International Immunopharmacology 73 (2019) 225–235

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Irisin pretreatment ameliorates intestinal ischemia/reperfusion injury in mice through activation of the Nrf2 pathway

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ARTICLE INFO

Keywords:
Irisin
Intestinal injury
Nrf2
Inflammation
Oxidative stress
Apoptosis

ABSTRACT

Intestinal ischemia/reperfusion (I/R) injury is a serious clinical event that may induce intestinal mucosal injury, whose major underlying mechanisms include reactive oxygen species (ROS) generation, release of inflammatory mediators and induction of apoptosis. Irisin is considered an agent with potent protection against many pathological injures. The aim of this study was to investigate the protective effect of irisin pretreatment on intestinal injury and explore its underlying mechanisms in a mouse model of intestinal I/R injury as well as a cell model (IEC-6 cell) of hypoxia/reoxygenation (H/R). The results showed that irisin pretreatment ameliorated I/R and H/R-induced injury *in vivo* and *in vitro*. In addition, irisin reduced the levels of tumor necrosis factor (TNF)-α, interleukin(IL)-1β and interleukin(IL)-6 in the intestine. Compared with the I/R group, irisin pretreatment effectively reduced malondialdehyde (MDA) and myeloperoxidase (MPO) levels, but increased superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities in the intestine, and significantly reduced oxidative stress. Furthermore, irisin pretreatment downregulated Bax and cleaved Caspase-3 at the protein level, and increased Bcl-2 protein amounts, significantly reducing apoptosis in the intestine of I/R mice. Moreover, both *in vivo* and *in vitro* results showed that irisin pretreatment significantly upregulated nuclear factor (erythroid-derived 2)-like 2 (Nrf2) protein. Meanwhile, Nrf2 siRNA treatment partially abrogated the protective effects of irisin pretreatment on H/R induced cellular damage, inflammatory response, oxidative stress, and apoptosis in IEC-6 cells. These findings suggest that irisin pretreatment improves I/R-induced intestinal inflammatory response, reduces oxidative stress and inhibits apoptosis, which could be, at least partially, associated with Nrf2 pathway activation.

1. Introduction

Intestinal ischemia/reperfusion (I/R) injury is a pathophysiological process closely associated with various clinical emergencies, including acute mesenteric ischemia, burn injury, sepsis and hemorrhagic shock, and characterized by high incidence and mortality rates [1–3]. Intestinal I/R induces not only local injuries in the intestine [4,5], but also multiple organ dysfunction syndrome (MODS) or even multiple organ failure (MOF) in distant organs, finally leading to death [6,7]. The underlying mechanisms are very complex, and include damage by oxygen radicals, release of large amounts of inflammatory factors, bacterial translocation and cell apoptosis. Protecting the intestinal mucosal barrier is one of the key targets in treating patients with intestinal I/R [1,5]. Oxidative stress and inflammatory response are very important mechanisms involved in intestinal epithelial cell damage and apoptosis in intestinal I/R injury. The regaining of O_2 by the organ could stimulate cells to generate large amounts of oxygen radicals, while excessive reactive oxygen species (ROS) could in turn activate various signaling pathways to induce cell apoptosis or necrosis, while aggravating inflammatory responses [8,9].

The nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a key factor of the endogenous antioxidant system, which is ubiquitously expressed in various tissues and cells [10]. In physiological conditions, Nrf2 is found in the cytoplasm and binds to the adaptor protein Keap1, and is rapidly degraded by the ubiquitin-proteasome system, which could maintain Nrf2 at relatively low activity. However, excessive oxidative stress can stimulate Nrf2 for nuclear translocation. In the nucleus, it binds to the antioxidant response element (ARE) in the upstream

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https://doi.org/10.1016/j.intimp.2019.05.011
Received 4 March 2019; Received in revised form 30 April 2019; Accepted 6 May 2019
Available online 17 May 2019

promoter region of many antioxidative genes to control the amounts of antioxidative proteins, such as phase II detoxification enzymes, therefore protecting tissues from damage caused by oxygen radicals and other toxins [11–13]. Several studies have demonstrated that the Nrf2 pathway participates in intestinal I/R injury, while enhancing endogenous antioxidant activities exerts important effects against intestinal I/R related damage [9,14–16]. Moreover, Nrf2 pathway is also considered to have a pivotal role in reducing the injuries of other organs, such as the heart [17],lung [18],liver [19],brain [20], and so on.

Irisin is a new peptide associated with metabolism, which plays important roles in white to brown fat conversion and energy expenditure regulation, and promotes heat production, ameliorates obesity and regulates glucose homeostasis [21]. Irisin inhibits ROS generation and regulates apoptosis related proteins, reducing the sizes of arterial plaques and improving inflammatory cell invasion to vascular walls, and therefore participating in the protection of endothelial cells [22]. Previous studies have shown that irisin levels represent a potential predictive factor of cardiovascular diseases. Blood irisin levels in patients with cardiac insufficiency tend to decrease, suggesting that irisin could play a role in lipid and glucose metabolic alterations in heart failure [23,24].A recent study has revealed that in the mouse model of ischemic cerebral infarction, the animals with elevated blood irisin levels have a relatively lower degree of cerebral damage and reduced inflammatory responses, as well as improved neurological functions, suggesting that irisin could exert neuroprotective effects [25]. In addition, several previous studies have already reported beneficial effects of irisin in various models of ischemia reperfusion injury, such as liver, lung and heart [26–28]. Recent studies have shown that irisin increases Nrf2 expression to regulate macrophage activities *in vitro* [29], and improves pulmonary function in chronic obstructive pulmonary disease (COPD) patients [30], which confirms its potential antioxidant effects. However, no studies have investigated whether irisin affects intestinal I/R injury and the potential implication of the Nrf2 pathway.

Based on previous findings, we hypothesized that irisin pretreatment could ameliorate intestinal I/R injury by activating the Nrf2 pathway. Therefore, this study aimed to investigate whether irisin pretreatment exerts protective effects in *in vitro* and *in vivo* models of intestinal I/R, and further explored the involvement of the Nrf2 pathway.

2. Materials and methods

2.1. Animals

Adult male C57BL/6 mice (6–8 weeks old; body weights of 22–25 g) were provided by the Animal Experimental Center of Southwest Medical University. The animals were kept at 22 ± 1 °C under a 12 h/12 h light-dark cycle, with free access to food and drinking water. Mice were acclimated for 7 days in the environment before the experiments. All animal studies were done in compliance with the approval of the institutional animal care and use Committee of Southwest Medical University and all experiments were conducted according to the National Institutes of Health guidelines.

2.2. Intestinal I/R model

The animals were randomly divided into 4 groups, including the sham (Sham), intestinal I/R (I/R), Irisin (10) + I/R and Irisin (100) + I/R groups. Before establishment of the intestinal I/R model, mice were fasted for 8 h, with free access to drinking water. For I/R model induction, the animals were anesthetized by intraperitoneal injection of ketamine/xylazine(10:1, 100 mg/mL, 0.1 mL per 100 g of mouse's body weight); then, a mid-abdominal incision was made, and the superior mesenteric artery (SMA) was isolated [7,31]. To generate intestinal I/R injury, an atraumatic microvascular clamp was used to clip the SMA for 30 min, after which the clamp was removed to allow reperfusion for 6 h. In the Sham group, the SMA was isolated but not clipped. Ropivacaine (0.125%) was administered to the incision for infiltration analgesia, which could alleviate postoperative pain. In the Irisin (10) + I/R group or Irisin (100) + I/R group, recombinant irisin (100 μL, 067-29A, Phoenix Pharmaceuticals, Inc. Burlingame, CA,USA) was intravenously administered to mice at a dose of 10 ng/g or 100 ng/g body weight, respectively, *via* the tail vein at 30 min before the operation, then intestine was subjected to I/R as above. The dose of irisin was decided according to our preliminary experiments and previous studies [25,28]. In the I/R group, the same volume of vehicle (0.9% saline) was intravenously injected as a control.

Table 1
Summary of primer sequences.

Gene	Primer sequences
Nrf2	Forward: 5′-AAAATCATTAACCTCCCTGTTGAT-3′ Reverse: 5′-CGGCGACTTTATTCTTACCTCTC-3′
β-actin	Forward: 5′-GCCATGTACGTAGCCATCCA-3′ Reverse: 5′-GAACCGCTCATTGCCGATAG-3′

2.3. Specimen collection

In each group, the blood of mice in deep anesthesia was obtained from the cardiac apex at 6 h after reperfusion. The blood was centrifuged at 4 °C, 3500 rpm for 15 min, and the supernatant was collected for further use. Intestinal segments of about 10 cm were also obtained at the same position, namely 5 cm from the terminal ileum, from each mouse. Then, 2 cm of the intestinal segment was fixed with 4% paraformaldehyde for 24 h for morphological observation, while the remaining 8 cm-segment was stored at −80 °C until use.

2.4. Cell culture and hypoxia/reoxygenation (H/R) model

The rat intestinal epithelial (IEC-6) cells were obtained from American Type Culture Collection(Cat. RL-1592; Manassas, VA, USA) and were used for the establishment of the H/R model, which mimics *in vivo* I/R injury [8,32]. The cells were cultured in Dulbecco's modified Eagle's medium(DMEM) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), at 37 °C in an incubator with 5% CO_2 and saturated humidity. For H/R treatment, the culture medium was changed to serum-free DMEM, and the cells were cultured in a microaerophilic system (Thermo Scientific,Waltham, MA) with 1% O_2, 5% CO_2, and 94% NO_2 for 6 h. Then, reoxygenation of the cells was conducted by incubation in normal conditions (95% air and 5% CO_2 at 37 °C) for another 6 h. The cells were collected for further use. In the irisin treatment groups, IEC-6 cells were pretreated with different doses of irisin (1, 10, and 100 ng/mL, respectively) (Sigma-Aldrich, MO,USA) before hypoxia treatment for different times (0, 3, 6, 12, and 24 h, respectively), after which H/R treatment was conducted. In the control group, the cells were rinsed and the culture medium was changed as in the H/R group, but culture was performed in normal conditions.

2.5. Cell transfection with Nrf2 siRNA

Nrf2 siRNA was obtained from GenePharma (Shanghai, China). For transfection, 1×10^5 IEC-6 cells were evenly seeded into 6-well plates, and transfection was performed at 55–65% confluence. According to the manufacturer's instructions, the transfection mixture containing Nrf2 siRNA and Lipofectamine 2000(Invitrogen, Carlsbad, CA) was added to the culture medium, and culture plates were shaken horizontally. The culture medium was changed to fresh culture medium after 6 h of transfection.

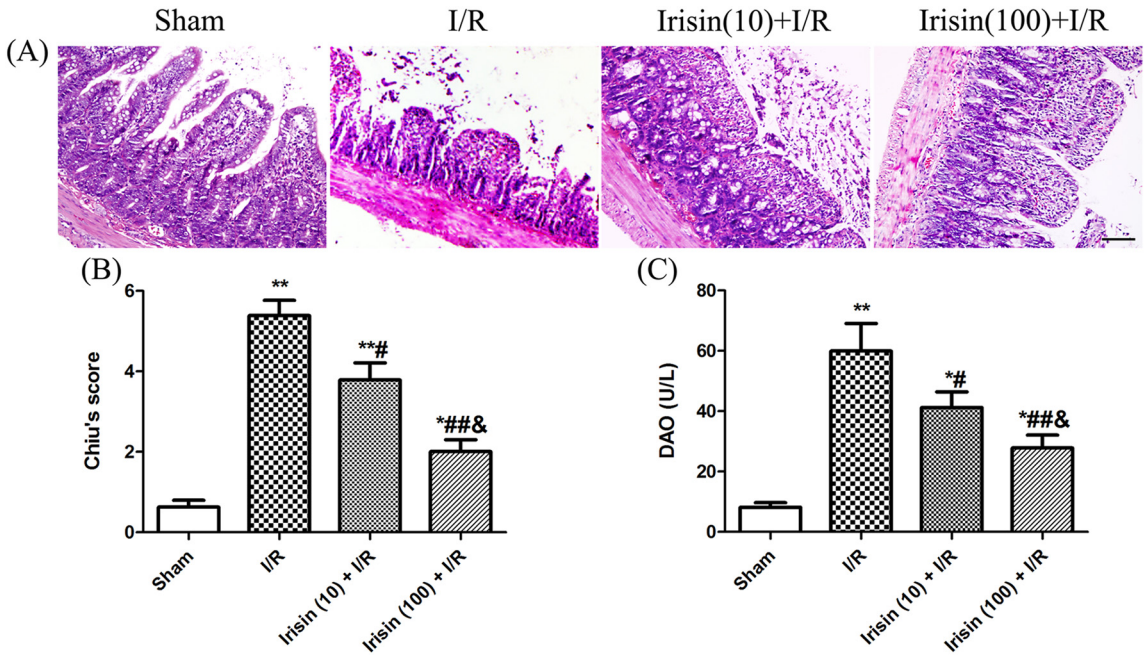

Fig. 1. Irisin protects against intestinal I/R injury in mice. (A) Pathological changes of intestinal mucosal tissues. Representative images of intestinal tissues (H&E staining, original magnification ×200).(B) Chiu scores for the intestinal mucosa. (C) Effects of irisin on serum DAO levels. Data are expressed as mean ± SD (n = 8). $^{*}P < 0.05$ *vs.* Sham group;$^{**}P < 0.01$ *vs.*Sham group;$^{\#}P < 0.05$ *vs.*I/R group;$^{\#\#}P < 0.01$ *vs.*I/R group;$^{\&}P < 0.05$ *vs.* Irisin (10) + I/R group. Scale bars = 50 μm.

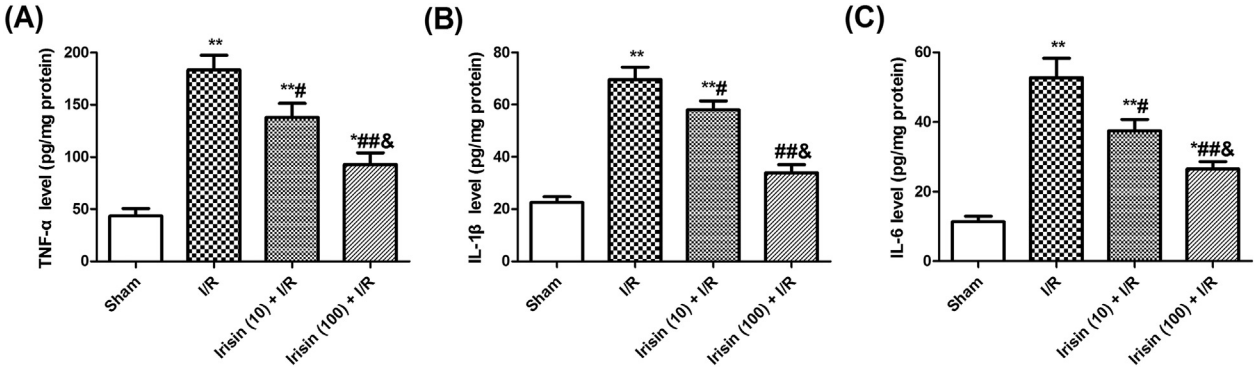

Fig. 2. Effects of irisin on inflammatory factors in intestinal I/R injury. Compared with Sham group values, intestinal TNF-α, IL-1β and IL-6 levels in the I/R group were significantly higher. Irisin significantly reduced TNF-α, IL-1β and IL-6 levels (A-C). Data are expressed as mean ± SD (n = 8). $^{*}P < 0.05$ *vs.* Sham group;$^{**}P < 0.01$ *vs.*Sham group;$^{\#}P < 0.05$ *vs.*I/R group;$^{\#\#}P < 0.01$ *vs.*I/R group;$^{\&}P < 0.05$ *vs.* Irisin (10) + I/R group.

2.6. Pathological examination of the intestinal mucosa

Intestinal tissues fixed with 4% paraformaldehyde were embedded in paraffin and sliced at 5 μm. Haematoxylin and eosin (H&E) staining was performed, and a light microscopy was used to observe the morphological changes of the intestinal mucosa. The Chiu scoring method was used to assess the degree of intestinal injury by two experienced pathologists blinded to the study [7,33].

2.7. Detection of serum diamine oxidase (DAO) activity

DAO is an intracellular enzyme with high activity, found in the cytoplasm of intestinal epithelial villus cells in all mammals. Measuring intestinal mucosal DAO activity could reflect the degree of intestinal mucosal injury [32,34]. In this study, serum DAO activity was measured with a specific assay kit (Nanjing Jiancheng Bioengineering Institute, China), according to the manufacturer's instructions.

2.8. Measurement of inflammatory factor levels

Intestinal tissue homogenates were prepared and centrifuged at 4 °C and 12,000 rpm for 15 min, and supernatants were obtained. In addition, IEC-6 cell culture supernatants were also obtained. Enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Inc. MN, USA) were used to measure the levels of TNF-α, IL-1β, and IL-6 in the supernatants of intestinal tissue samples and cells, according to the manufacturer's instructions.

2.9. Measurement of MPO activity

MPO is a specific enzyme of neutrophils, whose activity could reflect the degree of aggregation and invasion of neutrophils in intestinal tissues. MPO activity was measured with a specific MPO activity kit (Nanjing Jiancheng Bioengineering Institute, China), according to the manufacturer's instructions and previous studies [4,34].

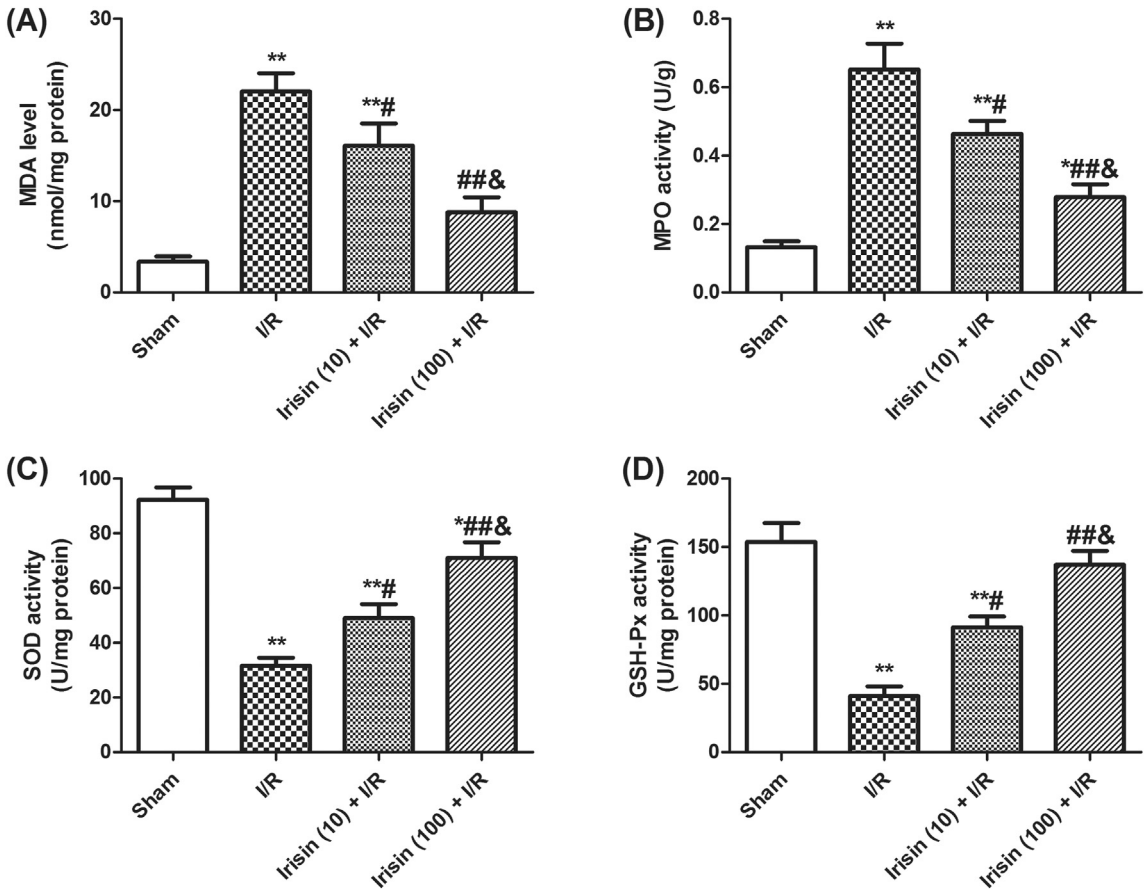

Fig. 3. Effects of irisin on MDA, MPO, GSH-Px and SOD levels in intestinal I/R injury. Compared with Sham group values, intestinal MDA and MPO levels were significantly elevated at 6 h after reperfusion in the I/R group, while GSH-Px and SOD activities were significantly decreased. Irisin significantly reduced MDA and MPO levels, and increased GSH-Px and SOD activities (A-D). Data are expressed as mean ± SD (n = 8). $^{*}P < 0.05$ *vs.* Sham group;$^{**}P < 0.01$ *vs.*Sham group;$^{\#}P < 0.05$ *vs.*I/R group;$^{\#\#}P < 0.01$ *vs.*I/R group;$^{\&}P < 0.05$ *vs.* Irisin (10) + I/R group.

2.10. Measurement of MDA, GSH-Px, and SOD levels

Supernatants from intestinal tissues and cells were obtained, and specific kits (Nanjing Jiancheng Bioengineering Institute, China) were used for the measurement of MDA levels, as well as GSH-Px and SOD activities [8], according to the manufacturer's instructions.

2.11. Western blot analysis

Lysis solution was added to intestinal tissues or IEC-6 cells to extract total protein, which was quantitated with the BCA protein quantification kit(Pierce Chemical Company, IL, USA). Equal amounts of total protein (50 μg) were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Biosciences, NJ, USA). After blocking with 5% skim milk for 1 h, the membranes were incubated with the corresponding primary antibodies (Nrf2, 1:1000; Bax, 1:500; Bcl-2, 1:500; β-actin, 1:1000)(Santa Cruz Biotechnology, Inc., CA,USA) and cleaved Caspase-3,(1:400, Cell Signaling Technology, Inc., MA, USA) at 4 °C overnight. Afterwards, HRP-labeled anti-rabbit IgG or anti-mouse IgG (1:2000, ZSGB-Bio, Beijing, China) was added, and the membranes were incubated for another 1 h. Protein bands were visualized using enhanced chemiluminescence (Amersham, Buckinghamshire, UK). β-actin was used as an internal reference for normalization. The Image J software (version 1.31; National Institutes of Health, Bethesda, ML, USA) was used for densitometric quantification.

2.12. Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from intestinal tissues and IEC-6 cells with TRIzol reagent (Takara, Dalian, China). Reverse transcription was performed using a first-strand cDNA synthesis kit (Fermentas International, Burlington, ON, Canada),according to the manufacturer's instructions. Then, obtained cDNA was amplified by a real-time PCR system (Agilent Technologies, Santa Clara, CA, USA) in the presence of a fluorescent dye (SYBR Green I; CWBIO, Beijing, China). β-actin was used as an internal reference for the quantification of Nrf2 gene expression level. The primer sequences are listed in Table 1.

2.13. CCK-8 assay

Cell viability was measured with the commercially available Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Inc. Tokyo, Japan), according to the manufacturer's instructions and previous studies [8,9]. IEC-6 cells were evenly seeded into 96-well plates, at a density of 2×10^3/mL and allowed to attach. Firstly, the effects of different reoxygenation times on cell viability were assessed. A mixture of 100 μL fresh culture medium and 10 μL CCK-8 reagent was added into each well, after 0, 3, 6, 12 and 24 h of reoxygenation, respectively. The cells were incubated at 37 °C for 2 h, cell viability was estimated, and the optimal reoxygenation time for subsequent experiments was selected. In the irisin time-dependent assay, 10 ng/mL irisin was added to each well at 0, 3, 6, 12 and 24 h, respectively, followed by model induction and cell viability evaluation. In the irisin dose-dependent assay, different doses of irisin (1, 10 and 100 ng/mL) were added to the

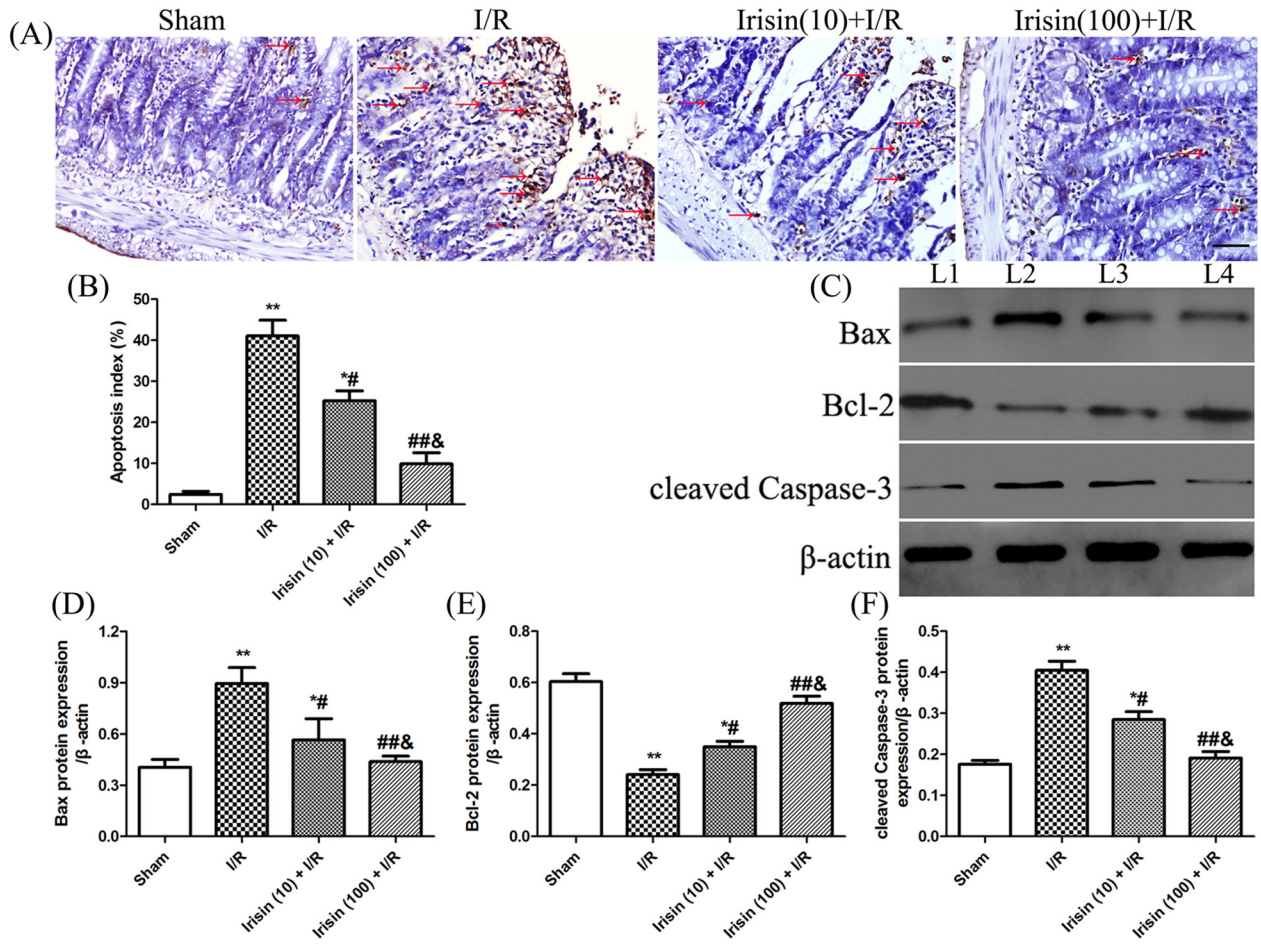

Fig. 4. Levels of Bax, Bcl-2 and cleaved Caspase-3 proteins and TUNEL positive cells in mouse intestinal tissues. (A) TUNEL positive intestinal epithelial cells (original magnification ×200). TUNEL-positive cells, indicating apoptosis, were stained dark brown in the nuclei, indicated by red arrows. (B)The quantitative analysis of apoptosis index among different groups. (C–F) Protein expression levels of Bax, Bcl-2 and cleaved Caspase-3 assessed by Western blot. Lanes 1 (L1): Sham group; Lane 2 (L2): I/R group; Lane 3 (L3): Irisin (10) + I/R group; Lane 4 (L4): Irisin (100) + I/R group. Data are expressed as mean ± SD (n = 8). $^{*}P < 0.05$ *vs.* Sham group;$^{**}P < 0.01$ *vs.*Sham group;$^{\#}P < 0.05$ *vs.* I/R group;$^{\#\#}P < 0.01$ *vs.* I/R group;$^{\&}P < 0.05$ *vs.* Irisin (10) + I/R group. Scale bars = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

culture medium to pretreat the cells for 6 h before hypoxia treatment, followed by H/R establishment and cell viability evaluation.

2.14. Assessment of cell apoptosis

The apoptosis of intestinal tissues and IEC-6 cells were detected by the terminal deoxynucleotidyl transferase-mediated dUDP-biotin nick end labeling (TUNEL) method with an assay kit (Roche, Indianapolis, IN) [4,7]. Deparaffinized intestinal tissue slices or IEC-6 cells were treated with TUNEL solution according to the manufacturer's protocol, as previously described [32]. Nuclei of IEC-6 cells were counterstained with 4′,6-diamidino-2-phenylindole(DAPI) (Sigma-Aldrich, MO,USA). For each sample, 5 non-overlapping visual fields were randomly selected for imaging, and the apoptotic index was calculated by the following equation:

Apoptotic index (%)

= number of TUNEL positive cells/number of total cells × 100

2.15. Role of Nrf2 in the protective effects of irisin pretreatment on the intestine

Before H/R treatment, IEC-6 cells were treated with irisin singly or combined with Nrf2 siRNA for 6 h. After treatment, the cells and supernatants were collected for subsequent measurements to further assess whether Nrf2 mediates the protective effects of irisin. Cell viability was measured by CCK-8 assay; lactate dehydrogenase (LDH) release in supernatants was measured with the LDH kit(Nanjing Jiancheng Bioengineering Institute, China). LDH leakage is one of the indexes reflecting cell membrane integrity and permeability [8]. The levels of TNF-α, IL-1β and IL-6 in supernatants were measured by ELISA. MDA levels, as well as GSH-Px and SOD activities were measured by chemical methods, and cell apoptosis was assessed by the TUNEL method.

2.16. Statistical analysis

The GraphPad Prism 7.0 statistical software (GraphPad Software, Inc., La Jolla, San Diego, CA, USA) was used for statistical analysis. Data are expressed as mean ± standard deviation (SD).One-way analysis of variance (ANOVA) and the least significant difference *post hoc* test were used for comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. In vivo findings

3.1.1. Pathological changes in the intestinal tissues

Slices were observed under a light microscope, and the Chiu scoring

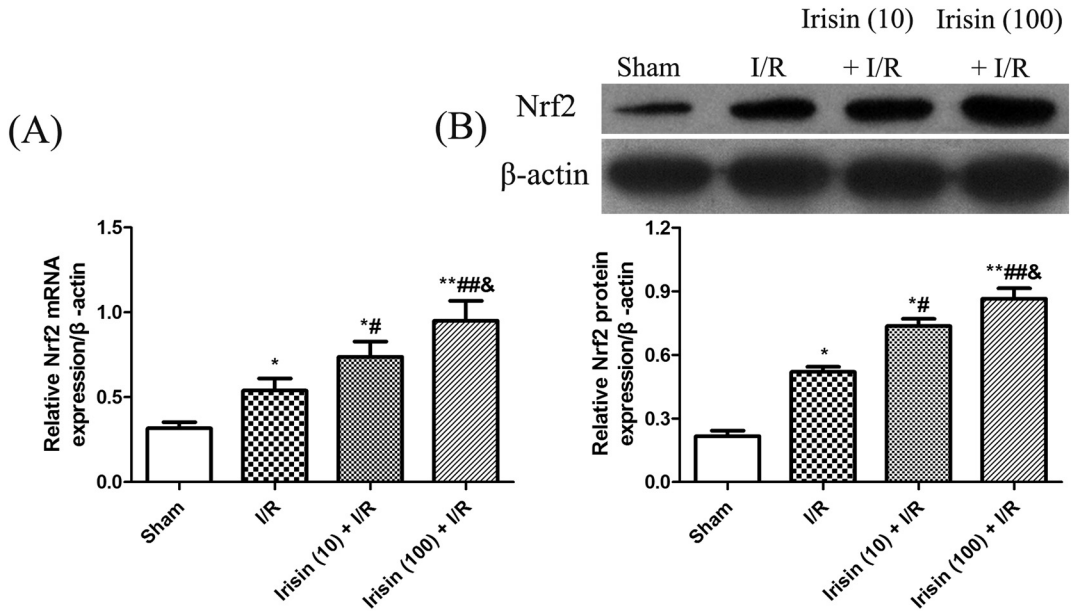

Fig. 5. Intestinal Nrf2 mRNA and protein expression levels assessed by qPCR (A) and Western blot (B). Compared with Sham group values, Nrf2 mRNA and protein levels in the I/R group were significantly increased. Meanwhile, Nrf2 mRNA and protein levels were significantly higher in the Irisin (10) + I/R and Irisin (100) + I/R groups compared with the I/R group. Data are expressed as mean ± SD (n = 8). $^{*}P < 0.05$ *vs.* Sham group;$^{**}P < 0.01$ *vs.*Sham group;$^{\#}P < 0.05$ *vs.*I/R group;$^{\#\#}P < 0.01$ *vs.*I/R group;$^{\&}P < 0.05$ *vs.* Irisin (10) + I/R group.

method was used for quantitative analysis of intestinal mucosal injury. The results showed normal intestinal mucosa morphology in the Sham group. However, severe mucosal injury, manifested by overt edema of intestinal villi, as well as intestinal wall vascular congestion and hemorrhage, were found in the I/R group. The severity of intestinal mucosal injury in the Irisin (10) + I/R and Irisin (100) + I/R groups was evidently lower compared with that of the I/R group. In agreement, Chiu scores in the Irisin groups were significantly lower than those of the I/R group. There were significant differences between the Irisin (10) + I/R group and Irisin (100) + I/R group ($P < 0.05$) (Fig. 1A–B).

3.1.2. Serum DAO activity

Compared with the Sham group, serum DAO activities in the I/R, Irisin (10) + I/R, and Irisin (100) + I/R groups were all significantly higher ($P < 0.05$). However, DAO activities in the Irisin groups were significantly lower than that of the I/R group ($P < 0.05$). DAO activities also differed significantly between the Irisin (10) + I/R group and Irisin (100) + I/R group ($P < 0.05$) (Fig. 1C).

3.1.3. Levels of inflammatory factors in intestinal tissues

Compared with Sham group values, the levels of TNF-α(A), IL-1β(B) and IL-6(C) in intestinal tissues were significantly higher in the I/R and both Irisin groups. Meanwhile, the levels of TNF-α, IL-1β and IL-6 in irisin groups were significantly lower than those of the I/R group ($P < 0.05$) (Fig. 2).

3.1.4. Levels of MDA, MPO, GSH-Px and SOD in intestinal tissues

Intestinal MDA, GSH-Px and SOD levels are excellent indexes reflecting lipid peroxidation and the antioxidant ability of tissues, while MPO activity is an inflammatory biomarker reflecting leukocyte infiltration. As shown in Fig. 3, the intestinal levels of MDA(A) and MPO (B) in the I/R group were significantly higher ($P < 0.05$), while SOD (C) and GSH-Px(D) activities were markedly lower ($P < 0.05$) compared with those of the Sham group. In addition, MDA and MPO levels in the Irisin (10) + I/R and Irisin (100) + I/R groups were significantly lower ($P < 0.05$), and GSH-Px and SOD activities were markedly higher ($P < 0.05$) compared with those of the I/R group. There were significant differences between the Irisin (10) + I/R group and Irisin (100) + I/R group ($P < 0.05$).

3.1.5. Apoptosis of intestinal cells

TUNEL staining and Western blot were used to assess intestinal cell apoptosis. Compared with the Sham group, the I/R group showed significantly higher apoptotic index in intestinal cells. In agreement, Bax and cleaved Caspase-3 protein levels in the I/R group were significantly higher, while Bcl-2 protein expression was significantly lower ($P < 0.05$) compared with Sham group values. In contrast, irisin pretreatment significantly reduced the apoptotic index, decreased cleaved Caspase-3 and Bax protein amounts, and increased Bcl-2 protein expression ($P < 0.05$). They also differed significantly between the Irisin (10) + I/R group and Irisin (100) + I/R group ($P < 0.05$) (Fig. 4A–F).

3.1.6. Expression levels of Nrf2 pathway effectors

Both qPCR and Western blot results showed that compared with the Sham group, I/R group had significantly higher Nrf2 mRNA and protein levels ($P < 0.05$). Meanwhile, Nrf2 mRNA and protein levels in the Irisin (10) + I/R and Irisin (100) + I/R groups were significantly higher than those of the I/R group ($P < 0.05$). There were significant differences between the Irisin (10) + I/R group and Irisin (100) + I/R group ($P < 0.05$)(Fig. 5A–B).

3.2. In vitro findings

3.2.1. Cell viability

The IEC-6 cell model of H/R was established *in vitro*, with cells pretreated with different concentrations of irisin for various times. CCK-8 assay showed that irisin pretreatment dose- and time- dependently ameliorated the effects of H/R in reducing cell viability ($P < 0.05$) (Fig. 6A–C).

3.2.2. Nrf2 levels in vitro

Western blot and qPCR showed that irisin dose-dependently increased Nrf2 protein and mRNA levels in IEC-6 cells after H/R stimulation, in agreement with *in vivo* findings ($P < 0.05$) (Fig. 6D–E).

Further study was performed to explore nrf2 whether or not mediates the protective effects of irisin, and prevents H/R induced cell

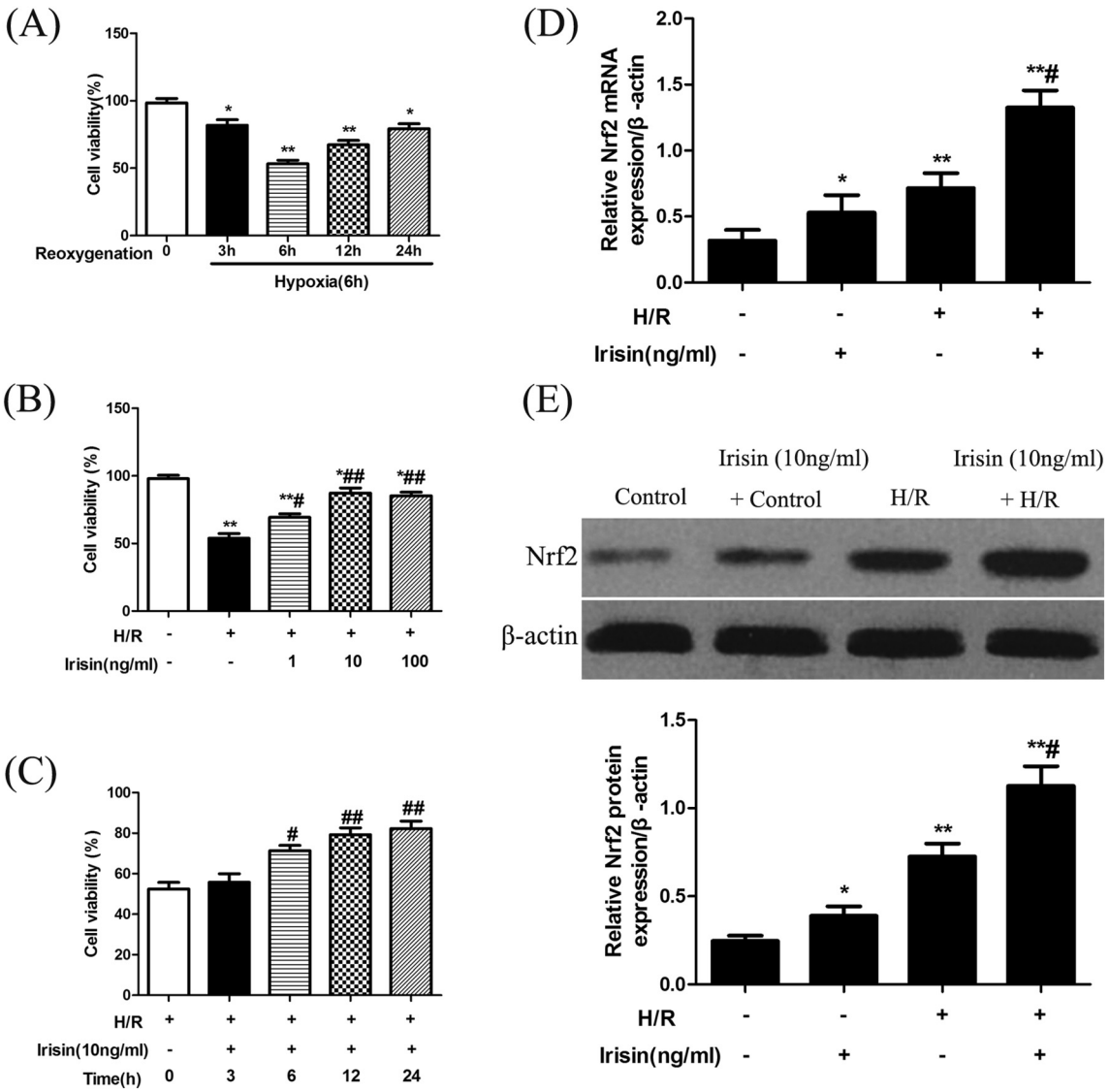

Fig. 6. Irisin improves IEC-6 cell survival and Nrf2 expression upon H/R stimulation *in vitro*. Cell viability was assessed by CCK-8 assay. (A) IEC-6 cells were exposed to a hypoxic environment for 6 h, and treated with reoxygenation for 0, 3, 6, 12 and 24 h, respectively. (B) IEC-6 cells were pretreated with irisin (1, 10 and 100 ng/mL, respectively) for 6 h, and subsequently exposed to hypoxia for 6 h and reoxygenation for 6 h. (C) IEC-6 cells were pretreated with irisin (10 ng/mL) for 0, 3, 6, 12 and 24 h, respectively, followed by H/R stimulation. (D-E) Nrf2 mRNA and protein levels in IEC-6 cells. Data are expressed as mean ± SD (n = 6). $^{*}P < 0.05$ *vs.* Control group;$^{**}P < 0.01$ *vs.* Control group;$^{\#}P < 0.05$ *vs.* H/R group;$^{\#\#}P < 0.01$ *vs.* H/R group.

apoptosis, inflammation and oxidative stress.

3.2.3. Cell apoptosis

TUNEL staining showed that compared with the control group, the H/R group had significantly increased apoptosis ($P < 0.05$). However, irisin treatment significantly reduced cell apoptosis levels, while pre-treatment with Nrf2 siRNA markedly decreased the protective effects of irisin on cell apoptosis ($P < 0.05$) (Fig. 7A–B). These findings showed that irisin exerted anti-apoptotic effects by increasing Nrf2 expression.

3.2.4. Cellular injury

Pretreatment with irisin effectively alleviated H/R induced cell viability decrease and LDH release. However, transfection with Nrf2 siRNA abrogated the protective effects of irisin($P < 0.05$) (Fig. 7C–D). These findings indicated that irisin could protect cells from H/R induced injury by increasing Nrf2 expression.

3.2.5. Inflammatory responses

H/R stimulation significantly increased the cellular levels of TNF-α, IL-1β and IL-6. Meanwhile, irisin pre-treatment ameliorated the increase of TNF-α, IL-1β and IL-6 induced by H/R stimulation. In contrast, Nrf2 siRNA transfection abrogated the anti-inflammatory effects of irisin($P < 0.05$)(Fig. 8A–C). These findings further demonstrated that irisin increased cellular Nrf2 expression, to exert anti-inflammatory effects.

3.2.6. Oxidative stress

MDA levels in cells were increased after H/R stimulation, while GSH-Px and SOD activities were decreased. Irisin pre-treatment ameliorated oxidative stress induced by H/R stimulation. However, Nrf2 siRNA reversed the antioxidant effects of irisin($P < 0.05$)(Fig. 8D–F). These findings demonstrated that Nrf2 mediated the protective effects of irisin on oxidative stress induced by H/R stimulation.

4. Discussion

This study adopted the traditional SMA ligation to induce *in vivo* I/R injury in mice and IEC-6 cells to establish an *in vitro* H/R model. The

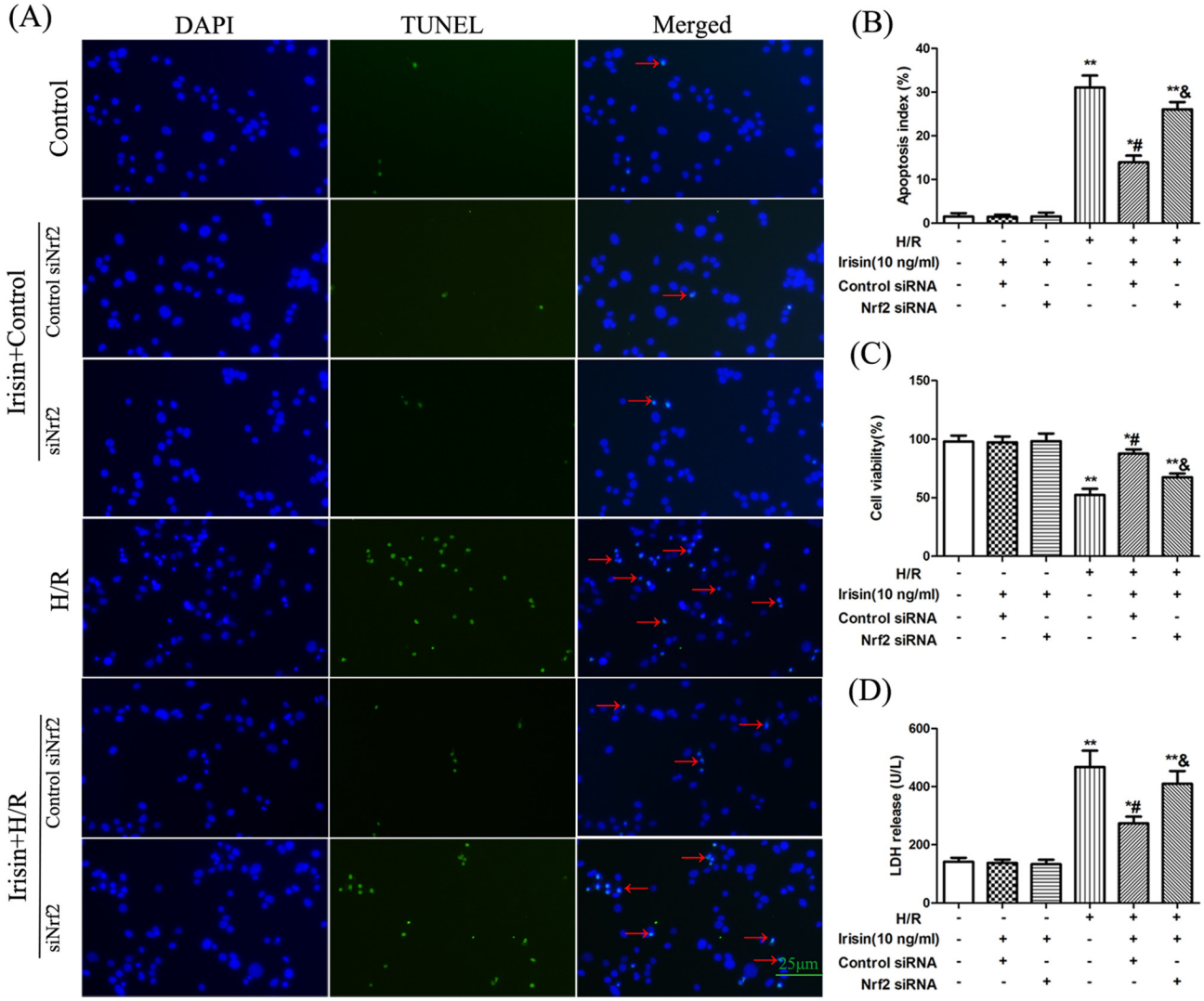

Fig. 7. Nrf2 activation protects IEC-6 cells from H/R induced cell damage. (A) Cell apoptosis assessed by the TUNEL method. IEC-6 cells were stained with TUNEL (green), and nuclei were stained with DAPI (blue). Colocalization (cyan; white arrows) demonstrated cell apoptosis (×400). (B)The quantitative analysis of apoptosis index among different groups.(C) Cell viability assessed by CCK-8 assay. (D)LDH release amounts. Data are expressed as mean ± SD (n = 6). $^{*}P < 0.05$ *vs.* Control group;$^{**}P < 0.01$ *vs.* Control group;$^{\#}P < 0.05$ *vs.* H/R group; $^{\&}P < 0.05$ *vs* H/R + Irisin + Control siNrf2 group. Scale bars = 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

results showed that intestinal I/R injury was accompanied with reduced intestinal mucosal integrity and increased apoptosis of intestinal epithelial cells. Irisin pretreatment upregulated Nrf2 in intestinal epithelial cells, ameliorated I/R induced changes of oxidative stress (including MDA, GSH-Px and SOD), and enhanced the expression of inflammatory factors (*e.g.* TNF-α, IL-1β, IL-6 and MPO), therefore exerting overt protective effects, in a dose- and time- dependent manner. Cell apoptosis is positively associated with disease progression in intestinal I/R injury. *In vivo* experiments confirmed that irisin could regulate apoptosis related proteins and reduce the apoptotic rate in intestinal epithelial cells, therefore protecting the intestine from injury. The *in vitro* study was in agreement with *in vivo* experiments. These findings suggest that irisin should have certain therapeutic effect in the treatment of intestinal I/R injury.

Intestinal I/R injury is a complex pathological damage with various factors involved. Early tissue ischemia could induce intestinal mucosal barrier dysfunction, while the subsequent reperfusion could lead to oxidative stress, cell apoptosis, and immune activation [1,5]. In addition, enterotoxin invasion could extensively activate systemic inflammatory responses, and finally lead to sepsis and MOF [35]. Recent studies have shown that energy failure, free radical induced damage, and cytokine production are the major mechanisms involved in intestinal mucosal injury [1,2,5]. Energy metabolism disturbance could affect the defensive functions and cell repair in the intestine, while insufficient oxygen could lead to electron transport failure in the mitochondria, which is the major source of oxygen radicals. Oxygen radicals play critical roles in the early stage of I/R injury [1]. Xanthine oxidase, which is abundant in the intestinal mucosa, catalyzes the generation of large amounts of oxygen radicals. Meanwhile, decreased SOD and catalase activities in tissues could also induce the aggregation of free radicals, therefore causing severe epithelial damage [36]. Previous studies have demonstrated that activating the Keap1/Nrf2 pathway alleviates intestinal I/R injury induced by oxidative stress [8,9]. Cytokines are also important mediators involved in signal transduction among cells upon I/R injury. Mutual promotion of oxidative stress and inflammatory mediators in the NF-κB pathway in monocytes/macrophages has been reported, and activation of this pathway induces the release of large amounts of pro-inflammatory mediators [4,37].

Irisin is a skeletal muscle-derived protein discovered in recent years. It belongs to the sarcosine family, and is closely associated with several diseases, including diabetes, atherosclerosis and obesity [22]. In recent

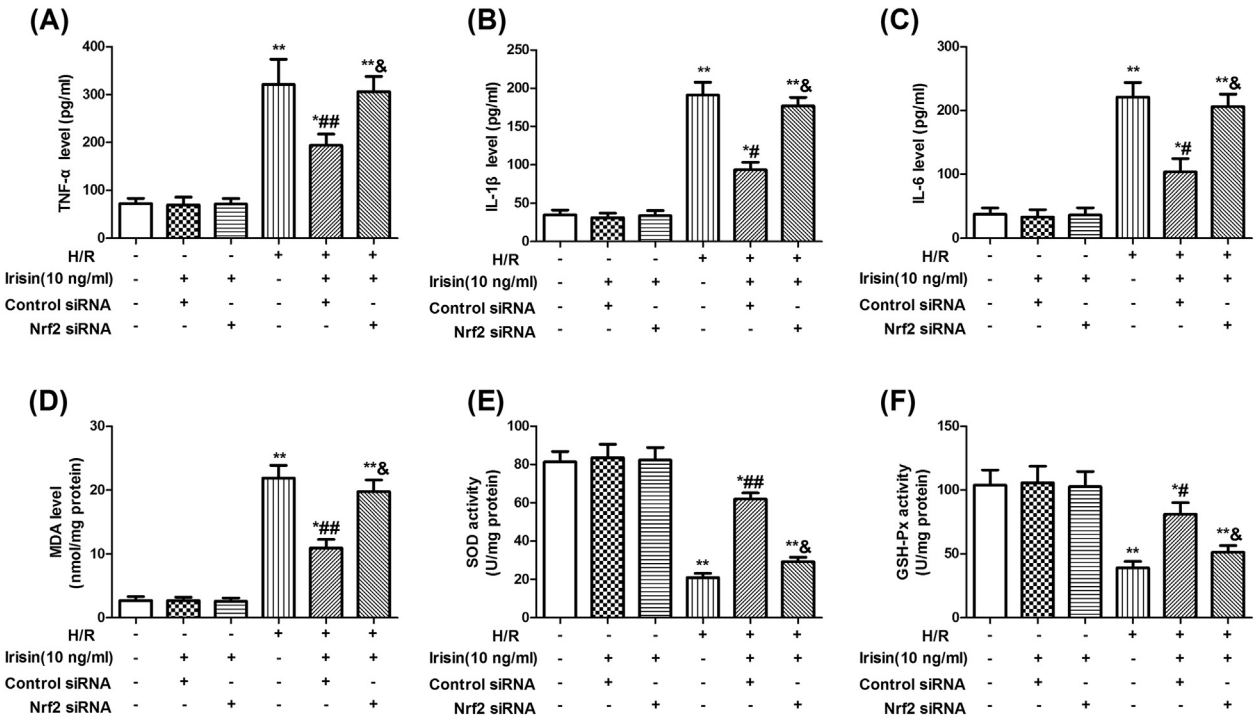

Fig. 8. Nrf2 activation protects IEC-6 cells from inflammatory responses and oxidative stress induced by H/R. (A-C) Levels of cellular inflammatory factors assessed by ELISA. (D-F) Oxidative stress levels in cells. Data are expressed as mean ± SD (n = 6). $^{*}P < 0.05$ *vs.* Control group; $^{**}P < 0.01$ *vs.* Control group; $^{\#}P < 0.05$ *vs.* H/R group; $^{\#\#}P < 0.01$ *vs.* H/R group; $^{\&}P < 0.05$ *vs* H/R + Irisin + Control siNrf2 group.

years, the indirect effects of irisin on ROS have been considered a hot research spot. Several studies have reported that elevated plasma irisin level is associated with alleviated oxidative stress in obese individuals [38] and pregnant women with gestational diabetes [39]. In addition, irisin also has protective effects on high-glucose induced damage in human umbilical vein endothelial cells, as well as COPD induced apoptosis of alveolar epithelial cells, whose mechanisms are associated with the potential of irisin in reducing oxidative stress in different biological environments [30]. In this study, we investigated whether irisin pretreatment could exert beneficial effects in intestinal I/R injury, and further demonstrated the role of the Nrf2 pathway in these protective effects.

Oxidative stress plays a key role in the development and progression of I/R injury. The level of free radicals increases sharply during reperfusion, which is accompanied by decreased activities of antioxidant enzymes. Aggregation of oxidation products could in turn induce severe damage to the body [1]. MDA is the product of lipid peroxidation, and its amounts could reflect the severity of peroxidation in the body. MPO is a reliable index reflecting neutrophil infiltration [40], while GSH-Px and SOD are important antioxidant factors [8,9]. In normal conditions, these three proteins are in a dynamic homeostatic state. The current findings showed that decompensated activation of oxidative stress occurs upon intestinal I/R injury, in which MDA and MPO levels are both increased evidently, while GSH-Px and SOD levels decrease significantly. Pre-treatment with irisin ameliorated the alterations of these three steps, as well as the expression of inflammatory factors, including TNF-α, IL-1β and IL-6, induced by leukocyte infiltration. *In vitro* experiments also showed that irisin significantly reduced H/R-induced inflammatory responses and oxidative stress. These findings demonstrated that irisin inhibits inflammatory responses, scavenges toxic free radicals, and regulates the activities of antioxidant enzymes, therefore exerting antioxidant effects.

Cell apoptosis involves various physiological and pathological mechanisms, and is positively associated with disease progression in I/R injury [1,4]. The Bcl-2/Bax ratio could determine cell apoptosis induction after stimulation, while cleaved Caspase-3 represents the key component amplifying the apoptosis cascade, and is activated at the early stage of cell apoptosis [9]. The current findings showed that compared with the I/R group, pretreatment with irisin evidently reduced the protein expression of cleaved Caspase-3, and increased the Bcl-2/Bax ratio. This suggested that irisin pretreatment upregulates the Bcl-2 protein and downregulates the Bax and cleaved Caspase-3 proteins, thus inhibiting apoptosis in intestinal epithelial cells and protecting the intestine.

The Nrf2/ARE signaling pathway is a critical endogenous antioxidant pathway, which regulates antioxidant enzymes at the transcriptional level [10]. Nrf2 exerts antioxidant, anti-inflammatory and anti-apoptotic effects, mainly by initiating the transcription of the downstream molecules [13,41]. The above results revealed that elevated Nrf2 expression was accompanied with decreased oxidative stress and cell apoptosis in the intestine, indicating that Nrf2 participates in the protective effects mediated by irisin pretreatment to a certain degree. To further demonstrate that irisin pretreatment exerted antioxidant and cell-protective effects in intestinal I/R injury *via* Nrf2, the Nrf2 gene was silenced by siRNA in IEC-6 cells in *in vitro* experiments. The results showed that the effects of irisin pretreatment in scavenging oxidative radicals, anti-inflammation, and anti-apoptosis were greatly attenuated, which further demonstrated that the protective effects of irisin on intestinal I/R injury were closely associated with Nrf2.

There were several limitations in the present study. Firstly, activation of the Nrf2 pathway effectively inhibited inflammation, oxidative stress, and apoptosis in intestinal I/R injury, which could be among the mechanisms by which irisin scavenges free radicals and reduces oxidation. However, the interlink between various signaling pathways associated with Nrf2 pathway remain to be determined. Secondly, the focus of the present study was on only the acute protection of irisin pretreatment in the intestines of mice subjected to I/R insult, while the chronic (long-term) impact of irisin are yet to prove. The optimal therapeutic windows of irisin for treatment required further exploration. Thirdly, irisin was not administered after I/R in the present study.

Interestingly, many previous studies have demonstrated that ischemic preconditioning(IPC) as well as other forms of preconditioning is a potent protective strategy against organic damages, including immunological and pharmacological preconditioning techniques [4,42–44]. The effective protection of irisin pretreatment has also been demonstrated [28,45]. Some researchers suggested that future studies should focus on the application of drugs to simulate the effect of intestinal IPC [1]. The effects of irisin post-treatment will be assessed in future studies. Finally, the present study is only a basic research, and the observed findings might be further detected by future studies in humans.

In summary, this study firstly investigated the protective effects of irisin pretreatment on intestinal I/R injury *in vivo* and *in vitro* studies. The findings demonstrated that irisin pretreatment ameliorates I/R-induced redox imbalance, inflammation and cell apoptosis in the intestine, processes in which activation of the Nrf2 pathway plays an important role. Irisin pretreatment has certain efficacy in treating intestinal I/R injury, and thus might provide a potential therapeutic alternative for this disease in clinical practice. However, more studies are needed to further investigate the effectiveness of irisin and the underlying mechanisms.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 81873930 and 81301055), and partly by the Research project of Health Department of Sichuan Province (No.130361 and No.140033).We would like to thank Dr. Ann Power Smith for critical reading and language revision of this manuscript.

Declaration of competing interest

The authors declare no conflicts of interest.

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