ORIGINAL RESEARCH

Ginsenoside Rg1 Alleviates Rat Liver Ischemia-Reperfusion Ischemia Through Mitochondrial Autophagy Pathway

Jie Lin, PhD; Zhe Qing, PhD; Hanfei Huang, PhD; Shikun Yang, MM, Zhong Zeng, PhD

ABSTRACT

Aim • The aim of this study was to elucidate the potential mechanism of Rg1 in alleviating hepatic ischemia-reperfusion (HIRI) through the mitophagy pathway.

Methods • The HIRI rat models were established and divided into 4 groups: the sham group, sham+Rg1 group, ischemia/perfusion (I/R) group and I/R+Rg1 group. Then the activities of aspartate transaminase (AST) and alanine aminotransferase (ALT) were detected by automatic serum analyzer. Meanwhile, cell apoptosis and changes in liver tissues were checked by TUNEL assay and histopathological analysis, respectively. The relative protein levels were detected by western blotting. Subsequently, cell counting Kit-8 assay and cytometric analysis were used to investigate cell viability and apoptosis of liver cells. Finally, the time points of the strongest mitochondrial autophagy were explored and the mitochondrial morphology was observed by the mitochondrial transmembrane potential (MMP) *in vivo* and *in vitro*.

Results • The mitophagy aggravated hepatocyte damage during liver I/R *in vivo*. In addition, Rg1 alleviated liver damage after liver I/R, maintained the stability of MMP and inhibited mitochondrial autophagy and signaling pathways during liver I/R *in vivo*. Furthermore, Rg1 could effectively increase cell viability, inhibit cell apoptosis and stabilize MMP after OGD/R injury *in vitro* Moreover, Rg1 exerted its protective effect on HIRI by regulating the PINK1/Parkin signaling pathway and the mitochondrial autophagy.

Conclusion • Rg1 could further improve its mechanism of alleviating HIRI in apoptosis and autophagy, 2 types of regulated programmed cell death via the mitochondrial pathway (*Altern Ther Health Med.* 2023;29(3):16-25).

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INTRODUCTION

Hepatic ischemia-reperfusion (I/R) injury is a common phenomenon in clinical surgery. After blood flow reconstruction, ischemic organs or tissues cannot restore normal function, but aggravate the destruction of tissue structure and further worsen organ function.[1] However, although the mechanism of liver I/R injury is still unknown, it is clear that it is a complex pathophysiological process involving multiple factors, which may include inflammatory response, apoptosis, autophagy, calcium overload, oxygen free radical production, stress response and so on.

The result of the occurrence and development of liver disease is hepatocyte death, including apoptosis, autophagy and necrosis.[2] Autophagy is involved in the whole process of I/R injury, and the level of autophagy is up-regulated.[3] In the whole process of I/R injury, autophagy mainly plays a role in 2 stages: ischemia and reperfusion, and the signal pathways mediating autophagy are also different in the ischemia and reperfusion stages. Moreover, autophagy plays an important role in both processes. Properly enhancing autophagy during liver ischemia can make cells adapt to environmental changes, enhance cell viability and protect liver function. However, during reperfusion, excessive autophagy directly leads to programmed cell death, intensifies the destruction of hepatocytes and aggravates the liver tissue injury after I/R.[4] Gujral, et al.[5] found that liver function could not be protected by inhibiting the activity of cysteine aspartate protease in rat liver injury, suggesting that autophagic cell death may play an important role in the injury process.

Lemasters, et al.[6] proposed the concept of "mitophagy" for the first time in 2005, and pointed out that under the stimulation of harmful conditions such as energy deficiency,

hunger and hypoxia, the double-layer membrane structure of autophagosomes recognized and specifically enclosed mitochondria with damaged structure and function in cells, and then fused them with lysosomes and degraded the damaged mitochondria. The release of some pro-apoptotic factors and reactive oxygen species (ROS) from mitochondria was reduced to prevent further cell damage and promote cell survival[7]; the decrease in mitochondrial transmembrane potential (MMP) and the opening of mitochondrial membrane permeability conversion pore (MPTP) could cause the occurrence of mitochondrial autophagy.[8,9]

Mitophagy is a special type of autophagy, which refers to specific autophagy by cells selectively removing excess or damaged mitochondria via autophagy.[10] Therefore, the core autophagy structure is still the same as other types of autophagy, which plays an important role in mitochondrial quality control and cell survival,[11] and the study of the relationship between mitophagy and HIRI has become a new research hotspot.

Rg1 has been proven to alleviate I/R injury by suppressing autophagy *in vivo* and *in vitro* in many diseases.[12-16] However, few studies of HIRI have been reported.[15]

The previous portion of this study showed that Rg1 alleviates HIRI through cyclohilin D (cypD)-mediated mitochondrial apoptosis. However, there are few studies on ginsenosides affecting liver ischemia re-injury via the regulation of autophagy. In our study, we assumed that in the process of HIRI injury in rats, excessive mitophagy aggravated the injury during the reperfusion period, while Rg1 could inhibit excessive mitophagy and protect the liver from I/R injury. The aim of this study was to clarify the potential mechanism of reducing HIRI via the mitophagy pathway; Thus, Rg1 can further improve its mechanism of alleviating HIRI in apoptosis and autophagy, and regulates programmed cell death, through mitochondrial pathway.

METHODS

Animals

This experimental protocol has been approved by the Animal Ethics Committee of Kunming Medical University, and the relevant procedures have been implemented in accordance with the requirements of the National Institutes of Health on the "Guidelines for the Feeding and Use of Laboratory Animals."

A total of 32 adult male Sprague-Dawley rats weighing 250g to 300g were obtained from the Experimental Animal Center of Kunming Medical University (Kunming, China). The rats were kept in separate cages with alternating 12h/12h dark/ light cycles, fasted for 8 h before surgery, but were allowed to drink freely.

Rat model of hepatic I/R. Rats were anesthetized with chloral hydrate (300mg/kg, intraperitoneal injection). After abdominal shaving, rats were disinfected with 70% alcohol, the abdominal cavity was opened with a midline incision, the liver was exposed with a micro abdominal expander, and then the blood flow of 70% (left lobe, left middle lobe and right middle lobe) of the rat liver was blocked with a micro vascular clamp to cause thermal ischemia. After 45 hours of thermal ischemia, the rats recovered and underwent reperfusion for 6 hours to establish a partial liver thermal IRI (ischemia reperfusion injury) model. After reperfusion, rats were killed under high-dose anesthesia, blood and liver tissue samples were obtained, and different preservation methods were selected according to the requirements of different indexes.

Administration of Rg1. Rg1 comes from Xiya reagent (purity≥98%, molecular formula: c42h72o14). A total of 32 rats were randomly divided into 4 groups with 8 rats in each group: (1) sham surgery group (sham group);with the exception of blocking liver blood flow, the rest other parameters were the same as in the ischemia/reperfusion (I/R) group; (2) sham + Rg1 group: completed the sham group surgery under the condition of Rg1 injection; I/R injury group, with partial (70%) ischemia of the liver for 45 minutes, and then reperfusion for 6 hours; (4) I/R + Rg1 group: in the case of Rg1 treatment, ischemia for 45 minutes, and then reperfusion for 6 hours. Rg1 was dissolved with 0.9% saline and injected into the tail vein of the rats 1 hour before surgery, with an injection dose of 20 mg/kg. In order to eliminate the influence of liquid, other groups were injected with the same volume of 0.9% saline solution.

Detection of AST and ALT activity. At the end of the experiment, blood samples were collected from the inferior vena cava of the rats, kept at room temperature for 2 h, centrifuged at 3000 rpm for 10 min at 4°C to obtain serum and stored at a temperature of approximately 80°C before evaluation. The activity levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by an automatic serum analyzer (H-7600; Hitachi Ltd, Tokyo, Japan).

TUNEL assay

The liver tissue fixed in 10% formalin buffer solution was taken out and embedded in paraffin (10 μm), dewaxed and dehydrated. According to the apoptosis Kit instructions (*in situ* cell death detection kit, pod, cat. No.: 11684817910, Roche, Switzerland), apoptosis in liver tissue was seen under optical microscope (Olympus Inc, Japan). It was noted that after fluorescence staining, those normal cells were stained blue, and the cells whose blue fluorescence overlapped with green fluorescence were positive cells. A total of 5 high-power fields (X400) were randomly selected for observation and counting in each section, and the AI apoptosis index was calculated: (apoptotic cells/total hepatocytes) × 100%; the average was taken as the result.

Histopathological analysis

After 6 h of reperfusion, the samples were collected and fixed in 10% neutral formalin buffer solution for >24 h, then 4 μm paraffin-embedded sections were cut and stained with hematoxylin-eosin. The slices were randomly selected, and the histopathological changes in the liver were evaluated by an optical microscope. According to the evaluation criteria

proposed by Suzuki, et al[16], the histological changes were scored 0-4 through the 3 aspects of sinus congestion, hepatocyte vacuolation and necrosis (congestion, vacuumization, necrosis). Scores were calculated for each animal liver specimen and then the average was taken as the result. All sections were evaluated by the same pathologist, who was blinded to the experimental specimens.

Isolation of mitochondria from rat liver

According to the published literature, mitochondria were isolated from rat liver tissue by differential centrifugation.[17] In short, within 1 hour after the rats were sacrificed, the liver tissue (1g) was washed with cold PBS, and 10 times the volume of cold mitochondrial separation reagent A (10 ml) was added for homogenization. The homogenate was centrifuged at 600 × g for 5 min at 4°C to collect the supernatant, and then further centrifuged at 11000 × g for 10 minutes under the same conditions. The supernatant was removed, and an appropriate amount of mitochondrial storage solution (0.4 mL) was added to resuspend the mitochondria, and the mitochondria were used within 4 hours. Protein content was determined by bicinchoninic acid (BCA) (Beyotime, China).

Western blot

Total protein was isolated by an RIPA lysis buffer (Cwbiotech, Bejing, China) containing proteinase inhibitors. Protein concentration was evaluated by BCA (Beijing ComWin Biotech Co., Ltd., Beijing, China) reagent. Afterwards, 20-μg protein samples were detached by 10% SDS-PAGE gels, followed by transfer to a PVDF membrane (Millipore). Blocked with 5% fat-free milk for 1 h, the PVDF membranes were probed with the antibodies at 4°C overnight. In this experiment, the primary antibody was diluted with antibody diluent (5% skim milk), LC3B (1:1000), PINK1 (1:1000), Parkin (1:2000), SQSTM1/p62 (1:2000), β-actin (1:3000); dilute the secondary antibody (goat anti-rabbit IgG 1:5000, goat anti-mouse IgG 1:5000). Image J image analysis software was utilized to analyze the gray value (IOD) of the target. Each sample was analyzed 3 times and the average value was use. The gray value of β-actin was used as the internal reference. The ratio of the IOD value of the sample to the β-actin IOD represents the protein relative expression level.

Cell-counting kit 8 assay

Cell viability of BRL-3A cells was tested by cell-counting kit 8 (CCK-8) (Beyotime, Shanghai, China). A 100-μl cell suspension was taken, and 1×10^3 cells/well were placed in 96-well plates. Then 10 μl CCK-8 reagent was added to each well, followed by incubation for 1.5 h at 37°C. Absorbance at 450 nm was measured by a microplate reader (Biotek, Winooski, Vermont USA) and the viability curve was plotted.

Flow cytometry analysis

After 48 h transfection, the cells were resuspended by adding Annexin V- binding buffer to adjust the cell density to 1-5 × 10^6/ml, mixed with 5 μl of Annexin V/FITC and incubated for 5 min in the dark at room temperature. Cells were then washed twice, and 10 μl of propidium iodide (PI) stain was added. Finally, FlowJo™ software (Ashland, Oregon USA) was used to analyze the results.

Selection of time point of the strongest mitochondrial autophagy *in vivo* and *in vitro*

Adult male Sprague-Dawley rats were obtained from the Experimental Animal Center of Kunming Medical University, and the HIRI rat model was established. After 45 min ischemia of the rat liver (70%), ALT and AST activities, LC3-II/LC3-I ratio, PINK1, Parkin and SQSTM1/P62 protein expression levels were established via liver tissue samples and serum samples at the end of reperfusion (0h) and 2h, 6h, 12h and 24h after reperfusion, respectively. The time point of maximum mitochondrial autophagy was evaluated based on liver function and expression level of key mitochondrial autophagy proteins.

The *in vitro* normal rat liver cell line (BRL-3A) was obtained from The Cell Bank (Kunming, China) of the Kunming Institute of Zoology, Chinese Academy of Sciences. The establishment of cell culture and the oxygen glucose deprivation/re-oxygenation (OGD/R) model was conducted. After 6h of deprivation, at the beginning of reoxygenation and 6h, 12h, 24h and 48h after reoxygenation, cell samples were obtained and the LC3-II /LC3-I ratio, PINK1, Parkin and SQSTM1/P62 protein expression levels were determined. The maximum mitochondrial autophagy flux was determined according to the expression level of key mitochondrial autophagy proteins, and the strongest mitochondrial autophagy time point was evaluated.

Specimens tested for mitochondrial transmembrane potential (MMP) *in vivo* and *in vitro*

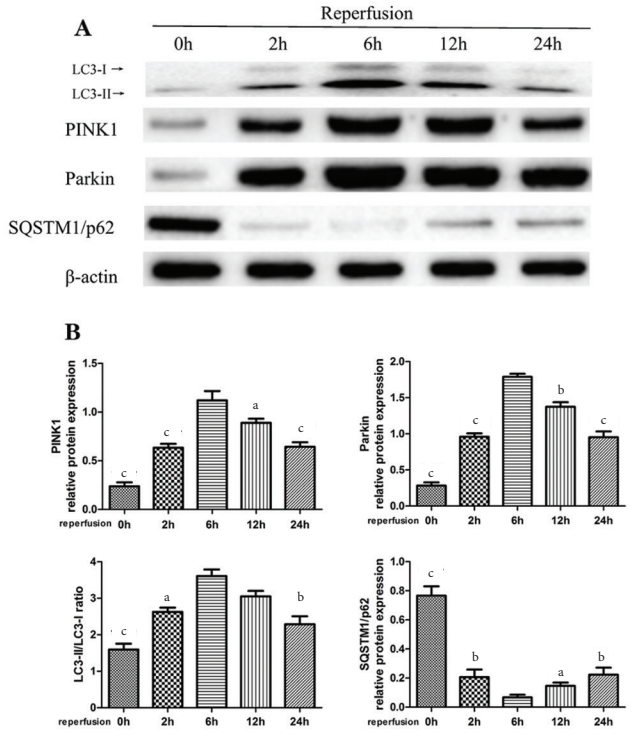
In vivo, the prepared JC-1 staining working solution was diluted 5 times with 1×JC-1 staining buffer. A total protein content of 0.1ml purified mitochondria (10-100 μg) was added with 0.9 ml of the 5-fold diluted JC-1 staining working solution.

In vitro, sufficient BRL-3A cells were collected after 4 hours of reoxygenation and washed twice with PBS. Then the cells were suspended in 500μ L JC-1 staining solution and incubated at 37°C in a 5% CO_2 incubator for 15 min. The cells were collected by centrifugation and resuspended in 500 μl incubation buffer for testing.

When JC-1 accumulates in the membrane, it generates red fluorescence, indicating that the MMP is relatively normal and the state of the cells is normal. In the depolarized mitochondria, JC-1 remains monomer in the cytoplasm and generates green fluorescence, suggesting that the MMP has decreased, and the cell is likely to be in the early stage of apoptosis. Therefore, the collapse of MMP can be mirrored by quantifying the reduction in the ratio of red/green fluorescence intensity.

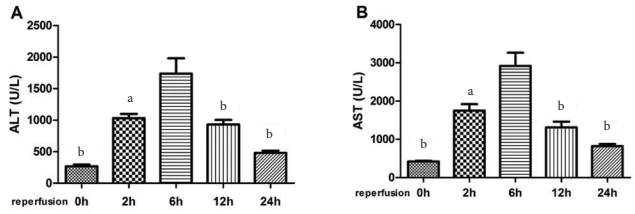
The results were analyzed by flow cytometry *in vitro* and *in vivo*. Finally, the results were observed with a fluorescence microscope or a laser confocal microscope.

Figure 1. LC3-II/LC3-I ratio, SQSTM1/p62, PINK1 and Parkin protein expression levels after different reperfusion times. Data are expressed as mean ± SD (n = 8).



[a]$P < .05$
[b]$P < .01$
[c]$P < .001$ vs the 6 h group

Figure 2. Effects of different reperfusion times on liver function and mitochondrial autophagy-related proteins in rats after 45 min partial liver ischemia. (2A, 2B) Changes in ALT and AST activity after different reperfusion times.



[a]$P < .01$
[b]$P < .001$ vs the 6 h group

Ultrastructure of mitochondria was observed by transmission electron microscopy

In vivo, 1 mm^3 tissue samples were cut from the middle lobe of rat liver and fixed overnight in 2.5% glutaraldehyde, washed with 0.1mol/L PBS 3 times, and then fixed with 1% osmium tetraoxide for 2h. After dehydration with graded ethanol, this was embedded in Epon resin. Ultrathin (65-80 nm) sections were prepared and stained with uranium acetate and lead citrate. A transmission electron microscope (TEM) (JEM-1011; Hitachi, Tokyo, Japan) was used to observe the mitochondria of liver cells.

In vitro, cells were taken from a Petri dish and centrifuged to remove the supernatant. 4% paraformaldehyde was added and the cells were fixed at 4°C for 4h. Then it was fixed in 2.5% glutaraldehyde for 1 h, and the cell clumps were taken out and cut into 1-mm^3 pieces. The remaining steps are the same as described for the liver tissue specimen processing.

Statistical Analysis

The *in vitro* experiment was repeated 5 times, while the *in vivo* experiment was repeated 8 times. SPSS 20.0 software was used for data analysis. One-way ANOVA was used for comparison of differences between the groups. Q test was used for further comparison between pairs, and rank-sum test was used for variance. Data illustrations were made using GraphPad Prism 5.0 software (GraphPad, San Diego, California USA). $P < .05$ was considered statistically significant.

RESULTS

Mitophagy Aggravates Hepatocyte Damage During Liver I/R *In Vivo*

To assess mitochondrial autophagy in the liver, we evaluated the protein expression levels of LC3-II/LC3-I ratio, PINK1, Parkin and SQSTM1/P62. As illustrated in Figure 1, the ratio of LC3-II/LC3-I and the protein expression levels of PINK1 and Parkin began to increase significantly after 2 hours of reperfusion compared with the beginning of reperfusion at the end of the ischemia period, and peaked at 6 hours, but gradually decreased after 12 h ($P < .05$). At 24 h, it was even close to the beginning of reperfusion, which was consistent with the results of liver function tests. On the contrary, the expression of SQSTM1/p62 protein decreased significantly at 2 h after reperfusion and gradually recovered after 12 h ($P < .05$). These results suggested that excessive mitochondrial autophagy after I/R aggravates liver damage, and the damage was the most severe after 6 h reperfusion.

ALT and AST Were Both Decreased After Rg1 Treatment

The effect of Rg1 was analyzed 6 hours after reperfusion. As presented in Figure 2, the use of Rg1 alone did not affect ALT or AST activity compared with the sham group ($P > .05$). ALT and AST activity were significantly increased after I/R ($P < .001$), while Rg1 preconditioning attenuated the activities of ALT and AST after I/R ($P < .01$).

***In* Vivo Rg1 Alleviates Liver Damage After Liver I/R and Maintains MMP Stability**

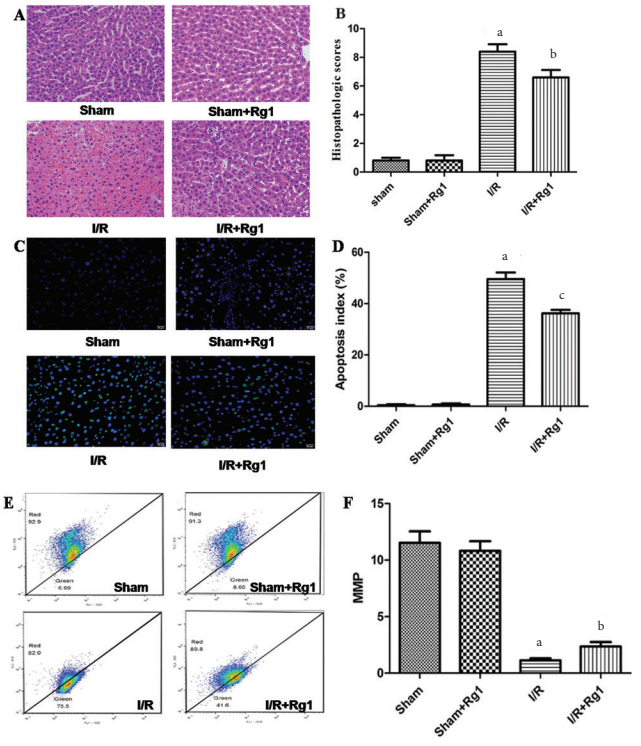
H&E staining and pathological examination indicated that the liver tissue in the sham group showed normal structure, while the liver tissue in the I/R group showed severe necrosis, sinus congestion and liver cell vacuolation. Suzuki's scoring standard was used to assess the hepatocyte damage, indicating that the livers of the rats in the I/R group were seriously damaged. However, Rg1 preconditioning

reduced the damage caused by I/R, and the Suzuki score was decreased ($P < .05$). Apoptosis was also analyzed by TUNEL analysis, as shown in Figure 3A-3D, and apoptotic cells were rare or even absent in the sham group. Compared with the sham group, the number of apoptotic cells and the apoptosis index were significantly increased in the I/R group ($P < .001$). While Rg1 pre-treatment significantly reduced the number of apoptotic cells and the apoptotic index caused by I/R ($P < .001$). As shown in Figure 3E-3F, compared with the sham group, the mitochondrial membrane potential in the I/R group was significantly reduced ($P < .001$). Compared with the I/R group, the use of Rg1 pre-treatment could effectively stabilize the mitochondrial membrane potential after I/R ($P < .05$). These results indicated that Rg1 could effectively alleviate HIRI and stabilize membrane potential.

In Vivo Rg1 Inhibits Mitochondrial Autophagy and Signaling Pathways During Liver I/R

As shown in Figure 4, compared with the sham group, the LC3-II/LC3-I ratio was significantly increased due to I/R ($P < .001$), and the protein level of SQSTM1/P62 protein was significantly decreased ($P < .01$). Rg1 attenuated this change trend. Compared with the I/R group, the ratio of LC3-II/LC3-I was significantly reduced ($P < .05$), and the expression of SQSTM1/p62 protein was significantly increased ($P < .05$). This suggested that Rg1 could inhibit autophagy. In order to confirm the western blotting results, the number of autophagosomes and lysosomes (mitochondrial autophagosomes) containing mitochondria was observed by TEM (Figure 4). Compared with the basal autophagosome levels in the sham group, liver I/R led to an increase in the number of mitochondrial autophagosomes, and Rg1 pre-treatment reduced the number of mitochondrial autophagosomes. To elucidate the mechanism by which Rg1 inhibits mitochondrial autophagy during liver I/R, we investigated the PINK1/Parkin signaling pathway. Compared with the sham group, I/R significantly increased the protein levels of PINK1 and Parkin ($P < .001$), but this increasing trend was significantly suppressed by Rg1 ($P < .05$) (Figure 4). These results suggested that Rg1 might reduce HIRI by inhibiting the activation of the PINK1/Parkin signaling pathway and reducing excessive mitochondrial autophagy after I/R.

Figure 3. The apoptotic cells were rare or even absent in the sham group. (3A), (3B). Histopathological changes (×400). (3C), (3D), (3E). Cell apoptosis detected by TUNEL (×400). Compared with the sham group, the number of apoptotic cells and apoptosis index were significantly increased in the I/R group ($P < .001$), while Rg1 pre-treatment significantly reduced the number of apoptotic cells and the apoptotic index caused by I/R ($P < .001$). (3F) Effect of Rg1 on liver tissue injury and MMP after liver I/R at 6 h after reperfusion. Compared with the sham group, the mitochondrial membrane potential of the I/R group was significantly reduced ($P < .001$).Data are expressed as mean ± SD (n = 8).



[a] $P < .001$vs the sham group
[b] $P < .05$
[c] $P < .001$ vs the I/R group

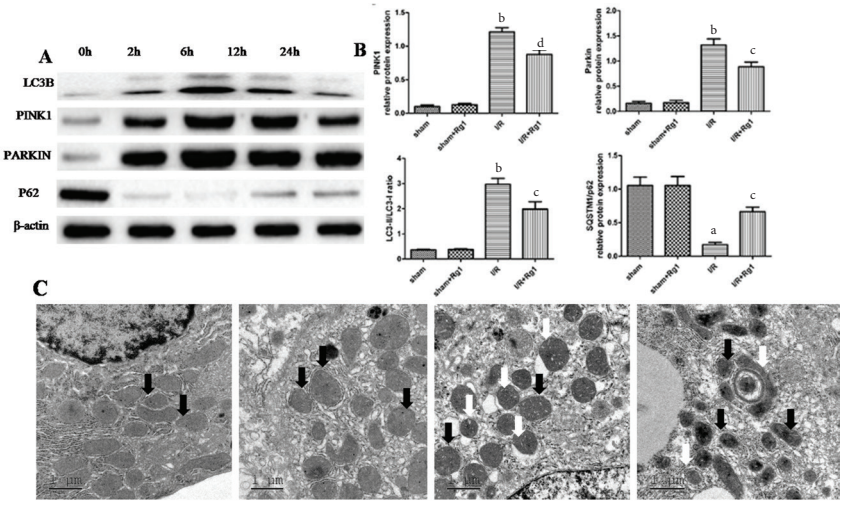
The Time Course of Changes in Mitochondrial Autophagy During OGD/R in BRL-3A Cells *In Vitro*

In vitro, cell samples were collected immediately after OGD/R and obtained again at oxygen-glucose reoxygenation at 6, 12, 24 and 48 h, respectively. Then, the ratio of LC3-II/LC3-I was evaluated, and the protein expression levels of PINK1, Parkin and SQSTM1/p62 were detected. As displayed in Figure 5, the ratio of LC3-II/LC3-I and the protein levels of PINK1 and Parkin were significantly higher at 6 h of reoxygenation and reoxygenation compared with the end of OGD, but gradually decreased at the beginning of 12 h of reoxygenation and glucose deprivation and even approached the end of OGD at 48 h. This indicated that mitochondrial autophagy reached the highest level after 6 h of oxygen-glucose reoxygenation, and the level of mitochondrial autophagy decreased after longer oxygen-glucose reoxygenation times.

Effects of Rg1 on Damage and Mitochondrial Transmembrane Potential of BRL-3A Cells After OGD/R Treatment *In* Vitro

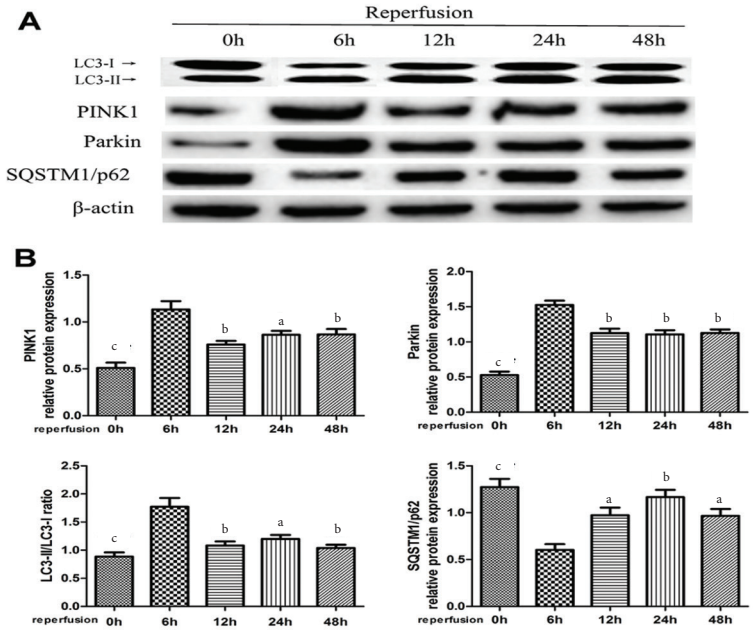
In vitro, the cell viability of BRL-3A cells was significantly decreased after OGD and oxygen-glucose reoxygenation for 6 h; Rg1 could effectively alleviate this situation. Compared with the OGD/R group, cell proliferation viability was significantly increased (Figure 6A). Similar to the results in our previous studies, Rg1 could still reduce apoptosis induced

Figure 4. Influence of Rg1 on the level of mitochondrial autophagy and signal pathway after liver I/R after 6 h of reperfusion. (4A, 4B) The ratio of LC3-II/LC3-I, the protein expression levels of SQSTM1/p62, PINK1 and Parkin in different experimental groups. Compared with the I/R group, the ratio of LC3-II/LC3-I was significantly reduced and the expression of SQSTM1/p62 protein was significantly increased. Moreover, compared with the sham group, I/R significantly increased the protein levels of PINK1 and Parkin, but this trend was significantly suppressed by Rg1. Data are expressed as mean ± SD (n = 8). (4C) The representative electron microscope (EM) images (×15 000) of mitochondrial structures in different experimental groups. Compared with the basal autophagosome levels in the sham group, liver I/R led to an increase in the number of mitochondrial autophagosomes, and Rg1 pre-treatment reduced the number of mitochondrial autophagosomes. The black arrows indicate normal mitochondria; white arrows represent the mitochondrial autophagosome.



[a]$P < .01$;
[b]$P < .001$ vs the sham group
[c]$P < .05$
[d]$P < .01$ vs the I/R group

Figure 5. Effect of different oxygen-glucose reoxygenation times on mitochondrial autophagy-related proteins after 6 h of OGD. 5A,5B) LC3-II/LC3-I ratio, SQSTM1/p62, PINK1 and Parkin protein expression levels after different reperfusion times. The ratio of LC3-II/LC3-I and the protein levels of PINK1 and Parkin were significantly higher at 6 oxygen-glucose reoxygenation compared with the end of OGD, but gradually decreased at the beginning of 12 h of reoxygenation and glucose deprivation and even approached the end of OGD at 48 h. Data are expressed as mean ± SD (n = 5).



[a]$P < .05$
[b]$P < .01$
[c]$P < .001$ vs the 6 h group

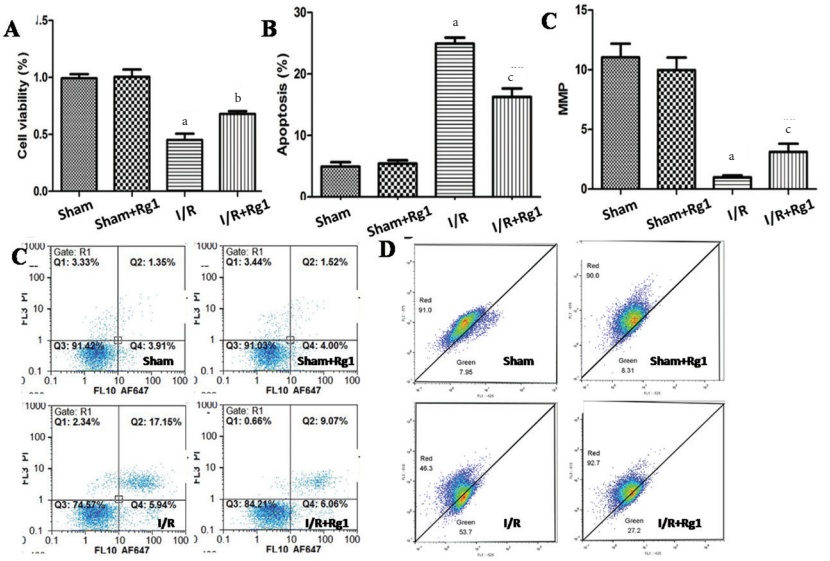
by OGD/R injury and stabilize mitochondrial transmembrane potential 6 h after oxygen-glucose reoxygenation. As shown in Figure 6B, 6C, compared with the control group, cell apoptosis in the OGD/R group was significantly increased ($P < .001$), and reduced by pre-treatment with Rg1 ($P < .001$). By detecting mitochondrial transmembrane potential, we obtained consistent results that Rg1 could effectively stabilize MMP loss caused by OGD/R injury (Figure 6D, 6E). This indicated that Rg1 could effectively increase cell viability, inhibit cell apoptosis and stabilize MMP after OGD/R injury.

Effects of Rg1 on Mitochondrial Autophagy and Signaling Pathways in BRL-3A Cells After OGD/R Injury *In Vitro*

To further determine whether Rg1 alleviates injury through the mitochondrial autophagy pathway in an *in vitro* OGD/R model, we detected the ratio of LC3-II/LC3-I, the protein levels of PINK1, Parkin and the SQSTM1/P62. As illustrated in Figure 7, compared with the control group, the LC3-II/LC3-I ratio, the protein levels of PINK1 and Parkin in the OGD/R group were significantly fortified, suggesting that the autophagy flux was significantly increased. On the other hand, the protein expression level of SQSTM1/p62 obviously decreased. Compared with the OGD/R group, Rg1 pre-treatment significantly suppressed the lc3-II/LC3-I ratio and the protein expression levels of PINK1 and Parkin.

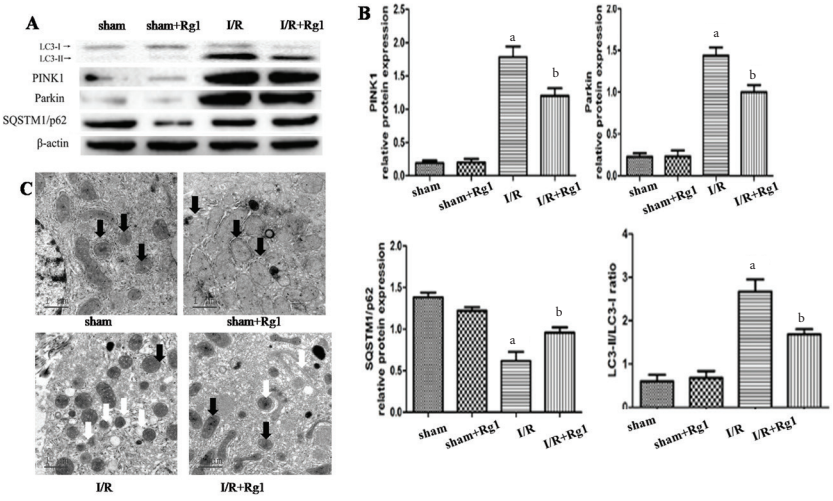
In order to confirm the detection results of mitochondrial autophagy-related proteins, we analyzed the number of mitochondrial autophagosomes in different experimental groups by TEM. Figure 7 shows that the number of mitochondrial autophagosomes increased significantly after OGD/R treatment. Compared with the OGD/R group, the number of mitochondrial autophagosomes decreased after pre-treatment with Rg1. These results indicated that the protective effect of Rg1 *in vitro* may be through the inhibition of PINK1/Parkin signaling pathway mediating the mitochondrial autophagy.

Figure 6. Effect of Rg1 on BRL-3A cell damage and MMP induced by OGD/R after 6 hours of oxygen-glucose reoxygenation. (6A) Cell proliferation activity of each experimental group; (6B, 6C) Flow cytometry analysis of BRL-3A cell apoptosis; (6D, 6E) Membrane potential levels in different experimental groups. Data are expressed as mean ± SD (n = 5).



[a]$P < .001$ vs the sham group
[b]$P < .05$
[c]$P < .01$ vs I/R group

Figure 7. Effect of Rg1 on the level of mitochondrial autophagy and signal pathways in BRL-3A cells induced by OGD/R after 6 h of oxygen-glucose reoxygenation (7A, 7B) The ratio of LC3-II/LC3-I, SQSTM1/p62, PINK1 and Parkin protein expression levels in different experimental groups. The data are expressed as mean ± SD (n = 5). (7C) The representative EM images (×15000) of mitochondrial structures in different experimental groups. Black arrows indicate normal mitochondria; white arrows indicate mitochondrial autophagosomes.



[a]$P < .001$ vs the sham group
[b]$P < .05$ vs the I/R group

DISCUSSION

In the ischemic phase, the damaged mitochondria are eliminated by hypoxia-induced mitochondrial autophagy, which prevents the release of pro-apoptotic factors such as ROS and cytochrome C from damaged mitochondria, reduces cell apoptosis and benefits cells survival.[18] During reperfusion, a large amount of ROS floods into the blood. Excessive ROS directly damages the mitochondria, leading to increased oxidative stress, and the opening of MPTP, resulting in a decrease in MMP, mitochondrial swelling and the activation of mitophagy mediated by PINKl/Parkin pathway.[19] Cathepsin B released after excessive activation of mitochondrial autophagy can induce mitochondrial-mediated apoptosis.[20] Zhou, et al.[21] found that melatonin effectively inhibited platelet activation and function by blocking mitophagy required by FUNDC1 and restoring PPARγ content in platelets, thus alleviating cardiac ischemia-reperfusion injury. Jin, et al.[22] found that dual-specific protein phosphatase 1 (DUSP1) repressed mitochondrial division required for mitochondrial fission factor (Mff) through the JNK pathway and Bnip3-related mitophagy reduced cardiac ischemia-reperfusion injury.

In general, insufficient or excessive mitochondrial autophagy is harmful, and the prevention of excessive mitophagy has a protective effect on ischemia-reperfusion injury within a certain period of time.

The PINKl/Parkin pathway is specific pathway to mammalian cell clearance of damaged mitochondria.[23] PINK1 (PTEN-induced putative kinase protein 1, PINK1) and Parkin proteins are encoded by the PARK6 and PARK2 genes, respectively.[24] PINK1 is a serine/threonine protein kinase that is primarily located in the outer membrane of the mitochondria.[25] In normal mitochondria, PINK1 first enters the mitochondrial membrane gap by interacting with the outer membrane translocation enzyme complex, and then interacts with the inner membrane translocation enzyme complex.[26] It is rapidly decomposed by presenilin-associated rhomboid-like protein (PARL) on the inner membrane, thus maintaining a low level of PINK1 and inhibiting mitophagy in normal mitochondria.[27] When the mitochondrial structure is damaged or dysfunctional, its transmembrane potential decreases and depolarizes, and the transport of PINK1 protein is blocked. Thus, PINK1 accumulates in large numbers on the mitochondrial outer membrane and collects Parkin protein from the cytoplasm to the mitochondrial membrane.[28]

Microtubule-associated protein light chain 3 (LC3) is an important marker on the membrane of autophagolysosomes, and LC3 exists in cytoplasm in 2 forms of LC3-I and LC3-II.[29,30] Conversion from LC3-I (free form) to LC3-II (phosphatidylethanolamine conjugated form) is the main step of autophagosome formation after ischemia-reperfusion, which is located in the autophagosomes, and the number of LC3-II is closely related to the number of autophagosomes.[31] The LC3 II/I ratio detected by western blotting can roughly reflect the level of autophagy.[32, 33]

Hypoxia increases ROS in mitochondria, which promotes the conversion of LC3-I to LC3-II and induces mitophagy.[34] LC3 II/I ratio is a reliable biomarker that can immediately reflect changes in autophagy.[35] P62 (SQSTM1/Sequestosome 1) is an adaptor protein of the ubiquitin system and an important receptor for mitochondrial autophagy, which plays a key role in the fusion of autophagosomes and lysosomes and mitochondrial aggregation.[36,37] In the PINKl/Parkin pathway of mitochondrial autophagy, P62 was recruited to damaged mitochondria by CHDH (choline dehydrogenase), and the two interact to stimulate mitophagy.[38]

In our study, we first conducted an *in vivo* and *in vitro* study. As per the previous study, we used 45 minutes of ischemia and 6 hours of OGD *in vivo* and *in vitro*, respectively, then performed various time reperfusions and post glucose oxygen at all time points. Some known key proteins, including LC3I, mitochondrial autophagy LC3II, P62, PINK1 and Parkin were detected and the liver function was also checked through animal experiments *in vitro* including. Our study found that in the *in vivo* experiment, mitochondrial autophagy reached its peak at 6 h after reperfusion, and mitophagy segments, the ratio of LC3-II/LC3-I and the protein expression levels of PINK1 and Parkin increased immediately after reoxygenation and resuscitation, reached a peak at 6 h, then began to decline and maintained at 48 h. The expression level of P62 protein, which was inversely proportional to autophagy flux, was the opposite. This indicated that the mitophagy level reached the highest peak 6 hours after oxygen-glucose reoxygenation.

We established that 6 h was selected as the duration of reperfusion and oxygen-glucose reoxygenation for Rg1 drug preconditioning in subsequent *in vivo* and *in vitro* studies.

In addition, our study outcomes revealed that compared with the sham group, the ratio of LC3-II/LC3-I and the protein levels of PINK1 and Parkin in the liver I/R group were significantly increased, while the P62 protein level was decreased. After pretreatment with Rg1, the ratio of LC3-II/LC3-I and protein levels of PINK1 and Parkin in liver tissue after I/R injury could be significantly reduced, and the protein level of P62 increased. At the same time, we found that Rg1 pretreatment could significantly reduce the elevation of ALT and AST caused by I/R injury, and the liver cell apoptosis index, histopathological injury and score performance were consistent with these indexes, suggesting that Rg1 pretreatment could alleviate the injury and have a protective effect. MMP was significantly reduced in the liver I/R group, which was inhibited by Rg1 preconditioning. TEM revealed that Rg1 reduced the number of mitochondrial autophagosomes, which was consistent with the western blotting results of mitochondrial autophagy-related proteins. These results indicated that excessive mitophagy aggravates liver damage during the reperfusion phase. Rg1 can alleviate liver injury after ischemia-reperfusion by reducing the level of mitochondrial autophagy.

Consistent with the *in vivo* studies, in *in vitro* experiments, after 6 h of deoxyglucose followed by reoxygenation of sugar

for 6 h, the ratio of LC3-II/LC3-I and the protein levels of PINK1 and Parkin were increased, indicating an increase in mitochondrial autophagy flux. Compared with the OGD/R group, Rg1 pre-treatment significantly reduced the expression level of mitochondrial autophagy-related proteins and increased the protein level of P62. This was contrary to the mitochondrial autophagy flux, indicating a significant decrease in the mitochondrial autophagy level.

The same results were obtained in the detection of cell proliferation activity, MMP and apoptosis. OGD/R led to a decrease in cell proliferation activity and MMP, and a significant increase in cell apoptosis, which were reversed by Rg1 pre-treatment. These findings suggested that Rg1 could protect the liver from IRI, and the potential mechanism might be that Rg1 prevents the opening of MPTP and the activation of the PINK1/Parkin signaling pathway, inhibits mitophagy, and ultimately alleviates HIRI.

Some studies[39] found that by knocking out the Ppif gene (encoding gene of CypD) in mice, MPTP opening can be effectively reduced, demonstrating that CypD is an important component of MPTP or an important factor in regulating MPTP opening. In rat cardiomyocytes without the CypD gene, starvation, the inducing factor of autophagy, could not induce autophagy, while cardiomyocytes under normal nutrition (non-starvation) showed excessive autophagy via overexpression of CypD. It is believed that CypD and MPT (mitochondrial membrane permeability) play important physiological roles in regulating autophagy and maintaining cell homeostasis.[40] CypD inhibitors could effectively reduce autophagy and thus reduce ischemia-reperfusion injury.[41] Studies on the aging process of *Podospora anserina* have found that the overexpression of CypD is closely related to the increase of autophagy and mitochondrial autophagy. CypD is a key factor in regulating the beneficial or harmful effects of autophagy induction, and the overexpression of CypD plays a harmful role under the influence of excessive external injury stimulation.[42] These results indicated that CypD plays an important role in regulating mitochondrial autophagy. Meanwhile, according to our previous research results, Rg1 could inhibit the expression of CypD.

CONCLUSION

In conclusion, Rg1 can alleviate hepatic ischemia-reperfusion injury. Part of its mechanism of action might be via inhibiting the protein expression of CypD, preventing the opening of MPTP, further inhibiting the activation of the PINKl/Parkin signaling pathway, thereby alleviating mitophagy during the reperfusion period, and finally exerting its protective effect. The level of mitophagy was reduced during the reperfusion phase of HIRI and alleviating ischemia-reperfusion injury has become a new strategy to improve liver function in patients after liver hepatectomy and transplantation.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This experimental protocol was approved by the Animal Ethics Committee of Kunming Medical University, and the relevant procedures have been implemented in accordance with the requirements of the National Institutes of Health on the "Guidelines for the Feeding and Use of Laboratory Animals."

CONFLICT OF INTEREST

None.

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AUTHOR CONTRIBUTIONS

Jie Lin and Zhe Qing contributed equally to the work

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