

Vitrification alters growth differentiation factor 9 and follicle-stimulating hormone receptor expression in human cumulus-mural granulosa cells

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Objective: Ovarian tissue vitrification is widely utilized for fertility preservation in prepubertal and adolescent female patients with cancer. The current literature includes reports of successful pregnancy and live birth following autografting. However, the effects of the vitrification process on cumulus-mural granulosa cells (C-mGCs)—somatic cells in ovarian tissue crucial for oocyte maturation and early embryonic development—remain unclear. This study was conducted to explore the impact of vitrification on the cellular function of C-mGCs by quantifying the expression of growth differentiation factor 9 (GDF-9), bone morphogenetic protein 15 (BMP-15), follicle-stimulating hormone receptor (FSHR), luteinizing hormone receptor (LHR), connexin 37, survivin, and caspase 3.

Methods: Mature and immature C-mGCs were obtained from 38 women with polycystic ovary syndrome who participated in an *in vitro* fertilization program. The C-mGCs were then divided into two groups: fresh and vitrified. The expression levels of target genes were assessed using real-time quantitative polymerase chain reaction.

Results: After vitrification, GDF-9 expression was significantly decreased among both mature and immature C-mGCs, with 0.2- and 0.1-fold changes, respectively ($p < 0.01$). Similarly, FSHR expression in the mature and immature groups was reduced by 0.1- and 0.02-fold, respectively, following vitrification ($p < 0.01$). The expression levels of the other genes, including BMP-15, LHR, connexin 37, survivin, and caspase 3, remained similar across the examined groups ($p > 0.05$).

Conclusion: Vitrification may compromise oocyte maturation through reduced GDF-9 and FSHR expression in C-mGCs after warming.

Keywords: Gene expression; Granulosa cells; Oocyte maturity; Oocyte quality; Vitrification

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Introduction

According to the Global Cancer Observatory, more than 19 million new cancer cases and 10 million cancer-related deaths are recorded annually [1,2]. While the incidence of cancer is age-related and increases sharply between 65 and 89 years, thousands of young people are diagnosed with cancer each year. Pharmaceutical companies have prioritized the acceleration of cancer treatment and therapy development. Currently, the relative survival rate for cancer is higher

than 80% and continues to improve [3-5]. However, sequelae are common among cancer survivors, especially young women, since many cancer treatments are gonadotoxic and can damage the ovaries [6]. For instance, chemotherapy agents induce apoptosis in primordial follicles, decrease anti-Müllerian hormone levels, and activate immature follicles, which can result in the loss of ovarian function [7]. Therefore, fertility preservation is a highly recommended approach for individuals with cancer.

Ovarian tissue vitrification is a promising option for fertility preservation in prepubertal and young women with cancer. This ultra-rapid cooling process allows hydrated cells and the extracellular milieu to solidify into a glass-like state without forming ice crystals [8,9]. A key advantage of this technique is that it eliminates the need for ovarian stimulation, thereby avoiding the risks associated with estrogen-dependent cancers. Additionally, it has been shown to preserve ovarian primordial follicles more effectively. Electron microscopy analysis has demonstrated that the majority of follicles in the ovarian cortex maintain their normal morphology and fine structure following vitrification [10,11]. Furthermore, the expression levels of apoptosis-related genes, such as Fas ligand (*FASL*), B-cell lymphoma 2 (*BCL2*), Bcl-2-associated X protein (*BAX*), tumor protein p53 (*TP53*), and caspase 3, along with the Bax:Bcl-2 ratio, have been reported to be similar between vitrified and non-vitrified ovarian tissue. The same is true for folliculogenesis-associated genes, including factor in the germline alpha (*FIGLA*), KIT ligand, growth differentiation factor 9 (*GDF-9*), and follicle-stimulating hormone receptor (*FSHR*). This indicates that vitrification aligns with the primary goal of ovarian preservation: to maintain and restore ovarian tissue function after grafting [11-13].

The ovary is a highly organized reproductive organ that contains oocytes and various somatic cells, including granulosa cells, thecal cells, and stromal cells. Among these somatic cells, cumulus-mural granulosa cells (C-mGCs) are particularly crucial [14,15]. C-mGCs mediate bidirectional communication between oocytes and the surrounding cells, which is essential for oocyte maturation. They also provide physical support and create an optimal microenvironment for appropriate oocyte development [15,16]. Furthermore, C-mGCs produce hormones that nourish the growing oocytes, making them a key determinant of oocyte maturity [17]. However, despite their fundamental role in maturation, little information is available regarding the impact of vitrification on the biological functions of C-mGCs.

Oocyte maturation is a complex process influenced by many intra- and extra-ovarian factors, which are regulated by numerous genes [18]. Among these factors, GDF-9 and bone morphogenetic protein 15 (BMP-15) are the most extensively studied and are known to regulate the growth and differentiation of granulosa and thecal cells. In turn, these cells provide the support required for normal oocyte de-

velopment [19,20]. Additionally, FSHR and luteinizing hormone receptor (LHR) have been shown to be involved in follicular maturation by inducing cumulus expansion and polar body extrusion [21]. Connexin 37, a member of the connexin family found in the gap junctions between cumulus cells and oocytes, is also reported to play a fundamental role in this process [22]. Consequently, the present study was conducted to investigate the largely unexplored impact of vitrification on the cellular function of C-mGCs by assessing the expression levels of GDF-9, BMP-15, FSHR, LHR, and connexin 37. We also measured the abundance of apoptosis-related genes (survivin and caspase 3), since follicular development and survival are known to be influenced by apoptotic events through the control of cell proliferation [23].

Methods

1. Study design and patient selection

This experimental study was conducted at Morula IVF Jakarta Clinic, Indonesia, between July 2020 and February 2022. A total of 38 patients were recruited based on the following inclusion criteria: women diagnosed with polycystic ovary syndrome (PCOS), aged 40 years or younger, and undergoing their first or subsequent *in vitro* fertilization (IVF) cycles. Participants were excluded if they were identified as poor responders or had endometriosis or adenomyosis. PCOS was diagnosed according to the Rotterdam criteria, which require the presence of two of the three following features: oligo-ovulation or chronic anovulation, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovarian morphology [24]. The study protocol received approval from the Ethical Committee of the Faculty of Medicine, Universitas Indonesia (KET-995/UN2.F1/ETIK/PPM.00.02/2019). Written informed consent was obtained from each participant.

2. Sample collection

Preliminary results from our prior research indicated that relying solely on granulosa cells to identify targeted genes was insufficient for quantification. Consequently, in the present study, cumulus cells were also harvested and analyzed (Figure 1). These cells were separated from the cumulus-oocyte complex (COC) via mechanical disruption using a 150 to 170 μm stripper pipette (CooperSurgical Fertility Solutions) following 30 seconds of immersion in hyaluronidase (Hyase; Vitrolife). Concurrently, granulosa cells were collected from the follicular fluid obtained during ovum pick-up procedures. The isolated C-mGCs from each follicle of an individual were pooled in 14-mL sterile tubes, where they were categorized by oocyte maturity status into mature and immature groups. These samples underwent Ficoll density gradient centrifugation to separate the C-mGCs from

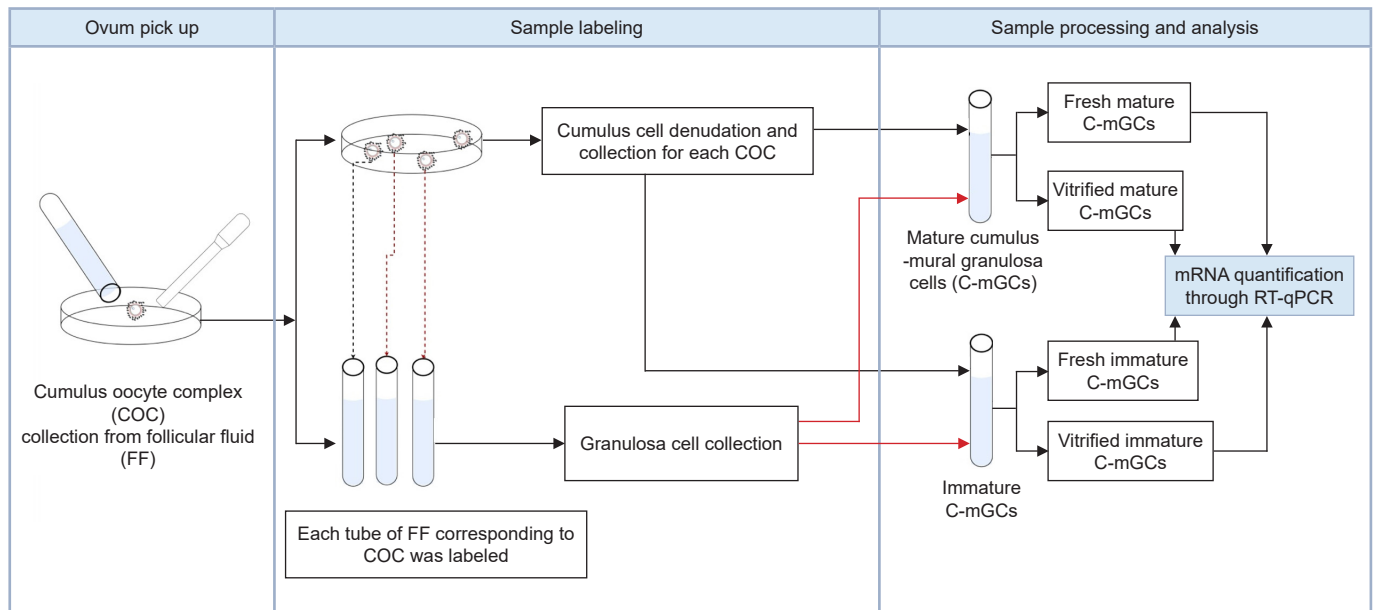


Figure 1. Schematic representation of the research workflow. C-mGC, cumulus-mural granulosa cell; RT-qPCR, real-time quantitative polymerase chain reaction.

other cell types. The process began with centrifugation at $115 \times g$ for 10 minutes, after which the supernatant was discarded and the cell pellet was resuspended in 6 mL of phosphate-buffered saline. This suspension was then centrifuged again at $115 \times g$ for 10 minutes, a step that was repeated two additional times. Following the final separation, the cell homogenate was transferred to a new 14-mL sterile tube containing 2 mL of Ficoll-Paque medium, maintaining a 1:3 ratio of Ficoll-Paque to homogenate. The mixture was then centrifuged at $155 \times g$ for 35 minutes.

After 35 minutes of centrifugation, four discontinuous density gradients were established, with the C-mGCs visible as a clear band in the second layer from the top. The layer containing the cells was carefully recovered with a pipette, resuspended in 6 mL of buffer medium, and then centrifuged again at $155 \times g$ for 10 minutes. The resulting supernatant was discarded, and the cell pellet was resuspended in 500 μ L of buffer medium and transferred to a 1.5-mL sample tube for storage. Before proceeding with further experiments, the number of C-mGCs was determined using a Neubauer counting chamber; to ensure an adequate RNA concentration, only samples with more than 100,000 C-mGCs were used. The mature and immature C-mGCs were then further assigned to fresh and vitrification groups.

3. Sample vitrification and warming

The stepped vitrification procedure utilized two solutions: equilibration solution 1 (VS1), containing 15% ethylene glycol (EG; Sig-

ma-Aldrich) supplemented with 10% human serum albumin (Vitro-life), and vitrification solution 2 (VS2), consisting of 15% EG, 15% dimethyl sulfoxide (DMSO; Sigma-Aldrich), and 20% human serum albumin. After calculating the concentration of C-mGCs, the remaining samples were centrifuged at $300 \times g$ for 1 minute, and the supernatant volume was reduced to approximately 50 μ L. The vitrification protocol was uniformly applied to all vitrification groups, beginning with the addition of 50 μ L of VS1 solution to a sample tube containing 50 μ L of resuspended samples (a 1:1 ratio). After 5 minutes in the equilibration solution, 40 μ L of VS2 solution was added to the sample and left exposed for 30 seconds. The combined sample and vitrification solution, with a total volume of 140 μ L, was then rapidly immersed in liquid nitrogen at -196°C and stored.

After a freezing period ranging from several days to 1 week, sample tubes were removed from liquid nitrogen storage and placed in a 37°C water bath for 120 seconds. The warming protocol involved a stepwise dilution using sucrose solutions (Sigma-Aldrich) at decreasing concentrations: 0.5, 0.25, and 0.125 M. The rehydration process was initiated by immersing the vitrified cells in 140 μ L of 0.5 M sucrose solution (a 1:1 ratio) for 1 minute. This was followed by centrifugation at $300 \times g$ for 1 minute, after which 100 μ L of the supernatant was removed and replaced with 180 μ L of 0.25 M sucrose solution (1:1 ratio). The cells were exposed to this solution for 1.5 minutes, then centrifuged again at $300 \times g$ for 1 minute, and 200 μ L of the supernatant was discarded. Subsequently, 160 μ L of 0.125 M sucrose solution (1:1 ratio) was added, and the cells were left in this

solution for 2.5 minutes. After a final centrifugation at 300 ×g for 1 minute following the 0.125 M sucrose exposure, the supernatant was removed. The cell pellet was then resuspended in G-MOPS solution (Vitrolife) and prepared for subsequent processing.

4. RNA extraction and complementary DNA synthesis

Total RNA was extracted using a High Pure RNA Isolation kit in accordance with the manufacturer’s protocol (Qiagen). The RNA concentration was measured at 260 nm, and 2 µg of total RNA was then reverse-transcribed into complementary DNA using ReverTra Ace quantitative polymerase chain reaction (qPCR) RT Master Mix with gDNA Remover (Toyobo) in line with the manufacturer’s instructions.

5. Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using a Techne PrimePro 48 PCR system (Cole-Parmer) with the QuantiTect SYBR Green PCR kit (Qiagen), following the manufacturer’s instructions. The primers listed in Table 1 were used to determine the relative expression of each target gene, which was normalized to the expression of human beta-actin (*ACTB*). The amplification protocol consisted of 40 cycles, with an initial denaturation at 95 °C for 5 minutes, followed by denaturation at 95 °C for 15 seconds, annealing at 57 °C for 30 minutes, and elongation at 72 °C for 30 seconds. For BMP-15, the annealing temperature was adjusted to 59 °C for 30 minutes.

Table 1. Primers designed for real-time quantitative polymerase chain reaction

Gene	Primer	Product size (bp)	Primer sequences (5'→3')
Connexin 37	Forward	200	CCATCTCCCACATCCGCTAC
	Reverse		GATCTTGCCATCTGACGCT
Caspase 3	Forward	130	TGCTATTGTGAGGCGGTTGT
	Reverse		TCCAGAGTCCATTGATTCGCT
Survivin	Forward	108	ACTTGCCCAAGTGGGTTTT
	Reverse		CAGAAAGGAAAGCGCAACCG
GDF-9	Forward	199	GGAATCCCAGTCAGGAAGCG
	Reverse		GGCCAAATGAAACCTCGTGC
BMP-15	Forward	196	GGCTCCTAGGGCATTCACTG
	Reverse		CCTCGGTTGGTCTGAGAGG
FSHR	Forward	166	TCTACCTGCACAAAGACAGT
	Reverse		AGAGAGGATCTCTGACCCCT
LHR	Forward	190	CTTGCTACCTCCCTGTCAA
	Reverse		ATGCTCCGGGCTCAATGTAT
ACTB	Forward	80	GTGTGGATTGGTGGCTCTAT
	Reverse		GACTCATCGTACTCTGCTTG

bp, base pairs; GDF-9, growth differentiation factor 9; BMP-15, bone morphogenetic protein 15; FSHR, follicle-stimulating hormone receptor; LHR, luteinizing hormone receptor; ACTB, human beta-actin.

6. Data analysis

The baseline characteristics of the participants were presented as number (percentage), median (min-max), or mean±SD for categorical and numerical variables depending on data distribution, respectively. The Kolmogorov-Smirnov test was employed to assess the normality of the distribution of participant characteristics. For bivariate analysis, the Mann-Whitney *U* test was utilized. The messenger RNA (mRNA) levels of the target genes were reported as the mean±standard error of the mean (SEM), acknowledging that the SEM does not depend on the assumption of normality [25,26]. All statistical analyses were conducted using SPSS ver. 20.0 (IBM Corp.) with a 95% confidence level.

Results

The participant and sample characteristics are summarized in Table 2. As shown, the mean age of the study participants was 32 years, with a mean body mass index of 24.6 kg/m². The mean anti-Müllerian hormone level and antral follicle count were 5.1 ng/mL and 14 follicles, respectively. Basal hormone levels, including follicle-stimulating hormone, luteinizing hormone, estradiol, and progesterone, fell within normal ranges. The mean numbers of C-mGCs in the mature and immature groups were 1,325×10³ and 445×10³ cells, respectively. Gene expression analysis revealed that GDF-9 expression in the mature and immature groups decreased significantly after vit-

Table 2. Baseline characteristics of the study population

Characteristic	Value
Female age (yr)	32±3
BMI (kg/m ²)	24.6±4.8
AMH (ng/mL)	5.1±3
AFC	14±6
Basal FSH (mIU/mL)	6±1.2
Basal LH (mIU/mL)	6 (2–24.1)
Basal estradiol (pg/mL)	30.4±12
Basal progesterone (ng/mL)	0.2±0.1
Characteristics of retrieved oocytes	
Number of retrieved oocytes	18±7
Number of mature oocytes	11 (6–32)
Number of immature oocytes	5 (3–16)
Characteristics of samples	
Number of C-mGCs	
Mature group (10 ³)	1,325 (100–17,900)
Immature group (10 ³)	445 (100–6,410)

Values are presented as mean±standard deviation or median (range). BMI, body mass index; AMH, anti-Müllerian hormone; AFC, antral follicle count; FSH, follicle-stimulating hormone; LH, luteinizing hormone; C-mGC, cumulus-mural granulosa cell.

rification, by 0.2- and 0.1-fold, respectively ($p < 0.01$) (Figure 2A). Similarly, the expression of FSHR in mature and immature C-mGCs decreased by 0.3- and 0.02-fold, respectively ($p < 0.01$). In contrast, BMP-15 expression remained relatively stable across all groups, although we noted a slight increase of 1.6- and 1.4-fold in the mature and immature groups, respectively, after warming ($p = 0.3$ and $p = 0.7$, respectively) (Figure 2A). In the fresh and vitrified groups, the expression levels of LHR ($p = 0.1$ and $p = 0.7$, respectively) (Figure 2A) and connexin 37 ($p = 0.7$ and $p = 0.2$, respectively) (Figure 2A) were not significantly altered. Similarly, the expression levels of survivin and caspase 3—markers associated with oocyte quality—were comparable after vitrification across all groups ($p = 0.6$ vs. $p = 0.8$ for survivin; and $p = 0.3$ vs. $p = 0.4$ for caspase 3) (Figure 2B).

Discussion

The present findings indicate that the expression levels of GDF-9

and FSHR in both mature and immature C-mGCs were significantly reduced after vitrification. To our knowledge, this is the first study to report the impact of vitrification on human C-mGCs. Furthermore, the results of this study were strengthened by separately analyzing mature and immature C-mGCs, thereby reducing potential interpretation bias. GDF-9 is an oocyte-secreted factor that plays a fundamental role in granulosa cell proliferation and oocyte maturation. A report on bovine COCs indicated that the expression level of GDF9 was lower in vitrified COCs than in a control group [27]. Similarly, research on sheep COCs revealed that GDF-9 expression was higher in the control group and reduced in the vitrified COCs [28]. The biological function of GDF-9 in granulosa cells is initiated via binding to the activated BMP type II receptor (BMPRII), which then recruits type I receptors. This process leads to Smad activation, mediated by activin receptor-like kinase 5 [29]. In sheep COCs, Ebrahimi et al. [28] demonstrated that the expression of BMPRII is significantly reduced after vitrification, suggesting that such treatment may alter BMPRII

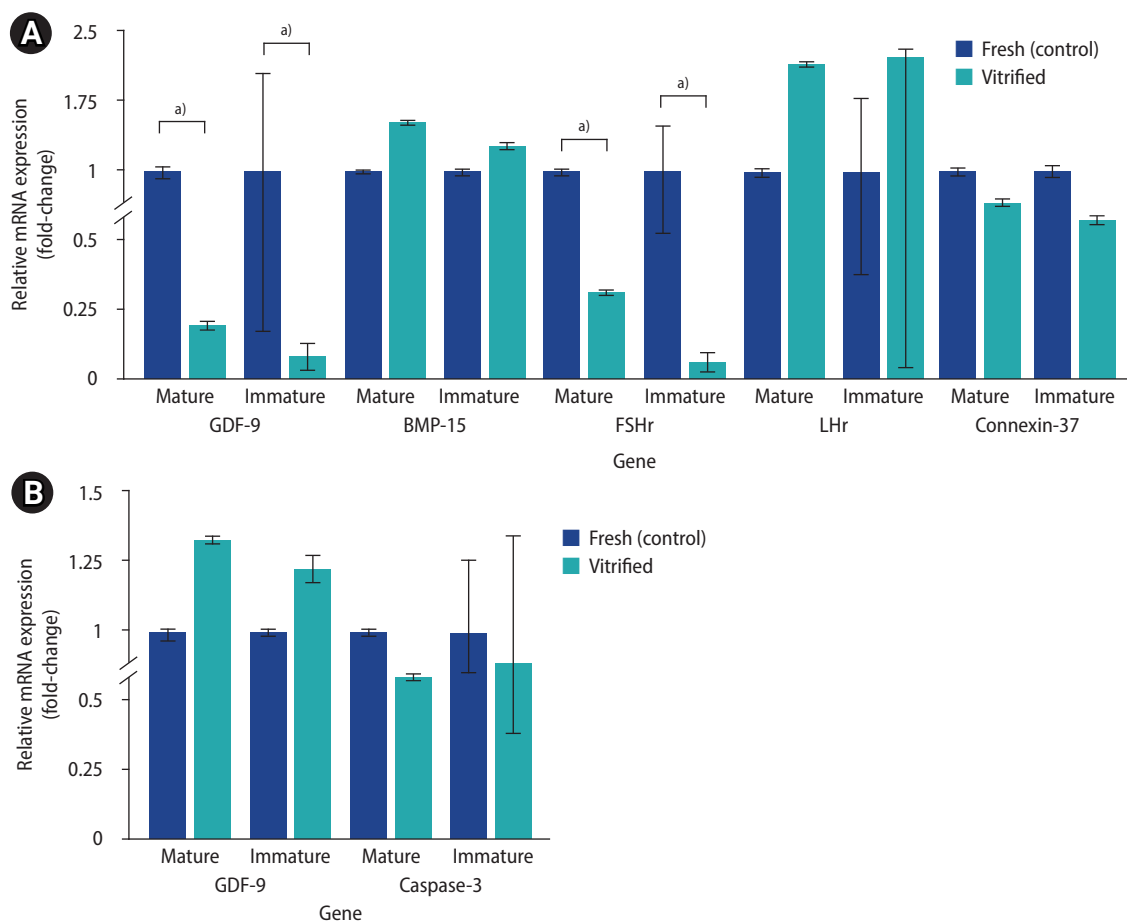


Figure 2. Relative messenger RNA (mRNA) expression of (A) genes associated with oocyte maturity (growth differentiation factor 9 [GDF-9], bone morphogenetic protein 15 [BMP-15], follicle-stimulating hormone receptor [FSHR], luteinizing hormone receptor [LHR], and connexin 37), and (B) genes associated with oocyte quality (survivin and caspase 3). The mRNA levels of the target genes were quantified relative to the amount of human beta-actin (*ACTB*) mRNA. All values are presented as the mean \pm standard error of the mean and were analyzed using the Mann-Whitney test at a 95% confidence level. ^{a)} $p < 0.01$.

expression in these cells. Meanwhile, a study on a psychosocial stress rat model showed that chronic stress during follicular development significantly downregulated GDF-9 mRNA expression, suggesting that GDF-9 is sensitive to changes in the intrafollicular microenvironment [30]. Consequently, we hypothesize that GDF-9 expression in human C-mGCs is also sensitive to environmental factors, and that vitrification may alter the function of GDF-9, presumably through the suppression of BMPRII expression.

FSHR is a region spanning 54 kb, comprised of 10 exons that encode the FSHR protein. It belongs to G protein-coupled receptor family 1 and is primarily localized on the surface of granulosa cells, playing a critical role in activating the aromatase gene and estrogen production [31,32]. However, results regarding the effect of vitrification on FSHR expression in pre- and post-vitrified bovine COCs have been inconclusive [27]. Information on how vitrification affects FSHR expression in C-mGCs remains scarce. Nevertheless, Shams Mofaraha et al. [11] observed that FSHR expression was lower in vitrified human ovarian tissues than in fresh tissues. Immunofluorescence staining has also revealed that the FSHR-positive area in ovarian tissue was significantly reduced after slow freezing and vitrification, although the levels were notably restored following xenotransplantation [33]. Considering that granulosa cells are believed to originate from the ovarian surface epithelium and thus share characteristics with ovarian tissue [34], we hypothesize that FSHR expression in human C-mGCs is thermally sensitive, ultra-rapid cooling during vitrification during vitrification altering its translational regulation, as evidenced by the reduced post-vitrified FSHR abundance observed in this study. Further research is required to clarify the relationship between vitrification and the dynamics of the gonadotropin receptor.

Regarding the quality markers of C-mGCs after warming, our study demonstrates that vitrification did not adversely affect the relative expression levels of caspase 3 and survivin. This result aligns with previous research. Wiweko et al. [35] reported that caspase-3 expression in isolated human preantral follicles was comparable between fresh and vitrified samples. In terms of survivin, to our knowledge, this is the first study to investigate its role as an antiapoptotic marker in post-vitrification granulosa cells; thus, no prior reports are available for comparison. Nevertheless, we hypothesize that the increased levels of survivin observed in this study may represent a compensatory response to counteract apoptotic events following vitrification, as indicated by the downregulation of caspase 3. These findings suggest that the vitrification and warming process effectively protected granulosa cells from potential damage caused by extreme cooling temperatures and osmotic shock. Additionally, the study demonstrated that a combination of 15% EG (v/v), 15% DMSO (v/v), and 0.5 M sucrose effectively maintained cell viability. EG and DMSO were chosen as cryoprotective agents (CPAs) due to their fre-

quent use in fertility cryopreservation. EG is preferred for its lower toxicity compared to other CPAs, while DMSO is favored for its ability to prevent ice crystal formation by increasing the intracellular solute concentration [36,37]. Supporting our findings, immunohistochemical analysis of cryopreserved avian gonads revealed a lower percentage of active caspase 3-positive cells when EG+DMSO was used compared to other CPA combinations [38].

Vitrification is a well-established technique for the cryopreservation of embryos and oocytes, which exist at a single-cell or structural level. In the early development of cryopreservation methods for more complex tissue samples than oocytes and embryos, the vitrification approach demonstrated lower efficiency. However, a recent meta-analysis [39] suggested that vitrification is superior to slow freezing, revealing reduced DNA damage in ovarian follicles and increased survival rates of stromal cells after implementing modifications to the vitrification protocol. Notably, the outcomes of vitrification can vary depending on the types of protocols [39,40] and samples, such as individual cells or tissues, the stage of ovarian follicular development, and the size of the ovarian tissue fragment [9,41]. The present study utilized C-mGCs from women diagnosed with PCOS following an IVF program. This approach was taken so that we could obtain C-mGCs from immature oocytes and minimize potential bias due to the varied clinical characteristics of the participants. Consequently, the results of this study may not be directly applicable to the vitrification of ovarian tissue, as C-mGCs are often vitrified along with ovarian tissue in the context of fertility preservation for prepubertal patients. Nevertheless, these findings serve as a valuable proxy, shedding light on the molecular impact of vitrification—a topic that has not been thoroughly investigated. This aligns with a prior study that used the human granulosa cell line human granulosa-like tumor cell line, contributing to a broader understanding of the subject [42].

This study had certain limitations, one of which was that the differentiation between mature and immature C-mGCs was determined solely by microscopic evaluation of oocyte maturation status. In the experiment, each sample of follicular fluid and its corresponding oocytes were carefully labeled. Although this labeling method is adequate for distinguishing granulosa cells from mature and immature COCs, the use of molecular markers for identification could provide a more definitive means of accurately categorizing C-mGCs. In conclusion, our study suggests that vitrification of C-mGCs may decrease the expression of GDF-9 and FSHR genes, but it does not appear to affect the expression of genes associated with oocyte quality, such as survivin and caspase 3.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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