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THE EFFECT OF ANTI-INFLAMMATORY, ANTIOXIDATIVE, AND NEUROPROTECTIVE CHARACTERISTICS OF ALOPERINE ON EXPERIMENTAL ACUTE SPINAL CORD INJURY IN A RAT MODEL

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ABSTRACT

Objective: Spinal cord injury (SCI) disrupts nerve axons with devastating neurological consequences. However, there is no effective clinical treatment. The purpose of this study was to investigate the effects of the anti-inflammatory, antioxidative, and neuroprotective characteristics of alverine on traumatic spinal injury in a rat model.

Materials and Methods: A total of 36 Wistar albino rats, each weighing 300-400 g, were divided into four treatment groups. In Group 1 (sham/control, n=9), only laminectomy was performed. In Group 2 (SCI, n=9), SCI was simulated after laminectomy. In Group 3 (SCI + saline, n=9), physiological saline solution was injected after SCI was induced. In Group 4 (SCI + aloperine), aloperine was administered after SCI was induced. SCI was established using the weight drop technique after laminectomy.

Results: Neurological examination scores were significantly better in the aloperine-treated group than in Groups 2 and 3. SCI significantly increased serum and spinal cord tissue glutathione peroxidase, total oxidant status, 8-hydroxiguanosine, and interleukin-6 levels. These levels were successfully reduced with alverine administration. Interleukin-10 and total antioxidant status levels also decreased with alverine administration. Increased histopathological spinal cord damage score and apoptotic index in Groups 2 and 3 were significantly decreased in Group 4.

Conclusion: Aloperine reduced apoptosis and increased anti-inflammatory and antioxidative mediator levels, which protected the SCI rat model against secondary nerve injury.

Keywords: Aloperine, antioxidant, inflammation, reactive oxygen species, spinal cord injury

INTRODUCTION

Spinal cord injury (SCI) accounts for 11.5-53.4 cases per million annually[1]. SCI causes varying degrees of loss of work and psychological effects among the patients and their relatives. Primary damage occurs at the time of injury, and there is no cure other than prevention. However, secondary damage occurs due to the accumulation of free oxygen radicals, cord ischemia, ionic imbalance, and cellular excitotoxicity[2]. Trauma causes neural ischemia, nerve compression, thrombosis, and vasospasm. Thus, it is important to manage this part of the injury by increasing cord perfusion and reducing reactive oxygen species (ROS)[3,4].

Aloperine (ALO) has been used to treat various neurological diseases. For example, it has been used in a cell model of Alzheimer's disease to reduce ROS and the resultant cell apoptosis[5]. In one study, ALO reduced inflammatory infiltration and tubular cell apoptosis, and protected the mice against renal injury[6]. In another study, ALO protected against oxygen-glucose deprivation and cultured rat hippocampal[7]. In an energy-deficient environment, ALO can diffuse across the blood-brain barrier[7]. Therefore, we hypothesized that ALO may protect a patient against cerebrospinal injury. Thus, in this study, we aimed to demonstrate the effects of ALO in terms of anti-inflammation, antioxidation, and anti-apoptosis.

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MATERIALS AND METHODS

Groups

Four study groups were created. In Group 1, only laminectomy was performed. In Group 2, SCI was mimicked after laminectomy. In Group 3, after SCI, a physiological saline solution was administered. In group 4, ALO was administered.

Anesthesia and Surgical Technique

Saline containing 10% acetic acid (2.5 mg/mL) was used to dissolve ALO (Kmaels, Shanghai, China)[6]. ALO (150 mg/kg) was administered intraperitoneally 2 and 24 hours after induction of the SCI model in Group 4[8]. Analyses involved western blotting, immunohistochemistry (IHC), terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining, assessment of neurological deficits, and enzyme activity assays.

Intraperitoneal injections of 10 mg/kg of ketamine (Ketalar; Parke-Davis, Eczacıbaşı, Turkey) and 50 mg/kg of xylazine (Alfazyne; Egevet, İzmir, Turkey) were used to anesthetize all rats. A midline incision was made between T7 and T12, and total laminectomy at T10 and T11 were performed. After the total laminectomy, a 10-g impact weight was dropped from a predetermined height of 25 mm onto the thoracic spinal cord in the SCI group. In Group 3, 150 mg/kg of 10% acetic acid solution was administered intraperitoneally. In Group 4, 150 mg/kg of ALO was intraperitoneally administered.

Two weeks after SCI, all animals were decapitated under deep anesthesia. The spinal cord was removed for histopathological examination and blood samples were drawn for biochemical analyses.

Biochemical Analysis

Blood samples were stored at -40 ˚C. Mechanical and ultrasonic homogenizers were used to thaw the tissue samples. Tissue levels of 8-hydroxiguanosine (8-OHG) (Sinogeneclon Co., Ltd.), glutathione peroxidase (GPx) (Sinogeneclon Co., Ltd., Hangzhou, China), interleukin (IL)-6 and IL-10 were determined from plasma and spinal cord samples using ELISA. The tissue protein levels, total antioxidant status (TAS), and total oxidant status (TOS) (RelAssay Diagnostics, Gaziantep, Turkey) were determined using spectrophotometry (ThermoScientific, Chicago, Illinois, USA).

Histopathological Investigations

The tissue samples were fixed using 10% (v/v) formaldehyde. Histopathological changes were graded between 0 and 3. Hemorrhage, edema, inflammation, and necrosis were scored as follows: 0, absent; 1, mild; 2, moderate; and 3, frequent[9].

TUNEL Assay

The apoptotic cells were labeled using the ApopTag *In Situ* Apoptosis Detection Kit (Millipore, Burlington, Massachusetts, USA). The terminal deoxynucleotidyl transferase modified the DNA fragments. In selective fields, the terminal TUNEL-positive neurons and the total number of neurons were counted[10].

Neurological Examination

The modified Tarlov scoring system and inclined plane test were used to evaluate neurological status[9]. A score of 0-5 was allocated. The rate included the following: no voluntary extremity movement, perceptible joint movement, active movement but an inability to sit without assistance, ability to sit but unable to jump, or a weak jump.

Statistical Analysis

SPSS (version 20.0; IBM Corp., Armonk, New York, USA) was used for all the data analyses. The Kruskal-Wallis test was used for comparisons. The Mann-Whitney U test was used to evaluate differences between the groups. A p-value <0.05 was considered statistically significant. The histopathological semiquantitative scoring system and Tukey's test were used to compare TUNEL-positive cell counts.

Ethics Statement

The study protocol was approved by the Necmettin Erbakan University KONÜDAM Experimental Medicine Application and Research Centering (decision no: 191518001, date: 19/10/2018). The study was conducted in conformance with the ethical and humane principles of research.

RESULTS

Biochemical Evaluation

The GPx, 8-OHG, TOS, and IL-6 levels significantly increased after SCI. The TAS and IL-10 levels significantly decreased after SCI. ALO treatment significantly decreased the plasma and spinal cord tissue levels of GPx, 8-OHG, TOS, and IL-6 and increased the levels of TAS and IL-10 (Tables 1 and 2).

TUNEL Assay

TUNEL-positive cells increased significantly in Group 2 (Figure 1B) and Group 3 (Figure 1C) than in Group 1 (Figure 1A). However, in Group 4 (Figure 1D), ALO significantly decreased the number of TUNEL-positive cells when compared with Group 3 (Figure 2).

Histopathological Evaluation

Hematoxylin-eosin staining revealed that the rats in Groups 2 and 3 had the most severe statistically significant SCI (Figure 3). The damaged area decreased in Group 4 (Figures 3 and 4). Rats in Group 1 had normal spinal cord histology (Figures 5A, 6A). Hemorrhage, necrosis, loss of myelin, axon degeneration, necrosis in the gray matter, and loss of Nissl bodies were observed in Groups 2 and 3 (Figures 5B, 5C, 6B, 6C). The severity of these findings decreased in Group 4 (Figures 5D, 6D).

Table 1. Comparison of serum IL-6, 8-OHG, IL-10, Gpx, TAS, and TOS levels

Pg/mL	Group 1	Group 2	Group 3	Group 4	p-value
IL-6	4.12±0.53	5.61±1.08	5.73±2.09	4.74±1.66	0.023
8-OHG	26.73±6.12	29.84±4.14	30.9±5.98	28.21±6.32	0.001
IL-10	10.38±2.46	8.62±4.52	8.47±3.44	9.40±3.38	0.012
Gpx	3.41±0.36	2.98±0.32	3.01±0.42	3.21±0.65	0.036
TOS	19.23±36.35	31.74±42.65	32.32±51.23	24.10±37.31	0.021
TAS	0.97±0.83	0.83±0.59	0.76±0.61	0.92±0.72	0.001

IL-6: Interleukin-6, 8-OHG: 8-hydroxyguanosine, IL-10: Interleukin-10, Gpx: Glutathione peroxidase, TAS: Total antioxidant status, TOS: Total oxidant status, pg/mL: Picograms per milliliter

Table 2. Comparison of tissue IL-6, 8-OHG, IL-10, Gpx, TAS, and TOS levels

Pg/mL	Group 1	Group 2	Group 3	Group 4	p-value
IL-6	16.32±5.19	18.83±6.59	19.36±7.95	17.32±6.29	0.016
8-OHG	26.57±5.36	28.13±5.21	29.36±6.35	27.81±7.65	0.024
IL-10	19.79±8.13	15.21±6.32	16.84±6.98	17.93±4.92	0.002
Gpx	3.61±0.83	2.86±0.68	2.91±0.36	3.41±0.65	0.001
TOS	22.14±26.63	28.84±39.87	32.19±47.36	26.115±37.31	0.002
TAS	0.31±0.57	0.20±0.25	0.18±0.45	0.28±0.63	0.001

IL-6: Interleukin-6, 8-OHG: 8-hydroxyguanosine, IL-10: Interleukin-10, Gpx: Glutathione peroxidase, TAS: Total antioxidant status, TOS: Total oxidant status, pg/mL: Picograms per milliliter

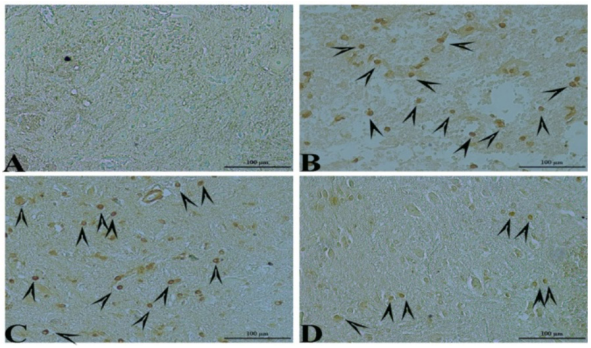

Figure 1. TUNEL-positive cells (black arrowheads) in samples from **(A)** Group 1, **(B)** Group 2, **(C)** Group 3, and **(D)** Group 4
TUNEL: Transferase dUTP nick-end labeling

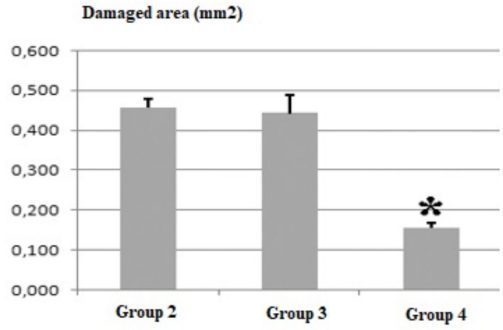

Figure 3. Evaluation of the damaged area measurements
*p<0.05 than in Groups 2 and 3

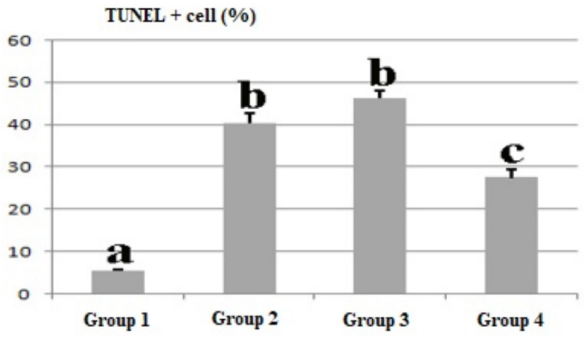

Figure 2. Comparison of the treatment groups with respect to TUNEL-positive cells. Different letters on the columns indicate that the means are significant compared to the others (p<0.05)
TUNEL: Transferase dUTP nick-end labeling

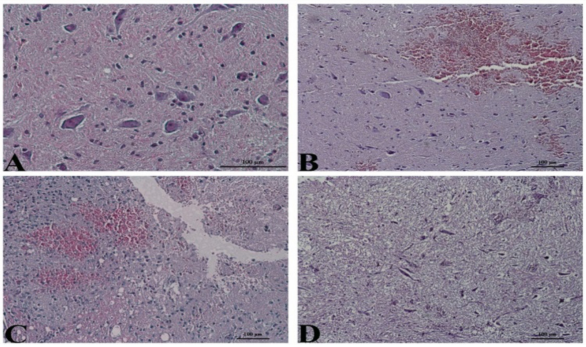

Figure 4. Hematoxylin-eosin stained tissue sections from rats in **(A)** Group 1, **(B)** Group 2, **(C)** Group 3, and **(D)** Group 4

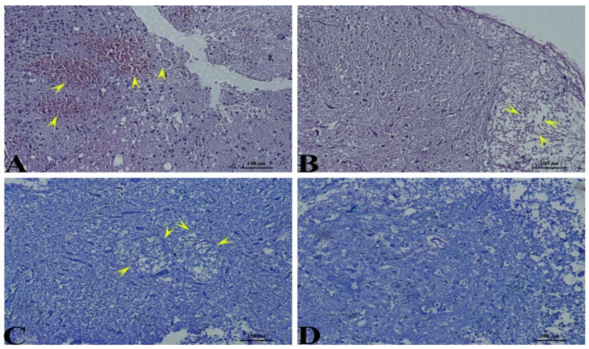

Figure 5. HE-stained sections from rats in Group 3 revealed **(A)** hemorrhage and necrosis (arrowheads) and **(B)** myelin loss and axon degeneration (arrowheads). Toluidine blue-stained sections demonstrate **(C)** vacuolization in the neuropil (arrowheads) and **(D)** neuron loss
HE: Hematoxylin-eosin

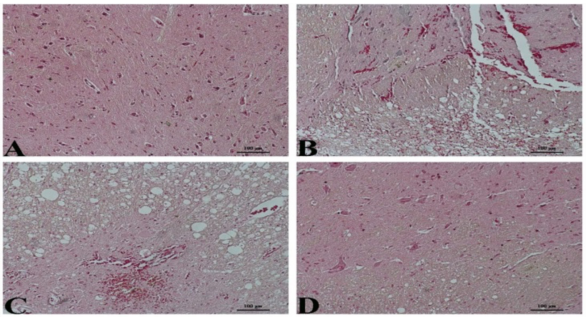

Figure 6. Masson Trichrome-stained tissue sections from rats in **(A)** Group 1, **(B)** Group 2, **(C)** Group 3, and **(D)** Group 4

Neurological Evaluation

Inclined plane score: The measurements obtained on days 1 and 7 in each group were as follows: Group 1, 58.6°±6.7° vs. 57.9°±8.2°; Group 2, 58.3°±5.3° vs. 38.7°±4.7°; Group 3, 59.2°±5.1° vs. 37.2°±6.3°; and Group 4, 58.3°±5.4 vs. 43.8°±6.5°.

On day 1, there was no significant difference in the angle between the groups. In Group 1, there was no significant difference in the angle between the first and seventh days. However, the angle at which the subjects could hold decreased on day 7 in all the other groups. There was no significant difference in the angle between Groups 2 and 3. However, Group 4 could hold at a significantly higher angle than Groups 2 and 3.

Drummond-Moore test: In our study, while the Drummond-Moore test score was 4 in all the rats before the experiment was performed, on day 7, the mean score was 4 points in Group 1, 1.3±0.3 points in Group 2, 1.2±0.2 points in Group 3, and 1.9±0.6 points in Group 4. The score in Group 4 was statistically higher than in Groups 2 and 3.

DISCUSSION

After mechanically inflicting primary damage to the tissue, trauma continues to inflict secondary damage, which includes inflammation, apoptosis, and oxidative stress-induced tissue damage[11]. Minimizing the effects of secondary injury on the central nervous system is important in trauma treatment[9,12]. Studies on spinal cord damage are ongoing.

Human SCI is the most convenient model for determining the mechanism of post-traumatic injury, its pathogenesis, and treatment of cellular and tissue damage[10]. In most human SCIs, the primary injury is at least two-sided and develops because of different movements. A combination of different forces causes an injury in humans[13]. Following a trauma, the point at which medical management needs to be switched to surgical treatment may vary. In animal experiments, the post-traumatic treatment process begins in the first hour[13]. In addition to trauma, vascular damage and ischemic injury may occur at different rates and uncontrollable levels[14]. Because we wanted to produce a standard effect in all the groups and exclude additional factors, we induced the SCI model in rats in our study using the weight drop method.

After a primary injury, secondary damage begins to develop within a few hours and it can continue to occur for weeks. It includes physiological responses to trauma, hypoxia, and ischemia. Local edema, formation of free radicals, and release of excitatory neurotransmitters are associated with increased oxidative stress. Additionally, increased inflammatory processes indirectly stimulate the apoptotic mechanisms[15]. Therefore, steroids, opioid antagonists, calcium channel blockers, volume expanders, Thyrotropin-Releasing Hormone, trilazad mesylate, and especially methylprednisolone are commonly used in the treatment of SCIs[16,17]. Although the primary injury due to a mechanical insult to the spinal cord cannot be treated, the effects of the secondary injuries, such as ischemia, inflammation, increased oxidative stress, the destructive effect of excitatory neurotransmitters, and programmed cell death, should be reduced[18,19]. The changes in the levels of inflammatory cytokines, which are involved in the secondary damage processes and treatment, and oxidative stress and changes at the tissue level are the result of all pathological processes. Cytokines are usually maintained at low levels under physiological conditions[20]. In the microenvironment of the central nervous system, cytokines are activated by glial cells following infection, trauma, or ischemia[21,22]. In a SCI model, systemic inflammation reportedly decreases after systemic IL-10 administration 30 minutes after injury[23].

Although several molecules have been used to assess oxidative stress in tissues and cells, TAS and TOS analyses allow us to obtain reliable, sensitive results easily, immediately, and cost-effectively using long-life reagents[24]. Furthermore, GPx prevents oxidative damage and determines the levels of 8-OHG[25]. Therefore, in our study, we compared the 8-OHG, GPx, TAS, and TOS levels with the oxidative stress levels of IL-10 and IL-6. In our study, we observed myelin loss and axonal degeneration following SCI; these are the most prominent indicators of neuron damage. Furthermore, we also identified neuropil vacuolization in the gray matter, necrotic neurons in

the gray matter, presence of Nissl bodies, and TUNEL-positive cells, which are histological indicators of apoptosis[26]. A histopathological scale (Malinowsky score) was used to assess cell damage under a microscope[27].

Zhou et al.[28] demonstrated the anti-allergic and anti-inflammatory effects of ALO in an animal model. Fan et al.[29] demonstrated that ALO significantly decreased the IL-1 and IL-6 levels and that it has anti-inflammatory effects. Yuan et al.[30] demonstrated that ALO significantly decreased IL-1b, IL6, and TNF-a levels in mice models of allergic contact dermatitis. In our study, the serum IL-6 levels were significantly higher in Groups 2 and 3 than in Group 1. Although the IL-6 level was higher in the mice administered ALO than in the sham group, the ALO administered was lower in group 4 than in the other groups in which SCI was induced. This difference was statistically significant. At the serum as well as the tissue level, the mice in Group 4 had significantly higher IL-6 levels than the mice in the sham group but lower IL-6 than mice in Groups 2 and 3[31].

In our study, we found a decrease in the inflammatory cascade, which is consistent with those of previous studies on ALO. This may be attributed to the effect on some cytokines, such as TNF-a and IL-1b, which are involved in initiating the inflammatory process. We did not examine these cytokines in our study.

Studies on ALO have mostly focused on the IL-10 level. Zhou et al.[32], in their experimental colitis model, found a significant increase in the IL-10 levels and improvement in colitis findings following ALO administration. Li et al.[33] stated that during the inflammatory process, which plays a role in endothelial damage, ALO administration increases the IL-10 level. In our study, the IL-10 levels were low in Groups 2 and 3 due to spinal trauma. Following ALO administration, the serum and tissue IL-10 levels were significantly higher in Group 4 than in Groups 2 and 3. Although the IL-10 level was the highest in both the serum and tissue samples in the sham group, where spinal damage had not occurred, its levels were the lowest in Group 3. Studies have demonstrated that ALO administration effectively prevents oxidative stress. Wu et al.[7] demonstrated a significant decrease in oxidative damage following ALO administration in patients with pulmonary hypertension. In the study by Hu et al.[6] ALO administration to mice with ischemic damage demonstrated an antioxidant effect and decreased cell damage. In our study, the TOS, an indicator of oxidative stress, was the lowest in the sham group. However, the TOS was significantly higher in Group 4 than in Groups 2 and 3. Additionally, we observed that the TAS was the highest in the sham group and the lowest in Group 3, indicating an antioxidant level. According to the trauma groups, the administration of ALO had a significantly higher TAS level. These TAS findings were confirmed at the enzymatic level based on the GPx and 8-OHG levels. In our study, the serum and tissue levels of 8-OHG were the lowest in the sham group and were the highest level in Group 3. Although it was significantly lower in Group 4 than in Groups 2 and Group 3, it was significantly higher than that in the sham group. Our study findings confirmed the hypothesis that ALO administration may improve the inflammatory and oxidative processes that develop after cord injury at the serum and tissue levels. Because the correction produced by the active substance in the serum values in experimental studies does not indicate improvement, a histological study should be performed to confirm this effect. Statistically, the sham group had the lowest levels of apoptotic cells according to the TUNEL test. In Groups 2 and 3, hemorrhagic and necrotic areas, loss of myelin, axon degeneration, neuropil in the gray matter, and necrosis were observed. Additionally, the Nissl bodies had disappeared. The decrease in the severity of these findings in Group 4 indicates that the antioxidant and anti-inflammatory effects of ALO treatment were successful. Although the pathologies concerning the nervous system occur at the tissue level, because they are reflected in physical activity, a treatment method can be considered valid if the biochemical and histological improvements affect motor functions. The Drummond-Moore test was performed to assess the neurological state. Because this test was performed on the 7th day before and after the damage, we did not observe any motor damage in the sham group. This indicates that we did not cause nerve tissue damage in the sham group. We also demonstrated that Group 4 had significantly higher scores on the Drummond-Moore test than Groups 2 and 3. The inclined plane test was also used to examine motor functions. Although there was no difference in the inclined plane test angle in the sham group before and after the experiment, the angles were similar in Groups 2 and 3, and they were significantly lower.

Study Limitations

This is the first study till date that investigated the effectiveness of ALO treatment in an SCI model and demonstrated its effect on the biochemical and histological results of motor functions. This study has some limitations. Creating a situation similar to SCI in humans is not possible. The trauma is unidirectional, the treatment starts on the first day, and ALO is administered as a single dose.

CONCLUSION

ALO has neuroprotective effects and prevents the degree of secondary cord damage following SCI via its antioxidative, anti-inflammatory, and antiapoptotic characteristics. It demonstrates promising results regarding its future applications in the clinic.

Ethics

Ethics Committee Approval: The study protocol was approved by the Necmettin Erbakan University KONÜDAM Experimental Medicine Application and Research Centering (decision no: 191518001, date: 19/10/2018).
Informed Consent: Informed consent is not required.

Authorship Contributions

Surgical and Medical Practices: E.S., F.H.Y., Concept: E.S., Y.K., G.C., D.A., Design: E.S., Y.K., G.C., D.A., Data Collection or Processing: E.S., D.A., F.H.Y., Analysis or Interpretation: E.S., Y.K., G.C., D.A., F.H.Y., Literature Search: E.S., M.Z.Y., C.İ.G., Writing: E.S., M.Z.Y., C.İ.G.

Conflict of Interest: The authors have no conflicts of interest to declare.

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