RETRACTED ARTICLE: *Lycium barbarum* polysaccharide alleviates oxygen glucose deprivation-induced PC-12 cells damage by up-regulating miR-24

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**ABSTRACT**

**Objective:** This research was aimed to detect the functions of *Lycium barbarum* polysaccharides (LBPs) on oxygen and glucose deprivation (OGD) injury and potential mechanisms at PC-12 cells.

**Methods:** CCK-8, flow cytometry and reactive oxygen species (ROS) assays were used to detect OGD, LBPs and miR-24 effects on cell viability, apoptosis, and oxidative stress. MiR-24 was transfected and tested by transfection and qRT-PCR. Moreover, the related-protein levels of apoptosis, autophagy and pathways were tested by Western blotting.

**Results:** LBPs significantly enhanced cell viability, inhibited cell apoptosis, autophagy and ROS level in OGD injury. In addition, miR-24 expression was declined in OGD-treated cells, while it was elevated when added LBPs. The preventive effects of LBPs on PC-12 cell damage induced by OGD were reversed by down-regulating miR-24. Furthermore, miR-24 inhibitor declined LBPs-induced change in Wnt/β-catenin and JAK1/STAT3 pathways in OGD-injured cells.

**Conclusions:** LBPs exhibited preventive effects via up-regulating miR-222 and activating Wnt/β-catenin and JAK1/STAT3 pathways in OGD-induced PC-12 cells.

**Introduction**

Ischemic stroke (IS) is a common acute cerebrovascular disease in the elderly. It is caused by blood flow fail to brain due to sudden vascular rupture or vascular occlusion in the brain, which results in insufficient supply of nutrient, serum, oxygen and glucose [1,2]. It accounts for about 85% of strokes with high mortality and disability worldwide, which results in a heavy burden to patients and society [3]. Nowadays, intravenous administration of alteplase within 4.5 h after mechanical thrombectomy or symptom onset is an effective therapy for IS [4,5]. Unfortunately, narrow treatment time window limits the widespread application of plasminogen activator therapy. Thus, it is urgent to further understanding the underlying mechanisms of stroke and seek for novel and effective strategies or medicines for IS treatment.

*Lycium barbarum* polysaccharides (LBPs) are the main bioactive molecule extracted from *Lycium barbarum*, a traditional Chinese medicine, which play key biological roles, such as neuroprotective, immunomodulatory, anti-aging and anti-oxidative functions [6]. Accumulating evidence have revealed LBPs exert anti-oxidative functions in oxidative liver injury [7] or doxorubicin-induced cardiotoxicity [8]. In addition, the beneficial roles of LBPs in anti-radiation or chemotherapy damages [9], hypoglycemia [10] and aging [11] have been reported. Deserved to be mentioned, the functions of LBPs in nervous system damage have been detected, LBPs can protect ganglion cells against retinal ischemia/reperfusion injury [12], partial optic transection injuries [13] and acute ocular hypertension [14]. Furthermore, LBPs play the preventive effects through anti-apoptotic and anti-oxidative mechanisms in cerebra ischemic injury [15,16]. However, whether LBPs can protect against PC-12 cell damage induced by oxygen and glucose deprivation (OGD) remains unclear.

MicroRNAs (miRNAs) are well-known as endogenous non-coding small-molecule RNAs, which are closely associated with various cardiovascular diseases [17]. MiR-24 is revealed to be involved in the development of cardiovascular disease as a hypoxia-sensitive miRNA by regulating the function of vascular endothelial cells [18]. The current research aimed to uncover the functions and mechanisms of LBPs in OGD-induced cell damage. We established OGD-induced cell damage model and then explored functions of the pretreatment of LBPs on cell viability, cell apoptosis, cell autophagy and reactive oxygen species (ROS) content in OGD-treated cells. Furthermore, whether miR-24 level related to the functions of LBPs in OGD-induced cell damage was investigated.

**Materials and methods**

**Cell culture, treatment and transfection**

PC-12 cells were obtained from Shanghai Obio Technology Co. (Shanghai, China), Ltd. DMEM (Dojindo, Tokyo, Japan)
with 10% fetal bovine serum (FBS, Dojindo) was used to culture cells. The standard incubation conditions were 5% CO₂, 37°C. The cell damage was induced by OGD for 16 h [19]. Before OGD, LBPs was administered at 0, 100, 300 or 500 μg/ml for 24 h [20]. In this step, glucose-free DMEM was instead of normal DMEM. Then, cells were cultured in a chamber with anaerobic (5% CO₂, 37°C) and normal medium was used to culture cells and cells were incubated with standard incubation conditions (5% CO₂ and 37°C). Cells with normal medium and standard incubation conditions were served as control. To detect the effects of miR-24 on OGD-induced cell damage after treatment with LBPs, OGD-treated cells were added LBPs and then miR-24 inhibitor or inhibitor NC (Thermo, Waltham, MA, USA) were transfected into cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

**Cell viability assay**

Cell viability was detected by Cell Counting Kit-8 (CCK-8, Dojindo). PC-12 cells with different treatments were grown in 96-well plates. Then, CCK-8 solution was appended into each well (1 h). The absorbances (450 nm) were read after incubation by microplate reader (Molecular Devices, USA).

**Apoptosis assay**

Annexin V-FITC Apoptosis Detection kit (Dojindo) was used to detect cell apoptosis. PBS collected and washed PC-12 cells with various treatments. Then cells were resuspended by 1 ml Binding Buffer, followed the incubation with PI and FITC–Annexin V (dark, 25°C, 15 min) and 1 ml Binding Buffer mixed. Finally, cells were detected by flow cytometer (BD).

**Quantitative real-time reverse transcription PCR (qRT-PCR)**

Total RNA was obtained by Trizol (Invitrogen, Gaithersburg, MD, USA). cDNA was acquired by reverse transcription of RNA by miRNA First-Strand Synthesis Kit or PrimeScript™ RT reagent Kit (Takara, Dalian, China). The RT-qPCR was performed by a SYBR Premix Ex Taq TM II (Takara) with primers as following: miR-24 forward, 5'-GAGGGGCCGATCGGCAGTCCGACCA-3' and reverse, 5'- AACGCTTCAAGATTGTGGT-3'; and U6 forward, 5'- AGGGGCCATCCAGATCTCC-3' and reverse 5'- AAGCTTTCCAAGATTGTGGT-3'. U6 was an internal reference and data were calculated with 2^△ΔCt method.

**ROS assay**

ROS levels were detected by 2,7-dichlorofluorescein diacetate (DCFH-DA) (Nanjing Jiancheng, Nanjing, China) by flow cytometry. The cells with various treatments were grown in 6-well plates. After washing with PBS, cells were cultured with 10 μM DCFH-DA in serum-free medium (dark, 37°C, 20 min). Subsequently, cells were collected using trypsin digestion method and resuspended in PBS. Lastly, a flow cytometer was used to measure the fluorescent intensities.

**Western blotting**

Protein samples extracted from cells were separated and transferred into polyvinylidene fluoride membranes. The 5% skim milk blocked the membrane. Besides, membrane was incubated with antibodies against Bax, cleaved-caspase-3, cleaved-caspase-9, p62, Beclin-1, LC3-I, LC3-II, TMT, β-catenin, total-JAK1 (t-JAK1), phosphorylated (p)-JAK1, t-STAT3, p-STAT3 (1:1000, Abcam, Cambridge, MA, USA) and β-actin (1:1000, Beyotime) overnight at 4°C. Then, membrane was reacted with secondary antibody (1:1000, Beyotime) (dark, 1 h). GAPDH was used as the control protein. Enhanced chemiluminescence (ECL) Plus reagent (Beyotime) was used to image blots. The band quantification was performed using Image J software.

**Statistical analysis**

Data statistical analysis was analyzed by GraphPad Prism 6 software. Data were expressed as the mean ± SD and analyzed by one-way ANOVA. A value of *p* < .05 was considered significant.

**Results**

**LBPs remitted cell damage induced by OGD**

In normal PC-12 cells, LBPs with different doses had no influence in cell viability (Figure 1(A)). Compared with control cells, OGD treatment significantly inhibited cell viability while LBPs treatment promoted cell viability in a dose-dependent method (*p* < .05, Figure 1(B)). Flow cytometry analysis revealed cell apoptosis was prominently enhanced in OGD-treated cells, LBPs treatment declined cell apoptosis in OGD-induced cells (*p* < .05, Figure 1(C)). Consistently, western blotting displayed OGD distinctly promoted the levels of Bax, cleaved-caspase-3 and cleaved-caspase-9, while LBPs treatment reversed their expression induced by OGD (*p* < .05, Figure 1(D)). In addition, ROS level was obviously enhanced after OGD, while LBPs treatment inhibited ROS level in OGD-induced cells (*p* < .05, Figure 1(E)).

**LBPs remitted OGD-induced cell autophagy**

Compared with control cells, the levels of p62 were significantly declined and Beclin-1, LC3-II and LC3-I expression were obviously enhanced in cells under OGD in PC-12 cells (*p* < .05, Figure 2). After treatment with LBPs, p62 expression was significantly enhanced. Beclin-1, LC3-II and LC3-I expression were obviously declined in OGD-induced cells (*p* < .05, Figure 2).

**LBPs promoted miR-24 expression**

Compared with control cells, OGD treatment significantly inhibited the miR-24 level, while LBPs treatment enhanced the miR-24 level in dose-dependent method in PC-12 cells (*p* < .01, Figure 3).
Relationship of miR-24 knockdown and the functions of LBPs in OGD-induced cell damage

Compared with cells with inhibitor NC, miR-24 level was significantly reduced in cells with miR-24 inhibitor ($p < .01$, Figure 4(A)). MiR-24 knockdown significantly inhibited cell viability in OGD-induced cell treated with LBPs ($p < .05$, Figure 4(B)). Flow cytometry results also revealed cell apoptosis was prominently enhanced in OGD-induced cells treated with LBPs and miR-24 inhibitor compared with cells with OGD-induced cells treated with LBPs and inhibitor NC ($p < .05$, Figure 4(C)). Consistently, western blotting showed miR-24 knockdown distinctly promoted the levels of Bax, cleaved-caspase-3 and cleaved-caspase-9 in OGD-induced cell treated with LBPs ($p < .05$, Figure 4(D)). ROS level was...
obviously enhanced after miR-24 knockdown in OGD-induced cells treated with LBPs \((p < .05, \text{Figure 4(E)})\). In addition, p62 expression was significantly declined and Beclin-1 expression and LC3-II and LC3-I were obviously enhanced in OGD-induced cells treated with LBPs and miR-24 inhibitor compared with cells with OGD-induced cells treated with LBPs and inhibitor NC \((p < .05, \text{Figure 4(F)})\).

**LBPs protected against OGD-induced cell damage through promoting wnt/β-catenin and JAK1/STAT3 pathways via up-regulating miR-24**

Compared with control cells, OGD treatment significantly inhibited the levels of wnt3a, β-catenin, p-JAK1 and p-STAT3, while LBPs treatment promoted their expression \((p < .05, \text{Figure S1(A,B)})\). Notably, miR-24 knockdown significantly inhibited the levels of wnt3a, β-catenin, p-JAK1 and p-STAT3 in OGD-induced cell treated with LBPs \((p < .05, \text{Figure S1(A,B)})\).

**Discussion**

In recently research, the functions and mechanisms of LBPs in OGD-induced PC-12 cell damage were uncovered. The results revealed LBPs significantly remitted PC-12 cells injury induced by OGD through promoting cell viability, inhibiting cell apoptosis and autophagy, down-regulating the levels of Bax, cleaved-caspase-3 and cleaved-caspase-9 and reducing ROS level in OGD-treated cells. In addition, we discovered LBPs elevated the miR-24 expression at a dose-dependent method in OGD-treated cells. Knockdown of miR-24 reversed the preventive effects of LBPs on cell damage caused by OGD. Furthermore, LBPs activated Wnt/β-catenin and JAK1/STAT3 signaling pathways by up-regulating miR-24 level in OGD-induced cells.

LBPs contains 6 monosaccharides, including xylose, rhamnose, mannose, galactose, arabinose and glucose, which exert multiple therapeutic functions such as anti-cancer, anti-inflammation, anti-fibrosis and antioxidant [21]. Noteworthily, LBPs have been evidenced to have neuro protective efficacy and are beneficial in cardiovascular and cerebrovascular diseases [21]. It is reported LBPs can induce cell autophagy to protect against peripheral neuropathy in streptozotocin-induced diabetic rats [22]. Shi et al. displayed LBPs enhanced cell viability in OGD-treated primary cultured cortical neurons [23]. In addition, Yu et al. have detected the functions of LBPs in OGD/reperfusion-treated primary hippocampal neurons and the results revealed the pretreatment of LBPs obviously enhanced cell viability, reduced cell apoptosis, cell autophagy and ROS [24]. Together with these findings, our results suggested LBPs could remit OGD-induced damage in PC-12 cells.

Furthermore, the underlying mechanisms of the functions of LBPs in OGD-induced cells were explored. MiR-24 has been identified to be highly expressed in myocardial tissues of infarction rats [25]. Besides, miR-24 is also significantly up-regulated in cerebral ischemia and is considered as potential diagnostic and therapeutic targets for post-ischemic injury [26]. On the contrary, our results discovered the miR-24 level was decreased in OGD-induced cells. Moreover, this research preliminary revealed LBPs pretreatment increased the miR-24 level, and the preventive effect of LBPs on PC-12 cell damage induced by OGD was reversed by knockdown of miR-24. Previous studies have suggested miR-24 can induce cell proliferation and inhibit cell apoptosis in cancers [27–29] as well as cardiomyocytes with ischemia/reperfusion injury [30]. Additionally, miR-24 is reported to participate in autophagy by regulating the expressions of LC3 and p62 in bladder...
These findings indicated LBPs might protect PC-12 cells against OGD-induced injury by up-regulating miR-24. Both Wnt/β-catenin and JAK1/STAT3 signaling pathways have been demonstrated to be implicated in the pathogenesis of IS [32,33]. Previous research has displayed Wnt/β-catenin signaling pathway participates in the neuroprotective effects in PC-12 cells induced by amyloid-beta protein [34]. Furthermore, Wnt/β-catenin pathway is reported to participate in neuron proliferation, apoptosis and autophagy [35,36]. Plenty of evidence has demonstrated the phosphorylation of JAK1 can activated STAT3, thereby exerting neuroprotective functions via reducing neuron apoptosis in OGD-treated cortical neurons [37] and rats with acute cerebral ischemia [38]. Herein, the results revealed Wnt/β-catenin and JAK1/STAT3 signaling pathways activated by LBPs in OGD-injured PC-12 cells, while miR-24 knockdown abolished the activation induced by LBPs. These findings indicated LBPs might function to OGD-injured PC-12 cells by up-regulating miR-24 and activating Wnt/β-catenin and JAK1/STAT3 pathways.

Conclusions

In conclusion, LBPs could remit OGD-induced injury in PC-12 cells, which might be achieved by up-regulating miR-24 and thereby regulating Wnt/β-catenin and JAK1/STAT3 pathways.
Disclosure statement
The authors have declared that no competing interests exist.

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References
[1] Van der Worp HB, van Gijn J. Acute ischemic stroke. N Engl J Med. 2007;357(6):572–579.
[2] Muir KW, Tyrrell P, Sattar N, et al. Inflammation and ischaemic stroke. Curr Opin Neurol. 2007;20(3):334–342.
[3] Feigin VL, Norrving B, Mensah GA. Global burden of stroke. Circ Res. 2017;120(3):439–448.
[4] Derex L, Cho T-H. Mechanical thrombectomy in acute ischemic stroke. Rev Neurol. 2017;173(3):106–113.
[5] Berkhemer OA, Fransen PS, Beumer D, et al. A randomized trial of intraarterial treatment for acute ischemic stroke. N Engl J Med. 2015;372(1):11–20.
[6] Liu W, Liu Y, Zhu R, et al. Structure characterization, chemical and enzymatic degradation and chain conformation of an acidic polysaccharide from Lycium barbarum L. Carbohydr Polym. 2016;147:114–124.
[7] Wu H-T, He X-J, Hong Y-K, et al. Chemical characterization of Lycium barbarum polysaccharides and its inhibition against liver oxidative injury of high-fat mice. Int J Biol Macromol. 2010;46(5):540–543.
[8] Xin Y-F, Wan L-L, Peng J-L, et al. Alleviation of the acute doxorubicin-induced cardiotoxicity by Lycium barbarum polysaccharides through the suppression of oxidative stress. Food Chem Toxicol. 2011;49(1):259–264.
[9] Gong H, Shen P, Jin L, et al. Therapeutic effects of Lycium barbarum polysaccharide (LBP) on irradiation or chemotherapy-induced myelosuppressive mice. Cancer Biother & Radiopharm. 2005;20(2):155–162.
[10] Zhu J, Liu W, Yu J, et al. Characterization and hypoglycemic effect of a polysaccharide extracted from the fruit of Lycium barbarum L. Carbohydr Polym. 2013;98(1):8–16.
[11] Tang T, He B. Treatment of d-galactose induced mouse aging with Lycium barbarum polysaccharides and its mechanism study. Afr J Trad Compl Alt Med. 2013;10(4):12–17.
[12] He M, Pan H, Chang R-C, et al. Activation of the Nrf2/HO-1 antioxidant pathway contributes to the protective effects of Lycium barbarum polysaccharides in the rodent retina after ischemia-reperfusion-induced damage. PloS One. 2014;9(1):e84800.
[13] Li H, Liang Y, Chiu K, et al. Lycium barbarum (wolfberry) reduces secondary degeneration and oxidative stress and inhibits JNK pathway in retina after partial optic nerve transection. PLoS One. 2012;7(10):e45469.
[14] Mi X-S, Feng Q, Lo ACY, et al. Protection of retinal ganglion cells and retinal vasculature by Lycium barbarum polysaccharides in a mouse model of acute ocular hypertension. PLoS One. 2012;7(10):e45469.
[15] Wang T, Li Y, Wang Y, et al. Lycium barbarum polysaccharide prevents focal cerebral ischemic injury by inhibiting neuronal apoptosis in mice. PLoS One. 2014;9(3):e90780.
[16] Rui C, Yuxiang L, Yinju H, et al. Protective effects of Lycium barbarum polysaccharide on neonatal rat primary cultured hippocampal neurons injured by oxygen-glucose deprivation and reperfusion. J Mol Hist. 2012;43(5):535–542.
[17] Barwari T, Joshi A, Mayr M. MicroRNAs in cardiovascular disease. J Am Coll Cardiol. 2016;68(23):2577–2584.
[18] Chen W, Ou H. Regulation of miR-24 on vascular endothelial cell function and its role in the development of cardiovascular disease. Sheng li xue bao: Acta physiologica Sinica. 2016;68(2):201–206.

Figure 5. Effects of miR-24 knockdown and/or LBPs on Wnt/β-catenin and JAK1/STAT3 pathway in OGD-induced cell damage. (A) The levels of wnt3a and β-catenin in control cells, cells treated with OGD, OGD+LBPs, OGD+LBPs+inhibitor NC, OGD+LBPs+miR-24 inhibitor by western blotting. (B) The levels of total-JAK1 (t-JAK1), phosphorylated (p)-JAK1, t-STAT3 and p-STAT3 in control cells, cells treated with OGD, OGD+LBPs, OGD+LBPs+inhibitor NC, OGD+LBPs+miR-24 inhibitor by western blotting. *p < .05 and **p < .01 compared with control group; #p < .05 compared with OGD group; &p < .05 compared with OGD+LBPs+inhibitor NC group.
[19] Vavilis T, Delivanoglou N, Aggelidou E, et al. Oxygen-glucose deprivation (OGD) modulates the unfolded protein response (UPR) and inflicts autophagy in a PC12 hypoxia cell line model. Cell Mol Neurobiol. 2016;36(5):701–712.

[20] Gao K, Liu M, Cao J, et al. Protective effects of Lycium barbarum polysaccharide on 6-OHDA-induced apoptosis in PC12 cells through the ROS-NO pathway. Molecules. 2014;20(1):293–308.

[21] Kwok SS, Bu Y, Lo A-Y, et al. A systematic review of potential therapeutic use of Lycium barbarum polysaccharides in disease. BioMed Res Int. 2019;2019:1.

[22] Liu S-Y, Chen L, Li X-C, et al. Lycium barbarum polysaccharide protects diabetic peripheral neuropathy by enhancing autophagy via mTOR/p70S6K inhibition in Streptozotocin-induced diabetic rats. J Chem Neuroanat. 2018;89:37–42.

[23] Shi Z, Zhu L, Li T, et al. Neuroprotective mechanisms of Lycium barbarum polysaccharides against ischemic insults by regulating NR2B and NR2A containing NMDA receptor signaling pathways. Front Cell Neurosci. 2017;11:288.

[24] Yu Y, Wu X, Pu J, et al. Lycium barbarum polysaccharide protects against oxygen glucose deprivation/reoxygenation-induced apoptosis and autophagic cell death via the PI3K/Akt/mTOR signaling pathway in primary cultured hippocampal neurons. Biochem Biophys Res Commun. 2018;495(1):1187–1194.

[25] Wang J, Huang W, Xu R, et al. Micro RNA-24 regulates cardiac fibrosis after myocardial infarction. J Cell Mol Med. 2012;16(9):2150–2160.

[26] Zhou J, Zhang J. Identification of miRNA-21 and miRNA-24 in plasma as potential early stage markers of acute cerebral infarction. Mol Med Rep. 2014;10(2):971–976.

[27] Zhang H, Duan J, Qu Y, et al. Onco-miR-24 regulates cell growth and apoptosis by targeting BCL2L11 in gastric cancer. Protein Cell. 2016;7(2):141–151.

[28] Yin Y, Zhong J, Li S-W, et al. TRIM11, a direct target of miR-24-3p, promotes cell proliferation and inhibits apoptosis in colon cancer. Oncotarget. 2016;7(52):86755.

[29] Pan B, Chen Y, Song H, et al. Mir-24-3p downregulation contributes to VP16-DDP resistance in small-cell lung cancer by targeting ATG4A. Oncotarget. 2015;6(1):317.

[30] Xiao X, Lu Z, Lin V, et al. MicroRNA miR-24-3p reduces apoptosis and regulates Keap1-Nrf2 pathway in mouse cardiomyocytes responding to ischemia/reperfusion injury. Oxid Med Cell Longev. 2018;2018:1.

[31] Yu G, Jia Z, Dou Z. miR-24-3p regulates bladder cancer cell proliferation, migration, invasion and autophagy by targeting DEDD. Oncol Rep. 2017;37(2):1123–1131.

[32] Libro R, Bramanti P, Mazzon E. The role of the Wnt canonical signaling in neurodegenerative diseases. Life Sci. 2016;158:78–88.

[33] Tang Y, Tong X, Li Y, et al. JAK2/STAT3-pathway is involved in the protective effects of epidermal growth factor receptor activation against cerebral ischemia/reperfusion injury in rats. Neurosci Lett. 2018;662:219–226.

[34] Wang J, Jing Y, Song L, et al. Neuroprotective effects of Wnt/β-catenin signaling pathway against Aβ-induced tau protein overphosphorylation in PC12 cells. Biochem Biophys Res Commun. 2016;471(4):628–632.

[35] Zhang L, Yang X, Yang S, et al. The Wnt/β-catenin signaling pathway in the adult neurogenesis. Eur J Neurosci. 2011;33(1):1–8.

[36] Shi Z-Y, Deng J-X, Fu S, et al. Protective effect of autophagy in neural ischemia and hypoxia: negative regulation of the Wnt/β-catenin pathway. Int J Mol Med. 2017;40(6):1699–1708.

[37] Chen H, Lin W, Zhang Y, et al. IL-10 promotes neurite outgrowth and synapse formation in cultured cortical neurons after the oxygen-glucose deprivation via JAK1/STAT3 pathway. Sci Rep. 2016;6(1):30459.

[38] Suzuki S, Tanaka K, Nogawa S, et al. Phosphorylation of signal transducer and activator of transcription-3 (Stat3) after focal cerebral ischemia in rats. Exp Neurol. 2001;170(1):63–71.