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Original Investigation

Effects of Resveratrol on Inflammation and Apoptosis After Experimental Spinal Cord Injury

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ABSTRACT

AIM: To determine the effects of resveratrol on inflammation and apoptosis after experimental spinal cord injury (SCI).

MATERIAL and METHODS: Eighteen Sprague-Dawley rats were randomly divided into three groups. All groups underwent thoracic laminectomy. The first group received no other intervention. The second and third groups suffered SCI via the aneurysm clip compression method, and additionally the third group received resveratrol. After euthanizing the rats, immunohistochemical analysis and biochemical parameters of tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1β were measured.

RESULTS: The resveratrol group had statistically significant lower levels of TNF-α, IL -1β, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) positive cells and higher number of glial and motor neuron cells.

CONCLUSION: Resveratrol proves to have remarkable neuroprotective effects on SCI in an experimental model in addition to its proven cardioprotective effects.

KEYWORDS: Neuroprotection, Neuroregeneration, Rat, Resveratrol, Spinal cord injury, TUNEL

INTRODUCTION

Spinal cord injury (SCI) is a catastrophic, multistep cascade that causes various symptoms and morbidities. It was first defined in the ancient Egyptian Edwin Smith Papyrus from 2500 BC (16) and was thought to be untreatable with mortality rates reaching up to 80% until the 20th century (9). Developments in imaging, surgery, medicine, and rehabilitation contributed to a considerable improvement in SCI management. Neuroprotection and neuroregeneration are subjects of intense interest given the inevitability of the primary injury caused by trauma (21).

SCI is categorized into two phases: the primary phase in which the direct trauma of contusion, shear injury, or compression causes a disturbance in the neural tissue and the secondary phase which starts within minutes after the initial trauma. In the secondary phase, various mechanisms such as inflammation, edema, ischemia, and hypoxemia cause gradual deterioration (12). Several studies on SCI focus on preventing the damage caused by the secondary phase.

3,5,4′-trihydroxy-trans-stilbene (Resveratrol), a stilbenoid, is a natural phenol and a phytoalexin found in grapes, raspberries, blueberries, and peanuts, which functions in response to

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trauma or injury (10). It was first discovered in Japan in 1939 (25), and has become popular and even labeled as the "miraculous molecule" at the beginning of the 21st century. Leading pharmaceutical brands have focused on the molecule; numerous studies have investigated its effects on cancer (4,6), Alzheimer's disease (26), heart diseases (24), diabetes (15), and depression (22). Subsequently, resveratrol has become popular along with red wine as it contains the highest level of trans-resveratrol (23).

Resveratrol diminishes the oxidation of low-density lipoproteins by chelating copper and phagocytosis of oxygen-free radicals. Furthermore, it disrupts platelet aggregation formation, thereby decreasing the rate of thrombus formation. It alters arachidonic acid metabolism and inhibits protein kinase activities, thereby proving its anti-inflammatory, anti-bacterial, and anti-apoptotic effects (27).

Numerous studies on resveratrol have focused on its effects after SCI (7,13,14,17,20,27,29). These studies have targeted resveratrol's effects on oxidative stress, anti-inflammatory process, and apoptosis. During the subacute phase of SCI, rapid and extreme oxidative stress causes necrosis of neurons, leading to diminished blood flow followed by an inflammatory response. In addition, apoptosis worsens the primary injury by obliterating neurons, oligodendrocytes, microglia, and astrocytes (29). Thus, the present study focuses on resveratrol's effects on tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), and motor neuron, glial cell, and apoptotic cell counts after experimental spinal trauma on rats.

MATERIAL and METHODS

The experimental procedures on the subjects were performed at the TICAM Experimental Animals Laboratory of Osmangazi University School of Medicine. Tissue preparation for histological analysis was carried out at the Department of Pathology, Hitit University, and immunohistochemical analysis was performed at the Department of Pharmacology, Osmangazi University. The experiment was approved by the Experimental Animals Ethical Committee of Osmangazi University (384/2014).

A total of 18 female Sprague-Dawley rats weighing 200–250 g were used. They were divided randomly and equally into three groups: control, trauma, and resveratrol. The animal subjects received 60 mg/kg of ketamine hydrochloride intraperitoneally (IP) (Alfamine 10%, Egevet Veterinary Services, Turkey) and 5 mg/kg of xylazin (Alfazyne 2%, Egevet Veterinary Services, Turkey) for general anesthesia under the control of a veterinary physician.

Experimental Procedure

Animals were fixed in the prone position on a board. A midline incision at about the T9 level was performed. After the T8–10 laminectomy, the spinal cord was exposed. The spinal cord of the control group did not undergo any further intervention. The spinal cord of those in the trauma and resveratrol groups was compressed using the aneurysm clip compression technique for 60 seconds; described first by Dolan in 1979 (8). Yasargil's aneurysm clip (FE 740 K) with a closing pressure of 1.43 N was used. The resveratrol group received 10 mg/kg of resveratrol IP after the trauma. At the end of the experiment, the animal subjects were given standard bait and free access to tap water and kept in separate cages at 22°C. The bladders were manually emptied, and bait was kept close to the rats as they were all paraplegic. They were euthanized after 24 hours under the same anesthesia through decapitation, and sections of the vertebral colon from T7 to T11 were removed for further analysis.

Enzyme-Linked Immunosorbent Assay (ELISA)

Blood was drawn from the animal subjects before euthanasia to measure inflammatory cytokines released in response to trauma. Blood samples were analyzed for TNF-α and IL-1β levels at the Department of Pharmacology, Osmangazi University, via ELISA (eBioscience, Bender MedSystems, Vienna, Austria).

Histological Analysis

All tissue samples were fixed for 24 hours in 10% formaldehyde at the Department of Pathology, Hitit University. Tissue samples were embedded in paraffin, and 5-μm slices were obtained. These sections were stained using hematoxylin-eosin (H&E), glial fibrillary acidic protein (GFAP), and terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) methods.

A light microscope (Nikon E200) under 5× magnification field (×400) was used; neurons and glial cells were counted under each magnification field. GFAP antibody (Biogen) was used via an automated immunohistochemistry stainer (Ventana). GFAP staining allows visualization of GFAP to confirm the presence of glial cells that increase in number secondary to trauma or malignancy (11). The TUNEL method allows the labeling of deoxyribonucleic acid (DNA) of cells that are undergoing apoptosis by binding TdT to DNA fragments, which are labeled with a marker. The number of apoptotic cells was counted under light microscope (Nikon E200, ×400). Analysis was carried out using computer software by pathologists blinded to the experiment.

Statistical Analysis

Sigmastat 3.5 (Sysstat Software Inc.) and IBM SPSS Statistics for Windows 21.0 (IBM Corp) were used to analyze the data. Descriptive statistics were presented as mean ± standard deviation. Given that the comparison of TNF-α and IL-1β levels yielded a normal distribution, one-way analysis of variance (ANOVA) was performed. Tukey's test was used for multiple comparison tests. Cell counts were compared by using Mann–Whitney U test.

Cell counts were compared using Mann–Whitney U test via IBM SPSS Statistics for Windows 16.0 to analyze the data obtained from histological analysis.

RESULTS

Biochemical Findings

TNF-α and IL-1β levels in all groups were measured to evaluate the extent of immune response and correlated apoptosis. The

resveratrol group had statistically significant lower level of cytokines than the trauma group.

Comparison results showed that the TNF-α means of the trauma group (684.167) is higher and statistically significant than those of the control (45.500) and resveratrol groups (194.167) ($p<0.001$). However, no statistically significant results were found between the TNF-α means of the resveratrol and control groups ($p=0.110$) (Tables I, II).

Comparison results showed that the IL-1β means of the trauma group (791.667) are higher and statistically significant than those of the control (29.167) and resveratrol groups (137.500) ($p<0.001$). However, no statistically significant results were found between the IL-1β means of the resveratrol and control groups ($p=0.027$) (Tables III, IV).

Histopathological Findings

Histological analyses of all animal subjects concerning motor neuron, glial cell, and apoptotic cell counts were performed. The resveratrol group had a statistically higher count of motor neuron and glial cells, but no TUNEL positive-stained cells.

Table I: One Way Analysis of Variance – TNF-α

	N	missing	Mean	SD	SEM
Control	6	0	45.500	10.858	4.433
Trauma	6	0	684.167	193.143	78.850
Resveratrol	6	0	194.167	57.829	23.608

Source of Variation	DF	SS	MS	F	p
Between Groups	4	1409587.533	352396.883	34.740	**<0.001**

The differences in the mean values among the groups are greater than would be expected by chance; there is a statistically significant difference (p=<0.001).

Table II: Comparison of Means in Accordance with TNF-α using ANOVA Tukey's Test

Comparison	Diff of Means	q	p
Trauma vs. Control	638.667	15.533	**<0.001**
Trauma vs. Resveratrol	490.000	11.917	**<0.001**
Resveratrol vs. Control	148.667	3.616	0.110

Table III: One Way Analysis of Variance – IL 1β

	N	missing	Mean	SD	SEM
Control	6	0	29.167	7.360	3.005
Trauma	6	0	791.667	93.095	38.006
Resveratrol	6	0	137.500	60.725	24.791

Source of Variation	DF	SS	MS	F	p
Between Groups	4	2128188.333	532047.083	156.400	**<0.001**

The differences in the mean values among the groups are greater than would be expected by chance; there is a statistically significant difference (p=<0.001).

Table IV: Comparison of Means in Accordance with IL-1β using ANOVA Tukey's Test

Comparison	Diff of Means	q	p
Trauma vs. Control	762.500	32.023	**<0.001**
Trauma vs. Resveratrol	654.167	27.473	**<0.001**
Resveratrol vs. Control	108.333	4.550	**0.027**

When the motor neuron cells in the trauma group (4.06) were compared with those of the control (13.05) and resveratrol (9.05) groups, the results were statistically significant (p= 0.001 and 0.006). However, when the control and resveratrol groups were compared, no statistically significant difference was found (p=0.472). Resveratrol protected the number of motor neuron cells significantly than the trauma group (Figure 1A-D).

When the glial cells in the trauma group (4.5) were compared with the control (13.5) and resveratrol (8.5) groups, the results were statistically significant (p=0.000 and 0.02). Moreover, the number of glial cells in the resveratrol group was lower and statistically significant than the control group (8.5 vs. 13.5) (p=0.02) (Figure 2A-C).

TUNEL-positive-stained apoptotic cells were observed mainly in the trauma group, whereas the control and resveratrol groups did not show any significant positive staining (Figure 3A-D).

■ DISCUSSION

The results obtained from this study sheds novel insights into the pathology and potential treatment of SCI. This study supports resveratrol's anti-inflammatory and anti-apoptotic effects through biochemical and immunohistochemical analyses. Resveratrol had a great effect on reducing pro-inflammatory cytokines TNF-α and IL-1β, and the effects were visualized through histological examination, which showed increased glial and motor neuron cell counts with decreased apoptosis compared with the trauma group.

Resveratrol diminishes the oxidation of low-density lipoproteins by eliminating oxygen-free radicals. It proves to have anti-inflammatory, anti-bacterial, and anti-apoptotic effects by altering arachidonic acid metabolism and inhibiting protein kinase activities (27). Moreover, a previous study has reported this effect along with alleviating hemorrhage and edema in neural tissue while also reversing tissue necrosis and apoptosis (29). In addition, another report revealed resveratrol's effect of partially restoring normal functions in neurons, most

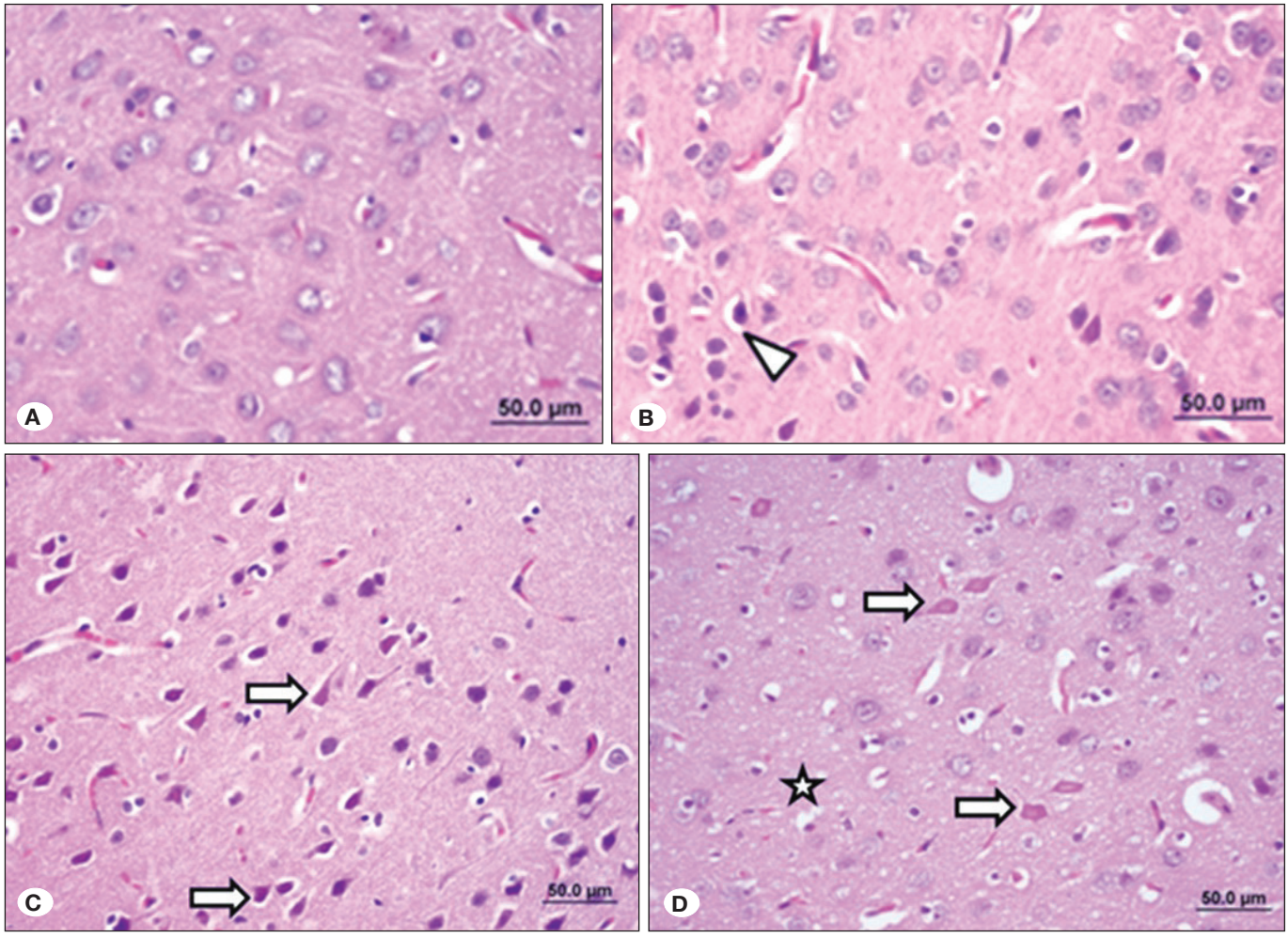

Figure 1: Hematoxylin & Eosin Stain: Motor Neuron Cell Count. Spinal cord tissue morphology with hematoxylin & eosin staining (H&E, scale bar: 50.0 µm). **A)** Control Group**:** Section through the spinal cord showing normal distribution of cells. **B)** Resveratrol Group: Generally normal distribution of cells along with minimal necrotic neurons (Δ) with pericapillary edema. **C,D)** Trauma Group: Significant numbers of necrotic neurons (arrow) along with cellular loss (star) are observed.

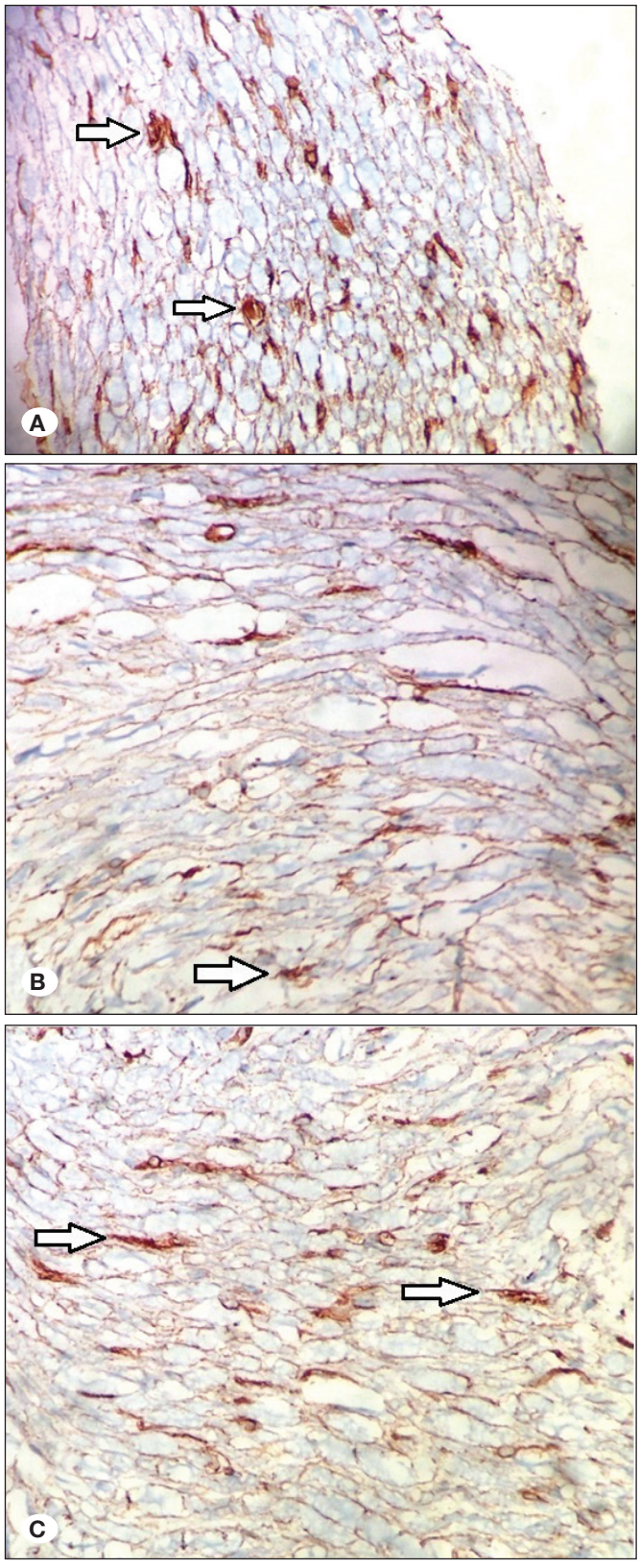

Figure 2: GFAP Stain: Glial Cell Count. Sections of the spinal cord stained with an immunoperoxidase stain for GFAP. **A)** Control group, **B)** Trauma group, **C)** Resveratrol Group. GFAP positive stains of glial cells are indicated by arrows.

notably in the areas of cellular nutrient supply and energy biosynthesis (17).

Higher doses of resveratrol (100 mg/kg IP) have shown promising anti-inflammatory effects by reducing malondialdehyde (MDA). The same study revealed that with an even higher dose (200 mg/kg IP), the antioxidant effects become more apparent by reducing xanthine oxidase and nitric oxide levels while increasing superoxide dismutase (SOD) activity and glutathione peroxidase (GSH) levels (3).

In the present experimental study on 18 rats, three randomized groups were used to evaluate the effects of resveratrol on spinal cord trauma. Biochemical analysis was performed to measure the levels of TNF-α and IL-1β, which have been increased after spinal trauma and majorly constituted the secondary phase of SCI, such as inflammation (1). The TNF-α means of the trauma group (684.167) was statistically higher than the resveratrol group (194.167) ($p< 0.001$). Resveratrol has been previously shown to reduce inflammation by decreasing TNF-α-induced activation of mitogen-activated protein kinases and nuclear factors that eventually reduced expression of intercellular adhesion molecule 1 (ICAM-1) and adhesion of monocytes (18). The biochemical parameters obtained in the present study in parallel with the recent literature has shown resveratrol's anti-inflammatory effects on spinal cord after trauma, which may prove to be beneficial in reducing the damage caused by the secondary phase of SCI.

A pilot study conducted on 23 patients with SCI revealed that both TNF-α and IL-1β levels were correlated with recovery status as patients with better overall outcome had lower levels of both cytokines (5). This is the reason that the present study focused only on these two cytokines. The results obtained in the present study also reported a statistically significant ($p<0.001$) lower value of IL-1β in the resveratrol group (137.500) than the control group (791.667). Another study that has focused on inflammation and apoptosis after SCI has stressed the importance of upregulated cytokines beginning as early as 30 minutes after the initial trauma starts an inflammatory cascade that develops exponentially, leading to the destruction of the remaining healthy neural tissues (28). Thus, several studies have focused on reducing the overexpressed inflammation after SCI, and the present study support resveratrol's effects on reducing both inflammation and apoptosis. A recent comprehensive paper proposed two different treatment modalities to evaluate the effects of resveratrol on traumatic brain injury on rats. Low- and high-dose treatments were given daily (50 and 100 mg/kg per day). Multiple biochemical analyses were performed to measure MDA, SOD, GSH, and 8-OHdG/106 dG (oxidative DNA damage) levels. All parameters were found to be higher in the trauma group, suggesting the protective and possible healing effect of resveratrol after traumatic brain injury. No statistically significant difference was found between the high- and low-dose treatments, suggesting that resveratrol may prove beneficial even at low doses (2). The results obtained from the present study proved the beneficial results of resveratrol even at 10 mg/kg dose.

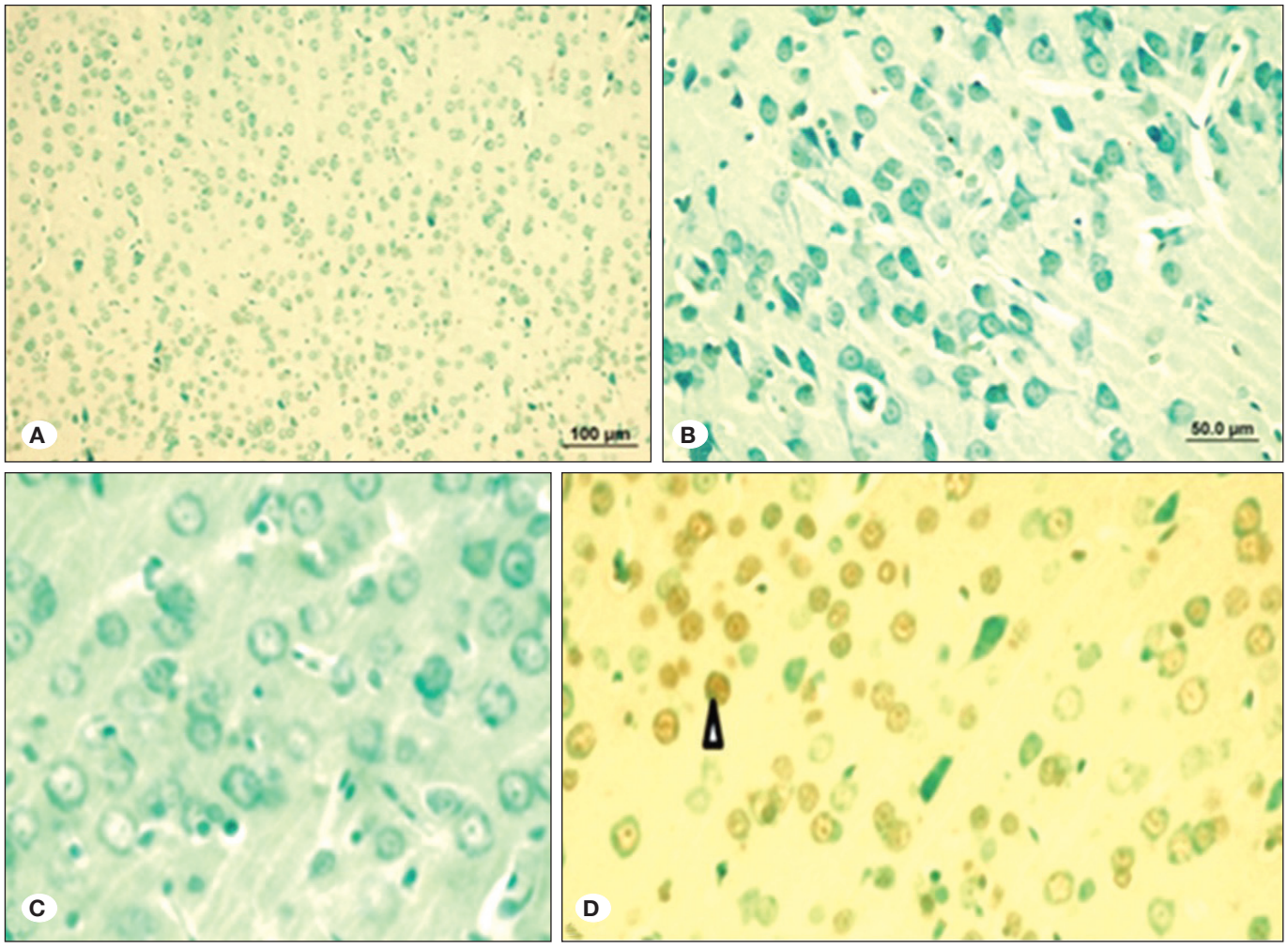

Figure 3: TUNEL Assay: Apoptotic Cell Count. TUNEL stained sections of the spinal cord. **A, B)** Resveratrol group: Negative staining of TUNEL (scale bar: 100, 50.0µm). **C)** Control group: Negative TUNEL staining (scale bar: 50.0µm). **D)** Trauma group: TUNEL positive stained cells indicated by arrow (scale bar: 50.0µm).

The TUNEL staining method allows the labeling of DNA of cells that are undergoing apoptosis by binding TdT to DNA fragments that are labeled with a marker. In a study using electron microscope identification and TUNEL staining, the resveratrol group showed a statistically significant decreased number of TUNEL-positive cells. Moreover, the resveratrol group showed the effects of improving abnormal cellular morphology by the inhibition or reduction in neuronal shrinkage, disappearance of mitochondrial ridges, cytoplasm vacuolization, and enlargement of the endoplasmic reticulum, which all contribute to a cell's path toward apoptosis (17).

Resveratrol has also been shown to inhibit the upregulation of pro-apoptotic factor Bax and terminal executing enzymes for substrate cleavage of caspase-3. Moreover, the downregulation of anti-apoptotic factor Bcl-2 after SCI was inhibited (17). Another study reported that resveratrol could prevent apoptosis in neurons by activating the PI3K/Akt pathway (30). These effects can be contributed to the anti-apoptotic effect of resveratrol. A larger series of subjects evaluated resveratrol's effects on transient cerebral ischemia in 60 rats; similar to the present study, TUNEL staining was obtained along with H&E, NeuN analysis, and Western Blot to determine Bcl-2, Bax, and caspase-3 levels. The rats received 30 mg/kg IP of resveratrol similar to the present study but were given treatment consecutively for 7 days before the procedure (19). This crucial study supports resveratrol's neuroprotective effects by inhibiting neuronal apoptosis. In addition, the administration of resveratrol before the procedure may prove its prophylactic effect on cerebral ischemia.

The present experimental study provides support for the anti-apoptotic mechanisms of resveratrol through histological analysis. The resveratrol group had no TUNEL-positive stains, whereas the trauma group had positive stains displaying cells that were undergoing apoptosis. In addition, H&E and GFAP staining showed that compared with the trauma group's motor neuron cell (4.06) and glial cell (4.5) counts, the treatment groups had a better outcome with 9.05 and 8.50, respectively. Both comparisons yielded a statistically significant result (p=0.001).

CONCLUSION

The present experimental study on rats following SCI focuses on major issues correlated with inflammation and apoptosis after the initial trauma, which constitutes the major portion of the secondary phase of SCI. Complications of SCI are catastrophic, and there is no definitive cure available. From a neurosurgical perspective, decompressing the spinal cord and stabilizing the vertebral column are long-term methods of helping the patient to recover. Many areas of multistep and complex physiological processes remain to be enlightened. Current therapeutic interventions aim at minimizing the inflammatory and apoptotic process after the initial trauma. The present experimental study supports the use of resveratrol in SCI given its anti-inflammatory and anti-apoptotic effects. Nonetheless, the present study is just a drop in the ocean as SCI is a boundless sea. The authors hope that the results obtained here support the development of new approaches in SCI treatment by providing a framework. Much work through clinical trials is needed to ascertain whether any of the therapies at hand are beneficial.

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