Effects of *Ganoderma lucidum* Polysaccharides on Different Pathways Involved in the Development of Spinal Cord Ischemia Reperfusion Injury: Biochemical, Histopathologic, and Ultrastructural Analysis in a Rat Model

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■ OBJECTIVE: **Inflammation and oxidative stress are 2 important factors in the emergence of paraplegia associated with spinal cord ischemia-reperfusion injury (SCIRI) after thoracoabdominal aortic surgery. Here it is aimed to investigate the effects of *Ganoderma lucidum* polysaccharide (GLPS) on SCIRI.**

■ METHODS: **Rats were randomly selected into 4 groups of 8 animals each: sham, ischemia, methylprednisolone, and GLPS. To research the impacts of various pathways that are efficacious in formation of SCIRI, tumor necrosis factor α, interleukin 1β, nitric oxide, superoxide dismutase levels, and catalase, glutathione peroxidase activities, malondialdehyde levels, and caspase-3 activity were measured in tissues taken from the spinal cord of rats in all groups killed 24 hours after ischemia reperfusion injury. The Basso, Beattie, and Bresnahan locomotor scale and inclined plane test were used for neurologic assessment before and after SCIRI. In addition, histologic and ultrastructural analyses of tissue samples in all groups were performed.**

■ RESULTS: **SCIRI also caused marked increase in tissue tumor necrosis factor α, interleukin 1β, nitric oxide, malondialdehyde levels, and caspase-3 activity, because of inflammation, increased free radical generation, lipid peroxidation, and apoptosis, respectively. On the other hand, SCIRI caused significant reduction in tissue superoxide dismutase, glutathione peroxidase, and catalase activities. Pretreatment with GLPS likewise diminished the level of the spinal cord edema, inflammation, and tissue injury shown by pathologic and ultrastructural examination. Pretreatment with GLPS reversed all these biochemical changes and improved the altered neurologic status.**

■ CONCLUSIONS: **These outcomes propose that pretreatment with GLPS prevents progression of SCIRI by alleviating inflammation, oxidation, and apoptosis.**

Key words
- *Ganoderma lucidum* polysaccharides
- Ischemia/reperfusion
- Neuroprotection
- Spinal cord

Abbreviations and Acronyms
BBB: Basso, Beattie, and Bresnahan
CAT: Catalase
ELISA: Enzyme-linked immunosorbent assay
GL: *Ganoderma lucidum*
GLPS: *Ganoderma lucidum* polysaccharide
GPx: Glutathione peroxidase
IL-1β: Interleukin 1β
MDA: Malondialdehyde
MP: Methylprednisolone
NO: Nitric oxide
ROS: Radical oxygen species
SCI: Spinal cord ischemia
SCIRI: Spinal cord ischemia-reperfusion injury
SOD: Superoxide dismutase
TEM: Transmission electron microscopy
TNF-α: Tumor necrosis factor α

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INTRODUCTION

Spinal cord ischemia-reperfusion injury (SCIRI), which may occur after thoracoabdominal aortic surgery, can lead to dramatic results such as paraplegia and even death. SCIRI is considered to have a 2-stage mechanism. Acute ischemia triggers the onset of inflammation and causes necrotic and/or apoptotic cell death in more severely injured neurons, and subsequent reperfusion aggravated the initial inflammation causing lipid peroxidation and radical oxygen species (ROS) production, resulting in delayed cell loss.[1] Understanding of the complex pathophysiology of ischemia-reperfusion injury is increasing, and in parallel to these advances, investigations for effective treatment options have accelerated in recent years, although there is not yet an efficacious treatment method to reduce neurologic damage caused by SCIRI.[2]

Recently, there has been evidence that free radical-scavenging polysaccharides obtained from mushrooms, fungi, and plants have strong abilities to prevent oxidative damage in living organisms.[3,4] *Ganoderma lucidum* (GL), possibly the most mainstream edible therapeutic mushroom, which has a place with the family Ganodermataceae of Polyporales, has been widely used in the therapy of various illnesses, including malignancy, hypertension, diabetes, and neurasthenia via its biologically active components.[5-11] The GL polysaccharide (GLPS) is one of the fundamental bioactive parts of GL and has been shown to have potent neuroprotective activity, as a result of its antiinflammatory, antioxidative, and antiapoptotic properties.[12-18] The efficacy of GLPS in SCIRI has not yet been shown.

In this study, the effects of GLPS on different pathways such as inflammation, oxidative stress, and apoptosis in SCIRI are evaluated by comparing it with methylprednisolone (MP), an agent shown to be effective in many previous studies.

METHODS

Preparation of GLPS

GLPS extract was obtained as a result of sequential extraction with hot water and precipitation with ethanol of the fruiting bodies of GL obtained from Çukurova University (Adana, Turkey), as described previously.[16,19] The oil and/or oily substances in the samples were removed by supercritical CO_2 extraction of GL species and subsequent atmospheric pressure extraction of triterpenoids with ethyl alcohol. The system was passed through a high-purity nitrogen stream to completely remove the ethyl alcohol residue in the reactor. For the boiling water extraction of polysaccharides at atmospheric pressure, the connection on the top cover where the safety disc of the reactor is placed was removed and 2 separate grinding back coolers were connected one on top of the other with a special adapter. 8 L of distilled water was added to the samples and extracted in boiling water for 4 hours. At the end of the period, the extract was collected by opening the valve under the reactor. This process was repeated twice more and all extracts were combined and concentrated to a certain volume (45–50 mL) in a vacuum rotary evaporator. The extract of the free protein denaturation, was treated by direct precipitation to remove extracted trichloroacetic acid (approximately 4 g). Whereas the precipitate formed as a result of this process was removed by centrifugation, the centrifuge was dialyzed in running water for 3 days and in purified water for 24 hours (ServaMembra Cell Dialysis Tubing; molecular weight cutoff, 3500 Da). After dialysis, the residue was collected in a flask, concentrated to a small volume in the rotary evaporator, and 4 times its volume ethanol was suffixed to the remaining fluid extract. To this mixture at 4°C overnight, the precipitate formed after standing at 6000 rpm at 4°C for 25 minutes was centrifuged. After centrifugation, the last residue (polysaccharide-containing solid) was washed with ethanol and acetone and then frozen with liquid nitrogen and dried in Freeze Dryer (Labconco 7670530 [Labconco Corp., Kansas City, Missouri, USA]) at −48 °C for 5 days.

Analysis of Molecular Weight and Structural Components

The primary structural properties of the extract were determined by high-performance liquid chromatography and nuclear magnetic resonance cross-polarization, and its molecular weight was shown by the high-pressure size exclusion chromatography multiangle laser light scattering system. According to the results, the extract we obtained has been shown to be a glycoprotein with a weight of 6.279×10^4 Da consisting of carbohydrate and protein in the β-D-glucan structure.

Experimental Groups

The examination was led as per the Guide for Care and Use of Laboratory Animals distributed by the U.S. National Institutes of Health (NIH distribution no. 85-23, reexamined 1996). The whole research procedure used in this examination was surveyed and endorsed by the ethical committee of the Ministry of Health Ankara Education and Research Hospital. Thirty-two adult male Sprague-Dawley rats weighing 350 ± 20 g were arbitrarily allocated to 4 groups, with 8 rats in each.

The groups were as follows:

Group 1: sham group ($n = 8$): laparotomy only without aortic cross-clamping. No treatment was given to this group. Rats were killed 24 hours after surgery and spinal cord tissues were acquired to decide regular spinal cord morphology and benchmark biochemical qualities.

Group 2: ischemia group ($n = 8$): rats undergoing transient global spinal cord ischemia (SCI). Rats received 2 mL of distilled water orally (as a vehicle) starting 7 days before occlusion until death.

Group 3: MP group ($n = 8$); as determined by previous studies MP (Prednol, Mustafa Nevzat, Turkey) was administered intraperitoneally to the injured rats at a single 30 mg/kg dose just after the closing the abdominal wall.[20,21]

Group 4: GLPS group ($n = 8$); as in group 3 after the selection of dosage based on previous studies, GLPS was administered orally at a dose of 400 mg/kg/day, starting 7 days before SCIRI was performed, until the rats were killed.[12,16]

Anesthesia and Surgical Procedure

With free access to food and water, rats were kept at 22°C −25°C with suitable moisture and a 12-hour light and 12-hour dark circulation, under environmentally controlled conditions. Rats were anesthetized, allowed spontaneous respiration, with 10 mg/kg

xylazine (Rompun, Bayer, Turkey), and 50 mg/kg ketamine (Ketalar, Parke Davis, Turkey) by intraperitoneal injection. After induction of anesthesia, rats were placed on a heating pad to maintain their body temperature at 37°C with rectal probes attached.

Induction of SCIRI was performed as previously described.[22] The aorta was separated from the surrounding tissues from the beginning of the left renal artery proximally to the point at which it bifurcated distally via the transperitoneal route after entry with an abdominal incision. Then, 200 IU/kg intravenous heparin was injected and 5 minutes later, the aorta was closed just below the left renal artery and just above the aortic bifurcation with 2 clamps (Yasargil FE 721 [Aesculap, Tuttlingen, Germany]) with 70 g closing force. At beginning of a 30-minute ischemia period that allowed adequate injury to occur,[23] it was visually confirmed that the femoral artery pulse disappeared and the aortic pulsation returned after the clamp was removed. At the end of the procedure, the abdominal incision sutured. The 30-minute ischemia period was chosen to achieve adequate injury.[23] Immediately after suturing the incision, 30 mg/kg intraperitoneal MP was administered. The rats were kept in a suitable environment until the time of killing 24 hours after the operation. In the sham-operated group, no aortic clamping was performed after laparotomy. The neurologic function of the rats was evaluated by Basso, Beattie, and Bresnahan (BBB) scores and inclined a plane test 24 hours after occlusion. Spinal cord samples from the L4-L6 segments of the rats were cut into 3 pieces of 5 mm to determine the light microscopic (cranial part), electron microscopic (middle part), and biochemical (caudal part) evaluation immediately after they were killed by injection of 200 mg/kg pentobarbital (Nembutal). Tissues to be used in biochemical analysis were stored at −80°C until the study date.

Biochemical Procedures

For removing upper clear supernatants to be used in analysis, the homogenized tissues in physiologic saline solution were centrifuged at 4000 rpm for 20 minutes.

Tissue Tumor Necrosis Factor α and Interleukin-1β Analysis

Tissue tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) levels as indicators of inflammation were calculated by enzyme-linked immunosorbent assay (ELISA) kits (Uscn Life Science Inc., Wuhan, China), according to the instructions for use and expressed as U/g protein.

Tissue Nitric Oxide Analysis

Tissue nitric oxide (NO) levels were measured as described by Miranda et al.[24] In this method, proteins of tissues homogenized in saline solution are precipitated using ethanol. The substances are separated for 15 minutes at 25°C to recover the supernatant. 0.5 mL supernatant, 0.5 mL vanadium (III) chloride (8 mg VCl_3/mL), and 0.5 mL freshly prepared Griess reagent (1% sulfanilamide, 2% phosphoric acid, and 0.1% N-1 naphthylethylene diamine dihydrochloride; 500 µL) mixture is incubated at 37°C for 30 minutes by vortexing. Then, the absorption at 540 nm is measured using a dual-beam spectrophotometer and the results are expressed as nmol/mg protein.

Tissue Superoxide Dismutase Analysis

Total (Cu-Zn and Mn) superoxide dismutase SOD (EC 1.15.1.1) activity of the supernatant in the ethanol phase was determined according to the description of Sun et al.[25] after adding 1.0 mL of ethanol/chloroform mixture (5/3, v/v) to the same volume of sample and centrifuging. The amount of enzyme that caused 50% inhibition of nitroblue tetrazolium reduction was defined as 1 unit of SOD and the enzyme activity was expressed as U/mg protein.

Tissue Catalase and Glutathione Peroxidase Analysis

The catalase (CAT) and glutathione peroxidase (GPx) activities as indicators of oxidative stress were calculated by ELISA kits (Uscn Life Science Inc.), according to the instructions for use and expressed as U/g protein.

Tissue MDA Analysis

Tissue malondialdehyde (MDA) levels were assessed by a method based on reaction with thiobarbituric acid as previously described by Ohkawa et al.[26] To a mixture of 100 mL of tissue homogenate and 50 mL of sodium dodecyl sulfate (8.1%) are added 375 µL acetic acid (pH 3.5, 20%) and 375 µL thiobarbituric acid (0.6%), which is vortexed and incubated at room temperature. After this mixture is heated in boiling water for 60 minutes and subsequently cooled to room temperature, 1.25 mL of butanol:pyridine (15:1) is added to each test tube. Then this vortexed mixture is centrifuged at 4000*g* for 5 minutes and the absorption of 750 µL organic layer is read at 532 nm in 1-mL cells. MDA concentrations were expressed as nmol/mg protein.

Tissue Caspase-3 Analysis

The caspase-3 activity as an indicator of apoptosis caspase-3 activity was calculated by ELISA kits (Uscn Life Science Inc.), according to the instructions for use and expressed as U/g protein.

Histopathologic Procedures

Sections of 5 µm thickness obtained from paraffin-embedded spinal cord tissue samples fixed with 10% buffered formalin for 24 hours were stained with hemotoxylin-eosin and then examined by a histopathologist blinded to the study design.

Pathologic findings such as edema, vascular occlusion, and inflammation and neurodegenerative findings such as nuclear pyknosis, nuclear hyperchromasia, cytoplasmic eosinophilia, and axonal edema were calculated using a semiquantitative system for histopathologic scoring of each tissue sample from 0 (none) to 3 (common). As a result, calculations were made according to the sum of the scores of 4 different parameters.[27]

The number of normal motor neurons in the anterior horn in each spinal cord tissue sample was counted in 3 sections and then averaged to achieve a more detailed grading of the neuronal injury.[27]

Ultrastructural Examination

Tissue samples that were purified from blood and meninges removed using a scalpel were fixed for 24 hours in 2.5% glutaraldehyde, washed in phosphate buffer, and then fixed in 1% osmium tetroxide in phosphate buffer (pH 7.4) for 2 hours, dehydrated to increasing alcohol concentrations. The tissues,

which were subsequently washed with propylene oxide, were embedded in epoxy resin. The LKB-Nova (LKB-Produkter AB, Bromma, Sweden) ultramicrotome was cut with a glass knife to obtain semithin sections of 2 μm thickness and ultrathin sections of approximately 60 nm thickness. Semithin sections were stained with methylene blue and ultrathin sections with uranyl acetate and lead citrate. After staining, semithin sections were examined using a Nikon Optiphot (Nikon Corporation, Tokyo, Japan) light microscope and ultrathin sections were examined using Jeol JEM 1200 EX (Jeol Ltd., Tokyo, Japan) transmission electron microscopy (TEM). As described by Kaptanoğlu et al.,[28] every 100 large diameter myelinated axons, medium diameter myelinated axons, and small diameter myelinated axons were counted, evaluated, and scored between 0 and 3, as follows: 0, ultrastructurally normal myelinated axon; 1, separation in myelin configuration; 2, interruption in myelin configuration; and 3, honeycomb appearance in myelin configuration.

Neurologic Evaluation

Immediately before the rats were killed, 2 independent observers blinded to the groups scored the animals' neurologic status using the BBB locomotor scale,[29] in which movement, weight support, and coordination were scored between 0 (motor activity absent) and 21 (normal status), and assessed their ability to maintain postural stability for 5 seconds at maximum inclination using the inclined plane test.[30]

Statistical Analysis

Data analysis was performed by using SPSS for Windows version 11.5 (SPSS Inc., Chicago, Illinois, USA). Whether the distributions of continuous variables were normally or not was determined by a Shapiro-Wilk test. A Levene test was used for the evaluation of homogeneity of variances. Data were shown as mean ± standard deviation or median (interquartile range), as applicable.

Whereas the mean differences among groups were analyzed by using 1-way analysis of variance, a Kruskal-Wallis test was applied for comparisons of the median values. When the P value from 1-way analysis of variance or Kruskal-Wallis test statistics are statistically significant, a post hoc Tukey HSD (honestly significant difference) or Conover nonparametric multiple comparison test was used to identify which group differed from which others.

A P value <0.05 was considered statistically significant.

RESULTS

Tissue TNF-α Levels

Because I/R injury leads to release of proinflammatory cytokines, tissue TNF-α levels in the ischemia group showed a statistically significant increase compared with other groups ($P < 0.001$). However, treatment with both GLPS and MP provides a significant reduction in tissue TNF-α levels because of their antiinflammatory effects ($P < 0.001$ for both). In comparison among treatment groups, MP and GLPS showed no statistical difference ($P = 0.822$).

Tissue IL-1β Levels

In the ischemia group, tissue IL-1β levels, an important mediator of the inflammatory response, showed a statistically significant increase compared with the other groups ($P < 0.001$). Tissue IL-1β levels showed a marked reduction with response to treatment in both GLPS and MP groups compared with the ischemia group ($P = 0.003$ and $P < 0.001$, respectively). In comparison among treatment groups, MP and GLPS showed no statistical difference ($P = 0.474$).

Tissue NO Levels

A statistically significant increase in tissue NO levels was determined in the ischemia group compared with other groups as a result of oxidative stress ($P < 0.001$). A marked reduction was established in tissue NO levels in both GLPS and MP groups compared with the ischemia group because of their antioxidative properties ($P < 0.001$ and $P = 0.002$, respectively). In comparison among treatment groups, MP and GLPS showed no statistical difference ($P = 0.248$).

Tissue SOD Activity

Tissue SOD activity, a powerful antioxidant enzyme, was significantly decreased in the ischemia group compared with the other groups ($P < 0.001$). Because of the antioxidative properties of both GLPS and MP, this decrease in the ischemia group markedly increased in both groups after treatment ($P = 0.004$ and $P < 0.001$, respectively). MP and GLPS groups did not show any statistical difference regarding tissue SOD activity ($P = 0.338$).

Tissue CAT Activity

Tissue CAT activity, which is the primary antioxidant defense component, showed a marked reduction in the ischemia group compared with others ($P < 0.001$). This reduction, which was induced by I/R damage, showed a statistically significant improvement after treatment with both GLPS and MP ($P < 0.001$ for both). No such difference was observed between the MP and GLPS groups ($P = 0.893$).

Tissue GPx Activity

After SCIRI, tissue GPx levels of the ischemia group decreased significantly compared with the other groups because of the decrease in endogenous antioxidant enzyme activity ($P < 0.001$). GLPS and MP treatments provided a marked recovery in reduced tissue GPx activity compared with the ischemia group ($P < 0.001$ for both). No such difference was detected between the MP and GLPS groups regarding tissue GPx activity ($P = 0.998$).

Tissue MDA Levels

Because of the induction of lipid peroxidation by SCIRI, tissue MDA levels in the ischemia group markedly decreased compared with others ($P < 0.001$). When the ischemia group was compared with treatment groups, a marked increase was found in tissue MDA levels in both GLPS and MP treated groups ($P = 0.001$ and $P = 0.003$, respectively). No difference was found among treatment groups ($P = 0.753$).

Tissue Caspase-3 Activity

As an indicator of apoptosis occurring after SCIRI, a marked increase in caspase-3 activity was detected in the ischemia group compared with others ($P < 0.001$). The decrease in tissue caspase-3 activities after treatment in both GLPS and MP groups compared with the ischemia group showed that both drugs effectively

prevented apoptotic cell death ($P = 0.001$ for both). There was no difference among the treatment groups in terms of effects on the apoptotic pathway ($P = 0.788$).

The results are summarized in Table 1.

Histopathologic Examination

The sham group showed normal tissue morphology under light microscopic evaluation (Figure 1A). In the ischemia group, widespread hemorrhage, congestion, and edema in both white and gray matter and neuronal degeneration accompanying inflammatory cell flow were observed (Figure 1B). Pathologic changes caused by I/R damage were significantly reduced in both MP and GLPS groups (Figure 1C and D).

The ischemia group pathologic scores were excessively higher than the sham, GLPS, and MP groups regarding statistical difference ($P < 0.001$, $P < 0.001$, and $P < 0.05$, respectively). The GLPS group showed a lower degeneration score than the MP group ($P = 0.02$).

The number of normal motor neurons in the anterior spinal cord of the ischemia group was markedly reduced compared with others ($P < 0.001$ for all). GLPS and MP groups did not show a statistical difference ($P = 0.949$). These results showed that the spinal cord tissue was largely protected from the pathologic effects of I/R damage in the treatment groups.

The results are summarized in Table 2.

Ultrastructural Examination

TEM examination of the sham group showed ultrastructurally normal gray and white matter morphology of the spinal cord, except for slight separations caused by delayed fixation of the tissue in a few of the large myelinated axons (Figure 2A).

TEM examination of the ischemia group showed detachment and disruption in the myelin configuration of all-sized myelinated axons. Perineural edema in the gray matter, swelling of the mitochondria in the neuron cytoplasm, and vacuolization were observed. The highest ultrastructural damage was observed in myelinated axons in this group (Figure 2B).

TEM examination of the MP group showed less perineural edema in the gray matter, swollen mitochondria, and vacuoles in the neuron cytoplasm compared with the ischemia group ($P < 0.001$). Separated myelin sheets were detected in the white matter, mostly in large and medium-sized axons (Figure 2C).

No ultrastructural changes were found by TEM examination of the GLPS group, except for a small amount of perineural edema and dilatations in the perinuclear cisterna in the gray matter. Small myelinated axons in the GLPS group had a mostly ultrastructurally normal appearance compared with the ischemia group ($P < 0.001$) (Figure 2D).

These results show that both GLPS and MP treatments have a protective effect on axonal myelination against SCIRI. In addition, the ultrastructural appearances of small and medium-sized myelinated axons were significantly better in the GLPS group than in the MP group as determined by scoring ($P < 0.01$) (Table 3).

Neurologic Outcome

The BBB score, which was 21 in all rats before SCIRI, showed a statistically marked decrease in the ischemia group compared with the other groups in the assessment just before the rats were killed ($P < 0.001$ for all). No such difference was detected among treatment groups following SCIRI ($P = 0.591$).

The recorded mean angles of the rats in the ischemia group, which were subjected to an inclined plane test after I/R injury, were statistically significantly lower compared with the other groups ($P < 0.001$). Increased mean angles were recorded in the inclined plane test for the GLPS and MP treatment groups compared with the ischemia group ($P < 0.001$ for both). But no statistical difference was found between the MP and the GLPS groups ($P = 0.97$).

BBB and inclined plane test results are summarized in Table 4.

Table 1. Biochemical Measurements

Variables	Sham	Ischemia	Methylprednisolone	Ganoderma	*P* Value
Tissue nitric oxide (nmol/g)	36.4 (20.82)*,†	83.7 (59.97)*,‡,§	48.7 (35.18)†,‡	44.3 (21.38)§	0.003
Tissue malondialdehyde (nmol/g)	1.9 (1.46)*,†,‖	8.7 (9.47)*,‡,§	3.9 (5.00)†,‡	4.3 (2.95)§,‖	0.002
Tissue superoxide dismutase (U/g protein)	0.56 (0.81)*,†	0.30 (0.17)*,‡,§	0.39 (0.36)†,‡	0.49 (0.25)§	0.006
Tissue tumor necrosis factor (U/g protein)	20.8 (11.64)*,†,‖	43.1 (16.37)*,‡,§	28.3 (8.61)†,‡	26.4 (9.50)§,‖	<0.001
Tissue glutathione peroxidase (U/g protein)	75.0±13.28*	27.6±7.87*,‡,§	54.6±14.41†,‡	55.6±11.80†,‡	<0.001
Tissue catalase (U/g protein)	1.31 (1.26)*,†,‖	0.16 (0.24)*,‡,§	0.69 (0.62)†,‡	0.79 (0.49)§,‖	<0.001
Tissue interleukin 1 (U/g protein)	22.2 (6.82)*,†,‖	77.0 (49.52)*,‡,§	35.4 (23.72)†,‡	39.3 (22.47)§,‖	<0.001
Caspase-3 (U/g protein)	155.4 (52.12)*,†,‖	881.4 (611.37)*,‡,§	420.6 (383.10)†,‡	415.7 (270.97)§,‖	<0.001

*Sham versus ischemia ($P < 0.01$).
†Sham versus methylprednisolone ($P < 0.05$).
‡Ischemia versus methylprednisolone ($P < 0.01$).
§Ischemia versus *Ganoderma* ($P < 0.01$).
‖Sham versus *Ganoderma* ($P < 0.05$).

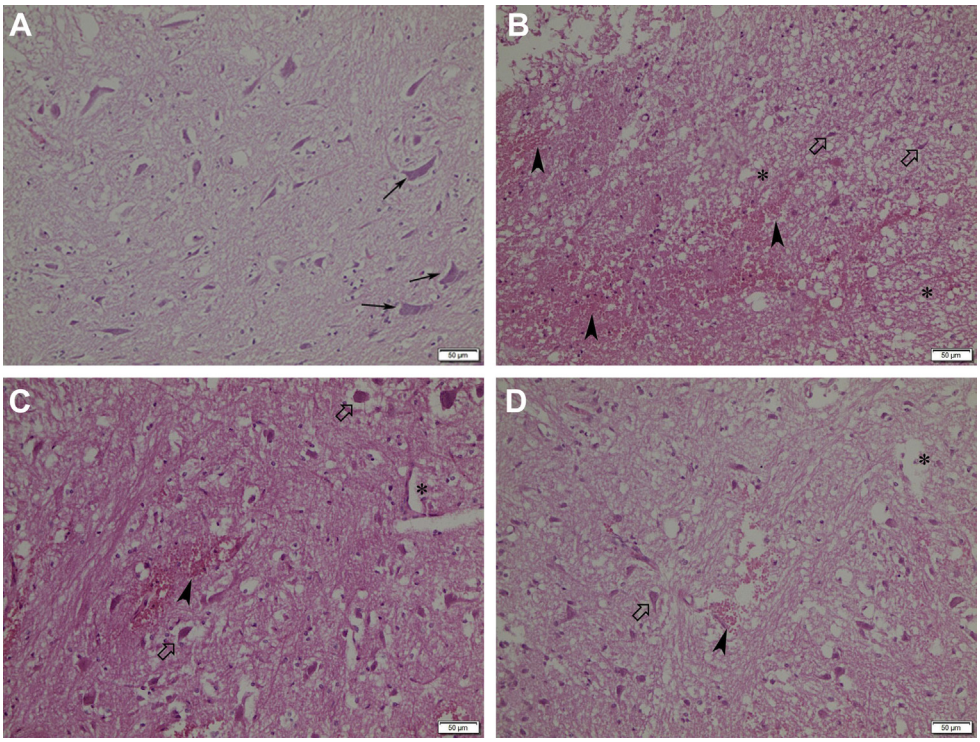

Figure 1. Photomicrographs of 5-mm-thick spinal cord tissue sections from the different treatment groups (hematoxylin-eosin, original magnification ×200). (**A**) Sham group showing normal spinal cord parenchyma with normal-appearing neurons (*filled arrow*). (**B**) Ischemia group showing diffuse hemorrhage and congestion (*arrowhead*), and widespread edema (*asterisk*) with highly degenerated neurons (*arrow*) in the gray matter. (**C**) Methylprednisolone group, showing mild edema (*asterisk*) and mild hemorrhagic congestion (*arrowhead*) with fewer degenerated normal neurons (*arrow*). (**D**) GL polysaccharides group, showing mild edema (*asterisk*) and minimal congestion (*arrowhead*) with fewer degenerated normal neurons (*arrow*).

Table 2. Histopathologic Parameters

Variables	Sham	Ischemia	Methylprednisolone	Ganoderma	*P* Value
Edema	0.0 (0.00)*,†,‡	2.0 (1.00)*,§,‖	1.0 (0.75)†,§	1.0 (0.75)‡,‖	<0.001
Congestion	0.0 (0.00)*,†,‡	2.0 (0.00)*,§,‖	1.0 (0.75)†,§	1.0 (0.00)‡,‖	<0.001
Inflammation	0.0 (0.00)*,†,‡	1.0 (0.75)*	1.0 (1.00)†	1.0 (1.00)‡	0.014
Degeneration	0.0 (0.00)*,†,‡	2.0 (0.00)*,§,‖	1.0 (0.75)†,§,¶	1.0 (1.00)‡,‖,¶	<0.001
Pathology score	0.0 (0.00)*,†,‡	6.0 (3.00)*,§,‖	4.0 (3.00)†,§	3.0 (2.00)‡,‖	<0.001
Number of normal neurons	45.6±4.14*,†,‡	21.1±2.03*,§,‖	35.4±3.58†,§	36.2±2.9‡,‖	<0.001

*Sham versus ischemia ($P < 0.001$).
†Sham versus methylprednisolone ($P < 0.001$).
‡Sham versus *Ganoderma* ($P < 0.05$).
§Ischemia versus methylprednisolone ($P < 0.05$).
‖Ischemia versus *Ganoderma* ($P < 0.001$).
¶Methylprednisolone versus *Ganoderma* ($P = 0.02$).

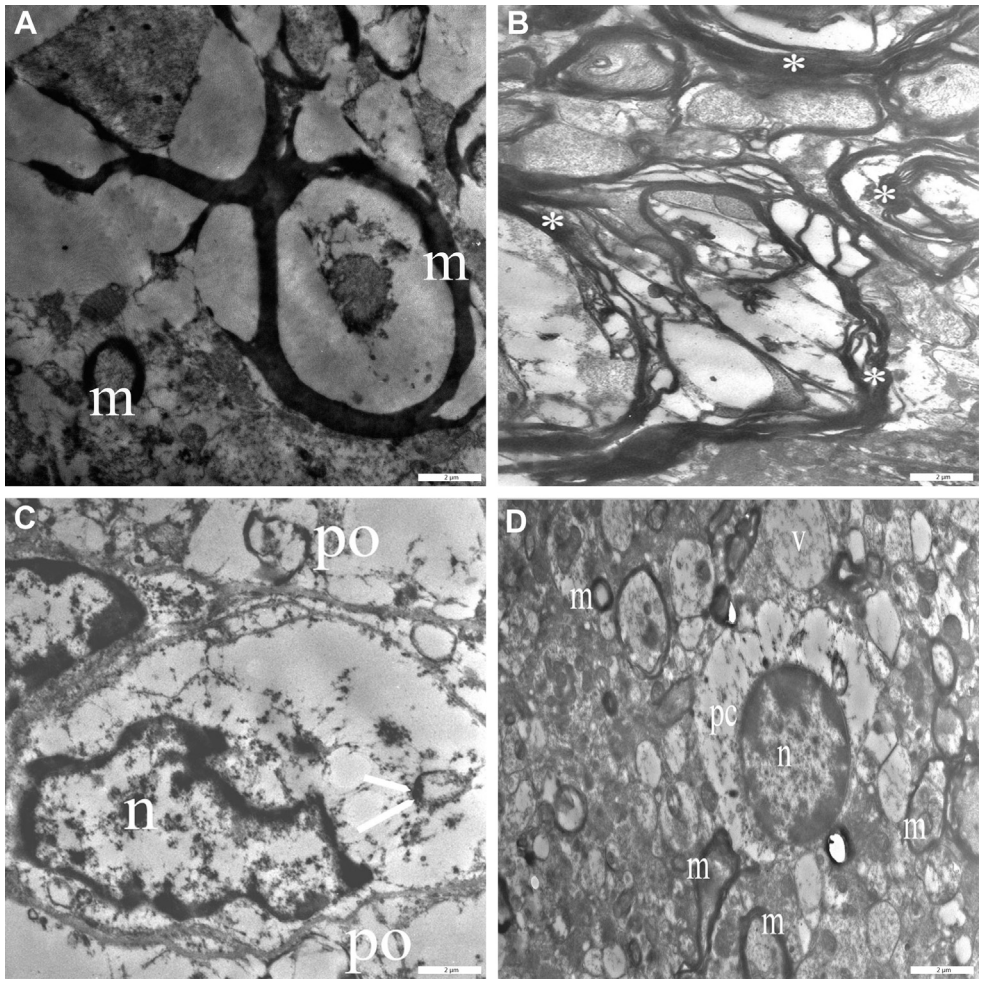

Figure 2. Transmission electron microscopy of the groups. (**A**) Transmission electron microscopic examination of the tissue samples of the sham group showed normal spinal cord ultrastructure in the gray and white matters. The mild separations in the myelin configuration observed in a few of the large myelinated axons may be explained by delayed fixation; *m*, ultrastructurally normal myelinated axon (bar represents 2 µm). (**B**) Electron micrograph of the ischemia group showing perineural edema and interruption in myelin configuration of the myelinated axons (*). (**C**) Electron micrograph of the methylprednisolone group showing perineural edema (*po*) with swollen mitochondria (*double arrow*); *n*, nucleus of neuron. (**D**) Electron micrograph of the *Ganoderma lucidum* polysaccharides group showing dilatations in perinuclear cisternae (*pc*), small vacuole (*v*) inside the cytoplasm of a neuron, and ultrastructurally normal myelinated axons (*m*).

DISCUSSION

SCIRI is a serious complication of thoracoabdominal aortic interventions, with the incidence varying between 1% and 32% in different series, and it can result in paraplegia or even death.[31,32] Numerous interwoven cascades such as lipid peroxidation, overproduction of ROS, inflammation, and apoptosis play an important role in these processes.[23,33-36] In thoracoabdominal aortic interventions, several protective and therapeutic strategies have been used to both increase the resistance of the neural structures to ischemia and to prevent secondary damage caused by reperfusion.[37-41] Despite many clinical and experimental studies carried out to obtain an effective treatment method, there is still no consensus on the exact management of SCIRI.

GL (Leyss. Old Fr.) Karst (Ling Zhi), which has been commonly used conventionally for a long time in East Asia and has recently become a popular dietary supplement ingredient in Western countries, is a type of mushroom belonging to the Ganodermataceae Polyporales family.[42,43] The polysaccharide (GLPS) is the main active constituent of GL and its use has been shown to be safe in vivo and clinically in previous toxicologic studies.[44-48] Previous studies have suggested that GL spores provide neuroprotective effects by making changes with upregulation and

Table 3. Electron Microscopy Results

Myelinated Axon	Sham	Ischemia	Methylprednisolone	Ganoderma	*P* Value
Small	0.0 (0.00)*,†,‡	115.0 (8.50)*,§,‖	30.0 (4.00)†,§,¶	31.0 (3.00)‡,‖,¶	<0.001
Medium-sized	0.0 (0.00)*,†,‡	140.0 (14.50)*,§,‖	91.0 (5.50)†,§,¶	72.0 (6.00)‡,‖,¶	<0.001
Large	4.0 (3.50)*,†,‡	169.0 (7.00)*,§,‖	115.0 (4.00)†,§	79.0 (7.50)‡,‖	<0.001

*Sham versus ischemia ($P < 0.01$).
†Sham versus methylprednisolone ($P < 0.01$).
‡Sham versus *Ganoderma* ($P < 0.01$).
§Ischemia versus methylprednisolone ($P < 0.01$).
‖Ischemia versus *Ganoderma* ($P < 0.01$).
¶Methylprednisolone versus *Ganoderma* ($P < 0.01$).

downregulation in protein expressions that provide axonal regeneration in damaged spinal motor neurons.[49] In addition, previous studies have shown that GL protects hippocampal neurons after I/R injury via its oxidation and inflammation mitigating properties.[14] Our previous results showed that GLPS pretreatment had neuroprotective effects through antiinflammatory, antioxidative, and antiapoptotic paths in spinal cord injury.[16] As far as we know, this is the first study to investigate GLPS efficacy on SCIRI.

Ischemia causes the initiation of an inflammatory cascade by activating various pathophysiologic signaling pathways, including cytokines such as TNF-α and IL-1β, ROS. These proinflammatory cytokines alert monocyte migration, macrophage activation, and neutrophil adhesion to the injury site.[50,51] Activated macrophages can also cause production of NO in relatively large amounts by their own inducible NO syntase in response to inflammation. NO, which has an antiinflammatory effect under normal physiologic conditions, can act as a proinflammatory mediator in prolonged ischemia caused by its overproduction. Excessive amounts of NO can exacerbate damage to the lipid, protein, and nucleic acid contents of neurons, similar to other free radicals.[52] All these elements contribute to aggravation of the inflammation and can result in a vicious circle that constantly exacerbates the injury. Therefore, breaking this circuit is of the utmost importance for the limitation of secondary injury and the regeneration of surviving neurons during the progression of SCIRI. It has been shown in past studies that the production of microglia-derived proinflammatory and cytotoxic factors such as NO, TNF-α, and IL-1β can be inhibited by GL extracts.[53] Zhang et al.[14] asserted that GL represses the expression of hippocampal proinflammatory cytokines such as TNF-α and IL-8 in cerebral I/R injury in rats. In addition, Wang et al.[54] reported that GLPSs significantly inhibit lipopolysaccharide solution-stimulated NO production against murine Raw 264.7 macrophages in a dose-dependent manner through downregulation of inducible NO synthase, IL-1, and TNF-α messenger RNA gene expression. Recently, it has been shown that GLPS inhibit the cytokine-induced NO production in mice through inhibition of iNOS protein expression.[55] Our previous results also showed that GLPS decreased TNF-α, IL-1β, and NO levels in traumatic SCI.[16] Consistent with the literature, our study also showed that TNF-α, IL-1β, and NO levels were markedly increased in spinal cord tissue after ischemia/reperfusion injury. In our study, marked reduction was observed in increasing proinflammatory cytokine and NO levels as a result of I/R damage, which shows the antiinflammatory effects of both GLPS and MP.

ROS are formed during normal metabolic activities of the cells and these molecules are neutralized by properly working antioxidant defense systems including SOD, CAT, and GPx. In the normal detoxification process, SOD, CAT, and GPx cocatalyze the enzymatic reactions in which the superoxide anion converts initially into hydrogen peroxide and oxygen, and then hydrogen peroxide into water and oxygen.[56,57] However, prolonged ischemic damage in SCIRI causes a high amount of ROS production that cannot be compensated by a diminished antioxidant defense system. Thus, an imbalance toward the pro-oxidative state can occur, which is referred to as oxidative stress. These excessive oxygen radicals have destructive effects on cellular proteins, DNA, and membrane lipids. Peroxidation of membrane lipids results in a large amount of MDA production.[58] Therefore, MDA is a reliable

Table 4. Neurologic Examination Results

Myelinated Axon	Sham	Ischemia	Methylprednisolone	Ganoderma	*P* Value
Basso, Beattie, and Bresnahan	21.0 (0.00)*,†,‡	2.0 (1.75)*,§,‖	8.0 (2.75)†,§	9.0 (3.00)‡,‖	<0.001
Inclined plane	77.4±5.32*,†,‡	37.7±5.06*,§,‖	61.2±7.99†,§	59.7±8.03‡,‖	<0.001

*Sham versus ischemia ($P < 0.001$).
†Sham versus methylprednisolone ($P < 0.001$).
‡Sham versus *Ganoderma* ($P < 0.001$).
§Ischemia versus methylprednisolone ($P < 0.001$).
‖Ischemia versus *Ganoderma* ($P < 0.001$).

marker of lipid peroxidation. In previous studies, it has been shown that GLPS increases antioxidant enzyme activities such as SOD, GPx, and CAT and decreases MDA levels with its dose-dependent antioxidative properties.[16,59] Similar to previous results, the results of our study showed that both GLPS and MP treatment significantly reversed the decrease in endogenous antioxidant enzyme activities and the increase in tissue MDA levels caused by SCIRI.

In the acute phase of ischemia, severely disrupted perfusion can cause rapid cell death through necrosis and/or apoptosis. Also, in some cells that are exposed to less severe ischemic damage and subsequent reperfusion, apoptotic pathways can be activated and result in delayed-cell death.[1,60] Caspase-3 is a key enzyme believed to be involved in both intrinsic and extrinsic pathways of apoptosis. It has been shown in many previous studies that the increase in caspase-3 activity observed in the pathophysiology of SCIRI provides the demonstration of apoptotic activity as a reliable marker.[60-63] Zhou et al.[12] reported that GLPS inhibits apoptotic neuronal cell death by downregulating caspase-3 activation in cerebral ischemic injury of rats. Our previous results also showed that GLPS prevented apoptotic cell death by effectively reducing the increased caspase-3 activity caused by SCI in rats.[16] As a result of our study, the antiapoptotic efficiency of GLPS was shown by inhibition of the increased caspase-3 activity caused by SCIRI with GLPS treatment.

To obtain more reliable and detailed results, we also performed a histopathologic and ultrastructural examination. In the white and gray matter of the spinal cord samples a day after SCIRI, there was edema, inflammatory cell infiltration, vascular congestion, and a decrease in the number of motor neurons in addition to morphologic changes of neurons such as loss of cytoplasmic features and cell volume, and pyknosis of the nucleus. Both GLPS and MP alleviated these pathologic changes.

In addition, TEM was used to evaluate the ultrastructural changes. Ischemia/reperfusion injury resulted in interruptions and separations in myelin configuration at the ultrastructural level especially in the large axons. Both GLPS and MP treatment provided partial improvement of ultrastructural pathologic changes. The results once again showed that both MP and GLPS were effective treatment methods against SCIRI and GLPS was associated with more successful results than MP.

Measurement of locomotor performance with BBB score evaluated the effectiveness of treatment on motor functions.[19] In the evaluation before the rats were killed, both GLPS and MP treatment significantly improved the decrease in SCIRI-induced neurologic examination scores and mean angles in the inclined plane test.

The routine use of high-dose MP in the treatment of spinal cord injury has recently become controversial because of its risks and side effect profile, which has necessitated the investigation of new pharmacologic treatment methods. Despite attempting to develop new pharmacologic agents against spinal cord injury, the only neuroprotective drug widely accepted is MP.[64] Because MP is the most frequently used pharmacologic agent in experimental models of spinal cord injury,[16,65,66] it was used for comparison with GLPS as a positive control group.

Nonetheless, this study has some limitations. First, the number of subject animals could be considered insufficient to apply these results to clinical use. Further studies with more rats are needed to confirm the results of this study. The study would be stronger if we measured the blood levels of GLPS after treatment and performed a dose-response study. Because this study was planned as a preliminary observation, we were not able to evaluate the long-term results because of the high mortality risk to the animals and the duration and dose were not included in the present study as a variable. In addition, although GLPS was more effective in histopathologic and ultrastructural examination, biochemical results showed no such difference between the GLPS and MP groups. So, we suggest that GLPS exerts neuroprotective activity by acting on different biochemical pathways from those investigated in our study.

This study shows that GLPS provides neuroprotection against SCIRI by increasing the activities of endogenous antioxidant enzymes as well as reducing lipid peroxidation, inflammatory cytokine production, free radical formation, and apoptosis. These results are also verified by histopathologic, ultrastructural, and functional evaluations. Nevertheless, further studies are needed for the establishment of different pathways related to its neuroprotective activity in a time-dependent and dose-dependent manner.

CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Ramazan Kahveci: Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Visualization. **Fatih Ozan Kahveci:** Validation, Formal analysis, Writing - review & editing. **Emre Cemal Gokce:** Conceptualization, Writing - review & editing. **Aysun Gokce:** Investigation, Resources, Writing - review & editing. **Üçler Kısa:** Investigation, Resources, Writing - review & editing. **Mustafa Fevzi Sargon:** Investigation, Resources, Writing - review & editing. **Ramazan Fesli:** Validation, Writing - review & editing. **Bora Gürer:** Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Visualization, Supervision.

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