Anti-apoptosis effects of codonolactone on cerebral ischemia-reperfusion injury

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ABSTRACT

Codonolactone is the main biologically active ingredient in *Atractylodes lancea*. Studies have shown various functions of codonolactone, while its protective effect against neurotoxicity caused by ischemic stroke is unclear. This study investigated the roles of codonolactone in inflammation, oxidative stress and apoptosis after cerebral ischemia-reperfusion (I/R) injury. Rats with codonolactone treatment, I/R treatment and the sham operation group were used in this study. After reperfusion for 24 hours, nerve damage was detected by nerve staining, and the neurological deficits of the rats were analyzed. The contents of superoxide dismutase (SOD), malondialdehyde (MDA), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in rat brain tissues were also determined. Western blot analysis was performed to determine the expression levels of Akt/Nrf2 pathway-associated proteins. Compared with the I/R group, the cerebral blood flow, infarct volume, brain water content, coronary blood flow and neurological deficits in the codonolactone treatment group, especially with the 80 mg/kg dosage, were significantly reduced. Codonolactone could significantly reduce the expression levels of caspase-3 and Bax, and significantly increase the expression levels of Bcl-2 after I/R. In addition, codonolactone could significantly reduce MDA content and the expression levels of TNF-α and IL-1β in ischemic brain tissues. It also significantly increased SOD activity, the expression levels of heme oxygenase-1 (HO-1) and the phosphorylation of Akt and Nrf2. Codonolactone ameliorated the cerebral I/R injury by improving anti-oxidant, anti-inflammatory activities and reducing apoptosis. Besides, the Akt/Nrf2 pathway was involved in the pharmacological action of the codonolactone.

Significance of this study

What is already known about this subject?

⇒ Codonolactone plays an important role in antitumor, cytotoxic, anti-oxidant, antibacterial and antigenic variability.
⇒ Studies have found that codonolactone can exert anti-inflammatory functions by inhibiting enzyme activities or medium release.
⇒ Previous studies have found that atractylenolide III was able to reduce complications associated with ischemia by inhibiting neuroinflammation.

What are our visions based on the known themes?

⇒ The current research of cordonolactone on cerebral infarction is still lacking, and it has an effect on cerebral infarction.
⇒ Codonolactones can affect cell apoptosis when they are antitumor factors, so can they affect cerebral ischemia-reperfusion (I/R) injury?
⇒ How does codonolactone regulate the inflammatory response?
⇒ Will it affect the inflammatory response of cerebral I/R injury?

What are the new findings?

⇒ Codonolactone could reduce infarct size, reduce neurological deficit, alleviate pathological changes caused by ischemia after reperfusion and had protective effect on cerebral I/R injury.
⇒ Codonolactone might downregulate the inflammatory response induced by reperfusion through downregulation of inflammatory factors.
⇒ Codonolactone could reduce the production of free radicals, exhibit good anti-oxidative damage and reduce lipid peroxidation.
⇒ Codonolactone could protect cerebral I/R injury by regulating the expression of apoptosis-related proteins.

INTRODUCTION

Ischemic stroke is one of the three major diseases in the world that are leading causes of deaths.[1] The cost for treatment and care for patients with ischemic stroke have become a heavy economic burden in modern society.[2]As the focus of medical basics and clinical researches, the mechanisms of various types of death of nerve cells caused by cerebral ischemia have been investigated.[3 4] The pathophysiological process of cerebral ischemic injury is gradually understood and recognized with extensive research studies. Although researchers have actively intervened in these mechanisms of ischemic stroke, there is no effective brain protection therapy available, except for early thrombolytic therapies such as tissue plasminogen activator and urokinase.[5 6] Therefore, researchers all over the world are actively looking for effective clinical

Significance of this study

How might these results change the focus of research or clinical practice?

⇒ This study provided an experimental basis for the clinical therapeutics of codonolactone in cerebral ischemic diseases treatment.

neuroprotective drugs to reduce the morbidity with acute ischemic stroke.[7] The pathogenesis and prevention of ischemic stroke will still be the focus of clinical medical research in the future.

If the recanalization of cerebral blood flow (CBF) exceeds the time limit of the reperfusion time window, brain damage can continue to increase,[8] leading to cerebral ischemia-reperfusion (I/R) injury.[9] Previous studies on the effects of cerebral I/R injury have been focusing on oxygen free radicals, excitatory amino acids and intracellular calcium overload.[10] Recent studies have found that brain tissues and cells are damaged by interruption of CBF and blood recanalization, which is a dynamic and rapid cascade reaction.[11] It is closely related to inflammation and cell apoptosis, and there is a connection network between the mechanisms.[12 13]

Codonolactone is a main bioactive component of *Atractylodes lancea*.[14] It has been reported that codonolactone plays an important role in antitumor, cytotoxic, antioxidant, antibacterial and antigenic variability.[15 16] A schematic diagram of the structure of codonolactone is shown in figure 1A. Although studies have found that codonolactone can exert anti-inflammatory functions by inhibiting enzyme activities or medium release,[15] the mechanism of the action of codonolactone on cerebral I/R is still unknown. Previous studies have found that atractylenolide III (a sesquiterpene lactone found in *Atractylodes macrocephala Koidz*) was able to reduce complications associated with ischemia through inhibiting neuroinflammation, which was mediated in part by JAK2/STAT3-dependent mitochondrial fission in microglia.[17] In this study, the rat middle cerebral artery occlusion (MCAO) reperfusion model was established to investigate the effects of codonolactone on inflammatory factors and anti-oxidant factors after I/R. Our findings will clarify the potential protective mechanisms of codonolactone in cerebral I/R injury, and provide new research data for understanding the mechanism of cerebral I/R injury and brain protection.

MATERIALS AND METHODS

Animals and chemical information

A total of 218 healthy male Sprague-Dawley rats (weighted 200–220 g, 7–8 weeks old, n=6) were purchased from the Experimental Animal Center of Zhejiang University. The chemical structure of codonolactone (purity≥98.0%) is shown in figure 1A. The codonolactone was purchased from Shanghai Pure One Biotechnology (Shanghai, China).

Focal cerebral ischemia-reperfusion model

The modified Longa method was used to establish the MCAO reperfusion model. Before operation, rats were fasting for 12 hours and allowed to drink water freely. After intraperitoneal injection (10 mL/kg) with 3.5% chloral hydrate, the rat was placed on the back of the neck and the midline incision was taken. The insertion plug (diameter 0.26–0.28 mm, length 5 cm) was stopped by the arterial bifurcation to the internal carotid artery 18–20 mm. After 2 hours of embolization, the embolism was withdrawn and reperfused for 1 day. In the sham group, the thread plug was not inserted, and the remaining steps were the same as above. The anus temperature (37°C±0.5°C) was maintained during the operation with a lamp. The animals were put back into their cages and fed freely after awakening. Nimodipine (4 mg/kg), purchased from Bayer Healthcare (Leverkusen, Germany), was dissolved in 0.9% sodium chloride (NaCl) and administered intraperitoneally at the onset of reperfusion.

Exclusion criteria: at the time point of 24 hours after reperfusion, neurological deficits were scored by one investigator who was blind to the experimental design. In order to exclude the interference of operative failures, the rats subjected to MCAO/R with no detectable neurological deficits were eliminated. The mortality rate was about 21%.

Drug administration and groups

Sham-operated group, I/R group and codonolactone group of rats were randomly divided. The sham operation group (n=60) was treated with MCAO without common carotid artery (CCA). Ligation was performed for 2 hours, followed by reperfusion for 24 hours in I/R group (n=60). In codonolactone treatment group (n=60), 5–80 mg/kg of codonolactone was used. Rats in I/R group and sham operation group received an equal volume of 0.9% NaCl at the same time point. After reperfusion for 24 hours, rats were decapitated. The specific experimental design of the study is shown in figure 1B.

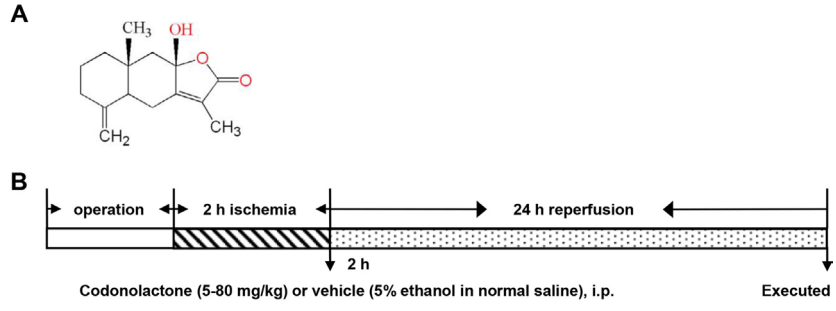

Figure 1 (A) Chemical structure of codonolactone. (B) Specific experimental design.

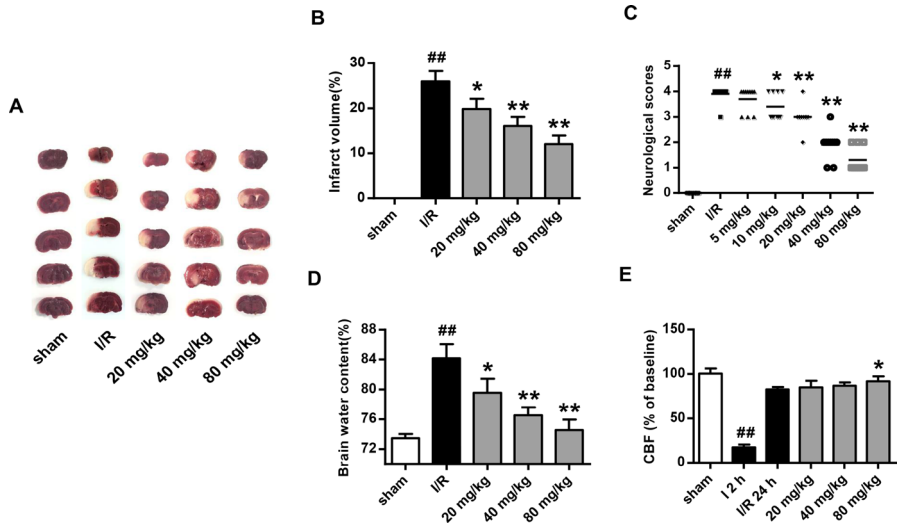

Figure 2 Codonolactone ameliorated neurological scores, cerebral blood flow (CBF) and reduced infarct volume and brain water content. Infarct volume (A–B), neurological deficit scores (C), brain water content (D), CBF (E) at ischemia-reperfusion (I/R) 24 hours, n=6 versus sham operation group, ##p<0.01; versus I/R group, *p<0.05, **p<0.01.

Assessment of infarct volume, neurological deficit, brain water content and cerebral blood flow

Rats were decapitated after 24 hours of cerebral I/R injury. Some of the rats were used to extract the cerebellum, olfactory bulb and lower brainstem, which were quick frozen. The others were used to extract the whole brain, which was sliced and stained with 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) for 30 min. White staining represents infarction and red staining represents normal brain. The area of cerebral infarction was measured by an image analysis system. The ratio of cerebral infarction volume to the total brain volume was calculated. Neurological deficits were scored according to the 5-point standard described in literature[18] after 24 hours of cerebral I/R in rats. Rats were decapitated after 24 hours of cerebral I/R, the cerebellum, olfactory bulb and lower brainstem were removed and weighed. After baking, the dry brain tissues were weighed. Brain water content (%)=(wet mass−dry mass)/wet mass×100%. A laser Doppler flowmeter was used to analyze the CBF.

Nissl staining

After rats were anesthetized with intraperitoneal injection of 35 mg/kg pentobarbital sodium, saline and then 4% paraformaldehyde phosphate buffer were used for transcardial perfusion in the rats at 24 hours or 7 days after reperfusion. The whole brain was extracted and fixed in 4% paraformaldehyde at 4°C for 2 hours. Coronal sections were sliced at 10 μm and stored at −20°C. Before using the brain slices, the sections were successively infiltrated with 0.1 M phosphate-buffered saline containing 20% sucrose overnight, and then with 25% sucrose overnight. The numbers of neurons with the features of ischemic cell change (eosinophilic cytoplasm, triangulated, shrunken cell bodies and pyknotic nuclei) and morphologically normal neurons were detected by Nissl staining kit (Beyotime Institute of Biotechnology, China). ImagePro Plus 5.1 software (Media Cybernetics, Bethesda, Maryland, USA) was used to count the neurons in 10 different fields in each region of brain slices. Dividing the sum of ischemic neuron number by the total number of neurons was used to calculate the percentage of ischemic neurons in the 10 defined areas.

Determination of antioxidant index

The rats were sacrificed by decapitation. The supernatant was aspirated and stored after centrifuging the brain tissues at 2500 g for 5 min. Frozen saline was used to homogenize the occipital lobe. The product of lipid peroxidation, malondialdehyde (MDA), was measured by a detection kit (Beyotime, China). Spectrophotometry at 532 nm was used to measure the absorbance of the supernatant. Total cerebral superoxide dismutase (SOD) activity was determined by a detection kit (Jiancheng Bio Institution, Nanjing, China). Spectrophotometry at 450 nm was used to measure the absorbance of the supernatant.

Western blot analysis

The supernatant of brain tissue was collected by centrifugation, and total proteins were extracted after 24 hours of reperfusion. BCA Protein Assay Kit was used to quantify the protein concentration, and then the protein was separated and transferred onto polyvinylidene difluoride membrane. The phosphatidylinositol 3 kinase (PI3K) inhibitor LY294002 (0.3 mg/kg; Cell Signaling Technology, Beverly, Massachusetts, USA) was solubilized in 0.9% NaCl which included 3% dimethylsulfoxide and intraperitoneal injected 20 min before the onset of ischemia (30230477; 24801159). The primary antibody of Nrf2 (1:500, Shidai, China), heme oxygenase-1 (HO-1) (1:500, Shidai, China), Akt (1:500, Shidai, China) and p-Akt (1:500, Shidai, China), Bcl-2 (1:1000, Shidai, China), cleaved caspase-3 (1:1000, Shidai, Shanghai, China) or Bax (1:1000, Shidai, China), interleukin-1β (IL-1β) (1:500, Shidai, China), tumor necrosis factor-α (TNF-α) (1:500, Shidai, China) and β-actin antibody (1:5000, Shidai, China) were used to incubate the membrane overnight. After that, antirabbit secondary antibody (1:5000, Shidai, China) was used to

incubate the membrane for 1 hour. Western blot analysis was performed as previously described in literature.[19]

H&E stain examination

The rats were sacrificed after 24 hours of reperfusion, and brain tissues were fixed with 4% formaldehyde. The brain tissues were dehydrated with different concentration gradients of alcohol and embedded in paraffin and cut into 5 mm sections. To detect morphological changes in neurons, the sections were stained with H&E according to the standard procedure, and subjected to Nissl staining using 0.1% cresyl violet acetate. The number of intact cells in the penumbra of the ischemic cortex by Nissl staining was counted through five randomly selected lesion regions.[20]

TdT-mediated dUTP Nick-End Labeling (TUNEL) assay

To test the DNA fragmentation associated with apoptosis, the TUNEL assay was performed. The brain slices in each group were prepared and the In situ Cell Death Detecting Kit (Roche Diagnostics, Penzberg, Germany) was used for achieving TUNEL staining. TUNEL staining was performed following the manufacturer's instructions. Apoptotic cells were identified as those with a brown-stained nucleus. Cells were counted in five randomly selected fields, and the apoptosis index was calculated as the percentage of positive cells to total cells. Samples were analyzed under a microscope and researchers were blind to different groups.[20]

Statistical method

SPSS V.19.0 statistical software was used to analyze the data, which were shown as the mean±SD. One-way analysis of variance was used to explore the differences among multigroup data, and then the least significant difference test was used for further analysis. $P<0.05$ indicated significant difference.

RESULTS

Codonolactone ameliorated neurological scores, cerebral blood flow and reduced infarct volume and brain water content

To assess the effect of codonolactone on brain I/R injury, MCAO/R model was established. Rats were treated with 20–80 mg/kg of codonolactone. When given 24 hours after occlusion, we found that the infarct size in I/R group was significantly increased compared with that in the sham group ($p<0.01$), while it was significantly reduced in codonolactone group (20–80 mg/kg) (figure 2A,B, $p<0.05$). In addition, we found that the neurological deficit scores in I/R group were significantly increased compared with that in the sham group (figure 2C, $p<0.01$, $n=10$), but significantly reduced in the codonolactone group in a dose-dependent manner compared with that in the I/R group ($p<0.05$). The brain water content in the I/R group was significantly increased than that in the sham group (figure 2D, $p<0.01$), while it was significantly less in the codonolactone group than that in the I/R group (figure 2D, $p<0.05$). As shown in figure 2E, CBF was markedly lower in I/R group than that in the sham group ($p<0.01$), while codonolactone could significantly increase CBF after reperfusion ($p<0.05$).

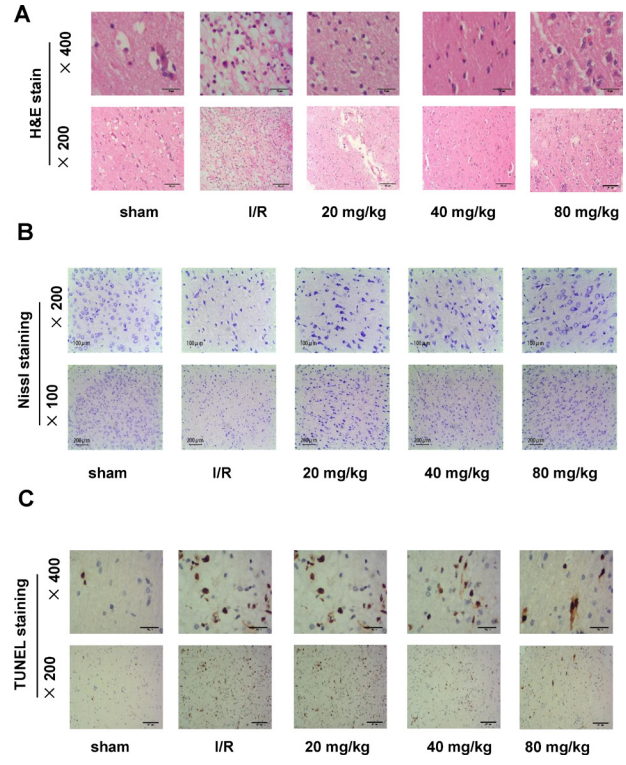

Figure 3 Codonolactone protected against ischemia-reperfusion (I/R)-induced brain tissue injury and weakened the bain tissue apoptosis. (A) H&E staining. (B) Nissl staining results. (C) TUNEL staining. n=4, original magnification 200× and 400×, respectively. The area in box was presented as magnification 400×; n=6 versus sham operation group, ##p<0.01; versus I/R group, *p<0.05, **p<0.01.

The effect of codonolactone on brain tissue injury and brain tissue apoptosis induced by I/R

We discussed the protective effect of codonolactone on rat brain tissue cell damage after I/R injury. H&E staining was used to check the tissue morphology changes. As shown in figure 3A, in the cerebral cortex, the cytoplasm of the sham group was average, and the nuclei had clear edges and orderly arrangement. Most of the neurons in the ischemic part of the cerebral cortex in the I/R group were atrophied and stained darker. Contrary to the I/R group, the residual neuron structure in the codonolactone treatment group was slightly atrophied, which was significantly better than that of the I/R group, and it had a certain improvement effect.

Nissl staining (figure 3B) showed that compared with the sham group, the intercellular space of the cells in the I/R group became larger, the cytoplasm was atrophy and deep staining appeared. Compared with the I/R group, the codonolactone treatment group had less cell atrophy, lighter staining and richer cytoplasm. In addition, TUNEL staining was performed to determine the effect of codonolactone on neuronal apoptosis.[20] The results showed that compared with I/R, the neuronal apoptosis in the codonolactone group was significantly improved, as shown in figure 3C, $p<0.05$. These results indicate that codonolactone can effectively reduce I/R damage.

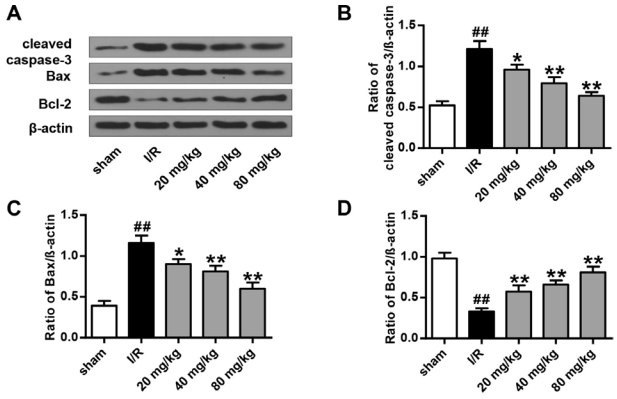

Figure 4 Codonolactone weakened the brain tissue apoptosis measure by western blot analysis. (A) Protein levels of cleaved caspase-3, Bax and Bcl-2. (B) Quantitative assessment of cleaved caspase-3. (C) Quantitative assessment of Bax. (D) Quantitative assessment of Bcl-2, n=6 versus sham operation group, ##p<0.01; versus ischemia-reperfusion (I/R) group, *p<0.05, **p<0.01.

We used western blot analysis to further evaluate the effect of codonolactone on ischemic cell apoptosis. Compared with the sham group, the expression levels of cleaved caspase-3 and Bax in the I/R group were significantly increased, while the expression level of Bcl-2 was significantly reduced (figure 4A–D, p<0.01). Compared with the I/R group, the expression levels of caspase-3 and Bax in the codonolactone group were significantly reduced, and the expression levels of Bcl-2 were significantly increased (p<0.05). These results indicate that codonolactone has anti-apoptotic effects.

Codonolactone inhibited oxidative stress and inflammatory cytokines

The pathophysiological mechanism underlying cerebral I/R injury is very complex. Such injuries are commonly caused by the release of inflammatory factors and oxidative damage. The levels of SOD1 and SOD in the sham group were higher than those in the I/R group as well as those in the codonolactone 20, 40 and 80 mg/kg groups (figure 5A–B, p<0.05). In addition, the expression levels of MDA, TNF-α and IL-1β in the I/R group were higher compared with that in the sham group, and codonolactone reduced the expression levels in a dose-dependent manner (figure 5C–E, p<0.05). These results indicated that the beneficial effects of codonolactone administration under cerebral I/R were associated with anti-oxidative function.

Codonolactone exerted neuroprotective function by activating the Nrf2/HO-1 pathway

To explore the effect of codonolactone on protecting the neurons after I/R, the expression levels of Akt, p-Akt, Nrf-2 and HO-1 in rat brain tissues were detected by western blot analysis and immunohistochemical staining. The results showed that the I/R injury increased the expression levels of proteins Nrf-2 and HO-1 (figure 6A, B and D), and decreased the expression levels of protein p-Akt/Akt (figure 6A,C) compared with that in the sham group. These findings indicated that the neuroprotection effect of

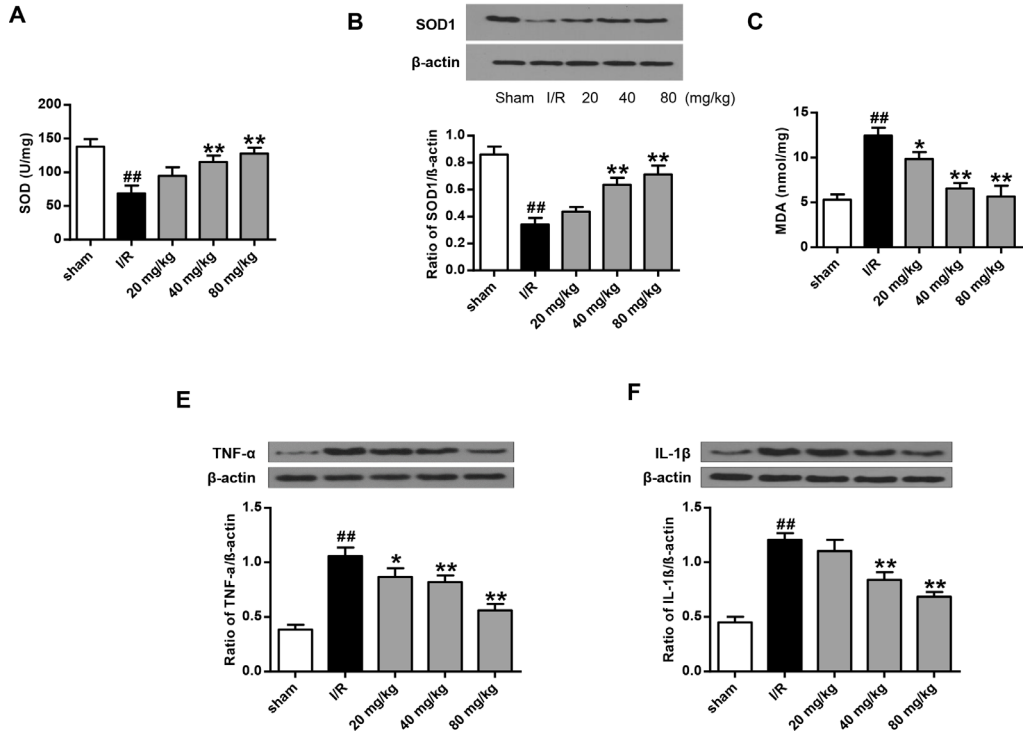

Figure 5 Codonolactone inhibited oxidative stress and inflammatory cytokines. (A) Superoxide dismutase (SOD), (B) superoxide dismutase 1 (SOD1), (C) malondialdehyde (MDA), (D) tumor necrosis factor-α (TNF-α), (E) interleukin-1β (IL-1β) (F) in rats of all experimental groups were determined by ELISA, n=6 versus sham operation group, ##p<0.01; versus ischemia-reperfusion (I/R) group, *p<0.05, **p<0.01.

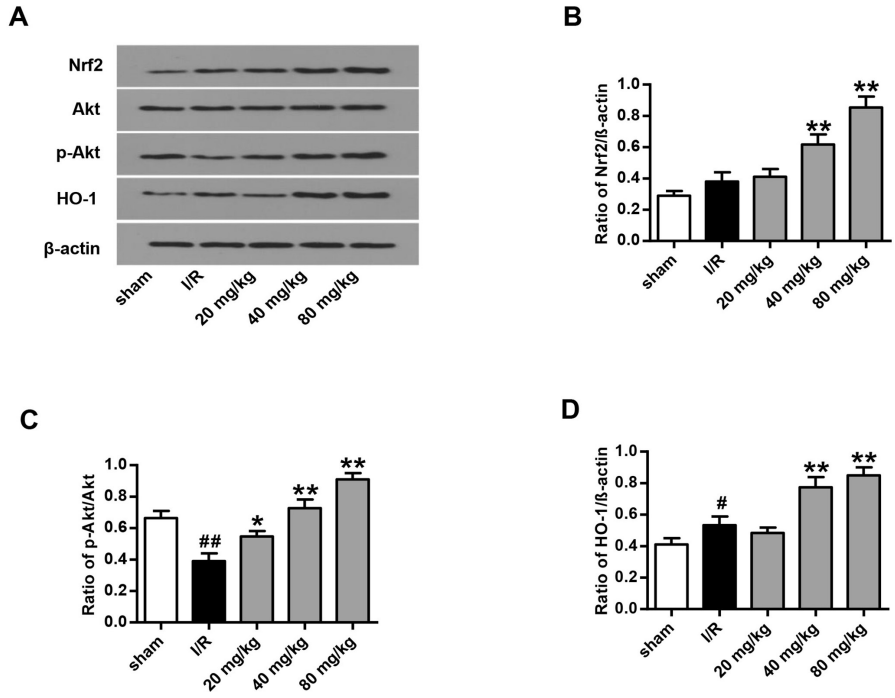

Figure 6 Codonolactone exerted neuroprotective effects by activating the Nrf2/HO-1 pathway. (A) Nrf2, Akt phosphorylation and HO-1protein levels. (B) Quantitative evaluation of Nrf2. (C) Quantitative assessment of Akt phosphorylation. (D) Quantitative assessment of HO-1, n=6 versus sham operation group, #P<0.05;##p<0.01; versus ischemia-reperfusion (I/R) group, *p<0.05, **p<0.01.

codonolactone was accompanied by the upregulation of the Akt/Nrf2/HO-1 pathway.

DISCUSSION

Stroke is an acute cerebrovascular disease caused by impaired blood supply.[21] After the cerebrovascular rupture, the overflowing blood enters the space around the brain cells, causing a stroke.[22 23] It is about three times of the incidence for ischemic stroke than that of hemorrhagic stroke.[24] Therefore, ischemic stroke has been a hot topic of research all over the world.[25 26] It has been reported that the recovery of blood supply can aggravate brain damage and reperfusion injury occurs after cerebral ischemia reaches a certain time limit.[27 28] The high incidence of such diseases is extremely harmful to human health. Therefore, it is very necessary to solve the harm caused by cerebral ischemia reperfusion injury. Codonolactone, a natural product, is the major bioactive component of *A. lancea*, and also found in a range of other medical herbs, such as *Codonopsis pilosula*, *Chloranthus henryi Hemsl* and *A. macrocephala Koidz*. Codonolactone has been well studied, while its functions in cerebral I/R injury are elusive. Our results showed that codonolactone could reduce infarct size, reduce neurological deficit, alleviate pathological changes caused by ischemia after reperfusion and exerted a protective function in cerebral I/R injury.

Inflammatory response is in the ischemic cascade that has been attracting extensive attentions.[29] Many inflammatory factors can be activated under hypoxic and ischemic conditions. They participate in the pathological process of cerebral ischemia damage earlier, further induce other related reactions, leading to neuronal death or apoptosis eventually.[30] TNF-α is an important promoter of many cytokines. It also directly promotes the accumulation of neutrophils to the injured area, leading to expansion of local inflammation and aggravating brain tissue damage.[31] IL-1β acts on vascular endothelial cells, causing changes in endothelial cell morphology and cytoskeletal structure, impairing its function and causing an increase in tissue factor-like coagulation activity.[32] After cerebral I/R injury, endothelial cells are activated by inflammatory cytokines such as IL-1β and TNF-α, which induce neutrophils to enter the ischemic brain tissue and cause inflammation, leading to cerebral edema and tissue necrosis.[33] It is known that one of the most important pathological mechanisms of ischemic stroke is oxidative stress.[34] The Nrf2/HO-1 signaling pathway involves in the pathophysiological response of oxidative stress and has become the main defense mechanism of cellular anti-oxidant stress.[35] Our results showed that codonolactone can downregulate IL-1β and TNF-α, and increase the level of SOD. These findings indicate that codonolactone may downregulate the inflammatory response induced by reperfusion by downregulating inflammatory factors, and affecting the oxidative response of I/R injury, thereby reducing the pathological damage of brain tissue. I/R-induced neurological damage can be attenuated by the activation of the PI3K/Akt signaling pathway.[36] The activity of Nrf2 can also be regulated by the PI3K/Akt signaling pathway.[37] Phosphorylation of Akt enhances the expression of downstream Nrf2 and its target proteins HO-1 and SOD1, thereby promoting endogenous anti-oxidant activity of cells.[38] In this study, it showed that codonolactone could activate the Akt/Nrf2/HO-1 pathway to exert anti-oxidant damage. The rate of lipid peroxidation and the degree of vascular damage, and the level of oxygen free radicals can be reflected by MDA content. As a

free radical scavenger, SOD plays an anti-oxidation role in cerebral ischemia and hypoxia. Our study also found that codonolactone can significantly inhibit the activity of SOD in rat brain tissues and increase the concentration of MDA, which indicates that codonolactone can reduce the production of free radicals, exhibits good anti-oxidant damage and reduces lipid peroxidation. Oxidative stress can lead to apoptosis. Under the action of I/R, apoptotic proteins such as Bax increase the permeability of mitochondrial membrane, which results in the release of substances in mitochondria, such as apoptotic inducible factors, into the cytoplasm, thus initiating endogenous apoptosis.[39] Bcl-2 can directly bind to Bax, which prevents Bax from promoting the release of apoptosis-inducing factor by mitochondria, thereby inhibiting apoptosis.[40] Caspase-3 is the ultimate performer of apoptosis and is an apoptotic effector protein.[41] Our study showed that codonolactone could increase the expression levels Bcl-2 and reduce the expression levels of proapoptotic protein Bax and apoptosis effector caspase-3 after cerebral I/R. It suggested that codonolactone could protect cerebral I/R injury by regulating the expression of apoptosis-related proteins.

CONCLUSION

Codonolactone could defend the brain from I/R injure by reducing inflammation, oxidative stress, apoptosis and the Akt/Nrf2 pathway. This study provided an experimental basis for the clinical therapeutics of codonolactone in cerebral ischemic diseases treatment.

Contributors FC: experimental design, literature review, experiments, data analysis and manuscript preparation. WL: definition of intellectual content, literature research experimental studies, manuscript preparation and editing. All authors read and approved the final manuscript. FC is responsible for the overall content as the guarantor.

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Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval All methods were carried out in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines. The study was carried out in compliance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and approved by the Animal Care and Use Committee of the First Affiliated Hospital of Wenzhou Medical University (No. W6326U).

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

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