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Modulatory effects of experimentally induced hyperthyroidism and melatonin on thymus gland immune function in male rat

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ABSTRACT

The current study aimed to investigate how L-thyroxine-induced hyperthyroidism and melatonin affect the immune system. The rats were divided into four groups, each with ten rats. The rats received the following treatments for four weeks: group I, control group; group II, melatonin group, treated with melatonin; group III, hyperthyroid group, treated with L-thyroxine; and group IV, hyperthyroid + melatonin group, treated with L-thyroxine and melatonin. Serum total T3 and T4 levels, $CD4^+$ and $CD8^+$ T lymphocytes analysis using flow cytometry, and relative gene expression of TNF-α, IL-10, IL-2, and IFN-γ were evaluated. The results revealed that hyperthyroidism substantially increased $CD4^+$ T cells while not affecting $CD8^+$ T cells percentage. Moreover, TNF-α, IL-2, and IFN-γ expressions were significantly upregulated in hyperthyroid rats, whereas IL-10 was not significantly altered. Melatonin administration to hyperthyroid rats significantly decreased thyroid hormone levels, increased $CD4^+$ and $CD8^+$ T cells percentage, upregulated IL-10, IL-2, and IFN-γ gene expression, and downregulated TNF-α gene expression. When compared to controls, the melatonin group exhibited a substantially higher percentage of $CD4^+$ and $CD8^+$ T cells and elevated levels of IL-10, IL-2, and IFN-γ gene expression; however, TNF-α gene expression had not changed appreciably. Therefore, it might be concluded that L-thyroxine and melatonin induce T lymphocyte proliferation and that melatonin also has anti-inflammatory properties.

1. INTRODUCTION

Hormones and cytokines are the main mediators of the intricate cross-talk between the endocrine and immune systems, which is carried out in both directions. In this context, numerous studies have demonstrated that thyroid hormones (TH) regulate immune cell development, proliferation, and function, proving the crucial role played by thyroid hormones in the immune system (Montesinos and Pellizas, 2019). The primary indication of thyroid hormone interactions with the immune system is through the existence of particular TH receptors on lymphocytes or through the changes in the immune system that occur due to fluctuations in TH levels (Arcos et al., 2010).

Hyperthyroidism is an abnormally high concentration of thyroid hormones in the tissues that result from increased thyroid hormone synthesis, excessive release of preformed thyroid hormones, or an extrathyroidal source, either endogenous or exogenous (Kumar et al., 2020). Increased metabolism and disturbance of other regular physiological system processes are characteristics of hyperthyroidism (Laskar and Singh, 2020). Previous studies have indicated that elevated levels of TH can exert an impact on the immune system. Hyperthyroidism is frequently associated with imbalanced immune responses, which include atypical antibody production (either boosted or reduced), increased polymorphonuclear leukocyte migration, increased proliferation of lymphocytes, and an increase in the generation of reactive oxygen species by macrophages, particularly hydrogen peroxide, and superoxide (Jara et al., 2017). Additionally, hyperthyroidism may result in reduced levels of antioxidant enzymes and an increase in pro-inflammatory markers (Zhao et al., 2020**;** Ashry et al., 2023).

Melatonin is a neurohormone mainly synthesized by the pineal gland. Also, it is produced by other tissues and organs, such as the retina, skin, and bone marrow (Kong et al., 2020)**.** Melatonin regulates a wide range of physiological processes**,** including circadian rhythms, reproduction, and immune responses (Anadón et al., 2021). Several studies conducted on melatonin have provided evidence for its antioxidative, anti-inflammatory, and immunomodulatory properties (Chitimus et al., 2020**;** Ahmad et al., 2023). Melatonin has the potential to regulate a variety of molecular pathways involved in processes such as proliferation, apoptosis, autophagy, metastasis, and inflammation in various pathological conditions (Zhao et al., 2019). In addition, research indicates that melatonin has a crucial role in modulating innate and adaptive immune reactions and maintaining the equilibrium of cytokines produced by T-helper 1 and T-helper 2 cells (Lin et al., 2017). The immunoregulatory activities of melatonin are partly attributed to its interaction with melatonin receptors present in immune cells. The enhancement of humoral and cellular

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immunity triggered by melatonin in mice is mediated through the melatonin receptors MT1 and MT2 (Cardinali and Esquifino, 2012). The current study aimed to investigate how L-thyroxine-induced hyperthyroidism and melatonin affect immune system function. This was done via analyzing the percentage of $CD4^+$ and $CD8^+$ T lymphocytes and the relative gene expression of TNF-α, IL-10, IL-2, and IFN-γ.

2. MATERIAL AND METHODS

2.1. Experimental animals

Forty male Sprague Dawley albino rats weighing 100-120 g were obtained from Vacsera, Egypt. Rats were maintained under standard laboratory conditions at 25 ± 2°C, with 50 ± 5% humidity, and a 12/12 h light/dark cycle. They were fed a regular pellet diet and received water *ad libitum*. All animal treatments were approved by the ethics committee of the Faculty of Veterinary Medicine, Benha University (BUFVTM16-03-23). The experiments were conducted after acclimatization for one week.

2.2. Chemicals

L-thyroxine (T4, Cat. no. T2376) was purchased from Sigma-Aldrich, Germany. T4 was reconstituted by solubilizing 50 mg in 1 mL 4M ammonia solution in methanol (779423, Sigma-Aldrich, Switzerland) per the manufacturer's recommendation. The T4 stock solution was stored at − 20 °C. T4 stock aliquots were diluted to produce the final concentrations at the time of administration. Melatonin was obtained from Nerhadou International Company. Melatonin was prepared daily by dissolving it in 100% ethanol (50 mg/ml) before diluting it with saline. A final ethanol concentration of less than 1% was achieved (Sirichoat et al., 2019).

2.3. Experimental design

Rats were assigned to four groups of ten rats each and treated as follows:

Group I, Control (Con): Rats were injected intraperitoneally with saline daily for 4 weeks.

Group II, Melatonin (Mel): Rats were injected IP with 10 mg/kg melatonin every day for 4 weeks (Luo et al., 2020).

Group III, Hyperthyroid (Hyper): Hyperthyroidism was induced in rats by daily IP injection with 250 µg/kg L-thyroxine for 4 weeks (Ayala et al., 2019).

Group IV, Hyperthyroid + Melatonin (Hyper+ Mel): Rats were treated as group III and received IP injection of 10 mg/kg melatonin every day for 4 weeks.

2.4. Blood and tissue samples

Rats were anesthetized with isoflurane and two blood samples were obtained from the retro-orbital venous plexus. The first sample was collected in EDTA tubes for flow cytometric analysis. The second sample was collected without anticoagulant and kept at room temperature for serum separation. The serum was stored at −20 °C until used in biochemical analysis. After cervical dislocation, the spleen was dissected and kept frozen at −80 ◦C until RNA extraction.

2.5. Measurement of thyroid hormones

The levels of serum total T3 and T4 were assessed using electro-chemiluminescent immunoassay (ECLIA) tests with a Cobas® analyzer (Elecsys, E170, Roche Diagnostics, Mannheim, Germany) as described previously by Yu et al. (2018).

2.6. Flow cytometry analysis of $CD4^+$ and $CD8^+$ T cells

Flow cytometry was done using a Beckman-Coulter Navios EX (Inc., USA) instrument for the measurement of $CD4^+$ and $CD8^+$ T lymphocytes. The cells were labeled with mouse anti-rat CD4 (Thermo Fisher, Germany, cat. no. MA5-44019) and mouse anti-rat CD8 (Thermo Fisher, Germany, Cat. no. PA5-79011) as primary monoclonal antibodies conjugated to mouse secondary antibody labeled with Alexa Fluor 488 fluorescent dye according to Pockley et al. (2015). Briefly, Blood was incubated with anti-CD4 and anti-CD8 monoclonal antibodies in binding buffer for 20 minutes, then washed with phosphate buffer saline and labeled with Alexa Fluor labeled secondary antibody. The cells were placed in the dark for 20 minutes. Following this incubation, 200 µL of red blood cell lysis buffer was added for 10 minutes, and samples were centrifuged at 500 ×g for 5 min. After removing the supernatants, the cells were washed twice with 2 mL FACS buffer, and the centrifuge procedure was repeated. Samples were then resuspended in FACS buffer and analyzed by flow cytometry. Navios EX software was used to analyze flow cytometry data. Logarithmic dot plots are used to display the lymphocyte population, and CD4 and CD8 positive cells were gated.

2.7. Real time PCR

Total RNA was extracted from the spleen using RNeasy Mini Kit (Qiagen, Germany, Cat. no. 74104) following the manufacturer's directions. The RNA was reverse transcribed into cDNA using RevertAid Reverse Transcriptase kit (200 U/µL) (Thermo Fisher, USA, Cat. no. EP0441) as per the manufacturer's guidelines. The primer sequences are presented in Table 1. Real-time PCR was conducted in a Stratagene MX3005 P Real-time PCR system utilizing the Quantitect SYBR Green PCR kit (Qiagen, Germany, Cat. no. 204141). Amplification curves and Ct values were obtained using Stratagene MX3005P software. The variance of gene expression on RNAs from the various samples was estimated by comparing the Ct from each sample to that from the control group using the "ΔΔCt" approach (Livak and Schmittgen, 2001) using the following ratio: (2-ΔΔct).

Whereas ΔΔCt = ΔCt gene of interest – ΔCt Control

ΔCt gene of interest = $Ct_{\text{target}} - Ct_{\text{β-actin}}$ and ΔCt Control = $Ct_{\text{target}} - Ct_{\text{β-actin}}$

Table 1 Primer sequence for Real Time PCR

Gene	Primer sequence (5' to 3')	
	Forward	Reverse
ß-actin	TCCTCCTGAGCGCAAGTACTCT	GCTCAGTAACAGTCCGCCTAGAA
TNF-α	CACCAGCTCTGAACAGATCATGA	TCAGCCCATCTTCTTCCAGATGGT
IL-10	GCGGCTGAGGCGCTGTCAT	CGCCTTGTAGACACCTTGGTCTTGG
IL-2	CCTGAGCAGGATGGAGAATTACA	TCCAGAACATGCCGCAGAG
IFN-γ	AACGCTACACACTGCATCTTGG	GACTTCAAAGAGTCTGAGG

2.8 Statistical analysis

All statistical analyses were processed through IBM SPSS Statistics Version 26. Statistically significant differences among groups were assessed using one-way ANOVA, with the Duncan test serving as a post-hoc test (Sarma and Vardhan, 2019). The results are presented as means ± SE, with significance considered at $P < 0.05$. The figures were drawn with GraphPad Prism software version 9.4.0.

3. RESULTS

3.1. Effect of hyperthyroidism and melatonin on serum thyroid hormones

The results indicated that the hyperthyroid group exhibited significantly ($p< 0.05$) elevated levels of tT3 and tT4 compared to the control group. A remarkable decrease in tT3 and tT4 levels was recorded in the hyperthyroid + melatonin

group compared with the hyperthyroid. However, no remarkable variation was detected between the melatonin and control groups (Fig. 1.).

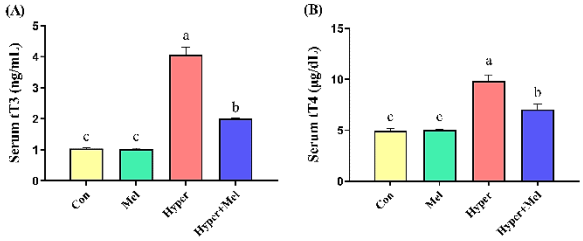

Fig. 1. Effect of hyperthyroidism and melatonin on serum tT3 (A) and tT4 (B) levels. Values are expressed as mean ± S.E.M. Values within the same column carrying different letters are significantly different at (p< 0.05).

3.2. Effect of hyperthyroidism and melatonin on CD4⁺ and CD8⁺ T cells percentage

As presented in Fig. 2., hyperthyroidism substantially (p < 0.05) increased $CD4^+$ T cells, while $CD8^+$ T cells did not differ appreciably from controls. The hyperthyroid + melatonin group exhibited a substantial boost in $CD4^+$ T cells compared to the hyperthyroid group. In addition, melatonin-treated rats had significantly higher $CD4^+$ and $CD8^+$ T cell percentages than controls.

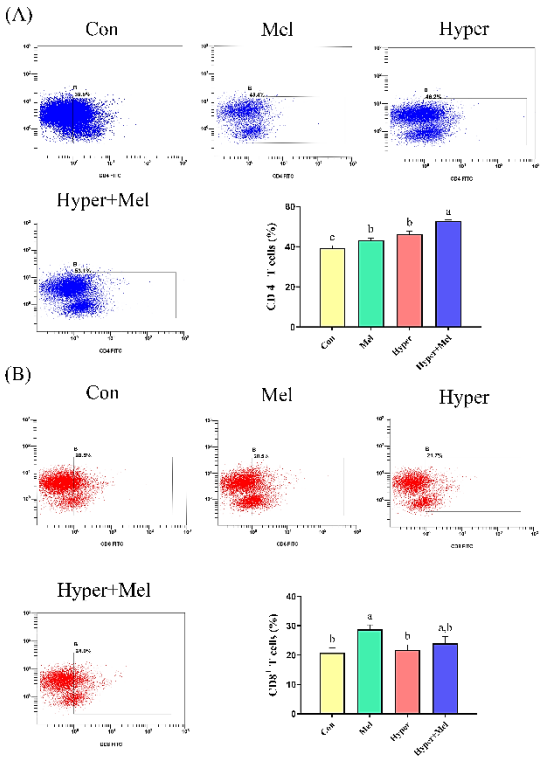

Fig. 2. Effect of hyperthyroidism and melatonin on the percentage of $CD4^+$ (A) and $CD8^+$ (B) T cells. Values are expressed as mean ± S.E.M. Values within the same column carrying different letters are significantly different at (p< 0.05).

3.3. Effect of hyperthyroidism and melatonin on relative gene expression of cytokines

The obtained data revealed a substantial (P< 0.05) upregulation of TNF-α expression in hyperthyroid rats compared with controls. Significant downregulation of TNF-α gene expression was detected in hyperthyroid rats co-administered with melatonin compared to hyperthyroid rats. However, no appreciable variation in TNF-α gene expression was demonstrated between the melatonin and control groups. Regarding IL-10, its gene expression showed no significant (p < 0.05) difference change in the hyperthyroid group compared to the control. IL-10 gene expression showed significant upregulation in the hyperthyroid+ melatonin group compared with the hyperthyroid. Also, IL-10 gene expression showed significant upregulation in melatonin-administered rats compared to control rats. IL-2 and IFN-γ gene expression showed significant (p < 0.05) upregulation in the hyperthyroid and melatonin groups compared to the control. Also, IL-2 and IFN-γ gene expressions showed significant upregulation in the hyperthyroid + melatonin group compared to the hyperthyroid (Fig. 3.)

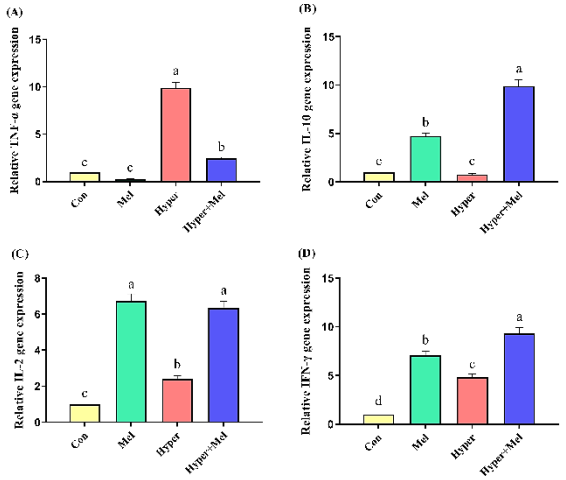

Fig. 3. Effect of hyperthyroidism and melatonin on TNF-α (A), IL-10 (B), IL-2 (C) and IFN-γ (D) relative gene expression. Values are expressed as mean ± S.E.M. Values within the same column carrying different letters are significantly different at (p < 0.05).

4. DISCUSSION

In recent decades, the interrelationship between melatonin and the thyroid gland has been a topic of research interest. Thyroid hormones and melatonin play a crucial role in immune function regulation (Laskar and Singh, 2018). In the present research, hyperthyroidism induction in rats was successful as evidenced by the substantial rise in tT3 and tT4 serum levels in hyperthyroid rats compared with controls. Melatonin co-administration significantly reduced thyroid hormone levels in hyperthyroid rats. This is in agreement with the findings reported by Laskar and Singh (2018). The mechanism driving the inhibitory impact of melatonin on thyroid hormones level during hyperthyroidism may involve pituitary-thyroid axis regulation. The anterior pituitary gland produces thyroid stimulating hormone (TSH), which triggers thyroid hormone production and secretion by activating the TSH receptor on thyroid epithelial cells (Jara et al., 2017). The findings by Laskar and Singh (2020) indicated that melatonin suppressed thyroid-stimulating hormone (TSH) secretion from the pituitary and downregulated protein expression of TSH receptors in the thyroid gland.

The thymus serves as the primary lymphoid organ where T cell progenitors from the bone marrow undergo an intricate maturation process. Eventually, this results in the mature naïve $CD4^+$ and $CD8^+$ T cells migrating into the circulation (Han and Zúñiga-Pflücker, 2021). $CD4^+$ helper T cells activate innate immune system cells, B cells, and cytotoxic T cells and have a vital role in regulating immune responses by releasing a diverse array of cytokines. $CD8^+$ cytotoxic T

cells have a pivotal role in immune defense against foreign antigens (Carrillo-Vico et al., 2013). Therefore, the analysis of blood $CD4^+$ and $CD8^+$ T cells was conducted to assess the potential influence of hyperthyroidism and/or melatonin on lymphocyte-mediated responses. In the present study, L-thyroxine had a stimulatory effect on $CD4^+$ T cells but did not affect $CD8^+$ T cells. This would be consistent with past research showing a large rise in the percentage of $CD4^+$ T cells in the blood of hyperthyroid rats (Robinson et al., 2014). In addition, rats given L-thyroxine had a high percentage of $CD4^+$ T cells in their thymus, according to Robinson et al. (2013). The current study also revealed that melatonin administration boosted the percentage of $CD4^+$ and $CD8^+$ T cells. T cells expressed the thyroid hormone receptor α (TRα) and TRβ at the mRNA and protein levels, respectively (Wenzek et al., 2022). Melatonin's action is mediated by MT1 and MT2 receptors which are associated with the membrane (Ferlazzo et al., 2020). MT1 receptors are found in various immune system components, including the spleen, thymus, $CD4^+$ T cells, $CD8^+$ T cells, and B cells (Singh and Jadhav, 2014). L-thyroxine and melatonin may promote T-cell proliferation by binding directly to their receptors.

Cytokines are small proteins that act locally or systemically to modify and regulate immune and inflammatory responses. To ascertain the importance of L-thyroxine and melatonin actions on immune response, we evaluated the expression of key cytokines in the spleen including the proinflammatory TNF-α (involved in inducing specific immune responses) the anti-inflammatory IL-10, IL-2(a cytokine necessary for lymphocyte proliferation and activation) and IFN-γ (important for cellular immunity). According to the current study, hyperthyroidism significantly upregulated TNF-α gene expression, indicating an elevated inflammatory response and a disturbed immune system. This result is supported by previous studies by Bahtiyar et al. (2020) and Salem et al. (2023), who revealed that L-thyroxine-induced hyperthyroidism was related to a rise in TNF- α level. Increased thyroid hormones markedly activate metabolism with excess reactive oxygen species production and impairment of antioxidant capacity. As a result of this imbalance, macromolecules such as DNA and proteins are exposed to oxidative damage (Ourique et al., 2013). Increased oxidative stress could stimulate the release of various cytokines, including interleukins and TNF-α (Perra et al., 2008)**.** This could account for the high expression of TNF-α seen in the hyperthyroid group. The current data revealed that melatonin administration to hyperthyroid rats resulted in an appreciable downregulation in TNF-α expression and, notably, upregulation of the anti-inflammatory IL-10. This result is supported by previous melatonin investigations, which have reported reductions in inflammation as a result of melatonin therapy (Hassanvand et al., 2019; Abdulwahab et al., 2021).

Our findings indicated that hyperthyroidism and melatonin treatment upregulated IL-2 and IFN- γ gene expression. These findings agreed with those of other researchers like Rubingh et al. (2020), who reported that hyperthyroid lymphocytes produced considerably more IL-2 and IFN-γ, and Vishwas and Haldar (2014), who found that melatonin administration raised IL-2 and IFN-γ levels in T cell cultures. Melatonin treatment enhanced MT1 receptor expression in the thymus, spleen, and bone marrow mononuclear cells. This suggests that elevated melatonin levels in the bloodstream can upregulate the expression of its receptor (Vishwas and Haldar, 2014)**.** Higher MT1 receptor expression may stimulate T lymphocyte cells to produce more cytokines such as IL-2 and IFN-γ.

5. CONCLUSION

In conclusion, L-thyroxine-induced hyperthyroidism, and melatonin both promote T lymphocyte proliferation and IL-2 and IFN-γ production. Melatonin co-administration revealed anti-inflammatory effects in rats with hyperthyroidism. These findings highlight the potential immunomodulatory effects of L-thyroxine and melatonin.

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