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ORIGINAL PAPER

Protective effects of thymoquinone against cholestatic oxidative stress and hepatic damage after biliary obstruction in rats

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Abstract The aim of this study was to examine the preventive and therapeutic effects of thymoquinone (TQ) against cholestatic oxidative stress and liver damage in common bile duct ligated rats. A total of 24 male Sprague–Dawley rats were divided into three groups: control, bile duct ligation (BDL) and BDL + received TQ; each group contain 8 animals. The rats in TQ treated groups were given TQ (50 mg/kg body weight) once a day orally for 2 weeks starting 3 days prior to BDL operation. To date, no more biochemical and histopathological changes on common bile duct ligated rats by TQ treatment have been reported. The application of BDL clearly increased the tissue hydroxyproline (HP) content, malondialdehyde (MDA) levels and decreased the antioxidant enzyme [superoxide dismutase (SOD), glutathione peroxidase (GPx)] activities. TQ treatment significantly decreased the elevated tissue HP content, and MDA levels and raised the reduced of SOD, and GPx enzymes in the tissues. The changes demonstrating the bile duct proliferation and fibrosis in expanded portal tracts include the extension of proliferated bile ducts into lobules, mononuclear cells, and neutrophil infiltration into the widened portal areas were observed in BDL group. Treatment of BDL with TQ attenuated alterations in liver histology. The immunopositivity of alpha smooth muscle actin and proliferating cell nuclear antigen in BDL were observed to be reduced with the TQ treatment. The present study demonstrates that oral administration of TQ in bile duct ligated rats maintained antioxidant defenses and reduces liver oxidative damage and ductular proliferation. This effect of TQ may be useful in the preservation of liver function in cholestasis.

Keywords Thymoquinone · Oxidative stres · Immunohistochemistry · Liver · Bile duct ligation · Rats

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Introduction

Cholestasis is characterized by an abnormal accumulation of bile acids, which is caused by defectiveness in the process of bile acid transport. It is the main feature of several chronically progressive liver disorders such as biliary atresia, primary biliary cirrhosis, and primary sclerosing cholangitis. The primary event of cholestasis has been implicated in the later development of hepatocellular injury, progressive hepatic fibrogenesis, cirrhosis, and death from liver failure (Guicciardi and Gores 2002). However, the mechanisms by which cholestasis cause hepatic changes and injury remain largely unclear. Evidence indicates that free radicals, oxidative stress, and lipid peroxidation are present in cholestatic damage (Sokol et al. 1991, 1998; Parola et al. 1996; Pastor et al. 1997). Studies further demonstrate the pro-oxidant potential of hydrophobic bile acids in hepatocytes (Baroni et al.1998; Sokol et al.1998). Therefore, it is believed that oxidative stress is a likely mediator for cholestatic damage and antioxidant therapy is a recommended therapeutic strategy.

Hepatic stellate cells (HSCs) are known to play a central role in hepatic fibrosis and accordingly have become a major therapeutic target in the treatment of liver cirrhosis (Bedossa et al. 1994; Friedman et al. 1985). Experimental bile duct ligation (BDL) induces a form of liver fibrosis,

which etiologically and pathogenically resembles biliary fibrosis in humans. Injury to hepatocytes results in the generation of lipid peroxides, which may have a direct stimulatory effect on matrix production by activated stellate cells (Serviddio et al. 2004). Complete biliary obstruction causes cholestatic injury to the liver, including hepatocellular necrosis and apoptosis, bile duct epithelial cell proliferation, stellate cell activation, and, eventually, liver fibrosis (Kountouras et al. 1984). Following cholestatic injury, the liver undergoes a tissue remodeling process that combines regeneration and fibrogenesis. Immunohistochemically, it was found that the majority of cells observed in the fibrosis regions were positive cells (spindle cells) for alpha smooth muscle actin (α-SMA). It is suggested that the spindle cells, probably transforming from Ito cells or myofibroblasts, play an important role in the pathogenesis of hepatic fibrosis (Kanter and Yener 2001).

There are many experimental and clinical data that show the important role of reactive oxygen species in the pathogenesis of hepatic damage and cholestasis produced by biliary obstruction (Sokol et al. 1991; Gonzalez-Correa et al. 1997; Hunt 1980). The bile acid concentrations increase in rats after BDL and this induces lipid peroxidation, probably related to the stimulation of phagocytic activity in polymorphonuclear phagocytes and inflammatory cells (Seto et al. 1998). Many experimental studies reported beneficial effects of antioxidants in cholestasis (Pastor et al. 1997; Cruz et al. 2003; Montilla et al. 2001). Thymoquinone (TQ) was isolated as the principal active ingredient from the volatile oil of the black seeds (Nigella sativa) (Mahfouz and El-Dakhakhny 1960). The seeds of the plant were shown to contain a fixed oil (>30% wt/wt) and a volatile oil (0.40–0.45%) (El-Alfy et al. 1975). The volatile oil has been shown to contain 18.4–24.0% TQ (2-isopropyl-5-methyl-1,4-benzoquinone) (Aboutabl et al. 1986). TQ has been shown to attenuate eicosanoid generation (Houghton et al. 1995), lipid peroxidation (Nagi et al. 1999), cisplatin nephrotoxicity (Badary 1999), ifosfamide Fanconi syndrome (Badary et al. 1997), tetrachloride hepatotoxicity (Al-Gharably et al. 1997), doxorubicin cardiotoxicity (Al-Shabanah et al. 1998), histamine release (Chakravarty 1993) and neuronal injury (Kanter 2011). In spite of these studies, there is no available information on the effect of TQ on biliary obstruction-induced liver injury.

In the present study, we examined whether TQ reduces biliary obstruction-induced liver injury by prevention of the oxidative stres, inflammation and fibrosis in rats. To assess the effect of TQ in bile duct ligated rats, we measured the tissue hydroxyproline (HP) content, malondialdehyde (MDA) levels and the antioxidant enzyme [superoxide dismutase (SOD), glutathione peroxidase (GPx)] activities.

Materials and methods

Animals

In this study, 24 healthy male Sprague–Dawley rats, weighing 200–250 g and averaging 16 weeks old were utilized. Food and tap water were available ad libitum. In the windowless animal quarter automatic temperature (21 ± 1°C) and lighting controls (12 h light/12 h dark cycle) was performed. Humidity ranged from 55 to 60%. All animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health.

Experimental procedure

TQ was obtained from Sigma Chemical Co., St. Louis, MO, USA. It was dissolved by the initial addition of DMSO, followed by the addition of normal saline (the final concentration of DMSO was <0.5%). The solution was administered at a dose of 50 mg/kg body weight once daily by using intragastric intubation for up to 2 weeks (Fararh et al. 2005).

Experimental groups

A total of 24 male Sprague–Dawley rats were divided into three groups: control, BDL and BDL+received TQ; each group contain 8 animals.

Experimental protocols

The rats were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) intraperitoneally (i.p.) and their bile duct (BD) were exposed through a midline abdominal incision. The BD was located and obstructive jaundice induced by a double ligation with 5/0 silk and transsection of the BD in the supraduodental part between the lowermost tributary of the bile duct and the uppermost tributary of the pancreatic duct. The rats in TQ treated groups were given TQ (50 mg/kg body weight) once a day orally for 2 weeks starting 3 days prior to BDL operation. Control and BDL untreated rats were also given with the same volume of saline as the BDL treated animals that received TQ. After 2 weeks of treatment, the animals were decapitated. Liver tissue samples were obtained for biochemical and histopathological investigation.

Biochemical analyses

Measurement of tissue hydroxyproline

The tissue samples taken for HP determination were washed with normal saline and dried in an oven set at

100°C for 72 h. The HP levels were determined spectrophotometrically by the Woessner's (1961) method.

Measurement of tissue malondialdehyde level

The MDA content of homogenates was determined spectrophotometrically by measuring the presence of thiobarbituric acid reactive substances (Uchiyama and Mihara 1978). Three milliliter of 1% phosphoric acid and 1 ml of 0.6% thiobarbituric acid solution were added to 0.5 ml of plasma pipetted into a tube. The mixture was heated in boiling water for 45 min. After cooling, the color was extracted into 4 ml of n-butanol. The absorbance was measured in spectrophotometer (Shimadzu UV-1601, Japan) with 532 nm. The amounts of lipid peroxides were calculated as thiobarbituric acid reactive substances of lipid peroxidation. The results were expressed as nanomole per g wet tissue (nmol/g wet tissue) according to a standard graph which was prepared from the measurements done with a Standard solution (1, 1, 3, 3-tetramethoxypropane). Protein measurements were made at all stages according to the Lowry et al. (1951) method.

Measurement of tissue superoxide dismutase activity

Total (Cu–Zn and Mn) SOD activity was determined according to the method of Sun et al. (1988). The principle of the method is based on the inhibition of NBT reduction by the xanthine–xanthine oxidase system as a superoxide generator. One unit of SOD is defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. SOD activity was expressed as units per milligram protein (U/mg protein).

Glutathione peroxidase activity

GPx activity was measured by the method of Paglia and Valentine (1967). The enzymatic reaction in the tube, which is containing following items: NADPH, reduced glutathione (GSH), sodium azide, and glutathione reductase, was initiated by addition of H_2O_2 and the change in absorbance at 340 nm was monitored by a spectrophotometer.

Histopathologic evaluation

At the end of the surgical procedure, the liver specimens were individually immersed in Bouin's fixative, dehydrated in alcohol and embedded in paraffin. Lobular architecture, presence of inflammation, necrosis and ductular proliferation were investigated. Lobular and portal inflammation, focal hepatocyte necrosis and interface activity were scored as in modified hepatitis activity index of Ishak et al. (1995). Fibrosis was assessed in sections stained with Masson's trichrome. The histopathological fibrosis were grouped as: no fibrosis (scored as 0), portal fibrosis (scored as 1), septal fibrosis (scored as 2), incomplete cirrhosis (scored as 3), and complete cirrhosis (scored as 4). Histopathological examination was carried out by a pathologist who had no prior knowledge of the animal groups.

Immunohistochemistry

The harvested liver tissues were fixed in Bouin's, embedded in paraffin and sectioned at 5 μm thickness. Immunocytochemical reactions were performed according to the ABC technique described by Hsu et al. (1981) The procedure involved the following steps: (1) endogenous peroxidase activity was inhibited by 3% H_2O_2 in distilled water for 30 min; (2) the sections were washed in distilled water for 10 min; (3) non-specific binding of antibodies was blocked by incubation with normal goat serum (DAKO X 0907, Carpinteria, CA) with PBS, diluted 1:4; (4) the sections were incubated with specific mouse monoclonal anti α-SMA antibody (Cat. # MS-113-P, Neomarkers, USA) and specific mouse monoclonal anti- proliferating cell nuclear antigen (PCNA) antibody (Cat. # MS-106-B, Thermo LabVision, USA), diluted 1:50 for 1 h at room temperature; (5) the sections were washed in PBS 3 × 3 min; (6) the sections were incubated with biotinylated anti-mouse IgG (DAKO LSAB 2 Kit); (7) the sections were washed in PBS 3 × 3 min; (8) the sections were incubated with ABC complex (DAKO LSAB 2 Kit); (9) the sections were washed in PBS 3 × 3 min; (10) peroxidase was detected with an aminoethylcarbazole substrate kit (AEC kit; Zymed Laboratories); (11) the sections were washed in tap water for 10 min and then dehydrated; (12) the nuclei were stained with hematoxylin; and (13) the sections were mounted in DAKO paramount. All dilutions and thorough washes between steps were performed using phosphate buffered saline unless otherwise specified. All steps were carried out at room temperature unless otherwise specified.

The number of α-SMA positive HSCs in each specimen was scored using the system developed previously (Schmitt-Graff et al. 1991) with modification. Ten randomly selected septa were scored for each specimen in every experiment as follows: 0 = no positive response; 1 = less than 10% of HSCs; 2 = 11–20% of HSCs; 3 = 21–40% of HSCs; 4 = more than 40% of HSCs.

Quantification of PCNA immunoreactive hepatic cells, including hepatocytes, in each specimen was also scored. Ten randomly selected septa were scored for each specimen in every experiment as follows: 0 = no positive response; 1 = less than 10% of hepatic cells; 2 = 11–20% of hepatic cells; 3 = 21–30% of hepatic cells; 4 = more than 30% of hepatic cells.

Statistical analysis

All statistical analyses were carried out using SPSS statistical software (SPSS for windows, version 11.0). All data were presented in mean (±) standard deviations (S.D.). Differences in measured parameters among the three groups were analyzed with a nonparametric test (Kruskal–Wallis). Dual comparisons between groups exhibiting significant values were evaluated with a Mann–Whitney *U* test. These differences were considered significant when probability was less than 0.05.

Results

Biochemical findings

The application of BDL clearly increased the tissue HP content, MDA levels and decreased the antioxidant enzyme (SOD, GPx) activities. TQ treatment significantly decreased the elevated tissue HP content, and MDA levels and raised the reduced of SOD, and GPx enzymes in the tissues (Fig. 1).

Histopathological findings

The architecture of hepatic lobules of the normal control rats was complete, and there was neither fibroplasia nor inflammatory cell infiltration. Acute biliary obstruction was accompanied by distorsion of liver parenchyma around portal triads when compared with the control rats (Fig. 2a, b). BDL rats showed a loss of hepatic structure, with proliferation of portal and periportal biliary ductules and disorganization of the hepatocyte plates, dilated portal spaces and areas of inflammatory infiltrate. Masson's trichrome revealed an increase of connective tissue in portal and periportal areas. However, in the rats of the BDL group, the lobules of the liver were separated and surrounded by collagen fibers, which resulted in apparent pseudo-lobules. Infiltration of inflammatory cells were also observed (Fig. 2b). Treatment of BDL rats with TQ attenuated alterations in liver histology (Figs. 2c, 3).

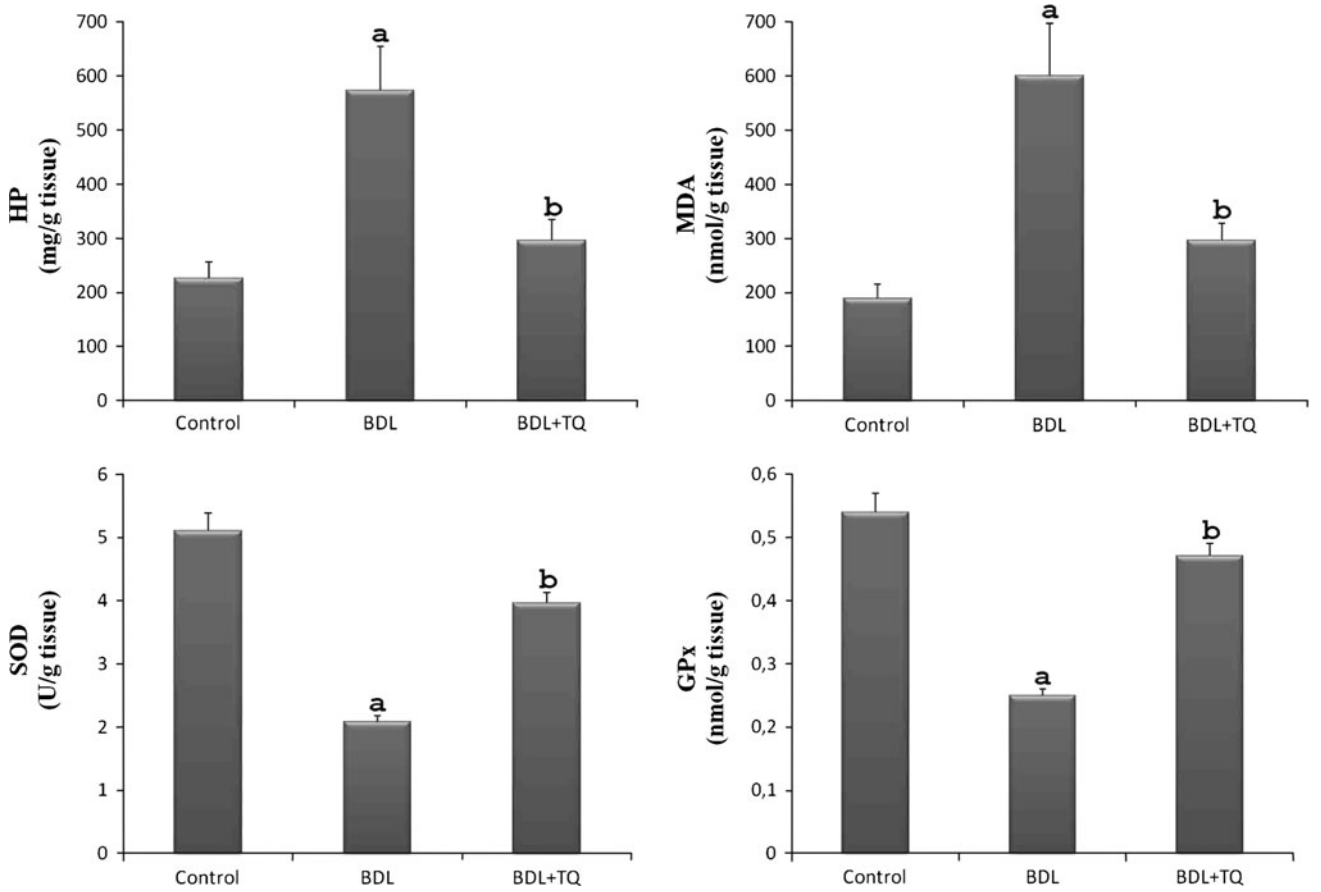

Fig. 1 Tissue HP (mg/g wet tissue) content, MDA levels, SOD and GSH-Px enzyme activities in control, BDL and BDL + TQ groups. Tissue HP and MDA ($P < 0.001$) levels were significantly increased, SOD and GPx ($P < 0.001$) enzyme activities were significantly decreased in BDL treated rats in comparison to sham. TQ treatment significantly ($P < 0.01$) decreased the elevated tissue MDA levels and increased of reduced SOD and GSH-Px ($P < 0.01$) enzyme activities in the liver tissues

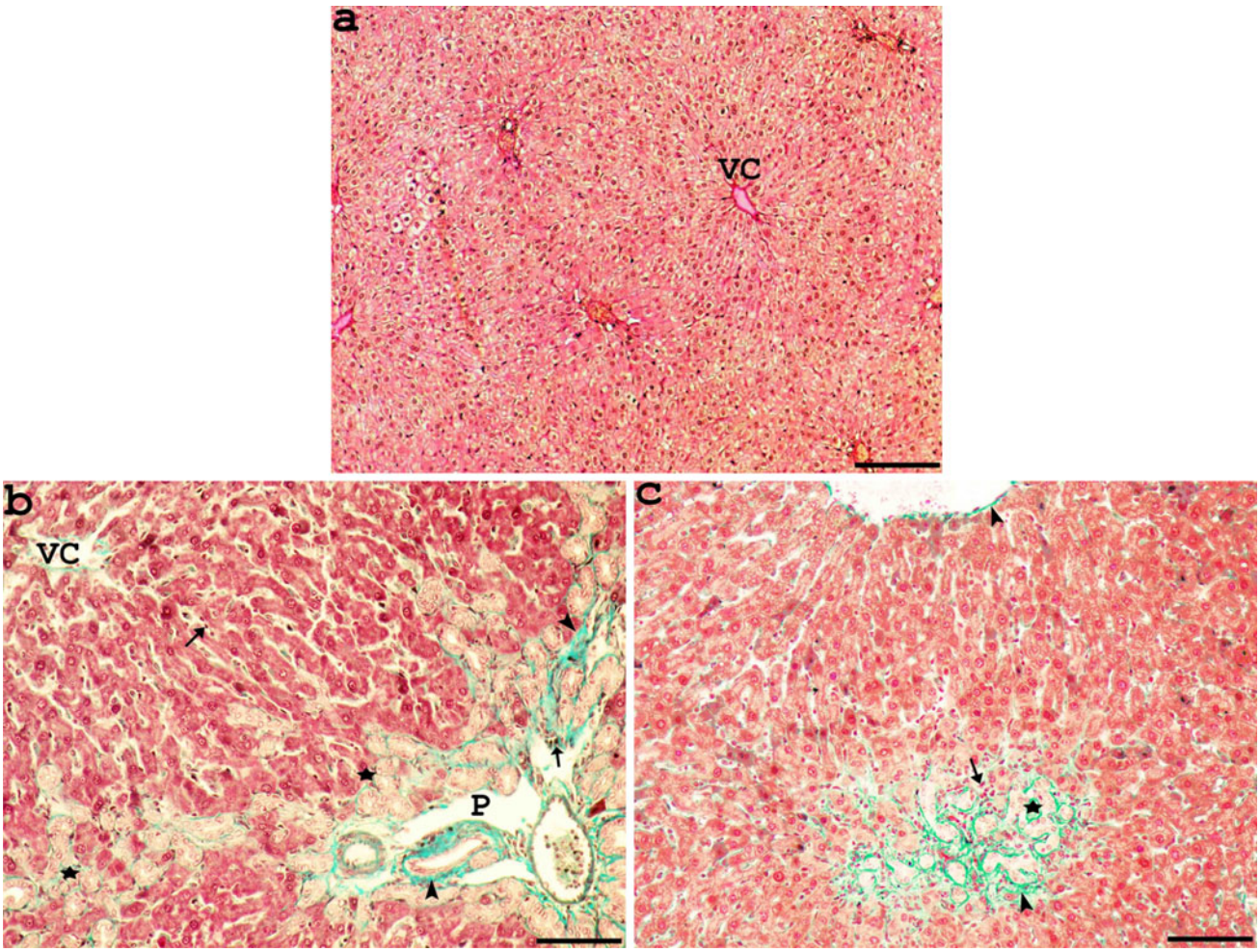

Fig. 2 Light microscopy of liver tissue in different groups. Masson trichrome: **a** in controls, normal liver architecture was seen; **b** after BDL, rat liver showing inflammatory cell infiltration, fibrosis and marked ductular proliferation; **c** TQ treatment reduced the inflammatory cell infiltration, fibrosis and ductular proliferation. (*VC*: vena centralis, *P*: Portal area, *Asterisk*: bile ducts, *Arrowhead*: collagen fibers, *Arrow*: inflammatory cells) (Masson trichrome, *scale bar*, a = 200 μm, b, c = 100 μm)

Immunohistochemical findings

The staining for α-SMA positive cells in the control livers was present only in vascular smooth muscle cells (Fig. 4a). Since α-SMA was expressed in activated HSCs, immunostaining for this protein was used to detect with the development of hepatic fibrosis. The positive stainings for α-SMA were greatly increased especially in vascular smooth muscle cells and sinusoids and also in the cells of portal ducts, fibrotic septa, perisinuses and around the proliferated bile ducts (Fig. 4b). The α-SMA positive cells in the BDL group were observed to be reduced with the TQ treatment (Figs. 4c, 5).

In control group, a few PCNA positive cells were observed in the hepatocytes (Fig. 6a). After BDL, the number of PCNA positive cells was markedly increased in the hepatocytes (Fig. 6b). Treatment of TQ significantly reduced the number of PCNA positive cells (Figs. 6c, 7).

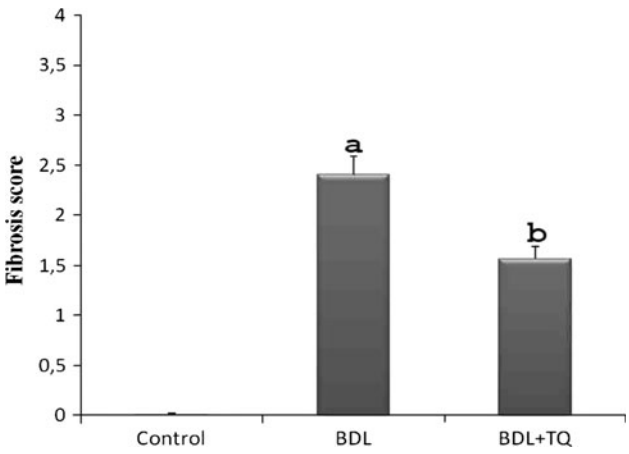

Fig. 3 Comparison of fibrosis in control, BDL and BDL + TQ groups. Fibrosis score ($P < 0.0001$) were significantly increased in BDL treated rats in comparison to control. TQ treatment significantly ($P < 0.01$) decreased the fibrosis score in the liver tissues

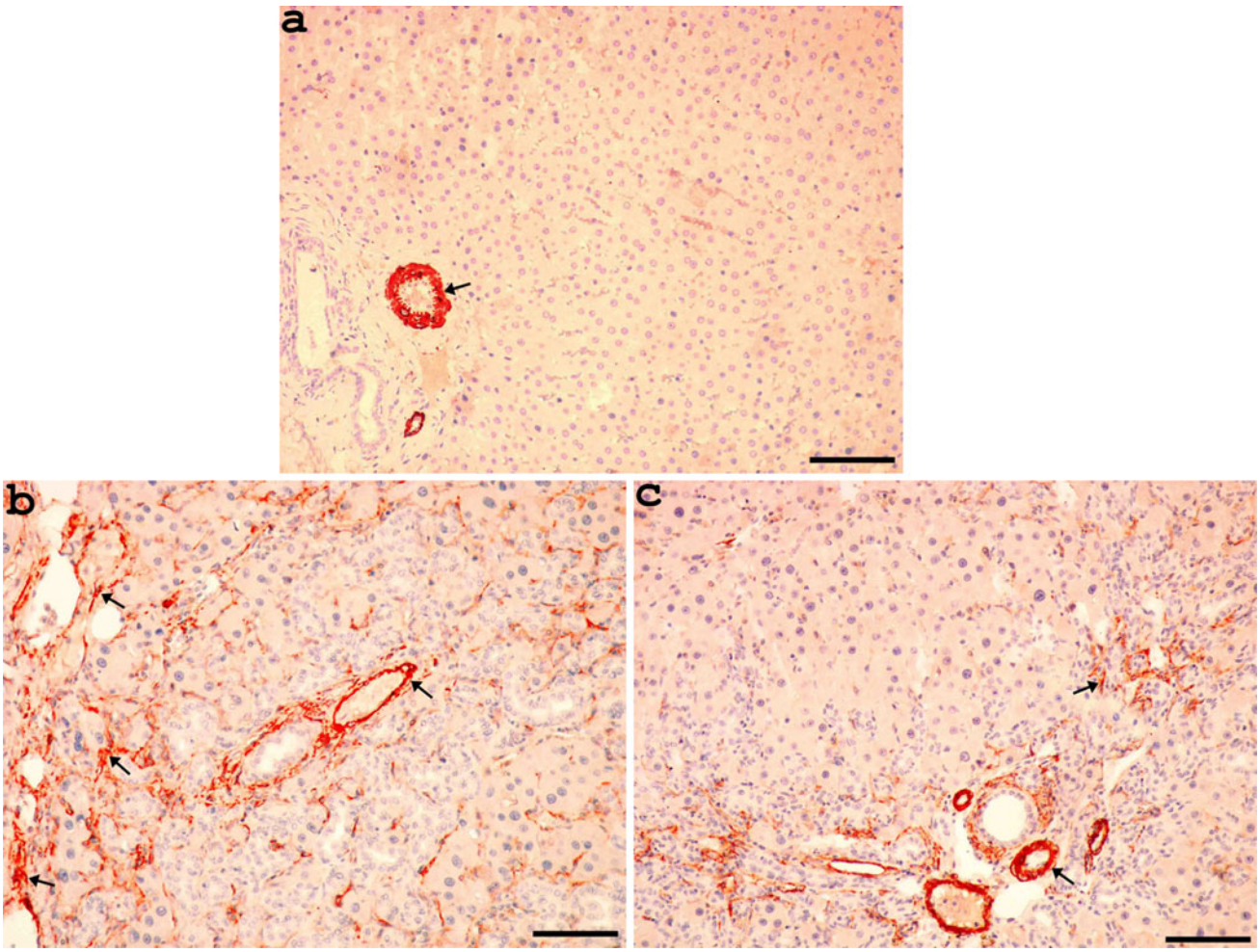

Fig. 4 Light microscopy of liver tissue in different groups. α-SMA: **a** in control, the staining for α-SMA positive cells was present only in vascular smooth muscle cells. **b** After BDL, the positive stainings for α-SMA were greatly increased especially in vascular smooth muscle cells and sinusoids and also in the cells of portal ducts, fibrotic septa, perisinuses and around the proliferated bile ducts. **c** The α-SMA positive cells reduced with the TQ treatment (*VC*: vena centralis, *Arrow*: α-SMA positive cells), (Immunoperoxidase, haematoxylin counterstain and *scale bar*, 100 μm)

Discussion

Cholestasis is characterized by an abnormal accumulation of bile acids, which is caused by defectiveness in the process of bile acid transport. It is the main feature of several chronically progressive liver disorders such as biliary atresia, primary biliary cirrhosis, and primary sclerosing cholangitis. The primary event of cholestasis has been implicated in the later development of hepatocellular injury, progressive hepatic fibrogenesis, cirrhosis, and death from liver failure (Guicciardi and Gores 2002).

In concordance with previos studies, bile duct obstruction induced an intense oxidative stress with depletion of different enzymes with antioxidant properties. In addition to oxidative stress and lipid peroxidation, virtually not only cholestasis but all liver diseases also are associated with a dense inflammatory response seen in our study. The present

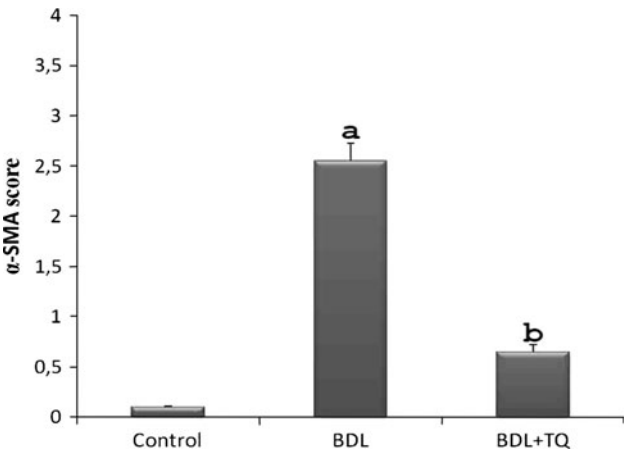

Fig. 5 Comparison of α-SMA in control, BDL and BDL + TQ groups. α-SMA score ($P < 0.0001$) were significantly increased in BDL treated rats in comparison to control. TQ treatment significantly ($P < 0.001$) decreased the fibrosis score in the liver tissues

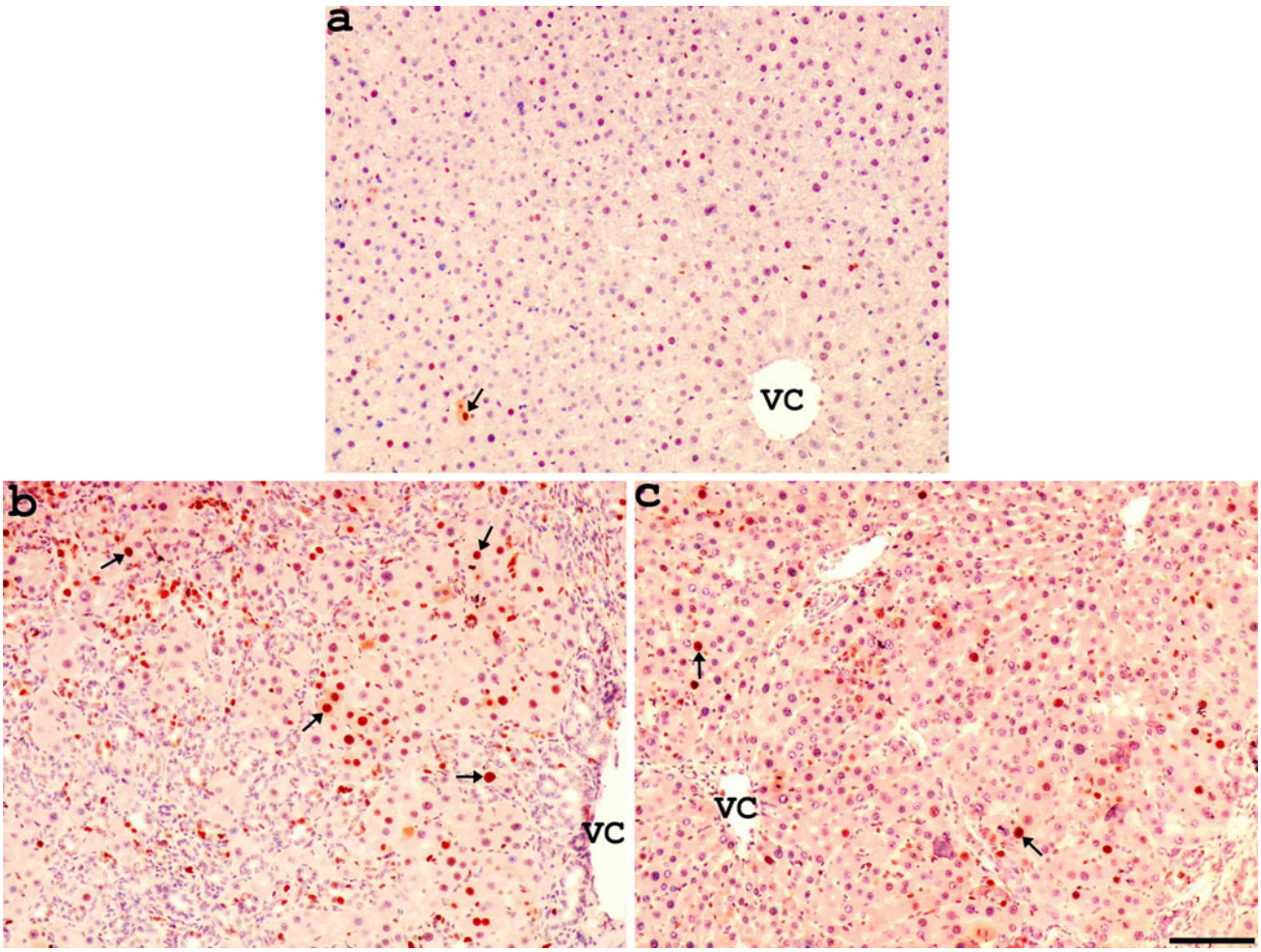

Fig. 6 Light microscopy of liver tissue in different groups. PCNA: **a** in control group, a few PCNA positive cells were observed in the hepatocytes; **b** after BDL, the number of PCNA positive cells was markedly increased in the hepatocytes; **c** treatment of TQ significantly reduced the number of PCNA positive cells (*VC*: vena centralis, *Arrow*: PCNA positive cells), (Immunoperoxidase and haematoxylin counterstain, *scale bar*, 100 μm)

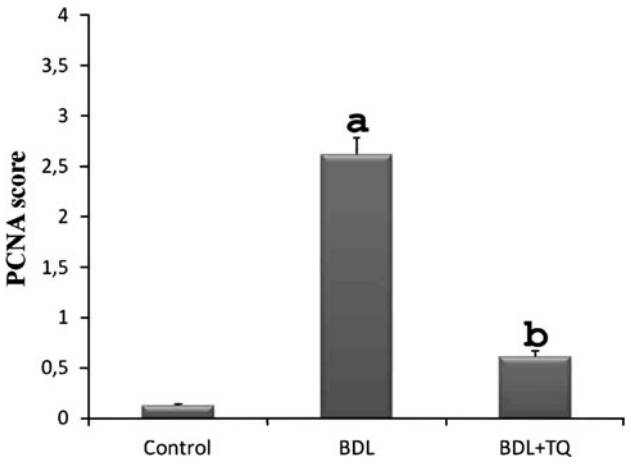

Fig. 7 Comparison of PCNA in control, BDL and BDL + TQ groups. PCNA score ($P < 0.0001$) were significantly increased in BDL treated rats in comparison to control. TQ treatment significantly ($P < 0.001$) decreased the fibrosis score in the liver tissues

study also showed that TQ was able to recover oxidant status, inflammatory response, and reduce liver injury observed in experimental extrahepatic cholestasis. Biliary obstruction is associated with an intense state of oxidative stress. It has previously shown that an important reduction of antioxidant defenses and exacerbation of lipid peroxidation in liver exist during extrahepatic BDL in rats (Tomur et al. 2011; Singh et al. 1992; Krahenbuhl et al. 1995; Parola et al. 1996; Alptekin et al. 1997; Tsai et al. 1998). In this study, MDA, which is the end product of oxidative injury and an indicator of lipid peroxidation, rapidly increased in the hepatic tissues in the BDL rats. The MDA levels were decreased significantly after TQ treatment. We also observed that the activities of SOD and GPx were significantly decreased in liver tissues 14 days after BDL when compared with control animals. TQ treatment markedly increased the hepatic SOD and GPx activities in

BDL rats. These data suggest that the protective effects of TQ are associated with the abated production of hepatic oxidative products and the improved endogenous antioxidant potential.

Obstruction of the biliary tree by BDL causes cholestasis. Persistent cholestasis induces bile duct proliferation and dilation, attracts lowgrade inflammatory infiltration, activates matrix-producing cells, and then leads to periportal and perineoductular fibrosis (Kanter 2010; Desmet et al. 1995). In our study, we found both a preventive effect and a therapeutic effect of TQ on BDL-induced hepatic fibrosis. The preventive effect was examined by 14 days' oral administration of TQ starting 3 days prior BDL, and the therapeutic effect was examined by 14 days' oral administration of TQ immediately after BDL. In this study, the most novel and relevant finding was that TQ supplementation was accompanied by the alleviation of bile duct proliferation and ductular reaction in this model and TQ was also able to reduce newly formed bile ducts. Since the proliferation of bile ducts is an early event in cholestasis-related changes, the attenuation of hepatic injury and fibrosis in BDL rats by TQ might be associated with alleviation of ductular reaction.

HSCs (previous called Ito cell, fat-storing or perisinusoidal cells) are fat-storing perisinusoidal cells anda re important cells in hepatic fibrosis. HSCs are activated during liver cell injury, including acute and chronic liver disease and then white blood-cell chemoattractant releases the various cytokines and oxygen free radicals. Also activated stellate cells supply the type 1 collagen and extracellular matrix and these materials were accumulated in the space of Disse. These processes are the beginning of hepatic fibrosis. α-SMA is present in smooth muscle cells near the biliary structure and is a stained cytoskelatal structure in the HSCs. The degree of α-SMA stain represent the extension of fibrosis, indirectly (Pinzani 1995; Lee 1995). In a study, numerous a-SMA positive cells were observed within the parenchyma of BDL rats. In BDL-QE rats, fibrosis was greatly reduced (Tieppo et al. 2009). Likewise Tieppo et al. and Aksu et al.'s (2009,2010) studies, we have found in our study that the positive stainings for α-SMA were greatly increased especially in vascular smooth muscle cells and sinusoids and also in the cells of portal ducts, fibrotic septa, perisinuses and around the proliferated bile ducts. The α-SMA positive cells in the BDL group were observed to be reduced with the TQ treatment.

Cellular proliferation is a compensatory pathological reaction to hepatic injury (apoptosis and necrosis), which can be evaluated by the detection of cell mitosis or proliferation related markers (Colozza et al. 2005). So far, the report of hepatocellular proliferation in BDL model is stil missing. In a study, Wen et al. (2011) observed numerous cells in the mitotic phase and double-nuclei cells in BDL mouse livers, indicating increased hepatocytic proliferation after cholestatic injury. On the other hand, the expression level of PCNA, a molecular marker highly associated with cell cycle and proliferation, was found to be significantly increased in BDL (Bhattacharyya et al. 2008; Tomur et al. 2011). Likewise, the above mentioned studies, we have found in our study that after BDL, the number of PCNA positive cells was markedly increased in the hepatocytes. Treatment of TQ significantly reduced the number of PCNA positive cells.

Our results reveal that TQ is highly effective in both preventing and reversing cirrhosis. In conclusion, we provide here new evidence that TQ exerts a preventive effect and a therapeutic effect on cholestasis-induced hepatic fibrosis through inhibition of the activation of HSCs, decreased lipid peroxidation and bile duct proliferation, and prevention of the oxidative stress. All these findings suggest that TQ may be a promising new therapeutic agent for cholestatic liver injury.

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