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Partial Ligation of the Common Bile Duct Results in Reversible Cholestasis in Rats

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The animal model of common bile duct ligation is very toxic; therefore, the aim of this study was to establish a new model of obstructive jaundice in rats with partial common bile duct obstruction. Male Sprague-Dawley rats were subjected to a sham operation or partial ligation of bile duct procedure. Serum biochemistry, liver histology, and expression of bile salt transporters were examined after surgery. Serum levels of aspartate aminotransferase, alkaline phosphatase, total bilirubin, and bile acids were significantly increased in the partial bile duct ligation group 3 days after surgery. However, these changes spontaneously normalized within 14 days after surgery in the partial bile duct ligation group compared with the sham group. Bile infarcts, ductular reaction, and abundant hepatocyte turnover were detected exclusively in the partial bile duct ligation group on postoperative day 3. However, these changes dramatically reversed 14 days after surgery. Bile salt transporter expression was significantly decreased at day 3 and gradually recovered in the following 2 weeks. In conclusion, the current rat model of obstructive cholestasis is reversible, representing the clinical characteristics of partial biliary obstruction, and may be used to investigate the effects of various therapeutic strategies on reversible acute cholestasis.

Key words: Cholestasis – Reversible cholestasis – Obstructive jaundice – Experimental study – Animal model

Cholestasis is associated with increased morbidity and mortality in patients undergoing hepatobiliary surgery.[1] Patients with obstructive jaundice are predisposed to postoperative complications,[2] or have decreased tolerance to ischemic liver injury. The mechanisms contributing to the poor outcome

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remain unknown. Understanding the underlying mechanisms would be helpful to develop effective treatments for patients with cholestatic liver diseases.[3] The model of common bile duct ligation (BDL) in rats or common BDL with or without cholecystectomy in mice is a well-described animal model of obstructive cholestasis.[4] However, this animal model is toxic, accompanied by rapid biochemical and histological changes leading to high mortality in rodents[5]; therefore, it is not appropriate for the investigation of the underlying mechanisms of obstructive cholestasis. In addition, complete obstruction of the common bile duct (CBD) does not occur frequently in humans. Partial obstruction of the CBD is more commonly seen in the clinical scenarios such as tumor invasion or intraoperative injury to the CBD. Hence, more appropriate animal models of cholestasis representing human disease are still needed. The current study presents a method for establishment of partial bile duct ligation (PBDL) in rats that is used to examine the potential mechanisms of cholestatic liver injury.

Materials and Methods

Animals

Male Sprague-Dawley rats (204–240 g) were purchased from the Animal Center of Nanjing Medical University (Nanjing, China). Animals were kept in controlled environmental conditions, maintained under a 12-hour light/dark cycle, and fed with commercial rat chow and tap water ad libitum. All experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of Nanjing Medical University.

Surgical procedures

All rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). After laparotomy, the CBD was mobilized. Rats were randomized to the PBDL or the sham group. In the PBDL group, a 0.014-inch guidewire (0.35-mm outer diameter, Boston Scientific, Boston, Massachusetts) was introduced into the CBD through the duodenal papilla from the anterior wall of the duodenum[6] and the CBD was firmly tied using an 8-0 prolene suture with the inner guidewire support. The needle was then pulled out from the ligature, which was left in place. The puncture site in the duodenum was then sutured by a purse string suture using an 8-0 prolene suture. In the sham-operated rats, a loose prolene ligature was placed around the CBD. The abdomen was closed in layers after irrigation. All rats were randomly sampled for ultrasonic examination and were killed on postoperative day (d)3 and d14.

Ultrasonic examination

Randomly selected animals were anesthetized with 1.5% inhaled isoflurane. Rat livers were examined by a high-frequency ultrasound apparatus (Vevo2100, VisualSonics, Toronto, Canada) with a 16-MHz transducer before relaporotomy.

Biochemical analysis

Blood samples taken from the inferior vena cava were snap-frozen and stored at –80°C for further biochemical analysis. Serum levels of alanine aminotransferase, alkaline phosphatase, total bilirubin, and bile acids were measured using standard analytical methods.

Histology and immunohistochemistry analysis

The liver tissues fixed in 4% neutral buffered formaldehyde were embedded in paraffin wax and 4-μm thick sections were prepared and stained with hematoxylin/eosin or Masson staining using standard histological techniques. Immunohistochemical analysis was performed as previously described.[7] Staining with Ki-67 was used to assess hepatocyte turnover with the anti-Ki-67 antibody (Abcam, Cambridge, Massachusetts). To quantify the activity of bile duct proliferation, bile ducts in 10-portal tract-centered areas were counted in high magnification (×200). Histological images were captured by a microscope equipped with a microscope camera controller (Nikon Eclipse 80i with Nikon Digital Sight DS-U3, Nikon Corp, Tokyo Japan).

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed as previously described.[8] Briefly, total RNA was extracted from 50 mg of liver tissue using a reagent (TRIzol, Invitrogen, Shanghai, China) and cDNA was synthesized using reagent kit (PrimeScript RT kit, Takara, Dalian, China). Quantitative RT-PCR was performed using a reaction mix (FastStart Universal SYBR Green Master, Rox, Roche Life Science, Indianapolis, Indiana) and sequence detection system (ABI PRISM 7500, Applied Biosystems, Life Technologies Corp, Carlsbad, California).

The relative gene expression of mRNA for Na+ taurocholate cotransporting peptide (Ntcp) and bile salt excretory pump (Bsep) was examined as the inverse log of the ΔΔCt and normalized to the reference gene, β-actin. All experiments were performed in triplicate. 5′-3′ Ntcp forward: TCTGCTCTCTTCCAACTCAATCC, reverse: GAGTT GAATGTTTTGGAATCCTGTT; 5′-3′ Bsep Forward:TGCCAAGGATGCTAATGCATAC, reverse: TCATCTGGCCTCCTCCTTCTC. Primers were synthesized by Invitrogen.

Western blotting

Total liver membranes were prepared as previously reported. [8] The protein concentration was determined using a bicinchoninic acid kit. Proteins were separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore Corporation, Billerica, Massachusetts), which were blocked in 5% nonfat dried milk and incubated overnight at 4°C with the appropriate primary antibodies as follows: rabbit polyclonal anti-Ntcp antibody (Abcam); rabbit polyclonal anti-Bsep antibody (Abcam); and glyceraldehyde-3-phosphate dehydrogenase antibody (Beyotime, Nantong, China) was used as an internal control. Electrochemiluminescence was performed with a commercial imaging system (ChemiImager 5500, Alpha Innotech Co, San Leandro, California).

Statistical analysis

All data are expressed as means ± SEM. All statistical analyses were conducted using statistical software (SPSS 13.0 for Windows, SPSS, Chicago, Illinois). Comparisons between 2 groups were made using Mann-Whitney *U* test. A value of $P < 0.05$ was considered statistically significant.

Results

PBDL led to partial obstruction of the CBD

All rats of both the PBDL group and the sham control group survived to the selected time points (postoperative d3 and d14). There was no significant difference in weight loss between the sham group (243.3 ± 4.4 g) and the PBDL group (233.5 ± 3.6 g) on postoperative d3 (n = 8 for each group, $P = 0.130$ by Mann-Whitney *U* test). However, rats in the PBDL group lost weight (276.6 ± 3.7 g) compared with the sham group (292.5 ± 4.1 g) in the second week (n = 8 for each group, $P = 0.021$ by Mann-Whitney *U* test). Ultrasound showed the dilatation of the CBD before relaparotomy (Fig. 1A). Intraoperative inspection confirmed the dilatation of the CBD proximal to the ligature (Fig. 1B). During the relaparotomy, the pressure of the dilated CBD was not measured due to technique failure. Patency of the CBD was confirmed by the presence of bile flow after dividing the CBD distal to the ligature (Fig. 1C).

PBDL induced cholestasis and hepatocellular injury

Biochemical analysis showed increased serum levels of alanine aminotransferase, alkaline phosphatase, total bilirubin, and total bile acids in the PBDL group 3 days after surgery compared with the sham group (Fig. 2). However, these parameters normalized within 14 days (Fig. 2), though the dilatation of the CBD was still present. Histological examination of the liver revealed bile infarcts in the PBDL group, which were absent in the control group (Fig. 3A) 3 days after surgery. Increased bile duct proliferation in the PBDL group (10.9 ± 0.9) was observed in comparison with the sham group (6.3 ± 0.5, $P < 0.001$ by Mann-Whitney *U* test, Fig. 3B). Similarly, more abundant hepatocyte turnover in response to partial biliary obstruction was detected in the PBDL group (0.484 ± 0.038, quantification by the percentage of Ki-67 positive nuclei) by Ki-67 immunostaining (Fig. 3C) than the sham group (0.032 ± 0.003, quantification by the percentage of Ki-67 positive nuclei) 3 days after surgery ($P < 0.0001$ by Mann-Whitney *U* test). However, these changes in the PBDL group were dramatically reversed 14 days after surgery without additional intervention. There was no difference between the 2 groups with respect to bile infarct, bile duct proliferation, and hepatocyte proliferation 14 days after surgery (data not shown). Consistently, fibrosis found by Masson staining was mild and comparable between 2 groups at both d3 and d14 (Fig. 3D).

PBDL resulted in changes of bile salt transporter expression

Ntcp uptakes bile acids from the portal blood into hepatocytes and Bsep pumps bile acids into bile.[9] To investigate the adaptive response of bile transporter expression to PBDL, the mRNA and protein expression levels of Ntcp and Bsep was examined. Ntcp and Bsep gene expression, quantified by qRT-PCR, were significantly decreased in the PBDL

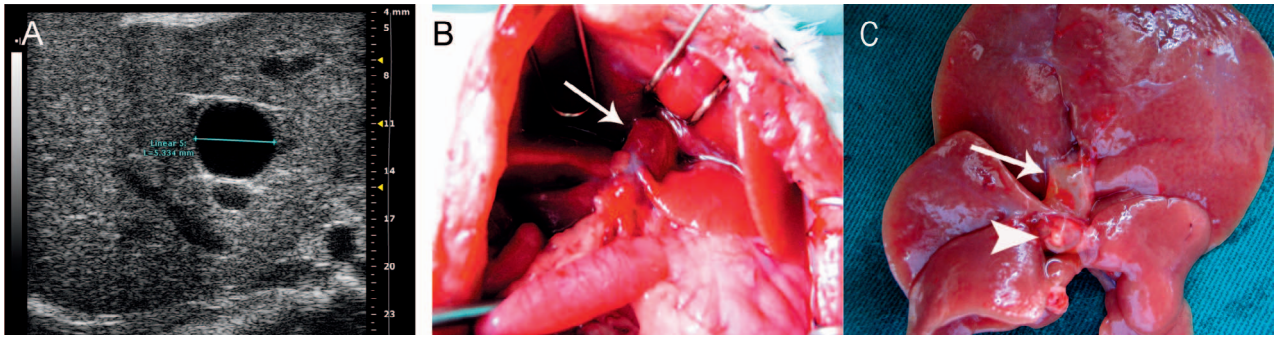

Fig. 1 Dilatation of the common bile duct was demonstrated by (A) ultrasound, (B) intraoperative inspection (arrow) and (C) the excised rat liver (arrow showing the proximal dilated bile duct; arrowhead showing the distal normal bile duct). Bar in (A) 5.3 mm.

group compared with the sham group; however, a tendency to recover was observed (Fig. 4A). Consistently, protein expression of Ntcp was significantly downregulated at all time points in comparison with the sham group, which was consistent with the qRT-PCR results (Fig. 4B). Protein expression of Bsep was significantly reduced 3 days after surgery in the PBDL group compared with the sham group; however, there was no significant difference between the two groups at day 14 (Fig. 4B).

Discussion

The principal finding of this study is that partial ligation of the CBD led to histologic and biochemical abnormalities, which were easily reversible. This model is different from the total ligation of BDL model in rats, which has been widely used to investigate biliary fibrosis and cholestatic liver injury.[10] Total ligation of BDL results in marked ductular reaction, bile infarcts, and biliary type fibrosis at an early stage.[4] This is a very toxic animal model that causes complete obstructive cholestasis and acute liver injury with a high postoperative mortality. The rapid obstructive changes observed in total ligation of BDL model in rats make it difficult to identify the underlying mechanisms of bile infarcts and ductular reaction.[5] Moreover, total obstruction of the CBD is not frequently observed in humans. Invasion to the CBD due to progressive Klatskin tumors or intraoperative incidental injury to the bile duct during open or laparoscopic cholecystectomy is the most common cause of partial biliary obstruction in clinical practice.[11,12] The current reversible cholestatic model induced by PBDL may better mimic the clinical scenario and be a more suitable animal model for further research of obstructive cholestasis as

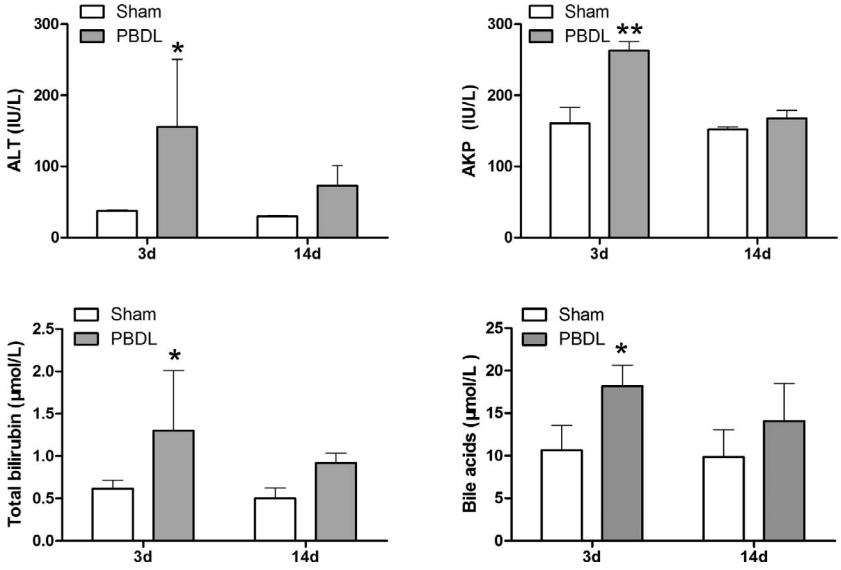

Fig. 2 Serum levels of alanine aminotransferase (A), alkaline phosphatase (B), total bilirubin (C), and bile acids (D) at different time points in the sham group and the PBDL group. Data are shown as mean ± SEM (6–8 rats per group). Comparisons between 2 groups were made by Mann-Whitney *U* test. * $P < 0.05$. ** $P < 0.01$.

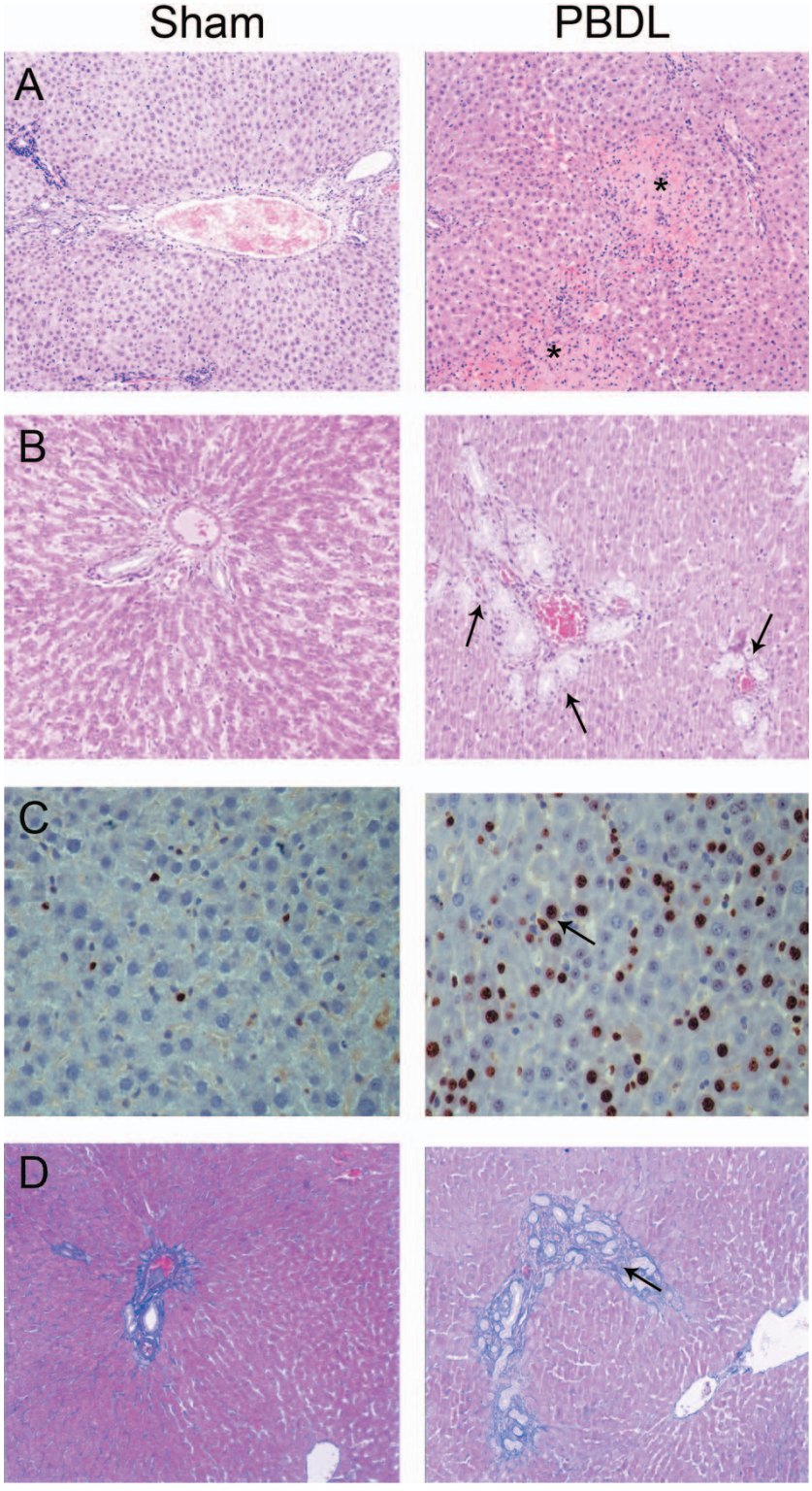

Fig. 3 Histologic examination of liver sections from the sham group and the PBDL group by light microscopy. (A) Hematoxylin and eosin (H & E) staining showed the presence of scattered bile infarcts (asterisks), which were not detected in the sham group, in the PBDL group (magnification ×100). (B) H & E staining showed increased bile duct proliferation (arrows) in the PBDL group compared with the sham group (magnification ×200). (C) Ki-67 immunostaining revealed increased hepatocyte turnover of Ki-67-positive hepatocytes (arrow) in the PBDL group compared with the sham group (magnification ×400). (D) Marked periductular fibrosis (arrow) was detected in the PBDL group in comparison with the sham group by Masson staining (magnification ×100).

pathological changes including bile infarcts and bile duct proliferation were mild.

The diameter of the CBD in rats weighing 250 to 300 g was reported to range from 0.6 to 1 mm by Martins *et al*[13] and 0.2 to 0.5 mm by Guyot *et al*[14] Theoretically, bile duct cannulation would result in biliary obstruction, or at least partial biliary obstruction to some degree. Interestingly, previous studies

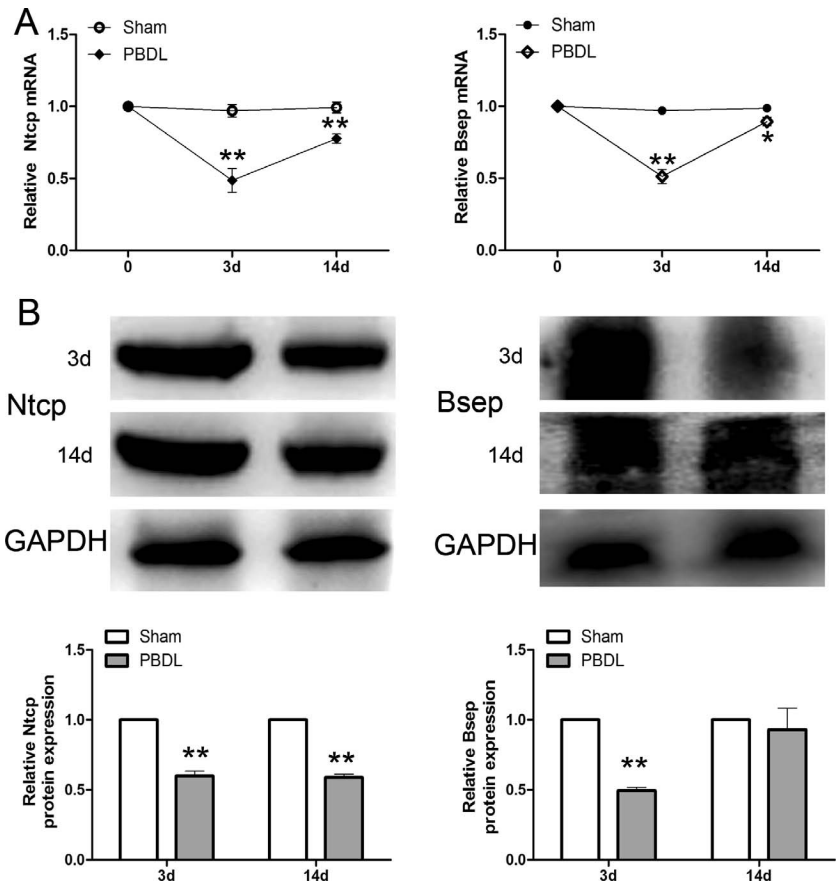

Fig. 4 Gene and protein expression for Ntcp and Bsep quantified by qRT-PCR (A) and Western blotting (B) in the sham group and the PBDL group. PCR results are expressed as mean ± SEM of at least triplicate measurements. Western blot data are mean ± SEM of 3 independent experiments, and are normalized to glyceraldehyde-3-phosphate dehydrogenase. Comparisons between 2 groups were made by Mann-Whitney *U* test. * $P < 0.05$. ** $P < 0.01$.

demonstrated that ligation of the peribiliary plexus with a bile duct cannulation tube (polyethylene tube, 0.28 mm inner diameter in mice or 0.3 mm in rats) did not cause bile duct dilation and obstructive cholestasis.[15–17] However, in the current rat model partial ligation with the support of the hard guidewire (0.36 mm outer diameter), which was then removed after securing a single suture, induced reversible cholestatic changes. The underlying reason for the discrepancy is unclear. However, it is likely that the bile duct cannulation tube protects the CBD from compression of edema and inflammation induced by surgery and the prolene suture. We cannot fully exclude the possibility that transient total obstruction of the common bile duct may occur due to the placement of the calibrating wire during the procedure or due to edema and inflammation of the common bile duct after the procedure.

Partial ligation of the CBD did not interrupt the extrahepatic peribiliary plexus, thus ischemia is not the cause of cholestasis in the current model.[16] Here cholestasis is believed to result from bile duct obstruction. Generally, ductular reaction is in response to increased biliary pressure.[10] Bile duct proliferation is also assumed to be an adaptation of cholestatic changes due to hepatic arterial ischemia[18] and facilitate the bile drainage.[17] However, bile duct proliferation regressed 2 weeks after surgery in the PBDL group. Changes in bile transporter expression observed in the current study may, to some extent, result in the functional modifications of bile salt secretion[16] and relieve the extent of cholestatic liver injury induced by bile salt retention. Additionally, dilatation of the CBD may decrease the biliary pressure; changes in bile transporters may decrease bile flow. However, this was not evidenced in the current study.

Likewise, the mechanism for the absence of bile infarcts on postoperative day 14 also remains elusive. Increased serum levels of bile acids and bilirubin, which are demonstrated to interrupt mitochondrial membrane permeability transition,[19] are supposed to be the mechanisms for bile infarct in the current model.[20] However, Shibayama[21] believed that some unidentified toxic bile constituents absorbed into systemic circulation from bile, other than bile acids and bilirubin, led to bile

infarcts. In addition, increased biliary pressure might be another possible mechanism for bile infarct. However, dilatation of the CBD was still present while bile infarcts were not detected 2 weeks after surgery. Furthermore, increased hepatocyte proliferation in response to cholestatic injury, to some degree, compensated hepatocyte necrosis and contributed to reversal of bile infarcts. Thus, the exact cause for cholestasis and the reversal of bile infarcts in the current model remains unknown. Further exploration of these underlying mechanisms may provide new directions for therapy of obstructive jaundice.

The initial purpose of establishing this model was to examine whether ligation of the bile duct to a diameter similar to the inner diameter of bile duct cannulation, which was used in previous studies to interrupt the extrahepatic peribiliary plexus,[15–17] induces partial biliary obstruction. This unexpected finding of reversible cholestasis in this current model allows for further investigation of the mechanisms and adaptive response of obstructive cholestasis characterized by ductular reaction and bile infarcts. Longer follow-up of this model might be of value in future analyses of the development and reversal of periductular fibrosis. Moreover, this model can be used to assess the effect of ischemia, infection or pharmacotherapy on hepatobiliary system in the setting of obstructive cholestasis.[22]

Conclusion

In conclusion, the technique presented here is a reproducible and simple surgical technique. This rat model especially is suitable for research of pathological changes induced by partial biliary obstruction in the clinical scenarios.

Acknowledgements

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