Article

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Therapeutic Effects of Baicalin Combined with Bone Marrow Stromal Cells on Hepatic Ischemia/Reperfusion Injury in Rats

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Background and Purpose: Hepatic ischemia-reperfusion (I/R) injury has been considered a primary hindrance in liver operation. This study explored the efficiency of baicalin combined with bone marrow stromal cells (BMSCs) in treating rats' liver I/R injury.

Methods: BMSCs hepatic differentiation was evaluated by determining the expression of hepatic markers, urea synthesis, and low-density lipoprotein (LDL) uptake after the identification of BMSCs. Hepatic injury was evaluated via Hematoxylin and Eosin (H&E) staining, LDH activity, and Reactive Oxygen Species (ROS) levels. Apoptosis in liver tissue was detected by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) and flow cytometry. The inflammatory cytokines levels were determined using enzyme-linked immunosorbent assay (ELISA). Oxidative stress damage was assessed using Superoxide Dismutase (SOD) activity. The target molecules expression was detected via Polymerase Chain Reaction (real-time PCR) and western blotting.

Key Results: Results showed that baicalin facilitated hepatic differentiation of BMSCs, as evidenced by enhancing expression of albumin (ALB), Cytokeratin-18 (CK-18), and alpha fetoprotein (AFP), urea production, and LDL uptake. As compared with BMSCs monotherapy, co-treatment with baicalin exhibited higher efficiency in attenuating liver injury. Moreover, combination with baicalin strengthened the inhibitory effect of BMSCs on apoptosis via reducing the expression of B-cell lymphoma-2 (Bcl-2) Associated X Protein (Bax) and cleaved caspase-3 but elevating the expression of Bcl-2. Additionally, oxidative stress and inflammatory response were restrained by combination with baicalin and BMSCs treatments. Finally, the promotion of Heme Oxygenase 1 (HO-1) expression and inactivation of the nuclear factor kappa-B (NF-κB) pathway were involved in the molecular mechanisms of this combined therapy.

Conclusions and Implications: In summary, combined therapy of baicalin and BMSCs showed higher efficiency in mitigating hepatic I/R injury than monotherapy, and this therapeutic action may depend on activating HO-1 and inhibiting the activation of NF-κB.

Keywords: bone marrow stromal cells; baicalin; hepatic I/R injury; NF-κB; HO-1 rats

Introduction

Hepatic ischemia-reperfusion (I/R) injury results from the reperfusion of blood after liver ischemia, which commonly happens in liver transplantation and resection [1]. The damage of liver structure and function caused by hepatic I/R injury may affect patient recovery and even cause death [2]. Although the mechanism causing hepatic I/R injury is not fully understood yet, oxidative stress and inflammation have been considered to be implicated [3]. During this injury, enhanced reactive oxygen species (ROS) generation leads to acute inflammatory cascade and subsequent apoptosis [4]. Therefore, it is important to identify effective measures to minimize the adverse consequences of hepatic I/R injury.

Mesenchymal stem cells (MSCs) have attracted attention, due to their regenerative, multipotent, and immunoregulatory capacities [5]. Mounting evidence has demonstrated that MSCs possessed effective efficacy in attenuating I/R injury of various tissues, including the brain [6], heart [7], intestine [8], and kidney [9]. Bone marrow-derived MSCs (BMSCs) have been reported to confer protection against hepatic I/R injury via facilitating liver regeneration, inhibiting apoptosis, oxidative stress, and inflammation [10–12]. As demonstrated previously, combined therapy of BMSCs and oxiracetam exhibited more effective efficacy in reducing cerebral I/R injury [6]. In addition, a

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recent study showed that melatonin enhanced the therapeutic efficacy of MSCs on renal I/R injury [13]. Given the above background, this study sought to find a explore a way to improve the therapeutic effects of BMSCs for liver I/R injury.

Baicalin is one of flavones extracted from *Radix Scutellariae.* It has multiple biological activities, such as anti-microbico, anti-inflammation, anti-oxidative, and antineoplastic efficacy, as a previous study verified [14]. A study by Zhu *et al.* [15] reported that baicalin treatment could promote survival, suppress apoptosis and inflammatory response of LPS-challenged MSCs. More importantly, anotherstudy demonstrated that combined treatment with baicalin and BMSCs exhibited a synergistic effect on alleviating carbon tetrachloride-induced hepatic fibrosis via promoting hepatocyte differentiation [16]. However, whether combination with baicalin and BMSCs can show superior therapeutic efficiency in relieving liver I/R injury is still unknown.

The aim of this study was to explore whether the combined therapy of baicalin and BMSCs has better efficacy than BMSCs alone in a rat hepatic I/R injury model and investigate its potential mechanisms. The ultimate hope was to provide theoretical foundation for the clinical application of combined therapy of baicalin and BMSCs in treating hepatic I/R injury in humans.

Materials and Methods

Cell Culture and Treatment

Rat BMSCs were bought from the Procell Life Science & Technology Co., Ltd (CP-R131, Wuhan, China) and identified using flow cytometry (C00162, Beckman Coulter, CA, USA). They were incubated in Procell medium containing 15% FBS (Fetal Bovine Serum) (10500, Thermo Fisher, Waltham, MA, USA). To induce hepatocyte differentiation, BMSCs were separated as follows:

- Group 1, the control;
- Group 2, 20 ng/mL hematopoietic growth factor (294-HG, HGF, R&D Systems, Minneapolis, MN, USA);
- Group 3, 10 ng/mL Recombinant Fibroblast Growth Factor 4 (7460-F4, FGF-4, R&D Systems, Minneapolis, MN, USA);
- Group 4, 400 μM baicalin (21967-41-9, Aladdin, Shanghai, China);
- Group 5, 400 μM baicalin (21967-41-9, Aladdin, Shanghai, China) and 20 ng/mL HGF (294-HG, R&D Systems, Minneapolis, MN, USA);
- Group 6, 400 μM baicalin (21967-41-9, Aladdin, Shanghai, China) and 10 ng/mL FGF-4 (294-HG, R&D Systems, Minneapolis, MN, USA).

BMSCs were cultured in differentiation media containing 1% insulin-transferrin-selenium (41400045, Thermo Fisher) and 100 nM dexamethasone (D4902, Sigma-Aldrich, Saint Loui, MO, USA) for 21 d and then collected for further evaluation.

Animal Model

Adult male Wistar albino rats weighing from 250 to 300 g were bought from SLAC laboratory Animal Co., Ltd (2018-0004, Shanghai, China) and separated into the following groups (n = 6):

- the sham group,
- the I/R injury group,
- the I/R injury+BMSCs group,
- the I/R injury+BMSCs+baicalin group.

Rats were acclimatized for one week and housed in a laboratory animal room under standard conditions (20–25 °C; 40–70% humidity).

As previously mentioned, a liver I/R injury model was established using Pringle's maneuver [17]. Firstly, 60 mg/kg phenobarbital sodium was injected intraperitoneally, to anesthetize the rats. Then, the porta hepatis was exposed via a midline incision. Hepatic ischemia was acheived using bulldog microvascular clamp for 45 min. Subsequently, reperfusion was carried out by removing the microvascular clamp and the abdominal incision was closed with sutures. The same surgical incision was made and closed with sutures on rats undergoing sham surgery.

For I/R injury+BMSCs group, the rats were injected with 1×10^6 BMSCs via portal vein immediately after reperfusion. For the combined therapy group, the rats received portal vein injection of 1×10^6 BMSCs and intraperitoneal injection of 200 mg/kg baicalin immediately after reperfusion. All rats were euthinased by 5% isoflurane anesthesia 24 h after reperfusion and blood and liver tissues were obtained for the following experiments. This research was conducted based on the Basic and Clinical Pharmacology and Toxicology policy for experimental and clinical studies [18]. The ethics of animal experiments was approved by the Experimental Animal Ethics Committee of Zhejiang Haikang Biological Products Co (ethical numbers: HKSYDWLL2021010).

Reactive Oxygen Species (ROS) Assay

Specify the volume of DCFH-DA (2′,7′-Dichlorodihydrofluorescein diacetate) was diluted in 10 μmol/L (serum-free culture medium; 1:1000). A well of a six-well plate was filled with no less than 1 mL diluted DCFH-DA and incubated at 37 °C for 20 min in a cell incubator. To adequately eliminate DCFH-DA which had not entered the cells, three washes with serum-free cell culture medium were performed. After that, cells were collected and detected using flow cytometry (C00162, Beckman Coulter, CA, USA).

Flow Cytometry

After 24 h of cell administration treatment, the cells were rinsed twice using cold PBS (70013032, Thermo Fisher, Waltham, MA, USA), resuspended in 1×10^6 cells/mL 1× binding buffer, and placed in a 5 mL culture tube with 100 μL solution, 5 μL Propidium Iodide (PI), 5

μL FITC Annexin V (FCABS254F, Sigma-Aldrich, Saint Loui, MO, USA). The cells were lightly vortexed and cultured in the dark at 25 °C for 15 min. Then, 400 μL of 1× binding buffer was added to each tube and analyzed with a flow cytometer (C00162, Beckman Coulter, CA, USA).

Western Blotting

RIPA Lysis Buffer (HY-16160, Beyotime, Haimen, China) containing sodium orthovanadate (S1873, Beyotime, Shanghai, China) and PMSF (78830-5G 329-98-6, Sigma-Aldrich, Saint Loui, MO, USA) was adopted for total protein extraction. Then, the protein samples were isolated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (89888, Thermo Fisher, Shanghai, China) and transferred to polyvinylidene fluoride membranes after quantification via an Enhanced BCA Protein Assay Kit (P0012S, Beyotime, Shanghai, China). Next, the membranes were blockedin 5% non-fat milk buffer for 30 min and tested them using primary antibodies against ALB (1:2000, Abcam, Cambridge, UK), CK-18 (1:2000, Abcam, Shanghai, China), AFP (1:1000, Abcam, Shanghai, China), Bax (1:500, Bioss, Beijing, China), Bcl-2 (1:1000, Bioss, Beijing, China), cleaved caspase-3 (1:1000, Cell Signaling Technology, Danvers, MA, USA), Phospho- NF-κB p65 (p-NF-κB p65) (1:200, Bioss, Beijing, China), NF-κB p65 (1:500, Bioss, Beijing, China), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000, Bioss, Beijing, China), and HO-1 (1:1000, Abcam, Beijing, China) at 4 °C overnight. Then, the membranes were cultured with Goat Anti-rabbit IgG (1:1000, Bioss) for 2 h at 37 °C. Finally, very sensitive ECL chemiluminescence kit (BeyoECL Moon) (P0018FS, Beyotime, Shanghai, China) were used to visualize the protein bands.

Low-Density Lipoprotein (LDL) Uptake

Dil-Acetylated Low Density Lipoprotein (Dil-Ac-LDL) (YB-0013, Yiyuan biotechnology, Guangzhou, China) at a concentration of 20 mg/L was added to the BMSCs with various treatments. Next, BMSCs were rinsed with PBS and stained with DAPI for 10 min after culturing for 4 h at 37 °C. The results were looked at with a fluorescence microscope (Scipu002552, Olympus, Tokyo, Japan). Red fluorescence was taken to indicate the LDL uptake.

Urea Synthesis Assay

To determine urea synthesis, the supernatant of BMSCs were collected and detected using the Urea nitrogen Activity Assay Kit (BC1530, Solarbio, Beijing, China). In brief, 60 μL of the sample was incubated with working reagent I (120 μL) and II (220 μL) for 10 min at 37 °C and then reacted with working reagent III (80 μL) and IV (60 μL) for 30 min at 37 °C. Results were detected at 630 nm on a microplate reader.

Real Time-Polymerase Chain Reaction (PCR)

To assess HO-1 and NF-κB p65 messenger RNA (mRNA) expression, total RNA was separated from liver tissues by Trizol reagent (15596018, Thermo Fisher, Shanghai, China). Subsequently, complementary DNA (cDNA) was synthesized using the All-in-One First-Strand cDNA Synthesis SuperMix (AE341-02, TransGen, Beijing, China). SG Fast Real-time Quantitative PCR Detecting System (qPCR) Master Mix (B639271, Sangon, Shanghai, China) was utilized for real-time PCR. And β-actin was employed as the endogenous gene to maintain normal target genes expression levels. Primers used are as follow:
HO-1:
Forward: 5′-TATCGTGCTCGCATGAACACTCTG-3′;
Reverse: 5′-GTTGAGCAGGAAGGCGGTCTTAG-3′;
β-actin:
Forward: 5′-CCACCATGTACCCAGGCATT-3′;
Reverse: 5′-CAGTGAGGCCAGGATAGAGC-3′.

Haematoxylin and Eosin (H&E) Staining

The liver injury's severity was assessed using H&E staining. The collected rat liver tissues were, briefly, fixed in paraformaldehyde, embedded in paraffin, and cut into 5-μM sections. A HE Staining Kit (G1120, Solarbio, Beijing, China) was used to stain the slices. Livers were scored according to the following parameters: number of thrombi, number of (micro)abscesses, presence and degree of inflammation, and presence and degree of necrosis. Each parameter was graded 0–3, with 0 being absent and 3 being severe. The degree of liver damage was determined using a pathology scoring method as previously described [19].

TUNEL Assay

Hepatic tissues were subjected to paraformaldehyde fixation, dehydration, and paraffin embedding. Then a TUNEL assay kit (ab66110, Abcam, Shanghai, China) was applied to detect the 5-μM sections to evaluate cells apoptosis. The images were obtained with a fluorescence microscope.

Detection of Cytokines and Liver Enzymes

The serum levels of TNF-α, IL-1β, IL-6, and IL-10 were determined via commercial Rat TNF alpha ELISA Kit (ab46070, Abcam, Shanghai, China), Rat IL-1 beta ELISA Kit (ab255730, Abcam, Shanghai, China), Rat Interleukin 6 (IL-6) ELISA Kit (ab234570, Abcam, Shanghai, China), and Rat Interleukin 10 (IL-10) ELISA Kit (ab100764, Abcam, Shanghai, China) following the protocol of the manufacturer, respectively. The superoxide dismutase (SOD) and lacate dehydrogenase (LDH) activities of liver tissues were detected using the SOD Activity Assay Kit (BC0175, Solarbio, Beijing, China) and LDH Activity Assay Kit (BC0685, Solarbio, Beijing, China).

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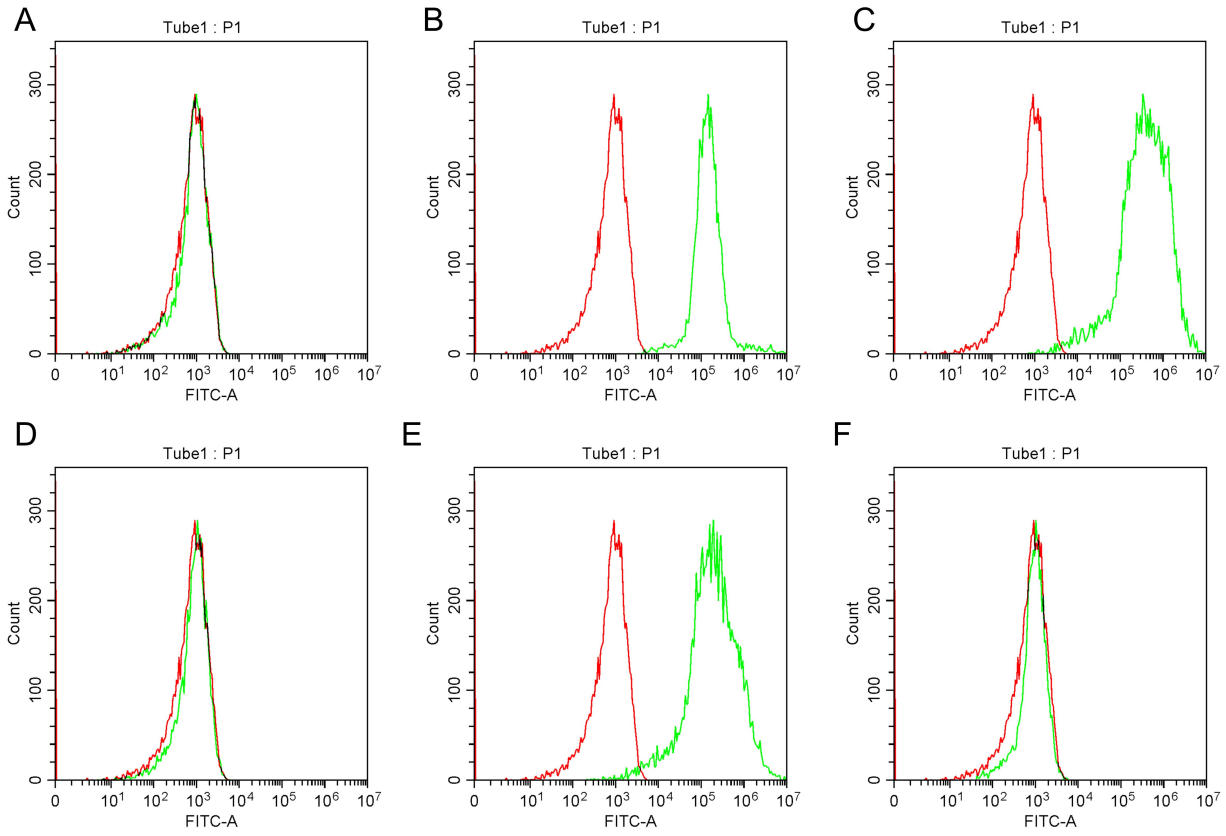

Fig. 1. BMSC cells were determined using flow cytometry and incubated by fluorescently labeled antibodies against (A) CD14, (B) CD29, (C) CD44, (D) CD45, (E) CD105, and (F) HLA-DR index measurements, through flow cytometry.

Statistical Analysis

The distribution of continuous data was checked for normality using Chapio-Wilk's test. Normally distributed continuous data were described as mean ± standard deviation and compared using one-way analysis of the variance. Continuous data that did not fit a normal distribution were described using as the median [inter-quartile range] and compared using non-parametic one-way analysis of the variance (non-parametric ANOVA). Categorical data were described as number (%) and compared using the chi-squared test, resorting to the Mann-Whitney test when the number of expected frequencies fell below 5 in 20 % or more of the cells. When a statistically significant difference was detected, each pair of the study groups was compared. Normally distributed continuous data were compared using student *t*-test. Continuous data that did not fit a normal distribution were compared using Wilkinson's signed rank sum test. Categorical data were compared using the chi-squared test, resorting to the Mann-Whitney test when the number of expected frequencies fell below 5 in 20 % or more of the cells. GraphPad Prism 8 was used for statistical analysis. A p-value < 0.05 was considered statistically significant.

Results

Baicalin Promoted the Hepatic Differentiation of BMSCs

Firstly, BMSCs were identified, as shown in Fig. 1. Then the effect of single treatment with baicalin, growth factors (FGF-4, HGF) or a combination of them on the differentiation of BMSCs into hepatocyte-like cells was evaluated. ALB, CK-18, and AFP protein levels were up-regulated ($p < 0.01$) after exposure to FGF-4 or HGF, which were further strengthened by combination with baicalin ($p < 0.001$), as shown in Fig. 2A–D. In addition, the amount of urea produced by BMSCs was enhanced by FGF-4, HGF, or baicalin treatment. This change was more obvious in combined baicalin treatment groups ($p < 0.01$), as shown in Fig. 2E. As presented in Fig. 2F, hepatocyte-like cells were further determined by LDL uptake. FGF-4, HGF, or baicalin treatment promoted LDL uptake, and co-treatment with baicalin and FGF-4/HGF exhibited higher LDL uptake ($p < 0.001$). Therefore, baicalin treatment enhanced the differentiation of BMSCs into hepatocyte-like cells.

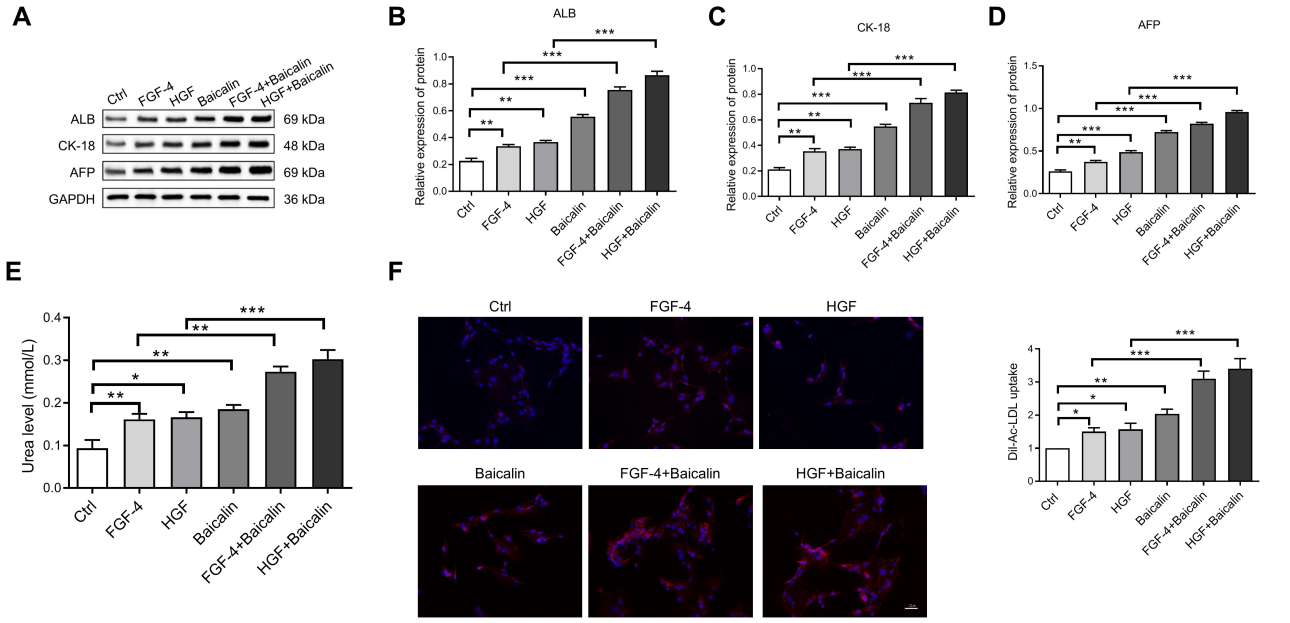

Fig. 2. Baicalin facilitated the differentiation of BMSCs into hepatocyte-like cells. (A–D) Western blotting for evaluating the protein levels of ALB, CK-18, and AFP in BMSCs with various treatments. (E) The level of urea in the supernatant of BMSCs was detected using a commercial kit. (F) Uptake of LDL by BMSCs after receiving multiple treatments were determined. Data were mean ± SD from 3 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Combined Baicalin and BMSCs Treatment Relieved Hepatic I/R Injury in Rats

Given that baicalin was shown to have the potential to promote hepatic differentiation of BMSCs, the protection of combined therapy of baicalin and BMSCs against hepatic I/R injury in rats was further evaluated. Liver histological examination showed that inflammatory infiltration and hepatocyte necrosis were found after I/R. The histological liver injury was attenuated by treatment with BMSCs, as evidenced by lower liver injury score ($p <$ 0.01) (this should have been mentioned and explained in full in the method), and this change was reinforced by co-treatment with baicalin and BMSCs ($p < 0.01$), as shown in Fig. 3A,B.

As shown in Fig. 3C, administration of BMSCs substantially reduced the elevation of LDH activity in the I/R group ($p < 0.001$), which was further lowered via co-treatment with baicalin ($p < 0.01$). In addition, the administration of BMSCs greatly reduced ($p < 0.001$) the elevated ROS levels in the I/R group, and the combination treatment with baicalin further reduced ($p < 0.01$) the ROS levels, as shown in Fig. 3D. In summary, these findings suggested that therapy with baicalin and BMSCs exhibited stronger protective impact ($p < 0.01$) on rats' hepatic I/R injury.

Apoptosis in Liver Tissues was Restrained by Combined Treatment with Baicalin and BMSCs

TUNEL staining revealed that the enhanced apoptosis in liver tissues caused by I/R injury was remarkably suppressed ($p < 0.001$) by BMSCs, and BMSCs-mediated inhibitory effect on apoptosis was strengthened ($p < 0.05$) by combined treatment with baicalin, as shown in Fig. 4A. Flow cytometry assays of apoptosis levels were also consistent with this result, as shown in Fig. 4B. Besides, the levels of proteins associated with apoptosis were assessed using western blot. As presented in Fig. 4C–F, the up-regulation of Bax and cleaved caspase-3 induced by I/R injury, and the down-regulation of Bcl-2 were partly reversed by BMSCs ($p < 0.05$). As expected, these changes were more obvious ($p < 0.01$) after co-treatment with BMSCs and baicalin. These findings suggested that combined treatment with baicalin and BMSCs might attenuate hepatic I/R injury via inhibiting apoptosis.

Co-treatment with Baicalin and BMSCs Suppressed Inflammation and Oxidative Stress in Liver Tissues

This information belongs to the method and there was no need to repeat here. Treatment with BMSCs restrained the increased secretion of TNF-α, IL-1β, and IL-6 ($p <$ 0.05), and decreased release of IL-10 ($p < 0.05$). Notably, BMSCs-mediated anti-inflammatory effects were enhanced by combination treatment with baicalin ($p < 0.05$), as shown in Fig. 5A–D).

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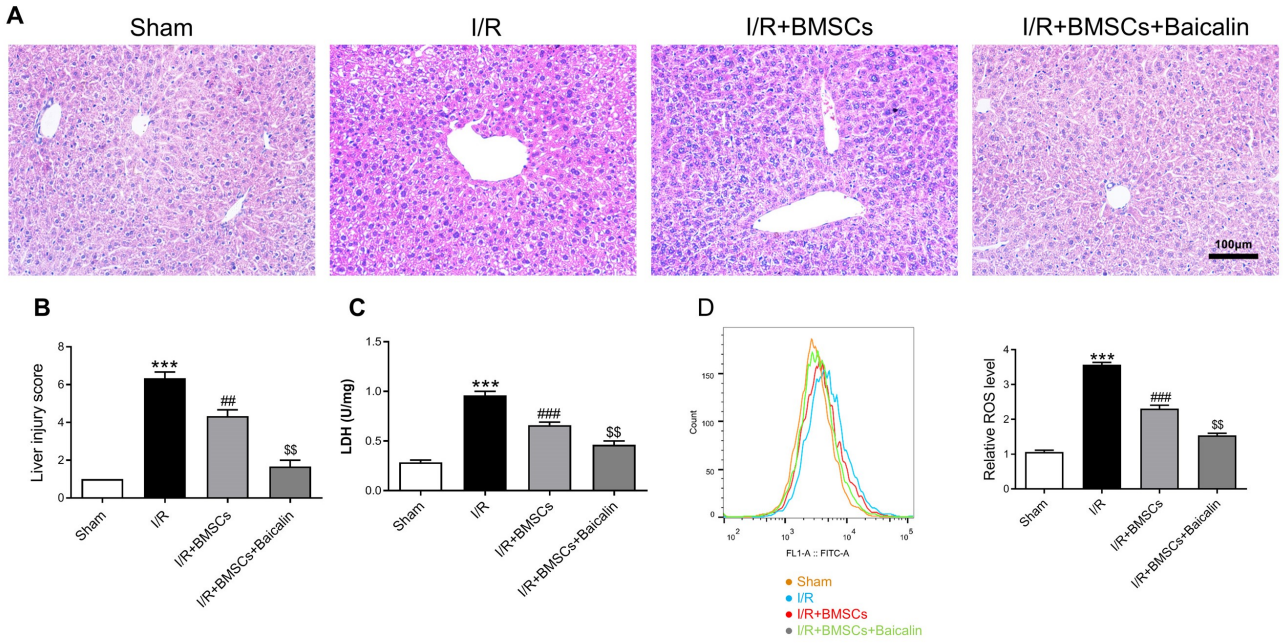

Fig. 3. Effect of combined baicalin and BMSCs treatment on rats' hepatic I/R injury. (A) H&E staining was used for assessing histological hepatic injury following I/R. (B) The liver injury score was shown. (C) Hepatic injury was assessed via LDH activity. (D) Liver injury was assessed by the levels of ROS. Data were mean ± SD from 3 independent experiments. $^{***}p < 0.001$ vs. the sham group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. the I/R group; $^{\$\$}p < 0.01$ vs. the I/R+BMSCs group.

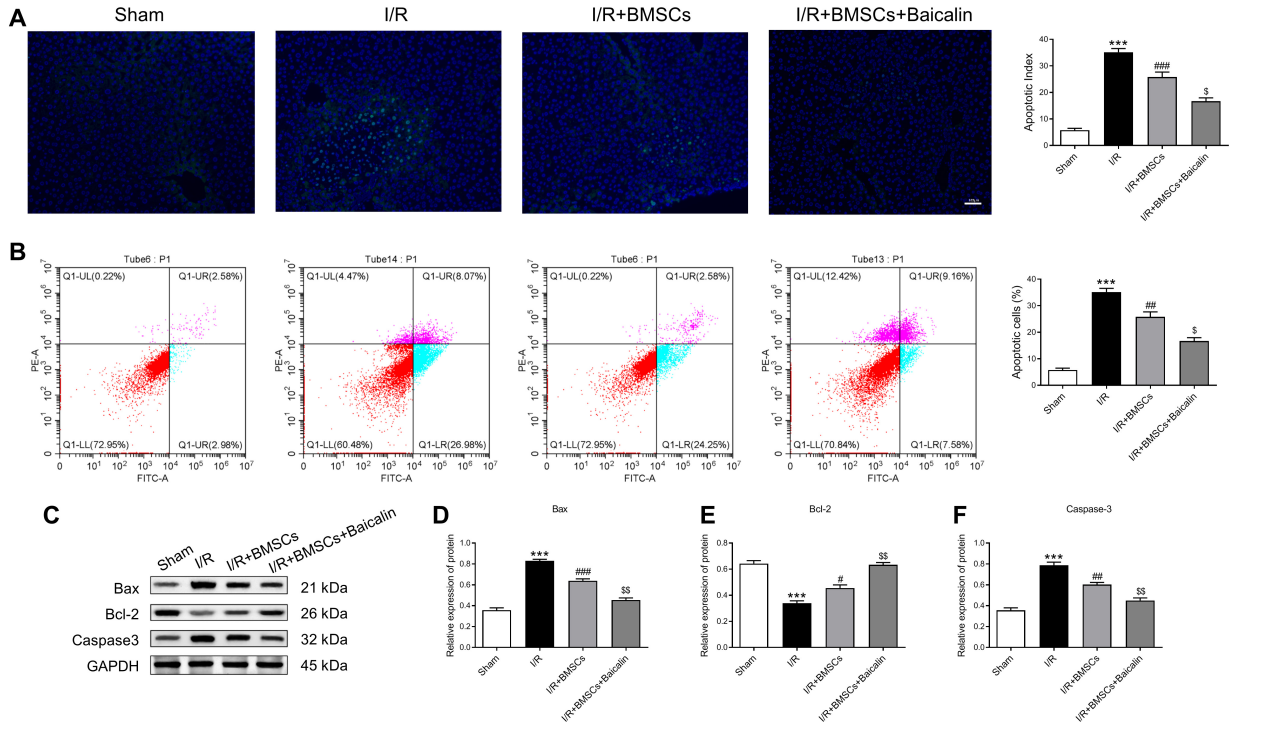

Fig. 4. Impact of combined baicalin and BMSCs treatment on apoptosis of liver tissues. (A) Apoptosis of liver tissues was determined using TUNEL. (B) Hepatocytes apoptosis was determined through flow cytometry. (C–F) The Bax, Bcl-2, cleaved caspase-3 protein levels in liver tissues were assessed by Western blot. Data were mean ± SD from three independent experiments. $^{***}p < 0.001$ vs. the sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. the I/R group; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ vs. the I/R+BMSCs group.

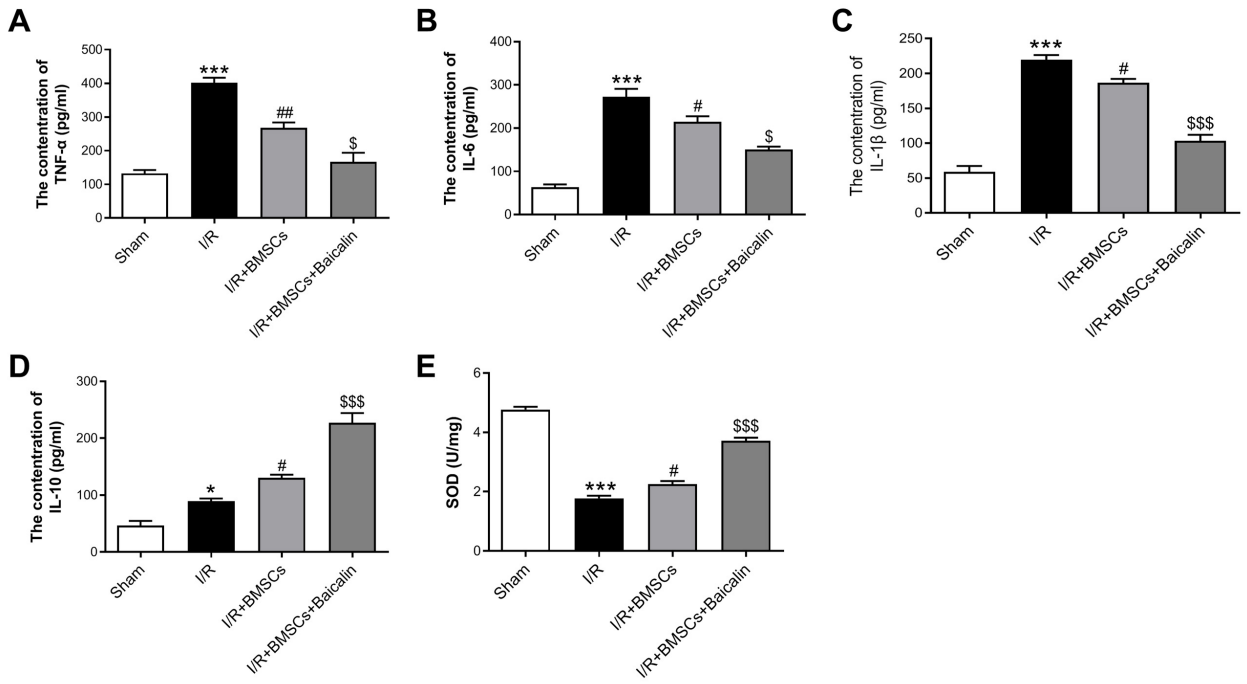

Fig. 5. Effect of co-treatment with baicalin and BMSCs on oxidative stress and inflammation of liver tissues. The serum levels of inflammatory cytokines (A) TNF-α, (B) IL-6, (C) IL-1β and (D) IL-10 were detected with ELISA kits. (E) The activity of SOD in liver tissues was measured. Data were mean $\pm$ SD of 3 independent experiments. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. the sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. the I/R group; $^{\$}p < 0.05$, $^{\$\$\$}p < 0.001$ vs. the I/R+BMSCs group.

The activity of antioxidant enzyme SOD was significantly reduced by I/R injury ($p < 0.001$), whereas BMSCs treatment could effectively enhance SOD activity. Compared with the BMSCs alone, co-treatment with baicalin and BMSCs showed higher SOD activity ($p < 0.001$), as shown in Fig. 5E). The above data revealed that co-treatment with baicalin and BMSCs might weaken liver I/R injury via repressing inflammation and oxidative stress.

Co-Treatment with Baicalin and BMSCs Activated HO-1 and Inhibited NF-κB Path

Real-time PCR showed that the mRNA expression of HO-1 from the liver tissues increased in the I/R group ($p < 0.001$), which was further elevated with combined treatment with BMSCs and baicalin in comparison to BMSCs alone ($p < 0.001$), as shown in Fig. 6A). Consistently, Western blotting analysis revealed that the p-NF-κB p65 protein level was decreased ($p < 0.05$), while HO-1 level was elevated when treated with combined BMSCs and baicalin ($p < 0.001$). The combined therapy was more effective as compared with individual treatment with BMSCs ($p < 0.01$), as shown in Fig. 6B–D). These results suggested that inactivation of NF-κB and activation of HO-1 were involved in the protective mechanisms of combined therapy of baicalin and BMSCs.

Discussion

The aim of this study was to determine an optimal treatment approach for liver I/R injury. Hepatic I/R injury is a severe obstacle during liver surgery, results in liver dysfunction and possibly death and lacks of effective and reliable treatments. For the purpose of identifying an optimal treatment, a combined application of effective drugs has been taken into consideration. BMSCs transplantation is recognized to improve liver I/R injury due to hepatocyte-like cells' regeneration [12]. Baicalin, an active component, has been demonstrated to protect against hepatic I/R injury via hindering oxidative stress, apoptosis, and inflammation [21]. This work is the first to investigate the favorable effects of combined application of baicalin and BMSCs on rat liver I/R injury. The results of this study suggested that a combination of baicalin and BMSCs presented higher efficiency in mitigating hepatic I/R injury through regulating NF-κB and HO-1 pathways.

Recent studies reported that one of the key mechanisms of hepatic I/R injury is apoptosis [22,23]. Apoptosis, a biological process, is regulated by multiple genes. In addition, mitochondrial apoptosis, as a crucial type of apoptosis, has been shown to contribute to hepatic I/R injury's pathogenesis [24]. During mitochondrial apoptosis, the pro-apoptotic proteins located on the mitochondrial membrane are released to the cytoplasm, which lead to subse-

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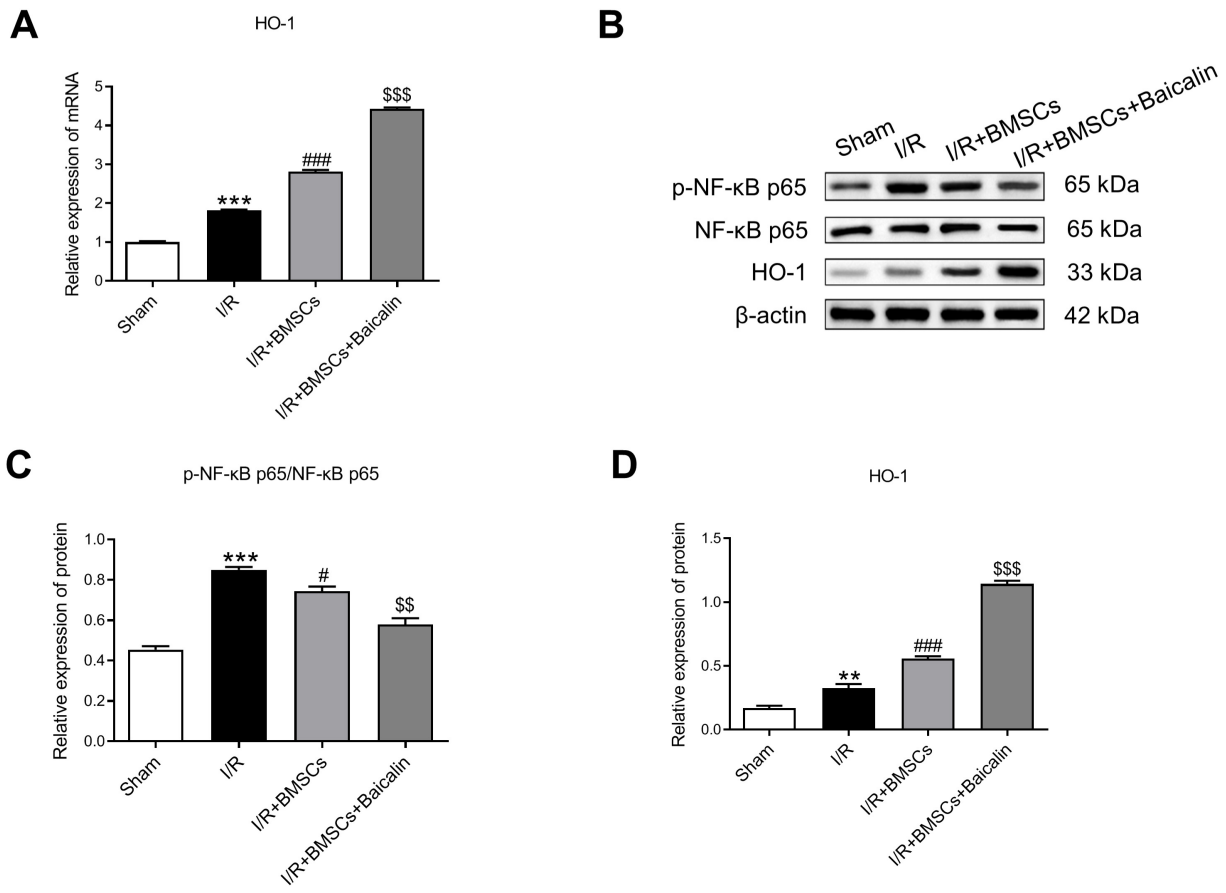

Fig. 6. Impact of combined baicalin and BMSCs treatment on NF-κB and HO-1 pathways. (A) The HO-1 mRNA expression of liver tissues was examined via real-time PCR. (B–D) The p-NF-κB p65, NF-κB p65 and HO-1 protein levels were assessed using western blotting. Data were mean ± SD of 3 independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the sham group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. the I/R group; $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs. the I/R+BMSCs group.

quent caspase activation-mediated apoptosis [25]. Besides, the dysregulation of Bcl-2 family proteins also affects the mitochondria membrane permeability and causes apoptosis. Baicalin has been reported to attenuate arsenic trioxide-induced apoptosis of endothelial cells [26]. Additionally, baicalin could protect against neuron apoptosis in Parkinson's disease rats [27]. In line with these previous studies, our data indicated that combined BMSCs and baicalin more effectively suppressed I/R injury-induced apoptosis via up-regulating Bcl-2/Bax ratio and inhibiting caspase-3 activation, as compared with single treatment.

It has been confirmed that inflammation contributes to hepatocellular damage during the pathological process of liver I/R injury [28]. Aberrant expression of inflammatory cytokines has close relation with the injury [29]. Moreover, I/R injury-mediated inflammation can trigger apoptosis [30]. Thus, repression of inflammatory response has been considered an essential treatment strategy for hepatic I/R injury. Previous research has proved that baicalin could restrain inflammation and ameliorate hepatic I/R injury [21]. The study findings further indicated that combined BMSCs and baicalin led to lower pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) levels and higher anti-inflammatory cytokine (IL-10) level.

After inflammation, oxidative stress has been indicated as another pivotal contributor to liver I/R injury's pathological process [31]. In response to the injury, enhanced production of reactive oxygen species may recruit and activate inflammatory cells, which further induces hepatocyte apoptosis [32]. Based on these observations, suppressing oxidative stress can be a promising approach for relieving I/R injury. In the present study, the antioxidant enzyme SOD activity was more efficiently enhanced by co-treatment with BMSCs and baicalin. Thus, we can conclude that combination with baicalin and BMSCs protects against liver I/R injury partly via suppressing oxidative stress and inflammation.

Growing evidence has demonstrated that HO-1 has beneficial impact on hepatic I/R injury. For instance, Atef *et al.* [33] showed that quercetin presented a protective effect from liver I/R injury through provoking the expression of HO-1. Besides, Yun *et al.* [34] indicated that HO-1

protected against liver I/R injury by repressing oxidative stress and inflammatory response. More importantly, earlier research has revealed that HO-1 pathway could be activated by baicalin [35] or BMSCs [36]. To explore the deeper protective mechanisms, HO-1 and NF-κB levels of live tissues were assessed. Consistent with these reports, this work found that combined baicalin and BMSCs further enhanced HO-1 expression after hepatic I/R injury, which was more efficient than treatment with BMSCs alone. NF-κB transcriptional factor has been regarded as a key responder to the stimulation of ROS and TNF-α [37]. Then, the phosphorylation of NF-κB further results in various pro-inflammatory cytokines' release, such as TNF-α and IL-1β, which leads to hepatic injury [3]. In our experiment, the increased p-NF-κB level following liver I/R injury was significantly reduced via combined treatment with baicalin and BMSCs. These findings suggested that modulating NF-κB and HO-1 pathways took part in the protective mechanisms of combined therapy of baicalin and BMSCs.

A limitation of our study is the mechanism was not investigated in depth enough. The advantages are the prospective design and the homogeneity of the study groups. In conclusion, combined therapy of baicalin and BMSCs presents showed higher efficiency in mitigating hepatic I/R injury than monotherapy. It is necessary to further study the mechanism of action of baicalin and BMSCs combined therapy to provide effective measures for clinical treatment of liver I/R injury.

Conclusions

This study provided evidence that combined therapy of baicalin and BMSCs exhibited better therapeutic efficacy in ameliorating liver I/R injury in rats via inhibiting apoptosis, oxidative stress, and inflammation. Mechanically, HO-1 activation and NF-κB inactivation were involved in the beneficial effects of this combined therapy. These results may provide an effective measure to treat hepatic I/R injury in the clinic. This will await further research in animals and then in humans.

Availability of Data and Materials

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

Author Contributions

YL and BZ—designed the experiment and wrote the paper; YL, BZ, JW and ZC—conducted experiments and performed data analysis. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

All animal experiments were approved by the Experimental Animal Ethics Committee of Zhejiang Haikang Biological Products Co., Ltd (number: HKSYD-WLL20210100).

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Conflict of Interest

The authors declare no conflict of interest.

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