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Combined effects of melatonin and valproic acid in cerebral ischemia via modulation of oxidative stress and melatonin receptors

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ABSTRACT

Background: Ischemic stroke is a major cause of mortality and disability worldwide, with limited effective neuroprotective treatments. Epigenetic modulation and oxidative stress regulation have emerged as promising therapeutic targets. This study aimed to evaluate the neuroprotective effects of melatonin and valproic acid, alone and in combination, in a rat model of cerebral ischemia.

Methods: Male Wistar rats were randomly divided into nine groups including sham, MCAO, vehicle, melatonin, valproic acid, and different combination treatment groups. Cerebral ischemia was induced using the middle cerebral artery occlusion (MCAO) method. Neurological deficits, infarct volume, brain edema, antioxidant activity, and gene expression of HDAC1, HDAC3, MT1, and MT2 were evaluated.

Results: Both melatonin and valproic acid significantly improved neurological outcomes, reduced infarct volume, and decreased brain edema compared to the MCAO group. Combination therapy, particularly at high doses, showed significantly greater neuroprotective effects than monotherapy. These effects were associated with downregulation of HDAC1 and HDAC3 expression, upregulation of MT1 and MT2 receptors, and increased antioxidant enzyme activity including catalase and superoxide dismutase levels.

Conclusion: Combined administration of melatonin and valproic acid exerts synergistic neuroprotective effects in cerebral ischemia through modulation of oxidative stress, epigenetic regulation, and melatonin receptor signaling. This combination may represent a promising therapeutic strategy for ischemic stroke.

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KEYWORDS
Cerebral ischemia; MCAO; melatonin; valproic acid; oxidative stress

Introduction

Stroke is a cerebrovascular disorder caused by interruption of cerebral blood flow. It occurs when cerebral vessels are occluded by thrombus formation or atherosclerotic processes, or when they rupture due to aneurysm or vascular malformations. Stroke is broadly classified into two main types: ischemic stroke (IS), resulting from reduced cerebral blood flow and subsequent neuronal injury, accounting for approximately 85% of all cases; and hemorrhagic stroke, caused by vessel rupture and representing about 15% of cases [1].

Due to the limited effectiveness of current pharmacological interventions for cerebral ischemia, recent research has increasingly focused on epigenetic regulation and chromatin remodeling as promising therapeutic strategies for mitigating ischemic brain injury [2]. Epigenetic modifications refer to heritable changes in gene expression that occur without alterations in DNA sequence. The nucleosome, as the fundamental unit of chromatin, consists of DNA wrapped around a histone octamer. Accordingly, histone acetylation and deacetylation play a central role in regulating chromatin structure and gene transcription, and it has been estimated that histone acetylation regulates approximately 2–10% of gene transcription [3]. Increasing evidence has demonstrated that inhibition of histone deacetylases (HDACs) exerts neuroprotective effects in both in vitro and in vivo models of ischemic stroke [4].

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Valproic acid (VPA), a short-chain fatty acid and HDAC inhibitor, is widely used as an anticonvulsant and mood stabilizer, particularly in epilepsy and bipolar disorder. Clinical observations have suggested a reduced incidence of ischemic stroke in patients receiving VPA [5]. Mechanistically, VPA exerts neuroprotective effects through multiple signaling pathways, including inhibition of caspase-3 activation and activation of pro-survival pathways such as ERK and Akt [6]. In addition, VPA has been reported to reduce oxidative stress in experimental models of cerebral ischemia [7].

Melatonin is an endogenous neurohormone synthesized by the pineal gland, with secretion regulated by the circadian light – dark cycle. In addition to its potent antioxidant properties, melatonin functions as a pleiotropic regulatory molecule acting through MT1 and MT2 receptors [8]. Because the brain has relatively low antioxidant defenses and is highly vulnerable to reactive oxygen species (ROS), oxidative stress plays a critical role in ischemia – reperfusion injury. ROS contribute to blood – brain barrier disruption, cerebral edema, mitochondrial dysfunction, and neuronal death.

Mitochondrial dysfunction is recognized as one of the earliest and most critical events following cerebral ischemia. Impaired mitochondrial respiration results in excessive ROS generation, ATP depletion, calcium overload, and activation of apoptotic pathways, thereby exacerbating neuronal injury. Therefore, preservation of mitochondrial integrity and attenuation of oxidative stress have emerged as important therapeutic targets for limiting ischemic brain damage [9–11].

Melatonin has been shown to counteract these pathological processes through direct free radical scavenging, preservation of mitochondrial function, modulation of antioxidant defense systems, and receptor-mediated mechanisms, thereby reducing oxidative injury following cerebral ischemia [10–12].

Importantly, although both valproic acid and melatonin have individually demonstrated neuroprotective effects in experimental ischemic stroke, they exert these effects through partially distinct yet complementary mechanisms. Valproic acid primarily mediates neuroprotection through HDAC inhibition, epigenetic regulation, activation of pro-survival signaling pathways, and attenuation of oxidative stress [7,13], whereas melatonin exerts potent antioxidant, anti-inflammatory, and mitochondria-protective effects through both receptor-dependent and receptor-independent mechanisms [10–12]. Interestingly, valproic acid has also been shown to epigenetically upregulate melatonin MT1 receptor expression [14], suggesting a potential interaction between HDAC inhibition and melatonin receptor signaling. Furthermore, experimental evidence indicates that melatonin may attenuate oxidative stress and neuronal toxicity associated with valproic acid under certain conditions [15]. Collectively, these findings provide a strong mechanistic rationale for investigating the combined administration of valproic acid and melatonin as a multi-target therapeutic strategy for ischemic stroke. However, despite these complementary mechanisms, their combined effects have not been systematically evaluated in experimental cerebral ischemia. Therefore, the present study was designed to determine whether combined administration of valproic acid and melatonin provides greater neuroprotective efficacy than either monotherapy and whether these effects are associated with modulation of oxidative stress and melatonin receptor signaling.

Materials and methods

In this study, a total of 252 adult male Wistar rats (aged approximately 6–8 weeks and weighing 240–260 g) were randomly selected. All experimental procedures were conducted in accordance with international guidelines for animal care and were approved by the Ethics Committee of Zanjan University of Medical Sciences. The animals were randomly assigned into nine groups ($n = 28$ per group).

The sham group underwent a neck incision, and the common carotid artery (CCA), internal carotid artery (ICA), and external carotid artery (ECA) were exposed and then sutured without induction of ischemia, in order to simulate surgical stress. The MCAO (model) group included animals subjected to middle cerebral artery occlusion without receiving any treatment. The vehicle group consisted of animals subjected to MCAO and injected with the same vehicle used for melatonin preparation (saline containing < 1–2% ethanol) to control for solvent effects. The VPA group (V) included animals subjected to MCAO and treated with valproic acid (300 mg/kg). The melatonin group (M) consisted of animals subjected to MCAO and treated with melatonin (10 mg/kg). The high-dose combination group (High) included animals subjected to MCAO and treated with valproic acid (300 mg/kg) and melatonin (10 mg/kg). The low-dose combination group (Low) included animals subjected to MCAO and treated with valproic acid (20 mg/kg)

and melatonin (4 mg/kg). The HV+LM group consisted of animals subjected to MCAO and treated with high-dose valproic acid (300 mg/kg) and low-dose melatonin (4 mg/kg). The LV+HM group included animals subjected to MCAO and treated with low-dose valproic acid (20 mg/kg) and high-dose melatonin (10 mg/kg).

Each experimental group consisted of 28 rats, which were randomly allocated into four subgroups (n = 7 per subgroup) for different outcome measures. Seven animals were used for neurological deficit scoring and infarct volume analysis using TTC staining, seven animals were used for brain edema measurement, seven animals were used for gene expression analysis (HDAC1, HDAC3, MT1, MT2) by qRT-PCR, and seven animals were used for biochemical assays including measurement of CAT and SOD in brain tissue.

All allocations were determined prior to experimentation to ensure proper randomization and avoidance of selection bias. All behavioral, histological, molecular, and biochemical assessments were performed by investigators blinded to the experimental group allocation. Tissue samples were coded until completion of data analysis, and group identities were revealed only after completion of statistical analysis.

All experimental procedures were conducted in accordance with international guidelines for animal care and were approved by the Ethics Committee of Zanjan University of Medical Sciences (Approval No. IR.ZUMS.AEC.1403.029).

Drug administration

Valproic acid was dissolved in normal saline (water) and administered at doses of 300 mg/kg and 20 mg/kg. Based on the average body weight of the rats (250 g), 75 mg (for 300 mg/kg) and 5 mg (for 20 mg/kg) of valproic acid were dissolved in 1.5 mL of saline, thoroughly mixed using a vortex device, and injected intraperitoneally 30 minutes after ischemia induction [16]. The selected dose range was based on previous preclinical studies demonstrating neuroprotective effects of valproic acid in rodent models of cerebral ischemia, allowing evaluation of both lower and higher effective pharmacological ranges.

Melatonin was initially dissolved in a minimal volume of ethanol to prepare a stock solution and then diluted with normal saline to obtain the final working solution. The final ethanol concentration in the injected solution was kept below 1–2% to avoid any solvent-related biological effects. Melatonin was administered at doses of 10 mg/kg and 4 mg/kg. Based on the average weight of 250 g, 2.5 mg (10 mg/kg) and 1 mg (4 mg/kg) of melatonin were prepared, thoroughly mixed, and injected intraperitoneally 30 minutes after ischemia induction [17]. The dose selection was based on previously reported effective neuroprotective doses of melatonin in experimental cerebral ischemia models, which typically fall within a narrow and well-established therapeutic range.

All injections were performed intraperitoneally. To ensure sterility and prevent contamination, a new syringe and needle were used for each injection, and the injection site was disinfected with alcohol.

Induction of cerebral ischemia

Cerebral ischemia was induced using a right middle cerebral artery occlusion (Rt. MCAO) model. Animals were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg). The rats were placed in a supine position, and a 2 cm midline incision was made in the neck. After separating the tissues, the right common carotid artery (CCA) was exposed and carefully isolated from the vagus nerve up to its bifurcation.

The internal and external carotid arteries were then identified. The external carotid artery was ligated distally, and the common carotid artery was ligated proximally. In this condition, the internal carotid artery remained the only extracranial branch supplying the right hemisphere.

A vascular clip was applied to occlude the distal part of the common carotid artery. A rounded-tip filament was then introduced through an incision in the CCA and advanced until resistance was encountered, corresponding to the origin of the middle cerebral artery from the Circle of Willis. This procedure effectively blocked blood flow to the right hemisphere. The filament remained in place for 60 minutes and was then gently withdrawn to allow reperfusion.

After removal of the filament, cerebral blood flow was restored via the Circle of Willis. The incision site was sutured, and the animal was placed in a clean cage at an appropriate temperature to prevent hypothermia. Respiratory status was monitored throughout recovery [18].

Neurological deficits assessment

24 hours after ischemia induction, animals were evaluated using the Bederson criteria and their neurological status was scored [18]. Score 0 indicated no observable neurological deficit. Score 1 (incomplete extension of the contralateral forepaw) was considered a mild focal neurological deficit. Score 2 (circling to the left) indicated a moderate focal neurological deficit. Score 3 (falling to the left side) represented a severe focal deficit. Score 4 indicated inability to walk spontaneously and a reduced level of consciousness. Score 5 included animals that died within 12 hours after surgery, provided that post-staining revealed extensive brain damage and death was solely due to stroke.

Brain removal

At the specified time after ischemia induction, animals were anesthetized with a high dose of chloral hydrate and then decapitated using a guillotine. A longitudinal incision was made on the scalp using surgical scissors. The skin was retracted laterally to expose the skull. After removal of excess tissues, a cut was made along the parietal suture using round-tip scissors (to avoid damage to brain tissue). The skull was removed using bone scissors, and the brain was separated from the cranial base using a spatula and placed in cold physiological saline at 4°C for 10 minutes.

Measurement of tissue damage volume

To evaluate the extent of tissue damage, sectioning and staining using TTC (Sigma-Aldrich, U.S.A.) were performed. The brain was removed from the refrigerator and placed in a brain matrix. Coronal sections with a thickness of 2 mm were cut from the frontal to the temporal region. The sections were placed sequentially in a Petri dish, TTC solution was added, and the slices were incubated at 37°C for 10 minutes. After incubation, viable tissues appeared red due to the presence of the mitochondrial enzyme succinate dehydrogenase, whereas infarcted tissues lacking this enzyme appeared white. Images were taken from the sections, and infarct volume was calculated using ImageJ software according to the following formula:

$$\text{Corrected infarct volume} = \text{Left hemisphere volume} - (\text{right hemisphere volume} - \text{infarct volume})$$

Measurement of brain edema

To assess brain edema, brain water content was calculated. After brain extraction, the olfactory bulb, cerebellum, and brainstem were removed. The right and left hemispheres were separated along the parietal fissure. The hemispheres were weighed to obtain wet weight (WW). Then, the tissues were dried in an oven at 120°C for 24 hours. After drying, they were weighed again to obtain dry weight (DW). Brain water content percentage was calculated using the following formula:

$$[(\text{WW} - \text{DW}) \div \text{WW}] \times 100$$

Gene expression analysis

All procedures were performed under a biological hood sterilized with 75% ethanol. Due to the susceptibility of RNA to degradation by RNase, all materials and equipment were RNase-free. Total RNA was extracted from brain tissue using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, U.S.A.) according to the manufacturer's protocol. RNA concentration and purity were assessed using the A260/A280 absorbance ratio.

Complementary DNA (cDNA) was synthesized using the PrimeScript™ RT reagent kit (Takara Bio Inc., Japan, Cat. No. RR037A). Quantitative real-time PCR (qRT-PCR) was performed using gene-specific primers (Table 1). Relative gene expression levels were normalized to β-actin as the housekeeping gene

Table 1. Primers sequences.

Genes	Primers
HDAC1	F: CTACATTTGCCACAGTCTCAAG
	R: TCGTACTGGAGAGTTCCGGTTT
HDAC3	F: GAGGAGGAGGAGGAGGAGAA
	R: CTTCTTCAGGGTGGTGGTGA
MT1	F: CTCCCATGCTATCTACAGTAGT
	R: GGCCAGATTCACCACAAATAAA
MT2	F: CATGTACGTTGCTATCCAGGC
	R: CTCCTTAATGTCACGCACGAT
B-actin	F: CATGTACGTTGCTATCCAGGC
	R: CTCCTTAATGTCACGCACGAT

and calculated using the 2^−ΔΔCt method, and results were expressed as fold change relative to the control group. Primer sequences are listed in Table 1.

Measurement of antioxidant enzymes in brain tissue

The ischemic right cerebral hemisphere was rapidly excised under cold conditions (4°C) and homogenized in ice-cold phosphate buffer (50 mM, pH 7.4) containing 0.1 mM EDTA for biochemical analysis. The homogenates were centrifuged at 10,000– 12,000 × g for 15–20 min at 4°C, and the supernatants were collected for biochemical analysis. Protein concentration was determined using the Bradford method (Bio-Rad, U.S.A.), and all enzyme activities were normalized to total protein content.

Superoxide dismutase (SOD) activity was measured based on its ability to inhibit the reduction of nitroblue tetrazolium (NBT) (Sigma-Aldrich, U.S.A.) by superoxide radicals generated in a riboflavin – methionine – light system. Riboflavin and methionine were obtained from Sigma-Aldrich (U.S.A.). The reaction mixture contained riboflavin, methionine, and NBT in phosphate buffer. The reaction was initiated by exposure to light, and the formation of formazan was measured spectrophotometrically at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of NBT reduction. Results were expressed as U/mg protein.

Catalase (CAT) activity was measured by monitoring the decomposition rate of hydrogen peroxide (H_2O_2) (Merck, Germany). The decrease in absorbance of H_2O_2 was recorded spectrophotometrically at 240 nm for 1–3 min. Enzyme activity was calculated using the molar extinction coefficient of H_2O_2 and expressed as U/mg protein.

Statistical analysis

Data were analyzed using SPSS software. After confirming normal distribution using the Shapiro – Wilk test, neurological deficit scores were analyzed using the non-parametric Mann – Whitney U test. All other quantitative data, including infarct volume, brain edema, gene expression, and biochemical parameters, were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. Data are presented as mean ± SEM, and a p-value <0.05 was considered statistically significant.

Findings

Neurological deficit score (NDS)

Neurological deficits were evaluated using the Bederson test following MCAO induction. Both valproic acid (V) and melatonin (M) significantly reduced neurological deficit scores compared to the model group ($p < 0.05$). High-dose co-administration of valproic acid and melatonin (High group) significantly reduced neurological deficits compared to the model group ($p < 0.01$). All combined treatment groups (High, HV+LM, and LV+HM) showed significant improvement in neurological scores compared to the model group ($p < 0.05$). The High group showed significantly lower neurological deficit scores compared to the Low group ($p < 0.05$), indicating a dose-dependent effect of combined therapy. In addition, the High group showed a significant reduction in neurological deficits compared to the melatonin alone group (M) ($p < 0.01$) (Figure 1).

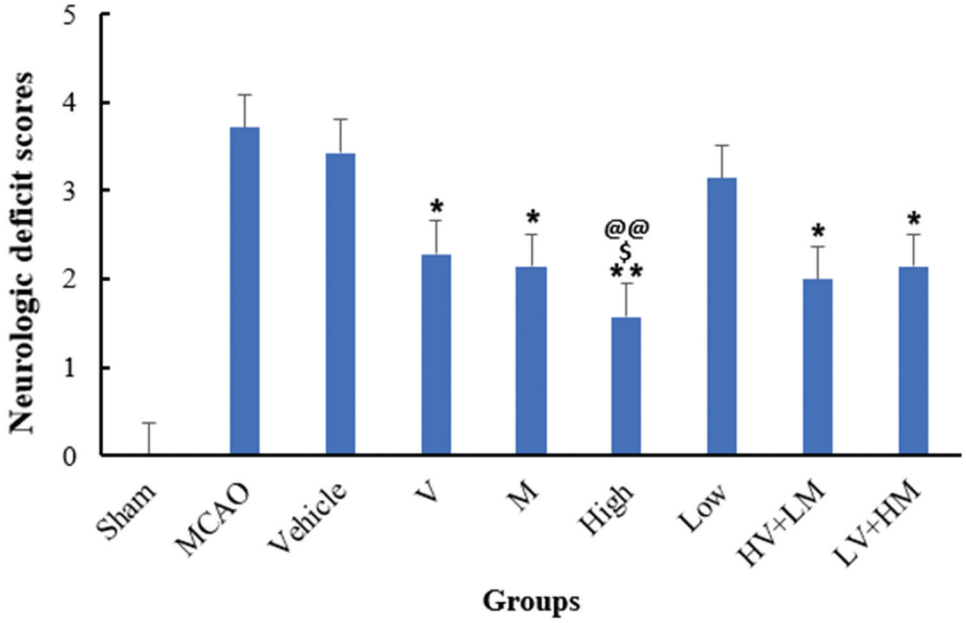

Figure 1. Neurological deficit scores (NDS). Neurological deficits were evaluated 24 hours after MCAO using the Bederson scoring system. M (Melatonin), V (Valproic Acid), Low (Low doses of Valproic Acid and melatonin), High (High doses of Valproic Acid and melatonin), HV+LM (High dose of Valproic Acid + Low dose of melatonin), LV+HM (Low dose of Valproic Acid + High dose of melatonin). $^{**}p < .01$, $^{*}p < .05$ compare to Model and Vehicle Groups, $\$p < 0.05$ compare to Low Group, $@p < 0.05$ compare to M Group.

Infarct volume

After staining with TTC, The damaded areas appear white and the healthy areas appear red (Figure 2(A)). Total infarct volume was significantly reduced in all treatment groups compared to the model and vehicle groups ($p < 0.001$). Valproic acid (V) and melatonin (M) alone significantly reduced infarct volume compared to the model group ($p < 0.001$).

Low-dose co-administration (Low group) also reduced infarct volume compared to the model group; however, this reduction was significantly smaller than that observed in the High group ($p < 0.001$). High-dose co-administration (High group) resulted in a significantly greater reduction in infarct volume compared to the Low group ($p < 0.001$). The High group also showed significantly lower infarct volume compared to both single-treatment groups (V and M) ($p < 0.001$). Both HV+LM and LV+HM groups significantly reduced infarct volume compared to the model group ($p < 0.001$), although their effects were less pronounced than the High group (Figure 2(B)).

Brain edema

Brain water content analysis showed a significant increase in the right hemisphere after MCAO compared to the left hemisphere and the sham group ($p < 0.001$). No significant difference was observed between the MCAO and vehicle groups ($p > 0.05$). Valproic acid (V) and melatonin (M) significantly reduced brain water content in the ischemic hemisphere compared to the model group ($p < 0.05$). High-dose co-administration (High group) significantly reduced brain edema compared to the model group ($p < 0.01$).

Both HV+LM and LV+HM groups also significantly reduced brain water content ($p < 0.01$ and $p < 0.05$, respectively). Low-dose combination (Low group) did not produce a significant reduction in brain edema. No significant differences were observed in the left hemisphere among groups (Figure 3).

HDAC1 and HDAC3 gene expression

MCAO significantly increased HDAC1 and HDAC3 expression compared to the sham group ($p < 0.001$). The vehicle group showed no significant difference compared to the model group. Valproic acid

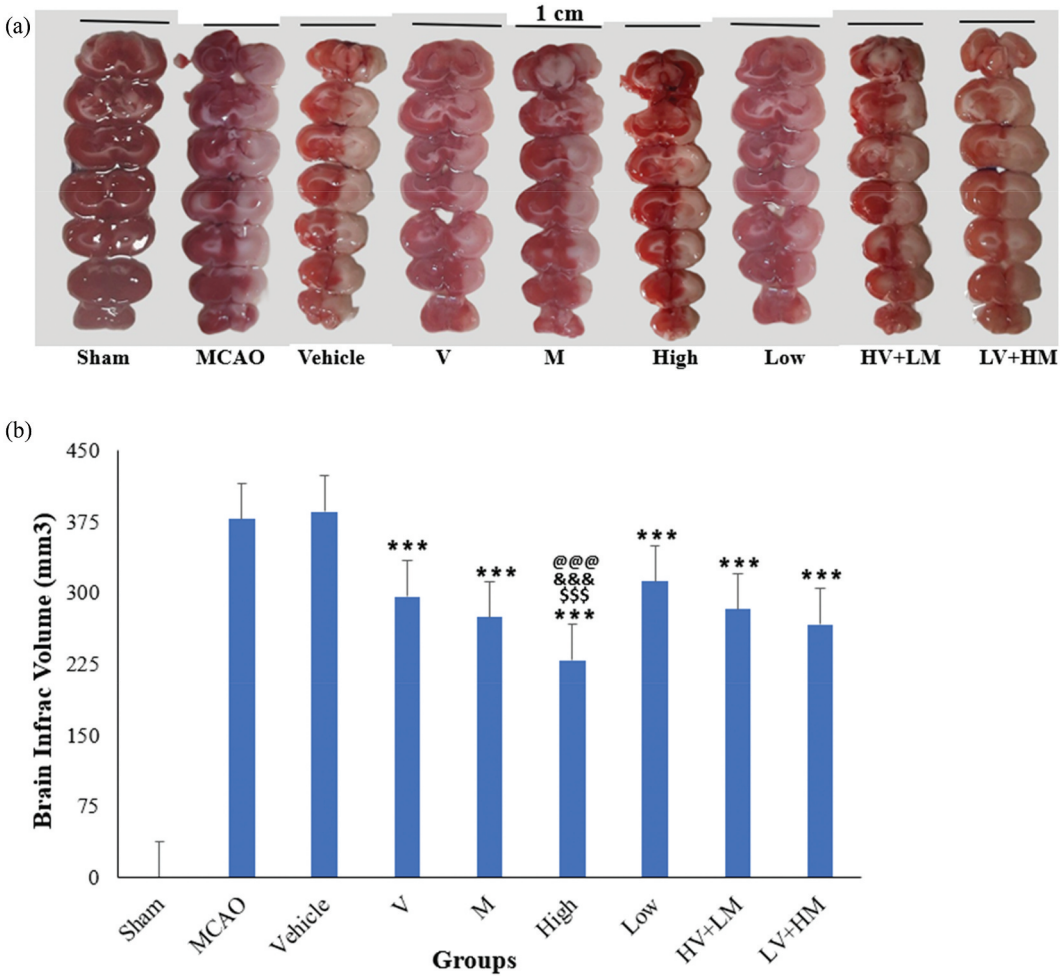

Figure 2. A. Brain tissue after staining with TTC. The red areas indicate healthy regions, and the white areas indicate ischemic brain injury. B. Infarct volume analysis. Total infarct volume was assessed using TTC staining. M (Melatonin), V (Valproic Acid), Low (Low doses of Valproic Acid and melatonin), High (High doses of Valproic Acid and melatonin), HV +LM (High dose of Valproic Acid + Low dose of melatonin), LV+HM (Low dose of Valproic Acid + High dose of melatonin). $^{**}p < .001$, compare to Sham Group, $^{*}p < .001$ compare to Model and Vehicle Groups, $\$\$p < 0.001$ compare to Low Group, $\&\&\&p < 0.001$ compare to V Group, $@@@p < 0.001$ compare to M Group.

(V) and melatonin (M) significantly reduced HDAC1 expression compared to the model group ($p < 0.05$). High-dose co-administration (High group) significantly reduced HDAC1 mRNA levels compared to the model group ($p < 0.001$) and also compared to single treatments (M and V) ($p < 0.05$) (Figure 4(A)).

For HDAC3, valproic acid (V, $p < 0.001$), melatonin (M, $p < 0.01$), and combination treatments (HV+LM and LV+HM, $p < 0.001$) all significantly reduced expression compared to the model group. High-dose co-administration (High group) showed significantly lower HDAC3 expression compared to the Low group ($p < 0.001$), indicating a dose-dependent effect. Low-dose combination (Low group) did not significantly reduce HDAC3 expression (Figure 4(B)).

MT1 and MT2 expression

MCAO significantly reduced MT1 (Figure 5(A)) and MT2 (Figure 5(B)) expression compared to the sham group ($p < 0.001$). Valproic acid (V) significantly increased MT1 ($p < 0.01$) and MT2 ($p < 0.05$) expression compared to the model group. Melatonin (M) significantly increased both MT1 and MT2 expression ($p < 0.001$). All combination groups (High, Low, HV+LM, LV+HM) significantly increased MT1 and MT2 expression compared to the model group ($p < 0.001$). High-dose co-administration (High group) showed

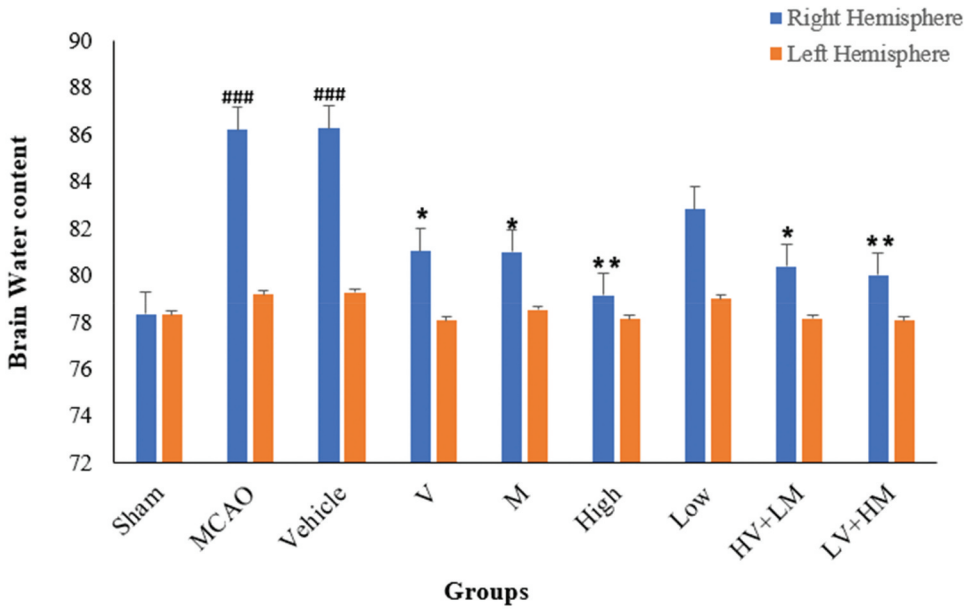

Figure 3. Brain edema (water content). Brain water content (%) was used to evaluate cerebral edema. M (Melatonin), V (Valproic Acid), Low (Low doses of Valproic Acid and melatonin), High (High doses of Valproic Acid and melatonin), HV +LM (High dose of Valproic Acid + Low dose of melatonin), LV+HM (Low dose of Valproic Acid + High dose of melatonin). $^{**}p < .001$ compare to Sham Group, $^{*}p < .01$, $^{\#\#\#}p < .05$ compare to Model and Vehicle Groups.

significantly higher MT1 and MT2 expression compared to the Low group ($p < 0.001$) and compared to single-drug treatments (M and V) ($p < 0.001$) (Figure 5(A,B)).

Antioxidant activity (CAT and SOD)

MCAO significantly reduced antioxidant activity compared to the sham group ($p < 0.001$). Catalase (CAT) activity was significantly increased following melatonin (M, $p < 0.001$) and valproic acid (V, $p < 0.05$) administration compared to the model group. High-dose co-administration significantly increased CAT activity compared to the model group ($p < 0.001$), while low-dose combination did not show a significant effect. Both HV+LM and LV+HM groups significantly increased CAT activity compared to the model group ($p < 0.01$). High-dose treatment showed significantly higher CAT activity compared to Low group ($p < 0.001$), as well as compared to V ($p < 0.001$) and M ($p < 0.01$) groups (Figure 6(A)).

Superoxide dismutase (SOD) (Figure 6(B)) levels were significantly increased following valproic acid (V, $p < 0.05$) and melatonin (M, $p < 0.01$) administration compared to the model group. High-dose co-administration (High group) significantly increased SOD levels compared to the model group ($p < 0.001$). The High group also showed significantly higher SOD ($p < 0.001$) levels compared to the Low group, indicating a dose-dependent effect of combined therapy (Figure 6(B,C)).

Discussion

The results of the present study demonstrated that administration of melatonin and valproic acid improves neurological outcomes following cerebral ischemia by enhancing antioxidant capacity and modulating the expression of HDAC genes and melatonin receptors. Among the treatment regimens, co-administration of melatonin and valproic acid at high doses produced the most pronounced neuroprotective effects, suggesting a potential additive or synergistic interaction between these two agents.

Cerebral ischemia is characterized by excessive production of reactive oxygen species (ROS) and a reduction in endogenous antioxidant defenses, leading to oxidative damage of lipids, proteins, and DNA, ultimately contributing to neuronal death and tissue injury. Therefore, strengthening antioxidant defense systems and reducing free radical formation represent key therapeutic strategies for improving

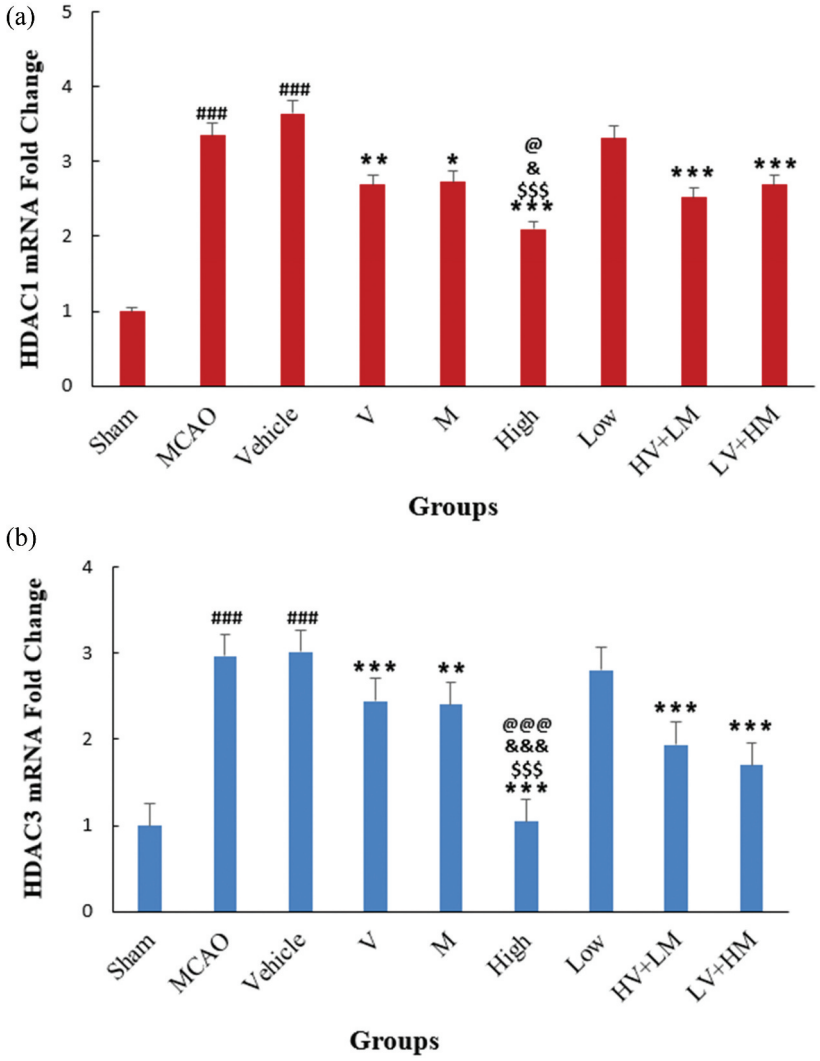

Figure 4. HDAC1 and HDAC3 mRNA expression. Relative HDAC1 gene expression was measured by qRT-PCR and normalized to β-actin.M (Melatonin), V (Valproic Acid), Low (Low doses of Valproic Acid and melatonin), High (High doses of Valproic Acid and melatonin), HV+LM (High dose of Valproic Acid + Low dose of melatonin), LV+HM (Low dose of Valproic Acid + High dose of melatonin). $^{**}p < .001$ compare to Sham Group, $^{*}p < .001$, $^{\#\#\#}p < .01$, $^{***}p < .05$ compare to Model and Vehicle Groups, $\$\$p < 0.001$ compare to Low Group, $\&\&\&p < 0.001$, $\&p < 0.05$ compare to V Group, $@@@p < 0.001$, $@p < 0.05$ compare to M Group.

ischemic outcomes [9]. Following ischemic insult, oxygen and glucose deprivation disrupts mitochondrial function and ATP production, resulting in excessive ROS generation during ischemia and subsequent reperfusion. Elevated ROS levels promote oxidative damage, activate inflammatory pathways, and contribute to disruption of the blood – brain barrier (BBB), cerebral edema, and neuronal apoptosis. In parallel, ischemic injury induces activation of immune cells and release of inflammatory mediators, which further amplify tissue damage and secondary neuronal injury [19]. In this context, melatonin, as a potent endogenous antioxidant, has been shown to reduce ROS production and attenuate oxidative stress associated with cerebral ischemia [10]. Its neuroprotective effects are largely mediated through MT1 and MT2 receptors, which activate intracellular signaling pathways involved in cell survival and antioxidant defense [11]. Consistent with these findings, the present study showed increased expression of MT1 and MT2 receptors, along with elevated levels of antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) following melatonin administration.

Valproic acid exerts its neuroprotective effects primarily through epigenetic-associated mechanisms, particularly via inhibition of histone deacetylases (HDACs). By inhibiting HDAC1 and HDAC3, valproic acid promotes histone acetylation and enhances the transcription of genes involved in neuronal survival and

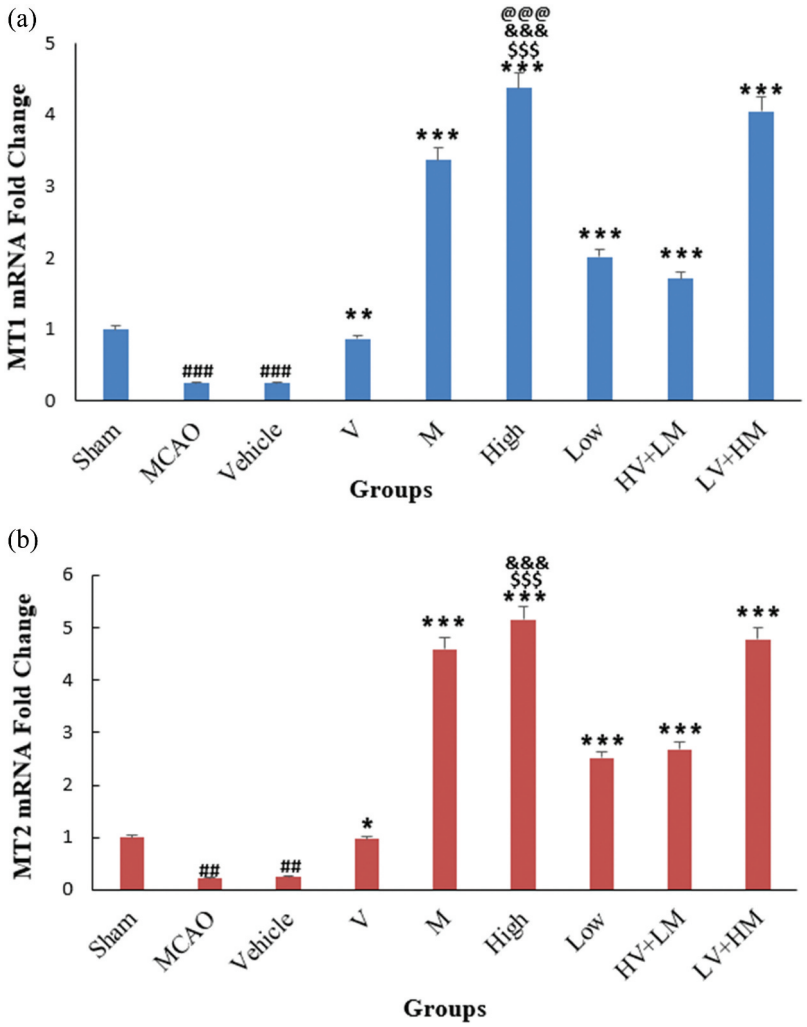

Figure 5. MT1 and MT2 receptor expression. Relative expression of MT1 and MT2 was assessed by qRT-PCR M (Melatonin), V (Valproic Acid), Low (Low doses of Valproic Acid and melatonin), High (High doses of Valproic Acid and melatonin), HV +LM (High dose of Valproic Acid + Low dose of melatonin), LV+HM (Low dose of Valproic Acid + High dose of melatonin). $^{**}p < .001$, $^{*}p < .01$ compare to Sham Group, $^{###}p < .001$, $^{***}p < .01$ compare to Model and Vehicle Groups, $\$\$p < 0.001$ compare to Low Group, $\&\&\&p < 0.001$, compare to V Group, $@@@p < 0.001$ compare to M Group.

plasticity, including BDNF. Recent evidence indicates that valproic acid exerts neuroprotective effects through multiple mechanisms, including modulation of HDAC activity, regulation of neuroplasticity-related pathways, attenuation of oxidative stress and inflammation, and improvement of mitochondrial function [20]. Valproic acid also mediates neuronal effects through epigenetic regulation by inhibiting histone deacetylases, thereby influencing gene transcription, neuronal signaling pathways, and cellular survival mechanisms [21]. In addition, valproic acid has been reported to regulate melatonin receptor expression through epigenetic-associated pathways, suggesting a possible link between HDAC inhibition and melatonergic signaling [14]. In agreement with these reports, the present study demonstrated that valproic acid significantly reduced HDAC1 and HDAC3 expression while simultaneously increasing MT1 and MT2 receptor expression, which was associated with improved neurological and histological outcomes.

Importantly, the combined administration of melatonin and valproic acid resulted in greater neuroprotective effects compared to either treatment alone. This enhanced efficacy may be attributed to complementary and interacting mechanisms. Valproic acid, through epigenetic modulation, may increase the expression of melatonin receptors, thereby potentially enhancing the responsiveness of neural tissue to melatonin. Conversely, melatonin may attenuate oxidative stress and mitochondrial dysfunction that may be associated with ischemic injury and pharmacological stress, thereby improving the overall

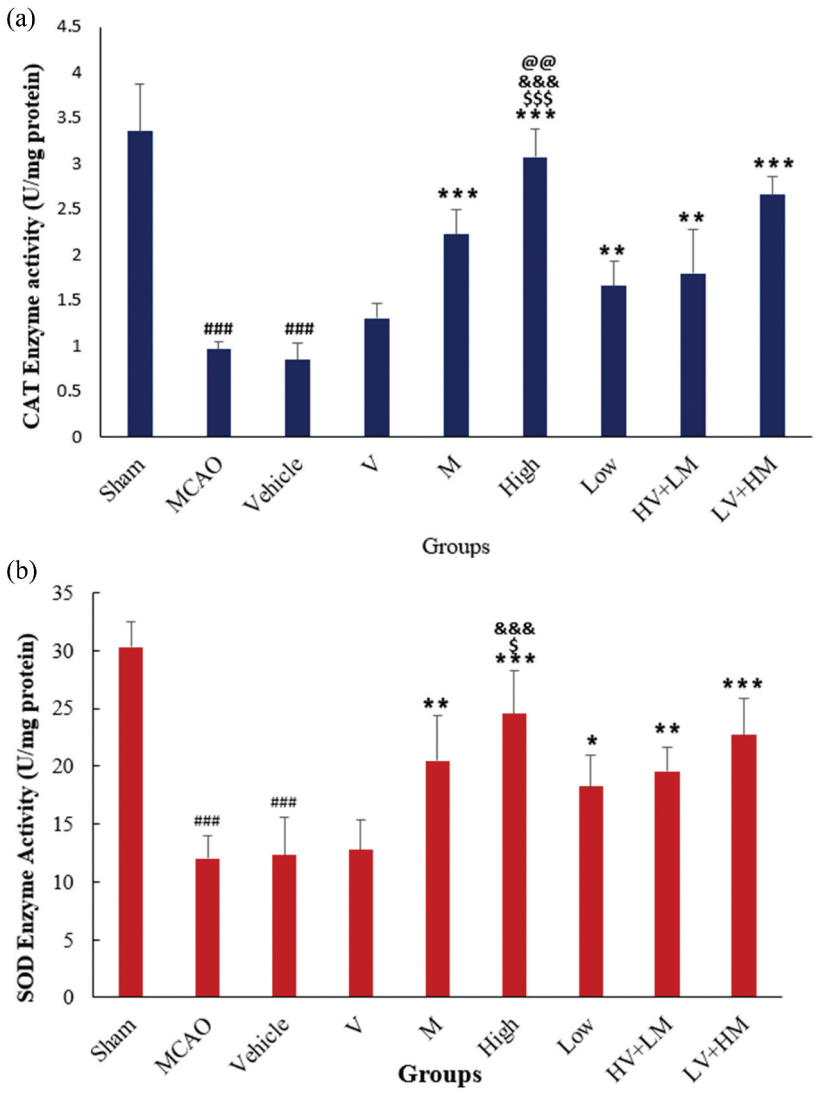

Figure 6. Antioxidant activity (CAT and SOD). Antioxidant enzyme activity was assessed by measuring CAT and SOD levels. M (Melatonin), V (Valproic Acid), Low (Low doses of Valproic Acid and melatonin), High (High doses of Valproic Acid and melatonin), HV+LM (High dose of Valproic Acid + Low dose of melatonin), LV+HM (Low dose of Valproic Acid + High dose of melatonin). $^{**}p < .001$ compare to Sham Group, $^{*}p < .001$, $^{\#\#\#}p < .01$, $^{***}p < .05$ compare to Model and Vehicle Groups, $ $\$p < 0.001$, $\$p < 0.01$ compare to Low Group, $\&\&\&p < 0.001$ compare to V Group, $@@@p < 0.001$ compare to M Group.

neuroprotective profile of the treatment. Therefore, the combination of these two agents may provide a complementary neuroprotective approach by simultaneously targeting epigenetic regulation and oxidative stress-related pathways, while modulation of melatonin receptor signaling may contribute to the enhanced effects observed following combined treatment [22]. Such interactions have been suggested in previous studies, where melatonin was shown to mitigate valproic acid-induced neurotoxicity [15] and enhance antioxidant enzyme activity in patients receiving valproate therapy [23].

Despite the promising findings, it is important to consider the potential limitations of valproic acid, particularly its dose-dependent adverse effects, including mitochondrial dysfunction and oxidative stress [24,25]. These concerns highlight the importance of optimizing dosing strategies and support the rationale for combination therapies that may reduce required doses while maintaining therapeutic efficacy. In this regard, melatonin may serve as a protective adjunct, potentially mitigating adverse effects of valproic acid while enhancing its neuroprotective properties.

In conclusion, the findings of this study suggest that the combination of melatonin and valproic acid exert enhanced neuroprotective effects in cerebral ischemia through coordinated modulation of oxidative

stress, epigenetic-associated regulation, and melatonin receptor signaling. This combined approach may represent a promising multi-target therapeutic strategy for the treatment of ischemic stroke, although further studies, particularly in clinical settings, are required to validate these findings.

A limitation of this study is that protein levels of HDAC1/3 and MT1/MT2 and histone acetylation status were not evaluated. In addition, oxidative stress assessment was limited to SOD and CAT activities, and MDA and MPO were not measured. Therefore, the conclusions should be interpreted with caution.

Author contributions

CRediT: **Parisa Firouzi:** Data curation, Formal analysis, Investigation, Validation; **Elham Ghasemloo:** Data curation, Methodology, Software, Writing – original draft, Writing – review & editing; **Meysam Forouzandeh:** Methodology, Writing – original draft, Writing – review & editing; **Mehdi Eskandari:** Data curation, Formal analysis, Supervision; **Mahin Ganjkhani:** Data curation, Investigation, Resources, Visualization; **Massomeh Hosseini:** Data curation, Software; **Ali Majdi:** Methodology; **Bahram Perseh:** Software; **Ali Rostami:** Conceptualization, Project administration, Validation; **Hossein Mostafavi:** Conceptualization, Investigation, Project administration, Resources, Supervision, Writing – review & editing.

Disclosure statement

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Ethics declarations

All experimental procedures involving animals were conducted in accordance with institutional guidelines and approved by the Ethics Committee of Zanjan University of Medical Sciences, Zanjan, Iran (Approval No: IR.ZUMS.AEC.1403.029).

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