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Tetramethylpyrazine accelerates the function recovery of traumatic spinal cord in rat model by attenuating inflammation

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ABSTRACT

In the present study, we explored the effects of tetramethylpyrazine (TMP), an alkaloid extracted from the Chinese herbal medicine *Ligusticum wallichii* Franchat (chuanxiong), on a rat model of contusion spinal cord injury (SCI). The contusion SCI model was induced in rats by a modified Allen's weight-drop method with a severity of $5 \text{ g} \times 50 \text{ mm}$ impacting on the T10 segment. In the TMP treatment group, rats were injected intraperitoneally (i.p.) with TMP (200 mg/kg), every 24 h for 5 days, starting half an hour after contusion SCI. The control group was treated with saline. Compared with the control group, the TMP group significantly ameliorated the recovery of hindlimb function of rats. TMP treatment significantly reduced the expression of macrophage migration inhibitory factor, nuclear factor κappa B, pro-inflammatory cytokine interleukin-18 and neutrophil infiltration. On the other hand, TMP enhanced the expression of inhibitor κappa B and anti-inflammation cytokine interleukin-10. In conclusion, our results demonstrate that TMP inhibits the development of inflammation and tissue injury associated with spinal cord contusion in rats which may improve the rats' hindlimb function.

1. Introduction

Acute spinal cord injury (SCI), usually caused by motor vehicle accidents, sports injuries, diving accidents and violence, is one of the most common and devastating injuries encountered at the spine surgery department. The injury has a high rate of prevalence in the younger population, and causes permanent disability or loss of movement and sensation. Current major treatment for SCI is the use of high doses of methylprednisolone (MP), which reduces edema of the spinal cord and secondary damages. However, MP has numerous side effects, and its therapeutic effects are controversial. There is insufficient evidence to support the use of MP as a standard treatment for acute SCI [1].

The pathophysiology of acute SCI comprises both primary and secondary injuries. The primary injury is an immediate injury to the spinal cord, causing irreversible damage. Secondary injury, which takes place over a period of weeks or even months, can cause severe and permanent functional deficits [2]. Thus, many clinical and experimental studies have been conducted on potential treatments for secondary injuries of SCI [3].

Inflammation plays an important role in the secondary injuries after traumatic SCI [4]. Many studies have shown that injury-induced inflammation can result in neuropathology and secondary

necrosis after traumatic SCI [5–7]. It had been reported that macrophage migration inhibitory factor (MIF), nuclear factor kappa B (NF-κB), inhibitor kappa B (I-kBα), P-selectin and interleukin (IL)-10 take part in the inflammation response after traumatic spinal cord injury [7–9].

TMP, also called ligustrazine, is an alkaloid extracted from the Chinese herbal medicine, *Ligusticum wallichii* Franchat (chuanxiong) [10]. For hundreds of years, TMP has been routinely used for the treatment of heart, kidney, and brain diseases [11–13]. In the central nervous system, TMP markedly reduced cerebral ischemia/reperfusion injury through suppression of inflammatory cell activation and proinflammatory cytokine production. It can also inhibit neuronal apoptosis and prevent neuronal loss [13,14]. Several studies have suggested that treatment with TMP before SCI has protective effects against spinal cord I/R injury by reducing apoptosis and inflammation [15,16].

Some studies have shown that the treatment between the primary and the secondary injury of SCI has the potential to either prevent or reduce the final neurological deficits [17,18]. Our previously published observations had addressed that TMP treatment accelerated spinal cord repair through up-regulating neurofilament protein expression and down-regulating caspase-3 expression following contusion SCI [19]. So we speculated that TMP may have anti-inflammatory effects on contusion SCI. Therefore, in the present study, we explored the effects of TMP on inflammatory responses after traumatic SCI in a rat model of contusion SCI.

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2. Material and methods

2.1. Animals

Adult male Sprague–Dawley (SD) rats, weighing 250 to 300 g, were provided by the Center of Experimental Animals, Central South University. All animal care, breeding, and testing procedures were approved by the Laboratory Animal Users Committee at Xiangya Hospital, Central South University, Changsha, China. All animals were housed in individual cages in a temperature and light ± dark cycle controlled environment with free access to food and water.

2.2. Establishment of contusion SCI rat model

Rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate (3 mg/ kg). The skin above the vertebral column was shaved and cleaned with antiseptics. A 15-mm midline skin incision was made, and the vertebral column was exposed. After the spinal thoracic region was exposed by separation of dorsal muscles to the side, the spinous processes of T8–T13 vertebrae were exposed. A laminectomy was performed at vertebral level T10, exposing the dorsal cord surface with the dura remaining intact. The exposed spinal cord segment (about 3 mm length) was subjected to a moderate vertical impact load using a modified Allen's weight drop apparatus (5 g weight at a vertical height of 50 mm, 5 g × 50 mm). The impact rod was removed immediately, and the wound was irrigated. Muscles and incision were sutured using 3–0 silk threads. Sham-operated animals received the same surgical procedures but sustained no impact injury. Penicillin G (4wu, i.m.) was injected into the quadriceps femoris during the surgery in each rat and then once a day in both hindlimbs for five days. The entire surgery was performed in a warm environment. Finally, the rats was housed in cages and given free access to food and water. Distended bladders were emptied by manual massage on the lower abdomen twice a day until voluntary emptying returned.

2.3. Experimental design

Rats were randomized into 3 groups: (1) sham group (N = 5), where rats were subjected to the surgical procedure except that the spinal cord injury was not applied; (2) control group (N = 40), where rats were subjected to SCI and treated intraperitoneally (i.p.) with saline (200 mg/kg); (3) TMP group (N = 40), where were rats subjected to SCI and treated i.p. with TMP (200 mg/kg), every 24 h for 5 days, starting half an hour after SCI. The control group and TMP group were divided into 8 time-period sub-group: 1 h, 3 h, 6 h, 12 h, 1 day, 3 days, 7 days, and 14 days (N = 5 each sub-group).

2.4. Basso–Beattie–Bresnahan (BBB) scores

Locomotor activity was evaluated at 1, 3, 7, 14 and 21 days post-injury, using the BBB scores, which measured the locomotor ability for 4 min. Two independent and well trained investigators observed the movement and scored the locomotor function according to the (BBB) scales, as described previously [20] in a blinded manner. The BBB scale is readily applicable to rat without modifications [21], especially when the score is below 16. The final score for each animal was obtained by averaging the values from both investigators. Before surgery the rats were placed individually in a molded plastic open field for 4 min to ensure that all subjects consistently obtained a maximum score of 21.

2.5. Immunohistochemical analysis

After receiving the functional behavioral testing, animals were given a lethal overdose of 10% chloral hydrate (300 mg/kg) and perfused intracardially with heparinized saline for 20 min, followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4. A 10-mm cord segment centered at the injury site was blocked from the vertebral column, placed in the same fixative overnight, and embedded in paraffin. For histological evaluation, the sections were stained with hematoxylin and eosin (H&E). At 1 h, 6 h, 12 h, and days 1, 3, 7, and 14 (n = 5 at each time point) after SCI, the tissues were fixed in 10% (w/v) PBS-buffered formalin and 10 mm sections were cut from paraffin embedded tissues and mounted on positively charged slides for immunohistochemical staining. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeabilized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Antigen retrieval was done by immersion of sections in 0.01 M citrate buffer, pH 6.0, in a steam bath (at 98 °C) for 25 min, followed by rapid cooling (20 min). Subsequently, endogenous peroxidase was inactivated by incubation in 3% H_2O_2 in dH_2O for 30 min at RT. After rinsing with 0.1 M phosphate-buffered saline (PBS), pH 7.4, sections were treated in 2% bovine serum albumin (BSA) in PBS (30 min, RT). Sections were incubated overnight (4 °C, overnight) with anti-MIF antibody, anti-NF-κB antibody, anti-I-kBα antibody, anti-P-selectin antibody, anti-IL-10 antibody and anti-IL-18 antibody (1:50) with 2% BSA. Sections were washed and incubated with goat anti-rabbit antibody (1:200) for 60 min at room temperature (RT) and subsequently treated with the avidin–biotin peroxidase complex for 1 h at RT. Finally, sections were incubated in 0.05% DAB plus 3% H_2O_2 in TBS for 10 min, rinsed in dH_2O, counterstained with hematoxylin, dehydrated, and mounted with a mounting medium. For negative controls the primary antibodies were replaced by PBS.

2.6. Cell counting

HIMAS-2000 image analysis software (Tongji Qingping Company, PRC) was used for the image analysis. Samples were viewed under BX-50 light microscopy (OLYMPUS, Japan). Images were captured using a digital camera (Nikon) attached to the microscope. Capture parameters were initially established and kept unmodified for all images. Cells were considered positively stained if their staining intensity was over five times of the background level [22]. Two sections from each specimen were randomly selected, and the total number of positive cells in three randomly selected view fields in each section was counted under 400× magnification. All measurements were performed by a pathologist blinded to the treatments.

2.7. Statistical analysis

Statistical analyses were performed by SPSS 13.0 software (The University of Cambridge, UK). Data values were expressed as means ± SD. Significant differences of immunohistochemical values between groups at specific time points were determined with Student's t test. The BBB scores between groups were analyzed by Wilcoxon rank sum test. A p-value of less than 0.05 was considered to be statically significant.

3. Results

3.1. TMP reduced the severity of spinal cord trauma

As shown in Fig. 1, histological features of normal spinal cord tissue were observed in the sham group. A significant damage to the spinal cord was observed in the control group compared with the sham group such as inflammatory cell infiltration, neuron edema and deformation, cavity formation and hemorrhage. Notably,