Protective effect of ginsenoside Rg1 on 661W cells exposed to oxygen-glucose deprivation/reperfusion *via* keap1/nrf2 pathway

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Abstract

● **AIM:** To construct an *in vitro* model of oxygen-glucose deprivation/reperfusion (OGD/R) induced injury to the optic nerve and to study the oxidative damage mechanism of ischemia-reperfusion (I/R) injury in 661W cells and the protective effect of ginsenoside Rg1.

● **METHODS:** The 661W cells were treated with different concentrations of $Na_2S_2O_4$ to establish OGD/R model *in vitro*. Apoptosis, intracellular reactive oxygen species (ROS) levels and superoxide dismutase (SOD) levels were measured at different time points during the reperfusion injury process. The injury model was pretreated with graded concentrations of ginsenoside Rg1. Real-time polymerase chain reaction (PCR) was used to measure the expression levels of cytochrome C (cyt C)/B-cell lymphoma-2 (Bcl2)/ Bcl2 associated protein X (Bax), heme oxygenase-1 (HO-1), caspase9, nuclear factor erythroid 2-related factor 2 (nrf2), kelch-like ECH-associated protein 1 (keap1) and other genes. Western blot was used to detect the expression of nrf2, phosphorylated nrf2 (pnrf2) and keap1 protein levels.

● **RESULTS:** Compared to the untreated group, the cell activity of 661W cells treated with $Na_2S_2O_4$ for 6 and 8h decreased ($P<0.01$). Additionally, the ROS content increased and SOD levels decreased significantly ($P<0.01$). In contrast, treatment with ginsenoside Rg1 reversed the cell viability and SOD levels in comparison to the $Na_2S_2O_4$ treated group ($P<0.01$). Moreover, Rg1 reduced the levels of caspase3, caspase9, and cytC, while increasing the Bcl2/ Bax level. These differences were all statistically significant ($P<0.05$). Western blot analysis showed no significant difference in the protein expression levels of keap1 and nrf2 with Rg1 treatment, however, Rg1 significantly increased the ratio of pnrf2/nrf2 protein expression compared to the $Na_2S_2O_4$ treated group ($P<0.001$).

● **CONCLUSION:** The OGD/R process is induced in 661W cells using $Na_2S_2O_4$. Rg1 inhibits OGD/R-induced oxidative damage and alleviates the extent of apoptosis in 661W cells through the keap1/nrf2 pathway. These results suggest a potential protective effect of Rg1 against retinal I/R injury.

● **KEYWORDS:** oxygen-glucose deprivation/reoxygenation; ginsenoside Rg1; oxidative stress; phosphorylated nrf2

INTRODUCTION

It is generally accepted that ischaemia-reperfusion (I/R)-induced tissue damage is not only related to the degree of reduced blood flow, but also to intracellular calcium overload, oxidative stress, inflammatory responses, neurotoxicity of excitatory amino acids, excessive nitric oxide synthesis, and disturbances in energy metabolism[1], ultimately causing tissue damage and neuronal cell necrosis/apoptosis with the process causing an increase in reactive oxygen species (ROS), including superoxide (O^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl radicals, leading to DNA fracture, lipid peroxidation and protein inactivation[2-3].

The mechanisms by which I/R injury to tissue occurs are complex and include primary injury in the early stages of ischaemia and secondary injury following reperfusion[4], which is further exacerbated by I/R caused by the restoration of tissue blood supply after ischaemia has occurred. It is a common cause of visual impairment and blindness in all forms

of ischaemic retinopathy (including central retinal artery occlusion, retinal vein occlusion, glaucoma, traumatic optic neuropathy (TON) and diabetic retinopathy)[5-6]. However, to date, no definitive neuroprotective treatment has been found to protect ganglion cells and photoreceptors in many optic nerve diseases, including glaucoma and TON, and there are no clinically approved drugs that are effective in rescuing retinal neurons in ischaemic retinopathy.

Ginsenoside Rg1 is one of the most important active ingredients in ginseng extracts, with a wide range of physiological activities and important medicinal values[7-8]. Rg1 has been found to have protective effects on various tissues and organs in the human body, with anti-apoptotic, anti-inflammatory and anti-aging effects. Previous studies have shown that Rg1 exerts various pharmacological effects through various mechanisms such as inhibition of apoptosis-related protein levels, down-regulation of inflammatory mediators and anti-oxidation, effectively exerting organ-protective effects against I/R induced damage[9-10]. Some studies have reported that Rg1 can reduce the release of reactive oxygen stress and lactate dehydrogenase in a hypoxia-reperfusion model of cardiomyocytes, thereby improving the ability of cardiomyocytes to resist I/R injury. However, the role of ginsenoside Rg1 in retinal ischemia or retinal I/R needs to be further investigated[11].

MATERIALS AND METHODS

Cell Cultures　Although it would be ideal to use a specific retinal ganglion cells (RGC) cell line to explain internal retinal degeneration *in vivo*, no specific RGC line currently exists in ophthalmology. Cell 661W line is thought to be a mouse immortalized cone photoreceptor cell line with multiple characteristics of retinal ganglion precursor-like cells, and this cell line has been widely used to study the characteristics of retinal ganglion cells; therefore, for our *in vitro* studies we used the murine retinal neuronal cell line 661W cells. Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco, 98 Thermo Fisher Scientific, USA) and 1% streptomycin-penicillin (HyClone, GE Healthcare Life 99 Science, USA). $Na_2S_2O_4$ (Sodium dithionite, 104 Macklin, China) was encapsulated and stored in a dry environment and dissolved in 1×phosphate buffer saline (PBS) as a master mix (0.5 mol/L) and then added in a gradient of different concentrations. Ginsenoside Rg1 was dissolved in dimethylsulfoxide (DMSO; MP 106 Biomedicals, USA) and stored in frozen portions, then added to Petri dishes in a concentration gradient.

Cellular Viability Analysis　Normally cultured 661W cells, cells cultured with ginsenoside Rg1, and cells treated with I/R were trypsinized and centrifuged. After resuspension, cells were placed in a MUSE Annexin V & Dead Cell Kit (Merck Millipore, Germany) and incubated for 20min at room temperature in the dark. The cell suspension was then well mixed and run on a MUSE cell analyser (Merck Millipore, Germany).

Cellular Oxidative Stress Assay and SOD Assay　Cellular oxidative stress assays were performed using the MUSE Oxidative Stress Kit (Merck Millipore, Germany): cell samples were collected from culture dishes and prepared at 1×10^6 cells/mL in 1× buffer. Subsequently, 10 μL of cell samples were incubated with 190 μL of Muse Oxidative Stress working solution for 30min at 37°C in the dark and run on a Muse Cell Analyzer (Merck Millipore, Germany).

Quantitative Real-time Polymerase Chain Reaction　Total cellular RNA was extracted using Trizol reagent (Invitrogen, USA) and nucleic acid quality control was performed to ensure the quality of RNA extraction. The cDNA was then transcribed using Prime Script™ RT reagent Kit and gDNA Eraser (TaKaRa, Japan). Real-time polymerase chain reaction (PCR) was performed using SYBR Green Master Kit (Roche, Switzerland) and recorded using the LightCycler 96 System. β-actin was used as an endogenous control. The list of DNA primers sequences is showed in Table 1.

Western Blotting　After treatment the cells were harvested and cellular proteins were isolated using standard procedures. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Protein-attached membranes were closed with 5% skimmed milk for 60min and then incubated overnight at 4°C with the following primary antibodies: rabbit anti-nuclear factor erythroid 2-related factor 2 (nrf2) antibody (BIOSS, China), rabbit anti-phospho-nrf2 (Ser40) antibody (BIOSS, China), rabbit anti-β-actin rabbit mAb (CST, USA). Rabbit anti-kelch-like ECH-associated protein 1 (keap1) antibody (BIOSS, China).

Statistical Analysis　All experiments were repeated at least three times. Each experimental group contained three independent cell samples. Statistical analysis was performed using IBM SPSS Statistics 22 software (IBM, USA). Data were expressed as mean±standard deviation (SD) and significance was determined by *t*-test or Bonferroni corrected one-way ANOVA. Graphs were plotted using GraphPad Prism 6 software (GraphPad software Inc, USA). $P<0.05$ was considered a statistically significant difference. GraphPad Prism 8 software (GraphPad Software Inc., USA) was used for 157 graphical plot.

RESULTS

Oxygen-Glucose Deprivation Induction of Cell Death in 661W Cells Using $Na_2S_2O_4$ *in Vitro*　To investigate the process of *in vitro* ischemia and hypoxia, we treated 661W cells for 50min using different concentrations (0, 2.5, 5.0, 10.0, 15.0, 20.0 mmol/L) of sodium bisulfite dissolved in PBS

Table 1 List of DNA primers sequences

Gene	Nucleotide sequence (5'-3')	Product length (bp)
GAPDH		120
F	GAGTCCACTGGCGTCTTCAC	
R	GTTCACACCCATGACGAACA	
KEAP 1		245
F	TGCCCCTGTGGTCAAAGTG	
R	GGTTCGGTTACCGTCCTGC	
HO-1		100
F	AAGCCGAGAATGCTGAGTTCA	
R	GCCGTGTAGATATGGTACAAGGA	
NRF2		140
F	TCTTGGAGTAAGTCGAGAAGTGT	
R	GTTGAAACTGAGCGAAAAAGGC	
P53		173
R	CGACTACAGTTAGGGGGCAC	
F	GGAGGAAGTAGTTTCCATAAGCCT	
BCL2		169
F	CAGCCAGGAGAAATCAAACAGAG	
R	GGAGAGCGTCAACAGGGAGA	
BAX		61
F	CCAAGAAGCTGAGCGAGTGTCT	
R	AGCTCCATATTGCTGTCCAGTTC	
CYTC		107
F	CCAAATCTCCACGGTCTGTTC	
R	ATCAGGGTATCCTCTCCCCAG	
NQO1		87
F	GCGAGAAGAGCCCTGATTGTACTG	
R	TCTCAAACCAGCCTTTCAGAATGG	
GCLC		102
F	ACATCTACCACGCAGTCAAGGACC	
R	CTCAAGAACATCGCCTCCATTCAG	
PTGS2		74
F	TGAGCAACTATTCCAAACCAGC	
R	GCACGTAGTCTTCGATCACTATC	

F: Forward; R: Reverse.

to simulate the oxygen-glucose deprivation (OGD) process (Figure 1A, 1B). Flow cytometric detection of the degree of cellular activity showed that cellular activity began to decrease at 10 mmol/L (67.0%±0.75%) and decreased in a waterfall fashion when the concentration was increased to 15 mmol/L (45.6%±2.78%) compared to the 10 mmol/L concentration. The cell activity decreased to 17.3%±9.67% when the concentration was increased to 15 mmol/L, and the cell debris increased significantly, making it impossible to complete the subsequent experiments.

We continued to investigate the drug toxicity of ginsenoside Rg1 on 661W cells (Figure 1C, 1D), as the drug concentration of ginsenoside Rg1 was increased (0, 20, 40, 60, 80, 100 μmol/L), there was no significant change in the activity of 661W cells in the low concentration drug group (0-60 μmol/L)

(92.6%±0.86%, 90.4%±1.42%, 89.9%±0.50%, 92.7%±0.42%), and the cellular activity decreased when the drug concentration was continued to increase (80 μmol/L 80.5%±2.20%, 100 μmol/L 63.4%±2.10%).The drug concentrations of 60 and 30 μmol/L were selected as the 661W cellular drug groups for the *in vitro* simulation process of 661W cellular I/R.

Effects of Different Doses of Ginsenoside Rg1 on Cell Viability During OGD/R Process On the previous basis, a simulated I/R process was performed, *i.e.* 15 mmol/L sodium hyposulphite treatment for 50min followed by the addition of serum-free medium (DMEM) for 0, 1, 2, 4, 6 and 8h. During this process, the cell activity underwent a process of first increase (0-4h) and then decrease (6 and 8h), and at the 6h stage, the cell activity decreased to 54.9%±1.76%, and the time was extended to the 8h stage, where the activity further decreased to 48.9%±1.59% (Figure 1E, 1F).

After constructing a stable 661W cell I/R model, in order to investigate the effect of ginsenoside Rg1 drug intervention, we pretreated 661W cells with gradient concentrations (0, 30, 60 μmol/L), specifically by adding the drug after cell apposition and incubating the cells with ginsenoside Rg1 0, 30, 60 μmol/L for 24h. When the cell density grew to 70%-80% of the optimal. The oxygen-glucose deprivation/ reperfusion (OGD/R) procedure was carried out at 6 and 8h when the apoptosis rate increased significantly, and it was found that as the concentration of ginsenoside Rg1 increased, the degree of apoptosis improved significantly (60.1%±1.14%, 70.1%±2.22%), and after 8h of damage, the drug intervention also improved significantly (60.3%±1.57%, 68.3%±2.19%). Based on these results, it was shown that ginsenoside Rg1 was able to delay the loss of cellular activity during I/R in 661W cells.

Effects of Different Doses of Ginsenoside Rg1 On Oxidative Stress during OGD/R Process To further explore the molecular effects of Rg1 on OGD/R in 661W cells *in vitro*, we used ROS and superoxide dismutase (SOD) to detect cellular redox status (Figure 2A). The results revealed a significant increase in the ratio of ROS(+) cells (55.2%±0.7%, 70.4%±0.8%) at the 6 and 8h stage of OGD/R compared to cells that had not been subjected to OGD/R treatment (3.5%±0.8%). However, the addition of Rg1 treatment resulted in a significant decrease in all ROS(+) cells, with the ratio decreasing to 50.1%±0.9% and 45.3%±1.1% at the 6h stage and from 70.4%±0.8% to 55.0%±1.4% and 48.2%±1.4% in the 8h (Figure 2B). In contrast, in the standardized SOD content assay, intracellular SOD increased compared to the control as the OGD/R 6, 8h phase progressed, while the administration of gradient concentrations of the drug was able to increase the relative intracellular SOD content (Figure 2C). The results indicate that Rg1 has an inhibitory effect on oxidative stress in 661W cells under *in vitro* conditions.
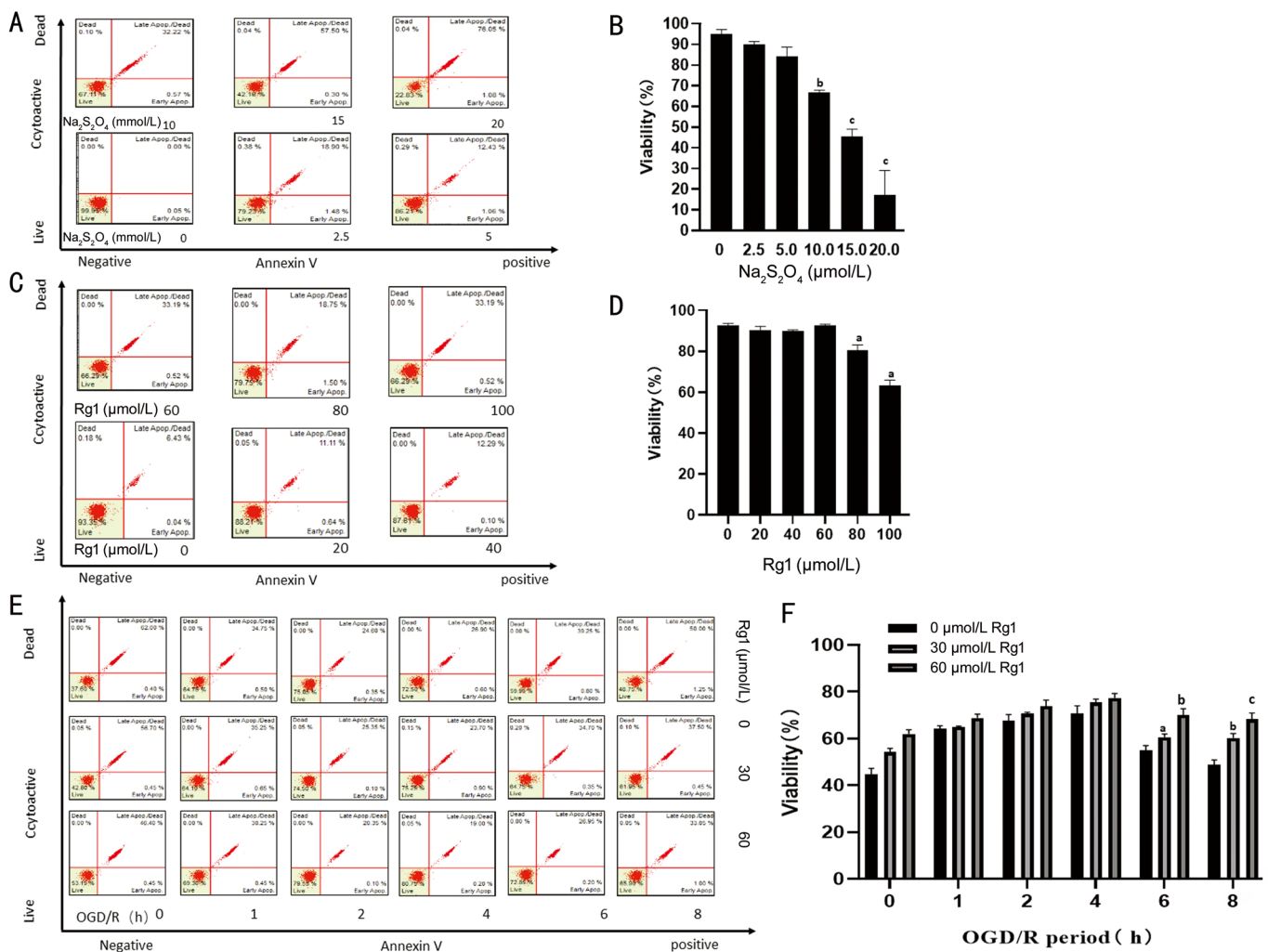
Figure 1 Establishment of retinal ischemia/reperfusion *in vitro* A, B: Flow analysis suggested a significant increase in the degree of apoptosis in 661W cells treated with increasing concentrations of sodium bisulfite for 50min; C, D: Effect of different ginsenoside Rg1 concentrations (0, 20, 40, 60, 80, and 100 umol/L) on the degree of apoptosis in 661W cells; E, F: Flow cytometric analysis of 661W cells showed that the degree of apoptosis decreased and then increased after the oxygen-glucose deprivation/reperfusion (OGD/R) process and decreased with increasing concentrations of ginsenoside Rg1. Comparison of apoptosis rates of cells with different concentrations of Rg1 at the 6 and 8h stages of OGD/R, [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$.

Effects of Ginsenoside Rg1 on the Expression of Keap1/Nrf2/HO-1 during OGD/R in 661W Cells Further molecular experiments were conducted to elucidate the changes in drug regulation in I/R injury in 661W cells. First, the expression of apoptosis-related expression factors [cyt C, B-cell lymphoma-2 (Bcl2)/Bcl2 associated protein X (Bax), caspase3, caspase9] was explored, as shown in Figure 3. Caspase 9 were differentially up-regulated, and the key point of apoptosis regulation, Bcl2/Bax, would be significantly reduced, suggesting that significant apoptotic processes occurred in 661W cells during I/R, and that administration of different gradient concentrations of drugs could inhibit the expression of apoptotic genes (cyt C, caspase3, caspase9) and regulate Bcl2/Bax to protect cells from apoptosis.

Keap1/nrf2 is thought to be an important part of regulating nrf2 expression, and P53 and heme oxygenase-1 (HO-1),

as important influential factors upstream and downstream of its regulation, are closely related to oxidative damage. In order to further investigate the related molecular changes, we also examined the changes of P53, keap1/nrf2 and HO-1 expression in 661W cells under the conditions of ctrl, ctrl+Rg1 60 µmol/L, OGD/R 6h, OGD/R 6h+Rg1 30 µmol/L, OGD/R 6h+Rg1 60 µmol/L, OGD/R 8h, OGD/R 8h+Rg1 30 µmol/L and OGD/R 8h+Rg1 60 µmol/L. The intermediate nrf2 molecule level was upregulated. However, keap1 was not significantly altered as a chaperone molecule in terms of gene and protein expression levels, and the mRNA expression level of total nrf2 was not significantly altered, whereas the level of phosphorylated nrf2 (pnrf2), which functions at the protein level, was altered, and elevated after drug intervention. Further quantitative analysis of pnrf2/nrf2 levels also revealed a significant increase in the pnrf2 ratio (Figure
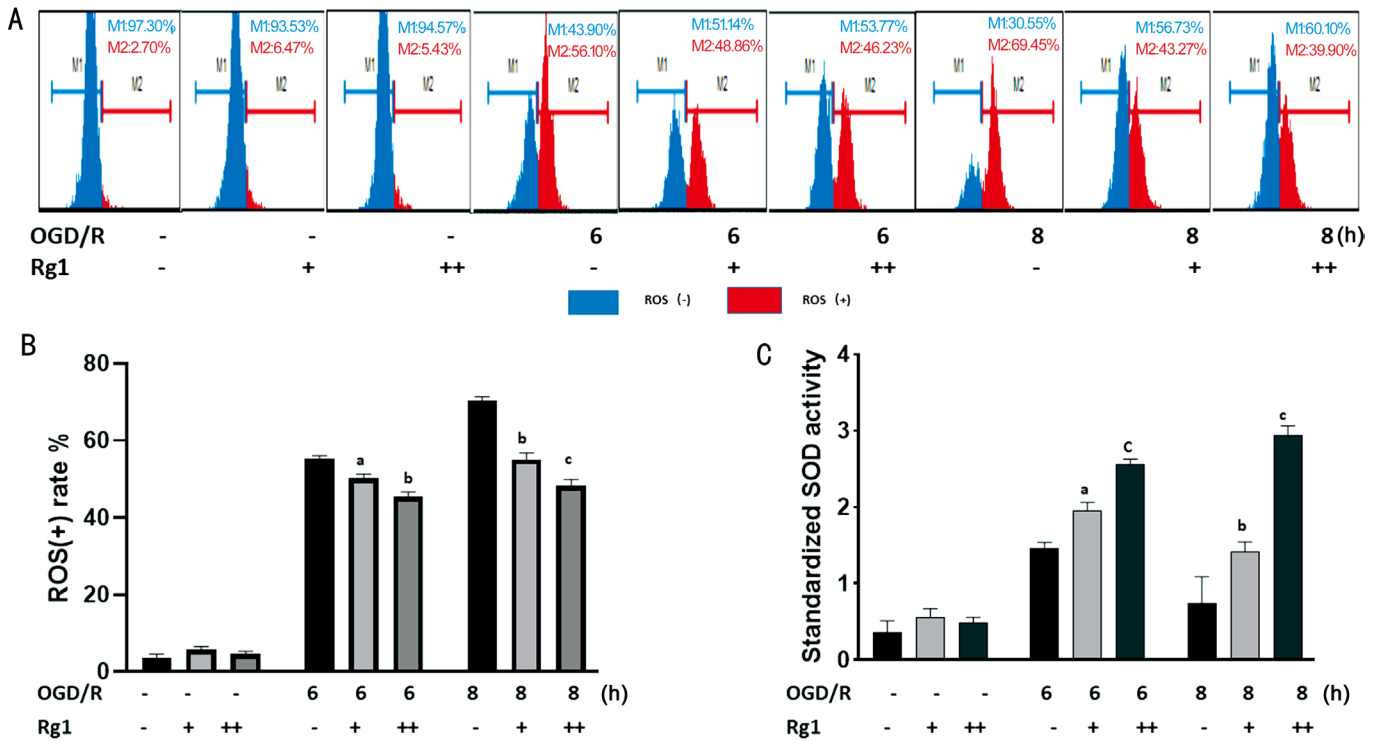
Figure 2 Oxidative stress was restricted by Rg1 treatments in 661W cells Ginsenoside Rg1 significantly inhibited the ROS levels of 661W cells at different oxygen-glucose deprivation/reperfusion (OGD/R) periods and elevated the SOD content accordingly, showing a concentration effect. Compared with OGD/R-, Rg1-, [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$. ROS: Reactive oxygen species; SOD: Superoxide dismutase; OGD/R: Oxygen-glucose deprivation/reperfusion.
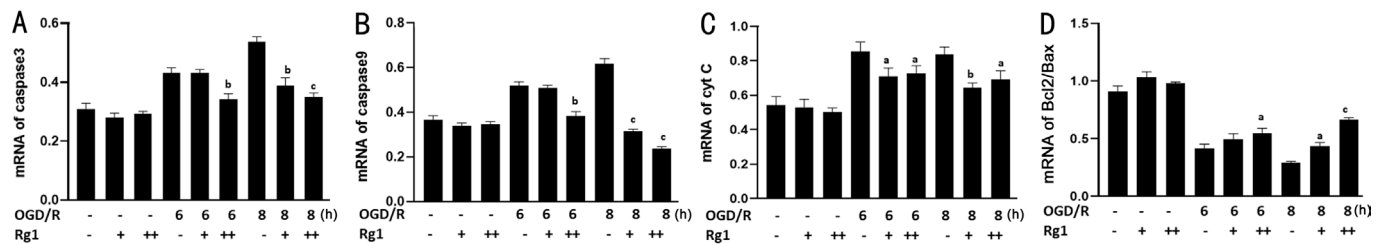

Figure 3 Ginsenoside Rg1 inhibited the apoptotic phenotype of OGD/R in 661W cells 661W cells showed increased expression of caspase3, caspase9 and cyt C during OGD/R, while Rg1 reversed this process. Rg1 alleviated the reduced Bcl2/Bax ratio under OGD/R. Comparison with OGD/R-, Rg1-, [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$. OGD/R: Oxygen-glucose deprivation/reperfusion.

4C-4E). It is suggested that 661W cellular processes undergo drug-regulated phosphorylation into the nucleus to regulate downstream gene expression, exerting a cytoprotective effect. Ginsenoside Rg1 administration significantly reduced the upstream P53 expression level (Figure 4K) but increased the downstream HO-1 expression level (Figure 4G), prostaglandin-endoperoxide synthase 2 (PTGS2), NADPH: quinone oxidoreductase 1 (NQO1) and gamma-glutamate cysteine ligase catalytic subunit (GCLC) did not participate in the overall protective effect of ginsenoside Rg1 (Figure 4H-4J).

DISCUSSION

In constructing models of I/R, previous studies have reported the use of triple gas medium to create an ischaemic hypoxic environment for cells[12-13], and phosphate buffer using sodium bisulphite to simulate ischaemia-hypoxia, the latter of which has been reported in the literature to deprive the cellular surroundings of oxygen and maintain a relatively hypoxic environment[14-15]. In this study, 661W cells exposed to OGD showed a decrease in cellular activity (45.1%±3.06%), and although there was an increase in cellular activity early on when reperfusion conditions were given, there was soon a trend towards a decrease at 6 and 8h. The OGD/R process disturbs the oxidative/antioxidative balance in the cells. The addition of Rg1 pretreatment significantly reduced ROS levels in 661W cells that had undergone OGD/R injury compared to the same stage, while providing an assay for SOD levels, it was also found that injury during this process was accompanied by an increase in SOD, suggesting that oxidative damage during cellular OGD stimulates the production of reductants, while
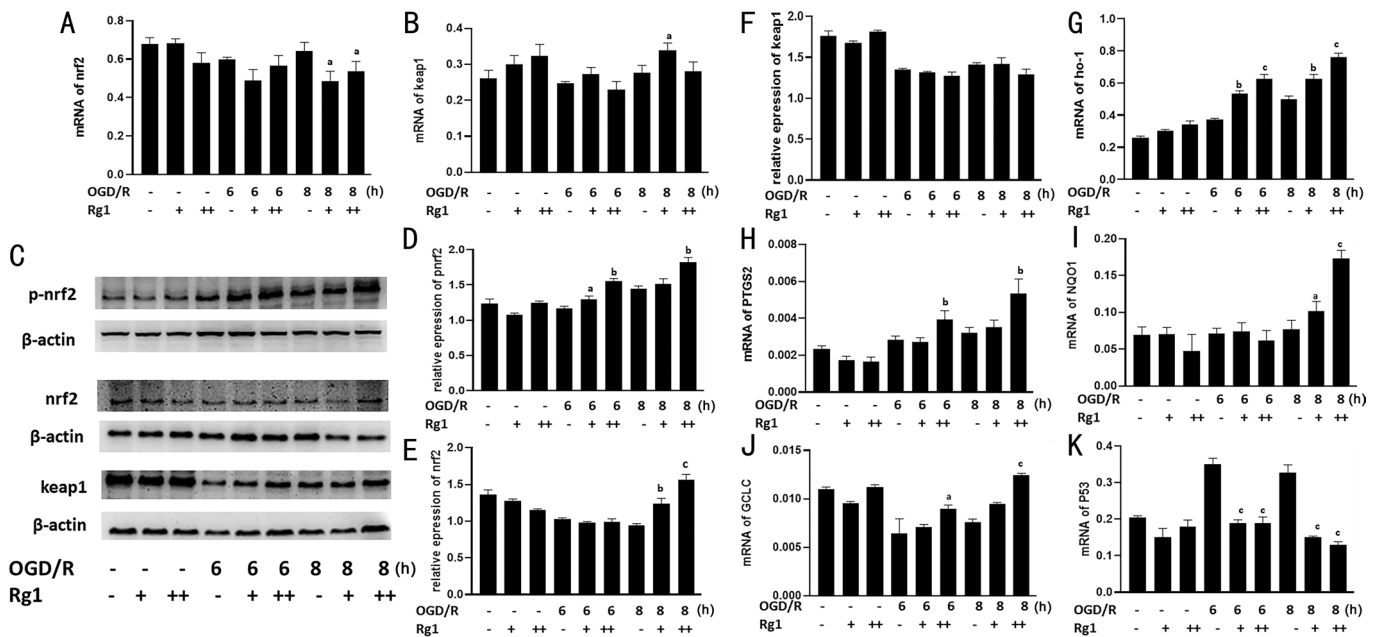
Figure 4 Rg1 activates the keap1/nrf2/HO-1 pathway of OGD/R in 661W cells A: Changes in nrf2 mRNA levels were not significant during OGD/R; B, C, D: Rg1 treatment of 661W cells increased protein levels of nrf2 in OGD/R as well as significantly increased the pnrf2/nrf2 ratio; C, E, F: Keap1 was not significantly altered during OGD/R in 661W cells; G, H, I, J: Ginsenoside Rg1 up-regulated expression of HO-1, PTGS2, NQO1 and GCLC during OGD/R in 661W cells; K: Rg1 treatment of 661W cells inhibited P53 expression during OGD/R. [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$. OGD/R: Oxygen-glucose deprivation/reperfusion.

the addition of ginsenoside Rg1 increased the accumulation of reductants.

661W cells, the current line of retinal cone cells for the study of mouse immortalisation, have a variety of characteristics of retinal ganglion precursor-like cells[16-17], and this cell line has been widely used to study the characteristics of RGC and is currently the most commonly used and only recognised retinal photoreceptor cell line[18]. As cells of neural origin, photoreceptors produce electrical signals that are transmitted to RGC *via* bipolar cells, and its state also influences RGC changes in I/R injury[17]. To investigate this I/R optic nerve injury this study used a cell line of 661W cells.

During retinal I/R, firstly blood flow failure in the body damages the circulatory system and subsequent vascular obstruction leading to primary ischaemia[18-19]. In the absence of nutrient supply, tissues are plunged into hypoxia, nutrient disturbance, calcium in-flow and accumulation of metabolites. Due to the microvascular system of the ocular retina leading to late reconstitution of circulation, late reperfusion process, due to the low concentration of antioxidants in the ischaemic cells[20], the production of ROS also increases and accumulates, and the imbalance of oxygen and nutrients in ischaemic cells brought about by reperfusion leads to a build-up of ROS, which in turn damages cellular structures[21]. Previous studies have shown that ROS-induced oxidative stress is the key to the pathophysiological mechanisms associated with retinal I/R injury[19]. In this study, ROS and SOD were used to detect

the redox status of cells. The results revealed a significant increase in the ratio of ROS(+) cells at the OGD/R 6, 8h stage. However the addition of Rg1 treatment resulted in a significant decrease in both ROS(+) cells. In contrast, in the standardized SOD content assay, as the OGD/R 6, 8h phase proceeded, intracellular SOD increased compared to the control group giving gradient concentrations of the drug was able to increase the relative intracellular SOD content. The results indicate that Rg1 has some inhibitory effect on oxidative stress in 661W cells under *in vitro* conditions.

Rg1 is one of the most important active ingredients in ginseng extracts and has a wealth of medicinal value and physiological activity[22-23]. In recent years, there is increasing evidence that ginsenoside Rg1 plays an important role in neurological, cardiovascular and hepatic studies[24-25]. Previous reports have found that ginsenoside Rg1 can exert estrogenic effects through activation of the estrogen receptor (ERα), upregulate the expression of Yes-associated protein (YAP), and reduce oxidative stress damage in mouse liver cells based on a mouse model of liver I/R injury[26-27]. In the case of neurodegenerative diseases, ginsenosides have been shown to counteract the apoptotic process in neuronal cells by inhibiting oxidative damage. However, the protective effect of these drugs on the retina is less reported. In this study, we simulated the OGD/R damage process in 661W cell line *in vitro* and investigated the protective effect of Rg1. The results showed that apoptosis was increased and Rg1 pretreatment had a protective effect on

OGD/R injury in 661W cells, and that the protective effect of Rg1 may be related to affecting downstream cytoprotective factors through the keap1/nrf2 pathway, improving cellular oxidative stress levels, and interacting with the P53-mediated apoptotic pathway.

Nrf2 is a transcription factor that is highly sensitive to redox changes and is responsible for promoting the expression of antioxidant genes in response to oxidative damage[28-29]. Under normal biological conditions, nrf2 binds to keap1 while limiting its ability to bind to antioxidant-associated elements (ARE)[30]. However, during oxidative stress events, nrf2 is released from keap1, degraded and bound to ARE, thereby activating nrf2 target genes, such as NQO1 and HO-1, to exert their inhibitory capacity against pro-inflammatory mediators[31-32]. In our study, we found that the keap1/nrf2/HO-1 pathway plays an important role in the protection of 661W cells from I/R injury, and the addition of ginsenoside Rg1 could regulate the phosphorylation process of nrf2, up-regulate its binding to ARE in the nucleus during antioxidant reperfusion injury, regulate SOD levels, affect the apoptotic process of cells during I/R injury, and ultimately protect cells from I/R injury. In the present study, we found that Rg1 had no significant toxic effect on 661W cells at doses below 60 μmol/L. Furthermore, the protection of Rg1 on 661W cells exposed to OGD/R was not dose-dependent.

In conclusion, we showed that OGD/R process down-regulated the expression of nrf2/HO-1, whereas Rg1 delayed the I/R injury of 661W cells by up-regulating the expression of nrf2/HO-1 cells from I/R injury. This study provides further molecular rationale for the application of ginsenoside Rg1 in ocular optic nerve I/R diseases such as traumatic optic nerve injury.

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