Effect of corilagin on cerebral ischaemia/reperfusion-induced cerebral injury in a rat model

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INTRODUCTION

Cerebral ischaemia induced stroke is the third leading cause of mortality and morbidity throughout the world. Within a few minutes after a stroke with cerebral ischaemia, necrosis occurs in cells, and within a few hours to days post-insult, neurons of the surrounding regions undergo injury [1]. Hence, for the prevention of neuronal damage, treatment is required within...
this period of time. Neuronal damage during cerebral ischaemia occurs due to activation of several mechanisms including autophagy, necrosis, and apoptosis [2].

The induction of inflammation and oxidative stress activate the apoptotic processes, and thereby damages neurons [3]. Oxidative stress reduces the synthesis of ATP, thereby disrupting mitochondrial functions, which then alters calcium concentrations in the brain tissue and results in the degeneration of neurons [4]. Moreover, during autophagy, the surviving cells become depleted of cellular components, which results in self-digestion of the damaged cells.

Autophagy is a protective mechanism for cells against starvation, stress, and ischaemia [5]. Although autophagic activity is required for neuroprotection, cell death may occur with excessive autophagy. Studies have shown that during cerebral ischaemia, apoptosis and autophagy were simultaneously activated [6]. A study found that apoptosis was activated in cerebral ischaemia due to autophagy through the upregulation of Beclin-1 [7]. Treatment with appropriate drugs protected the autophagic cells, thus suggesting that targeting autophagic cells could be a good choice for the effective management of stroke [8,9].

In recent years, alternative medicines such as herbal medicines have shown potential to prevent neuronal damage against cerebral ischaemia. *Caesalpinia coriaria* is traditionally used as an herbal medicine in China. An ellagitannin isolated from *C. coriaria*, known as corilagin, was reported to possess analgesic, hepatoprotective, anti-inflammatory, antitumor, and antioxidant activities [10-12]. It was reported that corilagin possesses analgesic activity by altering the glutaminergic system and anti-inflammatory activity by decreasing the production of pro-inflammatory cytokines and mediators, TNF-α, IL-1β, IL-6, NO (iNOS), and COX-2 at both the protein and gene levels, by blocking NF-κB nuclear translocation [13-16]. Thus, the present study evaluated the neuroprotective effects of corilagin on cerebral ischaemia.

**EXPERIMENTAL**

**Animals**

Sprague-Dawley rats (200 to 250 g) were used in this study. All the rats were housed under a controlled condition specified as per the guidelines. The animal experiments were approved by the institutional animal ethical committee of Xianning Central Hospital, The First Affiliated Hospital of Hubei University of Science and Technology, China (no. AHHU/IAEC/2016/11) and the given study followed the guidelines of Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) for experimentation and animal use [17].

**Induction of cerebral ischaemia**

All animals were anaesthetised by intraperitoneal (i.p.) injection of 10 % chloral hydrate (360 mg/kg). Later, the internal carotid artery (ICA), external carotid artery (ECA), and common carotid artery (CCA) were separated from other nerves and muscles. An incision was made on the ECA, and a 0.36 mm diameter round polylysine coated tip, on a 4-0 nylon monofilament, was inserted into the ECA to model cerebral arterial ischaemia. The parameters measured included heart rate, arterial blood gases, and blood pressure observed during the period of surgery. After 90 min of middle cerebral artery occlusion (MCAO), reperfusion was initiated by withdrawing the nylon monofilament. Thereafter, all the animals were separated into five groups including a control group that underwent surgery without inserting a monofilament, a MCAO group that received saline, a corilagin-treated group that received corilagin at 20 mg/kg, i.p., for the 7 days after reperfusion, a Tat-Beclin-1-treated group that received i.p. injections of 1.5 mg/kg of Tat-Beclin-1 on the 3rd and 6th days after MCAO, and a corilagin + Tat-Beclin-1-treated group that received corilagin for 7 days and Tat-Beclin-1 on the 3rd and 6th days after MCAO.

**Assessment of functional deficiency of neurons**

The functional deficiency of neurons was assessed after the treatment period by estimating the neurological deficit score. Neurological deficit scores were determined by observing different behaviours, and scoring was done as follows: 0, no deficiency of neurological function; 1, appearance of flexion in the forelimb; 2, failure to extend the right forelimb completely and the strength to resist a lateral push was obviously decreased; 3, forelimb flexion, rotating, and crawling towards the right side were impaired; and 4, unable or difficult to ambulate spontaneously.

**Determination of volume of brain infarct**

All animals were sacrificed by cervical dislocation and the brains were removed and sectioned into
2 mm sections. To stain the tissue sections, 0.5 % triphenyltetrazolium chloride (TTC) solution was used for 30 min at 37 °C. Thereafter, to fix the tissue sections, paraformaldehyde buffer (4 %) was used at room temperature for 12 h. TTC staining was done to assess the infarcted brain tissue areas. A red colour was shown as unstained tissues, and a pale colour indicated infarcted brain tissues. Imaging software was used to determine the volume of the infarct.

**Determination of water content of the brain**

Cerebral oedema was determined by comparing the wet weight with the dry weight of the tissue, thereby assessing tissue water content. All animals were sacrificed and brains were isolated. Immediately after isolation, the weights of the wet brain tissues were determined, and thereafter, the dry weights of the brain tissues were determined after drying the brain samples at 105 °C in an oven, for overnight.

**Western blot assay**

The infarct penumbra and tissues of its corresponding areas were separated from the isolated brain on ice. RIPA buffer that contained 150 mM NaCl, 1 % sodium deoxycholate, 50 mM Tris buffer, 0.1 % SDS, and Triton X 100 (1 %) was used to homogenize the tissue at 4°C for 45 min. The tissue homogenate was centrifuge at 13,000 RPM at 4 °C for 15 min, and the supernatant solution was separated. SDS-PAGE was used for the separation of proteins, after which samples were transferred to polyvinylidene fluoride (PVDF) membranes. Monoclonal rabbit antibodies against rat LC3 were used to label the tissue proteins, and thereafter, samples were rinsed with phosphate-buffered saline (PBS) solution with the detergent Tween® 20 (PBST). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG was used for the PVDF membranes, at room temperature for 60 min. After rinsing with PBST for 120 min, the reaction was visualized with enhanced-chemiluminescence (ECL) reagent.

**Immunofluorescence studies**

Sucrose solution (20 %) was used to dehydrate the isolated brains overnight, after which 20 mm thick sections of brain tissues were sectioned using a microtome. A PBS solution was used to rinse the tissue sections for 15 min at room temperature, and thereafter, 10 % goat serum was used to block the tissue sections for 40 min. Tissue sections were labelled at room temperature for 4 h with mouse antibody against rat glial fibrillary acidic protein (GFAP), NeuN, and monoclonal rabbit antibodies against rat LC3, plus a PBS control. After rinsing, Dylight® 488-conjugated anti-mouse IgG and Alexa Fluor 594-conjugated anti-rabbit IgG were incubated with tissue sections for 120 min. To stain nuclei, 6-diamidino-2-phenylindole (DAPI) stain was used for 5 min, and observed by fluorescence microscopy.

**Statistical analysis**

All data are shown as mean ± standard deviation (SD, n = 10), and were analysed by one-way ANOVA, followed by Dunnett’s post hoc test (Gradpad prism 6.1., CA, USA). P < 0.05 was considered statistically significant.

**RESULTS**

**Effects of corilagin on neurological defects**

The effects of corilagin on rat neurological defects induced by MCAO in cerebral ischaemia are shown in Figure 1. Cerebral ischaemia significantly (p < 0.01) enhanced the neurological deficit score in the MCAO- and Tat-Beclin-1-treated groups compared to the control group. However, treatment with corilagin significantly decreased the neurological deficit score (p < 0.05, p < 0.01) in cerebral ischemic rats compared to the MCAO group. Moreover, the neurological deficit scores were increased in the corilagin+tat-Beclin-1-treated group compared to the corilagin-treated group.

**Effect of corilagin on infarct volume**

The effect of corilagin on the infarct volume was determined by TTC staining as shown in Figure 2. Treatment with corilagin decreased the size of infarcted tissue compared to that of the MCAO group. There was a significant increase in the volume of infarcted tissue in the MCAO and Tat-Beclin-1 groups compared to the control group. However, treatment with corilagin significantly (p < 0.01) decreased the volume of infarcted tissue compared with that of the MCAO group.
Moreover, the percentage of infarct volume was increased in the corilagin+tat-Beclin-1-treated group compared with the corilagin-treated group.

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Effects of corilagin on cerebral oedema

Figure 3 shows that there was significant ($p < 0.01$) increase in the water content of the cerebral tissues in MCAO and Tat-Beclin-1 groups compared with that of the control group. However, treatment with corilagin significantly ($p < 0.01$) decreased the water content in the cerebral tissues of the ischaemic rats compared with the MACO group. Moreover, the water content in the cerebral tissue was increased in the corilagin + tat-Beclin-1-treated group compared with the corilagin-treated group.

Effect of corilagin on autophagic activity

The effects of corilagin on the level of autophagy were estimated by western blotting of the cerebral tissues from the cerebral ischaemic rats as shown in Figure 4. Treatment with corilagin significantly decreased the expression level of LC3-II in cerebral tissues of cerebral ischaemic rats compared with the MCAO group. However, the LC3-II/LC3-I ratio also decreased in cerebral ischaemic tissues compared with the MCAO group. The LC3-II and LC3-II/LC3-I ratio in the cerebral tissues increased in the corilagin+tat-Beclin-1-treated group compared with the corilagin-treated group.

Figure 3: Effect of corilagin on water content in the brain tissues of cerebral ischemic rats. Mean ± SD (n = 10); ** $p < 0.01$ vs control; * $p < 0.05$, ## $p < 0.01$ vs MCAO and @ $p < 0.05$, @@ $p < 0.01$ vs Tat-Beclin-1

Figure 4: Effect of corilagin on autophagy of cerebral tissues in cerebral ischemic rats. A. Western blot result, B. Expression of LC3-II, C. Ratio of LC3-II/LC3-I; Mean ± SD (n = 10); ** $p < 0.01$ Vs control; * $p < 0.05$, ## $p < 0.01$ Vs MCAO and @ $p < 0.05$, @@ $p < 0.01$ Vs Tat-Beclin-1
Effects of corilagin on the level of LC3 mRNA

The effect of corilagin on autophagy was assessed by measuring the level of LC3 mRNA using real time PCR as shown in Figure 5. In cerebral ischaemia, the level of LC3 mRNA was significantly enhanced ($p < 0.01$) in the MCAO and Tat-Beclin-1 groups, compared to the control group. Treatment with corilagin significantly reduced the level of LC3 mRNA in cerebral infarcted tissues compared with the MCAO group. Moreover, the level of LC3 mRNA in the cerebral tissue was increased in the corilagin+tat-Beclin-1-treated group compared with the corilagin-treated group.

Effect of corilagin on autophagy in neurons and astrocytes

The effect of corilagin on autophagy in brain tissues of cerebral ischaemic rats were determined by using antibodies against NeuN, GFAP, and LC3, and immunofluorescence as shown in Figure 6. There were significant increases in the percentages of LC3-II positive neurons in cerebral ischaemic tissues in the MCAO and Tat-Beclin-1 groups compared to the control group. Treatment with corilagin significantly decreased the percentage of LC3-II positive neurons compared to cerebral tissues from the MCAO- and Tat-Beclin-1-treated groups. However, the percentage of LC3-II positive astrocytes was significantly reduced in the corilagin-treated group compared to the MCAO group. Moreover, the percentage of LC3-II positive astrocytes in the cerebral tissue was increased in the corilagin+tat-Beclin-1-treated group compared to the corilagin-treated group (Figure 7).

DISCUSSION

Neuronal injury induced by cerebral ischaemia causes necrosis, apoptosis, and autophagy [18]. Autophagy was reported to provide a protective role for the survival of neurons, but excessive autophagy can result in cell death. Excessive autophagic activity during cerebral ischaemia also results in cell death. Studies have suggested that reversing autophagy can provide neuroprotection [19]. Thus, in this study, the neuroprotective effects of corilagin were assessed during cerebral ischaemia. Treatment with corilagin significantly decreased the neurological deficit score, infarct volume, and water content in the cerebral tissues of cerebral ischaemia rats compared to the MCAO and Tat-Beclin-1-treated groups. In yeast, after post-translational modification, LC3 is present in the autophagosomal membranes [20]. Studies have shown that autophagosome formation was confirmed by the conversion of LC3-I to LC3-II, which is formed during autophagy. Antibodies against LC3 have been used to estimate the autophagy-related formation of LC3-I and LC3-II [21-23]. However, the ratio of LC3I/LC3II also represents the degree of autophagy. Thus, in the present study, the effects of corilagin were assessed on the expressions of LC1 and LC3II to estimate the extent of autophagy. Treatment with corilagin significantly attenuated the expressions of LC1 and LC3II in the cerebral tissues of cerebral ischaemic rats.

In this investigation, the activation of autophagy was assessed after including an inducer on the 3rd and 6th days of treatment. Tat-Beclin-1 was reported to react with beclin-1 to initiate autophagy. The specific effects of corilagin on
Figure 6: Effect of corilagin on the expressions of LC3 in neurons and astrocytes in cerebral ischemic rats by immunofluorescence staining.

Figure 7: Effect of corilagin on the percentage of LC3-II positive neurons and astrocytes Mean ± SD (n=10); **p < 0.01 vs control; *p < 0.05, **p < 0.01 vs MCAO and ##p<0.05, @@p < 0.01 vs Tat-Beclin-1.
autophagy were estimated by co-administering tat-Beclin-1. The effects of corlagin were abolished by administering tat-Beclin-1 to a group of rats. Thus, in cerebral ischaemic rats, corlagin protected against neuronal damage by attenuating autophagy.

CONCLUSION

Corlagin attenuates the neuronal damage by decreasing the autophagy in cerebral tissue, thus suggesting that corlagin has potential to be used in the clinical management of stroke.

DECLARATIONS

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Conflict of interest

The authors have no conflict of interest with regard to this study.

Author contributions

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Feng Xu performed the experiments and wrote the report. Ai-Xia Li, Xin Li and Bao Hui Liu analysed the data and provided comments. Dong Ruan designed the project, supervised the experiments and wrote the manuscript. All authors have read and approved the final manuscript.

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