

Received: 2019.09.04

Accepted: 2019.10.28

Published: 2020.01.23

The Lipoxin A4 Receptor Agonist BML-111 Alleviates Inflammatory Injury and Oxidative Stress in Spinal Cord Injury

Authors' Contribution:

Study Design A

Data Collection B

Statistical Analysis C

Data Interpretation D

Manuscript Preparation E

Literature Search F

Funds Collection G

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Source of support: Departmental sources

Background: Spinal cord injury (SCI) has a high incidence and causes serious harm. Lipoxin A4 (LXA4) receptor agonist BML-111 was reported to regulate inflammation and oxidative stress. The goal of this study was to assess whether BML-111 could protect against SCI by suppressing inflammation and oxidative stress.

Material/Methods: We developed a rat SCI model, then BML-111 was intraperitoneally injected into SCI rats to observe the BML-111 function. The pathological changes of SCI were observed with hematoxylin and eosin (HE) staining. Motor function of rats were assessed by the modified Tarlov's scale. ELISA was used to assess the changes in levels of TNF- α , IL-1 β , and IL-6. Western blot analysis was performed to assess the expressions of TNF- α , IL-1 β , IL-6, Bcl2, Bax, and cleaved caspase3 in spinal cord tissue. TOS and TAS in rat serum were detected by xylenol orange method and ABTS method, respectively. The apoptotic cells in spinal cord tissue were observed with TUNEL assay.

Results: The results indicated that BML-111 effectively improved the SCI and motor function of rats. BML-111 treatment decreased the levels of TNF- α , IL-1 β , and IL-6 in serum and spinal cord tissue, as well as decreasing the levels of TOS and TAS and cell apoptosis.

Conclusions: BML-111 alleviated inflammation and oxidative stress in SCI rats.

MeSH Keywords: **Inflammation • Oxidative Stress • Spinal Cord Injuries**

Full-text PDF: <https://www.medscimonit.com/abstract/index/idArt/919883>

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Background

Spinal cord injury (SCI) can cause major impairment to spinal cord function (e.g., sensation, movement, and reflex) [1,2]. Approximately 23 people per million experience a new spinal cord injury every year worldwide [3]. About 90% of SCI patients had damage caused by motor vehicle injuries, falls, or violence [4]. Because of the high rate of disability after SCI, it is important to know how to take effective measures to reduce the disability rate of SCI patients and improve their quality of life.

Studies show that acute spinal cord injury (ASCI) consists of early mechanical injury and secondary injury. Neuronal apoptosis results from inflammation, and oxidative stress can induce ASCI [5,6]. Inhibition of inflammatory response and oxidative stress during secondary injury is beneficial to recovery of motor function in ASCI rats [7]. Lipoxin is an endogenous anti-inflammatory lipid medium, which is only synthesized in a small amount under physiological conditions and is significantly increased under pathological conditions such as inflammatory injury to promote the regression of inflammation [8–11]. Lipoxin A4 (LXA4) possesses a good anti-inflammatory effect on the respiratory tract, intestines, and nephridium [12–16]. It was observed that LXA4 alleviated inflammation and neuropathic pain after SCI [17,18]. Furthermore, LXA4 can reduce the level of oxidative stress in spinal cord ischemia and hypoxia injury [19]. The LXA4 receptor agonists suppressed the inflammatory activities to protect inflammatory diseases [20,21]. BML-111, which is a LXA4 receptor agonist, has reported to protect against acute lung injury by reducing inflammation and oxidative stress [22,23]. However, it is unknown whether BML-111 can alleviate inflammation and oxidative damage in SCI.

Here, a rat SCI model was constructed to explore the anti-inflammatory and antioxidant effects of BML-111 in the protection of SCI.

Material and Methods

Animals

Thirty male Sprague-Dawley (SD) rats (weight, 200–250 g) were brought from Shanghai Jiesijie Experimental Animal Co. All rats were housed with 5 rats per cage, with a temperature of $22\pm 2^{\circ}\text{C}$ and a controlled light/dark cycle. The rats had free access to water and standard rat chow. The experimental groups were the sham group (control), the SCI group (model), and the BML-111 treatment group (model+BML-111) ($n=10$). All experiments were approved by the Animal Ethics Committee of the Fourth People's Hospital in Chongqing City.

Animal surgery and drug administration

Thirty male SD rats were anesthetized by injecting 10% chloral hydrate into the peritoneum (400 mg/kg). The animals exhibited no signs of peritonitis after the administration of 10% chloral hydrate. T8 was used as the center for hair shaving and routine disinfection, followed by making a mid-line incision in the skin (about 18 mm long) to expose the T6–10 vertebra. Laminectomy was performed at the T8 level. An aneurysm clip with 20 g closing force was used to clamp the spinal cord at the T8 level. When flaccid paralysis occurred in the 2 hind legs and trunk, the clip was removed. The urinary bladders of rats were compressed by hand twice daily until spontaneous voiding occurred. Rats in the BML-111 treatment group received an intraperitoneal injection of BML-111 (1 mg/kg). Rats in the sham (control) group underwent the same operation as the model group, but they did not receive spinal cord compression. Rats in the sham group received an identical volume of DMSO solution by intraperitoneal injection. All rats were fed singly in a room at suitable temperature. After 28 days, all the rats were killed by cervical dislocation after intraperitoneal anesthesia with 350 mg/kg 7% chloral hydrate. The spinal cord tissue and serum was taken for follow-up experiments. When peritonitis and quadriplegia occurred in rats, they were euthanized.

Hematoxylin and eosin (HE) staining

Rats were anesthetized and killed at set time points. The left ventricle was infused with heparinized saline, until the perfusion was clear, then 4% paraformaldehyde perfusion was used to fix the spinal cord *in vivo*. Subsequently, isolated spinal cord tissues were fixed with 4% paraformaldehyde for 24 h and spinal cord tissues were then usually embedded in paraffin. Paraffin-embedded spinal cord tissues were cut into 5-mm section slices which were stained with hematoxylin-eosin (HE) dye and then sealed with resin. The spinal cord tissue pathological changes were observed by a biological microscope.

Motor function score

The motor function of rats was assessed according to the modified Tarlov's scale, in which 0 represents no lower-extremity movement, 1 indicates lower-extremity motion without gravity, 2 indicates lower-extremity motion against gravity, 3 indicates able to stand with assistance, 4 indicates able to walk with assistance, and 5 indicates normal.

Enzyme-linked immunosorbent assay (ELISA)

The levels of TNF- α , IL-1 β , and IL-6 in serum were analyzed by commercially-available ELISA kits (Boster, Wuhan, China). The experimental steps were performed according to the kit

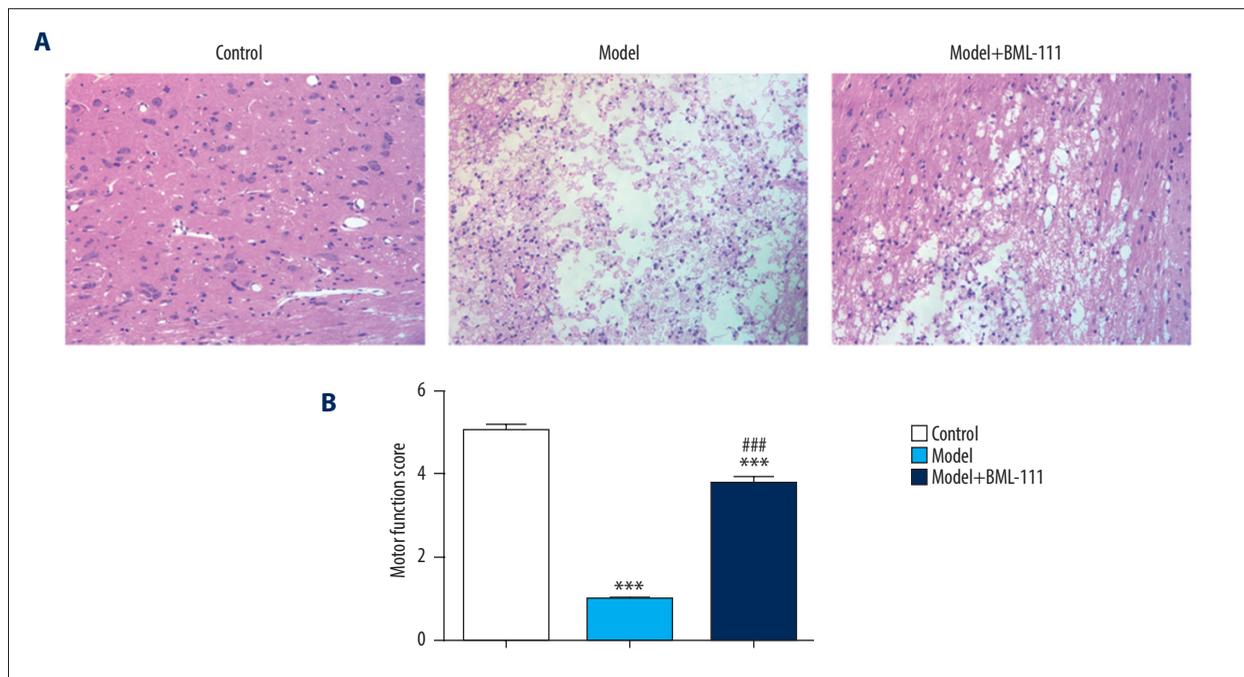


Figure 1. BML-111 alleviated spinal cord injury. (A) The pathological changes of spinal cord were detected by HE staining (magnification 200 \times). (B) The motor function of rats was assessed using the modified Tarlov's scale. *** $P < 0.001$ vs. control group. ### $P < 0.001$ vs. model group.

instructions. Finally, according to the standard curve, the levels of TNF- α , IL-1 β , and IL-6 in serum were calculated.

Western blot analysis

The 80–100-mg tissue samples were homogenized and lysed by RIPA. The same amount of protein was taken for SDS-PAGE electrophoresis in each group, and the protein was transferred to PVDF membranes. The membranes were sealed by 5% skim milk at 25 $^{\circ}\text{C}$ for 2 h and incubated with TNF- α (#3707; Cell Signaling Technology, Inc.; dilution, 1: 1000), IL-1 β (ab9722; Abcam; dilution, 1: 1000), IL-6 (ab9324; Abcam; dilution, 1: 1000), Bcl2 (ab196495; Abcam; dilution, 1: 1000), Bax (ab32503; Abcam; dilution, 1: 1000), cleaved caspase3 (ab49822; Abcam; dilution, 1: 1000), and caspase3 (ab44976; Abcam; dilution, 1: 1000) overnight at 4 $^{\circ}\text{C}$. Then, secondary antibody was incubated on the membrane at 37 $^{\circ}\text{C}$ for 1 h. The protein bands were visualized with BeyoECL Plus (Beyotime, Shanghai, China).

Detection of TOS and TAS

TOS in rat serum was detected by xylenol orange method [24] and TAS in rat serum was detected by ABTS method [25]. The levels of TOS and TAS in serum were analyzed by a 7600-020 automatic biochemical analyzer (Hitachi, Japan).

TUNEL assay

Paraffin sections were dewaxed with xylene. Apoptotic cells in spinal cord tissues were stained according to the TUNNEL kit instructions. Apoptotic cells were observed under a biological microscope.

Statistical analysis

SPSS 20.0 statistical software was used to conduct statistical analysis for the data. The normally distributed measurement data are represented by mean \pm standard deviation, and one-way analysis of variance (ANOVA) was used to compare multiple groups. $P < 0.05$ indicates a statistically significant difference.

Results

BML-111 alleviated spinal cord injury

The cell nucleus of spinal cord tissue in the model group showed obvious nucleus pyknosis, tissue edema, and inflammatory cell infiltration. In the model+BML-111 group, the nucleus pyknosis, tissue edema, and inflammatory cell infiltration were alleviated (Figure 1A). The motor function of rats in the model group was obviously decreased and lower-extremity motion without gravity was observed in rats. In the model+BML-111 group, the motor function of rats was obviously improved and rats were

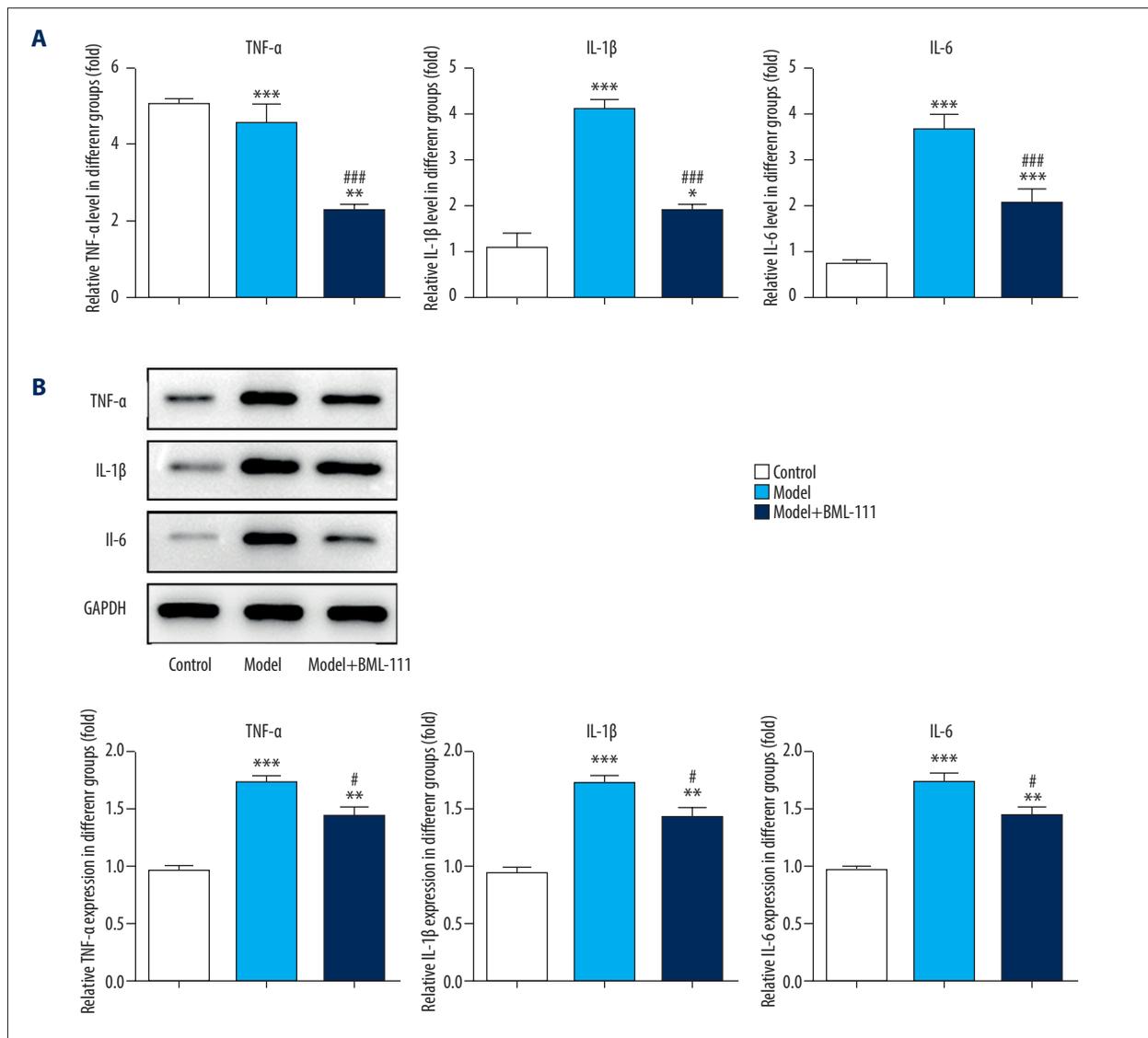


Figure 2. BML-111 reduced the expression of inflammatory factors in serum and spinal cord. (A) The expression levels of TNF- α , IL-1 β , and IL-6 in serum were detected by ELISA. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. control group. ### $P < 0.001$ vs. model group. (B) The expression levels of TNF- α , IL-1 β , and IL-6 in spinal cord were detected by Western blot analysis. ** $P < 0.01$ and *** $P < 0.001$ vs. control group. ### $P < 0.001$ vs. model group.

able to stand or walk with assistance, but while still significantly more impaired than rats in the control group (Figure 1B).

BML-111 reduced the expression of inflammatory factors in serum and spinal cord tissue

The inflammatory factors in the serum and spinal cord tissue were detected by ELISA (Figure 2A) and Western blot analysis (Figure 2B). In the model group, the levels of TNF- α , IL-1 β , and IL-6 in the serum and spinal cord tissue were obviously increased, but these levels were lower in the model+BML-111 group.

BML-111 alleviated oxidative stress levels in serum

As shown in Figure 3, in the model group, the TOS levels were obviously upregulated and the TAS levels were obviously downregulated in serum. In the model+BML-111 group, BML-111 effectively suppressed the TOS levels and increased the TAS levels in serum. Therefore, BML-111 alleviated oxidative stress levels in serum.

BML-111 decreased apoptosis of neuronal cells

In the TUNEL assay, the green fluorescent points indicate apoptotic cells. The results of Figure 4A indicate that the apoptosis

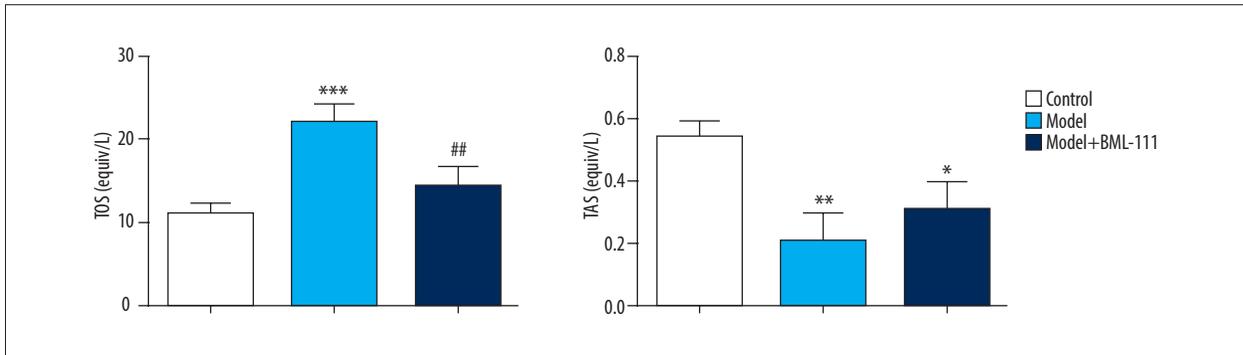


Figure 3. BML-111 alleviated oxidative stress levels in serum. The expression levels of TOS and TAS in serum were detected by use of a 7600-020 automatic biochemical analyzer.

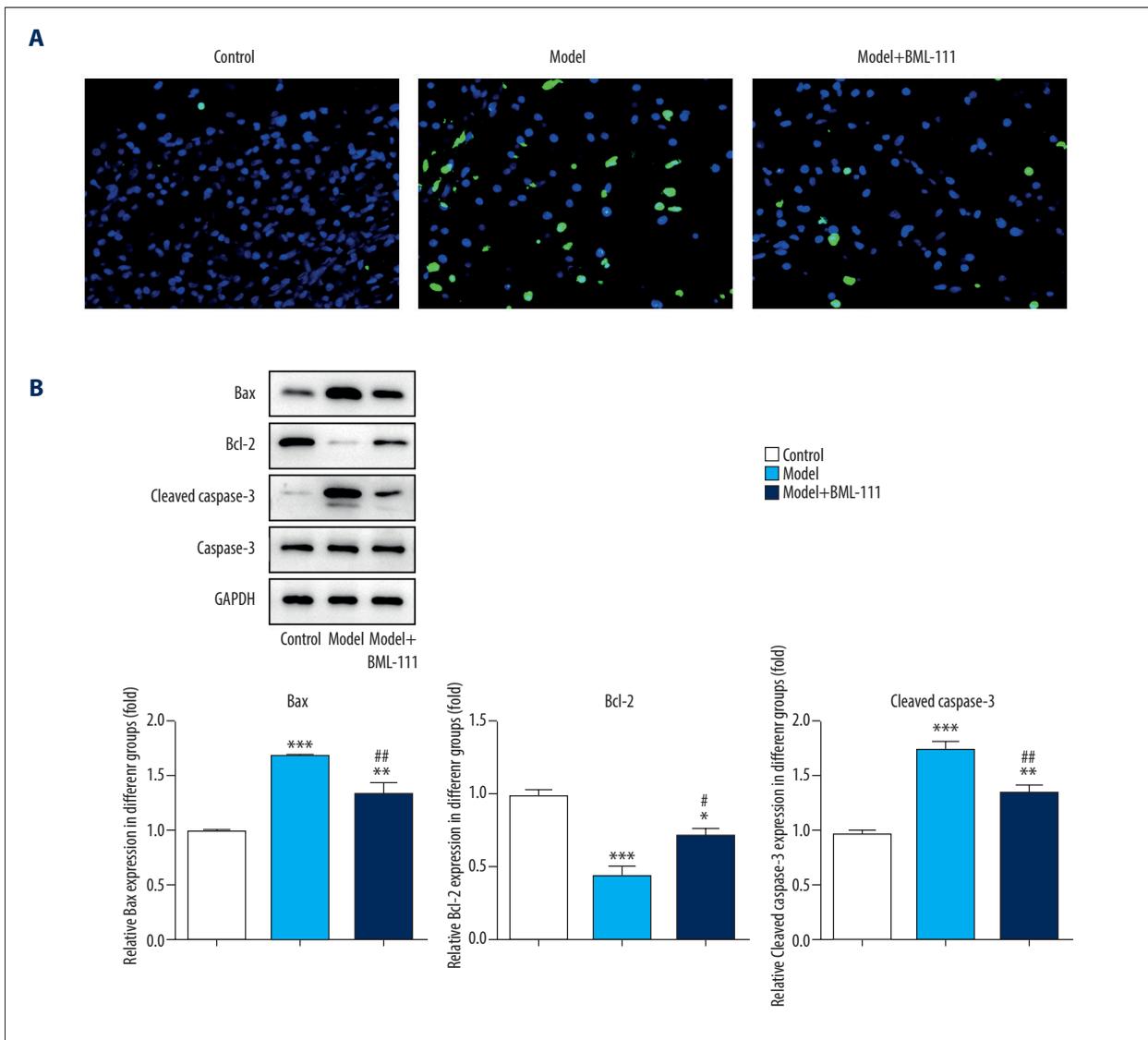


Figure 4. BML-111 decreased apoptosis of neuronal cells. (A) The apoptosis of neuronal cells was analyzed by TUNEL assay. (B) The apoptosis-related expression was detected by Western blot analysis. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control group. # $P < 0.05$ and ## $P < 0.01$ vs. model group.

of neuronal cells was increased in the model group and the apoptosis of neuronal cells was obviously decreased in the model+BML-111 group. As shown in Figure 4B, in the model group, the expression of Bcl-2 was decreased and the expression of Bax and cleaved caspase3 was increased. However, BML-111 promoted the expression of Bcl-2 and inhibited the expression of Bax and cleaved caspase3 in the model+BML-111 group. Therefore, BML-111 decreased apoptosis of neuronal cells.

Discussion

Neuronal apoptosis resulting from inflammation and oxidative stress is the main factor in the pathology of SCI. It is therefore essential to improve SCI by controlling the inflammation and oxidative stress. In the present study, SCI rats were treated with BML-111, a potential anti-inflammatory and antioxidant LXA4 receptor agonist, to assess its protective effects.

Many reports have demonstrated that BML-111 can effectively alleviate inflammation and oxidative injury in several diseases. BML-111 limited the inflammatory response and promoted anti-oxidative protein expression in liver injury [26]. BML-111 obviously decreased the inflammatory and pro-inflammatory factors in acute lung injury [27,28]. In a chronic obstructive pulmonary disease (COPD) animal model, BML-111 was shown to restrain NLRP3 inflammasome activation and suppress ROS production to exert its anti-inflammatory effect on COPD [29]. Chang et al. [30] indicated that BML-111 can mitigate the inflammatory response and apoptosis of renal tissue in acute renal injury. Pan et al. [31] found that increased levels of TNF- α and IL-1 β were reversed by BML-111 treatment to mitigate neuroinflammation in sepsis. This shows that BML-111 can improve inflammation and oxidative injury in various inflammatory diseases. In the present study, the function of BML-111 was demonstrated to be consistent with that reported in previous studies. We confirmed that BML-111 protected against SCI by reducing inflammation and oxidative injury, as evidenced by improvement of cell nucleus pyknosis, tissue edema, inflammatory cell infiltration, and motor function.

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TNF- α , IL-1 β , and IL-6 are common pro-inflammatory cytokines. Studies have confirmed that TNF- α , IL-1 β , IL-6, and other pro-inflammatory cytokines were increased significantly after SCI, which promoted inflammatory cell infiltration to further aggravate SCI [32–34]. IL-1 β can promote SCI progression by activating the NF- κ B [35]. Jia et al. [36] demonstrated that icariin protected against SCI by inhibiting the levels of IL-1 β , IL-6, and TNF- α . In the present study, BML-111 alleviated SCI by reversing the increase of TNF- α , IL-1 β , and IL-6 in serum and spinal cord tissue. In addition, Aras et al. [37] found that minocycline treatment significantly upregulated the levels of TAS and TOS to improve the total antioxidant status in SCI rats. In the present study, BML-111 improved SCI by effectively reversing the increased levels of TAS and TOS in serum. Hu et al. [38] found that SCI-induced oxidative injury can lead to neuron apoptosis, which subsequently induced changes in expression of apoptosis-related proteins. Other studies also demonstrated that SCI can induce cell apoptosis [39,40]. In addition, lycopene treatment was reported to protect against SCI by decreasing neuron apoptosis [38]. In our study, the SCI group showed increased neuron apoptosis, decreased Bcl2 expression, and increased expression of Bax and cleaved caspase3. Thus, BML-111 alleviated SCI by inhibiting neuron apoptosis.

Conclusions

BML-111 alleviated the spinal edema and the ultrastructure damage of spinal cord tissues, thereby recovering motor function. BML-111 reduced the levels of TNF- α , IL-1 β , and IL-6 in serum and spinal cord tissues, reduced the TOS level, and increased the TAS level to mitigate inflammation and oxidative injury. In addition, BML-111 protected against SCI by decreasing neuron apoptosis. The use of BML-111 to treat SCI may be valuable in clinical practice.

Conflicts of interest

None.

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