ORIGINAL ARTICLE

Additive effect of tetramethylpyrazine and deferoxamine in the treatment of spinal cord injury caused by aortic cross-clamping in rats

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Study design: Tetramethylpyrazine and deferoxamine are effective agents for nerve injury. Experiments in a Sprague–Dawley rat model of spinal cord injury were performed.

Objectives: This study investigated the effects of tetramethylpyrazine and deferoxamine on neurological outcome and spinal cord histopathology after transient spinal cord ischemia in rats.

Setting: Chongqing Medical University and Third Military Medical University, Chongqing, China.

Methods: Spinal cord ischemia was induced in Sprague–Dawley rats by infrarenal aortic occlusion for 30 min followed by 72 h of reperfusion. Animals were divided into a sham group with a sham procedure; control group with aortic occlusion, but no pharmacological intervention applied; and treatment group with aortic occlusion, treated with tetramethylpyrazine and deferoxamine. After 72 h of reperfusion, neurological status was evaluated in the animals. A histopathological study of spinal cords was performed, and glutamate level and metabotropic glutamate receptor-1 (mGluR1) mRNA expression were determined.

Results: All animals in the control group were completely paraplegic with 0% recovery. Tarlov criteria were significantly better in the animals treated with tetramethylpyrazine and deferoxamine than that in the control group ($P < 0.05$). Functional parameters were fully correlated with the morphological findings. Glutamate level was elevated in the control group, whereas it was significantly suppressed in animals treated with tetramethylpyrazine and deferoxamine treatment. The infrarenal artery occlusion significantly elevated the expression of mGluR-1 mRNA, whereas tetramethylpyrazine and deferoxamine greatly suppressed the expression of mGluR-1 mRNA.

Conclusion: The combination of tetramethylpyrazine and deferoxamine significantly reduced the incidence of paraplegia induced by spinal cord ischemia/reperfusion.

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Introduction

Spinal cord is the organ most sensitive to ischemia, and paraplegia from spinal cord injury is still incurable. Injured axons cannot regenerate into the central nervous system because of the 'nonpermissive' milieu, which comprises an abundance of growth-inhibitory molecules in the myelin and the glial scar, and a lack or insufficiency of growth-promoting factors at the neuronal growth cone or the somata.¹ Even though many efforts have been made to solve the problem, including surgical reconstruction, pharmacological intervention and mechanical methods, there remains

uncertainty regarding the efficacy of these adjuncts in preventing or reducing the incidence of spinal cord injury.

Pharmacological studies have demonstrated that tetramethylpyrazine (TMP) reduces ischemia/reperfusion damage of the spinal cord through multiple mechanisms,¹ and that deferoxamine (DFO) is an effective agent for the improvement of regeneration and functional recovery of nerve injury.² One of the critical mechanisms of spinal ischemia/reperfusion injury involves the release of glutamate,³ in which excessive glutamate results in the death of nerve cells.⁴ Additional studies have shown that the overexpression of metabotropic glutamate receptor-1 (mGluR-1) mRNA observed after ischemia/reperfusion suggests that mGluR-1 is involved in the process of ischemia/reperfusion injury.⁵

Both TMP and DFO are clinically available agents. We hypothesized that the possible additive effect of these agents

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might have clinical value. To date, no study has been designed to examine their combined use in spinal cord ischemia. Therefore, this study was designed to investigate the combined effect of TMP and DFO in the treatment of spinal cord ischemia/reperfusion injury caused by 30 min of aortic cross-clamping followed by 72 h of reperfusion in a rat model.

Materials and methods

All animal care procedures complied with the Convention on Animal Care, and the study was approved by the ethics committee of Chongqing Medical University.

Spinal cord ischemia model

In total, 24 male Sprague–Dawley rats, weighing 300–350 g, were randomly assigned to one of three groups: sham, control and treatment groups, with eight animals in each group. All rats were anesthetized by inhalation of 2% oxygen–isoflurane.

Body temperature was maintained at 37 °C with a heating pad. Regional cerebral blood flow was monitored by Laser Doppler Flowmetry (TSI, Vasamedics, MN, USA) as previously described.⁶ The model of transient spinal cord ischemia was established according to the previously reported method.⁷ In brief, the external jugular vein was cannulated for intravenous access for the administration of TMP and DFO, and for collecting blood samples for the analysis of blood gases and pH. With the rats in the prone position, the abdomen was opened through a vertical midline incision to expose the abdominal aorta. The aorta and its branches were meticulously dissected from the caudal to the left renal artery. A circumaortic occlusion device was placed across the aorta just distal to the left renal artery to occlude blood supply to the lumbar spinal cord. After aortic declamping, the abdominal wall was closed, and the animal was permitted to regain consciousness. Transient spinal cord ischemia was produced for 30 min, followed by 72 h of reperfusion.

In the sham group, the animals underwent the laparotomy only, but no aortic occlusion was performed. In the control and treatment groups, all animals underwent the aortic declamping procedure. In the control group, an intravenous infusion of 0.9% sodium chloride was administered, whereas in the treatment group, a dose of 50 mg kg⁻¹ per day of TMP (LAN Pharmaceutical, Shanghai, China) and 100 mg kg⁻¹ per day of DFO (APP Pharmaceuticals, Schaumburg, IL, USA) was administered intravenously at the onset of reperfusion, then every 24 h for 3 days.

After 72 h of reperfusion, all animals were evaluated for a neurological deficit score (NDS), and the spinal cord was quickly removed for morphological and mGluR-1 expression analyses.

Neurological assessment

A neurological assessment of the physical findings of hind limb neurological function was performed 72 h after reperfusion. The neurological assessment was performed by an independent observer when the animals were fully

conscious. NDS were assigned based on the previously reported Tarlov criteria.⁸ The Tarlov criteria were quantitated as follows: grade 0 = no voluntary function (complete paralysis), 0% recovery; grade I = movement of joints perceptible, 25% recovery; grade II = active movement of joints (inability to stand), 50% recovery; grade III = able to stand (unable to walk), 75% recovery; and grade IV = complete recovery (able to stand, walk and run), 100% recovery.

Morphological analysis

Specimens from spinal cords were embedded in paraffin and sectioned for light microscopic examination. Sections from the lumbar (L5) level of each spinal cord were stained with Hematoxylin and eosin. The histological examination was carried out in a masked manner. Neuronal injury was evaluated at × 200 magnification.

Determination of glutamate

At 72 h after reperfusion, the level of glutamate in serum was determined following the instruction of the colorimetric glutamate assay kit (BioAssay Systems, Hayward, CA, USA).

Determination of mGluR-1 RNA expression

The rats were killed at 72 h after reperfusion. The spinal cords were obtained, and quickly separated at 4 °C. RNA was extracted following the instructions in the TRIzol Kit (Invitrogen, Carlsbad, CA, USA). Semiquantitative reverse transcription-polymerase chain reaction was used to determine the expression of mGluR-1. The expression of β -actin was used as an internal control. The primer sequences used in the PCR amplification of mGluR-1 were 5'-AAAGGCTA TGGCATTGCGA-3' and 5'-CTCGATTAAGGCAACCAGCA-3'. The size of the mGluR-1 PCR product was 207 bp. The primer sequences used in the PCR amplification of β -actin were 5'-AGTGTGACGTTGACATCCGT-3' and 5'-GACTCATCGTAC TCCTGCTT-3'. The size of the β -actin PCR product was 240 bp. The PCR products were analyzed by gel electrophoresis, followed by image analysis (Pearson, NY, USA). A percentage of the integrated density value was calculated for each band.

Statistical analysis

All experimental data were analyzed using the SPSS 11.0 statistical software package (University of Cambridge, UK). The Mann–Whitney nonparametric test was used to analyze NDS. Differences in mGluR-1 expression were compared by using Student's *t*-test between two groups or by using one-way analysis of variance for multiple groups across time points, followed by Fisher's exact test. A *P*-value < 0.05 was considered statistically significant.

Statement of ethics

We certify that all applicable institutional and governmental regulations regarding the ethical use of animals were followed during the course of this research.

Figure 1 Neurological outcomes 72 h after reperfusion. * $p < 0.05$, compared between the sham and treatment groups. ** $p < 0.05$, compared between the control and treatment groups. At 72 h after reperfusion, the neurological defect score in the treatment group, which was administered with TMP and DFO, was significantly better than in the control group. All rats in the control group showed paralysis of the hind limbs with Tarlov score of grade 0, that is, no voluntary function (complete paralysis).

Results

Heart rate, arterial blood gases and pH, blood pressure and body temperature were stable and similar in all groups before, during and after the procedure. All animals completed the study.

Neurological outcomes

At 72 h after reperfusion, the animals in the sham group had normal motor function. The NDS in the group treated with TMP and DFO was significantly better than the NDS in the control group treated with 0.9% sodium chloride only. All rats in the control group showed paralysis of the hind limbs with a median Tarlov score of 0 (Figure 1).

Histology of the lumbar spinal cord

Histology of the L5 spinal cord segment was examined in all animals 72 h after reperfusion (Figure 2). The number of normal neurons in the anterior spinal cord was significantly greater in the group treated with TMP and DFO than that in the control group (Figures 2a and c). Spinal cords from the animals in the control group with paraplegia (grade 0) showed few normal neurons, and destruction of the motor neurons (Figure 2c). No sign of neuronal damage was observed in the spinal cord in the sham group with normal motor function (grade IV, Figure 2b).

Correlation between the neurological defect score and the numbers of normal neurons in the anterior spinal cord

There was a positive correlation between the neurological outcome and the numbers of normal neurons in the anterior spinal cord 72 h after reperfusion ($r = 0.776$, $P < 0.01$).

Glutamate levels

The level of glutamate was elevated from $3.63 \mu\text{mol l}^{-1}$ before infrarenal artery occlusion to $28.26 \pm 5 \mu\text{mol l}^{-1}$ at

Figure 2 Histopathology of the spinal cord 72 h after reperfusion in the three groups. Representative sections from each group are shown ($n = 8$ rats in each group). (a) The anterior horn spinal cord obtained from the treatment group with administration of TMP and DFO for 72 h after reperfusion. (b) The anterior horn spinal cord obtained from the sham group. (c) The anterior horn spinal cord obtained from the control group without administration of TMP and DFO for 72 h after reperfusion.

Figure 3 The level of glutamate measured at different time points. ** $P < 0.01$, compared between the sham and control groups. # $P < 0.05$, compared between the control and treatment groups. The level of glutamate was elevated after the artery occlusion. Treatment of TMP and DFO significantly suppressed the level of glutamate. The level of glutamate was not changed in the sham group.

15 min and $36.1 \pm 6 \mu\text{mol l}^{-1}$ at 30 min after the artery occlusion. Administration of TMP and DFO for 72 h following the occlusion significantly suppressed the level of glutamate compared with the control group, although this decreased level did not reach the level observed in the sham group. The level of glutamate was not changed in the sham group (Figure 3).

mGluR-1 mRNA expression

The infrarenal artery occlusion for 30 min followed by 72 h of reperfusion significantly elevated the expression of mGluR-1 mRNA in the control group, whereas TMP and DFO treatment greatly suppressed the expression of mGluR-1 mRNA (Figure 4).

Figure 4 Differences in the expression (integrated density value) of mGluR-1 mRNA. * $P < 0.05$, compared between the sham and treatment groups * $P < 0.05$, compared between the control and treatment groups. After the infrarenal artery occlusion for 30 min followed by reperfusion for 72 h, spinal ischemia/reperfusion significantly elevated the expression of GluR-1 mRNA in the control group, whereas TMP and DFO administration suppressed the expression seen in the treatment group. The expression in the sham group was not altered.

Discussion

Nerve traumas are frequently caused by work or traffic accidents and other violent events. Although these types of lesions do not usually represent a threat to the patient's survival, their consequences to quality of life and other related socioeconomic costs are significant. The effects of various pharmacological agents on spinal cord ischemia have been extensively examined; however, complete recovery and normalization of nerve function almost never occur, and the clinical outcome often remains unchanged.

TMP, a biomonomer with molecular formula $C_8H_{12}N_2 \cdot HCl \cdot 2H_2O$, is extracted from a traditional herbal medicine. TMP has been widely used in the treatment of ischemic stroke for years in Asian countries, and the benefit to ischemic nerve injury has been demonstrated in experimental research.¹ However, limited data exist regarding the use of TMP in spinal cord injury. In addition, a growing interest has arisen in the application of DFO in the treatment of neurodegeneration.⁹ Even though DFO has been shown to reduce neuroinflammation and local scar formation at the site of nerve repair,⁹ the basic biological mechanisms that underlie the effects of DFO on nerve regeneration are still largely to be elucidated.

This study showed that intravenous administration of TMP and DFO significantly reduced the spinal cord damage produced by occlusion of the infrarenal aorta, and exerted effects on nerve regeneration and recovery in the rat. There was significant improvement in the neurological outcomes of rats treated with TMP and DFO compared with the control group. The group treated with TMP and DFO had higher Tarlov scores than the scores of the control animals. The animals without administration of TMP and DFO showed paralysis of the hind limbs.

Histological examination confirmed the effects of TMP and DFO on the reduction of nerve cell damage in the

anterior horn motor neurons. Spinal cords in the control animals without administration of TMP and DFO had evidence of extensive spinal cord injury, with central gray matter necrosis, perikaryal swelling and vacuolization of anterior horn motor neurons. The number of normal neurons in the spinal cord showed a positive relationship between administration of TMP and DFO and final neurological function.

Motor neuronal cell death after spinal cord ischemia/reperfusion may involve molecular, cellular and genetic factors. Some studies have shown that TMP has antiplatelet activation and aggregation activity, dilates arterioles to improve microcirculation,¹⁰ scavenges free radicals and decreases lipid peroxidation.¹¹ On the other hand, it has been shown that oxidative stress induces apoptosis, and iron chelators may protect cells by enhancing DNA through molecular mechanisms.¹² It is interesting to note that DFO has been shown to have a positive effect in the treatment of neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases, suggesting that DFO has an effect on regeneration and functional recovery of nerve injury.²

One of the critical mechanisms of spinal ischemia/reperfusion injury involves the release of glutamate.^{1,3} The excessive glutamate results in the death of nerve cells.¹³ A large number of glutaminergic synapses make the central neuronal system particularly vulnerable to uncontrolled release of glutamate during ischemia and postischemia. A positive correlation between glutamate level and neurological defect has been shown in this study. Therefore, a reduction of glutamate level may be important for protection against neurological injury. Studies have confirmed that mGluR-1 is extensively involved in the excitatory toxicity of glutamate and the neuron injury or death secondary to spinal ischemia/reperfusion, and that the expression of mGluR-1 mRNA is greatly elevated in response to the ischemia.¹⁴ Activation of mGluR-1 promotes the postischemic injury of neurons,¹⁵ whereas the downregulation of mGluR-1 expression protects nerve cells against ischemic injury.¹⁶ Our study has shown that administration of TMP and DFO not only inhibits the release of glutamate but also downregulates the expression of mGluR-1 mRNA, suggesting that the decrease in spinal injury and the improvement of neurological defect achieved by administration of TMP and DFO are due to the inhibited release of glutamate and the downregulated expression of mGluR-1 mRNA.

Previous studies have shown that TMP reduces damage in the spinal cord by suppressing hypoxia-inducible factor-1 α and tumor necrosis factor- α ; and by activating caspase-3 expression,¹ antiplatelet activity,¹⁰ scavenging oxygen-free radicals and inhibiting the release of intracellular calcium.¹¹ DFO improves nerve regeneration² through the reduction of local hydroxyl radical formation.¹⁷ Therefore, in consideration of these previous studies together with our present findings, the potential mechanisms involved in the therapeutic effect of TMP and DFO may include multiple factors. The downregulation of mGluR-1 mRNA expression enhanced the tolerance to spinal ischemia in this study. This effect may involve factors such as calcium influx and calcium release,¹⁸ release of arachidonic acid¹⁹ and synthesis

of nitric oxide.¹⁹ Further study is required to examine these mechanisms in spinal cord ischemia/reperfusion injury.

Conclusion

In summary, the protective effects of TMP and DFO on spinal cord ischemia/reperfusion injury were characterized by a decrease in the spinal nerve damage and improvement of neurological defect. The mechanism behind the neuroprotection involved the downregulation of mGluR-1 mRNA and inhibition of the excessive release of glutamate resulting from spinal ischemia/reperfusion injury. TMP and DFO are clinically available agents, and therefore we anticipate that TMP combined with DFO might have important clinical application in the treatment of spinal cord ischemia-reperfusion injury.

Conflict of interest

The authors declare no conflict of interest.

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