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Spinal cord injury can be relieved by the polysaccharides of *Tricholoma matsutake* by promoting axon regeneration and reducing neuroinflammation

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Background **With an increase in the number of spinal cord injuries (SCIs) in China, severe dysfunction of the limb below the injured segment is prominent. Among the studies centered on the factors inducing SCIs, inflammatory response has a dramatic input on the pathogenesis of SCIs.**

Objectives **This study aimed to investigate the effects of *Tricholoma matsutake* polysaccharides (TMP) on function recovery following SCIs.**

Methods **The cell viability, neurite growth, NF-kappa B, TNFα and IL-6 production from hydrogen peroxide-treated PC12 cells were analyzed. In-vivo, a total of 36 male C57 mice were divided into sham group, SCI group and TMP group (100 mg/kg). The protective effects of TMP were evaluated by Basso mouse scale (BMS) scores, HE staining, immunofluorescence and Western blotting.**

Results **TMP promoted neurite growth and inhibited TNFα, IL-6 and NF-kappa B signaling in a concentration-dependent manner *in vitro*. Moreover, compared with the SCI group, the BMS scores and nerve regeneration showed a significant increase, while NF-kappa B signaling, TNFα and IL-6 production significantly decreased after TMP treatment.**

Conclusion **TMP has a protective effect against SCIs *in vitro* and *in vivo*, which may be a potential strategy for future application in clinical practice. *NeuroReport* 31: 1024–1029 Copyright © 2020 The Author(s). Published by Wolters Kluwer Health, Inc.**

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Keywords: antioxidants, spinal cord injuries, *Tricholoma matsutake* polysaccharides

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Introduction

In recent years, the incidence of spinal cord injuries (SCIs) has gradually increased in China, with 80% of men under 40 [1]. SCIs is often leading to severe dysfunction of the limb below the injured segment. The pathogenesis of SCIs involves tissue damage induced by a variety of factors, but inflammatory response plays a very important role in the pathogenesis of SCI [2]. At present, the prevention and treatment methods of SCIs include surgical intervention, hormone shock therapy, drug therapy and cell transplantation therapy. Notwithstanding, the incidence of SCIs in China keeps increasing year after year. Therefore, minimizing the degree of spinal cord disabilities and restoring spinal cord functions remain a hot issue in medical research.

Promoting the regeneration of neurons and inhibiting neurogenic inflammation are the primary options to improve SCIs [3,4]. *Tricholoma matsutake*, also known as Brazilian mushroom, is a precious edible and medicinal fungus, and its polysaccharides are easy to modify in structure, thereby increasing the potential value of the efficacy. *Tricholoma matsutake* polysaccharides (TMP) have strong anti-inflammatory and anti-oxidation effects in organisms [5,6]. It is studied that it can effectively inhibit skin aging by preventing the degradation of extracellular matrix [7]. In this study, we used cultured nerve cells and spinal cord clamp injury mice as animal models to observe the effects of TMP on the repair of nerve injury, and in addition to explore whether TMP could be used as a potential adjuvant therapy to improve the repair of SCIs.

Materials and methods

Cell culture

PC12 cells were seeded in Dulbecco's Modified Eagle's Medium (DEME) high glucose medium containing 10% fetal bovine serum and cultured in an incubator at 37°C in a 5% CO_2 atmosphere. The cells in the logarithmic growth phase were randomly divided into five groups: blank control group, hydrogen peroxide group, low dose of TMP (1 μg/ml) group, medium dose of TMP (10 μg/ml) group and high dose of TMP (100 μg/ml) group. TMP, low molecular polysaccharide mixture with a purity of 98%, was purchased from Watercress Biology, Lanzhou, China and dissolved in DMEM with a final concentration of 1 mg/ml.

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Cell viability evaluation

PC12 cells in the logarithmic growth phase were inoculated into a 96-well culture plate at a density of 1×10^7/L, 100 μl per well, and cultured in a CO_2 incubator for 24 h. Then, the hydrogen peroxide group was given the final concentration of 30 μmol/L of H_2O_2 for 12 h. After pretreatment with H_2O_2, the freshly prepared concentrations of 1, 10 and 100 μg/ml of TMPs were added, while the control group was added the same amount of high glucose DEME. The cell viability was evaluated by the cell counting kit-8 after culture at different intervals [8].

Neurite growth assay

After PC12 cells were cultured for 7 days, the culture medium was discarded, the cells were washed with PBS for 5 minutes × 3 times, and 4% paraformaldehyde was added to them at 4°C for 15 minutes. Then, five fields of view were randomly selected for each group under the 200-fold microscope. In each field of view, average length of neurites from 20 cells was calculated.

Mice spinal cord injury model

Thirty-six male C57 mice (20–25 g) were randomly divided into sham-operated group, SCI group, and TMP treatment group, making 12 mice in each group. The mice were anesthetized with a 10% chloral hydrate (5 ml/kg). After simple disinfection, the mid-line incision (about 2 cm long) was done, followed by dissection and retraction of the paravertebral muscles to reveal the T9–T11 vertebral spinous processes. The T10 lamina was removed with fiber scissors to expose the spinal cord and avoid injury to the dura mater, and mouse SCI was induced by microsurgical arterial clamp for 15 seconds to induce a dorsal injury. At the moment of injury, the mice were paralyzed; the lower limbs and the body retracted, and the lower limbs were paralyzed [9]. The sham-operated group underwent the same surgery except for the clamp. For the TMP group, the mice were intraperitoneally injected with TMP 100 mg/kg per day after successful SCI model. The mice were kept to maintain warm temperature after surgery; the bladder was squeezed to urinate and prevented urinary system infection. All animal experiments and procedures were approved by the Institutional Animal Care and Use Committees of the Second Affiliated Hospital of Nanchang University, China (2HNC-2015632).

The Basso mouse scale score

The recovery of the spinal cord function in each group of mice was evaluated by the Basso mouse scale (BMS) scoring criteria. The score range applied was 0–9 points: 0, no ankle movement; 1, slight ankle movement; 2, extensive ankle movement; 3, planter placing or dorsal stepping; 4, occasional plantar stepping; 5, frequent or consistent plantar stepping, no coordination; 6, frequent or consistent plantar stepping, some coordination, paws parallel at initial contact; 7, frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and rotated at lift off; 8, frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift off, and mild trunk instability; 9, frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift off, and normal trunk stability and tail always up. Each mouse was assessed for 4 minutes. If scores were different for the right and left legs, the averages were calculated.

Spinal histopathology

On the 7th day after the SCI in mice, anesthesia was intraperitoneally injected with 10% chloral hydrate (5 ml/kg). After perfusion of the left ventricle, a 1 cm long spinal cord located on the injured area was harvested and fixed with 4% paraformaldehyde for at least 48 h, after which they were dehydrated, embedded, sectioned and subjected to HE staining for observation of the morphology of mouse spinal cord tissue.

Immunofluorescence

After goat serum was added to the slides to block specific binding at 37°C for 1 h, rabbit-derived primary antibody p65 (1:200; Abcam, Dallas, Texas, USA), mouse-derived Gap-43, or Iba1 (1:100; Abcam) were added and incubated overnight at 4°C. The slides rinsed three times in PBS (10 minutes/per time) the next day. Then protecting from light, anti-rabbit or anti-mouse (Proteintech, Chicago, USA) fluorescent secondary antibody (1:200) was added to incubate at 37°C for 2 h. After the third time of rinsing in PBS (10 minutes/time), 50% glycerol was mounted, and the co-expression was observed by laser confocal microscopy.

Western blotting

Cells or spinal cord tissue were lysed with RIPA buffer containing protease inhibitors (Dingguo, Beijing, China). Rabbit anti-p65 (1:500), GAPDH (1:2000) were provided by Santa Cruz Biotechnology (Dallas, Texas, USA). A horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:5000; Boster, Wuhan, China) was used as a secondary antibody [10].

ELISA

The supernatant of cell culture or protein extract from mouse spinal cord using RIPA buffer was used for TNFα and IL-6 determination. ELISA kits (Abcam) were utilized to detect the secretion of TNFα (ab100785) and IL-6 (ab100772). Briefly, 0.2 ml of sample was added to each well of the microtiter plate and incubated at 4°C overnight. After the third wash, 0.2 ml of diluted mAb was added to each well and incubated for 2 h at room temperature. After the third wash, 0.2 ml of secondary antibody was added to each well and incubated for 2 h at room temperature. After the third wash, 0.2 ml of substrate was added to each well to develop color for 30 minutes, and the absorbance was read in a microplate reader at OD 450 nm. The concentrations were calculated according to the standard curves of TNFα and IL-6.

Statistics analysis

The statistical analysis was performed using the SPSS 13.0 software. The data were expressed as mean ± SD, and the one-way analysis of variance was used for comparison between groups. The test level (α) was 0.05.

Results

Tricholoma matsutake polysaccharides promotes neuronal proliferation and neurite growth

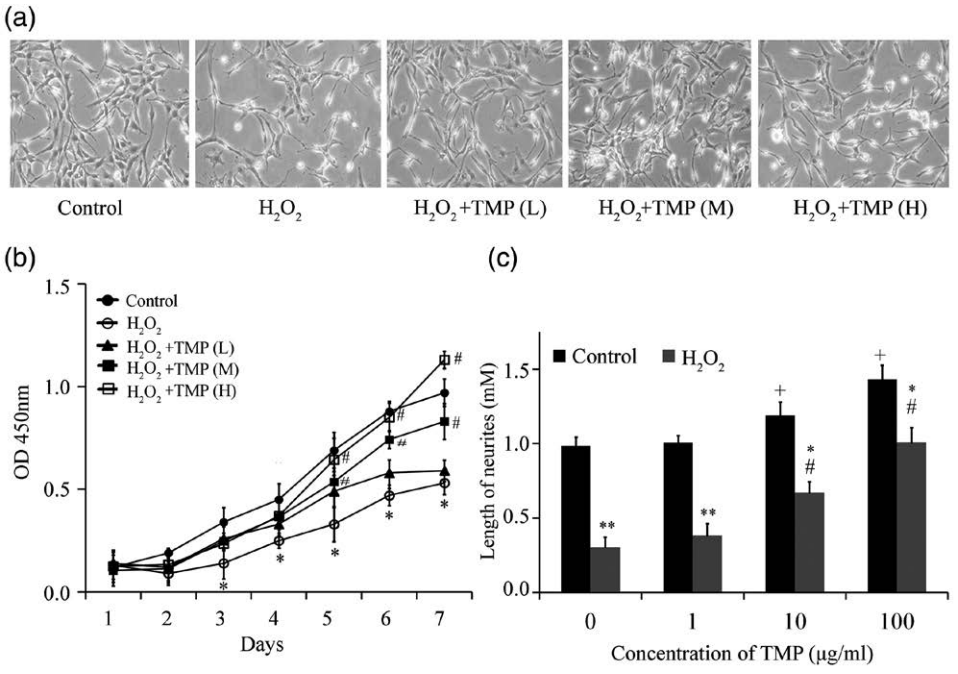
When normal neurons were cultured for 7 days, the neurites increased, elongated, and branched, forming a network of dense cells. After the treatment with H_2O_2 (30 μmol/L), the number of cells was significantly reduced, length of neurites were also reduced, and the length of neurites in medium and high doses of TMP groups was longer than that of the H_2O_2 group. The reticular connections between neurons were not different from those in the normal group. The protective effect was obvious except the low concentration group (Fig. 1a). Results with CCK-8 assay showed that, with the extension of culture time, the activity of neurons in each group increased, which was consistent with the increase in the number of cells perceived with morphological observation. The absorbance by the medium and high doses of TMP was significantly higher than that of the H_2O_2 group beginning from the fifth day ($P < 0.05$), and there was no significant difference when compared with the normal group (Fig. 1b), suggesting that TMP promote the proliferation of neuronal cells. After 7 days of culture, the neurites in the TMP groups were significantly more than that of the H_2O_2 group ($P < 0.01$). When compared with the other groups, the 100 mg/L group had the strongest promoting effect, and this lead to the suggestion that TMP could obviously promote neuronal neurite extension ($P < 0.01$, Fig. 1c).

Results from ELISA assay also showed that the significant increases in TNFα and IL-6 for the H_2O_2 group, which were inhibited by TMP in a concentration-dependent manner (Fig. 2a and b). Results from Western blotting showed that, compared with the control group, p65 in the H_2O_2 group was significantly increased, while decreased in a concentration-dependent manner after TMP treatments (Fig. 2c), and this is indicating that TMP exhibited stronger anti-inflammation effects.

Tricholoma matsutake polysaccharides improves spinal cord injuries in mouse models

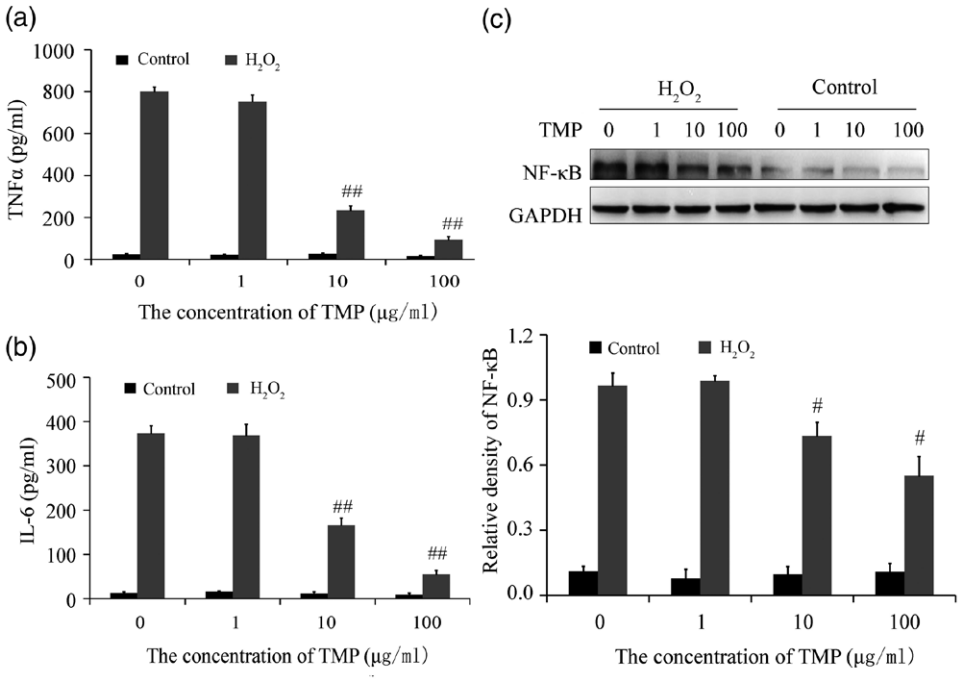
Compared with the sham-operated group, bilateral hind limb paralysis was seen in mice after SCI while there was an improvement in symptoms of bilateral hind limb paralysis in the TMP group compared with the model group. Seven days after SCI, two mice from the model group died, but no mice died in the sham-operated group and TMP treatment group.

Fig. 1



TMP promotes neuronal proliferation and neurites growth (n = 4). (a) The effect of TMP on neuronal growth after 7 days of culture; (b) the effect of TMP on the proliferation of neurons (n = 5); (c) the effect of TMP on length of neurites after 7 days of culture (n = 6). *$P < 0.05$ and **$P < 0.01$ as compared with the control group; #$P < 0.05$ compared with hydrogen peroxide group. +$P < 0.05$ as compared with the control group.

Fig. 2



TMP inhibits neuroinflammation (n = 4). The expression of TNFα (a) and IL-6 (b) in cell culture supernatant was identified using ELISA. (c) The expression of p65 protein in PC12 cells was determined using Western blot. #$P < 0.05$ and ##$P < 0.01$ compared with the hydrogen peroxide group.

In the entire evaluation process, the sham-operated group had a stable score of 9, while the SCI group and the TMP treatment group showed delayed paralysis immediately after the injuries. On days 1–7 after SCI, the BMS scores of the SCI group were lower than those of the sham-operated group ($P < 0.01$), while those of the TMP treatment group were higher than those of the model group from the 6th day after treatment ($P < 0.05$), giving rooms to the suggestion that TMP could improve the functional recovery of SCI in mice (Fig. 3a).

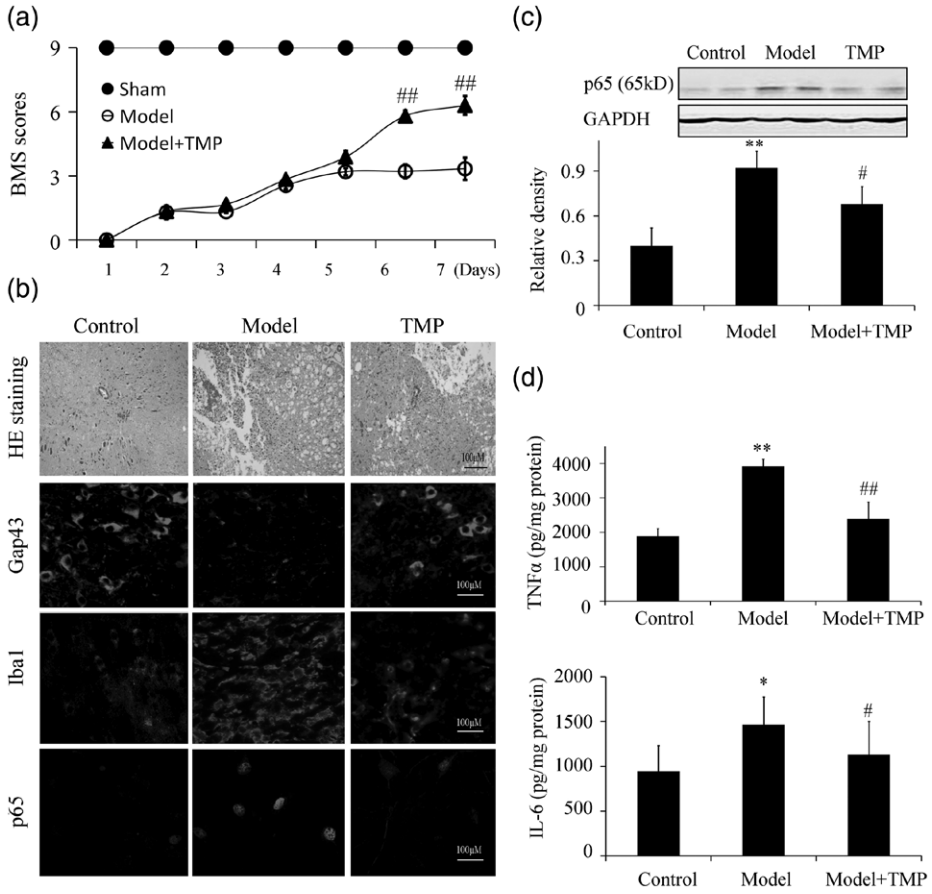
The results from HE staining showed that the morphology of the spinal cord was normal in the sham-operated group after 7 days. The morphological damage of the spinal cord in the model group could be alleviated by TMP treatment. The results from immunofluorescence staining showed that the expression of the neuronal axon growth-associated protein (GAP-43) in the model group decreased, the neuronal arrangement was distorted, and the morphology and structure of the cells were incomplete when compared with the sham-operated group. Against the model group, the expression of GAP-43 significantly increased in the TMP treatment group (Fig. 3b).

The results from the immunofluorescence assay showed that the microglia in the model group were obviously activated and p65 was expressed at a high level, while the expression of p65 in the treated group was lower than that of the model group (Fig. 3c). Results from Western blotting showed that, when compared with the sham-operated group, p65 in the model group was significantly increased, while p65 activity in the TMP treatment group showed a decreased rate (Fig. 3d). Results from ELISA assay also expressed a significant increase in TNFα and IL-6 for the model group, which were inhibited by TMP (Fig. 3d).

Discussion

A class of drugs represented by polysaccharides from mushroom species studied to exhibit the immune-regulatory, anti-tumor and anti-oxidant effects are drawing much attention for both local and international scholars. Xiong *et al.* [11] reported that neuroprotective effect of crude polysaccharide isolated from the fruiting bodies of *Morchella importuna* against H_2O_2-induced PC12 cell cytotoxicity by inhibiting cell apoptosis and reducing oxidative stress via downregulation of the NF-κB pathway and the p38-JNK pathway. Moreover, Tremella Polysaccharide Enteric-coated Capsules was approved by the Chinese Food and Drug Administration in 2002 for treatment of cancer patients with leukopenia induced by chemotherapy and radiotherapy. It acts on immunomodulation, antitumor, anti-oxidation, anti-aging, hypoglycemic, hypolipidemic, neuroprotection and other effects [12]. Based on the results from our study, compared with the normal group at the corresponding time points, TMP could significantly promote the survival of neurons at low

Fig. 3



TMP promotes nerve cell regeneration and inhibits inflammation (n = 12). (a) The effects of TMP on BMS scores of mice after SCI; (b) the effects of TMP on morphological changes, the expression of GAP-43 protein, the activation of glial cells and p65 in spinal cord tissue after SCI in mice (×200). (c) The effect of TMP on the expression of p65 protein in spinal cord tissue of mice after SCI was determined using Western blot; (d) the expression of TNFα and IL-6 in injured tissue was identified using ELISA. *$P < 0.05$ and **$P < 0.01$ compared with the control group; #$P < 0.05$ and ##$P < 0.01$ vs. the model group. BMS, Basso mouse scale.

and medium doses, showing a concentration-dependent manner. The reticular connections formed by neuronal processes are rich, and this is suggesting that different doses of TMP can promote nerve cell regeneration and maintain cell viability during acute phase. The mouse spinal cord clamp injury model was used to study the effect of TMP on functional recovery within a week of SCI. TMP could improve the BMS scores and the morphology of the spinal cord of mice within a week after SCI, which indicated that TMP could promote the recovery of motor function and morphology within that period of SCI occurring. GAP-43 was used to label the growth of neurons and neurites in spinal cord. TMP could promote the growth of neurons and neurites in injured spinal cord, suggesting that promoting the growth of neurons and neurites is one of the protective pathways of TMP on SCI in mice.

After the occurrence of SCI, excessive inflammatory cytokines such as TNFα and IL-6 are produced locally in the injury, which are important factors causing progressive spinal cord necrosis [13]. These inflammatory cytokines can mediate the accumulation of neutrophils, glial cells, and lymphocytes at the site of injury, destroy residual neurons, and form spinal cavity and glial scars [14]. Microglia accounts for approximately 20% of the glial cells in the brain. They constantly remove damaged nerves, plaques, and infectious substances in the central nervous system [15]. Using Iba1 labeled microglia, we found that mice in the model group were activated with microglia and the expression of them in the TMP treatment group were lower than those of the SCI group. In addition, the NF-kappa B signaling pathway is involved in the regulation of cellular inflammatory responses [16]. TMP can also reduce the activation of NF-kappa B signaling pathway and the production of inflammatory cytokines in spinal cord tissue, indicating that the inhibition of inflammation is one of the protective pathways of TMP on mouse SCI.

Undoubtedly, a large number of studies have shown that mushroom extract can enhance body immunity and is widely used in adjuvant treatment of tumors [17,18]. Our research focused only on acute infections, showing that polysaccharide extracts can also significantly inhibit inflammation and promote proliferation in the acute phase, while suggesting that polysaccharide has very strong immunomodulatory effects. However, the related mechanism begs for more diverging studies.

Conclusion

In conclusion, this study was able to find, the first time, that TMP can promote the regeneration of neuronal cells *in vivo* and *in vitro*, and can significantly inhibit the neuroinflammatory reaction, which provides scientific grounds for exploring the application of TMP in clinical treatment.

Acknowledgements

Ethics approval and consent to participate: All animal experiments and procedures were approved by the Institutional Animal Care and Use Committees of The Second Affiliated Hospital of Nanchang University, China (2HNC-2015632).

Conflicts of interest

There are no conflicts of interest.

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