

Protective effect of low dose of melatonin against cholestatic oxidative stress after common bile duct ligation in rats

Mukaddes Esrefoglu, Mehmet Gül, Memet Hanifi Emre, Alaattin Polat, Mukadder Ayse Selimoglu

Mukaddes Esrefoglu, Mehmet Gül, Department of Histology and Embryology, Faculty of Medicine, Inonu University, 44280 Malatya, Turkey

Memet Hanifi Emre, Alaattin Polat, Department of Physiology, Faculty of Medicine, Inonu University, 44280 Malatya, Turkey
Mukadder Ayse Selimoglu, Department of Pediatric Gastroenterology, Hepatology and Nutrition, Faculty of Medicine, Ataturk University, Erzurum, Turkey

Co-first-authors: Mukaddes Esrefoglu

Co-correspondents: Mukaddes Esrefoglu, Mehmet Gül
Correspondence to: Dr. Mukaddes Esrefoglu, Inonu Universitesi, Tıp Fakültesi, Histoloji ve Embriyoloji Anabilim Dalı, 44128 Malatya, Turkey. drmukaddes@hotmail.com

Telephone: +90-422-3410660 Fax: +90-422-3410036

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400.46±48.89 nm/g, respectively, whereas the values of BDL+melatonin group were 115.93±6.8, 0.74±0.02 μmol/g, and 290.38±32.32 nm/g, respectively. Melatonin treatment was associated with a significant recovery of MDA, GSH and NO_x levels.

CONCLUSION: We have concluded that oxidative stress is associated with the pathogenesis of cholestatic liver damage and NO contributes to oxidative damage. Melatonin, even at low dose, is an efficient agent in reducing negative parameters of cholestasis.

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Key words: Cholestasis; Melatonin; Oxidative stress; Free radicals; Hepatic injury

Abstract

AIM: To investigate the role of oxidative injury and the effect of exogenous melatonin administration on liver damage induced by bile duct ligation (BDL), and second, to evaluate the role of nitric oxide (NO), a free oxygen radical, in oxidative injury.

METHODS: Thirty-two Sprague-Dawley rats were assigned to four groups: sham operation (SO), BDL, BDL+melatonin, and BDL+vehicle. Cholestasis was achieved by double ligation of the common bile duct. Melatonin was injected intraperitoneally 500 μg/(kg·d) for 8 d. Hepatic oxidative stress markers were evaluated by changes in the amount of lipid peroxides, measured as malondialdehyde (MDA), and reduced GSH. Total nitrite (NO_x) concentrations were determined in hepatic homogenates. Histopathological examination was performed using a histological scoring system.

RESULTS: The histopathological changes including portal inflammation, necrosis, apoptosis, focal inflammation and fibrosis were severe in the BDL and BDL+vehicle groups. There were numerous large areas of coagulation necrosis. Histological Activity Index scores of these groups were significantly higher than that of the SO group. Treatment with melatonin reduced these alterations significantly. The degree of necro-inflammation and fibrosis showed significant difference between the BDL and BDL+melatonin groups. BDL was accompanied by a significant increase in MDA and NO_x, and a significant decrease in GSH levels. Mean±SE values of MDA, GSH and NO_x levels of SO group were 147.47±6.69, 0.88±0.33 μmol/g and 180.70±6.58 nm/g, respectively. The values of BDL group were 200.14±21.30, 0.65±0.02 μmol/g, and

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INTRODUCTION

Cholestasis, extrahepatic or intrahepatic, is a common pathophysiological process in many human diseases leading to the accumulation of toxic bile salts within the liver^[1-5]. It seems likely that the detergent action of bile salts is responsible for solubilization of plasma membranes and cell death, which in turn may lead to oxidative stress, oxidation of reduced glutathione (GSH), and lipid peroxidation^[6]. There is growing evidence suggesting that considerable impairment of oxidative stress regulation may play an important role in cholestatic liver injury^[7-11]. Acute bile-duct obstruction is characterized by increased lipid peroxidation and by marked decline in reduced GSH, a major cellular antioxidant^[12]. It is known that bile-duct ligation (BDL) results in a shift in the oxidant/prooxidant balance in favor of increased free radical activity^[12,13]. Enhanced production of reactive oxygen intermediates augments lipid peroxidation by disturbing oxidant-antioxidant balance in hepatic mitochondrial fraction. The damage pattern of free radicals interfering with hepatocytes suggests peroxynitrite-mediated liver injury^[14]. As a reactive-free radical, NO mediates the cytotoxicity caused by activated neutrophils and macrophages in the inflammatory response^[15,16].

In attempting to limit the oxidative damage, a number of antioxidants have been tested in experimental bile-duct obstruction models^[6,7,12]. It has been proposed that antioxidants,

which maintain the concentration of reduced GSH, may restore the cellular defense mechanism and block lipid peroxidation^[10]. Melatonin has been proved to have the greatest impact not only on oxidative stress, but also on systems of defense against free radicals, restoring the oxidative balance in treated experimental animals^[10,12]. Administration of melatonin at pharmacological doses has been shown to decrease free radical formation and lead to a substantial recovery of the major antioxidant enzymes, thus limiting oxidative damage to the liver^[12]. Recent evidences have shown that melatonin has protective effects on hepatic injury after extrahepatic BDL in rats^[10,11,17].

The aims of this study were first to investigate the role of oxidative injury and the effect of exogenous melatonin administration on liver damage induced by BDL, and second, to evaluate the role of NO in oxidative injury. Hepatic oxidative stress markers were evaluated by changes in the amount of lipid peroxides, measured as MDA and GSH. Since NO measurement is difficult in biological specimens, tissue nitrite (NO₂) and nitrate (NO₃) were estimated as an index of NO production. Additionally, a detailed histopathological examination using a histological scoring system was performed. To our knowledge, there has been only one study revealing histological results about the effects of melatonin on experimental cholestatic liver injury in rats^[17]. We suggest that NO contributes to oxidative stress and melatonin is a possible protective agent in biliary cholestasis and parenchymatous liver injury.

MATERIALS AND METHODS

Animals and experimental groups

Thirty-two adult male Sprague-Dawley rats weighing 250-290 g were used. Animals were housed under continuous observation in appropriate cages in a quiet temperature (21±2 °C) and humidity (60±5%)-controlled room in which a 12-12 h light-dark cycle was maintained. They were allowed free access to a commercial standard diet and water ad libitum. Rats were randomly assigned to four groups each containing eight rats as follows: sham operation (SO) (control), BDL, BDL+melatonin and BDL+vehicle. Sham-operated rats served as controls. Except in this group, biliary canals were ligated. Rats were fasted for 12 h before the operation, but were given water.

Animal experiments were performed in accordance with the guidelines for animal research from the National Institute of Health and were approved by the Committee of Animal Research at Inonu University, Malatya, Turkey.

Surgery procedure

Each rat was weighed and anesthetized by intraperitoneal administration of ketamin (70 mg/kg) and xylacaine (7 mg/kg). The abdomen was shaved and disinfected with 10% povidone iodine. Following a midline incision, the common bile duct was exposed and a double ligature with 3/0 silk was performed and the bile duct was sectioned between the ligatures. Abdominal muscles were closed with 3/0 silk and abdominal skin was closed with 2/0 silk. Sham surgery was identical to the ligation procedure, including locating and manipulating the common hepatic duct, except that the

bile duct was not ligated or sectioned. Rats were maintained on the retrospective preoperative diet after surgery. Melatonin (Sigma Chemical Co., St. Louis, MO) 500 µg/(kg·d) was dissolved in ethanol and further diluted in saline (0.9 mL/L NaCl) to give final concentration of 1%. It was given intraperitoneally at 10.00 a.m. daily, beginning 1 d before the operation and continuing for seven successive days. BDL+vehicle group received equal volume (1 mL) of diluted ethanol in saline (0.9 mL/L NaCl). Rats were killed on the 8th d after the surgery. After the macroscopic findings were noted, the livers were promptly removed and were processed for histological and biochemical examination. The right lobe of the liver was divided into two pieces. The first samples were placed in 40 g/L formaldehyde for histopathological examination by light microscopy. The second piece of liver tissues was washed thrice with cold saline solution, placed into glass bottles, labeled, and stored in a deep freeze (-85 °C) for MDA, NO, and GSH analyses.

Histological examination

Liver tissues were fixed in 40 g/L formaldehyde and were embedded in paraffin. For histopathological evaluation, 4-mm slides were stained with hematoxylin-eosin, Masson's trichrom, Periodic acid-Schiff (PAS), and Hall's stain for bile. Sections were scored by an independent observer blinded to the experimental protocol. The following lesions were scored according to Modified Histological Activity Index (HAI):^[18,19] portal inflammation, focal necrosis, confluent necrosis, piecemeal necrosis, apoptosis, focal inflammation and fibrosis. The number of biliary canals in five portal sites for each section was also noted.

Biochemical determination

Tissues were homogenized in four volumes of ice-cold Tris-HCl buffer (50 mmol/L, pH 7.4) using a homogenizer (IKA-Werke Ultra-Turrax T25 basic homogenizer, Germany) after cutting of the liver into small pieces. The malondialdehyde (MDA), nitric oxide (NO) and reduced GSH content of homogenates were determined spectrophotometrically.

Assessment of lipid peroxide formation Lipid peroxide was determined calorimetrically in a sample of the liver homogenates as thiobarbituric acid-reactive substances (TBA-RS) according to the method of Uchiyama and Mihara^[20]. The absorbances of the formed colored product at 520 and 535 nm were measured and the results were expressed as the difference in absorbance at the two wavelengths ($A_{535-520}$). MDA are expressed as micromole per gram wet hepatic tissue.

NO determination As NO measurement is very difficult in biological specimens, tissue nitrite (NO₂) plus nitrate (NO₃) concentrations were estimated as an index of NO production. The method for tissue NO₂ plus NO₃ levels based on the Griess reaction was used^[21]. Samples were initially deproteinized with Somogy reagent^[22]. Total nitrite (NO_x) (NO₂+NO₃) was measured after conversion of nitrate to nitrite by copperized cadmium granules by a spectrophotometer at 545 nm. Results are expressed as nanomole per gram wet hepatic tissue.

Reduced glutathione determination Liver tissues were deproteinated by the addition of trichloroacetic acid. D'TNB

[5,5'-dithiobis(2-nitrobenzoic acid)] was added to supernatants cleared by centrifugation (10 min, 3 000 r/min). The formation of 5-thio-2-nitrobenzoic acid, which is proportional to total GSH concentration, was monitored at 412 nm at 25 °C against reagent controls^[23]. The level of GSH was determined from the standard curve with commercially available GSH (Sigma Chemical Co.). GSH are expressed as micromole per gram wet hepatic tissue.

Statistical analysis

The results were statistically analyzed by the Kruskal-Wallis *H* test. The differences between groups were evaluated by the Mann-Whitney *U* test followed by *t* test with Bonferroni correction when indicated. $P < 0.05$ was considered significant. The results are expressed as the arithmetic mean \pm SE.

RESULTS

Survival and macroscopic findings

Twenty-four hours after operation, clinical condition of the animals of all groups except those of the SO group worsened, with decreasing activity, weight loss, yellowed ears and tails, darkened urine and pale feces. All of the animals survived until the end of the experiment. Jaundice was observed in the visceral and parietal peritoneum of all animals except those of the SO group. The livers were enlarged and the bile ducts above the obstruction point were dilated. Animals from the SO group showed no alteration after surgery and were killed on d 8.

Microscopic findings

Animals from the SO group presented no histological alterations. The liver specimens of the BDL and BDL+melatonin groups showed various degrees of lobular and portal changes. The degree of necro-inflammation (focal necrosis, confluent necrosis, piecemeal necrosis, focal and portal inflammation, and apoptosis) and fibrosis showed

significant difference between the BDL and BDL+melatonin groups ($P < 0.05$). HAI scores of the groups are summarized in Table 1. The number of bile ducts in five portal sites for each section did not show significant differences among the BDL, BDL+vehicle and BDL+melatonin groups. The number of bile ducts is summarized in Table 2. In the BDL and BDL+vehicle groups the histopathological changes including necrosis, bile duct proliferation, fibrosis and polymorphonuclear leukocyte (PNL) and lymphocyte infiltration were prominent (Figure 1A). There were numerous large areas of coagulation necrosis randomly distributed. The Masson's trichrom staining of liver tissue showed fibrotic reaction that was usually accompanied with inflammatory cells in the periphery of the majority of the portal space areas (Figure 1B). Many mitotic figures and apoptotic bodies were seen. There was a massive loss in the amount of glycogen in hepatocytes (Figure 1C). Although all of the liver sections were stained with Hall's bilirubin pigment staining technique, no bile pigment accumulation was demonstrated neither in canaliculi nor in bile ducts.

In the BDL+melatonin group, histopathological evidence of parenchymatous injury was markedly reduced (Figure 1D). Relatively small, scattered necrotic hepatocyte groups between normal-appearing parenchymal cells were observed. Portal fibrosis was not evident (Figure 1E). Bile duct proliferation (Figure 1E) and glycogen depletion were still prominent. Apoptotic bodies and mitotic figures were also observed.

Acute ligation of bile duct was accompanied by a significant increase in MDA and NO, and a significant reduction in GSH levels in hepatic homogenates. Melatonin administration significantly decreased MDA and NO levels, whereas significantly increased GSH levels ($P < 0.05$). MDA, GSH and NO_x concentrations measured in hepatic tissue are summarized in Table 3.

The histopathological analyses correlated with biochemical data.

DISCUSSION

When a large bile duct is obstructed at any point in its course, several microscopic changes follow^[24]. The morphological features of cholestasis depend on its severity, duration, and underlying cause^[1]. The ducts themselves dilate and are filled with abundant bile^[1,24]. Droplets of bile pigments can accumulate within hepatocytes^[1]. Within a few days of the onset of obstruction, alterations are found in the portal tracts. The portal tracts become swollen as a result of edema

Table 1 Scores for necro-inflammation (focal necrosis, confluent necrosis, piecemeal necrosis, focal and portal inflammation, and apoptosis) and fibrosis of groups using modified HIA grading system (mean \pm SE)

	Scores for necro-inflammation	Scores for fibrosis
SO	0.25 \pm 0.16	0 \pm 0
BDL	11.87 \pm 0.83 ^a	1.37 \pm 0.18 ^a
BDL+vehicle	11.12 \pm 1.00 ^a	1.35 \pm 0.20 ^a
BDL+melatonin	6.87 \pm 0.63 ^c	0.75 \pm 0.25 ^c

SO: sham operation; BDL: bile duct ligation; HAI: Histological Activity Index. ^a $P < 0.05$ vs SO. ^c $P < 0.05$ vs BDL, BDL+vehicle.

Table 2 Number of biliary canals in five portal sites for each specimen (mean \pm SE)

	Number of biliary canals
SO	9.37 \pm 0.49
BDL	40.37 \pm 2.50 ^a
BDL+vehicle	39.50 \pm 2.67 ^a
BDL+melatonin	39.87 \pm 2.01 ^a

SO: sham operation; BDL: bile duct ligation. ^a $P < 0.05$ vs SO.

Table 3 MDA, GSH and NO_x concentrations measured in hepatic tissue

	MDA (μ mol/g tissue)	GSH (μ mol/g tissue)	NO _x (nmol/g tissue)
SO	147.47 \pm 6.69	0.88 \pm 0.03	180.70 \pm 6.58
BDL	200.14 \pm 21.30 ^a	0.65 \pm 0.02 ^a	400.46 \pm 48.89 ^a
BDL+vehicle	211.12 \pm 19.98	0.63 \pm 0.04 ^a	411.66 \pm 77.32
BDL+melatonin	115.93 \pm 6.80 ^c	0.74 \pm 0.02 ^c	290.38 \pm 32.32 ^c

SO: sham operation; BDL: bile duct ligation; MDA: malondialdehyde; GSH: reduced glutathione; NO_x: NO₂ (nitrite) + NO₃ (nitrate). ^a $P < 0.05$ vs SO. ^c $P < 0.05$ vs BDL, BDL+vehicle. ^c $P < 0.05$ vs BDL.

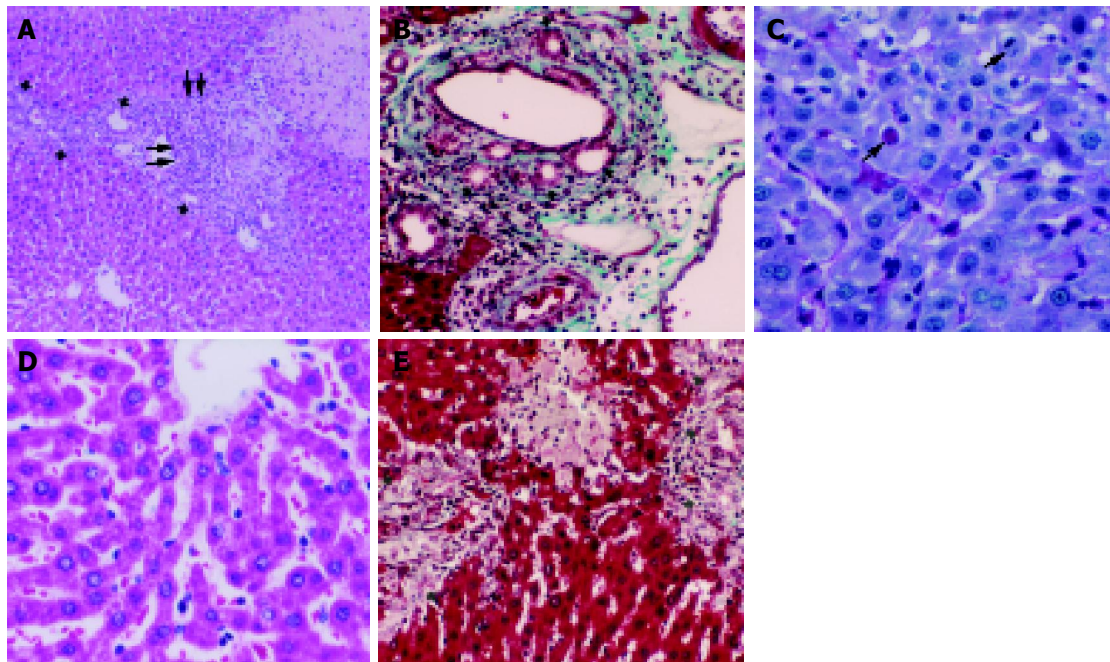


Figure 1 A: BDL group. Portal fibrosis (f) with bile duct proliferation (thick arrows) and cell infiltration (arrows), and a large area of coagulation necrosis (n) are observed. H-E, $\times 10$; B: BDL group. Portal fibrosis (f) with bile duct proliferation (arrows) and cell infiltration is observed. Portal vein (p) is highly enlarged. Masson's trichrom, $\times 20$; C: BDL group. A massive loss in the amount of glycogen in hepatocytes is seen. There are a mitotic figure (arrow) and an apoptotic body (double arrow) in the parenchyma. PAS, $\times 40$; D: BDL+melatonin group. Section is normal in histological appearance. Hepatocytes arranged as cords of cells radiating from central vein (c) are observed. H-E, $\times 40$; E: BDL+melatonin group. Portal fibrosis is not evident. Bile duct proliferation is obvious (arrows). A small area of necrosis (n) is present between proliferated ducts. Masson's trichrom, $\times 20$.

of the connective tissue and infiltration by PNL, a classical acute inflammatory reaction attributed to the action of toxic bile salts reabsorbed from the bile by small bile ducts^[2,24]. The bile stasis and backpressure due to obstruction induce proliferation of the duct epithelial cells and looping and reduplication of ducts, termed 'bile duct proliferation'^[11]. If the obstruction is not relieved, increasing fibrosis around ducts and extending between adjacent portal tracts develops^[1,2,24]. Biliary obstruction leads to the death of hepatocytes due to bile accumulation^[2,4]. Hepatic injury and cell death leads ultimately to liver fibrosis, cirrhosis, and cancer^[2].

In the present study, bile duct proliferation in all groups compared to the controls was a result of the biliary obstruction. The number of bile ducts in five portal sites for each section did not show significant differences among the BDL, BDL+vehicle and BDL+melatonin groups ($P > 0.05$) since obstruction was not relieved. Abdel-Aziz *et al*^[25], 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. We have observed many histopathological features of cholestasis such as focal necrosis, confluent necrosis, piecemeal necrosis, focal and portal inflammation, bile duct proliferation and fibrosis in our experimental model. HAI scores of the BDL and BDL+vehicle groups were significantly higher than that of the SO group. Moreover, the obstructive jaundice was demonstrated by the coloration of the skin, peritoneum, and urine, and the dilatation of the common bile duct above the obstruction point. On the other hand, although bilirubin is one of the easiest pigments to be identified in standard HE sections, we could not

observe it either in canaliculi or in bile ducts even though we used Hall's stain for bile salts. Bile pigment accumulation within hepatocytes, canaliculi or bile ducts has been supposed to be one of the characteristic features of cholestasis^[1]. As far as we know, in rodents the absence of bile salts after BDL has already been reported twice before in the studies of Prado *et al*^[4], and Trauner and Boyer^[26]. It is probably due to the induction of alternative detoxification and elimination pathways, which is very pronounced in rodents^[26]. We think that bile salt accumulation may not accompany the other characteristic features of cholestasis in rodents.

Cell degeneration and death in cholestasis have been reported to be related to retention of toxic bile salts^[2,4,6]. It seems that the detergent action of bile salts is responsible for solubilization of plasma membranes and cell death, which in turn may lead to oxidative stress^[6]. It is shown that the acute infusion of toxic bile salts responsible for cholestasis induces zone 1 hepatocellular necrosis^[5]. Padillo *et al*^[27], have observed marked necrosis and apoptosis induced by cholestasis. In the present study, we observed numerous areas of coagulation necrosis and many apoptotic bodies in the BDL and BDL+vehicle groups. However, since we have not been able to identify accumulation of bile salts within parenchyma, we are suspicious about the direct, inducing role of the bile salts on the hepatocellular injury after BDL although, we cannot fully exclude the role of these products. A possible explanation is that an oxidative stress initiated by bile salts would make hepatocytes more susceptible to the toxic effects of small amount of bile salts, of which great amounts have been detoxified, that would not ordinarily injure the hepatic cells.

We observed many mitotic figures both in hepatocytes and in ductular epithelial cells. Mitosis and fibrosis within the parenchyma are the signs of wound repair. Increased cell proliferation and increased secretion of collagen and the other matrix proteins are important compensatory mechanisms for the repair of the injured tissues^[28]. Mitosis in the ductular epithelium is the evidence of bile duct proliferation. The bile stasis and backpressure induce proliferation of the duct epithelial cells^[1]. Bülbüller *et al.*^[17] observed mitotic figures in parenchyma and proliferation of biliary canals 7 d after experimental biliary canal obstruction. After bile-duct obstruction, the deposition of connective tissue elements and formation of ductular proliferates rapidly set in^[29]. In our study, fibrous expansion of most portal areas was observed but portal to portal bridging was not present. So, HAI score for fibrosis was relatively low.

In obstructive jaundice, glucose metabolism undergo changes, the most common being the development of glucose intolerance^[17,30]. Recent studies have reported a decrease in serum glucose values^[17] and hepatic glycogen content^[31,32]. We have observed nearly a total loss in the amount of glycogen in hepatocytes. Loss of appetite, reduction of oral food intake, reduced glycogen synthesis, possibly related to endotoxemia, may probably be the causes of decrease in the hepatic glycogen content^[17,29,32].

Many studies have reported that there is a correlation between the intensity of biliary tract obstruction and increased free radical generation, as well as a direct correlation between the level of oxidative stress and the biochemical markers of liver injury^[6-11,14]. It has been well established that deleterious accumulation of lipid peroxides is correlated with marked impairment of soluble antioxidant defense mechanisms and cell necrosis^[5,9,14,33]. Acute BDL is associated with an increase in MDA levels and a decrease in GSH levels both in plasma and in tissue^[9,10,17,34-37]. Cruz *et al.*^[37] have reported an increase in MDA and a decrease in GSH levels in renal tissue after biliary obstruction. As observed in previous studies, biliary tract obstruction was accompanied by increased levels of lipid peroxidation and by the depletion of GSH, a ubiquitous antioxidant, in hepatic tissue in our study. On the other hand, there is increasing evidence that the enhanced production of reactive oxygen intermediates augments lipid peroxidation by disturbing the oxidant-antioxidant balance in hepatocytes. Since the levels of NO_x in the liver were significantly higher in the BDL and BDL+vehicle groups than in the SO group ($P<0.05$), we suggest that NO, probably via its derivative peroxynitrite, may contribute to oxidative damage. Engin *et al.*^[14] reported a significant increase in plasma NO₂, NO₃ and NO_x concentrations in inbred albino guinea pigs 24 h after common BDL.

A number of antioxidants have been tested in experimental bile-duct obstruction models in the attempt to limit the oxidative damage^[7,12]. Administration of melatonin at pharmacological doses has been shown to decrease free radical formation and lead to a substantial recovery of the major antioxidant enzymes, thus limiting oxidative damage to the liver^[10-12,17]. It is shown that melatonin administration reduces histopathological signs of liver injury such as focal

necrosis and apoptosis^[17,27]. There are a few studies about the protective effect of low dose of melatonin against oxidative stress^[27,37]. In the present study, histopathological evidence of parenchymatous injury was significantly reduced in the BDL+melatonin group ($P<0.05$). Portal fibrosis was not evident in this group. Additionally, melatonin administration prevented the GSH decrease and reduced significantly lipid peroxidation products. Montilla *et al.*^[10], reported significant differences in tissue and plasma MDA and GSH levels between non-treated and low dose of melatonin-treated rats. Lower levels of hepatic NO_x in the BDL+melatonin group than in the BDL group ($P<0.05$) may support the role of melatonin on reducing free radical generation.

As a conclusion, the present study confirms the association between hepatic injury and increased oxidative stress, possibly mediated by NO. We have proved that even low dose of melatonin is efficient reducing the negative parameters of cholestasis, the degree of oxidative stress, and provided a significant hepatoprotective effect against liver injury secondary to ligation of biliary duct. Finally, we suggest that further studies comparing higher doses of melatonin should be performed in order to determine if the effect of melatonin is dose-dependent.

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