Original Article

Sodium Danshensu protects against oxygen glucose deprivation/reoxygenation-induced astrocytes injury through regulating NOD-like receptor pyrin domain containing 3 (NLRP3) inflammasome and tuberous sclerosis complex-2 (TSC2)/mammalian target of rapamycin (mTOR) pathways

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Background: Cerebral ischemic stroke is a serious condition with high incidence, mortality, and associated disability. Currently, effective therapeutic options are available for ischemic stroke are limited. Accumulating evidence indicates that sodium Danshensu, mono sodium compound derived from Salvia miltiorrhiza, plays protective roles in ischemic stroke. However, the underlying protective mechanism of sodium Danshensu in cerebral ischemic stroke remains unknown.

Methods: In the current study, we explored the role and mechanism of sodium Danshensu on astrocytes exposed to oxygen-glucose deprivation/reoxygenation (OGD/R), which mimics the process of ischemia-reperfusion. The impact of sodium Danshensu on cell viability and apoptosis after OGD/R were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-dophenyl tetrazolium bromide (MTT) assay and flow cytometry. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) and western blot were used to detect the expression of target messenger RNA (mRNA) and proteins associated with apoptosis and autophagy. The release of lactate dehydrogenase (LDH) was determined, and the production of proinflammatory cytokines were detected using enzyme-linked immunosorbent assay (ELISA) kits.

Results: It was found that sodium Danshensu could significantly increase cell viability and decrease LDH release and apoptosis. Besides, it inhibited the production of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6. Sodium Danshensu also dose-dependently decreased protein and mRNA levels of nucleotide binding oligomerization NOD-like receptor pyrin domain containing 3 (NLRP3) and high mobility group box 1 (HMGB1), which play a crucial role in promoting ischemic stroke-induced cell injury. Moreover, sodium Danshensu dose-dependently upregulated Beclin 1 expression, downregulated P62 protein expression, and further increased LC3B-II/LC3B-I ratio through inducing autophagy in astrocytes. Additionally, we noticed that sodium Danshensu dose-dependently increased tuberous sclerosis complex-2 (TSC2) protein expression, while significantly reduced the levels of mammalian target of rapamycin (mTOR) in the presence of OGD/R insult.

Conclusions: These findings suggest that sodium Danshensu protects against OGD/R-induced injury by modulating the NLRP3 inflammasome and TSC2/mTOR pathways.

Keywords: Autophagy; tuberous sclerosis complex-2/mammalian target of rapamycin (TSC2/mTOR); NOD-like receptor pyrin domain containing 3 inflammasome (NLRP3 inflammasome); ischemic stroke; sodium Danshensu

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Introduction

Salvia miltiorrhiza, called Danshen in Chinese, is a common herbal medicine used widely to enhance blood circulation, dilate coronary arteries, and improve blood flow (1,2). Danshen and its derivatives have been discovered to have pro-angiogenic, anti-inflammatory, and antioxidant properties, as well as inhibit platelet adhesion and aggregation in cardiovascular ischemia (3-5). The National Medical Products Administration of China, a government department similar to the American Food and Drug Administration (FDA), has approved about 900 Danshen-related drugs (2). Sodium Danshensu [sodium D-(+)-β-(3,4-dihydroxyphenyl) lactate, C9H9O5Na, molecular weight 220], is an important water-soluble derivative of Danshen (6). *In vitro* and *in vivo* studies have suggested that sodium Danshensu exerts various pharmacological effects, such as anti-inflammatory, coronary artery relaxation, and myocardial ischemia-reperfusion injury prevention (7-9). In clinical application, danshensu sodium can be used for the prevention of hypoxic pulmonary hypertension and the treatment of psoriasis (10,11). Besides, sodium Danshensu actively enhances neurogenesis and collagen production in post-ischemic injury, and has potential therapeutic effect on ischemic stroke (1). The existing data suggest that sodium Danshensu exerts a strong protective effect on ischemic stroke; however, the underlying molecular mechanism of this effect remains to be further investigated.

Ischemic stroke, a cerebrovascular accident, is a severe brain disorder with a high incidence of disability, morbidity, and mortality (12,13). It is characterized by abnormal blood circulation and inadequate oxygen supply to the brain (14). Several factors have been identified as involved in the development and progression of ischemic stroke, including nutrient insufficiency, ischemic stress, and dysregulation in autophagy (15). Although considerable efforts have been made to advance our knowledge on ischemic stroke, the understanding of ischemic stroke remains limited. Until recently, an intravenous thrombolytic agent with a recombinant tissue plasminogen activator was the only recommended medication for treating acute stroke, but the narrow therapeutic window and danger of intracerebral hemorrhage make this therapy unsuitable for many individuals (16). In recent years, herbal extracts and their derivatives have shown extraordinary efficacy on ischemic stroke, such as baicalin, danshensu and isoquercetin, which can improve the cell damage caused by cerebral ischemia, but the side effects are limited (1,14,17,18). Therefore, exploring new herbal therapeutic options to manage ischemic stroke is warranted.

Astrocytes, the primary glial cells of the brain, are critical for maintaining normal brain structure and function through interacting with neurons, oligodendrocytes, and endothelial cells (15,19,20). Notably, reactive astrogliosis, known as astrocyte dysfunction, is a common pathological feature in focal ischemic stroke, and dysfunction in astrocytes induces neural injury and promotes stroke-induced disability (21-23). Astrocyte homeostasis is critical to neurons, especially during cerebral ischemia, which reduces ischemia-induced disabilities (24,25). Astrocyte autophagy flux plays a crucial role in astrocyte homeostasis (24,25). Notably, the *in vitro* culture of astrocytes under oxygen-glucose deprivation/reoxygenation (OGD/R) insult can resemble the process of ischemia-reperfusion (25). Therefore, it is suggested that astrocytes are highly associated with ischemic stroke, which makes astrocytes an ideal therapeutic target for the management of cerebral ischemic stroke.

In this study, we aimed to investigate the role of sodium Danshensu on ischemic stroke using an *in vitro* model of astrocytic cerebral ischemic stroke and explored the molecular mechanism underlying this protective effect. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-2143/rc).

Methods

Primary astrocyte isolation and culture

Primary astrocytes were isolated from 24-hour postnatal male Sprague-Dawley (SD) rats, according to a previously described technique with minor modification (26). The cells were cultured in a poly-d-lysine coated 75 cm^2 flask, and the seeded concentration was 1×10^6 cells/mL. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1%

antibiotics at 37 ℃ with 5% CO_2 supplement. The rats were obtained from Chengdu Dasuo Experimental Animal Co., LTD. Experiment was performed under a project license (No. CDYFY-IACUC-202208QR027) granted by ethics board of the First Affiliated Hospital of Nanchang University, in compliance with the First Affiliated Hospital of Nanchang University guidelines for the care and use of animals.

Primary astrocyte OGD/R modeling and drug treatment

The primary astrocyte OGD/R model was developed based on the previously described protocol with minor changes (22). Briefly, the primary astrocytes were collected and washed with phosphate-buffered saline (PBS). For OGD insult, the cells were grown in glucose- and FBS-free DMEM for 6 hours at 37 ℃ in an anoxic incubator. For reperfusion, the culture medium was changed back to high-glucose DMEM, containing 10% fetal bovine serum (FBS), and kept in a normoxic incubator for 24 hours. The cells in the control group were always cultured with normal high-glucose and FBS-containing DMEM in a regular oxygen incubator. To understand the effect of sodium Danshensu, the cells were exposed to OGD/R insult with or without sodium Danshensu (0, 25, 50, and 100 μM), except the control group.

3-(4,5-dimethylthiazol-2-yl)-2,5-dophenyl tetrazolium bromide (MTT) assay

A MTT (Sigma-Aldrich, St. Louis, MO, USA) assay was performed to evaluate cell viability. Following specific treatment, the primary astrocytes were plated in a 96 well plate at a density of 2×10^4 cells/well and incubated for 24 hours. The MTT (5 mg/mL) solution was added to each well and kept at 37 ℃ for 4 hours. The culture medium was then discarded, and 150 μL of dimethyl sulfoxide (DMSO) was applied to each well. Finally, the absorbance was recorded at 490 nm by a microplate reader (Bio-Rad Instruments, Hercules, CA, USA).

Flow cytometry assay

Apoptosis was analyzed by Annexin V/propidium iodide (PI) flow cytometry kit [Becton, Dickinson, and Co. (BD), Franklin Lakes, NJ, USA], following the manufacturer's protocol. After treatment, the cells were harvested and washed with 250 μL ice-cold PBS, followed by staining with Annexin V and PI for 30 minutes in the absence of light on ice. The cells were then washed with PBS to remove unbound antibodies and resuspended in 200 μL PBS buffer for flow cytometry. The apoptosis was detected via a flow cytometer (BD Bioscience, USA), and the data was analyzed by Flowjo software (BD, USA).

Estimation of caspase-3 activity

Caspase-3 activity was evaluated using a caspase-3 assay kit (Beyotime, Haimen, Jiangsu, China). Following treatment, the cells were collected, and a caspase-3 kit was utilized by following the manufacturer's protocol. The absorbance was measured at 405 nm by a spectrophotometer (Bio-Rad Instruments, USA), and the results were expressed as U/mL.

Enzyme-linked immunosorbent assay

The level of cytokines [tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6] secretion were quantified using commercialized enzyme-linked immunosorbent assay (ELISA) kits (Abcam, Cambridge, UK) by following manufacturer's instructions, and the absorbance was determined by spectrophotometer (Bio-Rad Instruments, USA).

Lactate dehydrogenase (LDH) assay

To determine the LDH release in the primary astrocytes, an LDH cytotoxicity detection kit (Beyotime, China) was used according to the manufacturer's instructions.

Western blotting

The total proteins were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Solarbio, Beijing, China), and the protein was quantified with a bicinchoninic acid (BCA) protein assay kit (Tanon, Shanghai, China). About 40 g of the proteins were separated according to the mass by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, the proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked with 5% nonfat milk, then incubated with the respective primary antibodies: NOD-like receptor pyrin domain containing 3 (NLRP3) (cat. no. Ab263899; 1:1,000; Abcam), high mobility group protein B1 (HMGB1) (cat. no. Ab18256; 1:1,000; Abcam), phosphorylated-p65 (p-p65) (cat. no.

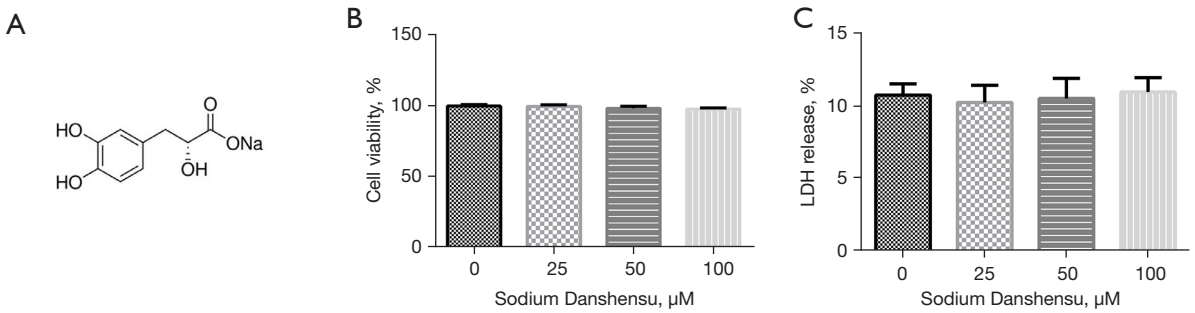

Figure 1 The effect of sodium Danshensu on cell cytotoxicity. (A) The chemical structure of sodium Danshensu; (B) the effect of sodium Danshensu on cell viability was determined by MTT assay; (C) the LDH release was determined by the LDH ELISA kit. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; LDH, lactate dehydrogenase; ELISA, enzyme-linked immunosorbent assay.

Ab76302; 1:1,000; Abcam), p65 (cat. no. 8242; 1:1,000; Cell Signaling Technology, Inc. (CST), Danvers, MA, USA), Beclin1 (cat. no. Ab207612; 1:1,000; Abcam), P62 (cat. no. 39749; CST), LC3B (cat. no. Ab192890; 1:1,000; Abcam), tuberous sclerosis complex-2 (TSC2) (cat. no. 3990; 1:1,000; CST), p-mammalian target of rapamycin (mTOR) (cat. no. Ab137133; 1:1,000; Abcam), mTOR (cat. no. Ab134903; 1:1,000; Abcam), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (cat. no. 5174; 1:1,000; CST) for 24 hours at 4 ℃. Subsequently, the membranes were washed, and the secondary antibody (cat. no. ab96899; 1:2,000; Abcam) was applied to conjugate for 2 hours at room temperature. Finally, the western blot images were visualized using enhanced chemiluminescence (ECL) and recorded by Gel Imaging System (Bio-Rad, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR was used to determine the NLRP3, high-mobility group box 1 (HMGB1), p-p65, p65, TSC2, and mTOR expression levels after sodium Danshensu treatment. The cells were collected, and the total RNA was extracted by using TRIzol (Invitrogen, Carlsbad, CA, USA) RNA extraction kit from astrocytes, and the RNA concentration was determined by a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). About 2 μg total RNA was used to synthesize complementary DNA using PrimeScript RT Reagent Kit (Takara Bio., Beijing, China). The qPCR reaction was conducted using SYBR Green PCR Master mix (Takara, Otsu, Japan) in a thermocycler (Thermo Fisher Scientific, USA). The relative messenger RNA (mRNA) levels of target genes were analyzed by $2^{-\Delta\Delta Ct}$ formula.

Statistical analysis

All experiments were repeated at least 3 times. The data analysis was conducted by using SPSS 20.0 software (IBM Corp., Armonk, NY, USA). The data were presented as mean ± SD. One-way analysis of variance (ANOVA) followed by Tukey's test was used to compare the difference among 3 or more groups.

Results

The effects of sodium Danshensu on cell cytotoxicity

The chemical molecular structure formula of sodium Danshensu is displayed in *Figure 1A*. To understand the toxic effect of sodium Danshensu, we treated primary cultured astrocytes with different amounts (0, 25, 50, and 100 μM) of sodium Danshensu for 24 hours. We found that sodium Danshensu had no considerable effect on cell viability and LDH production from primary astrocytes (*Figure 1B,1C*). It was implied that sodium Danshensu is not toxic to primary cultured astrocytes.

Sodium Danshensu protects OGD/R-induced primary astrocyte injury

Whether sodium Danshensu can prevent astrocytes injury, we established the OGD/R model according to previously reported protocols with minor modifications (22). The OGD/R group showed a substantial decrease in cell viability (*Figure 2A*), a significant increase in LDH release (*Figure 2B*), a significant increase in apoptosis (*Figure 2C,2D*), and a considerable increase in caspase-3 activity (*Figure 2E*) compared to the control group. We treated OGD/R

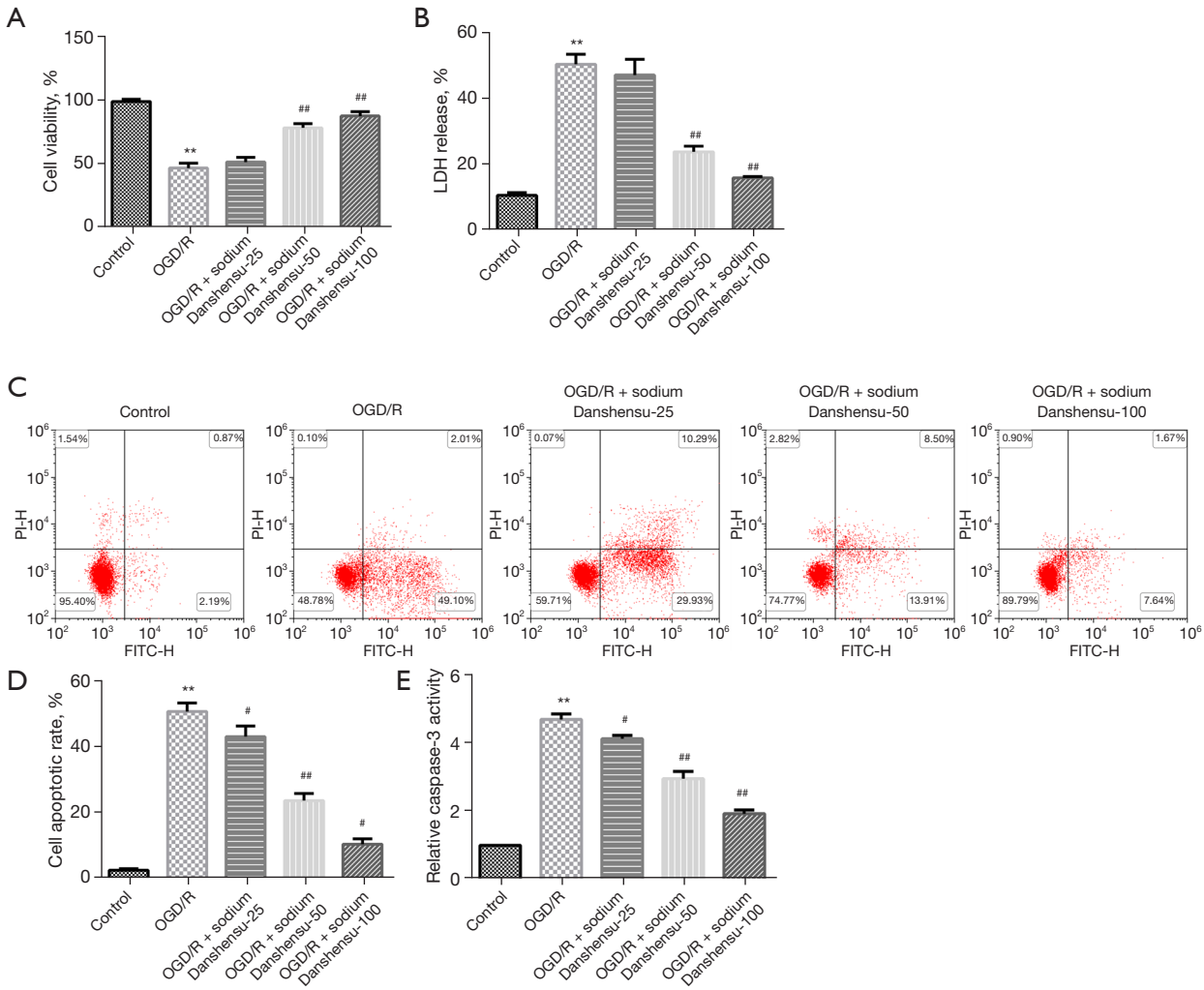

Figure 2 The protective effects of sodium Danshensu on OGD/R insult primary astrocytes. (A) The cell viability was reduced in the OGD/R group, determined by MTT assay; (B) the OGD/R group showed a significant increase in LDH release, which was evaluated by the LDH ELISA kit; (C,D) FCM was used to determine cell apoptosis; (E) caspase-3 activity was determined. OGD/R, oxygen glucose deprivation/reoxygenation; PI, propidium iodide; FITC, fluorescein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; LDH, lactate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; FCM, flow cytometry. **P<0.001, showed significant difference; #P<0.01, showed significant difference; ##P<0.05, showed significant difference.

exposed astrocytes with various doses of sodium Danshensu and found that sodium Danshensu dose-dependently decreased LDH release, increased cell viability, decreased apoptosis, and decreased caspase-3 activity in comparison to the OGD/R group. Besides, IL-6, IL-1β, and TNF-α secretion were markedly increased in the supernatant of the OGD/R group. While sodium Danshensu showed a dose-dependent decrease in IL-6, IL-1β, and TNF-α secretion compared with the OGD/R group (*Figure 3A-3C*). The mRNA expression level of TNF-α, IL-6, and IL-1β was the same pattern (*Figure 3D-3F*).

Besides, the protein and mRNA expression of NLRP3 and HMGB1 were considerably upregulated in primary astrocytes of OGD/R group (*Figure 4A-4C*). The p-p65 protein and p-p65/p65 ratio were also markedly enhanced (*Figure 4D,4E*), while p65 protein and mRNA expression were similar between the groups (*Figure 4D,4F*) in OGD/R group. On the other hand, sodium Danshensu showed a dose-dependent decreased in protein and mRNA levels of NLRP3 and HMGB1 (*Figure 4A-4C*). The p-p65

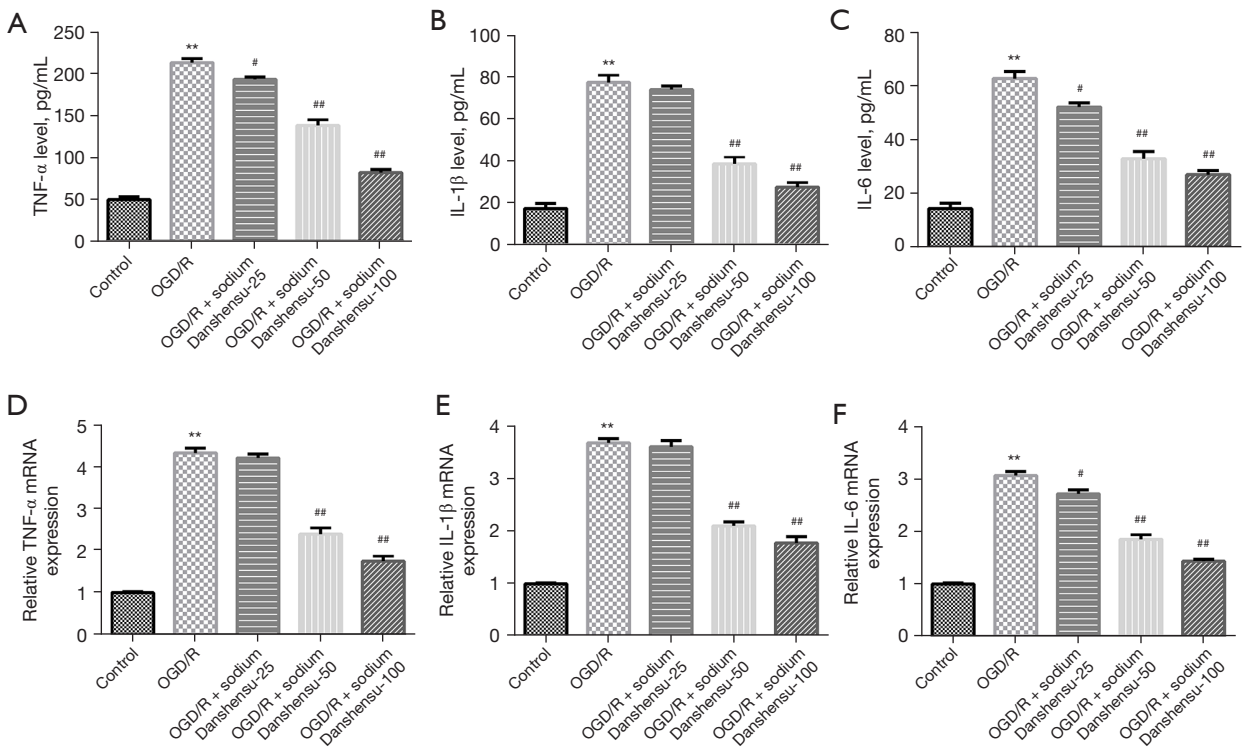

Figure 3 The effect of sodium Danshensu on cytokine production. (A-C) The proinflammatory cytokines were determined by ELISA kits, sodium Danshensu reduced TNF-α, IL-1β, and IL-6 secretion. (D-F) qRT-PCR analysis of TNF-α, IL-1β, and IL-6. OGD/R, oxygen glucose deprivation/reoxygenation; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6; ELISA, enzyme-linked immunosorbent assay; qRT-PCR, quantitative real-time polymerase chain reaction. **P<0.001, showed significant difference; #P<0.01, showed significant difference; ##P<0.05, showed significant difference.

protein and p-p65/p65 ratio were considerably reduced (*Figure 4D*,*4E*). Besides, the p65 protein and mRNA expression were incomparable after sodium Danshensu treatment (*Figure 4D*,*4F*). These results suggested that sodium Danshensu could protect astrocytes from ischemia/reperfusion insult.

Sodium Danshensu promotes OGD/R-induced autophagy in primary astrocytes

Autophagy flux has an inevitable function in astrocytes injury and highly associated with ischemic stroke. To understand the role of autophagy and sodium Danshensu in our *in vitro* OGD/R model, we studied the role of sodium Danshensu on astrocyte autophagy. After OGD/R insult, there was no considerable change in autophagy-associated protein Beclin 1 expression in primary astrocytes (*Figure 5A*,*5B*). While we noticed an increase in P62 protein expression (*Figure 5A*,*5C*) and LC3B-II/LC3B-I ratio (*Figure 5A*,*5D*) after OGD/R insult. Interestingly, sodium Danshensu dose-dependently upregulated Beclin 1 expression in primary astrocytes, decreased P62 protein level, and further increased LC3B-II/LC3B-I ratio compared to the OGD/R group, suggesting that autophagy was promoted after sodium Danshensu treatment.

To further understand the molecular mechanism, we studied the role of sodium Danshensu on different autophagy-related pathways and found TSC2/mTOR was the most obvious one. The TSC2/mTOR pathway is an important pathway in autophagy regulation. After OGD/R insult, we found there was no significant change in TSC2 protein in primary astrocytes (*Figure 6A*,*6B*), while the p-mTOR protein (*Figure 6A*) and p-mTOR/mTOR (*Figure 6C*) ratio were slightly reduced. Notably, sodium Danshensu showed a dose-dependently increased TSC2 protein and mRNA expression and further decreased p-mTOR protein levels and p-mTOR/mTOR ratio in astrocytes compared to the OGD/R group. These findings demonstrate that

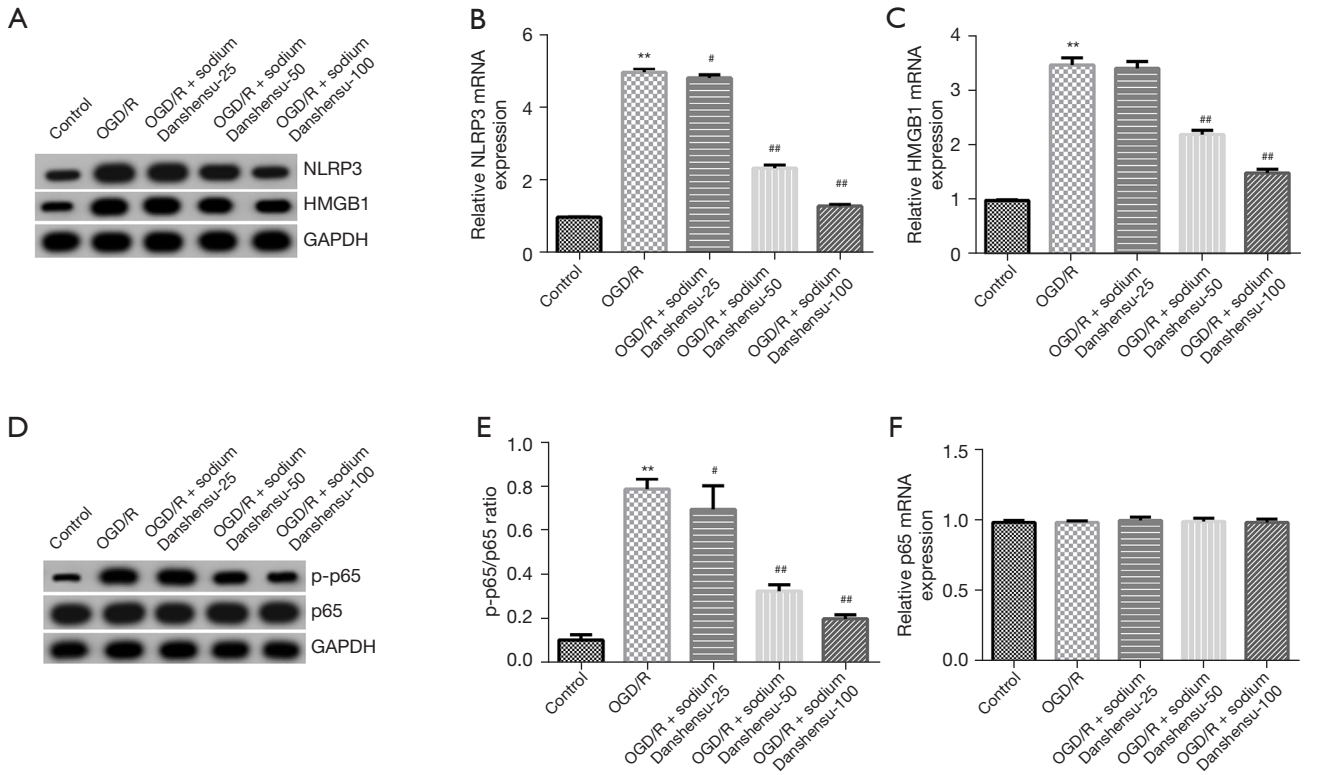

Figure 4 The role sodium Danshensu reduces NLRP3 inflammasome. (A-C) The protein and mRNA of NLRP3 and HMGB1 were significantly reduced in primary astrocytes after treatment, determined by western blot and qRT-PCR, respectively; (D,E) the p-p65 protein and p-p65/p65 ratio were considerably reduced after treatment; (D,F) the p65 protein and mRNA expression were similar after sodium Danshensu treatment. OGD/R, oxygen glucose deprivation/reoxygenation; NLRP3, nucleotide binding oligomerization domain-like receptor family pyrin domain protein 3; HMGB1, high mobility group protein B1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA; qRT-PCR, quantitative real-time polymerase chain reaction; p-p65, phosphorylated-p65. **P<0.001, showed significant difference; #P<0.01, showed significant difference; ##P<0.05, showed significant difference.

sodium Danshensu may promote autophagy in OGD/R exposed astrocytes by modulating the TSC2/mTOR pathway.

Discussion

Ischemic stroke is a leading cause of neurological morbidity and mortality globally, and the rate of death and disability due to stroke are gradually increasing (27,28). Hypoxia and ischemia in brain tissue after cerebral ischemia led to inadequate energy supply, which in turn promotes neuronal damage. In the absence of oxygen, mitochondrial function is dysregulated, leading to cellular damage, such as astrocyte injury, which is critical for neurons in ischemic stroke (29). Therefore, restoring cellular energy balance and astrocyte homeostasis are the key to the management of stroke. In this study, we investigated the role of sodium Danshensu in OGD/R-induced astrocyte injury. Our results shed light on the molecular mechanisms behind sodium Danshensu-mediated astrocyte protection during ischemic stroke.

Previous studies have demonstrated that sodium Danshensu has neuroprotective effects, and most importantly, can cross the blood brain barrier (18,30). It also has a strong capacity to increase superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity and decrease the level of malondialdehyde (MDA) and the reactive oxygen species (ROS), which make sodium Danshensu a potent antioxidant agent (30,31). Moreover, Danshensu and Danshen derivatives decrease inflammation, and they have protective roles in myocardial ischemia-reperfusion injury (7,32,33). These results suggested that sodium Danshensu could help to prevent subsequent

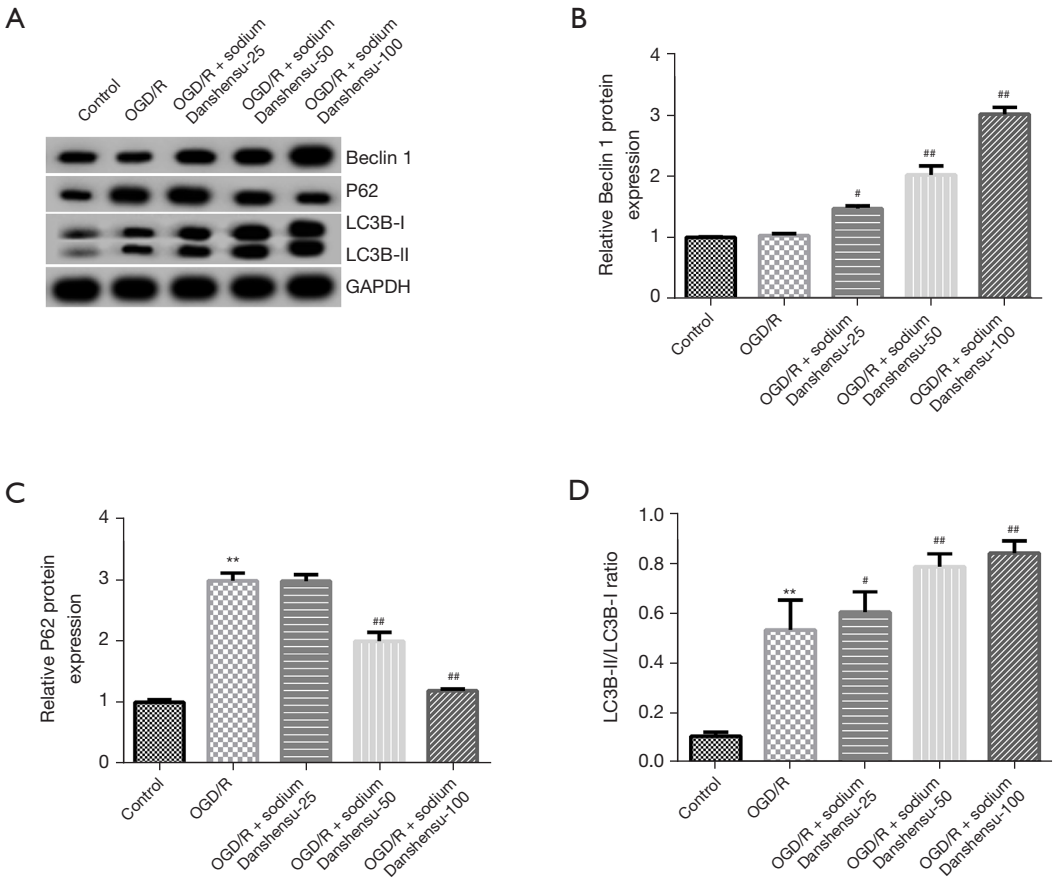

Figure 5 The impact of sodium Danshensu on astrocytes autophagy. (A,B) The mRNA and protein of Beclin 1 were dose-dependently upregulated after treatment; (A,C) P62 protein expression was downregulated in sodium Danshensu treatment group; (A,D) the LC3B-II/LC3B-I ratio was further increased by sodium Danshensu, the mRNA and proteins were analyzed by qRT-PCR, and western blot, respectively. OGD/R, oxygen glucose deprivation/reoxygenation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA; qRT-PCR, quantitative real-time polymerase chain reaction. **P<0.001, showed significant difference; #P<0.01, showed significant difference; ##P<0.05, showed significant difference.

damage responses after ischemia. However, the molecular mechanism was not elucidated, and further investigation is required.

In this study, we used primary astrocytes to understand the effect of sodium Danshensu in astrocyte injury caused by the OGD/R insult. Primary astrocytes exposed to OGD/R insult are widely used as an *in vitro* cell model of ischemic stroke (22,34,35). Here, we adopted the OGD/R model of primary astrocytes to mimic cerebral ischemia-reperfusion *in vitro*. According to the literature, the concentration range of danshansu sodium is 0.5–80 mg /L (360 μM), and in this concentration range, danshansu sodium can play the best therapeutic effect, with higher safety (35,36). To understand the toxic effect of sodium Danshensu, we cultured primary astrocytes with different doses of sodium Danshensu (0, 25, 50, and 100 μM) for 24 hours, and assessed the cell viability and LDH production, which revealed that sodium Danshensu did not affect the cell cytotoxicity.

A Previous study has demonstrated that ischemic stroke induces neural cell injury and promotes apoptosis (29). To understand whether sodium Danshensu protect astrocytes injury in OGD/R-induced ischemic model, we studied the effect of sodium Danshensu on an OGD/R model. The cell viability was markedly decreased, while LDH release, caspase-3 activity, and apoptosis were considerably enhanced in the model group. Importantly, sodium Danshensu dose-dependently increased cell viability, reduced apoptosis, caspase-3 activity, and LDH production. Besides, sodium

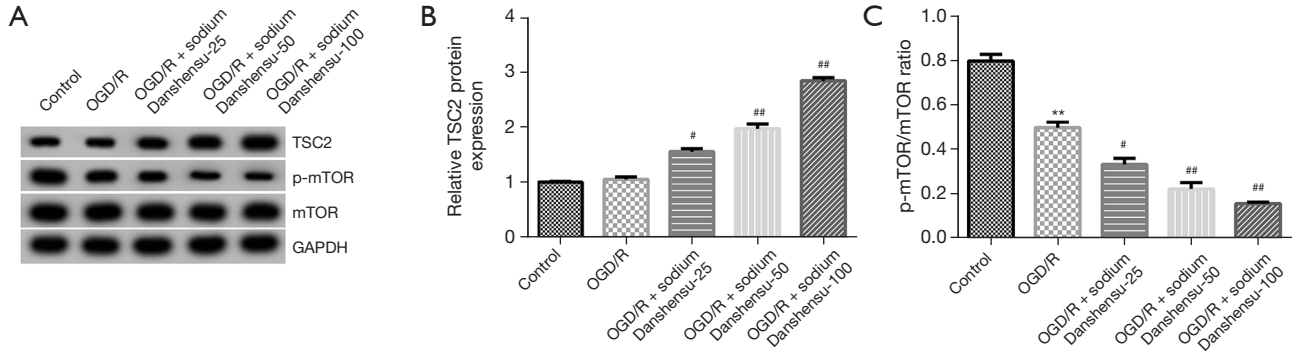

Figure 6 Sodium Danshensu regulates TSC2/mTOR pathway. (A,B) Sodium Danshensu showed a dose-dependent increase in TSC2 protein expression; (C) sodium Danshensu further decreased p-mTOR protein expression and p-mTOR/mTOR ratio in astrocytes compared with OGD/R group, the mRNA and proteins were evaluated by qRT-PCR and western blot, respectively. OGD/R, oxygen glucose deprivation/reoxygenation; TSC2, tuberous sclerosis complex-2; mTOR, mammalian target of rapamycin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA; qRT-PCR, quantitative real-time polymerase chain reaction. **P<0.001, showed significant difference; #P<0.01, showed significant difference; ##P<0.05, showed significant difference.

Danshensu lowered the secretion of ischemia-associated cytokines, such as TNF-α, IL-1β, and IL-6, which are usually upregulated in stroke and promote tissue injury (37). Moreover, sodium Danshensu showed a dose-dependent decrease in protein and mRNA expression of NLRP3 and HMGB1. NLRP3 inflammasome can be stimulated by a variety of molecules and events, such as reactive oxygen species, mitochondrial dysfunction, etc. (38). NLRP3 inflammasome can be induced and activated in cerebral arterial thrombosis, promoting brain cell death and playing an important role in brain cell injury. Inhibition of NLRP3 inflammasome activation improves cerebral ischemia outcome (39,40). Many traditional Chinese medicine components also regulate the activation of NLRP3 inflammasome. Currently, more than 20 traditional Chinese medicine monomers such as resveratrol, colchicine and chrysophanol are known to inhibit the formation of NLRP3 inflammasome and play a therapeutic role in ischemic stroke (41). Also, HMGB1 has an important function in ischemic stroke in that it activates innate immunity and promotes proinflammatory cytokine release. Besides, HMGB1 can activate the NLRP3 inflammasome and further promote neural injury (39,42). Additionally, the p-p65 protein and p-p65/p65 ratio were considerably reduced, which are usually upregulated after ischemia and tend to cooperate with cellular injury (43). These results suggest that sodium Danshensu can protect OGD/R-induced primary astrocyte injury via modulating the NLRP3 inflammasome.

Autophagy is a catabolic process in which cells eliminate destroyed impaired proteins and organelles via lysosome-dependent pathway to preserve the homeostatic balance (44). Autophagy plays controversial roles in ischemic brain injury (15). According to increasing evidence, autophagy has a protective effect in ischemic stroke (25). Importantly, activation of autophagy reduces inflammation and improves overall survival in rat models of cerebral ischemia (45,46). Therefore, modulating autophagy may be beneficial in preventing or treating ischemic stroke.

In recent years, many techniques and markers have been established for determining autophagy in cells and animals (37). Notably, Beclin 1 and LC3 are essential autophagy-related factors. *In vitro* astrocyte autophagy is indicated by the levels of autophagic substrate p62, and autophagy-associated protein of LC3-II, Beclin-1 expression (14,47). Our study found that sodium Danshensu dose-dependently upregulated Beclin 1 expression in primary astrocytes, reduced P62 protein expression, and further increased LC3B-II/LC3B-I ratio compared with the OGD/R group. To further elucidate the role of sodium Danshensu on astrocytes autophagy, we studied its effect on TSC2 and the mTOR signaling pathway, which is highly linked to autophagy. While TSC2 regulates the phosphorylation of the mTOR, which controls autophagy, TSC2 is now widely acknowledged to have a function in brain ischemia-reperfusion injury (34,48).

Interestingly, we noticed sodium Danshensu dose-dependently increased TSC2 protein expression and significantly reduced p-mTOR protein expression and

p-mTOR/mTOR ratio in astrocytes compared with the OGD/R group. However, the TSC2 protein in the control and OGD/R groups was similar. Altogether, our results suggested that sodium Danshensu mediated neuroprotection, which may be due to sodium Danshensu-induced astrocyte autophagy via modulating the TSC2/mTOR pathway. Therefore, in the following experiments, we can conduct *in vivo* experiments and use danshensu sodium to treat cerebral ischemia-reperfusion mice, so as to further explore the therapeutic effect of danshensu sodium on ischemic stroke.

Conclusions

In conclusion, our study provides experimental evidence for the protective effect of sodium Danshensu on cerebral OGD/R injury. It inhibits OGD/R-mediated astrocyte injury through modulating the NLRP3 inflammasome and TSC2/mTOR pathways. The present findings further our understanding of sodium Danshensu's neuroprotective effect and its underlying mechanism in ischemic stroke.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-2143/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-2143/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-2143/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. Experiment was performed under a project license (No. CDYFY-IACUC-202208QR027) granted by ethics board of the First Affiliated Hospital of Nanchang University, in compliance with the First Affiliated Hospital of Nanchang University's guidelines for the care and use of animals.

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