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Research Article

Effects of isotretinoin and acitretin on neuroregeneration in experimental spinal cord injury

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ABSTRACT

Objective: This study aimed to determine whether isotretinoin and acitretin have beneficial effects on neural tissue damage following acute spinal cord injury.

Methods: Thirty-six rats were randomly divided into 6 groups: control, sham spinal cord injury, spinal cord injury with isotretinoin 15 mg/kg for 14 days, spinal cord injury with isotretinoin 15 mg/kg for 28 days, spinal cord injury with acitretin 10 mg/kg for 14 days, and spinal cord injury with acitretin 10 mg/kg for 28 days. The damage to the spinal cord was formed by the clip compression technique. A neurological evaluation was conducted on days 1, 14, and 28. All rats were sacrificed following the treatment period, and samples of their spinal cords were collected for histopathological analysis.

Results: The inclined plane angle was significantly increased on the 14th and 28th days in the isotretinoin 15 mg and acitretin 10 mg groups, compared to the spinal injury group ($P = .049$ and $P = .009$, respectively). The Drummond–Moore criterion was significantly higher in the acitretin 10 mg group than in the injury group ($P = .026$). Cleaved Caspase-3 expression was similar in the isotretinoin 15 mg day 28 group and the control group ($P > .05$), but significantly decreased in the acitretin 10 mg 14th-day and acitretin 10 mg 28th-day groups compared to spinal injury isotretinoin 15 mg 14th-day and isotretinoin 15 mg 28th-day groups ($P < .05$).

Conclusion: This was the first study elaborating that isotretinoin and acitretin reduced neuronal apoptosis and improved functional recovery after spinal cord injury. These neuroprotective effects might open a window of opportunity for patients.

Introduction

Acute spinal cord injuries (ASCIs) occur due to high-energy traffic accidents, falls from height, work accidents, and sports injuries. Acute spinal cord injury is a serious health issue worldwide due to its high mortality and morbidity rates. It occurs most frequently in the cervical region and dorso-lumbar junction thus causing devastating health problems.[1,2] Individuals who were exposed to spinal cord injury (SCI) might continue their daily life or become tetraplegic and require respiratory support. Therefore, it affects the patient, his family, and the healthcare system due to treatment and caregiver costs, labor, and income losses.[3,4]

Primary and secondary damage mechanisms are involved in the formation of tissue damage in ASCI. Primary damage is unavoidable and occurs due to posttraumatic mechanical injury. Secondary damage develops as a result of the activation of endogenous apoptotic cell pathways triggered by primary damage and has not been fully resolved. Clinically manifested neurological dysfunction is mostly caused by secondary damage. The main goal of the treatment is stopping or slowing down the secondary damage cascade. Currently, there is no established effective treatment for SCI, and treatment options are limited to supportive care measures.[3-5]

Vitamin A (retinol) and its derivatives, commonly referred to as "retinoids," play an important role in the physiological development of vertebrate tissues through the control of expression of numerous genes that modulate cell proliferation, differentiation, and apoptosis. Isotretinoin and acitretin are synthetic vitamin A derivatives used to treat skin diseases.[6-8]

In this study, we aimed to elucidate whether isotretinoin and acitretin have positive effects on neural tissue damage after ASCI and also to investigate the difference between the therapeutic effects of isotretinoin and acitretin.

Materials and methods

This research was conducted in University Experimental Research Center Experimental Animals Laboratory and was supported by University Scientific Research Projects Unit. The ethics committee approval has been granted from the Ethics Committee of Düzce University, School of Medicine with protocol number: 2021/02/01. The study has been carried out in accordance with the EU Directive

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2010/63/EU for animal experiments and we tried to use the minimum number of animals.

Animals

A total of 36 Sprague–Dawley female adult rats, 2-4 weeks old, weighing 200-250 g were used. Animals were kept in cages and maintained under standard conditions with 12-hour light/dark cycles at room temperature (22 ± 2°C). All animals were housed 7 days before the tests for adaptation. The rats were randomly divided into 6 experimental groups consisting of 6 rats per each group for behavioral assessments.

Study groups

Group I (Control) (n=6): The group in which only laminectomy was performed at the T7-T9 level.

Group II (Sham) (SCI) (n=6): Laminectomy was performed at the T7-T9 level. After laminectomy, the dura was left intact and Tator and Rivlin clip compression model was applied to the medulla spinalis for 1 minute, and no treatment was given.

Group III (SCI+isotretinoin 14 days/15 mg/kg/day) (n=6): Laminectomy was performed at the T7-T9 level. Isotretinoin was given at a dose of 15 mg/kg/day by gavage for 14 days after compression with the aneurysm clip.

Group IV (SCI+isotretinoin 28 days/15 mg/kg/day) (n=6): Laminectomy was performed at the T7-T9 level. Isotretinoin was given at a dose of 15 mg/kg/day by gavage for 28 days after compression with the aneurysm clip.

Group V (SCI+acitretin 14 days/10 mg/kg/day) (n=6): Laminectomy was performed at the T7-T9 level. Acitretin was given at a dose of 10 mg/kg/day by gavage for 14 days after compression with the aneurysm clip.

Group VI (SCI+acitretin 28 days/10 mg/kg/day) (n=6): Laminectomy was performed at the T7-T9 level. Acitretin was given at a dose of 10 mg/kg/day by gavage for 28 days after compression with the aneurysm clip.

Spinal cord injury

All rats were examined for normal motor function before anesthesia. Anesthesia was induced by intramuscular administration of 50 mg/kg ketamine hydrochloride (Ketalar, Pfizer; Istanbul, Turkey) and 10 mg/kg xylazine (Rompun, Bayer; Istanbul, Turkey). The rats were placed in a prone position, their backs were shaved and cleaned with polyvidone iodine (Batticon; Adeka, Samsun, Turkey). A vertical skin incision was performed between T5 and T12 with reference to the interscapular distance and ribs, bilateral paravertebral muscles were dissected, a mastoid retractor was placed, and laminectomy was performed at the T7-T9 level. After laminectomy, the surgical wound was closed in the control group. Standard SCI was performed as follows: laminectomy was performed for 1 minute via a 70 g closing-force aneurysm clip (Yasargil FE 721Aesculap, Istanbul, Turkey), as described previously.[9] The Clip was removed after 1 minute in all groups (Figure 1). Following hemostasis, paravertebral muscles and the skin were primarily sutured with 3/0 Vicryl consistently regarding anatomical layers and rats were awakened normally at standard room temperature.

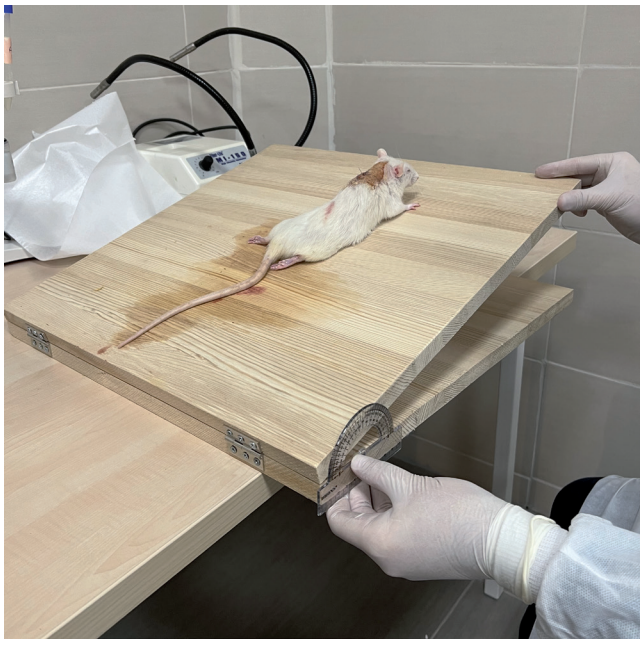

Figure 1. The table lifted at certain angles.

Evaluation of healing

Inclined plane and motor examination Drummond–Moore Criteria (Table 1) was utilized as damage score. This method was developed by Rivlin and Tator[10] in 1977. The highest angle at which the experimental animal stood without slipping on the inclined plane for 5 seconds is recorded as a score.[11] The rat was placed on the table parallel to the ground, and the table was lifted at certain angles (Figure 2). The angle at which the subject slipped was recorded on the 1st, 14th, and 28th days. In the evaluation with the Drummond–Moore criteria, motor examinations of the rats were performed on the 1st, 14th, and 28th days and were recorded.

Sample obtaining

The rats in groups I, II, III, and V were sacrificed at the end of the 14th day, and the rats in groups IV and VI were sacrificed at the end

HIGHLIGHTS

- Acute spinal cord injury (ASCI) is a serious health issue worldwide due to its high mortality and morbidity rates. Currently, there is no established effective treatment for spinal cord injury (SCI), and treatment options are limited to supportive care measures.This study aimed to elucidate whether isotretinoin and acitretin have positive effects on neural tissue damage after ASCI in.
- The results showed that the inclined plane angle was significantly increased on the 14th and 28th days in the isotretinoin 15 mg and acitretin 10 mg groups, and The Drummond–Moore criterion was significantly higher in the acitretin 10 mg group than in the injury group. Cleaved Caspase-3 expression was also significantly decreased in the acitretin 10 mg 14th-day and 28th-day groups compared to isotretinoin 15 mg 14th-day and 28th-day groups.
- This was the first study elaborating that isotretinoin and acitretin reduced neuronal apoptosis and improved functional recovery after SCI. These neuroprotective effects might open a window of opportunity for patients.

Table 1. Drummond–Moore criteria

0 Point	No motor muscle function in lower extremities, paraplegic
1 Point	Poor motor function in the lower extremity, weak movement against gravity
2 Points	Moderate lower extremity motor function, good strength against gravity, but unable to pull legs under the body
3 Points	Motor function very well, able to pull legs under body and jump, but not quite normal motor function
4 Points	Normal motor function

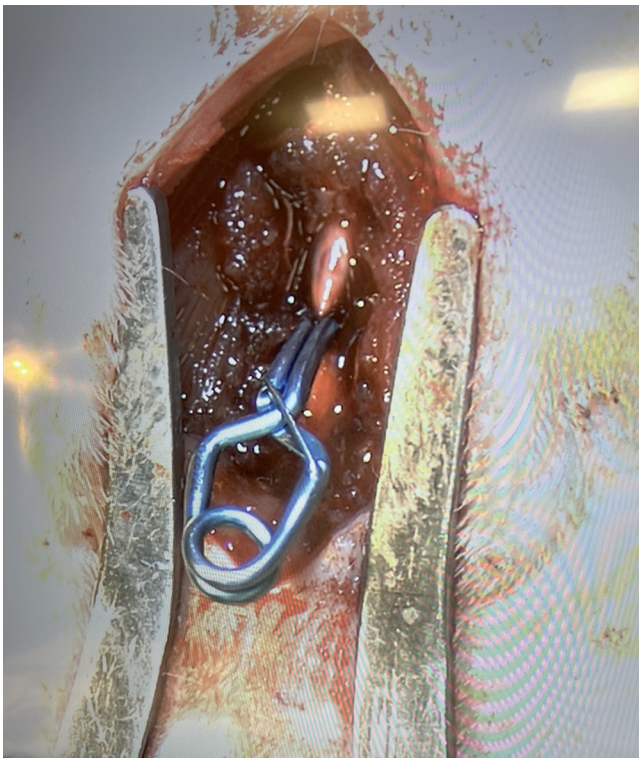

Figure 2. Closing-force aneurysm clip.

of their 28-day follow-up. We have utilized the previous skin incision, laminectomy, and clipping over the dura was reached, and the damaged cord was regularly removed.

Histopathological examination

All the spinal cord samples were fixed with 4% formaldehyde and embedded in paraffin blocks. Sections with 5 μm thickness were obtained from paraffin blocks on special silanized slides. Following deparaffinization, samples were marked with the immunohistochemical Cleaved Caspase-3. With this immunehistochemistry method, it was aimed to show the cells undergoing apoptosis. All histopathological analyses were made by light microscopy (Olympus Cx41-AxioCam Zeiss).

Statistical analysis

Statistical analysis of the evaluation of the inclined plane and Drummond–Moore criteria was performed using the Statistical Package for Social Sciences version 22.0 (IBM SPSS Corp.; Armonk, NY, USA) package program. Kruskal–Wallis test was used for group comparisons. Groups were evaluated with post hoc Bonferroni correction for multiple comparisons. Data are summarized by median, interquartile range, and minimum–maximum values. Categorical variables were compared with the Fisher–Freeman–Halton test and presented as numbers and percentages. In addition, immuno-histochemical findings of Cleaved Caspase proteins in different experimental groups were evaluated with the ImageJ program. Values were analyzed using GraphPad Prism 6 program, one-way analysis of variance followed by Holm–Sidak method. The *P*-value <.05 was accepted as the statistical significance level. In this study, the minimum sample size required to determine the statistical significance of the difference (effect size 0.685) between the control and treatment groups based on studies in the literature was determined as 6 rats in each group with 80% power and 5% type I error. Power analysis was done with G*Power v.3.1.9 package.

Results

The rats were randomly divided into 6 experimental groups consisting of 6 rats per group for behavioral assessments. At the end of the study, the rats were sacrificed in accordance with the protocol.

Motor examination outcomes on day 14

Inclined plane angle

The inclined plane angle was statistically lower in the spinal injury, isotretinoin 15 mg and acitretin 10 mg groups compared to the control group ($P < .001$; $P = .009$; and $P = .049$, respectively). The inclined plane angle was found to be significantly increased in the isotretinoin 15 mg and acitretin 10 mg groups compared to the spinal injury group ($P = .049$ and $P = .009$, respectively). No significant difference was found between the isotretinoin and acitretin groups in terms of inclined plane angle on the 14th day ($P = .527$) (Table 2), (Figure 3A and B).

Drummond–Moore criteria

The score was statistically lower in the spinal injury, isotretinoin 15 mg and acitretin 10 mg groups compared to the control group ($P < .001$; $P < .001$; and $P = .008$, respectively). The Drummond–Moore criteria scores of the isotretinoin 15 mg group were similar to the spinal injury group ($P = .351$) but it was observed that the acitretin 10 mg group had significantly higher scores than the spinal injury group ($P = .026$). No significant difference was found between the isotretinoin and acitretin groups in terms of Drummond–Moore criteria on the 14th day ($P = .678$) (Table 2) (Figure 3C and D).

Motor examination outcomes on Day 28

Inclined plane angle

Spinal injury, isotretinoin 15 mg and acitretin 10 mg groups had a statistically significantly lower inclined plane angle than the control group ($P < .001$; $P = .015$; and $P = .040$, respectively). It was determined that the inclined plane angle increased significantly in the isotretinoin and acitretin groups compared to the spinal injury group ($P = .040$ and $P = .015$, respectively). No significant difference was found between the isotretinoin and acitretin groups in terms of inclined plane angle on the 28th day ($P = .709$) (Figure 4A and B).

Drummond–Moore criteria

Drummond–Moore criteria scores were found to be statistically significantly lower in the spinal injury, isotretinoin 15 mg and acitretin 10 mg groups compared to the control group ($P < .001$; $P = .003$; and

Table 2. Fourteen-day values of inclined plane angle and Drummond–Moore criteria

	Control (n = 6)	Injury (n = 6)	Isotretinoin 15 mg (n = 6)	Acitretin 10 mg (n = 6)	*P*
Inclined plane angle	64.5 (1) [64-66]	44 (3) [42-46]	48.5 (1) [48-50]	49 (2) [48-50]	**<.001**
Drummond–Moore criteria	4 (0) [4-4]	0 (2) [0-3]	1 (0) [1-1]	1.5 (1) [1-2]	**<.001**

Data are median (IQR, interquartile range) and [minimum-maximum] values.
Statistical significance level was considered as $P < 0.05$

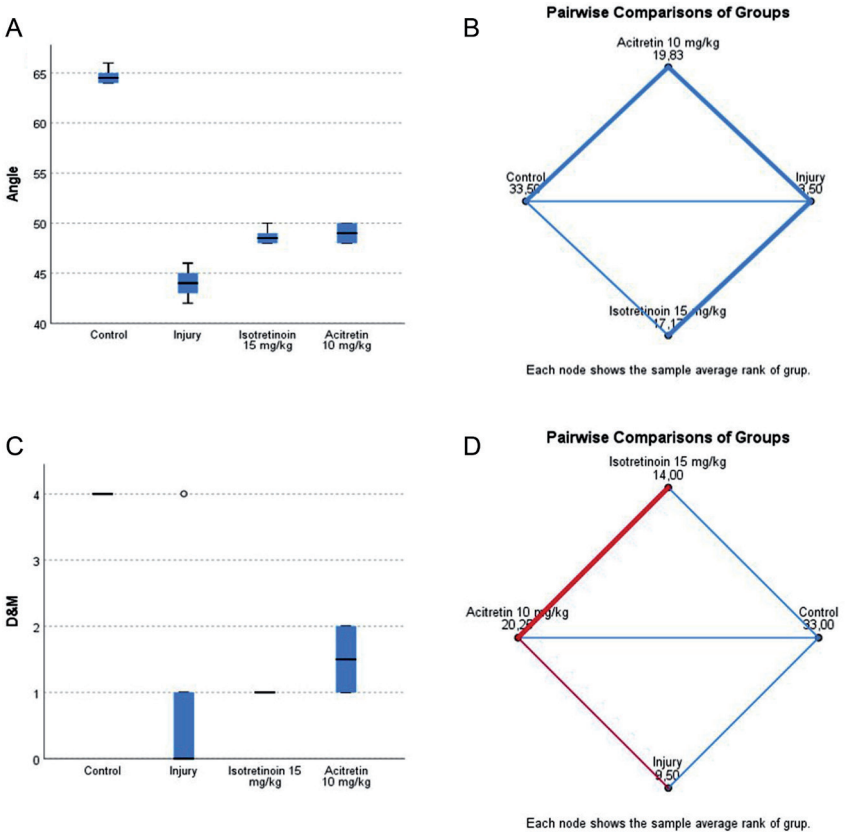

Figure 3. (A) Inclined plane angles of the groups on day 14. (B) Results from the multiple comparisons of inclined plane angles on day 14. (C) Drummond and Moore criteria scores on day 14. (D) Results from the multiple comparisons of Drummond and Moore criteria scores on day 14.

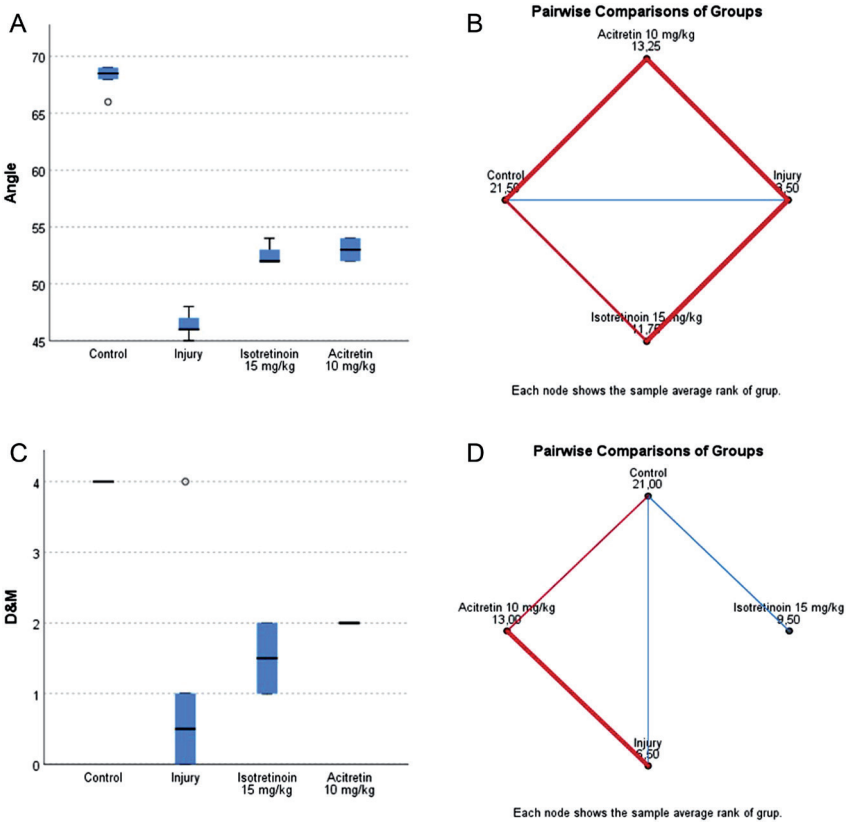

Figure 4. (A) Inclined plane angles of the groups on day 28. (B) Results from the multiple comparisons of inclined plane angles on day 28. (C) Drummond and Moore criteria scores on day 28. (D) Results from the multiple comparisons of the Drummond and Moore criteria scores on day 28.

Table 3. Twenty-eighth-day values of inclined plane angle and Drummond–Moore criteria

	Control (n = 6)	Injury (n = 6)	Isotretinoin 15 mg (n = 6)	Acitretin 10 mg (n = 6)	*P*
Inclined plane angle	68.5 (2) [66-69]	46 (2) [45-48]	52 (1) [52-54]	53 (2) [52-54]	**<.001**
Drummond–Moore criteria	4 (0) [4-4]	0.5 (2) [0-2]	1.5 (1) [1-2]	2 (0) [2-2]	**.001**

Data are median (WAG, interquartile range) and [minimum-maximum] values.
Statistical significance level was considered as $P < 0.05$

Table 4. Distribution of Drummond–Moore criteria at 14th and 28th days in groups, n (%)

Drummond–Moore criteria	Control (n = 6)	Injury (n = 6)	Isotretinoin 15 mg (n = 6)	Acitretin 10 mg (n = 6)	*P*
14th Day					**<.001**
0	0 (0.0%)	4 (66.7%)	0 (0.0%)	0 (0.0%)	
1	0 (0.0%)	1 (16.7%)	6 (100.0%)	3 (50.0%)	
2	0 (0.0%)	1 (16.7%)	0 (0.0%)	3 (50.0%)	
4	6 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
28th Day					**<.001**
0	0 (0.0%)	3 (50.0%)	0 (0.0%)	0 (0.0%)	
1	0 (0.0%)	2 (33.3%)	3 (50.0%)	0 (0.0%)	
2	0 (0.0%)	1 (16.7%)	3 (50.0%)	6 (100.0%)	
4	6 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	

Statistical significance level was considered as $P < 0.05$

$P = .040$, respectively). While the isotretinoin 15 mg and acitretin 10 mg groups were similar to the spinal injury group ($P = .442$ and $P = .096$, respectively), no significant difference was found between the isotretinoin and acitretin groups in terms of Drummond–Moore criteria scores on the 28th day ($P = .370$). (Tables 3 and 4) (Figure 4C and D).

Cleaved Caspase-3

No reaction was observed in the motor neurons via Cleaved Caspase-3 immunohistochemical staining of the control group (Figure 5). According to the immunohistochemical results, it was determined that Cleaved Caspase-3 expression increased statistically significantly compared to the control group ($P < .05$) in the spinal injury and isotretinoin 15 mg 14-day groups. Cleaved Caspase-3 expression significantly decreased in the isotretinoin 15 mg 28th-day group compared to the spinal injury group ($P < .05$). Cleaved Caspase-3 expression in the isotretinoin 15 mg 28th-day group was similar to the control group ($P > .05$). No statistically significant difference was observed between the isotretinoin 15 mg 14-day group and the isotretinoin 15 mg 28-day group ($P > .05$). Cleaved Caspase-3 was significantly decreased in the acitretin 10 mg 14th day and acitretin 10 mg 28th-day groups compared to the spinal injury, isotretinoin 15 mg 14th-day and isotretinoin 15 mg 28th-day groups ($P < .05$). Cleaved Caspase-3 expression of acitretin 10 mg 14th-day and acitretin 10 mg 28th-day groups was the same and no statistically significant difference was detected between them ($P < .05$) (Figure 6).

Discussion

The lack of a universally accepted treatment protocol in ASCI derives it necessary to understand all aspects of the pathophysiological process.[12,13]

The primary damage caused by posttraumatic mechanical injury refers to a compressive-contusion type injury that includes the first mechanical trauma which has disrupted the anatomical continuity of the body and is unavoidable.[14,15] Secondary damage occurs as a result of pathways triggered by primary damage that has not been fully resolved yet. During secondary damage, free radicals, lipid peroxidation, glutamatergic, cholinergic, and catecholaminergic neurotransmission systems, inflammation pathways, calcium and other ion channels, and activation of the apoptotic cascade play an

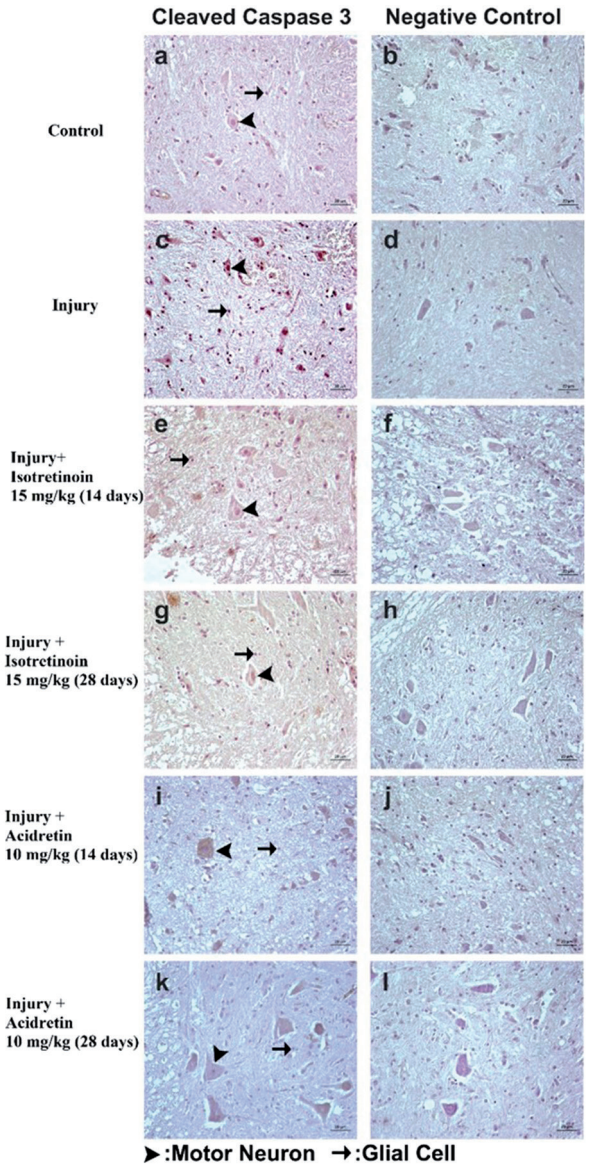

Figure 5. Control group, injury group, isotretinoin 15 mg/kg (14 days) group, isotretinoin 15 mg/kg (28 days) group, acitretin 10 mg/kg (14 days) group, acitretin 10 mg/kg (28 days) group with Cleaved Caspase-3 and negative control (×400).

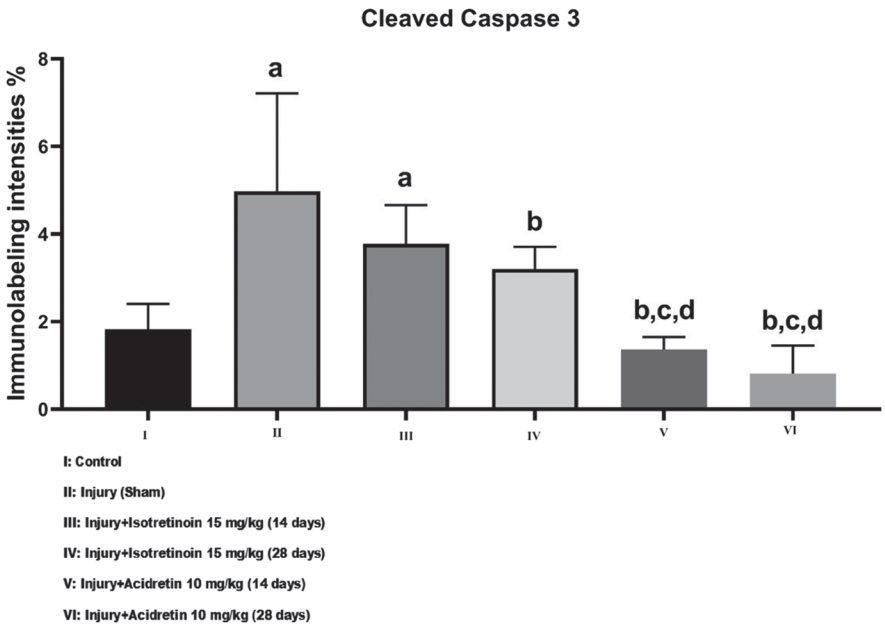

Figure 6. Cleaved caspase-3 antibody expression. (A) Significance level compared to the control group. (B) Significance level compared to the injury group. (C) Significance level compared to the 14th-day isotretinoin of 15 mg/kg/day group. (D) Significance level compared to the 28th-day isotretinoin of 15 mg/kg/day group.

important role.[14-16] As a result of the perfusion disorder that occurs, caspase-3 is activated in the cells and apoptosis becomes dominant.[17,18] Developing neurological dysfunction usually results from secondary damage rather than the primary injury.[14,15] Therefore, stopping or slowing down this secondary damage cascade that starts after injury is the main goal of clinical treatment. Neutrophils are the first immune cells to gather in the damaged area and activate other inflammatory cells and glial cells. Neutrophils secrete cytokines, proteases, and free radicals that result in neuron damage and death[19] thus, the suppression of inflammation would be effective in reducing the damage.[20] However, despite intensive experimental and clinical studies on this subject, no molecule with proven efficacy has yet been described. Therefore, laboratory and clinical studies at the molecular and cellular levels continue to reduce neural damage caused by trauma and to protect motor functions.

Acitretin and isotretinoin are synthetic retinoid derivatives of vitamin A. Oral and topical forms have been used successfully used in the prophylaxis and treatment of various dermatoses (cystic acne, psoriasis, hyperkeratotic skin diseases, genodermatoses, and benign and malignant skin tumors) since 1960.[21] Retinoid group drugs regulate cell proliferation and differentiation, modulate the immune system and has anti-inflammatory characteristics.[22,23] Isotretinoin shows anti-inflammatory effects by blocking different inflammatory pathways such as toll-like receptors, leukocyte migration, and Activator protein 1 (AP-1) pathway, decreasing tumor necrosis factor-α, interleukin (IL)-4, IL-17, and interferon-γ levels and reducing T-cell response.[24-27] Isotretinoin also reduces scar formation by decreasing MMP (matrix metalloproteinase)-9 and MMP-13 .[22] Acitretin primarily controls cell differentiation and maturation. It also has antiproliferative, anti-inflammatory, antikeratinization, and inhibitory effects on neutrophil chemotaxis.[28,29]

In this study, the anti-inflammatory effects of isotretinoin and acitretin have been investigated in SCI rat model. Different rat groups were enrolled considering that the short- and long-term effects may be different. The inclined plane angle was statistically significantly lower in the SCI, isotretinoin and acitretin groups compared to the control group. However, in the groups treated with isotretinoin and acitretin, the inclined plane angle increased significantly compared to the SCI group, but there was no difference between the isotretinoin and acitretin groups. This finding showed that isotretinoin and acitretin increased the inclined plane angle and improved motor functions. It was observed that this effect continued at 28 days, but there was no difference between the 14th and 28th days according to the Drummond–Moore criteria. As a result of the daily evaluation, it was determined that the isotretinoin group had similar results with the SCI group, while the acitretin group had significantly higher Drummond–Moore scores than the SCI group. There was no significant difference between isotretinoin and acitretin groups in terms of the 14th day Drummond–Moore criteria. It was observed that this effect was preserved on the 28th day, but there was no difference in terms of the 14-28 day results.

The cells going to apoptosis have been denoted in the Cleaved Caspase-3 immuno-histo-chemical staining. It was seen that the expression of Cleaved Caspase-3 in the isotretinoin 15 mg 28th-day group decreased statistically compared to the SCI group which was the same as the control group. The expression of Cleaved Caspase-3 in the acitretin 14th-day and acitretin 28th-day groups was statistically significantly reduced compared to the SCI, isotretinoin 14th-day and isotretinoin 28th-day groups. Cleaved Caspase-3 expression of acitretin 14th-day and acitretin 28th-day groups was similar with no statistically significant difference.

The results of this research elaborated that isotretinoin and acitretin reduced apoptosis in secondary injury in ASCI. This effect appeared to be independent of the duration of treatment. We assume that the motor improvement in the oblique plane and the Drummond–Moore criteria may be attributed to the anti-inflammatory, antiapoptotic, and immunoregulatory effects of isotretinoin and acitretin.

The limitations of this study are the lack of radiological and electromyographical evaluation after injury. Although our study suggests

that isotretinoin and acitretin reduced neuronal apoptosis and improved functional recovery after SCI, further in vitro studies and clinical trials are needed.

This was the first study elaborating that isotretinoin and acitretin reduced neuronal apoptosis and improved functional recovery after SCI. These neuroprotective effects might open a window of opportunity for patients.

Ethics Committee Approval: Ethics committee approval was received for this study from the Ethics Committee of Düzce University, School of Medicine (2022/02/01).

The ethics committee approval has been granted with protocol number: 2021/02/01. The study has been carried out in accordance with the EU Directive 2010/63/EU for animal experiments and we tried to use the minimum number of animals.

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

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Declaration of Interests: The authors have no conflict of interest to declare.

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