Original Article

The effects of prophylactic zinc and melatonin application on experimental spinal cord ischemia–reperfusion injury in rabbits: experimental study

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Study design: Experimental study.

Objectives: To determine the neuroprotective effects of zinc and melatonin on spinal cord ischemia–reperfusion (I/R) injuries of rabbits.

Setting: The Experimental Research Centre of Selçuk University, Konya, Turkey.

Methods: Twenty-four male rabbits underwent spinal cord ischemia by clamping the thoraco-abdominal aorta for 20 min. Twenty minutes before the aortic clamping, animals received zinc, melatonin or a combination of both. Neurological examination of the animals was performed three times during reperfusion period. The animals were killed 24 h after reperfusion. Spinal cord samples were taken for biochemical and histopathological evaluation.

Results: Pre-treated animals with zinc, melatonin or combination displayed better neurological outcomes than the I/R group ($P < 0.05$). Zinc, melatonin and combined treatment prevented spinal cord injury by reducing apoptosis rate ($P < 0.05$) and preserving intact ganglion cell numbers ($P < 0.05$). Zinc pre-treatment protected spinal cord by preventing malondialdehyde (MDA) formation ($P = 0.002$), increasing glutathione peroxidase (GPx) activity ($P = 0.002$) and decreasing xanthine oxidase enzyme activity ($P = 0.026$) at molecular level. Melatonin treatment also resulted with MDA formation ($P = 0.002$), increased GPx activity ($P = 0.002$) and decreased xanthine oxidase activity ($P = 0.026$).

Conclusion: The results of the study showed that prophylactic zinc and melatonin use in spinal cord I/R not only suppressed lipid peroxidation by activating antioxidant systems but also had significant neuroprotective effects by specifically improving the neurological and histopathological situation.

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Keywords: ischemia–reperfusion; zinc; melatonin; oxidative damage; rabbit

Introduction

Paraplegia, which results from spinal cord ischemia, can emerge as a result of postoperations concerning the thoracic and the thoracoabdominal aorta or the spinal cord.^{1,2} The neurological damage to the spinal cord due to ischemia–reperfusion (I/R) injury has an average incidence ranging from 3 to 33%.^{1,2,3} Free radicals are reactive molecules that emerge in traumatic brain injury and in the pathology of cerebral ischemia and they also constitute the mechanism known as oxidative stress.⁴

Melatonin, which is the main secretion product of the pineal gland, functions as a strong free radical scavenger and an antioxidant. Melatonin has especially significant neutralizing effect on hydroxyl radicals and stimulating

effect on glutathione peroxidase (GPx) activity.⁵ Furthermore, the increase in malondialdehyde (MDA) levels and the decrease in glutathione (GSH) levels related to ischemia are reversed with melatonin treatment.⁶ Compatible with these examinations, it was established that melatonin supports also significantly increased catalase and superoxide dismutase (SOD) activities, which have powerful antioxidant qualities.⁵ It was reported that the GPx, SOD activity decreased in relation to spinal injuries;⁷ also in a similar study it was reported that myeloperoxidase (MPO) activity increased as a result of spinal injury, but melatonin treatment suppressed the increased MPO activity and also corrected motor deficits.⁸ In another study, it was stated that the decrease in the Tarlov score as a result of spinal cord injury increased with melatonin treatment.⁹ In a

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study that investigated the relationship between spinal cord injury and xanthine oxidase (XO) activity, it was reported that the XO activity significantly increased following the injury.¹⁰ It was also stated that melatonin treatment had a protective role against lipid peroxidation by affecting pro-oxidant enzyme activity like XO and MPO.¹¹

Nitric oxide (NO) acts as an important mediator in glutamate toxicity, which develops after cerebral I/R. However, melatonin treatment reduces brain NO production by suppressing NO synthetase activity.¹² Application of zinc together with melatonin suppresses the effect that melatonin creates in NO production.¹³ It was reported that melatonin treatment prevented cell apoptosis and necrosis in parallel with lipid peroxidation.¹⁴

Zinc is also a strong antioxidant and it is acknowledged that it reduces oxidative stress and has neuroprotective effects.¹⁵ It is emphasized that zinc also plays an apoptosis-suppressing role with its metallothionein increasing effects.¹⁶ However, as zinc above normal levels is neurotoxic, chelation of excessive zinc has an improving effect on zinc neurotoxicity and a decreasing effect on neuronal injury.¹⁷

It was observed that the effects of melatonin on lipid peroxidation in spinal cord injuries were investigated in the previous studies. However, the effect of zinc has not been investigated in spinal cord injuries. The present study was conducted to determine the respective and combined neurological, biochemical and histopathological effects of zinc and melatonin in experimental spinal cord injuries.

Materials and methods

The present study was conducted at the Experimental Medicine Research and Application Center of Selçuk University after being approved by the board of research ethics of the mentioned institution (15 April 2005–09). The study was conducted on 28 male rabbits of New Zealand breed with average weights between 2.15 and 2.8 kg. The animal ages were between 32 and 48 weeks (mean: 40 weeks). The animals were kept at an average room temperature of 21°C and they were fed with standard food. Food and water were given freely.

Before the operation experimental animals were anesthetized intramuscularly with a combined dose of ketamine (70 mg/kg) (Ketalar, Parke-Davis Eczacibasi, Istanbul, Turkey) and xylazine (5 mg/kg) (Rompun, Bayer, Istanbul, Turkey). Additional required doses were administered during the operation. The animals were allowed to breathe room air without mechanical ventilation. Body temperature was kept close to 38°C using a heated operation table. An intravenous catheter (24 gauge) was placed in an ear vein and preoperatively cefazoline 10 mg/kg was administered as a single dose. Maintenance fluid of 0.9% NaCl was infused at a rate of 20 ml/h during the procedure.

The study groups were formed as follows

1. Sham group ($n = 4$): Only laparotomy was performed on the animals in this group.
2. I/R group ($n = 6$): Ischemia was performed for 20 min followed by reperfusion for 24 h.
3. I/R + zinc supplemented group ($n = 6$): The animals in this group were administered with a dose of 10 mg/kg zinc sulfate 20 min before the I/R period and then I/R was performed.
4. I/R + melatonin supplemented group ($n = 6$): After the animals were given a dose of 10 mg/kg melatonin, I/R was performed as in the previous group.
5. I/R + zinc + melatonin supplemented group ($n = 6$): After a combined dose of 10 mg/kg zinc and 10 mg/kg melatonin were administered to the animals in this group, I/R was performed.

I/R procedure

Spinal cord ischemia model was created as defined by DeGirolami and Zivin.¹⁸ The animals were placed in the supine position with the pelvis partially rotated to the right. After the surgical preparation, the vertical incision was made from left costal margin directed towards the pubis. The abdominal aorta was exposed through a retroperitoneal approach and mobilized from just inferior to the left renal vein down to the aortic bifurcation. Heparin (100 U/kg) was administered intravenously 5 min before aortic occlusion. Thoraco-abdominal aorta was clamped just below the renal vein with a 'Bulldog' atraumatic vascular clamp. A second similar clamp was placed above the aortic bifurcation for occluding iliac collateral circulation. Animals were exposed to ischemia for 20 min. At the end of occlusion time, the clamps were removed and restoration of the blood flow was observed. In the sham group, which formed the control group, only laparotomy was performed without applying clamp. The catheters were removed and the abdomen layers were sutured. When the animals came out from anesthesia, they were placed in their cages.

Postoperatively, the animals were observed for neurological signs for 24 h (their neurological examinations were recorded at the 1st, 8th and 24th hours) and their motor inefficiency and improvement rates were followed.

At the end of the experimental period, lumbar spinal cord tissue (L4–S1 segments) samples were taken and the changes in MDA, GPx, XO, NO and MPO levels were examined; in addition, L1–L3 segments were evaluated histopathologically for apoptosis and intact ganglion cell.

Neurological examination

Neurological evaluations were assessed by using Tarlov Scoring System. The animals were observed for 24 h after the I/R period and their neurological evaluations were performed at the 1st, 8th and 24th hours.¹⁹ In the

neurological evaluation, scores were classified as follows: 0: paraplegic; 1: seriously paraplegic; 2: functional movements present; 3: ataxic disconjugate movements; 4: minimal ataxic; 5: normal functions.

Biochemical analyses

Subjects and sample preparation Tissues were homogenized in 0.2 mM Tris-HCl buffer (pH 7.5) for NO, GPx, XO and MDA determinations. Hexadecyltrimethyl ammonium bromide (0.5%) was used for MPO determination. The tissues in the glass tubes were homogenized by ultrasonic homogenizer. This homogenate was used for NO and protein assay. Then the homogenate was centrifuged at 3220 r.p.m. for 30 min at 6°C. This supernatant was used for GPx, XO and protein assays. For tissue MPO determination, homogenized samples were centrifuged at 3220 r.p.m. for 45 min (+4°C). MPO and protein assays were determined in this supernatant. All samples were measured in duplicate.

Measurement of MDA

The MDA levels, as an index of lipid peroxidation, was determined by thiobarbituric acid reaction according to Uchiyama and Mihara.²⁰ The principle of the method depends on measurement of the pink color produced by interaction of the barbituric acid with MDA elaborated as a result of lipid peroxidation. The colored reaction 1,1,3,3 tetraethoxypropane was used as primary standard. The color absorbance was read at 530 nm.

Measurement of the GPx levels

The levels of GPx were determined by using an indirect method (Sigma, USA). The principle of the method is based on the oxidation of GSH to oxidized glutathione (GSSG) catalyzed by GPx, which is then coupled to the recycling of GSSG back to GSH utilizing glutathione reductase (GR) and β -nicotinamide adenine dinucleotide phosphate (NADPH). The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to NADP is indicative of GPx activity, as GPx is the rate-limiting factor of the coupled reactions.

Measurement of the XO activity

The XO activity is measured by the principle of the formation of uric acid from the xanthine. The addition of 100% TCA finalizes the reaction and formation of uric acid. The absorbance of uric acid was determined at 293 nm by the spectrophotometer and the amount of produced uric acid was determined in 30 min. The enzyme activity is given as U/mg protein.²¹

Measurement of nitrite and nitrate

The levels of nitrite and nitrate were determined by using a photometric end-point determination (Roche Diagnostic GmbH, Mannheim, Germany). The nitrate

is reduced to nitrite by reduced NADPH in the presence of the enzyme nitrate reductase. The nitrite formed reacts with sulfanilamide and *N*-(1-naphtyl)-ethylene-diamine dihydrochlorid to give a red-violet diazo dye. The diazo dye is measured on the basis of its absorbance in the visible range (550 nm).

Measurement of MPO activity

MPO activity was determined by a modification of the *O*-dianosidine.²² The assay mixture, in a cuvette of 1-cm path length, contained 0.2 ml 0.1 mol/l phosphate buffer (pH 7.1), 0.09 ml H₂O₂ (3%, v/v), 0.1 ml 0.02 mol/l *O*-dianosidine (freshly prepared in methanol) and 0.1 ml sample I a final volume of 2 ml. The sample was added last and the change in absorbance at 410 nm was followed for 30 min. All measurements were carried out in duplicate. One unit of MPO is defined as the increase in absorbance of 0.001 per min. The specific activity is given as U/mg of protein.²²

Histopathological examination

Tissue samples were fixed in 10% paraformaldehyde and prepared with autotechnicon and then embedded in paraffin. Slices (5 μ m) were obtained with a microtome and stained with hematoxylin and eosin (HE) and toluidine blue (TB). HE-stained specimens were examined under a Nikon Eclipse E400 light microscope. Apoptotic cells were seen as a picnotic, hiperchromatic nuclei and chromatolytic neurons (Figures 1, 2). For each specimen, the same area was photographed after staining by using a Nikon Coolpix 5000 photograph attachment. The photograph of Nikon micrometer microscope slide was also taken during the procedure. All photographs were then transferred into a PC and analyzed using Clemex Vision Lite 3.5 Image Analysis (Clemex Technologies Inc. Guimond, Longueuil, Canada) program. The length was calibrated by comparing

Figure 1 Histopathologic evaluation of apoptosis in the L2 segment of I/R spinal cord. Arrow indicates the hiperchromatic and picnotic appearance of apoptotic cell (HE)

Figure 2 Histopathologic evaluation of chromatolytic neurons in the L2 segment of I/R spinal cord. Arrows display chromatolytic neurons in spinal gray matter (HE)

the photograph of specimen with the photograph of Nikon micrometer microscope slide, which was taken under the same magnification. An area of 0.2 mm^2 was designated using Clemex Vision Lite 3.5 Image Analysis Program; then apoptotic cells were marked with the same Image Analysis Program in 0.2 mm^2 area. Damaged cells were not evaluated. The marked cells were counted automatically with the same Image Analysis Program. The reader was masked to the origin of the specimen. Each case was analyzed for four different areas.

In addition, the changes in the gray matter were evaluated in the TB-stained sections. Intact ganglion cell numbers were calculated using the same method described above in 0.1 mm^2 area.

Statistical analysis

The differences between groups in neurological examination were analyzed by one-way ANOVA tests, Tukey HSD by using SPSS 12.0[©] package program and *P*-values lower than 0.05 were accepted as significant.²³ The differences between groups in biochemical data were analyzed by Mann-Whitney *U*-test using SPSS 12.0[©] package program. Apoptotic cell numbers ($n/0.2\text{ mm}^2$) and intact ganglion cell numbers ($n/0.1\text{ mm}^2$) in unit area were analyzed by using one-way ANOVA.

Results

Neurological evaluation

Neurological evaluation was performed at the 1st, 8th and the 24th, respectively for each group (Table 1). It was established that there were remarkable differences between the IR group and the other groups. In the first hour, there was significant difference between group 1 and groups 2–5 ($P < 0.05$) (Figure 3, Table 1). Group 2 was significantly different from groups 1, 3, 4 and 5

Table 1 Neurological examination scores of the groups

Subject no.	1st hour	8th hour	24th hour
Group 1 – SHAM*			
1	5	5	5
2	5	5	5
3	5	5	5
4	5	5	5
Group 2 – IR**			
1	0	0	0
2	1	1	0
3	0	0	0
4	0	0	0
5	0	0	0
6	0	1	1
Group 3 : IR –zinc***			
1	2	2	3
2	2	4	4
3	2	4	4
4	2	4	4
5	1	2	2
6	3	4	4
Group 4 : IR –melatonin***			
1	1	1	2
2	4	4	4
3	1	2	2
4	2	2	3
5	2	4	4
6	0	1	1
Group 5:IR melatonin-zinc combined group***			
1	2	4	4
2	2	2	2
3	1	2	2
4	1	4	4
5	1	3	3
6	2	4	4

* $P < 0.05$ compared to groups 2–5 in 1st hour

** $P < 0.05$ compared to groups 1, 3, 4, 5 in 1st, 8th and 24th hours

*** $P < 0.05$ compared to groups 1 and 2 in 1st, 8th and 24th hour

($P < 0.05$). Although group 3 was significantly different from groups 1 and 2 ($P < 0.05$), the same was not found between group 4 and group 5 ($P > 0.05$). Although groups 4 and 5 were significantly different from groups 1 and 2 ($P < 0.05$), no statistical difference could be seen between group 3 and groups 4 and 5 ($P > 0.05$). Statistically similar findings were also observed at hours 8th (Figure 4, Table 1) and the 24th hours (Figure 5, Table 1). Neurologically, as a significant difference was observed between the IR group and the groups that were given zinc, melatonin and both combined ($P < 0.05$), it was determined that there were no significant differences among the zinc, melatonin and combined groups ($P > 0.05$).

Figure 3 Neurologic examination scores in the groups in 1 h. Data were analyzed with one-way ANOVA, Tukey HSD (group 1: control, group 2: I/R, group 3: I/R + zinc, group 4: I/R + mel, group 5: I/R + zinc + mel). * $P < 0.05$ compared to groups 2–5. ** $P < 0.05$ compared to groups 1, 3–5. *** $P < 0.05$ compared to groups 1 and 2

Figure 4 Neurologic examination scores in the groups at 8th hour. Data were analyzed with one-way ANOVA, Tukey HSD (group 1: control, group 2: I/R, group 3: I/R + zinc, group 4: I/R + mel, group 5: I/R + zinc + mel). * $P < 0.05$ compared to groups 2–5. ** $P < 0.05$ compared to groups 1, 3–5. *** $P < 0.05$ compared to groups 1 and 2

Biochemical evaluation

As shown in Table 2, there was a marked increase in tissue MDA levels in the I/R group compared to sham group ($P = 0.004$). On the other hand, treatment with

Figure 5 Neurologic examination scores in the groups at 24th hour. Data were analyzed with one-way ANOVA, Tukey HSD (group 1: control, group 2: I/R, group 3: I/R + zinc, group 4: I/R + mel, group 5: I/R + zinc + mel). * $P < 0.05$ compared to groups 2–5. ** $P < 0.05$ compared to groups 1, 3–5. *** $P < 0.05$ compared to groups 1 and 2

zinc, melatonin and combined therapy prevented MDA formation. Zinc-treated group MDA levels were close to the sham group. Further decrease was observed in melatonin-treated group and MDA levels were significantly lower than sham group ($P = 0.002$). Combined use of the antioxidants also reduced the MDA levels significantly when compared to I/R group ($P = 0.005$).

GPx activity was similar in the I/R group when compared to control. GPx activities were significantly increased in the groups given zinc. Similarly, melatonin treatment increased GPx activities over the control and sham groups. GPx activities significantly increased in groups given I/R + zinc, I/R + melatonin and I/R + zinc + melatonin when compared to the sham group ($P = 0.002$).

XO activities increased significantly in the I/R group in comparison to the sham group and supplemented groups ($P = 0.002$). Melatonin treatment suppressed XO activities in single or combined group. XO activities in antioxidant treated group were close to the sham group.

Tissue nitrate levels between sham and I/R group were not statistically different. However, all treatment group levels were lower than sham group. Only the decrease in the melatonin pretreatment was found statistically significant ($P = 0.004$). Tissue MPO levels were similar in all groups. There was no statistically significant difference between all groups.

Histopathological evaluation

The difference between the number of apoptotic cells in the unit area in group 1 and the other groups^{2–5} was

Table 2 Tissue biochemical analysis of the groups

Groups	MDA (nmol/l)	GPx (U/mg) pro	XO (mU/mg) Pro	Nitrite/nitrate (mg/l)	MPO (U/mg) Pro
Sham	202.9 ± 14.1	28.54 ± 2.8	18.2 ± 5	2.78 ± 1.28	83.9 ± 8.9
I/R	624 ± 351*	29.6 ± 14.8	53 ± 28 [^]	2.17 ± 0.7	86.1 ± 8.3
I/R + zinc	245.6 ± 52.5 [^]	42.2 ± 5.6**	15 ± 3.3	2.26 ± 0.7	77.9 ± 11.2
I/R + Mel	119.5 ± 18***	43.4 ± 11.7**	14 ± 10	1.32 ± 0.1*	69.5 ± 11.7
I/R + zinc + Mel	129.6 ± 41****	43 ± 7.6**	12 ± 8	1.66 ± 0.3	69.49 ± 4.8

I/R: ischemia/reperfusion, Mel: melatonin, Pro: protein

P*: 0.004 compared to sham*P*: 0.002 compared to I/R****P*: 0.002 compared to I/R[^]*P*: 0.002 compared to I/R + Mel^{^^}*P*: 0.026 compared to all other groups**Table 3** Apoptotic cells (*n*/0.2 mm²) and intact ganglion cell number(*n*/0.1 mm²)

	Control	I/R	Zinc + I/R	Melatonin + I/R	Melatonin + zinc + I/R
Apoptotic cells (<i>n</i> /0.2 mm ²)	0.69 ± 0.60*	6.04 ± 1.30**	2.79 ± 0.88***	3.05 ± 0.76***	2.50 ± 0.73***
Ganglion number (<i>n</i> /0.1 mm ²)	8.00 ± 0.73	1.83 ± 0.86 [^]	6.00 ± 1.22	6.60 ± 1.14	5.56 ± 1.31

P* < 0.05 compared to groups 2–5*P* < 0.05 compared to groups 1, 3–5****P* < 0.05 compared to groups 1 and 2[^]*P* < 0.05 compared to groups 1, 3–5

statistically significant (*P* < 0.05, Table 3). Group 2 had a significant difference in apoptotic cell numbers compared to all the other groups (*P* < 0.005). Although group 3 had a significant difference (*P* < 0.05) compared to group 1 and group 2, there were no differences statistically between group 3 and groups 4 and 5. Similarly, although group 4 had a significant difference (*P* < 0.05) in apoptotic cell numbers compared with groups 1 and 2, they showed no significant difference with groups 3 and 5. Although group 5, which received the combined supplement, had a significant difference when compared to group 1 and group 2 (*P* < 0.05), it was found that there were no significant differences between groups 3 and 4 and this group (*P* > 0.05, Figure 6).

When TB sections were evaluated, we observed significant decrease in the intact cell number in I/R group. Intact cell number was significantly higher in zinc-supplemented group when compared with I/R group (*P* < 0.05). Similarly, intact cell numbers were higher in both melatonin alone and with zinc group in comparison to I/R group (*P* < 0.05). There was no significant difference between the treated groups. Although the levels of treated groups were lower than the control levels, the difference was not statistically significant (Table 3, Figure 7).

Discussion

Paraplegia seems to be a serious complication of spinal trauma, thoracic or thoraco-abdominal surgery and

post-operative neurological deficits are observed in a high percentage of the cases.²⁴ As the result of clamping the thoracic aorta, the decrease in spinal blood flow considerably affects the experiment animals and human beings.²⁵ This I/R experimental model results in widespread spinal cord ischemia inferior L1 segments. Acute inflammatory response that occurs in the injured area causes the production of reactive oxygen types and results in lipid peroxidation. Prophylactic neuroprotective agents are widely used in experimental I/R models. In this study, the effects of zinc, melatonin and combined were tested by examining neurological outcome, various biochemical tests and histopathological specimen outcome of the spinal cord. The I/R group animals were totally paraplegic in 1st, 8th and 24th hours. Apoptotic cell numbers were significantly high in the I/R group. Antioxidant use improved the neurological outcome considerably in all groups. Improved neurological outcome paralleled to less apoptotic rate and higher protection in intact ganglion cells. Although antioxidant effects of melatonin are well established in the literature, zinc prophylactic effects on spinal cord I/R injury are the first in the literature. Cai *et al*²⁶ showed that zinc pretreatment has neuroprotective effects in radiation-induced apoptosis. There was no significant difference between zinc and melatonin. There were no additional protections when zinc and melatonin were used together.

When the findings of the present study are evaluated, it can be seen that there is a significant increase in lipid peroxidation in rabbits as a result of spinal cord I/R

Figure 6 Apoptotic cell numbers in the groups ($n/0.2\text{ mm}^2$). Data were analyzed with one-way ANOVA (group 1: control, group 2: I/R, group 3: I/R + zinc, group 4: I/R + mel, group 5: I/R + zinc + mel). * $P < 0.05$ compared to groups 2–5. ** $P < 0.05$ compare to groups 1, 3–5. *** $P < 0.05$ compare to groups 1 and 2

Figure 7 Intact ganglion cell numbers in the groups ($n/0.1\text{ mm}^2$). Data were analyzed with one-way ANOVA (group 1: control, group 2: I/R, group 3: I/R + zinc, group 4: I/R + mel, group 5: I/R + zinc + mel). * $P < 0.05$ compared to groups 1, 3–5

injury. Moreover, as neurological deficits and apoptosis emerge as an important outcome of I/R, it was found that prophylactic zinc and melatonin use have caused substantial improvements in these parameters.

In the study as a result of I/R application, significant increases were determined in MDA levels, which is an important indicator of tissue damage. Increases in MDA levels related to spinal cord I/R were also reported in the previous studies.^{5,6} However, the use of melatonin before the application considerably suppressed the increases in MDA levels during the I/R process. Melatonin, which is the main secretion product of the pineal gland, functions as a strong free radical scavenger and as an antioxidant. Melatonin has especially considerable neutralizing effect on hydroxyl radicals and stimulating effect on GPx activity.¹ Furthermore, the increase in MDA levels and the decrease in reduced GSH levels related to ischemia are reversed with melatonin treatment.⁶ Parallel to the free radical scavenger effect of melatonin, zinc supplementation caused significant decreases in MDA levels. The damage-decreasing effect of rare-earth elements in I/R damage has been mentioned before.²⁷ However, it has been established that there are no studies that have investigated the effectiveness of zinc supplementation on spinal cord I/R. In our study, zinc, which was used for the first time in such experiments, prevented the damage that occurred in the tissue as a result similar to melatonin. However, it was observed that the decreasing effect of zinc on MDA is weaker when compared to melatonin.

When the GPx values in the tissue were analyzed, it was found that this parameter increased remarkably in the supplemented groups when compared with sham and I/R groups. It was reported that GPx activity decreased⁷ or did not change²⁸ owing to spinal injuries. In our study, no significant differences were observed in GPx levels related to I/R compared with the sham group. The unchanged GPx levels in I/R group are compatible with several studies.²⁸ However, in a study, which states that there are decreases in the GPx levels related to spinal cord injury, longer-term changes (46–72 h) are mentioned when compared to our study. Different findings can be obtained in our study as we examined the changes only after 24 h. Nonetheless, GPx activity notably increased in the groups that were given antioxidants. However, none of the antioxidants efficiency was superior to another. It was observed that combined treatment did not provide any advantages over individually given ones. This situation shows that GPx level-increasing effects of melatonin and zinc are equal in our experimental conditions. Although there are not any studies on the effectiveness of zinc in spinal cord I/R injuries, it is stated that zinc supplementation causes an increase in the levels of antioxidants such as GSH and GPx.²⁹

When the XO activities in the spinal tissue were investigated, it was found that these parameters considerably increased especially in the I/R group. However, there was a remarkable decrease in these parameters in groups in which zinc, melatonin and melatonin + zinc applications were implemented. A significant increase in the XO levels following the I/R was reported also by other researchers.^{11,30,31} On the

other hand, it is stated that melatonin treatment, affecting pro-oxidant enzyme activity, has a protective role against lipid peroxidation.¹¹ As similar results were obtained in our study, it is compatible with the mentioned study. The fact that lower XO levels were obtained in the group with combined zinc and melatonin showed that the effect created by zinc was made more distinct by melatonin. It was reported that zinc decreased oxidative stress by having an effect as an antioxidant.¹⁵ Parallel to these, it was suggested that melatonin supplementation causes the antioxidant characteristic to emerge by increasing plasma zinc levels³² and at the same time by increasing Cu-Zn-SOD activity.³³

When the part of the study related to NO was evaluated, I/R procedure did not result with significant difference in NO levels. This situation may be the result of the shortness of I/R period. However, it was observed that there were significant decreases in the NO levels in the I/R + melatonin-supplemented group. In fact, it has been established that melatonin supplementation has decreased the serum nitrite and nitrate levels.¹³ However, it is stated that melatonin and zinc supplementation negatively affects the suppression on NO levels formed by melatonin. Although NO is defined as a mediator of especially the post I/R glutamate toxicity, it has been determined that melatonin supplementation decreases the brain NO production by suppressing NO synthetase.¹² In a similar study, melatonin supplementation in cerebral artery occlusion prevented antioxidant damage by suppressing neuronal NO synthetase and MPO activity.³⁴

When the spinal tissue MPO levels were examined, it was found that these parameters showed no increase after the I/R period. In the study that investigated the relationship between spinal cord injury and XO activity, it was reported that XO activity considerably increased following the injury.¹⁰ However, single and combined use of zinc and melatonin prevented the increases to a certain extent. In a study similar to the current one, it was reported that MPO activity increased as the result of spinal injury, but melatonin treatment improved the increased MPO activity by suppressing it.^{8,11} Although there are no studies that investigated the effects of spinal cord injury and zinc application on XO levels, there are studies that have investigated the effects of zinc on the levels of damage in different organs. These studies state that zinc supplementation functions as an antioxidant.

Apoptotic cell numbers were found to be increased in the I/R group animals. However, single and combined use of zinc and melatonin supplementation significantly suppressed the apoptotic cell number increase. In similar studies related to the topic, it has been reported that melatonin supplementation has prevented lipid peroxidation together with cell apoptosis and necrosis.¹⁴ It was also stated that the decrease in the Tarlov score, which occurred as the result of spinal cord injury, increased with melatonin treatment.⁹ It is also assumed that zinc as a strong antioxidant has neuroprotective effects by reducing oxidative stress.¹⁵ In addition, it is

also stated that zinc has a suppressing role on apoptosis with its increasing effects on metallothioneins.¹⁶ However, the existence of excessive zinc causes neurotoxicity.¹⁷ Chelation of excessive zinc improves zinc neurotoxicity and decreases the ensuing neural injury.¹⁷ In the present study, it was determined that melatonin and zinc supplementations caused the revelation of similar results in terms of apoptosis. This situation suggests that the dose of zinc used in our study was an adequate dose for neuron protection.

When the biochemical, histopathological and neurological evaluations of the results of the study are carried out, it can be established that the oxidative damage, which occurs in experimental I/R injury in rabbits, is considerably suppressed with prophylactic zinc and melatonin use. It is suggested in this study that zinc use and the dose applied was implemented for the first time in such an experimental model and its neuron protective role was introduced without causing neurotoxicity.

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Statement of interest

None.

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