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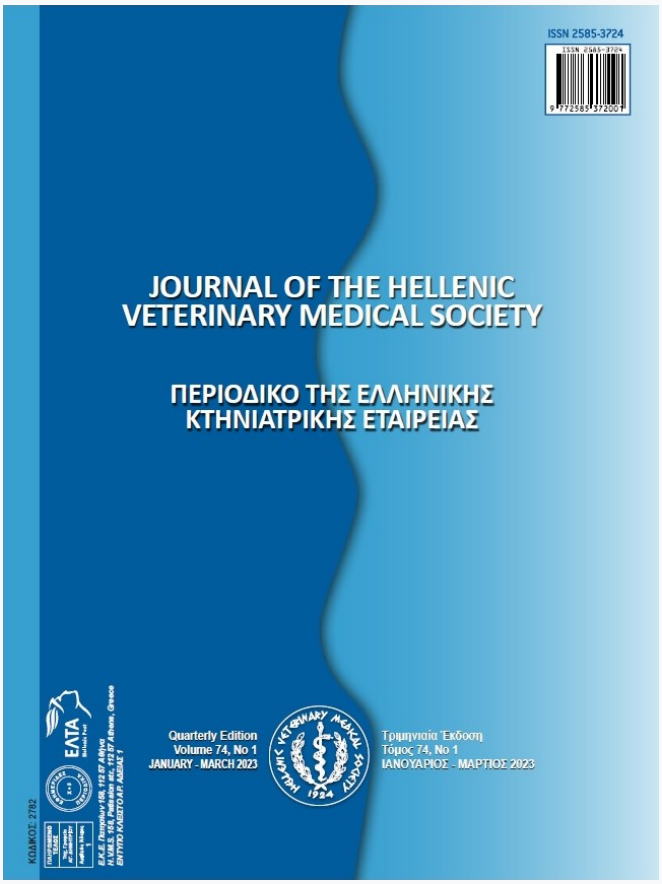

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Research article
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Effect of levothyroxine administration on oxidative stress and cytokine levels in rats with experimental hypothyroidism

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ABSTRACT: In this study, it was aimed to determine the effects of levothyroxine (L-T_4) administration on serum free triiodothyronine (fT_3), free thyroxine (fT_4), thyroid stimulating hormone (TSH) and serum total antioxidant capacity (TAC), serum total oxidant capacity (TOC), oxidative stress index (OSI) and superoxide dismutase (SOD) activity, liver malondialdehyde (MDA) and the effects of cytokines on tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) levels in rats with experimental hypothyroidism. In the study, 48, 39-day-old, male Wistar albino rats were randomly divided into 3 groups containing 16 rats in each as control, hypothyroid and levothyroxine (L-T_4) treated hypothyroid group. The rats in the control group were injected with 1 mL of saline and the rats in the hypothyroid group were injected via intraperitoneal (i.p.) route with 6-n-propyl thiouracil (PTU) at a dose of 10 mg/kg/day for 28 days. In the levothyroxine treated group, after induction of hypothyroidism 5 µg of levothyroxine diluted in 1 mL of sodium chloride (NaCl) administered via i.p. route for 15 days. At the end of the study, serum and liver samples were taken from the rats. In hypothyroidism group, serum fT_3, fT_4 and TAK levels as well as SOD ($P<0.001$) activity decreased statistically compared to the control group while serum TSH, TOC, OSI ($P<0.001$), liver MDA ($P<0.01$), TNF-α and IL-6 ($P<0.001$) levels increased significantly. The decreased serum fT_3, fT_4, TAC levels and SOD ($P<0.001$) activity in the hypothyroidism group increased with levothyroxine administration whereas the increased serum TSH, TOC, OSI and tissue MDA ($P<0.01$), TNF-α and IL-6 ($P<0.001$) levels were found to be decreased and approached to control levels. In conclusion, it has been shown that levothyroxine administration is effective against PTU-induced hypothyroidism in rats, inhibits lipid peroxidation and increases antioxidant status, minimizes the levels of inflammatory markers and protects against PTU-induced liver damage in rats. Because, the potential therapeutic use of levothyroxine in the treatment of hypothyroidism is thought to be appropriate.

Key words: Cytokine; hypothyroidism; levothyroxine; oxidative stress; rat.

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INTRODUCTION

Hypothyroidism is a condition that may result from insufficient production of thyroid hormones by the thyroid gland, or as a result of insufficient thyroid stimulating hormone (TSH) stimulation. Hypothyroidism that occurs due to the insufficiency of the thyroid gland is called primary hypothyroidism, and hypothyroidism that occurs due to insufficient TSH stimulation of the hypothalamus or pituitary gland is called secondary hypothyroidism (Özyardımcı Ersoy 2014; Derhem, 2019).

It has been reported that thyroid hormones regulate the oxidant/antioxidant balance in cells, and that excessive secretion of thyroid hormones leads to the formation of free radicals (Sajadian et al., 2016; Valcheva -Traykova and Bocheva, 2016; AbdelWahhab et al., 2019). When the energy requirements and the oxidant accumulations of the cells in hyperthyroidism were observed, it was determined that these facts play an important role in intracellular oxidative stress (Dariyerli et al., 2004). In addition, conflicting results indicating the effect of thyroid hormone therapy on cytokines were reported (Díez et al., 2002; Aksoy et al., 2013, Muthu et al., 2018; Zhou et al., 2018; Abdel-Wahab et al, 2019).

Hypothyroidism, which is a common endocrine system disease, is treated with levothyroxine, except in exceptional cases (Güngör et al., 2013). Although levothyroxine is a widely used drug in thyroid hormone replacement therapy, there is still no consensus on appropriate treatment protocols for hypothyroidism (Kılınç et al., 2015). In light of this knowledge, it has been known that the positive effects of levothyroxine administration to rats with hypothyroid has positive effects on serum free triiodothyronine (fT3), free tetraiodothyronine (fT4), TSH (Aydın et al., 2010; Wu et al., 2011; Ye et al., 2017; Głombik et al., 2021), total antioxidant capacity (TAC) (Salama et al., 2013; Ates et al., 2016; Muthu et al., 2018; Salami et al., 2019) levels, serum superoxide dismutase (SOD) (Rousset et al., 2004; Jena et al., 2012; Pan et al., 2013; Hosny et al., 2021; Panda et al., 2021) activities and tissue malondialdehyde (MDA) levels (Salama et al., 2013; Muthu et al., 2018; Abdel-Wahab et al., 2019; Salami et al., 2019; Panda et al., 2021). However, a limited number of studies have been found showing the effect of levothyroxine administration on serum total oxidant capacity (TOC), oxidative stress index (OSI) (Ates et al., 2016) and tissue cytokine (Chaalal et al., 2014; Hosny et al., 2021; Panda et al., 2021) levels in rats with hypothyroid. In this study, the effects of levothyroxine administration on serum fT_3, fT_4, TSH, TAC, TOC, OSI, SOD activity and tissue MDA, tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) levels in rats with experimental hypothyroidism were investigated in order to compensate for the lacking in this area and, it is thought that the obtained findings may contribute to the future studies on this subject.

MATERIAL AND METHODS

Animals and experimental design

Approval for the study was obtained from the Erciyes University Local Ethics Committee for Animal Experiments (ERÜ HADYEK) (dated 28.08.2019 and decision no: 19/148). For the study, 48, 39-day-old male Wistar albino rats obtained from Erciyes University Experimental Research and Application Center (ERÜ DEKAM) were used as the material. The rats were kept in polycarbonate cages (3-4 rats in each cage) on rough sawdust litter, under conventional animal housing conditions provided by the research center [controlled temperature (21±2°C), humidity (50±5%), air exchange (12 cycles per hour), light (12 hours light, 12 hours dark)]. The rats were randomly divided into 3 groups with 16 rats in each: the control group, the experimental hypothyroidism group, and the experimental hypothyroidism group treated with levothyroxine. During the experiments, standard rat chow was supplied *ad libitum* and tap water was supplied without restriction to the rats in all groups. A 500 µl of saline solution containing 1 ml of 0.1 N NaOH in 100 ml was injected via i.p route to each animal in the control group for 28 days. For 28 days, 6-n-propyl thiouracil (PTU, Sigma-Aldrich, St Louis, MO, USA), a thiolated uracil-derived anti-hyperthyroid drug that inhibits the conversion of thyroxine (T_4) to triiodothyronine (T_3) was injected via i.p route at 10 mg/kg body weight/day to the rats in the experimental hypothyroidism group after being dissolved and prepared in 0.1 NaOH. In the hypothyroidism group treated with levothyroxine, hypothyroidism was first induced in the animals. Subsequently, 5 µg of levothyroxine diluted in 1 ml of NaCl was injected to each animal via i.p. route for 15 days (Tahmez et al., 2000).

Sample collection and Biochemical analysis

At the end of the experiment, approximately of 3-4 ml of blood was taken from the hearts of the animals, after 12 hours fasting, by the puncture method into

tubes without anticoagulant. After the blood samples were kept at room temperature for approximately 45 minutes, they were centrifuged at 3000 rpm for 10 minutes and their sera were separated. The animals' liver tissues were taken at the end of the experiment. All the samples were kept at -80°C in until the fT_3, fT_4, TSH, TAC, TOC levels and SOD activities were determined in the serum samples and MDA, TNF-α and IL-6 levels were determined in the liver samples.

Serum fT_3 (Elabscience, cat. no: E-EL-0079), fT_4 (Elabscience, cat. no: E-EL-0122), TSH (Elabscience, cat. no: E-EL-R0976), TAC (Rel Assay, cat. no: RL0017), TOC (Rel Assay, cat. no: RL0024) levels and SOD (Rel Assay, cat. no: RLD0123) activities were determined and liver TNF-α (Elabscience, cat. no: E-EL-R0019) and IL-6 (Elabscience, cat. no: E-EL-R0015) levels were determined by enzyme linked immune sorbent assay (ELISA) according to the instruction of the manufacturers of the commercially available kits using an ELISA reader (μQuant, Bio-Tec, ELx50, USA). Liver MDA levels were determined with spectrophotometer (Shimadzu UV Model 1208) according to Janero (1990) method.

Preparation of liver tissue homogenates

The liver tissue taken for MDA and cytokine analyses was cleaned of blood and similar residues with distilled water, washed with cold 0.9% NaCl and dried with blotting paper. The dried tissues were wrapped in aluminum foils and stored at -80°C. During preparation for the analysis, the liver tissue was weighed to be approximately 0.1 g on a precision balance and a 1/10 phosphate buffer (0.9 mL) was added to it. It was first grounded with a glass homogenizer. The homogenate transferred into tubes. Tubes containing homogenate were centrifuged at 7.000 rpm at +4°C for 15 minutes, and the supernatants obtained were stored at -80°C until MDA, TNF-α and IL-6 analyses (Panda et al., 2012).

Statistical analysis

Statistical analysis of the data was done with the SPSS 20.0 package program for Microsoft. The difference between the groups was determined by one-way analysis of variance (ANOVA). When the F-score was significant, Duncan's multiple range test was used to determine which group originated the difference. All data was expressed as means ± standart error mean (SEM). Differences between groups were considered statistically significant at $P<0.05$.

RESULTS

fT_3, fT_4 and TSH levels: Compared to the control group, serum fT_3 ($P<0.001$) and fT_4 ($P<0.001$) levels in the hypothyroid group showed a statistically significant decrease, while serum TSH ($P<0.001$) levels showed a statistically significant increase. Administration of levothyroxine to the hypothyroid group reversed the decreased fT_3 and fT_4 levels and the increased TSH levels due to hypothyroidism to the levels of the animals in control (Table 1).

Levels of oxidant / antioxidant parameters: Liver MDA ($P<0.01$), serum TOC ($P<0.001$) and OSI ($P<0.001$) levels showed a statistically significant increase in the hypothyroid group compared to the control group. Levothyroxine administration to the hypothyroidism group reduced the increased liver MDA, serum TOC and OSI levels returning them closer to the values of the control group (Table 1).

Table 1. Serum fT_3, fT_4, TSH, TAC, TOC, OSI levels and SOD activity; tissue MDA, TNF-α and IL-6 levels in the control group and rats treated with PTU, PTU+L-T_4 (Mean±SEM).

	Parameters	Control n:16	PTU n:16	PTU+L-T_4 n:16	P
Serum	fT_3 (pg/ml)	4.38 ±0.16^b	0.94±0.13^a	4.01±0.11^b	***
	fT_4 (pg/ml)	42.70 ± 1.78^b	10.57 ± 1.23^a	40.79 ± 2.05^b	***
	TSH (pg/ml)	16.44 ± 0.81^a	42.465 ±1.83^c	28.64 ± 1.23^b	***
	SOD (U/ml)	34.65 ± 1.36^c	10.27 ± 1.43^a	22.04 ± 2.12^b	***
	TAC (mmol/L)	1.33±0.05^b	0.91 ± 0.02^a	1.24 ±0.02^b	***
	TOC (μmol/L)	7.76 ± 0.44^a	12.32 ±0.74^b	9.59 ± 0.84^a	***
	OSI	0.59 ± 0.04^a	1.35 ± 0.08^b	0.77 ± 0.07^a	***
Tissue	MDA (nmol/L)	4.65 ± 0.35^a	6.82 ± 0.55^b	5.45 ± 0.42^a	**
	TNF-α (pg/ml)	4114.78 ± 150.65^a	5178.58 ±88.86^c	4667.73 ± 190.52^b	***
	IL-6 (pg/ml)	745.2549 ± 16.57^a	929.85 ±19.24^c	843.17 ±83.87^b	***

**: $P<0.01$ ***: $P<0.001$

$^{a-c}$: The difference between values with different superscripts in the same raw is significant

Compared to the control group, serum SOD activity (P<0.001) and TAC (P<0.001) levels showed a statistically significant decrease in the hypothyroid group. Administration of levothyroxine to the hypothyroid rats increased serum SOD activity and T AC levels, bringing them closer to the control group levels (Table 1).

Cytokine levels: Compared to the control group, liver TNF-α and IL-6 levels showed a statistically significant increase in the hypothyroid group. With the administration of levothyroxine to the hypothyroid group, the increased liver TNF-α and IL-6 levels decreased significantly, approaching the values of the control group (Table 1).

DISCUSSION

It is known that thyroid hormones play an important role both in the normal growth and development of cells and in the regulation of protein, fat and carbohydrate metabolisms in adults (Rosen et al., 2021). Hypothyroidism may occur as a result of insufficient production of thyroid hormones by the thyroid gland or insufficient TSH stimulation (Özyardımcı Ersoy 2014; Derhem, 2019).

Various drugs (Levothyroxine (L-thyroxine), 6-n-propyl thiouracil (PTU), methimazole) are used in the experimental inducement of hypothyroidism and hyperthyroidism (Kumar et al., 2014; Abdel-Wahab et al., 2019; Ragone et al., 2020). The most commonly used of these are L-thyroxine and PTU. Since levothyroxine is effective in lowering TSH, it is used to treat primary hypothyroidism (Emerson, 2018). On the other hand 6-n-propyl thiouracil is known as a thioamide-derived drug that treats hyperthyroidism by increasing the secretion of thyroid hormones by the thyroid gland and inhibiting the 5'deiodinase enzyme, which converts T_4 to active T_3 (Nakamura et al., 2007). Therefore, PTU inhibits the synthesis of thyroid hormones, specifically, the conversion of T_4 to T_3 (Moriyama et al., 2007). Various researchers have shown that PTU causes primary hypothyroidism (Abdel-Wahab et al., 2019; Salami et al., 2019; Şahin et al., 2019; Głombik et al., 2021; Panda et al., 2021). In the presented study, it was also shown by measuring the TSH level in addition to fT_3 and fT_4 in the blood that a hypothyroidism model was formed. The fT_3 and fT_4 levels decreased significantly in animals treated with PTU, while TSH concentration increased as indicated several in previous studies by Ye et al., (2017); Abdel Wahhab et al., (2019); Şahin et al., (2019); Głombik et al., (2021); Panda et al. (2021). These changes are accepted as the most important findings showing the formation of hypothyroidism.

Aydın et al. (2010) found that 10 mg/kg body weight/day PTU administration to Wistar albino rats by gavage decreased fT_3 and fT_4 levels and increased TSH levels. They determined that administration of 1.5 mg/kg/mL levothyroxine to rats increased fT_3, fT_4 levels, decreased TSH levels and brought them back to normal levels.

Ye et al. (2017) reported that when Sprague-Dawley rats administered 2 µg of levothyroxine by gavage after adding 0.05% body weight/volume PTU to their drinking water, significantly decreased fT_3 and fT_4 levels with hypothyroidism were increased, and the elevated TSH level was decreased significantly. In a similar study on male Wistar Kyoto rats by Głombik et al. (2021) who induced hypothyroidism with 0.05% PTU in drinking water, 1.5 mg/kg/mL levothyroxine reversed the changes in fT_3 and fT_4 as well as TSH levels. In another study by Wu et al. (2011) who added 0.2% body weight/volume PTU to the drinking water of Wistar albino rats, significant increases in fT_3 and fT_4 and reduction in TSH levels due to administration of different doses of levothyroxine (20 µg/kg, 50 µg/kg and 100 µg/kg, i.p.) were reported. In this study, the decreased fT_3 and fT_4 levels with the inducement of hypothyroidism with 10 mg/kg body weight/day PTU in Wistar albino rats significantly increased while the increased TSH level with the inducement of hypothyroidism was decreased significantly with administration of 5 µg levothyroxine via i.p. route bringing them to normal levels.

Thyroid hormones such as thyroxine (T_4) and triiodothyronine (T_3) are reported to play a very important role in basal cellular metabolic rate and are considered to be the main regulators of energy metabolism, mitochondrial activity and oxygen consumption (Martinez et al., 2001; Mullur et al., 2014). In hypothyroidism, which is a common endocrine system disease, it is known that the metabolic rate slows down due to decreased hormone levels. It has been reported that slowed basal metabolism in hypothyroidism causes changes in reactive oxygen and nitrogen species and antioxidant defense system in humans and different living species (Venditti and Di Meo, 2006). In several previous studies, it has been emphasized that changes in thyroid hormone levels have significant effects on different organs (Faraji et al, 2016; Monnereau et al., 2013; Obradovic et al.,

2016) including liver, heart, skeletal muscles and brain and can induce oxidative stress (Baghcheghi et al., 2017; Beheshti et al., 2017). On the other hand, it was reported that in case of weakening of the antioxidant defense system, oxidative stress may increase thus oxidative damage may occur in lipid, protein and DNA (Kalyanaraman, 2013).

The level of malondialdehyde (MDA) is served as a reliable biomarker of lipid peroxidation (LPO) and usually served as a marker of LPO (Acaröz et al., 2018). In a study conducted with patients with hypothyroidism, it was reported that an increase in MDA values was observed whereas there was no change in SOD activity (Erdamar et al., 2008). On the other hand, Torun et al. (2009) found that MDA levels increased in the plasma and hippocampus tissues of hypothyroid patients and the activity of SOD, which is the main enzyme that plays a role in converting superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2), decreased. In studies where hypothyroidism was experimentally induced by adding PTU to the drinking water of rats, it was reported that serum, plasma and tissue (brain, kidney, liver and hippocampus) MDA levels increased in the hypothyroidism group (Pan et al., 2013; Salama et al., 2013; Muthu et al., 2018; Abdel-Wahab et al., 2019; Salami et al., 2019). In another study, it was found that the thiobarbituric acid (TBARS) level increased in the kidneys of male Wistar rats with hypothyroidism induced by the addition of 0.05% PTU to drinking water, while the mRNA expressions of SOD1 and SOD2 decreased (Jena et al., 2012).

It has been reported that the administration of various levels of levothyroxine to hypothyroidism induced rats with the addition of 0.05% PTU to drinking water decreased the elevated serum (Pan et al., 2013), liver (Panda et al., 2021), cerebral cortex (Hosny et al., 2021), hippocampus (Pan et al., 2013; Hosny et al., 2021) MDA levels and increased the reduced SOD activity. The primary cellular antioxidant enzymes which are considered as essential for life are SOD, catalase (CAT) and glutathione peroxidase (GPx), and since these enzymes are responsible for the detoxification of different types of reactive oxygen species (ROS), measuring their activities generally reflects the oxidation state (McCord et al., 1971; Arslan et al., 2021).

It has been reported that in ROS-mediated oxidative damage, generally, the SOD O_2^- is converted to H_2O_2, which is then detoxified by CAT and GPx (Paital, 2018). In the presented study, a significant reduction was also determined with levothyroxine application in the increased tissue MDA level with PTU application whereas an elevation was detected in the decreased serum SOD due to hypothyroidism. These findings suggest that the imbalance between the oxidant and antioxidant system may be a leading factor in the increased oxidative stress in hypothyroidism, and that levothyroxine administration may improve the oxidative stress marker to normal levels, thus increasing the antioxidant activities.

In addition to the regulating metabolism, thyroid hormones are also known to be effective in antioxidant enzyme synthesis and degradation (Costantini et al., 1998; Güngör et al., 2013). In hypothyroidism induced rats with 0.05% body weight/volume PTU in drinking water, serum TAC levels increased significantly (Muthu et al., 2018) but decreases were reported in liver and kidney TAC levels by Salama et al. (2013), and an increase in plasma MDA/TAC ratio was determined by Muthu et al (2018). In a study conducted on the 5th, 10th and 20th days after birth in the offspring of pregnant Wistar albino rats with experimental hypothyroidism by administration of 100 mg/L PTU in drinking water, it was reported that brain total antioxidant levels did not show any variation (Salami et al., 2019). In a study conducted in patients with hypothyroidism, no difference was determined in serum TAC levels by Torun et al. (2009). However, Ateş et al., (2016) reported decreases in serum TAC level and increases in TOC and OSI levels in patients with hypothyroidism. In addition, administration of levothyroxine to patients with hypothyroidism increases the reduced TAC levels and reverses the increased TOC and OSI values.

It has been emphasized that the high level of oxidative stress in hypothyroidism may be due to various reasons, and the most important reason may be chronic inflammation. It is thought that T and B lymphocytes play an active role in the pathogenesis of hypothyroidism and may cause an increase in reactive oxygen radicals by activating the NADPH oxidase (NOX) enzyme (Jackson et al., 2004; Bedard and Krause, 2007, Brieger et al., 2012). Indeed, this has been demonstrated in wild-type mice by demonstrating an increase in H_2O_2 and $O_2^{-\cdot}$ in T cells through stimulation of T cell receptors by antigens or mitogens (Devadas et al., 2002). In addition, since it is known that the excess of TSH hormone directly triggers oxidative stress in hypothyroidism, it is empha-

sized that the increased oxidant level in hypothyroidism may be related to the increase in TSH (Carmeli et al., 2008; Haribabu et al., 2013). However, it has been suggested that another reason for high oxidative stress in hypothyroidism may be the deficiency of thyroid hormones (Baser et al. 2014).

Thyroid hormones contribute to free oxygen radical scavenging by increasing non-enzymatic antioxidant molecules (He et al., 2017) that affect antioxidant enzyme levels such as SOD, CAT and GSPx (Fernandez et al., 1988) and may cause stimulation of mitochondrial non-degrading proteins, which are non-enzymatic antioxidant molecules (Rousset et al., 2004, Babu et al., 2011).

It has been reported that high oxidative stress in hypothyroidism may be due to excessive iodine intake and autoimmune response, and H_2O_2, an oxidant radical, is required for iodide oxidation during thyroid hormone synthesis in thyroid epithelial cells. It is stated that high iodine intake causes excessive H_2O_2 production and in this case, oxidant radicals begin to rise in the body (Burek and Rose, 2008). Excessive autoimmune response is also thought to increase oxidative stress by exaggerating inflammation or increase tissue damage, resulting in decreased thyroid hormone synthesis.

The relationship between hypothyroidism and plasma proinflammatory markers such as TNF-α and C-reactive protein (CRP) has been shown in some studies (Tuzcu et al., 2005; Dizdarevic-Bostandic et al., 2013; Hajje et al., 2014; Panda et al, 2021). However, conflicting results concerning the effect of thyroid hormone therapy on these markers have been reported in several other studies (Díez et al., 2002; Aksoy et al., 2013). In PTU-induced hypothyroid rats, increases in serum or plasma (Rizos et al., 2011; Muthu et al., 2018; Zhou et al., 2018) and hippocampus (Chaalal et al., 2014) TNF-α and IL-6 (Chaalal et al., 2014, Rizos et al., 2011) indicating an inflammatory state that may be due to oxidative stress (Zhou et al., 2018; Abdel-Wahab et al, 2019). In a study conducted on hypothyroidism induced Wistar rats, plasma TNF-α and IL-6 levels were significantly increased and 6 mg/ml levothyroxine administration resulted in further increases (Hajje et al., 2014). In another study conducted on hypothyroidism induced Wistar rats, liver TNF-α and IL-6 levels increased significantly, and levothyroxine (0.1 mg/kg/day, i.p.) administration had a reducing effect on cytokine levels (Panda et al., 2021). In contrast, in a study conducted on rats, it was found that treatment with levothyroxine could not restore the increase in hippocampal TNF-α level induced by PTU (Hosny et al., 2021).

The findings of the presented study clearly show that inflammatory markers are induced in hypothyroid rats; this inflammatory state was significantly reduced with levothyroxine treatment. The fact that levothyroxine administration reduces both TNF-α and IL-6 levels in hypothyroid rats suggests that levothyroxine may have an anti-inflammatory effect.

CONCLUSION

In this study, it has been revealed that 5 μg levothyroxine is effective against PTU-induced hypothyroidism in rats, inhibits lipid peroxidation and increases antioxidant status, and can protect against PTU-induced liver damage in rats by minimizing the levels of inflammatory markers. The findings obtained in the presented study suggest that the potential therapeutic use of levothyroxine in the treatment of hypothyroidism may be appropriate and may contribute to new studies to be conducted.

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CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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