ORIGINAL PAPER

The efficiency of CAPE on retardation of hepatic fibrosis in biliary obstructed rats

Ahmet Tomur · Mehmet Kanter · Ahmet Gurel · Mustafa Erboga

Abstract The aim of this study was to evaluate the possible protective effects of caffeic acid phenethyl ester (CAPE) against cholestatic oxidative stress and liver damage in the common bile duct ligated rats. A total of 18 male Sprague–Dawley rats were divided into three groups: control, bile duct ligation (BDL) and BDL + received CAPE; each group contain 6 animals. The rats in CAPE treated groups were given CAPE (10 μmol/kg) once a day intraperitoneally (i.p) for 2 weeks starting just after BDL operation. The changes demonstrating the bile duct proliferation and fibrosis in expanded portal tracts include the extension of proliferated bile ducts into lobules, inflammatory cell infiltration into the widened portal areas were observed in BDL group. Treatment of BDL with CAPE attenuated alterations in liver histology. The proliferating cell nuclear antigen and the activity of TUNEL in the BDL were observed to be reduced with the QE treatment. 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. CAPE treatment significantly decreased the elevated tissue HP content, and MDA levels and raised the reduced of SOD, and GPx enzymes in the tissues. The data indicate that CAPE attenuates BDL-induced cholestatic liver injury, bile duct proliferation, and fibrosis. The hepatoprotective effect of CAPE is associated with antioxidative potential.

Keywords CAPE · Immunohistochemistry · TUNEL · Liver · Bile duct ligation

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).

Bile duct ligation (BDL) induces a type of liver fibrosis, which etiologically and pathogenitically resembles the biliary fibrosis in the human beings. 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 and eventually liver fibrosis (Kountouras et al. 1984). Although the mechanisms of cholestasis-induced liver fibrosis are unclear, obstruction of bile flow causes the accumulation and retention of hydrophobic bile salts in the liver, which are toxicants to a

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number of cells, including hepatocytes and ductal biliary epithelial cells (Benedetti et al. 1997).

Caffeic acid phenethyl ester (CAPE) is an active component of honeybee propolis extracts and has been used for many years as a folk medicine. It has anti-inflammatory, immunomodulatory, antiproliferative, and antioxidant properties and have been shown to inhibit lipo-oxygenase activities as well as to suppress lipid peroxidation (Hepsen et al. 1997; Ilhan et al. 1999; Koltuksuz et al. 1999). Also, it has been previously shown that intraperitoneal administration of caffeic acid phenethyl ester in bile duct-ligated rats reduces intestinal oxidative (Ara et al. 2006). In the present study, we examined whether CAPE reduces biliary obstruction-induced liver injury by prevention of the oxidative stres, inflammation and fibrosis in rats. To assess the effect of CAPE 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, 18 healthy male Sprague–Dawley rats, weighing 250–300 g and averaging 12 weeks old were utilized. Food and tap water were available ad libitum. In the windowless animal quarter automatic temperature $(21 \pm 1°C)$ 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. CAPE (10 µmol/kg) was obtained from Sigma, St. Louis, MO, USA. Control group were injected with the same volume of saline as the BDL groups received.

Experimental groups

Eighteen Sprague–Dawley adult rats are enrolled in this study, and divided into three groups as control, bile duct ligation and BDL + CAPE (10 µmol/kg/day i.p.); each group contain six animals.

Experimental protocols

The rats were anesthetized with ketamine (50 mg/kg) and xylazine (5 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 4/0 silk and transsection of the BD in the supraduodental part between the lower-most tributary of the bile duct and the uppermost tributary of the pancreatic duct. In our study, ready-made CAPE was dissolved in absolute ethanol and further dilutions were made in saline. The rats in CAPE treated groups were given CAPE (10 µmol/kg/day i.p.) once a day for 2 weeks starting just after BDL operation. Control and BDL untreated rats were also given with the same volume of saline as the BDL treated animals that received CAPE. After 2 weeks of treatment, the animals were decapitated. Liver tissue samples were obtained for histopathological and biochemical investigation.

Histopathologic evaluation

At the end of the surgical procedure, the liver specimens were individually immersed in 10% neutral buffered formalin solution, 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). Ductular proliferation scores were grouped as no ductular proliferation (scored as 0), mild ductular proliferation restricted in the portal area (scored as 1), and marked ductular proliferation in the porta-portal bridges (scored as 2). 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 10% neutral buffered formalin solution, 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 × 0907, Carpinteria, CA) with PBS, diluted 1:4; (4) the sections were incubated with specific mouse monoclonal anti-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.

TUNEL assay

The TUNEL method, which detects fragmentation of DNA in the nucleus during apoptotic cell death in situ, was employed using an apoptosis detection kit (TdT-Fragel™ DNA Fragmentation Detection Kit, Cat. No. QIA33, Calbiochem, USA). All reagents listed below are from the kit and were prepared following the manufacturer's instructions. Five-μm-thick liver sections were deparaffinized in xylene and rehydrated through a graded ethanol series as described previously. They were then incubated with 20 mg/ml proteinase K for 20 min and rinsed in TBS. Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide. Sections were then incubated with equilibration buffer for 10–30 min and then TdT-enzyme, in a humidified atmosphere at 37°C, for 90 min. They were subsequently put into pre-warmed working strength stop/wash buffer at room temperature for 10 min and incubated with blocking buffer for 30 min. Each step was separated by thorough washes in TBS. Labelling was revealed using DAB, counter staining was performed using Methyl green, and sections were dehydrated, cleared and mounted.

The positive staining of PCNA, and TUNEL cell numbers were scored in a semiquantitative manner in order to determine the differences between the control group and the experimental groups in the distribution patterns of intensity of immunolabeling of lung tissue. The numbers of the positive staining were recorded as weak (±), mild (+), moderate (++), strong (+++) and very strong (++++). These analyses were performed in two sections from each animal at 400× magnification in at least ten different regions for each section.

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's et al. (1951) method.

Glutathione peroxidase activity

Glutathione peroxidase (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. 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).

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

Histopathologic findings

Animals of the control group did not present any histological alterations (Fig. 1a). The liver specimens of the BDL group showed prominent lobular and portal changes. In the BDL group, the histopathological changes including fibrosis and extension of proliferated bile ducts in expanded portal tracts include the into lobules, and periportal and parenchymal inflammatory cell infiltration were prominent (Fig. 1b). In the BDL + CAPE group, histopathological evidence of parenchymatous injury was markedly reduced. Portal fibrosis and bile duct proliferation were still prominent (Fig. 1c; Table 1).

Immunohistochemical findings

In control group, a few PCNA positive cells were observed in the hepatocytes (Fig. 2a). After BDL, the number of PCNA positive cells was markedly increased in the hepatocytes (Fig. 2b). Treatment of CAPE significantly reduced the number of PCNA positive cells (Fig. 2c; Table 2).

TUNEL findings

The number of TUNEL-positive cells in the control group was negligible (Fig. 3a). When liver sections were TUNEL stained, there was a clear increase in the number of positive cells in the BDL treated rats in the liver parenchyma
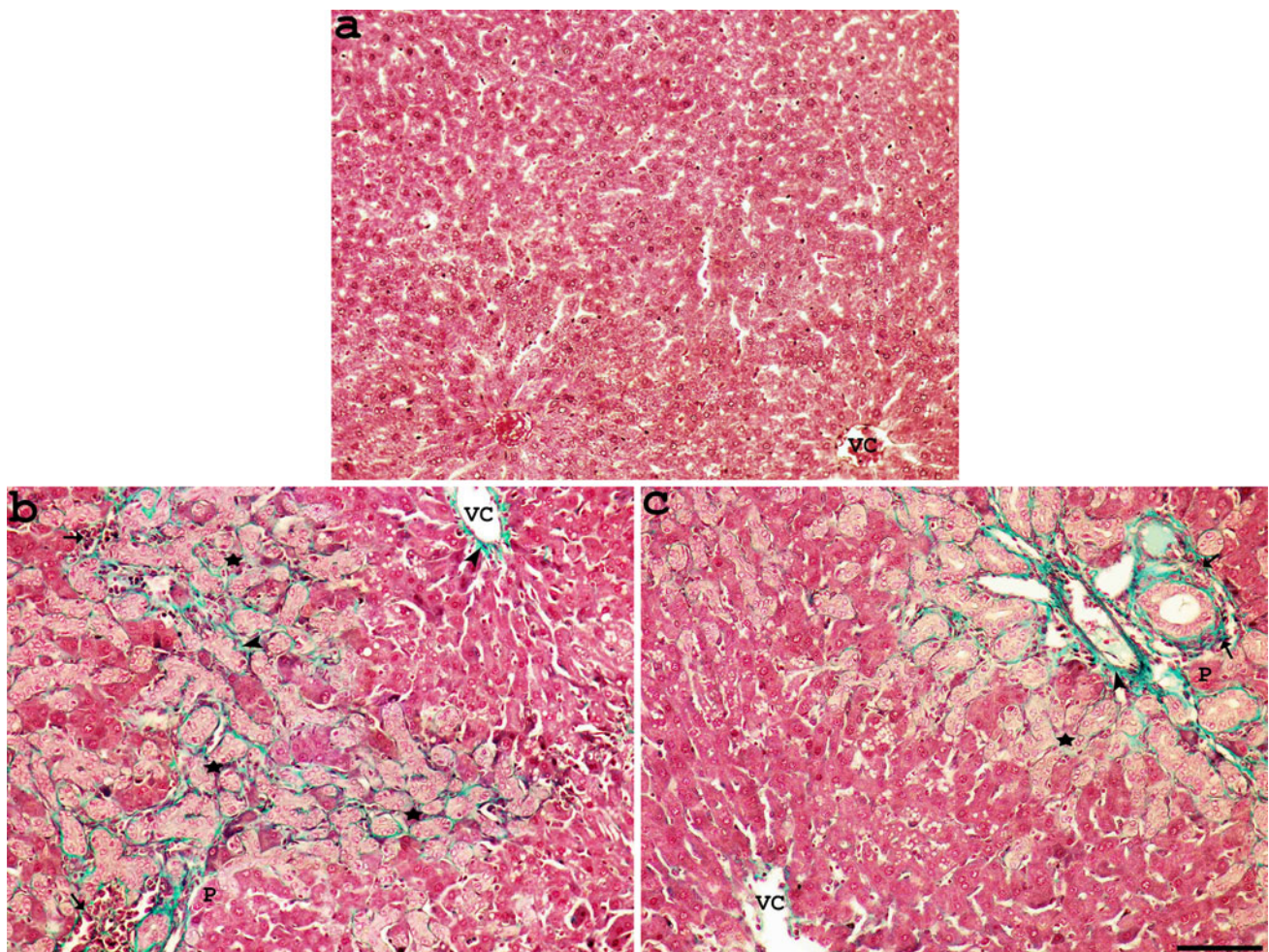

Fig. 1 Light microscopy of liver tissue in different groups. *Masson trichrome*: **a** In controls, normal liver architecture was seen; **b** After BDL, rat liver showing inflammatory cell infiltration, fibrosis and marked ductular proliferation; **c** CAPE treatment reduced the inflammatory cell infiltration, fibrosis and ductular proliferation. (*VC* vena centralis, *P* Portal area, *Asterisk* bile ducts, *Arrowhead* collagen fibers, *Arrow*: inflammatory cells) (Masson trichrome, *scale bar* 100 μm)

Table 1 Comparison of fibrozis, and ductular proliferation in control, BDL and BDL + CAPE groups

	Control	BDL	BDL + CAPE
Fibrosis	0.00 ± 0.00	2.33 ± 0.17[a]	1.51 ± 0.11[b]
Ductular proliferation	0.00 ± 0.00	1.91 ± 0.14[c]	0.79 ± 0.06[d]

Kruskal–Wallis test was used for statistical analysis. Values are expressed as means ± SD, $n = 6$ for each group

[a] $P < 0.001$ compared with control

[b] $P < 0.05$ compared with BDL

[c] $P < 0.0001$ compared with control

[d] $P < 0.01$ compared with BDL

(Fig. 3b). Treatment of CAPE markedly reduced the reactivity and the number of TUNEL positive cells (Fig. 3c; Table 2).

Biochemical findings

The application of BDL clearly increased the tissue HP content, MDA levels and decreased the antioxidant enzyme (SOD, GPx) activities. CAPE treatment significantly decreased the elevated tissue HP content, and MDA levels and raised the reduced of SOD, and GPx enzymes in the tissues (Table 3).

Discussion

Obstructive cholestasis in the liver occurs in various diseases in children, such as biliary atresia, gallstones, primary biliary cirrhosis, and sclerosing cholangitis. Biliary obstruction causes hepatocellular injury and leads to progressive hepatic fibrogenesis (Maher and McGuire 1990). In fibrogenesis, the rate of hepatic collagen synthesis
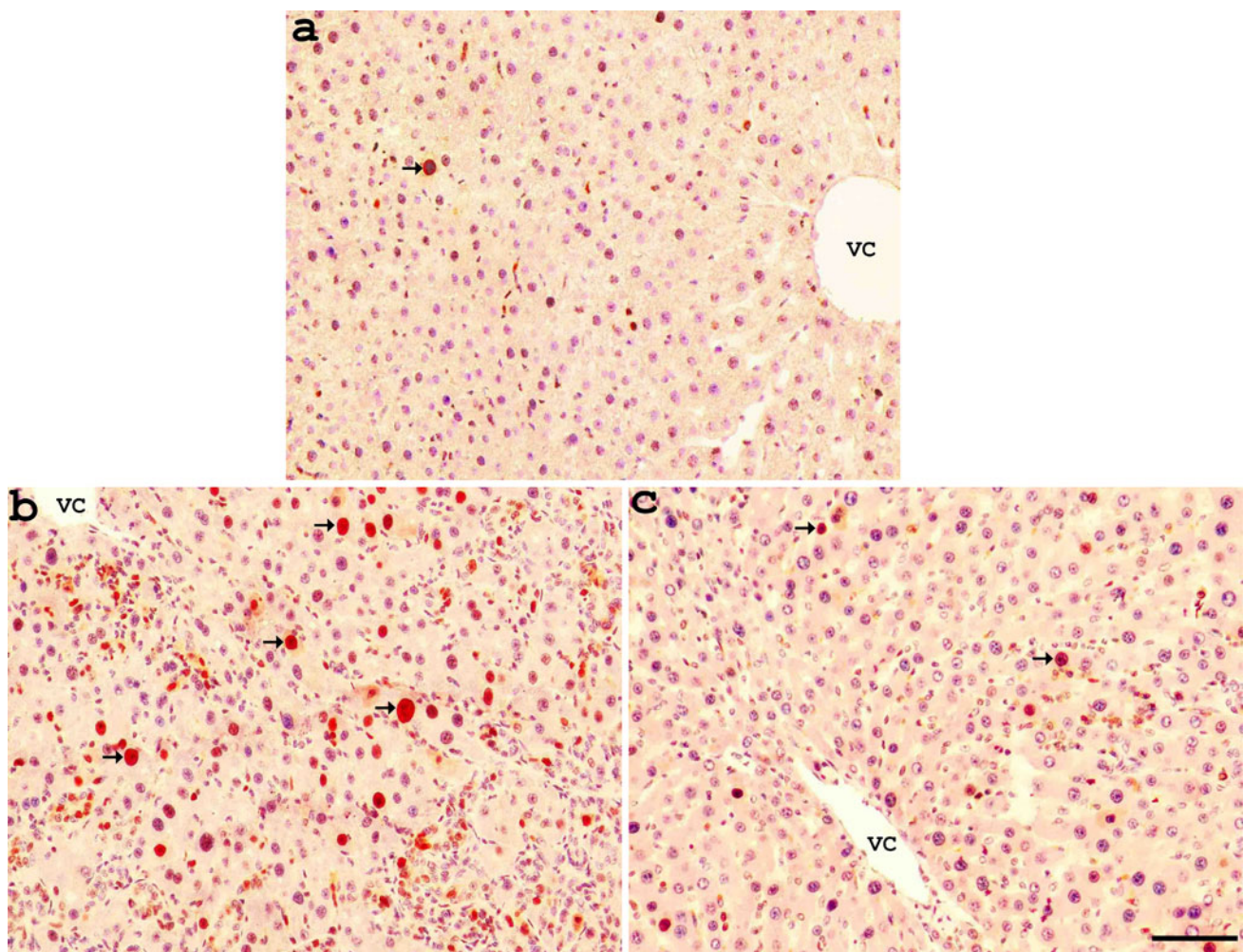

Fig. 2 Light microscopy of liver tissue in different groups. *PCNA*: **a** In control group, a few PCNA positive cells were observed in the hepatocytes; **b** After BDL, the number of PCNA positive cells was markedly increased in the hepatocytes; **c** Treatment of CAPE significantly reduced the number of PCNA positive cells (*VC* vena centralis, *Arrow* PCNA positive cells), (Immunoperoxidase and haematoxylin counterstain, *scale bar* 50 μm)

Table 2 Semiquantitative comparison of the PCNA and TUNEL cell numbers in liver tissues for each group control, BDL and BDL + CAPE groups (*n*: 6 for each group)

	Control	BDL	BDL + CAPE
PCNA	+	++++	++
TUNEL	±	+++	++

The numbers of the positive staining was recorded as absent (−), a few (±), few (+), medium (++), high (+++) and very high (++++)

increases (Morris et al. 1975), which is also found to be stimulated in hepatocytes exposed to free radicals (Liu et al. 2001).

Extrahepatic obstruction of bile outflow induces the retention of biliary constituents and triggers important pathophysiological changes in cholangiocytes that promote the rapid appearance of significant liver fibrosis (Strazzabosco et al. 2005). Bile duct ligation is associated with the development of oxidant injury, hepatic fibrosis, biliary cirrhosis, portal hypertension, and a hyperdynamic circulation that is a dynamic process implying different rates of progression or regression (Marley et al. 1999). It has been reported that BDL reduces antioxidant defenses and increases free radical formation (Karaman et al. 2006). Some studies have suggested the role of free radicals in the modulation of hepatic fibrogenesis, either directly or through lipid peroxidation (Parola et al. 1996). Hepatic fibrosis, which is usually initiated by hepatocyte damage, leads to recruitment of inflammatory cells and platelets, activation of Kupffer cells, and subsequent release of cytokines and growth factors (Kullak-Ublick and Meier 2000). These factors are probably related with the inflammatory processes and oxygen free radicals, which are known to cause tissue fibrosis (Strazzabosco et al. 2005; Karaman et al. 2006).
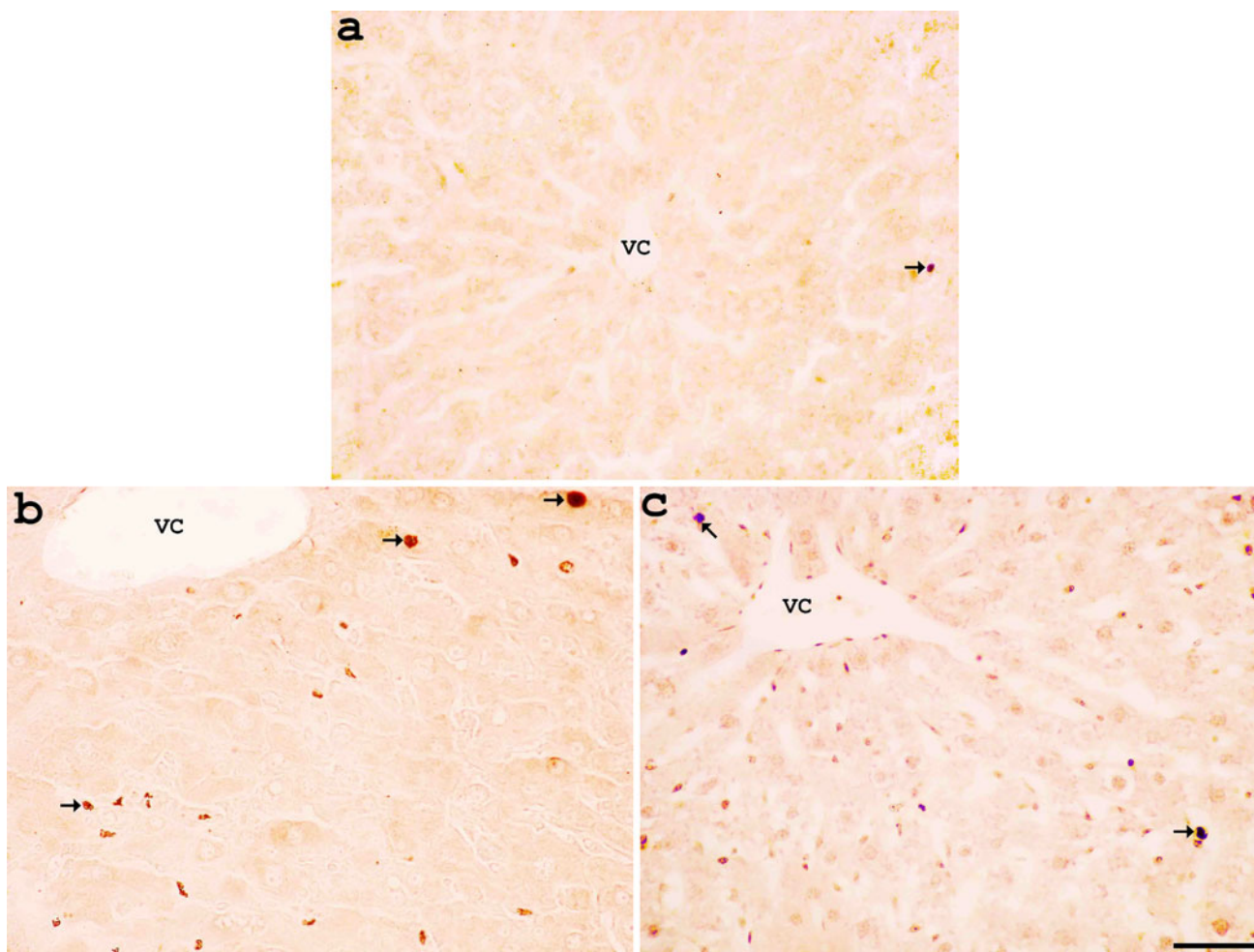

Fig. 3 Light microscopy of liver tissue in different groups. *TUNEL:* **a** In control group, a few TUNEL-positive cells are observed in the liver parenchyma; **b** After BDL, there was a clear increase in the number of positive cells in the liver parenchyma; **c** Treatment of CAPE markedly reduced the number of TUNEL positive cells (*VC* vena centralis, *Arrow* TUNEL-positive hepatocytes), (TUNEL, *scale bar* 50 µm)

Table 3 Tissue HP (mg/g wet tissue), MDA (nmol/g wet tissue), SOD (U/g protein) and GPx (nmol/g wet tissue) levels in groups ($n = 6$)

	Control	BDL	BDL + CAPE
HP	230.82 ± 31.52	579.21 ± 83.37[a]	301.17 ± 42.54[b]
MDA	191.71 ± 28.5	605.16 ± 98.0[a]	298.8 ± 34.04[b]
SOD	5.52 ± 0.30	2.53 ± 0.11[a]	4.36 ± 0.19[b]
GPx	0.57 ± 0.04	0.26 ± 0.01[a]	0.49 ± 0.03[b]

Kruskal–Wallis test was used for statistical analysis. Values are expressed as means ± SD, $n = 6$ for each group

[a] $P < 0.001$ compared with control

[b] $P < 0.05$ compared with BDL

In this study, 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. Our data are corroborated by previous studies reported by other investigators on BDL-induced hepatic fibrosis in animals. In this study we demonstrated the histologically that CAPE significantly reduced fibrosis in BDL rats.

Cholestasis results in the accumulation of hydrophobic bile acids, which may be responsible for hepatocellular apoptosis and necrosis, with apoptosis be ing a nearly ubiquitous response of the liver to injury (Galle et al. 1995; Kanter 2010). In addition to apoptosis, virtually all liver diseases are associated with an inflammatory response. The interplay between hepatocyte inflammation and apoptosis is complex. Concomitant with apoptosis and necrosis, liver cell proliferation, as a pathological compensation to injury, is common, and the pathogenesis is not well understood. Hence, a detailed description of these pathogenic changes is important for the interpretation of the available data as well as for the design of future studies.

BDL, markedly increased the percentage of TUNEL-positive cells, especially in the ductular proliferation areas and this increase was significantly suppressed by fluvastatin treatment. Several factors may influence the onset of hepatocyte cell death. Retention and accumulation of toxic hydrophobic bile salts within hepatocyte cause hepatocyte toxicity by inducing apoptosis (Corsini et al. 1993; Ogata et al. 2002). In our study, the number of TUNEL-positive cells in the control group was negligible. When liver sections were TUNEL stained, there was a clear increase in the number of positive cells in the BDL treated rats in the liver parenchyma. Treatment of CAPE markedly reduced the reactivity and the number of TUNEL positive cells.

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 bile duct ligation model is stil missing. In a study, Wen et al. (2010) 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 (Bhattacharyya et al. 2008), was found to be significantly increased in BDL mice. 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. In previous study (Mancinelli et al. 2009), coupled with increased apoptosis, chronic administration of CAPE to BDL rats decreased the number of PCNA positive cholangiocytes in liver sections. Our findings are consistent with the results of Mancinelli et al. (2009) concerning the effects of CAPE to BDL rats.

It has been shown that oxidative stres associated with lipid peroxidation is involved in the development of liver injury in the cholestatic liver disease. Pyrrolidine dithiocarbamate given intraperitoneally to the rats with BDL reduced the increases in hepatic MDA concentrations, and restored the decreases levels of hepatic antioxidant enzyme levels. These findings suggest that intraperitoneally administered pyrrolidine dithiocarbamate could exert a preventive effect on an enhancement of oxidative stress and hepatic lipid peroxidation during the progression of cholestatic liver injury in rats with BDL (Demirbilek et al. 2006). In the present study, the application of BDL clearly increased the tissue MDA levels and decreased the antioxidant enzyme (SOD, GPx) activities. CAPE treatment significantly decreased the elevated tissue MDA levels and raised the reduced of SOD, and GPx enzymes in the tissues.

In conclusion, these findings suggested that CAPE can reduce the hepatic damage in extrahepatic cholestasis by prevention of the oxidative stress, and the inflammatory process. All these findings suggest that CAPE may be a promising new therapeutic agent for cholestatic liver injury.

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