Water treadmill training attenuates blood-spinal cord barrier disruption in rats by promoting angiogenesis and inhibiting matrix metalloproteinase-2/9 expression following spinal cord injury

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Research

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Abstract

Background: The permeability of blood-spinal cord barrier (BSCB) is mainly determined by the junction complex between adjacent endothelial cells, including tight junctions (TJ) and adhesion junctions (AJ), which can be severely damaged after spinal cord injury (SCI). Exercise training is a recognized method for the treatment of SCI. The destruction of the BSCB mediated by matrix metalloproteinase (MMP) leads to inflammation, neurotoxin production, and apoptosis of neurons. The failure of effective regeneration of new blood vessels is also an important reason for delayed recovery after SCI. We introduced water treadmill training (TT) for the first time, which can help SCI rats successfully exercise and measured the effect of TT in promoting recovery after SCI and possible mechanisms involved.

Methods: Sprague-Dawley (200–250g) rats were randomly divided into three groups: Sham operated, SCI, and SCI + TT. Animals were sacrificed 7 d or 14 d post-surgery. The degree of neurological deficit as assessed by the Basso-Beattie-Bresnahan motor rating scale, tissue water content, BSCB permeability, apoptosis, protein expression and ultrastructure of vascular endothelial cells were assessed, Western blot, immunofluorescence and transmission electron microscopy.

Results: Our experiments showed that TT reduced the permeability of BSCB and decreased tissue structural damage. TT improved functional recovery significantly when compared with the SCI group; TJ and AJ proteins expression increased significantly after TT training and training reduced apoptosis induced by SCI. TT can promote angiogenesis and the expression of MMP-2 and MMP-9 was significantly inhibited by TT.

Conclusions: In this study, the results indicate that TT promotes functional recovery for the following reasons: (1) TT protects residual BSCB structure from further damage; (2) it promotes vascular regeneration; and (3) it inhibits the expression of MMP-2/9 to mitigate BSCB damage.

Background

Spinal cord injury (SCI) places a heavy burden on society and the economy [1]. Currently, it can only be relieved by surgery, but it cannot be cured [2]. The endothelial cells (EC), basement membranes, pericytes and the terminal foot process of astrocytes constitute the blood spinal cord barrier (BSCB), which normally provides protection to the parenchyma [3, 4]. After SCI, the barrier integrity is compromised by disruption of interendothelial tight and adherens junctions as well as by overall mechanical damage to vessels. This results in the infiltration of immune cells and neurotoxic products, causing the death of nerve cells, and permanent neurological dysfunction [5–7]. Therefore, it is crucial to identify any intervention that can effectively prevent the destruction of the BSCB after SCI.

Matrix metalloproteinases (MMP) are a family of zinc-containing peptidases that degrade and reshape the extracellular matrix and other extracellular proteins, and play a key role in barrier function [7, 8]. Studies have shown that MMPs exacerbate the destruction of the BBB/BSCB under pathological conditions, including SCI [8, 9]. Two important members of the MMP superfamily are MMP-2 and -9 [10].

MMP-9 can induce BSCB-related protein degradation, and upregulation of MMP-2 can lead to the initial opening of the BBB/BSCB [11, 12], while blocking MMP-9 activity can protect vascular permeability [9]. The expression of MMP-2/9 has been detected up to 7 d after SCI [13]. Vascular endothelial growth factor (VEGF) is a highly specific vascular endothelial cell growth factor that promotes vascular, proliferation, and angiogenesis [14]. It is a necessary angiogenic factor for embryonic development and neovascularization under many pathological conditions [15]. Several molecules have been proved to be angiogenic in vivo, but only VEGF is considered to be a secretory mitogen specific to vascular-ECs. At present, VEGF is the most likely target for the study of vascular growth dynamics [16, 17].

Previous studies have mainly focused on drug treatment after SCI [18, 19]. However, this often has specific side effects, affecting the quality of life of patients. Therefore, it is important for patients with SCI to find a safe, effective and healthy treatment. Exercise training is a non-traumatic rehabilitation method that can promote the functional recovery of paralyzed muscles [20-23]. Also, most studies have focused on the effect of drug therapy on the neurovascular system after SCI [24, 25], ignoring the protection and functional recovery of the vascular system by exercise training. Based on this fact, starting from clinical practice and simulating the exercise rehabilitation of patients, our experimental team designed and invented a water treadmill for the first time which is suitable for rats to exercise after SCI. Compared with swimming exercise, water treadmill training (TT) can make rats passively and forcibly exercise at the initial stage, and can actively follow the treadmill in the middle and later stages of training. The ordinary treadmill is difficult for rats to perform rehabilitation exercise due to its high resistance. Our water treadmill equipment is a combination of swimming and treadmill training, and makes up for the above shortcomings. However, the protective effect of TT on SCI has not been reported in the literature; its effects on the BSCB are not clear. The aim of these experiments was to measure the protective effect of TT on the BSCB after SCI and investigate the mechanisms involved.

Method

Antibodies

Anti-MMP-9, anti-MMP-2, anti-Tubulin were purchased from Proteintech (Rosemont, IL, USA). Anti-VEGF, anti-BrdU, anti-Laminin and anti- β -Actin were purchased from Abcam (330 Cambridge Science Park, Cambridge, UK). Anti-p120-Catenin, anti- β -Catenin, anti-ZO-1, anti-Occludin, anti-Claudin-5 and anti-CD31 were purchased from Affinity (OH, USA). In situ cell death detection kit was purchased from Roche Molecular Biochemicals.

Animals

A total of 123 adult male Sprague-Dawley rats (200–250g) were purchased from Shanghai Laboratory Animal Center. The protocols were approved by the Animal Research Committee of Wenzhou Medical University. All animals were housed in a controlled environment and regularly fed with food and water. Rats were randomly divided into the following three groups: Sham (n=41; group S); SCI (n=41; group M); SCI + TT (n=41; group TM).

SCI Model

Rats were anaesthetized with 2% pentobarbital sodium (30 mg/kg), then were shaved and a 2 cm incision was made to expose the T10 segment of the spinal cord [26]. The exposed site was impacted with a New York University (NYU) Impactor (10 g × 20 cm) in all groups except group S. Lower limb trembling contractions and tail wagging showed that SCI was successful. Finally, the wound was sutured, disinfected with iodophor, and the rats allowed to recover from the anesthetic. During the following days, the bladder was emptied manually every morning and evening.

Water treadmill training

Our research group provided the initial design for the water treadmill (Wenzhou Xinglong Stainless Steel Co., LTD, Zhejiang, China) and submitted it for a patent. Rats were given adaptive training for three days before SCI. The surface of the water was adjusted to the xiphoid process of the sternum of the rat, the water temperature was set at 30 °C, and the speed of the water treadmill was maintained at 10-15 meters per minute. Training started one day after SCI, for rats in the training group(TM) began and lasted for 7 d or 14 d (5 min/round, 3 rounds in total, 5 min interval between rounds) (see Additional file 1).

Behavioral tests

Two independent examiners who were blinded to the treatment groups conducted the Basso-Beattie-Bresnahan (BBB) motor rating scale in an open field test. The BBB score has a total of 21 points, and the higher the score, the closer the animal is to normal[27].

Evaluation of BSCB permeability

Water content

At 7 d or 14 d after SCI, 2% sodium pentobarbital was intraperitoneally injected to anesthetize the animals ($n=5$), they were perfused cardiac perfusion with 0.9% normal saline and 0.5 cm of the T10 spinal cord segment was removed. The degree of edema in this segment was assessed by the dry and wet weight method as previously reported [28, 29].

Evans blue dye assays

According to previously reported methods [4, 8], rats ($n=5$) were injected with Evans blue (EB)dye to assess BSCB permeability (4 ml/kg) by tail vein at 7 d or 14 d after SCI, followed by 2% sodium pentobarbital anesthesia 2 hours later and 0.9% saline perfusion. Tissue containing T10 was soaked in N, N'-dimethylformamide at 50°C for 72 hours. The concentration of dye in the samples was determined based on a standard curve ($\mu\text{g/g}$). Tissues were cut into 15 μm thick sections with a freezing microtome at -20°C, and then the sections analysed. Quantitative analysis of data was performed with ImageJ software.

Haematoxylin-eosin (HE) Staining

Briefly, T9-T11 spinal cord tissue was removed from rats at 7 or 14 d after SCI, and stored in 4% paraformaldehyde for 24 hours (4°C). The spinal cord was immersed in a 0.1 m phosphate buffer solution and a 30% sucrose solution overnight (4°C). Successive sections (15 µm thick) were frozen and stored for subsequent HE staining.

Western Blot Analysis

Tissues containing T10 segments were put into a collection tube containing a mixture of phenylmethanesulfonyl fluoride (PMSF) and RIPA lysis buffer (100:1) and then were microfuged at 12,000 rpm for 5 min at 4°C. We extracted the supernatant and calculated the protein concentration with a BCA protein assay kit. The mixed solution was heated to 100°C for 10 min. After electrophoretic transfer to membranes, they were incubated with the appropriate primary (Anti-p120-Catenin, anti-β-Catenin, anti-ZO-1, anti-Occludin, anti-Claudin-5, anti-MMP-9, anti-MMP-2, anti-VEGF, anti-Tubulin, β-Actin) and secondary antibodies and the signal was digitally quantified.

Immunofluorescence Staining

After drying sections, they were washed 3 times for 15 minutes. They were treated with nonimmune goat serum for 1 hour and incubated first with primary antibodies against rabbit anti-occludin antibody (1:100), rabbit anti-claudin-5 antibody (1:100), rabbit anti-p120-Catenin (1:200), rabbit anti-β-Catenin (1:100), anti-CD31(1:100), anti-BrdU (1:100) and anti-Laminin (1:100) at 4°C, and then with Alexa Fluor 488 Affinipure goat anti-rabbit IgG (H+L) (1:200, Yeasen, China) for 50 min at room temperature. Phosphate-buffered saline (PBS) was used in place of the primary antibody in the negative control. We use an in situ Cell Death Detection Kit to detect apoptotic cells. The nuclei were colored by Hoechst or DAPI. The fluorescence signal was observed with laser confocal microscopy, five fields on each of three slides per animal were randomly selected for visualization and analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Transmission Electron Microscopy(TEM)

Tissue was quickly removed and cut into 1 mm³ pieces on ice and soaked in 2.5% glutaraldehyde. The tissue was fixed with a 1% oxidizing fixative for 1 h and stained with 1% uranyl acetate for 2 h, and then embedded after dehydration in gradient acetone solution. After semi-thin sectioning and toluidine blue staining, ultrathin sections were cut and observed using Hitachi TEM.

Statistical Analysis

All experimental data are expressed as the mean ± standard deviation. Kolmogorov-Smirnov (K-S) test was used for a normality test, $p > 0.05$ indicated a normal distribution; Levene's test was used for a test of homogeneity of variance, $p > 0.05$ was considered homogeneity of variance, and vice versa. When comparing the two groups, a t-test was used. One-way ANOVA and Dunnett's test were used to evaluate

the data when comparing more than two groups of components. SPSS 16 statistical software for statistical analysis, and $p < 0.05$ was considered statistically significant.

Results

TT reduces the permeability of BSCB after SCI

We used an impactor to generate a model of SCI (**Fig. 1a**). Subsequently EB staining and analysis of spinal cord water content was assessed to determine the degree of tissue damage. The results showed that the water content increased significantly after SCI, and TT could reduce the oedema caused by SCI at both 7d and 14d after SCI (**Fig. 1b-c**) (**M vs. S:** $P < 0.001$, $P_{14} < 0.001$; **TM vs. M:** $P < 0.01$, $P_{14} < 0.001$). The amount of EB in the tissue increased significantly after SCI compared with that of group S, suggesting BSCB leakage (**M vs. S:** $P < 0.001$, $P_{14} < 0.001$). After TT training, infiltration of EB was significantly reduced. (**Fig. 1d-e**) (**TM vs. M:** $P < 0.001$, $P_{14} < 0.001$). The fluorescence intensity of EB after SCI was much higher than that in the S group. However, the fluorescence level of the TM group was significantly lower than that of the M group (**Fig. 1f-g**) (**TM vs. M:** $P < 0.001$, $P_{14} < 0.001$). All these data indicate that TT can reduce BSCB disruption.

TT decreased tissue structure damage and improved functional recovery after SCI

At 7 d and 14 d after injury, histological differences at T9-T11 spinal cord levels were observed with HE staining (**Fig. 2a**). The arrangement of tissues in group S was normal. In group M the grey matter and white matter were destroyed to varying degrees, which was accompanied by the death of multiple neurons (**Fig. 2e**). In group TM the results were significantly better than in group M. The quantitative analysis of the cavity area showed the same results (**Fig. 2b**) (**TM vs. M: coronal section,** $P < 0.01$, $P_{14} < 0.05$; **sagittal section,** $P < 0.01$, $P_{14} < 0.01$). The functional recovery of the S, M and TM groups was evaluated by BBB motor rating at 6 h, 1 d, 3 d, 7 d, and 14 d after SCI (**Fig. 2c**). The BBB scores of the TM group were significantly higher than those of the M group at 7 d and 14 d after SCI (**TM vs. M:** $P < 0.05$, $P_{14} < 0.05$). We further tested the relationship between apoptosis with TT at 7 d after SCI (**Fig. 2d**). The number of apoptotic cells (Tunel staining) in the epicenter increased significantly after SCI. Compared with the M group, the number of apoptotic cells in the TM group were significantly decreased (**M vs. S:** $P < 0.001$; **TM vs. M:** $P < 0.01$). These results show that TT can significantly improve functional recovery and tissue preservation.

TT prevents the loss of tight junction(TJ) and adhesion junction (AJ) proteins

To further determine the effect of TT on interendothelial junctions, AJ proteins (β -Catenin, p120-Catenin), TJ protein (Claudin-5, and Occludin) and ZO-1, were estimated by Western blot. According to the results (**Fig. 3a-c**), the expression of TJ and AJ proteins decreased to some extent after SCI compared to the levels of the control (**M vs. S: β -Catenin, p120-Catenin, Claudin-5, Occludin ZO-1, $P < 0.001$**). However, compared to the M group rats treated with TT showed higher TJ and AJ protein expression at 7 d (**Fig. 3b**)

(TM vs. M: β -Catenin, $P < 0.05$; p120-Catenin, $P < 0.01$; Claudin-5, $P < 0.001$; Occludin, $P < 0.01$; ZO-1, $P < 0.001$). Using Occludin/CD31/Hoechst (Fig. 3c); Section showing Claudin-5/CD31/Hoechst (Fig. 3d); p120-Catenin /CD31/Hoechst (Fig. 4a) and β -Catenin /CD31/Hoechst (Fig. 4b) staining to show the distribution of BSCB proteins after SCI and indicate that TT can reduce the degradation of Claudin-5/Occludin/p120-Catenin/ β -Catenin around the epicenter. These results suggest that TT can prevent the loss of TJ and AJ proteins after SCI.

TT promotes angiogenesis after SCI

We investigated the expression of VEGF protein in the epicenter at 7 d after SCI (Fig. 5a) after SCI. Compared with expression in group S, VEGF increased significantly after SCI (M vs. S: $P < 0.05$). Furthermore, TT significantly upregulated the VEGF expression compared to SCI alone (Fig. 5b-c) (TM vs. M: $P < 0.05$). Blood vessels co-labeled with 5-bromo-2-deoxyuridine (BrdU) and laminin in the ischemic penumbra (The light shaded part in Fig. 5a) were quantified. As shown in Fig. 5d and e, angiogenesis in the M group was significantly higher than it was in the S group at 7 d after injury (M vs. S: $P < 0.001$). Additionally, the neovascularization density of the TM group was increased compared with that in the M group, suggesting that TT can effectively promote angiogenesis (TM vs. M: $P < 0.001$).

TT inhibits the expression of MMP-2/9 after SCI

The expression level of MMP-2/9 protein was detected by Western blot. The results showed that TT could significantly inhibit the upregulation of MMP-2/9 after SCI (Fig. 6a-c) (TM vs. M: MMP-2, $P < 0.001$; MMP-9, $P < 0.01$). In addition, to determine the defects of vascular ECs, we analysed them by TEM. In group S, the vascular ECs of spinal cord showed a tight connection with a rivet-like structure, and a series of electron dense bands appeared between the plasma membranes, forming a relatively closed vascular lumen. The intercellular TJ was partially opened after SCI (M group) and compared with group S in the same period, the structure of the junction was disordered, the electron-dense zone was lighter, the ECs were atrophied and scar tissue was formed around the blood vessels (M vs. S: $P \neq 0.001$, $P_{14} \neq 0.001$). On the contrary, the rats in the TM group had more electron dense bands, and the ultrastructural changes were intermediate between S and M (Fig. 6e) (TM vs. M: $P \neq 0.001$, $P_{14} \neq 0.001$).

Discussion

In this study, our data indicate that TT can protect the integrity of BSCB and prevent further edema of spinal cord after impact injury. After TT training, the histological structure and motor function of the spinal cord was significantly improved. We showed that TT can promote angiogenesis, reduce apoptosis and inhibit the expression of MMP-2/9. Therefore, the vascular protection of the BSCB by TT may occur through the following mechanisms: (1) TT protects residual BSCB structure from further damage; (2) it promotes vascular regeneration; and (3) it inhibits the expression of MMP-2/9 to mitigate BSCB damage.

Emerging evidence indicates that exercise therapy can promote recovery after SCI [30-33]. It is well known that normal exercise training cannot be carried out in rats after SCI. We introduced water treadmill

training(TT), which simulates the clinical exercise treatment and can help rats successfully train on the treadmill in the early stage of SCI. TT for patients is mainly used in rehabilitation for motor system diseases [34, 35]. TT can reduce the resistance of forward movement, allowing patients to walk or run with a normal gait [36]. Additionally, the depth and speed of the TT can be adjusted, which facilitates the control of exercise intensity [36].

The mechanisms for mitigating damage after SCI were studied for the first time in our experiments using rats. Trauma, infection, tumor growth or obstruction of blood supply can cause edema of the central nervous system (CNS). Cytotoxicity and vasogenic edema are interdependent factors involved in the development of CNS edema. Prolongation of cytotoxic edema leads to vasogenic edema, and vice versa[37, 38]. Cytotoxic edema refers to the accumulation of water in intact cells which occurs when an anoxic state leads to the loss of energy-dependent solute homeostasis [39]. Vasogenic edema is the accumulation of fluid in the extracellular space around damaged BBB/BSCB [40]. The integrity of the BSCB is essential for the spinal cord to maintain normal function [41]. The destruction of the BSCB after SCI, leads to increased permeability, causing secondary damage [18, 42]. In our study, through the horizontal comparison of data of each group in the same time frame, we found that the degree of edema in the SCI group increased significantly compared to that of sham-operated group, accompanied by lower BBB motor scores, severe tissue structure damage and a large number of necrotic cells. However, these conditions were significantly improved after TT training and this may be related to a protective effect of TT on the BSCB. By longitudinal comparison of the BBB scores 7 d and 14 d after SCI, it was found that there was some recovery with time (Fig 2c). The partial recovery of these functions may be related to both the preservation of BSCB function and the formation of new blood vessels.

The BSCB protects the spinal cord by restricting the entry of plasma components and blood cells [9]. Following SCI, the destruction of the BSCB leads to increased microvascular permeability, inflammatory reaction, tissue edema, and neurotoxic products [43]. Tight, adhesion, and gap junctions connect the endothelial cells lining the microvessels in the spinal cord[3, 44].The dense network of TJs and AJs is destroyed after SCI, resulting in a decrease in the expression of TJ and AJ proteins. Our results show that the expression of p120-Catenin, β -Catenin, ZO-1, Occludin, and Claudin-5 were greatly reduced after SCI. However, their expression was significantly improved in the TT-treated spinal cord (Figs 3, 4). This shows that the residual BSCB structure is protected by TT.

In order to determine the protective mechanism of TT, we have carried out more in-depth research. Vascular endothelial growth factor (VEGF) stimulates the proliferation and survival of endothelial cells, the formation of vascular structures, nitric-oxide dependent vasodilatation and vascular leakage [17]. It has been reported that VEGF reaches a peak at 3 d, and there was still a large amount of expression at 7 d after SCI [45]. In addition, ischemia and injury can induce angiogenesis, which will provide oxygen and nutrition to the ischemic or diseased site, thus improving tissue repair and remodeling [46-48]. We used laminin as a vascular marker and BrdU as a proliferation marker [49]. The results showed that TT could promote angiogenesis after SCI, as shown by detecting the protein expression of VEGF. Quantitative analysis of neovascularization 7 d after SCI with co-labelling also showed that the number of

BrdU⁺/Laminin⁺ cells increased significantly after TT treatment in rats with SCI, which will may help to maintain the stability of BSCB (Fig 5).

The activation of MMP-2/9 after SCI plays an essential role in the destruction of the BBB/BSCB [9, 10] and the expression of MMP-2/9 can aggravate the damage[50]. In this study, 7 d after SCI, the expression of MMP-2/9 was up-regulated (Fig 6 a-c), with the potential to further aggravate the damage to BSCB. We were surprised to find that the expression of MMP-2/9 decreased significantly after TT training. Although we have not proved how TT down-regulates the expression of MMP-2/9, we believe that TT can effectively prevent the destruction of the BSCB, and part of the mechanism may be to inhibit the expression of MMP-2/9 after SCI.

As far as we know, this is the first time that TT has been applied to the treatment of SCI in rats. We found that TT can enhance the expression of TJ and AJ proteins after SCI. We also found that TT could promote angiogenesis and inhibit the expression of MMP-2/9, which may be an important mechanism for TT to maintain the stability of BSCB. These experimental results provide a better understanding of the possible mechanisms for TT in the treatment of SCI and provide a reliable basis for TT application in the future.

We acknowledge the limitations of our studies. In this experiment, we only used male rats, ignoring the possible differences caused by sex. Due to the limitation of experimental conditions, we could not observe changes at the functional level through electrophysiological techniques to understand the role of TT. The training period was relatively short and the specific mechanism by which TT inhibits the expression of MMP-2/9 remains to be further studied.

Conclusion

TT participates in protection of the BSCB after SCI by protecting residual BSCB structure from further damage. Furthermore, TT also promotes vascular regeneration and additionally, inhibits the expression of MMP-2/9 to mitigate BSCB damage (**Fig. 7**). However, TT-induced BSCB protection after SCI is a complex process that involves many key factors and remains to be fully elucidated. As mentioned in the limitations of our discussion, our next research direction will be an in-depth study of the upstream molecular mechanisms of MMP-2/9 and VEGF, using techniques such as in vivo electrophysiology to study the spinal cord at the functional level.

Declarations

Ethics approval and consent to participate

The protocols were approved by the Animal Research Committee of Wenzhou Medical University

Consent for publication

Not applicable

Availability of data and materials

The datasets supporting the conclusions of this article are available from the corresponding author on reasonable request

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YXW designed the experiments and wrote the first manuscript; XQF and LSC was responsible for data acquisition and analysis; YXL, ZKC, YJJ and CXL conceived the experiments; TWZ and YGH provided guidance; JSH supervised the whole process of the experiment, and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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Abbreviations

SCI, Spinal cord injury; TT, Water treadmill training; BSCB, Blood-spinal cord barrier; TJ, tight junction; AJ, adhesion junction; MMP, Matrix metalloproteinase; BBB, Basso-Beattie-Bresnahan motor scale; HE, haematoxylin-eosin; EB, Evans blue; WB, Western blot; TEM, Transmission electron microscopy; VEGF, Vascular endothelial growth factor; PBS, Phosphate-buffered saline; BrdU, 5-bromo-2-deoxyuridine; EC, Endothelial cell; CD31: Platelet endothelial cell adhesion molecule-1; CNS, Central nervous system.

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Figures

Figure 1

Treadmill training reduced the influx of water and Evans blue after SCI. (a) SCI model was performed by NYU Impactor (10g × 20cm). (b, e) Representative views of excised T10 cord and (c) Represent views of EB dye permeabilized into excised spinal cord after SCI. (d) Representative fluorescent images of Evans Blue Dye extravasation (scale bar = 200 μm). (e) Quantification of water content in S (Sham), M (SCI) and TM (SCI + TT) groups, columns represent mean ± SD, n=5. (f) Quantification of Evan's Blue in the tissue (ug/g), n =5. (g) Quantification of the fluorescence intensity, n=5. #p < 0.05 as M group versus S group, *p < 0.05 as TM group versus M group. (#p, *p < 0.05; ##p, **p < 0.01; ###p, ***p < 0.001)

Figure 2

TT decreased tissue structure damage and improved functional recovery after SCI. (a) HE stained sections at 7 d and 14 d after SCI. Scale bars are 500 μ m. (b) Quantification of the size of cavity area, columns represent mean $\pm$ SD, n=5. (c) BBB scores in S, M, TM groups. (d) TUNEL staining in the epicenter at 7 d after SCI. Scale bars are 500 μ m and 100 μ m. (e) Quantitative estimation of positive (apoptotic) cells. Columns represent mean $\pm$ SD, n= 5. (#p, *p < 0.05; ##p, **p < 0.01; ###p, ***p < 0.001)

Figure 3

TT prevents the loss of tight and adherens junction proteins. Representative western blots (a) and quantification (b) of TJ and AJ protein in each group, columns represent mean $\pm$ SD, n=5. (c) Double staining of Occludin/CD31/Hoechst and (d) Claudin-5/CD31/Hoechst. Red: Occludin /Claudin-5; Green: CD31; Blue: Hoechst. Scale bar, 50 μ m. (#p, *p < 0.05; ##p, **p < 0.01; ###p, ***p < 0.001)

Figure 4

(a) Double staining of p120-Catenin /CD31/Hoechst and (b) β -Catenin/CD31/Hoechst. Red: p120-Catenin/ β -Catenin; Green: CD31; Blue: Hoechst. Scale bar, 20 μ m.

Figure 5

TT promotes angiogenesis after SCI. (a) Schematic diagram of the regions sampled after SCI (b) Representative western blots and (c) quantification of VEGF/Tubulin, columns represent mean $\pm$ SD, n=5. (d) Quantification data of number of Brdu and Laminin co-stained cells, columns represent mean $\pm$ SD, n=5. (e) Double staining of Laminin(green)/Brdu (red) of sections from the spinal cord in each group rats. Scale bars are 20 μ m. (#p, *p < 0.05; ##p, **p < 0.01; ###p, ***p < 0.001)

Figure 6

TT inhibits the increased expression of MMP-2 and MMP-9 after SCI. (a-c) Represent western blots and quantification of MMP-2/9 7 d after SCI in each group, columns represent mean $\pm$ SD, n=5. (d) Quantification of percentage of broken junctions, n =3. (e) TEM showed the EC-EC junctions in S, M, TM. Arrows indicate TJ electron dense band and the open TJ, scale bars are 1.0 μ m. (#p, *p < 0.05; ##p, **p < 0.01; ###p, ***p < 0.001)