ELSEVIER Original Article

Lack of Protection of Ischaemic Preconditioning in the Rat Model of Major Hepatectomy With Ischaemia Reperfusion Injury

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OBJECTIVE: To investigate the effects of ischaemic preconditioning (IP) on residual liver regeneration after major hepatectomy without portal blood bypass in rats, and to verify whether it can protect the residual liver from ischaemia reperfusion (IR) injury.

METHODS: Ninety rats were randomized into three groups: Group PH, rats were subjected to 70% hepatectomy alone; Group IR, rats were subjected to 30 minutes of total hepatic ischaemia, and 70% hepatectomy was performed just before reperfusion; Group IP, rats were pretreated with IP (5/10 minutes). During the preoperative period and at 0.5, 6, 12, 24 and 48 hours after the operation, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured using an autoanalyser. Serum hyaluronic acid (HA) was measured by radioimmunoassay. Regenerated liver weight (RLW) of the rats was measured and the expressions of Ki-67 and cyclin D1 were determined by immunohistochemistry in remnant liver tissue.

RESULTS: There were no significant differences in serum AST and ALT levels in all the groups before the operation. After partial hepatectomy, AST and ALT levels increased rapidly. From 0.5 to 24 hours after operation, serum AST and ALT levels were significantly higher in IP group rats than in PH and IR rats ($p < 0.05$). There were no significant differences in serum HA levels in all the groups before the operation. After partial hepatectomy, HA levels increased rapidly, reaching peak values at 12 hours. In the early stage (during 12 hours) after the operation, HA level was significantly higher in IP rats than in PH and IR rats ($p < 0.05$). The RLW of the rats rapidly increased after partial hepatectomy, and significantly decreased in IP rats compared with PH and IR rats ($p < 0.05$). Cyclin D1 and Ki-67 expression in all groups before the operation were low and were not significantly different. After partial hepatectomy, they rapidly increased. The expression of Ki-67 and cyclin D1 reached a peak at 24 hours after the operation in PH rats, and they were significantly higher compared with IR and IP rats ($p < 0.05$). In groups IR and IP, the expression of cyclin D1 and Ki-67 reached peak values at 48 hours. A significant decrease ($p < 0.05$) was observed after 24 and 48 hours of reperfusion in group IP compared with groups PH and IR.

CONCLUSION: IP impairs residual liver regeneration after major hepatectomy without portal blood bypass in rats, and protection from IR injury disappears. IP-induced hyperperfusion may be the cause of reduced liver regeneration. [*Asian J Surg* 2008;31(3):140–7]

Key Words: cyclin D1, hyaluronic acid, ischaemia reperfusion injury, ischaemic preconditioning, liver regeneration

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Introduction

In liver operations (such as partial hepatectomy and liver transplantation), ischaemia reperfusion (IR) is inevitable. It may induce metabolism in hepatocytes, including damage to structure and function, resulting in postoperative organ dysfunction or even liver failure.[1,2] Ischaemic preconditioning (IP) is a process in which a brief episode of ischaemia and reperfusion effectively protect the organ against the longer subsequent ischaemia. The phenomenon was first described in the heart by Murry et al.[3] It has been documented that exposure of the liver to a short period of ischaemia (5–10 minutes) can induce the organism to have one kind of endogenous protection mechanism, which will predispose the liver to IR injury.[4,5] However, the mechanism of such preconditioning is poorly understood.

It is well known that the regenerative capacity of remnant liver tissue represents a key effect of treatment given to increase cell function and viability of liver tissue during partial hepatectomy.[5,6] Teoh et al used a murine model of partial hepatic ischaemia and demonstrated that the hepatoprotective effects of IP are associated with entry of hepatocytes into the cell cycle, a critical biological effect that favours survival of the liver against IR injury.[7] Bedirli et al reported that, in a model of portal blood bypass, IP could ameliorate the hepatic injury associated with IR and has a stimulatory effect on liver cell regeneration after partial hepatectomy.[8] However, portal blood bypass is complex, and the whole body heparin has a greater influence on the organism. Therefore, clinical application is difficult. We have studied portal clamping without portal blood bypass, which will later be used clinically. In this study, we examined the effects of IP on regeneration of the residual liver after major hepatectomy, and investigated whether IP could protect the residual liver from IR injury without portal blood bypass.

Materials and methods

Animals and surgical procedure

Ninety female Sprague-Dawley rats (body weight, 300 ± 25 g; Center of Animal Laboratories, Anhui Medical University, Hefei, China) were used for the experiments. The animals were housed individually in cages with a 12-hour light-dark cycle and allowed free access to standard rat chow and water before and after the experiments. The animals were fasted overnight before the experiments but were given free access to water. After induction of isoflurane/oxygen inhalation anaesthesia, the animals were laparotomized.

The animals were allocated to three groups. In the nonischaemic control group (PH group), a midline incision was done in the upper abdomen and the liver was freed from its ligament. The left lateral and median lobes were excised (70% hepatectomy). In the ischaemic group (IR group), a midline incision was made in the upper abdomen and the liver was freed from its ligament. Animals were subjected to 30 minutes of total hepatic ischaemia by cross-clamping the hepatic artery, the portal vein, and the common bile duct with a microvascular clamp. The left lateral and median lobes were excised (70% hepatectomy) just before reperfusion. In the IP group, a midline incision was made in the upper abdomen and the liver was freed from its ligament. IP was performed by subjecting the rats to 5 minutes of hepatic ischaemia followed by 10 minutes of reperfusion before 30 minutes of total hepatic ischaemia. The left lateral and median lobes were excised (70% hepatectomy) just before reperfusion.

After the operation, the animals were kept in individual cages. Five rats from each group were killed by exsanguination at each time point: preoperatively, and 0.5, 6, 12, 24 and 48 hours after operation; blood and liver samples were stored for enzymatic, biochemical and histological analyses. The residual liver lobes were removed and weighed.

Hepatocellular damage

We measured the extent of hepatocellular damage by enzymatic determination of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and hyaluronic acid (HA) levels. AST and ALT activities were measured using an autoanalyser. The expression of HA was measured using a radioimmunoassay kit (Beifang Biotechnology Institute, Beijing, China).

The residual liver tissues were preserved for 24 hours in 10% formalin and then embedded in paraffin for histopathological assessment. From the paraffin-embedded tissue blocks, 5-μm sections were cut and stained with haematoxylin and eosin (H&E) according to standard protocols. The severity of hepatic injury was evaluated.

Regenerated liver weight

The weight of the residual liver lobes was used to calculate the regenerated liver weight (RLW). RLW was assessed using the following equation: weight of residual liver lobes/body weight × 100 (vol%).

Immunohistochemistry

Cyclin D1 and Ki-67 were useful for monitoring proliferation in rat liver. They were determined by immunohistochemical analysis and densitometry, using an indirect enzyme-linked antibody method with the immunohistochemistry kits of Max-Vision™ (Maixin.Bio, Fuizhou, China). The proliferation index of cyclin D1-stained and of Ki-67-stained tissue were quantitatively assessed using Metamorph Image Analysis (Universal Imaging Corp., West Chester, PA, USA), determined as the integral optical density (high powered field, 200×).

Statistical analysis

All values were expressed as mean ± standard deviation. After testing for normality and equal variance across the groups, intergroup differences were assessed using the appropriate pair-wise comparison test (ANOVA). When a difference was found, specific differences were identified by the Dunn test. Statistical evaluation was carried out using SAS version 9.0 (SAS Institute Inc., Cary, NC, USA). Values of $p < 0.05$ were considered to be statistically significant.

Results

Expression of serum AST and ALT

The time course of AST and ALT changes in the animals are depicted in Figures 1 and 2. There were no significant differences in the levels of serum AST and ALT in all the groups before the operation ($p > 0.05$). After partial hepatectomy, the levels rapidly increased, reaching peak values at 12 hours in group PH, and at 6 hours in groups IR and IP. Serum AST and ALT levels were significantly higher in the IP group than in the PH and IR groups from 0.5 to 24 hours after operation ($p < 0.05$).

Expression of serum HA

Figure 3 shows the levels of serum HA. There were no significant differences in the levels of serum HA in all the groups before the operation ($p > 0.05$). After partial hepatectomy, they rapidly increased, reaching peak values at 12 hours. In the early stage (up to 12 hours) after operation, the levels of HA were significantly higher in the IP group than in the PH and IR groups ($p < 0.05$).

RLW

The RLW of the rats are shown in Figure 4. There were no significant differences in RLW in all the groups before

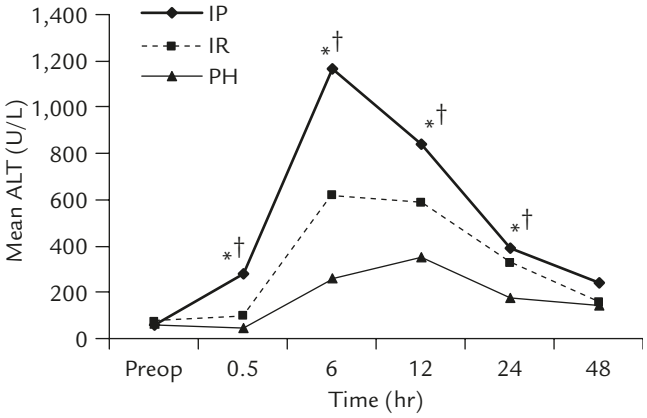

Figure 1. Levels of serum alanine aminotransferase (ALT) before and 0.5 to 48 hours after operation. *$p < 0.05$ versus IR and PHx groups; †$p < 0.05$ versus IP and IR groups.

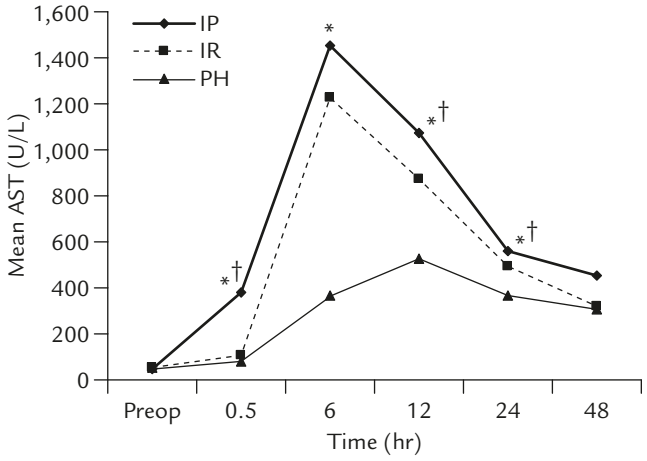

Figure 2. Levels of serum aspartate aminotransferase (AST) before and 0.5 to 48 hours after operation. *$p < 0.05$ versus IR and PHx groups; †$p < 0.05$ versus IP and IR groups.

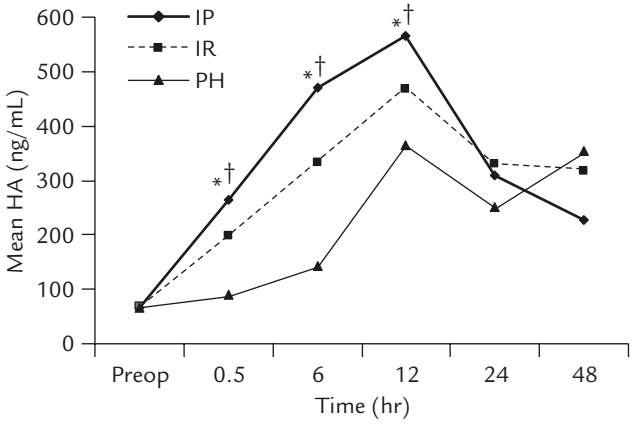

Figure 3. Levels of serum hyaluronic acid (HA) before and 0.5 to 48 hours after operation. *$p < 0.05$ versus IR and PHx groups; †$p < 0.05$ versus IP and IR groups.

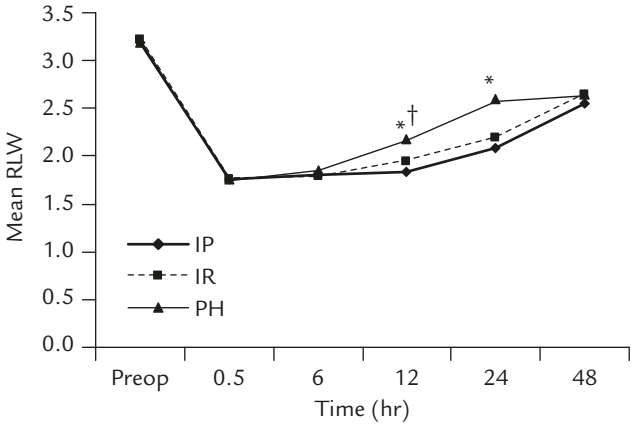

Figure 4. Regenerated liver weight (RLW) of the rats before and 0.5 to 48 hours after operation. *$p < 0.05$ versus IR and PHx groups; †$p < 0.05$ versus IP and IR groups.

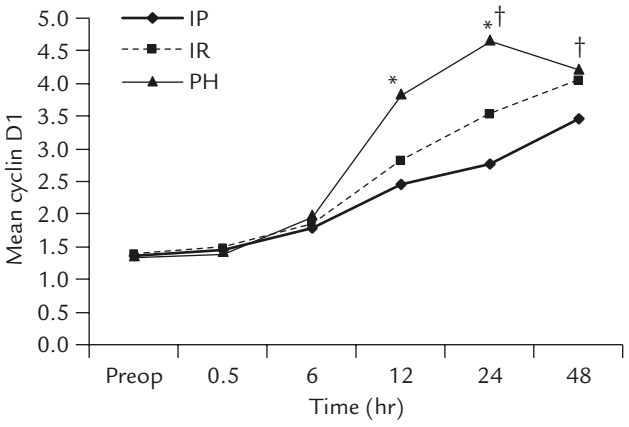

Figure 5. Expression of cyclin D1 before and 0.5 to 48 hours after operation. *$p < 0.05$ versus IR and PHx groups; †$p < 0.05$ versus IP and IR groups.

the operation ($p > 0.05$). RLW rapidly increased after partial hepatectomy. At 12 hours after operation, RLW was significantly lower in the IP group compared to the PH and IR groups ($p < 0.05$).

Expressions of cyclin D1 and Ki-67

The expressions of cyclin D1 and Ki-67 are shown in Figures 5 and 6. There were no significant differences in the expression of cyclin D1 and Ki-67 in all the groups before the operation ($p > 0.05$). After partial hepatectomy, they rapidly increased. The expressions of Ki-67 and cyclin D1 reached a peak at 24 hours after operation in group PH, and were significantly higher than those of groups IR and IP, which reached peak values at 48 hours ($p < 0.05$). A significant decrease in the expressions of cyclin D1 and Ki-67 was observed after 24 and 48 hours of reperfusion in group IP when compared with groups PH and IR ($p < 0.05$).

Histopathology

Hepatic pathological changes were insubstantial in group PH, and were characterized by vacuolization of hepatocytes, without apparent hepatic necrosis (Figures 7A and 7B). In the livers of group IR rats at the corresponding time, focal infarction with sinusoidal congestion and neutrophil accumulation was observed (Figures 7C–E). In contrast, H&E staining of liver tissue after IP in IP-treated control animals showed marked vacuolization of hepatocytes, bleb formation, and neutrophil accumulation. In addition, the extent of necrosis of the hepatic parenchyma and disintegration of the sinusoidal lining cells were significantly higher (Figures 7F–H). Cyclin D1 and Ki-67 immunohistochemical staining are shown in Figures 8 and 9, respectively.

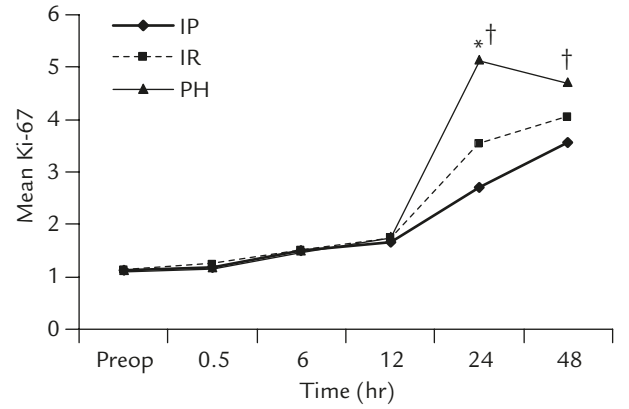

Figure 6. Expression of Ki-67 before and 0.5 to 48 hours after operation. *$p < 0.05$ versus IR and PHx groups; †$p < 0.05$ versus IP and IR groups.

Discussion

IP involves subjecting an organ to a short period of ischaemia before a more sustained period of ischaemia and subsequent reperfusion. Although it is believed that IP limits the detrimental effects of IR, studies do not conclude that short periods of ischaemia positively influence liver regeneration ability under the conditions of major tissue loss. It has been shown that periods of ischaemia beyond those used in models of preconditioning have deleterious effects.[1] Therefore, it is necessary to determine whether IP may stimulate hepatic regeneration of the residual liver.

A number of reports have documented that the expression of cyclin D1 and Ki-67 are indicators for hepatocyte proliferation. Cyclin D1 is undetected in quiescent rat liver. During the G1 phase, cyclin D1 is upregulated and forms complexes primarily with cdk4. The complexes initiate

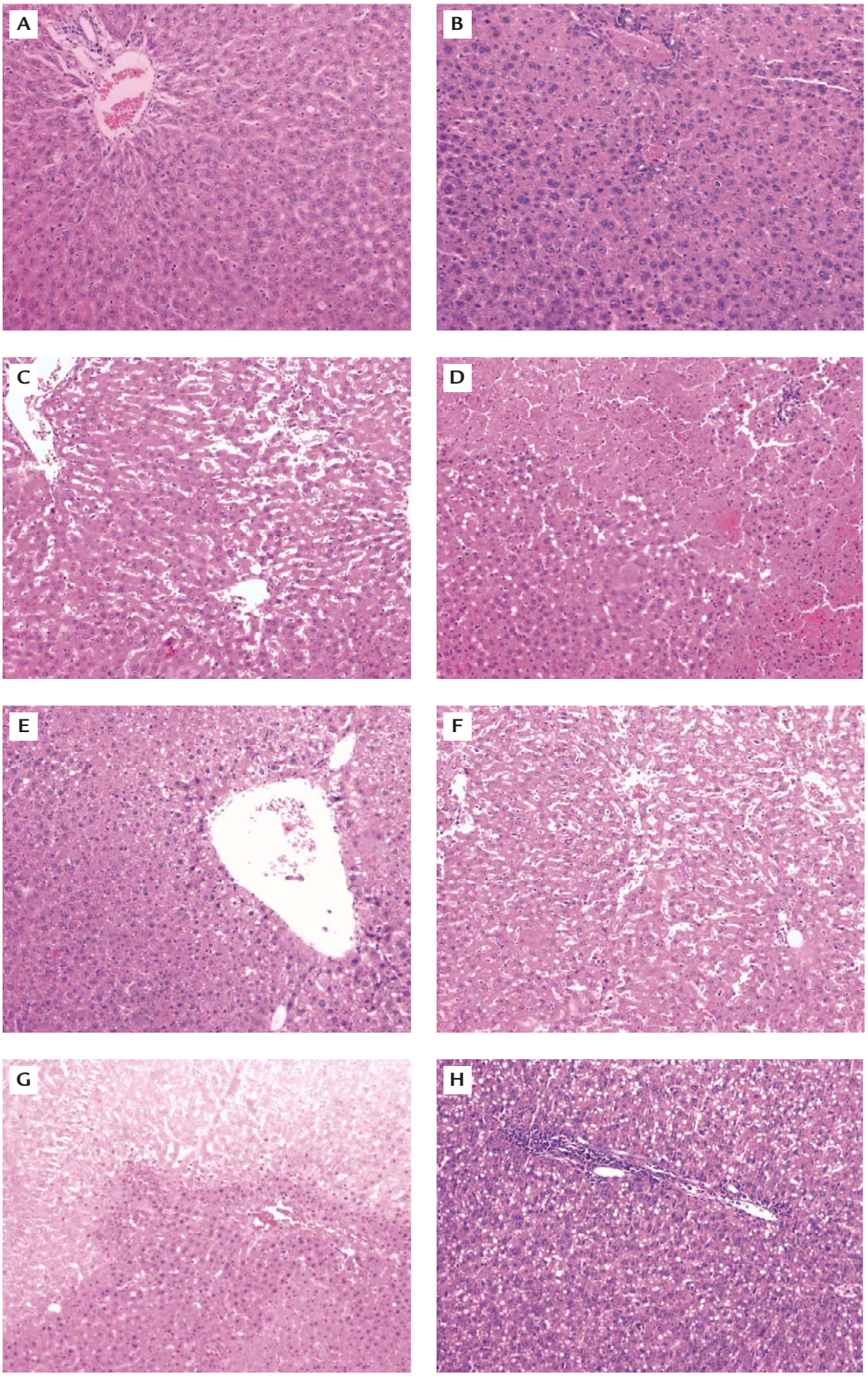

Figure 7. Hepatopathological staining of the liver (haematoxylin & eosin; original magnification, 100×). Group PH at: (A) 12 hours; (B) 24 hours. Group IR at: (C) 6 hours; (D) 12 hours; (E) 24 hours. Group IP at: (F) 6 hours; (G) 12 hours; (H) 24 hours.

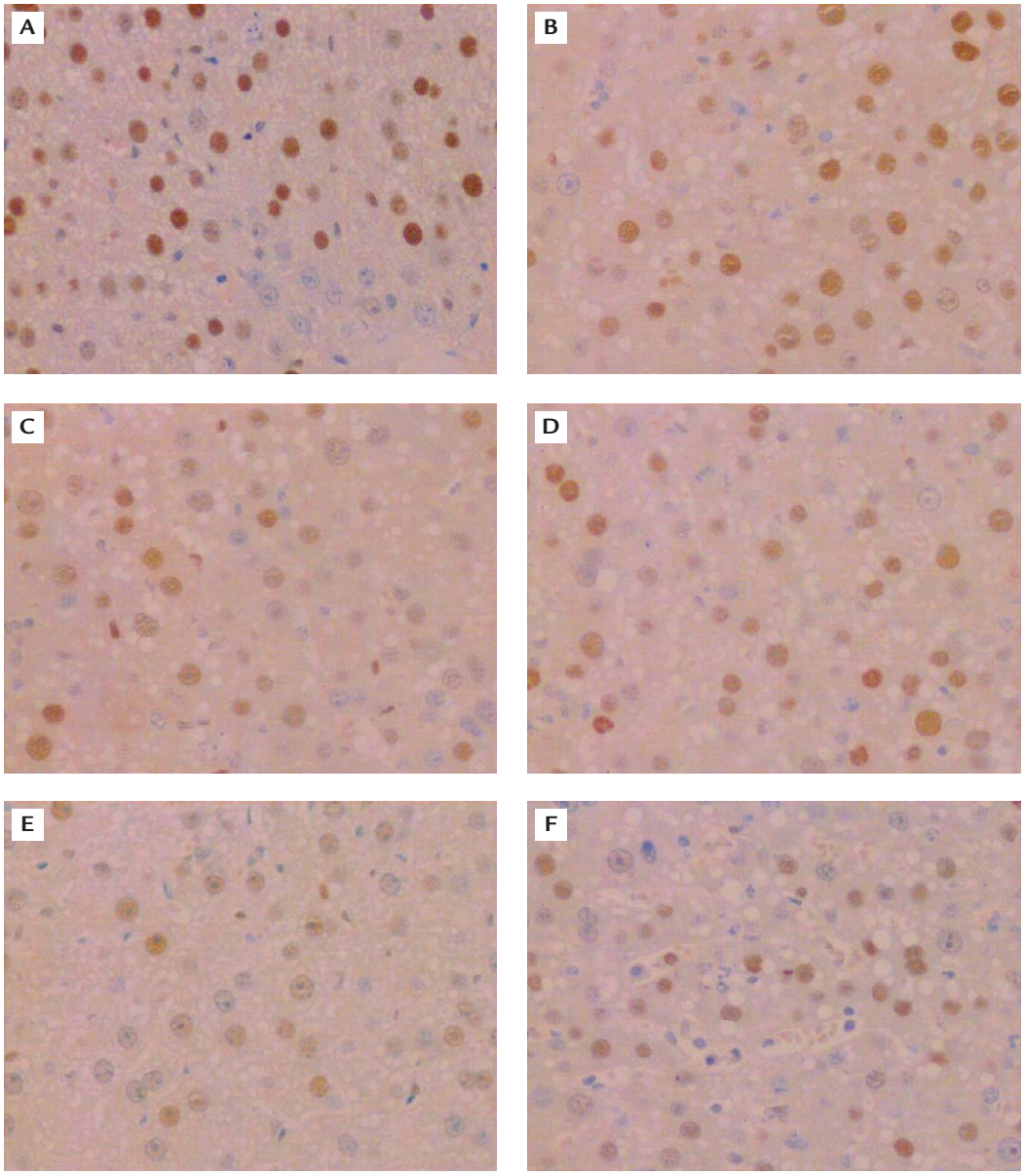

Figure 8. Immunohistochemical staining of cyclin D1-positive hepatocytes (original magnification, 200×). Group PH at: (A) 24 hours; (B) 48 hours. Group IR at: (C) 24 hours; (D) 48 hours. Group IP at: (E) 24 hours; (F) 48 hours.

phosphorylation of the retinoblastoma protein, activating genes required for entry into the S phase.[9,10] Ki-67 is a cell cycle nuclear protein that is expressed in the late G1 phase and throughout the S phase of the mitotic cycle. The amount of Ki-67 expression correlates with the degree of cell proliferation.[11] With regard to the expression of cyclin D1 and Ki-67 and the level of RLW, which are indicators of the regenerative capacity, there were significant differences observed among the IP, IR and PH groups, as measured by immunohistochemical analysis and densitometry. As shown in Figure 4, the expressions of Ki-67 and cyclin D1 were significantly lower in the IR group, reaching the peak later. IP also markedly suppressed these expressions, and the level of RLW was the same in groups IP and IR.

Damage to the structure and function of sinusoidal endothelial cells (SEC) is an important contributor to IR injury.[12] Serum HA is degraded in the SEC. When the liver structure is damaged, the synthesis of HA will increase and degradation decreases, which results in an increase in serum HA. Moreover, the degree of HA elevation and the extent of liver damage are positively correlated. Therefore, the level of serum HA is a sensitive indicator of damage of liver SEC. In our study, we found an early increase in serum HA in groups IP and IR, and serum HA level was significantly higher in IP rats than in PH and IR rats

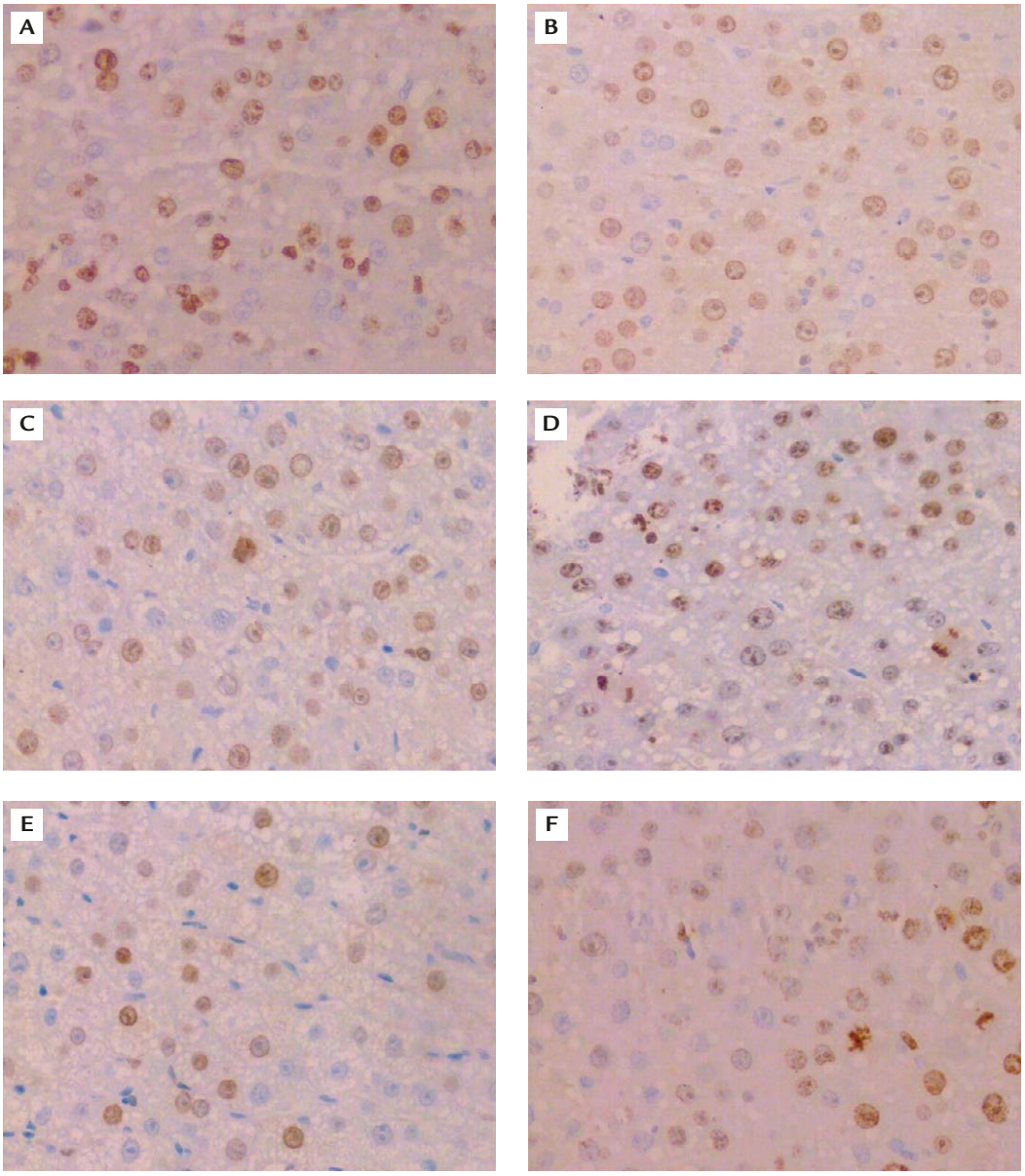

Figure 9. Immunohistochemical staining of Ki-67-positive hepatocytes (original magnification, 200×). Group PH at: (A) 24 hours; (B) 48 hours. Group IR at: (C) 24 hours; (D) 48 hours. Group IP at: (E) 24 hours; (F) 48 hours.

($p < 0.05$). In addition, IP markedly enhanced the increase in AST and ALT levels, and histologically aggravated the extent of liver lesion after partial hepatectomy under IR in the rats.

These results suggest that IP impairs residual liver regeneration after major hepatectomy without portal blood bypass in rats, and protection from IR injury disappears. Previous reports have demonstrated that mechanical forces that arise from changes in portal flow after partial hepatectomy are well defined and discussed as putative physiological trigger mechanisms for liver regeneration.[13] However, portal hyperperfusion has also been considered harmful. It may lead to damage of the SEC and hepatocyte ballooning with massive mitochondrial swelling, resulting in both enhanced injury and reduced metabolic and synthetic capacity of parenchymal cells.[14] The previous studies have confirmed that the protective effects of IP have been shown to be associated with improved hepatic tissue circulation and reduced cellular disturbances.[1,15] IP-induced nitric oxide release prevented the IR-induced increase in endothelins, favouring local vasodilatation and reduction of flow hindrance. Moreover, the decrease in reactive oxygen species production and in neutrophil-mediated injury by IP led to preservation of the architecture of the microcirculation. As a result, IP improved microcirculatory and sinusoidal perfusion, leading to an

increase in overall liver blood flow. Eipel et al demonstrated for the first time that IP-induced hyperperfusion may aggravate microvascular injury.[16] Work by Bedirli and coworkers showed that, with a portosystemic shunt at preoperation, IP appears to have unique protective properties in both types of injury in enhancing hepatocyte proliferation and preventing ischaemic cell death after combined ischaemia and hepatic resection.[8] In line with these, it is reasonable to confirm that the reduced regeneration potential in pretreatment of rats with IP may be associated with IP-induced hyperperfusion.

As a result of this study, one might suggest that the clinical application of IP is uncertain. In clinical liver resections with major tissue loss, IP should not be used to induce protection. However, further studies on the protection of IP in reduced-size liver transplantation (especially small-for-size liver grafts) are required to investigate this topic further.

In summary, this study shows that IP impairs residual liver regeneration after major hepatectomy in rats, and protection from IR injury disappears. Next, we need to clarify whether IP has a detrimental effect on hepatocyte proliferation in reduced-size liver transplantation.

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