Bile duct ligature in young rats: a revisited animal model for biliary atresia

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Abstract

Biliary atresia leads to cirrhosis in the vast majority of patients and constitutes the first cause of paediatric liver transplantation. Animal models allow us to understand the molecular basis and natural history of diseases. The aim of this study is to describe a surgically created animal model of biliary atresia with emphasis in long-term liver function. Forty-two 3-week-old Sprague-Dawley rats were randomly divided into two groups: bile duct ligature (BDL) and control. The animals were sacrificed on the 2nd, 4th, and 6th postoperative weeks. Blood samples were collected for liver function analysis. The spleen to body weight ratio was determined. Histopathological examination of liver tissue was performed by hematoxylin-eosin and Sirius red staining. Collagen quantification was determined by using colorimetric digital image analysis and was expressed as a percentage of total liver tissue area. Quantitative real-time polymerase chain reaction was performed to analyse gene expression levels of transforming growth factor-β1 (Tgfβ1) and apelin (Apeln) genes. Statistical analysis was performed where P<0.05 was considered significant. Animals from BDL group developed increasing cholestasis with clinical and laboratory features. Splenomegaly was detected at 4th and 6th week (P<0.05). Histological evaluation of the liver showed ductular reaction, portal fibrosis and bile plugs. Collagen area to total liver tissue area had a median of 2.5% in the control group and 6.5%, 14.3% and 37.7% in BDL rats at 2nd, 4th and 6th weeks, respectively (P<0.001). Tgfβ1 mRNA expression level was significantly higher at 6th week (P<0.001) in BDL group when compared to control. Apeln mRNA expression level was significantly higher at 4th and 6th week (P<0.001) and showed a positive linear correlation (r = 0.975, P<0.05) in BDL group when compared to control. Bile duct ligature in young rats is an animal model that recreates clinical, laboratory, histological and molecular findings of biliary atresia. Bile duct ligature constitutes a good animal model to investigate therapeutic approaches for modifying the progression of liver fibrosis in biliary atresia.

Introduction

Biliary atresia (BA) is a cholestatic neonatal disease of unknown etiology characterized by progressive, inflammatory, and fibrosclerosing cholangiopathy resulting in obstruction of both extrahepatic and intrahepatic bile ducts.1 Clinically, biliary atresia is classified into three groups: cystic biliary atresia, biliary atresia with other congenital malformations, and isolated biliary atresia.2 The incidence of isolated biliary atresia is higher in Asian countries and French Polynesia (1:2,700-3,500) compared to Europe and North America (1:5,000-18,000).3 Kasaì et al. described the classical portoenterostomy in 1957 and this has been the surgical treatment since then.4 However, despite surgery 68-80% of patients affected by biliary atresia develop progressive fibrosis leading to biliary cirrhosis and portal hypertension. Biliary atresia constitutes the most common diagnosis leading to pediatric liver transplantation, accounting for half of cases.5

Animal models play a key role in our understanding of the molecular basis and natural history of diseases. Three types of animal models have been reported in biliary atresia: spontaneous, secondary to viral infection and surgically created.6 The sea lamprey (Petromyzon marinus) is a spontaneous animal model of biliary atresia. The sea lamprey is a jawless vertebrate that during metamorphosis from larva to parasitic juveniles progressively loses the biliary system until complete biliary degeneration. However, sea lamprey does not develop cirrhosis during developmental biliary atresia.7

Intrapерitoneal inoculation of mice with rhesus rotavirus within the first 48 h of postnatal life leads to injury of the biliary epithelium and subsequent transient extrahepatic biliary obstruction and intrahepatic bile duct proliferation during the neonatal period. These features validate the use of the viral murine model as a tool to study the pathogenesis of human biliary atresia. However, in contrast to human biliary atresia, rhesus rotavirus animal model develops a much less intensive liver fibrosis and without portal hypertension.5,8 Other limitations of this model are: timing and dosing of virus application affect the reproducibility of the model; injection-related injury to abdominal organs is usually fatal; cannibalization of pups impedes the harvest of valuable specimens and sequential investigations cannot be performed in the same animal because pups are extremely unstable and too small for performing repeated biopsies and blood samplings. Moreover, the survival rate of pups in rhesus rotavirus mice is only 10% at 3 weeks of age.8

Bile duct ligature (BDL) is a surgically created animal model developed by Cameron and Oakley in 1932 that consist in ligation or excision of the common bile duct.4 BDL has been the most useful experimental model to study cholestatic disease and is an important model to study portal hypertension and hepatopulmonary syndrome due to the possibility of long-term follow up. BDL model has been performed mainly in adult rats.9,10 However, it is likely that natural history of cholestatic injury is different in adults compared to young animals.11

The aim of this study is to describe a surgically created experimental model of BA in young animals with emphasis in long-term liver function.

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Materials and Methods

Animals

Forty-two 3-week-old Sprague-Dawley rats (weighing 40.5-73.2 g, mean 53.2±7.9 g) were used in the experiments and divided into two groups: experimental group (n=21) and control group (n=21). The puppies were kept with their mothers until surgery at three weeks of age. They had access to drink and food ad libitum, circadian cycle of light-darkness 12:12 h, controlled humidity (40-70%) and temperature (21±2°C), with ventilation of 10 changes air/h.

The study was approved by the Institutional Bioethics Committee for Animal Research of Universidad de Valparaíso (BEA029-2014). It was carried out according to the International Guiding Principles regarding the use of laboratory animals.11

Surgical procedure

One hour before the operation, acetylsalicylic acid 0.1 mL (10 g/100 mL) was administered by oral route. The animal was placed in a transparent acrylic box especially designed for anesthesia induction of young rats. The rat was pre-oxygenated with 99.5±0.5 pure oxygen at 1 L/min for 30 s, and then anesthetized with isoflurane at 3-4% with an isoflurane funnel-fill vaporizer (Harvard Apparatus, Holliston, MA, USA) until assessed by the absence of voluntary movements, muscle relaxation and loss of response to stimuli reflexes. The abdomen was shaved and cleaned with chlorhexidine gluconate soap 2%. After that, the rat was attached to a heated small animal operating table (Harvard Apparatus). The anasthesia was maintained by a facial mask with oxygen and isoflurane 2%. The surgical procedure was performed under clean conditions, using surgical loupes (magnification 2.5x) and microsurgical instruments. A superior midline abdominal incision was performed under clean conditions, using isoflurane 2%. The surgical procedure was performed at 3-4% with an isoflurane funnel-fill vaporizer (Harvard Apparatus, Holliston, MA, USA) until assessed by the absence of voluntary movements, muscle relaxation and loss of response to stimuli reflexes. The abdomen was shaved and cleaned with chlorhexidine gluconate soap 2%. After that, the rat was attached to a heated small animal operating table (Harvard Apparatus). The anasthesia was maintained by a facial mask with oxygen and isoflurane 2%. The surgical procedure was performed under clean conditions, using surgical loupes (magnification 2.5x) and microsurgical instruments. A superior midline abdominal incision was performed under clean conditions, using isoflurane 2%

Blood samples were collected by puncture with a 21-gauge needle from the infrahepatic inferior cava vein at time of euthanasia. These were centrifuged at 1000 rpm by 3 min and analyzed by VetTest® Chemistry Analyzer (Idexx Laboratories, Westbrook, ME, USA), according to fabricant instructions, adjusting by species and age of animals. The following liver function tests were studied: albumin, alkaline phosphatase, aspartate transaminase (AST), alanine transaminase (ALT), gamma-glutamyl transpeptidase (GGT) and total bilirubin.

Determination of spleen index

The spleen was extracted and weighed (grams) in order to obtain an indirect measurement of portal hypertension expressed as “spleen index”: spleen weight/body weight x 100.

Histopathological examination

The left lateral lobe of the liver was subject to histological evaluation. The lobe was divided into two samples: one of them was kept in 4% buffered paraformaldehyde, embedded in paraffin, sectioned and stained with hematoxylin-eosin following conventional protocols to obtain the final block. The second sample was fixed in methacarn solution (methanol, chloroform, acetic acid; 6:3:1) during 4 h at 4°C. After that, samples were dehydrated in 100% ethanol, cleared in xylool and embedded in paraffin. Five μm thick histological sections were cut and stained in a Sirius-red solution (0.1% v/v) for 1 h. Sirius red is a strongly acidic and insoluble dye molecule that reacts with basic groups of collagen molecules. This is a sensitive and specific staining method that has the advantage of permitting easy and precise localization of the tissue components containing oriented collagen molecules, permitting a close morphological observation and allowing quantification methods.13 The slides were analyzed in a light microscope (Olympus® CX81, Olympus, Tokyo, Japan) and photographed (DP71 camera, Olympus). Images at 40x, 100x and 400x magnification were captured.

Collagen quantification

Ten non-overlapping photographs from random fields per section were taken from two slides per specimen at 100x magnification. Images were exported in .jpg format with a resolution of 1200 x 900 pixels in RGB color palette. Images were processed in an automated image analyzer (ImageJ version 1.48, U.S. National Institutes of Health, Bethesda, MA, USA). Threshold colors were used according to the Hue-Saturation-Brightness (HSB) combination to select all the positive Sirius red marking according to controls. Selected area was measured in square pixels and the staining intensity was measured in an 8-bit gray scale (0 to 256). The positive area fraction considering a complete picture of the same resolution was calculated. The process of analysis was automated using macrocode to process 10 images per cycle. Data were expressed as a percentage of total liver tissue area.

Gene expression

A sample of the left lateral lobe of the liver was frozen in liquid nitrogen and used for gene expression analysis. Total RNA was extracted using TRIzol® RNA isolation reagent (Ambion, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s recommendations. Template cDNA was obtained by reverse transcription of 2 μg of total RNA using MMLV retrotranscriptase (NEB, Ipswich, MA, USA). Reaction mixtures were incubated at 25°C for 10 min, 42°C for 50 min and 70°C for 10 min.

Relative quantification of gene expression levels for transforming growth factor-β1 (Tgfb1) and apelin (Apelin) genes were carried out by real-time quantitative PCR (qPCR) on EcoPCR real time system (Illumina, San Diego, CA, USA) using cDNA samples obtained as described before. For this purpose, SYBR® Select Master Mix (Thermo Fisher Scientific) was used according to manufacturer’s instructions. Specific primers were designed for amplification of each gene, and their sequences are described below. Comparative cycle threshold (Ct) values were obtained after qPCR reaction were performed. We used the Ct method to calculate relative mRNA expression. All quantifications were normalized by the correspon-
The expression of the same normalized genes in control tissues. Finally, gene expression differences were calculated using data obtained from groups according to the time elapsed after surgery. These differences were shown as “fold change” in the level of expression of both Apln and Tgfb1 at 2, 4 and 6 weeks after surgery compared with control group.

The sequences of the primers used in this study were as follows:

- Tgfb1 sense primer 5’-AGAGCCCTGGATACCAACTA-3’ and antisense primer 5’-GACCTTGCTGACTGTGTGT-3’;
- Apln sense primer 5’-TGTCCCTCATCCCGTTGTTTC-3’ and antisense primer 5’-AAGCCTACCTCCCTTCA-3’;
- B2m sense primer: 5’-CAGTTCACCACACCTCAGAT-3’ and antisense primer: 5’-TTTTGGGCTCCTTCAGATGAGTG-3’.

Statistical analysis

Data are expressed as median with interquartile range in parenthesis. Non-parametric Mann-Whitney Rank Sum Test was applied to evaluate differences among groups. Correlation coefficient Spearman Rank Order Correlation was also calculated at the assessment of gene expression and postoperative follow-up time. Kruskal-Wallis test was applied to determine differences among gene expression levels between postoperative weeks for each profibrogenic gene. A P value <0.05 was considered statistically significant. Statistical analysis was performed using STATA software ver. 12.0 (StataCorp LP, College Station, TX, USA).

Results

Rats in the control group weighed a median of 49.5 g (48.3-55.6) before surgery, and rats in the BDL group weighed a median of 52.6 g (46.6-58.1) before surgery (P=0.93). All the rats in the control group survived the experiments. In BDL group, 19/21 (90.5%) rats survived the experiments. In BDL group, cholestasis was clinically evident, characterized by icteric coloration of face muzzle, legs and tail. Additionally, yellowish pigmentation was observed in the gravel of boxes (choluria). At laparotomy, bile-stained tissues, ascites, hepatomegaly and cystic dilatation of bile duct stump was observed (Figure 1). All these findings were progressive in time. In addition, poor growth of hair in the surgical wound was observed. Table 1 shows postoperative weight gain, spleen index and collagen quantification. Weight was significantly affected by the BDL procedure during the second and fourth postoperative weeks compared to the control group. By the 6th postoperative week, no weight difference could be observed. Spleen index was significantly altered in the 4th and 6th postoperative weeks in the experimental group, meaning that a progressive splenomegaly was developed. Collagen quantification as a quantitative method for collagen deposition showed no changes in the control group over the weeks. In the BDL group, a highly significant collagen deposition was seen over the weeks on exponential rise, being highest on the 6th postoperative week.

Table 2 shows biochemical liver function analysis. Bilirubin was the only parameter that showed a significant change over the weeks in the experimental group when compared to the control animals. In BDL group, histological analysis of liver showed ductular reaction, portal fibrosis with collagen deposition and bile plugs. These findings were progressive in time after surgery (Figure 2). Tgfb1 mRNA expression level

![Figure 1. Intraoperative findings at 6 weeks after BDL procedure. Note fibrotic aspect of liver (L), enlarged spleen (S) and cystic dilation of common bile duct (CBD).]
was significantly higher at 6th postoperative week in BDL compared to control group (P<0.001), and showed a positive exponential correlation (Figure 3A). Apln mRNA expression level was significantly higher at 4th and 6th postoperative week in BDL compared to control group (P<0.001), and showed a positive linear correlation (r= 0.975; P<0.05) (Figure 3B).

**Discussion**

In the past years, a number of authors have stressed the differences in the cholestatic response between adults and young animals after bile duct ligation. Gibelli et al. studied a neonatal BDL model and demonstrated through semiquantitative and quantitative histological analyses a less intense ductal proliferation and inflammatory infiltrate when compared to the adult model. On the other hand, portal and periporal fibrosis were higher when compared to adult animals.14 Medeiros et al. showed a slower initial inflammatory response in young animals, but at the end of the study period there was a faster progression to regeneration nodule formation and cirrhosis in young animals.15 Omori et al. demonstrated that ductal proliferation and fibrogenesis were more intense in young rats compared to adult animals.16 These authors have demonstrated that histological and molecular findings in livers of young rats submitted to BDL were more similar to biliary atresia-related fibrosis. Therefore, it is advisable the use of neonatal or young animals as a surgical animal model to study cholestatic diseases of childhood. However, operating on rat pups is technically difficult because of the small size of biliary structures. Moreover, rat pups are more prone to die during anaesthetic procedures or after surgery. Previous reports have shown a 66.6% survival of newborn rats submitted to BDL.11 Even in adult rats, BDL has a high mortality (18.1-77.5%) attributed to sepsis and multiple abscesses.9,10 In our study, the survival rate of young rats was superior to previous reports. With appropriate anesthetic agent choice and hypothermia prevention, the animals are able to achieve elevated survival rates. We chose isoflurane as the anesthetic agent, because induction and recovery are more rapid compared to other volatile agents, like halothane, and because it induces less myocardial depression. Moreover, only a small percentage of isoflurane is metabolized in the liver. It is easy to adjust isoflurane depth during maintenance of anesthesia.16 In three-week-old rats, ligation of the common bile duct is technically easy to perform and rats can be kept without their mothers during the postoperative period avoiding cannibalism. Moreover, BDL in 3-week-old rats, recreates the histological and molecular features

|                          | Control median (IQR) (n = 6) | Bile duct ligature 2nd PO week median (IQR) (n = 5) | Bile duct ligature 4th PO week median (IQR) (n = 5) | Bile duct ligature 6th PO week median (IQR) (n = 5) |
|--------------------------|-------------------------------|---------------------------------------------------|---------------------------------------------------|---------------------------------------------------|
| Albumin (g/dL)           | 2.5 (2.5-2.5)                 | 2.4 (2.15-2.65)                                  | 2.1 (2.21)                                       | 2.1 (1.95-2.1)                                   |
| ALT (U/L)                | 70 (67.5-72.5)                | 87 (78.5-88.25)                                 | 130 (74-134)                                    | 70 (54-99.5)                                    |
| AST (U/L)                | 61 (43.5-78.5)                | 260 (155-353.5)                                 | 184 (160-201)                                   | 247 (169-276.5)                                 |
| Alkaline Phosphatase (U/L)| 574 (538.5-609)              | 415 (390-445.75)                                | 705 (542-724)                                   | 418 (334-506.5)                                 |
| GGT (U/L)                | 0 (0-0)                       | 22 (0-46.75)                                    | 31 (31-45)                                      | 23 (17.5-43.5)                                   |
| Bilirubin (mg/dL)        | 0.1 (0.1-0.1)                 | 2.75 (0.275-5.2)                                | 3 (1.94.4)                                      | 3.8 (3.6-4.65)                                   |

PO, postoperative; IQR, interquartile range; *P<0.05 versus control group.
of biliary atresia. Therefore, 3 weeks old rats have the advantages of both neonatal and adult rat models of bile duct ligation.\(^17\)

At diagnosis, biliary atresia typically presents with conjugated jaundice, pale stools, and dark urine. Failure to thrive is another clinical finding in biliary atresia that results from poor absorption of long-chain fats and the catabolic state.\(^1\) The rats in our study developed jaundice, choluria and acholia following surgical procedure. The catabolic state was inferred from poor growth hair in the abdominal surgical wound. Failure to thrive is noted early in the 2\(^{nd}\) postoperative week and maintained up to the 4\(^{th}\) postoperative week. Rat weight was similar in control and BDL rats on the 6\(^{th}\) postoperative week. We believe that this is due to the development of ascites, hepatomegaly and splenomegaly that can increase body weight as observed in BDL group.

Splenomegaly is not usually found in biliary atresia unless diagnosis is made late. Splenomegaly is a sign of portal hypertension.\(^1\) In our study, the spleen index was progressively higher as the postoperative weeks passed and as the cirrhosis and portal hypertension developed. Ascites is another feature of portal hypertension also present in this model. Therefore, BDL model in young rats recreate the complications associated to the progression of biliary atresia.

Biochemical variables in biliary atresia typically identify cholestatic liver function test, with increased bilirubin, alkaline phosphatase, gamma-glutamyl transferase, aspartate aminotransferase and alanine aminotransferase. At presentation, albumin value is typically normal.\(^1\) Our results show a similar pattern in BDL group compared to human biliary atresia. However, only bilirubin was significantly higher with near 40-fold elevation compared to control group. We believe that the other liver function tests were not significantly altered due to the limited number of samples studied.

Histopathological liver changes in biliary atresia include ductular reaction, portal fibrosis and bile plugs. Ductular reaction consists in proliferation of small interanastomosing ductules located at the periphery of portal tracts, and represents the most consistent indicator of the presence of biliary obstruction.\(^18\) Ductular reaction is typically accompanied by portal fibrosis and portal and periductal edema. Mild lymphocytic inflammation is usually present within portal tracts in biliary atresia. Other inflammatory cells include eosinophils, plasma cells, and macrophages. Bile plugs are frequently seen within dilated lumens of ducts and represent another useful diagnostic feature.\(^19\)

Liver fibrogenesis, is a dynamic wound healing-like process that leads to progressive accumulation of extracellular matrix components in an attempt to limit hepatic damage. Biliary atresia-related fibrosis is more rapid and aggressive than any other adult liver disease. Biliary fibrosis pattern is characterized by formation of portal - portal fibrotic septa surrounding liver nodules. This pattern develops by preserving connection between central vein and portal tract and is typically associated to intense proliferation of reactive bile ductules and periductular myofibroblasts.\(^19\)

In the present study, we found a progressive liver fibrosis over the weeks, with a massive fibrosis on the 6\(^{th}\) postoperative week. These findings demonstrate that BDL model in young rats clearly reproduces hepatic histological features of biliary atresia and specifically recreates progressive collagen deposition as observed in the human pathological condition.

TGF-β is a central regulator in chronic liver diseases that contributes to all stages of disease progression from initial liver injury through inflammation and fibrosis to cirrhosis and hepatocellular carcinoma. Three isoforms of TGF-β have been identified (β1, β2, and β3), but only TGF-β1 is linked to liver fibrogenesis.\(^21\) TGF-β1 is considered as a major profibrogenic cytokine.\(^22\) TGF-β1 regulates a wide variety of cellular processes in liver fibrogenesis,

Figure 3. mRNA expression level of transforming growth factor-β1 (Tgfb1) (A) and apelin (Apln) (B) genes in the liver of bile duct ligation group compared to control group by real-time quantitative polymerase chain reaction. The differences are expressed as “fold change”. Note massive overexpression of Tgfb1 on 6\(^{th}\) postoperative week (A) and progressive linear elevation of Apln (B). PO, postoperative; *P<0.001 BDL versus control group on each week; ‡P<0.001 BDL 6\(^{th}\) PO week versus BDL 2\(^{nd}\) PO week.

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including apoptosis of hepatocytes, enhances hepatoocyte destruction, and mediates hepatic stellate cell and fibroblast activation resulting in a wound healing response with extracellular matrix deposition. TGF-β1 also regulates activation and recruitment of inflammatory cells into injured liver, and transdifferentiation of some liver-resident cells. In our study, Tgfb1 gene expression had an exponential overexpression on the 6th postoperative week in accordance to what previous authors have reported. Apelin, the endogenous ligand of angiotensin-like-receptor 1, is an emergent peptide involved in liver disease. Recent studies have demonstrated that apelin is overexpressed in livers from both cirrhotic human and rats. In addition, apelin modulates splanchnic angiogenesis and portosystemic collateral vessel formation in an experimental model of portal hypertension in rats. Apelin expression in rodents has been evaluated in other non-cholestatic adult animal models. Chen et al. demonstrated that apelin is overexpressed in livers of biliary atresia patients according to progression of disease, markedly activated in end-stage cirrhosis. Significant linear correlations were observed between apelin mRNA level and liver fibrosis, serum total bilirubin and the grade of esophageal varices. Since apelin expression level accurately reflects the severity of hepatic fibrosis, Chen et al. proposed that it could be used as a prognostic factor in biliary atresia patients, to estimate the timing of liver transplantation. Our current results confirm these findings on progressive apelin expression. Moreover, these findings support the use of apelin as a fibrosis and prognostic marker, because the rise in Apln gene expression levels is significantly elevated in BDL animals as early as at 4th postoperative week, while the classic Tgfb1 gene expression levels are statistically different at 6th postoperative week.

In conclusion, bile duct ligature in young rats is a simple surgical model that recreates clinical, laboratory, histological and molecular findings of human biliary atresia. Bile duct ligature in young rats produces a progressive cholestatic injury, leading to liver fibrosis and related complications. Bile duct ligature in young rats is a good animal model to investigate therapeutic approaches against liver damage and portal hypertension in biliary atresia.

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