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Chrysin ameliorates cerebral ischemia/reperfusion (I/R) injury in rats by regulating the PI3K/Akt/mTOR pathway

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ABSTRACT

In this study, the effects of chrysin on cerebral ischemia by establishing middle cerebral artery occlusion (MCAO) in rat were investigated. In *vivo* experiments, the rats were orally administrated with clopidogrel or chrysin once daily for 7 days before the experimental of ischemia and the rats were divided into 5 groups: the sham group, the I/R group, I/R + clopidogrel group, I/R + chrysin (10 mg/kg), I/R + chrysin (20 mg/kg) group. Chrysin significantly ameliorated the I/R rats, evaluated by TTC staining, determination of brain wet to dry weight ratio and neurological deficits. Moreover, in serum and brain tissues of the I/R rats, chrysin also could effectively suppress the release of inflammatory cytokines, including levels of interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). In addition, chrysin could improve the SOD activity in the I/R rats. Mechanically, chrysin could activate the PI3K/Akt/mTOR pathway, inhibited inflammation and apoptosis. In oxygen-glucose deprivation and recovery (OGD/R)-induced SH-SY5Y cells in *vitro*. Chrysin markedly decreased the levels of TNF-α, IL-6 and IL-1β in supernatant of OGD/R-induced SH-SY5Y cells via activating PI3K/Akt/mTOR pathway. In conclusion, our study demonstrated that chrysin might be a potential therapeutic agent for cerebral ischemia.

1. Introduction

Currently, ischemic stroke annually affects approximate 15 million people and remains a high mortality in the world (Liang et al., 2011). As the brain needs a lot of oxygen to survive, cerebral blood flow decline may lead to deprives brain oxygen and glucose supply that cause cerebral ischemia. However, the recovery of blood supply may be induced cerebral ischemia and reperfusion injury (I/R), because of the recovery of blood will produce excessive reactive species (ROS) and inflammation, which exacerbate brain damage (Atif et al., 2009; Erbil et al., 2008). Cerebral I/R injury have many pathological processes, such as excitotoxicity, oxidative stress, inflammation and apoptosis. Therefore, extensive research is wanted to discover effective anti-inflammatory and anti-apoptosis agents, which can inhibit pro-inflammatory cytokine production, inhibit inflammation induced apoptosis, and ameliorate cerebral ischemia/reperfusion (I/R) injury (Granger and Kvietys, 2015).

Recently, more and more evidences have reported that ischemic stroke is associated with inflammation, oxidative stress and apoptosis. Inflammatory cytokines is linked with brain ischemia/reperfusion injury, such as IL-6, IL-1β and TNF-α which are governed by nuclear factor κB (NF-κB) (Lin et al., 2015; Wang et al., 2017). As a nuclear transcription factor, NF-κB plays a regulation effect on apoptosis and inflammation diseases (Xu et al., 2016; Zhan et al., 2016). Apoptosis after cerebral ischemia is one of the major pathways that lead to the process of cell death, caspase-dependent and caspase-independent signaling pathways are mainly roles in the apoptosis (Liao et al., 2016; Wu et al., 2017). Caspase-dependent pathway is regulated cytochrome *c* from mitochondria, which is regulated by Bax, Bcl-XL, Bcl-2 and so on. (Chen et al., 2016; Gao et al., 2016). Numerous studies have shown that phosphoinositide-3-kinase/serine threonine kinase signaling pathway (PI3K/Akt) plays a vital role in various physiological processes, including inflammation, apoptosis and oxidative stress (Abdel-Aleem et al., 2016; Wang et al., 2016). Meantime, in the brain, previous studies have shown that activation of the PI3K/Akt/rapamycin (PI3K/Akt/mTOR) pathway acts the crucial role on the effect of inflammation and

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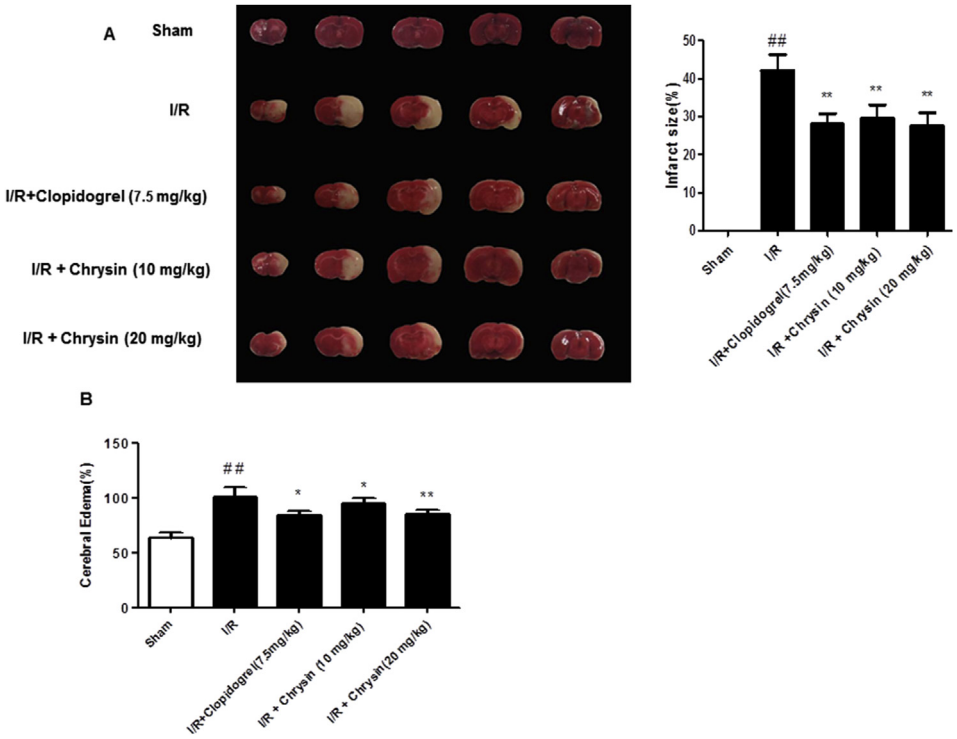

Fig. 1. Chrysin treatment decreased the cerebral infarction and edema in I/R rat. A. TTC staining of brain in I/R rat. **B.** cerebral edema in I/R rat. Data shown are representative examples from each treatment group of rats. The data was presented as means ± SDs. Compared with sham: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. Compared with I/R: $^{*}P < 0.05$,$^{**}P < 0.01$.

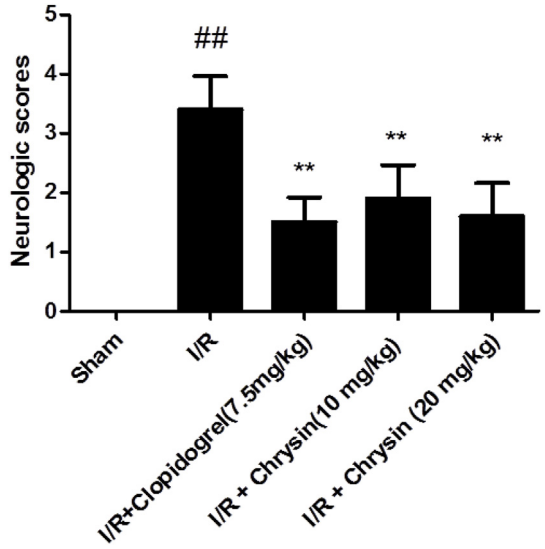

Fig. 2. Chrysin decreased neurological scores in I/R rat. The data was presented as means ± SDs. Compared with sham: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. Compared with I/R: $^{*}P < 0.05$,$^{**}P < 0.01$.

apoptosis (Li et al., 2015).

Chrysin, called 5, 7-dihydroxyflavone, is one of the most biologically active components of propolis flavone (Gai et al., 2004). Recently, there are many studies that have proved that chrysin reduces oxidative stress (Deldar et al., 2018), plays anti-inflammatory effects (Ramirez-Espinosa et al., 2017), possesses anti-apoptotic and anti-cancer (Kandemir et al., 2017; Salimi et al., 2017). It has been reported that chrysin improves murine inflammatory bowel diseases (Shin et al., 2009), suppresses LPS-stimulated proinflammatory responses (Ha et al., 2010) and processes the neuroinflammatory protection (Gresa-Arribas et al., 2010). Chrysin also prevents brain damage caused by global cerebral ischemia/reperfusion in a C57BL/J6 mouse model (Durak et al., 2016). However, the detailed mechanisms underlying the effects of chrysin protecting against cerebral I/R injury remain unclear. Therefore, in this study, the purpose of the present study was to detect the effect and its potential mechanism of chrysin on MCAO injury.

2. Materials and methods

2.1. Reagents

Chrysin (purity ≥98%) was obtained from National Institutes for Food and Drug Control (Beijing, China). Clopidogrel was obtained from Sanofi-Aventis. Enzyme-linked immunosorbent assay (ELISA) kits for the detection of IL-6, IL-1β, TNF-α and SOD were obtained from Nanjing KeyGEN Biotech. CO., LTD. (Nanjing, China). P-PI3K (#4228), PI3K (#4255), P-AKT (#4060), AKT (#2920), P–NF-κBP65 (#3033), NF-κBP65 (#8242), P-IκBα (#2859), IκBα (#4814), Bax (#2772), Bcl-2 (#3498), Caspase-3 (#9662), Caspase-9 (#9508) and GAPDH (#5174) antibodies were obtained from Cell Signaling Technology (MA, USA).

2.2. Animals

Animal study was approved by The Institutional Animal Care and Use Committee (IACUC) in Zhengzhou University (IAUCU number: LAC-2017-0025). Male Sprague-Dawley rats (60 rats, 250–280 g) were obtained from Shanghai SIPPR-BK laboratory animal Co. Ltd. (License number: SCXK (Hu) 2013–0016) and maintained under specific pathogen-free conditions in GLP laboratory in accordance with institutional guidelines. The animals were kept in a conventional animal facility with a 12 h light/12 h dark cycle circumstance at a constant temperature of 22–24 °C. The rats had free access to standard water and food pellets *ad libitum*.

2.3. Experimental protocol for middle cerebral artery occlusion (MCAO)/reperfusion

Rats were randomly separated into five groups (12 rats in each group): (1) Sham group, (2) model group (I/R), (3) I/R + clopidogrel (7.5 mg/kg) group, (4) I/R + chrysin (10 mg/kg) group, (5) I/

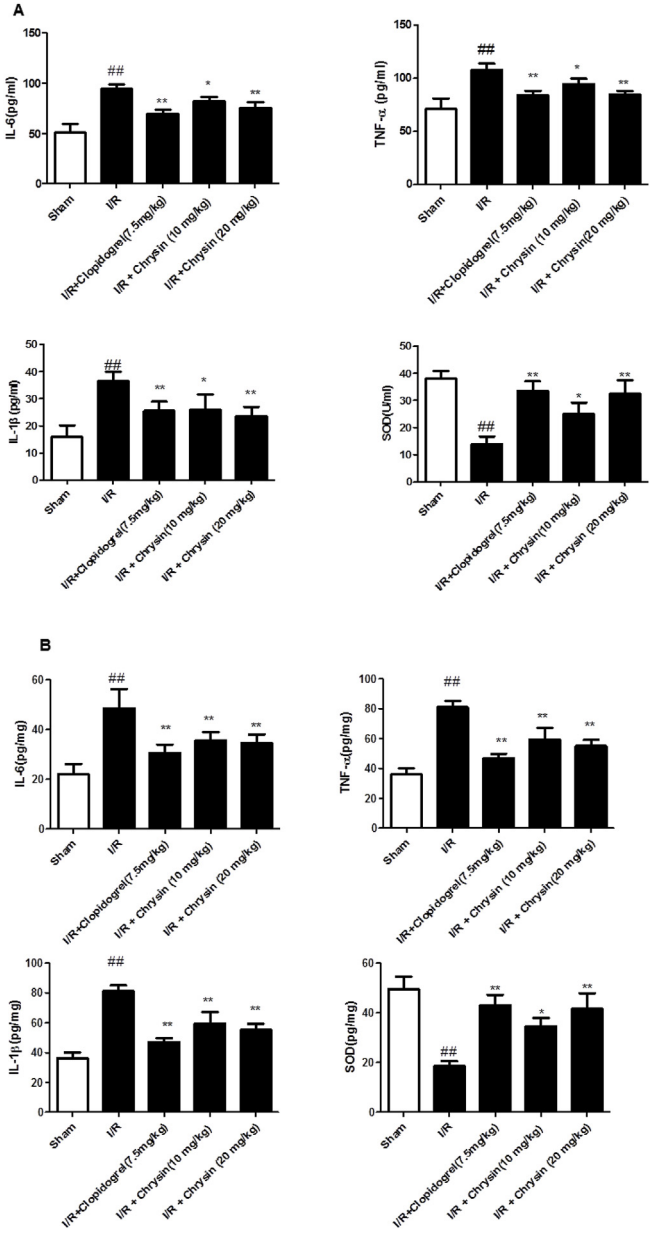

Fig. 3. Chrysin reduced production of proinflammatory cytokines and increased the SOD activity in serum and brain tissues in I/R rat. A. The serum levels of TNF-α, IL-6, IL-1β and SOD in I/R rat. **B.** The brain levels of TNF-α, IL-6 and IL-1β and SOD in I/R rat. The data was presented as means ± SDs. Compared with sham: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. Compared with I/R: $^{*}P < 0.05$, $^{**}P < 0.01$.

R + chrysin (20 mg/kg) group. Before the experimental of ischemia, the rats were orally administrated with clopidogrel or chrysin once daily for 7 days. Clopidogrel and chrysin were dissolved in 0.5% sodium carboxyl methyl cellulose (CMC-Na) for administration.

The rats were constituted with minor modification to a MCAO model as described previously. Firstly, individual rats were surgically exposed the internal carotid artery (ICA), right common carotid artery, and external carotid artery (ECA). Focal cerebral ischemia was induced by intraluminal occlusion of the right middle cerebral artery (MCAO) for 2 h followed by reperfusion dislodged nylon suture for 24 h. Sham-operated rats were subjected to the same surgical procedure, but the MCA was not occluded.

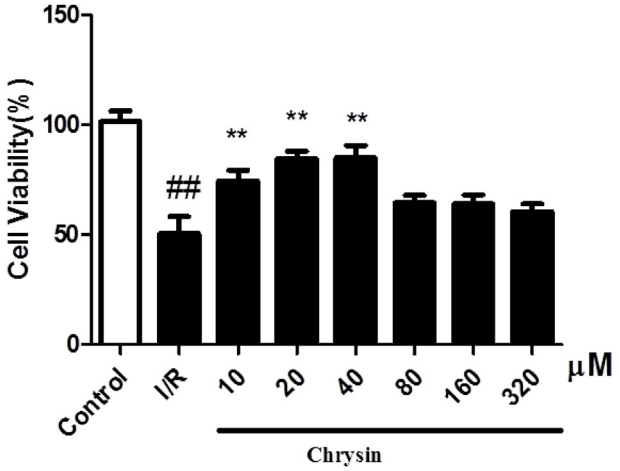

Fig. 4. The various concentrations of chrysin on OGD/R induced SH-SY5Y cells. The data was presented as means ± SDs. Compared with Control: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. Compared with OGD/R: $^{*}P < 0.05$, $^{**}P < 0.01$.

2.4. Evaluation of the neurological deficit

22 h after reperfusion, the neurological deficits of the rats were detected by using a five-point scale in a blinded manner: The system scoring is as follows: No deficit, 0; Failure to stretch the contralateral torso and forelimb fully, 1; Turning to the ipsilateral side when lifted by tail, 2; Falling to the affected side, 3; No spontaneous walking with depressed consciousness, 4.

2.5. Evaluating rat brain infarct area and cerebral edema

Rats were euthanized 24 h after ischemia reperfusion, and six slices of 2 mm coronal brain sections obtained from the entire brain were incubated in a 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) for 30 min at 37 °C. Normal brain tissues were stained brightly (red), whereas the infarcted area brain tissues remained unstained (white). Infarct volume was demarcated and calculated using an image processing software (Image-Pro Plus, Version 6.0).

2.6. Measurement of brain-water content

For measurement of brain-water content, the brains were rapidly excised onto ice after ischemia reperfusion. Immediately, weigh the brain sample to get the wet weight (ww) and then the brain tissue sample was dried at 60 °C for 48 h to get the dry weight (dw). After that, the brain water content was calculated as follows: The cerebral edema = [(ww − dw)/(ww)] × 100%.

2.7. Culture of SH-SY5Y cells

SH-SY5Y (human neuroblastoma cell line) was purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). The SH-SY5Y cells were maintained in DMEM/F12 medium containing with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/mL streptomycin at 37 °C under 5% CO_2.

2.8. MTT assay

After OGD/R model made, the SH-SY5Y cell viability was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma, St Louis, MO, USA) assays. Along with reoxygenation, SH-SY5Y cells were exposed to chrysin (10, 20, 40, 80, 160, 320 μM) for 6 h. Afterwards, the cells were added with 20 μl of

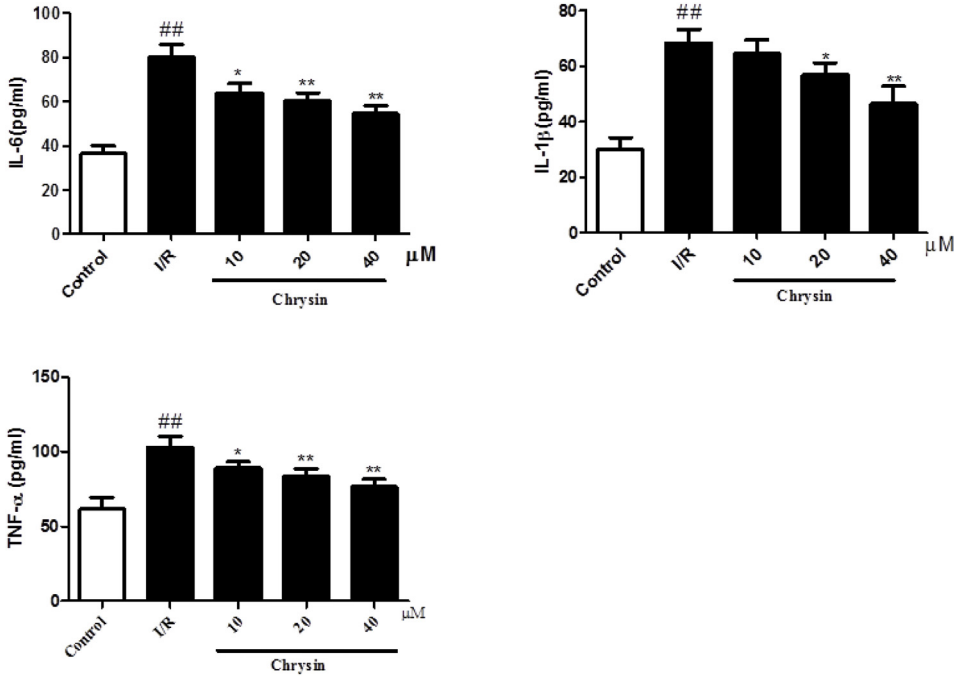

Fig. 5. Chrysin reduced production of proinflammatory cytokines TNF-α, IL-6, IL-1β in supernatant in OGD/R-induced SH-SY5Y cells. The data was presented as means ± SDs. Compared with Control:$^{\#}P < 0.05$, $^{\#\#}P < 0.01$. Compared with OGD/R: $^{*}P < 0.05$,$^{**}P < 0.01$.

MTT (5 mg/ml) working solution in each well followed an additional 4 h incubation at 37 °C. Then, the medium was removed and dye crystals were dissolved in 150 μl DMSO. The absorbance values were detected at 490 nm with a microplate spectrophotometer (Tecan, Switzerland). Results were presented as the percentage of the average absorbance of control group MTT assay in accordance with the manufacturer's instructions.

2.9. Building oxygen-glucose deprivation and recovery (OGD/R) mode in vitro

SH-SY5Y cells were seeded into 96-well plates at a density of 5×10^4 cells/ml in 100 μl culture medium for 24 h. Then the cells were transferred to an anaerobic chamber (Thermo Fisher Scientific) filled with 94% N_2, 5% CO_2 and 1% O_2 for 6 h in culture medium deprived of serum and glucose. Then the cells were incubated with various concentrations of chrysin for 6 h, dissolved in culture medium for 6 h. Meanwhile, the control and vehicle cells were incubated with the same volume culture medium.

2.10. Measurements of cytokines in serum, brain and supernatant

The contents of IL-6, IL-1β, and TNF-α in serum, brain and supernatant were detected with by an enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions (Nanjing KeyGEN Biotech. CO., LTD). The results of the levels of inflammatory cytokines were expressed as pg/ml or pg/mg.

2.11. Western blot

The rat brain tissues and SH-SY5Y cells were homogenized in ice-cold RIPA buffer containing 0.1% phenylmethylsulfonyl fluoride. The total protein content was quantified by Bicinchoninic acid (BCA) protein assay kits (Beyotime, Nanjing, China) Equal amounts of protein were loaded on 8%–12% SDS-polyacrylamide gel electrophoresis. The transferred PVDF membranes from SDS-polyacrylamide gel electrophoresis were blocked in skim milk at room temperature over 2 h. Then the PVDF membranes were incubated with the appropriate concentration of specific antibodies overnight at 4 °C. On the second day, PVDF membranes were incubated with second antibody at room temperature for 1 h after washing three times by TBST. The immunoreactive bands were interacted with an enhanced chemiluminescence (ECL) kit and visualized on a gel imaging system (Tanon Science & Technology Co., Ltd., China).

2.12. Statistical analysis

Data were normally distributed and presented as mean ± SDs. Results were analyzed by analysis of variance (ANOVA) with Tukey's post hoc test using SPSS 17.0 (SPSS Inc., USA). P value < 0.05 was considered to be significant.

3. Results

3.1. Chrysin decreased cerebral infarct size in I/R rats

In the first, the infarct size of brain was detected using 2,3,5-triphenyltetrazolium chloride (TTC) staining. As shown in Fig. 1, compared with sham group, the cerebral infarct area percentage of rats in the I/R model group was significantly increased ($p < 0.01$). Interestingly, there was a remarkably decreased in chrysin (10, 20 mg/kg) or clopidogrel (7.5 mg/kg) treatment groups compared with I/R group in the cerebral infarct area, suggesting that chrysin could ameliorate the rat in cerebral ischemia.

3.2. Chrysin decreased cerebral edema in I/R rats

Brain water content was examined as an index of cerebral edema with a wet/dry method, as revealed in Fig. 1. Notably, in the I/R group, the ratio of cerebral edema was significantly elevated compared to the sham group. Moreover, compared with those in I/R group, chrysin (10, 20 mg/kg) or clopidogrel (7.5 mg/kg) treatment group significantly reduced the cerebral edema. From these results, it was determined that chrysin processed the protective effect on brain water content.

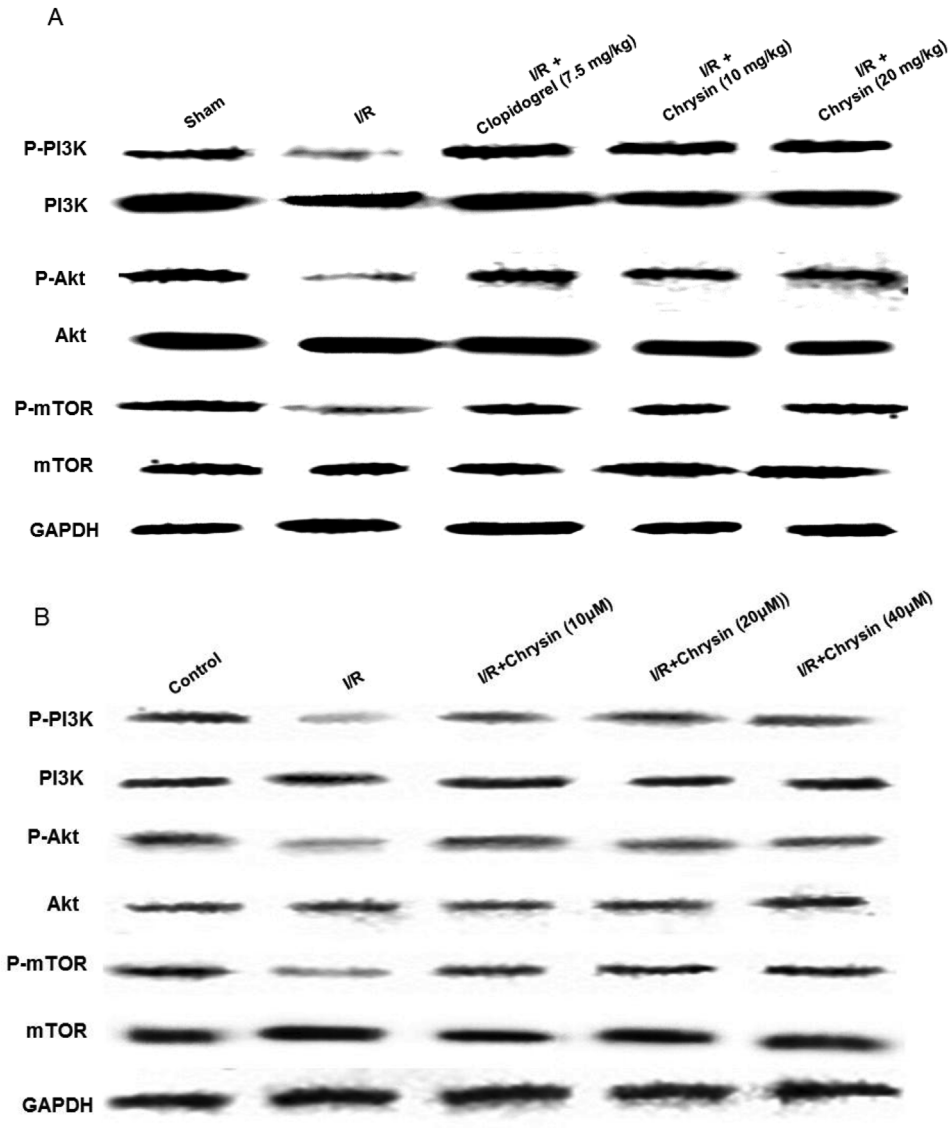

Fig. 6. Chrysin activated PI3K/Akt/mTOR pathway in rats and SH-SY5Y cells. A. Chrysin activated PI3K/Akt/mTOR pathway in I/R rats. The data was presented as means ± SDs. Compared with sham:$^{\#}P < 0.05$, $^{\#\#}P < 0.01$. Compared with I/R: $^{*}P < 0.05$,$^{**}P < 0.01$. **B.** Chrysin activated PI3K/Akt/mTOR pathway in OGD/R-induced SH-SY5Y cells. The data was presented as means ± SDs. Compared with Control:$^{\#}P < 0.05$, $^{\#\#}P < 0.01$. Compared with OGD/R: $^{*}P < 0.05$,$^{**}P < 0.01$.

3.3. Chrysin decreased neurological deficits scores in I/R rats

To detect the neurologic deficit, a five-point scale was used to score the animals. As shown in Fig. 2, there was a significantly increased neurologic score in the I/R-injury group compared to the sham group ($p < 0.01$). As expected, compared with I/R group, chrysin-treated animals drastically reduced the neurologic deficit score as well as the clopidogrel-treated group. Our data displayed that chrysin could relieve the neurological function on cerebral I/R injury.

3.4. Chrysin reduced production of proinflammatory cytokines and increased the SOD activity in serum and brain tissues

Next, the neuroinflammation triggered by MCAO surgery was evaluated according to the Elisa kits. As revealed in Fig. 3, the levels of IL-1β, IL-6 and TNF-α in I/R group were significantly increased in the serum and brain compared with the sham group. As expected, compared with those in I/R group, the high dose group of chrysin could notably suppress the release of inflammatory cytokines. Meanwhile, chrysin could also increase the activity of SOD in I/R group.

3.5. Chrysin on cell viability in OGD/R-challenged SH-SY5Y cells

To investigate the concentration of chrysin during the following experiment, the MTT assay was performed, as shown in Fig. 4. After OGD/R injury, the cell viability of SH-SY5Y cell was significantly decreased compared with that in control group. However, treatment with different concentrations of chrysin (10, 20, 40 μM) could effectively increase cell viability in OGD/R-induced cytotoxicity. However, the concentrations of 80, 160, 320 μM were of less significance. Herein, the concentrations of 10, 20, 40 μM was selected to conduct the investigation in vitro.

3.6. Chrysin reduced production of proinflammatory cytokines in the supernatant of OGD/R-challenged SH-SY5Y cells

Inflammation-induced damage occupies an important process in OGD/R damage. As shown in Fig. 5, as compared with control group,

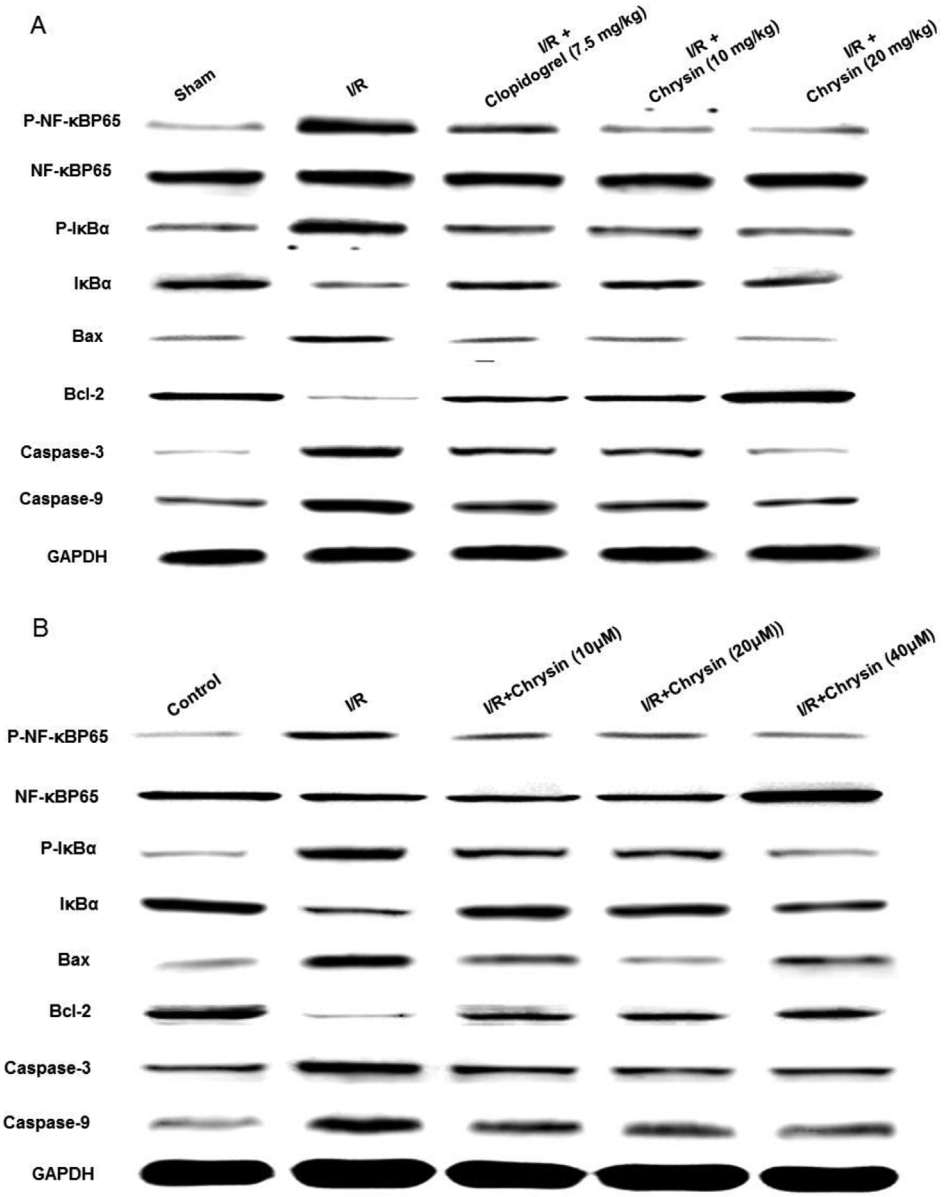

Fig. 7. Chrysin inhibited inflammation and apoptosis pathway-related proteins in rats and SH-SY5Y cells. A. Chrysin inhibited inflammation and apoptosis pathway-related proteins in I/R rats. The data was presented as means ± SDs. Compared with sham:$^{\#}P < 0.05$, $^{\#\#}P < 0.01$. Compared with I/R: $^{*}P < 0.05$,$^{**}P < 0.01$.**B.** Chrysin inhibited inflammation and apoptosis pathway-related proteins in OGD/R-induced SH-SY5Y cells. The data was presented as means ± SDs. Compared with Control:$^{\#}P < 0.05$, $^{\#\#}P < 0.01$. Compared with OGD/R: $^{*}P < 0.05$,$^{**}P < 0.01$.

the levels of inflammatory cytokines were increased in OGD/R group. Notably, the treatments with chrysin (10, 20, 40 μM) decreased the contents of TNF-α, IL-1β and IL-6 compared with control group.

3.7. Chrysin activated PI3K/Akt/mTOR pathway in rats and SH-SY5Y cells

As revealed in Fig. 6, I/R injury contributed to decrease P-PI3K, P-Akt and P-mTOR ($p < 0.01$) in I/R-induced rats and OGD/R-induced SH-SY5Y cells. By contrast, administrations of Chrysin effectively increased the expressions of P-PI3K, P-Akt and P-mTOR in brains and SH-SY5Y cells. The findings above suggested that Chrysin exhibited the protective effect on cerebral ischemia possibly via the PI3K/Akt/mTOR pathway.

3.8. Chrysin inhibited inflammation and apoptosis pathway-related proteins in rats and SH-SY5Y cells

As revealed in Fig. 7, I/R injury contributed to increase P–NF-κBp65, P-IκBα, Bax, Bax/Bcl-2, cleaved-caspase-3, cleaved-caspase-9. Besides, it decreased Bcl-2 compared with sham group. By contrast, administrations of clopidogrel and chrysin effectively decreased the expressions of P–NF-κBp65, P-IκBα, Bax, Bax/Bcl-2, cleaved-caspase-3, cleaved-caspase-9. Besides, it increased the expressions of Bcl-2 in brains.

Moreover, the treatments with Chrysin (10, 20, 40 μM) obviously decreased the levels of P–NF-κBp65, P-IκBα, Bax, Bax/Bcl-2, cleaved-caspase-3, cleaved-caspase-9 and increase Bcl-2 in OGD/R-induced SH-SY5Y cells. The findings above suggested that chrysin exhibited the protective effect on cerebral I/R possibly via the anti-inflammation and anti-apoptosis.

4. Discussion

Recently, more and more evidences emerged have indicated that cerebral ischemia triggers an inflammatory response characterized (Hao et al., 2019). After cerebral ischemia, there are many cytokines expression, inflammatory cell infiltration and oxygen free radical reaction in the ischemic injury area, which can accelerate cell damage after ischemia (Pei et al., 2015; Zhao et al., 2019a). Hence, novel effective therapeutic agents and wider application are urgent to be developed. In this study, it was indicated that chrysin could attenuate the deficit in neurological function, decreased cerebral infarct size and cerebral edema in ischemic rat, suggesting that chrysin was capable of attenuating the cerebral I/R injury.

Accumulating evidence reported that the levels of proinflammatory cytokines, TNF-α, IL-1β and IL-6, were elevated after I/R indicating that inflammation plays a vital function in ischemic/reperfusion injury (Zheng et al., 2019). In addition, we also demonstrated that chrysin could inhibited the high production of IL-6, IL-1β and TNF-α in MCAO-induced rats or OGD/R-induced SH-SY5Y cells. NF-κB is a major transcriptional regulator of inflammation. It can be rapidly activated in the acute phase of cerebral infarction, which is consistent with the elevated expression of NF-κB protein after cerebral ischemia in the present study. Some studies showed that NF-κB was implicated in the acute phase of cerebral ischemia because it led to the production of pro-inflammatory cytokines and the subsequent injuries (Sui, 2019; Zhao et al., 2019b). Regardless of transient or permanent cerebral ischemia model, drugs that inactivate NF-κB can significantly reduce the infarct volume and improve the neurological deficit, suggesting inhibition of NF-κB activation has a certain therapeutic effect on cerebral ischemia. Our results proved that chrysin treatment decreased the levels of p-IκBα, p–NF–κBp65 both in I/R-induced brains and OGD/R-induced SH-SY5Y cells.

Activation of the PI3K/Akt pathway has been proved to decrease inflammation genes and then protect vascular function (Sinha et al., 2004). The neuroprotective role of the PI3K/Akt pathway in cerebral ischemia has been widely studied. Activated Akt can rapidly activate some molecular functions including mTOR, as a multifunctional collection point, mTOR regulates cytotrophy, energy supply and promotes protein synthesis. Given the neuroprotective role of the PI3K/Akt pathway, we hypothesized that the PI3K/Akt/mTOR pathway may be inactivated after cerebral ischemia injury. In the paper, the results shown that chrysin could activate P-PI3K, P-Akt, P-mTOR in MCAO rats or OGD/R induced SH-SY5Y cells. As one of major pathways that lead to the process of cell death, apoptosis after cerebral ischemia. is directly controlled by Bcl-2 family, it could make Bax to translocate the cytosol into the mitochondria accompanying the release of cytochrome *c* (Kuwana et al., 2002). Activation of caspase-9 and caspase-3 further induced the occurrence of apoptosis to the development of ischemic infarction (Yao et al., 2012). Our results shown that chrysin could increase Bacl-2 and decrease Bax, cleaved-caspase-3 and cleaved-caspase-9 in MCAO rats or OGD/R-induced SH-SY5Y cells.

5. Summary

In conclusion, our data demonstrated that chrysin effectively attenuated the I/R-induced cerebral ischemia *in vivo* and in vitro. The underlying mechanisms might be associated with the anti-inflammatory and anti-apoptosis effects of chrysin through PI3K/Akt/mTOR pathway. These findings provided that chrysin could be developed as a therapeutic agent for cerebral ischemia/reperfusion (I/R) injury.

Conflicts of interest

The authors have no conflict of interest to declare.

Acknowledgements

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Abbreviations

MCAO	middle cerebral artery occlusion
I/R	ischemia/reperfusion
IL-6	interleukin-6
IL-1β	interleukin-1β
TNF-α	tumor necrosis factor-α
OGD/R	oxygen-glucose deprivation and recovery
NF-κB	nuclear factor κB
PI3K	phosphoinositide-3-kinase
FBS	fetal bovine serum
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
BCA	Bicinchoninic acid
ECL	enhanced chemiluminescence

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