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Therapeutic Potential of *Bacopa monnieri* L. in Sciatic Nerve Ligation: Modulation of Regeneration and Oxidative Stress

Siyatik Sinir Ligasyonunda *Bacopa monnieri* L.'nin Tedavi Potansiyeli: Rejenerasyon ve Oksidatif Stresin Modülasyonu

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ABSTRACT

Bacopa monnieri L. is a plant known for its neuroprotective properties with positive effects on neuronal regeneration and synaptic activity. In this study, the effects of *Bacopa monnieri* L. extract on the regeneration process in sciatic nerve injury, which is a peripheral nerve injury model, were investigated. Within the scope of this study, 18 Sprague–Dawley rats, each group containing six animals, were divided into three groups. Surgical ligation of the sciatic nerve was performed at the beginning of the experiment to induce sciatic nerve injury in the positive control and treatment groups. Following surgical intervention, the treatment group received a daily dose of 400 mg/kg extract for 15 days starting from the 14th day post surgery. The oxidative stress status in serum samples obtained from rats was investigated biochemically. Immunohistochemical measurements of tumor necrosis factor alpha and inducible nitric oxide synthase were also performed in the muscles surrounding the sciatic nerve. Upon examination of the results, it was observed that the treatment group exhibited a significant increase in antioxidant capacity compared to the control groups. Furthermore, the oxidant status in the treatment group was lower than that in the positive control group. Immunohistochemical examinations revealed an increase in tumor necrosis factor alpha- and inducible nitric oxide synthase immunoreactivity in the positive control group, while a decrease in the immunoreactivity of these inflammation markers was observed in the treatment group. In conclusion, *Bacopa monnieri* L. extract may contribute to recovery in peripheral nerve injury by regulating antioxidant and anti-inflammatory processes.

Keywords: Anti-inflammatory, antioxidant, *Bacopa monnieri* L., peripheral nerve injury, sciatic nerve ligation

ÖZ

Bacopa monnieri L. nöronal rejenerasyon ve sinaptik aktivite üzerinde olumlu etkileri olduğu bilinen nöroprotektif özelliğe sahip bir bitkidir. Bu çalışmada *Bacopa monnieri* L. ekstresinin periferik sinir hasarı modeli olan siyatik sinir hasarında rejenerasyon süreci üzerine etkileri araştırılmıştır. Çalışma kapsamında Sprague–Dawley ırkı 18 adet sıçan her birinde 6 hayvan bulunan 3 gruba ayrıldı. Pozitif kontrol ve tedavi gruplarına siyatik sinir hasarı oluşturmak amacıyla deneyin başlangıcında cerrahi olarak siyatik sinir ligasyonu uygulandı. Tedavi grubuna cerrahi uygulama sonrası 14. günden itibaren 15 gün boyunca 400 mg/kg/gün ekstrakt uygulandı. Sıçanlardan alınan serum örneklerinde oksidatif stres durumu biyokimyasal olarak araştırıldı. İmmunohistokimyasal olarak ise siyatik sinir çevresindeki kaslarda TNF-α ve iNOS ölçümü gerçekleştirildi. Elde edilen sonuçlar incelendiğinde kontrol gruplarına göre tedavi grubunda antioksidan kapasite önemli ölçüde artmıştır. Oksidan durumun ise tedavi grubunda PC grubundan daha düşük olduğu görülmüştür. Immunohistochemical incelemelerde PC grubunda TNF-α ve iNOS immunoreaktivitesini artarken tedavi grubunda bu inflamasyon belirteçlerinin immunoreaktivitesinin düştüğü belirlenmiştir. Sonuç olarak *Bacopa monnieri* L. ekstresi periferik sinir hasarında antioksidan ve antiinflamatuar süreçleri düzenleyerek iyileşmeye katkı sağlayabilir.

Anahtar Kelimeler: Antiinflamatuar, antioksidan, *Bacopa monnieri* L., periferik sinir hasarı, siyatik sinir bağlama

Introduction

The nervous system is an intricate network of nerves that orchestrates its operations through the transmission of messages to and from diverse anatomical regions. The system is capable of perceiving alterations in the surrounding environment that exert an influence on the physiological state of the organism. The classification of the nervous system entails two main divisions, namely the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS is composed of the spinal cord and brain, whereas the PNS is composed of nerves, which are densely packed bundles of elongated fibers, or axons. The primary role of these nerves is to establish functional connections between various organs of the body and the CNS (Vecchiarelli & Tremblay, 2023). The spinal cord's ventral and dorsal roots constitute the PNS. Dorsal root ganglia cells include motor and sensory neurons, respectively. The ventral horn and brainstem nuclei contain motor neuron cell bodies. Sensory and motor axons may reach distant organs (Zhang et al., 2021). The integrity of the injured nerve, the age of the affected individual, and the type of lesion sustained are all factors that contribute to the diverse functional settings experienced by each of them. Sciatic nerve ligation (SNL) represents a significant neurological disorder that presents a considerable risk to both human and animal patients also known as peripheral nerve injuries. These injuries can lead to profound and enduring functional and physiological impairments throughout an individual's lifetime (Dong et al., 2019). The term SNL refers to several forms of peripheral nerve stem or branch injury. Sciatic nerve ligation accounts for 1.5–4.0% of all trauma cases worldwide each year (Siemionow & Brzezicki, 2009). Sciatic nerve ligation elicits modifications in immune cells and the corresponding factors involved in immune regulation, leading to a series of immunoregulatory reactions within the cellular microenvironment. There are several immunomodulatory substances that have been identified, including interleukins (ILs) such as IL-1, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, and IL-17 (Xiaoting et al., 2013) (Wang et al., 2015), and tumor necrosis factor alpha (TNF-α) (Kiguchi et al., 2015). Sciatic nerve ligation often causes poor function, nerve damage, and sensory and motor impairment. Partial healing, muscle atrophy, prolonged pain, and significant weakening follow (Wang et al., 2019). In order to achieve reinnervation and establish connections with distal motor endplates, axons often necessitate regeneration over considerable distances, exhibiting a relatively slow rate of 1–3 mm per day (Slavin et al., 2021). Consequently, the process of regeneration requires an extended period of time, particularly when external aid is not available (R. Dong et al., 2019). Most medical procedures are microsurgical, offering direct repair, tension-free end-to-end sutures, or the best method utilizing autologous nerve grafts to close bigger gaps (Kornfeld et al., 2019; Rayner et al., 2020, 2021). However, it has drawbacks, including morbidity at the donor site, a lack of donors, impaired sensation, scarring, and the possibility of neuroma formation (Wang et al., 2019).

The use of animals in experiments allows for more precise control over the factors influencing regeneration and recovery, as well as the evaluation of novel techniques and treatment stances. In addition, animal models permit the implementation of scientific methodologies to examine molecular and cellular processes in a manner not possible in clinical research, as well as to evaluate the onset and progression of regeneration (Allodi et al., 2012). The experimental paradigm of sciatic nerve axotomy (SNA) has been employed in the field of neuroscience since the early 20th century. Santiago Ramón y Cajal, in his 1906 Nobel Prize acceptance speech, elucidated the utilization of SNA in his research. Cajal significantly contributed to the support of the neurotropic theory and the concept of nerve regeneration continuity by employing a thoughtful application of the model and providing detailed descriptions of the outcomes. As a result, towards the conclusion of the 19th century, SNA attained the status of a widely accepted experimental model in the two primary domains that would subsequently drive its utilization in the 20th century: nerve regeneration and neuropathic pain (Savastano et al., 2014).

Bacopa monnieri L. extract (BME), commonly referred to as "medhya rasayanas" in Ayurveda, has been widely employed as an Ayurvedic remedy in India for approximately three millennia. The main reason for this is that the plant has a large number of bioactive parts, such as alkaloids, glycosides, flavonoids, and "bacosides," which are complex mixtures of compounds with similar structures (Dowell et al., 2015). *Bacopa monnieri* L. extract, a botanical extract, has been found to contain a diverse array of chemical components, including brahmine, nicotinine, herpestine, bacosides A and B, saponins A, B, and C, β-sitosterol, betulinic acid, serine, aspartic acid, glutamic acid, and other various elemental components (Jeyasri et al., 2020). The therapeutic efficacy of a compound consisting of two saponins, specifically bacoside A and its optically rotated isomer bacoside B, has been scientifically validated. The saponins present in BME have been identified as its active constituents. Moreover, it has been recognized that bacoside A is comprised of four saponins, specifically bacopaside II, bacoside A3, bacopasaponin C, and the jujubogenin isomer of bacopasaponin C (Deepak & Amit, 2013). Bacopaside II and bacoside A3 demonstrate enhanced neuroprotective characteristics in comparison to the remaining two constituents, owing to their ability to lessen levels of intracellular reactive oxygen species (ROS) and enhance cellular viability (Bhardwaj et al., 2018). The *Bacopa* plant has been identified to facilitate the process of neuronal regeneration, promote the synthesis of neurons, restore synaptic activity, and enhance cognitive function (Mehta et al., 2022). Its benefits extend to enhanced cognitive performance and better memory (Shinomol et al., 2011).

In this study, the effect of BME on the regeneration process of peripheral nerve damage was investigated using biochemical and pathological methods.

Methods

Chemicals

Ketamine (Keta control, Doğa İlaç, İstanbul, Türkiye) and xylazine (Control 10%, Doğa İlaç, İstanbul, Türkiye) used for anesthetics. *Bacopa monnieri* L. extract was purchased from commercially (Naturevibe Botanicals).

Animals

Atatürk University Medical Experimental Research and Application Center (ATADEM) provided 18 male Sprague–Dawley rats weighing 250–300 g for the experiments. All rats were housed at room temperature of 21 ± 2°C, 12 hours of light and 12 hours of darkness, and housed in cages without specific pathogens, with access to food and water ad libitum. In experimental animal studies, the ARRIVE guidelines and the rules applicable from the eighth edition of the Guidelines for the Care and Use of Laboratory Animals were applied. Ethics committee approval was obtained from Atatürk University Animal Experiments Local Ethics Committee with the date June 22, 2023, and the number 110.

Experimental Groups
- Negative control (NC): healthy control group ($n = 6$)
- Positive control (PC): surgical SNL ($n = 6$)
- Treatment group (BME): 15 days 400 mg/kg BME ($n = 6$)

Model
Sciatic nerve ligation is an experimental model for inducing neuropathic pain and examining nerve injury in rats. In this model, triple suturing of surgically exposed sciatic nerves is performed, and then rats are monitored for signs of hypersensitivity, such as increased sensitivity to tactile, thermal, and chemical stimulation. It is also used to study the effects of acute nerve injury on the expression of various molecules and gene transcripts. 60 mg/kg ketamine and 10 mg/kg xylazine were used for anesthetic purpose.

In this study, SNL was used to assess the potential therapeutic efficacy of BME in acute nerve injury. Three branches of the sciatic nerve were ligated, and the pattern development was verified after a 14-day waiting period. Following the induction of SNL, BME was administered by gavage at a dose of 400 mg/kg (the treatment dose was chosen in accordance with the literature data) for 15 days beginning on day 14 (Kishore et al., 2016; Omura et al., 2005).

Biochemical Analysis
Serum was obtained from blood samples taken from rats and total antioxidant capacity (TAC) and total oxidant status (TOS) were analyzed. The analyses were performed in commercially available enzyme-linked immunosorbent assay kits and performed according to the kit protocol of the company (Rel Assay Diagnostics, Mega Tıp, Gaziantep, Türkiye) (Ferah Okkay et al., 2023).

Histopathological Analysis
Rats were necropsied and muscle tissue samples were preserved in neutral formalin solution at 10% concentration. The tissues were placed in paraffin blocks and subjected to standard alcohol–xylol preservation procedures (Toraman et al., 2023).

Immunohistochemical Analysis
To inactivate endogenous peroxidase, 5 µm sections from polylysine slides were subjected to a series of xylol and alcohol treatments before being washed with PBS and placed in 3% H_2O_2 for 10 minutes. The antigen retrieval solution was applied twice for 5 minutes each at 500 watts to release the antigen in the tissue.

Then muscle tissues were incubated with inducible nitric oxide synthase (iNOS) and TNF-α primary antibodies at appropriate dilution ratios determined at room temperature (Okkay et al., 2021).

Statistical Analysis
Data were analyzed with the GraphPad 9.5 program. Enzyme-linked immunosorbent assay results were analyzed using the one way analysis of variance test, one of the parametric tests, and multiple comparisons were made according to the post hoc Tukey test.

Results

Biochemical Parameters
Serum biochemistry was examined in terms of oxidative parameters TAS and TOS analyses. In the TOS test used to determine the level of oxidation, the both NC and Treatment groups showed a significant difference of $p < .001$ compared to the PC group (Figure 1). In the TAS analysis, in which the antioxidant level was measured, the NC and Treatment groups gave $p = .001$ and $p < 0.001$ differences compared to the PC group, respectively (Figure 2).

Histopathological Findings
Histological findings of the control group were normal. In the PC group, mild mononuclear cell infiltration was observed in the endomysium surrounding the muscle fibers. No histopathologic findings were observed (Table 1 and Figure 3).

Immunohistochemical Findings
Staining for TNF-α did not show a significant immune positivity in NC. Immune positivity was moderate in the treatment group, but PC was severe in the treatment group. Negative control and

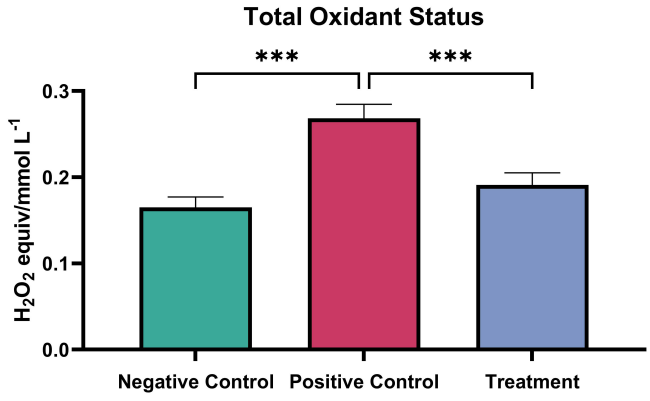

Figure 1.
Total Oxidant Status Analysis Showing Oxidation Level Analysis. Oxidative stress was significantly increased in the PC group.
****p < .001 differences between groups.*

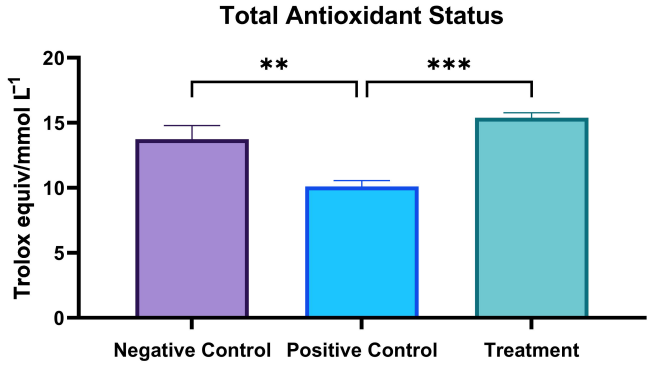

Figure 2.
Total Antioxidant Capacity Analysis Showing Antioxidation Level Analysis. Positive control group's antioxidant status is lower than the other groups.
***p = .001 differences between groups; ***p < .001 differences.*

Table 1.
Histopathologic Investigations Showed Statistically Significant Differences Between the Groups ($p < .05$).

Groups	Myositis
Negative control	0.16 ± 0.40^{a}
Positive control	1.16 ± 0.40^{b}
Therapeutics	0.33 ± 0.51^{a}

Note: ^{a,b}Indicate the difference between groups ($p < .05$).

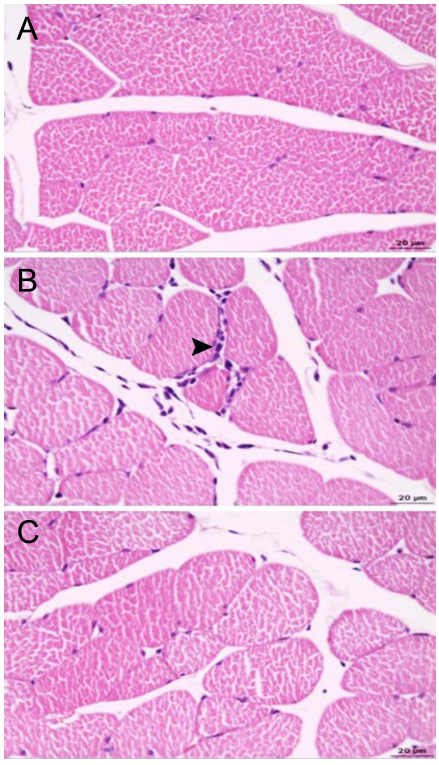

Figure 3.
(A) NC Group, Normal Histologic Appearance. (B) PC Group, Mild Myositis Consisting of Mononuclear Cells in the Endomysium (Arrowhead), (C) Treatment Group, Normal Histologic Appearance. Hematoxylin and eosin.

Table 2.
Immunohistochemical Examinations Revealed Statistically Significant Differences Between the Groups ($p < .05$).

Groups	TNF-α	iNOS
Negative control	0.16 ± 0.40[a]	0.00 ± 0.00[a]
Positive control	2.66 ± 0.51[b]	0.83 ± 0.40[b]
Treatment	1.83 ± 0.40[c]	0.16 ± 0.40[a]

Note: [a,b,c]Indicate the difference between groups ($p < .05$)

treatment groups did not have immune positivity in staining for iNOS, but PC group had mild immune positivity. Tumor necrosis factor alpha and iNOS positivity was found in muscle fibers and capillary endothelial cells (Table 2 and Figure 4).

Discussion

The peripheral nervous system has a very limited regeneration ability. For this reason, severe SNL often do not heal completely, resulting in motor and sensory defects. This condition, which negatively affects the quality of life of many people, continues with complications starting from mild neuropathic pain to complete loss of motor and sensory functions (Lopes et al., 2022). Despite this, the lack of a definitive cure prompts scientists to search for new treatments. The SNL model is the most commonly used model to evaluate motor and sensory nerve functions in peripheral nerve damage, due to the easy accessibility of rats for studies on PNS injuries, their elongation similar to humans, and their length and thickness suitable for surgical intervention (An et al., 2022).

In SNL, a series of pathophysiological events such as inflammation, oxidative stress, and excitotoxicity occur in the injured area. The immune response in SNL is characterized by a rapid flow of immune cells, particularly macrophages, leading to an early pro-inflammatory response followed by an anti-inflammatory response (Ferdowsi et al., 2023). As part of this process, cytokines such as IL-1β and TNF-α, which emerge as part of the inflammatory response, play an effective role in the development and maintenance of pain after peripheral nerve damage or inflammation (Nadeau et al., 2011).

Studies have shown an increase in IL-1β mRNA expression following peripheral nerve injury. The upregulation of pro-inflammatory cytokines such as IL-1β and TNF-α after nerve damage has been reported to play a crucial role as initiators of inflammation and in the development of neuropathic pain (Ren et al., 2011) (Ohtori et al., 2004). These findings indicate that IL-1β and TNF-α not only modulate postneural injury pain but also affect nerve repair and functional recovery (Nadeau et al., 2011).

In addition to inflammation, oxidative stress is also one of the main causes of neural damage in SNL (Zhang et al., 2013). The

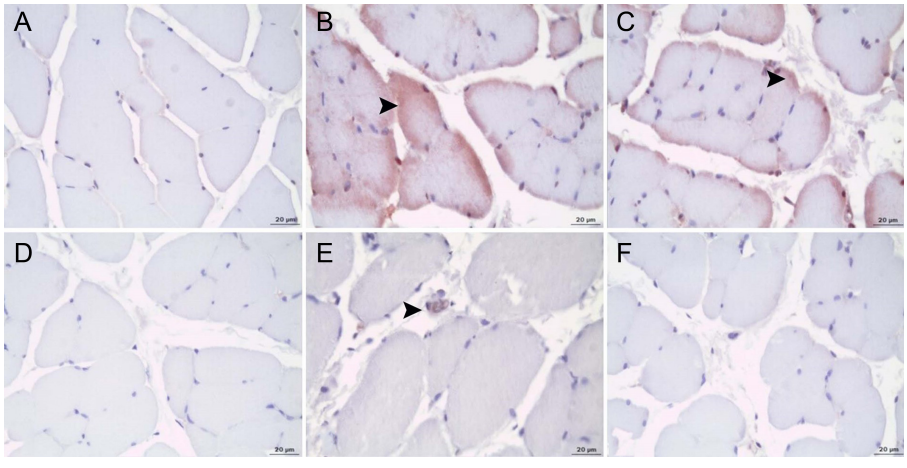

Figure 4.
(A) NC Group. Tumor Necrosis Factor Alpha Immune Negativity. (B) PC Group. Severe TNF-α Immunopositivity in Muscle Fibers (Arrowhead), (C) Treatment Group. Moderate TNF-α Immunopositivity in Muscle Fibers (Arrowhead), (D) NC Group. iNOS Immune Negativity. (E) PC Group. Mild iNOS Immunopositivity in Capillary Endothelial Cells (Arrowhead), (F) Treatment Group. iNOS Immune Negativity, IHC.
IHC = immunohistochemistry; iNOS = inducible nitric oxide synthase; NC = negative control; PC = postive control; TNF-α = tumor necrosis factor alpha.

level of oxidative stress is proportional to the different types of SNL (Wang et al., 2015). A recent study has shown that inhibition of oxidative stress after peripheral nerve injury can enhance functional recovery by directing the pathway toward the repair process and assisting in accelerated repair (Qian et al., 2018). Therefore, modulation of the inflammatory response and oxidative stress for enhancing peripheral nerve regeneration has been considered an effective therapeutic strategy.

Bacopa monnieri L. extract, due to its bioactive compounds such as saponins, alkaloids, betulinic acid, flavonoids, stigmasterol, and beta-sitosterol, is a commonly used plant in traditional medicine (Emsen et al., 2019). In silico and in vitro studies conducted with eight of the 52 active compounds in BME have shown its association with genes such as COX2 (Siklooksijenaz), iNOS, LOX (Lipoksijenaz), STAT3 (Signal transducer and activator of transcription), CCR1 (chemokine receptor type), and MMP9 (matrix metallopeptidase), which are induced by pro-inflammatory cytokines associated with pain and inflammation such as TNF-α, IL-1β, and interferon gamma (Jeyasri et al., 2022). Besides its anti-inflammatory and antioxidant effects, BME has been reported to have analgesic, antinociceptive, antipyretic, neuroprotective, pro-cognitive, neuropsychiatric, anticancer, and anticonvulsant effects (Sanyal et al., 2022).

In an in vitro study, Nemetchek et al found that BME exhibited anti-inflammatory effects by reducing the levels of IL-6, IL-1β, and TNF-α (Nemetchek et al., 2017). It has also been reported to have antioxidant properties by neutralizing free radicals, suppressing lipid peroxidation, and activating antioxidant enzymes (Jauhari et al., 2019); (P B & Padma, 2017). Another study found a decrease in SOD (superoxide dismutase), CAT (catalaz), and GSH (glutathione) levels in rats treated with BME (Vigneshwar et al., 2021).

In our study, it was determined that the level of the oxidation marker TOS was lower in the negative control group and treatment group compared to the positive control group. Consistently, the level of TAC was found to be higher in the negative control group and treatment group. This condition is thought to be associated with the bioactive compounds of the plant. A study supporting this found that bacopaside II and bacoside A3 in BME reduced the levels of intracellular ROS and exhibited antioxidant effects (Bhardwaj et al., 2018).

When reviewing the studies in the literature, in rats with modeled SNL, it has been reported that 21 days of oral treatment with BME resulted in a significant antinociceptive effect by attenuating allodynia and hyperalgesia (Shahid et al., 2017). This effect may be associated with the ability of BME to reduce the levels of cytokines such as TNF-α and IL-6. In another study, Kishore et al. found that in rats with streptozotocin-induced diabetic neuropathy, treatment with BME (100, 200, and 400 mg/kg) reduced the pain threshold, and the levels of TNF-α, transforming growth factor beta, and IL-1β were significantly lower in the treatment group compared to diabetic rats, with the dose of 400 mg/kg being closest to the cytokine levels in the control group (Kishore et al., 2017). In our immunohistochemical examinations, we did not detect immunopositivity in the negative control and treatment groups, while we observed mild immunopositivity in the positive control group. This indicates successful induction of inflammation resulting from SNL. Furthermore, the TNF-α and iNOS values examined immunohistochemically showed a significant increase in the positive control group compared to the negative control group, while the treatment group showed a significant decrease.

In another study, Starinets et al applied synaptamide (4 mg/kg/day) for the treatment of damage induced on the sciatic nerve and reported a significant decrease in IL-1β levels in the treatment groups. They also noted an increase in remyelination based on immunohistochemical staining (Starinets et al., 2023). In our histopathological study on myositis, significant differences were observed. In the positive control groups, inflammatory cell infiltrations consisting of mild mononuclear cells were observed in the endomysium surrounding the muscle fibers, while no histopathological findings were detected in the treatment group, indicating the effectiveness of BME treatment on myositis.

Our study determined that BME reduced SNL damage modeled in rats. Although there is ample evidence for the therapeutic effect of BME's bioactive compounds, further studies are needed for its clinical application.

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