



Effect of formononetin from *Trifolium pratense* L. on oxidative stress, energy metabolism and inflammatory response after cerebral ischemia-reperfusion injury in mice

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Abstract

Cerebral ischemia-reperfusion injury (CIRI) is one of the main diseases leading to death and disability. Studying the role and mechanism of drugs in mitigating CIRI is of great significance. In the present study, the effect of formononetin from *Trifolium pratense* L. on oxidative stress, energy metabolism impairment and inflammatory response after cerebral ischemia-reperfusion injury (CIRI) in mice were investigated. Formononetin was extracted from *Trifolium pratense* L. and was purified. The mice were treated with formononetin for six days. Then, the CIRI model was established. After 24 h of reperfusion, compared with model group, in formononetin group, the neurological deficit score, cerebral water content and cerebral infarction rate were significantly decreased, the brain tissue nitric oxide and malondialdehyde levels were significantly decreased, the brain tissue superoxide dismutase level was significantly increased, the brain tissue Na⁺-K⁺-ATPase, Ca²⁺-Mg²⁺-ATPase and Ca²⁺-ATPase activities were significantly increased, and the brain tissue tumor necrosis factor α , interleukin 1 β and interleukin 6 levels were significantly decreased. In conclusion, formononetin from *Trifolium pratense* L. can reduce the oxidative stress, energy metabolism impairment and inflammatory response in brain tissues, thus mitigating the CIRI in mice.

Keywords: formononetin; cerebral ischemia-reperfusion injury; oxidative stress; energy metabolism; inflammatory response.

Practical Application: Formononetin has beneficial effect on cerebral ischemia-reperfusion injury and may be developed as a potential candidate for prevention and treatment of cerebral ischemia-reperfusion injury.

1 Introduction

Ischemic stroke accounts for about 80% of cerebrovascular diseases. It is mainly due to the cerebral artery atherosclerosis, thrombus shedding and vascular damage, which lead to the cerebral artery embolism, resulting in the cerebral tissue ischemia and necrosis (Wang et al., 2019). Clinically, the cerebral blood circulation is improved by thrombolysis and use of anticoagulant drugs. However, due to the risk of bleeding and narrow treatment time window, the vast majority of patients cannot obtain effective treatment. In addition, the vascular recanalization leads to the cerebral ischemia-reperfusion injury (CIRI) (Zhang et al., 2021). At present, there is a lack of specific drugs for CIRI in clinic, so it is still a research focus to seek new drugs with high efficiency and low toxicity for CIRI. *Trifolium pratense* L. (red clover) is a medicinal plant widely distributed in Europe, United States, New Zealand and China. It is being marketed for use in alleviation of hot flashes and other menopausal symptoms (Kanadys et al., 2020). Formononetin is one of the main active components of *Trifolium pratense* L. (Medjakovic & Jungbauer, 2008). Formononetin belongs to isoflavones, and has a variety of pharmacological activities, such as tumor inhibition, anti-oxidation, anti-inflammation, anti-hypertension and estrogenic like effects (Chen et al., 2011; Sun et al., 2013; Lima Cavendish et al., 2015). Formononetin can prevent the myocardial from ischemia-reperfusion injury in rats by suppressing the ROS-TXNIP-NLRP3 pathway (Wang et al., 2020). It is found that many plants or their extracts have the

antioxidant, anti-inflammatory and other activities (Costa et al., 2020; Hu et al., 2021; Sultan et al., 2021). This study was to investigate the effect of formononetin from *Trifolium pratense* L. on oxidative stress, energy metabolism and inflammatory response after CIRI in mice.

2 Materials and methods

2.1 Extraction of formononetin from *Trifolium pratense* L. and purification

A 1 kg dry *Trifolium pratense* L. was crushed into powder, and was placed in the microwave extractor. Then, 5 L 80% ethanol water solution was added. The microwave-assisted extraction (power 50 W) was carried out for 1 h. After filtering and vacuum concentration, the residue was suspended with water. Ethyl acetate was added to extract. The extraction solution was concentrated by vacuum. The residue was dissolved in 5% Na₂CO₃ solution. Then, 1 mol/L hydrochloric acid was added to the solution for precipitating. The precipitate was up-loaded on the macroporous resin column, followed by gradient elution. The target elution solution was collected, and was concentrated to a certain degree. After standing, the crystals were precipitated out. The crystals were dissolved in ethanol by refluxing, followed by cooling and crystallization. After filtering, the final crystals were obtained, which were the final formononetin product.

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2.2 Grouping and treatment of animals

The flow chart of animal experiments was shown in Figure 1. Sixty clean-grade male KM mice (18-22 g) were divided into sham-operated, model, formononetin and nimodipine groups, 15 mice in each group. The formononetin group was administrated with 20 mg/kg formononetin by intraperitoneal injection. The nimodipine group was administrated with 2 mg/kg nimodipine by intraperitoneal injection. The other two groups were given the same volume of normal saline by intraperitoneal injection. The injection was performed once per day, and the treatment lasted for six continuous days.

2.3 Modeling of CIRI

One hour after the last drug injection, the CIRI model was established in model, formononetin and nimodipine groups. The mice were anesthetized with 2% sodium pentobarbital and fixed on the operating table. The hair in the middle of the neck was carefully shaved, and the skin was disinfected with iodophor alcohol. A 1.5 cm-long longitudinal incision was made. The common carotid artery, internal carotid artery and external carotid artery were exposed. The distal end of external carotid artery was ligated. An incision was made at the heat-proximal end of external carotid artery. A nylon thread was inserted through the incision into the internal carotid artery until encountering resistance. The nylon thread was fixed, and the skin was sutured. After 30 min of ischemia, the nylon thread was slowly pulled out, followed by reperfusion for 24 h. In sham-operated group, the operation steps were the same with other groups, but the nylon thread was not inserted into the internal carotid artery.

2.4 Evaluation of neurological deficit

After 24 h from the reperfusion, five mice in each group were taken. The neurological deficit scoring was blindly performed by a single examiner using a 5-point scale as follows (Longa et al., 1989): no neurological deficit, 0 point; failure to extend the right forepaw fully, 1 point; circling to the right, 2 points; falling to the right, 3 points; did not walk spontaneously and had depressed levels of consciousness, 4 points. The higher the score was, the more serious the neurological deficit was.

2.5 Measurement of cerebral water content

After neurological deficit scoring, five mice in each group were taken. The mice were killed and decapitated quickly. The ischemic hemisphere was separated. The residual blood on the surface was sucked dry with filter paper. The brain tissues were weighed on the electronic balance to obtain the wet mass. Then, the brain tissues were dried in oven at 105 °C to constant weight, and the dry mass was obtained. The brain water content was obtained as follows: water content (%) = [(wet mass - dry mass) / wet mass] × 100%.

2.6 Measurement of cerebral infarction rate

Five mice in each group were taken. After the mice were killed, the brain was carefully separated. After cleaning with normal saline, the brain tissues were frozen at -20 °C for 0.5 h. The coronal sections of brain were prepared. The sections were quickly immersed in 0.05% 2,3,5-triphenyltetrazole chloride solution (TTC) at 37 °C for staining. After 15 min, the sections were fixed in 10% formalin for 30 min. The TTC-stained sections were scanned and photographed. Image processing software was used to calculate the percentage of infarct area in the total brain, that was, the cerebral infarction rate.

2.7 Determination of biochemical indexes

Five mice in each group were taken. The brain tissues were separated, and were homogenized with ice-cold normal saline. After centrifugation, the supernatant was obtained. The oxidative stress indexes including nitric oxide (NO), malondialdehyde (MDA) and superoxide dismutase (SOD) were measured by chemical colorimetry. The energy metabolism indexes including Na⁺-K⁺-ATPase, Ca²⁺-Mg²⁺-ATPase and Ca²⁺-ATPase activities were detected using the corresponding kits. The inflammatory factors including tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β) and interleukin 6 (IL-6) were measured by enzyme linked immunosorbent assay.

2.8 Statistical analysis

Data presented were presented as mean ± SEM, and were analyzed using SPSS 19.0 statistical software package. The comparisons among various groups were made using one way analysis of variance followed by a Newman-Keuls post-hoc test. P < 0.05 was taken to represent the statistically significant difference.

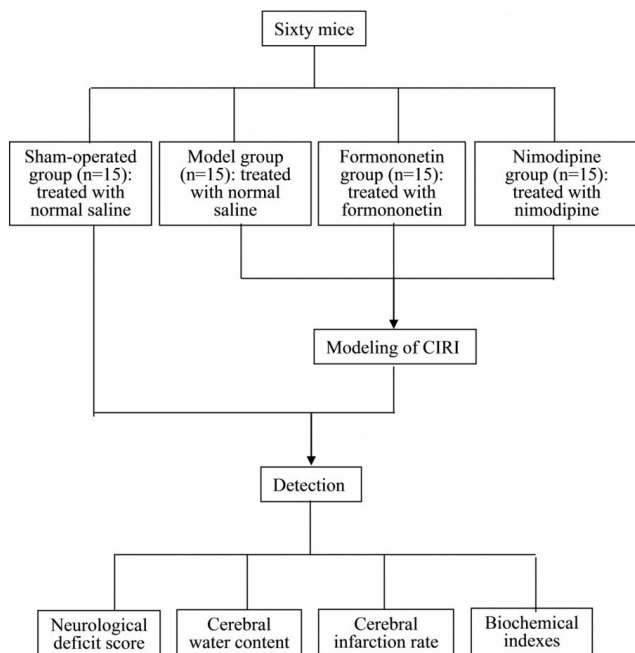


Figure 1. Flow chart of animal experiments.

3 Results

3.1 Effects of formononetin on neurological deficit score, cerebral water content and cerebral infarction rate in CIRI mice

After 24 h of reperfusion, the neurological deficit score, cerebral water content and cerebral infarction rate in model, formononetin and nimodipine groups were significantly ($P < 0.05$) higher than those in sham-operated group, respectively. Each index in formononetin and nimodipine groups was significantly ($P < 0.05$) decreased compared with model group, respectively (Figure 2).

3.2 Effects of formononetin on brain tissue oxidative stress indexes in CIRI mice

Figure 3 showed that, after 24 h of reperfusion, the brain tissue NO and MDA levels in model, formononetin and nimodipine groups were significantly ($P < 0.05$) higher than those in sham-operated group, respectively, and the brain tissue SOD level in model, formononetin and nimodipine groups was significantly ($P < 0.05$) lower than that in sham-operated group, respectively. Compared with model group, in formononetin and nimodipine groups the NO and MDA levels were significantly

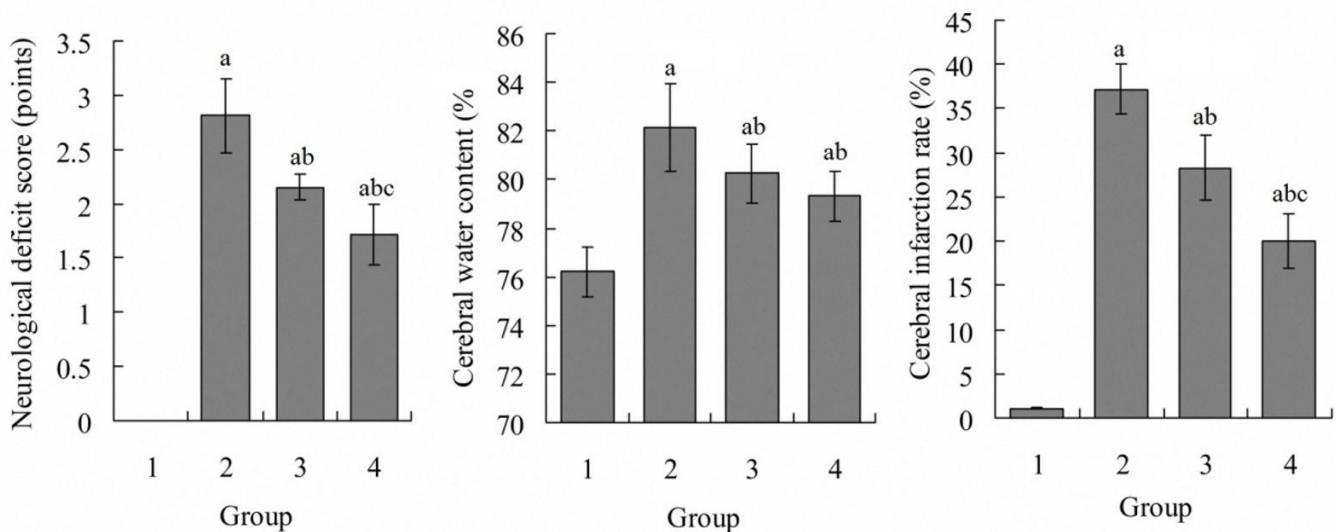


Figure 2. Comparisons of neurological deficit score, cerebral water content and cerebral infarction rate among four groups. ^a $P < 0.05$ vs. group 1; ^b $P < 0.05$ vs. group 2; ^c $P < 0.05$ vs. group 3. 1: sham-operated; 2: model; 3: formononetin; 4: nimodipine.

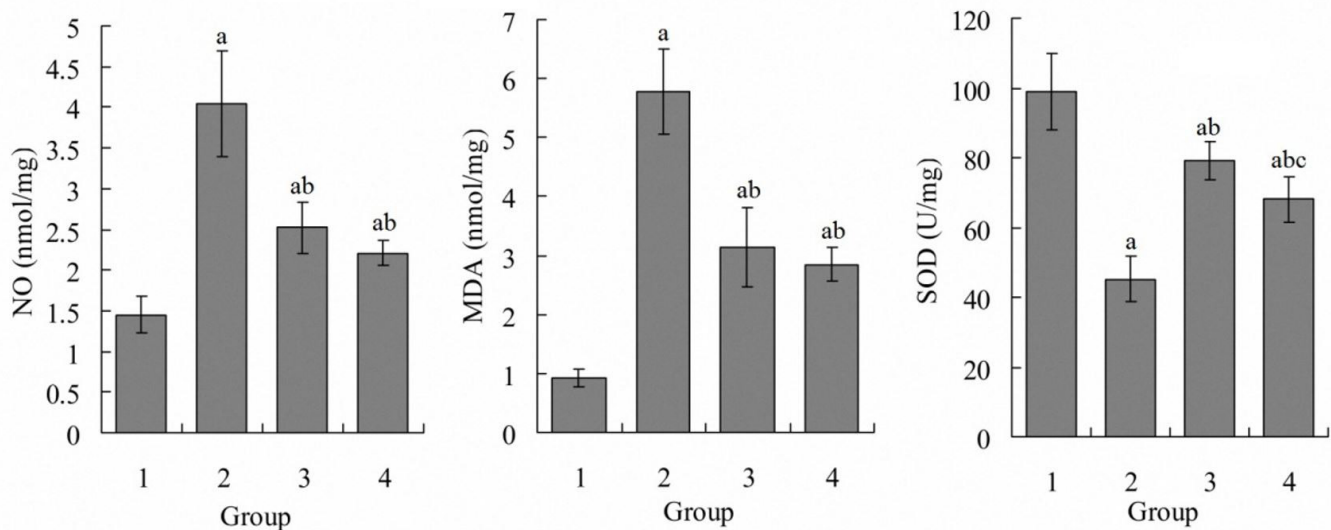


Figure 3. Comparisons of brain tissue NO, MDA and SOD levels among four groups. ^a $P < 0.05$ vs. group 1; ^b $P < 0.05$ vs. group 2; ^c $P < 0.05$ vs. group 3. 1: sham-operated; 2: model; 3: formononetin; 4: nimodipine. NO, nitric oxide; MDA, malondialdehyde; SOD, superoxide dismutase.

($P < 0.05$) decreased, respectively, and the SOD level was significantly ($P < 0.05$) increased, respectively.

3.3 Effects of formononetin on brain tissue energy metabolism indexes in CIRI mice

After 24 h of reperfusion, compared with sham-operated group, the $\text{Na}^+\text{-K}^+\text{-ATPase}$ and $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ activities in model, formononetin and nimodipine groups and $\text{Ca}^{2+}\text{-ATPase}$ activity in model and formononetin groups were significantly ($P < 0.05$) decreased, respectively. Compared with model group,

in formononetin and nimodipine groups the $\text{Na}^+\text{-K}^+\text{-ATPase}$, $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ and $\text{Ca}^{2+}\text{-ATPase}$ activities were significantly ($P < 0.05$) increased, respectively (Figure 4).

3.4 Effects of formononetin on brain tissue inflammatory factor levels in CIRI mice

As shown in Figure 5, after 24 h of reperfusion, the brain tissue $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ levels in model, formononetin and nimodipine groups and IL-6 level in model and formononetin groups were significantly ($P < 0.05$) higher than those in sham-

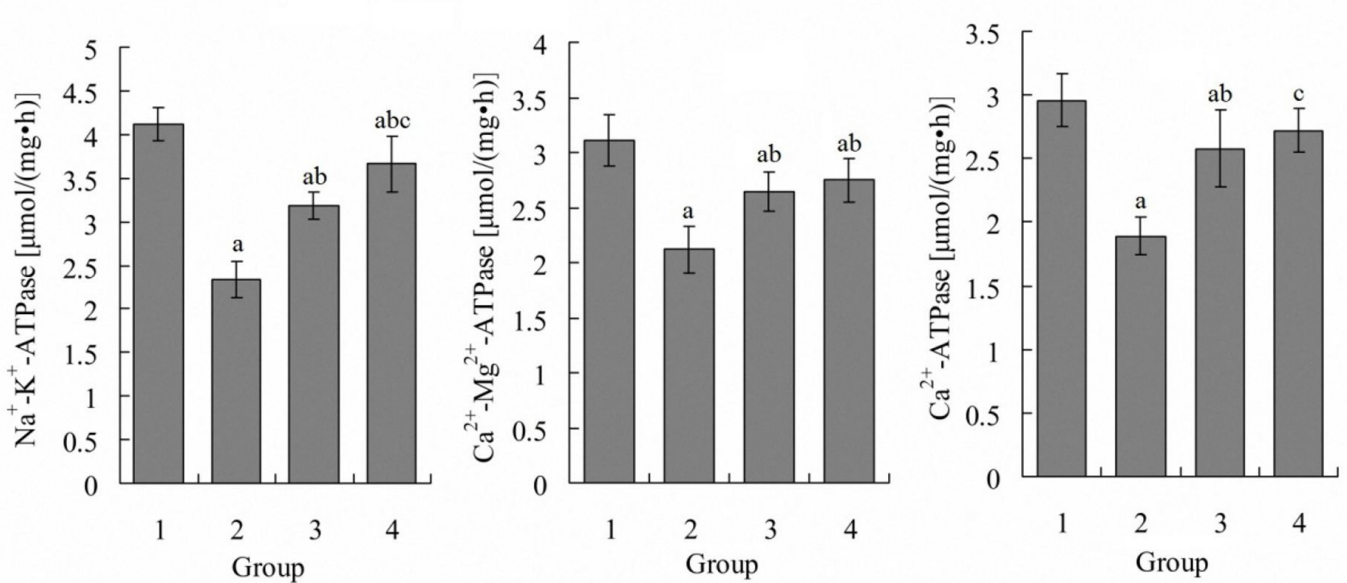


Figure 4. Comparisons of brain tissue $\text{Na}^+\text{-K}^+\text{-ATPase}$, $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ and $\text{Ca}^{2+}\text{-ATPase}$ activities among four groups. ^a $P < 0.05$ vs. group 1; ^b $P < 0.05$ vs. group 2; ^c $P < 0.05$ vs. group 3. 1: sham-operated; 2: model; 3: formononetin; 4: nimodipine.

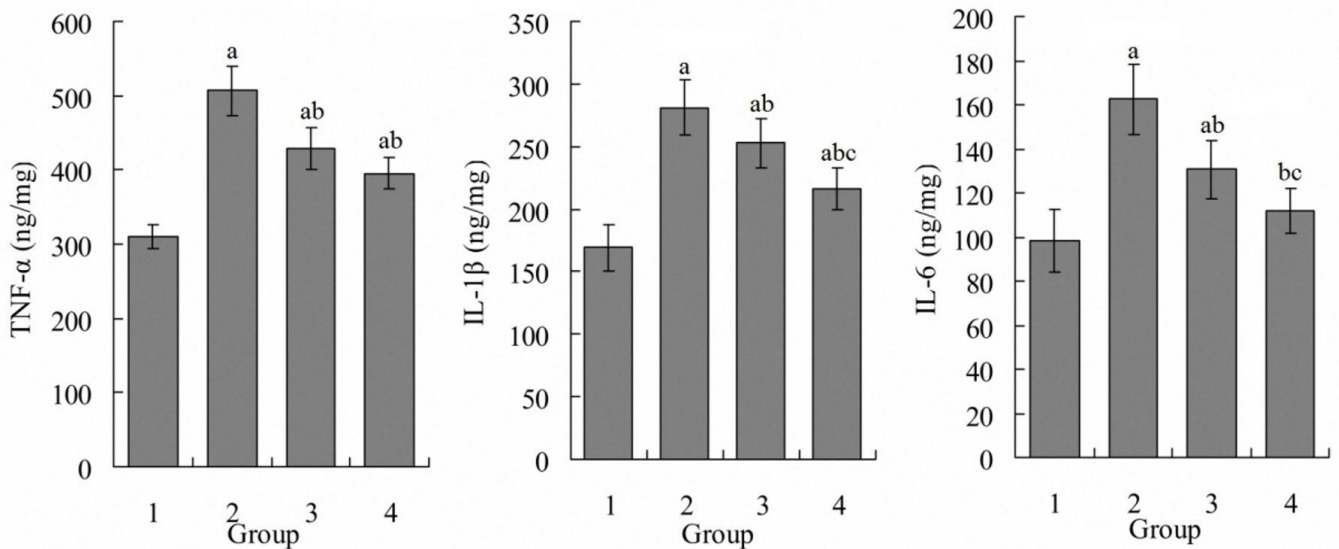


Figure 5. Comparisons of brain tissue $\text{TNF-}\alpha$, $\text{IL-1}\beta$ and IL-6 levels among four groups. ^a $P < 0.05$ vs. group 1; ^b $P < 0.05$ vs. group 2; ^c $P < 0.05$ vs. group 3. 1: sham-operated; 2: model; 3: formononetin; 4: nimodipine. $\text{TNF-}\alpha$, tumor necrosis factor α ; $\text{IL-1}\beta$, interleukin 1 β ; IL-6 , interleukin 6.

operated group, respectively. Each index in formononetin and nimodipine groups was significantly ($P < 0.05$) decreased, respectively, compared with model group.

4 Discussion

The incidence rate of cerebral ischemia is increasing year by year, and there is no effective intervention method in clinic. Therefore, the treatment of CIRI has become a hot spot in the research of cerebrovascular disease. Studying the role and mechanism of natural medicine in mitigating CIRI can provide a new strategy for the prevention of nerve injury caused by ischemic stroke, and it is of great significance for the effective treatment of cerebrovascular disease and reducing the mortality (Wu et al., 2010; Li et al., 2015). In this study, the protective effect of formononetin from *Trifolium pratense* L. against CIRI in mice was evaluated. Results showed that, after 24 h of cerebral ischemia-reperfusion, the neurological deficit score, cerebral water content and cerebral infarction rate in model group were significantly higher than sham-operated group. This indicates that, the CIRI was successfully established. Compared with model group, each index in formononetin group was significantly decreased. This demonstrates that, the pre-administration of formononetin can mitigate the CIRI in mice.

CIRI can cause the production of a large number of oxygen free radicals in brain tissue, among which NO is a research hotspot. In the early stage of cerebral ischemia, NO produced by endothelial nitric oxide synthase (NOS) has neuroprotective effect, but its effect is very small. In the late stage of ischemia, especially the reperfusion stage, NO produced by neuronal NOS and inducible NOS can damage the nerve tissues (Gürsoy-Özdemir et al., 2000; Dohare et al., 2008). MDA is the final product of lipid oxidation, and its level changes can indirectly reflect the changes of oxygen free radicals in tissues (Larsen et al., 2002). SOD, as the main enzyme of scavenging oxygen free radical, plays an important role in resisting oxygen free radical damage to cells (Kharb et al., 2000). In our study, after 24 h of reperfusion, the brain tissue NO and MDA levels in model group were significantly higher than sham-operated group, and the SOD level in model group was significantly lower than sham-operated group. This demonstrates that, the oxidative stress is involved in the CIRI of mice. Compared with model group, in formononetin group the NO and MDA levels were significantly decreased, and the SOD level was significantly increased. This confirms that, formononetin can reduce the oxidative stress in brain tissue, thus mitigating the CIRI in mice.

Na^+ - K^+ -ATPase, Ca^{2+} - Mg^{2+} -ATPase and Ca^{2+} -ATPase are the proteases that exist on the biofilm. Their biological functions include energy conversion, material transport, information transport, and maintenance of intracellular and extracellular ion balance. During ischemia-reperfusion injury, the oxidative phosphorylation ability of cells is decreased. The ATP synthesis is decreased, with increased energy consumption and decreased Na^+ - K^+ -ATPase activity. So, the Na^+ and K^+ transport ability of cell membrane is impaired (Huang et al., 2015). At the same time, the Ca^{2+} - Mg^{2+} -ATPase and Ca^{2+} -ATPase activities are decreased. The influx of Ca^{2+} is increased and the efflux is decreased, which causes the calcium overload, aggravating the secondary tissue

injury (Qiao et al., 2011; Yan et al., 2017). In this study, after 24 h of reperfusion, compared with sham-operated group, the Na^+ - K^+ -ATPase, Ca^{2+} - Mg^{2+} -ATPase and Ca^{2+} -ATPase activities in model group were significantly decreased. Compared with model group, each index in formononetin group was significantly increased. These findings indicate that, formononetin can reduce the energy metabolism impairment, which may be related to its protective effect on CIRI in mice.

More and more evidence shows that the inflammatory response plays an important role in ischemia-reperfusion injury (Oyama et al., 2004; Goebel et al., 2021). $\text{TNF-}\alpha$ is mainly produced by macrophages. It play a neurotoxic role by inhibiting NF- κ B pathway, promoting the expression of adhesion molecules, inducing the production of neurotoxic mediators, aggravating brain edema and increasing the permeability of blood-brain barrier (Dalvi et al., 2017). IL-1 β and IL-6 are also the important mediators of inflammatory response. The tissue ischemia-reperfusion activates the astrocytes and microglia to secrete a large amount of IL-1 β and IL-6, which further aggravate the tissue injury (Kim et al., 2013). Results of this study showed that, after 24 h of reperfusion, the brain tissue $\text{TNF-}\alpha$, IL-1 β and IL-6 levels in model group were significantly higher than sham-operated group. Each index in formononetin groups was significantly decreased, compared with model group. It is suggested that formononetin can mitigate the CIRI in mice by inhibiting the inflammatory response in brain tissues.

5 Conclusion

In conclusion, formononetin from *Trifolium pratense* L. can reduce the oxidative stress, energy metabolism impairment and inflammatory response in brain tissues, thus mitigating the CIRI in mice. Therefore, formononetin has beneficial effect on CIRI in mice and may be developed as a potential candidate for prevention and treatment of CIRI. Of course, our study is only a preliminary experiment on the protection of formononetin on CIRI. Maybe other mechanisms are involved in the action formononetin, but they are not investigated here. This is a limitation of our study, and should be solved in further researches.

Conflict of interest

The authors declare that they have no conflict of interest.

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