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Different Stages of Hyperthyroidism: Alterations in Proliferation, Apoptosis, and Histology of Female Rat Ovary

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ABSTRACT

The effects of different stages of hyperthyroidism on female estrous cyclicity, mediators of inflammation, proliferation, and apoptosis in rats' ovaries were evaluated in this study. Mild, moderate, and severe hyperthyroidism were induced in adult female rats by daily oral administration of L-thyroxin for 10, 20, and 30 days, respectively. Serum levels of thyroid stimulating hormone (TSH), free triiodotyrosine (FT3), progesterone and estradiol II were assayed. The ovarian levels of interleukin-6 (IL-6) and insulin-like growth factor-1 (IGF-1) were also estimated. The localization and expression of both proliferating cell nuclear antigen (PCNA) and BCl-2 were evaluated in ovaries by immunohistochemistry. Ovarian and uterine histological alterations were examined microscopically. Mild hyperthyroidism did not change serum level of estradiol II but significantly decreased serum progesterone level, while moderate and severe hyperthyroidism significantly decreased the serum level of the two hormones. Mild hyperthyroidism did not alter the ovarian and uterine histological structure, ovarian IL-6 concentration, and expressions of PCNA and BCl-2, but it produced a slight reduction in the ovarian IGF-1 concentration. Increasing the severity of hyperthyroidism produced ovarian inflammation and apoptosis, which is evident from the measured parameters and the histological changes. In conclusion, moderate and severe hyperthyroidism altered estrous cyclicity, inflammatory, proliferative, and apoptotic markers in rats' ovary.

Keywords: Hyperthyroidism - Apoptosis - Ovary - Proliferation

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INTRODUCTION

Infertility is the inability to conceive after one year of regular intercourse in women < 35 years and after six months in women > 35 years [1]. According to World Health Organization statistics, 37% of infertile couples are due to female infertility, 8% due to male infertility, and 35% due to infertility in both couples. Some reports revealed that ovulatory disorders (25%), endometriosis (15%), pelvic adhesions (11%), tubal blockage (11%), other tubal abnormalities (11%), and hyperprolactinemia (7%) are the common causes for female infertility. While other reports revealed that ovulatory disorders account for more than half of the female infertility causes [2].

It is well known that thyroid hormones can affect the oocytes, sperm, and embryo during fertilization, implantation and placentation. However, the association between hyperthyroidism and infertility is evidenced and scarce and sometimes conflicting. Infertility may occur in hyperthyroid females, but euthyroidism can restore these abnormalities [3]. Thyroid hormones are described to affect both reproduction and pregnancy [4] via thyroid hormone receptors (TR) and TSH receptors (TSHR) , which are present in the endometrium as well as ovarian granulosa and stromal cells during follicular development. Thyroid receptors are expressed throughout the different phases of the menstrual cycle: TRα1 and TRβ1 are expressed during the mid-luteal phase and increase during the secretory phase [5]. Thyroid disturbances are the most common endocrine disease in females at reproductive age [6]. Joshi et al. [7] reported that 5.8% of primary or secondary infertility is associated with hyperthyroidism. Hyperthyroid animals and humans have many reproductive abnormalities including menstrual disturbance, reduced fecundity, and elevated morbidity during pregnancy [8].

The mammalian ovary is a critical organ for reproduction by supporting the development of the oocyte. The ovarian follicle is the functional unit of the ovary and is necessary for the development of a mature oocyte and the production of steroid sex hormones [9], therefore, controlling the proliferation as well as apoptosis of the ovarian follicles is a vital and complicated process in which many factors are implicated.

Many eukaryotic organs including ovaries can express proliferating cell nuclear antigen (PCNA); a 36 kDa protein [10]. Localization of PCNA in the ovary varies. Oktay et al. [11] reported that the granulosa cells or oocytes of primordial follicles of rats did not express PCNA but its expression is elevated with the initiation of follicle growth. Another study of Picut et al. [12] found that the expression of PCNA protein is a marker for ovarian follicle counts. PCNA is important in many cellular processes including DNA replication & repair, sister-chromatid cohesion, DNA damage avoidance, cell cycle control, and cell survival [13]. Furthermore, PCNA is a key regulator of the growth of ovarian follicles [14].

Females are born with a fixed number of primordial follicles; the source of all oocytes needed for future ovulations [15]. Throughout growth, the pool of primordial follicles is gradually depleted due to death or their natural conversion into growing follicles. Most of the ovarian follicles undergo atresia, which is characterized by apoptosis of the granulosa cells and oocyte degeneration [16], with only a few oocytes surviving to be ovulated [17]. Over time, the ovarian reserve declined until females become infertile. Apoptosis is a key marker for determining the number of primordial follicles established and maintained within the ovary [18]. Aitken et al. [19] highlighted the role of intrinsic or B cell lymphoma-2 (BCl-2)-regulated intrinsic apoptotic pathway in the ovary. In the ovary, BCl-2 has many functions including the regulation of germ cell loss during ovarian development and the determination of primordial follicles numbers maintained in the adult ovary [20].

Insulin-like growth factor-1 (IGF-1) is a regulator of follicle function in the ovary. In granulosa cells, IGF-1 stimulates hormone secretion and follicular growth and stops programmed cell death of the mature follicles [21]. Several reports have shown that numerous cytokines are involved in regulating ovarian function [22], one of them is interleukin-6 (IL-6) is a pleiotropic cytokine known to activate acute phase proteins and maintain chronic inflammatory states in infertility [23]. This cytokine is elevated in patients with the acute pelvic inflammatory disease, idiopathic infertility, and patients with endometriosis [24].

Up till now, there are no investigations about the effects of different stages of hyperthyroidism on the female reproductive organs; ovary and uterus. So, the present study was undertaken to fulfill the effects of mild, moderate, and severe hyperthyroidism on a) The level of female sex hormones. b) Ovarian proliferating and apoptotic markers. c) The histological abnormalities in both ovary and uterus.

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MATERIALS AND METHODS

Animals

This study included 40 adult female Wistar rats (2 months old) bred in the breeding unit of the Egyptian Stock Holding Company for Biological Products of Vaccines, Sera and Drugs (VACSERA), Helwan, Egypt, weighing 180 ±10g, and with regular 4-day cycles. The rats were left for adaptation in the laboratory for one week before beginning the experimental work. Throughout the experimental period, rats were fed a standard pellet diet (20% casein, 15% corn oil, 55% corn starch, 5% salt mixture and 5% vitaminized starch, Egyptian Company of Oils and Soap, Kafr-Elzayat, Egypt) and *ad libitum*. The temperature in the animal house was kept at 23±2ºC with a relative humidity of 55± 5% under 12:12h light-dark cycle. The experimental protocol was approved by Research Ethics Committee (REC) for Animal Subject Research at National Hepatology & Tropical Medicine Institute (NHTMRI), 2017 (Serial No: 5-2017).

Induction of Hyperthyroidism

Hyperthyroidism was induced in adult female Wister rats by oral administration of L-thyroxin (Glaxos-Mithkline, Egypt) at a dose of 50 mg /100ml drinking water [25].

Study Design

The experiment was started in female rats from day 21 of weaning and were divided equally into four groups: Group I (Normal Control), orally injected with sterile saline; Group II (Mild Hyperthyroidism), orally injected with L-thyroxin daily for 10 consecutive days; Group III (Moderate Hyperthyroidism), orally injected with L-thyroxin daily for 20 consecutive days; Group IV (Severe Hyperthyroidism), orally injected with L-thyroxin daily for 30 consecutive days.

Blood Sampling and Collection of Body Organs

At the end of the experimental period, all rats were euthanized by intravenous injection with thiopental and subjected to a complete necropsy. Hematocrit capillaries were used to collect blood samples from the retroorbital system of each rat. Blood was incubated at 37°C for 30 min, then tubes were centrifuged at 2000 x g for 10 min and sera were collected, aliquoted, and stored at -20°C until analyses. Ovaries and uterus were excised, rinsed thoroughly in isotonic sterile saline containing heparin, and blotted dry with a filter paper. One ovary was kept in 10% formalin for histological and immunohistochemical examinations. The other ovary was kept intact in ice-cold sterile saline at -80˚C until analyses. The uterus was kept in 10% formalin for histological examination.

Histological Examination

Ovarian and uterine specimens of the different groups were stained by hematoxylin and eosin [26] and examined under the electric light microscope (Olympus, Japan).

Immunohistochemistry

Ovaries were fixed in 10% formaldehyde for 3 days followed by paraffin embedding, attachment of 5 mm paraffin sections to microscope slides, and heating at 60°C for 2 hours. After performing a de-paraffinization step, sections were re-hydrated via a series of graded ethanol/water solutions and then boiled in 10 mM citric acid (pH 6.0) at 100°C for 10 minutes followed by incubation in 3% hydrogen peroxide for 10 minutes. The tissues were blocked by 3% bovine serum albumin prepared in tween buffered saline (TBS) and incubated with a rabbit anti-rat PCNA monoclonal antibody (DAKO Japan Co, Tokyo, Japan; 1:200), or a rabbit anti-rat BCl-2 monoclonal antibody (DAKO Japan Co, Tokyo, Japan; 1:2000) overnight at 4°C. After rinsing thoroughly with TBS, the sections were incubated with a biotinylated and streptomycin labeled goat anti-rabbit immunoglobulin (Nichirei, Tokyo, Japan) for 15 minutes at room temperature. The expressions of PCNA and BCl-2 antigens in the ovarian sections were detected by the reaction of peroxidase with 3, 39-

diaminobenzidine tetrahydrochloride (Sigma, USA), counterstained with hematoxylin and analyzed using Olympus microscope and images were captured by a digital camera (Cannon 620).

Hormonal Profile

The concentration of serum-free triiodotyrosine (FT3) was measured using ELISA research kits provided from Diagnostic systems Laboratories (Taxes, USA). Thyroid stimulating hormone (TSH), progesterone, and estradiol II were assayed in serum using ELISA research kits provided by Biocheck, Inc (USA).

Preparation of Ovarian Homogenate

Ten percent of ovarian tissue homogenate was prepared by homogenization in ice-cold phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4), pH 7.4. The whole homogenates were centrifuged at 18000x g for 20 minutes at 4°C (Cooler Microfuge Laborzentrifugen, Sigma, Germany) to obtain the cytosolic fraction. The cytosolic fractions were then collected, aliquoted, and stored at -80˚C until analyses.

Ovarian Total Protein Measurement

The total protein concentration was determined in ovarian tissue according to the method of Bradford *et al*. [27].

Ovarian IGF-1 and IL-6 Measurements

The concentrations of both IGF-1 and IL-6 were assayed in the cytosolic fraction of ovarian homogenate using ELISA research kits provided from R&D Systems (USA) and Cusabio Technology LLC (China), respectively.

Statistical Analysis

Independent sample t-test was used to analyze statistically the individual data in the experimental groups using Statistical analysis was performed using the Statistical Package for Social Science version 20 for Windows (SPSS software package, Chicago, USA). P value was considered significant at $p < 0.05$.

RESULTS

Figure 1 illustrated a gradual increase in the body weight of the normal control rats attaining a weight gain of 4.82% at the end of the experimental period. On the other hand, induction of hyperthyroidism in rats by daily oral administration of L-thyroxin for 10, 20, and 30 days caused significant and gradual reductions in the rat body weights amounting to 4.34% ($p<0.001$), 13.69% ($p<0.0001$), and 17.86% ($p<0.0001$), respectively.

Induction of hyperthyroidism in groups II, III and IV caused significant reductions in serum TSH level (39.58, 96.52, and 98.6%, respectively, at $p<0.001$) together with significant elevations in serum FT3 level (23.94, 47.9, and 62%, respectively, at $p<0.001$), compared to the normal control rats (Table 1). Mild, moderate, and severe stages of hyperthyroidism caused significant reductions in serum progesterone level (20.17%, 41.18 and 53%, respectively, $p<0.001$), compared to the normal control group. Mild hyperthyroidism did not show any significant alteration in the level of serum estradiol II, however serum estradiol II level was significantly reduced in moderate and severe hyperthyroidism (9.38 and 14%, respectively, $p<0.001$), compared to the normal control rats (Table 1).

Table (2) showed that mild hyperthyroidism did not cause any significant disturbance in the concentration of ovarian IL-6 with a slightly significant reduction in the ovarian IGF-1 concentration (23.89% at $p<0.05$), compared to the normal control group. On the other hand, moderate and severe hyperthyroidism significantly elevated the concentration of ovarian IL-6 (42.3 and 100.8%, respectively, $p<0.001$) with significant reductions in the ovarian IGF-1 concentration (51.67 and 81%, respectively, $p<0.001$), compared to the normal control group.

Examination of H&E- stained sections of control rat's ovary revealed that the ovarian parenchyma was formed of cortex and medulla. In the ovarian cortex, the follicles were seen which were formed of a primary oocyte surrounded by clear zona pellucida and few follicular cells called corona radiata. The whole follicle was surrounded by theca interna, a secretory layer and theca externa, a fibrous layer (Figure 2.A). The corpus luteum was formed of both granulosa and theca lutein cells which were polyhedral cells containing large spherical nuclei and a large amount of vacuolated cytoplasm. The luteal cells were separated by spindle-shaped fibroblasts and blood capillaries. The stroma of the ovary was formed of connective tissue showing collagen fibers and blood vessels (Figure 2.A and Table 3).

Induction of mild hyperthyroidism for 10 days did not show any histological alterations in the ovaries of rats (Figure 2.B). However, the ovary of the moderate hyperthyroid group showed the beginning of degeneration in the ovarian follicles as well as the presence of many apoptotic bodies in the ovarian stroma (Figure 2.C). More deleterious effects were reported in severe hyperthyroid rats in the form of collapsed follicles and the presence of dilated and congested blood vessels in ovarian stroma (Figures 2.D). Most of the ovarian follicles in severe hyperthyroid rats were degenerated. The degeneration appeared in different forms; some follicles showed lysis of the oocyte and appearance of acidophilic material filling the cavity of the follicular antrum. The surrounding granulosa cells showed pyknosis of their nuclei and degeneration of their cytoplasm.

Uterine sections from the normal control group showed normal histology with regular columnar epithelial cells lining the uterine lumen and glands and normal blood vascularity. Fibrosis, necrosis, apoptosis or any other adaptive cellular changes were not observed in the control uterine tissue sections (Figure 3.A and B and Table 4). On the other hand, induction of mild hyperthyroidism in female rats caused vacuolations in the lumen epithelial cell and uterine glands (Figure 3.C). More histological abnormalities were seen in moderate hyperthyroid females in which congestion in uterine blood vessels and degeneration of the endometrial epithelial cells (Figure 3.D) were observed. Severe hyperthyroidism increased the thickness of endometrium lumen epithelium with the presence numerous apoptotic bodies. Moreover, many endometrium epithelial cells became apoptotic (Figure 3.E).

To investigate the localization of PCNA protein, a marker of cell proliferation in the ovary, immunohistochemical analysis was performed in the ovaries. Results showed that the PCNA was mainly localized in granulosa cells as well as oocyte of the ovarian follicle. The intensity of PCNA immunoreactivity in the control group was moderate (++) in the granulosa cells and weak or negative in the theca interna cells (Figure 4.A).

In mild and moderate hyperthyroid rats (Gr. II&III), PCNA was localized in granulosa cells, oocyte, and theca cells of the ovarian follicle with moderate immunoreactivity (++) (Figures 4B&C, respectively). The intensity of PCNA immunostaining increased to be severe (+++) in the granulosa cells and oocyte of the ovarian follicle of the hyperthyroid rats (Gr. IV) with moderate immunostaining reactivity in the theca cells (Figure 4.D).

Immunohistochemical analysis was performed on the ovaries to investigate the localization of the anti-apoptotic BCl-2 protein. Results showed that the immunostaining reactivity of BCl-2 in normal control group is moderate (++) in the granulosa cells of the ovarian follicle (Figure 5.A). The immunostaining of BCl-2 localization and intensity in the ovary of mild hyperthyroid rats (Gr. II) were similar to that of the normal control rats (Figure 5.B). The ovary of moderate hyperthyroid rats (Gr. III) had moderate immunoreactivity for BCl-2 in the granulosa cells and the theca cells of the ovarian follicle as well as corpus lutea (Figure 5.C). On the other hand, the ovary of severe hyperthyroid rats (Gr. IV) showed weak immunostaining intensity of BCl-2 in the granulosa cells of the ovarian follicle and corpus lutea with weak to negative immunostaining reactivity in the theca cells (Figure 5.D).

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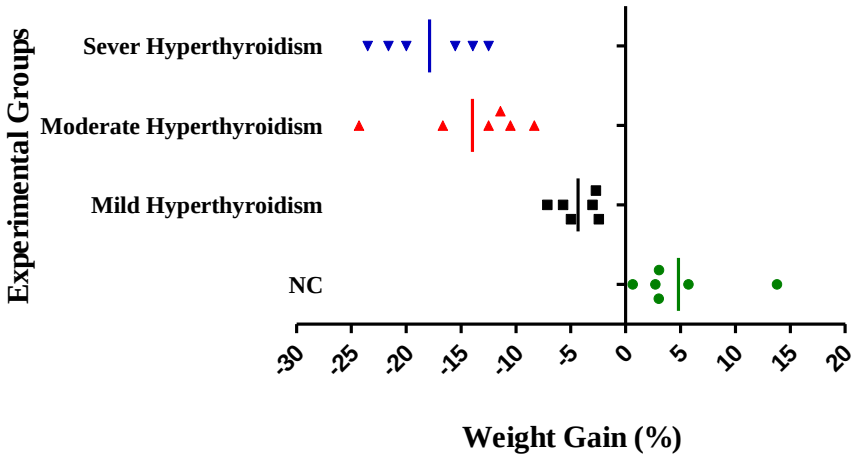

Figure 1: The percentage of the body weight gain of rats in the different stages of hyperthyroidism (10, 20, and 30 days), compared with the normal control group. For each group, each value represents the difference between the mean body weights of rats at a certain 10 days point compared to the mean body weight of the preceding 10 days point. The mean difference is significant at $p < 0.05$ using unpaired T-Test.

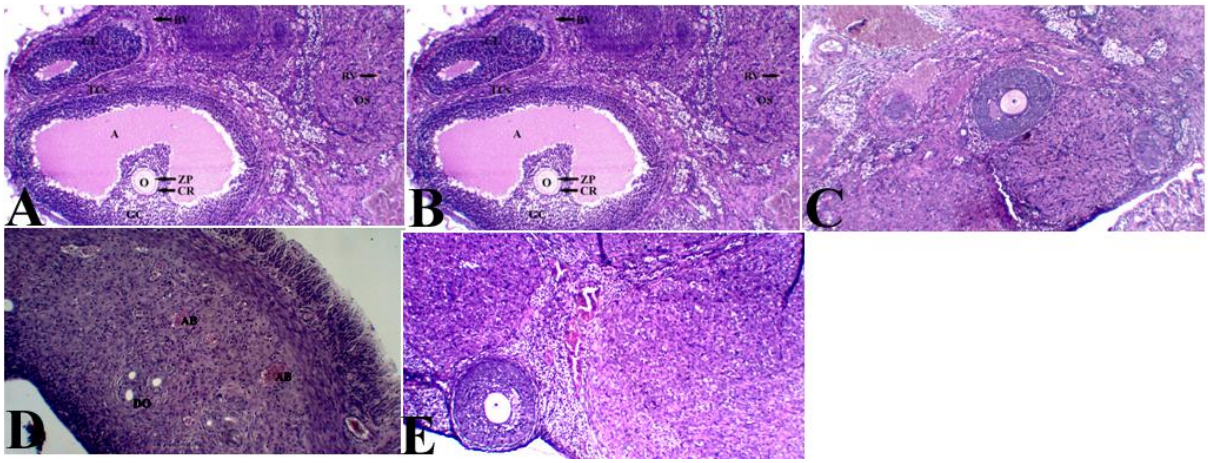

Figure 2: Photomicrographs of ovarian sections stained with hematoxylin and eosin (H&E, 200x). (A): Normal control. (B): Mild hyperthyroid rats. (C): Moderate hyperthyroid rats. (D): Severe hyperthyroid rats. Normal control rats as well as mild hyperthyroid rats showed normal blood vesssels (BV), corpus luteum (CL), ovarian stroma (S), and mature follicle consisting of theca cells (TCs), granulose cells (GC), antrum (A), and encentrically oocyte. The oocyte was surrounded by corona radiata (CR) and zona pellucida (ZP). Moderate hyperthyroid rats denoted a decrease in the number of corpora lutea and follicles with moderate degenerated oocytes (DO). In addition, many apoptotic bodies could be seen in the ovarian cortex. In severe hyperthyroidism, a collapsed follicle (arrow head) as well as dilated and congested blood vessels was seen. In addition, a collapsed follicle (arrow head) and multiple bleeding sites, dilated and congested blood vessels were observed.

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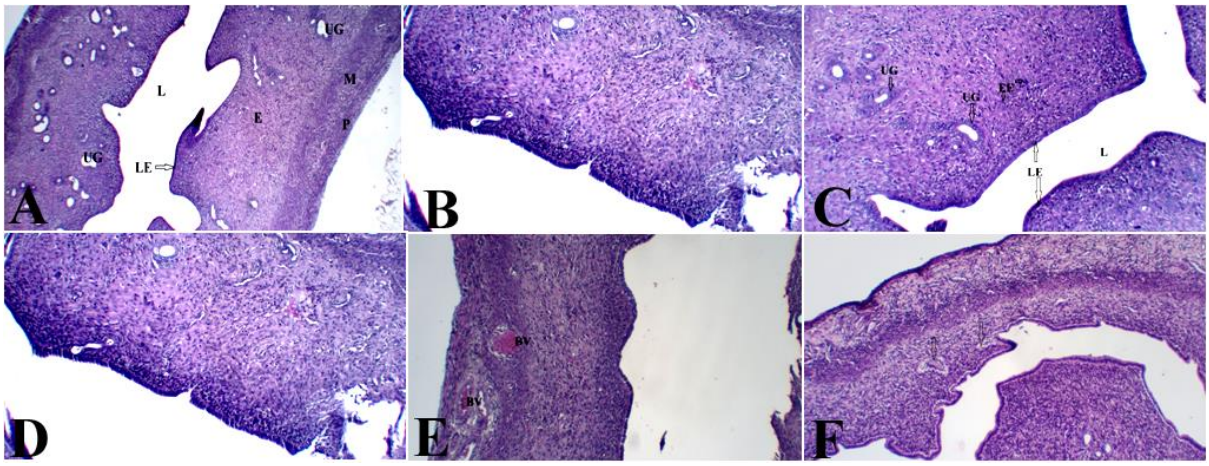

Figure 3: Photomicrographs of uterine sections stained with hematoxylin and eosin (H&E, 200x). (A)& (B): Normal control. (C): Mild hyperthyroid rats. (D)&(E): Moderate hyperthyroid rats. (F): Severe hyperthyroid rats. Normal control showed showing uterine endometrium (E), and lumen (L). The lumen of normal control uterus was characterized by the presence of normal epithelial cells (LE). Endometrium consisted of columnar epithelial cells (EE), normal oval nucleus and the presence of uterine glands (UG). Mild hyperthyroid rats showed had vacuolations (V) in the lumen epithelial cell and uterine glands. Moderate hyperthyroid rats showed congested blood vessels (BV) and degenerated endometrial epithelial cells (arrow). Severe hyperthyroid rats revealed the presence of endometrium with thickened lumen epithelium and numerous apoptotic bodies (AB). Moreover, many endometrium epithelial cells became apoptotic (AB).

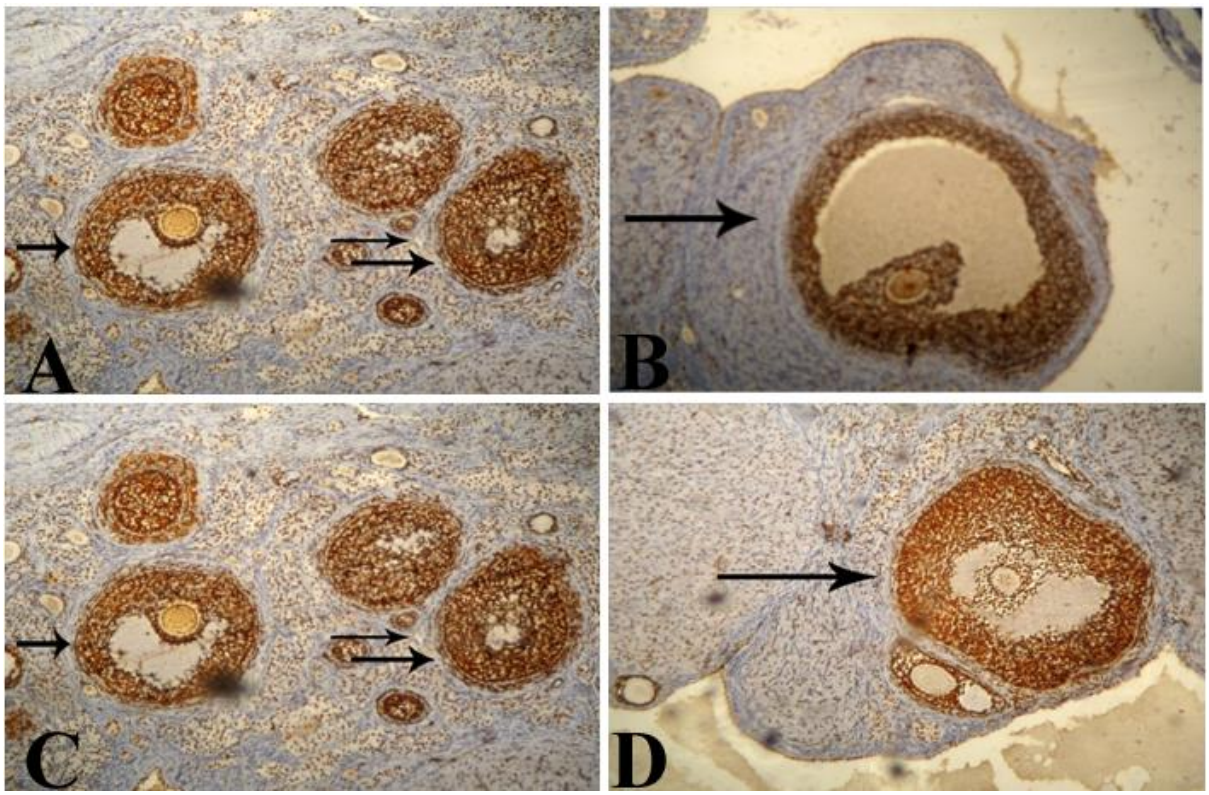

Figure 4: Photomicrographs of immunostaining for the expression of ovarian PCNA protein stained with hematoxylin and eosin (H&E, 200x). (A): Normal control. (B): Mild hyperthyroid rats. (C): Moderate hyperthyroid rats. (D): Severe hyperthyroid rats. Signals appear reddish-brown using DAB as substrate. In normal rats, PCNA antigen was localized mainly in the granulose cells as well as oocyte of the ovarian follicle (arrow) with moderate immunoreactivity. In addition, PCNA immunostatining reactivity was weak to negative in the theca cells. Mild hyperthyroid rats showed moderate immunoreactivity of PCNA antigen in the granulose cells, oocyte, and theca cells of the ovarian follicle (arrow). Moderate hyperthyroid rats denoted moderate immunostaining intensity in the granulose cells, oocyte, and theca cells of the ovarian follicle (arrow). Severe hyperthyroid rats showed severe immunostatining reactivity of PCNA antigen in in the granulose cells as well as oocyte of the ovarian follicle (arrow). In addition, PCNA immunostatining reactivity was moderate in the theca cells.

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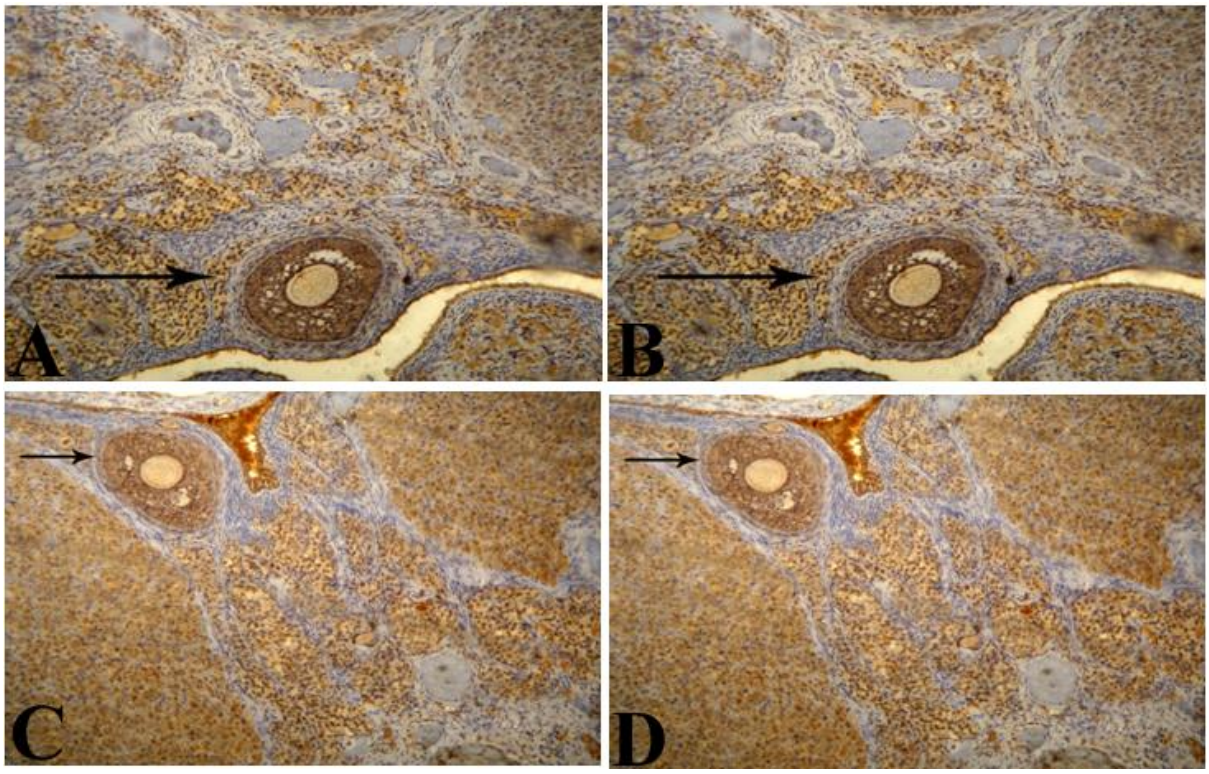

Figure 5: Photomicrographs of immunostaining for the expression of ovarian BCl-2 protein stained with hematoxylin and eosin (H&E, 100x). (A): Normal control. (B):Mild hyperthyroid rats. (C): Moderate hyperthyroid rats. (D): Severe hyperthyroid rats. Signals appear reddish-brown using DAB as substrate. Normal rats showed localization of BCl-2 antigen in the granulose cells of the ovarian follicle (arrow) with moderate immunostaining intensity. Furthermore, BCl-2 immunostatining reactivity was weak in the theca cells and corpus lutea. Mild hyperthyroidism denoted localization of BCl-2 antigen in the granulose cells of the ovarian follicle (arrow) with severe intensity. In addition, BCl-2 immunostatining reactivity was weak in the theca cells and corpus lutea. Moderate hyperthyroid rats revealed localization of BCl-2 antigen in the granulose cells of the ovarian follicle (arrow) with moderate intensity. In addition, BCl-2 immunostatining reactivity was moderate in the theca cells and corpus lutea. Severe hyperthyroid rats showed localization of BCl-2 antigen in the granulose cells of the ovarian follicle and corpus lutea (arrow) with weak intensity. Moreover, BCl-2 immunostatining reactivity was weak to negative in the theca cells.

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Table 1: Statistical significance of serum TSH, FT3, progesterone and estradiol II in all the studied groups

Groups	TSH (µIU/ml)	FT3 (ng/ml)	Progesterone (µIU /ml)	Estradiol II (µIU/ ml)
Normal Control				
Mean ±SD	2.88±0.15	1.42±.15	11.9±0.5	32±0.4
Mild Hyperthyroidism				
Mean ±SD	1.74±0.24	1.76±.13	9.5±0.7	30±2.49
P<	**0.001**	**0.001**	**0.001**	**NS**
-				
Moderate Hyperthyroidism				
Mean ±SD	0.1±0.07	2.1±0.11	7±0.52	29±0.92
P<	**0.001**	**0.001**	**0.001**	**0.001**
Severe Hyperthyroidism				
Mean +SD	0.038±0.02	2.3±0.76	5.6±0.6	27.5±0.4
P<	**0.001**	**0.001**	**0.001**	**0.001**

Table 2: Statistical significance of ovarian interleukin-6 and insulin like growth factor-1 concentrations

Groups	IL-6 (pg/mg protein)	IGF-1 (pg/mg protein)
Normal Control		
Mean±SD	2.6±0.3	18±3.1
Mild Hyperthyroidism		
Mean±SD	2.7±0.17	13.7±1.5
P<	**NS**	**0.05**
Moderate Hyperthyroidism		
Mean±SD	3.7±0.2	8.7±1.2
P<	**0.001**	**0.001**
Severe Hyperthyroidism		
Mean+SD	5.2±0.6	3.4±1.0
P<	**0.001**	**0.001**

Table 3: The severity of ovarian histological alterations all the studied groups

Groups	Degeneration	Number of Corpus Lutea and Follicles
NC	Absent	+++
Mild Hyperthyroidism	+	++
Moderate Hyperthyroidism	++	+
Severe Hyperthyroidism	+++	+

+++ indicates Severe, ++ indicates moderate, and + indicates mild.

Table 4: The severity of uterine histological alterations in all the studied groups

Groups	Degeneration	Apoptosis
NC	Absent	Absent
Mild Hyperthyroidism	Absent	Absent
Moderate Hyperthyroidism	++	++
Severe Hyperthyroidism	+++	+++

+++ indicates Severe, ++ indicates moderate, and + indicates mild.

DISCUSSION

Many reports discussed the association between infertility and hyperthyroidism in both humans and animal models [28, 29]. However, no studies had been investigated regarding the effects of different stages of hyperthyroidism on female reproductive organs; ovary and uterus. So, the present study was undertaken to evaluate the effects of different durations of hyperthyroidism (mild, moderate, and severe) on the ovarian and uterine histological abnormalities as well as ovarian proliferating, apoptotic, and inflammatory markers.

The present study revealed that daily oral administration of L-thyroxin either for 10, 20, and 30 days significantly elevated serum FT3 with a feedback reduction of serum TSH levels. Different stages of hyperthyroidism caused significant reductions in the body weight of rats. These results agreed with the previous report of Kong et al. [30] who reported that disturbances in the levels of serum thyroid hormones change the body weights, as body growth and organ development are regulated by thyroid hormones.

It is known that estradiol II and progesterone levels possess important roles in both uterine development and the menstrual cycle [30]. Moreover, in this study, we found that serum estradiol levels exhibited slight significant reductions in moderate and severe hyperthyroid rats with no change in the mild hyperthyroid rats, while serum progesterone levels were significantly reduced in all stages of hyperthyroidism. Zarifkar [31] reported that induction of hyperthyroidism by daily oral administration of levothyroxine (100µg/100g b.w) for 10 days significantly lowered serum estradiol and progesterone levels. These results can be explained by the previous reports of Tamura et al. [32] ,who stated that thyroid hormones suppress FSH-induced aromatase activity in granulosa cells and therefore inhibit ovulation.

Based on the fact that estradiol and progesterone have central roles in the cellular proliferation and differentiation of uterus in preparation for embryo implantation [33, 34], therefore, hyperthyroidism at different statuses might cause disturbances in the menstrual cycle and reduction of the uterine development by altering the levels of female sex hormones. Vasudevan et al. [35] reported that estrogen-mediated reproductive manner is inhibited by T3. Wei et al. [36] reported that hyperthyroidism alters estrous cyclicity and antioxidative status in the rat ovary through nitric oxide synthase (NOS) signaling pathway through raising ovarian nitric oxide, total NO synthase, inducible NOS and constitutive NOS activities. In addition, hyperthyroidism altered the oxidant/antioxidant status via elevating ovarian malondialdehyde contents as well as the activities of ovarian total superoxide dismutase, glutathione peroxidase, and catalase.

The histological examination of both ovary and uterus are in agreement with previous studies, Khair [37] reported that Eltroxin-induced hyperthyroid animals for 3 consecutive months result in degenerative changes in the ovarian follicles in the form of lysis of the primary oocytes of the growing follicles and degeneration of the granulosa cells. Wei et al. [36] revealed a decrease in the numbers of secondary and antral ovarian follicles with fewer corpora lutea in hyperthyroid rats. These histological abnormalities could be explained by the previous studies of Kong et al. [30] and López et al. [38] who evidenced the presence of thyroid hormone nuclear receptor α/β (TRα/β) in the ovarian granulosa cells and different cell types in uterus, respectively, and therefore involved in the regulation of folliculogenesis and steroidogenesis.

The present study revealed that increasing the severity of hyperthyroidism increased inflammation and reduced proliferation of the ovaries by altering the concentrations of ovarian IL-6 and IGF-1. This results are in accordance with previous studies ,where Mysliwiec et al. [39] suggested that IL-6 elevation plays a crucial role in thyrotoxicosis-related disturbances of bone turnover in mice. Also Slowik et al. [40] reported a significant elevation in serum IL-6 concentration in patients with Grave's hyperthyroidism. A recent study of Juri et al. [41] reported clear differences in the levels of different cytokines including IL-6 in the tears of healthy subjects and patients with Graves' hyperthyroid patients.

Peritoneal fluid macrophages express IL-6 and soluble IL-6 receptors in which IL-6 exerts its effects in pelvic inflammatory states [42]. Banerje et al. [43] demonstrated a direct relation between elevating IL-6 concentration and the deterioration in the morphology of the microtubule and chromosomal alignment in metaphase-II mouse oocytes.

The maintenance of the integrity of the mature oocyte spindle is important for cell division and embryo formation [44]. Indeed, abnormal spindle dynamics mediated by IL-6 elevation results in aneuploidy, impaired fertilization, early loss of pregnancy [45], low reproductive outcome under certain pathological conditions such as in patients with endometriosis and pelvic inflammation [46], alteration in fallopian tube thus affecting ovum pick up and implantation [41] or inhibition of estradiol II and progesterone secretion from ovarian granulose cells [22].

IGF-1 is an intra-ovarian growth factor that is involved in regulating follicular cells proliferation [47], stimulating granulosa cell proliferation, producing progesterone [48], and stimulating estradiol-17b (E2) production in the granulosa cells [49]. Rochel et al. [50] reported a significant reduction in plasma IGF-1 concentration in hyperthyroid cats. Thus, induction of disturbances in ovarian IL-6 and IGF-1 due to hyperthyroidism could be attributed the disturbances in the levels of progesterone and estradiol II as well as the histological alterations in both ovary and uterus.

Cell proliferation is important for normal reproductive processes [51], including ovarian development, follicle progression from the primordial stage to the primary, secondary and tertiary or Graafian stages and ovulation [52]. Follicular proliferation and apoptosis are regulated by many hormones, including thyroid hormones [53]. However, less than 1% of the ovarian follicles reach the ovulation stage in mammals because of degeneration and atresia of more than 99% of ovarian follicles [54]. Apoptosis is regulated by the expression of numerous genes including BCl-2 gene family. BCl-2 is anti-apoptotic protein [55] since it can inhibit Bax-induced apoptosis by forming heterodimers with it [56]. The immunohistochemical results denoted the incidence of apoptosis in the ovarian granulose cells, corpus lutea, and theca cells of hyperthyroid rats (moderate and severe).

PCNA is an auxiliary protein of DNA polymerases δ, so it is a proliferative marker [57]. Results revealed increasing the immunoreactivity of PCNA protein in the ovarian granulosa cells by increasing the severity of hyperthyroidism. However, expression of PCNA cannot be attributed to oocytes proliferation because the oocyte is arrested meiotically [58]. PCNA is involved in DNA repair [57] suggesting that PCNA may be expressed by nonproliferating cells. Although no new DNA synthesis occurs in the growing oocyte, activation of DNA polymerases is possibly happened to repair genetic material damage in the oocytes selected to grow [13]. Xu et al. [59] found that PCNA is present in oocytes of the primordial follicles suggesting PCNA role in earlier stages of folliculogenesis. The increased PCNA expression in oocytes during the initiation of primordial follicle formation and the death of the two-thirds of oocytes during primordial follicle assembly [60] suggest a role of PCNA in the regulation of oocyte fate, i.e., death or survival to form primordial follicles.

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REFERENCES

1. Committee P, Society A. Definitions of infertility and recurrent pregnancy loss: A committee opinion. Fertil Steril 2013;99:63. doi:10.1016/j.fertnstert.2012.09.023.
2. Unuane D, Tournaye H, Velkeniers B, Poppe K. Endocrine disorders & female infertility. Best Pract Res Clin Endocrinol Metab 2011;25:861–73. doi:10.1016/j.beem.2011.08.001.
3. Mintziori G, Kita M, Duntas L, Goulis DG. Consequences of hyperthyroidism in male and female fertility: pathophysiology and current management. J Endocrinol Invest 2016;39:849–53. doi:10.1007/s40618-016-0452-6.
4. Cramer DW, Sluss PM, Powers RD, McShane P, Ginsburg ES, Hornstein MD, et al. Serum prolactin and TSH in an in vitro fertilization population: Is there a link between fertilization and thyroid function? J Assist Reprod Genet 2003;20:210–5. doi:10.1023/A:1024151210536.
5. Colicchia M, Campagnolo L, Baldini E, Ulisse S, Valensise H, Moretti C. Molecular basis of thyrotropin and thyroid hormone action during implantation and early development. Hum Reprod Update 2014;20:884–904. doi:10.1093/humupd/dmu028.
6. Davis LB, Lathi RB, Dahan MH. The Effect of Infertility Medication on Thyroid Function in Hypothyroid Women Who Conceive. Thyroid 2007;17:773–7. doi:10.1089/thy.2007.0065.
7. Joshi J V, Bhandarkar SD, Chadha M, Balaiah D, Shah R. Menstrual irregularities and lactation failure may precede thyroid dysfunction or goitre. J Postgrad Med 1993;39:137–41.
8. Poppe K, Velkeniers B, Glinoer D. Thyroid disease and female reproduction. Clin Endocrinol (Oxf) 2007;66:309–21. doi:10.1111/j.1365-2265.2007.02752.x.
9. Saitou M, Yamaji M. Primordial germ cells in mice. Cold Spring Harb Perspect Biol 2012;4. doi:10.1101/cshperspect.a008375.
10. Zhang Z, Shen B, Wang Y, Chen Y, Wang G, Lin P, et al. Molecular Cloning of Proliferating Cell Nuclear Antigen and Its Differential Expression Analysis in the Developing Ovary and Testis of Penaeid Shrimp Marsupenaeus japonicus. DNA Cell Biol 2010;29:163–70. doi:10.1089/dna.2009.0958.
11. Oktay K, Schenken RS, Nelson JF. Proliferating cell nuclear antigen marks the initiation of follicular growth in the rat. Biol Reprod 1995;53:295–301. doi:10.1095/biolreprod53.2.295.
12. Picut CA, Swanson CL, Scully KL, Roseman VC, Parker RF, Remick AK. Ovarian follicle counts using proliferating cell nuclear antigen (PCNA) and semi-automated image analysis in rats. Toxicol Pathol 2008;36:674–9. doi:10.1177/0192623308317428.
13. Stoimenov I, Helleday T. PCNA on the crossroad of cancer. Biochem Soc Trans 2009;37:605–13. doi:10.1042/BST0370605.
14. Langerak P, Nygren AOH, Krijger PHL, van den Berk PCM, Jacobs H. A/T mutagenesis in hypermutated immunoglobulin genes strongly depends on PCNAK164 modification. J Exp Med 2007;204:1989–98. doi:10.1084/jem.20070902.
15. Bristol-Gould SK, Kreeger PK, Selkirk CG, Kilen SM, Mayo KE, Shea LD, et al. Fate of the initial follicle pool: Empirical and mathematical evidence supporting its sufficiency for adult fertility. Dev Biol 2006;298:149–54. doi:10.1016/j.ydbio.2006.06.023.
16. Tilly JL. Commuting the death sentence: How oocytes strive to survive. Nat Rev Mol Cell Biol 2001;2:838–48. doi:10.1038/35099086.
17. Campos-Junior PHA, Assuncao CM, Carvalho BC, Batista RITP, Garcia RMG, Viana JHM. Follicular populations, recruitment and atresia in the ovaries of different strains of mice. Reprod Biol 2012;12:41–55. doi:10.1016/S1642-431X(12)60076-X.
18. Jones RL, Pepling ME. Role of the antiapoptotic proteins BCL2 and MCL1 in the neonatal mouse ovary. Biol Reprod 2013;88:46. doi:10.1095/biolreprod.112.103028.
19. Aitken RJ, Findlay JK, Hutt KJ, Kerr JB. Apoptosis in the germ line. Reproduction 2011;141:139–50. doi:10.1530/REP-10-0232.
20. Ke F, Bouillet P, Kaufmann T, Strasser A, Kerr J, Voss AK. Consequences of the combined loss of BOK and BAK or BOK and BAX. Cell Death Dis 2013;4. doi:10.1038/cddis.2013.176.
21. Mehta B, Chimote M, Chimote N, Nath N, Chimote N. Follicular-fluid anti-Mullerian hormone (FF AMH) is a plausible biochemical indicator of functional viability of oocyte in conventional in vitro fertilization (IVF) cycles. J Hum Reprod Sci 2013;6:99. doi:10.4103/0974-1208.117168.

22. Salmassi A, Lü S, Hedderich J, Oettinghaus C, Jonat W, Mettler L. Interaction of interleukin-6 on human granulosa cell steroid secretion. J Endocrinol 2001;170:471–8. doi:10.1677/joe.0.1700471.
23. Gabay C. Interleukin-6 and chronic inflammation. Arthritis Res Ther 2006;8. doi:10.1186/ar1917.
24. Papaleo E, Ottolina J, Vigana P, Brigante C, Marsiglio E, De Michele F, et al. Deep pelvic endometriosis negatively affects ovarian reserve and the number of oocytes retrieved for in vitro fertilization. Acta Obstet Gynecol Scand 2011;90:878–84. doi:10.1111/j.1600-0412.2011.01161.x.
25. Osonuga IO, Olowookorun MO, Iquot IS, Akinola BO. Influence of Thyroxine on Blood Parameters and Liver Enzymes in Adult Male and Female Rats 2014;17:53–6.
26. Downie T. Theory and Practice of Histological Techniques Edited by J.D. Bancroft & A. Stevens, Churchill Livingstone, Edinburgh, 740 pages, ??55.00. Histopathology 1990;17:386–386. doi:10.1111/j.1365-2559.1990.tb00755.x.
27. Bradford MM. A rapid and sensitive method for the quantifcation of microgram quantities of protein utilizing the principle of protein– dye binding. Anal Biochem 1976;72:248–254. doi:10.1016/0003-2697(76)90527-3.
28. Idris MA, Idris OF, Sabahelkhier MK. The Effects of Induced Hyperthyroidism on Plasma FSH and LH Concentrations in Female of Wistar Rats 2012;1:55–7.
29. Nasir S, Khan MM, Ahmed S, Alam S, Ziaullah S. Role of Thyroid Dysfunction in Infertile Women With Menstrual Disturbances. Gomal J Med Sci 2016;14:20–4.
30. Kong L, Wei Q, Fedail JS, Shi F, Nagaoka K, Watanabe G. Effects of thyroid hormones on the antioxidative status in the uterus of young adult rats. J Reprod Dev 2015;61:219–27. doi:10.1262/jrd.2014-129.
31. Zarifkar A. Effects of levo thyroxine administration on ovulation rate and sex hormone levels in prepubertal and adult rats 2003;17.
32. Tamura K, Hatsuta M, Watanabe G, Taya K, Kogo H. Inhibitory regulation of inhibin gene expression by thyroid hormone during ovarian development in immature rats. Biochem Biophys Res Commun 1998;242:102–8. doi:10.1006/bbrc.1997.7919.
33. O'Brien JE, Peterson TJ, Ming HT, Lee EJ, Pfaff LE, Hewitt SC, et al. Estrogen-induced proliferation of uterine epithelial cells is independent of estrogen receptor α binding to classical estrogen response elements. J Biol Chem 2006;281:26683–92. doi:10.1074/jbc.M601522200.
34. Al-Asmakh M. Reproductive functions of progesterone receptors. Recent Prog Horm Res 2007;12:147–52. doi:10.1210/rp.57.1.339.
35. Vasudevan N, Ogawa S, Pfaff D. Estrogen and thyroid hormone receptor interactions: physiological flexibility by molecular specificity. Physiol Rev 2002;82:923–44. doi:10.1152/physrev.00014.2002.
36. Wei Q, Fedail JS, Kong L, Zheng K, Meng C, Fadlalla MB, et al. Thyroid hormones alter estrous cyclicity and antioxidative status in the ovaries of rats. Anim Sci J 2017. doi:10.1111/asj.12950.
37. Khair NS TS. Effect of Thyroid Disorders on the Adult Female Albino Rats (Histological and Histochemical Study). J Cytol Histol 2012;5. doi:10.4172/2157-7099.1000245.
38. López Navarro E, Ortega FJ, Francisco-Busquets E, Sabater-Masdeu M, Álvarez-Castaño E, Ricart W, et al. Thyroid Hormone Receptors Are Differentially Expressed in Granulosa and Cervical Cells of Infertile Women. Thyroid 2016;26:466–73. doi:10.1089/thy.2015.0416.
39. Mysliwlec J, Zbucki R, Winnicka MM, Sawicki B, Nikolajuk A, Kaminski K, et al. A crucial role of interleukin-6 in the pathogenesis of thyroxicosis-related disturbances of bone turnover in mice. Horm Metab Res 2007;39:884–8. doi:10.1055/s-2007-992776.
40. Slowik M, Urbaniak-Kujda D, Bohdanowicz-Pawlak A, Kapelko-Slowik K, Dybko J, Wolowiec D, et al. CD8+CD28-lymphocytes in peripheral blood and serum concentrations of soluble interleukin 6 receptor are increased in patients with graves' orbitopathy and correlate with disease activity. Endocr Res 2012;37:89–95. doi:10.3109/07435800.2011.635622.
41. Juri J, Kozmar A, Kusa S, Jazbec A. The levels of 12 cytokines and growth factors in tears : hyperthyreosis vs euthyreosis 2018:6–13.
42. Rose-John S, Scheller J, Elson G, Jones S a. Interleukin-6 biology is coordinated by membrane-bound and soluble receptors: role in inflammation and cancer. J Leukoc Biol 2006;80:227–36. doi:10.1189/jlb.1105674.
43. Banerjee J, Sharma R, Agarwal A, Maitra D, Diamond MP, Abu-Soud HM. Il-6 and mouse oocyte spindle. PLoS One 2012;7. doi:10.1371/journal.pone.0035535.
44. Eichenlaub-Ritter U, Vogt E, Yin H, Gosden R. Spindles, mitochondria and redox potential in ageing oocytes. Reprod Biomed Online 2004;8:45–58. doi:10.1016/S1472-6483(10)60497-X.

45. Saito H, Seino T, Kaneko T, Nakahara K, Toya M, Kurachi H. Endometriosis and oocyte quality. Gynecol. Obstet. Invest., vol. 53, 2002, p. 46–51. doi:10.1159/000049424.
46. Papathanasiou A, Djahanbakhch O, Saridogan E, Lyons RA. The effect of interleukin-6 on ciliary beat frequency in the human fallopian tube. Fertil Steril 2008;90:391–4. doi:10.1016/j.fertnstert.2007.07.1379.
47. Spicer LJ, Stewart RE. Interactions among basic fibroblast growth factor, epidermal growth factor, insulin, and insulin-like growth factor-I (IGF-I) on cell numbers and steroidogenesis of bovine thecal cells: role of IGF-I receptors. Biol Reprod 1996;54:255–63. doi:10.1095/biolreprod54.1.255.
48. Silva JR V, Figueiredo JR, van den Hurk R. Involvement of growth hormone (GH) and insulin-like growth factor (IGF) system in ovarian folliculogenesis. Theriogenology 2009;71:1193–208. doi:10.1016/j.theriogenology.2008.12.015.
49. Butler ST, Pelton SH, Butler WR. Insulin increases 17β-estradiol production by the dominant follicle of the first postpartum follicle wave in dairy cows. Reproduction 2004;127:537–45. doi:10.1530/rep.1.00079.
50. Rochel D, Burger M, Nguyen P, Jaillardon L. Insulin-like growth factor type 1 concentrations in hyperthyroid cats before and after treatment with thiamazole. J Feline Med Surg 2017:1098612X1769473. doi:10.1177/1098612X17694735.
51. Stouffer RL, Xu F, Duffy DM. Molecular control of ovulation and luteinization in the primate follicle. Front Biosci 2007;12:297–307. doi:10.2741/2065.
52. Yin M, Wang X, Yao G, Lü M, Liang M, Sun Y, et al. Transactivation of microRNA-320 by microRNA-383 regulates granulosa cell functions by targeting E2F1 and SF-1 proteins. J Biol Chem 2014;289:18239–57. doi:10.1074/jbc.M113.546044.
53. Nelson ER, Habibi HR. Thyroid receptor subtypes: Structure and function in fish. Gen Comp Endocrinol 2009;161:90–6. doi:10.1016/j.ygcen.2008.09.006.
54. Maeda A, Inoue N, Matsuda-minehata F. The Role of Interleukin-6 in the Regulation of Granulosa Cell Apoptosis During Follicular Atresia in Pig Ovaries 2007;53.
55. Parborell F, Pecci A, Gonzalez O, Vitale A, Tesone M. Effects of a gonadotropin-releasing hormone agonist on rat ovarian follicle apoptosis: regulation by epidermal growth factor and the expression of Bcl-2-related genes. Biol Reprod 2002;67:481–6.
56. Sugino N, Suzuki T, Kashida S, Karube A, Takiguchi S, Kato H. Expression of Bcl-2 and Bax in the human corpus luteum during the menstrual cycle and in early pregnancy: regulation by human chorionic gonadotropin. J Clin Endocrinol Metab 2000;85:4379–86. doi:10.1210/jcem.85.11.6944.
57. Wood RD, Shivji MKK. Which DNA polymerases are used for DNA-repair in eukaryotes? Carcinogenesis 1997;18:605–10. doi:10.1093/carcin/18.4.605.
58. Hirshfield AN. Development of Follicles in the Mammalian Ovary. Int Rev Cytol 1991;124:43–101. doi:10.1016/S0074-7696(08)61524-7.
59. Xu B, Hua J, Zhang Y, Jiang X, Zhang H, Ma T, et al. Proliferating cell nuclear antigen (PCNA) regulates primordial follicle assembly by promoting apoptosis of oocytes in fetal and neonatal mouse ovaries. PLoS One 2011;6. doi:10.1371/journal.pone.0016046.
60. Pepling ME. From primordial germ cell to primordial follicle: Mammalian female germ cell development. Genesis 2006;44:622–32. doi:10.1002/dvg.20258.