Original paper

Effects of folic acid on dyslipidemia and serum homocysteine in a rat model of cholestasis and hepatic fibrosis

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The liver is the major site for storage and metabolism of folate. Folate deficiency is common in many liver diseases and causes severe effects on cellular metabolism and increases oxidative stress and the homocysteine (Hcy) level. The objective of this research was to investigate the effects of folic acid on dyslipidemia and serum Hcy concentrations in an experimental rat model of cholestasis. Eighty-one male Wistar rats were divided into nine groups: control, sham-operated, folic acid control, bile duct-ligated (BDL), and BDL+ folic acid groups. In folic acid treated groups, folic acid (1, 5, and 10 mg/kg body weight) was given orally for 28 days. After taking blood and liver samples, plasma lipid profiles and Hcy and hepatic reduced and oxidized glutathione concentrations were measured. Histopathological features of cholestasis were assessed by Masson's trichrome staining. Treatment of folic acid in BDL rats significantly prevented the progression of hepatic fibrosis and improved the serum and liver biochemical changes. These results suggest that folic acid protects the liver against cholestasis by reducing serum Hcy and by its antioxidant properties. Folic acid can be an important therapeutic intervention in dyslipidemia caused by cholestasis.

Key words: bile duct ligation, folic acid, hepatic fibrosis, dyslipidemia, homocysteine.

Introduction

Cholestasis is a liver disease, which if untreated or not prevented, causes liver fibrosis and cirrhosis and eventually death [1]. As a result of bile duct ligation (BDL), toxic bile acids accumulate in the liver, causing oxidative stress, inflammation, DNA damage, cell death, hyperplasia of the bile duct, and activation of myofibroblasts [2, 3]. Increased accumulation of collagen in the extracellular matrix (ECM) causes hepatic fibrosis [2]. Hepatic fibrosis along with hepatic dysfunction alters homocysteine (Hcy) metabolism [4]. A number of studies have shown that there is a relationship between Hcy levels and hepatic diseases [5]. Increased Hcy results in elevated levels of HMG-CoA reductase and increased cholesterol biosynthesis through the activation of several transcription factors and leads to the accumulation of lipids in the liver and hypercholesterolemia [6]. On the other hand, the liver maintains cholesterol homeostasis by producing bile and converting excess cholesterol into fatty acids [7]. Acute or chronic impairment of the liver function may lead to hyperlipidemia [8]. Recent advances in medical sciences have made the treatment and pre-

vention of hepatic fibrosis achievable [9]. Folic acid (vitamin B_9) is a synthetic, oxidized form of folate with high bioavailability [10]. Because it is not synthesized in humans, oral supplementation of folate is necessary [11]. Folate is not only a structural component of cells but is also essential for the regulation of Hcy, DNA and RNA metabolism, and methylation of multiple proteins [10]. The liver is the main organ for folate storage and metabolism, and bile flow plays an important role in the folate hepatobiliary circulation [12]. Because liver disease causes defects in the formation of 5-methyl tetrahydrofolate (5-MTHF), the active metabolite of folate, [13] folate deficiency is a common occurrence in many liver diseases [10]. In addition to severely affecting cellular metabolism, folate deficiency causes structural changes in the DNA, neurological diseases, increased oxidative stress in the liver tissue, membrane defects due to hypomethylation of phospholipids, and increased Hcy levels [10, 12]. The main objective of this study was to assess the effects of folic acid supplementation on changes in liver pathology, dyslipidemia, and serum homocysteine concentrations in a rat model of hepatic fibrosis induced by BDL.

Material and methods

Animals

Eighty-one male Wistar rats weighing 260-320 g with an average age of 12 weeks were used in this study. To avoid stress and to adapt to the environment, all animals were transferred to an environment of standard experimental conditions (22 ±2°C, 56% ±2 humidity, 12 h light/dark cycle) 7 days before tests. Animals were given free access to food pellets and water. The study and protocols were approved by the ethics committee of the Islamic Azad University. The study was conducted in accordance with the guidelines of the International Committee for the Protection of Laboratory Animals (the United States National Institutes of Health, 1985).

Experimental procedures

Animals were classified into the following nine experimental groups (n = 9): (1) control; (2) sham-operated (these animals underwent the same surgical procedure without interruption of the bile duct to investigate the potential stress associated with surgery); (3-5) folic acid control; (6) BDL; (7-9) BDL plus folic acid. Folic acid treatment groups were given folic acid orally (1, 5, and 10 mg/kg body weight) once a day by gavage for 28 days. The treatment began after BDL. Folic acid (Sigma, Louis, USA) was freshly dissolved in 0.5 ml sterile 0.9% saline just before the experiment. Control groups received 0.5 ml sterile 0.9% saline as vehicle.

Bile duct ligation method

Bile duct ligation was performed according to standard protocols [14] as follows: (I) deep anesthesia with intra-peritoneal administration of ketamine (90 mg/kg body weight) and xylazine (10 mg/kg body weight); (II) after disinfection, the abdominal cavity was opened by midline incision; (III) the common bile duct was found and blocked by 4-0 silk suture in two places (toward the end of the intra-hepatic bile duct and before the entrance of the pancreatic duct); then, the space between the two sites was discontinued; (IV) 3 cc of saline was poured into the abdominal cavity, the muscle was sutured with absorbable 4-0 vicryl and the skin incision was closed with non-absorbable 3-0 nylon suture; (V) oxy-tetracycline spray was used to prevent the rats from chewing the stitches. All animals survived throughout the experiment.

Preparation of samples

After 28 days of treatment, animals were fasted for 14 h and anesthetized by diethyl ether. Immediately after anesthesia, blood samples were collected via cardiac puncture and the livers were excised. After clotting, blood samples were centrifuged for 20 minutes at 37°C. The serum collected was used for lipid profiling and to quantify Hcy levels. The left lobe of the liver was cut into two pieces after washing with saline. One piece was immediately frozen for assessing the reduced glutathione (GSH) and oxidized glutathione (GSSG) levels, and the other piece was fixed with 10% formalin for histological evaluation of hepatic fibrosis.

Plasma lipid profiles and total homocysteine (Hcy) analysis

Levels of triglycerides (TG), total cholesterol (TC), and high-density lipoprotein (HDL) were measured by colorimetric assay kits (Pars Azmoon, Tehran, Iran) according to the manufacturer's instructions. An autoanalyzer (Selectra2, Netherlands) was used to analyze samples. Low-density lipoprotein (LDL) concentration was calculated by the Friedewald formula [15]. Serum total Hcy concentrations were determined by HPLC (high performance liquid chromatography) equipped with a fluorometric detection system [16].

Assay of hepatic reduced (GSH) and oxidized (GSSG) glutathione concentrations

GSH and GSSG concentrations were determined by standard fluorometric methods as follows: For GSH, approximately 250 mg of hepatic tissue was homogenized in 3.75 ml of cold EDTA buffer. The homogenate was centrifuged for 20 minutes at 8000 rpm and 4°C. Glutathione concentration in the supernatant was estimated by measuring the absorbance at 412 nm

after diluting the sample with EDTA buffer and O-phthalaldehyde (OPT). The concentration of GSH was expressed as nmol/mg protein [4]. To measure the amount of GSSG, one portion of the supernatant was incubated for 45 min with 2-vinyl pyridine. After the incubation, the sample was added to a reaction mixture containing 0.2 mol/l sodium phosphate buffer (PBS), 0.2 mmol/l NADPH, 0.1 mmol/l DTNB, and 1.25 units of glutathione reductase. Following incubation for 10 minutes at room temperature, the absorbance of the mixture at 412 nm was measured [17].

Histopathological evaluation

Masson's trichrome staining was performed on the paraffin-embedded, formalin-fixed liver tissue to assess fibrosis and other histopathological features of cholestasis [18]. Images of the stained samples from 5 non-overlapping fields were acquired with the help of an optical microscope (Motic, Spain) and were examined semi-quantitatively. The METAVIR scoring system was used to determine fibrosis as follows: (0) normal liver; (1) expansion into some portal areas; (2) incomplete expansion with only one septa; (3) incomplete cirrhosis with several well-formed but thin septa; and (4) complete cirrhosis with thick septa and ductular reaction [19]. All other features of cholestasis were evaluated as follows: Inflammatory cell infiltration: (0) lack of inflammation; (1) presence of local inflammatory cells in less than 25% of hepatic tissue; (2) presence of local inflammatory cells in 25-50% of hepatic tissue; (3) local, but extensive, presence of inflammatory cells in 51-75% of hepatic tissue; (4) global inflammatory cell infiltration in hepatic tissue [20]. Necrosis: (0) no cell damage; (1) local damage on less than 25% of the hepatic tissue; (2) local damage on 25-50% of the hepatic tissue; (3) extensive but localized damage to hepatic tissue; and (4) global necrosis of hepatic tissue [21]. Bile duct hyperplasia: (0) no ductal hyperplasia; (1) hyperplasia limited to the portal space and present in less than 25% of each hepatic lobule; (2) hyperplasia limited to the septa and present in 25-50% of each hepatic lobule; (3) extensive but localized hyperplasia; and (4) global extensive hyperplasia [2, 22].

Statistical analysis

Statistical analysis was conducted using one-way ANOVA followed by Tukey's test. Results were expressed as means ± SEM. All statistical analyses were performed using the SPSS software version 20. $P < 0.05$ was considered statistically significant.

Results

Effect of folic acid on histopathology of livers in bile duct ligated rats

Results of histopathological examination are shown in Table I. Hepatocytes, sinusoids, and portal area in rats of control, sham, and treated control groups had normal histopathological features. Incomplete cirrhosis of the lobular septa (grade 3) with bile duct hyperplasia, infiltration of inflammatory cells, and tissue necrosis were observed in the livers of BDL rats (Fig. 1). Folic acid treatment reduced collagen levels and limited the fibrosis to the portal space and a small number of lobular septa (grade 1-2). Other features

Table I. Histopathological scores of hepatic fibrosis under different doses of folic acid in BDL rats

Groups	Index			
	Necrosis	Infiltration of inflammatory cells	Bile duct hyperplasia	Fibrosis
Normal control	0	0	0	0
Sham operated	0	0	0	0
Folic acid (mg/kg) control				
1	0	0	0	0
5	0	0	0	0
10	0	0	0	0
BDL control	3.0 ±0.26***	3.0 ±0.00***	3.0 ±0.00***	3.0 ±0.29 ***
BDL+ folic acid (mg/kg)				
1	3.0 ±0.25***	2.2 ±0.21***$^{+}$	2.3 ±0.21***$^{+}$	2.2 ±0.17***$^{+}$
5	2.0 ±0.25***$^{+}$	1.8 ±0.30***$^{++}$	1.0 ±0.00***$^{+++}$	1.8 ±0.30***$^{++}$
10	1.0 ±0.00***$^{+++}$	1.3 ±0.21***$^{++}$	1.0 ±0.00***$^{+++}$	1.0 ±0.00***$^{+++}$

Data are expressed as mean ± S.D. (n = 9)

****$p < 0.001$ in comparison with normal control group*

$^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ in comparison with BDL control group.

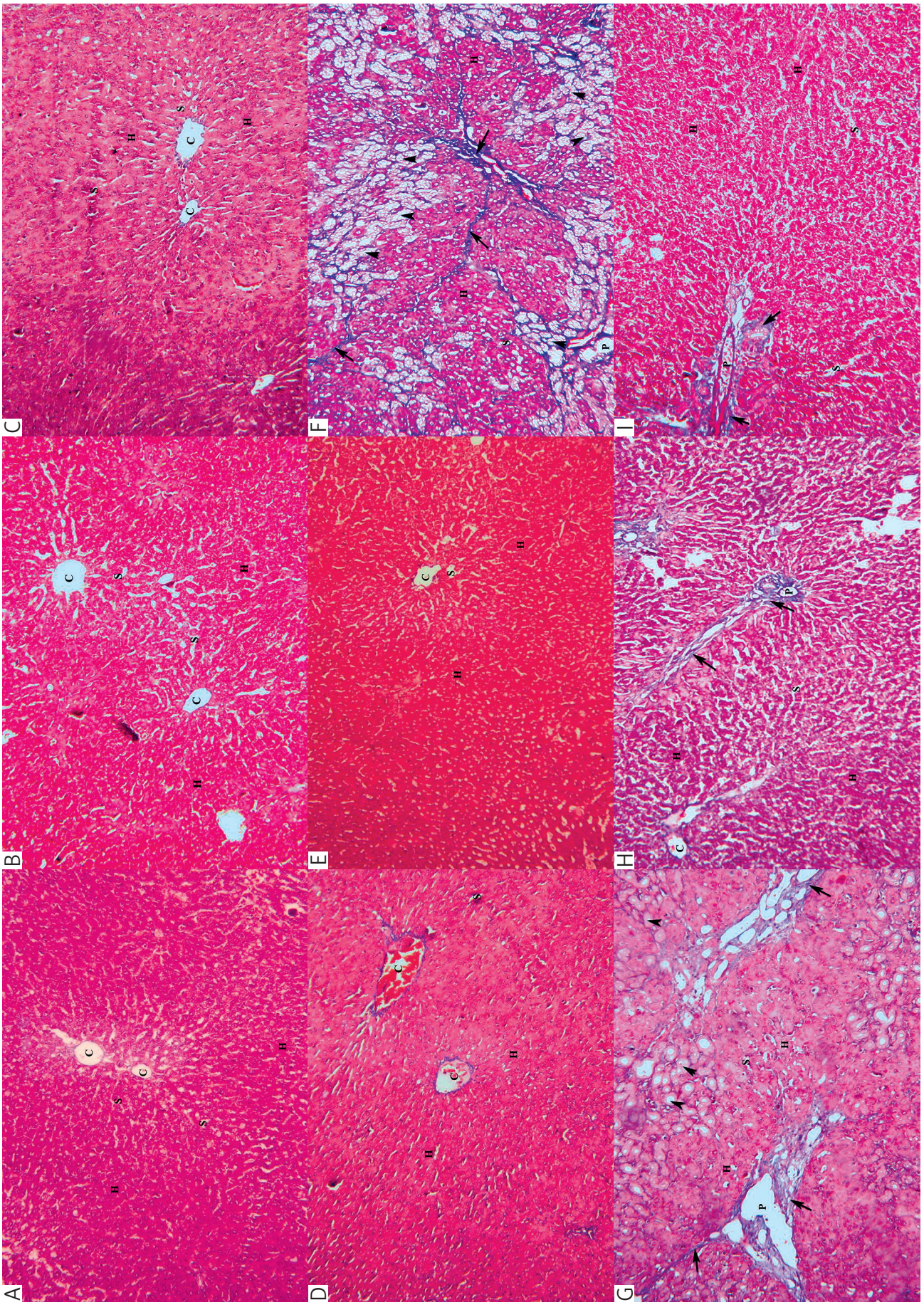

Fig. 1. Histopathological images of hepatic fibrosis in normal and BDL rats. (A and B) normal control and sham operated groups, respectively; (C, D and E) folic acid (1, 5 and 10 mg/kg body weight, respectively) treated control groups; (F) BDL group; (G,H and I) BDL rats treated with folic acid (1, 5 and 10 mg/kg body weight, respectively) groups; (C) central vein; (H) hepatocyte; (S) sinusoidal area; and (P) peri-portal area. (Arrow head) bile duct hyperplasia; and (arrow) incomplete fibrotic septa are shown in BDL rats (Masson's trichrome, ×10)

Table II. Effects of folic acid on serum Hcy and hepatic concentrations of GSH, GSSG and GSH : GSSG in control and BDL rats.

Groups	Index			
	Hcy (M/L)	GSH (nmol/mg protein)	GSSG (nmol/mg protein)	GSH : GSSG
Normal control	4.22 ±0.10	33.33 ±1.20	3.01 ±0.15	11.17 ±0.66
Sham operated	4.38 ±0.15	34.00 ±1.32	3.12 ±0.13	11.02 ±0.72
Folic acid (mg/kg) control				
1	4.13 ±0.12	36.16 ±1.49	2.98 ±0.10	13.41 ±0.34*
5	3.77 ±0.19**	37.17 ±1.83	2.67 ±0.11	14.06 ±0.93*
10	3.35 ±0.18***	39.17 ±1.58**	2.52 ±0.16	15.88 ±1.28*
BDL control	5.95 ±0.35***	18.66 ±2.19***	6.32 ±0.25***	2.89 ±0.36***
BDL + folic acid (mg/kg)				
1	5.75 ±0.20***	20.66 ±1.78**	6.33 ±0.27***	3.27 ±0.29***
5	4.78 ±0.21^{+}	29.00 ±1.86^{++}	4.20 ±0.29**$^{++}$	7.38 ±0.77**$^{++}$
10	4.43 ±0.15^{++}	34.67 ±2.58^{++}	3.57 ±0.28^{+++}	10.05 ±1.19^{+++}

Data are expressed as mean ± SD (n = 9).
Hcy – homocysteine; GSH – reduced glutathione; GSSG – oxidized glutathione; GSH : GSSG – GSH to GSSG ratio
**p < 0.05, **p < 0.01 and ***p < 0.001 in comparison with normal control group*
$^{+}$p < 0.05, $^{++}$p < 0.01 and $^{+++}$p < 0.001 in comparison with BDL control group

of extra-hepatic cholestasis were also significantly less evident in folic acid-treated animals. The 10 mg/kg body weight dose was the most effective in reducing hepatic fibrosis induced by cholestasis.

Effects of folic acid on hepatic concentrations of GSH and GSSG

Compared with the control group, BDL rats showed significantly increased GSSG and decreased GSH resulting in a decreased GSH to GSSG ratio, indicating increased oxidative stress in the BDL group (Table II). Treatment of the BDL rats with 5 and 10 mg/kg body weight doses of folic acid significantly increased GSH levels and the GSH to GSSG ratio, and reduced the level of GSSG in the liver. In normal rats, at all doses, folic acid significantly increased the GSH to GSSG ratio. Additionally, compared with the control group, a single 10 mg/kg body weight dose of folic acid caused a significant increase in the hepatic GSH concentration. These results suggested that folic acid participated in GSH metabolism.

Effect of folic acid in plasma total Hcy concentration

Four weeks after the BDL, serum Hcy concentration was significantly high in BDL rats compared with that in the control animals (Table II). Treatment of BDL rats with 5 and 10 mg/kg body weight doses of folic acid restored the serum Hcy concentration to normal. Compared with non-treated control animals, at these doses, folic acid significantly reduced the Hcy levels in the control animals.

Effects of folic acid in plasma lipid profiles

Hypolipidemic effects of folic acid supplementation are shown in Table III. The results showed that compared with the control rats, BDL caused a significant increase in serum triglyceride, cholesterol, and LDL, and a significant decrease in serum HDL concentrations. At 5 and 10 mg/kg body weight doses, folic acid significantly reversed these changes in lipid profiles induced by BDL. Additionally, a 10 mg/kg body weight dose of folic acid significantly reduced the levels of TG, TC, and LDL, and increased the level of HDL in treated control rats compared to those in the control group. The 5 mg/kg body weight dose of folic acid in treated control rats only caused a significant reduction in serum LDL concentration compared with that in the control group.

Discussion

The results of the present study show that 28-day treatment of folic acid supplementation significantly prevents the progression of hepatic fibrosis in BDL rats. Bile duct-ligated causes bile duct hyperplasia, extended fibrosis to the portal tract, and extensive infiltration of inflammatory cell into the liver parenchyma. HSC also causes the accumulation of collagen in the ECM during tissue repair responses, such as fibrosis [23]. Some studies suggest that HSC stimulates leukocyte chemotaxis by producing chemotactic factors and adhesive molecules such as MIP-2, ICAM-1, and VCAM-1. It has been found that leukocytes, potentially by producing pro-fibrotic mediators such

Table III. Effects of folic acid on plasma lipid profiles in control and BDL rats.

Groups	Index			
	TG (mg/dl)	**TC** (mg/dl)	**HDL** (mg/dl)	**LDL** (mg/dl)
Normal control	61.33 ±1.49	57.00 ±1.63	21.01 ±0.97	23.33 ±0.42
Sham operated	64.50 ±1.80	56.00 ±1.59	22.00 ±1.06	21.33 ±0.42
Folic acid (mg/kg) control				
1	64.01 ±1.77	55.00 ±1.53	22.10 ±1.12	20.33 ±0.43
5	66.16 ±1.75	53.17 ±1.11	23.67 ±0.99	16.33 ±0.76***
10	56.83 ±1.40 *	51.33 ±1.15*	25.01 ±0.58 *	14.83 ±0.94***
BDL control	116.01 ±6.57 ***	78.00 ±2.86***	13.00 ±1.21***	41.33 ±2.59 ***
BDL + folic acid (mg/kg)				
1	102.00 ±4.66 ***	73.00 ±2.84***	12.17 ±1.01**	40.50 ±3.06***
5	83.16 ±5.74 **$^{++}$	62.00 ±3.59^{++}	19.10 ±1.67 $^{+}$	26.00 ±4.20 $^{+}$
10	85.83 ±4.65 **$^{++}$	61.00 ±1.18^{+++}	19.83 ±1.28 $^{++}$	24.00 ±1.63 $^{++}$

Data are expressed as mean ± SD (n = 9).
TG – triglyceride; TC – total cholesterol; HDL – high-density lipoprotein; LDL – low-density lipoprotein
$*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ in comparison with normal control group
$^{+}p<0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ in comparison with BDL control group

as TGF-β1, affects HSCs and enhances ECM synthesis in various liver diseases [24]. Folic acid plays an important role as a potent antioxidant by scavenging free radicals and reducing oxidative stress, [25] to reduce the hepatic tissue damage in rats treated with carbon tetrachloride, [26] receiving a high-fat diet, [17] under chronic treatment with ethanol, [27] and receiving methotrexate [12]. Studies have shown that antioxidant supplements can significantly inhibit hepatic necrosis [28]. Thus, folic acid has a hepatoprotective effect and significantly prevents cholestatic liver fibrosis. By limiting oxidative damage, folic acid reduces the severity of the inflammatory response and prevents fibrogenesis. Glutathione acts as a potent non-enzymatic antioxidant in the liver [17] and is a major component of the cellular defense mechanism against oxidative stress in BDL rats [22].

Several factors may have contributed to the reduced activity of GSH in BDL rats: (I) it may be inhibited by superoxide anions; (II) lipid hydrogen peroxides may inhibit glutathione peroxidase enzyme by binding to its GSH binding site; [22] (III) it can be affected by the flow of bile thiol or mediated by the induction of pro-inflammatory cytokine; (IV) reduced availability of amino acids required for GSH synthesis or defects in the trans-sulfuration pathway [29]. By eliminating free radicals and increasing acetaldehyde oxidation, folic acid prevents the inactivation of GSH [30]. Folic acid may act as a coenzyme for enzymes involved in the trans-sulfuration pathway and increases GSH synthesis and GSH levels [26] The lower GSH to GSSG ratio in BDL rats indicates oxidative stress [4, 17]. Folic acid significantly improves this ratio by reducing the amount of oxidized glutathione and maintains the antioxidant defense in the liver [17]. The finding that folic acid, at the doses used in this study, significantly increases the GSH to GSSG ratio in healthy rats suggests that folic acid may have antioxidant effects. There is a direct relationship between Hcy levels, oxidative stress, DNA damage, and apoptosis [22]. *In vitro* studies suggest that Hcy may induce hepatic fibrosis. Elevated plasma Hcy levels have been reported in cirrhosis of the liver in humans and in experimental models treated with ethanol and carbon tetrachloride [4]. Our data are not consistent with those obtained by Ebrahimkhani *et al.* (2005) [4]. Several studies have shown that folate deficiency or mutations in enzymes involved in Hcy catabolism can result in increased Hcy levels [31]. Our results indicate an association of Hcy concentration with the tissue GSH concentration [32]. It is likely that folate stimulates GSH through the transsulfuration pathway from Hcy [10] and protects the liver by reducing serum Hcy [5]. Increased free radical damage in cholestasis can impair lipid uptake and metabolism and decrease the synthesis or activity of enzymes involved in the metabolism of lipoproteins [33]. Hyperlipidemia results from an increase in lipid synthesis or a decrease in their metabolism [8]. Hypercholesterolemia can result from defects in the clearance of cholesterol and bile salts through bile flow and increase cholesterol in the blood stream [34]. The decrease in HDL during cholestasis can be attributed to increased HDL clearance or its decreased synthesis [35]. Folic acid is involved in various steps of lipid metabolism and reduces free fatty acid concentrations in the blood by inhibiting adipose tissue lipolysis [36]. As a result,

fewer fatty acids for the production of cholesterol, triglyceride, and LDL are transported to the liver, resulting in reduced synthesis of the lipids. These results are consistent with findings from experimental models treated simultaneously with carbon tetrachloride and folic acid [26]. A reduction in serum cholesterol levels can result in a decrease in intra-hepatic circulation of bile acids and prevent production of excessive bile [37]. Minimizing the liver damage caused by toxic bile acids can inhibit the alteration of lipid metabolism [38]. On the other hand, the accumulation of cholesterol during cholestasis leads to an increase in cholesterol content of the hepatic cell membrane, dysfunction in integral protein of membrane and a decrease in membrane fluidity [3]. Folic acid prevents this and helps to maintain the integrity of the hepatic cell membrane. Folic acid may increase protein synthesis by reducing damage to tissue, [39] and consequently increase HDL synthesis in BDL rats. Also, increased HDL can have a protective effect on liver cells [33] and prevent further damage. Decreased cholesterol synthesis and increased oxidation of free radicals involved in the oxidation of LDL [40] following treatment with folic acid can reduce LDL levels.

In conclusion, folic acid prevents the progression of hepatic fibrosis and protects the liver against cholestasis by reducing serum Hcy and by its antioxidant properties. Our results indicate that folic acid could be used as an important therapeutic intervention in dyslipidemia caused by cholestasis.

The authors declare no conflict of interest.

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