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Basic & Translational

Treatment with milk thistle extract (*Silybum marianum*), ursodeoxycholic acid, or their combination attenuates cholestatic liver injury in rats: Role of the hepatic stem cells

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Original Article

ABSTRACT

Background/Aims: Cholestasis, which results in hepatic cell death, fibrosis, cirrhosis, and eventually liver failure, is associated with oxidative stress. The aim of this study was to evaluate the effects of milk thistle (MT, *Silybum marianum*) and ursodeoxycholic acid (UDCA) or their combination on the activation of hepatic stem cells and on the severity of cholestasis liver injury in rats.

Materials and Methods: Under anesthesia, bile ducts of female Sprague Dawley rats were ligated (BDL) or had sham operation. BDL rats were administered saline, UDCA (15 mg/kg/d), MT (600 mg/kg/d), or UDCA+MT by gavage for 10 days. On the 11th day, rats were sacrificed and blood and liver samples were obtained. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), hepatic malondialdehyde (MDA) levels, and myeloperoxidase (MPO) activity were measured. Hepatic injury, a-smooth muscle actin expression, and stem cell markers c-kit, c-Myc, Oct3/4, and SSEA-1 were histologically determined.

Results: Histological scores, serum ALT, and hepatic MDA levels were higher in BDL group than in the sham rats, while all treatments significantly reduced these levels. The reduction in ALT was significantly greater in UCDA+MT-treated group than in other treatment groups. c-Kit, c-Myc, Oct3/4, and SSEA-1 were increased in saline-treated BDL group with respect to sham-operated control group, and these markers were significantly reduced in all treatment groups.

Conclusion: In addition to a modulatory effect on the stem cell-induced regenerative response of the liver, UDCA, MT, and their combination demonstrated similar anti-inflammatory and antiproliferative effects on cholestasis-induced hepatic injury.

Keywords: Bile duct ligation, milk thistle (*Silybum marianum*), oxidative stress, primary biliary cirrhosis, stem cell factor, ursodeoxycholic acid

INTRODUCTION

Chronic liver diseases affecting about 600 million people around the World represent a major health issue with high mortality and morbidity (1). Cholestasis, a common cause of chronic liver disease, is characterized by impaired bile secretion that can result from a functional defect in bile formation by the hepatocytes or from an impaired bile flow via the ducts. Oxidative stress and inflammation induced by cholestasis may lead to apoptosis, fibrosis, and eventually to cirrhosis and hepatic failure (2). Ursodeoxycholic acid (UDCA), a dihydroxy bile acid, is believed to replace endogenous bile acids and decrease the cytotoxicity associated with primary biliary cirrho-

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sis (PBC). In order to replace toxic bile acids with non-toxic, UDCA was suggested to be effective in the treatment of cholestatic diseases, by increasing the secretion capacity of hepatocytes and modulating the immunoregulatory processes. In accordance with these observations, Food and Drug Administration in U.S. has approved the use of UDCA in the treatment of intrahepatic cholestasis, PBC, and other cholestatic syndromes. Despite these reported therapeutic effects, the use of UDCA during the early stages of biliary obstruction is limited. Although various combinations of immunomodulating drugs with UDCA have been investigated in several studies, these combinations have shown a limited efficacy or have totally failed to be effective. Therefore, there is still a need to develop efficacious treatments for chronic cholestatic diseases (3,4).

Milk thistle or *Silybum marianum* contains the potent substance named silymarin, which is a traditional herbal remedy that had been widely used in liver diseases for nearly 2000 years. Studies have presented that silymarin acts against oxidative injury, fibrosis, inflammation, tumor formation, atherosclerosis, whereas it plays a role in stabilizing membranes, modulating immune functions, and regenerating hepatic tissue. MT decreases the formation of free radicals, inhibits lipid peroxidation, and acts as an antioxidant. Furthermore, MT exerts antifibrotic effects by inhibiting the binding of toxic agents to membrane receptors of hepatocytes (5,6). It was reported that treatment with silymarin (400 mg/d) in combination with UDCA (450 mg/d) provided an enhanced improvement in the hepatic functions of 30 patients with chronic ethylic hepatopathy (7).

In recent years, activation, distribution, and in vivo roles of hepatic stem cells have been extensively identified, and the hepatic stem cells were suggested to play an essential role in liver regeneration. Upon hepatic injury, the regenerative response of proliferating mature hepatocytes is remarkable, with the contribution of liver progenitor cells to cellular turnover and regeneration. There is increasing strong evidence indicating that oval cells are the progeny of liver stem cell compartment, and following the injury, initially oval cells with ovoid nuclei and high nuclear/cytoplasmic ratios appear in the small portal zone (1,8). It has been suggested that a novel ligand/receptor system, namely, stem cell factor and c-kit system, activates hepatic stem cells during the early phase. Although the impact of stem cells on liver regeneration in humans has been controversial, it was demonstrated in rats with hepatic damage that stem cell factor and c-kit increase along with stem (oval) cell activation (9). On the basis of the aforementioned studies, we aimed to investigate whether MT or UDCA or their combination has a therapeutic impact on the cholestatic liver injury in rats and to evaluate the involvement of hepatic stem cell activation.

MATERIALS AND METHODS

Animals

Adult female Sprague Dawley rats (200-250 g) were obtained from Marmara University Animal Center (DEHAMER), in Istanbul, Turkey. Rats were housed in cages kept at standard temperature (21°C±1°C) and humidity (45%-55%), and a 12:12-h light/dark cycle was applied. Animals had free access to tap water and were fed a rodent laboratory diet *ad libitum*. The experimental protocols, which were designed in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and the ARRIVE guidelines, were approved by the Animals Care and Use Committee of Marmara University (Ref. no. 63.2013. Mar).

Experimental Protocols and Surgery

The rats were randomly divided into five groups, each consisting of six rats. (i) sham-operated control group, (ii) saline-treated bile duct ligated (BDL) group, (iii) UDCA (15 mg/kg/d; Sigma; St. Louis, MO)-treated BDL group, (iv) MT (600 mg/kg/d; Solgar; Leonia, USA)-treated BDL group, and (v) UDCA (15 mg/kg/d) and MT (600 mg/kg/d) combination-treated BDL group. Rats were anesthetized with ketamine (100 mg/kg, intraperitoneally, i.p.) and chlorpromazine (0.75 mg/kg, i.p.) during surgical procedures. In the BDL groups, the common bile duct was reached through a midline laparotomy. In order to induce cholestasis, bile duct was ligated at the hilum of the liver with double 3-0 silks and resected between the two ligatures, so that bile flow was prevented without blocking the flow of pancreatic juice (10). The incision was then closed, and the animal was allowed to recover. In the sham-operated group, rats were operated similarly, but bile ducts were not ligated. Starting at the 24th hour of surgery, BDL rats were administered saline, UDCA, MT, or UDCA+MT by orogastric gavage for 10 days. On the 11th day of the experiment, blood was obtained by cardiac puncture, and liver samples were collected and stored for biochemical and histopathological analyses.

Biochemical Analysis

By centrifuging the blood at 3,000´*g* for 10 min, supernatant was obtained. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using commercial kits (Abcam Co.; Cambridge, USA) by a semi-automatic analyzer (EMP 168 Vet Biochemical analyzer; Shenzhen Emperor Electronic Technology Co. Ltd., Shenzhen, China).

Measurement of Hepatic Malondialdehyde Level

Lipid peroxidation was determined by the measurement of malondialdehyde (MDA) levels in the liver tissue samples that were homogenized in ice-cold KCl (150 mM). The level of lipid peroxidation was stated as MDA equivalents given in nmol MDA/g tissue (11).

Measurement of Myeloperoxidase Activity

Myeloperoxidase (MPO) is the active enzyme that is involved in the phagocytic action of neutrophils and macrophages. The assay for the measurement of MPO activity was performed as it was stated in previous studies (12). One unit (U) of MPO activity was stated as the quantity of the MPO in per g tissue weight that results in a 1.0/min difference in the absorbance read at 460 nm wavelength and at 37°C.

Histopathologic Examination

All histological and histochemical evaluations were performed by a histologist in a double-blind manner. Liver samples were fixed in formalin solution (10%) for 24 h and were then embedded in paraffin. Five-micrometer-thick tissue sections were stained with hematoxylin-eosin (H&E; hematoxylin Gill III, Cat. no. 1.05174; eosin solution 0.5% alcoholic; Cat. no. 1.02439, Merck, Darmstadt, Germany) and Van Gieson stains to evaluate Ishak modification of hepatitis activity index. Using a modified grading method, the severity of the necroinflammatory process and fibrosis was determined by scoring periportal necrosis with or without bridging necrosis (0-10), intralobular degeneration and focal necrosis (0-4), portal inflammation (0-4), and fibrosis (0-6). In addition, scoring of fibrosis was made as follows: 0, no fibrosis; 1, fibrous expansion in a few portal tracts, with or without short fibrous septa; 2, fibrous expansion in most of the portal tracts, with or without short fibrous septa; 3, fibrous expansion in most of the portal tracts with some portal to portal bridging; 4, fibrous expansion in portal tracts with significant portal to portal and portal to central bridging; 5, significant portal to portal and/or portal to central bridging and some nodules; 6, cirrhosis (13). For each rat, five serial sections and at least five adjacent fields were analyzed in each section at magnifications of x40, x100, and x200.

Immunohistochemical Analysis

Hepatic 5-µm-thick tissue sections that were stained with H&E were incubated overnight at 60°C. Following the immersion in xylene and rehydration in a series of ethanol solutions, sections were rinsed with both distilled water and phosphate-buffered saline solution (PBS, P4417; Sigma-Aldrich, St. Louis, MO) and then bathed with citrate buffer (pH=7.6) in a microwave oven. Following washing with PBS, sections were marked with a Dako Pap pen (S2002-Dako; Glostrup, Denmark) and incubated in hydrogen peroxide solution (3%) for 5 min to suppress endogenous peroxidase activity. Sections were washed in PBS, incubated in non-immune serum for 60 min, and then incubated overnight with primary antibodies such as c-Myc (1:100; Santa Cruz, cat no sc-70469), c-kit (1:100 cat no sc-168; Santa Cruz, California, USA), Oct3/4 (1:100 cat no sc-5279; Santa Cruz, California, USA), and stage-specific embryonic antigen 1 (SSEA-1; 1:100 cat no sc-21702; Santa Cruz, California, USA) at 40°C in a humidity chamber. Sections were rinsed with PBS and then incubated with biotinylated secondary antibody, with streptavidin conjugated to horseradish peroxidase in PBS (sc-2053; Santa Cruz Biotechnology,California, USA) and washed again with PBS. For immunostaining, sections were then incubated with aminoethyl carbazole and with primary antibodies such as collagen type I (1:200, cat no ab34710; Abcam, Cambridge, United Kingdom) for 1 h at 25°C in the humidity chamber. Following incubation with the secondary antibody (RE715K NovoLink polymer detection system; Novocastra, Newcastle, United Kingdom), the counterstaining of the sections was done with Mayer's hematoxylin. Deparaffinized sections were incubated with primary antibodies for fibronectin (1:100, clone 568; Novocastra, Newcastle, UK), FGF (1:200, cat no ab8880; Abcam, Cambridge, UK), and β-catenin (ready to use, cat no GTX15180; Genetex, Irvine, USA) in fully automatic system (Ventana Benchmark Ultra, Arizona, USA) on the basis of the streptavidin-biotin method for immunohistochemistry. In negative controls, the primary antibodies were omitted (14).

Alpha-smooth muscle actin (α-SMA), which is a stained cytoskeletal structure in the hepatic stellate and smooth muscle cells, was analyzed by immunohistochemistry using the Leica-UK Bond Max IHC Stainer and Bond Polymer Refine Detection wDAB (Leica Biosystems, UK). The protocol included in situ deparaffinization and high pH epitope retrieval (ER1, citrate; for 20 min); incubation with primary antibodies, such as α-SMA (60 min, Clone: 1A4; CellMarque, USA), polymer (15 min) and post-polymer (15 min); and addition of 3,3'-diaminobenzidine as the chromogen (10 min), followed by hematoxylin counterstaining (5 min). All mounted (Shandon EZ-Mount, USA) sections were microscopically evaluated (Olympus BX51, USA). The presence of a brown precipitate was regarded as a positive reaction for the primary antibodies. Semiquantitative analysis of immunoreactivity (for c-Myc, c-kit, Oct3/4, SSEA-1) was calculated using the following equation: $H\text{-score}=\sum P_i\ (i+1)$. In this formula, i stands for the intensity of labeling (weak, moderate, and strong, inserted as 1, 2, and 3), and for each intensity, P_i represents the percentage (0%-100%) of labeled cells (15).

Histomorphometric Analysis

Using α-SMA staining, the number of Ito cells transformed in myofibroblasts was counted by histomorphometric analysis. Five serial sections were taken from each block, and 10 images were captured with a 200× objective from the areas with the highest cellular densities (16). The cells were tagged and counted via the semi-automatic image analysis system (University of Texas Health Science Center at Santorino, TX, USA, image tool for Windows version 1.28 program). Measurements were performed by two independent histologists who were blinded to the experimental groups (15).

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 6.0 (GraphPad Software; San Diego, CA, USA). Data are expressed as means±SEM. Analysis of variance followed by Tukey's multiple comparison tests and Student's t test were used to analyze the data, and $p<0.05$ was regarded as statistically significant.

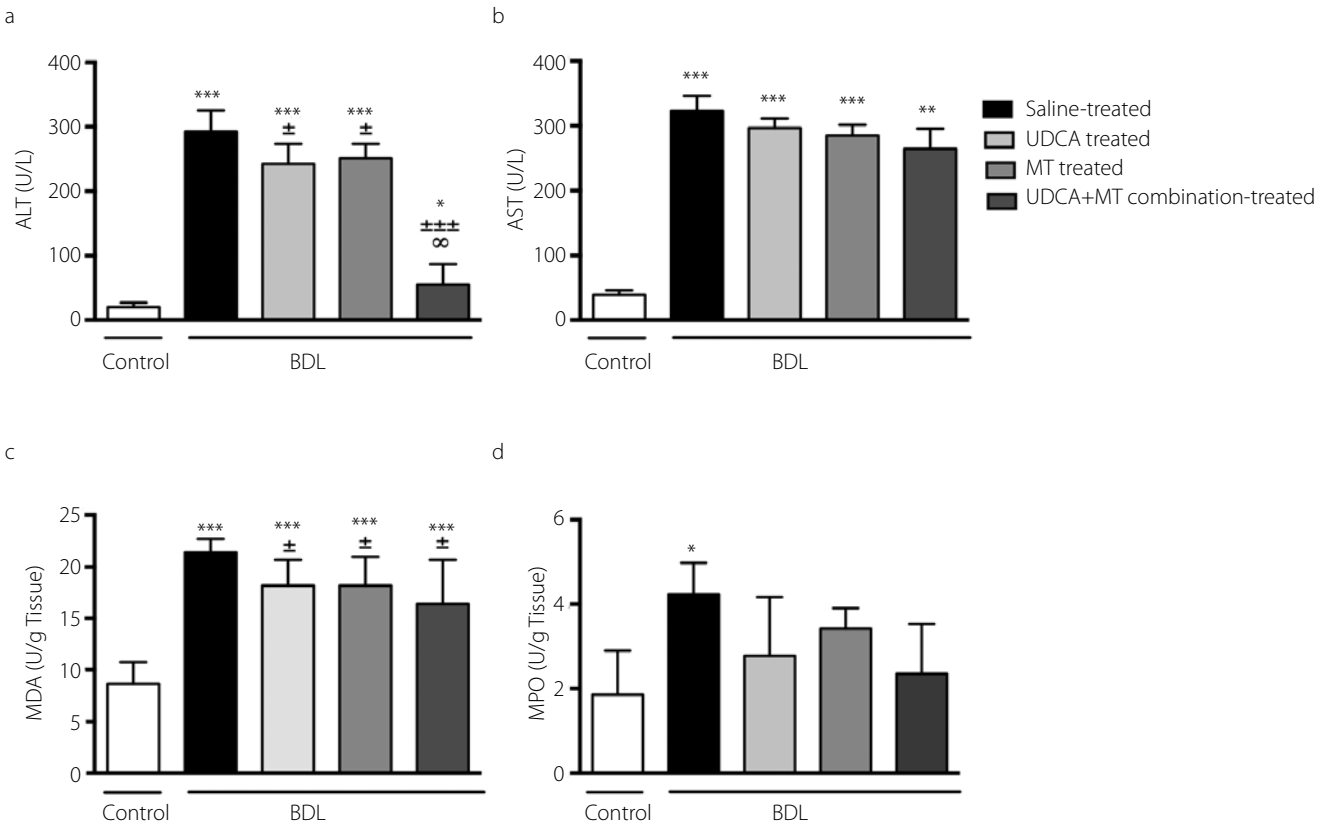

Figure 1. a-d. Biochemical analysis. Serum levels of (a) ALT and (b), (c) hepatic levels of MDA, (d) MPO activity in rats with sham operation (control) or BDL rats treated with saline, UDCA, MT, or their combination
*p<0.05
**p<0.01
***p<0.001 compared with sham-operated group; ±p<0.05, ±±±p<0.001 compared with saline-treated BDL group; ∞ compared with p<0.05 BDL+MT group and BDL+UDCA group

RESULTS

Effects of UDCA, MT, or UDCA+MT Treatments on Serum ALT and AST Levels in BDL Rats

Serum ALT levels were increased significantly in saline-treated BDL group as compared with sham-operated control group (p<0.001; Figure 1a). With respect to saline-treated BDL group, serum ALT levels were significantly decreased in BDL groups that were treated with either UCDA or MT or its combination (p<0.05-0.01), whereas the decrease in serum ALT level was greater in the combined treatment group than those in the individual treatments (p<0.05). Similar to ALT, serum AST levels were also significantly increased in all the BDL groups as compared with that of the control group (p<0.01-0.001), but none of the treatments had a significant effect on elevated serum AST levels (Figure 1b).

Effects of UDCA, MT, or UDCA+MT Treatments on Hepatic MDA Level and MPO Activity in BDL Rats

Compared with the control group, hepatic MDA levels were higher in all BDL groups (p<0.001), but treatment with either UDCA or MT or their combination reduced the elevated MDA levels (p<0.05; Figure 1c). Hepatic MPO activity was increased in saline-treated BDL group with respect to sham-operated group (p<0.05), but the MPO activity in the hepatic tissues of the BDL groups with either three treatments was not different than that of the control (Figure 1d).

Effects of UDCA, MT, or UDCA+MT Treatments on BDL-Induced Morphological Changes

When the severity of necroinflammatory process and fibrosis were graded in H&E-and Van Gieson-stained tissues (Figure 2), the portal inflammation score was significantly increased in all groups with BDL as compared with the control group (p<0.01) without any significant difference among the treatment groups (Figure 3d). However, increased scores for periportal and interlobular necrosis (p<0.001) and lobular fibrosis (p<0.01) in saline-treated BDL group were reduced in all treatment groups (p<0.01) (Figure 2,3).

Effects of UDCA, MT, or UDCA+MT Treatments on α-SMA Expression

Compared to control group, α-SMA expressions were higher in saline-treated BDL group (p<0.001), but treatments with either UDCA (p<0.001) or MT (p<0.05) or their combination (p<0.001) reduced the α-SMA expression for hepatic stellate cell activation (Figure 2, 3e).

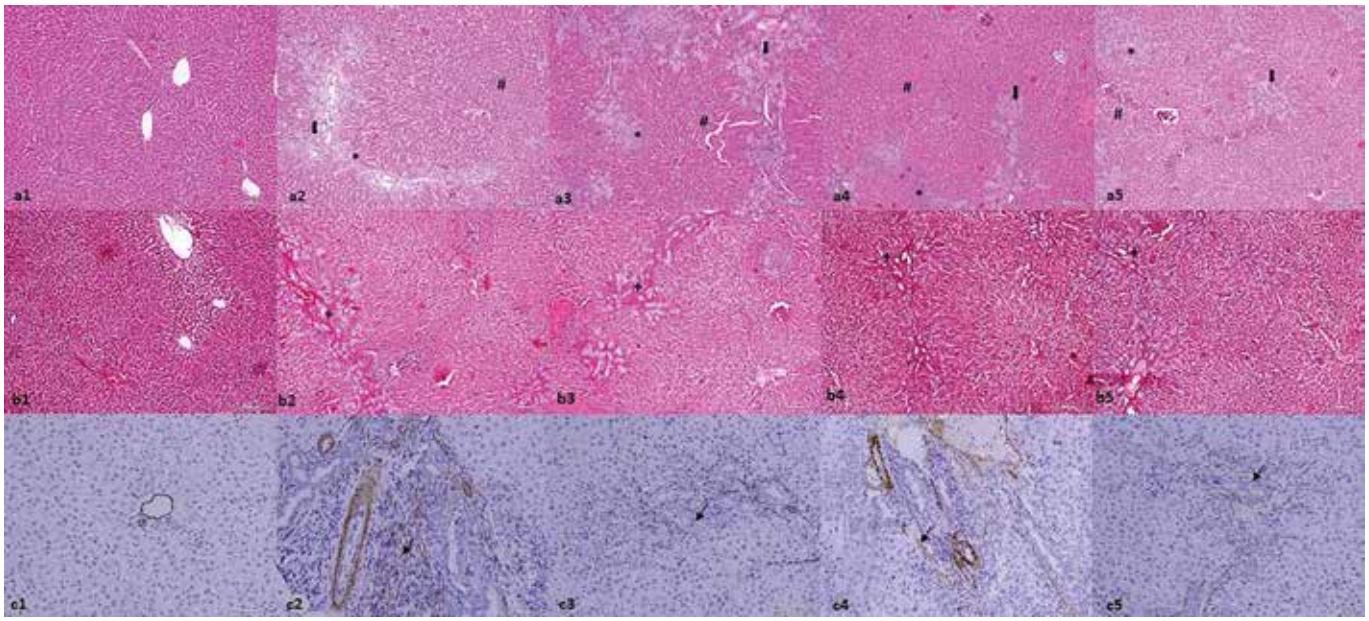

Figure 2. a-c. Histopathological analysis. H&E staining ×200: (a1) sham-operated control group, (a2) saline-treated BDL group; (a3) BDL+MT group, (a4) BDL+UDCA group; (a5) BDL+UDCA+MT group. *periportal necrosis; #,intralobular necrosis; thick arrow, ductular proliferation. Van Gieson's staining (VG) ×100: (b1) sham-operated control group; (b2) saline-treated BDL group; (b3) BDL+MT group; (b4) BDL+UDCA group; (b5) BDL+UDCA+MT group. +,fibrosis area. α-SMA staining ×200: (c1) sham-operated control group; (c2) saline-treated BDL group; (c3) BDL+MT group; (c4) BDL+UDCA group; (c5) BDL+UDCA+MT group. Thin arrow. expression of α-SMA in cells

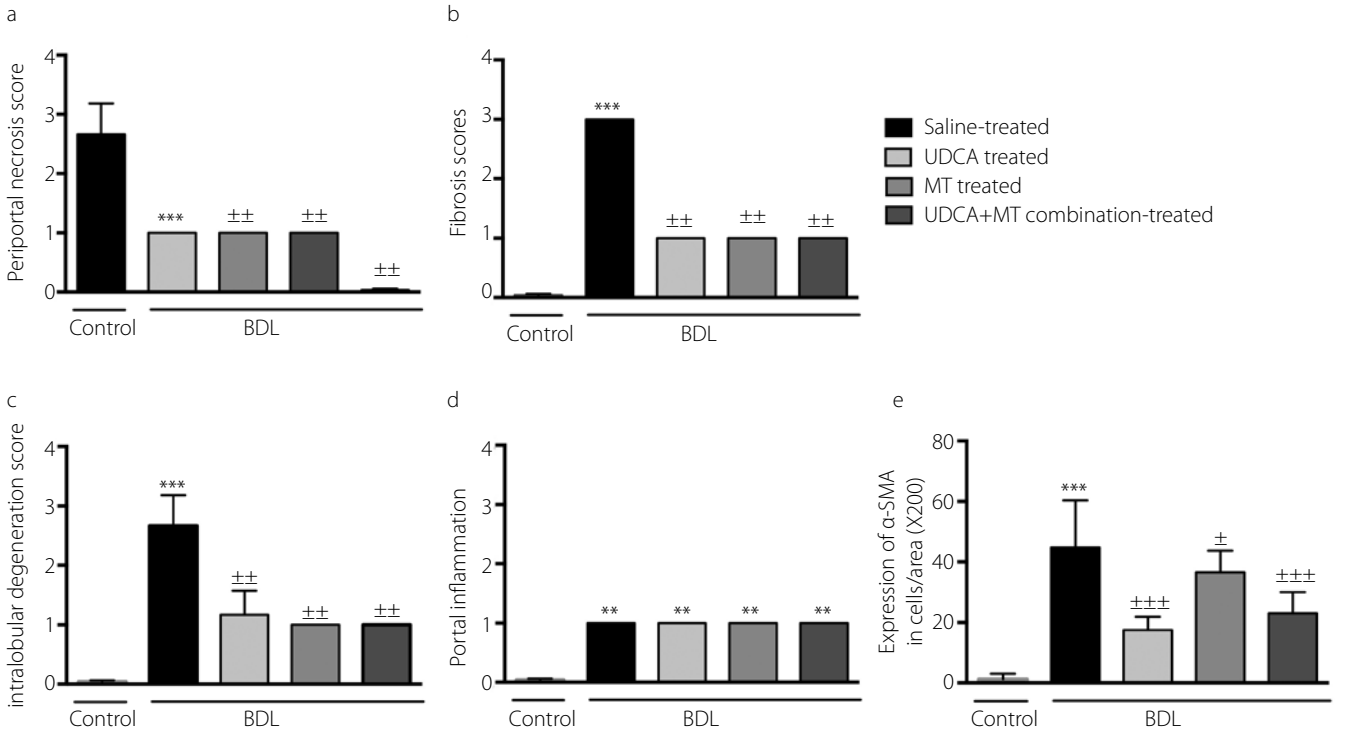

Figure 3. a-e. Hepatitis activity index. (a) Periportal necrosis with or without bridging necrosis (0-10), (b) fibrosis (0-6), (c) intralobular degeneration and focal necrosis (0-4), (d) portal inflammation (0-4), (e) expression of α-SMA in cells/area (×200) in rats with sham operation (control) or BDL rats treated with saline, UDCA, MT, or their combination

**p<0.01, ***p<0.001 compared with sham-operated group; ±p<0.05, ±±p<0.01, ±±±p<0,001 compared with saline-treated BDL group

Effects of UDCA, MT, or UDCA+MT Treatments on Markers for Stem Cells

Immunoreactivity of transcription factors c-Myc, Oct3/4, c-kit, and SSEA-1 was increased in all BDL groups as compared with the control group (p<0.05-0.001) (Figure 4, 5). On the other hand, c-kit, c-Myc, Oct3/4, and SSEA-1 scores were significantly reduced by UDCA, MT, or UDCA+MT treatments (p<0.05-0.001).

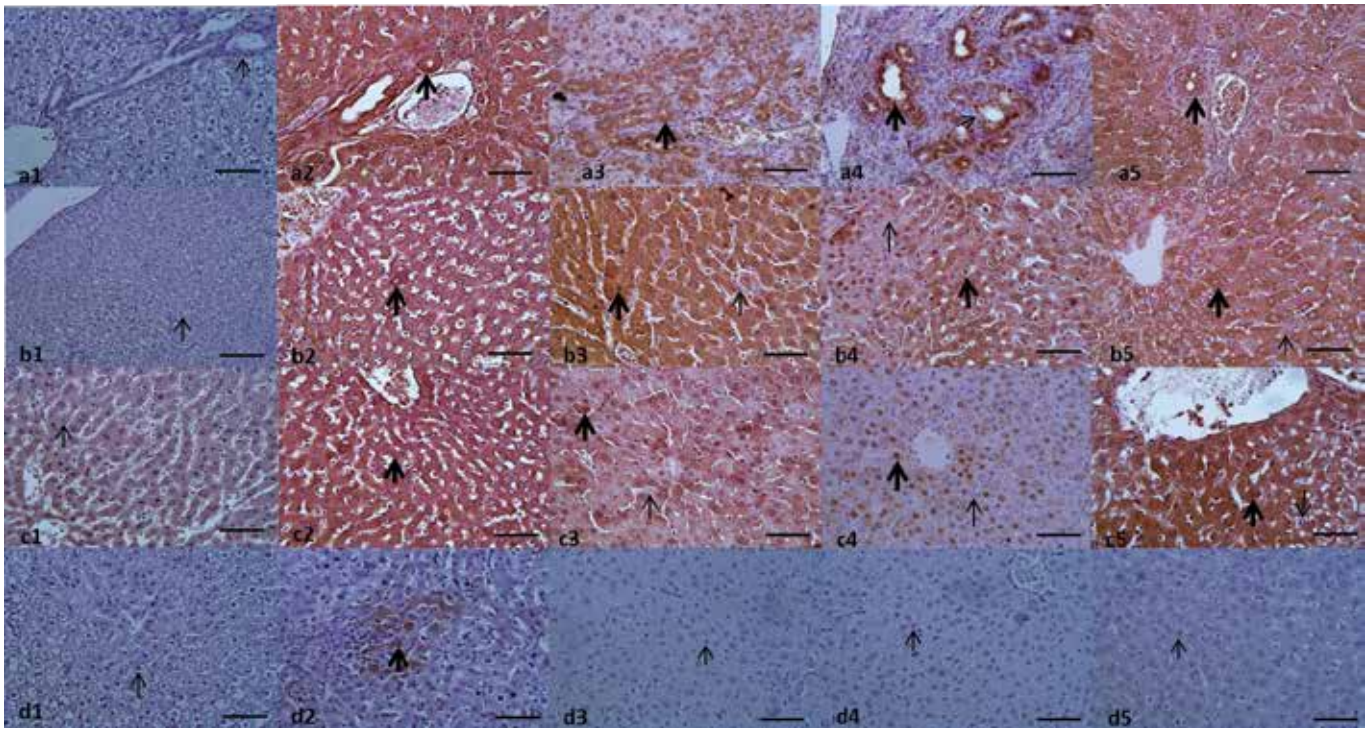

Figure 4. a-d. Immunohistochemical expressions of stem cell markers. c-kit (CD117) immunoreactivity ×200: a1: sham-operated control; a2: saline-treated BDL; a3: BDL+MT; a4: BDL+UDCA; a5: BDL+UDCA+MT; c-Myc immunoreactivity ×200: b1: sham-operated control; b2: saline-treated BDL; b3: BDL+MT; b4: BDL+UDCA; b5: BDL+UDCA+MT; Oct3/4 immunoreactivity ×200 (D): c1: sham-operated control; c2: saline-treated BDL; c3: BDL+MT; c4: BDL+UDCA; c5: BDL+UDCA+MT; SSEA-1 immunoreactivity ×200 (D): d1: sham-operated control; d2: saline-treated BDL; d3: BDL+MT; d4: BDL+UDCA; d5: BDL+UDCA+MT. Thin arrows, negative staining; thick arrows, positive staining.

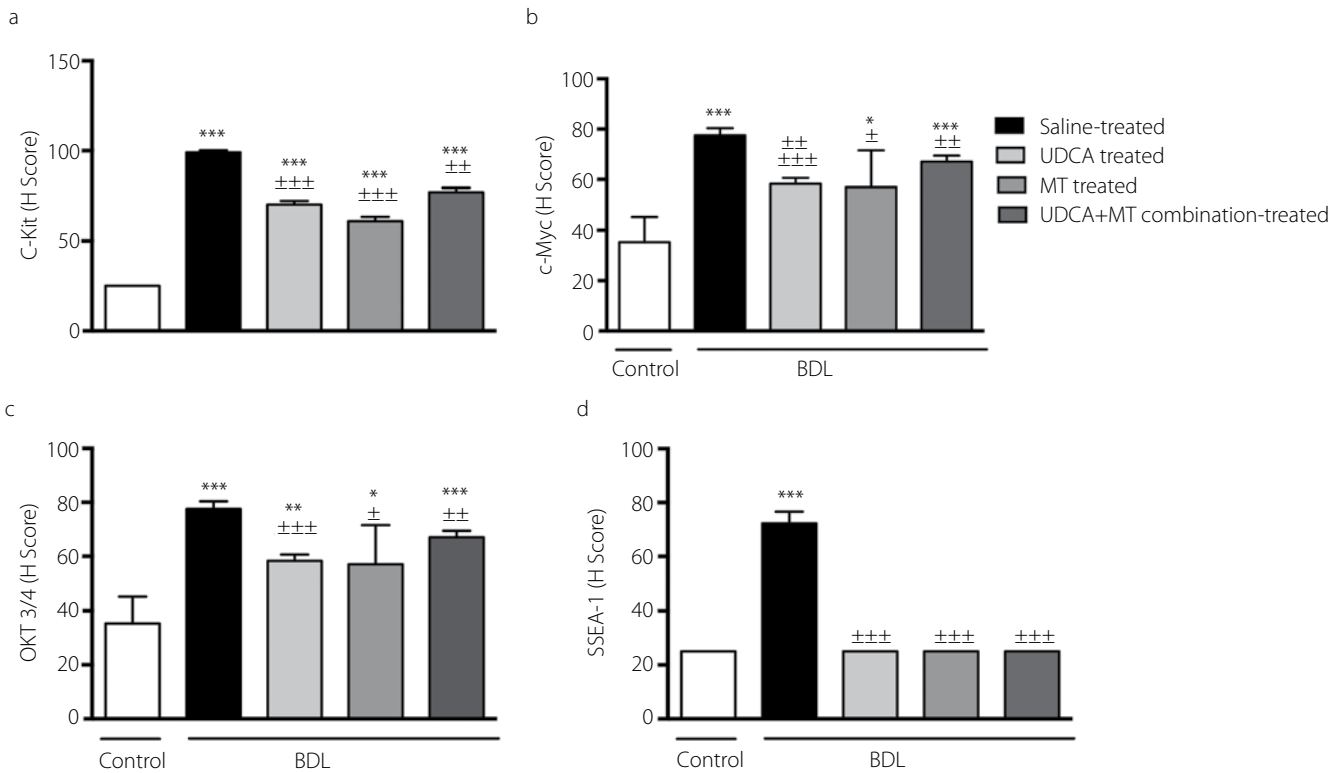

Figure 5. a-d. Immunoreactivity of stem cell markers. (a) c-kit (CD117) immunoreactivity, (b) c-Myc immunoreactivity, (c) Oct3/4 immunoreactivity, (d) SSEA-1 immunoreactivity in rats with sham operation (control) or BDL rats treated with saline, UDCA, MT, or their combination

*p<0.05, **p<0.01, ***p<0.001 compared with sham-operated group; ±p<0.05, ±±p<0.01, ±±±p<0.001 compared with saline-treated BDL group

DISCUSSION

The widely used BDL model was proven to result in cirrhosis, which involves proliferated bile duct epithelial cells, oxidative stress, and necrosis in hepatocytes along with activated stellate cells (17). In our study, ALT, AST, MDA levels and MPO activity were elevated, whereas serious histopathological deterioration along with increased stem cell markers has occurred, indicating cholestatic liver injury in BDL rats. Our findings also demonstrated that the supplementation of MT (600 mg/kg/d) with UDCA (15 mg/kg) has achieved similar effects to UDCA or MT treatment alone in BDL rats by (i) decreasing serum levels of ALT and hepatic MDA levels; (ii) improving liver histology, with reduced periportal necrosis, interlobular degeneration and focal necrosis, fibrosis and expression of α-SMA; and (iii) reducing stem cell markers.

The accumulation of toxic bile acids due to chronic cholestasis may lead to liver necrosis, fibrosis, and cirrhosis, accompanied by increased levels of liver enzymes (4). Accordingly, levels of hepatic enzymes (AST and ALT) were elevated in all BDL animals in our study, whereas none of the treatment regimens had an impact on AST levels. On the other hand, ALT levels were depressed by either MT or UDCA treatment, but a greater improvement was reached by the combined therapy. Similar to our results, the treatment with UDCA has been reported to reduce the bile salt toxicity and decrease the AST and ALT levels in cholestatic conditions, such as in PBC. The hydrophilic UDCA reduces the hydrophobicity of the bile acid pool, which occurs due to 7β-epimerization of chenodeoxycholic acid, through the action of colonic bacteria and occurs at low concentrations (3%) in human bile acid. In addition to its extensive use in Chinese medicine for centuries, clinical trials using UDCA have demonstrated its beneficial effects against various cholestatic conditions (18). However, harmful effects of high-dose UDCA have also been reported in recent studies in patients with primary sclerosing cholangitis (4). On the other hand, MT treatment has been known to have beneficial effects in patients with liver disease in classical Greek medicine. In our study, the administration of UDCA or MT or their combination reduced the serum ALT levels, whereas the reduction in ALT level was significantly more when the combination was given. Similarly, Hajiani (19) reported that administration of a 12-week regimen consisting of vitamin E and MT reduced serum ALT and AST levels in patients with nonalcoholic fatty liver disease. However, the mechanism responsible of MT-induced alleviation of hepatic disease has not been fully evaluated. Our results confirm that the antioxidant actions of UDCA and MT could be partially involved in their protective mechanisms.

Oxidative stress has been demonstrated to have a major role in cholestatic liver injury and apoptosis, whereas an elevated tissue MDA level is considered as an indicator of increased lipid peroxidation and oxidative stress. In our study, MDA levels were elevated at the 11th day of bile duct ligation. In line with our study, increased lipid peroxidation levels were reported at 7 or 14 days of biliary obstruction (20). UDCA was reported to reduce MDA levels by decreasing reactive oxygen species resulting from the increased hydrophobic bile acids, due to suppressed oxidative mitochondrial metabolism and Kupffer cell stimulation (21). In line with other study, our current findings demonstrate that UDCA treatment decreased high MDA levels, suggesting its antioxidant action against the cytotoxicity of hydrophobic bile acids (22). UDCA was also shown to increase the level of glutathione- and thiol-containing proteins and consequently protected hepatocytes and hepatic mitochondria against oxidative stress. Similar to UDCA, the administration of MT or their combination in our study has also decreased the MDA levels. MT was suggested to prevent liver damage by inhibiting the leakage of enzymes and maintaining the integrity of the plasma membrane. Although the mechanisms involved in MT-induced reduction in MDA are not clearly defined yet, MT was reported to increase the level of superoxide dismutase for scavenging free radicals. The activity of polymorphonuclear cells as the major source of free radicals in the hepatic tissue was elevated by BDL (23). However, despite the elevations, MPO activity was similar to that of the control group, suggesting that the either of the treatments had slight and similar suppressive effects on hepatic MPO activity.

The formation of reactive oxygen species plays a major role in the pathogenesis of cholestasis-induced progressive fibrosis, which occurs due to accumulation of extracellular matrix proteins and by stimulating collagen expression in hepatic stellate cells (24). Histological examination revealed periportal necrosis, portal inflammation, interlobular degeneration, and focal necrosis and fibrosis in the BDL group, whereas UDCA, MT, or their combination provided significant improvements in all histological scores, but portal inflammation scores were the same for all BDL groups. In parallel with the previous reports indicating that the degree of α-SMA stain demonstrates indirectly the extension of fibrosis, α-SMA expression was increased in saline-treated BDL group (25). However, its expression was reduced significantly with the administration of UDCA or MT or their combination. Previously, antifibrotic effects of UDCA and MT have been demonstrated (4,6,7,23,26,27). It was suggested that UDCA exerts an antifibrotic effect by reducing transforming growth factor-beta 1 expression, transforming growth factor type 1 receptor activation, and activation of the other components of the signaling pathways that were shown in the rat hepatic stellate cell cultures. Furthermore, UDCA reduces oxidative stress and fibrosis due to its inhibitory effect on glutathione synthesis in hepatic stellate cells and cell activation (4,7,27). On the other hand, MT treatment was reported to decrease hepatic collagen content and hepatic fibrosis score in rats with carbon tetrachloride-induced liver fibrosis. Jeong et al. (26) have demonstrated the anti-inflammatory effects, hepatic stellate cell activation, mast cell stabilization, and eventually antifibrotic feature of MT (26). Thus, when taken with the results of other studies, our findings suggest that UDCA, MT, or their combination have antifibrotic effects that involve the inhibition of oxidative hepatic injury.

Stem cells have the potential to give rise to change into any kind of cell and have the ability of self-renewal. Although stem cells in different organs have different potentials for development, all stem cells are supposed to maintain the balance among the self-repair and differentiation. When an injury occurs, the changes in signaling pathways of stem cells result in the regulation of the gene transcription to allow self-renewal and differentiation. Stem cells usually have similar characteristics with the reprogrammable pluripotent somatic stem cells (iPSCs) and embryonic stem cells. Stage-specific embryonic antigens (SSEA) have been newly defined as a key player in identifying cells endowed with pluripotent and stem cell characteristics (28,29). Identification of the stem cells can be made by immunohistochemical staining of the markers expressed during early stages (e.g., SSEA-1) or gene products (e.g., Oct4, alkaline phosphatase, etc.) (1,15,30). In our study, elevated SSEA-1 levels in the BDL group were reversed by all treatment regimens, suggesting the inhibitory action of the treatments on stem cell-induced hepatic fibrosis. c-Myc, Sox-2, Oct3/4, and Klf-4 genes are used to study the pluripotent characteristics of iPSCs (31). Among these genes, the Oct3/4 is the octamer-binding transcription factor with the ability of recognizing the ATTTGCAT octamer in the DNA. Initially described as a protein found in unfertilized egg, Oct3/4 is the most important transcription factor of the embryonic stem cell, and it is also expressed in human and rodents (32,33). In our study, the transcription factor Oct3/4 was increased in BDL group treated with saline, whereas the increase was depressed in all treatment groups, implicating that treatments provide a proper progression of differentiation. Similarly, increased immunoreactivity of the other oncogenic transcriptional factor c-Myc was also suppressed by all the treatments. C-Myc plays a role in the histone acetylation and is one of the important factors in cell cycle, apoptosis, signal transduction, transcriptional, and post-transcriptional regulatory mechanisms (15,34). Taken together with the improved histological scores in the BDL groups treated with UDCA, MT, or their combination, it can be suggested that the antifibrotic actions of the drugs may be related with their suppressive effects on the stem cell activity.

Although the activation, expansion, and *in vivo* roles of hepatic stem cells have been identified, their role in the liver regeneration has been controversial for a long time (35). Lately, stem cell factor and c-kit system was postulated to be involved in the initial activation of stem cells in the liver, and the c-Kit(+) cells were increased in portal tracts of cirrhotic patients, suggesting that c-kit(+) cells may be the progenitor cells of the liver (1,8,9). Similar to other transcriptional factors, c-Kit was also increased by the induction of BDL in the current study, whereas each of the treatments reduced the immunoreactivity of c-Kit. Since hepatic stem cell transformation and progenitor cell transformation are involved in the pathogenesis of some hepatic cancers and c-kit expression was verified in hepatocellular carcinoma cell lines, the regulation of proliferative activity of both the tumorous and nontumorous hepatic cells appears to be associated with c-kit expression (36,37).

Because of their oncogenic potential, the use of iPSCs in regenerative medicine has been limited. Our current findings revealed that stem cells, which are expected to be responsible for the repair of the damaged hepatic cells, were increased in rats with cholestasis, whereas this increase was modulated by either UDCA, MT, or combined treatment. In addition to their modulatory effect on hepatic regeneration, all treatments resulted in similar improvements in the hepatic function (ALT) and morphology and depressed lipid peroxidation (MDA). Considering that the liver is particularly unique in terms of regeneration, further experimental and clinical studies are necessary to clarify the supportive effects of MT and UDCA treatments on the stimulated stem cell activity in hepatic injury.

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