EXPERIMENTAL STUDY

Histopathological and immunohistochemical investigation of the effect of Shilajit in rats with experimental spinal cord injury

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ABSTRACT

BACKGROUND: This experimental study was designed to investigate the histopathological and immunohistochemical effects of Shilajit in rats with experimentally induced spinal cord injury (SCI).

METHODS: The rats were divided into three groups: Control group: The group in which spinal cord damage was created but no drug was administered. Low-dose group: This is the group in which intraperitoneal Shilajit is given at a dose of 150 mg/kg at the 1st h, 1st day, 2nd day, and 3rd day after spinal cord damage was induced. High-dose group: This is the group in which intraperitoneal Shilajit is given at a dose of 250 mg/kg at the 1st h, 1st day, 2nd day, and 3rd day after spinal cord damage was induced. Thin sections taken from the spinal cord after euthanasia were sent for histopathological and immunohistochemical examination.

RESULTS: Histopathological examination of the high-dose group showed lower amounts of morphological findings compared to the low-dose group and control group. While a significant CD68 immune reaction was observed in the control group of rats with spinal injury, the positive immune reaction was found to be significantly decreased in the Shilajit-applied groups.

CONCLUSION: It is thought that the use of Shilajit in SCI will reduce the effects of secondary damage in SCI and that its administration to such patients will have positive effects on the results.

Keywords: Experimental study; primary injury; secondary injury; shilajit; spinal cord paralysis; spinal cord injury.

INTRODUCTION

Spinal cord injury (SCI) is a major neurological disorder that results in severe motor loss (i.e., paralysis), sensory impairment, and autonomic dysfunction. It is brought on by mechanical trauma.[1] While falls and violence are the second and third most common causes of SCI after traffic accidents, sports and leisure activities are another very frequent cause.[2] Following an SCI, the pathways that carry information from the brain to the body may be hampered or even completely blocked, resulting in total loss of sensation, movement, reflexes, full or partial loss of sphincter function below the damage level, and sometimes paralysis. These effects substantially reduce the quality of life.[3] It has a tremendous effect on people and society due to permanent disabilities brought on by sensory loss and stroke, which puts a heavy burden on health-care systems across the world.[4]

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SCI is a terrible condition that frequently leads to autonomic dysfunction below the level of damage, lifelong muscular paralysis, and loss of feeling. The inability of damaged neurons in the central nervous system (CNS) to develop new axons and form functional connections is primarily responsible for the persistence of dysfunctions. Long-distance connections may be restored following injury to any area of the spinal cord, and this has been studied using the SCI model.[5]

The term "regeneration" is frequently used to describe any structural alteration that results in functional recovery in the nervous system. This includes the regrowth of axons or dendrites, the remodeling of synapses, and even the responses of glial cells and their ancillary structures, such as myelin.[6] Sprouting or axonal development from healthy neurons, is another kind of axonal growth that occurs after damage. An undamaged neuron can grow additional branches from an existing axon to a denervated area in response to injury. Unlike regeneration, which must develop at or around the damaged site, sprouting can start distal to the site of the injury. Although regeneration is more challenging to achieve in the CNS, sprouting happens naturally and may be boosted experimentally. Both regeneration and sprouting can help functional recovery.[7]

The traditional approach to treating SCI centers on surgical stabilization of the damaged region, pharmacological management to avoid subsequent injury, and rehabilitation to both prevent function loss and assist in function restoration.[8] Other contemporary therapies include the use of steroids for neuroprotection (although the usage of methylprednisolone sodium succinate has reduced due to lack of effectiveness) and mean arterial pressure therapy to increase spinal cord perfusion.[9] However, because they do not promote spinal cord regeneration, these therapies have had mixed results.

Shilajit is a naturally occurring leaching from high mountain rocks, notably those in the Himalayas, that is, produced by the slow microbial deterioration of plant matter and contains humic components such as minerals and fulvic acid. It has been widely employed in Ayurvedic medicine for ages to boost energy. Because of its anti-inflammatory and antioxidant properties, it aids in the treatment of many illnesses.[10]

The purpose of the current study was to investigate Shilajit's impact on rats with experimental spinal cord damage in terms of histopathology and immunohistochemistry.

MATERIALS AND METHODS

The study was carried out with the permission of the Van Yüzüncü Yıl University Animal Experiments Local Ethics Committee dated May 27, 2021 and no 2021/05-13.

Animal Material

A total of 30 Wistar Albino male rats, each weighing 200–250 g, were used in the study. Adult, pathogen-free, male Albino Wistar rats were obtained from Van Yüzüncü Yıl University Experimental Medicine Application and Research Center. Animals were fed adibitum and kept in light for 12 h and dark for 12 h per day. The average temperature of the living spaces is 26°C and 60% relative humidity.

To provide general anesthesia before the operation, xylazine hydrochloride (Rhompun, Bayer Turkish Chemical Industry, Istanbul) 10 mg/kg and ketamine hydrochloride (Ketalar, Pfizer PFE İlaçları AŞ, Istanbul) 50mg/kg were administered intraperitoneally to the rats before the operation.

Operation Method

The operation area of the rats taken under general anesthesia was shaved and sterilization was applied. SCI was created in rats in all groups according to the modified Allens method. For this purpose, a 2 cm incision was made in the midline at the level of the 8th thoracic vertebra of the rats, the spinous process and lamina of the T8 were removed and the spinal cord was exposed. Then, a weight of 10 g was dropped on the spinal cord from a distance of 5 cm and left on it for 3 min after the operation, the layers were sutured in accordance with its anatomy. After the operation, 5 mL intraperitoneal saline infusion was made (Fig. 1).

Trial groups

• All rats (30) were randomly divided into three groups (10 rats in each group) after the operation

• Group 1 (Control Group): It is the group in which spinal cord damage was induced but no drug was administered. SCI was induced in 10 rats in this group and no post-operative medication was administered

• Group 2 (Low-dose group): This is the group in which intraperitoneal Shilajit is given at a dose of 150 mg/kg at the post-operative 1st h, 1st day, 2nd day, and 3rd day after spinal cord damage was induced. In this group, 10 rats were given intraperitoneal Shilajit at a dose of 150 mg/kg at the 1st h, 1st

Figure 1. (a,b,c): Showing experimentally induced-spinal injury in rats.

day, 2nd day, and 3rd day post-operative after SCI.

• Group 3 (High-dose group): This is the group in which intraperitoneal Shilajit is given at a dose of 250 mg/kg at the post-operative 1st h, 1st day, 2nd day, and 3rd day after spinal cord damage was induced. In this group, 10 rats were given intraperitoneal Shilajit at a dose of 250 mg/kg at the 1st h, 1st day, 2nd day, and 3rd day post-operative after SCI.

After the rats were taken to general anesthesia on the 14th day, they were euthanized by blood collection from the heart. Thin sections taken from the spinal cord after euthanasia were sent to the laboratory for histopathological and immunohistochemical examination.

Histopathological Examination

Obtained medulla spinalis were fixed 10% buffered formalin solution. A transversal sample was taken from the medulla spinalis and embedded in paraffin blocks after routine tissue follow-up. Tissue sections of 4 µm were taken from paraffin blocks using a rotary microtome. The tissue sections were stained with hematoxylin-eosin and immunohistochemical methods and then examined under a light microscope and photographed (Nikon 80i, DS-RI2; Nikon, Tokyo, Japan). The stained slides were evaluated by two specialists who were blinded to the study groups. The degree of morphological changes was evaluated by scoring as: Slight, moderate, or severe.[11,12]

Immunohistochemical Examination

Prepared tissue sections were stained with CD68 antibody according to the immunoperoxidase method. Tissue sections were prepared for staining, blocked with 3% H2O2, antigen retrieval solution (citrate buffer), and protein-blocking (non-immune serum), sequentially. Then, the CD68 primary antibody was dripped onto each tissue section and incubated overnight at +4°C and then incubated with a biotinylated secondary antibody. Tissue sections were incubated in streptavidin-peroxidase for 20 min and then reacted with diaminobenzidine (DAB). After the DAB reaction, all tissues were stained with Mayer's hematoxylin for background staining. Appropriate negative and positive controls were used to confirm the staining process. Slides used as negative controls were incubated in phosphate-buffered sodium instead of primary antibodies. Tissue sections were evaluated, viewed, and photographed under a light microscope (80i; DS-RI2, Nikon, Tokyo, Japan). Immunohistochemical results were evaluated according to the intensity and extent of staining in the tissue as follows: Mild (+); moderate (++); or intense (+++).[13]

RESULTS

Experimental Inducing Spinal Damage in Rats

Histopathological Findings

Group 1 (Spinal injury): A marked inflammatory reaction consisting of capillary vascularization, edema, myelin degeneration (vacuolation), macrophages, and neutrophil leukocytes was observed in the spinal injury area. However, no significant connective tissue proliferation was observed (Figures 2a and b). In other parts of the spinal area, especially among the motor neurons, acute inflammatory cell infiltration was noted.

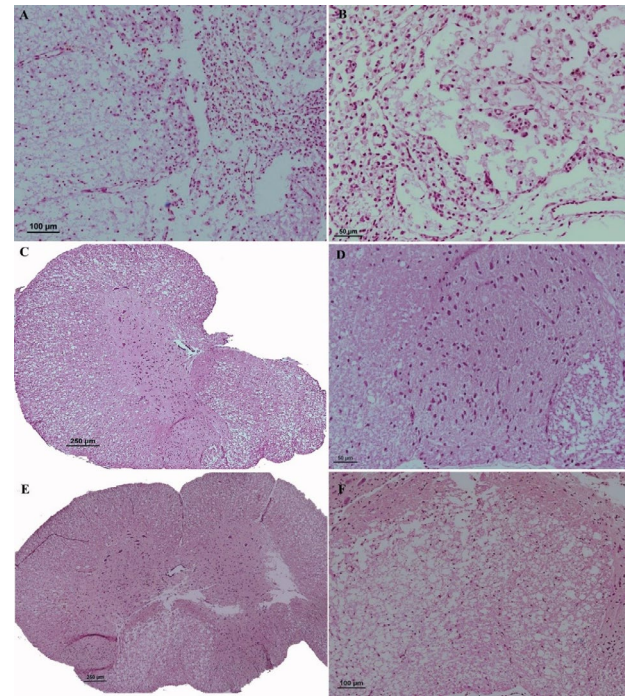

Figure 2. Cross-section of the medulla spinalis stained with hematoxylin and eosin (H&E): **(a,b)** Group I (Control; Spinal Injury): In the area of spinal injury, a marked inflammatory reaction consisting of vascular proliferation, macrophages and neutrophil leukocytes is observed. **(c,d)** Group II (Spinal Injury + 150 mg/kg Shilajit): Mild leukocyte infiltration and degeneration are observed in the spinal cord. **(e,f)** Group III (Spinal Injury + 250 mg/kg Shilajit): Almost similar findings observed in group II are also observed in this group.

Group 2 (Spinal damage + 150 mg/kg Shilajit): In the area of spinal injury, the findings observed in Group 1 were also observed in Group 2, albeit at milder levels (Figures 2c and d).

Group 3 (Spinal damage + 250 mg/kg Shilajit): It was noted that morphological changes were significantly less shaped in this group (Figures 2e and f). Morphological findings (mild, moderate, and severe) between groups were scored in Table 1.

Immunohistochemical Findings

While a significant CD68 immune reaction was observed in the spinal injury group, it was determined that the positive immune reaction was significantly reduced in the Shilajit applied groups (Figure 3). Immune reaction scores between groups; pronounced (+++), moderate (++), and mild (+) are shown in Table 2.

DISCUSSION

SCI can result in a variety of complicated pathophysiological reactions that are often separated into the original injury and subsequent injury stages.[14] Secondary injury frequently results in the loss of function of the wounded region in addi-

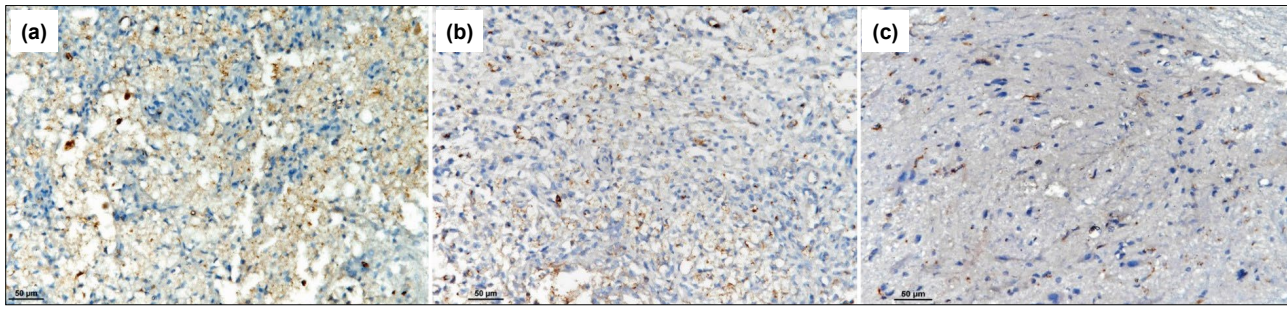

Figure 3. CD68 immunolocalization in cross-sections of the rat medulla spinalis. **(a)** Group I (Spinal Injury) showing strong nuclear expression of CD68. **(b)** Group II (Spinal Injury + 150 mg/kg Shilajit) showing weak nuclear CD68 immunoreactivity. **(c)** Group III (Spinal Injury + 250 mg/kg Shilajit) showing less CD68 immunoreactivity compared to the other two groups.

tion to physical damage such as original injury, rupture of the blood–spinal cord barrier, axonotmesis, and destruction of neurons and glial cells.[15] Depending on the intensity and kind of the main injury, secondary damage follows and can persist for a few days to years. It significantly hinders the recovery of spinal cord functions following injury.[16]

Both cellular component-specific mechanisms and extrinsic variables external to the damage site contribute to the failure of healing after SCI.[17] Astrocytes and microglia work in concert with other cell types, including but not limited to macrophages, oligodendrocyte progenitor cells, pericytes/fibroblasts, ependymal and endothelial cells, to form the glial/fibroblastic scar.[18]

Understanding the pathophysiology of spinal cord injuries has advanced significantly, yet different treatment methods have specific benefits and drawbacks.[19] The first issue is figuring out how to avoid the series of circumstances connected to the secondary spinal damage phase. Regenerating damaged spinal cord tissue and reestablishing lost connection constitute the second obstacle. SCI has a dynamic and intricate pathophysiology that involves several interconnected molecular and metabolic processes.[20]

Different therapies have been developed to influence either a

Table 1. Comparison of results between three different suture material

Morphological Findings	Group 1	Group 2	Group 3	P value
Inflammatory cell infiltration	10/10[a]	10/10[b]	10/10[b]	*
Slight	3	7	10	
Moderate	4	3	–	
Severe	3	–	–	
Vacuolation (myelin degeneration)	10/10[a]	10/10[b]	10/10[b]	*
Slight	2	7	8	
Moderate	5	3	2	
Severe	3	–	–	

[a,b]Values in a row with no common superscript letter are significantly different *p<0,05.

Table 2. Semiquantitative evaluation of immunoreactivity in all the groups in immunohistochemical examination with CD68 antibody.

Immunohistochemical Findings	Group 1	Group 2	Group 3	P value
CD68 immunoreactivity	10/10[a]	10/10[b]	10/10[b]	
+	1	6	8	
++	2	4	2	*
+++	7	–	–	

[a,b]Values in a row with no common superscript letter are significantly different *p<0.05.

single component of occurrences or multiple events simultaneously.[21] Therapies that either directly or indirectly regulate and manage synchronous pathways aid in the recovery of this terrible illness. The best strategies work best to overcome SCI-related difficulties.[22]

In preclinical investigations, several possible treatments have shown effectiveness up to phase IV, but some have also revealed inappropriate dosages or drawbacks, including undesirable pharmacokinetics, a brief half-life, and undesirable pharmacodynamic characteristics. Designing appropriate drug delivery systems that can combine one or more medications, which directly alter drug bioavailability and specificity, decrease adverse drug effects, target neuroprotection and neuroregeneration, and extend therapeutic effects, can help solve these problems. Numerous cellular and genetic techniques have also shown promise in reducing the harmful consequences of SCI, in addition to medications and active substances.[23]

Combination treatment utilizing stem cells and neuroprotective or neuroregenerative drugs exhibits the potential to provide positive results, and some new techniques have been effective in eliminating or minimizing negative effects.[24]

Shilajit has a wide range of advantages, as suggested by studies with strong scientific backing and earlier sources. It has stress-relieving, memory and energy-boosting, anti-inflammatory, antidiabetic, spermatogenic, neuroprotective, antiulcer, and wound healing properties. The presence of humic acid, fulvic acid, dibenzo-pyrons, dibenzo-pyrones, chromoproteins, and trace elements is primarily responsible for these pharmacological actions.[25]

In a study by Kangari et al., Shilajit showed that Alg hydrogel accelerated the differentiation of human fat-derived mesenchymal stem cells into osteoblasts without changing their physical properties.[26] Another study showed that Shilajit was given systemically to individuals with tibial and femoral fractures, and compared to placebo groups, this expedited bone formation.[27]

SCI has evolved as one of the most severe illnesses, having a significant influence on global health-care systems. Sadly, there is no long-term treatment for SCI. It would be helpful to develop a unifying approach that leverages neuroprotective and neuroregenerative strategies to target multiple pathways simultaneously.

The present study aimed to show some possible effects histopathologically and immunohistochemically using Shilajit, which has been used conventionally in Ayurvedic medicine for centuries, in our experimental study in which we created spinal cord damage in the hope of benefiting from its neuroprotective and neuroregenerative effects.

Worldwide research, both clinical and experimental, is ongoing in spinal cord injuries. The research is progressing in a multidisciplinary manner to try to prevent secondary damage and accelerate the regenerative process. No cure has been found for this devastating disease, which affects many people worldwide, socially, economically, physically, and spiritually. In our experimental study, some promising results were obtained, albeit partially, in these patients. It has been concluded that Shilajit, which has been used and successfully demonstrated in many animal experiments before, may be useful in SCI histopathologically and immunochemically.

CONCLUSION

In experimental SCIs, in line with morphological and immunohistochemical findings, it was concluded that Shilajit application inhibited anti-inflammatory reactions at significant levels in direct proportion to the dose increase used, and partially reduced myelin degenerations.

Ethics Committee Approval: This study was approved by the Faculty of Veterinary Medicine, Van Yüzüncü Yıl University Ethics Committee (Date: 02.07.2023, Decision No: 2021/05-13).

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Authorship Contributions: Concept: E.Ç.; Design: E.Ç.; Supervision: T.S.; Resource: İ.Ü.; Materials: Ö.F.K.; Data collection and/or processing: T.S.; Analysis and/or interpretation: Ö.F.K.; Literature search: M.E.A.; Writing: E.Ç.; Critical review: Ö.A.

Conflict of Interest: None declared.

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DENEYSEL ÇALIŞMA - *ÖZ*

Deneysel omurilik yaralanması olan sıçanlarda Shilajit'in etkisinin histopatolojik ve immünohistokimyasal olarak incelenmesi

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AMAÇ: Bu çalışma, deneysel olarak omurilik yaralanması (SCI) oluşturulan sıçanlarda Shilajit'in histopatolojik ve immünohistokimyasal etkilerini araştırmak üzere tasarlandı.

GEREÇ VE YÖNTEM: Sıçanlar üç gruba ayrıldı. Kontrol grubu: Spinal kord hasarı oluşturulan fakat herhangi bir ilaç uygulanmayan gruptur. Düşük doz grubu: Spinal kord hasarı oluşturulduktan sonra 150 mg/kg dozunda ameliyat sonrası 1. saatte, 1. günde 2. günde ve 3. günde intraperitoneal Shilajit verilen gruptur. Yüksek doz grubu: Spinal kord hasarı oluşturulduktan sonra 250 mg/kg dozunda ameliyat sonrası 1. saatte, 1. günde 2. günde ve 3. günde intraperitoneal Shilajit verilen gruptur. Ötenazi sonrasında spinal kordan alınan ince kesitler ise histopatolojik ve immünohistokimyasal incelemeye gönderildi.

BULGULAR: Yüksek doz grubunun histopatolojik incelemesinin morfolojik bulgular açısından düşük doz grubu ve kontrol grubuna kıyasla daha düşük miktarlarda olduğu gözlendi. Spinal hasarlı sıçanların kontrol grubunda belirgin derecede CD68 immün reaksiyonu gözlenirken, Shilajit uygulanan gruplarda ise pozitif immün reaksiyonun anlamlı düzeyde azaldığı saptandı.

SONUÇ: Omurilik yaralanmasında Shilajit kullanılmasının sekonder hasarın etkilerini azaltacağı ve bu hastalarda tedavi olarak verilmesinin sonuçlar üzerinde olumlu etkiler yapacağını düşünmekteyiz.

Anahtar sözcükler: Spinal kord yaralanması; shilajit, omurilik felci, deneysel çalışma; primer hasarlanma; sekonder hasarlanma.

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