Lycium barbarum polysaccharide protects against neurotoxicity via the Nrf2-HO-1 pathway

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Abstract. The incidence of neurodegenerative diseases including Alzheimer's and Parkinson's disease has markedly increased over the past few decades. Oxidative stress is considered to be a common pathophysiological condition resulting in neurotoxicity. Lycium barbarum polysaccharide (LBP) is the major active component of Lycium barbarum L., which exhibit potent antioxidant activity. The current study investigated the neuroprotective effects of LBP in H_2O_2-treated PC12 cells *in vitro* and in $CoCl_2$-treated rats *in vivo*. It was determined that LBP concentration-dependently reversed the H_2O_2-induced increase in reactive oxygen species (ROS) levels, decrease in cell viability, increase in TUNEL-stained cells, increase in caspase-3 and -9 activity and decrease in mitochondrial membrane potential, indicating the amelioration of mitochondrial apoptosis. Furthermore, LBP inhibited the H_2O_2-induced decrease in nuclear factor erythroid 2-related factor 2 (Nrf)2 and heme oxygenase (HO)-1 expression and binding of Nrf2 to the promoters of HO-1. Silencing of Nrf2 and inhibition of HO-1 by zinc protoporphyrin IX (ZnPP) reversed the protective effects of LBP against H_2O_2-resulted neurotoxicity in PC12 cells. In $CoCl_2$-treated rats, it was demonstrated that LBP decreased brain tissue apoptosis, reduced the time spent by rats finding the platform site, decreased escape latencies and reduced the distance traveled to find the platform. In addition, LBP inhibited the $CoCl_2$-induced decrease of Nrf2 and HO-1 expression. Administration of ZnPP also suppressed the protective effects of LBP against $CoCl_2$-resulted neurotoxicity in rats. Thus, the current study indicated that LBP exhibits protective effects against neurotoxicity by upregulating Nrf2/HO-1 signaling. These data may increase understanding regarding the neuroprotective activities of LBP.

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Introduction

The incidence of neurodegenerative diseases, such as Alzheimer's disease, has increased dramatically over the past few decades (1,2). Although the molecular mechanism of neurodegenerative diseases is not well understood, oxidative stress is considered to be a common pathophysiological condition that results in neurotoxicity (3,4). The redox system consists of a free radical/reactive oxygen species (ROS)-generating system, oxidants and antioxidant systems (5). Following damage to the antioxidant system or an increase in ROS generation, the balance of the redox status may be disrupted, leading to the development of oxidative stress (5). The central nervous system is particularly susceptible to ROS-induced damage and numerous studies have demonstrated that oxidative stress is closely associated with the development of neurodegenerative diseases. Greilberger *et al* (6) compared the redox state of the blood between healthy individuals and patients with neurodegenerative diseases. Compared with healthy controls, there were significant elevations in malondialdehyde, protein carbonylation and oxidized albumin levels in patients with neurodegenerative diseases, indicating that there is an association between oxidative stress and the development of neurodegenerative diseases (6). Furthermore, 4-hydroxynonenal-modified proteins have been detected in patients with mild cognitive impairments and throughout the course of Alzheimer's disease (7). In addition, oxidative stress may serve an important role in dopaminergic neurotoxicity (8) and dopamine metabolism-induced alterations in redox status may increase the risk of the onset or progression of Parkinson's disease (9).

The fruits of Lycium barbarum L. (family Solanaceae), more commonly known as Goji berry or wolfberry, have been used in traditional Chinese herbal medicine for thousands of years. Lycium barbarum polysaccharide (LBP) is the major active component found in the fruits. It has been demonstrated that LBP possesses various biological activities, including antioxidant, anti-cancer, anti-inflammatory, anti-aging and immune-regulatory activities (10-15). In particular, it has recently been demonstrated that LBP exhibits potent neuroprotective effects *in vivo* and *in vitro* (16-18).

Bie *et al* (16) reported that LBP improved bipolar pulse current-induced microglia cell injury via modulation of autophagy. Furthermore, LBP improved traumatic cognition by reversing the imbalance of apoptosis/regeneration in hippocampal neurons following stress (17). Wang *et al* (18) determined that LBP prevented focal cerebral ischemic injury by inhibiting neuronal apoptosis in mice. However, the mechanism of the neuroprotective effect of LBP remains unclear.

The present study aimed to investigate the mechanism of the LBP-exerted protective effect against oxidative stress-induced neurotoxicity *in vivo* and *in vitro*. The results demonstrated that LBP exhibited neuroprotective activity via activation of nuclear factor erythroid 2-related factor 2 (Nrf) 2/heme oxygenase (HO)-1 signaling.

Materials and methods

Reagents. β-actin (sc-130300) and Nrf2 (sc-722) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and the HO-1 (RT1270) antibody was purchased from Epitomics; Abcam (Cambridge, MA, USA). MTT and $CoCl_2$ were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Lipofectamine® 2000, the intracellular superoxide probe dihydroethidium (DHE) and Rhodamine (Rho) 123 were all purchased from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Cell culture and treatment. PC12 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.), 100 μg/ml penicillin and 100 U/ml streptomycin at 37˚C with 5% CO_2 in a humidified incubator. Throughout the experimental treatments, cells were exposed to H_2O_2 in the presence or absence of LBP in serum-free DMEM.

Cell transfection. To test the role of Nrf2 in LBP-exerted protective effect, PC12 cells were transfected with small interfering RNA (siRNA) of Nrf2 and control siRNA before the treatment of H_2O_2 and LBP. Nrf2 siRNA and control siRNA were purchased from Santa Cruz Biotechnology (sc-156128). Transfection was conducted using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Determination of cell viability. Cells were seeded in a 96-well plate at a density of 1×10^4 cells/well. Cells in the exponential phase were exposed to 0, 50, 100, 200 or 400 μM H_2O_2 and/or 125, 250, 500, 800 or 1,000 μg/ml LBP for 24 h. In some experiments, cells were transfected with Nrf2 siRNA of Nrf2 and control siRNA for 48 h prior to treatment. Cells were then exposed to H_2O_2 and/or LBP in the presence of zinc protoporphyrin IX (ZnPP) (10 μM; Sigma-Aldrich; Merck KGaA), an inhibitor of HO-1. Following the experiments, cell viability was determined by MTT assay. The purple formazan was dissolved in dimethyl sulfoxide. Absorbance was measured at 550 nm and the results are presented as folds of the control cells without H_2O_2 treatment.

Determination of apoptosis. Cells (5×10^5) were seeded in culture dishes and treated with 400 μM H_2O_2 in the presence or absence of 125, 250 or 500 μg/ml LBP with or without ZnPP for 24 h. In some experiments, cells were transfected with siRNAs for 48 h prior to treatment. Following treatment, cells were fixed with 4% formaldehyde for 15 min at room temperature. Following washing with PBS, the cells were covered with proteinase K solution for 15-20 min. After another PBS wash, the cells were covered with the TUNEL reaction mixture (Roche Applied Science, Penzberg, Germany) and incubated for 1 h in the dark. DAPI counterstaining (10 min at room temperature) was followed by a final PBS wash, and tissue sections were then examined and photographed using confocal microscopy. The average number of fluorescent dots in three images from each treatment group was calculated. Results were expressed as folds of the control.

Determination of caspase activity. Activities of caspase 3 and 9 were determined using a Caspase 3 Activity Assay kit (C1115) and Caspase 9 Activity Assay kit (C1157; Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol.

Determination of ROS levels. ROS levels were measured using oxidation-sensitive probes. Cells were incubated with 10 μM DHE for 20 min at 37˚C in the dark and were subsequently observed under a confocal fluorescence microscope. In addition, cells were also incubated with 10 μM 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA, an intracellular hydrogen peroxide probe) (Sigma-Aldrich; Merck KGaA) for 30 min at 37˚C in the dark. Subsequently, cells were washed twice with PBS and analyzed using a flow cytometer (BD Accuri™ C6, BD Biosciences, Franklin Lakes, NJ, USA) and BD Accuri C6 software (BD Biosciences). The results were expressed as folds of the control.

Mitochondrial membrane potential. After the treatment of H_2O_2 and LBP, cells were stained with Rhodamine 123 (10 μM) for 20 min at 37˚C in the dark. Then the fluorescence was observed under a confocal fluorescence microscope.

Determination of transcription activity. A reporter gene assay was conducted to evaluate the transcriptional activity of Nrf2. PC12 cells (5×10^4) were seeded into 12-well plates. Subsequently, cells were transfected with 0.2 μg antioxidant response element (ARE)-Luc constructs (Beyotime Institute of Biotechnology, Haimen, China) using Lipofectamine 2000, according to the manufacturer's protocol. pRL-null plasmid (50 ng) encoding Renilla luciferase was included in all samples to ensure transfection efficiency. A total of 48 h after transfection, cells were treated with 400 μM H_2O_2 in the presence or absence of 125, 250 or 500 μg/ml LBP for 24 h. Firefly and Renilla luciferase activities were detected sequentially using the Dual-Glo™ Luciferase assay system (Promega Corporation, Madison, WI, USA).

Chromatin immunoprecipitation (ChIP) assay. Cells were treated with 400 μM H_2O_2 in the presence or absence of 500 μg/ml LBP for 24 h. Subsequently, a ChIP assay was conducted to examine the binding of Nrf2 in HO-1 promoters.

Following the experiment, cells were washed, fixed in 1% formaldehyde for 10 min at room temperature and sonicated (on 3 sec and off 10 sec for 10 times at room temperature) to shear chromatin. Nrf2 antibody (dilution 1:50) was added to the cleared lysate and binding was allowed to proceed at 4˚C overnight. The complex was eluted and the released DNA was amplified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) as stated below.

RNA isolation and RT-qPCR. Total RNA was isolated from cells and tissues using Total RNA Isolation kit (Tiangen Biotech, Co., Ltd., Beijing, China) according to the manufacturer's instructions. A total of 0.5 μg RNA was reverse transcribed using the First Strand cDNA synthesis kit (Takara Bio, Inc., Otsu, Japan). qPCR was performed to precisely quantify Nrf2 and HO-1 using SYBR Green reagent (Takara Bio, Inc.). Amplification was performed with an initial step at 94˚C for 5 min, followed by 40 cycles of denaturation at 94˚C for 30 sec, annealing at 63˚C for 30 sec and extension at 72˚C for 10 sec. The $2^{-\Delta\Delta Cq}$ method was used to determine gene expression compared with the endogenous controls (GAPDH) (19). The primers for qPCR analysis were as follows: Nrf2, forward, 5'-ATTGCCTGTAAGTCCTGGTCA-3', reverse, 5'-ACTGCTCTTTGGACATCATTTCG-3'; HO-1, forward, 5'-TTCCTGGACTGATCCCAATTCTG-3', reverse, 5'-CTTGGAAGCCACAGAAATGCAG-3'; GAPDH, forward, 5'-GCACCGTCAAGGCTGAGAAC-3', reverse, 5'-TGGTGAAGACGCCAGTGGA-3'.

Protein extraction and western blotting. Following treatment, cells were lysed and cytoplasmic and nuclear proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction reagents (Thermo Fisher Scientific, Inc.). Protein concentration was determined using the BCA assay. Lysates containing 20 μg protein were boiled at 100˚C for 5 min, separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to PVDF membranes. Membranes were blocked in 8% skim milk in TBS buffer for 30 min at 37˚C. Subsequently, membranes were incubated with the appropriate primary antibodies (Nrf2, HO-1 and β-actin, dilution: 1:500) overnight at 4˚C and washed four times with TBST for 10 min each time. Following washing, membranes were further incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit antibody, dilution: 1:5,000) (32260; Invitrogen, Thermo Fisher Scientific, Inc.) for 30 min at 37˚C and then washed another four times. Membranes were visualized using Immobilon Western Chemiluminescent HRP Substrate (Merck KGaA). Bands were captured using a VersaDoc image analysis system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and quantified with Quantity One software v.4.62 (Bio-Rad Laboratories, Inc.).

Animal treatment. All animal care was conducted in accordance with the guidelines for the Care and Use of Laboratory Animals (15) and the principles presented by School of Medicine, Xi'an Jiaotong University (Shaanxi, China). The present study was approved by the Ethics Committee of Xi'an High-Tech Hospital. All efforts were made to reduce suffering and the number of animals used. A total of 64 adult male Wistar rats weighing 250-300 g (8-10 weeks old) were obtained from the Animal Centre of the School of Medicine, Xi'an Jiaotong University. Rats were housed in a controlled environment, with a 12-12 h light/dark cycle, controlled humidity (50%) and temperature (24˚C), and had free access to standard laboratory rat food and water.

Rats were randomly divided into four groups with 16 animals in each group: Control group, $CoCl_2$ group, $CoCl_2$+LBP group and $CoCl_2$+LBP+ZnPP group. Surgery was conducted as previously reported (20-22). Control group: Rats received saline solution. $CoCl_2$ group: Rats received 50 mM $CoCl_2$. $CoCl_2$+LBP group: Rats received 50 mM $CoCl_2$ and LBP (100 mg/kg) injection. $CoCl_2$+LBP+ZnPP group: Rats received 50 mM $CoCl_2$, LBP (100 mg/kg) injection and ZnPP (10 mg/kg) injection. Briefly, rats were placed in a stereotaxic apparatus and received a unilateral lesion by drilling a small hole on the skull in the frontoparietal cortex at Bregma-1.30 mm. Subsequently, a sterile solution of $CoCl_2$ (50 mM) or an equivalent volume of saline solution was injected in the right hemisphere, 1 mm below the pia level in the cerebral cortex (layers 3-4) with a Hamilton syringe. The $CoCl_2$ solution was adjusted to reach a physiological osmolarity of 310 mOsm/kg. Following the injection, the lesion site was repaired using the temporal muscle and the attached fascia. During the surgery period, the body temperature of the animals was maintained using a heating pad. Surgical procedures were performed under anesthesia induced with 8% (v/v) sevofluorane inhalation (the dose was selected by preliminary experiments). Concurrent preemptive analgesia was used and a precision vaporiser was used to perform anaesthesia and each animal was put in a separate cage for recovery. Following 2 days recovery, rats in $CoCl_2$+LBP group and $CoCl_2$+LBP+ZnPP group received intraperitoneal LBP (100 mg/kg) and rats in $CoCl_2$+LBP+ZnPP group received ZnPP (10 mg/kg) injection once a day for 10 days. After the treatment, 8 rats in each group were sacrificed using intraperitoneal injection of an overdose of sodium pentobarbital (200 mg/kg; Merck KGaA). Frozen brain sections (5 μm) were cut and then fixed in 4% formaldehyde overnight at room temperature for the determination of apoptosis.

Morris water maze (MWM) test. Immediately after the treatment, an MWM test was conducted to evaluate spatial learning and memory abilities, as previously reported (22,23). The maze was a black circular pool (120 cm in diameter and 45 cm high) divided into four quadrants, with a depth of 30 cm and filled with clear tap water at a temperature of 27±0.5˚C. A black platform 10 cm in diameter was located 2 cm below the surface of the water approximately in the middle of one of the four quadrants. Each rat's path in the pool was recorded with Videomex Water Maze Monitoring Software BL-420 (Chengdu Taimeng Technology Co., Ltd., Chengdu, China) and analyzed off-line. Experiments were conducted in a soundproof room and the light source and surrounding environment remained unaltered between each experiment.

During the first 5 days, rats underwent four swimming trials per day, with each trial period limited to 60 sec. In the trials, the time taken to reach the platform, the swimming distance and the speed were recorded. If the rat did not find the platform within the set time, the computer would stop tracking and record the time as 60 sec. If the platform was found within the 60 sec, the rat would be allowed to remain

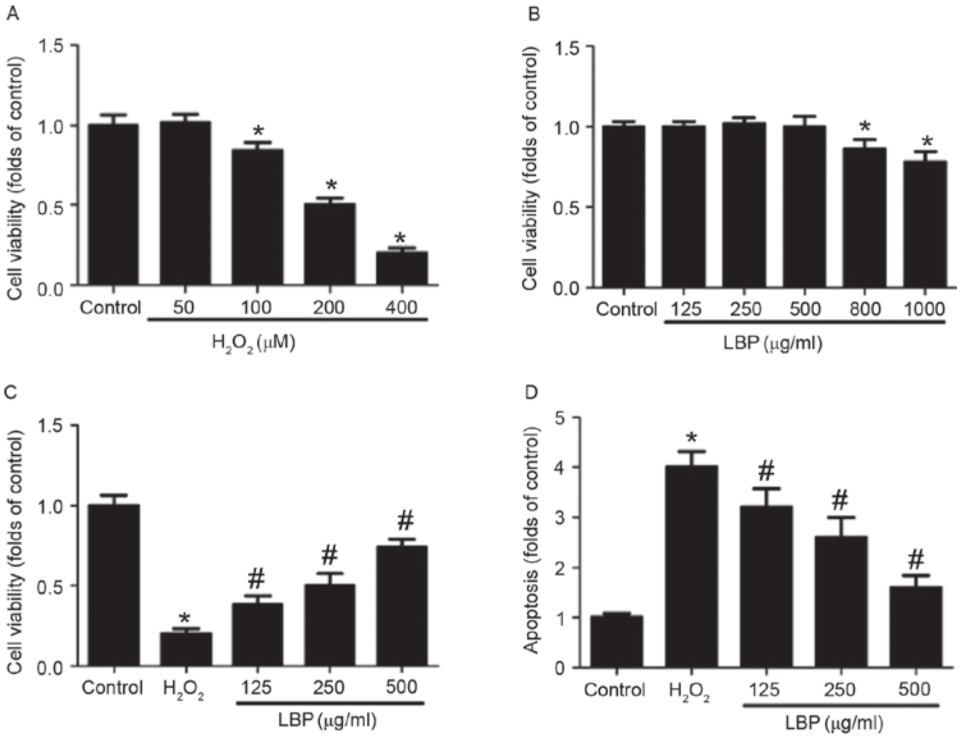

Figure 1. Effect of LBP on H_2O_2-induced apoptosis. PC12 cells were incubated with (A) 0, 50, 100, 200 or 400 μM H_2O_2 or (B) 0, 125, 250, 500, 800 or 1,000 μg/ml LBP for 24 h. Cell viability was determined by MTT assay and the results are presented as folds of control. (C) PC12 cells were incubated with 400 μM H_2O_2 in the presence or absence of 125, 250 or 500 μg/ml LBP for 24 h. Cell viability was determined by MTT assay and the results are presented as folds of control. (D) Apoptosis was determined by TUNEL staining and the results are presented as folds of control. *P<0.05 vs. control; $^{\#}$P<0.05 vs. H_2O_2 treatment. LBP, Lycium barbarum polysaccharide.

on the platform for 10 sec. Otherwise, the rat would be guided to the platform to remain for 10 sec. On day 6, a probe trial test was conducted. The platform was removed on day 6 for a 60 sec exploration test to record the crossing index to the previous platform site.

Statistical analysis. Statistical analysis was performed using GraphPad Software 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). The results are presented as the mean ± standard error of the mean. The statistical significance of differences between groups was analyzed via one-way analysis of variance followed by a Dunnett's t-test for multiple comparisons. P<0.05 was considered to indicate a significant difference.

Results

LBP inhibits H_2O_2-induced mitochondrial apoptosis in PC12 cells. PC12 cells were incubated with 0, 50, 100, 200 or 400 μM H_2O_2 for 24 h and the results indicated that 100-400 μM H_2O_2 significantly decreased cell viability in a concentration-dependent manner (P<0.05; Fig. 1A). Treatment with 400 μM H_2O_2 decreased cell viability to 20% of the control; thus 400 μM H_2O_2 was used to induce oxidative injury in PC12 cells in subsequent experiments. PC12 cells were incubated with 0, 125, 250, 500, 800 or 1,000 μg/ml LBP for 24 h and the results indicated that LBP significantly decreased cell viability in a concentration-dependent manner at concentrations >500 μg/ml (P<0.05; Fig. 1B). Thus, a concentration of 125-500 μg/ml LBP was selected to examine the effect of LBP on neurotoxicity in PC12 cells in subsequent experiments.

LBP dose-dependently reversed the decrease in cell viability induced by H_2O_2 (P<0.05; Fig. 1C). Furthermore, LBP treatment decreased TUNEL-positive cell numbers in H_2O_2-treated cells in a concentration-dependent manner (P<0.05; Fig. 1D). H_2O_2-induced increases in caspase-3 and -9 activity were significantly reversed by LBP in a concentration-dependent manner (P<0.05; Fig. 2A and B). Mitochondrial membrane potential was determined using Rho 123 staining and fluorescence was observed using a confocal microscope. The results demonstrated that LBP markedly inhibited the H_2O_2-induced decrease in Rho 123 fluorescence, indicating a reversal in the decrease of the mitochondrial membrane potential (Fig. 2C). ROS levels were determined by DHE and DCFH-DA staining. The H_2O_2-induced increase in DHE staining was markedly inhibited by LBP (Fig. 2D). Furthermore, LBP significantly suppressed the H_2O_2-induced increase of DCFH-DA-positive cell numbers in a concentration dependent manner (P<0.05; Fig. 2E). These results indicate that LBP concentration-dependently inhibits H_2O_2-induced mitochondrial apoptosis.

LBP inhibits the H_2O_2-induced decrease of Nrf2/HO-1 signaling in PC12 cells. The potential mechanism responsible for the LBP-induced protective effects against H_2O_2-induced neurotoxicity in PC12 cells was subsequently evaluated. Levels of Nrf2 and HO-1 mRNA and protein were measured. The results demonstrated that the H_2O_2-induced decreases in Nrf2 and HO-1 mRNA expression were significantly reversed by LBP in a concentration-dependent manner (P<0.05; Fig. 3A and B). LBP also markedly reversed the decrease in Nrf2 and HO-1 protein expression induced by H_2O_2 (Fig. 3C).

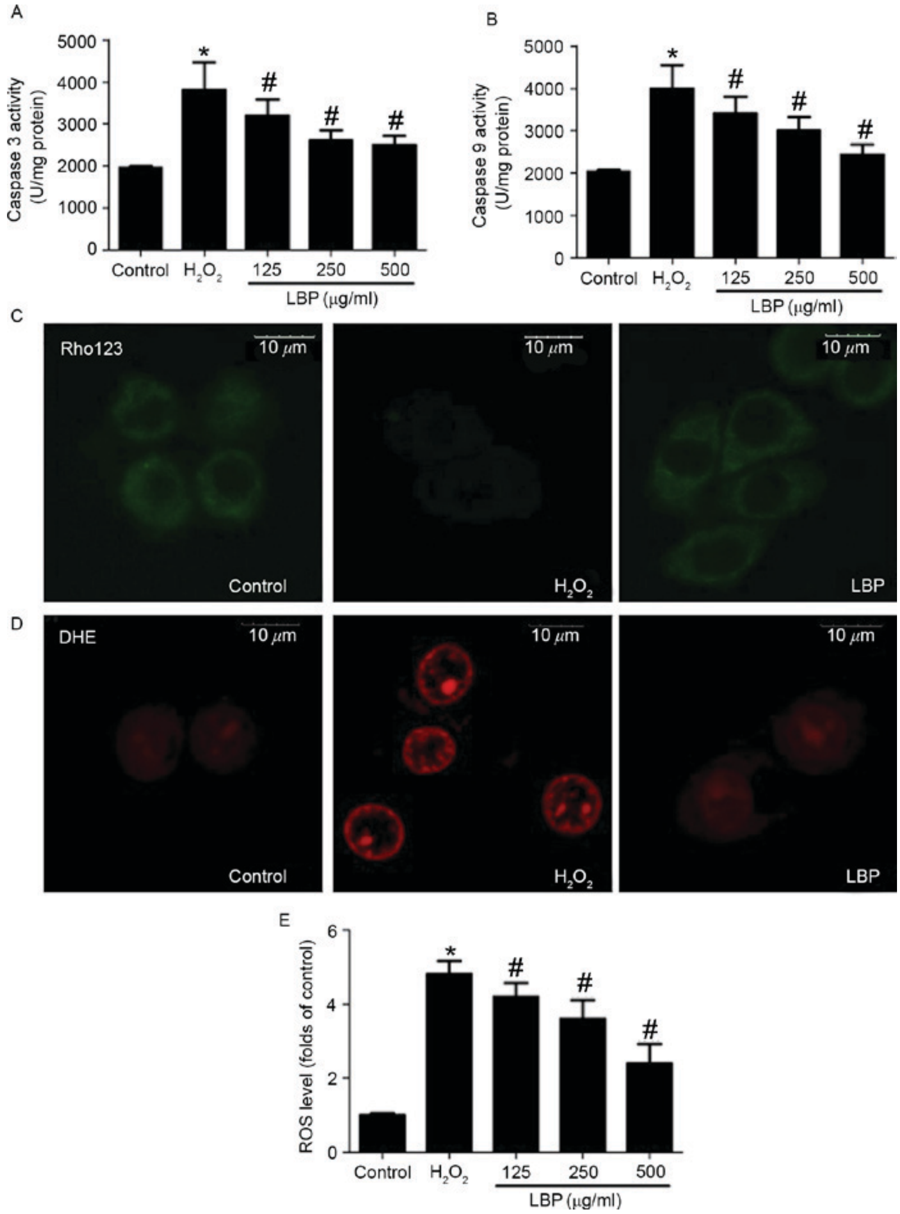

Figure 2. Effect of LBP on the H_2O_2-induced mitochondrial apoptotic pathway. PC12 cells were incubated with 400 μM H_2O_2 in the presence or absence of 125, 250 or 500 μg/ml LBP for 24 h. (A) Caspase-3 and (B) -9 activities were measured using commercial kits and expressed as U/mg protein. (C) PC12 cells were incubated with 400 μM H_2O_2 in the presence or absence of 500 μg/ml LBP for 24 h. Mitochondrial membrane potential was detected using Rho123 staining. Representative images are presented. ROS levels were determined by DHE and DCFH-DA staining. (D) For DHE staining, a representative image is presented. (E) For DCFH-DA staining, fluorescence was analyzed by flow cytometry and the results are presented as folds of control. $^*P<0.05$ vs. control; $^\#P<0.05$ vs. H_2O_2 treatment. LBP, Lycium barbarum polysaccharide; ROS, reactive oxygen species; DCFH-DA, 7-Dichlorodihydrofluorescein-diacetate; DHE, dihydroethidium; Rho123, Rhodamine 123.

ARE-luciferase activity was significantly reduced by H_2O_2 (P<0.05), however this reduction was reversed by LBP (P<0.05; Fig. 3D). The results of the ChIP assay identified that LBP significantly reversed the H_2O_2-induced decrease of Nrf2 binding to the promoters of HO-1 (P<0.05; Fig. 3E). Cells were transfected with siNrf2 and then exposed to H_2O_2 in the presence or absence of LBP. The results determined that the LBP-induced increase of HO-1 expression in H_2O_2-treated cells was significantly inhibited by Nrf2 silencing induced by siNrf2 (P<0.05; Fig. 3F). These results suggest that Nrf2/HO-1 signaling is involved in the LBP-induced protective effects against neurotoxicity induced by H_2O_2 and indicate that LBP inhibits the H_2O_2-induced decrease of Nrf2/HO-1 signaling in PC12 cells. To determine whether the upregulation of Nrf2/HO-1 signaling is involved in the protective effect of LBP, cells were transfected with siNrf2, then exposed to H_2O_2 in the presence or absence of LBP with or without ZnPP, an inhibitor of HO-1. It was demonstrated that in H_2O_2-treated cells, the LBP-induced decrease in ROS levels (Fig. 4A), increase in cell viability (Fig. 4B) and decrease in cell apoptosis (Fig. 4C) was significantly reversed by siNrf2 and ZnPP (all P<0.05). These results indicate that Nrf2/HO-1 signaling is involved in the LBP-induced protective effects against neurotoxicity induced by H_2O_2 in PC12 cells.

Neuroprotective effect of LBP on $CoCl_2$ in vivo. Subsequently, the neuroprotective effect of LBP against $CoCl_2$ *in vivo* was evaluated. Rats were injected with $CoCl_2$ to construct an

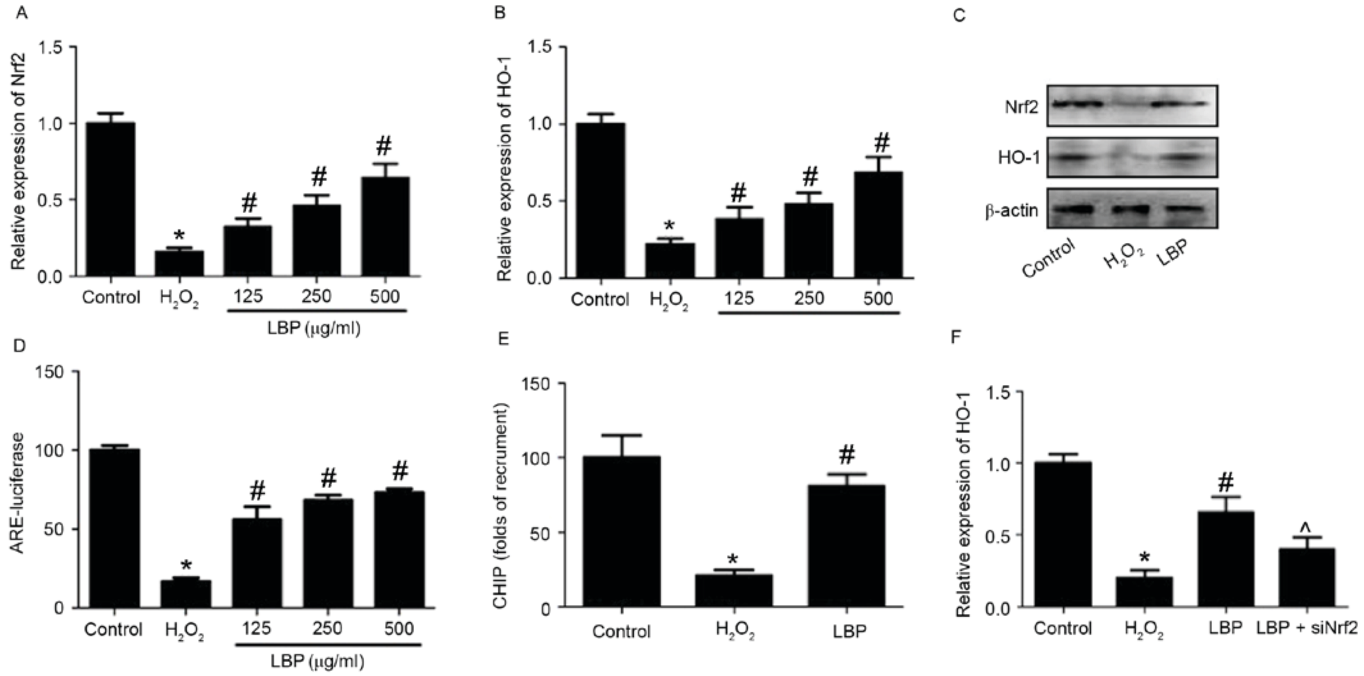

Figure 3. Effect of LBP on the H_2O_2-induced decrease of Nrf2/HO-1 signaling. PC12 cells were incubated with 400 μM H_2O_2 in the presence or absence of 125, 250 or 500 μg/ml LBP for 24 h. (A and B) mRNA and (C) protein expression of Nrf2 and HO-1 was determined by RT-qPCR and western blotting, respectively. (D) Transcriptional activity of Nrf2 was detected by ARE-luciferase activity. (E) Direct regulation of HO-1 by Nrf2 was determined using a ChIP assay. (F) PC12 cells were transfected with siNrf2 for 48 h and incubated with 400 μM H_2O_2 in the presence or absence of 500 μg/ml LBP for 24 h. HO-1 mRNA expression was determined by RT-qPCR. *P<0.05 vs. control; $^\#$P<0.05 vs. H_2O_2 treatment; ^P<0.05 vs. LBP treatment. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; LBP, Lycium barbarum polysaccharide; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; ChIP, Chromatin immunoprecipitation.

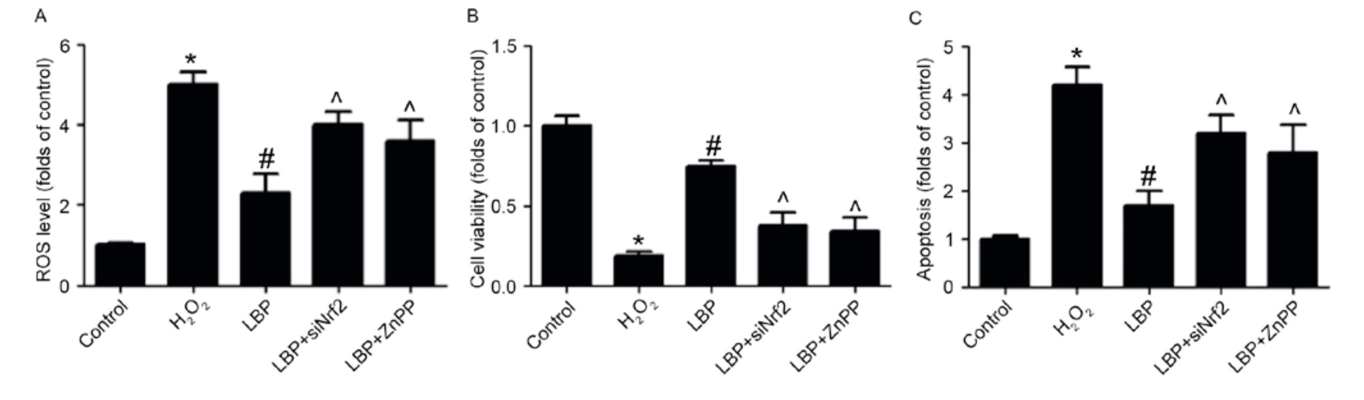

Figure 4. Role of Nrf2/HO-1 signaling in LBP-induced effects on H_2O_2-treated cells. PC12 cells were transfected with siNrf2 for 48 h and subsequently incubated with 400 μM H_2O_2 in the presence or absence of 500 μg/ml LBP with or without ZnPP for 24 h. (A) ROS levels were determined by staining with DCFH-DA. (B) Cell viability was determined by an MTT assay and the results are presented as folds of the control. (C) Apoptosis was determined by TUNEL staining and the results are presented as folds of the control. *P<0.05 vs. control; $^\#$P<0.05 vs. H_2O_2 treatment; ^P<0.05 vs. LBP treatment. siNrf2, small interfering Nrf2 RNA; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; ZnPP, zinc protoporphyrin IX; LBP, Lycium barbarum polysaccharide; DCFH-DA, 7-Dichlorodihydrofluorescein-diacetate; ROS, reactive oxygen species.

animal model of neurotoxicity and subsequently received LBP with or without ZnPP. Following treatment, a MWM test was conducted to evaluate the spatial learning and memory abilities of rats. The $CoCl_2$-induced decrease of Nrf2 expression was significantly inhibited by LBP (P<0.05) and ZnPP did not significantly affect Nrf2 expression in LBP-treated rats (Fig. 5A). LBP reversed the $CoCl_2$-induced decrease of HO-1 expression in rat brains (P<0.05; Fig. 5B), however this effect was significantly attenuated by ZnPP (P<0.05; Fig. 5B). A $CoCl_2$-induced increase in the number of TUNEL-positive cells was inhibited by LBP, indicating a reduction in apoptosis (P<0.05; Fig. 5C). However, ZnPP significantly reversed the anti-apoptotic effect of LBP in $CoCl_2$-treated rats (P<0.05; Fig. 5C). During the MWM test, rats in $CoCl_2$ group spent more time finding the platform site, indicated by a significantly lower crossing index compared with control rats (P<0.05; Fig. 5D). Compared with the $CoCl_2$ group, rats in LBP group spent significantly less time finding the platform site (P<0.05; Fig. 5D), however this decrease in time spent to find the platform site induced by LBP was reversed by ZnPP (P<0.05; Fig. 5D).

Rats in the $CoCl_2$ group exhibited significantly longer escape latencies on test days 3, 4, and 5 than those in the control

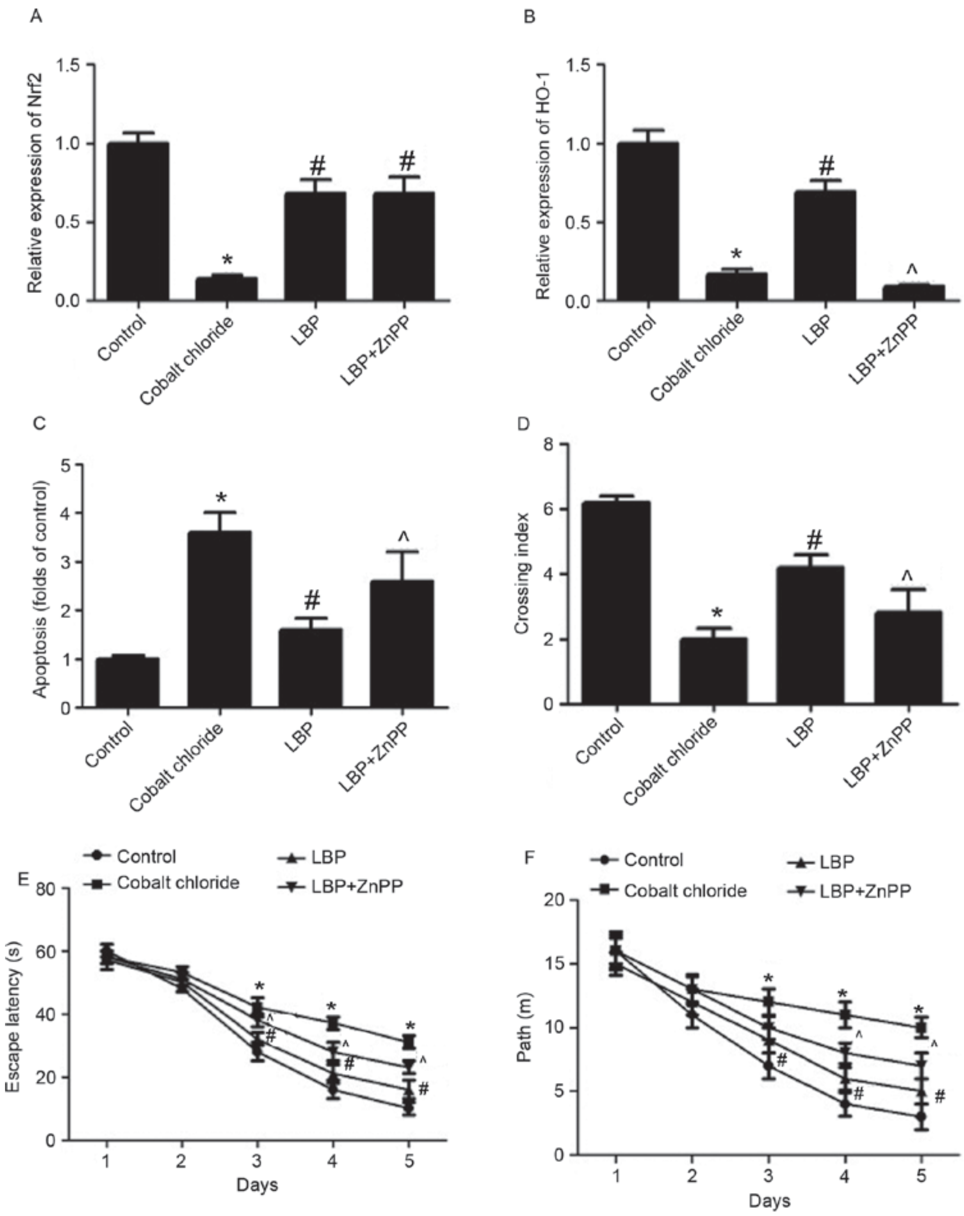

Figure 5. Neuroprotective effect of LBP in rats against $CoCl_2$. Rats were treated with $CoCl_2$ and subsequently received intraperitoneal LBP (100 mg/kg) or saline solution with or without ZnPP (10 mg/kg). The expression of (A) Nrf2 and (B) HO-1 mRNA in brain tissue was determined by reverse transcription-quantitative polymerase chain reaction and the results are presented as folds of the control. (C) Apoptosis was determined by TUNEL staining and results were shown as folds of the control. (D) A MWM test was conducted to evaluate spatial learning and memory abilities of rats. Development of spatial memory for platform location during the probe trials following 5 days training during the MWM test. The crossing index represents the average frequency of swims over the platform site in the target quadrant. (E) Average escape latencies. (F) The distance traveled to find the platform. *P<0.05 vs. control; #P<0.05 vs. H_2O_2 treatment; ^P<0.05 vs. LBP treatment. Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; ZnPP, zinc protoporphyrin IX; MWM, morris water maze; LBP, Lycium barbarum polysaccharide.

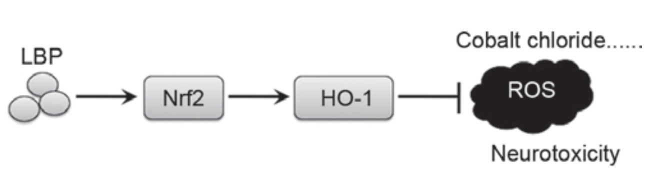

Figure 6. Mechanism of the neuroprotective effect of LBP. LBP, Lycium barbarum polysaccharide; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; ROS, reactive oxygen species.

group (P<0.05; Fig. 5E). Compared with the $CoCl_2$ group, rats in the LBP group had shorter escape latencies on test days 3, 4, and 5 (P<0.05; Fig. 5E); however this decrease in escape latencies induced by LBP was reversed by ZnPP (P<0.05; Fig. 5E). The distance traveled by the rats in each group to find the platform was also gradually reduced by training and the changing patterns of traveling distance induced by LBP and ZnPP were largely consistent with those for the latency (Fig. 5F). The results demonstrated that LBP may protect against $CoCl_2$-induced neurotoxicity *in vivo* and that this may involve the regulation of Nrf2/HO-1 signaling (Fig. 6).

Discussion

There has been a marked increase in the incidence of neurodegenerative diseases including Alzheimer's and Parkinson's disease over the last few years (1,2). Previous studies have suggested that oxidative stress serves a crucial role in the development of different types of neurotoxicity (24-26). It has been demonstrated that LBP, the major active component of the Lycium barbarum L. fruit, possesses potent antioxidant activity (27-29), thus the current study examined the potential neuroprotective effect of LBP.

In the current study, *in vitro* experiments were performed on PC12 cells that had undergone H_2O_2-induced neurotoxicity to evaluate the neuroprotective effect of LBP. In addition, $CoCl_2$-mediated hypoxic neurotoxicity was established in rats to examine the neuroprotective effect of LBP *in vivo*. It was demonstrated that LBP exhibits potent neuroprotective activity, as indicated by the increase in cell viability and decrease in mitochondrial apoptosis in H_2O_2-treated PC12 cells, as well as the decreased apoptosis in rat brain tissue, decreased time to find the platform site, shorter escape latencies and a shorter distance traveled to find the platform in $CoCl_2$-treated rats.

There have been few studies assessing the neuroprotective effects of LBP. It has been demonstrated that LBP improves bipolar pulse current-induced microglia cell injury via modulation of autophagy (16). Furthermore, LBP improved traumatic cognition by reversing the imbalance of apoptosis/regeneration that occurs in hippocampal neurons following the induction of stress (17). Wang *et al* (18) indicated that LBP prevents focal cerebral ischemic injury by inhibiting neuronal apoptosis in mice. Additionally, Rui *et al* (30) determined that LBP protects rat primary cultured hippocampal neurons against injury induced by oxygen-glucose deprivation and reperfusion. Li *et al* (31) demonstrated that LBP reduces neuronal damage, disruption of the blood-retinal barrier and oxidative stress in retinal ischemia/reperfusion injury. Based on the aforementioned results and the results of the current study, LBP may exhibit potent neuroprotective activity.

The antioxidant activities of LBP may serve an important role in the protective effects against neurotoxicity, as reflected by the significant reduction in ROS levels that occurs in H_2O_2-treated PC12 cells. Nrf2 is a key transcription factor that determines redox status by regulating numerous antioxidant enzymes (32,33). HO-1 is an important target gene of Nrf2; HO-1 exhibits antioxidant, anti-apoptotic and anti-inflammatory properties via bilirubin/biliverdin and carbon monoxide, the products of HO-1-catalyzed heme degradation (34). It has been demonstrated that disruption to the Nrf2-ARE pathway contributes to the development of neurotoxicity and neurodegenerative diseases (35-37). Activation of Nrf2/HO-1 signaling may be an important method of protecting against neurotoxicity (38-40). It has been indicated that dietary LBP stimulates the Nrf2/ARE pathway and ameliorates insulin resistance induced by a high-fat diet (41). Furthermore, activation of the Nrf2/HO-1 antioxidant pathway may contribute to the protective effects of LBP in the rodent retina following ischemia-reperfusion-induced damage (42). In the present study, the potential role of Nrf2/HO-1 signaling in the neuroprotective effects of LBP was examined. LBP inhibited the reduction of Nrf2/HO-1 signaling in H_2O_2-treated PC12 cells and $CoCl_2$-treated rats. Nrf2 silencing significantly inhibited the protective effects of LBP in H_2O_2-treated PC12 cells. Furthermore, administration of ZnPP suppressed the protective effects of LBP in H_2O_2-treated PC12 cells and $CoCl_2$-treated rats. These results demonstrate that the Nrf2/HO-1 pathway may, at least partly, be responsible for the neuroprotective effects of LBP.

In conclusion, the current study demonstrated that LBP exhibits protective effects against neurotoxicity via upregulation of Nrf2/HO-1 signaling. Enhancement of Nrf2/HO-1 signaling contributed to an improvement of oxidative stress and the amelioration of apoptosis. These data may improve understanding of the neuroprotective activities of LBP.

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