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Effect of Melatonin on Hepatic Fibrogenesis, Vitamin C and Hydroxyproline Levels in Liver of Ethanol-Fed Rats

Abstract: Pineal-gland-derived melatonin (N-Acetyl 5-Methoxytryptamine), which is a known antioxidant hormone, is known to have inhibitory effects on cell growth and proliferation. Ethanol causes increases in lipid peroxidation, superoxide formation and collagen synthesis resulting in fibrosis in the liver due to alcohol intake. The aim of this study was to examine the effect of exogenously administered melatonin on liver tissue damage resulting from alcohol intake. A total of 30 male wistar albino rats were divided into three groups; Group 1 (control), Group 2 (ethanol-administration), and Group 3 (ethanol +melatonin administration). All the rats were fed with a specially modified diet for one week. At the end of the 1st week, the control group rats were given isocaloric sucrose, while Group 2 and Group 3 rats were given gradually increasing amounts of ethanol to induce liver damage. The third group also received melatonin (10 mg/kg/day) together with ethanol. Biochemical and histopathological findings revealed the formation of liver damage after the ethanol application was completed. There was a significant increase ($p < 0.001$) in the hydroxyproline levels in the livers of the Group 2 rats (8.57 ± 1.30 mg/gr tissue) when compared to those in the control group (4.90 ± 0.98 mg/gr tissue) whereas hydroxyproline levels in the livers of rats which had received melatonin together with ethanol was similar to those in the control group, indicating melatonin's protective effect. In addition, ascorbic acid (an important vitamin required for collagen synthesis) levels were reduced drastically ($p < 0.001$) in Groups 2 and 3, when compared to those in the control group. The livers of rats given melatonin and ethanol had significantly higher ($p < 0.001$) ascorbic acid levels than those of the Group 2 rats. In conclusion, exogenously given melatonin has a partial protective effect on development of fibrosis in alcohol-damaged liver.

Key Words: Hydroxyproline, ascorbic acid, alcoholic fibrosis, melatonin.

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Introduction

The pineal-gland-derived hormone melatonin has attracted interest particularly with regard to its role as a mediator of photoperiodic information, a role that leads to a kind of physiological pleitropy (1). The pineal gland acts as a general synchronizing, stabilizing and moderating organ for several physiological processes. Melatonin has anti-aging properties and appears to modify growth. It also known functions as an immunomodulator, and has oncostatic and antioxidative effects (1,2). In addition, melatonin is involved in the inhibitory control of cell proliferation, and removal of the pineal gland, the main source of melatonin accelerates the growth of cells. Melatonin has been shown to have an antimitotic effect only when it is administered in the late afternoon (3). Recent research has shown elevated collagen contents in the abdominal cavities of pinealectomized rats and suggests that a reduced level of melatonin causes an elevation of collagen accumulation in the tissue (4). Melatonin has also been found to stimulate synthesis of prostaglandin E_1, which is an inhibitor of collagen production (4,5). Janus *et al.* reported that collagen levels were modified by melatonin hormone in intact skin (6).

Tissue repair during wound healing occurs through granulation and connective tissue. Collagen, the main protein in granulation tissue, is composed of repeated units of 3 polypeptide chains containing glycine-proline. In addition to regularly repeated glycine-proline, hydroxyproline formation takes place by hydroxylation of

specific proline units. If hydroxyproline formation on the specific prolines is not carried out, collagen is still synthesized but it will not be as stable as that containing hydroxyprolines. This may result in complications during wound healing (4). Hydroxylation of proline is catalyzed by proline hydroxylase which needs ascorbic acid, a cofactor in the presence of oxygen (7). Suresh *et al.* reported that tissue vitamin C levels were reduced in alcohol-induced liver damage in guinea pigs. Interestingly, ascorbic acid degradation was lowered as a response to alcohol administration in guinea pigs so that tissue vitamin C levels were not totally diminished (8,9). Balanced degradation of synthesized collagen is also important for normal wound healing. Collagenases are the enzymes responsible for the degradation of collagen. Both synthesis and degradation need to be carried out simultaneously so that the formation of scars can be avoided (10,11).

Irregularities in collagen synthesis and degradation in the liver, which has a capacity for very rapid regeneration, may also be observed. This is similar to other diseases causing fibrotic changes in the liver tissue. Hepatic fibrosis or increased collagen content of the liver can lead to hemodynamic and functional abnormalities that, when extensive, may be life-threatening. The formation of fibrosis and granulation tissue occupies the places of normal hepatocytes, which then leads to dysfunction of the liver (10,11). When damage in hepatocytes due to alcohol is evaluated, it is important to check alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels and especially the gamma glutamil transpeptidase (γ-GT) level, which is an indicator of alcohol-dependent toxicity. In addition, the roles of zinc (Zn, a trace element with an important role in wound healing) and copper (Cu, a co-factor of lysil oxidase that plays a role in collagen synthesis) were also evaluated in alcohol-induced liver damage by measuring Zn and Cu levels. Liver fibrosis due to alcohol and viral and tumoral factors is generally evaluated histopathogically.

Pineal-gland-derived melatonin (N-acetyl 5-methoxytryptamine), which is a known antioxidant hormone, is known to have inhibitory effects on cell growth and proliferation. It has been reported in several studies utilizing pinealaectomized animals that melatonin reduces collagen levels in granulation tissue during wound healing (2, 4,12). The aim of the present study was to examine the modulatory effect of melatonin on hydroxyproline and ascorbic acid in liver fibrosis, steatosis and necrosis caused by ethanol administration.

Material and Methods

Animals: A total of 30 male Wistar Albino rats (160 to 200 g) were used in the study. All the animals received humane care in compliance with the guidelines of the Fırat University Research Council criteria. The rats were kept in specially prepared air-conditioned cages which received 12 hours day light per day. The rats were divided into three groups (10 rats per group).

Ethanol-Induced Liver Damage Model: Every rat in each group was given a liquid diet as modified by De Carli and Lieber (13). This liquid-only diet was administered for a one-week period. At the end of the first week, isocaloric sucrase solution was added to the liquid diet of one group. Ethanol (8g/kg/body weight) was added to the liquid diet of the other two groups. The amount of ethanol intake was then increased to 1g/kg per week and over eight weeks it was increased to 15 g/kg/body weight.

Melatonin (Sigma Chem Co., St Louis, U.S.A.) was freshly dissolved in absolute ethanol + 0.9 % NaCl (1:10) and injected ip once daily into the rats of one of the groups that was being given ethanol at 5-6 p.m. for 8 weeks. The other group that was receiving ethanol in the same period was injected with isotonic saline as a placebo. After eight weeks, the rats were sacrificed by decapitation. Blood samples were taken, and the plasma was separated and kept at –20°C for the measurement of biochemical parameters. Liver tissue samples were taken for the examination of hydroxyproline and ascorbic acid levels and for histopathological examination. The liver tissue homogenates were prepared with a homogenizer (Ultra-Tunnax T25, Germany).

The levels of plasma ALT, AST, alkaline phosphatase (ALP), and γ-GT were determined with a Technicon RA-XT autoanalyser (Technicon Corp., U.S.A.).

Determination of Hydroxyproline levels: Liver tissue (10 mg) was homogenized in 1 ml of 10 N HCl with a homogenizer. This homogenate was used directly for hydroxyproline analysis. Hydroxyprolines attached to strong acid cations were exchanged with resins. Then the resins were washed with distilled water to remove substances which may have caused interference. Peptide bonds in the resins were broken at 100 °C for 16 hr. The hydroxyprolines were then oxidized with a pirol derivate. Following incubation at 60 C° for 25 min, the samples were kept at room temperature for 30 min to stabilize the color, and the optical density of the developed color was read at 560 nm with a Schimadzu UV 1201 (Schimadzu Corp., Japan) spectrophotometer. The results were expressed as mg/g tissue (14,15).

Determination of Ascorbic Acid: Samples were treated with 0.1 M HClO$_4$ and centrifuged at 4000 rpm for 10 min. The supernatants were utilized in high performance liquid chromatography (Cecil 1100, HPLC) according to the method of Tawazzi *et al.* (16).

Histopathological Examination: Liver-tissue sections were fixed in 10% formalin saline and processed in paraffin blocks. Sections from the blocks were stained with Hematoxylen-Eozin, Reticulum and Masson's Trichrome. Light microscopic examinations were carried out at magnifications of x40, x100, x200 and x400. The microscopic examinations were performed in a blind fashion. Histopathological findings, of portal tract lesions, focal necrosis, fibrosis and fatty degeneration, were expressed qualitatively with + sign(s) (+:light, ++: moderate +++: severe) according to the dissemination of pathologies (17). Other findings were reported as present or absent.

Statistical analysis: Statistical evaluations were performed using ANOVA (Kruskal Wallis) tests for biochemical parameters, liver tissue hydroxyproline and ascorbic acid levels. Fischer's Exact Chi-square test was used for histopathological parameters (mononuclear cell infiltration, fibrosis, focal necrosis and fatty degeneration).

Results

Animals fed with ethanol for 8 weeks gained weight at a slower rate than the control rats. The average of the differences between the weights of the groups was not statistically significant (p>0.05, Table 1).

As shown in Figure 1, although hepatic hydroxyproline levels were significantly higher (p<0.001) in the liver tissue of the group which was fed athanol (8.57±1.3 mg/ g wet tissue) than those in the control group (4.90±0.98 mg/g wet tissue), they were

significantly higher in the liver of Group III (6.54±1.72 mg/g wet tissue) than in that of Group II (p<0.01).

The control group plasma ALT values were significantly higher in the ethanol group than those control (p<0.01) whereas the difference between the ethanol group and ethanol+melatonin group was statistically significant (p<0.05). Plasma γ-GT levels were higher in Group II (7.55±2.19 U/L) than in the control group (3.98±1.51 U/L) (p<0.05). Melatonin caused a significant reduction (p<0.05) in the γ–GT levels of Group III (5.17±2.13 U/L) when compared to Group II, but they were still higher than those in the control group.

As shown in Figure 2, ascorbic acid levels were significantly lower in the liver of the ethanol group (0.016±0.007mg/g tissue) (p<0.001) and ethanol+melatonin group (0.054±0.009 mg/ g tissue) (p<0.01) than in the control group (0.156±0.021 mg/g tissue). Ascorbic acid levels in the ethanol+melatonin group were significantly higher than those in the ethanol group (p<0.01).

In the histopathological examination, it was found that different grades of fatty liver developed in all the rats that had received alcohol (Table 3, Figure 3). The severity of steatosis, the necrotic focus and the number of inflammatory cells and fibrosis were significantly lower in the ethanol+melatonin group. The histopathological results are shown in Table 3 and Figure 4.

Discussion

Liver fibrosis or cirrhosis is characterized by hyper accumulation of fibrous tissue components and it is commonly observed in later or terminal states of chronic hepatic diseases, e.g, hepatitis and heamochromatosis (18). Chronic intoxication with ethanol is probably the most common cause of liver fibrosis. Chronic alcohol consumption has hepatotoxical effects on human beings.

Groups	n	Initial (g. range)	Final (g. range)	Difference*
Control Group I	10	181.45±11.57 (160-200)	201.19±21.41 (195-209)	19.74±5.94 (5-35)
Ethanol Group II	10	178.69±17.55 (160-185)	196.19±19.38 (190-205)	17.50±4.83 (5-35)
Ethanol+Melatonin Group III	10	176.91±16.86 (165-185)	195.28±17.35 (190-210)	18.17±7.91 (10-30)

Table 1. The average weights of the rats at the beginning and the end of the study. Data were expressed as mean±SD and the differences between the body weights of the groups were not statistically significant (NS, p>0.05) (ANOVA , Kruskal Wallis).
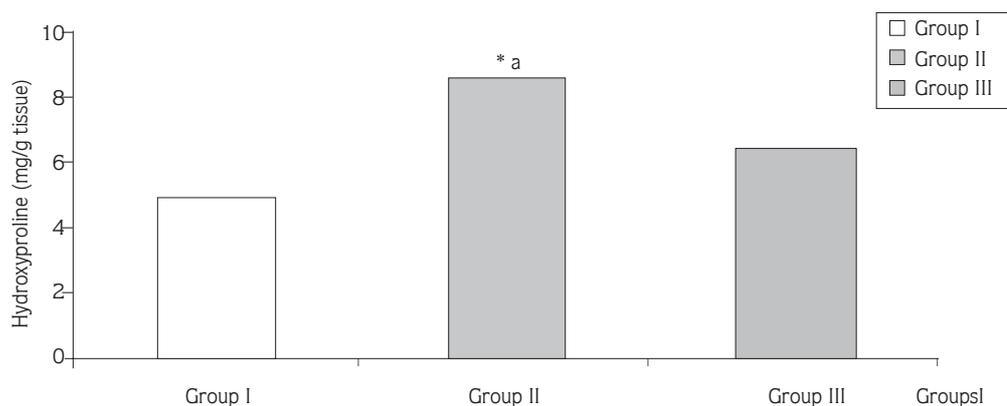
Figure 1. Hydroxyproline levels in liver tissues of Group I (control), Group II (Ethanol-induced) and Group III [Ethanol+Melatonin (10 mg kg-1)], * p<0.001 (Control vs Ethanol), ap<0.01 (Ethanol vs Ethanol+Melatonin). Data were expressed as mean±SD   (ANOVA, Kruskal Wallis).
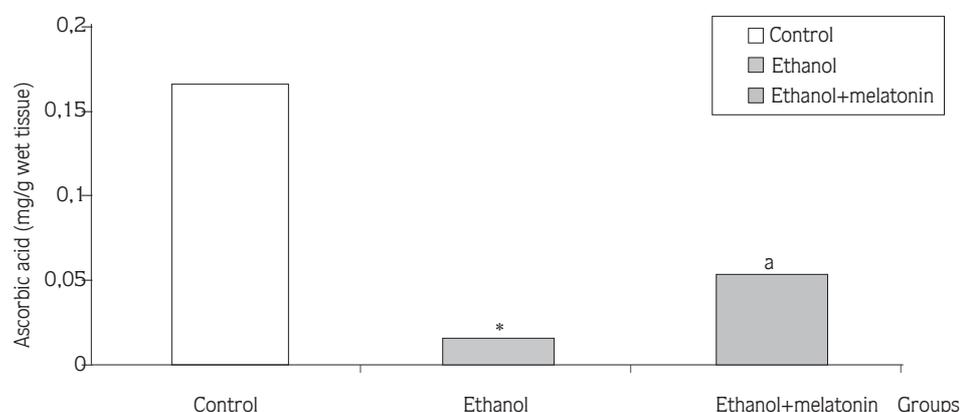
Figure 2. Ascorbic acid levels in liver tissues of Group I (Control), Group II (Ethanol) and Group III (Ethanol+Melatonin 10 mg kg^{-1}), *p<0.001 (Control vs Ethanol), ^{a}p<0.01 (Control vs Ethanol+Melatonin). Data were expressed as mean±SD (ANOVA, Kruskal Wallis).

It causes steatosis, alcoholic hepatitis, fibrosis and cirrhosis (19). Experimental and clinical data suggest that acetaldehyde, which is an intermediary product of ethanol oxidation, is responsible for stimulation of collagen biosynthesis in the liver. Liver fibrosis is accompanied by a significant increase in collagen content in this organ (18,19). Collagen is involved in wound healing via formation of hydroxylation. However, the balance of collagen is important in wound healing. If an uncontrolled accumulation of collagen cannot be degraded by collagenases, it will result in fibrosis formation instead of normal healthy hepatocytes. Elevated tissue collagen levels can be measured directly or can be indirectly evaluated by the measurement of hydroxyproline levels (14). The development of fibrosis has been studied in detail. It has been reported that long-term ethanol feeding selectively reduces hepatic mitochondrial glutathione content by impairing the mitochondrial uptake of this thiole. Progressive and selective depletion of mitochondrial glutathione is demonstrated in the experimental model of alcoholic liver disease and is associated with mitochondrial lipid peroxidation and the progression of liver damage (*i.e*, centrilobuler liver necrosis along with fibrosis) (20,21,22). In addition, ethanol is thought to cause increased lipid peroxidation by means of acetaldehyde stimulating the superoxide formation from the neutrophils and the collagen synthesis. There are a number of substances (selenium, vitamin A and E, traditional medicines, etc.) used for therapy according to the etiology of liver fibrosis (23,24). In the present study, we examined AST, ALT and GGT levels in order to evaluate alcohol-induced liver damage. It has been reported that administration of ascorbic acid lowers increased γ-GT levels due to alcohol-induced

Table 2.   The liver tissue hydroxyproline and ascorbic acid levels and some biochemical parameters in the plasma of the Control, Ethanol and Ethanol+ Melatonin groups.

Parameters	Control I	Ethanol II	Ethanol+Melatonin III	P value	
ALT (U/L)	76.41±11.21	271.87±51.65	154.13±20.18	p<0.01 p<0.05	I-II II-III
AST (U/L)	176.24±19.35	307.95±76.89	374.58±51.68	P<0.05	I-II, I-III
γ-GT (U/L)	3.98±1.51	7.55±2.19	5.17±2.13	p<0.05	I-II, I-III II-III
Zn (mg/dl)	129.17±7.76	118.07±10.59	125.02±12.41	p<0.05	I-II, I-III II-III
Cu (mg/dl)	637.51±32.98	618.76±27.03	658.97±26.82	p>0.05	NS

Table 3.   Histopathological changes in the liver tissue of Group I (Control), Group II (Ethanol) and Group III (Ethanol+Melatonin 10 mg kg^{-1}) rats.

Histopathologica Findings	Control n:10		Ethanol n:10		Ethanol + Melatonin n:10	
	+	%	+	%	+	%
In portal tract						
MNC infiltration						
Slight (+)	-	-	1	10	5	50
Medium (++)*	-	-	6	60	3	30
Severe (+++)	-	-	3	30	2	20
Fibrosis						
Slight (+)	-	-	-		5	50
Medium (++)**	-	-	8	80	4	40
Severe (+++)	-	-	2	20	1	10
Focal necrosis						
Slight (+)	-	-	3	30	6	60
Medium (++)	-	-	7	70	4	40
Severe (+++)	-	-	-		-	
Fatty degeneration						
Slight (+)	-	-	-	-	4	40
Medium (++)**	-	-	7	70	5	50
Severe (+++)*	-	-	3	30	1	10

* p<0.05 (Ethanol vs Ethanol+Melatonin), ** p<0.01(Ethanol vs Ethanol+Melatonin) Fischer's Exact Chi-square test was used for histopathological parameters (mononuclear cell infiltration (MNC), fibrosis, focal necrosis and fatty degenaration).

damage (9,25). In the present study, we observed a meaningful reduction in vit. C levels and an increase in γ-GT levels in Group II. On the other hand, in Group III, there was a significant increase in Vit. C levels accompanied with a meaningful decrease in γ-GT levels. Our histopathological observations revealed the development
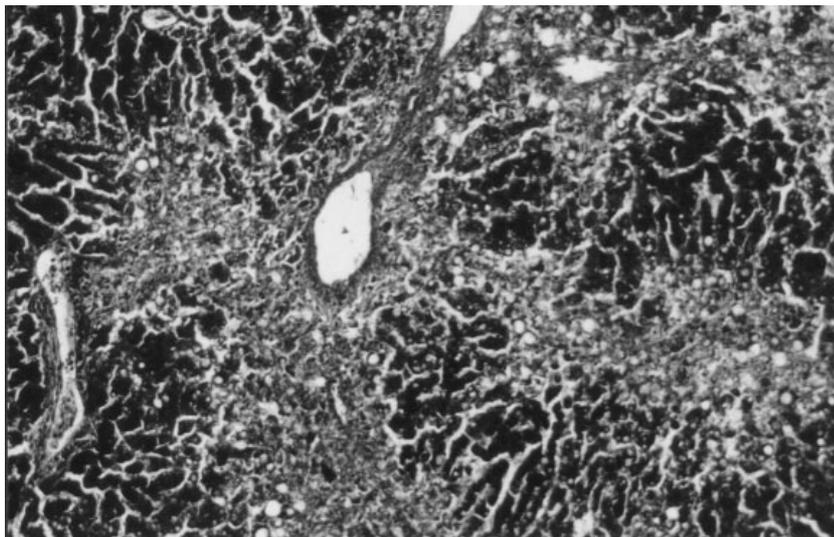
Figure 3. The histopathogical changes of liver tissue in the ethanol-induced rats (Masson Trichrome, X 100).

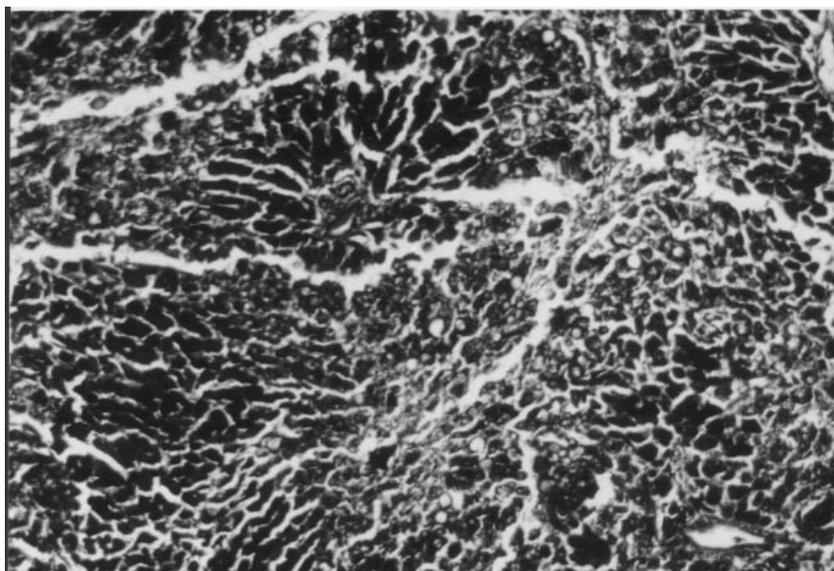
Figure 4. The histopathogical changes of liver tissue in the rats treated with ethanol+melatonin (Masson Trichrome, X 100).

of liver steatosis and fibrosis in the liver tissue of rats which had consumed ethanol for 8 weeks.

Melatonin has anti-aging properties and appears to modify growth, in addition to its other known functions. It has immunomodulatory, oncostatic and antioxidative effects. In addition, melatonin is involved in the inhibitory control of cell proliferation. Melatonin has exhibited a protective effect on the development of fibrosis (2,12). In the present study, increased liver tissue hydroxyproline levels in the ethanol group were significantly reduced in the group treated with melatonin (p<0.001). In addition, ascorbic acid levels were significantly lower in the liver of the ethanol group (p<0.01) and ethanol+melatonin group (p<0.01) than in controls. It has been reported that when ascorbic acid levels are decreased due to alcohol intake in guinea pigs, degradation of ascorbic acid is lowered as a response (8). Interestingly, when ascorbic acid and alcohol are taken together, tissue ascorbic acid levels seem to increase (9). In our study, we observed a decrease in the vit. C levels of Group II. Since degradation of vit. C was reduced, we believe that the amount of ascorbic acid is adequate as a co-factor of proline hydroxylase to carry out hydroxylation of proline. Melatonin seemed to increase vit. C levels in Group III.

In a study examining the effect of octreotide on liver-tissue hydroxyproline levels, a meaningful correlation between reduced tissue hydroxyproline levels and fibrosis area was reported (26). Collagen contents in wound healing were shown to increase in pinealectomized rats. When exogenous melatonin was administered to these animals, collagen levels were reduced (26).

Lopez et al. (27) reported that there was a higher hydroxyproline level in the pancreas of rats given ethanol than in that of rats in the control group. Zhang et al. (24) examined the effect of a traditional Chinese medical agent, selenium, vit E on CCl$_4$/ethanol-induced liver fibrosis and found that hepatic fibrosis was partially prevented by the antioxidative effect of vit E and selenium. Similarly, Brown et al. (23) reported that vit E has an antioxidant effect on LPO in hepatic fibrosis induced by iron supplementation. Melatonin is more potent (approximately twice as much) than vit E and shows its antioxidant effects not only on LPO levels, but also on antioxidant enzymes and prostaglandin synthesis (23,28).

According to our results, melatonin seems to reduce increased liver-tissue hydroxyproline levels in ethanol-induced liver fibrosis. Therefore, melatonin may be utilized as a therapeutic agent in chronic liver diseases for the prevention of fibrosis formation or its progression.

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