Original Article

Granulosa Cell Conditioned Medium Enhances The Rate of Mouse Oocyte *In Vitro* Maturation and Embryo Formation

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Abstract

Objective: *In vitro* maturation (IVM) and cryopreservation of oocytes are two important parts of assisted reproductive technology (ART), but their efficacy is low. This study aimed to improve the quality of *in vitro* vitrified-warmed maturated oocytes using granulosa cell conditioned medium (GCCM).

Materials and Methods: In the experimental study, fresh/non-vitrified and vitrified-warmed mouse germinal vesicle (GV) oocytes (as F and V) were *in vitro* maturated using basal medium (BM) and also BM supplemented with 50% GCCM as treated groups (GM), and categorized as FBM, FGM, VBM and VGM groups, respectively. The rate of successful IVM (MII oocyte formation), mitochondrial membrane potential and the viability of MII oocytes were determined using inverted microscopy, JC-1 and trypan blue staining. Then, the rate of *in vitro* fertilization (IVF) and subsequent two-cell embryo formation was calculated. Finally, the expression levels of *Oct4, Sox2, Cdk-2, Gdf9*, *Integrin beta1* and *Igf2* were analyzed using real-time polymerase chain reaction (PCR) in MII oocytes and two-cell embryos.

Results: These analyses showed that GCCM significantly increased the IVM rate, oocyte meiotic resumption and mitochondrial membrane potential (P<0.05). In addition, the rate of IVF and two-cell embryo formation was significantly higher in FGM and VGM compared to FBM and VBM (P<0.05). Interestingly, GCCM significantly affected the expression of the studied genes.

Conclusion: Our findings suggest that GCCM might be useful for improving the efficiency of IVM and the subsequent IVF outcomes.

Keywords: Conditioned Medium, *In Vitro* Fertilization, *In vitro* Maturation, Vitrification

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Introduction

Assisted reproductive technology (ART) needs ovarian hyperstimulation to increase the number of oocytes. Oocyte cryopreservation is an important and promising adjunct to ART and helps to preserve women's fertility (1). Oocyte cryopreservation enhances the cumulative live-birth rate and promotes the formation of donor banks. It is also important for allowing patient synchronization and better management of medical risks, especially in women who want to freeze oocytes for medical reasons such as cancer, autoimmune diseases and medical conditions causing ovarian insufficiency (2). *In vitro* maturation (IVM) is another important part of ART, which involves the removal of oocytes from an ovary before they are fully developed, and allowing them to finish their maturation *in vitro* (3). IVM has benefits for patients with high antral follicle count (AFC) and/or polycystic ovarian syndrome (PCOS), and helps reduce the risk of ovarian hyperstimulation syndrome (OHSS) as well (4). Despite these benefits, the efficiency of *in vitro* fertilization (IVF) and the viability of embryos derived from *in vitro* maturated oocytes are lower than desired, highlighting the importance of further optimization of the IVM technique.

It is well known that both oocyte cytoplasm maturation (e.g. increasing mitochondrial potential) and nuclear maturation (e.g. progression of meiosis to metaphase II) are required for a successful fertilization and subsequent embryo development (5). The disappearance of the nuclear membrane can be considered as a distinctive sign for the resumption of meiosis morphologically, which is called germinal vesicle breakdown (GVBD) (6). During oocyte maturation *in vivo*, a series of mutual interactions play

roles between oocytes and their surrounding granulosa cells (7). Paracrine communication and gap junctions are the most active bidirectional interactions between oocytes and granulosa cells. Interestingly, granulosa cells-to-oocyte connections are mediated by paracrine signals, whereas the majority of oocyte-to-granulosa cells interaction take place via gap junctions (8, 9). For example, cAMP transferred from granulosa cells to oocytes induces CDK1 phosphorylation and MPF activation, thereby help oocyte to resumes meiosis (10). Moreover, granulosa cell conditioned medium (GCCM) contains various cytokines and growth factors such as epidermal growth factor (EGF), insulin-like growth factor (IGF) and transforming growth factor beta (TGFβ) (11, 12), which stimulate oocyte meiotic resumption via activating MPF subunits (i.e. *Cyclin b1* and *Cdk1*) (13). In addition, it was reported that bovine cumulus-oocyte complex conditioned medium significantly increases IVM of canine oocytes, particularly via nuclear maturation (14). Very recently it was showed that IL-6 concentration in single-blastocyst conditioned medium is linked to embryo quality, depending on the blastulation time (15). Moreover, conditioned media obtained from mesenchymal stem cells has been reported to induce preantral follicle growth, oocyte maturation and subsequent embryo development (16). All these studies suggest a vital role for granulosa cells and GCCM in oocyte maturation and the likelihood of IVF, embryo formation and implantation.

Based on such previous studies, we hypnotized that GCCM could improve the efficiency of IVM, IVF and subsequent embryo formation in mice. Therefore, the aim of this study was to investigate the effects of GCCM on IVM and embryo formation of fresh and vitrified-warmed mouse oocytes.

Materials and Methods

Animals and sample preparation

In the experimental study, Naval Medical Research Institute (NMRI) mice, originally derived from the Royan Institute, were housed in a conditioned environment (20-25°C), humidity (40-60%) and with 12 hours light:12 hours dark cycles. The GV-stage oocytes, preantral follicles and epididymal sperm were obtained from 6-8 week-old and 14 day-old female ovaries, and 10-12 week-old male mice, respectively. All animal experiments were accomplished under Ethics Committee of Royan Institute (IR.ACECR.ROYAN.REC.1398.102).

Granulosa cell culture and preparation of conditioned medium

Preantral follicles (100-120 μm) were isolated mechanically from 14 day-old female mice using 29 gauge insulin needles, placed into minimum essential medium alpha (α-MEM, Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Waltham, Massachusetts, USA) and 1% follicle-stimulating hormone (Merck, Darmstadt, Germany), and incubated at 37°C and 5% CO_2. After 3 days, non-adherent cells were removed through washing twice with phosphate-buffered saline (PBS) and the remaining adherent cells were cultured in the medium mentioned above until passage 3-4. When the cultured cells reached 70% confluency (approximately 28 days), GCCM was collected, filtered and stored at -20°C until subsequent using for IVM.

GV oocyte isolation and vitrification

Granulosa denuded GV-stage oocytes were obtained from the ovaries of 6-8-week-old NMRI female mice through mechanical dissection of the antral follicles.

To vitrify GV-stage oocytes, 3 droplets of 30 μl of equilibrium solution (ES) medium were placed separately in a dish, and then the collected GV oocytes were washed 3 times in those 3 ES droplets. The oocytes were kept in the third droplet for 5 minutes, and then were transferred to vitrification solution (VS) medium and washed 3 times again in VS medium. It should be noted that washing the oocytes in the second and third droplets and transferring them to cryotop were all done in one minute. Then the cryotops were transferred into the liquid nitrogen tank (-196°C). After 1 day, cryotops were taken out and oocytes were warmed through a specific warming process. To warm the oocytes, three media called W1, W2 and W3 were used. The oocytes were separated from the cryotop and nine washings were performed in three droplets of W1 for less than 1 minute. Then the oocytes were washed in W2 and W3, and for each they were left for 3 minutes in the last droplet. Then, oocytes were transferred to recovery medium containing α-MEM+FBS 10% and placed for 30 minutes in the incubator.

In vitro maturation

For IVM of vitrified-warmed GV-stage oocytes (V) and freshly isolated GV-stage oocytes (F), both types of oocytes were cultured in base medium (BM) and 50% filtered GCCM. In these experiments, α-MEM supplemented with human chorionic gonadotropin (HCG, 7.5 IU, Merck, Darmstadt, Germany), FSH (100 mIU) and FBS (10%) was considered as BM; and BM plus 50% GCCM was referred to as GM. Accordingly, IVM was accomplished in four groups: i. Fresh GV-stage oocytes cultured in the BM (FBM), ii. Fresh GV-stage oocytes cultured in GM (FGM), iii. Vitrified-warmed GV-stage oocytes cultured in the BM (VBM) and iv. Vitrified-warmed GV-stage oocytes cultured GM (VGM). The cultured oocytes in the four listed conditions were evaluated using inverted microscopy (Nikon-TS100) and first polar body extrusion was considered as the maturation criterion [also called metaphase II (MII) phase].

Trypan blue staining

In vitro matured oocytes (MII oocytes) were stained using 0.4% trypan blue (TB). Then, they were washed 3 times in PBS droplets and finally observed using inverted light microscopy. TB-negative MII oocytes and embryos were considered as viable. In contrast, cells with damaged membranes were stained and appeared with a distinctive blue color and were determined as dead cells.

Hoechst staining

Oocytes or two-cell embryos were placed in 70% paraformaldehyde. After 10-15 minutes, they were transferred into a Hoechst staining (Sigma, St Louis, MO, USA) droplet (20 µl). After 6 minutes, the oocytes or two-cell embryos were placed on slides, and observed using fluorescent microscopy (Olympus BX).

JC-1 staining: mitochondria membrane potential activity

To investigate the mitochondria membrane potential (ΔΨm) and localization of mitochondria, the *in vitro* matured oocytes was stained with JC-1 (Sigma, St Louis, MO, USA). JC-1 is a cationic dye that accumulates in energized mitochondria. Low and high concentrations of JC-1 yield green and red (to orange) fluorescence, respectively. Accordingly, A high ratio of red/green fluorescence points to an increase in ΔΨm, as well as mitochondria accumulation in the oocytes. To detect ΔΨm, the *in vitro* matured MII oocytes were stained with JC-1 as previously described (13). Briefly, the *in vitro* matured MII oocytes were stained with JC-1 for 18 hours. Then, they were treated with 0.25 µl/ml JC-1 for 30 minutes in an incubator. Next, they were washed with PBS and evaluated under a fluorescent microscope (Olympus IX71). Finally, Image J software (Java 1.8.0_172 (64-bit) was used to quantify the red/green ratio.

In vitro fertilization procedure

To study the effect of GCCM on the fertilization level and efficiency of embryo formation, the *in vitro* matured oocytes were subjected to IVF method. Briefly, the sperms were collected from the cauda epididymis of 6-8-weeks-old male NMRI mice, capacitated in T6+bovine serum albumin (BSA, 15 mg/ml) medium for 40-60 minutes in an incubator. Then, they were added to the *in vitro* matured oocytes in droplets containing T6 media supplemented with 10% BSA. After 4-6 hours, two pronuclei embryos (2PN) were isolated and washed carefully with IVF medium and transferred to the developmental droplets containing T6 medium supplemented with 10% BSA (4 mg/ml). The two-cell embryos were evaluated after 18 24-hours.

Gene expression following IVM and IVF

To analyze the expression levels of the genes involved in meiotic resumption (*Gdf9, Cdk1, Cyclin b1)* and embryo development (*Oct4, Sox2, Igf2, Cdk2, Integrin beta1)*, total RNA was extracted using PicoPure Kit (TaKaRa Bio Inc., Shiga, Japan) from the oocytes matured in the four previously mentioned conditions (FBM, FGM, VBM and VGM) and their subsequent two-cell embryos. The quality and concentration of RNA in each sample was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific). The possible genomic DNA contamination was removed by DNase I (Invitrogen) treatment for 15 minutes at room temperature. The total RNA was used for cDNA synthesis using a Takamed Kit (TaKaRa Bio Inc., Shiga, Japan), according to the manufacturer's instructions. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed using ABI Step One Plus Real-Time PCR detection system, in triplicate for each sample and each gene. The relative gene expression was calculated using $2^{-\Delta\Delta Ct}$ method. *Gapdh* was used as the internal control (housekeeping gene) to normalize the expression of genes (the primer sequences are provided in Table 1).

Table 1: The list of primers used for real-time polymerase chain reaction

Gene	Primer sequence (5´-3´)
Gdf9	F: TGAACAACTCTGCCTCTTCC
	R: ATGCTAAACACTCCGTCCTC
Cdk1	F: GACAAAGGAACAATCAAACTGG
	R: GCAAATATGGTCCCTATACTCC
Cyclin B	F: GTTGATAATCCCTCTCCAAGCC
	R: CTGCTCTTCCTCCAGTTGTC
Oct4	F: AGAACCTTCAGGAGATATGCAAA
	R: AGAACCATACTCGAACCACAT
Sox2	F: GCTGGGAGAAAGAAGAGGAG
	R: ATCTGGCGGAGAATAGTTGG
Integrin β1	F: GACACTCAGATACAACCA
	R: AGGTAGTAGAGATCAATAGGG
Igf2	F: AGTTCTGCTGCTGCTTATTG
	R: CTACCTGGCTAGTCATTGG
Cdk2	F: CCTGCTTATCAATGCAGAGGG
	R: TGCGGGTCACCATTTCAGC

Statistical analysis

All quantifications were performed using at least three independent replicates. In the present study, descriptive and inferential statistics were used to analyze the data. Descriptive statistics were used to prepare tables, draw charts and calculate statistical indices and one-way analysis of variance was used to analyze the data. Data were analyzed by GraphPad Prism 8 software (Dotmatics,

USA) and presented as proportions or mean ± standard deviation (SD). Differences with P<0.05 were considered significant.

Results

GCCM increases the rate of *in vitro* maturation

TB staining after IVM revealed that *in vitro* matured oocytes in all four groups (FBM, FGM, VBM and VGM) were viable (Fig.1A). The analysis of the first polar body release showed that its rate was significantly higher in VGM (75.8 %) compared to the groups FBM (53%) and VBM (43.5%, P<0.05, Fig.1B). In addition, the rate of the first polar body release was significantly higher in FGM (72.6%) compared to VBM (43.5%, P<0.05, Fig.1B). Moreover, determination of the number of oocytes, which degraded their nucleus and progressed to the MI and MII stages, showed that the meiotic resumption rate was significantly higher in FGM and VGM than in either one of FBM or VBM (P<0.05, Fig.1C).

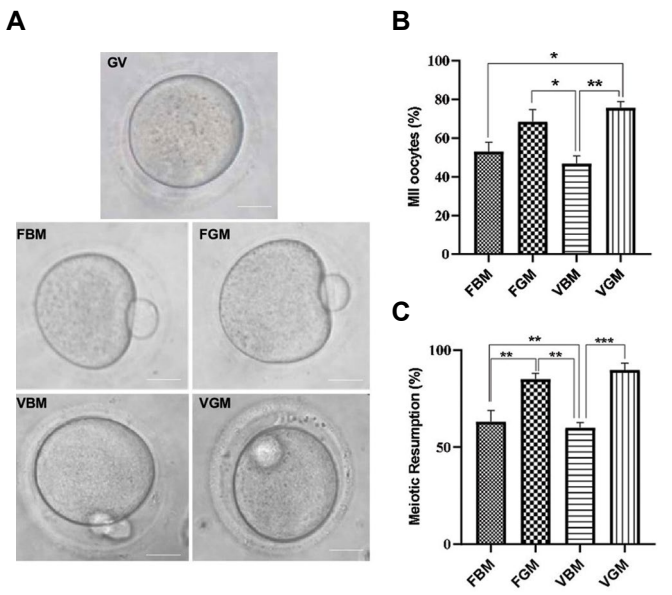

Fig.1: The effect of GCCM on IVM of mouse vitrified-warmed GV oocytes. **A.** Trypan blue staining of oocytes at GV-stage and after IVM (magnification: ×40), **B.** The rate of polar body release of *in vitro* maturated oocytes in the different experimental groups, and **C.** The rate of meiotic resumption of in vitro maturated oocytes in the experimental groups. Data are expressed as mean ± SD. *; P<0.05, **; P<0.01, ***; P<0.001, GCCM; Granulosa cell conditioned medium, IVM; *In vitro* maturation, GV; Germinal vesicle, FBM; *In vitro* matured of fresh/non-vitrified GV-stage oocytes in the basal medium, FGM; *In vitro* matured fresh/non-vitrified GV-stage oocytes in 50% GCCM, VBM; *In vitro* matured vitrified GV-stage oocytes in the basal medium, and VGM; *In vitro* matured vitrified GV-stage oocytes in 50% GCCM (scale bars: 80 µm).

GCCM increased the mitochondrial membrane potential (ΔΨm) activity

Fluorescence microscopy revealed that the JC-1 green signal, which indicates low ΔΨm, was observed in the cytoplasm of the *in vitro* matured oocytes in all experimental groups (FBM, FGM, VBM and VGM) (P<0.05, Fig.2A). Interestingly, the red signal of JC-1, which indicates high ΔΨm, was clearly observed in the cytoplasm of the matured oocytes in FGM and VGM, but it was not significant (P<0.05, Fig.2B). The quantification of red to green signals (Red/Green) showed insignificant differences among all experimental groups. However, the ratio of Red/Green was higher in VGM compared to FBM, FGM and VBM. Moreover, VBM showed the lowest ratio of Red/Green compared to the other groups (P<0.05, Fig.2B).

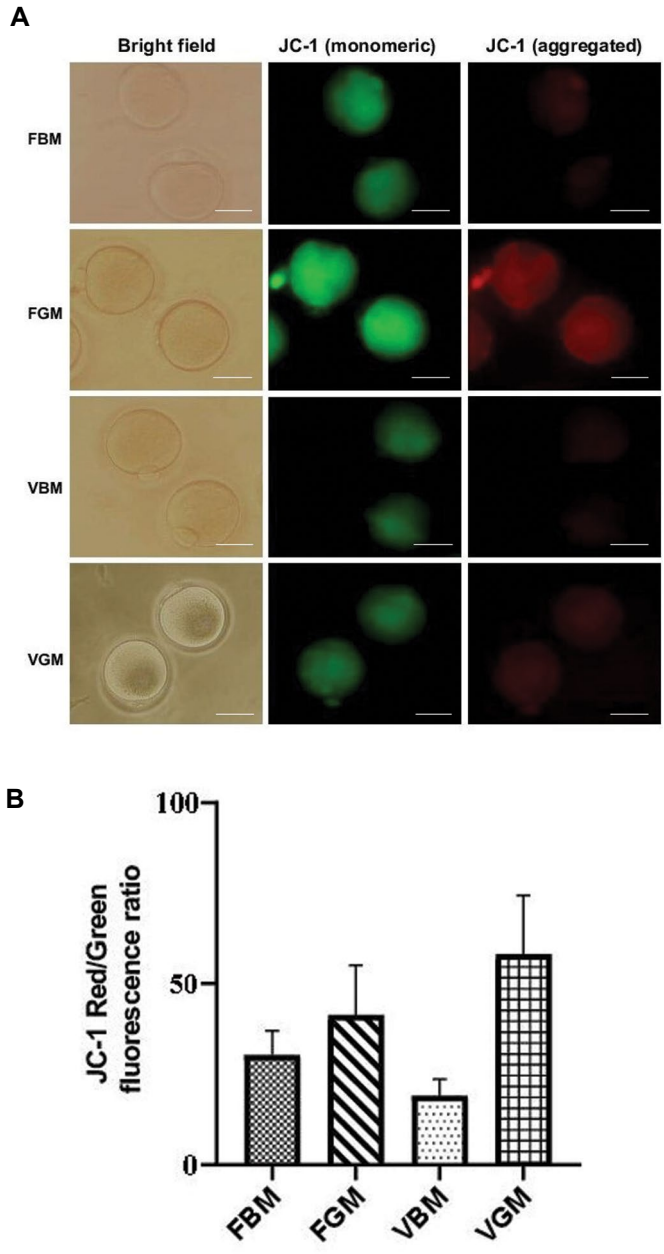

Fig.2: Effects of GCCM on mitochondrial membrane potential (ΔΨm) of *in vitro* maturated vitrified-warmed mouse GV oocytes. **A.** JC-1 staining to assay ΔΨm of *in vitro* maturated oocytes; Green indicates monomeric form of JC-1 generated at low ΔΨm and Red indicates JC-1-aggregated form generated at high ΔΨm) and **B.** The ratio of Red/Green fluorescenceof *in vitro* maturated oocytes in the experimental groups. Data are expressed as mean ± SD (P=0.9, P=0.1). GCCM; Granulosa cell conditioned medium, GV; Germinal vesicle, FBM; *In vitro* matured of fresh/non-vitrified GV-stage oocytes in the basal medium, FGM; *In vitro* matured fresh/non-vitrified GV-stage oocytes in 50% GCCM, VBM; *In vitro* matured vitrified GV-stage oocytes in the basal medium, and VGM; *In vitro* matured vitrified GV-stage oocytes in 50% GCCM (scale bars: 40 µm).

GCCM improves the rate of IVF and embryo formation

Light microscopy showed the MII oocytes, fertilized eggs and two-cell embryos derived from all experimental groups had normal morphology and structure (Fig.3A). The analysis of the second polar body release (as a sign of fertilization) showed that the rate was significantly higher in FGM (68.84 % ± 5.88) compared to FBM (52.23% ± 8.50) and in VGM (77.96% ± 9.29) compared to VBM (62.29% ± 6.70) ($P<0.05$, Fig.3B). Interestingly, the highest rate of IVF was observed in VGM. Analyzing the second polar body release over time revealed that IVF occurred significantly faster in VGM (4.5 hours) and FGM (5.2 hours) compared to VBM (6.6 hours) and FBM (6.4 hours) ($P<0.05$, Fig.3C).

Moreover, the evaluation of two-cell embryo formation revealed that FGM (71.79% ± 8.92) and VGM (76.08% ± 5.32) had higher rate of two-cell embryo formation than that in FBM (54.28% ± 11.66) and VBM (57.89% ± 6.55, $P<0.05$, Fig.3D). Similar to the results obtained from IVF analysis, the highest and lowest rates of two-cell embryo formation were observed in VGM and FBM, respectively (Fig.3B, D). The evaluation of the first cleavage over time demonstrated that FBM- and VBM-derived zygotes needed significantly longer time to complete their first cleavage (Fig.3E).

GCCM has a biphasic effect on gene expression during IVM and embryo formation

The relative expression of *Cyclin B1* (the regulatory subunit of MPF), *Cdk1 (*the catalytic subunit of MPF*)* and *Gdf9* (a growth factor involved in oocyte maturation) was analyzed in all experimental groups (FBM, FGM, VBM and VGM). It was found that *Cdk1* expression was significantly higher in VBM compared to FBM and VGM, but not to FGM ($P<0.05$, Fig.4A). In addition, relative expression of *Cyclin B1* and *Gdf9* was not statistically higher in VGM compared to the other groups (Fig.4A).

In addition, the expression levels of *Oct4* and *Sox2* (transcription factors controlling pluripotency), *Cdk2* (an important factor for cell division), *Itgb1* (integrin beta1; an adhesion molecule involved in implantation) and *Igf-2* (a key growth factor for fetal growth) were examined in two-cell embryos in all experimental groups. The highest expression levels of *Itgb1* and *Cdk2* were observed in VGM-derived embryos ($P<0.05$, Fig.4B). These two genes showed the lowest level of expression in FBM-derived embryos. The highest levels of *Oct4* and *Sox2* expression were detected in the VBM-derived embryos ($P<0.05$, Fig.4B); nonetheless, their expression patterns were different among other groups. It was also found that *Igf-2* expression was insignificantly higher in FBM-derived embryo (Fig.4B) compared to the other groups.

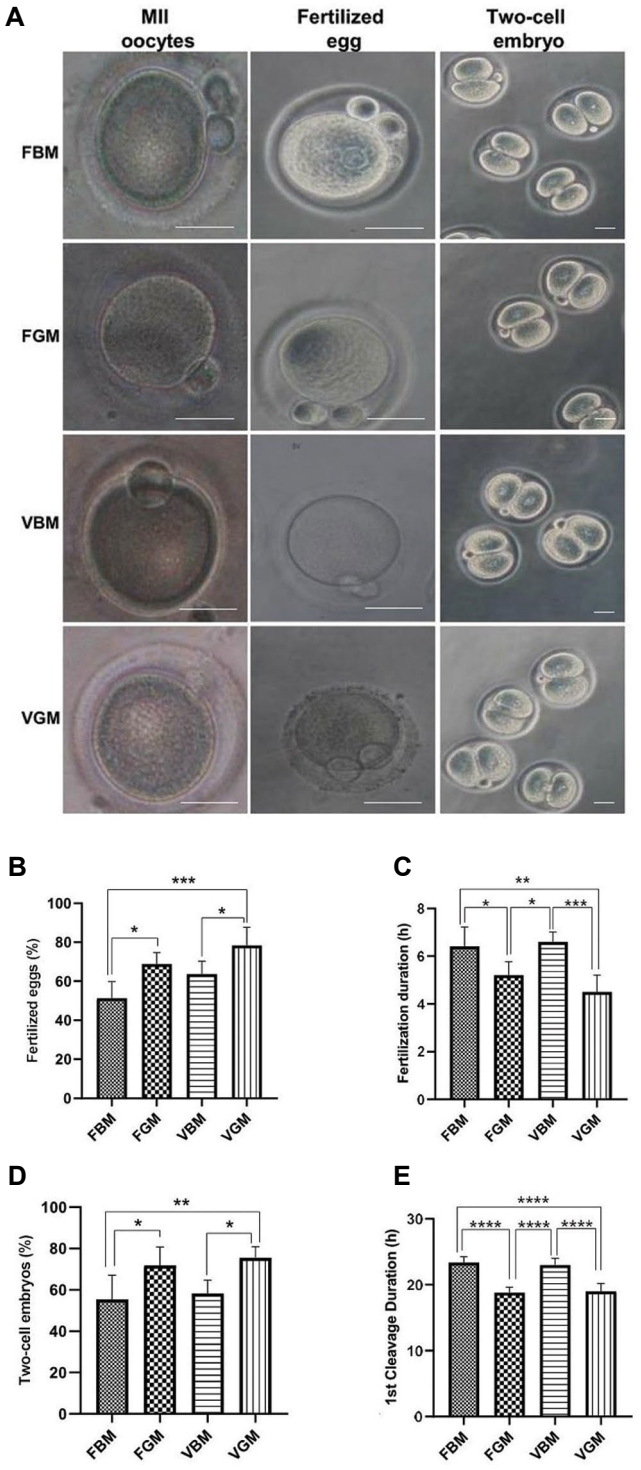

Fig.3: The effect of GCCM applied in IVM of mouse vitrified-warmed GV oocytes on subsequent IVF and two-cell embryo formation. **A.** The morphology of MII oocytes in the experimental groups before and after fertilization and at two-cell embryo stage (magnification 40X for MII oocytes and fertilized eggs, 10X for two-cell embryos). **B.** IVF rate in the experimental groups. **C.** The amount of time taken by the experimental groups to accomplish IVF. **D.** The rate of two-cell embryo formation in the experimental groups. **E.** The amount of time taken by the zygotes derived from the experimental groups to complete the first cleavage. Data are expressed as mean ± SD. *; P<0.05, **; P<0.01, ***; P<0.001, ****; P<0.0001), GCCM; Granulosa cell conditioned medium, IVM; *In vitro* maturation, GV; Germinal vesicle, IVF; *In vitro* fertilization, MII; Metaphase II, FBM; *In vitro* matured of fresh/non-vitrified GV-stage oocytes in the basal medium, FGM; *In vitro* matured fresh/non-vitrified GV-stage oocytes in 50% GCCM, VBM; *In vitro* matured vitrified GV-stage oocytes in the basal medium, and VGM; *In vitro* matured vitrified GV-stage oocytes in 50% GCCM (scale bars: 40 µm).

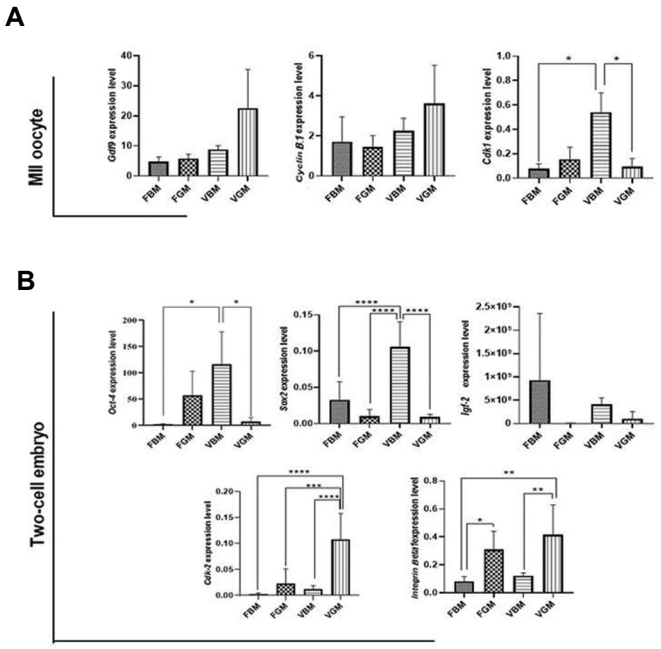

Fig.4: The effect of GCCM on gene expression of *in vitro* matured vitrified-warmed mouse GV oocytes. **A.** The relative gene expression analysis at MII oocytes and **B.** Derived two-cell embryos. Data are expressed as mean ± SD. *; P<0.05, **; P<0.01, ***; P<0.001, ****; P<0.0001, GCCM; Granulosa cell conditioned medium, GV; Germinal vesicle, MII; Metaphase II, FBM; *In vitro* matured of fresh/non-vitrified GV-stage oocytes in the basal medium, FGM; *In vitro* matured fresh/non-vitrified GV-stage oocytes in 50% GCCM, VBM; *In vitro* matured vitrified GV-stage oocytes in the basal medium, and VGM; *In vitro* matured vitrified GV-stage oocytes in 50% GCCM.

Discussion

Today, many different therapeutic strategies are available to treat or control different types of cancer. However, these strategies prolong the life expectancy in cancer patients, but they also reduce their fertility. In recent years, we and other researchers have focused on oncofertility and creating conditions outside the body to preserve and improve fertility in cancer patients. Vitrification is a promising technique for cryopreservation of oocytes and embryos collected from cancer patients. Unfortunately, vitrification increases the level of free radicals in oocytes, induces irreversible damage to the oocyte cell membrane and cytoplasm, decreases antioxidant capacity, mitochondrial membrane potential and ATP levels, and thus reduces fertilization rate and embryo development (17-19). Therefore, improvements in vitrification conditions or other cryopreservation techniques and enhancement of oocyte survival and maturation rate will ameliorate the current situation regarding the reduced fertilization rate and embryo development. In this study, we attempted to take a step towards making improvements in vitrified-warmed oocyte maturation using GCCM, which seems to have critical factors such as IL-6, IGF1, EGF and TGFβ, involved in oocyte maturation and the subsequent processes such as fertilization and embryo formation. In addition, granulosa cells secrete the factors that help the subsequent stages of fertilization like implantation and fertility maintenance. These secretions indicate different functions such as the effects of steroids and follicle stimulating hormone (FSH) on granulosa cells, and lead to estradiol production. Granulosa cells also secrete progesterone after the ovulation stage, which protects against possible pregnancy. The activity of the matrix metalloproteinase (MMP) protein, which is involved in uterine tissue regeneration, embryo development and cervix regeneration during pregnancy, is controlled by tissue inhibitors of metalloproteinases (TIMPs), which are only found *in vitro* by stimulating the granulosa cells with HCG. In addition to the known proteins that are found in the secretion of granulosa cells, proteins with unknown functions are also detected (20-22).

In the present study, the effects of GCCM on IVM of fresh/non-vitrified and vitrified GV-stage oocytes, mitochondrial membrane potential, IVF and two-cell embryo formation were evaluated.

Our results showed that GCCM enhanced the meiotic resumption and maturation in the fresh/non-vitrified GV oocytes (VGM) and vitrified-warmed GV oocytes (VGM). It has been reported that the expression levels of *Gdf9* (23) and *Cdk1* (24) decrease significantly after oocyte vitrification. We found that the relative expression levels of *Cyclinb1* and *Gdf9* were higher, but not significantly, in VGM compared to other groups, whereas *Cdk1* had a significantly higher expression level in VBM compared to other groups. Very recently, it was shown that GDF9 improves meiotic resumption in sheep oocytes (25). Importantly, vitrification-induced damage to the boundary between the nucleus and the cytoplasm results in the early activation of the *Cdk1* gene and the sudden entry of the oocyte into metaphase II (26). Interestingly, we found that *Cdk1* expression was significantly lower in VGM compared to VBM, suggesting that GCCM acts very precisely to promote oocytes to enter metaphase. Although, previous studies demonstrated that oocyte vitrification causes defects in oocyte chromosomal structure and arrangement (27, 28), it is possible that GCCM reduces the destructive effects of vitrification on chromosomal structure and arrangement. To provide a cryopreservation methodology with no destructive impact on embryo and gamete, is one of the most important challenges in the field of cryobiology. In our experiences, when dealing with cryopreservation of the reproductive samples, especially oocytes and embryos, we commonly observe this exciting result. It is accepted that when different treatments are integrated with cryopreservation in oocytes or embryos, the results are in favor of the cryopreserved samples against "fresh" or non-cryopreserved ones. There are several studies that represent a similar idea (29, 30). It is clear that there are many factors involved in this phenomenon, but only some of them have been understood by now. Rodriguez-Wallberg et al. (31), have compared the laboratory and clinical outcomes of embryo and gamete cryopreservation with non-cryopreserved samples. They concluded thatcryopreservation of embryos and gametes is a developing technology that makes frozen reproductive samples comparable to fresh ones.

Moreover, it has been reported that the conditioned media

obtained from embryonic stem cells (32), mesenchymal stem cells (16), and cumulus-oocyte-complexes (33) improve the rate of IVM in different species, suggesting that factors contained in such conditioned media regulate mechanisms involved in oocyte maturation.

We also found that the rate of IVF and embryo formation was significantly increased in FGM and VGM, compared to FBM and VBM, indicating that GCCM contains factors involved in the fertilization process and early embryo development. Our findings also suggest that GCCM eliminates the negative effects of vitrification on IVF and embryo formation and development. These results are supported by previous studies that demonstrated GCCM improves embryo formation and development through the blastocyst stage (12, 34). To better understand the effects of GCCM on fertilization and early embryo development, we analyzed the expression of *Oct4* and *Sox2* (transcription factors regulating pluripotency), *Cdk-2* (an important kinase regulating cell division), *Igf-2* (a key growth factor for fetal growth) and *Integrin beta1* (an important adhesion molecule for implantation) at the two-cell embryo stage. We found that GCCM reduces the expression of pluripotency regulating factors *Oct4* and *Sox2*. It will be interesting and important to investigate the next developmental stages in embryos derived from FGM and VGM. It has been shown that inhibition of maternal-*Oct4* expression has no effect on normal mouse embryo development (35). Moreover, a deliberate increase of *Sox2* gene expression in two-cell embryos impairs embryonic development during pre-implantation stages (36). *Integrin beta1* is another important factor that is necessary for embryo formation and development, and *Integrin beta1*-null embryos don't form inner cell mass (ICM) and die in the preimplantation phase (37). In the present study, there was a higher expression level of *Integrin beta1* in FGM and VGM, suggesting an inducing role for GCCM in *Integrin beta1* expression, which might support further development of embryos. Additional studies are needed to find cell and molecular mechanisms whereby GCCM exerted these effects on IVM, IVF, embryo formation and gene expression. However, it seems that secreted growth factors and cytokines such as IL-6, IGF1, EGF and TGFβ may be one of the more likely candidates, as it has been previously demonstrated that IVM medium supplemented with IGF-1 improves the rate of oocyte maturation, fertilization, and embryo formation (38), and IL-6 improves the quality of mouse embryos and the rate of hatching (39). In addition to above mentioned growth factors, GCCM might have different types of exosomes and miRNAs that could affect embryo quality and development, as it has been recently shown that human embryo culture media and blastocoel fluid have different soluble RNAs that might be used as biomarkers for embryo quality and viability (40).

Conclusion

Taken together, the results presented here indicate that supplementation of GCCM in IVM improve the meiotic resumption in both fresh and vitrified-warmed GV oocytes, and increase the oocyte maturation, fertilization and 2-cell embryo formation rates in GV oocytes derived from vitrification. Overall, it can be concluded that GCCM improves the vitrification outcomes in mouse GV oocytes.

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Authors' Contribution

R.F., M.T.; Contributed to the design and implementation of the research. Z.B., N.H.; Carried out the experiment, collected, analyzed data, and wrote the manuscript. M.T.; Took the lead in writing the manuscript. E.Z.; Contributed to set up IVM and IVF tests. R.F., M.T., D.G.M.; Provided critical feedback and helped to finalize the manuscript structure. All authors read and approved the final manuscript.

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