Original Article

Ozone reduces ischemic damage after a stroke by regulating the autophagy of astrocytes

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Background: Stroke is a common and damaging disease of brain tissue, and has high morbidity, disability, and mortality rates. Ozone (O_3) is an isomer of oxygen and can be applied to ozonate the isolated blood in specific containers outside the body and return it to the body. O_3 can also alter the activity and function of multiple cellular components, thus affecting blood viscosity and altering hemodynamics. However, the question of whether O_3 has clinical value in the treatment of stroke requires further investigation. This study sought to evaluate the protective effect and possible mechanism of O_3 in brain injury after stroke.

Methods: First, oxygen-glucose deprivation/reoxygenation (OGD/R)-induced human glioblastoma cell (A172) and middle cerebral artery occlusion (MCAO) rat models were established. Second, the levels of the associated ribonucleic acids and proteins were analyzed using reverse-transcription real-time-quantitative polymerase chain reaction, Western Blot, or immunofluorescence assays. Third, the concentration of glutamate and lactate dehydrogenase (LDH) were assessed using enzyme-linked immunoassays.

Results: The results showed that glial fibrillary acidic protein was upregulated in the OGD/R A172 cells. O_3 decreased LDH and increased glutamate levels in the OGD/R A172 cells, which suggests that O_3 reduced brain damage in the *in vitro* stroke model. We also showed that O_3 attenuated brain infarction in the *in-vivo* stroke model. Further, we found that O_3 alleviated stroke-induced brain damage by reducing the apoptosis of astrocytes. Further, the B-cell lymphoma 2 inhibitor propofol alleviated stroke-induced brain damage.

Conclusions: Thus, O_3 notably alleviated stroke-induced brain damage by inhibiting the apoptosis of astrocytes in the OGD/R-induced human glioblastoma cell and MACO rat models.

Keywords: Ozone; stroke; ischemic damage; propofol

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Introduction

In vascular disease, stroke is caused by cerebral atherosclerosis or atrial fibrillation embolism (1). It is a common disease among the elderly and has high morbidity, disability, and mortality rates. The rapid infusion of the tissue plasminogen activator by triggering thrombolysis has a significant therapeutic effect, but only about 5–10% of patients can dissolve thrombolysis and reach the stroke unit within 4.5 hours of their initial symptoms (2). In the remaining 90% of patients, anti-coagulant, anti-

hypertensive, and anti-polymerizable drugs can only be used with caution (3). Thus, new treatment methods for stroke patients need to be explored.

Chen *et al.* found that ozone (O_3) treatment reduced hypoxia-reperfusion injury in a mouse model (4). This treatment method not only rapidly improves the oxygenation of the penumbra area, but also induces many metabolic modifications, such as the upregulation of antioxidant enzymes, which enhances the release of nitric oxide (NO) and carbon monoxide (CO) as vasodilators and enhances local adenosine release (5-8). Thus, preliminary preclinical studies need to be conducted to examine the role of gaseous oxygen-O_3 mixtures as potential therapeutic agents for stroke.

A study has shown that cerebral ischemia can open the hemispherical channel of connexin in the astrocyte network and damage the integrity of the blood-brain barrier by losing ion gradients and releasing "danger signals" [including adenosine triphosphate (ATP) and uridine triphosphate (UTP)] (9). Thus, the upregulation of glial fibrillary acidic protein (GFAP) protein can serve as a sign of nerve damage. As the largest number of cells in the brain, astrocytes not only protect and support the central nervous system, but also participate in a series of functions such as water transport, neurotransmitter metabolism and release, and synthesis of neural active substances. In the event of ischemic stroke, astrocytes can produce a variety of protective effects on injured neurons. However, a study has shown that the proliferation of astrocytes after ischemia has a "double-edged sword" effect (10). Additionally, Han *et al.* found that the autophagy of astrocytes is also a sign of astrocyte activation; thus, the autophagy of astrocytes can serve as a sign of nerve damage (11).

In this study, we established brain injury models to verify the effect of O_3 therapy on stroke brain injury. Our experimental data show that O_3 therapy significantly reduced brain damage after stroke. Further, we found that B-cell lymphoma 2 (Bcl-2) inhibitors had certain practical significance in the treatment of stroke. We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-6456/rc).

Highlight box

Key findings

- *In-vivo* and *in-vitro* studies showed that ozone (O_3) reduced stroke damage.

What is known and what is new?

- O_3 treatment reduced hypoxia-reperfusion injury in a mouse model.
- O_3 reduced brain damage by reducing astrocyte apoptosis.

What is the implication, and what should change now?

- Medical O_3 can be applied in ischemic stroke therapy, and its therapeutic mechanism is related to apoptosis.

Methods

Cell culture

The human astrocytoma cell line (A172) was purchased from ATCC® and cultured in Dulbecco's Modified Eagle Medium (Gibco) containing 10% fetal bovine serum (Hyclone) at 37 ℃ with 5% carbon dioxide (CO_2).

Construction of the OGD/R cell model

The A172 cells were inoculated in 96-well plates at 2.5×10^4 cells/mL. After 24 h of normal culture, the cells were washed with sugar-free Earle's solution and cultured at 37 ℃ under hypoxic conditions (95% nitrogen + 5% CO_2). After 0, 2, 4, 8, and 12 h, the sugar-free Earle's solution was replaced with normal cell culture medium, and the cells continued to be cultured for 24 h under normoxic conditions.

O_3 preparation

A high-precision medical O_3 meter (HUMAZON, Germany) was used to obtain the O_3. The concentration range was 1–56 μg/mL, and the gas flow rate was 0–1,300 mL/min, with a minimum adjustable concentration of 1 μg/mL.

Cell treatment

The OGD/R A172 cells were treated with 20, 30, 60, 90, 120, or 150 μg/mL of O_3.

Experimental animals

A total of 30 specific pathogen free (SPF)-grade male Sprague-Dawley rats (weighing 300±20 g) were purchased from the Animal Center. The animals were fed in strict accordance with the animal husbandry rules. The animals were housed at a room temperature of 24±2 ℃ with 50–60% humidity, and had free access to water and food.

Construction of the MCAO rat model

The experiments began after 7 days of adaptive feeding. The rats were anesthetized with 3% sodium pentobarbital (50 mg/kg) by injection. The middle cerebral artery occlusion (MCAO) model was established in all the rats except those in the sham-operated group. The hair on the neck of each rat was removed, after which the skin was disinfected with 75% ethanol, and a 2-cm incision was made in the midline of the neck. Subsequently, the muscles were bluntly separated. A "V"-shaped incision was made at a distance of about 6 mm from the bifurcation of the common carotid artery (CCA), and a wire plug was inserted from the CCA to the internal carotid artery and into the cerebral artery. The plug was fixed for 2 h and then removed, and blood flow was restored to complete the modeling. The sham-operated group was operated on as described above, but no bolus was inserted. After waking up, the rats could not fully extend their left forelimbs when lifting their tails, and turned to their opposite side when crawling, which indicated the success of the model. This animal experiment was approved by the Institutional Animal Ethics Committee (No. 2003008) and in compliance with the Animal Care Guidelines for the Care and Use of Guizhou Medical University. A protocol was prepared before the study without registration.

Animal grouping

The SD rats were randomly divided into the sham-operated (control) group, MCAO model group, MCAO + O_3 group, MCAO + propofol group, and MCAO + O_3 + propofol group. O_3 concentration selection 20 μg/mL, if the concentration is higher than, the body of rats may be damaged. Propofol was intraperitoneally injected with 30 mg/kg for 30 min.

2,3,5-triphenyte-trazoliumchloride (TTC) staining

After anesthesia, the rat brain tissues were isolated, and the brain tissues were cut into 2-mm-thick coronal sections by freezing. Subsequently, the brain sections were processed with 2% TTC for 30 min, protected from light at 37 ℃. After staining, the brain sections were placed in 4% paraformaldehyde and fixed overnight at 4 ℃. The following day, the sections were observed under the microscope and photographed. The white area indicated the infarcted area, and the red area indicated the non-infarcted area. The infarct area of the sections was calculated using IPP6.0.

ELISA analysis

After anesthesia, fresh cerebral cortical tissue on the ischemic side was taken from the rats after cardiac perfusion, the surface appendages were removed, then it was washed with cold saline to make brain homogenate at a concentration of 10%. For the cells, we collected the supernatant of the cell culture fluid. The lactate dehydrogenase (LDH) and glutamate content were measured as per the instructions of each kit.

Western blot

The total protein was extracted with radio-Immunoprecipitation Assay (RIPA) lysate from the brain tissue or A172 cells of each group. The BCA method was used to determine the concentration of the proteins, and 30 μg of the protein samples was separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis and transferred to the polyvinylidene fluoride (PVDF) membranes using the wet-transfer method. The membranes were blocked with 5% skim milk powder and incubated with primary antibody (1:1,000) at 4 ℃ overnight. The primary antibody was then washed away, the membranes were invubated with the goat anti-rabbit secondary antibody (Abcam, 1:5,000) at room temperature for 1h. After electrochemical luminescence (ECL) visualization, the relative expression levels of the target bands were derived by the gray scale ratio with the internal reference. The primary antibodies included anti-glial fibrillary acidic protein (GFAP) (Abcam, ab4674), anti-GAPDH (Abcam, ab76523), anti-Bcl-2 (Abcam, ab692), and anti-LC3B (Abcam, ab192890).

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total ribonucleic acid (RNA) was extracted from the brain tissue or A172 cells using the Trizol method. The RNA was reverse transcribed using the SYBR®PremixExTaqTM kit (Takara). Subsequently, PCR amplification was conducted using the SYBR Green PCR kit (Applied Biosystems). The $2^{-\Delta\Delta Ct}$ method was used to analyze the results. The primer sequences were as follows: GFAP: 5'-CACCTACAGGAAATTGCTGGAGG-3' (forward), 5'-CCACGATGTTCCTCTTGAGGTG-3' (reverse); Bcl-2: 5'-CCTGTGGATGACTGAGTACCTG-3' (forward),

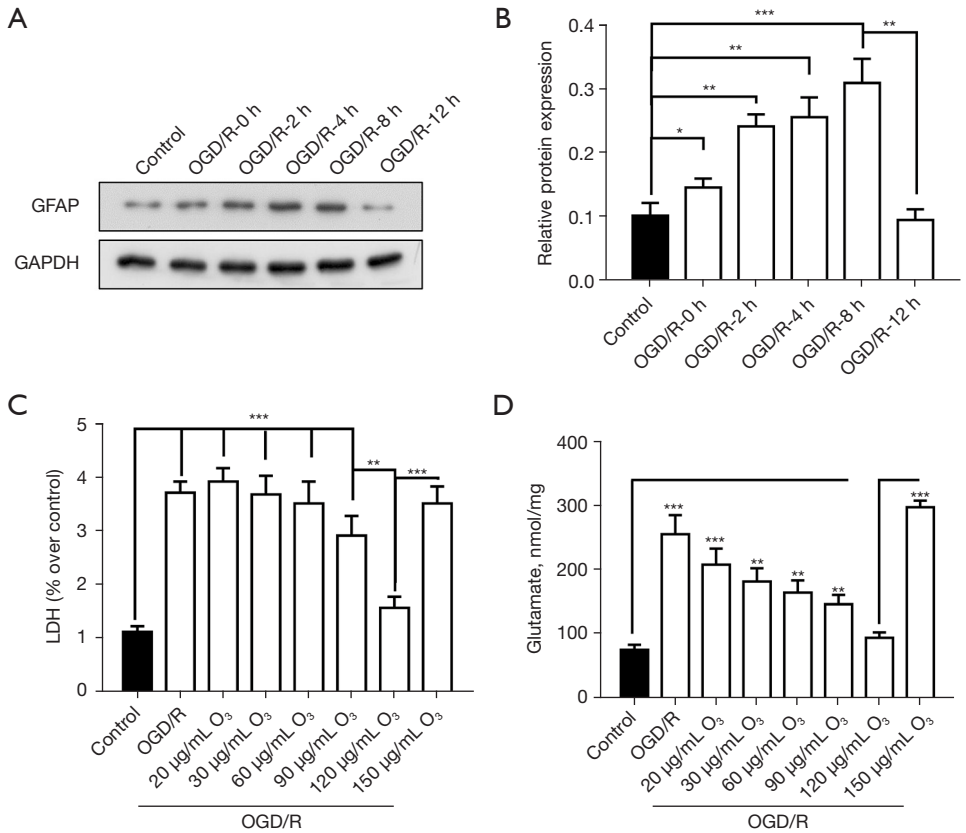

Figure 1 O_3 reduced brain damage in the *in vitro* stroke model. (A) Western blot analysis of GFAP expression in the OGD/R treated A172 cells whole-cell lysates from the brain slices were analyzed by immunoblots with the indicated antibodies; (B) statistics for the Western blot analysis results in A; (C) LDH content measurement of the O_3 treated A172 cells; (D) glutamate content measurement of the O_3 treated A172 cells. The data are representative of 3 independent experiments and were analyzed by the unpaired *t*-test. Error bars denote SD. *P<0.05; **P<0.01; ***P<0.001. LDH, lactate dehydrogenase; SD, standard deviation.

5'-AGCCAGGAGAAATCAAACAGAGG-3' (reverse); ATG3: 5'-TAAGGCTGACGCTGGAGGTGAA-3' (forward), 5'-GTGCTCAACTGTTAAAGGCTGCC-3' (reverse); GAPDH: 5'-GAAGGTGAAGGTCGGAGTC-3' (forward), 5'-GAAGATGGTGATGGGATTTC-3' (reverse).

Immunofluorescence staining

Brain tissue sections from each group were fixed with 4% paraformaldehyde and placed in 30% sucrose solution for dehydration. Frozen sections (–20 ℃, stored in antifreeze solution) were hydrated with phosphate-buffered saline for 20 min. After rinsing, the sections were blocked with 10% goat serum for 1 h and incubated with the primary antibody (GFAP, 1:20) overnight at 4 ℃. After washing, the sections were treated with goat anti-rabbit immunoglobulin G-fluorescein isothiocyanate (lgG-FITC) (1:200) for 1 h. After washing, the sections were sealed with glycerol. The expression and distribution of GFAP were observed under a fluorescence microscope. The results were quantitatively analyzed by ImageJ software.

Statistical analysis

All the above experiments were independently repeated 3 times, and the obtained data are expressed as the mean ± standard deviation (SD). The data were analyzed using SPSS 21.0 software (IBM-SPSS Inc., Chicago, IL, USA). One way ANOVA was used for comparison between multiple groups, and LSD or snk-q was used for pairwise comparison between groups. A P value <0.05 denotes a statistically significant difference.

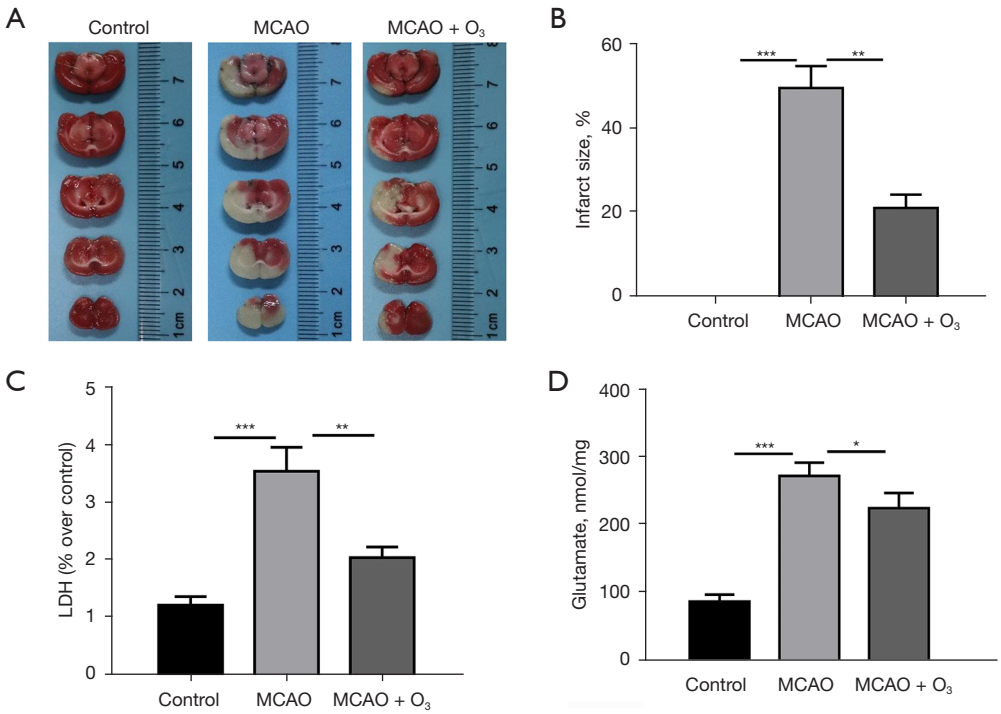

Figure 2 O_3 reduced brain infarction after stroke *in vivo*. (A) Representative images of TTC-stained brain slices. n=5 animals/group; (B) statistics of TTC-stained analysis results in A; (C) LDH content measurement of the O_3 treated tMCAO rat brain slices; (D) glutamate content measurement of the O_3 treated tMCAO rat brain slices. The data are representative of 3 independent experiments and were analyzed by unpaired *t*-test. Error bars denote SD. *P<0.05; **P<0.01; ***P<0.001. TTC, 2,3,5-triphenyte-trazoliumchloride; tMCAO, transient middle cerebral artery occlusion.

Results

O_3 reduces brain damage in the in-vitro stroke model

We applied the experimental methods of Frosini *et al.* to establish the *in-vitro* optimal ischemia-like [oxygen-glucose deprivation and reoxygenation (OGD/R)] experimental conditions (12). As *Figure 1A,1B* show, by detecting the changes in GFAP expression, we determined that the best time for OGD/R was 8 hours. Consistent with this, the contents of the tissue damage markers of LDH and glutamate increased significantly after OGD/R treatment (*figure 1C,1D*). We also found that O_3 significantly reduced the LDH and glutamate content of the A172 cells treated with OGD/R (*figure 1C,1D*). Thus, O_3 may reduce brain damage after stroke.

O_3 reduces brain infarction after stroke in vivo

To further evaluate the therapeutic significance of O_3 for stroke, we used a transient middle cerebral artery occlusion (tMCAO) mouse model to evaluate the effect of O_3 on brain damage after stroke *in vivo* (13). As *Figure 2A,2B* show, O_3 significantly reduced the degree of brain damage in the tMCAO mouse model. In addition, we found that O_3 significantly reduced the LDH and glutamate content of the A172 cells treated with OGD/R (*figure 2C,2D*). Further, the Western blot and immunofluorescence results showed that O_3 significantly reduced stroke-induced GFAP activation *in vivo* (*figure 3A-3D*). These data indicated that O_3 might reduce brain damage after stroke.

O_3 alleviates stroke-induced brain damage by reducing the autophagy of astrocytes

A study (14) has reported that autophagy in astrocytes induces the activation of astrocytes, which is common after stroke and causes brain damage. Thus, we speculated that the effect of O_3 in alleviating brain damage might be related to the autophagy of astrocytes. The RT-qPCR results showed that O_3 significantly reduced stroke-induced autophagy gene

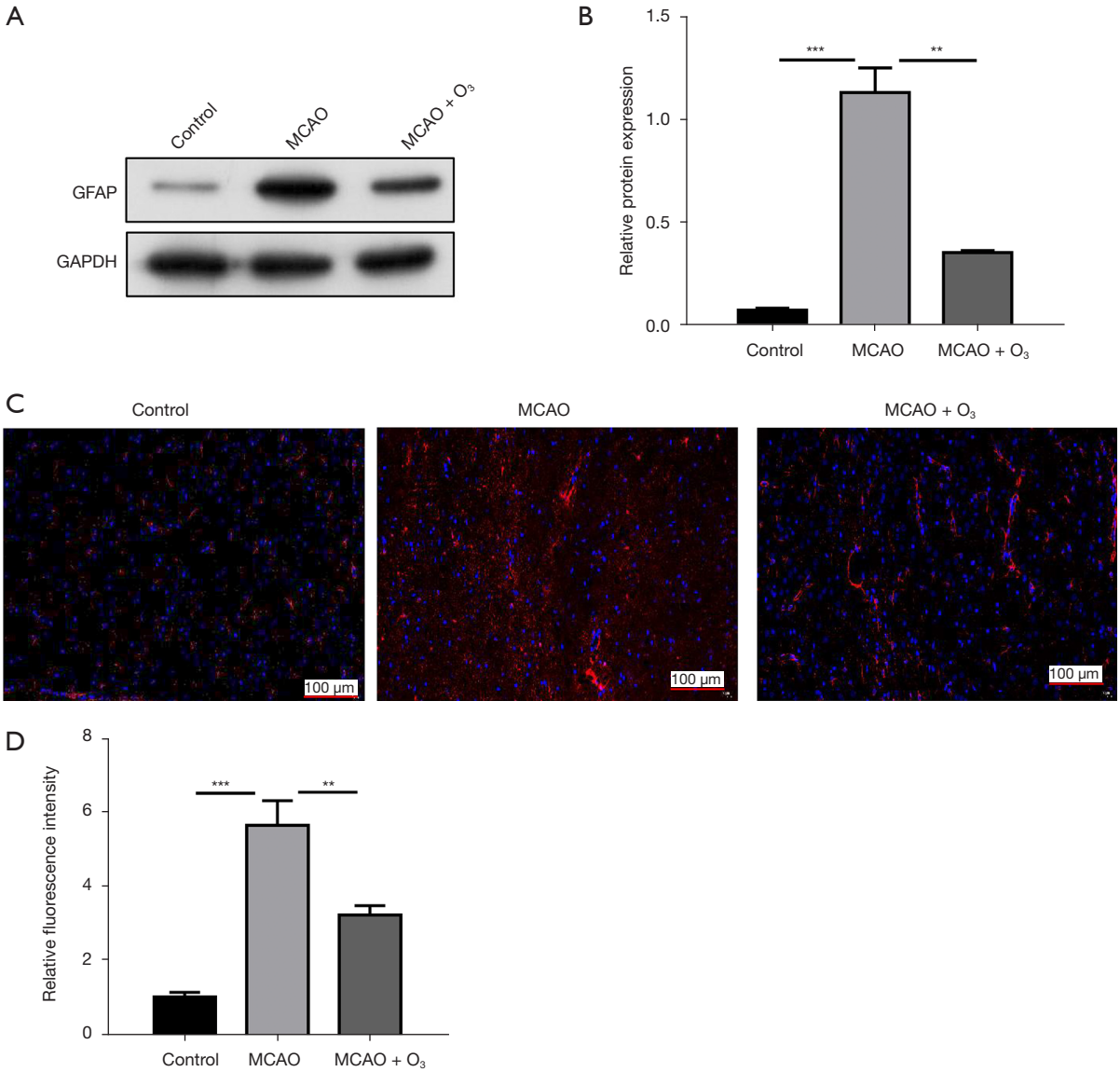

Figure 3 O_3 reduced the activation of astrocytes after stroke *in vivo*. (A) Western blot analysis of GFAP expression in the O_3 treated tMCAO rat brain slices; whole-cell lysates from the brain slices were analyzed by immunoblots with the indicated antibodies; (B) statistics of the Western blot analysis results in A; (C) immunofluorescence analysis of GFAP expression in the O_3 treated tMCAO rat brain slices, GFAP (red), nucleus (blue), scale bars, 100 μm; (D) statistics of the immunofluorescence analysis results in A. The data are representative of 3 independent experiments and were analyzed by unpaired *t*-test. Error bars denote SD. **P<0.01; ***P<0.001. tMCAO, transient middle cerebral artery occlusion; SD, standard deviation.

activation (*figure 4A*). The Western blot experimental results showed that O_3 significantly reduced the protein levels of Bcl-2 and LC3II (*figure 4B,4C*). In addition, the ectopic expression of Bcl-2 reduced the rescuing effect of O_3 on brain injury (*figure 4D-4F*). These results suggested that O_3 plays a neuroprotective role by inhibiting stroke-induced autophagy.

Propofol (the BCL-2 inhibitor) alleviates stroke-induced brain damage

The above experimental data indicated that O_3 inhibited the key protein of autophagy, Bcl-2. We speculated that Bcl-2 inhibitors might also be effective in alleviating stroke-induced brain damage. We used the Bcl-2 inhibitor propofol to carry

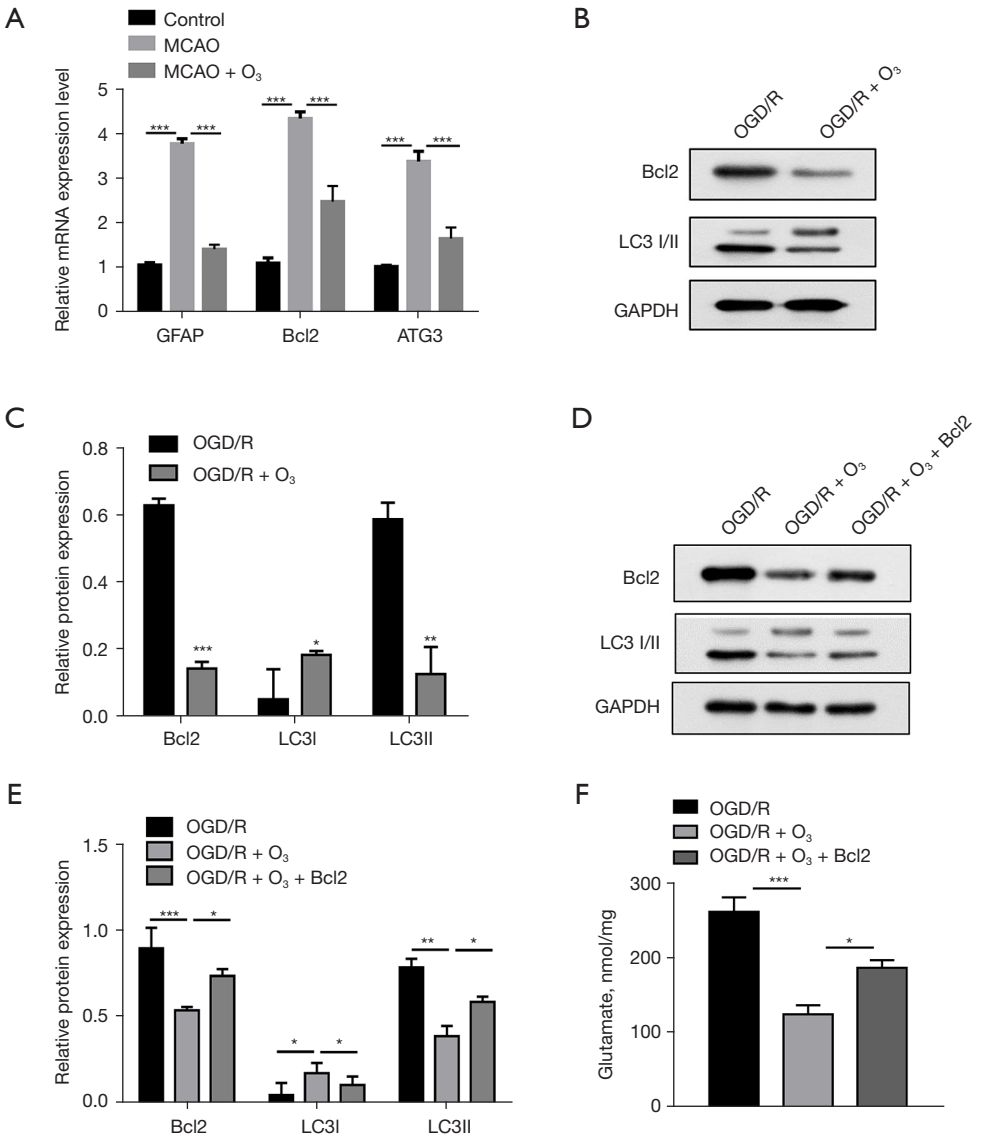

Figure 4 O_3 alleviated stroke-induced brain damage by reducing the autophagy of astrocytes. (A) GFAP, Bcl-2, and ATG3 mRNA expression level in the ozone treated tMCAO rat brain slices; (B) Western blot analysis of Bcl-2 and LC3 I/II expression in the OGD/R treated (with or without ozone) A172 cells; whole-cell lysates from the astrocytes were analyzed by immunoblots with the indicated antibodies; (C) statistics of the Western blot analysis results in B; (D) western blot analysis of Bcl-2 and LC3 I/II expression of the different group; (E) statistics of the Western blot analysis results in D; (F) glutamate content measurement of the different groups in. The data are representative of 3 independent experiments and were analyzed by unpaired *t*-test. Error bars denote SD. *P<0.05; ***P<0.001. tMCAO, transient middle cerebral artery occlusion.

out the relevant experimental verification in the tMCAO mouse model (15). We found that propofol inhibited the increase in LC3II protein expression induced by stroke (*figure 5A,5B*). In addition, the combined effect of O_3 and propofol further inhibited stroke-induced autophagy (*figure 5A,5B*). The LDH test results showed that propofol significantly alleviated the stroke-induced brain injury (*figure 5C*). Further, the combined effect of O_3 and propofol provided a better cerebral protective effect after stroke (*figure 5C*).

Discussion

Stroke, which is an important part of cardiovascular and cerebrovascular diseases, poses a serious threat to people's

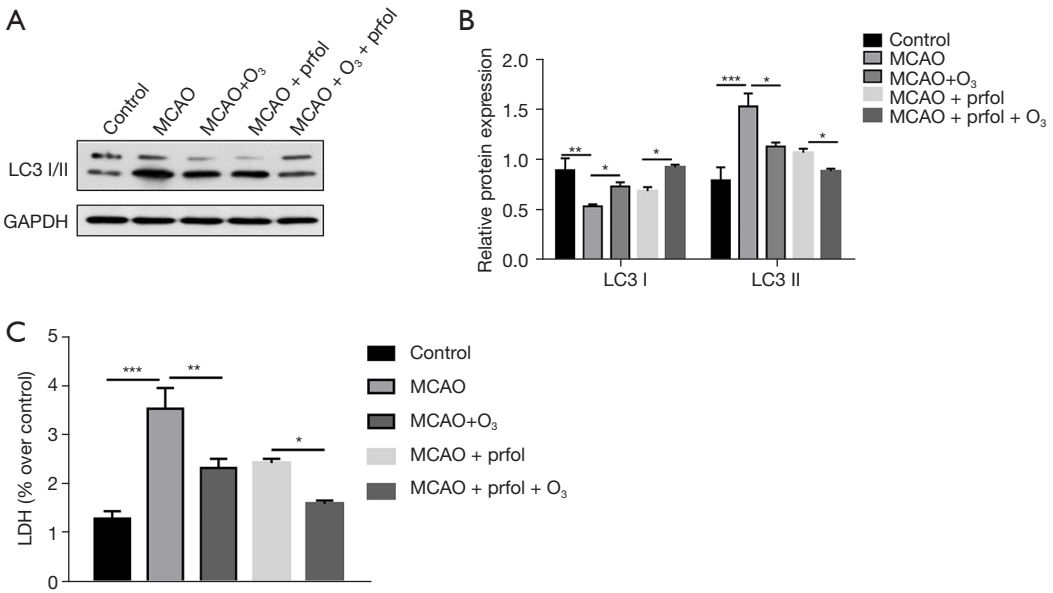

Figure 5 Propofol alleviated stroke-induced brain damage. (A) Western blot analysis of LC3 I/II expression of the rat brain slices of different groups; whole-cell lysates from the brain slices were analyzed by immunoblots with the indicated antibodies; (B) Western blot analysis results in A; (C) LDH content measurement of the rat brain slices of different group as indicated. The data are representative of 3 independent experiments and were analyzed by unpaired *t*-test. Error bars denote SD. *P<0.05; **P<0.01; ***P<0.001. LDH, lactate dehydrogenase; SD, standard deviation.

health (15-18). There has been great progress in research on the molecular mechanisms and clinical treatment of stroke in recent years; however, the effective treatment options that can be administered to stroke patients in clinical practice remain very limited. Thus, the study of new and effective treatments for stroke is of great clinical significance.

In this study, consistent with the findings of Clavo *et al.* (19), we found that O_3 significantly alleviated stroke-induced brain damage. In addition, our RT-qPCR and Western blot results suggested that O_3 plays a protective role in the brain by regulating the autophagy of astrocytes. However, the relevant molecular mechanisms were not thoroughly examined in this study. The ozone concentration commonly used in the treatment of osteoarthritis and lumbar disc herniation is 30–50 μg/mL. The common concentration of O_3 in large autohemotherapy is 20–30 μg/mL. High concentration of O_3 may cause damage to neurons in rats, so it is particularly important to select an appropriate concentration of O_3 so that the treatment of O_3 can play a greater role without harming the body tissues. In this experiment, the concentration of O_3 is 20 μg/mL, if the concentration is higher than that, the body of rats may be damaged.

Previous studies have shown that O_3 is related to the reactive oxygen species (ROS) pathway (20,21). In this present study, we found that O_3 had a regulatory effect on Bcl-2 gene expression. Additionally, research has revealed that there is crosstalk between Bcl-2 and the ROS pathway in autophagy (22). Thus, we speculated that O_3 may play a protective role after stroke through the ROS pathway. This conjecture has great research value and will be one of the main directions of our follow-up research. Our results also showed that Bcl-2 inhibitors had a neuroprotective effect after stroke. In addition, O_3 and Bcl-2 inhibitors had a significant combined neuroprotective effect. However, when evaluating the neuroprotective effect of Bcl-2 inhibitors in this study, the evaluation targets used were insufficient. Our follow-up research will further improve the relevant evaluation indicators.

In addition, our combination of O_3 and propofol provides better brain protection after stroke. Propofol is an antioxidant, which may affect many aspects of oxidative stress. In the free radical reaction system, the phenol structure can provide a hydrogen atom, which can reduce the damage to the body caused by excessive free radicals. The trace plasma concentration of propofol can play an

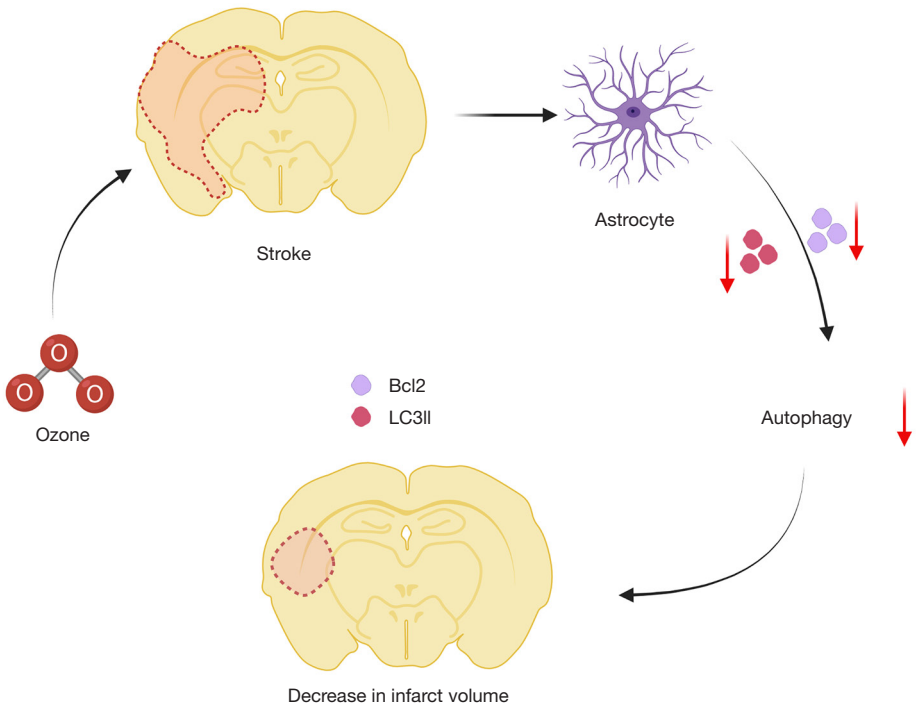

Figure 6 Diagram showing how O_3 protects against brain injury in a MCAO rat model. O_3 plays a neuroprotective role after stroke by regulating autophagy. MCAO, middle cerebral artery occlusion.

antioxidant role in protecting cell membrane. Because of the fat solubility of propofol, it is easier to accumulate on the lipid bilayer of cells, thus improving the ability of cells to resist oxidative damage. The rat cerebral cortex astrocytes were exposed to peroxide to form an oxidative stress model. After 2 hours of propofol treatment, it was found that the Glu release was significantly lower than that of the untreated group (14,20). Therefore, the combination of O_3 and propofol in this study has a better protective effect on stroke.

Conclusions

Overall, this study demonstrated that O_3 had a protective effect on the brain after stroke an *in vitro* and *in vivo* models. In addition, we verified that O_3 plays a neuroprotective role after stroke by regulating autophagy (*Figure 6*). Our findings may provide a theoretical basis for the treatment of stroke patients in the future.

Acknowledgments

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-6456/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-6456/dss

Conflicts of Interest: All authors have completed the

ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-6456/coif). 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. This animal experiment was approved by the Institutional Animal Ethics Committee (No. 2003008) and in compliance with the Animal Care Guidelines for the Care and Use of Guizhou Medical University.

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