Artemether confers neuroprotection on cerebral ischemic injury through stimulation of the Erk1/2-P90rsk-CREB signaling pathway

Shuai Li, Tangming Peng, Xia Zhao, Marta Silva, Linlin Liu, Wenshu Zhou, Ligang Chen, Wenhua Zheng

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ABSTRACT

Ischemic stroke is one of the leading causes of death and disability among adults. Despite the economic burden of the disease, available treatment options are still very limited. With the exception of anti-thrombolytics and hypothermia, current therapies fail to reduce neuronal injury, neurological deficits and mortality rates, suggesting that the development of novel and more effective therapies against ischemic stroke is urgent. In the present study, we found that artemether, which has been used in the clinic as an anti-malarial drug, was able to improve the neurological deficits, attenuate the infarction volume and the brain water content in a middle cerebral artery occlusion (MCAO) animal model. Furthermore, artemether treatment significantly suppressed cell apoptosis, stimulated cell proliferation and promoted the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), P90rsk and cAMP responsive element-binding protein (CREB). Artemether protective effect was attenuated by PD98059, an ERK1/2 inhibitor, administration. Similarly, in oxygen-glucose deprivation/reperfusion (OGD/RP) cell models, artemether pre-treatment induced the suppression of the intracellular ROS, the down-regulation of LDH activity, the reduction of caspase 3 activity and of the apoptosis cell rate and reversed the decrease of mitochondrial membrane potential. As with MCAO animal model, artemether promoted the activation of Erk1/2-P90rsk-CREB signaling pathway. This effect was blocked by the inhibition or knockdown of ERK1/2. The present study provides evidences of the neuroprotective effect of artemether unraveling its potential as a new therapeutic candidate for the prevention and treatment of stroke.

1. Introduction

Ischemic stroke is one of the leading causes of death and disability among adults. Despite the economic burden of the disease, available treatment options are still very limited. With the exception of anti-thrombolytics and hypothermia, current therapies fail to reduce neuronal injury, neurological deficits and mortality rates, suggesting that the development of novel and more effective therapies against ischemic stroke is urgent. In the present study, we found that artemether, which has been used in the clinic as an anti-malarial drug, was able to improve the neurological deficits, attenuate the infarction volume and the brain water content in a middle cerebral artery occlusion (MCAO) animal model. Furthermore, artemether treatment significantly suppressed cell apoptosis, stimulated cell proliferation and promoted the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), P90rsk and cAMP responsive element-binding protein (CREB). Artemether protective effect was attenuated by PD98059, an ERK1/2 inhibitor, administration. Similarly, in oxygen-glucose deprivation/reperfusion (OGD/RP) cell models, artemether pre-treatment induced the suppression of the intracellular ROS, the down-regulation of LDH activity, the reduction of caspase 3 activity and of the apoptosis cell rate and reversed the decrease of mitochondrial membrane potential. As with MCAO animal model, artemether promoted the activation of Erk1/2-P90rsk-CREB signaling pathway. This effect was blocked by the inhibition or knockdown of ERK1/2. The present study provides evidences of the neuroprotective effect of artemether unraveling its potential as a new therapeutic candidate for the prevention and treatment of stroke.

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nitroprusside (SNP) [8,9]. Artemether is an artemisinin derivative with a higher anti-malarial activity than artemisinin. This compound can easily pass through the blood-brain barrier and it is used more frequently in the clinic [10–12]. Besides its strong anti-malarial activity, it has also potential as an anti-cancer [13,14], anti-allergic or anti-inflammatory [15,16], anti-viral [17], anti-helminths and anti-protozoan parasitic drug [18,19]. In addition, several studies have also being reporting its potential use in oxidative stress suppression [5,9,20,21]. Unfortunately, the protective effects and the molecular mechanism by which artemether affects ischemic stroke was not investigated. ERK1/2 pathway is a key signal component and plays an important role in the activation and regulation of cell proliferation, cell survival and cell apoptosis [22]. Acute ischemic stroke injury is accompanied by changes in the intracellular signaling cascade involved in neuronal survival such as the ERK1/2 pathways [23,24]. Artemisinin was reported to protect RPE cells and neuronal cells against oxidative stress-induced apoptosis through ERK pathways which makes artemisinin and its derivatives as potential candidates for the ischemic stroke [8,9]. A series of pathological events are occurring during acute ischemic stroke due to the loss of oxygen and energy supply, which results in irreversible neuronal and brain tissue damage [25]. Reactive oxygen species (ROS) accumulation has been described to be associated with brain injury after ischemic stroke [26]. The rapid increase of ROS production after acute ischemic stroke disrupts the endogenous antioxidant defense mechanisms, leading to tissue damage. ROS can destroy cell macromolecules, leading to autophagy, apoptosis and necrosis [27,28]. In addition, the rapid recovery of the blood flow increases tissues oxygenation levels, further increasing the production of ROS leading to reperfusion injury [29–31]. Therefore, it is very important to develop new therapeutic approaches that may lead to the reduction of oxidative damage. Artemisinin and artemether were reported to protect against oxidative stress-induced apoptosis in neurodegenerative disease in vitro and in vivo [32–34], further supporting the potential use of artemether in the treatment of ischemic stroke.

In this study, we assessed the protective effect of artemether in a classical middle cerebral artery occlusion (MCAO) stroke animal model and in an OGD/RP cellular model and found that artemether reduced cell injury by stimulation of the Erk1/2-P90rsk-CREB signaling pathway. These results provide evidences that artemether may play a critical role in the protection against cerebral ischemic injury supporting its potential use in the treatment of ischemic stroke.

2. Materials and methods

2.1. Materials

Monofilament nylon sutures were purchased from Beijing Sunbio Biotech Co. Ltd. (Beijing, China), 2,3,5-triphenyltetrazolium chloride (TTC, Cat# T8877) were obtained from Sigma Aldrich (St. Louis, MO, USA). Dulbecco’s Modified Eagle’s Medium (DMEM) with or without glucose, Fetal Bovine Serum (FBS), Bovine Serum Albumin (BSA) and 0.25% Trypsin were obtained from Gibco (Invitrogen Corporation). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Hoechst 33,342 were purchased from Molecular Probes (Eugene, OR, USA). Artemether (ARTE), BrdU (5-Bromo-2-deoxyuridine), Penicillin/Glutamine, Fetal Bovine Serum (FBS), Bovine Serum Albumin (BSA) and 2,3,5-triphenyltetrazolium chloride (TTC, Cat# T8877) were obtained from Sigma Aldrich (St. Louis, MO, USA). Artemisinin and its derivatives as potential candidates for the ischemic stroke [8,9]. Monofilament nylon suture (diameter 0.21 mm) with a circular tip was inserted through the right external carotid artery (ECA) and internal carotid artery (ICA) and moved forward slightly to occlude the left middle cerebral artery (MCA). After exposing and separating the right CCA, the ECA and ICA, the monofilament suture was inserted to the right common carotid artery (CCA). MCAO model was also established in male Sprague Dawley (SD) rats weighting 160–180 g using the same method. In short, after exposing and separating the right CCA, the ECA and ICA, the ECA was ligated and a monofilament nylon suture (diameter 0.31 mm) was inserted into ICA until resistance was encountered to prevent the origin of the middle cerebral artery. Cerebral blood flow (CBF) was measured in the area of the MCA by a laser doppler flowmetry (RWD, Shenzhen, China) and the detector was fixed on the skull above the cortex. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health.

2.3. Neurological evaluation

Neurological deficit scores were evaluated by an observer blinded to the experimental groups. Zea-Longa Scale was used to assess the neurological function of MCAO models as previously described [36]: grade 0, normal (no apparent neurological deficits); grade 1, failure to entirely extend the contralateral forelimb; grade 2, circling continuously to the contralateral side but standard posture at rest; grade 3, falling to the injured side; grade 4, no spontaneous autonomic activity and a sluggish level of consciousness; grade 5, death. Animals with grades of 0, 1 or 5 were withdrawn from the study.

2.4. Grip strength and sensory function measurement

Grip strength meter (Bioseb, USA) and a hot plate (Panlab Harvard Apparatus, Barcelona) were used to assess the grip strength and the
recovery of the sensory function of the animals at days 1, 3, 7, 14 and 28 after MCAO. The assays were performed as previously reported [37,38].

Briefly, for the measurement of the grip strength, the animal’s forelimbs were placed on the grid of the grip strength meter. The animals were then pulled away from the grid until losing the grip. The grip strength of each animal was displayed on the screen and recorded. The hot plate test has been widely used to assess the sensory function by measuring the thermal withdrawal latency (the latency of paw licking) of the forelimbs. Briefly, the surface of the hot plate apparatus was set to 50 °C, and then the animals were gently lifted by the tail, so that their forelimbs touched the plate surface. The latency of paw licking was recorded.

2.5. Morris water maze test

The MWM was used to test the learning and memory abilities of the animals 1 month after the surgical procedures. The assays were performed as reported previously [39] and included a 4-day hidden platform test and a 1-day spatial probe test. Briefly, the animals were allowed to swim for 60 s before getting to the platform for the hidden platform test. The time to reach the platform (escape latency) was recorded. Then, the spatial probe test was carried out 24 h after the last time of the hidden platform test and the percentage of time spent on the target quadrant and the number of crossings in the target quadrant area were measured.

2.6. Tissue samples preparation

Animals were sacrificed 24 h after MCAO procedure by decapitation and the brains were immediately extracted. Brains used for TTC staining were immediately used, brains used for SOD, MDA and western blotting analysis were immediately used or stored in –80 °C. Brains used to determine the water content were immediately weighed after dissection and dried for 48 h. Brains used for HE Staining, Nissl Staining, TUNEL assay, immunofluorescence and immunohistochemical analysis were post-fixed overnight in 4% paraformaldehyde (PFA). After fixation, the samples were dehydrated and embedded in paraffin using standard methods [32]. Coronal 4 μm sections were cut and kept at 37 °C for 24 h, followed by deparaffination and hydration.

2.7. 2, 3, 5-triphenyltetrazolium chloride (TTC) staining

TTC (2,3,5-triphenyltetrazolium chloride) staining was used to evaluate the size of the infarct volume in MCAO model groups. Coronal brain sections (2 mm) were put into 2% TTC stain in saline at 37 °C and gently stirred to ensure an homogeneous staining exposure for 15 min. Excess TTC was drained off, and the slices were fixed in 4% PFA during overnight. TTC stained and post-fixed brain slices were then scanned by a photo scanner. TTC stains viable brain tissue in deep red and the infarcted tissue remains unstained. Infarct and total brain volumes were quantified with an Image J analysis system. Brain infarct volume was expressed as a percentage of total brain tissue volume. All studies were performed for a minimum of 5 sections per sample, with 5 animals in each group.

2.8. Brain water content

We observed brain water content using the wet–dry method [40,41]. The right hemisphere of the dissected brains was immediately collected and weighed (wet tissue weight) 24 h after MCAO. Afterwards, the brain samples were dried in an oven at 95 °C for 48 h and weighed again (dry tissue weight). The brain water content was calculated as [(wet tissue weight – dry tissue weight)/wet tissue weight] × 100% (4 animals per group).

2.9. HE staining, Nissl Staining and TUNEL assay

HE and Nissl stainings were conducted according to routine protocols [32]. Nissl staining was used to detect surviving neurons. The survival index was calculated as follows: survival index (%) = (number of surviving neurons/total number of neurons) × 100%. The apoptotic response was assessed by TUNEL assay using the commercially available TUNEL Apoptosis Assay kit (Beyotime Institute of Biotechnology, Shanghai, China). The assay was performed according to the instructions provided by the manufacturer. The apoptosis index in the peri-infarct region was calculated as follows: apoptosis index (%) = (number of apoptotic neurons/total number of neurons) × 100%. All studies were performed for a minimum of 5 sections per sample, with 4 animals in each group.

2.10. BrdU labelling

Animals from each group received intraperitoneal injections of BrdU (50 mg/kg/time) with an interval of 2 h from reperfusion to sacrifice. To detect BrdU-labeled cells, brain sections were subjected to double immunofluorescence labeling for BrdU and NeuN. Following incubation with primary antibodies (Rabbit anti-NeuN, 1:500; mouse anti-BrdU, 1:500), the corresponding secondary antibody was used for NeuN (TRITC-conjugated anti-rabbit IgG, 1:500) and BrdU (FITC-conjugated anti-mouse IgG, 1:500). The images were captured with a fluorescence microscope (Nikon A1 confocal microscope). All studies were performed for a minimum of 5 sections per sample, with 3 animals in each group.

2.11. Immunofluorescence and immunohistochemical analysis

Immunofluorescence and immunohistochemical analyses were performed as previously described [32]. Briefly, after incubation with antigen retrieval solution and 3% H2O2 for 30 min, the slides were rinsed with PBS and incubated with the primary antibodies (Rabbit anti-Nestin, 1:500, Mouse anti-BrdU, 1:500) overnight at 4 °C. For negative controls, the primary antibody was replaced by nonimmunized serum. The following day, the slides were rinsed and incubated with the corresponding secondary antibody (TRITC-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG) for 1 h followed by three washes in PBS for 15 min. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI), and images were acquired with a Nikon A1 confocal microscope. For IHC analysis, the slides were incubated with biotinylated secondary antibody anti-rabbit IgG (1:500) at room temperature for 2h, processed using the avidin-biotin complex (Boster Biological Technology, Wuhan, China) and stained with 3,3′-diaminobenzidine (DAB) and hematoxylin. The slides were then examined and photographed using a microscope (EVOS FL Imaging System). All studies were performed for a minimum of 5 sections per sample, with 4 animals in each group.

2.12. Determination of superoxide dismutase, malonaldehyde

Brain samples were processed with a immunoprecipitation assay (RIPA) lysis buffer. Total superoxide dismutase (SOD) assay kit and lipid peroxidation malonaldehyde (MDA) assay kit were used to measure SOD, MDA contents, respectively. The assays were performed according to the instructions provided by the manufacturers (4 animals per group).

2.13. Cell culture

PC12 cells were obtained from the Cell Bank of Sun Yat-sen University (Guangzhou, China). The cells were grown in DMEM Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 units/ml penicillin, and maintained at 37 °C in an humidified atmosphere of 5% CO2.

Primary cultured cortical neurons were prepared from brains of
newborn C57BL/6 mice as previously described [42], and cultured for 7–8 days in neurobasal medium supplemented with 2% B27, 10 U/ml penicillin, and 10 μg/ml streptomycin at 37 °C in 5% CO₂ humidified atmosphere.

### 2.14. Establishment of OGD/RP model

For OGD treatment, cells were rinsed once with warm glucose-free DMEM (Gibco), and then placed in an incubator loaded with mixed gas containing 5% CO₂, 1% O₂ and 94% N₂ for 2 h, 4 h, 6 h and 8 h at 37 °C before reperfusion (re-oxygenation). For reperfusion, cells were refreshed with normal culture medium for 22 h, 20 h, 18 h and 16 h at 37 °C in an atmosphere of 5% CO₂, 21% O₂ and 74% N₂. Control groups were incubated in glucose-containing DMEM with equal refreshment at 37 °C in an atmosphere of 5% CO₂, 21% O₂ and 74% N₂ all the time.

### 2.15. MTT assay

Cells viability was assessed using MTT assay according to the commonly used protocols in our laboratory [43,44]. Cells were seeded in 96-well plates (5x10^3 cells/well) with 0.5% FBS. After exposure of cells to 2 h, 4 h, 6 h and 8 h of OGD followed by 22 h, 20 h, 18 h and 16 h of reperfusion, cell viability was measured by MTT assay after 24 h. To assess the effect of artemether pre-treatment, Cells were seeded in 96-well plates (5x10^3 cells/well) with 0.5% FBS and were pretreated with different concentrations of artemether (10–100 μM) or 0.1% DMSO for 2 h before OGD. After 4 h of OGD, cells were refreshed with normal culture medium and cell viability was tested by MTT assay 24 h later.
Cells were incubated with MTT (0.5 mg/mL) for 3 h at 37 °C. After this period, the medium is drawn and substituted by DMSO (100 µL). The absorbance at 570 nm was measured by BIO-RAD680 microplate reader (Thermo Fisher, MA, USA). The experiment was repeated 3 times.

2.16. Measurement of LDH

Cells cytotoxicity was evaluated by measuring the activity of lactate dehydrogenase (LDH) released into the medium according to commonly used protocols in our laboratory [42, 43]. Briefly, cells were seeded into 96-well plates (1x10^4 cells/well). After artemether or DMSO treatments, the activity of LDH released into the medium was determined using Cyto Tox-ONE TM Homogeneous Membrane Integrity Assay (Promega, USA), according to the instructions of the manufacturer. Fluorescence intensity was measured using an Infinite M200 PRO multimode microplate at excitation at 560 nm and emission at 590 nm wavelengths. The released LDH values were normalized to the percentage of the control group.

2.17. Caspase 3 activity detection

The activity of caspase 3 was measured as previously described [43, 44]. Briefly, after artemether treatment, the cells were extracted with lysis buffer and detection solution (sample: lysis buffer: Ac-DEVD-pNA = 5:4:1) was added followed by 2 h incubation. The absorbance was measured at 405 nm using a BIO-RAD680 microplate reader (Thermo Fisher, MA, USA). All values of caspase 3 activity (%) were normalized to the percentage of the control group.

2.18. Measurement of reactive oxygen species (ROS), mitochondrial membrane potential (Δψm) and cell apoptosis

These experiments were measured as previously described [42, 43]. Intracellular reactive oxygen species (ROS) production was assessed by DCFH-DA reagent, according to the manufacturer’s instructions. Protein concentration was measured using a BCA protein assay kit (Thermo scientific), according to the manufacturer’s instructions. Protops were resolved by SDS-PAGE (polyacrylamide gel electrophoresis) and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked in 5% non-fat milk in PBST for 1 h and probed with selective primary antibodies (Bcl2, Bax, Cleaved Caspase 3, P-ERK, T-ERK, P-P90 rsk, P-CREB, T-CREB at a 1:1000 dilution, actin, GAPDH at a 1:1500 dilution) overnight at 4 °C. Membranes were washed and incubated with horseradish peroxidase conjugated anti-rabbit secondary antibody (CST) at a dilution of 1:5000 for 1 h at room temperature. Immunoblotting was performed using an ECL detection kit reagent.

2.20. ERK1/2 silencing by siRNA

Gene silencing of ERK1/2 was performed as previously described [45]. Interfering RNA (siRNA) were synthesized by GenePharm (Shanghai). The sequence of ERK1 gene was 5′-GGGCCUAUAUAUACACUTT AUGCUGAUGUACUGGCCCTT-3′; and the sequence of ERK2 gene was 5′-CCUGAGGAAUUAAGUAUTT AUACUUAUAUCCUCAGGT-3′. Briefly, PC 12 cells were cultured in DMEM medium containing with 10% FBS plus antibiotics, and were maintained at 37 °C in a humidified, 5% CO2 atmosphere. The next day, cells were transfected with specifically synthesized siRNA or scrambled siRNA using Lipofectamine 2000 for 6 h. Then, media was replaced with DMEM plus 10% serum for 48 h, cells were collected for protein expression analyses or MTT assays.

2.21. Statistical analysis

All the data are presented as mean ± SEM. Each experiment was performed in triplicates. Statistical differences were analyzed by one-way ANOVA (Analysis of variance) in combination with a post-hoc test, and p values < 0.05 were considered statistically significant.
3. Results

3.1. Artemether attenuated the infarction volume in MCAO model

TTC staining is a commonly used indicator for evaluating cerebral ischemic injury [46]. Analysis of the stained coronal brain sections revealed that artemether treatment significantly reduced the cerebral ischemia infarction volume in MCAO mice in a dose dependent manner (Fig. 1B and C). Temporal analysis revealed that the neuroprotective effect of artemether, at a dose of 10 mg/kg, is delimited by a therapeutic window that ranges from 0 to 4 h post ischemia (Fig. 1D and E). Moreover, the neuroprotective effect of artemether was also present in MCAO rat model. Artemether (10 mg/kg) treatment significantly improved the cerebral ischemia infarction volume of rats with cerebral ischemic injury (Fig. 2A, B).

3.2. Artemether improved the neurological deficits and reduced brain edema in MCAO mouse model

The neurological deficits were evaluated before and 24 h after artemether administration. Results revealed that artemether treatment significantly improved the neurological deficits of MCAO mice and rats, as measured using Zea-Longa scale, 24h after reperfusion (Fig. 1F and G and Fig. 2C). Assessment of the brain water content revealed that MCAO mice presented significantly increased brain water contents when compared with the animals from the control group. Artemether treatment significantly reduced the brain edema when compared with the MCAO model animals (Fig. 1H).

3.3. Artemether improved the grip strength, the recovery of sensory function and the learning and memory abilities in C57 mice after MCAO

The grip strength and the recovery of the sensory function were...
evaluated at days 1, 3, 7, 14 and 28 after artemether treatment. Obtained results revealed that artemether treatment significantly improved the grip strength and the recovery of sensory function of MCAO mice after reperfusion (Fig. 3A and B). Assessment of the learning and memory abilities one month after the surgical procedure, revealed that MCAO mice presented learning and memory deficits when compared with the animals from the blank group. Artemether treatment for one month significantly attenuated the learning and memory deficits when
compared with the MCAO model animals as denoted by the decrease of the escape latency and by the increase of the percentage of time spent on the target quadrant and the number of crossings in the target quadrant area presented by treated animals in comparison with the MCAO model animals (Fig. 3D–F).

3.4. Artemether attenuated MCAO-induced cell injury

Morphological analysis revealed that MCAO caused neuronal vacuolar degeneration that was reduced by artemether treatment (10 mg/kg) (Fig. 4A-HE). MCAO also induced alterations in the number of Nissl bodies in the peri-infarct region. Specifically, it induced a significant decrease in the number of Nissl bodies compared to control animals that was reversed by artemether treatment (10 mg/kg) (Fig. 4A-Nissl, B). MCAO mice also presented a significantly higher number of TUNEL-positive cells in the peri-infarct region, an increased Bax/Bcl-2 expression ratio and cleaved caspase 3 expression level when compared to control animals. The number of apoptotic cells was significantly decreased by the treatment with artemether (10 mg/kg) (Fig. 4A-TUNEL, C). The MCAO-induced increase of Bax/Bcl-2 expression ratio and cleaved caspase 3 expression level were also significantly decreased upon artemether treatment (5, 10 and 20 mg/kg) (Fig. 4F–H).

3.5. Artemether promoted neuronal proliferation in MCAO mouse model

Artemether treatment (10 mg/kg) significantly increased the expression levels of the neural stem cell marker (nestin) in the brain peri-infarct region (Fig. 4A-Nestin, D). Further assessment of BrdU/NeuN positive neurons revealed that artemether treatment (10 mg/kg) promoted the neuronal proliferation in this area (Fig. 4A-BrdU + NeuN, E).

Fig. 5. Artemether decreased the levels of malonaldehyde (MDA) and increased the levels of superoxide dismutase (SOD) after MCAO. (A) The levels of malonaldehyde (MDA) and (B) superoxide dismutase (SOD) were determined using an enzyme-linked immunosorbent assay. Three independent experiments were performed in triplicate. *p < 0.05, **p < 0.01 were considered significantly different.

Fig. 6. Artemether stimulated the phosphorylation of Erk1/2-P90rsk-CREB after MCAO. (A) The phosphorylation of ERK, P90rsk and CREB was detected by western blotting. (B, C, D) Quantification of western blotting results. Three independent experiments were performed in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001 were considered significantly different.
3.6. Artemether attenuated MCAO-induced oxidative stress

Lipid oxidation occurs whenever oxidative stress occurs. MDA is a natural product of lipid oxidation in organisms, so it can be used to detect lipid oxidation levels. The results revealed that the MDA levels were significantly increased in the brains of MCAO model group. Upon artemether treatment (5, 10, 20 mg/kg), MDA levels significantly decreased (Fig. 5A). In contrast, assessment of SOD contents revealed that the level of SOD was significantly decreased in the brain of MCAO model group. Artemether treatment induced a significant increase of SOD contents in the brains of MCAO animals (Fig. 5B).

3.7. Artemether stimulated Erk1/2-P90\textsuperscript{rsk}-CREB signaling pathway in MCAO mouse model and this effect was blocked by PD98059

Previous studies reported that up-regulation of ERK 1/2 signaling activity has a positive neuroprotective effect in ischemia stroke in vivo and in vitro models [47,48-50]. In this study, MCAO induced a down-regulation of the phosphorylation of ERK1/2, P90\textsuperscript{rsk} and CREB, the downstream proteins of ERK1/2. Upon artemether treatment the phosphorylation levels of ERK1/2, P90\textsuperscript{rsk} and CREB significantly increased (Fig. 6). Moreover, animals pretreatment with the ERK1/2 inhibitor PD98059, resulted in the inhibition of the neuroprotective effect of artemether on the infarction volumes (Fig. 7A and B) and neurological deficits (Fig. 7C), with an apparent direct correlation with the down-regulation of Erk1/2-P90\textsuperscript{rsk}-CREB signaling activities (Fig. 7D–G).

3.8. Artemether attenuated OGD/RP-induced cell cytotoxicity

OGD conditions during 4, 6 and 8 h induced a significant decrease in the cells viability compared with the control group. Attending to the significant impact of OGD conditions for 4 h followed by 20 h of reperfusion on cell viability (~60%), this protocol was selected to establish the OGD/RP model in all the experiments (Fig. 8A). Assessment of the possible neuroprotective effect of artemether on OGD/RP-induced cell death revealed that artemether pre-treatment improved cell viability in a dose-dependent manner (10–100 μM) (Fig. 8B). Measurement of LDH release, Caspase 3 activity, MDA and SOD contents revealed that artemether (30 μM) conferred neuroprotection towards OGD/RP-induced necrotic cell death, cell apoptosis and oxidative stress (Fig. 8C–F).

3.9. Artemether reduced intracellular ROS, reversed mitochondrial membrane potential (Δ\text{ψ}_m) and decreased cell apoptosis in OGD/RP-induced cell injury

Cells exposure to OGD for 4 h and reperfusion for 20 h induced a significant increase in the production of intracellular ROS and a significant decrease of cells mitochondrial membrane potential compared with control cells that were reversed by artemether pre-treatment (10–100 μM) (Fig. 9A–C). Further investigation of the neuroprotective effect of artemether upon OGD/RP-induced apoptosis, revealed that artemether pre-treatment (10–100 μM) significantly reversed the OGD/RP-induced increase of cell apoptosis (Fig. 9D and E). In accordance with these nuclei morphological changes, FACS analyses of Annexin-V-FITC labeled cells also indicated that artemether inhibited OGD/RP-induced apoptosis. Furthermore, the OGD/RP -induced increase of Bax/Bcl-2 expression ratio was also decreased upon artemether pre-treatment with different concentrations (Fig. 9H and I).

3.10. Erk1/2-P90\textsuperscript{rsk}-CREB signaling pathway is involved in the neuroprotective effect of artemether on OGD/RP-induced cell injury

Assessment of the involvement of Erk1/2-P90\textsuperscript{rsk}-CREB signaling pathway in the neuroprotective effect of artemether on OGD/RP-induced cell injury, revealed that similarly to the MCAO experimental
model, artemether pre-treatment also induced a significant increase in the phosphorylation of ERK1/2, P90rsk and CREB (Fig. 10). In addition, the neuroprotective effect of artemether in the cells injured by OGD/RP was blocked upon inhibition of ERK1/2 with PD98059 (Fig. 11A). Knock-down of ERK1/2 further validated these findings as it robustly eliminated the cell protective tendency of artemether (Fig. 11B and C). Additional experiments revealed that treatment of cells with PD98059 prevented artemether-induced decrease of intracellular ROS and cell apoptosis and reversal of mitochondrial membrane potential (Δψm) (Fig. 11D–G).

3.11. Artemether conferred neuroprotection against OGD/RP induced cell injury in primary cultured cortical neurons

The possible neuroprotective effect of artemether on OGD/RP-induced cell injury in primary cultured cortical neurons was assessed. MTT results revealed that artemether pre-treatment improved cell viability in a dose-dependent manner (3–30 μM) (Fig. 12A) and that this neuroprotective effect was blocked upon inhibition of ERK1/2 with PD98059 (Fig. 12B). Measurement of LDH release revealed that artemether (10 μM) conferred neuroprotection towards OGD/RP-induced cell injury.
Fig. 9. Artemether decreased mitochondrial membrane potential, the production of ROS and cell apoptosis in PC12 cells exposed to OGD/RP conditions. PC12 cells were pre-treated with varying concentrations of arte- 

mether or 0.1% DMSO (vehicle control) for 2 h and then incubated with or without OGD conditions for 4 h followed by 20 h of reperfusion. (A) Representa- 

tive images of DCFH-DA assay and of cells stained with JC-1 dyes depicting intracellular ROS and the changes of mitochondrial mem- 

brane potential (△ψm), respectively. (B) Quantification of intracellular ROS levels. (C) Quantification of the red to green fluorescence intensity ratio representing the loss of mitochondrial membrane potential. (D) Representative images of Hoechst 33,342 staining. The apoptotic cells with condensed chromatin are indicated by an arrow head. (E) Quantification of apoptotic cell’s nuclei. (F,G) Determina- 

tion of cell apoptosis by flow cytometry. (H) Bcl-2 and Bax levels were detected by western blotting. (I) Quantification of Bax/Bcl-2 ratio. Three independent experiments were performed in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001 were considered significantly different. (For interpreta- 

tion of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
necrotic cell death and this effect was also blocked upon inhibition of ERK1/2 with PD98059 (Fig. 12C). Moreover, artemether (10 μM) reduced intracellular ROS levels, reversed mitochondrial membrane potential (Δψm) and decreased cell apoptosis and these effects were blocked upon inhibition of ERK1/2 with PD98059 (Fig. 12D–I).

Assessment of the involvement of Erk1/2-P90rsk-CREB signaling pathway, revealed that similarly to the PC12 cell model, artemether pre-treatment also induced a significant increase in the phosphorylation of ERK1/2, P90rsk and CREB in primary cultured cortical neurons, and these effects were blocked upon inhibition of ERK1/2 with PD98059 (Fig. 12J–M).

4. Discussion

This work describes for the first time, the neuroprotective effect of artemether, a derivative of artemisinin, on ischemic stroke. Stroke is an age-related disease and the second most common cause of death [51, 52]. A complex series of biochemical and molecular mechanisms that include excitotoxicity, calcium overload, oxidative stress, inflammation and apoptosis are involved in cerebral ischemia-induced impairment, resulting in fatal neuronal death [53]. So far, all therapies, except anti-thrombolitics and hypothermia approaches, have failed to significantly reduce neuronal injury, neurological deficits, and mortality rates following cerebral ischemia, suggesting that the development of novel therapies against stroke is urgently needed. Oxidative stress is one of the most important factors that contribute to ischemic injury. Reactive aldehydes generated from oxidized lipids, such as malondialdehyde (MDA), have been detected in almost all tissues that are subjected to ischemia [54]. Several studies have shown that artemisinin confers neuroprotection through the resistance to oxidative stress induced by hydrogen peroxide (H2O2), sodium nitroprusside (SNP), β-amyloid (Aβ) and glutamate [33,55–57]; and artemether (one of artemisinin derivatives) has been reported to be a potential therapeutic agent against AD [32]. However, there are no reports of artemether related to ischemic stroke. Hence, this work aimed to assess the protective effect of artemether in a MCAO stroke animal model and in an OGD/RP cellular model, which have been widely used for studying cerebral ischemia [58, 59]. The establishment of the MCAO experimental animal model required the animals to remain deeply anesthetized for a long period of time. Pentobarbital was the anesthetic of choice has previously described in other studies reporting the use of the same experimental animal model [60–62]. Despite being reported to reduce blood pressure (BP), thereby increasing the tolerance to an ischemic insult, this effect is highly dependent on the route of administration being less pronounced with IP administration rather than IV administration [63]. Pentobarbital has also been reported to have a hypothermic effect [64], therefore its administration was performed slowly and a heating pad was used to maintain the body temperature at 37 ± 0.5 °C throughout the procedure.

Our results showed that artemether administration to MCAO animal model resulted in a substantial functional recovery, related to the reduction of the brain infarction volume and water content, reduced oxidative stress levels and cell apoptosis, and promotion of cell proliferation. Recently, the role of mitochondria has attracted much attention with regards to cerebral ischemic injury [65–67]. During ischemia, hypoxia depletes intracellular ATP, inactivates oxidative phosphorylation and leads to a compensatory transformation of anaerobic metabolism [68]. Re-introduction of oxygen by reperfusion significantly increases the production of destructive reactive oxygen species...
superoxide and hydrogen peroxide from mitochondria \[67, 69\] damaging cellular lipids, proteins and DNA \[70\]. This leads to the destruction of mitochondrial ATP supply inducing the opening of mitochondrial permeability transition pore, thereby inducing cell death \[71, 72\]. Therefore mitochondrial oxidative damage is a major contributor for ischemic stroke injury. In line with these evidence, our results showed that exposure of cells to OGD/RP conditions caused the collapse of mitochondrial membrane potential, increased ROS levels and up-regulated Bax/Bcl2 ratio, an indicator of cellular apoptosis. Artemether pretreatment significantly suppressed these changes. Similarly, it also induced a significant change of MDA levels and SOD activity in both in vitro and in vivo models, suggesting that artemether’s antioxidant activity may be involved in its neuroprotective effect on stroke. These results are consistent with our previous studies reporting the antioxidant

**Fig. 11.** The effect of artemether was blocked by ERK1/2 inhibition or silencing. (A) PC12 cells were pretreated with 25 \(\mu\)M PD98059 (ERK1/2 inhibitor) for 40 min, followed by incubation with artemether 30 \(\mu\)M or 0.1% DMSO (vehicle control) for 2 h and then incubated with or without OGD conditions for 4 h followed by 20 h of reperfusion. Cell viability was measured by MTT assay. (B) Cells were transfected with si-CTRL or si-ERK1/2 and the knock-down efficiency of Erk1/2 was assessed by western blotting. (C) Transfected cells followed were treated with 30 \(\mu\)M artemether or 0.1% DMSO (vehicle control) for 2 h and then incubated with or without OGD conditions for 4 h followed by 20 h of reperfusion. Cell viability was measured by MTT assay. (D) Representative images of ROS, mitochondrial membrane potential and apoptosis on cells pretreated with 25 \(\mu\)M PD98059 (ERK1/2 inhibitor) for 40 min, artemether 30 \(\mu\)M or 0.1% DMSO (vehicle control) for 2 h and incubation with or without OGD conditions for 4 h followed by 20 h of reperfusion. (E, F, G) Quantification of ROS, mitochondrial membrane potential and cell apoptosis. Three independent experiments were performed in triplicate. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\) were considered significantly different.
Fig. 12. Artemether conferred neuroprotection against OGD/RP-induced cell injury in primary cultured cortical neurons. Primary cultured cortical neurons were prepared and grown according to the commonly used protocols in our laboratory. (A) Cells were pre-treated with varying concentrations of artemether (3–30 μM) or 0.1% DMSO (vehicle control) for 2 h and then incubated with or without OGD conditions for 4 h followed by 20 h of reperfusion. Cell viability was measured by MTT assay. Cells were pre-treated with 20 μM PD98059 (ERK inhibitor) for 30 min before pretreatment with 10 μM artemether or 0.1% DMSO (vehicle control) for 2 h, and then incubated with or without OGD conditions for 4 h followed by 20 h of reperfusion. Cell viability and LDH release were measured by MTT (B) and LDH assays (C). (D) ROS and mitochondrial membrane potential detected by DCFH-DA and JC-1 dye, respectively. (E, F) Quantification of ROS and mitochondrial membrane potential. (G, H) Representative images of apoptosis detected by Hoechst 33,342 and quantification of cell apoptosis. (I) Measurement of Caspase 3 activity. (J) The phosphorylation of ERK, P90rsk and CREB was detected by western blotting. (K, L, M) Quantification of western blotting results. Experiments were performed in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001 were considered significantly different.
activity of artemether in Aβ-induced injury model and the antioxidant activity of artemisinin [8,9,32,42,43].

Assessment of the involved signaling pathways revealed that ERK1/2 signaling correlated with the neuroprotective effect of artemether in MCAO animal and OGD/RP cellular models through activation of Erk1/2-P90rsk-CREB signaling cascade. ERK1/2, one of the most extensively studied mitogen-activated protein kinases (MAPK) family members, is involved in the regulation of several cellular processes such as neuronal plasticity, migration and cell survival [73,74]. Evidence also suggest it has a key role in mediating oxidative stress-induced apoptosis [75,76]. Moreover, up-regulation of ERK1/2 signaling activity is associated to a neuroprotective effect in in vivo and in vitro ischemia stroke models [48,49]. Our results suggest that activation of Erk1/2-P90rsk-CREB pathway plays a critical role in artemether mediated neuroprotective effect against cerebral ischemic injury. Upon inhibition of ERK1/2, artemether’s protective effect against neurological deficits, brain infarction volumes, cell viability, intracellular ROS, mitochondrial membrane potential and cell apoptosis was significantly reduced (Fig. 11).

Artemisinin and its analogues (arts) are used as first-line anti-malarials having also anti-cancer properties. They kill malaria parasites and cancer cells by releasing free radicals which damage the mitochondria and cause apoptosis [77,78]. Arts also target the translationally controlled tumor protein (TCTP) promoting its downregulation and cell apoptosis [79–81]. These reports look contradictory to our present findings describing artemether’s neuroprotective effect on stroke. However, our findings were verified in different cellular and animal models with multiple standard evaluation methods. Moreover, the present findings are consistent with our several previous reports describing the protective effect of arts on different neuronal cells against other insults including H2O2, SNP, corticosterone and β-amyloid [8,33,55]. Importantly, our findings are also supported by studies from other groups in the field. For example, it was reported that artemisinin inhibited glutamate-induced apoptosis in hippocampal neuronal HT-22 cells [57]. Artemisinin also effectively inhibited neuronal apoptosis and improved cognition and memory via regulating histone acetylation and JNK/ERK1/2 signaling [82]. In a different study, artemisinin attenuated doxorubicin-induced cardiotoxicity and hepatotoxicity in rats [83]. Artemisinins treatment also promoted the decrease of the number of TUNEL positive cells in myocardial tissue section [84].

Why arts kill malaria parasites/cancer cells while protecting neuronal cells from different insults is not clear at present but possible in several ways. First, arts usually need high concentrations to kill cancer cells while lower concentrations are used for neuronal protection [85]. Second, the mechanisms in anti-malaria/anti-cancer effect of arts may be different from the ones involved in their neuroprotective effect. In the case of anti-malaria/cancer, arts release free radicals and damage the mitochondria, while for neuronal protection arts reduce the accumulation of ROS, reverse the reduction of mitochondria membrane potential and inhibit apoptosis via activation of ERK, AMPK and P38 [9,34,86]. Third, different artemisinin analogues also have different effects. Some arts are more toxic while others are more protective. For example, artemisinin and artemether are more protective while dihydroartemisinin and artesunate are more toxic and used more often in anti-cancer studies [87,88]. Finally, the effect of arts may be different in different cell types. Arts are more toxic for cancer cells and have a low toxicity on normal cells. These phenomena are also seen in other active components of Chinese traditional medicine such as aloe-emodin [89], astragaloside IV (AS-IV) [90] and berberine [91]. Nevertheless, more studies are necessary to fully uncover the different mechanisms underlying the anti-malaria/cancer and neuronal protection of arts in the future.

In conclusion, our study suggests that artemether has a neuroprotective effect against cerebral ischemic injury via activation of Erk1/
2-P90rsk-CREB pathway (Fig. 13). Obtained results support the potential use of artemether in the development of more effective therapeutic approaches against ischemic stroke.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of the manuscript entitled “Artemether confers neuroprotection on cerebral ischemic injury through stimulation of the Erk1/2-P90rsk-CREB signaling pathway”.

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