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Original Article

HSP70 alleviates spinal cord injury by activating the NF-kB pathway

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Abstract

Objectives: Spinal cord injury (SCI) is an acute traumatic lesion of neurons in the spinal cord which has a high prevalence in the world, and has no effective surgical treatment. HSP70 is a molecular chaperone protein, serves a protective role in several different models of nervous system injury. The aim of the present study was to investigate the anti-inflammatory role of HSP70 in spinal cord injury and explore its mechanism. **Methods**: *In vivo* and *in vitro* models were constructed to mimic SCI. The Basso Mouse Scale (BMS) was applied to assess SCI degrees of the mouse model. Immunofluorescence (IF) was used for visualizing HSP70 and Iba1 in the spinal cord. Western blot assay was employed to quantify HSP70 and p65, and ELISA was for IL-1β and TNF-α. **Results**: The results showed that HSP70 expression decreased after SCI. HSP70 and Iba1 showed a decrease of co-localization in SCI mice. Further studies revealed that p65 was upregulated during the process of SCI. Overexpression of HSP70 inhibited the expression of p65 both *in vitro* and *in vivo*, and promoted the recovery of SCI mice. **Conclusions**: HSP70 was involved in the pathological process of spinal cord injury, HSP70 alleviated the spinal cord injury via inhibiting NF-κB signaling pathway.

Keywords: HSP70, NF-κB Signaling Pathway, Spinal Cord Injury

Introduction

Spinal cord injury (SCI) is an acute traumatic lesion of neurons in the spinal cord and cauda equina, which begins with a sudden mechanical force and leading to neuronal death, resulting in motor, sensory, or autonomic dysfunction[1,2]. SCI happens often in traffic accidents, altitude falling, and violence, making it a leading cause of permanent disability in young adults[3-5]. SCI also brings lots of complications with it, including chronic pain, orthostatic hypotension, and galactorrhea, which severely decrease life qualities of patients[6-8]. Treatment most currently used for SCI shows poor effect, making it one of the most challenging research topics to find the new pathological mechanism and new treatment target[9]. Inflammation plays key roles in the pathological process. Inflammatory reaction such as inflammatory cells infiltration, and release of inflammatory cytokines caused by SCI building blocks for the repair and treatment of injured tissues[10]. Microglial is the main immune cells which dominate the inflammatory response in SCI in humans[10,11]. Reduction of microglial activation attenuates the spinal cord injury[12]. Hence, understanding of the regulatory mechanism microglial activation related inflammation in SCI is very important.

Heat-shock-protein 70 (HSP70) is a member of heat shock proteins family, functions mainly as intracellular molecular chaperone of proteins and protection effect on cells in stressful conditions such as ultraviolet radiation, bacterial infections or inflammation[13]. HSP70 was reported to show protective effect in various injury-related diseases, including acute kidney injury[14], acute lung injury[15], and liver injury[16]. In SCI rats, HSP70 was reported to decrease in the ventral horn, and increased in the rats recovered from SCI[17], indicating HSP70 may play beneficial effects in protecting SCI. However, whether HSP70 could inhibit the inflammatory

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response during SCI, and its specific molecular mechanism hasn't been fully elucidated yet. Here we constructed mice SCI models and investigate the role of HSP70 during SCI. We found that HSP70 played protective effect in SCI by inhibiting the inflammatory response through regulating NF-κB activation.

Materials and methods

Spinal cord injury model

Animal care and experimental manipulation were approved by the Committee on Animal Care and Use of ethical committee of the Third Affiliated Hospital of Xi'an Jiaotong University. All animal handling practices followed Directive 2010/63/EU of the European Union "on the protection of animals used for experimental purposes". A total of forty-two male C57BL/6 mice, weighing 22±2 g, were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China). After adapted to new environment for 1 week, the mice were randomly divided into different groups with 6 mice in each group, and the mice were subjected to spinal cord injury model as reported previously[18,19]. Briefly, mice were anesthetized and the spinal cord was exposed via laminectomy and SCI was produced by extradural compression of the spinal cord using an aneurysm clip (using the aneurysm clip applicator oriented in the bilateral direction) with a closing force of 24 g for 1 min, at the T6 to T7 level. Mice in the sham group were only subjected to laminectomy. After surgery, the muscles and skin were sutured in layers.

For the detection of HSP70 and NF-κB expression courses, 18 mice were subjected to the SCI, then the mice were sacrificed at 1st, 3rd, 14th day after the operation, and the spinal cord tissues were collected.

In order to examine the role of HSP70 in SCI, 24 mice were randomly divided into four groups: sham group, SCI group, SCI+HSP70 overexpression group (SCI+HSP70 OE group), SCI+HSP70 overexpression+ p65 overexpression group (SCI+HSP70 OE+p65 OE group). The lentivirus harboring HSP70 or p65 were inject into the lesion site of SCI mice at a concentration of 8×10^5 units/μl. The HSP70 lentivirus and the control lentivirus were obtained from Genepharma (Shanghai, China). Tissues collection were performed by dissecting L4-6 fragments of spinal cords and kept in liquid nitrogen for other experiments.

Behavioral tests

The Basso Mouse Scale (BMS) was applied to evaluate the motor ability of the mouse model as described previously[20]. All mice were allowed to acclimatize to the apparatus for several days before modeling. Baseline BMS scores were measured of the enrolled mice before SCI induction. After SCI was induced, the mice were observed for 4 min everyday by 2 independent observers in a double-blind manner. BMS score of each hindlimb was recorded and analyzed.

Immunofluorescence staining

Mice were perfused transcardially with ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH=7.2) at 0 and 14 days after injury. The spinal cords were removed and fixed in 4% paraformaldehyde. Fifteen-micrometer sections were prepared on a cryostat. Longitudinal section was made in order to show the structure of injured spinal cord well. The sections were blocked by using normal goat serum with 0.2% Triton-X 100 for 30 min at room temperature, and then incubated with Mouse Anti-HSP70 antibody (abcam) and Rabbit Anti-Iba1 antibody (abcam) overnight at 4°C and followed by staining with the corresponding secondary antibodies (Alexa Fluor 488 or 594, 1:800, Jackson Immuno Research) for 1 h at 37°C.

Cell culture

The BV2 immortalized murine microglial cell line was obtained from American Type Culture Collection. The murine BV2 microglia were cultured in DMEM supplemented with 10% FBS, 1% penicillin and streptomycin, and were maintained in a humidified incubator with 5% CO_2. Cells were treated with LPS (0.5 μg/ml) in serum-free DMEM for 6, 12 and 24 h. Then the cells were lysed and the cell lysate were used for western blot analysis.

Western blot analysis

Total protein was extracted from spinal cord tissues or cells after different treatments by using a Total Protein Extraction Kit (Synthgene Biotech, Nanjing, China) according to the manufacturer's protocol. The concentration of the extracted total protein was measured with a BCA Protein Assay Kit (Synthgene Biotech, Nanjing, China) according to the manufacturer's protocol. 40 μg total protein was then electrophoresed, and transferred onto PVDF membranes, blocked with 5% milk and incubated with primary antibodies against HSP70 (1:1000, abcam, Shanghai, China), p65 (1:1000, abcam, Shanghai, China), p-p65 (1:1000, abcam, Shanghai, China), Iba1 (1:1000, abcam, Shanghai, China), and GAPDH (1:1000, abcam, Shanghai, China). Following primary antibody incubation, membranes were incubated with HRP-conjugated secondary antibodies (1:5000, abcam, Shanghai, China). Protein bands were visualized using a HiSignal™ ECL WB Detection Kit (Synthgene Biotech, Nanjing, China) according to the manufacturer's protocol.

Enzyme linked immunosorbent assay

The expression levels of inflammatory cytokines including IL-1β and TNF-α in myelid tissue samples were measured using specific ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instruction.

Statistical analysis

Data are representative of three independent experiments and values are expressed in mean ± Standard Error of Mean (SEM). The significance of the differences in mean values

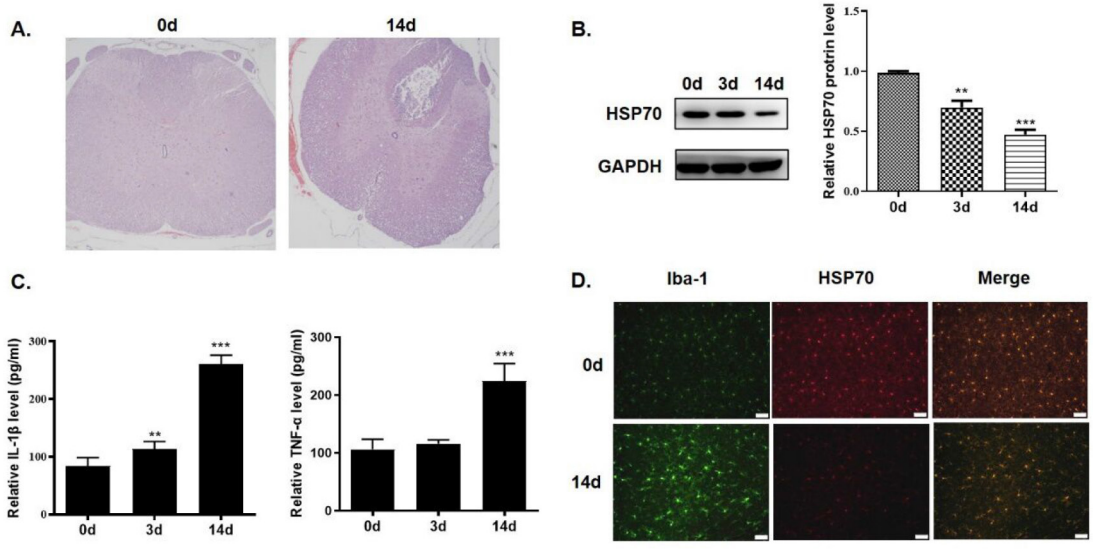

Figure 1. The expression of HSP70 decreases in the injured spinal cord. (A) H&E staining of spinal cord of sham group rats (Sham) and of spinal cord injury group rats (SCI) 14 days after Surgery. (B) HSP70 expression was detected by western blot at indicated day after SCI. Densitometric analysis of the expression levels of HSP70 were analyzed by ImageJ. (C) IL-1β and TNF-α level in the spinal cord was detected by Elisa at indicated day after SCI. (D). Immunofluorescence staining of Iba1 and HSP70 in spinal cord of normal mice and mice with SCI at 14 day (14d). n=6. Data are presented as the mean ± sem. **p<0.01, ***p<0.001.

between and within multiple groups was examined by one-way ANOVA followed by Duncan's multiple range test. Statistical significance was set at $p<0.05$.

Results

The expression of HSP70 decreases in the injured spinal cord

We established mouse spinal cord injury model. As shown in Figure 1A, the mice showed sever spinal cord injury in the SCI group. To investigate the expression of HSP70 in the process of SCI, we performed western blot and found that the expression of HSP70 decreased gradually at the day 1 to day 14 (Figure 1B). We then detected the inflammatory cytokines IL-1β and TNF-α level in the tissue homogenate. As shown in Figure 1C, IL-1β and TNF-α level in the spinal cord increased gradually at the day 1 to day 3.

As microglial is one of the main immunocytes activated after the spinal cord injury, we further performed confocal microscopy to investigate the expression of microglial maker, Iba1. As shown in Figure 1D, expression of Iba1 increased significantly at day 3 after the SCI. Furthermore, HSP70 showed a significant decrease of the expression and colocalization with Iba1.

HSP70 attenuates LPS-induced inflammation in microglia.

We then investigated the anti-inflammatory role of HSP70 *in vitro*. In microglial BV-2 cells, LPS induced significant decrease of HSP70 expression, which could be blocked by overexpression of HSP70 (Figure 2A, B). LPS induced the enhanced expression of IL-1β and TNF-α, while overexpression of HSP70 inhibited LPS-induced increase of IL-1β and TNF-α, indicating the anti-inflammatory effect of HSP70 (Figure 2C, D). NF-κB signaling pathway play a central role in regulating the inflammatory response[21]. We then investigated if NF-κB signaling was involved in the anti-inflammatory effect of HSP70. As shown in Figure 2E and F, phosphorylation of p65 increased during the process of SCI, indicating the activation of NF-κB signaling. We then isolated the nucleus and detected the change of p65 in the nucleus. In BV-2 cells, LPS induced increased nuclear translocation and phosphorylation of p65, which was inhibited by overexpression of HSP70 (Figure 3G-I).

HSP70 attenuates inflammatory response in microglia by inhibiting NF-κB activation

To further investigate the mechanism of the anti-inflammatory effect of HSP70 and its relationship with p65, we overexpressed HSP70 with or without p65 overexpression by using HSP70 and p65 overexpression lentivirus. We found that LPS increased the nuclear p65 level, which were inhibited by HSP70 overexpression (Figure 3A, B). Overexpression of p65 reversed the nuclear level of p65 inhibited by HSP70 (Figure 3A, B). HSP70 reduced the increased phosphorylation of p65 induced by LPS, and overexpression of p65 abolished the effect of HSP70 (Figure 3A, C). LPS decreased the HSP70 expression and overexpression of p65 showed no effect on the expression of HSP70 expression (Figure 3A, D). Besides, HSP70 inhibited the expression of LPS-induced

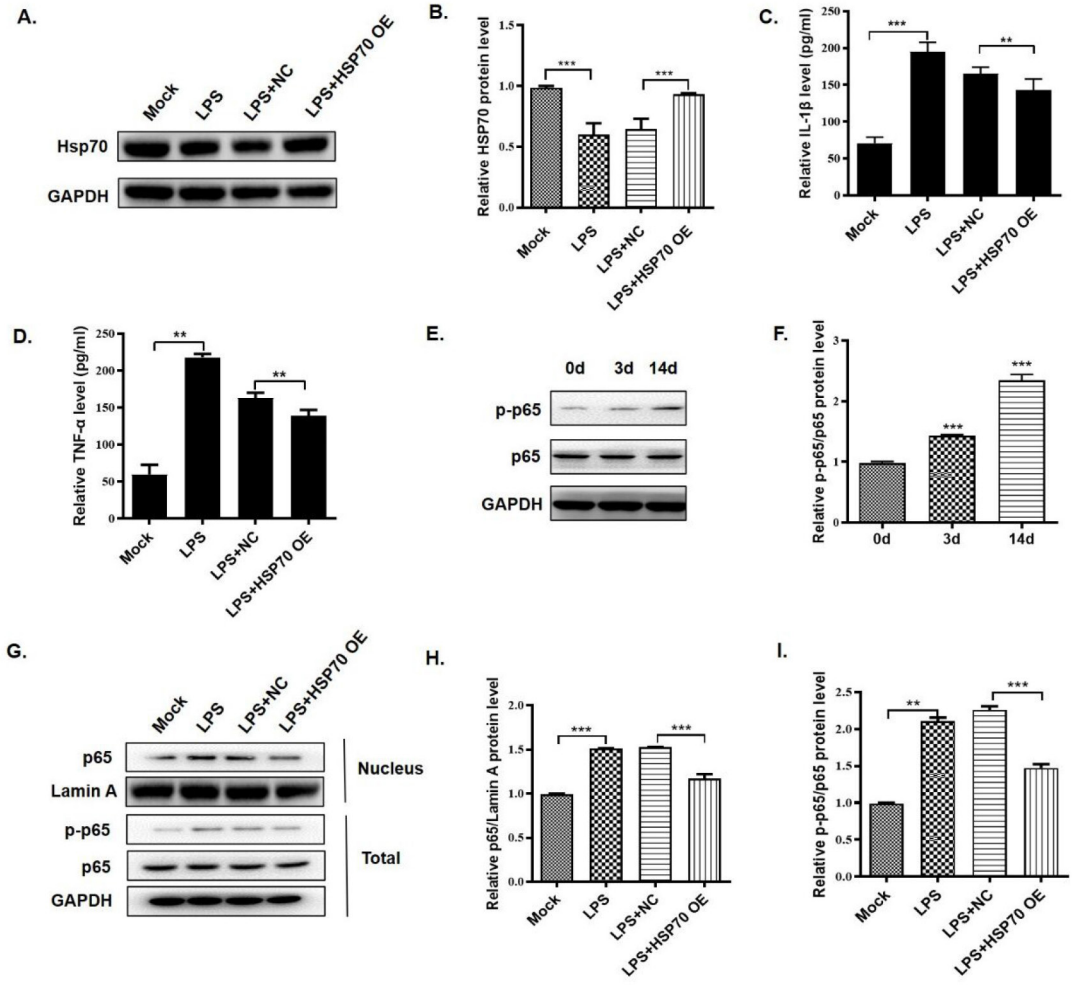

Figure 2. HSP70 attenuates LPS-induced inflammation in microglia. BV-2 cells treated with LPS (500 ng/mL) for 24 h with or without overexpression of HSP70. (A) HSP70 expression was detected by western blot. (B) Densitometric analysis of the expression levels of HSP70 were analyzed by ImageJ. (C, D) IL-1β and TNF-α level in the culture medium was detected by Elisa. (E) p65 protein expression and its phosphorylation were detected by western blot at indicated day after SCI. (F) Densitometric analysis of the expression levels of HSP70 were analyzed by ImageJ. (G) p65 expression in nucleus and phosphorylation level in BV2 cells were detected by western blot. (H, I) Densitometric analysis of the expression levels of nucleus p65 and p65 phosphorylation were analyzed by ImageJ. n=6. Data are presented as the mean ± sem. **p<0.01, ***p<0.001.

inflammatory cytokines TNF-α and IL-1β expression, while overexpression of p65 inhibited HSP70's anti-inflammatory effects (Figure 3E).

Overexpression of HSP70 alleviates the SCI in mice

To further investigate the effect of HSP70 on SCI, we performed behavioral experiment. HSP70 and p65 overexpression lentivirus were injected into the lesion site separately or jointly at a concentration of 8×10^5 units in a volume of 1 μl after SCI to overexpress HSP70 with or without overexpression of p65. Open-field locomotor function was tested using the BMS, from 1 day post injury through to the day 8. While motor function was only slightly impaired in sham-operated mice, a significant increase in hind limb motor disturbances was observed in the SCI operated mice, while overexpression of HSP70 showed a significant recovery in the motor function. (Figure 4A). Overexpression of p65 inhibited the recovery effect of HSP70 overexpression (Figure 4A). Overexpression of HSP70 inhibited the increased TNF-α and IL-1β expression in the spinal cord of SCI mice, while overexpression of p65 abrogated the effect of HSP70 (Figure 4B). Western blot showed that HSP70 expression decreased in the SCI and SCI+HSP70 OE+p65 OE group (Figure 4B, C). HSP70 overexpression decreased p65 phosphorylation in SCI group, indicating that HSP70 inhibited SCI-induced NF-κB activation (Figure 4B, D).

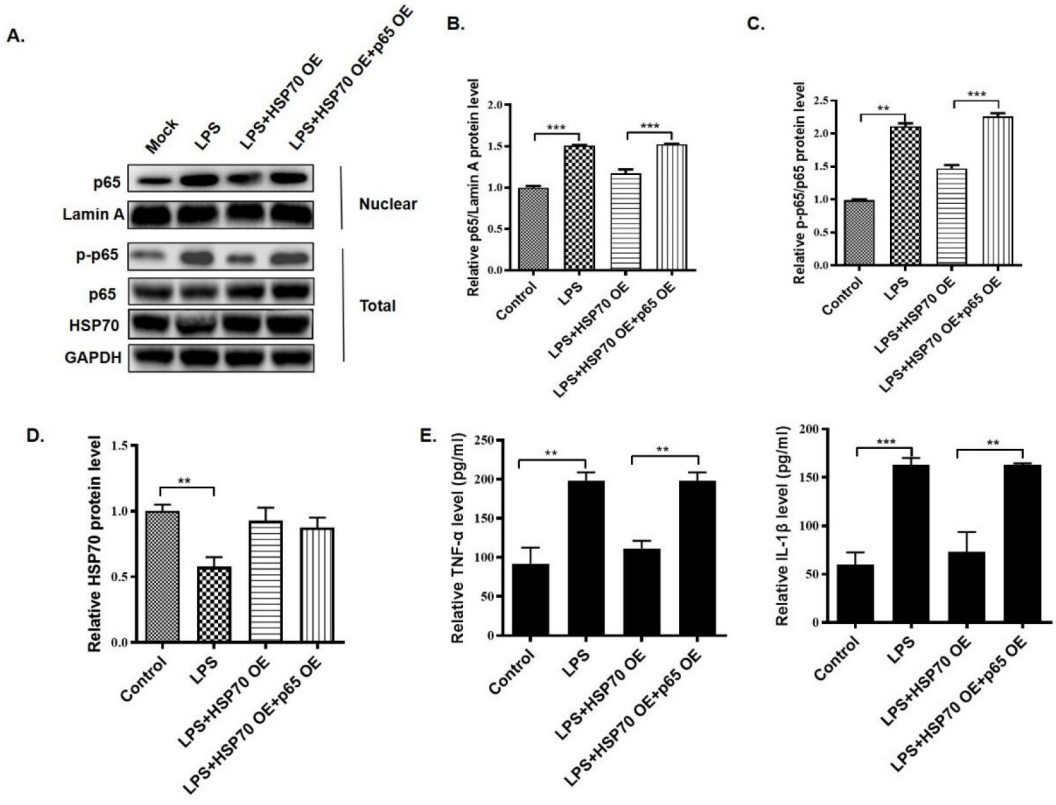

Figure 3. HSP70 attenuates inflammatory response in microglia by inhibiting NF-κB activation. BV-2 cells treated with LPS (500 ng/mL) for 24 h with or without overexpression of HSP70, or overexpression of HSP70 and p65 jointly. (A) HSP70 expression, p65 expression in nucleus and phosphorylation level in BV2 cells were detected by western blot. (B, C, D) Densitometric analysis of the expression levels of nucleus p65, p65 phosphorylation, and HSP70 were analyzed by ImageJ. (E) IL-1β and TNF-α level in the culture medium was detected by Elisa. n=6. Data are presented as the mean ± sem. **p<0.01, ***p<0.001.

Discussion

The estimated of spinal cord injury is 40 to 80 cases per million population. Spinal cord injury has a high personal and socio-economic impact[22-24]. Recovery of motor function after spinal cord injury for most people is not optimistic, and the nonsurgical therapies is rare in clinic treatment. The medicine used most for SCI patients is corticosteroid methylprednisolone which inhibits the early inflammatory reaction after SCI in human beings[25]. However, the severe adverse side effects including the increased risk of wound infection and metabolic complications of methylprednisolone inhibits its application for treating SCI patients, making it unqualified for being a standard therapy for SCI[26]. So, searching for new potential therapeutic targets and developing new drugs for SCI is of vital importance. In the present studies, we constructed the mice spinal cord injury model to investigate the mechanism and pathogenesis of SCI, and showed that HSP70 plays important roles in the pathogenesis of SCI.

It is reported that the molecular events that mediate the pathogenesis of SCI are logical targets for pharmacological manipulation and include glutamate accumulation, aberrant calcium fluxes, free radical formation, lipid peroxidation and generation of arachidonic acid metabolites[27]. Recently, molecular events related to glial activation and secondary inflammation have attracted more and more attention. It was reported that there is inflammation reaction following SCI, and reducing inflammation decreased the functional deficit after SCI[28,29]. In the present study, we found gradual increase of TNF-α and IL-1β level, indicating the increased inflammation response during SCI. HSP70 is a molecular chaperone protein, that serves a protective role in several different models of nervous system injury, especially on neurodegenerative disease[30]. However, its role for neuroprotection is debatable[31,32]. In the present study, we found an increase in Iba1 protein expression with decrease colocalization with HSP70 during the process of SCI by using confocal microscopy, suggesting that HSP70 reversely correlated with the activation of microglia.

NF-κB signaling pathway shows a significant activation following SCI. Whereas, targeting NF-κB signaling reduces inflammatory infiltration, apoptosis and improves mobility of SCI rats[33,34]. NF-κB activation was reported to induce spinal

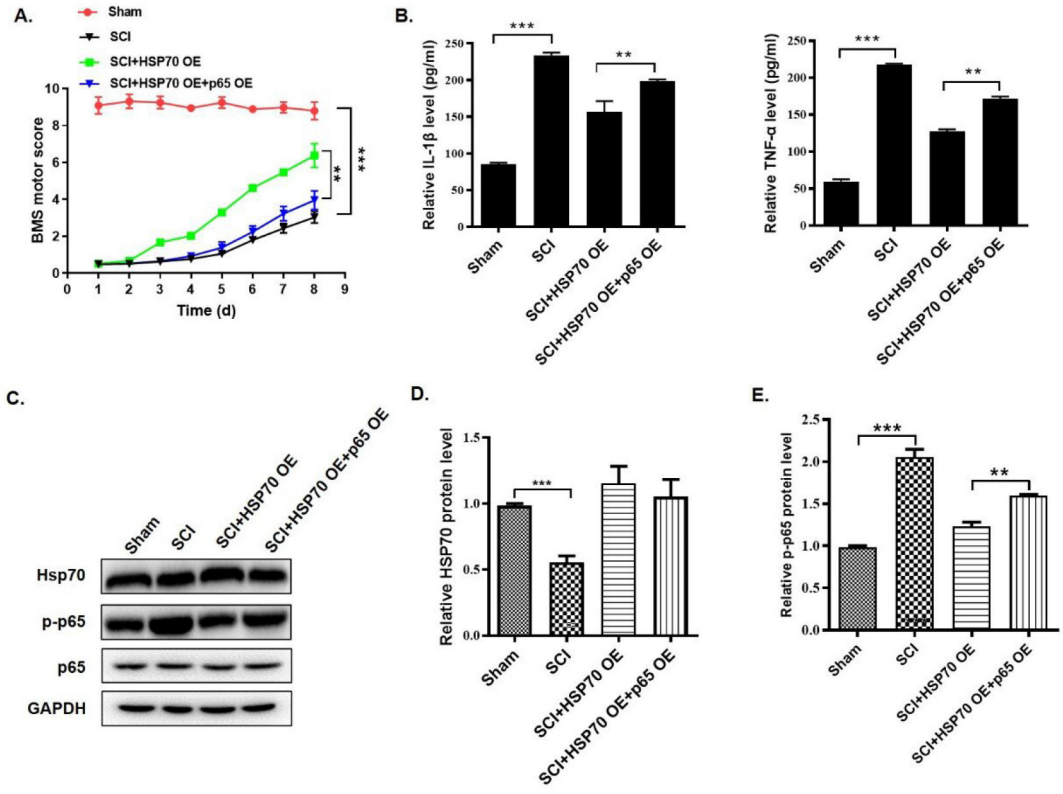

Figure 4. Overexpression of HSP70 alleviates the SCI in mice. (A) Basso Mouse Scale (BMS) recovery curves illustrate the extent of gross functional locomotor recovery in mice following SCI. (B) IL-1β and TNF-α level in the spinal cord was detected by Elisa at indicated day after SCI. (C) Western blot analysis of HSP70, p65, p-p65 and GAPDH protein. (D, E) ImageJ densitometric analysis of the expression levels of indicated proteins. n=6. Data are presented as the mean ± sem. *P<0.05.

cord microglia activation leading to neuropathic pain following spinal cord injury[35]. Suppressing microglial activation attenuates spinal cord injury-induced pain, and inhibition of microglial proliferation showed improvement of hind-limb motor function of SCI rats[36,37]. In the present studies, we found that HSP70 protein expression decreased after SCI, and in BV2 cells induced by LPS. Further studies showed that overexpression of HSP70 decreased the nuclear p65 protein level and p65 phosphorylation *in vitro*, and improve the mice recovery of motor function *in vivo*. Overexpression of p65 abolished the effect of HSP70 *in vitro* and *in vivo*. All the results indicated that HSP70 alleviated spinal cord injury, and its mechanism might be related to inhibition of NF-κB signaling pathway.

In summary, this study identified that HSP70 is involved in the pathological process of spinal cord injury, and upregulation of HSP70 protein expression alleviated the spinal cord injury via inhibition of NF-κB signaling pathway. These findings may provide a new approach to the treatment of spinal cord injury in the future.

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