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

Feng Xu¹, Ai-Xia Li², Xin Li³, Bao Hui Liu⁴, Dong Ruan¹*

¹Department of Neurosurgery, Xianning Central Hospital, The First Affiliated Hospital of Hubei University of Science and Technology, ²Department of Encephalopathy, Xianning Hospital of Traditional Chinese Medicine, ³Computer Science and Technology College, Hubei Science and Technology University, Xianning 437100, ⁴Department of Neurosurgery, Renmin Hospital, Wuhan University, Wuhan 430060, Hubei Province, China

*For correspondence: Email: DaisyCeszie@yahoo.com; Tel/Fax: 0086-715-8896172

Sent for review: 1 July 2017
Revised accepted: 19 November 2017

Abstract

Purpose: To evaluate the neuroprotective effects of corilagin in cerebral ischaemia-induced cerebral injury in a rat model.

Methods: Cerebral ischaemia was induced by middle cerebral artery occlusion (MCAO). The animals were separated into five groups, including a control group that underwent surgery without inserting a monofilament; MCAO group that received saline; corilagin-treated group (20 mg/kg corilagin, intraperitoneally for 7 days after reperfusion); Tat-Beclin-1-treated group (intraperitoneal injection of 1.5 mg/kg of Tat-Beclin-1 on the 3rd and 6th days after MCAO); and corilagin + Tat-Beclin-1-treated group (corilagin for 7 days and Tat-Beclin-1 on the 3rd and 6th days after MCAO). At the end of the treatments, neurological deficit, brain oedema, and volume of infarct were determined in all the animals. Moreover, the level of autophagy in infarcted tissues was evaluated by immunofluorescence, real-time PCR, and western blotting.

Results: There was a significant decrease in neurological deficit, brain oedema, and volume of infarcted tissue in corilagin-treated group when compared with MCAO- and Tat-Beclin-1-treated groups. Treatment with corilagin attenuated the autophagy of astrocytes and neurons in cerebral infarcted tissue, as demonstrated by immunofluorescence, quantitative PCR, and western blotting data.

Conclusion: Corilagin has a protective effect against neuronal damage in cerebral ischaemic rats by decreasing neurological deficit score, infarct volume, and water content of cerebral tissue. Corilagin attenuates autophagy in cerebral tissue, thus protecting cerebral ischaemic rats from neuronal damage.

Keywords: Corilagin, Cerebral ischaemia, Autophagy, Tat-Beclin-1, Neuronal damage, Astrocytes, Neurological deficit score, Infarct volume

This is an Open Access article that uses a funding model which does not charge readers or their institutions for access and distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0) and the Budapest Open Access Initiative (http://www.budapestopenaccessinitiative.org/read), which permit unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited.

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, anticancer, and antioxidant activities [10-12]. It was reported that corilagin possessed 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.

Determination of LC3 mRNA levels

TRIzol® reagent was used to extract total RNA from the ischaemic penumbra tissue homogenates. As per the manufacturers’ guidelines, 1 mg of total RNA was used to synthesize the first-strand of complementary DNA (cDNA). Primers and SYBR Green I were used to perform real time PCR. Amplification of PCR products was done using a 20 mL reaction and the Mastercycler® ep Realplex system (Eppendorf, Ontario, Canada).

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 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.

**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.

**Figure 1:** Effect of corilagin on neurological defect in cerebral ischemic rats. Mean ± SD (n = 10); ** p < 0.01 Vs control; * p < 0.05, **p < 0.01 vs MCAO, @ p < 0.05, @@ p < 0.01 vs Tat-Beclin-1

**Figure 2:** Effect of corilagin on volume of infarcted brain tissues in 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
Effects of corilagin on cerebral oedema

Figure 3 shows that 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 MCAO 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 @@ $p < 0.01$ vs Tat-Beclin-1](image1)

![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](image2)
Figure 5: Effect of corilagin on the level of LC3 mRNA in cerebral ischemic rats by real time PCR. 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 LCI and LC3II to estimate the extent of autophagy. Treatment with corilagin significantly attenuated the expressions of LCI 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 potentials to be use in the clinical management of stroke.

DECLARATIONS

Acknowledgement

The authors would like to thank Xianning Central Hospital, The First Affiliated Hospital of Hubei University of Science and Technology, China for providing the facilities for this work.

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.

REFERENCES

1. Klowka SP, Wintermark M, Engelhorn T, Fiebach JB. Acute stroke magnetic resonance imaging: current status and future perspective, Neuroradiol 2010; 52: 189–201.
2. Olsen TS, Larsen B, Herning M, Skriver EB, Lassen NA. Blood flow and vascular reactivity in collaterally perfused brain tissue: evidence of an ischemic penumbra in patients with acute stroke, Stroke 1983; 14: 332–341.
3. Lo EH. A new penumbra: transitioning from injury into repair after stroke. Nat Med 2008; 14: 497–500.
4. Edinger AL, Thompson CB. Death by design: apoptosis, necrosis and autophagy. Curr Opin Cell Biol 2004; 16: 663–669.
5. Nixon RA. Autophagy in neurodegenerative disease: friend foe or turncoat? Trends Neurosci 2006; 29: 528–535.
6. Nixon RA. The role of autophagy in neurodegenerative disease. Nat Med 2013; 19: 983–987.
7. Wong YC, Holzbaur EL. Autophagosome dynamics in neurodegeneration at a glance, J Cell Sci 2015; 128: 1259–1267.
8. Rami A, Langhagen A, Steiger S. Focal cerebral ischemia induces upregulation of Beclin1 and autophagy-like cell death. Neurobiol Dis 2008; 29: 132–141.
9. Li L, Tian J, Long MK, Chen Y, Lu J, Zhou C, Wang T. Protection against experimental stroke by ganglioside GM1 is associated with the inhibition of autophagy, PLoS One 2016; 11: e0144219.
10. Liu W, Shang G, Yang S, Huang J, Xue X, Lin Y, Zheng Y, Wang X, Wang L, Lin R, Tao J, Chen L. Electroacupuncture protects against ischemic stroke by reducing autophagosome formation and inhibiting autophagy through the mTORC1-ULK1 complex-Beclin1 pathway, Int. J. Mol. Med. 2016; 37: 309–318.
11. Rios JL, Recio MC, Giner RM and Mäñez S. An update review of saffron and its active constituents. Phytother Res 1996:10: 189-193.
12. Hosseinzadeh H and Talebzadeh F. Anticonvulsant evaluation of safranal and crocin from Crocus sativus in mice. Fitoterapia 2005; 76:722-724.
13. Hosseinzadeh H, Karimi G, Naipoor M. Antidepressant effects of Crocus sativusstigma extracts and its constituents, crocin, and safranal, in mice. J Med Plants 2004; 3:48-58.
14. Nemati H, Boskabady MH, Ahmadzadef Vostakolaei H. Stimulatory effect of Crocus sativus (saffron) on beta2-adrenoceptors of guinea pig tracheal chains. Phytomedicine 2008; 15:1038-1046.
15. Abdullaev Fl, Espinosa-Aguirre JJ. Biomedical properties of saffron and its potential use in cancer therapy and chemoprevention trials. Cancer Detect Prev 2004; 28:426-432.
16. Hosseinzadeh H, Sadeghnia HR, Ziaee T, Danaee A. Protective effect of aqueous saffron extract (Crocus sativus L.) and crocin, its active constituent, on renal ischemia-reperfusion-induced oxidative damage in rats. J Pharm PharmSci 2005; 8: 387-393.
17. Guide for the Care and Use of Laboratory Animals: Eighth Edition Committee for the Update of the Guide for the Care and Use of Laboratory Animals; National Research Council. 2010; ISBN: 0-309-15401-4.
18. Nikoletopoulou V, Markaki M, Palkaras K, Tavamarakis N. Crosstalk between apoptosis, necrosis and autophagy, Biochim. Biophys. Acta 2013; 1833: 3448–3459.
19. Puyal J, Clarke PG. Targeting autophagy to prevent neonatal stroke damage, Autophagy 2009; 5: 1060–1061.
20. Zhang L, Hu X, Luo J, Li L, Chen X, Huang R, Pei Z. Physical exercise improves functional recovery through
mitigation of autophagy, attenuation of apoptosis and enhancement of neurogenesis after MCAO in rats, BMC Neurosci. 2013; 14: 46.

21. Zhang L, Niu W, He Z, Zhang Q, Wu Y, Jiang C, Tang C, Hu Y, Jia J. Autophagy suppression by exercise pretreatment and p38 inhibition is neuroprotective in cerebral ischemia, Brain Res. 2014; 1587: 127–132.

22. Shao ZQ, Liu ZJ. Neuroinflammation and neuronal autophagic death were suppressed via Rosiglitazone treatment: new evidence on neuroprotection in a rat model of global cerebral ischemia, J. Neurol Sci. 2015; 349: 65–71.

23. Baek SH, Noh AR, Kim KM, Akram M, Shin YJ, Kim ES, Yu SW, Majid A, Bae ON. Modulation of mitochondrial function and autophagy mediates carnosine neuroprotection against ischemic brain damage, Stroke 2014; 45: 2438–2443.