Potential antioxidant & anti-inflammatory protective effect of melatonin receptor agonist in hypo & hyperthyroid rat model

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Abstract

Background: Hormonal instability is linked to inflammation and oxidative stress (OS) through common pathways. Thyroid hormones have been found to play a major part in the antioxidant balance among many hormonal factors, since OS has been linked to both hypothyroidism and hyperthyroidism in humans and animals. A number of research studies have demonstrated the antioxidant, anti-inflammatory, and protective properties of melatonin receptor agonist Ramelteon (RML). Accordingly, **the aim of the current study was to** examine the potential antioxidant and anti-inflammatory properties of RML on OS induced by thyroid dysfunction (hyperthyroidism, hypothyroidism) in a rat model

Methods: Thirty adult albino male rats (fed ordinary laboratory diet along the four weeks' study), were randomly divided into 5 groups; ***control group***, ***experimentally-induced hypothyroid group*** (rats received a single dose of 2 mg carbimazole/100g daily diluted in drinking water by oral gavage for four weeks), ***hypothyroid group with administration of RML*** (10 mg/kg/day diluted in drinking water, *via* oral gavage for four weeks), ***experimentally- induced hyperthyroid group*** (rats received a single daily dose of 2µg/ml L-thyroxin diluted in drinking water by oral gavage for four weeks) and ***hyperthyroid group with administration of RML*** (10 mg/kg/day diluted in drinking water, *via* oral gavage for four weeks). All rats' body mass indices (BMIs) were assessed at the end of the experiment. **Results:** The final BMI index, serum glucose, insulin, HOMA-IR, serum T3, serum T4, serum TSH, serum total cholesterol, serum triglycerides, serum low density lipoprotein, serum tumor necrosis factor alpha (TNF-α), serum interleukin-6 (IL-6), and serum oxidative stress markers, such as serum malondialdehyde (MDA) and reduced glutathione (GSH), superoxide dismutase (SOD), were found to differ statistically significantly between the hypothyroid and hyperthyroid groups. In terms of final BMI index, serum glucose, insulin, HOMA-IR, total cholesterol, triglycerides, low density lipoprotein, serum IL-6, TNF-α, and serum oxidative stress markers (MDA), (GSH), and (SOD), ramelteon significantly reduced these levels. This suggests that ramelteon has anti-inflammatory and antioxidant properties in thyroid dysfunction (hyperthyroidism, hypothyroidism). **Conclusions:** Ramelteon may be able to diminish the oxidative and metabolic damage caused by hypo- and hyperthyroidism. Its protective anti-obesity, hypoglycemic, hypolipidemic, antioxidant and anti-inflammatory properties may contribute to its useful effects.

Keywords: *Ramelteon; oxidative stress; thyroid disorders*

Introduction

Systemic inflammation and hormonal instability are related to oxidative stress (OS). Thyroid hormones play important roles in antioxidant modulation, as revealed in several many studies **[1]**. It has been demonstrated that OS is linked to hyper and hypo-thyroidism. In most cells production of OS is correlated with ATP synthesis and related to cell respiration rate **[2]**. Oxidative damage arises when the equilibrium of OS synthesis and elimination is upset **[3]**. In all types of mammalian cells, thyroid hormones (THs) play a vital function in regulation of metabolism & respiration rate **[4]**. In general terms, some enzymes activity, as superoxide dismutase, increases by TH stimulation beside the rate of OS. Other enzymes as catalase and glutathione peroxidase can be reduced **[5]** or elevated by TH stimulation. On the other hand, a reduction of TH activity can be decrease OS but also, depresse the antioxidant activity **[6]**. Numerous researches established the preventive, anti-inflammatory, and antioxidant mechanisms of ramelteon (RML), a melatonin receptor agonist. Ramelteon has the ability to regulate the TNF-α/NF-κB pathway, which could potentially impact inflammatory processes such the production of cytokines **[7]**. Its capability to eliminate free radicals has been discovered and this could play a crucial role in inhibiting OS **[8]**.

Material & methods

Thirty mature male albino rats weighing between 180 and 200 grams, belonging to the local strains obtained from Zagazig University's Faculty of Veterinary Medicine. The animals were kept in 5 steel wire cages in a temperature- and light-controlled room with 12-hour light-dark cycle. They were fed a typical pellet lab chow and had unlimited access to tap water. Before the trial began, rats were housed under observation for a period of fifteen days. The institutional animal care and use committee (ZU-IACUC) of Zagazig University's faculty of medicine accepted the experimental protocol. The approval number for the committee is ZU-IACUC/3/F/11/2020. Following the acclimation phase, rats were split into five equal groups at random. **(Control group)**; rats received regular saline oral gavage therapy for four weeks (study period) and were fed a normal diet consisting of {25.8% protein, 11.4% fat and 62.8% carbohydrates}. The rats were housed in distinct, clean containers that were acquired from Zagazig Agriculture College. **(experimentally-induced hypothyroid group)**; for four weeks, rats were given a single oral gavage dose of 2 mg carbimazole/100g diluted in drinking water **[9]**. **(Hypothyroid group with administration of RML)** rats were received (10 mg/kg/day diluted in drinking water, *via* oral gavage for four weeks)**[10]**, **(experimentally-induced hyperthyroid group)**; rats got L-thyroxin at a dose of 2μg/ml diluted in drinking water for four weeks**[11]**. **(Hyperthyroid group with administration of RML)** rats were received (10 mg/kg/day diluted in drinking water, *via* oral gavage for four weeks. All rats fed ordinary laboratory diet along the four weeks' study. At the end of the experimental protocol, Rat span was determined by measuring the distance between the nose & anus, and the BMI index which is equal to body weight (gm)/length2 (cm2) was computed [12]. Under ether anesthesia, blood **samples** were taken from the tail vein **[13]**. The samples were then allowed to clot for 30 min before being centrifuged for 15 min at 3000 rpm. The serum was then separated and kept at -80^{o}C for estimation of biochemical parameters.

Biochemical measurements: Serum levels of glucose, insulin, free T3, free T4, TSH, total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL), interleukin 6 (IL6), and tumor necrosis factor alpha (TNFα) were measured using commercial ELISA kits (Sigma,

Aldrich). Also, we measured malondialdehyde (MDA), reduced glutathione (GSH), and superoxide dismutase (SOD). Insulin resistance was assessed using the calculation (HOMA-IR) = (Fasting glucose in mg/dl x Fasting insulin in μIU/ml)/405 **[14]**. LDL (mg/dl) was determined using the formula [TC] – [(HDL) + (TG / 5)] **[15]**.

Statistical analysis: The findings were presented in the form of mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and Tukey HSD for Post hoc multiple comparisons were utilized to compare means for statistical significance. That was accomplished with the help of the program IBM Statistical Package for Social Sciences (SPSS) Version 26 Software for Windows (SPSS, Inc., Chicago, IL, USA). P values < 0.05 were used to determine significance.

RESULTS

The results in Table 1 indicated that there was a statistically significant difference (P<0.05) in BMI between hypothyroid, hyperthyroid and control groups, hypothyroid group had the highest final BMI and hyperthyroid had the lowest final BMI. BMI in hypothyroid group administrated RML showed insignificant change compared with hypothyroid group. BMI in hyperthyroid group administrated RML showed insignificant change compared with hyperthyroid group.

Concerning serum glucose level, it was a significantly higher (P<0.05) in both hyper & hypothyroid groups in comparison to that in the control group. Serum glucose level in hypothyroid group administrated RML showed a significant decrease when compared to hypothyroid group. Serum glucose level in hyperthyroid group administrated RML showed significant decrease compared with hyperthyroid group.

In hyperthyroid group, insulin level was significantly higher (P<0.05) than hypothyroid group and control group. Hyperthyroid group administrated RML showed significant reduction in serum insulin level compared with hyperthyroid group.

HOMA-IR was a significantly higher (P<0.05) in both hyper & hypothyroid groups in comparison to that in control group. HOMA-IR in both groups administrated RML showed significant decrease (P<0.05) in comparison with those corresponding groups.

Moreover, Free T3 and Free T4 were significantly high (P<0.05) in hyperthyroid group and significantly low in hypothyroid group in comparison to that in control group. Free T3 and Free T4 in both hypothyroid and hyperthyroid groups showed insignificant change compared with hypothyroid and hyperthyroid groups administrated RML. TSH in hypothyroid group was significantly higher (P<0.05) than control group and hyperthyroid group.

The results in Table 2 revealed that in hypothyroid group, TC, TG and LDL were higher significantly (P<0.05) than hyperthyroid group and control group. In hypothyroid group administrated RML, TC, TG and LDL were lowered significantly (P<0.05). In hyperthyroid group, TC, TG and LDL were significantly lower (P<0.05) than hypothyroid group. In this group administrated RML, TC, TG and LDL were significantly elevated (P<0.05). HDL showed no significant changes between groups.

The results in figure1 revealed that in hypothyroid and hyperthyroid groups, serum MDA was higher significantly (P<0.05) than control group. In both hypo & hyperthyroid groups administrated RML serum MDA showed significant decrease (P<0.05) in comparison with those corresponding groups.

The results in figure2 revealed that in hypothyroid group serum reduced glutathione was higher significantly (P<0.05) than control and hyperthyroid groups**.** In hypothyroid group administrated RML serum reduced glutathione was significantly lower than hypothyroid group. A significant reduction (P<0.05) in reduced glutathione levels in hyperthyroid rats compared to

those of controls. Administration of RML in hyperthyroid group showed a significant elevation of serum reduced glutathione level when compared with hyperthyroid group.

SOD activities in hyperthyroid rats significantly increased when compared with both hypothyroid and control rats. In both hypo & hyperthyroid groups administrated RML serum SOD activities showed significant reduction (P<0.05) in comparison with those corresponding groups.

The results in figure3&4 revealed that serum TNF-α and IL-6 level in hypo & hyperthyroid groups were significantly higher in comparison to control group**,** also, in hyperthyroid group serum TNF-α was revealed to be significantly higher than that in both control and hypothyroid group. Serum IL-6 and TNF-α level in both hypothyroid & hyperthyroid groups administrated RML showed significant decrease in comparison with with those corresponding groups.

Table (1): Biochemical parameters in all examined groups

Parameter	(Control group)	(Hypothyroid group)	Hypo+ RML	(Hyperthyroid group)	Hyper+RML
Final BMI (g/cm2)	0.71±0.072 A	0.93±0.054 B	0.91±0.066 B	0.45±0.042 D	0.43±0.072 D
Blood glucose (mg/dl)	85.2±2.54 A	229.3±4.3 B	190.7±3.71 C	259.3±5.0 D	175.3±1.34 E
Serum Insulin (µIU/ml)	10.1±0.4 A	14.46±0.8 B	12.1±0.7 C	20.04±0.3 D	16.3±0.2 E
HOMA-IR	2.14±1.37 A	8.80±1.34 B	5.74±1.17 C	12.06±7.57 D	7.34±1.47 E
Free T3 (pg/dl)	2.91±0.6 A	0.7±0.04 B	0.8±0.02 B	9.7±0.7 C	8.7±0.7 C
Free T4 (ng/dl)	1.4±0.3 A	0.7±0.05 B	0.7±0.03 B	5.9±0.49 C	4.9±0.68 C
TSH (mIU/ml)	1.5±0.2 A	4.3±0.8 B	4.1±0.5 B	.09±0.04 C	.08±0.06 C

Groups with different letters are statistically significant (P<0.05). **BMI**, body mass index; **HOMA-IR,** homeostasis model assessment insulin resistance; **TSH** thyroid stimulating hormone.

Table (2): Lipid profile measurements in all studied groups

Parameter	Control group	Hypothyroid group	Hypo+ RML	Hyperthyroid group	Hyper+RML
TC(mg/dl)	110±3.3 A	144.6±4.5 B	126.3±2.6 C	86.9±4.5 D	91.1±5.3 E
TG(mg/dl)	64±2.6 A	89.1±2.5 B	71±2.3 C	48±2.1 D	52±2.9 E
HDL(mg/dl)	31.5±2.3 A	30.2±2.6 A	30.5±2.1 A	32.1±2.2 A	31.9±2.4 A
LDL(mg/dl)	59.1±3.4 A	88.5±2.7 B	73.5±2.1 C	43±2.2 D	48±2.4 D

Groups with different letters are statistically significant (P<0.05). **TC**, total cholesterol, **TG**, triglycerides; **HDL**, high density lipoprotein; **LDL**, low density lipoprotein**..**

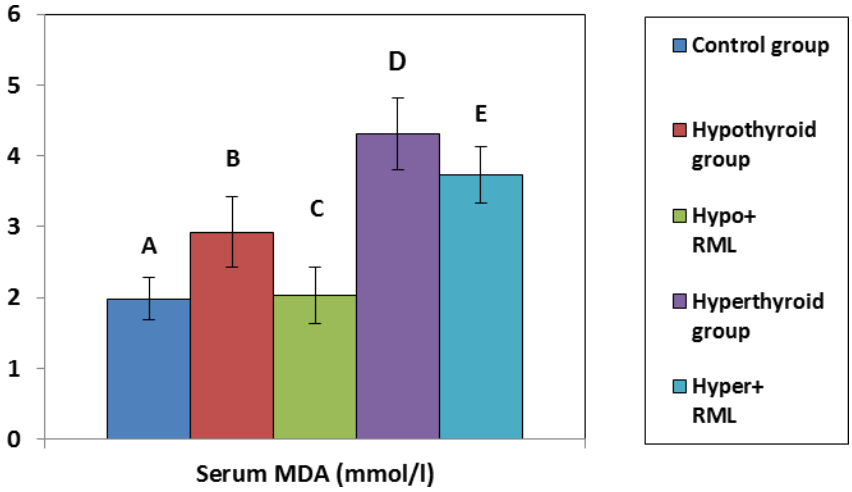

Figure1 showed serum MDA level in all studied groups. Groups with different letters are statistically significant (P<0.05)

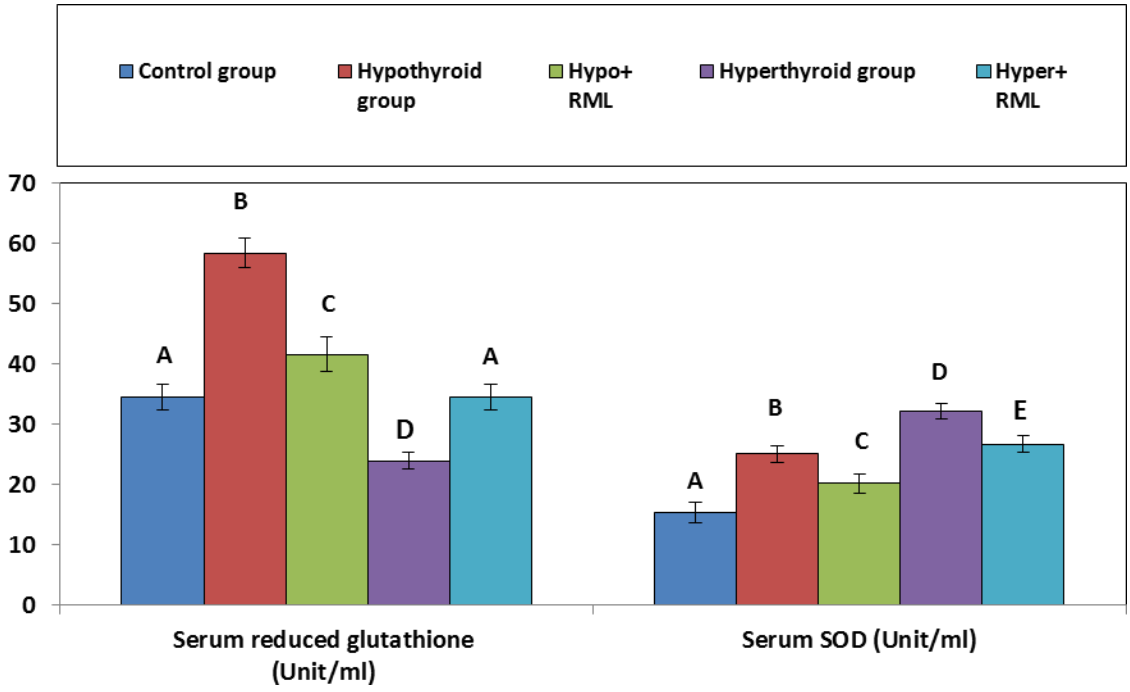

Figure 2 showed serum reduced glutathione & SOD levels in all studied groups. Groups with different letters are statistically significant (P<0.05)

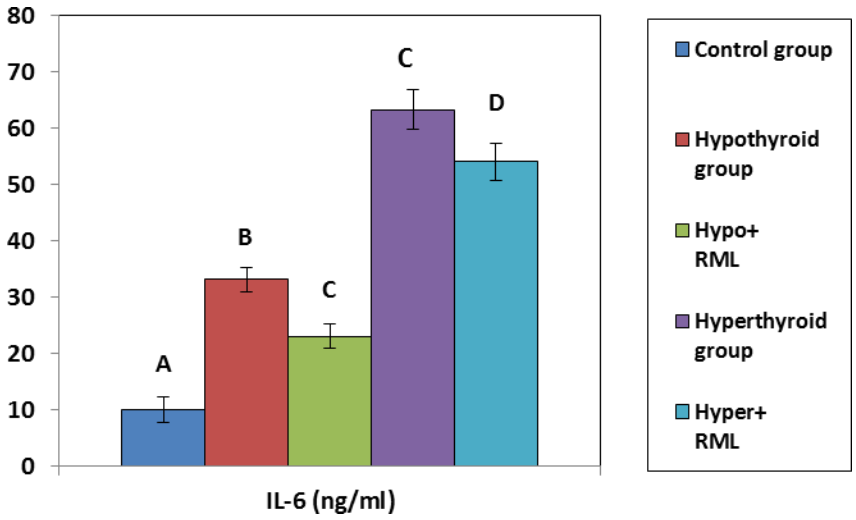

Figure 3 showed serum IL-6 level in all studied groups. Groups with different letters are statistically significant (P<0.05)

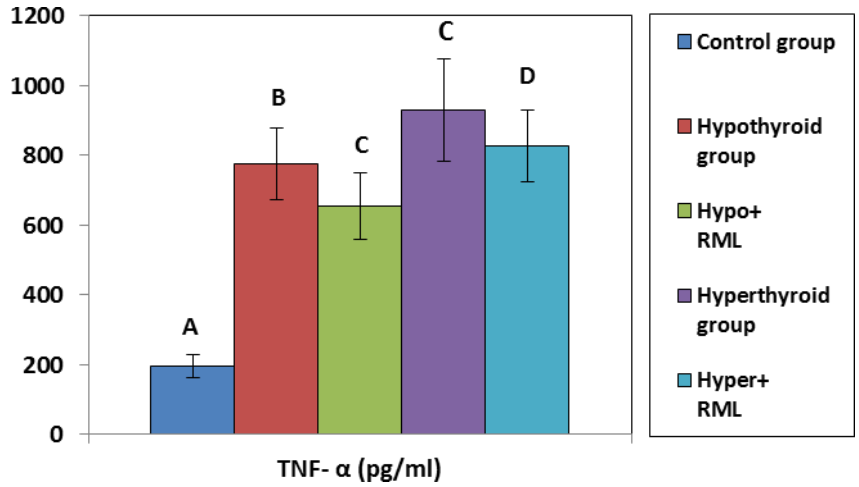

Figure 4 showed serum TNF-α level in all studied groups. Groups with different letters are statistically significant (P<0.05)

DISCUSSION

This study was done to evaluate the probable anti-inflammatory & anti-oxidant special effects of RML on OS in rat model of thyroid dysfunction (hyperthyroidism, hypothyroidism). The thyroid gland was the study's gland of choice because dysfunction of the gland indicates a serious decline in our bodies' oxidative and metabolic states.

Our findings indicate that, there was a statistically significant rise in final BMI among hypothyroid group. **Venditti et al. [17] and Yeldu and Ishaq [16]** corroborated these findings. However, the final BMI index was considerably lower in the hyperthyroid group than in the control group. According to **Kim and Lee [18],** there was a considerable rise in mean weight following the study period, with an inverse association observed between the hyperthyroid and

control groups. Levothyroxine raises metabolic rate, but it may also cause rats to eat more, which could explain why the hyperthyroid group may have gained weight.

The findings of this experiment showed that insulin sensitivity declined in both the hypothyroid and hyperthyroid groups, as evidenced by a large rise in HOMA-IR. This was followed by a significant increase in serum insulin & glucose levels. This was according to **Yeldu and Ishaq [16]**, who discovered that in hyperthyroid situations, oxidative stress effect on hepatic lipid peroxidation & cell damage indicators {ALT, LDH and ALP activities} is linked to a decline in pancreatic function. The primary cause of OS is the glucose metabolic imbalance that our investigation has already documented **[19]**. Furthermore, **Vazquez-Anaya et al. [20]** verified the link between insulin resistance and hypothyroidism. A decline in insulin-mediated glucose elimination and an inability of insulin to enhance blood flow to the hyperthyroid were identified as the causes of insulin resistance **Ormazabal et al.[21]**.

The current study found that when RML was administered to the hypothyroid group, their serum glucose level significantly decreased in comparison to hypothyroid group, and when RML was administered to the hyperthyroid group, their blood glucose level significantly decreased in comparison to the hyperthyroid group. This was in agreement with the findings of **Tetsuji et al. [22]** who demonstrated that ramelteon lowers blood glucose levels in diabetic individuals. An agonist of melatonin is ramelteon. Numerous fundamental researches have looked into how melatonin affects glucose metabolism and its methods of action. Consequently, researches have demonstrated that melatonin lowers insulin release as well as blood glucose levels by promoting skeletal muscles GLUT-4 expression through PI-3 or IRS-1 kinase-mediated pathway, melatonin enhances glucose transport. **[23]**. Insulin release is inhibited when melatonin binds to the MT1 and MT2 receptors of β-cells, lowering c-AMP & c-GMP levels, correspondingly **[24]**. Additionally, it has been demonstrated that melatonin enhances insulin resistance by lowering the amount of free fatty acids, inflammatory cytokine production as well as OS **[25]**. Apart from this fundamental research, melatonin also revealed enhancing glucose metabolism in type 2 diabetes individuals. **Hussain et al.** found that after three months of a high dose of melatonin, patients' HbA1c decreased from 7.63% to 7.13% **[26]**. Additionally**, Garfinkel et al**. showed that in patients who took prolonged-release melatonin for 5 months HbA1c dropped to 8.47% **[27]**.

According to our research, HOMA-IR significantly reduced in hypothyroid group administered RML when compared to hypothyroid group. This was in agreement with the findings of **Ayako et al. [28]** who documented that ramelteon could induce reduction in energy intake due to activation of AMPK. In obese rats, ramelteon therapy also reduced responses of inflammation. Additionally, ramelteon therapy reduced BAT, which is involved in controlling insulin sensitivity throughout the body and glucose tolerance.

In the current investigation, the hyperthyroid group had lower levels of TG, TC, and LDL than control group, whereas the hypothyroid group had significantly greater levels than the latter. Regarding HDL, no discernible variation was discovered between the groups under investigation. The identical study findings were described by **Oktay et al. [29]**.

In this study, TC, TG, and LDL were noticeably lower in the hypothyroid group administered RML than in hypothyroid group. This was in agreement with the findings of **Christina P.C. et al.[30]**, who discovered that ramelteon lowers LDL, TG, and TC levels in obese persons. Improved lipid metrics have been attributed to RML's antioxidant qualities, altered gut microbial populations, and increased insulin sensitivity

In the present study, free T3 and T4 elevated significantly in hyperthyroid group relative to control, while the opposite was observed in the hypothyroid animals. Likewise, earlier findings

as published by **Najafi et al. [31]**. It was made clear by **Minakhina et al. [32]** that hypothalamic-pituitary-thyroid axis is regulated the activity of thyroid gland. The authors mentioned TSH as a key thyroid growth factor and linked it to their role in the synthesis, release, and processing of thyroid hormones.

In the current research, the hyperthyroid rats' levels of OS indicators, such as MDA and superoxide dismutase (SOD), were higher significantly than those of control group. Furthermore, in comparison to control group, reduced glutathione was significantly elevated in hypothyroidism and significantly decreased in hyperthyroidism. Prior researches have demonstrated that hyperthyroidism is concomitant with an elevation of free radicals **[29]**. Lipid peroxidation products were found to be substantially greater in hyperthyroid rats, according to **Yeldu and Ishaq's research [16]**. When comparing the lowered glutathione levels of hyperthyroid rats to those of controls, a notable decrease was noted. Furthermore, the mean MDA levels were elevated considerably in hyperthyroid group by **Najafi et al. [31]**. The elevated rate of lipid peroxidation and metabolism may be the cause of this. Additionally, consuming more oxygen causes the heart and skeletal muscles to experience higher levels of OS, which leads to lipid peroxidation & production of hydroperoxide **[17]**.

According to the present study, the hypothyroid group's serum MDA level dropped in comparison to hyperthyroid group. According to the study by **Erdamar et al. [33]**, following treatment with propylthiouracil (PTU), MDA levels rose in the hypo & hyperthyroid groups while they decreased in hyperthyroid group. Furthermore, **Chesere et al.'s study [34]** shown that lipid peroxidation levels rose during T3 administration but did not change following hypothyroidism development. Increased SOD activity in hyperthyroidism suggests that OS is present as a result of mitochondrial oxidation. Another function of the SOD is to convert (O2-) to inorganic hydroperoxide (H2O2), which is then reduced by CAT and GPx enzymes **[17]**.

Serum MDA and serum SOD significantly decreased in hypothyroid group administered RML in the present study when compared to hypothyroid group. Additionally, compared to hyperthyroid group, the serum MDA and SOD levels in the hyperthyroid group administered RML significantly decreased. This was in agreement with the findings of **Geng et al. [35]** who discovered that melatonin agonist is a potent antioxidant that scavenges {reactive oxygen species} (ROS), lowers MDA levels, and promotes the production of antioxidant enzymes. Melatonin receptor activation, which can trigger the synthesis of numerous antioxidative enzymes via a number of signaling pathways, may be the mechanism by which melatonin agonists protect against oxidative stress **[36]**. However, new researches also show that, in some circumstances, melatonin agonists might function as prooxidants **[36]**. Melatonin agonist prooxidant effects are generally depending on concentration, cell type and length of treatment; these effects have been documented in vitro **[36]**. Studies have demonstrated that melatonin agonist's strong antioxidant potential, while concurrent observations have indicated that it possesses prooxidant properties **[37]**. The antioxidant activity of mealatonin and melatonin receptor agonist was investigated by **Russell et al. [38]**. According to their report, melatonin has several antioxidant properties, including the ability to scavenge free radicals, stimulate antioxidative enzymes, increase the mitochondrial oxidative phosphorylation and diminish electron leakage, which in turn lowers free radicals generation.

In the current study, the hyperthyroid group had higher TNF-α and IL-6 levels. This was in agreement with the findings of **Baldissarelli et al. [39]** which discovered that thyrotoxicosis increases OS and production inflammatory cytokines. Nonetheless, **Zhou et al. [40]** demonstrated that TNF-α & IL-6 levels were continuously rising in hypothyroidism, suggesting

that the elevated pro-inflammatory cytokine levels are likely related to. Rats with hypothyroidism that were treated with levothyroxine (L-T4) had significantly lower serum levels [41]. According to Marfella et al. [42], patients with subclinical hypothyroidism who received L-T4 had lower plasma levels of IL-6 and TNF-α than those who did not get treatment.

Serum IL-6 &TNF-α level in the hypothyroid group receiving RML was significantly decreased as compared to hypothyroid group. Additionally, hyperthyroid group administered RML demonstrated significant drop in serum IL-6 & TNF-α levels when compared to hyperthyroid group, which was consistent with **Reza et al's findings [43]**. It has been shown that the Nrf2 pathway's activation is connected with the melatonin agonist's advantageous effects. The Nrf2 pathway shows a critical effect in tissue injury by binding of the transcription factor Nrf2 to DNA antioxidant response element (ARE) which responsible for its antioxidant effect **[45]**. Additionally, it was found that Nrf2 pathway is up-regulated by melatonin agonist suppresses IL-6 production. Decreased IL-6levels suppress the expression of STAT3 and its related genes linked to inflammation. Thus, melatonin agonists can also exert their cardio-protective effects indirectly by inhibiting IL-6 levels. **Tugba et al** established that RML anti-inflammatory effects were connected to melatonin agonist activity **[46]**.

Additionally, it is known that anti-inflammatory effects of melatonin were owing to prevention of NF-κβ signaling pathway **[46]**. It was also known that NF-κβ inhibition followed TNF-α inhibition. Preceding researches had demonstrated that TNF-α influences the JAK-STAT pathway in cells. **[47**]. Moreover, melatonin receptors activation could inhibit JAK2/STAT3 signal pathway, causing cytokine & chemokine secretion **[48]**. Also, **Celinski et al. [49]** found that pro-inflammatory cytokines such TNF-α were lowered when melatonin receptors were activated. Evidence from studies of acute ocular inflammation **[50]**, rats' cerebral toxicity caused by methotrexate **[51]** and the human model's brain cytotoxicity increased by isoflurane **[52]** suggested that RML could reduce pro-inflammatory cytokines. Furthermore, it was previously established that RML provided neuro-inflammatory protection in endotoxin rat model by blocking TNF-α, NF-κβ, and IL-1β **[53]**. In line with earlier research, our findings imply that RML prevented the increase in cytokines caused by thyroid conditions, thereby reducing organ dysfunction.

CONCLUSIONS

Based on the previously mentioned data, it is possible to conclude that Ramelteon may have the potentiality to ameliorate metabolic & oxidative state of hypo & hyperthyroidism as it capable to exert anti-obesity, hypoglycemic, hypolipidemic, antioxidant and anti-inflammatory potentially protective effects.

Conflict of Interest: None

Financial Disclosures: None

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