Recovery of the Cholangiocytes After Ischemia and Reperfusion Injury: Ultra-Structural, Hystological and Molecular Assessment in Rats

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Introduction: **Ischemia–reperfusion (I/R) injury of the liver is a common area of interest to transplant and hepatic surgery. Nevertheless, most of the current knowledge of I/R of the liver derives from the hepatocyte and little is known of what happens to the cholangiocytes. Herein, we assess the sequence of early events involved in the I/R injury of the cholangiocytes.** ***Methods:*** **Sixty Wistar rats were randomized in a *SHAM* group and I/R group. Serum biochemistry, histopathology, immunohistochemistry, transmission electron microscopy (TEM) and laser capture microdissection (LCM) were used for group comparison.** ***Results:*** **There was peak of alkaline phosphatase 24 h after IR injury, and an increase of aspartate aminotransferase and alanine aminotransferase after 6 h of reperfusion, followed by a return to normal levels 24 h after injury. The I/R group presented the liver parenchyma with hepatocellular degeneration up to 6 h, followed by hepatocellular necrosis at 24 h. TEM showed cholangiocyte injury, including a progressive nuclear degeneration and cell membrane rupture, beginning at 6 h and peaking at 24 h after reperfusion. Cytokeratin-18 and caspase-3-positive areas were observed in the I/R group, peaking at 24-h reperfusion. Anti-apoptotic genes Bcl-2 and Bcl-xl activity were expressed from 6 through 24 h after reperfusion. BAX expression showed an increase for 24 h.** ***Conclusions:*** **I/R injury to the cholangiocyte occurs from 6 through 24 h after reperfusion and a combination of TEM, immunohistochemistry and LCM allows a better isolation of the cholangiocyte and a proper investigation of the events related to the I/R injury. Apoptosis is certainly involved in the I/R process, particularly mediated by BAX. (J Clin Exp Hepatol 2018;8:380–389)**

Ischemia reperfusion (I/R) injury is a set of clinical, histological, and molecular manifestations resulting from an ischemic process, followed by a slow return of the blood flow to a tissue.[1] In patients who undergo liver surgery, a major cause of mortality is the hepatic failure usually due to insufficient functional liver mass combined to the effects of I/R injury in the remnant liver.[2] In the transplant setting, there are also several inflammatory and immunological mechanisms known to be involved with the pathology caused by I/R. Thus, a major focus of liver research is the investigation of how the I/R process develops, in order to achieve a better control and/or to avoid it altogether, either by pharmacological agents or through new strategies that preserve a functional liver parenchyma.[3]

Much of the current knowledge about I/R injury in the liver comes from the use of animal models, mainly focused on the study of changes in the hepatocytes.[4] Nevertheless, little is known about what happens to cholangiocytes during I/R injury.[5] Biliary tree damage due to I/R has been poorly studied, mostly during liver surgery.[6–10] I/R might cause biliary complications and graft non-function after transplantation or liver failure after major liver resection in patients.[10] Murine studies have shown a deleterious effect of I/R injury directly on the biliary epithelium.[6,7] In a study of organ preservation for transplantation in humans, I/R injury changed the basal lamina of the cholangiocytes, leading to severe invasion of this membrane by leukocytes.[8] Changes in the bile flow after I/R injury and an increased rate of apoptosis with a decreasing turnover of cholangiocytes have also been described in rats.[9] However, these studies did not detail the sequence of early events or the apoptotic molecular pathways that impact the severity of I/R injury in the cholangiocytes.

Keywords: ischemia–reperfusion, cholangiocytes, apoptosis, rats
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Abbreviations: ALKP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BIL: bilirubin; CK18: cytokeratin-18; GGT: gamma-glutamyl transferase; HPC: hepatic progenitor cells; I/R: ischemia–reperfusion; LCM: laser capture microdissection; PCNA: proliferating cell nuclear antigen; TEM: transmission electron microscopy; VEGF: vascular endothelial growth factor

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Abnormalities in the apoptotic process are involved in the pathogenesis of various hepatobiliary diseases such as acute and chronic viral hepatitis, chronic cholestatic diseases, autoimmune hepatitis, liver graft rejection, alcohol-associated injury and chemicals, I/R injury, metabolic diseases, hepatocellular carcinoma and cholangiocarcinoma.[11] Nevertheless, the clinical implications of the abnormalities of the apoptotic process with liver disease are not fully understood. The late manifestation of intrahepatic bile duct ischemia in murine models have been better defined,[9] but little is known about the early alterations in the period of I/R of the cholangiocytes.

There are recent findings on the mechanisms of the adaptation of cholangiocysts to the damage, with particular emphasis on the molecular pathways susceptible to the therapeutic intervention, as well as the morphogenic pathways that have been shown to regulate the response to biliary lesions.[12] We believe that a better understanding of the initial changes triggered by I/R injury in the cholangyocites, and the role of apoptosis within this process, could lead to new therapeutic options for biliary diseases.

In this context, the aim of this study was to analyze the sequence of the events involved in the early phase of I/R injury of the cholangiocytes.

METHODS

Protocols, Animals and Surgery

This study was approved by the Institutional Animal Care and Use Committee of Hospital Israelita Albert Einstein (Protocol number 1431-11). All animals received care according to Guide for the Care and Use of Laboratory Animals criteria.

Sixty male Wistar rats, weighing from 200 to 250 g, were caged with food and water *ad libitum* in the Animal Experimentation and Training Surgery Center of the Hospital Israelita Albert Einstein. The animals were randomized by drawing into two groups: (a) Control Group (*SHAM*) in which animals underwent laparatomy with abdominal organ manipulation without I/R of the liver; (b) Group IR in which rats underwent a laparotomy, the hepatoduodenal ligament was dissected and the blood supply to the portal triad was occluded with an atraumatic vascular clamp for 60 min. Animals were sacrificed at the end of ischemia and after 6, 12, 24, 48 h, or 7 days of reperfusion. Inhaled anesthesia (isoflurane concentration, 1.5–3%; oxygen flow 0.5 L/min) was used in these experiments. Analgesia protocol used i.p. Tramal® (2–5 mg/kg). Rats were euthanized under isofluorane anesthesia after the corresponding time of reperfusion. Blood was drawn from the vena cava into a heparinized syringe. The liver was rapidly removed and rinsed in saline, and sections were placed in a phosphate-buffered 10% formalin solution and embedded in paraffin for histological evaluations or snap-frozen in liquid nitrogen for molecular analysis.

Serum Biochemistry

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALKP), bilirubin (BIL), and gamma-glutamyl transferase (GGT) were measured in an automated bench-top dry chemistry analyzer (IDEXX Laboratories Ltd., UK), according to the manufacturer's instructions.

Liver Histopathology

The fixation procedure was carried out by immersing the liver samples in 10% formalin for 24 h, after which they were embedded in Paraplast. For histopathology liver sections were cut and 5-μm tissue slides were stained with hematoxylin–eosin.

Immunohistochemistry Analysis

5-μm liver sections were submitted to antigen retrieval in citrate acid solution at pH 6.0 in a microwave at 700 W for 12 min. Endogenous peroxidase was blocked with 3% H_2O_2 in methanol for 5 min. The blocking of nonspecific reactions was performed by Protein Block (*Dako*) for 30 mins. Immunohistology staining with cytokeratin 18 (*LifeSpan, 1:200, Seattle, WA, USA*) and cleaved caspase-3 (*NovusBio, 1:200, Littleton, CO*) was performed to characterize biliary epithelial, total and apoptotic cells, respectively. Briefly, histology sections were revealed by diaminobenzidine, according to the manufacturer's instructions (*EnVisionTM and Dual Link System HRP, Burlingame, CA*). The images were captured by an AxioCam (*Carl ZEISS Vision, Hallbergmoos, Germany*) and analyzed with the utilization of KS400 software (*Carl ZEISS Vision, Hallbergmoos, Germany*).

Transmission Electron Microscopy (TEM)

Liver fragments were fixed in 2.5% glutaraldehyde. Then, the tissues were fixed in the same solution for 12 h at 4 °C. The specimens were postfixed in 2% osmium tetroxide solution, rinsed in distilled water, and immersed in 2% tannic acid solution for 1 h at room temperature. Then, the tissues were dehydrated in an increasing series of ethanol and propylene oxide and embedded in Spurr resin. The thick sections were made with a Porter Blum ultramicrotome using glass knives and stained with toluidin blue solution for light microscopy analysis. For thin sections a Ultra-Cut Reichert ultramicrotome with diamond knife was used. The ultrathin sections were mounted on 200 and 300 mesh grids, counterstained with uranyl acetate and lead citrate, and examined by transmission electron microscopy (*Jeol, JSM1010 at 100 kV*).

Laser Capture Microdissection (LCM)

The tissue was sectioned (12 μm) on a cryostat (*Jung CM3000, Leica*) and mounted on membrane-coated 1 mm PEN slides (*Zeiss-Jena, Germany*). Before use slides were treated with RNase decontamination solution for

30 min as recommended by the manufacturer. Once the first section had been mounted, the slide was kept inside the cryochamber (−20 °C) while the next section was cut. Four sections were mounted on each slide. Cutting and mounting was performed as quickly as possible to ensure that all sections adhered properly to the membrane. The unfixed sections were stored immediately at −80 °C. Slides were stained with cresyl violet, according to the manufacturer's instructions (*LCM Staining Kit—LifeTech, CA, USA*), and the bile ducts were evaluated. A laser capture microscope (*PALM—Zeiss, Jena, Germany*) was used for the analysis. Tissue sections were dehydrated and overlaid with a thermoplastic membrane mounted on an optically transparent cap.

For LCM staining of the tissue, we utilized three sequential 30-s baths in different concentrations of alcohol, finished by cresyl violet for 30 s. Five additional 30-s baths in different concentrations of alcohol were then finalyzed with xylene. Slides were then dried at room air temperature for 5-min prior to reading. Caps containing 20 μl lysis buffer (*Single Cell-to-CT™— LifeTech, CA, USA*) were placed above the selected area containing cells of interest. The cells were captured by focal melting of the membrane through laser activation. The approximate time required to collect all four areas from one slide (4 sections) was 20 min. The tissue strips that were in the RNA stabilization solution were directly stored at −80 °C with the cap down. Strips in lysis buffer were vortexed for 45 s, spun down, and stored at −80 °C.

Single Cell Real-Time RT-PCR

Cell groups collected in lysis buffer were defrosted and pooled with the same cell lysates from different livers. Total RNA was extracted using the Single Cell-to-CT™ (*LifeTech, CA, USA*) following the manufacturer's instruction. The volume in each tube was brought up to 10 μl by adding Single Cell DNase I/Single Cell Lysis solution, and then the contents were incubated at room temperature for 5 min. Following cDNA synthesis by performing reverse transcription in a thermal cycler (25 °C for 10 min, 42 °C for 60 min and 85 °C for 5 min), gene expression primers were mixed with preamplification reaction mix based on the instructions from the kit (95 °C for 10 min, 14 cycles of 95 °C for 15 s, 60 °C for 4 min, and 60 °C for 4 min). Taqman assays for 18S (4352930E), Bcl-2 (Rn_99999125m1), Bid (Rn_00674175m1), Mcl-1 (Rn_00821024g1), Bad (Rn_00575519m1), Bcl-xl (Rn_00437783m1) and Bax (Rn_02532082g1) were obtained from Life Technologies (Applied Biosystems, Carlsbad, CA). The products from the preamplification stage were used for the real-time RT-PCR reaction (50 °C for 2 min, 95 °C 10 min, and 40 cycles of 95 °C for 5 s and 60 °C for 1 min).

Statistical Analysis

GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, *CA*) was used for statistical analysis. Parametric data were analyzed by one-way ANOVA followed by a Tukey–Kramer test for multiple comparisons. In all experiments,

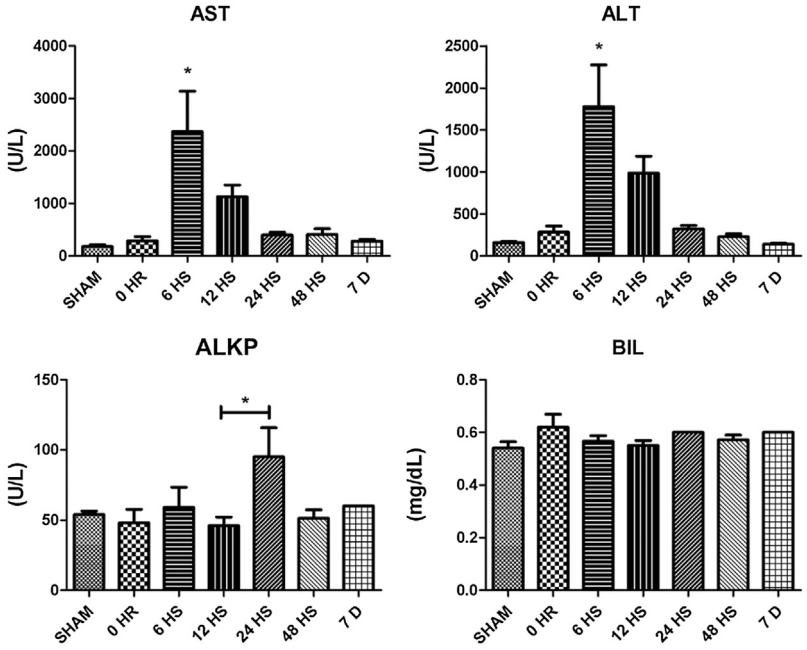

Figure 1 Biochemistry analysis of liver enzymes after liver I/R in rats. *There was statistical difference compared to SHAM group considering $P < 0.05$ (ANOVA). *Only in ALKP there was difference between groups of 12–24 h ($P < 0.05$ ANOVA). AST: aspartate aminotransferase; ALT: alanine transferase; ALKP: alkaline phosphatase; BIL: total bilirubin; GGT: gamma glutamyl transferase. SHAM ($n = 5$); 0 HR ($n = 5$); 6 HS ($n = 8$); 12 HS ($n = 10$); 24 HS ($n = 8$); 48 HS ($n = 8$); 7 D ($n = 5$).

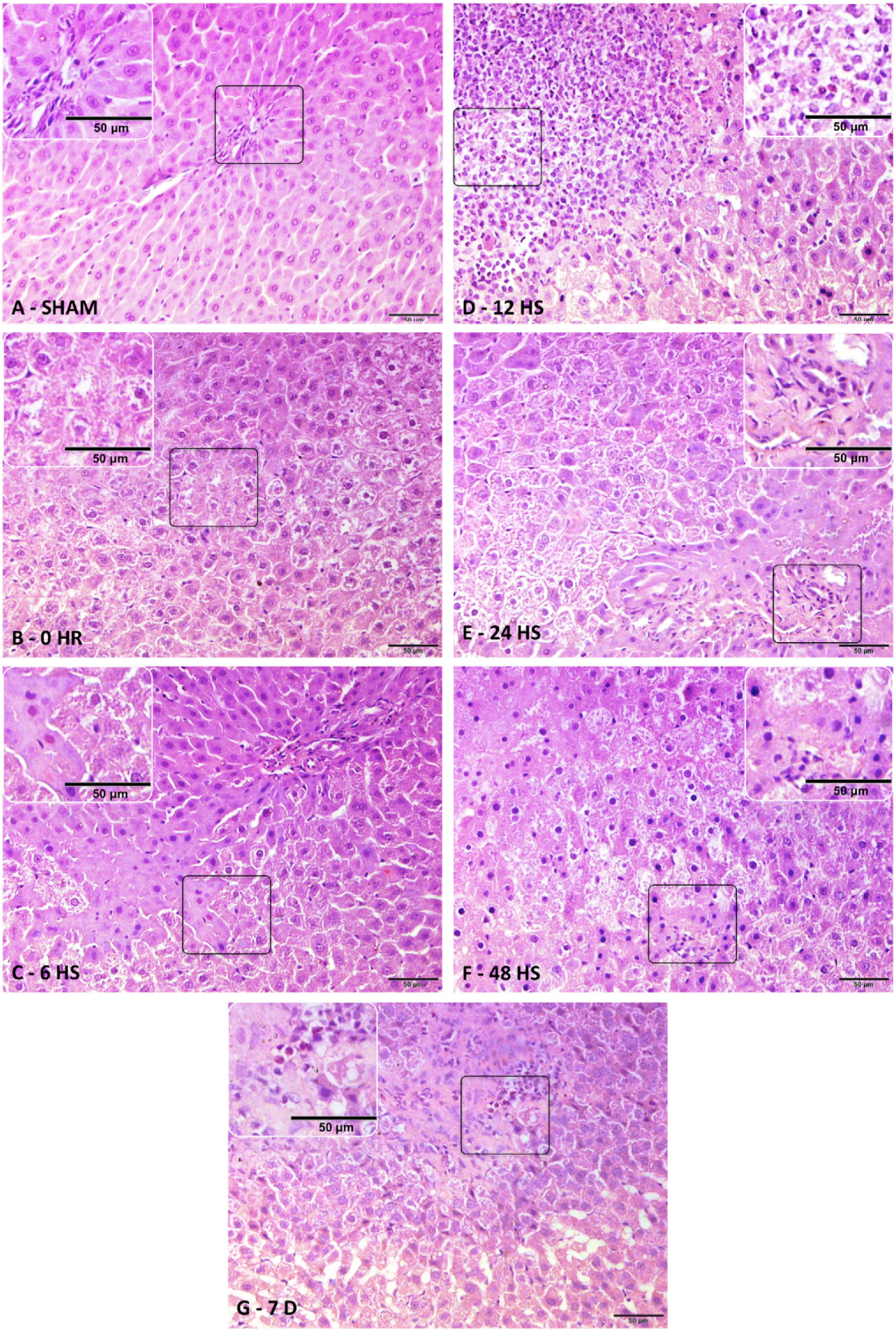

Figure 2 Hepatic histopathology following I/R. *Sham animals underwent laparatomy with abdominal organ manipulation without I/R of the liver (A). The remaining images show livers from rats were sacrificed at the end of ischemia (60 min) (B) or after 6 h (C), 12 h (D), 24 h (E), 48 h (F) or 7 days (G) of

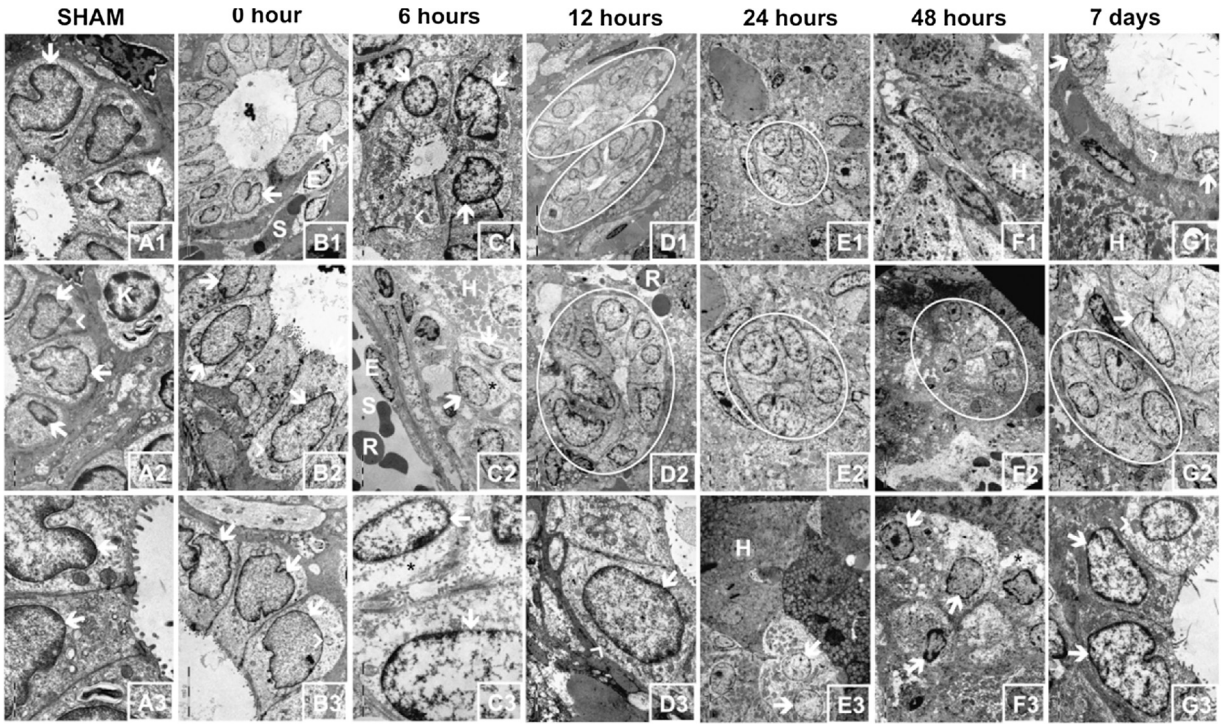

Figure 3 Representative transmission electron micrographs of liver rats after I/R. *White arrows point to cholangiocytes joining to form larger bile ducts in different periods of reperfusion. Circle mark bile ducts. Damaged liver cells indicate a pathological process: edema, swelling of mitochondria, cristolysis, flocculated matrix (arrowhead—intact mitochondria in SHAM group), nuclear chromatin clumping (white arrow—intact chromatin in SHAM group), margination and fragmentation—apoptosis (6–48 h group), RER stripping, dense deposits in mitochondria, peroxysomes and matrix, absence of cytoplasmic process lining (*), loss of microvilli (D2-3, F2-3—intact microvilli in SHAM group), and necrosis (H: hepatocyte, R: red blood cells, K: Kupffer cells, E: endothelial cells, S: sinusoid).

$P \leq 0.05$ was considered significant. Data are presented as the mean ± SD.

RESULTS

Serum Biochemistry

Figure 1 shows the liver function tests after reperfusion. Analysis of the aminotransferases demonstrated an increase of AST (2141.6 ± 1992.68 U/L) and ALT (1778.1 ± 1310 U/L) after 6 h of reperfusion followed by a return to normal levels 24-h after unclamping. There was no change in BIL levels in all animals. There was a peak of ALKP 24-h after I/R injury.

Liver Histopathology and Transmisssion Electronic Microscopy (TEM)

Figure 2 shows that the rats in the I/R group had hepatocellular degeneration in their liver parenchyma up to 6-h after reperfusion, followed by hepatocellular necrosis at 24-h. Different degrees of ballooned cells, areas of necrosis, and inflammatory infiltration and congestion were also present throughout all times after reperfusion. Identified bile ducts (black arrow) revealed few variations such as a mild ductular reaction after 6-h of reperfusion.

Figure 3 shows the ultrastructure of the liver. TEM showed cholangiocyte injury, including a progressive nuclear degeneration and cell membrane rupture according to time of reperfusion, beginning at 6-h and peaking at 24-h after reperfusion.

Immunohistochemistry Analysis

As shown in Figure 4, cytokeratin 18 and anti-caspase 3-positive areas (brown areas) were observed in the I/R group, peaking at 24-h reperfusion when compared with the SHAM group. There was no biliary staining after 7-days by anti-cytokeratin 18 and anti-caspase 3 markers.

reperfusion. Sham animals show normal hepatic histology (A). A pattern of reperfusion damage is evident by congestion and hepatocellular necrosis in the pericentral and middle zones, with relative sparing of the periportal areas (B, C, F, G). After ischemic time the number of neutrophils in the inflammatory infiltrate could be observed in the middle zone around the central vein (D, G). The ischemic liver sections were prepared and stained with H&E.

Laser Microdissection and Gene Expression

By using LCM we were able to isolate RNA from the cholangiocytes to study the apoptotic gene profile. Figure 5 shows a panel of anti- and pro-apoptotic genes studied after RNA isolation by LCM. Anti-apoptotic genes Bcl-2 and Bcl-xl activity occurs from 6- to 24-h after reperfusion. Pro-apoptotic genes BAX, BIM, and BAD peaked at 24-h. We demonstrated that BAX expression showed an increase 24-h after reperfusion (18.23 ± 15.7%) that was statistically significant when compared to controls, but other pro-apoptotic markers did not differ.

DISCUSSION

Studies of I/R injury in the liver do not describe in detail the cascade of events leading to cholangiocyte damage. Moreover, they have not used specific techniques for cell isolation or advanced microscopy. We aimed to understand the early sequence of events and the impact of apoptosis during the I/R injury of rat cholangiocytes using modern cell isolation and advanced microscopy analysis.

Studies with cholangiocytes are recent, only in 1988 Alpini et al. showed that the biliary ductules/ducts in the rat seem to contribute little to bile secretion, they do appear to have the property of secreting a bicarbonate-rich fluid both spontaneously and under the stimulating effect of the hormone secretion. However, the proper role of this bicarbonate-rich fluid in I/R injury of the cholangiocyte was not clear.[13]

Most of I/R studies in murine models have described microscopic changes in hepatocytes such as edema, inflammatory infiltration, and sinusoidal dilation as found in our study.[14,15] In contrast, models that induce cholangiocyte fibrosis and proliferation have shown the additional presence of bile duct edema, regeneration, balooning, and neutrophil or mononuclear infiltration.[16] These findings were not seen in our analysis and must be further investigated. Future studies might focus on developing a model of acute bile duct injury, therefore allowing proper study of the sequence of the events involved with I/

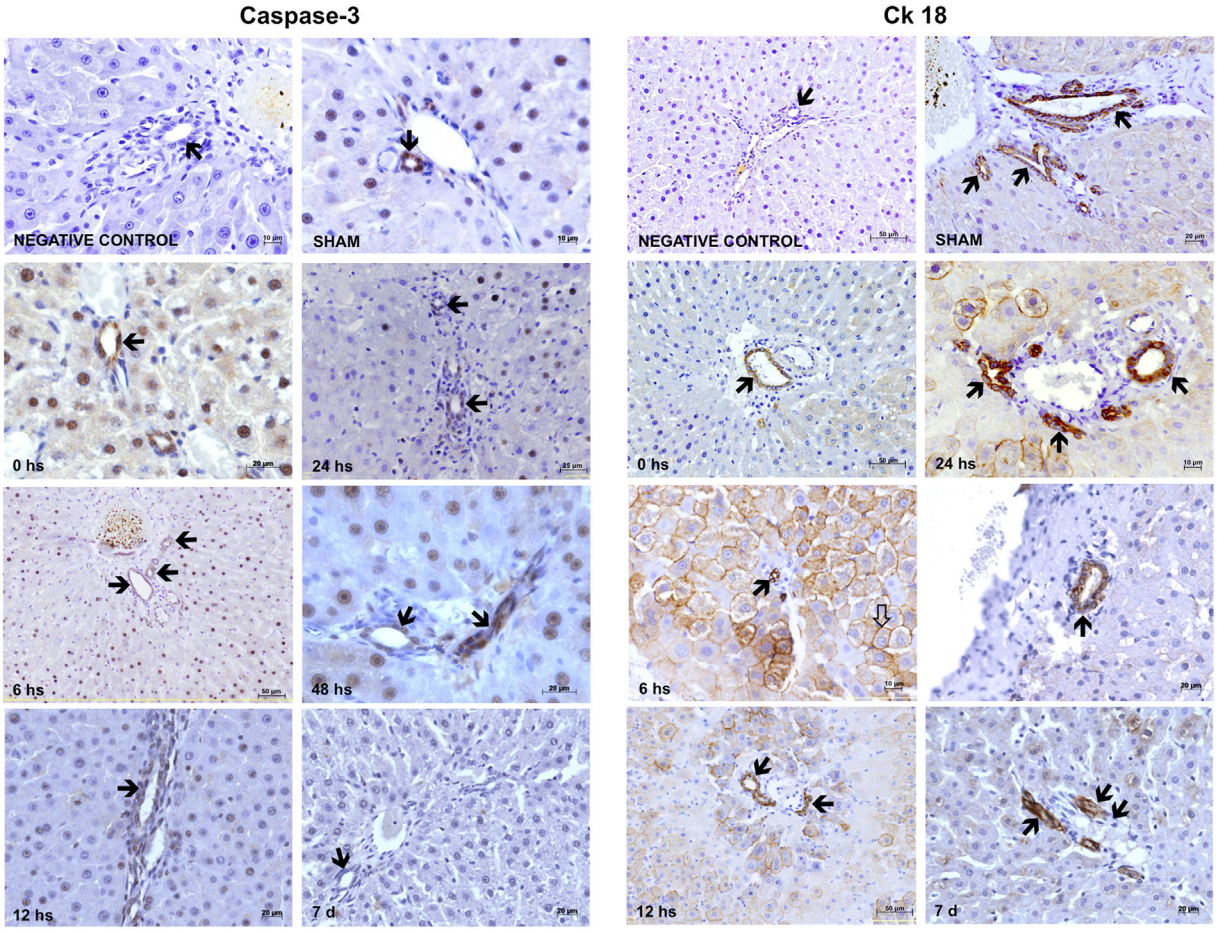

Figure 4 Immunohistochemistry of the rat livers showing cytoplasmic labeling of cholangiocytes and hepatocytes with caspase-3 and cytokeratin-18 (CK18). *Black arrows show cholangiocytes, unfilled arrows show parallel marking in the hepatocytes.

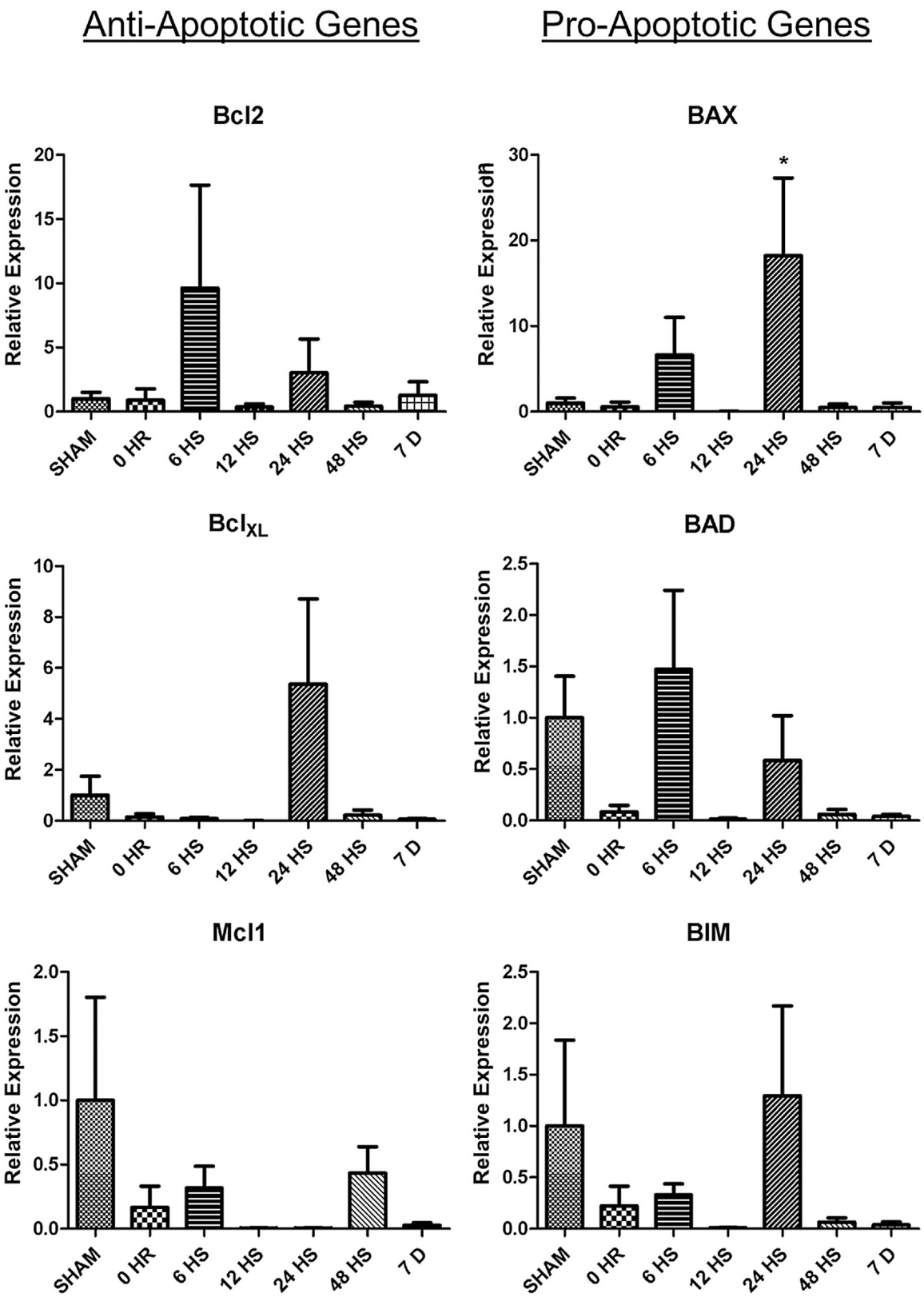

Figure 5 Expression of anti-apoptotic and pro-apoptotic genes from cholangiocytes after IR injury. *There was a difference between the groups analysis at $P < 0.05$ (ANOVA).

R injury, cholestasis and their potential relationship with human hepatobiliary diseases.[16–18]

A single study used TEM to analyze cholangiocytes after I/R in a murine model of liver transplantation.[19] We chose also to use TEM to better understand the sequence of events after I/R specifically related to the cholangiocytes. Our findings indicate that the injury to bile ducts peaks around 24-h after reperfusion, suggesting that early treatment may prevent bile duct lesions or at least subsequently revert I/R injury.

Another study investigated the role of apoptosis of cholangiocytes after I/R in a murine model,[9] employing TUNEL immunohistochemistry assays. Cholangiocyte proliferation was evaluated *in situ* by morphometry of liver sections stained for cytokeratin-19 and proliferating cell nuclear antigen (PCNA). Two days after I/R apoptosis was observed in the large intrahepatic bile ducts and their number was reduced. Seven days after I/R the index of apoptotic cholangiocytes decreased and the number of intrahepatic bile ducts began to increase returning to nearly normal on day 28.[9]

Our findings confirm that apoptosis is involved in I/R injury to cholangiocytes. In addition, we find here that apoptotic events are present at the onset of reperfusion, lasting for 24-h. Apoptosis and cholangiocyte injury are not present few days after reperfusion in rodents. Whether this pattern of cell injury and recovery is maintained in larger animals and humans remain unknown and must be tested in future studies.

Other studies have revealed how, in the course of chronic liver diseases, hepatic progenitor cells (HPC) and cholangiocytes are activated constituting a neuroendocrine compartment regulated by many different agents including hormones, neuropeptides, cytokines, and growth factors. The authors conclude that understanding how we can manage the proliferation of HPC and cholangiocytes could represent a challenge for the treatment of acute and chronic liver diseases.[20] Studies with melatonin (an over-the-counter drug used for curing sleep disorders) have shown important clinical implications because it may be an important therapeutic tool for managing the cholangiocyte hyperplasia in biliary disorders.[21] Scientists also described the beneficial effects of vascular endothelial growth factor (VEGF) in the management of cholangiopathies. The reduction of the blood supply through the hepatic artery (ischemic lesions of bile ducts after liver transplantation, primary sclerosing cholangitis) impairs the proliferative and the repairing capacities of damaged ducts. Therefore, cholangiocyte proliferation can be regulated by neuropeptides, hormones, and growth factors including VEGF.[22] Other research groups show that, likely by an autocrine mechanism, VEGF also plays a major role in modulating cholangiocyte proliferation in response to cholestasis.[23] Others studies raise the possibility for utilizing the inhibition of angiogenesis and VEGF signaling for the treatment of various biliary tract diseases ranging from biliary fibrosis and cholangiocarcinoma to liver cysts in patients with polycystic liver disease.[24]

In others studies with rat bile duct ligation, showed that the peribiliary plexus undergoes marked proliferation, thus supporting the increased nutritional and functional demands from the proliferated bile ductal system. However, the proliferation of the peribiliary plexus only occurs after that of the bile ductal system.[25]

Isolation of cholangiocyte RNA by LCM permitted us to obtain additional information. This technique is powerful to obtain under the microscope, a pure subgroup of targeted cells or even a single cell, quickly and precisely, successfully tackling the problem of tissue heterogeneity in molecular analysis.[26] Once again, the use of LCM is a step forward in studying in studying I/R to the cholangiocyte, in contrast with previous studies that did not use advance techniques of cell isolation.

We found activity of the anti-apoptotic genes Bcl-2 and Bcl-xl as opposed to the pro-apoptotic expression of BAX and BAD, from 6- through 24-h after reperfusion. There was a clear measurable preponderance of the pro-apoptotic profile, especially concerning expression of BAX. Although the role of BAX has been much explored in models of I/R injury to hepatocytes, we found no data relating to cholangiocytes.[27,28] BAX is part of the Bcl-2 pro-apoptotic protein family[29] and in murine cells, most of BAX is found in the cytosol, but upon initiation of apoptotic signaling, Bax undergoes a conformational shift becoming organelle membrane-associated, especially the mitochondrial membrane.[30,31] BAX has been further linked to mitochondrial outer membrane permeabilization that leads to caspase activation.[32] Future experiments will test whether the BAX activity correlates with our TEM findings.

The holy grail of understanding I/R injury is the quest to develop therapies that could prevent or treat the damaged cells. For decades, innumerable substances have been employed to ameliorate hepatic I/R, mostly focused on the hepatocyte.[33,34] Few studies proposed treatment for cholangiocyte damage caused by I/R.[9,35,36] We suggest that BAX inhibitor drugs might prevent apoptosis in this setting and could be tested as a treatment for I/R. Caution in this setting is needed since over induction of apoptosis might deregulate the cell cycle and therefore increase the cancer risk.[37]

In summary, we have found that I/R injury to cholangiocytes occurs from 6 to 24-h after reperfusion and can not be clearly identified by regular optic microscopy. A combination of TEM, immunohistochemistry, and LCM allowed us to view and analyze the cholangiocytes separately from hepatocytes to investigate the events occurring during I/R. Apoptosis is certainly involved in the I/R process and our results indicate that BAX is involved.

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AUTHORS' CONTRIBUTION

Thiago P. Aloia: design, data collection, analysis, writing, review of the manuscript.

Bruno Cogliati: design, analysis and review of the manuscript.

Janaina M. Monteiro: data collection.

Anna C. Goldberg: analysis and review of the manuscript.

Paolo R. Salvalaggio: design, data collection, analysis, writing, review of the manuscript.

CONFLICTS OF INTEREST

The authors have none to declare.

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