D.

Memantine is known to protect brain tissue from apoptosis and excitotoxicity through NMDA-type glutamate receptors following TBI (26). Moreover, supplementing certain vitamins might enhance the efficacy of drugs.

Accordingly, we investigated whether Vit D supplementation amplifies the effects of memantine. Our findings indicated that Vit D has supportive and protective effects against the rmTBI model. Administration of Vit D before and after trauma significantly reduced mTOR, TRPM2 expression, MMP-2 and MMP-9 levels, as well as DNA damage in the hippocampal and cerebral cortex of young rats with rmTBI. However, decreased GABA receptor levels in the trauma group were altered in Groups II, IV, V, and VI, with the most protective effect observed in Group VII.

Studies have highlighted various intracellular signaling cascades in the pathogenesis of rmTBI. Increasing evidence suggests that mTOR signaling is an important control mechanism of synaptic plasticity.

Recent studies have suggested that the neurodevelopmental processes associated with neuropsychiatric disorders involve the mTOR signaling cascade (18). Phosphorylation of mTOR and its downstream targets increased within 30 minutes post-TBI in adult rats (3). Our study observed a significant induction of mTOR expression in the trauma group, whereas combined memantine and Vit D administration markedly reduced mTOR expression levels in brain tissue and hippocampal regions. The immunomodulatory and antiproliferative properties of Vit D, particularly its hormonal form, 1,25-dihydroxy Vit D (1,25(OH)2D). These effects highlight the potential use of Vit D in the treatment of various disorders. By promoting DNA damage-inducible transcript 4 (DDIT4) and regulating the development and DNA damage response 1 (REDD1), two key components of the mTOR signaling cascade, 1,25(OH)2D can modulate the activity of these molecules (29). Our results align with these findings, with decreased mTOR expression observed in the brain cortex and hippocampus of Group VII. Alongside mTOR signaling outcomes, we also assessed DNA damage levels in Group VII. Another study highlighted the hippocampal neuroprotection induced by early inhibition of mTORC1 with a single dose of rapamycin in a TBI model (34).

Following TBI, there is an observed increase in reactive oxygen species (ROS), apoptotic cell death, and lipid peroxidation (17). These molecules are responsible for the secondary tissue damage. Vit D is a known antioxidant that protects cells against ROS (5). Our results also confirmed that Vit D alone significantly reduced TOS levels in the brain.

In response to rmTBI, several physiopathological cascades occur, including excitotoxicity and oxidative stress. we investigated the impact of Vit D on the expression levels of the TRPM2 channel, a cation-permeable ion channel activated by oxidative stress, in both the hippocampal and cerebral cortex regions. Consistent with previous studies (9), we found significantly increased TRPM2 expression levels in these areas. However, memantine and Vit D supplementation notably decreased these levels. Particularly, Vit D supplementation, both pre- and post-trauma, regulated TRPM2 expression levels in control group animals compared to injury model rats. Inflammation, cell death mediated by inflammation, and oxidative stress have all been shown to activate TRPM2 channels. Inflammation has a key role in rmTBI because it promotes neurotoxicity. We found similar histopathological findings in in the brain cortex of the animals in the trauma group. TRPM2 channels regulate intracellular calcium (Ca2+) levels, which increase in response to oxidative stress, subsequently heightening the susceptibility to apoptotic cell death. TRPM2 channels participate in neuroinflammation following tissue damage, as in (27).

MMPs are a broad family of zinc-dependent endopeptidases secreted by various cell types, including astrocytes, endothelial cells, and neurons. They are primarily responsible for apoptosis and remodeling of the extracellular matrix (1). Their levels typically rise during pathophysiological conditions to break down elements of the extracellular matrix and interfere with the blood-brain barrier. MMPs dysregulate apoptotic cascades following tissue damage by triggering specific receptors and mediators. Additionally, hormones, cytokines, and growth factors can regulate MMP activation, which may vary during development and adolescence.

Oxidative agents, known as ROS, readily target guanine bases in DNA, leading to the formation of 8-hydroxydeoxyguanosine (8-OHdG). This compound tends to pair with thymidine instead of cytosine. Therefore, 8-OHdG is commonly viewed as an indicator of mutations resulting from oxidative damage.

Increased ROS levels can cause DNA damage and strand fracture. ROS readily attack DNA's guanine bases to produce 8-OHdG. Generally, 8-OHdG is considered as a biomarker of oxidative stress-related mutagenesis (33-35). We measure 8-OHdG levels from nucleic acids extracted from the brain tissue relative to dG to assess DNA damage (4). Elevated 8-OHdG levels have been observed in patients with neurodegenerative diseases (30). In our study, 8-OHdG/106 dG levels were significantly higher in the trauma group. However, administering Vit D both before and after trauma, particularly in combination with memantine, significantly reduced 8-OHdG/106 dG levels. These findings suggest a protective role for Vit D against mutations.

Enzymes repair damaged DNA without 8-OHdG, although postmitotic neurons have limited DNA repair capacity and may be particularly vulnerable to modified nucleotide damage. Dysfunctional DNA repair can trigger apoptosis in neurons (22). Our study showed that rmTBI could increase 8-OHdG accumulation due to oxidative damage and further disrupt the DNA damage mechanism.

These findings indicate that brain trauma caused marked histopathological lesions in the cortex, but not in the hippocampus or cerebellum. Immunohistochemical findings showed increased expressions of mTOR and TRPM while GABA expressions decreased in the brain cortex, hippocampus, and cerebellum. Memantine and Vit D treatments resulted in amelioration, with the most effective improvement observed in the Pre Vit D + Post trauma Mem + Vit D group.

CONCLUSION

Vit D administration plays a crucial role in juvenile rats. Supplementation with Vit D before and after trauma enhances the effectiveness of memantine by regulating intracellular signaling cascades and reducing oxidant molecule levels. Administering Vit D can thereby reduce DNA damage levels. Practical and easy use of Vit D and memantine supplementation may offer better outcomes against the rmTBI model in juveniles.

Declarations

Funding: This study did not receive any financial support from private or official institutions and was personally funded.

Availability of data and materials: Authors can confirm that all relevant data are included in the article and/or its supplementary information files.

Disclosure: Each author hereby declares that their interests do not conflict.

AUTHORSHIP CONTRIBUTION

Study conception and design: IG
Data collection: MEA, OA
Analysis and interpretation of results: OO, HHA
Draft manuscript preparation: HA, IG
Critical revision of the article: IG, HHA
All authors (IG, HA, MEA, OO, HHA, OA) reviewed the results and approved the final version of the manuscript.

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