BOLETIN LATINOAMERICANO Y DEL CARIBE DE PLANTAS MEDICINALES Y AROMÁTICAS
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Articulo Original / Original Article

Protective effect of chrysin on ischemia-reperfusion injury in rats and the relation with oxidative stress and excessive autophagy

[Efecto protector de la crisin sobre la lesión por isquemia-reperfusión en ratas y su relación con el estrés oxidativo y la autofagia excesiva]

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Section Biological activity

Received: 23 August 2024
Accepted: 14 November 2024
Accepted corrected: 4 January 2025
Published: 30 July 2025

Citation:
Deng C, Yang Q, Yang C, Gu W
Protective effect of chrysin on ischemia-reperfusion injury in rats and the relation with oxidative stress and excessive autophagy
Bol Latinoam Caribe Plant Med Aromat
24 (4): 506 - 513 (2025)
https://doi.org/10.37360/blacpma.25.24.4.35

Abstract: To explore the protective effect of chrysin on cerebral ischemia-reperfusion injury (CIRI) in rats and mechanisms. Fifty-four rats were divided into sham-operated, model and treatment groups. The treatment group was treated with 100 mg/kg chrysin for five days. Then, the CIRI model was established by middle cerebral artery occlusion in model and treatment groups. After modeling, compared with model group, in treatment group the neurological deficit score, brain water content and brain infarction percentage were decreased, the brain tissue superoxide dismutase level was increased, the malondialdehyde level was decreased, the nuclear factor-erythroid 2 related factor 2 and heme oxygenase-1 protein expression levels were increased, and the LC3-II and Beclin-1 protein levels were decreased (all $p<0.05$). The chrysin pretreatment may alleviate the CIRI in rats by reduction of brain tissue oxidative stress and inhibition of excessive autophagy, the significant causes of morbidity and mortality in neurological disorders.

Keywords: Chrysin; CIRI; Oxidative Stress; Autophagy; Rats

Resumen: Este estudio tiene como objetivo explorar el efecto protector de la crisina sobre la lesión cerebral por isquemia-reperfusión (CIRI) en ratas y los mecanismos involucrados. Cincuenta y cuatro ratas fueron divididas en grupos de modelo de operación simulada y tratamiento. El grupo de tratamiento recibió 100 mg/kg de crisina durante cinco días. Luego, se estableció el modelo de CIRI mediante oclusión de la arteria cerebral media en los grupos modelo y de tratamiento. Después de la modelación, en comparación con el grupo modelo, en el grupo de tratamiento se observaron disminuciones en el puntaje de déficit neurológico, contenido de agua cerebral y porcentaje de infarto cerebral; se incrementó el nivel de superóxido dismutasa en el tejido cerebral; se redujo el nivel de malondialdehído; y se aumentaron los niveles de expresión de proteínas del factor nuclear eritroide 2 relacionado con el factor 2 y la heme oxigenasa-1, mientras que los niveles de proteínas LC3-II y Beclin-1 disminuyeron (todos $P < 0.05$). El pretratamiento con crisina puede aliviar la CIRI en ratas mediante la reducción del estrés oxidativo en el tejido cerebral y la inhibición de la autofagia excesiva, causas significativas de morbilidad y mortalidad en trastornos neurológicos.

Palabras clave: Crisina; CIRI; Estrés oxidativo; Autofagia; Ratas

INTRODUCTION

Ischemic stroke is an acute cerebrovascular disease seriously endangering human health. It is characterized by high incidence, high mortality and high disability, and has a significant impact on society (Feske, 2021). In clinical practice, the treatment for ischemic stroke usually involves intravenous thrombolysis and blood supply restoration. However, the restoration of blood supply in ischemic brain tissue can lead to the production of reactive oxygen species (hydroxyl radicals (·OH), superoxide anions ($O^{-2}\cdot$), singlet oxygen (O_2), hydrogen peroxide (H_2O_2), et al.), infiltration of inflammatory cells and programmed cell death, exacerbating the brain tissue injury. This series of processes is collectively known as cerebral ischemia-reperfusion injury (CIRI) (Herpich & Rincon, 2020; Lv *et al*., 2020). Research has shown that the etiology and pathogenesis of CIRI are complex and diverse, and the oxidative stress, inflammation, apoptosis, and other factors are widely involved in it (Su *et al*., 2017; Franke *et al*., 2021; Xu *et al*., 2021). Chrysin is a flavonoid compound extracted from *Oroxylum indicum*, a Verbenaceae family plant (Nagasaka *et al*., 2018). It is also abundant in propolis and is an important active ingredient in propolis (Salari *et al*., 2022). The chemical name of chrysin is 5,7-dihydroxyflavonoid. Chrysin has extensive pharmacological activity in anti-tumor, anti-inflammatory, antioxidant and insecticidal aspects (Cespedes-Acuña *et al*., 2015; Mani & Natesan, 2018; Stompor-Gorący *et al*., 2021;). In addition, it can restrain ferroptosis in CIRI (Shang *et al*., 2024). However, until now there is no report on the protective effect of chrysin on CIRI based on the mechanisms related to oxidative stress and autophagy. Therefore, the objective of this study was to explore the protective effect of chrysin on the CIRI in rats and the action mechanisms related to oxidative stress and autophagy.

MATERIALS AND METHODS

Animal grouping and treatment

Fifty-four healthy male Sprague Dawley rats (220-260 g; Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China) were randomly divided into sham-operated, model and treatment groups, with 18 rats in each group. The rats in treatment group were treated with chrysin (Chengdu Mansite Biotechnology Co., Ltd, Chengdu, China) by gavage with dosage of 100 mg/kg (based on the results of pre-experiments), once a day. The rats in sham-operated and model groups were synchronously treated with equal volume of normal saline by gavage. The treatment in three groups was performed for a total of five days. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee of our hospital.

Establishment of CIRI model

Based on pre-operative health assessment, the CIRI model of rats was established using the middle cerebral artery occlusion (MCAO) method according to the reported methods (Zhao *et al*., 2016; Liu *et al*., 2020), with some revisions. After 1 h from the last administration, the rats were anesthetized with isoflurane and were fixed in supine position. The right common carotid artery (CCA), external carotid artery (ECA), internal carotid artery (ICA), and pterygopalatine artery were separated. The CCA and ICA were clamped with arterial clips. A small incision was made at position 2 cm near the bifurcation of ECA and ICA. A 0.26-0.28 mm nylon thread was slowly inserted into the ICA. The ICA clips were removed. The nylon suture was continuously inserted for depths of 18-20 mm until there was a slight sense of resistance. The CCA arterial clips were removed. After 2 h of occlusion for ischemia, the nylon thread was removed for reperfusion. In sham-operated group, the surgical operations were the same as those in other groups, excepting insertion of nylon thread into ICA. After surgery, the rats are placed back in cages for normal feeding. No rat died during the modeling process.

Scoring of neurological dysfunction

After 3, 12 and 24 h from ischemia, the neurological dysfunction of rats was evaluated using the neurological deficit scoring, which was related to the clinical outcomes (Bieber *et al*., 2019): 0 point: no neurological deficit symptom; 1 point: the rats could not fully extend the left front claw; 2 points: the rats turned around to the left side; 3 points: the rats tilted to the left side; 4 points: the rats could not spontaneously walk, with loss of consciousness; 5 points: the rats died.

Measurement of brain water content

After 24 h from ischemia, six rats were taken from each group. After anesthesia, the rats were sacrificed. The brain tissues were taken and weighed to obtain the wet mass. Then, the brain tissues were dried at 100°C to constant weight to obtain the dry mass. The

brain water content (%) was calculated by the ratio of (wet mass - dry mass) to wet mass.

Measurement of brain infarction percentage

Six rats were taken from each group. After anesthesia, the rats were sacrificed. The brain tissues were taken. The brain tissue slices were prepared, three repetitive slices for each rat. The slices were stained with 2% 2,3,5-triphenyltetrazolium chloride solution, followed by fixing with 10% paraformaldehyde. The normal brain tissue appeared rose red, while the infarcted brain tissue appeared white. The brain slices were photographed and analyzed using Image J software. The brain infarction percentage was calculated.

Detection of brain tissue superoxide dismutase and malondialdehyde levels

The other six rats were taken from each group. After anesthesia, the rats were sacrificed. Partial brain tissues were taken and weighed. The brain tissue homogenate was prepared. After centrifuging at 3500 r/min for 10 min, the supernatant was obtained. The superoxide dismutase (SOD) and malondialdehyde (MDA) levels were determined using the corresponding kits, respectively.

Western blotting

Remaining brain tissues of rats were taken and weighed. The brain tissues were homogenized with RIPA lysate. After centrifugation, the supernatant was obtained. The protein was determined using bicinchoninic acid method. The sodium dodecyl sulfonate polyacrylamide gel electrophoresis was performed, and then the separated protein was transferred to the polyvinylidene fluoride membranes. After blocking with 1% bovine serum albumin, the membranes were incubated with primary antibodies of nuclear factor-erythroid 2 related factor 2 (Nrf2), heme oxygenase-1 (HO-1), LC3-II and Beclin-1, respectively, at 4°C overnight. After rinsing with Tris buffer saline Tween for three times, the membranes were incubated with the second antibody horseradish peroxidase-labeled anti-IgG at 37°C for 1.5 h. The visualization was accomplished using the enhanced chemiluminescence reagent. The gray value of the band was measured using Image J software. The ratio of target protein gray value to internal reference (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) gray value was used to reflect its expression level.

Statistical analysis

Statistical analysis was performed using SPSS 22.0 software. The data were presented as mean±standard deviation and analyzed using one-way analysis of variance (ANOVA) followed by the Least Significant Difference test. $p<0.05$ indicated the statistically significant difference.

RESULTS

Neurological deficit score

After 3, 12 and 24 h from ischemia, the neurological deficit scores in model and treatment groups were significantly higher than that in sham-operated group, respectively ($p<0.05$). Compared with model group, the neurological deficit score in treatment group at each time point was significantly decreased ($p<0.05$) (Figure No. 1).

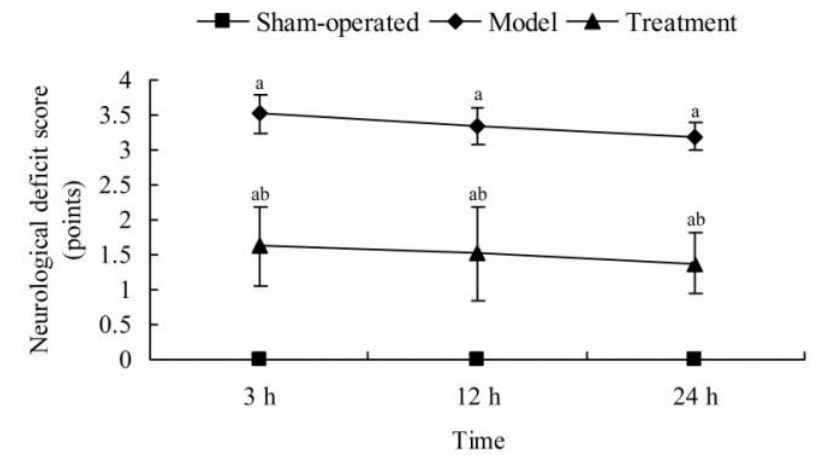

Figure No. 1

Comparison of neurological deficit score of rats among three groups (n=18) (3 h: F = 427.610, $p<0.001$; 12 h: F = 294.165, $p<0.001$; 24 h: F = 614.537, $p<0.001$). $^{a}p<0.05$ compared with sham-operated group; $^{b}p<0.05$ compared with model group

Brain water content and brain infarction percentage
After 24 h from ischemia, compared with sham-operated group, the brain water content and brain infarction percentage in model and treatment groups were significantly increased, respectively (p<0.05). Compared with model group, each index in treatment group was significantly decreased (p<0.05) (Figure No. 2).

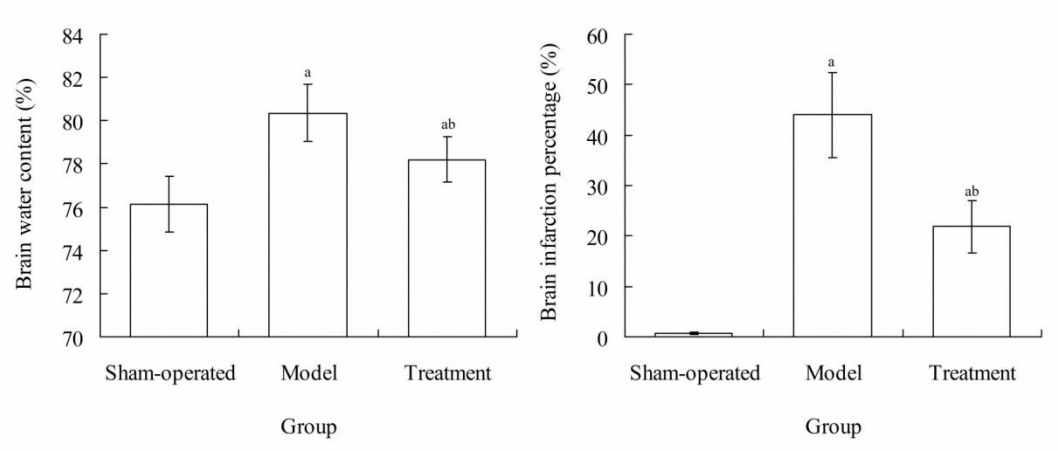

Figure No. 2
Comparison of brain water content and brain infarction percentage among three groups (n=6) (brain water content: F = 17.528, p<0.001; brain infarction percentage: F = 85.951, p<0.001). [a]p<0.05 compared with sham-operated group; [b]p<0.05 compared with model group

Brain tissue SOD and MDA levels
After 24 h from ischemia, the brain tissue SOD level in model and treatment groups was significantly lower than that in sham-operated group, respectively (p<0.05), and the brain tissue MDA level in model and treatment groups was significantly higher than that in sham-operated group, respectively (p<0.05). Compared with model group, in treatment group the SOD level was significantly increased, and the MDA level was significantly decreased (p<0.05) (Figure No. 3).

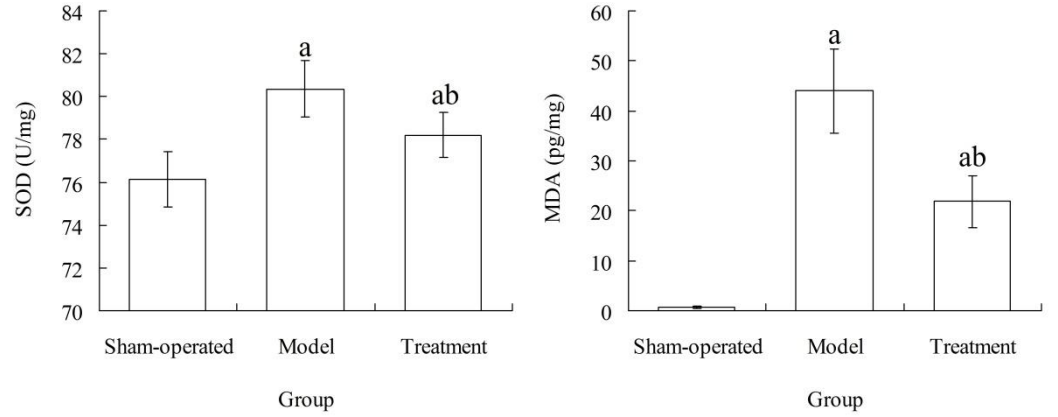

Figure No. 3
Comparison of brain tissue SOD and MDA levels among three groups (n = 6) (SOD: F = 17.528, p<0.001; MDA: F = 85.951, p<0.001). [a]P < 0.05 compared with sham-operated group; [b]p<0.05 compared with model group. SOD, superoxide dismutase; MDA, malondialdehyde

Brain tissue Nrf2 and HO-1 protein levels

As shown in Figure No. 4, after 24 h from ischemia, compared with sham-operated group, the brain tissue Nrf2 and HO-1 protein levels in model and treatment groups were significantly decreased, respectively (p<0.05). Compared with model group, each index in treatment group was significantly increased (p<0.05).

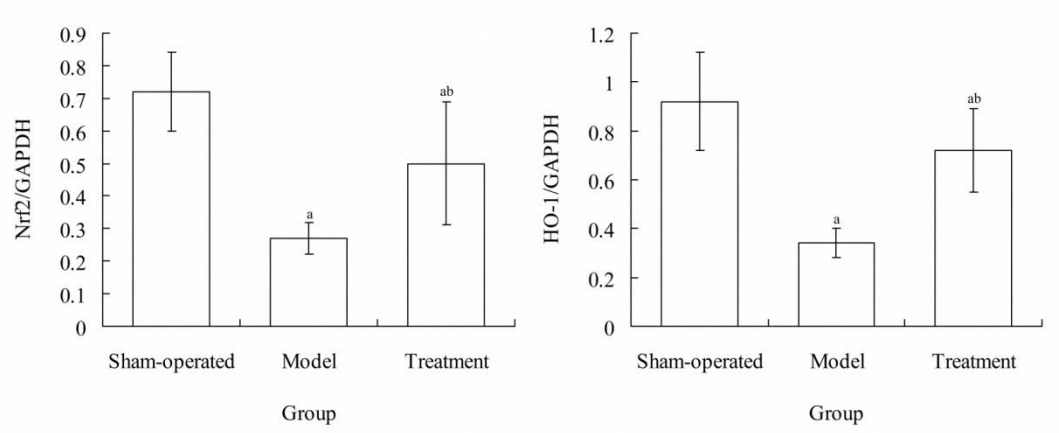

Figure No. 4

Comparison of brain tissue Nrf2 and HO-1 protein levels among three groups (n=6) (Nrf2/GAPDH: F = 17.196, p<0.001; HO-1/GAPDH: F = 21.550, p<0.001). [a]p<0.05 compared with sham-operated group; [b]p<0.05 compared with model group. Nrf2, nuclear factor-erythroid 2 related factor 2; HO-1, heme oxygenase-1

Brain tissue LC3-II and Beclin-1 protein levels

After 24 h from ischemia, the brain tissue LC3-II and Beclin-1 protein levels in model and treatment groups was significantly higher than those in sham-operated group, respectively (p<0.05). Compared with model group, in treatment group the LC3-II and Beclin-1 levels were significantly decreased, respectively (p<0.05) (Figure No. 5).

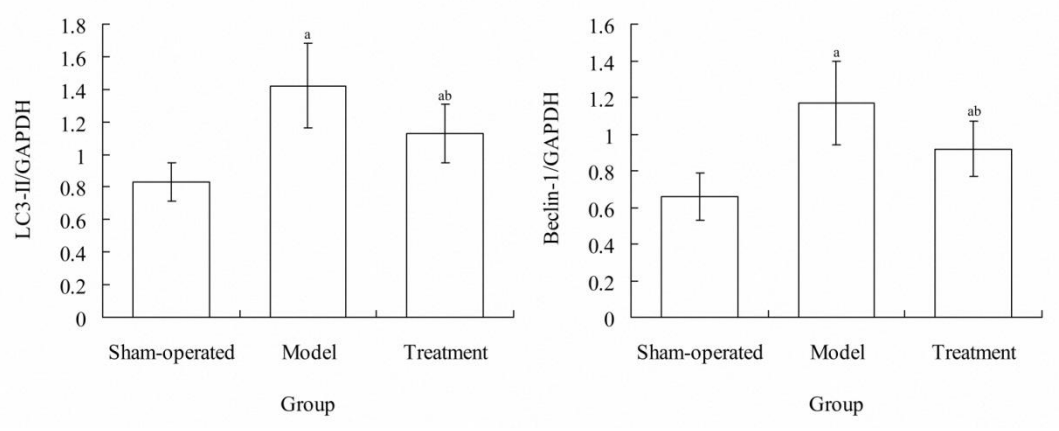

Figure No. 5

Comparison of brain tissue LC3-II and Beclin-1 protein levels among three groups (n=6) (LC3-II/GAPDH: F = 13.694, p<0.001; Beclin-1/GAPDH: F = 12.683, p<0.001). [a]p<0.05 compared with sham-operated group; [b]p<0.05 compared with model group

DISCUSSION

CIRI is a clinical crisis in which the restoration of brain tissue blood supply after ischemia leads to the exacerbation of brain tissue injury. It is often more common in cases such as brain injury, extracorporeal circulation surgery, heart transplantation, and shock. CIRI has a high incidence, with extremely poor prognosis (Wang *et al*., 2014; Jin *et al*., 2019). How to prevent and alleviate the CIRI has become an urgent issue to be solved in clinical practice. This study explored the protective effect of chrysin on CIRI in rats. Results showed that, compared with model group, in treatment group the neurological deficit score, brain water content and brain infarction percentage were obviously decreased. This indicates that the chrysin pretreatment can alleviate the CIRI in rats.

Oxidative stress is an important mechanism of CIRI. After cerebral ischemia, the antioxidant defense system is damaged, and a large amount of reactive oxygen species accumulate. The reperfusion allows the ischemic brain tissue to regain oxygen supply. This can activate the xanthine oxidase and produce a large amount of superoxide anion free radical. The free radicals attack the neuronal membranes and microvasculature, resulting in microcirculation disorders and neuronal damage (Liu *et al*., 2020; Me *et al*., 2022). SOD exists broadly in all types of organisms, which can scavenge oxygen free radical and protect cells from oxidative damage (Warner, 1994). MDA is a metabolic product of fatty acid peroxidation reaction when oxygen free radicals attack biofilms. Its content can indirectly reflect the level of oxygen free radicals and the strength of lipid peroxidation reaction (Yiğit *et al*., 1998). Previous studies have shown that, chrysin can interact with oxidative stress, thus exerting the protective effects (Yang *et al*., 1998; Li *et al*., 2014). In our study, compared with model group, in treatment group the brain tissue SOD level was significantly increased, and the MDA level was significantly decreased. It is suggested that, the alleviative effect of chrysin on CIRI in rats may be related to its reduction of oxidative stress in brain tissue.

Nrf2 is an important regulatory factor involved in cellular redox reactions and plays a crucial role in the occurrence of many diseases, such as neurodegenerative diseases, tumors, aging, etc. (Tonelli *et al*., 2018). The antioxidant effect of Nrf2 is promoted by its binding to antioxidant response elements (ARE) and promoting the expression of downstream SOD, catalase, and glutathione peroxidase (Vasconcelos *et al*., 2019). HO-1 is a regulatory protein of the Nrf2/ARE pathway. Its activation can initiate the protective mechanisms in antioxidant, anti-inflammatory and anti-cell damage action (Zhang *et al*., 2021). HO-1 can promote the oxidative degradation of heme and neutralize the excess reactive oxygen species. The up-regulation of its expression is an important mechanism for cells to adapt to the oxidative stress (Wang *et al*., 2019). Previous study has shown that, chrysin can upregulate the brain tissue Nrf2 and HO-1 expressions, thus exerting the neuro-protective effect (Mishra *et al*., 2021). Results of our study showed that, compared with model group, in treatment group the brain tissue Nrf2 and HO-1 protein levels were significantly increased. This indicates that, the chrysin pretreatment can enhance the brain tissue Nrf2 and HO-1 expressions, thus exerting the antioxidant effect to alleviate the CIRI in rats. This is similar with above study.

Autophagy is a highly conserved mechanism of cellular self clearance and recycling that exists in eukaryotes. It is involved in the occurrence and development of CIRI (Mei *et al*., 2020). LC3-II and Beclin-1 are considered marker proteins for autophagy formation. LC3-II is a tissue protein located on the surface of precursor autophagic vesicles and autophagic vesicle membranes, and it is involved in the formation of autophagosomes (Tanida *et al*., 2008). Beclin-1 can mediate the initiation stage of autophagy and the formation of autophagosomes, and regulates the localization of other autophagic proteins on the autophagy precursor membrane (Xu & Qin, 2019). It is found that, chrysin dampens the induction of autophagy-related genes of Beclin-1 and LC3-II (Lee *et al*., 2019). In this study, compared with mode group, in treatment group the brain tissue LC3-II and Beclin-1 protein levels were significantly decreased. This indicates that, the alleviative effect of chrysin on CIRI in rats may be related to its inhibition of autophagy in brain tissue, which is a therapeutic potential for recovery and rehabilitation of post-CIRI.

CONCLUSIONS

In conclusion, the chrysin pretreatment can alleviate the CIRI in rats. The potential action mechanism may be related to its reduction of brain tissue oxidative stress and inhibition of excessive autophagy. However, due to experimental conditions and sample size, this study has certain limitations. Further research is needed to determine a more precise mechanism of chrysin on CIRI, and the clinical practice of chrysin should be studied in future

researches.

ACKNOWLEDGEMENTS

This work was supported by the Research Project of Hubei Polytechnic University in 2024 (No. 24xjz05Q).

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