

Hepatoprotective and antioxidant effects of lycopene in acute cholestasis

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Background/aim: Lycopene, which is suggested to be a potent antioxidant, may play a protective role in diseases related to oxidative stress. In order to understand the effects of lycopene in the pathogenesis of cholestasis, we investigated the effects of lycopene on oxidative stress parameters and DNA damage induced by experimental biliary obstruction in the liver tissues and the lymphocytes of Wistar albino rats.

Materials and methods: The animals were randomized into 3 groups. The sham group was subjected to a sham operation, the BDL group was subjected to bile duct ligation (BDL), and the BDL+L group was subjected to BDL and treated with 10 mg/kg body weight of lycopene. After 7 days of treatment, the liver functions, oxidative stress parameters, and DNA damage were evaluated.

Results: The lycopene treatment significantly ameliorated the liver function parameters in BDL rats. It significantly reduced malondialdehyde and nitric oxide levels and enhanced reduced glutathione levels and catalase, superoxide dismutase, and glutathione S transferase activities in the BDL rats. The lycopene treatment also decreased DNA damage as assessed by comet assay in the lymphocytes and hepatocytes of the BDL rats.

Conclusion: These results suggest that lycopene might have protective effects on acute cholestasis.

Key words: Lycopene, cholestasis, DNA damage, oxidative stress, peripheral lymphocytes, liver

1. Introduction

Many clinical conditions, including strictures of the bile duct, gallstone complications of pancreatitis, or biliary surgery, are cited among etiologies that lead to a decrease in biliary flow, causing cholestasis (1). Cholestasis leads to acute hepatic injury and results in progressive fibrosis (2). The production of free oxygen radicals has been suggested to play an important role in the pathogenesis of cholestasis. One of the significant endpoints leading to liver damage arising from cholestasis is considered to be intrahepatic accumulation of toxic bile salts and inflammatory cells (3). A validated experimental model of acute cholestasis in rats can be presented by bile duct ligation (BDL) (4).

Lipid peroxidation may result from the excessive production of reactive oxygen species (ROS), which may

then interrupt the integrity of the cellular membranes and cause further hepatic injury in cholestasis (5–7). In cholestasis, the decline in the activity of glutathione peroxidase (GSH-Px), as well as the decline in reduced glutathione (GSH), may damage the antioxidant defense system (7). Altered gene expression and DNA base damage are the results of ROS; these may lead to the development of cancer and mutation (8).

Useful effects of antioxidants in cholestasis have been reported in several experimental models (9). Being a natural antioxidant carotenoid, lycopene is suggested to be one of the most powerful antioxidants (10). Animal studies have indicated a large margin of safety for lycopene. An oral 90-day toxicity study in Wistar albino rats demonstrated the daily NOAEL of lycopene to be 586

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mg/kg body weight for males and 616 mg/kg body weight for females (11).

In many studies, lycopene's possible protective role against some oxidative stress-related organ toxicities and diseases through the protecting of biomolecules such as DNA, proteins, and lipids has been reported (10,12–14). In addition to the antioxidant activity of lycopene, other possible mechanisms, such as the activation of the immune system and metabolic pathways and the modulation of gap junction communication, can also play important roles in its protective activity (15).

In our previous study, we identified changes in the oxidative stress parameters and DNA damage in the peripheral lymphocytes, the whole blood cells, and the liver and kidney tissues of rats given a daily dose of 100 mg/kg lycopene for 14 days in an experimental obstructive jaundice model (16). In this study, we aimed to evaluate the protective effects of a 7-day treatment of 10 mg/kg lycopene on oxidative stress and DNA damage in the lymphocytes and the liver cells of bile duct-ligated Wistar albino rats.

2. Material and methods

2.1. Animals and experimental design

A total of 24 healthy adult male Wistar albino rats (*Rattus norvegicus*) weighing 200–250 g, housed in stainless steel cages under a controlled temperature (22 °C) and humidity (55%–60%) and with a 12-h light/dark cycle, were used in this study. Standard laboratory rat feed containing 21% protein and fresh drinking water were given ad libitum before and after the operation. The animals were treated humanely with regard to alleviation of suffering, and the study protocol was designed according to the ethical standards for animal use and approved by the Ankara Education and Research Hospital Ethics Committee of Animal Use (25-29/01/2011).

All surgical procedures were performed under anesthesia by intraperitoneal injection of 80 mg/kg ketamine hydrochloride (Ketalar, İstanbul, Turkey), plus 10 mg/kg xylazine. A midline incision was made under sterile techniques. After a midline laparotomy of 1–2 cm, the common bile duct was identified, doubly ligated using 5-0 silk sutures, and transected at the level 0.7–0.8 cm distal to the last bifurcation (17).

The animals were randomized into 3 groups with 8 in each group. The sham group was subjected to a sham operation and treated with 0.5 mL of maize oil intragastrically (i.g.) once daily. The BDL group was subjected to BDL and was treated with 0.5 mL of maize oil i.g. once daily. The BDL+L group was subjected to BDL and was treated with a suspension of 10 mg/kg body weight of lycopene (Sigma, St. Louis, MO, USA) in 0.5 mL of maize oil i.g. once daily, as described in a previous study

(18). All treatments started 3 days before the operation and continued 7 days after the operation.

Blood samples via cardiac puncture were collected in preservative-free heparin tubes for biochemical evaluation and DNA damage analysis. The heparinized blood samples were kept in the dark at 4 °C and were processed within 6 h. The liver tissues were carefully dissected from their attachments and were totally excised. Excised tissues were divided into 3 parts for histopathological examination, DNA damage analysis, and determination of antioxidant parameters.

2.2. Biochemical analyses

Plasma from heparinized blood samples was examined for direct bilirubin (D-Bil), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT), and alkaline phosphatase (AP) using spectrophotometric analysis as the indicator of the hepatic functions with standard diagnostic kits (Roche Diagnostics, Mannheim, Germany) and a Roche modular P800 clinical chemistry analyzer.

2.3. Determination of oxidative stress parameters in the liver tissues

The liver tissue was extracted following a homogenization and sonication procedure (19). The levels of malondialdehyde (MDA), GSH, nitric oxide (NO), superoxide dismutase (SOD), catalase (CAT), and glutathione-S-transferase (GST) in the liver tissue homogenates were analyzed.

MDA levels, as a biomarker of lipid peroxidation, were determined spectrophotometrically by measuring thiobarbituric acid-reactive substances (TBARS). The results were expressed as pmol/mg protein.

GSH levels were determined spectrophotometrically with a GSH assay kit (Cayman Chemicals Co., Ann Arbor, MI, USA) at 405 nm according to the manufacturer's instructions. NO levels were determined using a nitrate/nitrite colorimetric assay kit (Cayman Chemicals Co.) at 550 nm according to the manufacturer's instructions. Results were expressed as nmol/mg tissue.

The determination of tissue CAT, SOD, and GST level activities was performed with a CAT colorimetric assay kit (Sigma-Aldrich, St Louis, MO, USA) at 520 nm, a SOD assay kit (Cayman Chemicals Co.) at 440 nm, and GST assay kit (Cayman Chemicals Co.) at 340 nm, respectively, according to the manufacturer's instructions. Results were expressed as U/mg protein.

Protein concentrations of the tissue homogenates were determined using the method described by Lowry et al. (20).

2.4. Determination of DNA damage

Single-cell gel electrophoresis (comet assay) was employed to assess the DNA damage. We followed the fundamental alkaline technique of Singh et al. (21), which was further

described by Anderson et al. (22) and Collins et al. (23). The separation of lymphocytes from the whole heparinized blood was done using the Ficoll-Hypaque density gradient and centrifugation (24). Afterwards, the cells were washed using PBS buffer. We put a small piece of liver tissue into 1 mL of cold Hanks Balanced Salt Solution (HBSS) with 20 mM EDTA/10% DMSO, and then we cut the tissue into tiny pieces. The pieces were allowed to settle, and the supernatant containing the single-cell suspension was taken. The concentrations of lymphocytes and the hepatocytes using PBS buffer were adjusted to around 2×10^6 cells/mL. The cells were inserted into agarose gel and, in order to form a comet, were lysed. Fragmented DNA strands were pulled out using electrophoresis (alkaline conditions). The details of comet assay protocol were published previously (16).

2.5. Histopathological evaluation

Excised tissue specimens were fixed in 10% formalin processed in graded alcohol and xylene and were then embedded in paraffin. The sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome according to the standard procedure for routine histopathological examination under a light microscope (Olympus BX51, Hamburg, Germany). The histological examination was performed by an experienced histologist who was uninformed about of the treatment groups.

Portal inflammation, focal necrosis, and ductal proliferation were examined according to the modified histological activity index score proposed by Knodell et al. (25).

2.6. Statistical analysis

Statistical analysis was performed with SPSS 15.0 for Windows. The differences between the means of data were compared by one-way variance analysis (ANOVA) test and post hoc analysis of group differences was performed by least significant difference (LSD) test. The Kruskal-Wallis H test was used in comparing parameters displaying abnormal distribution between groups. The results were

given as the mean \pm standard deviation, and $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Chemical findings

The plasma biochemical parameters as markers of hepatocellular damage are shown in Table 1. D-Bil, AST, ALT, GGT, and AP levels were found to be significantly higher in the BDL group ($P < 0.001$) and the lycopen-treated BDL group ($P < 0.001$) compared to the sham group. However, these parameters in the BDL+L group were found to be significantly lower than in the BDL group ($P < 0.001$).

3.2. Oxidative stress parameters in the liver tissues

Hepatic MDA and NO levels were found to increase significantly in the BDL group compared to the sham group ($P < 0.001$) (Table 2). These levels were found to be significantly decreased in the BDL+L group compared to the BDL group ($P < 0.001$) (Table 2). There was no significant difference between the sham group and the BDL+L group ($P = 0.786$ for MDA levels; $P = 0.812$ for NO levels) (Table 2).

GSH levels were found to decrease significantly in the BDL group compared to the sham group ($P = 0.008$). These levels were found to be significantly increased in the BDL+L group compared to the BDL group ($P = 0.012$) (Table 2). There was no significant difference between the sham group and the BDL+L group ($P = 0.681$).

The levels of hepatic CAT, SOD, and GST were found to decrease significantly in the BDL group compared to the sham group ($P < 0.001$) (Table 2). These levels were found to be significantly lower in the BDL group than the BDL+L group ($P < 0.001$ for CAT and SOD levels; $P = 0.011$ for GST levels) (Table 2). However, there was no significant difference in these enzyme levels between the sham and the BDL+L groups ($P = 0.641$ for CAT level; $P = 0.875$ for SOD levels; $P = 0.750$ for GST levels) (Table 2).

Table 1. Plasma biochemical measurements.

	Sham group	BDL group	BDL+L group
D-Bil (mg/dL)	0.07 \pm 0.03 ^a	15.1 \pm 4.2 ^b	7.5 \pm 1.5 ^c
AST (U/L)	114.0 \pm 3.9 ^a	672.0 \pm 102.8 ^b	319.7 \pm 141.8 ^c
ALT (U/L)	64.4 \pm 18.1 ^a	276.7 \pm 68.3 ^b	142.7 \pm 42.6 ^c
GGT (U/L)	3.3 \pm 3.1 ^a	80.6 \pm 13.5 ^b	26.4 \pm 15.3 ^c
AP (U/L)	341.2 \pm 66.9 ^a	772.0 \pm 107.5 ^b	582.0 \pm 74.1 ^c

The results are given as the mean \pm standard deviation for 8 rats in each group. Values with superscripts of different letters differ significantly ($P < 0.05$) from each other within the same row.

Table 2. Hepatic MDA, GSH, NO, CAT, SOD, and GST levels.

	Sham group	BDL group	BDL+L group
MDA (pmol/mg protein)	73.2 ± 10.9 ^a	107.2 ± 14.9 ^b	64.5 ± 16.9 ^a
GSH (nmol/mg tissue)	3.0 ± 1.3 ^a	0.9 ± 0.9 ^b	2.4 ± 1.0 ^a
NO (nmol/mg tissue)	68.0 ± 8.9 ^a	113.8 ± 17.5 ^b	73.2 ± 3.6 ^a
CAT (U/mg protein)	135.0 ± 21.4 ^a	69.8 ± 7.5 ^b	116.6 ± 9.2 ^a
SOD (U/mg protein)	76.0 ± 12.3 ^a	35.4 ± 6.6 ^b	71.5 ± 11.0 ^a
GST (U/mg protein)	0.5 ± 0.1 ^a	0.2 ± 0.1 ^b	0.4 ± 0.1 ^a

The results are given as the mean ± standard deviation for 8 rats in each group. Values with superscripts of different letters differ significantly ($P < 0.05$) from each other within the same row.

3.3. Assessment of DNA damage

BDL seemed to induce DNA damage in the lymphocytes and the hepatocytes because all the parameters studied were found to increase in the BDL group compared to the sham group ($P < 0.001$) (Figure 1). Lycopene seemed to significantly reduce the DNA damage, since all of the parameters studied in all samples were found to decrease significantly in the BDL+L group when compared to the BDL group ($P < 0.001$).

3.4. Histopathological findings

The histological structure of the liver was observed to be normal in the sham group, as seen in Figure 2A and Figure 2B. There was edema and widening of the portal tracts due to extensive bile ductal proliferation (Figure 2C). Inflammatory infiltration, including neutrophil leukocytes, in the portal tracts was also prominent in the BDL group (Figure 2C). Focal necrosis was observed in the BDL group (Figure 2D). Portal inflammation and

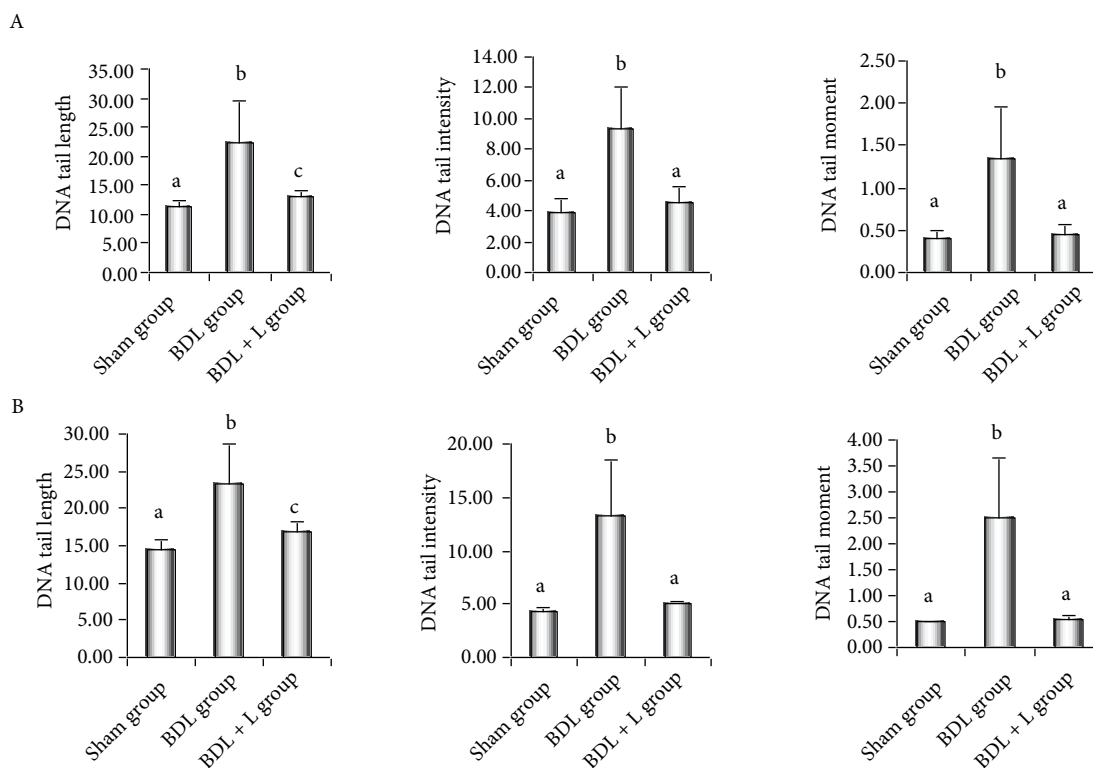


Figure 1. The effects of lycopene on cholestasis-induced DNA damage in the lymphocytes and hepatocytes of rats. DNA damage was expressed as DNA tail length, DNA tail intensity, and DNA tail moment in the lymphocytes (A) and the hepatocytes (B) of the experimental groups. Results were given as the mean ± standard deviation for 8 rats in each group. Values with superscripts of different letters differ significantly ($P < 0.05$).

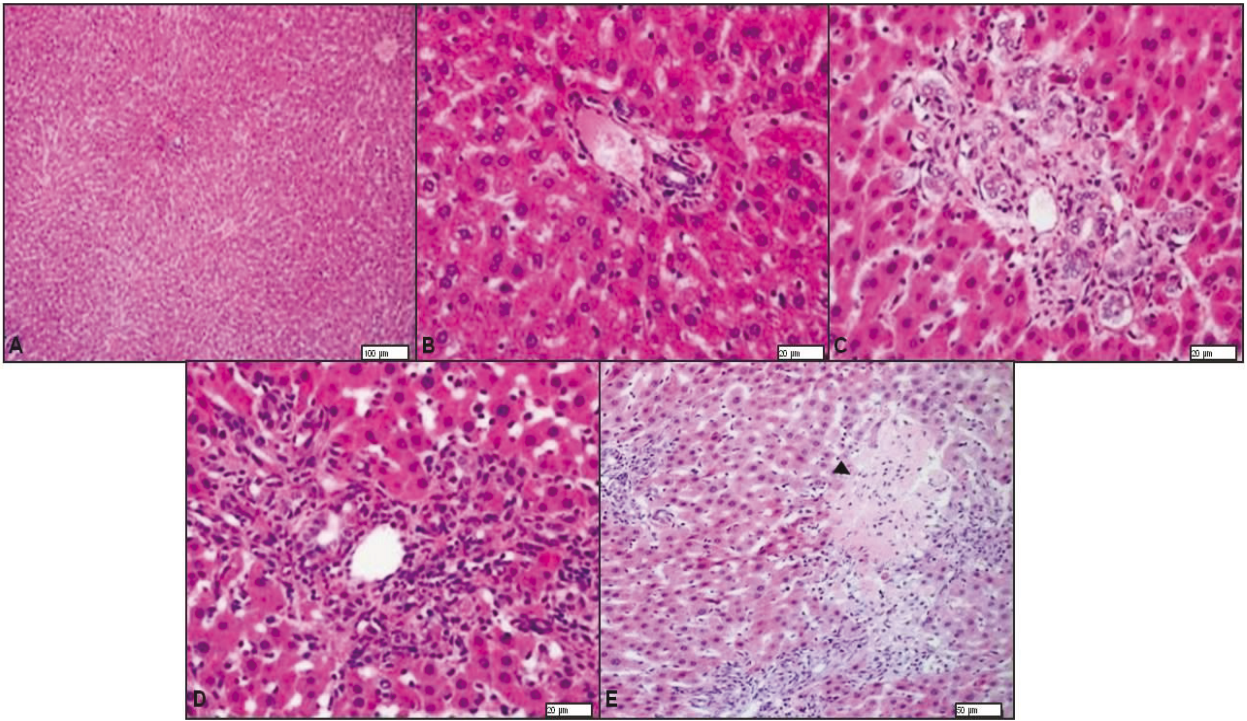


Figure 2. Histopathologic section of hepatic tissue. **A)** Normal liver histology from the sham group (H&E, $\times 100 \mu\text{m}$); **B)** a portal tract from the sham group (H&E, $\times 20 \mu\text{m}$); **C)** portal inflammation including neutrophil leukocytes and bile ductal proliferation in the BDL group (H&E, $\times 20 \mu\text{m}$); **D)** a necrotic focus near the portal tract in the BDL group (H&E, $\times 20 \mu\text{m}$); **E)** portal inflammation and bile ductal proliferations (arrowhead) in the BDL+L group (H&E, $\times 50 \mu\text{m}$).

ductal proliferation were markedly attenuated in the BDL+L group compared to the BDL group (Figure 2E). The frequency of necrotic focus seemed to be reduced in the BDL+L group compared to the BDL group (Figure 2C and Figure 2E). The number of inflammatory cells in the portal areas and leukocyte permeation of the bile duct epithelium also seemed to decrease in the BDL+L group compared to the BDL group (Figure 2E).

The mean grades of liver portal inflammation were found to be 0.29 ± 0.49 , 1.86 ± 0.38 , and 0.71 ± 0.45 in the sham, BDL, and BDL+L groups, respectively. Liver portal inflammation was found to increase significantly in the BDL group compared to the sham group ($P < 0.001$). Lycopene treatment seemed to reduce liver portal inflammation significantly in the BDL+L group compared to the BDL group ($P = 0.016$). However, portal inflammation was found to increase in the BDL+L group compared to the sham group ($P < 0.035$) (Table 3).

The mean grades of liver lobular inflammation were found to be 0.57 ± 0.53 , 1.71 ± 0.49 , and 1.14 ± 0.64 in the sham, BDL, and BDL+L groups, respectively. The liver lobular inflammation was found to increase significantly in the BDL group ($P < 0.001$) and the BDL+L group ($P = 0.027$) compared to the sham group. However, lycopene

treatment did not significantly reduce the liver lobular inflammation in the BDL+L group compared to the BDL group ($P = 0.561$) (Table 3).

The mean grades of ductal proliferation in the portal area were found to be 0.43 ± 0.53 , 2.86 ± 0.90 , and 1.86 ± 0.83 in the sham, BDL, and BDL+L groups, respectively. It was found that bile ductal proliferation in the portal area increased significantly in the BDL group ($P < 0.001$) and the BDL+L group ($P = 0.009$) compared to the sham group. Bile ductal proliferation was found to decrease significantly in the BDL+L group compared to the BDL group ($P = 0.034$) (Table 3).

4. Discussion

Different biologic targets such as DNA, lipids, and proteins may be inflicted with damage by oxygen-derived free radicals, which are highly reactive intermediates (26). Tissue injury may occur because of the free radicals that cause lipid peroxidation, and accumulation of lipid peroxidation products in human tissues is cited among the major reasons behind tissue dysfunction (27). Nevertheless, tissues are equipped with a range of defense mechanisms, and scavenger antioxidant molecules can provide protection against the damage caused by free radicals (28).

Table 3. Evaluation of portal inflammation, lobular inflammation, and bile ductal proliferation in the liver tissues of rats.

	Sham group	BDL group	BDL+L group
Liver portal inflammation	0.29 ± 0.49 (0–1) ^a	1.86 ± 0.38 (1–2) ^b	0.71 ± 0.45 (0–1) ^c
Liver lobular inflammation	0.57 ± 0.53 (0–1) ^a	1.71 ± 0.49 (1–2) ^b	1.14 ± 0.64 (0–2) ^b
Bile ductal proliferation	0.43 ± 0.53 (0–1) ^a	2.86 ± 0.90 (2–4) ^b	1.86 ± 0.83 (1–3) ^c

The results are given as the mean ± standard deviation (min–max) for 8 rats in each group. Values with superscripts of different letters differ significantly ($P < 0.05$) from each other within the same row.

An increase in bilirubin fractions and hepatic plasma enzymes leads to the significant assumption that cholestasis raises hepatic oxidative stress. These increases in bilirubin fractions and hepatic plasma enzymes are symptoms of cholestasis, hepatic dysfunction, and liver injury (29,30). In our study, AP, AST, GGT, D-Bil, and ALT levels indicating hepatic functions tended to increase remarkably in cholestasis, providing evidence that liver functions were affected by cholestasis. Daily administration of lycopene (25 mg kg⁻¹ day⁻¹) for 14 days before the experiments notably decreased the liver injury markers (GGT, ALT, AST, and lactate dehydrogenase) caused by ischemia reperfusion injury in rats (30). It was also reported that 40 mg/kg lycopene decreased ALT levels in rats treated with mercuric chloride (31). In our study, we also found that lycopene treatment (10 mg kg⁻¹ day⁻¹ for 7 days) reduced the plasma hepatocellular damage parameters (D-Bil, AST, ALT, GGT, and AP) in rats with cholestasis.

MDA, which is an outcome of the lipid peroxidation process, is accumulated in tissues. This serves as an indication of the scale of oxidative stress and lipid peroxidation. Damage to important cellular components resulting from free radicals and peroxides can be prevented by GSH, which is an endogenous antioxidant (32). A rise in hepatic, systemic, and renal MDA formation has been linked to cholestasis. Orellana et al. (33) reported that cholestasis-induced rats had increased GSH and MDA levels in liver tissues, which demonstrated the production of oxidative stress by cholestasis. In our study, we found a decline in GSH levels and a rise in MDA levels in the liver tissue in the bile duct ligated rats, and that finding is in line with the study of Gonzalez-Correa et al. (29) determining the effects of S-adenosyl-L-methionine on hepatic oxidative stress using a model of extrahepatic biliary obstruction of 7 days' duration.

Our study showed that MDA levels were decreased while GSH levels were increased in the liver tissue of BDL rats because of the 7-day lycopene treatment. One earlier study, being in line with our findings, showed a remarkable decline in retinol, lycopene, alpha- and beta-carotene, total carotenoid, and lutein levels in cholestatic liver diseases

such as sclerosing cholangitis and primary biliary cirrhosis compared to the control groups (34). It was reported that a 14-day administration of lycopene (25 mg kg⁻¹ day⁻¹) decreased the liver MDA levels in rats with the ischemia reperfusion injury (30). Similarly, Deng et al. (31) reported that 40 mg/kg lycopene administered once a day for 2 days notably decreased the liver MDA levels in rats in mercury chloride-induced hepatotoxicity conditions.

The intestinal mucosal barrier in cholestasis is disturbed by high-level biliary salts, which then cause the transfer of enteric bacteria to the mesenteric lymph nodes and liver. This results in endotoxemia, which is the reason behind increased NO synthesis by inducible NO synthase (35). Both in patients with primary biliary cirrhosis and in experimental cholestasis (5,35), the overproduction of NO was found (35). Among causes of the rapid development of hepatic dysfunction in cholestasis and hepatocellular injury, there is the rise in plasma and hepatic levels of NO and cytokines. We observed that NO level increased in the liver tissue of rats with cholestasis, while indicating that the lycopene treatment contributed to the decrease in BDL-induced NO level.

It is noteworthy to mention the crucial function of antioxidant enzymes such as CAT, SOD, and GST in cellular defense against free radicals. Oxidative stress in cholestasis also damages the liver antioxidant system (36). It was found that the livers of BDL rats had decreased levels of GST, SOD, CAT, GSH, and GSH-Px (37). It was also reported in some types of chemical-induced damage that antioxidant enzyme levels such as GGT, SOD, CAT, and GSH-Px were increased (38). In our study, levels of antioxidant enzymes such as GST, SOD, and CAT in the liver tissue were decreased in cholestasis. This could be interpreted as an indication of oxidative imbalance of the tissue. We observed that the increases in GST, SOD, and CAT levels in the liver tissues of BDL rats were provided with lycopene treatment. This shows that lycopene might have a significant activity in maintaining antioxidant enzymes. Our findings are in accordance with recent studies indicating the useful effects of lycopene (14,16,18).

Oxidative DNA damage can be notably reduced by the ingestion of certain vegetables or fruits. It was observed that lycopene had preventive effect against oxidative damage to cell membranes and DNA, and that it remarkably alleviated histological changes caused by free radicals in the rat liver and in many cells (39,40). To assess whether consumption of vegetables containing different carotenoids might protect against DNA damage and oxidative DNA damage in 23 healthy, nonsmoking males aged 27–40, Pool-Zobel et al. (41) found that a 2-week-long supplementation with tomato juice (40 mg/day lycopene), carrot juice (22.3 mg/day beta-carotene and 15.7 mg/day alpha-carotene), or 10 g of spinach powder (11.3 mg/day lutein) resulted in significantly decreased DNA strand breaks in the lymphocytes (41). The findings of Zhao et al. (42) indicated a reduction in endogenous DNA damage following a 57-day intake of supplements such as beta-carotene, lycopene, and lutein and a combination of all three. Our study, on the other hand, found increased DNA damage in the lymphocytes and liver tissue cells in BDL rats. However, DNA damage caused by cholestasis was prevented by lycopene, thus confirming earlier studies (40–42).

Toxic bile salt accumulation in the liver tissue may be one of the causes of increased free radical production in cholestasis. It is considered to be one of the significant causes in the development of liver damage resulting from cholestasis. Kisseleva and Brenner (43) reported that a radical increase in liver and serum bile acid levels

resulted in the proliferation of bile ducts, liver toxicity, and fibrosis, which develop into cirrhosis. Dirlik et al. (7) also found that following common BDL, the ductal proliferation grows increasingly, and it reaches the top level after 5 days in rats. Cholestatic liver disease appears with hepatic inflammation, which is one of its significant traits; proliferation of the biliary epithelial cells, portal tract fibrosis, portal tract edema, and neutrophil infiltration in the portal tracts are among the inflammatory characteristics of obstructive cholestasis (44). In this study, we found that in the rats with cholestasis, focal necrosis, liver portal inflammation, and bile ductal proliferation advanced after 7 days, indicating that cholestasis furthered the liver tissue damage.

To conclude, we indicated the significant role of oxidative stress in the development of cholestasis. We further showed the protective effect of lycopene in cholestasis, noting that lycopene is among the most potent chemicals to prevent free radicals. Lycopene treatment may be a useful tool to reduce tissue injury associated with cholestasis-induced oxidative stress. Therefore, it is obvious that this can pave the way for possible useful effects of the use of lycopene to combat patient disorders associated with cholestasis in the clinic and during follow-up. However, there is still a need to shed light on the in vivo mechanisms of lycopene and for further study with the intention of exploring the effectiveness of lycopene on other organs, in addition to the liver, in the course of cholestasis.

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