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Original Article

Neuroprotective mechanism of crocin via PI3K/Akt/mTOR signaling pathway after cerebral infarction: an *in vitro* study

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Abstract: Objectives: To explore the potential neuroprotective mechanism of crocin after cerebral infarction. Methods: The murine hippocampal neuronal cell line HT-22, was used as the study model, with a control group, OGD-group, low-dose crocin group, middle-dose crocin group, and high-dose crocin group. Except for the control-group, cells in the other groups were treated with OGD for 6 h, in which 1 μg/mL, 2 μg/mL and 5 μg/mL of crocin were added in low-dose group, medium-dose group and high-dose group, respectively. Subsequently, the OGD cells were cultured for another 6 h. CCK-8 assay was carried out to detect the cell viability of each group, flow cytometry was used to detect cell apoptosis, immunofluorescence was conducted to detect the expression of reactive oxygen species, and Western Blot was performed to detect the protein expression of p-PI3K, p-Akt, p-mTOR, LC-3 I, LC-3 II, and Beclin-1. Results: After hypoxia-reoxygenation treatment, the viability of HT22 cells was remarkably decreased, the apoptosis rate and expression of ROS were significantly increased, the protein expression of p-PI3K, p-Akt and p-mTOR were reduced, while the expression of LC-3 II/I and Beclin-1 were increased. After crocin treatment, the activity of hypoxic reoxygenated cells increased, the apoptosis rate decreased, the expression of reactive oxygen species dropped, the protein expression of p-PI3K, p-Akt and p-mTOR increased, and the expression of LC-3 II/I and Beclin-1 decreased. Conclusion: At the cellular level, crocin can inhibit autophagy by activating the PI3K/Akt/mTOR pathway, and reduce the level of oxidative stress, thus playing a neuroprotective role.

Keywords: Crocin, PI3K/Akt/mTOR signaling pathway, cerebral infarction, neuroprotection

Introduction

Cerebral infarction is one of the most frequent clinical diseases and a main cause of disability. Therapies for cerebral infarction can be classified into two strategies: reperfusion and neuroprotection [1, 2]. Currently, the clinical time window for thrombolytic therapy is only 3-6 h, and there is a risk of intracranial hemorrhage, limiting its clinical application. Therefore, early combination of “reperfusion” and “neuroprotection” is the most ideal treatment strategy [3].

Recent studies indicate that excessive activation of autophagy after cerebral infarction is one of the major factors which lead to the neuronal death. Autophagy refers to the process in which cells degrade the cytoplasm or organelles in lysosomes after being stimulated, and the degradation products are ultimately recycled [4]. The degradation products of autophagy can provide raw materials for the synthesis of new biomolecules for reconstruction of cell structure and the generation of energy [5, 6]. Physiologically, low-level autophagy exists in cells for preserving homeostasis. However, in pathological or physiological conditions that are beyond a certain regulatory range, the autophagy level will be strongly enhanced in the cells, which might lead to cell damage [7]. Thus moderate autophagy has a neuroprotective effect in the nervous system, while excessive or insufficient autophagy can damage cells and even lead to its death.

Although autophagy regulation is very complex, some important regulatory signaling pathways have been identified. Among those important regulation pathways, the PI3K/Akt/mTOR signaling pathway is considered as a key upstream

pathway that regulates autophagy [8]. It has been reported that there is a tight correlation between the PI3K/Akt/mTOR signaling pathway and autophagy regulation in cerebral infarction [9].

Crocin is one of the effective components of Crocus sativus, which has drawn attention recently due to its specific bio-activity in neurons and/or brains both *in vitro* and *in vivo* [10]. For example, the crocin can reduce lipopolysaccharide-induced anxiety as well as depressive-like behaviors by its anti-oxidative properties. Crocin also has also been showed to improve microcirculation *in vivo*, indicating a protective effect in hypoxia and ischemia [11, 12]. Moreover, it has been reported that crocin has a significant protective effect on cardiomyocytes which are ischemic and hypoxic. Crocin has shown protective effects on myocardial tissue via the reduction of cardiomyocyte apoptosis and alleviation of oxidative stress [13]. However, its neuroprotective mechanism is poorly understood.

This study aimed to explore whether the neuroprotective effects of crocin after cerebral infarction are regulated through the PI3K/Akt/mTOR signaling pathway.

Materials and methods

Cultivation of cell lines and construction of the OGD cell model

Regular culture conditions: murine hippocampal neuronal cell line, HT-22 (Shanghai Huiying Biotechnology Co., Ltd.), was cultured in DMEM (Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA) in a 5% CO_2 incubator at 37°C. The sub-culture was carried out when the cells grew to about 90% in the flask.

The Oxygen Glucose Deprivation (OGD) model was developed as describe before [4]. Briefly, the HT22 cells were incubated in an anoxic incubator which was full of N_2 and CO_2 (95:5, v/v) for 6 h using simulated ischemic solution. Then the medium was replaced with regular culture medium and incubated in regular culture conditions for 24 h. The ODG model, a method to mimic ischemic conditions *in vitro*, has been used in many studies [14, 15] was applied in this study. The method is simply leaving cells in conditions with reduced O_2 level and without glucose supplement. The important hallmark is that ODG will cause the death of or damage to cells, which can be detected by the cell viability assay [14]. In our study, we found the cell viability in the ODG group was only about 30% of the cell viability in control group (normal culture conditions), which was very similar with the 25% reported in the literature [14]. Thus, we believe our cell model was successfully developed.

Cell proliferation detected by CCK-8 assay

HT-22 cells were seeded on a 96-well plate at the density of 10^4 cells per well for 24 h. The cells were grouped into the control-group, OGD-group, low-dose crocin group, medium-dose crocin group and high-dose crocin group. Except for the control group, cells in the other groups were treated with OGD for 6 h, in which 1 μg/mL, 2 μg/mL and 5 μg/mL of crocin (purity 98%, Nanjing Jingzhu Biotechnology Co., Ltd.) were added in low-dose group, medium-dose group and high-dose group, respectively. After 6 hours of OGD modeling, the cells were continuously cultured under regular cell culture conditions for another 6 h. After reoxygenation, we replaced medium and added 10 μl of CCK-8 (Wuhan Boster Bioengineering Co., Ltd.) into each well. Cells were incubated for another 2 h. The cell viability was accessed by reading the absorbance at 450 nm wavelength.

Cell apoptosis detected by flow cytometry

After treatment, the cells were digested with 0.25% trypsin and collected, followed by centrifugation at 1000 r/min for 5 min under 4°C. After discarding the supernatant, the cells were collected and resuspended with staining buffer which contained 200 μl of annexin V-FITC binding solution and 5 μl annexin V-FITC. After 15 min incubation without light, cells were centrifuged and washed by PBS twice. PI staining solution was added into the cell suspension and incubated on ice for 10 min. Subsequently, the cells were rinsed twice, resuspended, and tested on the machine.

Detection of reactive oxygen species

After treatment, cells were rinsed with PBS, mixed with the diluted Cm-H2DCFDA dye, and incubated at 37°C for 30 min in dark. Finally, the cells were rinsed 3 times and fixed with 4%

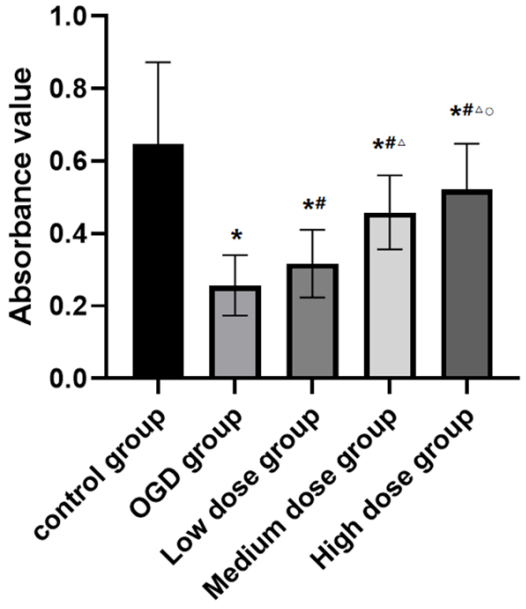

Figure 1. Comparison of cell proliferation between the groups. Cell viability of HT22 cells after hypoxia-reoxygenation was remarkably reduced comparing to that of the control-group (*P<0.05*). Note: Compared with control group, **P<0.05*; Compared with OGD group, *$^{\#}$P<0.05*; Compared with Low dose group, *$^{\Delta}$P<0.05*; Compared with Medium dose group, *$^{\circ}$P<0.05*.

paraformaldehyde for 15 min. The level of reactive oxygen species was measured and photographed by a fluorescence microscope.

Detection of protein expression by Western Blot

After treatment, cells were lysed using precooled RIPA buffer with protease inhibitor. The supernatant of the cell lysate was collected after centrifugation at 4°C and 13,000 g for 5 min. Protein amounts were quantified by BCA kit. A total of 20 μg of protein was separated on 4-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene (PVDF) membranes. Membranes were blocked with 5% non-fat milk, and primary antibodies including anti-p-PI3K (9029; Abcam; 1:2000), anti-p-Akt (32027; Abcam; 1:1000), anti-p-mTOR (43966; Abcam; 1:2500), anti-LC-3 I (ab89443; Abcam; 1:3000), anti-LC-3 II (ab89443; Abcam; 1:3000), anti-Beclin-1 (ab89443; Abcam; 1:2000), and anti-beta-actin dehydrogenase (GAPDH; ab181602, Abcam; 1:10000) were applied. Then, after 3 washes, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies, and films were developed with ECL reagents.

Statistical analysis

Data collected in the study were processed with SPSS 25.0. Data were expressed by ($\bar{x}\pm s$). ANOVA was used for multi group comparison and LSD post hoc test was adopted for the comparison between two groups after ANOVA. The difference was statistically significant at *P<0.05*.

Results

Crocin protected cells after hypoxia-reoxygenation

As shown in **Figure 1**, cell viability of HT22 cells after hypoxia-reoxygenation was remarkably reduced compared to that of the control-group (*P<0.05*). After the cells were exposed to crocin, the cell viability increased significantly and the increase in cell viability occurred in a dose-dependent manner. The highest cell viability was found in the high-dose group. Importantly, the cellular viability in the high dose crocin treated group was close to the control group, showing a great effect of crocin in protecting cells from hypoxia-reoxygenation.

Crocin inhibited cell apoptosis after hypoxia-reoxygenation

The apoptosis rate of HT22 cells in the OGD group was significantly higher than that in the control group (*P<0.05*). The apoptosis rate was significantly decreased after treatment with crocin. Specifically, the anti-apoptotic effect of crocin occurred in a dose-dependent manner (**Figure 2**).

Crocin reduced the reactive oxygen species level after hypoxia-reoxygenation

The expression of reactive oxygen species in HT22 cells after hypoxia-reoxygenation was much higher than that in the control group (*P<0.05*, **Figure 3**). The ROS is shown in green in **Figure 3B**, and the level of ROS was decreased after treatment with crocin (*P< 0.05*). As the crocin concentration increased, the expression of ROS in cells decreased more significantly.

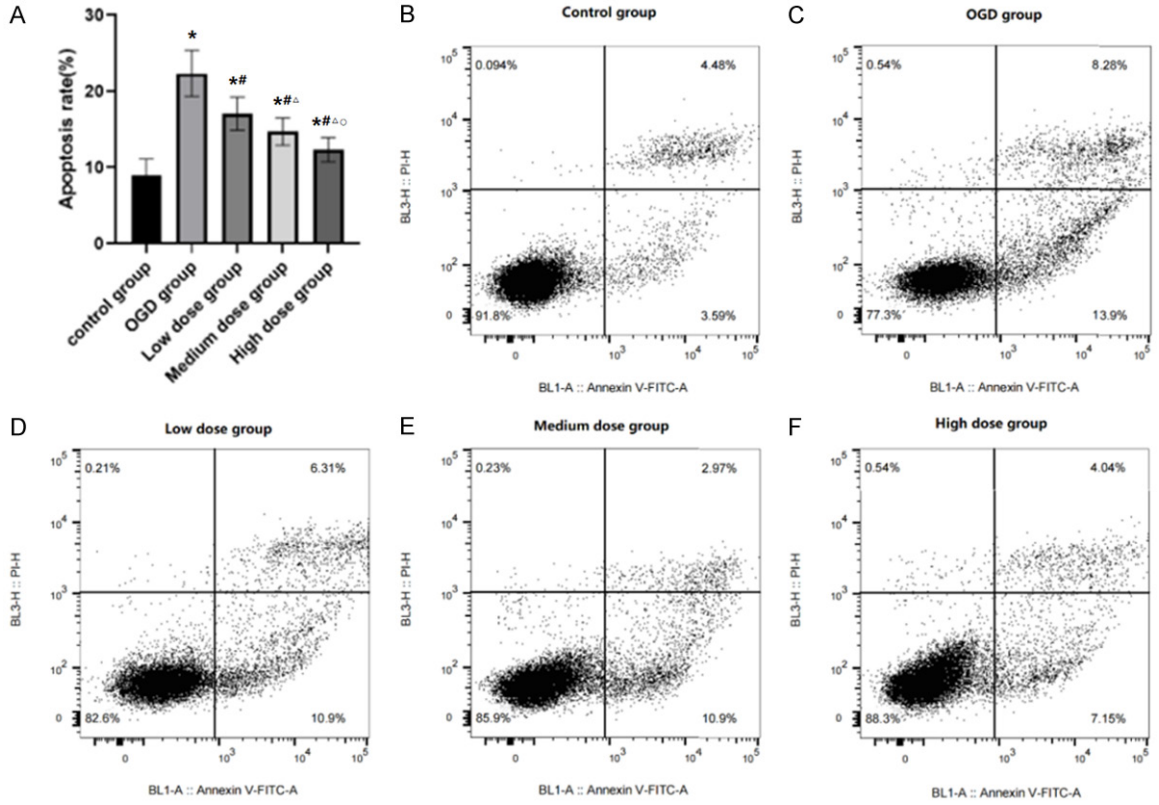

Figure 2. Comparison of apoptosis between the groups. A: The apoptosis rate of HT22 cells in the OGD group was significantly higher than that in the control group (*P<0.05*). The apoptosis rate was significantly decreased after treatment with crocin. Specifically, the anti-apoptotic effect of crocin showed a dose-dependent manner. B: Control group. C: OGD group. D: Low dose group. E: Medium dose group. F: High dose group. Note: Compared with control group, $^{*}P<0.05$; Compared with OGD group, $^{\#}P<0.05$; Compared with Low dose group, $^{\Delta}P<0.05$; Compared with Medium dose group, $^{\circ}P<0.05$.

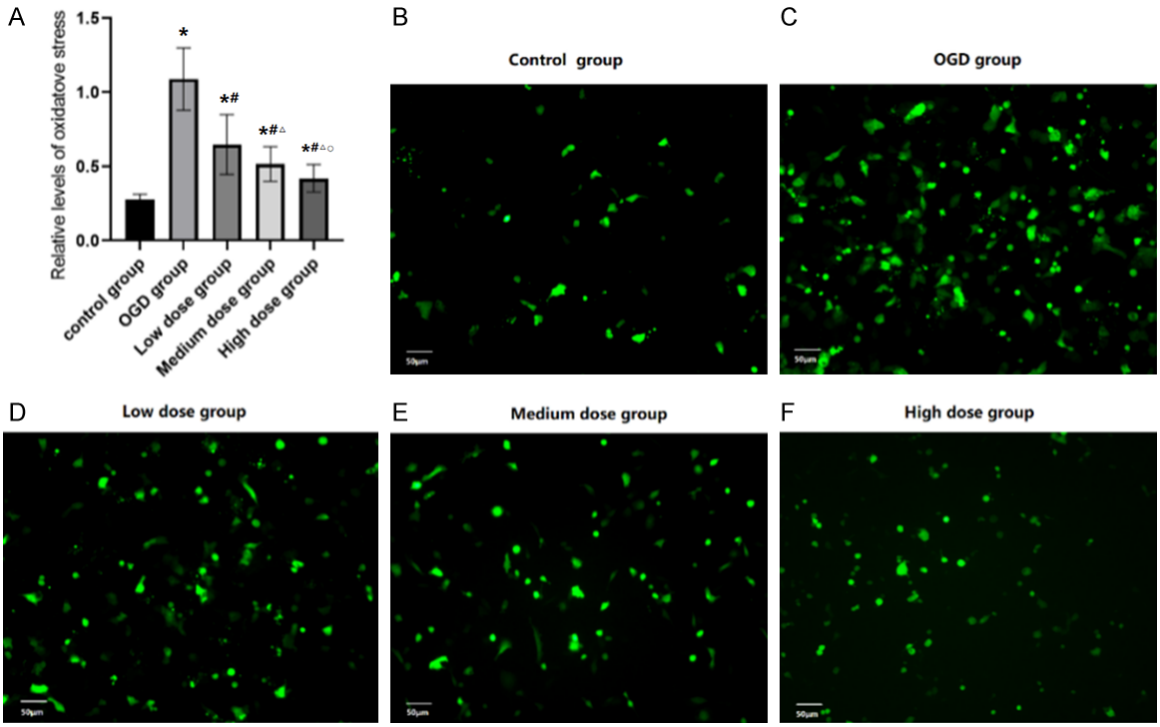

Figure 3. Comparison of reactive oxygen species expression in cells between groups (×400). (A) The expression of reactive oxygen species in HT22 cells after hypoxia-reoxygenation was much higher than that in control group (P<0.05). The ROS is showing in green in (B), and the level of ROS was decreased after treatment with crocin (P<0.05). As the crocin concentration increased, the expression of ROS in cells decreased more significantly. (B) Control group. (C) OGD group. (D) Low dose group. (E) Medium dose group. (F) High dose group. Note: Compared with control group, $^{*}P<0.05$; Compared with OGD group, $^{\#}P<0.05$; Compared with Low dose group, $^{\Delta}P<0.05$; Compared with Medium dose group, $^{\circ}P<0.05$.

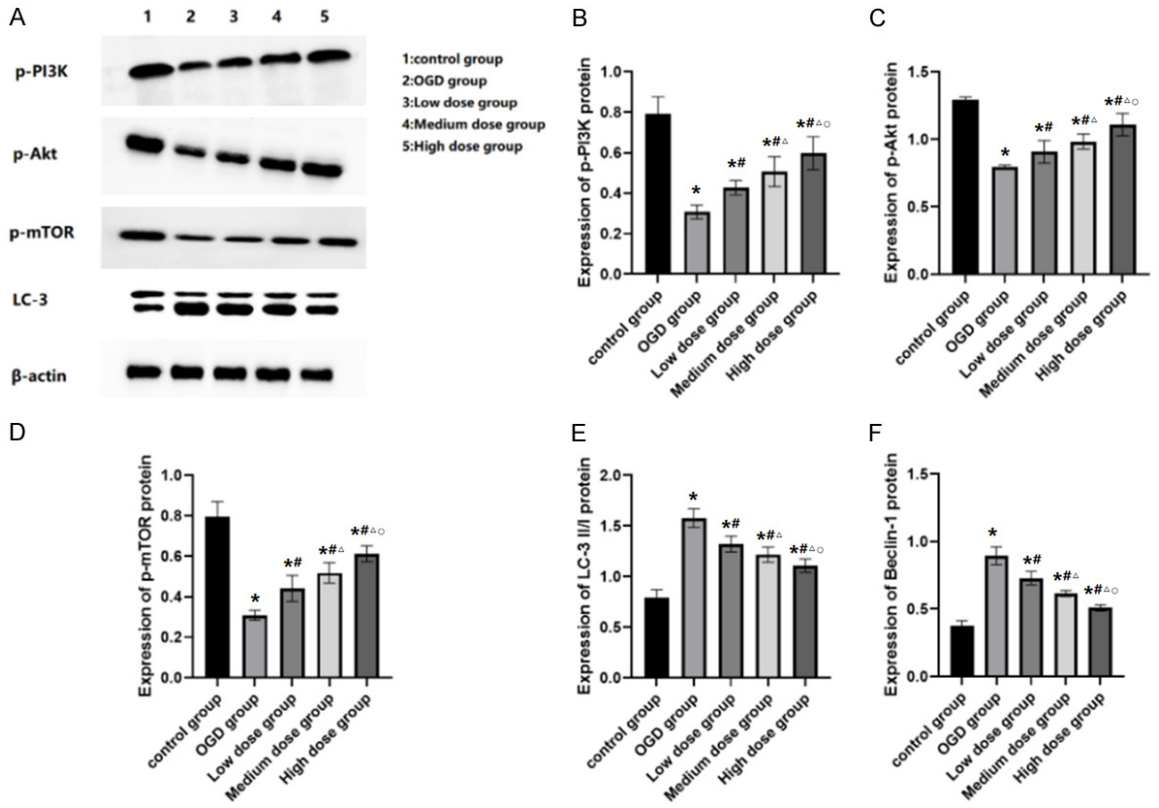

Figure 4. Comparison of cell protein expression between groups. A: WB protein expression bands. B: The expression levels of p-PI3K in HT22 after hypoxia-reoxygenation were clearly lower than those in the control group (P<0.05). The expression levels of p-PI3K in cells treated with crocin were notably higher than those in OGD-group (P<0.05). C: The expression levels of p-Akt in HT22 after hypoxia-reoxygenation were clearly lower than those in the control group (P<0.05). The expression levels of p-Akt in cells treated with crocin were notably higher than those in OGD-group (P<0.05). D: The expression levels of p-mTOR in HT22 after hypoxia-reoxygenation were clearly lower than those in the control group (P<0.05). The expression levels of p-mTOR in cells treated with crocin were notably higher than those in OGD-group (P<0.05). E: The expression levels of LC-3 II/I after hypoxia-reoxygenation were clearly higher than those in the control group (P<0.05). The expression levels of LC-3 II/I in cells treated with crocin were notably lower than those in OGD-group (P<0.05). F: The expression levels of Beclin-1 after hypoxia-reoxygenation were clearly higher than those in the control group (P<0.05). The expression levels of Beclin-1 in cells treated with crocin were notably lower than those in OGD-group (P<0.05). Note: Compared with control group, $^{*}P<0.05$; Compared with OGD group, $^{\#}P<0.05$; Compared with Low dose group, $^{\Delta}P<0.05$; Compared with Medium dose group, $^{\circ}P<0.05$.

Crocin inhibited autophagy

The expression levels of p-PI3K, p-Akt and p-mTOR in HT22 cells after hypoxia-reoxygenation were clearly lower while the expression levels of LC-3 II/I and Beclin-1 were clearly higher than those in the control group (all *P<0.05*).

The expression levels of p-PI3K, p-Akt and p-mTOR in cells treated with crocin were notably higher while the levels of LC-3 II/I and Beclin-1 were notably lower than those in OGD-group (all *P<0.05*). And the increase or decrease was all in a dose-dependent manner (**Figure 4**).

Discussion

Cerebrovascular disease is one of the most frequent diseases seen in clinical neurology, in which acute ischemic cerebrovascular disease

or cerebral infarction, accounts for the majority of cerebrovascular diseases [16]. Due to its high mortality and disability rate, this disease has brought heavy burden to both society and families. At present, only thrombolytic therapy (rt-PA) in the super-acute phase of cerebral infarction (3-6 h) has shown a definite effect, but due to the narrow time window of thrombolytic therapy and many complications, the application of thrombolytic therapy has been greatly restricted [17]. At present, the main therapeutic methods for cerebral infarction in clinical focus is on ischemia reperfusion and nerve cell protection, and the combination of the two therapeutic modes is generally used in clinical empirical therapy [18]. Although there are lots of Chinese patent medicines showing quite positive clinical effects for promoting blood circulation and removing blood stasis, their mechanisms have not been explored, which limits the promotion in patients with cerebral infarction worldwide [19]. Novel therapeutic methods are urgently needed, and it is worth exploring the potential mechanism of those active compounds including crocin.

Crocin is the main ingredient of crocus sativus. Studies have shown that it has remarkable cardio-cerebrovascular protective effect and antioxidant activity [20]. In this research, it has been discovered that after hypoxia and reoxygenation treatment, the cellular viability of HT22 cells was significantly decreased. At the same time, the apoptosis rate was increased. Taken together, those results indicated that hypoxia and reoxygenation treatment can lead to damage in celsl. In fact, this model has been used as an *in vitro* model of cerebral infarction in the literature [21]. To further understand this model, several experiments have been carried out to detect the ROS level and explore the signaling pathways. The expression of reactive oxygen species was significantly increased after hypoxia and reoxygenation treatment. We observed decreased levels of p-PI3K, p-Akt and p-mTOR after hypoxia and reoxygenation treatment. However, LC-3 II/I and Beclin-1 were increased after hypoxia and reoxygenation treatment, indicating that autophagy was activated. Our results showed similar results as stated in the literature in which hypoxia and reoxygenation treatment caused oxidative and autophagy stress on cells [22].

A balance between ROS and redox are critical in maintaining cell health. Too much ROS not only resulted in the change of mitochondrial membrane permeability, but also accelerated the autophagy. It has been showed that crocin significantly inhibited the generation of ROS in hippocampal neurons of OGD rats [23]. In this study, after the treatment of crocin, the ROS level in cells (OGD group with crocin treatment) were significantly lower when compared with to that in OGD group. Moreover, the level of ROS in the OGD group with high crocin treatment is close to that in the control group. This indicated the strong anti-oxidative effects of crocin and this strong anti-oxidative effect of crocin reduced the apoptosis rate after hypoxia and reoxygenation treatment.

The PI3K/Akt signaling pathway exerts vital functions in the survival and progression of cells. The downstream molecules of PI3K, in most mammalian cells, regulate cell autophagy and proliferation. One of the most important downstream molecules in PI3K is mTOR, a protein kinase that regulates autophagy [24]. After cerebral infarction, autophagy selectively retains and highly regulates its own processes to eliminate the detrimental products produced by lysosomes [25]. During autophagy, factors such as LC3, Beclin-1, ATG5 and ATG3 regulate the process and intensity of autophagy [26]. As reported, neuronal injury or death caused by excessive autophagy is highly associated with the LC3-II and Beclin-1 level [27]. In this study, LC3-II/I and Beclin-1 levels were highly expressed in the OGD group, indicating that autophagy activity was greatly increased after OGD modeling, and the inhibition of cell activity and promotion of apoptosis may be related to over-activated autophagy behavior [28]. The autophagy in the OGD cell model with crocin treatment was inhibited. Interestingly, the p-PI3K, p-Akt and p-mTOR expressions increased while LC-3 II/I and Beclin-1 expressions decreased. Those changes in autophagy occurred in a dose-dependent manner. Crocin can promote p-pi3k, p-Akt and p-mTOR expression levels, but it inhibits the autophagy-related proteins LC3-II/I and beclin-1, indicating that the protective effect of crocin on neural cells may involve in the activation of PI3K/Akt/mTOR as well as the autophagic inhibition. However, lack of *in vivo* experiments in the study might be a limit. It is suggested that fol-

low-up studies will further analyze the effect of crocin on cerebral infarction modeled animals and verify the mechanisms to contribute to the development and application of new clinical drugs.

In summary, crocin can inhibit autophagy by activating the PI3K/Akt/mTOR pathway at the cellular level, and reduce oxidative stress, thus playing a neuroprotective role.

Disclosure of conflict of interest

None.

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