

Apoptosis and proliferation of intrahepatic bile duct after ischemia-reperfusion injury

Wen-Hui Xu, Qi-Fa Ye and Sui-Sheng Xia

Wuhan, China

BACKGROUND: In orthotopic liver transplantation, ischemic-reperfusion is one of the most important factors that cause the incidence of biliary complication. The aim of this study was to investigate the effects of ischemia reperfusion on epithelial cells apoptosis and proliferation of intrahepatic bile duct (IBD) ($>20 \mu\text{m}$).

METHODS: 30-minute warm ischemia was applied to rat livers respectively, and experiment was performed on days 2, 7, 14, 28 after reperfusion. Apoptosis was determined in situ by morphology and TUNEL, and cholangiocyte proliferation was evaluated in situ by morphometry of liver sections stained for cytokeratin-19 (CK-19) and by proliferating cellular nuclear antigen staining in liver sections.

RESULTS: Two days after ischemia reperfusion, apoptosis of cells was observed in large intrahepatic bile ducts ($>20 \mu\text{m}$) ($5.6\% \pm 1.2\%$), but the number of large intrahepatic bile ducts reduced (0.32 ± 0.06). Seven days after ischemia reperfusion, the apoptosis index of cholangiocytes decreased to $1.2\% \pm 0.3\%$, and the number of intrahepatic bile ducts began to proliferate and returned to nearly normal on day 28.

CONCLUSION: Ischemia reperfusion causes a decrease in the number of intrahepatic bile ducts ($>20 \mu\text{m}$) as a result of a higher rate of apoptosis and absence of initial proliferation.

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KEY WORDS: apoptosis; proliferation; intrahepatic bile duct; epithelial cells

Introduction

Intrahepatic bile duct epithelial cells or cholangiocytes line the complex three-dimensional network of bile ducts within the liver.^[1-3] This ramified net-

work consists of the canals of Herring or conduits adjacent to the canalicular domain of hepatocytes and interlobular bile ducts. Bile, after secretion from hepatocytes, drains through progressively larger septal, segmental, and hepatic ducts into the extrahepatic bile ducts before reaching the duodenum. Cholangiocytes play a key role in the modification of bile of canalicular origin by a series of spontaneous and hormone-regulated processes. Cholangiocytes in normal liver are mitotically quiescent.^[4] Cholangiocytes, however, markedly proliferate in response to specific pathological stimuli such as ischemia and toxin.^[5,6]

In rat liver, the injury produced by ischemia reperfusion is centrilobular in nature, leading to membrane disruption and hepatocyte necrosis.^[7,8] The model of ischemia reperfusion-induced hepatocyte necrosis is well defined. Previous studies have shown transient hepatocyte loss followed by hepatocyte proliferation.^[9] No information exists regarding the effect of ischemia reperfusion on proliferative and apoptosis processes of the intrahepatic bile duct. We evaluated in situ apoptosis and necrosis of both cholangiocytes and hepatocytes by TUNEL morphological light-microscope analysis and proliferative capacity of small bile ducts by CK-19 and proliferating cellular nuclear antigen (PCNA) staining in liver sections.

Methods

Rats

Male Sprague-Dawley rats weighing 150 ± 30 g were used in all experiments. The model of hepatic ischemia and reperfusion was prepared as described previously.^[30] The rats were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg). A midline laparotomy was performed and an atraumatic clip was used to interrupt the arterial and portal venous blood supply to the liver. After 30 minutes of hepatic ischemia, the clip was removed to initiate hepatic reperfusion. Sham control rats underwent the same protocol, but without vascular occlusion.

The rats were killed after reperfusion for 2, 7, 14, 28 days respectively, and liver tissue and blood samples were taken for histological analysis. Formalin-fixed tis-

Author Affiliations: Institute of Organ Transplantation, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China (Xu WH, Ye QF and Xia SS)

Corresponding Author: Wen-Hui Xu, MD, Institute of Organ Transplantation, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China (Tel: 86-27-83604840; Email: jzxwh@yahoo.com.cn)

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sue samples were embedded in paraffin, and cut in 5 μm sections. Replicate sections were either stained with hematoxylin and eosin (HE) for evaluation of necrosis and apoptosis or stained with the deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (Apop Tag Peroxidase in situ apoptosis detection kit). The number of apoptotic cholangiocytes were counted in 20 high power fields ($\times 400$). Reagents were purchased from Sigma Chemical Co., USA, The mouse cytokeratin-19 (CK-19) antibody was purchased from Beijing Zhongshan Biological Technology Company, Beijing, China.

In situ morphological studies

In situ morphological evaluation of cholangiocyte apoptosis was performed. Liver blocks of 1 to 2 cm were taken from each animal and fixed in 10% buffered formalin at pH 7.4. Sections of 5 μm thickness were cut from two liver blocks for each rat, HE stained and examined for apoptosis under a light microscope.

TUNEL assay

Paraffin sections were dewaxed by routine method and incubated for 10 minutes with 3% H_2O_2 . Each section was incubated for 10 minutes with 3% H_2O_2 to neutralize the activity of endogenous catalase, and then washed for 10 minutes with PBS. After digestion with 20 $\mu\text{g}/\text{ml}$ of protease K at 37 $^\circ\text{C}$ for 15 minutes, the section was incubated with TUNEL reaction mixture at 37 $^\circ\text{C}$ for 2 hours. After that, it was incubated with normal coat serum at 37 $^\circ\text{C}$ for 30 minutes, and then washed for 15 minutes with PBS. Each section was incubated with Converter-AP solution at 37 $^\circ\text{C}$ for 40 minutes, washed for 15 minutes with PBS, and then stained with DAB/ H_2O_2 at 37 $^\circ\text{C}$ for 3 minutes and counterstained with hematoxylin for 30 minutes. All sections were washed in tap water, dehydrated, clarified and mounted

Assessment of cholangiocyte proliferative capacity

In paraffined liver sections from control and ischemia reperfusion rats, the number of bile ducts was counted after staining for CK-19, a specific marker for rat cholangiocytes. Immunohistochemistry for CK-19 was performed according to the manufacturer's instructions.

In situ immunohistochemistry for proliferating cellular nuclear antigen (PCNA)

Liver sections of 5 μm thickness were cut from two liver blocks from normal or ischemia reperfusion rats and processed for immunohistochemistry using the monoclonal mouse antibody, and anti-PCNA (Dako, Kyoto, Japan). Control sections were similarly prepared except for omission of the primary antibody.

Statistical analysis

Data were expressed as mean \pm SEM. Statistical evaluation was performed by unpaired Student's *t* test when the two groups were analyzed. A *P* value less than 0.05 was considered statistically significant.

Results

Morphological and biochemical characteristics of ischemia reperfusion induced model of hepatic damage on day 2 after ischemia reperfusion showed necrosis in zone 3 and micro-macrovacuolar steatosis of hepatocytes. Histological changes of the liver were similar to those of controls on days 14 and 28 after ischemia-reperfusion. Ischemia reperfusion induced transient damage of hepatocytes, and on the day after ischemia, a transient, significant increase was noted in the serum levels of AST, alkaline phosphatase, total bilirubin as compared with those in normal rats (Table 1). The increased serum levels on day 2 following ischemia reperfusion returned to normal 1 to 2 weeks after reperfusion.

Measurement of apoptosis in cholangiocytes

Apoptosis of cholangiocytes was not observed in normal rat liver. On day 2 after ischemia reperfusion it was observed mainly in the intrahepatic bile duct ($>20 \mu\text{m}$).^[9,10] Morphologically necrosis was not detected in cholangiocytes on day 2 after ischemia reperfusion. With the prolongation of reperfusion, the number of apoptotic cholangiocytes decreased. On day 2 after ischemia reperfusion, apoptosis was observed in 5.6% \pm 1.2% of cholangiocytes. On day 7, only 1.2% \pm 0.3% of cholangiocytes were TUNEL positive. On days 14 and 28, cholangiocytes returned to normal.

Assessment of cholangiocyte proliferative capacity

In situ immunohistochemistry for CK-19, a cholangiocyte-specific marker showed only 2 to 3 ducts in a normal rat liver section. The number of intrahepatic bile ducts ($>20 \mu\text{m}$) was markedly decreased on day 2 after ischemia reperfusion and increased gradually to the pretreatment level on day 28 after ischemia reperfusion

Table 1. Serum enzyme levels in control rats and ischemia-reperfusion rats

Treatment (d)	AST	Alkaline phosphatase	Total bilirubin
Control	38.42 \pm 4.65	13.64 \pm 0.83	0.48 \pm 0.05
2	82.15 \pm 3.60 *	23.17 \pm 2.34 *	0.85 \pm 0.09 *
7	35.84 \pm 4.13	15.85 \pm 2.06	0.76 \pm 0.06 *
14	36.27 \pm 3.66	14.49 \pm 2.11	0.64 \pm 0.05
28	38.56 \pm 2.86	15.32 \pm 1.82	0.56 \pm 0.04

* : *P* < 0.05. Serum enzyme levels of ischemia reperfusion rats differed from those of control rats.

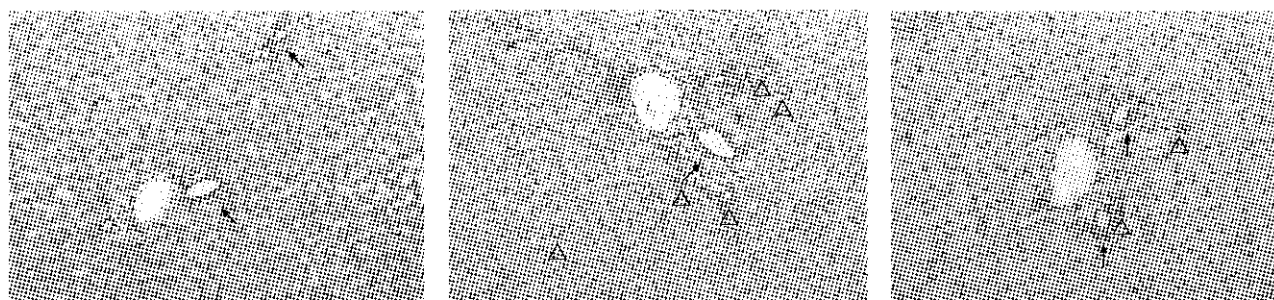


Fig. 1. In situ immunohistochemistry for CK-19 in formalin fixed sections obtained from normal rats on days 2, 7, and 28 after reperfusion (original magnification $\times 200$). \uparrow : large bile duct ($>20 \mu\text{m}$); Δ : small bile duct ($\leq 20 \mu\text{m}$).

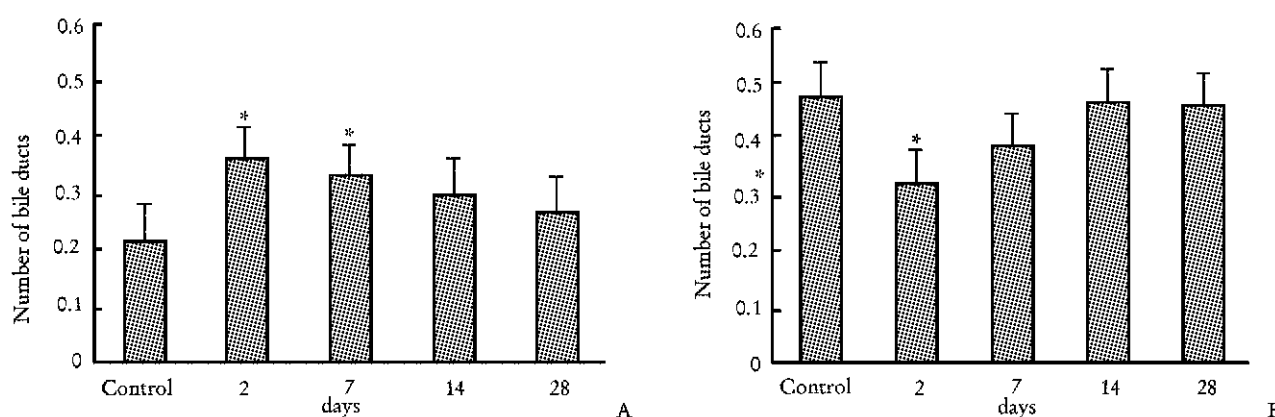


Fig. 2. Morphometric quantitative measurement of intrahepatic bile ducts mass. A; a marked increase in the density of intrahepatic bile ducts after ischemia reperfusion. B; a marked decrease on day 2 and then returned to nearly normal on days 14 and 28 after ischemia reperfusion. *; $P < 0.05$ compared with control.

(Figs. 1 and 2).

In situ immunohistochemistry for PCNA

On days 7 and 14 liver sections after ischemia reperfusion showed positive staining for PCNA in cholangiocytes lining bile ducts. On day 28 after ischemia reperfusion, only a few cholangiocytes were PCNA-positive in normal rats.

Discussion

Intrahepatic bile ducts occupy a central anatomic position within the liver, conducting bile from individual hepatocytes into a larger, single extrahepatic bile duct. Cholangiocytes, the cells that line the intrahepatic bile duct, form a simple epithelium and have ultrastructural properties. Comprising only 2%–5% of liver cells, cholangiocytes alkalize the bile through net bicarbonate secretion and account for up to 40% of the bile volume.^[10,11] In a number of cholestatic liver diseases, including primary biliary cirrhosis, primary sclerosing cholangitis, and post transplantation liver failure, bile ducts represent a focal

site of injury. Unlike hepatic parenchymal cells, bile ducts are selectively perfused by the hepatic artery. Accordingly, interruption of this oxygen-rich perfusate has been postulated to be an initiating event in many duct-oriented disease processes. The capacity for liver ischemia to initiate targeted bile duct injury is particularly apparent following hepatic artery thrombosis or prolonged ischemia related to liver transplantation.^[12-14] For example, retrospective analysis of liver function after transplantation revealed that livers with cold ischemic period under 11.5 hours had a low incidence of bile duct stricture while those with the period greater than 11.5 hours before transplantation had an incidence of 33% for bile duct stricture and dilatation.^[14,15]

Although the late manifestations of intrahepatic bile duct ischemia are well defined, little is known about the early alterations in the period of ischemia reperfusion. These studies attempted to examine the effects of ischemia reperfusion on the apoptotic, proliferative processes of the intrahepatic bile duct. In this study we found a decreased number of bile ducts two days after ischemia reperfusion. Ischemia-reperfusion-induced-hepatic damage was reported to be primarily caused by transient hepa-

tocellular necrosis,^[16-18] but in our study ischemia-reperfusion-induced bile duct damage was primarily caused by apoptosis of cholangiocytes, which is a reaction to ischemia reperfusion-induced liver injury. The application of reliable markers of apoptosis demonstrated apoptosis of cholangiocytes. Moreover no morphological evidence was found on necrosis of bile ducts on day 2 after ischemia reperfusion. Necrotic cholangiocytes may be rarely missed in situ morphological study of bile ducts after ischemia reperfusion. Analysis of bile ducts showed that apoptosis is the primary mechanism for cholangiocyte loss after ischemia reperfusion in this study. These findings propose that ductopenia in the intrahepatic bile duct after ischemia reperfusion injury is primarily caused by apoptosis of cholangiocytes, rather than by necrosis of hepatocytes. Thus, it is likely that cholangiocytes, as compared with hepatocytes, have a higher threshold for ischemia reperfusion because they only manifest a milder form of injury in apoptosis. The different sensitivities of hepatocytes and cholangiocytes to ischemia reperfusion may be a result of different blood supply or the presence of Bcl-2 (a known protector against apoptosis) in cholangiocytes, but not in hepatocytes.^[19,20]

In contrast to hepatocytes, cholangiocytes have a delayed proliferative response (not until day 7 after ischemia reperfusion), which is considered a result of selective damage and apoptosis. Bile ducts are selectively perfused by the hepatic artery, and they are more resistant to a lethal anoxic injury than hepatocytes. At the same time, ischemia reperfusion damages liver sinusoidal endothelial cells, resulting in a longer non-thorough ischemia even after reperfusion. It has been shown^[21] that cholangiocyte became dysfunctional with loss of secretin-receptor gene expression and secretin-induced cAMP synthesis in an animal model of experimental duct injury.^[21] Alpini^[22] found that proliferating cholangiocytes required at least 7 days in a bile duct ligation rat model before they responded to secretin in vivo. But the expression of secretin receptor and secretin-stimulated cAMP synthesis in purified cholangiocytes did not always result in enhanced secretin-induced bile flow or bicarbonate secretion in vivo.^[22,23] Moreover, proliferation of cholangiocyte lasted longer than that of hepatocyte, which may be due to their differing cell cycle and timing of DNA synthesis (eg, more prolonged G1 phase).^[24,25]

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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Though this be madness, yet there is method in 't.

— William Shakespeare