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Protective effects of melatonin against carbon tetrachloride-induced hepatotoxicity in rats: a light microscopic and biochemical study

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The aim of this study was to examine the protective effects of melatonin against CCl_4-induced hepatotoxicity in the rat. Twenty-four male Wistar rats were divided into three groups. Group I was used as a control. Rats in group II were injected every other day with CCl_4 for 1 month, whereas rats in group III were injected every other day with CCl_4 and melatonin for 1 month. At the end of the experiment, all animals were killed by decapitation and blood samples were obtained. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total and conjugated bilirubin levels were determined. For histopathological evaluation, livers of all rats were removed and processed for light microscopy. All serum biochemical parameters were significantly higher in animals treated with CCl_4 than in the controls. When rats injected with CCl_4 were treated with melatonin, significantly reduced elevations in serum biochemical parameters were found. In liver sections of the CCl_4-injected group, necrosis, fibrosis, mononuclear cell infiltration, haemorrhage, fatty degeneration and formation of regenerative nodules were observed. Additionally, apoptotic figures, microvesicular steatosis and hydropic degeneration in hepatocytes were seen in this group. In contrast, the histopathological changes observed after administration of CCl_4 were lost from rats treated with CCl_4 and melatonin. Except for mild hydropic degeneration of the hepatocytes, a normal lobular appearance was seen in the livers of this group. The results of our study indicate that melatonin treatment prevents CCl_4-induced liver damage in rats. Copyright © 2004 John Wiley & Sons, Ltd.

KEY WORDS — carbon tetrachloride; hepatotoxicity; melatonin; rat; light microscopy

INTRODUCTION

Various pharmacological and chemical substances are known to result in liver damage. One of these chemicals is carbon tetrachloride (CCl_4)[1] which is a xenobiotic producing hepatotoxicity in humans as well as in animals.[2]

The hepatotoxic effect of CCl_4 is thought to result from its reductive dehalogenation by the P-450 enzyme system to the highly reactive free radical, trichloromethyl radical, CCl_3. This radical quickly adds molecular oxygen to form a trichloromethylperoxy radical. Removal of hydrogen atoms from unsaturated fatty acids by such radicals creates carbon-centred, lipid radicals. These lipid radicals quickly add molecular oxygen to form lipid peroxyl radicals, thereby initiating the process of lipid peroxidation. Unless scavenged by radical scavengers, these lipid peroxyl radicals in turn remove hydrogen atoms from other lipid molecules, thereby propagating the process of lipid peroxidation.[3–5]

The pineal gland and its main hormone, melatonin (N-acetyl-5-methoxytryptamine), are known to be involved in a variety of physiological processes

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including the regulation of endocrine rhythms,[6] antigonadotropic effects,[7] neuroprotective effects,[8] and stimulation of the immune function.[9] There is also evidence that melatonin may regulate smooth muscle tone.[10] Besides these functions, many recent *in vitro* studies have shown that melatonin functions effectively as an antioxidant, i.e. a hydroxyl radical and a peroxyl radical scavenger.[11–17] It has also been shown that when animals and tissues are subjected to lipid peroxidation, melatonin gives a substantial protection against the oxidative destruction of lipids.[18–20]

The aim of this study was to examine the protective effects of melatonin against CCl_4-induced hepatotoxicity in rats at biochemical and histological levels.

MATERIALS AND METHODS

Animals and treatments

Adult male Wistar albino rats (weighing 170–220 g) supplied by Firat (Euphrates) University Medical Faculty Experimental Research Unit were randomly divided into three groups of eight animals. All animals received humane care in compliance with the guidelines of Firat University Research Council's criteria. The rats were kept in plexiglas cages (four animals per cage) where they received standard chow and water *ad libitum* in an air-conditioned room with automatically-regulated temperature ($22 \pm 1°C$) and lighting (07.00 to 19.00 hours). All rats were allowed to acclimatize for 1 week prior to experimentation. Control rats (group I) received pure olive oil (1 ml subcutaneously (sc)) alone. Rats in group II were injected with CCl_4 ($0.5\,ml\,kg^{-1}$ body weight per 1 ml olive oil sc; EM Science, Cherry Hill, NJ, USA) every other day for 1 month. Rats in group III received melatonin ($25\,mg\,kg^{-1}$ body weight per 1 ml 10% ethanol sc; Sigma, St. Louis, MO, USA) with a subcutaneous injection of CCl_4 every other day for 1 month.

Determination of serum biochemical parameters

All animals were killed by decapitation at the end of the experiment. Blood samples were collected in tubes, allowed to clot and the serum was removed by centrifugation at 3000 r.p.m. for 10 min. All serum samples were sterile, haemolysis-free, and were kept at 4°C before determination of the biochemical parameters.

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total biluribin and conjugated biluribin levels were measured with an AU600 multiparametric analyser (Olympus, Hamburg, Germany).

Histopathological analysis of liver

The livers of all rats were removed immediately after collection of the blood and fixed in neutral formalin solution (10%). Tissue specimens were embedded in paraffin wax and sectioned (thickness, 5 μm), for histopathological evaluation. After staining with haematoxylin and eosin (H&E) and Masson's trichrome, they were examined with an Olympus BH2 light microscope. The liver preparations were evaluated by a pathologist, who had not been informed which particular group the specimens belonged to.

Statistical analysis

Quantitative data are expressed as mean ± standard error of mean (SEM). Comparisons between groups were performed with the Kruskal–Wallis's test for unpaired comparisons followed by the Mann–Whitney rank sum test. All analyses were performed with SPSS 11.0 for windows software. P values of <0.05 were considered significant.

RESULTS

Biochemical findings

The means of the biochemical parameters and statistical comparisons of the groups are presented in Table 1.

In CCl_4-injected group, serum AST, ALT, ALP, total biluribin and conjugated biluribin were significantly increased as compared to those in the control group ($p < 0.05$). When rats injected with CCl_4 were treated with melatonin, significantly attenuated elevations in serum AST, ALT, ALP, total biluribin and conjugated biluribin were found ($p < 0.05$). Additionally, ALT levels were returned to the control values in this group.

Histopathological findings

The protective effects exerted by the melatonin against CCl_4-induced liver hepatotoxicity as revealed by the biochemical data, were confirmed by conventional histopathological examination.

When the liver sections belonging to rats in the control group were examined, they were found to have a normal histological appearance (Figure 1). Livers of rats treated with CCl_4 showed classic cirrhotic histology. Coagulative necrosis, massive fibrosis, mononuclear cell infiltration, haemorrhage, fatty degeneration and formation of regenerative nodules were all observed. Additionally, apoptotic figures, microvesicular steatosis and hydropic degeneration in hepatocytes were seen in this group (Figures 2, 3 and 4).

Table 1. Serum biochemical parameters and statistical comparisons of the groups

Parameter	Control		CCl_4		CCl_4 + melatonin	
	n	Mean ± SE	*n*	Mean ± SE	*n*	Mean ± SE
AST ($U l^{-1}$)	8	295.00 ± 35.37	8	843.50 ± 125.36[a]	8	299.00 ± 6.20[c]
ALT ($U l^{-1}$)	8	63.50 ± 3.39	8	741.00 ± 133.53[a]	8	63.50 ± 3.69[c]
ALP ($U l^{-1}$)	8	240.00 ± 23.74	8	816.25 ± 56.29[a]	8	370.00 ± 23.84[b,c]
T. bilirubin ($mg\,dl^{-1}$)	8	0.25 ± 0.01	8	0.50 ± 0.04[a]	8	0.32 ± 0.03[e]
C. bilirubin ($mg\,dl^{-1}$)	8	0.09 ± 0.01	8	0.19 ± 0.01[a]	8	0.11 ± 0.02[d]

[a]Statistical difference according to control group at $p = 0.001$.
[b]Statistical difference according to control group at $p = 0.011$.
[c]Statistical difference according to CCl_4 group at $p = 0.001$.
[d]Statistical difference according to CCl_4 group at $p = 0.002$.
[e]Statistical difference according to CCl_4 group at $p = 0.011$.

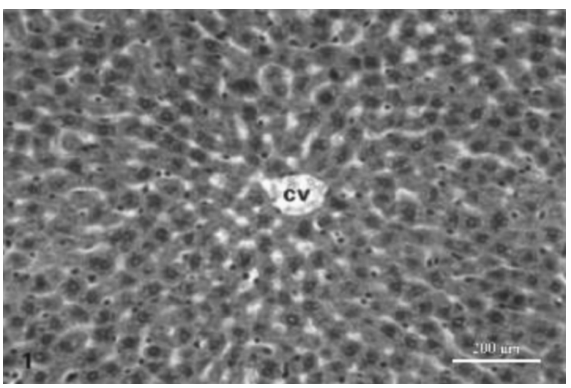

Figure 1. Liver section from control group showing a normal histological appearance. cv, central vein. H&E; Scale bar, 200 µm

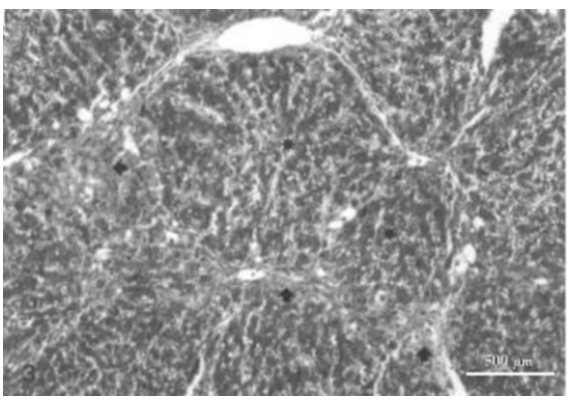

Figure 3. Massive fibrosis (arrows) and regenerative nodules (asterisks) were seen in the liver section of CCl_4-treated rats. Masson's trichrome; scale bar, 500 µm

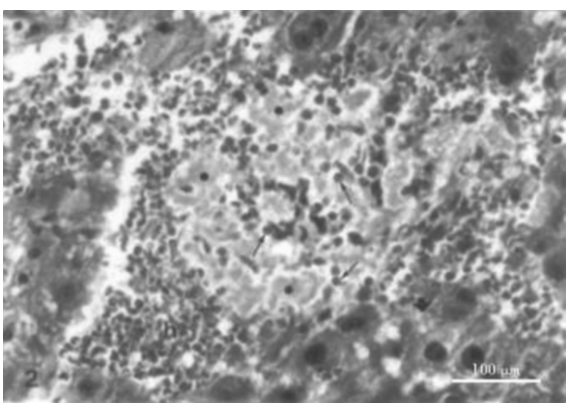

Figure 2. Liver section in rats treated with CCl_4 showing a classic cirrhotic appearance with the presence of coagulative necrosis (asterisks), mononuclear cell infiltration (small arrows), haemorrhage (h), microvesicular steatosis and hydropic degeneration (large arrow). Masson's trichrome; scale bar, 100 µm

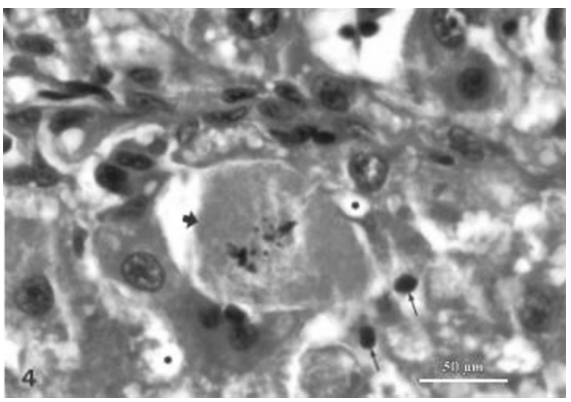

Figure 4. Apoptotic hepatocyte (large arrow), mononuclear cell infiltration (small arrows) and fatty degeneration (asterisks) were observed in rats treated with CCl_4. H&E; scale bar, 50 µm

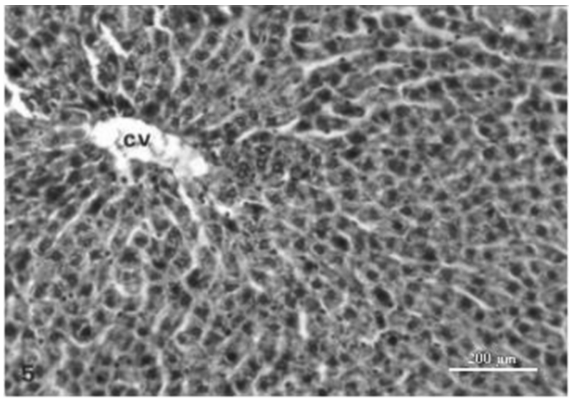

Figure 5. Liver section of rats treated with CCl_4 and melatonin. Except for mild hydropic degeneration of hepatocytes, a normal lobular appearance of the liver was seen in this group. cv, central vein. H&E; scale bar, 200 µm

However, the histopathological changes observed after administration of CCl_4 were lost from rats treated with CCl_4 and melatonin. Except for mild hydropic degeneration of the hepatocytes the liver had, a normal lobular appearance in this group (Figure 5).

On the basis of our biochemical and histopathological findings, it is concluded that melatonin treatment prevents CCl_4-induced liver damage in rats.

DISCUSSION

Various pharmacological and chemical substances that belong to intrinsic or idiosyncratic groups of hepatotoxins, may induce a level of hepatic damage varying from asymptomatic hepatic functional disturbance to widespread liver necrosis. CCl_4 , which is an intrinsic hepatotoxin, was used for the purpose of inducing hepatic damage in this study, because carbon tetrachloride has previously been shown to exert its toxic effects on the liver.[21,22] The administration of CCl_4 to rats caused severe liver injury which was recognized histopathologically together with an increase in the activities of serum hepatic enzymes AST and ALT, which are indices of liver cell damage.[23]

The biochemical mechanism involved in the development of CCl_4 hepatotoxicity have long been investigated. It is generally believed that it is due to lipid peroxidation caused by the carbon trichloromethyl radical, CCl_3. CCl_4 is biotransformed by cytochrome P450 to the trichloromethyl free radical which elicits membrane lipid peroxidation and disturbs Ca^{2+} homeostasis to produce hepatocellular injury.[5]

Previous experimental studies have shown that CCl_4 administration caused an increase in serum AST, ALT and ALP levels.[23–31] The hepatotoxicity of CCl_4 was confirmed in this study by significant elevation of serum AST, ALT, ALP, total biluribin and conjugated biluribin. Additionally, we observed histopathological changes indicating liver damage after CCl_4 administration. In our light microscope examination, livers of rats treated with CCl_4 showed classic cirrhotic histology. Coagulative necrosis, massive fibrosis, mononuclear cell infiltration, haemorrhage, fatty degeneration and formation of regenerative nodules were observed. Furthermore, apoptotic figures, microvesicular steatosis and hydropic degeneration in hepatocytes were seen in this group. Similarly, it has previously been reported that CCl_4 caused necrosis,[21,22,24,28,31–34] fibrosis,[23,24,27,28,34–36] mononuclear cell infiltration,[23,24,27] steatosis and foamy degeneration of hepatocytes, an increase in mitotic activity[23] and cirrhosis[24,28] of the liver. Furthermore, it has also been reported that CCl_4 causes apoptosis in the liver.[23,28,37–39] In terms of histopathological changes which CCl_4 administration caused in the liver, our findings are in agreement with previous studies.

Melatonin is known to function as an antioxidant *in vitro* and *in vivo*.[11–20] In addition, melatonin has been shown to protect against acute hepatic injuries induced by endotoxic shock and ischaemia–reperfusion in which lipid peroxidation induced by active oxygen species is involved, through its antioxidant action.[18,40,41] It has also been reported that melatonin has protective effects against CCl_4-induced oxidative damage in the liver and kidney of rats.[18,26] In the present study, melatonin treatment was found to prevent the progression of liver injury in rats intoxicated with CCl_4 since melatonin significantly decreased the elevated levels of serum AST, ALT, ALP, total biluribin and conjugated biluribin, which are indices of liver cell damage found after CCl_4 administration. Additionally, the protective effects exerted by melatonin against CCl_4-induced liver hepatotoxicity which were, revealed by the biochemical data, were confirmed by conventional histopathological examination. By light microcopy, it was determined that the histopathological changes observed after administration of CCl_4 were reversed in rats treated with CCl_4 and melatonin. Apart from mild hydropic degeneration of the hepatocytes, the liver had a normal lobular appearance in this group.

In conclusion, the results obtained in the present study indicate that melatonin treatment prevents

CCl_4-induced liver damage in rats, possibly through its antioxidant action.

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