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RESEARCH ARTICLE

Effects of Tarantula cubensis Extract on Experimental Acute Spinal Cord İnjury in Rats

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ABSTRACT

Spinal cord injury (SCI) is a critical health problem that occurs after spinal trauma. In this study, the possible effects of Tarantula cubensis extract in the acute period were evaluated. For the rat trauma model, 28 Sprague dawley rats were used. All rats were divided into 4 groups (n=7); Control, injury (IG), prednisolone treatment (PG), and Tarantula cubensis extract treatment (TCG). Laminectomy was applied to all rats except the control group, and an aneurysm clamp was applied to the spinal cord for 60 seconds. BBB (Basso, Beattie, Bresnahan Locomotor Rating Scale) for clinical improvement assessment, histopathological (inflammatory response, neuron viability), and the amount of biochemical parameters 8-hydroxydeoxyguanosine (8-OHdG), myeloperoxidase (MPO), transforming growth factor beta 1 (TGF β-1), tumor necrosis factor alpha (TNF-α), interleukin 2 (IL-2) were evaluated. According to histopathological evaluations; less damage and inflammation were observed in the TCG group compared to the IG group. There was a statistical similarity between TCG and PG (p=0.005) BBB score TCG values were higher than PG and IG rats. MPO level was found to be significantly lower than TCG group IG and PG in laminectomy rats (p=0.001). TGF β-1 (ng-l), which is an anti-inflammatory cytokine, was measured at the highest level of TCG (p=0.001). TNF-α (ng-l) levels were higher in TCG, PG, and IG compared to the control group. IL-2 (ng-l) levels were decreased in TCG, PG, and control groups compared to the IG group (p=0.05). Tarantula cubensis extract has been determined neuroprotective effect by showing anti-inflammatory activity.

Keywords: Anti-inflammatory effect, Neuroprotection, Spinal cord injury, *Tarantula cubensis* extract

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Tarantula cubensis Ekstraktının Sıçanlarda Deneysel Akut Omurilik Yaralanması Üzerine Etkileri

ÖZ

Omurilik yaralanması (SCI), omurganın ciddi yaralanmalarından sonra ortaya çıkan kritik bir sağlık sorunudur. Bu çalışmada Tarantula cubensis ekstraktının akut dönemdeki olası etkileri değerlendirilmiştir. Sıçan travma modeli için 28 Sprague dawley kullanıldı. Tüm ratlar 4 gruba ayrıldı (n=7): Kontrol, hasar grubu (IG), prednizolon tedavisi (PG) ve Tarantula cubensis ekstraktı tedavisi (TCG). Kontrol grubu dışındaki tüm ratlara laminektomi uygulandı ve spinal korda 60 saniye anevrizma klempi uygulandı. Klinik iyileştirme değerlendirmesi, BBB (Basso, Beattie, Bresnahan Locomotor Rating Scale), histopatolojik (inflamatuar yanıt, nöron canlılığı) ve biyokimyasal değerlendirmede için 8- hydroxydeoxyguanosine (8-OHdG), myeloperoxidase (MPO), transforming growth faktör beta 1 (TGF β-1), tümor Nekroz Faktör Alfa (TNF-α), interleukin-2 (IL-2) değerlendirildi. Histopatolojik değerlendirmelere göre; TCG grubunda IG grubuna oranla daha az hasar ve inflamasyon gözlendi. TCG ve PG arasında istatistiksel olarak benzerlik vardı (p=0.005). BBB skoru TCG değerleri PG ve IG'ye göre yüksek bulundu. Laminektomi yapılan sıçanlarda MPO düzeyi TCG'de IG ve PG'den anlamlı düzeyde düşük bulundu (p=0.001). Antiinflamatuvar bir sitokin olan TGF β-1 (ng-l) TCG'de en yüksek seviyesinde ölçüldü (p=0.001). TNF-α (ng-l) seviyeleri TCG, PG ve IG'de kontrol grubuna göre daha yüksekti. Ancak kendi aralarında anlamlı bulunmamışlardır. IL-2 (ng-l) düzeyleri TCG, PG'de IG grubuna göre azalmıştı (p=0.05). Sonuç olarak; Tarantula cubensis ekstraktının antiinflamatuar aktivite göstererek omurilik dokusunda nöroprotektif etkisi olduğu belirlenmiştir.

Anahtar Kelimeler: Antiinflamatuar etki, Nöroproteksiyon, Omurilik yaralanması, *Tarantula cubensis* ekstraktı

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INTRODUCTION

Spinal cord injury (SCI) is a considerable health problem that can result in paralysis. This situation, which affects many people, can occur as a result of spinal injuries, especially in traffic accidents. While spontaneous recovery of SCI can be seen in non-mammalian species, this is very limited in humans (Bloom, 2014). Leukocyte infiltration and hemorrhage may occur due to primary damage to the nerve tissue and increased neuron damage. Therefore, tissue stabilization and nerve tissue regeneration are the main goals after injury. Steroids are currently used as a first choice in the treatment of spinal cord patients (Bracken et al. 1992; Walters et al. 2013). These drugs suppress the inflammatory response and reduce the leukocyte infiltration into the damaged tissue. However, it was determined in experimental studies that they did not provide sufficient improvement in treatment and were ineffective at high doses after trauma (Tator and Koyanagi, 1997). It has also been stated that steroids can cause hyperglycemia in metabolic diseases and worsen pneumonia situations (Sultan et al. 2020). The fact that the current treatment options do not result in full recovery in SCI makes it necessary to apply new treatment protocols and drugs. It has been reported that some useful results regarding SCI have been obtained in previous studies. These; Schwann cells differentiated from adipose stem cells (Zaminy et al. 2013). growth factor treatment (Chehrehasa et al. 2014), anti-CD 11cell treatment (Geremia et al. 2017), Cyclooxygenase-2 (Cox-2) selective inhibitör parecoxib treatment (Yuksel et al. 2019), human bone marrow-derived stem cells treatment (Munter et al. 2019), melatonin hormone (Zhang et al. 2019), platelet rich plasma(Salarinia et al. 2017) and quetiapine treatment (Aytar et al. 2018). However, the unique structure of the nerve tissue and the variability of trauma responses do not always produce positive outcomes in humans.

Tarantula cubensis extract is used especially in veterinary medicine. It is known that it has favorable outcomes on postpartum complications, and foot and breast diseases, and it is used in oral papillomatosis with its immunomodulatory effects (Coskun, 2017). *Tarantula cubensis* extract D6 has been reported to have positive effects on peripheral sciatic nerve damage (Kizilay et al. 2019), wound healing (Gul Satar et al. 2017), and aflatoxin-induced injury in rats (Karabacak et al. 2015). It is necessary to present new approaches to spinal cord injury, which is a very serious health problem. This study examined the possible effects of *Tarantula cubensis* extract in the treatment of acute SCI in rats.

MATERIALS AND METHODS

Animal Study

This experimental study was performed with the permission of Adnan Menderes University Animal Experiments Local Ethics Committee (ADU-HADYEK 64583101/2018/004). For the animal study 220-250 g weight 28 female Sprague Dawley rats were randomly divided into 4 groups (n=7). These were Control (CG), Injury group (IG), Prednisolone treatment group (PG), and *Tarantula cubensis* extract treatment group (TCG). Laminectomy was applied to all groups except the control group. Methylprednisolone (Precord liyo, Kocak Pharma/Turkey) was intraperitoneally administered at a dose of 1 mg/kg and Tarantula cubensis extract (Theranekron D6, Ricter Pharma/Austria) was administered intraperitoneally (IP) at 0.3 ml/animal (Kizilay et al. 2019). An equal volume of physiological saline was injected into the control and injury groups. In the treatment groups (PG and TCG), drug administrations were performed at the 4th hour after injury. The urinary bladders were emptied by applying handle massage every 6 hours to the rats who underwent surgical procedures until the end of the 72 th hours of the study. The reason why male animals were not used in the study was urinary incontinence problems that could develop after SCI.

Laminectomy

The laminectomy procedure was performed as stated by previous studies (Rivlin and Tator, 1978). Ketamine (50mg/kg/bw) and xylazine (5 mg/kg/bw) anesthesia was applied to the rats who underwent a surgical procedure. The dorsum of rats were cleaned with betadine solution following to clipping the hairs. After the skin incision, resection of the muscle and fascia tissues was performed. Thoracic (T 7-9) spine-level laminectomy was performed without damaging the dura mater by using the clip compression method. In clip compression application, an aneurysm clamp (Mizuho Aneurysm Clip, Mizuho, Japan) was applied for 60 seconds (Figure 1). The area was then closed by suturing the muscle and skin. After applying antiseptic to the skin, the rats were observed in clean cages. There was mortality observed after the surgical procedures. Paracetamol (100 mg/kg/bw) was added to the drinking water of the operated rats until the first 12 hours to reduce pain.

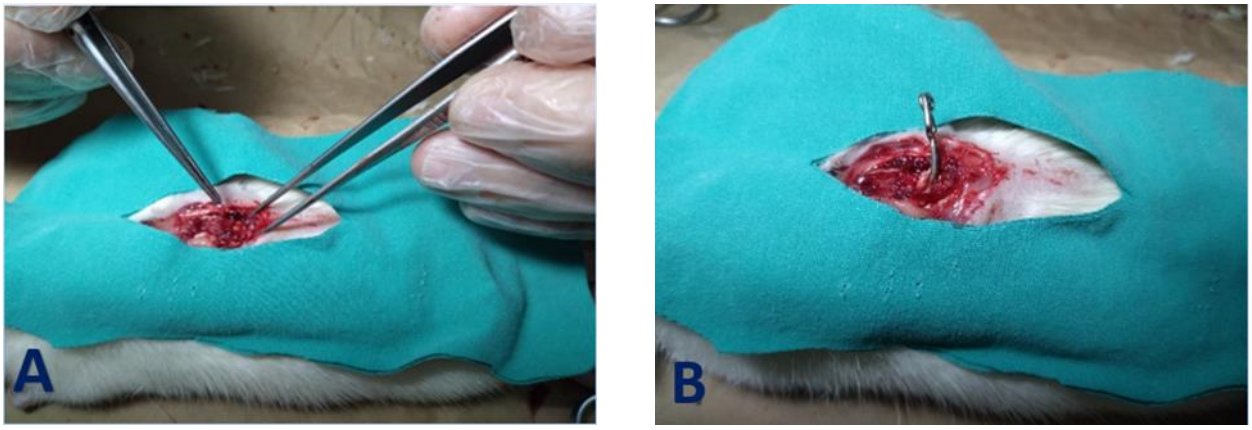

Figure 1: Laminectomy procedure on spinal cord injury (**A**) and application of aneurysm clamp for 60 seconds (**B**) in a rat model. (**A**); Following the general surgery principles, the spinal cord is opened in rats under anesthesia until the spinal cord is seen. Laminectomy is completed without damaging the dura mater. (**B**); Aneurysm clamp is applied for 60 seconds. Afterward, the operation was completed using the appropriate suture material. All surgical procedures were completed in accordance with animal welfare.

Biochemical analyzes

At the end of the study (72nd hour), intracardiac blood samples were taken and serums were separated with a refrigerated centrifuge device (Sigma, 3K30). Samples were stored at -20 ˚C degrees until analysis. A commercial test kit was used to determine the total protein level in blood serum samples (A2300, Archem Health Ind. Co., Turkey). Myeloperoxidase (MPO) activity, 8- Hydroxydeoxyguanosine (8-OHdG), TNF-α (Tumor Necrosis Factor Alpha), IL-2 (Interleukin 2), and Transforming growth factor beta 1 (TGF beta-1) levels were measured in blood serum. A commercial elisa test kit was used for MPO activity (Colorimetric, Cat No: ab105136). For measuring 8-OHdG Elisa Kit (Cat no: E-EL-0028) also TGF beta 1 ELISA Kit (Cat no: ab119558), TNF-α ELISA Kit Bt-lab (Cat no: E0764Ra), IL-2 Bt-lab ELISA Kit (Cat no: E0123Ra) was used. All tests were analyzed by a subject expert on 96 Elisa plates with 3 replicates of each sample. ELISA tests; microplate reader: BIOTEK ELX800-Auto strip washer: BIO TEK EL X 50 it was done on the device.

Physical examination and Basso, Beattie, Bresnahan (BBB) Locomotor Rating Scale

Locomotor testing was performed at 24, 48, and 72 hours after SCI trauma. In the evaluations, muscle and joint movements, paraplegia level, reflexes, general condition, and movement codifications were examined. The result of the evaluation, the BBB test with a scale of 0-21 was used (Basso et al. 1995). The examinations were video recorded for 1 minute. The results were evaluated by 2 different experts.

Histopathology evaluation

All rats were euthanized by the cervical dislocation method under anesthesia (Ketamine and Xylazine combination) 72 hours after the operation. The spinal cords were harvested immediately, and tissue segments (4-6 cm long) were placed in 10% formaldehyde solution. Serial transverse sections were made from the laminectomy areas from the materials after 24 hours of fixation, the pieces were placed in cassettes and routinely followed for 16 hours in an automatic tissue tracking device. After routine follow-up, serial sections of 4 µm were taken from the samples embedded in paraffin blocks with a microtome (Thermo Shandon HM 355S). Prepared slides were stained with a commercial kit of Hematoxylin-Eosin staining. Samples were examined with a light microscope (Olympus BX53, Japan). Photo samples were recorded with a high-resolution video camera (Olympus BX61, Japan).

Histopathological evaluation; the samples were examined by three expert pathologists. The degree of ischemic damage and leukocyte leak in motor neurons in the ventral horns of the spinal cord was evaluated. Neurons showing cellular swelling or loss of eosinophilic cytoplasm, nucleus, and Nissl bodies were considered damaged. Neurons with thin chromatin, prominent nucleolus, and cytoplasmic Nissl bodies were defined as alive. The viability index was calculated as the ratio between the number of viable motor neurons in the entire spinal cord section and the number of all motor neurons for each rat (viability index = Number of viable neurons / total number of neurons). In addition, the inflammatory response was graded semi-quantitatively by calculating the number of leukocytes seen infiltrating the tissue in randomly selected gray matter areas: '0' for none, '1' for less than 20, '20 to 50' for '2', "3" for more than 50 detected leukocytes (Boga et al. 2006).

Statistical analysis

Biochemical parameters were expressed as the mean ± SE (standard error) for the 7 rats in each group. SPSS for Windows Version 20 was used for statistical analysis of different groups. Statistical significance among groups was tested using the Kruskal-Wallis test for parameters (pathological parameters) that were not normally distributed and using one-way ANOVA for normally distributed parameters (other parameters). A significant difference is accepted when $p<0.05$ (Conover, 1980).

RESULTS

Biochemical Analyzes Results

According to the results of 8-OHdG blood serum levels, a statistically significant difference was found between the treatment groups and the control group ($p=0.04$). In the tests performed with the ELISA assay, the highest result was found in TCG. There was also a significant difference between the group's PG and TCG.

MPO activity increased with laminectomy and its level decreased in the treated groups. One of the most important results we found in the tests is MPO activity. Because TCG levels decreased statistically significantly in PG and IG groups (Table 1).

TGF β-1 level was the lowest in the control group. It was determined that the level of laminectomy was increased in groups (IG, PG, and TCG). TGF β-1 level was similar between PG and IG, the highest level was determined in TCG ($p=0.001$).

There was no statistically significant difference between TNF-α blood levels PG, IG, and TCG. These values were found to be higher than the control group ($p=0.025$). IL-2 levels of PG, TCG, and control groups showed a statistically significant decrease in the IG group ($p=0.05$).

Table 1. Biochemical parameters from blood serum taken from rats at the end of the study (72nd hour). 8-hydroxy-2'-deoxyguanosine damage marker (8-OHdG), myeloperoxidase (MPO) activity, transforming growth factor beta-1 (TGF β-1) levels, TNF-α (Tumor Necrosis Factor Alpha), IL-2 (Interleukin 2)

Groups (n=7)	Parameters				
	8-OHdG (ng/l)	**MPO (ng/ml)**	**TGF β-1 (ng/l)**	**TNF-α (ng/l)**	**IL-2 (ng/l)**
Control	5.90±0.07^{c}	0.41±0.02^{d}	57.62±1.42^{c}	35.95±3.44^{b}	26.38±5.42^{b}
Injury	5.94±0.09^{c}	2.27±0.08^{a}	62.38±0.95^{b,c}	73.49±4.22^{a}	46.37±8.35^{a}
Prednizolon	6.95±0.50^{b}	1.50±0.05^{b}	68.43±4.04^{b}	64.96±6.83^{a}	28.20±3.03^{b}
Tarantula cubensis	10.01±0.37^{a}	1.21±0.06^{c}	79.08±2.30^{a}	68.77±8.18^{a}	25.56±4.28^{b}
p	0.040	0.001	0.001	0.025	0.050

a, b, c, d: Different letters in the same column indicate statistically significant difference.

Histopathological Results

Spinal cord ventral horns in animals in the control group had a high viability index, and the inflammatory response detected in gray matter was mild. Diffuse ischemic harm was observed in the ventral horns of spinal cords from animals in the IG group, consistent with the low viability index. In the same samples, the inflammatory response in the gray matter was severe. The viability index of the spinal cords taken from the animals belonging to the PG and TCG groups was at similar rates, and neuronal ischemic damage was observed to be lower (focal) in these groups. Although these results were lower than the control group, they were statistically significantly higher than the Injury group (Figure 2). The lowest rate of inflammatory response was seen in TCG after the control group (Table 2). The statistical similarity in both groups indicates anti-inflammatory activity.

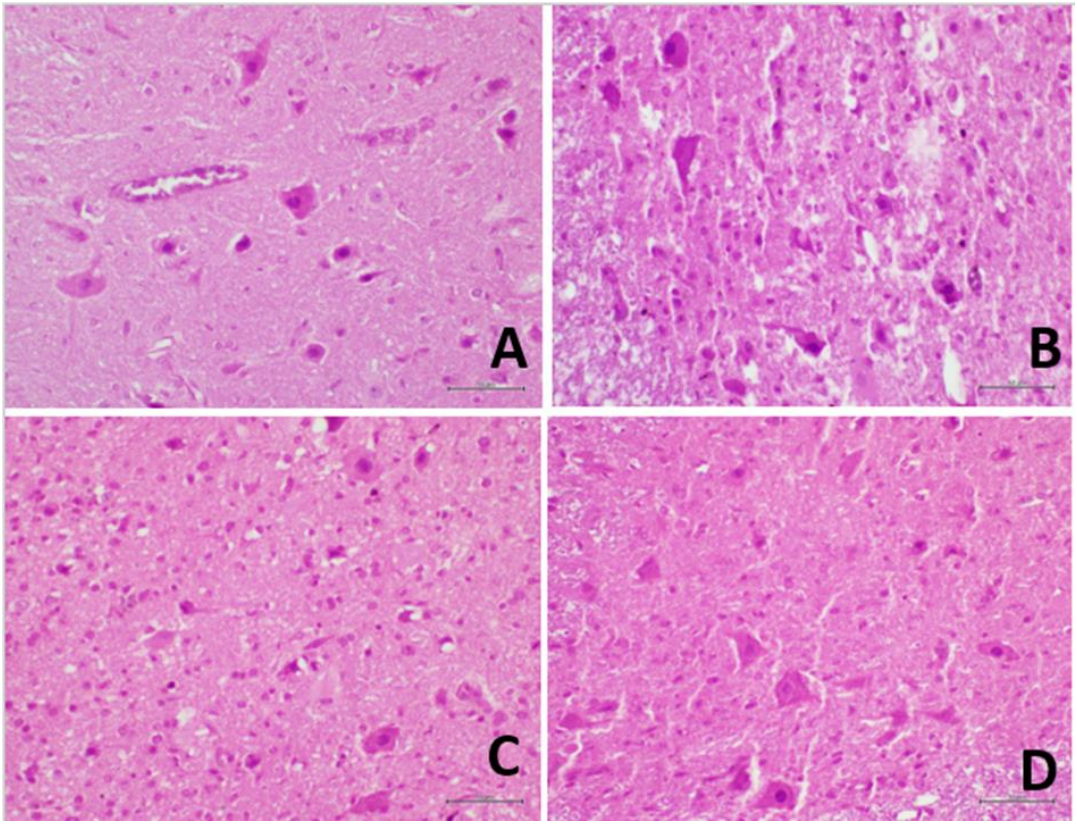

Figure 2: Hematoxylin-eosin staining (HxE, 400x magnification) in rat spinal cords after 72 hours. **(A)** Control group, normal spinal nerve formations, non-inflammation and damage. **(B)** Injury group (IG), high rate of leukocyte infiltration and inflammation, decrease in viable motor neurons and nissl bodies, cavitation onsets. **(C)** Prednisolone treatment group (PG), less inflammation, cavitation. **(D)** Tarantula cubensis extract treatment group (TCG), low inflammation rate, low leukocyte rate. There are similarities in histopathology evaluation between the PG and TCG groups.

Table 2. Histopathological evaluation of spinal cord tissue. Viability index = number of viable neurons /total number of neurons. Inflammatory response; **'0'** for none, **'1'** for less than 20, 20 to 50 for **'2'**, **"3"** for more than 50 leukocytes.

Groups (n=7)	**Parameters**	
	Neuron Viability (%)	**Inflammatory response**
Control	95.00±0.77[a]	1.00±0.00 [c]
Injury group	54.00±1.41 [c]	2.60±0.24 [a]
Prednisolone group	70.40±1.72 [b]	1.60±0.24 [b]
Tarantula cubensis group	74.00±2.44 [b]	1.20±0.20 [b,c]
p	0.001	0.005

a, b, c, d: Different letters in the same column indicate statistically significant differences.

Physical Examinations and BBB Scale Results

In the BBB score evaluation, paraplegia was present in all animals in the first 24 hours, and no significant difference was observed. The values on the 24th hour are IG, PG, and TCG, respectively; It was found to be 1±0.00, 1.50±0.22, 1.66±0.21. 48 th hour values are; It was determined as 1.50±0.22, 2.66±0.33, 4.16±0.33. At the end of the study (72nd hour); It was determined as 2.33 ±0.21, 3.5 ±0.34, and 5.83±0.3 (Figure 3).

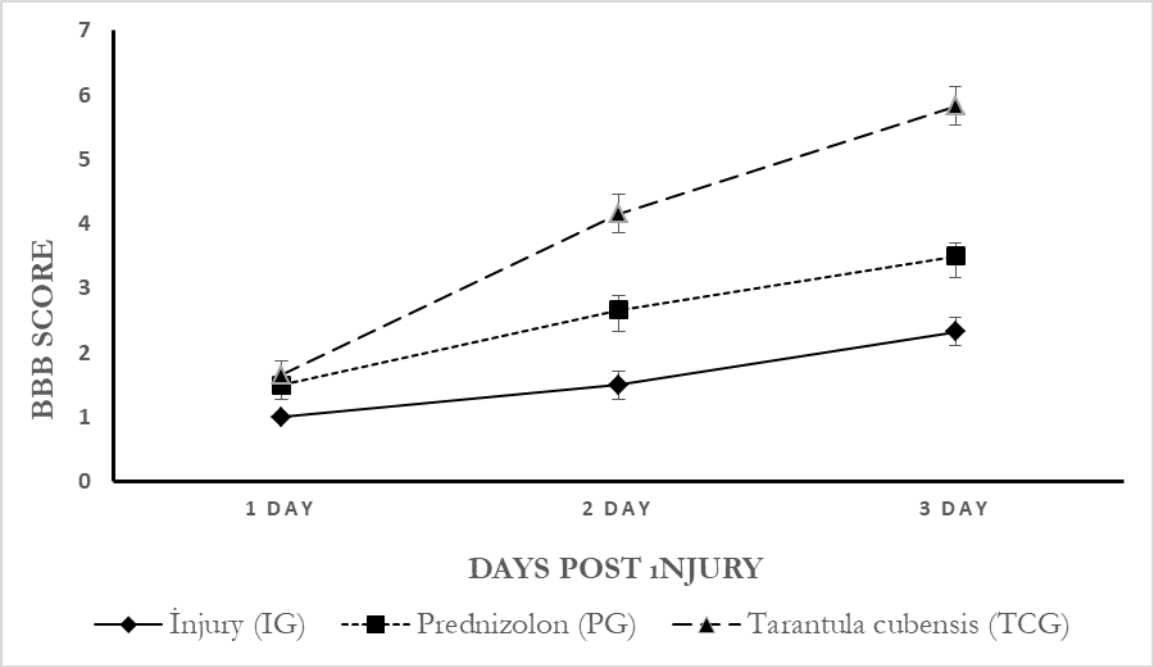

Figure 3. Clinical improvement scale applied to rats that underwent laminectomy; Beattie, Bresnahan (BBB) Locomotor Rating Scale. Tarantula cubensis extract group (TCG), Prednisolone treatment group (PG) and Injury group (IG). BBB values are (0; poor-21; healthy).

DISCUSSION

SCI has a complex damage mechanism and results in incomplete healing (Gensel and Zhang, 2015). Preventing the acute phase response in spinal cord injuries may help prevent chronic cavitations and glial scarring (McDonald et al. 2002). Although previous experimental animal studies have offered new approaches, steroids are now used as the only option (Bracken et al. 1992). The first 12 hours after SCI trauma is extremely important. It has been reported that hemorrhage, axonal necrosis, and demyelination develop after acute SCI, and leukocyte infiltration paradoxically disrupts the situation (Kakulas, 1984; Wallace et al. 1987). In this study, it was observed that there was a statistically significant similarity between the PG and TCG in the histopathological evaluation of the acute period after trauma. Likewise, there was a similar situation in the inflammatory response and neuron viability values (Table 2). Although only hematoxylin-eosin was evaluated in this study, a clear similarity was observed between PG and TCG in niss body ratios and leukocyte rates (Figure 2). İnflammation and leukocyte infiltration were observed at a lower rate in TCG compared to IG. In another previous study, Tarantula cubensis extracts are consistent with the results obtained in the peripheral nerve tissue trauma model (Kizilay et al. 2019). Although the mechanism of action of Tarantula cubensis extracts is unclear, appears to have neuroprotective effects on the nervous tissue.

TNF-α and IL-2 are important proinflammatory cytokines and their levels increase in inflammatory reactions (Gensel and Zhang, 2015). In this study, TNF-α showed a non-statistical decrease in TCG compared to IG. This is attributed to the fact that TNF-α remains high in the first hours of the injury and then decreases in the 72nd hour (Table 1). IL-2 levels are high in IG and low in PG and TCG, showing statistical significance ($p=0.05$). This is important evidence that prednisolone and Tarantula cubensis extract significantly reduce secondary damage and inflammation of SCI. TGF β-1 tends to increase in cases of damage or ischemia of nerve tissues (Klempt et al. 1992). It has been reported in experimental studies that it also plays a role in neuronal regeneration (Abe et al. 1996). Also, it is a cytokine with anti-inflammatory properties. TGF β-1 levels have not been examined in any experimental study before. In this study, the changes in TGF levels were statistically significant between the groups and increased in the TCG compared to the other groups. Although this indicates the role of Tarantula cubensis extracts in the recovery of neuronal damage, it requires further studies. 8-OHdG is a general marker of DNA damage and its level increases in case of damage. In this study, the blood DNA damage level was higher in the TCG in the acute period compared to the others. This can be explained by the fact that he is an alcoholic extract. Alcohol is used as a general solvent in homeopathic medicines. However, it is recommended to be used at low rates due to its cytotoxicity (Chirumbolo et al. 2015). This confirms the hypothesis of reviewing and reducing the alcohol content in homeopathic medicines. MPO activity is due to the increase in leukocytes penetrating the tissue after trauma. This situation creates more ROS (Reactive oxygen species) compounds in the environment and complicates the

recovery of axonal damage. Previous studies have shown that MPO is a very important marker in the acute period after trauma (Geremia et al. 2012). In this study, it was observed that the MPO activity, which increased significantly after trauma, was at the lowest level in the TCG, and it decreased statistically significantly even compared to the PG. This is evidence that Tarantula cubensis extracts significantly reduce leukocyte infiltration.

BBB scale has been reported to be important in clinical improvement in acute experimental SCI studies (Basso et al. 1995). In this study, no significant difference was found between the groups in the first 24 hours after laminectomy. A better improvement in TCG was observed at 48 and 72 hours compared to PG and CG. It was observed that Tarantula cubensis extract supports clinical improvement and can do this better than prednisolone (Figure 3). The only problem with SCI damage does not occur in the neuronal tissue. Post-traumatic proinflammatory response and leukocyte increase may also reveal distant tissue damage such as lung (Gris et al. 2008). In this study, the anti-inflammatory effect of Tarantula cubensis extracts and tissue responses similar to prednisolone were observed on the damaged spinal cord. In addition, it reduces MPO and IL-2 activity in the blood, increases TGF β-1 levels, and shows an important inflammatory response suppression feature. It is thought that similar effects may also occur in distant tissue damage.

CONCLUSIONS

Tarantula cubensis extract significantly reduces neuronal degeneration acute SCI, has anti-inflammatory activity, and increases clinical improvement. It is suggested that this study should be performed at different doses and durations (long-term), and the combined effects of steroids should be revealed.

Ethical Approval: This experimental study was performed with the permission of Adnan Menderes University Animal Experiments Local Ethics Committee (ADU-HADYEK 64583101/2018/004).

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Authors Contribution Rate: The authors declare that they have contributed equally to the article.

Conflict of Interest: Authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Limitations the Study: In this study, Caspase 3 tissue staining could not be performed due to budget constraints.

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