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Research Paper

Effect of hepatic ischemia-reperfusion on bile canalicular F-actin microfilaments in rats ☆

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Abstract

Objective: To investigate the effect of hepatic ischemia-reperfusion(I/R) on bile canalicular F-actin microfilaments in rats. **Methods:** A rat model of hepatic ischemia-reperfusion was employed and the ischemia time was 35 min. The activity of serum alanine aminotransferase (ALT), aspartate aminotransferase(AST), γ-glutamyl transferase(GGT) and the level of total bilirubin(TBIL) were measured. Changes in the bile canaliculi were observed by transmission electron microscope. The modification of F-actin microfilaments was quantified by using FITC-Phalloidin and analyzed by confocal laser scanning microscopy imaging. **Results:**Modifications of F-actin staining were consistent with the observations made by transmission electron microscopy. The staining of F-actin was normal in hepatocytes before reperfusion but decreased significantly after reperfusion, and there was a marked loss of canalicular microvilli after reperfusion, which coincided with abnormal serum GGT and TBIL levels. **Conclusion:**Reperfusion, not short-term ischemia, induced a disruption of F-actin microfilaments and a loss of microvilli. These modifications could lead to the impaired bile secretion by damaging canalicular contraction, and could be the main mechanisms of cholestasis after hepatic ischemia-reperfusion in rats.

Keywords: ischemia-reperfusion; cholestasis; F-actin

INTRODUCTION

Hepatic ischemia-reperfusion often leads to cholestasis[1-3]. However, the mechanism of this cholestasis is not yet fully understood. F-actin microfilaments are distributed along the plasma membrane, particularly in the region of bile canaliculi, and these microfilaments play an important role in bile secretion during canalicular contraction[4-5]. At present, domestic and international reports on the changes of bile canalicular F-actin microfilaments in hepatic ischemia-reperfusion are rare. We therefore designed experiments to study the modification of bile canalicular F-actin microfilaments, quantified by using FITC-Phalloidin and analyzed by confocal laser scanning microscopy imaging, with the goal of determining the mechanism of cholestasis following hepatic ischemia-reperfusion in rats.

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MATERIALS AND METHODS

Materials

Thirty-six adult male SD rats weighting 200~250 g were obtained from the Experimental Animal Center of Xi'an Jiaotong University, and were cared for in accordance with the Guide for the Care and Use of Laboratory Animals. FITC-Phalloidin and antifade solution were purchased from Alexis Corp. (China). Transmission electron microscopy(H-600, Hitachi Corp., Japan) was provided by the Electron Microscope Centre of Medical School of Xi'an Jiaotong University. Confocal laser scanning microscopy imaging(Model:TCS Sp2, Leica Microsystems, Germany) was performed by the Laboratory Centre of Medical School of Xi'an Jiaotong University.

Experimental group

Thirty-six rats were randomly allocated to six groups of 6 rats each. ① Control group: Sham operation.

② Ischemia group:The hepatic pedicle was occluded for 35 minutes, but not reperfused. ③I/R2h group: The hepatic pedicle was occluded for 35 minutes, and reperfused for 2 hours. ④ I/R 6 h group: The hepatic pedicle was occluded for 35 minutes, and reperfused for 6 hours. ⑤I/R 24 h group:The hepatic pedicle was occluded for 35 minutes, and reperfused for 24 hours. ⑥ I/R 48 h group:The hepatic pedicle was occluded for 35 minutes, and reperfused for 48 hours.

Animal model and tissue sampling

Rats were fasted for 12 hours and given free access to water before the operation. The animals were anesthetized by intraperitoneal injection of 3% sodium pentobarbital(30 mg/kg). A midline laparotomy was performed and the hepatic pedicle was dissociated. Complete hepatic ischemia was caused by clamping the hepatic pedicle with an atraumatic vascular clamp for 35 min. After 35 min of hepatic ischemia, the clamp was removed to initiate hepatic reperfusion. Tissues were obtained from the rats at the scheduled times. At the end of the protocol, blood(1 ml) was obtained by cardiac puncture and prepared for serum. The hepatic tissue was harvested immediately. Some sections were cut into 1 mm^3 cubes at 4℃, and fixed in 2.5% glutaraldehyde for electron microscopy. Some samples were washed free of excess blood with ice-cold saline, wiped free of saline, and prepared for cryopreservation. These liver samples were frozen in liquid nitrogen at −155℃, and then stored at −80℃ for F-actin analysis. Another group of tissue samples were fixed in neutral formalin.

① Determination of liver function indicators: Determination of serum alanine aminotransferase(ALT), aspartate aminotransferase(AST), γ-glutamyl transferase(GGT) and total bilirubin(TBIL). Serum was sent to the Clinical Laboratory of the Second Affiliated Hospital of Medical School of Xiản Jiaotong University and the above indicators determined using a 7600 automatic biochemical analyzer(Hitachi Corp., Japan).

② Histological/ultrastructural studies: The hepatic tissue, fixed in formalin, was embedded in paraffin, sectioned(3 μm thick), and stained with hematoxylin-eosin(HE). Pathological changes in the hepatic tissue were observed by optical microscopy. The hepatic tissue sections(1 mm^3) fixed in 2.5% glutaraldehyde were post-fixed in 1% osmium tetroxide fixative, dehydrated in acetone, embedded in Epon-812 resin, and then sections stained with uranyl acetate and lead citrate for 15 min. Bile canalicular ultrastructure was observed by transmission electron microscopy.

③Detection of F-actin[6]:The hepatic tissue stored at −80℃ was cut into 8 μm sections using a cryotome at −28℃. Sections were washed in phosphate-buffered saline(PBS) and dried. After fixation in 3.7% polyformaldehyde for 10 minutes at room temperature, the sections were washed in PBS 3 times. Subsequent steps were performed, avoiding light. The sections were incubated for 1 hour at room temperature with FITC-Phalloidin, dissolved in methanol and diluted to 10 μg/ml in PBS, and washed 3 times in PBS(15 minutes per wash) to remove excess fluorescence. Slides were mounted with antifade solution(1,4-phenylenediamine and glycerol in an aqueous buffer solution). Images were observed and collected by confocal laser scanning microscopy to determinate the mean specific fluorescence intensity. The intensity of fluorescence was expressed as the ratio between the total fluorescence of the area (total specific fluorescence) and the surface of this area (mean specific fluorescence). The mean values of bile canalicular membranes and basolateral membranes for each group were processed.

Statistical analysis

Experimental data were expressed as means ± standard deviation. Differences between the groups were examined by using analysis of variance and Dunnett's test. When variances were non-homogeneous, the non-parametric Rank Sum test was used. The *P*-value of < 0.05 was considered to be statistically significant.

RESULTS

Changes in the levels of serum ALT, AST, GGT and TBIL

Compared with the control group, the ALT and AST levels in all the other groups were significantly increased ($P < 0.05$). This was most obvious in the 6 hour reperfusion group. Compared with the control group, the GGT and TBIL levels in the reperfusion groups were significantly increased($P < 0.05$), and unlike the ALT and AST levels, the GGT and TBIL levels continued to increase with increasing duration of reperfusion (***Table 1***).

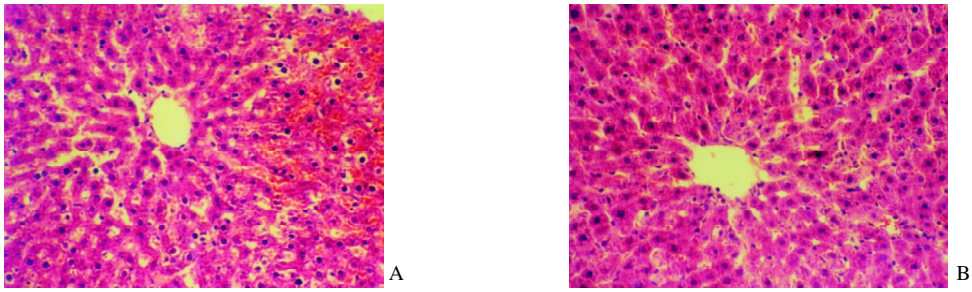
Table 1 **The levels of serum ALT, AST, GGT and TBIL** ($\bar{x} \pm s, n = 6$)

Group	ALT(U/L)	AST(U/L)	GGT(U/L)	TBIL(μmol/L)
Control	34.20 ± 8.91	93.53 ± 16.76	5.58 ± 2.69	5.13 ± 2.02
Ischemia	101.09 ± 10.53*	344.93 ± 27.65*	6.37 ± 1.96	5.98 ± 1.47
I/R 2h	366.67 ± 24.88*	915.09 ± 43.34*	16.56 ± 2.70*	9.14 ± 2.04*
I/R 6h	401.45 ± 27.43*	1 073.61 ± 51.99*	23.41 ± 3.97*	11.28 ± 2.02*
I/R 24h	288.46 ± 16.85*	532.64 ± 31.72*	26.28 ± 4.78*	15.94 ± 2.84*
I/R 48h	74.70 ± 13.07*	201.17 ± 20.07*	27.01 ± 5.40*	16.68 ± 2.72*

Compared with the control group, *$P < 0.05$.

Pathological changes in the hepatic tissue observed by optical microscopy

The hepatic tissue morphology in the control group exhibited normal morphology(***Fig. 1A***). The hepatic tissue did show mild inflammatory reactions in the each of the reperfusion groups, and this was most apparent in the 6 hour reperfusion group. This was manifest as cytoplasmic vacuolations, pyknotic nuclei and some necrotic hepatocytes(***Fig. 1B***).

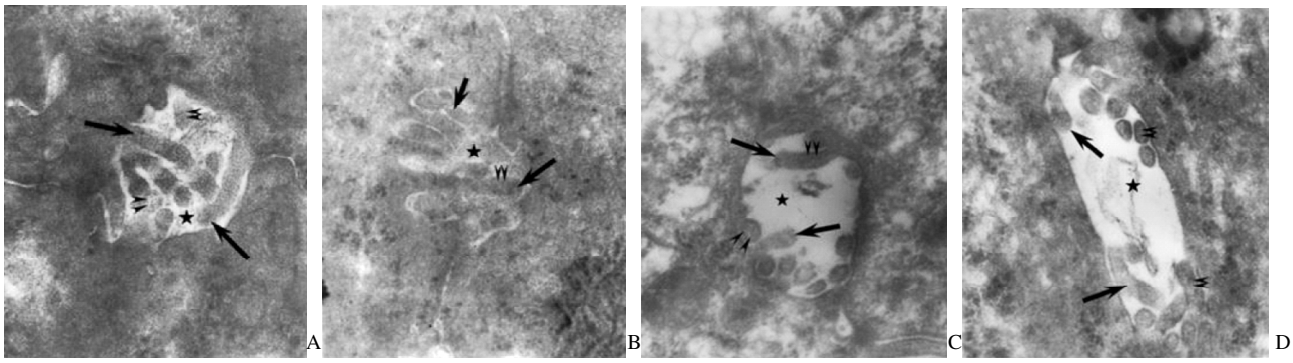


A: normal morphology of the hepatic tissue. B:after reperfusion for 6 hours.

Fig. 1 The hepatic tissue morphology(HE, 200 ×)

Changes in bile canaliculi observed by transmission electron microscopy

Normal bile canaliculi cross-sectional appearance in rats was observed by transmission electron microscopy in the control group. The apical membranes of the cells of the bile canaliculus were rich in microvilli. The microvillar cores formed by actin microfilaments were clear. Hepatocyte membranes surrounding bile canaliculi formed tight junctions. The connecting complexes of tight junctions and desmosomes kept the bile canaliculus lumen small(***Fig. 2A***). After 35 min of hepatic ischemia, microvilli still extended into the lumen of the bile canaliculus, but they were swollen and poorly defined(***Fig. 2B***). After reperfusion for 2, 6, 24 and 48 hours, more obvious morphological changes occurred in bile canaliculi. Bile canalicular lumina were expanded and became exfoliated of microvilli. The tight junction was widened occasionally. Cholestasis appeared to occur in the bile canaliculi(***Fig. 2C,2D***).



A:normal morphology of a bile canaliculus. B:after 35 min of hepatic ischemia. C:after reperfusion for 6 hours. D:after reperfusion for 48 hours. *indicates the bile canalicular lumen. Arrows show microvilli. Double arrows show F-actin microfilaments in microvilli.

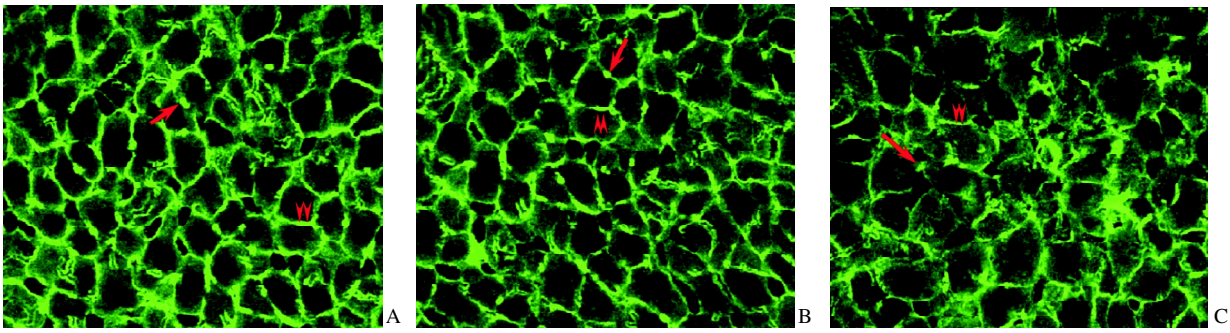
Fig. 2 Bile canalicular ultrastructure(electron microcopy, 50 000 ×)

Localization of F-action

In the control group, the fluorescence staining of F-actin was seen along the basolateral and bile canalicular membranes. F-actin was seen as bright small fluorescent dots in bile canalicular cross sections(***Fig. 3A***). In the ischemia group, the fluorescence staining was quite similar to that of the control group(***Fig. 3B***). However, after reperfusion for 2, 6, 24 and 48 hours, the staining of F-actin was clearly decreased. The fluorescence of hepatocyte basolateral membranes was disorganized and the fluorescence of bile canalicular membranes was decreased(***Fig. 3C***).

Quantitative analysis of bile canalicular membranes F-actin

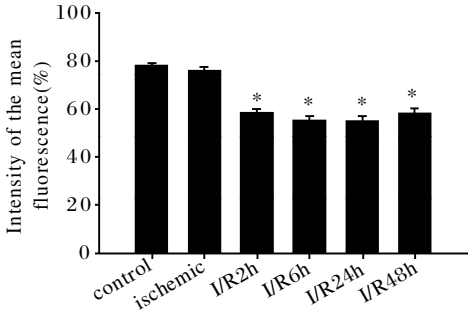
Confocal laser scanning microscopy images were studied quantitatively by measuring the intensity of the mean specific fluorescence of bile canalicular membranes. However, since the fluorescence staining of basolateral membranes after reperfusion was lower than the detection threshold of the analytical method, it was not possible to compare the mean specific fluorescence of basolateral membranes of the different groups.



A:normal morphology of F-actin. B:after 35 min of hepatic ischemia. C:after reperfusion for 6 hours. Double arrows indicate hepatocyte basement membranes.Arrow indicates bile canalicular membranes.

Fig. 3 Localization of F-actin using FITC-Phalloidin and confocal laser scanning microscopy(400 ×)

Fig. 4 shows that no significant difference in the intensity of the mean fluorescence of bile canalicular membranes F-actin was observed between the ischemia group and the control group($P > 0.05$). The intensity of the mean fluorescence of bile canalicular membranes F-actin between the 2, 6, 24 and 48 hour reperfusion groups all differed significantly from that of the control group($P < 0.05$, ***Fig. 4***).



Compared with the control group, $*P < 0.05$.

Fig. 4 Intensity of the mean fluorescence of bile canalicular membranes F-actin in each study group

DISCUSSION

F-actin is assembled from the G-actin monomers in an ATP-dependent manner[7], and is a key component of the cytoskeleton structure[8]. Tsukada *et al.*[4] found that bile canalicular microvilli contain many F-actin microfilaments which extend from the plasma membrane to the lumen and constitute the cores of the bile canalicular microvilli. Bile canaliculi are the smallest pipelines of bile secretion, and play an important role in the process of this secretion[4,9]. Bile secretion in the liver relies on bile canalicular contraction, and the continuous, effective and one-way canalicular contractions in the area around the portal tract can effectively push bile into the bile ducts[10-11]. Research has shown that continuous polymerization and depolymerization of F-actin microfilaments implement bile canalicular contraction, and further promote bile secretion[12]. It has been proved that structural alterations of F-actin microfilaments correlate with the loss of bile canaliculi integrity[13] and damage the bile canalicular contraction, thus leading to cholestasis[14]. Cholestasis often occurs in various surgical procedures, with shock, and in the early period after liver transplantation[1,3]. It has been reported by Geuken *et al.*[3] that bile salt secretion recovers before phospholipid secretion early after tranplantation, and the increased bile salt concentration causes bile duct injury. From the clinical point of view, it is of great importance that we understand the mechanism of cholestasis caused by the abnormal changes of F-actin microfilaments after hepatic ischemia-reperfusion.

In this study, we observed that the levels of serum ALT and AST peaked with 6 hours of reperfusion, and then declined with longer periods of reperfusion. On the other hand, the GGT and TBIL levels in each group gradually increased with increasing duration of reperfusion. The ALT and AST results suggest that a reversible injury and cholestasis occurred in the hepatocytes after mild warm ischemia-reperfusion injury. With the damaged hepatocytes gradually restored, there would probably still be cholestasis. It has also been reported that the levels of serum ALT and AST peak rapidly after an orthotopic liver transplantation, and then rapidly decline, while the GGT and TBIL levels continue to rise, and peak in two weeks [1,3]. Our results also suggested that there was cholestasis for a certain period of time after hepatic ischemia-reperfusion. At the same time, the pathological examination of the hepatic tissue in this study confirmed that the inflammatory response was mild in the rat model of hepatic ischemia for 35 minutes and reperfusion, and only a few hepatocytes were necrotic.

The present experimental results indicated that the changes of the fluorescence staining were in line with

the ultrastructural changes observed by electron microscopy. After reperfusion for 2, 6, 24 and 48 hours, the F-actin staining was reduced and the fluorescence disorganized. The intensity of the staining was significantly lower than that of the control group and the luminal microvilli were exfoliated. After 35 min of hepatic warm ischemia, the fluorescence staining was not significantly changed compared with the control group, and canalicular microvilli still existed. These morphological results paralleled the abnormal changes of the serum GGT and TBIL after reperfusion. These present results show:①F-actin microfilament destruction and the collapse of the cytoskeleton after hepatic ischemia-reperfusion in rats, suggesting that these changes lead to an impaired contractile function of bile canaliculi and an obstacle for bile secretion, thus resulting in cholestasis. ② Bile canalicular microfilament F-actin is sensitive to reperfusion, while insensitive to the short-term(35 min) warm ischemia alone. Benkoel *et al.*[6] found that compared to the control group, a 25% reduction of fluorescence was observed in the F-actin of bile canalicular membranes in the early reperfusion period, and in contrast, the staining was similar to that of the control group after cold ischemia. Cutrin *et al.*[1] confirmed that large numbers of bile canalicular microvilli are exfoliated in the early period of reperfusion after liver transplantation, and these changes are similar to bile canaliculi and microvilli perfused with cytochalasin B, whereas bile canalicular microvilli have no significant changes after cold ischemia. This was similar to our experimental results.

The alteration of F-actin microfilaments after reperfusion is probably the result of F-actin disruption by reactive oxygen species(ROS). A large amount of reactive oxygen occurs in the early period of hepatic ischemia-reperfusion[12]. Studies using *in vitro* rat hepatocytes confirmed that ROS could lead to actin rearrangement which was mediated by Ca^{2+}-dependent PKC, rather than by direct oxidation, and oxidative stress is known to lead to increased concentrations of Ca^{2+} and disintegration of F-actin mediated by Ca^{2+}-dependent PKC[15]. In addition, the damage to F-actin microfilaments may have been related to the changes of calcium balance and the ATP consumption[16]. Hepatic ischemia-reperfusion injury created a calcium disequilibrium, which lead to a calcium overload in hepatocytes[17]. The hepatocyte calcium overload activated intracellular phospholipase C, phospholipase A2 and Ca^{2+}-dependent protease, thereby damaging the actin cytoskeleton.When the mitochondrial calcium is overloaded, calcium combines with phosphate producing insoluble calcium phosphate, leading to impaired respiratory function, disordered energy metabolism and reduced ATP production. At the same time, calcium can activate certain ATPases and accelerate the ATP consumption. Thus the depletion of the ATP could cause F-actin destruction, resulting in cytoskeleton damage and loss of microvilli[18]. In our experiments,bile canalicular changes were observed by confocal laser scanning microscopy imaging and electron microscopy.

Gutmayer *et al.*[19] reported that the actin cytoskeleton plays an important role in protecting the plasma membrane from damage caused by the high concentration of bile salts. Cholestasis could itself cause an increase in the concentration of canalicular bile salts. Destruction of the cytoskeleton may cause damage of the bile canalicular membranes, further exacerbating cholestasis.

It is worth mentioning that although there is oxidative stress in short-term hepatic warm ischemia(e.g. 35 minutes in this experiment), the level of stress is minor, not enough to cause obvious injury[20]. In addition, during the ischemic period, there is almost no change in the calcium concentration in the hepatocytes, but the calcium concentration increases sharply in the reperfusion period. This may be the reason that F-actin microfilaments did not exhibit significant changes after 35 minutes of ischemia. Fukai *et al.*[20] found that the degree of oxidative stress which occured in the warm ischemia period was greater with the extension of ischemic time in the rat segmental(70%) hepatic ischemia-reperfusion experiment. Therefore, we speculate that with the extension of the warm ischemia time, changes may occur in F-actin microfilaments during the ischemic period. This suggests that during the implementation of liver transplantation, partial hepatectomy, etc, we should shorten the hepatic warm ischemia time to the greatest degree possible, and thus reduce any injury induced by oxidative stress to F-actin microfilaments, and reduce the possibility of postoperative cholestasis.

Cholestasis, which occurred in the rat 35 minute hepatic ischemia and reperfusion model, was reversible[2]. Due to the limited time frame of the present experiments, we could not ascertain the morphological and functional recovery of the damaged F-actin microfilaments, microvilli and bile canaliculi, or the long-term effects of bile secretion. This will require further research.

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