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Melatonin reduces dimethylnitrosamine-induced liver fibrosis in rats

Abstract: Increased deposition of the extracellular matrix components, particularly collagen, is a central phenomenon in liver fibrosis. Stellate cells, the central mediators in the pathogenesis of fibrosis are activated by free radicals, and synthesize collagen. Melatonin is a potent physiological scavenger of hydroxyl radicals. Melatonin has also been shown to be involved in the inhibitory regulation of collagen content in tissues. At present, no effective treatment of liver fibrosis is available for clinical use. We aimed to test the effects of melatonin on dimethylnitrosamine (DMN)-induced liver damage in rats. Wistar albino rats were injected with DMN intraperitoneally. Following a single dose of 40 mg/kg DMN, either saline (DMN) or 100 mg/kg daily melatonin was administered for 14 days. In other rats, physiologic saline or melatonin were injected for 14 days, following a single injection of saline as control. Hepatic fibrotic changes were evaluated biochemically by measuring tissue hydroxyproline levels and histopathogical examination. Malondialdehyde (MDA), an end product of lipid peroxidation, and glutathione (GSH) and superoxide dismutase (SOD) levels were evaluated in blood and tissue homogenates. DMN caused hepatic fibrotic changes, whereas melatonin suppressed these changes in five of 14 rats ($P < 0.05$). DMN administration resulted in increased hydroxyproline and MDA levels, and decreased GSH and SOD levels, whereas melatonin reversed these effects. When melatonin was administered alone, no significant changes in biochemical parameters were noted. In conclusion, the present study suggests that melatonin functions as a potent fibrosuppressant and antioxidant, and may be a therapeutic choice.

Veysel Tahan[1], Resat Ozaras[2], Billur Canbakan[1], Hafize Uzun[3], Seval Aydin[3], Beytullah Yildirim[1], Huseyin Aytekin[1], Gulsen Ozbay[4], Ali Mert[2] and Hakan Senturk[1]

Departments of [1]Internal Medicine, [2]Infectious Diseases, [3]Biochemistry and [4]Pathology, Cerrahpasa Medical Faculty, Istanbul University, Istanbul, Turkey

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Address reprint requests to Veysel Tahan, Cavusdere Cad. Huzur Camii Sokak, Akman Apt. No:1/1 Daire: 9. Uskudar/ Istanbul, 34672 Turkey.
E-mail: veytahan@yahoo.com

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Introduction

Liver fibrosis is one of the main components of cirrhosis and characterized by the accumulation of collagen and extra cellular matrix proteins in the space of Disse [1–3]. These proteins are produced in damaged liver by the stellate cells. Collagen type I, III and IV, fibronectin, laminin, and proteoglicans [1, 4] are the extra cellular proteins produced during this process, where collagen type I and III [3, 5] are most abundantly found. Collagen deposits in liver are not only demonstrated by histologic cross sections, but also quantitatively detected via measuring the tissue levels of hydroxyproline, which is the end product of collagen metabolism. Several lines of evidence have suggested the important role of oxidative stress in the etiopathogenesis of liver fibrosis [1, 2, 6–14]. Furthermore, oxidative stress aggravates liver fibrosis via stellate cell activation [15], and lipid peroxidation stimulates the collagen gene transcription in cell culture [1, 16, 17].

Particularly collagen type I and III accumulate mostly during liver fibrosis in human beings. Similar to alcoholic liver fibrosis, type III collagen accumulation is more remarkable after dimethylnitrosamine (DMN) administration in rats [1, 18, 19]. An effective treatment for liver fibrosis is still needed. Nevertheless, recent experimental studies have reported partial success against liver fibrosis in the DMN induced rats with hepatocyte growth factor [20], estradiol [6, 21], malotilate [22], halofuginone [23], retinyl palmitate [11], lipoic acid [24], interferon gamma [25], and sho-saiko-to, a Japanese herbal therapeutic [1, 26].

Melatonin (N-acetyl-5 methoxytryptamine), a secretory product of the pineal gland is a powerful endogenous antioxidant [27–30]. Exogenous application of this molecule leads to a remarkable decline in oxidative stress via directly neutralizing with the hydroxyl radicals. In addition, melatonin is also indirectly effective by enhancing the levels of potential antioxidants such as glutathione peroxidase (GPx), superoxide dismutase (SOD), and glutathione (GSH) [31–38]. Melatonin may reduce fibroblast proliferation and collagen synthesis. Reduced melatonin has been shown to enhance collagen accumulation in tissues. These studies also underlined the antifibrotic effects of melatonin as well as its properties in controlling collagen levels in wound granulation tissue [39, 40]. Stimulation of endogenous melatonin secretion achieved by continuous exposure of animals to dark [41], have been found to reduce the amount of collagen in granulation tissue during scar formation delaying superficial wound healing [39].

Increased collagen levels in both intact skin and wound have been observed following pinealectomy, whereas exogenous application of melatonin has caused the opposite effect [39]. In pinealectomized rats, excessive fibrotic tissue formation has been reported to result in adhesions between the abdominal organs, as well as fixation of liver and spleen beneath the diaphragm [40]. In one study, performed on pinealectomized rats, adventitial and periadventitial fibrosis formation in the renal arterioles, has been demonstrated [42]. Cytostatic effect of melatonin is because of its ability to inhibit cell proliferation, and this feature may also lead to an inhibition on the oxidative stress caused by polymorphonuclear leukocytes, and on the fibrogenic activity of stellate cells.

In this study, the possible fibrosuppressant effect of melatonin on a liver fibrosis model in rats was evaluated as a new therapeutic choice.

Materials and methods

All experimental protocols were approved by Istanbul University Animal Care and Use Committee. Male Wistar albino rats, 3.5–4 months old, weighing 190–220 g obtained from Istanbul University Animal Research Laboratory were kept at a constant temperature (22 ± 1) with 12 h light and dark cycles fed chow (Eris Chow Industry, Istanbul, Turkey) ad libitum. All rats had free access to tap water. All animals received humane care in compliance with the National Institutes of Health criteria for care of laboratory animals.

Forty-two rats were divided into four groups. A single injection of 40 mg/kg DMN (Aldrich Chemical Co, Deisenhofen, Germany) intraperitoneally was given to 14 rats and another 14 rats received DMN + melatonin (100 mg/kg/day). The control rats received saline or melatonin only. Melatonin (Sigma Chemical Co, Deisenhofen, Germany) was given for 14 days at 08.00 hours whereas the DMN animals were given physiologic saline.

On the 14th day, animals were killed by decapitation and trunk blood was collected and centrifuged (500 g, 10 min, 4°C). Liver samples were quickly obtained. GPx levels in erythrocytes and biochemical parameters were studied immediately. For the remaining studies, serum and liver tissue samples were stored at −80°C. Liver samples were weighed and homogenized in 0.15 M NaCl for lipid peroxidation parameters and for the other studies, and homogenates of 20% were obtained. Tissue homogenates were sonicated two times at 30 s intervals. Homogenization and sonication were performed at 4°C. After sonication, homogenates for lipid peroxidation and biochemical studies were centrifuged at 500 g for 10 min and at 12 500 g for 15 min. Aliquots of the supernatants were used for both studies [43]. The assayed parameters were expressed per mg protein. Protein content of the aliquots was determined by the method of Lowry et al. [44].

Biochemical measures

Lipid peroxidation was measured by the thiobarbituric acid method, a modified form of the procedure described by Beuge and Aust [45]. Serum and homogenized liver tissue SOD levels were measured by using commercial kits (Randox-Ransod, Crumlin, Co. Antrim, UK). Erythrocyte and homogenized liver tissue GSH levels were measured by using 5,5′-ditiobis-2-nitrobenzoic acid method [46]. Erythrocyte GPx level was also measured by using commercial kits (Randox-Ransel, Crumlin, Co. Antrim, UK). Biochemical parameters were determined by an autoanalyzer (Hitachi 717, Tokyo, Japan). Hemoglobin level was measured manually by cyanmethemoglobin method.

Wet liver tissue hydroxyproline levels were measured by the method of Kivirikko et al. [47] by using commercial kits (Organon Tec, B.V., Boxel, Holland).

Histologic evaluation

Liver histologic sections were fixated with 10% formaldehyde and slides, made from paraffin-embedded blocks were stained by haemotoxylene and eosin. To determine the fibrotic tissue component, Masson-Tricrom and Gomory reticulum staining methods were applied.

Statistical analysis

All results are expressed as mean ± standard errors. Comparisons between the groups were performed by Kruskal–Wallis variance analysis and a $P < 0.05$ was accepted as statistically significant.

Results

The mean liver hydroxyproline level of the DMN group was significantly higher than in the DMN + melatonin treated rats, in the saline treated rats or in the melatonin only treated rats; however, mean hydroxyproline levels in the DMN + melatonin rats were still higher than either the saline rats or the melatonin only treated rats (Fig. 1).

The mean plasma and liver MDA levels of the DMN treated rats were significantly higher than those in the DMN + melatonin animals; they were also higher than levels in the saline and melatonin injected animals (Figs 2 and 3). The mean hepatic SOD levels of DMN rats were significantly higher than the DMN + melatonin animals,

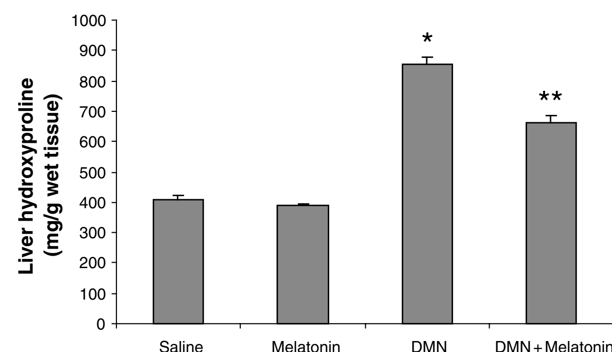

Fig. 1. Tissue hydroxyproline (mg/g wet tissue) levels. $^{*}P < 0.001$ versus DMN + melatonin, saline and melatonin only treated rats. $^{**}P < 0.001$ versus saline and melatonin only treated rats (DMN, dimethylnitrosamine).

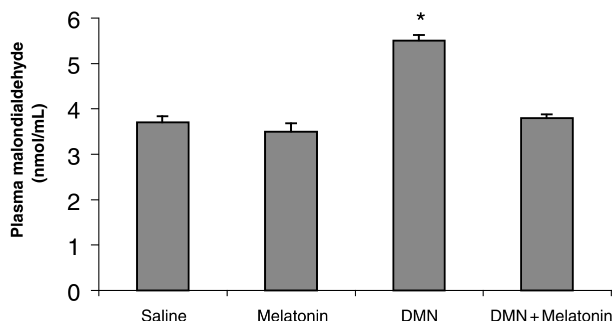

Fig. 2. Plasma malondialdehyde (nmol/mL) levels. *$P < 0.001$ versus other three groups (DMN, dimethylnitrosamine).

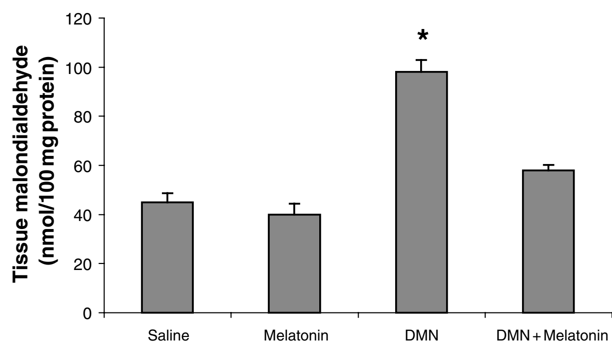

Fig. 3. Tissue malondialdehyde (nmol/100 mg protein) levels. *$P < 0.001$ versus other three groups (DMN, dimethylnitrosamine).

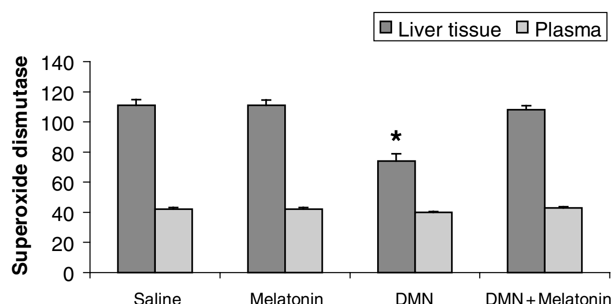

Fig. 4. Tissue (U/100 mg protein) and serum (U/mL) superoxide dismutase levels. *$P < 0.001$ versus other three groups (DMN, dimethylnitrosamine).

the saline rats and the the melatonin only injected rats, while the mean plasma SOD levels were not statistically different (Fig. 4).

The mean erythrocyte and liver GSH levels of DMN rats were significantly higher than in the DMN + melatonin animals, the saline rats, and melatonin only treated rats (Fig. 5). The mean erythrocyte GPx levels were also significantly higher in the DMN animals relative to the other groups (Fig. 6). While plasma alkaline phosphatase (ALP) levels in DMN treated rats were significantly higher than in the DMN + melatonin animals as well as in the control groups (Table 1), no difference was observed

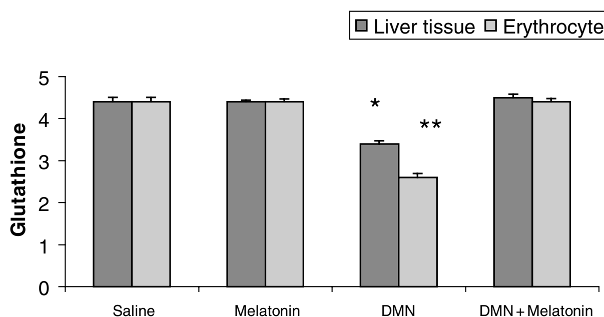

Fig. 5. Tissue (mg/g protein) and erythrocyte (mg/g Hb) glutathione levels. *$P < 0.001$ versus other three groups. **$P < 0.001$ versus other three groups (DMN, dimethylnitrosamine).

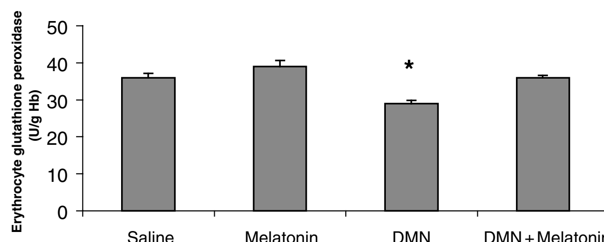

Fig. 6. Erythrocyte glutathione peroxidase (U/g Hb) levels. *$P < 0.001$ versus other three groups (DMN, dimethylnitrosamine).

between groups in reference to other biochemical parameters.

In histologic examination, while a single administration of DMN caused hepatic fibrotic changes in all the DMN injected rats (14/14) (Fig. 7A,C), these fibrotic changes were suppressed in liver histopathology of 5/14 the DMN + melatonin treated rats ($P < 0.05$) (Fig. 7B).

Discussion

Lipid peroxidation is an indicator of tissue damage and accelerates collagen synthesis by stimulating stellate cells [48]. Free radicals and biomolecular reaction products promote phagocytic and myofibroblastic activities. Fibrosis caused by the lipid peroxidation and its products decreases after the administration of antioxidants in animal models [10, 15]. Stellate cells are activated by MDA and this activation is blocked by antioxidants [1, 17]. Melatonin has been found to possess higher antioxidant efficiency than vitamin E and GSH, which are known as powerful antioxidants [38, 49].

Ostrowska et al. [50] revealed a negative correlation between melatonin and urine hydroxyproline levels in fasting rats. Drobnik and Dabrowski [51] studied tissue hydroxyproline levels in the pinealectomized rats, and demonstrated increased amounts of collagen in both scarred and healthy skin; in contrast, they observed markedly decreased collagen accumulation in both tissues after melatonin administration at a dose of 30 μg/100 g body weight.

Table 1. Biochemical parameters measured in the current study

Mean ± standard error	Saline	Melatonin	DMN	DMN + melatonin	*P*
AST (U/L)	119 ± 2.4	124 ± 3.4	149 ± 4.5	147 ± 3.5	> 0.05
ALT (U/L)	67 ± 2.1	64 ± 2.6	66 ± 2.9	68 ± 2.2	> 0.05
Alkaline phosphatase (U/L)	723 ± 27	744 ± 25	920 ± 31	825 ± 21	< 0.01
Total bilirubin (mg/dL)	0.13 ± 0.02	0.14 ± 0.02	0.13 ± 0.01	0.13 ± 0.01	> 0.05
Total protein (g/dL)	7.13 ± 0.08	7.13 ± 0.09	6.93 ± 0.09	7.18 ± 0.06	> 0.05
Albumin (g/dL)	4.07 ± 0.05	4.11 ± 0.06	4.11 ± 0.08	4.09 ± 0.06	> 0.05

DMN, dimethylnitrosamine.
Plasma alkaline phosphatase levels in the DMN injected rats were significantly higher than the DMN + melatonin ($P < 0.001$) treated rats, the saline rats and the melatonin rats ($P < 0.01$, for both).

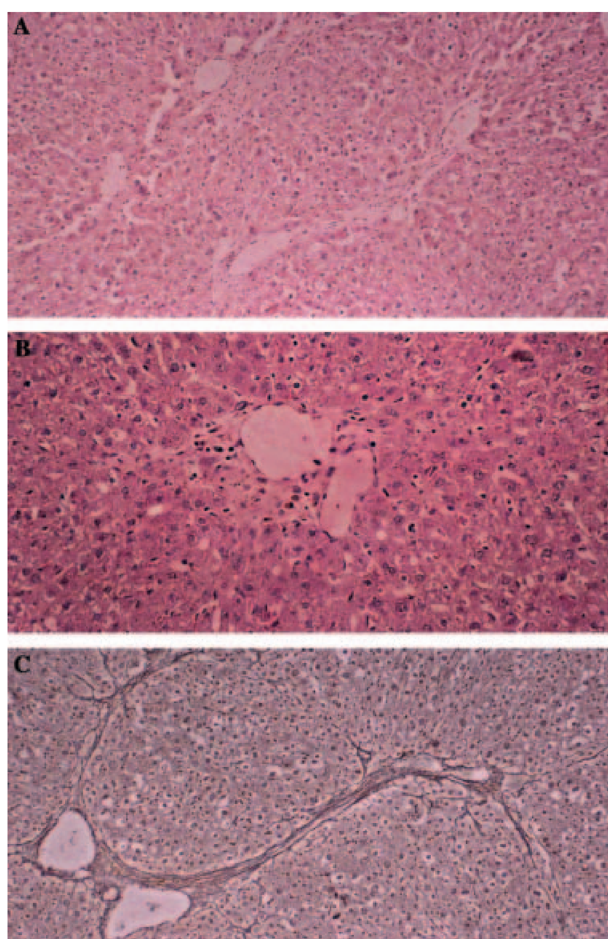

Fig. 7. (A) Dimethylnitrosamine (DMN)-induced damage in the liver. There is extreme cellular loss with pigment-laden macrophages and fibroblasts (HE, ×100). (B) Melatonin improved DMN-induced liver damage. This figure shows only focal cellular damage in the DMN + melatonin rats (HE, ×200). (C) DMN induced fibrous septa, connecting portal areas to each others and lobule centers in the DMN-treated rats (Gomori's reticulum staining, ×100).

Several lines of evidence support that melatonin has either the regulatory role in maintaining collagen levels or the inhibitory role on collagen accumulation [39, 40, 51]. Some researchers suggested a relationship between primary biliary cirrhosis and melatonin deficiency [40] because of the demonstration of increased pigmentation, increased ALP and cholesterol levels and accelerated fibrosis in pinealectomized rats. Another experimental study in pinealectomized rats demonstrated a relationship between liver fibrosis and pinealectomy [52]. Because of its circadian rhythm melatonin has nocturnal increase, and plays a protective role against possible formation of stasis related emboli, by inhibiting the thrombocyte aggregation, which is induced by collagen and arachidonic acid [53].

In patients with scleroderma, cutaneous collagen synthesis increases during summer, and this situation has been related to seasonal reduction in pineal gland activity [40]. Prolonged methysergide treatment causes increased fibrosis in tissues, predominantly in retroperitoneal region; this was attributed to the negative effect of methysergide on serotonin, which is a known melatonin precursor. Suppression of melatonin synthesis and secretion was reported in cases with methysergide related heart and lung fibrosis [54, 55].

Our study is the first to demonstrating partial protective effect of melatonin on liver fibrosis. We found significantly lower hydroxyproline levels in the DMN + melatonin treated rats compared with the rats that received only DMN. There were not statistically significant differences between the saline and melatonin only treated animals.

Histologic evaluation of liver specimens of all 14 subjects in the DMN-only treated rats revealed replacement of the lost parenchyma by a repair tissue characterized by macrophages filled with degradation pigments, fibroblasts, collagen fibers, and mononuclear inflammation cells, altogether constituting a central-to-central and central-to-portal bridging structure. While in nine of 14 DMN + melatonin rats, we failed to demonstrate a difference in their histologic structure when compared with the DMN rats in reference to tissue damage and fibrosis, in five of 14 parenchyma loss and the repair tissue were minimal, documenting a significant protective effect of melatonin on hepatic histology. Liver histology was normal in the saline and melatonin treated control groups.

Oxidative stress plays an important role in the formation of liver fibrosis via increasing the stellate cell activation and collagen synthesis. MDA, the end product of lipid peroxidation has been shown to activate the collagen producing stellate cells. Kaya et al. [31] reported suppression of lipid peroxidation in rats with co-administration of melatonin and cyclophosphamide. Interestingly, melatonin administration prior to the same procedure prevented development of oxidative damage. In

our study, liver and plasma MDA levels in the DMN rats were significantly higher than the other three groups. Consistent with the previous studies, melatonin suppressed MDA generation at a rate close to the control levels. Pastor et al. [56] studied a rat model in which secondary biliary cirrhosis developed 28 days after the experimentally created bile duct obstruction. In this model, decreased levels of GPx, GSH, and SOD, and significantly elevated levels of MDA in the rat liver were noted. Cabre et al. [57] demonstrated increased lipid peroxidation and decreased liver GPx and GSH levels, as well as a negative correlation ($r = -0.47$; $P < 0.001$) between lipid peroxidation and GPx in a liver fibrosis model created by carbon tetrachloride (CCl_4) administration in rats. Melatonin has also been reported to reduce free radical damage by secondarily elevating GPx activation [34, 58–60]. We found significantly higher GPx levels in the DMN rats when compared with the other three groups.

In reference to liver biochemistry, serum ALP levels were higher in the DMN rats, than in the other three groups. In addition, plasma AST levels in the DMN and DMN + melatonin rats were significantly higher than the controls whereas other biochemical parameters did not reveal any significant difference. Ohta et al. [61] investigated the effects of melatonin on a rat model with CCl_4 induced acute hepatitis and in the study each rat received CCl_4, one of the groups received melatonin at pharmacological doses of 10, 50, and 100 mg/kg. In the CCl_4-only group had significantly increased AST, ALT levels, and liver lipid peroxidation with decreased GSH levels when compared with the controls. However, this study was confined only to the acute phase and fibrosis formation was not particularly studied. In our experiment, liver fibrosis formation was evaluated both qualitatively by histology and quantitavely with hydroxyproline levels. This is the first study demonstrating the effect of melatonin on hydroxyproline levels in liver tissue. Hydroxyproline levels significantly decrease in all rats of the DMN + melatonin group while histologic protection was evident in five of 14 rats. In the DMN + melatonin rats, no significant difference was demonstrated between the histologically responsive and unresponsive rats in reference to the biochemical parameters.

In conclusion, daily melatonin injections at pharmacological doses proved to be effective against liver damage in a rat liver fibrosis model induced by a 14-day DMN regimen. The protective action of melatonin may relate to its antioxidant activity.

Acknowledgments

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