Melatonin ameliorates liver fibrosis induced by bile-duct ligation in rats

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Background: Hepatic stellate cells, the main mediators in the pathogenesis of fibrosis, are triggered by free radicals and produce collagen. Melatonin is a powerful physiologic scavenger of hydroxyl radicals. It is also involved in the inhibitory regulation of the collagen content in tissue. There is no effective treatment available for liver fibrosis. Our objective was to evaluate the effects of melatonin on liver fibrosis induced by bile-duct ligation (BDL) in rats.

Methods: We divided male Wistar rats ($n = 32$) into 4 groups. Two groups received BDL and 2 groups received sham operations. One of the BDL groups and one of the sham groups were administered melatonin (100 mg/kg/day via intraperitoneal injection), and the controls were given vehicle only. After 1 month, we biochemically evaluated the changes in hepatic fibrosis by measuring tissue collagen levels and histopathologic examination. We evaluated the levels of malondialdehyde (MDA), glutathione (GSH), luminal and lucigenin in tissue homogenates, and we studied proinflammatory cytokines in serum using commercially available kits.

Results: Bile-duct ligation caused hepatic fibrotic changes, whereas melatonin suppressed these changes in 5 of 8 rats ($p < 0.001$). Bile-duct ligation resulted in increased collagen, MDA, luminal and lucigenin levels and decreased GSH levels, whereas melatonin reversed these effects.

Conclusion: We found that melatonin functions as an effective fibrosuppressant and antioxidant, and the results suggest that it can be used as a therapeutic option.

Contexte : Les cellules de Kupffer dans le foie, qui sont les principaux médiateurs de la pathogenèse de la fibrose, sont déclenchées par des radicaux et produisent du collagène. La mélatonine est un puissant capteur physiologique des radicaux hydroxyles. Elle joue aussi un rôle dans la régulation inhibitrice du contenu en collagène des tissus. Il n'y a pas de traitement efficace disponible contre la fibrose hépatique. Nous voulions évaluer les effets de la mélatonine sur la fibrose hépatique causée par une ligature du canal biliaire (LCB) chez les rats.

Méthodes : Nous avons réparti des rats Wistar mâles ($n = 32$) en 4 groupes. Les sujets de 2 groupes ont subi une LCB et les sujets de 2 autres groupes, une opération factice. Un des groupes qui ont subi la LCB et 1 de ceux qui ont subi une intervention factice ont reçu de la mélatonine (100 mg/kg/jour par injection intrapéritonéale) et les sujets témoins ont reçu l'excipient seulement. Après 1 mois, nous avons évalué les changements biochimiques de la fibrose hépatique en mesurant les concentrations de collagène dans les tissus et nous avons procédé à un examen histopathologique. Nous avons évalué les concentrations de malondialdéhyde (MDA), de glutathion (GSH) et de luminol et de lucigénine dans des homogénats de tissus, et nous avons étudié les cytokines pro-inflammatoires dans le sérum au moyen de trousses vendues dans le commerce.

Résultats : La ligature du canal biliaire a causé des changements de la fibrose hépatique tandis que la mélatonine a supprimé ces changements chez 5 rats sur 8 ($p < 0,001$). La ligature du canal biliaire a entraîné une élévation de la concentration de collagène, de MDA, de luminol et de lucigénine et une diminution des concentrations de GSH, tandis que la mélatonine a inversé ces effets.

Conclusion : Nous avons constaté que la mélatonine est un fibrolytique et antioxydant efficace et les résultats indiquent qu'elle peut servir comme traitement possible.

liver fibrosis is a major feature of many chronic liver injuries and is one of the main components of cirrhosis. It is characterized by the accumulation of collagen and extracellular matrix proteins in the space of Disse. Activation of hepatic stellate cells (HSCs) and the initiation of unbalanced synthesis of collagen are the main components in the pathogenesis that leads to fibrosis.[1–3]

Accumulation of bile acids and inflammatory cells in liver tissue causes free radical production in biliary obstruction.[4,5] Several lines of evidence have suggested the important role of oxidative stress in the etiopathogenesis of liver fibrosis.[1,2,5] Furthermore, oxidative stress aggravates liver fibrosis via HSC activation,[2,6] and lipid peroxidation stimulates transcription of the collagen gene.[7]

Melatonin (*N*-acetyl-5-methoxytryptamine), a secretory product of the pineal gland, is a powerful endogenous antioxidant. Exogenous application of this molecule leads to a remarkable decline in oxidative stress and inflammation because of its ability to scavenge hydroxyl radicals and cytokines.[2,8] In addition, melatonin also enhances the levels of potential antioxidants such as glutathione peroxidase, superoxide dismutase and glutathione (GSH) by an indirect mechanism.[2,8] There are studies in the literature that report the antifibrotic effects of melatonin as well as its properties in controlling collagen levels in tissues.[2,9–12] Further, the cytostatic effects of melatonin on polymorphonuclear leukocytes and HSCs may lead to the inhibition of both free radical damage and fibrogenic activity.[2]

At present, no clinically effective therapy for liver fibrosis is available. We hypothesize that melatonin could be a potential therapeutic candidate for fibrotic liver diseases. In this study, the possible fibrosuppressant effect of melatonin on liver fibrosis induced by bile duct ligation (BDL) in rats was evaluated as a novel therapeutic choice.

METHODS

Materials

We purchased melatonin from Sigma Chemical Co., cytokine kits (enzyme-linked immunosorbent assay [ELISA]), rat interleukin (IL)-1b and tumour necrosis factor (TNF)-α from R&D Systems Europe, and ELISA and rat IL-6 from Diaclone Research.

Experimental animal procedures

We obtained full approval for this study from the local ethics committee on animal research, and the study was performed according to the criteria of international guidelines for animal research. The experimental protocol was performed at Marmara University Animal Research Laboratory. All animals received humane care in compliance with the National Institutes of Health criteria for laboratory animals and had free access to standard rat chow and water.

We obtained 32 Wistar rats (aged 3.5–4.5 mo, 200–240 g) from the Institutional Animal Research Laboratory. Animals were kept at a constant temperature (mean 22°C, standard deviation [SD] 1°C) with 12-hour light and dark cycles, in the same unit. The animals were allowed to acclimatize to their new conditions for 1 week before the study began.

Thirty-two male Wistar rats underwent BDL or sham operation under general pentobarbital anesthesia. Two groups of rats underwent BDL, and the control rats underwent sham operations. Briefly, the common bile duct was exposed after laparotomy. Two double knots were placed proximally and distally, and the part of the bile duct between the 2 double knots was excised. In the sham group, the abdomen was closed without BDL. In total, 16 rats underwent BDL and 16 received sham operations. One of each of the operation groups was given melatonin (100 mg/kg/day via intraperitoneal injection), and the controls were given the vehicle. The 4 groups were as follows: saline group (sham operation and injected with saline, *n* = 8); melatonin group (sham operation and injected with melatonin, *n* = 8); BDL group (BDL and injected with saline, *n* = 8); and BDL–melatonin group (BDL and injected with melatonin, *n* = 8).

After 1 month, the animals were fasted overnight and sacrificed. We obtained serum samples by centrifugation of the trunk blood (4024 *g*, 10 min, 4°C). We removed and weighed the livers. After obtaining samples for histologic assessment, the livers were snap frozen in liquid nitrogen. The serum and liver samples were stored at –80°C for laboratory assays. All histopathological and biochemical tests were performed blinded.

Tissue homogenization

We weighed the liver samples and homogenized them in 0.15 m NaCl to determine the amount of reactive oxygen species (ROS). The liver tissue homogenates were diluted with 0.15 m NaCl up to 20%. The tissue homogenates were sonicated 2 times for 30-second intervals at 4°C. After sonication, we centrifuged the homogenates at 4024 *g* for 10 minutes or at 20 124 *g* for 15 minutes. Aliquots of the supernatants were used for the subsequent studies. We determined the protein content of the tissue aliquots by the Lowry method.[13]

Biochemical parameters

We determined the plasma levels of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT) and bilirubin by use of a spectrophotometer (Roche 917 R autoanalyzer) and commercial kits.

Oxidative stress parameters

Malondialdehyde measurements
We performed measurements of thiobarbituric acid reactive species (TBARS) using the method described by Yagi.[14] We homoginized the liver tissue in an icy 10% trichloroacetic acid solution and then centrifuged the mixture. The superficial liquid portion was mixed with equal volume of TBARS (0.67%) and heated at 90°C for 15 minutes. We measured TBARS in nmol/g of tissue according to the absorbance at 532 nm.

Chemiluminescence measurements
We measured reactive oxygen metabolites (ROM) at room temperature via a chemiluminescence technique using a Mini Lumat LB 9506 luminometer (EG&G). Samples were placed into 2 mL of a 0.02-mol HEPES buffer (pH 7.4) containing 0.5 mol of phosphate-buffered saline. For measurement of ROM, we used 0.2 nmol of concentrated lucigenin (specific for superoxide radicals) or luminal ($HOCl^-$, H_2O_2, OH^-). We performed serial measurements at 15-second intervals for 5 minutes, and we calculated the area under the curve and relative light units; we also corrected for fresh tissue weight (relative light units per mg tissue area under the curve).[15,16]

Glutathione level measurements
We measured the levels of glutathione (GSH) using a spectrophotometer with Boyne and Ellman's reagent and method.[17] We calculated the results as μmol GSH/g tissue.

Cytokine tests

We analyzed the serum levels of TNF-α, IL-1β and IL-6 using commercially available enzyme immunoassay kits. The principal method for the immunoassay was the same for all cytokines. The analyses of all samples, standards and controls were performed in duplicate. The coefficients of intra-assay variation for TNF- α, IL-1β, and IL-6 were 4.2%, 4.1% and 4.0%, respectively; the interassay variations were 6.7%, 7.9% and 6.9% ($n = 10$), respectively.

Biochemical collagen content determination

We assessed the liver collagen content by use of the colorimetric method described by Lopez de Leon and Rojkind.[18] In this analysis, collagenous protein are coloured red by Sirius red (36554-8, 2610-10-8; Aldrich Chemical) and noncollagenous proteins are coloured green by fast green (14280; Merck). We obtained 15 μm–thick liver slices from each paraffin block and layered the slices on glass slides. We deparaffinized the slices and performed the assay as originally described.[18] We calculated the collagen content using the formula described by the authors as μg collagen/mg protein.[18]

Liver tissue sampling

We explored the left, middle and right lobes of each liver. Six different slices ($5 \times 5 \times 5$ mm) were fixed in 10% buffered formalin, routinely processed and blocked in paraffin for the detection of the collagen content by image analysis.[3]

Histopathology

The histopathologic examinations were performed blinded. We stained 5-μm liver sections with hematoxylin–eosin and Masson trichrome–Gomori reticulin stains. The grading of necroinflammatory activity and the staging of fibrosis were performed according to Knodell's criteria.[19]

Statistical analysis

All results are expressed as mean and standard errors. We analyzed numerical data using the Kruskal–Wallis test. Significant differences revealed by multigroup comparisons were further analyzed by use of the Dunn test. Nominal data were analyzed by the χ^2 test or the Fisher exact test with Yates correction. We considered p values less than 0.05 to be significant.

RESULTS

Melatonin lowered the levels of AST, ALT, ALP, GGT and bilirubin in the BDL–melatonin group compared with the BDL group (Table 1). The levels of albumin were not statistically different among the groups. The mean levels of liver tissue collagen, malondialdehyde (MDA), luminal and lucigenin in the BDL group were significantly higher than in the BDL–melatonin group; they were also higher than in the saline- and melatonin-injected groups (Figs. 1, 2 and 3). The mean hepatic level of GSH in the BDL group was significantly lower than in the BDL–melatonin, saline and melatonin groups (Fig. 4). The average levels of IL-1β, IL-6 and TNF-α in the BDL group were significantly higher than in the BDL–melatonin, saline and melatonin groups (Fig. 5). The rats in the saline and melatonin groups had normal liver histology (Fig. 6A). Bile-duct ligation caused prominent liver fibrosis in the untreated BDL group, and BDL alone led to grade 3 liver fibrosis. The bridging fibrosis in the portoportal and portocentral areas was prominent in the BDL group (Fig. 6B). Melatonin treatment improved fibrosis in 5 of 8 rats BDL–melatonin model ($p < 0.001$). These animals showed stage 2 fibrosis (Fig. 6C).

DISCUSSION

At present, there is no effective treatment of liver fibrosis available for clinical use. Some experimental studies have reported partial success with substances such as pegylated interferon[20] and N-acetylcysteine[1] in this setting. An

Table 1. Biochemical parameters in rats that underwent bile-duct ligation or sham operation

Parameter	Treatment; mean (standard error)				
	Sham + saline	Sham + melatonin	BDL + saline	BDL + melatonin	p value
Aspartate transaminase, U/L	158 (14)	162 (12)	371 (36)	261 (27)	0.008
Alanine transaminase, U/L	66 (8)	59 (6)	97 (8)	74 (7)	0.006
Alkaline phosphatase, U/L	464 (214)	377 (237)	1747 (299)	775 (365)	0.007
γ-glutamyl transpeptidase, U/L	8 (2)	10 (5)	81 (23)	43 (17)	0.008
Total bilirubin, mg/dL	0.2 (0.04)	0.2 (0.03)	13.6 (4.1)	7.7 (3.3)	< 0.001
Direct bilirubin, mg/dL	0.02 (0.01)	0.02 (0.01)	10 (2.2)	5.3 (1.7)	< 0.001
Albumin, g/dL	3.9 (0.8)	3.9 (0.7)	3.8 (0.7)	3.9 (0.8)	0.65

BDL = bile-duct ligation.
Plasma aspartate transaminase, alanine transaminase, alkaline phosphatase, γ-glutamyl transpeptidase and bilirubin levels in the BDL rats were significantly higher than the BDL + melatonin rats ($p < 0.05$), the saline rats and the melatonin rats ($p < 0.01$, for both).
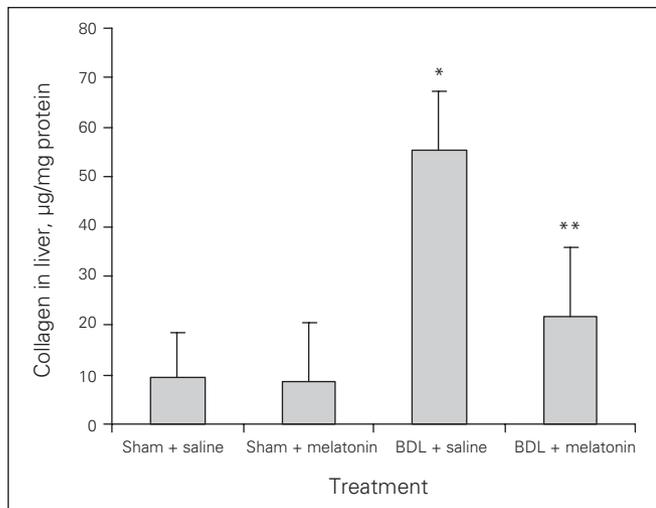
Fig. 1. Levels of tissue collagen in rats after bile-duct ligation (BDL) or sham operation and treatment with saline or melatonin. Results are means and standard errors. *$p < 0.001$ versus BDL and melatonin, saline and melatonin-only treated rats. **$p < 0.001$ versus saline and melatonin-only treated rats.
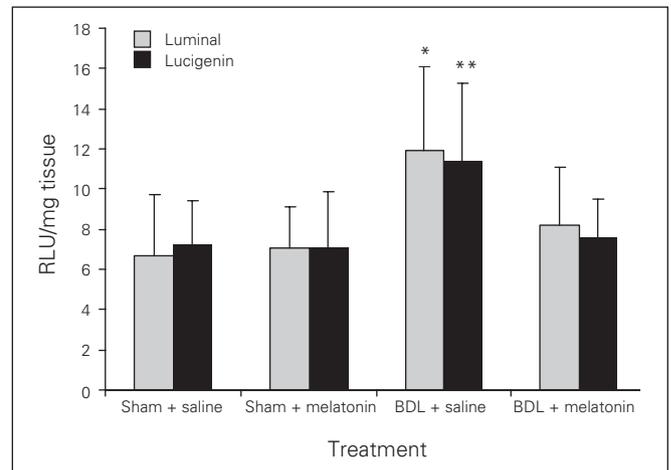

Fig. 3. Tissue luminal and lucigenin levels after bile-duct ligation (BDL) or sham operation and saline or melatonin administration. Results are means and standard errors. *$p < 0.001$ versus other 3 groups, **$p < 0.001$ versus the other 3 groups.
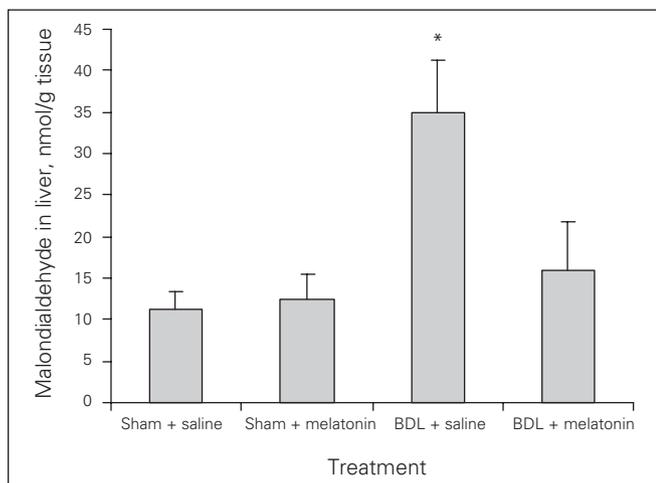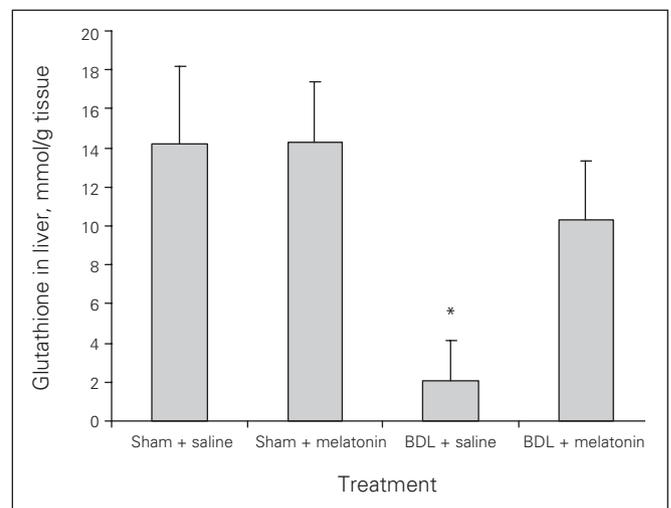

Fig. 2. Levels of tissue malondialdehyde after bile-duct ligation (BDL) or sham operation and saline or melatonin administration. Results are means and standard errors. *$p < 0.001$ versus other 3 groups.

Fig. 4. Tissue glutathione levels after bile-duct ligation (BDL) or sham operation and saline or melatonin administration. Results are means and standard errors. *$p < 0.001$ versus other 3 groups.

effective therapeutic approach against the development of hepatic fibrosis is still needed.

Current data indicate that oxidative stress is related to the activation of HSCs, which are the central mediators in the pathogenesis of liver fibrosis.[21,22] Hepatic stellate cells are activated by MDA, and this activation is blocked by antioxidants.[2] Lipid peroxidation is an indicator of tissue damage and accelerates collagen synthesis by stimulating HSCs.[23] Fibrosis caused by lipid peroxidation and its products decreases after the administration of antioxidants in animal models.[2,21]

Melatonin studies are ongoing into its antiaging and anticancer effects. Its best known effect is as a sleeping pill and a way to fight jet lag.[24,25] Recently, our group reported that melatonin is a potent hepatoprotective agent in a non-alcoholic steatohepatitis rat model.[8] In the present study, we aimed to study some of the basic oxidative stress markers and antioxidant levels to see if melatonin has an effect in an acceptable fibrosis model of BDL. Melatonin improved oxidative stress by increasing GSH levels and decreasing MDA, luminal and lucigenin levels in this model.

Cytokines are also involved in triggering and activating HSCs, which produce collagen.[26] In addition to reducing oxidative stress parameters, melatonin significantly decreased the levels of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) in the BDL group compared with the BDL–melatonin group.

Several lines of evidence suggest that melatonin has either a regulatory role in maintaining collagen levels or an inhibitory role in collagen accumulation.[2,9,10] In patients with scleroderma, cutaneous collagen synthesis increases during summer, which has been related to a seasonal reduction in the activity of the pineal gland, thus suggesting a relation between fibrosis and melatonin.[9]

A previous study from our group was the first to show the partial protective effect of melatonin on liver fibrosis in an experimental model involving dimethylnitrosamine (DMN)
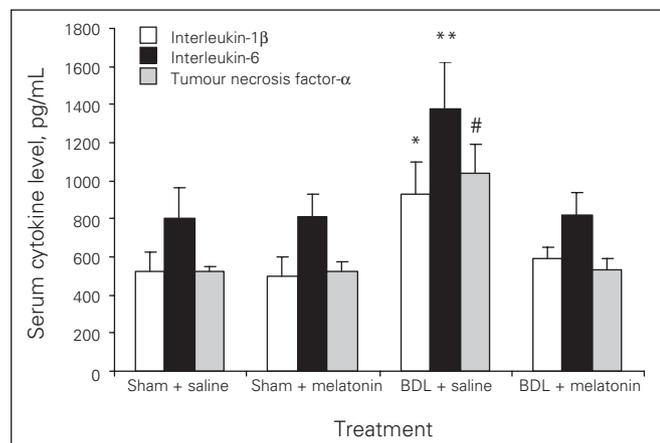
in rats.[2] We found significantly lower hydroxyproline levels in the DMN- and melatonin-treated rats compared with the rats that received only DMN. Histologic evaluation of liver specimens from all 14 rats in the BDL-only group revealed a central-to-central and central-to-portal bridging structure. The parenchymal loss and generation of repair tissue was minimal in 5 of 14 DMN–melatonin rats, suggesting a
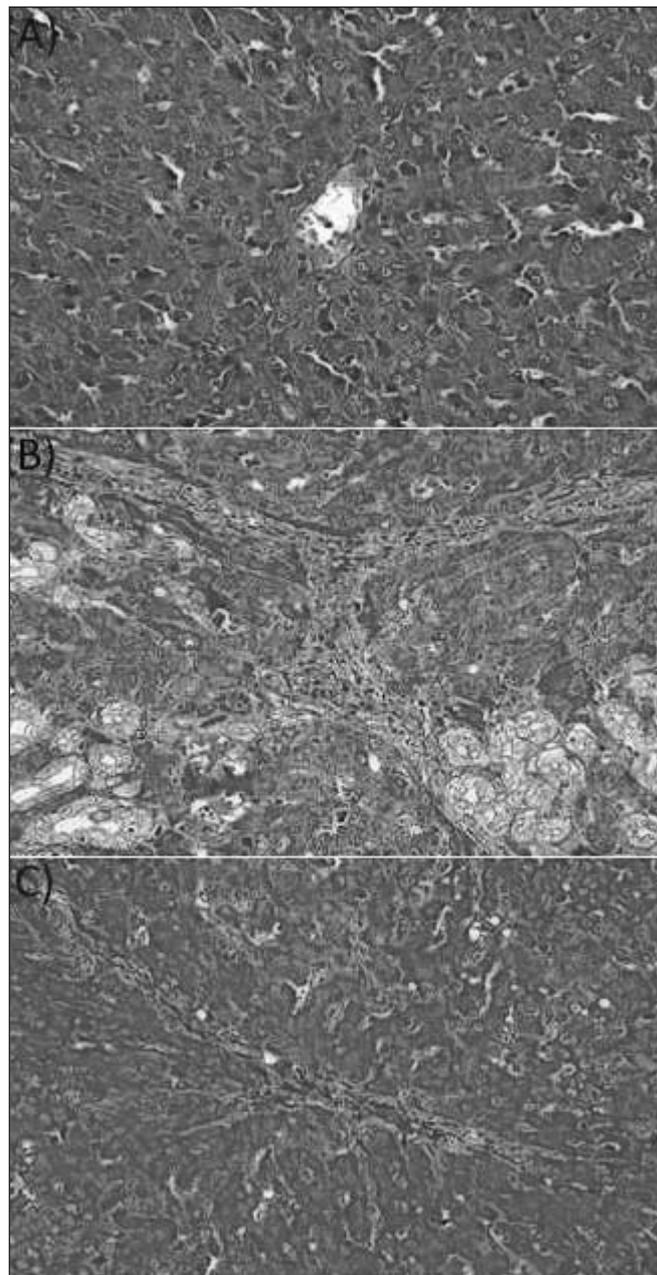
Fig. 6. (**A**) Normal liver architecture was observed in the saline and melatonin groups (hematoxylin and eosin staining, original magnification ×40). (**B**) Damage induced by bile-duct ligation (BDL) in the liver. Stage 3 fibrosis with prominent portoportal and portoseptal bridging was seen in the BDL rats (Trichrome, original magnification ×40) (**C**) Administration of melatonin improved liver damage induced by BDL. In this figure, only stage 2 fibrosis can be seen in the BDL–melatonin group (Trichrome, original magnification ×40).

Fig. 5. Serum cytokine levels after bile-duct ligation (BDL) or sham operation and saline or melatonin administration. Results are means and standard errors. *p < 0.001 versus other 3 groups, **p < 0.001 versus the other 3 groups; #p < 0.001 versus the other 3 groups.

significant protective effect of melatonin on hepatic histology.[2]

Pastor and colleagues[27] reported that decreased levels of GSH were significantly correlated with elevated levels of MDA in a BDL model. Our findings are in agreement with their results. In terms of liver biochemistry, the serum levels of AST, ALT, ALP, GGT and bilirubin were higher in the BDL group than in the other 3 groups.

In a recent study, Hong and colleagues[11] showed protective antifibrotic effects of melatonin on hepatic fibrosis induced by carbon tetrachloride in experimental rats. They concluded that the protective effect of melatonin on hepatic fibrosis may be related to its antioxidant activities. In our study, we wanted to evaluate the free radical scavenging, anti-inflammatory and antifibrotic activities of melatonin in an acceptable fibrosis model of BDL. We evaluated formation of liver fibrosis both qualitatively by histology and quantitatively by measuring collagen levels. We found that the collagen levels were significantly decreased in all rats in the BDL–melatonin group, whereas histologic protection was evident in 5 of 8 rats. In the BDL–melatonin rats, we found no significant difference to biochemical parameters between the histologically responsive and unresponsive rats.

In conclusion, we found that daily melatonin injections at pharmacologic doses are effective in reducing liver damage in rats with BDL-induced hepatic fibrosis. The protective action of melatonin may be related to its antioxidant, anti-inflammatory and antifibrotic activities.

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