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Original Investigation

Histopathological Evaluation of the Effects of CAPE in Experimental Spinal Cord Injury

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ABSTRACT

AIM: Spinal cord injuries negatively affect the individuals and the life quality of their families due to neurological deficits caused by trauma. The prevalence of spinal cord injury is 15-45/1 million in the world. Caffeic acid phenethyl ester (CAPE) is the most active component of propolis and has neuroprotective, anti-oxidant and anti-apoptotic effects. Our aim was to determine the effects of CAPE on the prevention of secondary injury and to compare with methylprednisolone.

MATERIAL and METHODS: Forty rats were divided into 4 groups. The control group did not undergo surgery (Group I), only trauma group (Group II), trauma+CAPE treatment group (Group III), and trauma+methylprednisolone treatment group (Group IV). Histopathological assessment was performed with two staining methods as hematoxylin-eosin (HE) and terminal deoxynucleotidyl Transferase Biotin - dUTP Nick End Labeling (TUNEL). The groups were statistically compared.

RESULTS: The apoptotic cells decreased in treatment groups compared with the trauma. CAPE has more anti-apoptotic effect than methylprednisolone. The histological difference between the Group II, and Groups III and IV was statistically significant.

CONCLUSION: CAPE has a positive effect on spinal cord injuries by preventing apoptosis.

KEYWORDS: Apoptosis, CAPE, Propolis, Spinal cord injury, TUNEL

INTRODUCTION

Spinal cord injuries (SCI) negatively affect the individuals and the life quality of their families (25,28). Trauma causes considerable functional and serious economical losses. It is mostly seen in the young population between 15 and 25 years of age (1,23,29). Another important issue is the fact that society and medical staff do not have adequate experience on the management of SCI (18). The causes of spinal cord injuries are motor vehicle accidents (50%), falling and work accidents (30%), crimes (11%), sports injuries and vascular surgeries (19,24). The male/female rate is 4/1 (22).

There is no significant epidemiological study in our country about spinal cord injuries. Every year 1600-2000 severe SCI cases are reported and its annual prevalence is estimated to be 12.7/1.000.000 (13). It is well known that the prevalence of spinal cord injuries is 15-45 per 1 million in the world (10).

The biochemical and histopathological cascade mechanisms in the spinal cord following the primary injury are called secondary injury and these mechanisms lead to progressive neuronal death after trauma. There is no medical or surgical treatment for primary injury. All studies on the SCI are intended to prevent secondary injury (9,23,28,29).

The national acute spinal cord injury studies (NASCIS I, II, III) showed that high dose of methylprednisolone (MP) within the first 8 hours after trauma provides neurological recovery (23,28). The positive effects (in motor and sensory functions)

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of MP in the first 8 hours after SCI continue for 6 weeks, 6 months or one year after injury (16,30).

Propolis is a substance that is collected by honeybees from herbal sources and its biological and pharmacological effects have been studied for several years (15). Caffeic acid phenethyl ester (CAPE) is the most active component of propolis. It is also previously proven that CAPE has neuroprotective, antioxidant and antiapoptotic effects (8,9). CAPE is one of the strongest lipophilic antioxidants (15).

Today, no effective treatment method has been developed for the secondary injury following SCI. Partial benefit has been obtained from medical treatment but complete recovery in the neurological deficits has not been reported following SCI in the literature (23).

In this study, our aim was to determine the effects CAPE on the prevention of neurological deficits caused by secondary injury after SCI and to compare CAPE with MP.

MATERIAL and METHODS

This study was carried out in Experimental Animals Laboratory of Osmangazi University (TICAM) and histopathologic examinations were performed in Laboratory of Histology and Embryology. This study was approved by the Animal Ethics Committee of Osmangazi University.

Forty adult Sprague Dawley female rats weighing 200 to 250 grams were used in this study. They were divided randomly in 4 groups (10 rats per group) as control group without any surgical procedure or treatment (Group I), only trauma group without any treatment (Group II), trauma and CAPE treatment group (Group III) and, trauma and MP treatment group (Group IV) (Table I). Yasargil aneurysm clip (code no. FE 740 K) with a strength of 24 g (Aesculap AG, Germany) has been applied to trauma groups (Group II,III, IV) following T9-T11 laminectomy. The duration of epidural clip application was 60 seconds. Paraplegia was observed in all rats after the trauma, except control group.

CAPE (Sigma –Aldrich Co., Steinheim, Germany), being the active component of propolis, has been dissolved in saline solution in powder form (0.9% NaCL, Eczacıbaşı-Baxter, Istanbul) and dimethyl Sulfoxide (DMSO) (Sigma –Aldrich Co., Steinheim, Germany) diluted at the rate of 0.06 mg/1mg. CAPE solution has been administered intraperitoneally (IP) at a dose of 10 µmol/kg in all rats of Group III (26). A 30 mg/kg loading dose of MP was administered IP in the rats of Group IV. After the loading dose, four-dose of 5.4 mg/kg MP was administered IP in the same rats every 6 hours in one day.

As analgesia, 10 mg/kg paracetamol (Perfalgan, 10 mg/mL infusion, Bristol Myers SQUIIB, France) was administered IP to the rats, 48 hours after the surgery. Forty-eight hours later, the rats underwent re-operation, and spinal cord samples were removed en bloc for histopathological examinations. The samples were inserted into a 10% formaldehyde solution for fixation. Following the surgery, all rats were sacrified.

Histopathological assessment was performed with two staining methods as hemotoxylin-eosine (HE) and Terminal deoxynucleotidyl Transferase Biotin - dUTP Nick End Labeling (TUNEL). TUNEL method was used to determine the apoptosis. Millipore kit (TUNEL Apoptosis Detection Kit, Merc Millipore Headquarters, Billerica, MA, 01821) was preferred for TUNEL staining.

IBM SPSS Statistic 21.0 and Sigmastat 3.5 data analysis programmes were used for the statistical analysis. Descriptive data were shown as average ± standard deviation. Kruskal Wallis One Way Analysis Test was used since the groups are non-normally distributed in the comparison of necrosis, vacuolization and general damage. Student-Newman-Keuls was used in all groups as multiple comparison tests. Kruskal Wallis Oneway Analysis test was used since the numbers of apoptotic cells are non-normally distributed and Tukey test was used for pairwise comparisons.

Sections stained by HE were examined by an experienced histologist under optical microscope (Olympus BH-2). Intact and necrotic cells were examined carefully in the spinal cord sections. A scoring from one to three has been made based on the extensity of necrosis. (+) means area of necrosis, vacuolization and hemorrhage was regional, (++) means area of necrosis, vacuolization and hemorrhage covered almost half of the spinal cord, (+++) means necrotic cells were mostly observed in the spinal cord section.

Apoptotic cells marked by TUNEL method were counted by the same observer by the use of Olympus BX 51 (Japan) light microscope with the BAB Pro2000 program. Pictures of sections have been taken. Apoptotic cells were counted at the four sections of each subject and the mean numbers with standard deviations were calculated.

For statistical assessment, $p<0.001$ value was considered significant. Graphs were created by the use of Microsoft Excel.

Table I: Study Groups and Procedures

	Trauma	Treatment	Dose
Group I (Sham)	-	-	-
Group II (Only trauma)	+	-	-
Group III	+	CAPE	10µmol/kg
Group IV	+	Methylprednisolone	30 mg/kg 5.4 mg/kg

RESULTS

Histological Findings

Based on the HE staining; In Group I, white matter structure of spinal cord and axons and myelin sheaths were observed. Multipolar motor neurons located at anterior horn in grey substance were seen normal together with their euchromatic nucleus, distinctive nucleolus and cell bodies (Figure 1A-D). Furthermore, axons and surrounding myelin sheaths were observed in normal histological structure in the spinal cord longitudinal section (Figure 2A, B). In Group II, intensive degeneration and distensions in the axons and myelin sheaths surrounding axons in white substance and partly rupture in the axons were observed in the longitudinal sections of spinal cord. In Group III, there was a considerable decreased size of damage in the spinal cord. Grey substance, white substance, canalis centralis and ependyma cells and multipolar neurons at the anterior horn in grey substance were observed to have a near-normal histological structure (Figure 3A-D). In Group IV, spinal cord had a near-normal histological structure including axons and myelin sheaths at white substance, euchromatic nucleus, distinctive nucleus in the motor neurons, tigroid areas arising from ergostoplasmic reticulum in perikaryon at the white substance. But dark basophilic cell, nucleus and nucleolus could not be distinguished among a few cells (Figure 4A-D).

Based on the TUNEL staining; In Group I, all structures of the spinal cord were TUNEL-negative (Figure 5A, B). In Group II, TUNEL-positive staining was remarkable in the ependymal cells and glial cells of the spinal cord (Figure 6A-D). In Group III, the number of TUNEL-positive ependymal cells was partially decreased as compared with Group II and the TUNEL-positive glial cells were partially located especially in the posterior horn of grey substance. In Group IV, there was a remarkable decrease in number of TUNEL-positive ependymal cells and glial cells which were located especially in the posterior horn of grey substance as compared with Group II (Figure 7A, B).

Statistical Comparison

Considering the extensity of necrosis, a statistically significant difference was observed with Kruskal Wallis Oneway Analysis test ($p<0.001$). The groups were compared by Student-Newman-Keuls Method. There was a significant difference between the Group II, and Groups III and IV ($p>0.001$).

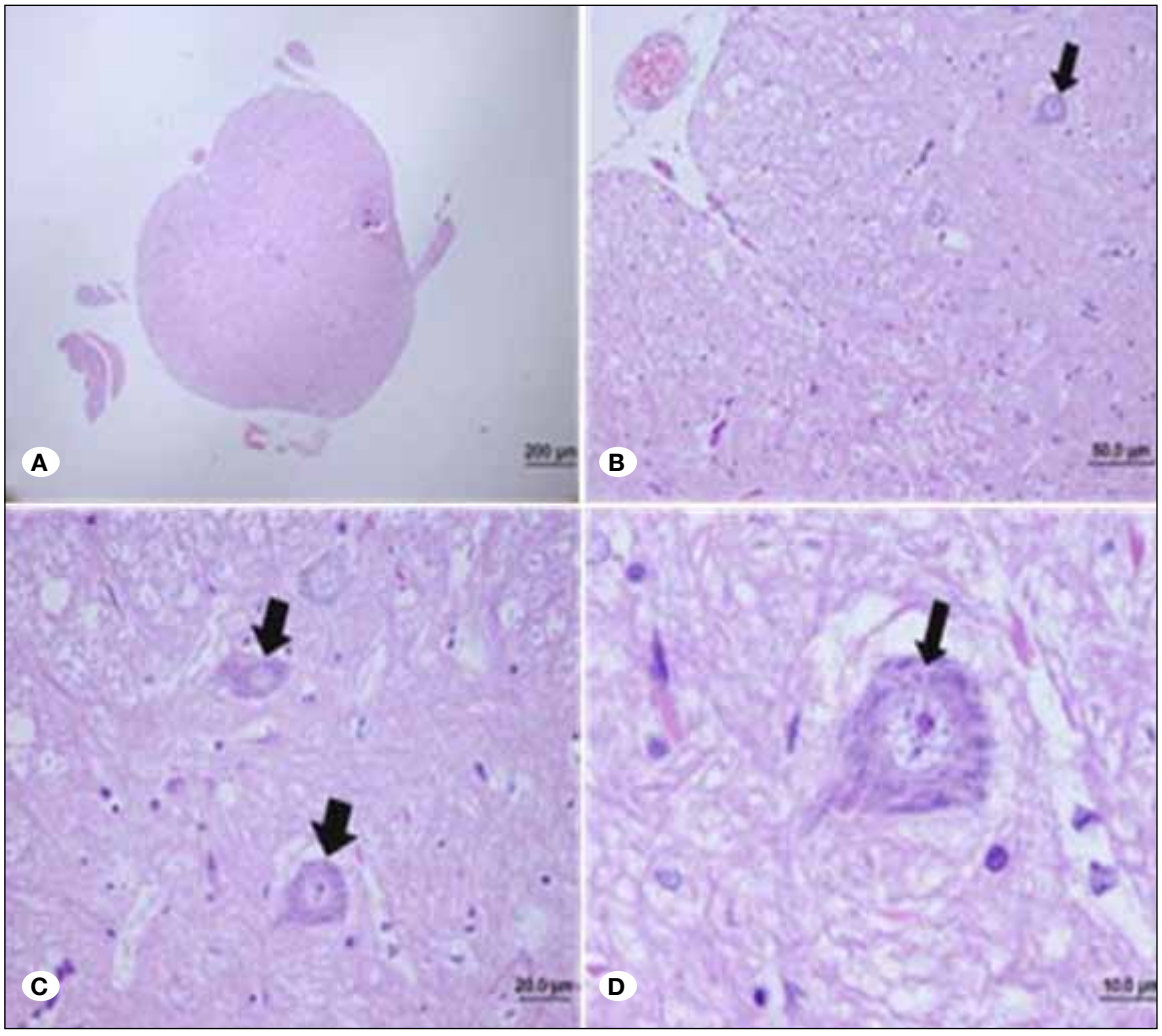

Figure 1A-D: In the control group (Group I), white matter structure of spinal cord and axons and myelin sheathes and multipolar motor neurons (→) located at anterior horn in grey substance seem normal together with euchromatic nucleus, distinctive nucleolus and cell bodies. (bar: 200μm, bar: 50.0μm, bar: 20.0μm, bar: 10.0μm, HE).

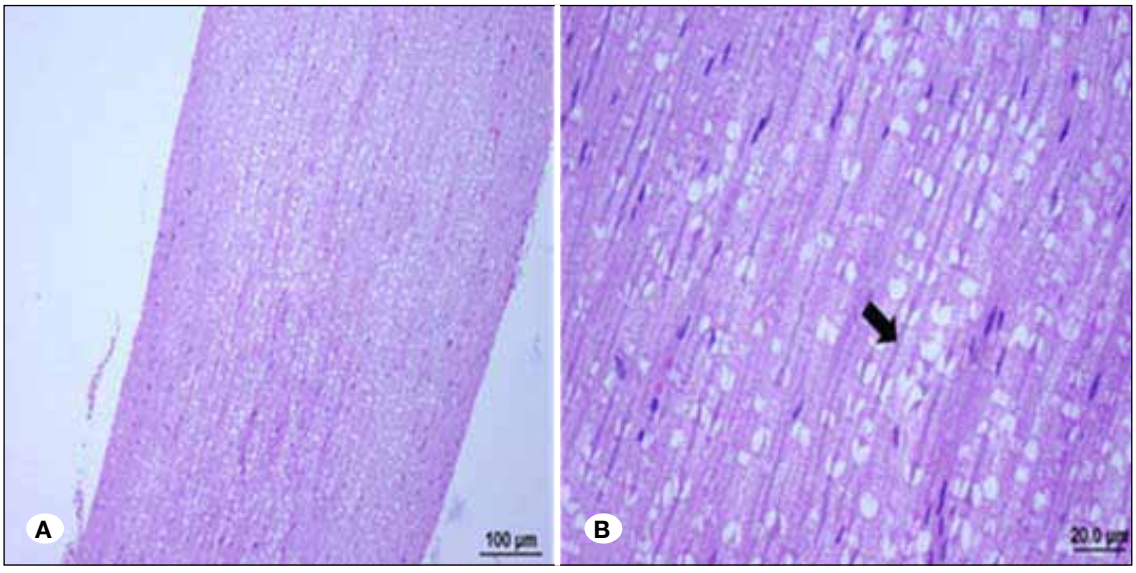

Figure 2A, B: Axons (→) and surrounding myelin sheaths are observed to have almost normal histological structure at the spinal cord in longitudinal section was observed in the control group (Group I). (bar:100µm, bar:20.0µm, HE).

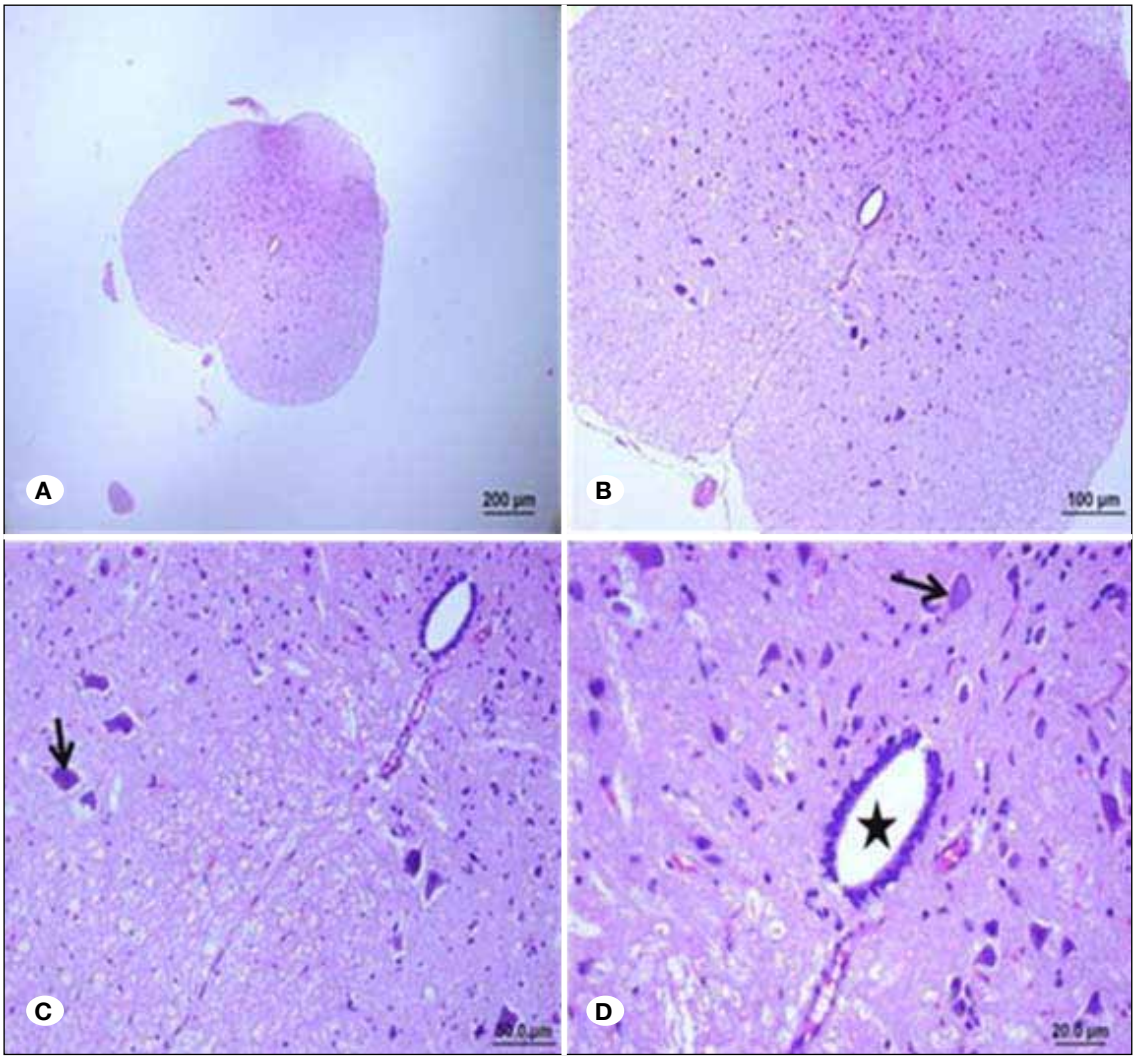

Figure 3A-D: In the Group III (CAPE treatment group), less damage is seen. Grey substance, white substance, canalis centralis (*) and ependymal cells lining canalis centralis and multipolar neurons at anterior horn in grey substance (→) are observed to have a near-normal histological structure (bar:200µm, bar:100µm, bar:50.0µm, bar:20.0µm, HE).

CAPE treatment produced similar results with MP when used after SCI and decreased the necrosis within the spinal cord after trauma.

There was a statistically significant difference in vacuolization between the groups with Kruskal Wallis One-way Analysis Test ($p<0.001$). The groups were compared by Student-Newman-Keuls Method. A statistically significant difference was found between Group II, and Groups III and IV ($p>0.001$). CAPE treatment produced similar results with MP when used routinely after SCI and decreased the vacuolization within the spinal cord after trauma.

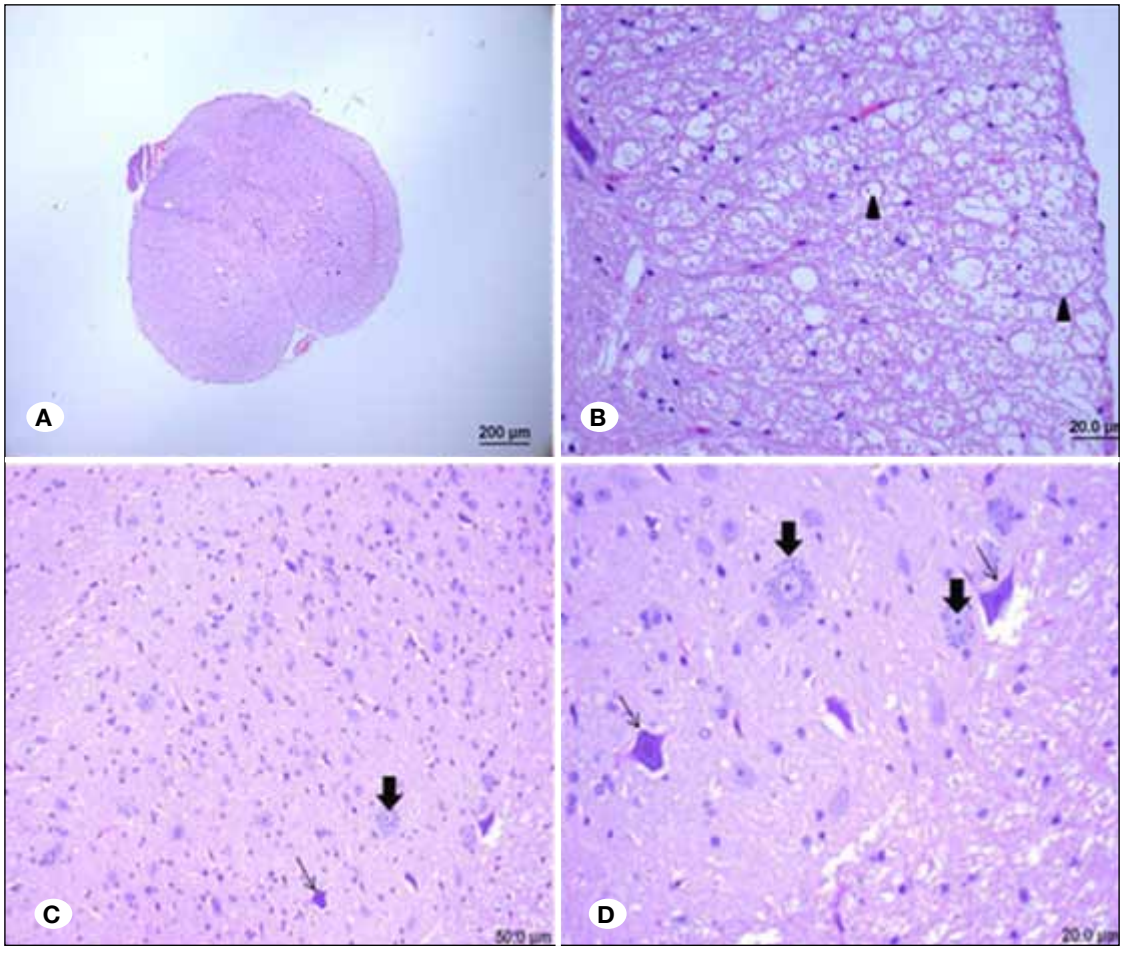

Figure 4A-D: In the Group IV (MP treatment group) medulla spinalis is observed to have near-normal histological structure (thick arrow) including axons and surrounding myelin sheaths at white substance (▶) and euchromatic nucleus, distinctive nucleus in the motor neurons and tigroid areas arising from ergostoplasmic reticulum in perikaryon at white substance. It is remarkable that dark basophilic cell, nucleus and nucleolus could not be distinguished among a few cells (→) (bar:200µm, bar:20.0µm, bar:50.0µm, HE).

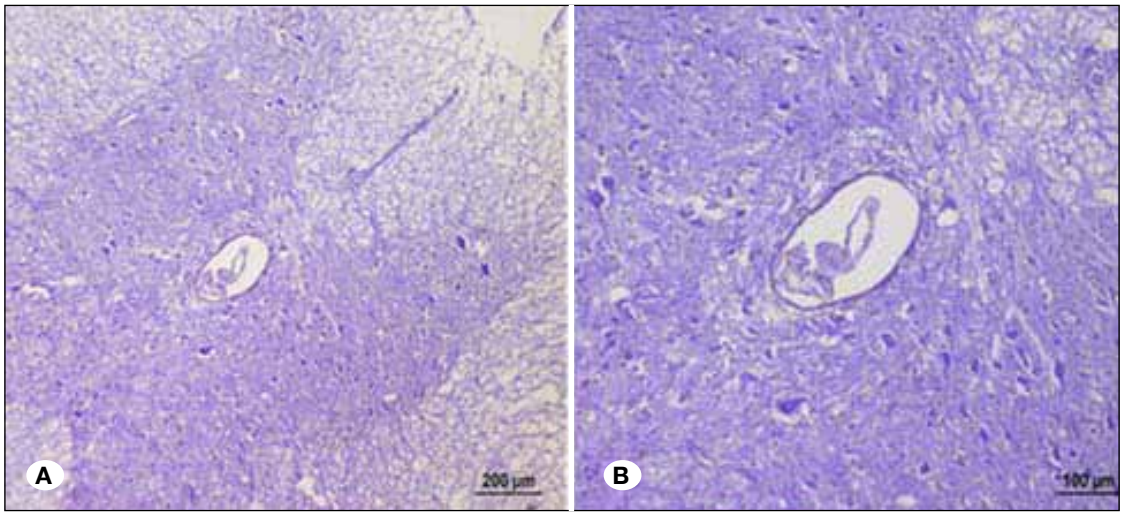

Figure 5A,B: All structures at spinal cord in Group I are TUNEL-negative (bar:200µm, bar:100µm, TUNEL).

Considering the extensity of hemorrhage, a statistically significant difference was observed with Kruskal Wallis Oneway Analysis Test ($p<0.001$). The groups were compared by Student-Newman-Keuls Method. A significant difference was determined between the Group II, and Groups III and IV ($p>0.001$). Based on this comparison, it seems that CAPE was as effective as MP in the treatment of SCI.

The numbers of apoptotic cells were non-normally distributed. Kruskal Wallis Oneway Analysis test was used to compare the apoptotic cells and there was a statistically significant difference ($p<0.001$). The groups were compared by Tukey test. The results showed that CAPE and MP prevented apoptosis after SCI and decreased the cell damage. Furthermore, CAPE

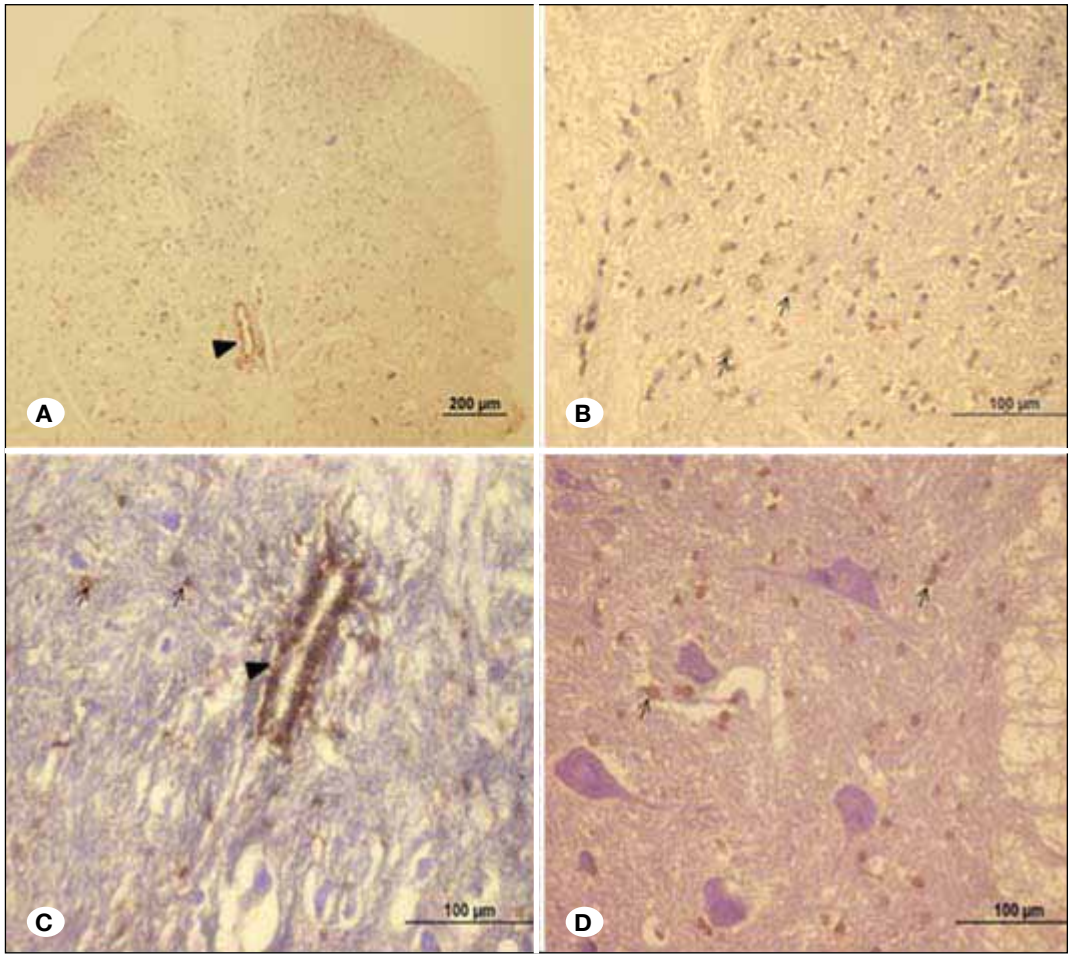

Figure 6A-D: Ependymal cells (▶) and glia cells (arrow) at the spinal cord are TUNEL-positive in Group II (only trauma group) (bar:200µm, bar:100µm, TUNEL).

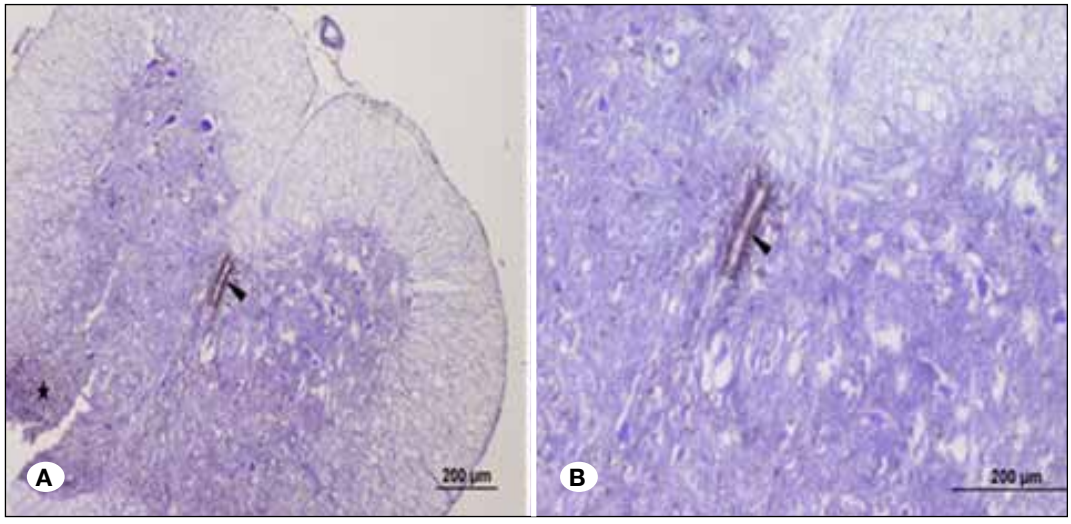

Figure 7A, B: In Group III, cells stained with TUNEL positive are partially ependymal cells (▶). The number of these cells are decreased as compared with Group II. TUNEL staining was partially positive at glial cells (*) located especially at posterior horn in grey substance. The number of these cells are remarkable at the spinal cord image of the group treated with MP), (bar:200µm, TUNEL).

was more effective in suppressing apoptosis than MP and this was statistically significant ($p<0.001$).

DISCUSSION

Spinal cord injuries are important health problems today. They have a high prevalence especially among the young and productive people, and cause great economic losses (1,4,7,11). Notably, MP, gangliosides, opiate receptor antagonists and nimodipine are the medications which have been approved and used actually for the treatment of SCI (2,11,23). Currently, administration of MP in the first 8 hours following SCI is known to be most useful treatment option. Many pharmacological agents have been tried in large, prospective, randomized and controlled clinical studies. But these agents are not in clinical use despite their success in laboratory studies. Now, the prevention from the development of secondary injury is experimentally possible thanks to the neuroprotective treatments (12,15,25,26,27).

Pharmacological agents were compared with MP in the literature. In a recent study, combined medication was found to be superior to the use of MP alone (27). It was also shown that the intravenous bone marrow stem cells administration accelerates spinal cord regeneration and remyelination in SCI model, cerebrovascular cases and degenerative brain diseases (17). Therefore, stem cells, which may reproduce and immigrate to the spinal cord, is thought to recover the irreversible neurological deficits after SCI (10).

CAPE is the most active form of propolis. It prevents neutrophil migration to the inflammation region (19,23). Antiapoptotic characteristics of propolis are effective on the prevention of tumor formation. This effect is the results of the action of CAPE and other active component called as flavanoid (7,15,17,21). CAPE has been also shown to be effective on the ischemia-reperfusion models of the brain, kidney and liver (5,15). Prevention of apoptosis decreases injury and cancer development in different organs like skin and liver (23).

Functional improvement in locomotor activity after the use of propolis has been reported in experimental SCI models (9). Propolis have a great number of active components and it has positive effects on SCI over CAPE (15). Therefore, in our study, we used CAPE, an active form of propolis, as a medical treatment of SCI.

The decrease in spinal cord blood flow has been tought as a most important cause of injury. Endorphins decrease the blood flow in the spinal cord and they are the responsible of vasoconstriction after the SCI (10,11). Based on this mechanism, opiate antogonists have been introduced to block the vasoconstrictive effects of endorphines, to increase the spinal cord blood flow and to prevent neurological injury (6).

Seckin et al. (22) showed that overdose MP therapy increases regional blood flow and decreases lipid peroxidation in rats. Hypotension develops in rats after SCI as long as the dose of MP increases (22). Similar with these findings, we observed that the number of apoptosis and necrotic cells decreased in MP treatment group as compared with only trauma group. Cell death due to lipid peroxidation is important in secondary injury and this was not observed in our MP treatment goup since MP prevents lipid peroxidation. But MS has serious side effects like gastrointestinal hemorrhage and infection. This lead researchers to develop new effective medications for the treatment (23). In our study, we used CAPE which has no side-effects like MP and it was targeted to prevent the destruction in the injured area of spinal cord.

Ilhan et al. (9) created SCI model on the rabbits and showed that CAPE prevents free radical formation and endothelial cell damage. Effects of CAPE on apoptosis are dose-dependent (8,9). Serum cytokine levels also rise together with the histopathological changes in spinal cord ischemia-reperfusion injury. Similarly, no hemorrhage, vacuolization and necrosis development have been observed in the spinal cord 24 hr after the trauma (4).

MP is globally in use in the treatment of SCI because of its antioxidant, anti-inflammatory and antiedema effects (11). It decreases neural tissue damage and cell death in acute and subacute periods of the SCI, as also shown in our study (3,24).

Apoptosis is a parameter which is currently used in experimental SCI studies (12,14,20,21,23,27). MP and ginkgolid B are effective on the prevention of apoptosis and decrease secondary injury after SCI (27,30). Their effects are over JAK/STAST signaling pathway (26). In our study, we also showed the effects of MP on apoptosis histologically.

CONCLUSION

CAPE has a positive effect on experimental spinal cord injury in rats. The decrease in numbers of apoptotic cells with CAPE treatment was higher than the decrease with MP treatment. A distinctive anti-apoptotic effect on SCI was shown by CAPE treatment. MP is the most effective agent on resolving the edema, but CAPE has been found to be superior in preventing apoptosis. Further studies are needed for the clinical reflections of this information.

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