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EFFECT OF HYPERTHYROIDISM ON THYROID RECEPTOR GENE EXPRESSION LEVELS IN HYPOTHALAMIC AND PITUITARY TISSUES IN PREPUBERTAL AND PUBERTAL MALE RATS

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Abstract
The present study has been designed to investigate hyperthyroidism on thyroid receptor gene expression levels in hypothalamic and pituitary tissues in prepubertal and pubertal male rats. The present study has been conducted in the animal house of the college of veterinary medicine, AL-Qadisiya University during the period extended from Feb-July,2022. Thirty-two premature (aged 40 days) and thirty-two mature (aged 70 days) male rats. Divided to 4 groups each 16 rats 2 subgroups 10 and 20 days as following, Pre-pubertal control group (C-pre): 16 premature male rats (aged 40 days) will be orally administered for 20 days with distilled water. Pre-pubertal hyperthyroid group (H-pre): 16 premature male rats (aged 40 days) will be orally administered for 20 days with thyroxine (T4) in drinking water (0.002% w/v) beside intragastric gavage of 200 T4 µg/kg body weight. Post-pubertal control group (C-pre): 16 mature male rats (aged 70 days) will be orally administered for 20 days with distilled water. Premature hyperthyroid group (H-pre): 16 mature male rats (aged 70 days) will be orally administered for 20 days with thyroxine (T4) in drinking water (0.002% w/v) beside intragastric gavage of 200 T4 µg/kg body weight. At the end of each treated and control subgroup period, males were anaesthesized (by injection of 0.3ml ketamine + 0.1 ml of xylazine/ kg b.w. *ip*), dissected and blood samples were obtained from abdominal vein in non-heparinized tubes. Hypothalamic, testicular and pituitary sample from each male has been obtained for evaluation of mRNA expression levels of GAPDH as housekeeping gene and TR-α and TR-β genes using qRT-PCR technique based on Syber Green dye. Hypothalamic, testicular and pituitary samples obtained from all groups recorded significant higher RNA concentrations at 10 and 20 day periods compared with control. Significant elevation of both TR-α and TR-β genes expression levels (fold changes) have been shown in thyroxine treated males in comparison with control, started after 10 days of treatment and continued in its elevation after 20 days of treatment. In comparison between period, both genes showed a significant difference between periods of control group, whereas thyroxine treated group recorded significant gradual elevation of both genes as the treatment period progress at 10^{th} and 20^{th} days.
Keywords: TR-α gene, TR-β gene, thyroxine.

Introduction

Thyroid hormones (THs; thyroxine, T_4 and triiodothyronine, T_3), known as regulators of metabolism, development and growth (Mullur *et al*.,2014), play an important role in proper development and function of the reproductive system, particularly in pubertal onset (Doufas and Mastorakos,2000). It was shown that conversion of T_4 to bioactive T_3 is increased with entry into

puberty (Marwaha *et al*.,2012), thus delayed pubertal onset is often observed clinically in children with hypothyroidism; sometimes, precocious puberty in case of extreme hypothyroidism (Cabrera *et al*.,2013). Pubertal onset is regulated in part by a brain-dependent process, whereby increased pulsatile secretion of hypothalamic gonadotropin-releasing hormone (GnRH) leads to the activation of pituitary-gonadal axis to awake the entire reproductive system (Ebling,2005). There are some explanations on how abnormal thyroid status leads to pubertal disorders based on the multilevel interactions of the two neuroendocrine systems, the hypothalamus-pituitary-thyroid (HPT) axis and the hypothalamus-pituitary-gonadal (HPG) axis. First, elevated levels of thyrotropin-releasing hormone in hypothyroidism induce hyperprolactinemia and alter GnRH pulsatile secretion, which lead to a delay in luteinizing hormone (LH) response, thus result in delayed puberty (Dittrich *et al*.,2011). Second, increased thyroid-stimulating hormone (TSH) levels activate gonadal function by stimulating follicle-stimulating hormone (FSH) receptor expressed in gonads, because the structure of FSH and TSH receptors is very similar, which is responsible for precocious puberty (Niedziela and Korman,2001). Although there is some debate whether GnIH can directly act on the pituitary in some species, GnIH decreases the synthesis and/or release of pituitary gonadotropins, LH and FSH in many species (Kriegsfeld *et al*.,2010; Son *et al*.,2012). Together, these findings suggest that GnIH is a key regulatory factor of the HPG axis to govern the neuronal activities of GnRH and kisspeptin, and eventually gonadotropin secretion. From a developmental standpoint, both GnIH expression and neuronal activation decreased markedly in the early prepubertal stage in the dorsomedial hypothalamic nucleus of female mice (Semaan and Kauffman,2015; Xiang *et al*.,2015).

Materials and Methods

Experimental design

Thirty-two premature (aged 40 days) and thirty-two mature (aged 70 days) male rats will be allocated to the following experimental groups:

1. Pre-pubertal control group (C-pre): 16 premature male rats (aged 40 days) will be orally administered for 20 days with distilled water.
2. Pre-pubertal hyperthyroid group (H-pre): 16 premature male rats (aged 40 days) will be orally administered for 20 days with thyroxine (T4) in drinking water (0.002% w/v) beside intragastric gavage of 200 T4 µg/kg body weight.
3. Post-pubertal control group (C-pre): 16 mature male rats (aged 70 days) will be orally administered for 20 days with distilled water
4. Premature hyperthyroid group (H-pre): 16 mature male rats (aged 70 days) will be orally administered for 20 days with thyroxine (T4) in drinking water (0.002% w/v) beside intragastric gavage of 200 T4 µg/kg body weight.

Each group will be allocated to two subgroups as follow:

A. Subgroup 1: 8 males will be sacrificed after 10 days of treatment.
B. Subgroup 2: 8 males will be sacrificed after 20 days of treatment.

After each treatment period, the following objectives will be examined Male rats have been monitored throughout the experimental periods. At the end of each treated and control subgroup

period, male rats were anaesthesized (by injection of 0.3ml ketamine + 0.1 ml of xylazine/ kg b.w. *ip*), dissected samples were obtained from abdominal vein in non-heparinized tubes. Testicular, pituitary and hypothalamic samples from each male have been obtained and kept directly at –70 ºC for evaluation of mRNA expression levels of GAPDH as housekeeping gene and TR-α and TR-β genes using qRT-PCR technique based on Syber Green dye.

Primers

The primers that used in this study were GAPDH gene primer as a Housekeeping gene, TR-α and TR-β genes primers as targetgene expression. These primers were designed by using NCBI- Gene Bank data base and Primer 3 design online. The primers were used in the quantification of gene expression levels by using qRT-PCR technique based SYBER Green DNA binding dye, which supported from (Bioneer company, Korea).

Results

Relative quantification of *TR-α* and *TR-β* genes expression (testicular) Pre-pubertal 10 days

In the present study, significant elevation ($p<0.05$) of both in testicular ***TR-α and TR-β*** genes expression levels (fold changes) have been shown in thyroxin treated males in comparison with control, started after 10 days of treatment (figure 1).

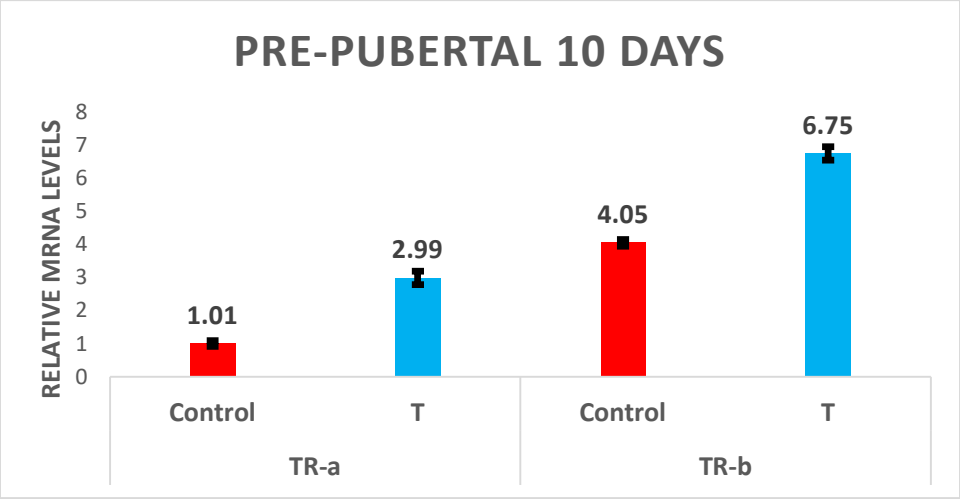

Figure (1): effect of thyroxin on expression level (fold changes) of testicular *TR-α and TR-β* pre-pubertal at 10 days of treatment in male rats.

Post-pubertal 10 days

In the present study, significant elevation ($p<0.05$) of both in testicular ***TR-α and TR-β*** genes expression levels (fold changes) have been shown in thyroxin treated males in comparison with control, started after 10 days of treatment (figure 2).

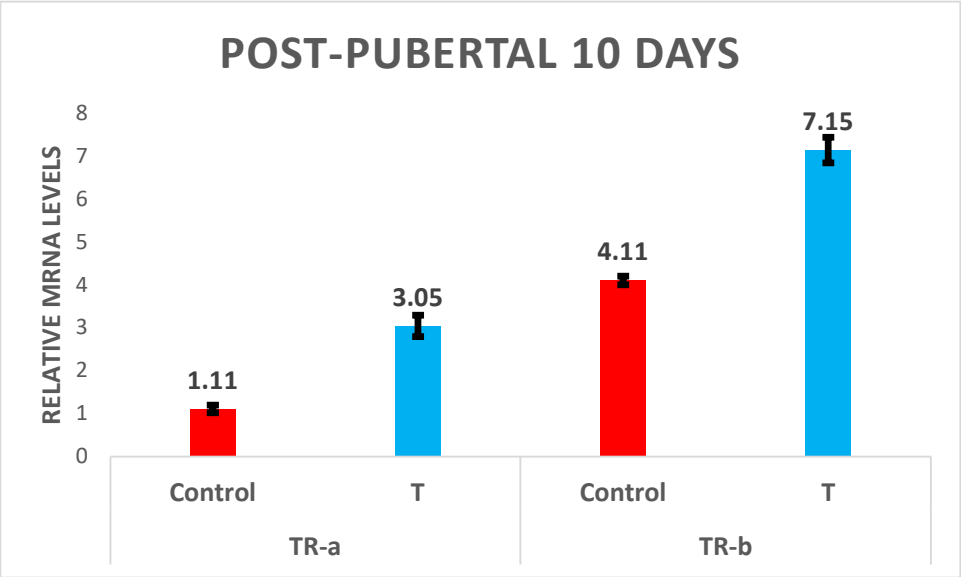

Figure (2): effect of thyroxin on expression level (fold changes) of testicular *TR-α and TR-β* post-pubertal at 10 days of treatment in male rats.

Pre-pubertal 20 days

In the present study, significant elevation ($p<0.05$) of both in testicular ***TR-α and TR-β*** genes expression levels (fold changes) have been shown in thyroxin treated males in comparison with control, started after 20 days of treatment (figure 3).

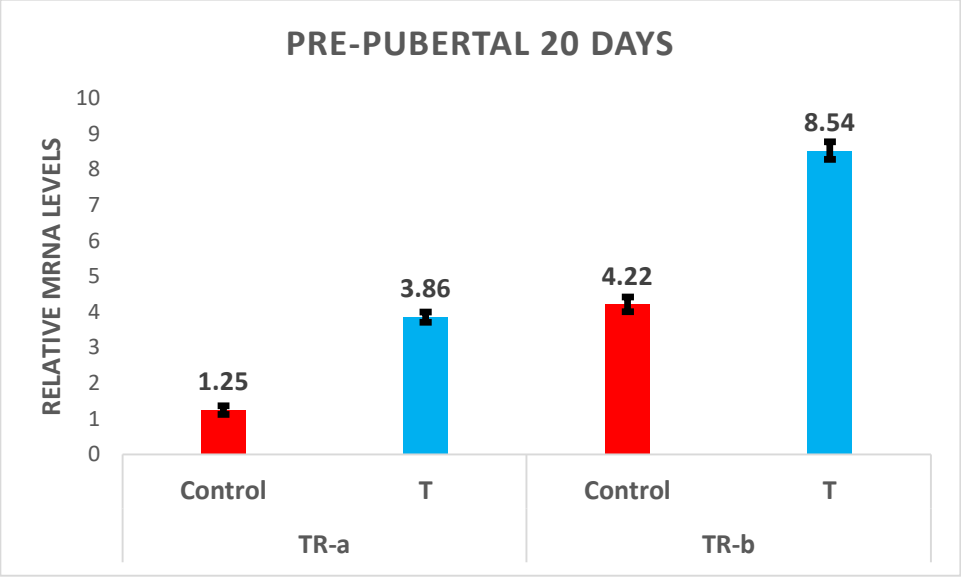

Figure (3): effect of thyroxin on expression level (fold changes) of testicular *TR-α and TR-β* pre-pubertal at 20 days of treatment in male rats.

Post-pubertal 20 days

In the present study, significant elevation ($p<0.05$) of both ***TR-α and TR-β*** genes expression levels (fold changes) have been shown in thyroxin treated males in comparison with control, started after 20 days of treatment (figure 4).

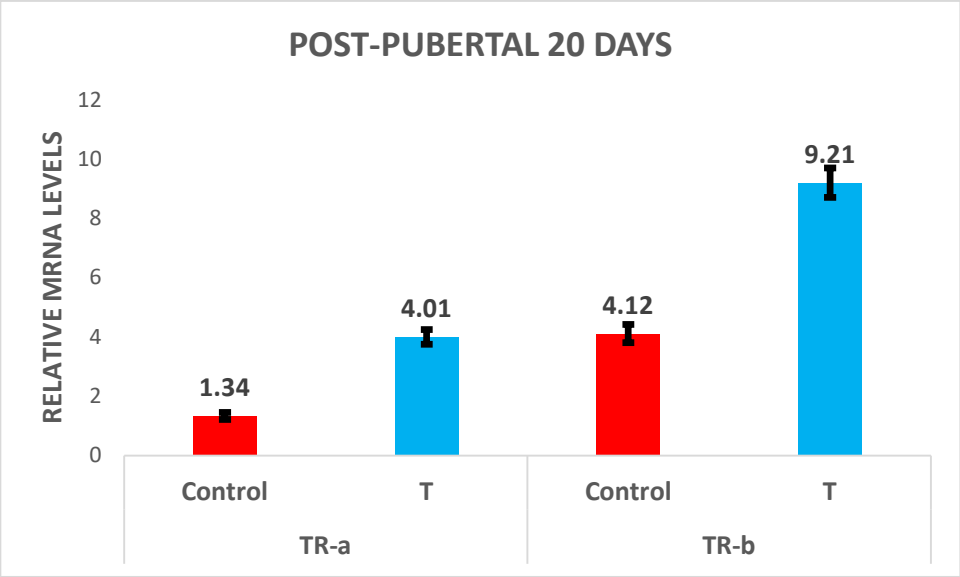

Figure (4): effect of thyroxin on expression level (fold changes) of testicular *TR-α and TR-β* post-pubertal at 20 days of treatment in male rats.

Relative quantification of TR-α and TR-β genes expression (Pituitary)

Pre-pubertal 10 days

In the present study, significant elevation ($p<0.05$) of both in pituitary ***TR-α and TR-β*** genes expression levels (fold changes) have been shown in thyroxin treated males in comparison with control, started after 10 days of treatment (figure 5).

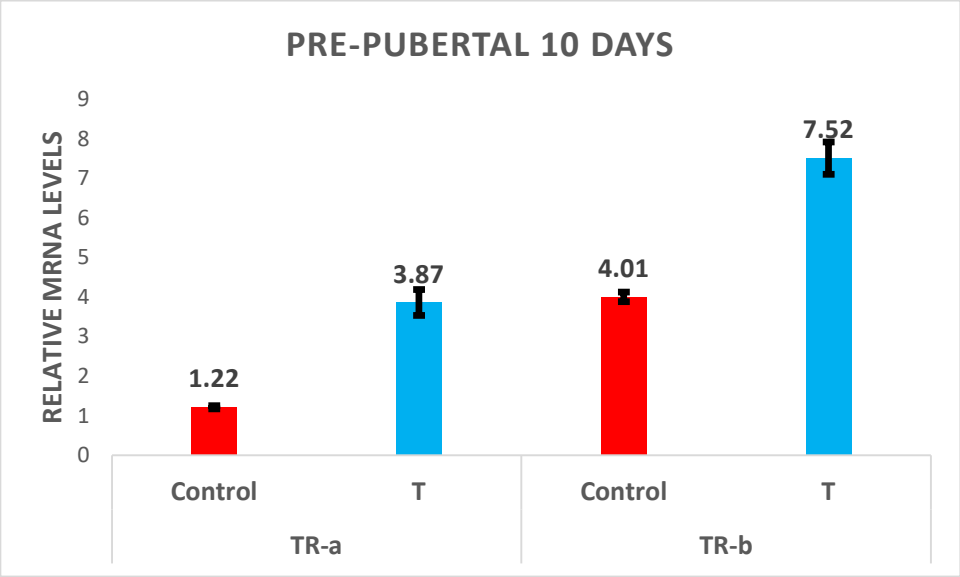

Figure (5): effect of thyroxin on expression level (fold changes) of pituitary *TR-α and TR-β* pre-pubertal at 10 days of treatment in male rats.

Post-pubertal 10 days

In the present study, significant elevation ($p<0.05$) of both in pituitary ***TR-α and TR-β*** genes expression levels (fold changes) have been shown in thyroxin treated males in comparison with control, started after 10 days of treatment (figure 6).

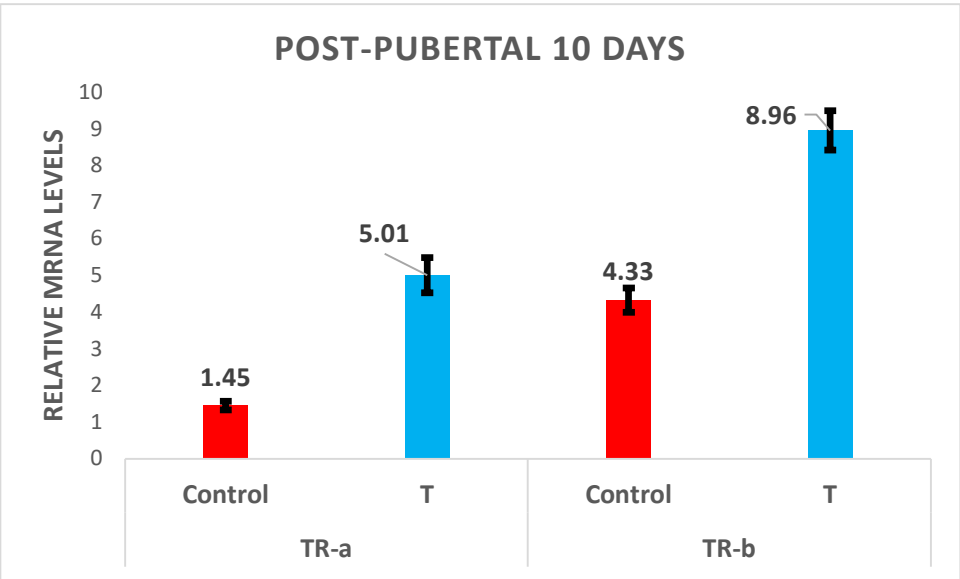

Figure (6): effect of thyroxin on expression level (fold changes) of pituitary *TR-α and TR-β* post-pubertal at 10 days of treatment in male rats.

Pre-pubertal 20 days

In the present study, significant elevation ($p<0.05$) of both in pituitary ***TR-α and TR-β*** genes expression levels (fold changes) have been shown in thyroxin treated males in comparison with control, started after 20 days of treatment (7).

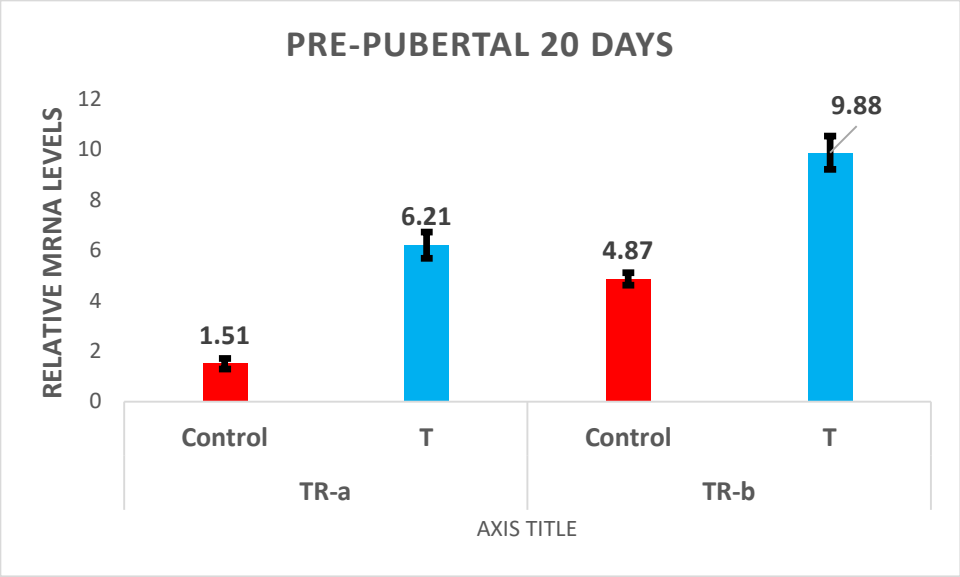

Figure (7): effect of thyroxin on expression level (fold changes) of pituitary *TR-α and TR-β* pre-pubertal at 20 days of treatment in male rats.

Post-pubertal 20 days

In the present study, significant elevation ($p<0.05$) of both in pituitary ***TR-α and TR-β*** genes expression levels (fold changes) have been shown in thyroxin treated males in comparison with control, started after 20 days of treatment (figure 8).

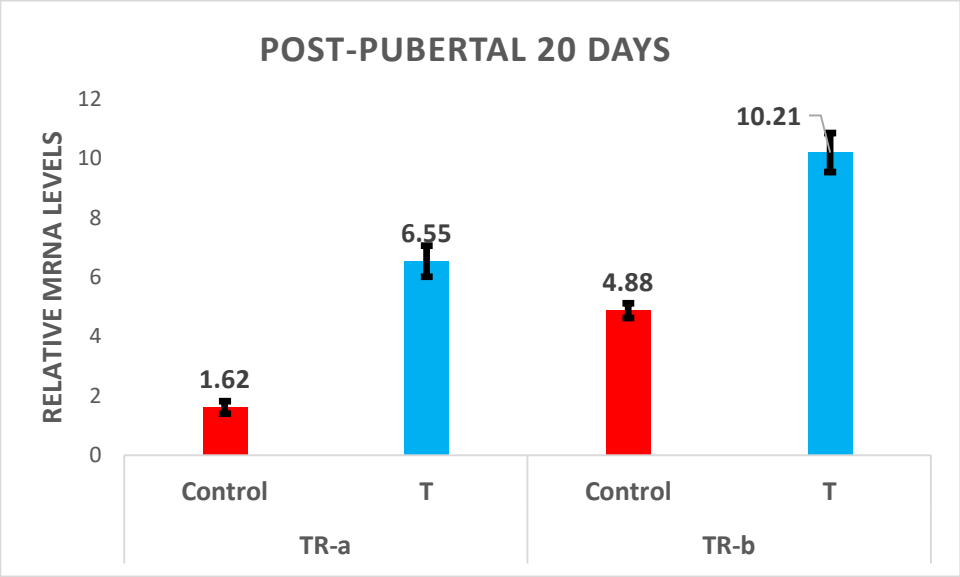

Figure (8): effect of thyroxin on expression level (fold changes) of pituitary *TR-α and TR-β* post-pubertal at 20 days of treatment in male rats.

Relative quantification of TR-α and TR-β genes expression (Hypothalamic) Pre-pubertal 10 days

In the present study, significant elevation ($p<0.05$) of both in hypothalamic ***TR-α and TR-β*** genes expression levels (fold changes) have been shown in thyroxin treated males in comparison with control, started after 10 days of treatment (figure 9).

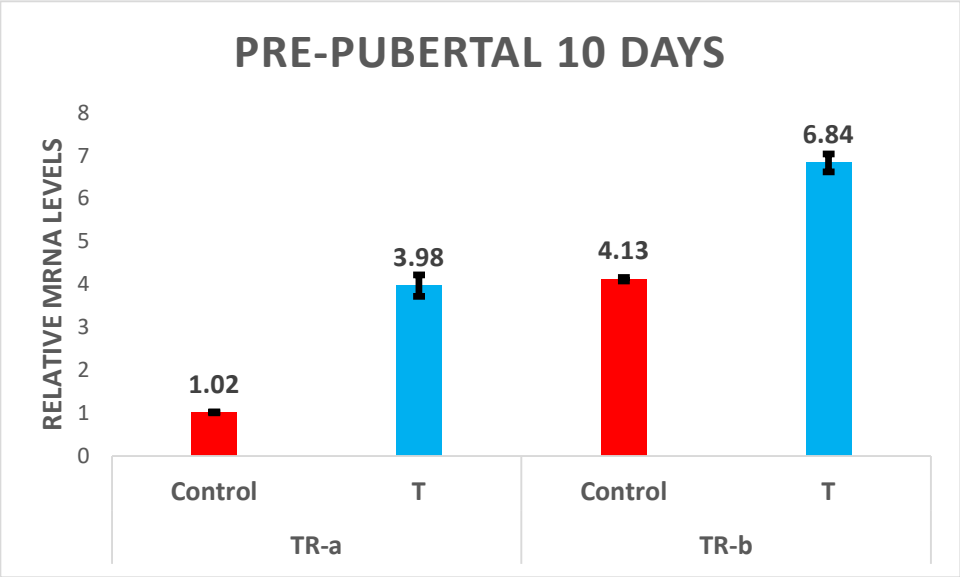

Figure (9): effect of thyroxin on expression level (fold changes) of hypothalamic *TR-α and*

***TR-β* pre-pubertal at 10 days of treatment in male rats.**

Post-pubertal 10 days

In the present study, significant elevation ($p<0.05$) of both in hypothalamic ***TR-α and TR-β*** genes expression levels (fold changes) have been shown in thyroxin treated males in comparison with control, started after 10 days of treatment (figure 10).

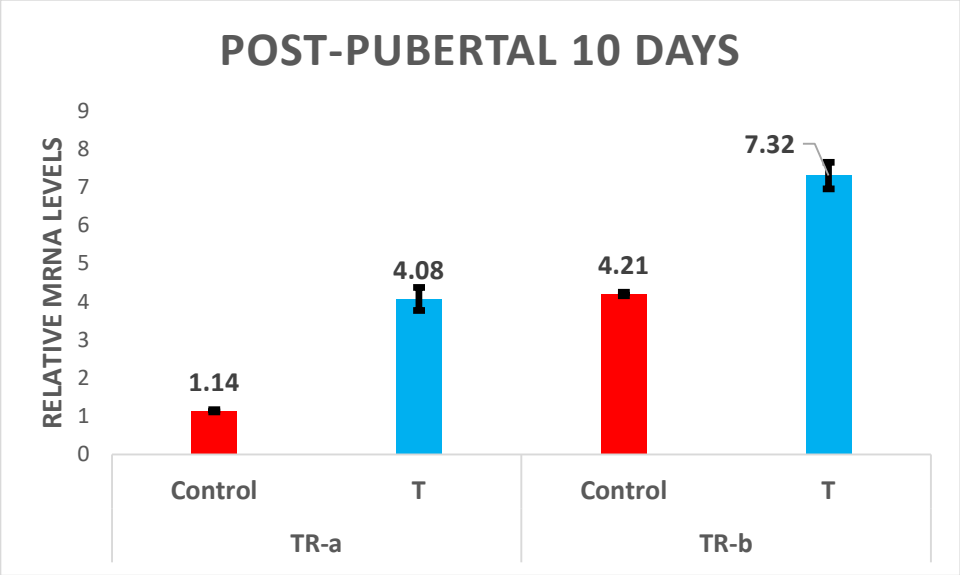

Figure (10): effect of thyroxin on expression level (fold changes) of hypothalamic *TR-α and TR-β* post-pubertal at 10 days of treatment in male rats.

Pre-pubertal 20 days

In the present study, significant elevation ($p<0.05$) of both in hypothalamic ***TR-α and TR-β*** genes expression levels (fold changes) have been shown in thyroxin treated males in comparison with control, started after 10 days of treatment (figure 11).

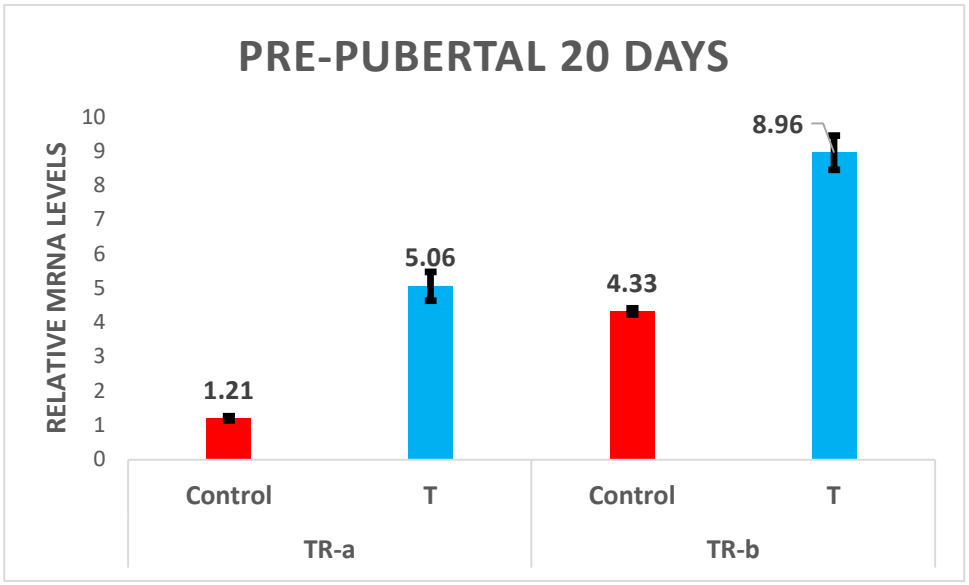

Figure (11): effect of thyroxin on expression level (fold changes) of hypothalamic *TR-α and TR-β* pre-pubertal at 20 days of treatment in male rats.

Post-pubertal 20 days

In the present study, significant elevation ($p<0.05$) of both in hypothalamic ***TR-α and TR-β*** genes expression levels (fold changes) have been shown in thyroxin treated males in comparison with control, started after 10 days of treatment (figure 12).

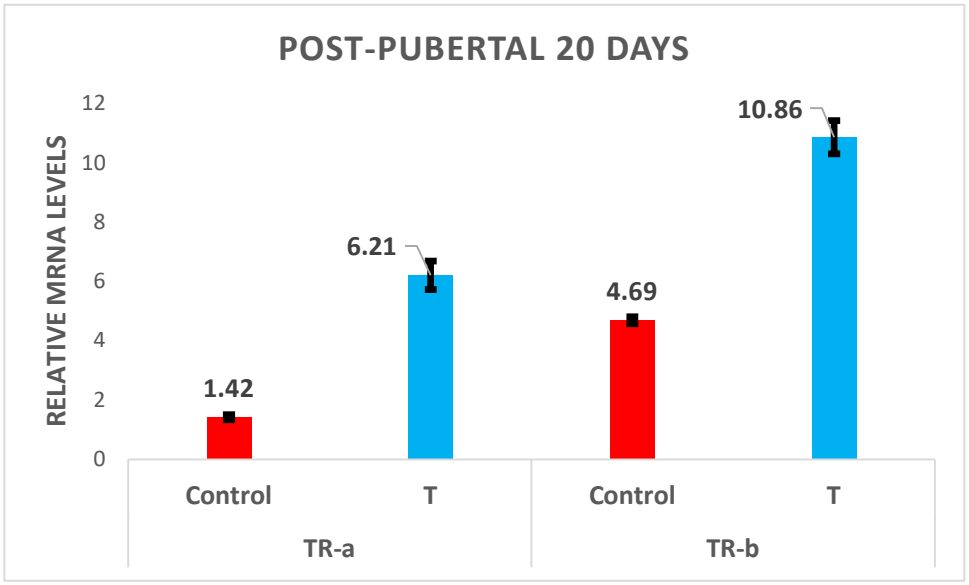

Figure (12): effect of thyroxin on expression level (fold changes) of hypothalamic *TR-α and TR-β* post-pubertal at 20 days of treatment in male rats.

Discussion

All male rats showed normal activity and body health throughout the five periods of the present study. This finding indicated that drenching of thyroxine at the given dose (??? mg/kg b.w.) and given periods (10, 20) has no side effects effect on body functions in normal rats. This study was conducted for the purpose of verifying the effect of thyroxine on pre-pubertal and post-pubertal male rats for two periods of 10 m and 20 days. The results showed that there is an effect of thyroxine on both pre-pubertal and post-pubertal alike in both time periods.

All tissue samples used in the present study gave a high concentration of total RNA and appeared quantitatively enough to proceed in quantitative real-time reverse transcriptase PCR. In the present study, significant elevation ($p<0.05$) of both pituitary TR-α and TR-β gene expression levels (fold changes) have been shown in thyroxin-treated males in comparison with control, starting after 10 days of treatment. Normal reproduction highly depends on the interaction between the gonadal axis and the thyroid.

In the initial investigations describing the presence of specific thyroid hormone nuclear binding sites, tissue-enriched extracts and growing rat testes were employed; these results were revolutionary since they challenged the long-held belief that the testes is unresponsive to thyroid hormone (Marchlewska et al., 2015). Several molecular approaches, including RT-PCR (mRNA expression), in situ hybridisation, western blotting, and immunohistochemistry, demonstrated that the functioning TR isoforms TR1 and TR1 are present in testicular cells. In the testes of rats and humans, an ontogenetic pattern of TRs expression was identified (Hernandez & Martinez, 2020). These investigations demonstrated that TR1 is absent from the testes of both humans and rats and that the active TR1 isoform is expressed in varying amounts at various stages of development. Tissues are the primary target cells for T3 activity in the testes, with expression peaking throughout the late foetal and early neonatal stages (Hernandez & Martinez, 2020). Recent literature reviews indicate that active TR isoforms, including TR1, are present in Leydig cells, peritubular cells, and germ cells during neonatal development and the adult testes (Rodrigues et al., 2022). These results demonstrated that T3 binding capacity is not completely lost in adult testes, even though its expression is highest during perinatal development and after that declines.

Observations of reproductive function and thyroid hormone levels in experimental animals are always correlated. This study focused on how thyroid hormones may alter the development of puberty in young rats. In this study, we evaluated the morphology and thyroid receptor gene expression levels in hypothalamic in the testes of hyperthyroidism pubertal male rats that were later treated with thyroid hormones (Hernandez & Martinez, 2020). This is the first prospective study to assess the efficacy of TSH, T3 and T4 in treating hyperthyroidism patients. Previous research has demonstrated that an imbalanced thyroid can retard growth in both newborn and adult rats; our findings confirm this idea. A metabolic feedback loop links thyroid hormone levels to fat storage. When the thyroid hormone is produced in excess, it frequently accelerates the body's basic metabolic rate, resulting in an increased energy supply and, paradoxically (Xing et al., 2014).

Testosterone levels were higher in the hyperthyroidism and lower in the hypothalamic and hypothalamic +T4 groups, demonstrating that thyroid hormones affect Leydig cell function. In contrast, total testosterone levels increase with hyperthyroidism but decrease with hypothyroidism, decreasing LH and FSH levels and total serum testosterone. Testosterone levels in neonatal hypothyroid animals had no deleterious effect on adult testicular function, showing that the tissue is more important than their quantity in determining differences in testosterone output (Kyritsi & Kanaka-Gantenbein, 2020).

We also noticed a change in testicular morphology after 20 days of thyroid failure in premature mice. Like Gnocchi et al. (2016) we discovered that the diameter of the seminiferous tubules rose in the hypothalamic and hypothalamic +T4 groups but decreased in the hyperthyroidism. The hyperthyroidism and hypothalami groups had more tissues than the control, but the hypothalamic +T4 group contained fewer. Previous research has demonstrated that the number of Leydig cells in neonatal rats aged 16 days' increases with hyperthyroidism and decreases with hypothyroidism. Another study discovered that adult rats with hypothyroidism had larger testicles, generated more sperm, and had a greater number of Leydig cells, but their steroidogenic function was diminished. In contrast to hyperthyroidism, hypothyroidism has been shown to suppress the growth of tissues during the pre-adolescent stages (Stagi et al., 2010; Wagner et al., 2008).

Rodrigues et al. (2022) reported that mice and rabbits given excessive doses of thyroid hormone had testicles and seminal vesicles that were considerably smaller. In newborns with induced hypothyroidism, developmental impairments were identified.

Prior research revealed no link between thyroid hormones and sperm quality. TRab has already localised to the testicles by the time rats are 20 days old, consistent with prior research in both humans and rats suggesting that particular TRs present in tissues impact the effects of thyroid hormone (Kushchayeva et al., 2019).

PCNA is required for RNA replication and repair in proliferating eukaryotic cells, and its expression decreases with cell inactivity. Here, we assessed how well PCNA localisation works for analysing germ cell proliferation in the rat testes after being subjected to various thyroidal therapies (Stagi et al., 2010). Accordingly, by demonstrating the variations in PCNA expression between the groups, our findings unmasked the effects of thyroid hormones in testes. The decreased PCNA expression and the increase in oxidative stress were previously linked in research conducted on rats with hyperthyroidism. Therefore, tissue proliferation appears to be prolonged in neonatal hypothyroidism, and the number of Tissues, testes weight, and daily sperm production are all elevated after recovery to euthyroidism. Consistent with observations for hyperthyroidism and hypothalamic groups, we found that PCNA expression was higher in the control group than in the hypothyroid group (Kyritsi & Kanaka-Gantenbein, 2020). In contrast, the hypothalamic +T4 group showed normal expression of PCNA, which may have been the result of the modified effects of T4. PCNA shows high staining in spermatogonia of the control and hypo- T+T4 groups, which is consistent with the idea that the effectiveness of spermatogenesis is tied to the proliferative activity of spermatogonia and germ cell losses during meiosis and spermiogenesis (Oliveira et al., 2020).

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