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Effect of lycium barbarum polysaccharides on high glucose−induced retinal ganglion cell apoptosis, gene expression and delayed rectifier potassium current

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ABSTRACT

Objective: To study the effect of lycium barbarum polysaccharides (LBP) on high glucose-induced retinal ganglion cell apoptosis, gene expression and delayed rectifier potassium current. **Methods:** RGC-5 retinal ganglion cell lines were cultured and divided into control group, high glucose group and LBP group that were treated with normal DMEM, high-glucose DMEM as well as high-glucose DMEM containing 500 ng/mL LBP respectively. After treatment, the Annexin V-FITC/PI kits were used to measure the number of apoptotic cells, fluorescence quantitative PCR kits were used to determine the expression of apoptosis genes and antioxidant genes, and patch clamp was used to test delayed rectifier potassium current. **Results:** 12, 24, 36 and 48 h after intervention, the number of apoptotic cells of high glucose group was significantly higher than that of control group, and the number of apoptotic cells of LBP group was significantly lower than that of high glucose group (P<0.05); 24 and 48 h after intervention, c-fos, c-jun, caspase-3, caspase-9, Nrf-2, NQO1 and HO-1 mRNA expression as well as potassium current amplitude (I_K) and maximum conductance (G_{max}) of high glucose group were significantly higher than those of control group while half maximum activation voltage ($V_{1/2}$) was significantly lower than that of control group (P<0.05); c-fos, c-jun, caspase-3 and caspase-9 mRNA expression as well as I_K and G_{max} of LBP group were significantly lower than those of high glucose group, while Nrf-2, NQO1 and HO-1 mRNA expression as well as $V_{1/2}$ of LBP group were significantly higher than those of high glucose group (P<0.05). **Conclusions:** LBP can reduce the high glucose-induced retinal ganglion cell apoptosis and inhibit the delayed rectifier potassium current amplitude.

1. Introduction

Type 2 diabetes mellitus is a the most common endocrine system disease worldwide, its incidence has been rising year by year, and in the development and change of type 2 diabetes, microvascular complications and macrovascular complications will cause harm to the patients' quality of life and life safety and influence the disease outcome. Diabetic retinopathy is a common microvascular complication in patients with type 2 diabetes, and is also a clinical common cause of blindness[1,2]. At present, the research on the mechanism of diabetic retinopathy believes that the retinal ganglion cell apoptosis and damage caused by high glucose environment is closely related to the occurrence of retinopathy[3]. Lyceum barbarum polysaccharides (LBP) are the traditional Chinese medicine extracts with cytoprotection, anti-inflammatory and anti-oxidative stress effect[4,5]. In the following study, the effect of LBP on high glucose-induced retinal ganglion cell apoptosis, gene expression and delayed rectifier potassium current was analyzed.

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2. Materials and methods

2.1. Experimental materials

RGC-5 retinal ganglion cell lines were bought from Xingzhi Biotechnology Co., LTD., normal DMEM, high-glucose DMEM, fetal bovine serum and 0.125% trypsin for cell culture were all purchased from Gibco Company, Annexin V-FITC/PI kits were purchased from R&D Company, and the fluorescence quantitative

PCR kits were purchased from Shanghai Kemin Biotechnology Co., LTD.

2.2. Cell culture and treatment methods

RGC-5 were cultured in normal DMEM containing 10% fetal bovine serum, the cells were digested with 0.125% trypsin when they grew with adherence and the density reached 90%, the sub-cultured cells with logarithmic growth phase were collected for treatment and divided into control group, high glucose group and LBP group, and the treatment methods were as follows: (1) the control group were treated with common DMEM, and the glucose concentration was 5.5 mmol/L; (2) the high glucose group were treated with high glucose DEME, and the glucose concentration was 25.0 mmol/L; (3) the LBP group were treated with the high-glucose DMEM containing 500 ng/mL LBP.

2.3. Cell apoptosis detection methods

The cells for apoptosis detection were inoculated in 6-well culture plate and treated with different conditions for 12, 24, 36 and 48 h, then the culture medium was abandoned, 0.125% trypsin was added for digestion, the cells were collected, washed with PBS twice, then re-suspended, added in 5 μL AnnexinV-FITC staining fluid, incubated away from light for 10 min, centrifuged and re-suspended again, 5 μL PI staining fluid was added, and the number of apoptotic cells in 100 cells were determined in flow cytometer.

2.4. Gene mRNA expression detection methods

The cells for gene mRNA detection were inoculated in 12-well culture plate and treated with different conditions for 24 and 48 h, then the culture medium was abandoned, the cells were washed with PBS twice and then added in Trizol lysis buffer to extract the total RNA in cells, it was reverse-transcribed into cDNA for fluorescence quantitative PCR amplification, the amplified genes included c-fos, c-jun, caspase-3, caspase-9, Nrf-2, NQO1, HO-1 and GAPDH, and GAPDH was used as reference to calculate the c-fos, c-jun, caspase-3, caspase-9, Nrf-2, NQO1 and HO-1 mRNA expression.

2.5. Delayed rectifier potassium current detection methods

The cells for delayed rectifier potassium current detection were inoculated in 6-well culture plate and treated with different conditions for 24 and 48 h, then the culture medium was abandoned, the electrode liquid was added, and then whole-cell patch clamp was used to record potassium current and analyze potassium current amplitude (I_K), half maximum activation voltage ($V_{1/2}$) and maximum conductance (G_{max}).

2.6. Statistical analysis

SPSS20.0 software was used to input and analyze data, measurement data analysis among three groups was by variance analysis, pair-wise comparison was by *LSD-t* test and $P<0.05$ indicated statistical significance in differences.

3. Results

3.1. Number of apoptotic cells among groups

12, 24, 36 and 48 h after intervention, analysis of the number of apoptotic cells among groups is as follows: the number of apoptotic cells of high glucose group was significantly higher than that of control group, and the number of apoptotic cells of LBP group was significantly lower than that of high glucose group. Differences in pair-wise comparison of the number of apoptotic cells were statistically significant among groups 12, 24, 36 and 48 h after intervention ($P<0.05$) (Table 1).

Table 1

Comparison of the number of apoptotic cells among groups (n=5, $\bar{x}\pm s$).

Groups	12 h	24 h	36 h	48 h
Control group	6.92±0.89	7.28±0.91	7.11±0.84	7.30±0.88
High glucose group	10.37±1.35*	22.32±3.28*	31.37±4.92*	48.50±5.84*
LBP group	7.52±0.91&	11.37±1.36&	18.38±2.03&	25.62±3.28&

*: compared with control group, $P<0.05$; &: compared with high glucose group, $P<0.05$.

3.2. Apoptosis gene expression among groups

24 and 48 h after intervention, analysis of apoptosis genes c-fos, c-jun, caspase-3 and caspase-9 expression among groups is as follows: c-fos, c-jun, caspase-3 and caspase-9 mRNA expression of high glucose group were significantly higher than those of control group, and c-fos, c-jun, caspase-3 and caspase-9 mRNA expression of LBP group were significantly lower than those of high glucose group. Differences in pair-wise comparison of c-fos, c-jun, caspase-3 and caspase-9 mRNA expression were statistically significant among groups 24 and 48 h after intervention ($P<0.05$) (Table 2).

Table 2

Comparison of apoptosis gene expression among groups (n=5, $\bar{x}\pm s$).

Groups	Intervention time (h)	c-fos	c-jun	Caspase-3	Caspase-9
Control group	24	1.02±0.14	1.05±0.11	0.98±0.10	0.97±0.12
	48	0.97±0.08	0.94±0.12	1.03±0.14	0.94±0.07
High glucose group	24	1.78±0.22*	1.81±0.24*	1.72±0.19*	1.87±0.22*
	48	2.65±0.28*	2.77±0.29*	2.91±0.35*	2.72±0.29*
LBP group	24	1.33±0.16&	1.41±0.18&	1.31±0.14&	1.38±0.16&
	48	1.71±0.22&	1.80±0.19&	1.75±0.20&	1.69±0.18&

*: compared with control group, $P<0.05$; &: compared with high glucose group, $P<0.05$.

3.3. Antioxidant gene expression among groups

24 and 48 h after intervention, analysis of antioxidant genes Nrf-2, NQO1 and HO-1 expression among groups is as follows: Nrf-2, NQO1 and HO-1 mRNA expression of high glucose group were significantly higher than those of control group, and Nrf-2, NQO1 and HO-1 mRNA expression of LBP group were significantly higher than those of high glucose group. Differences in pair-wise comparison of Nrf-2, NQO1 and HO-1 mRNA expression were statistically significant among groups 24 and 48 h after intervention (P<0.05) (Table 3).

Table 3

Comparison of antioxidant gene expression among groups (n=5, $\bar{x}\pm s$).

Groups	Intervention time (h)	Nrf-2	NQO1	HO-1
Control group	24	1.03±0.14	1.06±0.12	0.99±0.10
	48	0.97±0.12	1.04±0.11	1.08±0.12
High glucose group	24	1.64±0.22*	1.59±0.19*	1.52±0.17*
	48	2.15±0.26*	1.98±0.20*	2.05±0.26*
LBP group	24	2.08±0.29&	1.97±0.22&	2.21±0.28&
	48	2.89±0.35&	2.77±0.31&	2.82±0.34&

*: compared with control group, P<0.05; &: compared with high glucose group, P<0.05.

3.4. Delayed rectifier potassium current among groups

24 and 48 h after intervention, analysis of delayed rectifier potassium current parameters I_K, $V_{1/2}$ and G_{max} among groups was as follows: $V_{1/2}$ of high glucose group was significantly lower than that of control group while I_K and G_{max} were significantly higher than those of control group; $V_{1/2}$ of LBP group was significantly higher than that of high glucose group while I_K and G_{max} were significantly lower than those of high glucose group. Differences in pair-wise comparison of I_K, $V_{1/2}$ and G_{max} were statistically significant among groups 24h and 48h after intervention (P<0.05) (Table 4).

Table 4

Comparison of delayed rectifier potassium current among groups (n=5, $\bar{x}\pm s$).

Groups	Intervention time (h)	I_K (pA/pE)	$V_{1/2}$ (mV)	G_{max}
Control group	24	56.75±7.75	22.31±3.09	0.42±0.06
	48	58.33±6.92	22.93±2.78	0.44±0.05
High glucose group	24	81.32±9.62*	12.48±1.74*	0.72±0.09*
	48	85.37±9.91*	10.97±1.35*	0.76±0.08*
LBP group	24	66.53±7.85&	17.63±1.94&	0.61±0.06&
	48	59.57±6.39&	19.78±2.34&	0.54±0.08&

*: compared with control group, P<0.05; &: compared with high glucose group, P<0.05.

4. Discussion

Retinal ganglion cell damage under high glucose environment is an important pathological link in diabetic retinopathy, and relieving the retinal ganglion cell damage caused by high glucose is the important target for the prevention and treatment of diabetic retinopathy. LBP are the water-soluble polysaccharides extracted from Chinese wolfberry, and have cytoprotection, anti-apoptotic, anti-inflammatory, antioxidant and a variety of other biological functions[4,5]. In the cell models of glaucoma, LBP has inhibiting effect on the retinal ganglion cell apoptosis induced by high pressure, and can improve the retinal ganglion cell function[6]. However, there is no report about the value of LBP for the treatment of diabetic retinopathy, and it is also not clear whether LBP can relieve retinal ganglion cell damage caused by high glucose. In the study, in order to define the LBP effect on the retinal ganglion cell damage caused by high glucose, the retinal ganglion cell apoptosis was analyzed at first, and the results showed that the number of apoptotic cells of high glucose group was significantly higher than that of control group (P<0.05), and the number of apoptotic cells of LBP group was significantly lower than that of high glucose group (P<0.05). This means that high glucose conditions can induce retinal ganglion cell apoptosis, and LBP intervention can reverse the cell apoptosis caused by high glucose, and has significant cytoprotection effect.

The c-fos in FOS family and the c-jun in JUN family are the important apoptosis signaling molecules, and c-fos/c-jun pathways can regulate the retinal ganglion apoptosis. The c-fos and c-jun expression in cells are mutually coupled, and external stimulation could increase the c-fos and c-jun expression and promote their transfer into the nucleus to induce the expression and activation of pro-apoptotic molecules caspase-3 and caspase-9, and promote apoptosis[7,8]. In the study, analysis of the changes in apoptosis gene expression under the high glucose environment showed that c-fos, c-jun, caspase-3 and caspase-9 mRNA expression of high glucose group were significantly higher than those of control group (P<0.05). This means that high glucose can increase the caspase-3 and caspase-9 expression mediated by c-fos/c-jun to induce cell apoptosis. In order to further clarify the LBP influence on apoptosis gene expression, the apoptosis gene expression levels were compared between high glucose group and LBP group, and the results showed that c-fos, c-jun, caspase-3 and caspase-9 mRNA expression of LBP group were significantly lower than those of high glucose group (P<0.05). This means that LBP can inhibit the high caspase-3 and caspase-9 expression mediated by c-fos/c-jun under high glucose environment.

In addition to inducing cell apoptosis through c-fos/c-jun pathways, high glucose environment can also activate oxidative stress response to cause cell damage[9,10]. The feature of the oxidative stress caused by high glucose is that the reactive oxygen species are excessively produced and exceed the scavenging capacity of antioxidant system for them, and reactive oxygen species can lead to the oxidation of a

variety of compositions in retinal ganglion cells, and cause damage to cell structure and function[11,12]. The massively produced reactive oxygen species in retinal ganglion cells can activate the antioxidant pathways mediated by Nrf-2, and cause the Nrf-2 and Keap1 to dissociate, enter into the nucleus and be combined with the specific sequence in the DNA to start the expression of antioxidant enzymes NQO1 and HO-1. NQO1 can catalyze quinone dual electron reduction reaction and reduce the generation of reactive oxygen species, and HO-1 can remove the ROS to reduce oxidative stress reaction[13,14]. In the study, analysis of above Nrf-2 antioxidant pathway in retinal ganglion cells showed that Nrf-2, NQO1 and HO-1 mRNA expression of high glucose group were significantly higher than those of control group, and Nrf-2, NQO1 and HO-1 mRNA expression of LBP group were significantly higher than those of high glucose group (P<0.05). This means high glucose environment can cause the compensatory activation of Nrf-2 antioxidant pathways in retinal ganglion cells, and LBP can further increase the Nrf-2 antioxidant pathways and enhance the antioxidant capacity of retinal ganglion cells.

Research on retinal ganglion cell apoptosis in recent years has found that the apoptosis process is mostly associated with the increased potassium efflux and decreased intracellular potassium concentration, and the abnormal opening of potassium channels on cell membrane is closely related to the changes in intracellular and extracellular potassium distribution[15-17]. In the study, patch clamp technique was used to record the load current of retinal ganglion cell potassium under high glucose environment, and the results showed that $V_{1/2}$ of high glucose group was significantly lower than that of control group while I_K and G_{max} were significantly higher than those of control group (P<0.05). This means that high glucose environment can promote the potassium efflux in retinal ganglion cells and increase the outward potassium current. Further analysis of the influence of LBP on intracellular and extracellular potassium distribution and the load current of potassium showed that $V_{1/2}$ of LBP group was significantly higher than that of high glucose group while I_K and G_{max} were significantly lower than those of high glucose group (P<0.05). This means that LBP can inhibit high glucose-induced potassium efflux in retinal ganglion cells.

To sum up, it is believed that high glucose can induce retinal ganglion cell apoptosis, increase the caspase-3 and caspase-9 expression mediated by c-fos/c-jun as well as the NQO1 and HO-1 expression mediated by Nrf-2, and also increase the potassium efflux; LBP can inhibit the retinal ganglion cell apoptosis caused by high glucose environment, and also regulate the apoptosis gene and anti-apoptotic gene expression as well as the potassium effux.

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