Investigation of the neuroprotective effect of kefir in experimental spinal cord injury

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ABSTRACT

In this study, the antioxidant, anti-inflammatory, and neuroprotective effects of kefir were investigated in spinal cord injury that was experimentally created on rats with a compression trauma model. A total of 56 Wistar-Albino male rats were used in the study. Daily freshly prepared 18 ml/kg/day of kefir was given by oral gavage to animals 7 days before and during the trauma and during the trauma. Spinal cord injury was created according to the weight drop method. On the 1st and 7th days before euthanasia, intracardiac blood was collected for analysis, and then they were sacrificed. The damaged spinal cord segments were examined biochemically, immunohistochemically, and histopathologically. When compared to the sham groups, kefir had a positive effect in the preconditioning and treatment groups by decreasing spinal cord bleeding, edema, myelin sheath damage, liquefactive necrosis, neuronal necrosis, selectivity of canalis centralis, and gitter cell levels significantly. When compared to the sham groups, kefir was found to have a positive effect in the treatment groups by decreasing the neuron specific enolase (NSE), ionized calcium binding adapter molecule 1 (IBA-1), inducible nitric oxide synthase (INOS), cyclooxygenase 2 (COX-2) and myelin basic protein (MBP) levels significantly on the 1st and 7th days, and by increasing the glial fibrillary acidic protein (GFAP) level significantly. As a result, it was demonstrated that kefir had a protective and therapeutic effect on spinal cord injury.

Introduction

Acute spinal injuries have increased recently with the rapid growth of the industry, transportation, and construction industries (23). Spinal cord injury has two mechanisms, which are primary and secondary. Primary injury refers to mechanical damage, and secondary injury refers to the progressive cell damage that occurs after the trauma (25, 35). Spinal cord injuries (SCI) could lead to a complete and permanent loss of neurological function (38, 39). Despite the advancement of current drug and surgical techniques, there is no surgical technique or therapeutic agent that would provide a complete recovery in cases of spinal cord injury (16, 18). Therefore, researchers are still looking for new medical treatments for the treatment of spinal injuries (23). Guven et al. (17) emphasized that ultrastructural studies are needed to develop kefir as a promising therapeutic agent to be used in spinal cord injury. Kefir has a polysaccharide structure with a whitish yellowish color. It owes its strong antioxidant characteristic to the high amount of lactic acid bacteria it involves (5). In addition, kefir is reported to have anti-inflammatory (4, 30), antibacterial (29), antitumoral (26), immunological (11), cholesterol-lowering (27, 36), and antiapoptotic effects (10, 27, 29).

Brain-Derived Neurotrophic Factor (BDNF) is considered an important protein that affects brain function as well as the peripheral nervous system. In addition to preventing cell deaths, it demonstrates a neuroprotective effect under adverse conditions such as cerebral ischemia, hypoglycemia, neurotoxicity, and glutamatergic

stimulation by supporting neuronal differentiation, maturation, and survival in the nervous system (3, 21).

Spinal cord (medulla spinalis) damage causes important social and economic problems and there is no clear solution regarding its definitive treatment. There are drugs used today, but new searches continue because their efficacy cannot be fully demonstrated and they have serious side effects. With many studies, it is aimed that the individual, who has lost his active life as a result of the damage and lost his work force, can return to social life again. This study, which was planned based on previous experimental studies on the usability of this neuroprotective effect for treatment in cases with neural tissue damage such as trauma and ischemia, was planned to investigate the antioxidant, anti-inflammatory, and neuroprotective effects of kefir in spinal cord damage induced by an experimental trauma model in rats. The present study is a prerequisite study, and both the protective efficacy of kefir on the damaged spinal cord tissue and the therapeutic efficacy after trauma were evaluated.

Materials and Method

In this study, 56 healthy male Wistar-Albino rats that were 10-12 weeks old and weighed 300-400g were used. The animals were placed in individual cages and divided into seven equal groups with eight animals in each group. Experimental applications were carried out in accordance with the conditions for the care and use of laboratory animals (12 hours of light; 12 hours of darkness, and 24±3°C, in individual cages). During the experimental applications, rats were fed on commercial rat food (pellet food) including 22.5% HP, 2750 Kcal/kg, and tap water ad libitum.

The animals with spinal cord injury were given 18 ml/kg/day (13) oral gavage of freshly prepared kefir. Kefir grains were washed with distilled water and inoculated in UHT (Ultra High Temperature) whole milk. After each preparation of the beverage, the grains were filtered by sieving the fermented milk and washed again for later use. When the grains were not used, they were maintained in milk at 4°C. Kefir was prepared by adding 5% kefir grains sterile milk and fermenting at 25°C for 24 hours. The number of yeast cells was found to be $1.65x10^7$ (log10 cfu/g).

Animals: Group I; (Control group) The control group received no treatment and was used as a reference. Group II; (Sham-A group) trauma was created only and they were sacrificed on the 1st day. Group III; (Sham-B group) trauma was created only and they were sacrificed on the 7th day. Group IV; (Preconditioning A) were given 18ml/kg/day PO kefir for 7 days before the trauma, and they were sacrificed one day after the trauma was created. Group V; (Preconditioning B) It was the preconditioning group that was given 18ml/kg/day PO kefir for 7 days before the trauma, continued to be fed on 18ml/kg/day PO kefir for 7 days after the trauma, and were sacrificed on the 7th day. Group VI; (Treatment A) They were given 18ml/kg/day PO kefir after the trauma and were sacrificed on the 1st day. Group VII; (Treatment B) They were given 18ml/kg/day PO kefir for 7 days after the trauma and were sacrificed on the 7th day. The animals were determined to be healthy during the clinical examinations performed before the trauma. Their neurological examinations were evaluated through the Modified Tarlov Scale and finger opening tests. After the trauma, neurological examinations of the related groups were repeated on the 1st, 3rd, and 7th days.

Creation of spinal cord damage: In all surgical groups, 10 mg/kg Xylazine hydrochloride intraperitoneal (ip) (Alfazyne 2% injection 50 mL, EGE-VET, Türkiye) and 50 mg/kg ketamine hydrochloride (ip) (Alfamine 10% injection 50 mL EGE-VET, Türkiye) were given for general anesthesia. After the general anesthesia rats were identified in the sternal position, the back part was shaved and antisepsis was provided with povidone-iodine. Paravertebral muscles were reached after crossing the skin and subcutaneous tissues with a two-cm incision at the T5-T12 level with reference to the interscapular distance. The paravertebral muscles were dissected and the vertebral laminae were reached through the spinous process. The spinal cord was exposed at the T7-T10 level by total laminectomy preserving dura mater integrity.

Spinal cord injury was created using the weight drop method by dropping a 10g metal bar with a 3mm diameter from a height of 10 cm (100 g/cm). Rats were made paraplegic. Following hemostasis, paravertebral muscles and skin were sutured in accordance with their anatomical layers. While animals were under general anesthesia on the 1st and 7th days before euthanasia, intracardiac blood was collected for analysis, and then they were sacrificed. After sacrification, sections were taken from the proximal and distal of the trauma for tissue analysis. The samples were examined biochemically, immunohistochemically, and histopathologically.

Neurological examination: In the study, neurological examinations were performed before the sacrification in groups 2, 4, and 6, which underwent a surgical procedure and were sacrificed on the first day; and they were performed on the 1st, 3rd, and 7th days in groups 1, 3, 5 and 7, which underwent a control and surgical procedure and were sacrificed on the seventh day.

Modified Tarlov Scale was used for clinical motor examination and classified as follows; 0:complete

paralysis in the back extremities, no movements in the back extremities, no weight bearing; 1:noticeable back limb movements, no weight bearing; 2: frequent and / or strong back limb movements, pronounced posterior limb movements that do not result in weight overlay or locomotion; 3:back extremities support body weight, can take one or two steps; 4: there is a slight loss in walking; 5: normal walking (12).

Finger opening test used for a neurological examination. The rat was lifted from its back and its back extremities were suspended. Opening of the fingers was observed and the reflexes were classified as follows: 0: fingers not opened; 1: fingers slightly opened; 2: fingers fully opened (12).

Biochemical analyses: Plasma was extracted from the serous fluid and stored in the freezer (-24 °C). In the samples, BDNF (Brain Derived Neurotrophic Factor) levels were calculated by ELISA using commercial kits.

Histopathological and immunohistochemical assessments: Macroscopic changes that could occur in the spinal cord after the experimental trauma were noted and photographed. For histopathological examinations, the spinal cord extracted was placed in 10% buffered formalin stained with Hematoxylin Eosin (HE). For immunohistochemical examination Mouse specific HRP (ABC) (Abcam, ab128971) kit was used and the recommended procedure was applied in the kit. Endogenous peroxidase activity was eliminated by maintaining 3% H_2O_2 methanol for 30 minutes. Depending on the type of antibodies, either 10 minutes 45°C Proteinase K (Abcam, ab64220) (NSE, GFAP, IBA-1 antibodies) or temperature was applied as antigen retrieval (pH 6.0, % 0,1 Tween) (3x5 minutes) (INOS, MBP, COX-2 antibodies). Sections were incubated with the blocking serum of the kit at 37˚C for 10 minutes to prevent non-specific antigenic binding. Anti-Iba-1 antibody (Abcam, ab108539, 1/100, 15 minutes room temperature), Anti-iNOS antibody (Abcam, ab3523, 1/100, +4°C overnigth), Anti COX-2, C-Terminal antibody (Sigma, SAB4502491-100UG, 1/200, 2 hours 37˚C), Anti-GFAP antibody (Abcam, ab7260, 1/100, 1 hour 45˚C), Anti-NSE C Terminal antibody (Sigma, SAB4500768-100UG, 1/100, 1 hour 45˚C) and Anti-MBP antibody (Sigma, ab40390, 1/100, 1 hour 45˚C) were used in order to identify the lesions that could occur in the spinal cord. This was followed by treatment steps with biotinized serum (goat serum, at 37˚C for15 min) and streptavidin peroxidase (at 37˚C for 20 min). The AEC (RED) Substrate kit (Zymed Laboratories inc. Cat. No: 00-2007) (NSE, IBA1, INOS, COX-2) and DAP (3,3′-diaminobenzidine tetrahydrochloride, ScyTek Laboratories, Logan, UT) (GFAP, MBP) were used as the chromogene. The samples were counterstained with Harris hematoxylin. All steps were carried out in a moist camera environment, preventing the sections from drying out. Phosphat Buffer Saline (PBS, pH 7.4) was used in the washes. After all the microscopic results obtained were examined under a light microscope, their microphotographs were taken.

Statistical Analysis: Statistical analyses were performed using SPSS (version 17) software. Data were given as mean ± standard error (SE). The differences between the groups were analyzed with the One Way ANOVA Bonferroni Test. General Linear Model, Repeated Measures, multiple comparisons bonferroni test was used to evaluate time-dependent differences in neurological examination findings. Statistical significance was interpreted according to $P<0.05$ level.

Results

Neurological examination findings: MTS and the finger opening test were performed while evaluating the neurological examination (Day 1 was evaluated as acute; days 3 and 7 were evaluated as subacute).

In terms of MTS values, significant differences were found between the control groups and, the sham groups, preconditioning groups and treatment groups on the 1st day (P=0.000, Table 1). In the neurological examination performed on the first day, the MTS values in the preconditioning groups were higher compared to the sham and treatment groups. MTS values on the 3rd and 7th days in the preconditioning (group 5) and treatment (group 7) groups were higher compared to the sham (group 3) value. MTS values in the treatment group were higher compared to the preconditioning groups on the 3rd and 7th days. Looking at the MTS values, it can be argued that kefir provides clinical improvement (Table 1).

In terms of finger opening tests, significant differences were found between the control groups and, the sham, preconditioning and treatment groups on the 1st day (P=0.000). On the first day, finger opening test values in the preconditioning groups were higher compared to the sham and treatment groups. The fact that the finger-opening test values in the preconditioning group were higher compared to the sham and treatment groups on the 1st day showed that preconditioning played a protective role in preventing peracute injury. On the 7th day, finger opening test values in the preconditioning (group 5) and treatment (group 7) groups were found to be higher compared to the sham (group 3) value (Table 1). Looking at the finger opening test values, it can be argued that kefir provides a clinically moderate benefit in both the peracute and subacute periods.

Table 1. Effects of kefir on Modified Tarlov Test and Finger Opening Test in experimental spinal cord injury.

Groups	Modified Tarlov 1. day	Modified Tarlov 3. day	Modified Tarlov 7. day	Finger extension test 1. day	Finger extension test 3. day	Finger extension test 7. day
	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE
Group 1: Control A	5.00±0.00	5.00±0.00	5.00±0.00	2.00±0.00	2.00±0.00	2.00±0.00
Group 2: Sham A	1.66±0.33*			0.16±0.16*		
Group 3: Sham B	1.71±0.47*	1.14±0.55**	1.42±0.57*	0.28±0.18*	0.28±0.28*	0.42±0.29*
Group 4: Preconditioning A	1.85±0.67*			0.57±0.29*		
Group 5: Preconditioning B	2.50±0.42**	1.87±0.54*	2.28±0.60*	0.87±0.22*	0.25±0.16*,β	0.85±0.34*,&
Group 6: Treatment A	1.25±0.55*			0.00±0.00*,≠		
Group 7: Treatment B	2.00±0.68*	2.00±0.75**	3.14±0.55	0.50±0.26*	0.62±0.26*	0.71±0.18*

A: Group sacrificed on day 1, B: Group sacrificed on day 7
*: When compared with the control group, the differences between the groups were found significant (P <0.005-0.000).
**:When Compared with the control group, the differences between the groups were found significant (P <0.05).
≠: Finger extension test, on the first day, the differences between the Peconditioning Group B and treatment A groups were found to be significant (P <0.05).
β: When compared to the first day, the differences in the finger extension test were found significant (P <0.05).
&: When compared to the third day, the differences in the finger extension test were found significant (P <0.05).

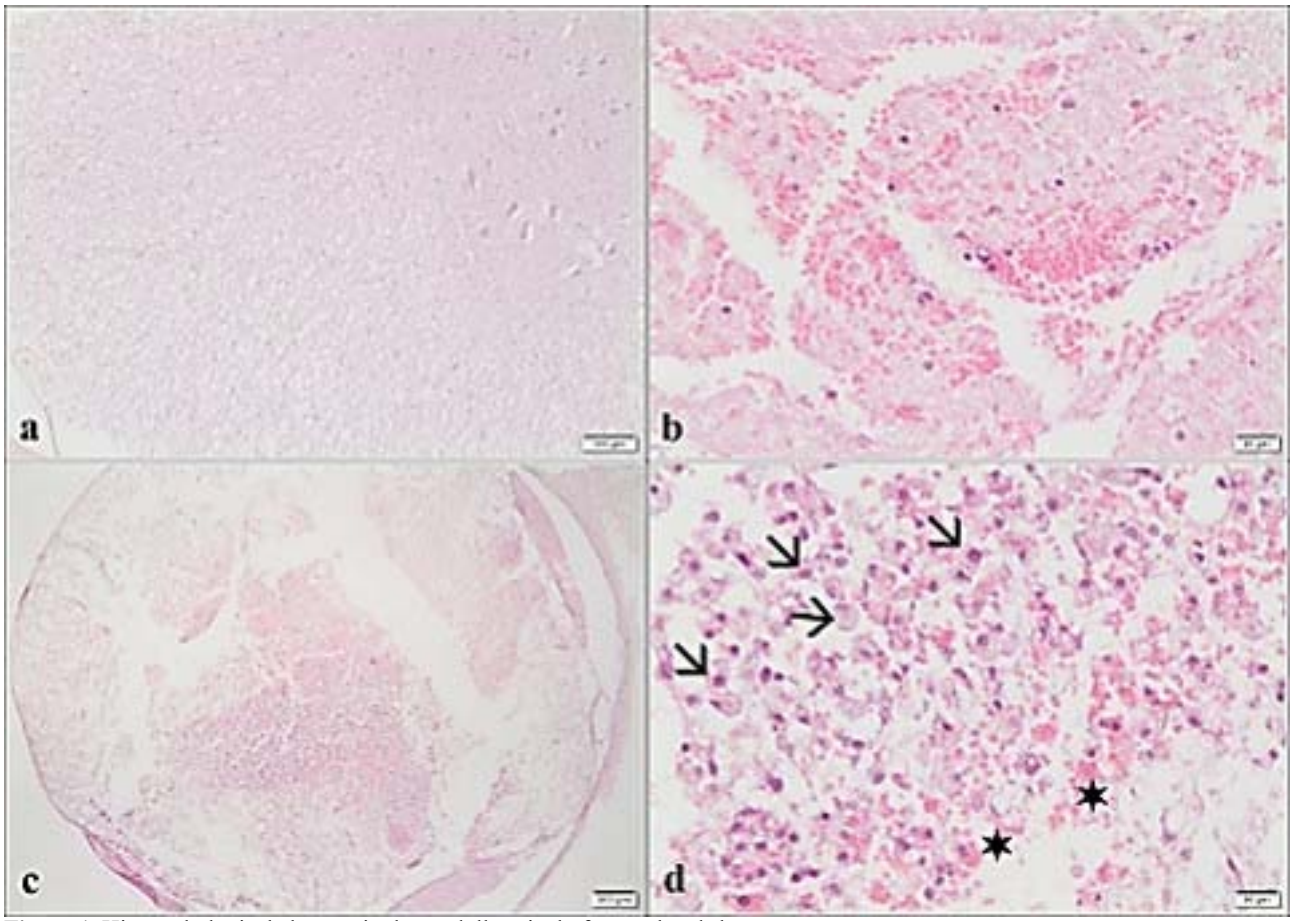


Figure 1. Histopathological changes in the medulla spinal of control and sham groups.
a. Medulla spinalis normal gray matter and white matter structure, Control group, HE, ×100 µm.
b. Hemorrhage and microglial cells in both gray and white matter, Sham A group, HE, ×20 µm.
c. Large necrosis sites in the transverse section, vacuolization in myelin sheaths and damaged canalis centralis, Sham B group, HE, ×200 µm.
d. Intensely gitter cells (arrows) and hemorrhage (stars), Sham B group, HE, ×20 µm.

Histopathological results: The cells in the anterior horn of the spinal cord were examined for damage. For this reason, the cells that have lost their eosinophilic cytoplasm and nucleus were considered dead neurons due to ischemic damage. Cells with cytoplasmic Nissl bodies, thin chromatin, and a prominent nucleolus were considered alive. Histopathological findings observed were classified as none (0), mild (1), moderate (2) and severe (3).

Group I; control group: The spinal cord had normal gray and white matter structure (Figure 1a). No

morphological changes were observed. Group II; (Sham-A): There was prevalent edema, hemorrhage in both gray and white matter, vacuolization in myelin sheaths and neuronal necrosis; and the structure of neurons lined with ependymal cells were observed to be damaged. A small number of microglia cells were noted (Figure 1b). Group III; (Sham-B): They were similar to Group 2, with more severe morphological changes in the damage zone (Figure 1c). In addition, intensive gitter cells were noted in this group (Figure 1d). Group IV (Preconditioning A): There was localized edema, hemorrhage, vacuolization in myelin sheaths and neuronal necrosis; and the structure of neurons lined with ependymal cells were observed to be damaged. Group V (Preconditioning B): There was no hemorrhage, liquefactive necrosis and gliosis; however, there was edema, hemorrhage, damage and vacuolization in myelin sheaths and neuronal necrosis; and the damage in the structure of neurons lined with ependymal cells were observed to decrease (Figure 2a and Figure 2b). Group VI (Treatment A): Moderate edema, hemorrhage, vacuolization in myelin sheaths and neuronal necrosis were also present in this group. It was observed that while the structure of the canal where ependymal cells were lined was preserved in some parts it was damaged in some other parts (Figure 2c). Group VII (Treatment B): The edema, hemorrhage, vacuolization in myelin sheaths and neuronal necrosis were milder compared to Group VI. It was observed that the structure of the canal lined with ependymal cells was preserved in part and deteriorated in another part (Figure 2d).

Histopathological changes shaped in the spinal cord are summarized in Table 2.

When compared to the sham groups, it was observed that kefir had positive effects in the preconditioning and treatment groups by significantly reducing spinal cord bleeding, edema, myelin sheath damage, liquefactive necrosis, neuronal necrosis, selectivity of the canalis centralis and gliosis/gitter cell levels (P=0.000, Table 2). On the other hand, kefir demonstrated a therapeutic effect on the 7th day by reducing liquefactive necrosis and gliosis/gitter cell levels more than the first day in the preconditioning and treatment groups (P=0.000, Table 2).

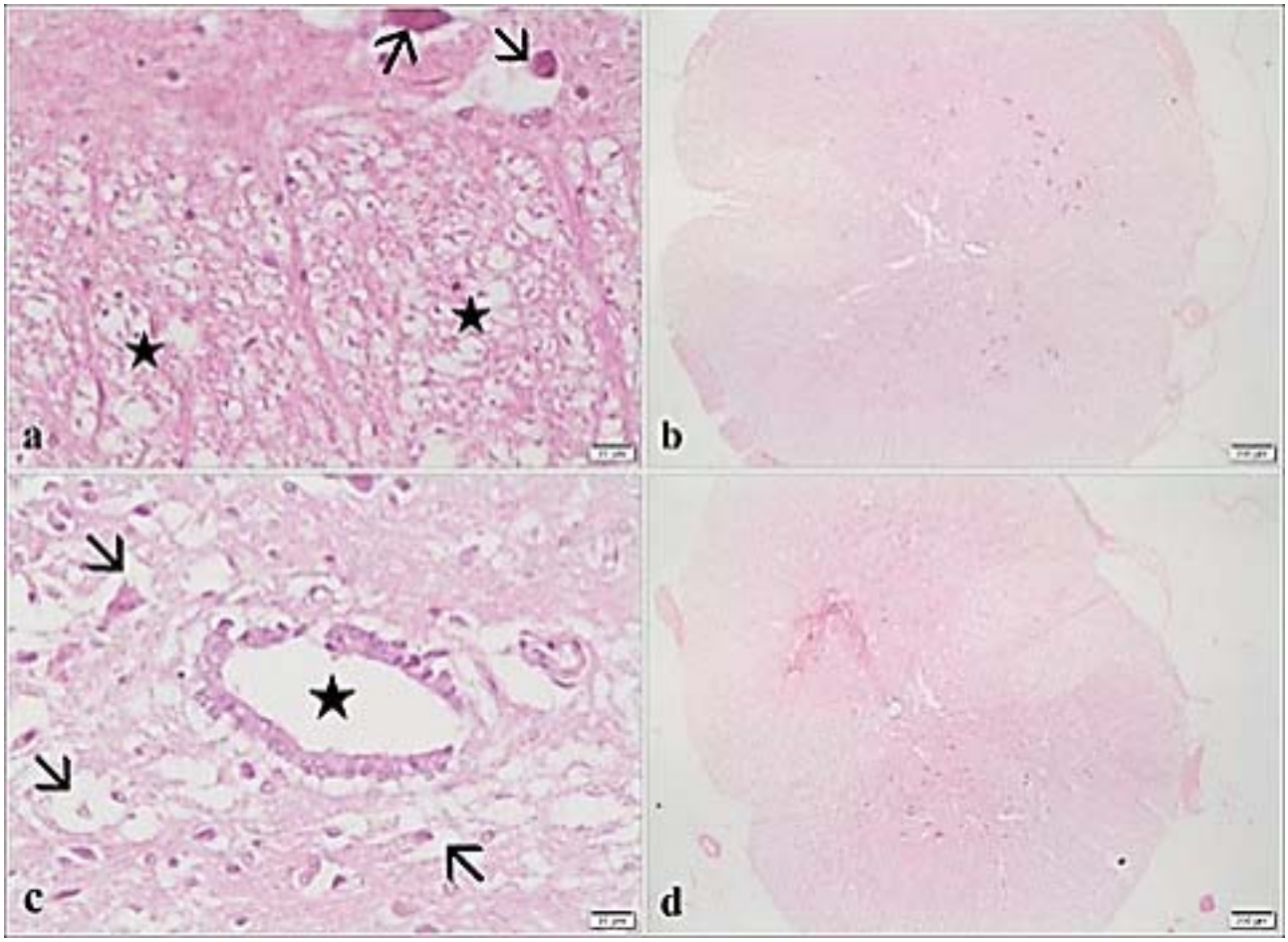


Figure 2. Histopathological changes in the medulla spinal of the preconditioning and treatment groups.
a. Basophilic necrotic neurons (arrows) with loss of nuclei, damage and vacuolization (stars) in myelinated sheaths, Preconditioning B group, HE, ×20 µm.
b. Canalis centralis structure is preserved, Preconditioning B group, HE, ×200 µm.
c. Pericellular edema (arrows) and condition of the canalis centralis (star), Treatment A group, HE, ×20 µm.
d. The appearance of the spinal cord, Treatment B group, HE. ×200 µm.

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Table 2. Histopathological changes in the spinal cord.

Groups	Haemorrhage	Edema	Myelin Sheath Damage	Liquefaction Necrosis	Necrosis in Neurons	Canalis Sentralis Not Selected	Gliosis-Gitter Cells
	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE
1:Control	0.00±0.00	0.00±0.00	0.12±0.12	0.00±0.00	0.00±0.00	0.12±0.12	0.00±0.00
2:Sham A	2.00±0.18	1.75±0.16	1.87±0.22	2.00±0.18	1.62±0.18	2.25±0.16	0.62±0.18
3:Sham B	2.25±0.25	2.25±0.16	2.75±0.16	2.62±0.26	2.87±0.12	2.87±0.12	2.50±0.37
4:Preconditioning A	1.75±0.16	1.75±0.25	1.50±0.26 $^{\beta}$	1.75±0.31$^{\neq}$	0.87±0.22$^{*,\beta}$	2.25±0.16	0.25±0.16
5:Preconditioning B	0.25±0.16$^{€,\beta}$	1.00±0.00$^{*,\beta}$	1.00±0.00$^{*,\beta}$	0.37±0.18$^{€,\beta}$	1.00±0.00$^{\beta}$	1.00±0.00$^{€,\beta}$	0.00±0.00$^{\beta}$
6:Treatment A	1.50±0.26	2.00±0.18	2.25±0.16	1.87±0.12	1.75±0.16$^{\beta}$	1.12±0.35$^{€,\beta}$	0.25±0.16$^{\beta}$
7:Treatment B	1.12±0.22$^{\beta}$	1.00±0.00$^{*,\beta}$	1.00±0.00$^{*,\beta}$	0.00±0.00$^{€,\beta}$	0.75±0.16$^{€,\beta}$	0.50±0.26$^{€,\beta}$	0.12±0.12$^{\beta}$

A: First day, B: Seventh day.
Compared with the Sham A group: * : $P<0.05$; € : $P<0.005$.
Compared with the Sham B group: ≠ : $P<0.05$, β : $P<0.005$.
Compared with the Preconditioning A group: α : $P<0.05$, ¥ : $P<0.005$.
Compared with the Preconditioning B group: $: $P<0.05$, & : $P<0.005$.

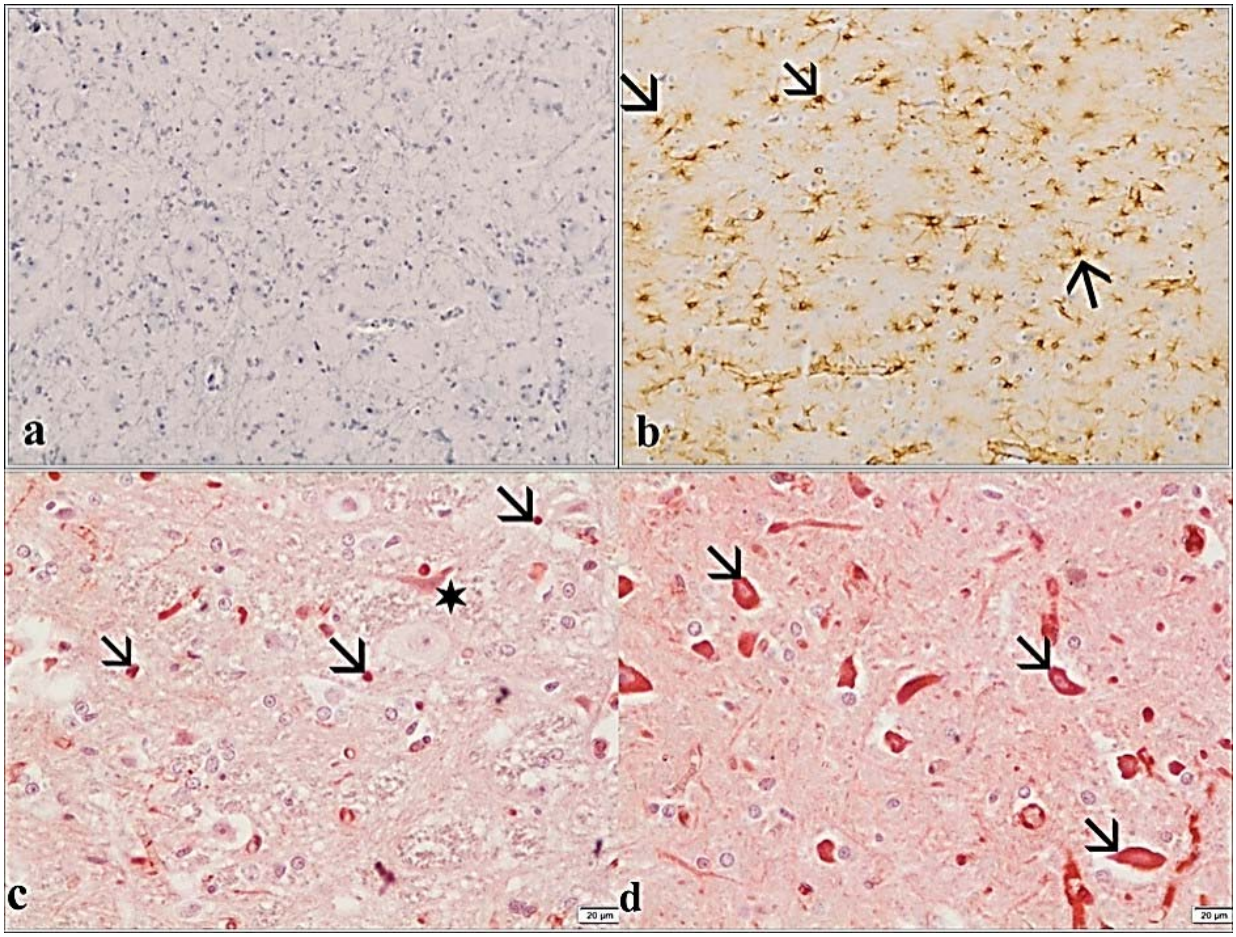


Figure 3. Immunohistochemical findings for GFAP, COX-2 and NSE antibodies.
a. Medulla spinalis GFAP negative, Control group DAB, ABC, ×100 µm.
b. Medulla spinalis GFAP positive astrocytes (arrows), Preconditioning B group, DAB, ABC, ×20 µm.
c. COX-2 positive microglia (arrows) and mild positive neuron cytoplasm in medulla spinalis, Sham A group, AEC, ABC, ×20 µm.
d. NSE positive neurons (arrows) in medulla spinalis, Sham A group, AEC, ABC, ×20 µm.

Immunohistochemical results: While immunoreactivity was not observed against GFAP, NSE, INOS and IBA-1 antibodies in the control group (Figure 3a); in the uninjured central nervous system, COX-2 positive staining in neurons and MBP positive staining in cords in myelin sheaths were observed.

GFAP positive fibrous astrocytes attracted attention most intensely in the 5th and 7th groups, as a result of the increase in the severity of the lesion and the repair in the astrocytes in parallel with the increase in the experimental period in the groups where experimental spinal cord injury was created (Figure 3b). Weaker immunoreactivity was

observed in Groups 3, 4, and 6, respectively, whereas immunosuppressive staining was not observed in Group 2.

The strongest immunoreactivity against the antibody used to demonstrate the COX-2 protein levels increasing after the spinal cord injury in astrocytes and microglia was observed in Groups 3, 2, 6, 4, 7 and 5, respectively. In addition, immunoreactivity was noted in non-damaged neuron cytoplasms (Figure 3c).

The strongest staining against the NSE antibody used to determine neuronal damage was observed in groups 3, 2, 6 and 4, respectively (Figure 3d). While there was moderate immune reactivity related to neuronal damage in group 7 compared to other groups and group 5, there was a significant decrease in the number of immune positive cells in group 5.

Immunohistochemical staining was performed for the presence of myelin basic protein (MBP) to determine the destruction of myelin sheaths. The strongest staining was observed particularly in the areas with severe damage. In the myelin sheaths with granular and irregular appearance among the healthy myelin sheaths in the form of cords, the strongest immunoreactivity was observed in the animals that belonged to the 3rd and 2nd groups, respectively (Figure 4a). Immunoreactivity in the form of wires and organized myelin sheaths as a result of more minimal myelin fragmentation due to reduced damage was also noticed in groups 5, 7, 4 and 6, respectively (Figure 4b).

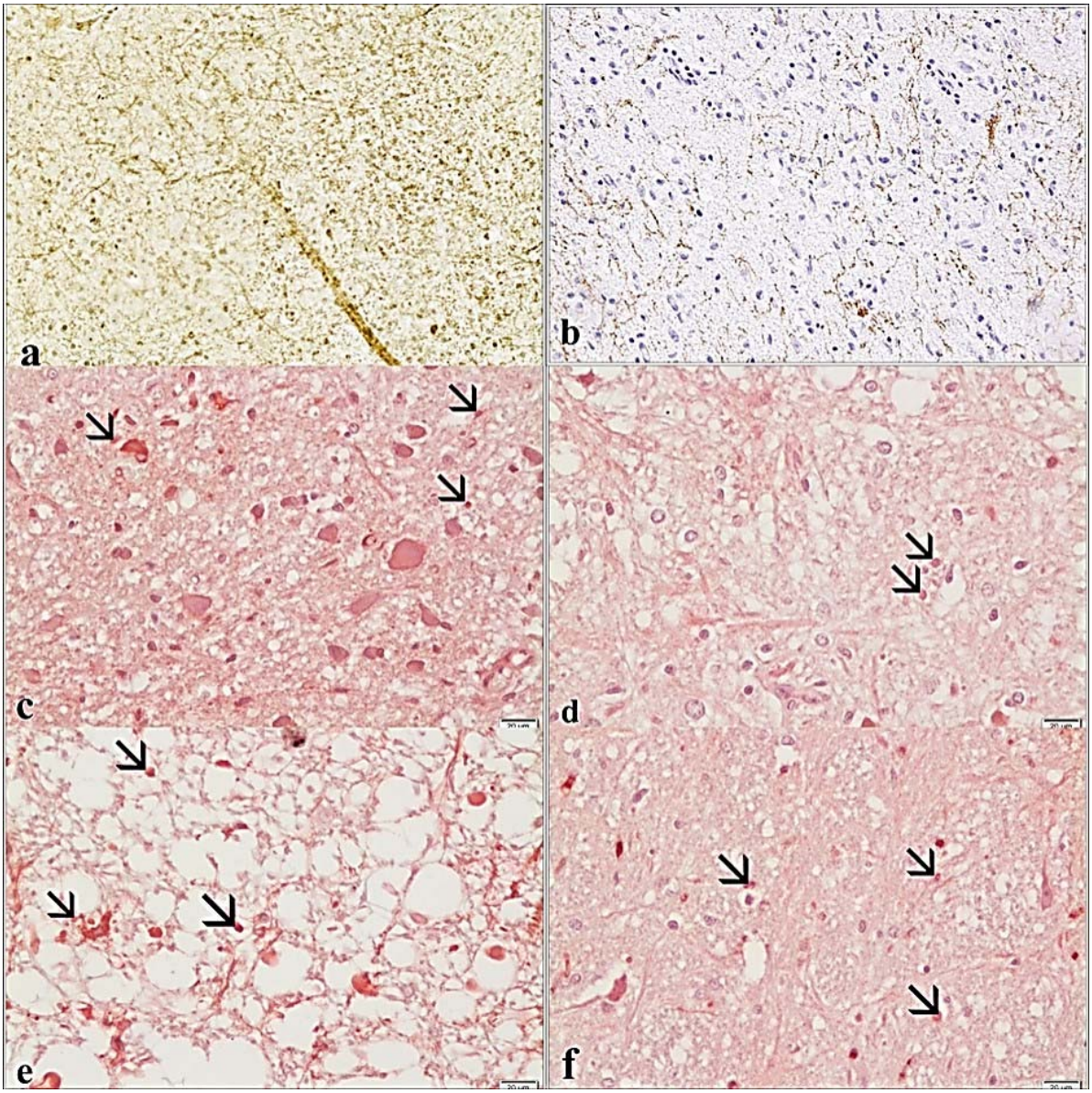


Figure 4. Immunohistochemical findings regarding MBP, IBA-1 and INOS antibodies.
a. Medulla spinalis MBP positive myelin sheaths, Sham B group, DAB, ABC, ×100 µm.
b. MBP weak positive myelin sheaths in medulla spinalis, Preconditioning A group, DAB, ABC, ×20 µm.
c. IBA-1 positive microglia (arrows) in medulla spinalis, Sham A group, AEC, ABC.
d. IBA-1 weak positive microglia (arrows) in medulla spinalis, Treatment A group, AEC, ABC, ×20 µm.
e. Medulla spinalis INOS moderate positive microglia (arrows), Sham B group, AEC, ABC, ×20 µm.
f. Medulla spinalis INOS weak positive microglia (arrows), Treatment B group, AEC, ABC, ×20 µm.

Table 3. Immunohistochemical changes in the spinal cord.

Groups	GFAP	NSE	IBA	iNOS	COX-2	MBP organized	MBP unorganized
	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE
1: Control	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	$3.00\pm0.00^{\alpha}$	3.00±0.00	0.00±0.00
2:Sham A	0.00±0.00	2.75±0.16	1.25±0.16	1.00±0.18	2.00±0.00	2.00±0.18	2.12±0.12
3:Sham B	0.62±0.18	3.00±0.00	1.75±0.16	$1.87\pm0.12^{*}$	$2.75\pm0.16^{€}$	$1.12\pm0.12^{€}$	1.87±0.12
4:Preconditioning A	$0.62\pm0.18^{*}$	$1.62\pm0.18^{€,\beta}$	$0.87\pm0.22^{\neq}$	$0.75\pm0.16^{\beta}$	1.62 ± 0.18^{B}	$0.75\pm0.16^{€}$	$1.12\pm0.12^{€,\beta}$
5: Preconditioning B	$2.00\pm0.00^{€,\beta}$	$0.50\pm0.18^{€,\beta}$	$0.37\pm0.18^{*,\beta}$	$0.37\pm0.18^{\beta}$	$0.87\pm0.12^{€,B}$	$2.00\pm0.00^{\beta}$	$0.50\pm0.18^{€,\beta}$
6:Treatment A	0.50±0.18	$2.00\pm0.00^{€,\beta}$	$0.62\pm0.26^{\beta}$	$0.62\pm0.26^{\beta}$	1.75 ± 0.16^{B}	$0.62\pm0.18^{€}$	$1.00\pm0.00^{€,\beta}$
7:Treatment B	$2.00\pm0.00^{€,\beta}$	$1.00\pm0.00^{€,\beta}$	$0.50\pm0.18^{\beta}$	$0.50\pm0.18^{\beta}$	$1.00\pm0.00^{€,B}$	$0.87\pm0.12^{€}$	$0.87\pm0.12^{€,\beta}$

A: First day, B: Seventh day, α : Immunoreactivity in neurons
Compared with the Sham A group: * : $P<0.05$; € : $P<0.005$
Compared with the Sham B group: ≠ : $P<0.05$, β : $P<0.005$.

Table 4. Effects of Kefir on Serum BDNF (ng / ml) in Experimental Spinal Cord Injury.

Groups	Group 1: Control	Group 2: Sham A (1.day)	Group 3: Sham B (7.day)	Group 4: Preconditioning A (1.day)	Group 5: Preconditioning B (7.day)	Group 6: Treatment A (1.day)	Group 7: Treatment B (7.day)
Mean	63.94	12.06	31.14	30.96	33.41	26.09	36.58
SE	±19.93	± 5.51	±14.32	±11.10	±16.57	±10.77	±13.84

IBA-1 and INOS positive cells were observed in the microglia cells around the vacuolizations in the damage area in groups 3, 2, 4, 6, 7 and 5, respectively, ranging from minimal to mild according to the severity of immunoreactivity (Figures 4c, 4d, 4e, 4f). INOS immunoreactivity was observed in some blood vessel endothelial cells among these microglial cells (Groups 3, 2, 4.6, respectively).

Immunohistochemical changes in the spinal cord were graded as none (0), mild (1), moderate (2) and severe (3).

When kefir was compared with the sham groups, it was found that the preconditioning groups showed a positive effect by significantly decreasing the NSE, IBA, INOS, COX-2, and unorganized-MBP levels and increasing the GFAP level on the 1st and 7th days and increasing spinal cord (P=0.000).

Compared to sham groups, it was found that kefir demonstrated a positive effect in treatment groups by decreasing the levels of NSE, IBA-1, INOS, COX-2 and MBP significantly on the 1st and 7th day and increasing the GFAP level (P=0.000, Table 3). On the other hand, kefir had a therapeutic effect by decreasing the levels of NSE, IBA-1, INOS, COX-2, and MBP more than day 1 on the 7th day in the preconditioning and treatment groups (P=0.005, Table 3).

Serum BDNF findings: When compared to the control group, Serum BDNF levels decreased significantly in the Sham A-B, Preconditioning A-B and Treatment A-B groups. When compared to Sham and B groups, kefir increased BDNF levels in preconditioning and treatment groups on the 1st and 7th days. Compared to the Sham A group, kefir increased BDNF levels on the 1st day more than the 7th day in the preconditioning and treatment groups (P=0.292, Table 4).

Discussion and Conclusion

Pathological injury mechanisms after SCI are mostly focused on primary and secondary injuries. Primary trauma of the spinal cord leads to irreversible primary injury (33, 37). In contrast, primary injury cascade reactions lead to reversible secondary injury with more serious levels of injury compared to primary injuries (24). Acute spinal cord injuries could result in severe central nervous system damage as well as motor and sensory dysfunction, and it has a high rate of disability (23). Despite the importance of motor dysfunction repair in SCI patients (34), the basic mechanisms have not been demonstrated yet and more basic research is required. In the study, the values in the preconditioning group were found to be higher compared to the sham and treatment groups in the neurological evaluation with the MTS. In terms of the finger opening test, the differences between the Preconditioning B and Treatment A groups were significant in the neurological evaluation on the 1st day (P=0.000). Many studies have reported that antioxidants

could delay the progression of neurodegeneration (1). Zhang et al. (40) applied compression from the T8 level to the spinal cord in rats and demonstrated locomotor healing in the MTS with combined anti-inflammatory therapy. In our study, it can be argued that the MTS values in the preconditioning group were higher than those in the other groups; and kefir contributed to the clinical improvement after trauma. Based on these findings, it can be argued that the preconditioned application of kefir provides clinical benefits. While there was a decrease in the finger opening test values after the trauma, an increase was observed in the groups that were fed kefir. Similar to the findings of our study, Şirin et al. (35) reported that there was a significant decrease in finger opening test values after spinal cord injury.

BDNF mainly promotes the survival and regeneration of neurons, and it has been defined in many brain regions including the bulbus olfactorius, cortex, hippocampus, basal forebrain, mesencephalon, hypothalamus, brainstem, and spinal cord (2). After SCI, the requirement for BDNF is increased (8, 15). Therefore, we believe that BDNF could be useful in identifying spinal cord injury and in monitoring the post-treatment process and prognosis. When compared to the control group, Serum BDNF levels decreased significantly in the Sham A-B, Preconditioning A-B, and Treatment A-B groups. When compared to Sham and B groups, kefir increased BDNF levels in preconditioning and treatment groups on the 1st and 7th days. When compared to the Sham A group, kefir demonstrated a therapeutic effect by increasing the BDNF levels in the preconditioning and treatment groups more on the 1st day compared to the 7th day (P=0.292).

Spinal cord injury always initiates an inflammatory response characterized by the infiltration of leukocytes and the synthesis of cytokines and chemokines. This excessive inflammation induced by a spinal cord injury induces degeneration of neurons and apoptosis of oligodendrocytes, causing a progressive injury. Astrocytes are one of the first cell populations to detect spinal cord injury. Astrocytes participate in repairing damaged parts of the brain; the presence of a significant increase in fibrous astrocytes during astrocytosis could be detected by strong GFAP expression in the brain tissue (6, 7). Intense astrocytosis does not occur within a short time following the spinal cord injury, and it is considered a chronic process. Similarly, myelin damage occurs immediately at the time of the injury; however, it does not start to appear morphologically within the few days following the trauma and it becomes visible with the sustained release of the metabolites applied to the damage (28). In the study, the GFAP and MBP expressions differed between the groups particularly in parallel with the increases in trauma and kefir application periods. However, it cannot be argued that kefir application provides full protection both in protecting myelin sheath structure and in sporrting astrocytosis. We attribute this to the short duration of both trauma and the kefir application in order to better observe morphological lesions. In the study, spinal cord damage lesions from kefir application that continued for 7 days before and after formation were observed less frequently compared to other groups, both in neurological examinations and at the histopathological level.

The rise of neuron-specific enolase (NSE), known to play a role in the pathogenesis of hypoxic-ischemic brain injury, has been blamed for neuronal damage following spinal cord injury. For this reason, NSE is believed to be an important marker that directly evaluates functional damage in neurons (19). While a large number of NSE positive neurons were observed in the group that did not receive kefir after the trauma, a significant reduction in immunoreactivity was also noted despite complete protection was not provided with the inclusion of kefir (P=0.000).

Traumatic spinal cord injury directly causes axonal and myelin damage as well as migration of inflammatory cells to the inflamed region (28). Miller et al. (28) reported that focal hemorrhage and necrosis were observed at the 1st hour following the trauma and stated that there was an increase in IBA-1 and INOS expression in macrophages and microglia in the area of damage. Monocytes and microglia begin to multiply around the primary lesion after 48 hours following the spinal cord injury. Neutrophils are no longer present after 3 days. They are replaced by a large number of monocytes and microglia (40). Microglia activation is a common incidence in spinal cord trauma and is claimed to cause tissue damage during the elaboration of proinflammatory agents. INOS is an inducible enzyme found in macrophages and endothelial cells. INOS release is very low in the brain and often cannot be expressed. However, the production of cytokines causes INOS expression in microglia and astrocytes, resulting in continuous and high levels of nitric oxide production, which could lead to further tissue damage, especially with toxic byproducts such as peroxynitrite. Nitric oxide is also toxic to neurons and is responsible for neuronal death (28).

IBA-1, which plays a role in the rearrangement of the actin cytoskeleton,is a cell surface marker directly associated with microglia activation, migration, and phagocytosis (22). Following the spinal cord injury, INOS and IBA-1 positive staining is observed in the microglia cells and macrophages around the vacuolizations and disintegrated myelin sheaths; however, immunoreactivity was observed to decrease due to the anti-inflammatory effect of kefir.

There are two forms of the cyclooxygenase enzyme called COX-1 and COX-2. In experimental acute spinal cord injury, the production of COX-2, mRNA and proteins

is identified to increase between 2 and 48 hours, and it was determined that COX-2 inhibition would contribute to the results of spinal cord injury selectively. In the central nervous system without damage, the presence of COX-2 in the neurons was demonstrated immunohistochemically (31); however, it was reported that the increase in COX-2 related to the damage accelerated neuronal death and the neuroinflammatory response resulting from the production of prostaglandin E2 (PGE_2) (14, 32). It is reported that COX-2, which is normally observed in neurons, is released with the injury from astrocytes and microglia, respectively (9, 20). In this study, severe COX-2 immunoreactivity was observed in groups in which trauma was created but no kefir application was made. Despite the fact that the complete protective effect of kefir, which is reported to have an anti-inflammatory effect (4, 30), was not observed, it was able to provide a reduction in the COX-2 release even with the application for 7 days before and after the experiment.

As a result, histopathological examinations concluded that kefir had a positive effect in preconditioning and treatment groups by decreasing spinal cord bleeding, edema, myelin sheath damage, liquefactive necrosis, neuronal necrosis, the selectivity of canalis centralis and gitter cell levels significantly. Immunohistochemical examinations concluded that kefir had a positive effect in the treatment groups by decreasing the NSE, IBA-1, INOS, COX-2, and MBP levels on the 1st and 7th days and significantly increasing the GFAP level. This was supported by the increase that was observed in serum BDNF levels. In addition, no side effects or negative consequences have been reported for the consumption of kefir, which is a probiotic substance; on the contrary, it is reported to have antioxidant, anti-inflammatory, antiapoptotic, antitumoral, cholesterol-lowering, and neuroprotective effects. In the present study, it can be argued that kefir reduces the negativities formed at the cellular level against traumatic spinal cord damage; however, it cannot be argued that it provides complete protection. We believe that this may be related to the shortness of the period of kefir consumption. Therefore, there is a need for further studies, in which kefir would be applied for a longer time and in greater amounts. It is predicted that the consumption of this beverage, which is easily accessible and easy to prepare, would have protective effects on spinal cord injuries.

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Conflict of Interest

The authors have no conflict of interest to declare.

Author Contributions

ZY, ŞYÖ, TK, BÖ, MEA and HA conceived and planned the experiments. ZY, MEA and HA carried out the experiments. ZY, ŞYÖ, TK and BÖ planned and carried out the simulations. ŞYÖ, TK and BÖ contributed to sample preparation. ZY, ŞYÖ, TK, BÖ, MEA and HA contributed to the interpretation of the results. ZY, ŞYÖ, TK and MEA took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was approved by the Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee (2017/10-2).

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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