Research Report

Supplement zinc as an effective treatment for spinal cord ischemia/reperfusion injury in rats

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ABSTRACT

Objective: Brain-derived neurotrophic factor (BDNF) plays a key role in the pathophysiology process and therapy of spinal cord injury (SCI). Accordingly, zinc regulates the expression of BDNF and its receptor in the central nervous system, the mechanism of which is still unknown. The present study investigates whether supplement zinc could reduce neurological damage in a rat model, with spinal cord ischemia–reperfusion (I/R) injury and how the effect of zinc transporter 1 (ZnT-1) was involved.

Methods: 100 Sprague–Dawley male rats were randomly and evenly divided into four groups. They were subjected to spinal cord ischemia by clamping the abdominal aorta for 45 min. Rats in the zinc-deficient dietary model group (ZD), zinc-adequate dietary model group (ZA), and zinc-high dietary model group (ZH) were given free access to purified diet, containing 5, 30, or 180 mg Zn/kg. Sham operation rats were subjected to laparotomy without clamping of the aorta and were fed by ZA diet (30 mg Zn/kg). Neurological function was scored by Tarlov’s score. The spinal cord segments (L5) were harvested for histological examination, auto-metallographic (AMG) analysis, myeloperoxidase (MPO) activity analysis, expression of ZnT-1 and BDNF.

Results: The rats in the ZH group have shown the higher neurological scores, slighter histological changes and the attenuated MPO activity, compared with those in the ZD and ZA groups at the four observation time points (p<0.05). The AMG staining density in the ZH group was significantly higher than that of ZD group in 14 days later after the operation. Compared with other groups, ZH group’s expression of Zn-T1 and BDNF were significantly increased, and was positively correlated with the same time points after surgery (Spearman rho=0.403, p=0.0152.) Conclusion: These findings suggest that zinc supplement can significantly reduce the spinal cord I/R injury in rats. The mechanism may be related with restraining the MPO activity and increasing of ZnT-1, which promoted the synthesis and release of BDNF.

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1. Introduction

I/R injury of the spinal cord causes paraplegia, which is a serious complication after thoracic or thoracoabdominal aortic surgery. The specific mechanism of spinal cord I/R injury is not clear now, while it has been recognized that oxygen free radical mediated lipid peroxidation, calcium overload, excitability amino acids, and prostaglandins play important roles in SCI mechanism. A few studies have taken measures to protect against I/R injury, and achieved some good results (Deniro and Al-Mohanna, 2012; Jiang et al., 2009; Yang et al., 2011).

As an essential trace element, zinc plays a key role in multiple metabolic and signaling pathways, which can resist ischemia reperfusion injuries (Bulbuloglu et al., 2011). Supplemental zinc can be used during recovery to improve cognitive and behavioral deficits associated with brain injury (Cope et al., 2012). It is recently demonstrated in the study that zinc supplement could reduce neurological damage after acute SCI, and promote the recovery of spinal cord function. Related with increasing of Zn-T1, the mechanism may promote the synthesis and release of BDNF and play an essential role in modulating spinal zinc homeostasis (Su et al., 2012; Wang et al., 2011a, b; Wang et al., 2011). However, few studies have described the protective effect of dietary zinc on spinal cord I/R. The current study, therefore, was designed to test the neuroprotective effects of zinc supplement in a spinal cord I/R model in rats, and furthermore to preliminarily verify the possible mechanisms of this neuroprotection.

2. Results

2.1. Neurological function assessment

The four groups (1, 3, 7, and 14 days after the operation) of the individual Tarlov’s score are shown in Fig. 1. The sham-operated rats had a normal motor function of hind limbs throughout the observation period. A 45-min aortic occlusion resulted in severe lower extremity neurologic deficits in ZD and ZA groups, and the neurologic status of ZH group were significantly superior to ZD and ZA groups at the four observation time points ($p<0.05$). The Tarlov score of ZD group was significantly lower than that of ZA group 14 days later after the operation ($p<0.05$).

2.2. Histologic assessment

Paraffin-embedded sections of lumbar spinal cords stained with hematoxylin and eosin for light microscopic examination are shown in Fig. 2 A–D, and the results of counting viable motor neurons are summarized in Fig. 2 E. No sign of spinal cord damage was observed in sections in the sham-operated rats, and many large motor neurons were presented in the anterior horn.

![Fig. 1 – Motor functions of the hind limbs assessed with Tarlov’s score on day 1, 3, 7, and 14 after spinal cord ischemia/reperfusion injury in rats. ZH = zinc-high dietary model group (180 mg Zn/kg); ZA = zinc-adequate dietary model group (30 mg Zn/kg); ZD = zinc-deficient dietary model group (5 mg Zn/kg); and Sham = sham-operated group (30 mg Zn/kg). *$p<0.05$ versus ZH group. *$p<0.05$ versus ZA group.](image-url)
A 45-min aortic occlusion induced severe neuronal damage on the animals of the ZD group on 14th day after ischemia, as indicated by frank necrosis, vacuolization, and disappearance of Nissl bodies and nuclei in the motor neuron cells. Slighter histological changes were found in the lumbar spinal cords of rats in the ZH group, and the intact motor neurons were preserved to a much greater extent (\(p < 0.05\) versus ZD and ZA groups).

### 2.3. Autometallography analysis

The AMG patterns were particularly dense in the gray matter of the dorsal horn, which was coincident with previous reports (see Fig. 3) (Jo et al., 2000; Su et al., 2012). AMG staining in the spinal cord is shown in brown. The staining density in the ZH group was significantly higher than that of ZD group on 14th day after the operation.

### 2.4. Myeloperoxidase (MPO) activity analysis

Spinal cord ischemia/reperfusion resulted in a significant increase in spinal cord MPO activity compared with sham-operated rats (see Fig. 4) (\(p < 0.05\)). In three spinal cord I/R groups, the MPO activity was significantly attenuated in the ZH group compared with ZA and ZD groups (\(p < 0.05\)).
2.5. Immunohistochemical analysis of BDNF

The expression of BDNF of rat spinal cord is shown in Fig. 5, 2 weeks after operation by immunohistochemistry methods. Immunohistochemical staining showed that zinc supplement caused a significant up-regulation of BDNF protein expression in the ZH group and ZA group, compared with ZD group and sham-operated rats.

2.6. Determination of Zn-T1 mRNA and BDNF mRNA by real-time PCR

The mRNA levels of Zn-T1 (see Fig. 6A) and BDNF (see Fig. 6B) were very low throughout the observation period in ZD group and sham-operated group. In ZA group, Zn-T1 mRNA and BDNF mRNA were increased along with the experimental process gradually as compared with the sham-operated group 3 d, 7 d and 14 d after surgery (*p < 0.05). Compared with other groups, Zn-T1 mRNA and BDNF mRNA were significantly increased in ZH group. The Zn-T1 mRNA and BDNF mRNA in ZH group were detected maximal increase in 7 d after surgery, and then they were decreased in 14 d after surgery.

Concentrations of Zn-T1 and BDNF were positively correlated in ZH and ZA group at the same time points after surgery, attested by the determination of the Spearman index (Spearman rho = 0.403, p = 0.0152. Spearman rho = 0.376, p = 0.0171.).

3. Discussion

It is found in our previous work that zinc supplement exerts neuroprotection against acute spinal cord injury in rats.
The possible mechanism of the phenomenon is that overexpression of ZnT-1 promoted the synthesis and release of BDNF, which this mechanism would have resulted in the corresponding biological reaction and promoted the recovery of neural function of injured spinal cord (Su et al., 2012; Wang et al., 2011a, 2011b). This study demonstrates a neuroprotective effect of zinc against spinal cord ischemia/reperfusion injury in rats. The improvements of rats consuming zinc exhibited neurological scores, reduced tissue destruction, and oxidative stress.

Zinc is an essential mineral which is required for various cellular functions. Although beneficial effects of zinc have been demonstrated in I/R injuries of various organs, it has rare effect on spinal cord I/R injuries.

Recent studies have suggested that free radicals-derived lipid peroxidation play an imperative role in spinal cord ischemia–reperfusion injury of the spinal cord (Yilmaz et al., 2012; Zhu et al., 2012). Plenty of studies indicated that zinc can mitigate the harm done by free radicals by antagonizing lipid peroxidation (Mazani et al., 2013). Apoptosis of motor neurons in spinal cord after transient ischemia is an important mechanism of postoperative paraplegia (Sakurai et al., 2005; Wu et al., 2012). In this study, slighter histological changes were found in the lumbar spinal cords of rats in the ZH group, and more intact motor neurons were preserved compared with ZD and ZA groups. Myeloperoxidase (MPO) is most abundantly expressed in neutrophil granulocytes (S.J. Chen et al., 2012). Recent studies have reported an association between myeloperoxidase levels and the severity of ischemia/reperfusion injury (Y. Chen et al., 2012; Zhang et al., 2012).

Our research shows that MPO was significantly increased in the spinal cord of the I/R group compared with sham-operated rats ($p<0.05$), while the increase of MPO was prevented by zinc supplement especially in ZH group ($p<0.05$). As for the protective effect of zinc supplement, our results suggest that it may be, in part, dependent on its effects of anti-apoptosis and inhibitory on neutrophil infiltration (Kumar et al., 2012).

ZnT-1, a zinc exporter localized at the plasma membrane of neurons, plays a very important role in zinc homeostasis (Sankavaram and Freake, 2012; Sun et al., 2007). A recent study has indicated that ZnT-1 may have an important role in the ischemic myocardium through its ability to interact with Raf-1 kinase (Beharier et al., 2012). Previous studies have shown that ZnT-1 expression should be regulated by dietary zinc after SCI (Su et al., 2012), and present study has shown the expression level of ZnT-1 in ZH dietary group becomes obviously higher than other groups. Moreover, immunohistochemical analysis has shown that zinc supplement caused a significant up-regulation of BDNF protein expression in the ZH group and ZA group when compared with ZD group and sham-operated rats. BDNF is a member of the “neurotrophin” family of growth factors, which can support the survival of existing neurons, encourage the growth of new neurons, and reduce neuronal apoptosis (Acheson et al., 1995; Li et al., 2012; Sasaki et al., 2009). We hypothesized that zinc supplement could reduce the spinal cord ischemia/reperfusion injury in rats through increasing Zn-T1, which promotes and up-regulates the protective neurotrophic factor such as BDNF.
What is more meaningful is that the mRNA levels of Zn-T1 and BDNF were increased in ZH and ZA groups over time, and there is a positive correlation between them (Spearman \( \rho = 0.403, \ p = 0.0152 \)). Spearman \( \rho = 0.376, \ p = 0.0171 \). But the ratios of Zn-T1/GAPDH mRNA and BDNF/GAPDH mRNA were not significantly different between ZH and ZA groups on 14th day. The death rate of rats was higher on the 14th day, so we did not know the changes of Zn-T1 and BDNF after that. Excess zinc, which is released in the synaptic clefts, causes the apoptotic death of the target neurons. 180 mg may be overdosing. The best dose of zinc for the treatment of spinal cord I/R injury and the precise underlying mechanism requires further studies.

Recent clinical trials have shown that zinc has beneficial effects on glycemic and postmenopausal osteoporotic control in adults. Postmenopausal osteoporotic women may benefit from 220 mg zinc sulfate daily supplement (Mahdaviroshan et al., 2013). The glucose concentration of pre-diabetic patients who are treated with zinc sulfate monohydrate 55.096 mg daily was lower than that of the control group (Ranasinghe et al., 2013). We will evaluate the effects of zinc supplementation in those with spinal cord injury, which will provide the necessary groundwork for future large-scale clinical trials.

The study had demonstrated the positive role of zinc supplementation in spinal cord ischemia/reperfusion injury in rats, which may be related with its antioxidant effects and enhance the overexpression of BDNF. However, the best dose of zinc for the treatment of spinal cord I/R injury and the precise underlying mechanism requires further studies.

4. Experimental procedure

4.1. Animals care

The study was approved by the Ethics Committee of Liaoning Medical University, China and was carried out at the Institute of Orthopedics, the First Affiliated Hospital of Liaoning Medical University. All study procedures were conducted in agreement with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

100 healthy, clean, Sprague–Dawley male rats, aged 2–3 months, weighing (250 ± 25) g were provided by the Laboratory Animal Center, Liaoning Medical University. Animals were housed in individual stainless steel cages in a room with a 12–12 h light–dark schedule. The temperature was adjusted to 23 ± 1 °C for the duration of the experiment with a relative humidity of (50–70%). Animals received sterilized food and water ad libitum.

4.2. Experimental design

A total of 100 rats were randomly and evenly divided into four groups (Su et al., 2012): sham-operated group (Sham, 30 mg Zn/kg), zinc-high dietary model group (ZH, 180 mg Zn/kg), zinc-adequate dietary model group (ZA, 30 mg Zn/kg), and zinc-deficient dietary model group (ZD, 5 mg Zn/kg). Rats were anesthetized with a 4% chloral hydrate solution (360 mg/kg i.p.). Core body temperature was maintained at 37 °C with the aid of a heating lamp and a heating pad. Spinal cord I/R model was induced, as described by Akgun et al. (2004).

The abdominal aorta was reached through midline laparotomy. Sham operation rats were subjected to laparotomy without clamping of the aorta. In other three groups, animals were produced by occlusion of the abdominal aorta 0.5 cm below the left renal artery by non-traumatic vascular clamp for 45 min, followed by 24 h, 3 d, 7 d and 14 d of reperfusion. Following surgery, 1.0 ml saline was administered subcutaneously in order to replace the blood volume lost during the surgery and Penicillin (0.2 ml/kg) was administered subcutaneously to prevent infection at the end of the surgery. As for the animals, they were allowed free access to water during recovery. Sham-operated groups were feed by ZA diet (30 mg Zn/kg), and other three groups were given free access to purified diet containing either 5, 30, or 180 mg Zn/kg.
The animals also had no significant health problems for weeks after surgery.

### 4.3 Neurological function assessment

Neurological function was scored by assessment of the motor functions of the hind limbs from 24 h to 2 weeks (Day 1, Day 3, Day 7, Day 14) after surgery by using previously published methods from Tarlov’s score (Tarlov, 1972). The motor functions of the hind limbs were graded as follows: (0) no voluntary hind limb movement; (1) movement of joints perceptible; (2) active movement but unable to sit without assistance; (3) able to sit but unable to hop; (4) weak hop; and (5) complete recovery of hind limb function. The procedure was repeated five times and all behavior scores testing were implemented in the single-blind.

### 4.4 Spinal cord histological study

All rats were killed on 14th day after the spinal cord ischemia/reperfusion using lethal dose of pentobarbital injection (75 mg/kg i.p.) and sections of the ischemic spinal cord were harvested for subsequent study rapidly (Damiani et al., 2003). A section of tissue was frozen in liquid nitrogen and stored at −80 °C until its further use. The remaining samples were fixed in 4% paraformaldehyde/phosphate buffered saline (PBS), followed by paraffin-embedding for sectioning. Paraffin-embedded sections of lumbar spinal cords (L5) were stained with hematoxylin and eosin for the light microscopic examination.

The intact motor neurons in the ventral gray matter were counted by a blinded observer by using light microscopy in three sections for each rat, and the results were averaged. These cells were considered to be “necrotic or dead” if the cytoplasm was diffusely eosinophilic and “viable or alive” if the cells demonstrated basophilic stippling (contained Nissl substance) (Mutch et al., 1993; Shi et al., 2007). The ratio of dead to total anterior spinal cord neurons was calculated.

### 4.5. Autometallography analysis

The distribution of zinc ions in spinal cord were detected by autometallography (AMG) according to the method described previously (Danscher et al., 2004; Su et al., 2012). Briefly, rats were killed 14 days later after the spinal cord ischemia/reperfusion. The slices of tissue were immersed in a solution, i.e., 3% glutaraldehyde and 0.1% sodium sulfide in a 0.1 M Sorensen’s phosphate buffer (pH 7.4). The immersion jars were placed on a water bath shaker and kept at 4 °C for 3 days. After that the slices were carefully rinsed three times in 0.1 M phosphate buffer for 10 min. The slices were placed in Farmer-cleaned jars and poured with the AMG developer. The jars were placed in a water bath at 26 °C and covered by a light-tight hood. The development was stopped by replacing the developer with 5% sodium thiosulfate after 60 min and the slices were rinsed carefully for 5 min in several washes of distilled water after 10 min. The glass slides were counterstained with a 0.1% toluidine blue solution, dehydrated in alcohol to xylene, and finally embedded in DEPEX and observed by the optical microscope (Danscher and Stoltenberg, 2006).

### 4.6. Myeloperoxidase (MPO) activity analysis

The spinal cord samples were frozen and stored in a −70 °C freezer for assays of tissue MPO activity according to the manufacturer’s instructions (Sigma-Aldrich, USA) (Khalatbary and Ahmadvand, 2011). The absorbance of the supernatant was measured by spectrophotometry at 650 nm. The MPO activity was expressed as units of MPO/mg of proteins.

### 4.7. Immunohistochemical analysis of BDNF

After blocking in 10% normal goat serum for 1 h, the frozen sections of spinal cord were incubated with rabbit anti-BDNF antibody (1:1000, Upstate, USA) at 4 °C for 72 h. Controlling sections were processed with same amount of PBS instead of primary antibody. The sections were then washed in PBS three times and incubated with biotinylated goat anti-rabbit IgG (1:300, Invitrogen, USA) at 37 °C for 30 min. Washed several times in PBS, the sections were incubated in the avidin–biotin–peroxidase complex (1:300, ABC Elite, Invitrogen, USA) at 37 °C for 45 min. The horseradish peroxidase reaction was developed in 0.1 M Tris-buffered saline (pH 7.4) containing 0.05% 3, 3P-diaminobenzidine (DAB), 0.3% nickel sulfate, and 0.01% H2O2. The sections were then dehydrated, mounted onto gelatin/chrome alum-coated glass slides, and coverslipped (Geng et al., 2010). Images were acquired using laser scanning confocal microscope.

### 4.8. Determination of Zn-T1 mRNA and BDNF mRNA by real-time PCR

Real-time PCR was performed by using an Applied Biosystems 7500 Real-Time PCR System (Foster City, CA, USA). According to the manufacturer’s protocol, total cellular RNA from frozen spinal cord samples was purified by using TRizol RNA isolation reagent. A non-template control (NTC) was run in order to exclude the presence of genomic DNA, and all experiments were performed five times by using GAPDH (Chemicon International Temecula; CA, USA) as the internal reference. The BDNF, Zn-T1 and GAPDH primer sequences had described previously (Wang et al., 2011a,b). The selection of the primers was performed using a Lasergene (DNA Star Inc., WI, USA) program.

### 4.9. Statistical analysis

Data were collected in Microsoft Excel 2003 and statistically analyzed by using SPSS 11.0. All data were expressed as the mean ± SD. For statistical analysis, the Tarlov scale was analyzed with the Kruskal–Wallis test, and other data were assessed by using the one-way analysis of variance and the post-hoc Tukey’s test. p < 0.05 was considered to indicate significant differences.

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