

Original Article

HSP70 alleviates PC-12 cell oxidative damage caused by ROS through activation of Wnt/beta-catenin signaling pathway in spinal cord injury

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Abstract: Spinal cord injury (SCI) is a severe and worldwide clinical problem, researches focusing on SCI for overall analysis and understanding of the mechanism will be of great importance. In the present study, we sought to explore the protective effects of HSP70 on H₂O₂ induced the PC-12 cell proliferation reducing and apoptosis increasing. The 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide (MTT), flow cytometry and qRT-PCR were used to test the influence of HSP70 on cells viability, apoptosis and the ROS activity. The results showed that HSP70 promoted cell proliferation and reduced cell apoptosis, protected against PC-12 from ROS damage. Western blot results proved that overexpression of HSP70 could activate the downstream Wnt/beta (β)-catenin signaling pathway, inhibit apoptosis factor expression. In conclusion, HSP70 promoted cell proliferation and reduced cell apoptosis, protected PC-12 cells from ROS damage via Wnt/ β -catenin signaling pathway. This paper preliminary studied the role of HSP70 in nerve cells oxidative damage, which might provide theoretical basis for the treatment of SCI.

Keywords: Spinal cord injury, HSP70, ROS, cell apoptosis, cell viability

Introduction

Spinal cord injury (SCI) is an important clinical problem worldwide, and the pathogenesis of SCI is complicated and still not very clear [1]. SCI is a severe injury, inducing both immediate mechanical damage and subsequent tissue degeneration, the latter often involves of the occurrence of ischemia, edema, electrolyte disorder and other physiological and biochemical reactions [2, 3]. The neuroinflammation following SCI plays an important role during the secondary injury phase, which perpetuates neurodegeneration and cytotoxicity within the injured spinal cord [4]. Ischemia hypoxia and inflammation will promote production and release of reactive oxygen species (ROS), and large accumulation of ROS causes local tissue oxidative damage, nerve cells apoptosis and necrosis [5-7]. Looking for effective drugs to reduce secondary spinal cord neuron death has been a research hot spot for a long time.

Heat shock protein-70 kDa (HSP70) is one of the important member of heat shock transcription factor (HSFs) family, ubiquitously expressed in the cytosol, endoplasmic reticulum and mitochondria [8, 9]. HSP70 supports intracellular homeostasis and prevents protein damage after the temperature increasing and other stressful environmental stimuli [10]. Moreover, HSP70 is one kind of highly conserved molecular chaperone, it was also been reported to be expressed in different species, organization, as wells as many kinds of cells [11, 12]. It has been reported that HSP70 was up-regulated in keloid fibroblasts and keloid tissue, and the overexpression of it might be closely related to the excessive collagen production by keloid fibroblasts [13]. Additionally, animal experiments have proved that HSP70 participated in the inflammatory response and represented pharmacological targets of the intestinal anti-inflammatory drugs [14]. Under normal circum-

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stances, HSP70 was low-expressed, while in the condition of heat challenge, anoxia and other harmful stress, expression of HSP70 was induced to improve capability of anti-stress [15, 16].

Previous studies have made great efforts in the functional analysis of HSP70, but there was not an overall analysis or expression profiling of HSP70 in SCI. In this study, we focused on researching the role of HSP70 in PC-12 cells growth and development, which will be helpful to determine the functional characteristics of HSP70 in SCI progress. First, PC-12 cells were cultured carefully and been used throughout the study. Then, cells were stimulated by H_2O_2 to construct nerve oxidative damage model. Next, the MTT assay was used to test cell viability after overexpression of HSP70 in the H_2O_2 treated PC-12 cells. Meanwhile, apoptosis of PC-12 cells was measured by flow cytometry, separately. Finally, western blot results showed that the overexpression of HSP70 activated downstream Wnt/ β -catenin signaling pathway, inhibited apoptosis factor expression. The results showed that overexpression of HSP70 promoted cell viability, decreased cells apoptosis and ROS activity. Finally, the Wnt/ β -catenin signaling pathway was confirmed to be responsible for the HSP70 regulation in PC-12. Our findings provided theoretical basis and new insights to the research and treatment of SCI.

Materials and methods

Cell culture and H_2O_2 treatment

The PC-12 cells (Kunming Institute of Zoology, Kunming, China) that been kept by our laboratory were seeded onto flasks at a density of 1×10^4 cells/ml. It was maintained in DMEM within 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin, in a humidified incubator containing 5% CO_2 , at 37°C. Culture medium was changed every other day. For the H_2O_2 injury model, the cells were seeded in cell culture multi-well plates and treated with fresh medium containing 500 μ M H_2O_2 for 24 h [17].

MTT assay

The cell viability was determined using 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay as the described standard methods.

Apoptosis analysis

Flow cytometry analysis was used to test the apoptotic cells by using Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, China). Treated PC-12 cells were washed twice with cold PBS and resuspended in buffer. Flow cytometer (Beckman Coulter, USA) was used to differentiate apoptotic cells from necrotic cells of the adherent and floating cells.

Overexpression of HSP70

Adenovirus-CMV-HSP70 (Ad-HSP70) was purchased from Vector Biolabs. An adenovirus empty vector was used as a control (Ad-Control). Ad-Control or Ad-HSP70 were transfected into cells at 2.5 MOI for 24 h before H_2O_2 treatment [18].

Intracellular ROS assay

ROS was measured by flow cytometry using 2, 7-dichlorofluorescein diacetate (DCFH-DA) (Nanjing Jiancheng, China). The cells were seeded as described above, and co-incubated with serum-free culture medium containing 10 μ M DCFH-DA for 20 min, at 37°C, in dark. After washing with PBS, all samples that be collected by trypsin digestion method were centrifuged and the supernatants were removed. The cells were resuspended in 500 μ l PBS and the fluorescent intensities were measured by flow cytometer.

The qRT-PCR

Total RNA was isolated from transfected cells by using TRIzol reagent (Invitrogen) and treated with DNase I (Promega). Reverse transcription was performed by using the Multiscribe RT kit (Applied Biosystems) and random hexamers or oligo (dT). The reverse transcription conditions were 10 min at 25°C, 30 min at 48°C, and a final step of 5 min at 95°C [18]. For HSP70, the forward primer and reverse primer were 5'CTCGTACACCTGGATCAGCA'3 and 5'CGTGGAGGAGTTCAAGAGGA'3, respectively. For GAPDH, the forward primer and reverse primer were 5'GCACCGTCAAGGCTGAGAAC'3 and 5'TGGTGAAGACGCCAGTGGA'3, respectively.

Western blot

The protein samples used for western blotting were extracted using RIA lysis buffer (Beyotime

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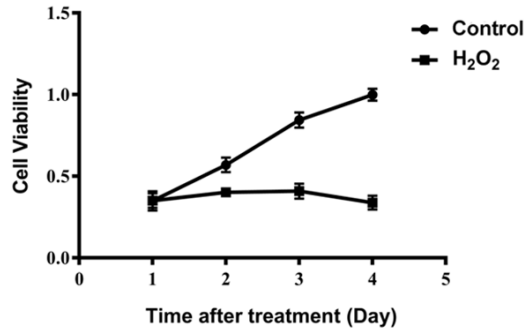


Figure 1. H₂O₂ decreased cell viability. The PC-12 cells were treated with H₂O₂ to induce oxidative injury. The cells viability was monitored after 1, 2, 3, 4 days, and the oxidative injury model was proved to be established successfully.

Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Guangzhou, China). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The western blot system was established using a Bio-Rad Bis-Tris Gel system according to instructions. Rabbit-anti-mouse HSP70 antibody was purchased from Santa Cruz (Santa Cruz, CA, USA); GAPDH antibody was purchased from Sigma. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1,000, and incubated with the membrane at 4°C overnight, followed by been washed and incubated with secondary antibody marked by horseradish peroxidase for 1 h at room temperature. After rinsing, the polyvinylidene fluoride (PVDF) membrane carried blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system, and then 200 µl immobilon of Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

Statistical analysis

All experiments were repeated three times. The results were presented as the mean ± standard deviation (SD). Statistical analyses were performed using SPSS 19.0 statistical software. Student's t-test was used for pairwise comparisons, one-way analysis of variance (ANOVA) was used for multi group comparisons. A *P* value of < 0.05 was considered statistically significant.

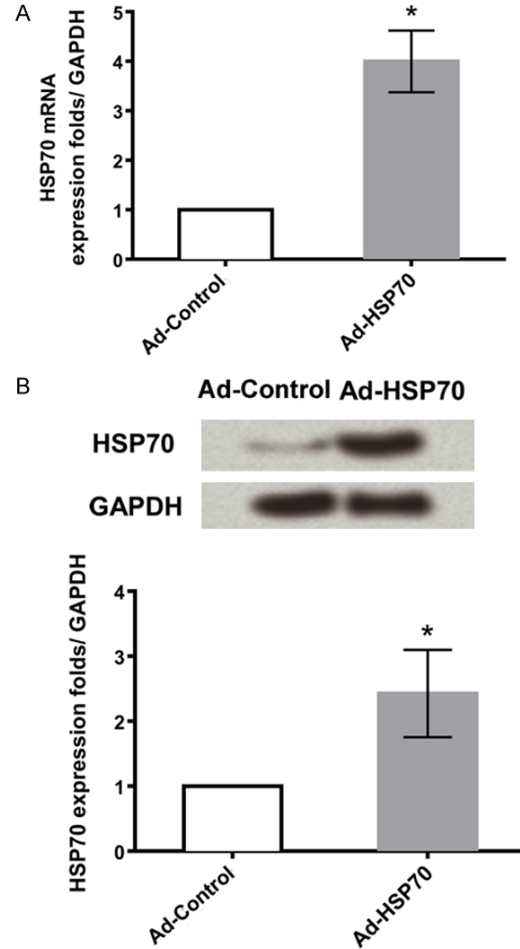


Figure 2. Ad-CMV-HSP70 successfully interfered expression of HSP70. The transfection of Ad-CMV-HSP70 upregulated the HSP70 expression in both mRNA (A) and protein levels (B). GAPDH acted as an internal control. *, *P* < 0.05.

Results

H₂O₂ induced PC-12 oxidative injury

First, we constructed H₂O₂ injury model in this part of work. PC-12 was treated with H₂O₂ to simulate neural cell oxidative damage, then the cell viability was monitored. The results showed that H₂O₂ caused cell oxidative damage and cell viability was declined (**Figure 1**).

Ad-CMV-HSP70 upregulated HSP70 expression

PC-12 cells were transfected with Ad-CMV-HSP70. The qRT-PCR and Western Blot results shown in **Figure 2A** and **2B** confirmed the over-expression of HSP70 after the transfection.

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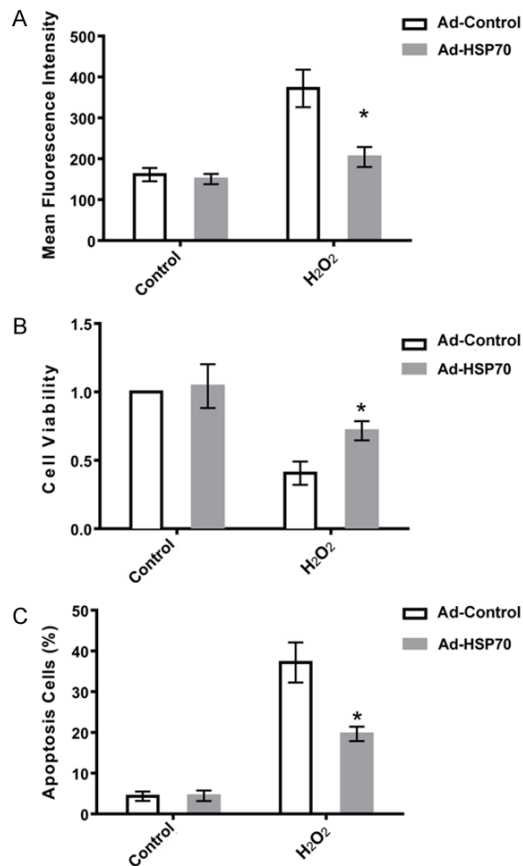


Figure 3. Effects of HSP70 on cell viability and apoptosis induced by H_2O_2 treatment of PC-12 cells. The PC-12 cells were transfected with Ad-CMV-HSP70 to upregulate expression of HSP70. The ROS level (A), cell viability (B) and cell apoptosis (C) were measured in PC-12 cells. *, $P < 0.05$.

Overexpression of HSP70 decreased the ROS activity of H_2O_2 treated cells

To make clear the effect of HSP70 on the ROS activity, PC-12 cells were transfected with Ad-CMV-HSP70 to upregulate the HSP70 expression. Then, cells were treated with H_2O_2 and measured ROS activity. As the results shown in **Figure 3A**, the ROS activity was tested before and after HSP70 overexpression, respectively. The ROS activity were increased after H_2O_2 treatment in both Ad-Control and Ad-HSP70 groups, while the Ad-HSP70 inhibited the increase trend at the same time ($P < 0.05$). Thus, overexpression of HSP70 inhibited ROS activity.

Overexpression of HSP70 increased cell viability after treated with H_2O_2

PC-12 cells were transfected with Ad-CMV-HSP70 to upregulate the HSP70 expression,

followed by H_2O_2 treatment in both Ad-Control and Ad-HSP70 groups. MTT assay was constructed to confirm the protective effects of HSP70 on cell viability. We found that H_2O_2 treatment decreased the cell viability, while the overexpression of HSP70 reduced the damaged caused by H_2O_2 and promoted cell viability (**Figure 3B**).

Overexpression of HSP70 decreased the apoptosis of cells treated with H_2O_2

The effect of HSP70 on cell apoptosis was measured by flow cytometry. We found that H_2O_2 treatment increased the cell apoptosis, while the overexpression of HSP70 protected cells from apoptosis which caused by H_2O_2 (**Figure 3C**). Thus, overexpression of HSP70 protected PC-12 cells from H_2O_2 -induced cell damage through inhibiting apoptosis.

The protective effect of HSP70 on PC-12 was related to Wnt/ β -catenin pathway

We explored whether Hsp70 was dependent upon Wnt/ β -catenin. Western Blot assay was used to detect the related factors through Wnt/ β -catenin and apoptosis pathway. Wnt3a, β -Catenin, Wnt5a expression in the Wnt/ β -catenin pathway were tested. Meanwhile, the activation of caspase3 and Bcl-2 which have been recognized as important factors that drive apoptosis were also measured in this study. The results showed that overexpression of HSP70 activated downstream factors of Wnt/ β -catenin signaling pathway, and inhibited expression of apoptosis factors (**Figure 4**). Thus, HSP70 might protect PC-12 cells from H_2O_2 induced damage by related with Wnt/ β -catenin signaling pathway.

Discussion

SCI leads to neurological complications and eventually paraplegia or quadriplegia, which is major contributor to SCI morbidity [19]. In addition, the underlying pathophysiological mechanism of SCI is very complicated. Although the understanding of SCI has been certainly increased, SCI treatment has always been a challenge for clinical practitioners and scientists [20].

Heat shock proteins represent key roles in the body's defense against various damaging elements, as well as the potential pathogenesis and treatment of several diseases [21]. HSPs

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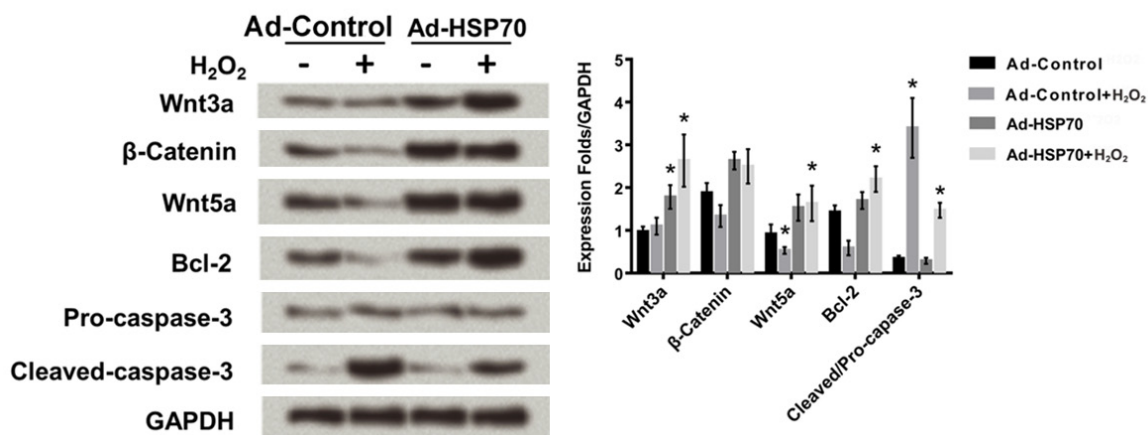


Figure 4. HSP70 protected PC-12 cells via Wnt/ β -catenin pathway. The expression levels of Wnt3a, β -Catenin, Wnt5a, Pro-caspase-3, Cleaved-caspase-3 and Bcl-2 were tested by western blotting. HSP70 activated downstream factors of Wnt/ β -catenin signaling pathway, and inhibited expression of apoptosis related factors. GAPDH acted as an internal control. *, $P < 0.05$.

have been proved to be present in all cells and were upregulated during inflammation, several studies have showed the immunoregulatory effects of HSPs and their protective effects on inflammation [22]. As an important member of molecular chaperone family, Hsp70 involved in the proper folding of various proteins, the tumor cell survival, and increasing the drug resistance of various cancer types [23, 24]. However, there was no clear understanding between the HSP70 expression and SCI. In this study, we carried out a preliminary exploration about it.

Many accumulated evidences showed that ROS excessive was a prominent feature in many neurodegenerative diseases, which eventually contributed to neuronal cells injured or died [25, 26]. Many researches have confirmed that H₂O₂ could lead to oxidative stress and induced the apoptosis of cells [27]. So we used H₂O₂ to induce neuronal cell damage of PC-12 cells and tested the bioactivity of PC-12 cells after HSP70 interference *in vitro* in order to investigate the correlation between HSP70 and SCI. In the present study, the ROS levels in PC-12 cells were increased after H₂O₂ treatment, and this increasing trend was inhibited in HSP70 overexpression group. Thus, overexpression of HSP70 inhibits ROS activity. MTT and flow cytometry assay results confirmed that HSP70 could promoted PC-12 cells viability, and inhibit cells apoptosis after H₂O₂ damage.

The Wnt/ β -catenin signaling pathway is an evolutionarily conserved pathway that plays key

roles in embryonic development and adult homeostasis, including differentiation and proliferation [28-30]. It has been demonstrated that heme oxygenase-1, which was involved in biological processes including anti-oxidant and anti-apoptosis, prevented renal tubulointerstitial fibrosis by regulating Wnt/ β -catenin signaling [31]. In the further research of this study, we monitored the expression changes of Wnt3a, β -Catenin, Wnt5a, the activation of caspase3 and Bcl-2, and finally found that HSP70 activated downstream of Wnt/ β -catenin signaling pathway, and inhibit expression of apoptosis related factors. So, it could be concluded that HSP70 protected PC-12 against oxidative injury via relating to Wnt/ β -catenin signaling pathway.

In conclusion, HSP70 promoted cell viability and reduced cell apoptosis, also protected PC-12 against ROS damage via Wnt/ β -catenin signaling pathway. This paper preliminary studied role of HSP70 in nerve cells oxidative damage, which might provide theoretical basis for the treatment of SCI.

Disclosure of conflict of interest

None.

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