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Protective effects of epigallocatechin gallate against ischemia reperfusion injury in rat skeletal muscle via activating Nrf2/HO-1 signaling pathway

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ABSTRACT

Aims: Previous studies have demonstrated that epigallocatechin gallate (EGCG) had certain protective effects on myocardial and renal ischemia reperfusion (I/R) injury. We aimed to research the special effects and underling mechanisms of EGCG on skeletal muscle I/R injury.
Main method: We established an experimental rat model of I/R skeletal muscle injury and treated with different doses of EGCG. Hematoxylin eosin staining, TUNEL assay, ELISA, qRT-PCR and Western blotting were used to evaluate the effects of EGCG.
Key findinds: EGCG significantly improved skeletal muscle function of I/R injury rats. Moreover, EGCG had positive effects on decreasing apoptosis of skeletal muscle tissues, alleviating oxidative stress damage and suppressing the production of inflammatory cytokines. Further, EGCG had positive effects on activating Nrf2/HO-1 signaling pathway.
Significance: EGCG might be a powerful candidate compound for alleviating I/R injury in rat skeletal muscle.

1. Introduction

Ischemia-reperfusion (I/R) injury refers to the phenomenon that the injury of tissues and organs is aggravated after the organs recover blood flow on the basis of ischemia [1–5]. I/R injury is common in organs and tissues, and skeletal muscle is no exception especially in the field of orthopedic trauma [6,7]. The tolerance of various tissues to ischemia varies greatly, and skeletal muscle is the least tolerant tissue of limbs. Besides, skeletal muscle is the most sensitive to ischemia in human limbs [8]. Many clinical events, such as vascular injury of extremities, arterial embolism, primary arterial thrombosis, limb replantation, compartment syndrome, overtime application of tourniquet and so on, may lead to severe skeletal muscle ischemia [9–11]. After the re-establishment of circulation, reperfusion injury not only results in the second injury of local tissues, but also leads to secondary injury of distant organs, resulting in high disability rate and mortality [12]. However, reperfusion injury is unavoidable in this case because the limbs of patients must be preserved by re-establishing blood circulation. Therefore, it is of great clinical significance to study the underlying mechanisms of I/R injury of skeletal muscle and alleviate the reperfusion injury of skeletal muscle.

The mechanisms of I/R injury of skeletal muscle is very complex and has not been fully elucidated. At present, the most recognized mechanisms are injury of oxygen free radicals, calcium overload, interaction between endothelial cells and neutrophils [13,14]. In addition, it is also related to energy metabolism disorder, no reflux phenomenon, the role of endothelia and cell apoptosis. There are many methods to protect skeletal muscle from I/R injury, such as increasing oxygen and energy supply of tissues and organs, reducing the burden and energy consumption of injured organs or tissues and so on [15–17]. However, no matter what method is adopted, once the tissue is truly ischemic, the protective effect is limited. In the past 10 years, with the increasing research on improving tissue ischemic tolerance with I/R injury, researchers have begun to pay attention to mitigating I/R injury of skeletal muscle by regulating the tolerance of tissue cells to ischemia and hypoxia, which has also become the research hotspot in the field of wound repair [18,19].

In recent years, many scholars have found that traditional Chinese medicine has certain effects on preventing and treating I/R injury of skeletal muscle, for example, Schisandrin B treatment prevented hind limb I/R skeletal muscle injury possibly by attenuating oxidative stress and inflammation via p38MAPK, ERK1/2 and NF-κB pathways [20,21]. Caffeic acid phenethyl ester protect against skeletal muscle I/R injury in rats via regulation of antioxidant, anti-inflammatory and free radical

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scavenger properties [22]. In addition, Lutein protected against I/R injury in rat skeletal muscle by modulating oxidative stress and inflammation [23]. EGCG, as one of the extracts in green tea, widely exists widely in nature with positive activities of anti-oxidation, anticancer, improving neurological dysfunction, cardiovascular protection and reducing cholesterol loss [24–26]. A large amount of studies have shown that EGCG has protective effects against I/R injury. For instance, EGCG reduceed I/R injury in isolated perfused rabbit hearts [27]. Moreover, EGCG exhibited neuroprotective effects against focal cerebral I/R injury in rats through attenuation of inflammation [28]. EGCG attenuated myocardial injury induced by I/R in diabetic rats and in H9C2 cells under hyperglycemic conditions [29]. However, the role and possible mechanisms of EGCG in skeletal muscle I/R injury have not been understood yet. Therefore, the present study was designed to explore the protective effects and possible signaling pathways of EGCG on I/R injury in rat skeletal muscle.

2. Materials and methods

2.1. Experimental animals

A total of 30 male Sprague-Dawley (SD) rats weighting 250–280 g were purchased Selleck Ltd. (Shanghai, China)., and all animals were housed under standard environmental conditions at controlled temperature (22 ± 2 °C), humidity (50 ± 10%), and light (12 h light/dark cycle) with free access to standard diet and water. All procedures for animal care and use were in accordance with the National Institute of Health (NIH) guidelines, and approved by the Institutional Animal Care and Use Committee of SYXK (su) 2018–0124(20180326).

2.2. Establishment of I/R injury model in rat skeletal muscle

The skeletal muscle I/R injury model rats were established following the protocols described as previous [30]. In brief, a total of 24 rats were anesthetized by 3% pentobarbital sodium intraperitoneally. Afterwards, the skin on the right hind leg was sheared and sterilized with 70% alcohol, cut open along the knee until completely expositing the joint. Subsequently, the ischemia model was established by closing femoral artery and vein together with proper size of non-invasive vascular clamp for 4 h. Further, the skeletal muscle I/R injury model was established by reopen the clamp at 6 h after ischemia. Moreover, the sham operation was also performed upon the similar procedures without using vascular clamp. For the saline and drug administration groups, 500 mL/kg saline and EGCG (50, 100 and 150 mg/kg) were given intravenously at 5 min prior to the reperfusion, respectively.

2.3. Hematoxylin eosin (HE) staining

The skeletal muscle slices were stained with hematoxylin for 5 min, then rinsed for 1 min, and returned to blue by 1% ammonia (30 s). Afterwards, slices were flushed with running water (1 min). Furthermore, slices were stained by 0.5% HE (for 1 min), rinsed (for 30 s), made into transparent, and finally mounted with neutral gum.

2.4. TUNEL assay

The apoptosis of paraffin-embedded skeletal muscle sections was detected by TUNEL assay. Briefly, the sections were dewaxed, and permeabilized with proteinase K for 15 min at room temperature. Afterwards, the sections were treated with 3% H_2O_2 to block endogenous peroxidases and incubated with equilibration buffer and terminal deoxynucleotidyl transferase enzyme. Finally, sections were incubated with antidigoxigenin-peroxidase conjugate. Tissue peroxidase activity was evaluated through DAB application. Sections were examined under a light microscope.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Skeletal muscle tissue and serum were collected to measure the IL-1, IL-6 and TNF-α concentrations using an ELISA kit according to the manufacturer's instructions (uscn-SEA064R and uscn-SEA563Ra, respectively).

2.6. Determination of anti-oxidant enzyme activities

The activity of malondialdehyde (MDA), superoxide dismutase (SOD) determined from cell lysates using commercial kits (SOD-525, Catalase-520 and GPx-340 from OxisResearchTM, Portland, OR, USA) following the manufacturer's instructions.

2.7. RNA extraction and real-time PCR

Total RNA was extracted from skeletal muscle tissues using TRIzol Reagent following the manufacturer's suggestions. The mRNA expression levels of Bcl-2, Bax, NF-κB, ICAM-1, Keap1, Nrf2 and HO-1 were amplified by qRT-PCR using the SybrGreen reagent (Takara, Dalian, China) on a Stratagene 3005p system (Agilent Technologies, Mississauga, Canada). The housekeeping gene GADPH was the internal standards, respectively, and $2^{-\Delta\Delta CT}$ method was used to measure the relative expression level. Primer sequences used were designed as follows: Bcl-2 forward, 5′-TGAACCGGCA TCTGCACAC-3′, Bcl-2 reverse, 5′-CGTCTTCAGAGACAGCCAGGAG-3′, Bax forward, 5′-AGAC ACCTGA GCTGACCTTGGAG-3′, Bax reverse, 5′-GTTGAAGTTGCCATCAGCAA ACA-3′, NF-κB forward, 5′-CTGTTTCCCCTCATCTTTCC-3′, NF-κB reverse, 5′-GTGCGTCTTAGTGGTATCTGT G-3′, ICAM-1 forward, 5′-AGG TATCCATCCATCCACA-3′, ICMA-1 reverse, 5′-GCCACAGTTCT CAAAG CACA-3′, Akt forward, 5′-AATACCTGGTGTCGGTCTCA-3′, Akt reverse, 5′-TCGAGCTC ATCCTAATGGAG-3′, GSK-3β forward, 5′-TAGGAACAC CAACAAGGGAGC-3′, GSK-3β reverse, 5′-CGAGCGTGAGGAGGGAT AAG-3′, Keap-1 forward, 5′-GTGGAGACAGAGACCTGGACTTT C-3′, Keap-1 reverse, 5′-TGTCAAGCGGGTCACTTCACTC-3′, Nrf2 forward, 5′-TTCCTCTGCTGC CATTAGTCAGTC-3′, Nrf2 reverse, 5′-GCTCTTCC-ATTTCCGAGRCACTG-3′, HO-1 forward, 5′-C ACCAGCCACACAGCAC TAC-3′, HO-1 reverse, 5′-CACCCACCCCTCAAAAGACA-3′, GAPDH forward, 5′-GATGCTGGTGCTGAGTATGRCG-3′, GAPDH reverse, 5′-GTGG TGCAGGATGCATT GCTCTGA-3’.

2.8. Western blotting assay

Proteins from isolated skeletal muscle tissues were extracted according to the manufacturer's protocol, and the protein concentrations were determined using BCA Protein Assay Kit. Equal amounts of protein loaded on 12% SDS-PAGE for electrophoresis separation. The protein bands were transferred to PVDF membranes and then incubated at 4 °C overnight with primary antibodies, including Bax (ab32503; 1: 1000), Bcl-2 (ab196495; 1: 1000), Cleaved caspase-3 (ab49822; 1: 1000), Cleaved caspase-9 (ab2324; 1: 1000), NF-κB (ab16502; 1: 1000), ICAM-1 (ab171123; 1: 1000), p-Akt (ab384493; 1: 1000), Akt (ab8805; 1: 1000), p-GSK-3β (ab131097; 1: 1000), GSK-3β (ab131356; 1: 1000), Keap1 (ab119403; 1: 1000), Nrf2 (ab137550; 1: 1000), HO-1 (ab13248; 1: 1000), GAPDH (ab1816023; 1: 2000) and Lamin B (#13435; 1: 2000). Subsequently, the protein bands were incubated with HRP-conjugated secondary antibody at room temperature for another 1 h. The membrane was imaged by using the ChemiDocTMMP imaging system, Image J software was used to analyze the gray value and GAPDH or LaminB was selected as the internal reference. Antibodies in this experiment were purchased from Abcam and Cell Signaling Technology.

2.9. Statistical analysis

Graph Pad Prism 5.0 statistical software was used to perform all

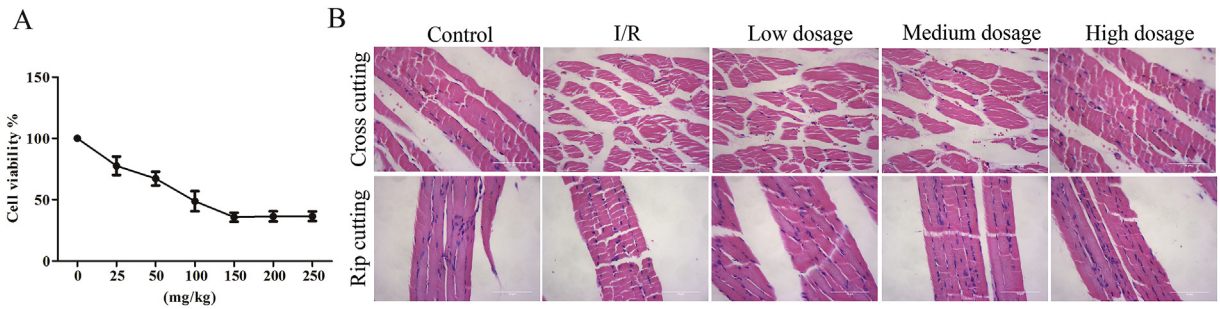

Fig. 1. Determine the experimental dose of EGCG and effects of EGCG on pathological changes of skeletal muscles in I/R injury rats. HE staining from different groups was performed in skeletal muscle tissues. The results were expressed as the mean ± SD of three independent experiments.

statistical analyses. Measurement data were represented as X ± SD; Statistical differences between means among multiple groups were analyzed by one-way ANOVA followed by a Bonferroni post hoc analysis. A value of $P < 0.05$ indicated that the difference was statistically significant.

3. Results

3.1. Determine the experimental dose of EGCG and effects of EGCG on pathological changes of skeletal muscle in I/R injury rats

In order to determine the appropriate EGCG concentration, we conducted cytotoxicity experiments, and the results were shown in Fig. 1A. We found that the cytotoxicity hardly changed when the dose reached 150 mg/kg, so we selected 50, 100, 150 mg/kg as the study dose in our study. Then, we investigated the protective effects of EGCG against I/R injury in rat skeletal muscle, firstly, HE staining assay was performed to explore the role of EGCG in pathological changes of skeletal muscles in rats. As shown in Fig. 1B, the skeletal muscle cells in normal group were normal in outline and intact in membrane, myofibrils were arranged neatly and the structures were clear. Compared with those in normal group, the structure of skeletal muscle fibers was abnormal, and muscle fibers arranged disorderly and were thickened and swollen. Moreover, the integrity and continuity of some muscle fibers were destroyed and muscle fibers may become thinner and were broken or the gap between muscle fibers was widened and inflammatory infiltration appeared between muscle fibers. Futhermore, after treatment with EGCG, the pathological changes of skeletal muscles in rats have been improved very well in a dose dependent manner and 150 mg/kg was optimal dose. These data suggested that EGCG had protective effects of EGCG against ischemia reperfusion injury in rat skeletal muscle.

3.2. Effects of EGCG on apoptosis of skeletal muscle in I/R injury rats

Apoptosis of skeletal muscle cells plays an important role in development and progress of I/R injury in rat skeletal muscle [31], so in the present study, TUNEL assay was performed to evaluate the effects of EGCG on apoptosis of skeletal muscle cells. The data of Fig. 2A demonstrated that in the normal group, the nuclei of skeletal muscle cells were round or oval in shape with clear edges and uniform staining, while in I/R injury group, apoptotic bodies were observed in the skeletal muscle cells of rats, which was that the nucleus broke up into round bodies of different sizes and were surrounded by cell membranes with irregular margins. Interestingly, EGCG obviously suppressed apoptosis of skeletal muscle cells induced with I/R injury in a dose dependent manner. In addition, qRT-PCR was performed to determine the effects of EGCG on the mRNA expressions of Bax and Bcl-2 related to apoptosis, as expected, the results of Fig. 2B indicated that the mRNA expression of Bax was up-regulated and the mRNA expression of Bcl-2 was down-regulated in in I/R injury group compared with those in normal group, and EGCG restored the effects of I/R injury on the mRNA expressions of Bax and Bcl-2. Further, western blotting assay was carried out to examine the apoptosis-related proteins such as Bax, Bcl-2, Cleaved caspase-3 and Cleaved caspase-9, and the data suggested that the levels of Bax, Cleaved-caspase-3 and Cleaved-caspase-9 proteins were significantly increased and the protein level of Bcl-2 was obviously decreased in I/R injury group related to those in normal group. However, compared with those in I/R injury group, EGCG remarkably suppressed the protein levels of Bax, Cleaved-caspase-3 and Cleaved-caspase-9 and promote the protein level of Bcl-2 (Fig. 2C). These findings indicated that EGCG suppressed apoptosis of skeletal muscle in I/R injury rats.

3.3. Effects of EGCG on oxidative stress of skeletal muscle in I/R injury rats

Oxygen-derived free radicals (ODFR) and lipid peroxidation were the main mechanisms of I/R injury [32]. To investigate the effects of EGCG on the oxidative stress of skeletal muscle in I/R injury rats, the contents of MDA and SOD were measured and the data of Fig. 3 showed that decreased SOD, and the increased MDA were observed in I/R injury group compared with those in normal group. However, treatment with EGCG significantly increased the MDA activities, and notably attenuated the SOD levels. The data demonstrated that EGCG could relieve oxidative stress of skeletal muscle in I/R injury rats.

3.4. Effects of EGCG on inflammatory responses of skeletal muscle in I/R injury rats

I/R injury was usually accompanied by local inflammation. IL-6, TNF-α and IL-1β were important cyto-inflammatory factors, and the expression levels of these factors reflected the severity of inflammatory reaction and degree of skeletal muscle injury during I/R [33]. As shown in Fig. 4A, in tissues of skeletal muscle the levels of IL-6, TNF-α and IL-1β in I/R injury group were notably higher than those in the normal group. The results in skeletal muscle serum were consistent with those in tissue at Fig. 4B. Moreover, treatment with EGCG significantly decreased the pro-inflammatory factors levels including IL-6, CK, TNF-α and IL-1β compared to those in I/R injury group.

NF-κB was a new member of the transcription factor family discovered in recent years, and was the important nuclear transcription factor in cells, playing a criucal role in the transcriptional regulation of cellular information mediated by many cell stimuli, and being involved in the expression and regulation of many genes. Moreover, NF-κB was a key transcription factor regulating the expression of inflammatory factors in I/R injury [34]. Therefore, qRT-PCR and western blotting assays were performed to explore whether NF-κB was involved in inflammatory responses in I/R injury of skeletal muscle and EGCG affected the expression of NF-κB. As expected, the data of Fig. 4C and D showed that the mRNA and protein levels of NF-κB and ICAM-1 were obviously up-regulated in I/R injury group compared with those in normal group, while treatment with EGCG significantly suppressed the

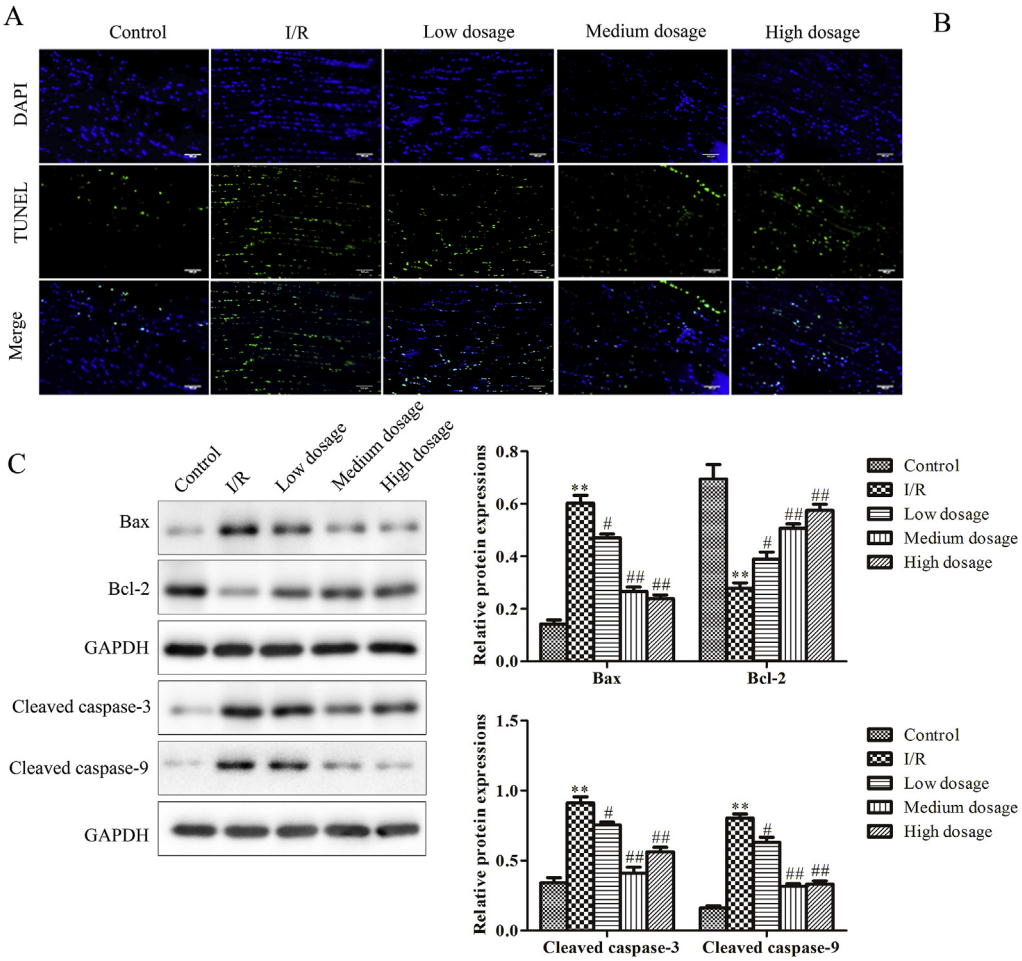

Fig. 2. Effects of EGCG on apoptosis of skeletal muscles in I/R injury rats. (A) TUNEL assay was performed to investigate the apoptosis of skeletal muscle tissues. (B) RT-qPCR was performed to evaluate the levels of Bax and Bcl-2. (C) Western blotting was used to detect the expression levels of Cleaved caspase-3, Cleaved caspase-9, Bcl-2 and Bax in skeletal muscle tissues. Then the band intensity was quantified by Image J software. The results were expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. normal group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. I/R injury group.

mRNA and protein levels of NF-κB and ICAM-1 induced with I/R injury. The data indicated that EGCG alleviated inflammatory responses of skeletal muscle in I/R injury rats.

3.5. Effects of EGCG on Nrf2/HO-1 signaling pathway of skeletal muscle in I/R injury rats

In order to further investigate the molecular mechanisms underlying the protective effects of EGCG on I/R injury in rat skeletal muscle, we evaluated the regulation of EGCG on Nrf2/HO-1 signaling pathway [35]. Firstly, we performed RT-qPCR assay to evaluate the effects of EGCG on the mRNA expressions of Keap1, Nrf2 and HO-1 and western blotting assay was carried out to determine the effects of EGCG on the protein levels of p-Akt, Akt, p-GSK-3β, GSK-3β, Keap1 (Cytoplasm), Nrf2 (Cytoplasm) and HO-1. As shown in Fig. 5A and B, the mRNA expressions of Keap1 and Nrf2 were down-expressed, and the mRNA

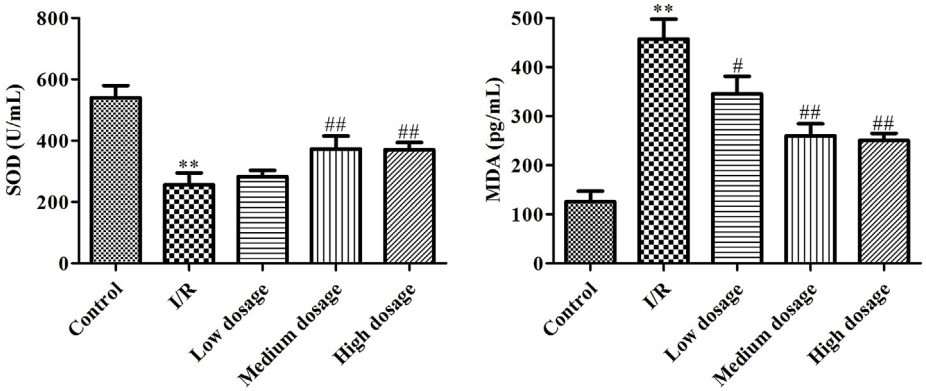

Fig. 3. Effects of EGCG on oxidative stress of skeletal muscles in I/R injury rats. The activities of MDA and SOD in skeletal muscle tissues were tested. The results were expressed as the mean ± SD of three independent experiments. $^{*}P < 0.5$, $^{**}P < 0.01$ vs. normal group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. I/R injury group.

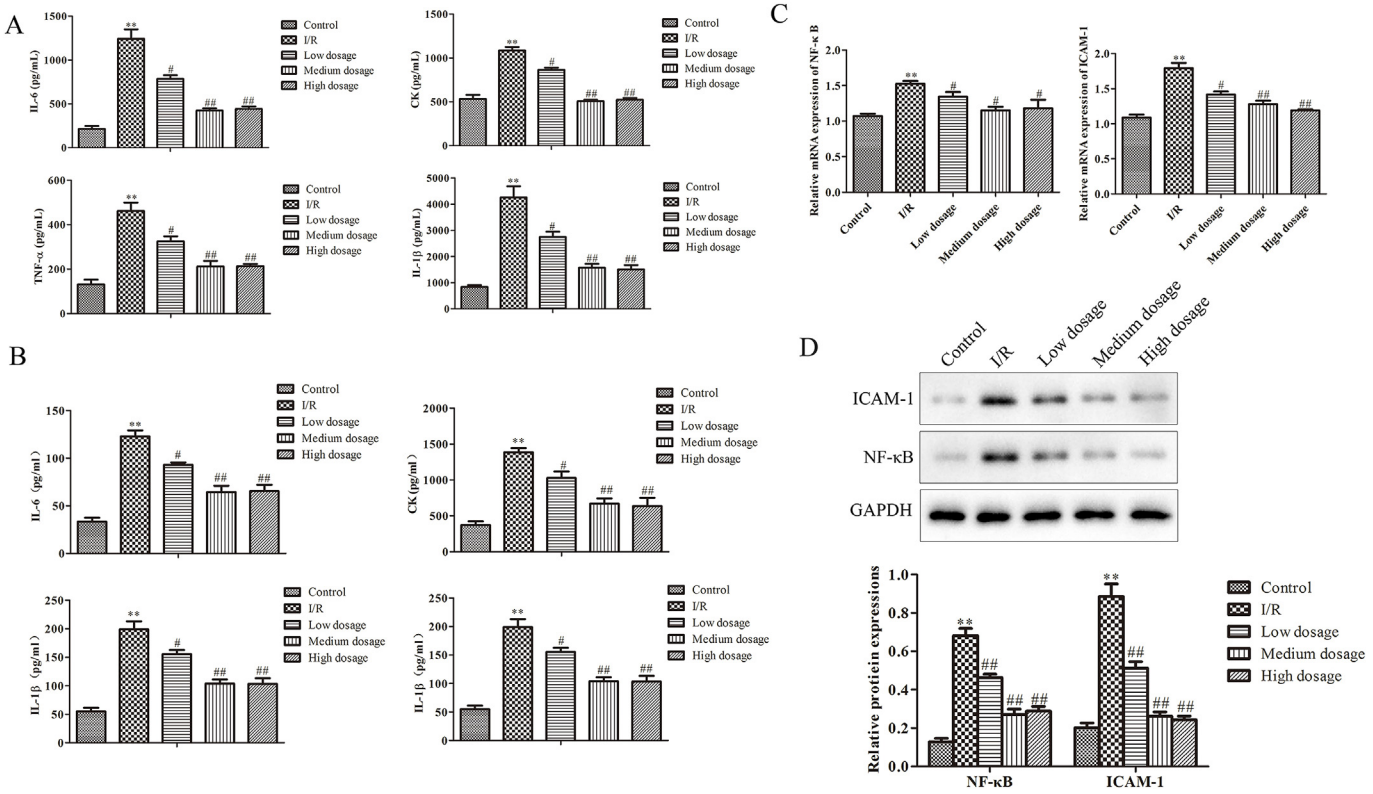

Fig. 4. Effects of EGCG on inflammatory responses of skeletal muscles in I/R injury rats. (A) The production of IL-6, CK, TNF-α and IL-1β was examined in skeletal muscle sreum. (B) The production of IL-6, CK, TNF-α and IL-1β was examined in skeletal muscle tissues. (C), (D) RT-qPCR and Western blotting were used to detect the expression levels of NF-βB and ICAM-1 in skeletal muscle tissues. Then the band intensity was quantified by Image J software. The results were expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. normal group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. I/R injury group.

expression of HO-1 was higher-expressed in I/R injury group than those in the normal group, and the protein levels of p-Akt, p-GSK-3β, Keap1 (Cytoplasm) and Nrf2 (Cytoplasm) were down-expressed, and the protein expression of HO-1 was up-expressed in I/R injury group when compared with those in the normal group. Moreover, treatment with EGCG significantly rescued these phenomenon induced with I/R injury. Further, western blotting assay was performed to examine the effects of EGCG on the expressions of nuclear proteins such as Keap1 and Nrf2, and the results of Fig. 5C showed that the protein levels of Keap1 (Nuclear) and Nrf2 (Nuclear) were obviously increased in I/R injury group than those in the normal group, while EGCG significantly inhibited the protein levels of eap1 (Nuclear) and Nrf2 (Nuclear) induced with I/R injury. Taken together, EGCG protected I/R injury partially through regulation of Nrf2/HO-1 signaling pathway.

4. Discussion

As early as 1960s, Blebea put forward the concept of I/R injury, which can not only cause tissue injury, but also aggravate the injury after blood reperfusion [36]. Skeletal muscle I/R injury refers to different degrees of skeletal muscle in ischemic region caused by abnormal metabolism of local skeletal muscle tissues and cells during a period of interruption of blood supply to skeletal muscles. Moreover, when the blood is perfused again, the abnormal metabolites of muscle tissue and cells will further increase the damage of damaged skeletal muscle tissue and cells. This dual injury further deteriorates limb function, and the accumulation of harmful metabolites leads to multiple organ dysfunctions [37,38]. At present, many clinical events causes different degrees of I/R injury of skeletal muscle and further seriously affect the prognosis and quality of life of patients.

EGCG is a kind of catechin with the highest content and the strongest biological activity extracted from green tea, accounting for 50%–75% of the total amount of green tea catechin [39]. EGCG has many biological activities and pharmacological effects, such as anti-inflammation, anti-oxidation, free radical scavenging, anti-mutagenesis and anti-tumorigenesis. Within the therapeutic dose range, there was no significant adverse reaction of EGCG to normal tissue cells [40,41]. Previous studies have shown that EGCG had protective effects against I/R injury. For example, EGCG attenuated myocardial and renal I/R injury [42,43]. However, whether EGCG had protective effects on I/R injury of skeletal muscle have not been reported. We prepared the I/R injury model in rat skeletal muscle and administrated with EGCG, and we found EGCG protected the pathological changes of skeletal muscles in I/R injury rats.

After I/R of skeletal muscle, the body produces a large number of reactive oxygen species (ROS), including superoxide anions, hydroxyl radicals and hydroxides, which are mainly produced by intracellular mitochondria [44,45]. Normally, the body produces a small amount of ROS, which sterilized and maintained the balance of oxidation and reduction *in vivo*, and was cleared by SOD [46]. A large number of ROS affected the normal metabolism and function of cells, affect the function of mitochondria, lead to calcium overload, cause inflammation and apoptosis and necrosis, so oxidative stress was an important regulator of skeletal muscle I/R injury. The expression and activity of SOD were also severely affected when the organism was severely stimulated, which made the production of ROS more dramatic *in vivo* [47]. In the experiment, the oxidative stress of EGCG group was significantly lower than that of I/R injury group. The results showed that EGCG improved the activities of MDA and reduced the production of SOD. Therefore, the natural antioxidant activity of EGCG might be an important mechanism to alleviate I/R injury of skeletal muscle.

In addition, after I/R injury of skeletal muscle, a large number of cytokines including TNF-a, IL-1β, IL-6 and IL-10 was produced, and a large number of adhesion molecules including ICAM-1 and selectin also

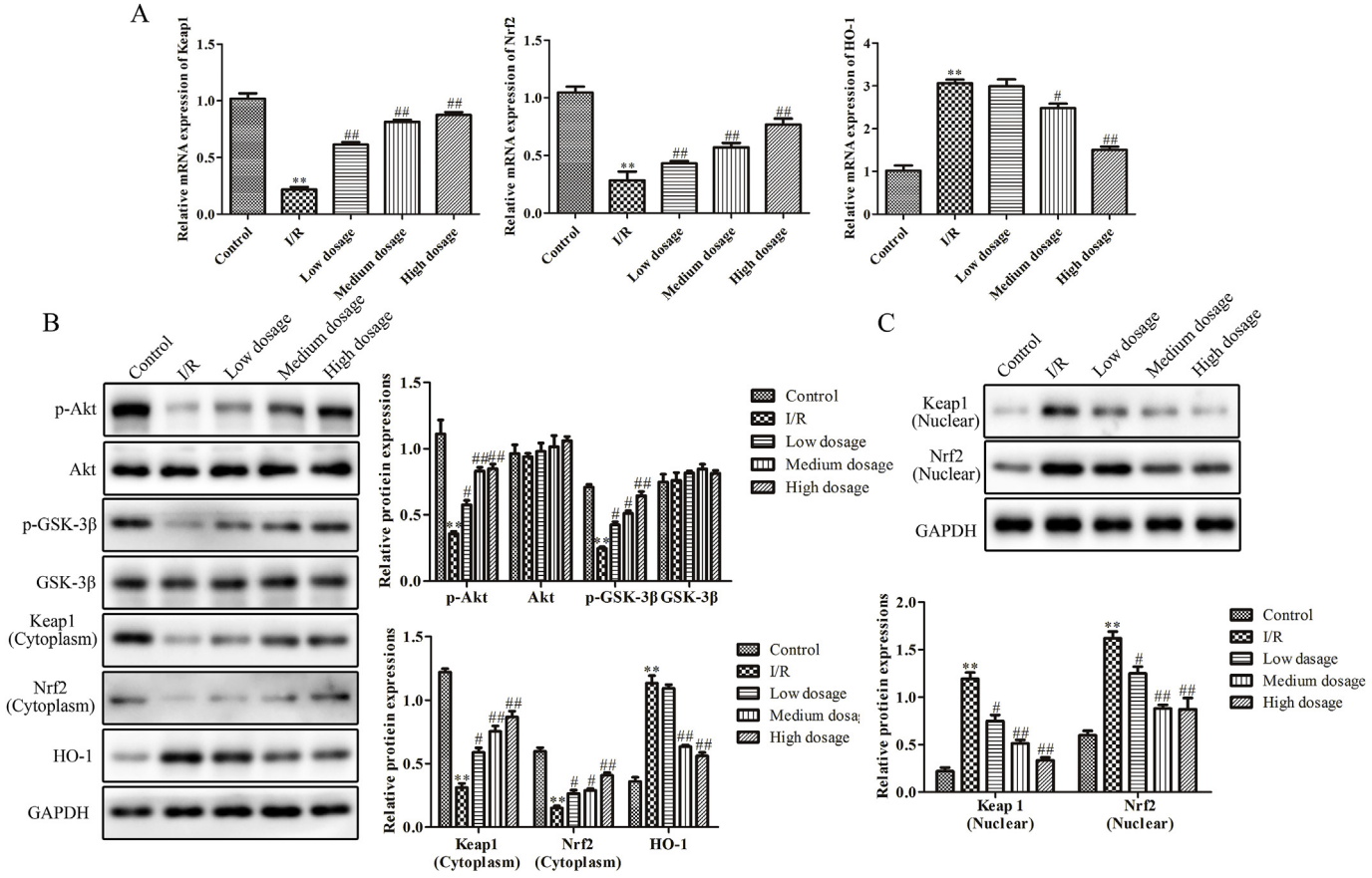

Fig. 5. Effects of EGCG on Nrf2/HO-1 signaling pathway of skeletal muscles in I/R injury rats. RT-qPCR (A) and western blotting (B and C) assay was performed to evaluate the Nrf2/HO-1 signaling pathway. Then the band intensity was quantified by Image J software. The results were expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. normal group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. I/R injury group.

was produced [48]. TNF-a and IL-1β are important inflammatory factors, which produces many biological effects, such as activating inflammatory cells including leukocytes and monocytes, stimulating the release of other inflammatory factors, activating NF-κB signaling pathway, increasing the expressions of inflammatory factors and adhesion molecules, increasing the adhesion between inflammatory cells and blood vessels, and destroying vascular endothelium [49]. To conclude, inflammatory factors TNF-a and IL-1β aggravate I/R injury of skeletal muscle. In our study, EGCG significantly decreased the pro-inflammatory factors levels including IL-6, TNF-α and IL-1β, and suppressed the mRNA and protein levels of NF-κB and ICAM-1 induced with I/R injury. Generally speaking, EGCG reduces the level of inflammatory factors, and reduces the invasion of inflammatory cells and control the inflammatory response, which may be another important aspect of the protective effect of EGCG on skeletal muscle I/R injury.

Apoptosis is not only involved in the normal growth of skeletal muscle cells, but also in the pathogenesis of many skeletal muscle diseases [50]. During the normal growth of skeletal muscle cells, apoptosis plays an important role in regulating the number of skeletal muscle cells. Bcl-2 is the main anti-apoptotic substance in the body, antagonizing the pro-apoptotic substance Bax, inhibiting cytochrome C from mitochondria into the cytoplasm, inhibiting the activation of Caspase family proteins and so on [51]. Bax is one of the main pro-apoptotic substances in the body, which can form heterodimers with Bcl-2, and the change of the ratio of them was very important to the apoptotic effects [52]. In the present study, EGCG remarkably reduced the expression of Bax, Cleaved caspase-3 and Cleaved caspase-9 and increase the expression of Bcl-2. Further, TUNEL staining intuitively responded to EGCG to reduce the apoptosis of skeletal cells. Therefore, EGCG reduceed the apoptosis of skeletal muscle cells, protect skeletal muscle cells and alleviate I/R injury of skeletal muscle.

Under basic conditions, most Nrf2 is coupled with Keap1 in the cytoplasm and degraded by ubiquitinated protease pathway. In addition, a small amount of Nrf2 existed in the nucleus in an activate state, which mediated the transcription of downstream antioxidant genes in combination with ARE, and maintained the balance of redox function. When the body was stimulated by ROS, the molecular conformation of Keap1 was changed. Nrf2 was uncoupled with Keap1, transferred from cytoplasm to nucleus, regulated the expressions of a large number of antioxidant molecules, and improved the overall antioxidant capacity of the body [53]. Recent studies have found that GSK-3β negatively regulated Nrf2 expression as a Keap1-independent pathway. GSK-3β degraded Nrf2 after ubiquitination by transferring Nrf2 from nucleus and phosphorylating it [54]. Akt/GSK-3β signaling pathway was a classical signaling pathway that mediates cell survival and played an important role in I/R injury of brain, heart and kidney [55]. Among various antioxidant enzymes induced by Nrf2, HO-1 was considered to be the most easily induced and most studied enzyme. HO-1, as one of the three isoenzymes in HO family (HO-1, HO-2 and HO-3), was a rate-limiting enzyme involved in heme metabolism. In normal physiological state, HO-1 was expressed in a small amount, while HO-1 expression was significantly up-regulated in stress state [56,57]. Under the action of cytochrome P450 reductase and NADPH, HO-1 catalyzed the degradation of heme to carbon monoxide, biliverdin and iron ions, which had anti-inflammatory, anti-oxidative and anti-apoptotic effects, constituting the body's endogenous protective barrier [58,59]. Our studies demonstrated that EGCG promoted Akt phosphorylation in I/R skeletal muscle, accelerated GSK-3β phosphorylation, weakened the negative regulation of GSK-3β on Nrf2, induced the expression of downstream signal molecule HO-1, alleviated oxidative stress injury and apoptosis,

and ultimately exerted skeletal muscle protection.

5. Conclusion

EGCG as a kind of catechin isolated from green tea, was confirmed to be a potent compound against skeletal muscle I/R injury accomplished by regulation of apoptosis, oxidative stress and inflammatory responses of skeletal muscles in I/R injury rats via activating Nrf2/HO-1 signaling pathway. We also confirmed that 150 mg/kg is the optimal concentration for EGCG by using intravenously. Therefore, EGCG might be a powerful candidate compound for alleviating I/R injury in rat skeletal muscle.

Declaration of competing interest

All authors declare that there are no conflicts of interest in the manuscript.

Abbreviations

EGCG	epigallocatechin gallate
I/R	ischemia reperfusion
SD	Sprague-Dawley
NIH	National Institute of Health
HE	hematoxylin eosin
ELISA	Enzyme-linked immunosorbent assay
MDA	malondialdehyde
SOD	superoxide dismutase
ODFR	Oxygen-derived free radicals
qRT-PCR	quantitative real time polymerase chain reaction
ROS	reactive oxygen species

Author contributions

Y.Z. conceived the work, designed and performed the experiments, analyzed the data, and wrote the manuscript. X.H.L., X.J.F., and Z.Y.M. designed and performed the experiments and analyzed the data. Y.J. and Y.L.Y. conceived the work, wrote the manuscript, and critically reviewed and supervised the study.

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