Original Article

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Effect of lycopene on the blood-spinal cord barrier after spinal cord injury in mice

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Summary **The current study aimed to investigate the effect of lycopene on the blood-spinal cord barrier (BSCB) after spinal cord injury (SCI) in a mouse model. Lycopene inhibited lipid peroxidation and oxidative DNA damage as a highly efficient antioxidant and free radical scavenger. Lycopene (4 mg/kg/d) was administrated immediately following SCI. The permeability of the BSCB and water content in the spinal cord tissue were evaluated. Additionally, levels of expression of tight junction proteins and heme oxygenase-1 (HO-1) were determined with Western blotting. An enzyme-linked immunosorbent assay analysis of spinal cord tissue homogenates was performed 48 h after SCI to evaluate the expression of inflammation-related cytokines. In addition, recovery of motor function was assessed 1 d, 2 d, 5 d, 10 d, and 15 d after SCI using the Basso Mouse Scale to score locomotion. Compared to the group with an untreated SCI, mice with an SCI treated with lycopene had significantly reduced spinal cord tissue water content and BSCB permeability. Furthermore, motor function of mice with an SCI was also greatly improved by lycopene administration. The expression of the proinflammatory factors TNF-α and NF-kB increased markedly 48 h after SCI, and their upregulation was significantly attenuated by lycopene treatment. The expression of molecules that protect tight junctions, zonula occluden-1 and claudin-5, was upregulated by lycopene treatment after SCI. Taken together, these results clearly indicate that lycopene attenuated SCI by promoting repair of the damaged BSCB, so lycopene is a novel and promising treatment for SCI in humans.**

Keywords: Lycopene, spinal cord injury, blood-spinal cord barrier, tight junction

1. Introduction

Spinal cord injury (SCI) is a severe trauma of the central nervous system (CNS) that results in loss of blood vessels and disruption of the blood-spinal cord barrier (BSCB) (*1,2*). Previous studies focused largely on alleviating neurological manifestations of the injury while ignoring pathological changes in the spinal cord (*3*). The compression forces induced by SCI can rupture blood vessels, destroy endothelial cells and pericytes, disrupt the BSCB, and cause production of numerous molecules that result in vascular disruption (*4,5*).

Maintaining spinal cord microcirculation in required to sustain the normal functioning of spinal cord nerve cells. After injury, damaged blood vessels allow an influx of blood cells including leucocytes, monocytes, and macrophages, contributing to additional loss of nervous tissue in some way. Blood is toxic to nervous tissue probably because degradation of hemoglobin releases catalytic metal ions, it produces free radicals, and it thereby leads to lipid peroxidation. Previous researchers focused on improving sensory motor function. However, they ignored the microvascular reaction of the spinal cord or alterations to the properties of the BSCB. Pharmaceuticals may have a considerable effect on microcirculation (*6*). Lycopene, a carotenoid mostly

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found in tomatoes and tomato products, possesses potent neuroprotective (*7*), antiproliferative, anticancer (*8*), and hypocholesterolemic activity (*9*). Lycopene reduces proinflammatory cytokine and chemokine-expressing macrophages (*10*), it protects cultured hippocampal neurons against Aβ and glutamate toxicity (*11*), it protects against microglial activation in focal cerebral ischemia in rats (*7*), and it attenuates cognition impairment in the elderly (*12*). Lycopene is an agent that may prove useful in managing neurodegenerative disorders because of its ability to cross the blood-brain barrier (BBB) (13) and its strong antioxidant properties (*14*). Furthermore, lycopene has been shown to reverse neurobehavioral deficits in rats (*15*). However, the mechanism by which lycopene affects the integrity of the BSCB after SCI remains unclear. The current study used a mouse model of SCI due to impact to investigate the mechanism by which lycopene acts on the BSCB.

2. Materials and Methods

2.1. *Animals*

Male C57BL mice weighing 22-25 g (Vital River Laboratory Animal Technology Co. Ltd, Beijing, China) were housed under standard 12-h light/dark conditions and received food *ad libitum*. All procedures involving experimental animals were approved by the Laboratory Animal Care and Ethics Committee of Tianjin Central Hospital.

2.2. *Induction of an SCI*

Mice were anesthetized using pentobarbital sodium (80 mg/kg body weight). The modified weight-drop model was used to produce an SCI (*16*). A longitudinal incision was made along the midline of the back to expose the paravertebral muscles, which were dissected away to expose the T10 vertebra. The laminae were removed. The animal was subjected to an impact of 50 g/mm (a 5-g weight dropped from a height of 10 mm) to the dorsal surface of the spinal cord. Afterwards, the muscles were sutured and the incision was closed. Following the surgical procedure, the rats were placed in a warming chamber. In the SCI groups, the cord was compressed for 1 min. Following surgery, 0.2 mL of saline was administered subcutaneously in order to replace the blood volume lost during surgery. The mice were singly housed at 25°C in a temperature-controlled room for 48 h. During this period, the animals' bladders were manually voided twice a day. Sham-injured animals were only subjected to laminectomy.

2.3. *Experimental design*

Mice were randomly divided into three groups: (a) A sham group in which the T10 vertebra was removed but no impact was applied and that was treated with saline containing 5% ethanol (n = 29); (b) A group in which an SCI was produced and treated with saline containing 5% ethanol (n = 29); and (c) A group in which an SCI was produced and treated with lycopene (n = 29) at a dose of 4 mg/kg/d (*17*).

2.4. *Evaluation of the permeability of the BSCB*

Evans blue (EB) leakage was assessed using an established protocol (*18*). EB (Sigma-Aldrich, USA, 2% w/v in saline) was injected intraperitoneally. Mice were perfused transcardially with saline and rinsed thoroughly until no more blue dye flowed out of the right atrium 3 h after the injection. The tissues (epicenter ± 0.5 cm) were dehydrated at 60°C for 48 h. Afterwards, they were weighed and extracted in 60% trichloroacetic acid for 72 h. To measure EB dye, the tissues were then centrifuged at 14000× g for 8 min. A standard curve with EB dye was plotted and fluorescence intensity was measured in the supernatant using a spectrophotometer at an excitation wavelength of 620 nm and an emission wavelength of 680 nm. All measurements were within the range of detection established by the standard curve. R^2 of the standard curve was 0.99. The concentration of dye was calculated as the ratio of absorbance relative to the tissue volume.

2.5. *Evaluation of the water content in spinal cord tissue*

Lycopene treatment (4 mg/kg/d) was administered within 30 min of surgery and then every 24 h. Forty-eight h after SCI, the injured spinal cord (epicenter ± 0.5 cm) was extracted and dried at 80°C for 48 h. Water content in spinal cord tissue was calculated according to the following formula: spinal cord water content (%) = (wet weight-dry weight)/wet weight × 100%.

2.6. *Functional testing*

Functional recovery after SCI was assessed using Basso Mouse Scale (BMS) (*19*). Mice with an SCI were tested 1 d, 2 d, 5 d, 10 d, and 15 d after SCI, and they were scored in an open-field environment by trained investigators.

2.7. *Enzyme-linked immunosorbent assay*

To detect the levels of inflammation-related cytokines, the rats were sacrificed 48 h after SCI and the injured spinal cord was used to prepare spinal cord tissue homogenates (epicenter ± 0.5 cm). Levels of TNF-α and NF-kB were determined using specific enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Shanghai, China).

2.8. *Western blotting*

Spinal cord samples (epicenter ± 0.5 cm) were collected and total protein was prepared with a lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). Protein levels were determined using a protein assay kit (BCA, Pierce, Rockford, USA). Protein was separated with 10% SDS-PAGE and transferred onto PVDF membranes. The membranes, blocked with 5% BSA for 1 h, were incubated with antibodies against heme oxygenase-1 (HO-1) (1:100, Santa Cruz, USA), zonula occluden-1 (ZO-1) (1:100, Santa Cruz, USA) and claudin-5 (1:100, Santa Cruz, USA). The primary antibodies were detected using horseradish peroxidase-conjugated rat anti-rabbit, rat anti-mouse, or rabbit anti-goat IgG secondary antibodies (1:500, Santa Cruz, USA). The bands were visualized using enhanced chemiluminescence and relative band intensities were quantified using Image Pro Plus 7.0 (Media Cybernetics, Silver Spring, MD).

2.9. *Statistical analysis*

All data are presented as the mean ± SD. Data on the two groups were compared using a paired student's *t* test. One-way analysis of variance and Tukey's post-hoc tests were used to compare groups. $p < 0.05$ was considered statistically significant.

3. Results

3.1. *Effect of lycopene on motor function*

Functional recovery of overground locomotion was observed 2 d, 5 d, 10 d, and 15 d after SCI in all groups, and observations indicated that motor function of the group with an SCI treated with lycopene improved significantly compared to that of the group with an untreated SCI ($p < 0.05$). At 24 h, there were no significant differences in the motor function of the group with an untreated SCI and the group with an SCI treated with lycopene (Figure 1).

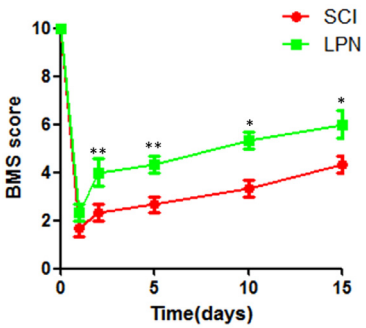

Figure 1. Lycopene improved functional recovery. Motor function of the group with an untreated SCI and the group with an SCI treated with lycopene was assessed using the BMS score ($n = 8$). Two d, 5 d, 10 d, and 15 d after SCI, motor function of the group with an SCI treated with lycopene was better than that of the group with an untreated SCI. $^{*}p < 0.05$, $^{**}p < 0.01$.

3.2. *Effect of lycopene on the water content in the spinal cord*

Water content in the spinal cord was detected to evaluate whether lycopene has protective action by reducing the level of edema. Water content in the spinal cord after injury increased significantly in the group with an untreated SCI compared to that in the sham group (untreated SCI: 0.72 ± 0.02 *vs*. sham: 0.47 ± 0.04, $p < 0.001$). Water content in the spinal cord decreased in the group with an SCI treated with lycopene in comparison to that in the group with an untreated SCI (untreated SCI: 0.72 ± 0.02 *vs*. SCI treated with lycopene: 0.62 ± 0.06, $p < 0.05$) (Figure 2).

3.3. *Effect of lycopene on the permeability of the BSCB*

To determine whether lycopene reduced the increase in permeability induced by compression injury, the level of EB in injured tissue was determined. The level of EB in the group with an untreated SCI increased significantly compared to that in the sham group (SCI: 0.48 ± 0.15 μg/mg *vs*. sham: 0.01 ± 0.03 μg/mg, $p < 0.001$). After lycopene treatment, the level of EB in the spinal cord tissue decreased significantly compared to that in the group with an untreated SCI (untreated SCI: 0.48±0.15 μg/mg *vs*. SCI treated with lycopene: 0.25 ± 0.10 μg/mg, $p < 0.05$) (Figure 3).

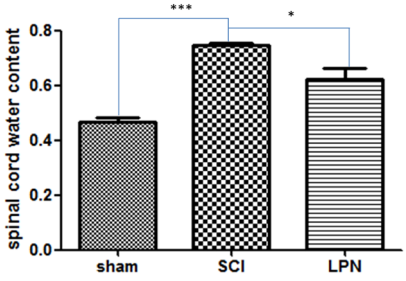

Figure 2. Lycopene reduced edema of the spinal cord after SCI. Water content in the spinal cord was determined ($n = 6$). $^{*}p < 0.05$, $^{***}p < 0.001$.

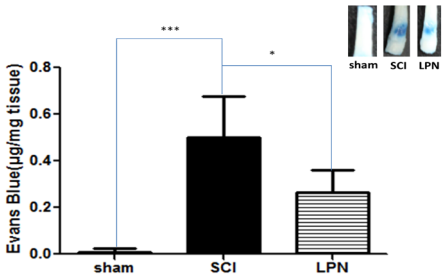

Figure 3. As indicated by EB levels, lycopene reduced the permeability of the BSCB. Shown here are photographs of the sham group, the group with an untreated SCI, and the group with an SCI treated with lycopene. The length of each tissue specimen was 1 cm. The fluorescence intensity of EB in the three groups was quantified ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$.

3.4. *Effect of lycopene on the expression of pro-inflammatory cytokines after SCI*

Changes in levels of TNF-α and NF-kB in the spinal cord were detected with ELISA. Compared to the sham group, the group with an untreated SCI had marked increased levels of expression of TNF-α (untreated SCI: 14.03 ± 0.60 pg/mg *vs*. sham: 9.94 ± 0.05 pg/mg, $p < 0.001$) and NF-kB (untreated SCI: 24.03 ± 0.55 pg/mg *vs*. sham: 11.26 ± 0.38 pg/mg, $p < 0.001$). Compared to the group with an untreated SCI, the group with an SCI treated with lycopene had markedly decreased levels of expression of TNF-α (untreated SCI: 14.03 ± 0.60 pg/mg, lycopene: 11.59 ± 0.51 pg/mg, $p < 0.05$) and NF-kB (untreated SCI: 24.03 ± 0.55 pg/mg, SCI treated with lycopene: 14.92 ± 0.88 pg/mg, $p < 0.001$) (Figure 4).

3.5. *Effect of lycopene on expression of tight junction (TJ) proteins and the protein HO-1*

Proteins related to the BSCB, *i.e.* ZO-1, claudin-5, and HO-1, were detected. Expression of TJ proteins was upregulated by lycopene 48 h after SCI. The levels of expression of the proteins ZO-1 and claudin-5 were much lower in the group with an untreated SCI than those in the sham group 48 h after SCI. However, lycopene alleviated the abnormal disruption of ZO-1 and claudin-5 in the group with an SCI treated with lycopene. The group with a treated SCI had a significantly increased level of HO-1 protein 48 h after SCI compared to the sham group ($p < 0.001$). The level of HO-1 decreased in the group with an SCI treated with lycopene in comparison to that in the group with an untreated SCI ($p < 0.05$) (Figure 5).

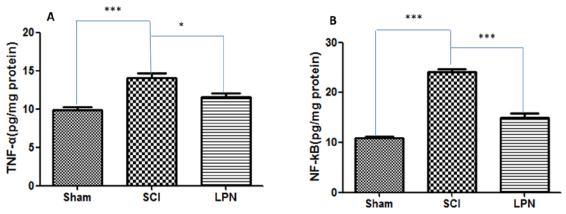

Figure 4. Effect of lycopene on the concentrations of inflammation cytokines after SCI. Injured spinal cord tissue was used to prepare spinal cord tissue homogenates (epicenter ± 5 mm) 48 h after SCI, and levels of TNF-α **(A)** and NF-kB **(B)** were determined using ELISA ($n = 6$). $^{***}p < 0.001$.

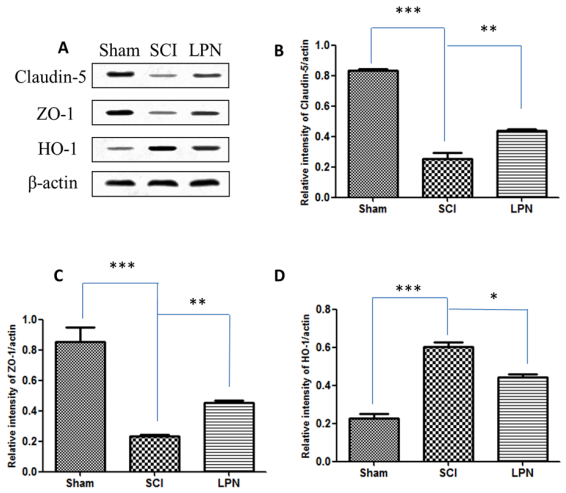

Figure 5. Effect of lycopene on expression of the proteins ZO-1, claudin-5, and HO-1 at the injured spinal cord 48 h after SCI ($n = 3$). (A) Expression of claudin-5, ZO-1, and HO-1 in the three groups. **(B-D)** The relative intensity of claudin-5, ZO-1, and HO-1 expression in the three groups. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

4. Discussion

As a member of the carotenoid family, lycopene has numerous potential health benefits, most of which are based on its antioxidant properties. Previous researchers focused on improving sensory motor function, but microvascular reactions of the spinal cord or alterations to the properties of the BSCB were neglected. Any alterations to the BSCB may change the spinal cord microfluid environment, leading to functional disturbance. Reducing the breakdown of the BSCB may protect the nervous system. However, how lycopene acts on the integrity of the BSCB and microcirculation after SCI remains unclear.

Spinal cord edema largely explains the morbidity and mortality of SCI (*20*). Spinal cord edema induces openings in the BSCB, leading to an exchange of harmful substances between blood and tissue. Disruption of the BSCB elicits marked local inflammation with the infiltration of neutrophils and macrophages, leading to cell death and permanent neurological disability (*21,22*). The current study found that lycopene significantly reduced the level of edema after SCI.

TJs are located at the apical end of the inter-endothelial space and are connected to adherens junctions near the basal end of the inter-endothelial space (*23*). TJ proteins, such as transmembrane proteins claudin-5 and the peripheral membrane protein ZO-1, are essential to the normal functioning of TJs (*24*). Changes in the distribution and expression of TJ-associated proteins are reported to be closely related to the permeability of the BSCB after an SCI (*25,26*). In the current study, expression of ZO-1 and claudin-5 was detected and lycopene treatment significantly increased the expression of ZO-1 and claudin-5 compared to levels in the group with an untreated SCI. This indicated that lycopene treatment increased the integrity of the BSCB after SCI by regulating ZO-1 and claudin-5.

Neuroinflammation leads to breakdown of the BSCB, which is implicated in the pathogenesis of CNS disease. The expression of proinflammatory cytokines, including TNF-α and NF-kB, at the site of injury has been found to regulate the permeability of the BSCB following SCI (*27,28*). Exposure of the endothelium

to proinflammatory cytokines interrupts the BBB by disorganizing cell-cell junctions (*29*). In the current study, the effect of lycopene on the inflammatory mediator TNF-α and its regulatory factor NF-kB was investigated. Results revealed that lycopene treatment significantly decreased the activation of TNF-α and NF-kB compared to levels in the group with an untreated SCI. These results indicate that lycopene inhibited the generation of inflammatory mediators, thereby limiting disruption of the BSCB and eventually resulting in amelioration of pathological changes caused by SCI.

HO-1 is involved in the rate-limiting step in the oxidative degradation of heme. HO-1 expression is upregulated in response to oxidative stress, which catalyzes the degradation of pro-oxidant heme to carbon monoxide, iron, and bilirubin (*30*). HO-1 expression is an adaptive and protective response to oxidative stress. HO-1 can be induced by a variety of stimuli and various agents that cause oxidative stress, and it protects cells from the oxidative damage caused by reactive oxygen species (*31*). Lycopene has been found to protect against oxidation of lipids, proteins, and DNA *in vivo*. Therefore, the current study used lycopene to detect an anti-oxidant effect after SCI, and the results indicated that lycopene significantly decreased the expression of HO-1.

In conclusion, the current findings indicate that lycopene could potentially serve as a treatment to reduce the severity of SCI. These results should help in studying the mechanism and treatment of SCI in humans. This study found that lycopene acts on the BSCB after SCI. Lycopene improved motor function, it reduced the permeability of the BSCB, it increased the number of tight junctions, and it reduced inflammatory factors. However, how lycopene acts on the components of the BSCB (endothelial cells, pericytes, and astrocytes) remains unclear. Future studies should concentrate on the key molecules mediating neurovascular links among the various types of cells of the BSCB and pharmaceuticals should be developed to treat SCI and other CNS conditions.

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