Research Article

Laminarin Alleviates the Ischemia/Reperfusion Injury in PC12 Cells via Regulation of PTEN/PI3K/AKT Pathway

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Objective. To investigate the protective effect of laminarin on PC12 cells damaged by oxygen glucose deprivation/reoxygenation (OGD/R) and its molecular mechanism.

Methods. PC12 cells in the logarithmic phase were randomly divided into the control group, OGD/R group, and OGD/R+laminarin (0.5, 2.5, and 5 μg/ml) group. CCK-8 activity assay kit was used to detect cell viability. ELISA kit was performed to examine the levels of proinflammatory factors (TNF-α, IL-1β, and IL-6) and oxidative stress markers (ROS, LDH, and MPO). In addition, flow cytometry was employed to determine cell cycle and apoptosis. The expression of cell proliferation-related proteins (PCNA and Ki67), apoptosis-related proteins (Bcl-2, Bax, and Caspase-3), and PTEN/PI3K/AKT pathway-related proteins was evaluated by Western blot.

Results. Compared with the control group, the cell viability was decreased significantly in the OGD/R group. CCK-8 results showed that laminarin could attenuate the damage of PC12 cell viability induced by OGD/R in a concentration-dependent manner. Meanwhile, the highest concentration of 5 μg/ml laminarin could significantly promote the viability of PC12 cells and the expression of PCNA and Ki67 than the OGD/R group. Additionally, ELISA assays showed that laminarin significantly inhibited the expression of proinflammatory factors (TNF-α, IL-1β, and IL-6) and oxidative stress markers (ROS, LDH, and MPO). Flow cytometry results demonstrated that laminarin promoted the cell cycle. And laminarin upregulated the expression of apoptotic protein Bcl-2, while downregulated the expression of apoptotic proteins Bax and Caspase-3. Finally, laminarin significantly suppressed the expression of PTEN and facilitated the expression of PI3K and p-AKT compared to the OGD/R group.

Conclusion. Laminarin could alleviate the OGD/R-induced PC12 cell neuronal injury via promoting cell activity and cycle and inhibiting inflammation, oxidative stress, and apoptosis. The mechanism may be related to the downregulation of PTEN protein and the activation of the PI3K/AKT pathway.

1. Introduction

Ischemic stroke is an acute cerebrovascular disease that can seriously affect patients’ quality of life [1, 2]. According to statistics, about 1.5 million people die of ischemic stroke in China every year. More importantly, the disease was the first cause of death in China in 2017 [3]. Studies have shown that stroke patients will have a sustained brain injury due to insufficient oxygen and glucose supply in the brain. There is no effective treatment at present. Clinically, stroke patients mainly recover the blood supply of ischemic brain tissue as soon as possible through thrombolysis to alleviate symptoms. However, limited perfusion time window and rapid blood perfusion will lead to further pathological damage of ischemic tissue, namely, hypoxia reperfusion (OGD/R) injury [4, 5]. Studies have confirmed that neuronal OGD/R injury is a complex physiological process involving reactive oxygen species (ROS), proinflammatory mediators, and apoptotic genes [6]. Therefore, it is imperative to develop new neuroprotective drugs or treatment strategies to improve the quality of life of stroke patients. Laminaria polysaccharide is an active component that extracts and isolates from...
the dried leaves of Laminaria japonica, a plant of the Laminaria family. Its chemical composition includes β-1,3-glycosidic bonds and β-1,6-glycosidic bonds [7]. Studies have shown that Laminaria polysaccharide has a variety of physiological activities such as antioxidant, antilipid, antibacterial, and antitumor, which is of great development value [8]. Studies have reported that laminarin can reduce the development of renal interstitial fibrosis by reducing endoplasmic reticulum stress-mediated apoptosis [9]. Therefore, we speculate that laminarin may also play a corresponding protective role in hypoxic-reperfusion nerve cell injury. Therefore, this study is aimed at exploring the protective effect and potential molecular mechanism of laminarin at different concentrations on OGD/R-induced PC12 nerve cell injury by establishing an OGD/R-induced PC12 nerve cell model.

2. Materials and Methods

2.1. Main Reagents. Laminaria polysaccharide is extracted from kelp. The polysaccharide content was ≥90%. The PC12 cell line was purchased from the Shanghai Institute of cell research, the Chinese Academy of Sciences. DMEM high glucose medium and 10% fetal bovine serum were purchased from GIBCO company of the United States. Penicillin/streptomycin and ROPA cell lysates were purchased from the American Becton company. DMEM high glucose medium and 10% fetal bovine serum were from the Beijing Zhongshan Jinqiao company. DMEM high glucose medium containing 10% fetal bovine serum and 1% penicillin/streptomycin, and HBR-labeled Goat anti-rabbit secondary antibody were purchased from the Beijing Zhongshan Jinqiao company. Other common molecular biology-related antibody reagents and consumables were provided by Thermo Fisher Scientific, USA.

2.2. PC12 Cell Culture and OGD/R Model Construction. PC12 cells were cultured in DMEM high glucose medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. The culture conditions were set at 37°C, 5% CO2 content, and 95% relative humidity. The degree of cell fusion reached more than 90% for subculture. More than three generations of PC12 cells were taken for follow-up experimental research. The DMEM high glucose medium of logarithmic PC12 cells was first discharged and then washed with PBS for 3 times. A Sugar-free Earle equilibrium salt solution was added to the cells. The cells were then transferred to a 37°C hypoxia incubator (95% N2, 5% CO2, and 1% O2) for 6 hours. Then, the equilibrium solution in the cell culture plate was replaced with DMEM high glucose medium containing 10% fetal bovine serum and placed in a cell culture box at 37°C and 5% CO2 for 24 hours for reoxygenation.

2.3. Experimental Grouping. The extracted Laminaria polysaccharide was diluted into 0.5, 2.5, 5, and 10 μg/ml of serum-free DMEM high glucose culture medium polysaccharide solution. The polysaccharide solution has been ensured to be used in the ultraclean table for sterilization by 0.22 μm pore size filter. According to the experimental requirements, PC12 cells were divided into 5 groups: blank control group (PC12 cells not treated with OGD/R), OGD/R group (PC12 cells treated with OGD/R), OGD/R+laminarin (0.5 μg/ml) group, OGD/R+laminarin (2.5 μg/ml) group, and OGD/R+laminarin (5 μg/ml). The cells in each administration group were incubated for 2 hours before OGD/R modeling.

2.4. Cell Proliferation Activity Was Detected by Cell Counting Kit-8 (CCK-8). PC12 cells in each group were inoculated into 96-well plates at the density of 3 × 10^3 cells/well. These cells were cultured in a cell incubator at 37°C and 5% CO2 for 24 hours and 20% CO2. Then, 10 μl of CCK-8 solution was added, and cells were continuously incubated for another 2 hours. The microplate reader (Thermo Fisher Scientific, USA) was used to detect the absorbance of PC12 cells at 450 nm.

2.5. Detection of Proinflammatory Factors (TNF-α, IL-1β, and IL-6) and Oxidative Stress Indicators (ROS, LDH, and MPO) by Enzyme-Linked Immunosorbent Assay (ELISA). PC12 cells in each group were inoculated into 96-well plates at the density of 3 × 10^3 cells/well according to the operating instructions of the ELISA kit. Cells were cultured for 24 hours before collecting the cell supernatant of each group. The expression of TNF-α, IL-1β, IL-6, ROS, LDH, and MPO was examined by ELISA. The absorbance value was measured by a microplate reader.

2.6. Cell Cycle and Apoptosis Were Detected by Flow Cytometry. Cell cycle. PC12 cells in each group were inoculated into 6-well plates at the density of 1 × 10^6 cells/well and cultured for 48 hours. After culture, the cells were digested with 0.25% trypsin. The cells were then fixed with 70% ethanol at 4°C overnight. Finally, the cells were centrifuged at 1000 r/min for 5 min, and ethanol was discarded. 500 ml PI was added, and cells were incubated in the dark at 37°C for 45 min. The cell cycle was detected by flow cytometry.

Apoptosis. PC12 cells in each group were inoculated into 6-well plates at the density of 1 × 10^6 cells/well and cultured for 48 hours. After culture, the cells were digested with 0.25% trypsin. The cells were centrifuged at 1000 r/min, and the supernatant was discarded. The cells were resuspended 200 μl combined solution to adjust the cell concentration to 1 × 10^6 cells/ml. 5 μl PI and 10 μl Annexin V-FITC were added to the suspension. Cells were incubated in the dark for 15 min at room temperature. Apoptosis was detected by flow cytometry.

The FlowJo software (Tree Star, USA) was used to analyze apoptosis and cell-cycle data.

2.7. The Expression Levels of PCNA, Ki67, Bax, Caspase-3, PTEN, PI3K, and p-AKT Were Detected by Western Blot. The total protein of PC12 cells in each group...
was extracted by RIPA cell lysate, and the protein concentration was detected by BCA protein quantitative kit. The extracted total protein was separated by 10% SDS-PAGE and transferred to the PVDF membrane. These membranes were sealed with 5% skimmed milk powder solution for 2 h before mixing with corresponding primary antibody PCNA (ab92552; Abcam), Ki67 (ab16667; Abcam), Bcl-2 (ab32124; Abcam), Bax (ab32503; Abcam), Caspase-3 (cat. No. 9662; Cell Signaling Technology, Inc.), PTEN (ab32199; Abcam), PI3K (ab32089; Abcam), p-AKT (cat. No. 4060; Cell Signaling Technology, Inc.), and GAPDH (ab9485; Abcam). Cells were incubated overnight at 4°C. These membrane proteins were then incubated with HBR-labeled secondary antibodies (cat. No. 7074; Cell Signaling Technology, Inc.) at room temperature for 1 h. ECL chemical kit was used for protein imaging. The value analysis of protein bands was performed through the Image J software. GAPDH was used as the internal reference.

2.8. Statistical Analysis. All data in this experiment were expressed by mean ± standard deviation. All experiments were repeated 3 times, and the data were analyzed with GraphPad prism 7.0. The data difference between the two groups was analyzed by an independent sample t-test. One-way ANOVA analyzed the difference between the three groups and above. P < 0.05 was considered to be statistically significant.

3. Results

3.1. Effects of Laminaria Polysaccharide at Different Concentrations on the Viability of PC12 Cells Induced by OGD/R. We first detected the effect of Laminaria polysaccharide at the concentration of 0.5, 2.5, 5, and 10 μg/ml on the viability of PC12 cells. As shown in Figure 1(a), 10 μg/ml laminarin could significantly reduce cell viability compared to the control group (P < 0.05). The above results suggest that the concentration of Laminaria polysaccharide around 0.5-5 μg/ml has no toxic effect on the viability of PC12 cells. Therefore, 0.5-5 μg/ml Laminaria polysaccharide was used to construct the subsequent OGD/R model. CCK-8 and Western blot were used to detect the viability of PC12 cells induced by OGD/R. As shown in Figure 1(b), PC12 cell viability in the OGD/R group decreased significantly (P < 0.05) than the control group. Laminarin could promote the viability of PC12 cells in a concentration-dependent manner (P < 0.05, P < 0.01, and P < 0.001) compared to the OGD/R group. The recovery of PC12 cell viability was the most obvious at 5 μg/ml Laminaria polysaccharide. As shown in Figure 1(c), the expression of PCNA and Ki67 in the OGD/R group decreased significantly (P < 0.05). In addition, the expression levels of PCNA and Ki67 protein in the laminarin group increased gradually with the increase of concentration, and the difference was statistically significant (P < 0.05, P < 0.01, and P < 0.001) than the OGD/R group. The above results showed that Laminaria polysaccharide could play a protective role in alleviating the decline of PC12 cell viability induced by OGD/R.

3.2. Effects of Different Concentrations of Laminaria Polysaccharide on Inflammatory Response and Oxidative Stress Level of PC12 Cells Induced by OGD/R. We then detected the effect of different concentrations of Laminaria polysaccharide on OGD/R-induced proinflammatory factors (TNF-α, IL-1β, and IL-6) and oxidative stress indicators (ROS, LDH, and MPO) in PC12 cells by ELISA. As shown in Figure 2(a), the expressions of TNF-α, IL-1β, and IL-6 in the OGD/R group were significantly increased (P < 0.001) compared with the control group, and laminarin could inhibit TNF-α, IL-1β, and IL-6 (P < 0.05, P < 0.01, and P < 0.001) in a concentration-dependent manner compared with the OGD/R group. In addition, as shown in Figure 2(b), the expressions of ROS, LDH, and MPO in the OGD/R group were significantly increased (P < 0.001) compared with the control group, and the levels of ROS, LDH, and MPO in cell supernatant decreased gradually with the increase of laminarin concentration (P < 0.05, P < 0.01, and P < 0.001) compared to the OGD/R group. The above results showed that Laminaria polysaccharide could alleviate the inflammatory response and oxidative stress of PC12 cells induced by OGD/R.

3.3. Effects of Laminaria Polysaccharide at Different Concentrations on PC12 Cell Cycle Induced by OGD/R. The cell cycle was detected by flow cytometry. As shown in Figure 3, the proportion of S-phase cells in the OGD/R group increased significantly than the control group (P < 0.001). The proportion of G0/G1 phase cells decreased significantly (P < 0.05, P < 0.01, and P < 0.001), while the proportion of G2/M phase cells did not change significantly. Compared with the OGD/R group, the proportion of S-phase cells decreased significantly (P < 0.001), and the proportion of G0/G1 phase cells increased substantially in a concentration-dependent manner (P < 0.05, P < 0.01, and P < 0.001). The above results showed that Laminaria polysaccharide could reduce the S-phase arrest of PC12 cells induced by OGD/R and improve the inhibitory effect of OGD/R on the PC12 cell cycle.

3.4. Effects of Laminaria Polysaccharide at Different Concentrations on Apoptosis of PC12 Cells Induced by OGD/R. Meanwhile, apoptosis was detected by flow cytometry and Western blot. Compared with the control group, the apoptosis rate of the OGD/R group was significantly increased (P < 0.001) (Figure 4(a)). Compared with the OGD/R group, laminarin could inhibit apoptosis in a concentration-dependent manner (P < 0.05, P < 0.01, and P < 0.001) (Figure 4(a)). Compared with the control group, the expression levels of apoptotic proteins Bax and Caspase-3 in the OGD/R group were significantly increased (P < 0.001), while the expression level of the antiapoptotic protein Bcl-2 was significantly decreased (P < 0.001) (Figure 4(b)). With the increase of laminarin concentration, the expression levels of proapoptotic protein Bax and Caspase-3 in PC12 cells gradually decreased, while the expression level of antiapoptotic protein Bcl-2 gradually increased (P < 0.05, P < 0.01, and P < 0.001) (Figure 4(b)).
The above results showed that Laminaria polysaccharide could inhibit the apoptosis of PC12 cells induced by OGD/R.

3.5. Effects of Laminaria Polysaccharide at Different Concentrations on the Expression Levels of PTEN, PI3K, and p-AKT Proteins in PC12 Cells Induced by OGD/R. To further study the potential mechanism of Laminaria polysaccharide on OGD/R-induced PC12 cell injury, we detected the expression level of PTEN/PI3K/AKT pathway-related proteins by Western blot. As shown in Figure 5, the protein expressions of PI3K and p-AKT in the OGD/R group were significantly decreased ($P < 0.001$) compared with the control group, while the protein expression of PTEN was significantly increased ($P < 0.001$), and laminarin could upregulate the protein expression of PI3K and p-AKT in a concentration-dependent manner while inhibiting the protein expression of PTEN ($P < 0.05$, $P < 0.01$, and $P < 0.001$). These results suggest that Laminaria polysaccharide can effectively protect PC12 cells from OGD/R-induced injury. Its mechanism may be related to the activation of the PI3K/AKT pathway and the downregulation of PTEN protein.

4. Discussion

At present, the treatment options for ischemic stroke are not satisfactory. Therefore, there is an urgent need to develop new treatment schemes to improve the treatment effect, especially for high-risk patients [10, 11]. This study found that laminarin can reduce OGD/R-induced nerve cell injury by activating PI3K/AKT pathway and downregulating PTEN protein expression. The results indicate that laminarin is a promising candidate drug for treating ischemic stroke, which is worthy of in-depth study.

Due to the characteristics of multitarget and multichannel treatment, studies have confirmed that traditional Chinese medicine significantly promotes the recovery of ischemic stroke [12–15]. For example, in the mouse model of glucose deficiency and hypoxia, tanshinone can effectively
reduce the area of cerebral infarction in mice [16]. In addition, Ginkgo biloba extract can effectively reduce brain oxygen uptake and consumption in elderly patients with cerebral ischemia to improve the balance between brain oxygen supply and consumption [17]. These studies have confirmed the critical protective effect of traditional Chinese medicine on hypoxic-ischemic brain injury. Laminaria polysaccharide has been widely studied as a kind of macro-molecular polysaccharide with many biological functions, including antioxidation and antitumor. However, the role of laminarin in hypoxic-reperfusion nerve injury has not been reported. In this study, Laminaria polysaccharide can...
promote the activity of PC12 nerve cells induced by OGD/R in a concentration-dependent manner and reduce the inhibitory effect of OGD/R on the activity of PC12 cells. This result suggests the protective effect of laminarin on ischemia-reperfusion injury.

Cerebral hypoxia reperfusion injury involves multiple pathological processes, including neuroinflammation, oxidative stress, and apoptosis [18–20]. Excessive ROS is widely considered the leading cause of reperfusion injury of microvessels and parenchymal organs in ischemic tissues. After the blood supply of ischemic tissue is restored, excessive free radicals will attack the cells of the tissue and cause damage [21]. At the same time, tissue ischemia can induce proinflammatory cytokines (TNF-α, IL-1β, and IL-6), further aggravating ROS levels [22]. In addition, ischemia-reperfusion injury can also activate cell death programs, including apoptosis, autophagy, and necrosis. There is increasing evidence that excessive proapoptotic proteins (Bax and Caspase-3) and oxidative stress products (LDH and MPO) are produced in cerebral ischemic injury [23, 24]. Previous studies have shown that cinnamon extract transcinnamaldehyde can protect PC12 cells from OGD/R stimulation through antiapoptosis and antioxidant stress [25]. Therefore, targeting inflammation, oxidative stress, and apoptosis has been proved to be a feasible treatment option for cerebral hypoxia reperfusion injury. In this study, we found that OGD/R can induce PC12 neuronal proinflammatory factor (TNF-α, IL-1β, and IL-6) expression and increase the levels of oxidative stress indexes (ROS, LDH, and MPO). At the same time, laminarin could reverse the above effects in a concentration-dependent manner. In addition, the results of flow cytometry showed that laminarin

![Flow cytometry results](image1)

**Figure 4:** Effect of different concentrations of laminarin (0.5, 2.5, and 5 μg/ml) on the apoptosis of PC12 cells induced by OGD/R. (a) Flow cytometry was used to detect the effects of different concentrations of laminarin (0.5, 2.5, and 5 μg/ml) on apoptosis of PC12 cells induced by OGD/R. (b) Western blot assay was performed to detect the effects of different concentrations of laminarin (0.5, 2.5, and 5 μg/ml) on the protein expression levels of Bcl-2, Bax, and Caspase-3 in PC12 cells induced by OGD/R. Note: 1: OGD/R group, 2: OGD+0.5 μg/ml laminarin group, 3: OGD+2.5 μg/ml laminarin group, and 4: OGD+5 μg/ml laminarin group. Compared with the control group, ***P < 0.001; compared with the OGD/R group, **P < 0.01, and ***P < 0.001.
could alleviate the blocking and proapoptotic effects of OGD/R on the s cycle of PC12 cells. These results suggest that Laminaria polysaccharide can improve ischemia-reperfusion nerve cell injury by regulating inflammatory factors, oxidative stress-related indexes, and cycle-related proteins.

Finally, we studied the possible molecular mechanism of laminarin in protecting nerve cells from ischemia-reperfusion injury. PTEN is a tumor suppressor gene with bispecific phosphatase activity, mainly responsible for regulating cell growth and apoptosis signal transduction [26]. Many studies have shown that PTEN gene knockout can protect against ischemia-reperfusion nerve injury [27]. For example, SETD5-AS1 can increase cerebral infarction volume and neurological impairment in ischemic stroke mice by inducing PTEN overexpression [28]. Andrographolide of traditional Chinese medicine can protect hippocampal neurons in rats with chronic cerebral ischemia injury by inhibiting PTEN levels [29]. In addition, PI3K/AKT is a vital pathway closely related to apoptosis after cerebral hypoxia and ischemia. PTEN can promote cell activity damage by antagonizing PI3K/AKT pathway to induce cell-cycle arrest [30]. Studies have shown that Panax notoginseng saponins improve hypoxic-ischemic neonatal brain injury by activating the PI3K/AKT pathway [31]. Curcumin can target vascular endothelial growth factors by activating PI3K/AKT to prevent cerebral hypoxic-ischemic injury in neonatal rats [32]. In this study, Laminaria polysaccharide can effectively inhibit the PTEN level of PC12 cells induced by OGD/R and promote the levels of PI3K and p-AKT, suggesting that Laminaria polysaccharide can reduce nerve cell injury after ischemia-reperfusion by regulating PTEN/PI3K/AKT pathway.

In conclusion, laminarin can inhibit OGD/R-induced inflammation, oxidative stress, and apoptosis of PC12 cells by regulating PTEN/PI3K/AKT pathway. These results provide new insights into the protective mechanism of laminarin against cerebral hypoxia and ischemia and indicate that laminarin is a promising choice for treating ischemic stroke. However, this study has not verified the effect of Laminaria polysaccharide on brain injury in hypoxic mice, which will be further studied in a follow-up study.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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References


