Original Article

Study of the Effect of Hypothyroidism on the Apoptotic Index in Rat Ovarian Follicles, Using the TUNEL Technique

Mahmoud Moghaddam-Dorafshani , M.Sc.[1], Medi Jalali, Ph.D.[1*], Mohammad Reza Nikravesh, Ph.D.[1], Ali Reza Ebrahimzadeh , Ph.D.[1]

1. Anatomy and Cell Biology Department, Medical Faculty, Mashhad University of Medical sciences, Mashhad, Iran
***Corresponding author, E-mail address:** JalaliM@mums.ac.ir*

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Professor Mehdi Jalali has 26 years of experience as an educator, supervisor, and research scientist in gross anatomy, embryology and histology at Mashhad University of Medical Sciences, Mashhad, Iran. Dr. Jalali conducts research in developmental biology with specific emphasis on developmental immunohistochemistry.

Abstract

Introduction: Among the endocrine diseases, hypothyroidism is the commonest after diabetes.Thyroid hormones (T3,T4) are essential for genital organs function. Apoptosis process in ovarian cells plays a significant role of development in ovarian follicles. The aim of the present study was determine the apoptotic index induced by hypothyroidism in rat ovarian follicles.

Materials and Methods: In the present study, twenty female mature wistar rats were used with age of 2.5 months and weight of 200-250 (g). Rats were divided into test and control groups. In test group chemical hypothyroidism induced by propylthiouracil (PTU;500 mg/L) in drinking water. The control group only received normal drinking water. After three weeks the rats were killed and their ovaries removed and were fixed for tissue preparation. TUNEL technique were used for determine of apoptosis. Cells count done by stereological method. Data were analyzed by t-test and one-way ANOVA followed by Tukey test. Significance was accepted at $P<0.05$.

Results: The findings showed that the apoptotic index had a significant decrease in late antral and graffian follicles ($P=0.000$) and no significant decrease in preantral and early antral follicles ($P>0.05$) in hypothyroid group. All granulosa cells were TUNEL-positive in primary follicles but no cell was seen in primordial follicles in groups.

Conclusion: The results of the present study showed that the hypothyroidism may be vigorous decreased of apoptotic index in antral and graffian follicles. Hypothyroidism increased the number of luteal bodies and decreased the number of graffian follicles in ovarian tissue.

Keywords: Hypothyroidism, Apoptosis, Ovarian Follicle, Thyroid Gland, Rats

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Introduction

After diabetes, hypothyroidism is the most common endocrine disease whose incidence increases with age. This disease is mainly caused by disorders in thyroid gland that lead to decreases in triiodothyronine (T_3) and thyroxin (T_4) production and secretion, resulting in primary hypothyroidism [1-3]. Levothyroxine is a thyroid hormone prescribed when the thyroid gland does not produce an adequate amount of hormones [4,5].

Female fertility is dependent upon the precise development of ovarian tissue, special regulation of oocytes, and the maturity, multiplication and discrimination of somatic cells during folliculogenesis. This process is regulated by intragonadal and extragonadal factors. The intragonadal factors begin follicular growth and organize oocyte development, granulosa cells and theca cells ingredients in follicles. Four main types of follicles are present in the ovary: primordial, primary, secondary and graffian [6-8].

Mattheij et al. researched the ovary-pituitary axis following reduction in thyroid hormones in mature female rats by iodine-131 radioidectomy. According to the results, hypothyroid rats had irregular, long menstrual cycles, increased plasma progesterone and decreased ovulation [9].

Apotosis is the planned cell death that occurs naturally during different stages of morphogenesis, both in fetal tissues and during adulthood. The rate of apoptosis increases in cells under certain pathologic conditions such as genetic changes (BCL_2, ligand Fas, P53), heat, exposure to ionizing rays, toxic substances, hormonal and growth factor depri-vations, and genetic mutations. The cell that undergoes apoptosis morphologically becomes round and wrinkled in shape and its nuclear chromatin becomes compressed and marginal. When DNA is analyzed, the cell nucleus will be broken into several blocks or chromatin bodies, called apototic bodies which are finally eliminated by phagocytizing cells [10,11].

The necessity of apotosis is widely accepted in multicellular organisms. According to studies, the process of apoptosis in ovarian tissue cells plays a significant role in the development of ovarian follicles. This process occurs within three stages during ovarian development: oogonium before birth, follicular atresia and lutealysis [12,13]. This study aims to determine the apoptotic index that results from reductions in the thyroid hormones, T_3 and T_4, in ovarian follicles.

Materials and Methods

All study procedures were approved by the Medical Ethics Committee Mashhad University of Medical Sciences Mashhad,Iran. The present research is an experimental and interventional study.The number of female mature wistar rats were selected in 2.5 months of the age and body weight of 200-250 (g). Animals were maintained in standard animal house conditions (12 hr light:12 hr dark) with adequate water and food, at a temperature of 24±1°C. The experimental group (n=10) was given a solution of propylthiouracil 500 (mg/L) (PTU; Iran Hormone Company, Iran) in drinking water. Their hypothyroidism was confirmed by radioimmunoassay (RIA). PTU causes rapid decline in thyroid hormones under hypothyroid conditions [4,15] Controls (n=10) received ordinary drinking water.

Radioimmunoassay (RIA)

In order to ensure hypothyroidism , the level of thyroid hormones in the rats' blood plasma is measured in RIA method. Three weeks after Reception of the drug, were venesected from their angular eye vein to 1-2 mL. After centri-

fuging the blood's serum was determined using kit (Iran,IRMA co.) in RIA method. At the end of the period, the ovaries all of the groups were dissected and transferred to fixation solution.

Histological analysis

Tissue samples were fixed in paraformaldehyde (4%) that was solved in phosphate buffer saline(100 mL) (PBS) for 14 to 16 h, dehydrated in ascending grades (20%-100%) of alcohol for 45 min to 1 h, then cleared in alcohol-xylene (50:50) and xylene (three times). Tissues were then fixed in paraffin, samples were cut in 5 µm sections with a microtome and placed on poly L-lysine slides. Slides were deparaffinized and hydrated in descending grades of alcohol. Tissues were analyzed by the TUNEL technique and viewed with an optical microscope.

TUNEL immunohistochemical technique

Apotosis in tissue was done by TUNEL proxidase kit (In situ cell death detection Kit-POD,Roch,Germany). The Sections were deparaffinized, hydrated and then incubated for 15 minutes in room humid temperature with 20 (g/mL) K protein kinase. The slides were then incubated with reactive TUNEL mixture consisting terminal deoxynucleotidyl transferase (Enzyme Solution 450µL, Lable Solution 50µL) for sixty minutes in temperature of 37 °C.Then dUTP(Deoxyuridine Triphosphate) conjugated by dioxygen proxidase was added and the slides were covered with a lid. Afterwards Dioxygen and Hydrogen Peroxide (Converter-POD) was added to the samples. The slides were incubated for 30 minutes and DAB(Diaminobenzidine) was added (DAB powder 6mg , PBS 10mL , H2O2 3% 10µL).The slides were stained by hematoxylin. Apoptotic cells will appear in brown [16-20].

Morphological evaluation of ovarian follicles

The ovarian follicles were classified to following groups:[21-24].

1) Primordial Follicle, the oocytes is surrounded by a layer of squamous follicular cells.
2) Intermediate Follicle, the oocytes is surrounded by squamous and cuboid cells.
3) primary follicles ,the oocytes is 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. This follicle itself includes two stages.
5) In primary stages called Early antral
6) In final stages called late antral
7)Tertiary (Graffian) the selected follicle with a space bigger than two-third of follicle's volume.

Stereology technique

Apoptotic index were calculated in stereological method [25-31]. (Table1).

Statistical analysis

The data were analysed by Life science , Image j, Image Tools3 and SPSS 16 softwares. T-test, ANOVA and Tukey tests were done and the results that valued $P<0.05$ were regarded as significant.

Results

Radioimmunoassay Test and ovarian tissues weight

Statistical analysis on the data showed a significant decrease in the rate of thyroid hormones in hypothyroid group ($P<0.001$), (Table 2).

In macroscopic study ,the ovarian tissues were weighted whit digital scale in control and hypothyroid groups. Statistical analysis showed a significant decrease in hypothyroid group ($P<0.001$),(Table 2; Fig. 1).

Table 1. Method for determining apoptotic index

Equation	Description
$N_v = \frac{\Sigma Q^-}{\Sigma F \,.\, a(F) \,.\, t}$	N_v numerical density in volume unit (mm^{-3}), ΣQ-total counted marked nuclei cross-section, ΣF total frames related to the desired structure, $a(F)$ area of each frame considering the magnification (mm^2) and t dissector depth (mm).
$V_R = t \,.\, a(P) \,.\, \sum_{i=1}^{n} P$	V_R reference volume (mm^3), t distance of two sections (cross-section thickness) (mm), $a(P)$ area related to spot (mm^2), n number of cross-sections and $\sum_{i=1}^{n} P$ total spots hit with the desired structure.
$V_v = \frac{\Sigma P_f}{\Sigma P_R}$	V_v volume fraction or volume density, ΣP_f total spots hit with follicle and ΣP_R reference volume (mm^3) .
$V_f = V_v \,.\, V_R$	V_f follicle volume (mm^3), V_v volume fraction or volume density and V_R reference volume (mm^3).
$N = N_v \,.\, V_f$	N total number of marked nuclei in the follicle, N_v numerical density in unit volume (mm^{-3}) and V_f follicle volume (mm^3).
$AI = \frac{n\,follicle^+ \,.\, n\,cell^+ \,.\, 100}{n\,total_f \,.\, n\,total_c}$	AI apoptotic index, $n\,follicle^+$ number of follicles with dying cells , $n\,cell^+$ number of dying cells in each follicle , $n\,total_f$ total number of follicles and $n\,total_c$ total number of cells in each follicle.

Table 2. T3 and T4 hormone serum levels and weight of ovaries in control and hypothyroid groups.

	Group	Mean (%)	SD
T3**	Control	85.90	14.47
	Hypothyroid	54.30*	9.79
T4**	Control	4.60	0.45
	Hypothyroid	3.22*	0.68
Ovary weight (g)	Control	0.07	0.00
	Hypothyroid	0.04*	0.01

*Significant values compared with control group, P<0.05.
**Results according to RIA analysis.

Apoptotic index in ovarian follicles

The results are expressed as follow:

The studies on primordial follicles showed zero apoptotic index and no TUNEL-positive cells in control and hypothyroid groups (Figs. 3,4-B).

The apoptotic index was equal 100% in control and hypothyroid groups. This means that all of the cells were TUNEL- positive in Primary follicles. The standard deviation (SD) between data averages was zero in both groups (P>0.05), (Figs. 3,4-C).

The results of the apoptotic index showed no significant difference between groups in preantral and early antral follicles (P>0.05). TUNEL–positive cells were seen in theca layer cells(Table3, Figs.3,4-D,E).

The results of the apoptotic index showed a significant decrease in hypothyroid compared to control groups in late antral and graffian follicles (P=0.000). TUNEL-positive cells were seen in antrum margin clearly (Table3, Figs.3-F,G,H,I and 4-F,G,H,I).

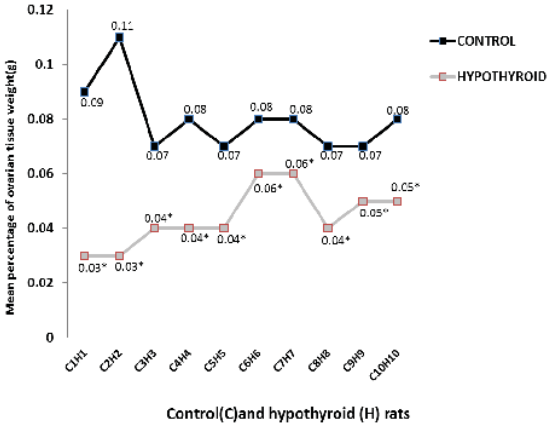

Figure 1. Ovarian tissues weight (g) in control and hypothyroid groups.

*Significant values compared with control group, P<0.05, (Control (C), Hypothyroid (H) rats)

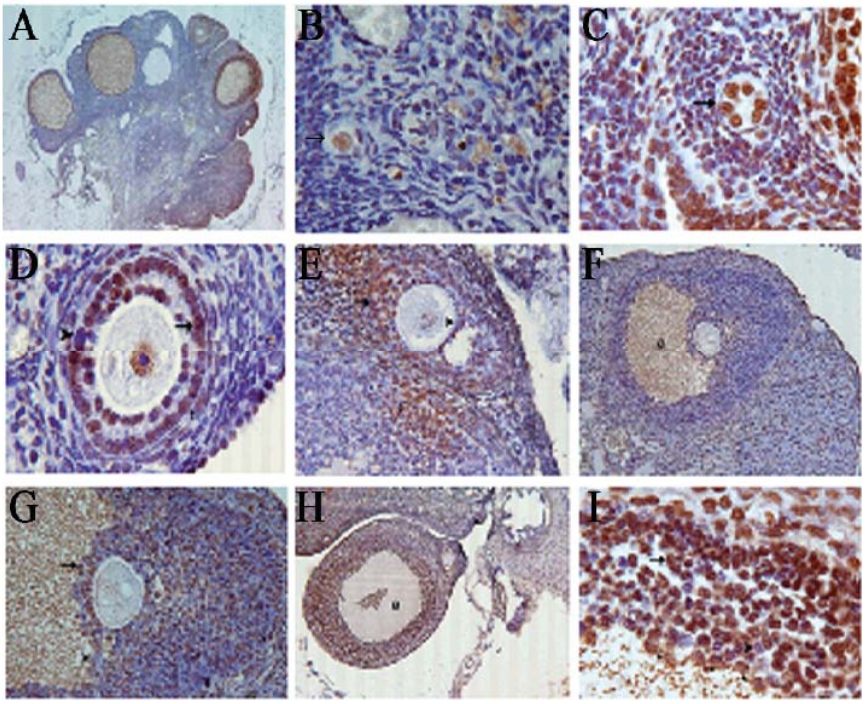

Figure 3. Photomicrograph of rat ovarian follicles in control group after TUNEL immunohistochemical technique. TUNEL-positive nuclei are seen in brown. A) General image of ovarian tissue, spots in brown indicate TUNEL-positive cells in ovarian tissue. B) Primordial follicle (arrow), absence of TUNEL-positive cells in squamous cells of follicle. C) Primary follicle (arrow), granulosa cuboid TUNEL-positive cells are clearly visible. D) Preantral follicle, granulosa TUNEL-positive cell nucleus (arrow) and TUNEL-negative cell nucleus (arrow tip), TUNEL-positive cells are seen in theca layer (t). E) Early antral follicle with small antrum, granulosa TUNEL-positive nucleus (arrow) and TUNEL-negative cell nucleus (arrow tip). F) Late antral follicle with two-thirds antrum of the follicle volume(a). G) Same as (F) image, but at higher than magnification, Granulosa TUNEL-positive cell nucleus (arrow) and TUNEL-negative cell nucleus (arrow tip). H) Graffian follicle with very large antrum that is over two-thirds of the follicle volume (a),I) Same as (H) image but at higher than magnification, granulosa TUNEL-positive cell nucleus (arrow) and TUNEL-negative cell nucleus (arrow tip), TUNEL-positive cells are clearly visible along the antrum (asterisk). magnification: x4 (A); x100 (B-D and I); x40 (E and G); x20 (F); x10 (H).

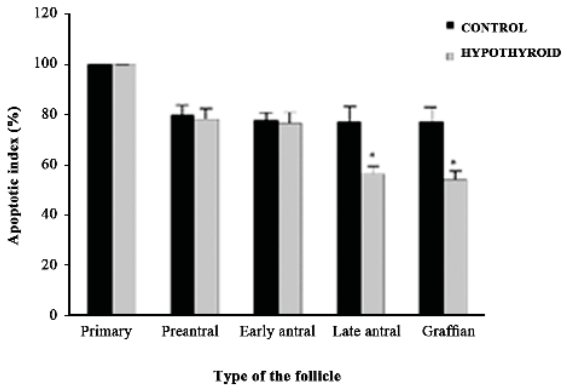

Figure 2. Apoptotic index of ovarian follicles in control and hypothyroid groups.
*Significant values compared with control group, P=0.000.

Analysis of the apoptotic index

Studying the apoptotic index of ovarian follicles in control and hypothyroid groups showed the most degree in primary follicle and the least in graffian follicle. Apototic index of graffian follicle showed a significant decrease in hypothyroid compared to control groups. The trend of apoptotic index decrease of primary to graffian follicle in control group.

ANOVA statistical analysis and Tukey test showed a significant difference of apoptotic

index between primary follicle and other types of the follicles in control group (P=0.000), While the difference was not significant in other types of ovarian follicles. Also a significant difference was seen between primary follicle and other types of the follicles in hypothyroid group. The results of other ovarian follicles in hypothyroid group were as follows: apoptotic index of preantral follicle showed a significant difference in late antral and Graffian follicles, while the difference was not significant in Early antral follicle. Apoptotic index of Late antral follicle showed no significant difference of Graffian follicle , while it showed a significant difference the other types of follicles. The apoptotic index of Graffian follicle showed a significance difference of primary follicle in control group. But it showed a significant difference of preantral and early antral follicles in hypothyroid group (Table4; Fig. 2).

Table 3. Apoptotic index of ovarian follicles in control and hypothyroid groups.

Type of follicle	Group	Mean (%)	SD
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

*Significant values compared with control group, P<0.05.

Numbers of graffian follicles and luteal bodies

According to our results, there was a significant decrease in the number of graffian follicles in the hypothyroid group (P=0.000). There was a significant increase in the number of luteal bodies in the hypothyroid group compared to the control group (P=0.000), (Table 5; Figs. 3, 4-A).

Table 4. Significant levels of apoptotic index of ovarian follicles in control and hypothyroid groups

	Primordial		Primary		Preantral		Early antral		Late antral		Graffian	
	Control	Hypothyroid	Control	Hypothyroid	Control	Hypothyroid	Control	Hypothyroid	Control	Hypothyroid	Control	Hypothyroid
Primordial			+	+	+	+	+	+	+	+	+	+
Primary	+	+			+	+	+	+	+	+	+	+
Preantral	+	+	+	+			-	-	-	+	-	+
Early antral	+	+	+	+	-	-			-	+	-	+
Late antral	+	+	+	+	-	+	-	+			-	-
Graffian	+	+	+	+	-	+	-	+	-	-		

+: Significant difference; -: No significant difference

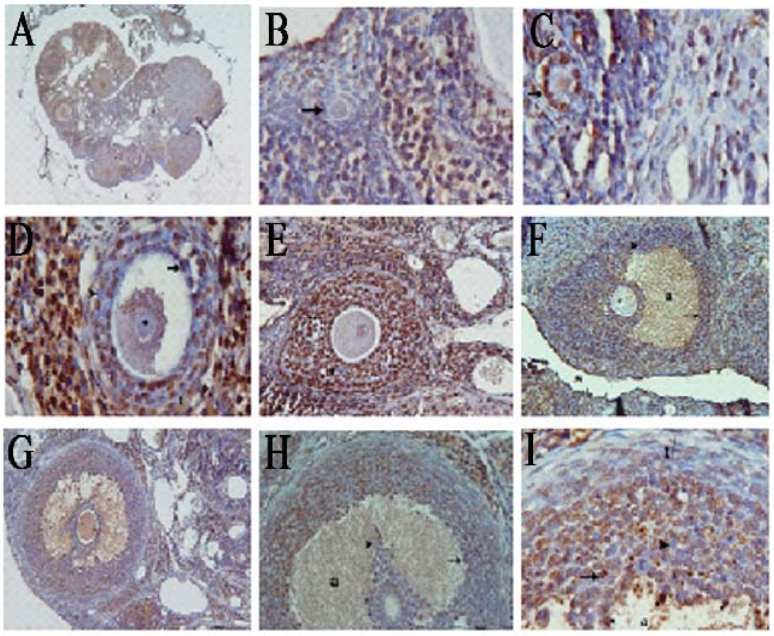

Figure 4. Photomicrograph of rat ovarian follicles in hypothyroid group after TUNEL immunohistochemical technique. TUNEL-positive nuclei are seen in brown. A) General image of ovarian tissue. B) Primordial follicle (arrow), absence of TUNEL-positive cells in squamous cells of follicle. C) Primary follicle (arrow), granulosa cuboid TUNEL-positive cells are clearly visible. D) Preantral follicle, granulosa TUNEL-positive cell nucleus (arrow) and TUNEL-negative cell nucleus (arrow tip), TUNEL-positive cells are seen in theca layer (t), oocyte nucleus (asterisk) is clearly visible. E) Early antral follicle with sporadic small holes between granulosa cells, granulosa TUNEL-positive nucleus (arrow) and TUNEL-negative (arrow tip). F) Late antral follicle with two-thirds antrum of the follicle volume(a), granulosa TUNEL-positive nucleus (arrow) and TUNEL- negative (arrow tip). G) Graffian follicle with large antrum more than two-thirds of the follicle volume. H) Same as (G) image, but at higher than magnification with large antrum (a), granulosa TUNEL-positive cell nucleus (arrow) and TUNEL-negative cell nucleus (arrow tip). I) Same as (H) image but at higher than magnification, granulosa TUNEL-positive cell nucleus (arrow) and TUNEL-negative cell nucleus (arrow tip), TUNEL-positive cells are clearly visible along antrum (a) (asterisk). magnification: x4 (A); x100 (B-D and I); x40 (E and H); x20 (F); x10 (G).

Table 5. Number of graffian follicles and luteal bodies in control and hypothyroid groups.

	Group	Mean (%)	SD
Graffian follicles (n)	Control	5.00	0.81
	Hypothyroid	1.90*	0.73
Luteal bodies (n)	Control	2.50	0.85
	Hypothyroid	7.80*	1.22

*Significant values compared with control group, P=0.000

Discussion

The most common cause of hypothyroidism world wide is attributed to iodine deficiency. In regions that have adequate iodine, autoimmune diseases such as Hashimoto's thyroiditis is among the most common cause of hypothyroidism [1-3]. The ovary regulates the evolution of mature oocytes as well as the release and production of hormones such as estrogen, progesterone and inhibin which are vital for evolution during maturity, preparation of the uterus for pregnancy, and during implantation and the primary stages of pregnancy. This is indicative of the crucial role of oogonia in forming primordial follicles [32,33]. Experience has shown that normal sexual behavior and related physiological aspects are subject to the

presence of a balanced level of thyroid hormones [34,35].

Armada et al. conducted a study to determine whether infertility due to hypothyroidism was the result of changes to function of the pituitary gland or resulted from the ovary. Their findings showed a decrease in numbers and thickness of the luteal body in hypothyroid rats. In addition, there was a significant decrease in the number of graffian follicles in the hypothyroid rats. The researchers have determined that morphological changes could be the outcome of excess production of prolactin (PRL) in the pituitary gland which prevents secretion and function of gonadotropin hormone (GnH) [36]. Various vital function mechanisms, including reproductive functions have a neural-hormonal nature [37]. The hypothalamic-pituitary axis and its impact in increasing gonadotropins can influence reproductive activities [38].

Hosoda et al. have shown that infertility in studied rats resulted from a thyroid function deficiency. Sufficient thyroid secretion (T_3) was highly important for natural function of reproduction in female rats. Their results have shown that the thyroid hormone is not the requisite for mating and delivery. The thyroid hormones play a crucial role in evolution and function of reproductive organs during maturity [39].

According to research, the hypothalamic–pituitary axis begins to form from weeks 4 and 5 of embryonic development and completes formation by weeks 30 to 35 of pregnancy [40]. The hypothalamic–pituitary axis establishes a relationship with gonads, and assists with feedback and control related to hormonal secretion and balance [41]. Existing evidence indicates that iodine deficiency and ensuing reduction in thyroid hormones can be manifested as reductions in fertility, miscarriage, and fatal defects that include defects in the evolution and function of this system [42,43].

Apoptosis plays a crucial role in regulating follicular growth and causes atresia in follicles. Kiess et al. have researched apoptosis hormone control by studying the effect of different hormones, including steroids and growth factors on the prostate, ovaries, testes, and mammary glands. The findings showed that steroid hormones played a crucial role in regulating apoptosis in organs and some endocrine glands caused apoptosis adjustment. Growth factors and estrogens cause ovarian follicles to survive, whereas androgens and gonadotropins induce apoptosis in follicles [44]. According to the literature, thyroid hormones play a crucial role in apoptosis of ovarian follicular cells.

In the present study, the apoptotic index was zero in the primordial follicles from both groups. Thus, according to this result apoptosis in ovarian follicular cells begins with the onset of primary follicular formation. The absence of apoptotic cells in primordial follicles has proven the hypothesis that about 99% of the follicle supply degenerated during the early stages of primary follicle formation [8]. According to the apoptotic index, there was a reducing trend of primary to graffian follicles in both groups. This shortage was clearly evident in the late antral and graffian follicles of the hypothyroid group.

The results have proven that GnH are an important controlling factor in the apoptosis process in ovarian follicles. These hormones regulate caspase-3, which has a basic role in apoptosis induction in granulosa cells [44]. There is an increase in PRL hormone production and reduced secretion of gonadotropins in hypothyroidism [36]. It has been stated that there is more iodine uptake in ovarian follicular fluid than any other organs except for the thyroid gland and increase by estrogens and hypothyroid state [45]. The results of the present study showed decreased apoptotic index in follicles with large antrum (late antral and

graffian) which supported the results of previous studies. Although the apoptotic index of the graffian follicle selected for ovulation decreased at a normal level in the control group, there was a significant decrease due to deficiency of GnH in the hypothyroid group. In particular, the apoptotic index of selected graffian follicles showed a significant decrease compared to control group, considering large antrum containing follicular fluid for preserving and stability of follicle.

According to the results we have presumed that a reduction of thyroid hormones can lead to significant decreases in the apoptotic index in only follicles with large antrum. In the current study, hormone reduction has caused a significant decrease in the apoptotic index in some types of follicles compared to the control group.

Since most primary follicles had an apoptotic index of 100% in both groups, it might be concluded that they were unaffected by hypothyroidism. Within the subgroups of the primary follicle, the preantral follicle had the highest apoptotic index followed by the early antral follicle. Although hypothyroidism causes disorders in the reproductive process, it was possible that adjustments occurred in a way that the selected follicle could enter the ovulation stage. Microscopic images of the ovarian tissue showed a increase in antral follicles in the control group, as well as the corpus luteum were clearly observed in the hypothyroid group.

It can be presumed that the developing trend toward graffian follicles in the hypothyroid group occurred at a faster rate compared to the control group. Studies have proven the presence of thyroid hormones, particularly T_3 in follicular fluid and the existence of their receptors in granulosa cells. The our findings prove of the hypothesis proposed in this research [45]. Experimental studies should be undertaken to evaluate the contents of follicular fluid, both molecularly and biochemically to clearly determine the factors that create these effects.

The results of present study propose a hypothesis that decreases in thyroid hormones may cause extensive hormonal changes. In turn, these changes cause the factors present in follicular fluid, particularly in antral follicles with large antrum, to undergo changes and make the process of follicular growth pass quickly. Therefore the follicle will enter the next stage without making required potentiality and ovules are produced of healthy or morphologically defective. Increase in luteal bodies can also be an outcome of these events.

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