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Hydrogen-rich saline protects against hepatic injury induced by ischemia-reperfusion and laparoscopic hepatectomy in swine

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ABSTRACT

Background: Hydrogen-rich saline (HRS) has antioxidative, anti-inflammatory and anti-apoptotic properties. We investigated the effects of hydrogen on hepatic ischemia-reperfusion (I/R) and laparoscopic hepatectomy in swine.

Methods: Twenty-one healthy Bama miniature pigs were randomly divided into the sham group, ischemia-reperfusion injury (IRI) group, HRS-5 (5 mL/kg) group, and HRS-10 (10 mL/kg) group. HRS was injected through the portal vein 10 min before reperfusion and at postoperative day 1, 2 and 3. The roles of HRS on oxidative stress, inflammatory response and liver regeneration were studied.

Results: Compared with the IRI group, HRS treatment attenuated oxidative stress by increasing catalase activity and reducing myeloperoxidase. White blood cells in the HRS-10 group were reduced compared with the IRI group ($P < 0.01$). In the HRS-10 group, interleukin-1 beta, interleukin-6 and tumor necrosis factor alpha, C-reactive protein and cortisol were downregulated, whereas interleukin-10 was upregulated. In addition, HRS attenuated endothelial cell injury and promoted the secretion of angiogenic cytokines, including vascular endothelial growth factor, angiopoietin-1 and angiopoietin-2. HRS elevated the levels of hepatocyte growth factor, Cyclin D1, proliferating cell nuclear antigen, Ki-67 and reduced the secretion of transforming growth factor-beta.

Conclusions: HRS treatment may exert a protective effect against I/R and hepatectomy-induced hepatic damage by reducing oxidative stress, suppressing the inflammatory response and promoting liver regeneration.

Introduction

Laparoscopic hepatectomy was first reported by Reich in the 1990 s [1]. Laparoscopic liver resection offers certain advantages, including reduced blood loss and pain, rapid recovery and reduced postoperative adhesion [2–4]. The volume of the liver is substantially reduced after partial hepatectomy, and hepatic ischemia-reperfusion (I/R) injury is an inevitable problem [5]. Hepatic I/R can be divided into warm I/R and cold I/R injury (IRI), warm IRI is manifested as hepatocellular damage, while cold IRI is mainly sinusoidal endothelial cells damage and microcirculation interruption, and cold IRI is usually accompany with warm IRI during transplantation surgery. Although many agents have shown protective effects against liver IRI in animal models, only a few have been tested in clinical trial. Methylprednisolone and epoprostenol have been demonstrated to reduce oxidative damage and inflammatory response induced by hepatic I/R. New modalities for the treatment of IRI are necessary [6], and it is also necessary to discover a protective strategy in a model of I/R combined with hepatectomy [5,7–9].

In recent years, studies on hydrogen have been performed in several diseases, such as cerebral infarction, IRI and organ transplantation. Besides its antioxidative, anti-inflammatory and anti-apoptotic effects [10–12], Ohsawa and colleagues found that hydrogen has anti-oxidative effect by directly eliminating hydroxyl radical and peroxynitrite [10]. Subsequent studies demonstrated that hydrogen regulates oxidative stress, inflammation, apoptosis and dysmetabolism by reducing reactive oxygen species (ROS), modulating signal transduction systems, and regulating the expression of downstream genes [13]. However, the mechanisms remain to be elucidated.

The hydrogen gas is membrane permeable and capable to spread into the cytosol and organelles. Hydrogen is effective in liver diseases, including liver IRI, nonalcoholic steatohepatitis and acetaminophen-induced hepatotoxicity, by suppressing oxidative stress, inhibiting inflammation and influencing hepatic

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regeneration [14,15]. Hydrogen may attenuate portal hyperperfusion injury, protect the nuclear DNA, maintain ATP synthesis and protect the mitochondrial membrane potential [16,17]. It has been demonstrated that hydrogen inhalation alleviates hepatic IRI. We have successfully applied laparoscopy to establish I/R combined with 50% and 70% hepatectomy models, and we examined the effects of hydrogen-rich saline (HRS) on endoplasmic reticulum stress and apoptosis after liver injury [18–20]. In this study, we investigated the biological effects of hydrogen on I/R combined with hepatectomy in a swine model.

Methods

Animals

Twenty-one healthy Bama miniature pigs were used for this study. The pigs were provided by the Bama miniature pigs Farm of College of Life Sciences (Harbin, China). The pigs were housed under steady temperature (20 °C) and a 12-h light/dark cycle. All animals were fed piglet diet (Shenzhen Jinxinnong Feed, China) and tap water *ad libitum*. This study was approved by the Animal Care and Use Committee of Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences (SQ-2017-420).

Preparation and estimation of HRS

Hydrogen was produced from a hydrogen-generator (QL-500, Shandong Success Hydrogen Energy Co., China) and dissolved in physiological saline under 0.4 MPa pressure for 4 h. HRS was freshly prepared every time before the surgery to ensure a hydrogen concentration greater than 0.6 mmol/L. The content of hydrogen was confirmed using a dissolved hydrogen portable meter (ENH-1000, Trustlex Inc., Osaka, Japan).

Surgical procedure

Twenty-one healthy Bama miniature pigs were randomly divided into the sham group ($n=6$), IRI group ($n=6$), HRS-5 group (5 mL/kg, $n=3$) and HRS-10 group (10 mL/kg, $n=6$). Carbon dioxide pneumoperitoneum was established in all three groups, and the four-port purely laparoscopic technique was used. The sham group was subjected to a flipped liver lobe and no hepatectomy was performed. In the IRI, HRS-5 and HRS-10 groups, we divided the falciform and left triangular ligaments. After the dissection of the cystic duct, gallbladder artery and portal vein, a tourniquet with needle was used to penetrate the parenchyma of the right lobe to stop the blood flow for 60 min. During hepatic ischemia, central venous catheters were placed through the portal vein. A triple row of silk sutures that penetrated the parenchyma at the root of the left lobes. A monopolar hook electrode was used for hepatic transection, and the resected lobes were placed in a specimen bag and removed. In the HRS-5 and HRS-10 groups, HRS were injected through the portal vein 10 min before reperfusion and at postoperative day 1, 2, and 3 (5 mL/kg and 10 mL/kg). The IRI and sham groups were injected with the same dose of saline. Liver tissues were collected by laparoscopy at preoperative and postoperative day 1, 3, and 7, and blood samples were collected through the anterior vein.

Analysis of hepatic biochemical parameters

Hepatic biochemical parameters such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), total bilirubin (T-Bil) and total protein (TP) were measured with the commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instructions.

Blood tests

Blood samples were collected to test the white blood cell, neutrophil and lymphocyte using an automated hematology system (MEK-7222K, Nihon Kohden, Tokyo, Japan).

Levels of antioxidative enzymes and MPO in serum

Serum samples were used to analyze the levels of antioxidative enzymes and myeloperoxidase (MPO), catalase (CAT), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities; malondialdehyde (MDA) and MPO concentrations were determined using Nanjing Jiancheng Bioengineering Institute reagents (Nanjing, China).

The CAT activity was determined based on the consumption of H_2O_2. The GSH-Px activity was measured based on the principle that GSH-Px could catalyze glutathione (GSH) and H_2O_2 into oxidized glutathione (GSSG) and H_2O. The levels of SOD were measured by the xanthine oxides method, and the MDA levels were analyzed by the method of thiobarbituric acid (TBA). The MPO activity was expressed as the degrading 1 μmol of H_2O_2 per min. All of the procedures were performed in accordance with the manufacturer's instructions.

Immunohistochemical analysis

Livers were removed and fixed in 4% paraformaldehyde for 48 h, dehydrated, cleared, and embedded in paraffin. Then, 4-μm sections were blocked with 3% H_2O_2 for 10 min and the antigens were retrieved in a microwave for 8 min. The sections were blocked with bovine serum albumin (BSA) and incubated with anti-proliferating cell nuclear antigen (PCNA) (1:100, Abcam, Cambridge, UK), anti-Ki-67 (Novus, Saint Charles, USA), anti-CD45 and anti-CD68 (Sanying, Wuhan, China) overnight at 4 °C. After washed with PBS, the sections were incubated with rabbit anti-goat IgG labeled with biotin (Beijing ZSGB-Biotechnology Co., Ltd., Beijing, China). Streptavidin labeled with horseradish peroxidase was added and incubated at room temperature for 20 min. The antigens were visualized with diaminobenzidine (DAB). The sections were counterstained with hematoxylin for 20 s and fixed with neutral balsam. Sections were quantified with Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

Hematoxylin-eosin staining

Liver tissue samples were removed and fixed in 4% paraformaldehyde for 24 h, dehydrated and embedded in paraffin. Then, 4-μm sections were stained with hematoxylin and eosin, treated with acidic alcohol, immersed in running water, stained with eosin and evaluated by light microscope examination. Liver necrosis and vacuolar degeneration were scored by pathologists according to the classification of Suzuki and Levene [21,22]. Classification of liver necrosis scores: 0, none necrosis; 1, minimal necrosis; 2, mild necrosis; 3, moderate necrosis; 4, severe necrosis. Classification of vacuolar degeneration scores: 0, no vacuolation; 1, a few vacuoles around the hepatic lobules and the central vein; 2, half of hepatocytes vacuolation; 3, all hepatocytes vacuolation

ELISA assays

Interleukin-1 beta (IL-1β, CK-E50117), interleukin-6 (IL-6, CK-E50113), interleukin-10 (IL-10, CK-E50120), tumor necrosis factor alpha (TNF-α, CK-E50077), C-reactive protein (CRP, CK-E50055), cortisol (COR, CK-E50093), vascular endothelial growth factor (VEGF, CK-E95062), angiopoietin-1 (ANG1, CK-E50049),

Table 1
Gene-specific primers used for qPCR.

Gene	Primer sequence
HGF	Forward 5′-TGATCAACTCAGACGGCCTA-3′ Reverse 5′-AGCCCCAGCACATATTTCAG-3′
Cyclin-D1	Forward 5′-AAGTGCGTGCAGAAGGAAAT-3′ Reverse 5′-AGGAAGCGGTCCAGGTAGTT-3′
TGF-β	Forward 5′-CCGTCATTAGTGCGTCAGTTCT-3′ Reverse 5′-TTGCAGCCCACAAAAAGCA-3′
TNF-α	Forward 5′-ACCAGCCAGGAGAGAGACAAG-3′ Reverse 5′-AGCGTGTGAGAGGGAGAGAGT-3′
IL-1β	Forward 5′-TCTCCAGCCAGTCTTCATTGT-3′ Reverse 5′-GCCATCAGCCTCAAATAACAG-3′
IL-6	Forward 5′-AGCAAGGAGGTACTGGCAGA-3′ Reverse 5′-AAGACCGGTGGTGATTCTCA-3′
IL-10	Forward 5′-GGGAGGATATCAAGGAGCACG-3′ Reverse 5′-CTTGGAGCTTGCTAAAGGCAC-3′
β-actin	Forward 5′-TCTGGCAACCACACCTTCT-3′ Reverse 5′-TGATCTGGGTCATCTTCTCAC-3′

HGF: hepatocyte growth factor; TGF-β: transforming growth factor-beta; TNF-α: tumor necrosis factor alpha; IL-1β: interleukin-1 beta; IL-6: interleukin-6; IL-10: interleukin-10.

angiopoietin-2 (ANG-2, CK-E50047), hyaluronic acid (HA, CK-E50088), hepatocyte growth factor (HGF, CK-E50105), Cyclin D1(CK-E50143) and transforming growth factor-beta (TGF-β, CK-E50076) levels were determined using an ELISA Kit (Suzhou Calvin Biotechnology Co., Suzhou, China).

Quantitative real-time PCR analysis of mRNA levels

Total RNA was isolated from livers using the TRIzol reagent according to the manufacturer's instructions (Invitrogen, Shanghai, China). First-strand cDNA was synthesized using the ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). Quantitative real-time PCR was performed on a LightCycler 480 Real-Time PCR System (Roche Applied Science, Penzberg, Germany) in a 10-μL final reaction volume containing 5 μL FastStart Universal SYBR Green Master (Roche Applied Science, Penzberg, Germany), 1 μL cDNA, 0.3 μL of each primer and 3.4 μL ddH_2O. The PCR program was performed as follows: 95 °C for 15 s; 40 cycles of 95 °C for 15 s, 60 °C for 60 s, and 60 °C for 30 s. Primers used in the present study were synthesized by Sangon Biotech (Shanghai, China) (Table 1).

Statistical analysis

Data were reported as the mean ± standard deviation (SD). Statistical differences within each group were identified by one-way analysis of variance (ANOVA) tests with Turkey's post hoc test, using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). A $P < 0.05$ was considered statistically significant.

Results

Effects of HRS on liver injury and function

The levels of ALT, AST and T-Bil in serum were increased significantly in the IRI, HRS-5 and HRS-10 groups during 6 h to day 3 after surgery. After the injection of HRS through the portal vein, the increases in serum ALT, AST and T-Bil levels were significantly inhibited compared with those of the IRI group, and the AST and T-Bil levels in the HRS-10 group were significantly lower than those in the HRS-5 group at day 1 (Fig. 1A–C). The LDH levels in the IRI and HRS-10 groups were significantly increased at 6 h and day 1 compared with that of the sham group, and LDH level was significantly reduced after 10 mL/kg HRS treatment at day 1 compared with the IRI group (Fig. 1D). In addition, ALP levels in the IRI and HRS-10 groups were increased significantly at day 1, while TP levels were decreased significantly from 6 h to day 3 in the IRI group. However, there were no significant differences in ALP and TP levels between the HRS-10 group and the IRI group (Fig. 1E and F).

Effects of HRS on histopathologic changes

Liver histopathologic changes were observed in all three groups. Liver tissues from the sham group exhibited normal liver lobule structures and clear hepatic cords, no hepatic injury or necrosis was observed. However, after ischemia-reperfusion and hepatectomy, livers from the IRI group demonstrated extensive hemorrhage at day 1 (Fig. 1G), the area of necrosis mainly located in zone 2 and zone 3, few hepatocytes necrosis in zone 1, and CD45 and CD68 positive cells were observed (Fig. 2A and G). Severe inflammatory cell infiltration was observed at day 3 (Figs. 1H, 2B and H), and livers exhibited minimal hepatic vacuolar degeneration at day 7 (Fig. 1I) and few inflammatory cells (Fig. 2C and I). In contrast, histopathologic changes following HRS treatment included reduced hepatic edema at day 1 (Fig. 1J and K). After treatment with HRS, the positive cells of CD45 and CD68 were significantly less than those of the IRI group at day 1 and 3 (Fig. 2D, E, J, L–N). The portal region was approximately normal at day 7 (Figs. 1L, 2F and L). Liver histology showed that the scores of necrosis and vacuolar degeneration in the HRS-10 group were lower than that in the IRI group, but there were no significant differences between the two groups (Table 2). The results suggested that HRS reduced hepatic degeneration and inflammatory cell infiltration and protected against liver injury.

Effects of HRS on peripheral blood cells

We used blood cell analyzer to determine the effects of HRS on peripheral blood white blood cell, neutrophil and lymphocyte. As shown in Table 3, 6 h after the surgery, the increases of white blood cell in the HRS-10 group was significantly slower compared with the IRI group. The IRI group displayed the most apparent changes among all the time points. White blood cell and lymphocyte counts were significantly elevated in the IRI group within 6 h to day 1 after reperfusion, whereas neutrophil levels were significantly elevated within 6 h to day 3 ($P < 0.01$). After treatment with HRS, white blood cell counts were significantly reduced at 6 h ($P < 0.01$) and day 1 compared with the IRI group ($P < 0.05$). These results indicate that HRS plays a role in alleviating systemic injury.

Table 2
Pathological score of liver.

Groups	Necrosis			*P* value	Vacuolation			*P* value
	1d	3d	7d		1d	3d	7d	
IRI	2.83 ± 0.72	2.17 ± 0.75	0.67 ± 0.52	>0.05	2.67 ± 0.52	1.83 ± 0.41	0.83 ± 0.41	>0.05
HRS-10	2.33 ± 0.52	1.50 ± 0.55	0.50 ± 0.55		2.17 ± 0.41	1.33 ± 0.52	0.33 ± 0.52	

IRI: ischemia-reperfusion injury; HRS: hydrogen-rich saline.

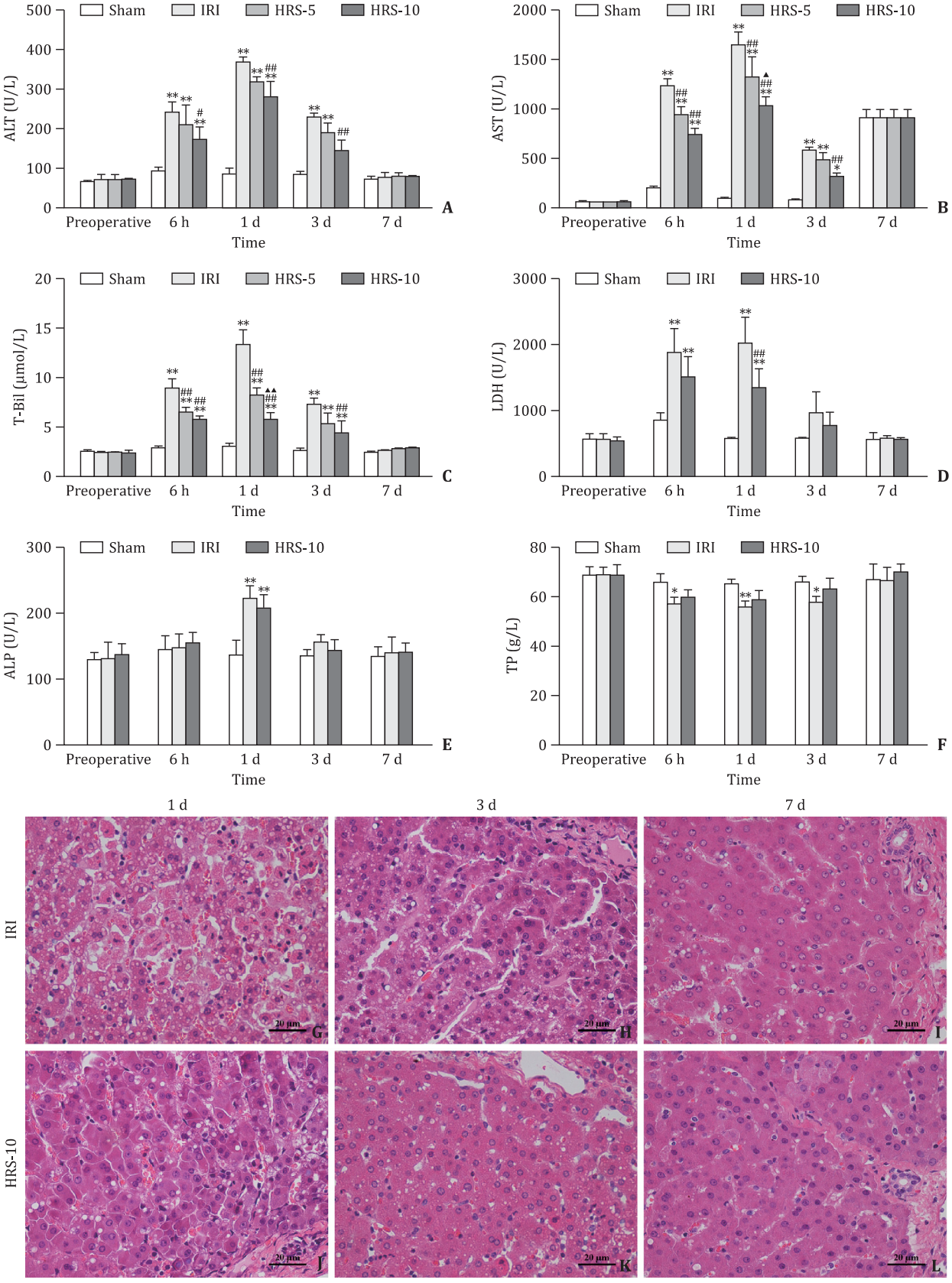

Fig. 1. Effects of HRS on hepatic biochemical parameters and histopathologic changes. **A–F**: Serum hepatic biochemical parameters ALT, AST, T-Bil, LDH, ALP and TP: **G–I:** Liver hematoxylin-eosin staining of the IRI group at day 1, 3 and 7 (original magnification × 400); **J–L**: Liver hematoxylin-eosin staining of the HRS-10 group at day 1, 3 and 7 (original magnification × 400). *: $P < 0.05$, **: $P < 0.01$, vs. the sham group. #: $P < 0.05$, ##: $P < 0.01$, vs. the IRI group. ▲: $P < 0.05$, ▲▲: $P < 0.01$, vs. the HRS-5 group. HRS: hydrogen-rich saline; IRI: ischemia-reperfusion injury; ALT: alanine aminotransferase; AST: aspartate aminotransferase; T-Bil: total bilirubin; LDH: lactate dehydrogenase; ALP: alkaline phosphatase; TP: total protein.

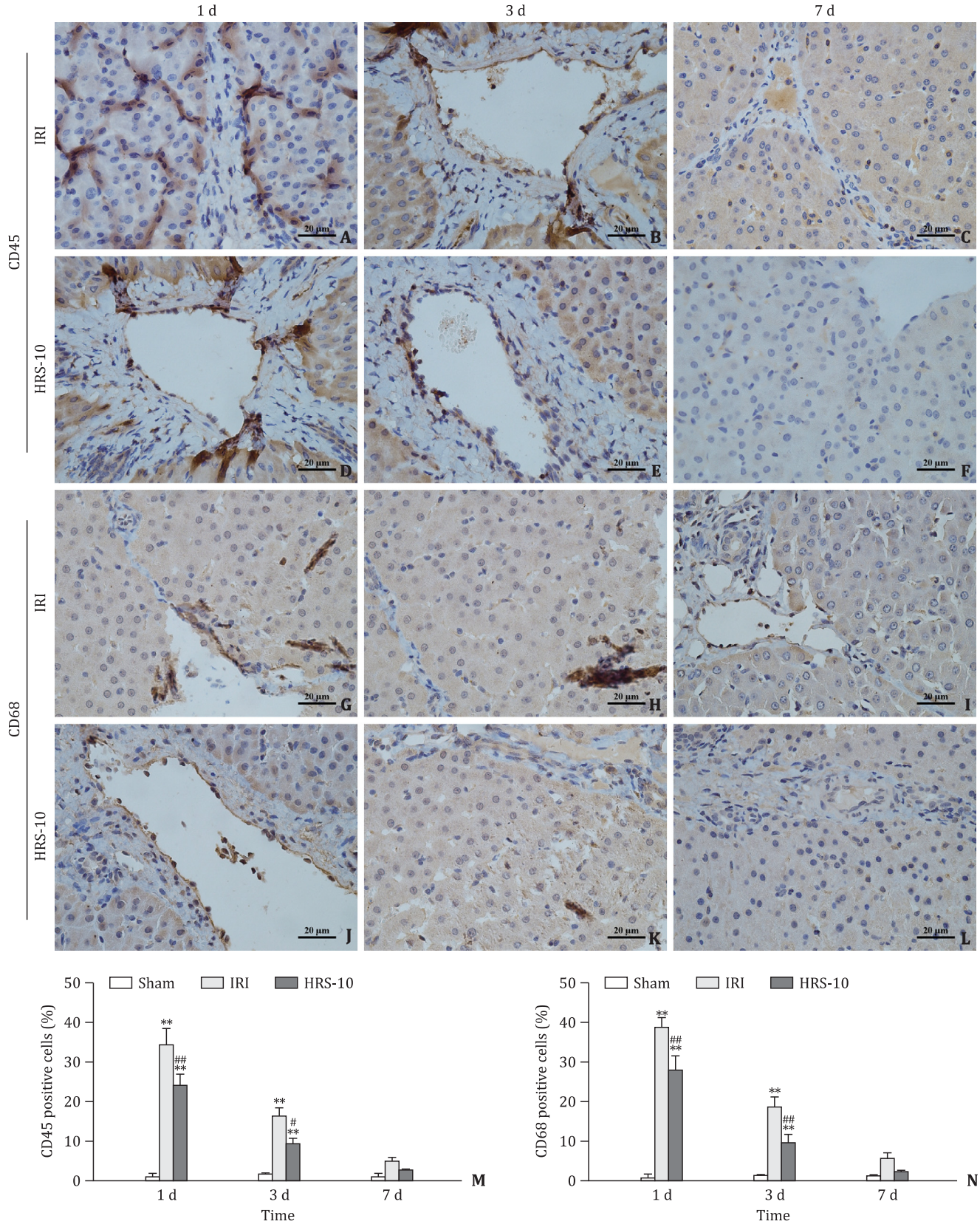

Fig. 2. HRS treatment reduces CD45 and CD68 positive cells in liver. Immunohistochemical staining of CD45 (**A**–**C** and **D**–**F**) and CD68 (**G**–**I** and **J**–**L**) in the IRI group and the HRS-10 group at day 1, 3 and 7 (original magnification × 400); **M** and **N:** CD45 and CD68 positive cells. *: $P < 0.05$, **: $P < 0.01$, vs. the sham group. #: $P < 0.05$, ##: $P < 0.01$, vs. the IRI group. HRS: hydrogen-rich saline; IRI: ischemia-reperfusion injury.

Table 3
Effects of HRS on peripheral blood.

Variables		Sham ($n=6$)	IRI ($n=6$)	HRS-10 ($n=6$)
White blood cell ($\times 10^9$/L)	Preoperative	19.13 ± 4.32	16.50 ± 3.95	18.68 ± 3.55
	6 h	31.20 ± 7.50*	43.73 ± 4.83**,▲	29.45 ± 7.10*,**
	1 d	22.03 ± 3.26	33.57 ± 10.14**,▲	24.33 ± 5.68*
	3 d	19.42 ± 3.91	27.08 ± 5.93	25.10 ± 4.26
	7 d	16.28 ± 2.88	20.92 ± 3.57	17.38 ± 2.91
Neutrophil ($\times 10^9$/L)	Preoperative	7.85 ± 0.94	7.07 ± 1.20	6.68 ± 1.16
	6 h	18.92 ± 3.26**	25.78 ± 4.69**,▲▲	21.23 ± 3.94**
	1 d	14.27 ± 1.96**	24.25 ± 3.52**,▲▲	20.22 ± 1.04**,▲▲
	3 d	8.53 ± 3.02	17.23 ± 3.07**,▲▲	13.30 ± 0.61**
	7 d	9.27 ± 1.55	9.42 ± 1.21	8.92 ± 1.36
Lymphocyte ($\times 10^9$/L)	Preoperative	7.20 ± 2.57	8.83 ± 4.57	8.18 ± 1.92
	6 h	15.23 ± 1.91**	21.12 ± 3.29**,▲	15.93 ± 0.90**
	1 d	11.48 ± 1.96	18.75 ± 3.45**,▲▲	15.58 ± 3.08**
	3 d	10.87 ± 2.95	13.65 ± 1.41	10.25 ± 2.25
	7 d	7.17 ± 3.34	8.40 ± 1.54	8.70 ± 1.61

* : $P<0.05$, vs. preoperative at different time points;
** : $P<0.01$, vs. preoperative at different time points;
▲ : $P<0.05$, vs. the sham group;
▲▲ : $P<0.01$, vs. the sham group;
⋆ : $P<0.05$, vs. the IRI group;
⋆⋆ : $P<0.01$, vs. the IRI group. HRS: hydrogen-rich saline; IRI: ischemia-reperfusion injury.

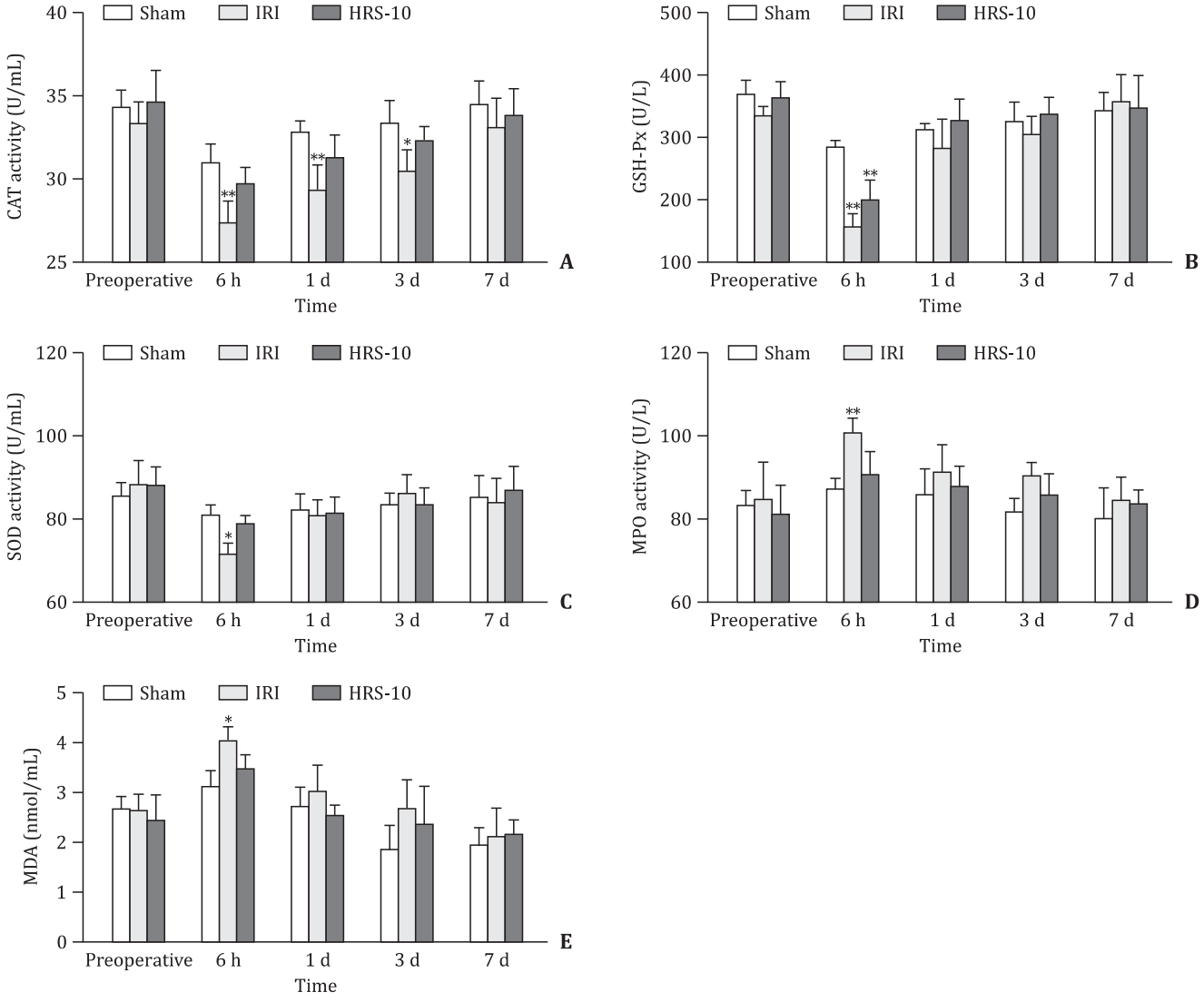

Fig. 3. Effects of HRS-mediated reductions in oxidative stress in serum. CAT, GSH-Px, SOD and MPO activities (**A**–**D**) and MDA levels (**E**). The results were expressed as mean ± SD. *: $P<0.05$, **: $P<0.01$, vs. the sham group. CAT: catalase; GSH-Px: glutathione peroxidase; SOD: superoxide dismutase; MPO: myeloperoxidase; MDA: malondialdehyde; IRI: ischemia-reperfusion injury; HRS: hydrogen-rich saline.

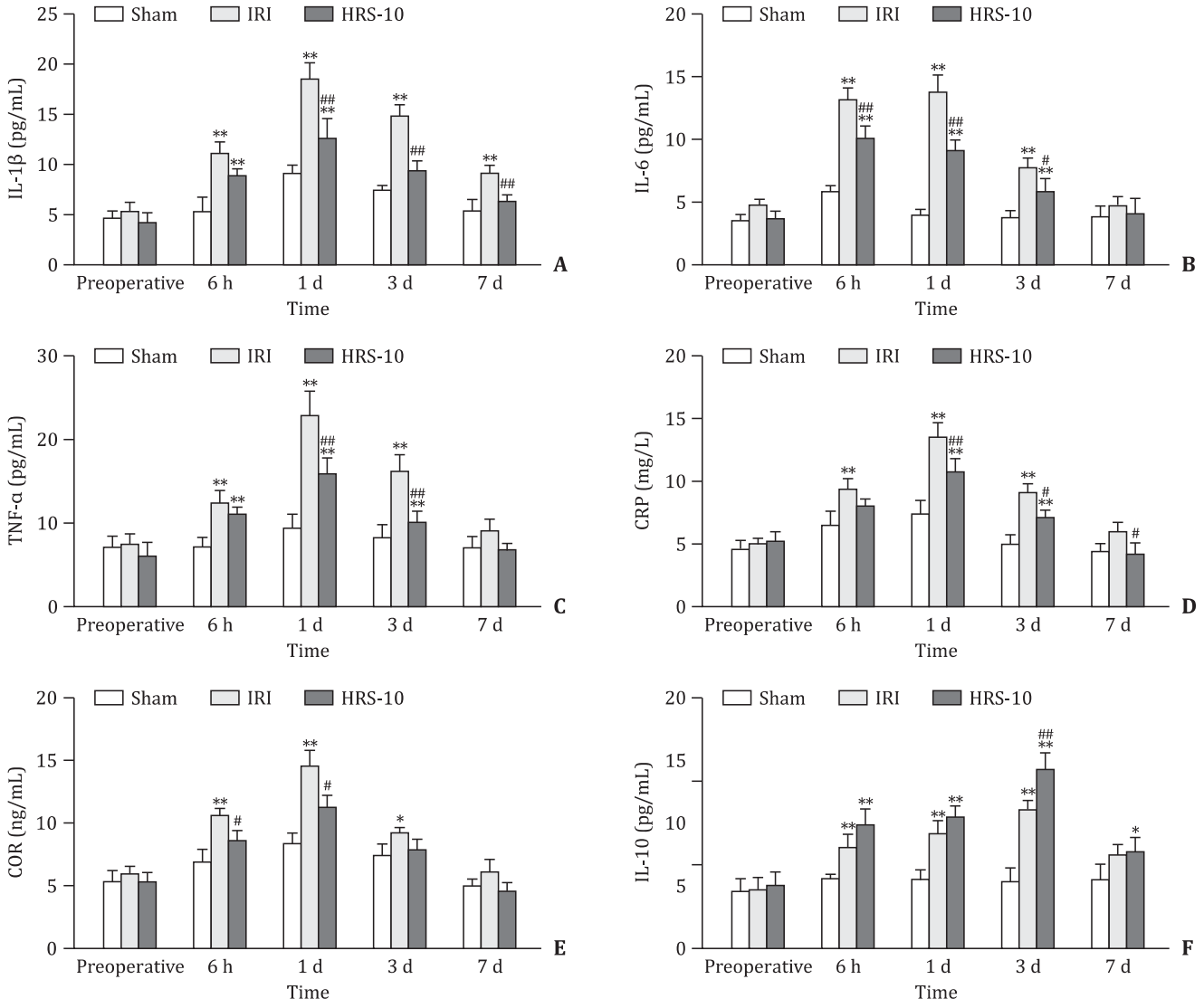

Fig. 4. Effects of HRS on the serum inflammatory response. HRS treatment reduces the expression of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α (**A–C**), inflammatory mediators CRP and COR (**D** and **E**), and increases anti-inflammatory cytokine IL-10 (**F**) in serum. The data were expressed as mean ± SD, *: $P < 0.05$, **: $P < 0.01$, vs. the sham group. #: $P < 0.05$, ##: $P < 0.01$, vs. the IRI group. IL-1β: interleukin-1 beta; IL-6: interleukin-6; TNF-α: tumor necrosis factor alpha; CRP: C-reactive protein; COR: cortisol; IRI: ischemia-reperfusion injury; HRS: hydrogen-rich saline.

Effects of HRS on oxidative stress

To evaluate the effects of HRS on oxidative stress, we measured concentrations of the antioxidant enzymes, MPO and MDA. Compared with the sham group, we observed a significant CAT reduction at 6 h and day 1 ($P < 0.01$) in the IRI group. The levels of CAT were increased at 6 h after HRS treatment compared with the IRI group ($P < 0.01$, Fig. 3A). GSH-Px levels were significantly decreased in the IRI and HRS-10 groups at 6 h ($P < 0.01$, Fig. 3B). Moreover, serum SOD activity was the lowest in the IRI group at 6 h, and was significantly lower than the sham group ($P < 0.05$, Fig. 3C). Additionally, IRI combined with hepatectomy induced sharp increases in MPO at 6 h ($P < 0.01$, Fig. 3D). MDA levels were elevated at 6 h in the IRI group compared with the sham group ($P < 0.05$, Fig. 3E). HRS application decreased the increases in MPO and MDA levels, but there were no significant differences between the IRI group and the HRS-10 group. These results indicate that HRS suppresses oxidative stress by elevating the CAT levels and reducing the production of MPO.

Effects of HRS on inflammatory response

Serum IL-1β, IL-6 and TNF-α levels were increased during 6 h to day 3 compared with the sham group (Fig. 4A–C). After HRS treatment, the levels of these cytokines were reduced compared with the IRI group. Similarly, CRP and COR levels were significantly reduced in the HRS-10 group compared with the IRI group (Fig. 4D and E). Moreover, IL-10 serum levels were elevated and peaked at day 3 after reperfusion. HRS treatment increased IL-10 levels at all time points compared with the IRI group (Fig. 4F).

Moreover, TNF-α, IL-1β, IL-6 and IL-10 mRNA expressions were significantly increased in the IRI group ($P < 0.01$). HRS treatment significantly reduced the increased levels of TNF-α, IL-1β, and IL-10 mRNA expressions compared with the IRI group (Fig. 5A–C). Anti-inflammatory cytokine IL-10 levels were elevated after reperfusion in both the IRI and HRS-10 groups. After HRS treatment, IL-10 mRNA expression was significantly increased compared with that of the IRI group at day 1 and 3 ($P < 0.01$, Fig. 5D).

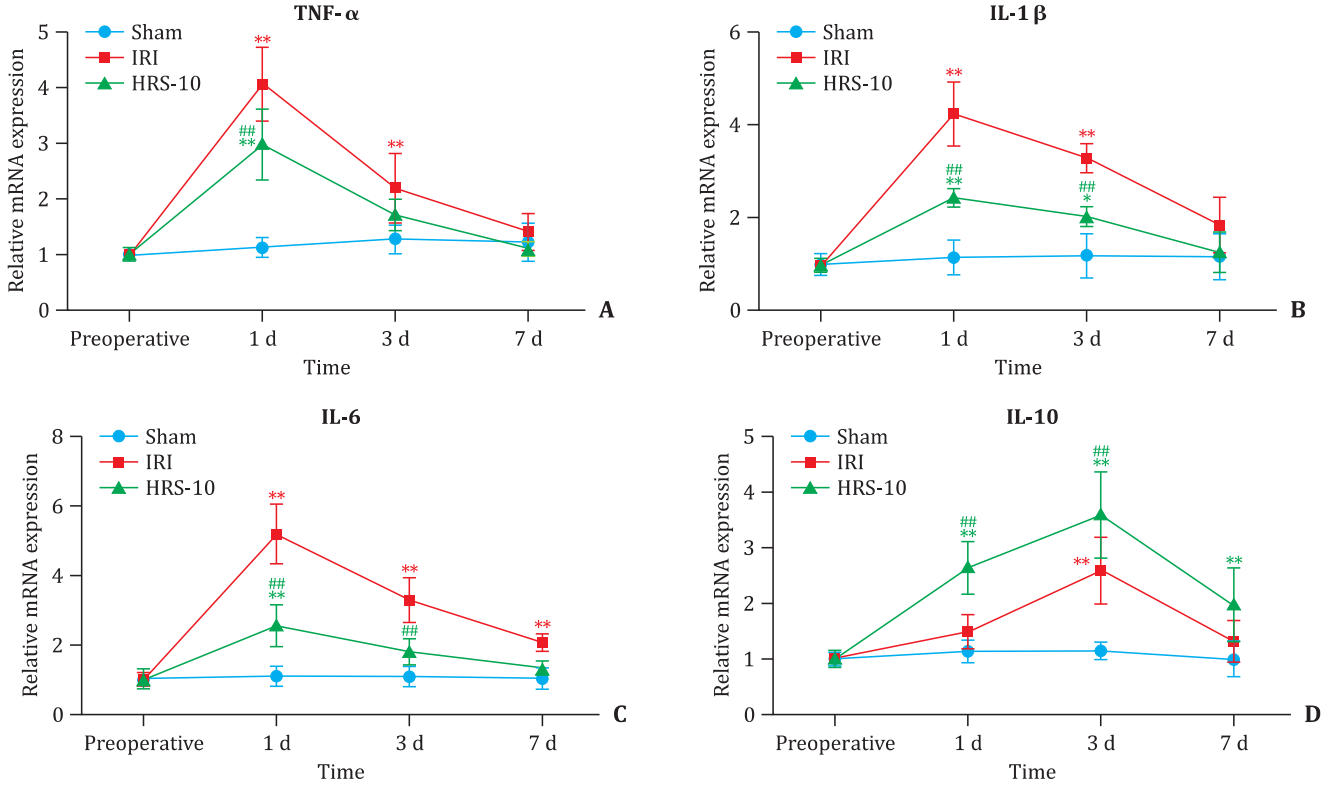

Fig. 5. Quantitative real-time PCR analysis of inflammatory cytokines in liver tissues. HRS treatment decreases the expression of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 mRNA (**A**–**C**) and increases anti-inflammatory cytokine IL-10 mRNA (**D**) in liver tissues. The data were expressed as mean ± SD, *: $P < 0.05$, **: $P < 0.01$, vs. the sham group. ##: $P < 0.01$, vs. the IRI group. TNF-α: tumor necrosis factor alpha; IL-1β: interleukin-1 beta; IL-6: interleukin-6; IL-10: interleukin-10; IRI: ischemia-reperfusion injury; HRS: hydrogen-rich saline.

Effects of HRS on angiogenic factors and HA

As shown in (Fig. 6), serum levels of VEGF, ANG-1 and ANG-2 in both the IRI group and the HRS-10 group were significantly increased after surgery, and peaked at day 3. VEGF and ANG-1 levels were returned to normal at day 7. HRS treatment significantly increased serum VEGF, ANG-1 and ANG-2 levels at day 3 ($P < 0.01$, Fig. 6A–C). After hepatectomy, serum HA levels in the IRI group were significantly increased at day 1 and 3, and were significantly reduced after HRS treatment ($P < 0.01$, Fig. 6D). In addition, the changes of angiogenic factors in the liver were similar to those in the serum. HRS treatment significantly increased the levels of VEGE and ANG-2 at day 3, and ANG-1 levels significantly elevated at day 1 and 3 in the HRS-10 group compared with the IRI group ($P < 0.01$, Fig. 6E–G).

Effects of HRS on growth factors

The mRNA and protein levels of HGF and Cyclin D1 in the IRI group and the HRS-10 group markedly increased at day 1 and began to decrease at day 3. After treatment with HRS, the mRNA and protein expression of HGF in the liver was increased significantly at day 1–3, while Cyclin D1 was increased significantly at day 1 compared with the IRI group ($P < 0.01$, Fig. 7A, B and D–E). Additionally, TGF-β mRNA and protein levels were significantly elevated at day 1 and 3 in the IRI and HRS-10 groups. Compared with the IRI group, TGF-β levels were significantly reduced in livers with HRS treatment at day 3 ($P < 0.01$, Fig. 7C and F). These results suggest that HRS may regulate the expression of growth factors in the liver.

Effects of HRS on liver regeneration

PCNA and Ki-67 levels (Fig. 8) were increased significantly at day 1 and 3 after hepatectomy in the IRI and HRS groups. The maximal PCNA and Ki-67 labeling indexes were observed in pigs subject to HRS treatment at day 1 and returned to normal level at day 7. HRS treatment significantly enhanced the expression of PCNA at day 1 and 3 compared with the IRI group ($P < 0.01$, Fig. 8M), whereas the expression of Ki-67 in the two groups showed significant difference at day 1 ($P < 0.05$, Fig. 8N).

Discussion

Laparoscopic liver resection has been widely substituted for hepatic surgery with low morbidity and mortality [23]. Hepatic IRI is inevitable during hepatic surgery and is a key factor associated with postoperative hepatic failure. Our previous studies have shown that HRS treatment attenuates apoptosis induced by hepatic I/R and hepatectomy [20], and inhibits the activation of endoplasmic reticulum stress signaling pathways [19]. In the present study, we demonstrated that HRS treatment significantly reduced ALT and AST, and T-Bil. The HRS-10 group showed more effective liver protection than the HRS-5 group, and previous study has proved that treatment with hydrogen gas significantly suppressed hepatic IRI in a concentration dependent manner [17].

The pathophysiological process of IRI is complex and consists of several steps. In a hepatectomy, liver function becomes insufficient in the remnant liver, and IRI leads to ROS and inflammatory cytokines secretion, leading to hepatic necrosis [24]. Our previous studies have shown that HRS treatment reduces apoptosis and endoplasmic reticulum stress induced by hepatic I/R and

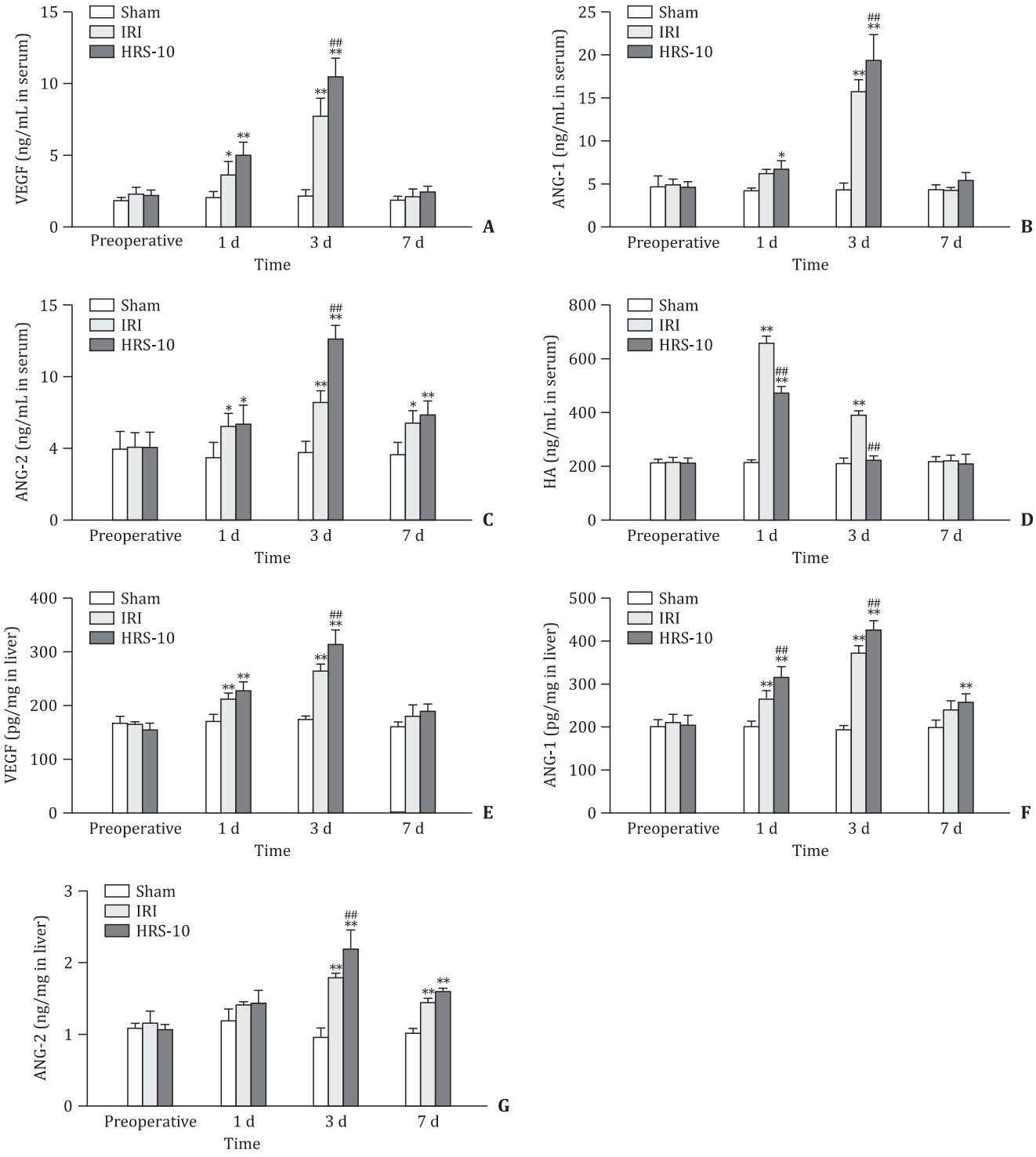

Fig. 6. Effects of HRS on serum angiogenic factors. HRS treatment enhances the expression of angiogenic factors VEGF (**A** and **E**), ANG-1 (**B** and **F**) and ANG-2 (**C** and **G**) in serum and liver tissues, and reduces serum HA levels (**D**). The data were expressed as mean ± SD. *: $P < 0.05$, **: $P < 0.01$, vs. the sham group. ##: $P < 0.01$, vs. the IRI group. VEGF: vascular endothelial growth factor; ANG: angiopoietin; HA: hyaluronic acid; IRI: ischemia-reperfusion injury; HRS: hydrogen-rich saline.

hepatectomy [19,20]. Therefore, the present study investigated the effects of HRS on liver function and regeneration in a swine model after hepatic I/R and subsequent hepatectomy.

The early stage of IRI is mainly caused by superoxide radicals. The superoxide radical is catalyzed by SOD through dismutation; its product, H_2O_2 is converted to H_2O and free oxygen by GSH-Px and CAT [25]. MDA is widely accepted as a marker of lipid peroxidation and plays an essential role in oxidative damage. MDA can alter cellular membrane characteristics and indirectly reflect the level of oxygen radicals [11]. Hydrogen has systemic anti-oxidative activity because of its effective scavenging of ROS. Previous research has reported that hydrogen may increase SOD, GSH-Px and CAT activities, restore depleted GSH and downregulate the generation of MDA [26]. In the present study, pretreatment with HRS increased serum CAT, SOD and GSH-Px levels and reduced the concentration of MDA.

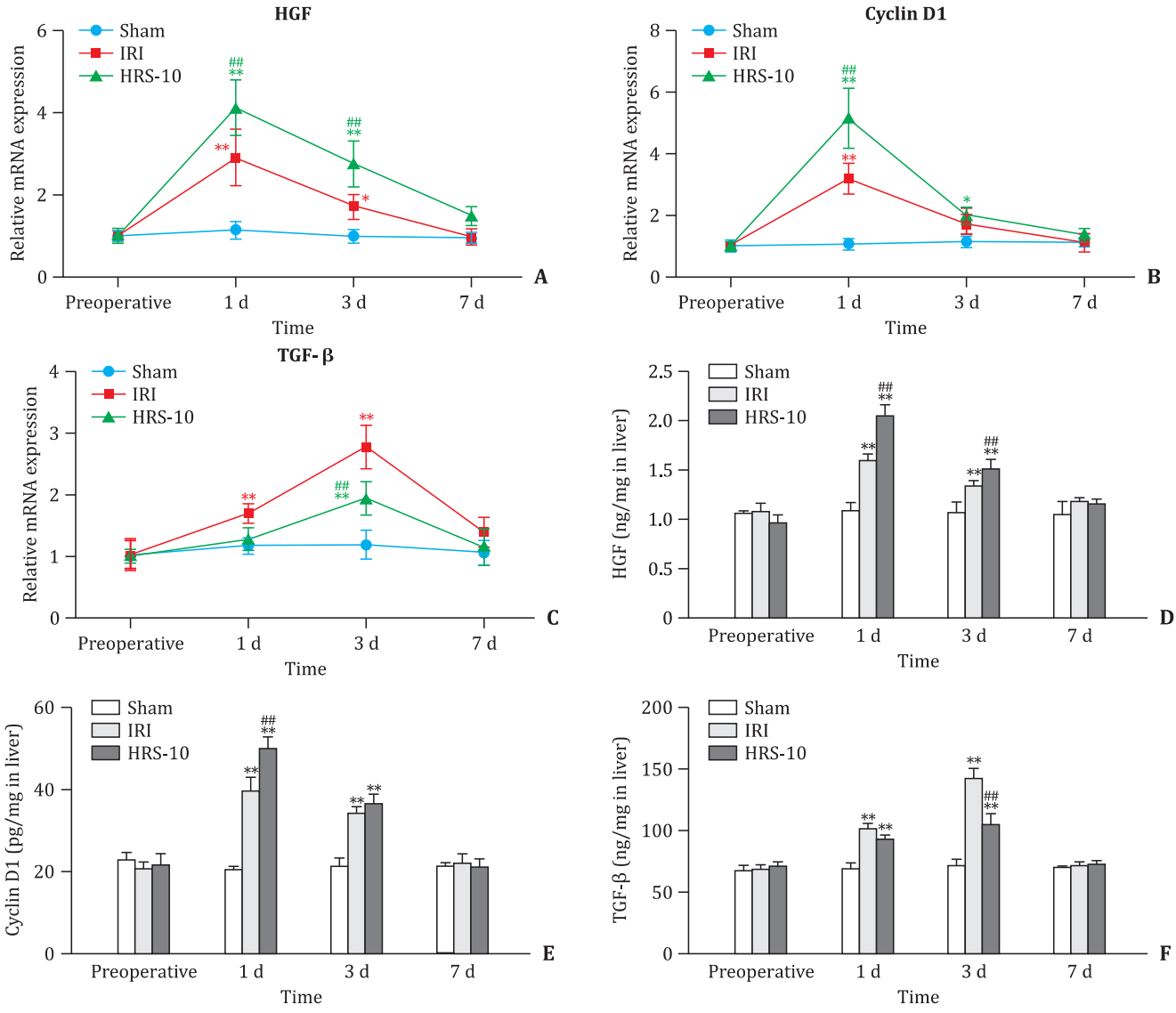

Fig. 7. Expression of genes involved in liver regeneration. HRS treatment increases the expression of growth factors HGF (**A** and **D**) and Cyclin D1 (**B** and **E**) protein and mRNA in liver tissues, and inhibits the expression of TGF-β (**C** and **F**). The data were expressed as mean ± SD. *: $P < 0.05$, **: $P < 0.01$, vs. the sham group. ##: $P < 0.01$, vs. the IRI group. HGF: hepatocyte growth factor; TGF-β: transforming growth factor-beta; IRI: ischemia-reperfusion injury; HRS: hydrogen-rich saline.

MPO is stored primarily in polymorphonuclear neutrophils and monocytes, plays a fundamental role in the generation of ROS and serves as an indicator of inflammation. The increase of MPO reflects neutrophil infiltration. MPO is released into extracellular fluid during the inflammatory response and catalyzes H_2O_2 into hypochlorous acid (HClO) [27]. In the present study, serum MPO activity in the HRS-10 group was lower than that in the IRI group. We observed extensive hepatic necrosis, hemorrhage and CD45 and CD68 positive cells in the IRI group, and HRS treatment attenuates hepatic damage and the inflammatory response. This finding is supported by Guo's study, which demonstrated that HRS therapy reduces the levels of MPO in serum and kidneys and inhibits the release of inflammatory mediators [28]. Regarding the increased inflammatory reaction in serum, we observed that white blood cell, neutrophil and lymphocyte counts were significantly increased at 6 h and day 1 in the IRI and HRS-10 groups compared with preoperative time point. In addition, the increased total white blood cell count in IRI swine was significantly decreased after HRS treatment at 6 h and day 1. Neutrophil and lymphocyte counts were also reduced in the HRS-10 group. However, no significant differences were noted between the IRI group and the HRS-10 group.

Previous studies have demonstrated that liver IRI triggers pro-inflammatory immune responses and systemic inflammation. Hydrogen attenuates the impact of oxidative stress and inflammation that protects the liver against the injury of oxidative stress [29]. TNF-α, IL-1β and IL-6 are biomarkers of the inflammatory reaction. During hepatic I/R, Kupffer cells are activated and release TNF-α and oxygen free radicals, whereas macrophages release IL-1β and IL-6 [30]. A previous study reported that the excessive release of pro-inflammatory cytokines aggravates the damage, and downregulation of pro-inflammatory cytokines may alleviate IRI [31]. In the present study, HRS significantly reduced serum TNF-α, IL-1β and IL-6 levels compared with the IRI group. The inhibition of inflammatory mediators by HRS has been reported in alcohol, doxorubicin and acetaminophen-induced liver injury models [26,32,33]. Moreover, similar changes in TNF-α, IL-1β and IL-6 mRNA expressions were also observed in liver tissues. Previous studies have shown that postoperative CRP level is consistent with the magnitude of surgical injury, and can be used as a marker for assessing postoperative inflammatory response [34,35]. Our results indicate that CRP levels are significantly decreased in pigs treated with HRS, indicating that HRS can alleviate the inflammatory response,

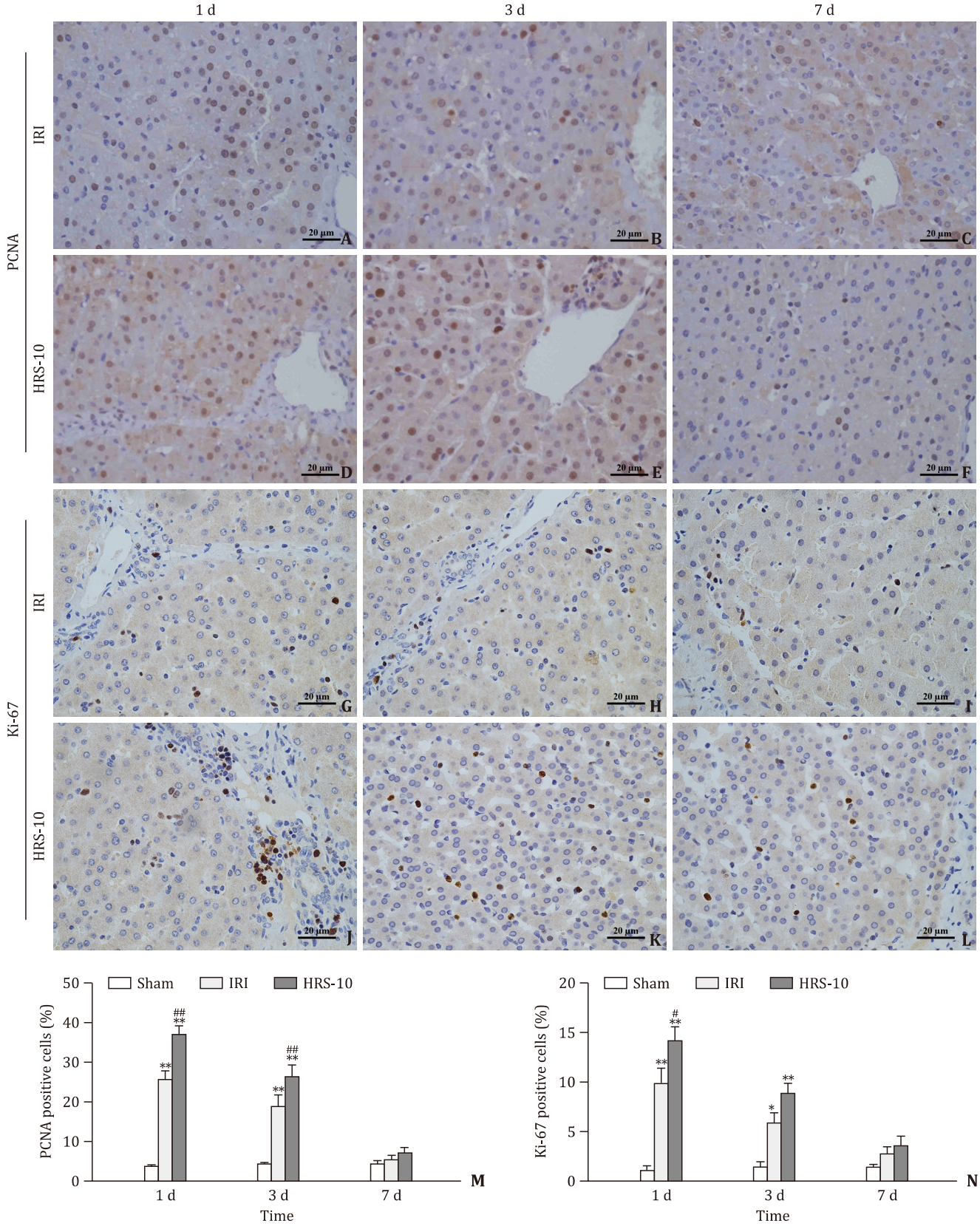

Fig. 8. Evaluation of liver regeneration in pigs treated with HRS (original magnification × 400). HRS treatment increases the expression of nuclear antigen PCNA and Ki-67 proteins in liver. Immunohistochemical staining of PCNA (**A–C** and **D–F**) and Ki-67 (**G–I** and **J–L**) in the IRI group and the HRS-10 group at day 1, 3 and 7. PCNA (**M**) and Ki-67 (**N**) positive cell rates. The data were expressed as mean ± SD. **: $P < 0.01$, vs. the sham group. #: $P < 0.05$, ##: $P < 0.01$, vs. the IRI group. PCNA: proliferating cell nuclear antigen; HRS: hydrogen-rich saline; IRI: ischemia-reperfusion injury.

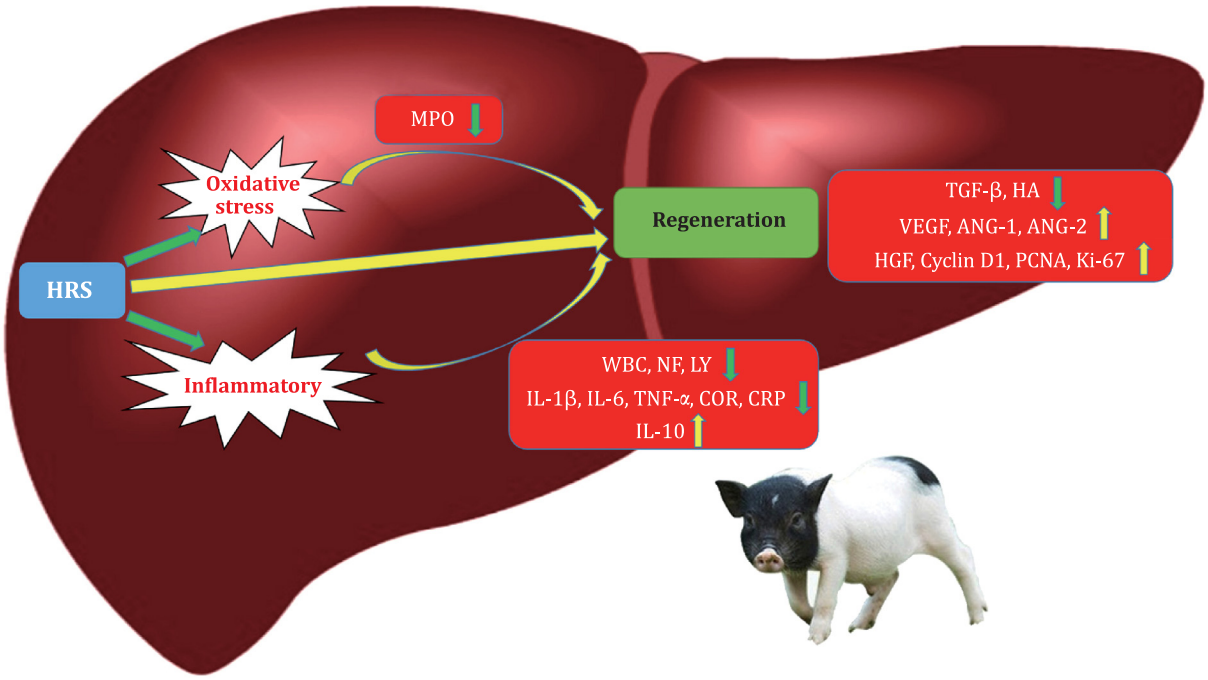

Fig. 9. Effects of HRS on I/R and hepatectomy. HRS: hydrogen-rich saline; IRI: ischemia-reperfusion injury.

as previously described in intestinal inflammation model [36]. COR is a steroid hormone produced by the zone fasciculate. COR is released in response to stress and suppresses the immune system by reducing TNF-α and IL-1β levels and upregulates IL-10 levels [37]. COR is metabolized in the liver, and increased COR levels reflect hepatic damage [38]. Our study demonstrated that HRS treatment reduces COR in IRI swine. The anti-inflammatory cytokine IL-10 plays an important role during the inflammatory response. IL-10 inhibits the production of TNF-α, IL-1β and IL-6 [39]. Consistent with the previous studies [40,41], our results confirmed that HRS treatment significantly increased IL-10 serum levels and mRNA expression in liver.

VEGF, ANG-1 and ANG-2 are important regulators of angiogenesis. VEGF has been reported to play a dual role in the process of I/R. In the early stage of reperfusion, the elevation of VEGF may increase vascular permeability and aggravate inflammatory response. At the same time, VEGF can increase the blood perfusion and oxygen supply of ischemia tissue, making the body more easily adapt to ischemia and hypoxia and its role in accelerating neovascularization also promotes the regeneration of liver [42]. ANG-1 stimulates the sprouting process of blood vessels and promotes the maturation of new blood vessels, and ANG-2 plays an important role in promoting angiogenesis when interacting with VEGF [43]. Our results indicate that treatment with HRS significantly upregulated the secretion of angiogenic factors. This finding is contrary to the role of HRS in inhibiting pathologic corneal neovascularization in a corneal alkali-burn model [44], and this may be related to the inhibition of the expression of inflammatory mediators by highly concentrated angiogenic factors [45]. Moreover, hydrogen may attenuate severe damage to the sinusoidal endothelial cells (SECs) of the remnant liver after hepatectomy [16]. The uptake and elimination of HA mainly occurs in hepatic SECs. Increasing concentrations of serum HA reflect SECs damage [46]. In our study, the significantly reduced HA level in the HRS group indicates that hepatic SEC damage is reduced in pigs treated with HRS after I/R and hepatectomy injury.

Previous study has shown that treatment with hydrogen could facilitate hepatocyte mitosis and promote liver regeneration [47]. HGF is a mitogenic agent for hepatocytes that exhibits significant effects on liver regeneration after hepatectomy [48]. HGF stimulates DNA synthesis and regulates cell growth by binding to the c-Met receptor, which activates a tyrosine kinase signaling cascade [49]. Moreover, Cyclin D1 is a marker for progression through the G1 phase of the cell cycle. During the G1 phase, Cyclin D1 is synthesized rapidly and is degraded as the cell enters into S phase [50]. TGF-β is an important cytokine that inhibits the proliferative response after partial hepatectomy [51]. Our study showed that HRS treatment increased HGF and Cyclin D1 expression and reduced TGF-β levels, and Yu's study has proved that lactulose can nhance hydrogen in promoting liver regeneration [52]. The data suggested that HRS may promote hepatocyte proliferation after hepatic IRI and hepatectomy.

PCNA is an auxiliary factor of DNA polymerase that regulates DNA replication and the cell cycle. During DNA replication, PCNA acts as a homotrimer and helps increase the processivity of leading strand synthesis. In addition, Ki-67 protein is thought to be exclusively expressed in proliferating cells. Therefore, hepatocyte proliferation status can be detected by labeling PCNA and Ki-67 proteins [53]. Studies have demonstrated that HRS can increase the levels of liver PCNA and Ki-67 [11,15]. Our data showed that HRS treatment significantly increased the expression of PCNA and Ki-67 compared with the IRI group, consisting with the trend of regeneration related factors IL-6 and TNF-α. The results indicate that HRS may promote hepatocyte regeneration.

In conclusion, our study demonstrates that HRS attenuates I/R and hepatectomy-induced hepatic injury via reducing oxidative stress, decreasing the inflammatory response and facilitating liver regeneration. HRS reduces ROS, inflammatory cells, pro-inflammatory cytokines and increases anti-inflammatory cytokines. In addition, HRS treatment attenuates ESCs damage, and promotes angiogenesis and hepatocyte mitosis Fig. 9. The data indicates that HRS has a potential therapeutic effect on hepatic I/R injury and subsequent hepatectomy. HRS pretreatment may alleviate postoperative injury of liver surgery, thus HRS is a promising therapeutic option for extracorporeal circulation perfusion to reduce the complications after liver transplantation.

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Contributors

GYS and WHB designed the research. GYS wrote the manuscript. GYS, ZQZ, LH and BG performed the experiments. GYS and JZH analyzed the data. All authors contributed to the design and interpretation of the study and to further drafts. WHB is the guarantor.

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Ethical approval

This study was approved by the Animal Care and Use Committee of Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences (SQ-2017-420).

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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