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Neuroprotective effects of testosterone on ischemia/reperfusion injury of the rabbit spinal cord

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ABSTRACT

Aim: Previous studies demonstrated the neuroprotective effects of testosterone, but no previous study has examined the neuroprotective effects of testosterone on spinal cord ischemia/reperfusion injury. The purpose of this study was to evaluate whether testosterone could protect the spinal cord from ischemia/reperfusion injury.

Methods: Rabbits were randomised into four groups of eight animals as follows: group 1 (control), group 2 (ischemia), group 3 (methylprednisolone) and group 4 (testosterone). In the control group only a laparotomy was performed. In all other groups, the spinal cord ischemia model was created by the occlusion of the aorta just caudal to the renal artery. Levels of malondialdehyde and catalase were analysed, as were the activities of caspase-3, myeloperoxidase, and xanthine oxidase. Histopathological and ultrastructural evaluations were performed. Neurological evaluation was performed with the Tarlov scoring system.

Results: After ischemia-reperfusion injury, increases were found in caspase-3 activity, myeloperoxidase activity, malondialdehyde levels, and xanthine oxidase activity. In contrast, decreases in catalase levels were observed. After the administration of testosterone, decreases were observed in caspase-3 activity, myeloperoxidase activity, malondialdehyde levels, and xanthine oxidase activity, whereas catalase levels increased. Furthermore, testosterone treatment showed improved results concerning histopathological scores, ultrastructural score and Tarlov scores.

Conclusions: Our results revealed for the first time that testosterone exhibits meaningful neuroprotective activity following ischemia-reperfusion injury of the spinal cord.

Introduction

Severe neurological injury still represents one of the most catastrophic complications after thoracoabdominal aneurysm surgery, and results from the ischemia/reperfusion (I/R) injury of the spinal cord. This devastating injury may cause paraplegia up to 40% of the patients [1]. Optimal neuroprotection is the cornerstone for success of such surgeries.

Ischemic injury consists of inadequate blood supply to the spinal cord, which is aggravated by reperfusion, and results in neuronal damage [2]. The mechanisms that underlie I/R injury are complex and multifactorial. Hypoxia with energy failure, excitoxicity, oxidative stress, inflammation, lipid peroxidation and apoptosis appear to be the most important mechanisms that cause neuronal damage after spinal cord I/R injury [2–6].

Testosterone (TES), the gonadal sex steroid hormone has various effects on numerous body tissues, including central nervous system (CNS) [7]. Testosterone, due to its lipophilic structure, can pass the blood brain barrier and influence neuronal cells [8]. Testosterone acts via androgen receptors, which are found in neurons throughout the CNS [7,9,10]. Many of the theuropathic effects of TES including those on libido, cognition and mood are mediated through the CNS [11].

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One of the less known actions of TES is neuroprotection. Activation of androgen pathways cause neuronal differentiation, and increase in neurite overgrowth in cultured cells [12–14]. Other experiments in rodents suggest that TES causes an increase in neuronal stromal size, neuritic growth, plasticity and synaptogenesis in both motor neurons of spinal nucleus of the bulbocavernousus [15,16] and pelvic autonomic neurons [17]. Furthermore, Ogata et al. reported that TES had protective effects on spinal cord against neuronal damage induced by glutamate, and reduced the extent of spinal cord damage [18]. On the other hand, TES has shown to have antioxidant and antiapoptotic effects, which may further cause neuroprotective effects [10,19,20].

There are no previous studies that examine the neuroprotective effects of TES in spinal cord I/R injury. To complement previous neuroprotection studies, the purpose of this study was to evaluate whether TES could protect the spinal cord after I/R injury in rabbits. We also compared the TES with methylprednisolone (MP), which has been widely used for spinal cord injuries [21,22].

Materials and methods

Experimental groups

Animal care and all experiments were conducted following the European Communities Council Directive of November 24, 1986 (86/609/EEC) concerning the protection of animals for experimental use. All experimental procedures used in this investigation were reviewed and approved by the ethical committee of the Ministry of Health Ankara Education and Research Hospital Committee of Animal Ethics. Thirty-two adult male New Zealand white rabbits, weighing 2800–3500 g, were randomly divided into the following four groups (eight rabbits in each group):

Group 1: Control group ($n = 8$): laparotomy only. Rabbits underwent laminectomy, and non-ischemic spinal cord samples were obtained immediately after the surgery. No treatment was given to this group.

Group 2: Ischemia group ($n = 8$); Rabbits underwent transient global spinal cord ischemia. The rabbits received 2 cm^3 of saline (0.9% NaCl) intravenously immediately after the occlusion clamp was removed. The animals then underwent laminectomy, and spinal cord samples were removed 24 h post-ischemia.

Group 3: Methylprednisolone (MP) group ($n = 8$); Treated similarly to group 2, but the rabbits received a single intravenous 30 mg/kg dose of MP (Prednol, Mustafa Nevzat, Turkey) immediately after the occlusion clamp was removed. This dosage of the MP was selected based on earlier studies [2,23,24].

Group 4: Testosterone (TES) group ($n = 8$); treated similarly to group 2, but the rabbits received a single intraperitoneal dose of 15 mg/kg TES (Sustanon 250, Schering-Plough, Istanbul, Turkey; containing testosterone propionate 30 mg, testosterone phenylpropionate 60 mg, testosterone isocaproate 60 mg, and testosterone decanoate 100 mg) immediately after the occlusion clamp was removed. This dosage of TES was selected based on past studies [25,26]. The mixture of four TES esters with different half-life (testosterone propionate, testosterone phenylpropionate, testosterone isocaproate, testosterone decanoate) was preferred to provide more stable serum TES levels.

Anesthesia and surgical procedures

The animals were kept at an optimal (18–21 °C) room temperature, fed a standard diet and kept under a 12-h light–dark cycle. Free access to food and water was provided. The animals were anesthetised by intramuscular administration of 70 mg/kg ketamine (Ketalar, Parke Davis Eczacıbaşı, Turkey) and 5 mg/kg xylazine (Rompun, Bayer, Turkey) and were allowed to breathe freely. Body temperatures were measured using an anal thermometer (Digital Fever thermometer, Becton Dickinson, NJ, USA) and were maintained at 37 °C with a heating pad. Animals were placed in the supine position for the surgery. After sterile preparation, a 10-cm midline incision was made, and the abdominal aorta was exposed through a transperitoneal approach. Heparin (Nevparin, Mustafa Nevzat, Turkey) at a dose of 150 U/kg was administered intravenously 5 min before clamping for anticoagulation. Approximately 1 cm below the renal artery, the aorta was clamped under a surgical microscope using an aneurysm clip with 70 g of closing force (Yasargil, FE721, Aesculap, Germany). The cross clamp time was 20 min. At the end of the occlusion period, the clips were removed and restoration of blood flow was visually verified. The drugs were administered immediately after the clamp was removed. The rabbit aortic cross-clamping method, which was used in this study, is a useful method for these procedures. The 20 min ischemia period was chosen to achieve adequate injury [27]. The rabbits were allowed free access to food and water 2 h after surgery. Crede's manoeuvre was performed on animals with a neurogenic bladder at least two times a day. The animals were sacrificed 24 h after the operation by injection of 200 mg/kg pentobarbital (Nembutal, Oak Pharmaceuticals, Lake Forest, IL, USA). Spinal cord segments between L2 and L5 were carefully removed by laminectomy and used for the biochemical, histopathological and ultrastructural analyses. Blood (10 cm^3) was taken from the left ventricle for biochemical analysis. The blood samples were centrifuged at $1000 \times g$ for 5 min, and the upper clear supernatants were removed for analysis. All serum and tissue samples were stored at −80 °C until analysed. On the day of the analysis, the tissues were homogenised in physiologic saline solution and centrifuged at $1780 \times g$ for 20 min. The serum samples obtained as the upper clear supernatants of the centrifuged blood were used for the biochemical analyses.

Tissue caspase-3 activity

Caspase-3 activity was measured using an ELISA kit (Cusabio, Hubei, China), and the ELISA procedures were performed according to the manufacturer's instructions. This assay employs the quantitative sandwich enzyme immunoassay technique. Antibodies specific for caspase-3 had been pre-coated onto a microplate. Standards and samples were pipetted into the wells, and any caspase-3 present was bound by the immobilised antibody. After removal of any unbound substances, a biotin-conjugated antibody specific for caspase-3 was added to the wells. After washing, avidin-conjugated horseradish peroxidase (HRP) was added to the wells. Any unbound substances had been removed by the three washes with washing buffer. Following the washing procedure, the avidin-enzyme reagent was added to the wells. The colour develops in proportion to the amount of caspase-3 bound in the initial step. When the colour development stopped, the intensity of the colour was measured at 450 nm. Caspase-3 concentrations were calculated by comparing the absorbance values of the samples with those of standard caspase-3 solutions. The results are expressed in ng/ml.

Serum and tissue myeloperoxidase (MPO) activity

MPO activity was measured using an ELISA kit (Cusabio, Hubei, China), and the ELISA procedures were performed according to the manufacturer's instructions. This assay employs the competitive inhibition enzyme immunoassay technique. The microtitre plate provided in this kit was pre-coated with an antibody specific to MPO. Standards or samples were added to the appropriate microtitre plate wells with Biotin-conjugated MPO. A competitive inhibition reaction was initiated between the MPO (from the standards or the

samples) and the Biotin-conjugated MPO with the pre-coated antibody specific for MPO. With greater amounts of MPO in the samples, lower amounts of antibodies are bound by the Biotin-conjugated MPO. After washing, avidin-conjugated HRP was added to the wells. Then, the substrate solution was added, and the colour developed to indicate the amount of MPO in the sample. When colour development stopped, the intensity of the colour was measured at 450 nm. The MPO concentrations were calculated by comparing the absorbance values of the samples with those of standard MPO solutions. The results are expressed in ng/ml.

Tissue malondialdehyde (MDA) analyses

Tissue MDA levels were determined using a method based on reaction with thiobarbituric acid (TBA). Briefly, the samples were mixed with two volumes of cold saline solution containing 0.001% butylated hydroxytoluene (BHT) and 0.07% sodium dodecyl sulfate (SDS). Then, 1 ml of the samples was added to 500 μl of 0.01 μl NH2SO4 and 500 μl of the thiobarbituric acid reagent (0.67% thiobarbituric acid in 50% acetic acid) to precipitate protein. Then, the samples were heated in boiling water for 60 min. After cooling, an equal volume (2 ml) of *n*-butanol was added to each test tube and mixed. The mixture was centrifuged at 1780 × *g* for 10 min at room temperature. The absorbance of the organic layer was read at 535 nm in a 1 ml cell (Molecular Devices Corporation, Sunnyvale, CA, USA). The malondialdehyde concentrations were calculated by comparing the absorbance values of the samples with those of standard MDA solutions. The MDA concentrations are expressed in nmoles per gram of wet weight tissue.

Tissue catalase (CAT) analyses

Catalase levels were determined by measuring the absorbance decrease of hydrogen peroxide (H_2O_2) at 240 nm [28]. In the activity calculations, an extinction coefficient of H_2O_2 was used for CAT. The results are expressed in IU/ml.

Serum xanthine oxidase (XO) activity

Serum XO activity was measured by the method of Prajda and Weber, where activity is measured by the determination of the amount of uric acid formed from xanthine [29]. Serum samples (100 μl) were incubated for 30 min at 37 °C in 3 ml of the phosphate buffer (pH 7.5, 50 nM) containing xanthine (4 mM). The reaction was stopped by the addition of 0.1 ml 100% (w/v) TCA, and the mixture was then centrifuged at 1780 × *g* for 20 min. Uric acid was determined in the supernatant by measuring the absorbance at 292 nm against a blank and expressed as mIU/ml. A calibration curve was constructed using 10–50 mU/ml concentrations of standard XO solutions (Sigma X-1875, Sigma-Aldrich, St. Louis, MO). One unit of activity was defined as 1 μmol of uric acid formed per minute at 37 °C and pH 7.5.

Histopathological procedures

The cord specimens obtained at 24 h post-injury were prepared for histological study. Each cord segment was immersed in 4% paraformaldehyde in 0.1 mol/l phosphate buffer and stored at 4 °C. The specimens were then embedded in paraffin, cut into 5 μm thick sections and stained with hematoxylin-eosin (H&E). The specimens were examined under a light microscope by a neuropathologist, who was blinded to the study design. Five different fields of the grey matter of the spinal cord were evaluated using a 40× objective lens.

A semi-quantitative scoring system, ranging between 0 and 3, was used for grading the histopathological changes in the spinal cord tissues of all samples. Six different parameters (haemorrhage, congestion, necrosis, oedema, neuronal loss and inflammation) were assessed histopathologically and were scored as follows: 0 = absent, 1 = mild, 2 = moderate, and 3 = common. The pathological score for each spinal cord was calculated by averaging the scores of these six parameters [2].

In addition, a more detailed assessment of the degree of ischemic neuronal injury was also performed. For this analysis, the number of normal motor neurons in the anterior horn of the spinal cord (anterior to a line drawn through the central canal perpendicular to the vertebral axis) was counted. For each animal, three sections were evaluated using a 40× objective. An average normal motor neuron count for the sections from each animal was then determined. Neurons that contained Nissl substances in the cytoplasm, loose chromatin and prominent nucleoli were considered normal viable neurons [30].

Ultrastructural examinations

Tissue samples were cleared of blood using a scalpel and the meninges were carefully removed. The tissue samples were then fixed in 2.5% glutaraldehyde for 24 h, followed by washing in phosphate buffer (pH: 7.4). They were next post-fixed in 1% osmium tetroxide in phosphate buffer (pH: 7.4) for 2 h and dehydrated with increasing concentrations of alcohol. Then, the tissues were washed with propylene oxide and embedded in epoxy-resin embedding media. Semi-thin sections approximately 2 μm thick and ultra-thin sections approximately 60 nm thick were cut with a glass knife on a LKB-Nova ultramicrotome (LKB-Produkter AB, Bromma, Sweden). The semi-thin sections were stained with methylene blue and examined with a Nikon Optiphot (Nikon Corporation, Tokyo, Japan) light microscope. Following this examination, the tissue blocks were trimmed, and ultra-thin sections were prepared using the same ultramicrotome; these sections were stained with uranyl acetate and lead citrate. After staining, all of the ultra-thin sections were examined using a Jeol JEM 1200 EX (Jeol Ltd., Tokyo, Japan) transmission electron microscope. The electron micrographs were taken by the same transmission electron microscope at 5000× magnification. A total of 100 large-sized myelinated axons, 100 medium-sized myelinated axons and 100 small-sized myelinated axons were evaluated for every sample. They were scored from 0 to 3 and counted, and the data are presented as the mean values, as described by Kaptanoğlu et al. [31].

The scoring system was as follows:

0: Ultrastructurally normal myelinated axon.
1: Separation in myelin configuration.
2: Interruption in myelin configuration.
3: Honeycomb appearance in myelin configuration.

Neurologic evaluations

The neurologic statuses of the animals were scored 24 h after the procedure by assessing hind-limb neurologic function using the modified Tarlov Scoring System [2,24]. A score from 0 to 5 was assigned to each animal as follows: 0 = no voluntary hind-limb movement, 1 = movement of joints perceptible, 2 = active movement but unable to sit without assistance, 3 = able to sit but unable to hop, 4 = weak hop and 5 = complete recovery of hind-limb function. A medical doctor blinded to the experimental groups performed the neurologic evaluations.

Statistical analyses

Data analysis was performed using SPSS for Windows, version 11.5 (SPSS Inc., Chicago, IL, United States). The Shapiro Wilk test

was used to determine if the continuous variables were normally distributed, and the Levene test was used for the evaluation of the homogeneity of the variances. Where applicable, the data are shown as the means ± standard deviation or the medians (IQR). The mean differences among groups were analysed using One-Way ANOVA. Otherwise, the Kruskal Wallis test was applied for comparison of the median values. When the p value from the One-Way ANOVA or the Kruskal Wallis test was statistically significant, post hoc Tukey HSD or Conover's non-parametric multiple comparison tests were used to evaluate the differences between the various groups. A p value less than 0.05 was considered statistically significant.

Results

Tissue caspase-3 analyses

There was a statistically significant difference between the control and the ischemia groups in the means of their caspase-3 activity ($p < 0.001$). When the TES group was compared with the ischemia group, there was a statistically significant decrease in caspase-3 activities ($p < 0.001$). Similar to the TES group, the MP group also showed a statistically significant decrease in caspase-3 activities when compared to the ischemia group ($p = 0.001$). There was no statistically significant difference between the TES and the MP groups ($p = 0.698$).

Serum and tissue myeloperoxidase (MPO) analysis

Statistically significant differences were observed between the control and the ischemia groups with regard to the mean serum and tissue MPO levels ($p < 0.001$ for both). Treatment with TES led to a statistically significant decrease in the MPO levels both in serum and tissue when compared to the ischemia group ($p < 0.001$ for both). As in the TES group, the MP group showed a statistically significant difference in their serum and tissue MPO levels when compared to the ischemia group ($p < 0.001$ for both). There were no statistically significant differences between the MP and the TES groups for both the serum and tissue MPO levels ($p = 0.654$ and $p = 0.096$, respectively).

Tissue malondialdehyde (MDA) analyses

When the mean tissue MDA levels were compared between the control and the ischemia group, statistically significant differences were observed ($p < 0.001$). When we compared the ischemia and the TES groups, there was a statistically significant difference between these groups ($p = 0.001$). As in the TES group, a comparison between the MP and the ischemia groups revealed a statistically significant difference in the MDA levels ($p < 0.001$). When the TES and the MP groups were compared, no statistical significance was found ($p = 0.98$).

Tissue catalase (CAT) analyses

When the mean tissue CAT levels of the control group were compared with the ischemia group, there were statistically significant differences observed ($p < 0.001$). Treatment with TES was associated with statistically significant increases in the tissue CAT levels ($p < 0.001$). As in the TES group, MP was also associated with statistically significant increases in the CAT levels in tissue ($p < 0.001$). There were no differences between the MP and the TES groups in the tissue CAT levels ($p = 0.516$).

Serum xanthine oxidase (XO) analyses

Serum XO activity was linked to a statistically significant increase in the ischemia group compared with the control group ($p < 0.001$). In the TES group, serum XO activity was significantly decreased when compared to the ischemia group ($p < 0.001$). Similar to the TES group, the XO activity of the MP group showed a statistically significant decrease compared to the ischemia group ($p = 0.004$). Furthermore, in the TES group XO levels were decreased to statistically significantly lower levels when compared to the MP group ($p = 0.001$).

All of the biochemical results are summarised in Table 1.

Histopathological procedures

Light microscopic examinations of the spinal cord samples from the control group were normal (Fig. 1a). In the ischemia group (Fig. 1b), diffuse haemorrhage and congestion in the grey matter were observed 24 h after the I/R injury. There was marked necrosis and widespread oedema in both the white and grey matter. In the damaged areas, there were infiltrating polymorphonuclear leukocytes, lymphocytes, and plasma cells observed. Neuronal pyknosis, a loss of cytoplasmic features and cytoplasmic eosinophilia, were also observed in the ischemia group. In the TES group as well as in the MP group, the cord tissues were protected well from I/R injury (Fig. 1c and d).

When the pathological scores were compared, the ischemia group showed statistically higher scores than the control group ($p < 0.001$). In the TES group, the pathological score was significantly lower than that for the ischemia group ($p = 0.019$). In the MP group, the pathology score was significantly lower than the ischemia group ($p < 0.001$). The difference between the MP and the TES groups was not statistically significant ($p = 0.104$) (Fig. 2a).

In the ischemia group the number of normal motor neurons in the anterior spinal cord was significantly decreased compared with that of the control group ($p < 0.001$). In the TES group, the number of normal motor neurons in the anterior spinal cord was

Table 1
Biochemical alterations among the groups.

Variables	Control	Ischemia	MP	TES	p-Value
Tissue caspase-3 (ng/ml)	$1.43 \pm 0.85^{a,b}$	$5.01 \pm 1.18^{a,c,d}$	$3.72 \pm 0.79^{b,c,e}$	$1.97 \pm 0.46^{d,e}$	<0.001
Serum MPO (ng/ml)	$1.34\ (0.61)^{a,b,f}$	$4.22\ (1.28)^{a,c,d}$	$2.59\ (0.67)^{b,c}$	$2.58\ (0.58)^{d,f}$	<0.001
Tissue MPO (ng/ml)	$2.47\ (0.81)^{a}$	$4.83\ (0.77)^{a,c,d}$	$3.17\ (1.50)^{c}$	$2.39\ (1.15)^{d}$	<0.001
Tissue MDA (nmol/g tissue)	2.39 ± 0.98^{a}	$5.21 \pm 0.94^{a,c,d}$	3.18 ± 0.86^{c}	3.35 ± 0.70^{d}	<0.001
Tissue CAT (IU/ml)	$168.36\ (77.99)^{a,b,f}$	$53.55\ (34.00)^{a,c,d}$	$117.12\ (29.28)^{b,c}$	$114.70\ (24.59)^{d,f}$	<0.001
Serum XO (mIU/ml)	$0.05\ (0.07)^{a,b}$	$0.36\ (0.15)^{a,c,d}$	$0.12\ (0.03)^{b,c,e}$	$0.05\ (0.03)^{d,e}$	<0.001

[a] Control vs. Ischemia ($p < 0.001$).
[b] Control vs. MP ($p < 0.001$).
[c] Ischemia vs. MP ($p < 0.05$).
[d] Ischemia vs. Testosterone ($p < 0.05$).
[e] MP vs. Testosterone ($p < 0.01$).
[f] Control vs. Testosterone ($p < 0.05$). MP = methylprednisolone, TES = testosterone, MPO = myeloperoxidase, MDA = malondialdehyde, CAT = catalase, XO = xanthine oxidase.

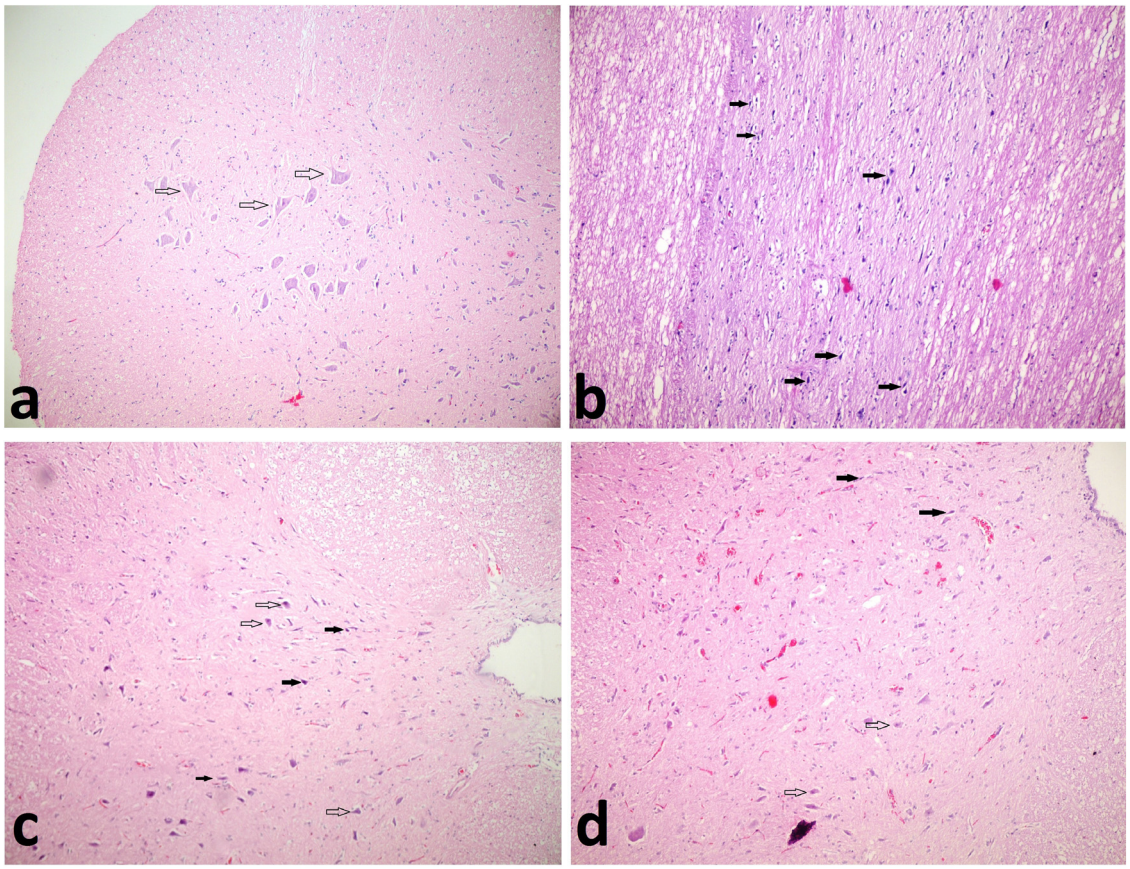

Fig. 1. Photomicrographs of spinal cord tissue sections from the different study groups (H&E, ×10 obj.).
(a) Control group showing normal spinal cord parenchyma. Normal neurons are marked with hollow arrows.
(b) Ischemia group, showing degenerated neurons (filled arrows) in the edematous surface.
(c) Testosterone group showing less degenerated neurons (filled arrows), and more normal neurons (hollow arrows). The spinal cord tissues were significantly protected from injury.
(d) Methylprednisolone group, showing less degenerated neurons (filled arrows); note the normal neurons (hollow arrows).

significantly higher when compared with that of the ischemia group ($p < 0.001$). Similar to the TES group, the MP group showed a statistically significant higher number of normal neurons when compared with the ischemia group ($p < 0.001$). The comparison between the TES and the MP groups did not show a statistical difference ($p = 1$). Histopathologically, both TES and MP protected the spinal cord from I/R injury (Fig. 2b).

Ultrastructural examinations

In the transmission electron microscopic examination of the tissue samples of the control group, no ultrastructural pathological changes were observed in the grey and white matter of the spinal cord. The neurons were ultrastructurally normal in appearance, and the intracellular organelles, nuclei, membranes and perineuronal tissues did not show any pathological changes. However, in a few of the large-sized myelinated axons mild separations were observed in a small part of the myelin sheath. This may be related to delayed fixation of the tissue. The rest of the large-sized myelinated axons and the whole medium- and small-sized myelinated axons were found to be ultrastructurally normal (Fig. 3a).

The transmission electron microscopic examination of the ischemia group showed separations in the myelin configuration of small-sized, medium-sized and large-sized myelinated axons. When all of the groups were compared, the ischemia group showed the greatest ultrastructural damage in the myelinated axons (Fig. 3b).

In the MP group, separations were observed in the myelin configuration of the medium-sized and large-sized myelinated axons. Additionally, separations in the myelin configuration in a few of the small-sized myelinated axons were observed (Fig. 3c).

In the TES group, separations were observed in the myelin configuration of large-sized myelinated axons. All of the small-sized and most of the medium-sized myelinated axons of this group were ultrastructurally normal (Fig. 3d). The ultrastructural appearances of the myelinated axons of the TES group were better than those of the MP group, which were also observed after the scoring.

Compared to the control group, the ischemia group showed greater disruption in the small-sized myelinated axons ($p = 0.008$). When compared to the ischemia group, both TES and MP protected the small-sized myelinated axons from disruption ($p = 0.008$ for both). When the TES group was compared to the MP group, the TES group revealed statistically significant improvements in terms of protecting small-sized myelinated axons ($p = 0.008$).

In the ischemia group, medium-sized myelinated axons are more injured when compared to the control group ($p = 0.008$). Similarly, there was a significant difference between both the TES and the MP groups when compared to the ischemia group, in which both TES and MP treatments protected the medium-sized axons from I/R injury ($p = 0.008$ for both). In addition, when the TES group was compared to the MP group, the TES group revealed statistically significant improvements in terms of protecting medium-sized myelinated axons ($p = 0.008$).

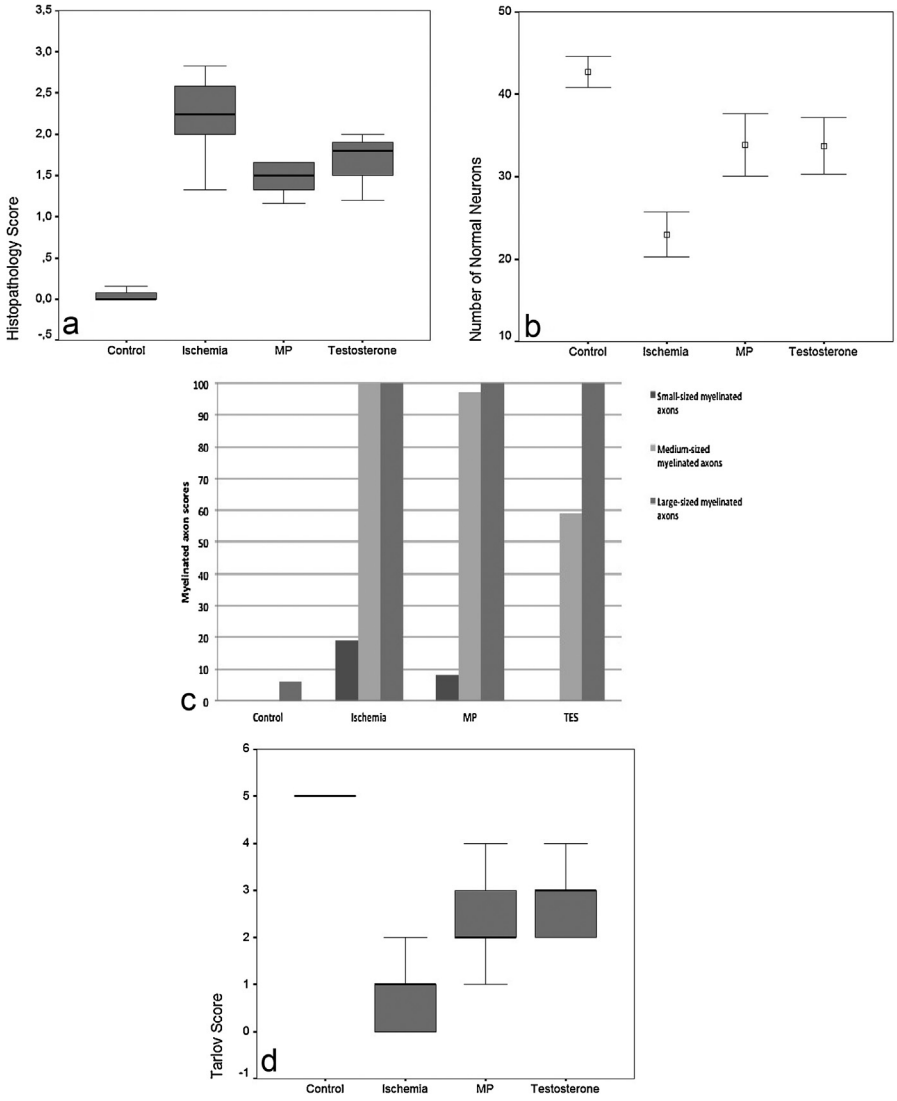

Fig. 2. Graphs showing the histopathological (a, b), ultrastructural (c) and neurological examination results (d) of the groups.
(a) Comparison of the histopathology scores among the groups. Five different fields of spinal cord grey matter were evaluated using a 40× objective. The horizontal lines in the middle of each box indicates the median, while the top and bottom borders of the box mark the 25th and 75th percentiles, respectively. The whiskers above and below the box mark the maximum and minimum levels observed.
(b) Comparison of the number of normal neurons in the anterior horn among the groups. The box in the middle of each whisker indicates the arithmetic mean, and the whiskers above and below the box mark the +1 SD and −1 SD levels, respectively.
(c) Bar graph showing the results of the ultrastructural analysis.
(d) Comparison of the Tarlov scores among the groups. The horizontal lines in the middle of each box indicates the median, while the top and bottom borders of the box mark the 25th and 75th percentiles, respectively. The whiskers above and below the box mark the maximum and minimum levels observed.
MP: methylprednisolone, TES: testosterone.

Further, large-sized axons were more damaged after the I/R injury in the ischemia group than the control group. Unfortunately, neither MP nor TES protected the large-sized axons of the spinal cord from I/R injury (Fig. 2c).

Neurologic evaluations

The mean Tarlov score of the ischemia group was significantly lower than that of the control group ($p < 0.001$). However, the mean Tarlov score of the TES group was significantly higher than that of the ischemia group ($p < 0.001$). The mean Tarlov score of the MP group was also significantly higher than that of the ischemia group ($p = 0.002$). There were no statistically significant differences in Tarlov scores between the TES group and the MP group ($p = 0.36$) (Fig. 2d).

Discussion

Paraplegia following spinal cord injury is an unpredictable and disastrous complication related to the operations on the descending and thoracoabdominal aorta. The main cause of spinal cord dysfunction is believed to be the result from the primary injury, due to hypoperfusion during aortic cross-clamping, and the secondary injury caused by the reestablishment of the blood flow [32]. The primary injury occurs when the blood supply of the spinal cord is diminished due to loss of nutrients and oxygen. Numerous biochemical pathways are activated and cause generation of reactive oxygen species, apoptosis, necrosis, lipid peroxidation, excessive aminoacid release and inflammation [33,34]. After the primary injury, reestablishment of the blood flow, which is called reperfusion, causes further additional damage to the spinal cord and results in a decline of function (i.e., secondary injury) [32].

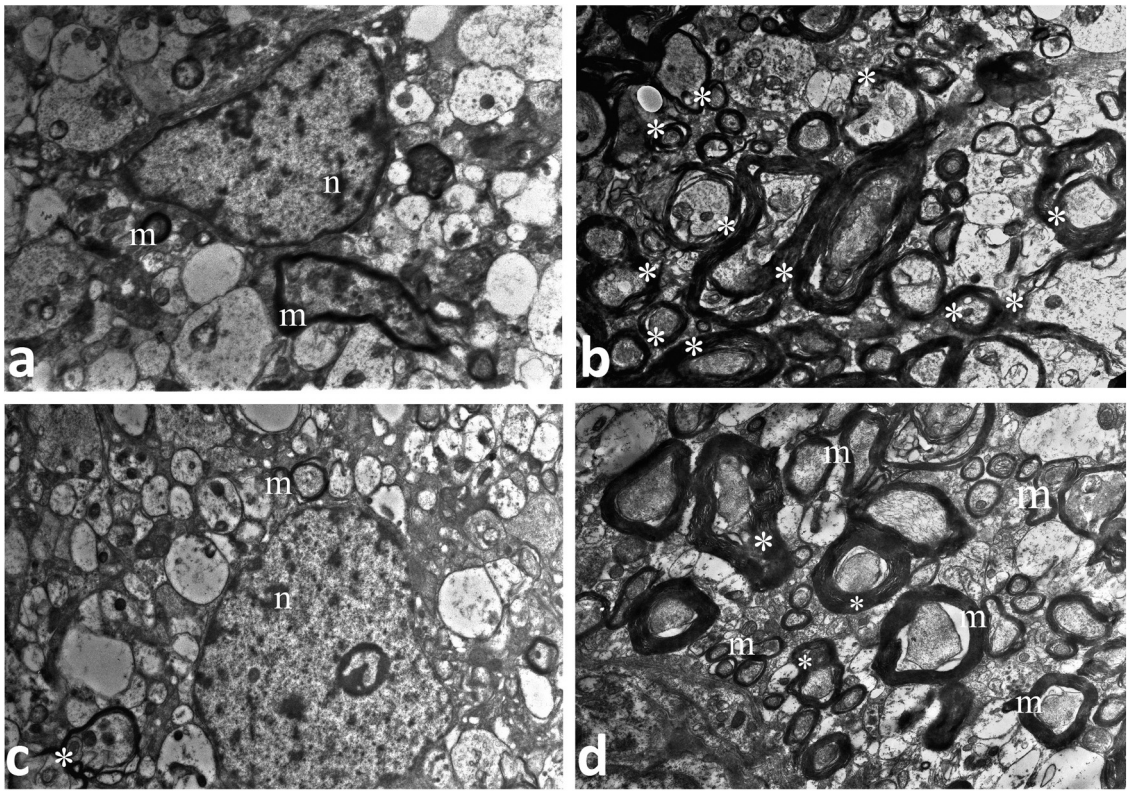

Fig. 3. Transmission electron microscopy of the groups.
(a) Electron micrograph from the control group showing ultrastructurally normal nucleus of a neuron (n) and non-damaged myelinated axons (m) (original magnification ×5000).
(b) Electron micrograph from the ischemia group showing the small and medium-sized axons with separations in myelin configuration (*) (original magnification ×5000,).
(c) Electron micrograph from the MP group showing separations in myelin configurations (*) in medium-sized myelinated axons. Notice the ultrastructurally normal neuronal nucleus (n) and non-damaged small-sized myelinated axon (m) (original magnification ×5000).
(d) Electron micrograph from the TES group showing medium-sized myelinated axons with mild separation in myelin configuration (*) and ultrastructurally normal small-sized and medium-sized myelinated axons (m) (original magnification ×5000). MP: methylprednisolone, TES: Testosterone.

Gonadal sex steroid hormones provide protection from many of the pathophysiological changes seen after spinal cord injury by reducing the inflammation and free oxygen radical generation [35]. Testosterone, the gonodal sex steroid hormone, has various effects on almost all tissues including CNS. Testosterone is physiologically secreted by testes and adrenal glands, and transported by the sex hormone binding globulins and albumin. Only 0.5–2% of TES is circulated freely in the blood [8].

Testosterone acts via androgen receptors. Androgen receptors are found in neurons throughout the CNS [9]. Regulation of androgen receptor protein and mRNA by androgens has been observed in mammalian brains, and CNS is reported to be an androgen responsive tissue [36]. Testosterone in its free form can cross the blood brain barrier and influence neuronal cells [8]. Testosterone might act directly through the androgen pathway or indirectly via conversion to estrogen [7]. Testosterone shares the ability for neuroprotection with estrogen, but moreover TES induces its neuroprotective effects through separate mechanisms [37].

Treatment with TES provides a wide array of neuroprotective and neurotheuropathic effects [38]. Recent data suggest that TES may exert neurotrophic actions, increase expression of nerve growth factor [39], and mediate promotion of neurite growth [40]. Kujawa et al. reported that TES exerts accelerate effects on facial nerve regeneration through a receptor-mediated mechanisms [41]. Testosterone accelerates both axon regeneration and functional recovery after axotomy [42]. Testosterone treatment attenuates the synaptic striping after motoneuron injury and preserves the central input to the motoneurons [43]. Furthermore, TES shows neuroprotective effects via antioxidant properties and via inhibiting apoptotic pathways [44,45].

In the spinal cord, treatment with TES revealed neuroprotective and neurotrophic effects on spinal cord motoneurons and their target musculature after spinal cord injury [35]. Also, TES treatment improves motor function in patients with spinal cord injury. Patients treated with TES had higher discharge motor scores. The result has been ascribed to either improved strength through the anabolic effects of TES on skeletal muscle or its neuroprotective and neuroregulating effects [46].

The neuroprotective effects of TES are directly manifest through the pathophysiological mechanisms such as apoptosis, necrosis, neuronal death, free radical generation and inflammation, and are all seen after I/R injury of the spinal cord. Therefore, we hypothesised that TES may have a neuroprotective effect in spinal cord I/R injury.

Apoptosis is one of the most important causes of neuronal death following spinal cord injury [47]. Apoptosis is mediated by members of the cysteine protease family known as caspases. Caspase-3 is an interleukin-converting enzyme and has been suggested to be the principle effector of the apoptotic cascade [48]. Previous studies demonstrated that caspase-3 is a reliable marker of apoptosis in spinal cord I/R injury [2,24]. As expected, in this study caspase-3 activity was found to be increased after spinal cord I/R injury. Treatment with TES and MP caused a decrease in caspase-3 activity demonstrating their antiapoptotic properties. Previous studies also showed that androgen metabolites determine neuron number via regulating apoptosis [20,49]. Furthermore, androgen neuroprotection involves a mitogen-activated

protein kinase/extracellular signal regulated kinase (MAP/ERK) signaling pathway that inactivates pro-apoptotic factor Bcl-2-associated with death protein [50].

We demonstrated that spinal cord I/R injury caused a significant increase in MPO activity in both serum and the spinal cord tissue. Myeloperoxidase activity is a reliable marker of neutrophil infiltration to the injured tissue, and is associated with the number of neutrophils infiltrating the spinal cord and their activity [23,51]. Our findings showed that both TES and MP reduced the MPO activity indicating the existence of anti-inflammatory activity of TES. Similar to our findings, anti-inflammatory effects of TES had been described in rat prostate epithelial cells [52], and in human benign prostatic hyperplasia cells [53]. Also, TES revealed anti-inflammatory effects on intestinal I/R injury in rats [54].

The CNS consists of lipids, which are easily damaged by free-radical induced lipid peroxidation [24]. Following spinal cord ischemia and during reperfusion, lipid peroxidation occurs in cell membranes and is one of the main pathophysiological mechanisms involved in secondary injury [21]. Malondialdehyde levels increase after spinal cord I/R injury and demonstrate the involvement of lipid peroxidation thus supporting the presence of reperfusion injury [2,24]. As consistent with previous published data, this study also showed that I/R injury significantly caused an increase in MDA levels. Both TES and MP treatments protected the spinal cord from lipid peroxidation by lowering the MDA levels.

Reactive oxygen species has an important key role in mediating secondary injury caused by reperfusion [55]. Catalase is one of the antioxidant enzymes that has the capacity to scavenge reactive oxygen species [56]. Due to elevated oxidative stress during spinal cord I/R injury CAT levels were diminished. After administration of TES or MP, CAT levels increased significantly revealing the antioxidant activity of both drugs. Also, previous studies demonstrated the antioxidant activity of TES [10,19,54,57]. Furthermore, XO is another important source of reactive oxygen species [58]. After I/R, XO levels were elevated indicating an oxidative stress [2]. In our study, we demonstrated that both TES and MP reduced the XO levels, which reveals further evidence for their antioxidant activity.

Light microscopic evaluation of the spinal cord samples revealed that I/R injury caused haemorrhage, marked oedema, and necrosis diffusely in the effected tissues. Also the number of normal motor neurons was significantly decreased. Histopathological evaluation of the samples relevant to the TES and MP groups revealed that both drugs protected spinal cord tissues from injury with better morphological results and higher numbers of normal motor neurons when compared to the ischemia group.

To evaluate tissue samples in detail, we performed ultrastructural analysis using a transmission electron microscope. The ultrastructural evaluation of the tissues revealed that I/R injury caused significant disruption in small, medium and large-sized myelinated axons. Both TES and MP treatments protected the small- and medium-sized myelinated axons from I/R injury. Unfortunately the studied drugs could not protect large-sized myelinated axons.

Moreover, the 20 min ischemia period caused paraplegia in all animals. Both TES and MP treatments protected spinal cord from I/R injury and improved neurological function as determined by Tarlov scores.

All biochemical, histopathological, ultrastructural and functional analysis results of the presented study suggest that TES and MP have neuroprotective effects on preserving normal spinal cord morphology and ultrastructure by inhibiting apoptosis, reducing inflammation, lipid peroxidation and oxidative stress.

There were some limitations of this study. Non-castrated intact male animals can have variable endogenous androgen levels, based on time of day and environmental stress, as evidenced by the wide range of androgen values reported. Further study models with castrated groups are needed to conclude better results for the effects of TES on spinal cord I/R injury. The number of rabbits in each group and the time periods of biochemical, histopathological and functional evaluations should be augmented. Also, to provide more stable serum TES levels, the mixture of four TES esters with different half-life was used in this study; dose-dependent results of each TES ester can also be investigated.

Conclusions

Our biochemical, histopathological, ultrastructural and neurological examination findings showed, for the first time, that TES could protect spinal cord from I/R injury. Moreover, TES has been shown to be at least as effective as MP. More studies based on these findings may be helpful for further evaluation of this promising medication for I/R injury of the spinal cord.

Conflict of interest statement

Conflict of interest: None.
Bora Gürer: None.
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