Determination of VASA and IFITM3 immunolocalization in experimental hyperthyroidism in rat ovaries at the stage of diestrus

Abstract

Aims: Hyperthyroidism occurs due to overproduction of thyroid hormone. We aim to investigate the relationship of VASA and IFITM3 with hyperthyroidism by demonstrating immunolocalization at the stage of diestrus in hyperthyroid rat ovaries.

Materials and Methods: To induce hyperthyroidism, rats were given 250 ug/kg/day of L-thyroxine by subcutaneous injection for 21 days. The control group was a saline solution injected only. Ovarian tissues of euthanized rats were removed after cardiac blood was collected. Results: It was determined that VASA is intensely expressed in oocytes and theca layers of growing follicles, and IFITM3 is strongly expressed in germinal epithelium and oocytes.

Conclusion: The intense expression of VASA is most likely due to the abundant ATP in the mitochondria of the oocyte cytoplasm. The effect of VASA and IFITM3 expressions in hyperthyroidic ovary compared to the control group shows the link between hyperthyroidism and follicle development.

Keywords**:** VASA, IFITM3, ovary, hyperthyroidism, diestrus

1. Introduction

Hyperthyroidism is a condition that occurs due to excessive production of thyroid hormone in the thyroid gland. Hyperthyroidism causes weight loss, increased heart rate, rhythm disorders, sleep disorders, increased appetite, tension, quick irritation, tremors, sweating, heat intolerance, changes in the menstrual cycle and intestinal peristalsis, thinning of the skin, thinning of the hair and increased brightness, and this as well as causing infertility. This disease is usually diagnosed by determining thyroxine (T4), triiodothyronine (T3), and thyroid-stimulating hormone levels [1, 2]. Since VASA and IFITM3 are essential proteins for the homing and maturation of germ cells, their levels may change in patients with hyperthyroidism.

VASA is an ATP-dependent RNA helicase, participating in multiple biological processes. It has been widely used as a germ cell marker [3]. The VASA gene (also called DDX4) is highly conserved among species. It is expressed in various model organisms such as fly, worm, frog, mouse, and human especially, in germ cell precursors [4]. Although little is known about the role of VASA in germ cell specification, it is considered to be one of the oldest markers in the determination of primordial germ cells (PGC) and is highly conserved among taxa, especially in mammals [5].

Interferon-induced transmembrane proteins (IFITM); is a family of small membrane proteins with immunological and developmental roles. Five types of IFITM have been identified in humans, including IFITM1, IFITM2, IFITM3, IFITM5, and IFITM10 [6, 7]. The family of interferon-mediated transmembrane protein (IFITM / MIL / Fragilis) genes encode cell-surface proteins capable of regulating cell adhesion and affecting cell differentiation. IFITM3 expressed in PGC might be necessary for PGC migration and the formation of female germ cells [7]. Early studies have identified the possible roles of IFITM1, IFITM2, and IFITM3 in development, apoptosis, cell proliferation, and cell signaling [8]. There is an

evident correlation between VASA and IFITM3 expression and folliculogenesis condition or female fertility [9].

In this study, the immunoreactivity of VASA and IFITM3 in rat ovaries with hyperthyroidism was demonstrated and its relationship with hyperthyroidism was investigated. Since the VASA and IFITM3 antibodies used in the study play a role in the development of follicle, and the diestrus stage is the stage in which follicular development begins again, the study was preferred at this stage. Also, the possibility that thyroid hormones may be effective in follicle structure is a cause of our study in this stage. Explain the roles of VASA and IFITM3 molecules in rat ovaries with hyperthyroidism, and explain the processes of growth, embryogenesis, and cell proliferation. Besides, the negative effects of hyperthyroidism on reproduction can be monitored and may help to better understand the diseases and to develop treatment methods.

The study aimed was to determine whether hyperthyroidism has an effect on VASA and IFITM3 expression in rat ovaries. To date, there is no other study investigating the expression of VASA and IFITM3 in rat ovarian hyperthyroidism.

2. Materials And Methods

2.1. Animals and experimental groups

The local committee of Sivas Cumhuriyet University approved all experimental protocols (Approval No: 65202830-050.04.04). Twenty adult female rats weighing 200-220 grams and 16 weeks old Wistar albino genus, grown in the Experimental Animals Laboratory of Sivas Cumhuriyet University, were used. The animals were fed with standard pellet feed and tap water during the experiments. The animals used in the study were grown at room temperature, 12 hours light-12 hours dark periods. Vaginal smear samples were taken from the experimental and control groups and the animals were in the diestrus stage. The diestrus stage rats were divided into two groups as control (n = 8) and hyperthyroidism (n = 12). To create

hyperthyroidism in animals, 250 ml of L-thyroxine hormone (Sigma, USA, T2376) was injected by subcutaneous injection, 250 µg/kg/day for 21 days, and control group rats were injected with saline solution for 21 days. Rats were weighed and 3 mg/kg xylazine HCl + 90 mg/kg ketamine HCl was anesthetized by intraperitoneal administration. After cardiac blood was taken for biochemical evaluation, 200 mg/kg of thiopental sodium ($C_{11}H_{17}N_2NaO_2S$) was administered by the intraperitoneal route to animals in both groups.

2.2. Determination of the diestrus stage

Vaginal smears were obtained by lavage using a few drops of physiological saline. The smears were dried, stained with 1% toluidine blue, and examined by a light microscope (Olympus BX51 (Tokyo, Japan) to determine the stage of the diestrus cycle [10].

2.3. Free T4 level measurement

Plasma T4 levels were measured from cardiac blood from each rat belonging to the hyperthyroid and control groups. Blood samples were centrifuged at 2000-2500 rpm for 10 minutes following ingestion. The plasma samples were then stored at -20 °C. T4 levels of all groups were measured by the Elisa method.

2.4. Histology

After euthanasia, the ovarian tissues were fixed in 4% paraformaldehyde at +4 ° C for 24 hours, followed by dehydration and clearing.

Immunohistochemistry

For immunohistochemical staining, the deparaffinized and redehydrated tissue sections were inactivated the endogenous peroxidase by incubation with 3 % H_2O_2 for 10 minutes. To recover antigen, these sections were put into citrate buffer (DIAPATH, Martinengo, Italy, pH:6) and heated in the microwave oven twice. The slides were then washed with phosphate buffer solution (PBS) at pH 7.2 - 7.6, twice. Non-specific binding sites were blocked with Ultra V Block (ThermoFisher Scientific, Fremont, CA, USA) for 20 minutes. After the

redundant liquid was discarded, the sections were incubated with primary antibody VASA (Abcam ab13840) (1:100) ve IFITM3 (Abcam ab15592) (1:100) at room temperature for 90 minutes and washed with PBS. Then the slides were incubated with biotinylated secondary antibody (ThermoFisher Scientific, Fremont, CA, USA) for 20 minutes and washed with PBS, followed by incubation with streptavidin-HRP (Thermo Scientific, USA) for 20 minutes and washed with PBS. The antibody binding sites were visualized by incubation with an AEC chromogen (Life technologies, USA) solution. The slides were counterstained for 1 minute with hematoxylin and then dehydrated with sequential ethanol for sealing and microscope (Olympus BX51 (Tokyo, Japan)) observation.

2.5. Semiquantitative scoring method

The intensity of VASA and IFITM3 expressions in ovarian tissues in the experimental and control groups was determined by the semiquantitative scoring method [11] and the scoring was shown in Table 1. All sections were examined by two observers independent of each other, according to the intensity of immunostaining of VASA and IFITM3 antibodies in ovarian tissue.

Table 1. The scoring of the intensity in immunostaining of VASA and IFITM3 antibodies

Score	Immunostaining Intensity
0	no
1	very very weak
2	very weak
3	weak
4	Mild
5	Moderate
6	Strong
7	very strong
8	super strong
9	hiper strong

2.6. Statistical Method for T4 Levels

The data obtained from our study were uploaded to SPSS (data 22.0) program and since the parametric test assumptions were performed in the evaluation of the data (Kolmogorov-Smirnov), the significance tests were used for the differences between the two groups in the independent groups and the error level was taken as $p < 0.05$ level.

3. Results

3.1. Free T4 levels

When the control group and the experimental group were compared statistically for free T4 levels, the change with L-thyroxine was by chance greater than expected; there is a statistically significant change ($p=0.01$). This result shows that hyperthyroidism occurs in experimental group rats (Figure 1).

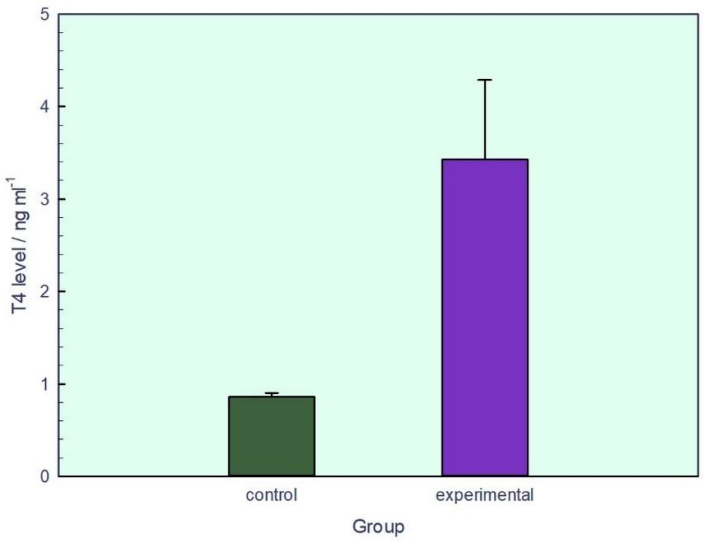

Figure 1: The bar graphs of free T4 levels.

3.2. Determination of the diestrus stage

Nucleated epithelial cells, cornified squamous epithelial cells, and leuokocytes are seen in Figure 2, so they were identified as the diestrus stage, which is one of the oestrus stages.

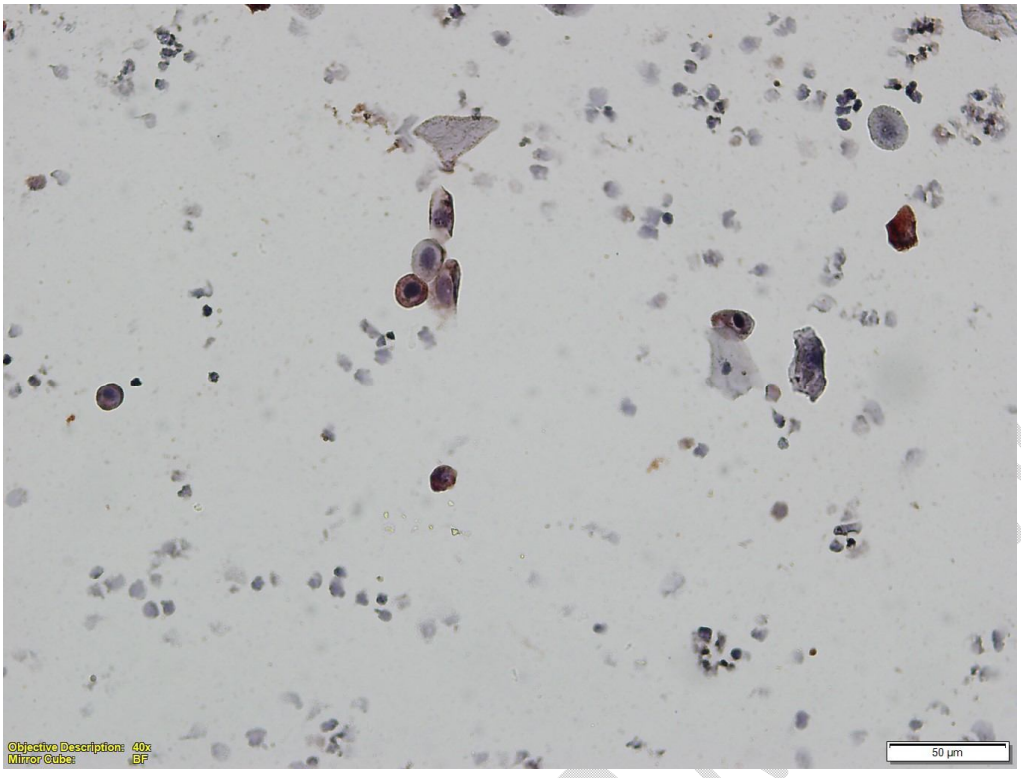

Figure 2: Diestrus cycle; nucleated epithelial cells, cornified squamous epithelial cells, and leukocytes.

Immunohistochemical results. VASA and IFITM3 immunolocalizations in the experimental and control groups are shown in Figures 3 and 4.

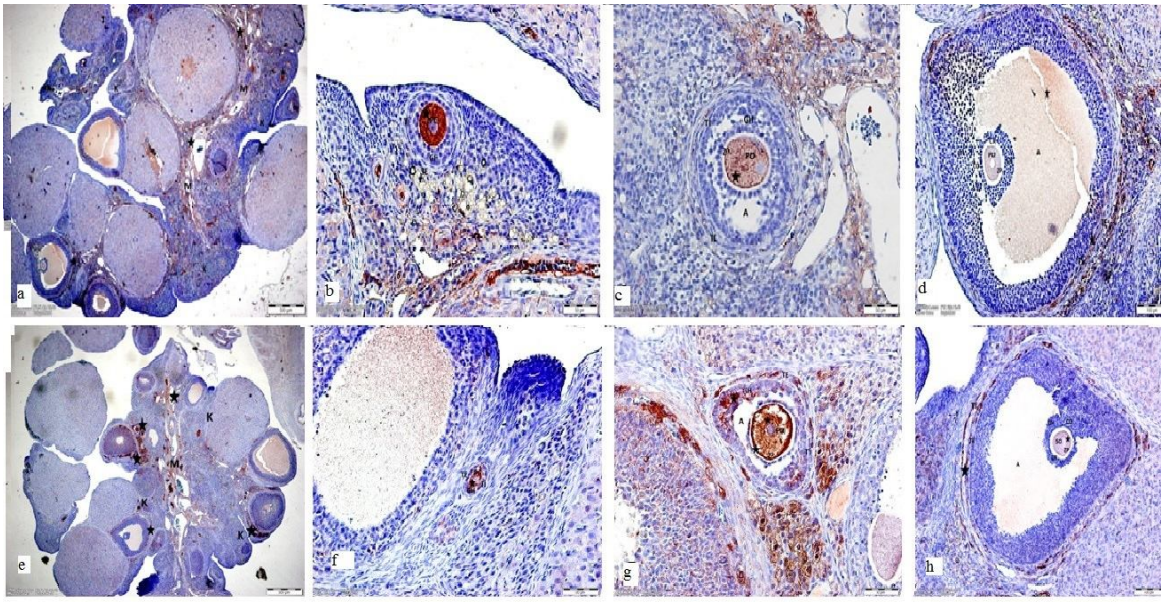

Figure 3: Immunolocalization of VASA in the control (a-d) and experimental groups (e-h)

a) Ovarian general image, **b)** Primary follicle, **c)** Secondary follicle, **d)** Graafian follicle,

e) Ovarian general image, **f)** Primary follicle, **g)** Secondary follicle, **h)** Graafian follicle.

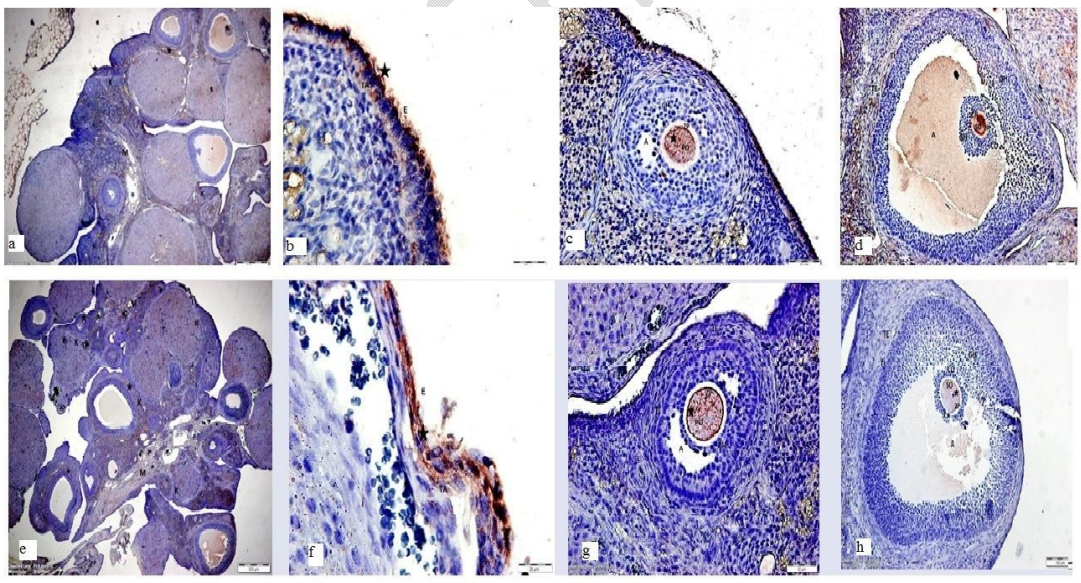

Figure 4: Immunolocalization of IFITM3 in the control group (a-d) and experimental groups (e-h).

a) Ovarian general image, **b)** Germinal epithelium, **c)** Secondary follicle, **d)** Graafian follicle,

e) Ovarian general image, **f)** Germinal epithelium, **g)** Secondary follicle, **h)** Graafian follicle.

The immunolocalizations of VASA and IFITM3 in rat ovaries were evaluated by semiquantitative scoring for the control and experimental groups. The scores are given in Table 2 for VASA and IFITM3, respectively.

Table 2. Mean scores of VASA and IFITM3 antibodies of the immunostaining intensity with standard deviations (SD) in the control and experimental groups

	VASA		IFITM3	
Species	Control Mean ± SD	Experimental Mean ± SD	Control Mean ± SD	Experimental Mean ± SD
Epithelium	0.0 ± 0.00	0.4 ± 0.49	8.8 ± 0.40	8.8 ± 0.40
Tunica Albuginea	0.0 ± 0.00	0.2 ± 0.40	4.2 ± 1.17	3.4 ± 0.49
Cortex				
Cortex blood vessels	6.4 ± 1.02	6.2 ± 0.75	4.6 ± 0.49	4.6 ± 0.49
Medulla				
Fibroblast-like cell	8.2 ± 0.75	6.0 ± 0.63	3.8 ± 0.75	3.6 ± 0.49
Medulla blood vessels	8.6 ± 0.49	8.4 ± 0.80	5.8 ± 0.40	3.4 ± 0.49
Primordial Follicles				
Primordial Follicles Cells	0.0 ± 0.00	0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Primary Oocyte	7.6 ± 0.49	6.8 ± 0.75	0.0 ± 0.00	0.0 ± 0.00
Primary Follicles				
Granulosa Cells	0.0 ± 0.00	4.6 ± 1.02	0.4 ± 0.49	0.0 ± 0.00
Primary Oocyte	8.6 ± 0.49	8.8 ± 0.40	5.4 ± 0.49	8.8 ± 0.40
Theca Interna	0.0 ± 0.00	0.8 ± 0.75	0.2 ± 0.40	0.0 ± 0.00
Theca Externa	0.0 ± 0.00	1.0 ± 0.89	0.2 ± 0.40	0.0 ± 0.00
Secondary Follicles				
Primary Oocyte	4.8 ± 0.75	8.8 ± 0.40	8.4 ± 0.49	8.8 ± 0.40
Zona Pellucida	5.2 ± 0.75	8.8 ± 0.40	8.6 ± 0.49	8.8 ± 0.40
Granulosa Cells	0.0 ± 0.00	6.8 ± 0.40	0.0 ± 0.00	0.0 ± 0.00
Antrum	0.0 ± 0.00	0.2 ± 0.40	0.0 ± 0.00	0.0 ± 0.00
Theca Interna	0.4 ± 0.49	5.6 ± 0.49	0.4 ± 0.49	0.0 ± 0.00
Theca Externa	0.4 ± 0.49	5.6 ± 0.49	0.4 ± 0.49	0 0 ± 0.00
Graafian Follicle				
Secondary Oocyte	2.6 ± 0.49	2.4 ± 0.49	8.8 ± 0.40	2.6 ± 0.49
Zona Pellucida	2.6 ± 0.49	2.4 ± 0.49	8.8 ± 0.40	2.8 ± 0.40
Granulosa Cells	0.0 ± 0.00	0.2 ± 0.40	0.4 ± 0.49	0.8 ± 0.75
Antrum 2	3.2 ± 0.75	0.4 ± 0.49	5.6 ± 0.49	0.0 ± 0.00
Corona Radiata	0.8 ± 0.75	0.4 ± 0.49	0.2 ± 0.40	0.0 ± 0.00
Cumulus Oophorus	0.8 ± 0.75	0.2 ± 0.00	0.2 ± 0.40	0.0 ± 0.00

Theca Interna	5.2 ± 0.75	6.0 ± 0.63	3.4 ± 0.49	0.0 ± 0.00
Theca Externa	0.0 ± 0.00	6.0 ± 0.63	3.4 ± 0.49	0.0 ± 0.00
Corpus Luteum				
Blood Vessels	8.2 ± 0.75	6.6 ± 0.00	5.6 ± 0.49	4.8 ± 0.75
Theca- Lutein Cells	3.6 ± 0.80	2.6 ± 0.00	3.2 ± 0.75	2.6 ± 0.49
Granulosa-Lutein Cells	4.0 ± 0.89	2.8 ± 0.00	6.4 ± 0.49	2.6 ± 0.49

In the immunolocalization scoring of VASA and IFITM3 in rat ovaries, there was a score difference in some follicles between the control and experimental groups, while some did not have immunolocalization. Bar graphs are drawn and presented in Figure 5 to better evaluate follicles that differ in immune staining intensity.

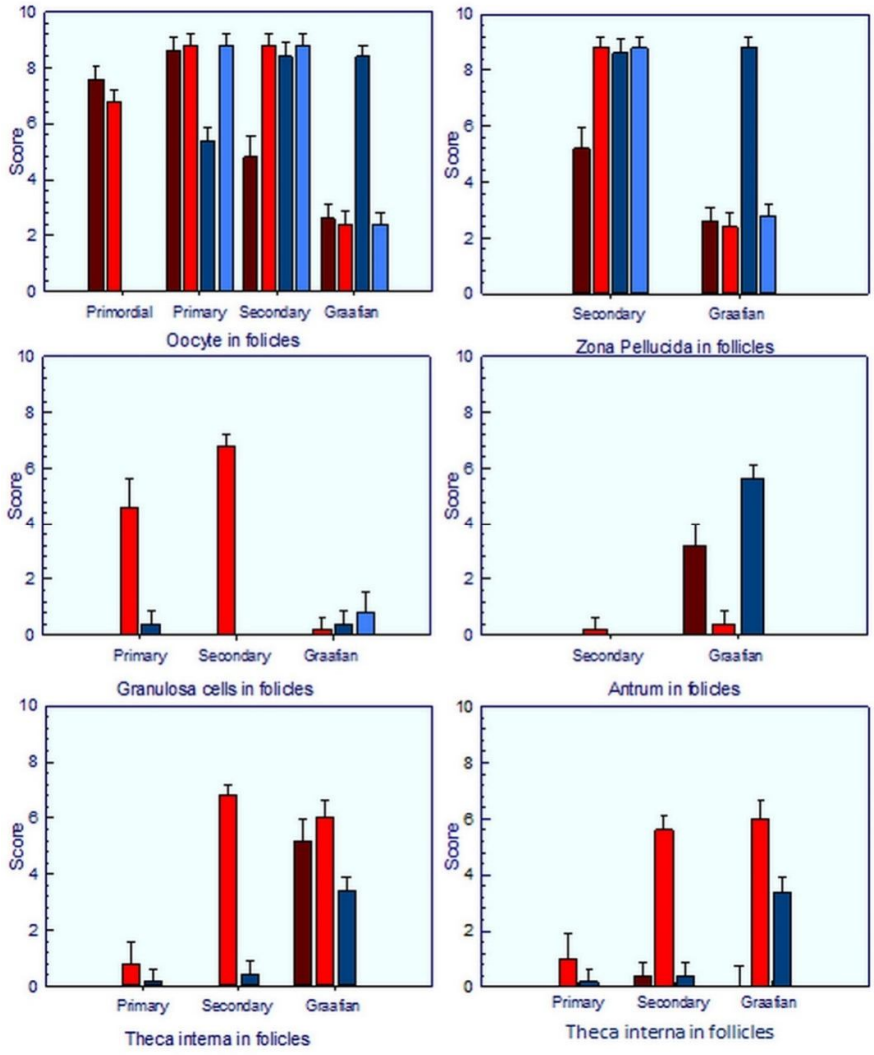

Figure 5. The bar graphs of immunostaining scoring in the sections for VASA and IFITM3, ■; control group for VASA, ■; experimental group for VASA, ■; control group for IFITM3, ■; experimental group for IFITM3.

4. Discussion

In the reproductive age women, the second most common endocrinological disorder is thyroid diseases. The prevalence of primary or secondary infertility associated with hyperthyroidism was reported as 5.8% [12]. The human VASA gene is specifically expressed in the germ cell lineage and can only be detected in tissues of the ovary and testis in adults. VASA has a rich history useful as a marker of germ cell precursors and is thought to act as a positive translation regulator in the identification and maintenance of germ cell precursors. Recent studies also show that VASA is involved in regulating cell cycle [13]. Interferon-inducible transmembrane proteins have a variety of roles, including control of cell proliferation, promotion of homotypic cell adhesion, protection against viral infection, improvement of bone matrix maturation and mineralization, and mediation of germ cell development [14]. IFITM3, also known as 1-8U, is an important member of the IFN-inducible transmembrane protein family [15]. The expression IFITM3 identifies cells capable of becoming PGC as early as E6.25 and may play a role in germ cell development by promoting the formation of a separate cell population that separates hypothetical cell adhesion from somatic cells and possibly hypothetical PGC. Since the thyroid hormones are a common domain affecting the whole body, the mechanisms of action on ovarian functions are not fully understood. However, the detection of specific binding sites for thyroxine in human and mouse oocytes suggests that thyroid hormones have a direct effect on oocytes and follicular structure [16].

Since we aimed to determine the effect of hyperthyroidism on VASA and IFITM3 expression in rat ovaries, we preferred to study at the stage of diestrus, where VASA and

IFITM3 play a role in follicle development. Because diestrus is the phase when follicular development begins again. Besides, the possibility of thyroid hormones to be effective in the follicle structure is one of the reasons for choosing the diestrus stage. When the experimental group was compared with the control group, it was observed that although there was no histological change in the histological structure of oocyte, granulosa cells, and germinative epithelium, there were differences in terms of immunostaining in the oocyte, zona pellucida, and theca layers. While immunolocalization of VASA was extensively expressed in the oocyte of primordial, primary, and secondary follicles, in the tertiary follicle decreased. These immunostaining intensity differences are also seen numerically in our scoring. In one study, VASA was detected in the oocyte cytoplasm in all non-apoptotic follicles and VASA was concluded that high of levels protein in the ovarium may prepare oocytes to continue meiosis. It was observed that the expression density of VASA in the ovary decreased with follicular development [17]. VASA may play a role in meiosis division, development of follicles. Mitochondria play an important role in the development of the oocyte cytoplasm. The task of mitochondria is to synthesize ATP. VASA, an ATP binding RNA helicase, may have high expression in this region due to the excess mitochondria in the oocyte.

We found that IFITM3 localization is more concentrated in oocytes, epithelium, and cytoplasmic areas, while VASA is concentrated in oocytes and theca layer in semiquantitative scoring. Li Zhou et al. tested DDX4 and Fragilis (IFITM3) expression in the ovaries of 4-5 week old mice by the immunohistochemical method to identify female germline stem cells. DDX4 was expressed in all stages of ovarian germ cells. Fragilis is expressed at a high level in early-stage germ cells, while Fragilis signals are weak in secondary oocytes and later stages [18]. The expression of VASA in the oocyte decreased as the follicle grew in the experimental and control groups in our study. In contrast to this study, however, IFITM3 was not localized in primordial follicles in the experimental and control groups. The lack of localization in these

follicles may be due to the low effectiveness of IFITM3 in the early stages of development. Expression of IFITM3; although the follicle in oocytes increased in the control group, the decrease in expression in the oocyte as follicle grew in the experimental group suggested that hyperthyroidism decreased IFITM3 expression and could affect follicle development. IFITM3 was expressed in the germinal epithelium, but there was not much difference in the immunohistochemical staining of the germinal epithelium of ovaries of hyperthyroid and healthy animals. IFITM3, which has tasks such as inhibiting cell proliferation, can be induced by type I and type II IFN. Type I IFNs are also inducible in epithelial cells, thus suggesting that IFITM3 may be localized in the germinal epithelium. In cytoplasmic maturation of oocytes; with the formation of the first polar body, the perivitelline cavity expands and forms. The number of mitochondria increases and structural changes occur. With the release of cortical granules from Golgi complexes, the ooplasm becomes granular. Initially, the central mitochondria have a peripheral position with the development of the oocyte. Mitochondria are known to play a key role in cytoplasmic maturation. Mitochondria play an active role in intracellular metabolic events, cell differentiation, and cell proliferation [19]. We think that the expression of VASA, which is ATP binding RNA helicase, in the oocyte cytoplasm, may be due to the presence of mitochondria. Thyroid hormones increase the number and activity of mitochondria. When thyroxine or triiodothyronine is administered to an animal, it increases in size as well as the number of mitochondria in most body cells. The total surface area of the mitochondria increases, almost in direct proportion to the increase in the animal's metabolic rate. Therefore, one of the main functions of thyroxine can be said to increase the number and activity of mitochondria, which increases the rate of formation of adenosine triphosphate (ATP) to provide energy to cellular functions. Since there is no LH receptor in the stromal cells to be recognized in the theca cells, the formation of the theca cell layer occurs without the LH effect. The granulosa cells of the surrounding follicle become two or more layered

shapes. Then the theca differentiation factors (TDF) released from these cells, the LH receptors of the theca precursor cells, and the steroidogenic enzymes (CYP11A, 3ß-HSD, and CYP17) required for androgen biosynthesis stimulate the mRNA expressions [20]. VASA, which was expressed in the theca layer of the secondary follicle, was more intensely expressed in the theca layer of the tertiary follicle from the sections and the scoring. Although VASA expression was increased in the monolayer in the experimental group compared to the control group, IFITM3 was not localized in the monolayers. The reason that IFITM3 is not expressed in the monolayer may be since IFITM3 does not recognize the LH receptors and TDF to which it will bind, whereas the localization of VASA in the monolayer may be due to VASA recognizing LH receptors and TDF.

It can be said that the dense expression of the VASA antibody in oocytes and theca layers is dependent on ATP synthesized by mitochondria abundantly in the oocyte cytoplasm, LH receptors are found in the theca layer, increased VASA expression in theca layers of follicles growing compared to the control group is caused by hyperthyroidism at the LH level and VASA is caused by LH receptors and hyperthyroidism increases VASA expression. Besides, it can also be said IFITM3 is induced by both type I and type II of IFN (Type I IFNs are mostly produced by dendritic cells, but can also be induced in cell types such as epithelial cells), and IFITM3 expression in the germinal epithelium is induced in epithelial cells to result from the stimulation of IFN.

Decreased oocyte cytoplasm expression with ovarian follicle development with hyperthyroidism may indicate that hyperthyroidism decreased IFITM3 expression compared to the control group.

5. Conclusion

It has been concluded that expression of VASA and IFITM3 in the oocyte, zona pellucida, antrum, theca interna and theca externa of the follicles may be effective in the follicular development.

Ethical Approval:

Animal Ethic committee approval has been collected and preserved by the author(s)

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