INCREASE OF GLYCOSAMINOGLYCANS AND METALLOPROTEINASES 2 AND 9 IN LIVER EXTRACELLULAR MATRIX ON EARLY STAGES OF EXTRAHEPATIC CHOLESTASIS

Pedro Luiz Rodrigues GUEDES¹, Maria Christina Marques Nogueira CASTAÑON², Márcia Regina NAGAOKA³ and Jair Adriano Kopke de AGUIAR¹

ABSTRACT - Context - Cholestasis produces hepatocellular injury, leukocyte infiltration, ductular cells proliferation and fibrosis of liver parenchyma by extracellular matrix replacement. Objective - Analyze bile duct ligation effect upon glycosaminoglycans content and matrix metalloproteinase (MMPs) activities. Methods - Animals (6-8 weeks; n = 40) were euthanized 2, 7 or 14 days after bile duct ligation or Sham-surgery. Disease evolution was analyzed by body and liver weight, seric direct bilirubin, globulins, gamma glutamyl transpeptidase (GGT), alkaline phosphatase, alanine and aspartate aminotransferases, histopathology and glycosaminoglycans content. Results - Cholestasis caused cellular damage with elevation of globulins, GGT, Alk-P, ALT, AST. There was neutrophil infiltration observed by the increasing of myeloperoxidase activity on 7 (P = 0.0064) and 14 (P = 0.0002) groups which leads to the magnification of tissue injuries. Bile duct ligation increased pro-MMP-2 (P = 0.0667), MMP-2 (P = 0.0003) and MMP-9 (P < 0.0001) activities on 14 days indicating matrix remodeling and establishment of inflammatory process. Bile duct ligation animals showed an increasing on dermatan sulfate and/or heparan sulfate content reflecting extracellular matrix production and growing mitosis due to parenchyma depletion. Conclusion - Cholestasis led to many changes on rats' liver parenchyma, as so as on its extracellular matrix, with major alterations on MMPs activities and glycosaminoglycans content.


INTRODUCTION

On 2012, in Brazil, hepatic diseases of many etiologies caused almost 250,000 hospital internalizations, with over 2,000 deaths, burdening Brazilian government in over 500 million reais (6). During chronic stages a massive substitution of liver parenchyma by scar tissue occurs, causing hepatic fibrosis, which is the common endpoint for most types of chronic liver injury, considered an irreversible process (31). There are many causes of chronic liver inflammation that invariably gives rise to tissue fibrosis, which can be considered a deregulated fibroproliferative response ultimately affecting tissue architecture and function because of extracellular matrix (ECM) accumulation (42).

A widespread experimental model for liver injury is carbon tetrachloride (CCL₄) injection which causes transitory effects such as loss of Ca²⁺ homeostasis, lipid peroxidation, toxin release and apoptotic events followed up by tissue regeneration (18, 27, 29). Although, this solvent may cause unspecific toxicity, including central nervous system depletion, respiratory failure and death (3), and animal response to CCL₄ is variable, with high mortality taxes besides the long period to induce cirrhosis (8 to 12 weeks). Beyond that, nowadays, human CCL₄ intoxication is very rare (28).

Extrahepatic cholestasis induction is an alternative to study liver fibrosis, where morphological changes similar to human biliary cirrhosis occur without needling of pathology process activation by any exogenous toxin. Cholestasis results on high concentrations of biliary salts in hepatocytes, inducing hepatocellular death by apoptosis (12, 21, 34, 35). Common bile duct ligation (BDL) have been used to induce extrahepatic cholestasis on rats, promoting liver injury, intra-hepatic epithelial biliary cells proliferation, myofibroblastic differentiation of hepatic stellate cells (HSCs) and ECM deposition (8, 39).

Current treatment of liver fibrosis is limited to withdrawal of the agent, but it is not always feasible. Therefore, it is important to establish disease’s pathway to figure out targets to liver recovery (25). The aim of this study was to comprehend cholestatic liver disease by analyzing changes on hepatic parenchyma and ECM.

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¹ Departamento de Bioquímica, Universidade Federal de Juiz de Fora – UFJF, Juiz de Fora, MG; ² Departamento de Morfologia, UFJF; ³ Departamento de Biociências, Universidade Federal de São Paulo - UNIFESP (Baixada Santista), Santos, SP, Brasil.
Correspondence: Jair Adriano Kopke de Aguiar, Universidade Federal de Juiz de Fora, Rua José Lourenço Kelmer, s/n - Campus Universitário Bairro São Pedro - CEP: 36036-900, Juiz de Fora, MG, Brasil. E-mail: jair.aguiar@ufjf.edu.br
METHODS

Materials
Ketamine 10% (Syntec, Cotia, SP, Brazil); Xylazine 2% (Syntec, Cotia, SP, Brazil); Nylo’n-5-0 suture lines (Shalon, Sao Luis M. Belos, GO, Brazil); Catgut 5-0 suture lines (Technofo, Goiania, GO, Brazil); Biochemical diagnosis kit (LabtestDiagnostica, Lagoa Santa, MG, Brazil); Cetyltrimethylammonium bromide (CTAB, Sigma-Aldrich Co., St. Louis, MO, USA); o-dianisidine hydrochloride (Sigma-Aldrich Co., St. Louis, MO, USA); Acrylamide (Ludwig Biotechnology Ltd., Porto Alegre, RS, Brazil); N,N’-methylenebisacrylamide (Neon Comercial Ltda., Sao Paulo, SP, Brazil); Tris(hydroxymethyl)aminomethane (Bio-solve Valkenswaard, Netherlands); Triton x-100 (Vetec Fine Chemicals Ltda., Duque de Caxias, RJ, Brazil); Gelatin (Sigma-Aldrich Co., St. Louis, MO, USA); Papain from papaya latex (Sigma-Aldrich Co., St. Louis, MO, USA); Q-Sepharose fast flow anion exchange resin (GE Healthcare Life Sciences, Uppsala, Sweden).

Animals
We used 6 to 8 weeks old male Wistar rats (n = 40) with four animals kept on each plastic cage for 3 days before surgical procedure. All animals were housed in a room maintained at 23 ± 1°C with 12h light/dark cycles and had free access to standard chow and tap water. The Animal Experiments Committee of Federal University of Juiz de Fora approved this study according to the “Guide for the Care and Use of Laboratory Animals” (protocol number 025/2013).

Common bile duct ligation
All surgical procedures were performed under intraperito-neally ketamine (90 mg/kg) and xylazine (10 mg/kg) anesthesia with clean surgical techniques as previously described(7,31). Briefly, obstructive jaundice was induced by midline laparotomy and common bile duct exposure followed by double ligation and section between stitches. Sham-operated animals were submitted to a similar procedure, without BDL. After surgery, BDL (n = 25) and Sham-operated (n = 15) animals were randomly divided in 3 groups with different induction time (2, 7 or 14 days), being euthanized by deep anesthesia (ketamine 180 mg/kg) and exsanguination by the end of the experiment.

Biochemical analysis
During euthanasia, blood was drawn by infrahepatic inferior vena cava puncture in test tubes and serum samples by centrifugation, frozen and kept at −80°C until analysis. Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (alk-P), γ-glutamyl transpeptidase (GGT), direct bilirubin (DB) and globulins were measure by automated standardized colorimetric procedures using a Labmax 240 analyzer (LabtestDiagnostica, Brazil), in accordance with technical manual.

Histopathological examination
Excised liver specimens were fixed in formalin (10% on PBS 0.05 M pH 7.4) and embedded with paraffin. Hematoxy-lin and Eosin (H&E) staining was performed according to standard procedures. For morphometric analysis, we used image analyzer Axiovision version 4.5 (Zeiss, Germany).

Gelatinase zymography
Activities of matrix metalloproteinase (MMP) pro MMP-2, MMP-2 and MMP-9 were measured as previously described(26, 37). Briefly, liver homogenates were prepared with electrophoresis loading buffer and saccharose 0.25 M. Thereafter, samples were subjected to electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with copolymerized gelatin (2 mg/mL; Sigma-Aldrich Co., St. Louis, MO, EUA). After electrophoresis, gels were washed with 2% Triton X-100 for 1 h (3 times, 20 min each) and incubated for 24 h in enzyme assay buffer (50 mM Tris-HCl, pH 8.2, 5 mM CaCl2, and 1µM ZnCl2) to enzymatic activity bands development. After incubation, gels were fixed and stained in 40% methanol, 40% acetic acid and 0.1% (wt/v) Coomassie Blue R-250 for 1 h, and then unstained. The gelatinolytic activities were detected as transparent bands against stained gelatin background, MMPs were identified by their molecular weights compared to standards. To measure band intensities densitometric analysis was carried out using TotalLab Quant®. Activities were corrected by protein content and data are expressed as arbitrary densitometric units (ADU)/µg of proteins.

Neutrophil infiltration
Liver neutrophil infiltration was quantified by measuring myeloperoxidase (MPO) activity as described previously(9). Briefly, liver homogenate was prepared in 50 mM phosphate buffer (pH 6.0) containing 0.5% cetyltrimethylammonium bromide (CTAB; Sigma-Aldrich Co., St. Louis, MO, EUA), sonicated for 10 s and freeze-thawed three times, and centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was added to o-dianisidine dihydrochloride 2 mg/mL (Sigma-Aldrich Co., St. Louis, MO, EUA) and hydrogen peroxide (H2O2 10 mM), and the change in absorbance at 460 nm was measured. MPO activity was calculated using the molar extinction coefficient (ε=11.48 mM⁻¹.cm⁻¹)(11). One unit of MPO was defined as the amount that degraded 1 µmol of hydrogen peroxide per minute at 25°C. Results are expressed as specific activity (mUE/mg protein).

Glycosaminoglycan extraction and analysis
GAGs were extracted by proteolysis of tissue samples with papain (1 mg/mL in 0.05 M phosphate-cysteine buffer pH 6.5 and 20 mM EDTA – 1 mL/100 mg of tissue) and trichloroacetic acid and ethanol precipitation as previously described(4). Samples were purified by anionic ion-exchange chromatography on Q-sepharose fast flow and submitted to agarose gel electrophoresis (5 µL) in 0.05 M 1,3-diaminopropane-acetate buffer (PDA), pH 9.0(9). After fixation with CTAB, drying and toluidine blue staining, GAGs were
quantified by densitometry of gel slabs (Scanner Epson Expression 1680, model G780B, Helena Laboratories). Measure of bands intensities was carried out using Quick Scan 2000© software (Quick Scan 2000 WIN version 1.14/00, Copyright 2001, Helena Laboratories).

**Statistical analysis**

Statistical analysis were performed using GraphPadPrism 6.01 for Windows. Normality of the data was verified by the Shapiro-Wilk test, the comparison of data distributed on the curve of normality was performed by one-way ANOVA with Tukey’s multiple comparison post-hoc test. Data are expressed as mean± standard error of the mean (SEM).

**RESULTS**

After surgery, we observed weight gain for animals of both groups (Sham and BDL) after 7 or 14 days of experiment. Although initially, we have noticed a significant weight loss for induced 2 days group animals (~7-fold; \( P = 0.0257 \)) (Figure 1A). BDL rats presented higher liver weight with increasing values for 7 (\( P = 0.0305 \)) and 14 (\( P<0.0001 \)) days groups (Figure 1A and B).

To access cholestatic process installation, serum levels of DB, ALT, AST, GGT, Alk-P and globulins were determined in both Sham and BDL groups (Figure 2). All BDL animals presented high DB levels (\( P<0.0001 \)) (Figure 2A). ALT activity was also higher on induced animals reaching maximum levels on 2 days group (\( P<0.0001 \)) and lower but rising values on 7 and 14 days groups. AST increased on every group when compared to Sham-operated animals, with highest activities on 2 days (\( P<0.0001 \)) (Figure 2A, B). GGT, Alk-P and Globulins presented increasing values on 2, 7 and 14 days groups of BDL animals, while Sham groups remained on basal levels (Figure 2D, E and F).

**FIGURE 1.** Changes on rats’ liver after bile duct ligation (BDL). (A) Data description of weight variation during experiments, liver mass and liver/body weight ratio. (B) Rats’ liver weight variation (\( *P = 0.0305, **P<0.0001 \)). Values expressed as mean ± SEM.

**FIGURE 2.** Biochemical serum parameters of liver injury. (A) Direct bilirubin (mg/dL); (B) Alanine Transaminase (U/L); (C) Aspartate Transaminase (U/L); (D) Gamma Glutamyl Transpeptidase (U/L); (E) Alkaline Phosphatase (U/L); (F) Globulins (g/dL). Values expressed as mean ± SEM.
Histopathological analysis of liver specimens showed increasing ductular proliferation on portal triads of BDL animals in all groups, with presence of fibrotic septum on 14 days group (Figure 3A f). Morphometric measures of ductular proliferation on portal areas are shown on Figure 3B. It was also observed neutrophil infiltration on these tissues, confirmed by myeloperoxidase (MPO) activity, with higher levels for BDL than Sham animals on both seven ($P = 0.0064$) and 14 ($P = 0.0002$) days groups (Figure 3A, B).

Cholestasis induced a rise on MMPs activities on liver homogenates. MMP-2 activity presented increasing values on 7 ($P = 0.0023$) and 14 ($P = 0.0003$) days groups while Sham activities remained constant (Figure 4A). BDL reflected even on pro MMP-2 levels, with higher activities on zymograms of induced animals on 14 days group ($P = 0.0677$; Figure 4B). It was also observed an increasing MMP-9 activity on BDL samples on each experimental group reaching approximately 4-fold the control on 14 days group ($P<0.0001$; Figure 4C).

FIGURE 3. Tissue alterations on bile duct ligation (BDL) animals. (A) Histopathological changes on rats’ liver (H&E, original magnification 200x, bars = 50 µm). Sham groups maintained normal parenchyma architecture (a, b, c), while BDL animals presented increasing ductular proliferation on 2, 7 and 14 days groups (arrows on d, e, f), confirmed by (B) morphometric measure of portal triads. Initial injuries on tissue cause chemokines liberation leading to neutrophil infiltrate, confirmed by (C) myeloperoxidase (MPO) activity on liver homogenates, increasing on BDL animals from 2, 7 and 14 days groups. Values expressed as mean ± SEM.

FIGURE 4. Matrix metalloproteinases (MMPs) activities on liver homogenates. Homogenates (10 µg of protein) were submitted to SDS-PAGE (10%) with substrate (gelatin 2 mg/mL). After incubation and Coomassie Blue staining, activities of (A) MMP-2, (B) pro MMP-2 and (C) MMP-9 were quantified by bands densitometry on gel slabs. Values expressed as mean ± SEM.
Dermatan sulfate (DS) and heparan sulfate (HS) are the major GAG found in the liver with smaller amounts of chondroitin sulfate (CS) (Figure 5A). BDL animals showed higher hepatic content of DS and HS than Sham-operated ones (Figure 5C, D) causing an elevation on total GAG content (Figure 5E).

Rats submitted to early extrahepatic cholestasis presented weight loss on 2 days group when compared to Sham-operated, what might have happened because of most stressing surgical procedure (Figure 1A). Increasing on liver weight of BDL animals (7 and 14 days groups) may be due to tissue’s hyperplasia, ductular proliferation, edema and/or increased hepatic ECM (Figure 1B)²,¹⁴,¹⁵.

Raise on DB levels of cholestatic rats (Figure 2A) is the first sign of bile retention and responsible for primary injuries on liver(¹⁰). Bile salts damage hepatocytes membranes, what changes cellular permeability causing a rise on serum aminotransferases’ levels. BDL animals showed increasing activity in both enzymes: ALT levels raised on 2 days group when compared to Sham-operated animals, showing acute response to injuries, most probably due to surgery stress, while AST activity showed increasing pattern on 7 and 14 days groups. These changes, so as in globulins levels, indicate lesions on liver parenchyma after ligation. Significantly increased GGT and Alk-P activities point to biliary injury due to severe obstructive cholestasis, because of damage on epithelial cells of bile ducts (Figure 2C, D)⁴⁰.

We observed classic histopathological features on animals submitted to BDL, with increasing substitution of liver parenchyma by epithelial bile duct cells on portal triad (Figure 3A and B). Ductular proliferation and ECM deposition are responsible for major tissue changes resulting on formation of fibrotic septum, first sign of tissue fibrosis (Figure 3A)⁸,²₃. Apoptotic bodies of hepatocytes activate Kupffer cells, which secrete cytokines and chemokines perpetuating the signal by recruiting leucocytes to the injury sites, among them neutrophils, which is confirmed by MPO activity, enzyme present on azurophilic granules of these cells, that showed increased on 7 (P = 0.0064) and 14 (P = 0.0002) days groups of BDL animals (Figure 3C)¹³,²₄.

Pro-inflammatory and fibrogenic mediators secreted led to early changes on ECM and consequent activation of hepatic stellate cells (HSCs), vitamin A storing cells activated to a profibrogenic myofibroblastic form responsible for breaking and renovation of ECM in substitution to liver parenchyma. Active HSC controls expression and regulation of MMPs and its inhibitors (TIMPs)¹¹,²⁵.

BDL rats of 14 days group presented higher MMP-2 activity than control (Figure 4B) maybe due to HSCs activation, with previous evidence of increasing expression and activity of this enzyme on chronic liver diseases and cirrhosis. This group also presented augmentation on pro MMP-2 activity, what may be a sign of rising on enzyme expression (Figure 4A), as demonstrated on hepatic fibrosis induced by CCl₄ (⁵⁰). MMP-9 activity was also higher for BDL animals (Figure 4C), what might be related to inflammatory process of the liver, since this enzyme is usually co-located to Kupffer cells¹¹,⁴¹.

Activation of HSC with rising on MMPs activities result on ECM remodeling, causing major changes on its constitution. Previous authors cited changes on collagen types I, III, IV, V, VI, and many non collagenous components, including fibronectin, laminin, tenascin, undulin,and entactin on fibrotic

Animal models have been widely used to comprehend mechanisms underlying acute and chronic liver injury¹⁰,³⁰,³¹. In our study, we analyzed early stages of BDL on rats’ liver cells and ECM in order to understand effects of extrahepatic cholestasis and its pathological changes. During cholestasis bile duct walls become thin and stiff and hepatic gap junctions disappear with formation of large crater-like fenestrae in ductular epithelium and development of focal necrosis, causing leakage of toxic substances such as hydrophobic bile acids with parenchymal damage and induction of liver injury¹⁶,²².

**DISCUSSION**

**FIGURE 5.** Glycosaminoglycans content on cholestatic liver. After proteolytic digestion of the tissue and purification by anionic ion exchange chromatography (Q-Sepharose fast flow) liver samples were submitted to (A) agarose gel electrophoresis on PDA buffer (pH = 9.0). Samples from Sham animals are 2x concentrated (B) Chondroitin sulfate (µg/mg of tissue); (C) Dermatan sulfate (µg/mg of tissue); (D) Heparan sulfate (µg/mg of tissue); (D) Total GAG content (µg/mg of tissue). Bile duct ligation (BDL) animals presented increasing on DS and HS content reflecting ECM production and ductular proliferation due to parenchyma depletion. Values expressed as mean ± SEM.
livers\textsuperscript{(36)}, but GAGs content alterations was not described. As in our study, previous works show that the main GAGs found on liver are dermatan sulfate and heparan sulfate with smaller amounts of chondroitin sulfate\textsuperscript{(17, 19, 32, 33)}. Cholestasis resulted on a great rise of dermatan sulfate and heparan sulfate content (Figure 5). DS is mainly found on ECM proteoglycans (PGs) reflecting its degradation and reassembly, while HS is characteristic of cell surface PGs, reflecting cellular proliferation, in this case ductular epithelial cells.

Net deposition that occurs during hepatic fibrosis has generated a great deal of interest in liver’s ECM changes. Results of our study are in agreement with previous reports indicating marked alterations in MMPs activity and tissue injury on early stages of extrahepatic cholestasis. Nevertheless, we have also demonstrated an important modification on ECM content, suggesting that initial destruction of this structure leads to further substitution of liver parenchyma.

CONCLUSIONS

We have shown that important information can be gained by analyzing ECM to access liver’s injury. The whole process of tissue depletion after bile salts accumulation ends up to MMPs activity, which leads to ECM renovation and expansion that causes fibrosis of the liver with major changes on GAGs content.
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