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ORIGINAL ARTICLES

Histological and biochemical alterations in early-stage lobar ischemia-reperfusion in rat liver

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Abstract

AIM: To investigate the structural and biochemical changes in the early stage of reperfusion in the rat livers exposed to lobar ischemia-reperfusion (IR).

METHODS: The median and left lobes of the liver were subjected to 60 min ischemia followed by 5, 10, 30, 45, 60 and 120 min reperfusion. Blood samples were taken at different time intervals to test enzyme activities and biochemical alterations induced by reperfusion. At the end of each reperfusion period, the animals were killed by euthanasia and tissue samples were taken for histological examination and immunohistochemistry.

RESULTS: Cell vacuolation, bleb formation and focal hepatitis were the most important changes occur during ischemia. While some changes including bleb formation were removed during reperfusion, other alterations including portal hepatitis, inflammation and the induction of apoptosis were seen during this stage. The occurrence of apoptosis, as demonstrated by apoptotic cells and bodies, was the most important histological change during reperfusion. The severity of apoptosis was dependent on the time of reperfusion, and by increasing the time of reperfusion, the numbers of apoptotic bodies was significantly enhanced. The amounts of lactate dehydrogenase, alanine aminotransferase, aspartate aminotransferase, creatinine and urea were significantly increased in serum obtained from animals exposed to hepatic IR.

CONCLUSION: Inflammation and subsequent apoptotic cell death were the most important changes in early-stage hepatic reperfusion injury, and the number of apoptotic bodies increased with time of reperfusion.

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Key words: Lobar ischemia; Liver; Reperfusion injury; Apoptosis; Immunohistochemistry

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INTRODUCTION

Reperfusion of a previously ischemic tissue is associated with additional injury that leads to structural and functional alterations in many organs including the liver. The hepatic injury that occurs during reperfusion has been shown to be the major problem associated with stroke, shock, cirrhosis, liver surgery and transplantation[1-4]. The mechanisms of reperfusion-induced pathological and functional alterations are under intensive investigation, but the results of different studies are controversial. Some studies suggest that the reintroduction of oxygen to the ischemic (hypoxic) tissues stimulates the production of reactive oxygen species (ROS), which contribute to cell damage. Others have argued that the liver tissues are basically resistant to the oxidative stress followed by ischemia-reperfusion (IR)[5-7]. However, many studies have shown that the ischemic livers undergo moderate[8,9] to severe structural and functional alterations by IR[10,11].

It has been shown that the injury induced during

reperfusion has a biphasic pattern that consists of an early stage that starts upon reoxygenation and a delayed phase. The early stage is associated with hepatocellular damage during 2-6 h after reperfusion (reoxygenation), and the delayed phase occurs 18-24 h after reperfusion and is accompanied with a massive neutrophil infiltration[12-15]. The injury in early stage (acute phase) is mediated by ROS, but the damage in the delayed stage (subacute phase) is associated with the inflammatory responses mediated by neutrophil activity. It is thought that ROS formation during reperfusion induces a cascade of cellular events that eventually leads to hepatocellular injury, including inflammation, necrosis, and/or apoptosis[16-19]. However, the detailed mechanisms of cell death and the structural alterations induced during different stages of reperfusion injury are not yet completely determined. Some studies have reported that the morphological changes induced by reperfusion are predominantly limited to non-parenchymal cells[20-23], whereas others have shown that some changes are seen in parenchymal cells[8,24].

While necrosis has been shown to be the cause of hepatic IR injury, many studies have shown that programmed cell death or apoptosis is the cause of cell death during liver reperfusion after long-term ischemia[20,25,26]. However, the role of apoptosis as the main cause of the injury and the level of morphological changes induced by this type of cell death have not been determined in detail.

The present study was designed to characterize the features of the injury induced in the early stage of reperfusion in rat liver. The ischemia was established by a lobar model and the ischemic liver was exposed to different reperfusion times. The hepatic alterations were assessed by both histological and biochemical observations.

MATERIALS AND METHODS

Animals and experimental groups

Female Sprague-Dawley rats weighing 230-280 g were used in all experiments. The animals were group-housed with a 12-h light-dark cycle and fed a standard laboratory diet. All experiments were performed according to the standard procedures outlined by our institutional guidelines. Rats were fasted overnight for at least 16 h prior to the experiments, but access to water was uninterrupted.

A group of four animals were subjected to 60 min lobar ischemia only. They were sacrificed at the end of this period and then liver tissue samples were taken for histology and immunohistochemistry (IHC). Seven groups of animals underwent 60 min ischemia followed by 5, 10, 15, 30, 54, 60 or 120 min reperfusion. A sham-operated group was selected for each test groups as a control.

In vivo (lobar) models of IR

This term refers to the model in which the ischemia is induced in the anesthetized animals through application of a vascular clamp simultaneously to branches of the hepatic portal vein, hepatic artery and bile duct. The reperfusion is commenced by removal of the clamp, thus restoring normal blood flow. Anesthesia was induced by a single intraperitoneal (i.p.) injection of ketamine (80 mg/kg) plus xylazine (10 mg/kg). After injection of 300 U of heparin *via* the femoral vein, the right jugular veins were catheterized by polyethylene tubing for blood sampling and infusion of normal saline solution to replace the removed blood. After laparotomy, the median and left lobes of the liver were removed from the abdominal cavity. Then, *in vivo* lobar ischemia was induced by clamping the left branches of the hepatic portal vein, hepatic artery and bile duct with a microvascular occlusion clip for a period of 60 min. This, caused occlusion of all blood vessels supplying the median and left lobes of the liver, which is reported to produce approximately 70% (partial) liver ischemia[27]. Upon release of the clamp, reperfusion was commenced and the blood flow was continued for different times as described above. Control (sham-operated) groups underwent the same surgical procedure, except that the blood supply to the liver lobes was not interrupted.

Biochemical assays

Blood samples were taken at different times before ischemia, during ischemia and after reperfusion. The plasma was separated by centrifuging the blood, which was kept in the freezer until analysis. The release of lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes, as well as the level of glucose, urea and creatinine were measured by a Hitachi 747 analyzer (Boehringer Mannheim).

Histological examination

Small pieces of liver were taken from both left and median hepatic lobes. Parts of samples were fixed in 10% formalin for light microscopy. Paraffin embedded sections of 5-μm thickness were stained with hematoxylin and eosin and/or periodic acid-Schiff. The remaining samples were fixed with 3% glutaraldehyde/4% paraformaldehyde in 0.1 mmol/L sodium cacodylate buffer for electron microscopy. They were transferred into sodium cacodylate buffer on the following day and then stored at 4℃ until processing.

IHC

IHC was carried out to detect the presence of apoptosis protease-activating factor 1 (APAF-1) as a marker of apoptosis induction in tissue samples. Serial embedded sections were prepared from formalin-fixed samples, which were cut at a thickness of 3 μm and dried at 37℃ overnight. IHC was performed by the avidin-biotin complex (ABC) procedure, including heat-induced epitope-retrieval and enzymatic antigen-retrieval procedures. Incubation with the primary antibody (NCL-APAF-1; Novocastra; 1:100 dilutions) was carried out in a moist chamber at 37℃ for 1 h. Negative controls were treated identically, with the primary antibody omitted; positive controls consisted of normal hepatic tissue.

Table 1 Data analysis of different biochemical tests obtained from livers subjected to 60 min ischemia followed by 30-120 min reperfusion

Time of sampling	Glucose (μg/dL)	Urea (μg/dL)	Creatinine (μg/dL)	AST (IU/L)	ALT (IU/L)	LDH (IU/L)
Before ischemia	261.33 ± 36.16	17.66 ± 2.88	0.55 ± 0.058	54.82 ± 19.82	78.2 ± 24.3	153.75 ± 0.11
	n = 5	*n* = 5	*n* = 5	*n* = 5	*n* = 5	*n* = 16
During ischemia	189.66 ± 18.8	18.33 ± 1.2	0.57 ± 0.075	12.74 ± 9.51	18.19 ± 4.39	39.69 ± 8.58
	n = 5	*n* = 5	*n* = 7	*n* = 5	*n* = 5	*n* = 16
After reperfusion	160.83 ± 10.63	26.50 ± 1.66	2.62 ± 1.29	61.58 ± 9.12	142.28 ± 30.94	200.52 ± 60.52
	n = 14	*n* = 14	*n* = 13	*n* = 10	*n* = 7	*n* = 23

The values are expressed as the mean ± SE taken from at least 12 samples in test.

Table 2 Comparison of data obtained from biochemical analysis of blood samples taken from the animals exposed to 60 min ischemia followed by different times of reperfusion

	P-value					
Sampling phase	Glucose	Urea	Creatinine	AST	ALT	LDH
Before ischemia/ during ischemia	0.38	0.72	0.79	0.043	0.049	0.034
Before ischemia/ after reperfusion	0.31	0.05[a]	0.006[a]	0.08	0.043[a]	0.008[a]
During ischemia/ after reperfusion	0.17	0.05[a]	0.05[a]	0.08	0.005[a]	0.014[a]

[a]$P < 0.05$.

Statistical analysis

Data from biochemical assays are expressed as the mean ± SD obtained from at least four experiments in each group. They were analyzed by ANOVA using the SPSS program and the significance of the differences between groups were tested by Tukey's post-hoc test, with $P < 0.05$ considered statistically significant. Pathological changes in both untreated and treated groups were scored semi-quantitatively from + (mild) to ++++ (severe).

RESULTS

Biochemical changes induced by reperfusion injury

There were significant changes in enzyme release and blood urea and creatinine level in the animals exposed to hepatic IR, compared to the controls. The data obtained from analysis of biochemical assays are summarized in Table 1, and comparison of the results at different times of reperfusion is shown in Table 2. As seen in the tables, while enzyme release was significantly reduced during ischemia ($P < 0.05$), the level of glucose, creatinine and urea did not change in the blood of animals exposed to lobar hepatic ischemia. However, plasma level of LDH, AST, creatinine and urea was significantly increased during reperfusion (Table 2).

Morphological findings

The histological changes induced by IR were examined, based on portal inflammation, focal inflammation, sinusoidal congestion, cytoplasmic vacuolation, bleb formation, apoptotic cells and apoptotic bodies production. The liver samples from the sham-operated group did not show significant histological alterations,

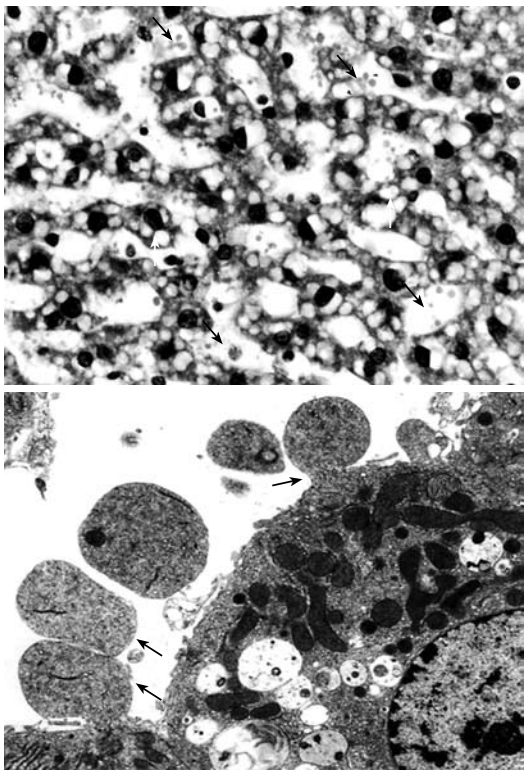

Figure 1 Zone 3 cytoplasmic vacuolation and hepatocyte bleb formation in the liver subjected to 60 min ischemia only. A: The white arrow shows cytoplasmic vacuolation and the black arrows show the cytoplasmic blebs formation with light microscopy; B: The arrows shows the cytoplasmic blebs with electron microscopy.

similar to those of the non-operated control group. Changes observed in the liver exposed to lobar ischemia alone were limited to mild to moderate focal hepatitis, sinusoidal congestion, vacuolation and bleb formation (Figure 1).

During reperfusion, whilst some changes including blebbing of hepatocytes were improved, induction of portal hepatitis and mild apoptosis were added at 5 min of reperfusion. By increasing the time of reperfusion, the induction of portal and local hepatitis were reduced, but the amount of apoptosis was moderately increased, so that at 30 min reperfusion, the presence of apoptotic cells but not apoptotic bodies was the most important change in the majority of tissue samples (Figure 2A). Sixty minutes ischemia followed by 45 or 60 min reperfusion caused an increased amount of apoptosis

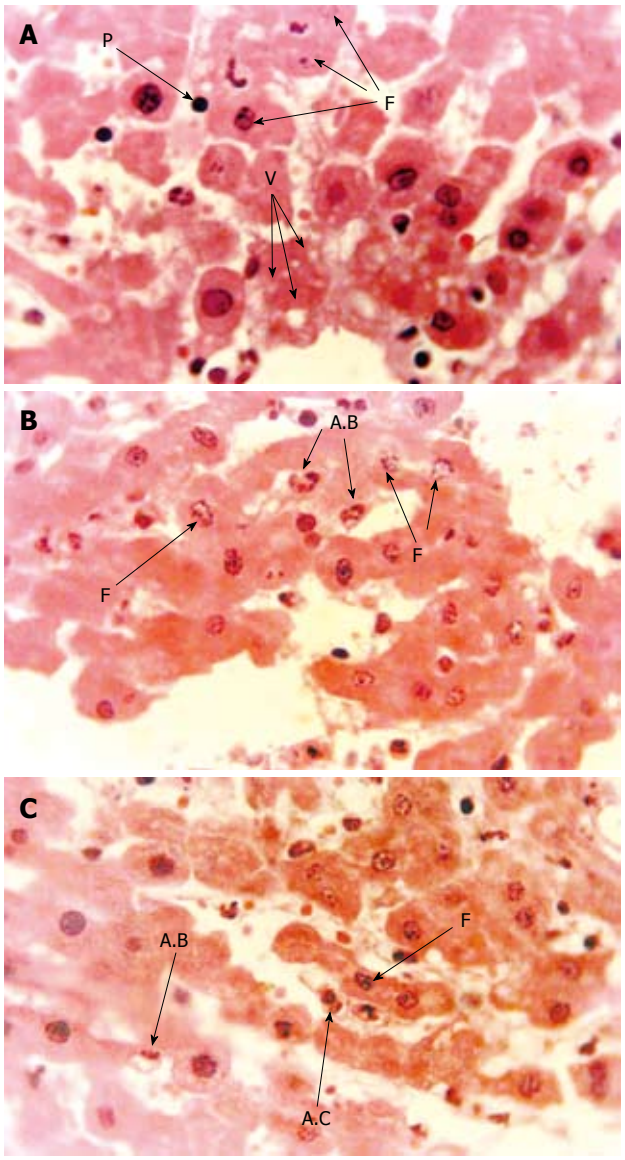

Figure 2 Histological changes in the livers exposed to 60 min lobar ischemia followed by different times of reperfusion. A: Nuclear pyknosis (P), nuclear fragmentation (F) and cytoplasmic vacuolation in the liver exposed to 60 min ischemia followed by 30 min reperfusion; B: Apoptotic bodies (A.B) and nuclear fragmentation (F) in the liver exposed to 60 min ischemia followed by 60 min reperfusion; C: Nuclear fragmentation (F), apoptotic cell (A.C) and apoptotic bodies (A.B) in the liver exposed to 60 min ischemia followed by120 min reperfusion.

with phagocytic apoptotic bodies and sinusoidal congestion (Figure 2B). However, in the livers that underwent ischemia and 60 or 120 min reperfusion, phagocytic apoptotic bodies were seen in most tissue samples (Figures 2C and 3). To organize different groups exposed to different reperfusion times, they were classified into three durations: short time, 5 and 10 min; middle time, 30, 45 and 60 min; and long time (120 min). Statistical analysis of the histological alterations and the severity of hepatic changes in the liver subjected to 60 min ischemia followed by short, middle and long times of reperfusion in comparison with the changes induced by 60 min ischemia only are summarized in Figure 4.

The occurrence of apoptosis was confirmed by

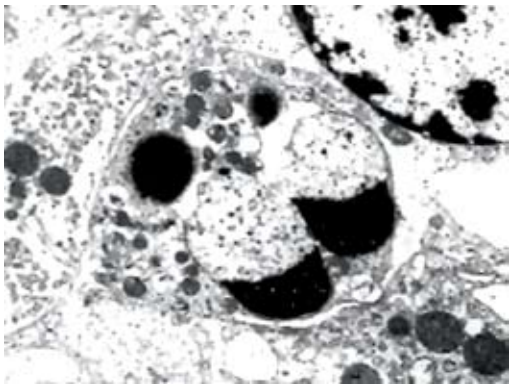

Figure 3 Apoptotic bodies of an endothelial origin apoptotic cell phagocyted by a hepatocyte in the liver exposed to 60 min ischemia followed by 60 min reperfusion.

IHC, in which the expression of APAF-1 was positive in the stained sections of livers exposed to IR, and the presence of apoptosis and/or apoptotic bodies was seen by light and electron microscopy (Figure 5A). Representative staining patterns for APAF-1 expression showed that the occurrence of apoptosis was limited to the pericentral area (Figure 5B). The presence of cells labeled with brown color indicated that the expression of APAF-1 was increased during 5-15 min of reperfusion. This showed that the occurrence of apoptosis occurred upon the initiation of reperfusion. However, during 30-60 min of reperfusion, the presence of cells with a brown-colored cytoplasm was accompanied with apoptotic bodies in which, when reperfusion time was increased (120 min), the number of apoptotic bodies also increased (Figure 5B).

DISCUSSION

In the present study, an *in vivo* lobar (partial) model of rat liver IR injury was established by clamping the vessels to the left lateral and median hepatic lobes, which account for 70% of the rat liver mass. This hepatic insult is similar to the clinical situation when the liver is rendered ischemic during total vascular exclusion for liver resection[28]. We tried to illustrate the pathological profile of the liver exposed to 60 min ischemia followed by different periods of 5, 10, 30, 45, 60 and 120 min reperfusion. It was found that cell vacuolation, bleb formation and focal hepatitis were the most important changes induced by *in vivo* lobar ischemia in rat liver. However, during reperfusion, not only some changes including bleb formation was reduced, but some other alterations including portal hepatitis, inflammation and the induction of apoptosis, occurred. It appears that the occurrence of apoptosis, as demonstrated by the formation of apoptotic cells and bodies, is the most important histological change during the early stage of reperfusion. The severity of apoptosis was dependent on the time of reperfusion, so that by increasing the time of reperfusion, the number of apoptotic bodies was significantly enhanced. To accompany these changes,

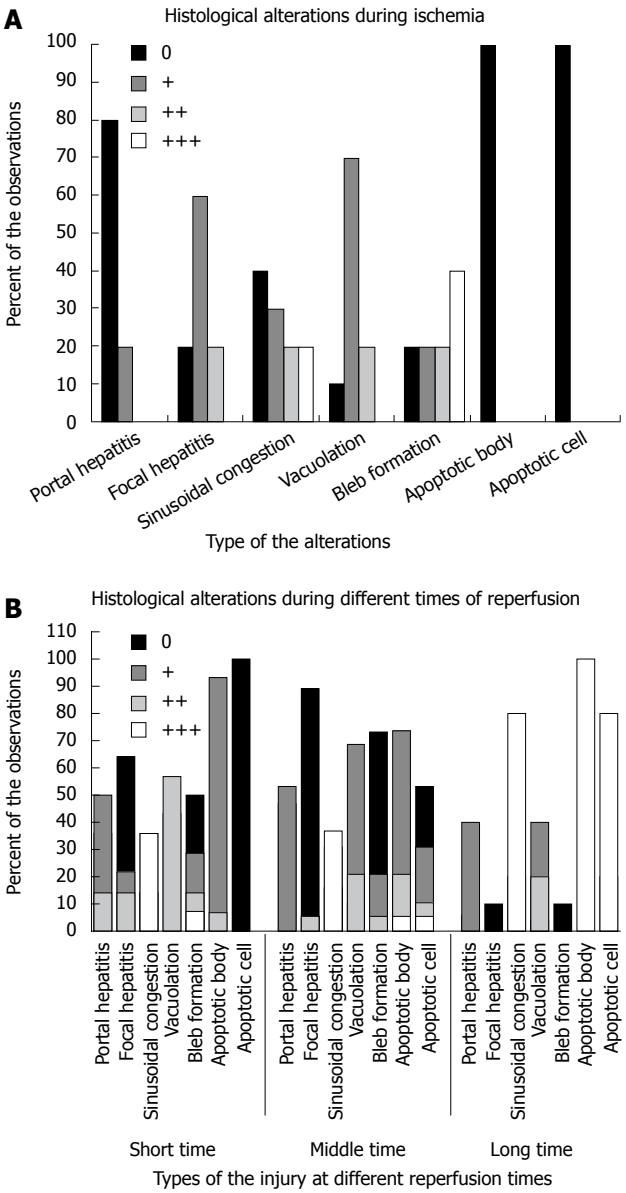

Figure 4 A summary of statistical analysis of histological alterations in the livers exposed to ischemia or ischemia-reperfusion. A: Hepatic changes in the liver exposed to 60 min lobar ischemia only; B: Changes in the liver subjected to 60 min ischemia followed by short, middle and long times of reperfusion in which: 0= Normal or no changes, +: Mild injury, ++: Moderate injury, +++: Severe injury; 0 < A.C/A.B ⩽ 2 = +, 2 < A.C/A.B ⩽ 5 = ++, 5 < A.C, A.B = +++.

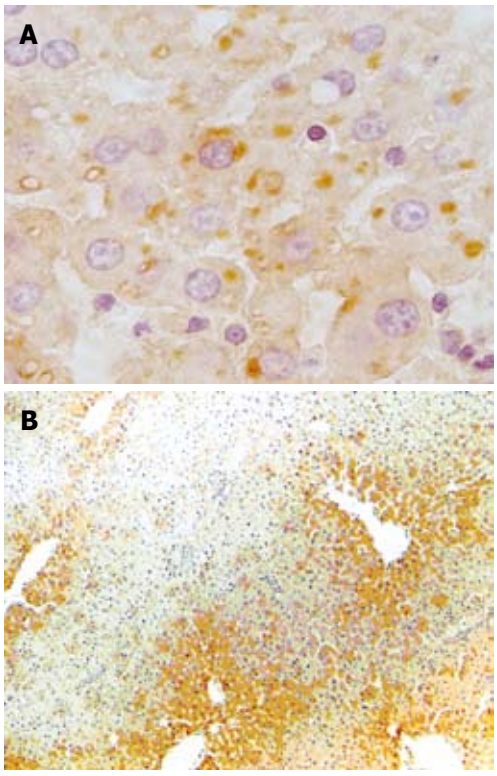

Figure 5 Confirmation of apoptosis by IHC assay in the staining sections of livers exposed to ischemia-reperfusion and the presence of apoptosis cells and/or apoptotic bodies. A: Representative staining patterns for *APAF-1* positive, illustrating the occurrence of apoptosis in the sections of the liver exposed to 60 min ischemia followed by long time of reperfusion; B: *APAF-1* positive staining that shows the high level of apoptosis incidence in the pericentral area of the liver exposed to 60 min ischemia followed by long time of reperfusion.

the serum level of LDH, ALT, creatinine and urea was significantly increased in rats exposed to hepatic IR, which indicates the induction of cell injury in the liver and other organs, including the kidneys.

Hepatic IR injury is a common pathological phenomenon that may be induced after severe liver trauma, extensive hepatic lobe excision, liver transplantation, and shock. By initiation of reperfusion injury, a series of functional, humoral and structural alterations occur in the liver tissues that directly influence the prognosis of patients[3-5,9]. The mechanisms by which the reperfusion injury induces pathological and functional alterations and the methods of intervention have been under intensive investigation. However, the detailed pathological mechanisms of liver injury induced by this phenomenon are complex and not yet fully understood. Different *in vivo* and *in vitro* models have been used to establish the pathological process of hepatic IR injury, such as the liver transplantation model, the partial warm or cold IR model, and the total hepatic IR model[6,28-30].

There is evidence that the pathogenesis of reperfusion injury involves a series of events, including Kupffer cell activation, cytokine release, neutrophil activation, increased expression of adhesion molecules, sinusoidal endothelial cell death, and hepatocyte injury[15,19,31-33]. Among these, the inflammatory process and activation of Kupffer cells, which result in the release of excessive quantities of cytokines and ROS formation, play a major role[4,17]. The inflammatory aspect of the injury includes cellular and humoral components. A growing body of evidence, primarily from animal models of IR and preliminary human studies, has revealed that the inflammatory mechanisms may play a major role in the pathogenesis of the injury induced by reperfusion. It has been shown that hepatocyte injury followed by reperfusion is partly dependent on Kupffer cell activation and production of inflammatory mediators. This occurs in a biphasic pattern that consists of acute-phase (ROS-mediated) and subacute-phase (neutrophil-mediated) damage[8,19,20,24].

Our findings strongly suggest the occurrence of inflammation and the subsequent cell death by apoptosis as an important morphological change observed in the early stage (acute phase) of reperfusion. It is proposed that in the early stage of hepatic reperfusion injury, these inflammatory reactions and the different stress processes that follow may result in the activation of the apoptotic pathway mediated by mitochondria. This may lead to an increased number of apoptotic cells and apoptotic body formation, which is associated with a reduction in the total number of parenchymal cells, thus damaging the hepatic tissues and resulting in liver dysfunction[20,27].

The role of apoptosis as a cell death mechanism in reperfusion injury has been shown by some studies previously[20,34,35]. Although other alterations including hepatocyte blebbing, sinusoidal congestion, and portal and focal hepatitis were shown have been seen in liver histology, the integrity and organizational arrangement of the hepatic acinus remains intact. This suggests that the liver parenchyma is able to resist against these types of insults[9]. This has been confirmed with further studies that have demonstrated that parenchymal cells are not susceptible to damage under some conditions of ischemia-reperfusionIR[21-23].

In the present study, the occurrence of apoptosis was confirmed by IHC of the liver for APAF-1 expression. Positive APAF-1 staining in most sections of liver exposed to IR confirmed the role of apoptosis as the main cause of cell death in early-stage hepatic reperfusion injury. APAF-1 has been identified as a key protein that plays an essential role in the induction of apoptosis in different mammalian cells. In response to apoptotic stimuli, such as ROS, Ca^{2+} and cytokines released by reperfusion, APAF-1 binds to cytochrome c and procaspase 9, to yield a complex entitled the apoptosome. Activation of procaspase 9 through an autocatalytic process initiates a cascade of downstream effector caspases, which finally leads to mitochondrial apoptosis. The mitochondrial/cytochrome c apoptotic pathway and the expression of APAF-1 have attracted close attention of researchers to determine the induction of apoptosis[36-39]. Using IHC, we found that the occurrence of apoptosis was started in the initial phase of reperfusion, and it was completed as the reperfusion time increased. This was shown by the abundance of apoptotic bodies phagocytosed by macrophages or neighboring hepatocytes during long periods of reperfusion. Increased expression of APAF-1 in zone 3 of the liver indicated the greater susceptibility of this area of the liver to reperfusion injury.

In conclusion, we showed that inflammation and apoptosis were the major histological alterations induced by early-stage reperfusion injury in the liver exposed to lobar ischemia. It appears that apoptosis is the most important histological change induced in the early stage of hepatic reperfusion injury, during which the number of apoptotic bodies was increased with the time of reperfusion.

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COMMENTS

Background

Reperfusion of a previously ischemic tissue is associated with additional injury that leads to structural and functional alterations in many organs, including the liver. The mechanisms of reperfusion-induced pathological and functional alterations are under intensive investigation, but the results of different studies are controversial.

Research frontiers

The injury induced during reperfusion evolves in a biphasic pattern that consists of an early stage that starts with reoxygenation and a delayed phase. The early stage is associated with hepatocellular damage at 2-6 h after reperfusion, and the delayed phase occurs at 18-24 h after reperfusion, and is accompanied by massive neutrophil infiltration. The injury in the early stage is mediated by reactive oxygen species (ROS), but the damage in the delayed stage is associated with the inflammatory responses mediated by neutrophil activity. It is thought that ROS formation during reperfusion induces a cascade of cellular events that eventually leads to hepatocellular injury. However, the detailed mechanisms of cell death and the structural alterations induced during different stages of reperfusion injury are not yet completely understood.

Innovations and breakthroughs

In the present study, the authors demonstrated that the occurrence of inflammation and the subsequent cell death by apoptosis were the most important changes in the early stage of hepatic reperfusion injury.

Applications

By characterizing the feature of the injury induced in the early stage of reperfusion, this study may represent a future strategy for therapeutic intervention of reperfusion injury induced under different conditions, such as stroke, shock, cirrhosis, liver surgery and transplantation.

Terminology

Lobar ischemia refers to the model in which the ischemia is induced in the anesthetized animals through application of a vascular clamp simultaneously to branches of the hepatic portal vein, hepatic artery and bile duct.

Peer review

The authors examined the effects of 60 min lobar ischemia followed by different periods of 5, 10, 30, 45, 60 and 120 min reperfusion. It was found that the occurrence of apoptosis, as demonstrated by apoptotic cells and bodies, was the most important histological change during reperfusion. The severity of apoptosis was dependent on the time of reperfusion, such that by increasing the time of reperfusion, the number of apoptotic bodies was significantly enhanced.

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