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Protective effects of melatonin and β-D-glucan against acetaminophen toxicity in rats

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Abstract

The aim of this study was to investigate the possible protective effects of melatonin and β-D-glucan against AA-induced liver injury in rats. Forty (Spraque–Dawley male) rats were randomly divided into 5 experimental groups: sham (S), acetaminophen only (AA, 900 mg/kg), melatonin (10 mg/kg) + AA (MLT), β-D-glucan (50 mg/kg) + AA (β), and melatonin + β-D-glucan + AA (MLT+β) groups. All of the rats were killed on day 11 of the experiment. Histopathological changes and biochemical parameters including levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and liver tissue malondialdehyde (MDA), activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were determined to assess the liver function. MDA levels were the highest in the AA group whereas activities of SOD, CAT, and GPx in the liver tissue were found as lowest in this group. MDA activities were significantly lower in the MLT+β group than in the AA group. Only GPx activities in the MLT+β group were significantly higher than those in the MLT and β groups. The serum AST and ALT levels were increased significantly following treatment with AA ($p < 0.001$). Pretreatment with the antioxidant compounds decreased AST levels significantly but again, the levels were still significantly higher than the sham levels ($p < 0.001$). There were no statistically significant differences in the microscopic damage between the S, MLT, β, and MLT+β groups ($p > 0.05$). We concluded that combination of melatonin and β-D-glucan may be attributed to scavenging free radicals and stimulating the antioxidant enzymes.

Key words: acetaminophen toxicity, melatonin, β-glucan, rat, liver

Introduction

Acetaminophen (AA) is mostly used as an analgesic and antipyretic, it is known to cause liver injury when administered in large quantities, and may have lethal in humans and experimental animals [1]. Previous studies have shown that AA-linked liver injury associated with reactive oxygen radical species (ROS) [2-4]. Lipid peroxidation mediated by ROS is highly destructive and can be harmful to the cell membranes [5]. Evidence that antioxidants reduce acetaminophen –induced (AA-induced) hepatotoxicity supports the notion that oxidative stress plays a major role in this pathogenic process [6]. Several antioxidant agents are reported to suppress the adverse events associated with high doses of AA; however, no data are available regarding the preventive action of combined melatonin and β-glucan treatment.

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β-Glucans are glucose polymers derived from *Saccharomyces cerevisiae* and have beneficial effects, including tumoricidal properties and ability to enhance immune responses [7]. In addition, β-glucan has anti-inflammatory and antioxidant properties [8] While several mechanisms have been suggested for the preventive effects of β-D-glucan, the dominant hypothesis depends on the antioxidant properties of this molecule [9]. Melatonin, chief hormone of the pineal gland, scavenges free radicals and activates various antioxidative enzymes and defence mechanisms. It also regulates the gene expression of some preventive enzymes and decreases lipid peroxidation [10]. In addition, melatonin has anti-inflammatory effects and inhibits neutrophils activation by free radicals [11]. Melatonin exerts beneficial effects against oxidative injury caused by diverse toxic agents in tissues including liver tissue [12]. Therefore, Kanno et al [13]. Suggested that due to powerful antioxidant and free radical scavenging effects of melatonin it might be a preventive agent against AA toxicity.

In this study, we investigated whether a combination of β-D-glucan and melatonin prevented AA-induced hepatotoxicity more effectively than did either of the drugs

alone in rats, by performing biochemical and histological analyses.

Materials and Methods

Animals

We obtained 40 male Spraque–Dawley albino rats (age, 10–12 weeks; weight, 200–260 g) rats from Inonu University Laboratory Animals Research Center, Malatya, Turkey (2011/A-105). The rats were kept in a room with a temperature of 21 ± 2°C and relative humidity of 60% ± 5% under a 12-h light/dark cycle. The animals were housed in plastic cages (50 × 35 × 20 cm, 8 animals per cage). Experiments were carried out according to the standards of animal research issued by the National Health Research Institute and with the approval of the Inonu University Ethical Committee.

Drug Administration

AA was purchased from Sandoz Chemical, Istanbul, Turkey. AA was first dissolved in water at a temperature of 70°C, and then, the mixture was cooled to 37°C prior to administration. A single dose of 900 mg/kg AA was injected intraperitoneally (i.p.) [1]. Melatonin was obtained from Sigma Chemical, St Louis, MO, USA. It was dissolved in ethanol and diluted in saline to a final concentration of 5% ethanol. A single dose of melatonin (10 mg/kg, i.p.) was injected 30 min before AA injection.[5] The microparticulate form of β-D-glucan was prepared from *Saccharomyces cerevisiae* yeast (Mustafa Nevzat Drug Co, Istanbul, Turkey) daily for the 11 days of administration. β-D-Glucan was suspended in saline and a dose of 50 mg/kg/day was administered by intragastric gavage for 10 days prior to, and 30 min after, AA injection.[6] The dosage of AA, melatonin and β-D-Glucan were chosen according to previous dose-response studies which has been reported to cause liver injury and marked antioxidative [12,13].

Experimental Design

Rats were randomly divided into 5 groups of 8 animals, as follows: sham (S), i.p. 0.9% NaCl saline only; acetaminophen only (AA); melatonin + AA (MLT); β-D-glucan + AA (β); and melatonin + β-D-glucan + AA (MLT+β). All of the rats were sacrificed on day 11 of the experiment. The liver was removed from each rat. At the same time, hepatic tissue was stored in 10% formalin for histological examination and the other part of the liver tissue was put in liquid nitrogen and stored at -70°C until biochemical analysis. Blood samples were taken from the inferior vena cava to determine the serum levels of oxidants and antioxidants. All specimens were coded in the research laboratory and were evaluated by individuals who were blinded to group assignments.

Biochemical Analysis

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were determined to assess the liver function by using AST, ALT commercial kits (Roche Diagnostics, GmbH, Mannheim, Germany) in Roche-Hitachi Modular Auto-analyzer (Roche Diagnostics GmbH, Mannheim, Germany). The hepatic tissue samples were stored at -70°C.

Determination of Enzyme Activities

SOD activity was determined according to the method of Sun et al [7]. CAT activity was measured according to the method of Aebi [8]. GPx activity was measured by the method of Paglia and colleagues [9]. MDA activity contents of homogenates were determined spectrophotometrically [10]. Protein contents were measured according to the method of Lowry et al [14].

Histological Analysis

Liver samples were fixed in 10% formalin and embedded in paraffin. Paraffin-embedded specimens were cut into 4-µm–thick sections and mounted on slides. For evaluation using a Leica DFC 280 light microscope (Leica Micros Imaging Solutions Ltd, Cambridge, UK), slides were stained with hematoxylin and eosin (H–E). For immunohistochemical staining, serial sections (4 µ thick) were cut on polylysine-coated slides, rehydrated in distilled water, placed in citrate buffer (pH 7.6) and heated in a microwave oven 700 watt for 20 min. After washing with phosphate-buffered saline (PBS), the slides were placed in 0.3% H_2O_2 for 7 min and washed again with PBS. They were then rinsed in PBS and incubated with biotinylated goat anti-polyvalent for 10 min and streptavidin peroxidase for 10 min at room temperature. Sections were stained with chromogen and substrate for 15 min, and then counter-stained with Mayer's hematoxylin for 1 min. After staining, slides were rinsed in tap water and dehydrated. Hepatocytes were counted in 20 different fields at 40× magnification. All hepatocytes in these fields were examined and classified as either uncolored or colored.

Statistical Analysis

The sample size was based on a power analysis. At least 7 rats were required in each group to detect a of MDA stay difference of 30 % between the groups with a type I error of 0.05 and a type II error of 0.20. Data were presented as median (min–max) and mean ± SD. SPSS 21.0 software package was used for the analysis of data. The assumption of normal distribution was confirmed using the Kolmogorov-Smirnov test. Homogeneity of variance was tested by Levene's statistic. The Kruskal-Wallis H test was used when the assumption of normality was not provided. The Mann Whitney *U* test with Bonferroni correction was used for multiple comparisons. Statistical significance was set at $p < 0.05$.

Results

The oxidant and antioxidant parameters are presented in Table 1. Briefly, serum MDA levels were the highest in the AA group (p = 0.001). MDA levels were significantly

lower in the MLT+β group than in the AA group ($p < 0.05$). However, antioxidant enzyme activities of liver tissue, including SOD, CAT, and GPx, were significantly lower in the AA group than in other groups ($p < 0.05$). These antioxidant enzyme activities were significantly higher in the group MLT+ β-D-glucan than AA group ($p < 0.05$). Only GPx activities in group MLT+β-D-glucan were significantly higher when compared to MLT and β-D-glucan groups ($p < 0.05$).

The AST levels were increased significantly following treatment with AA ($p < 0.001$). Pretreatment with the antioxidant compounds decreased AST levels significantly but again, the levels were still significantly higher than the sham levels ($p < 0.001$). Similar results were obtained for ALT levels; this enzyme which was increased by AA treatment ($p < 0.001$). Pretreatment with the antioxidant compounds decreased AST levels significantly but again, the levels were still significantly higher than the sham levels ($p < 0.001$). Pretreatment with MLT+ β-D-glucan group decreased ALT levels significantly down to the control levels, demonstrating the protective effect of these antioxidant agents against the liver damage induced by AA ($p < 0.001$).

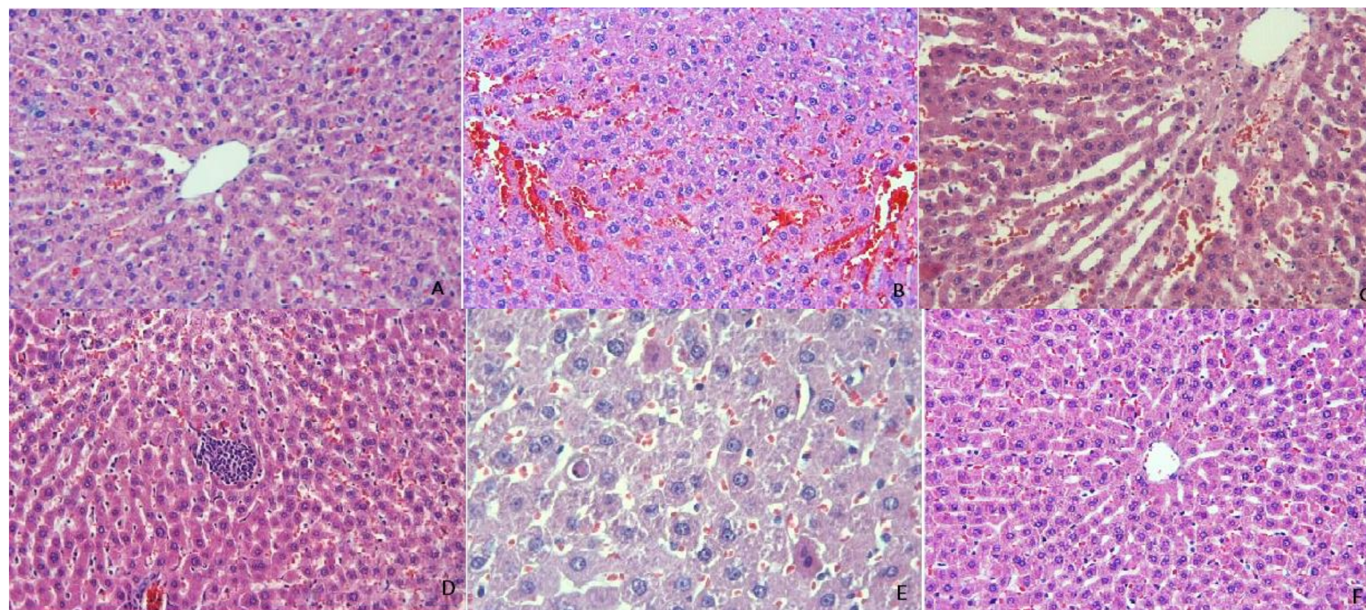
Table 1. Enzyme activities in liver tissue

Groups (n=8)	MDA ^{y} (nmol/g tissue)	SOD ^{x} (U/g protein)	GPx ^{x} (U/g protein)	CAT ^{y} (k/g protein)
S	32.7 ± 5.6^{b}	57.4 (54.4-66.6)^{b}	5.6 (5-8.9)^{b,c}	65.7± 9.3^{b}
AA	48.5 ± 3.5^{a}	32.9 (25.8-37.7)^{a}	4.5 (3.6-5.5)^{a}	37.4± 3.9^{a}
MLT	37.1 ± 4.4^{b}	43.6 (32.2-43.1)^{b}	5.5 (4.9-7.6)^{b,c}	52.0± 7.6^{b}
β-glucan	34.3 ± 3.7^{b}	44.5 (33.8-42.8)^{b}	6.1 (6.1-9.3)^{b,c}	51.9± 5.3^{b}
MLT+ β-D-glucan	32.3 ± 4.8^{b}	46.8 (33.8-40.7)^{b}	8.1 (6-15.5)^{a,b}	57.3±4.1^{b}

^{x} median (min-max); ^{y} Mean+/-SD. S = sham; AA = acetaminophen; MLT = melatonin. MDA= malondialdehyde; SOD= superoxide dismutase; GPx= glutathione peroxidase; CAT= catalase ^{a}:$p < 0.05$ compared to sham group. ^{b}:$p < 0.05$ compared to AA group. ^{c}:$p < 0.05$ compared to MLT+ β group.

Histological Changes

The histological appearance is presented in Figure 1A-H. The S group samples had a normal histological appearance, and showed hepatocytes extending from the central vein to the periphery. In the AA group sinusoidal dilatation, congestion, hemorrhage, and infiltration into the portal area were observed. Histological analysis revealed a lower degree of damage in the MLT, β, and MLT+β groups than in the AA group. There were no statistically significant differences in the microscopic damage between the S, MLT, β, and MLT+β groups ($p > 0.05$).



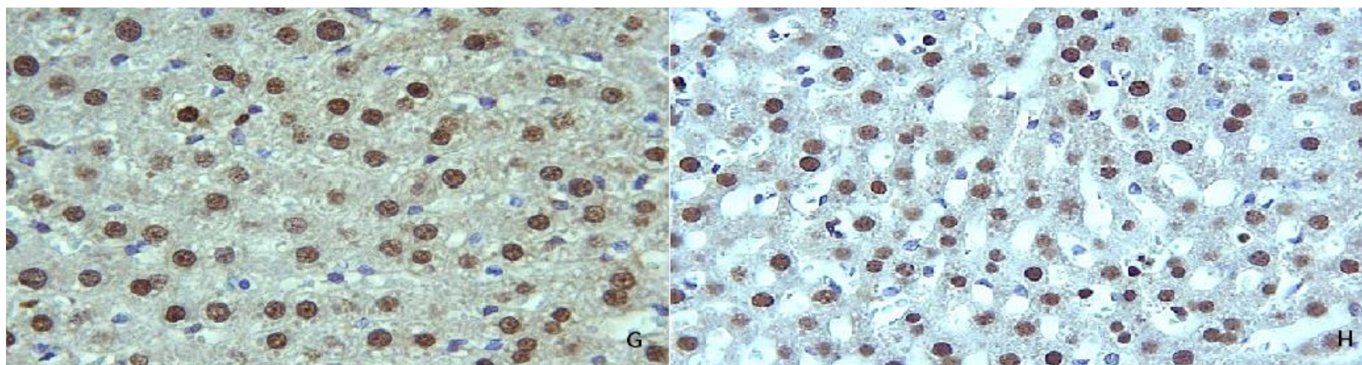


Figure A-H. The histological appearance

Discussion

AA is an efficient analgesic–antipyretic drug when used in therapeutic doses. Nevertheless, an overdose can induce serious liver injury in experimental animals and in humans [15]. Oxidative stress is a significant factor in the development of AA hepatotoxicity. Metabolic biotransformation of AA includes capability restricted conjugation trail besides a minor metabolic pathway in which cytochrome P450 oxidizes AA [16] At sufficiently high doses of AA, GSH supplies are exhausted. This depletion of GSH supply is the primary influence on the degree of AA-induced hepatic injury.

Lipid peroxidation is thought to be closely associated with AA-induced tissue injury, and MDA is a well-established indicator of the degree of lipid peroxidation. In this study, there was an increase in the MDA content of livers of rats damaged by AA overdose [17]. In the current study, both melatonin and β-D-glucan when administered alone, suppressed the AA-induced increase in lipid peroxidation in liver tissue. It is likely that melatonin reduces membrane injury owing to its ability to scavenge the free radicals that induce lipid peroxidation. Sener et al [18]. concluded that melatonin prevented MDA production and effectively protected liver tissue against oxidative injury. Toklu et al [19]. showed that β-D-glucan treatment also reduced MDA production, and their findings are similar to our biochemical results. Parallel to our MDA results, melatonin, β-D-glucan, and melatonin plus β-D-glucan applications caused to amelioration of the antioxidant enzymes. Also, there was no difference among the therapeutics groups for ameliorating levels.

The GSH antioxidant system plays an important role in the detoxification of the liver and is involved in dealing with liver damage caused by a variety of hepatotoxins [20]. GSH is a necessary component of the antioxidant system, and cellular GSH is important for the maintenance of cellular redox states through the direct scavenging of radical species or participation in reactions catalyzed by antioxidant enzymes, such as GPx [21]. In the current study GPx levels were found as reduced in the AA-treated rats. This circumstance is in accordance with the degree of liver injury. Pre-treatment with melatonin or β-D-glucan reduced the damage caused by AA-induced hepatotoxicity by reducing oxidative stress and increasing the GPx, SOD and CAT antioxidant activities. Because melatonin or β-D-glucan are known free-radical scavengers, the idea that free radicals are involved in the pathogenesis of AA-induced hepatotoxicity is supported [22]. Melatonin is a strong direct free radical scavenger and an indirect antioxidant through the induction of antioxidant enzymes. Melatonin protects rats against AA-stimulated hepatotoxicity, likely through the prevention of oxidative stress, including lipid peroxidation and protein oxidation . Matsura et al [23]. reported that pre-administered oral melatonin dramatically suppresses AA-stimulated hepatotoxicity. Similarly, β-D-glucan protected hepatic tissues against AA-induced oxidative injury [10]. The present study clearly indicates that exogenously administered melatonin and β-D-glucan combination exhibit a potent hepatoprotective effect in cases of AA overdose probably as well as its antioxidant activity.

AST and ALT concentrations are commonly used as indirect biochemical indices of liver injury. In the present study, hepatotoxic effects caused by acute administration of AA produced significant increases in the levels of AST and ALT, probably resulting from cell membrane damage. Exogenously administered melatonin and β-D-glucan treatment improved ALT levels significantly down to the control levels, demonstrating the protective effect of these antioxidant agents against the liver damage induced by AA.

Our histological investigation suggested that an overdose of AA caused severe pathological changes in the liver, including sinusoidal dilatation, congestion, hemorrhage, and infiltration into the portal area. Pretreatment with melatonin and β-D-glucan ameliorated the AA-induced histological changes. These results may be due to the antioxidant effect of these drugs. Our results are in agreement with a recent study that described histological liver alterations following the administration of an overdose of AA [10]. One of the limitations of the current

study is that the sample size is too small and the other one is examination period is too short. However, in the current study we focused the possible protective effects of combined therapy against well-established AA-induced liver injury. It is clearly showed that melatonin and β-D-glucan combination can attenuate liver injury caused by AA overdose in the light of biochemical findings and histopathological observations.

In conclusion, administration of melatonin or β-D-glucan prior to AA-overdose increased the GPx, CAT, and SOD activity of liver tissue in rats. The present study showed for the first time that a combination of melatonin and β-D-glucan has a protective effect against AA-induced hepatic injury, as determined by biochemical and histopathological evaluations. However, further investigation is needed in order to determine the exact mechanism of melatonin and β-D-glucan protection against AA-induced hepatotoxicity.

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