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Original Research Article

Ginsenoside Rg1 improves ischemic brain injury by balancing mitochondrial biogenesis and mitophagy

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Abstract

Purpose: *To study the effects of ginsenoside Rg1 on mitochondrial dysfunction induced by ischemic stroke.*
Methods: *Human neuroblastoma SK-N-SH cells, subjected to oxygen-glucose deprivation (OGD), were divided into six groups: control group, OGD group, 3 OGD + Rg1 groups (6.25, 12.5 and 25 μM), and Rg1 (25 μM) group. Apoptosis rate, intracellular production of reactive oxygen species (ROS), and mitochondrial transmembrane potential (MTP) in the OGD cells treated with different concentrations of Rg1 were determined. The mRNA and protein expression levels of mitochondrial biogenesis-related transcription factors and autophagy-related proteins were determined by reat time-polymerase chain reaction (RT-PCR) and Western blotting.*
Results: *ROS production was significantly increased in OGD SK-N-SH cells ($p < 0.01$), but this was reversed by Rg1 treatment ($p < 0.05$). Rg1-treated cells had significantly higher MTP when compared with OGD cells ($p < 0.01$). Rg1 treatment led to significant increases in mRNA and protein expression levels of PGC1-α, NRF-1, and TFAM-1 ($p < 0.01$). Moreover, Rg1 treatment inhibited apoptosis in SK-N-SH cells, and up-regulated autophagy-related proteins in t neuronal injury model. Treatment with autophagy inhibitors decreased the mitochondrial protective effects exerted by Rg1 in OGD SK-N-SH cells.*
Conclusion: *Rg1 improves mitochondrial dysfunction by regulating autophagy in mitochondria. Thus, it may offer protection from brain injuries caused by cerebral ischemia.*

Keywords: *Cerebral ischemia, Ginsenoside Rg1, Mitochondrial dysfunction, Mitophagy*

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INTRODUCTION

Stroke is the third most deadly disease in the world and a major source of disability [1]. Ischemic stroke, also known as cerebral ischemia, accounts for about 65-80 % of strokes, and is caused by lack of cerebral blood flow due to a variety of factors [1,2]. Cell autophagy is extensively involved in various stages of cerebral ischemia [3]. Autophagy is a self-regulating defensive adaptation process in eukaryotic cells [3,4]. It is expected to provide raw materials for cell repair and reconstruction to meet the normal turnover and cyclic utilization of cytoplasmic components. Thus, it has an important regulatory role in maintaining the stability of the intracellular environment, cell growth, and development [3,4].

The mitochondrion is an important organelle that produces energy and regulates cell signaling and apoptosis. It is also the main source of reactive oxygen species (ROS) in the cell [5]. The ROS generated from damaged mitochondria may be released into the cytoplasm, where they induce functional and morphological changes, resulting

in imbalance in intracellular redox metabolism and eventually extensive mitochondrial damage [5,6]. Hence, the timely removal of damaged mitochondria is very vital for the normal growth of cells. Mitochondrial autophagy (mitophagy) is a process discovered in recent years, in which the damaged mitochondria are removed by a selective autophagy pathway so as to decrease ROS and promote cell survival by maintaining steady state of intracellular redox status [7,8]. If mitochondrial autophagy is inhibited, damaged mitochondria will produce excess ROS and trigger on cell death [7,8]. At present, the role of this adaptive metabolic mechanism in the pathogenesis of cerebral ischemia is the subject of research interest to many investigators [9].

Ginsenoside Rg1 is one of the main components of ginseng extract and pharmacologically active compound in clinical applications [10]. It has antioxidant and anti-aging properties, and has been used to alleviate nerve function impairment, and for the treatment of cerebrovascular diseases [10]. Recently studies reported that ginsenosides inhibit mitochondrial dysfunction via regulation of imbalances in mitochondrial dynamics [11,12].

This study was aimed at investigating the effect of Rg1 on mitochondrial dysfunction due to cerebral ischemia.

EXPERIMENTAL

Cell culture and Rg1 preparation

Human neuroblastoma SK-N-SH cells were obtained from Sigma-Aldrich (USA). The cells were cultured at 37 °C in Dulbecco′s Modified Eagle′s Medium (DMEM; Invitrogen, USA) containing 2 mM glutamine and 10 % Fetal Bovine Serum (FBS) in a humidified environment containing 5 % CO_2. Rg1 solution (100 mM; Meilunbio, China) was prepared in dimethylsulfoxide and diluted to the indicated final concentrations with DMEM medium before treatment.

Oxygen-glucose deprivation (OGD)

In order to subject SK-N-SH cells to OGD, the cell culture media was replaced by glucose-free DMEM (Invitrogen, USA). Then, the cells were placed in an incubator containing 95 % N_2 and 5 % CO_2 for 4 min. The culture chamber was then sealed and the cells were incubated for 4 h at 37°C. Cells in the control group were cultured in glucose-containing DMEM in a normoxic condition for 4 h. The SK-N-SH cells were pretreated either with vehicle or Rg1 (6.25, 12.5, and 25 μM) for 24 h and then subjected to OGD. Cells were divided into six groups: cells treated with vehicle (control group), cells treated with OGD (OGD group), cells treated with OGD and different concentrations of Rg1 (6.25, 12.5 and 25 μM); and cells treated with 25 μM Rg1.

Apoptosis assay

Apoptosis in OGD-treated neuronal injury model exposed to different concentrations of Rg1 was determined using annexin V assay (BD, USA) according to the manufacturer's protocol. The apoptotic cells were identified by flow cytometry.

Measurement of ROS

Intracellular production of ROS was determined using a DCFDA Cellular ROS detection assay kit (Abcam, UK). The fluorescence intensity of carboxy-H_2DCFDA (excited at 488 nm) was measured using a FACSCanto II cytometer (BD, USA), to determine the ROS level in each group.

Measurement of mitochondrial transmembrane potential (MTP)

A TMRE MTP assay kit (Abcam, UK) was used to determine MTP. The cells were incubated in a medium containing 10 μM tetramethylrhodamine (TMRE, ethyl ester) for 20 min at 37°C (in this assay, mitochondria with decreased membrane potential are not stained by TMRE). Then, the cells were transferred to a clear 96-well plate, and the TMRE staining was measured with a microplate spectrophotometer (excitation at 549 nm and emission at 575 nm).

Western blot analysis

Cells were harvested and washed with cold phosphate buffer solution for twice. Then, the cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, USA). The total protein concentrations were determined using Pierce™ BCA protein assay kit (Thermo Fisher Scientific, USA). The proteins (25 μg) were subjected to 10 % SDS-PAGE and then electro-transferred onto a nitrocellulose membrane. The transferred proteins were then probed with primary antibodies against PGC1- α, NRF1, Tfam, LC3B II, p62, and beclin-1 (Abcam, USA). β-actin was loaded as an internal reference. Then the membranes were further probed with goat anti-rabbit IgG-HRP secondary antibody (1:2000; Abcam, USA). Bands were developed using Pierce™ electro-chemiluminescence (ECL) substrate (Thermo Fisher Scientific, USA).

RT-PCR

RNA was extracted by with RNeasy Plus Micro Kit (QIAGEN, USA), according to the manufacturer's instructions. Then, the reverse transcription of the extracted RNA was performed with the SuperScript® IV First-Strand Synthesis System (Invitrogen, USA). RT-PCR was performed in Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, USA), using 20 ng template in 25 μL reaction volume with 2 x Power SYBR® Green PCR Master Mix (Invitrogen, USA) and gene specific primer pairs for PGC1- α, NRF1, Tfam, LC3B II, p62, beclin-1, and β-actin. The PCR program setting was as follows: 95°C, 10 min; 45 cycles of 95°C, 15 s, and 60 °C, 1 min. The gene expression levels for all samples were normalized to β-actin mRNA expression using the comparative Ct method.

Statistical analysis

All the experiments were done in triplicates and the SD was calculated. Analysis of variance (ANOVA) and multiple comparisons using Dunnett's test were applied to compare the differences between groups. $P < 0.05$, $p < 0.01$, or $p < 0.001$ was considered statistically significant.

RESULTS

Rg1 treatment inhibited OGD-induced apoptosis in SK-N-SH cells

An *in vitro* neuronal injury model was successfully established by subjecting the human neuroblastoma SK-N-SH cells to OGD. As shown in Figures 1A and Figure 1B, OGD treatment alone significantly increased the apoptotic rate of SK-N-SH cells ($p < 0.01$, when compared with the control group. However, this increase in apoptosis was significantly and dose-dependently inhibited by pre-treatment with Rg1 ($p < 0.05$).

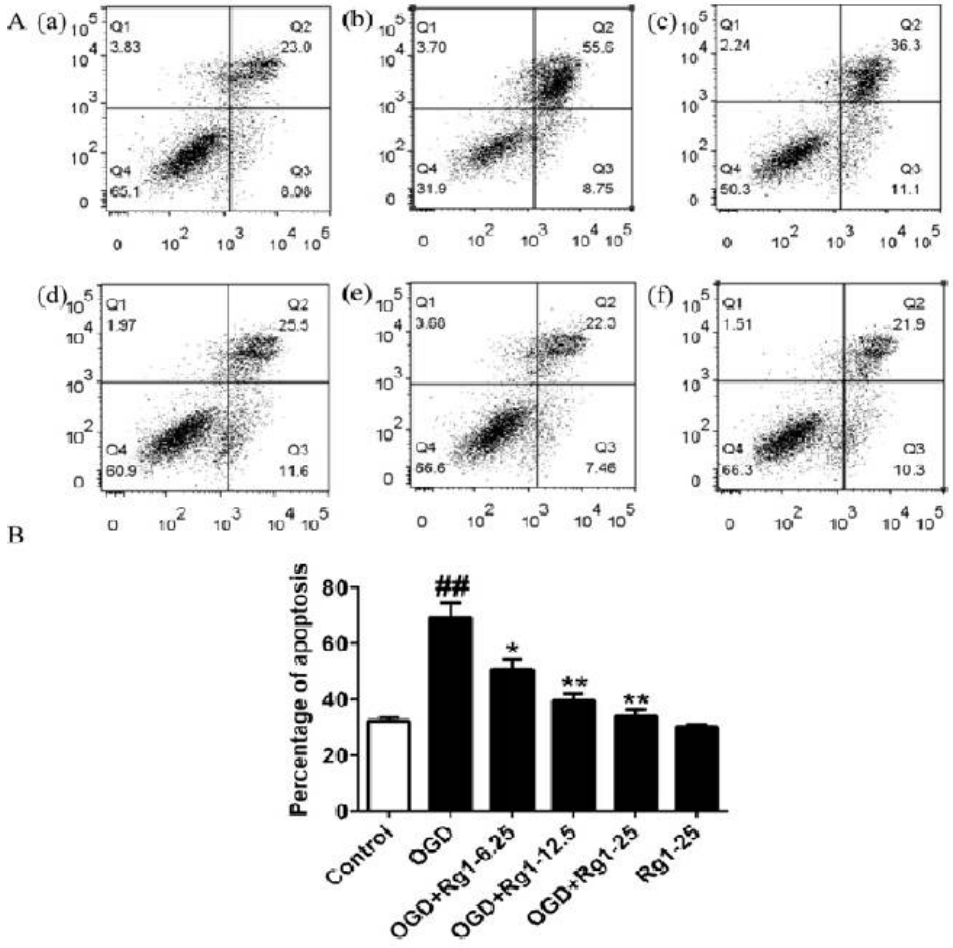

Figure 1: Inhibition of OGD-induced apoptosis in SK-N-SH cells by Rg1 treatment (A) Representative dot plots of apoptosis rate measured by flow cytometry. (a) control group; (b) OGD group; (c) OGD + 6.25 μM Rg1 group; (d) OGD + 12.5 μM Rg1 group; (e) OGD + 25 μM Rg1 group; (f) 25 μM Rg1 group. (B) Quantitative analysis of percentage of apoptotic death. ## $p < 0.01$, versus the control group; *$p < 0.05$, **$p < 0.01$ versus the OGD group

Rg1 treatment alleviated OGD-induced ROS production in SK-N-SH cells

In order to study the effect of Rg1 on ROS production in neuronal injury model, a DCFDA cellular ROS detection assay was applied. The results showed that ROS production was significantly increased in SK-N-SH cells subjected to OGD ($p < 0.01$ relative to the control group; Figure 2). The Rg1 treatment also significantly attenuated ROS production in the neuronal injury model when compared to the OGD group ($p < 0.05$ at 12.5 and 25 μM Rg1 treatments).

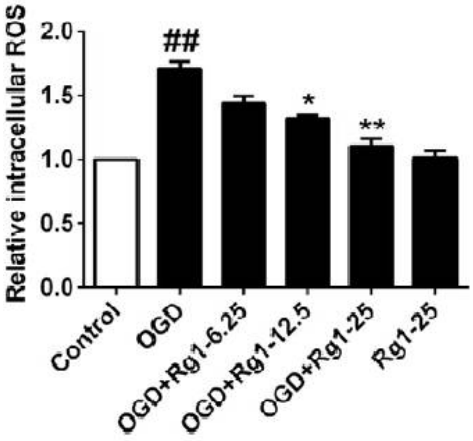

Figure 2: Effect of Rg1 on intracellular ROS production in OGD SK-N-SH cells. ## $p < 0.01$, versus the control group; * $p < 0.05$, ** $p < 0.01$, versus the OGD group

Effect of Rg1 treatment on MTP in SK-N-SH cells with OGD

Results showed that OGD treatment significantly decreased MTP in the SK-N-SH cells, when compared with the control ($p < 0.01$, Figure 3). However, treatment with Rg1 reversed the OGD-induced decreases in MTP by bringing about significant and concentration-dependent increases in MTP, relative to the OGD ($p < 0.01$ at 12.5 and 25 μM of Rg1).

Effect of Rg1 treatment on mitochondrial biogenesis in neuronal injury model

The effect of Rg1 on mitochondrial biogenesis in neuronal injury model was further determined. SK-N-SH cells underwent OGD showed a slightly increased pattern of expressions of the mitochondrial biogenesis related transcription factors PGC1-α, NRF-1, and TFAM-1, as determined by RT-PCR (Figure 4B), as well as increases in protein levels detected by Western blotting (Fig. 4A). Moreover, Rg1 (25 μM) treatment on SK-N-SH cells further up-regulated the mitochondrial biogenesis-associated transcription factors, as demonstrated by RT-PCR and Western blotting in the neuronal injury model ($p < 0.01$, versus the OGD group).

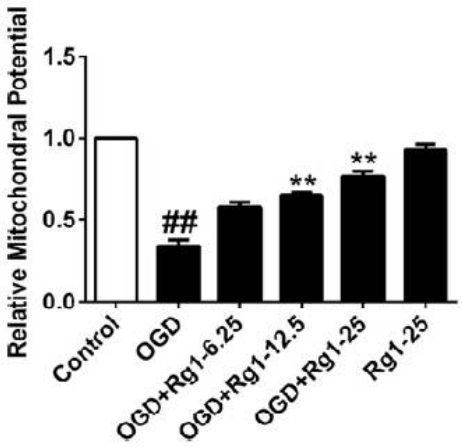

Figure 3: Effect of Rg1 on MTP in SK-N-SH cells subjected to OGD. ##p < 0.01, versus the control group; * $p < 0.05$, ** $p < 0.01$, versus the OGD group

Effect of Rg1 on expressions of autophagy proteins

The effect of Rg1 on the expressions of autophagy-related proteins (LC3B-II, p62, and Beclin-1) was studied *in vitro*. The results showed that the expression levels of LC3B-II and Beclin-1 were significantly up-regulated in Rg1-treated OGD SK-N-SH cells, relative to the control group and the OGD-only group ($p < 0.01$ and $p < 0.05$, respectively; Figures 5A and Figure 5B). However, Rg1 treatment had no appreciable effect on p62 levels. In addition, Rg1 treatment failed to reverse the OGD-induced decrease in MTP when the autophagy inhibitors 3-methyladenine (3MA) and wortmannin (wort) were applied (Figure 5C, $p < 0.01$, versus the OGD + Rg1 group). These results indicate that Rg1 exerts mitochondrial protective effect on OGD-treated SK-N-SH cells via activation of autophagy.

DISCUSSION

Ischemic stroke is a serious threat to human health, and easily leads to ischemic neuronal damage, cognitive and physical dysfunction and high mortality [1,13]. Currently, ischemic stroke is effectively treated by injecting recombinant tissue plasminogen activator for thrombolysis, but due to the limitations of time window (within 4-5 h after incidence), only few ischemic stroke patients are lucky to receive this therapy [14]. Therefore, it has become necessary to explore new therapeutic targets and drugs for the treatment of stroke.

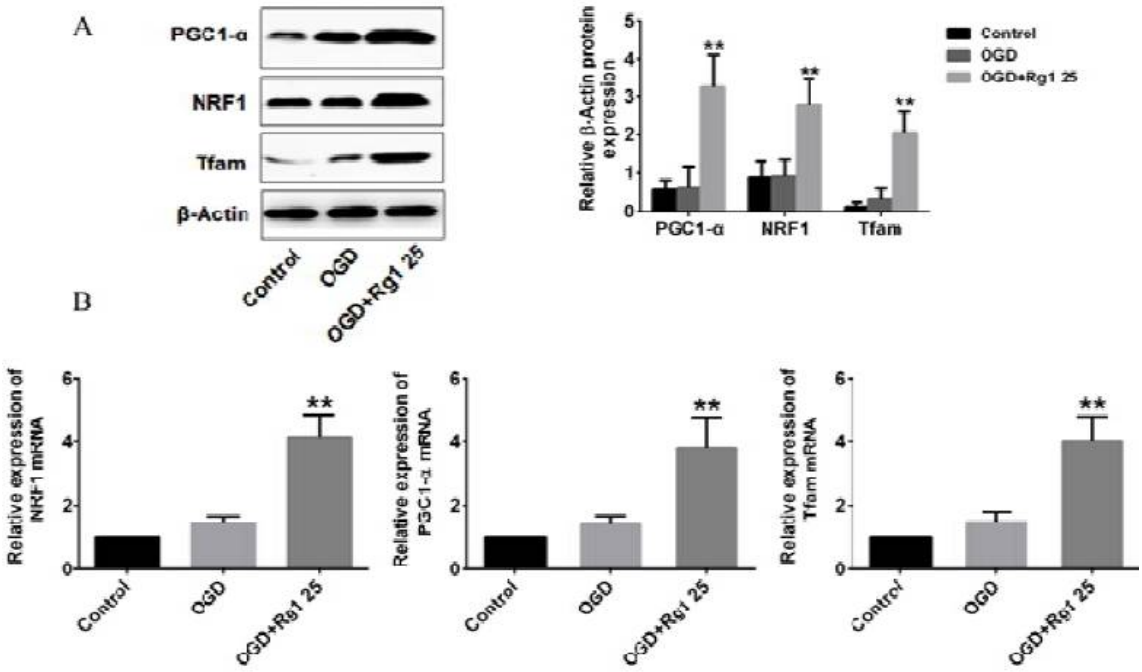

Figure 4: Effect of Rg1 treatment on expressions of mitochondrial biogenesis in neuronal injury model. Western blot (A) and RT-PCR (B) analysis of the expression levels of mitochondrial biogenesis- related transcription factors PGC1-α, NRF-1, and Tfam-1 in OGD-treated SK-N-SH cells. ** $p < 0.01$, versus the OGD group.

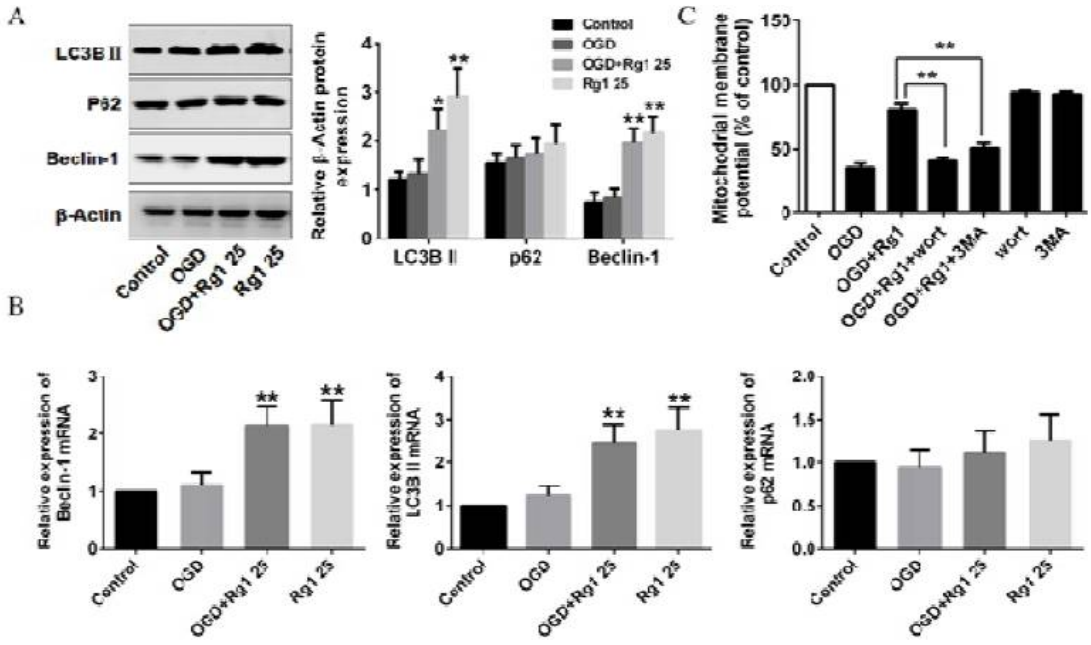

Figure 5: Protective effect of Rg1 on mitochondrial function in OGD-treated SK-N-SH cells via autophagy activation. Western blot (A) and RT-PCR (B) analyses of the expression levels of autophagy-related proteins LC3B II, p62, and Beclin-1. (C) Rg1 treatment failed to reverse the OGD-induced decrease in MTP when autophagy inhibitors were applied; *$p < 0.05$, **$p < 0.01$, compared to the OGD group or as indicated in the figure.

Energy metabolic disorder is an early pathologic response to cerebral ischemic injury [2]. The mitochondrion is the most important energy organelle of cells, providing most of the energy needed for cellular activities, including metabolism and excitatory transmission of cerebral neuronal cells [8,9]. During ischemia and hypoxia, the mitochondria produce a large number of ROS. This results in lipid peroxidation of cell and organelle membranes, loss of membrane integrity and permeability, and ultimately cell death [5,6,9,13].

Excessive ROS can lead to sustained opening of mitochondrial permeability transition pore (MPTP) and membrane damage, impaired

mitochondrial Ca^{2+} recycling, decline in MTP, and activation of mitochondrial apoptotic pathway [5,6,9,13].

Ginseng is a drug commonly used for the treatment of cardiovascular and cerebrovascular diseases. It contains ginsenoside, ginseng polysaccharide, proteins, polypeptides, and amino acids, but ginsenoside is the most important active ingredient [10]. In the present study, Rg1 inhibited apoptosis and ROS production in SK-N-SH cells subjected to OGD. In addition, Rg1 treatment increased MTP, when compared with cells subjected to OGD only. Changes in MTP result in impairment of ATP production, which leads to further production of ROS, damage of mitochondrial membrane or even damage of the entire cell [15,16]. Therefore, Rg1 may alleviate mitochondrial dysfunction induced by OGD via promoting ATP production in this neuronal injury model.

In vitro ischemia-reperfusion experiments of mouse cortical neurons showed that cortical neurons had decreased mtDNA after ischemia reperfusion, with decreased mitochondrial mass and lowered generative capacity [17,18]. Animal ischemia-reperfusion model studies have shown that promotion of mitochondrial production, maintenance of stability of mitochondrial function and increasing energy supply can reduce the incidence of cerebral infarction and neuronal apoptosis [17,19]. Therefore, the effect of Rg1 on the mitochondrial biogenesis-associated transcription factors was studied in the SK-N-SH cells subjected to OGD. It was found that Rg1 treatments consistently promoted mitochondrial biogenesis.

Mitochondrial autophagy can delay and decrease the accumulation of mtDNA mutations [7]. When the body is exposed to adverse external stimuli, selective mitochondrial autophagy may occur [7]. Mitochondrial damage may lead to the loss of function and thus trigger mitochondrial autophagy, which is an important way of removing damaged mitochondria to prevent ROS accumulation [7]. In recent years, many experiments have shown that cerebral ischemia is closely related to mitochondrial autophagy. A study by Carloni *et al* showed that improving mitochondrial autophagy can perfect cell morphology and reduce tissue necrosis, thus enhancing neuro-protection [20]. The present study found that Rg1 can un-regulate the autophagy-related proteins and activate autophagy in the neuronal injury model studied.
The mitochondrial protective function exerted by Rg1 decreased after application of autophagy inhibitors *in vitro*. Cell autophagy has destructive effect, so the inhibition of autophagy can protect neurons [21]. Therefore, autophagy may be a double-edged sword: under mild physiological stress, the activation of mitochondrial autophagy has a protective effect, while in severe ischemia and hypoxia, it may be excessively activated, leading to neuronal damage and necrosis [21]. Therefore, the regulation of autophagy and inhibition of signaling pathways of various damaged mitochondria may be new targets of neuroprotective agents.

CONCLUSION

The results obtained in this study demonstrate that Rg1 can relieve mitochondrial dysfunction by regulating autophagy in mitochondria. Thus, it has the potential to be developed as a therapeutic agent for brain injuries caused by cerebral ischemia.

DECLARATIONS

Acknowledgement

None.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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