



Evaluating the Expression of Wnt Pathway Related Genes in Mouse Vitrified Preantral Follicles: An Experimental Study

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Abstract

Background: Wnt signaling pathway plays critical role in ovarian follicle development. Therefore, the aim of this study was to evaluate the effects of vitrification on the expression of Wnt pathway related genes in preantral follicles (PFs).

Methods: Isolated PFs (n=982) of 14-16 day old female mice (n=45: 15 for each group) were divided into fresh (n=265), toxicity (n=272), and vitrified (n=265). The mRNA levels of Wnt2, Wnt4, Lrp5 and Fzd3 were evaluated by real-time PCR on the 2nd and 6th days of culture period. One-way ANOVA was conducted to analyze the data. Post hoc Tukey's HSD was used for multiple comparisons and p-value less than 0.05 was considered statistically significant.

Results: The developmental parameters of fresh PFs were significantly higher than those of vitrified (p<0.001). There were no differences between fresh and vitrified PFs on the 2nd day of culture (p<0.001). Wnt4 expression levels decreased significantly in vitrified groups compared with fresh ones (p<0.001). Fzd3 and Lrp expression levels increased significantly in vitrified groups compared with those in the fresh group on the 2nd day (p<0.001). On the 6th day of culture period, the expression levels of Wnt2 and Fzd3 increased significantly in vitrified group compared to those of fresh group (p<0.001). Moreover, the expression levels of Wnt4 and Lrp increased significantly in toxicity groups compared to those of the control group (p<0.001).

Conclusion: Vitrification increase the expression levels of Wnt2, Lrp and Fzd3 genes of PFs during *in vitro* culture.

Keywords: Cryopreservation, Folliculogenesis, Ovary, Wnt/ β -catenin pathway.

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Introduction

Cancer patients who are exposed to chemo and radiotherapy often become infertile (1, 2). There are several methods to preserve their fertility, including freezing or vitrification of ovarian tissue, follicles, oocytes, and embryos (3). Among them, vitrification of preantral follicles (PFs) has many advantages over other methods and is an alternative approach (4). Nevertheless, vitrification of ovarian follicles is technically challenging because of their larger size and long process of folliculogenesis. In addition, the varie-

ty of injuries may occur during the vitrification depending on the size, shape, permeability, quality, and sensitivity which result in distribution of cytoskeleton, release of cortical granules, and zona hardening (5). Also, it can disintegrate the plasma membrane and change the mRNA production, cause DNA damage and cell death (6). However, some investigations have reported controversial results regarding this issue. Many studies have been conducted on the effects of cryopreservation on gene expression. Even though there are

contradictory results (5, 7-11), in some cases, negative effects of vitrification on the molecular structure of the cell did not immediately appear after warming and occasionally do not cause degeneration (12). In this regard, Mofarahe et al. (13) showed that vitrification of ovarian tissue has no detrimental effect on the expression of folliculogenesis related genes, while *in vitro* cultivation changes the expression of those genes. This is in agreement with observations of Gumus et al. (14) who showed that the expression level of the genes related to follicular development did not change after vitrification. Also, other studies showed there was no significant difference in expression levels of apoptosis related genes between vitrified and fresh ovaries (9).

The wingless-type mouse mammary tumor virus integration site family (Wnt) pathway including the Wnt/ β -catenin dependent pathway and β -catenin-independent pathway triggers intracellular biochemical cascades which play critical roles in the regulation of many developmental mechanisms, including differentiation, proliferation, migration of cells, and homeostasis (15). A series of investigations have showed the potential role of Wnt signaling in the regulation of folliculogenesis such as follicular development, ovulation, formation of corpus luteum, and steroidogenesis in rodent ovaries (16-18). Most studies on Wnt molecules during ovarian follicle development were focused on Wnt2, Wnt4, Wnt3, and FZ receptors. It was demonstrated that Wnt4 is essential for normal function of reproduction system such as activation of regulatory genes related to granulosa cell function, follicle maturation, and ovulation (16). Also, Wnt2 has been shown to be expressed only in granulosa cells of preantral and antral follicles which are regulated by ovarian steroid hormones (19). In addition, Fzd2, Fzd3, and Fzd9 have been identified in human cumulus cells (20-23). Lrp5 and Lrp6 bind to Wnt proteins and interact with Fzd receptors. It was shown that Lrp5 acts as a common receptor for Wnt signal transduction and its overexpression activates the Wnt pathway and developmental defects are the consequence of its mutations (24).

Although previous studies showed the effects of vitrification on apoptosis, and some related genes of follicle development, there is still no report on the effects of vitrification on the related genes of Wnt pathway. Therefore, the aim of this study was to investigate the effect of vitrification on the

expression of Wnt pathway related genes in preantral follicles (PFs) during *in vitro* culture.

Methods

All chemicals in the present study were obtained from Sigma Aldrich (UK) except those mentioned in the text.

Animals: In this experimental study, 6-8 week old male and female NMRI mice were purchased from Pasteur Institute of Iran and were acclimatized to the animal house conditions at least for one week. To ensure pregnancy, females and males were kept in pairs throughout the breeding period. Female offspring of 14 to 16 days (n=45) old were used for the study. The mice were kept under standard conditions of 12 hr light/dark cycle at 20-24°C with 40-50% humidity and sufficient food and water. Experiments of the present study were performed according to guidelines of Ethics Committee of the School of Biology, Damghan University, Iran between June 2018 and November 2018. All experiments were approved by the ethics committee of Damgha University, Damghan, Iran (IR.DU.REC.1398.004).

Isolation of preantral follicles: Mice were sacrificed by cervical displacement and the ovaries were removed and placed in 200 μ l drops of α -MEM medium containing 10% of the fetal bovine serum (FBS; Sigma-Aldrich, UK), 2.2 g/l sodium bicarbonate, 100 ml/IU penicillin, and 75 μ g/ml streptomycin with 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), under mineral oil and placed in an incubator at 37°C with 5% CO₂, and 98% humidified air for at least 30 min to adapt to the new condition. PFs were mechanically isolated using 29G needle attached to insulin syringe under stereo microscope (Olympus, Japan). PFs (n=982) with a diameter of 140 to 160 μ m, a central healthy oocyte, and 2 to 3 layers of granulosa cells were selected and divided into three groups: 1) fresh (Control; n=265), PFs were transferred to the culture medium immediately after the isolation; 2) vitrification (n=272), PFs were vitrified/warmed and transferred to the culture medium; and 3) toxicity (n=265), PFs were exposed to all the vitrification/warming procedure stages except exposure to liquid nitrogen and eventually transferred to the culture medium. All three groups were cultured in incubator at 37°C with 5% CO₂, and 98% humidified air for 12 days.

Vitrification and warming: Vitrification of PFs was carried out based on the method of Hatami et al. (25) with some modifications. Equilibration solution (ES) was α -MEM supplemented with dimethyl sulfoxide (DMSO) 7.5% (V/V), ethylene glycol (EG) 7.5% (V/V), and vitrification solution (VS) was α -MEM supplemented with FBS 20% (V/V), DMSO 15% (V/V), EG 15% (V/V), and sucrose 0.5 M. PFs were exposed to ES and VS for 5 min and 30 s, respectively. After that, PFs were placed on the tip of Cryolock® and immersed in the liquid nitrogen for at least one week. In the warming procedure, vitrified PFs were immediately exposed to descending solutions of 1 M, 0.5 M, and 0.25 M sucrose for 30 s, 3 min, and 3 min, respectively. Finally, PFs were washed in phosphate buffer solution and transferred to the culture medium.

In vitro culture of PFs: PFs were cultured in 25 μ l droplets of α -MEM enriched with 5% FBS, 0.1 IU/ml human follicle stimulating hormone (hFSH), 1% insulin, transferrin and selenium (ITS), 10 ng/ml epidermal growth factor (EGF), 2.2 g/L sodium bicarbonate, 100 IU/ml penicillin, and 75 μ g/ml streptomycin in an incubator at 37°C with 5% CO₂, 20% O₂, and 98% humidified air up to 12 days. The culture medium was replaced every other day with a fresh medium. The diameter of the PFs was calculated by the mean perpendicular diameter of follicles under stereomicroscope (Nikon, Japan) on the first day, the second and the fourth days of the culture period. Morphological changes and survival rates of PFs were studied on days 2, 4, 6, 8, 10, and 12. The PFS with dark appearance, without oocyte, or those which stopped growing, were considered as degenerated follicles. Transparent areas among the granulosa cells surrounding the oocytes were considered as antral cavity. Ovulation was induced by changing the medium with enriched culture medium supplemented with 0.1 ml/IU human chorionic gonado-

tropin (hCG) on the 12th day and the released oocytes were classified as germinal vesicle (Nuclear envelope), when the germinal vesicle (GV) breakdown of the oocyte occurs and MI oocytes undergo the process of germinal vesicle breakdown (GVBD); MII oocytes refer to those extruded the first polar body.

Real-time PCR: In order to evaluate the relative expression of the chosen genes (Wnt2, Wnt4, Fzd3, and Lrp5), the PFs in optimal conditions were isolated on days 1 and 6 of the culture period (The initial time of culture was considered day 0). Total RNA was extracted from PFs (n=60 in each group) using the TRIzol reagent (Invitrogen, USA) based on the producer's guidance. Samples with an A260/A280 ratio of 1.8 to 2.0 were used for reverse transcription. The cDNA was synthesized using cDNA synthesis kit (Takara, Japan) based on the producer's instructions. Briefly, 500 ng of total RNA (6.5 μ l), 2 μ l PCR buffer, 0.5 μ l oligo-dt primer, 0.5 μ l random hexamer, and 0.5 μ l of RT-enzyme were mixed. Then, they were incubated at 37°C and 85°C for 20 min and 10 s, respectively. The gene specific primers were designed using AlleleID tool (Premier Biosoft, USA). The elongation factor 1 (Ef1) gene was used as the housekeeping gene (Table 1). Real-time PCR was performed on a Rotor-Gene 6000 machine (Corbett Life Science, USA) using SYBR Green RT-PCR kit (Takara, Japan). Thermal program of the real-time PCR was set to initial denaturation for 15 s at 94°C, followed by 40 cycles of 10 s at 94°C, 30 s at 60°C, and 30 s at 72°C. The specificity of PCR products was confirmed by both melting curve analysis and agarose gel electrophoresis. PCR efficiency for each gene was determined according to standard curves. Relative quantification analysis was performed using $2^{-\Delta\Delta CT}$ method with the Rotor-Gene 6000 series software v1.7 (Corbett Life Science, USA).

Statistical analysis: All data were analyzed by

Table 1. List of primers

Primers	Accession number	Sequence	Tm (°C)	Product size
Wnt2-F	NM_023653	5 TGTAGATGCCAAGGAGAGGAA 3	87.57	131
Wnt2-R		5 AGGAGCCACTCACACCAT 3	97.55	
Wnt4-F	NM_009523	5 GAATGACACAAGGGGTGG 3	97.55	113
Wnt4-R		5 CACACTTCTCCAGTTCTCC 3	67.56	
Lrp5-F	NM_008513	5 GTATGACACTGGTAGACA 3	41.51	187
Lrp5-R		5 ATGTAATCGCTATATTGAGTC 3	01.52	
Fzd3-F	NM_021458	5 ATTGTAATTGGATGTTCTCTTGG 3	30.55	279
Fzd3-R		5 CGGCTCTCATTCACATCTCT 3	87.57	
Ef1-F	NM_010106	5 AGTCGCCTTGGACGTTCTT 3	67.56	124
Ef1-R		5 CCGATTACGACGATGTTGATGTG 3	65.60	

SPSS software v24 (IBM, USA). One-way ANOVA was conducted to compare the rates of growth, survival, antrum formation, and oocyte maturation. Post hoc Tukey's HSD was used for multiple comparisons. For real-time PCR data, post hoc Dunnett's test was used. A p-value less than 0.05 was considered statistically significant. The data are presented as mean $\pm$ SD.

Results

The mean diameters of PFs and the rate of developmental parameters of the PFs are summarized in tables 2 and 3. On the second day, replication and growth of granulosa cells started and PFs were attached to the bottom of the culture dishes. After the fourth day, the growth rate increased and the appearance of the PFs became irregular and diffuse and their diameter could not be easily measured. From the sixth day onwards, transparent spaces among granulosa cells were created which were considered as antrum cavities. During the culture period, PFs with a natural appearance, bright color, healthy oocyte, and clear zona pellucida were regarded as healthy PFs (Figure 1). Otherwise, any changes were considered as degenerated PFs.

At the beginning of the culture period, the mean

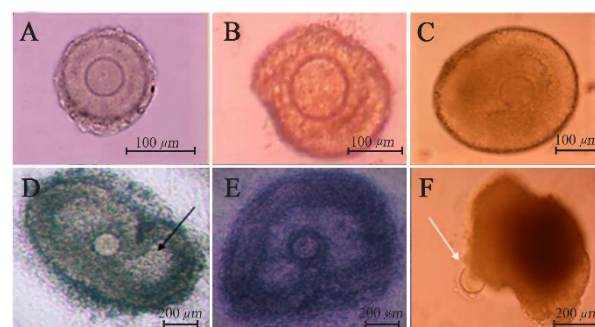


Figure 1. Images of mouse PFs during *in vitro* culture; PFs on the first day (A), 4th day (B), 6th day (C), 8th day (D), and 10th day (E) ovulated oocyte; white arrow (F) antrum cavity; black arrow

diameter of PFs was not significantly different among three experimental groups (Table 2). However, the mean diameters of PFs in control and toxicity groups were significantly higher than the vitrification group on the second ($p=0.005$) and fourth days ($p<0.001$) of culture period, whereas, no significant difference was observed between control and toxicity groups (Table 2).

The rates of survival ($p<0.001$), antrum cavity formation ($p<0.001$), and ovulation ($p<0.001$) in the vitrification group were significantly lower than control and toxicity groups (Table 3). How-

Table 2. Diameter of PFs during the cultivation period

Groups	No	Initial time	Day 2	Day 4
Control	265	150.50 $\pm$ 2.38	199.25 $\pm$ 4.03 #	298.75 $\pm$ 5.91 #
Toxicity test	272	149.50 $\pm$ 12.9	197.75 $\pm$ 4.57 #	295.00 $\pm$ 8.29 #
Vitrification	265	149.00 $\pm$ 1.41	188.50 $\pm$ 2.08 *,#	242.75 $\pm$ 2.99 *,#

* Indicates a significant difference in the same column ($p<0.05$).

Indicates a significant difference compared with initial time in the same row ($p<0.05$)

Table 3. Rates of developmental parameters of PFs during the cultivation period

Groups	No	Survival (n) mean $\pm$ SD	Antrum (n) mean $\pm$ SD	Ovulation (n) mean $\pm$ SD	Oocyte development #		
					GV (n) mean $\pm$ SD	MI (n) mean $\pm$ SD	MII (n) mean $\pm$ SD
Control	265	226 85.75 $\pm$ 2.22	225 81.75 $\pm$ 2.06	207 75.00 $\pm$ 0.82	34 12.50 $\pm$ 2.08	51 18.50 $\pm$ 1.73	122 44.25 $\pm$ 1.50
Toxicity test	272	234 86.00 $\pm$ 2.16	221 81.50 $\pm$ 5.92	202 74.25 $\pm$ 2.99	37 13.75 $\pm$ 1.89	51 18.75 $\pm$ 3.10	118 43.50 $\pm$ 4.43
Vitrification	265	207 78.0 $\pm$ 1.83 *	180 68.0 $\pm$ 0.82 *	160 60.75 $\pm$ 3.86 *	45 17.25 $\pm$ 3.59 *	36 13.50 $\pm$ 1.0 *	79 24.25 $\pm$ 3.86 *

* Indicates a significant difference in the same column ($p<0.05$)

The stages of oocyte development were assessed after induction of ovulation with the medium containing hCG on the 12th day of cultivation period

GV: Germinal vesicle oocyte, MI: Metaphase I oocyte, MII: Metaphase II oocyte

ever, there was no significant difference between control and toxicity groups (Table 3). The number of GV ($p=0.075$), MI ($p=0.011$), and MII ($p<0.001$) oocytes in the control and toxicity groups were significantly higher than those of the vitrification group (Table 3), while there was no significant difference between control and toxicity groups (Table 3).

Gene expression analysis: The relative expression of Wnt2 and Wnt4 genes as regulators of ovarian follicle growth was assessed in this study (21, 22, 26, 27). The results of real-time PCR showed significant differences among relative Wnt2 mRNA expression levels in all experimental groups ($F(2,6)=77.95$; $p<0.001$). In the toxicity group, Wnt2 mRNA level significantly increased at 24 hr of the culture period, compared to the control group, while there was no significant difference between the vitrification and control groups. Also, mRNA level of Wnt4 at 24 hr of the culture period was significantly different among groups ($p<0.001$). Relative expression levels of Wnt4 decreased significantly in the vitrification group compared to the control group, while there was no significant difference between that of toxicity and control group. In addition, the relative mRNA level of Fzd3 was evaluated as it is involved in follicle and oocyte development (17, 20). The results showed the relative expression of Fzd3 decreased significantly in the toxicity group compared to the control group, while it significantly increased in the vitrification group compared to the control at 24 hr of the culture period ($F(2,6)=50.75$; $p=0.000$). Furthermore, the relative expression of Lrp5 was evaluated when receiving Wnt signals (20). Results of relative expression level of Lrp5 mRNA showed a significant decrease in the toxicity group than that of the control group, while it significantly increased in the vitrification group compared with control ($p<0.001$) (Figure 2).

On the 6th day of culture, the relative expression level of Wnt2 mRNA in the vitrification group significantly increased in comparison to the control group, while there was no significant difference between that of the toxicity and control groups. The mRNA level of Wnt4 significantly decreased on the 6th day of culture period in the vitrification group compared to the control group, while no significant difference was observed between the toxicity and control groups ($p=0.004$). Furthermore, the expression level of Fzd3 mRNA

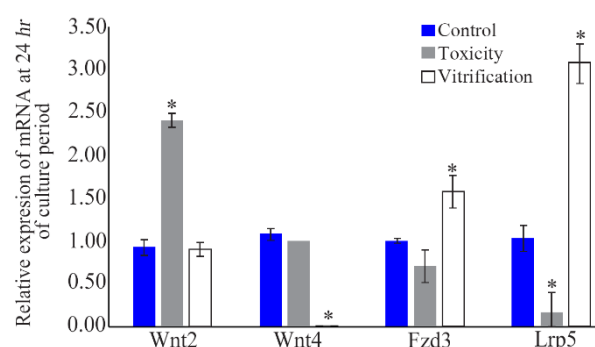


Figure 2. Relative mRNA expression of Wnt2, Wnt4, Fzd3, and Lrp5 in the PFs (n=60) at the 24th hr of culture period. The data are presented as relative fold changes (Mean $\pm$ SD) of three independent experiments.

* Shows significant difference compared to the control group ($p<0.05$)

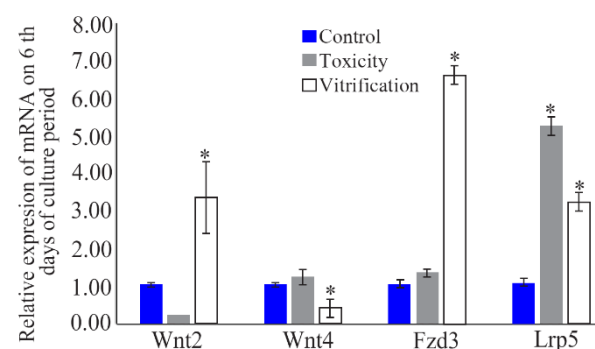


Figure 3. Relative mRNA expression of Wnt2, Wnt4, Fzd3, and Lrp5 in the PFs (n=60) on the 6th day of culture period. The data are presented as relative fold changes (Mean $\pm$ SD) of three independent experiments.

* Shows significant difference compared with control group ($p<0.05$)

in the vitrification group significantly increased compared to the control group on the 6th day of culture period, while there was no significant difference between toxicity group compared with control ($p<0.001$). Also, the expression level of Lrp5 mRNA in the vitrification and toxicity groups significantly increased in comparison with that of the control group ($p<0.001$) (Figure 3).

Discussion

The results of the present study indicated that the rate of growth, antrum formation, and oocyte maturation in the vitrification group significantly decreased compared with the control group, whereas no significant differences were observed in aforementioned rates in toxicity (The stage of liquid

nitrogen immersion was removed) and control group. In recent years, many studies have been conducted on the effects of vitrification on the ovarian tissue, PFs, oocyte, and embryo while contradictory results were obtained. Vitrification induces various types of injuries such as extracellular damage, the premature release of oocyte cortical granules, zona hardening, and changes in genes expression levels which in turn reduces growth and developmental competence (5-8, 10, 11, 28-30). In this regard, in a study on ovarian tissue, it was found that the expression of Zp3 and AMH genes significantly decreased in the vitrification group (29). In addition, Isachenko et al. (11) demonstrated that GAPDH gene expression in ovarian tissue significantly decreased after vitrification. Also, Asadzadeh et al. (10) reported similar results that the expression of some of the genes related to matrix metalloproteinase significantly changed after ovarian tissue vitrification. Their findings are in agreement with the results of the present study that showed the expression of Wnt pathway related genes expression changes in the vitrification and toxicity groups during the *in vitro* culture period.

Several Wnt molecules have been known in ovarian tissue to regulate target gene expression during follicle development and they play critical role in normal ovarian function and fertility (16, 31-33). The present study showed the expression of Wnt pathways related genes except Wnt2 in the vitrified group was significantly different compared with the control group at 24 *hr* of *in vitro* culture.

It was demonstrated that gene of Wnt2 is expressed in granulosa cells during all stages of ovarian follicle development (19, 22). Several stages of granulosa cell proliferation are controlled through Wnt2/ β -catenin signaling. Wnt2 controls β -catenin pathway through gathering the cytoplasm of GSK3 β and PCNA in mouse granulosa cells to enhance DNA synthesis (22). In this regard, Dalman et al. showed the Wnt3, B-Catenin, Fzd2, and Gsk3 β expression significantly changed in cryopreserved ovarian tissue (8). Choi et al. in 2008 reported that PCNA gene expression decreased during follicle development from the primitive stage to the initial stage, which is attributed to the reduced proliferation of granulosa cells (34). Also, Wnt2 regulates gap junction intercellular communication between granulosa cells which is essential for development of preantral follicles (27). It was suggested that in addition to Wnt2,

compensatory activity of other molecules is required for maturation of the ovarian follicles and granulosa cell proliferation (33). Also, in agreement with our results, it was demonstrated that in the cryopreserved ovarian tissues, the expressions of CDK4, cyclin D2, CDK2, and cyclin E also decreased at 0 and 24 *hr* of *in vitro* culture period, but increased after 48 *hr* of *in vitro* culture which indicates that the freezing of ovarian tissues may postpone the PFs development by suppressing the proliferation of ovarian granulosa cells through disturbing the cell cycle regulators (CDK4, cyclin D2, CDK2, and cyclin E) (34). It was shown that suppressing Wnt4 gene expression leads to decreased health of antral follicles in mice (21) which indicates that Wnt4 is essential for ovarian follicles growth and maturation. Wnt4 has been demonstrated to control the steroidogenic activity of granulosa cells (20). In this regard, Gifford suggested that β -catenin facilitates Wnt4 action in regulation of steroidogenesis and ovarian antral follicle maturation (33).

The present study revealed that mRNA level of Fzd3 significantly decreased in the toxicity group compared to the control group, while it considerably increased in the vitrification group at 24 *hr* of the culture period. In this regard, gene of FZ receptors has been indicated to be expressed in granulosa cells during follicular development and corpus luteus formation (33) and that follicles which suppress the expression of Fz receptor genes stop the development and maturation of follicles in early developmental stage (17). However, Fzd3 and Lrp5 genes are involved in the early message transmission of Wnt pathway and Fzd3 and Lrp5 receptors have been shown to be expressed at different phases of follicle development, while Lrps are members of the LDL receptors family with numerous biological functions in follicular cells of ovarian tissue (18, 19, 23, 32, 33); therefore, the increased expression of these two genes in the vitrified group may indicate that the follicles can keep the activation of Wnt pathway. On the other hand, expression of Wnt4 gene decreased significantly at 24 *hr* and on the 6th day of culture period in the present study. Wnt proteins act as ligand in activating the Wnt pathway; thus, the reduction of Wnt4 expression in this study may indicate that the Wnt pathway is impaired in vitrified group. Expression of Wnt2 gene was not significantly different at the beginning of *in vitro* culture, but increased significantly on the 6th day of culture. This significant increase of Wnt2 gene expression

during culture period may show that follicular development from preantral to preovulatory stage is associated with an increase in granulosa cells proliferation and FSH receptors. Thus, it seems that increased number of FSH receptors is associated with Wnt2 gene expression.

Conclusion

In conclusion, this study suggests that follicle vitrification changes the pattern of Wnt signaling pathway related genes expression, which may be the cause of the reduction in follicle survival and development. Although many genes are involved in follicle viability and development, blocked follicular growth after vitrification cannot be directly attributed to altered Wnt pathway related genes expression. Also, expression of these genes in the toxicity group had a different pattern. Therefore, it can be concluded that cryoprotectant agents can alter the patterns of Wnt signaling pathway related gene expression.

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Conflict of Interest

None of the authors has any financial or other potential conflict of interest.

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