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Effects of curcumin on acute spinal cord ischemia-reperfusion injury in rabbits

Laboratory investigation

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Object. The object of this study was to conduct a prospective, randomized, laboratory investigation of the neuroprotective effects of curcumin functionally, biochemically, and histologically in an experimental acute spinal cord ischemia-reperfusion injury on rabbits.

Methods. Eighteen rabbits were randomly assigned to 1 of 3 groups: the sham group, the ischemia-reperfusion group, or the curcumin group. Spinal cord ischemia was induced by applying an infrarenal aortic cross-clamp for 30 minutes. At 48 hours after ischemia, neurological function was evaluated with modified Tarlov criteria. Biochemical changes in the spinal cord and plasma were observed by measuring levels of malondialdehyde (MDA), advanced oxidation protein products (AOPP), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), nitrite/nitrate, and tumor necrosis factor-α (TNF-α). Histological changes were examined with H & E staining. Immunohistochemical staining with antibodies against caspase-3 was performed to evaluate cell apoptosis after ischemia.

Results. In the curcumin group, neurological outcome scores were statistically significantly better compared with the ischemia-reperfusion group. In the ischemia-reperfusion group, MDA, AOPP, and nitrite/nitrate levels were significantly elevated in the spinal cord tissue and the plasma by the induction of ischemia-reperfusion. The curcumin treatment significantly prevented the ischemia-reperfusion–induced elevation of nitrite/nitrate and TNF-α. In addition, the spinal cord tissue and the plasma SOD, GSH, and CAT levels were found to be preserved in the curcumin group and not statistically different from those of the sham group. Histological evaluation of the tissues also demonstrated a decrease in axonal damage, neuronal degeneration, and glial cell infiltration after curcumin administration.

Conclusions. Although further studies including different dose regimens and time intervals are required, curcumin could attenuate a spinal cord ischemia-reperfusion injury in rabbits via reducing oxidative products and proinflammatory cytokines, as well as increasing activities of antioxidant enzymes and preventing apoptotic cell death. *(http://thejns.org/doi/abs/10.3171/2013.12.SPINE1312)*

Key Words • **antioxidant** • **curcumin** • **ischemia-reperfusion injury** • **neuroprotection** • **oxidative stress** • **spinal cord injury**

Curcumin, a polyphenol derived from the herbal remedy and dietary spice turmeric, possesses diverse antiinflammatory and anticancer properties following oral or topical administration.[29,40] Extensive research within the past decade has established curcumin as a pleotropic molecule, which is useful for neurodegenerative, cardiovascular, pulmonary, metabolic, arthritic, and autoimmune diseases.[24] Cemil et al.[6] showed neuroprotective effects of curcumin in an experimental spinal cord ischemia model.

During surgery of abdominal aortic aneurysms, prolonged aortic cross-clamping may result in interruption of blood flow and ischemic complications that will cause paraplegia, with a reported prevalence of 4%–40%.[23] Some patients experience acute spinal cord dysfunction following thoracic aortic occlusion as a result of acute spinal cord ischemia from hypoperfusion during cross-clamping.[16]

In the English literature, the protective potential of curcumin has not yet been investigated in spinal cord ischemia-reperfusion injury induced by abdominal aortic occlusion. The aim of this study was to evaluate the

Abbreviations used in this paper: AOPP = advanced oxidation protein products; CAT = catalase; GSH = glutathione; MDA = malondialdehyde; SOD = superoxide dismutase; TBARS = thiobarbituric acid reactive substances; TNF = tumor necrosis factor.

This article contains some figures that are displayed in color online but in black-and-white in the print edition.

neuroprotective effects of curcumin on the acute phase of spinal cord ischemia-reperfusion injury. In this study, we investigated the acute-phase protective efficacy of curcumin in terms of functional, biochemical, histological, and immunohistochemical outcomes of acute spinal cord ischemia-reperfusion in rabbits.

Methods

Animal Care

New Zealand male rabbits weighing 350–450 g were housed in polycarbonate cages and given free access to food and tap water. None of the animals had any neurological abnormalities before anesthesia and surgery. Anesthesia was induced using 50 mg/kg of intramuscular ketamine hydrochloride (Pfizer) and 10 mg/kg of xylazine (Bayer). The body temperature of the rabbits was continuously monitored with a needle-type thermistor inserted into the paravertebral muscles at the L-3 and L-4 levels, and was maintained at approximately 38°C with a heating lamp and an underbody heating pad. The rabbit model of spinal cord ischemia and reperfusion described by Guven et al.[10] was used to perform spinal cord ischemia and reperfusion. All rabbits were assigned a number from 1 to 18, randomly chosen by the senior author (G.K.), and all medication and surgical procedures were performed by a surgeon (E.C.) who was blinded to the groups. The senior author was only the participant who was not blinded and as a result he was never in direct contact with the animals. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health. The Animal Experiment Committee of Gazi University School of Medicine approved this study.

Operative Technique

Rabbits were placed supine. Using a transperitoneal approach, the aorta was isolated and dissected carefully from the beginning of the left renal artery down to the aortic bifurcation. During surgery no aortic or caval hemorrhage occurred. Each animal received 200 U heparin intravenously 5 minutes before occlusion. The aorta was then cross-clamped at 2 sites using 2 aneurysm clips with a closing force of 70 grams (Yasargil FE 721, Aesculap). The proximal clipping site was just below the left renal artery exit, and the distal clipping site was just above the aortic bifurcation. After occlusion, pulsation of the femoral artery disappeared. Clips were removed after 30 minutes and aortic pulsation was verified.

Eighteen rabbits were randomly assigned to 3 groups as follows: ischemia-reperfusion group, 0.9% NaCl solution (n = 6); curcumin group, 200 mg/kg curcumin (n = 6); and sham group (n = 6). Because this experimental study was focused on reversibility of acute effects of ischemia-reperfusion injury on the spinal cord, drugs or saline were immediately administered intraperitoneally just after the aortic occlusion was released.[6] Animals in the sham group underwent a surgical procedure, but no aortic occlusion performed.

Postoperative Period and Euthanasia

The wound was closed in layers after the operation. After the injury, bladder massage was performed twice a day to stimulate autonomic urinary reflex. Rabbits were killed 2 days after the surgery. Long-term follow-up was not performed for 2 reasons. First, this study focused on the short-term effects of a single-dose antiinflammatory drug that was applied just after ischemia-reperfusion injury while in the acute phase of injury. Second, because this was an investigation of changes in the acute phase of injury, the local ethics committee did not allow long-term follow-up of the animals in the state of paraplegia and urinary dysfunction.

Neurological Function Evaluation

Rabbits were evaluated blindly by a third investigator (B.C.) for hindlimb motor function at 2 hours, 1 day, and 2 days after surgery. Modified Tarlov criteria were used to score neurological function as follows: 0, no movement in hindlimbs; 1, perceptible movements of joints; 2, good movement of joints but inability to stand; 3, active support with uncoordinated gait; 4, coordination of the forelimbs and hindlimbs during gait with lack of control of the ankle or foot and alterations in the base of support; and 5, complete recovery.[10]

Histological Evaluation

The spinal cord was excised between L-3 and L-5 and divided into 2 equal parts. Cranial portions were stored immediately at –70°C for biochemical analysis, and caudal portions of the spinal cord were fixed in 10% buffered formalin for approximately 10 days before being set in paraffin. Spinal cords were then embedded with paraffin, and serial 5-μm thick coronal sections were collected. To assess histological changes, the sections were further subjected to H & E staining. Slides were evaluated under a light microscope (DMI 4000 B, Leica) by a histologist (G.T.K.) blinded to the groups. Axonal damage, neuronal degeneration, and astrocyte and microglia infiltration were analyzed for histological changes. Axonal damage was graded as follows: G-0 (normal), G-1 (mild swelling and vacuolization in axons), or G-2 (severe swelling and vacuolization in axons). The intensity of degenerated neurons was calculated in each field on the following scale: 0 = no degeneration, and 10 = complete degeneration. In each field, microglia and astrocyte infiltration was graded as follows: + = normal, ++ = slight infiltration, +++ = moderate infiltration, ++++ = severe infiltration.[15]

Immunohistochemical Evaluation

Tissue samples from each experimental group were fixed in neutral formalin for 72 hours and processed for paraffin embedding. Sections of 4–5 mm thickness were processed for polylysine microscope slides. For immunohistochemical examination, slides were stored in a microwave oven in 0.01 M citrate buffer (Lab Vision Corp.). Endogenous peroxidase activity was blocked in 3% hydrogen

peroxide (Lab Vision Corp.). Epitopes were stabilized by application of serum blocking solution (Lab Vision Corp.). Each slide was incubated with caspase-3 (rabbit monoclonal antibody). After incubation, the biotinylated goat antipolyvalent secondary antibody (Lab Vision Corp.) was applied. Then, streptavidin peroxidase (Lab Vision Corp.) was applied to the slides. 3-amino-9-ethylcarbazole (Lab Vision Corp.) was used as chromogen (a coloring agent). Afterward, the slides were counterstained with Mayer hematoxylin and examined with a light microscope.

Biochemical Analyses

Malondialdhyde (MDA) was estimated using the thiobarbituric acid reactive substances (TBARS) test as described previously.[5,18,36] Briefly, TBARS formation was quantitated using 1,1,3,3-tetraethoxypropane as standard, and the absorbances of the TBARS were read at 532 nm using a Schimadzu UV 1601 spectrophotometer. Levels of advanced oxidation protein products (AOPPs) were measured by a spectrophotometric method (Schimadzu UV 1601 spectrophotometer) in the presence of potassium iodide at 340 nm[35] and calibrated with chloramine-T solutions. The AOPP levels were expressed in micromoles chloramine-T equivalents per liter.

The tissue protein levels were determined by the colorimetric method of Bradford.[4] Nitrite/nitrate levels were determined by a colorimetric method (photometric end point determination kit, Cayman Chemical). The assay principle used was that nitrate is reduced to nitrite by decreased nicotinamide adenine dinucleotide phosphate in the presence of the enzyme nitrate reductase. The nitrite formed reacts with sulfanilamide and *N*-(1-naphtyl)-ethylene-diamine dihydrochloride to give a red-violet diazo dye. The diazo dye is measured on the basis of its absorbance in the visible range of 540 nm.[3,22]

The superoxide dismutase (SOD) activity measurements were performed by inhibiting the SOD activity using nitro blue tetrazolium reduction. Xanthine-xanthine oxidase was used as a superoxide generator, and 1 IU was defined as the quantity of SOD required to produce 50% inhibition.[31]

Catalase (CAT) activity measurements were determined by a colorimetric method using the Oxis Research Bioxytech Catalase kit. The assay principle was based on determination of the hydrogen peroxide decomposition through measuring the absorbance changes at 520 nm per minute.[14]

The tissue glutathione (GSH) levels were determined by a modified Ellman method.[1] Briefly, after centrifugation at 3000 rpm for 10 minutes, 0.5 ml of supernatant was added to the 2 ml of 0.3 M $Na_2HPO_4 0.2H_2O$ solution. Next, a 0.2-ml solution of dithiobisnitrobenzoic acid solution (0.4 mg/ml in 1% sodium citrate) was added, and the absorbance at 412 nm was measured immediately after mixing. The GSH levels were calculated using an extinction coefficient of 13,000 M^{-1} cm^{-1}.

The plasma GSH levels were determined as total sulfhydryl;[18] 0.5 ml of each sample was mixed with 1 ml of a solution containing 100 mM Tris-HCl, pH 8.2, 1% sodium dodecyl sulfate, and 2 mM ethylenediaminetetraacetic acid. The mixture was incubated for 5 minutes at 25°C and centrifuged to remove any precipitate. 5,5-dithiobis (2-nitrobenzoic acid)/DTNB 0.3 mM was then added to each reaction volume and incubated for 15 minutes at 37°C. The absorbance of each sample was determined at 412 nm. The total sulfhydryl levels were calculated assuming a molar extinction coefficient of 13,000 M^{-1} cm^{-1} at 412 nm. The plasma tumor necrosis factor-α (TNF-α) level was determined by a solid phase sandwich enzyme-linked immunosorbent assay (BioSource Immunoassay Kit, BioSource International, Inc.).

Statistical Analysis

Data were analyzed by SPSS (version 16.0, SPSS Inc.) and expressed as mean ± SD. Comparisons were made using the Mann-Whitney U-test. Differences among the groups were assessed using the Kruskal-Wallis test. A p value < 0.05 was considered statistically significant. Significant differences between histological grades were assessed using the chi-square test.

Results

Neurological Function Evaluation

The evaluation of neurological function in each group is summarized in Fig. 1. The results showed that Tarlov scores in the curcumin group were not statistically different than those in the sham group ($p > 0.05$). The surgical procedure markedly decreased Tarlov scores in the ischemia-reperfusion group. Animals in the ischemia-reperfusion group had significantly worse postoperative neurological outcomes than animals in the other groups ($p < 0.05$).

Histological Evaluation

The histological structure of the specimens from the sham group was normal, whereas the ischemia-reperfusion group demonstrated significant tissue destruction (p

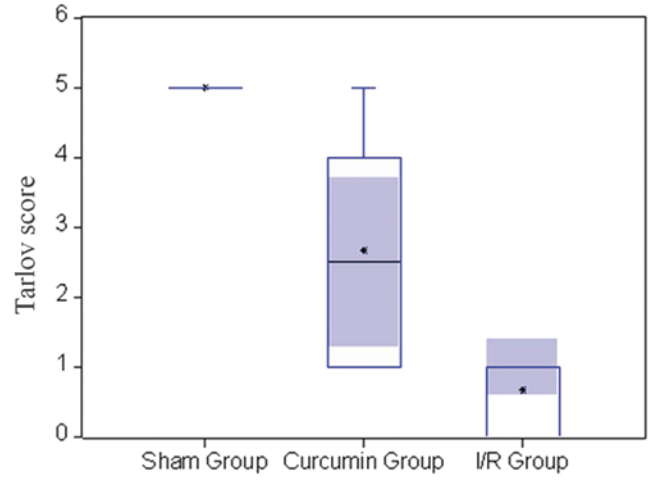


Fig. 1. Box-and-whisker plot showing that neurological function as assessed using the Tarlov score was higher in the curcumin group than in the ischemia-reperfusion group. The median is depicted by the *line* through the center of the box, while the mean is represented by the *asterisk*. *Shaded regions* display approximate confidence intervals for the median. I/R = ischemia-reperfusion.

< 0.05). Axonal damage, neuronal degeneration, and glial cell infiltration parameters were significantly lower in the curcumin group compared with the ischemia-reperfusion group ($p < 0.05$). There was no statistically significant difference between the sham group and the curcumin group. Table 1 lists the histological evaluation results.

Immunohistochemical evaluation revealed no caspase-3 immunoreactivity in the sham group. In contrast, increased caspase-3 immunoreactivity was shown in the ischemia-reperfusion group (Fig. 2 upper), whereas decreased caspase-3 immunoreactivity was observed in the curcumin group (Fig. 2 lower).

Biochemical Analyses

Tissue Changes. In the ischemia-reperfusion group, spinal cord tissue MDA, AOPP, and nitrite/nitrate levels were significantly elevated by the induction of ischemia-reperfusion; however, curcumin treatment significantly prevented the ischemia-reperfusion–induced elevation. Consistent with these effects, ischemia-reperfusion caused a significant decrease in tissue SOD, GSH, and CAT levels as compared with that of the sham group; while in the curcumin group, spinal cord tissue SOD, GSH, and CAT contents were found to be preserved and not statistically different from that of the sham group (Table 2).

Plasma Changes. Ischemia-reperfusion injury significantly increased the mean plasma MDA, AOPP, nitrite/nitrate, and TNF-α levels in the ischemia-reperfusion group. Consistent with these effects, ischemia-reperfusion caused a significant decrease in mean plasma SOD, GSH, and CAT levels as compared with those of the sham group. When rats were treated with curcumin, mean plasma SOD, GSH, and CAT levels were significantly increased and were not statistically different than those in the sham group (Table 3).

Discussion

In this study, we demonstrated that curcumin treatment significantly attenuated motor dysfunction and histological damage on the acute-phase spinal cord changes following ischemia-reperfusion injury. In addition, biochemical analysis of the plasma and spinal cord tissue verified its systemic and local effects. Previously, Cemil et al.[6] reported the neuroprotective effects of curcumin on experimental spinal cord injury induced by aneurysmal clipping. Thereafter, Lin et al.[19] performed hemisection on the spinal cord and showed curcumin reduction of robust RANTES production in reactivated astrocytes both in vitro and in vivo may contribute to its neuroprotection and potential application in spinal cord ischemia. Finally, they reported that curcumin inhibited apoptosis and neuronal loss, quenched astrocyte activation, and significantly improved neurological deficits. Sahin Kavaklı et al.[25] reported that curcumin effectively protects spinal cord tissues against oxidative damage by using the weight drop–induced spinal cord injury rat model. Sanli et al.[28] showed protective effects of curcumin by ultrastructural analysis of the rat spinal cord.

Curcumin, a phenolic pigment, is extracted from the rhizome of the turmeric family, including turmeric, curcuma, and rhizomes, all of which belong to traditional Chinese medicine. Curcumin has been widely used as a

TABLE 1: Results of the histopathological evaluation in the 3 groups

Group	Rat No.	Neuronal Degeneration*	Axonal Damage†	Glial Cell Infiltration‡
sham group	1	1/10	G-0	+
	2	2/10	G-1	++
	3	2/10	G-0	+
	4	1/10	G-0	+
	5	0/10	G-0	+
	6	0/10	G-1	+
curcumin group	1	4/10	G-1	+++
	2	5/10	G-1	++
	3	2/10	G-0	+
	4	2/10	G-1	++
	5	1/10	G-0	+
	6	2/10	G-0	+
ischemia-reperfusion group	1	9/10	G-2	++++
	2	9/10	G-2	+++
	3	9/10	G-1	+++
	4	8/10	G-2	++++
	5	7/10	G-2	+++
	6	8/10	G-1	++++

* Scale: 0 = no degeneration; 10 = complete degeneration.
† G-0 = normal; G-1 = mild swelling and vacuolization in axons; G-2 = severe swelling and vacuolization in axons.
‡ + = normal; ++ = slight infiltration; +++ = moderate infiltration; ++++ = severe infiltration.

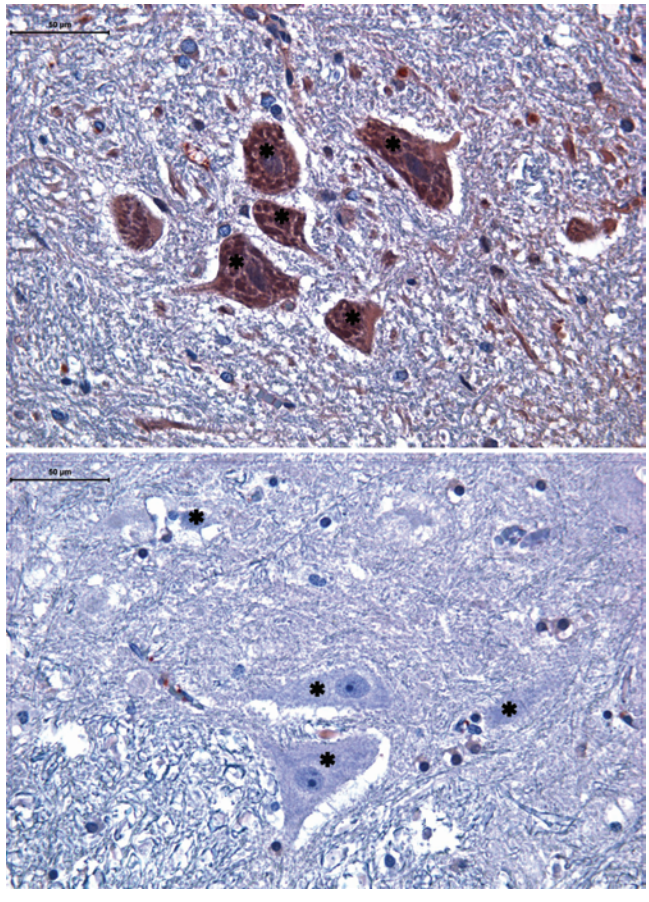


Fig. 2. Photomicrographs showing immunoperoxidase-hematoxylin–stained spinal cord section from the ischemia-reperfusion group **(upper)** and the curcumin group **(lower)**. Increased immunoreactivity was shown in the neurons in the ischemia-reperfusion group, whereas decreased immunoreactivity was shown in the curcumin group. *Asterisk* = neural cell. Bar = 50 μm.

natural food additive and dye due to its color stability and low toxicity. Curcumin is the main pharmacologically active component in extracts from the turmeric family and plays roles in anti–free radical, antioxidation, and antiinflammatory processes. A small molecular weight ($C_{21}H_{20}O_6$, 368.37) and, more importantly, liposolubility enable it to cross the blood-brain barrier into the CNS. Recent studies have shown that curcumin relieves brain injury and reduces cerebral edema in rats with hypoxic-ischemic injuries.[32] Although curcumin shows some protective effects in rat hypoxic-ischemic brain damage,[34] underlying mechanisms have yet to be elucidated.[37]

Spinal cord injury after surgery for thoracic aortic aneurysms remains an unpredictable and disastrous complication.[20] The main cause of acute spinal cord dysfunction is believed to be the result of spinal cord ischemia from hypoperfusion during aortic cross-clamping.[8] In spite of the extensive efforts to understand the pathophysiological mechanisms following spinal cord ischemia-reperfusion, no clinical neuroprotective treatment is currently available.[13] After spinal cord ischemia-reperfusion was performed, several mechanisms emerged, including the initiation of acute oxidative stress and the release of many immune mediators such as interleukins and chemotactic factors.[9] Ischemia-reperfusion injury results in cellular metabolic alterations and inflammatory responses, which lead to marked increases in the production of reactive oxygen species.[39] Formation of reactive oxygen species and lipid peroxidation are prominent events believed to contribute to neuronal dysfunction and cell loss following ischemic injury to the CNS.[2] Malondialdehyde is the most abundant aldehyde resulting from lipid peroxidation,[11] and the AOPP level reflects free radical generation and the degree of protein oxidation.[35] Several endogenous antioxidant enzymes, including SOD, GSH peroxidase, and CAT could detoxify reactive oxygen species following spinal cord ischemia.[33] Tumor necrosis factor-α, which is a potent proinflammatory cytokine, is synthesized immediately after injury.[12] It has been well demonstrated that the blocking of TNF-α confers neuroprotection and improves functional recovery following experimental spinal cord ischemia.[17] Nitric oxide (NO) plays a physiological role in neuronal signal transmission and vessel dilation and is involved in the secondary injury that occurs following spinal cord ischemia.[7] However, excessive NO production has cytotoxic effects and induces neuronal apoptosis.[21] Apoptosis is the process of programmed cell death that involves a series of biochemical events leading to characteristic cell morphology and death, which is critical for removing unnecessary cells and maintaining normal cell functions during development. Apoptosis occurs not only during development but also after brain and spinal cord ischemia.[38] Caspase-3, a frequently activated

TABLE 2: Biochemical analysis of the spinal cord tissue changes in the sham and experimental groups*

Variable	Sham Group	Ischemia-Reperfusion Group†‡	Curcumin Group§
MDA (nmol/g tissue)	3.115 ± 0.882	6.683 ± 1.765	3.221 ± 0.799
AOPP (nmol/mg protein)	15.358 ± 2.138	25.693 ± 3.994	16.469 ± 1.527
nitrite/nitrate (μmol/g tissue)	21.566 ± 9.757	48.815 ± 5.180	28.170 ± 8.089
SOD (U/mg protein)	7.28 ± 1.586	3.478 ± 0.777	6.351 ± 1.106
CAT (nmol/min/mg protein)	4.921 ± 2.606	1.597 ± 0.458	4.689 ± 1.624
GSH (μmol/g tissue)	0.575 ± 0.116	0.339 ± 0.081	0.631 ± 0.100

* All data given as mean ± SD.
† $p < 0.05$ compared with sham group.
‡ $p < 0.05$ compared with curcumin group.
§ $p > 0.05$ compared with sham group.

TABLE 3: Biochemical analysis of the plasma changes in the sham and experimental groups*

Variable	Sham Group	Ischemia-Reperfusion Group†‡	Curcumin Group§
MDA (nmol/L)	2.729 ± 0.882	8.0156 ± 3.957	2.712 ± 1.190
AOPP (μmol/L)	64.128 ± 20.886	136.068 ± 33.306	61.643 ± 19.778
nitrite/nitrate (μmol/L)	5.444 ± 2.394	13.950 ± 4.126	7.405 ± 2.671
SOD (U/ml)	1.110 ± 0.308	0.445 ± 0.157	0.992 ± 0.406
CAT (U/ml)	10.157 ± 8.118	4.258 ± 0.839	8.562 ± 6.927
GSH (nmol/ml)	210.089 ± 67.764	132.009 ± 43.921	225.3 ± 40.144
TNF-α (pg/ml)	7.722 ± 1.314	13.153 ± 1.203	7.615 ± 1.141

* All data given as mean ± SD.
† $p < 0.05$ compared with sham group.
‡ $p < 0.05$ compared with curcumin group.
§ $p > 0.05$ compared with sham group.

death protease, is a crucial mediator of programmed cell death and catalyzes the specific cleavage of many key cellular proteins.[21] Because of this, caspase-3 has become the primary focus in apoptosis studies.

In our measurements, spinal cord tissue and plasma MDA and AOPP levels increased significantly in the ischemia-reperfusion group compared with the others. The increased generation of oxidative protein product is correlated with the degree of the produced free radicals.[30] Administration of curcumin significantly decreased spinal cord tissue plasma MDA and AOPP levels. These results are compatible with previous reports that were reported by Cemil et al.,[6] Sahin Kavaklı et al.,[25] and Sanli et al.[28] In addition, we found that SOD, GSH, and CAT were significantly increased in spinal cord tissue and plasma in the curcumin group. Similar results have been shown in a traumatic spinal cord injury model in a series of studies of oxidative stress and antioxidant defense potential.[6,25] Furthermore, our study showed that nitrite/nitrate and TNF-α levels increased significantly in spinal cord tissue and plasma in the ischemia-reperfusion group of rabbits, and in the curcumin group there was a significant decrease in levels of nitrite/nitrate and TNF-α. Because of these results, curcumin may reduce neuronal apoptosis after ischemia-reperfusion–induced spinal cord damage. Results of the neurological assessment and histological evaluation in this study supported the biochemical results. The rabbits in the curcumin group showed better histological grades and neurological results than the rabbits in the ischemia-reperfusion group. In addition, decreased caspase-3 immunoreactivity was noted in the curcumin group. The results of our study clearly demonstrate that curcumin has protective effects in spinal cord ischemia-reperfusion injury.

A potential limitation of our study is that we only examined the efficacy of curcumin up to 48 hours after reperfusion because the acute phase of spinal cord injury occurs at this time.[26] Also, the local ethics committee did not allow long-term follow-up of animals in the state of paraplegia and urinary dysfunction. In the clinical setting, however, paraplegia may develop 1–5 days after spinal cord ischemia, which must not be understimated.[27]

Conclusions

This study demonstrated that curcumin treatment significantly attenuated acute spinal cord ischemia-reperfusion injury. The protective effects of curcumin treatment were associated with decreased levels of oxidative products and proinflammatory cytokines, as well as increased activities of antioxidant enzymes. Different dose regimens of curcumin with different experimental settings, including long-term follow-up with a maintenance dosage regimen, may reveal better histological, biochemical, and ultrastructural results.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: Yildirim, Kurt, Celtikci. Acquisition of data: Yildirim, Kaplanoglu. Analysis and interpretation of data: Kurt, Cemil. Drafting the article: Yildirim, Kurt, Cemil. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Yildirim. Administrative/technical/material support: Yildirim, Kurt, Celtikci. Study supervision: Yildirim, Kurt, Cemil.

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