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Neuroprotective effects of mildronate in a rat model of traumatic brain injury

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ABSTRACT

Objective: Traumatic brain injury (TBI) is one of the most common preventable causes of mortality and morbidity. Inflammation, apoptosis, oxidative stress, and ischemia are some of the important pathophysiological mechanisms underlying neuronal loss after TBI. Mildronate is demonstrated to be beneficial in various experimental models of ischemic diseases via anti-inflammatory, antioxidant, and neuroprotective mechanisms. This study aimed to investigate possible antioxidant, anti-inflammatory, antiapoptotic, and neuroprotective effects of mildronate in a rat model of TBI.

Methods: A total of 46 male rats were divided into three groups of control, saline-treated TBI, and mildronate-treated TBI. Both TBI groups were subjected to closed-head contusive weight-drop injuries followed by treatment with saline or mildronate (100 mg/kg) administered intraperitoneally. The forebrain was removed 24 h after trauma induction, the activities of myeloperoxidase (MPO) and caspase-3, levels of superoxide dismutase (SOD), luminol- and lucigenin-enhanced chemiluminescence were measured, and histomorphological evaluation of cerebral tissues was performed.

Results: Increased MPO and caspase-3 activities in the vehicle-treated TBI group ($p < 0.001$) were suppressed in the mildronate-treated TBI group ($p < 0.001$). Similarly, increase in luminol and lucigenin levels ($p < 0.001$ and $p < 0.01$, respectively) in the vehicle-treated TBI group were decreased in the mildronate-treated TBI group ($p < 0.001$). Concomitantly, in the vehicle-treated TBI group, TBI-induced decrease in SOD activity ($p < 0.01$) was reversed with mildronate treatment ($p < 0.05$). On histopathological examination, TBI-induced damage in the cerebral cortex was lesser in the mildronate-treated TBI group than that in other groups.

Conclusion: This study revealed for the first time that mildronate, exhibits neuroprotective effects against TBI because of its anti-inflammatory, antiapoptotic, and antioxidant activities.

Introduction

Traumatic brain injury (TBI) is responsible for 30%–40% of injury-related deaths and is the leading cause of neurological disabilities [1]. Primary injury that occurs during the event causes direct mechanical damage to neurons, glial cells, and blood vessels. Secondary injury begins within minutes following the physical impact and activates several signaling cascades that cause further cell death. Some of these secondary cascades include inflammation, apoptosis, oxidative stress, neurotransmitter excitotoxicity, mitochondrial dysfunction, and ischemia [2,3]. Although secondary injuries may require hours or years to disappear, TBI is the most common preventable cause of functional and long-term disability [4].

In the last decade, TBI prevention efforts have often been focused on potential therapeutic agents aimed at preventing neuronal cell loss; however, none of the experimental agents have been approved for clinical use [2]. Within the first 12 h of trauma [5,6], cerebral blood flow (CBF) is severely impaired and has been considered to be the major mechanism underlying ischemic brain damage observed in all types of TBI [7].

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Mildronate (3-[2,2,2-trimethylhydrazinium]-propionate, MET-88, meldonium, quaterine) is an anti-ischemic agent produced in the early 1980s [8]. When first designed, mildronate was intended to decrease fatty acid oxidation and limit the production of cytotoxic products in ischemic shock by inhibiting carnitine biosynthesis and accumulation of toxic acyl-carnitines in ischemic tissues [9,10]. Thus, mildronate directs cells to use glucose, thereby increasing their survival in ischemic conditions [9,11].

In addition to its proven cardiovascular efficacy, several studies have focused on the neuroprotective action of mildronate and its impact on cerebral circulation and ischemic processes [12–21]. Mildronate markedly decreases brain edema by regulating brain hemodynamics and optimizing oxygen balance, thereby maintaining the availability of energy metabolites under ischemic conditions [12,13]. In animal studies, mildronate showed positive effects on neuronal outcomes following ischemic brain damage [15], increased tolerance to anoxia-reoxygenation [16], and demonstrated antiepileptic activity [14]. In addition, mildronate was shown to decrease neuroinflammation, increase neuronal regeneration and prevent apoptosis in several experimental models [17–21].

Although the neuroprotective effects of mildronate have been previously demonstrated in ischemic and neurodegenerative diseases of the brain, they have not been studied in TBI. In the present study, the neuroprotective activity of mildronate was investigated in a rat model of mild TBI for the first time.

Materials and methods

Experimental groups

Animal care and all experiments were conducted in concordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) on the protection of animals for experimental use. All experimental procedures used in this investigation were reviewed and approved by the Marmara University Animal Care and Use Committee (35.2017.mar). Forty-six adult male Wistar albino rats weighing 250–400 g were used. Animals were housed in an air-conditioned room with 12-h light and dark cycles, maintained at constant temperature (22 ± 2 °C) and relative humidity (65%–70%). Rats were fed standard laboratory chow and had free access to water.

The rats were randomly assigned to three groups as follows:

1. Sham-operated control group (n = 16): Rats underwent only a skin incision and received a single intraperitoneal dose of saline (0.9% NaCl, 0.1 ml/100 g) immediately after surgery. Nontraumatized brain samples were obtained 24 h after surgery; some samples were used for biochemical analysis (n = 10), whereas others were stored in formaldehyde solution for histopathological analysis (n = 6).
2. Saline-treated TBI group (n = 16): Rats underwent TBI as described below and received a single intraperitoneal dose of saline immediately after TBI. Brain samples were removed 24 h after injury and used for biochemical (n = 10) or histopathological (n = 6) analysis.
3. Mildronate-treated TBI group (n = 14): Rats received a single intraperitoneal dose of mildronate (100 mg/kg; JSC Grindeks, Riga, Lithuania) immediately after TBI. The selected dose of mildronate was based on that used in previous studies [15,19,21–24]. Brain samples were used for biochemical (n = 8) and histopathological (n = 6) analysis 24 h after TBI.

Anesthesia and induction of TBI

The animals were anesthetized by an intraperitoneal injection of 10 mg/kg xylazine (Rompun, Bayer, Turkey) and 50 mg/kg ketamine (Ketalar, Parke Davis, Turkey) combination and were allowed to breathe spontaneously. A moderate brain injury model, described by Marmarou et al. [25] and modified by Ucar et al. [26], was applied for head trauma. Rats were placed in prone position on the table and supported on a 10-cm foam bed that provided deceleration after impact. A midline incision was made on the head, and the coronal and lambdoid sutures were identified. A metallic disc of 10-mm diameter and 3-mm thickness was fixed to the cranium using bone wax between the two cranial sutures and the midline. Trauma was applied at the point where the disc was placed on the midline. A lead object weighing 300 g was allowed to fall freely from a height of 70 cm through a copper tube on to the metal disc over the skull of the rat. After the induction of injury, the metallic disc was removed, the surgical area was cleaned, and the skin was sutured.

All the animals were decapitated 24 h after trauma, and the brains were carefully removed. Samples of forebrain tissues were obtained in a standardized manner by excising the same parts of the brain for the analysis of each parameter.

Biochemical analyses

Measurement of myeloperoxidase activity in brain tissue

Myeloperoxidase (MPO), an enzyme that is mainly located in the azurophilic granules of polymorphonuclear leukocytes, is commonly used to demonstrate the accumulation of neutrophils in tissues [27]. Tissue MPO activity was evaluated by measuring the hydrogen peroxide-dependent oxidation of o-dianizidine dihydrochloride. Tissues (0.2–0.4 *g*) were homogenized in potassium phosphate buffer containing hexadecyl-trimethylammonium bromide and centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was discarded and, the pellet was rehomogenized with 50-mmol/l K_2HPO_4 containing 0.5% (w/v) hexadecyl-trimethyl ammonium bromide and 10-mmol/l ethylenediaminetetraacetic acid (EDTA, Sigma Aldrich, USA). One unit alteration of enzyme activity measured at 460 nm and 37 °C was determined to be the amount of the MPO present per gram of tissue [28].

Measurement of tissue caspase-3 activity

To determine the levels of caspase-3 activity in the brain tissue, a commercial kit (Rat Caspase 3, Casp-3 ELISA Kit, Kat.No: SL0152Ra, Sunlong Biotech Co. Ltd., China) was used according to the manufacturer's instructions. Supernatants of tissue homogenates were used for measuring the activity of the apoptotic marker caspase-3.

Determination of tissue superoxide dismutase activity

Superoxide dismutase (SOD) activity in the brain samples was measured according to a previously described method [29]. Briefly, measurements were performed in cuvettes containing 2.8 ml of 50-mM potassium phosphate (pH 7.8) with 0.1-mM EDTA, 0.1 ml of 0.39-mM riboflavin in 10-mM potassium phosphate (pH 7.5), 0.1 ml of 6-mM o-dianisidine·2HCl in deionized water, and tissue extract (50–100 µl). Cuvettes with all their components were illuminated with 20-W Sylvania Grow Lux fluorescent tubes that were placed 5 cm above and to one side of the cuvettes, maintaining a temperature of 37 °C. Absorbance was measured at 460 nm with a Shimadzu UV-02 model spectrophotometer. A standard curve was prepared using bovine SOD (Sigma–Aldrich; S-2515-3000 U, USA) as a reference. Absorbance readings were obtained before and 8 min after illumination, and the net absorbance was calculated.

Chemiluminescence measurements in brain tissue

Chemiluminescence (CL) is a direct, noninvasive method for the measurement of reactive oxygen radicals that utilizes luminol and lucigenin as enhancer probes. When added to *in vitro* biological systems, luminol and lucigenin produce high levels of excited products. Excited electrons from these compounds generate radiating light energy or CL that can be detected by a luminometer. Luminol detects radicals such as hydroxyl ions, hydrogen peroxide, and hydrochloric acid, whereas lucigenin is selective to superoxide anions [30]. Reactive oxygen species (ROS) were numerically measured after the addition of 0.2 mM of enhancers (Sigma Aldrich, USA). The final concentrations of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and lucigenin (bis-*N*-methylacridinium nitrate) probes (Sigma Aldrich, USA) [31] were measured at room temperature using a luminometer (Junior LB 9509 luminometer; EG&G Berthold, Germany). Counts were obtained at 1-min intervals for 5 min, area under the curve was determined, and data expressed as relative light units after counts were normalized to the weight of the brain tissue sample. Results were expressed as relative light units/mg tissue (rlu/mg).

Histopathological examination

Animals were anesthetized with ketamine and xylazine as described previously and perfused through the aorta with 4% paraformaldehyde in phosphate buffer (pH 7.4). After decapitation, the brains of the animals were removed from the skull and fixed in the same fixative for 24 h at 4 °C. Tissues were then embedded in paraffin and 5-µm-thick coronal sections were created using a rotary microtome. The sections were stained with hematoxylin and eosin stain. Finally, sections were examined under a photomicroscope (Olympus BX51, Japan). The severity of neuronal damage in the cortex was scored semiquantitatively as follows: 0 = no damage, 1 = mild damage, 2 = moderate damage, and 3 = severe damage. Pyknotic nuclei and intense staining of the shrunken neuronal perikarya were considered in scoring the degree of neuronal degeneration. Histopathology was scored on a 9-point scale, including subscores for neuronal degeneration, perivascular edema, and vascular edema that were assigned scores of 0–3 each [32].

Statistical analysis

Data were analyzed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA) and expressed as means ± SEM. Analysis of biochemical data was performed using one-way analysis of variance (ANOVA). Values of $p < 0.05$ were considered to be statistically significant.

Results

Biochemical evaluation

Myeloperoxidase activity (U/g tissue)

MPO activity was significantly higher in brain samples of the saline-treated TBI group than in those of the control group ($p < 0.001$), revealing that cerebral MPO activity increases after TBI. Tissue MPO activity in the mildronate-treated TBI group was significantly lower than that in the saline-treated TBI group ($p < 0.001$); however, it did not differ from that in the control group (Fig. 1A) (Table 1).

Caspase-3 activity (nmol/mg protein)

Similarly, caspase-3 activity was significantly higher in the saline-treated TBI group than in the control group ($p < 0.001$). However, treatment with mildronate significantly inhibited the TBI-induced increase in caspase-3 activity ($p < 0.001$; Fig. 1B).

Superoxide dismutase activity (U/mg protein)

Compared with the control group, tissue SOD activity decreased following TBI ($p < 0.01$). However, mildronate treatment totally abolished this decrease in cerebral SOD activity ($p < 0.05$; Fig. 1C).

Chemiluminescence measurement (rlu/mg)

CL of both luminol and lucigenin were higher in the saline-treated TBI group than in the control group ($p < 0.001$ and $p < 0.01$, respectively). Mildronate treatment abolished the elevations in

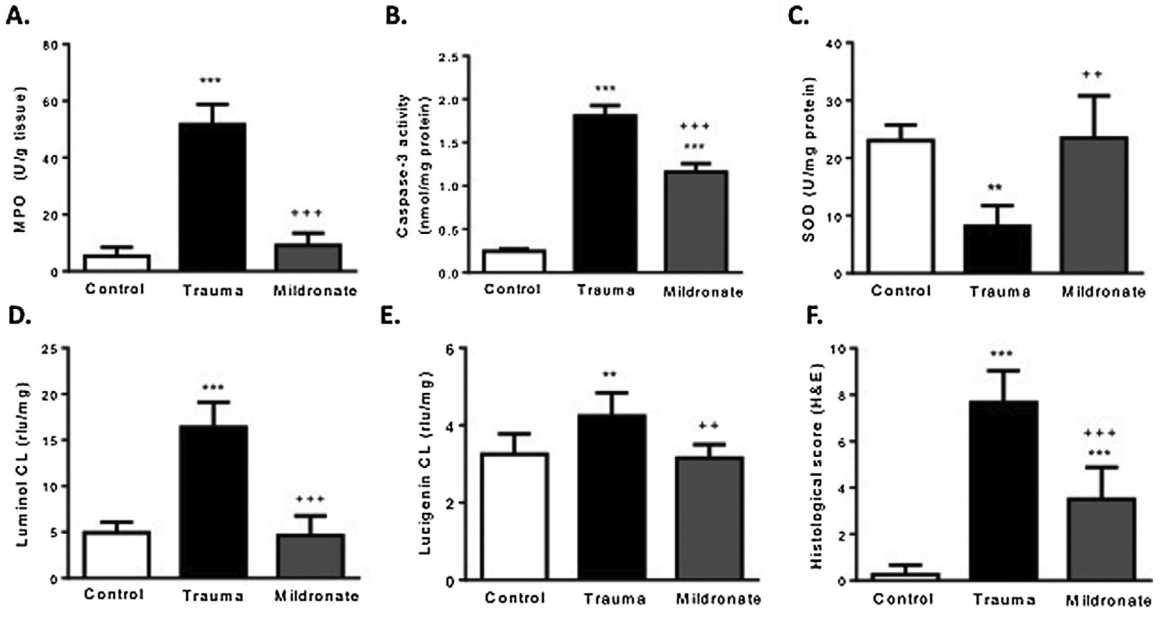

Fig. 1. Bar graphs showing tissue myeloperoxidase activity (A), caspase-3 activity (B), superoxide dismutase activity (C), luminol levels (D), lucigenin levels (E), and histological scores (F) of the various study groups. Values are expressed as mean ± SD.
(** $p < 0.01$, *** $p < 0.001$ compared with the control group; ++ $p < 0.01$, +++ $p < 0.001$ compared with the saline-treated TBI group).

Table 1
Biochemical results and histology scores.

Parameters	Control	TBI	Mildronate	p-value
Tissue MPO (U/g tissue)	5.29 ± 2.95	51.69 ± 6.67	9.11 ± 3.94	$p < 0.001$
Tissue Caspase-3 (nmol/mg)	0.24 ± 0.03	1.81 ± 0.11	1.16 ± 0.09	$p < 0.001$
Tissue SOD (U/mg protein)	23.02 ± 2.43	8.19 ± 3.09	23.49 ± 6.32	$p < 0.01$
Luminol (rlu/mg)	4.92 ± 1.09	16.37 ± 2.56	4.61 ± 1.98	$p < 0.001$
Lucigenin (rlu/mg)	3.25 ± 0.49	4.24 ± 0.56	3.15 ± 0.32	$p < 0.01$
Histology score	0	7.67 ± 1.25	3.5 ± 1.26	$p < 0.001$

MPO: Myeloperoxidase; SOD: Superoxide dismutase, TBI: Traumatic Brain Injury.

both luminol and lucigenin CL levels ($p < 0.001$ and $p < 0.01$, respectively; Fig. 1D, -E).

Histopathological evaluation

Normal neuropil structure and neurons with uniform morphology, large nuclei, and distinct nucleoli were prominent in the cortices of control rats (Fig. 2A). Compared with the control group, hemorrhage and edema were observed in the saline-treated TBI group. Furthermore, neuronal damage, pyknotic cell nuclei, irregularity of cell structures, and cytoplasmic deterioration were detected (Fig. 2B). The histological scores of the saline-treated TBI group showed a significant increase compared with those of the control group ($p < 0.001$; Fig. 1F). Following mildronate treatment, neurons with less damage and more normal morphological structure were observed and the effects of trauma on neuropil structure were less prominent (Fig. 2C). Compared with the saline-treated TBI group, the mildronate-treated TBI group had a significantly low histological score ($p < 0.001$; Fig. 1F).

Discussion

Over the last two decades, greater understanding of the pathophysiology of TBI, improvement in intensive care services, and technological advancements in monitoring and follow-up of patients have decreased mortality rates and increased survival in TBI patients [33]. However, despite extensive experimental studies reporting promising results, no pharmacological treatment has demonstrated clinical effectiveness following TBI [34–36]. Therefore, the treatment of TBI yet attracts serious interest and numerous experimental studies continue to be conducted to unveil a possible therapeutic agent. The present study revealed that mildronate, an anti-ischemic agent, exhibits neuroprotective effects against TBI because of its anti-inflammatory, antiapoptotic, and antioxidant activities when administered immediately after trauma.

Hemorrhage, edema, deterioration of the blood-brain barrier, and decrease in CBF are early events that occur immediately after trauma. These early events are followed by hypoperfusion, impaired transport of metabolic precursors, hypoxia, and ischemic tissue damage [2]. Because neurons depend almost completely on aerobic metabolism as their primary energy source, cerebral perfusion and oxygenation are critical for neuronal function. In the early period, decrease in CBF following TBI can result in inadequate oxygenation of brain tissue [37]. Therefore, maintaining optimum levels of cerebral perfusion and CBF is crucial in the treatment of TBI patients [38–40].

Several clinical trials have suggested that decrease in CBF after TBI results in ischemia [39]. Mildronate is an anti-ischemic cardioprotective agent [41,42] that is reported to be beneficial in the treatment of neurological diseases [9,12,15]. In a transient middle cerebral artery occlusion model of stroke in rats, a 100-mg/kg dose of mildronate decreased L-carnitine levels in the brain and increased gamma-butyrobetaine (GBB) levels, in addition to improving performance on behavioral tests [15]. Furthermore, clinical studies have shown that mildronate has positive effects on cerebral hemodynamics [14,43]. Rumaks et al. [24] concluded that mildronate decreases the infarct area after cerebral ischemia in a rat model.

Vasospasm after TBI decreases cerebral perfusion, resulting in ischemia [44]. Therefore, treatment of vasospasm after head trauma decreases TBI-related morbidity and mortality [45]. Mildronate is a rapid-acting vasorelaxant [39]. It activates secondary messenger systems by binding to membrane receptors and triggers the expression of genes involved in vasospasm [46]. In addition, mildronate blocks the effect of nitric oxide synthase inhibitors, resulting in an increase in the concentration of nitric oxide, which is a potent vasodilator [47]. Because of its anti-ischemic and vasospasm-decreasing effects, mildronate was preferred in this study because it is considered to be an effective neuroprotective agent in TBI.

The anti-ischemic agent mildronate was produced in the 1980s. It inhibits carnitine biosynthesis and cytotoxic degradation products of fatty acid β-oxidation in ischemic tissues [48]. Effects on ischemic myocardial tissue occur via GBB hydroxylase inhibition and decrease in fatty acid β-oxidation [9,12]. Mildronate acts by decreasing the level of carnitine, which aids the transfer of long chain fatty acids into the mitochondrial matrix for β-oxidation. Furthermore, it regulates glycolysis and pyruvate dehydrogenase activity by controlling the ratio of acetyl coenzyme A to coenzyme A in mitochondria [22,49,50]. Mildronate inhibits L-carnitine biosynthesis by increasing the amount of GBB. This results in the inhibition of fatty acid oxidation. When fatty acid oxidation is inhibited in ischemic tissues, glucose is used as the energy substrate [14,51,52].

The administration of mildronate in healthy people decreases plasma carnitine levels without causing serious side effects [50]. Unlike other organs, the mammalian brain depends on glucose rather than fatty acids as its main source of energy. Because

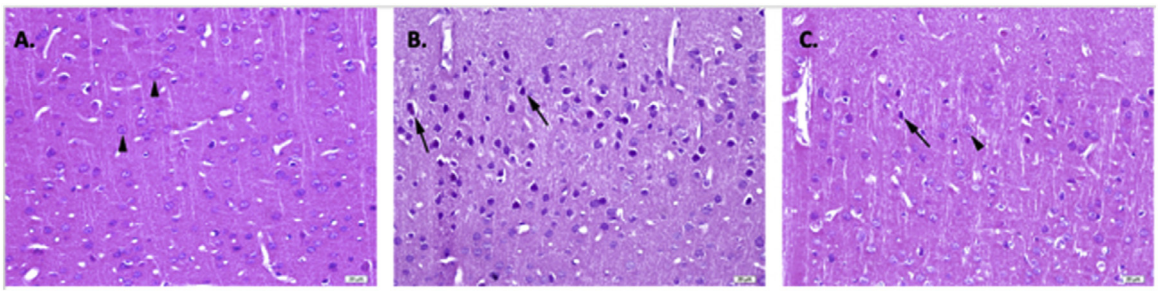

Fig. 2. Photomicrographs showing hematoxylin and eosin staining in the cortices of experimental groups. Control group (A) showed regular neuronal morphology and neuropil structure. Evident degeneration of neurons was observed in the saline-treated TBI group (B). In the mildronate-treated TBI group (C), degenerated neurons were decreased. Arrowhead: neurons with normal morphology; arrow: degenerated neurons, bars: 20 μm.

carnitine and GBB hydroxylase are produced in brain cells [53], the effects of mildronate on CNS are considered to be fatty acid independent [12]. Mildronate is proposed to mediate its effects on the brain through membrane receptors and secondary messengers of these receptors, thus triggering DNA replication, repair, and methylation. In addition, it triggers RNA polymerase activity in neuronal cells [12].

Although the brain is considered to be immune privileged, neuroinflammation is an important component of cell death after TBI [54,55]. The anti-inflammatory effect of mildronate has been reported both in CNS and other tissues [8,17,19,24,48]. Moreover, the neuroinflammation-decreasing effect of mildronate has been reported in an ischemic stroke model in rats [24].

MPO activity is an important indicator of neutrophil infiltration at the site of tissue damage [56,57]. MPO activity is an important indicator of inflammation and is proportional to the number and activity of infiltrating neutrophils [57]. It is used to demonstrate neuroinflammation in several studies, and decrease in MPO activity has been evaluated as a potential marker for neuroprotection [56,58,59]. In this study, MPO activity was significantly higher in the saline-treated TBI group than in the control group. Decrease in MPO activity in the mildronate-treated TBI group is an indication of the drug's anti-inflammatory effects.

Following TBI, two types of cell death are observed: necrosis and apoptosis. Experimental studies show that TBI results in intranuclear DNA fragmentation, which is considered to be a sign of apoptosis [60]. Apoptosis is activated by secondary damage pathways such as excitatory amino acids, increased intracellular calcium, and free radicals. Intrinsic mechanisms and caspases in the first 6 h following trauma actively trigger apoptosis [61]. In mammalian cells, caspase-3, an interleukin-converting enzyme, is an important and reliable indicator of apoptotic activity [62]. Sakurai et al. [63] showed the appearance of caspase-3 immunoreactivity in the first 15 min following ischemia. In addition, apoptotic activity occurs in the first 24 h following brain injury [34,63,64]. Caspase-3 activity is shown to be increased in several experimental TBI studies, rendering it a reliable indicator of apoptosis. Accordingly, decreased activity reflects antiapoptotic activity [34–36]. Mildronate has been shown to decrease caspase-3 activity and cellular apoptosis in ischemic states [8,18]. The presented study demonstrated that TBI caused a significant increase in caspase-3 activity, which was attenuated by mildronate treatment. This indicates that mildronate exerts antiapoptotic effects against TBI.

Cytoskeletal impairments, cellular swelling, and neuronal damage occur after TBI. This mechanical damage causes intense calcium influx into the cell. Increased intracellular calcium affects the mitochondria, which is the seat of ROS production, and ultimately results in oxidative stress in the cell [65,66]. In addition, high oxygen consumption capacity, dense ROS production, and metals such as iron that increase ROS production cause the brain to be at the highest risk of oxidative stress exposure [67,68]. When the tissue is exposed to oxidative stress, compensatory mechanisms increase the production of antioxidant enzymes. However, increased oxidative stress after TBI is much higher than the antioxidant activity and the net result is oxidative damage [69]. TBI-mediated molecular damage depletes antioxidant enzymes because of the high level of oxidative activity. Accordingly, animal studies showed a decrease in SOD levels 1 h and 24 h after trauma [33–35,70,71]. Similarly, this study revealed decreased activity of SOD in the saline-treated TBI group because of increased oxidative stress. Following mildronate treatment, SOD levels increased significantly compared with the saline-treated TBI group.

Luminol and lucigenin enhanced CL levels, which are good indicators of oxidative stress, were increased in the saline-treated TBI group compared with that in the control group because of increased oxidative stress, as shown previously [72]. With mildronate treatment, both luminol- and lucigenin-enhanced CL levels were significantly lower than that in the mildronate-treated TBI group. Accordingly, in this group, increased SOD and decreased lucigenin and luminol CL levels were indicative of antioxidant activity.

All histology preparations in the control group had neurons with normal morphological appearance and neuropils. In the saline-treated TBI group increased hemorrhage, edema, neuronal damage, pyknotic cell nuclei, irregular cell structures, and cytoplasmic deterioration were observed. Perivascular and perineuronal edema was greater in the saline-treated TBI group than in the control group. Damage and swelling were lower in the mildronate-treated TBI group than in the saline-treated TBI group. When histological scores were compared, the higher histological scores in the saline-treated TBI group significantly decreased following mildronate treatment. The histological data of the study supported the anti-inflammatory, antiapoptotic, and antioxidant properties of mildronate. Mildronate showed neuroprotective activity after TBI by preserving normal brain morphology.

Several studies have shown the neuroprotective efficacy of mildronate; however, it has not been shown in a TBI model [8,12,14–21,24,37]. Mildronate exerts anti-ischemic, vasorelaxant, antiapoptotic, anti-inflammatory, and antioxidant properties and is considered to be an important neuroprotective agent against neuronal tissue damage following TBI.

However, there are some limitations to this study. Different trauma forces with different weight–height modifications at different time intervals and severities could be studied. Number of subjects in the groups and dose diversity can be increased. The biochemical parameters that are used can be varied. Furthermore, ultrastructural analysis with electron microscopic examination can enrich the study. Another limitation of this study is the lack of functional outcomes and behavioral tests.

Conclusion

This study demonstrated the anti-inflammatory, antiapoptotic, and antioxidant properties of mildronate after TBI for the first time. Possible neuroprotective mechanisms of mildronate were discussed. Mildronate has been found effective in preventing neural damage following TBI. Further experimental and clinical studies are required to validate the use of mildronate in the treatment of TBI.

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