Neuroprotective Effects of Idebenone on hydrogen peroxide (H2O2)-induced Oxidative Damage in Retinal ganglion cell-5 (RGC-5) Cells

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Research article

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Abstract

Purposes To investigated the neuroprotective effect of Idebenone against H2O2-induced oxidative damage in RGC-5 cells. Methods RGC-5 cells were treated with different concentrations (5, 10, 20μM) of idebenone for 12h prior to addition of 300μM H2O2 for 12 h. The apoptosis of RGC-5 cells were detected by flow cytometry. The changes of mitochondrial membrane potential were detected by JC-1 staining. The autophagy in RGC-5 cells was observed by transmission electron microscopy, and the expression level of autophagy-related protein light chain3, Beclin-1 and mitochondrial membrane potential-related protein Cyt-c in RGC-5 cells were measured by Western blot analysis. Results Flow cytometry showed that the apoptosis rates in control group, H2O2 group and H2O2-treatment with Idebenone pretreatment groups were (6.48±0.55)%, (27.34±0.51)%, (22.88±0.52)%, (15.45±0.81)%, (12.59±0.58)%, respectively($F = 559.7$, $P < 0.0001$). After incubation with H2O2, the number of autophagosomes increased significantly, while which was decreased in H2O2-treatment with Idebenone pretreatment groups. After incubation of RGC-5 cells with H2O2, the mitochondrial membrane potential was significantly decreased, while idebenone could prevent the decrease of MMP. Contrast with control group, LC3 II /I, the expression levels of Beclin-1 and Cyt-c in H2O2 group increased significantly($P<0.05$); while contrast with H2O2 group, LC3 II/I, the expression of Beclin-1 and Cyt-c in H2O2-treatment with Idebenone pretreatment groups was significantly decreased($P<0.05$). Conclusion Idebenone may have protective effects on RGC-5 cells suffering from oxidative damage induced by H2O2 through improving antioxidant capacity, reducing mitochondrial membrane potential decline and the activity of autophagy.

Introduction:

Glaucoma is a neurodegenerative disease which characterized by the progressive death of Retinal ganglion cells(RGCs). Glaucoma can lead to blindness second to Cataract[1]. The injury of optic nerve is the main damage of Glaucoma. However, the specific pathogenesis of glaucomatous optic neuropathy (GON) has not been fully elucidated.

Recently, researchers discover that oxidative stress is also a key injury factor in GON[2]. Under normal circumstances, reactive oxygen species(ROS) play roles as signaling molecules in vivo. But oxidative stress occurs once the increased ROS content beyond clearance ranges of the body. The imbalance between oxidation and antioxidant capacity is considered to be an important feature of early retinal damage and glaucoma pathology[3].According to research, H_2O_2 can induce apoptosis in RGC-5 cells in vitro. Cyt-c is not only a substance transfer by breath Chain electron transfer, but also the principal protein of regulating apoptosis[4]. Mitochondria is the main source of ROS and also the attack target of ROS[4], which is an important factor in the injury of glaucoma optic nerve[6-9]. In addition, increased ROS can lead to the decline of MMP, mitochondrial dysfunction occurs, then Cyt-c released from mitochondria lead to apoptosis[6-9]. Mitochondrial damage will in turn leads to more reactive oxygen species and aggravates oxidative stress. Therefore, mitochondria are important factors in determining cell survival and apoptosis, and play an essential role in glaucomatous optic nerve injury[8, 9].

In addition to the mitochondrial pathway of the apoptotoic pathway, autophagy is also has an impact on the survival of RGCs[11]. Autophagy is a rather conserved lysosomal pathway which is highly regulated by complex factors, such as hypoxia, hunger,some specific medicine. A recent study found that ROS can also mediate autophagy[9]. Autophagy plays a major role in the renewal of cell components and the maintenance of intracellular rings and the stability of the environment[12]. Under normal circumstances, autophagy plays a key role in maintaining cellular homeostasis. However, some recent experimental studies point out that the autophagy is associated with death. Autophagy may be another form of cell death[13].

Idebenone is an analog of coenzyme Q10, which may be a promising therapeutic strategy for ameliorating glutamate excitotoxicity and oxidative stress in glaucomatous neurodegeneration[14]. It has been proved that coenzyme Q10 can treat mitochondrial disease[15]. We aim to explore whether Idbenone has the antioxidant damage and protective effects on RGCs, and provide some references for therapeutic intervention in glaucomatous optic nerve injury.

Materials And Methods

1. Cell line culture

The RGC-5 cells (bought from Fudan IBS) is a mouse retinal ganglion progenitor cell line. In our study, RGC-5 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin and 100g/mL streptomycin. And cells were cultured at 37℃ in an incubator with 5% CO_2. The medium was altered every other day. When the cells grow about 80% confluent, they will be passaged at a ratio of 1:4 using 0.25% Trypsin. Usually, the period of passage is two or three days.

2. H_2O_2-treatment RGC-5 cells and Idebenone pretreatment

RGC-5 cells were placed in a 6 well flat-bottomed plates at a concentration of 2×10^4 cells/well and randomly divided into five groups: control group(without H_2O_2 or Idebenone treatment), H_2O_2 group (300μM H_2O_2 without Idebenone pretreatment), H_2O_2-treatment with Idebenone pretreatment groups (5, 10, 20μM Idebenone). After cultured for 24 h, injury was induced by exposing these RGC-5 cells to 300μM H_2O_2 for 12 h after pretreatment with Idebenone(5, 10 and 20μm) for 12h.

3. Cell Apoptosis analysis

RGC-5 cells were collected via EDTA-free trypsin, washed with cold phosphate buffered saline (PBS) twice, and resuspended in Annexin V-binding buffer. The cells were fluorescently labeled by addition of 5μL of Annexin V-FITC, 5μL of propidium iodide (PI), and 100 μL of binding buffer (BD Biosciences). After incubation in the dark for 15 min at room temperature, the cells were washed once with binding buffer

and re suspended in 400 μL of binding buffer prior to flow cytometric analysis using a BD FAC Scan flow cytometer (BD Biosciences).

4.Detection of mitochondrial membrane potential

MMP was detected by JC-1 staining and fluorescence microscope. The cells were treated according to the procedure of the JC-1 Kit (Kai Ji Biotechnology, Nanjing, China).

5.Transmission electron microscopy(TEM)

The intracellular autophagosomes and autolysosomes were observed by TEM. The cells were washed three times in PBS and fixed with 2.5% PBS buffered glutaraldehyde for 1 hour at 4 °C, then 1% OSO_4 was dehydrated again in the gradient ethanol series for 1 hour and placed flat in Araldite. Ultrathin sections (1μM, thickness) were cut with uranyl acetate and lead citrate and double stained. At last, ultrathin sections were observed using the TEM(JEOL-1230, Tokyo, Japan).

6.Western Blotting Assay

Firstly, cells at a density of 2×10^5cells/mL were plated onto 100-mm plates and incubated at 37℃ for 24 hours. After experimental treatment, RGC-5 cells were rinsed and lysated in mammalian cell lysis reagent (Sigma Aldrich) containing protease inhibitor cocktail (Sigma-Aldrich). Samples (20μg) were loaded onto a 12% SDS-PAGE gel and electrophoresed at 150 V for about 1 hour. The proteins were then transferred to the polyvinylidene difluoride membrane (Millipore, California, USA). And after being blocked with 10% nonfat milk for 1 hour, Cyt-c antibody (Kai Ji Biotechnology, Nanjing, China), autophagy-related protein light chain3 (LC3) A/B (1:1000; Cell Signaling Technology, # 12741, USA) and anti-rabbit Beclin-1 monoclonal antibody monoclonal antibody (1:1000; Cell Signaling Technology, #3495 , USA) were applied at 4°C overnight. Then incubation with HRP-labeled Goat anti-rabbit IgG or anti-mouse secondary antibody (1:5000; Cell Signaling, USA) for 1.5h. At last, bands were visualized by incubation with SuperSignal® West Pico Chemiluminescent substrates. Blots were analyzed by the ImageJ software. The protein levels were normalized by GAPDH.

7.Statistical Analysis

Statistical analysis Data analysis was performed using SPSS 20.0. All experimental data were presented as mean±SD and measured at least three separate experiments. Differences between groups were evaluated by t test and one-way ANOVA. Values of $P < 0.05$ were considered as statistically significant.

Results

1.Idebenone Protected RGC-5 Cells against Apoptosis Induced by H_2O_2

The result of Annexin V-FITC / PI flow cytometry showed that the apoptotic rates of control group, H_2O_2 group and H2O2-treatment with Idebenone pretreatment groups (5, 10 and 20μm) with H_2O_2 were

□6.48±0.55□%, □27.34±0.51□%, □22.88±0.52□%, □15.45±0.81□% and □12.59±0.58□%. And the results were analyzed by one-way ANOVA and t test, F=559.7, P<0.0001, and the difference was statistically significant(P < 0.01).

Fig.1. The results of apoptosis detected by Flow Cytometry. Each chart consists of four areas: Q1 region represents those necrotic cells; Q2 region represents the late stage apoptosis; Q3 region represents the early stage apoptosis; Q4 region represents viable cells. Q2 region adds Q3 region represents the total apoptosis of cells. The numbers in each region represent the proportion of cells in the region of total cells.

Fig.2. Statistical analysis of apoptotic rate measured by flow cytometry.(**P<0.01VS control group,## P<0.01VS H_2O_2 group, n=4).

2. Idebenone prevent the decrease of MMP induced by H_2O_2

The changes of MMP (ΔψM) were measured by JC-1 kit. Orange represents the MMP is normal, the green color represents a decrease in MMP. Control group shows normal Orange, while the H_2O_2 group becomes green mostly. H2O2-treatment with Idebenone pretreatment groups are between control group and H_2O_2 group, and as the concentration of Idebenone increases, green decreases and orange grows. The results show that there was a loss of ΔψM after incubation with 300μM H_2O_2 for 12 hours, while idebenone can prevent the decline of MMP.

Fig.3. The changes of MMP measured by JC-1 kit□×100□. A. the control group; B.H_2O_2 group; C. Idebenone5μm+ H_2O_2; D. Idebenone10μm + H_2O_2; E. Idebenone20μm + H_2O_2.

Idebenone attenuates autophagy induced by H_2O_2

Autophagy in each group of cells were observed by transmission electron microscopy (Fig.4). A small amount of autophagosomes and autophagosomes were observed in the normal control group. Compared with the normal control group, the autophagosomes and autophagosomes in the H_2O_2 group were significantly increased. However, compared with the H_2O_2 group, intracellular autophagosomes and autophagosomes in the Idebenone intervention group (Idebenone 5μM + H_2O_2 group, Idebenone 10μM + H_2O_2 group, Idebenone 20μM + H_2O_2 group) were reduced. And the decreasing trend is more obvious as the concentration of idebenone increases.

Fig.4. Transmission electron microscopy image of RGC-5s. Autophagosomes (red arrows) and autolysosomes (yellow arrows) are noted in RGC-5s.

4.The results of Western blotting

Activation of Cyt-C and the levels of autophagy-related proteins such as LC3 and Beclin-1 were quantified by Western blotting. Compared with the contract group, the relative LC II/I expression, the relative Beclin-1 expression and the activation of Cyt-c are all statistically significant(P<0.01). Compared with the H_2O_2

group, the relative LC II/I expression, the relative Beclin-1 expression and the activation of Cyt-c of groups with various concentrations of Idebenone are all statistically significant($P<0.05$). In short, experimental results imply that H_2O_2 leads to significant increase in the activity of autophagy and leads to a significant decrease in MMP, however Idebenone could reduce the activity of autophagy and decrease in MMP.

Fig.5. Relative expression of LC3 II/I, Beclin-1 and Cyt-c. A.The expression of LC3, Beclin-1, Cyt-c were detected by Western blotting; B.the relative expression of LC3II/I; C.the relative expression of Beclin-1; D.the relative expression of Cyt-c. 1.Control group; 2.H_2O_2 group; 3.Idebenone5μm+H_2O_2 group; 4.Idebenone10μm+H_2O_2 group;5.Idebenone20μm+H_2O_2 group. (**$P < 0.01$, vs. control group; #$P < 0.05$, ##$P<0.01$vs. H_2O_2 group).

Discussions

Oxidative stress plays a major role in the pathogenesis of glaucoma. Recent studies have shown that mitochondria and autophagy are all involved in the progressive death of retinal ganglion cells in the course of glaucoma. In this study, we use exogenous H_2O_2 to break the balance of oxidation and antioxidation of the cell, to explore the effect of idebenone on hydrogen peroxide-induced autophagy and to explore whether idebenone has antioxidant and protective effects on RGC-5 cells. LC3 a marker protein for autophagy. When autophagy is activated, LC3-I is reacted with phosphorus under the action of ubiquitin-like enzyme Lipoethanolamine coupling to produce LC3-II. LC3-I is located in the cytoplasm, while LC3-II is located in the inner and outer membranes of the autophagosome. And the lipidated form of LC3 (LC3-II) can be considered as the autophagosome marker protein[16, 17]. In addition, autophagy related protein Beclin-1 also plays a critical role in the progress of autophagy[18]. For instance, the discovery of Beclin-1's interacting partners has resulted in the identification of Bcl-2 as a central regulator of autophagy and apoptosis[19]. The expression of LC3-II/I and Beclin-1 can reflect the activity of autophagy.

Idebenone has antioxidant ability to prevent lipid peroxidation and ROS production in multiple systems[19], such as brain, brown fat.etc. It's waiting to study whether idebenone has the same protective effects in oxidative damage on retinal ganglion cells. Our experimental results suggest that Idebenone can reduce the apoptosis of RGC-5 cells caused by hydrogen peroxide; H_2O_2can reduce the MMP and lead to apoptosis and autophagy of RGC-5 while idbenone can prevent the decline of MMP and reduce the activity of autophagy. Our study indicate that idbenone has antioxidant and protective effects on RGC-5 cells. But we must emphasize even under normal circumstances, apoptosis and autophagy exist all the time. According to the experimental research on autophagy in recent years, the amount of autophagy has different effects on the survival number of cells. However, the concrete mechanism of autophagy that induced by H_2O_2 in RGCs has not been elucidated. Researches has shown that autophagy could regulate biological processes by mediating the related signaling protein degradation[25, 26]. Apoptosis and autophagy are not completely independent, they interaction each other in many identical molecular adjustment mechanisms [19, 27]. According to the results of our study, maybe, we can

prevent or mitigate oxidative damage from two aspects, antioxidant such as maintenance of retinal ganglion cell mitochondrial functions and control the level of autophagy. It may can provide a promising new idea for the optic nerve protection of glaucoma.

Declarations

Acknowledgement

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Disclosure Statement

The authors have no financial or competing interests concerning the present study.

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Figures

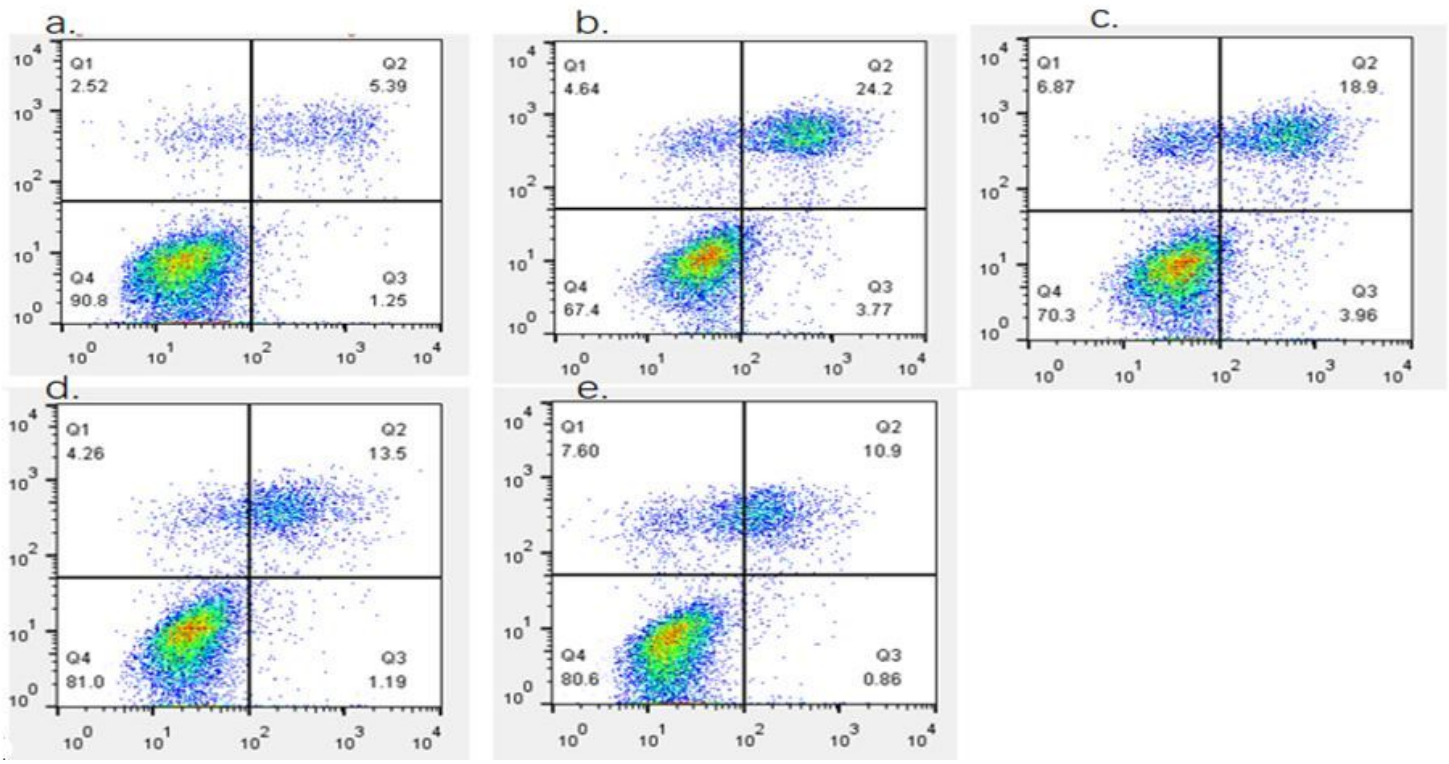

Figure 1

The results of apoptosis detected by Flow Cytometry. Each chart consists of four areas: Q1 region represents those necrotic cells; Q2 region represents the late stage apoptosis; Q3 region represents the early stage apoptosis; Q4 region represents viable cells. Q2 region adds Q3 region represents the total apoptosis of cells. The numbers in each region represent the proportion of cells in the region of total cells.

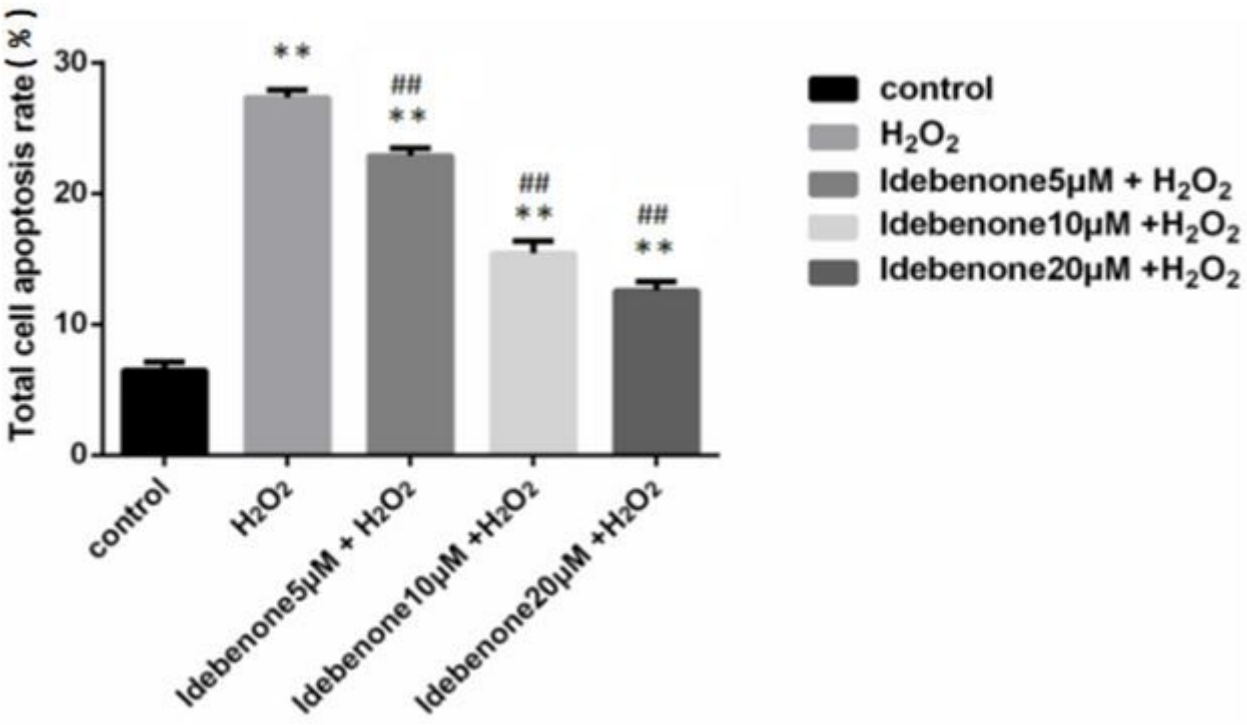

Figure 2

Statistical analysis of apoptotic rate measured by flow cytometry.(**P<0.01VS control group,## P<0.01VS H2O2 group, n=4).

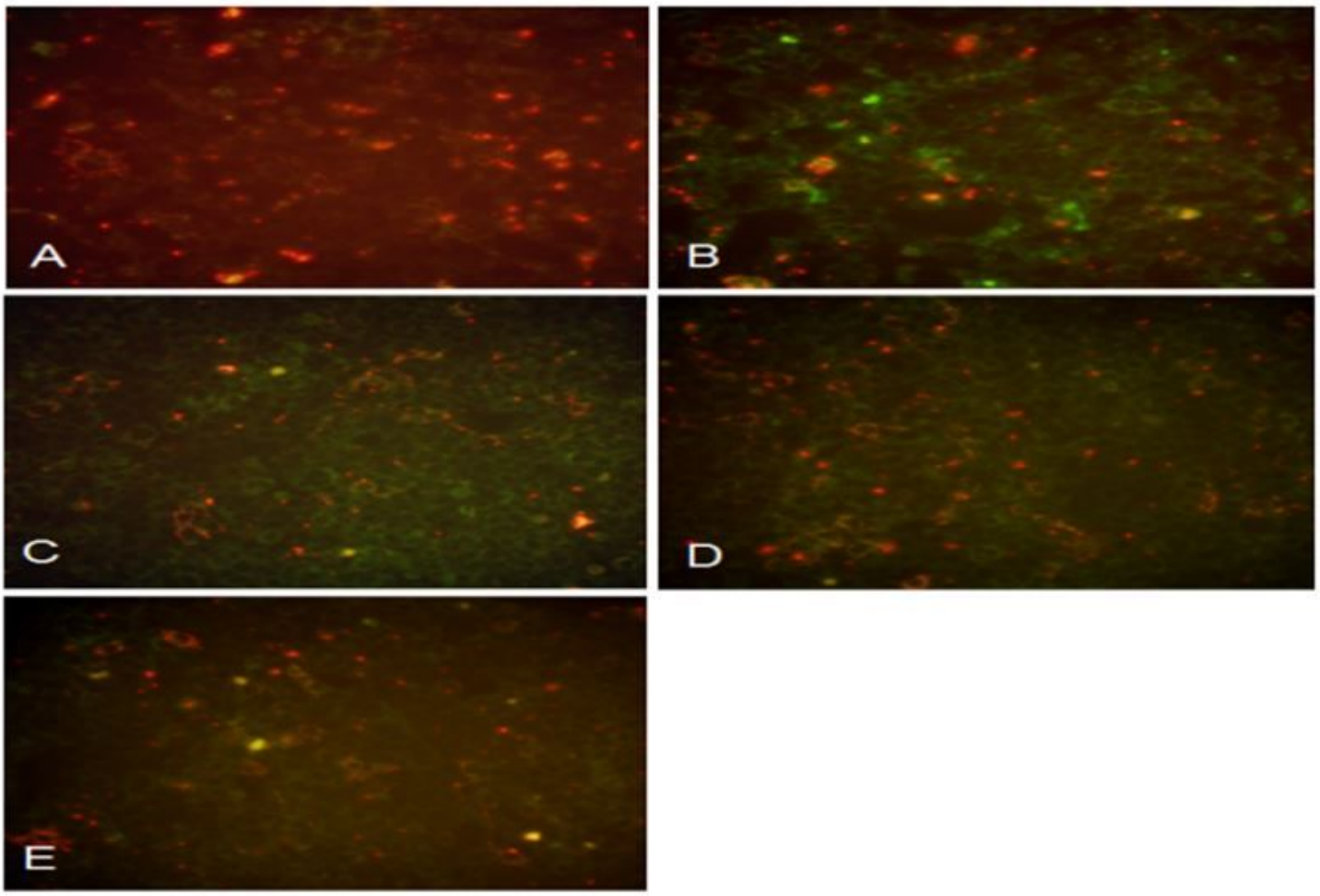

Figure 3

The changes of MMP measured by JC-1 kit□×100□. A. the control group; B.H2O2 group; C. Idebenone5μm+ H2O2; D. Idebenone10μm + H2O2; E. Idebenone20μm + H2O2.

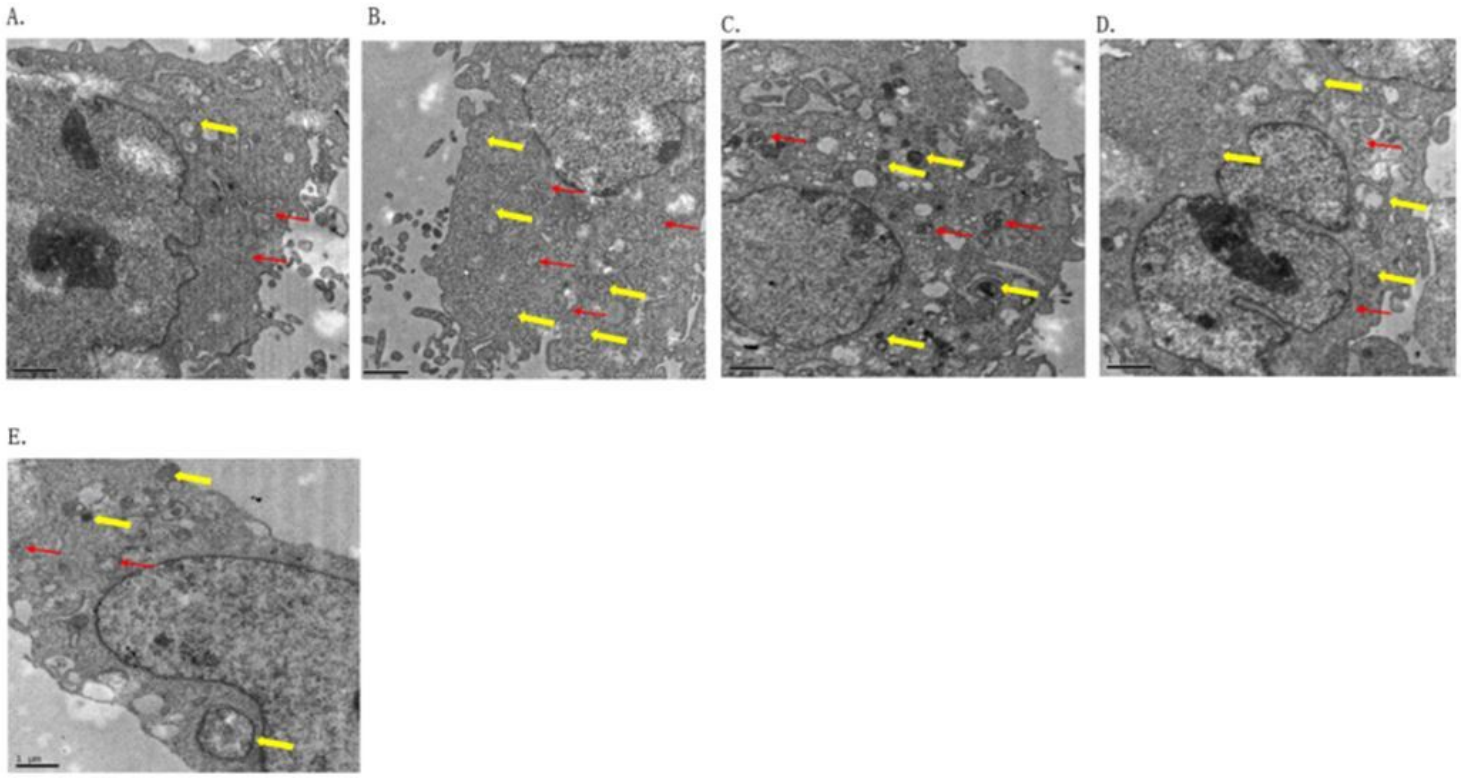

Figure 4

Transmission electron microscopy image of RGC-5s. Autophagosomes (red arrows) and autolysosomes (yellow arrows) are noted in RGC-5s.

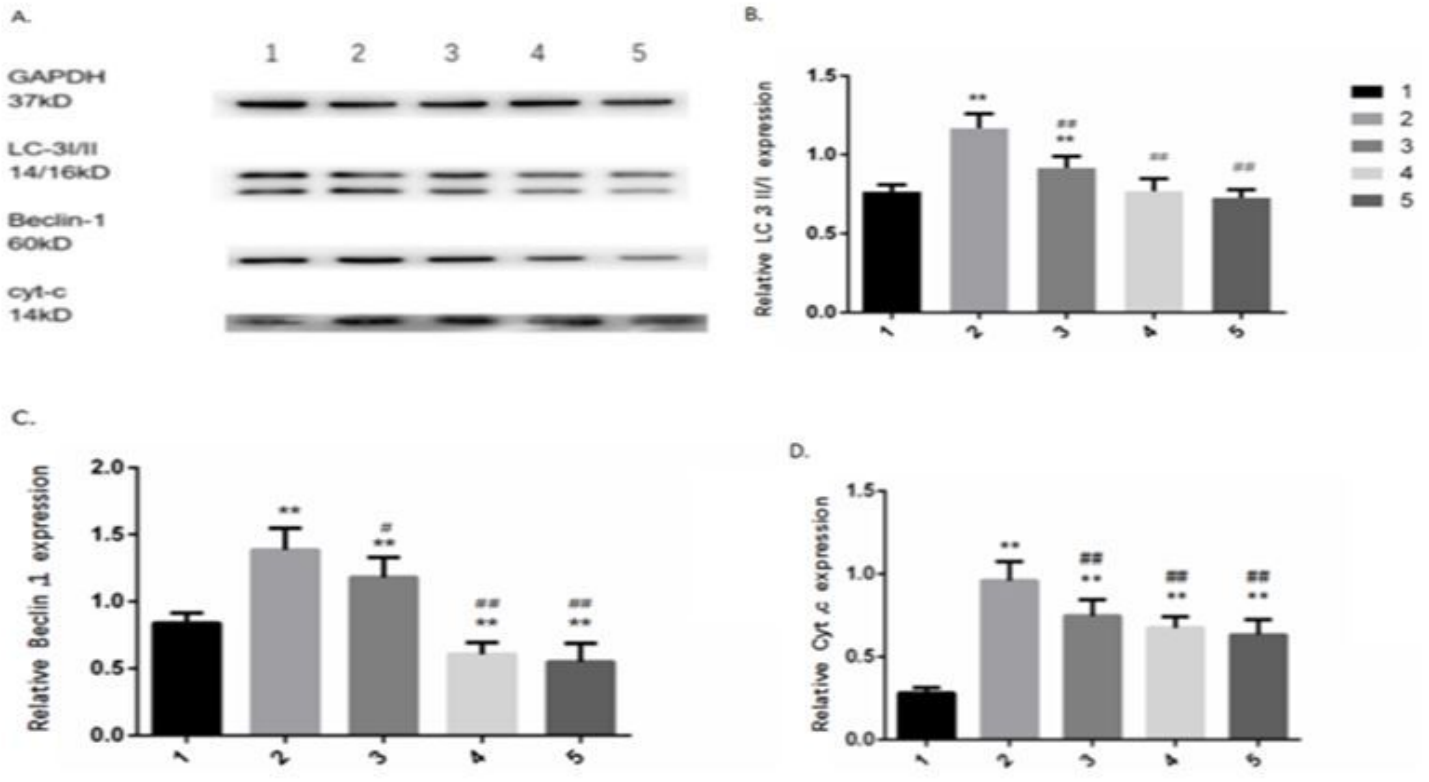

Figure 5

Relative expression of LC3 II/I, Beclin-1 and Cyt-c. A.The expression of LC3, Beclin-1, Cyt-c were detected by Western blotting; B.the relative expression of LC3II/I; C.the relative expression of Beclin-1; D.the relative expression of Cyt-c. 1.Control group; 2.H2O2 group; 3.Idebenone5μm+H2O2 group; 4.Idebenone10μm+H2O2 group ;5.Idebenone20μm+H2O2 group. (**P < 0.01, vs. control group; #P < 0.05, ##P<0.01vs. H2O2 group).