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Ashwagandha is protective of impaired motor-coordination in experimental CI/R damage in rats

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Abstract

Aim: Cerebral ischemia (CI) is a condition in which metabolic stress increases when blood flow to a part of the brain is interrupted, resulting in oxygen and glucose deprivation. Later, during the treatment process, the ischemic tisue is reperfused, causing more neurological damage than the ischemic process. For this reason, developing protective practices in individuals at high risk of CI is much more important than developing treatment practices. Ashwagandha (ASW), an extract of the W.somnifera plant, is an agent with antioxidant, anti-inflammatory, antiapoptotic and neuroprotective effects. It has been reported in the literature that ASW is effective against neuropathological damage with different experimental models. In this study, it was aimed to explain the protective effect of ASW against CI/reperfusion damage.

Materials and Methods: 30 *Spraque Dawley* male rats were divided into 3 groups as Sham, CI, ASW+CI (n=10). 60 min CI was created using the intraluminal filament technique and sacrificed at the end of the 24-hour reperfusion period. ASW (200 mg/kg/day) was administered orally for 7 days before CI. Motor coordination was evaluated with rota-rot test before and after CI. Infarct area was determined by triphenyltetrazolium chloride (TTC) staining. Kruskal-Wallis test was used to compare the differences between the groups. $p<0.05$ was considered statistically significant.

Results: Neurological evaluations of the experimental groups were scored as ASW+CI (2), CI (3.5) and Sham (0). Infarct areas were calculated as Sham (0), CI (% 45.7) and ASW+CI (% 32.6). Post-ischemia rota-rot test results evaluating motor coordination were measured as Sham (280 sec), CI (15 sec) and ASW+CI (86 sec). Compared with the sham group, the increase in neurological score and ischemic area and the decrease in rota rot time in the CI and ASW+CI groups are statistically significant ($p\leq0.05$). Compared with the ASW+CI group, the decrease in neurological score and ischemic area and the increase in rota rot time are statistically significant ($p\leq0.05$).

Conclusion: ASW has protective effects against CI/R injury. 7-day ASW application before ischemia reduced the ischemic area. It reduced neurological damage, regulated motor coordination and maintained the neuronal death/survival balance against CI/R injury.

Introduction

Stroke is a process in which permanent effects occur or are temporarily exposed to an area of the brain as a result of blockage or bleeding in the vessels feeding the brain. Stroke is the leading cause of morbidity and mortality worldwide. Stroke, which is the second cause of death worldwide, is the main cause of disability in older ages [1]. 4/5 of stroke cases are ischemic. Cerebral ischemia (CI) causes oxygen and energy deprivation, followed by the formation of reactive oxygen species, glutamate release, intracellular calcium accumulation, and induction of inflammatory processes [2]. Two main approaches are accepted in the treatment of CI: neuroprotection and reperfusion. Reperfusion is the reperfusion and oxygenation of tissue exposed to ischemia. Reperfusion of ischemic tissue paradoxically causes much more severe damage to the tissue than the damage caused by ischemia. The ischemic cas-

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cade series of events leads to irreversible tissue damage [3].

The reperfusion process of stroke treatment is to use thrombolytic drugs or mechanical devices to recanalize occluded vessels. The only medical treatment for acute ischemic stroke is to provide intravenous thrombolysis with recombinant tissue plasminogen activator (rtPA) [4]. However, this treatment has a high risk of hemorrhagic complications. In addition, rtPA treatment is applied in the acute phase of the CI process and the patient group is small. Therefore, it is much more important to develop agents that protect against ischemia/reperfusion injury and reduce post-ischemic damage in individuals at high risk of stroke. Various experimental animal stroke models are being tested to develop new agents for CI treatment, to investigate pathophysiology and therapeutic approaches, and to mimic the processes [5]. In humans, CI varies in terms of causes and anatomical localization. Experimental ischemic stroke models applied in animals are quite well controlled, allow for more sensitive analyses, and are standardized in a reproducible manner. Perfusion injury, which is important in the pathophysiology of vascular stroke, cannot be modeled in in vitro models. Therefore, animal models are preferred for research studies. Since molecular, genetic, biochemical and physiological studies usually require direct invasive access to brain tissue, animal models are necessary for the evaluation of stroke pathophysiology and drug effects [6]. Selecting an appropriate experimental stroke model to be used in the development of new therapeutic agents can increase the success of preclinical stroke studies. The cerebral vascular structure of the rat, which is mentioned in many studies in the literature and which we used in our study, is similar to that of humans, its body size is ideal for monitoring physiological parameters. The size of the rat brain tissue facilitates suitability for fixation procedures and reproducibility [7]. The most common method for focal ischemic stroke is intraluminal occlusion (MCAO) of the middle cerebral artery, which is used in approximately half of stroke studies [8]. The severity of the damage developing in the brain varies depending on the duration of ischemia and subsequent reperfusion. The most important factor in neuronal survival after stroke may be the induced defense mechanisms rather than the ischemia duration. In approaches aimed at reducing treatment costs, the development of protective systems rather than treatment for individuals at high risk of stroke has recently gained more importance in this respect. Preinduction of defense mechanisms against neuronal damage may be effective not only in CI/R damage but also in protective treatments against other neuropathological diseases [9].

Ashwagandha (ASW), widely used in ayurvedic medicine, is the commercial form of the plant extract Withania somnifera. ASW contains components with adoptogenic effects. One of these components, Withanolide A, reduces neurodegeneration in the hippocampus region by increasing glutathione biosynthesis in neuronal cells. In cerebral ischemia, cells are exposed to excessive stimulation of glutamate receptors, cellular Ca^{2+} overload, and DNA damage that increases the production of oxygen radicals, leading to the gradual death of neuronal cells. Withanolide A significantly reduces the loss of endothelial cell viability, the increase in proinflammatory IL-1B, IL-6, IL-8, TNF-α, cyclooxygenase-2 (COX) gene expression. Previous studies have found that Withanolide A significantly reduces the effects of 7-ketocholesterol and the formation of reactive oxygen species (ROS). It is also thought that it reduces the expression of Factor 2/thrombin, Factor 8, von Willebrand factor and thromboxane A synthase genes related to blood coagulation and may reduce coagulation complications with this effect. Withanolide A has been found to suppress ROS formation and NO production in BV-2 microglia cells through induction of the Nrf2-heme oxygenase-1 pathway [10]. Withanolide A also reduces the expression of lipopolysaccharide-induced inflammatory cytokines by inhibiting the mitogen-activated protein kinase and NF-κB pathways. Another active ingredient found in ASW is Withaferin A (WFA). It has been reported that low-dose (1 mg/kg) WFA reduces cardiomyocyte apoptosis by reducing caspase-3 activity, while high-dose (5 mg/kg) WFA has the opposite effect. WFA increases the protein expression of Bcl-2, an antiapoptosis agent. It is known that during brain ischemia/reperfusion, high amounts of free radicals are formed, especially in the hippocampus area, and this leads to cell death [11]. This is the main reason for cognitive dysfunction after stroke. In experimental studies with acute stroke models, it has been shown that increased antioxidant capacity provides protection against the negative effects of free radical production during ischemia and reperfusion [12].

ASW exhibits neuroprotective activity by significantly reducing MDA levels and increasing SOD, CAT and GPx activity. It particularly acts by increasing the number of healthy neurons in the CA1 hippocampus region and decreasing the number of TUNEL positive neurons. In conclusion, ASW may exhibit strong neuroprotective activity against oxidative stress-related injuries caused by global cerebral ischemia/reperfusion in rats, probably through radical scavenging and antioxidant activities. It improves mitochondrial dysfunction, apoptosis and cognitive impairment. In light of these studies, in our research, the effects of ASW given before CI on neurological deficit scoring, rotarod and grip strength test results were compared. In addition, the effectiveness of Ashwagandha, a natural adaptogen, against the pathophysiology of CI/R damage in neuronal cells and the behavior and healing process was also evaluated in this study.

Materials and Methods

Animals and drugs

A total of 30 male *Spraque Dawley* rats (8 weeks old, 280-300 g) were obtained from Inonu University Experimental Animal Research Center in Malatya, Turkey. All rats used in the study were treated in accordance with the Experimental Animal Care and Use Guide throughout the study. All rats were randomly assigned to groups by blinding method. All application procedures and sacrification process of the study were approved by Inonu University Faculty of Medicine Animal Research Ethics Committee with the approval document numbered 2023/12-3. Rats were housed at 19-23°C, 12/12 h dark-light cycle, and with ad libitum access to water and standard laboratory chow. The study group and the number of rats in each group

(sample size) were determined according to the power analysis based on the values specified in the neuropathological analyses. According to this analysis [13], the Type I error amount (α) was calculated as 0.05, the power of the test (1-β) as 0.8, and the effect size as 0.82 (large). When the number of groups was 3, the minimum sample size required to find a significant difference between the groups for the rota rot test was at least 10 in each group. A total of 30 rats were included in the study, 10 animals in each group.

- *Group 1-Sham Group:* Animals in this group underwent sham surgery (all surgical operations outside the vascular capacity were performed). Animals in this group received oral administration of 1 ml of visible saline once a day for a week, starting one hour after Sham CI surgery. After 24 hours after the surgical operations, the rats were sacrificed by taking blood samples and performing a rotarod test and neurological evaluation. Brain tissues were removed for analysis.

- *Group 2-CI Group:* Animals in this group received oral 1 ml of visible saline for a week. On the 7th day, a 60-minute temporary focal CI was induced followed by reperfusion. After the 60-minute focal CI, a 24-hour reperfusion process was started. At the end of the 24-hour reperfusion, the rats were sacrificed by taking blood samples and performing a rotarod test and neurological evaluation. Brain tissues were removed for analysis.

- *Group 3-ASW +CI Group:* Animals in this group received oral dose of 200 mg/kg/day [14, 15] of ASW for a week. On the 7th day, a 60-minute temporary focal CI was induced followed by reperfusion. After the 60-minute focal CI, a 24-hour reperfusion process was started. At the end of the 24 hours reperfusion, the rats were sacrificed by taking blood samples and performing a rotarod test and neurological evaluation. Brain tissues were removed for analysis.

Creation of CI/R model

Anesthesia was induced in rats with intraperitoneal administration of 70 mg/kg ketamine and 8 mg/kg xylazine. Throughout the experiment, the body temperatures of the subjects were maintained at a constant range of 36.5-37 °C, monitored by a rectal temperature probe (RWD, Life Science, Guangdong, China). For continuous monitoring of regional cerebral blood flow, was tracked using a Laser-Doppler (Moor Instruments, Axminster, Devon, UK) blood flow meter. The focal ischemia model was created by occluding the right middle cerebral artery using the intraluminal filament (MCAO) technique [16]. After 60 min of ischemia, the monofilament (MSRC42B200PK50, RWD, Life Science, Guangdong, China) was withdrawn to initiate reperfusion [17]. The Sham CI/R group underwent the same surgical operation without filament insertion. At the end of the required period, the rats were sacrificed under anesthesia brain tissues were collected. Brain, tissues were TTC analyses.

Neurological deficit assessment

This scoring will be applied to all animals in the groups 24 hours after reperfusion begins using Longa's method [16]. According to this scoring;

0 =Normal neurological examination,

1=Flexion in the trunk and contralateral forelimb when lifted by the tail,

2=Normal posture at rest but drawing a circle in the contralateral direction while walking,

3=Falling in the contralateral direction while resting,

4=Inability to walk spontaneously. The relationship between the ischemic area and behaviors of the experimental groups was evaluated with neurological deficit scoring.

Rotarod test

The Rotarod device consists of a rod that rotates around itself at an adjustable speed. The essence of this test is to measure the time that the animal can stay on a rod that rotates at an increasing speed without falling. In order for the rats to get used to the rotarod device, which will help evaluate the resistance power, balance and coordinated movements after the animals have been stroked and the treatment applied, they were given pre-training at 5 rpm for 15 minutes for 3 days before the creation of the CI [18]. After the tests started 24 hours after reperfusion, all animals were placed in the opposite direction of the rotating rod, and the Rotarod device was increased from 4 rpm to 40 rpm within 5 minutes and the time that the rats could stay on the device (rod) was measured. The trial period did not exceed 5 minutes and the animals were rested for 5 minutes between each trial. The experiment was repeated 5 times for each animal, and the average of all trials was taken. With this test, the differences in motor coordination and balance changes of the experimental groups were evaluated. All neurological examinations and behavioral tests performed in the study were evaluated without knowing the treatment and ischemia groups.

Measurement of infarct area

On the third days following reperfusion, rats were euthanized, and their frozen brain tissues were 5 sectioned into 2 mm thick coronal slices. The brain sections were then incubated in a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, USA) in PBS at 37°C for 45 minutes. This resulted in normal brain tissues being stained red, while infarcted tissue appeared white or pasty. Infarct volumes were calculated using Image J software [19]. The percentage of infarct areas was calculated using the following formula:

The contralateral hemisphere - noninfarct area of the ipsilateral hemisphere/non-infarct area of the ipsilateral hemisphere×100

Statistical analysis

The data obtained in the study were analyzed using a program developed by the faculty members of the Department of Biostatistics and Medical Informatics, Inonu University Faculty of Medicine, and made available as an open access program [20]. In the analysis of the data, checks were

made to prevent missing and incorrect data and excessive variables/outliers, and corrections were made when necessary. The compliance of the variables examined with the normal distribution and the homogeneity of the variances were checked with the Levene test. When these assumptions were met, the difference between the group means was made using one-way analysis of variance, and when the variances were homogeneous, multiple comparisons were made using Tukey HSD, and when they were not homogeneous, using Tamhane T2. In cases where normality assumptions were not met, Kruskal-Wallis H was used, and in multiple comparisons, Conover was used. Quantitative data results were given as mean±SD. $p<0.05$ was considered statistically significant.

Results

Neurological deficit findings

Neurological deficits of the experimental groups were measured at 24 hours of reperfusion and the groups were scored according to their physical appearance and adequacy (Figure 1). No restrictive movements were observed in the Sham group, and the Sham was scored as 0. The neurological deficit score of the CI group was 3.5±0.5 and the ASW+CI group was scored as 2±1. When the CI and ASW+CI groups were compared with the Sham group, the difference between them was statistically significant. When the CI group was compared with the ASW+CI group, the improvement in the ASW+CI group was statistically significant ($p<0.05$).

Rotarod test findings

In the post-ischemia rota-rot test (Figure 2) in which motor coordination was evaluated. Rats in the Sham group remained in balance for an average of 280±18 seconds,

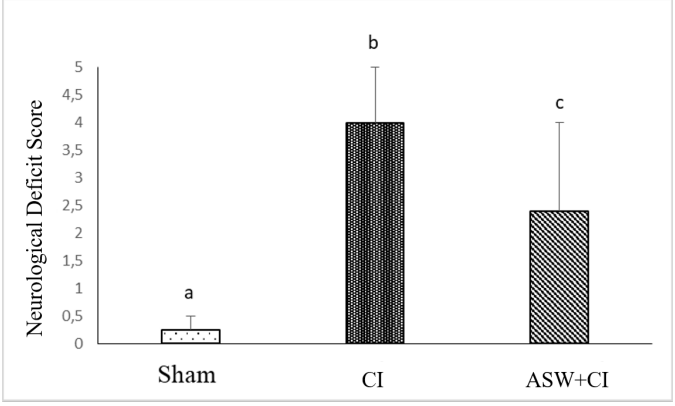

Figure 1. Neurological deficits of the experimental groups were measured at 24 hours of reperfusion and the groups were scored according to their physical appearance and adequacy. Different letters a, b, c, were statistically different from each other $p < 0.05$. The Kruskal-Wallis H test was used to evaluate results of the groups. When significant differences were detected between groups, multiple pair wise comparisons were made using the Mann-Whitney U test with Bonferroni correction ($p<0.05$).

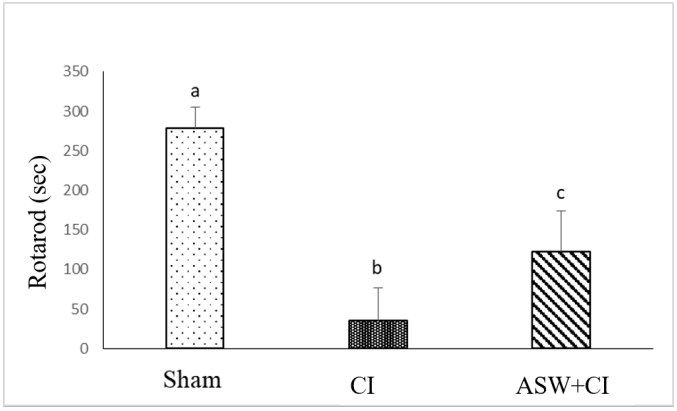

Figure 2. In the post-ischemia rota-rot test (Figure 2) in which motor coordination was evaluated, rats. The groups marked with different letters a,b and c were statistically different from each other ($p<0.05$). Differences between groups designated with the same letters are not statistically significant. The Kruskal-Wallis H test was used to evaluate results of the groups. When significant differences were detected between groups, multiple pair-wise comparisons were made using the Mann-Whitney U test with Bonferroni correction ($p<0.05$).

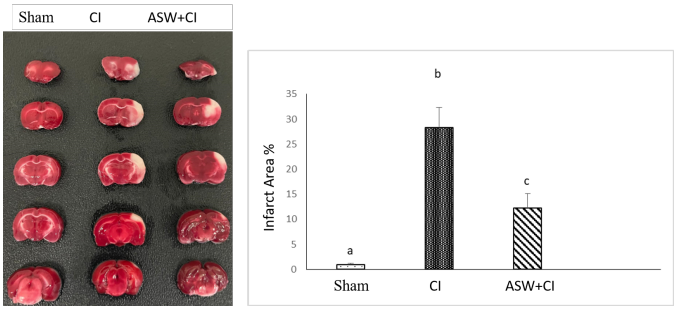

Figure 3. Infarct areas were determined with TTC staining .Groups marked with different letters a, b and c were statistically different ($p<0.05$). Differences between groups designated with the same letters are not statistically significant. The Kruskal-Wallis H test was used to evaluate the infarct area results of the groups. When significant differences were detected between groups, multiple pair-wise comparisons were made using the Mann-Whitney U test with Bonferroni correction ($p<0.05$).

while rats in the CI group remained in balance for an average of 15±7 seconds and rats in the ASW+CI group remained in balance for 86±35 seconds. When compared with the Sham group, the decrease in the balance time of the CI and ASW+CI groups is statistically significant. When the ASW+CI group is compared with the CI group, the increase in the balance time of rats in the ASW+CI group is statistically significant ($p<0.05$).

Evaluation of infarct area

According to our findings, infarct areas were determined with TTC staining in Figure 3. No infarct area was observed in the brain of sham-operated rats.Infarct area increased by 45.7% in the CI group compared to the Sham group, while it was 32.6% in the ASW+CI group treated with ASW, and the decrease of 13.1% in the ASW+CI group was statistically significant ($p<0.05$).

Discussion

Ischemic stroke is a neurological dysfunction in the brain caused by inadequate cerebral blood flow due to narrowing or blockage of blood vessels. CI, the most common cerebrovascular disease, is one of the most common causes of death worldwide [21]. In addition to the high rate of stroke-related deaths, it is the leading cause of acquired disability in adulthood. Caring for sick individuals creates a significant financial burden for both society and healthcare systems. Therefore, in addition to treatment-oriented studies, it is more important to develop preventive or protective practices for individuals at high risk of stroke. Cerebral ischemic stroke is a cerebrovascular disease that causes cell death and brain infarction due to arterial occlusion, which triggers various biochemical events including neuronal excitotoxicity, oxidative stress, inflammation, and apoptosis [22, 23].

Stroke can cause various neurological disorders such as speech disorders, cognitive perception errors, and dementia that impair the quality of life of patients, and sometimes lead to death. Current clinical therapeutic treatments for stroke include thrombolysis, reduction of bed rest due to stroke, and prevention of recurrent attacks [24]. However, the ability of these treatments to promote neuronal regeneration and restore brain function is limited. However, these treatments can slow the progression of neuronal damage, thereby improving patient quality of life and prolonging life. The main challenge in the treatment of poststroke neuronal dysfunction is postmitotic neurons with minimal regenerative capacity [25]. Mitochondrial dysfunction is the first-stage marker of neuronal death after ischemic stroke [26]. Therefore, preserving neuronal mitochondrial functions in CI/R injury is a promising treatment strategy targeted by researchers [27]. In CI/R injury, the absence of glucose and oxygen at the required level disrupts the energy balance in cerebral tissue, leading to the progression of pathological processes such as inflammatory response, oxidative stress, necrosis, apoptosis, and other harmful phenomena [28, 29]. During reperfusion, the reestablished blood flow also causes excessive production of reactive oxygen species (ROS), which seriously disrupts the homeostasis and function of mitochondria [27] Therefore, it is important to identify agents targeting mitochondria for the treatment of CI/R injury.

Researchers and clinicians aim to develop various strategies to improve the regeneration of neuronal cells and increase the rate of cell survival in order to enhance functional recovery after stroke. In the treatment process, two main groups of drugs are used according to their mechanisms of action; thrombolytics and neuroprotective agents. Neuroprotective agents are drugs that age the ischemic cascade, prevent secondary damage, and reduce the loss of vulnerable neurons in the ischemic penumbra. These agents, including antioxidants, neuronal stimulants, calcium channel antagonists, and free radical scavengers, employ a variety of mechanisms to protect neuronal cells against CI/R damage [30]. Although many neuroprotective agents have shown promise in experimental CI/R animal models, activation of multiple pathways in the pathophysiological process remains a significant challenge for researchers and clinicians to translate findings to clinical studies.

ASW has strong clinical and experimental evidence for treating CI. Many neuropathological studies in the literature have shown that ASW treatment can improve neurological functions and neuropathological damage in animal models [31]. Our results are quite consistent with the studies in the literature. In our study, we examined its effectiveness against CI/R injury using the effective dose application one week before, which has been shown to have neuroprotective activity [14]. Our results show that the group using ASW before ischemia improved neurological deficit scores and locomotor activities. In the measurement of ischemic areas, the ischemic areas in the ASW group were measured smaller. ASW is effective in changing the frequency of excitation by increasing the plasticity in neuronal cells. It is an effective agent in reducing and preventing structural damage in neuronal cells and preventing functional disorders [32]. This activity may contribute to the recovery of neuronal cells after CI/R injury. In our study, the improvement in rotarod results of ASW application is due to the increase in plasticity. The increase in neuronal plasticity increased the balance time of rats.

ASW is an adaptogen with hypoglycemic and hypolipidemic effects. It has also been shown to help learning and memory [33]. It is known to have anti-inflammatory, antiplatelet aggregation, anxiolytic, anti-convulsive and neuroprotective effects [34]. These effects make ASW a target for use as a potential protective agent against complications resulting from neuronal damage such as ischemia.

The aim of CI treatment is to start reperfusion as soon as possible. However, the reperfusion process causes much more neuronal damage than the ischemia process. In the literature, this paradoxical damage process is attributed to increased free oxygen radicals during the reperfusion process. Strengthening the antioxidant defense system against increased free oxygen radicals, which are not the only cause of neuronal damage pathways but an important pathophysiological process, can be an effective preventive treatment method [35]. ASW can be used as a protector against ischemia damage due to its antioxidant activity, as stated in the literature [36]. The positive results of ASW application in our study may be due to its antioxidant activity. It is due to the neuroprotective activity of the small ischemic area in ASW-treated rats. Increased oxidative stress in brain neuron cells is one of the main reasons for the deterioration of cognitive functions. Increasing antioxidant capacity against increased oxidative damage in pathophysiological processes such as ischemia, reperfusion or hyperglycemia is effective in reducing the limitations in cognitive functions. The improvements in the rotarod test in ASW-applied rats in this study are an indication of

this [37].

In CI/R injury, autophagy and apoptosis processes are also active pathways in neuronal damage and physical limitations. In ischemia studies, the antiapoptotic and autophagic effects of protective agents accelerate the treatment process [9]. ASW is effective in reducing the ischemic area and neurological deficit due to its antiapoptotic [38] and autophagic [39] activities stated in the literature. Although we did not evaluate it in our study, ASW has shown a protective effect against CI/R damage due to its healing effect on many pathways that are effective in neuropathological processes.

Conclusion

ASW has protective and therapeutic effects against CI/R damage. The use of ASW before ischemia is effective in reducing neuronal damage caused by CI/R damage. Its protection against neuronal damage may be due to its antioxidant, anti-apoptotic and autophagic activities. It can be used as a potential agent in the treatment and prevention of not only CI/R damage but also many central and peripheral nervous system diseases caused by neuronal damage.

Conflict of interest

The authors declare that they have no conflict of interest to disclose.

Ethical approval

This study was carried out with the approval of the Ethical Committee of Experimental Animals of the Faculty of Medicine at Inonu University (2023/12-3). The authors have no ethical conflicts to disclose.

Authors' contributions

T.K.,C.EI.,B.FN.,B.IO., C.M.,C.O. and B.B. conducted experiments and analysed the data.

T.K., C.CB., C.D., C.E., B.S., K.S. data curation.

T.K. wrote the manuscript.

T.K. project administration.

All authors have read and agreed to the published final version of the manuscript.

T.K. is the guarantor of this work and takes responsibility for the integrity of the data.

Informed consent

Informed consent was obtained from all subjects involved in the study. This study was presented as an oral presentation at the 9th National Medical Student Congress, hosted by Inonu University Faculty of Medicine, within the scope of the 14th Evidence-Based Medical Practice Days.

Data availability

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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