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Protective Effects of Melatonin and β-D-Glucan Against Liver Injury in Rats – a Comparative Study

Działanie ochronne melatoniny i β-D-glukanu w uszkodzeniach wątroby u szczurów – studium porównawcze

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Objectives. The aim of this study was to investigate the possible protective effects of melatonin and β-D-glucan against ischemia-reperfusion (IR) injury in rats.
Materials and Methods. Forty rats were randomly divided into 5 groups, each consisting of 8 animals, as follows. Sham group [S], IR group [C], IR + β-Glucan group [β], IR + melatonin group [MLT], IR + melatonin + β-Glucan group [MLT + β]. The rats in the C, β, MLT and MLT + β groups were subjected to IR for 60 min each. Melatonin (10 $mg \cdot kg^{-1}$) was intraperitoneally injected for a single dose 30 min before IR. β-Glucan (50 $mg \cdot kg^{-1} \cdot day^{-1}$) was orally administered for 10 days to rats. All of the rats were killed on day 11, and histological changes in the liver and tissue levels of oxidants and antioxidants were evaluated.
Results. Malondialdehyde [MDA] level were significantly higher in the C group compared to the S group ($p = 0.007$). MDA level were significantly higher in the β group compared to the MLT and MLT + β groups ($p = 0.007$). Tissue antioxidant markers (superoxide dismut ase [SOD], glutathione-peroxidase [GPx], and catalase [CAT]) were significantly lower in the C group than the S group ($p < 0.05$). SOD levels were simply not significant in the β group compared to the MLT and MLT + β groups. CAT and GPx activities were significantly higher in the β group compared to the MLT and MLT + β groups ($p = 0.004$).The histological damage ameliorated in β, MLT and MLT + β groups compared to C group.
Conclusion. Our results suggest that melatonin and β-glucan combination pretreatment suppressed oxidative stress and increased antioxidant levels in an experimental rat model of liver IR injury (**Adv Clin Exp Med 2013, 22, 5, 621–627**).

Key words: liver, ischemia reperfusion injury, melatonin, β-glucan, oxidative stress.

Słowa kluczowe: wątroba, szkodzenie niedokrwienno-reperfuzyjne, melatonina, β-glukan, stres oksydacyjny.

Liver ischemia-reperfusion injury (IRI) occurs during transplantation and major hepatic surgery [1]. IRI may contribute to postoperative liver dysfunction and negatively affect graft function in liver transplant patients following vascular clamping [2]. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are protective against IRI. For these reasons, treatment with exogenous antioxidants, particularly in the early stages of

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reperfusion, markedly reduces the severity of liver IRI [3].

β-glucans have shown beneficial effects, including anti-inflammatory, antioxidant properties and enhanced immune response [4, 5]. Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine produced by the pineal gland in a circadian rhythm, and it is one of the most powerful stimulation of antioxidative enzymes, including glutathione reductase and superoxide dismutase [6, 7].

Liver IRI is a persistent problem that cannot be prevented during liver surgery and transplant [8, 9]. Multi pharmacologic preconditioning is a new strategy to reduce IRI. Because different pathways in various cell types are involved in I/R-injury, it is conceivable to use multiple drugs to prevent IRI [10]. In the present study, we aimed to investigate if a combination of β-D-glucan and melatoninwould be better than drugs used alone against liver IRI in rats by using biochemical and histological analyses for the evaluation of the extent of oxidative damage.

Material and Methods

Animals

The authors obtained 40 Spraque-Dawley male rats weighing 210–240 g from Inonu University Laboratory Animals Research Center, Malatya, Turkey. The rats were kept in a room that was 21 ± 2°C with relative humidity of 60% ± 5% and 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.

Experimental Design

Rats were randomly divided into 5 groups, each consisting of 8 animals, as follows. (1) *Sham group* [S]: sham-operated animals only a midline laparotomy was performed, and the abdomen was closed without any further procedure. (2) *IR group* [C]*:* animals were subjected to 60 min of ischemia followed by 60 min of reperfusion. (3) *IR + β-Glucan group* [β]*:* animals received oral β-glucan and were subjected to 60 min of ischemia followed by 60 min of reperfusion. (4) *IR + melatonin group* [MLT]: animals received i.p. melatonin and were subjected to 60 min of ischemia followed by 60 min of reperfusion. (5) *IR + melatonin + β-Glucan group* [MLT + β]: animals received i.p. melatonin and oral β-glucan and were subjected to 60 min of ischemia followed by 60 min of reperfusion.

Glucan Administration

The micro particulate form of β-glucan was prepared from *Saccharomyces cerevisiae* yeast (Mustafa Nevzat Drug Co., Turkey). 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, IR procedure [11].

Melatonin Administration

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 IR procedure. The authors determined the melatonin dose on the basis of the results from recent studies [12].

Surgical Procedure

Following a 12-hour fasting period, rats were anesthetized with intraperitoneal ketamine (40 mg/kg) and xylazine (10 mg/kg) and subjected to surgery. The abdominal region was sterilized with povidine-iodine solution, and the abdomen was explored through a midline minimal laparotomy using minimal dissection. In the sham group, only a midline laparotomy was performed, and the abdomen was closed without any further procedure. In the C, β, MLT and MLT + β groups, total hepatic ischemia was induced for 60 min by clamping the hepatic artery, portal vein, and bile duct, using a non-traumatic vascular clamp. The liver was reperfused for 60 min by removing the clamp. After declamping, the authors confirmed that hepatic blood flow had been restored before the closure of the incision. During the surgery, body temperature was maintained at approximately 37.5°C with a heating lamp. Fluid loss was replaced by intraperitoneal injection of 3 mL warm (37°C) saline before abdominal closure. At the end of 60 min of reperfusion, the abdomen was reopened. Subsequently, the animals were killed, and hepatectomy was performed. The liver tissues obtained were stored at –80°C for biochemical analyses. At the same time, hepatic tissue was stored in 10% formalin for histological examination. All specimens were coded and evaluated by the same individuals, who were blinded to group assignments.

Determination of Enzyme Activities

Determination of Superoxide Dismutase Activity

Total SOD activity was determined according to the method of Sun et al. [13]. The principle of the method is the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine–xanthine oxidase system as a superoxide generator. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. SOD activity was given as units per gram protein (U/g protein).

Determination of Catalase Activity

Catalase activity was determined according to Aebi's method [14]. The principle of the assay is based on the determination of the rate constant (k, s^{-1}) or the H_2O_2 decomposition rate at 240 nm. Results are given as k per gram protein (k/g protein).

Determination of Glutathione Peroxidase Activity

Glutathione peroxidase activity was measured by the Paglia and Valentine method [15]. An enzymatic reaction in a tube containing NADPH, reduced glutathione (GSH), sodium azide, and glutathione reductase, was initiated by addition of H_2O_2, and the change in absorbance at 340 nm was observed by a spectrophotometer. Activity was given in units per gram protein (U/g protein).

Determination of Malondialdehyde Activity

The MDA contents of homogenates were determined spectrophotometrically [16], by measuring the presence of thiobarbituric acid reactive substances. 3 mL of 1% phosphoric acid and 1 mL 0.6% thiobarbituric acid solution were added to 0.5 mL of homogenate pipetted into a tube. The mixture was heated in boiling water for 45 min. After the mixture had cooled, the colored part was extracted into 4 mL of n-butanol. The absorbance was measured by a spectrophotometer (UV-1601; Shimadzu, Kyoto, Japan) at 532 and 520 nm. The amount of lipid peroxides was calculated as thiobarbituric acid reactive substances of lipid peroxidation. The results were given in nanomoles per gram tissue (nmol/g tissue) according to a prepared standard graph.

Determination of Protein Content

Protein contents were measured according to the method of Lowry et al. [17].

Histological Analysis

Liver tissues fixed in 10% formaldehyde solution were embedded in paraffin. The 4-μm sections were stained with hematoxylin-eosin (H-E). Score of liver damage severity was semi-quantitatively assessed as follows: disruption in radial arrangement around central vein, sinusoidal dilatation, congestion, intracellular vacuolization and nuclear changes. The sections were examined by a histologist who was unaware of the treatment group by using a Leica DFC 280 light microscope and the Leica QWin Plus analysis system (Leica Micros Imaging Solution Ltd., Cambridge, UK).

Statistical Analysis

The data is expressed as median (min–max). Normal distribution was confirmed using the Shapiro-Wilk test. Statistical analyses were performed using the Kruskal-Wallis *H* test or two-tailed unpaired Student's *t*-test with SPSS for Windows (SPSS Inc., Chicago, IL, United States), as appropriate. If the results of the Kruskal-Wallis *H* test were significant, multiple comparisons were evaluated by the Conover test. A difference was considered significant at $p < 0.05$.

Table 1. Enzyme activities in liver tissue

Groups	n	MDA (nmol/g tissue)	SOD (U/g protein)	GPx (U/g protein)	CAT (k/g protein)
S	8	24.28 (18.3–31.6)[b]	47.43 (40.1–55.4)[b]	1.37 (1.1–1.6)[b]	31.72 (21.7–42.3)[b]
C	8	43.17 (38.4–48.4)[a,x]	28.72 (22.7–34.6)[a,x]	0.68 (0.4–0.9)[a,x]	6.25 (3.5–9.7)[a,x]
MLT	8	30.13 (24.8–36.6)[b]	36.25 (30.6–42.9)[b]	1.03 (0.7–1.1)[b]	15.54 (11.2–21.8)[b]
β	8	34.28 (26.2–42.4)[b, x]	35.58 (32.4–39.3)[b]	1.24 (0.8–1.4)[b,x]	24.45 (18.4–30.7)[b,x]
MLT+β	8	28.57 (22.5–34.3)[b]	37.49 (33.5–42.1)[b]	1.11 (0.9–1.2)[b]	17.23 (14.3–22.4)[b]

The data were presented as median (min–max).S – sham; C – ischemia/reperfusion; MLT – ischemia/reperfusion + Melatonin intraperitoneal; β – ischemia/reperfusion + β-glucan orally; MLT + β – ischemia/reperfusion + Melatonin + β-glucan; MDA – malondialdehyde; SOD – superoxide dismutase; GPx – glutathione peroxidase; CAT = catalase; k = rate constant (k, s–1).
[a] $p < 0.05$ compared to sham group, [b] $p < 0.05$ compared to IR group, [x] $p < 0.05$ compared to MLT+ β group.

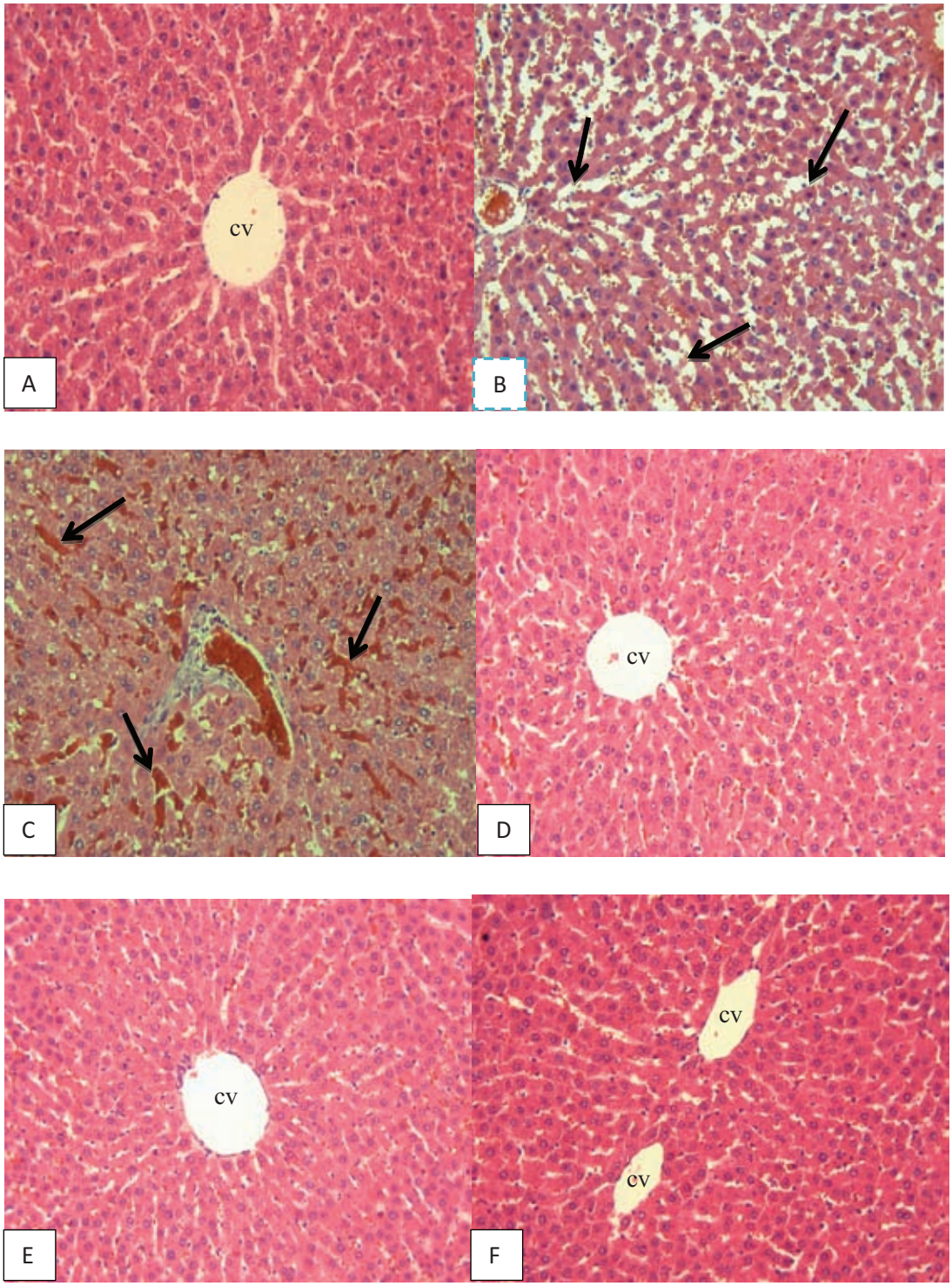

Fig. 1. A) Appearance the central vein (cv) and cordons of hepatocytes in liver sham group. **B)** Sinusoidal dilatation (arrows) in IR (control) group. **C)** Sinusoidal congestion (arrows) in IR (control) group. **D)** The view of the central vein and cordons of hepatocytes in IR + melatonin (MLT). **E)** The histological structure of central vein and cordons of hepatocytes in IR + β-glucan (β). **F)** The central vein and cordons of hepatocytes in IR + combination (MLT + β). H-E × 20

Results

As shown in table 1, MDA level were significantly higher in the C group compared to the S group ($p = 0.007$). MDA level were significantly higher in the β group compared to the MLT and MLT + β groups ($p = 0.007$). Tissue antioxidant markers [SOD, GPx and CAT] were significantly lower in the C group than the S group ($p < 0.05$). SOD levels were not significantly in the β group compared to the MLT and MLT + β groups. CAT and GPx activities were significantly higher in the β group compared to the MLT and MLT + β groups ($p = 0.004$).

Histological Changes

The histological characteristics of all groups are given in Figure 1A–F. The liver tissue in the S group showed a normal histological appearance, except light degenerative changes such as minimal sinusoidal dilatation (Fig. 1A). However, in the C group, sinusoidal dilatation, congestion, hemorrage were observed when compared with regular S group (Fig. 1B and 1C). On the other hand, the histological damage ameliorated in β, MLT and MLT + β groups compared to C group (Fig. 1D, 1E and 1F).

Discussion

To the best of our knowledge, the effect of MLT + β combination on the liver IRI has not been previously reported. Also, these results indicate that administration MLT + β have a combined stronger effect than MLT or β-glucanalone in reducing lipid peroxidation, which was apparent from the biochemical and histological findings.

IR is an inevitable event during liver surgery, including transplantation and tumor resection [18]. Because IRI is a complex process involving numerous intracellular signaling pathways, mediators, cells, and pathophysiological disturbances [19], the extent of IR-associated liver injury is a major factor directly affecting graft survival and post-transplantation function. During transplantation, both cold and warm ischemia occur. However, the authors used warm ischemia in our surgical protocol.

Many investigators have reported that lipid peroxidation is thought to be closely associated with liver IRI pathogenesis [20]. MDA is a by-product of oxidant-induced lipid peroxidation and protein oxidation [21]. In the present study, we observed that MDA levels were increased after IRI. However, MDA levels almost returned to control levels with β-glucan and melatonin combination treatment.

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 [22, 23]. In the present study, there was lower GPx activity in rat livers by IRI than in livers that were not IRI. Pre-treatment with melatonin or β-D-glucan reduced the damage caused by IRI by reducing oxidative stress and increasing antioxidant activity. Because melatonin or β-D-glucan are known free-radical scavengers [8, 24] the idea that free radicals are involved in the pathogenesis of IRI is supported. Melatonin is a strong direct free radical scavenger and an indirect antioxidant through the induction of antioxidant enzymes [25, 26].

GPx further catalyzes the transformation of hydrogen peroxide to form water [27]. CAT is an antioxidant defense enzyme and a potent H_2O_2 scavenger. It prevents the formation of highly toxic hydroxyl radicals when SOD is insufficient to neutralize ROS [28]. Thus, the production of CAT provides additional antioxidative activity against oxidative stress during IR. CAT has been shown to have beneficial effects toward the end of the ischemic period [29, 30]. The delivery of CAT protein successfully prevented liver IRI in mice [31]. The authors found that β-glucan pretreatment significantly increased CAT and GPx activities compared to the MLT + β G group, and this may explain the observed ROS decrease.

SOD has been widely studied among the endogenous antioxidant. SOD catalyzes the dysmutation of superoxide anion to hydrogen peroxide and oxygen, but hydrogen peroxide still produces liver oxidative injury. In many different reports, however, SOD activity was not affected, suggesting that this enzyme is a less sensitive predictor of oxidative stress [32, 33]. The authors found that MLT or β-glucan pretreatment were not significantly increased SOD activity compared to the MLT + β G group and this may result from MLT and β G combination treatment indirectly reducing oxidative damage.

The histological investigation suggested that IR caused severe pathological alterations in the liver, including edema, vascular congestion, hemorrhage, and leukocyte infiltration. Pretreatment with β, MLT and MLT + β combination ameliorated the IR-induced histological changes that were attributed to its antioxidant efficacy. As a result, we may explain these results due to the antioxidant effect of β-glucan and melatonin.

The limitations of the study were explained as follow: Firstly, the sample size used in this study was small. Secondly, we did not evaluate β-glucan and melatonin doses or longer time-dependent response to IRI. However, we established that β-glucan and melatonin attenuates IRI even if it is incomplete protection.

In conclusion, the biochemical and histological findings in this study indicate that β-glucan and melatonin combination pretreatment is useful for preventing tissue damage in liver IRI. The protective effects of β-glucan and melatonin combination may be related to its antioxidant actions, such as clearing ROS and increasing antioxidant enzyme activities. So, β-glucan and melatonin combination may be used in human studies in the future. Additional studies should be performed in order to evaluate the effects of β-glucan and melatoninon IRI in greater detail.

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