Sodium Tanshinone IIA Sulfonate Promotes Spinal Cord Injury Repair by Inhibiting BSCB Disruption In Vitro and In Vivo

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Research

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Abstract

Background: Spinal cord injury (SCI) leads to microvascular damage and the destruction of blood spinal cord barrier (BSCB), which progresses to secondary injuries like apoptosis and necrosis of neurons and glia, culminating in permanent neurological deficits. BSCB restoration is the primary goal of SCI therapy, although very few drugs can repair the damaged barrier structure and permeability. Sodium tanshinone IIA sulfonate (STS) is commonly used to treat cardiovascular disease. We found that STS restored BSCB integrity and promoted microvessel recovery 7 days after SCI in a mouse model. However, the therapeutic effects of STS on damaged BSCB in the early stage of SCI remained uncertain.

Methods: we exposed spinal cord microvascular endothelial cells (SCMECs) to H₂O₂ and treated them with different doses of STS. The mice received intraperitoneal injection of STS after SCI *in vivo* model. Spinal cord tissue was taken 1 and 3d post-SCI. HE, Nissl staining, BSCB permeability, and the expression levels of tight junction (TJ) and adherens junction (AJ), MMP2, MMP9, NeuN, and C-caspase-3 were analyzed.

Results: In addition to protecting the cells from H₂O₂-induced apoptosis, STS also reduced cellular permeability. In the *in vivo* model of SCI as well, STS reduced BSCB permeability, relieved tissue edema and hemorrhage, suppressed MMPs activation and prevented TJ and AJ the loss of proteins.

Conclusions: Our findings indicate that STS treatment promotes SCI recovery, and should be investigated further as a drug candidate against traumatic SCI.

1. Background

Spinal cord injury (SCI) can cause permanent functional defects in the limbs through primary and secondary injuries[1, 2]. Primary injury is the result of the initial trauma that leads to structural disturbance, whereas secondary injuries consist of inflammation, apoptosis, axonotmesis and formation of glial scars, which eventually culminate to progressive secondary hemorrhage in the central nervous system (CNS)[3, 4]. The blood spinal cord barrier (BSCB) has a similar function to that of the blood-brain barrier (BBB)[5, 6]. It comprises of specialized endothelial cells that are attached via tight junction-associated (TJ) and adherens junction-associated (AJ) protein complexes, and enclosed by the end feet of astrocytes[7]. The BSCB blocks the entry of circulating toxins and pathogens into the CNS[8], and SCI-induced damage to the BSCB allows influx of endogenous inflammatory factors and pathogens into the spinal cord tissues, which further aggravates SCI[9, 10]. Therefore, BSCB integrity is crucial for motor function recovery after SCI.

The spinal cord microvascular endothelial cells (SCMECs) are essential for maintaining the structural integrity and normal physiological function of the BSCB[11–13]. The impermeability of the BSCB depends on the TJ and AJ proteins that are expressed by the SCMECs, and form a barrier which keeps solutes and cells from entering into the inner environment[14]. The activation of matrix metalloproteases (MMPs), especially MMP-9 and MMP-2, is the key pathological basis of BSCB disruption[15–17]. MMP9

causes proteolytic degradation of the BBB, and blocking MMP9 activity can attenuate vascular permeability[18]. In fact, restoring BSCB integrity is the primary therapeutic approach for SCI.

Tanshinone IIA is a major bioactive component of *Salvia miltiorrhiza*, which is used in several traditional Chinese medicine formulations[19]. However, the poor water solubility and low bioavailability of Tanshinone IIA limits its clinical applications[20, 21]. Sodium tanshinone IIA sulfonate (STS) has greater water solubility and can effectively promote blood circulation, remove blood stasis, and activate channels and collateral[22, 23]. STS is widely used for treating coronary artery disease, angina pectoris and myocardial infarction[24]. Studies show that STS can not only exert neuroprotective effects but also repair the BBB in stroke patients[25–27]. Our preliminary results indicated that STS restored BSCB function and rescued microvessels 7 days after SCI[28]. However, the therapeutic effects of STS in the early stages post-SCI remain to be elucidated.

To this end, we analyzed the effects of STS on H₂O₂-treated SCMECs, and found that it restored BSCB integrity, prevented loss of TJ and AJ proteins, inhibited MMPs activation, and exerted neuroprotective effects in the early stage of SCI.

2. Materials & Methods

2.1. Reagents

Endothelial growth medium 2 was purchased from LONZA, (California, USA). DMEM, fetal bovine serum (FBS) and trypsin were purchased from Gibco (New York, USA). Cell counting kit-8 (CCK-8) and BCA assay kit were bought from Beyotime Biotechnology (Shanghai, China). Evans Blue and fluorescein isothiocyanate (FITC)-dextran were purchased from Aladdin (Shanghai, China), and STS (purity > 99% using HPLC) from Chengdu Herbpurify Co. Ltd. (Chengdu, China).

2.2. SCMECs culture and viability test

SCMECs were maintained in EGM-2 supplemented with 5% FBS and antibiotics (1% penicillin and 1% streptomycin) under 5% CO₂ at 37 °C. The medium was replaced every 2 days. The cells were cultured in the presence of 25–400 µM H₂O₂ for 12 h, and 10 µl CCK-8 reagent was added to each well. After incubating for 2 h, the absorbance at 450 nm was detected using a microplate reader (Bio Red, USA) to determine the proportion of viable cells. The cells were exposed to the suitable H₂O₂ concentration, followed by 0, 5, 10 and 20 µM STS. Cell viability was evaluated by the CCK-8 assay as described. All experiments were performed in triplicate.

2.3. Cell permeability measurement

The STS-treated cells were seeded into Transwell chambers containing 0.4 µm pore size membrane in a 24-well plate at the density of 2×10^4 cells/200 µl/well. The lower chambers were filled with 500 µl medium, and the cells were cultured for 24 h. Once the cells adhered, the medium was replaced with

sugar-free medium and the cells were incubated for 12 h with 200 μM H_2O_2 in the dark. To each well, 4 μl FITC-dextran (50 $\mu\text{g}/\mu\text{l}$) was added and the cells were incubated for 4 h. Finally, 200 μl medium was aspirated from each lower chamber, and fluorescence intensity (excitation wavelength 485 nm, emission wavelength 520 nm) was measured using a microplate reader.

2.4. Establishment of SCI model

Ten weeks old male C57BL/6 mice were provided by Guangdong Medical Laboratory Animal Center (Foshan, China, Certificate No.44005800011215). After anesthetizing with 1% (w/v) pentobarbital sodium (40 mg/kg), the mice were subjected to laminectomy at the T9-T10 level to expose the spinal cord without damaging the dura. A contusion was made using a pneumatic impact device as described by Allen et al. For the sham operated controls, T10 laminectomy was performed without impact injury. All animals were treated with antibiotics to prevent local and urinary infection. Bladder massage was performed to facilitate defecation post-surgery if needed. The STS-treated group received intraperitoneal injection of 20 mg/kg STS 1 h after SCI and once daily thereafter, whereas the untreated model and sham-operated groups were injected with the same volume of 0.9% sodium chloride.

2.5 Evans blue assay

The permeability of the BSCB was assessed by the Evans blue dye extravasation method[29]. Briefly, the mice were injected intravenously with 150 μl of 2% Evans blue dye in saline on days 1 and 3 post-SCI. The mice were anesthetized 3 h later and euthanized by intra-cardiac perfusion with PBS and 4% paraformaldehyde (PFA). The spinal cords were dissected, and cut into 30 μm cross sectional slices using an Ultra-Thin Semiautomatic Microtome (Leica RM2255, Germany). The fluorescence intensity was observed under a microscope.

2.6 Water content of spinal cord

Spinal cords were extracted as described from five random mice per group on days 1 and 3 post-SCI. One centimeter-long pieces were cut from the injured site and weighed. The tissues were then dried in an oven at 60°C for 48 h, and the dehydrated specimens were weighed. The water content of the spinal cord was calculated as (wet weight - dry weight)/wet weight.

2.7 Tissue preparation

Mice were anesthetized and perfused by cardiac puncture with 0.1M PBS and 4% PFA on days 1 and 3 post-SCI. The spinal cords were removed, and some segments were embedded in OTC and cut into 10- μm -thick frozen sections, and others were fixed with 10% formaldehyde, embedded with paraffin and cut into 5- μm -thick sections. For molecular assays, the mice (n = 5/group per time point) were perfused with 0.1M PBS and 10 mm spinal cord segments including the lesion site were isolated and frozen at - 80 °C.

2.8 Western blotting

The protein content of cell lysates and tissue homogenates were measured using the BCA assay kit. Equivalent amounts of protein per sample were separated in 10% SDS-PA gels, and transferred to PVDF

membranes (Millipore). After blocking with 5% skim milk in 0.5% Tris-buffered saline Tween (TBST) for 1 h at room temperature, the membranes were incubated overnight with rabbit anti- β -catenin (1:1000; Abcam), rabbit anti-occludin (1:1000; Abcam), rabbit anti-Bcl2 (1:1000; Abcam), rabbit anti-Bax (1:1000; Cell Signaling Technology), mouse anti-c-caspase-3 (1:1000; Cell Signaling Technology), rabbit anti-MMP-2 (1:1000; Abcam), rabbit anti-MMP-9 (1:1000; Abcam) and rabbit anti-GAPDH (1:1000; Cell Signaling Technology) primary antibodies at 4 °C. The bands were then probed with horseradish peroxidase (HRP)-conjugated rabbit/mouse secondary antibodies (1:1000; Abcam). The experiment was repeated thrice.

2.9 Histopathology and immunofluorescence

Paraffin-embedded sections were stained with hematoxylin and eosin (HE) and Nissl's dye according to standard protocols. Tissue cryosections and cells grown on glass coverslips were washed thrice with PBS, fixed with 4% PFA, permeabilized with 0.3% Triton X-100 and blocked with 10% goat serum. After incubating overnight with primary antibodies against occluding (1:200, Abcam), NeuN (1:200, Abcam) and C-caspase3 (1:200, Abcam) at 4 °C, the samples were washed thrice with PBS, and probed with AlexaFluor 488 donkey anti-rabbit/mouse secondary antibodies (1:200) for 1 h at room temperature. The tissue sections or cells were rinsed again with PBS, counterstained with DAPI for 5 min, rinsed, and sealed with a coverslip. The slides were observed under a fluorescence microscope.

2.10 Statistical analysis

SPSS 24.0 software was used for statistical analysis. The data were expressed as mean $\pm$ standard deviation (SD), and compared using unpaired Student's T-Test. P-values < 0.05 were considered statistically significant.

3. Results

3.1. STS protects SCMECs from H₂O₂-induced damage and restores barrier function

The chemical structure of STS is shown in Fig. 1A. The SCMECs were treated with varying concentrations of H₂O₂, and 200 μ M was selected for subsequent experiments. As shown in Fig. 1B, STS increased the viability of the H₂O₂-treated cells in a dose-dependent manner. Furthermore, STS treatment also decreased the permeability of the SCMECs following H₂O₂ exposure, as indicated by reduced uptake of FITC-dextran (Fig. 1C). The TJ and AJ proteins between SCMECs maintain the barrier function of BSCB, and their loss is directly related to increased permeability[30]. As shown in Fig. 1E-G, H₂O₂ treatment significantly decreased the levels of occludin and β -catenin in SCMECs, and both were restored by STS in a dose-dependent manner. Overactivation of the MMPs in the extracellular matrix of injured spinal cord tissues degrades the BSCB and leads to a massive influx of white blood cells, which further aggravates the secondary injury[31]. Consistent with this, both MMP2 and MMP9 levels increased significantly after H₂O₂ treatment, and were reduced to near baseline levels by STS (Fig. 1H-K). Taken together, STS can

reverse the H_2O_2 -induced hyperpermeability of SCMECs by blocking MMP activation and maintaining the TJ and AJ proteins. Furthermore, 200 μM H_2O_2 markedly increased the expression of pro-apoptotic proteins such as Bax and cleaved Caspase-3 but reduced that of Bcl-2. In contrast, STS reversed the apoptotic program in the H_2O_2 -treated cells in a dose-dependent manner (Fig. 2A-C). Consistent with this, TUNEL staining confirmed that STS reduced the percentage of apoptotic SCMECs following H_2O_2 exposure (Fig. 2D).

3.2 STS maintains BSCB integrity after SCI by preventing MMP activation and loss of junction proteins

BSCB disruption following SCI was evaluated by Evans blue extravasation and intraspinal hemorrhage assay. As shown in Fig. 3A-B, the injured spinal cords showed increased Evans blue dye extravasation on days 1 and 3 post-surgery compared to the sham-operated controls, indicating that SCI triggered BSCB disruption. However, STS treatment sharply reduced the extent of Evans blue dye extravasation on both time points, which suggested a protective effect of STS on the BSCB. The total amount of Evans blue in the spinal cord tissues and its fluorescence intensity in the sham-operated, vehicle-treated and STS-treated groups were also quantified and were consistent with the images (Fig. 3C-D). MMP-2 and MMP-9 play a key role in BSCB disruption post SCI by degrading the supporting ECM. As shown in Fig. 3E-G, MMP-2 and MMP-9 protein levels markedly increased on days 1 and 3 after injury compared to the sham-operated control, and were downregulated by STS. In addition, the in-situ expression of occludin and β -catenin were also low in the injured spinal cord, and restored by STS treatment (Fig. 5A-D). Thus, STS protects the BSCB after SCI by preventing loss of junction proteins and inhibiting MMP activity.

3.3 STS reduced secondary hemorrhage and edema after SCI

Surface bleeding was observed in the injured spinal cords after 1 and 3 days of contusion, and was markedly alleviated in the STS-treated group versus the vehicle-treated control (Fig. 4A-B). HE staining of the spinal cord lesions indicated few inflammatory cells in the sham-operated group, along with normal tissue structure, proper cellular arrangement in each layer, and absence of any swelling or bleeding. In contrast, the injured spinal cords had extensive hemorrhagic areas, swollen and disrupted nerve cells, massive infiltration of inflammatory cells, widened interstitial spaces and severe edema. STS treatment not only reduced the hemorrhagic areas but also alleviated the other pathological changes (Fig. 4C). Furthermore, the water content in the spinal cord tissues also increased significantly after injury compared to the sham-operated controls, and was decreased by STS (Fig. 4D).

3.4 STS reduced neuronal apoptosis in the spinal cord lesions

Any injury to the spinal cord triggers apoptosis around the trauma epicenter[32–34]. Consistent with this, induction of SCI in our model significantly increased the levels of Bax protein and downregulated that of Bcl-2 on days 1 and 3 d post-injury. In contrast, STS treatment enhanced Bcl-2 expression and inhibited

Bax (Fig. 6A-B). TUNEL staining also showed a marked increase in the number of apoptotic cells after injury, which was alleviated by STS treatment (Fig. 6C-D). Furthermore, co-staining for NeuN (green) and cleaved caspase-3 (red) indicated that the number of apoptotic neurons in particular increased after SCI, whereas STS intervention led to a decline in neuronal apoptosis (Fig. 7A). The total cleaved caspase-3 protein level also decreased significantly in the injured spinal cords from the STS-treated animals (Fig. 7B-C). Consistent with the above, SCI led to a significant loss of motor neurons as revealed by Nissl staining, which was recovered by STS (Fig. 7D). Taken together, STS can alleviate the pathological effects of SCI by reversing neuronal loss.

4. Discussion

Spinal cord injury (SCI) often leads to permanent loss of limb function defect due to disruption of the BSCB and the ensuing secondary injuries[35, 36]. The BSCB is a semi-permeable interface of blood vessels that surrounds the spinal cord, and consists of the basement membrane, endothelial cells, pericytes, and astrocytes endplate foot processes[37]. The BSCB protects the spinal cord microenvironment from both endogenous and exogenous factors[38, 39]. Functional impairment of the BSCB is a key pathological event in SCI, and leads to edema and secondary nerve injury. The disrupted BSCB allows blood flow into the spinal cord, which increases the risk of infection and inflammation. In addition, the infiltrating blood cells may produce neurotoxic substances that damage the neurons and synapses, eventually leading to glial cell and neuronal apoptosis. BSCB dysfunction caused by endovascular treatment increase patient mortality, while its functional recovery can significantly reduce secondary nerve injury after SCI. Sarah et al. showed that the BSCB is damaged within an hour of SCI, and remains unrepaired for 5 days. Peak BSCB permeability is usually observed 24 hours after SCI, and early functional recovery of BSCB can significantly alleviate secondary injury[40].

STS is a water-soluble derivative of Tanshinone IIA, one of the most pharmacologically active monomers extracted from *Salvia miltiorrhiza*. Both STS and Tanshinone IIA can induce vasodilation, inhibit inflammatory response and prevent atherosclerosis and cardiac injury[41–43]. Jun Cheng et al.[44] found that STS prevented LPS-induced proinflammatory cytokine expression and secretion by inhibiting the NF- κ B signaling pathway in HUVECs. However, the potential therapeutic effect of STS on BSCB disruption in the early stages of SCI have not been demonstrated so far. We established an *in vitro* model of SCI-related BSCB disruption by exposing the SCMECs to H₂O₂, and evaluated the effects of STS on the viability and permeability of SCMECs. STS not only decreased the uptake of FITC-dextran but also protected the SCMECs from H₂O₂-induced toxicity.

MMP-9 and MMP-2 mediate BSCB breakdown during SCI by degrading the AJ and TJ proteins, and suppressing MMP-9 activity inhibits vascular permeability[45]. MMP-9 activity increases rapidly within the first 24 h post-injury and its peak levels correlate with maximum BSCB disruption[46]. In addition, increased level of MMP-2 is also related to the degree of SCI[47]. STS treatment reversed the H₂O₂-induced increase in MMPs and reduction in occludin and β -catenin synthesis in the SCMECs, and also inhibited the apoptosis program. Taken together, STS can maintain the functional integrity of SCMECs

under pathological stimuli. In the *in vivo* model of SCI as well, 20 mg/kg STS reduced the synthesis of both MMPs and increased the levels of junction proteins. The permeability of the BSCB was assessed using Evans blue, which has a high affinity for serum albumin and therefore cannot pass through the BSCB under normal conditions. However, a damaged barrier allows the tracer dye to stain the spinal cord tissues. We found that SCI induction significantly increased Evan blue extravasation, which was reduced by STS treatment. Thus, STS can preserve the structural integrity of the BSCB after SCI by inhibiting the proteolytic action of MMPs and promoting synthesis of TJ and AJ proteins.

SCI triggers extensive neuronal apoptosis and vascular damage, which leads to the loss of motor neurons and a reduction in the number of blood vessels[48–50]. In addition, the progressive failure of capillary function leads to hemorrhaging[51]. The number of apoptotic neurons in the injured spinal cord declined sharply after STS administration, which also reduced capillary fragmentation and internal bleeding compared to the vehicle-treated control. To summarize, STS reduces the permeability of damaged BSCB in the early stage of SCI, alleviates secondary injuries and hemorrhage, and accelerates functional recovery.

5. Conclusion

STS prevents BSCB disruption and hemorrhage in the early stages of SCI by inhibiting MMPs activation and promoting synthesis of TJ and AJ proteins. Furthermore, STS treatment protected the motor neurons from apoptosis and accelerated functional recovery. This is the first report to demonstrate the therapeutic efficacy of STS in the early stages of SCI, and should be investigated further as a candidate drug against traumatic SCI.

Abbreviations

SCI: Spinal cord injury

BSCB : Blood spinal cord barrier

STS: Sodium tanshinone IIA sulfonate

SCMECs : Spinal cord microvascular endothelial cells

TJ : Tight junction

AJ : Adherens junction

BBB : Blood-brain barrier

MMPs : Matrix metalloproteases

DMEM☐Dulbecco's modified eagle medium

FBS: Fetal bovine serum

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee of Guangzhou University of Chinese Medicine and performed according to the “NIH Guide for the Care and Use of Laboratory Animals”. No work on humans was included in this manuscript.

Availability of data and materials

The primary data for this study is available from the authors on direct request.

Consent for publication

All authors have agreed to publish this article.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DL and XL contributed to conception and design. DL and XL analyzed the data. DL drafted the manuscript. XL, JZ, YH, and JL contributed critical revision of the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

STS prevents loss of TJ and AJ proteins and the expression of MMP-2/MMP-9 in H₂O₂-treated SCMECs. (A) The molecular structure of STS. (B) Viability of SCMECs treated with different concentrations of H₂O₂. (C) The percentage of surviving SCMECs in the indicated groups. (D) Representative fluorescence images showing FITC-dextran uptake in the differentially treated SCMECs. (E-G) Representative immunoblot showing expression levels of β-catenin and occludin in the different groups. (H-J) Representative immunoblot showing expression levels of MMP-2 and MMP-9 in SCMECs treated with or without STS under H₂O₂. Data indicates mean ± SEM of three independent experiments; *P < 0.05, **P < 0.01 and ***p<0.001 by one-way ANOVA.

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Figure 2

STS reduces apoptosis in H₂O₂-treated SCMECs. (A) Representative immunoblot showing expression levels of Bcl-2, Bax and C-caspase 3 in the indicated groups. (B, C) Quantification of protein bands with GAPDH as the loading control. (D) Representative immunofluorescence images of TUNEL (green)-stained SCMECs treated as indicated. Scale bar = 25 μm. Data indicates mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01 and ***p<0.001 by one-way ANOVA.

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Figure 3

STS inhibits BSCB disruption and MMP-2/MMP-9 expression after SCI. (A) Representative images of whole spinal cords taken on days 1 and 3 post-SCI induction showing Evans Blue staining. Scale bar = 1 mm. (B) Representative confocal images of the transverse spinal cord sections of days 1 and 3 showing Evans Blue extravasation. Scale bar = 500µm. (C) Quantification of Evans Blue content in the spinal cord (µg/g) of the indicated groups. (D) Quantification of the fluorescence intensity of Evans Blue in each group at 1 and 3 days after SCI. (E) Representative immunoblots showing expression levels of MMP-2 and MMP-9 at 1 and 3 days after SCI. GAPDH was used as the control. (F, G) Quantification of relative MMP-2 and MMP-9 protein levels. Data indicates mean ± SEM (n = 5). *P < 0.05, **P < 0.01 and ***p<0.001 by one-way ANOVA.

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Figure 4

STS reduces secondary hemorrhage and edema. (A-B) Hemorrhagic area on the surface of injured cords from STS-treated and vehicle control mice at 1 and 3 days after injury. Scale bar = 1 mm. (C) Representative images of HE-stained spinal cord tissues showing hemorrhage and edema. Scale bar = 50µm. (D) The water content of the spinal cord in different groups at 1 and 3 days after injury. Data indicates mean values $\pm$ SEM, n = 5. *P < 0.05, **P < 0.01 and ***p<0.001 by one-way ANOVA.

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Figure 5

STS attenuates BSCB disruption by preventing loss of TJ and AJ proteins. (A) Representative images showing immunofluorescence staining of TJ protein occludin (red). Nuclei are counterstained with DAPI (blue). Scale bar = 20μm. (B) Representative immunoblot showing levels of AJ protein β-catenin and occludin. (C, D) Quantification of relative protein levels. Data indicates mean ± SEM, n = 5. *P < 0.05, **P < 0.01 and ***p<0.001 by one-way ANOVA .

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Figure 6

STS reduces apoptosis after injury. (A) Representative immunoblot showing expression levels of Bcl-2 and Bax in the spinal cord segment at the contusion epicenter at 1 and 3 days after SCI. (B) Quantification of relative Bcl-2/Bax protein levels. (C) Representative images showing TUNEL (green)-stained sections from the injured spinal cord of the indicated groups. Scale bar = 50 μ m. (D) Quantitative estimation of apoptotic and TUNEL cells. Data indicates mean $\pm$ SEM, n = 5. *P < 0.05, **P < 0.01 and ***p<0.001 by one-way ANOVA.

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Figure 7

STS prevents neuron loss after SCI. (A) Representative immunofluorescence images showing co-localization of C-caspase3 (red) and NeuN (green) in the indicated groups. Scale bar = 50 μ m. (B) Representative immunoblot showing expression levels of C-caspase 3 in the spinal cord segment at the contusion epicenter. GAPDH was used as the loading control. (C) Densitometric analysis of C-caspase 3 band. (D) Representative images showing Nissl staining of spinal cord sections (scale bar = 50 μ m). Data indicates mean $\pm$ SEM, n = 5. *P < 0.05, **P < 0.01 and ***p<0.001 by one-way ANOVA.

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