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BIOCHEMICAL ANALYSIS FOR NEUROPROTECTIVE EFFECTS OF GANODERMA LUCIDUM IN EXPERIMENTAL RAT SPINAL CORD TRAUMA MODEL

Denizhan Divanlıoğlu[1], Ece Miser Salihoğlu[2], Murat Korkmaz[3], Ahmet Eren Seçen[1], Özgür Öcal[1], Göksal Günerhan[1], Deniz Belen[1], Sevgi Akaydın[2], Ali Dalgıç[1]

[1]*Turkish Ministry of Health, Ankara City Hospital, Clinic of Neurosurgery, Ankara, Turkey*
[2]*Gazi University Faculty of Pharmacy, Department of Biochemistry, Ankara, Turkey*
[3]*Bakırçay University, Çiğli Training and Research Hospital, Clinic of Neurosurgery, İzmir, Turkey*

ABSTRACT

Objective: Injury of the spinal cord is studied in two separate mechanisms as primary and secondary injuries. During the secondary injury, spinal cord damage and related neurological defects could increase mostly because of oxidative damage. Treatments targeting this process are promising for reducing neuronal damage. Ganoderma lucidum (GL) has the potential to suppress the inflammatory response and oxidative stress. The aim of this study is to determine the neuroprotective effect of traditional GL hot-water extract during the secondary spinal cord injury (SCI) period on an experimental rat spinal trauma model by measuring the biochemical parameters.

Materials and Methods: A total of 34 rats were distributed randomly into 4 groups as trauma, vehicle, low-dose medication group (low-DMG) and high-dose medication group (high-DMG). A modified Walsh-Tator clip was applied extradurally to form an experimental SCI model. GL liquid extract was performed in medication groups with low and high (10 times higher) oral doses. Spinal cord specimens were collected after 5 days of treatment for biochemical analysis.

Results: In the low-DMG, both diphenypicrylhydrasyl (DPPH) and malondialdehyde (MDA) values were found statistically negligible when compared with the trauma group. Comparison of the Low-DMG and vehicle group showed a significant change in DPPH value, but an insignificant change in MDA value. A statistically significant positive change in both DPPH and MDA values was found in High-DMG when compared to trauma and vehicle groups.

Conclusion: Prevention of secondary SCI is very important, since the neurological condition of the patients may get worse during this period. Dose-dependent positive results were obtained in the favor of GL in terms of both antioxidant efficacy and prevention of lipid peroxidation after SCI. The results of this biochemical study is supporting the previous studies and showing that GL has the potential of reducing posttraumatic oxidative damage in the spinal cord when given at the appropriate dose.

Keywords: Ganoderma lucidum, spinal injury, rat model, oxidative damage, neuroprotection

INTRODUCTION

Spinal cord injuries (SCIs), which may cause permanent disabilities, have special importance for both prevention and treatment. Injury of the spinal cord could be studied in two separate mechanisms as primary and secondary injuries. The mechanical injury that occurred right in the time of spinal trauma is named as primary injury[1,2]. Injury by the hemodynamic, metabolic, biochemical and apoptotic mechanisms initialized after hours or days following the trauma is called secondary injury[1,3-5]. In most cases, spinal cord damage and related neurological defects increase during the secondary injury period[6,7]. Treatments targeting this inflammation process are promising to attenuate neuronal damage.

There are lots of theories on the development of secondary SCI such as neurogenic shock, excitotoxicity, electrolyte imbalance, inflammation, immunological injury, vascular injury, increased intracellular calcium, free-radical development, endogenous opioids and apoptosis[5,8]. Oxidative stress and inflammation are two subjects considered to be very important for secondary SCI development[9]. The lipid peroxidation process is known to be the main reason for the cell membrane degradation leading to irreversible neuronal injury[10,11]. Peroxidation of fatty acids reveals an intermediate product called malondialdehyde (MDA) which can be used for measuring the level of lipid

Address for Correspondence: Denizhan Divanlıoğlu, Turkish Ministry of Health, Ankara City Hospital, Clinic of Neurosurgery, Ankara, Turkey
Phone: +90 505 467 02 99 **E-mail:** ddivanlioglu@gmail.com **Received:** 28.05.2021 **Accepted:** 12.07.2021
ORCID ID: orcid.org/0000-0003-0267-196X

peroxidation[10]. Although MDA is not a specific or quantitative indicator, it correlates with lipid peroxidation levels and could be measured by using thiobarbituric acid as applied in this study[10,12].

Ganoderma lucidum (GL) is one of the well-known mushrooms, named "Red Reishi" or "Lingzhi", which was being used for over 2000 years especially in far eastern countries because of the belief that the mushroom provides long and healthy life for the user[13,14]. This belief was supported by many scientific studies identifying active ingredients of GL such as bioactive triterpenes, polysaccharides and immunomodulatory proteins which have potent anti-tumor, anti-inflammatory and cytotoxic effects for malignant cells[15-18]. Besides, GL has the potential to suppress the inflammatory response and oxidative stress with its antioxidant, immunomodulatory and steroid-like bioactive ingredients.

The aim of this study is to investigate the neuroprotective potency of traditionally used hot-water extract of GL and if any, its' dose-dependent effects on oxidative stress by measuring the biochemical parameters, during the secondary SCI period.

MATERIALS AND METHODS

After ethical committee approval (2011-123-485/26.10.2011) from Ankara University Ethical Committee of Animal Experiments was taken, the experimental study and animal care were carried out at the Animal Experiments Laboratory of Ankara Hospital. Biochemical study was performed at the Pharmacology Department Laboratory of Gazi University. GL 30% extract was obtained from Erkel Food Industry and Trade Ltd., which was produced with the authorization of The Republic of Turkey Ministry of Food, Agriculture and Livestock (food production authorization no: G34-6054-00002-4). All the subjects were obtained from Saki Yenilli Laboratory of Experimental Animal Production as 240 – 260 grams weighted, male, Wistar-Albino rats.

Experiment and Surgical Procedure

Daily care was given to a total of 34 rats in a suitable standard environment at room temperature without any food or water restrictions. The subjects were distributed randomly into 4 groups as follows:

Group 1: Trauma group (n=8): Only spinal trauma was applied to the subjects in this group. No additional treatment was given other than standard care.

Group 2: Vehicle group (n=8): Subjects in this group were given 1 mL of distilled water in addition to standard care.

Group 3: Low-dose medication group (Low-DMG, n=9): The recommended daily human dose of GL hot-water extract was administered to the subject of this group in proportion of their body surface areas[19-21].

Group 4: High-dose medication group (High-DMG, n=9): A ten times more of the recommended daily human dose of GL hot-water extract was administered to the subject of this group in proportion of their body surface areas[19-21].

Under veterinary supervision, all the subjects were abstained from oral intake 6 hours before the anesthesia process. According to their previously calculated body surface areas, 2% xylazine hydrochloride (10 mg/kg) and 5% ketamine hydrochloride (50 mg/kg) were applied by intraperitoneal injection[21]. After appropriate surgical disinfection microsurgical procedure was started in the prone position with an interscapular midline incision. Bilateral subperiosteal blunt dissection and retraction of paravertebral muscles were followed by 3 adjacent thoracic laminectomies under extreme care for avoiding dura mater and SCI (Figure 1). A modified Walsh-Tator clip with 35 grams of closing pressure was extradurally applied for 60 seconds to all cases to form an experimental SCI as described by Rivlin and Tator[22]. A line-shaped bruising over the spinal dura mater was observed in all subjects before primary closure (Figure 1).

After the surgical procedure, all the subjects were observed as paraplegic as expected. Two hours after recovery, besides standard care without food or water restrictions, 1 mL of GL hot-water liquid extract in distilled water was started to both medication groups by orogastric cannula in two equal doses a day. Although a standardized dosage for GL hot-water extract is absent, the suggested effective dose for human usage is 1.5 g to 9 g daily for different ailments, which can be divided into 2 or 3 doses[19,20]. Recommended mean oral dose for human daily usage was proportioned to body surface area of the subjects as 13.3 mg/m^2/day (0,3%) for low-DMG and ten times more of the recommended dose, 133 mg/m^2/day (3%) for high-DMG[19,20,23]. Unlike the trauma group, an additional 1 mL of distilled water was given to the vehicle group besides standard care. No adverse effects were observed due to GL treatment.

All subjects remained paraplegic without any observable neurologic improvement during the study before, the spinal cord specimens were collected after 5 days of postoperative follow-up. The procedure was started under general anesthesia with a thoracotomy and left cardiac ventricle catheterization and followed by injection of 25 mL 0.1 M phosphate-buffered saline solution to clean the spinal cord specimens from blood elements. After cardiac arrest developed, previous surgical incisions were reopened. For each subject, a total of 2 cm spinal cord segment, centering the previously injured region was harvested and the specimens were immediately stored at -80º C without any contaminants.

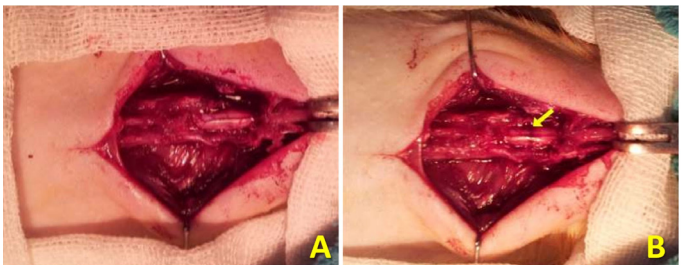

Figure 1. Spinal cord dissection after laminectomy procedure **(A)** and extradural marking (arrow) of spinal cord injury created with clip compression **(B)**

Biochemical Study

A ten times diluted solution of 0.01 M isotonic phosphate buffer at pH: 7.4 was prepared (9 gr NaCl, 2,575 gr $Na_2HPO_4.7H_2O$, 0.274 gr $NaH_2PO_4.2H_2O$, distilled water). Spinal cord samples were weighed and 10 µL of the buffer solution per sample weight in milligrams were added. Weights of the collected spinal cord samples were given in Table 1. Prepared samples were homogenized twice under ice-cooling using Ultra Turnax® T18 Basic Homogenizer at 14.000rpm for 30 seconds. The homogenates were centrifuged at 17,000 g, +4° C for 20 minutes, and 250 µL of the supernatants were separated for the biochemical study.

The samples were hydrolyzed in a 50 µL NaOH solution at 60° C for 30 minutes using an incubator and acidified by using 125 µL perchloric acid solution. After re-centrifugation at 14,000 g for 10 minutes, 250 µL of supernatants were separated in glass tubes. Then the samples were incubated with 25 µL 2.4-dinitrophenylhydrazine for 10 minutes and twice extraction with hexane was performed before the organic phases were dried at -40° C using nitrogen[24-26].

For total MDA quantity measurement, 20 µL of each sample was studied with 310 nm wavelength using high performance liquid chromatography (HPLC) at 30° C with a flow rate of 0.6 mL/min[26]. For antioxidant capacity determination 1.2 mg 2.2-diphenyl-1-picrylhydrasyl (DPPH) and 50 mL MetOH radical solutions were used. For 10 µL of each sample a 20 µL of MetOH was added on and the obtained solution was centrifuged at 15,000 g for 20 minutes. Obtained supernatant (15 µL) was mixed with DPPH solution (195 µL) and incubated for 30 minutes in total darkness. Then the samples and DPPH radical solution were studied for absorbance values at 515 nm wavelength using HPLC. Inhibition % values were calculated using "Inhibition $\%=A_{DPPH}-A_{sample}/A_{DPPH}x100$" formula[24,25].

Statistical Analysis

Obtained data from HPLC wavelength peak areas representing MDA levels and absorbance inhibition levels of the DPPH treated samples were calculated using the Statistical Package for the Social Sciences (SPSS v16.0.0) software. A Levene's test was used for the evaluation of homogeneity of variances and the study groups were compared with the independent samples t-test, including mean and standard deviations in %95 confidence intervals. P-value ⩽0.05 was considered as statistically significant and group comparisons were summarized in Table 2.

RESULTS

DPPH levels: For the Low-DMG, differences in DPPH levels were found statistically negligible when compared with the trauma group (p>0.05). Low-DMG and vehicle groups comparison showed a significant DPPH difference (p⩽0.05). High-DMG showed an evident statistically significant difference compared to the trauma group for the DPPH levels (p⩽0,05). High-DMG and vehicle group comparisons were also significant for DPPH levels (p⩽0.05). Comparisons for DPPH levels of High-DMG and Low-DMG showed an insignificant difference (p>0.05).

MDA levels: Comparison of Low-DMG and trauma groups were insignificant for MDA values (p>0.05). Low-DMG and vehicle groups comparison was also insignificant for MDA (p>0.05) values. For high-DMG, differences in MDA levels were found statistically significant when compared to the trauma group (p⩽0.05). High-DMG and vehicle group comparisons were also significant for MDA values (p⩽0.05). Comparisons for MDA levels of high-DMG and low-DMG showed an insignificant difference (p>0.05).

DISCUSSION

SCIs have special importance for both prevention and treatment especially for causing personal and social health problems. Most of the patients suffer from permanent incapacities, severe labor loss, and high expenses for hospitalization followed by long-term rehabilitation programs. Preventing the primary spinal injury is golden for sure but, the patients are always at the secondary SCI period at the time of hospital admission. Relatively fortunate patients with incomplete SCI on hospital admission frequently get worse over hours or days during the

Table 1. Weights of the collected spinal cord samples in miligrams

Sample	High-DMG	Low-DMG	Vehicle Group	Trauma Group
1	174.0	221.7	156.5	190.4
2	95.6	114.4	196.8	314.6
3	161.8	180.0	154.3	209.7
4	188.8	167.0	205.0	141.3
5	230.2	204.7	152.6	140.3
6	232.9	209.6	209.4	138.0
7	151.9	248.8	63.5	168.4
8	201.2	115.0	225.6	202.0
9	154.3	134.6	-	-
Mean	176.7	177.3	170.5	188.1

DMG: Dose medication group

Table 2. Statistical comparisons of the experiment groups

	Groups	N	Mean	Standard Deviation	Standard Error Mean	Sig.
DPPH	Low-DMG	9	10.5509	2.84696	0.94899	p>0.05
	Trauma	8	11.2185	5.57615	1.97147	
MDA	Low-DMG	9	4.8560	1.33605	0.44535	p>0.05
	Trauma	8	2.8867	1.09488	0.38710	
DPPH	Low-DMG	9	10.5509	2.84696	0.94899	p≤0.05
	Vehicle	8	13.6134	7.12992	2.52081	
MDA	Low-DMG	9	4.8560	1.33605	0.44535	p>0.05
	Vehicle	8	3.8158	1.52369	0.53871	
DPPH	High-DMG	9	13.4827	2.31020	0.77007	p≤0.05
	Trauma	8	11.2185	5.57615	1.97147	
MDA	High-DMG	9	2.6651	0.57380	0.19127	p≤0.05
	Trauma	8	2.8867	1.09488	0.38710	
DPPH	High-DMG	9	13.4827	2.31020	0.77007	p≤0.05
	Vehicle	8	13.6134	7.12992	2.52081	
MDA	High-DMG	9	2.6651	0.57380	0.19127	p≤0.05
	Vehicle	8	3.8158	1.52369	0.53871	
DPPH	High-DMG	9	13.4827	2.31020	0.77007	p>0.05
	Low-DMG	9	10.5509	2.84696	0.94899	
MDA	High-DMG	9	2.6651	0.57380	0.19127	p>0.05
	Low-DMG	9	4.8560	1.33605	0.44535	

DPPH: Diphenypicrylhydrasyl, MDA: Malondialdehyde, DMG: Dose medication group

secondary injury period which makes the treatment of secondary injury so important[1,3,5,7,8,16,27]. Besides lots of factors that were accused for the development of secondary SCI, it is known that oxidative stress has a significant role in this mechanism, which makes it a good target for new treatment modalities[9]. This is the main reason that this study was focused on oxidative injury mechanisms.

There are many experimental spinal trauma models developed so far. One of these standardized methods is the clip-compression method described by Rivlin and Tator[28]. In this study, this clip-compression method was preferred because of its high reliability and similitude according to human spinal injuries[22,28,29]. By this method, ischemia and neuroinflammation related free oxygen radical formation and lipid peroxidation can be created. During and after the application of clip compression, a perfusion defect occurs in all circulatory levels (arteriolar, venular and capillary) similar to the changes that occur during human spinal traumas[1,30].

Although GL was being used traditionally for thousands of years especially in the far eastern countries, it was recognized and began to be researched in western medicine in the 1980s. These studies revealed a variety of biological activities for this mushroom such as anti-neoplastic, anti-inflammatory, immunomodulatory, immunotherapeutic, hepatoprotective and ACE inhibitory effects[31-37]. Pharmacologically active ingredients of GL are triterpenes, which have similar molecular structures like steroid hormones and polysaccharides (especially β-d-glucan). These pharmacologically active molecules were recognized both in vitro and in vivo studies for preventing free oxygen radical generation and reducing their cellular oxidative damage[32,36,38]. This antioxidant effect is highest in Terpenes such as ganoderic acid A, B, C and D, lucidenic acid B and ganodermanontriol[36]. At the same time, it was shown that the "GL peptide" can effectively eradicate hydroxyl and superoxide radicals dose-dependently[39]. Shi et al.[40] studied hot water extracts of eight different mushrooms for their potential of reducing hydrogen peroxide (H_2O_2) mediated oxidative stress and found that GL has a high antioxidant feature and it has the potential to protect cellular DNA from oxidative damage. In a following *in vitro* study by Abdullah et al.[41], an antioxidant index (AI) was generated for hot water extracts of different mushroom species and GL (IC_{50}=50 µg/mL) was found to be the most potent with a 30.1% AI.

There are various studies showing that GL treatment reduces oxidative damage both in vitro and *in vivo*[42-44]. These findings were also similar in the central nervous system, especially studied on cerebral oxidative injury models[45,46], and previous spinal trauma models. In a detailed clip-compression spinal trauma experiment on rats, pre-isolated GL polysaccharides (GLPS) were administered before SCI. This biochemical, histopathological and ultrastructural study by Gokce et al.[47], showed positive results on reducing oxidative damage in favor

of crude GLPS. In another detailed study by Kahveci et al.[48], ischemia-reperfusion injury was created in the spinal cord by clamping the aorta, and pre-isolated GLPS were given to the subjects before SCI. Successful results were obtained with GLPS in preventing oxidative damage[48]. In a weight-dropping spinal cord injury rat model by Ekinci et al.[49], isolated GLPS were used after SCI. Biochemical and histopathological evaluations showed that, the use of GLPS had positive results in preventing oxidative damage[49]. In all three SCI studies mentioned above, subjects were administered pre-isolated GLPS at a constant dose of 400 mg/kg/day. In the first two studies, GLPS treatment was started 7 days before the SCI and spinal cord tissues were obtained 24 hours after the injury. In the last study mentioned, GLPS treatment was applied for 7 days after SCI. As mentioned before, polysaccharides, triterpenoids and peptides in GL were also determined to be bioactive. Therefore, in our study, unlike these studies, traditionally consumed hot-water extract of GL was tested at different doses without pre-isolating GLPS. In daily practice, since the treatment of spinal cord injuries is usually started after the injury, it was preferred to start the GL application after the SCI was created in the experiment. In previous biochemical experiments, the levels of different tissue free oxygen radicals and antioxidants were measured and the positive effects of the use of GL were shown[47-49]. In our study, in addition to the MDA level measurement used for the evaluation of lipid peroxidation, the antioxidant capacity of spinal cord tissue after SCI was tried to be tested by measuring the DPPH level.

To the subjects in low-DMG, the average recommended GL hot-water extract dose for humans was applied, in proportion to the rats, while ten times more dose was applied to the subjects in high-DMG[19,20]. In this study, supporting the previous studies, it was found that the GL treatment after SCI, increases the absorbance values of the DPPH treated spinal cord samples, and decreases the MDA levels. These positive results could only be obtained in the high dose medication group when compared to the trauma and vehicle groups. It was determined that, only DPPH change is statistically significant for the comparison of the Low-DMG and the vehicle group but, it was insignificant for MDA values. In the comparison of high-DMG and low-DMG, insignificant results were found for both DPPH and MDA values. These findings suggest that GL treatment also had some efficacy at low doses, but since it is not affecting MDA values, the efficacy could be considered as inconsistent and negligible. Since, traditional GL hot-water extract started in a sufficient dose range during the secondary SCI period prevents the oxidative damage, it was thought to have therapeutic potential for secondary SCI. Previous studies have shown that the efficacy of methylprednisolone (MP), the commonly used drug in the treatment of spinal injury, and GL are similar[47,49]. However, various and sometimes serious side effects can be observed due to MP therapy[50]. There are no serious side-effects encountered previously for GL both in animal and human experiments[23,51,52]. Besides its potential, the lack of significant side effect even at high doses makes GL more clinically valuable. The common handicap of experimental studies on this subject, including ours, is the limited number of subjects. Studies with more subjects are needed to establish a treatment protocol for human use.

CONCLUSION

In this study, the antioxidant neuroprotective effect of GL was biochemically investigated in a spinal cord injury model created by clip compression in rats. After SCI was administered, traditional GL hot water extract was given for 5 days to the subjects in two different dose groups and spinal cord samples were compared with control groups. Positive dose-dependent results were obtained by GL treatment in terms of both tissue antioxidant efficacy and prevention of lipid peroxidation. The results of the study is supporting the previous work, and showing that GL has the potential of reducing oxidative stress that causes secondary SCI when given at the appropriate dose. As a result, it was determined that GL traditional hot-water extract has a preventive effect on oxidative damage when applied in the secondary SCI period in a dose-dependent manner.

Ethics

Ethics Committee Approval: The study was approved by the Ankara University Ethics Committee of Animal Experiments (2011-123-485/26.10.2011).

Informed Consent: Animal study, spinal cord injury.

Author Contributions

Surgical and Medical Practices: D.D., M.K., D.B., A.D., Concept: D.D., M.K., D.B., A.D., Design: D.D., E.M.S., M.K., D.B., S.A., A.D., Data Collection or Processing: D.D., E.M.S., M.K., G.G., S.A., D.B., A.D., Analysis or Interpretation: D.D., E.M.S., A.E.S., Ö.Ö., G.G., S.A., D.B., A.D., Literature Search: D.D., E.M.S., A.E.S., Ö.Ö., G.G., D.B., A.D., Writing: D.D., A.E.S., D.B., A.D.

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