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Original Article

CHOLESTATIC LIVER FIBROSIS IN A RAT MODEL OF BILE DUCT LIGATION: EVALUATING BIOCHEMICAL VERSUS HISTOPATHOLOGICAL CHANGES

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ABSTRACT

Objective: Bile duct ligation (BDL), chronic liver injury model, was extensively used in studying mechanisms of fibrogenesis and antifibrotic agents. Considering the liver regenerative capacity and the diverse results from BDL, the present study aimed to evaluate the biochemical and histopathological changes over 10 weeks following BDL assessing if BDL-induced changes remain in a deterioration state or improve at a certain stage.

Methods: Sham operation and BDL were conducted in Male Wistar rats. Serum AST, ALT, total bilirubin and albumin and hepatic hydroxyproline (HYP), reduced glutathione (GSH) and malondialdehyde (MDA) were measured in sham-operated (n=3) and BDL-rats (n=6) at 0, 1, 2, 4, 6, 8 and 10 weeks following operation. Liver tissue was also processed for histopathological analysis (H&E and Sirus red staining).

Results: Progressive liver injury (H&E) and collagen deposition (Sirus red and HYP) in BDL-rats were observed starting from the first week post-operation and reached their maximum with early signs of cirrhosis on the 10th week of BDL. Severe and sustained cholestatic injury appeared in 2 weeks (increased ALT, AST, bilirubin along with decreased albumin ($P<0.001$) compared to sham-operated rats). AST peaked on first week, however, bilirubin, ALT and MDA peaked on the 4th week ($P<0.001$) then gradually decreased compared to their peaks.

Conclusion: The relative improvement in liver function/cholestasis following their peaks in BDL model despite progression of fibrosis and hepatic injury require investigators using this model to consider not only biochemical, but also histopathological findings to guarantee an accurate interpretation of their results.

Keywords: Bile duct ligation, Fibrosis, Sirus red, ALT, AST, Hydroxyproline.

INTRODUCTION

Chronic liver diseases are common and potentially life threatening for humans. These involve progressive injury and regeneration of liver parenchyma leading to fibrosis and subsequently liver cirrhosis [1]. Massive deposition of extracelluler matrix components in the liver as wound-healing response to repeated liver injury results in liver fibrosis [2, 3]. At some stages of the disease, the liver regenerates to respond to a loss of liver mass or liver injury retrieving some of its lost functions[3]. Minimum 50% hepatocyte loss and presence of hepatocytes replicative senescence are necessary triggers for liver progenitor cells (LPC) activation. Inhibition of the LPC proliferation and impairment of liver regeneration was achieved after 70% partial hepatectomy [4]. Studying fibrogenesis and possible antifibrotic drugs would therefore require a model that most closely mirror advanced human fibrotic liver diseases.

Different rodent models, developed to mimic human hepatic fibrosis, are valuable to understand disease etiology and pathology, as well as to test therapeutic approaches that cannot be safely applied to humans [5, 6]. Bile duct ligation (BDL) model has attracted many investigators studying (i) the underlying cellular and molecular mechanisms of fibrogenesis [7, 8] and (ii) the assessment of the efficacy of various antifibrotic agents[9]. It is considered as a representative chronic liver injury model that is mediated by biliary obstruction and subsequent cholestasis [10]. Double ligation of the common bile duct with dissection between the ligatures causes abnormal flux of bile acids and bilirubin in the liver. Toxic hydrophobic bile salts subsequently accumulate within hepatocytes causing successive inflammatory reactions, hepatocyte death and peri ductular fibrosis [11, 12]. Models of BDL employed at different time points up to 6 weeks following BDL revealed diverse results [13-15]. A 3 or 4-week BDL-rat model is the most commonly used by many investigators testing possible antifibrogenic drugs in prophylactic or curative study designs [16-18]. Nonetheless, some treatment regimens may require prolonged duration and so two important questions come up: (i) Do the changes induced by BDL remain in a deterioration state or improve at a certain stage because of self regeneration? (ii) Is the study designed in a way that guarantees the correct/accurate interpretation of results? The current study was designed therefore to evaluate the BDL model over 10 weeks following BDL and verify the suitable duration of BDL at which the efficiency of possible anti-fibrotic drugs can be tested to exclude model specific artifacts and to strengthen the data obtained.

MATERIALS AND METHODS

Male Wistar rats weighting 250±30 g (n=57) were obtained from animal facility of the National Institute for Vaccination, Helwan, Egypt. The animals were kept in a temperature-and humidity-controlled environment in a 12-h light-dark cycle. Rats were acclimatized for 1 week before any experimental procedures and were allowed free access to water and standard rodent chow at all times. All animal experiments received approval from the Ethical Committee of the Faculty of Pharmacy, Zagazig University, Egypt (No. P11/2/2013).

Experimental model

Rats were generally anesthetized with an intraperitoneal injection of ketamine hydrochloride (50 mg/kg) and diazepam (3 mg/kg). Under aseptic conditions, double ligation at the common bile duct and complete cutting at midpoint were conducted in 36 rats (BDL). The other 21 rats were sham-operated; received an identical laparotomy and isolation of the common bile duct without ligation [19].

Blood samples were obtained via retro-orbital bleeding from either sham-operated (n=3 rats) and BDL rats (n = 6 rats) at time of operation as well as 1, 2, 4, 6, 8 and 10 weeks thereafter. Serum

samples were frozen at-20 °C for subsequent measurement of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, total bilirubin and albumin. All rats were euthanized following blood withdrawal and liver tissues were harvested and washed in normal saline. Each liver was divided into three parts. Two parts were quickly frozen in liquid nitrogen (-170 °C) and stored at-20 °C for subsequent determination of hepatic hydroxyproline (HYP), reduced glutathione (GSH), malondialdehyde (MDA) content. The third part of liver tissue samples was immediately fixed in 10% formalin at 4 °C and embedded in paraffin for subsequent histopathological analysis.

Liver function tests

Serum concentrations of liver enzymes (ALT and AST), albumin and total bilirubin were assayed using commercially available kits provided by Spinreact, Gerona, Spain following manufacturer's instructions.

Hepatic hydroxyproline content

Hydroxyproline content determination was performed spectrophotometrically using Ehrlich reagent [20]**.** Briefly, liver tissue was homogenized in 6 N HCl and was incubated at 120°C for 12 hrs (hydrolysis). The filtered acid hydrolysate (5 µl) was applied to 96-wells plate and 5 µl of citric/acetate buffer and 100 µl chloramine T solution were added. The plate was incubated for 20 minutes at room temperature before the addition of 100 µl Ehrlich solution. The plate was then incubated for 15 minutes at 65°C and The O. D was read at 550 nm using microplate reader (Sunrise plate reader, Austria). The concentration of hydroxyproline in the hydrolyzed samples was determined from hydroxyproline standard curve which was established using serial dilutions of 1 mg/ml hydroxyproline (sigma, St. Louis, Mo).

Hepatic lipid peroxide and reduced glutathione (GSH) content

Liver tissue was homogenized in ice-cold phosphate buffered saline, pH 7.2 to prepare 10 % homogenate. The resultant homogenate was used for spectrophotometric determination of MDA and GSH using Biodiagnostics kits (Biodiagnostics Co., Giza, Egypt) following manufacturer's instructions.

Histopathological examination

The paraffin-embedded liver tissue was sectioned (4 mm thickness) and stained with H&E using standard histological techniques. All slides were blindly examined. Sirius Red staining was performed by incubating slides in 0.1% Sirius Red F3B for 1 h, washing twice in acidified water, dehydrating thrice in 100% ethanol, and then clearing in xylene. The percent of fibrosis were measured using image J software and expressed as a percentage of total analyzed areas. Histopathological scoring of the liver was done according to the Metavir score. The Metavir score is a semiquantitative classifications system consisting of an activity and a fibrosis score: The fibrosis score is assessed on a five point scale (0 = no fibrosis, 1 = portal fibrosis without septa, 2 = few septa, 3 = numerous septa without cirrhosis, 4 = cirrhosis). The activity score was graded according to the intensity of necroinflammatory lesions (A0 = no activity, A1 = mild activity, A2 = moderate activity, A3 = severe activity)[21].

Statistical analysis

All statistical analyses were done using Windows-based Statistical Package for Social Sciences software (SPSS version 14.0, SPSS Inc, Chicago, IL). Results were expressed as mean±SD. Statistical difference were sought using Student's t-test or one-way analysis of variance (ANOVA) followed by LSD post-hoc test (if more than two sets of data were being compared), taking $p<0.05$ as statistically significant.

RESULTS

Histopathological findings

The progression of liver injury over 10 weeks following BDL was initially evaluated by histological examination (fig. 1). The sham group (fig. 1A) showed normal hepatocytes arranged in branching cords radiating from the central vein (CV). The nuclei are central and rounded and the cytoplasm was granular and acidophilic. Normal sinusoidal space and periportal area were also observed. No inflammatory activity could be seen. BDL exhibited progressive increase in the level of bile duct proliferation (arrows) and cellular inflammatory response (arrow heads) after 1 week (fig. 1B), 2 weeks (fig. 1C), 4 weeks (fig. 1D), 6 weeks (fig. 1E), 8 weeks (fig. 1F) and reached its maximum levels at 10 weeks after BDL (fig. 1G). Early signs of cirrhosis were detected in liver sections of rats on week 10 post BDL. Congested blood vessels were also noticed in all operated groups (asterisk).

Marked degree of hepatocytes injury was noticed. Hepatocytes were vacuolated in (fig. 1D) and some of them had pkynotic shrunken nuclei after 6 to 10 weeks of BDL (fig. 1E to fig. 1G). Karyolysis was observed in 8 to 10 weeks after BDL. Connective tissue (CT) started to appear at the 6 weeks and increased progressively by 10 weeks of BDL**.**

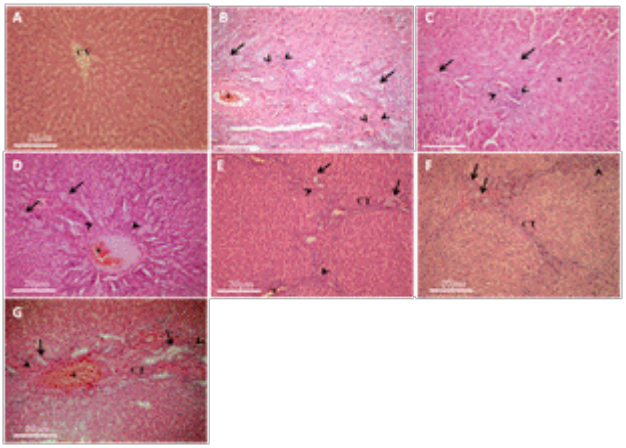

Fig. 1: Liver tissue sections from sham operated rats (A) and rats with BDL, on post-operative week 1 (B), week 2 (C), week 2 (D), week 6 (E), week 8 (F) and week 10 (G), stained with hematoxylene and eosin. Bile duct proliferation (arrows) and cellular inflammatory response (arrow heads) and Congested blood vessels (asterisk). Results are representative of three animals at each time point; original magnification X 100

Fibrogenic response in BDL-induced liver injury

Sirus red staining of liver tissue sections was utilized to assess for the extent of fibrosis. Sham animals showed the lowest level of collagen deposition, as anticipated (fig. 2A). A significant progressive increase in collagen deposition in BDL-rats was observed after 1 week (fig. 2B), 2 weeks (fig. 2C), 4 weeks (fig. 2D), 6 weeks (fig. 2E), 8 weeks (fig. 2F) and reached its maximum levels at 10 weeks after BDL (fig. 2G).

The quantitation of fibrosis area (fig. 2H) revealed a time-dependent progression of fibrosis in BDL rats compared to sham-operated rats ($P<0.001$). Metavir scoring at different time points following BDL is illustrated in fig. 2I. In confirmation, a time-dependent progressive increase in hepatic HYP content was perceived in BDL-rats throughout 10 weeks duration following operation ($P<0.01$ at fist week following BDL and $P<0.001$ thereafter) as compared to corresponding sham-operated rats (fig. 3).

Serum biochemical tests indicating cholestatic liver injury

Serum biochemical tests monitored over 10 weeks (fig. 4) demonstrated that BDL-operated rats developed severe and sustained cholestatic injury characterized by significant increases liver enzymes (ALT and AST), bilirubin along with decrease in serum albumin compared to sham-operated rats ($P<0.001$). The peak of serum ALT and AST were observed at week 4 (412%) and at week 1 (615%) from corresponding sham groups, respectively. Such peaks in ALT and AST were followed by significant gradual decreases in enzyme activities compared to their peak values ($P<0.001$).

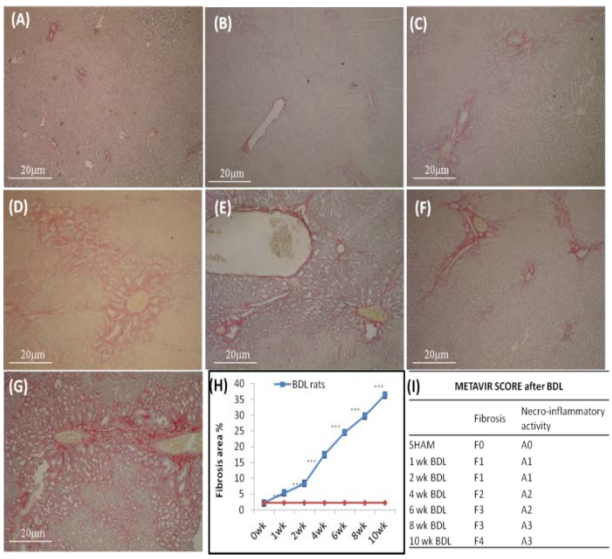

Fig. 2: Liver tissue sections from sham operated rats (A) and rats with BDL, on post-operative week 1 (B), week 2 (C), week 2 (D), week 6 (E), week 8 (F) and week 10 (G), stained with sirus red. Representative photographs of liver sections from three different animals at each time point, and three randomly selected areas from each section were analyzed. (H) Sirus red stained liver tissue sections from rats with sham operation, or with BDL at different time points after surgery (weeks 1, 2, 4, 6, 8 and 10) were measured using image J software and expressed as a percentage of total analyzed area (% fibrosis). Results are means±SD from 9 consecutive fields; ***P<0.001 as compared to sham groups. Original magnification X 100. (I) Metavir scoring at different time point following BDL

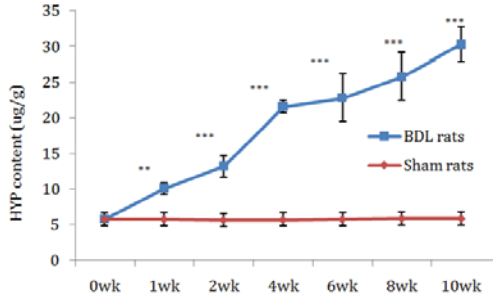

Fig. 3: Hydroxy proline (HYP) content in liver tissues of BDL- and sham-operated rats at different time durations after BDL. Results were expressed as mean±SD, n=3 and 6 for sham and BDL groups respectively. *P<0.05, **P<0.01, ***P<0.001 as compared to sham groups

At all time points ALT and AST activities in BDL-rats were higher than those in sham-operated rats (P<0.001). Serum total bilirubin attained its maximum level (7.7±0.46 mg/dl) after 4 weeks of operation. This peak was followed by significant gradual reduction thereafter. Conversely, the decrement in serum albumin was readily detected after 2 weeks of BDL (P<0.001).

Hepatic MDA and GSH contents

The level of MDA, a marker of lipid peroxidation, increased by 300% and 276% in the 2nd and 4th weeks after BDL respectively as compared to the sham groups (P<0.001). Afterward, there was a gradual decrease in hepatic MDA (P<0.001 compared to MDA peak at 4th week). Unlike other parameters, hepatic MDA content in BDL-rats was similar to that of sham-operated rats at weeks 8 and 10 postoperative (fig. **5A)**. Conversely, BDL-induced cholestasis resulted in a notable time-dependent depletion of total GSH content all the way through the 10 weeks duration compared to the sham group (fig. 5B).

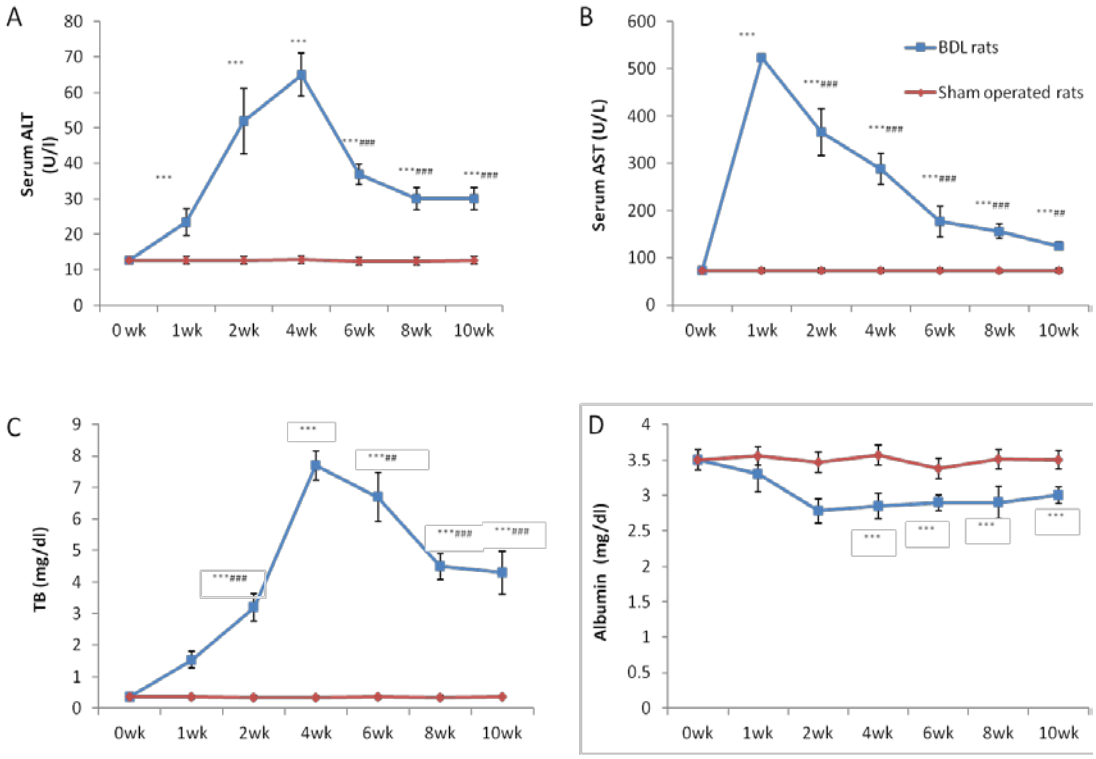

Fig. 4: Serum biochemical tests of cholestatic liver injury induced by BDL. Serum concentration of (A) ALT, (B) AST, (C) total bilirubin and (D) albumin were measured in BDL-rats and sham-operated rats at different time points up to 10 weeks following surgery. Results were expressed as mean±SD and n=3 and 6 for sham and BDL groups respectively. ***P<0.001 as compared to sham groups and ###P<0.001 as compared to peak value

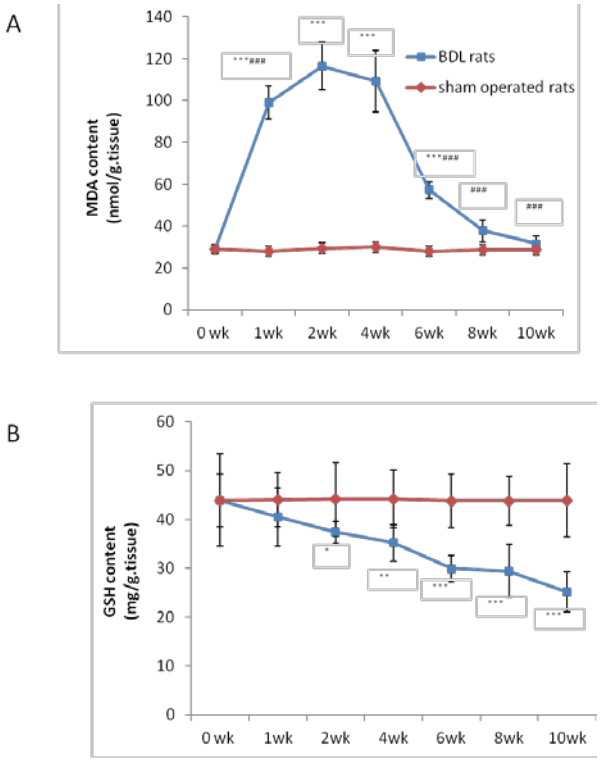

Fig. 5: Hepatic MDA (A) and GSH (B) content in BDL-and sham-operated rats at different time durations after BDL. Results were expressed as mean±SD, n=3 and 6 for sham and BDL groups, respectively. *P<0.05, **P<0.01, ***P<0.001 as compared to sham groups and $^{\#\#\#}$P<0.001 as compared to peak value

DISCUSSION

Bile duct ligation (BDL) is the most common model used to induce obstructive cholestatic injury in mice and rats. Biliary obstruction can lead to hepatocellular injury, which is followed by bile duct proliferation, fibrosis and cirrhosis. Depending on the obstruction time, an acute or chronic hepatic damage can take place [22]. Different studies of BDL model revealed diverse results regarding their outcomes of either fibrosis and/or cirrhosis [13-15,23]. In the current study BDL provided an acute obstructive jaundice in two weeks, and progressed to fibrosis in 4 to 10 weeks. Cirrhosis started to show early signs by the end of experiment at week 10 post operation. Previous study BDL model in rats over 6 weeks similarly reported acute obstructive jaundice in two weeks, but progressed to cirrhosis in 4 or 6 weeks [24]. All murine models of cholestatic liver fibrosis show several characteristics leading to liver injury including direct damage of the biliary epithelial cells induced by obstruction, immune responses leading to infiltration of mononuclear cells and periductular inflammation [6]. In consistence with these, the present study showed that BDL operation caused progressive increase in bile duct proliferation and cellular inflammatory response started as early as 1 week postoperation and lasts till week 10 postoperative. Percent of hepatic fibrosis area increased with formation of a fibrous connective tissue bridge between the portal tracts in BDL-rats. These findings were in agreement with other studies [16,25-27]. Liver fibrosis increased in a time-dependent fashion throughout 10 weeks following BDL. It is well known that liver fibrosis is characterized by excessive accumulation of extracellular matrix (ECM) proteins such as type I and type IV collagen within the perisinusoidal space of Disse [28]. The accumulation of collagen, was assessed by a significant increase in hepatic hydroxyproline content in BDL-rats which gradually increased from the 1st week of BDL operation till reach its maximum at the end of our experiment in the 10th week of BDL operation.

Although histological changes were progressive following complete blockage of bile acid flow; up to week 10 postoperative, biochemical changes were not in complete harmony. Serum total bilirubin level dramatically elevated till reached its peak after 4 weeks of BDL operation. It also induced severe inflammation in both blood and hepatic tissue, determined by drastic increases of hepatocyte aminotransferases (ALT peaked on week 4, while AST peaked on week 1 of BDL). The decrements of serum total bilirubin and hepatic aminotransferases following their peaks are in consistence with previous studies [29,30]. There is accumulating evidence that several hepatic transporters undergo adaptive regulation in response to cholestatic liver injury. This adaption are presumed to minimize the hepatic retention of bile acids and other potentially toxic substrates and this may explain the decline in total bilirubin seum level started on week 6 of BDL [31]. It is quite possible that this response only reflects a non-effective consequence to the accumulation of toxic bile constitutes in hepatocytes [32].

Moreover, Hepatocytes are able to develop number of secondary adaptive changes to minimize the detrimental effects of toxic biliary compounds retained as a consequence of the secretory failure. This may explain the decline of serum ALT and AST following their peaks [33]. A BDL models over a period of 6 weeks showed ALT peak on week 2 following BDL and decline started on week 3 throughout the 6 weeks postoperative [15]. As early as 5 days postoperative, ALT and bilirubin were reported to max out and declines were observed 10 weeks after BDL[29]. Likewise, ALT and AST were reported to attain their peaks at day 2 and day 1 respectively after BDL [34]. Another 4 weeks model wasn't able to show any peaks in AST [35] that was observed on 1st week in our model.

The decline in serum albumin level subsequent to BDL is in agreement with reported studies [36]·[37]. Henriksen and colleuges [38] reported that patients with advanced cirrhosis almost always have hypoalbuminemia caused both by decreased synthesis by the hepatocytes and water and sodium retention that dilutes the content of albumin in the extracellular space. Other factors likely contribute to the development of hypoalbuminemia, including an increased transcapillary transport rate [39].

Hepatic oxidative stress was previously reported in different BDL models [17,40-44] Previous studies reported a status of oxidative stress, depletion of reduced GSH[17,40,41] and increase in lipid peroxides induced by accumulated bile acids in BDL models[42-44] as seen in our model. The time-dependent depletion of hepatic reduced GSH may occur through the detergent action and cytotoxicity of the retained bile salts which is partly responsible for the plasma membrane damage seen in BDL models leading to further oxidative stress resulting in extensive release of reactive oxygen species that most likely attributed to a lack of adequate reactive oxygen species scavengers leading to this depletion in hepatic reduced GSH content [45].

Concurrently, hepatic MDA peaked after 4 weeks of BDL then unexpectedly declined starting from week 6 thereafter till reached normal on weeks 8 and 10. This result is in harmony with previous reported study[46]. Such decline in MDA could be due to its consumption in cross linking of collagen as MDA has been illustrated to possess fibroblast-stimulating properties in culture. Collagen production was amplified by 2–3 times in cultured fibroblasts treated with MDA [47].

CONCLUSION

Cholestasis liver injury induced by BDL induced progressive changes in the hepatic architecture, namely inflammation and fibrosis. The liver function tests and hepatic MDA peeked at different time points following BDL and then gradually declined. Previous studies on rat model of BDL and our study revealed the possibility of marked strain differences in fibrosis susceptibility and therefore the importance of conducting preliminary groups of BDL at different time points. Depending on the study aims, investigators should choose the duration of their injury model based on these preliminary experiments, nevertheless other's reports, taking in consideration not only biochemical, but also histopathological findings. Well-designed study protocol is control groups are mandatory to avoid any ambiguous data and guarantee an accurate interpretation of study's results.

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CONFLICT OF INTERESTS

All authors have no conflicts of interest to declare.

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