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Original Paper

Lycium Barbarum Polysaccharides Alleviates Oxidative Damage Induced by H_2O_2 Through Down-Regulating MicroRNA-194 in PC-12 and SH-SY5Y Cells

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Key Words
Spinal cord injury • Oxidative stress • Lycium barbarum polysaccharides • MiR-194 • PI3K/AKT

Abstract
Background/Aims: Currently, scientists attempt to improve outcome of spinal cord injury (SCI) via reducing secondary injury during SCI. Oxidative stress is critical for pathophysiology of secondary damage, thus we mainly focused on the anti-oxidant effects of *Lycium barbarum* polysaccharides (LBPs) on PC-12 and SH-SY5Y cells as well as the underlying mechanisms. ***Methods:*** Oxidative stress was induced by H_2O_2 stimulation. Effects of LBPs on cell viability, apoptosis, and expression of proteins associated with apoptosis and autophagy in H_2O_2-induced cells were assessed by CCK-8 assay, flow cytometry assay and Western blot analysis, respectively. Then, expression of miR-194 was determined by qRT-PCR. Expression of miR-194 was dysregulated, and whether LBPs affected H_2O_2-treated cells through modulating miR-194 was verified. The expression of key kinases in the PI3K/AKT pathway and the intracellular levels of ROS and NO were testified by Western blot analysis and flow cytometry with fluorescent probes. ***Results:*** H_2O_2-induced decrease of cell viability and increases of apoptosis and autophagy in PC-12 cells were mitigated by LBPs treatment. Next, we found that miR-194 expression was both down-regulated by LBPs treatment in PC-12 and SH-SY5Y cells. More experiments consolidated that influence of LBPs on H_2O_2-treated cells was reversed by miR-194 overexpression while was augmented by miR-194 inhibition. LBPs elevated the phosphorylated levels of PI3K and AKT and reduced levels of ROS and NO through miR-194. ***Conclusion:*** LBPs alleviated H_2O_2-induced decrease of cell viability, and increase of apoptosis and autophagy through down-regulating miR-194. Moreover, LBPs activated the PI3K/AKT pathway and reduced oxidative stress through miR-194.

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Introduction

Spinal cord injury (SCI), a devastating event, is mainly caused by trauma which is related to sport and recreational activities, falls, motor-vehicle accidents, and *etc* [1]..Annually, more than 250000 individuals are suffering with SCI and the new cases are exceeding 15000 [2]. With the fact that there is no effective therapy for SCI till now, huge numbers of scientists attempt to figure out novel and better drugs for SCI treatments.

Despite of the primary mechanical injury, SCI evokes a multifactorial and complex pathophysiological process that is also termed secondary injury [3]. Considering the irreversible property of the primary injury, scientists focus on exploring strategies to reduce secondary injury to adjacent tissues, in order to improve outcome of SCI treatments [4]. Oxidative stress is critical for pathophysiology of secondary damage [5]. In the cell membranes of the CNS, there are a great amount of lipids which are easily vulnerable to free radical. Moreover, the neurons in the CNS contain low levels of antioxidant enzymes [6]. Therefore, the neurons in the CNS are vulnerable to oxidative damage due to the low antioxidant ability and limited scope for up-regulation when the oxidative stress is enhanced [6]. The above descriptions indicate that oxidative stress is a promising therapeutic target for SCI treatments [7].

The fruit of *Lycium barbarum*, also known as wolfberry, is a homology of medicine and food in Chinese medicine [8]. *Lycium barbarum* polysaccharides (LBPs) are the major active ingredients of *Lycium barbarum*, which showed eyesight preservative [9], immune-modulatory [10], and anti-tumorous [11] functions. Intensive literatures have identified the protective function of LBPs against oxidative stress in various cells and tissues. For example, a previous study has stated that oxidative stress induced by hepatic cadmium in rats is reduced by LBPs [12]. Likewise, oxidative stress induced by exhaustive exercise of skeletal muscle in rats is also ameliorated by LBPs [13]. Recently, the neuroprotective role of LBPs has been reported [14]. However, the possible role of LBPs in SCI progression as well as the underlying mechanisms are yet poorly understood, which limits the application of LBPs in SCI treatments.

MicroRNAs (miRNAs/miRs) are small non-coding RNAs that participate in numerous biological processes, including SCI [15, 16]. A previous study has reported that the anti-oxidant capacity of LBPs is closely associated with miR-210 [17], broadening our horizons on the interaction between LBPs and miRNAs. miR-194 is a widely reported regulatory miRNA that either presents tumor suppressive or oncogenic roles in diverse cancer cells [18, 19]. It once reported as a biomarker for postmenopausal osteoporosis [20]. Up-regulated miR-194 expression was observed in H_2O_2-treated human retinal pigment epithelium cells [21]. Likewise, expression of miR-194 was also up-regulated by stimulation of H_2O_2 in villous 3A cytotrophoblast cells [22]. Literatures presented above indicated that miR-194 is dysregulated in cells under oxidative stress.

Since oxidative stress is a promising therapeutic target for SCI treatments and LBPs showed an anti-oxidative role, we aimed to explore the effects of LBPs on neuronal cells with H_2O_2 stimulation. Considering the involvements of miR-194 in oxidative stress, we further explored the regulatory mechanism of LBPs focusing on the interaction between LBPs and miR-194. This study may provide theoretical basis for the expansion of LBPs in the treatments of SCI.

Materials and Methods

Cell culture and oxidative stress

Rat pheochromocytoma PC-12 cells, obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China), were cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (all HyClone, Logan, UT, USA). Human neuroblastoma SH-SY5Y cells, obtained from American Type Culture

Collection (ATCC; Manassas, VA, USA), were cultivated in Eagle's Minimum Essential Medium (EMEM): Ham's F-12 medium (1:1) containing 10% FBS. For cell culture, cells (1×10^4 cells/ml) were plated into the flasks and maintained at 37°C in a humidified incubator, which was filled with a mixture of 5% CO_2 and 95% air. Culture medium was replaced by fresh medium every day, and cells were passaged using trypsin (0.25%, HyClone) upon reaching 80% confluency. Oxidative stress was induced by incubating cells with the presence of H_2O_2 (50-300 μM) for 6 hr at 37°C.

Preparation of LBPs and cell treatments

LBPs lyophilized powder was obtained from Shanghai Kang Zhou Funqi Polysaccharide Co., Ltd. (Shanghai, China). Ascertained by the phenol-sulfuric acid method as described previously [23], the polysaccharide content in the LBPs powder reached to 95%. For experimental use, LBPs powder was freshly dissolved in basal medium, and cells were incubated with diverse concentrations of LBPs (100-500 μg/ml) for 24 hr prior to H_2O_2 treatments.

Cell viability assay

Cell viability was determined by Cell Counting Kit-8 (CCK-8) assay. In brief, 5×10^3 cells were seeded into each well of 96-well plates, followed by incubation overnight (37°C, 5% CO_2). Then, transfected or untransfected cells were treated with H_2O_2 or LBPs + H_2O_2 as designed. Subsequently, the culture medium was replaced by basal medium containing 10% CCK-8 solution (Dojindo Molecular Technologies, Gaithersburg, MD, USA), and cells were incubated at 37°C for additional 1 hr. Absorbance at 450 nm was examined by using a Microplate Reader (Bio-Rad, Hercules, CA, USA).

Apoptosis assay

Cell apoptosis was measured by flow cytometry assay. In brief, cells were plated into 6-well plates with a density of 1×10^5 cells per well and incubated overnight. Then, transfected or untransfected cells were treated with H_2O_2 or LBPs + H_2O_2 as designed. Cells were collected and suspended in binding buffer from the FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen, Carlsbad, CA, USA). According to the manufacturer's instructions, 5 μL FITC annexin V and 0.1 μg PI were added into cells, and cells were incubated at room temperature for 15 min. Finally, these stained cells (1×10^5 events) were subjected to a flow cytometer (MoFlo™ XDP, Beckman Coulter Inc., Indianapolis, IN, USA). Percentage of apoptotic cells was analyzed by using Kaluza® Analysis Software.

miRNA transfection

Cells (5×10^5 cells per well) were seeded into 6-well plates, and given basal medium for 24 hr. The miR-194 mimic, miR-194 inhibitor and their corresponding negative control (NC) (50 nM; GenePharma Co., Shanghai, China) were transfected into cells with 5 μl Lipofectamine 3000 in basal medium. After incubation for 48 hr, cells were harvested and used for subsequent experiments.

Analysis of miR-194 expression

Expression of miR-194 was assessed by quantitative reverse transcription PCR (qRT-PCR). Total cellular RNAs were extracted using RNA pure Rapid Extraction Kit (Bioteke Corporation, Beijing, China) as recommended by the manufacturer. Then, miRNAs were converted to cDNA using the TaqMan™ MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Subsequently, real-time PCR was performed on the Applied Biosystems 7700 Sequence Detection System using a TaqMan® Universal PCR Master Mix (Thermo Fisher Scientific) according to the manufacturer's recommendations. The thermocycling parameters were as follows: 10 min at 95°C for polymerase activation, followed by 40 cycles consisting of 95°C for 15 s and 60°C for 60 s. U6 was used as the internal control. Relative quantification analysis was conducted using the $2^{-\Delta\Delta Ct}$ method [24]. All experiments were carried out three times independently, and each sample was analyzed in triplicate.

Western blot analysis

After treatments, proteins were extracted using RIPA buffer (Beyotime, Shanghai, China). The contents of protein samples were examined by using the Bradford protein assay. Protein samples (40 μg per lane) were separated by SDS-PAGE, and then were transferred to nitrocellulose membranes. Those membranes were incubated in 5% skimmed milk for blocking of nonspecific proteins, followed by incubation at 4°C overnight with antibody against B cell lymphoma-2 (Bcl-2, ab196495), Bcl-2-associated X protein (Bax, ab182733), pro caspase-3 (ab90437), active caspase-3 (ab49822), microtubule-associated protein 1 light chain 3B (LC3B; ab48394), p62/sequestosome 1 (p62; ab91526), Beclin-1 (ab62557), total (t)-phosphatidylinositol-3-kinase (PI3K) (ab191606), phospho (p)-PI3K (ab182651), β-actin (ab8227, all Abcam, Cambridge, UK), caspase-9 (9508), t-AKT (9272) or p-AKT (9271, all Cell Signaling Technology, Beverly, MA, USA). Afterwards, membranes were washed in Tris-buffered saline containing 0.1% Tween-20 (TBST), and incubated with HRP-conjugated secondary antibody (goat anti-rabbit ab97051 or goat anti-mouse ab6789, Abcam). Membranes were washed in TBST and reacted with the Amersham ECL Prime Western blotting detection reagent (GE Healthcare, Piscataway, NJ, USA). The ImageJ software (National Institutes of Health, Bethesda, MA, USA) was utilized for measurements of relative expression folds.

Measurement of Intracellular reactive oxygen species (ROS)

Intracellular ROS was monitored by using the 2',7'-dichlorofuorescin diacetate (DCFH-DA) fluorescent probe (Beyotime). DCFH-DA is converted to DCFH by intracellular esterases and then converted to highly fluorescent dichlorofluorescein (DCF) by ROS. After treatments, PC-12 cells were incubated with 10 μM DCFH-DA at 37°C for 30 min. Then, cells were washed twice with PBS and detected by using a flow cytometer (MoFlo™ XDP) with excitation at 488 nm and emission at 525 nm.

Measurement of Intracellular NO

Intracellular NO was monitored by using the 3-amino,4-aminomethyl-2',7'-difluorescein diacetate (DAF-FM DA) fluorescent probe (Beyotime). DAF-FM DA is converted to DAF-FM by intracellular esterases and then reacts with NO to produce a fluorescent compound. After treatments, PC-12 cells were incubated with 5 μM DAF-FM DA at 37°C for 30 min. Then, cells were washed twice with PBS and detected by using a flow cytometer (MoFlo™ XDP) with excitation at 495 nm and emission at 515 nm.

Statistical analysis

Results were presented as the mean ± standard deviation (SD) of three independent experiments. Statistical analysis was undertaken using Graphpad Prism 5 software (GraphPad, San Diego, CA, USA). The *P*-values were calculated using the one-way analysis of variance (ANOVA) or unpaired two-tailed *t*-test. Differences were considered to be significant at $P < 0.05$.

Results

Decrease of cell viability and increase of apoptosis induced by H_2O_2 are mitigated by LBPs treatments in PC-12 cells

To optimize the dosage of H_2O_2, PC-12 cells were incubated with 0, 50, 100, 150, 200 or 300 μM H_2O_2, followed by assessments of cell viability. Compared with the non-treated cells, cell viability was significantly decreased by 100 μM ($P < 0.05$), 150 and 200 μM (both $P < 0.01$), and 300 μM ($P < 0.001$) of H_2O_2, whereas 50 μM H_2O_2 showed no statistically significant anti-survival effects (Fig. 1A). According to the results of cell viability, the concentration of H_2O_2 was ascertained to be 200 μM in subsequent experiments. Then, percentage of apoptotic cells and expression of apoptosis-associated proteins were examined. As compared to the control group, 200 μM H_2O_2 markedly increased the percentage of apoptotic cells ($P < 0.01$, Fig. 1B). Together, expression level of anti-apoptotic Bcl-2 was reduced, whereas expression levels of pro-apoptotic Bax, cleaved caspase-3 and cleaved caspase-9 were elevated by H_2O_2 treatments (Fig. 1C). Those results presented above illustrated that H_2O_2 stimulation reduced cell viability and facilitated apoptosis.

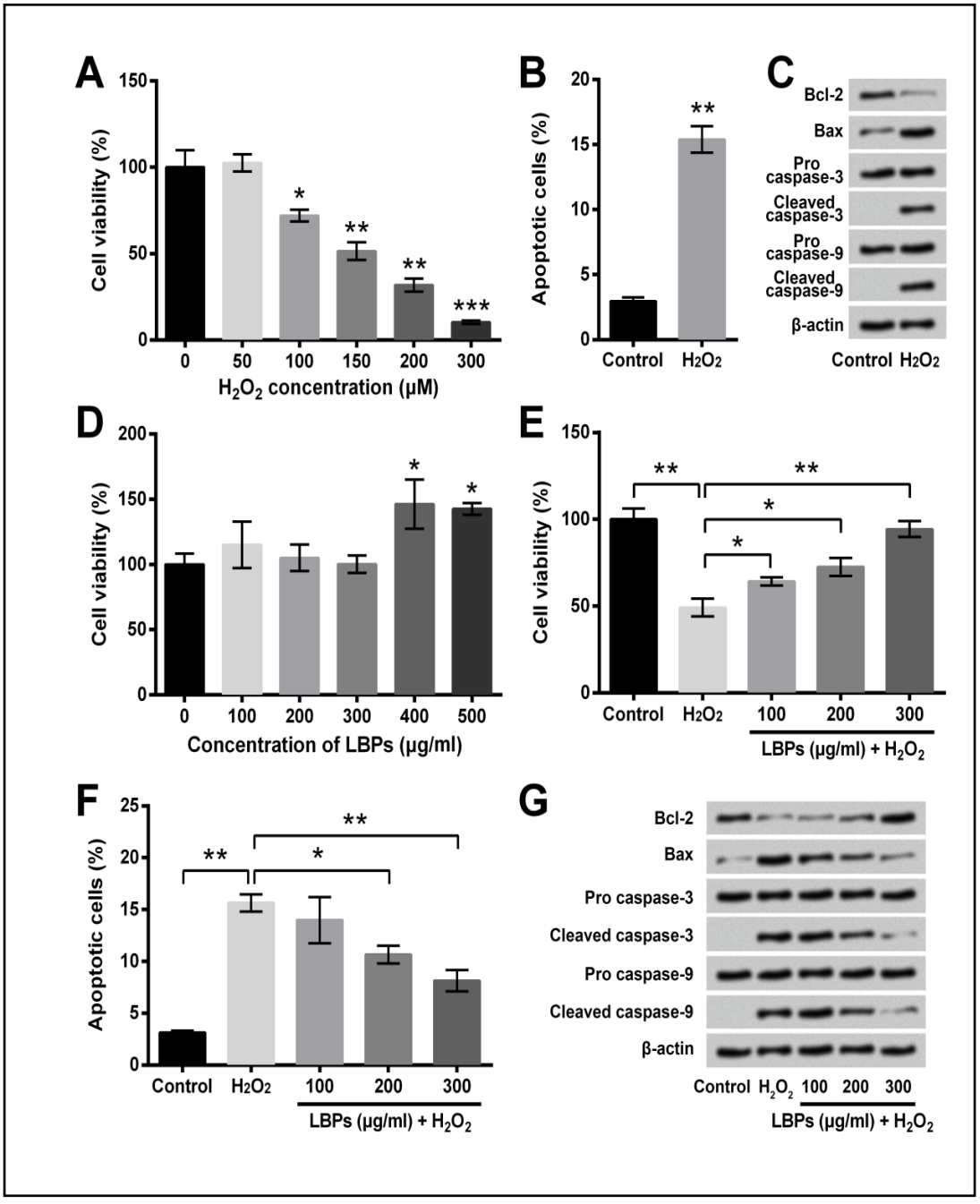

Fig. 1. Effects of LBPs on H_2O_2-induced decrease of viability and enhancement of apoptosis in PC-12 cells. A. Cells were stimulated with 0, 50, 100, 150, 200 or 300 μM H_2O_2, and cell viability was measured by CCK-8 assay. Cells were treated with 200 μM H_2O_2, and non-treated cells were acted as control. Percentage of apoptotic cells (B) and expression of apoptosis-related proteins (C) were tested by flow cytometry assay and Western blot analysis, respectively. D. Cells were stimulated with 0, 100, 200, 300, 400 or 500 μg/ml LBPs, and cell viability was examined. Cells were treated with 200 μM H_2O_2 alone or with the presence of 100, 200 or 300 μg/ml LBPs, and non-treated cells were acted as control. Then, cell viability (E), percentage of apoptotic cells (F) and expression of apoptosis-related proteins (G) were assessed. Data presented are the mean ± SD (n = 3). *, P<0.05; **, P<0.01; ***, P<0.001. LBPs, Lycium barbarum polysaccharides.

Afterwards, we focused on the effects of LBPs on H_2O_2-induced alterations. First of all, cells were stimulated with diverse concentrations of LBPs (0, 100, 200, 300, 400 and 500 μg/ml) and cell viability was measured to confirm the adequate dosage of LBPs. As evidence from Fig. 1D, cell viability was dramatically enhanced by 400 or 500 μg/ml LBPs (both $P < 0.05$), whereas cell viability was remained unchanged under stimulation of 100-300 μg/ml LBPs. Thus, maximal concentration of LBPs for subsequent experiments was 300 μg/ml. Results in Fig. 1E showed cell viability was notably increased by 100-200 μg/ml (both $P < 0.05$) or 300 μg/ml ($P < 0.01$) of LBPs relative to the H_2O_2 group. Conversely, percentage of apoptotic cells was prominently lowered by 200 μg/ml ($P < 0.05$) or 300 μg/ml ($P < 0.01$) of LBPs relative to the H_2O_2 group (Fig. 1F). Likewise, 200-300 μg/ml LBPs observably reversed H_2O_2-induced alterations of apoptosis-associated proteins (Fig. 1G). Results indicated that H_2O_2-induced alterations of viability and apoptosis could be reversed by LBPs stimulation in PC-12 cells.

Autophagy induced by H_2O_2 is attenuated by LBPs treatments in PC-12 cells

LC3B, Beclin-1 and p62 are three major autophagy markers. Expression of these proteins in PC-12 cells treated with H_2O_2 or LBPs + H_2O_2 was tested to evaluate the alteration of autophagy. After H_2O_2 treatments, expression of LC3B-II and Beclin-1 was notably up-regulated while

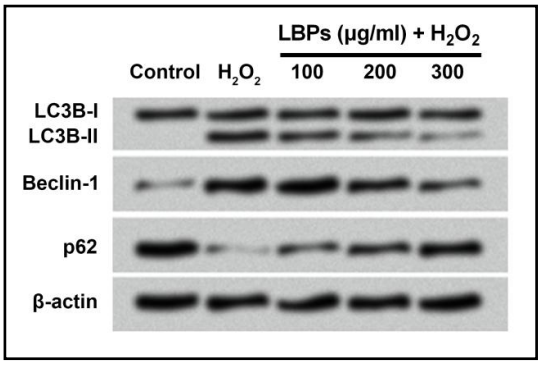

Fig. 2. Effects of LBPs on H_2O_2-induced autophagy in PC-12 cells. Cells were treated with 200 μM H_2O_2 alone or with the presence of 100, 200 or 300 μg/ml LBPs, and non-treated cells were acted as control. Then, expression of autophagy-related proteins was measured by Western blot analysis. LBPs, Lycium barbarum polysaccharides.

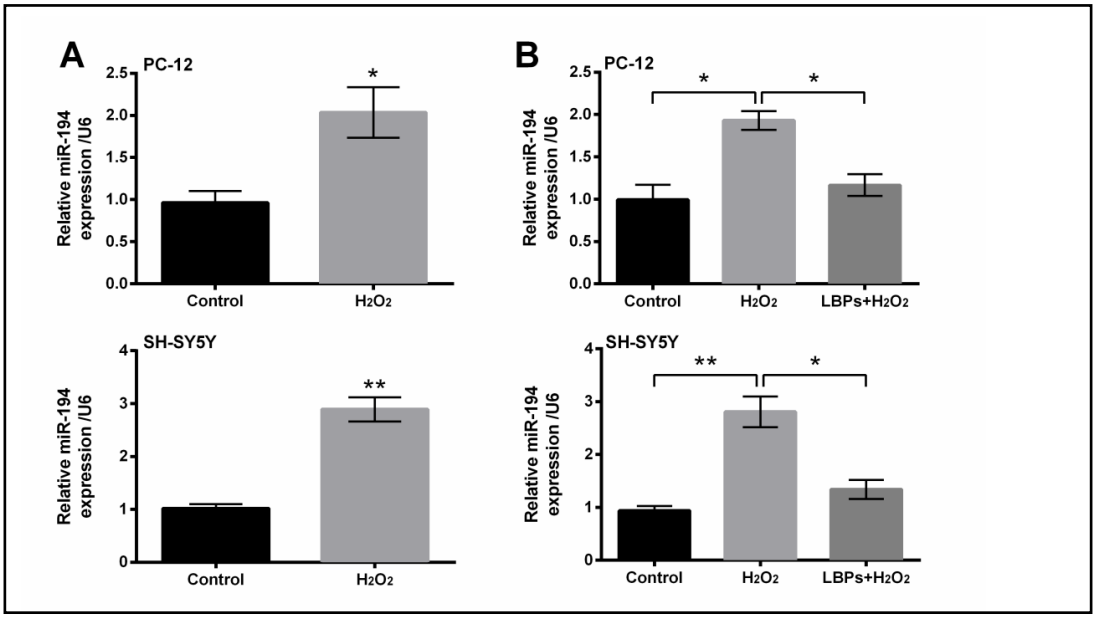

Fig. 3. Effects of LBPs on H_2O_2-induced alteration of miR-194 expression in PC-12 and SH-SY5Y cells. Expression of miR-194 was assessed by quantitative reverse transcription PCR. A. Cells were treated with 200 μM H_2O_2, and non-treated cells were acted as control. B. Cells were treated with 200 μM H_2O_2 alone or with the presence of 300 μg/ml LBPs, and non-treated cells were acted as control. Data presented are the mean ± SD (n = 3). *, P<0.05; **, P<0.01. LBPs, Lycium barbarum polysaccharides.

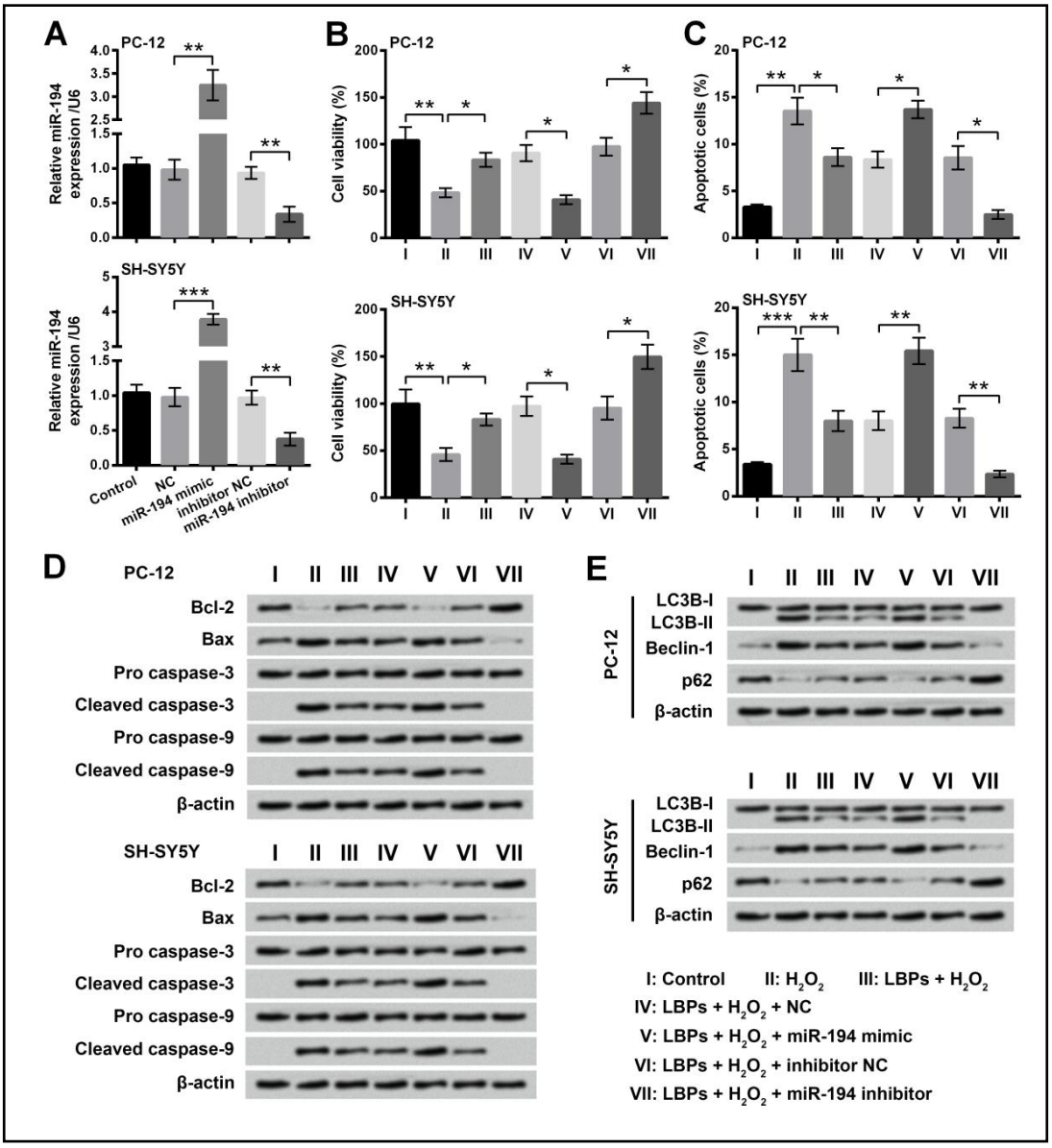

Fig. 4. Effects of abnormally expressed miR-194 on viability, apoptosis and autophagy of PC-12 and SH-SY5Y cells treated with H_2O_2 and LBPs. A. Cells were transfected with NC, miR-194 mimic, inhibitor NC or miR-194 inhibitor, and non-transfected cells were acted as control. Expression of miR-194 was assessed by quantitative reverse transcription PCR. Transfected and non-transfected cells were treated with 200 μM H_2O_2 alone or with the presence of 300 μg/ml LBPs, and non-treated cells were acted as control. Then, cell viability (B), percentage of apoptotic cells (C), and expression of apoptosis-related proteins (D) and autophagy-related proteins (E) were determined by CCK-8 assay, flow cytometry assay and Western blot analysis, respectively. Data presented are the mean ± SD (n = 3). *, P<0.05; **, P<0.01; ***, P<0.001. LBPs, Lycium barbarum polysaccharides; NC, negative control of miR-194 mimic; inhibitor NC, negative control of miR-194 inhibitor.

expression of p62 was down-regulated, suggesting that H_2O_2 induced autophagy in PC-12 cells (Fig. 2). Moreover, those alterations of autophagy markers, induced by H_2O_2, were observably mitigated by 200-300 μg/ml LBPs, suggesting that LBPs could reverse H_2O_2-induced autophagy of PC-12 cells.

Expression of miR-194 is down-regulated by LBPs in PC-12 and SH-SY5Y cells

We assumed that LBPs might affect miR-194 expression in PC-12 cells, thus the miR-194 levels in cells treated with H_2O_2 or LBPs + H_2O_2 were measured. In Fig. 3A, miR-194 levels in the H_2O_2 group were remarkably higher than that in the control group ($P < 0.05$ or $P < 0.01$). Moreover, LBPs significantly negatively regulated expression of miR-194 relative to the H_2O_2 group (both $P < 0.05$, Fig. 3B). Results described above indicated that miR-194 might participate in the modulation of LBPs in PC-12 cells.

LBPs affect H_2O_2-treated PC-12 and SH-SY5Y cells through down-regulating miR-194

Subsequent experiments were done to affirm whether LBPs affect H_2O_2-treated PC-12 and SH-SY5Y cells through modulating miR-194. First of all, miRNAs were introduced to alter the expression level of miR-194. In Fig. 4A, miR-194 level in cells transfected with miR-194 mimic was dramatically higher than that of the NC group ($P < 0.01$ or $P < 0.001$). Meanwhile, miR-194 level in cells transfected with miR-194 inhibitor was markedly lower than that of the inhibitor NC group (both $P < 0.01$). Then, we interestingly found influences of LBPs on cell viability (Fig. 4B), percentage of apoptotic cells (Fig. 4C), and expressions of proteins associated with apoptosis (Fig. 4D) and autophagy (Fig. 4E) were observably reversed by

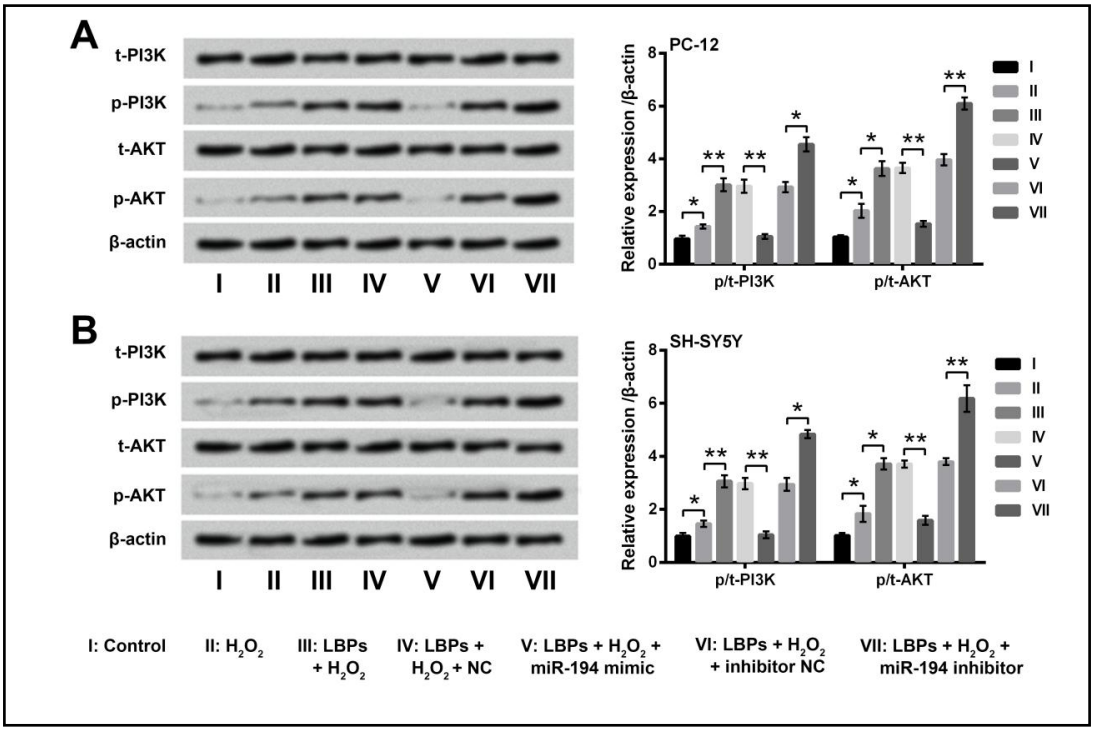

Fig. 5. Effects of LBPs on activation of the PI3K/AKT pathway in PC-12 and SH-SY5Y cells treated with H_2O_2 and LBPs. Transfected and non-transfected cells were treated with 200 μM H_2O_2 alone or with the presence of 300 μg/ml LBPs, and non-treated cells were acted as control. Then, expression of proteins associated with the PI3K/AKT pathway in PC-12 cells (A) and SH-SY5Y cells (B) was measured by Western blot analysis. Data presented are the mean ± SD (n = 3). *, P<0.05; **, P<0.01. LBPs, Lycium barbarum polysaccharides; NC, negative control of miR-194 mimic; inhibitor NC, negative control of miR-194 inhibitor.

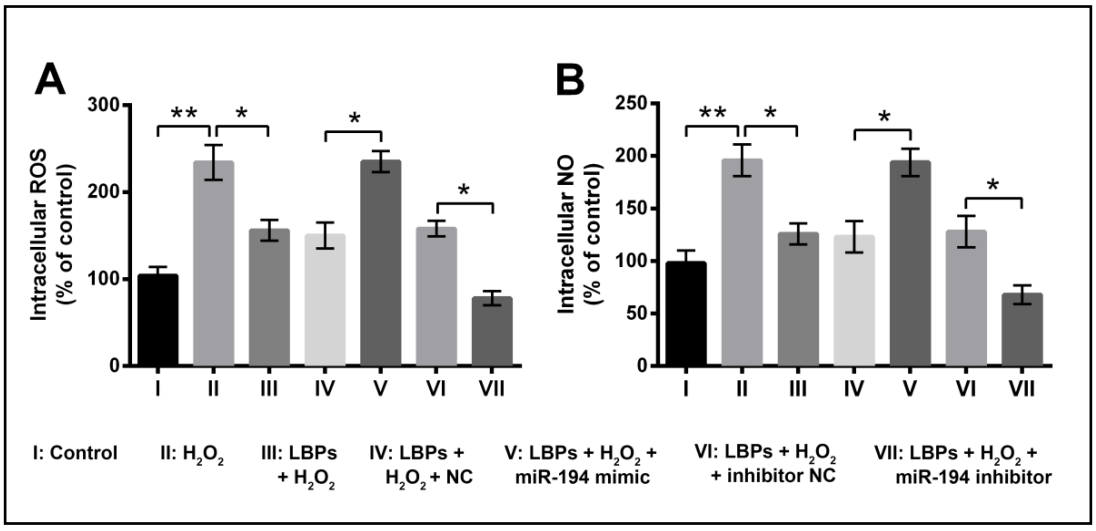

Fig. 6. Effects of LBPs on oxidative stress in PC-12 treated with H_2O_2 and LBPs. Transfected and non-transfected cells were treated with 200 μM H_2O_2 alone or with the presence of 300 μg/ml LBPs, and non-treated cells were acted as control. Then, intracellular ROS level (A) and NO level (B) were measured by using DCFH-DA fluorescent probe and DAF-FM DA fluorescent probe, respectively. Data presented are the mean ± SD (n = 3). *, P<0.05; **, P<0.01. LBPs, Lycium barbarum polysaccharides; NC, negative control of miR-194 mimic; inhibitor NC, negative control of miR-194 inhibitor.

miR-194 overexpression relative to the LBPs + H_2O_2 + NC group ($P < 0.05$ or $P < 0.01$). Those influences of LBPs were significantly augmented by miR-194 inhibition relative to the LBPs + H_2O_2 + inhibitor NC group ($P < 0.05$ or $P < 0.01$). Those results indicated LBPs might affect H_2O_2-treated PC-12 and SH-SY5Y cells through down-regulating miR-194.

LBPs activate the PI3K/AKT pathways via down-regulation of miR-194

The PI3K/AKT pathway is a crucial pathway that is closely related to cell survival and apoptosis [25]. Thus, we analyzed the phosphorylation of key kinases in the PI3K/AKT pathway to explore the underlying mechanisms of LBPs-associated regulations. Western blot results in PC-12 cells (Fig. 5A) and SH-SY5Y cells (Fig. 5B) showed phosphorylated levels of PI3K and AKT were dramatically enhanced by LBPs treatments as compared to the H_2O_2 group ($P < 0.05$ or $P < 0.01$). Furthermore, the LBPs-induced enhancements of p/t-PI3K and p/t-AKT were both significantly reversed by miR-194 overexpression relative to the LBPs + H_2O_2 + NC group while were notably augmented by miR-194 inhibition relative to the LBPs + H_2O_2 + inhibitor NC group (all $P < 0.01$). Results talked above proved that LBPs might activate the PI3K/AKT pathways via down-regulation of miR-194 in PC-12 and SH-SY5Y cells.

LBPs show a potential anti-oxidative capacity in PC-12 cells via miR-194

Effects of LBPs on oxidative stress in PC-12 cells were analyzed through the levels of intracellular ROS and NO. By using fluorescent probes, we found intracellular ROS (Fig. 6A) and NO (Fig. 6B) levels were markedly enhanced by H_2O_2 stimulation (both $P < 0.01$), and the enhancements were observably reversed by LBPs treatments (both $P < 0.05$). Furthermore, we also found effects of LBPs on ROS and NO levels were prominently reversed by miR-194 overexpression while were significantly augmented by miR-194 inhibition (all $P < 0.05$). Results illustrated that LBPs reduced oxidative stress through down-regulating miR-194.

Discussion

Currently, several strategies such as decompression of the spinal cord, vertebral stabilization and managements with methylprednisolone, are applied to treat SCI [26]. However, the outcome is far away from satisfaction. Herein, we attempted to explore potential drugs for SCI treatments through studying the antioxidant drug in PC-12 and SH-SY5Y cells. Accordingly, we identified that H_2O_2-induced increases of apoptosis and autophagy, and decrease of cell viability were all alleviated by LBPs stimulation. Then, we interestingly found that miR-194 was up-regulated by H_2O_2, whereas the up-regulation was eliminated by LBPs stimulation. Furthermore, miR-194 overexpression could reverse the effects of LBPs on viability, apoptosis, autophagy, activation of the PI3K/AKT pathway, and oxidative stress in H_2O_2-treated PC-12 cells, whereas those effects of LBPs were augmented by miR-194 inhibition.

Oxidative stress is a crucial factor in the injurious secondary pathological process of SCI. Increased production of ROS and/or low ability of biological system to detoxify ROS may result in oxidative stress [27]. Since H_2O_2 which can diffuse through biological membranes via aquaporins is a major source of ROS generation, we induced oxidative stress in cells utilizing H_2O_2.

Apoptosis leads to further neural injury and functional loss during secondary SCI [28]. PC-12 cells are sensitive to oxidative stress, and PC-12 cell viability is decreased, along with elevated cell apoptosis under H_2O_2 stimulation [29]. Therefore, we firstly studied the effects of LBPs on PC-12 cell viability and apoptosis. Results showed H_2O_2-induced alterations of cell viability and percentage of apoptotic cells were both attenuated by LBPs, suggesting the neuroprotective role of LBPs in PC-12 cells. Mitochondrial dysfunction is associated with oxidative stress-induced pathology [30]. The mitochondrial pathway is also termed as intrinsic or Bcl-2-regulated pathway, in which Bcl-2 proteins and diverse caspases were involved [31]. In our study, H_2O_2 stimulation down-regulated Bcl-2 expression while up-regulated expression of Bax, active caspase-9 and active caspase-3. Meanwhile, alterations of those proteins were mitigated by LBPs, which illustrated LBPs reduced apoptosis through repressing the intrinsic apoptosis pathway in PC-12 cells. The anti-apoptotic role of LBPs in H_2O_2-induced PC-12 cells was coincident with that in human lens epithelial cells [32].

Autophagy is an evolutionarily conserved catabolic process, by which intracellular proteins and aging organelles are degraded to maintain cytoplasmic homeostasis [33]. An extensive body of evidence suggests a crosstalk between autophagy and SCI [34, 35]. Therefore, we explored the effects of LBPs on autophagy in H_2O_2-induced PC-12 cells. The post-translational modifications from LC3B-I to LC3B-II as well as Beclin-1 are essential for the generation of pre-autophagosomal structure [36]. p62 can recognize ubiquitin (unbiquitinated proteins or bacteria) and target them to the autophagosomes, followed by degradation [37]. In our study, up-regulated expression of LC3B-II and Beclin-1 as well as down-regulated expression of p62 collectively illustrated H_2O_2 induced autophagy in PC-12 cells. Proteins are modified post-translationally in cells under oxidative stress, which may contribute to protein aggregation. Hence, excessively aggregated proteins results from oxidative stress may be a reason for the enhanced autophagy. Following results showed LBPs stimulation could repress autophagy. A possible explanation was that LBPs might alleviate H_2O_2-induced cell injury thereby reduced aggregation of toxic or oxidized proteins, resulting in repression of autophagy.

In the process of SCI, Zhou *et al.* have reported miR-199b silence aggravated acute SCI in microglial cells [15]. Fu *et al.* also stated that SCI-induced inflammation and oxidative were attenuated by miR-30a-5p [16]. We supposed that LBPs might protect cells against H_2O_2-induction through regulating miR-194 expression due to the up-regulation of miR-194 in H_2O_2-treated cells. Consistent with previous literatures [21, 22], the expression of miR-194 in PC-12 and SH-SY5Y cells was up-regulated after H_2O_2 stimulation in our study. In addition, the up-regulation was abrogated by LBPs, indicating the possible involvements of miR-194 in the LBPs-associated regulations. More experiments showed effects of LBPs on viability,

apoptosis and autophagy in H_2O_2-induced PC-12 and SH-SY5Y cells were reversed by miR-194 overexpression while were augmented by miR-194 inhibition, verifying that LBPs might function through down-regulating miR-194.

We explored the possible involved signaling pathway. The PI3K/AKT cascade is a major signal transduction pathway that exerts pro-survival and anti-apoptotic functions [38]. Additionally, it can halt excessive autophagy [39]. The PI3K/AKT pathway was activated by H_2O_2, and was further activated by LBPs stimulation in our study, which was consistent with a previous literature [40]. Moreover, the activation was abrogated by miR-194 overexpression while was enhanced by miR-194 inhibition, indicating that LBPs might affect H_2O_2-induced PC-12 and SH-SY5Y cells through miR-194-mediated activation of the PI3K/AKT pathway. Collectively, we speculated a possible regulatory pathway. Protein aggregation induced by H_2O_2 promoted autophagy to maintain cytoplasmic homeostasis. Meanwhile, H_2O_2 stimulation up-regulated miR-194 expression, accompanying by decreased cell viability and increased apoptosis. Then, LBPs activated the PI3K/AKT pathway via down-regulating miR-194 expression. The activated PI3K/AKT pathway thereby elevated cell viability and repressed apoptosis and autophagy.

Considering oxidative stress is a crucial factor in SCI progression, we finally explored the effects of LBPs on intracellular ROS and NO levels as well as the influence of dysregulated miR-194 in LBPs-treated cells. Consistent with previous studies [12, 13], LBPs showed an anti-oxidant capacity in PC-12 cells under H_2O_2-stimulation. We also proved that LBPs-induced reduction of oxidative stress was reversed by miR-194 overexpression while was augmented by miR-194 inhibition, indicating that down-regulation of miR-194 might be a rational explanation for the anti-oxidant capacity of LBPs.

Conclusion

To sum up, oxidative stress-induced alterations of cell viability, apoptosis and autophagy were all mitigated by LBPs stimulation. Furthermore, we found LBPs might affect H_2O_2-induced PC-12 and SH-SY5Y cells through miR-194-mediated activation of the PI3K/AKT pathway. We figured out a possible regulatory mechanism for the anti-oxidant capacity of LBPs in PC-12 and SH-SY5Y cells. Results in this study may provide novel therapeutic drug for clinical treatments of SCI as well as the possible theoretical basis. More evidences performed *in vivo* are needed in the future to reinforce the conclusion in this study.

Disclosure Statement

Authors declare that there is no conflict of interest.

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