Injury, Int. J. Care Injured 46 (2015) 2146–2155

Contents lists available at ScienceDirect

Injury

journal homepage: www.elsevier.com/locate/injury

Neuroprotective effects of *Ganoderma lucidum* polysaccharides against traumatic spinal cord injury in rats

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ARTICLE INFO

Article history:
Accepted 5 August 2015

Keywords:
Ganoderma lucidum
Methylprednisolone
Neuroprotection
Polysaccharides
Spinal cord injury
Trauma

ABSTRACT

Introduction: *Ganoderma lucidum* (*G. lucidum*) is a mushroom belonging to the polyporaceae family of Basidiomycota and has widely been used as a traditional medicine for thousands of years. *G. lucidum* has never been studied in traumatic spinal cord injury. The aim of this study is to investigate whether *G. lucidum* polysaccharides (GLPS) can protect the spinal cord after experimental spinal cord injury.
Materials and methods: Rats were randomized into five groups of eight animals each: control, sham, trauma, GLPS, and methylprednisolone. In the control group, no surgical intervention was performed. In the sham group, only a laminectomy was performed. In all the other groups, the spinal cord trauma model was created by the occlusion of the spinal cord with an aneurysm clip. In the spinal cord tissue, caspase-3 activity, tumour necrosis factor-alpha levels, myeloperoxidase activity, malondialdehyde levels, nitric oxide levels, and superoxide dismutase levels were analysed. Histopathological and ultrastructural evaluations were also performed. Neurological evaluation was performed using the Basso, Beattie, and Bresnahan locomotor scale and the inclined-plane test.
Results: After traumatic spinal cord injury, increases in caspase-3 activity, tumour necrosis factor-alpha levels, myeloperoxidase activity, malondialdehyde levels, and nitric oxide levels were detected. After the administration of GLPS, decreases were observed in tissue caspase-3 activity, tumour necrosis factor-alpha levels, myeloperoxidase activity, malondialdehyde levels, and nitric oxide levels. Furthermore, GLPS treatment showed improved results in histopathological scores, ultrastructural scores, and functional tests.
Conclusions: Biochemical, histopathological, and ultrastructural analyses and functional tests reveal that GLPS exhibits meaningful neuroprotective effects against spinal cord injury.

Introduction

Spinal cord injury (SCI) is one of the most devastating health problems leading to neurological dysfunction. An initial insult to the spinal cord, which is called the primary injury, causes an interruption in signal transmission in concert with the loss of sensory, voluntary motor, and autonomic functions, and dysregulation of various reflex functions as a result of mechanical injury; this process initiates secondary injury [1,2]. Secondary injury causes a number of cellular and biochemical cascades and leads to further injury in the spinal cord after the primary injury. Secondary injury has always been therapeutic target of the most experimental studies to reduce further damage and promote remyelination [3–5].

Pharmaceutical products isolated from mushrooms have been regarded as a panacea for a variety of diseases such as cancers,

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http://dx.doi.org/10.1016/j.injury.2015.08.017
0020–1383/

immunologic disorders, and neurodegenerative diseases [6–8]. *Ganoderma lucidum* (*G. lucidum*) is a mushroom belonging to the polyporaceae family of Basidiomycota and has widely been used as a traditional medicine for thousands of years, particularly in Asian countries [9]. The fruiting bodies, cultured mycelia, and spores of *G. lucidum* contain a variety of bioactive chemical substances such as polysaccharides, triterpenoids, and proteins [8,10]. Different experimental studies and modern clinical trials suggest that these active compounds isolated from its fruiting body 'Lingzhi' have anti-inflammatory, anti-oxidant, anti-tumour, and immunomodulatory activities [11–14]. Previous studies showed that *G. lucidum* polysaccharides (GLPS) could have neuroprotective effects and increase the cell viability of cerebral cortical neurons exposed to ischaemia/reperfusion injury in a rat model [15,16]. This evidence suggests that GLPS might be of therapeutic benefit and could be a promising treatment candidate for SCI. To the best of our knowledge, GLPS had not yet been studied in SCI.

The aim of this study is to investigate whether GLPS protect the spinal cord from apoptosis, inflammation, and oxidative stress in rats after experimental traumatic SCI. We also compared GLPS with methylprednisolone (MP), which had been widely researched in traumatic SCI.

Materials and methods

Preparation of extract of G. lucidum

Fruiting bodies of *G. lucidum* were obtained from Çukurova University Chemistry Lab (Adana, Turkey). Identification of the bioactive component from the fruiting body of local *G. lucidum* strains was completed using a sequential extraction procedure. As a preliminary clean-up step, the samples were cut into small pieces after they were dried at 95–100 °C and eluted with supercritical CO_2 atmosphere at 50 °C, 3300 psi pressure, and ethanol, respectively, to remove apolar components such as fatty acids. Next, extraction of triterpenoids from these local *G. lucidum* strains was carried out with ethanol under reflux. In the last step, *G. lucidum* water-soluble polysaccharides and polysaccharide–protein complexes were extracted from fruiting bodies using hot water-extraction, as described previously [17]. Then, the extract was eluted with trichloroacetic acid (~4 g) to remove the protein substances by denaturation. Next, the precipitate was dried in a freeze dryer (Labconco 7670530, Labconco Corporation, USA) at −48 °C for five days to yield crude polysaccharides and to obtain the powder used for rat feed. We note that the powder was first subjected to chemical analysis before use.

Primary structural composition was determined by high-performance liquid chromatography (HPLC). Crude polysaccharides were dissolved in 4% H_2SO_4 and incubated at 100 °C for 6 h. After incubation, the samples were cooled to room temperature and centrifuged at 3000 rpm for 20 min. Twenty microliters of the resulting aqueous sample solutions were filtered through a 0.45 μm syringe filter and injected on two columns connected to the HPLC system. The preliminary calibration of the column was conducted using dextrans with different molecular weights. The ultraviolet detection wavelength was set at 280 nm.

Molecular weight characterizations were determined by the High-Pressure Size Exclusion Chromatography-Multi Angle Laser Light Scattering system. The data and chromatograms were recorded and processed using ASTRA software (Wyatt Technology, Version 4.70.07.).

Structural features were elucidated by ^{13}C nuclear magnetic resonance (NMR) Cross Polarization/Magic Angle Spinning (CP-MAS) recorded on a 300-MHz NMR spectroscopy (Bruker Biospin Corp., USA).

Chemical analysis revealed that GLPS contained 59.4% carbohydrate (55.35% D-glucose, 2.37% D-mannose, 1.68% D-galactose) and 30.33% protein. The glycosyl composition analysis indicated that glucose, galactose, and mannose were the major carbohydrates found in GLPS (Fig. 1) and the average molecular weight of GLPS was determined to be 6.279×10^4 Da. The ^{13}C NMR CP-MAS spectral signals of GLPS indicated that GLPS had a backbone consisting of glycoprotein in the form of β-D-glucan.

Experimental groups

The experimental protocol was performed in accordance with the guidelines of 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 ethical committee of the Ministry of Health Ankara Education and Research Hospital. Forty adult male Wistar Albino rats with a mean age of 8 months, weighing 250 ± 30 g were used. The rats were randomly assigned to five groups with eight rats per group.

The groups were as follows:

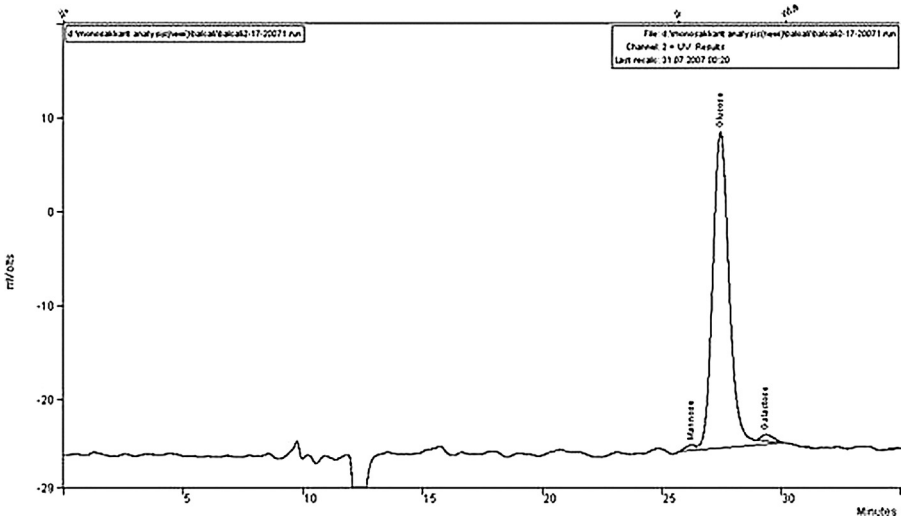

Fig. 1. High-performance liquid chromatogram of composition of *G. lucidum* polysaccharides (Man: mannose, Glc: glucose, Gal: galactose).

Group 1: Control ($n = 8$); no surgical procedure was performed. Non-traumatized spinal cord samples were obtained from the control group to determine normal spinal cord morphology and baseline biochemical values.
Group 2: Sham ($n = 8$); rats underwent only a simple laminectomy. Non-traumatized spinal cord samples were removed after 24 h.
Group 3: Trauma ($n = 8$); rats underwent SCI, as described below. After laminectomy, spinal cord samples were removed 24 h after injury. Rats received 2 ml of distilled water by gavage for 7 days before SCI induction, and administration was continued until sacrifice.
Group 4: GLPS ($n = 8$); similar to group 3, but rats received 400 mg/kg daily dose of GLPS by gavage for 7 days before SCI induction, and administration was continued until sacrifice.
Group 5: MP ($n = 8$); similar to group 3, but rats received a single intraperitoneal dose of 30 mg/kg MP (Prednol, Mustafa Nevzat, Turkey) immediately following SCI.

Anaesthesia and SCI procedure

All rats were kept under environmentally controlled conditions at 22–25 °C, with appropriate humidity and a 12 h light cycle and granted free access to food and water.

The animals were anesthetized with an intraperitoneal injection of 10 mg/kg xylazine (Rompun, Bayer, Turkey) and 50 mg/kg ketamine (Ketalar, Parke Davis, Turkey), and allowed to breathe spontaneously. A rectal probe was inserted, and the animals were positioned on a heating pad that maintained their body temperature at 37 °C.

The rats were placed in a prone position. A T5–T9 midline skin incision was made, and the paravertebral muscles were dissected. From T6 to T8, the spinous processes were removed, and a laminectomy was performed. The dura was left intact. An aneurysm clip with 70 g closing force (Yasargil FE 721, Aesculap, Germany) was applied to the T7 level of the spinal cord for 1 min [18,19]. At the end of the procedure, the clip was removed, and the surgical wound was closed in layers with silk sutures. The drugs were administered intraperitoneally immediately after the wound was closed. The animals were killed 24 h after the operation via an injection of high-dose (200 mg/kg) pentobarbital (Nembutal, Oak Pharmaceuticals, Lake Forest, IL, USA). Next, spinal cord samples (15 mm) were obtained from the operated area and immediately extracted on ice and divided into three equal parts. Cranial parts of the tissue samples were used for light microscopic evaluation, the middle parts were used for electron microscopic evaluation, and the caudal parts were cleaned of blood with a scalpel, snap-frozed in liquid nitrogen without additives and then transferred to tissue archiving freezer (−80 °C) and stored at for biochemical analysis.

Biochemical procedures

For the analysis, the tissues were homogenized in a physiologic saline solution and centrifuged at 4000 rpm for 20 min. Then, the upper clear supernatants were removed for the analysis.

Tissue caspase-3 analysis

Caspase-3 activity was measured using an ELISA kit (Uscn Life Science Inc., China). The ELISA procedures were performed according to the manufacturer's instructions. The results were expressed as ng/mg protein.

Tissue tumour necrosis factor-alpha analysis

The tissue tumour necrosis factor-alpha (TNF-α) level was measured using an ELISA kit according to the manufacturer's instructions (Uscn Life Science Inc., Wuhan.). The results are expressed as pg/mg.

Tissue myeloperoxidase analysis

The myeloperoxidase (MPO) activity was measured using an ELISA kit (Uscn Life Science Inc., Wuhan.). The ELISA procedures were carried out according to the manufacturer's instructions. The results are expressed as pg/mg.

Tissue malondialdehyde analysis

The tissue malondialdehyde (MDA) levels were determined by a method based on the reaction with thiobarbituric acid (TBA), as described previously by Ohkawa et al. [20] The MDA concentrations were expressed as nmol/mg protein.

Tissue nitric oxide analysis

Tissue nitric oxide (NO) levels were determined using the method described by Miranda et al. [21]. 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 was determined according to the method described by Sun et al. [22]. The principle of the method is based on the inhibition of nitrobluetetrazolium reduction using a xanthine–xanthine oxidase system as a superoxide generator. The SOD activity was expressed as U/mg protein.

Histopathological procedures

The spinal cord tissues of all rats in all of the groups were embedded in paraffin and fixed with 10% buffered formalin for 24 h. Give μm-thick serial sections were cut from the paraffin blocks using a microtome and stained with haematoxylin–eosin (H&E) for routine histopathological observations. All of the tissue sections were observed under a light microscope by a neuropathologist who was blinded to the study design.

A semiquantitative scoring system, ranging between zero and three, was used for grading both histopathological changes (oedema, vascular congestion, and inflammation) and neuronal degenerative signs (nuclear pyknosis, nuclear hyperchromasia, cystoplasmic eosinophilia, and axonal oedema) in all of the spinal cord tissue samples. Four different histopathologically assessed parameters were scored as follows: 0: absent, 1: mild, 2: moderate, and 3: common. The pathological score for each spinal cord was calculated based on the sum of the scores of these four different parameters [23].

To assess the degree of neuronal injury in more detail, the number of normal motor neurons in the anterior horn of the spinal cord (anterior to a line drawn through the central canal perpendicular to the vertebral axis) was counted in three sections for each animal and then averaged.

Ultrastructural examination

Tissue samples were cleared of blood using a scalpel, and the meninges were carefully removed. The tissue samples were fixed in 2.5% glutaraldehyde for 24 h, washed in phosphate buffer (pH: 7.4), post-fixed in 1% osmium tetroxide in phosphate buffer (pH: 7.4) for 2 h and dehydrated in increasing concentrations of alcohol. Next, the tissues were washed with propylene oxide and embedded in epoxy-resin embedding media. Semi-thin sections approximately 2 μm in thickness and ultra-thin sections approximately 60 nm in thickness were cut with a glass knife on a LKB-Nova (LKB-Produkter AB, Bromma, Sweden) ultramicrotome. The semi-thin sections were stained with methylene blue and

examined using a Nikon Optiphot (Nikon Corporation, Tokyo, Japan) light microscope. Following this examination, the tissue blocks were trimmed; their ultra-thin sections were made using the same ultramicrotome and stained with uranyl acetate and lead citrate. Following staining, all of the ultra-thin sections were examined using a Jeol JEM 1200 EX (Jeol Ltd., Tokyo, Japan) transmission electron microscope. The electron micrographs were taken by the same transmission electron microscope. Every 100 large-diameter myelinated axons, medium-diameter myelinated axons, and small-diameter myelinated axons were evaluated and scored from 0 to 3, as described by Kaptanoğlu et al. [24].

The scoring system was as follows:

0: Ultrastructurally normal myelinated axon
1: Separation in the myelin configuration
2: Interruption in the myelin configuration
3: Honeycomb appearance in the myelin configuration

Neurological evaluation

The neurologic status of the animals was scored 24 h after the procedure based on assessment using the Basso, Beattie, and Bresnahan (BBB) locomotor scale and the inclined-plane test.

The open-field locomotor test assessed the movement, weight support, and coordination of the rats, and the results were scored using the BBB locomotor scale, where 0 indicates no motor activity and 21 indicates a normal performance [25]. To summarize, animals were allowed to walk around freely in a circular field for 5 min, and the movements of the animals' hind limbs were closely observed. The animals' ability to maintain postural stability was assessed with the inclined-plane test. The rats were placed on the inclined plane, and the maximum inclination at which the rat could maintain its position for 5 s was recorded as the final angle [26]. Two independent examiners who were blinded to the experimental protocols observed the rats during the tests.

Statistical analysis

Data analysis was performed using SPSS for Windows, version 11.5 (SPSS Inc., Chicago, IL, USA). To determine if the distributions of continuous variables were normally distributed or not, we used the Shapiro–Wilk test. The Levene test was used to evaluate the homogeneity of variances. The data are shown as mean ± standard error of the mean (SEM).

While the differences in normally distributed variables among groups were analysed using one-way ANOVA, the Kruskal–Wallis test was applied in not normally distributed data. When the p value from one-way ANOVA or the Kruskal–Wallis test statistics were statistically significant, the post hoc Tukey HSD or Conover's non-parametric multiple comparison test were used to determine which group differed from which other groups.

A p value less than 0.05 was considered statistically significant.

Results

Tissue caspase-3 activity

The expression of caspase-3 activity showed a marked increase in the trauma group compared with both the control and sham groups ($p < 0.001$ for both). Treatment with GLPS resulted in a statistically significant decrease in caspase-3 activity compared with the trauma group ($p = 0.003$). As in the GLPS group, caspase-3 activity was statistically significantly decreased in the MP group compared with the trauma group ($p = 0.001$). However, there were no statistically significant differences between the control and the sham groups ($p = 0.952$), or between the MP and the GLPS groups ($p = 0.698$).

Tissue TNF-α levels

The tissue TNF-α levels of the trauma groups showed a statistically significant increase compared with both the control and sham groups ($p < 0.001$ for both). Both GLPS and MP treatments significantly reduced tissue levels of TNF-α compared with the trauma group ($p < 0.001$). There were no significant differences between the control and the sham groups ($p = 0.952$), or between the MP and the GLPS groups ($p = 0.905$).

Tissue MPO activity

When the tissue MPO activities of the control and sham groups were compared with those of the trauma group, a statistically significant difference was observed ($p < 0.001$ for both); these data showed that after SCI, tissue MPO activity was increased. Treatment with GLPS significantly decreased the tissue MPO activity ($p = 0.007$). As in the GLPS group, MP treatment also significantly decreased the MPO activity in the spinal cord ($p = 0.025$). There were no significant differences between the control and sham groups ($p = 0.405$), or between the GLPS and the MP groups ($p = 0.612$).

Tissue MDA levels

When the trauma group was compared with the control and sham groups, the MDA values were significantly increased ($p < 0.001$ for both). Treatment with GLPS significantly decreased the MDA levels compared with the trauma group ($p < 0.001$). As in the GLPS group, MP treatment also caused a significant decrease in MDA levels ($p = 0.003$). There were no statistically significant differences between the control and sham groups ($p = 0.286$), or between the GLPS and the MP groups ($p = 0.512$).

Tissue NO levels

Tissue NO levels in the trauma group were significantly higher than in both the control and sham groups ($p < 0.001$ for both). *G. lucidum* treatment induced a significant decrease in tissue NO levels compared with the trauma group ($p = 0.01$). As in the GLPS group, MP treatment significantly decreased tissue NO levels compared with the trauma group ($p = 0.005$). No statistically significant differences were found between the control and sham groups, or between the MP and the Ganoderma groups in terms of tissue NO values ($p = 0.149$ and $p = 0.8$, respectively).

Tissue SOD activity

Following SCI, tissue SOD activity decreased significantly when both the control and sham groups were compared with the trauma group ($p < 0.001$ for both). Treatments with both GLPS and MP significantly increased the tissue SOD activity compared with the trauma group ($p = 0.001$ for both). There were no significant differences between the control and sham groups ($p = 0.777$), or between the GLPS and MP groups ($p = 0.988$).

The biochemical results of the study are summarized in Table 1.

Histopathological evaluation

Light microscopic examinations of the spinal cord samples from the control and the sham groups revealed nothing remarkable (Fig. 2a and b). Histological alterations, such as marked necrosis, oedema, polymorphonuclear leucocytes, lymphocytes, and plasma

Table 1
Biochemical results relevant to the study groups.

Variables	Control	Sham	Trauma	MP	GLPS	*p*-Value
Caspase-3 (ng/mg-protein)	242.53 ± 30.85[a]	251.56 ± 38.86[b]	958.78 ± 119.41[a,b,c,d]	410.79 ± 83.44[c]	410.79 ± 67.76[d]	<0.001[†]
Tissue TNF-α (pg/mg)	21.27 ± 1.46[a]	21.93 ± 1.13[b]	41.63 ± 2.78[a,b,c,d]	22.80 ± 1.63[c]	22.10 ± 1.19[d]	<0.001[†]
Tissue MPO (pg/mg)	141.56 ± 10.28[a]	154.11 ± 12.08[b]	317.81 ± 34.53[a,b,c,d]	211.75 ± 12.17[c]	208.04 ± 19.34[d]	<0.001[†]
Tissue MDA (nmol/mg-protein)	1.19 ± 0.18[a]	1.47 ± 0.23[b]	5.68 ± 0.58[a,b,c,d]	2.22 ± 0.21[c]	2.05 ± 0.27[d]	<0.001[†]
Tissue NO (nmol/mg-protein)	44.41 ± 4.48[a]	34.07 ± 5.79[b]	83.79 ± 8.81[a,b,c,d]	54.85 ± 6.37[c]	55.43 ± 5.96[d]	<0.001[‡]
Tissue SOD (U/mg-protein)	1.69 ± 0.18[a]	1.70 ± 0.17[b]	0.41 ± 0.02[a,b,c,d]	1.28 ± 0.09[c]	1.31 ± 0.10[d]	<0.001[†]

GLPS: *Ganoderma lucidum* polysaccharide, MP: methylprednisolone, TNF-α: tumour necrosis factor-alpha, MPO: myeloperoxidase, MDA: malondialdehyde, NO: nitric oxide, SOD: superoxide dismutase.
[†] Kruskal–Wallis test.
[‡] One-way ANOVA test.
[a] Control vs trauma ($p < 0.05$).
[b] Sham vs trauma ($p < 0.001$).
[c] Trauma vs MP ($p < 0.01$).
[d] Trauma vs GLPS ($p < 0.05$).

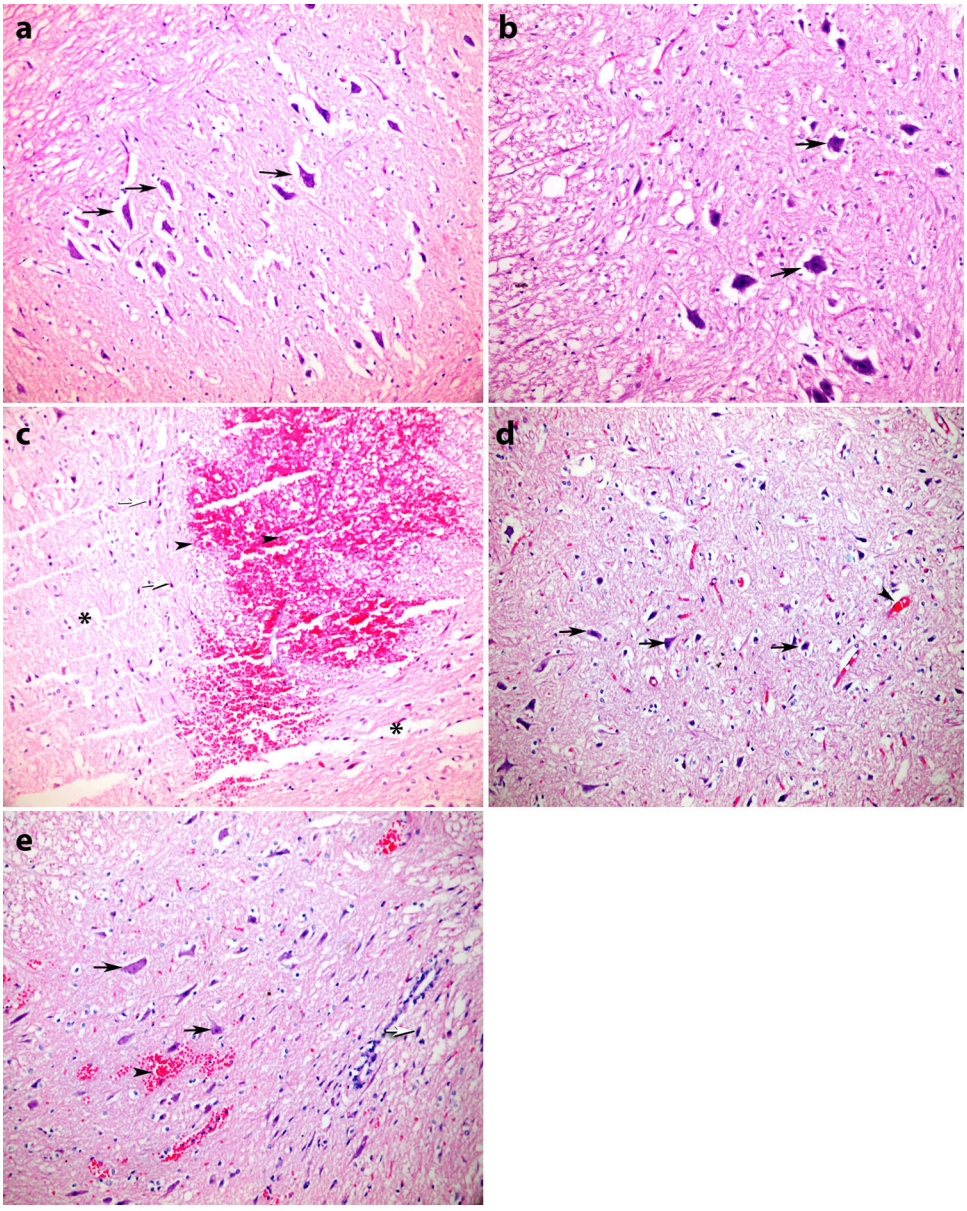

Fig. 2. Photomicrographs of 5-μm-thick spinal cord tissue sections from the different treatment groups (H&E, ×200). (a) Control group revealing spinal cord parenchyma with normal neurons (arrow). (b) Sham group showing normal-appearing non-degenerated neurons (arrow). (c) Trauma group, showing extremely spread oedematous (*) and haemorrhagic (arrow head) spinal cord parenchyma with highly degenerated neurons (hollow arrow). (d) GLPS group, showing less degenerated neurons and more normal-appearing neurons (arrow). The spinal cord tissue was well protected from injury, revealing less haemorrhagic congestion (arrow head). (e) MP group, revealing normal-appearing neurons (arrow) with less degenerated neurons (hollow arrow) and mild haemorrhagic (arrow head) – oedematous spinal cord parenchyma (GLPS: *Ganoderma lucidum* polysaccharide, MP: methylprednisolone).

cells infiltrations, were found in the trauma group. In addition, diffuse haemorrhage and congestion, neural pyknosis, loss of cytoplasmic features, and cytoplasmic eosinophilia were observed in the trauma group (Fig. 2c). This pathological evidence detected in the trauma group was attenuated in the GLPS group, as well as in the MP group (Fig. 2d and e).

When the pathological scores of the experimental groups were analysed, the trauma group exhibited significantly higher scores compared with the control and sham groups ($p < 0.001$ for both). Rats in the GLPS and MP groups showed significantly lower scores than rats in the trauma group ($p = 0.001$ and $p = 0.021$, respectively). There was no significant difference between the GLPS and the MP groups ($p = 0.254$) (Fig. 3a).

Furthermore, the number of normal motor neurons in the anterior horn of the spinal cord was evaluated. The trauma group had a significantly decreased number of motor neurons compared both with the control and sham groups ($p < 0.001$ for both). The number of normal motor neurons in the GLPS group was significantly higher compared with the trauma group ($p < 0.001$). Similarly, the MP group had a significantly higher number of normal motor neurons compared with the trauma group ($p < 0.001$). A comparison between the GLPS and the MP groups did not reveal any statistically significant difference in the number of normal motor neurons ($p = 0.588$) (Fig. 3b).

Ultrastructural examination

In the ultrastructural examination of the grey matter in the control group, no ultrastructural pathology was detected. In the ultrastructural examination of the white matter, mild separations in the myelin configuration were observed in a very few of the medium-sized and large-sized myelinated axons. Small-sized myelinated axons were normal ultrastructurally. The small differences in myelin configuration observed in very few of the medium-sized and large-sized myelinated axons might be explained by delayed fixation (Fig. 4a).

In the transmission electron microscopic examination of the tissue samples of the sham group, no pathological ultrastructural changes were observed in the grey and white matters of the spinal cord; the neurons were ultrastructurally normal in appearance. The intracellular organelles, nuclei, and membranes of the neurons were normal ultrastructurally. The perineuronal tissues did not show any pathological changes. However, mild separations were observed in a small part of the myelin sheath for only a few of the large-sized myelinated axons. This finding may be related to the delayed fixation of the tissue. The rest of the large-sized myelinated axons and all of the medium- and small-sized myelinated axons were normal ultrastructurally (Fig. 4b).

In the transmission electron microscopic examination of the tissue samples of the trauma group, separations and interruptions in myelin configuration were observed in the small-sized, medium-sized, and large-sized myelinated axons. In the structural examination of the grey matter, swollen mitochondria and vacuoles were present inside the cytoplasm of neurons. Additionally, perineural oedema was observed (Fig. 4c).

In the GLPS group, a mild degree of perineural oedema was present in the structural examination of the grey matter. Swollen mitochondria and small vacuoles were seen. In the structural examination of the white matter, separations in myelin configuration were observed in a very few small-sized myelinated axons. Additionally, separations in myelin configuration were present in some of the medium- and large-sized myelinated axons. Interruptions in myelin configuration were not detected in the myelinated axons (Fig. 4d).

In the transmission electron microscopic examination of the tissue samples of the MP group, swollen mitochondria and vacuoles were observed inside the cytoplasm of neurons in the ultrastructural examination of the grey matter. Additionally, perineural oedema was present. In the ultrastructural examination of the white matter, separations in myelin configuration were found in small-sized, medium-sized, and large-sized myelinated axons (Fig. 4e).

The trauma group showed more disruption in the small-, medium-, and large-sized myelinated axons compared with the control and sham groups ($p < 0.001$). GLPS and MP treatments both protected the axons of all sizes from traumatic SCI compared with the trauma group (small-sized myelinated axons: $p < 0.001$ and $p = 0.014$; medium-sized myelinated axons: $p < 0.001$ and $p = 0.008$; large-sized myelinated axons: $p < 0.001$ and $p = 0.008$, respectively). Furthermore, GLPS yielded better results for the small-, medium-, and large-sized myelinated axons compared with the MP group ($p < 0.006$, $p = 0.008$, and $p = 0.008$, respectively). The ultrastructural examination and histopathological results relevant to the study groups are listed in Table 2.

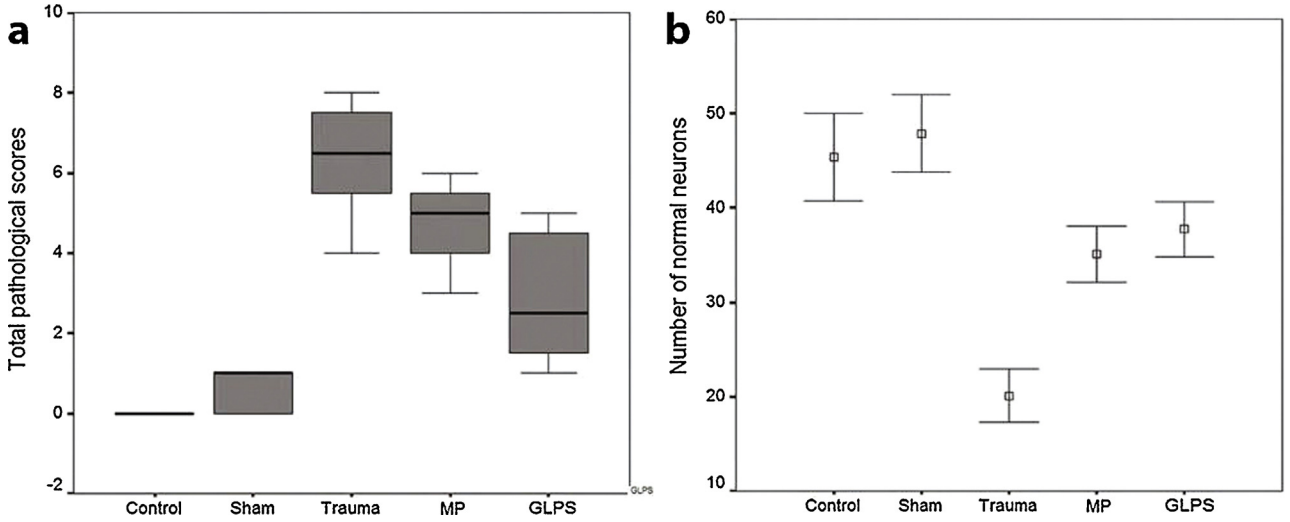

Fig. 3. (a) Bar graph representing the pathology scores of the study groups. The horizontal lines in the middle of each box indicate the median; the top and bottom borders of the box mark the 25th and 75th percentiles, respectively. The whiskers above and below the box correspond to the maximum and minimum values. (b) Bar graph representing the number of normal neurons in the study groups. The box in the middle of each whiskers indicates the arithmetic mean; the whiskers above and below the box mark the +1 SD and −1 SD levels, respectively.

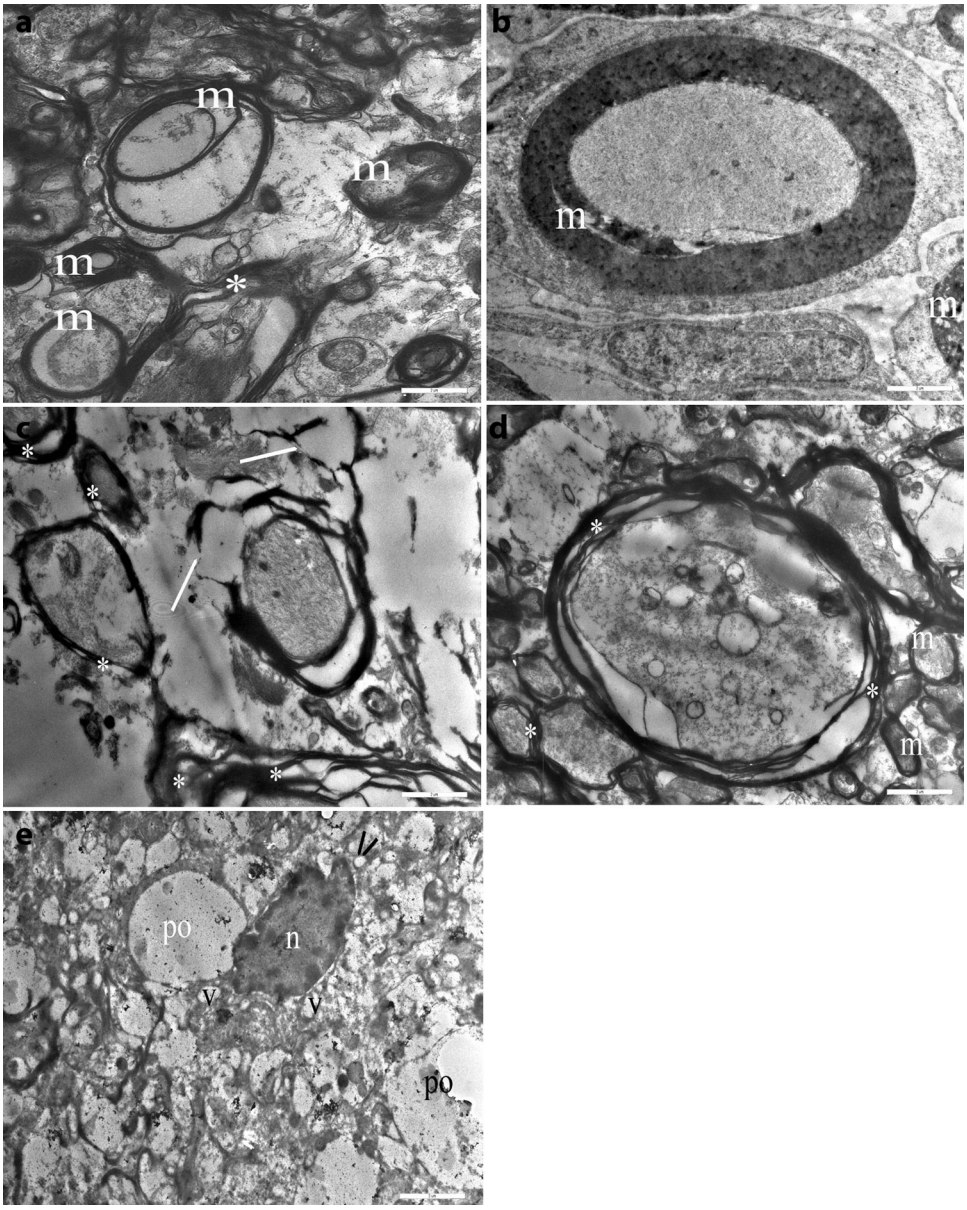

Fig. 4. Transmission electron microscopy of the groups. (a) A transmission electron microscopic examination of the tissue samples of the control group showed normal cord ultrastructure. The mild separations in the myelin configuration observed in very few of the medium-sized and large-sized myelinated axons may be explained by delayed fixation. m: ultrastructurally normal myelinated axon; *: mild separation in myelin configuration. (b) Electron micrographs of the sham group revealed no ultrastructural pathology. m: ultrastructurally normal myelinated axon. (c) Electron micrograph of the trauma group showing separations (*) and interruptions (arrow) in the myelin configuration of myelinated axons. (d) Electron micrograph of the GLPS group showing ultrastructurally normal myelinated axons (m) and myelinated axons with separations in the myelin configuration (*). (e) Electron micrograph of the MP group showing perineural oedema (po) and vacuoles (v) inside the cytoplasm of a neuron and swollen mitochondria (double arrow).

Neurological outcome

All the animals had an initial BBB score of 21. Following SCI, the mean BBB score of the trauma group was decreased statistically significantly compared with the control and sham groups ($p < 0.001$ for both). Both the GLPS and the MP groups showed better BBB scores compared with the trauma group ($p = 0.001$ and $p = 0.04$, respectively). There was no significant difference between the control and sham groups ($p = 1$) or between the GLPS and MP groups ($p = 0.141$).

Following SCI, the mean angle recorded in the inclined-plane test was significantly lower in the trauma group than in the control and sham groups ($p < 0.001$ for both). Both GLPS and MP treatments revealed better angles in the inclined-plane test compared with the trauma group ($p < 0.001$ for both). There was no significant difference between the control and sham groups ($p = 0.202$) or between the GLPS and MP groups ($p = 0.679$).

The results of the neurological examinations relevant to the study groups are summarized in Table 3.

Discussion

Initial traumatic insult to spinal cord with the damaged vertebral bones and surrounding muscle tissue constitutes the primary phase of SCI and results in disruption of axons and excessive death of neuronal-glial cells and causes much of the damage that cannot be reversed [27]. The cascade of secondary injury, comprises complex pathological processes that include inflammation [28], excitotoxicity, lipid peroxidation [29], and apoptosis [30].

Table 2
Electron microscopy and histopathological results relevant to the study groups.

Myelinated axon	Control	Sham	Trauma	MP	GLPS	*p*-Value
Small sized	$0.00 \pm 0.00^{a,b,c}$	$0.00 \pm 0.00^{d,e,f}$	$85.60 \pm 1.50^{a,d,g,h}$	$77.40 \pm 1.81^{b,e,g,i}$	$29.00 \pm 0.71^{c,f,h,i}$	$<0.001^{\dagger}$
Medium sized	$0.00 \pm 0.00^{a,b,c,j}$	$12.20 \pm 0.86^{d,e,f}$	$121.80 \pm 4.22^{a,d,g,h}$	$88.20 \pm 1.11^{b,e,g,i}$	$68.80 \pm 1.39^{c,f,h,i}$	$<0.001^{\dagger}$
Large sized	$4.20 \pm 0.86^{a,b,c,j}$	$16.00 \pm 1.14^{d,e,f}$	$140.40 \pm 3.23^{a,d,g,h}$	$97.80 \pm 0.58^{b,e,g,I}$	$76.40 \pm 2.16^{c,f,h,I}$	$<0.001^{\dagger}$
Pathology score	$0.00 \pm 0.00^{a,b,c}$	$1.25 \pm 0.37^{d,e,f}$	$8.75 \pm 0.53^{a,d,g,h}$	$4.62 \pm 0.50^{b,e,g}$	$3.25 \pm 0.70^{c,f,h}$	$<0.001^{\dagger}$
Number of normal neurons	$45.38 \pm 1.65^{a,b,c}$	$47.88 \pm 1.44^{d,e,f}$	$20.13 \pm 1.01^{a,d,g,h}$	$35.13 \pm 1.04^{b,e,g}$	$37.75 \pm 1.03^{c,f,h}$	$<0.001^{\ddagger}$

MP: methylprednisolone, GLPS: *Ganoderma lucidum* polysaccharide.
† Kruskal–Wallis test.
‡ One-way ANOVA test.
[a] Control vs trauma ($p < 0.001$).
[b] Control vs MP ($p < 0.001$).
[c] Control vs GLPS ($p < 0.001$).
[d] Sham vs trauma ($p < 0.001$).
[e] Sham vs MP ($p < 0.001$).
[f] Sham vs GLPS ($p < 0.01$).
[g] Trauma vs MP ($p < 0.05$).
[h] Trauma vs GLPS ($p < 0.001$).
[i] MP vs GLPS ($p < 0.01$).
[j] Control vs sham ($p < 0.01$).

G. lucidum has been used for thousands of years in the Eastern Asia for preventive medicine [31]. Polysaccharides, which have been isolated from *G. lucidum* fruting bodies, have been reported to possess antioxidant [32], immunomodulatory [33], and antitumour characteristics [34]. Moreover, these polysaccharides have also been reported to have protective effects against cerebral ischaemic injury [35]. *G. lucidum* may mediate neuronal functions by modulating the activities of different signalling pathways. Recent data suggest that GLPS induced the neuronal differentiation of pheochromocytoma cell cultures and protected the neurons from apoptosis by a possible involvement of the Erk1/2 and the CREB signalling pathways [6]. Additionally, *G. lucidum* extract reduced the expressions of proinflammatory and cytotoxic factors from the activated microglia, and effectively protected the dopaminergic neurons against inflammatory and oxidative damage [36]. Furthermore, Zhang et al. suggested that spores of *G. lucidum* preserved the injured spinal motor neurons through up- or down-regulating the expression levels of the proteins which play important roles in axonal regeneration [37]. These results implied that the polysaccharide extracts isolated from *G. lucidum* has neural protection and antioxidant properties. To the best of our knowledge, the neuroprotective effects GLPS has never before been studied in traumatic SCI.

In the present study, we successfully isolated polysaccharides from the fruiting bodies of *G. lucidum* and employed this crude polysaccharide extract. To obtain this powder, triterpenoids and protein complexes were removed by hot water extraction, followed by ethanol precipitation, reserve dialysis, and protein depletion, as described above in the Materials and Methods section. After its primary structural features and molecular weight were characterized, we performed an experimental study to determine the effect of this crude polysaccharide extract that we obtained from *G. lucidum* against SCI. Spinal cord-injured rats were pretreated with 400 mg/kg GLPS for 7 days; this dose was selected based on a previous study [35].

MP, a steroidal antioxidant and anti-inflammatory agent, has been in the use for the treatment of acute spinal cord injury [38,39]. The use of MP is under debate in recent years to whether MP should be used as the drug treatment. Although some authors conclude that MP is not a standard care for all patients. The current clinical treatment still insists of using high-dose MP as a treatment option until it is supplanted by future evidence based therapies. In this study, MP group was used for comparison with GLPS.

Both necrosis [40,41] and apoptosis [42,43] cause neuronal death following SCI. Apoptotic death of neurons in the spinal cord following trauma lead to impairment in impulse conduction through the axon myelin structural unit [42,44]. Caspase-3 is an interleukin-converting enzyme that has been implicated as the principal effector of apoptosis in mammalian cells [45]. Therefore, caspase-3 is a reliable marker for indicating apoptotic activity [46]. Furthermore, a recent study showed that GLPS exerted neuroprotective effects in in vivo and in vitro models of stroke via modulation of the Bcl-2/Bax-2 ratio and the inhibition of caspase-3 activity [35]. In the present study, caspase-3 activity was significantly increased in the injured spinal cord samples following SCI. The activity of caspase-3 was significantly downregulated in

Table 3
Neurological examination results relevant to the study groups.

Variable	Control	Sham	Trauma	MP	GLPS	*p*-Value
BBB	$21.00 \pm 0.00^{a,b,c}$	$21.00 \pm 0.00^{d,e,f}$	$2.25 \pm 0.16^{a,d,g,h}$	$6.13 \pm 0.35^{b,e,g}$	$7.63 \pm 0.18^{c,f,h}$	$<0.001^{\dagger}$
INCLINED PLANE	$76.38 \pm 2.04^{a,b,c}$	$69.50 \pm 2.16^{d,e,f}$	$34.50 \pm 1.64^{a,d,g,h}$	$53.88 \pm 2.51^{b,e,g}$	$58.00 \pm 2.54^{c,f,h}$	$<0.001^{\ddagger}$

MP: methylprednisolone, GLPS: *Ganoderma lucidum* polysaccharide, BBB: Basso, Beattie, and Bresnahan.
† Kruskal–Wallis test.
‡ One-way ANOVA test.
[a] Control vs trauma ($p < 0.001$).
[b] Control vs MP ($p < 0.001$).
[c] Control vs GLPS ($p < 0.01$).
[d] Sham vs trauma ($p < 0.001$).
[e] Sham vs MP ($p < 0.001$).
[f] Sham vs GLPS ($p < 0.01$).
[g] Trauma vs MP ($p < 0.05$).
[h] Trauma vs GLPS ($p < 0.001$).

the GLPS and MP groups. The results of our study revealed that both GLPS and MP treatments significantly inhibited apoptosis in the injured spinal cord segments.

Leucocytes achieve transendothelial migration via the CD11b integrin-ICAM-1 interaction, and therefore enter the spinal cord and induce cell damage by releasing reactive oxygen products, proteases, elastase, and cytokines that comprise the inflammatory component of the secondary injury cascade [47]. The inflammatory activity and quantity of leucocytes in the injured area is arranged by MPO activity [48]. Furthermore, the assays of proinflammatory cytokines, such as TNF-α and MPO, are useful quantitative indicators of the inflammatory extent [49]. As a result, we measured MPO and proinflammatory cytokine TNF-α levels to evaluate neuroinflammation after SCI. The mean MPO and TNF-α levels increased in the trauma group compared with the control and sham groups. Treatment with GLPS and MP significantly suppressed the production of MPO and TNF-α, which is likely related to the anti-inflammatory activity of GLPS.

Oxidative stress following SCI produces free radicals and initiates lipid peroxidation activity in the damaged neural tissue [50]. In our study, spinal cord levels of MDA, which are stable products of lipid peroxidation, increased significantly after traumatic SCI. Both GLPS and MP administration decreased the levels of MDA by inhibiting lipid peroxidation.

Oxidative stress is accompanied by antioxidant enzyme depletion and excess production of oxygen free radicals and NO at the site of injury [51]. By damaging lipids, proteins, and nucleic acids, oxygen free radicals can cause cytotoxicity [52]. Furthermore, oxidative stress plays a major role in the progression of spinal cord lesions after the primary injury [53]. Since the central nervous system consists largely of lipids, neuronal tissues such as the spinal cord are highly vulnerable to oxidative injury. Oxidative stress leads to oxidation of polyunsaturated fatty acids and proteins in the neurons, damages the DNA helix, and causes cell death and consumption of antioxidant enzymes that are capable of scavenging reactive oxygen species [54]. Due to elevated oxidative stress in the spinal cord, SOD levels were shown to decrease [23]. Chen et al. [55] reported that GLPS exhibited a noticeable antioxidant activity. Additionally, Zhao et al. [16] reported that GLPS exhibits a remarkable protection against hypoxia/reoxygenation injury in rat cortical neurons by reducing MDA levels of reactive oxygen species production, and increasing SOD activity. In this study, we also demonstrated that after traumatic SCI, NO levels were increased and SOD levels were decreased due to highly elevated oxidative stress in the spinal cord. On the other hand, GLPS and MP treatments decreased the NO levels and increased the antioxidant enzyme SOD levels in the traumatized tissue. This finding is thought to be evidence for the antioxidant effect of GLPS and MP.

In order to gain more detailed data and compare them with the biochemical results, a histopathological examination was carried out. Neuronal degeneration, cellular oedema, haemorrhage/congestion, and inflammation were analysed and scored for histological changes and the number of normal motor neurons in the anterior horn of the spinal cord was counted. In the trauma group, diffuse haemorrhage and congestion, cavitation, and oedematous regions were observed in grey matter. Additionally, the traumatized segments were infiltrated by polymorphonuclear leukocytes and lymphocytes and there was marked necrosis and degeneration in the motor neurons. Scores determined according to these histopathological parameters revealed that the trauma group had statistically higher scores than the control and sham groups. Both the GLPS and the MP groups had significantly lower scores compared with the trauma group. Moreover, the GLPS group exhibited better histomorphological characteristics than the MP group. Furthermore, the number of normal motor neurons in the anterior horn of the GLPS and the MP groups was significantly increased compared with the trauma group.

In addition, we investigated the myelin configuration of the axons in the white matter and the neurons in the grey matter of spinal cord using transmission electron microscopy. All small-, medium-, and large-sized myelinated axons were significantly disturbed after traumatic SCI. Both GLPS and MP treatments preserved the axons of all sizes from traumatic SCI; however, scoring of the myelinated axons in the treatment groups revealed that GLPS was significantly more effective than MP in preserving the small-, medium-, and large-sized myelinated axons.

Both agents, GLPS and MP, inhibited lipid peroxidation, and demonstrated anti-inflammatory activity prevented oxidative stress and apoptosis following spinal cord injury. However GLPS treatment was associated with better histopathological and ultrastructural results than MP. There were lack of a significant difference for biochemical parameters between GLPS and MP. We assume that this discrepancy may be due to the presence of different pathways affected by GLPS, so that GLPS had further neuroprotective activity through other biochemical pathways than the pathways that were investigated in this study.

The functional efficiency of the treatment was evaluated by locomotor performance with BBB scores [25]. All rats had a BBB score of 21/21 before the trauma, and the traumatic SCI caused a significant decrease in BBB scores within 24 h. GLPS and MP treatments both revealed better BBB scores compared with the trauma group. Additionally, the mean angle in the inclined-plane test was decreased following the traumatic SCI. As expected, both GLPS and MP increased the mean angle in the inclined-plane test. As a result of these functional tests, both GLPS and MP treatments following SCI protected the spinal cord and improved neurological functioning.

The results of this study suggest that GLPS has beneficial effects for preserving normal spinal cord morphology, ultrastructure, and function by inhibiting apoptosis and reducing inflammation and oxidative stress.

However, this study has some limitations. The number of rats in each group may be modified to produce stronger conclusions. When the results of the GLPS groups were compared with the MP group, despite histopathological and ultrastructural results, the knowledge of treatment with GLPS has beneficial effects as MP. The dose-dependent results can be additionally investigated. As, this study focused on the early changes occurring during the first 24 h following injury, thereby not providing information regarding clinical outcome, a delayed biochemical and histopathological assessment may yield stronger results for further studies. Furthermore, in this study GLPS were administrated 7 days before the trauma. The pretreatment studies therefore do not realistically reflect the daily practice of SCI. As a result, additional studies of GLPS are necessary to prove the effect of this treatment when administered after SCI.

Conclusions

In conclusion, this study is the first investigation to evaluate the antioxidant, anti-inflammatory, anti-apoptotic activity, and neuroprotective effects of GLPS in a SCI model in rats; GLPS represents the main bioactive compound of the traditionally valued *G. lucidum* fungus. GLPS treatment improves early functional and biochemical results as much as MP and yields better ultrastructural findings and histomorphological results than MP. Although the results of the present study have provided some interesting data confirming the useful effect of GLPS after SCI, pathways, which are activated through the GLPS treatment, must be revealed in future studies in order to explain the exact mechanism underlying the neuroprotective activity.

Conflict of interest

None.

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