Astragalin alleviates ischemia/reperfusion-induced brain injury via suppression of endoplasmic reticulum stress

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Abstract. Excessive apoptosis and neuronal dysfunction are pathological features of ischemic stroke. Previous studies have demonstrated that astragalin (AST) exerted both anti-apoptotic and anti-inflammatory effects in several types of disease, although its potential effect in ischemic stroke remains unclear. The purpose of the present study was to investigate the effects of AST on cerebral ischemia/reperfusion (I/R)-induced brain injury and the underlying mechanisms. Brain injury was assessed in an experimental rat model using measurement of neurological scores and inflammatory factors. To assess the role of AST in I/R-induced brain injury and the potential mechanism of action, SH5Y were treated with thapsigargin and AST. Apoptotic rate and ER stress levels were measured by western blotting, reverse transcription-quantitative PCR and immunofluorescence staining. It was discovered that AST significantly improved long-term neurological outcomes in rats following cerebral I/R injury, through the attenuation of the expression levels of apoptotic proteins (Bax and cleaved-caspase-3) and the release of inflammatory cytokines, as well as upregulating the expression levels of the anti-apoptotic protein Bcl-2. Furthermore, AST attenuated the expression levels of the endoplasmic reticulum (ER) stress-related protein, glucose-regulated protein, 78 kDa, as well as its downstream apoptotic mediators (CHOP and caspase-12). Thapsigargin-induced ER stress activation and apoptosis were also attenuated by AST in an *in vitro* neuronal cell culture model. In conclusion, these results suggested that AST may protect against I/R-induced brain injury, thus, highlighting its therapeutic potential in patients with ischemic stroke.

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Introduction

Cerebral ischemia-reperfusion (I/R) is a devastating cerebrovascular disease, accounting for significant morbidity and mortality rates worldwide, with an estimated 41.6% death rate every year (1). The prevention and treatment of I/R injuries remains an important challenge in clinical practice (1). Various pathophysiological processes have been discovered to be tightly involved with cerebral I/R injury, with excessive inflammation and neuronal apoptosis among the most critical responses (2,3). However, the specific mechanisms triggered by I/R injury remain unclear, although numerous mechanisms are known to contribute to neuronal death.

There is increasing evidence to suggest that endoplasmic reticulum (ER) stress is tightly involved with the pathophysiological processes of numerous types of central nervous system disease, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and Huntington's disease (4,5). Moreover, ER stress has been noted to serve an essential role in cerebral I/R injury (6). The ER is the primary site for the synthesis, processing and transportation of functional proteins, and has a vital role in the maintenance of cellular homeostasis (7). ER function has been identified to be inhibited by several factors, including unfolded protein accumulation and an imbalance of Ca^{2+} homeostasis, both of which contribute to ER stress (7). The accumulation of misfolded proteins within the ER initiates the unfolded protein response (UPR) (8), which is regulated by several signaling pathways, including activating transcription factor 6 (ATF6) and protein kinase RNA-like endoplasmic reticulum kinase (PERK), contributing to apoptotic activity and neuronal apoptosis (9).

Astragalin (AST), also known as kaempferol-3-O-glucoside, is a flavonoid compound that is isolated from various traditional herbs, such as leaves of persimmon or Rosa agrestis (10). AST has been discovered to exert inhibitory effects on apoptosis and inflammation in the treatment of various types of disease; for example, AST was reported to improve antioxidant activity and inhibit the inflammatory responses during spermatogenesis in a streptozotocin-induced diabetes model (11), while another study revealed that AST regulated the activity of ER stress in varicocelized rats (12). However, to the best of our knowledge, it remains unclear whether AST serves a role in ischemic stroke. The present study aimed to determine the effect of AST and its potential mechanism of action in cerebral

I/R injury *in vivo* and *in vitro*. The effects of AST on cerebral I/R injury were hypothesized to be mediated through the regulation of inflammation and apoptosis.

Materials and methods

Animal model. Male Sprague-Dawley rats (300-320 g; 8-weeks-old; n=30) were purchased from the Animal Center of the Chinese Academy of Sciences (Shanghai, China) Animals were housed at 23±2˚C and 40-70% humidity with a 12-h light/dark cycle and *ad libitum* access to food and water. Rats (n=10) were assigned into three groups: i) Sham; ii) I/R; and iii) I/R + AST. The cerebral I/R injury model was developed according to the intraluminal occlusion method previously described (13). Briefly, animals were anesthetized using 50 mg/kg pentobarbital. A nylon thread (0.3 mm) was then embedded into the external carotid artery and extended to the middle cerebral artery. The thread was removed to induce I/R injury after 2 h of ischemia. In the sham group, the thread was extended to the middle cerebral artery and immediately removed. Animals in the I/R + AST group received 50 mg/kg/day of AST orally, beginning immediately post operation (14). Rats in the other groups received equal volumes of saline. The animals were sacrificed 3 days after brain I/R injury for further research (western blotting and PCR) (n=5 in each group). All animal research was approved by the Animal Care and Use Committee of Hainan Medical University (Haikou, China).

Neurological score. Neurological outcomes were measured using a foot fault test, as previously described (15). The animals were trained for 3 days pre-operation; an initial neurological outcome was then recorded for use as a baseline at day 0. The neurological scores (15) (left forelimb score + foot fault score) of each group (Sham, I/R, I/R + AST; n=15) were subsequently recorded at 1, 3, 5, 7, 14 and 28 days post operation.

Chemicals and reagents. AST (purity >98%) was acquired from Chengdu Must Bio-Technology Co., Ltd. (Fig. 1). Thapsigargin (TG), a commonly used inducer of ER stress (6), was acquired from Sigma-Aldrich; Merck KGaA. Primary antibodies against CHOP (cat. no. 2895), glucose-regulated protein 78 kDa (GRP78; cat. no. 3183), Bax (cat. no. 2772), Bcl-2 (cat. no. 3498), cleaved (c)-caspase-3 (cat. no. 9661) and GADPH, and a caspase-3 activity kit and anti-mouse (cat. no. 7076) and anti-rabbit (cat. no. 7074) horseradish peroxide (HRP)-conjugated secondary antibodies (1:10,000) were acquired from Cell Signaling Technology, Inc. An *in situ* cell death detection kit (cat. no. PH0534) was acquired from Roche Diagnostics. Other reagents were acquired from Sigma-Aldrich; Merck KGaA, unless otherwise specified.

Cell culture, drug treatment and Cell Counting Kit-8 (CCK-8) assay. The human-derived neuroblastoma SH-SY5Y cells were acquired from the American Type Culture Collection. SH-SY5Y cells were passaged every 3-5 days in DMEM (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in a cell incubator (5% CO_2, 37˚C).

To determine the effects of AST against TG-induced apoptotic activity, cells were seeded into a 96-well plate ($8x10^3$ cells/well). Cells were subsequently incubated with a range of AST concentrations (0-200 μM) with or without TG (10 μM) for 8 h at 37˚C. Following the incubation, 10 μl CCK-8 dye was added into every well for 1 h at 37˚C, and the optical density (450 nm) was detected on a microplate spectrophotometer according to the manufacturer's protocol.

Western blotting. SH-SY5Y cells were subsequently incubated with AST concentrations (50 μM) with or without TG (10 μM) for 24 h at 37˚C. Ischemic cortex and neuronal cell culture samples were homogenized in RIPA lysis buffer (Sigma-Aldrich; Merck KGaA) supplemented with PMSF (1:100) and quantified using a BCA protein assay kit to assess protein concentration. Proteins (50 μg) were resolved on an 8 and 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Samples were then incubated with 5% skimmed milk for 90 min at room temperature. Subsequently, the membranes were incubated with the following primary antibodies at 4˚C overnight: Anti-Bax (1:1,000), anti-Bcl-2 (1:1,000), anti-c-caspase-3 (1:1,000), anti-GRP78 (1:1,000), anti-CHOP (1:1,000) and anti-GAPDH (1:5,000). Following the primary antibody incubation, the membranes were incubated with a HRP-conjugated secondary antibody (1:10,000) for 60 min at room temperature. Finally, protein bands were detected using western blotting detection reagents (MAC Gene Technology Ltd.) by an imaging system (Bio-Rad Laboratories, Inc.). Data was analyzed using the Image Lab 3.0 software (Bio-Rad Laboratories, Inc.).

Apoptosis analysis. SH-SY5Y cells were planted onto cover slips in 6-well plates at a density of $5x10^5$ cells/ml. Cells were subsequently incubated with AST concentrations (50 μM) with or without TG (10 μM) for 24 h at 37˚C. Apoptotic activity was detected by both a TUNEL kit (and caspase-3 activity kit. Briefly, SH-SY5Y cells ($6x10^4$/ml) were incubated with precooled 4% paraformaldehyde for 1 h, followed by 3% (v/v) H_2O_2 for 15 min and then 0.1% Triton X-100 for 8 min at room temperature. Specimens were analyzed using an TUNEL kit solution at 37˚C for 2 h and nuclei were visualized following incubation with DAPI for 8 min at room temperature. TUNEL positive cells were analyzed using a Nikon Eclipse Ti confocal microscope (magnification, x10; Nikon Corporation), and the percentage of apoptotic cells was counted in three random high-power fields of three different slides and analyzed using GraphPad Prism (Version 8.0, GraphPad Software, Inc.). Caspase-3 activity was detected as a marker of apoptosis using a Caspase-3 Activity assay kit according the manufacturer's protocol.

Immunofluorescence assay. SH-SY5Y cells were planted onto cover slips in 6-well plates at a density of $5x10^5$ cells/ml. Cells were subsequently incubated with AST concentrations (50 μM) with or without TG (10 μM) for 24 h at 37˚C. Following drug treatment, cells were incubated with 4% paraformaldehyde for 1 h, 0.5% Triton X-100 for 8 min, then 5% BSA (Sigma-Aldrich; Merck KGaA) for 45 min. Samples were subsequently incubated with an anti-caspase 12 primary antibody (1:200; cat. no. 62484; Abcam) overnight at 4˚C. Following the

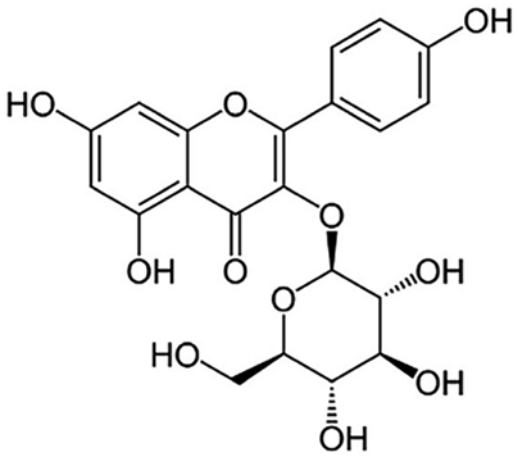

Figure 1. Chemical structure of astragalin.

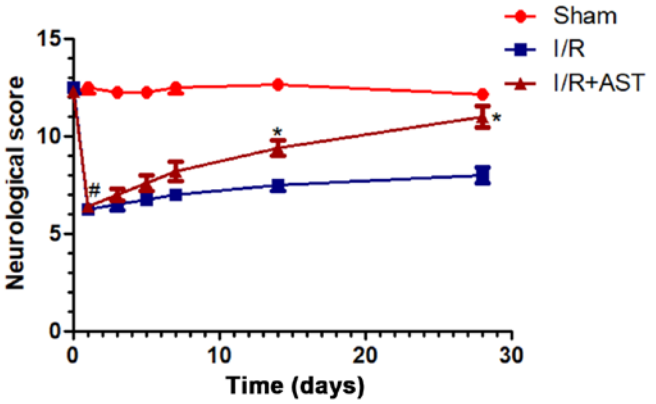

Figure 2. AST improves the long-term neurological outcomes *in vivo*. Data are presented as the mean ± SD, n=5/group. #P<0.05 vs. sham group, *P<0.05 vs. I/R + AST group. AST, astragalin; I/R, ischemia/reperfusion.

primary antibody incubation, the sections were incubated with Donkey Anti-Rabbit IgG-TRITC secondary antibody (1:500; cat. no. 7980; Abcam) for 1 h at room temperature. Nuclei were visualized by incubation with DAPI for 8 min at room temperature. Samples were then visualized using a Nikon Eclipse Ti microscope (magnification, x40; Nikon Corporation).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the ischemic cortex of rats using TRIzol® reagent (Sigma-Aldrich; Merck KGaA). RNA was then quantified by spectrophotometry by calucluating the optical density (OD)260 and OD280 ratio. Total RNA(1 μg) was reverse transcribed into cDNA using a PrimeScript RT reagent kit (Takara Bio, Inc.) as follows: 2 μl 5X PrimeScript buffer, 0.5 μl PrimeScript RT Enzyme Mix, 2 μl total RNA, 5 μl RNase Free distilled H_2O. The reaction was carried out in water bath at 37˚C for 15 min, and then at 85˚C for 15 sec. qPCR was subsequently performed using a SYBR Premix Ex Taq mixture (Takara Bio, Inc.). The expression levels of target genes in the ischemic cortex were quantified using the $2^{-\Delta\Delta Cq}$ method (16). PCR primers were as follows: IL-1β forward, 5'-AATGACCTGTTCTTTGAGGCTGAC-3' and reverse, 5'-CGAGATGCTGCTGTGAGATTTGAAG-3'; IL-6 forward 5'-AGGAACGAAAGTCAACTCCATCTG-3' and reverse, 5'-GGCAGTGGCTGTCAACAACATC-3'; TNF-α forward, 5'-AGTCCGGGCAGGTCTACTTT-3' and reverse, 5'-TTCAGCGTCTCGTGTGTTTC-3'; IL-8 forward, 5'-CAGAGACTTGGGAGCCACTC-3' and reverse 5'-GCTGAAATTATCCACCCTGATT-3'; cyclooxygenase (COX)-2 forward, 5'-TGAGTACCGCAAACGCTTCTC-3' and reverse, 5'-TGGACGAGGTTTTTCCACCAG-3'; inducible nitric oxide synthase (iNOS) forward, 5'-ATGGAACAGTATAAGGCAAACACC-3' and reverse, 5'-GTTTCTGGTCGATGTCATGAGCAAAGG-3'; β-actin forward, 5'-GAGAGGGAAATCGTGCGT-3' and reverse, 5'-GGAGGAAGAGGATGCGG-3'.

Statistical analysis. Each experiment was performed at ≥3 times and data are represented as the mean ± SD. All statistical significances between groups were calculated using a one-way ANOVA and the Tukey's post hoc test using Prism (Version 8.0, GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

AST improves long-term neurological outcomes in rats with cerebral I/R injury. To investigate the effects of AST on cerebral I/R injury, animals were examined following I/R injury with and without AST treatment. Compared with sham group, neurological scores markedly decreased in I/R model rats at 1-day post operation, and then recovered slowly during one-month post operation (Fig. 2). Notably, AST administration significantly improved neurological scores by day-14 and day-28 post operation compared with the I/R group, which indicated that AST administration may improve the long-term neurological outcomes in rats with cerebral I/R injury.

AST suppresses mRNA expression levels of inflammatory cytokines induced by cerebral I/R injury in vivo. The mRNA expression levels of the inflammatory factors, IL-1β, IL-6, IL-8, TNF-α, COX-2 and iNOS, were analyzed by RT-qPCR. Significant inflammatory-related changes were evident in the rats with I/R injury; the expression levels of the inflammatory factors were significantly upregulated in the I/R group compared with the sham group (Fig. 3). In contrast, the I/R + AST group demonstrated significantly downregulated mRNA expression levels of inflammatory cytokines compared with the I/R group, which indicated that AST may regulate inflammatory gene responses in the cerebral I/R injury model.

AST administration downregulates the expression levels of apoptotic-related proteins in vivo. To determine whether AST could affect the protein expression levels of apoptosis-related proteins following I/R injury, the expression levels of apoptosis-related proteins [apoptotic protein (Bax and c-caspase3) and anti-apoptotic protein Bcl-2] were analyzed using western blotting analysis. The I/R group exhibited significantly (~4 fold) upregulated Bax expression levels compared with the sham group, which were subsequently significantly downregulated in the I/R + AST treatment group (Fig. 4A and B). Furthermore, the I/R + AST group exhibited a significant upregulation in the expression levels of the anti-apoptotic protein Bcl-2 compared with the I/R model group (Fig. 4A and C). Finally, the expression levels of c-caspase-3 were significantly upregulated in the

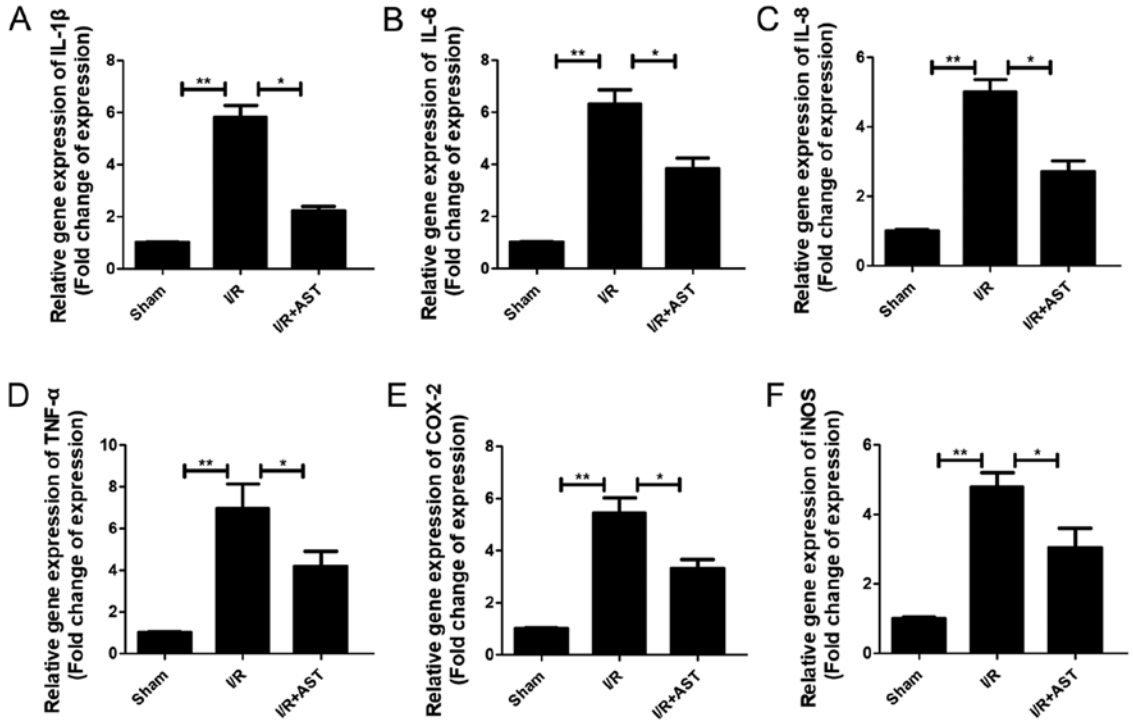

Figure 3. AST suppresses the release of inflammatory cytokines in cerebral I/R injury model rats. The relative mRNA expression levels of (A) IL-1β, (B) IL-6, (C) IL-8, (D) TNF-α, (E) COX-2 and (F) iNOS in cerebral I/R injury model rats with or without AST treatment (50 μM) were analyzed using reverse transcription-quantitative PCR. Data are presented as the mean ± SD, n=5/group. *P<0.05, **P<0.01. AST, astragalin; COX-2, cyclooxygenase-2; I/R, ischemia/reperfusion; iNOS, inducible nitric oxide synthase.

I/R group compared with the sham group, but subsequently significantly downregulated in the I/R + AST group compared with the I/R group (Fig. 4A and D). Together, these findings suggested that AST may downregulate the apoptotic rate in a cerebral I/R injury model.

AST attenuates ER stress and related apoptotic protein expression levels induced by cerebral I/R injury in vivo. To determine whether the protective effects of AST were tightly associated with its regulation of ER stress, the expression levels of the ER stress-related proteins GRP78 and CHOP in the rats following AST treatment were compared with the rats that did not receive AST treatment. The I/R group exhibited a significant upregulation of GRP78 (Fig. 5A and B) and CHOP (Fig. 5A and C) expression levels compared with the sham group, which were subsequently significantly downregulated following the administration of AST in both cases. These findings indicated that ER stress may be associated with the protective effects of AST in rats with cerebral I/R injury.

AST improves survival in TG-treated SH-SY5Y cells. CCK-8 assays were used to analyze the cytoprotective role of AST on ER stress-stimulated SH-SY5Y cells. Preliminary analyses revealed that AST did not exhibit any significant cellular toxicity at doses of up to 200 μM (Fig. 6A). Thus, to exclude the potential toxic effects of AST on cells, 0-100 μM AST was used in all subsequent experiments. To further investigate whether the cytoprotective effects of AST were related to its regulation of ER stress, SH-SY5Y cells were treated with TG, a commonly used inducer of ER stress. The viability of TG-treated SH-SY5Y cells was significantly decreased compared with the control group; this viability was significantly rescued by the administration of AST (25-100 μM; Fig. 6B). Together, these data indicated that AST may exert a cytoprotective effect on TG-treated SH-SY5Y cells.

AST inhibits TG-induced ER stress and downregulates apoptotic protein expression levels in SH-SY5Y cells. Western blotting analysis discovered that the TG-treated cells exhibited significantly upregulated expression levels of GRP78 and the downstream apoptotic protein CHOP compared with the control cells. Notably, the upregulation of both GRP78 and CHOP expression levels was significantly reversed following the administration of AST (Fig. 7A-C). Immunofluorescence analysis of caspase-12 expression levels suggested there was higher level of caspase12-positive puncta in TG-induced apoptotic cells, which was reversed by the treatment of AST (Fig. 7D). Together, these data provided strong evidence to suggest that AST treatment may attenuate ER stress.

AST inhibits TG-induced apoptosis in vitro. To further investigate the association between ER stress and the anti-apoptotic effects of AST in the cerebral I/R injury model, the expression levels of apoptotic proteins were analyzed in SH-SY5Y cells. Western blotting analysis revealed that AST administration partially reversed the significant upregulation of Bax and downregulation of Bcl-2 expression levels observed in TG-treated SH-SY5Y cells compared with the control cells (Fig. 8A-C). Analysis of caspase-3 activity suggested there was higher apoptotic activity in TG-induced apoptotic cells compared with that of the control group, which was reversed by the treatment of AST (Fig. 8D). TUNEL staining and subsequent

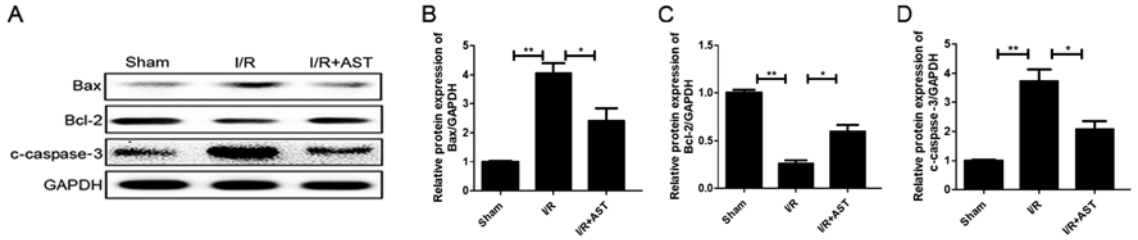

Figure 4. AST administration attenuates the apoptotic levels *in vivo*. (A) Western blotting was used to analyze Bax, Bcl-2 and c-caspase-3 expression levels in cerebral I/R injury model rats with or without AST treatment (50 μM). Semi-quantification of the expression levels of (B) Bax, (C) Bcl-2 and (D) c-caspase-3 from part (A). Data are represented as the mean ± SD, n=5/group. *P<0.05, **P<0.01. AST, astragalin; c-, cleaved; I/R, ischemia/reperfusion.

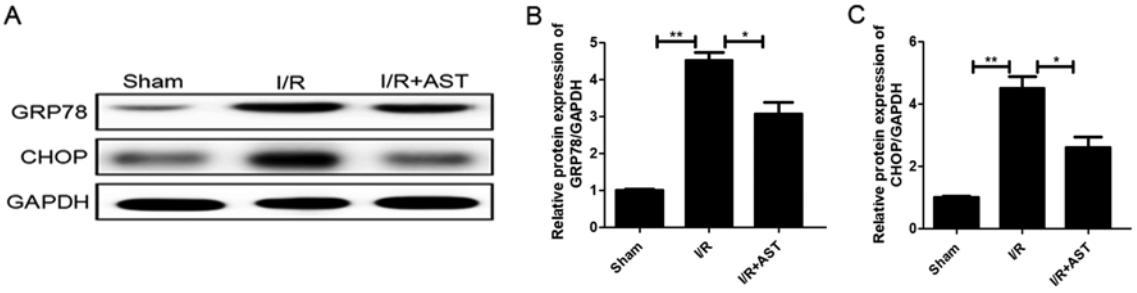

Figure 5. AST downregulates the expression levels of endoplasmic reticulum stress- and apoptosis-related proteins *in vivo*. (A) Western blotting was used to analyze the expression levels of GRP78 and CHOP in cerebral I/R injury model rats with or without AST treatment (50 μM). Semi-quantification of the expression levels of (B) GRP78 and (C) CHOP from part (A). Data are represented as the mean ± SD, n=5/each group. *P<0.05, **P<0.01. AST, astragalin; I/R, ischemia/reperfusion; GRP78, glucose regulated protein, 78 kDa.

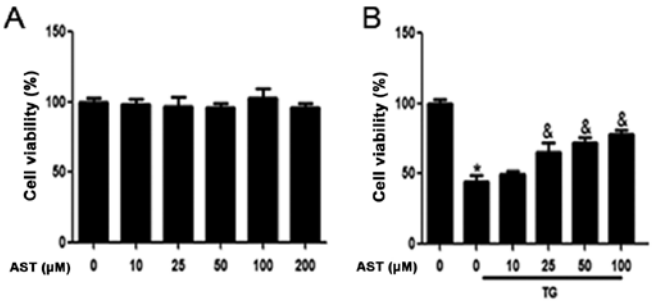

Figure 6. AST improves the survival of TG-induced SH-SY5Y cells. (A) CCK-8 assays were performed to determine the viability of cells treated with a series of concentrations of AST. (B) CCK-8 assays were performed to determine the cell viability of AST-treated chondrocytes stimulated by TG (10 μM). Data are represented as the mean ± SD, n=5 in each group. *P<0.05 vs. 0 μM AST; $^{\&}$P<0.05 vs. 0 μM AST + TG group. AST, astragalin; TG, thapsigargin; CCK-8, Cell Counting Kit-8.

semi-quantification demonstrated that AST treatment significantly reduced the numbers of apoptotic cells compared with TG-treated SH-SY5Y cells, which were significantly increased compared with the control cells (Fig. 8E and F). Together, these results suggested that the anti-apoptotic roles of AST may be related to the regulation of ER stress.

Discussion

Cerebral I/R remains one of the most devastating cerebrovascular events, which results in significant mortality and long-term disability, with an estimated 41.6% death rate every year (1), thereby exerting a heavy social and economic burden. It has been hypothesized that the only effective approach for the treatment of cerebral I/R injury is restoration of blood perfusion within an effective time window (17). Various studies have reported that the restoration of blood flow initiated a complex series of molecular events, including oxidative stress, inflammation, mitochondrial dysfunction and ER dysfunction, ultimately contributing to cerebral I/R injury (18,19). Cerebral I/R injury may also occur in the perioperative period, especially in elderly or critically ill patients (1). Thus, the development of an effective strategy to both treat and prevent cerebral I/R injury has become the primary focus of I/R research (2,3).

Numerous studies have demonstrated that the neurological damage that occurs during cerebral I/R injury may be enhanced under various complex pathophysiological conditions such as inflammation and apoptosis, which subsequently regulates the cell death-related signaling pathway (2,20). Previous studies have revealed that excessive inflammation and neuronal apoptosis were critical factors for cerebral I/R injury (2,3,21). In fact, several inflammatory mediators (TNF-α, IL-1β and iNOS) were discovered to damage microvessels, further enhancing the expression of inflammatory factors through the activation of leukocytes and causing secondary neuronal damage (22,23). It has been demonstrated that inflammation has an essential role in cerebral I/R injury (24,25). In an analysis of 37 patients with ischemic stroke, peak IL-6 plasma concentrations were identified to be closely related to brain infarct volume and neurological outcomes (26). Treatment with recombinant IL-33 reduced the infarct area, attenuated microglia activation and decreased proinflammatory macrophage infiltration in a mouse model of middle cerebral artery occlusion (MCAO) (27). Infarct size and neurological outcomes

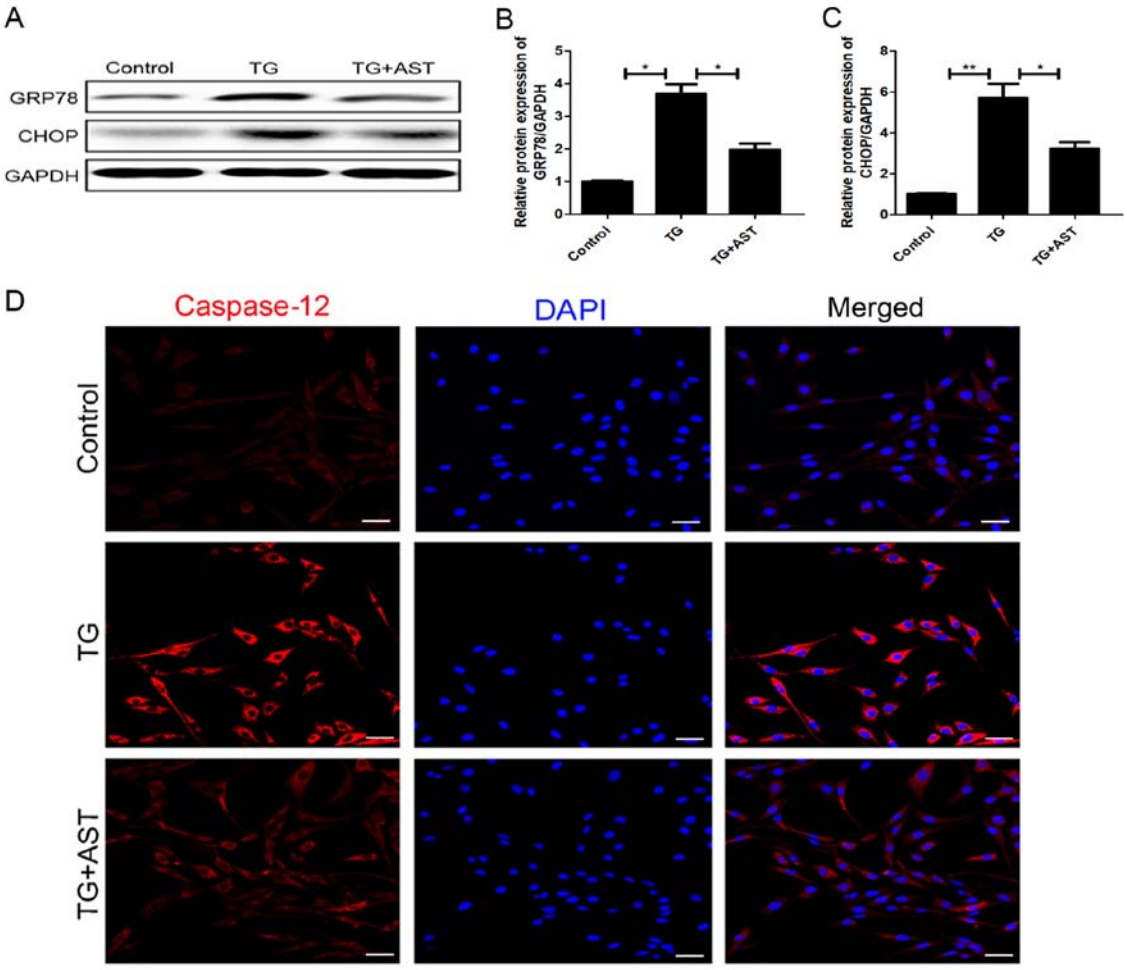

Figure 7. AST inhibits TG-induced endoplasmic reticulum stress and subsequent apoptosis *in vitro*. (A) Western blotting was used to analyze the expression levels of GRP78 and CHOP in TG (10 μM)-induced SH-SY5Y cells with or without AST (50 μM) treatment. Semi-quantification of (B) GRP78 and (C) CHOP expression levels from part (A). (D) Immunofluorescence staining of caspase-12 protein expression levels (red) and nuclei labeled with DAPI (blue). Scale bar, 50 μm. Data are represented as the mean ± SD, n=5 in each group. *P<0.05, **P<0.01. AST, astragalin; TG, thapsigargin; GRP78, glucose regulated protein, 78 kDa.

were also improved in Toll-like receptor 4-deficient mice of cerebral I/R injury (28).

The enhancement of neuronal apoptotic activity was discovered to be a main feature of cerebral I/R injury (29). The suppression of growth arrest through RNA interference was demonstrated to markedly upregulate apoptotic protein expression levels and enlarge the infarct size in rats with MCAO (29). Previous studies have also noted the roles of AST in the regulation of inflammation and apoptosis in various types of disease; for example, AST improved diabetes-induced spermatogenic dysfunction in male mice through the regulation of antioxidant activities and inflammation (11). Similar effects were also observed in varicocelized Sprague-Dawley rats, in which AST promoted spermatogenesis by regulating inflammation, oxidative stress, ER stress and apoptosis (12). From a mechanistic standpoint, AST has been illustrated to confer various cardioprotective effects through a combination of anti-oxidative, anti-apoptotic and anti-inflammatory activities (30). Among IL-1β-stimulated human chondrocytes, these effects were mediated by the suppression of various inflammatory factors through the regulation of NF-κB and MAPK signaling (31). The present study observed significant cell death following cerebral I/R injury, which was significantly attenuated by treatment with AST. AST also significantly downregulated the expression levels of the pro-apoptotic proteins Bax and c-caspase-3, inhibited caspase-3 activity and upregulated the expression levels of the anti-apoptotic protein Bcl-2 following I/R. Moreover, AST suppressed the release of inflammatory cytokines, resulting in improved long-term neurological outcomes of cerebral I/R injury *in vivo*. Together, these results demonstrated the neuroprotective effects of AST against cerebral I/R injury *in vivo* and *in vitro*.

Numerous studies have proved that ER stress contributed to apoptotic activity following cerebral I/R injury (6,32-35). These effects appeared to be partially mediated by the upregulation of ER stress markers, including CHOP, eukaryotic initiation factor 2α and GRP78 (6,32). Previous work has demonstrated that ER stress exerted a positive effect on neuronal apoptosis and the detection of cellular damage in ischemic stroke (33,34). GRP78 is an ER resident protein triggered by microenvironmental damage that disturbs ER function (35). There is increasing evidence to suggest that GRP78 may regulate the UPR in the ER, and that it is a critical regulator of cellular apoptosis under stress

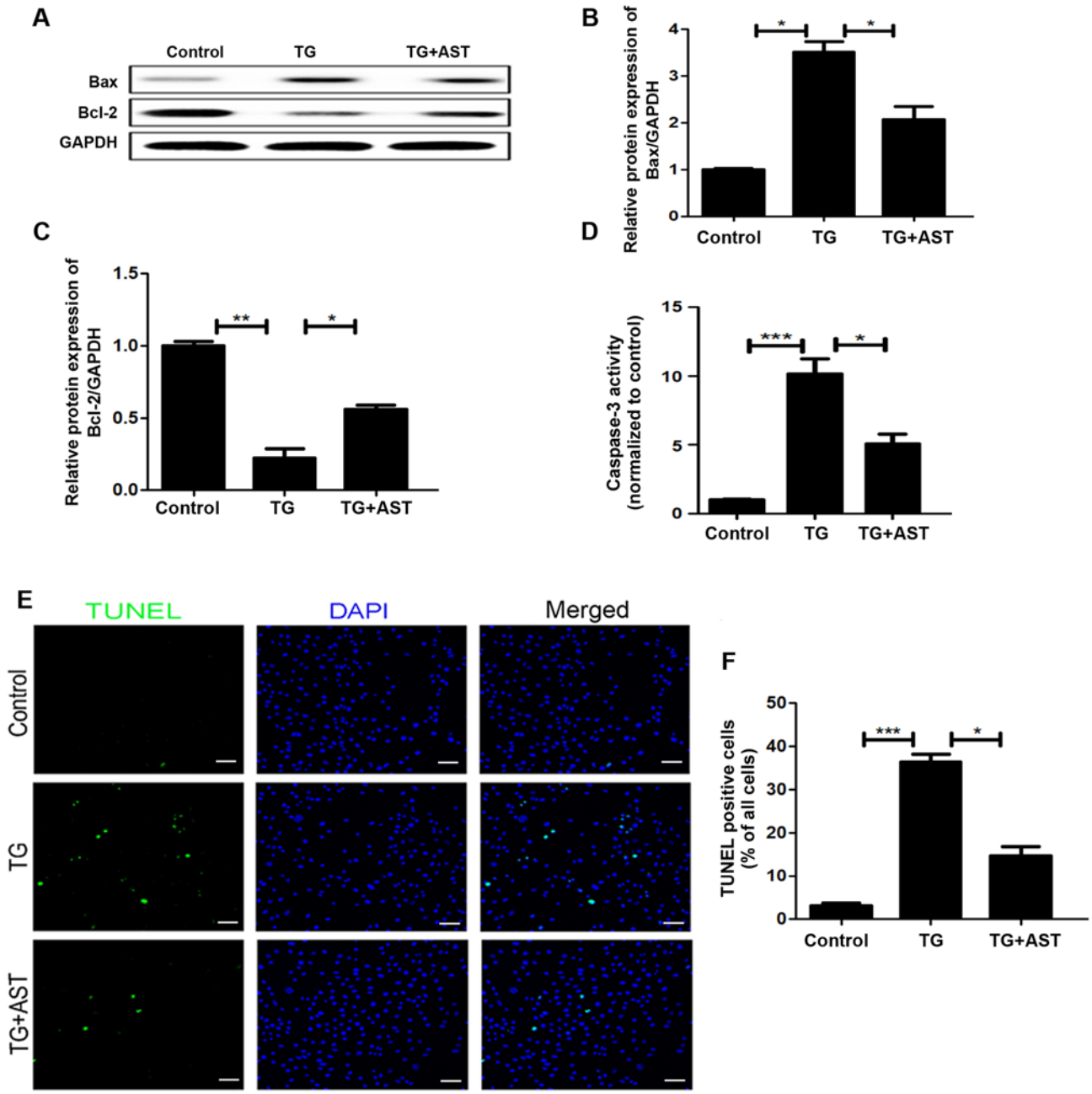

Figure 8. AST inhibits TG-stimulated increases in apoptotic levels in SH-SY5Y cells. (A) Western blotting was used to determine the expression levels of Bax and Bcl-2 in TG (10 μM)-induced SH-SY5Y cells with or without AST treatment (50 μM). Semi-quantification of the expression levels of (B) Bax and (C) Bcl-2. (D) Caspase-3 activity in TG (10 μM)-induced SH-SY5Y cells with or without AST treatment (50 μM). (E) TUNEL assay was used to analyze the levels of apoptosis in SH-SY5Y cells of each groups as treated above. Scale bar, 50 μm. (F) Semi-quantification of the results of the TUNEL assay from part (E). Data are represented as the mean ± SD, n=5/group. *P<0.05, **P<0.01, ***P<0.001. AST, astragalin; TG, thapsigargin.

conditions (36). There is also accumulating evidence to support the essential role of CHOP during apoptosis which is triggered by ER stress (37). Under conditions of ER stress, caspase-12 becomes dissociated from the membrane, leading to the elevated production of c-caspase-12, which then activates various apoptotic signaling mechanisms such as PERK/ATF6 (38-40). The UPR activates the PERK/ATF6 signaling pathway and enhances chaperone expression 9GRP78 and protein disulfide isomerase (PDI)], which results in the elevated production of CHOP and caspase-12, as well as increased apoptosis (39,40). In the present study, the neuroprotective effects of AST were analyzed along with its potential association with ER stress and apoptotic signaling. The present findings demonstrated that the administration of AST downregulated the expression levels of GRP78, CHOP and caspase-12, indicating a potential mechanism for AST. To further investigate the effect of ER stress on cerebral I/R injury, TG, a well-established inducer of ER stress, was used in an *in vitro* neuronal cell culture model. AST treatment significantly reduced TG-stimulated ER stress related-protein (GRP78, CHOP and caspase-12) in SH-SY5Y cells. Furthermore, AST treatment partially reversed the upregulation in Bax and downregulation in Bcl-2 expression levels, contributing to

the significant attenuation of neuronal apoptosis. These data suggested that ER stress may be tightly associated with the protective effects of AST.

However, some limitations should be noted in the current study. AST has been demonstrated to have a concentration-dependent protective effect on other types of disease, such as spermatogenesis and osteoarthritis (11,30). However, only one drug dose was used in the present animal study and the mechanism was not clarified, thus other concentrations and in-depth investigations into the mechanisms need to be further studied.

In conclusion, the findings of the present study indicated that the administration of AST may improve long-term neurological outcomes in cerebral I/R injury model rats. These protective effects were regulated through the inhibition of neuronal apoptosis, the inflammatory response and ER stress following injury. *In vitro* analyses further supported these observations: AST significantly attenuated ER stress and apoptosis in a neuronal cell culture model. Thus, these results suggested that the regulation of ER stress by AST may be associated with neuronal apoptosis and neurological recovery, highlighting the potential use of AST as a therapeutic candidate in the treatment of cerebral I/R injury.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DL and WC designed the study and supervised the experiments. DL and WW contributed to the statistical analysis, data interpretation and manuscript preparation. DL and YG performed the experiments and data interpretation. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Animal Care and Use Committee of Hainan Medical University (approval no. 201819; Haikou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Moskowitz MA, Lo EH and Iadecola C: The science of stroke: Mechanisms in search of treatments. Neuron 67: 181-198, 2010.
2. Radak D, Katsiki N, Resanovic I, Jovanovic A, Sudar-Milovanovic E, Zafirovic S, Mousad SA and Isenovic ER: Apoptosis and acute brain ischemia in ischemic stroke. Curr Vasc Pharmacol 15: 115-122, 2017.
3. Dziedzic T: Systemic inflammation as a therapeutic target in acute ischemic stroke. Expert Rev Neurother 15: 523-531, 2015.
4. Xiang C, Wang Y, Zhang H and Han F: The role of endoplasmic reticulum stress in neurodegenerative disease. Apoptosis 22: 1-26, 2017.
5. Sprenkle NT, Sims SG, Sanchez CL and Meares GP: Endoplasmic reticulum stress and inflammation in the central nervous system. Mol Neurodegener 12: 42, 2017.
6. Nakka VP, Gusain A and Raghubir R: Endoplasmic reticulum stress plays critical role in brain damage after cerebral ischemia/reperfusion in rats. Neurotox Res 17: 189-202, 2010.
7. Secondo A, Petrozziello T, Tedeschi V, Boscia F, Vinciguerra A, Ciccone R, Pannaccione A, Molinaro P, Pignataro G and Annunziato L: ORAI1/STIM1 interaction intervenes in stroke and in neuroprotection induced by ischemic preconditioning through store-operated calcium entry. Stroke 50: 1240-1249, 2019.
8. Hetz C: The unfolded protein response: Controlling cell fate decisions under ER stress and beyond. Nat Rev Mol Cell Biol 13: 89-102, 2012.
9. Lee JY, Maeng S, Kang SR, Choi HY, Oh TH, Ju BG and Yune TY: Valproic acid protects motor neuron death by inhibiting oxidative stress and endoplasmic reticulum stress-mediated cytochrome C release after spinal cord injury. J Neurotrauma 31: 582-594, 2014.
10. Kim MS and Kim SH: Inhibitory effect of astragalin on expression of lipopolysaccharide-induced inflammatory mediators through NF-κB in macrophages. Arch Pharm Res 34: 2101-2107, 2011.
11. Han XX, Jiang YP, Liu N, Wu J, Yang JM, Li YX, Sun M, Sun T, Zheng P and Yu JQ: Protective effects of Astragalin on spermatogenesis in streptozotocin-induced diabetes in male mice by improving antioxidant activity and inhibiting inflammation. Biomed Pharmacother 110: 561-570, 2019.
12. Karna KK, Choi BR, You JH, Shin YS, Cui WS, Lee SW, Kim JH, Kim CY, Kim HK and Park JK: The ameliorative effect of monotropein, astragalin, and spiraeoside on oxidative stress, endoplasmic reticulum stress, and mitochondrial signaling pathway in varicocelized rats. BMC Complement Altern Med 19: 333, 2019.
13. Nan D, Jin H, Deng J, Yu W, Liu R, Sun W and Huang Y: Cilostazol ameliorates ischemia/reperfusion-induced tight junction disruption in brain endothelial cells by inhibiting endoplasmic reticulum stress. FASEB J 33: 10152-10164, 2019.
14. Soromou LW, Chen N, Jiang L, Huo M, Wei M, Chu X, Millimouno FM, Feng H, Sidime Y and Deng X: Astragalin attenuates lipopolysaccharide-induced inflammatory responses by down-regulating NF-κB signaling pathway. Biochem Biophys Res Commun 419: 256-261, 2012.
15. Metz GA and Whishaw IQ: Cortical and subcortical lesions impair skilled walking in the ladder rung walking test: A new task to evaluate fore- and hindlimb stepping, placing, and co-ordination. J Neurosci Methods 115: 169-179, 2002.
16. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
17. Rabinstein AA: Treatment of acute ischemic stroke. Continuum (Minneap Minn) 23: 62-81, 2017.
18. Bakthavachalam P and Shanmugam PST: Mitochondrial dysfunction-silent killer in cerebral ischemia. J Neurol Sci 375: 417-423, 2017.
19. Ma XD, Song JN, Zhang M, An JY, Zhao YL and Zhang BF: Advances in research of the neuroprotective mechanisms of cerebral ischemic postconditioning. Int J Neurosci 125: 161-169, 2015.
20. Dong J, Yuan X and Xie W: Pentoxifylline exerts anti-inflammatory effects on cerebral ischemia reperfusioninduced injury in a rat model via the p38 mitogen-activated protein kinase signaling pathway. Mol Med Rep 17: 1141-1147, 2018.
21. Cheng CY, Kao ST and Lee YC: Ferulic acid ameliorates cerebral infarction by activating Akt/mTOR/4EBP1/Bcl2 antiapoptotic signaling in the penumbral cortex following permanent cerebral ischemia in rats. Mol Med Rep 19: 792-804, 2019.

22. Ji K, Xue L, Cheng J and Bai Y: Preconditioning of H2S inhalation protects against cerebral ischemia/reperfusion injury by induction of HSP70 through PI3K/Akt/Nrf2 pathway. Brain Res Bull 121: 68-74, 2016.
23. Moritz M, Pfeifer S, Balmayor ER, Mittermayr R, Wolbank S, Redl H and van Griensven M: VEGF released from a fibrin biomatrix increases VEGFR-2 expression and improves early outcome after ischaemia-reperfusion injury. J Tissue Eng Regen Med 11: 2153-2163, 2017.
24. Jin R, Liu L, Zhang S, Nanda A and Li G: Role of inflammation and its mediators in acute ischemic stroke. J Cardiovasc Transl Res 6: 834-851, 2013.
25. He F, Zhang N, Lv Y, Sun W and Chen H: Lowdose lipopolysaccharide inhibits neuronal apoptosis induced by cerebral ischemia/reperfusion injury via the PI3K/Akt/FoxO1 signaling pathway in rats. Mol Med Rep 19: 1443-1452, 2019.
26. Smith CJ, Emsley HC, Gavin CM, Georgiou RF, Vail A, Barberan EM, del Zoppo GJ, Hallenbeck JM, Rothwell NJ, Hopkins SJ and Tyrrell PJ: Peak plasma interleukin-6 and other peripheral markers of inflammation in the first week of ischaemic stroke correlate with brain infarct volume, stroke severity and long-term outcome. BMC Neurol 4: 2, 2004.
27. Zhang SR, Piepke M, Chu HX, Broughton BR, Shim R, Wong CH, Lee S, Evans MA, Vinh A, Sakkal S, *et al*: IL-33 modulates inflammatory brain injury but exacerbates systemic immunosuppression following ischemic stroke. JCI Insight 3: e121560, 2018.
28. Caso JR, Pradillo JM, Hurtado O, Lorenzo P, Moro MA and Lizasoain I: Toll-like receptor 4 is involved in brain damage and inflammation after experimental stroke. Circulation 115: 1599-1608, 2007.
29. Liu B, Zhang YH, Jiang Y, Li L, Chen Q, He GQ, Tan XD and Li CQ: Gadd45b is a novel mediator of neuronal apoptosis in ischemic stroke. Int J Biol Sci 11: 353-360, 2015.
30. Qu D, Han J, Ren H, Yang W, Zhang X, Zheng Q and Wang D: Cardioprotective effects of astragalin against myocardial ischemia/reperfusion injury in isolated rat heart. Oxid Med Cell Longev 2016: 8194690, 2016.
31. Ma Z, Piao T, Wang Y and Liu J: Astragalin inhibits IL-1β-induced inflammatory mediators production in human osteoarthritis chondrocyte by inhibiting NF-κB and MAPK activation. Int Immunopharmacol 25: 83-87, 2015.
32. Noh MR, Kim JI, Han SJ, Lee TJ and Park KM: C/EBP homologous protein (CHOP) gene deficiency attenuates renal ischemia/reperfusion injury in mice. Biochim Biophys Acta 1852: 1895-1901, 2015.
33. Qi W, Cao D, Li Y, Peng A, Wang Y, Gao K, Tao C and Wu Y: Atorvastatin ameliorates early brain injury through inhibition of apoptosis and ER stress in a rat model of subarachnoid hemorrhage. Biosci Rep 38: BSR20171035, 2018.
34. Yu Y, Zhou H, Xiong Y and Liu J: Exosomal miR-199a-5p derived from endothelial cells attenuates apoptosis and inflammation in neural cells by inhibiting endoplasmic reticulum stress. Brain Res 1726: 146515, 2020.
35. Suyama K, Watanabe M, Sakabe K, Okada Y, Matsuyama D, Kuroiwa M and Mochida J: Overexpression of GRP78 protects glial cells from endoplasmic reticulum stress. Neurosci Lett 504: 271-276, 2011.
36. Wang C, Jiang K, Gao D, Kang X, Sun C, Zhang Q, Li Y, Sun L, Zhang S, Guo K and Liu Y: Clusterin protects hepatocellular carcinoma cells from endoplasmic reticulum stress induced apoptosis through GRP78. PLoS One 8: e55981, 2013.
37. Cai XH, Li XC, Jin SW, Liang DS, Wen ZW, Cao HC, Mei HF, Wu Y, Lin ZD and Wang LX: Endoplasmic reticulum stress plays critical role in brain damage after chronic intermittent hypoxia in growing rats. Exp Neurol 257: 148-156, 2014.
38. Voccoli V, Mazzoni F, Garcia-Gil M and Colombaioni L: Serum-withdrawal-dependent apoptosis of hippocampal neuroblasts involves Ca^{++} release by endoplasmic reticulum and caspase-12 activation. Brain Res 1147: 1-11, 2007.
39. Li YH, Tardif G, Hum D, Kapoor M, Fahmi H, Pelletier JP and Martel-Pelletier J: The unfolded protein response genes in human osteoarthritic chondrocytes: PERK emerges as a potential therapeutic target. Arthritis Res Ther 18: 172, 2016.
40. Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA and Yuan J: Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature 403: 98-103, 2000.