Protective Effect of Ischemic Preconditioning against Liver Injury after Major Hepatectomy Using the Intermittent Pringle Maneuver in Swine

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Key Words

Reperfusion injury · Ischemic preconditioning · Intermittent clamping

Abstract

Objective: To investigate whether ischemic preconditioning (IP) protects the liver against ischemia-reperfusion injury (I/R-I) after major hepatectomy through intermittent hepatic pedicle clamping (IC) in a swine liver resection model. **Background:** Although many studies have reported a protective effect of IP against continuous hepatic ischemia, it has not been elucidated whether IP protects the liver against I/R-I after hepatectomy using IC. This is the first study to evaluate the effect of IP in a swine major hepatectomy model using IC. **Methods:** Pigs ($n = 12$) were divided into 2 groups (IP or non-IP). In the IP group, livers were subjected to IP (10 min ischemia and 10 min reperfusion) before liver resection using IC (15 min ischemia and 5 min reperfusion). A left hemihepatectomy was then performed using IC in both groups. Hemodynamic changes and plasma concentrations of aspartate aminotransferase, lactate dehydrogenase, lactic acid and hyaluronic acid were measured at 60, 120 and 180 min after hepatectomy. Apoptosis (TUNEL staining and electron microscopy), plasma tumor necrosis factor- α (TNF- α) and $\text{NO}_2^-/\text{NO}_3^-$ were evaluated for 180 min after hepatectomy. **Results:** There were no significant differences in body weight, blood loss, resected liver weight, Pringle time or hemodynamic changes between the 2 groups. IP significantly

reduced plasma aspartate aminotransferase levels for 180 min after hepatectomy (IP: 135.8 ± 13.5 vs. non-IP: 199 ± 16.8 IU/l; $p = 0.018$). In the non-IP group, apoptotic changes in sinusoidal endothelial cells were observed with increased plasma TNF- α levels. IP protected liver injury from increase in plasma TNF- α ($p = 0.042$). Significantly fewer apoptotic cells were seen in the IP than in the non-IP group ($p = 0.002$). Plasma levels of lactate dehydrogenase, lactic acid and $\text{NO}_2^-/\text{NO}_3^-$ in the IP group tended to be lower than those in the non-IP group. **Conclusions:** IP prior to hepatectomy with IC resulted in less hepatic injury and apoptotic cell death than in livers not subjected to IP. IP with IC has the potential to improve the clinical postoperative course of patients undergoing hepatectomy.

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Introduction

The Pringle maneuver, involving compression of the hepatoduodenal ligament, interrupts most of the blood flow to the liver, but produces profound hepatic ischemia and intestinal congestion unless the clamping is frequently released. Since this maneuver was first reported by Pringle [1] in 1908, it has been used clinically during hepatectomy to reduce blood loss. However, ischemia-reperfusion injury (I/R-I) resulting from the Pringle maneuver is one of the pathogenetic factors involved in postoperative liver dysfunction and hepatic failure, especially

when the liver is steatotic and cirrhotic [2, 3]. Liver resection is widely performed for hepatocellular carcinoma in patients with chronic liver disease due to hepatitis C or hepatitis B [4–7]. Since Makuuchi et al. [8], who were the first to advocate the usefulness of the hemihepatic pedicle clamping during hepatectomy for diseased liver, various modified Pringle maneuvers such as hemihepatic or intermittent hepatic pedicle clamping (IC) have been tried as candidates for an optimal treatment for deranged liver patients. The IC procedure has been accepted in both experimental and clinical settings [2, 9].

Murry et al. [10] were the first to report that a short period of ischemia prior to prolonged ischemia reduced the size of the subsequent myocardial infarct. This phenomenon, known as ischemic preconditioning (IP), has also been reported to protect the liver in patients undergoing hepatectomy. In a rat model, we have already demonstrated that the protective effect of IP was greater against damage induced by intermittent ischemia than that induced by continuous ischemia [11]. However, it is still unknown whether IP might not only reduce I/R-I after continuous but also after intermittent hepatic ischemia in a liver resection model.

If further ischemic tolerance could be promoted by performing IP before IC, this would be more advantageous for clinical surgery, especially in patients with diseased liver. Therefore, we evaluated the effect of IP in a swine hepatectomy model by monitoring hemodynamic and biochemical parameters as well as histological examinations.

Methods

Male white pigs were used in this study, which was performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Dokkyo University School of Medicine. The pigs were allowed free access to water and standard foods before undergoing surgical procedures.

Surgical Procedures

A chevron incision was performed under general anesthesia. On preliminary examination, we first performed occlusion of the portal vein and hepatic artery without a veno-veno bypass, but all pigs adopted severe hypotension due to intestinal congestion within 5 min after pedicle clamping, and we could not confirm any hepatic resection in this condition. In humans, collateral vessels develop between the portal and systemic vein, and hepatectomy using hepatic pedicle clamping can be safely performed without intestinal congestion and severe hypotension. In pigs, these collateral vessels are very poor; therefore, to prevent splenic and intestinal congestion during prolonged occlusion of the portal vein and hepatic artery, a veno-veno bypass was created between the

main splenic vein and the right internal jugular vein by using a venous cannula (V122-16; Stöckert, Munich, Germany) [12]. The hepatic pedicle and all hepatic ligaments were isolated, and total liver ischemia was performed by clamping the hepatic pedicle using a vessel tape. During clamping, a veno-veno bypass was used for preventing bowel congestion and closed during declamping. After clamping the hepatic pedicle, a left hemihepatectomy was performed using IC (cycles of 15 min ischemia and 5 min reperfusion). Liver transection was achieved by the crush-clamping method using Pean forceps. During liver transection, each of the exposed Glisson's vessels was ligated with 2-0 or 3-0 silk. Immediately after hepatectomy, the hepatic pedicle was declamped and complete hemostasis was confirmed by suture closure.

During this procedure, hemodynamic parameters (systolic and diastolic arterial pressure, heart rate) were monitored by an arterial line, and intraoperative blood loss was measured. All pigs received Ringer solution during this procedure.

Experimental Groups

The pigs were divided into 2 groups: (1) an IP group ($n = 6$; IP was performed before hepatectomy using IC) and (2) a non-IP group ($n = 6$; hepatectomy was performed using IC without prior IP). IP was defined as total hepatic ischemia for 10 min followed by reperfusion for 10 min prior to hepatectomy.

Measurements and Sampling Protocol

Blood samples were obtained from the arterial line immediately after laparotomy, after hepatectomy, and at 60, 120 and 180 min after reperfusion. We evaluated serum aspartate aminotransferase (AST), lactate dehydrogenase (LDH), lactic acid (LA), hyaluronic acid (HA), tumor necrosis factor- α (TNF- α) and NO₂/NO₃. Hepatic tissues were obtained from the right lobe after reperfusion at 180 min to evaluate histological findings.

Serum AST, LDH, LA and HA were measured using standard clinical methods for automated analysis (Model 7170; Hitachi Inc., Tokyo, Japan). Plasma TNF- α levels were examined by enzyme-linked immunosorbent assay using a commercial porcine TNF- α /TNFSF2 immunoassay kit (R&D Systems Inc., Minneapolis, Minn., USA). Plasma NO₂/NO₃ levels were determined by a commercial nitrate/nitrite colorimetric assay kit (Cayman Chemical Company, Ann Arbor, Mich., USA).

Histological Examination

Tissue samples were obtained from the remnant liver 180 min after hepatectomy, fixed with 10% formalin for 24 h, and embedded in paraffin. Three-micrometer-thick sections were stained with hematoxylin and eosin and analyzed by the in situ terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method using an apoptosis in situ detection kit (Wako Pure Chemical Inc., Osaka, Japan) according to the manufacturer's instructions. The mean numbers of apoptotic cells per 10 high-power fields were calculated for the 2 groups and then compared.

Immediately after removal, other hepatic tissues were fixed with 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.3) for 1.5 h at 4°C, postfixed with OsO₄ in 0.15 M phosphate buffer (pH 7.3) for 1.5 h at 4°C, dehydrated, and embedded in Epon812 resin. Ultrathin sections were cut using a Super Nova ultramicrotome (Reichert-Jung, Vienna, Austria), double-stained with uranyl acetate and lead citrate, and examined with a JEM-1210 electron microscope (JEOL, Tokyo, Japan).

Fig. 1. Heart rate (a) and blood pressure (b) revealed no significant differences between the IP and non-IP group at 3 h.

Fig. 2. The serum AST levels in both groups increased gradually during the experimental period and significant differences were observed at 3 h after reperfusion. * $p = 0.018$.

Table 1. Characteristics of pigs undergoing hepatectomy with IC and using IP

	Non-IP (n = 6)	IP (n = 6)
BW, kg	24.2 ± 3.11	24.8 ± 2.28
Blood loss, ml	57.5 ± 45.74	60.0 ± 35.59
IC time, min	30.6 ± 1.78	33.25 ± 2.87
RLW, g	259.2 ± 49.99 (40.7%)	288.0 ± 55.08 (45.6%)

BW = Body weight; IC = intermittent hepatic pedicle clamp; RLW = resected liver weight.

Statistical Analysis

All values are expressed as means $\pm$ SD. All parameters were evaluated using Student's t test or the χ^2 test. Differences between the 2 groups were evaluated using analysis of variance and considered significant at $p < 0.05$.

Results

There were no significant differences in body weight, IC time, blood loss or resected liver weight between the IP and non-IP groups (table 1).

Effect of IP on Hemodynamics

Heart rate was higher immediately after hepatectomy than during the period of laparotomy, and then decreased gradually. There were no significant differences in heart rate between the 2 groups (fig. 1a). Systolic blood pressure was reduced after hepatectomy and was then maintained at the same level thereafter, there being no significant differences between the 2 groups (fig. 1b).

Effect of IP on Serum AST, LDH, LA and HA Levels

The serum AST levels in both groups increased gradually during the experimental period and significant differences were observed at 180 min after reperfusion (IP: 135.8 ± 13.5 vs. non-IP: 199 ± 16.8 IU/l; $p = 0.018$). At other observation points, the AST level in the IP group tended to be lower than that in the non-IP group (fig. 2). The serum LDH levels in the IP group were maintained after reperfusion, whereas those of the non-IP group in-

Fig. 3. **a** There were no significant differences in serum LDH levels between the IP and non-IP group at 3 h. **b** LA levels in the IP group were lower than those in the non-IP group at 2 and 3 h after hepatectomy, the intergroup differences were not significant at 3 h.

Fig. 4. Immediately after hepatectomy, the serum HA levels of the IP and non-IP group increased and then gradually decreased. However, there were no significant differences in the serum HA levels between the IP and non-IP group at 3 h.

creased gradually after hepatectomy. However, there were no significant differences in serum LDH levels between the 2 groups (fig. 3a). Immediately after hepatectomy, the serum LA levels in both groups elevated and then decreased gradually thereafter. Although the LA levels in the IP group were lower than those in the non-IP group at 120 and 180 min after hepatectomy, the intergroup differences were not significant (fig. 3b). Immediately after

hepatectomy, the serum HA levels in both groups increased and then gradually decreased. However, there were no significant differences in serum HA levels between the groups (fig. 4).

Effect of IP on Plasma TNF- α Levels and NO₂/NO₃

The levels of plasma TNF- α in the IP group tended to be lower than those in the non-IP group ($p = 0.042$; fig. 5a). The NO₂/NO₃ levels in the IP group tended to be lower than those in the non-IP group. However, the differences in plasma NO₂/NO₃ levels between the groups were not significant during the first 180 min after hepatectomy ($p = 0.222$; fig. 5b).

Protective Effect of IP against Liver Apoptotic Cell Death

TUNEL staining at 180 min after hepatectomy revealed significantly fewer TUNEL-positive cells in the IP group than in the non-IP group (IP group: 31.0 ± 14.1 vs. non-IP group: $65.4 \pm 11.5/10$ fields; $p = 0.002$; fig. 6a, b).

Electron microscopy revealed apoptosis and necrosis of hepatocytes and sinusoidal endothelial cells in the non-IP group, but such changes were rarely observed in the IP group. In the non-IP group, nuclear chromatin condensation occurred frequently in sinusoidal endothelial cells, and early nuclear chromatin condensation was seen occasionally in hepatocytes. Necrotic hepatocytes showing expanded mitochondria were rarely observed (fig. 7).

Fig. 5. a Plasma TNF- α levels after hepatectomy for 3 h. The levels in the IP group tended to be lower than those in the non-IP group. * $p = 0.042$. **b** The NO₂/NO₃ levels in the IP group tended to be lower than those in the non-IP group. The differences in plasma NO₂/NO₃ levels between the groups were not significant during the first 3 h after hepatectomy. $p = 0.222$.

a IP

Non-IP

Fig. 6. a TUNEL staining after hepatectomy for 3 h revealed fewer TUNEL-positive cells (arrows) in the IP than the non-IP group. $\times 200$. **b** Microphotometric evaluation of TUNEL-positive hepatocytes in TUNEL-stained tissue after reperfusion for 3 h. Values are expressed as means $\pm$ SD. $n = 6$ in both groups. The differences between the groups are statistically significant. * $p = 0.002$.

Fig. 7. Electron microscopy revealed apoptotic and necrotic features of hepatocytes and sinusoidal endothelial cells in the non-IP group. Nuclear chromatin condensation occurred frequently in the hepatocytes (arrows).

Discussion

This is the first experimental study to evaluate the effect of IP plus IC during major hepatectomy in a swine model. IC has been advocated because of its safety during hepatectomy in patients with diseased liver, and it has been suggested that the beneficial effects of IC may be related to the phenomenon of IP [7]. Although the effect of IP has only been reported after continuous hepatic pedicle clamping (CC), its protective effect against liver injury after IC has not yet been discussed. We hypothesized that IP may exert protective effects against liver injury after hepatectomy and conducted a trial using not only CC but also IC.

It is still unclear whether CC or IC is appropriate for liver resection. CC is more effective than IC for decreasing the amount of blood loss during hepatic resection. However, postoperative liver enzyme and bilirubin levels are significantly higher after CC than after IC, especially in patients with liver abnormality [2]. Furthermore, our previous experimental study using a rat model suggested that IC had a better impact on I/R-I than CC [11]. Consequently, IC was defined as repeated clamping for 15 min followed by reperfusion for 5 min on the basis of this experimental study. Using this procedure, clinical liver resection has been performed safely at our institution. There is evidence suggesting that hepatic ischemia lasting for more than 15 min activates calpain proteases [13].

Furthermore, Belghiti et al. [2] have reported a lower rate of complication with IC than with continuous portal triad clamping. On the other hand, the optimal duration of IP is still unclear [14]. Previous studies have suggested that several durations of IP are optimal: 5 min clamping followed by 30 min reperfusion, 5 or 10 min of ischemia followed by 10 or 15 min of reperfusion, or 10 min of ischemia and 10 min of reperfusion. In this study, we specified an ischemic period of 10 min followed by reperfusion for 10 min before hepatic pedicle clamping [11, 14–19]. This method was found to be the most beneficial after warm ischemia *in vivo* and prolonged exposure to hypoxia *in vitro*, and Clavien et al. [15] have reported that 10 min clamping and 10 min reperfusion by IC exerts a protective effect against liver injury in humans [20, 21]. Therefore, IP with IC was the most effective procedure protecting for I/R-I.

The serum AST level has been used to evaluate liver damage after liver resection, and is considered to objectively reflect the degree of liver damage [22]. In the present study, serum AST levels were significantly reduced by IP after hepatectomy at 180 min. Levels of other parameters, including serum LA and LDH, tended to be lower in the IP than in the non-IP group. These results indicate that IP protects against liver injury after hepatectomy using IC. As sinusoidal endothelial cells take up HA from blood, the serum HA level has been used to evaluate the function of these cells, and patients with liver cirrhosis or posthepatectomy patients frequently show an increased serum HA level [23, 24]. In the present study, serum HA levels were similar in both the IP and non-IP groups at each observation point, and IP was effective for protecting sinusoidal endothelial cell function. Although the pathogenesis of liver injury after liver resection using the Pringle maneuver is multifactorial, operative stress such as blood loss and Pringle time play an important role in liver damage during hepatectomy. In our previous study, we found that blood loss and Pringle time did not differ between the 2 groups, and that IP did not affect blood loss or Pringle time. These results suggest that IP promotes liver tolerance through a factor other than reduction of blood loss and Pringle time.

Because of its transient and volatile nature, NO is difficult to measure directly. However, NO is largely oxidized to $\text{NO}_2^-/\text{NO}_3^-$, and the concentration of these anions is often used as a quantitative measure of NO production. NO acts by maintaining perfusion of the hepatic microcirculation and modulates liver injury through its vasodilatory and anti-inflammatory effects. Experimental studies suggest an association of NO with

liver injury in relation to I/R-I. NO has been reported to play a protective role against tissue injury through several mechanisms, including inhibition of leukocyte adhesion to the endothelium, platelet aggregation and a vasodilative action [25]. Several studies have shown that I/R-I is partially mediated by activation of Kupffer cells. Our results also suggest that $\text{NO}_2^-/\text{NO}_3^-$ was induced to a greater extent in the non-IP group than in the IP group, and that injury to Kupffer cells or microvessels might have induced $\text{NO}_2^-/\text{NO}_3^-$. The decreased $\text{NO}_2^-/\text{NO}_3^-$ levels at 180 min after reperfusion support the possibility that IP had a protective effect against liver damage.

TNF- α is one of the important extracellular mediators involved in liver damage after hepatic ischemia [26, 27]. Apoptosis is initiated by TNF- α binding to TNF receptor 1, which activates caspase 8 through the TNF receptor-associated death domain, resulting in induction of DNA fragmentation by the activated caspase cascade [28]. In our study, the serum TNF- α levels at 180 min after hepatectomy were significantly lower in the IP group than in the non-IP group. Moreover, TUNEL positivity and features of apoptosis such as nuclear chromatin condensation were rarely observed in the IP group with reduced serum TNF- α levels. These results suggest that IP exerts a protective effect by interfering with the increase in serum TNF- α , thus preventing apoptotic death.

Conclusion

We confirmed that IP can exert a protective effect against liver injury not only after liver resection using CC but also after IC. Our study suggested that apoptotic liver damage appeared at an early stage following liver resection using IC, it also confirmed that IP had a protective effect against apoptotic liver damage. Although the protective effect obtained by using IP plus IC is not maximal, our strategy has the potential to improve the clinical postoperative course of patients undergoing liver resection. A clinical study in humans will be required to confirm whether IC followed by IP improves the postoperative condition or not. Thus we have confirmed that liver resection using IP plus IC is considerably superior to the use of IC without IP.

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