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Effects of hypothyroidism on proliferation and programmed cell-death in rat ovarian granulosa cells

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Abstract

Hypothyroidism is the most common endocrine disease, after diabetes. Thyroid hormones are essential for genital organs function. In this study, we aimed to determine the apoptotic and cell proliferation indexes resulting from reduced thyroid hormones in rat ovarian follicles. For the purpose of this study, 20 female mature Wistar rats were divided into test and control groups. The test group underwent chemical thyroidectomy by receiving 500 mg/l propylthiouracil added to drinking water.

The control group only received ordinary drinking water. After three weeks, the rats were sacrificed and their ovaries were removed and fixed for tissue preparation. Triphosphate-biotin nick end-labeling (TUNEL) and proliferating cell nuclear antigen (PCNA) immunohistochemical techniques were applied to determine apoptosis and cell proliferation variations. Our findings revealed that apoptotic index significantly diminished in large antral follicles. There were no significant differences between the two groups in terms of primary and pre-antral follicles. No TUNEL-positive cell was noted in primordial follicles in the both groups. Cell proliferation index revealed a significant decrease in follicular growth of pre-antral to graafian follicles in the hypothyroid group. PCNA-positive cells were not observed in primordial follicles in the both groups. The results of the study suggested that reduced thyroid hormones lead to a wide range of hormonal changes, and factors existing in follicular fluid, especially in large antral follicles, undergo transformations that affect apoptotic and cell proliferation indexes. The process of follicular growth occurs by entrance of follicles to the next growth phase without inducing sufficient potentiality, and the produced ovules might be healthy or morphologically defected.

Keywords: Apoptosis, Cell proliferation, Folliculogenesis, Hypothyroidism, Ovarian follicle, Rat

Introduction

Hypothyroidism mainly originates from disorders of thyroid gland leading to reduced thyroxin (T4) and triiodothyronine (T3) production and secretion (Harrison, 2008. Bharaktya, Griffing, 2010. Guyton, 2011). Levothyroxine, as a thyroid hormone, is prescribed when thyroid gland does not produce sufficient level of hormones (Imberti et al., 2010. Radaeli, Diehl, 2011).

Female fertility depends on proper development of ovarian tissue, oocyte regulation and maturation, as well as proliferation and differentiation of somatic cells during folliculogenesis, which is controlled in two levels; first, the intragonadal factors, which initiate follicular growth and regulate development of oocytes, granulosa cells, and components of theca cells, located in two rows proximal to follicles; second, the extragonadal factors.

Thus, every month the number of follicles grows, but in humans, solely one follicle matures and prepares for ovulation, while this number ranges between three and seven in rodents. Four main types of follicles are recognized in ovarian tissue, namely, perimordial, primary, secondary, and graafian follicles (Elvin, Matzuk, 1998. Codon et al., 2001. Ruoss et al., 2009).

The researchers studied ovarian-pituitary axis after reduction of thyroid hormones in mature female rats. The results showed irregular long menstrual cycles, elevated levels of plasma progesterone, and reduced ovulation in hypothyroid rats (Mattheij et al., 1995).

Apoptosis is programmed cell-death, occurring naturally in different stages of morphogenesis in fetal and adult tissues. Some pathologic conditions such as heat, exposure to ionized rays and toxic substances, hormonal and growth factor deprivation, genetic mutations, as well as genetic transformation of BCL2, Fas ligand, and P53 increase the rate of cellular apoptosis (Saraste, 1999. Sjostrom, Bergh, 2001).

The necessity of apoptosis in multicellular organisms is widely accepted. According to former studies, apoptosis plays a significant role in development of ovarian follicles. This process occurs in three stages of ovarian tissue development, namely, oogenesis before birth, follicular atresia, and luteolysis (Tilly, 1996. Tingen et al., 2009).

The level of DNA synthesis is an indicator of cell proliferation (Hirshfield, 1989. Hall, Levison, 1990). Sensitive methods for detecting markers of cell proliferation are Ki-67 and proliferation cell nuclear antigen (PCNA) in G1 phase of cell cycle. PCNA is a nuclear protein that plays a key role in regulating cell cycle. Cycklin D protein and PCNA are necessary for controlling S phase. This complex may be transformed by various growth factors and hormonal stimuli (Liu et al., 1989. Xiong et al., 1991. Xiong et al., 1992).

The scientists demonstrated that PCNA is a sensitive marker of early stages of follicular growth, and its expression and DNA synthesis are matched to some extent (Kutluk et al., 1995). Thus, in the present study, we aimed to determine apoptotic and cell proliferation indexes resulting from reduced levels of thyroid hormone in rat ovarian follicles.

Materials and Methods

Animal preparation

This experimental, interventional study was performed on 60 female mature Wistar rats aged 2.5 months with body weight of 200-250 g. The animals were kept in a climate-controlled room under a 12 hr alternating light/dark cycle at 24±1°C with enough food and water. The experimental group included 10 alive rats receiving 500 mg/L propylthiouracil (PTU) (Iran Hormone Co.) in drinking water for three weeks. Hypothyroidism was confirmed in this group by radioimmunoassay (RIA) test. PTU causes rapid decline of thyroid hormones in hyperthyroid diseases (Gottesfeld et al., 1984. Rassouli et al., 1991). Ten alive rats receiving ordinary drinking water were assigned to the control group. Based on experimental studies, the samples comprised of removed ovaries from 20 rats.

Radioimmunoassay Test (RIA)

To confirm hypothyroidism, the level of thyroid hormones in plasma is measured in RIA method. Three weeks after receiving the drugs, 1-2 ml blood was drawn from the rats' angular eye vein using sterile glass capillary tubes. After centrifuging, the separated blood serum was determined using kit (IRMA Co., Iran) via RIA method. At the end of the period, the sample ovaries were dissected and transferred to fixation solution.

Samples preparation for histological study

The tissue samples were fixed in 4% paraformaldehyde soluted in buffer phosphate (PBS) for 14 hr, the samples were dehydrated by alcoholic solutions and were molded by paraffin embedding. After deparaffinization and hydration by alcoholic solutions with descending grades, the samples were tested using TUNEL and PCNA immunohistochemical methods and were studied by optical microscope.

TUNEL immunohistochemical method

Tissue apoptosis was assessed by TUNEL proxidase kit (in situ cell death detection Kit-POD, Roch, Germany). The slices were deparaffinized, hydrated, and then incubated for 15 minutes at humid room temperature with 20 g/ml K protein kinase. The slides were incubated with reactive TUNEL mixture consisting terminal deoxynucleotidyl transferase (450 µL of Enzyme Solution, 50 µL of Lable Solution) for sixty minutes at 37°C. Afterwards, deoxyuridine triphosphate (dUTP) conjugated by dioxygen proxidase was added and the slides were covered with a lid, and then dioxygen and hydrogen peroxide (Converter-POD) were added to the samples. The slides were incubated for 30 minutes and diaminobenzidine (DAB) was added (6 mg DAB powder, 10 mL PBS, and 10 µ H2O2 3%). The slides were washed by PBS three times and were stained with hematoxylin. Apoptotic cells appeared in brown color (Gavrieli et al., 1992. Ichimura et al., 1995. Kraupp et al., 1995. Marlangue et al., 1995. Clarke et al., 2000).

PCNA immunohistochemical method

PCNA kit (Zymed Co., USA) was used as the primary stain, similar to a successful study on rodent ovaries (Picut et al., 2008). Our tissue preparation methods differ from that study only in that we counterstained with hematoxylin for 60 seconds rather than 3 minutes and we used 1:100 dilution of PCNA (instead of 1:400) as was recommended by the stain supplier.

The preparation sequence was 1) Disparaffination and hydration, 2) Heat-induced Antigen retrieval for 60 minutes with TRIS-EDTA buffer solution (PH 9), 3) Endogenous peroxidase was blocked using 0.3% H2O2 in distilled water for 15 minutes at room temperature, 4) Washing in distilled water for 10 minutes followed by buffer wash (PBS), 5) Incubation at room temperature for 60 minutes with primary antibody (mouse monoclonal PCNA concentrate, dilution 1:100, clone PC 10 BIOCARE) followed by buffer wash (PBS), 6) Incubation using Dual Link HPR (DAKO Envision) for 30 minutes at room temperature followed by buffer wash (PBS), 7) Application of DAB chromogen (DAKO) for 10 minutes followed by wash in distilled water, 8) Counterstain nuclear-Mayer hematoxylin for one minute followed by 10 minutes under running water, 9) Dehydration in alcohol, 10) Application of Xylene, and 11) Mounting on a standard coverslipped slide.

Morphological ovarian follicles

The ovarian follicles were classified into (Pedersen, 1970. Hirshfield et al., 1978. Peters et al., 1978) 1) Primordial follicle (the oocytes are surrounded by a layer of squamous follicular cells), 2) Intermediate follicle (the oocytes are surrounded by squamous and cuboid cells), 3) Primary follicles (the oocytes are surrounded by cuboid cells), 4) Preantral follicle (the spaces are seen between cells sporadically), antral follicle (the space is extending between the cells finally taking one-third of the follicle's volume), which includes two stages of 5) Early antral and 6) Late antral, and 7) Tertiary ([graafian] the selected follicle with a space larger than two-third of follicle's volume).

Stereology technique

Apoptotic and cell proliferation indexes were calculated using stereological analysis (Gundersen et al., 1988. West et al., 1991. Gundersen et al., 1999. Rassouli et al., 2000. Melo et al., 2002. Charleston et al., 2003). In this technique, the disector density of the particles is measured in a three-dimensional space. Some pairs of incisions were randomly selected from among the prepared incisions with equal spaces (random systematic sampling), and the distance between the first and second slices in each pair of selected slices (the disector depth) was determined in a way that it was less than the size of the smallest counted particle. In so doing, the selected particle was cut by one of the parallel cuts in each pair.

On a parallel incision (reference incision), a number of sampling frames with defined area were randomly placed on a reference incision and other sampling frames were placed on the second frame with exactly the same location as the first one. Marked particles' cross-section was observed within unbiased frames of the first incision, no trace in the frames of the second incision was counted.

Thereafter, the second incision was considered as the reference, and the particles with marked cross-section within the frames were placed on it and counted with no trace in the frames of the first incision. In this way, the number of marked particles in the two dissectors were counted. A similar process was performed for other parallel incisions. Afterwards, the number of marked nuclei was quantified using stereological analysis and the following equations (Table 1).

Table 1: Stereology technique of apoptotic and cell proliferation indexes

Equation	Description
$N_v = \frac{\sum Q^-}{\sum F \times a(F) \times t}$	Nv numerical density in volume unit (mm-3), ∑Q⁻ total counted marked nuclei cross-section, ∑F total frames related to the desired structure, a(F) area of each frame considering the magnification (mm2) and t dissector depth (mm)
$V_R = t\,.\,a(P)\,.\sum_{i=1}^{n} P$	VR reference volume (mm3), t distance between two section (Cross-section thickness)(mm), a(P) area related to spot (mm2), n number of cross-sections and $\sum_{i=1}^{n} P$ total spots hit with the desired structure.
$Vv = \frac{\sum Pf}{\sum Pr}$	Vv volume fraction or volume density, ∑Pf total spots hit with follicle and ∑PR reference volume (mm3).
$V_f = V_v \times V_R$	Vf follicle volume(mm3), Vv volume fraction or volume density and VR reference volume (mm3)
$N = N_v\,.\,V_f$	N total number of marked nuclei in the follicle, Nv numerical density in unit volume (mm-3) and Vf follicle volume (mm3)
$AI, PI = \frac{n\,follicle^{+}\,.\,n\,cell^{+}\,.\,100}{n\,total_f\,.\,n\,total_c}$	AI apoptotic index, PI cell proliferation index, n follicle+ number of follicles with dying cells or proliferating cells, n cell+ number of dying cells or proliferating cells in each follicle, n totalf total number of follicles and n totalc total number of cells in each follicle

Statistical analysis

The data were analysed using Life science, Image j, Image Tools3, and SPSS version 16. Normality was controlled to check any variation among means. T-test, ANOVA, and Tukey's tests were run, and P-value less than 0.05 was considered statistically significant.

Results

RIA test

The results are shown in Table 2. Statistical analysis showed a significant reduction in the level of thyroid hormones in hypothyroid group (P<0.001).

Table 2: Mean serum levels of T3 and T4 hormones and weight of ovarian tissue in the control and hypothyroid groups

	group	Mean	Std Deviation
T3 RIA test	Control	85.90	14.479
	hypothyroid	54.30*	9.799
T4 RIA test	control	4.60	0.452
	hypothyroid	3.22*	0.684
weight rat ovary	control	0.078	0.007
	hypothyroid	0.044*	0.010

*Significant values compared with control group P<0.05

Weight of ovarian tissue

In macroscopic studies, the ovarian tissues were weighted with a digital scale in the control and hypothyroid groups. The results of which are shown in Table 2. T-test reflected a significant decrease in weight of ovarian tissue of hypothyroid group (P<0.001).

Calculating of apoptotic and cell proliferation indexes

TUNEL and PCNA techniques were conducted in both groups, and then were studied for quantifying cells, apoptotic and cell proliferation indexes, and the number of graafian follicles and luteal bodies in stereological method. The results are expressed as follows:

Primordial follicle

The studies on primordial follicles showed zero apoptotic and cell proliferation indexes, which signifies that no TUNEL–positive and PCNA-positive cells appeared in the control and hypothyroid groups (Figure 3).

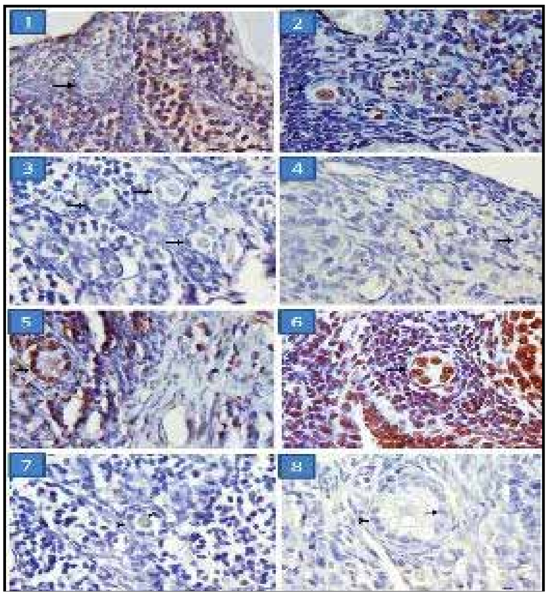

Figure 3: Optical photomicrograph of rat ovarian primordial follicles (arrow) after triphosphate-biotin nick end-labeling (TUNEL) technique (1=hypothyroid, 2=control) and proliferating cell nuclear antigen (PCNA) technique (3=hypothyroid, 4=control); absence of TUNEL and PCNA–positive cells in squamous cells in follicles; primary follicles after TUNEL technique (5=hypothyroid, 6=control) and PCNA technique (7=hypothyroid, 8=control); TUNEL and PCNA–positive cells nuclei (arrow), TUNEL and PCNA–negative cells nuclei (arrow head); magnification: 100x.

Primary follicle

The apoptotic index was approximately 100% in the studied samples. This means that almost all cells were TUNEL-positive in primary follicles. Since the standard deviation between data was zero in control and hypothyroid groups, the T-test statistical analysis was not done on the data. PCNA-positive cells were scattered in the follicles. Index of cell proliferation was observed in the samples. Statistical analysis of the data showed no significant differences between the groups (P>0.05; Table 3; Figure 3).

Table 3: Mean of apoptotic and cell proliferation indexes of ovarian follicles as well as the number of graffian follicles and luteal bodies in the control and hypothyroid groups

	Type of the follicle	group	Mean	Std Deviation
Apoptotic Index	Preantral	Control	79.85	6.87
		hypothyroid	78.30	0.80
	Early antral	control	77.57	3.90
		hypothyroid	76.69	6.27
	Late antral	control	77.28	6.83
		hypothyroid	56.68*	1.64
	Graffian	control	77.11	5.76
		hypothyroid	54.26*	4.05
Cell Proliferation Index	Primary	Control	42.12	15.56
		hypothyroid	37.76	15.33
	Preantral	Control	31.27	3.41
		hypothyroid	16.34*	1.30
	Early antral	control	24.32	2.14
		hypothyroid	13.06*	3.38
	Late antral	control	23.33	8.09
		hypothyroid	10.28*	2.56
	Graffian	control	39.65	3.18
		hypothyroid	15.29*	1.76
	Number of graffian follicles	control	5.00	0.816
		hypothyroid	1.90*	0.738
	Number of luteal bodies	control	2.50	0.850
		hypothyroid	7.80*	1.229

*Significant values compared to control group P<0.05

Preantral follicle

The results of apoptotic index calculation showed no significant differences between the groups (P>0.05). TUNEL–positive cells were observed in theca layer cells. The results of cell proliferation index showed a significant decrease in hypothyroid compared to the control group (P=0.000). PCNA-positive cells were observed for the first time in theca layer (Table 3; Figure 4).

Early antral follicle

Apoptotic index calculation presented no significant differences between the two groups (P>0.05). TUNEL–positive cells were seen in theca layer cells. The cell proliferation index showed a significant decrease in hypothyroid group, compared to the controls (P<0.01). Some scattered PCNA-positive cells were observed among the granulosa cells (Table 3; Figure 4).

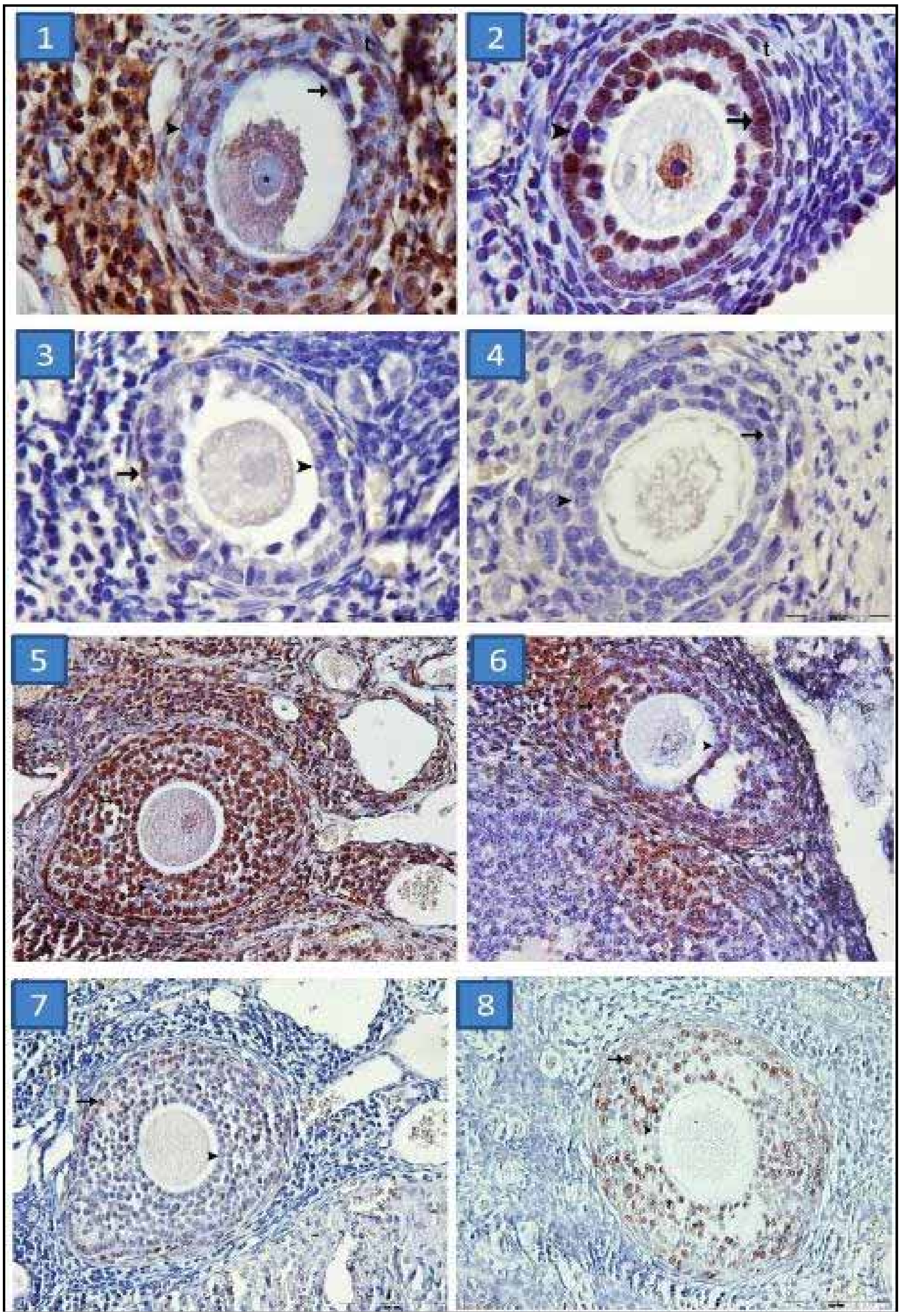

Figure 4: Optical photomicrograph of rat ovarian preantral follicles after triphosphate-biotin nick end-labeling (TUNEL) technique (1=hypothyroid, 2=control) and proliferating cell nuclear antigen (PCNA) technique (3=hypothyroid, 4=control); TUNEL and PCNA–positive cell nuclei (arrow), TUNEL and PCNA–negative cell nuclei (arrow head); TUNEL-positive cells are seen in techa layer(t), magnification x100. Early antral follicles containing small antrum after TUNEL technique (5=hypothyroid, 6=control) and PCNA technique (7=hypothyroid, 8=control). TUNEL and PCNA–positive cell nuclei (arrow), TUNEL and PCNA–negative cell nuclei (arrow head); magnification: 40x.

Late antral follicle

The results of apoptotic index calculation exhibited a significant diminiuation in the hypothyroid group compared to the control group (P=0.000). TUNEL-positive cells were clearly observed in antrum margin. Cell proliferation index showed a significant decrease in the hypothyroid group, compared to the control group (P<0.01). PCNA-positive cells were observed in granulosa cells and theca layer (Table 3; Figure 5).

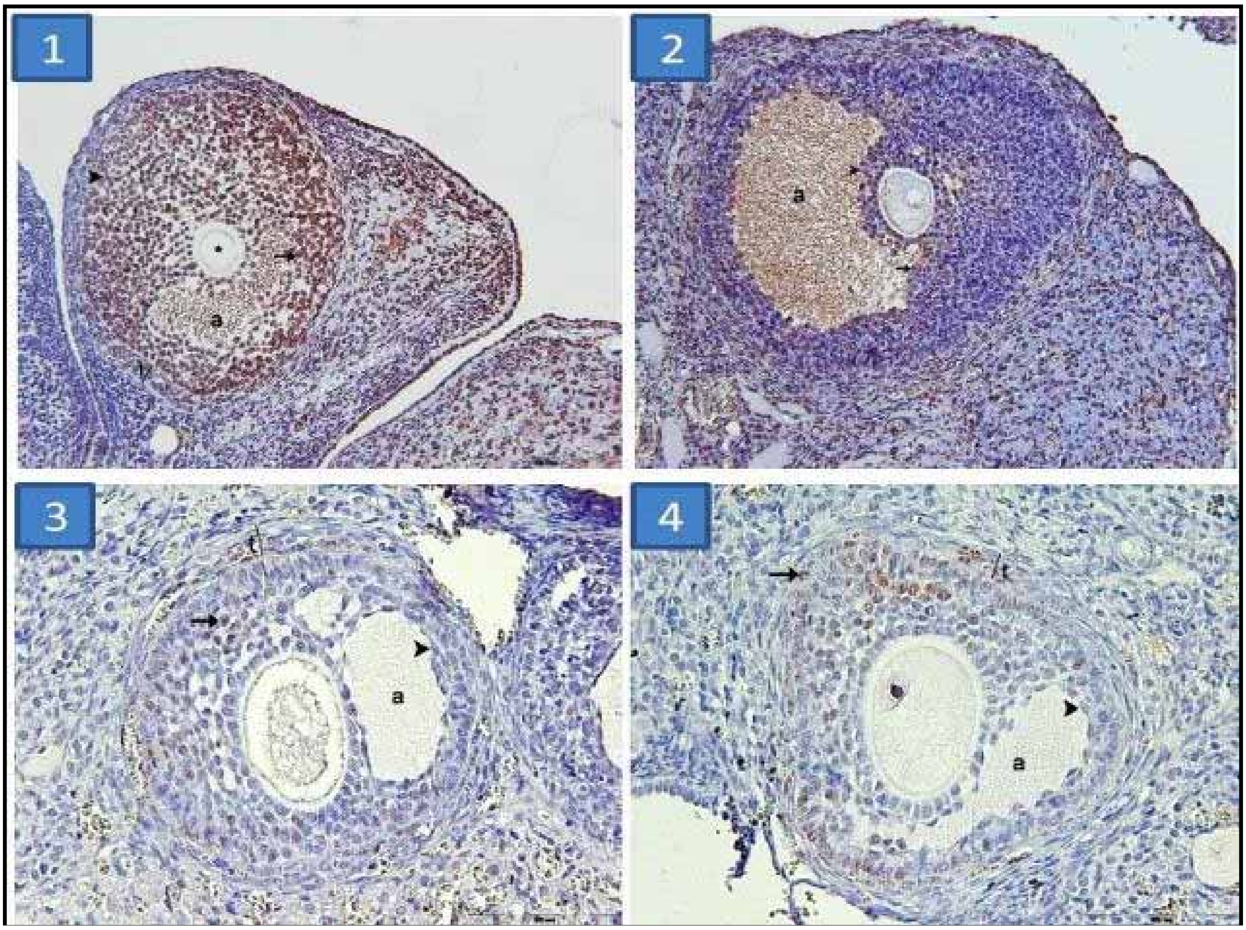

Figure 5: Optical photomicrograph of rat ovarian late antral follicles containing antrum about two-third of follicle volume(a) after triphosphate-biotin nick end-labeling (TUNEL) immunohistochemical technique (1=hypothyroid, 2=control) and proliferating cell nuclear antigen (PCNA) immunohistochemical technique (3=hypothyroid, 4=control). TUNEL and PCNA–positive cells nuclei (arrow), TUNELand PCNA–negative cells nuclei (arrow head); magnification: 20x.

Graaffian Follicle

The results of apoptotic index calculation in these follicles showed a significant decline in hypothyroid group compared to the control group (P=0.000). T-test analysis of cell proliferation index calculation in these follicles reflected a significant reduction in hypothyroid group compared to the control group (P=0.000; Table 3; Figure 6).

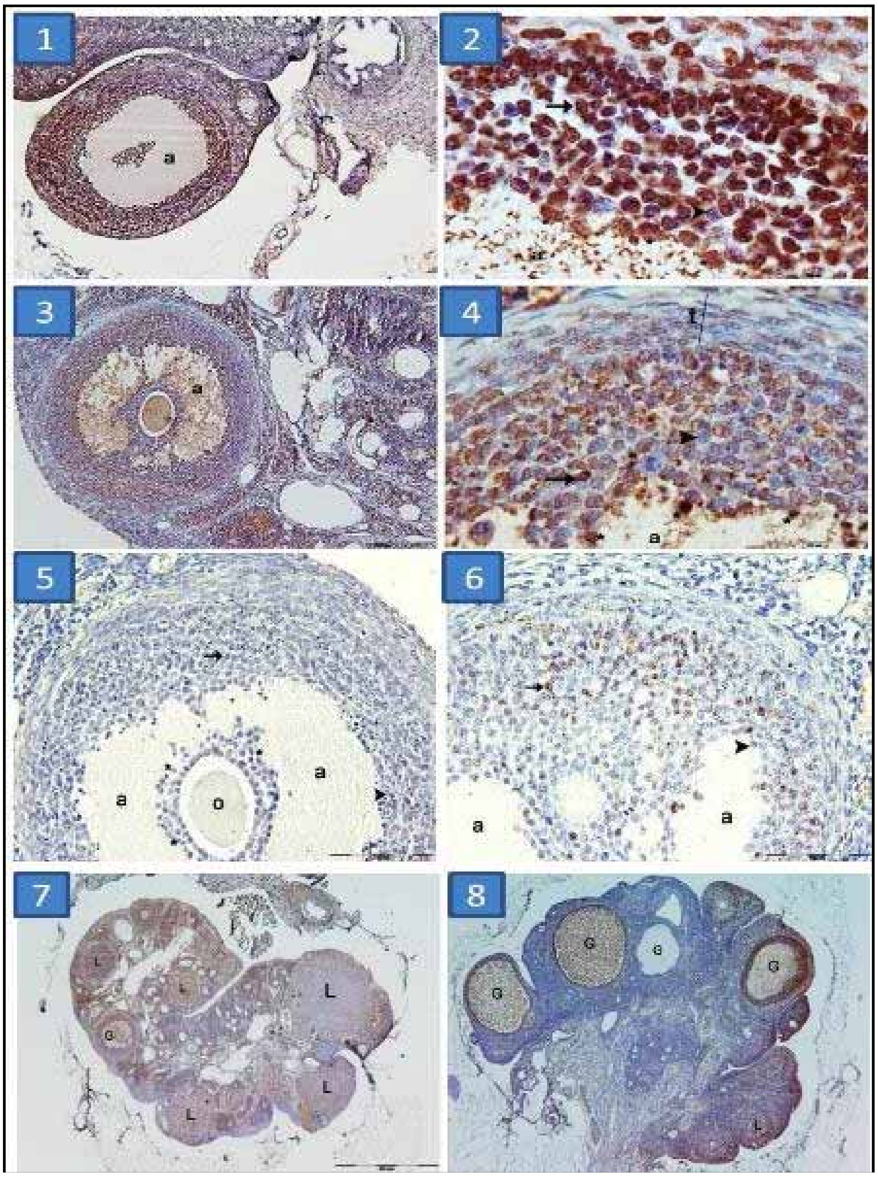

Figure 6: Optical photomicrograph of rat ovarian graffian follicles containing large antrum more than two-third of follicle volume(a) after triphosphate-biotin nick end-labeling (TUNEL) technique (1,2=control, 3,4=hypothyroid) and proliferating cell nuclear antigen (PCNA) technique (5=hypothyroid, 6=control). TUNEL and PCNA–positive cell nuclei (arrow), TUNEL and PCNA–negative cell nuclei (arrow head). Magnification: 10x, 100x. General rat ovarian tissue. 7) hypothyroid group, there are afew antral follicles (G) and lots of luteal bodies (L). 8) control group, there are afew luteal bodies (L) and lots of antral follicles (G); magnification: 4x.

Number of luteal bodies and graaffian follicles

Quantification of the number of graafian follicles revealed a significant decrease in hypothyroid group compared to the control group (P=0.000). T-test analysis of the number of luteal bodies showed a significant increase in hypothyroid group in comparison with the control group (P=0.000; Table 3; Figure 6).

Discussion

Study of the control and hypothyroid groups showed that primary and graafian follicles had the highest and lowest levels of apoptotic indexes of ovarian follicles, respectively (Figure 7). Moreover, evaluation of a variety of ovarian follicles indicated that primary and late antral follicles had the highest and lowest cell proliferation indexes, respectively (Figure 8). PCNA was observed in primary follicles for the first time. Small amounts of PCNA are expressed in the early growth stages of primary follicle in granulosa cells. Furthermore, in corpus luteum, there were some cells with PCNA. In theca layer, PCNA expression was noted in pre-antral follicles for the first time. Despite the observation of PCNA expression in oocytes of growing follicles, cell division cannot be concluded. It is known that oocytes are stopped at diplotene stage in mammalian cell cycle. Our results revealed that pre-antral and early antral follicles have the highest rate of cell proliferation.

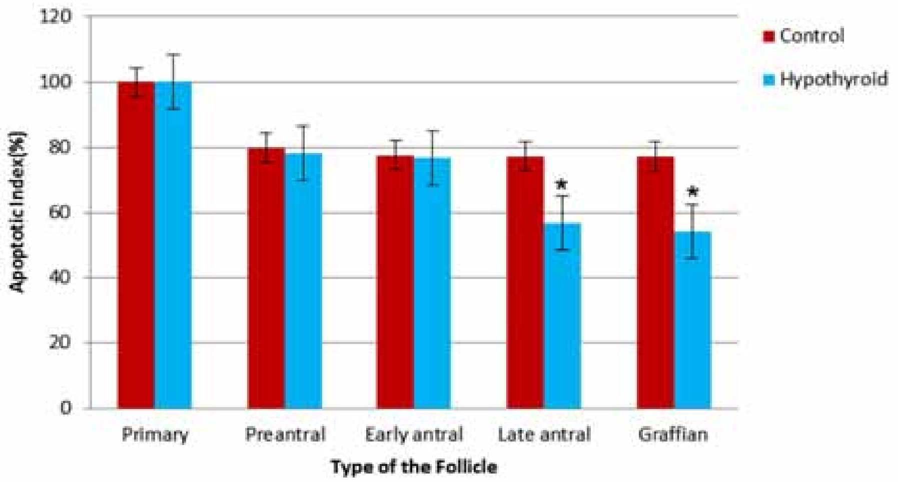

Figure 7: Comparison of mean of apoptotic index of ovarian follicles in the control and hypothyroid groups (*Significant values compared to the control group P<0.05)

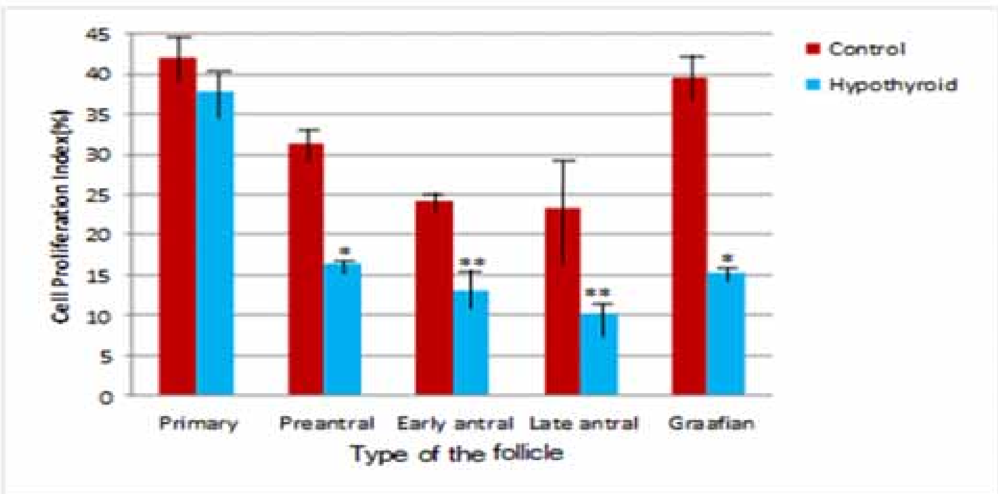

Figure 8. Comparison of mean of cell proliferation index of ovarian follicles in the control and hypothyroid groups (*Significant values compared to the control group P=0.000, **Significant values compared to the control group P<0.01)

It is known that normal reproductive behavior and its relevant physiologic aspects are subject to having a balanced level of thyroid hormones, and hypothyroidism is accompanied with reproductive dysfunction (Bloom et al., 1975. Bourget et al., 1987. Jannini et al., 1995. Takagi et al., 2007). The researchers investigated whether infertility in hypothyroidism results from ovarian or pituitary functional changes; they proposed that hypothyroidism leads to excess production of prolactin hormone, which prevents secretion of gonadotropins and induces the observed alterations (Armada et al., 2001). Increased gonadotropins can influence reproductive function (Krassas, Pontikides, 2004. Saita et al., 2005).

The researchers pinpointed that infertility was due to deficiency of thyroid function in rats. They prpounded that thyroid hormone is not a prerequisite for mating and delivery (Hosoda et al., 2008). According to previous studies, hypothalamus–pituitary axis establishes a relationship between gonads and helps feedback control associated with hormone secretion and regulation (Porterfield, Henderson, 1993. Galikoglu et al., 1996. Krassas, perros, 2003). The researchers investigated the effect of different hormones including steroid and growth factors on some important organs such as prostate, ovaries, testicles, and mammary glands. Their findings demonstrated that after apoptosis in granulosa cells, atresia begins in ovarian follicles (Kiess, Gallaher, 1988).

The present study showed that apoptotic index of primordial follicles was zero in both groups. In fact, it indicates that apoptosis is initiated at the beginning of formation of primary follicles in ovarian follicular cells. Absence of apoptotic cells in primordial follicles proves that 99% of folliclular supplies are exhausted in the early stages of formation of primary follicles (Codon et al., 2001). Apoptotic index exhibited a reducing trend in both groups from primary to graafian follicles. This shortage was evident in late antral and graafian follicles of the hypothyroid group.

In hypothyroidism, prolactin hormone production increases, which in turn, causes reduced secretion and activity of gonadotropins (Armada et al., 2001). Previous studies revealed that iodine uptake was higher in ovarian follicular fluid following theroid gland than any other body part (Slebodzinshi, 2005). Therefore, the results of the present study concerning reduced apoptotic index in large antral follicles (late antral and graafian) was in line with the results of former studies. It indicates that although the apoptotic index of graafian follicle, recruited for ovulation, decreased in the control group, a significant decline was noted in the hypothyroid group due to deficiency of gonadothropin hormones. In the recruited graafian follicles, considering the large antrum and containing follicular fluid for preservation and stability of follicles, apoptotic index showed a significant reduction compared to the control group.

It was assumed that reduction of thyroid hormones significantly dwindled apoptotic index only in large antral follicles. The reduction of hormones cause a significant decrease of apoptotic index in all types of follicles compared to the control group. In this study, most primary follicles showed apoptotic index of 100% in both groups; thus, hypothyroidism might not affect these follicles. After primary follicles, the highest apoptotic index was noted in pre-antral follicles followed by early antral follicles.

DNA synthesis is measured as an indicator of cell proliferation (Kutluk et al., 1995). PCNA is a non-histone protein, which plays an auxiliary role for DNA polymerase delta (Hirshfield, 1986. Jaskulski et al., 1988. Chang et al., 1990. Hall et al., 1990). The present study determined that cell proliferation index was zero in primordial follicles of both groups. PCNA in primary follicles is mainly expressed in ovarian follicular cells. According to the results of the present study, apoptotic cells do not exist in primordial follicles. Therefore, the hypothesis that 99% of follicular supplies are degenerated in the initial stages of primary follicle formation is confimed (Nelson et al., 1985).

Considering our results and the lack of PCNA expression in primordial follicles, increased accumulation of primary granulosa cells in early stages of follicular growth might result from convergence of follicle adjacent cells in ovarian stroma. Cell proliferation index from primary follicle to late antral follicle declined in the control group, while graafian follicle significantly increased. Previous studies proved that hormonal stimuli may lead to variation in the expression of PCNA (Jaskulski et al., 1988. Chang et al., 1990). Cell proliferation index of graafian follicles decreased significantly in the hypothyroid group, compared to the control group. Reduced thyroid hormone levels might cause decreased cell proliferation index in large antral follicles. The highest rate of cell proliferation might be observed in primary and pre-antral follicles.

According to the results of the current and previous studies, PCNA expression begins after the start of follicular growth. Absence of PCNA in granulosa cells indicated that the cell may be in phase M of cell cycle. This result shows that PCNA expression is not synchronous with cell division (Kutluk et al., 1995). According to the present study, cell proliferation index and weight of the ovaries significantly lessened in the hypothyroid group (Dijkstra et al., 1996).

Although hypothyroidism impairs reproductive function, some adjustments can be done to reach the selected follicle to ovulation stage. Conducting an experimental study on follicular fluid from molecular and biochemical aspects is recommended to determine the factors inducing the aforementioned impacts.

Conclusion

In the present study, it was found that low levels of thyroid hormones might cause extensive hormonal variations that cause the factors existing in follicular fluid, especially in large antral follicles, undergo changes and precipitate the process of follicular growth. Therefore, the follicle will enter the next stage without having the required potentiality and consequently, the produced ovule might be healthy or morphologically defected. Our outcomes indicated that based on the initial growth phases of follicular granulosa cells, PCNA expression was found in primary follicles, therefore, the initial increase in granulosa cells of growing follicles might be due to adjacent cells to follicle and in ovarian stroma.

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