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Title: Immunohistochemical demonstration of mTOR and p-mTOR expression in frozen and thawed ovarian tissue.

Short title: mTOR and p-mTOR expression in ovarian tissue.

Abstract

Purpose: The Ovarian cryopreservation is highly useful method is minimizing toxic due to prefered for protect fertility. Mammalian target of rapamycin (mTOR) pathway is critical to cell growth, proliferation, autophagy, nutrient signaling, and survival. Our aim was to investigate the mTOR and Phosphorylated (p-mTOR) expressions before and after frozen of the ovary by immunohistochemical method.

Material and methods: Control (C; n=6) group and vitrification and thawing group (V/T; n=6) groups of ovaries. C group fixed for 48 hours in buffered formalin. V/T group were frozen in liquid nitrogen at -196ºC. One week later the tissues were thawed. Both groups performed paraffin tracking. Following the Hematoxylin and Eosin staining of the ovaries, a follicle count was made. Immunohistochemistry examination for mTOR and p-mTOR expression were performed.

Results: Follicle numbers were not statistically different in the groups (p>0.05). The strong expression of mTOR was observed in follicles in C and V/T group ovary. Positive p-mTOR expression was observed in oocytes and granulosa cells in the control group, whereas the expression was negative in the V/T group.

Conclusion: Cryopreservation methods did not induce to alter mTOR expression in ovarian tissue. However, the absence of p-mTOR activity associated with oocyte quality in frozen ovarian tissues suggested that cryopreservation effected the quality of oocyte.

Keywords: Ovary, Cryopreservation, Vitrification, mTOR, p-mTOR.

Makale başlığı: Dondurulmuş ve çözülmüş ovaryum dokusunda mTOR ve p-mTOR ekspresyonunun immünohistokimyasal olarak gösterilmesi.

Kısa başlık: Ovaryum Dokusunda mTOR ve p-mTOR ekspresyonu.

Öz

Amaç: Ovarian kriyoprezervasyon, doğurganlığı korumak için tercih edilen toksik etkileri en aza indiren oldukça faydalı bir yöntemdir. Mammalian target of rapamycin (mTOR) yolağı hücre büyümesi, çoğalması, otofaji, besin sinyalizasyonu ve hayatta kalması için kritik öneme sahiptir. Amacımız immünohistokimyasal yöntemle overin dondurulmasından önce ve sonra mTOR ve Fosforile (p-mTOR) ekspresyonlarını araştırmaktır.

Gereç ve yöntem: Kontrol (C; n=6) grubu ve vitrifikasyon ve çözdürme grubu (V/T; n=6) over grupları. C grubu tamponlu formalin içinde 48 saat fikse edildi. V/T grubu -196ºC'de sıvı nitrojen içinde dondurulduu. Bir hafta sonra dokular çözüldü. Her iki grupta da parafin takibi yapıldı. Ovaryumların Hematoksilen ve Eozin ile boyanmasını takiben folikül sayımı yapıldı. mTOR ve p-mTOR ekspresyonu için immünohistokimya incelemesi yapıldı.

Bulgular: Folikül sayıları gruplar arasında istatistiksel olarak farklı değildi (p>0.05). C ve V/T grubu overlerdeki foliküllerde güçlü mTOR ekspresyonu gözlendi. Kontrol grubunda oositlerde ve granüloza hücrelerinde pozitif p-mTOR ekspresyonu gözlenirken, V/T grubunda ekspresyon negatifti.

Sonuç: Kriyoprezervasyon yöntemleri over dokusunda mTOR ekspresyonunu değiştirmemiştir. Bununla birlikte, dondurulmuş ovaryum dokularında oosit kalitesi ile ilişkili p-mTOR aktivitesinin olmaması, kriyoprezervasyonun oosit kalitesini etkilediğini düşündürmüştür.

Anahtar kelimeler: Ovaryum, Kriyoprezervasyon, Vitrifikasyon, mTOR, p-mTOR.

Introduction

Ovarian operations or cancer treatments, oocyte depletion may occur early, leading to the loss of critical functions during the rest of life. In recent years, works on cryopreservation of mature oocytes have been under way to prevent early infertility. However, since a mature oocyte has a very complex structure, not many successful results could be obtained, as it was damaged during the process. Therefore, the studies into ovarian tissue freezing have gained momentum in recent years in an attempt to preserve immature oocytes, especially in pre-pubertal girls and to protect fertility, mostly in cancer patients [1]. Freezing and subsequent transplantation of ovarian tissue has been shown to be sufficiently effective and feasible to preserve fertility in women. However, challenges remain in terms of graft survival, significant follicular loss and delays in revascularisation [2]. Studies have shown that vitrification is the most effective and simple method in cryopreservation. So far, approximately 60 babies have been born as a result of transplantation of frozen ovarian tissue [1]. These results indicate that this method, which is fairly useful clinically, is of great value in maintaining fertility, particularly in young patients. Hormone functions and fertility have also been showed to recover when vitrified/thawed ovarian tissue is transplanted [3]. However, optimal vitrification protocols, which prevent the formation of ice crystals known to cause physical and mechanical damage to cells for tissue preservation, are used to protect ovarian tissues for infertility treatments [4]. Despite this, deaths have been observed in ovarian follicle cells during vitrification and other freezing protocols, resulting in a decrease in the number of follicles.

The development and maturation of oocytes is organized by a large number of intracellular and endocrine signaling molecules, one of which is Mammalian Target of Rapamycin (mTOR) protein. As members of the P13K / Akt / mTOR complex, mTOR plays a pivotal role in numerous biological processes, including survival, growth, and the synthesis of large macromolecules The functions of mTOR in the ovary have been revealed by recent scientific studies, and this was possible through mTOR inhibitors. For instance, it has been shown that the proliferation of granulosa cells in healthy mice is inhibited when synthetic mTOR inhibitors are used [5-7]. The dysfunction of mTOR brings about an increase in pathological conditions such as cancer, obesity, type II diabetes and neurodegeneration [8-10].

mTOR must be phosphorylated to be active, and p-mTOR (2481 and 2448) are intrinsic mTOR-activating catalysts. It is noteworthy that p-mTOR and mTOR having many functions such as autophagy, proliferation in the ovary. The expression and distribution of them have been investigated in ovarian follicle development, but not in

frozen ovarian tissue up till now. In this study, we tried to gain an insight into the expression of mTOR and p-mTOR in frozen ovarian tissue through the vitrification method immunohistochemically.

Materials and methods

Formation of experimental groups

Ethics approval was obtained from Pamukkale University Animal Experimentation Ethics Committee (approval date: 08.11.2016 and approval number: PAUHADYEK-2016/24). Twelve healthy Wistar-Albino female rats were obtained from Pamukkale University Experimental Surgery Practice Center. Rats were selected at eight weeks of age, with a weight range of 150-160 g, and divided into two groups: a control group (C; n=6) and a vitrification and thawing group (V/T; n=6). (C; n=6) group and vitrification and thawing group (V/T; n=6). Rats were kept in a 12-hour light / dark cycle regardless of water and nutrient restrictions. After rats were sacrificed by applying anesthesia, their ovaries were removed.

Vitrification and thawing protocol

The vitrification protocol was based on the experiment protocol in the study by Nasrabadi et al. [11]. According to the conventional vitrification seventh protocol in the study, 7.5% Ethyl Glycol (EG), 7.5% Dimethyl sulfoxide (DMSO), 0.5 M sucrose, 20% Fetal Bovine Serum (FBS) are mixed to obtain the vitrification solution. After the ovaries dissected from rats were in Phosphate-buffered saline (PBS), they were placed in 1.8 ml cryovials to which conventional vitrification seventh protocol solution was added. Tissues were placed in cryovial, kept in liquid nitrogen vapor for 10 minutes, and then put into liquid nitrogen tank. Thawing protocols were applied to the ovaries kept in liquid nitrogen tank for one week. To thaw the vitrified ovaries, they were first warmed to room temperature for 10 seconds, then transferred in a water bath at 25°C for 10 seconds. Sucrose was then added at concentrations of 1 M, 0.5 M and 0.25 M, along with DPBS at room temperature for a period of 10 minutes [11]. Then the ovaries were embedded into 10% buffered formalin as the tissue fixation solution. After 48 hours of fixation, tissue follow-up process was administered.

Hematoxylin & eosin staining and follicle counting method

After the rats were decapitated and both ovaries of them were removed, these ovaries were fixed in 10% of formaldehyde for 24 hours. Following the fixation, the ovaries were embedded in the paraffin blocks. Then, 5 μm thick sections from the paraffin blocks were stained with hematoxylin and eosin for ovarian histology and follicle counting. The follicles were classified as atretic and normal, and the normal ones were

classified according to their developmental phases. Primordial, primary, secondary, developing, tertiary and atretic follicles were counted in the 1st, 5th, and 10th sections [12]. Accordingly, 1. Primordial follicles: Oocyte is surrounded by a single-layer flat epithelium. 2. Primary follicle: Oocyte is surrounded by a single layer of cubic or very few cell layers. 3.Secondary follicle: Oocyte is surrounded by multi-layer granulosa cell layer with antrum 4.Tertiary follicle: Oocyte is surrounded by multilayered cubic epithelium and has a large antrum.

Immunohistochemical staining

Immunostaining was performed using the avidin-biotin peroxidase complex method. After deparaffinization and rehydration, citrate buffer (pH 6.0) was used for antigen retrieval through microwave. Later, endogenous peroxidase activity was blocked with 3% hydrogen peroxide inside methanol at room temperature for 15 minutes. The sections were then incubated at 4°C overnight with primary antibodies (mTOR:#29725, cell signal, Danvers, Massachusetts, USA, 1/100, p-mTOR:#D0617, Santa Cruze, Dallas, Teksas, ABD, 1/100). After washing the primary antibodies, the sections were incubated with secondary antibody (1:1000) at room temperature for 60 minutes, followed by its incubation with the avidin-biotin complex for 30 minutes. Immunohistochemical reactions were visualized with diaminobenzidine chromogen under a light microscope. For opposite staining, Mayer's hematoxylin was administered for 15 minutes. The staining intensities within these areas were determined by two investigators in a blindly. Immunohistochemical staining was evaluated in accordance with the following scale:(+++): strong staining, (++): moderate staining, (+): weak staining, (/+/-): very weak staining, (-): negative, (/): no established pattern The semi-quantitative evaluation of mTOR and p-mTOR expression levels in the C and V/T groups was conducted utilising the H-score formula: H SCORE=∑Pi (I+1). The scale of staining intensity is delineated (0 = no expression, 1 = mild, 2 = moderate, and 3 = strong). Pi is defined as the percentage of cells that have been stained for each intensity [13].

Statistical analysis

Subsequent to the follicle counting, the within-group differences were analyzed with Independent Samples T-test and Mann-Whithey U test. Statistical analysis were performed using SPSS 21 package program, and the significance level was $p<0.05$. The H scores were analyzed using Tukey post hoc tests to reveal the differences between the groups.

Results

Histological findings

In the sections, primordial follicles, primary follicles, secondary follicles and tertiary follicles were normal in appearance in the ovary of C group (shown in Figure 1a, 2). Granulosa layer, theca interna and externa layers were seen regularly and firmly attached to oocyte. In the V/T group, follicles were also observed in all stages of development in the ovary. Majority of the follicles were normal in structure (shown in Figure 1B, 2). However in some of the primary and primordial follicles vacuoles were seen between oocyte and granulosa cell layer. In the secondary follicles, thinning of granulosa layer as well as cell accumulation in lumen were identified. And also it was interesting that some follicles lost their normal shape and became oval (shown in Figure 1B, 2).

The follicle count of each group is presented in Table 1. As the size of the follicles increased, their number decreased following the vitrification procedure, but that decrease was not statistically significant ($p>0.05$). No significant difference was noted between all groups regarding primordial follicle, primary follicle, secondary follicle, tertiary follicle and atretic follicles ($p>0.05$) (Table 1).

Immunohistochemical findings

H score analyzes are illustrated in Table 2. The distrubition and localization of mTOR expression in C and V/T groups was shown in Figure 3. Immunohistochemical staining showed strong expression of mTOR in granulosa cells of pimordial, primary, secondary and tertiary follicles as well as in oocytes within C and V/T groups ovarian tissues. But that increase was not statistically significant between groups ($p>0.05$). Oocytes demonstrated more diffuse and stronger immunostaining compared to granulosa cells. What was also notable is that the degree and location of the reaction were the same in the C and V/T group. Also cytoplasmic and prevalent mTOR activity was also observed in the corpus luteum (shown in Figure 3).

Positive expression was observed in oocytes and granulosa cells in the C group, whereas the expression was negative in the V/T group ($p=0.002$). In the C group, p-mTOR expression reacted moderately in oocytes and weakly in granulosa cells (shown in Figure 4).

Discussion

In this study, we investigated that the expression of mTOR and p-mTOR in frozen ovarian tissue through the vitrification method immunohistochemically. To the best of our knowledge, this study was the first in investigating mTOR and p-mTOR expression in a

vitrified tissue. The degree of expression and location of mTOR protein in ovarian tissue where C and V/T group were applied were similar. However positive p-mTOR expression was observed in the C group, whereas the expression was negative in the V/T group.

In a study, the number of primary follicles was lower in the frozen/thawed group compared to the control group (p<0.05). There was no significant difference between the groups in the number of secondary follicles [14]. In ovarian tissue transplantation, the main problem is follicular loss due to follicular activation. Rapamycin, an mTOR inhibitor, has been shown to prevent follicular activation induced by ovarian cryopreservation in mause transplantation models. Rapamycin was observed to prevent follicle proliferation stimulated by cryopreservation through the AKT and mTOR pathways [15]. Another study found that activation of the mTOR pathway in tissues was effectively inhibited by short-term incubation with rapamycin just prior to freezing during tissue cryopreservation. An increase in the ratio of primordial follicles and a decrease in apoptosis were observed after transplantation [2]. In our study, the number of primordial, primary, secondary and tertiary follicles decreased in the V/T group, and the number of atretic follicles and corpus luteum was high. But it was not found statistically significant.

A study using recombinant AavLEA1 protein in the cryopreservation of ovarian tissue reported that this protein protected ovarian follicles, increased their proliferative capacity and reduced apoptosis [16]. In frozen-thawed ovarian autografts in rats, Adipose-derived stem cells (ASCs) therapy has maintained the viability of ovarian grafts and increased the expression of genes related to apoptosis and inflammatory cytokines. ASC application has supported a decrease in caspase-3 expression in the theca interna layer of follicles and increased cell proliferation in the granulosa layer without altering VEGF [17]. In our study, one of the culture media used for cryopreservation was FBS, which contains numerous proteins. Contrary to previous studies, there was a decrease in follicle count in the V/T group compared to the C groups in our study, although not statistically significant.

Food deficiency and various hormones play a role in mTOR activity. mTOR is of pivotal importance in cytogenesis of meiosis in oocytes, and pSer2448 or pSer2481(p-mTOR) is indicative of intrinsic mTOR catalytic activity in dividing cells [18-21]. The V/T of ovarian tissue has become more of an issue recently, becoming a glimmer of hope in preserving fertility, especially for young cancer patients [9, 22-24]. Furthermore, minimizing follicle loss in V/ T ovaries will accelarate the clinical use of the technique. Here we elucidated the role of the mTOR and its phosphorylated p-mTOR proteins, which are effective not only in cell proliferation and differentiation but also in triggering autophagy, in V/T ovaries.

In a study, the effects of sericin, an antioxidant, on the freezing and thawing of mouse ovarian tissue were investigated. The study found that after thawing, 1% sericin reduced oxidative stress and could protect ovarian tissue during freezing and thawing via the PI3K/AKT/mTOR signalling pathway [25]. However, in our study, the cryoprotectants used did not affect mTOR expression but inhibited p-mTOR expression.

The investigators have reported that mTOR may have a function in cytokinesis and that in particular the p-mTOR activity in cumulus cells may play a role in the production and secretion of meiosis-inducing products. The proliferative effect of cumulus cells during meiosis has been reported to occur in the formation of high quality oocytes that can go further to the oocytes [21]. Our study revealed that mTOR was strongly expressed in both granulosa cell cytoplasm and oocytes in pimordial, primary, secondary and tertiary follicles within both C and V/T groups ovaries. The expression in the oocyte turned out to be stronger than the granulosa cells and was observed in all the follicles at every stage of development. What is also remarkable is that the expression in oocytes took place both in the ooplasm and in the nucleus. The degree and location of the mTOR reaction did not differ in the ovarian tissues which the control and vitrification procedures were carried out. Nevertheless, the p-mTOR expression did differ in both groups. Positive expression was observed in oocytes and granulosa cells in the C group, while both oocytes and granulosa cells were negative in the V/T group. On the other hand, p-mTOR expression reacted moderately in the oocytes and weakly in granulosa cells in the C group.

High levels of p-mTOR expression have been identified in cancer cells; for instance, the p-mTOR is expressed intensively in liver, colon and pancreatic cancers [26-28]. Generally, the p-mTOR expression was evaluated in cancerous tissues, but Kogasaka et al. [29] were the first to investigate the expression and distribution in normal tissues. They studied the expression of pSer2448 and p2481 mTOR in oocyte and follicle development in vitro and in vivo. They found that the expression of pSer2448 mTOR or pSer2481 mTOR in oocytes and cumulus cells was widespread in the cytoplasm during the interphase phase, around the chromosomes in the prophase, and around the spindle strands in the metaphase and telophase [29]. In our study the p-mTOR expression, only detected in the C group, was also apparent in both the oocyte and granulosa cell cytoplasm. However, the p-mTOR expression in the V/T group was not detected in either oocyte or granulosa cell nuclei. In other words, the p-mTOR is heavily involved in high quality oocyte formation. The last but not the least, the p-mTOR was positively expressed only in the C group in our study. The negative oocytes in which the vitrification is applied may be indicative of poor quality of oocytes.

In conclusion, the degree of expression and location of mTOR protein in ovarian tissue were similar in both groups. Positive expression of mTOR leads to the inhibition of autophagy, while its negative expression activates autophagy. The positive expression of mTOR in both groups demonstrated that vitrification did not induce autophagy in the ovary. However, when we consider that p-mTOR is associated with oocyte quality in line with the literature, the absence of p-mTOR positive expression in the vitrification ovaries suggests that vitrification deteriorates oocyte quality and may reduce fertilization capacity.

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Contributions of the authors to the article: U.D., M.S.U., N.C. and G.A.M. designed the experiments; U.D. and N.C. performed experiments and collected data; U.D., N.C., and G.A.M. discussed the results and strategy; G.A.M. Supervised, directed and managed the study; U.D., M.S.U., N.C. and G.A.M. Final approved of the version to be published.

Conflict of interest: No conflict of interest was declared by the authors.

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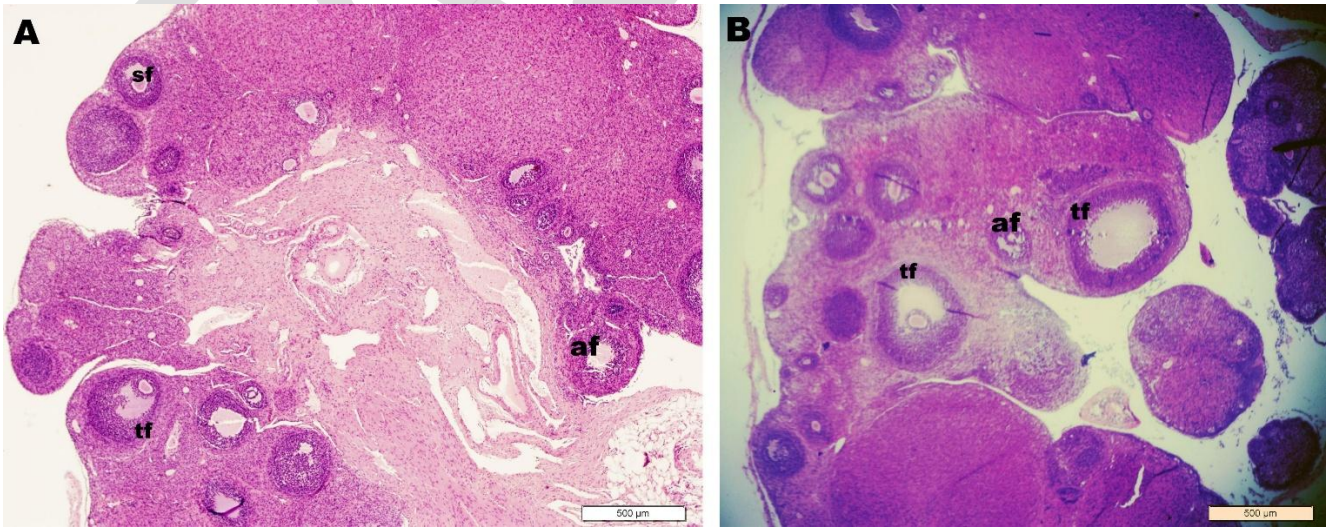

Figure 1. Control; (A) and Vitrification and Thawing group; (B) ovarian tissue

Secondary follicle; (sf), tertiary follicle; (tf), corpus luteum;(cl), atretic follicle; (af). Hematoxylin &eosin. Bar A, B: 500 µm

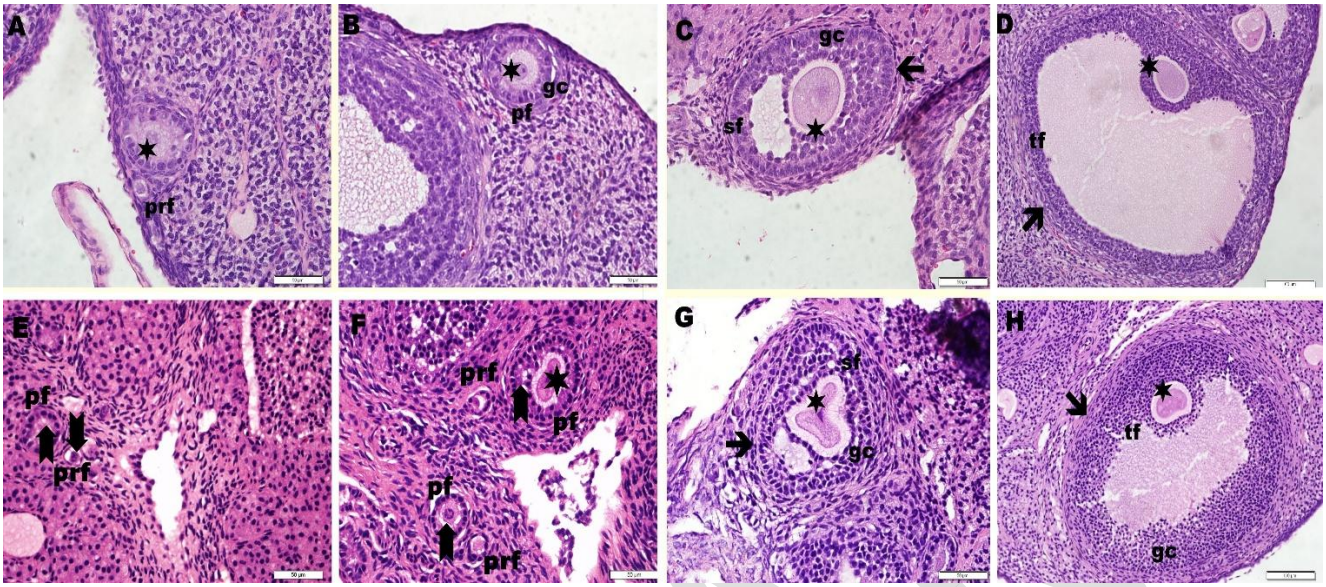

Figure 2. In Control group ovary tissue (A-D) and Vitrification and Thawing group ovary (E-H) tissue

Primordial follicle; (prf), primary follicle; (pf), secondary follicle; (sf), tertiary follicle; (tf), corpus luteum; cl, theca cell; (arrow), vacuoles; (thick arrow), oocyte: (star) Hematoxylin & eosin. Bar A, B, C, E, F, G: 50µm, D, G: 100 µm

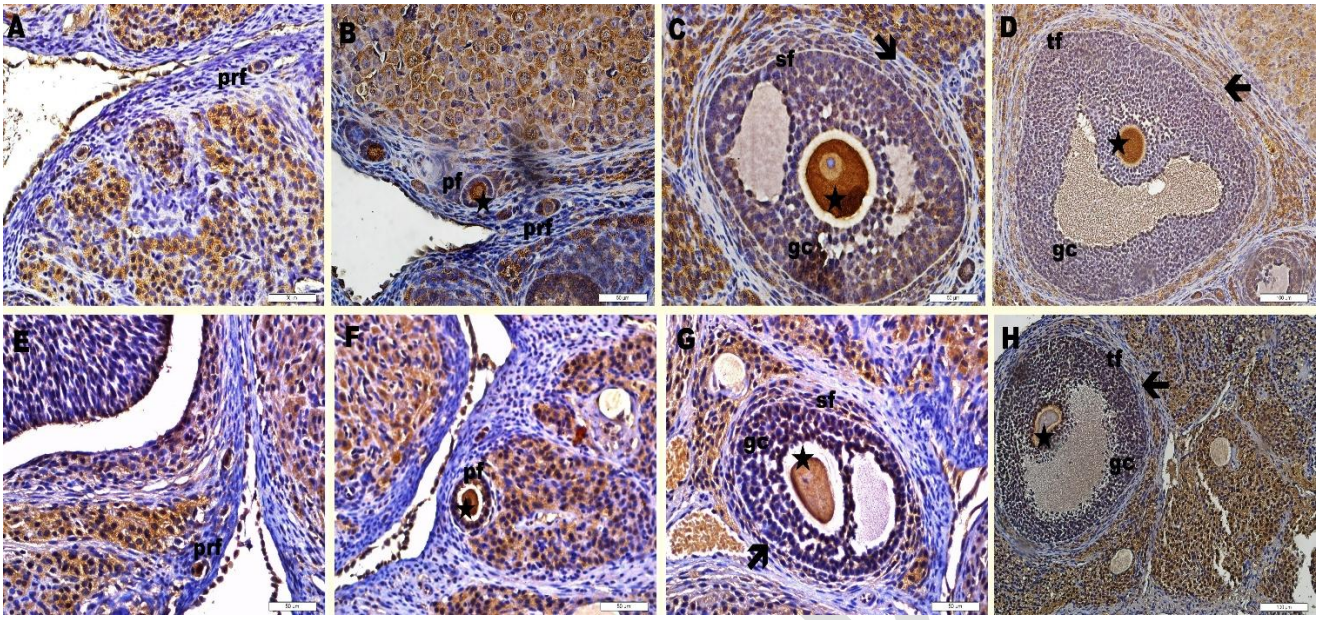

Figure 3. The expression of mTOR in control group ovary tissue (A-D) and Vitrification and Thawing group ovary (E-H) tissue

Primordial follicle; (prf), primary follicle; (pf), secondary follicle; (sf), tertiary follicle (tf), granulosa cell; gc, theca cell;(arrow), oocyte: (star). Immunoperoxidase&Hematoxylin. Bar A, B, C, E, F, G: 50µm, D, G: 100 µm

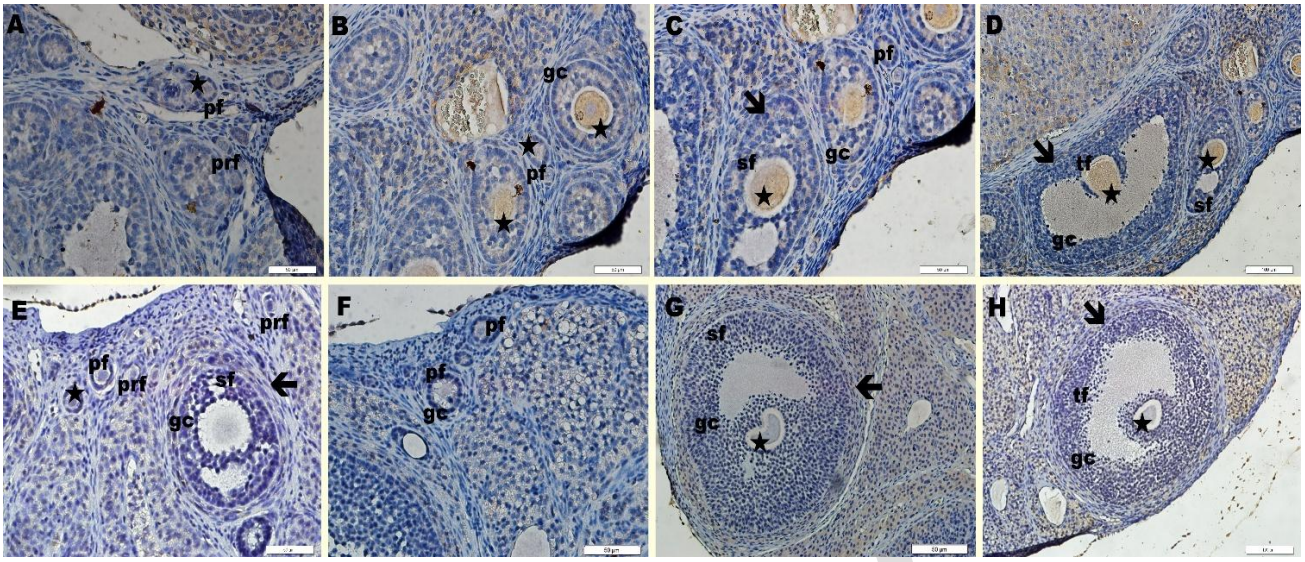

Figure 4. The expression of p-mTOR in control group ovary tissue (A-D), Vitrification and Thawing group ovary tissue(E-H)

Primordial follicle; (prf), primary follicle; (pf), secondary follicle; (sf), tertiary follicle (tf), granulosa cell; gc, theca cell;(arrow), oocyte: (star). Immunoperoxidase&Hematoxylin. Bar A, B, C, E, F, G: 50µm, D, G: 100 µm

Table 1. Follicle count in rat ovaries of experimental groups

	C Mean ±SD	V/T Mean ±SD	*p* value
Primordial Follicles	9.75±4.75	9.25±5.29	0.805
Primary Follicles	4.75±1.00	3.33±1.20	0.265
Secondary Follicles	7.00±3.79	5.33±2.01	0.179
Tertiary Follicles	2.50±2.62	2.41±0.90	0.853
Atretic Follicles	8.00±2.21	9.50±3.00	0.177
Corpus Luteum	6.50±2.57	7.66±3.14	0.331

p<0.05 was considered statistically significant
C: Control, V/T: vitrification and thawing group, SD: Standard deviation

Table 2. HSCORE values of mTOR and p-mTOR immunoreactivity in oocytes of primordial, primary, secondary and tertiary follicles in groups

	mTOR			p-mTOR		
	C mean±SD	V/T mean±SD	*p* value	C mean±SD	V/T mean±SD	*p* value
Primordial follicles	236.50±30.38	226.16±33.12	0.417	99.00±15.15	10.00±0.00	0.002
Primary follicles	226.66±18.16	219.83±16.09	0.423	105.83±11.03	10.00±0.00	0.002
Secondary follicles	226.00±20.15	218.00±17.43	0.336	102.83±9.45	10.00±0.00	0.002
Tertiary follicles	240.00±13.66	218.00±13.31	0.420	103.33±16.40	10.00±0.00	0.002

p<0.05 was considered statistically significant
C: Control, V/T: vitrification and thawing group, SD: Standard deviation

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