Original Article
Impact of catalpol on retinal ganglion cells in diabetic retinopathy

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Abstract: Diabetic retinopathy (DR) is one of the most important complications of diabetes mellitus (DM), and also the main cause of blindness. Retinal ganglion cells (RGCs), the main cells forming vision in retina, are damaged in DR. It was reported that catalpol played an important role in nervous repair and remodeling. This study aims to investigate the role of catalpol in DR and provide theoretical basis and reference for DR clinical treatment and clarifying DR pathogenesis. RGCs cultured in vitro were randomly divided into normal control, high glucose group, and catalpol group. RGCs in catalpol group received 2 mm catalpol under high glucose circumstance. Cell proliferation was detected by MTT assay. LDH activity, SOD activity, and ROS content were tested by colorimetric method. Inflammatory factors TNF-α and IL-1β secretion was determined by ELISA. NF-κB expression was measured by Real time PCR and Western blot. Under high glucose circumstance, GRCs survival rate decreased, NF-κB level elevated, TNF-α and IL-1β secretion enhanced, LDH and ROS content increased, and SOD activity reduced obviously compared with normal control \( (P < 0.05) \). Catalpol significantly increased RGCs survival rate, declined LDH and ROS content, enhanced SOD activity, reduced TNF-α and IL-1β secretion, decreased NF-κB mRNA and protein expression compared with high glucose group \( (P < 0.05) \). Catalpol can prevent DR through regulating oxidation/antioxidation balance, inhibiting inflammatory factors and NF-κB signaling pathway to promote GRCs survival.

Keywords: Catalpol, diabetic retinopathy, retinal ganglion cell, antioxidation, NF-κB

Introduction

Diabetes mellitus (DM) is a type of endocrine disease threatened to human health. DM complication is the important factor to cause multiple organ damage and even death [1]. As one of the most serious complications, diabetic retinopathy (DR) shows high incidence. As an important factor of acquired blinding disease, DR cause great harm to DM patients [2]. WHO survey demonstrated that DM incidence increased daily worldwide, and it may reach 1 billion within 10 years [3, 4]. Several studies revealed that DR was closely associated with retinal microvascular system damage caused by high glucose environment [5]. It was found that retinal nerve cell damage appeared earlier than microvascular lesions in DR. It had occurred dysfunction in DM patients without retinal microvascular lesions. Electroretinogram (ERG) may show abnormity, weakened dark adaptation ability, and visual field damage [6, 7]. As the earliest differentiated nerve cells in the retina, retinal ganglion cells (RGCs) are the main cells to form vision. Thus, GRCs death is the main reason to cause visual function irreversible damage in DR occurrence and development [8]. DR has complicated pathogenic factors, while RGCs oxidation by free radical is one of the mechanisms. Therefore, how to effectively remove oxygen free radical and maintain oxidation/antioxidation balance is a hotspot [9, 10].

Catalpol is the main effective component of rehmanniae in scrophulariaceae plants. It belongs to the small molecular weight iridoid glycoside compound [11]. It was confirmed that catalpol had many biological activities, including anti-tumor, antifungal, antivirus, anti-
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Table 1. Primer sequences used in PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward 5'-3'</th>
<th>Reverse 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>AGTGCCAGCCTCCTCATAG</td>
<td>CGTTGAACTTGCGTGAG</td>
</tr>
<tr>
<td>NF-κB</td>
<td>CTCATCTAGCGGAACATTGG</td>
<td>GCACATTCTCTCCAGCG</td>
</tr>
</tbody>
</table>

Materials and methods

Reagents and instruments

RGC-5 cells were purchased from ATCC. Catalpol was from Chinese pharmaceutical and biological products identification. DMEM medium, EDTA, penicillin-streptomycin were from Hyclone. B27, CNTF, BDNF, and glutamine were from Sigma. DMSO and MTT were from Gibco. Enzyme-EDTA and epidermal growth factor were from Sigma. PVDF membrane was from Pall Life Science. Western blot related reagents were from Beyotime. ECL reagents were from Amersham Biosciences. Rabbit anti-human NF-κB primary antibody and HRP tagged IgG secondary antibody was from Cell Signaling. RNA extraction kit and reverse transcription kit were from Axygen. SOD activity detection kit was from Nanjing Jiansheng Biotechnology institute. Other reagents were from Sangon. Labsystem Version 1.3.1 microplate reader was from Bio-Rad.

RGC-5 cells culture and grouping

RGC-5 cells were seeded in the dish at 1×10⁵/cm² and maintained in serum free DMEM medium (100 U/ml penicillin and 100 µg/ml streptomycin) together with 50 µg/L CNTF, 1:50 B27, 40 µg/L CNTF, and 5.6 mmol/L glucose. The medium was changed every other day, and the cells were passaged every 2-3 days. RGC-5 cells were randomly divided into three groups, including normal control that maintained in normal condition, high glucose group that cultured in 55 mmol/L glucose, and catalpol group treated by 2 mm catalpol for 48 h under high glucose circumstance [15].

MTT assay

The cells were seeded in 96-well plate at 3000/well with five repetition in each group. After the cell adhering to the wall, 20 µl MTT at 5 g/L was added to each well and incubated for 4 h. 150 µl DMSO was added after removing the supernatant and vibrated for 10 min. The plate was read at 570 nm on microplate reader for cell proliferation calculation.

LDH and SOD activity detection

SOD activity was tested according to the manual. The protein was extracted and water bathed at 95°C for 40 min. After cooling, the protein was centrifuged at 4000 rpm for 10 min. Tissue ethanol was extracted by ethanol-chloroform mixture (volume ratio 5:3) to detect LDH and total SOD activity.

ROS content detection

After water bathed at 95°C for 40 min and cooling, the cells were centrifuged at 4000 rpm for 10 min. Under 37°C, the homogenate was incubated in 2', 7'-dichlorofluorescein diacetate (DCF-DA) for 15 min. After centrifuged at 10000 rpm for 15 min, the sediment was resuspended in PBS and incubated at 37°C for 60 min. ROS level was measured by spectrophotometer.

ELISA

ELISA was applied to test inflammatory factors TNF-α and IL-1β level in cell supernatant according to the manual. 50 µl standard substances were diluted and added to five repeat holes at each concentration to draw the standard curve. 50 µl sample was added to each well with three replicates. The plate was vibrated at room temperature for 30 s and washed for 5 times. After incubated at 37°C for 30 min, the plate was washed for 5 times. 50 µl color developing agent A and 50 µl color developing agent B were added and incubated at 37°C for 10 min. After added by 50 µl stop buffer, the plate was read at 405 nm on microplate reader for OD value within 15 min. OD value and standard substance concentration were used to draw the standard curve for TNF-α and IL-1β content calculation.
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Real-time PCR

Total RNA was extracted from LECs by Trizol and reverse transcribed to cDNA using the related primers (Table 1). Real-time PCR was applied to detect the target genes. The reaction consists one cycle of 52°C for 1 min, followed by 35 cycles of 90°C for 30 s, 58°C for 50 s, and 72°C for 35 s. GAPDH was used as reference, and the results were calculated 2^(-ΔΔCt) method.

Western blot

RGC-5 cells in each group were cracked on ice for 15-30 min and ultrasonicated for 4×5 s to extract protein. After centrifuged at 10000 g and 4°C for 15 min, the protein was moved to a new Ep tube and store at -20°C. The protein was separated by 10% SDS-PAGE electrophoresis and transferred to PVDF membrane. After blocked by 5% skim milk for 2 h, the membrane was incubated in primary antibody at 1:1000 and 4°C over night. Then the membrane was incubated with secondary antibody at 1:2000 for 30 min and washed by PBST. At last, the membrane was treated with chemiluminescent agent for 1 min and imaged on X-ray. Protein image processing system and Quantity one software were used for data analysis. All experiments were repeated for four times.

Statistical analysis

SPSS16.0 was adopted for data analysis. All data were presented as mean ± standard deviation (X ± S). One-way ANOVA was applied for comparison. P < 0.05 was considered as statistical significance.

Results

Catalpol impact on RGC-5 survival in high glucose circumstance

MTT assay was applied to test catalpol impact on RGC-5 cell survival under high glucose circumstance. It was showed that RGC-5 cell survival rate significantly decreased in high glucose environment compared with normal control (P < 0.05). Catalpol treatment obviously enhanced RGC-5 survival rate in high glucose compared with high glucose group (P < 0.05).

Table 2. Catalpol impact on oxidative stress index in RGC-5 under high glucose environment

<table>
<thead>
<tr>
<th>Index</th>
<th>Control</th>
<th>High glucose group</th>
<th>Catalpol group</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROS</td>
<td>62 ± 17.15</td>
<td>211 ± 27.31*</td>
<td>153 ± 21.22*#</td>
</tr>
<tr>
<td>SOD</td>
<td>121 ± 15.26</td>
<td>51 ± 13.2*</td>
<td>98 ± 16.17*#</td>
</tr>
</tbody>
</table>

*P < 0.05, compared with control; #P < 0.05, compared with high glucose group.
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(Figure 1). It indicated that catalpol can promote RGCs proliferation and survival in high glucose circumstance.

Catalpol impact on LDH activity in RGC-5

LDH activity detection revealed that neuron damage induced LDH activity obviously enhanced in high glucose environment (P < 0.05). Catalpol markedly reduced LDH activity induced by high glucose (P < 0.05) (Figure 2).

Catalpol impact on oxidative stress index in RGC-5 under high glucose environment

ROS and SOD content measurement showed that under high glucose circumstance, ROS level significantly elevated, while SOD content declined in RGC-5 cells (P < 0.05). ROS production was obviously restrained, while SOD content increased in RGC-5 after catalpol treatment compared with high glucose group (P < 0.05) (Table 2). It suggested that catalpol played a protective role on RGCs by regulating oxidative stress and cell survival.

Catalpol impact on inflammatory factor secretion in RGC-5 cells under high glucose circumstance

ELISA detection demonstrated that inflammatory factors TNF-α and IL-1β secreted by RGC-5 cells markedly elevated under high glucose environment (P < 0.05). Catalpol significantly suppressed TNF-α and IL-1β secretion compared with high glucose group (P < 0.05) (Figures 3 and 4). It indicated that catalpol can weaken inflammatory factor secretion by RGC-5 in high glucose environment, so as to alleviate inflammation.

Catalpol impact on NF-κB mRNA expression in RGC-5 cells under high glucose circumstance

Real time PCR results showed that NF-κB mRNA overexpressed in RGC-5 cells under high glucose circumstance (P < 0.05). Catalpol treatment markedly inhibited NF-κB mRNA expression compared with high glucose group (P < 0.05) (Figure 5).

Catalpol impact on NF-κB protein level in RGC-5 cells under high glucose circumstance

Western blot was further adopted to test NF-κB protein expression. Similar with NF-κB mRNA expression, NF-κB protein level significantly increased in RGC-5 cells under high glucose circumstance (P < 0.05). Catalpol obviously downregulated NF-κB protein level compared with high glucose group (P < 0.05) (Figures 6 and 7). It confirmed that catalpol can improve DR as it downregulated NF-κB mRNA and protein expression in RGC-5 under high glucose environment.
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Discussion

DR is a common DM microvascular complication. It seriously impairs DM patients healthy and brings heavy burden to family and society by causing progressive lesions [16]. Though medical technology continuous progresses, DR treatment effect still fails to achieve satisfactory results. High blood glucose environment in DM patients may affect optic nerve function lesions and injury at first. RGCs mainly focus on visual signal processing, transmission and processing. However, because of its special structural features, RGCs axon is easily damaged in the disease, leading to irreversible retinal damage, thus resulting in retina and other organs structure and function abnormalities [17, 18]. DR belongs to chronic inflammatory disease, and RGCs oxidation by free radicals is one of its pathogeneses. On the other hand, high blood glucose can make RGCs dysfunction through regulating inflammatory factors. Neuron damage may release a large amount of inflammatory factors, further broke the balance between pro-inflammation and anti-inflammation which aggravates neuron damage [19].

Our results confirmed that high glucose may cause RGCs damage, decrease cell survival, and enhance NF-κB expression. NF-κB expression may activate corresponding target genes, including immune receptors, adhesion molecules, inflammatory cytokines, and acute reactive protein, so as to regulate immune reaction and amplify inflammatory response [20].

High glucose can enhance ROS and LDH production, and decrease SOD activity. Increased ROS produced may lead to tissues and organs oxidative damage, while SOD is an important antioxidant enzyme to scavenge oxygen free radicals. It plays a vital role in oxidation/antioxidation balance, thus its vitality indirectly reflects organ-
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...ism ability of removing oxygen free radicals [21]. Catalpol is extracted from traditional Chinese medicine with many pharmacological activities. It has critical role in anti-inflammation and redox balance, and also provides neuron protective effect in the nervous system diseases, such as senile dementia and Parkinson’s disease [22]. However, the role of catalpol on DR and related mechanism has not been elucidated. Our results demonstrated that catalpol can decrease LDH and ROS content, enhance SOD activity, promote oxidation/antioxidation balance recover, decline TNF-α, IL-1β, and NF-κB expression, and increase RGCs survival.

In conclusion, our study verified catalpol can prevent DR through regulating oxidation/antioxidation balance, inhibiting inflammatory factors and NF-κB signaling pathway to promote GRCs survival. It provides reference and new target selection for DR prevention in clinic.

Acknowledgements

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Disclosure of conflict of interest

None.

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References

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