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PROTECTIVE EFFECTS OF MELATONIN AGAINST THIOACETAMIDE-INDUCED LIVER FIBROSIS IN RATS

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The aim of this study was to determine the effect of melatonin on thioacetamide (TAA) induced liver fibrosis in rats. The antifibrotic effects of melatonin were assessed by determining activity indirect markers of fibrosis: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), and proinflammatory cytokines: interleukin 6 (IL-6), interleukin-1beta (IL-1 β), tumour necrosis factor alpha (TNF- α), transforming growth factor-beta (TGF- β) and platelet-derived growth factor (PDGF). Parameters of oxidative stress: oxidised glutathione (GSSG), reduced glutathione (GSH) and presaged activity of paraoxonase 1 (PON-1), an antioxidative enzyme were determined. Inflammatory changes and fibrosis extent were evaluated histologically. Experiments were carried out in Wistar rats. Animals were divided into 4 groups: I - controls, water *ad libitum* for 12 weeks, group II - TAA, 300 mg/L *ad libitum* for 12 weeks, III - melatonin, 10 mg/kg b.w. intraperitoneally (i.p.) daily for 4 weeks, IV - TAA, 300 mg/L *ad libitum* for 12 weeks followed by melatonin, 10 mg/kg/b.w. i.p. daily for 4 weeks. Results of serum determinations demonstrated significantly lower activity of AST, ALT and AP in the group receiving TAA followed by melatonin compared to the group receiving only TAA. Immunoenzymatic findings on effect of melatonin on concentration of proinflammatory cytokines confirmed these data. Biochemical examinations in liver homogenates revealed statistically significant improvement (concentration of GSH increases and concentration of GSSG decreases) in animals with TAA-induced liver damage receiving melatonin. Moreover, the activity of PON-1 toward phenyl acetate and paraoxon was increased in liver homogenates and serum in the group receiving TAA followed by melatonin compared to the TAA group without melatonin treatment. Microscopic evaluation disclosed inhibitory effects of melatonin on inflammatory changes and extent of liver fibrosis.

Key words: *fibrosis, liver, melatonin, oxidative stress, inflammatory cytokines, paraoxonase-1, paraoxon, phenyl acetate*

INTRODUCTION

The development of liver fibrosis, consequently resulting in cirrhosis, is attributable to various etiological factors, including hepatotropic viruses A, B, C, D, E, metabolic diseases, alcohol, drugs and xenobiotics, autoimmune diseases, and genetic disorders such as Wilson disease or haemochromatosis. Irrespective of the causative factor, liver fibrosis is always the response to organ damage and impairs the stability between synthesis and degradation of connective tissue components. On the one hand, the process can be considered beneficial as it reduces the damaged areas and segregates the healthy tissue from the affected one; on the other hand, it enhances liver failure leading to massive rearrangement of the hepatic parenchyma (1, 2).

Recently, marked advances in earlier diagnosis of fibrosis, monitoring of its course and novel antifibrotic therapies have been observed. Thanks to them, the disease can be inhibited or possibly even reversed (3, 4).

Melatonin (N-acetyl-5-methoxytryptamine) is one of the numerous new antifibrotic drugs, which is worth considering. For many years, melatonin has been known as the hormone produced only by the pineal gland; however, recent findings demonstrate its presence in the gastric and intestinal mucosa as well as in the liver from which it is directly secreted to the bile. Melatonin is produced in the gastrointestinal tract *via* the separate pathway independent of the synthesis in the pineal gland (5).

Antioxidative and anti-inflammatory properties of melatonin are crucial. An outstanding antioxidative effect of melatonin is attributable to the structure of its molecule, i.e. the presence of O-methyl and N-acetyl groups, which enables it to function as a hydrophilic and hydrophobic antioxidant. Melatonin deactivates the hydroxyl radical, singlet oxygen, nitrogen oxide, hydrogen peroxide and inhibits the peroxidation of lipids (6). Moreover, it affects the synthesis of glutathione, the essential cellular antioxidant, stimulating its synthesis and regeneration (7). Furthermore, melatonin reduces the level of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF- α),

interleukin-6 (IL-6), transforming growth factor beta (TGF- β), and interleukin 1beta (IL-1 β) (8, 9). The effects of melatonin are largely associated with its availability and easy cell penetration; thanks to them, the intracellular concentration of melatonin is often higher than that in body fluids, although its concentration in the body is substantially lower than for instance the concentration of glutathione (10). Considering the antiradical and anti-inflammatory action of melatonin as well as the fact that its highest content was found in the gastrointestinal tract, attempts have been made to use it in inflammatory diseases of the pancreas, liver, intestines or oesophagus (11, 12). The study conducted by Konturek and Brzozowski has demonstrated beneficial effects of melatonin on gastric ulcer; by stimulating the synthesis of nitric oxide, it facilitates the healing of erosions and ulcer niches (5). Moreover, melatonin is safe for patients; no severe adverse side effects of its use were observed, except for occasional drowsiness and abdominal pain. Likewise, no lethal dose, 50% (LD50) was obtained since the highest dose of melatonin, 800 mg, did not cause the death of animals (13).

The objective of the present study was to evaluate the effects of melatonin on liver fibrosis in an animal model. Fibrosis was induced with thioacetamide (TAA), the compound causing fibrosis comparable to fibrogenic changes observed in the human liver.

The evaluation of antifibrotic effects of melatonin was based on indirect (AST, ALT, AP) and direct markers of fibrosis (proinflammatory cytokines: IL-6, IL-1 β , TNF- α , TGF- β , platelet-derived growth factor AB (PDGF-AB). Moreover, the following were determined: oxidative stress parameters, reduced glutathione (GSH), oxidized glutathione (GSSG), and the activity of paraoxonase-1 (PON-1), a less known enzyme exhibiting antioxidative properties. Subsequently, inflammatory lesions and severity of liver fibrosis were assessed histopathologically.

MATERIAL AND METHODS

Animals, experimental conditions, division into groups

The experimental study was carried out on male Wistar rats weighing 200 – 220 g obtained from the breeding farm of laboratory animals (Brwinow n/Warsaw). During the 5-day acclimatisation and throughout the experiment animals were

kept in the animal house of the Department of Experimental and Clinical Pharmacology, Medical University of Lublin supervised by the Veterinary Inspectorate in Pulawy. Rats were kept under constant environmental conditions (22°C, 12-hour day/night cycles), received the standard diet (LSM granulated feed, Agropol, Motycz n/Lublin) and had free access to water. Animals were randomly allocated to groups. Experiments were conducted between 9 am and 3 pm. The biological material used in experiments was sent to the Utilization Plant "BACUTIL" Pulawy, the Zaslaw branch.

The division of animals into groups is presented in Table 1. The first group (I) included animals receiving water *ad libitum* for 12 weeks. The second group (II) received fibrosis-inducing thioacetamide (TAA) *ad libitum* (Signa-Aldrich), which was administered at a dose of 300 mg/L for 12 weeks. The third group (III) was administered with melatonin intraperitoneally at a dose of 10 mg/kg/ b.w. daily for 4 weeks. Melatonin was dissolved in dimethyl sulfoxide (Sigma). The fourth group (IV) was given TAA *ad libitum* in a dose of 300 mg/L for 12 weeks followed by i.p. melatonin in a dose of 10 mg/kg/b.w. for 4 weeks. After the experiment, animals were decapitated using a guillotine. Blood was sampled for biochemical determinations and livers were collected for histopathological and immunoenzymatic tests.

Biochemical examinations

Serum alanine (ALT) and aspartate aminotransferase (AST) as well as alkaline phosphatase (AP) were determined by the kinetic method in the ADVIA 1800 analyser (Siemens). ALT and AST were measured using NADH and Tris buffer (according to IFCC) whereas AP using p-nitrophenyl phosphate and AMP buffer. Determinations of enzymes were performed in the ALAB Laboratory in Lublin, Poland.

Immunoenzymatic determinations of tumor necrosis factor- α , interleukin-6, transforming growth factor- β , interleukin-1 β , platelet-derived growth factor-AB

The concentrations of cytokines in individual study groups were determined in serum using ELISA. Determinations were conducted according to the manufacturer's instructions. The following ELISA kits were used:

Table 1. Study groups and drug administration design.

Group	Drug	Number of animals	Dose, time and route
I	Drinking water	8	12 weeks, <i>ad libitum</i>
II	Thioacetamide (TAA)	8	300 ml/L, 12 weeks, <i>ad libitum</i>
III	Melatonin	8	10 mg/kg/b.w., 4 weeks, i.p.
IV	TAA + melatonin	8	300 ml/L, 12 weeks, <i>ad libitum</i> + melatonin 10 mg/kg/b.w., 4 weeks, i.p.

Table 2. Effects of melatonin on ALT, AST and AP in serum.

Variable [U/L]	I (CON)	II (TAA)	III (MEL)	IV (TAA + MEL)
ALT	62.88	105.38***	45.13	63.13###
AST	158.88	271.63***	62.75	174.88###
AP	201.50	435.88***	121.13	222.88###

I (CON) - control rats, II (TAA) - thioacetamide-treated rats, III (MEL) - melatonin-treated rats, IV(TAA + MEL) - thioacetamide-treated followed melatonin-treated rats. Statistically significant are indicated as: ***P < 0.001 vs. CON, ###P < 0.001 vs. TAA.

Rats TNF- α - Immunoassay kit (R&D)
 Rats IL-6 - Immunoassay kit (R&D)
 Rats TGF- β - Immunoassay kit (R&D)
 Rats IL-1 - Immunoassay kit (R&D)
 Rats PDGF-AB - Immunoassay kit (R&D).

Serum samples were added to a 96-well microplate coated with monoclonal antibodies against TNF- α , IL-6, TGF- β , IL-1 β , and PDGF-AB. During incubation at room temperature, cytokines were immobilised by solid-phase-bound antibodies. Subsequently, the unbound factors were rinsed away and the conjugate bound to a specific enzyme was added to the wells. The wells were rinsed to remove the unbound conjugate part. In order to determine the amount of proteins in the sample, the enzyme substrate enabling colorimetric measurements of cytokine concentrations was added. Absorbance was read at 450 nm using a Victor plate reader (Perkin Elmer, USA). The computer coupled to the reader using the Work Out 2 software plotted the calibration curve and calculated the concentrations in samples. Concentrations were expressed in pg/ml.

Determination of reduced glutathione and oxidised glutathione concentrations in liver homogenates

The concentrations of GSH and GSSG were determined colorimetrically in supernatants from liver homogenates. The BIOXYTECH kit (OxisResearch TM, USA) was used. The individual stages of experiment were carried out according to the manufacturer's instructions. The results were expressed as nmol/g tissue.

Determinations of paraoxonase 1 activity in blood serum

The PON-1 activity towards paraoxon (paraoxonase activity) and phenyl acetate (arylesterase activity) was determined. PON-1 hydrolyzes paraoxon (diethyl-p-nitrophenol phosphate) to p-nitrophenol. In each sample, an increase in p-nitrophenol was determined spectrometrically based on 412 nm wavelength light absorption (14).

The following procedure was used for determinations: 800 ml of the reagent containing 2 mM paraoxon, 50 mM Tris-HCL, pH

= 8 and 1 mM CaCl₂ was added to 20 μ L of serum. An increase in absorbance was measured for 1 minute (a1). Simultaneously, an absorbance increase was measured in the blind sample without serum (a2), which reflected spontaneous hydrolysis of paraoxon; the latter was subtracted from the former (a2-a1).

The activity of PON-1 was calculated based on the specific absorbance of p-nitrophenol, i.e. 18290 M⁻¹cm, and expressed in U/ml (1 U of enzyme hydrolyses 1 nM of paraoxon during 1 minute). Absorbance was measured using a UV-Vis Shimadzu 1610 spectrophotometer.

An increase in phenol concentration was determined by measuring the absorbance of light of 270 nm wavelength (14). The determination of arylesterase activity of the enzyme was based on absorbance increase at 270 nm during 1 minute. To the serum sample of 10 μ L, 3 ml of the reagent containing 2 mM phenyl acetate, 2 mM CaCl₂ and 50 mM Tris-HCL, pH 4 was added. The absorbance increase in the blind sample without serum was subtracted from the value of absorbance increase in the examined sample. The enzyme activity was calculated based on the specific absorbance of phenol (E 270 = 1310 M⁻¹cm⁻¹) and expressed in U/ml.

Determinations of paraoxonase 1 activity in liver homogenates

The PON-1 activity towards phenyl acetate and paraoxon was determined. The weighted liver sections were homogenized in 10 volumes of 50 mM Tris-HCL, pH = 8 containing 2 mM CaCl₂ and centrifuged at 10,000 rev/min for 10 minutes. The PON-1 activity towards phenyl acetate was determined in the supernatant. The absorbance increase at 412 nm was read after 2 minutes. The activity of tissue PON-1 towards phenyl acetate was expressed in U/mg protein. The protein concentrations were determined according to the Lowry method (15). The PON-1 activity towards paraoxon was determined in the supernatant and expressed in U/mg protein. The absorbance increase was read after 2 minutes.

Histological examinations

The liver sections for examinations under a light microscope were collected from the right lobe and fixed in 10% buffered formalin, pH 7.4. The sections were embedded in paraffin

Table 3. Effects of melatonin on proinflammatory cytokines (TNF- α , IL-6, TGF- β , IL-1 β , PDGF-AB) in serum.

Variable [pg/ml]	I (CON)	II (TAA)	III (MEL)	IV (TAA + MEL)
TNF- α	21.91	69.67***	21.13	28.26###
IL-6	23.20	65.49***	20.80	28.47###
TGF- β	48678.34	69625.41***	50168.43	39104.32###
IL-1 β	737.91	922.42***	661.92	592.26###
PDGF-AB	292.77	500.57***	304.35	274.15###

I (CON) - control rats, II (TAA) - thioacetamide-treated rats, III (MEL) - melatonin-treated rats, IV (TAA + MEL) - thioacetamide-treated followed melatonin-treated rats. Statistically significant are indicated as: ***P < 0.001 vs. CON, ###P < 0.001 vs. TAA.

Table 4. Effects of melatonin on GSH and GSSG in liver homogenates.

Variable [nmol/g tissue]	I (CON)	II (TAA)	III (MEL)	IV (TAA + MEL)
GSH	368.20	261.44***	341.55	300.13###
GSSG	115.59	154.77***	112.31	118.01###

I (CON) - control rats, II (TAA) - thioacetamide-treated rats, III (MEL) - melatonin-treated rats, IV (TAA + MEL) - thioacetamide-treated followed melatonin-treated rats. Statistically significant are indicated as: ***P < 0.001 vs. CON, ###P < 0.001 vs. TAA.

Table 5. Effects of melatonin on serum PON1 activity towards paraoxon and phenyl acetate.

Variable [U/ml]	I (CON)	II (TAA)	III (MEL)	IV (TAA + MEL)
PON1 activity towards paraoxon	222.90	147.02***	210.40	226.44###
PON1 activity towards phenyl acetate	164.24	103.22***	144.67	141.29##

I (CON) - control rats, II (TAA) - thioacetamide-treated rats, III (MEL) - melatonin-treated rats, IV (TAA + MEL) - thioacetamide-treated followed melatonin-treated rats. Statistically significant are indicated as: ***P < 0.001 vs. CON, ###P < 0.001 vs. TAA.

Table 6. Effects of melatonin on liver homogenate PON1 activity towards paraoxon and phenyl acetate.

Variable [mU/mg protein]	I (CON)	II (TAA)	III (MEL)	IV (TAA+ MEL)
PON1 activity towards paraoxon	1032.13	883.32**	986.36	1139.41###
PON1 activity towards phenyl acetate	3088.87	2238.24***	2409.47	2620.77##

I (CON) - control rats, II (TAA) - thioacetamide-treated rats, III (MEL) - melatonin-treated rats, IV (TAA + MEL) - thioacetamide-treated followed melatonin-treated rats. Statistically significant are indicated as: **P < 0.01 vs. CON; ***P < 0.001 vs. CON; ###P < 0.001 vs. TAA.

blocks, cut and stained with haematoxylin + eosin (H + E) and Masson's Trichrome. The preparations were evaluated under a light microscope Olympus BX45. The absence or presence of inflammation and its activity were determined in livers of each study group. Furthermore, the presence and nature of necrosis (piecemeal, bridging), extent of proliferation of Brown-Kupffer cells and cholestasis in hepatocytes were evaluated. The 5-point Scheuer scale was applied (16).

The above-mentioned scale was also used to assess the extent of fibrosis, i.e. its absence or otherwise, severity and type. Moreover, degenerative lesions were examined: vacuolar, micro- and macro- vesicular steatosis and the presence of "balloon" cells (16).

Statistical analysis

The results were statistically analysed. The differences between the control and groups of animals receiving the examined substances were checked. The values of parameters were presented as a mean. Normality of distribution was checked using the Shapiro-Wilk test. The inter-group differences were analysed using ANOVA and the NIR test. $P < 0.05$ was considered as significant. The significance of differences was denoted as „*“. If the probability of conclusion was accepted at $P < 0.01$, the differences were considered more significant and marked as (**); if at $P < 0.001$, the differences were considered highly significant and marked as (***). The database and statistical analysis were conducted using Statistica 9.1 software (StatSoft, Poland). The cumulative statistical analysis was presented in Tables 2-6.

RESULTS

Biochemical results

The activities of ALT, AST and AP were determined in serum; the findings were presented in Fig. 1A-1C.

The mean activities of enzymes were found to be significantly higher in group (II) which received TAA for 12 weeks (ALT 105.38, AST 271.63, AP 435.88) compared to controls ($P < 0.001$) (ALT 62.88, AST 158.88, AP 201.50) and significantly lower in group (III), which was administered with melatonin at a dose of 10 mg/kg/b.w. for 4 weeks compared to controls ($P < 0.001$) (ALT 45.13, AST 62.75, AP 121.13). Furthermore, significantly lower enzyme activities were demonstrated in group IV (TAA for 12 weeks followed by melatonin at a dose 10 mg/kg/b.w. for 4 weeks) in comparison to the group which received TAA for 12 weeks (II) ($P < 0.001$) (ALT 63.13, AST 174.88, AP 222.88). No statistically significant differences were found between group I untreated controls and IV, which was administered with TAA for 12 weeks followed by melatonin at a dose 10 mg/kg/b.w. for 4 weeks. The levels of enzymes in the both groups were comparable.

Immunoenzymatic results

The concentrations of cytokines involved in the process of liver fibrosis were analysed, i.e. TNF- α , IL-6, TGF- β , IL-1 β , PDGF-AB. The results were listed in Fig. 2A-2E.

The mean cytokine concentrations were significantly higher in the group that was given TAA for 12 weeks (II) compared to controls ($P < 0.001$). The values were respectively 69.67 and 21.91 for TNF- α , 65.49 and 23.20 for IL-6, 69625.41 and 48678.34 for TGF- β , 922.42 and 737.91 for IL-1 β , 500.57 and 292.77 for PDGF-AB.

No statistically significant differences were found between the group which was administered with melatonin for 4 weeks (III) vs. the control group; concentrations of cytokines were comparable, except for IL-1 β , the level of which was significantly lower compared to controls ($P < 0.05$) (II-1 β : 661.92 for the melatonin group and 737,91 for the control group). The mean concentrations of all cytokines examined were lower in the group which was administered with TAA for 12 weeks followed by melatonin at a dose 10 mg/kg/b.w. for 4 weeks (IV) than in the group which was given TAA for 12 weeks

(II) ($P < 0.001$). The values were respectively 28.26 and 69.67 for TNF- α , 28.47 and 65.49 for IL-6, 39104.32 and 69625.41 for TGF- β , 592.26 and 922.42 for IL-1 β , 274.15 and 500.57 for PDGF-AB.

Concentrations of reduced glutathione and oxidized glutathione

The concentrations of GSH and oxidized glutathione GSSG were determined in liver homogenates. The results are presented in Fig. 3A, 3B.

The mean concentrations of GSH were significantly lower in the group which was administered with TAA for 12 weeks (II) ($P < 0.001$) and in the group that was given melatonin (III) compared to controls ($P < 0.01$). The values were 261.44, 341.55 and 368.20, respectively. Moreover, the mean concentrations of GSH were found to be significantly higher in the group which was administered with TAA for 12 weeks followed by melatonin at a dose of 10 mg/kg/b.w. for 4 weeks (IV) than in the group with TAA for 12 weeks (II) ($P < 0.001$) (300.13 and 261.44, respectively).

The concentrations of GSSG determined in liver homogenates were significantly higher in the group that was given TAA for 12 weeks (II) than in controls ($P < 0.001$). The values were 154.77 and 115.59, respectively. The significant

differences in GSSG concentrations were observed in the group which was administered with TAA for 12 weeks followed by melatonin at a dose of 10 mg/kg/b.w. for 4 weeks (IV) compared to the group that was given TAA for 12 weeks (II) ($P < 0.0001$). These values were 118.01 and 154.77, respectively. The mean concentration of GSSG in this group was lower than in group II.

Results of determinations of paraoxonase 1 activity in serum

The results of determinations of PON-1 activity in rat serum were listed in Fig. 4A, 4B.

The significantly lower mean PON-1 activities towards phenyl acetate ($P < 0.001$) and paraoxon ($P < 0.001$) were observed in the group which was administered with TAA for 12 weeks (II) compared to controls. The respective values were 103.22 and 164.24 for phenyl acetate and 147.02 and 222.90 for paraoxon, respectively.

The significantly higher mean PON-1 activities towards phenyl acetate ($P < 0.001$) and paraoxon ($P < 0.001$) were observed in the group which was given TAA for 12 weeks followed by melatonin at a dose of 10 mg/kg/b.w. for 4 weeks (IV) compared to the group which was administered with TAA for 12 weeks (II). Here the respective values were 141.29 and 103.22 for phenyl acetate and 226.44 and 147.02 for paraoxon, respectively.

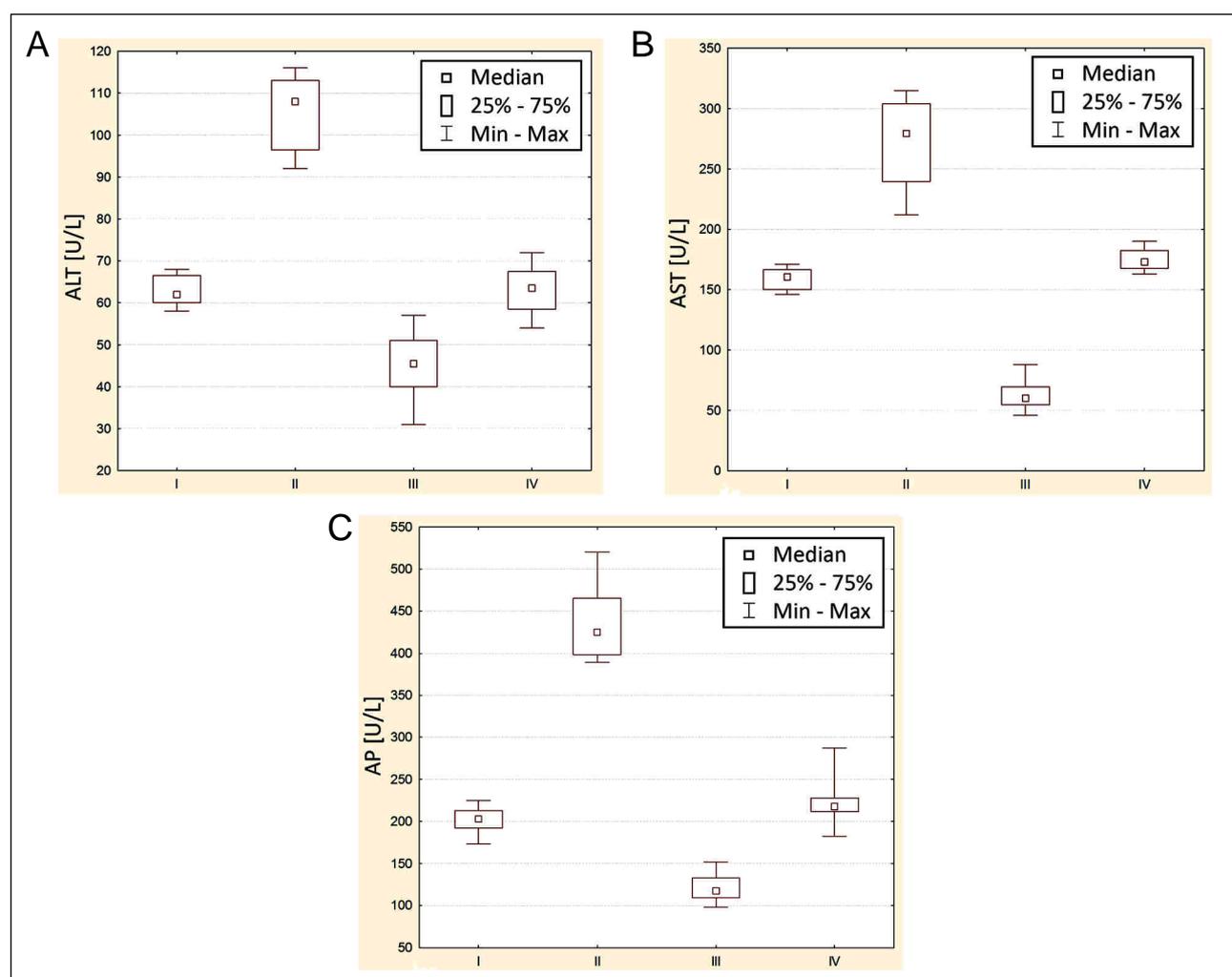


Fig. 1. Effects of melatonin on alanine aminotransferase - ALT: (A), aspartate aminotransferase - AST: (B) and alkaline phosphatase - AP: (C) in serum from thioacetamide-treated rats. Group I - CON (control rats), II - TAA (thioacetamide-treated rats), III - MEL (melatonin-treated rats), IV - TAA + MEL (thioacetamide-treated rats followed melatonin).

Results of determinations of paraoxonase 1 activity in liver homogenates

The results of determinations of PON-1 activity in liver homogenates were gathered in Fig. 4C, 4D.

The mean PON-1 activities towards paraoxon and phenyl acetate were significantly lower in the group which was administered with TAA for 12 weeks (II) than in controls ($P < 0.01$). The values were 883.32 and 1032.13 for paraoxon and 2238.24 and 3088.87 for phenyl acetate, respectively. Moreover,

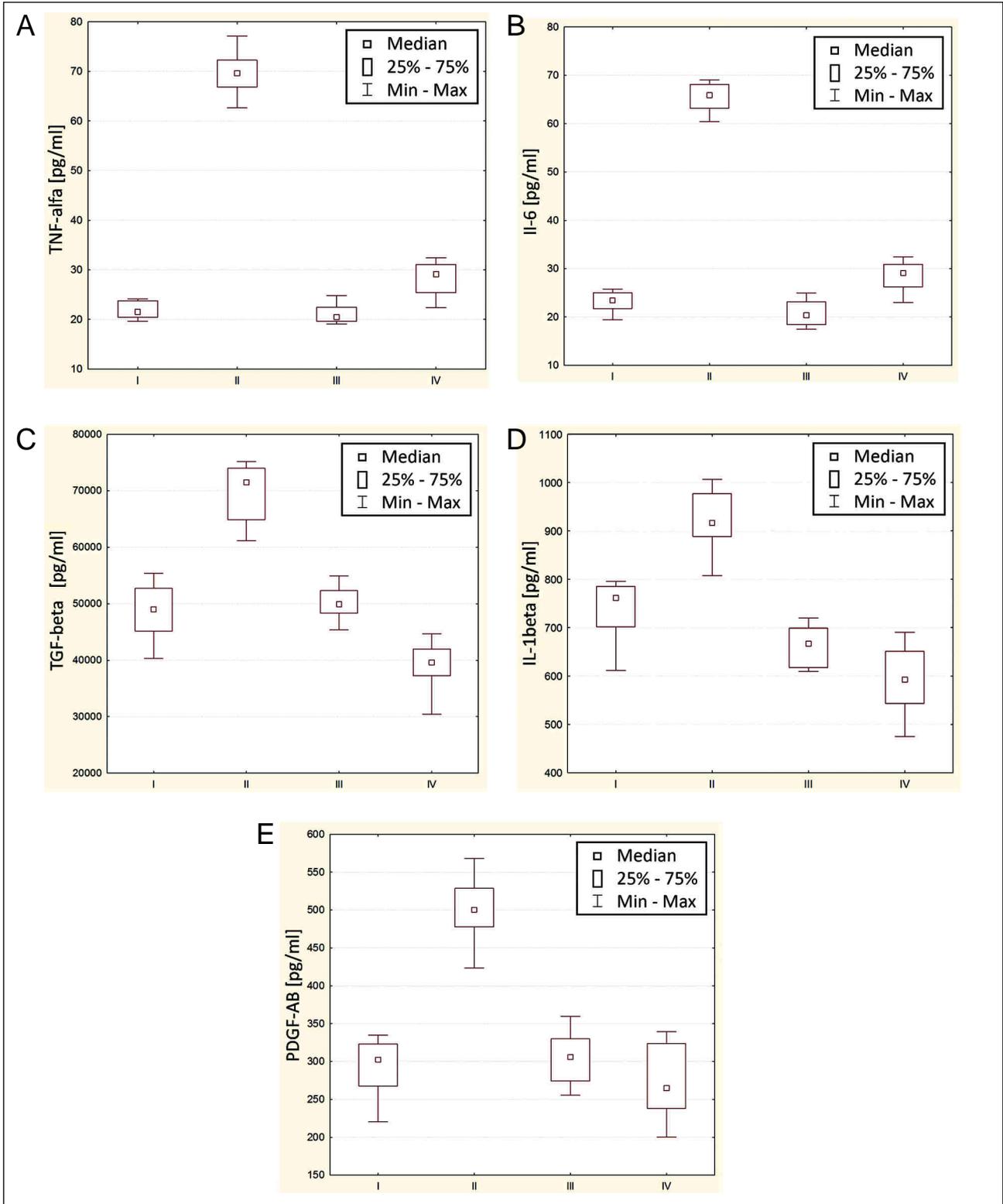


Fig. 2. Comparison of serum cytokines concentrations in study groups. (A): TNF- α , (B): IL-6, (C): TGF- β , (D): IL-1 β , (E): PDGF-AB. Group I - CON (control rats), II-TAA (thioacetamide-treated rats), III - MEL (melatonin-treated rats), IV - TAA + MEL (thioacetamide-treated rats followed).

the mean PON-1 activities towards paraoxon and phenyl acetate were significantly higher in the group that was given TAA for 12 weeks followed by melatonin at a dose of 10 mg/kg/bw for 4 weeks (IV) than in the group which was given TAA for 12 weeks (II) ($P < 0.001$). And here the respective values were 1139.41 and 883.32 for paraoxon and 2620.77 and 2238.24 for phenyl acetate, respectively.

Histological findings

The microscopic liver picture in controls (I) was normal. A slight passive congestion was demonstrated in central veins of lobules. No inflammatory cells or fibrosis elements were found (Fig. 5A, 5B).

The microscopic liver picture in the group receiving TAA for 12 weeks (II) was characterized by the presence of marked inflammatory lesions, features of degeneration and necrosis of hepatocytes as well as locally intensified fibrosis. Piecemeal necrosis was observed in hepatic lobules involving hepatocytes in the region of boundary lamina of biliary ducts and bridging necrosis affecting the laminae of hepatocytes (Figs. 6A and 7A). The inflammatory activity was considered substantial, 3rd and 4th degree of severity (Fig. 9). Fibrosis was staged as 3 and 4 (Fig. 10). Vacuolar degeneration and "balloon" cells of markedly oedematous cytoplasm were found in the cells (Fig. 6A). Micro- and macro-vesicular steatosis was demonstrated in the cytoplasm of hepatocytes (Fig. 6A); in the foci of enhanced inflammation, features of hepatocyte regeneration were observed in the form of enlarged nuclei and the presence of hepatocytes with two nuclei (Fig. 7A).

In animals receiving melatonin at a dose of 10 mg/kg/b.w. for 4 weeks (group III), clusters of few mononuclear cells were observed (mainly lymphocytes) and slight-intensity oedema within the biliary ducts.

In the group receiving TAA for 12 weeks and subsequently melatonin at a dose of 10 mg/kg/b.w. for 4 weeks (IV), decreased severity of microscopic changes was observed compared to the group receiving TAA (II). Slight inflammatory biliary tract was detected as well as focal vacuolar degeneration and steatosis of hepatocytes (Fig. 6B). Cholestasis was found in single hepatocytes (Fig. 7B). Moreover, fibrosis was observed in the biliary ducts with bridging fibrosis (in 7/8 animals - stage 1 and 2, in 1 rat - stage 3 according to Scheuer) (Fig. 8B). In boundary

lamina of hepatocytes, piecemeal necrosis was observed focally accompanied by inflammatory infiltration (Fig. 6B).

DISCUSSION

Despite considerable advances in medicine, liver fibrosis remains the disease for which there is no satisfactory pharmacotherapy. Therefore, each novel therapeutic option put forward is meticulously analysed. At present, melatonin produced by the pineal gland is considered the drug of antifibrotic properties. The use of melatonin in liver fibrosis is connected with its potent antioxidative and anti-inflammatory action.

Administered exogenously, melatonin reduces oxidative stress by direct neutralization of hydroxyl radicals. Indirectly, it is effective in increasing the level of antioxidants essential for the body, such as glutathione peroxidase or glutathione (7). In experimental studies, liver fibrosis is commonly induced with thioacetamide, which is readily metabolised to reactive acetamide and TAA-S-oxide. The metabolites formed combine covalently with macromolecules of the hepatic tissue leading to accumulation of fatty acids, damage of proteins and DNA and formation of reactive oxygen species (ROS). All these compounds impair the endogenous antioxidative system in the liver and are responsible for persistent oxidative stress (18, 19). In our study, the serum levels of AST, ALAT, and AP of animals receiving TAA were found to be markedly increased compared to controls. These findings were confirmed by disturbed microscopic images showing features of degeneration, necrosis, inflammatory infiltration and elements of fibrosis. The level of GSH decreased whereas the expression of proinflammatory cytokines (TNF- α , IL-1 β , TGF- β , PDGF-AB, IL-6) increased.

Melatonin used at a dose of 10 mg/kg/b.w. markedly improved enzymatic parameters, reduced the level of proinflammatory cytokines and increased the level of glutathione. Our findings are consistent with the results published by Friedman and Henderson, who used melatonin at a dose of 1 mg/kg/b.w. and observed elevated levels of the main antioxidative enzymes, i.e. superoxide dismutase (SOD) and catalase (CAT) as well as decreased levels of α -actin of smooth muscles in rat livers. The authors believed that the inhibition of α -actin accumulation was associated with

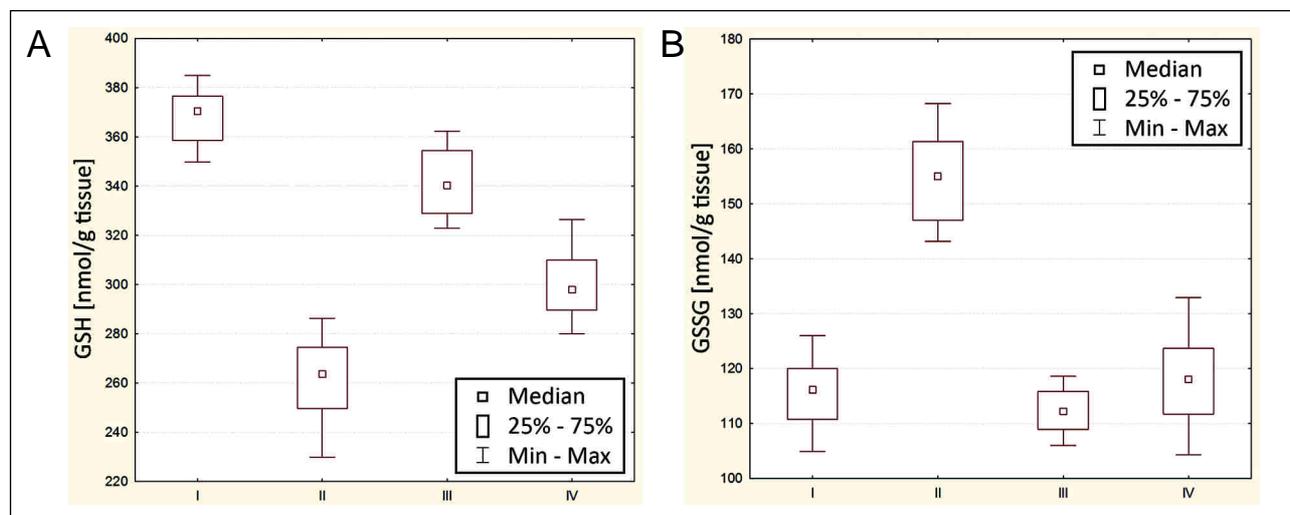


Fig. 3. Concentrations of reduced glutathione - GSH: (A) and oxidized glutathione - GSSG: (B) in liver homogenates (nmol/g tissue) with the effects of melatonin on parameter changed induced with TAA. Group I - CON (control rats), II - TAA (thioacetamide-treated), III - MEL (melatonin-treated rats), IV - TAA + MEL (thioacetamide-treated rats followed melatonin).

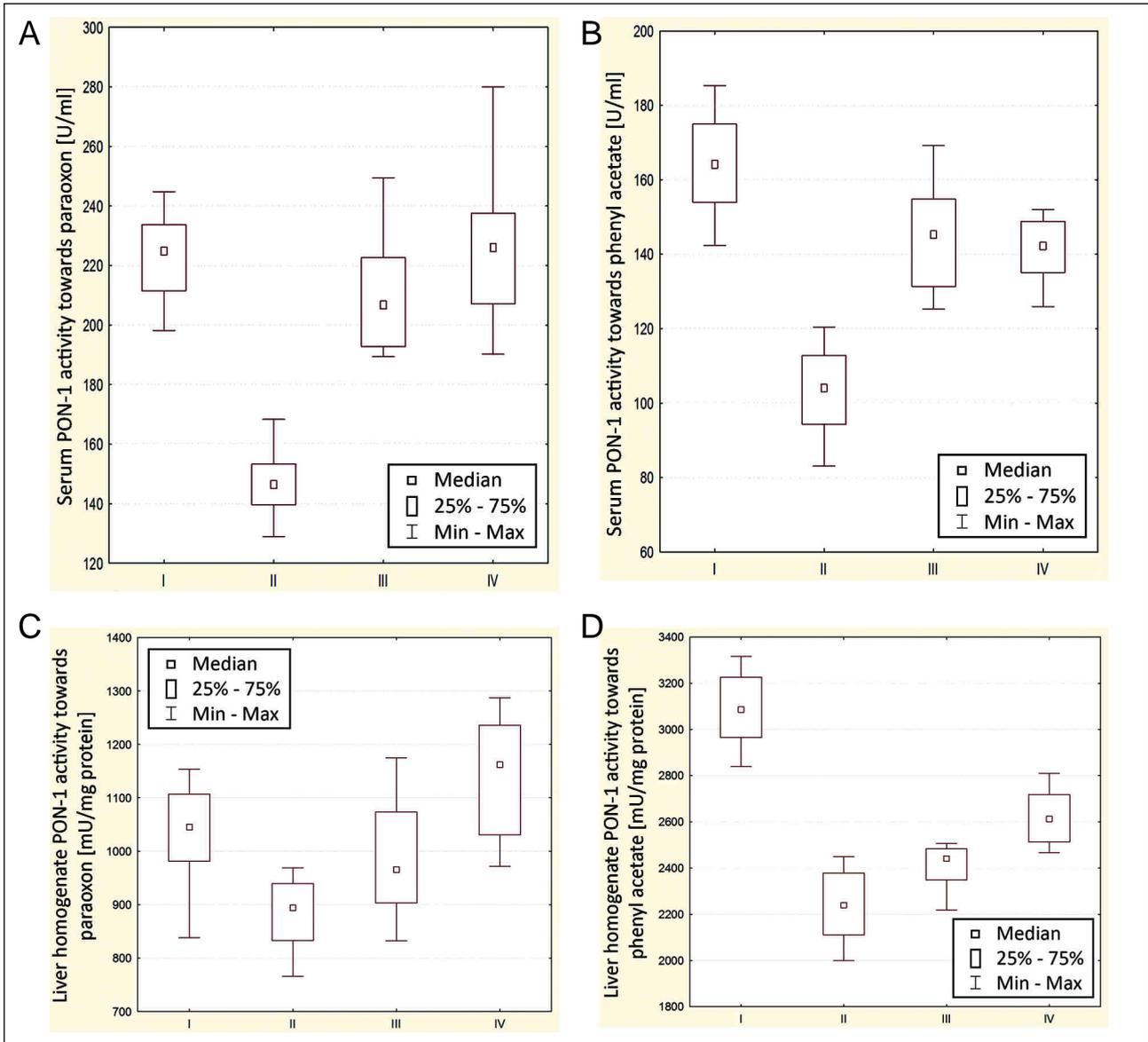


Fig. 4. Serum paraoxonase 1 (PON-1) activity towards paraoxon (A) and towards phenyl acetate (B) with effects of melatonin on parameter changes induced with TAA. (U/ml). Liver homogenate PON-1 activity towards paraoxon (C) and towards phenyl acetate (D) with effects of melatonin on parameter changes induced with TAA. Group I - CON (control rats), II - TAA (thioacetamide-treated rats), III - MEL (melatonin-treated rats), IV - TAA + MEL (thioacetamide-treated rats followed melatonin).

suppressed activity of stellate cells, the major function of which is to produce extracellular matrix, prostaglandins, growth factors and proinflammatory cytokines (20, 21). Similar findings were reported by Tahan (7). Using another hepatotoxic factor, CCL4, he demonstrated that melatonin administered at a dose of 25 mg/kg/b.w. reduced elevated levels of AST, ALT, and bilirubin (22). In nonalcoholic fatty liver disease (NAFLD) and also nonalcoholic steatohepatitis (NASH), in which insulin-resistance and oxidation-related abnormalities lead to ROS formation followed by cascade synthesis of proinflammatory cytokines (TNF- α , TGF- β , IL-6, IL-8) and release of end-products of lipid peroxidation (MDA) (23), long-term administration of melatonin normalized the levels of aminotransferases (24). The clinical report in patients with liver cirrhosis and portal hypertension demonstrated marked improvement after the use of melatonin or its precursor tryptophan (25). The similar research in patients with non-alcoholic fatty liver disease was conducted by

Celinski *et al.* According to their study, after 14 months of therapy melatonin and tryptophan attenuate the levels of proinflammatory cytokines (IL-1 β , IL-6, TNF- α) and improve parameters of fat metabolism (decrease in plasma levels of triglycerides, LDL - cholesterol) (26). According to the information available, one of the first manifestations of liver fibrosis is the release of proinflammatory cytokines TNF- α and IL-1 β by Kupffer cells and stimulated multiplication of hepatic stellate cells (HSCs). The progression of fibrogenesis leads to increased synthesis of collagen, reduced activity of anti-oxidative enzymes and increased release of nuclear factor-kappa B (NF- κ B), i.e. the factor of transcription connected with enhanced expression of proinflammatory genes. Melatonin not only reduced the accumulation of collagen but also alleviated the inflammatory response in the liver by preventing NF- κ B translocation to the nucleus and its binding to DNA. Protective effects of melatonin administered at a dose of 10 or 10 mg/kg/b.w. is

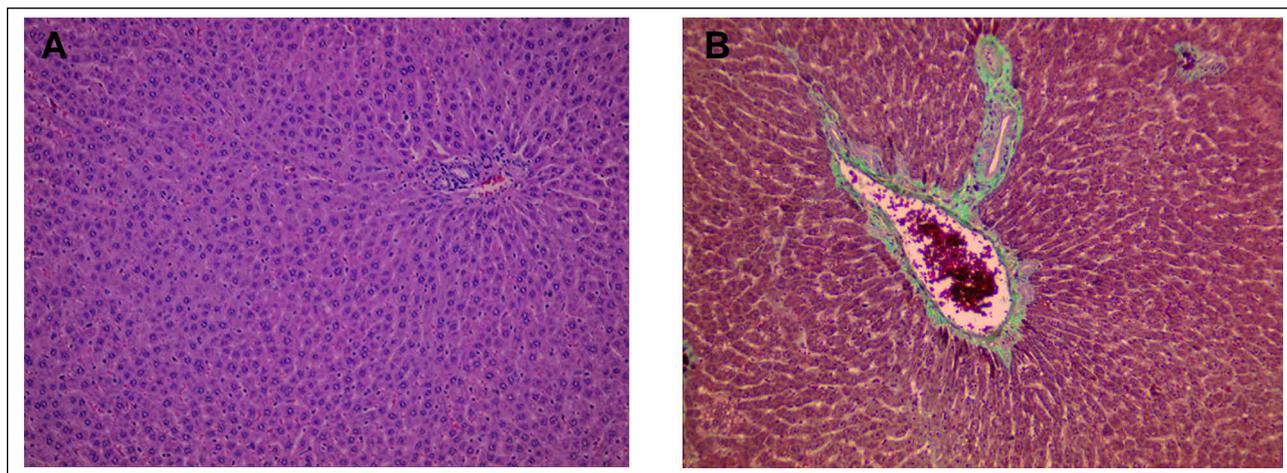


Fig. 5. Light microscopy, CON group (control rats). (A): Liver lobule with mild oedema within the periportal tract. H + E \times 400. (B): Normal hepatocytes architecture and mild congestion within the periportal vein. Masson's Trichrome \times 400.

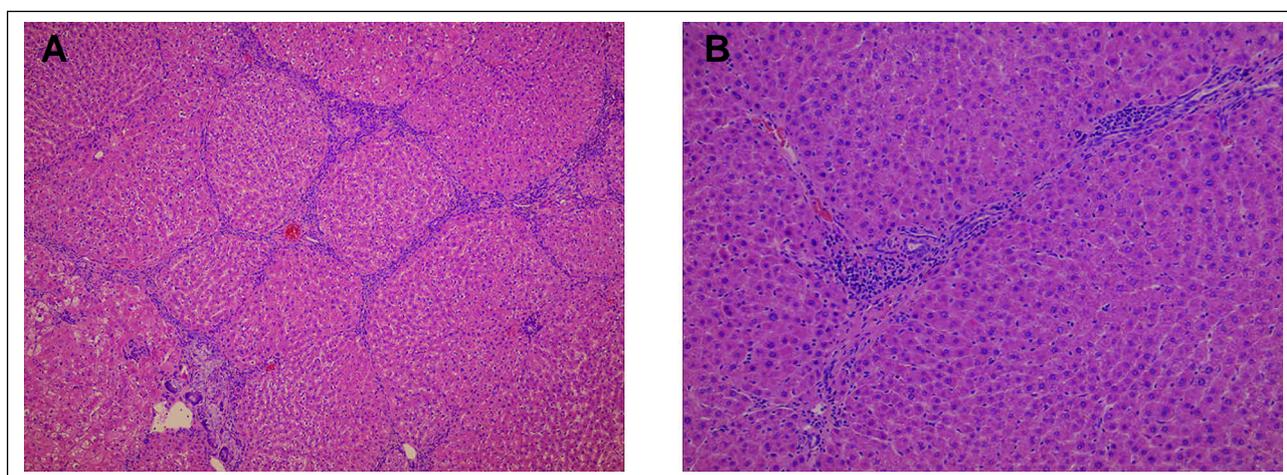


Fig. 6. Light microscopy. (A): TAA group (thioacetamide- treated rats) Nodular appearance of the liver lobule. Bridging necrosis with accompanied mononuclear inflammatory cells. Signs of vacuolar degeneration of hepatocytes with balloon cells visible. Focal proliferation of biliary ductules within the periportal tracts. H + E \times 200. (B): TAA + MEL group (thioacetamide- treated followed melatonin treated rats). Chronic inflammatory infiltrate within the periportal tract entering focally the liver lobule. Vacuolar degeneration of single hepatocytes H + E \times 400.

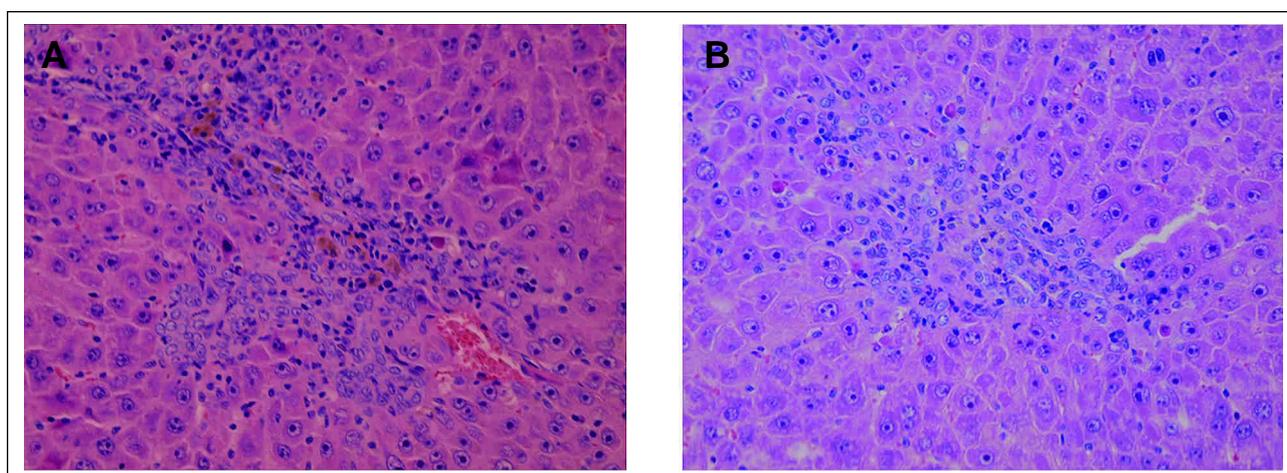


Fig. 7. Light microscopy. (A): TAA group (thioacetamide -treated rats). Piecemeal necrosis of hepatocytes with intense inflammation entering the lobule. Bile stasis within the single hepatocytes. Proliferation of Brown-Kupffer cells. (B): TAA + MEL group (thioacetamide-treated followed melatonin-treated rats). Focal necrosis of hepatocytes within the liver lobule with mild chronic inflammatory infiltrate. Signs of regeneration of hepatocytes. Mild proliferation of Brown-Kupffer cells. H + E \times 400.

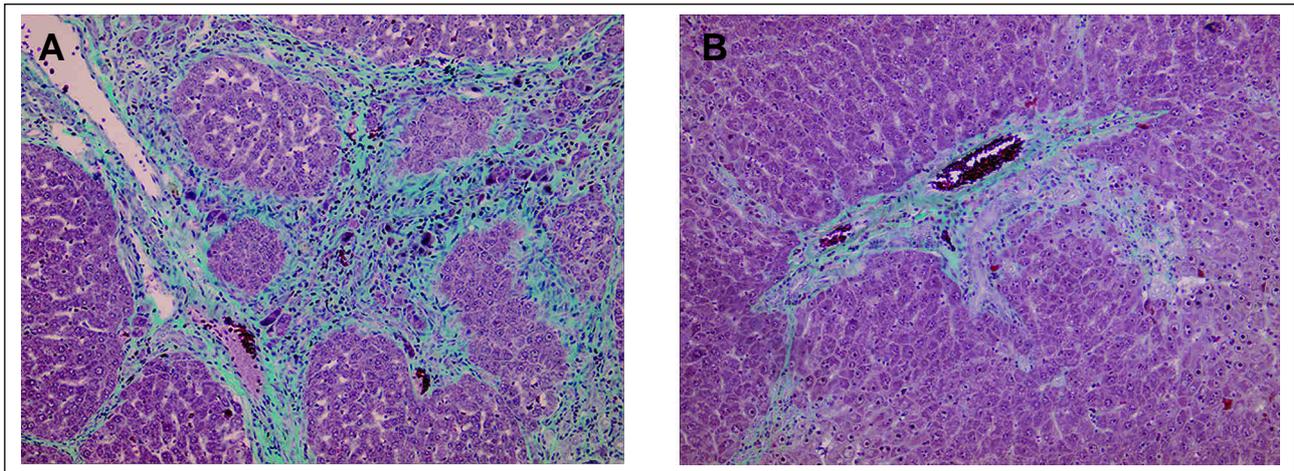


Fig. 8. Light microscopy. (A): TAA group (thioacetamide-treated rats). Intense fibrosis dividing the liver lobule into the nodules. (B): TAA + MEL group (thioacetamide-treated followed melatonin-treated rats). Mild fibrosis within the periportal tract entering focally the liver lobule. Masson's Trichrome $\times 400$.

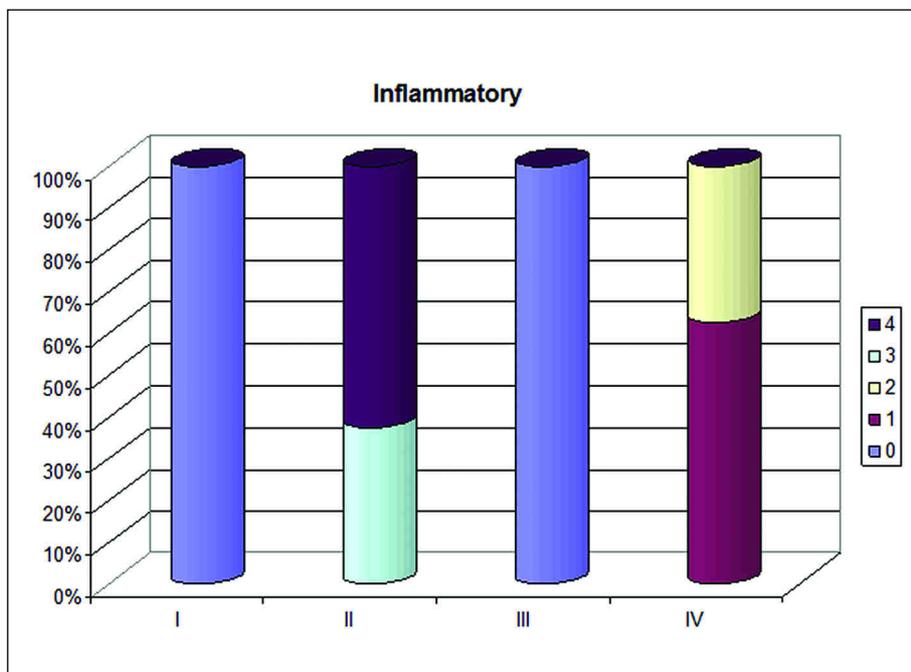


Fig. 9. Activity of inflammation expressed in % and staged on the Scheuer scale (0-1-2-3-4).

0 - no of inflammatory changes in portal spaces, 1 - low activity, slight infiltrations in portal spaces, low intra-lobular inflammatory activity, boundary laminae preserved, 2 - moderate activity, single dose foci of piecemeal necrosis, single necrotic foci within the lobules, 3 - moderate activity, piecemeal necrosis affecting the minority of boundary lamina circumference in all portal spaces, 4 - high activity, piecemeal necrosis affecting the majority of boundary lamina circumference, high intra-lobular inflammatory activity with bridging necrosis. Group I - CON (control group), II - TAA (thioacetamide-treated rats), III - MEL (melatonin-treated rats), IV - TAA + MEL (thioacetamide-treated rats followed melatonin).

likely to be associated both with antioxidative action and inhibition of NF- κ B release, thus reduced production of proinflammatory cytokines (27). In another study determining the concentration of malondialdehyde (MDA), the end-product of lipid peroxidation, its level increased after the use of such fibrosis-inducing factors as TAA and CCL4. The results published by Hong and Arand explicitly revealed that melatonin at dose of 5 or 10 mg/kg/b.w. substantially blocked the elevated levels of MDA, i.e. reduced lipid peroxidation acting as an antioxidant (28, 29). Melatonin is a direct as well as indirect antioxidant, hence it enhances the action of antioxidative enzymes in the liver. Montilla P. *et al.* observed that the biliary duct ligation in rats was associated with decreased levels of GSH in serum and liver homogenates as well as with considerably reduced levels of antioxidative enzymes, e.g. glutathione peroxidase (30). Similar conclusions were reached by Tahan *et al.* in the liver fibrosis model in rats (7). The literature data reveal that doses of

melatonin used in various liver injuries vary greatly, ranging from 100 micrograms to 100 mg/kg; nevertheless, in all cases, hepatoprotective effects of the agent are observed (31, 32). In our study, melatonin was used in a dose of 10 mg/kg/b.w. daily for 4 weeks. The evaluation of AST, ALT and AP activities in serum demonstrated protective effects of melatonin in the group of animals receiving TAA and melatonin, which was visible in significantly suppressed mean activities of the enzyme compared to the group receiving TAA. Slightly different results were presented by Cruz A. who also induced fibrosis with TAA administered for 1 and 3 months. Melatonin reduced the level of AP and bilirubin only in animals receiving the agent for one month. Moreover, values of AP and bilirubin determined in serum increased in animals administered with TAA for a month and decreased after 3 months. According to the authors, this difference is likely to be associated with the fact that an increase in the level of transaminases is a relevant marker of acute liver damage, yet not its advanced stage (33).

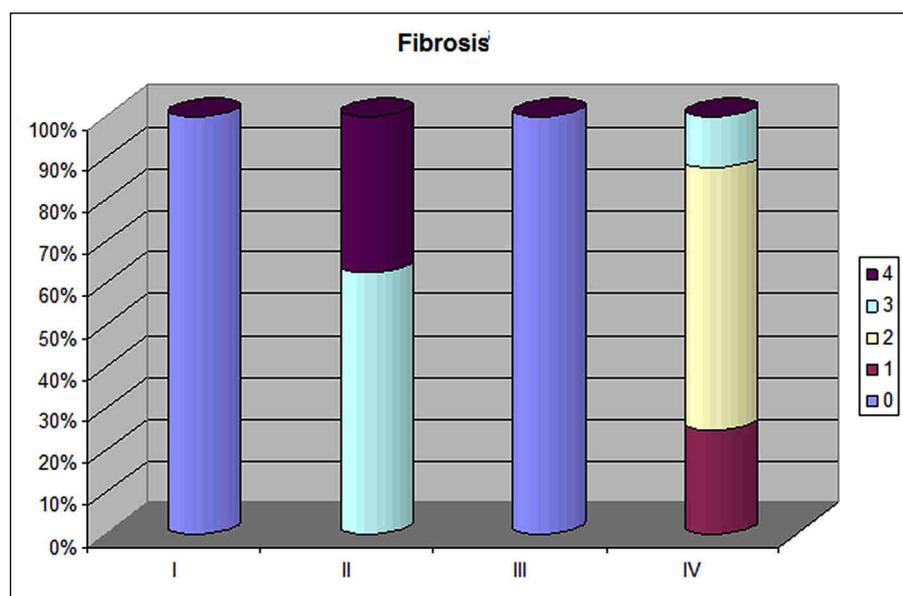


Fig. 10. Severity of fibrosis in study groups expressed in % and staged on the Scheuer scale (0-1-2-3-4). Extent of fibrosis according to Scheuer: 1 - normal, single collagen fibre in portal spaces, 2 - fibrosis within the portal ducts, single bridging fibrosis with the lobule structure preserved, 3 - numerous fibrous bridges with disturbed lobule architecture with regenerative reaction, 4 - diffuse fibrosis or cirrhosis. Group I - CON (control rats), II - TAA (thioacetamide-treated rats), III - MEL (melatonin-treated rats), IV - TAA + MEL (thioacetamide-treated rats followed melatonin).

In the further part of the study, concentrations of cytokines involved in the pathogenesis of liver fibrosis were analysed. The levels of TNF- α , IL-6, TGF- β , IL-1 β and PDGF-AB were immunoenzymatically determined. TNF- α is the main cytokine of inflammatory response produced by monocytes and activated macrophages. It affects the immune processes inducing the release of IL-1 β and IL-6 from macrophages. In the liver, TNF- α is not only the mediator of inflammation but is also involved in the mechanisms repairing parenchyma by activating the proliferation of hepatocytes (34). Mice with TNF- α deficiency exposed to CCL4 demonstrated slight inflammatory and fibrotic changes (35). Another cytokine, IL-6, also secreted by monocytes and macrophages, facilitates the development of inflammatory reaction and is involved in proliferation and programmed cell death. One of the most potent pro-fibrogenic mediators is TGF- β , produced by HSCs, Kupffer cells and endothelial cells. It is responsible for increased synthesis of collagen type I, III and IV as well as fibronectin and proteoglycans. Enhanced expression of TGF- β was observed in the necrotic-inflammatory regions and in fibrous septa bridges (36). IL-1 β activates the synthesis of IL-6 while PDGF activates the transformation of stellate cells. Our immunoenzymatic results regarding the effects of melatonin on the expression of proinflammatory cytokines explicitly demonstrate significantly reduced concentrations of TNF- α , IL-6, IL-1 β , TGF- β , and PDGF-AB compared to the TAA group.

Many experimental studies emphasise the role of oxidative stress in the mechanisms of liver fibrosis and cirrhosis (27). Oxidative stress is defined as increased production of reactive oxygen species (ROS) that are not sufficiently removed due to impaired anti-oxidative mechanisms, which leads to progressive organ failure. Due to their high reactivity, ROS easily react with the essential molecules (proteins, lipids, DNA) and are involved in the transmission of response signal, e.g. to growth factors or cytokines. In the experiment conducted by the Tahan team (7), fibrosis was induced with a single administration of dimethylnitrosamine followed by melatonin (14 days). Changes in the hepatic tissue were estimated by measuring the level of malondialdehyde (MDA), glutathione and superoxide dismutase. The findings in animals receiving melatonin demonstrated significantly lower levels of MDA but elevated levels of reduced glutathione and glutathione peroxidase (7).

In our study, biochemical examinations of liver homogenates showed that the administration of melatonin resulted in considerably reduced concentrations of reduced glutathione (GSH) and increased concentrations of oxidized glutathione (GSSG) in animals with TAA-induced liver damage receiving melatonin.

Nowadays, non-invasive markers for evaluation of the extent of liver damage in humans are of great interest. Once found, they would accelerate the establishment of diagnosis, enable earlier institution of therapy and non-invasive control of treatment. For these reasons, researchers started to be interested in the enzyme of antioxidative properties, which has been used for a long time as an enzyme hydrolyzing phospho-organic compounds yet could acquire a new significance when used as a marker of fibrotic-inflammatory changes. It was noticed that in patients with chronic liver diseases such as inflammation or cirrhosis, the activity of PON-1 in serum is reduced even by 50% and the reduction is proportional to the extent of organ damage (37-40). The above data led to determinations of melatonin effects on the level of PON-1 in serum and liver homogenates in the group of animals administered with TAA. The determinations of PON-1 activity in serum brought about satisfactory results regarding an increase in its activity towards paraoxon and phenyl acetate compared to the TAA group. The findings explicitly spoke in favour of protective action of melatonin; on the other hand, they would suggest possible use of serum PON-1 determinations as an additional marker evaluating the extent of fibrosis or anti-fibrotic therapy.

The microscopic examinations in the TAA⁺ melatonin group revealed decreased severity of microscopic changes compared to the TAA group. Small inflammatory infiltrations of slight and moderate intensity were found in the biliary ducts. Fibrosis present in the biliary ducts was assessed as stage 1 and 2 according to the Scheuer scale. The microscopic findings were consistent with the results reported by Tahan *et al.* and Cruz *et al.* (7, 33). The present study demonstrated improved function of the liver, reduced severity and incidence of morphologic changes and normalized biochemical and immunoenzymatic parameters in rats receiving TAA and melatonin compared to animals exposed to TAA. Further experimental studies and clinical trials are required to confirm the predictive value of serum PON-1 activity determinations.

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REFERENCES

- Bataller R, Brenner DA. Liver fibrosis. *J Clin Invest* 2005; 115: 209-218.
- Bonis PA, Friedman SL, Kaplan MM. Is liver fibrosis reversible? *N Engl J Med* 2001; 344: 452-454.
- Desmet VJ, Roskams T. Cirrhosis reversal: a duel between dogma and myth. *J Hepatol* 2004; 40: 860-867.
- Friedman SL, Sheppard D, Duffield JS, Violette S. Therapy for fibrotic diseases: nearing the starting line. *Sci Transl Med* 2013; 5: 167sr1.
- Konturek SJ, Konturek PC, Brzozowski T, Bubenik GA. Role of melatonin in upper gastrointestinal tract. *J Physiol Pharmacol* 2007; 58 (Suppl. 6): 23-52.
- Gulcin I, Buyukokuroglu ME, Kufrevioglu O. Metal chelating and hydrogen peroxide scavenging effects of melatonin. *J Pineal Res* 2003; 34: 278-281.
- Tahan V, Ozaras R O, Canbakan B, et al. Melatonin reduces dimethylnitrosamine-induced liver fibrosis. *J Pineal Res* 2004; 37: 78-84.
- Lahiri S, Singh P, Singh S, Rasheed N, Palit G, Pant KK. Melatonin protects against experimental reflux esophagitis. *J Pineal Res* 2009; 46: 207-213.
- Sidhu S, Pandhi P, Malhotra S, Vaiphei K, Khanduja KL. Melatonin treatment is beneficial in pancreatic repair process after experimental acute pancreatitis. *Eur J Pharmacol* 2010; 628: 282-289.
- Reiter RJ, Tan DX, Manchester LC, Pilar Terron M, Flores LJ, Koppisevi S. Medical implications of melatonin: receptor-mediated and receptor independent actions. *Adv Med Sci* 2007; 52: 11-28.
- Jaworek J, Konturek SJ, Leja-Szpak A, et al. Role of endogenous melatonin and its MT2 receptor in the modulation of caerulein-induced pancreatitis in the rat. *J Physiol Pharmacol* 2002; 53: 791-804.
- Konturek SJ, Zayachkivska O, Havryluk XO, et al. Protective influence of melatonin against acute esophageal lesions involves prostaglandins, nitric oxide and sensory nerves. *J Physiol Pharmacol* 2007; 58: 361-377.
- Barchas J, Da Costa F, Spector S. Acute pharmacology of melatonin. *Nature* 1967; 214: 919-920.
- Ayub A, Mackness MI, Arrol S, Mackness B, Patel J, Durrington PN. Serum paraoxonase after myocardial infarction. *Arterioscler Thromb Vasc Biol* 1999; 19: 330-335.
- Lowry OH, Rosenbrough NJ, Farr AJ, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193: 265-275.
- Scheuer PJ. Classification of chronic viral hepatitis: a need for reassessment. *J Hepatol* 1991; 13: 372-374.
- Tahan V, Artug O, Akin H, et al. Melatonin ameliorates methionine choline deficient diet induced nonalcoholic steatohepatitis in rats. *J Pineal Res* 2009; 46: 401-407.
- Muller D, Sommer M, Kretschmar M, et al. Lipid peroxidation on thioacetamide-induced macronodular cirrhosis. *Arch Toxicol* 1991; 65: 199-203.
- Abul H, Mathew TC, Dashti HM, Al-Bader A. Level of superoxide dismutase, glutathione peroxidase and uric acid in thioacetamide-induced cirrhotic rats. *Anat Histol Embryol* 2002; 31: 66-71.
- Friedman SL. Mechanism of hepatic fibrogenesis. *Gastroenterology* 2008; 134: 1655-1669.
- Henderson NC, Forbes SJ. Hepatic fibrogenesis: from within and outwith. *Toxicology* 2008; 254: 130-135.
- Kus J, Ogeturk M, Oner H, Sahin S, Yekeler H, Sarsilmaz M. Protective effects of melatonin against carbon tetrachloride-induced hepatotoxicity in rats: a light microscopic and biochemical study. *Cell Biochem Funct* 2005; 23: 169-174.
- Fierbinteanu-Braticevici C, Negreanu L, Tarantino G. Is fatty liver always benign and should not consequently be treated? *J Physiol Pharmacol* 2013; 64: 3-9.
- Gonciarz M, Gonciarz Z, Bielanski W, et al. The effect of long-term melatonin treatment on plasma liver enzymes levels and plasma concentrations of lipid and melatonin in patients with nonalcoholic steatohepatitis: a pilot study. *J Physiol Pharmacol* 2012; 63: 35-40.
- Celinski K, Konturek PC, Slomka M, et al. Altered basal and postprandial plasma melatonin, gastrin, ghrelin, leptin and insulin in patients with liver cirrhosis and portal hypertension without and with oral administration of melatonin or tryptophan. *J Pineal Res* 2009; 46: 408-414.
- Celinski K, Konturek PC, Slomka M, et al. Effects of treatment with melatonin and tryptophan on liver enzymes, parameters of fat metabolism and plasma levels of cytokines in patients with non-alcoholic fatty liver disease - 14 months follow up. *J Physiol Pharmacol* 2014; 65: 75-82.
- Wang H, Wei W, Wang NP, et al. Melatonin ameliorates carbon tetrachloride-induced hepatic fibrogenesis in rats via inhibition of oxidative stress. *Life Sci* 2005; 77: 1902-1915.
- Hong RT, Xu JM, Mei Q. Melatonin ameliorates experimental hepatic fibrosis induced by carbon tetrachloride in rats. *World J Gastroenterol* 2009; 15: 1452-1460.
- Aranda M, Albendea CD, Lostale F, et al. In vivo hepatic oxidative stress because of carbon tetrachloride toxicity: protection by melatonin and pinoline. *J Pineal Res* 2010; 49: 78-85.
- Montilla P, Cruz A, Padillo FJ, et al. Melatonin versus vitamin E as protective treatment against oxidative stress after extra-hepatic bile duct ligation in rats. *J Pineal Res* 2001; 31: 138-144.
- Manda K, Bhatia AL. Prophylactic action of melatonin for cyclophosphamide-induced oxidative stress in mice. *Cell Biol Toxicol* 2003; 19: 367-372.
- Sigala F, Theocharis S, Sigalas K, et al. Therapeutic value of melatonin in an experimental model of liver injury and regeneration. *J Pineal Res* 2006; 40: 270-279.
- Cruz A, Padillo FJ, Torres E, et al. Melatonin prevents experimental liver cirrhosis induced by thioacetamide in rats. *J Pineal Res* 2005; 39: 143-150.
- Schwabe RF, Brenner DA. Mechanisms of liver injury. TNF- α -induced liver injury: role of IKK, JNK and ROS pathways. *Am J Physiol Gastrointest Liver Physiol* 2006; 290: 583-589.
- Wei YH, Jun L, Qiang CJ. Effect of losartan, an angiotensin II antagonist, on hepatic fibrosis induced by CCL4 in rats. *Dig Dis Sci* 2004; 49:1589-1594.
- Bayer EM, Herr W, Kanzler S, et al. Transforming growth factor beta-1 in autoimmune hepatitis: correlation of liver tissue expression and serum levels with disease activity. *J Hepatol* 1998; 28: 803-811.
- Marsillach J, Aragones G, Mackness M, et al. Decreased paraoxonase-1 activity is associated with alterations of high-density lipoprotein particles in chronic liver impairment. *Lipids Health Dis* 2010; 9: 46.
- Marsillach J, Camps J, Ferre N, et al. Paraonase-1 is related to inflammation, fibrosis and PPAR delta in experimental liver disease. *BMC Gastroenterol* 2009; 9: 3.
- Ferre N, Camps J, Prats E, et al. Serum paraoxonase activity: a new additional test for the improved evaluation of chronic liver damage. *Clin Chem* 2002; 48: 261-268.

40. Kedage V, Mutigi MS, Shetty MS, *et al.* Serum paraoksonase1 activity status in patients with liver disorders. *Saudi J Gastroenterol* 2010; 16: 79-83.

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