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Immunohistochemical distribution of glutathione peroxidase and its gene expression via RT-PCR in the liver tissue of melatonin-administered mice

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Abstract: The purpose of this study is to investigate the effect of exogenous administration of melatonin, which is a strong antioxidant agent, on the gene expression of glutathione peroxidase, which is one of the antioxidant enzymes, in the livers of mice and also to examine the immunohistochemical localization of the enzyme. The 24 male Swiss albino mice used in the study were divided into 3 groups: experimental (n = 8), sham (n = 8), and control (n = 8). A 10 mg/kg dose of melatonin was administered intraperitoneally to the experimental group for 4 weeks. Only ethanol and a serum physiological solution were applied to the sham group. No administration was done for the control group. It was observed that the expression level of the glutathione peroxidase 1 enzyme in the experimental group showed a statistically significant increase ($P < 0.05$) compared to the sham and control groups. In the immunohistochemical examinations, it was seen that immunoreactivity was especially intensified in the hepatocytes around Kiernan's space and in the hepatocytes around the vena centralis, and that reaction was generally present both as cytoplasmic and nuclear reactions in the hepatocytes.

Key words: Melatonin, liver, glutathione peroxidase 1, RT-PCR, immunohistochemistry

1. Introduction

Antioxidant enzymes such as glutathione peroxidase (GPx), catalase, and superoxide dismutase (SOD) are of great importance in the capture of reactive oxygen species and in the protection of the cells against oxidative stress. GPx has various forms containing or not containing selenium (1). The decrease in the activity of this enzyme in cells causes severe cell damage and an increase in hydrogen peroxide (2). In a study conducted by Simmons and Jamall (3), it was reported that GPx is the most important antioxidant enzyme in the liver.

In recent years, various pharmacological agents with antioxidant properties have been used in order to strengthen the endogenous defense system, and melatonin is one such agent (4,5). In the literature, the antioxidant property of melatonin was first specified by Ianăş et al. (6) in 1991. By courtesy of this property, melatonin prevents the oxidative damage that occurs as a result of lipid peroxidation in the tissues (7). The antioxidant effect of melatonin is categorized under 3 main headings: direct antioxidant effect, antioxidant enzyme-mediated effect, and prooxidant enzyme-mediated effect (5). At pharmacological and physiological levels, the antioxidant enzyme-mediated effect of melatonin increases the gene expressions or activities of some of the antioxidant enzymes and thus prevents the damages that may occur as a result of oxidative stress (8).

Considering these points, the purpose of this study was to determine the effect of melatonin, which was administered exogenously and is a strong antioxidant agent, on the gene expression of the GPx 1 enzyme, which is also an antioxidant enzyme in the liver tissue of mice, via histological, immunohistochemical, and RT-PCR methods.

2. Materials and methods

This study was conducted after receiving approval from the Kafkas University Animal Experiments Local Ethics Committee (session with date 14.05.2009 and number 03).

Twenty-four adolescent male Swiss albino mice were used in the study. They were divided into 3 groups and melatonin (10 mg kg^{-1} day^{-1}) dissolved in ethanol and diluted by normal saline was injected intraperitoneally in the experimental group (n = 8) for 28 days. Ethanol and normal saline were applied intraperitoneally in the sham group (n = 8) every day in the same quantity as administered to the experimental group for 28 days. No administration was performed in the control group (n = 8).

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At the end of the 4-week (28-day) experimental period, euthanasia was applied via cervical dislocation and then liver tissue samples were taken. The samples taken for molecular analyses were kept at 4 °C after being homogenized in TRI reagent. The samples to be used for histological examinations were blocked in paraffin after the application of routine histological processes.

Hematoxylin and eosin, Crossman's triple staining, and periodic acid–Schiff (PAS) staining processes were applied to the sections taken from the paraffin blocks (9). In order to examine the immunoreactivity of GPx 1 in the liver tissue, the avidin-biotin-peroxidase complex technique (10) was used. The tissues were subjected to incubation for 1 h at room temperature in anti-GPx 1 (abcam-ab22604; 1:1000 dilution). As the chromogen, 3-amino-9-ethyl carbazole was used. Hematoxylin was used for negative staining. GPx 1 immunoreactivity in the tissues was specified by in a range of 0 to +3 according to the intensity grades of staining. In order to specify whether the immunoreactivity in the tissues was specific to GPx 1 or not, a negative control application was performed.

For molecular analysis, total RNA isolation was performed by using TRI reagent (Sigma T9424) that was obtained as a result of the modification of the guanidine isothiocyanate/phenol-chloroform method described by Chomczynski and Sacchi (11). The amount of RNA in 1 µL was measured at 260 nm wavelength with a spectrophotometer. In order to obtain mRNA from the total RNA, samples were kept at 70 °C for 5 min and at 4 °C for 2 min using Oligo dT primers and sterile, nuclease-free (NF) ddH_2O. In order to obtain cDNA from the mRNAs obtained, a mixture including the Moloney murine leukemia virus (MMLV) reverse transcriptase (M1701, Promega) enzyme was prepared. The MMLV mixture was prepared in a total volume of 25 µL for each sample with 1.6 µL of MMLV-RT enzyme, 8 µL of MMLV-RT enzyme buffer, 8 µL of dNTPs (final concentration of each: 10 mM), 1 µL of RNasin (N2511, Promega), and 6.4 µL of NF water. The prepared master mix was added to tubes containing mRNA and then kept at 37 °C for 1 h, at 95 °C for 5 min, and at 4 °C for a minimum of 2 min in the PCR device. At the end of this program, cDNA was obtained. In order to find the gene that we were looking for by reproducing the cDNA obtained, gene-specific primers and a mixture consisting of such primers were prepared. While base sequences for the GPx 1 gene were forward 5'-CCT CAA GTA CGT CCG ACC TG-3' and reverse 5'-CAA TGT CGT TGC GGC ACA CC-3' (12), for the β-actin gene, forward primer 5'-TCA TGA AGT GTG ACG TTG ACA TCC GT-3' and reverse primer 5'-CCT AGA AGC ATT TGC GGT GCA CGA TG-3' (13,14) were used. This mixture, which included Taq DNA polymerase enzyme (Sigma D1806), dNTP, and gene-specific primers, was placed into PCR tubes and our cDNA samples were added, and then the mixture was processed at 28 cycles for the GPx 1 gene and 30 cycles for the β-actin gene at 94 °C for 1 min, at 50 °C for 1 min, and at 72 °C for 1.5 min. After the cycles were completed, tubes were kept at 72 °C for 10 min and 4 °C for a minimum of 2 min, and then PCR products were taken. These PCR products were treated on 1.5% agarose gel for 1 h at 100 V in an electrophoresis device. The results were photographed under UV light. The densitometric measurements of the bands belonging to β-actin and GPx 1 products were performed with the Kodak MI Ready program. The densitometric findings of GPx 1 were normalized with reference to β-actin and statistical analyses were conducted.

For the statistical analysis, Minitab 12.1 (15,16) was used. ANOVA testing was applied for intergroup differences and the Tukey test, one of the multiple comparison tests, was used for finding the source of the differences in multiple groups (16). In the statistical analysis, the confidence interval was specified as 0.05.

3. Results

In terms of the histological structure of the liver, similar results were observed in the mice in the control, experimental, and sham groups and no different result was found. As a result of the PAS staining performed in all the groups, glycogen accumulations that were stained with a pink-red color were determined in the cytoplasm of the hepatocytes (Figures 1 and 2).

In the immunohistochemical examination of the liver tissue samples of all 3 groups, it was observed that GPx 1 immunoreactivity was specific and there was no immunoreactivity in Glisson's capsule, other connective tissue areas, endothelium cells, Kupffer cells, or the bile duct (Figures 3 and 4). In the tissues, hepatocytes

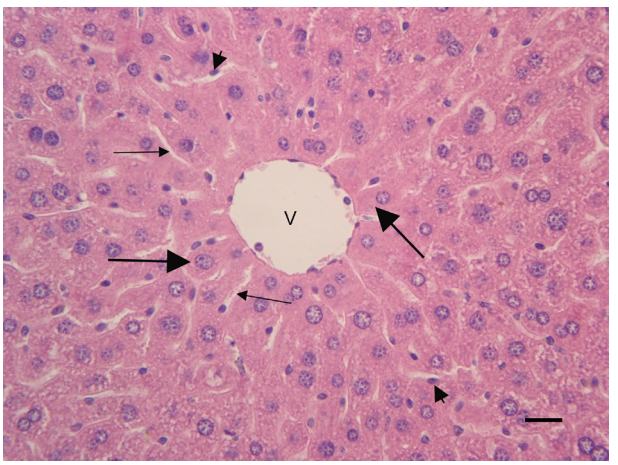

Figure 1. General view of the mice in the control group. V: vena centralis, thin arrow: sinusoid, thick arrow: hepatocyte, arrow head: Kupffer cells. H&E. Bar: 25 µm.

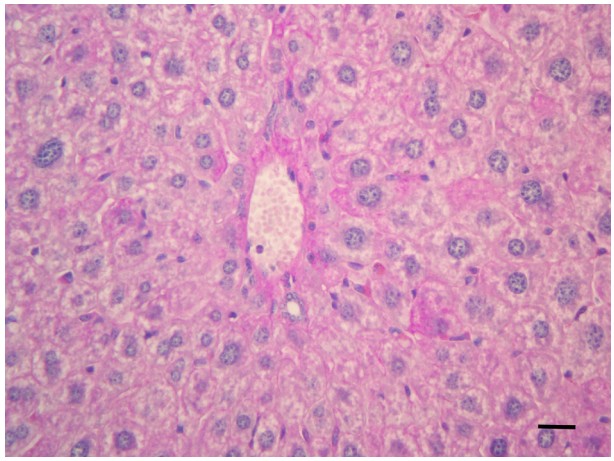

Figure 2. Glycogen accumulation in the liver tissue of the experimental group. PAS. Bar: 25 µm.

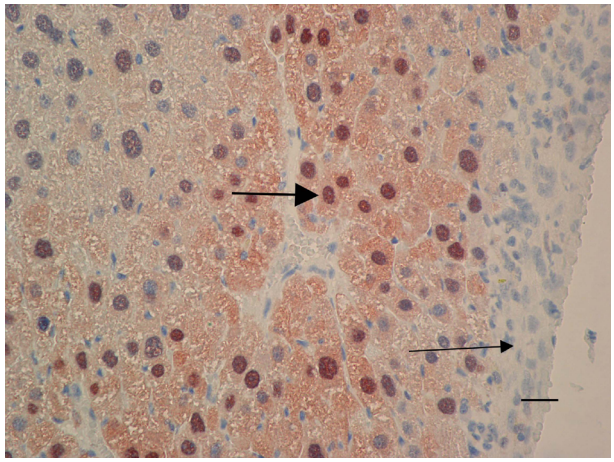

Figure 3. Status of GPx 1 immunoreactivity in the experimental group. Thick arrow: positive immunoreactivity in the hepatocytes, thin arrow: negative immunoreactivity in the liver capsule. Bar: 25 µm.

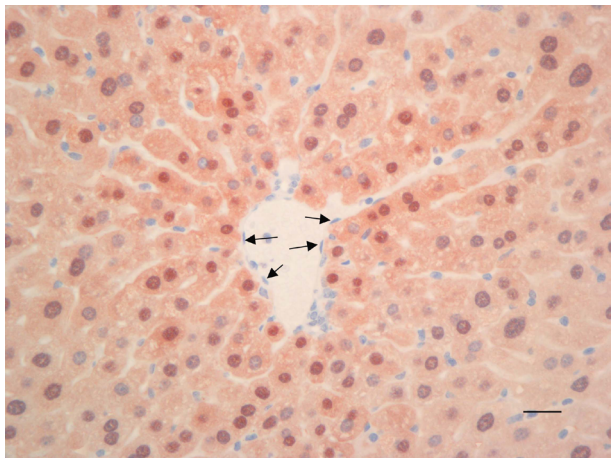

Figure 4. GPx 1 immunoreactivity in the tissue of the control group. Arrows: endothelium cells. Bar: 25 µm.

showing reaction intensity varying between 0 and +3 were seen. The immunoreactivity of GPx 1 was observed only in cytoplasmic form in some of the hepatocytes, but it was mostly in both cytoplasmic and nuclear forms and there was no reaction seen in some rare hepatocytes. In some binucleate hepatocytes in the tissue samples, it was observed that one of the nuclei gave a reaction but the other did not. Immunoreactivity was especially strong in the hepatocytes around the vena centralis and Kiernan's space, with a weaker reaction in other areas (Figures 5 and 6). These results were common for all 3 groups and there were no differences between the groups. In the negative controls, used in order to determine whether GPx 1 immunoreactivity was specific or not in the liver sections taken from all the groups, there was no GPx 1 immunoreactivity.

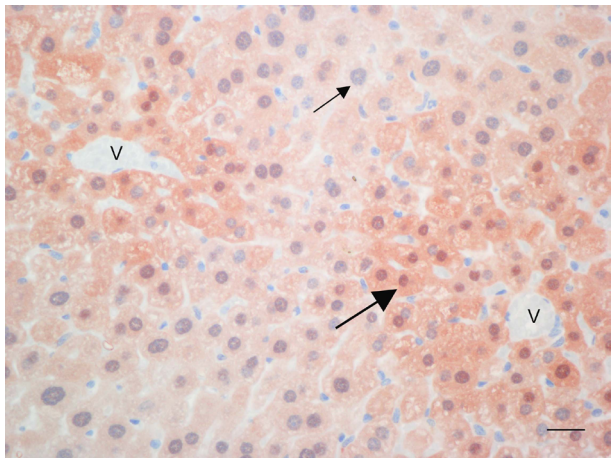

Figure 5. GPx 1 immunoreactivity around vena centralis in the liver tissue of the control group. V: Vena centralis, thick arrow: intense immunoreactivity, thin arrow: weak immunoreactivity. Bar: 25 µm.

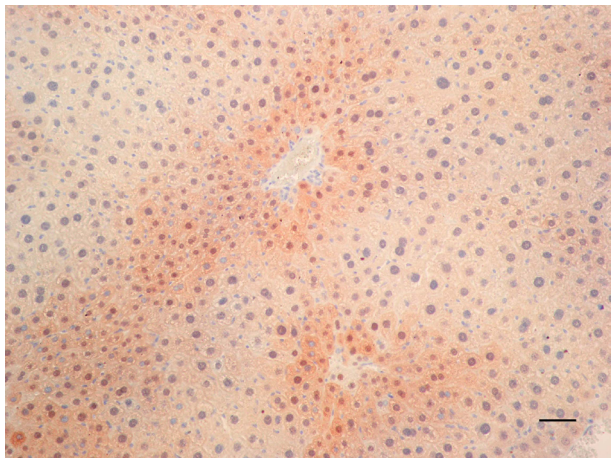

Figure 6. GPx 1 immunoreactivity in the liver tissue of the sham group. Bar: 50 µm.

In order to specify the effect of melatonin on the gene expression of GPx, molecular analyses of the liver samples of the experimental, control, and sham groups were performed. The Table illustrates the comparison of GPx 1 gene expression levels in the liver tissues among the experimental, control, and sham groups.

While no statistical significant difference was found between the control and sham groups in terms of GPx 1 gene expression level in the liver tissue, a statistically significant ($P < 0.05$) increase was found in the experimental group compared to the control and sham groups.

Figure 7 illustrates RT-PCR results of all groups.

4. Discussion

This study investigated the effect of the exogenous administration of melatonin, an antioxidant agent, on the immunohistochemical localization of the GPx 1 enzyme in the liver and on gene expression of this enzyme in the liver.

The histological structure of the liver was examined and it was determined that the general histological findings were in line with information from the classical literature (17) in all groups.

Deprem (18) reported that GPx 1 immunoreactivity in the liver of mice was seen both as cytoplasmic and nuclear in the hepatocytes. He also specified that immunoreactivity was especially denser in the hepatocytes around Kiernan's space and the vena centralis and there was no immunoreactivity in the endothelium cells and connective tissue areas. In their study, Asayama et al. (19) investigated the intracellular distribution of cellular GPx in rat hepatocytes and observed that localization was present in the nucleus and in the cytoplasm. In another study, Asayama et al. (20) examined the immunohistochemical localization of GPx in the lungs of rats and observed that GPx was present in various compartments in the nucleus and in the cytoplasm, similar to the liver. In the immunohistochemical study conducted by Yoshimura et al. (21) in rat livers, they observed that GPx was especially localized in the cytoplasm of hepatocytes and the reaction was stronger in the periphery of hepatic lobules in the portal areas. In the same study, it was observed that GPx was intensely localized in the parenchyma cells of the liver and no staining was seen in the endothelium cells and Kupffer cells around the sinusoids. In the immunohistochemical study conducted by Murakoshi et al. (22) in liver, they determined that GPx was more intense in the hepatocytes in the portal zones of the hepatic lobules. They attributed

Table. Comparison of GPx 1 gene expression level in livers between the groups.

Groups	n	GPx 1 Gene Expression level ($\bar{X} \pm S\bar{X}$)	F
Experimental	5	2.528 ± 0.291^{a}	
Control	5	1.496 ± 0.284^{b}	5.31*
Sham	5	1.478 ± 0.198^{b}	

$P = 0.022$, *: $P < 0.05$. Values with different superscripted letters are significantly different.
$\bar{X}$: Mean value, $S\bar{X}$: standard error.

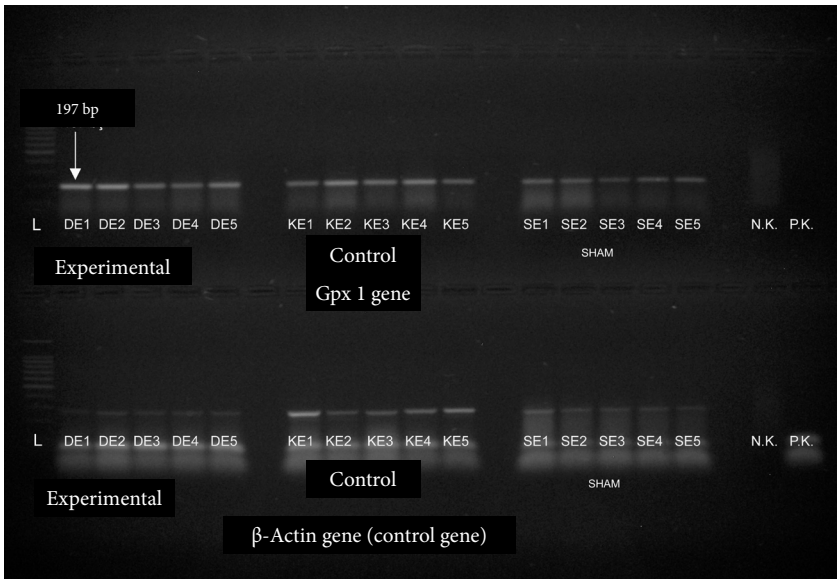

Figure 7. GPx 1 gene RT-PCR results. L: 100-bp DNA ladder, N.K.: negative control, P.K.: positive control, product size: 197 bp.

this to the fact that lipid peroxidation is intense in such areas and GPx is effective in decreasing lipid peroxides.

In our study, it was seen that GPx 1 immunoreactivity was cytoplasmic in some of the hepatocytes, nuclear in some of the hepatocytes, and commonly both cytoplasmic and nuclear. These results support the results of Deprem (18), Asayama et al. (19,20), and Yoshimura et al. (21). Furthermore, in some of the binucleate hepatocytes, GPx 1 immunoreactivity was positive in one of the nuclei and negative in the other. We think that this situation may be associated with the fact that one of the nuclei is functional and the other is not functional in this period. Our results are in line with those of Deprem (18), Yoshimura et al. (21), Asayama et al. (23), and Murakoshi et al. (22) and showed that the enzyme was especially more intense in the hepatocytes around Kiernan's space and the vena centralis. We think that this localization was present because of the intensity of the metabolic activity in this area, and thus the excess of free radical formation and the function of GPx 1 as a barrier here. In line with the results of Deprem (18) and Yoshimura et al. (21), it was observed that there was no immunohistochemical reaction in the endothelium cells or the Kupffer cells. Moreover, no immunoreactivity was observed in the capsule surrounding the liver.

In recent years, it has been shown that melatonin, an antioxidant agent, has various genomic movements and regulates the expression of various genes. Additionally, it was specified that melatonin also affects both the antioxidant enzyme activity and the cellular mRNA level of these enzymes (24). Lankoff et al. (25) stated that melatonin increases SOD, catalase, and GPx activity and thus may be protective against oxidative stress. In a study by Mayo et al. (26), they observed that melatonin increased mRNA levels for both SOD and GPx in neuronal cell lines. The results of the study conducted by Kotler et al. (8) evidently revealed that melatonin that is administered exogenously increases mRNA levels for GPx, ZnSOD, and MnSOD in the brain cortex of rats. In another study (27), it was found that melatonin increased glutathione, glutathione transferase, and GPx activities in the liver and kidneys. Bharti et al. (28) specified that cerebral epiphyseal proteins and melatonin activated and regulated hepatic and renal antioxidant mechanisms and increased GPx and SOD activity in the liver. In their study, Mauriz et al. (29) indicated that melatonin added to potable water (20 mg/L) eliminated oxidative stress in the livers of elderly rats and contributed to the regulation of catalase and CuZn-SOD and GPx gene expressions.

In the study conducted by Gómez et al. (30) to determine the effect of aluminum on the prooxidant activity and effect of melatonin administration on the gene expression of antioxidant enzymes in the rat hippocampus, they specified that melatonin increased SOD and CAT mRNA levels and there was no change in GPx activity.

Our results, different from those of Gómez et al. (30) but similar to those of Mayo et al. (26), Kotler et al. (8), Bharti et al. (28), and Swiderska-Kolacz et al. (27), revealed that melatonin statistically significantly increased ($P < 0.05$) the GPx 1 gene expression in the liver tissues of mice in the experimental group compared to the control and sham groups.

As a consequence, in terms of the immunohistochemical localization of GPx 1, it was observed that there was no difference between the experimental, control, and sham groups, and, as a result of the molecular examinations performed, the expression level of the GPx enzyme in the samples of the experimental group showed a statistically significant increase compared to the control and sham groups. The results obtained from this study support that melatonin can be used as a protective and treatment-supporting agent in ailments that cause the increase of free radicals.

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