Turk Neurosurg, 2023

Original Investigation

DOI: 10.5137/1019-5149.JTN.37599-22.2

Received: 19.01.2022
Accepted: 19.07.2022

Published Online: 15.09.2023

Investigation of Neuroprotective Effect of Shilajit Extract in Experimental Head Trauma Model Created in Rats

Adil Can KARAOGLU[1], Ibrahim Burak ATCI[2], Nail DEMIREL[2], Okan TURK[2], Canan HURDAG[5], Ozgur BARAN[3], Nuriye Guzin OZDEMIR[2], Ayhan KOCAK[4], Muhammet Teoman KARAKURT[2]

[1]Diyarbakır Selahaddin Eyyubi State Hospital, Department of Neurosurgery, Diyarbakir, Turkey
[2]Istanbul Training and Research Hospital, University of Health Sciences Faculty of Medicine, Department of Neurosurgery, Istanbul, Turkey
[3]Diyarbakir Gazi Yasargil Training and Research Hospital, Department of Neurosurgery, Diyarbakir, Turkey
[4]Taksim Training and Research Hospital, Department of Neurosurgery, Istanbul, Turkey
[5]Demiroglu Bilim University, Department Histology and Embriology, Istanbul, Turkey

Corresponding author: Ibrahim Burak ATCI ✉ drburakatci@hotmail.com

ABSTRACT

AIM: To investigate the neuroprotective effect of shilajit extract in experimental head trauma.

MATERIAL and METHODS: Three groups of 33 Sprague Dawley Albino strain male rats were included in the study. Group 1 (n=11): trauma but not treated. Group 2 (n=11): trauma and treated with 0.5 mL / rat saline Group 3 (n=11): 150 mg / kg shilajit extract was administered intraperitoneally in the treatment of trauma. Following the head trauma, the indicated treatments were applied to the 2nd and 3rd groups at the first, twenty-four and forty-eighth hours. Brain tissues and blood samples were taken after the control animals were sacrificed at the 72nd hour in all groups after trauma. Sections prepared from cerebral cortex and ca1 region were examined with hematoxylin eosin and luxol fast blue staining. Total antioxidant capacity, total oxidant capacity and oxidative stress index were measured from blood samples taken after routine procedures.

RESULTS: The number of red neurons and the severity of edema were significantly higher in both the cerebral cortex and the ca1 region in the group treated with trauma only and in the group administered saline after trauma compared to the group that received shilajit extract after trauma. The total antioxidant capacity increased significantly in blood samples taken only from the group treated with trauma and saline in post-trauma treatment compared to the group given post-traumatic shilajit extract, while shilajit extract given due to traumatic brain injury significantly decreased the total oxidant capacity and oxidative stress index values compared to the other groups.

CONCLUSION: As a result; Shilajit extract has been shown to have a neuroprotective effect in the treatment of acute traumatic brain injury. Our study showed that shilajit may be a useful option in the treatment of secondary brain injury, in humans.

KEYWORDS: Experimental head trauma, Shilajitis, Neuroprotection, Rats

INTRODUCTION

Head trauma is currently one of the most serious health problems in our country and all over the world. According to various studies, at least 2.5 million people in the United States are admitted to hospitals because of head trauma in a year (14). Traumatic brain injury (TBI), which occurs after head trauma, continues to be a serious health problem, despite the developments in today's modern medicine. After TBI develops, primary brain damage first occurs in the central nervous system (CNS). However, it is not possible to blame only the primary injury for the damage caused by the trauma. Secondary damage is a condition that can occur hours or days

Adil Can KARAOGLU : 0000-0001-9140-3005
Ibrahim Burak ATCI : 0000-0002-0317-4159
Nail DEMIREL : 0000-0001-8303-1504
Okan TURK : 0000-0002-9514-6891
Canan HURDAG : 0000-0003-3336-0830
Ozgur BARAN : 0000-0001-8021-3580
Nuriye Guzin OZDEMIR : 0000-0002-2702-4526
Ayhan KOCAK : 0000-0002-3389-1941
M. Teoman KARAKURT : 0000-0002-7729-3070

after a primary brain injury and develops because of many different pathophysiological mechanisms. The mechanisms involved in secondary damage include neurotransmitter release, free radical formation, calcium-dependent cell damage, gene activation, mitochondrial dysfunction, and inflammation (12,13). Secondary cell damage, some of which are pypreventable, shifts the prognosis to a negative direction to a large extent.

Brain tissue exposed to trauma can return to its normal physiology as long as it can be protected from secondary damage, which is mostly caused by oxidants (15). Mechanisms that inhibit oxidative agents have been shown to positively affect pathological neurological conditions due to hypoxia or stroke in the CNS with their healing properties (7,8).

Various drugs are used in experimental head trauma models to prevent secondary damage, especially after head trauma. In developed countries, various studies by healthcare professionals and pharmacological organizations have been conducted on the importance of shilajit in the treatment of different diseases because of its neuroprotective, anti-inflammatory, and antioxidant roles (6). Asia Shilajit (Mumiyo) contains 20% minerals, 15% protein, 5% lipids, 5% steroids and also some carbohydrates, alkaloids, and amino acids (4,12). Several therapeutic effects of this substance are as follows: memory enhancing, neuroprotective, anti-inflammatory and antioxidant roles (6). The biological effect of Shilajit has been attributed to its di-benzo-alpha-pyrone, humic acid and folic acid contents (1,2). Based on the various benefits of shilajit, we hypothesized that the application of this substance could be effective in healing post-head trauma injuries. Therefore, this study investigated the neuroprotective effect of this substance following TBI.

MATERIAL and METHODS

Animals

A total of 33 male Sprague–Dawley albino rats, each 8–12 weeks old, with an average weight of 280–320 g and reared through internal feeding, were obtained from the Bezm-i Alem University Experimental Animal Research Laboratory. The rats were fed at room temperature (20 ± 2°C) in a 12-h light and dark environment. They were fed standard pellet rat food and provided with easy access to water. Ethics committee approval for the study was obtained from the Experimental Research Ethics Committee of Bezm-i Alem University (No: 2020/158; Date: 26.10.2020).

Working Groups

Rats were randomly selected into three groups, with 11 rats in each group.

Group I (n=11): the group that was traumatized and not treated

Group II (n=11): the group in which trauma was applied and 0.5 mL/rat saline was administered intraperitoneally in the treatment

Group III (n=11): the group in which trauma was applied, and 150 mg/kg shilajit extract was administered intraperitoneally in the treatment.

Trauma Model

The rats were anesthetized by administering 5–10 mg/kg of xylazine HCL (Rompun®-Bayer İlaç San, Turkey) and 50 mg/kg of ketamine (Keta-Control®-Doğa İlaç San, Turkey) intraperitoneally. The rats were observed in their cages for a while to deepen their anesthesia. Then, the rats were taken from their cages, weighed one by one using precision scales,and placed in the apparatus. During the experiment, the rats were subjected to severe head trauma using the experimental trauma model developed by Marmarou et al. in 1994 (11). In this model, the main logic of the trauma tool is to drop a 250 gram made of metal into the skulls of the rats with minimum friction through a cylindrical tube with the effect of gravity. When the rats were ready for trauma, they were placed on a foam mattress in the prone position and fixed with plasters from their extremities to prevent them from slipping during trauma. Severe head injury was induced by dropping a weight of 250 g from a height of 1 m. The rats were immediately removed from the lower end of the tube with the foam mattress to prevent re-impact after the first impact.

Establishment of working groups and execution of the study

A total of 33 experimental animals were randomly selected into three groups:

Group I (n=11): The rats were observed in their cages for 72 h after trauma. At the end of the 72nd hour, anesthesia was applied again, and the sternum of the rats was opened using scissors to expose the heart. A small incision was made in the right atrium. Blood was drained from one side, and SF perfusion started from the left ventricle on the other side. After all the blood was drained, formaldehyde infusion was made from the left ventricle, and the whole brain tissue was fixed. Afterward, the skull bone was removed, and the brain tissue was liberated and placed in alcohol solution.

Group II (n=11): After head trauma, 0.5 mL/rat saline was administered intraperitoneally to the rats that were taken into their cages at the 1st, 24th, and 48th hour of the trauma. The rats were observed in their cages until the 72nd post-traumatic hour. At the 72nd hour, an appropriate dose of anesthesia was administered again, and the brains of the rats were processed in the order of the above-mentioned procedures.

Group III (n=11): A total of 150 mg/kg of shilajit was administered intraperitoneally to the rats that were taken to their cages at the 1st, 24th, and 48th hour of the trauma. The rats were observed in their cages until the 72nd post-traumatic hour. At the 72nd hour, an appropriate dose of anesthesia was administered again, and then the brains of the rats wereprocessed in the order of the above-mentioned procedures.

Group C (n=1): One rat brain of the same age, same breed,and same weight, decapitated for another training study and without any trauma, was used.

Evaluation Methods

Histological examination

After the brain tissues taken for histological examination were fixed in a neutral buffered formalin solution, they were dehydrated in an alcohol series, starting from 70% to 100%,and cleared in xylene. The tissues, which were kept in paraffin overnight in an oven at 60°C, were turned into paraffin blocks at room temperature.

Sections 3–4 µm thick were taken from the paraffin blocks. Hematoxylin and eosin (H&E) staining was applied to these sections to examine the general tissue morphology, and Luxol fast blue staining was used to define the basic neuron structure. The preparations were examined and photographed under a light microscope (Olympus BX53) at ×200 and ×400 magnifications for morphological evaluation after closure.

Biochemical Examination

Total antioxidant capacity (TAS) and total oxidant capacity (TOS)

The biochemical parameters used in our study were examined using the Rat Total Antioxidant Status ELISA Kit and the Rat Total Oxidant Status ELISA Kit of the Bioassay Technology Laboratory. Seventy-two hours after trauma, blood samples from the heart were taken from the rats in all study groups during scarification, placed into a solvent-free tube, and stored at 2–8°C to be studied within five days. On the working day, the kit and sample were naturally left at room temperature for 20–30 min. After allowing the serum to clot for 20–30 min at room temperature, it was placed in a centrifuge device at 2,000–3,000 RPM for 20 min. The analysis was completed in accordance with the kits' operating instructions.

Calculation of the oxidative stress index (OSI)

OSI = TOS (µmol.H2O2.equivalent/L) × 100

TAS (µmol.Trolox.equivalent/L)

Statistical Analysis

The results of the research were analyzed using GraphPad-Prism (version 5.0 for PC; GraphPad Software Inc.). The data obtained were calculated as the mean ± standard error. After testing the conformity of the distribution of the groups to anormal distribution, a one-way analysis of variance(ANOVA)–Kruskal–Wallis test was performed because the histopathological data were not normally distributed. Data were presented as the median (25%–75%). $p<0.05$ was considered significant. Statistical analysis of the biochemical data was performed using Tukey's multiple comparisons test after the one-way ANOVA–Kruskal–Wallis test. $p<0.05$ was considered significant.

RESULTS

Histological Findings

When the H&E-stained cerebral cortex of the control group was examined using light microscopy, the neocortex (III–Vzones), nucleus, and prominently visible nucleolus were observed in the cytoplasm of large neurons (pyramidal) located in the CA1 and CA1 regions of the hippocampus. When the neuron structures located in these regions of the experimental and placebo groups were examined, red neurons were observed to show ischemic damage after 12–48 h. These red neurons with pycnotic nuclei, nuclear loss, and eosinophilic cytoplasm were especially striking in the G1 and G2 groups. Red neurons and edema significantly increased in the experimental and placebo groups compared to the control group (Figure 1, 2; Table I). When the cerebral cortex and CA1 regions of the G3 tissue sections in the treatment group were compared with the same regions in the experimental group, the pyramidal neuron structures were normal and the amount of edema decreased significantly ($p<0.001$).

Luxol fast blue staining was applied to all groups to examine the Nissl bodies, which are responsible for protein production in the perikaryons of pyramidal neurons, under a light

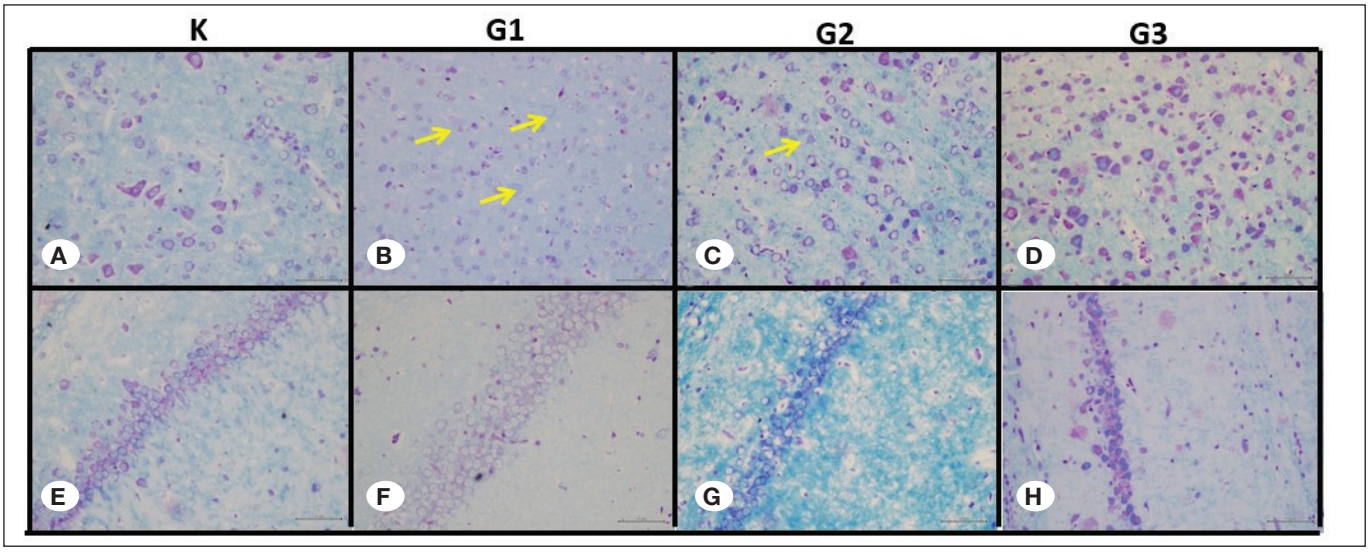

Figure 1: Presence of Red neurons (black arrows) indicating acute injury is indicated by edema (*) (**A-D:** cerebral cortex x20; **E-H:** Ca1 region x40).

microscope. When the cerebral cortex (III–V zones) and CA1 regions of the control group and the experimental group (G1) were compared, the violet-stained cytoplasm of the pyramidal neurons in the control group was paler (yellow arrows) than that in the cells in the experimental group. When G2 was compared with the control group, the Nissl bodies of the motor neurons decreased, and staining close to the control in the treatment group (G3) was observed (Figure 3, 4; Table II).

Table I: Distribution of Red Neurons by Groups and Median Values of Groups

	G1	G2	G3	C	p
Red neuron	8 (7-9)	6 (5-8)	2 (2-3)	1 (1-1)	G3-G1**, p=0.0013 C-G1***, p<0.0001 C-G2***, p<0.0001

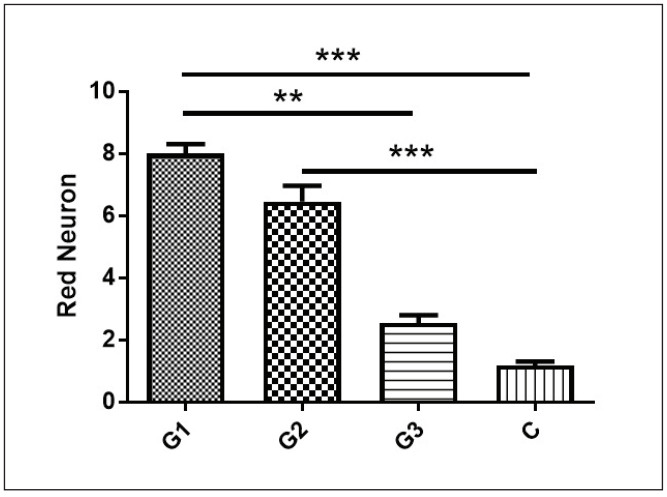

Figure 2: Statistical distribution of red neurons.

Biochemical findings

TAS, TOS, and OSI findings

In this study, when the TAS, TOS, and OSI values in the blood samples taken from the experimental groups were examined, the statistics of the TAS values in the trauma group were compared to those in the control group.

The values of TOS and OSI increased significantly ($p<0.001$). The mean ± standard error values of the TAS, TOS,and OSI values are presented in Table III (Figure 5).

DISCUSSION

According to the literature, a closed head trauma model has been created with many antioxidant substances, and its positive effects against secondary damage have been investigated.

Shilajit is mainly composed of humic substances, including fulvic acid, which makes up 60%–80% of the total nutraceutical compound and some oligoelements, mostly selenium. The curative properties attributable to shilajit are considered to be due to the potent antioxidant effect of fulvic acid and possible systemic effects as a complementary activator (3).

Many studies have examined the antioxidant properties of shilajit.

In 2010, Kumar et al. randomized 225 one-day-old chicks into groups of 15 chicks each. Basal diet, plant-based diet, shilajit-based diet, and selenium and vitamin E-weighted diet were given to groups 1–4, respectively. Oxidative stress was created by contacting the remaining chicks with lead, and basal diet, herbal diet, shilajit, and vitamin E–selenium complex were given to groups 1–4, respectively. The chicks were observed for 4–6 weeks. At the end of the follow-up period, blood samples were taken for glutathione peroxidase, reductase, and catalase. A statistically significant

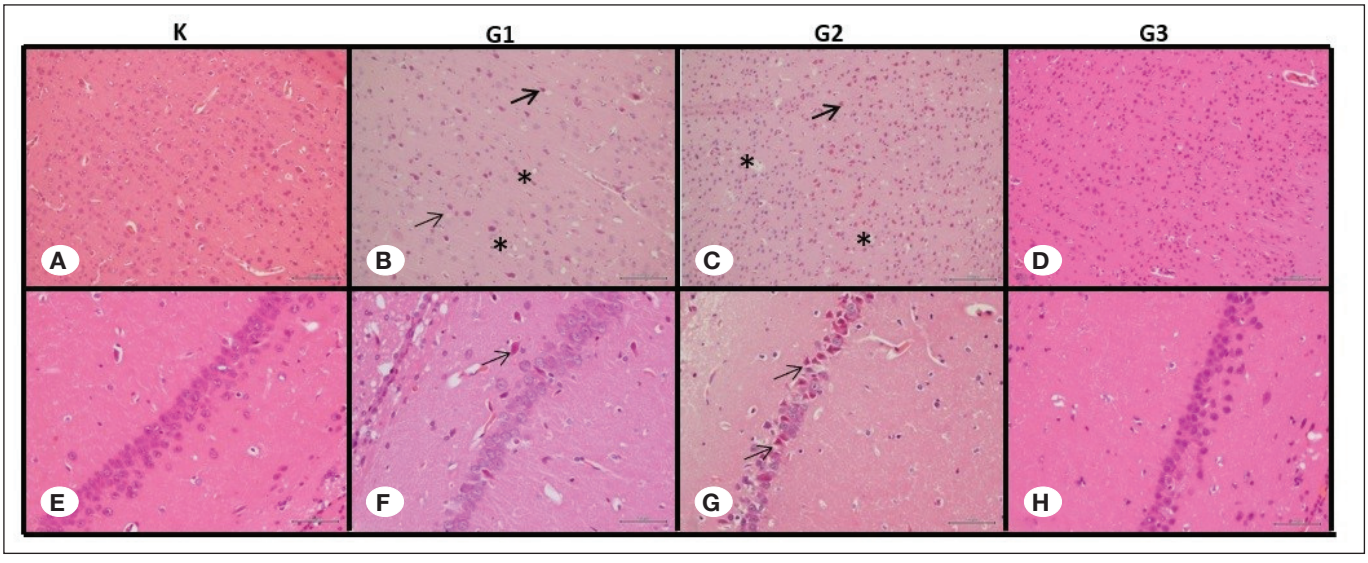

Figure 3: The loss of nuclei and Nissl particles of pyramidal neurons is indicated by (yellow arrows) (**A-D:** Cerebral cortexx40; **E-H:** Ca1 region x40).

Table II: Distribution of Edema by Groups and Median Values of the Groups

	G1	G2	G3	C	p
Edema	6 (6,7)	6 (5-7)	2 (1-2)	1 (1-2)	G3-G1**, p=0.0019
					C-G1***, p<0.0001
					G3-G2**, p=0.0024
					C-G2***, p<0.0001

Table III: Mean ± Standard Error Values of TAS, TOS and OSI Values

Vartables	Group 1	Group 2	Group 3
TAS (Trolox equivalent/L)	1.68 ± 0.14	0.85 ± 0.03	1.51 ± 0.07
TOS (μmol H2O2 Eqv./L)	8.11 ± 0.19	13.51 ± 0.27	11.43 ± 0.19
OSI (Arbitrary Unit)	4.89 ± 0.36	15.68 ± 0.79	8.13 ± 0.71

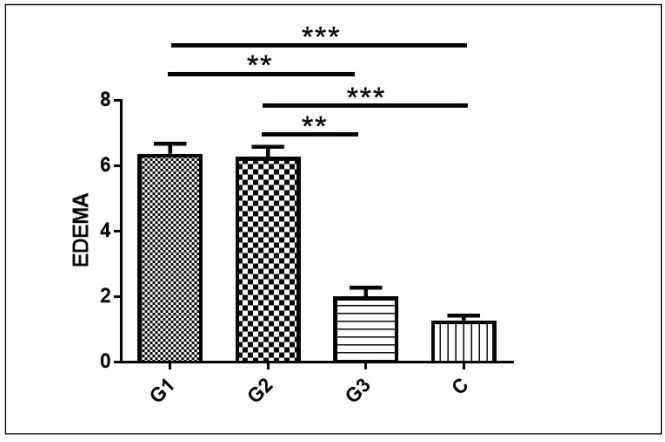

Figure 4: Statistical distribution of edema severity.

improvement was found in the shilajit group in the parameters indicating oxidative damage, and the author found that shilajit had a particularly strong antioxidant activity (10).

In 2021, Ghasemkhani et al. examined the effects of an omeprazole, saline, and shilajit complex on rats with gastric ulcers caused by aspirin. Shilajit was hypothesized to reduce leukocyte infiltration in tissues and improve oxidative stress parameters. Shilajit was found to exhibit a protective effect on tissues by reducing oxidative stress factors (5).

Only one study has shown the effectiveness of shilajit in treating head trauma. In Khaksari et al.'s study, rats were randomly divided into five groups, and shilajit was injected into two groups at doses of 150 and 250 mg/kg/rat. The dose was repeated at 1, 24, 48, and 72 h. During the observation period of the animals, intracranial pressure measurements were recorded in terms of motor movements. Decapitation was performed, and the brains were taken for histochemical analysis. Brain edema and blood–brain barrier permeability were determined using Evans blue dye. The results were statistically evaluated. In comparing the control group, sham group, and hypertonic saline group to the rat groups given 150 and 250 mg/kg of shilajit, the groups given shilajit had statistically significantly reduced brain edema. In comparing the control group, sham group, and hypertonic saline group to the rat groups given 150 and 250 mg/kg of shilajit, the groups given shilajit repaired the permeability of the blood–brain barrier in a statistically significant manner. However, the neurological follow-up of the group that was given shilajit was positive compared to that of the other groups, and intracranial pressure decreased with the application of shilajit. When the groups receiving shilajit were compared, positive effects were found to increase with an increase in dose (9).

In our study, red neurons were examined for acute injury assessment after head trauma. The p value for G3–G1 was 0.0013, which was considered statistically significant. When group C and group 1 were compared, the p value was 0.0001, which was not considered statistically significant. When group C and group 2 were compared, the p value was 0.0001, which was not considered statistically significant. As a result, the

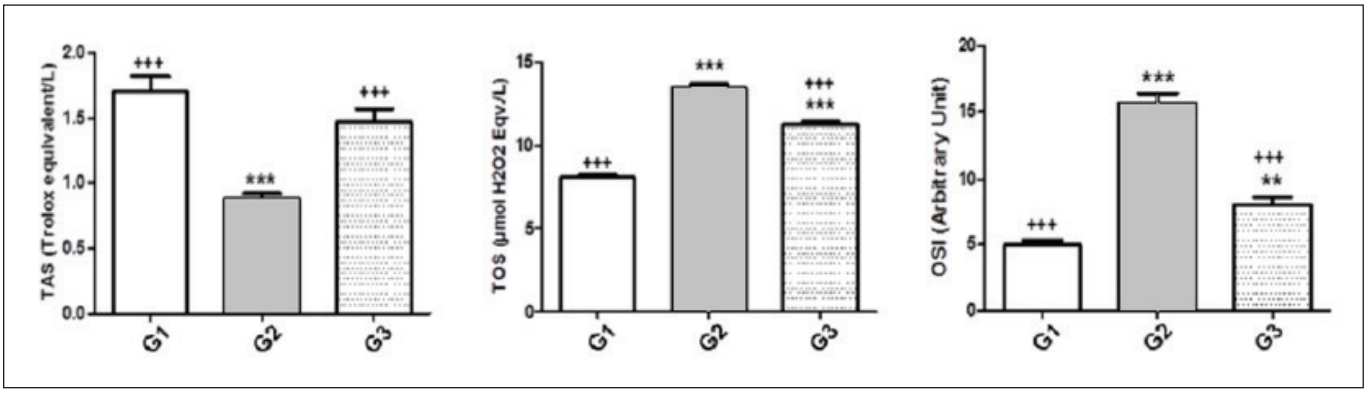

Figure 5: TAS, TOS and OSI values in blood samples taken from experimental groups. While TAS values decreased significantly due to TBI compared to the Control Group, Shilajit Given to the Trauma Group significantly increased this decreased value. While Tos and Osi values increased significantly due to TBI compared to the Control Group, Shilajit Given to the Trauma Group significantly decreased this increase. ** p<0.01, *** p<0.001 Compared to Control Group, +++*** p<0.001 Significance Value Compared to Trauma Group.

red neurons decreased in the H&E staining group in the group receiving shilajit.

When brain edema due to blood–brain barrier disruption was compared between the groups, the p value for G3–G1 was 0.0019, which was considered statistically significant. When group C and group 1 were compared, the p value was 0.0001, which was not considered statistically significant. When group C and group 2 were compared, the p value was 0.0001, which was not considered statistically significant. When groups 3 and 2 were compared, the p value was 0.0024, which was considered statistically significant. Again, the group that received shilajit was found to be statistically significant compared to the damascus and control groups. Thus, shilajit was found to have a reducing effect on cerebral edema.

However, in the biochemical analysis of the blood taken from the animals, the shilajit group caused a statistically significant improvement in the parameters, indicating the level of oxidative stress caused by trauma. This shows that shilajit has anti-inflammatory and antiedema effects and a strong neuroprotective effect.

In our study, 150 mg/kg of active substance was used in rats with head trauma, and it is the first study in the literature. The limitations of our study are that the efficacy of lower doses was not experimentally demonstrated and compared with each other.

CONCLUSION

In our study, the neuroprotective efficacy of shilajit was investigated in order to limit the diffuse damage in experimental diffuse closed head trauma. As a result of the histopathological and biochemical analyzes performed, a statistically significant difference was found in the biochemical parameters showing the severity of red neuron development, brain edema and trauma, and a positive effect was detected in the group that received shilajit compared to the other groups.

In order to investigate the neuroprotective efficacy of shilajit in head trauma, it can be tried to use different doses and durations for future studies. However, we think that shilajit can be used in the treatment by showing a positive effect on the secondary effects of trauma in diffuse head trauma.

ACKNOWLEDGMENTS

This study was produced from the thesis of Adil Can Karaoglu.

AUTHORSHIP CONTRIBUTION

Study conception and design: IBA, ADK

Data collection: IBA, ADK

Analysis and interpretation of results: IBA, ADK

Draft manuscript preparation: ND, OT, CH, OB, NGO, AK, MTK

Critical revision of the article: ADK, IBA, ND, OT, CH, OB, NGO, AK, MTK

Other (study supervision, fundings, materials, etc...): ND, OT, CH, OB, NGO, AK, MTK

All authors (ACK, IBA, ND, OT, CH, OB, NGO, AK, MTK) reviewed the results and approved the final version of the manuscript.

REFERENCES

1. Agarwal SP, Khanna R, Karmarkar R, Anwer MK, Khar RK: Shilajit: A review. Phytother Res 21:401-405, 2007
2. Bhattacharya SK, Ghosal S: Effect of Shilajit on rat brain monoamines. Phytother Res 6:163-164, 1992
3. Carrasco-Gallardo C, Guzmán L, Maccioni RB: Shilajit: A natural phytocomplex with potential procognitive activity. Int J Alzheimers Dis 2012:674142, 2012
4. Garedewa A, Feist M, Schmolz E, Lamprecht I: Thermal analysis of mumiyo, the legendary folkremedy from the Himalaya region. Thermochimica Acta 417(2):301-309, 2004
5. Ghasemkhani N, Tabrizi AS, Namazi F, Nazifi S: Treatment effects of Shilajit on aspirin-induced gastric lesions in rats. Physiol Rep 9(7):e14822, 2021
6. Goel RK, Banerjee RS, Acharya SB: Antiulcerogenic and antiinflammatory studies with shilajit. J Ethnopharmacol 29:95-103, 1990
7. Huh PW, Belayev L, Zhao W, Clemens JA, Panetta JA, Busto R, Ginsberg MD: Neuroprotection by LY341122, a novel inhibitor of lipid peroxidation, againstfocal ischemic brain damage in rats. Eur J Pharmacol 389:79-88, 2000
8. Jenneth B, Galbraith S: Head Injuries: Pathology and Natural History of Head Injury. An Introduction to Neurosurgery, 4th ed. London: William Heinemann, 1983:214-233
9. Khaksari M, Mahmmodi R, Shahrokhi N, Shabani M, Joukar S, Aqapour M: The effects of shilajit on brain edema, intracranial pressure and neurologic outcomes following the traumatic brain injury in rat. Iran J Basic Med Sci 16(7):858-864, 2013
10. Kumar MR, Reddy AG, Anjaneyulu Y, Reddy GD: Oxidative stress induced by lead and antioxidant potential of certain adaptogens in poultry. Toxicol Int 17(2):45-48, 2010
11. Marmarou A, Anderson RL, Ward JD, Choi SC, Young HF, Eisenberg HM, Foulkes MA, Marshall LF, Jane JA: Impact of ICP instability and hypotension on outcome in patients with severe head trauma. Special Supplements 75(1S):S59-S66, 1991
12. Mirza MA, Alam MN, Faiyazuddin M, Mahmood D, Bairwa R, Gulam M: Shilajit: An ancient panacea. Int J Curr Pharmaceut Rev Res 1:2-11, 2010
13. Pervez M, Kitagawa RS, Chang TR: Definition of traumatic brain injury, neurosurgery, trauma orthopedics, neuroimaging, psychology, and psychiatry in mild traumatic brain injury. Neuroimaging Clinics 28(1):1-13, 2018
14. Wickwire EM, Schnyer DM, Germain A, Williams SG, Lettieri CJ, McKeon AB, Scharf SM, Stocker R, Albrecht J, Badjatia N, Markowitz AJ, Manley GT: Sleep, sleep disorders, and circadian health following mild traumatic brain injury in adults: Review and research agenda. J Neurotrauma 35(22):2615-2631, 2018
15. Wilson JX, Gelb AW: Free radicals, antioxidants, and neurologic injury: possible relationship to cerebral protection by anesthetics. J Neurosurg Anesthesiol 14:66-79, 2002