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Effect of experimentally induced prepubertal hyperthyroidism on postubertal reproductive activity in male rats

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Abstract

This study was carried out to investigate the effect of induced prepubertal hyperthyroidism on the reproductive functions of male rats at the pubertal stage. Hyperthyroidism was induced by supplementing thyroxin in drinking water (0.002% w/v) and drenching of 200 µg/kg body weight. Sixty immature males (aged 50 days) were allocated to control and hyperthyroid (PH) groups, administered with distilled water and thyroxin, respectively. Each group was subdivided into three subgroups, sacrificed after 15 days (C15 and PH15), after administration for 15 days and left without treatment for 15 days (C15+ and PH15+), or after 30 days (C30 and PH30). After each period, body weight and relative weight of genital organs were recorded. Serum concentrations of thyroid stimulating hormone (TSH), thyroxin (TT4), triiodothyronine (TT3), follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone was assessed. The expression levels of testicular *inha* and thyroid hormone receptor (THR) genes were analyzed. Histopathological examination of testis was studied. Compared with control, PH group male rats showed decreased body weight gain and genital organ weights at all experimental periods, increased levels of serum TT4, TT3, and LH, decreased levels of TSH, FSH, and testosterone, and lower expression levels of testicular *inha* and THR genes. Testicular sections of PH group male rats, showed reduced germinal epithelium, vacuolation, and decreased the number of spermatocytes and Sertoli cells compared with control. In conclusion, the disturbed fluctuations of sex steroid hormones due to prepubertal hyperthyroidism might cause retardation of the testes' development.

Introduction

Gonadotropins (Gn) are the primary regulators of testicular function, but the relationship between thyroid hormones and gonadotropins is controversial. Unaltered luteinizing hormone (LH) and follicle stimulating hormone (FSH), declined LH and FSH, raised LH and FSH, reduction in LH with unaltered FSH, and increased FSH with normal LH are the five types of circulating gonadotropins observed in hypothyroid and hyperthyroid diseases [1]. These inconsistencies are attributed to differences in the age of the animals used for the experiment, the length, severity, and mode of induction of

hypothyroidism or hyperthyroidism (acute or chronic), and the species used for the experiment [2]. Thyroid hormones have a vital role in many physiological activities [3], including the proper reproductive function of animals, as they are essential in controlling the development and regulation of the metabolic activities of male and female reproductive organs particularly the gonads [4]. Thyroid hormones (T4 and T3) perform their actions either directly through nuclear thyroid receptors or indirectly by interactions with various hormones and growth factors, such as testosterone and insulin-like growth factors (IGF), or by manipulating the release of hypothalamic gonadotropin releasing hormone (GnRH) and/or pituitary

gonadotropic hormones (FSH and LH) (5). Moreover, it has been suggested that many cases of low fertility or infertility in animals could be related to thyroid dysfunction (6). Hyperthyroidism refers to a high production of THs, which must be differentiated from thyrotoxicosis, since thyrotoxicosis refers to the exposure of body tissues to high concentrations of thyroid hormones. However, hyperthyroidism is mainly the cause of thyrotoxicosis (7). Increased antibodies usually cause hyperthyroidism against TSH receptors in domestic animal species (8). Specific evidence suggests a link between hyperthyroidism and infertility (1), with some studies indicating that many males and females with hyperthyroidism suffer from primary or secondary infertility (8,9).

Because thyroid dysfunctions are associated with several physiological alterations, including reproductive dysfunctions in animals, this study aimed to evaluate the relationship of prepubertal hyperthyroidism on pituitary and testis physiology at puberty.

Materials and methods

Experimental animals

The current study has been conducted in the animal house of the College of Veterinary Medicine, the University of Al-Qadisiyah, following the National Research Council's Gide for the Care and Use of Laboratory Animals. The experiment was approved by the Ethical Council of College of Veterinary Medicine, University of Al-Qadisiyah. Premature male rats aged 50 days and weighted 110-120 g were used in the current study. Male rats were housed under controlled temperature 22-23ºC, dark and light (12D:12L), and feeding *ad lebitum*.

Induction of hyperthyroidism

Hyperthyroidism in premature male rats was induced by administering thyroxin (0.002% w/v) in drinking water and intragastric gavage of thyroxin 200 µg/kg body weight. Hyperthyroidism was ensured by the assessment of serum levels of T3 and T4.

Blood sampling and sera preparation

After general anesthesia with ketamine and xylazine, trunk blood samples from female rats were obtained from the abdominal vein into the non-heparinized tube. Immediately, the serum was isolated, by centrifugation of blood samples at 5000 rpm for 10 minutes, then aspired into an Eppendorf tube and reserved at -20°C until hormonal assay (10).

Experimental protocol

Sixty premature male rats (aged 50 days and weighted 110-120 g) were weighted (to record the initial weight) and equally distributed to control and hyperthyroid (PH) groups; daily administrated with distilled water or thyroxin (0.002% w/v with drinking water and 200 µg/kg body weight by gavage), respectively. Each group was subdivided into three subgroups (10 males each). First subgroup males (C15 and PH15) were sacrificed after daily administration for 15 days (at day 65 of age), and second subgroup males (C15+ and PH15+) were sacrificed after daily administration for 15 days and left without administration for another 15 days (at day 80 of age), and third subgroup males (C30 and PH30) were sacrificed after daily administration for 30 days (at day 80 of age).

After each treatment period, the final weight of the males was recorded, then sacrificed, anesthetized, dissected, and venous blood samples were obtained. The relative weight of genital organs (testis, epididymis, seminal vesicle, and prostate) was recorded. The TSH, total T3, total T4, FSH, LH, and T were assessed according to the manufacturer's instructions (Kits from China and Shanghai Biological Co. Ltd, China). Samples from testicular tissue were obtained for molecular analysis of testicular *inha* and THR gene expression levels by using qRT-PCR. Samples from other testis were also obtained for histopathological examination (11).

Total RNA extraction

TRIzol® reagent kit was used for total RNA extraction from male rats' pituitary and testicular tissues. The extraction procedure was achieved according to the instruction of the manufacturer company (Bioneer, Korea).

Estimating quantity and purity of extracted total RNA yield

Nanodrop spectrophotometer (THERMO. USA) was used to measure the quantity of extracted RNA. To control the quality of the extracted RNA, the concentration (ng/µL) and the purity of RNA were measured, as described by the manufacturer (Promega company, USA).

DNase I treatment

Using DNase I enzyme kit, the trace amounts of genomic DNA were removed from the eluted total RNA (accordingly to the procedure described by Promega company, USA).

cDNA synthesis

DNase-I treated total RNA samples were utilized in the cDNA synthesis stage by employing the AccuPower® RocktScript RT PreMix kit (Bioneer company, Korea).

qRT-PCR master mix preparation

It was done by using AccuPower[TM] Green Star Real-Time PCR kit depending on SYBER Green dye determination of gene amplification in Real-Time PCR system (Bioneer company, Korea).

The data analysis of qRT-PCR

The levels of the relative quantification gene (fold change) Δ^{CT} Livak approach was used to test the obtained data of qRT-PCR for studied and housekeeping genes. This approach was described according to Livak and Schmittgen (12). The amount gained from the qRT-PCR experiment and the relative quantification procedure was normalized to meaningful biologic data. In this approach, the calibrator is one of the studied samples like control samples, where the relative values of the expression level were produced by dividing every normalized target value (CT values) using the normalized target value of the calibrator.

Statistical analysis

Data analysis was performed using the GraphPad Prism-Version 5 (SAS Institute, Inc., USA). Results were expressed as mean ± standard deviation. One-way ANOVA was used with Newman- Keuls (13) to calculate the significant differences among means. P<0.05 is considered significant.

Results

As illustrated in table 1, body weight gain and relative weights of testis, epididymis, prostate, and seminal vesicle of PH group male rats revealed a significant decrease (P<0.05), at all experimental periods (PH15, PH15+, and PH30), in comparison with the corresponding subgroups of control males in a dose-dependent manner.

When compared with the corresponding subgroups of control, PH group male rats recorded a significant decrease (P<0.05) in serum TSH concentration at all experimental periods (Figure 1). In comparison between the subgroups, control subgroups showed no significant (P>0.05) changes between each other, while PH30 recorded the lowest (P<0.05) concentration among PH subgroups, followed by PH15, whereas PH15+ showed the highest (P<0.05) concentration among them. Serum TT3 and TT4 concentrations of PH subgroups (Figure 1) revealed a significant (P<0.05) increase in corresponding subgroups of control. When comparing the PH subgroups, the serum TT3 level of the PH30 subgroup recorded significantly higher level (P<0.05), and that of the PH15+ subgroup was the lowest (P<0.05) level in comparison with the PH15 subgroup. However, the TT4 level of Ph15 and PH15+ subgroups recorded no significant (P>0.05) between each other, but they were significantly (P<0.05) higher than PH30.

In comparison with control subgroups, PH subgroup male rats recorded a significant decrease (P<0.05) in serum FSH and T concentrations at all periods (Figure 2), and a significant increase (P<0.05) of LH concentration at PH15 and PH30 periods but not changed at PH15+ period (Figure 2). In comparison between the subgroups, the FSH concentrations of control subgroups showed no significant (P>0.05) difference between each other, while the levels of PH15 and PH15+ subgroups recorded no significant (P>0.05) between each other, but they were significantly (P<0.05) higher than PH30. Serum LH level of the PH30 subgroup recorded a significantly higher level (P<0.05), and that of the PH15+ subgroup was the lowest (P<0.05) level in comparison with the PH15 subgroup. However, the T level of the PH15+ subgroup recorded the higher level, and PH30 recorded the lower level in comparison with PH 15 subgroup.

Table 1: The effect of prepubertal hyperthyroidism on the weight of reproductive organs relative

Parameters	Periods	Groups	
		C	PH
Body weight (g)	15 d	135.5 ± 2.498 Ca	117.8 ± 2.033 Cb
	15 d+	164.4 ± 2.891 Ba	139.6 ± 2.318 Ab
	30 d	168.3 ± 3.127 Aa	126.7 ± 2.188 Bb
Testes (g/100g)	15 d	1.115 ± 0.114 Aa	1.003 ± 0.075 Ab
	15 d+	0.853 ± 0.089 Ba	0.718 ± 0.079 Bb
	30 d	0.888 ± 0.093 Ba	0.633 ± 0.083 Bb
Epididymis (g/100g)	15 d	0.733 ± 0.095 Ba	0.424 ± 0.043 Bb
	15 d+	0.822 ± 0.101 Aa	0.631 ± 0.051 Ab
	30 d	0.813 ± 0.089 Aa	0.349 ± 0.062 Bb
Seminal vesicle (g/100g)	15 d	0.632 ± 0.079 Aa	0.489 ± 0.033 Bb
	15 d+	0.687 ± 0.089 Aa	0.588 ± 0.041 Ab
	30 d	0.652 ± 0.083 Aa	0.401 ± 0.038 Cb
Prostate (g/100g)	15 d	0.421 ± 0.066 Aa	0.232 ± 0.022 Bb
	15 d+	0.452 ± 0.058 Aa	0.328 ± 0.028 Ab
	30 d	0.454 ± 0.069 Aa	0.210 ± 0.031 Bb

Data were presented as Mean ±SD. Different small letters denote significant differences (P<0.05) between groups for each period. Different capital letters denote significant differences (P<0.05) between periods for each group.
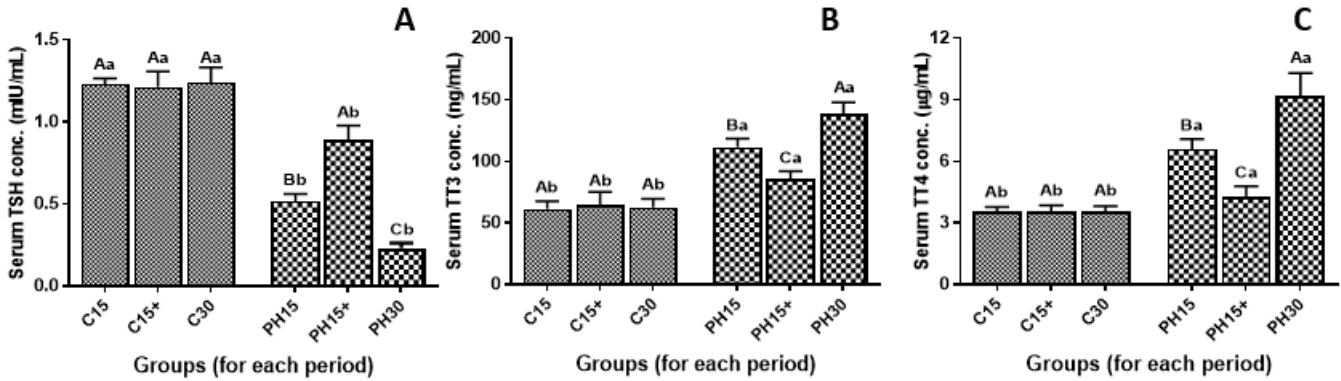
Figure 1: Effect of prepubertal hyperthyroidism on serum TSH (A), TT3 (B), and TT4 (C) concentrations in male rats during puberty.
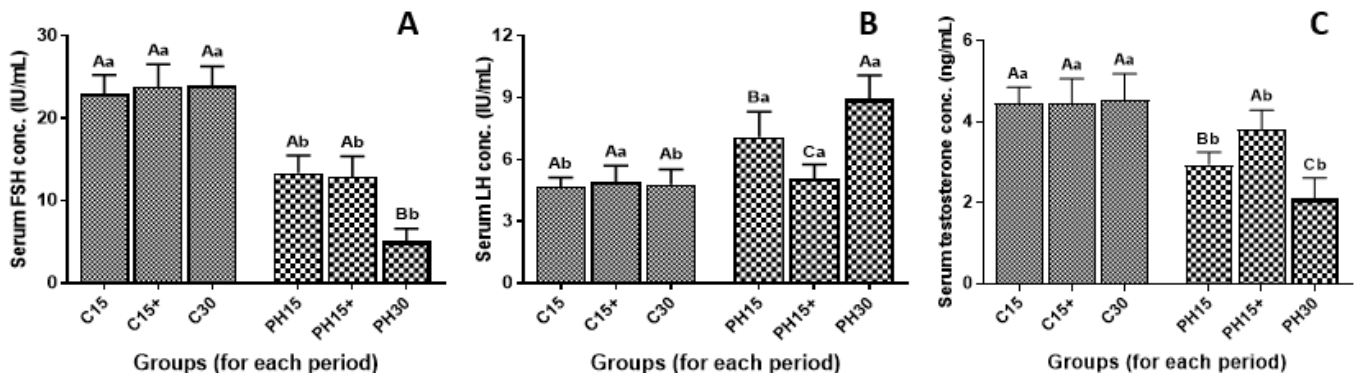

Figure 2: Effect of prepubertal hyperthyroidism on serum FSH (A), LH (B), and testosterone (C) concentrations in male rats during puberty.

In the control group of male rats, significant elevation ($P<0.05$) of testis *inh-α* gene expression level (fold changes) has been shown in the C15+ and C30 periods in comparison with the C15 period, but they showed no significant difference ($P>0.05$) when compared with each other. Whereas PH15 and PH30 periods revealed no significant difference ($P>0.05$) between each other, they were significantly ($P<0.05$) lower than PH15+. The comparison between the groups revealed that the PH group showed a significant decrease ($P<0.05$) in all of the experimental periods compared with the corresponding periods of the control group (Figure 3). As illustrated in figure 3, in both experimental groups, male rat testes showed significant elevation ($P<0.05$) of testis THR gene expression levels (fold changes) in at 30d period (C30 and PH30) compared with 15 and 15+ periods (C15 and C15+ or PH15 and PH15+). When comparing the experimental periods for each group, the results showed no significant differences ($P>0.05$) between the two corresponding periods (C15 and PH15, C15+ and PH15+, or C30 and PH30).

After 15 days of treatment, histological sections obtained from the testes of control (C15) and hyperthyroid

group (PH15) group male rats, revealed the typical architecture of immature seminiferous tubules in the C15 subgroup, and immature seminiferous tubules with decreased number of Sertoli cell nucleus, germinal epithelial cells, spermatogonium, primary spermatocytes and interstitial cells in PH15 subgroup. On the other hand, after 15 days of treatment and 15 days without treatment, testes sections of control (C15+) and hyperthyroid (PH15+) groups, show normal development with empty seminiferous tubules in the C15+ subgroup. In contrast, subgroup PH15+ shows empty seminiferous tubules with decreased number of Sertoli cells, spermatogonia, primary spermatocytes and spermatids. Testis sections of control (C30) and hyperthyroid (PH30) groups, after 30 days of treatment, revealed developed testicular tissue with typical architecture in the C30 subgroup, whereas the PH30 subgroup revealed a low density of sperms inside dilated seminiferous tubules, with decreased number of secondary spermatocytes, Sertoli cell nuclei, and Leydig cells (Figure 4).
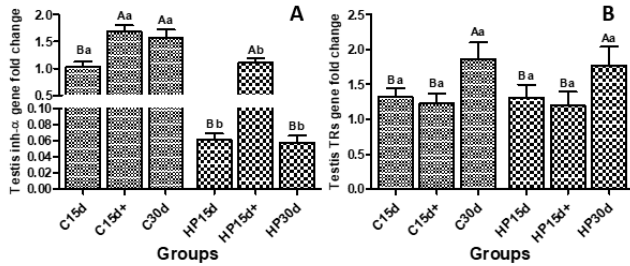
Figure 3: Effect of prepubertal hyperthyroidism on testicular *inha* (A) and THR (B) gene expression levels in male rats during puberty.
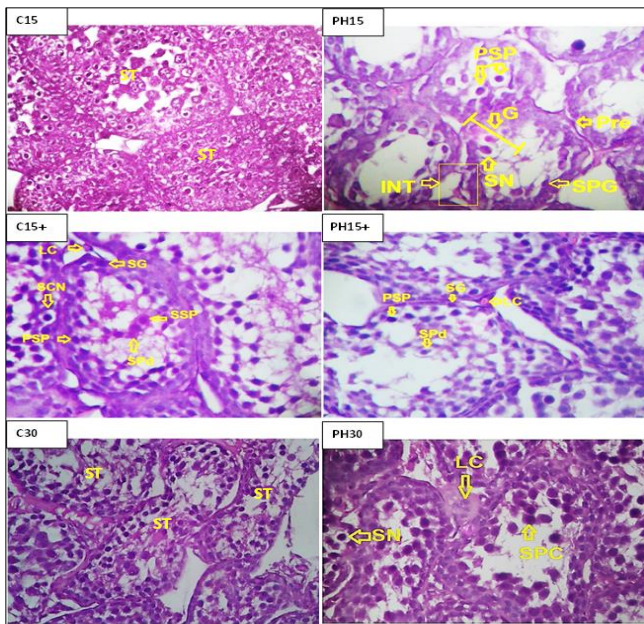

Figure 4: Cross sections of testes from control and hyperthyroid rats after 15 days of treatment (C15 and PH15), 15 days of treatment, 15 days without treatment (C15+ and PH15+), and 30 days of treatment (C30 and PH30). C15: shows immature seminiferous tubules (ST). Ph15: shows immature seminiferous tubules, decreased number of Sertoli cell nucleus (SN), germinal epithelial cells (G), spermatogonium (SPG), primary spermatocytes (PSP), and interstitial cells (INT). C15+: shows Sertoli cell nuclei (SCN), spermatogonium (SG), primary spermatocytes (PSP), secondary spermatocytes (SSP), spermatids (SPd), and Leydig's cells (LC). PH15+: shows decreased number of Sertoli cells, spermatogonia (SG), primary spermatocytes (PSP), and spermatids (SPd). C30: shows seminiferous tubules (ST). PH30: shows dilated and low density of sperms inside the seminiferous tubules, decreased the number of secondary spermatocytes (SPC), Sertoli cell nuclei (SN), and Leydig's cells (LC). H&E, 400X.

Discussion

Body weight

General findings revealed that the disturbances that occur in the manufacture and secretion of thyroid hormones affect almost most of the functions of the body's systems at the pre- and post-pubertal stage, including the functions of the reproductive system, as it is known that thyroid hormones have direct or indirect roles in the regulation of metabolic processes and energy production within the body (14). Because thyroid hormones have several essential roles in the regulation of growth, development, differentiation, and the metabolism of different macromolecules such as lipids, proteins, and carbohydrates, the significant decrease in body weight gain in PH subgroups in comparison to control subgroups could be due to elevated thyroid hormone levels (8,15), leading to pathophysiologic changes in most body systems as a metabolic syndrome. According to Singh and Beigh (15), a high BMR leads to a negative energy balance which leads to weight loss. Meanwhile, with the reduction in metabolic rates, the male rats did not gain the expected weight as in control.

Genital organ relative weights

The decreased relative weight of the genital organs, particularly the testicles, in the control and treatment male rats with age seems normal if we know that the increase in body weight was greater than the increase in the weight of the genital organs, especially since the animals were in the process of growth. However, the comparison between the two groups for each stage of the experiment showed a decrease in the relative weights of the genital organs of the treatment group. This indicates that hyperthyroidism negatively affect the growth and development of reproductive organs. All stages of growth and development of male genital organs require the impact of balanced thyroid gland activity; therefore, this retardation of reproductive organ weights might be related to abnormalities of thyroid hormones and/or their receptors on their tissue cells. The injection of slightly supra-physiological T4 dosages to immature male rats aged less than 4 weeks resulted in a propensity toward early maturity and a shortening development period. Larger TH doses resulted in a propensity toward early maturity and smaller testes and seminal vesicles in both mice and rabbits (16). It has been postulated that testicular proliferation, spermatogenesis and steroidogenesis are under the coordinative action of pituitary gonadotropins, metabolic hormones (GH and thyroid hormones), and local growth factors (17). In the present study, decreased testicular weight could also be attributed to increased thyroid hormone effect directly on the pituitary gland secretion of gonadotropin or indirectly through PRL elevation. The present study recorded a decline in serum FSH levels in hyperthyroid male rats. Therefore, the dysfunction of the

pituitary-testicular axis could play a role in decreased testicular tissue proliferation.

Thyroid stimulating hormone and thyroid hormones

The elevated levels of serum TT4 and TT3 and declined level of serum TSH in hyperthyroid male rats in comparison with control results from the suppression of pituitary TSH secretion as a result of negative feedback role by exogenous administration of T4. Although T4 is the primary hormone produced by the follicular cells of the TG which have a long half-life, it is less biologically active in comparison with T3 which is primarily synthesized outside of the thyroid gland with a short half-life (18). Therefore, hyperthyroid male rats recorded a lower ratio of T3 to T4 compared to the control.

Reproductive hormones

In hyperthyroid male rats, the results revealed decreased levels of serum FSH, which might affect the growth, development, and functional status of the testes which could decrease the spermatogenic and steroidogenic activity of the testis, and a decrease in the ability of Leydig cells to synthesize androgens (mainly testosterone). Thyroid hormones, on the other hand, regulate the growth and differentiation of testicular tissues (19). Moreover, although LH levels increased, the present study demonstrated a significant decline in serum testosterone levels in hyperthyroid male rats. Due to the changes in reproductive hormones, it can be confirmed that hyperthyroidism has an essential association with retard testicular function and male fecundity via affecting the reproductive axis (20,21). The testes and pituitary gland are among the target tissues for thyroid hormones. Thyroid hormones regulate the testes' growth, development, and metabolic activities. In addition to stimulating the secretory activity of the anterior lobe of the pituitary gland, any imbalance in the concentration of thyroid hormones or decreased sensitivity of their receptors leads to a defect in testicular growth and development, as TGs are increasingly being recognized as playing a vital role in male reproductive activities (16,22). TRs are distributed in most body tissues, including testicular tissue, where any down-regulation of thyroid receptors, decrease Sertoli cells, Leydig cells, and germ cells and lower male fertility (14). T3 appears to be a constituent of the neurological and endocrine systems that governs seasonal cycle of reproduction in different species of animals (23), as well as its responsibility in the cessation of reproduction at the conclusion of the reproductive season (22). Similarly, Krassas and Perros (24) suggested that hyperthyroidism negatively impacts seminal human parameters. According to Rijintjes *et al.* (25), hyperthyroidism inhibits Leydig cell formation and negatively impacts spermatogenesis in rat research. Therefore, it is clear that the decreased serum levels of FSH and LH in treated rats are not indicative of an intrinsic inability of the pituitary to maintain gonadotropin levels within the range observed in control males. Instead, the normally decreased FSH and LH concentrations in treated males appear to reflect at least in part an alteration in the pituitary' responsiveness to GnRH stimulation and, possibly, pituitary and/or hypothalamic sensitivity to gonadal feedback. The changes occurring in the expression levels of the testicular THR genes in the current study are related to the changes in the levels of thyroid hormones, as the level recorded a significant increase in hyperthyroid female rats. However, it seems that the disturbance could be related to the sensitivity of TRs to THs, since the function of thyroid hormones, particularly T3, either through genomic action inside the nucleus by increasing the level of protein synthesis, or through non-genomic action outside the nucleus (26). The current results showed a decline in the expression levels of the *inha* gene in testicular tissues of hyperthyroid male rats, which could be related to the decline of FSH production, as it has been postulated that the production of inhibin is in Sertoli cells under the direct influence of the FSH.

Testicular histophysiology

Normal reproductive processes require normal proliferative processes of testicular tissue (27). Indeed, the effects of T4 on spermatogenesis are conflicting, but it would appear that T4 does not exert a direct effect on spermatogenesis in mature rams (28). Furthermore, maximum Sertoli cell proliferation corresponds to maximum T3 binding capacity in the testis, implying that the Sertoli cell is the primary target of T3 activity. T3 does, however, play a function in seminiferous epithelial development, and investigations in rodents have revealed that T3 is a crucial component in Leydig cell maturation. T3 is required for mesenchymal cells to differentiate into Leydig progenitor cells, and it works in concert with other hormones, including LH and IGF-I to stimulate Leydig cell development (29).

Conclusion

In conclusion, the disturbed fluctuations of sex steroid hormones due to prepubertal hyperthyroidism might cause disturbances, in sexual desire and the retardation of the testes' development. Thus, the low reproductive outcomes in hyperthyroid male rats could result from inhibiting testosterone secretion.

Conflict of Interest

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تأثير فرط الدرقية المستحث تجريبيا قبل البلوغ على تكاثر ذكور الجرذان بعد البلوغ

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فرع الفسلجة والأدوية والكيمياء الحياتية، كلية الطب البيطري، جامعة القادسية، العراق

الخلاصة

أجريت الدراسة الحالية للتحري عن تأثير فرط الدرقية قبل البلوغ في الوظائف التكاثرية لذكور الجرذان في مرحلة البلوغ. تم استحداث فرط الدرقية عن طريق إعطاء الثيروكسين مع مياه الشرب (٠,٠٠٢٪ وزن / حجم) مع تجريع الثيروكسين (٢٠٠ ميكروجرام / كغم من وزن الجسم). تم توزيع ٦٠ جرذا ذكرا بعمر ٥٠ يوما على مجموعتي السيطرة والمعاملة (٣٠ لكل مجموعة)، جرعت الماء المقطر والثيروكسين، على التوالي. قسمت كل مجموعة الى ثلاث مجموعات ثانوية، تمت التضحية بالأولى بعد ١٥ يوما من المعاملة، وتركت ١٥ يوما أخرى بدون معاملة، أو بعد ٣٠ يوما من المعاملة. في نهاية مرحلة علاجية، تم تخدير وتشريح ١٠ ذكور من كل مجموعة وجمع منها عينات دم لغرض حساب تراكيز الهرمون محفز الدرقية والثيروكسين ٣ الكلي والثيروكسين ٤ الحر والهرمون محفز الجريب والهرمون المصفر والتستوستيرون. كما أخذت عينات من أنسجة الخصى لغرض

المعاملة وانخفاض تعبير جيني مستقبلات الثيروكسين و *inha* في أنسجة الخصى. أظهرت مقطع أنسجة الخصى لذكور جرذان مجموعة المعاملة انخفاض في الظهارة الجرثومية، وخلخلة، وانخفاض عدد الخلايا المنوية وخلايا سيرتولي بالمقارنة مع السيطرة. يستنتج أن التقلبات المضطربة لهرمونات الستيرويد الجنسية بسبب فرط نشاط الغدة الدرقية قبل البلوغ قد تسبب تأخرًا في نمو الخصيتين.

قياس مستوى تعبير جين مستقبلات هرمونات الدرقية وجين *inha*. وأخذت عينات أخرى لغرض الدراسة النسجية المرضية. عند المقارنة مع السيطرة، بينت نتائج مجموعة فرط الدرقية انخفاضا في الكسب الوزني والأوزان النسبية لأعضاء التكاثر في جميع مراحل الدراسة، مع زيادة تركيز هرمونات الثايرونين ثلاثي اليود والثيروكسين والهرمون المصفر في مصل الدم ونقصان تركيز الهرمون محفز الدرقية والهرمون محفز الجريب والتستوستيرون في مجموعات