Acteoside attenuates hydrogen peroxide-induced injury of retinal ganglion cells via the CASC2/miR-155/mTOR axis

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Background: Loss of retinal ganglion cells (RGCs), which eventually leads to optic nerve atrophy and vision loss, is the main cause of glaucoma and traumatic optic neuropathy. Acteoside is the effective component of Yunnan Kudingcha, which has been reported to exert neuroprotective effects and protects RGCs from injury. However, the underlying mechanisms of acteoside in RGC injury remain largely elusive.

Methods: Human RGCs was treated with hydrogen peroxide (H_2O_2). The expression of miR-155 and lncRNA CASC2 in RGC-5 cells was measured by RT-qPCR. The viability of RGCs was determined by the MTT assay. Flow cytometry and TUNEL staining were used to detect cell apoptosis. The malondialdehyde (MDA) content and superoxide dismutase (SOD) activity were determined using ELISA kits. The mTOR and autophagic proteins were measured by western blot.

Results: We identified the expression of miR-155 was upregulated in H_2O_2-treated RGCs, and enhanced miR-155 promoted RGC autophagy and apoptosis. Acteoside administration reduced miR-155 expression and abolished miR-155-mediated RGC injury. The expression of CASC2 was decreased in H_2O_2-treated RGCs. Acteoside administration could increase CASC2 expression and CASC2 overexpression reverses the effect of miR-155 overexpression on acteoside treatment-RGCs. Mechanistically, we discovered that highly expressed miR-155 promoted RGC autophagy and apoptosis via the mTOR pathway. In addition, acteoside attenuated RGC autophagy and apoptosis via the miR-155/mTOR axis. Together, these results identify a mechanism by which acteoside attenuates H_2O_2-induced RGC apoptosis and autophagy via the CASC2/miR-155/mTOR axis.

Conclusions: Acteoside protects RGC-5 cells against H_2O_2-induced cell injury via the CASC2/miR-155/mTOR axis. These results provide new insights for early medical interventions in patients with glaucoma.

Keywords: Retinal ganglion cells; acteoside; mTOR; apoptosis; autophagy

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Introduction

Glaucoma, a neurodegenerative eye disease that leads to blindness, is attributed to retinal ganglion cell (RGC) loss (1). Emerging studies have demonstrated that Chinese traditional medicines such as Lycium barbarum, Ginkgo biloba (2), and tetramethylpyrazine (3) play critical roles in treating glaucoma. Acteoside, as an effective component of Yunnan Kudingcha, has been reported to attenuate the apoptosis of RGCs and glaucoma-induced optic atrophy (4). However, the underlying mechanism of acteoside in RGC apoptosis is still unclear.

MicroRNAs (miRNAs) are a class of conserved small

non-coding RNAs that function as key players in glaucoma development, diagnosis, and treatment (5-7). In addition, the role of miRNAs in the activity regulation of RGCs has been well characterized. For example, miR-93-5p modulates NMDA-induced RGC autophagy via the AKT/mTOR pathway in glaucoma (8). Furthermore, miR-141-3p inhibits RGC apoptosis in glaucoma mice (9), and inhibition of miR-149 could suppress RGC apoptosis in glaucoma mice (10). A study also indicated that miR-155 is induced in neurodegenerative diseases including glaucoma (11). Moreover, miR-155 has been reported to inhibit retinal function (12). These studies suggest that miR-155 may be involved in glaucoma development.

It is recognized that the mTOR signaling pathway contributes to the activation of RGCs (13,14). In addition, mounting evidence demonstrates that miR-155 can modulate pathological states including cardiomyopathy (15), cancer (16), and alcoholic liver disease (17) via targeting the mTOR signaling pathway. Yet, the function of the miR-155/mTOR axis in glaucoma is unknown.

In the present study, we established hydrogen peroxide (H_2O_2)-induced RGC injury and determined the role of miR-155 in acteoside-mediated glaucoma treatment. miR-151 was induced in H_2O_2-treated RGCs. Inhibition of miR-155 alleviated H_2O_2-induced RGC apoptosis and autophagy. Acteoside not only attenuated apoptosis and autophagy of RGCs, but also suppressed miR-155 expression. Moreover, we confirmed that acteoside protected RGCs from injury via miR-155. We further demonstrated that miR-155 could directly target mTOR, and the mTOR signaling pathway was responsible for acteoside/miR-155-mediated RGC apoptosis and autophagy. Thus, our study revealed a novel mechanism by which acteoside protected against RGC injury via the miR-155/mTOR axis.

We present the following article in accordance with the MDAR reporting checklist (available at https://dx.doi.org/10.21037/atm-21-5630).

Methods

Cell culture and treatment

RGCs (RGC-5 cell line) were obtained from American Type Culture Collection (ATCC, YB-ATCC-7491) and maintained in Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin. RGCs were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37 ℃. To establish oxidative stress injury in RGCs, the RGCs were treated with 200 μM H_2O_2 as previously described (18). Acteoside (purity ≥98%, Sigma Chemical Co., MO, USA) was used to protect RGC-5 cells against H_2O_2-induced cell injury with 3 mg/mL concentration as our previous reported (19).

Cell transfection

The miR-155 inhibitor, miR-155 mimic, CASC2 overexpression vector (oe-CASC2) and corresponding control (NC inhibitor, NC mimic and oe-NC) were synthesized and purified by RiboBio. RGCs were transfected with the oe-CASC2 (2 μg/μL), oe-NC (2 μg/μL), miR-155 inhibitor (50 nM), NC inhibitor (50 nM), miR-155 mimic (100 nM) and NC mimic (100 nM) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction.

Cell viability measurement

The viability of RGCs was measured by the MTT assay using the MTT Cell Proliferation and Cytotoxicity Assay Kit (C0009S, Beyotime). Briefly, RGC-5 cells were seeded in a 96-well plate (2×10^5/well) and treated with H_2O_2 for 24 h. Subsequently, 10 μL MTT solution was added to RGCs and incubated at 37 ℃ for 4 h. The absorbance at 570 nm was measured.

Cell apoptosis measurement

The indicated RGCs were treated with H_2O_2 for 24 h and subjected to flow cytometry analysis using the Annexin V-FITC Apoptosis Detection Kit (C1062S, Beyotime) for cell apoptosis detection. Briefly, RGCs were harvested and mixed with 5 μL Annexin V-FITC and 10 μL propidium iodide (PI). After incubation for 30 min, RGCs were analyzed with flow cytometry.

Western blot assay

Total protein was isolated from RGCs using RIPA Lysis Buffer (P0013B, Beyotime) and the concentration of protein was measured by the BCA Protein Assay Kit (P0012, Beyotime). Equal amounts of proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred to PVDF membranes (ISEQ00010, Millipore). After blocking with non-fat milk, the membranes were incubated with the

following primary and secondary antibodies: mTOR (ab2732, Abcam, 1:1,000), LC3 (ab2775, Cell Signaling Technology, 1:2,000), and p62 (ab91526, Abcam, 1:2,000). GAPDH (ab9485, Abcam, 1:5,000) served as the loading control.

RNA isolation and quantitative real-time PCR (RT-qPCR) assay

Total RNA was extracted from RGCs using Trizol reagent (15596026, Invitrogen), and cDNA was synthesized using the miRNA 1st Strand cDNA Synthesis Kit (MR101-01, Vazyme). RT-qPCR was then carried out with SYBR Green Premix (DRR041A, Takara) and analyzed with the $2^{-\Delta\Delta Ct}$ method (20). The cycling conditions were as follows: 50 ℃ for 2 min, 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min. The primers used in this study were as follows: miR-155, forward: 5'-GGAGGTTAATGCTAATCGTGATAG-3'; reverse: 5'-GTGCAGGGTCCGAGGT-3'. U6, forward: 5'-TCGGCAGCACATATACTAA-3'; reverse: 5'-CGCTTCACGAATTTGCGTGT-3'. U6 was used as the endogenous control.

Malondialdehyde (MDA) content and superoxide dismutase (SOD) activity assays

The MDA content and SOD activity in RGCs were determined using commercially available ELISA kits (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. Absorbance was determined using a microplate spectrophotometer (BioTeke).

TUNEL staining

RGCs were treated or transfected and then cultured 48 h. After rising twice with PBS, the cells were fixed with 4% paraformaldehyde for 15 min at 37 ℃. 1×10^4 cells were prepared in 96-well plates and then subjected to Click-iTTM Plus TUNEL Assay Kit (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instructions. Following DAPI staining, the apoptotic cells were measured by using Nikon Eclipse 80i microscope (Nikon Corporation).

Statistical analysis

All data are presented as the mean ± SE from at least 3 biological replicates, and the differences between any 2 groups or multiple groups were compared using unpaired *t*-tests.

Results

MiR-155 is induced upon H_2O_2 treatment of RGC-5 cells

In order to determine the role of miR-155 in RGC-5 cell injury. We established RGCs oxidative stress injury model, as previous reported that RGC-5 cells were treated with different concentrations of H_2O_2 for 12 h (21), 16 h (3) and 24 h (18), which 200 μM H_2O_2 significantly reduced RGC-5 cells viability at 12, 16 and 24 h. Thus, we selected 200 μM H_2O_2 treated RGC-5 cells. As shown in *Figure 1A*, H_2O_2 treatment inhibited RGC-5 cell viability. The content of MDA, an oxidative stress marker, was elevated, while the activity of SOD, an anti-oxidative enzyme, was reduced, in the H_2O_2 treatment (*Figure 1B,1C*). H_2O_2 treatment also promoted RGC-5 cell autophagy (*Figure 1D*) and apoptosis (*Figure 1E,1F*). In addition, miR-155 expression was induced in H_2O_2-treated RGC-5 cells (*Figure 1G*).

MiR-155 inhibition alleviates H_2O_2-induced RGC-5 cell injury

To further identify the function of miR-155 in H_2O_2-induced RGC-5 cell injury, we transfected cells with an miR-155 inhibitor or NC inhibitor as the control (*Figure 2A*). After H_2O_2 treatment, RGC-5 cell viability, oxidative stress, apoptosis, and autophagy were determined. As shown in *Figure 2B-2G*, miR-155 inhibition protected RGC-5 cells from H_2O_2-induced injury.

Acteoside attenuates H_2O_2-induced RGC-5 cell injury via modulating miR-155

Our previous study indicated that acteoside was involved in glaucoma treatment and could inhibit autophagic apoptosis of RGC-5 cells (4). We next explored the relationship between acteoside and miR-155. Acteoside administration decreased miR-155 expression in H_2O_2-treated RGC-5 cells (*Figure 3A*). We also overexpressed miR-155 via miR-155 mimic transfection, and NC mimic was the corresponding control (*Figure 3B*). miR-155 overexpression abolished the protective effect of acteoside on cell viability, oxidative stress, apoptosis, and autophagy (*Figure 3C-3H*). These findings demonstrated that acteoside attenuates H_2O_2-induced RGC-5 cell oxidative stress and injury via miR-155.

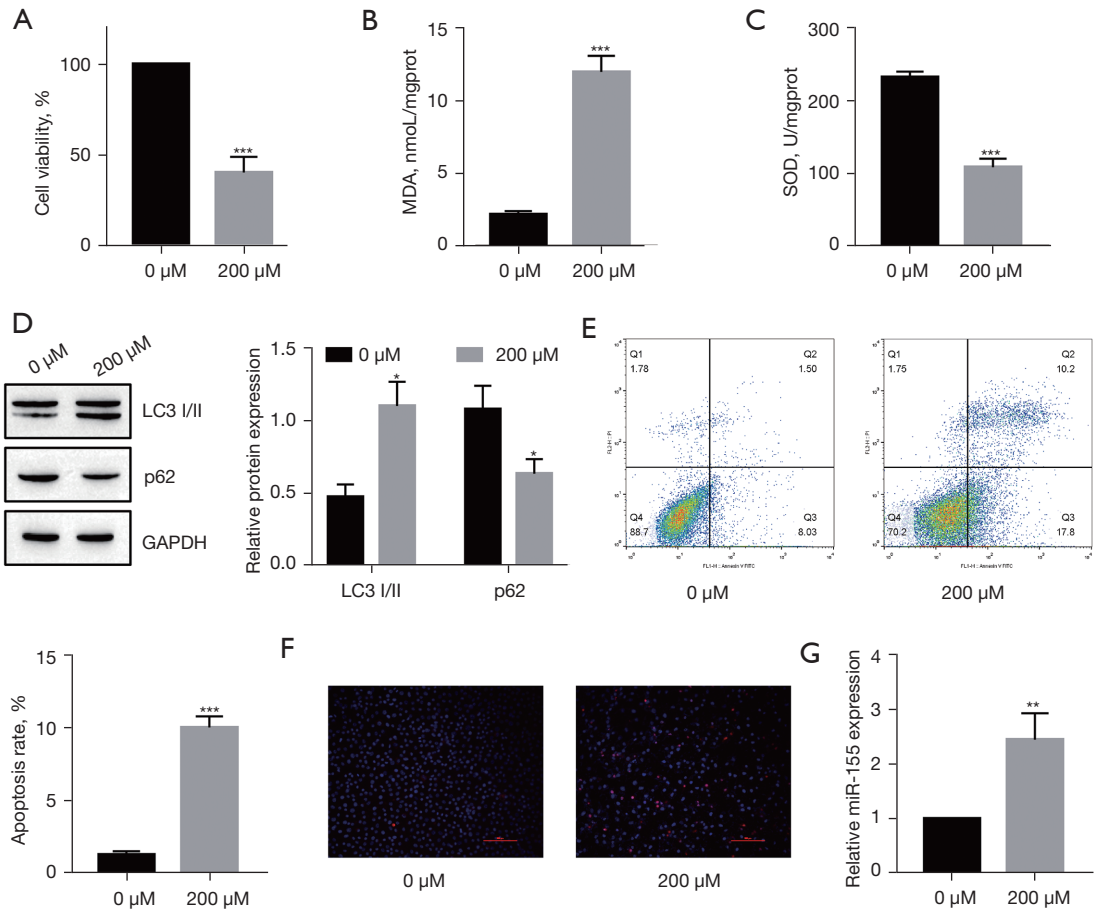

Figure 1 MiR-155 is induced upon H_2O_2 treatment of RGC-5 cells. (A) Cell viability of 0 μM- or 200 μM-treated RGC-5 cells was measured by the MTT assay; (B) MDA content; (C) SOD activity; (D) expression of autophagic proteins in 0 μM- or 200 μM-treated RGC-5 cells was measured by western blot; (E) apoptosis of 0 μM- and 200 μM-treated RGC-5 cells was measured by flow cytometry; (F) TUNEL staining of RGC-5 cells (scale bar =200 μm); (G) expression of miR-155 in 0 μM- and 200 μM-treated RGC-5 cells was measured by RT-qPCR. *P<0.05; **P<0.01; ***P<0.001. MDA, malondialdehyde; SOD, superoxide dismutase.

LncRNA CASC2 targets miR-155 in RGC-5 cells

The bioinformatics software starBase (http://starbase.sysu.edu.cn/) was used to identify potential targets of miR-155, and lncRNA CASC2 was selected due to its role in acteoside attenuates H2O2-induced RGC-5 cell injury. The binding sites between miR-155 and WT or MUT CASC2 3'-UTR are shown in *Figure 4A*. The expression of CASC2 in H_2O_2-induced RGC-5 cells was subsequently analyzed, its expression had a significantly downregulated (*Figure 4B*). To confirm whether miR-155 could bind to CASC2 3'-UTR, a luciferase reporter vector containing the WT or MUT 3'-UTR of CASC2 was constructed. Overexpression of miR-155 markedly inhibited the luciferase activity of the WT reporter vector but did not affect the luciferase activity of the mutant reporter vector in RGC-5 cells (*Figure 4C*). Furthermore, overexpression of miR-155 also significantly suppressed CASC2 expression (*Figure 4D*). These results suggested that miR-155 directly targeted the *CASC2* gene.

Acteoside attenuates H_2O_2-induced RGC-5 cell injury via modulating CASC2/miR-155 axis

We also overexpressed CASC2 via CASC2 overexpression vector oe-CASC2 (*Figure 5A*). The results indicated that acteoside attenuates H_2O_2-induced RGC-5 cell oxidative stress and injury, which was reversed with overexpression of miR-155, whereas the overexpression of CASC2 repressed the effect of acteoside on the cell viability, oxidative stress, apoptosis, and autophagy (*Figure 5B-5G*). These findings

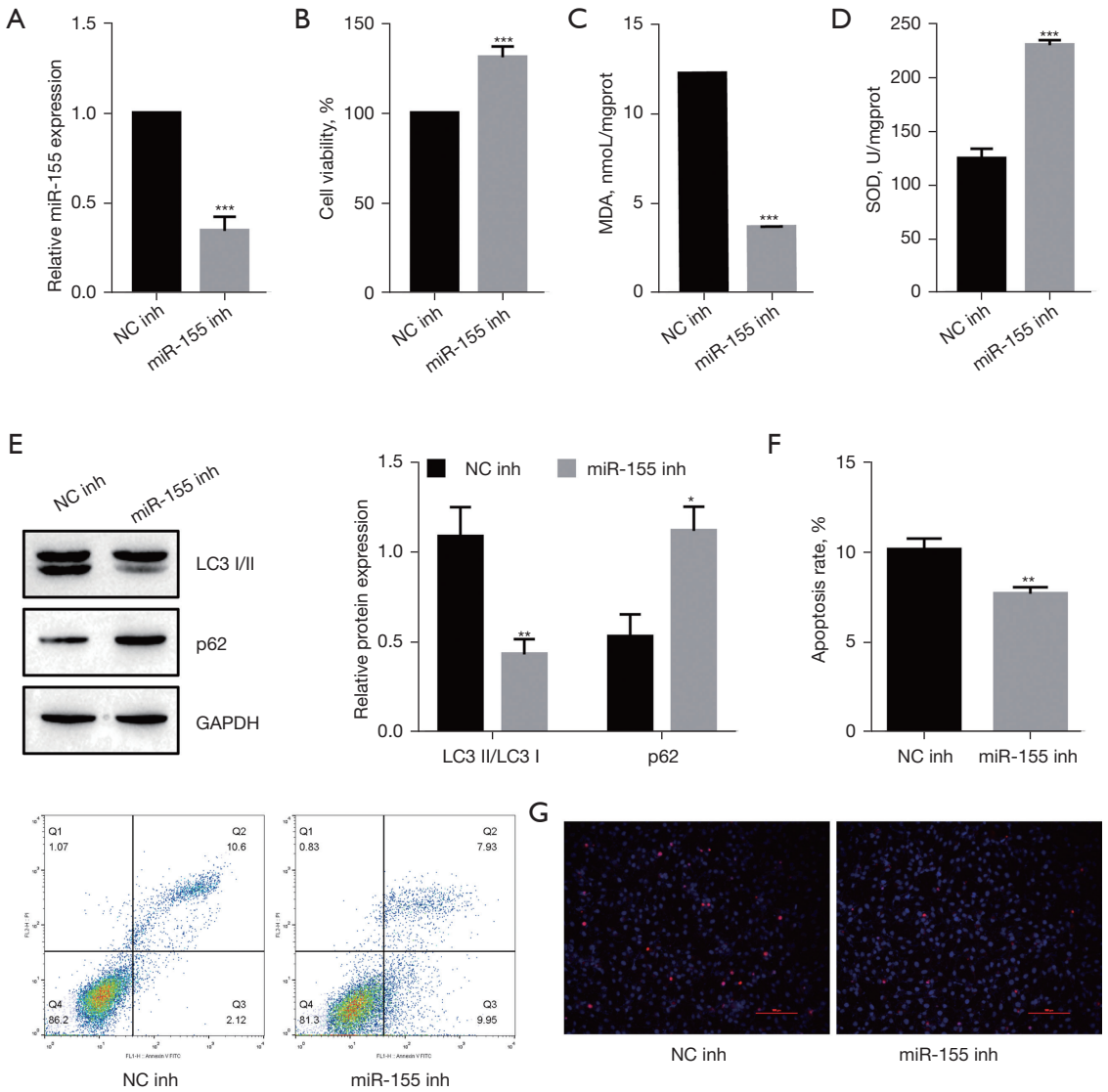

Figure 2 MiR-155 inhibition alleviates H_2O_2-induced RGC-5 cell injury. (A) Expression of miR-155was measured by RT-qPCR; (B) the cell viability was measured by the MTT assay; (C) MDA content was measured by ELISA; (D) SOD activity was measured by ELISA; (E) the expression of autophagic proteins was measured western blot; (F) apoptosis of RGC-5 cells was measured by flow cytometry; (G) TUNEL staining of RGC-5 cells. Scale bar =200 μm. *P<0.05; **P<0.01; ***P<0.001. RT-qPCR, quantitative real-time PCR; MDA, malondialdehyde; SOD, superoxide dismutase.

demonstrated that acteoside attenuates H_2O_2-induced RGC-5 cell oxidative stress and injury via CASC2/miR-155 axis.

Acteoside protects RGC-5 cells against H_2O_2-induced cell injury via the miR-155/mTOR axis

Given that miR-155 functions via mTOR signaling (22) and mTOR was involved in RGC damage and glaucoma development (8), we next determined the role of mTOR in the protective effect of acteoside/miR-155 on RGC-5 cells. It was found that mTOR expression was decreased in H_2O_2-treated RGC-5 cells (*Figure 6A*), and miR-155 inhibition could induce mTOR expression (*Figure 6B*). In addition, acteoside administration promoted mTOR expression while miR-155 overexpression abolished the promotive effect of acteoside on mTOR expression (*Figure 6C*). We further employed an mTOR agonist and inhibitor, HY-B0795 and rapamycin, to determine the relationship between acteoside

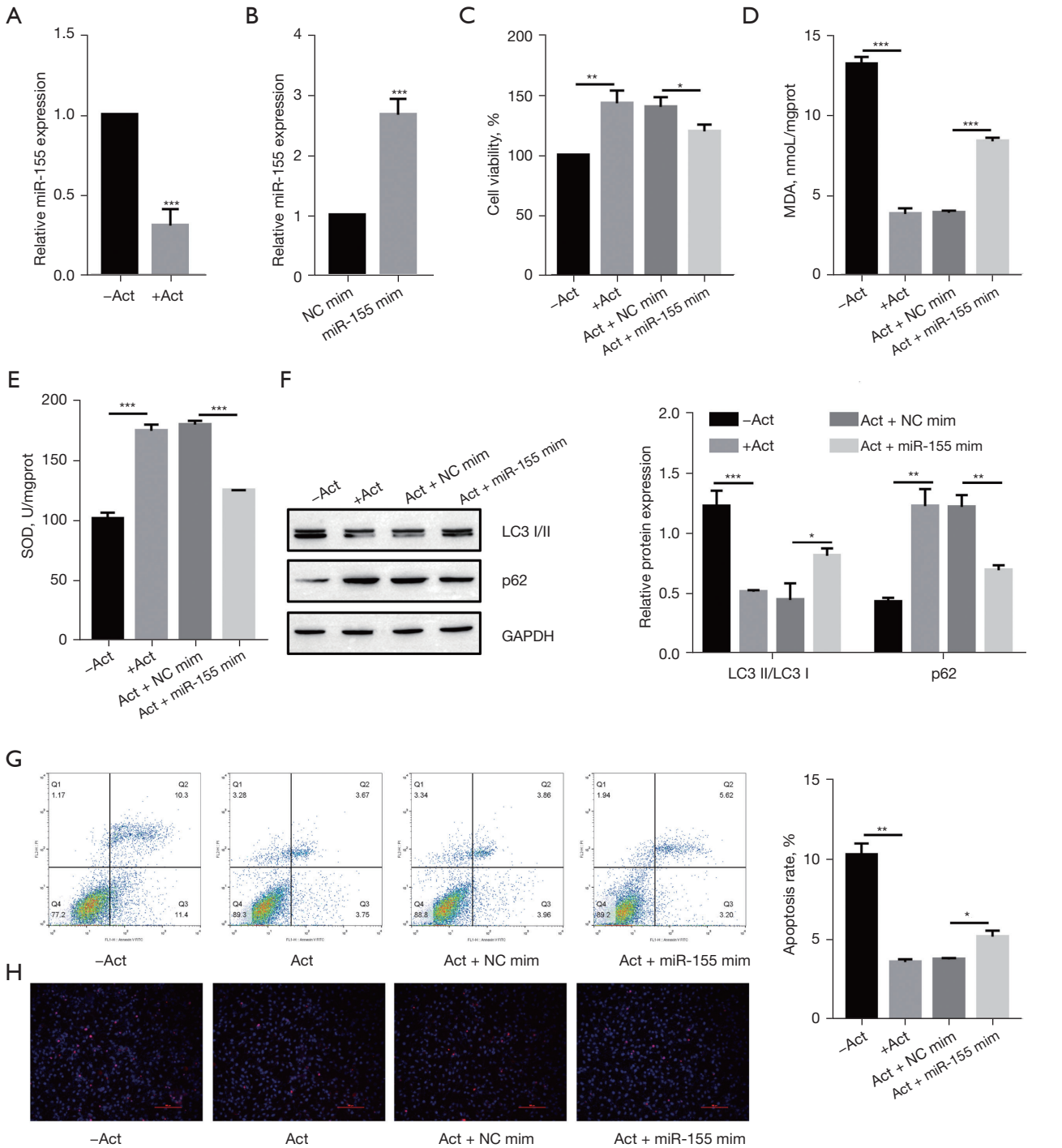

Figure 3 Acteoside attenuates H_2O_2-induced RGC-5 cell injury via modulating miR-155. (A) Expression of miR-155 in acteoside-treated RGC-5 cells was measured by RT-qPCR; (B) expression of miR-155 in RGC-5 cells was measured by RT-qPCR. (C) the viability of RGC-5 cells was measured by the MTT assay; (D) MDA content was measured by ELISA; (E) SOD activity was measured by ELISA; (F) the expression of autophagic proteins in RGC-5 cells was measured by western blot; (G) apoptosis of RGC-5 cells was measured by flow cytometry; (H) TUNEL staining of RGC-5 cells. Scale bar =200 μm. *P<0.05; **P<0.01; ***P<0.001. RT-qPCR, quantitative real-time PCR; MDA, malondialdehyde; SOD, superoxide dismutase.

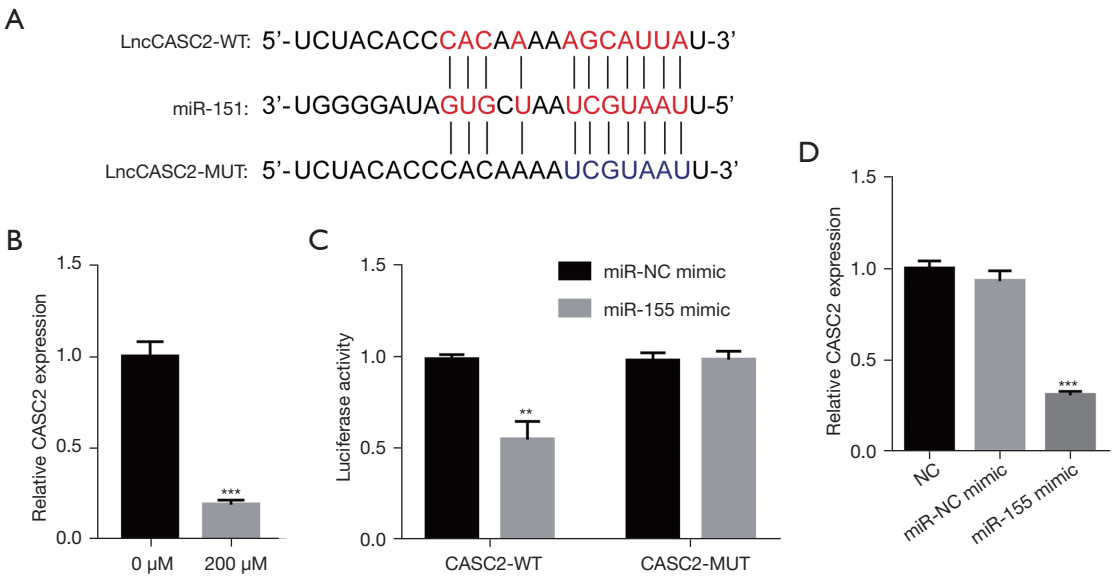

Figure 4 CASC2 targets miR-155 in RGC-5 cells. (A) Schematic representation of the binding site of miR-155 in CASC2-WT and in CASC2-MUT; (B) RT-qPCR was used to determine the expression of CASC2; (C) dual-luciferase reporter assays were used to demonstrate that CASC2 can target miR-155; (D) RT-qPCR was used to determine the expression of CASC2. **P<0.01; ***P<0.001. RT-qPCR, quantitative real-time PCR.

and mTOR. As shown in *Figure 6D-6I*, HY-B0795 and rapamycin promoted and reduced the protective effect of acteoside on attenuating RGC injury, respectively. Thus, these findings suggest that acteoside protects RGC-5 cells against H_2O_2-induced cell injury via miR-155/mTOR.

Discussion

Glaucoma is the second leading cause of blindness worldwide, which is characterized by optic nerve atrophy and visual field defects. It is believed that RGC injury is the main cause of glaucoma (23,24). It is reported that miRNAs could be promising biomarkers for glaucoma, and aberrant expression of miRNAs is involved in glaucoma development and treatment (6,25). For example, miR-93-5p is downregulated in NMDA-induced RGCs and a glaucoma rat model, and miR-93-5p overexpression suppresses RGC autophagy in NMDA-induced glaucoma (8). miR-200a is decreased in glaucoma and plays a protective role in improving glaucoma-induced optical nerve injury (26). Furthermore, miR-149 downregulation suppresses RGC apoptosis in glaucoma mice (10), and a previous study showed that miR-155 is upregulated in glaucoma (11). Notably, the expression of miR-155 is upregulated in the H_2O_2-induced cells, including vascular smooth muscle cells (27), human umbilical vein endothelial cells (28), and human embryonic kidney 293 cells (29), and then silencing of miR-155 can attenuate H_2O_2-induced cell injury (27). Here, we established RGC injury via 200 μM H_2O_2 administration and found that miR-155 was induced in H_2O_2-treated RGCs. Thus, the miR-155 was investigated in subsequent assays. Moreover, inhibition of miR-155 promoted RGC viability while repressing RGC apoptosis and autophagy. Emerging studies have paid much attention to the therapeutic effects of Chinese traditional medicine on glaucoma (2,30). Our previous work indicates that acteoside, which is the effective component of Yunnan Kudingcha, plays a critical role in glaucoma via inhibiting autophagy-induced apoptosis in RGCs (4). Here, we found that acteoside inhibits miR-155 expression in H_2O_2-treated RGCs. Importantly, miR-155 overexpression inhibits the protective effect of acteoside on attenuating RGC injury, but overexpression of CASC2 finally reverses the inhibitor effect of miR-155 overexpression on H_2O_2-treated RGCs.

Although miRNAs play roles in glaucoma, the involved mechanisms are different. Zhang *et al.* suggested that miR-141-3p inhibits retinal neovascularization in glaucoma mice via the DOK5-mediated MAPK signaling pathway (9). Su *et al.* demonstrated that miR-21a-5p modulates

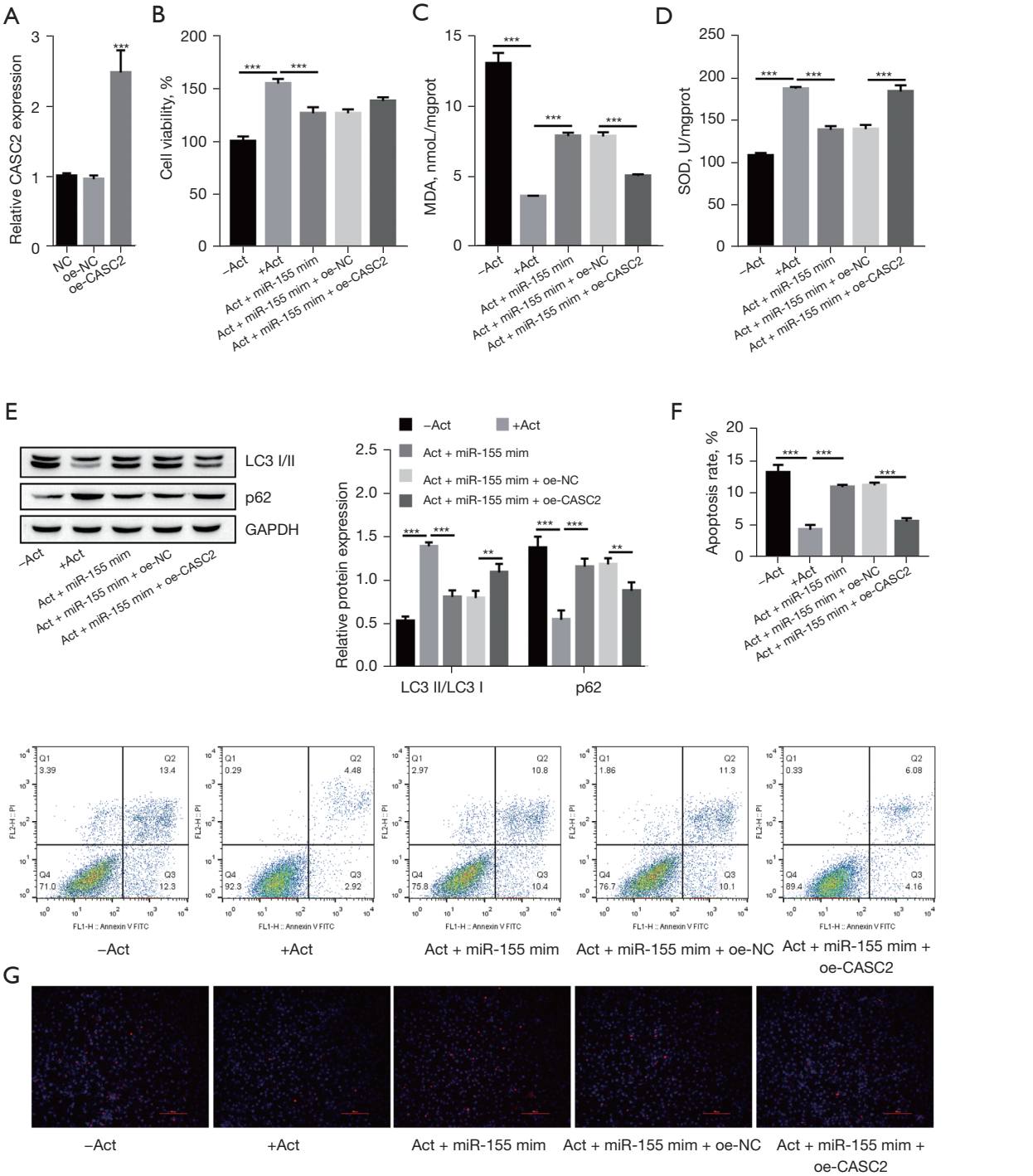

Figure 5 Acteoside attenuates H_2O_2-induced RGC-5 cell injury via modulating CASC2/miR-155 axis. (A) Expression of miR-155 in oe-CASC2-transfected RGC-5 cells was measured by RT-qPCR; (B) the viability of RGC-5 cells was measured by the MTT assay; (C) MDA content was measured by ELISA; (D) SOD activity was measured by ELISA; (E) the expression of autophagic proteins in RGC-5 cells was measured by western blot; (F) apoptosis of RGC-5 cells was measured by flow cytometry; (G) TUNEL staining of RGC-5 cells. Scale bar = 200 μm. **P<0.01; ***P<0.001. RT-qPCR, quantitative real-time PCR; MDA, malondialdehyde; SOD, superoxide dismutase.

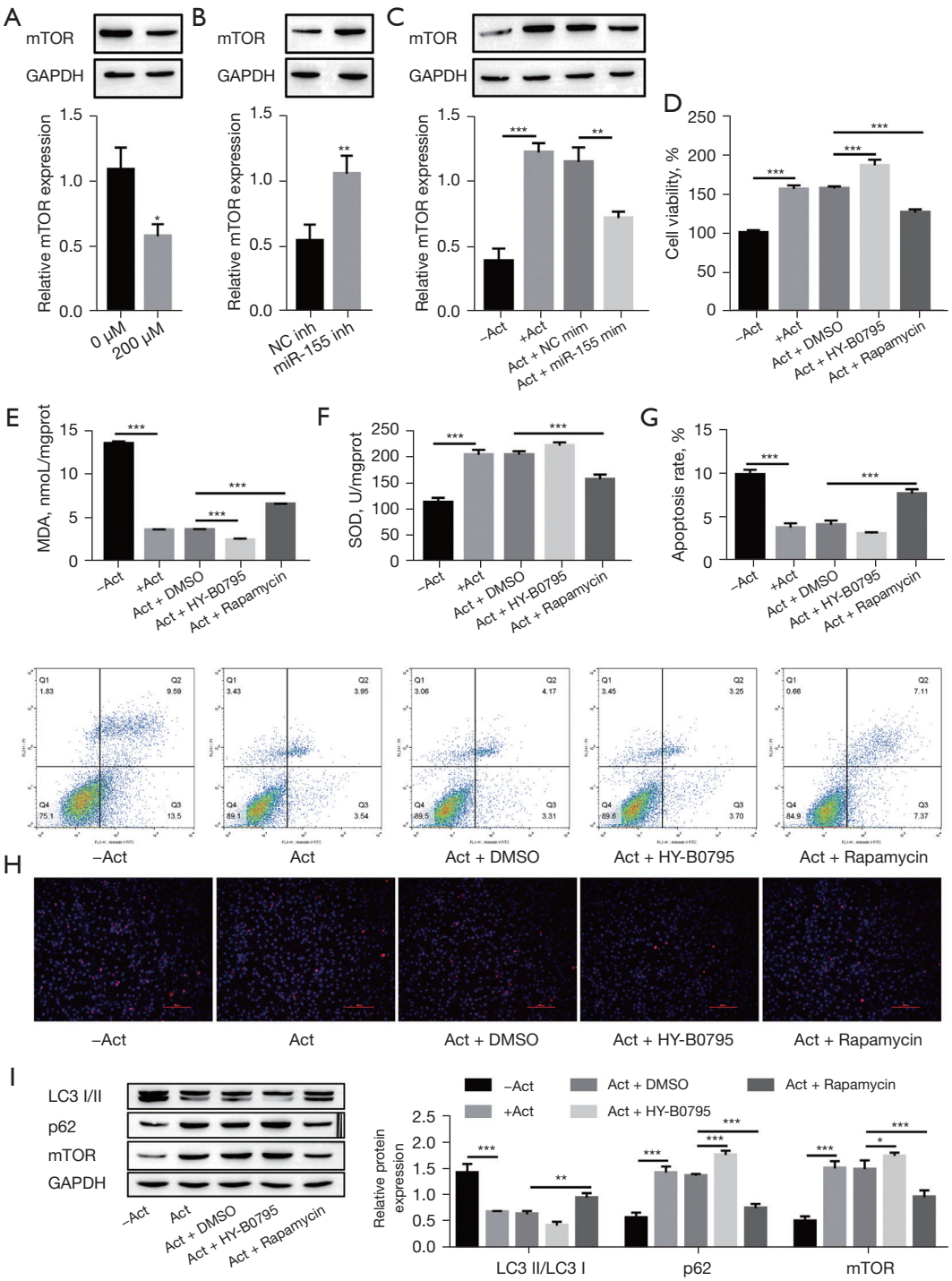

Figure 6 Acteoside protects RGC-5 cells against H2O2-induced cell injury via the miR-155/mTOR axis. (A) The expression of mTOR in 0 μM- or 200 μM-treated RGC-5 cells was measured by western blot; (B) the expression of mTOR in RGC-5 cells was measured by western blot; (C) the expression of mTOR in RGC-5 cells was measured by western blot; (D) the viability of RGC-5 cells was measured by the MTT assay; (E) MDA content was measured by ELISA; (F) SOD activity was measured by ELISA; (G) apoptosis of RGC-5 cells was measured by flow cytometry; (H) TUNEL staining of RGC-5 cells; (I) the expression of mTOR and autophagic proteins in RGC-5 cell were measured by western blot. Scale bar =200 μm. *P<0.05; **P<0.01; ***P<0.001. MDA, malondialdehyde; SOD, superoxide dismutase.

mesenchymal stem cell-mediated neuroprotective effects on acute glaucoma via targeting PDCD4 (31). Several studies show that the mTOR signaling pathway is involved in miR-155-mediated regulation (16,22). In addition, mTOR contributes to glaucoma development and is associated with RGC activity (13,32,33). Here, we found that the expression of mTOR was downregulated in H_2O_2-treated RGCs. Acteoside administration or miR-155 inhibition increased mTOR expression. However, miR-155 overexpression abolished the promotive effect of acteoside on the expression of mTOR. The mTOR inhibitor rapamycin reversed the protective effect of acteoside on the attenuation of RGC injury. Taken together, our findings demonstrate that acteoside attenuates RGC injury via the miR-155/mTOR axis, which is of significance for glaucoma treatment.

Conclusions

We report that acteoside repressed RGC injury via targeting the CASC2/miR-155/mTOR axis. The expression of miR-155 was induced in H_2O_2-treated RGCs. Induced miR-155 expression promoted RGC apoptosis and autophagy. Acteoside administration could suppress miR-155 expression and miR-155 overexpression abolished the protective effect of acteoside on RGCs. The expression of CASC2 was decreased in H_2O_2-treated RGCs. Acteoside administration could increase CASC2 expression and CASC2 overexpression reverses the effect of miR-155 overexpression on acteoside treatment-RGCs. Enhanced miR-155 repressed the mTOR signaling pathway while acteoside activated the mTOR signaling pathway. The mTOR inhibitor rapamycin rescued the protective effect of acteoside on RGCs. Therefore, our study describes a novel mechanism by which acteoside protects RGC-5 cells against H_2O_2-induced cell injury via the CASC2/miR-155/mTOR axis.

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Footnote

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Data Sharing Statement: Available at https://dx.doi.org/10.21037/atm-21-5630

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://dx.doi.org/10.21037/atm-21-5630). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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