Oestrogen Receptor Alpha and Progesterone Receptor in Uterus and Ovaries on Different Days of The Oestrus Cycle in Hyperthyroid Rats

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ABSTRACT

Background: The present study evaluated, in different oestrus cycle phases, the oestrogen receptor alpha (ERα) and progesterone receptor (PgR) in rates with hyperthyroid ovaries and uterus. **Materials and Methods:** Sixty-four adult female wistar albino rats were used. Animals were randomly separated into eight different groups, four of which constituted the control group and the other four of which were the actual experimental group. Hyperthyroidism was induced by daily s.c. injection of L-thyroxine (L-T4, 250 μg/kg) for 21 days. **Results:** Hyperthyroidism induced decreased plasma TSH levels and increased plasma free T4 levels ($P < 0.001$). In addition, it increased plasma oestrogen levels in all cycle phases, and plasma progesterone levels increased in different cycle phases, excluding the oestrus phase, in rats ($P < 0.01$). By the end of the experiment, ERα percentages were decreased in oestrus days in uterine luminal epithelium; however, ERα percentages were increased in metoestrus phases in hyperthyroid rats. ERα was elevated in proestrus, metoestrus and dioestrus phases in the corpus luteum, and hyperthyroidism also increased ERα percentages in theca folliculi. In uterine luminal epithelium, the hyperthyroidism group showed higher expressions of PgR ($P < 0.05$) in oestrus and metoestrus phases and decreased oestrus phase compared to control rats. **Conclusions:** As a result, we conclude that both the levels of plasma estrogen and progesterone as well as the differences in the distributions of relevant receptors in ovarian and uterus tissues have an impact on female infertility due to hyperthyroidism.

Key Words: Hyperthyroidism, oestrogen receptor alpha, progesterone receptor, ovary, uterus

INTRODUCTION

Hyperthyroidism can cause malfunctions in many systems. It is the second most common cause of endocrine diseases after diabetes in world.[1] Hyperthyroidism is often the result of an autoimmune disease with a not clearly detectible cause. It is ten times more common in women than in men.[2] The main complications of hyperthyroidism are short temper, sleeplessness, exophthalmia, urticaria, vitiligo, myopathy, hepatomegalia, gynaecomastia, decreased fertility, and irregular menses (amenorrhea or polymenorrhea).[1,3,4] Although both decreased fertility and irregular menses in women with hyperthyroidism have been studied extensively, there are many aspects that have not yet been considered.

Hyperthyroidism causes functional and structural changes in genitalia of humans (ovaries and uterus) as well as in animals.[5] In women diagnosed with hyperthyroidism, common malfunctions of the ovarian cycle are amenorrhea, oligomenorrhea, and decreased levels of or disappearance of lutein hormone (LH) in the midst of the cycle.[6]

Immunohistochemical staining is the method used to detect the dispersion of ER and PgR in the human uterus and ovaries during menstrual cycles.[7] Immunohistochemical staining is also used to show oestrogen and PgR in ovaries and uteruses of rats.[8]

There are two sub-types of ER: ERß and ERα. ERß can be observed in the cell nuclei of granuloza cells as well as

in primary, secondary and mature follicles. An authentic retention of ERß can be observed in theca cells, luteal cells, germinal epithelium and oocytes and nucleus. A little staining is also observed in atretic follicles or there is no staining at all. ERα is observed in germinal epithelium, interstitial cells and theca cells.[9]

This study aims to demonstrate the change in the distribution of oestrogen and PgR due to the effects of hyperthyroidism in ovarian and uterine tissues of female rats that were infected with hyperthyroidism using L-Thyroxine during different phases of the oestrus cycle.

MATERIAL AND METHODS

Experimental animals
Sixty-four mature female Wistar-albino rats with the same biological and physiological features were used for this study. These rats were bred in the Research Department of Experimental Animals in Trakya University, and their weights vary between 200-250 gr. The study was approved by the Institutional Animal Ethical Committee of the Trakya University, Edirne, Turkey (permission number TUHDYEK-2012/02). During this experiment, all subjects were fed with pellet feed (Purina), which contains drinking water and 21% pure protein in an optimum laboratory atmosphere (22 ± 1 ^{0}C, 12 hour lightness/darkness cycle). Eight groups, four control groups and four experimental groups were formed. At the beginning of the experiment, all subjects were numbered and weighed.

Groups
Control Groups (n=8)
I.   Group: Proestrus Control Group
II. Group: Oestrus Control Group
III. Group: Metoestrus Control Group
IV. Group: Dioestrus Control Group
     Experimental Groups (n=8); 250 μg/kg/day of thyroxin hormone (L-Thyroxine, Sigma) was injected subcutaneously for 21 days
I.   Group: Proestrus
II. Group: Oestrus
III. Group: Metoestrus
IV. Group: Dioestrus

Tissue sample collections
Starting on day 21 of the experiment, a vaginal smear was taken with a moistened swab every 24 hours, after wiping the vulva region with 70% ethyl alcohol. This smear was then spread on a microscope slide. The smear samples were fixed using 70% methanol and dried at room temperature. Preparates which were dyed with 1% Toluidine blue (Sigma-USA) for five minutes were washed with distilled water and dried. Then, they were covered with Canada balsam (Fluka-Germany). The photos of oestrus phase were taken with a CX31 Olympus light microscope and a C-5060 Olympus camera.

After the vaginal smear samples were taken from the experimental and control groups, the rats with the relevant cycles were weighed. After they were intraperitoneally (i.p.) anesthetized with 50 mg/kg ketamine (Ketalar-Eczacıbaşı/Turkey) and 10mg/kg xylazine (Rompun-Bayer/Turkey) and dissected, uterine, ovarian and thyroid samples were taken together and evaluated. These uterine, ovarian and thyroid samples were fixed in a 10% neutral formaldehyde buffered fixation.

Light microscopic examination
Uterine, ovarian and thyroid tissues were first fixed in 10% neutral formaldehyde buffered fixator for 72 hours and then blocked in paraffin. Five μm (micrometre) thick sections were taken from these blocks using Leica RM-2245. These sections, taken to demonstrate the histological structural changes in thyroid gland, uterus and ovaries, were stained with Haematoxylin + Eosin (H+E). They were examined with a light microscope (Olympus CX31-Japan), and the findings were photographed.

Immunohistochemical examination
At the end of the experiment, the rats were decapitated. Ovarian and uterine tissues were removed, fixed in 10% neutral formaldehyde buffered solution and, following routine laboratory methods, were embedded in paraffin. Five-micrometre paraffin tissue sections were mounted on poly-L-lysine slides. The slides were air-dried, and the tissue was deparaffinized. The avidin-biotin complex method was used for immunohistochemical staining, as previously reported.[10] The sections were incubated with a specific monoclonal anti-ERα antibody (1:100; ab37438 Abcam, USA) and an anti-PR (1:40; ab2764-PR-AT 4,14 Abcam, USA). Diaminobenzidine (DAB) was used as a chromogen, and the sections were counterstained with hematoxylin. Ovarian and uterine tissue sections were examined under light microscopy (400), and the number of immunoreactive positive cells were counted in random high-power fields using an Olympus BX51 (Japan) light microscope. Ten serial sections were examined by light microscopy on the preparations obtained from each rat.

Biochemical examination
TSH (mIU/ml), Free T_4 (ng/ml), progesterone (ng/ml) and oestrogen pg/ml) values in the plasma was measured in triplicate using 100 μL samples of each plasma with commercially available chemical immunoassay kits (Siemens, Immulite 2000).

Statistical analysis

Statistical analysis of variance was carried out using SASv 12.0 package programme. The differences between and among groups were identified using Duncan's test. All data were noted as average (±) and standard deviation (SD). In addition, anti-PgR immunoreactive cells were semi-quantitatively determined in corpus luteum and granulosa cells in ovarian. Semi-quantitative evaluation of anti-PgR immunoreactive cells density in ovarian is made as follows: none (-), rare (±), little (+), medium (++), much (+++), very much (++++).

RESULTS

Vaginal smear results were present Figure 1.

Body Weights

The weights of animals in both groups before and after the experiment were evaluated. The weights of the animals in the hyperthyroid group decreased, while the weights of the animals in the control group increased (P < 0.05, Table 1).

TSH and Free T4 Levels

At the end of the experiment, it was found that TSH value in the hyperthyroid group decreased by a statistically significant amount compared to the TSH value of the control group (P < 0.001; Table 1). In addition, free T_4 levels were higher in the experimental group by a statistically significant amount in comparison to those of the control group (P < 0.001; Table 1).

Plasma Oestrogen and Progesterone Levels

Plasma oestrogen levels were significantly higher in the experimental groups in comparison to the control groups during all menstrual cycle phases (P < 0.01 or P < 0.001; Table 2). Progesterone levels are statistically lower in proestrus experimental group (P < 0.01) when compared

to those of the control group; progesterone values are statistically high in experimental metestrus and diestrus groups when compared to the control groups (P < 0.001 and P < 0.005, respectively; Table 3).

Light Microscopic Findings
Thyroid gland findings

Examining the light microscopic findings from H&E staining sections taken from thyroid tissues of the rats in experimental and control groups revealed normal thyroid follicle structure in the control group. However, the thyroid follicles of the animals in the experimental group were larger than those in the control group. Furthermore, the follicle epithelium was in a flat shape in the experimental group, whereas in the control group, its shape varied between plasmatic and cubic (Figure 2).

Table 1: Body weights and blood TSH and free T_4 levels in the groups

	Control	Hyperthyroid
Blood TSH (mIU/ml)	0,051±0.001	0,004±0.02*
Blood free T_4 (ng/dl)	1,63±0.4	3,3±1.2*
First body weight (g)	187.3±12.5	189.8±14.7
Final body weight (g)	192.9±13.1	171.1±19.1**

*P<0.001; compared with control group, **P<0.05; compared with first value

Table 2: Blood oestrogen levels (pg/ml)

Cycle phases	Control	Hyperthyroid
Prooestrus	67,4±9.2	79,5±14.2*
Oestrus	46,4±11.3	57,4±7.5**
Metoestrus	51,7±8.4	58,9±12.4*
Dioestrus	44,6±10.2	57,8±9.1**

*P<0.01; compared with control group, **P<0.001; compared with control group

Table 3: Blood progesterone levels (ng/ml)

Cycle phases	Control	Hyperthyroid
Prooestrus	26,3±5.6	21,7±6.4*
Oestrus	20,7±4.8	21,2±5.1
Metoestrus	31,9±9.3	41,3±14.3**
Dioestrus	31,7±11.2	36,4±8.7***

*P<0.01; compared with control group, **P<0.001; compared with control group, ***P<0.005; compared with control group
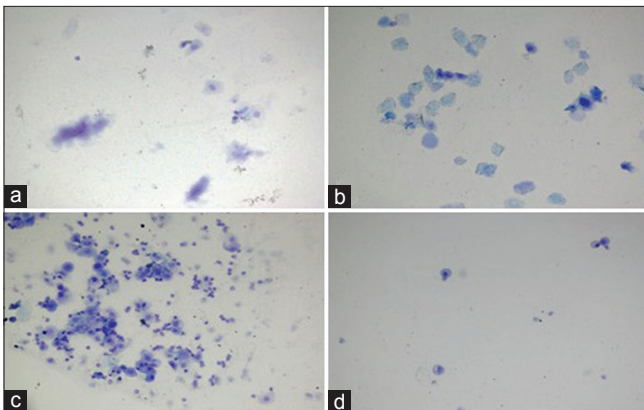
Figure 1: Vaginal smear results. a. Proestrus, b. Oestrus. c. Metoestrus. d. Dioestrus. TB staining, 400X
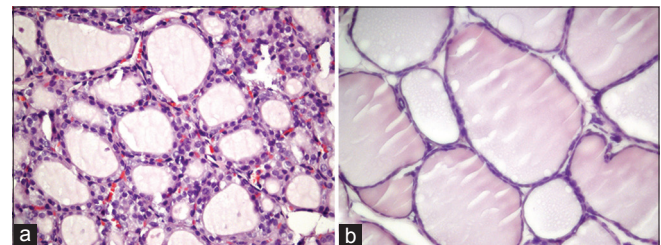
Figure 2: Thyroid histological results. a: In controls, normal thyroid architecture was seen; b: Hyperthyroid group, Hematoxylin & Eosin, 400X

Immunohistochemical Findings

Positively staining cells at eight different sections in four regions, including corpus luteum, germinal epithelium, granulosa cells and theca layer where oestrogen and progesterone were positively staining in the ovary, were counted and noted in per area percentages. Glandular epithelium and stromal cells in both the control and experimental groups were counted in eight different sections and areas, and it was noted in percentages in every cycle during which oestrogen and progesterone were positively staining in the uterus. This examination was conducted by two different researchers independently.

ER Distribution Findings in Uterus

There was no statistically change in uterine glandular epithelium in proestrus and dioestrus groups between control and experimental groups during immunohistochemical staining (P>0.05). The number of ERα decreased in the rats in the experimental group with a statistical significance when compared to the control group (P < 0.05). In addition, during the metoestrus phase, glandular epithelium in the experimental group increased with a statistical significance when compared to the control group (P < 0.01; Figure 3a, b; Figure 4).

The number of ERα-positively stromal cells in oestrus phase decreased in the experimental group with a statistical significance when compared to the control group in uterus (P < 0.005 Figure 5). There were no statistically significant differences between the experimental and control groups during other stages of the oestrus cycle (P>0.05; Figure 5).
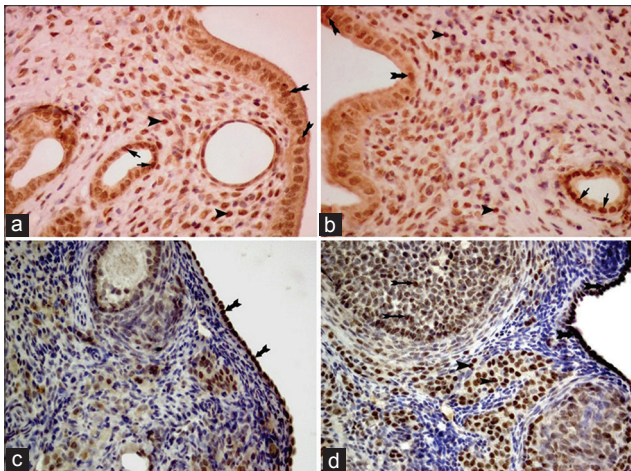

Figure 3: Uterine and ovarian histological ERα results: (a) Control uterus (Oestrus); b: Hyperthyroid uterus (Proestrus). ERα-positive epithelial cells (thick arrow), ERα-positive stromal cells (arrowhead), ERα-positive glandular epithelial cells (thin arrow). c: Control ovarian (Proestrus); d: Hyperthyroid ovarian (dioestrus); 400X, ERα-positive germinal epithelial cells (thick arrow), ERα-positive ovarian stromal cells (arrowhead), ERα-positive cells granulosa cell (thin arrow). Immunoperoxidase, Hematoxylin counterstain, 400X.

ER Distribution Findings in Ovarian

ERα was statistically increased in all cycle phases in the experimental groups, excluding oestrus, compared to the control groups at corpus luteum (P < 0.05; P < 0.01; Figures 6). The ovarian germinal epithelium of the control group and the experimental group were very close together, as were almost all stained oestrogen-positive cells. Phases of menstrual cycle ovarian granulosa cells in the experimental groups compared to those of the control groups did not differ significantly. In the proestrus and metoestrus phases, theca follicle ERα positive cell numbers increased significantly in the experimental groups compared to the control groups (P <0.05; Figure 7).

PgR Distribution Findings in Uterus

PgR immunohistochemical staining revealed a statistically significant decrease in the number of PgR in uterine lumen and glandular epithelium cells during the proestrus phase of the menstrual cycle in the experimental group when compared to that of the control group. Additionally, during oestrus and metoestrus phases, there was a statistically significant increase in the experimental groups when compared to the control groups (respectively; P < 0.001 and
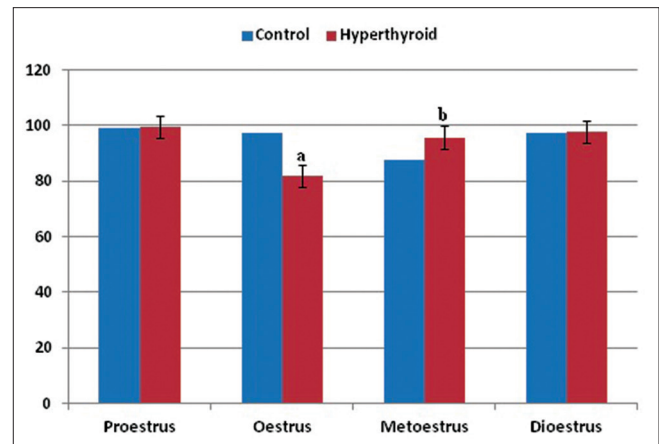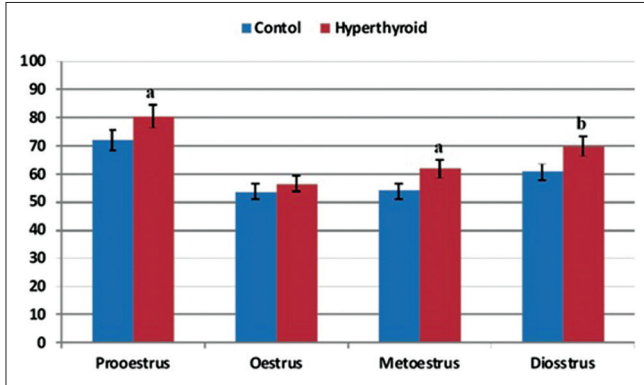

Figure 4: Uterine glandular epithelium cells percentages ERα. a: P < 0.005; compared to control group; b: P < 0.01; compared to control group
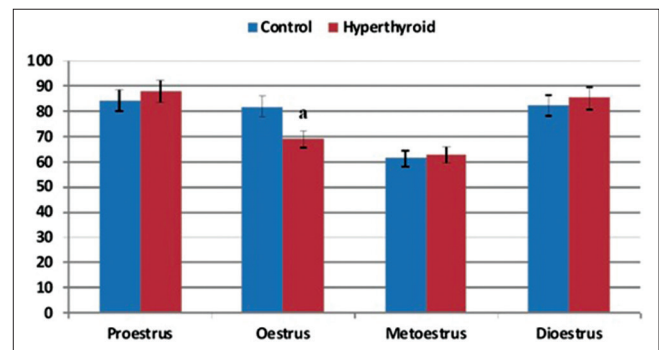
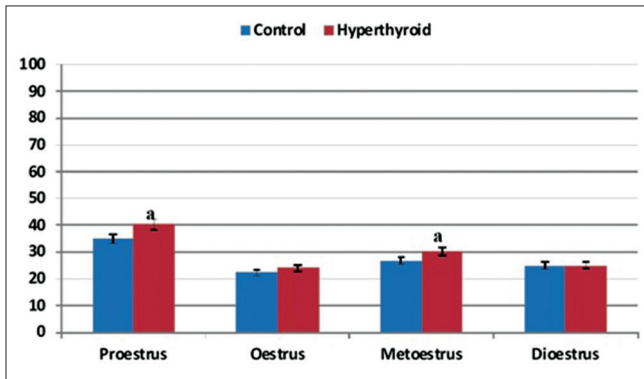**Figure 5:** Percentage of ERα-positive cells in uterine stromal cells. a: P < 0.005; compared to control group.
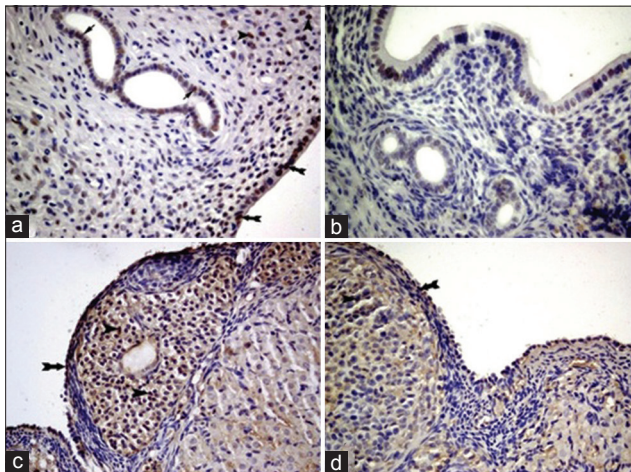
P < 0.005). There was no difference between experimental and control groups during the dioestrus phase (P>0.05; Figure 8a,b; 9).

Figure 6: Distribution of percentages of corpus luteum of the ER. a: P < 0.05; compared to control group; b: P < 0.01; compared to control group.

Figure 7: ERα-positive cells in ovarian theca layer percentages. a: P < 0.05; compared to control group.

Figure 8: Uterus and ovarian histological PgR results. a: Control uterus (Diestrus); 400X. b: Hyperthyroid metestrus uterus. c: Hyperthyroid metestrus ovary d: Control ovary. PgR-positive stromal cells (arrowhead), PgR-positive epithelium cells (thick arrow), PgR-positive glandular epithelium (thin arrow). Immunoperoxidase, Hematoxylin counterstain, 400X

PgR Distribution Findings in Ovarian

Although there is an increase in the experimental group when compared to the control group in all phases of the cycle PgR immunoreactive of ovarian germinal epithelium, this increase is at a statistically significant level during the oestrus, metoestrus (P < 0.05) and dioestrus (P < 0.005) phases (Figure 8c,d; 10). Table 4 shows semi-quantitative evaluation results of corpus luteum and granulosa cells immunoreactive of PgR in ovarian.

Table 4: Distribution of PgR-immunopositive cells in corpus luteum and granulosa cells

Group and phase	Corpus luteum*	Granulosa cells
Control proestrus	-	±
HT proestrus	±	+
Control oestrus	-	-
HT oestrus	±	±
Control metoestrus	-	+
HT metoestrus	±	++
Control dioestrus	±	-
HT dioestrus	+	-

HT: Hyperthyroid; *: None (-), rare (±), little (+), medium (++), much (+++), very much (++++)
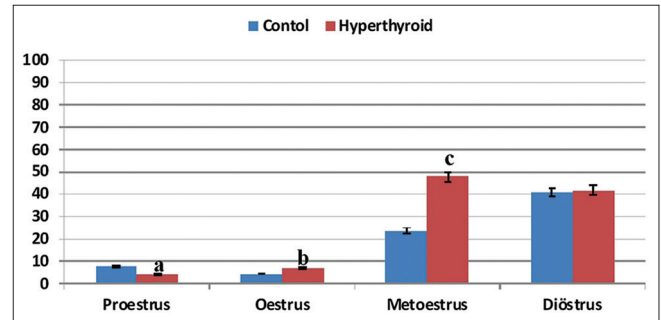
Figure 9: Distribution of percentages in the uterine gland epithelial cells of PgR. a: P < 0.001;compared to control group; b: P < 0.005; compared to control group; c: P < 0.001; compared to control group.
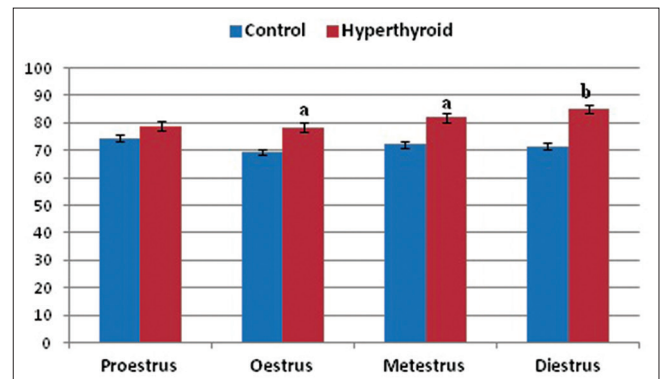
Figure 10: Distribution of percentages in the ovarian germinal epithelial cells of PgR. a: P < 0.05; compared to control group; b: P < 0.005; compared to control group.

DISCUSSION

Thyrotoxicosis is a syndrome caused by an excess of free thyroid hormones, with multiple etiologies and manifestations, which can affect all body systems.[11] The causes of illnesses include hyperthyroidism and thyrotoxicosis with low levels of radioactive uptake. The most common causes of hyperthyroidism are Basedow-Graves disease, toxic multinodular goitre and toxic adenoma.

Many studies indicate that TSH which is among free T_3 and free T_4 blood plasma concentrations and TSH which is used to identify the clinical table of hyperthyroidism are unusually low, and free T_3 and free T_4 are above usual limits. The study on rats by Özguner et al. demonstrates that TSH levels are statistically lower when compared to the control group, and free T_4 values are statistically higher when compared to the control group.[12] These findings are similar to this study's findings.

High blood thyroid hormone concentrations in patients with hyperthyroidism affect different organs and systems; therefore, findings and symptoms from the affected organs and tissues are observed.[13] Thyroid hormones can affect many systems in patients, such as reproductive functions.[12] It is debated whether these functions are a direct result of its effect on reproductive organs or its effect on the hypophysis axis.

Hyperthyroidism causes infertility in genital systems either directly, through the organs' own receptors, or indirectly, through the hypophysis axis. Studies on the effects of hyperthyroidism on infertility usually include not only its effects on the menstrual cycle but also its effects during pregnancy. High doses of thyroxin in neonatal rats causes delays in vaginal widening and the first oestrus.[14] Thyrotoxicosis before puberty can delay sexual maturity and the first menstrual cycle.[6] Similarly, menarche age of females with thyrotoxicosis is higher when compared to individuals with no endocrine disease.[15]

Although ovulatory menstrual cycles occur in women with thyrotoxicosis, menstrual disturbances are common. For this reason, when women with thyrotoxicosis do not have regular menstrual period, pregnancy should be considered. The most common anomalism is oligomenorrhea, and oligomenorrhea can turn into amenorrhea.[6] This situation is first indicated in a classic study by Von Bassedow.[16] Polymenorrhea is less common than hyperthyroidism. The frequency of these menstrual disturbances varies widely. In another study, Joshi et al. indicate that 64.7% of all women with hyperthyroidism suffer from menstrual irregularities, and 17% of them do not have any diseases.[17]

In an experimental study, De Moraes et al. found that the plasma value of progesterone hormone is high before oestrus, whereas its value becomes almost normal after oestrus.[18] In this current study, plasma progesterone values of the control group during the phases of the menstrual cycle are parallel with the previous studies on rats.[9,19] Plasma progesterone levels are low in the group with hyperthyroidism during proestrus phase of the cycle when compared to the levels of the control group. However, these levels are almost the same as those in the control group during oestrus phase, while they are statistically high during metoestrus and dioestrus phases.

Similar to the studies by Jacobs et al. (1980) and Kudolo et al. (1984), the existence of ERα ve PgR distributions in ovarian was observed in germinal epithelium, granulosa cells, corpus luteum and connective tissue between follicles in different rates during menstrual cycle in our study.[20,21] This finding was evaluated as proof that steroid hormones can have regulatory effects on follicle development through their receptors, and at the same time hyperthyroidism affects ERα and PgR localisation.

In immunohistochemical studies related to ERα and PgR distribution in uterus, the existence of receptors was detected in epithelium cells, connective tissue cells and smooth muscle cells. The existence of receptors was also observed in endothelium cells, perivascular connective tissue cells and blood cells.[22]

This current study finds that the localisation of oestrogen and PgR in uterus differs according to the phases of the menstrual cycle. In the experimental group with hyperthyroidism, ERα ve PgR distribution in stromal cells and glandular epithelium differs according to the phases of the menstrual cycle when compared to the control group.

Previous studies have demonstrated that the oestrogen hormone in epithelial cells and uterine stroma affects the expression of oestrogen and PgRs. Moreover, some studies show that high follicular oestrogen levels can be parallel with high levels of oestrogen and PgRs.[23] Another study indicates that while oestrogen increases its own receptors and PgRs, progesterone can decrease not only its own level but also ER levels.[24]

In the semi-quantitative evaluation of PgR in ovarian, it has been observed that PgRs in corpus luteum of control groups are negative during proestrus, oestrus and metoestrus phases. In addition, their levels are highest during the dioestrus phase in the group with hyperthyroidism. In the immunohistochemical evaluation, PgRs in granulosa cells are negative during oestrus and dioestrus phases in the control group, during dioestrus phase in the group with hyperthyroidism (Table 4).

CONCLUSION

In this current study, ERα is detected in varying proportions, depending on the menstrual phases, in ovarian tissue of the control and experimental groups. Its uptake is observed in germinal epithelium, theca cells, granulosa cells and corpus luteum, while there is a partial uptake in antrum. There is a statistically significant increase in the receptor numbers in theca cells and corpus luteum during the phases of the menstrual cycle in the group with hyperthyroidism when compared to the control group. There is no statistically significant increase in germinal epithelium and granulosa cells. The increase in ERα in different regions of ovarian in the experimental group is assessed as a result of the increase in the receptor concentration of oestrogen plasma concentration. These result in irregularities in the menstrual cycle, due to changes in the number of ERα and PgR as hyperthyroidism, which have been shown to be an important factor in infertility.

ACKNOWLEDGEMENTS

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