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Effect and mechanism of Tetramethylpyrazine in repair of sciatic nerve injury in rats

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Abstract

Objective Observing the effects of Tetramethylpyrazine (TMP) on the expression of Collagen IV and Laminin in neurovascular basement membrane and the apoptosis of vascular endothelial cells, and to study the mechanism of TMP in the treatment of sciatic nerve injury.

Results Compared with the NS group, the TMP group had a significant increase in the sciatic nerve function index ($P < 0.01$).The miss times in TMP group was significantly lower than that in NS group ($P < 0.01$). The HE staining results of the TMP group showed irregular arrangement of some neuronal axons and Schwann cells, and more edema and rupture of cells. The proliferation of glial cells and inflammatory cells was significantly increased in TMP group. The results of immunohistochemistry showed that the expression of type IV collagen and laminin in the TMP group group was distributed around the blood vessels, vascular endothelial cells, basal membrane and glial cells after SNI. The expression of type IV collagen and laminin in TMP group increased significantly($P < 0.05$). Immunofluorescence showed that compared with NS group, the apoptosis rate of TMP group was significantly decreased ($P < 0.01$). Flow cytometry results showed that compared with the NS group, the number of CECs in the TMP group was significantly decreased ($P < 0.01$).

Conclusions TMP can effectively improve the sciatic nerve functional index (SFI) of Sprague Dawley (SD) rats, enhance the proliferation of sciatic nerve vascular endothelial cells, reduce apoptosis, promote the expression of Collagen IV and Laminin in sciatic nerve microvascular basal membrane components, thereby promoting angiogenesis and improving lower limb function in rats.

Keywords Tetramethylpyrazine, Sciatic nerve injury, Endothelial cells, Circulating endothelial cells; angiogenesis

Introduction

Sciatic nerve injury (SNI) has the characteristics of high disability rate and difficult recovery of nerve function [1], and secondary lesions will spread to the surrounding normal tissues, resulting in chronic loss of function [2]. The etiology of secondary damage in SNI is not yet clear, but microcirculation disturbance is considered to be one of the important reasons for secondary damage [3–5]. Tetramethylpyrazine (TMP) can inhibit platelet aggregation, prevent thrombosis, dilate arterioles, enhance myocardial contraction, inhibit apoptosis, and reduce inflammatory reactions [6–10]. This study aimed to evaluate the efficacy of TMP in improving microcirculation and explore its mechanism in the treatment of sciatic nerve injury. The report is as follows.

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Research materials and methods

Experimental animal

Male Sprague Dawley (SD) rats aged 8–10 weeks, weighing 200–250 g, were provided by Chengdu Dashuo Biotechnology Co., Ltd. It was raised in the animal experimental center of Southwest Medical University, and the ambient temperature was manually controlled at 22–24 ℃, and the relative humidity was 60 − 80%.

Experimental reagent

Tetramethylpyrazine injection was purchased from Henan Furen Huaiqingtang Pharmaceutical Co., LTD; Bovine fetal serum was purchased from Biyuntian Inc; Primary antibody dilutions were purchased from Abcam biotechnology, USA; PBS solution was purchased from Beijing solabo Technology Co., Ltd; Streptomycin mixture was purchased from Beijing Solaibao Technology Co., LTD. Hanks solution was purchased from Beijing Solaibao Technology Co., LTD. Hoechst33258 dye solution was purchased from Beijing Solaibao Technology Co., LTD; TUNEL kit was purchased from Shanghai Roche Pharmaceutical Co., LTD. Anti-collagen IV antibody was purchased from Abcam Biotechnology Co., USA; Anti-Laminin antibodies were purchased from Abcam Biotechnology Co., USA.

Experimental apparatus

-80℃ low temperature refrigerator purchased from Qingdao Haier Group; Ordinary vertical refrigerator purchased from Qingdao Haier Group; The centrifuge was purchased from Shanghai Lu Xiangyi Centrifuge Instrument Co., LTD. Flow cytometer was purchased from THERMO FISHER Technology (China) Co., LTD; Laser confocal microscope was purchased from THERMO FISHER Technology (China) Co., LTD. The shaker was purchased from Hangzhou Qiwei Instrument Co., LTD. Electric thermostatic water temperature box was purchased from Shanghai Qixin Scientific Instrument Co., LTD. Ordinary light microscope was purchased from OLYMPUS Corporation; The oven was purchased from Shanghai Qixin Scientific Instrument Co., LTD.

Animal modeling

Male adult Sprague Dawley (SD) rats were randomly divided into sham operation group (NC group, $n=40$), Tetramethylpyrazine treatment group (TMP group, $n=40$) and normal saline group (NS group, $n=40$). Only sciatic nerve was exposed in NC group, and sciatic nerve crush injury model was made in TMP group and NS group by hemostatic forceps clamp.Before operation we used 1.5% Pentobarbital 3 ml/kg for intraperitoneal injection anesthesia to ensure that the rats remained anesthetized during the operation, in the preparation of sciatic nerve injury in SD rats. The anesthesia was successful when the corneal reflex disappeared, the respiration was stable, the muscles were relaxed, and the plantar stimulation was unresponsive. As shown in Fig. 1, at 0.5 cm below the femoral tubercle, parallel to the direction of the sciatic nerve stem, an incision about 1–1.5 cm long was cut, and the piriformis muscle was blunt separated to expose the left sciatic nerve. Except for the NC group, animals in other groups were vertically clamped with the same specification of hemostatic forceps for three consecutive times, each time for 10s, with an interval of 10s. The width of crush injury of nerve trunk is about 4 mm. TMP group received intraperitoneal injection of 2% Tetramethylpyrazine 200 mg / kg once a day for 5 days from 30 min after operation; NS group was treated with the

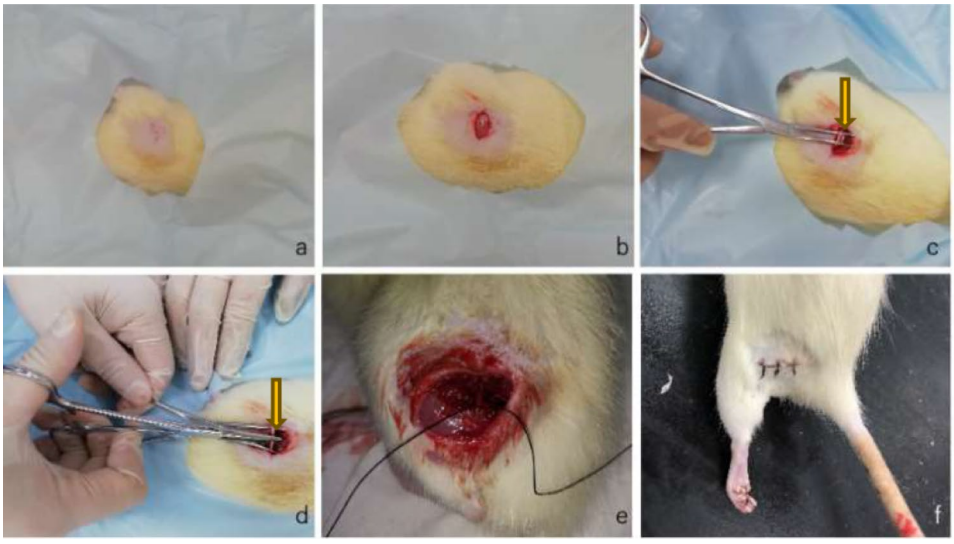

Fig. 1 Establishment of rat sciatic nerve injury model, The sciatic nerve crush injury model was prepared by exposing the sciatic nerve, and then clamping the sciatic nerve. The model was divided into three groups, $n=40$ for each group

same amount and frequency of normal saline (10 ml/kg) as the control treatment. The rats in each group were sacrificed on the 1st, 3rd, 7th and 14th day after operation. Rats were sacrificed using 1.5% Pentobarbital 40 ml/kg, and tissue specimens were subsequently removed.

Indicators of experiment

Animal behavior indicators

Sciatic nerve function index (SFI) The bilateral hind feet of rats were stained with ink and placed in the walking box without interfering with their movement trajectory in the walking box. The three variables of the foot prints of the affected side (E) and the healthy side (n) of rats were measured and calculated using Bain formula [11]. SFI=-100 in case of complete nerve apraxia and SFI=0 in case of completely normal sciatic nerve function.

Grid crawling experiment For the grid made of iron wire, the distance between each small grid is 2.5 cm × 2.5 cm, and the total length is 100 cm. The metal grid is fixed on the support at 45 degrees to the ground, and the rat is placed at the lower end of the grid. The experiment is completed when the hind limb of the rat leaves the grid. The number of times the rat steps empty during the period is recorded.

Hematoxylin-eosin stain

Take slices of sciatic nerve tissue from each group of rats, perform HE staining after dewaxing with xylene, and observe the pathological changes of tissues between each group under a microscope after loading.

Immunohistochemistry

After soaking in 10% formaldehyde for 24 h, the sciatic nerve tissue of each group of mice was taken for embedding and sectioning. Immunohistochemical reactions were performed according to the instructions of the mouse Anti collagen IV antibody kit and the mouse Anti Lamin antibody kit. Compare and observe the antigen distribution in the lung tissue of each group of mice under an optical microscope, and score each group.

Statistics analysis

Statistical analysis was conducted using SPSS 22.0 software, with data recorded as mean±standard deviation ($\bar{x} \pm s$). When the data conforms to a normal distribution and the variance is homogeneous, t-test was used, and LSD method was used for inter group comparison; If the data does not follow a normal distribution, use the rank sum test (U-test). The test result is indicated by $P<0.05$, indicating a statistically significant difference.

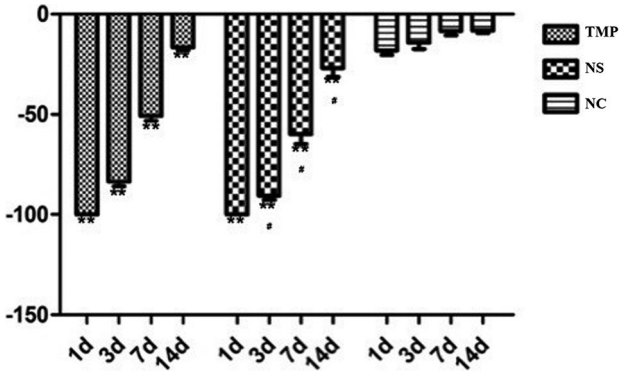

Fig. 2 Sciatic nerve function index results The sciatic nerve function index of TMP, NS and NC groups. Data show mean ± standard error ($n=40$, each time point). *$p<0.05$. Note. Compared with the NC group during the same period, *$p<0.05$, **$p<0.01$; Compared with the TMP group during the same period, #$p<0.01$

Results

Results of sciatic nerve function index in rats

As shown in Fig. 2; Table 1, the hind limbs of each group of rats were normal before the experiment; The SFI results of TMP and NS groups were significantly lower than those of NC group ($P<0.01$). There was no significant difference between the TMP group and the NS group ($P>0.05$). During the experiment, there was a significant decrease ($P<0.01$) in the TMP group compared to the NS group in the NC group; Compared with the NS group, the SFI value of the TMP group was significantly increased ($P<0.01$). On the 7th day, the SFI values of the TMP group and NS group improved significantly compared to before, but there was a significant decrease compared to the NC group ($P<0.01$). On the 14th day, the TMP group was close to but still lower than the NC group ($P<0.01$); The TMP group showed a significant increase compared to the NS group, and the difference was statistically significant ($P<0.01$).

Grid crawling experiment

As shown in Fig. 3; Table 2, in the comparison of 1d, 3d, and 7d, rats showed varying numbers of left lower limb

Table 1 Sciatic nerve function index

Group	Result ($\bar{X} \pm S$)			
	1d	3d	7d	14d
NC	-18.70±2.04	-14.19±2.95	-8.79±2.29	-8.09±1.15
TMP	-100**	-83.61±2.10**	-50.74±2.20**	-16.52±1.30**
NS	-100**	-89.40±3.02**,#	-59.79±4.46**,#	-26.89±3.95**,#

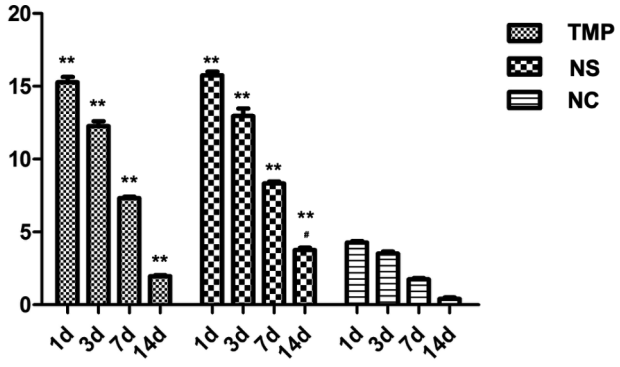

Fig. 3 Results of rat grid crawling experiment. The times of left lower limb treading of TMP, NS and NC groups. Data show mean ± standard error ($n=40$, each time point). *$p<0.05$. Note. Compared with the NC group during the same period, *$p<0.05$, **$p<0.01$; Compared with the TMP group during the same period, #$p<0.01$

treading. There was no significant difference in the number of empty stepping times between the TMP group and the NS group ($P>0.05$), while there was a significant difference in the number of empty stepping times between the TMP group and the NS group compared to the NC group ($P<0.01$); As time went on, the number of treadmills in each group of rats gradually decreased, and the NC group almost returned to normal. On the 14th day, there was a significant difference in the number of treadmills between the TMP group and the NS group ($P<0.01$), and there was still a significant difference compared to the NC group ($P<0.01$); There was a significant difference ($P<0.01$) between the results on day 14 and day 1 in each group, indicating that the lower limb function of the rats gradually recovered.

Hematoxylin-eosin stain

As shown in Fig. 4, the axons and Schwann cells of the NC group neurons are arranged neatly, with less infiltration of inflammatory cells and intact neural morphology. In the TMP group, some neuronal axons and Schwann cells were irregularly arranged, with more edema and rupture of cells. Over time, inflammatory cells infiltrated, glial cells proliferated, and over time, the neural axons and Schwann cells gradually returned to normal. The axons and Schwann cells of the NS group were irregularly arranged, with more edema and rupture of the cells, and the proliferation of glial cells and inflammatory cells was not as significant as that of the TMP group.

Immunohistochemistry

Using Image J software to analyze the immunohistochemical staining intensity score, the results are shown in Figs. 5 and 6. The expression of type IV collagen and laminin in the TMP group and NS group is basically consistent after sciatic nerve injury in rats, and they are mainly distributed around blood vessels, endothelial cells, basement membrane, and glial cells; The expression of NC group is mainly located around blood vessels. However, there was a significant difference between the TMP group and the NS group. The positive expression of type IV collagen and laminin in the TMP group was significantly stronger than that in the NS group. Among them, the TMP group showed strong positivity on day 1, day 3, and day 7, while the 14 day group showed moderate positivity; The NS group showed moderate positivity on the 1st, 3rd, and 7th day, while the 14th day group showed weak positivity. NC group analysis result is negative.

Immunofluorescence

As shown in Fig. 7A-B; Table 3, the proportion of apoptotic cells in the TMP group significantly increased compared to the NC group on day 1, day 3, and day 7, with statistical significance ($P<0.01$); The proportion of apoptotic cells in the NS group significantly increased compared to the NC group at 1 day, 3 days, 7 days, and 14 days ($P<0.01$); There was no significant difference between the TMP group and the NS group at 1 day, but the apoptosis rate of the TMP group was significantly lower than that of the NS group at 3, 7, and 14 days ($P<0.01$), indicating that TMP has an inhibitory effect on endothelial cell apoptosis.

Detection of circulating endothelial cells using flow cytometry

As shown in Fig. 8-A-B and Table 4, CECs were detected in all three groups. The number of CECs detected in the TMP group and NS group was significantly higher than that in the NC group at 1 day, 3 days, and 7 days after modeling ($P<0.01$). On day 1, there was no significant difference between the TMP group and the NS group ($P>0.05$); The number of CECs in the 3rd and 7th day NS groups was significantly higher than that in the TMP group ($P<0.01$); There was no significant difference in the number of CECs among the three groups on the 14th day ($P>0.05$). CECs are direct and specific indicators that can reflect endothelial damage in living blood vessels.

Table 2 Number of empty steps in rat grid crawling experiment

Group	Experimental time			
	1d (times)	3d (times)	7d (times)	14d (times)
NC	4.28 ± 0.66	3.52 ± 0.57	1.76 ± 0.71	0.4 ± 0.57
TMP	15.28 ± 1.43**	12.28 ± 1.40**	7.32 ± 0.97**	1.96 ± 0.77**
NS	15.76 ± 1.45**	12.96 ± 1.34**	8.32 ± 0.97**	3.76 ± 0.71**,#

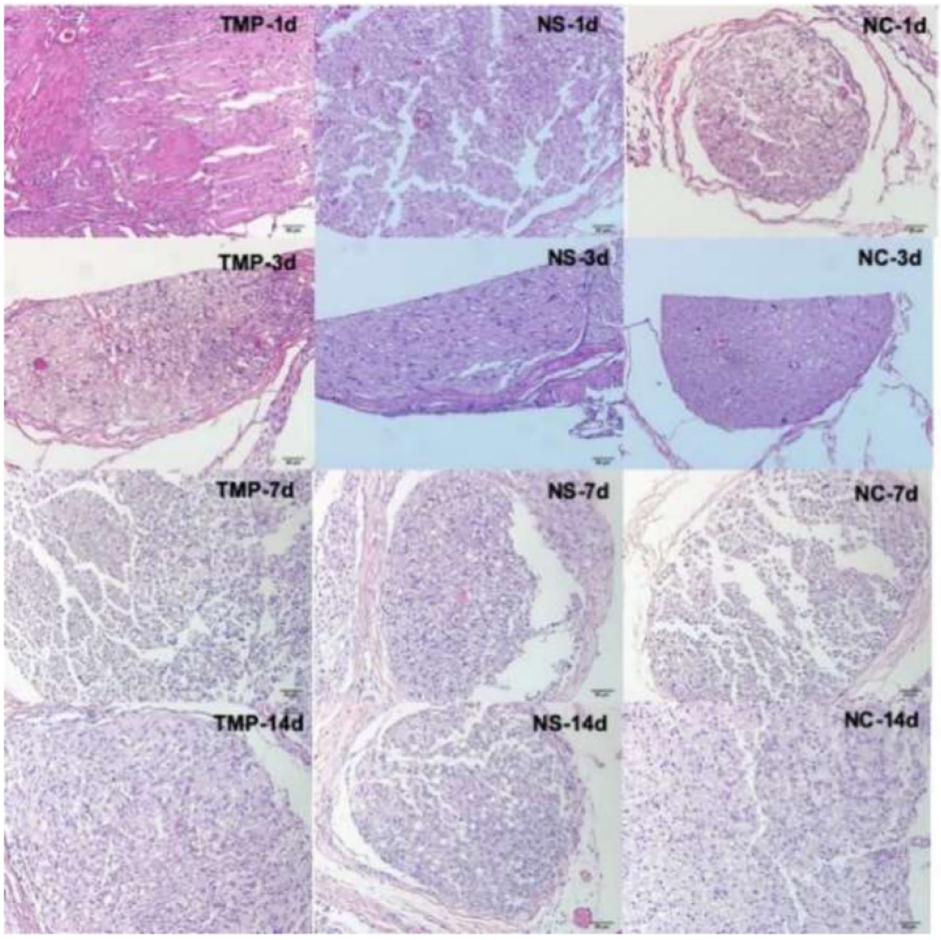

Fig. 4 HE staining light microscopy observation results (× 200). The pictures show the tissue of TMP, NS and NC groups under HE staining at different time periods ($n = 10$, each time point)

The results demonstrate that TMP can reduce the number of CECs in the bloodstream and alleviate endothelial damage.

Discussion

The repair of peripheral nerve injuries is currently one of the key concerns in the field of surgical trauma. The area of sciatic nerve injury and adjacent normal nerve tissue are in a continuous state of low blood flow perfusion. Although the peripheral nerves have low energy requirements and can quickly adapt to anaerobic metabolism, most injuries are reversible [12, 13]. However, severe ischemia and hypoxia can easily lead to an increase in metabolic demand, and at the same time, nerve cells with poor hypoxia tolerance may experience ischemic necrosis [14, 15].

Tetramethylpyrazine is a bioactive monomer found in Ligusticum chuanxiong, which can inhibit free radical production, scavenge oxygen free radicals, and enhance endogenous superoxide dismutase (SOD) activity [16]. With the deepening of pharmacological research, TMP has definite therapeutic effects in the treatment of cardiovascular and cerebrovascular diseases, and its clinical application is becoming increasingly widespread, achieving good therapeutic effects [17]. At present, experimental research on the protective effect of TMP on animal sciatic nerve injury models is increasing year by year. Its protective mechanisms involve inhibiting apoptosis, reducing inflammation, and promoting neurotrophic function, however, there are few research reports on the improvement of microcirculation by TMP in sciatic nerve injury, and the specific mechanism of its improvement of microcirculation is not yet clear. In the research results of TMP treatment for spinal cord injury [18–20], it was found that the number and volume fraction of new blood vessels in the injured and adjacent segments of the spinal cord were significantly increased in the TMP treatment group. It can be inferred that promoting vascular regeneration may be an important pathway for TMP to improve microcirculation disorders after SNI in the treatment of sciatic nerve injury.

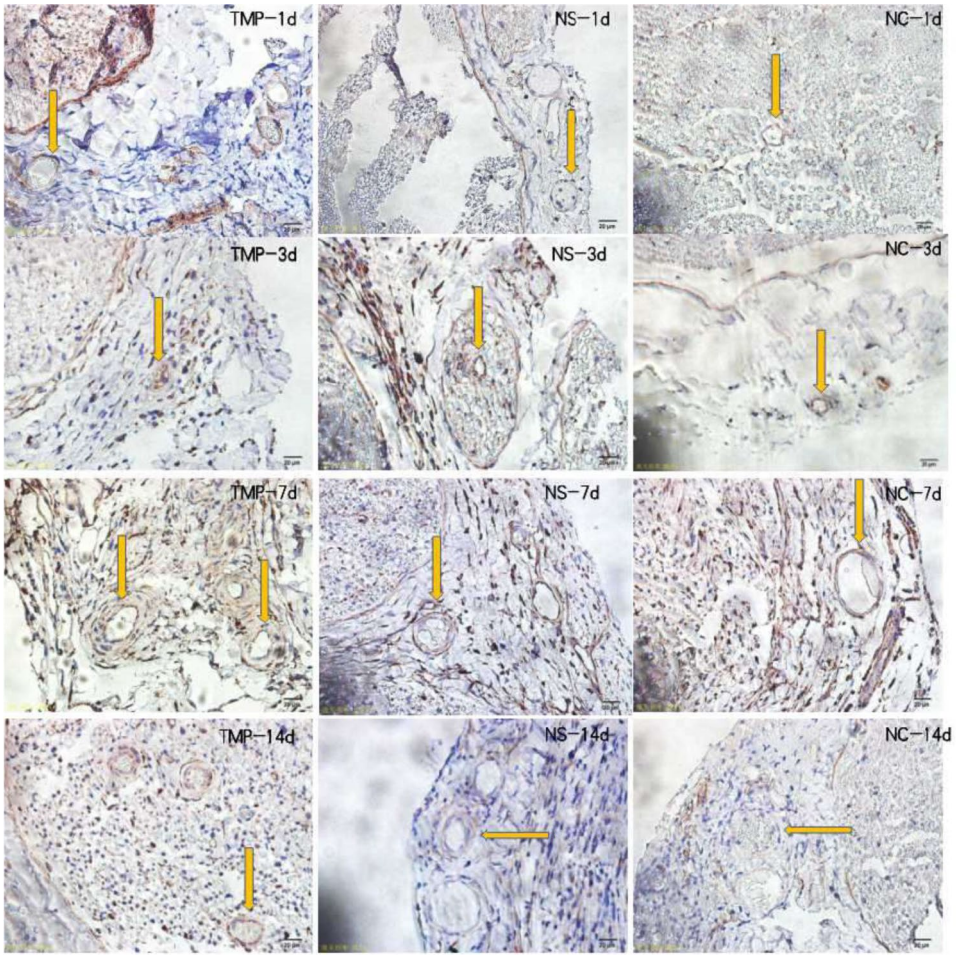

Fig. 5 Expression results of Collagen IV in vascular basement membrane (× 400). The pictures show the expression of type IV collagen of TMP, NS and NC groups under HE staining at different time periods ($n = 10$, each time point). Note. The yellow arrow represents the Collagen IV surrounding the vascular basement membrane

Luo Wei et al. [21] Concluded that TMP injection can promote nerve regeneration after end-to-end anastomosis of peripheral nerve injury by establishing a model of end-to-end anastomosis of sciatic nerve fracture in 12 rats and treating it with TMP. Li Yanjiang et al. 22 conducted a study on the promotion of nerve regeneration after allogeneic sciatic nerve transplantation in 40 Wistar rats as donors and 40 SD rats as recipients by pretreatment with TMP. The results of the study showed that a certain concentration of TMP had a protective effect on the pre treated sciatic nerve of rats and could promote nerve regeneration after allogeneic transplantation.

Collagen IV and Laminin are secreted by vascular endothelial cells and are the main components of the basement membrane in mammals. They are widely distributed in various tissues throughout the body and play a role in structural connectivity [23]. After sciatic nerve injury, microcirculation disorders occur in the nerves, ischemia and hypoxia occur within the nerves, and the blood nerve barrier (BNB) is disrupted, causing nerve edema. The axons and myelin sheaths of the neurons undergo a series of changes [24]. Neuroischemia and edema can cause infiltration of fibroblasts, and the increased synthesis and secretion of Collagen IV and Laminin will form new basement membrane components, separating the damaged area from other parts of the nerve and reducing inflammatory cell infiltration. The new vascular basement membrane components can restore blood nerve barrier function [25]. The expression of Collagen IV and Laminin in the TMP group was significantly enhanced compared to the NC group, with expression distributed in endothelial cells, basement membrane, and glial cells, while the expression in the NC group was mainly located around blood vessels. Moreover, the positive expression of Collagen IV and Laminin in the TMP group was higher than that in the NS group during the experimental time.

Circulating endothelial cells (CECs) are specific and direct quantitative indicators for evaluating vascular injury in vivo [26]. The peripheral blood CECs in the

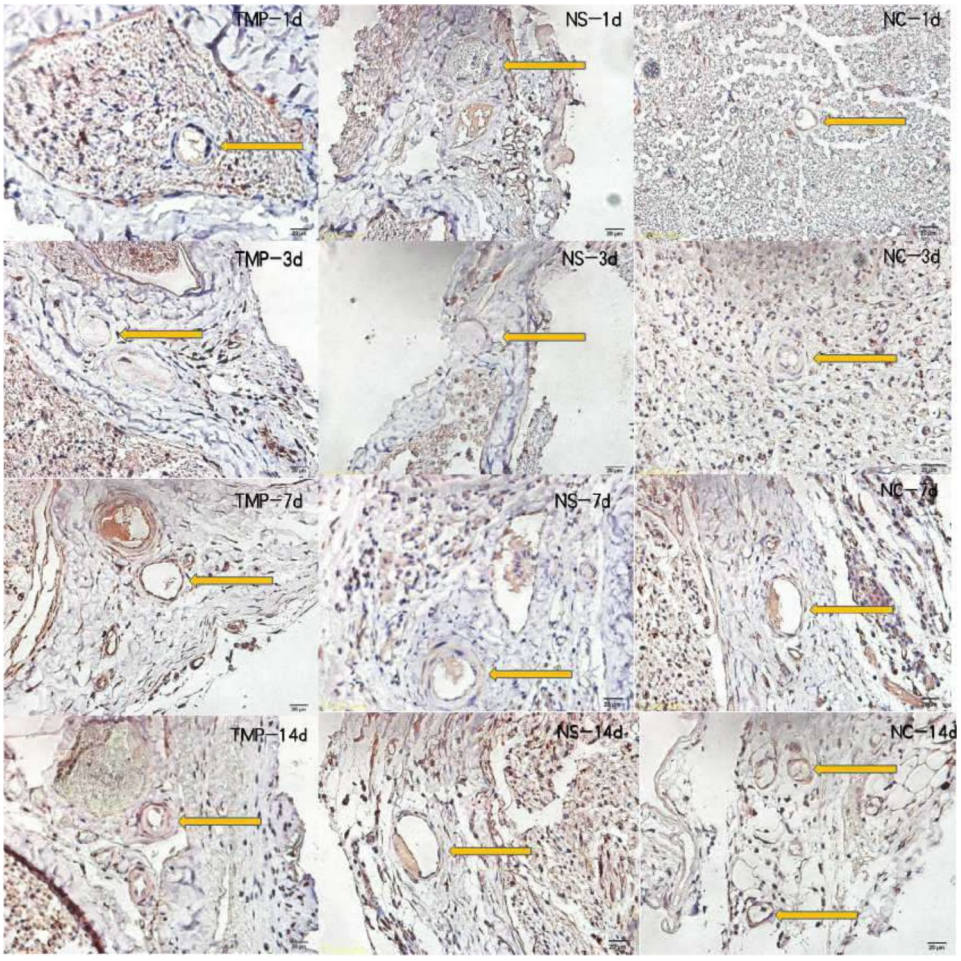

Fig. 6 Expression results of vascular basement membrane Laminin (× 400). The picture shows the expression of laminin of TMP, NS and NC groups under Immunohistochemistry at different time periods ($n = 10$, each time point). Note. The yellow arrow represents the Laminin surrounding the vascular basement membrane

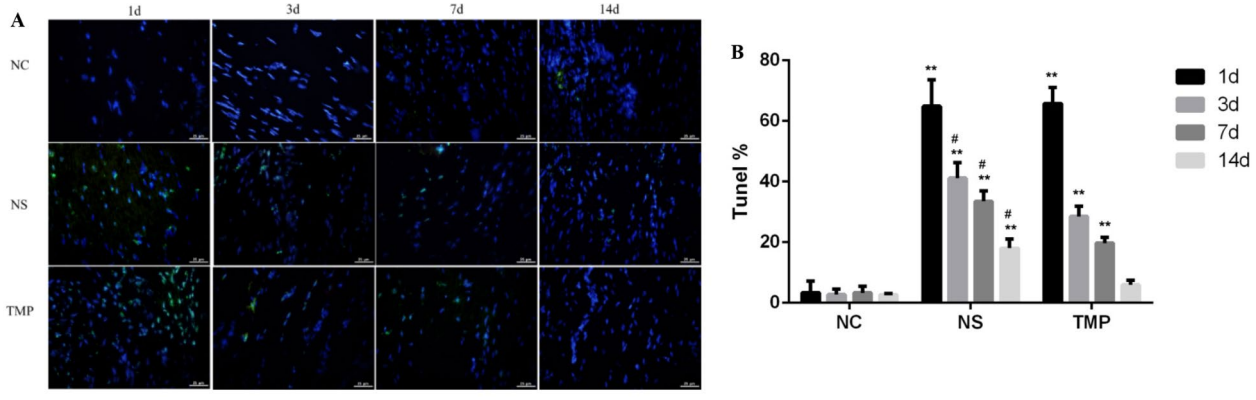

Fig. 7 Immunofluorescence detection of ECs apoptosis results. The picture **A** and **B** shows the proportion of apoptotic cells of TMP, NS and NC groups under Immunofluorescence at different time periods. Data show mean ± standard error ($n = 10$, each time point). *$p < 0.05$. Compared with the NC group during the same period, *$P<0.05$, **$P<0.01$; Compared with the TMP group during the same period, #$P<0.01$

Table 3 Immunofluorescence cell apoptosis results

Group (C)	Result ($\bar{X} \pm S$)			
	1d (pieces)	3d (pieces)	7d (pieces)	14d (pieces)
NC	3.36 ± 3.78	2.73 ± 1.82	3.31 ± 2.15	2.70 ± 0.38
TMP	65.80 ± 5.24**	28.50 ± 3.42**	19.67 ± 1.87**	5.99 ± 1.48
NS	64.87 ± 8.77**	41.19 ± 5.10**,#	33.49 ± 3.46**,#	18.02 ± 3.00**,#

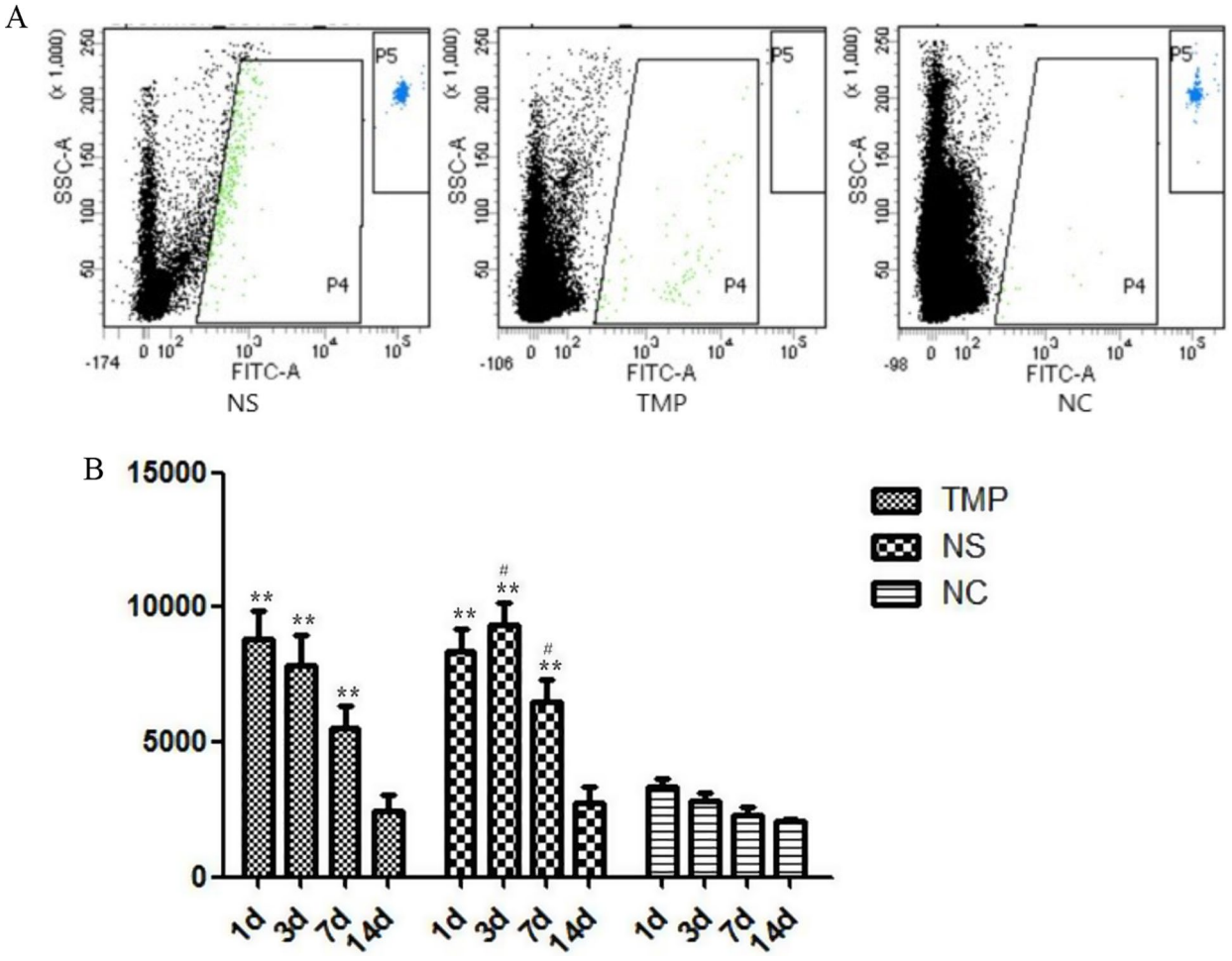

Fig. 8 Flow cytometry detection of CECs quantity results (pieces/ml). The picture **A** and **B** shows the number of CECs in TMP, NS and NC groups detected by flow cytometry at different time periods. Data show mean ± standard error ($n = 10$, each time point). *$p < 0.05$ Compared with the NC group during the same period, *$p<0.05$, **$p<0.01$; Compared with the TMP group during the same period, #$p<0.01$

Table 4 Flow cytometry detection of CECs quantity results

Group (C)	Result ($\bar{X} \pm S$)			
	1d (pieces/ μl)	3d (pieces/ μl)	7d (pieces/ μl)	14d (pieces/ μl)
NC	3.39 ± 0.26	2.86 ± 0.24	2.28 ± 0.27	2.06 ± 0.08
TMP	8.83 ± 0.99**	7.83 ± 1.11**	5.50 ± 0.80**	2.46 ± 0.58
NS	8.41 ± 0.77**	9.33 ± 0.81**,#	6.52 ± 0.75**,#	2.78 ± 0.52

TMP group of rats were significantly less than those in the NC group but more than those in the NS group, which can prove that TMP can reduce the number of circulating endothelial cells in the blood, alleviate the degree of endothelial damage, and the immunofluorescence results suggest that TMP can inhibit cell apoptosis. Therefore, it can be inferred that TMP may promote angiogenesis, inhibit endothelial cell apoptosis, improve microcirculation, and thus treat SNI by regulating the expression

of type IV collagen and laminin in the neurovascular endothelium.

At present, the cellular mechanism by which TMP regulates collagen components is not yet clear. The effects of TMP on Collagen IV and Laminin in the later stage, as well as its effects and mechanisms on Collagen I and Collagen XVIII after injury, still need to be further clarified in future research, in order to further explore the mechanism of TMP in the treatment of peripheral nerve injuries.

Conclusions

Tetramethylpyrazine can promote the expression of collagen IV and laminin in the microvascular basement membrane of rat sciatic nerve, promote angiogenesis, inhibit endothelial cell apoptosis, improve microcirculation, enhance sciatic nerve function index, and treat sciatic nerve injury in rats.

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Author contributions
Yang Li participated in study design and suggested the idea of the research. Naiqiang Zhuo, was responsible for the whole process of research. Yujie Li collected test data. Guang Wang analyzed the data and carried out the statistics. Yao Li drafted and revised the manuscript. All authors read and approved the final manuscript.

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Data availability
Data is provided within the manuscript.

Declarations

Ethics approval and consent to participate
The study was approved by the Institutional Animal Care and Use Committee of Southwest Medical University.

Consent for publication
Not applicable. Availability of data and material.

Competing interests
The authors declare no competing interests.

Conflict of interest
The authors declare no conflict of interest.

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