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Comparative effects of vitamin D and methylprednisolone against ischemia/reperfusion injury of rabbit spinal cords

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ABSTRACT

Introduction: Recent studies have demonstrated the neuroprotective and immunomodulatory effects of 1,25-dihydroxyvitamin D3 (calcitriol), but no previous study has examined these effects on spinal cord ischemia/reperfusion (I/R) injury. Therefore, the present study aimed to evaluate whether calcitriol protects the spinal cord from I/R injury.

Methods: Rabbits were randomized into four groups of eight animals: group 1 (laparotomy control), group 2 (ischemia control), group 3 (30 mg/kg intraperitoneal methylprednisolone at surgery), and group 4 (0.5 μg/kg, intraperitoneal calcitriol for 7 days before I/R injury). The rabbits in the laparotomy control group underwent laparotomy only, whereas all rabbits in the other groups were subject to spinal cord ischemia by aortic occlusion for 20 min, just caudal to the renal artery. Malondialdehyde and catalase levels, myeloperoxidase and xanthine oxidase activities, and caspase-3 concentrations were analyzed. Finally, histopathological, ultrastructural, and neurological evaluations were performed.

Results: After I/R injury, increases in malondialdehyde levels, myeloperoxidase and xanthine oxidase activities, and caspase-3 concentrations were found ($p < 0.001$ for all); by contrast, catalase levels decreased ($p < 0.001$). Calcitriol pretreatment was associated with lower malondialdehyde levels ($p < 0.001$), reduced myeloperoxidase (serum, $p=0.018$; tissue, $p < 0.001$) and xanthine oxidase ($p < 0.001$) activities, and caspase-3 concentrations ($p < 0.001$), but increased catalase levels ($p < 0.001$). Furthermore, calcitriol pretreatment was associated with better histopathological, ultrastructural, and neurological scores.

Conclusion: Calcitriol pretreatment provided significant neuroprotective benefits following spinal cord I/R injury.

1. Introduction

Spinal cord ischemia/reperfusion (I/R) injury is an unexpected complication of thoracoabdominal surgery that can cause paraplegia (Crawford et al., 1986). Neurological complications due to spinal cord ischemia are related to the duration, intensity, and maintenance of blood flow and reperfusion injury itself (Acher et al., 1990). Two major pathophysiologic mechanisms underlie spinal cord injury (Tator and Fehlings, 1991): primary injury, which is caused by necrotic cell death, and secondary tissue injury, which results from apoptotic cascades, inflammation, lipid peroxidation, and free oxygen radical formation, and is activated within hours of the primary injury (Safi et al., 2003; Kwon et al., 2002; Zhou et al., 2016). To counter these pathophysiological insults, various therapeutic strategies have been used, including cerebrospinal fluid drainage (Safi et al., 2003), hypothermia treatment (Dietrich et al., 2011), and use of shunts (Brodbelt and Stoodley, 2003)

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and experimental pharmacological agents (Gokce et al., 2015; Gürer et al., 2015; Kertmen et al., 2013; Sanli et al., 2012; Yilmaz et al., 2012).

Vitamin D is a neuroactive steroid that has receptors in the central nervous system (Eyles et al., 2005; Garcion et al., 2002; McGrath et al., 2001). The active form of vitamin D, which is known as calcitriol (1-α,25-dihidroxyvitamin D3), exerts its effects through vitamin D receptors (VDRs) that are present in neurons, Schwann cells, glial cells, and brain macrophages in the central nervous system, spinal cord, and peripheral nervous system (Baas et al., 2000; Cornet et al., 1998; Glaser et al., 1999). Due to this global expression in the central and peripheral nervous systems, vitamin D meets the criteria of a neurosteroid (Brown et al., 2003; Eyles et al., 2005; Garcion et al., 2002; McGrath et al., 2001; Stumpf et al., 1982). When calcitriol binds to a VDR, a membranous VDR (mVDR) is activated (Haussler et al., 2010). Calcitriol can bind to VDRs and mVDRs and behave as any other neurosteroid during neurotransmitter release and neuronal regulation (Cornet et al., 1998). Consequently, calcitriol may play important roles in the central and peripheral nervous systems, exerting neuroprotective effects through its action at VDRs (Garcion et al., 1999, 2002; Kalueff et al., 2004).

Vitamin D also exerts neuroprotective effects by mimicking the effects of calcium-binding proteins, decreasing calcium-related neurotoxicity, inhibiting L-type calcium channels, regulating glutathione metabolism, regulating antioxidant activity, decreasing nitric oxide synthase expression, stimulating neurotrophins and neuritogenesis, and regulating cytokine release (Bemiss et al., 2002; Brown et al., 2003; Cantorna et al., 1998; Cantorna and Mahon, 2004; Garcion et al., 2002; Kalueff et al., 2004). Previous studies have shown the neuroprotective, antioxidant, anti-apoptotic, and anti-inflammatory effects of vitamin D; however, to date, no study has shown the effects of calcitriol on rabbit spinal cord I/R injury. Therefore, in this study, we investigated the neuroprotective effects of calcitriol on rabbit spinal cord I/R injury. We specifically compared the effects of calcitriol with those of methylprednisolone because this steroid has established benefits in the treatment of spinal cord injury (Diaz-Ruiz et al., 2000; Kanellopoulos et al., 1997).

2. Materials and methods

2.1. Experimental groups

For experimental use, animals were taken care of and treated in accordance with the European Communities Council Directive, November 24, 1986 (86/609/EEC). Each aspect of this study was assessed and endorsed by the ethical board of trustees of the Ministry of Health Ankara Education and Research Hospital Committee of Animal Ethics (23.11.2010-103). Thirty-two adult male New Zealand white rabbits weighing 2800–3750 g were arbitrarily separated into the following four groups:

Group 1: Laparotomy control group (n=8). Rabbits underwent laminectomy, and non-ischemic spinal cord samples were obtained immediately after surgery. This group received no treatment.

Group 2: Ischemia control group (n=8). Rabbits underwent transient global spinal cord ischemia. The same volume of saline (2 cc 0.9% NaCl) was intraperitoneally infused instantly after the occlusion clamp was evacuated. The animals then underwent laminectomy, and spinal cord samples were evacuated 24 h post ischemia.

Group 3: Ischemia plus methylprednisolone group (n=8). Rabbits were treated like those in group 2 but also received a single intraperitoneal dose of 30 mg/kg methylprednisolone (Prednol, Mustafa Nevzat, Turkey) after the occlusion clamp was evacuated. This methylprednisolone dose was chosen based on previous studies (Gokce et al., 2015; Gürer et al., 2015; Kertmen et al., 2013; Sanli et al., 2012; Yilmaz et al., 2012).

Group 4: Ischemia plus calcitriol group (n=8). Rabbits were given preoperative intraperitoneal injections of 0.5 μg/kg calcitriol (Calcitriol, Abbot, ABD) for 7 days. On the seventh day, 1 h after calcitriol injection, they underwent the same surgical procedure performed for the rabbits in group 2. This calcitriol dose was chosen based on previous studies (Chabas et al., 2013; Kim et al., 2005; Seif and Abdelwahed, 2014; Sezgin et al., 2013; Sinanoglu et al., 2012).

2.2. Anesthesia and surgical procedures

Rabbits were fed ad libitum standard chow and water at ideal room temperature (18–21 °C) and were under a 12 h light/12 h dark cycle. They were anesthetized with an intramuscular dose of 70 mg/kg ketamine (Ketalar, Parke Davis Eczacıbaşı, Turkey) and 5 mg/kg xylazine (Rompun, Bayer, Turkey) and were permitted to spontaneously inhale. Body temperatures were measured using an anal thermometer (Digital Fever thermometer, Becton Dickinson, NJ, USA) and were maintained at 37 °C with a warming cushion. Rabbits were placed in the supine position for undergoing surgery. After sterilization, a 10-cm midline incision was made, and the abdominal aorta was approached through the transperitoneal route. Heparin (150 U/kg) was intravenously administered 5 min before clamping for anticoagulation. Around 1 cm beneath the renal artery, the aorta was clipped using an aneurysm clip with a closing force of 70 g (Yasargil, FE721, Aesculap, Germany) under a surgical microscope. The cross-clamping time was 20 min. At the end of the occlusion period, the clamps were removed and restoration of blood flow was visually checked.

The rabbit aortic cross-clamping technique used in this study is an established and valuable system (Gokce et al., 2015; Gürer et al., 2015; Kertmen et al., 2013; Yilmaz et al., 2012), with the 20 min ischemia period providing satisfactory damage. Rabbits were permitted free access to food and water 2 h after surgery. Credé's maneuver was performed at least twice a day for rabbits with a neurogenic bladder. All rabbits were sacrificed 24 h after surgery by the infusion of high-dose pentobarbital (200 mg/kg) (Nembutal, Oak Pharmaceuticals, Lake Forest, IL, USA). Spinal cord fragments between the L2 and L5 levels were precisely uprooted by laminectomy and were used for biochemical, histopathological, and ultrastructural investigations.

Blood (10 cm^3) was taken from the left ventricle for the biochemical examination and was centrifuged at 1000 × *g* for 5 min; the upper clear supernatant was used for analysis. All serum and tissue test samples were stored at −80 °C until further analysis. On the day of analysis, the tissue was homogenized in physiologic saline (1/5 w/v) using a homogenizer (B. Braun Melsungen AG 853202, Melsungen, Germany) and centrifuged at 1780 × *g* for 20 min; the protein level of the clear supernatant was studied by Lowry's method and adjusted to equal concentrations before analyses. Serum samples from the upper clear supernatant of centrifuged blood were used for the biochemical examination. The study design is summarized in Fig. 1.

2.3. Tissue caspase-3 concentration

Caspase-3 concentration was measured by performing enzyme-linked immunosorbent assay (ELISA) (ELISA kit; Cusabio, Hubei, China). ELISA was performed in accordance with the manufacturer's guidelines. This technique uses the quantitative sandwich protein immunoassay. Antibodies specific for caspase-3 were pre-coated onto a microplate. Standards and samples were pipetted into wells, allowing pre-coated antibodies to bind caspase-3 present in samples and standards, if any. After removing unbound substances, a biotin-conjugated antibody specific for caspase-3 was added to the wells. After washing, avidin-conjugated horseradish peroxidase was added to the wells. Any unbound substances were removed by three washes with a washing buffer, before the avidin-protein reagent was added to the wells. The intensity of the color developed was proportional to the concentration of caspase-3 bound in the initial step. At the point when color improvement stopped, the intensity of the color was measured at 450 nm. Caspase-3 concentrations were ascertained by comparing the

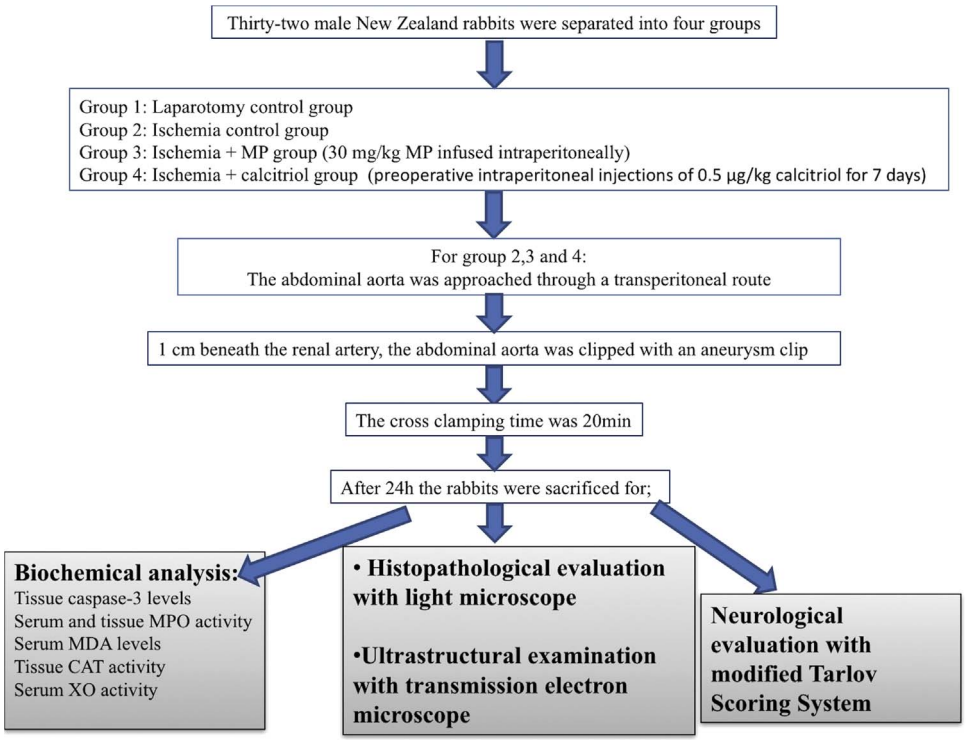

Fig. 1. Flow diagram summarizing the study design. CAT = catalase, MDA = malondialdehyde, MPO = myeloperoxidase, MP = methylprednisolone, XO = xanthine oxidase.

absorbance values of the samples with those of standard caspase-3 solutions. The results are expressed in ng/mL.

2.4. Serum and tissue myeloperoxidase analyses

Myeloperoxidase (MPO) activity was measured by competitive inhibition ELISA (Cusabio, Hubei, China), according to the manufacturer's instructions. The provided microtiter plate was pre-covered with an antibody specific for MPO. Standards or samples were added to appropriate microtiter plate wells with biotin-conjugated MPO, and a competitive inhibition reaction was started between MPO (from standards or samples) and biotin-conjugated MPO with the pre-coated antibody specific for MPO. With greater amounts of MPO, lower amounts of antibodies are bound by biotin-conjugated MPO. After washing, avidin-conjugated horseradish peroxidase was added to the wells; the substrate solution was then added, and the color was allowed to develop to show the amount of MPO in the sample. When color development stopped, color intensity was measured at 450 nm. MPO activities were calculated by contrasting the absorbance estimates of the samples with those of the standard MPO solutions. The results are presented in ng/mL.

2.5. Serum malondialdehyde analyses

Serum malondialdehyde (MDA) levels were determined using thiobarbituricacid (TBA). Briefly, specimens were blended with two volumes of cold saline liquid containing 0.001% butylated hydroxytoluene and 0.07% sodium dodecyl sulfate. At that point, 1 mL of the samples was added to 500 μL of TBA with 0.01 μL of NH_2SO_4 (0.67% TBA in half-acidic corrosive) to precipitate proteins. At that point, the specimens were warmed in boiling water for 1 h, cooled, and mixed with an equivalent volume (2 mL) of n-butanol. 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 MDA levels were determined by comparing the absorbance values of the samples with those of the standard MDA solutions and were expressed in nanomoles (nM).

2.6. Tissue catalase analyses

Catalase (CAT) levels were determined by measuring the rate of absorbance decrease of hydrogen peroxide (H_2O_2) at 240 nm (Aebi, 1984). In the calculations of CAT levels, extinction coefficients of H_2O_2 (40.98 L mol^{-1} cm^{-1} at 240 nm) were used. The results are presented in IU/mL.

2.7. Serum xanthine oxidase analyses

Serum xanthine oxidase (XO) activity was measured by the technique of Prajda and Weber (1975), where activity is measured by the determination of the amount of uric acid formed from xanthine. Serum samples (100 μL) were incubated for 30 min at 37 °C in 3 mL of phosphate buffer (pH 7.5, 50 nM) containing xanthine (4 mM). The reaction was halted by the addition of 0.1 mL 100% (w/v) trichloroacetic acid, and the mixture was then centrifuged at 1780 × *g* for 20 min. Uric acid levels were determined in the supernatant by measuring absorbance at 292 nm against a blank and are presented in 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.

2.8. Histopathological evaluation

Spinal cord samples obtained at 24 h post injury were prepared for histological evaluation. Each cord section was submerged in 4% paraformaldehyde with 0.1 mol/l phosphate buffer and stored at 4 °C. The samples were then embedded in paraffin, cut into 5-μm thick sections, and stained with hematoxylin and eosin. The samples were inspected under a light microscope by a neuropathologist who was blinded to the study plan. Five distinct fields of spinal cord gray matter were assessed using a 40× objective. A semi-quantitative scoring system, ranging from 0 to3, was used to evaluate histopathological changes in the spinal cord tissue of all samples. Six distinct parameters (hemorrhage, congestion, necrosis, edema, neuronal loss, and inflammation) were histopathologi-

cally evaluated and scored as follows: 0 = negligible, 1 = mild, 2 = moderate, and 3 = common. The histopathology score for every spinal cord sample was ascertained by averaging the scores of these six parameters (Sanli et al., 2012). Furthermore, a point-by-point appraisal of the level of ischemic neuronal injury was performed in a similar manner. For this investigation, the quantity of typical 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 determined. For every rabbit, three areas were assessed using a 40× objective. An average normal motor neuron count for the areas from every rabbit was then decided. Neurons that contained Nissl substances, loose chromatin, and prominent nucleoli were considered normal viable neurons (Umehara et al., 2010).

2.9. Ultrastructural evaluation

Tissue samples were cleared of blood using a surgical blade, and the meninges were precisely removed. Tissue samples were then altered in 2.5% glutaraldehyde for 24 h, followed which they were washed in phosphate buffer (pH =7.4). Next, they were post-fixed in 1% osmium tetroxide in phosphate buffer (pH=7.4) for 2 h and dehydrated with increasing concentrations of alcohol. At that point, 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 blade on an LKB-Nova ultra-microtome (LKB-Produkter AB, Bromma, Sweden). Semi-thin sections were stained with methylene blue and analyzed under a Nikon Optiphot (Nikon Corporation, Tokyo, Japan) light microscope. Obtained after this examination, tissue blocks were trimmed, and ultra-thin sections were prepared using the same ultra-microtome; these segments were stained with uranyl acetic acid and lead citrate. After staining, all ultra-thin sections were analyzed under a Jeol JEM 1200 EX (Jeol Ltd., Tokyo, Japan) transmission electron microscope. Electron micrographs were obtained from the same transmission electron microscope at 5000× magnification. A total of 100 large myelinated axons, 100 medium myelinated axons, and 100 small myelinated axons were assessed per sample; they were scored from 0 to 3 and counted. Data were then represented as mean values, as shown by Kaptanoglu et al. (2002) The scoring system was as follows: 0 = normal ultrastructure of myelinated axons, 1 = separation in myelin configuration, 2 = interruption in myelin configuration, and 3 = honeycomb appearance of myelin configuration.

2.10. Neurological evaluation

The neurological statuses of rabbits were scored 24 h after surgery by evaluating the hindlimb neurological function using the modified Tarlov scoring system (Gokce et al., 2015; Gürer et al., 2015; Kertmen et al., 2013; Yilmaz et al., 2012). A score of 0–5 was allocated to each rabbit: 0 = no voluntary hindlimb movement; 1 = perceptible joint movement; 2 = active movement but unable to sit without help; 3 = able to sit but unable to hop; 4 = week hop; and 5 = complete recovery of hindlimb function. A medical doctor blinded to the experimental groups performed the neurological evaluation.

2.11. Statistical analysis

Data analysis was performed using SPSS for Windows, Version 11.5 (SPSS Inc., Chicago, IL, USA). The Shapiro–Wilk test was used to determine whether continuous variables were normally distributed, and Levene's test was used to assess the homogeneity of variances. Where applicable, data are presented as mean ± standard deviation or as median (interquartile range). Mean differences among the groups were analyzed by one-way analysis of variance (ANOVA). Otherwise, the Kruskal–Wallis test was used for the comparison of medians. At the point when the p-value from ANOVA or the Kruskal–Wallis test was statistically significant, either Tukey's post-hoc honest significant difference test or Conover's nonparametric comparison tests were used to assess the differences among the groups. P-values less than 0.05 were considered statistically significant.

3. Results

3.1. Tissue caspase-3 analyses

There was a significant difference between the laparotomy control and ischemia control groups in their mean caspase-3 concentrations ($p < 0.001$), with I/R injury clearly increasing caspase-3 concentrations in the injured tissue. When either the ischemia plus methylprednisolone or ischemia plus calcitriol group was compared with the ischemia control group, a significant decrease incaspase-3 concentration was observed ($p=0.001$ for both). Moreover, there was no significant difference between the ischemia plus methylprednisolone and ischemia plus calcitriol groups ($p=0.871$), suggesting that treatment with either calcitriol or methylprednisolone inhibits apoptosis after I/R injury (Fig. 2A, Table 1)

3.2. Serum and tissue MPO analyses

Significant differences were observed in mean serum and tissue MPO activities between the laparotomy control and ischemia control groups ($p < 0.001$, for each comparison). Although I/R injury raised serum and tissue MPO activities, compared with the ischemia control group, treatment with either methylprednisolone ($p < 0.001$, for serum and tissue MPO) or calcitriol ($p=0.018$ for serum MPO; $p < 0.001$ for tissue MPO) significantly decreased MPO activities. Again, there were no significant differences in serum and tissue MPO activities between the ischemia plus calcitriol and ischemia plus methylprednisolone groups ($p=0.471$ for serum MPO; $p=0.981$ for tissue MPO) (Fig. 2B-C, Table 1). Thus, increased activities of the inflammatory marker MPO were shown to decrease with calcitriol and methylprednisolone treatment, showing the anti-inflammatory actions of these drugs.

3.3. Serum MDA analyses

A significant difference was found between the laparotomy control and ischemia control groups in mean serum MDA levels ($p < 0.001$), indicating that serum MDA levels increased by I/R injury. Comparison of the ischemia control group with either the ischemia plus methylprednisolone or ischemia plus calcitriol group revealed that treatment significantly decreased MDA levels ($p < 0.001$ for both). When the ischemia plus methylprednisolone and ischemia plus calcitriol groups were compared, no statistically significant difference was found ($p=0.859$) (Fig. 2D, Table 1). Thus, both calcitriol and methylprednisolone appeared to prevent lipid peroxidation in spinal cord I/R injury.

3.4. Tissue CAT analyses

There was a significant difference between the laparotomy control group and the ischemia control group in mean tissue CAT levels ($p < 0.001$), indicating that tissue CAT levels decreased after I/R injury. Compared with the ischemia control group, tissue CAT levels were significantly increased in the ischemia plus methylprednisolone and ischemia plus calcitriol groups ($p < 0.001$ for both), and there were no significant differences between the ischemia plus methylprednisolone and ischemia plus calcitriol groups ($p=0.995$) (Fig. 2E, Table 1). CAT levels decreased due to oxidative stress after I/R injury, and calcitriol and methylprednisolone exerted an antioxidant effect by increasing CAT levels.

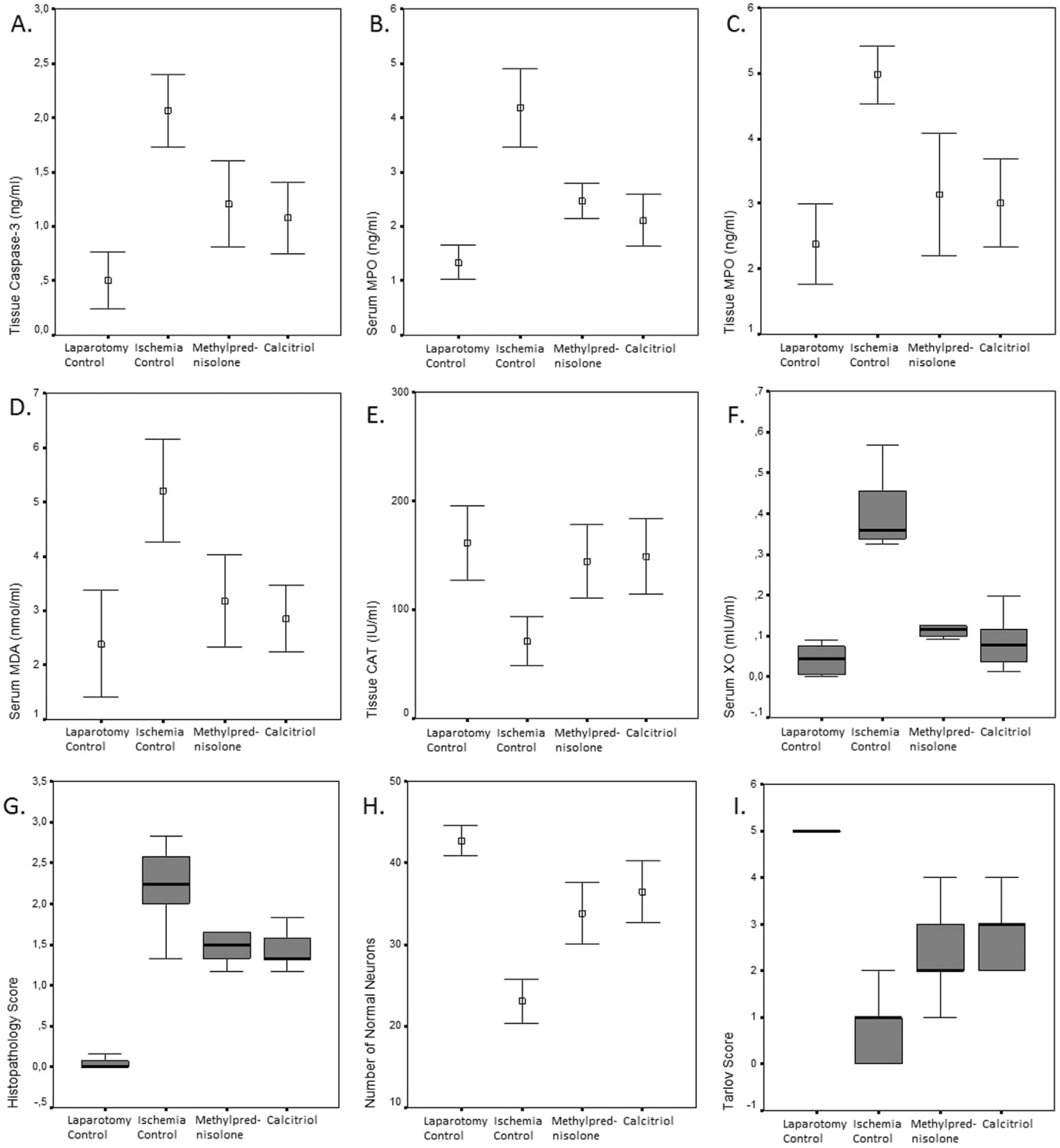

Fig. 2. Box and whisker plots of tissue levels of key markers of I/R injury.(A) Tissue caspase-3 concentrations*, (B)Serum MPO activities*, (C) Tissue MPO activities*, (D) Tissue MDA levels*, (E) Tissue CAT levels*, (F) Tissue XO activities**, (G) Histopathology score graphic**, (H) Normal neuron number graphic*, (I) Tarlov score graphic**. *: The box in the middle of each whisker shows the arithmetic mean, while the whiskers above and below the box indicate the +1 SD and −1 SD, respectively. **: The horizontal lines in the middle of each box represent the median, while the top and bottom borders of the box represent the 25th and 75th percentiles, respectively. The whiskers above and below the box represent the highest and lowest levels observed. CAT = catalase, I/R = ischemia/reperfusion, MDA = malondialdehyde, MPO = myeloperoxidase, XO = xanthine oxidase.

3.5. Serum XO analyses

Serum XO activity was associated with a significant increase in the ischemia control group compared with in the laparotomy control group ($p < 0.001$). Compared with the ischemia control group, serum XO activity significantly decreased in the ischemia plus methylprednisolone and ischemia plus calcitriol groups ($p=0.001$ for both); again, however, there was no significant difference between the ischemia plus methylprednisolone and ischemia plus calcitriol groups ($p=0.053$). After I/R injury, increased activities of the inflammatory marker XO appeared to be reduced by the secondary anti-inflammatory actions of calcitriol and methylprednisolone (Fig. 2F, Table 1).

Table 1
Biochemical results in the experimental groups.

Parameters	Laparotomy Control	Ischemia Control	Ischemia plus Methylprednisolone	Ischemia plus Calcitriol	p
Tissue Caspase-3 (ng/mL)	0.50 ± 0.26[a,b,e]	2.07 ± 0.33[a,c,d]	1.20 ± 0.40[b,c]	1.08 ± 0.34[d,e]	< 0.001
Serum MPO (ng/mL)	1.33 ± 0.32[a,b,e]	4.19 ± 0.71[a,c,d]	2.47 ± 0.33[b,c]	2.11 ± 0.48[d,e]	< 0.001
Tissue MPO (ng/mL)	2.38 ± 0.61[a]	4.98 ± 0.45[a,c,d]	3.14 ± 0.94[c]	3.01 ± 0.68[d]	< 0.001
Serum MDA (nM/mL)	2.39 ± 0.98[a]	5.21 ± 0.94[a,c,d]	3.18 ± 0.86[c]	2.84 ± 0.61[d]	< 0.001
Tissue CAT (IU/mL)	161.43 ± 34.71[a]	70.95 ± 22.10[a,c,d]	144.94 ± 34.23[c]	148.85 ± 35.06[d]	< 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]	0.08 (0.09)[d]	< 0.001

Abbreviations: CAT= catalase, MDA= malondialdehyde, MPO= myeloperoxidase, XO= xanthine oxidase.
[a] Laparotomy vs Ischemia ($p < 0.001$).
[b] Laparotomy vs ischemia plus methylprednisolone ($p < 0.001$).
[c] Ischemia vs ischemia plus methylprednisolone ($p < 0.05$).
[d] Ischemia vs ischemia plus calcitriol ($p < 0.05$).
[e] Laparotomy vs ischemia plus calcitriol ($p < 0.05$).

3.6. Histopathological evaluation

Light microscopy results of the spinal cord samples from the laparotomy control group were normal (Fig. 3A). In the ischemia control group, diffuse hemorrhage and congestion were observed in the gray matter 24 h after I/R damage, and there was marked necrosis and diffuse edema in the white and gray matter. In the injured areas, there were invading polymorphonuclear leukocytes, lymphocytes, and plasma cells. Neuronal pyknosis, loss of cytoplasmic elements, and cytoplasmic eosinophilia were additionally seen in the ischemia control group (Fig. 3B).

In the ischemia plus methylprednisolone and ischemia plus calcitriol groups, spinal cord tissue was protected from I/R damage (Fig. 3C–D). When the histopathology scores were analyzed, the ischemia control group demonstrated significantly higher scores than the laparotomy control group ($p < 0.001$). The histopathology scores were significantly lower in the ischemia plus methylprednisolone and ischemia plus calcitriol groups than in the ischemia control group ($p=0.001$ and $p=0.002$, respectively). There was no significant difference between the ischemia plus methylprednisolone and ischemia plus calcitriol groups ($p=0.622$) (Fig. 2G).

In the ischemia control group, the number of normal motor neurons in the anterior spinal cord was significantly lower than that in the laparotomy control group ($p < 0.001$). In the ischemia plus methylprednisolone and ischemia plus calcitriol groups, the number of normal motor neurons in the anterior spinal cord was significantly higher than that in the ischemia control group ($p < 0.001$ for both). However, there was no significant difference between the ischemia plus

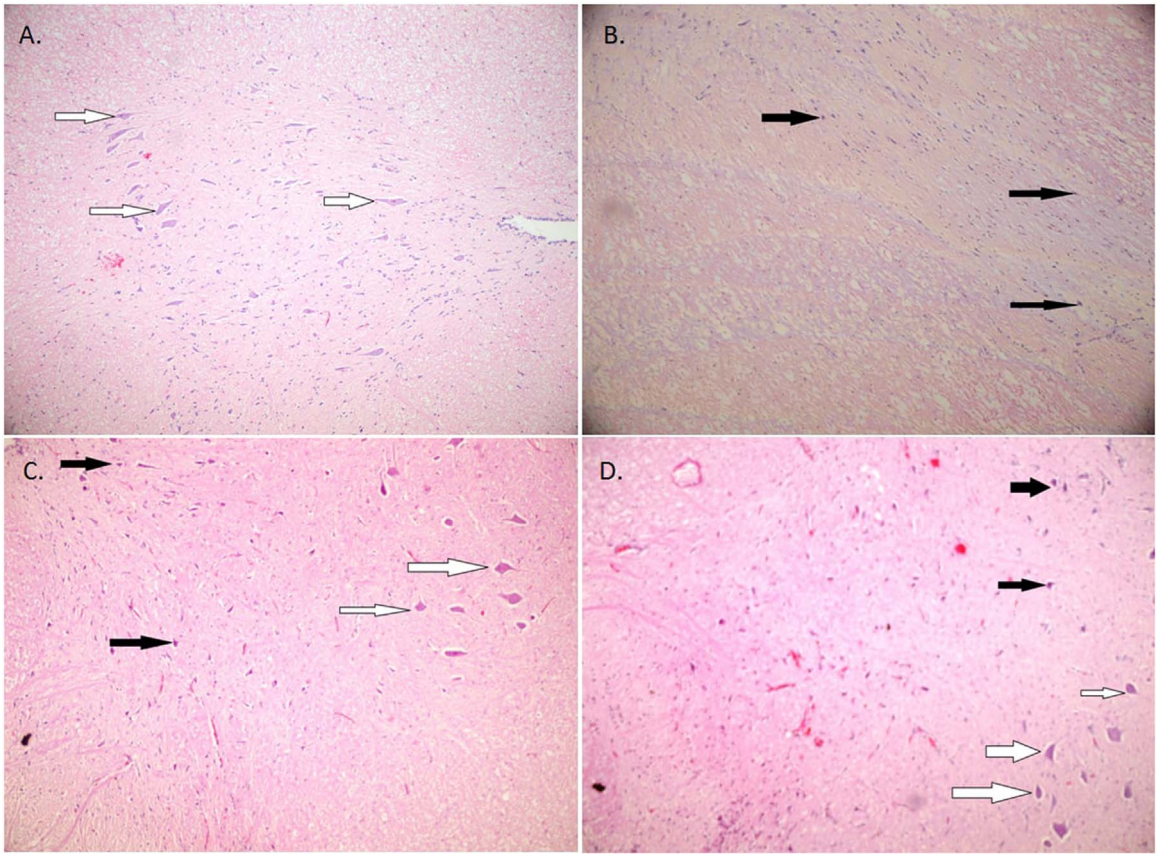

Fig. 3. Photomicrographs of 5-μm spinal cord tissue sections from each study group. Images are shown with hematoxylin–eosin staining under a 10× objective. (A) Laparotomy control, showing normal spinal cord parenchyma. Normal neurons are indicated with arrows.(B) Ischemia control, showing degenerated neurons (filled arrows) on the edematous surface. (C) Methylprednisolone intervention, showing less degenerated neurons (filled arrows); note the normal neurons (hollow arrows). (D) Calcitriol intervention, showing less degenerated neurons (filled arrows) and more normal neurons (hollow arrows). Spinal cord tissue was protected from injury by calcitriol pretreatment.

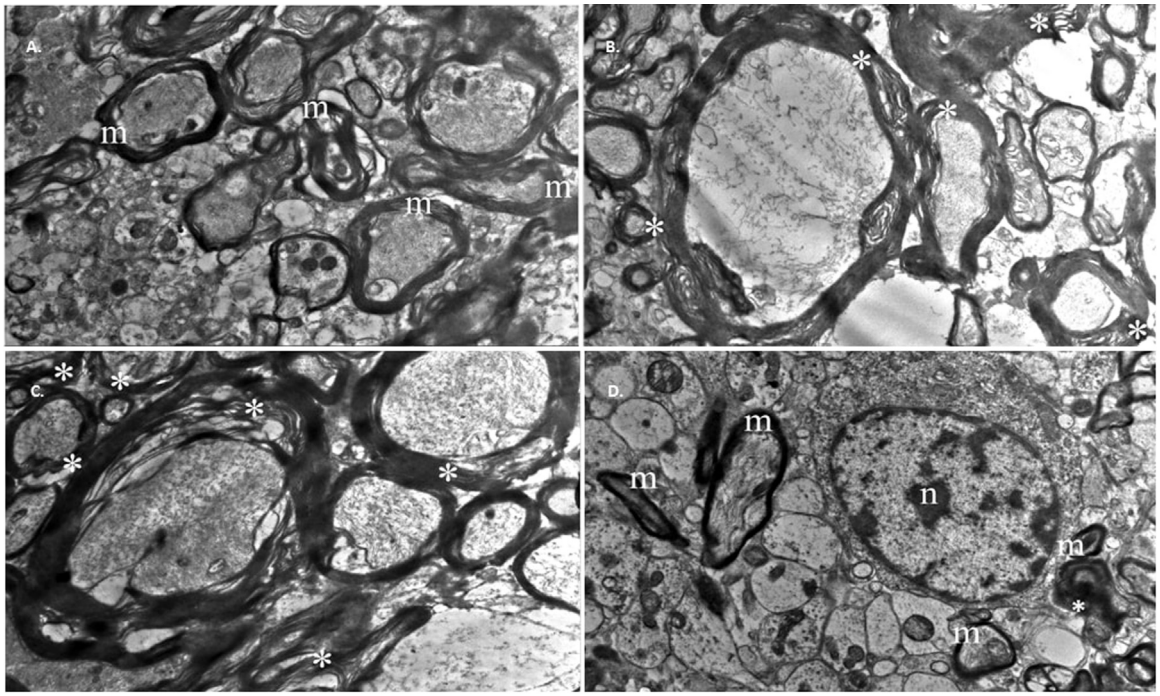

Fig. 4. Representative transmission electron micrographs for each group.(A) Laparotomy control, showing ultrastructurally normal myelinated axons (m) (original magnification 5000×, scale bar =2 μm). (B) Ischemia control, showing small, medium, and large axons with separations in myelin configuration (*) (original magnification =5000×, scale bar =2 μm). (C) Methylprednisolone intervention, showing separations in myelin configurations (*) in medium and large myelinated axons (original magnification =5000×, scale bar =2 μm). (D) Calcitriol intervention, showing large myelinated axons with separations in myelin configuration (*) and ultrastructurally normal, small myelinated axons (m) and neuronal nuclei (n) (original amplification =5000×, scale bar =2 μm).

methylprednisolone and ischemia plus calcitriol groups (p=0.357). Histopathologically, both calcitriol and methylprednisolone appeared to protect the spinal cord from I/R injury (Fig. 2H).

3.7. Ultrastructural evaluation

No ultrastructural pathological changes were seen in the gray or white matter of the spinal cord of the tissue from rabbits in the laparotomy control group under transmission electron microscopy. All neurons had normal ultrastructural appearances, with no pathological changes observed in the intracellular organelles, nuclei, membranes, or perineuronal tissues. However, in a couple of large myelinated axons, mild separations were seen in some myelin sheaths, possibly because of delayed tissue fixation. The remaining large myelinated axons and all medium and small myelinated axons appeared normal (Fig. 4A). By contrast, microscopy in the ischemia control group indicated separations in the myelin configuration of the small, medium, and large myelinated axons. When all groups were compared, the ischemia control group showed the greatest ultrastructural injury of myelinated axons (Fig. 4B).

In the ischemia plus methylprednisolone group, separations were seen in the myelin configuration of the medium and large myelinated axons and separations were observed in the myelin configuration of some small myelinated axons (Fig. 4C). In the ischemia plus calcitriol group, separations were seen in the myelin configurations of large myelinated axons, but all small and most medium myelinated axons appeared normal (Fig. 4D). Moreover, the ultrastructural appearances of the myelinated axons in the ischemia plus calcitriol group were better than those in the ischemia plus methylprednisolone group. The ischemia control group had more prominent disturbances in small myelinated axons than the laparotomy control group (p=0.008). Compared with the ischemia control group, the ischemia plus methylprednisolone and ischemia plus calcitriol protected the small myelinated axons from interruption (p=0.008 for both). When the ischemia plus calcitriol group was compared with the ischemia plus methylprednisolone group, significant improvements were seen in the protection of small myelinated axons (p=0.008).

Compared with the laparotomy control group, I/R injured the medium myelinated axons (p=0.008) in the ischemia control group. There were significant differences between the ischemia control group and the ischemia plus methylprednisolone and ischemia plus calcitriol groups, with both treatments protecting medium axons from I/R injury (p=0.008 for both). The ischemia plus calcitriol group showed significant enhancement in securing medium myelinated axons compared with the ischemia plus methylprednisolone group (p=0.008). Further, large myelinated axons were more injured in the ischemia control group after I/R damage than in the laparotomy control group (p=0.008). Unfortunately, neither methylprednisolone nor calcitriol protected the large axons of the spinal cord from I/R damage (Table 2).

3.8. Neurological evaluation

The mean Tarlov score in the ischemia control group was significantly lower than that in the laparotomy control group ($p < 0.001$). Consistent with the other analyses, the mean Tarlov scores in the ischemia plus methylprednisolone and ischemia plus calcitriol groups were significantly higher than that in the ischemia control group (p=0.002 and p=0.001, respectively). Clinically, there was also no significant difference in Tarlov scores between the ischemia plus methylprednisolone and ischemia plus calcitriol groups (p=0.622) (Fig. 2I).

4. Discussion

The central nervous system, including the spinal cord, has limited glycogen stores and limited capacity for anaerobic metabolism, making it very sensitive to ischemia. When blood flow to tissues decreases, cellular energy stores are therefore rapidly depleted, triggering ischemic cascades that lead to cell death. Reperfusion of ischemic tissues causes increased apoptotic activities, accelerated inflammatory reac-

Table 2
Electron microscopic results.

Myelinated Axons	Laparotomy Control	Ischemia Control	Ischemia plus Methylprednisolone	Ischemia plus Calcitriol	p
Small	0.0 (0.0)[a,b]	19.0 (6.5)[a,c,d]	8.0 (1.5)[b,c,e]	0.0 (0.0)[d,e]	< 0.001
Middle	0.0 (0.0)[a,b,f]	100.0 (0.0)[a,c,d]	97.0 (3.5)[b,c,e]	59.0 (6.5)[d,e,f]	< 0.001
Large	6.0 (2.5)[a,b,f]	100.0 (0.0)[a]	100.0 (0.0)[b]	100.0 (0.0)[f]	< 0.001

[a] Laparotomy vs Ischemia (p=0.008).
[b] Laparotomy vs Ischemia plus methylprednisolone (p=0.008).
[c] Ischemia vs Ischemia plus methylprednisolone (p=0.008).
[d] Ischemia vs Ischemia plus calcitriol (p=0.008).
[e] Ischemia plus methylprednisolone vs Ischemia plus calcitriol (p=0.008).
[f] Laparotomy vs Ischemia plus calcitriol (p=0.008).

tions, and free radical formation, which in turn cause secondary injury because of the sudden and excess presence of oxygen (Gokce et al., 2015; Gürer et al., 2015; Kertmen et al., 2013; Yilmaz et al., 2012). Spinal cord injury results from two main mechanisms. Primary injury occurs because of oxygen deprivation when blood flow to the spinal cord is interrupted, and it is irreversible. However, many secondary biochemical cascades are activated by primary injury (Cassada et al., 2001; Fan et al., 2011). Apoptosis, inflammation, lipid peroxidation, increased amino acid release, reactive oxygen species accumulation, and increased excitatory neurotransmitter injury occur during secondary injury (Gokce et al., 2015; Gürer et al., 2015; Kertmen et al., 2013; Yilmaz et al., 2012).

After surgical procedures involving the descending and thoracoabdominal aorta, paraplegia is an unexpected complication of spinal cord I/R injury (Hasturk et al., 2009). Preventive and therapeutic approaches for spinal cord ischemia following thoracic aortic operations mainly aim to augment the hemodynamics of spinal cord blood flow, minimize the duration of cord ischemia during the operation, make the spinal cord less susceptible to infarction, and detect spinal cord ischemia early to permit immediate intervention (Bicknell et al., 2009). Various therapeutic strategies have been used, including cerebrospinal fluid drainage (Safi et al., 2003), hypothermia treatment (Dietrich et al., 2011), and use of shunts (Brodbelt and Stoodley, 2003) and experimental pharmacological agents (Gokce et al., 2015; Gürer et al., 2015; Kertmen et al., 2013; Sanli et al., 2012; Yilmaz et al., 2012). The rabbit aortic cross-clamping technique used in this study is a useful method for replicating the features of ischemic spinal cord injury (Gürer et al., 2015; Ilhan et al., 2004; Kertmen et al., 2013; Lukácová et al., 1996). The 20 min ischemia and 24 h reperfusion durations were selected to cause adequate injury (Zivin and DeGirolami, 1980) and resulted in paraplegia in all rabbits in the ischemia control group.

Calcitriol has many biological actions in addition to calcium metabolism (Abrams et al., 2013). Indeed, it has roles in immunomodulation, cell proliferation and differentiation, and cell growth cycle regulation (Baeke et al., 2010; Bouillon et al., 2008; Chen et al., 2007; Haussler et al., 2010). Several studies have shown that calcitriol is a potent neuroprotective agent (Brewer et al., 2001; Chen et al., 2003; Garcion et al., 2002; Ibi et al., 2001; Kajta et al., 2009; Wang et al., 2000). Balabanova et al. (1984) reported that the concentrations of calcidiol (i.e., 25-hydroxyvitamin D) and calcitriol in the cerebrospinal fluid were 8.3 ng/mL and 25 pg/mL, respectively; moreover, calcitriol can cross the blood–brain barrier and bind to its specific receptor (Kalueff et al., 2004). Recently, vitamin D insufficiency has been associated with pain and peripheral nerve conduction disorders (Kuru et al., 2015)that were reversible with vitamin D supplementation (Akyuz et al., 2017). Finally, although calcitriol has been reported to be neuroprotective in experimental models of cerebral ischemia (Atif et al., 2013; Balden et al., 2012; Fu et al., 2013; Ibi et al., 2001; Kajta et al., 2009; Losem-Heinrichs et al., 2005; Wang et al., 2000), this has not been shown in a rabbit spinal cord I/R model.

After spinal cord I/R injury, apoptosis is one of the main factors that cause cellular death (Gürer et al., 2015). Acute ischemia results in a decrease in blood flow that depletes ATP and causes necrosis (Hayashi et al., 1998; Sakurai et al., 2003), with both mild and major I/R able to initiate apoptosis (Hearse and Boli, 1992; Lou et al., 1998) via cysteine protease family enzymes known as caspases (Emery et al., 1998). Of these, caspase-3 is one of the most important apoptotic enzymes that transform interleukin to its mature form (Li et al., 2000). Previous studies of spinal cord I/R injury have shown that caspase-3 is a reliable indicator of apoptosis (Gokce et al., 2015; Gürer et al., 2015; Kertmen et al., 2013; Yilmaz et al., 2012), and Sakurai et al. (2003) showed that the caspase-3 concentration increased after 15 min of spinal cord ischemia. In turn, this increased caspase-3 concentration initiates DNA fragmentation after ischemic events (Li et al., 2000). Previous studies have shown that calcitriol has anti-apoptotic activities (Hwang et al., 2013; Kajta et al., 2009; Regulska et al., 2007), with evidence that it decreases *Bax* and increases *Bcl-2* expression levels at the molecular level. The effects on these genes support the anti-apoptotic activity of calcitriol after I/R injury (Park et al., 2012). In our study, we found that caspase-3 concentrations increased 24 h after spinal cord I/R injury, as an indicator of apoptotic cell death. Calcitriol and methylprednisolone treatments were associated with decreased spinal cord caspase-3 concentrations and protected the spinal cord from apoptotic injury.

The inflammatory response after spinal cord I/R injury contributes to the observed damage (Fan et al., 2011; Hasturk et al., 2009). Neutrophils, macrophages, and monocytes play crucial roles in the inflammatory response and are major contributors to neuronal damage after reperfusion (Reece et al., 2004). Microglial cells that reside in the spinal cord release proinflammatory cytokines and cause inflammatory cells to enter the spinal cord tissue (Li et al., 2011), and proinflammatory cytokines and adhesion molecules cause progressive neuronal injury (İlhan et al., 2004). Therefore, it has been posited that spinal cord I/R injury can be prevented using anti-inflammatory substances (Cassada et al., 2001; Fan et al., 2011; Reece et al., 2004). MPO activity is increased by the number and activity of neutrophils that migrate to the spinal cord (Taoka et al., 1997). In our study, MPO activity increased in the serum and spinal cord following I/R injury, and calcitriol and methylprednisolone decreased that MPO activity. Accordingly, increased MPO activity after I/R injury may increase the number of neutrophils and their inflammatory activity, while treatment with calcitriol decreased MPO activity by providing an anti-inflammatory activity. This is consistent with previous research showing the anti-inflammatory activity of calcitriol (Balden et al., 2012; Garcion et al., 1997; Kalueff et al., 2004; Lee et al., 2014; Shih et al., 2011; Zhou et al., 2016). Like other glucocorticoid hormones, calcitriol exerts a marked immunosuppressive effect when it binds to intracellularly located VDRs when it behaves as a ligand-dependent transcription factor (Green and Chambon, 1988) and inhibits B-and T-lymphocyte proliferation (Koren et al., 1989).

Neuronal damage from spinal cord I/R injury involves oxygen free radicals and lipid peroxidation. Increases in oxygen free-radicals, for example, have been shown to result in cellular death (Bethea and Dietrich, 2002). Moreover, during normal cellular oxidative respiratory

activities, potentially toxic oxygen free radicals are formed, and antioxidative molecules play crucial roles in maintaining homeostasis (Lou et al., 1998). Destruction of the cellular membrane, which contains a high amount of polyunsaturated fatty acids and oxygen free radicals, is the most important step in neuronal damage (Demopoulos et al., 1980; Solaroglu et al., 2005). Lipid peroxidation occurs at the lipid bilayer and is a major factor in secondary spinal cord I/R injury (Diaz-Ruiz et al., 2000). MDA is a waste product of polyunsaturated fatty acid degeneration and can reliably show the extent of lipid peroxidation (Pandey and Rizvi, 2010). After spinal cord I/R injury, MDA levels increase to address lipid peroxidation secondary to reperfusion injury (Christie et al., 2008). Consistent with this, we showed that MDA levels excessively increased in the ischemia control group. Calcitriol and methylprednisolone treatments prevented the increase in MDA levels associated with spinal cord I/R injury, allowing us to conclude that both treatments can decrease lipid peroxidation in the spinal cord.

Reperfusion after ischemia causes secondary injury because of the effects of reactive oxygen species (Chan, 1996). CAT is an antioxidant enzyme that has a buffering effect on these molecules (İlhan et al., 2004), with the levels and activities of enzymes such as CAT decreasing during states of excessive oxidative stress that cause severe molecular damage (Gokce et al., 2015; Gürer et al., 2015; Kertmen et al., 2013; Yilmaz et al., 2012). Following brain ischemic injury, calcitriol has been shown to increase glial hemooxygenase-1 levels and increase the degradation of free heme to the endogenous anti-oxidants biliverdin and bilirubin (Losem-Heinrichs et al., 2004). Physiological concentrations of calcitriol inhibit gamma-glutamyl transpeptidase in embryonic astrocytes and increase the potent antioxidant glutathione (Lin et al., 2003). Besides, calcitriol inhibits the entrance of oxygen free radicals (e.g., H_2O_2) into the cell (Ibi et al., 2001). As expected, CAT levels decreased after I/R injury in this study, and calcitriol and methylprednisolone treatments increased CAT levels. This supports the argument that both substances have high antioxidant properties. XO is another reliable enzyme for assessing the degree of oxidative stress and increase in oxygen free radicals (Hille and Nishino, 1995). In our study, increased XO activity was observed in the I/R group, and calcitriol and methylprednisolone decreased XO activity. Thus, we conclude that calcitriol has a crucial role in neuroprotection through its antioxidant activity on CAT and XO.

We observed serious damage under light microscopy in the ischemia control group, with evidence of obvious hemorrhage, increased edema, and necrosis caused by I/R injury. Polymorphonuclear leukocytes, lymphocytes, and plasma cells, which are known inflammatory markers, were seen to infiltrate injured areas. In the ischemia control group, the normal motor neuron number decreased compared with the number in the laparotomy control group. The histopathologic results in the ischemia plus calcitriol and ischemia plus methylprednisolone groups were better than those in the ischemia control group, with the number of motor neurons preserved in both treatment groups. However, light microscopy alone is insufficient to confirm the neuroprotective effects of calcitriol, so we used transmission electron microscopy to show the numerous segregations in small, medium, and large myelinated axons. Treatment with calcitriol or methylprednisolone protected small and medium axons but could not protect large axons. Crucially, protection was better in the ischemia plus calcitriol group than in the ischemia plus methylprednisolone group.

In this study, we also assessed functional outcomes. The neurological functions of rabbits were evaluated using the Tarlov scoring system. Although all rabbits had some degree of paresis, the severity was clearly ameliorated in the ischemia plus calcitriol and ischemia plus methylprednisolone groups, and this functional recovery correlated with the biochemical and histopathological results. Thus, calcitriol and methylprednisolone not only protected the normal morphology and ultrastructural elements of the spinal cord from I/R injury (by inhibiting apoptosis, inflammation, and lipid peroxidation, and by decreasing oxidative stress) but also improved functional outcomes.

Methylprednisolone is an antioxidant and anti-inflammatory agent that has traditionally been used in spinal cord injury (Kwon et al., 2004). Despite this, several authors are questioning its routine clinical use and preferred choice of use in control groups in animal experiments (Gokce et al., 2015; Gürer et al., 2015; Kertmen et al., 2013; Yilmaz et al., 2012). Therefore, we compared calcitriol with methylprednisolone as an active control and also compared it with a laparotomy control group and an ischemia control group.

There were some limitations to this study. For example, few animals were included per group, which reduced the reliability of our results. In addition, different dosage regimens, with more detailed grouping could have been used. Investigations were also performed 24 h after injury, which limit the clinical relevance of the results. More detailed biochemical and histopathological evaluations are therefore needed, including changes over time. Furthermore, to determine the extent of ischemic spinal cord injury, biochemical markers such as creatine kinase and lactate dehydrogenase could have been studied. Finally, we used calcitriol treatment before spinal cord injury, which could decrease its practical use, particularly in emergency situations. Further studies are needed to clarify the role of calcitriol treatment in spinal cord I/R injury.

5. Conclusion

Biochemical, histopathological, ultrastructural, and functional analyses revealed that calcitriol provides meaningful neuroprotection against spinal cord I/R injury. Furthermore, calcitriol had a comparable effect to methylprednisolone. However, further experimental and clinical studies are needed to confirm these promising neuroprotective effects.

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References

Abrams, S.A., Coss-Bu, J.A., Tiosano, D., 2013. Vitamin D: effects on childhood health and disease. Nat. Rev. Endocrinol. 9, 162–170.
Acher, C.W., Wynn, M.M., Archibald, J., 1990. Naloxen and spinal fluid drainage as adjuncts in the surgical treatment of thoracoabdominal aortic aneurysm. Surgery 108, 755–762.
Atif, F., Yousuf, S., Sayeed, I., Ishrat, T., Hua, F., Stein, D.G., 2013. Combination treatment with progesterone and vitamin D hormone is more effective than monotherapy in ischemic stroke: the role of BDNF/TrkB/Erk1/2 signaling in neuroprotection. Neuropharmacology 67, 78–87.
Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–126.
Akyuz, G., Sanal-Toprak, C., Yagci, I., Giray, E., Kuru-Bektasoglu, P., 2017. The effect of vitamin D supplementation on pain, quality of life, and nerve conduction studies in women with chronic widespread pain. Int. J. Rehabil. Res. 40, 76–83.
Baas, D., Prüfer, K., Ittel, M.E., Kuchler-Bopp, S., Labourdette, G., Sarlième, L.L., Brachet, P., 2000. Rat oligodendrocytes express the vitamin D(3) receptor and respond to 1,25-dihydroxyvitamin D(3). Glia 31, 59–68.
Baeke, F., Gysemans, C., Korf, H., Mathieu, C., 2010. Vitamin D insufficiency: implications for the immune system. Pediatr. Nephrol. 25, 1597–1606.
Balabanova, S., Richter, H.P., Antoniadis, G., Homoki, J., Kremmer, N., Hanle, J., Teller, W.M., 1984. 25-Hydroxyvitamin D, 24, 25-dihydroxyvitamin D and 1,25-dihydroxyvitamin D in human cerebrospinal fluid. Klin. Wochenschr. 62, 1086–1090.
Balden, R., Selvamani, A., Sohrabji, F., 2012. Vitamin D deficiency exacerbates experimental stroke injury and dysregulates ischemia-induced inflammation in adult rats. Endocrinology 153, 2420–2435.
Bemiss, C.J., Mahon, B.D., Henry, A., Weaver, V., Cantorna, M.T., 2002. Interleukin-2 is one of the targets of 1,25-dihydroxyvitamin D3 in the immune system. Arch. Biochem. Biophys. 402, 249–254.
Bethea, J.R., Dietrich, W.D., 2002. Targeting the host inflammatory response in traumatic spinal cord injury. Curr. Opin. Neurol. 15, 355–360.
Bicknell, C.D., Riga, C.V., Wolfe, J.H., 2009. Prevention of paraplegia during thoracoabdominal aortic aneurysm repair. Eur. J. Vasc. Endovasc. Surg. 37, 654–660.
Brewer, L.D., Thibault, V., Chen, K.C., Langub, M.C., Landfield, P.W., Porter, N.M., 2001. Vitamin D hormone confers neuroprotection in parallel with downregulation of L-type calcium channel expression in hippocampal neurons. J. Neurosci. 21, 98–108.

Brodbelt, A.R., Stoodley, M.A., 2003. Post-traumatic syringomyelia: a review. J. Clin. Neurosci. 10, 401–408.
Brown, J., Bianco, J.I., McGrath, J.J., Eyles, D.W., 2003. 1,25-dihydroxyvitamin D3 induces nerve growth factor, promotes neurite outgrowth and inhibits mitosis in embryonic rat hippocampal neurons. Neurosci. Lett. 343, 139–143.
Bouillon, R., Carmeliet, G., Verlinden, L., van Etten, E., Verstuyf, A., Luderer, H.F., Lieben, L., Mathieu, C., Demay, M., 2008. Vitamin D and human health: lessons from vitamin D receptor null mice. Endocr. Rev. 29, 726–776.
Cantorna, M.T., Mahon, B.D., 2004. Mounting evidence for vitamin D as an environmental factor affecting autoimmune disease prevalence. Exp. Biol. Med. 229, 1136–1142.
Cantorna, M.T., Woodward, W.D., Hayes, C.E., DeLuca, H.F., 1998. 1,25-dihydroxyvitamin D3 is a positive regulator for the two anti-encephalitogenic cytokines TGF-beta 1 and IL-4. J. Immunol. 160, 5314–5319.
Cassada, D.C., Gangemi, J.J., Rieger, J.M., Linden, J., Kaza, A.K., Long, S.M., Kron, I.L., Tribble, C.G., Kern, J.A., 2001. Systemic adenosine A2A agonist ameliorates ischemic reperfusion injury in the rabbit spinal cord. Ann. Thorac. Surg. 72, 1245–1250.
Chabas, J.F., Stephan, D., Marqueste, T., Garcia, S., Lavaut, M.N., Nguyen, C., Legre, R., Khrestchatisky, M., Decherchi, P., Feron, F., 2013. Cholecalciferol (vitamin D_3) improves myelination and recovery after nerve injury. PLoS One 8, e65034.
Chan, P.H., 1996. Role of oxidants in ischemic brain damage. Stroke 27, 1124–1129.
Chen, K.B., Lin, A.M., Chiu, T.H., 2003. Systemic vitamin D3 attenuated oxidative injuries in the locus coeruleus of rat brain. Ann. NY Acad. Sci. 993, 313–324.
Chen, S., Sims, G.P., Chen, X.X., Gu, Y.Y., Chen, S., Lipsky, P.E., 2007. Modulatory effects of 1,25-dihydroxyvitamin D3 on human B cell differentiation. J. Immunol. 179, 1634–1647.
Christie, S.D., Comeau, B., Myers, T., Sadi, D., Purdy, M., Mendez, I., 2008. Duration of lipidperoxidation after acute spinal cord injury in rats and the effect of methylprednisolone. Neurosurg. Focus. 25, E5.
Cornet, A., Baudet, C., Neveu, I., Baron-Van Evercooren, A., Brachet, P., Naveilhan, P., 1998. 1,25-Dihydroxyvitamin D3 regulates the expression of VDR and NGF gene in Schwann cells in vitro. J. Neurosci. Res. 53, 742–746.
Crawford, E.S., Crawford, J.L., Safi, H.J., Coselli, J.S., Hess, K.R., Brooks, B., Norton, H.J., Glaeser, D.H., 1986. Thoracoabdominal aortic aneurysms: preoperative and intraoperative factors determining immediate and long-term results of operations in 605 patients. J. Vasc. Surg. 3, 389–404.
Demopoulos, H.B., Flamm, E.S., Pietronigro, D.D., Seligman, M.L., 1980. The free radical pathology and the microcirculation in the major central nervous system disorders. Acta Physiol. Scand. Suppl. 492, 91–119.
Diaz-Ruiz, A., Rios, C., Duarte, I., Correa, D., Guizar-Sahagun, G., Grijalva, I., Madrazo, I., Ibarra, A., 2000. Lipid peroxidation inhibition in spinal cord injury: cyclosporin-A vs methylprednisolone. Neuroreport 11, 1765–1767.
Dietrich, W.D., Levi, A.D., Wang, M., Green, B.A., 2011. Hypothermic treatment for acute spinalcord injury. Neurotherapeutics 8, 229–239.
Emery, E., Aldana, P., Bunge, M.B., Puckett, W., Srinivasan, A., Keane, R.W., Bethea, J., Levi, A.D., 1998. Apoptosis after traumatic human spinal cord injury. J. Neurosurg. 89, 911–920.
Eyles, D.W., Smith, S., Kinobe, R., Hewison, M., McGrath, J.J., 2005. Distribution of the vitamin D receptor and 1 alpha-hydroxylase in human brain. J. Chem. Neuroanat. 29, 21–30.
Fan, L., Wang, K., Shi, Z., Die, J., Wang, C., Dang, X., 2011. Tetramethylpyrazine protects spinal cord and reduces inflammation in a rat model of spinal cord ischemia-reperfusion injury. J. Vasc. Surg. 54, 192–200.
Fu, J., Xue, R., Gu, J., Xiao, Y., Zhong, H., Pan, X., Ran, R., 2013. Neuroprotective effect of calcitriol on ischemic/reperfusion injury through the NR3A/CREB pathways in the rat hippocampus. Mol. Med. Rep. 8, 1708–1714.
Garcion, E., Nataf, S., Berod, A., Darcy, F., Brachet, P., 1997. 1,25-Dihydroxyvitamin D3 inhibits the expression of inducible nitric oxide synthase in rat central nervous system during experimental allergic encephalomyelitis. Brain Res. Mol. Brain Res. 45, 255–267.
Garcion, E., Sindji, L., Leblondel, G., Brachet, P., Darcy, F., 1999. 1,25-dihydroxyvitamin D3 regulates the synthesis of gamma-glutamyltranspeptidase and glutathione levels in rat primary astrocytes. J. Neurochem. 73, 859–866.
Garcion, E., Wion-Barbot, N., Montero-Menei, C.N., Berger, F., Wion, D., 2002. New clues about vitamin D functions in the nervous system. Trends Endocrinol. Metab. 13, 100–105.
Glaser, S.D., Veenstra, T.D., Jirikowski, G.F., Prüfer, K., 1999. Distribution of 1,25-dihydroxyvitamin D3 receptor immunoreactivity in the rat olfactory system. Cell. Mol. Neurobiol. 19, 613–624.
Gokce, E.C., Kahveci, R., Atanur, O.M., Gürer, B., Aksoy, N., Gokce, A., Sargon, M.F., Cemil, B., Erdogan, B., Kahveci, O., 2015. Neuroprotective effects of Ganoderma lucidum polysaccharides against traumatic spinal cord injury in rats. Injury 46, 2146–2155.
Green, S., Chambon, P., 1988. Nuclear receptors enhance our understanding of transcription regulation. Trends Genet. 4, 309–314.
Gürer, B., Kertmen, H., Kasim, E., Yilmaz, E.R., Kanat, B.H., Sargon, M.F., Arikok, A.T., Ergüder, B.I., Sekerci, Z., 2015. Neuroprotective effects of testosterone on ischemia/reperfusion injury of the rabbit spinal cord. Injury 46, 240–248.
Hasturk, A., Atalay, B., Calisaneller, T., Ozdemir, O., Oruckaptan, H., Altinors, N., 2009. Analysis of serum pro-inflammatory cytokine levels after rat spinal cord ischemia/reperfusion injury and correlation with tissue damage. Turk. Neurosurg. 19, 353–359.
Haussler, M.R., Haussler, C.A., Whitfield, G.K., Hsieh, J.C., Thompson, P.D., Barthel, T.K., Bartik, L., Egan, J.B., Wu, Y., Kubicek, J.L., Lowmiller, C.L., Moffet, E.W., Forster, R.E., Jurutka, P.W., 2010. The nuclear vitamin D receptor controls the expression of genes encoding factors which feed the “fountain of youth” to mediate healthful aging. J. Steroid Biochem. Mol. Biol. 121, 88–97.
Hayashi, T., Sakuria, M., Abe, K., Sadahiro, M., Tabayashi, K., Itoyama, Y., 1998. Apoptosis of motor neurons with induction of caspases in the spinal cord after ischemia. Stroke 29, 1007–1013.
Hearse, D.J., Boli, R., 1992. Reperfusion induced injury: manifestations, mechanisms, and clinical relevance. Cardiovasc. Res. 26, 101–108.
Hille, R., Nishino, T., 1995. Flavoprotein structure and mechanism. 4. Xanthine oxidase and Xanthine dehydrogenase. Faseb. J. 9, 995–1003.
Hwang, H.S., Yang, K.J., Park, K.C., Choi, H.S., Kim, S.H., Hong, S.Y., Jeon, B.H., Chang, Y.K., Park, C.W., Kim, S.Y., Lee, S.J., Yang, C.W., 2013. Pretreatment with paricalcitol attenuates inflammation in ischemia-reperfusion injury via the up-regulation of cyclooxygenase-2 and prostaglandin E2. Nephrol. Dial. Transplant. 28, 1156–1166.
Ibi, M., Sawada, H., Nakanishi, M., Kume, T., Katsuki, H., Kaneko, S., Shimohama, S., Akaike, A., 2001. Protective effects of 1 alpha,25-(OH)(2)D(3) against the neurotoxicity of glutamate and reactive oxygen species in mesencephalic culture. Neuropharmacology 40, 761–771.
Ilhan, A., Yilmaz, H.R., Armutcu, F., Gurel, A., Akyol, O., 2004. The protective effect of nebivolol on ischemia/reperfusion injury in rabbit spinal cord. Prog. Neuropsychopharmacol. Biol. Psychiatry 28, 1153–1160.
Kajta, M., Makarewicz, D., Ziemińska, E., Jantas, D., Domin, H., Lasoń, W., Kutner, A., Łazarewicz, J.W., 2009. Neuroprotection by co-treatment and post-treating with calcitriol following the ischemic and excitotoxic insult in vivo and in vitro. Neurochem. Int. 55, 265–274.
Kalueff, A.V., Eremin, K.O., Tuohimaa, P., 2004. Mechanisms of neuroprotective action of vitamin D(3). Biochemistry 69, 738–741.
Kanellopoulos, G.K., Kato, H., Wu, Y., Dougenis, D., Mackey, M., Hsu, C.Y., Kouchoukos, N.T., 1997. Neuronal cell death in the ischemic spinal cord: the effect of methylprednisolone. Ann. Thorac. Surg. 64, 1279–1285.
Kaptanoglu, E., Palaoglu, S., Surucu, H.S., Hayran, M., Beskonakli, E., 2002. Ultrastructural scoring of graded acute spinal cord injury in the rat. J. Neurosurg. 97, 49–56.
Kertmen, H., Gürer, B., Yılmaz, E.R., Sanlı, A.M., Sorar, M., Arıkök, A.T., Sargon, M.F., Kanat, M.A., Ergüder, B.I., Sekerci, Z., 2013. The protective effect of low-dose methotrexate on ischemia-reperfusion injury of the rabbit spinal cord. Eur. J. Pharmacol. 714, 148–156.
Kim, Y.O., Li, C., Sun, B.K., Kim, J.S., Lim, S.W., Choi, B.S., Kim, Y.S., Kim, J., Bang, B.K., Yang, C.W., 2005. Preconditioning with 1,25-dihydroxyvitamin D3 protects against subsequent ischemia-reperfusion injury in the rat kidney. Nephron. Exp. Nephrol. 100, e85–e94.
Koren, R., Liberman, U.A., Maron, L., Novogrodsky, A., Ravid, A., 1989. 1,25-Dihydroxyvitamin D3 acts directly on human lymphocytes and interferes with the cellular response to interleukin-2. Immunopharmacology 18, 187–194.
Kuru, P., Akyuz, G., Yagci, I., Giray, E., 2015. Hypovitaminosis D in widespread pain: its effect on pain perception, quality of life and nerve conduction studies. Rheumatol. Int. 35, 315–322.
Kwon, B.K., Oxland, T.R., Tetzlaf, W., 2002. Animal models used in spinal cord regeneration research. Spine 27, 1504–1510.
Kwon, B.K., Tetzlaff, W., Grauer, J.N., Beiner, J., Vaccaro, A.R., 2004. Pathophysiology and pharmacologic treatment of acute spinal cord injury. Spine J. 4, 451–464.
Lee, J.W., Kim, S.C., Ko, Y.S., Lee, H.Y., Cho, E., Kim, M.G., Jo, S.K., Cho, W.Y., Kim, H.K., 2014. Renoprotective effect of paricalcitol via a modulation of the TLR4-NF-κB pathway in ischemia/reperfusion-induced acute kidney injury. Biochem. Biophys. Res. Commun. 444, 121–127.
Li, M., Ona, V.O., Chen, M., Kaul, M., Tenneti, L., Zhang, X., Stieg, P.E., Lipton, S.A., Friedlander, R.M., 2000. Functional role and therapeutic implications of neuronal caspase-1 and -3 in a mouse model of traumatic spinal cord injury. Neuroscience 99, 333–342.
Li, C., Zhao, R., Gao, K., Wei, Z., Yin, M.Y., Lau, L.T., Chui, D., Yu, A.C., 2011. Astrocytes: implications for neuroinflammatory pathogenesis of Alzheimer's disease. Curr. Alzheimer Res. 8, 67–80.
Lin, A.M., Fan, S.F., Yang, D.M., Hsu, L.L., Yang, C.H., 2003. Zinc-induced apoptosis in substantia nigra of rat brain: neuroprotection by vitamin D3. Free. Radic. Biol. Med. 34, 1416–1425.
Losem-Heinrichs, E., Görg, B., Redecker, C., Schleicher, A., Witte, O.W., Zilles, K., Bidmon, H.J., 2005. 1alpha,25-dihydroxy-vitamin D3 in combination with 17beta-estradiol lowers the cortical expression of heat shock protein-27 following experimentally induced focal cortical ischemia in rats. Arch. Biochem. Biophys. 439, 70–79.
Losem-Heinrichs, E., Görg, B., Schleicher, A., Redecker, C., Witte, O.W., Zilles, K., Bidmon, H.J., 2004. A combined treatment with 1alpha,25-dihydroxy-vitamin D3 and 17beta-estradiol reduces the expression of heat shock protein-32 (HSP-32) following cerebral cortical ischemia. J. Steroid Biochem. Mol. Biol. 89–90, 371–374.
Lou, J., Lenke, L.G., Ludwig, F.J., O'Brien, M.F., 1998. Apoptosis as a mechanism of neuronal cell death following acute experimental spinal cord injury. Spinal Cord 36, 683–690.
Lukácová, N., Halát, G., Chavko, M., Marsala, J., 1996. Ischemia-reperfusion injury in the spinal cord of rabbits strongly enhances lipid peroxidation and modifies phospholipid profiles. Neurochem. Res. 21, 869–873.
McGrath, J., Feron, F., Eyles, D., Mackay-Sim, A., 2001. Vitamin D: the neglected neurosteroid? Trends Neurosci. 24, 570–572.
Pandey, K.B., Rizvi, S.I., 2010. Markers of oxidative stress in erythrocytes and plasma during aging in humans. Oxid. Med. Cell. Longev. 3, 2–12.
Park, J.W., Cho, J.W., Joo, S.Y., Kim, C.S., Choi, J.S., Bae, E.H., Ma, S.K., Kim, S.H., Lee, J., Kim, S.W., 2012. Paricalcitol prevents cisplatin-induced renal injury by suppressing apoptosis and proliferation. Eur. J. Pharmacol. 683, 301–309.

Prajda, N., Weber, G., 1975. Malignant transformation-linked imbalance: decreased xanthine oxidase activity in hepatomas. FEBS. Lett. 59, 245–249.
Reece, T.B., Okonkwo, D.O., Ellman, P.I., Warren, P.S., Smith, R.L., Hawkins, A.S., Linden, J., Kron, I.L., Tribble, C.G., Kern, J.A., 2004. The evolution of ischemic spinal cord injury in function, cytoarchitecture, and inflammation and the effects of adenosine A2A receptor activation. J. Thorac. Cardiovasc. Surg. 128, 925–932.
Regulska, M., Leśkiewicz, M., Budziszewska, B., Kutner, A., Jantas, D., Basta-Kaim, A., Kubera, M., Jaworska-Feil, L., Lasoń, W., 2007. Inhibitory effects of 1,25-dihydroxyvitamin D3 and its low-calcemic analogues on staurosporine-induced apoptosis. Pharmacol. Rep. 59, 393–401.
Safi, H.J., Miller, C.C., 3rd, Huynh, T.T., Estrera, A.L., Porat, E.E., Winnerkvist, A.N., Allen, B.S., Hassoun, H.T., Moore, F.A., 2003. Distal aortic perfusion and cerebrospinal fluid drainage for thoracoabdominal and descending thoracic aortic repair: ten years of organ protection. Ann. Surg. 238, 372–380.
Sakurai, M., Nagata, T., Abe, K., Horinouchi, T., Itoyama, Y., Tabayashi, K., 2003. Survival and death-promoting events after transient spinal cord ischemia in rabbits: induction of Akt and caspase3 in motor neurons. J. Thorac. Cardiovasc. Surg. 125, 370–377.
Sanli, A.M., Serbes, G., Sargon, M.F., Çalişkan, M., Kilinç, K., Bulut, H., Sekerci, Z., 2012. Methothrexate attenuates early neutrophil infiltration and the associated lipid peroxidation in the injured spinal cord but does not induce neurotoxicity in the uninjured spinal cord in rats. Acta Neurochir. 154, 1045–1054.
Seif, A.A., Abdelwahed, D.M., 2014. Vitamin D ameliorates hepatic ischemic/reperfusion injury in rats. J. Physiol. Biochem. 70, 659–666.
Sezgin, G., Oztürk, G., Güney, S., Sinanoğlu, O., Tunçdemir, M., 2013. Protective effect of melatonin and 1,25-dihydroxyvitamin D3 on renal ischemia-reperfusion injury in rats. Ren. Fail. 35, 374–379.
Shih, P.K., Chen, Y.C., Huang, Y.C., Chang, Y.T., Chen, J.X., Cheng, C.M., 2011. Pretreatment of vitamin D3 ameliorates lung and muscle injury induced by reperfusion of bilateral femoral vessels in a rat model. J. Surg. Res. 171, 323–328.
Sinanoglu, O., Sezgin, G., Ozturk, G., Tuncdemir, M., Guney, S., Aksungar, F.B., Yener, N., 2012. Melatonin with 1,25-dihydroxyvitamin D3 protects against apoptotic ischemia-reperfusion injury in the rat kidney. Ren. Fail. 34, 1021–1026.
Solaroglu, I., Kaptanoglu, E., Okutan, O., Beskonakli, E., Attar, A., Kilinc, K., 2005. Magnesium sulfate treatment decreases caspase-3 activity after experimental spinal cord injury in rats. Surg. Neurol. 64, S17–S21.
Stumpf, W.E., Sar, M., Clark, S.A., DeLuca, H.F., 1982. Brain target sites for 1,25-dihydroxyvitamin D3. Science 215, 1403–1405.
Taoka, Y., Okajima, K., Uchiba, M., Murakami, K., Kushimoto, S., Johno, M., Naruo, M., Okabe, H., Takatsuki, K., 1997. Role of neutrophils in spinal cord injury in the rat. Neuroscience 79, 1177–1182.
Tator, C.H., Fehlings, M.G., 1991. Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. J. Neurosurg. 75, 15–26.
Umehara, S., Goyagi, T., Nishikawa, T., Tobe, Y., Masaki, Y., 2010. Esmolol and landiolol, selective beta1-adrenoreceptor antagonists, provide neuroprotection against spinal cord ischemia and reperfusion in rats. Anesth. Analg. 110, 1133–1137.
Wang, Y., Chiang, Y.H., Su, T.P., Hayashi, T., Morales, M., Hoffer, B.J., Lin, S.Z., 2000. Vitamin D(3) attenuates cortical infarction induced by middle cerebral arterial ligation in rats. Neuropharmacology 39, 873–880.
Yilmaz, E.R., Kertmen, H., Dolgun, H., Gürer, B., Sanli, A.M., Kanat, M.A., Arikok, A.T., Bahsi, S.Y., Ergüder, B.I., Sekerci, Z., 2012. Effects of darbepoetin-α in spinal cord ischemia-reperfusion injury in the rabbit. Acta Neurochir. 154, 1037–1043.
Zhou, K.L., Chen, D.H., Jin, H.M., Wu, K., Wang, X.Y., Xu, H.Z., Zhang, X.L., 2016. Effects of calcitriol on experimental spinal cord injury in rats. Spinal Cord 54, 510–516.
Zivin, J.A., DeGirolami, U., 1980. Spinal cord infarction: a highly reproducible stroke model. Stroke 11, 200–202.