Experimental and clinical studies on the management of bile duct cancer

Kloek, J.J.

Citation for published version (APA):

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Cholestasis enhances liver ischemia/reperfusion-induced coagulation activation in rats

Hepatol Res 2010; 40: 204-215

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Summary

INTRODUCTION
Cholestasis is associated with increased morbidity and mortality in patients undergoing major liver surgery. An additional risk is induced when vascular inflow occlusion is applied giving rise to liver ischemia/reperfusion (I/R) injury. The role of the coagulation system in this type of injury is elusive. The aim of this study was to assess activation of coagulation following hepatic I/R injury in cholestatic rats.

METHODS
Wistar rats were randomized into two groups and subjected to bile duct ligation (BDL) or sham laparotomy. After 7 days, both groups underwent 30 minutes partial liver ischemia. Animals were sacrificed before ischemia or after 6h, 24h, and 48h reperfusion.

RESULTS
Plasma AST and ALT levels were higher after I/R in cholestatic rats (\(P<0.05\)). Hepatic necrosis, liver wet/dry ratio and neutrophil influx were increased in the BDL group during 48h reperfusion (\(P<0.05\)). Liver synthetic function was decreased in the BDL group as reflected by prolonged prothrombin time after 6h and 24h reperfusion (\(P<0.05\)). I/R in cholestatic rats resulted in a 12-fold vs. 7-fold (\(P<0.01\)) increase in markers for thrombin generation and a 6-fold vs. 2-fold (\(P<0.01\)) increase in fibrin degradation products, (BDL vs. control, respectively). In addition, the cholestatic rats exhibited significantly decreased levels of antithrombin (AT) III and increased levels of the fibrinolytic inhibitor plasminogen activator inhibitor (PAI-1) during reperfusion.

CONCLUSIONS
Cholestasis significantly enhances I/R-induced hepatic damage and inflammation that concurs with an increased activation of coagulation and fibrinolysis.
Introduction

The only curative option for patients with malignant disease at the liver hilum, such as cholangiocarcinoma, is radical surgical resection consisting of a (extended) hemihepatectomy. Most of these patients present with cholestasis in consequence to biliary tract obstruction, which constitutes a significant risk factor in major liver surgery. Cholestasis is associated with impaired hepatic synthetic function as well as increased risk of sepsis and enhanced susceptibility to ischemia/reperfusion (I/R) injury of the liver. The latter results from the vascular inflow occlusion frequently applied to reduce blood loss during parenchymal dissection (Pringle maneuver).

At the (micro)circulatory level, hepatic I/R injury leads to endothelial damage that ultimately culminates in perfusion failure. The influx of oxygen during the onset of the reperfusion episode promotes the production of thrombogenic reactive oxygen species (ROS) and the release of pro-inflammatory cytokines. The hypothesis is that I/R-induced thrombosis during the reperfusion period is in part activated by tissue factor (TF) expression on the luminal surface of endothelial cells, which in turn triggers the initiation of the coagulation cascade and the formation of fibrin. Endothelium that has been damaged as a result of I/R is susceptible to interactions with circulating leukocytes that, in concert with TF-expressing Kupffer cells, exacerbate the procoagulant state following an ischemic insult.

The disequilibrium of the coagulation system may be even more pronounced when I/R injury occurs in cholestatic livers, since a pro-inflammatory state is manifest a priori in these livers as a result of an increased translocation of endotoxin. Furthermore, the presence of ROS-producing cells and a decreased rate of fibrinolytic protein synthesis may intensify the procoagulant imbalance. In contrast, a reduced rate of (hepatic) coagulation factor synthesis and a compromised clearance of pro-fibrinolytic factors such as tissue plasminogen activator (tPA) adds counterweight to the procoagulant imbalance. Overall this could result in a situation in which thrombo-embolic events are more prevalent in cholestatic livers, but less extensive than in normal livers.

Since the hemostatic mechanisms in the presence of posthepatic cholestasis are relatively unclear, this study was performed to assess the effect of activation of secondary hemostasis as a result of hepatic I/R injury in cholestatic rats. Understanding of the coagulation system in cholestatic livers subjected to I/R potentially leads to therapeutic interventions that may improve peri- and post-operative hemostasis, and decrease the incidence of complications related to cholestasis.

Methods

Cholestasis model and animal experiments

Animals

Following approval by the institute's animal ethics committee, male Wistar rats (Harlan, Horst, The Netherlands) weighing 250–275g were acclimatized for one week under standardized laboratory conditions in a temperature-controlled room with 12-h dark/light cycles. Animals were given free access to water and standard chow (Hope Farms, Woerden, The Netherlands).

Experimental design

The animals (n=60) were randomized into two groups. In the first group, posthepatic cholestasis

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was induced at t=0 days by bile duct ligation (BDL) as described below. The second group underwent a sham laparotomy. At t=7 days, both groups were subjected to 30min partial liver ischemia and 6h, 24h, 48h reperfusion, after which the rats were sacrificed. To assess the direct effects of BDL and sham operation, rats (n=12) were sacrificed before the induction of ischemia at t=7 days.

**Cholestasis induction**

Rats were anesthetized by inhalation of a mixture of O₂/air (1:1 v/v, 2 L/min) containing 2-2.5% isoflurane (Forene, Abbott Laboratories, Queensborough, UK). Isoflurane was specifically selected because it undergoes little biotransformation, is almost completely eliminated with exhaled air, and does not affect liver microsomal enzymes\(^{18}\). Post-operative analgesia was achieved by subcutaneous administration of buprenorphine (0.1mg/kg, Temgesic, Schering-Plough, Utrecht, The Netherlands). Cholestasis was induced by ligation of the common bile duct as described previously\(^{5,19}\). In the control group the bile duct was mobilized without ligation.

**Ischemia/reperfusion injury**

During I/R anesthesia was administered as described above. The animals were intubated to monitor end-tidal CO\(_2\) so as to assure adequate ventilation and maintenance of physiological pH throughout the procedure. The rectal temperature was maintained at 37.0±0.2°C by means of a heating lamp and pad. Partial ischemia was induced by clamping the afferent vessels to the median and left lateral lobes (approximately 70% of total liver mass) for 30min, which was confirmed visually by blanching of the respective lobes. The right lateral and caudate lobes retained portal and arterial inflow and venous outflow, preventing intestinal venous congestion. In the BDL group, biliary decompression was performed at the end of the ischemic period to mimic the clinical situation. To this end, the tip of the drain was opened and inserted into the duodenum\(^{5,19}\). The anastomosis was secured by a purse string suture applied to the wall of the duodenum using 6-0 silk suture (Ethicon, Johnson&Johnson, St.-Stevens-Woluwe, Belgium). In all procedures the abdomen was closed in two layers using a running 4-0 vicryl suture (Ethicon) and the animals were allowed to wake up.

**Blood and tissue sampling**

Isoflurane-anesthetized animals were sacrificed before ischemia and after t=6, 24, and 48h reperfusion (n=6 per group and time point). Biopsies from the median and left lateral lobes were immediately snap frozen in liquid nitrogen and stored at -80°C, fixed in 4% buffered formalin, or processed for dry/wet weight ratio determination. Blood was drawn from the caval vein after 30min reperfusion and directly after sacrifice collected into heparin or citrate-containing tubes and centrifuged at 5,000×g for 15min at 4°C. Plasma was aspirated and stored at -80°C for biochemical analyses.

**Laboratory methods**

**Cholestasis and hepatocellular damage**

To confirm BDL, total bilirubin and alkaline phosphatase (AP) were measured by routine laboratory assays. Similarly, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) were assayed in plasma as markers for hepatocellular damage by
conventional clinical chemistry. Measurements were performed before BDL or sham laparotomy, before ischemia, and at t=6, 24, and 48h reperfusion.

**Inflammation**

Hepatic myeloperoxidase (MPO) activity was determined as a measure of neutrophil influx according to a method modified from Krawisz et al.Liver tissue was homogenized on ice in 5 mM sodium phosphate buffer (pH=6.0) and centrifuged at 10,000×g for 10min at 4°C. The supernatant was aspirated and the pellet was resuspended in 0.5% buffered hexadecyltrimethyl ammonium bromide (Sigma, St. Louis, MO) with 10 mM EDTA (Sigma), samples were subjected to 3 freeze/thaw cycles, broken up in a propeller stirrer, sonicated for 10s, and centrifuged as in the previous step. After heating for 2h at 60°C to remove heat-labile proteins, the supernatant was mixed with o-dianisidine dihydrochloride (Sigma) and 0.001% H₂O₂ (Sigma). Changes in absorbance were measured spectrophotometrically at 450nm during 10min. MPO activity was expressed as units per mg protein (BCA protein assay kit, Pierce, Rockford, USA).

**Necrosis and edema**

Formalin-fixed and paraffin-embedded (FFPE) tissue from liver lobes subjected to ischemia was sectioned, and stained by hematoxylin and eosin. The extent of necrosis was assessed by two blinded investigators in 10 random histological regions at 100x magnification divided over multiple histological sections per liver and expressed as a percentage of the liver surface in the field of view. Edema was quantified by the dry/wet weight ratio of the post-ischemic median liver lobe. Biopsies were weighed immediately after animal sacrifice and incubated at 60°C until no further reductions in weight occurred. The degree of edema was computed by (1−(dry weight/wet weight))·100%.

**Tissue factor immunofluorescence staining**

FFPE 5 μm tissue sections were cut and mounted to coated glass-slides and dried for 24h at 37°C. Following deparaffinization and rehydration, sections were labeled overnight with rabbit anti-mouse TF polyclonal antibodies (1.3μg/mL, final concentration), cloned as described by Weijer et al. Biotin-conjugated goat anti-rabbit polyclonal antibodies incubated for 60min at room temperature (RT) were used as secondary antibody. TF was detected by streptavidin-FITC (DAKO, Glostrup, Denmark). Sections were counterstained with DAPI (Vector, Vectashield, Burlingame, USA).

A Leica BMS000 fluorescence microscope equipped with the Nuance spectral imaging system (CRi, Woburn, MA) was used for analysis of TF immunohistochemistry. Based on subtle spectral differences of true FITC signal and autofluorescence caused by formaldehyde fixation, spectral imaging is capable of unmixing all fluorescence signals. For unmixing a long pass FITC filter set (K3, 460–650nm) and the A4 filter set (420–500nm) for DAPI were used. Spectral data cubes were acquired at 10nm intervals and analyzed with Nuance 2.6 software. Spectral libraries from DAPI and autofluorescence were acquired from blank slides (without immunostaining). The spectral library from FITC plus autofluorescence was acquired from TF-stained slides and subtracted from the autofluorescence library, yielding a calculated spectrum for FITC only. Then images were unmixed in true FITC (pseudo-colored green), autofluorescence (pseudo-colored grey) and DAPI (pseudo-colored blue).
Coagulation and fibrinolysis
Fibrinogen and prothrombin time (PT) were measured directly after animal sacrifice by routine clinical chemistry (required 1mL plasma and therefore impossible to perform at 30m reperfusion). Citrate-anticoagulated plasma samples that had been stored at -80°C were used for the determination of thrombin, antithrombin (ATIII), fibrin degradation products (FDP), plasminogen activator activity (PAA), and plasminogen activator inhibitor-1 (PAI-1). Thrombin generation was deduced from thrombin-antithrombin (TAT) complexes as measured by ELISA (TAT Enzygnost, Behring, Marburg, Germany). ATIII was quantified by an automated amidolytic technique according to methods previously described23. FDPs were quantitated by ELISA (Asserachrom D-Di, Diagnostica Stago, Asnières-sur-Seine, France). PAA was measured by an automated amidolytic assay24. Briefly, 25μL of plasma was mixed with 0.1M Tris-HCl, pH=7.5, 0.1% (v/v) Tween-80, 0.3mM S-2251 (Chromogenix, Mölndal, Sweden), 0.13μM plasminogen (Chromogenix) and 0.12mg/mL cyanogen bromide-digested fibrinogen fragments (Chromogenix) to a final volume of 250mL. The amount of plasmin formed under these conditions is proportional to the PAA concentration and can be detected spectrophotometrically following the conversion of the chromogenic substrate. PAI-1 activity was measured with an amidolytic method previously described25. Plasma was incubated with a fixed excess of tPA (40 IU/mL) (Chromogenix) for 10min at RT. The residual tPA activity was assayed by incubation with 0.13μM plasminogen, 0.12mg/mL cyanogen bromide-digested fibrinogen fragments, and 0.1mM S-2251 (Chromogenix). Under these conditions, the plasmin generated is inversely proportional to the amount of PAI-1 present. Both PAA and PAI-1 activity were measured using normal rat plasma as reference.

Statistics
Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, Chicago, IL). Results are presented as mean±SEM. Differences were tested using the Mann-Whitney U test. A two-tailed P value of <0.05 was considered statistically significant.

Table 1. Confirmation of biliary obstruction and decompression

<table>
<thead>
<tr>
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<th>Plasma total bilirubin [μmol/L]</th>
<th>Plasma alkaline phosphatase [U/L]</th>
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<tr>
<td></td>
<td>Pre-BDL</td>
<td>Pre-I/R</td>
</tr>
<tr>
<td>Control</td>
<td>1.2 ± 0.2</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>BDL</td>
<td>1.2 ± 0.2</td>
<td>173 ± 12*</td>
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Values are expressed as mean ± SEM (n=6, *=p<0.01 vs. control, #=p<0.01 vs. pre-I/R BDL).
Results

Biliary obstruction and decompression

Plasma bilirubin and AP levels at different time points are presented in Table 1. Baseline bilirubin and AP levels did not differ between the control group and the BDL group before sham laparotomy or cholestasis induction, respectively. During the 7-day postsurgical period there were no significant changes in bilirubin and AP levels in the control group, whereas these had increased significantly in the BDL group ($P<0.01$). The increased bilirubin levels indicate that BDL was an effective model.

Internal biliary drainage at the end of the ischemic period resulted in a substantial decrease in plasma bilirubin levels within 6h of reperfusion, albeit that these remained elevated throughout the entire reperfusion period compared to the control group ($p<0.01$). Plasma AP kept rising up to 24h reperfusion in the BDL group, but declined at 48h reperfusion to baseline levels while remaining higher than in the control group ($P<0.05$). The decrease in plasma bilirubin and AP confirmed a successful drainage procedure.

Local inflammation

Hepatic neutrophil influx as assessed by MPO activity and hepatic edema served as indicators of the local inflammatory response. MPO levels were elevated in cholestatic livers compared to the control group ($P<0.01$, Fig. 1A), and increased approximately 5-fold and 20-fold after 6 and 24h reperfusion, respectively. Following 48h reperfusion, the extent of neutrophil influx had receded to baseline levels. The inflammatory response in the control group increased significantly during 6h and 24h reperfusion ($P<0.05$ vs. control pre-ischemia), but was less then 20% of the MPO levels in the BDL group after 24h reperfusion ($P<0.01$). The hepatic edema values corresponded to MPO activity and were increased up to 48h reperfusion in the cholestatic group as compared to the control group ($P<0.01$, Fig. 1B). Both parameters indicated a significantly higher inflammatory response in the BDL group versus control.

Figure 1. Hepatic myeloperoxidase (MPO) activity (A) and edema (B) were significantly increased in the cholestatic group before ischemia and during reperfusion compared to the control group. Values are expressed as means±SEM (n=6, *=p<0.05 and **=p<0.01 vs. control, #=p<0.05 vs. pre-I/R control).
Bile duct ligation (BDL) significantly enhanced hepatic ischemia/reperfusion (I/R) injury that was measured at baseline (pre-I/R) and at 6, 24, and 48h reperfusion. (A) Histological evaluation of necrosis expressed as a percentage of the liver surface in the field of view (n=10 random regions per section, multiple sections per liver), (B) aspartate aminotransferase (AST), (C) alanine aminotransferase (ALT), and (D) lactate dehydrogenase (LDH). Black bars, BDL I/R; white bars; control I/R. Values are expressed as means±SEM (n=6, **p<0.05 and ***p<0.01 vs. control, #p<0.05 vs. pre-I/R control). Representative H&E-stained sections demonstrating necrotic regions at 6 and 24h reperfusion are depicted in E.
The impact of cholestasis on I/R injury

To determine the effect of cholestasis on post-ischemic liver injury, the extent of hepatic necrosis and plasma AST, ALT, and LDH concentrations were assessed before ischemia and during reperfusion (Fig. 2). Cholestatic livers showed parenchymal necrosis before ischemia, which was absent in control livers. The reperfusion phase was associated with significant increases in the extent of necrosis in both groups vs. pre-I/R, which were more profound in the BDL group than in control animals ($P<0.05$, Fig. 2A). Plasma AST and ALT were significantly increased before ischemia in the cholestatic group (ATP $<0.01$), indicating increased hepatocellular damage as a result of cholestasis per se. After 30min ischemia, plasma AST and ALT peaked at 6h after the start of reperfusion, and were elevated in the cholestatic group at all time points as compared to the control group ($P<0.01$, Figs. 2B,C). Plasma LDH was significantly increased only at 6h reperfusion in the cholestatic group in comparison to the control group (Fig. 2D). Representative slides of necrosis are shown in Fig. 2E.

TF expression

The expression of TF, which triggers the initiation of the coagulation cascade, was determined as an indicator of I/R-induced thrombosis (Fig. 3). At 6h reperfusion, TF expression was more prominent in cholestatic rats compared to the control group which probably resulted in an increased activation of the coagulation system.

Activation of coagulation

Activation of coagulation and consequent thrombin generation was biochemically assayed in plasma by measuring TAT complexes and ATIII concentration. ATIII is a circulating serpin (serine protease inhibitor) synthesized in the liver, and is the most important plasma inhibitor of thrombin and factor Xa. The pre-I/R plasma concentration of ATIII and TAT complexes were similar in BDL and control animals (Figs. 4A,B), suggesting that cholestasis did not impair hepatic synthesis function with respect to this serpin. I/R injury induced a decrease in ATIII in both groups during reperfusion, with a 60% reduction in ATIII concentration in the BDL group vs. a 20% reduction in the control group after 6h reperfusion ($P<0.01$), suggesting that post-ischemic cholestatic animals sustained more activation of coagulation during the reperfusion phase. This was paralleled by...
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substantial increases in TAT complex formation from the onset of reperfusion in both groups. At 48h reperfusion, ATIII concentrations were restored to pre-I/R levels, while TAT complexes had returned to baseline levels.

Furthermore, PT was measured as a global net marker of coagulation factor concentrations reflecting the short-term synthetic function of the liver, with particular emphasis on vitamin K-dependent coagulation factors and coagulation factor consumption. Prior to I/R, PT did not differ between the BDL and control groups. I/R resulted in prolonged PT at 6 and 24h reperfusion, which was significantly increased in BDL rats vs. control rats ($p<0.05$, Fig. 4C). At 48h, PT values in both groups were slightly higher than pre-I/R, but no differences existed between cholestatic and control animals. The prolonged PT during the first 24h of reperfusion may indicate a greater rate of clotting factor utilization, i.e., a more extensive activation of secondary hemostasis in cholestatic animals, which is in agreement with the ATIII and TAT data.

Fibrin formation and fibrinolysis
Since activation and inhibition of fibrinolysis play a prominent role in the hemostatic balance as well, plasma concentrations of fibrinogen, PAA, FDPs, and PAI-1 were examined. It was expected that augmented thrombin formation during reperfusion would lead to increased fibrin formation and, if sufficiently extensive, cause a reduction in circulating fibrinogen. Indeed, at 6h reperfusion

Figure 4. Bile duct ligation (BDL) significantly exacerbated hepatic ischemia/reperfusion (I/R)-induced coagulation activation as measured by plasma levels of (A) antithrombin III (ATIII), and (B) thrombin-antithrombin (TAT)-complexes. The prothrombin time (PT, C) was measured for the indirect assessment of clotting factor plasma concentrations and the short-term synthetic function of the liver. Values are expressed as means±SEM ($n=6$, $**p<0.05$ and $***p<0.01$ vs. control, $#p<0.05$ vs. pre-I/R control).
the fibrinogen levels had declined 10% and 30% in control and cholestatic animals, respectively \( (P<0.01, \text{Fig. 5A}) \). I/R injury-affected endothelial cells release tPA and urokinase-type plasminogen activator (uPA) that mediate the conversion of plasminogen to plasmin. Plasmin formation will result in enzymatic cleavage of fibrin strands, leading to FDP generation. Figs. 5B and C show that both PAA (reflecting tPA and uPA activity) and FDPs were significantly elevated after 30min reperfusion. These phenomena were more prominent in the BDL group than in the control group. Since circulating levels of PAI-1 were similar in cholestatic and control animals at this time interval (Fig. 5D), the higher plasma concentrations of PAA in combination with increased FDP production suggest that clot formation and clot breakdown occur at an elevated rate in BDL animals.

At 6h reperfusion, PAA in the BDL group had declined to the level of control animals, albeit the PAA remained increased with respect to pre-I/R. In contrast, the concentration of circulating FDPs had more than doubled in the BDL group compared to a 60% increase in control animals at 6h reperfusion (vs. 0.5h). PAI-1 levels peaked at 6h reperfusion in both groups and high plasma levels continued to be detectable up to 24h reperfusion.
levels prevailed in the BDL group after 24h reperfusion, further underscoring the high degree of homeostatic disequilibrium during cholestasis. The fibrinogen concentration was restored to 90% of baseline in the BDL group after 24h of reperfusion, whereas it almost doubled in control animals with respect to baseline.

At 48h reperfusion, the FDP concentrations had substantially declined, although values remained 100% and 50% above pre-I/R conditions in the BDL and control group, respectively. This trend was opposite for fibrinogen, which had normalized to pre-I/R levels in cholestatic animals and had declined to baseline levels in the control group. Similarly, plasma levels of PAA were normalized after 48h of reperfusion, although PAI-1 was still increased compared to baseline ($P<0.01$). Furthermore the PAI-1 values in the BDL group remained higher in comparison to the control group ($P<0.01$).

**Discussion**

Major liver resections in patients with posthepatic cholestasis are more frequently associated with complications than similar surgical procedures performed in normal liver parenchyma. Several factors lie at the basis of the amplified extent of surgery-related complications, including an exacerbated I/R injury to the remnant liver in cases where the Pringle maneuver is employed. In an attempt to characterize the dynamics of coagulation during the manifestation of liver damage following I/R in cholestatic rats, hepatocellular damage and inflammation were juxtaposed to a number of parameters pertaining to secondary hemostasis and fibrinolysis.

This study demonstrates that cholestasis significantly enhances I/R-induced hepatic damage and inflammation that concurs with an increased activation of coagulation and fibrinolysis. All damage parameters assayed, except LDH, differ from control levels in cholestatic animals seven days after BDL, indicating that significant pathophysiological conditions prevailed prior to ischemia. However, all parameters related to secondary hemostasis and fibrinolysis suggest no activation of coagulation and fibrinolysis during the pre-ischemic period, despite the fact that necrosis and inflammation are considered to be thrombogenic. Consequently, a stronger stimulus than present in cholestatic rat livers is required for activating coagulation and fibrinolysis. Evidently, once the triggers become too abundant, such as in hepatic I/R, hemostasis shifts to a procoagulant and profibrinolytic state that is much more enhanced than in control livers. The augmented procoagulant and profibrinolytic states in BDL rats during reperfusion likely stem from factors associated with a compromised liver and its incapacity to cope effectively with severe physiological stress.

It should be noted that the pre- and post-ischemic patterns in regard to damage, coagulation, and fibrinolysis are species-specific in that these differ in mice and in humans. For example, Georgiev and co-workers observed that cholestasis protects against I/R injury in mice. They proposed that hyperbilirubinaemia leads to a decreased inflammatory response and consequently to reduced injury. However, they could not confirm this hypothesis in a selective BDL model in which the same protective effect was found but plasma bilirubin levels were not affected. As an alternative protective mechanism, they noted that cholestatic mice failed to activate nuclear factor kappaB and TNF-$\alpha$ synthesis, two mediators of post-ischemic liver inflammation. In our study, which was performed in rats, a marked inflammatory response persisted in the presence of cholestasis, suggesting that species differences with respect to the inflammatory responses to cholestasis.
A second important finding is that both coagulation and fibrinolysis during reperfusion were significantly enhanced in cholestatic rat livers. Clot formation as well as clot breakdown occurred at an intensified rate, as evidenced by the almost twofold greater generation of FDPs in cholestatic rats compared to control animals. It should be noted that clearance of FDPs was possibly compromised in cholestatic animals, which in turn could have amplified the measured FDP concentration. The presence of microthrombi may in turn exacerbate hepatic damage by causing localized infarctions in the liver (no-reflow phenomenon). Luminal occlusion by fibrin deposits has been reported as a significant finding in rat livers subjected to 120 min ischemia and may cause detrimental effects in cholestatic rat livers exposed to 30 min ischemia since the FDP concentrations in these two models were comparable. It is unclear from the current study whether cholestasis exaggerated the I/R-induced procoagulant response, or whether the exaggerated injury elicited enhanced coagulation.

Additional hepatic damage may stem from lowered ATIII concentrations, augmented leukocyte influx, and increased PAI-1 levels in cholestatic rats. ATIII is capable of attenuating neutrophil-endothelial cell interactions and reducing hepatic fibrin deposition. Furthermore, ATIII has been shown to directly inhibit the lipopolysaccharide-induced activation of monocytes and neutrophils in vitro and may thus indirectly diminish intrahepatic cytokine release and ROS production. In cholestatic rats ATIII levels were significantly more decreased during reperfusion compared to non-cholestatic rats, probably due to an impaired hepatic synthesis function and a stronger ATIII consumption. Decreased levels of ATIII during the reperfusion phase hence resulted in a decline of its protective properties, which may in part explain the extensive liver damage found in cholestatic rat livers following I/R, insofar as inflammation plays a pivotal role in I/R injury.

Inflammation-induced hepatic I/R damage is mediated by pro-inflammatory cytokines released by KCs and activated neutrophils. Cholestasis is a priori associated with chronic KC activation and an increased neutrophil influx during reperfusion as evidenced by the augmented MPO activity. Cytokines activate monocytes and endothelial cells to increase the expression of TF and downregulate the anticoagulant activity of the latter cells by decreasing the expression of glycosaminoglycans and thrombomodulin. The expression of TF by neutrophils and KC’s contributes to the local prothrombotic environment, which is characteristic for ischemic tissue.

In cholestatic rats TF expression was more prominent, which probably resulted in enhanced activation of the coagulation system. Furthermore, TF mainly co-localized with necrotic areas, suggesting that TF expression and hence the degree of coagulation disturbances are closely related to the grade of liver damage.

At the fibrinolytic end, PAI-1 has been shown to enhance I/R injury by inhibiting tPA-mediated thrombolysis of microthrombi in ischemically-compromised tissue. Additionally, a deficiency in PAI-1 has been correlated to a reduction in neutrophil diapedesis in livers of cholestatic mice not exposed to I/R, and thus to an attenuated hepatic inflammatory response. The dysfunctionally high levels of PAI-1 in BDL rats during reperfusion may therefore have contributed to significantly increased liver damage through the inhibition of thrombolysis and increased inflammation. Apparently, hepatocellular injury in normal livers was limited to such an extent that the increased levels of PAI-1 exerted only a moderate effect on liver damage during reperfusion.

In the final analysis, an increased neutrophil influx, strongly decreased ATIII plasma concentration, and increased levels of PAI-1 during the reperfusion phase strongly suggest that...
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the extensive damage in cholestatic rat livers following I/R may be attributable to an exacerbated activation of the coagulation and fibrinolytic systems. Consequently, pharmacological intervention by for instance administration of ATIII, possibly in combination with defibrinogenation, constitute potential strategies in the peri-operative management of cholestatic patients requiring extensive liver resection. In light of our findings, the Pringle maneuver may need to be employed more conservatively until effective countermeasures with respect to the hemostatic system have been clinically validated for this class of patients.
References


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