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Original Article

Effects of tetramethylpyrazine combined with bone marrow mesenchymal stem cells transplantation on recovery of neural function in rats with spinal cord injury

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Abstract: Objective: This study aims to investigate and analyze the effects of combined use of tetramethylpyrazine and bone marrow mesenchymal stem cells (BMSCs) transplantation on neurological recovery in rats with spinal cord injury (SCI). Methods: Male SD rats (aged from 3 to 4 weeks) were selected. BMSCs were isolated and cultured *in vitro*, and 3rd generation BMSCs were collected for follow-up experiments. Adult Wistar male rats were divided into the control group (sham-operated rats without spinal cord injury), the model group (spinal cord injured rats without BMSCs or drug treatment), the BMSCs group (spinal cord injury rats with BMSCs treatment) and the combination group (spinal cord injury rats with BMSCs and drug treatments). The changes of hindlimb motor function in each group were compared between 1 week and 2 weeks after surgery. At 2 weeks after surgery, the levels of neuroelectrophysiological sensory evoked potential (SEP) and motor evoked potential (MEP) were compared in each group. In addition, immunohistochemistry was used to analyze the changes of glial fibrillary acidic protein (GFAP) and nerve growth factor (NGF) in the spinal cord of rats in each group. Results: Compared with the control group, the motor function of the other groups was significantly reduced (all $P<0.05$). The latency of neural electrophysiological sensory evoked potentials and motor evoked potentials was significantly increased, and the amplitude was significantly reduced (all $P<0.05$). The positive rates of GFAP and NGF were decreased significantly (all $P<0.05$). Compared with the model group, the BMSCs and the combination groups could significantly restore the motor function of rats, and could significantly reduce the latency period of rats for electrophysiological sensory evoked potentials and motor evoked potentials. Moreover, it could improve the amplitude of rat's neural electrophysiology sensory evoked potentials and motor evoked potentials (all $P<0.05$). The combined effects of BMSCs and ligustrazine hydrochloride were significantly better than that of BMSCs only (all $P<0.05$). The effects of BMSCs on recovery of spinal cord motor function in injured rats were more significant. Compared with the model group, the positive expression rates of GFAP and NGF in the BMSCs group and the combination group were significantly higher (all $P<0.05$). Compared with BMSCs group, the positive expression of GFAP and NGF of rates in the combination group was significantly higher (all $P<0.05$). Conclusion: Tetramethylpyrazine combined with bone marrow mesenchymal stem cells transplantation could significantly restore the neurological function of rats with spinal cord injury.

Keywords: Tetramethylpyrazine, bone marrow mesenchymal stem cells, neural function, spinal cord injury

Introduction

Due to economic development and the popularization of transportation, spinal cord injury (SCI) becomes a common serious trauma with high morbidity and mortality [1]. Even if the SCI patients receive timely treatment, most patients still have long-term neurological sequelae [2]. Therefore, the treatment of SCI has always been a difficult and serious issue in the world [1].

Traditionally, it is believed that neurons are not regenerative, and adult injured neural tissue has limited ability to repair itself or does not possess structurally meaningful self-repairing function [3]. Relevant literature has shown that mesenchymal stem cells (MSCs) have biological characteristics such as multiple germ layer differentiation and are ideal transplant materials [4]. Stem cell transplantation can promote the repair of spinal cord injury. Therefore, stem cell transplantation has become a new method

for the treatment of SCI. Stem cells include bone marrow mesenchymal stem cells (BMSCs), embryonic stem cells, and neural stem cells [5]. BMSCs also have a strongpotential of multi-differentiation and proliferation. They can be used for autologous stem cell transplantation without immunological rejection. Thus, BMSCs are considered as ideal stem cells for the treatment of SCI [6].

As an extract, tetramethylpyrazine is collected from ligusticum wallichii, a traditional Chinese medicine. Its main active substance is a kind of alkaloid. It is widely used in various ischemic and traumatic diseases in neurology, including blood-brain barrier, antiplatelet, expansion of arterioles, improvement of microcirculation and the blood flow in the central nervous system [7, 8]. Tetramethylpyrazine can protect neurons by elevating the expression of glial fibrillary acidic protein in astrocytes *in vivo* [9]. Data suggest that tetramethylpyrazine be combined with bone marrow mesenchymal stem cell transplantation to repair spinal cord injury and also induce transformation of bone marrow stromal cells. However, it is still unclear whether the combination of BMSCs injection with tetramethylpyrazine is superior to the BMSCs single treatment in the recovery of nerve function.

This study aims to investigate the effects of bone marrow mesenchymal stem cell transplantation in combination with tetramethylpyrazine on neurological recovery in rats with spinal cord injury. After constructing a rat model of spinal cord injury, we performed different treatments on the rats after surgery (without any treatment, injection of BMSCs, injection of BMSCs plus concommitant tetramethylpyrazine). In addition, neurological function in each group was evaluated by measuring the index of neurological recovery in rats after surgery.

Materials and methods

Animal information

All animals in this study were specific pathogen-free rats (Wistar rats and SD rats), and experimental animals were selected from the Experimental Animal Center of Sun Yat-sen University of Medical Sciences. Fifty-eight male Wistar rats (8-week-old) were selected (160±23 g); ten SD young rats were selected (18±2 g). All rats were fed in a humidity environment with constant temperature (circulate 12 h day and night, free drinking and feeding). All animal experiments were approved by the Ethics Committees of Shandong Provincial Qianfoshan Hospital.

BMSCs culture and identification

Bone marrow mesenchymal stem cells were isolated and cultured by the whole bone marrow adherence method [10]. Based on the rat body weight, 2% pentobarbital was intraperitoneally injected at 35 mg/kg in rats and the rats were anesthetized. Rat skin was disinfected with 75% ethanol and the femur and tibia were removed aseptically. Then the femur and tibia were soaked in 75% ethanol for 10 min. The sample was transferred to a clean bench and dried in a sterile petri dish. The ends of the two bones were cut off, and the two bone marrow cavities were exposed. The marrow cavity was washed by serum-free Dulbecco's modified Eagle's medium (repeat 2-3 times), and the solution was centrifuged at 1,000 rpm for 5 minutes. After centrifugation, the supernatant was discarded and the cells were suspended in DMEM medium containing 10% fetal bovine serum. The cells were counted on the counting plate, and the cell density was adjusted to 5,000 cells/mL in a culture flask. The culture was incubated at 37°C (5% CO_2, wet saturation). After 24 hours, the medium was changed. Then the medium was changed every 3 days. After 80% to 90% of the cells covered the wall of the culture medium, cells were digested with 0.25% trypsin. The third-generation BMSCs were collected for flow cytometry detection, and were tested for CD34, CD44, CD45, CD90, CD11b, and CD106 cell surface markers. Positive CD44, CD90, CD106 and negative CD34, CD45, CD11b indicated that the BMSCs cells were successfully isolated. Then the third-generation BMSCs cells were used for subsequent experiments.

Grouping and treatment

All adult male Wistar rats included in the study were divided into control group, model group (SCI rats without BMSCs or drug administration), BMSCs group (SCI rats with postoperative

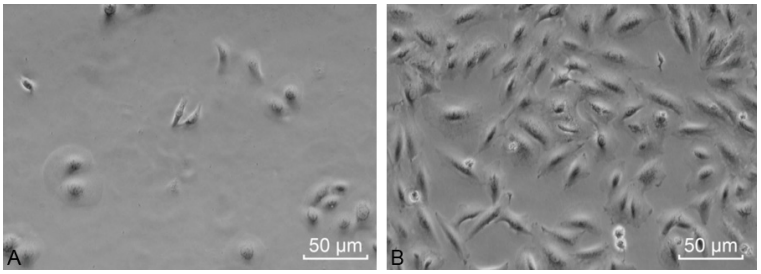

Figure 1. Morphological observation of primary (A) and third generation (B) BMSCs (200×). BMSCs, bone marrow mesenchymal stem cells.

tail vein injection of BMSCs), combination group (spinal cord injured rats with BMSCs and ligustrazine hydrochloride injection).

Spinal cord injury model construction

Spinal cord injury (SCI) model was constructed using the modified Allen method [11]. After anaesthetizing the rat by intraperitoneal injection of 10% chloral hydrate (300 mg/kg), the rat was taken in the prone position and fixed firmly. The back fur was cut off and then the skin of the operating area was disinfected by povidone iodine routinely. In the aseptic condition, the modified Allen method was used as follows. After centering on the spinous process of segment T11, a 3 cm longitudinal incision was made in the middle of the spine of the rat. The skin, subcutaneous tissue, and fascia were cut layer by layer. In the T10-12 segments, the muscles on the two side of spinous process were dissected, and the suprascapular and the interspinous ligament were removed. At the same time, some spinous processes and laminae of the T10 and T12 segments, and all the spinous processes and laminae of the T11 were completely resected. Adrenaline solution and gentamycin salt water was used to clean the wound surface. A circular blow zone centering on the T11 spinal cord with a diameter of 5 mm was exposed. After hemostasis was confirmed, a soft plastic sheet was tightly attached to the dura mater. At a height of 5 cm from the spinal cord of the rat, a impact rod (10 g) was vertically dropped directly above the soft plastic sheet to cause spinal cord injury. After the impact, the rat's body and the two hindlimbs immediately showed a retractable flapping, and there was a flaccid paralysis in the two hind limbs. The rat-tail immediately slapped spastically. The results indicated successful modeling. The model-successful rats were hemostatic and sutured. After the suturing, penicillin (twice daily, 200,000 U/time) was injected into the muscle of each rat, in order to prevent infection (continuous injection in 3 d). Urinary bladder extrusion was performed every 8 hours after surgery to help the rats urinate until they urinate normally. The rats in the control group were treated the same as those in the model group except that they did not hit the spinal cord.

Motor function rating

In order to observe the effect of different treatment methods on motor function recovery in rats, we performed inclined plate test, modified Tarlov grading, and Basso Beattie Bresnahan (BBB) scores for each group of rats [12, 13]. One and two weeks after surgery, the recovery of motor function in bilateral limbs was evaluated in all groups. The double-blind method was used in this experiment, ie two researchers observed and recorded independently. Finally, the data from the two researchers were recorded and processed by a statistician to reduce subjective errors. Oblique plate test was employed as follows. Rats were placed on the same smooth plate. The body axis was perpendicular to the longitudinal axis of the inclined plate. The plate was started from 0 and lifted gently (increasing by 5 at a time). Rats were able to maintain a functional value with a maximum angle of 5 s. Improved Tarlov classification method was measured as follows. According to the activities of hindlimbs, weights and walking, the motor function was divided into 6 levels, in which the hindlimb had no activity, and the weight was level 0; the normal walking was level 5; level 1 to 4 was classified based on the level 0 and 5. BBB scoring was evaluated as follows. The BBB scoring system classifies the hindlimb activities into 22 levels, including 0 in the hind limbs, 21 in the function, and 1 to 20 in the function. The basic content includes the number and range of joint activities, the tolerance and covariance of the hind limbs, and the movements of the fore and hind paws and the tail.

Table 1. Results of ramp test, BBB and Tarlov scores

Item	Control group	Model group	BMSCs group	Combination group
Ramp angle				
One week after surgery	40.29±4.03	14.56±1.22$^{\#}$	17.23±1.54$^{\#,\&}$	20.65±2.03$^{\#,\&,\$}$
Two weeks after surgery	41.22±4.20*	23.22±2.01$^{*,\#}$	25.64±2.19$^{*,\#,\&}$	29.98±2.11$^{*,\#,\&,\$}$
BBB score				
One week after surgery	21.65±2.06	1.65±0.23$^{\#}$	2.44±0.21$^{\#,\&}$	3.96±0.41$^{\#,\&,\$}$
Two weeks after surgery	22.65±2.16*	3.56±0.39$^{*,\#}$	5.68±0.54$^{*,\#,\&}$	9.52±0.94$^{*,\#,\&,\$}$
Tarlov score				
One week after surgery	42.06±4.03	14.26±1.23	18.56±1.44$^{\#,\&}$	21.65±2.12$^{\#,\&,\$}$
Two weeks after surgery	43.15±4.05*	18.45±1.86$^{*,\#}$	21.23±1.65$^{*,\#,\&}$	29.65±2.06$^{*,\#,\&,\$}$

Note: *indicates P<0.05 for comparison with 1 week after surgery; $^{\#}$indicates P<0.05 for comparison compared with the control group; $^{\&}$indicates P<0.05 for comparison with the model group; $^{\$}$indicates P<0.05 for comparison with the BMSCs group. BBB, Basso Beattie Bresnahan; BMSCs, bone marrow mesenchymal stem cells.

Animal neuroelectrophysiology sensory evoked potential (SEP) detection

SEP in each group of rats was measured on the DANTEC KEPONT evoked potential electromyography at 15 days after surgery [14]. First, the rats were anesthetized with 10% chloral hydrate (0.3 mL/kg). Concentric needle electrodes were used to monitor the sensory regions of the scalp and the coronal plane of the posterior limbs. The reference electrode was placed in the gastrocnemius muscle of the hindlimb, and the grounding electrode was placed in the anterior pole. After the stimulation of the posterior tibial position of the rat's limb, the nerve indicating electrode was measured. The intensity of nerve electrode stimulation was set at 2-5 mA with a frequency of 3 Hz and amplitude of 10 ms. The latency of P1 and P2 waves in the central nervous system of rats and the nerve potential N1 around the limbs were recorded. Spinal cord injury was defined by the following criteria [14]. Electromyogram showed that the amplitude of the stimulation potential in the rat was significantly reduced, and the waveform differentiation was not clear or disappeared, and the latency was prolonged significantly.

Animal Motion evoked potential (MEP) detection

At 15 days after surgery, the MEP of each group of rats was measured using the DANTEC KEPONT evoked potential electromyography. After the rats were anesthetized, the reference electrodes were placed in both the biceps of the hind limbs and the tendon of the rat. The distance between the anodes was set at 1 cm, and transcranial magnetic stimulation was performed using a MAG.2 and S.70 round coil. The coil was placed in the middle of the rat's head, and the maximum magnetic field intensity of 2 Tesla was output, and the stimulation intensity was 20% to 70%. The rat's head electrode and the compound muscle action potential of the hindlimb were recorded. The stable P1 and N1 waves and P1 and N1 wave latency were also recorded. Spinal cord injury was defined by the following critera [14]. Electromyogram showed that the amplitude of the stimulation potential in the rat was significantly reduced, and the waveform differentiation was not clear or disappeared, and the latency was prolonged significantly.

Immunohistochemistry

Six rats in each group were selected randomly. After sacrifice, the injured spinal cord tissue was taken and fixed with 40 g/L paraformaldehyde to make paraffin sections. Immunohistochemistry (SABC) staining was used to detect GFAP and NGF, and the reference kit (BBSW042, Baoankang Biotechnology, China) was used following the instructions. At 60°C for 1 h, the tissue sections were deparaffinized by conventional xylene, and dehydrated with a gradient of ethanol. Then the sections were soaked in 3% H_2O_2 for 10 min. The sections were washed with distilled water. The autoclave was repaired at 90 s, cooled at room temperature, and the sections washed with PBS gently. After adding 5% BSA blocking solution, the sections were incubated at 37°C for 30 min. Diluted rabbit anti-GFAP 1 (1:1000, ab8260, Abcam, USA)

Table 2. Rat SEP test in each group

Item	Control group	Model group	BMSCs group	Combination group
Latency period	15.24±0.16	39.64±0.32$^{\#}$	29.68±2.16$^{\#,\&}$	19.36±0.19$^{\#,\&,\$}$
Amplitude	5.42±0.55	1.16±0.12$^{\#}$	2.02±0.11$^{\#,\&}$	3.56±0.36$^{\#,\&,\$}$

Note: $^{\#}$indicates P<0.05 for comparison with with the control group; $^{\&}$means P<0.05 for comparison with the model group; $^{\$}$means P<0.05 for comparison with the BMSCs group. BMSCs, bone marrow mesenchymal stem cells; SEP, sensory evoked potential.

Table 3. Rat MEP test in each group

Item	Control group	Model group	BMSCs group	Combination group
Latency period	6.14±0.62	26.12±1.47$^{\#}$	18.36±1.06$^{\#,\&}$	9.46±0.95$^{\#,\&,\$}$
Amplitude	7.64±0.77	1.03±0.10$^{\#}$	2.16±0.16$^{\#,\&}$	4.98±0.42$^{\#,\&,\$}$

Note: $^{\#}$indicates P<0.05 for comparisonwith the control group; $^{\&}$means P<0.05 for comparison with the model group; $^{\$}$means P<0.05 for comparison with the BMSCs group. BMSCs, bone marrow mesenchymal stem cells; MEP, motor evoked potential.

and NGF (1:500, ab6199, Abcam, USA) were incubated overnight. The sections were then washed with PBS. Biotin (HRP)-conjugated goat anti-rabbit IgG (HY90046, Hengyuan Biotechnology, China) was diluted with instructions as secondary antibody for blocking, and incubated at 37°C for 30 min. After washing the sections with PBS, streptavidin (anti-biotin) peroxidase solution (Zhongshan Biotechnology, China) was added and incubated at 37°C for 30 min. Then the sections were washed twice with PBS, and DAB was added to (Boosen Biotechnology, China) develop color at room temperature. The slices were soaked in hematoxylin for 5 minutes and rinsed with water. After washing with 1% hydrochloric acid for 4 seconds and double distilled water for 20 minutes, the GFAP and NGF protein positive cells showed brown-yellow colour. The average optical density of GFAP and NGF positive coloration was measured with PROPLUS image software (Media CyrnNeTeCo, USA) by quantitative analysis of GFAP and NGF expression on mean optical density.

Statistical analysis

SPSS 21.0 software was used for statistical analysis. Measurement data is expressed as mean ± standard deviation ($\bar{x}$ ± sd). Measurement data with normal distribution was conducted with t test and expressed by t. The comparison of the data between the groups was performed using a one-way ANOVA. P<0.05 indicates statistically significant difference.

Results

BMSCs morphology observation

When BMSCs were cultured for 24 h, a few cells began to adhere to the wall. Most of them appeared in pairs, and were oval and had strong refraction. After 72 hours of culture, the growth of spheroid and spindle cells was observed. At 5 days, spindle cells and polygonal cells were found. The number of bone marrow stromal cells in the flask significantly increased under the microscope. Cells from 1st to 3rd generations were active and proliferating. The cells were mainly monolayer cells. The cells on the 1st and 2nd day were flat, spindle-shaped and irregular. The nucleus and nucleolus were clearly visible and the cells were mainly parallel or vortex. The 3rd generation cells were uniform in shape (**Figure 1**). The flow cytometry detection of rat bone marrow mesenchymal stem cells showed surface markers, which showed that the 3rd generation BMSCs cells positively expressed CD44, CD90 and CD106, while the expression of CD11b, CD34 and CD45 were negative. The results confirmed successful isolation and culture of BMSCs.

Recovery of postoperative motor function in each group

In order to study the recovery of motor function in each group, oblique plate experiments, BBB scores, and Tarlov scores at one and two weeks after surgery were performed (**Table 1**). Compared with one week postoperatively, the critical angle of oblique plate, BBB score, and Tarlov score were significantly higher in the model group, BMSCs group, and combination group at the end of two weeks (all P<0.05). Compared with the control group, the sloping plate critical angle, BBB score, and Tarlov score significantly decreased in the other groups (all P<0.05). Compared with the model group, the critical angle of the oblique plate, BBB score, and Tarlov score in the BMSCs group and the

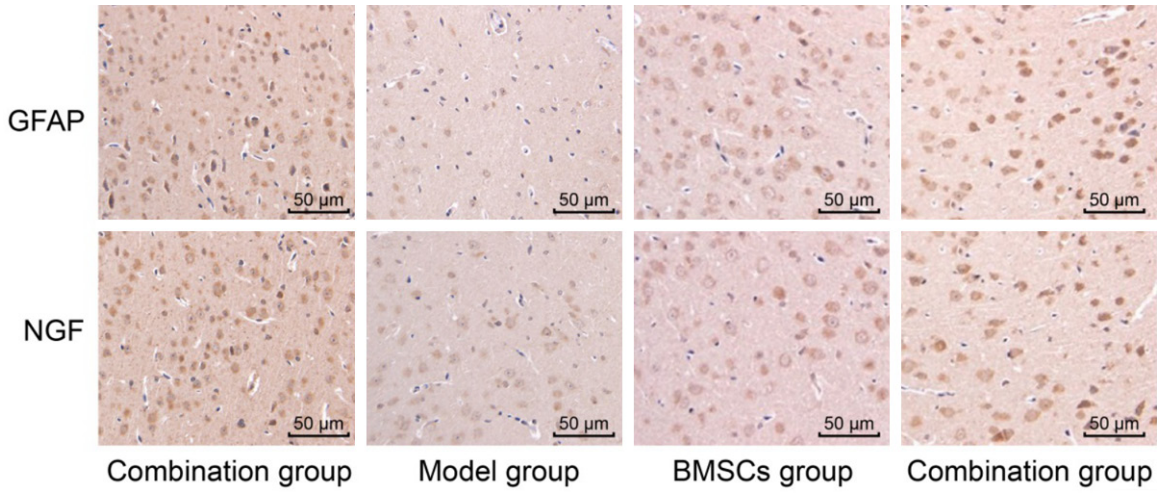

Figure 2. Immunohistochemical detection of positive expression of GFAP and NGF in each group (200×). GFAP, glial fibrillary acidic protein; NGF, nerve growth factor.

combined group significantly increased, and the critical angle of oblique plate, BBB score, and Tarlov score were better-recovered in the combined group (all P<0.05).

SEP in rats in each group

At 2 weeks after surgery, SEP in each group of rats was detected to observe the recovery of neurophysiological responses in rats (**Table 2**). Compared with the control group, the latency of SEP increased significantly in the other groups and the amplitude decreased significantly (all P<0.05). Compared with the model group, the latency of SEP in the BMSCs group and the combination group significantly reduced, and the amplitude significantly increased (P<0.05). Compared with the BMSCs group, the latency period of SEP in the combined group was significantly lower and the amplitude was significantly higher (P<0.05).

MEP in each group

At 2 weeks after surgery, MEP test in each group of rats were performed to observe and record the changes in latency and amplitude of MEP (**Table 3**). Compared with the control group, the latency of MEP increased significantly in the other groups and the amplitude decreased significantly (P<0.05). Compared with the model group, the latency of MEP in the BMSCs group and the combination group significantly reduced, and the amplitude significantly increased (P<0.05). Compared with the BMSCs group, the latency period of MEP in the combined group was significantly lower and the amplitude was significantly higher (P<0.05).

Immunohistochemistry

In order to confirm the recovery of neurological function furtherly, the positive expression levels of GFAP and NGF in each group of rats were detected by immunohistochemistry (**Figure 2**). Compared with the control group, the positive expression rates of GFAP and NGF in the other groups were significantly lower (P<0.05). Compared with the model group, the positive expression rates of GFAP and NGF in the BMSCs group and the combination group were significantly higher (P<0.05). The positive expression rate of GFAP and NGF in the combined group was significantly higher than that in the BMSCs group (P<0.05). The results indicated that the combination of tetramethylpyrazine injection and BMSCs had a significant effect on recovery of spinal cord injury.

Discussion

BMSCs are more convenient for drawing and *in vitro* culture, and have advantages of fewer rejection reactions. In recent years, BMSCs have gradually become an important source of clinical neural injury treatment [15]. As pluripotent stem cells, BMSCs are mainly derived from human bone marrow tissue. It is capable of multilineage differentiation and can be differentiated into many different cells when induced

by associated factors [16]. In a previous study, Alam et al. found that BMSCs could be converted into neurons, and could be used as a cell bridge to promote the growth of human spinal cord nerves and repair damaged nerves [17]. Due to the above characteristics, BMSCs have become an important source of stem cells for clinical treatment of spinal cord injury. Tetramethylpyrazine is mainly extracted from wallichii, a traditional Chinese medicine ligusticum. Its main component is tetramethyl pyrazine [18]. Animal studies have confirmed that it can protect against nerve injury and is currently widely used in the treatment of cardiovascular and cerebrovascular related diseases [19]. Lu et al. confirmed that tetramethylpyrazine has anti-inflammatory effects, could relieve cerebral ischemia-reperfusion inflammation in a study [20]. Qian et al. found that tetramethylpyrazine could repair spinal cord neurons damaged by SD rats and promote the recovery of their behavioral functions through animal studies [21].

We used tetramethylpyrazine combined with BMSCs transplantation to treat rats with spinal cord injury. The results confirmed that the sloping plate critical angle, BBB, and Tarlov scores of BMSCs and tetramethylpyrazine combined rats were significantly improved compared to other groups. It was suggested that the combination of the two treatments was more conducive to promoting the recovery of motor and neurobehavioral functions in rats. In a previous study, Lin et al. also confirmed the benefit of BMSCs transplantation in the improvement of neurological deficits in rats. That study indicated that BMSCs transplantation of SCI rat downregulated peripheral white matter Nogo-A ion, thereby repaired damaged nerves in rats [22]. Further examination of each group of electrophysiological conditions found that the SEP and MEP in the combination group were significantly better than those in the control group. The results indicated that tetramethylpyrazine is of value in repairing injured nerves of rats, which is helpful to promote the recovery of damaged nerve function and improve neurobehavioral function in rats.

GFAP is mainly distributed in the central nervous system as astrocytes which constitute an important part of the human cytoskeleton, and it also plays an important role in maintaining cell tension [23]. As a nerve growth-related factor, NGF is widely distributed in various tissues of the human body including the brain, and plays an important role in promoting the growth and development of neurons and ensuring the function of the nervous system [24, 25]. To confirm the recovery of neurological function in rats furtherly, immunohistochemistry was used to detect the positive expression of GFAP and NGF in each group. We found that the positive expression GFAP and NGF increased significantly in the combined group. It suggested that tetramethylpyrazine could help improve spinal cord microcirculation and promote the recovery of neurotrophic factors. This might be related to the improvement of neurological impairment function in the combined group of rats. However, relevant drug toxicology and human related experiments are still needed to further verify the experimental results in the future.

In conclusion, tetramethylpyrazine combined with bone marrow mesenchymal stem cell transplantation could significantly improve the neurological function of rats with spinal cord injury. This study brings new insights into spinal cord injury in clinical practice and deserves further application.

Disclosure of conflict of interest

None.

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