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HISTOPATHOLOGICAL AND BIOCHEMICAL EFFECTS OF COLCHICINE AND TRIMETHYLCOLCHICINIC ACID ON LIVER FIBROSIS INDUCED BY BILE DUCT LIGATION IN ALBINO RATS

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ABSTRACT

The present study investigated the effect of colchicine and trimethylcolchicinic acid on liver fibrosis induced by common bile duct ligation in albino rats. Bile duct ligated rats showed cirrhosis where liver architecture completely distorted with diffused fibrosis and generation of abnormal nodules. Hepatocytes with nuclear changes and highly proliferated bile ductules were also detected. Alpha smooth muscle actin positive cells increased markedly. Biochemical results also showed elevation in serum transaminases (AST, ALT) and alkaline phosphatase activities as well as total bilirubin concentration. Most of these effects were gradually reversed with colchicine and trimethylcolchicinic acid treatment. Both colchicine and trimethylcolchicinic acid were nearly similar in there therapeutic effect when used at the same dose and under similar conditions. Moreover, higher doses of trimethylcolchicinic acid were more efficient and safer than colchicine in treating liver fibrosis in the experimental animals.

Keywords: Colchicine, trimethylcolchicinic acid, bile duct ligation, liver, cirrhosis.

INTRODUCTION

Liver fibrosis is the excessive accumulation of extracellular matrix proteins (ECM) in the liver (Bataller and Brenner, 2005). Patients with liver fibrosis can be asymptomatic for 15-20 years with morbidity and mortality only occurring after progression to cirrhosis (Friedman, 2003; Bataller and Brenner, 2005). Cirrhosis is a diffuse process characterized by fibrosis and the conversion of normal liver architecture into structurally abnormal nodules (Anthony *et al*., 1978).

Hepatic fibrosis was historically thought to be a passive and irreversible process due to the collapse of the hepatic parenchyma and its substitution with a collagen-rich tissue (Schaffner and Klion, 1968; Popper and Uenfriend, 1970). Currently, it is considered a model of wound-healing response to chronic liver injury (Albanis and Friedman, 2001). During the last 40 years, research focused on the cellular origin of enhanced hepatic matrix synthesis within the damaged liver tissue (Ramadori and Saile, 2004). In 1980s, hepatic stellate cells (HSCs) were identified as the main collagen-producing cells in the liver (Friedman *et al*., 1985).

During liver injury, HSCs get activated and their number drastically increases (van de Bovenkamp, 2006), migrate and accumulate at the sites of tissue repair, expressing myogenic markers as alpha-smooth muscle actin (α-SMA), secreting large amounts of ECM and regulate ECM degradation (Bataller and Brenner, 2005). Thus, HSCs participate pathophysiologically in both fibrogenesis and fibrolysis "tissue remodeling" (Gressner and Weiskirchen, 2006).

It has been reported that colchicine, alkaloid obtained from various species of *Colchicum*, usually *Colchicum autumnale* (Robbers *et al.*, 1996), protects the liver of experimental animals against CCl_4, galactosamine and acetaminophen intoxication (Mourelle *et al.*, 1988; Mourelle and Meza, 1989; Muriel *et al.*, 1993 respectively). In addition, Kershenobich *et al*. (1988) and Muntoni *et al*. (2010) have shown some beneficial effects of colchicine in the treatment of cirrhotic patients. However, diarrhea, nausea and vomiting are the common side effects of colchicine even at therapeutic doses (Sweetman, 2007). Therefore, colchicine should be given at very low doses because of its toxicity that attributed to its ability to bind with microtubule protein (tubulin). Trimethylcolchicinic acid (TMCA) is a colchicine derivative, which does not bind with tubulin (Zweig and Chignell, 1973). Therefore, higher doses of TMCA may be more effective and harmless than colchicine in treating liver cirrhosis. The effect of TMCA on liver fibrosis was studied by few investigators (Cedillo *et al*.1996 and Muriel *et al*., 2005). The aim of this work is to compare the histopathological, immunohistochemical, and biochemical effects of colchicine and trimethylcolchicinic acid on liver fibrosis induced by common bile duct ligation in albino rats.

MATERIALS AND METHODS

Chemicals used

Colchicine was obtained from El-Nasr Pharmaceutical Chemical Company (ADWIC), Egypt, as white tablets

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each contain 500µg of colchicine. Trimethylcolchicinic acid was obtained from Sigma Chemical Company, USA, in the form of yellow powder. Colchicine and TMCA were dissolved in distilled water and were orally given to the experimental animals. Colchicine was administered at a dose level of 50µg/kg body weight/day (Poo *et al.*, 1993) while TMCA was administered at two dose levels; low dose (50µg/kg/day) and high dose (150µg/kg body weight /day).

Experimental design

Adult female albino rats (*Rattus norvegicus),* of an average body weight 200±10g were used. They were obtained from the animal house of The National Organization for Drug Control and Research (NODCAR). Animals were kept in the laboratory under almost constant condition of temperature (24±2°C) for 2 weeks before and throughout the experimental work. They were fed on standard rodent diet manufactured specially for laboratory purposes, as well as some vegetables. Water was given *ad libitum*. Biliary cirrhosis was induced surgically through double ligation and division of the common bile duct under light ether anesthesia.

Animals were divided into two main groups bile duct ligated group (BDL) and sham operated group. After 4 weeks from surgery 6 animals were dissected from each group and the remaining animals were divided as following. 1- BDL group divided into: non-treated subgroup, 50µg/kg body weight/day colchicine treated subgroup, 50µg/kg body weight/day TMCA treated subgroup and 150µg/kg body weight/day TMCA treated subgroup. 2- Sham operated group divided into: non-treated subgroup, 50µg/kg body weight/day colchicine treated subgroup and 150µg TMCA treated subgroup. All these subgroups were treated for 4 weeks.

Histological and immunohistochemical studies

Six animals from each subgroup were sacrificed 6 and 8 weeks after surgery. Small liver slices were rapidly removed, fixed in Bouin's fluid and stained with hematoxylin and eosin for histological examination under light microscopy. Other liver slices were fixed in Zenker's solution and stained with Mallory trichrome technique (Weesner, 1968) for demonstration of collagen fibers. Fibrous tissue area was measured by computerized image analysis system "*Image Proplus version 5*" in each subgroub. For analysis, six power fields per each liver section were taken at X 40 (4 objective X 10 ocular). The percent of fibrotic tissue area per examined liver sections were calculated in each group (Froh *et al.*, 2007). Alpha-smooth muscle actin positive cells were visualized immunohistochemically using liver slices fixed in 10% formalin.

Biochemical study

Blood was collected according to the retro-orbital plexus method (Schermer, 1967). Serum activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) were determined according to Reitman and Frankel (1957), while serum alkaline phosphatase (ALP) activity was measured according to Belfield and Goldberg (1971). Total bilirubin concentration was determined according to Jendrassik and Gróf (1938). For statistical analysis, one-way ANOVA was used in order to compare the group's means. Differences were considered significant for $P<0.05$.

RESULTS

Histopathological observations

In sham operated groups, no histological alterations were detected (Fig.1). On the other hand, all examined liver sections taken from BDL animals after 4 and 6 weeks showed cirrhosis. The hepatic architecture was completely distorted, liver lobules nearly disappeared and were replaced with macro and micro nodules separated by wide fibrous tissue area admixed with highly proliferated and dilated bile ductules (Fig. 2). Hepatic cells appeared with variable changes in their nuclei including hypertrophy, marginal chromatin, pyknosis, clumped, crescent shape, and irregular nuclear envelope. Mild to moderate inflammation and prominent enlarged Kupffer cells were seen in the fibrous tissue stroma.

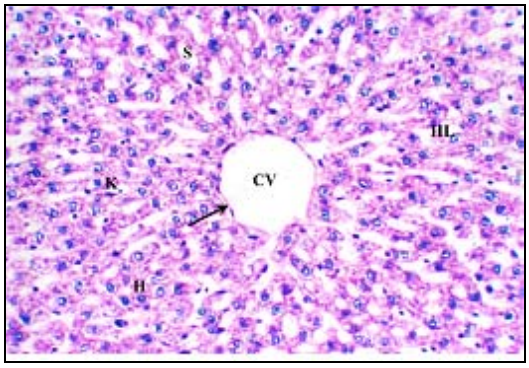

Fig. 1. Liver section of sham operated rat showing hepatic lobule (HL), central vein (CV) with it's endothelial cell lining (arrow), polygonal hepatic cells (H), sinusoids (S) and Kupffer cells (K), (H&E, X 200).

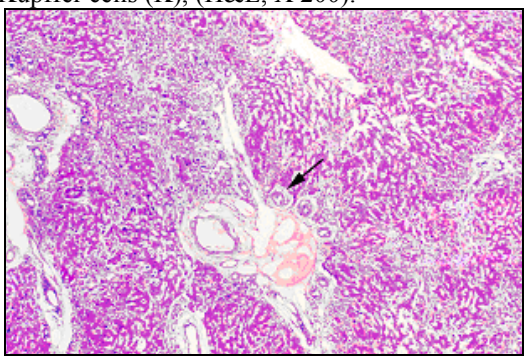

Fig. 2. Liver section of common bile duct ligated rat for 4 weeks duration showing complete distortion of hepatic architecture and hyper proliferated bile ductules (arrow), (H&E, X 40).

In BDL groups treated daily with either 50µg colchicine/kg or 50µg TMCA/kg for 2 weeks**,** 50% of examined liver sections still showing evidence of marked loss of hepatic lobular architecture. However, in the remaining 50% of examined cases, lobular architecture started to be distinguished (Figs. 3, 4). Treating BDL rats daily with 50µg colchicine/kg for 4 weeks, more progress in retaining well organized hepatic lobular architecture was seen in 50% of examined liver sections with appearance of thin fibrous band partitions invaded with inflammatory cells and marked reduction in number of the proliferated bile ductules. In addition, treating BDL animals daily with 50µg TMCA/kg for 4 weeks, showed also diffused well formed hepatic lobules in two thirds of the treated animals.

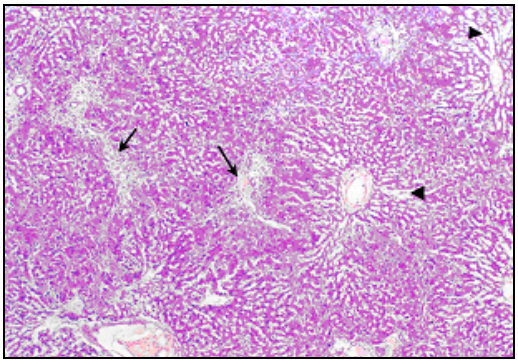

Fig. 3. Section of liver of BDL rat treated with 50µg colchicine/kg for 2 weeks. Distinguished hepatic lobules separated by moderately widened fibrotic portal areas (arrow) and widely dilated sinusoidal spaces (arrow head) are prominently seen, (H&E, X 40).

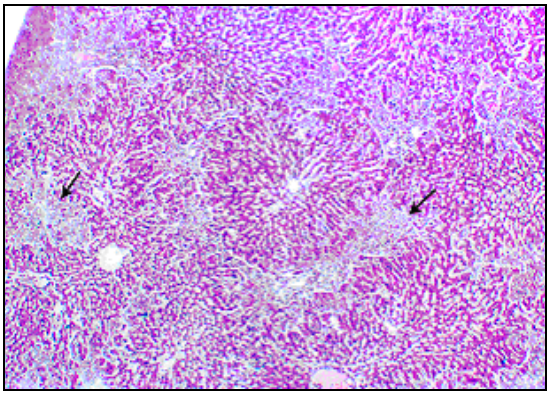

Fig. 4. Section of liver of BDL rat treated with 50µg TMCA/kg for 2 weeks showing well recognized hepatic lobular architecture in most areas. Mild fibrotic portal areas with bile ductular proliferation are seen (arrow), (H&E, X 40).

Examination of BDL rats treated with 150µg TMCA/kg/day for 2 weeks revealed moderate improvement in the hepatic lobular architecture in 84% of animals. In the remaining 16% of cases, hepatocellular lobulation was much well formed, with localized portal areas and more or less normal appearing nuclei .When BDL rats were treated with 150µg TMCA/kg/day for 4 weeks, well formed hepatocellular lobulation with slightly dilated sinusoids was seen in all examined cases. The hepatic lobules were separated with only thin bands of fibrous tissue, infiltrated with moderately dense inflammatory cells. Few bile ductules were occasionally seen in portal areas (Fig. 5) and most of the hepatocytes appeared with normal nuclei.

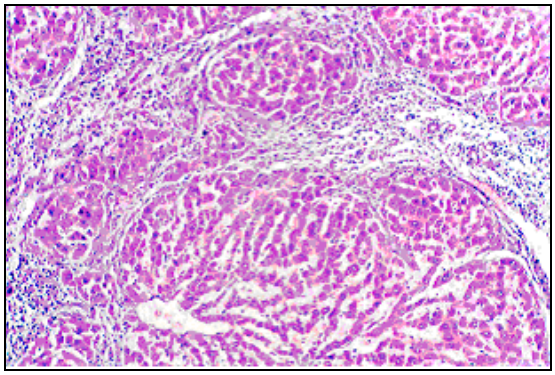

Fig. 5. Section of liver of BDL rat treated with 150µg TMCA/kg for 4 weeks showing well formed hepatic lobules separated by thin fibrous bands invaded with moderately dense inflammatory cells, (H&E, X 100).

Image analysis data of liver sections stained with Mallory trichrome technique showed that area of fibrosis decreased significantly ($P<0.05$) in BLD animals treated with 150µg TMCA/kg/day (Fig. 6).

Immunohistochemical observations

The activated stellate cells in liver sections were determined by immune-staining of α-SMA, a definitive marker of activated HSC (myofibroblasts). In liver sections of sham operated rats, α-SMA was normally expressed only in the intermediary layer of portal tract vessels and in some cells around the terminal liver venules. α-SMA positive cells increased markedly and stained as clusters lay within or in the vicinity of accumulated fibers in BDL animals for 6 weeks (Fig.7).

In BDL rats treated with 50µg colchicine/kg for 4 weeks, α-SMA positive cells were decreased as compared with BDL group (Fig. 8). On the other hand, liver sections of BDL rats treated with 50µg TMCA/kg for 4 weeks occasionally showed tiny areas of mild to moderate brownish deposit. α-SMA positive cells were markedly reduced as compared to either BDL group or 50µg colchicine treated BDL group (Fig. 9). Finally, liver sections of BDL rats treated with 150µg TMCA/kg for 4 weeks showed wide expanding areas of hepatic tissue completely negative of α-SMA positive cells.

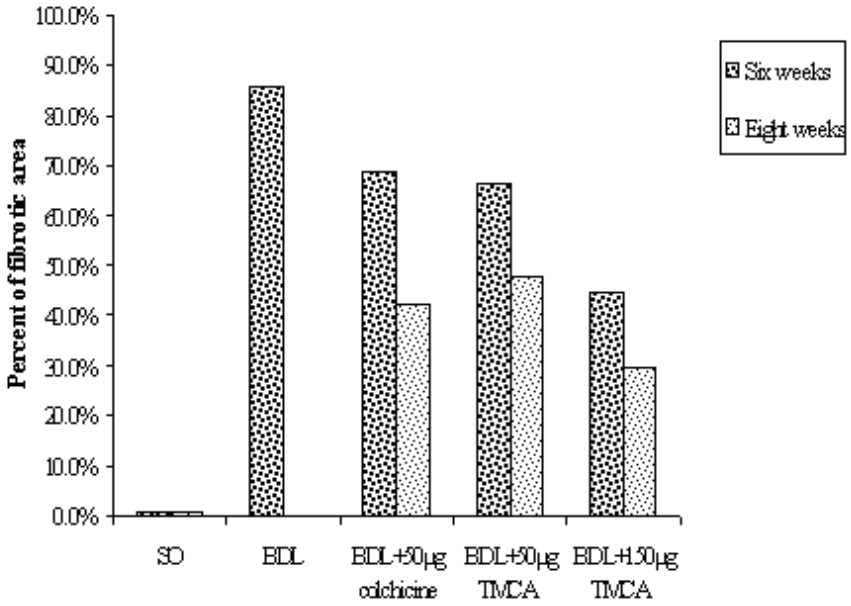

Fig. 6. Histogram showing percent of fibrotic tissue area per examined liver sections in different experimental groups at different time intervals.

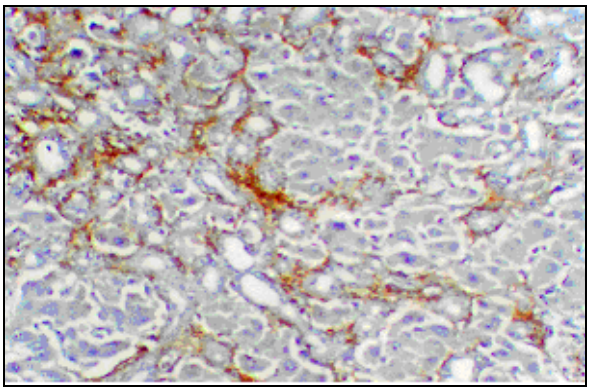

Fig. 7. Liver section of BDL rat for 6 weeks. Clusters of α-SMA positive cells lie within accumulated fibers, and encircling the proliferated bile ductules, (X 200).

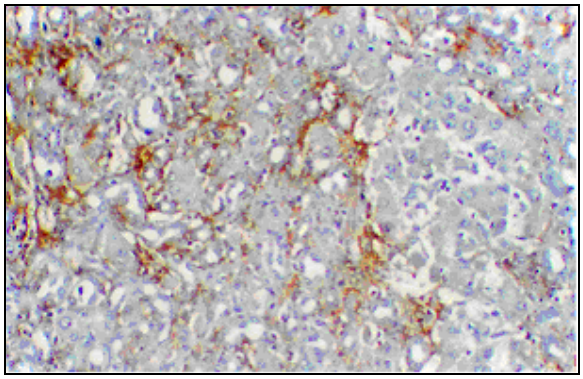

Fig. 8. Liver section of BDL rat treated with 50µg colchicine/kg for 4 weeks showing moderate α-SMA positive cells, (X 200).

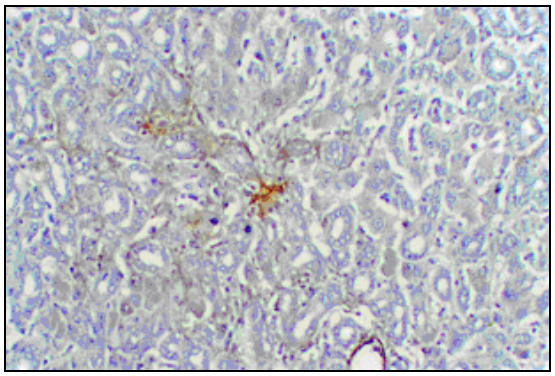

Fig. 9. Liver section of BDL rat treated with 50µg TMCA/kg for 4 weeks showing tiny brown deposit, (X 200).

Biochemical results

Data in figures 10 and 11 showed the change in activity of AST and ALT in different experimental groups. There was no significant changes in AST and ALT activities between sham operated none treated rats and those received 50µg colchicine or 150µg TMCA/kg. Bile duct ligated animals for either 4 or 6 weeks showed significant increase in serum AST and ALT activities when compared with sham operated group at the same duration. Colchicine or TMCA-treated BDL rats significantly decreased the elevated serum AST and ALT activities when compared with BDL rats. Before the end of 8 weeks of BDL, all of the BDL rats failed to survive, while BDL groups treated with colchicine or TMCA succeeded to survive till the end of the experiment. They not only

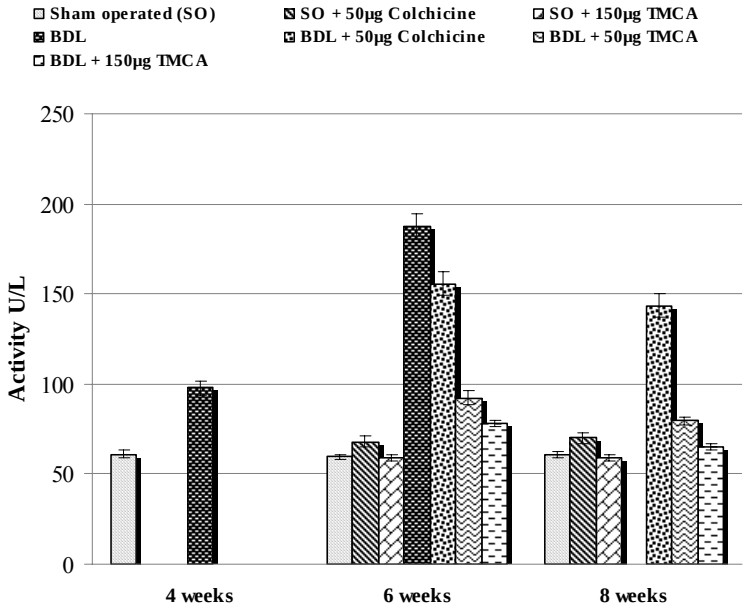

Fig. 10. Serum AST activity in different experimental groups.

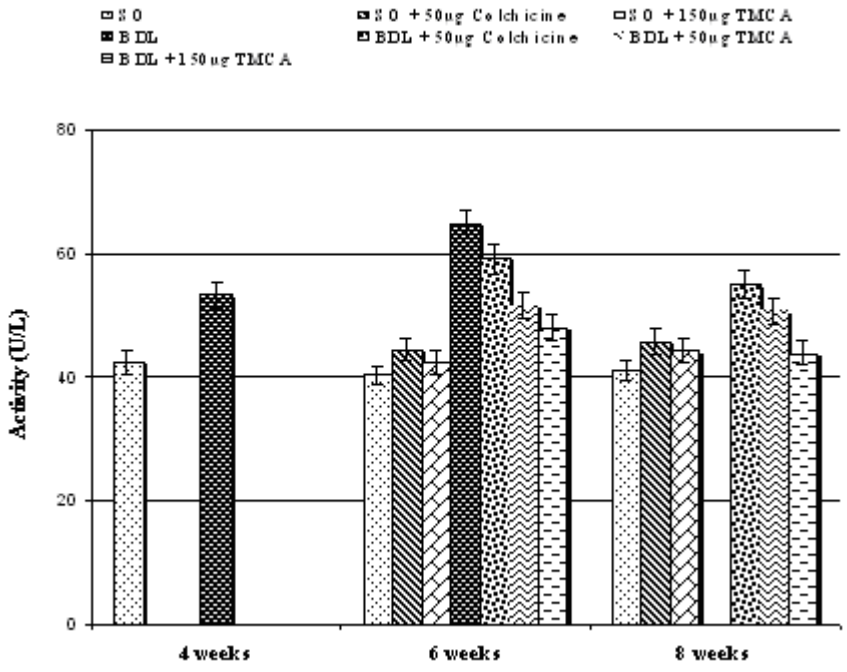

Fig. 11. Serum ALT activity in different experimental groups.

succeeded to survive but also showed marked reduction in transaminases activities.

Concerning the change in alkaline phosphatase activity, bile duct ligation for 4 or 6 weeks significantly increased serum ALP activity when compared with sham operated group at the same durations. Treatment of BDL animals with 50µg colchicine or 50µg TMCA/kg failed to reduce elevated serum ALP activity when compared with BDL rats. On the other hand, treatment of BDL rats with 150µg TMCA/kg significantly reduced elevated ALP activity when compared with BDL animals for 4 and 6 weeks respectively (Fig. 12).

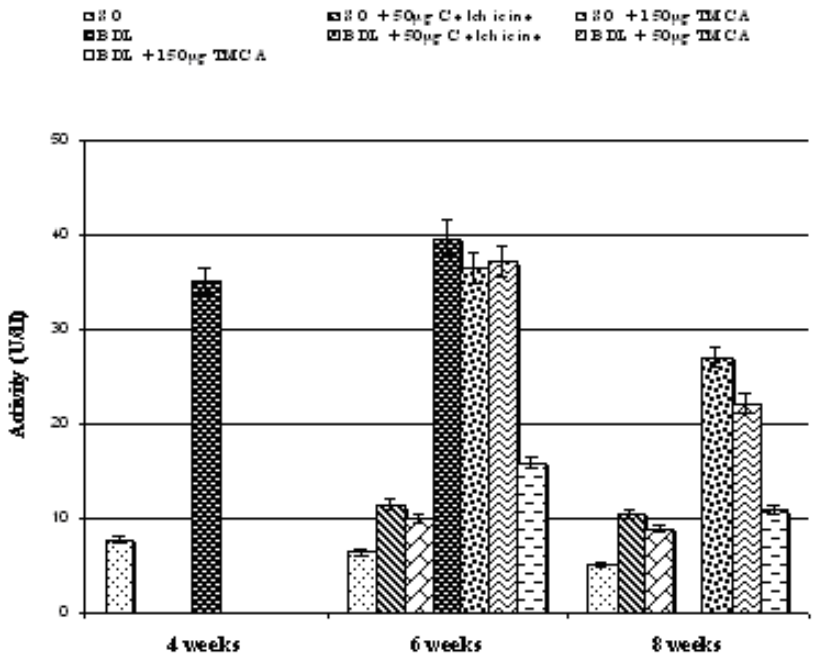

Fig. 12. Change in serum ALP activity in different experimental groups.

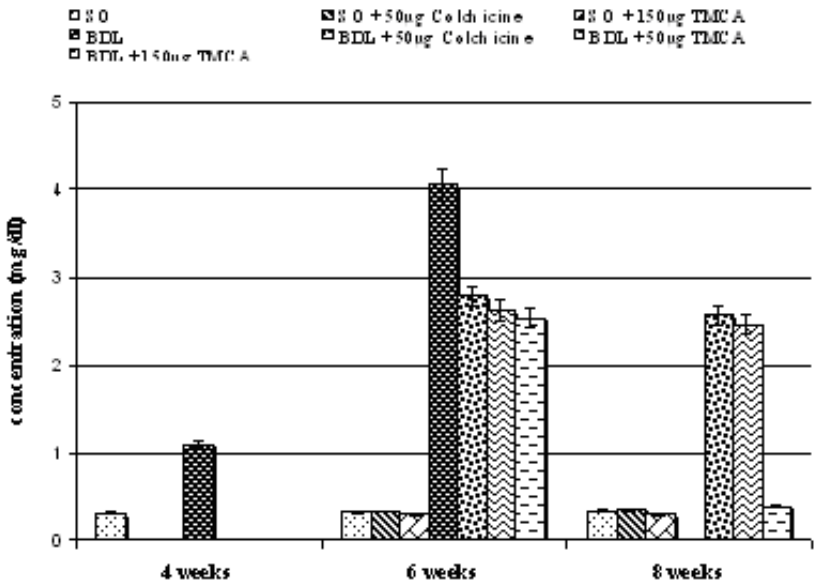

Fig. 13. Serum total Bilirubin concentration in different experimental groups.

Data in figure 13 showed that there was no significant difference in serum total bilirubin concentration between sham operated (non-treated) group and sham operated groups treated with either 50µg colchicine or 150µg TMCA/kg at the same period. Biliary obstruction increased serum total bilirubin concentration over sham operated group in the different periods. Treatment of BDL rats with 50µg colchicine or 50µg TMCA showed significant decrease in the elevated bilirubin concentration and 150µg TMCA was more efficient than 50µg colchicine and 50µg TMCA in reducing elevated total bilirubin concentration.

DISCUSSION

The animal model of common bile duct ligation has attracted considerable attention, because it simulates many histopathological features of human chronic biliary fibrosis and cirrhosis (Guyot *et al*., 2006). Results obtained in the present work revealed that biliary

obstruction in rats induced liver cirrhosis. Kountouras *et al.* (1984) reported that biliary obstruction for 4 weeks, developed cirrhosis in the majority of the animals. Histopathological changes displayed by livers of BDL rats in the present work included distortion of the hepatic lobular architecture, marked dilatation and proliferation of bile ductules with accentuated collagen deposits, degeneration, frequent areas of necrosis, enlarged Kupffer cells and dilated blood vessels. Extracellular matrix deposition increased with time and after 6 weeks of BDL fibrous tissue area expanded to occupy 85.7% of the examined liver sections. Most of these histopathological features were also previously detected by Muriel and Deheza (2003) and Prado *et al.* (2003).

Crawford (1999) reported that extrahepatic biliary obstruction results in cholestasis. The morphological features of cholestasis in human frequently include bile pigment accumulation within the hepatic parenchyma that can take on a wispy appearance (feathery or foamy degeneration) and in dilated bile canaliculi. Rupture of canaliculi, leads to bile extravasation which is phagocytosed by Kupffer cells. The author added that obstruction of the biliary tree induces distention of upstream bile ducts. The bile stasis and back pressure induce proliferation and reduplication of ducts. The labyrinthine ducts further slow the bile flow and favor the formation of concrements, which obstruct the ductal lumens.

Unrelieved obstruction leads to portal tract fibrosis which extends into and subdivides the parenchyma and develop to cirrhosis (biliary cirrhosis). In this respect, Prado *et al.* (2003) observed enlarged Kupffer cells filled with bile in long-term cholestatic rats.

In the present work, mild to moderate inflammation was seen which was also noticed by Abdel-Aziz *et al.* (1990) and Soylu *et al*. (2006). The authors observed extensive bile duct proliferation and formation of periportal fibrosis, with only slight inflammation and necrosis in their experimental model of extrahepatic cholestasis in rats.

Treatment of BDL rats with 50µg colchicine/kg for 2 weeks showed reappearance of only few scattered hepatic lobules in 50% of the cases. In bile duct ligated rats treated daily with the same dose of colchicine for 4 weeks, progress in retaining well organized hepatic lobular architecture was clearly seen. The fibrous tissue area decreased to form around 42% of the examined liver sections and appeared invaded with inflammatory cells. Similar results were obtained by Poo *et al*. (1993) who reported that early treatment of BDL rats with colchicine (50µg/kg/day, p.o.) for 4 weeks significantly increased hepatocyte and sinusoidal volume fractions than in BDL rats that received placebo. In addition, bile duct volume fractions, connective tissue fractions and portal pressure were significantly lowered than those in BDL rats received placebo. Jiang *et al.* (1996) observed that when mice infected with *Shistosoma japonicum* were treated with colchicine (0.25µg/g body weight), thick layer of inflammatory cells was found in granuloma in the liver. They suggested that colchicine can stimulate monocytes which phagocytized collagen and leads to disruption and inhibition of collagen accumulation. Colchicine is an agent that disrupts microtubules formation and inhibits collagen transport and synthesis (Rojkind and Kershenobich, 1975; Dumont *et al.*, 1994). Moreover, Warnes (1991) added that in experimental animals and *in vitro,* colchicine inhibited collagen synthesis and also increased collagen degradation by activating collagenase.

Treating BDL rats with 50µg TMCA/kg, daily for 2 weeks showed reappearance of hepatic lobules in 50% of cases. Fibrous tissue area slightly decreased to form 66% of the examined liver sections, but it was not significantly reduced as compared with colchicine treatment (68%) at the same duration. By treating BDL rats with 50µg TMCA for further 2 weeks, more improvement in liver architecture was noticed. Fibrous tissue area decreased but it is still greater than that in 50µg colchicine treated group at the same time. Colchicine and TMCA were nearly similar in their effect at the same dose level and under similar condition.

When TMCA was administered at a dose level of 150µg/kg/day for 4 weeks to sham operated rats, no side effects was noticed on the contrary with colchicine treatment (50µg/kg) under the same conditions. This indicated that higher dose of TMCA (150µg/kg) is more safe than colchicine (50µg/kg). When BDL rats treated with 150µg TMCA/kg, for 2 weeks, well formed hepatocellular lobulation and mild bile ductular proliferation was noticed in 84% of examined cases. On the other hand, after 4 weeks of treating BDL rats with 150µg TMCA/kg, all examined cases showed improvement in hepatic lobular architecture. The hepatic lobules separated with only a thin fibrous tissue bands densely infiltrated with inflammatory cells. Few bile ductules were occasionally seen and fibrous tissue area reduced more to occupy only 29.7% of the examined liver section. These results indicated that administration of TMCA at higher doses is more efficient than colchicine in treating liver cirrhosis.

Immunohistochemical data denoted that an intermediate filament protein, α-smooth muscle actin, is expressed by activated HSCs. This marker was used to identify activated HSCs. After BDL for 6 weeks, α-SMA positive cells dramatically increased in number and diffused throughout the liver tissue. They formed layers around proliferated bile ductules. This was in agreement with Kinnman *et al*. (2000) who reported that the main cell population involved in extracellular matrix deposition

following ligation of the common bile duct is activated hepatic stellate cells, reaching the periductular region by chemotaxis and migration. In the same respect, Gibelli *et al*. (2008) added that the expression of α-SMA by the stellate cells began to appear on the 5th day post BDL, and the expression increased, reaching maximum level on the 28th day.

Bile duct segments isolated from cholestatic rats exert a potent chemotactic action on HSCs (Kinnman *et al*., 2000). Cholangiocytes are known to express platelet derived growth factor in BDL model (Grappone *et al.*, 1999). Platelet derived growth factor expression may explain local accumulation of matrix-producing cells in proximity to newly formed bile ducts, as a result of chemotaxis and proliferation (Pinzani *et al.*, 1989; Kinnman *et al.,* 2001).

Colchicine treated BDL rats for 4 weeks showed decrease in α-SMA positive cells as compared with BDL group. This was in agreement with Lee *et al.* (2004) who found that treatment of cirrhotic rats with colchicine significantly decreased the number of α-SMA positive cells. They also added that colchicine at the concentration of 30-100 nM *in vitro,* notably inhibited the induction of α-SMA protein and transforming growth factor β1, indicating that colchicine directly inhibited activated stellate cells. Treating BDL rats with 50µg TMCA/kg, for 4 weeks markedly reduced the number of α-SMA positive cells. They appeared as tiny areas of mild brownish deposits. This reduction was more than the reduction observed with colchicine treatment at the same duration. BDL rats treated with 150µg TMCA/kg showed completely negative wide expanding areas of hepatic tissue. Mild linear brownish deposits were infrequently seen encircling hepatic nodules close to the hepatic capsule. These results may be explained by inactivation of hepatic stellate cells with TMCA. Iredale *et al*. (1998) added that they could not exclude the possibility of some HSCs to undergo reversion from an activated to a quiescent phenotype. However, their data suggested that the majority of loss of α-SMA positive cells is mediated via apoptosis.

Biochemical results showed significant elevation in serum alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase activities and total bilirubin concentration in BDL animals for 4 and 6 weeks when compared with the values in sham operated group at the same durations. These elevations reflect the severity of hepatic injury and cholestasis that observed in the histopathological examination in the present study.

Total bilirubin was highly increased after 4 and 6 weeks of BDL rats because obstruction of the common bile duct impaired bile flow into the intestine producing cholestasis in the liver and regurgitation into the blood stream. Crawford (1999) reported that elevated serum alkaline phosphatase is one of the characteristic findings in cholestasis, as this enzyme present in bile duct epithelium and in the canalicular membrane of the hepatocytes. Several studies recorded marked increase in serum aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase activities and bilirubin concentration in rats following BDL for 4 weeks (Marra *et al*., 2005; Fernandez-Martinez *et al*., 2006; Soylu *et al*., 2006; Reyes-Gordillo *et al.,* 2008).

The biochemical findings indicated that TMCA is better than colchicine in reducing elevated serum enzymes activities and bilirubin concentration, when used at the same dose for the same duration. Moreover, Cedillo *et al.* (1996) found that both colchicine and TMCA administered orally at a dose level of 10µg/rat/day were able to significantly reverse serum γ-GTP and alkaline phosphatase activities elevated by CCl_4- induced cirrhosis. Treating BDL animals with 150µg TMCA/kg showed marked reduction in the elevated AST, ALT, ALP activities and total bilirubin concentration when compared with BDL rats.

Thus, it is concluded from the results obtained in the present work that, trimethylcolchicinic acid at higher doses seems to be a better option than 50µg colchicine in the treatment of liver cirrhosis.

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