

Baicalein pre-treatment alleviates hepatic ischemia/reperfusion injury in mice by regulating the Nrf2/ARE pathway

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Abstract. Hepatic ischemia-reperfusion injury (HIRI) is caused by blood flow recovery following ischemia. Baicalein (BAI), a natural antioxidant used in traditional Chinese medicine, eliminates excessive free radicals and protects the structure of the cell membrane. However, its protective mechanism against HIRI is still unclear. The present study investigated underlying mechanism using a mouse HIRI model. Liver injury was evaluated using serum levels of alanine aminotransferase and aspartate aminotransferase, and hematoxylin-eosin staining was performed to evaluate the pathological changes in liver tissue. Apoptosis of hepatocytes was detected by TUNEL staining. The expression levels of reactive oxygen species (ROS), malondialdehyde (MDA) and superoxide dismutase (SOD) in the liver were detected to evaluate oxidative stress. Western blotting was performed to assess expression levels of nuclear factor E2-related factor 2 (Nrf2)/antioxidant response elements (ARE) pathway proteins in liver tissue. BAI pre-treatment significantly decreased elevation of serum aminotransferase levels induced by IR and alleviated histological damage to the liver. BAI decreased production of ROS and MDA in liver tissue induced by IR and increased the activity of SOD. At the same time, BAI inhibited apoptosis of liver cells induced by oxidative stress. Furthermore, BAI promoted the translocation of Nrf2 into the

nucleus and increased the expression of total heme oxygenase-1 and NAD(P)H dehydrogenase quinone-1. The Nrf2 inhibitor ML385 reversed the protective effect of BAI on HIRI. These results indicated that BAI served a protective effect in HIRI by regulating the Nrf2/ARE pathway.

Introduction

Hepatic ischemia-reperfusion injury (HIRI) occurs in hepatectomy, severe liver trauma surgery and liver transplantation (1). It results in postoperative liver dysfunction and failure (2). Reactive oxygen species (ROS) are one of the key factors influencing HIRI (3,4). ROS are continuously produced during hepatic ischemia-reperfusion and excessive levels ROS damage hepatocytes (5,6). In response to ROS damage, liver cells undergo inflammation and apoptosis (7). The liver forms a complex oxidative stress response system and upregulates expression levels of protective genes, such as heme oxygenase-1 (HO-1), to decrease injury to hepatocytes when exposed to ROS (8). Previous studies have shown that pre-treatment drugs, such as sulforaphane and baicalein (BAI), can also regulate the antioxidant pathway in the liver (9,10).

BAI is one of the most abundant flavonoids and the primary active component of *Scutellaria baicalensis* (11,12). Previous studies have confirmed that BAI has anti-bacterial, ROS scavenging, anti-inflammatory and anti-tumor effects (13-16). Moreover, accumulated data have shown that BAI protects against several types of liver disease, such as alcoholic liver disease (17), non-alcoholic fatty liver disease (18,19), chemical-induced liver fibrosis (20) and immunological liver injury (21). Although certain studies by Liu *et al* (22,23) addressed the beneficial effects of BAI on HIRI, no *in vivo* study has determined the mechanism underlying the protective effects of BAI on HIRI via the nuclear factor E2-related factor 2 (Nrf2)/antioxidant response element (ARE) pathway.

Nrf2 has been found to regulate antioxidant proteins by interacting with ARE, which is a key endogenous antioxidant stress pathway (9). There are >200 coding endogenous protective genes regulated by the Nrf2/ARE signaling pathway (1). These protective genes include antioxidant and phase II

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detoxification enzymes, such as catalase, HO-1, superoxide dismutase (SOD), and NAD(P)H dehydrogenase quinone (NQO)-1 and -2 (24-26). According to previous studies, the Nrf2/ARE pathway serves a key role in protection against HIRI in mice (8,27,28). However, whether the Nrf2/ARE pathway also contributes to the protection provided by BAI against HIRI remains unclear. The present study investigated the role of the Nrf2/ARE pathway in the protection effect provided by BAI against HIRI and its underlying mechanism.

Materials and methods

Animals. A total of 72 male C57BL/6 mice (age 6-8 weeks; body weight, 19-21 g) were obtained from the Animal Center of Guangxi Medical University (Nanning, China). Three mice were housed per individual standard cages under controlled conditions (22±1°C, 60±10% relative humidity and 12 h light/dark cycles), with free access to water and food. BAI was obtained from Yuanye Bio (cat. no. S25956), with a >98% purity (Fig. 1A). Nrf2 inhibitor ML385 (cat. no. SML1833) was obtained from Sigma-Aldrich (Merck KGaA). Mice were randomly assigned into different groups (n=6/group) as follows: i) Sham, following intraperitoneal injection of DMSO, the abdominal cavity was opened and closed without IR; ii) BAI (100 mg/kg), following intraperitoneal injection of BAI (100 mg/kg; dissolved in DMSO), the abdominal cavity was opened and closed without IR; iii) IR, DMSO was injected intraperitoneally before IR; iv) BAI (10 mg/kg) + IR, BAI (10 mg/kg) was injected intraperitoneally before IR; v) BAI (50 mg/kg) + IR, BAI (50 mg/kg) was injected intraperitoneally before IR; vi) BAI (100 mg/kg) + IR, BAI (100 mg/kg) was injected intraperitoneally before IR; vii) ML385 (30 mg/kg) + IR, ML385 (30 mg/kg) was injected intraperitoneally before IR; and viii) ML385 (30 mg/kg) + BAI (100 mg/kg) + IR, ML385 (30 mg/kg) and BAI (100 mg/kg) were injected intraperitoneally before IR. The IR and BAI (100 mg/kg) + IR groups were further divided into reperfusion subgroups, with 6 mice per subgroup, (1, 6 and 24 h). Mice were euthanized at 1, 6 and 24 h post-HIRI. All animals were treated humanely and all animal experiments were approved by the Animal Experimental Ethics Committee of the First Affiliated Hospital of Guangxi Medical University (Nanning, China).

HIRI model. General anesthesia was induced in all mice by continuous spontaneous inhalation of 2-5% volatile anesthetic isoflurane via a mask. A mouse model of HIRI was established as described by Tsung *et al* (29). The midline incision of the upper abdomen was performed to separate and expose the hepatic portal. The branches of the left and middle portal veins and hepatic arteries were clipped using a non-invasive vascular clamp, resulting in 70% warm ischemia. The color of the liver lobe in the blocked area changed from bright red to pale white, indicating successful induction of hepatic ischemia. After 60 min, the clamp was released and blood flow was restored. In the Sham and BAI (100 mg/kg) groups, only the liver was exposed and blood flow was not blocked. After 1, 6 and 24 h reperfusion, mice were anesthetized via inhalation of 3% isoflurane for 3-5 min and 0.8-1 ml blood samples from the inferior vena cava and liver tissue were collected. Following blood collection, all mice were euthanized in a

closed chamber via inhalation of 5% volatile isoflurane for 10 min. Mouse death was confirmed by cessation of heartbeat and nerve reflex. The procedure is shown in Fig. 1B.

Liver function assessment. Blood samples were stored at room temperature (RT) for 1 h and then were centrifuged at 1,413 x g, 4°C for 15 min. Serum was collected and stored at -80°C before testing. The levels of alanine aminotransferase (ALT) (cat. no. C001-e; Changchun Huili Biotech Co., Ltd.) and aspartate aminotransferase (AST) (cat. no. C002-e, Changchun Huili Biotech Co., Ltd.) were determined using a 7180 Biochemical Analyzer (Hitachi, Ltd.).

Histopathological observation and evaluation of liver injury. Ischemic liver lobes were fixed in 10% formalin at RT for 24 h and embedded in paraffin. Then, 5 µm thick paraffin slices were dewaxed with xylene twice (10 min each), fully hydrated with ethanol twice (5 min each) and rinsed with tap water. Sections were stained with hematoxylin solution (kit cat. no. C0105S; Beyotime Institute of Biotechnology) at RT for 3-5 min, rinsed with tap water and treated with 1% hematoxylin differentiation solution at RT for 6-25 sec. Sections were treated with 0.6% ammonia water solution for bluing at RT for 90 min, rinsed with tap water and immersed in 85 and 95% ethanol at RT for 5 min each. Sections were stained with 0.5% eosin dye (kit cat. no. C0105S; Beyotime Institute of Biotechnology) at RT for 5 min. The sections were then dehydrated and hyalinized in xylene before being sealed in neutral gum. Liver damage was determined under a light microscope (Olympus Corporation) (magnification, x400). The degree of liver injury was evaluated by two independent pathologists blinded to the experimental groups according to the classification standard described by Suzuki *et al* (30). This Suzuki grading standard consists of five grades (0-4) according to vacuolization of hepatocyte cytoplasm, necrosis and tissue congestion.

TUNEL detection of apoptosis. TUNEL was used to detect hepatocyte apoptosis (Colorimetric TUNEL Apoptosis Assay kit; cat. no. C1098; Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Ischemic liver lobes were fixed in 10% formalin at RT for 24 h and embedded in paraffin. The 5 µm thick paraffin slices were dewaxed with xylene twice for 10 min each, hydrated with ethanol twice for 5 min each, followed by dehydration using 95, 90, 80 and 70% ethanol (5 min each). Proteinase K working solution was added at 37°C for 20 min. Sections were immersed in 3% H₂O₂ at RT for 20 min, then washed three times with PBS (pH 7.4) in a Rocker device. A total of 50 µl TUNEL reaction mixture was added at 37°C for 1 h. Streptavidin-horseradish peroxidase and Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) were mixed at a ratio of 1:200, added to cover the tissue and incubated at 37°C for 30 min. The slides were placed in PBS (pH 7.4) and washed with shaking on a decolorizing shaker three times (5 min each). Then, diaminobenzidine coloring solution (0.2 ml) provided in the kit was added to marked tissue at RT for 5 min. Sections were counterstained with hematoxylin staining solution at RT for 1 min and washed in water, dried and sealed with neutral resin (cat. no. G8590; Beijing Solarbio Science & Technology Co., Ltd.). TUNEL-positive

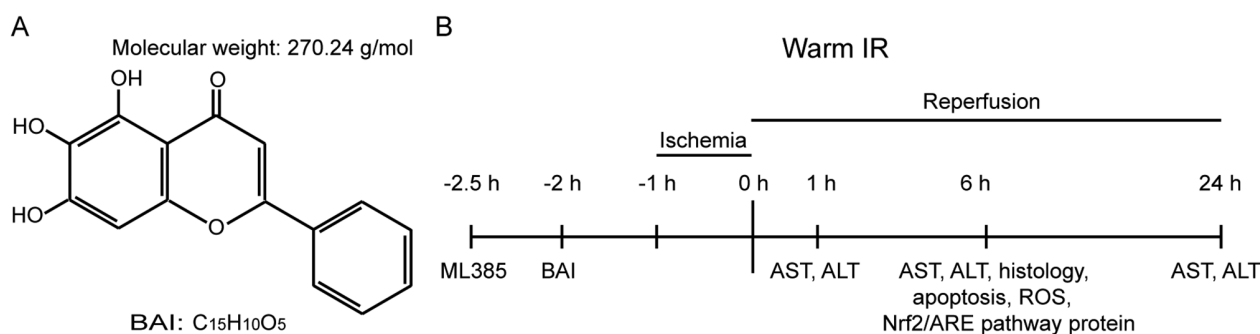


Figure 1. Structure of BAI and schematic diagram of the experimental protocol. (A) Chemical structure of BAI. (B) Experimental model design. BAI, baicalein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ROS, reactive oxygen species; Nrf2, nuclear factor E2-related factor 2; ARE, antioxidant response elements; IR, ischemia/reperfusion.

hepatocytes (brown-yellow nucleus) were counted in >3 high power histological fields under a light microscope (Olympus Corporation; magnification, x400) and the percentage was calculated.

Oxidative stress evaluation. Ischemic liver tissue was perfused with normal saline three times at 4°C for 3 min and ground into 10% homogenate. The homogenate was centrifuged at 1,413 x g and 4°C for 10 min. The total protein concentration of the supernatant was determined via BCA method. The content of SOD (cat. no. S0103; Beyotime Institute of Biotechnology) and malondialdehyde (MDA; cat. no. S0131S; Beyotime Institute of Biotechnology) in liver tissue homogenate from each group was determined according to the manufacturer's instructions. Frozen sections (10 μm) were prepared from fresh liver tissue and the ROS content was determined by a fluorescent dye dihydroethidium (DHE) (cat. no. D7008; Sigma-Aldrich; Merck KGaA). Tissue at RT was marked liquid blocker pen. Spontaneous fluorescence quenching reagent was added at RT for 5 min before washing in running tap water for 10 min. ROS staining solution (DHE 10 μmol/l) was added to the tissue area at 37°C for 30 min in the dark. Sections were washed three times with PBS (pH 7.4) in a Rocker device (5 min each), incubated with DAPI solution (cat. no. G1012; Wuhan Servicebio Technology Co., Ltd.) at RT for 10 min in the dark and washed three times with PBS (pH 7.4) in a Rocker device (5 min each). Samples were mounted with anti-fade mounting medium and images were captured using a fluorescence microscope (magnification, x400). DAPI glows blue under UV at excitation wavelength 330–380 nm and emission wavelength 420 nm; DHE itself displays blue fluorescence (absorption/emission, 355/420 nm) in cell cytoplasm while the oxidized form ethidium displays red fluorescence (absorption/emission, 510/590 nm) upon DNA intercalation. Nuclei appear blue by labeling with DAPI. ROS-positive cells were labelled by fluorescein are red.

Western blotting. Total protein of HO-1 and NQO-1 from each group was extracted using RIPA lysis buffer (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.). The level of Nrf2 nucleoprotein was measured in nuclear lysate by a nuclear protein extraction kit (cat. no. R0050; Beijing Solarbio Science & Technology Co., Ltd.). The protein concentration was determined using a BCA kit (cat. no. P0010S; Beyotime

Institute of Biotechnology). A total of 20 μg/lane protein samples were separated using 10% SDS-PAGE and transferred to PVDF membranes (cat. no. IPVH00010; Merck KGaA). Membranes were blocked with 5% skimmed milk at RT for 1 h. The membranes were incubated with primary antibodies at 4°C overnight. The primary antibodies were as follows: Anti-Nrf2 (1:1,000; cat. no. T55136; Abmart Pharmaceutical Technology Co., Ltd.), anti-HO-1 (1:1,000; cat. no. PY5393; Abmart Pharmaceutical Technology Co., Ltd.), anti-NQO-1 (1:1,000; cat. no. T56710; Abmart Pharmaceutical Technology Co., Ltd.), anti-Histone H3 (1:1,000; cat. no. T56587; Abmart Pharmaceutical Technology Co., Ltd.) and anti-GAPDH (1:20,000; cat. no. 10494-1-AP; ProteinTech Group, Inc.). Membranes were then washed three times in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) and incubated with horseradish-conjugated goat-anti-rabbit secondary antibody (1:10,000; cat. no. SA00001-2; ProteinTech Group, Inc.) at RT for 1 h. Membranes were washed again. ECL reagent (cat. no. P0018FM, Beyotime Institute of Biotechnology) was used to visualize the bands. Bands were detected with a GeneSys System (Bio Rad Laboratories, Inc.). Band intensities were measured using Image J v1.8.0 software (National Institutes of Health).

Statistical analysis. SPSS 13.0 (SPSS, Inc.) and GraphPad Prism 5.0 software (GraphPad Software, Inc.) were used for statistical analysis and presentation of the data. Shapiro-Wilk test confirmed that data were normally distributed. The data are presented as mean ± SD of at least three replicates. One- or two-way ANOVA followed post hoc Tukey's or Holm-Sidak test, respectively, were performed for multiple group comparisons. Two-sided P<0.05 was considered to indicate a statistically significant difference.

Results

BAI ameliorates liver injury induced by IR. The activity of ALT and AST in serum and histological examination were used to assess liver injury. Levels of AST and ALT in the IR group were significantly increased, indicating impaired liver function (Fig. 2A and B). Serum ALT and AST levels were significantly decreased by pre-treatment with medium- and high-dose BAI (50 and 100 mg/kg) before IR, which indicated that BAI exerted a significant protective effect on liver func-

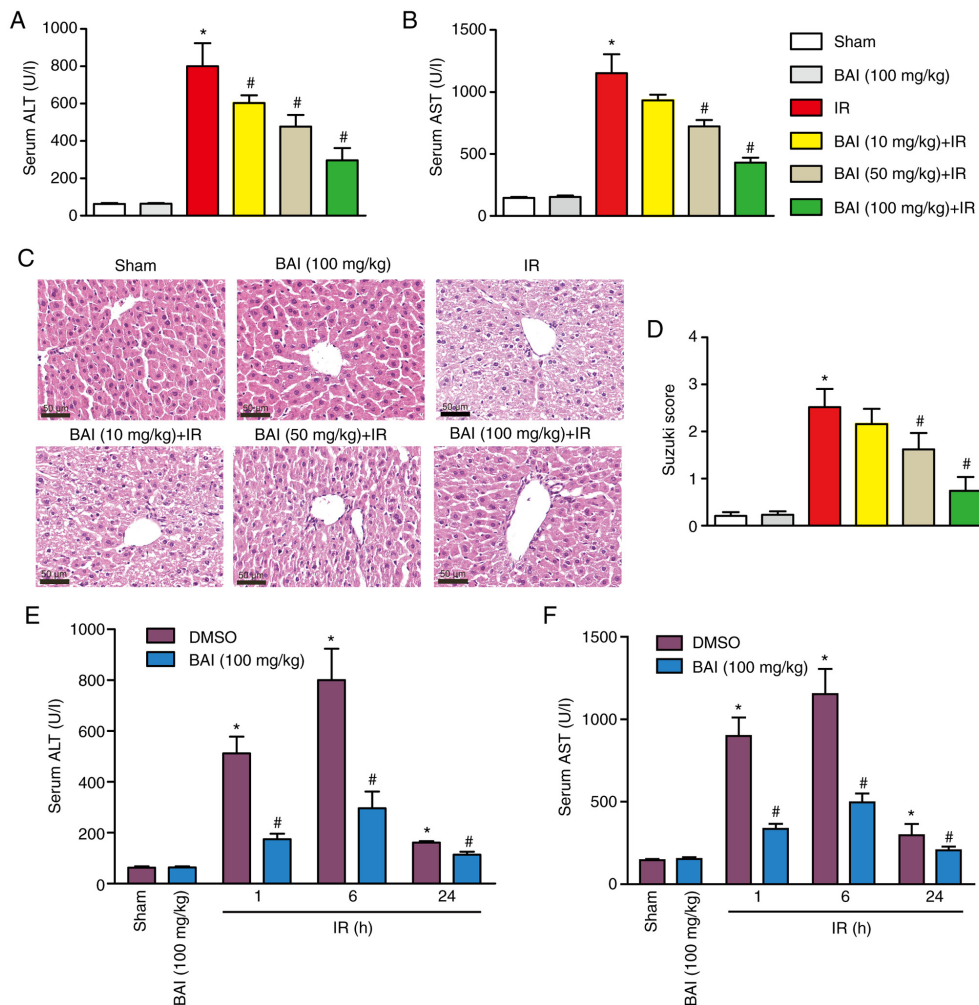


Figure 2. BAI ameliorates liver damage induced by IR. Mice were pre-treated with either vehicle (DMSO) or BAI at 10, 50 or 100 mg/kg. Serum levels of (A) ALT and (B) AST were assessed and (C) Hematoxylin and eosin staining was performed 6 h after hepatic IR. Scale bar=50 μ m. (D) Suzuki score. * P <0.05 vs. Sham; # P <0.05 vs. IR. The effect of BAI on serum (E) ALT and (F) AST levels in mice subjected to Sham or ischemia treatment followed by reperfusion. * P <0.05 vs. Sham; # P <0.05 vs. DMSO at the same time point. BAI, baicalein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; IR, ischemia/reperfusion.

tion. Consistent with the alterations in serum ALT and AST levels, compared with the Sham and BAI (100 mg/kg) group, a larger necrotic area was observed in the IR group by HE staining (Fig. 2C). Compared with the I/R group, pre-treatment with medium- and high-dose BAI (50 and 100 mg/kg) before IR significantly decreased liver necrosis. Pathological results were verified by Suzuki score (Fig. 2D). Suzuki scores indicated that the average injury level in the I/R group was significantly higher than that in the Sham and BAI (100 mg/kg) groups. However, compared with the I/R group, the BAI (50 mg/kg) + IR and BAI (100 mg/kg) + IR groups showed significant alleviation of average injury levels, while there was no significant difference between injury levels in the I/R and BAI (10 mg/kg) + IR groups. Following reperfusion (1, 6 and 24 h), pre-treatment with BAI significantly decreased the levels of ALT and AST (Fig. 2E and F) compared with the corresponding IR group. These results suggested that BAI protected the liver from HIRI.

BAI decreases oxidative stress and hepatic apoptosis following HIRI. The aforementioned results indicated that medium- and high dose BAI (50 and 100 mg/kg) had protec-

tive effects on HIRI and 100 mg/kg had a better protective effect. The levels of ALT and AST peaked at 6 h after IR; therefore, this time point was selected for subsequent experiments. To determine the protective effect of 100 mg/kg BAI, both blood and liver tissue samples from the Sham, BAI (100 mg/kg), IR and BAI (100 mg/kg) + IR groups were used for further study. To assess the effect of BAI on oxidative damage to the liver, liver tissue was stained with DHE probe to detect liver ROS content. Compared with the Sham and BAI (100 mg/kg) groups, IR liver tissue samples showed notably higher expression levels of intracellular ROS (Fig. 3A). Pre-treatment with BAI suppressed ROS levels. Pre-treatment with BAI (100 mg/kg) before IR significantly decreased liver cell apoptosis compared with the IR group (Fig. 3B and C). Moreover, compared with the Sham and BAI (100 mg/kg) groups, the MDA content in liver tissue of the IR group was significantly increased; this was downregulated significantly by BAI (100 mg/kg) pre-treatment (Fig. 3D). By contrast to MDA, the activity of SOD in liver tissue was decreased significantly in the IR group compared with the Sham group (Fig. 3E). Pre-treatment with BAI increased the activity of SOD in liver tissue independently of IR.

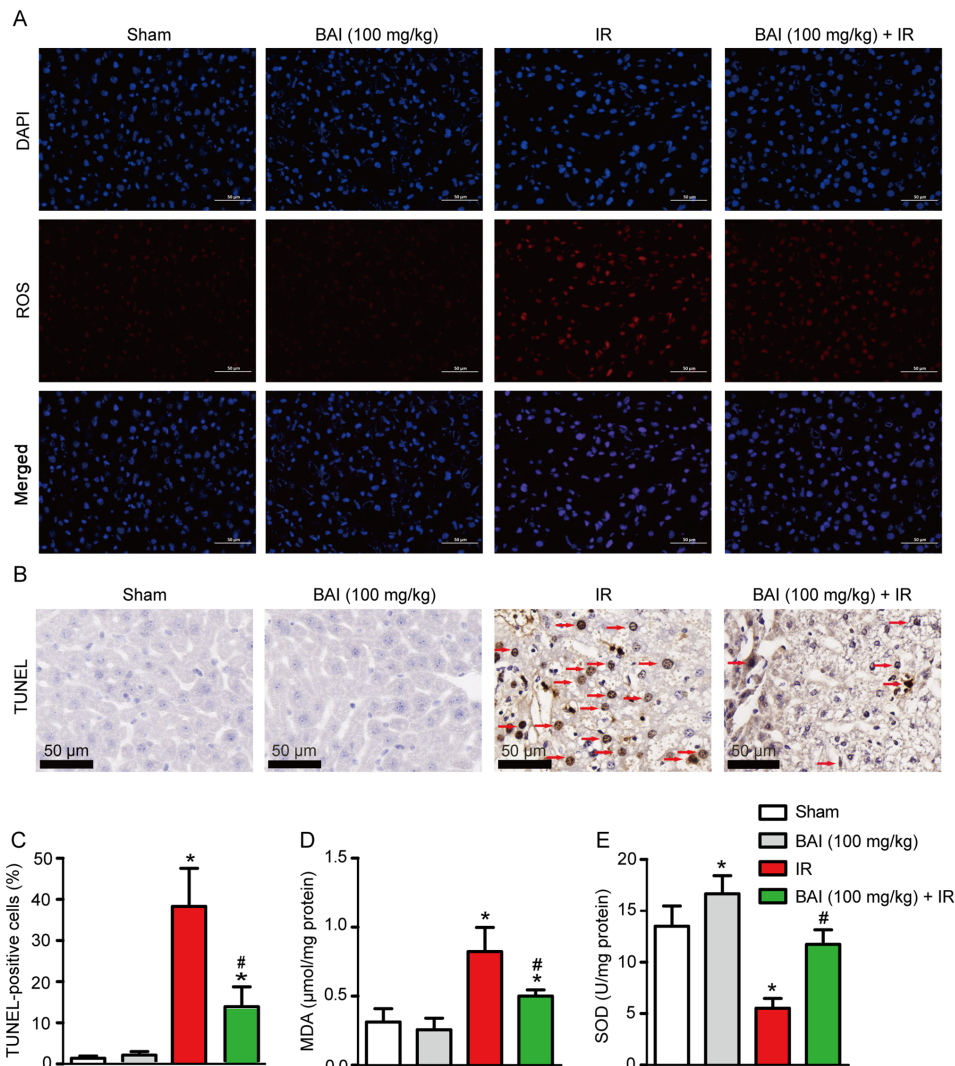


Figure 3. BAI decreases oxidative stress and hepatic apoptosis following I/R. Samples were collected 6 h after liver reperfusion in the I/R and BAI (100 mg/kg) + IR groups. (A) ROS (red) in liver tissue. (B) Representative images of TUNEL staining. Arrows indicate positive cells. Scale bar=50 μm. (C) Percentage of TUNEL-positive hepatocytes. (D) MDA content and (E) SOD activity were measured in liver tissue. *P<0.05 vs. Sham; #P<0.05 vs. IR. BAI, baicalein; IR, ischemia/reperfusion; ROS, reactive oxygen species; MDA, malonaldehyde; SOD, superoxide dismutase.

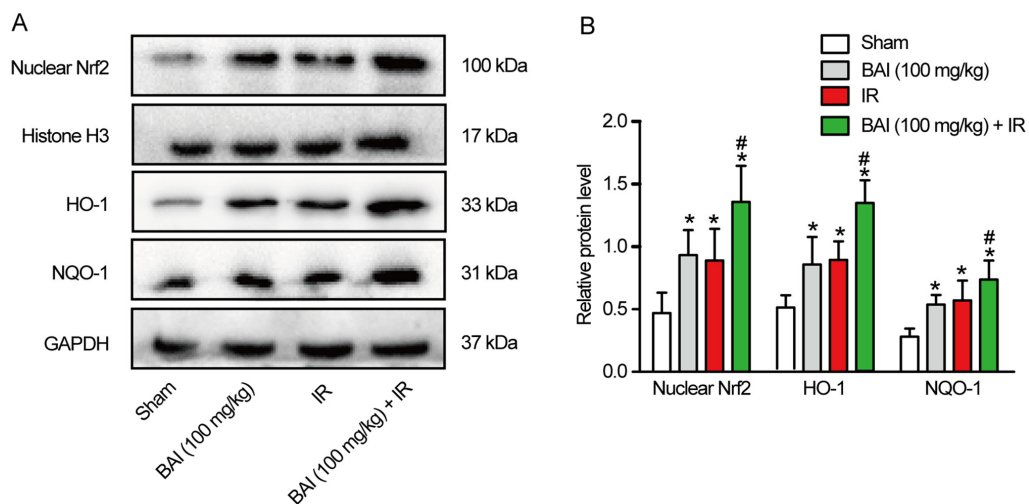


Figure 4. BAI upregulates Nrf2/ARE pathway protein expression in the liver. Samples were collected 6 h after liver reperfusion in the IR and BAI (100 mg/kg) + IR groups. (A) Levels of Nrf2 nucleoprotein, total HO-1 and NQO-1 protein were analyzed by western blotting. Histone H3 and GAPDH were used as the internal controls. (B) Nrf2 nucleoprotein relative to histone H3 and total HO-1 and NQO-1 protein relative to GAPDH. *P<0.05 vs. Sham. #P<0.05 vs. IR. BAI, baicalein; IR, ischemia/reperfusion; Nrf2, nuclear factor E2-related factor 2; ARE, antioxidant response elements; HO-1, heme oxygenase-1; NQO-1, NAD(P)H dehydrogenase quinone-1.

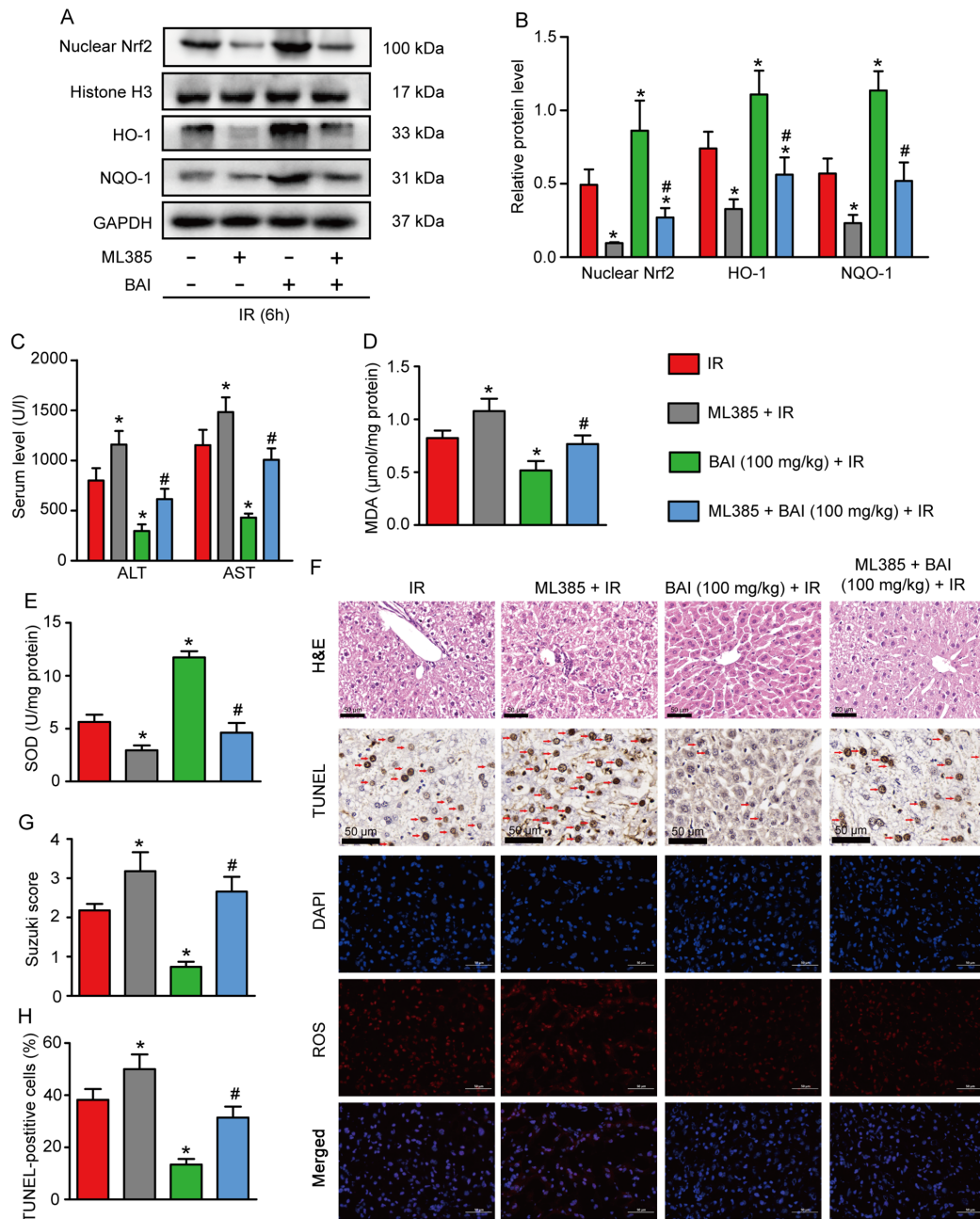


Figure 5. Nrf2/ARE pathway inhibitor reverse the protective effect of BAI on hepatic IR injury. Samples were collected 6 h after liver perfusion in all groups. (A) Levels of Nrf2 nucleoprotein, total HO-1, and NQO-1 protein were analyzed by western blotting. GAPDH and histone H3 were used as the internal controls. (B) Nrf2 nucleoprotein expression relative to histone H3 and HO-1 and NQO-1 protein expression relative to GAPDH. (C) Serum ALT and AST levels. (D) MDA content and (E) SOD activity were measured in liver tissue. (F) Pathological damage in the liver was measured by HE and TUNEL staining and ROS activity was measured by fluorescent-labeled DHE staining. Apoptotic cells are indicated by red arrows. Scale bar=50 μm. (G) Suzuki score. (H) Percentage of TUNEL-positive hepatocytes. *P<0.05 vs. IR; #P<0.05 vs. IR + BAI 100 mg/kg. BAI, baicalein; IR, ischemia/reperfusion; Nrf2, nuclear factor E2-related factor 2; ARE, antioxidant response elements; HO1, heme oxygenase-1; NQO-1, NAD(P)H dehydrogenase quinone-1; MDA, malonaldehyde; SOD, superoxide dismutase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HE, hematoxylin and eosin; ROS, reactive oxygen species.

BAI upregulates Nrf2/ARE pathway protein expression levels in liver. Western blotting was used to detect the expression levels of Nrf2/ARE pathway-associated protein. Compared with the Sham group, the expression levels of Nrf2 nucleoprotein and total HO-1 and NQO-1 protein increased significantly in the IR and BAI (100 mg/kg) groups (Fig. 4A and B). Compared with the IR group, the expression levels of Nrf2 nucleoprotein and total HO-1 and NQO-1 protein in the BAI (100 mg/kg) + IR group were further increased following pre-treatment with BAI. However, there was no significant

difference in the expression levels of Nrf2 nucleoprotein and total HO-1 and NQO-1 protein between the IR and BAI (100 mg/kg) groups.

Nrf2/ARE pathway inhibitor reverses the protective effect of BAI on HIRI. To confirm the role of the Nrf2/ARE pathway in BAI-treated mice following HIRI, the Nrf2 inhibitor ML385 was used. Compared with the IR group, the levels of Nrf2 nucleoprotein and total HO-1 and NQO-1 protein in the ML385 + IR group decreased significantly (Fig. 5A and B). Moreover, compared with

the BAI 100 mg/kg+ IR group, the expression of Nrf2 nucleoprotein and total HO-1 and NQO-1 protein in the ML385 + BAI (100 mg/kg) + IR group was significantly decreased. ML385 also aggravated liver injury (Fig. 5C, F and G), hepatocyte apoptosis (Fig. 5F and H) and oxidative stress injury (Fig. 5D-F). These data suggested that the Nrf2/ARE pathway served a significant role in the protective effect of BAI against HIRI.

Discussion

BAI is the most abundant flavonoid in *S. baicalensis* and a key component of traditional Chinese medicine (31). Previous studies have shown that BAI attenuates HIRI via inhibition of the NF- κ B pathway and induction of autophagy (22,23). Here, BAI pre-treatment significantly attenuated HIRI. BAI significantly decreased the elevated levels of AST and ALT and ameliorated the necrotic area of hepatocytes induced by HIRI. Additionally, pre-treatment with BAI inhibited oxidative damage and hepatocyte apoptosis. Pre-treatment with BAI promoted activation of the Nrf2/ARE pathway and alleviated liver injury during HIR, while Nrf2 inhibitor ML385 partially reversed the protective effect of BAI on HIRI. The present data indicated that BAI served a protective role in HIRI by regulating the Nrf2/ARE pathway to alleviate oxidative damage.

Multiple factors are involved in the occurrence and development of HIRI, such as oxygen free radical production, cell apoptosis, inflammatory reaction and calcium overload (32,33). Oxidative stress induced by large amounts of ROS during reperfusion not only serves an important role in the early stage of hepatocyte injury but also participates in the later stage of inflammation (1). Excessive ROS beyond the clearance capacity of the liver damage lipids and proteins in cells, leading to cell death (34). The present results showed that the levels of ALT and AST in serum were increased following HIRI. Histological examination revealed that HIRI resulted in hepatocyte apoptosis, congestion and necrosis. This damage may be due to the formation of lipid peroxides by combination of excessive ROS and lipids in the cell membrane, resulting in destruction of the hepatocyte membrane (35,36). The end product of lipid peroxides is MDA, which reflects the degree of cell peroxidation (37). SOD scavenges free radicals, which decreases oxidative damage caused by IR (38). BAI exhibits a well-known antioxidant capacity (39). Dong *et al* (10) found that BAI alleviates liver oxidative damage induced by high-level glucose. Pre-treatment with BAI increased the activity of SOD and decreased levels of ROS and MDA in liver tissue. Furthermore, pre-treatment with BAI significantly decreased the elevated levels of AST and ALT and ameliorated the necrotic area of hepatocytes induced by HIRI. These results showed that the protective effect of BAI against HIRI was mediated by alleviating oxidative damage to the liver.

The underlying mechanism of HIRI is complicated and not fully understood. Our previous study showed that [D-Ala2, D-Leu5]-Enkephalin significantly inhibits HIR-induced oxidative stress by activation of the Nrf2/HO-1 pathway (27). Numerous endogenous antioxidant protection genes, such as SOD (25) and phase II detoxification enzymes, such as HO-1, are regulated by the Nrf2/ARE pathway (40). HO-1 and NQO-1 are two widely studied phase II enzymes regulated by Nrf2 that serve an important role in antioxidation (38,41). Qin *et al* revealed that some

hepatic metabolic enzyme genes of the Nrf2/ARE pathway, such as NQO1 and HO-1, were activated in BAI-treated hepatocytes (42). Shi *et al* also demonstrated that pre-treatment with BAI activates the Nrf2/ARE pathway to alleviate acetaminophen-induced oxidative damage (43). Kim *et al* (44) reported that baicalin alleviates liver IR injury by upregulating HO-1 expression. At the same time, baicalin serves an anti-inflammatory and antiapoptotic role by inhibiting activation of NF- κ B and caspases 3 and 8 (44). Baicalin and BAI are primary flavonoids extracted from the dry roots of *S. baicalensis* (45). The molecular formula of BAI is $C_{15}H_{10}O_5$, while that of baicalin is $C_{21}H_{18}O_{11}$. The two molecules have similar pharmacological effects, such as anti-inflammatory, antioxidant and antiapoptotic effects. Qin *et al* (31) showed that BAI positively regulates the Nrf2/Kelch-like ECH-associated protein 1 (Keap1) pathway via Keap1-independent and -dependent pathways. In accordance with previous studies (9,27), HIR lead to an increase in expression levels of Nrf2 nucleoprotein and total HO-1 and NQO-1 protein, which indicated that the antioxidant pathway was activated when the liver was subjected to IR. Moreover, pre-treatment with BAI further increased the expression levels of Nrf2 nucleoprotein and downstream antioxidative enzymes, such as SOD, HO-1 and NQO-1. These results indicated that pre-treatment with BAI promoted Nrf2 nuclear translocation and increased the antioxidant capacity of the liver. The protective role of BAI in HIRI was disrupted by the Nrf2 inhibitor ML385, which supported the key role of Nrf2/ARE in BAI-provided protection against HIRI.

Several *S. baicalensis*-derived mixtures or pure compounds have been approved as clinical therapeutic drugs in China; for example, BAI capsules are used to treat hepatitis (46). Hepatitis patients often develop liver cancer, and these patients with liver cancer and hepatitis often need extensive hepatectomy (47). HIRI is a common pathological process occurring in extensive hepatectomy (1). Therefore, it is worth studying whether BAI can be used before surgery in patients with hepatitis related liver cancer to decrease ischemia-reperfusion injury, as it is not only able to decrease ischemia-reperfusion injury, but also to decrease the adverse effect of hepatitis on the perioperative period. Nevertheless, there are certain limitations to the present study. The role of Keap1, an endogenous inhibitor of Nrf2, was not investigated. The dose selection of BAI was based on previous research (22). The maximum dose of BAI was 100 mg/kg, which exhibited the most notable protective effect. Whether a higher BAI dose has a better protective effect requires further study. Based on the pharmacokinetics of BAI *in vivo*, the time and frequency of preoperative BAI should also be investigated in future.

The present findings showed that alleviation of oxidative stress by regulating the Nrf2/ARE pathway contributed to the protective effect of BAI against HIRI and BAI may be a promising therapeutic drug for the management of HIRI.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SH, GY and YZh designed the study. YZh, ZT, YZe, SC and GH performed the experiments. HH, YZe, JW, YZh, ZT and CQ analyzed the data. YZh and ZT wrote the original draft of the manuscript. SH and GY revised the manuscript. YZh and ZT confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Animal Experimental Ethics Committee of the First Affiliated Hospital of Guangxi Medical University (approval no. 2017-KY-81771674-002). All the animals used were treated humanely.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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