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Original Article

Molecular mechanism of high glucose-induced mitochondrial DNA damage in retinal ganglion cells

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Abstract

Mitochondrial DNA damage in retinal ganglion cells (RGCs) may be closely related to lesions of glaucoma. RGCs were cultured with different concentrations of glucose and grouped into 3 groups, namely normal control (NC) group, Low-Glu group, and High-Glu group. Cell viability was measured with cell counting kit-8, and cell apoptosis was measured using flow cytometry. The DNA damage was measured with comet assay, and the morphological changes of damaged mitochondria in RGCs were observed using TEM. Western blot analyzed the expression of MRE11, RAD50, and NBS1 protein. Cell viability of RGCs in Low-Glu and High-Glu groups were lower than that of NC group in 48 and 96 h. The cell apoptosis in NC group was 4.9%, the Low-Glu group was 12.2% and High-Glu group was 24.4%. The comet imaging showed that NC cells did not have tailings, but the low-Glu and high-Glu group cells had tailings, indicating that the DNA of RGCs had been damaged. TEM, mitochondrial membrane potential, ROS, mitochondrial oxygen consumption, and ATP content detection results showed that RGCs cultured with high glucose occurred mitochondrial morphology changes and dysfunction. MRE11, RAD50, and NBS1 protein expression associated with DNA damage repair pathway in High-Glu group declined compared with Low-Glu group. Mitochondrial DNA damage caused by high glucose will result in apoptosis of retinal ganglion cells in glaucoma.

Keywords: Glaucoma, Retinal ganglion cells, High glucose, Mitochondria, DNA damage.

1. Introduction

Glaucoma is one of the most common blindness diseases in the world. Its pathological manifestations are progressive retinal ganglion cells (RGCs) apoptosis and irreversible degeneration of the optic nerve. The onset of glaucoma is urgent, and the condition is serious. The patient will be easily blind if not treated in time [1,2]. At present, the pathogenesis of glaucoma is not fully understood [3,4]. It has been reported that many patients with glaucoma will also have diabetes. The increased blood viscosity due to the elevation of blood glucose in diabetic patients affects the ocular vascular microcirculation and aqueous humor circulation, leading to increased intraocular pressure to induce glaucoma [5].

Diabetes can increase the apoptosis rate of retinal neurons. More researches have indicated that retinal neurons begin to undergo apoptosis before microvascular lesions occur. RGCs are the earliest retinal cells to be affected, and the apoptosis rate is significantly increased [6,7]. Functional tests in people with early diabetes can also detect the loss of retinal nerve cells. RGCs are nerve cells in the final segment of the retina and their dendrites are the first to be affected during the development of diabetes [8]. With the development of the disease, RGCs begin to undergo apoptosis, similar to central nerve cells, with a small amount of apoptosis in the early stage. The surrounding cells can fill the space by expanding their cell body and dendrites to compensate for their functions. However, with the increase in the number of cell apoptosis, a large number of blank areas will appear and the function will be decompensated [9]. RGCs are the only cells in the retina that can transmit visual signals to the visual center through axons [10]. With the progression of diabetes, increased apoptosis cells can accelerate the loss of RGCs, which will lead to the decline of visual acuity or even permanent loss [11,12].

Progressive death of RGCs is the main pathological basis of glaucoma [13]. Studies have found that mitochondrial dynamics damage may be closely related to RGCs damage of glaucoma [14,15]. Mitochondrial dynamics refers to the continuous division, fusion, movement, transportation and autophagy of mitochondria in cells, which have important effects on the survival, metabolism and maintenance of physiological functions of cells [16]. Although we currently have a preliminary understanding of the process of regulating mitochondrial dynamical damage in RGCs, the molecular mechanism of mitochondrial DNA damage in RGCs is still limited. The present study used diabetic RGCs model induced by high glucose to investigate the related mechanism of cellular molecules.

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2. Materials and methods

2.1. Materials

Mouse retinal ganglion cells (RGCs) were obtain from Chinese Academy of Sciences; fetal calf serum (GIBCO, Rockville, MD, USA); penicillin and streptomycin (100×) (Solarbio, Beijing, China); 0.25% trypsin (Solarbio, Beijing, China); primary antibody MRE11, RAD50, NBS1 (Beyotime Biotechnology, Shanghai, China); CCK-8 (SAB); confocal microscope (Olyplus, Tokyo, Japan); enzyme label analyzer (Beijing Pulang New Technology Co., Ltd, Beijing, China); Western blot instruments (Eppendorf, Hamburg, Germany); flow cytometry (Thermo Fisher, Waltham, MA, USA).

2.2. Methods

2.2.1. Cell viability

RGCs were cultured with DMEM containing 10% FBS and 1% penicillin and streptomycin (100×) in 5% CO_2 incubator at 37°C to prepare 1-5 $\times 10^4$ cell/mL cell suspension. Cell suspension (100 μL) was cultured in 96-well plates and grouped into 3 groups, namely normal control group (NC), low glucose group (Low-Glu) and high glucose group (High-Glu). Cell counting kit-8 (CCK-8) and serum-free essential basic medium were mixed according to 1:10 volume ratio, and 100 μL of mixture was added into the RGCs after culturing 0, 24, 48 and 96 h. The RGCs were continuously incubated in an incubator for 1 h at 37°C and determined by enzyme plate analyzer. The OD value of each test well plate is subtracted from the OD value of the zeroing well plate or the control well plate. The OD value of each repeating well plate is averaged.

2.2.2. Cell apoptosis

The RGCs cells were divided into 3 groups, namely NC group, Low-Glu group and High-Glu group. The cell culture medium of NC group was supplemented with normal saline. The cell culture medium of Low-Glu group was supplemented with 15 mmol/L glucose solution. The cell culture medium of High-Glu group was supplemented with 35 mmol/L glucose solution. Each group of cells was cultured for 96 h. All cells precipitate were collected and Annexin V-FITC solution and PI were added for culturing 10-20 min. Cell apoptosis was measured using flow cytometry. Annexin V-FITC is green fluorescence and its corresponding detection channel is flow cytometer FL1. Propidium iodide (PI) is red fluorescence and its corresponding detection channel is flow cytometer FL2.

2.2.3. Comet assay

RGCs (1×10^5 cells/mL) were put in EP tube at 37°C water bath. The dissolved 1% normal melting point agarose (NMPA) was spread on the slide and condensed at 4°C for 10 min. The low melting point agarose (LMPA) was heated to dissolve the agarose completely and 1.0% concentration was prepared. 100 μL cell suspension was dissolved in 100 μL 1% LMPA, then dropped on the first layer of glue, and placed at 4°C to condense for 15 min to solidify the agarose. After the agarose cell gel solidified, the slides were slowly placed into the 4°C dyeing tank to crack for 20 h [17]. Electrophoresis was performed at 4°C for 20 min with 25 V voltage and 300 mA. The slides were removed, and each slide was stained with 10 μg/mL PI for 20 min in a dark condition, and then decolorized with distilled water for 10 min. Green light (wavelength: 515-560 nm) was excited and photographed under a fluorescence microscope. The main detection indexes are tail DNA (%) and olive tail moment (OTM). Tail DNA% represents the percentage of tail DNA in total DNA content. OTM is the ratio of the difference between the tail optical density center and the head optical density center to tail DNA content.

2.2.4. Morphological changes of mitochondria

RGCs (1×10^5 cell/mL) were cultured in 96-well plate and divided into 3 groups. The cell culture medium of NC group was supplemented with normal saline. The cell culture medium of Low-Glu group was supplemented with 15 mmol/L glucose solution. The cell culture medium of High-Glu group was supplemented with 35 mmol/L glucose solution. Each group of cells was cultured for 96 h. The ultrastructural and morphological changes of mitochondria in RGCs were observed by transmission electron microscopy. The prepared cell solution was diluted with ultra-pure water and dropped on the copper grid, then stained with phosphotungstic acid (5%, w/v) and air-dried at room temperature. The morphology of mitochondria in RGCs was characterized on H7600 Transmission Electron Microscopy (TEM).

2.2.5. Detection of mitochondrial membrane potential

The JC-1 fluorescence probe method was used to detect the mitochondrial membrane potential in each group of cells, and the measurement process was strictly carried out according to the instructions of the reagent kit. A final concentration of 10 μmol/L JC-1 was added to each group of cells for incubation at room temperature in dark for 30 minutes and measured by flow cytometry.

2.2.6. Mitochondrial ROS detection

After the incubation period, all cells were harvested and washed with PBS. The cells were further incubated with 10 μM DCFH-DA at 37°C for 20 min in dark. Cells were washed in PBS and analyzed by flow cytometry.

2.2.7. Mitochondrial oxygen consumption

A solution includes 125 mmol/L sucrose, 65 mmol/L KCl, 10 mol/L Hepes, 2 mmol/L inorganic phosphate, 2 mmol/L Mg Cl2, 100 μMol/L EGTA and 0.01% bovine serum albumin. The solution was continuously stirred using a Clark-type electrode with a computer interface at 37°C to calculate the tightness of the coupling between mitochondrial respiration and phosphorylation.

2.2.8. Mitochondrial ATP generation

For the measurement of ATP level, cells were extracted by lysis buffer. After centrifugation at 12,000 g for 5 min at 4°C, the supernatants were transferred to a new 1.5 ml tube for ATP analysis. The luminescence from a 100 μL sample was assayed in a luminometer together with 100 μL of ATP detection buffer. A standard curve of ATP concentrations (10 nM- 1 μM) was prepared from a known amount. Then, the ATP was detected according to the standard curve.

2.2.9. Western blot analysis of MRE11, RAD50, NBS1 expression

RGCs were collected and added RIPA lysate (containing protease inhibitor) to fully crack on ice

for 3-5 min. The lysate was transferred into a 1.5 mL centrifuge tube and centrifuged with 10000 r/min for 5 min at 4°C. Supernatant was absorbed and put into 1.5 mL centrifuge tubes to detect the concentration of protein. Prepare 8% SDS-PAGE electrophoresis and the concentrated glue run for 60 min under 80 V. Separation glue run for 120 min under 120 V, then transferred membrane 150 min under 120 V, and sealed for 60 min. Primary antibodies rat anti-human primary antibody MRE11, RAD50, NBS1 (1:500) were incubated with the RGCs at 4°C overnight. Second antibody HRP labeled sheep anti-rat secondary antibody (1:5000) was incubated at room temperature for 1 h. Image J software analyzed the gray values of the strips.

2.3. Statistical analysis

All data were analyzed using GraphPad prim 8.0 (IBM, Armonk, NY, USA). The results were shown mean ± standard deviation (SD) after One-way analysis of variance (ANOVA). $P< 0.05$ was considered a significant difference.

3. Results

3.1. Cell viability

The activities of cells were shown by measuring the OD value of each group. The number of OD values of living cells is determined at 450 nm. The larger the OD value is, the stronger cell activity is. From 0 to 24 h, it showed no statistically significant difference in cell viability of RGCs in NC, Low-Glu, and High-Glu groups. However, in 48 h and 96 h, it showed a significant difference among the three groups. Cell viability of RGCs in Low-Glu and High-Glu groups was lower than that of NC group. Cell viability of RGCs in High-Glu groups was lower than that of Low-Glu group (Fig. 1).

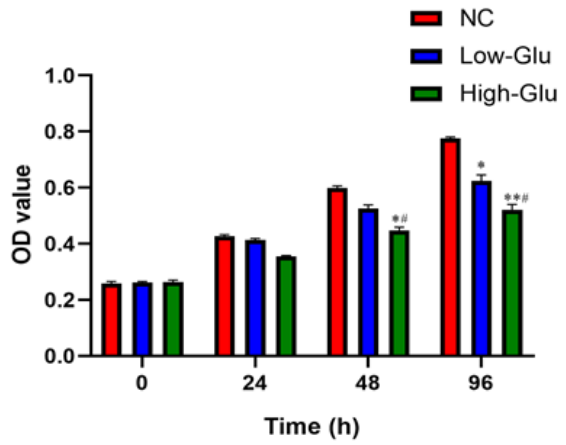

Fig. 1. Cell viability of RGCs in different groups after culturing at different times. $^{*}P<0.05$, $^{**}P<0.01$, compared with NC group; $^{\#}P<0.05$ compared with Low-Glu group.

3.2. Cell apoptosis

There were 4 areas, and area in top right corner (UR) presented the late apoptotic cells, and area in lower right corner (LR) presented the early apoptotic cells. The sum of the percentages of apoptotic cells (UR+LR) indicated the total cell apoptotic ratio. The sum percentage of the late and the early apoptotic cells in NC group was 4.9%, the Low-Glu was 12.2% and High-Glu was 24.4% (Figure 2A-B).

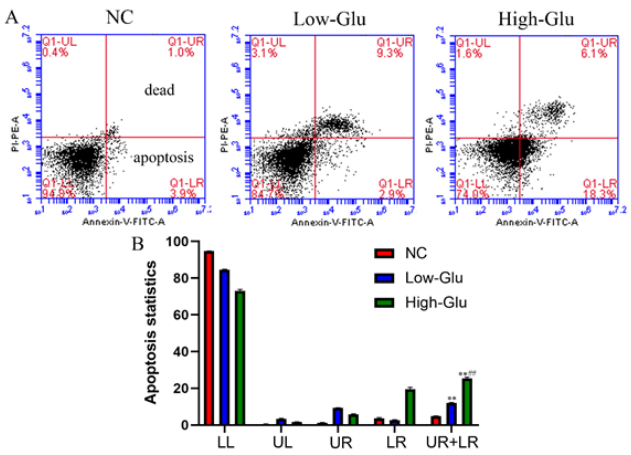

Fig. 2. Cell apoptosis properties of RGCs. (A) Cell apoptosis of different groups in RGCs. (B) Statistical analysis of apoptosis. $^{**}P<0.01$, compared with NC group; $^{\#\#}P<0.01$ compared with Low-Glu group.

3.3. Comet assay detects DNA damage

Comet assay was used to evaluate mitochondrial DNA damage. Typical comet imaging of each group is shown in Figure 3. No tail was found in RGCs of NC group, but tail was found in Low-Glu and High-Glu groups. It indicated that RGCs had different degrees of DNA damage, and the High-Glu group was the most serious. The comet experimental imaging was analyzed by CASP software. Tail DNA% and OTM were used as the index data of DNA

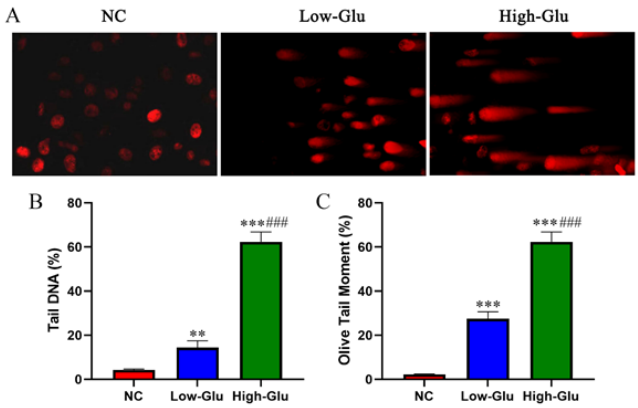

Fig. 3. Comet assay of RGCs. (A) Comet imaging in different groups; (B) Tail DNA (%) in different groups. $^{**}P<0.01$, $^{***}P<0.001$, compared with NC group; $^{\#\#\#}P<0.001$, compared with Low-Glu group. (C) Olive tail moment (%), $^{***}P<0.001$, compared with NC group; $^{\#\#\#}P<0.001$, compared with Low-Glu group.

Table 1. Tail DNA and olive tail moment in comet assay of each group. (Mean±SD).

Group	Tail DNA (%)	OTM (%)
NC	4.27 ±0.36	2.31 ±0.13
Low-Glu	14.36 ±1.85**	27.49 ±3.14**
High-Glu	30.24 ±2.74$^{***\#\#}$	62.34 ±4.47$^{***\#\#}$

$^{**}P<0.01$, $^{***}P<0.001$, compared with NC group; $^{\#\#}P<0.01$, compared with Low-Glu group.

damage, as shown in Table 1.

3.4. Morphological changes of mitochondria

Mitochondria in normal control group had uniform size, complete structure, orderly arrangement, and good integrity of intercellular connections. However, after high glucose treatment, the mitochondria in RGCs were different in size and disordered in arrangement, and the cell structure had degenerative changes. The number of mitochondria was relatively reduced, and most of them had become vacuolated. Mitochondrial density in High-Glu group also declined and the integrity of intercellular connections in High-Glu group was worse than that of NC and Low-Glu group, as shown in Figure 4A. The decrease in mitochondrial membrane potential is one of the characteristic events of cells in the early stage of apoptosis. Compared with the control group, the mitochondrial membrane potential of cells significantly decreased after high glucose treatment in this study, as shown in Figure 4B. These results indicated that high glucose caused mitochondrial dysfunction and induced cell apoptosis.

3.5. Mitochondrial function assessment

The production of ROS by mitochondria in High-GLU group cells was significantly higher than that in Low-GLU group, as shown in Figure 5A. ROS, as a free radical, disrupts mitochondrial function. Further experimental detection showed a decrease in mitochondrial oxygen consumption in High-GLU group, as shown in Figure 5B. Mitochondria utilize oxygen to participate in ATP production, and a decrease in oxygen consumption will result in a decrease in ATP production. Therefore, ATP production in the High-GLU group was significantly increased, as shown in Figure 5C.

3.6. Expression of MRE11, RAD50, NBS1 protein

MRN is a complex discovered during the study of DNA double-stranded damage repair pathway. It consists of three proteins, namely MRE11, RAD50 and NBS1. MRN complex plays an important role in the process of double-stranded DNA damage repair, participating in each link of DNA damage repair response such as initiation of repair, initiation of signal transduction and assistance in repair process. Western blot detected the protein expression of MRE11, RAD50 and NBS1 in each group, as shown in Figure 6. The protein content of MRE11, RAD50 and NBS1 decreased after RGCs incubating with glucose for 96 h, especially the High-Glu group. Compared with NC group, there was a significant difference in Low-Glu group and High-Glu group, $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$. The protein content of MRE11, RAD50, and NBS1 in High-Glu group was lower than that of Low-Glu group, $^{\#}P<0.05$.

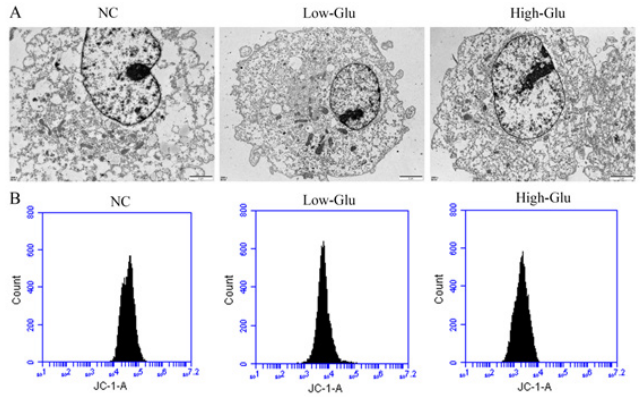

Fig. 4. (A) The morphological changes of mitochondria in RGCs were observed by transmission electron microscopy. Arrows indicated mitochondria. (B) Mitochondrial membrane potential was detected by flow cytometry.

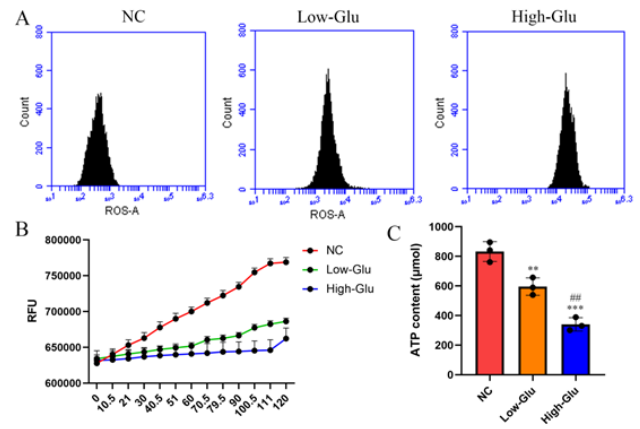

Fig. 5. (A) ROS detection in mitochondrion by flow cytometry; (B) ATP content (μmol); (C) Mitochondrial oxygen consumption. $^{**}P<0.01$, $^{***}P<0.001$, compared with NC group; $^{\#\#}P<0.01$, compared with Low-Glu group.

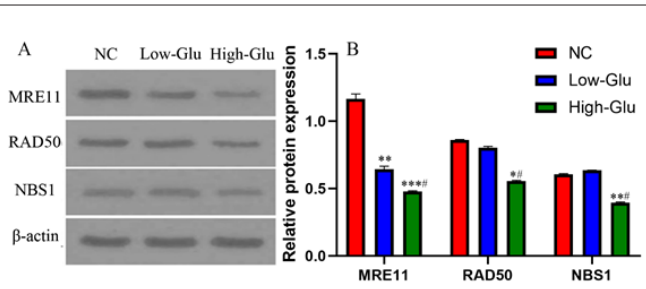

Fig. 6. Protein level of MRE11, RAD50, NBS1 in different groups determined by western blotting (Mean±SD, n=3). (A) Gray value; (B) Statistical analysis. $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$ compared with NC group; $^{\#}P<0.05$, compared with Low-Glu group.

4. Discussion

Diabetes can increase the apoptosis rate of retinal neurons. A large number of studies have confirmed that retinal neurons begin to undergo apoptosis before microvascular lesions occur. RGCs are the earliest retinal cells to be affected, and the apoptosis rate is significantly increased [10,11]. We found that the total cell apoptotic ratio in High-Glu was 24.4%, much higher than that of the NC group (4.9%). With the enhancement of the glucose concentration, RGCs begin to undergo apoptosis. RGCs are nerve cells in the final segment of the retina and their dendrites are the first to be affected during the development of diabetes [12]. However, with the increase in the number of cell apoptosis, a large number of blank areas will appear and the function will be decompensated [13]. RGCs are the only cells in the retina that can transmit visual signals to the visual center through axons [14]. With the progression of diabetes, increased apoptosis cells can accelerate the loss of RGCs, which will lead to the decline of visual acuity or even permanent loss [15-17].

Mitochondria, caspase protease family, signal transduction system, apoptosis-related genes, oxygen free radicals et al all participate in the occurrence of RGCs

apoptosis. Mitochondria are also the source and object of oxidative stress and energy, so they play a decisive role in cell apoptosis. Mitochondria are the integrated elements of apoptosis pathway and the executors of cell death [18]. Moreover, mitochondrial dysfunction was evaluated by detecting mitochondrial ATP production, mitochondrial oxygen consumption, and mitochondrial ROS. The main physiological function of mitochondria is to generate ATP through respiratory chain reactions, providing energy to cells. When high sugar induces cells to produce a large amount of ROS, it can damage the mitochondrial membrane, disrupt mitochondrial structure, and further reduce APT synthesis.

In this study, it was found that the structure of mitochondria in RGCs cultured with high glucose was incomplete, and the mitochondria were often vacuolated. In addition, the intercellular connection morphology was incomplete, among which the most serious was the cells in high-Glu group. These results indicated that high glucose induces morphological changes and abnormal function of mitochondria. Mitochondria are the main sites of intracellular oxidative phosphorylation and ATP formation, which provide the main energy for cells. Therefore, mitochondrial dysfunction is the key factor to induce cell apoptosis [19].

The special structure of mitochondrial DNA makes it vulnerable to attack. Double-stranded DNA damage is the main cause of genomic instability and cell death. MRE11/RAD50/NBS1 is an important complex for the detection and repair of double-stranded DNA [20]. Replication, mitosis, and inheritance in cell metabolism can cause DNA damage after toxic chemicals and radiation therapy [21,22]. MRN complex plays an important role in the process of DNA damage repairment, participating in each link of DNA damage repair response such as initiation of repair, initiation of signal transduction and assistance in repairment [23]. In recent years, it has been found that inhibiting the activity of MRN complex and hindering the repair of DNA damage in tumor cells can achieve the therapeutic effect of cancer [24]. In this research, western blot detected the lowest expression of MRE11/RAD50/NBS1 in the High-Glu group. It is further proved that high glucose can damage the mitochondrial DNA structure of retinal ganglion cells in glaucoma, affect mitochondrial function and induce cell apoptosis. DNA is easy to be damaged by free radical because it is not protected by related proteins and is not easy to be repaired after damage. DNA oxidative damage and its mutations will hurt oxidative phosphorylation genes, and make enzymes related to the respiratory chain abnormal. This process results in mitochondrial oxidative phosphorylation dysfunction and promotes the production of reactive oxygen species, which further unbalances the respiratory chain and eventually leads to cell death [25].

The optic nerve damage in diabetic glaucoma is the result of multiple factors such as hyperglycemia and superoxidation damage. There is no evidence for retinal ganglion cells to regenerate. Retinal neuroprotective therapy is directly aimed at saving retinal ganglion cells. Therefore, controlling blood glucose in diabetic patients and improving mitochondrial activity will become a new target for the treatment of glaucoma, which will also be a direction of future study by glaucoma researchers.

5. Conclusion

High glucose induces mitochondrial DNA damage in retinal ganglion cells and the morphological changes in mitochondria. The expression of important proteins MRE11/RAD50/NBS1 was decreased in DNA damage recovery. Therefore, DNA is easily damaged by free radicals and is difficult to repair after damage, leading to retinal ganglion cell apoptosis.

Ethics approval and consent to participate

The ethical approval was obtained from the Ethics Committee of The First People's Hospital of Guiyang.

Consent to publish

All of the authors have Consented to publish this research.

Availability of data and materials

The data are free access to and available upon request.

Competing interests

All authors declare no conflict of interest.

Authors' contributions

Each author has made an important scientific contribution to the study and has assisted with the drafting or revising of the manuscript.

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