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Effect of Hydroxytyrosol on Prdx6 Expression in Diabetic Rat Liver

Hidroksitirozolün Diyabetik Sıçan Karaciğerinde Prdx6 Ekspresyonu Üzerindeki Etkisi

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ABSTRACT

Aim: Oxidative stress caused by hyperglycemia, which is the most important complication of diabetes mellitus, causes liver damage. Hydroxytyrosol is a polyphenolic compound abundant in olive oil that protects the liver against oxidative damage. Peroxiredoxin 6 (Prdx6) is an anti-oxidative enzyme known to exist in the liver. The aim of this study was to investigate the effect of hydroxytyrosol on Prdx6 expression in diabetes-induced liver injury.
Material and Methods: Male Wistar rats were grouped into four as the control group (n=10), hydroxytyrosol group (n=10), streptozotocin group (n=10), and hydroxytyrosol+streptozotocin group (n=10). Blood glucose levels of the animals were measured after streptozotocin injection and at the end of the experiment. The general structure of the liver was examined with a hematoxylin-eosin stain. Prdx6 protein expression was determined with an immunohistochemical method.
Results: In the streptozotocin+hydroxytyrosol group, blood glucose level was found to be lower when compared with the streptozotocin group ($p<0.001$), and histopathological findings in hepatocytes were found to decrease. Prdx6 expression was found to be similar in the control and hydroxytyrosol groups ($p=0.590$). However, it was found to be higher in streptozotocin and streptozotocin+hydroxytyrosol groups ($p<0.001$). Prdx6 expression of the streptozotocin+hydroxytyrosol group was found lower than the streptozotocin group ($p<0.001$).
Conclusion: Hydroxytyrosol, the anti-oxidative activity of which has been proven in many studies, was found to relieve blood glucose levels in diabetic rats, cause histopathological changes in hepatocytes, and decrease anti-oxidative Prdx6 expression. This decrease suggested that instead of inhibiting Prdx6, hydroxytyrosol reduced oxidative stress irrespective of Prdx6.
Keywords: Diabetes mellitus; liver; hydroxytyrosol; Prdx6; streptozotocin.

ÖZ

Amaç: Diabetes mellitusun en önemli komplikasyonu olan hipergliseminin sebep olduğu oksidatif stres, karaciğer hasarına neden olmaktadır. Hidroksitirosol, zeytinyağında bol miktarda bulunan ve karaciğeri oksidatif hasara karşı da koruyan polifenolik bir bileşiktir. Peroksiredoksin 6 (Prdx6), karaciğerde varlığı bilinen bir anti-oksidatif enzimdir. Bu çalışmanın amacı, hidroksitirozolün diyabete bağlı karaciğer hasarındaki koruyucu rolünde Prdx6 ekspresyonunun etkisini araştırmaktı.
Gereç ve Yöntemler: Erkek Wistar ratlar kontrol grubu (n=10), hidroksitirozol grubu (n=10), streptozotosin grubu (n=10) ve hidroksitirozol+streptozotosin grubu (n=10) olmak üzere dört gruba bölündü. Hayvanların kan glukoz seviyesi streptozotosin enjeksiyonu sonrasında ve deney sonunda da ölçüldü. Karaciğerin genel yapısı ise hematoksilen-eozin boyasıyla incelendi. Prdx6 proteinin ekspresyonu immunohistokimya yöntemi ile belirlendi.
Bulgular: Streptozotosin+hidroksitirozol grubunda kan glukoz seviyesi streptozotosin grubu ile kıyaslandığında daha düşük olarak bulundu ($p<0,001$) ve hepatositlerdeki histopatolojik bulgularda ise azalma olduğu saptandı. Prdx6 ekspresyonu kontrol ve hidroksitirozol gruplarında benzer olarak bulundu ($p=0,590$). Ancak streptozotosin ve streptozotosin+hidroksitirozol gruplarında onlardan daha yüksek olduğu saptandı ($p<0,001$). Streptozotosin+hidroksitirozol grubu Prdx6 ekspresyonu streptozotosin grubuna göre daha düşük olarak bulundu ($p<0,001$).
Sonuç: Birçok çalışmada antioksidatif etkinliği kanıtlanmış olan hidroksitirozol diyabetik ratlarda kan glukoz seviyesini düşürdüğü, hepatositlerdeki histopatolojik değişikliklere neden olduğu ve antioksidatif Prdx6 ekspresyonunu azalttığı bulunmuştur. Bu azalma bize, hidroksitirozolün doğrudan Prdx6'yı inhibe etmesinden ziyade, Prdx6'dan bağımsız bir şekilde oksidatif stresi azaltmasına bağlı olarak gerçekleşiyor olabileceğini düşündürdü.
Anahtar kelimeler: Diyabetes mellitus; karaciğer; hidroksitirozol; Prdx6; streptozotosin.

INTRODUCTION

Diabetes mellitus (DM) is widely observed in the world. In addition, DM is a complex metabolic disease (1-3). DM is characterized by hyperglycemia. This is due to a lack of insulin secretion or insulin resistance (3). It is thought that approximately 220 million people in the world have DM. World Health Organization (WHO) predicts that if no solution is found for the treatment of this disease, the number of patients will double by 2030 (4). Both environmental and genetic factors support the occurrence of DM. The most striking main symptom of DM is hyperglycemia (5). Uncontrolled hyperglycemia in DM causes excessive free radical formation, which in turn causes oxidative stress by disrupting the oxidant-antioxidant balance (6). Deterioration in carbohydrate, protein, lipid, and nucleic acid metabolism caused by increased free radicals causes oxidative stress and inflammation. Oxidative stress and inflammation cause hepatopathy in the liver and damage to different organs (7). The liver is an organ with intense free radical reactions. Therefore, oxidative stress markers are quite high in the liver in the early stages of diabetes (3). The oxidative stress-induced liver injury affects insulin binding to the insulin receptor on the liver cell surface and insulin signaling (8). While this situation affects glucose and lipid balance negatively, it may also cause a large number of metabolic disorders (4). Insulin resistance in DM also causes the accumulation of free fatty acids, which is associated with steatosis, inflammatory steatohepatitis, cirrhosis, and liver failure (9). DM has also been associated with many diseases such as liver enzyme disorders, cirrhosis, non-alcoholic liver disease, and acute liver failure. Moreover, death due to diabetic liver disease is also remarkable (10).

Peroxiredoxins (Prdxs) are multifunctional enzymes. This enzyme family is found in many organisms. They have important roles in protecting cells against oxidative damage (11). The Prdxs family consists of six antioxidant enzymes. Prdx6 is the last member of this family (12). Prdx6 differs from other family members in that it has one conserved cysteine residue and has the ability to bind and reduce phospholipid hydroperoxides (13). While Prdx6 is widely expressed in all tissues, it is expressed in higher levels in the liver, pancreas, and kidney (14). Since the liver is a detoxifying organ and neutralizes circulating oxidants, higher expression of Prdx6 is observed in hepatocytes (15). Prdx6 has antioxidant protective properties against reactive oxygen species (ROS) in liver tissue (14). Prdx6 inhibits the oxidative damage caused by ischemia-reperfusion, hypoxia, and a high-fat diet in the liver (16-18).

Hydroxytyrosol (HT) is the most active biological extract found in olives and olive oil. HT has both in vivo and in vitro antioxidant, anti-inflammatory, and neuroprotective effects (19,20). HT shows its anti-inflammatory effects by inhibiting the expression of inflammatory cytokines (21) and MMP9 and COX2 in active monocytes (22). HT, which is a powerful scavenger against free radical species, has a protective effect from oxidative stress (20). HT has protective effects also on the liver (23). HT shows anti-fibrotic and anti-inflammatory effects by regulating oxidative stress in the liver (24). HT has also been shown to have a protective effect against liver damage (25). Another study has shown a combined HT and Vitamin E application to reduce fibrosis associated with non-alcoholic fatty liver disease (26).

When the aforementioned studies are examined, it can be seen that HT and Prdx6 are important in protecting the liver from oxidative damage. However, HT's effects on Prdx6 expression in liver damage due to oxidative stress caused by diabetes are not known. In this study, we investigated the effects of HT, which is known to have antioxidant and anti-inflammatory effects in olive and olive oil, on the expression of Prdx6, which is an antioxidant enzyme in diabetes-induced liver damage, in the diabetic rat model.

MATERIAL AND METHODS

Animals and Chemicals

Forty male Wistar rats with a weight of 250-300 gr were used in the study. The animals were housed at a room temperature of 24±2 ºC and a humidity of 50±5 % in 12-hour light:12-hour dark periods. The animals were provided with food and water ad libitum. Four groups were formed. These were control (only sterile distilled water injection), HT, streptozotocin (STZ), and STZ+HT. Before starting the study, the animals were included by looking at their blood glucose levels. 55 mg/kg STZ (Sigma, S0130) was dissolved in sterile distilled water and administered intraperitoneally as a single dose to STZ and STZ+HT group animals. The blood glucose levels of animals were measured 48 hours after the injection. Those with a blood glucose level of ≥250 mg/dL were considered diabetic. Next, 30 days 10 mg/kg HT (Sigma/Cayman-70604) was administered intraperitoneally to HT and STZ+HT groups. Blood glucose levels were measured from tail blood on days 3 and 30 after the STZ injection. At the end of the experiment, the animals were sacrificed and their liver tissues were removed.

Tissue Preparation

In all groups, after liver tissues were removed, they were fixated in 4% formaldehyde for 24 hours for immunohistochemistry. They were then dehydrated with ethyl alcohol series of 70%, 80%, and 90%, respectively. Next, they were cleared with xylene and blocked by embedding in paraffin.

Morphological Evaluation

4 µm thick sections were taken on a normal slide from the paraffin blocks of each group. After they were deparaffinized and rehydrated, the slides were stained with hematoxylin and eosin. The slides were subsequently followed back and covered with entellan. The hematoxylin and eosin-stained slides were evaluated histopathologically.

Immunohistochemistry

Sections of 4 µm thickness from paraffin blocks were taken on positively charged slides. The sections were deparaffinized in xylene and rehydrated by keeping them in decreasing-grade alcohols. Sections were boiled in citrate buffer (pH 6.0) in a 750-watt microwave oven for 7 minutes. It was then cooled to room temperature. Sections were washed 3 times for 5 minutes (3x5') with phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and washed 3x5' with PBS. Sections were blocked with UltraV block (Thermo Scientific™ TL-125-UB) for 7 min at room

temperature in a humidified chamber. They were then incubated with the Prdx6 primary antibody (1:300 dilution, Abcam; ab73350, Rabbit) at +4 ºC overnight. The next day, the sections were washed 3x5' with PBS. It was incubated with biotinylated secondary antibody (Thermo Scientific™ TL-125-PB) for 60 min at room temperature and washed 3x5' with PBS. It was then incubated with streptavidin (Thermo Scientific™ TL-125-PH) for 15 minutes at room temperature. Sections were washed 3x5' with PBS. Immunoreactivity was developed with diaminobenzidine (DAB). Slides were counterstained with hematoxylin, traced back, and sealed with entellan.

Immunohistochemistry and hematoxylin and eosin-stained sections were photographed under an Olympus Cx41 microscope with an AxioCam Zeiss digital camera. After 10 photos at 400X magnification were taken from each of the immunostaining slides, the intensity of the immunostaining was quantitatively analyzed using ImageJ software (http://imagej.nih.gov/ij/).

Statistical Analysis

All data were analyzed with GraphPad Prism 9 (GraphPad Software) in terms of statistical significance. After the normality of data obtained with ImageJ was evaluated with the Shapiro-Wilk test, descriptive statistics were expressed as mean±standard deviation. Data were analyzed with One-way ANOVA followed by the Holm-Sidak method for multiple comparisons. Blood glucose levels were analyzed by two-way repeated measures ANOVA with applied Tukey's multiple comparisons tests. A p value of <0.05 was considered statistically significant.

RESULTS

Blood Glucose Results

In the study, blood glucose levels of day 3 and 30 measurements were similar in the control and HT groups which were not given STZ injection (p=0.757, p=0.902, respectively, Table 1, Figure 1). In STZ and STZ+HT groups, blood glucose levels were significantly increased when compared with control and HT groups in day 3 and 30 measurements (p<0.001, Table 1, Figure 1). No statistically significant difference was found between groups on day 3 blood glucose measurements in STZ and STZ+HT groups (p=0.999, Table 1, Figure 1). However, in day 30 measurements, the STZ+HT group's blood glucose level was significantly lower when compared with the STZ group (p=0.015, Figure 1). On day 30 STZ group's blood glucose level increased compared to the day 3 STZ group's blood glucose level (p<0.001, Figure 1). However, the blood glucose levels of the day 3 and day 30 STZ+HT groups were similar (p>0.999, Figure 1).

Morphological Evaluation Results

The histological structure of the cells in the control and HT groups was normal (Figure 2a-d). In the STZ group, it was found that the intensity of eosinophilic staining decreased in the cytoplasm of hepatocytes and the microvesicular white areas were found to increase (Figure 2e,f). There were no histopathologic findings in hepatocytes and other cells in the STZ+HT group (Figure 2g,h).

Immunochemistry Results

Prdx6 immunohistochemical staining was both cytoplasmic and nuclear. Expression of Prdx6 in the liver was remarkable mostly in the cytoplasm and nuclei of hepatocyte cells. Prdx6 expression of control and HT groups was similar (p=0.590, Table 1, Figure 3A-3B). In control and HT groups, nuclear expression was higher than cytoplasmic expression (Figure 3A-a,c). However, cytoplasmic expression was higher in the hepatocytes lining the central vein. Cellular distribution of control and

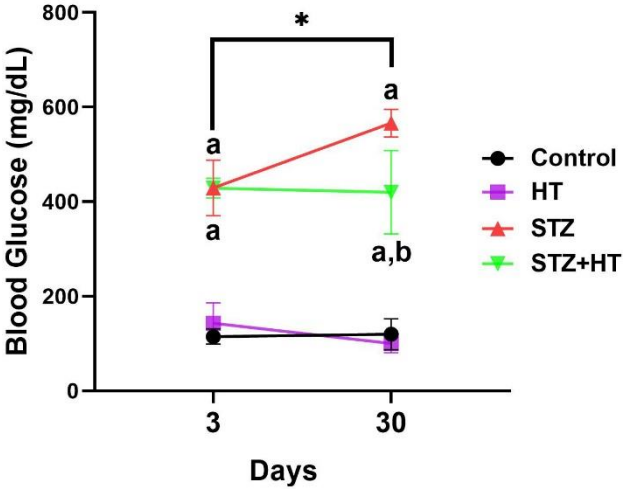

Figure 1. Blood glucose levels on days 3 and 30

HT: hydroxytyrosol, STZ: streptozotocin, statistical significance (p<0.05) compared with [a]: control, [b]: HT, and [c]: STZ, and [*]: between the day 3 and 30 STZ groups

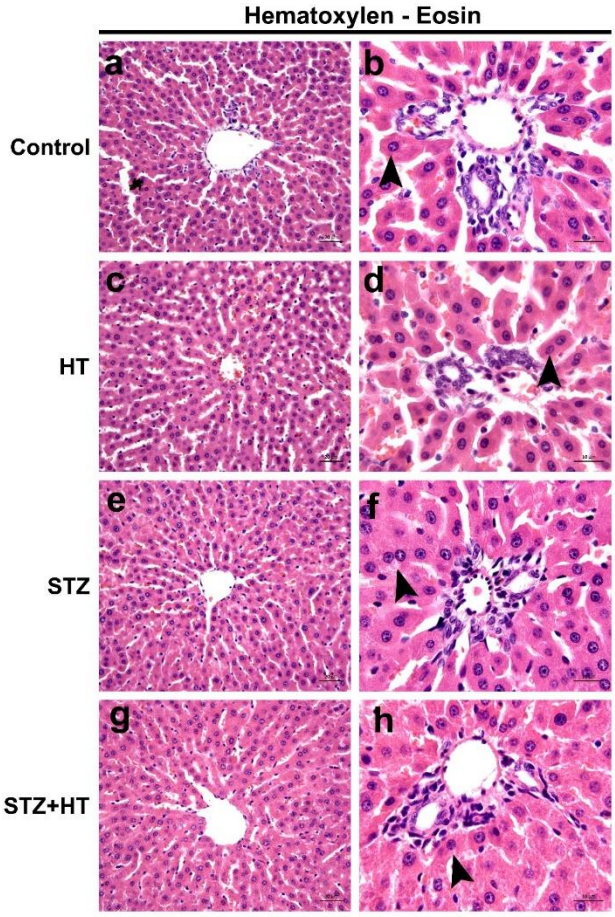

Figure 2. Representative micrographs of morphological evaluation of the liver by hematoxylin & eosin staining

HT: hydroxytyrosol, STZ: streptozotocin, black arrowhead: hepatocyte cells. a, c, e, and g photos were magnified at 400X; while b, d, f, and h were magnified at 1500X

Table 1. Comparison of the blood glucose and Prdx6 measurements between groups

	Control	HT	STZ	STZ+HT	p
Prdx6	4.858±0.414	4.753±0.507	7.125±0.518*	6.222±0.444*#	**<0.001**
Blood glucose					
Day 3	114.8±16.18	142.8±43.36	429.0±58.75*	428.8±20.97*	**<0.001**
Day 30	119.6±37.72	100.2±19.31	566.0±29.36*	419.8±88.11*#	**<0.001**
General	117.2±24.47	121.5±38.80	497.5±84.44*	424.3±60.57*	**<0.001**

Prdx6: peroxiredoxin 6, HT: hydroxytyrosol, STZ: streptozotocin, statistical significance ($p<0.05$) compared with *control and HT, and #STZ.

HT groups Prdx6 expression was positive in hepatocyte cells and negative in Kupffer cells. While the expression of endothelial and bile duct cells was very intensely positive in the control group, it was intensely positive in the HT group (Table 2, Figure 3A-a,c). Prdx6 expression of STZ and STZ+HT groups was higher than the control and HT groups ($p<0.001$, Table 1, Figure 3A-3B). The expression pattern of STZ and STZ+HT groups was both cytoplasmic and nuclear (Figure 3A-e,g). In the STZ group, nuclear and cytoplasmic staining was more intense than in the other groups. In addition, the cytoplasmic Prdx6 expression around the central vein was more intense when compared with the control and HT group (Figure 3A-e). In the STZ group, the cellular distribution of Prdx6 was very intensely positive in hepatocyte cells, and weakly positive in endothelial and bile duct cells, while it was negative in Kupffer cells (Table 2, Figure 3A-e). It was noteworthy that Prdx6 expression was decreased in the STZ+HT group when compared with the STZ group ($p<0.001$, Table 1, Figure 3A-3B). The cytoplasmic staining pattern of this group was decreased (Figure 3A-g). In addition, cytoplasmic staining of Prdx6 around the central vein was the most intense in this group (Figure 3A-g). In the STZ+HT group, the cellular distribution of Prdx6 was intensely positive in hepatocyte and endothelial cells, and positive in bile duct cells, while it was negative in Kupffer cells (Table 2, Figure 3A-g).

DISCUSSION

Diabetes mellitus is a metabolic disease characterized by hyperglycemia that occurs when the body cannot produce or use enough insulin (27). Hyperglycemia, which occurs as a result of diabetes, causes damage due to oxidative stress in many organs including the liver (7). HT is a polyphenolic compound that is abundant in olive and olive oil and which plays a protective role in the liver (23). In diabetic animals which were administered HT, a decrease was observed in blood glucose levels and histopathological findings in the cytoplasm of hepatocytes were found to be relieved. This shows that HT can have a protective role against liver damage due to diabetes.

When previously conducted studies are examined, it can be seen that HT and Prdx6 have been shown to have a role in protecting the liver against oxidative stress (14,24). Reactive oxygen species have an important role in the pathogenesis of liver diseases (28). After all, the liver is one of the important organs affected by reactive oxygen species. Therefore, it is very sensitive against the effects of oxidative damage caused by hyperglycemia (29). Prdx6 expression is high in hepatocytes which have a role in

Table 2. Semi-quantitative distribution of Prdx6 labeling

Cells	Control	HT	STZ	STZ+HT
Hepatocyte cells	+	+	+++	++
Endothelial cells	+	+++	(+)	++
Bile duct cells	++	++	(+)	+
Kupffer cells	-	-	-	-

Prdx6: peroxiredoxin 6, HT: hydroxytyrosol, STZ: streptozotocin, label 0: negative; (+): weak positive; +: positive; ++: dense positive; +++: very dense positive

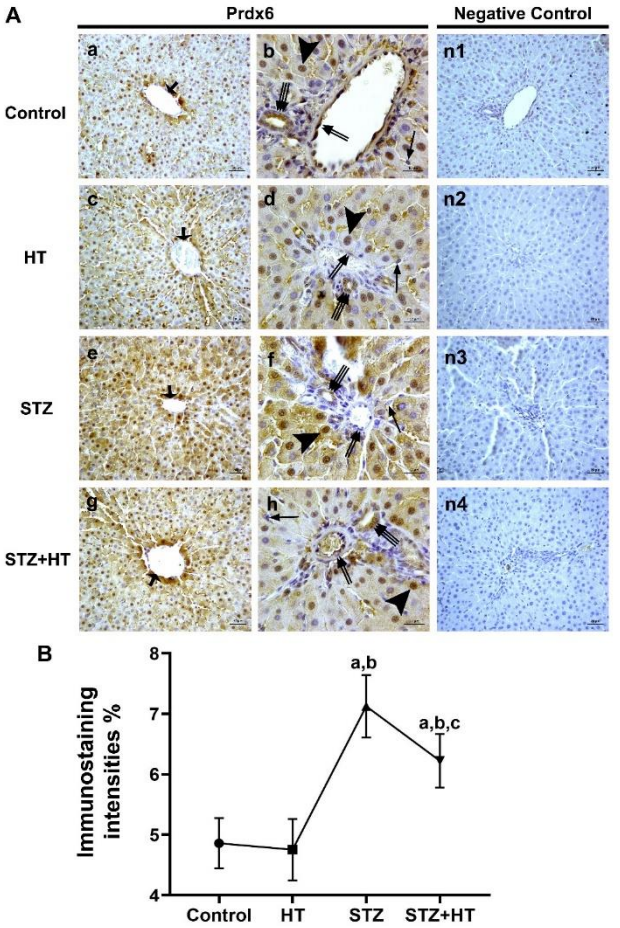

Figure 3. A. Representative micrographs of immunohistochemical analysis of Prdx6, **B.** Quantitative analysis graph of immunohistochemical staining intensity

Prdx6: peroxiredoxin 6, HT: hydroxytyrosol, STZ: streptozotocin, thick short arrow: central vein, arrowhead: hepatocyte cells, arrow: Kupffer cells, double arrow: endothelial cells, triple arrow: bile duct cells, statistical significance ($p<0.05$) compared with [a]: control, [b]: HT, and [c]: STZ. a, c, e, g, n1, n2, n3, and n4 photos were magnified at 400X, while b, d, f, and h were magnified at 1500X

detoxification and inhibiting oxidants in the blood (15). Prdx6 also protects the liver from reactive oxygen species (14). Similarly, HT shows anti-fibrotic and anti-inflammatory effects by balancing oxidative balance in the liver (24). Prdx6 is protective against ischemia-reperfusion, hypoxia, and damage due to oxidative stress triggered by a high-fat diet (16-18). Prdx6 also supports fatty acid oxidation in rats fed with a high-fat diet (30). These studies show that HT and Prdx6 protect the liver against oxidative damage caused by oxidative stress. The reason why there were no differences in Prdx6 expression between control and HT groups in the present study may be the fact that since the absence of diabetes-induced hyperglycemia is not considered a deterioration in oxidant-antioxidant balance, this may not affect Prdx6 expression. In addition, the fact that HT did not change Prdx6 expression in the HT group when compared with the control suggested HT may not have any inhibiting or stimulating effect on Prdx6 expression in the liver. However, in the STZ group, as a result of increased oxidative stress due to hyperglycemia, the expression of Prdx6, which is an important antioxidant enzyme, may have increased significantly. The result that Prdx6 expression decreased when HT was administered in the STZ+HT group was also interesting. The reason for this decrease may be due to the fact that HT reduces oxidative stress caused by diabetes-induced hyperglycemia irrespective of Prdx6. We thought that Prdx6 expression, which increased as a result of oxidative stress due to STZ-induced diabetes, may have decreased in parallel with the decrease in oxidative stress with the administration of HT. As a result, HT, which is an important protector of the liver against oxidants, may not be able to reduce or eliminate the oxidative stress in the liver caused by diabetes-related hyperglycemia through the Prdx6 enzyme. While HT regulates the oxidative balance in the liver, Prdx6 expression in liver cells may be decreased due to the decrease in oxidative stress.

CONCLUSION

HT can reduce blood glucose and improve histopathological findings by reducing oxidative stress due to hyperglycemia through an anti-oxidative pathway or pathways in diabetic rats. However, the decrease of Prdx6 after HT administration to diabetic animals suggested that HT might suppress oxidative stress by using other anti-oxidative pathways in liver cells rather than this pathway.

Ethics Committee Approval: The study was approved by the Experimental Animals Local Ethics Committee of Düzce University (21.12.2022, 12/04).

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