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Improvement of Mouse Preantral Follicle Survival and Development following Co-Culture with Ovarian Parenchyma Cell Suspension

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Abstract

Background: The parallel and continued improvements in both infertility treatment and the management of malignancy cases have brought to the forefront the potential for fertility preservation. Using ovarian follicular resources can effectively improve reproductive capacity and prevent infertility. The primary aim of this research was to try to generate an appropriate *in vivo* environment for the growth of the mouse follicles. Hence, the possible effects of the ovarian parenchyma cell suspension were explored on the growth and maturation of preantral follicles *in vitro*.

Materials and Methods: In this experimental study, ovarian parenchymal cells were mechanically dissociated from preantral follicles of 12-14 days-old NMRI mice and then divided into 5 experimental groups (G1: Control, G2: Fresh follicle with fresh parenchyma cell suspension, G3: Vitrified-warmed follicle with fresh parenchyma cell suspension, G4: Fresh follicle with frozen-thawed parenchyma cell suspension, and G5: Vitrified-warmed follicle with frozen-thawed parenchyma cell suspension). The diameter of the follicles and immature oocytes, viability, antrum formation, resumption of meiosis, *in vitro* fertilization (IVF), and *Gdf9*, *Bmp6*, and *Bmp15* gene expression were examined on different periods.

Results: The diameter of the follicles and the oocytes on days 4 and 8, as well as the survival rate of the follicles up to day 12, were significantly higher in G2 and G4 compared to the Ctrl group (G1: 73.66%, G2:87.99%, G3: 82.70%, G4: 94.37%, and G5: 78.59%). Expression of growth marker genes for G3, and G5 groups was significantly higher than other groups, which indicated the protective effects of parenchyma cell suspension on follicles damaged by vitrification solutions.

Conclusion: The growth, survival, and maturation of preantral follicles could be enhanced by co-culturing them with ovarian parenchyma cells. Further studies are needed to optimize the conditions for a successful parenchyma cell suspension-induced *in vitro* maturation (IVM) to occur in infertility clinics.

Keywords: Co-Culture, Fertility Preservation, *In vitro* Culture, Ovarian Tissue, Preantral Follicle

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Introduction

Female infertility may arise as a consequence of aging, various ovulation blocking diseases such as pelvic inflammatory disease, endometriosis, fibroids, and exposure to various treatments for oncological conditions. Hence, fertility preservation in women provides a realistic chance for potential future pregnancies. Improvements in both infertility and cancer treatments, allow for fertility preservation in cancer patients being treated with radio-chemo therapeutics. Therefore, there is an increasing demand to prevent or decrease the loss of fertility in young female cancer patients who are undergoing fertility-destroying, chemo or radiation therapy. Ovarian hyperstimulation and multiple oocyte collection, as per a full *in vitro* fertilization (IVF) cycle, offers cancer patients a chance of preserving their fertility. It's also well known that the number of ovarian reserves is reduced during life,

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so using ovarian follicular resources can effectively improve reproductive efficacy for a longer period of time and to prevent infertility (1-3).

In vitro ovarian follicle culture has been used to investigate ovarian pathology and to determine how ovaries or oocytes are affected by exposure to various toxic chemicals, with the purpose of obtaining fertilizable oocytes from primordial ovarian follicles. Numerous studies have shown that malignancy, chemotherapy, or radiation therapy in women, are likely causes of harm in their reproductive tissues and are often associated with premature ovarian failure/insufficiency (POF/POI) and infertility (4, 5). Due to the failure of the usual culture systems to satisfy the metabolic requirements of the growing follicles and the lack of paracrine connection with the surrounding stromal tissue, prolonged follicular cultures frequently result in atresia. The complex procedure of folliculogenesis is one of the primary reasons that small follicle development is not sustained or promoted under normal culture conditions (3); besides the endocrine, autocrine and paracrine glands, and complex cell-cell interactions facilitate this process. It is well known that folliculogenesis plays an essential role in normal ovarian functioning, because it allows for ovulation and the synthesis of the vital sex hormones including estradiol and progesterone. The mammalian ovary is composed of ovarian follicles, where each follicle has a single oocyte surrounded by granulosa cells, enclosed within a basement membrane. The opportunity to track and estimate the growth rate and follicle size during the culture phase is provided by the culture of intact ovarian follicles. Therefore, in vitro culture of follicles is considered as an excellent option to evaluate different aspects of follicular growth and development (2, 6). Moreover, it may also be regarded as an approach to examine fertility maintenance (3, 7).

Multiple efforts have been completed to create a healthy oocyte from individual follicles *in vitro*, leading to significant achievements and advances in preantral follicle culture from small mammals (8-10). Previous studies have shown that preantral follicles have been cultured with various cells, including mesenchymal stem cells, adipose-derived stem cells, and granulosa and theca cells (11-16). The present study uses ovarian parenchyma tissue in a two-dimensional culture medium to evaluate the survival and growth rates of preantral follicles. Embryonic stem cells with asymmetric divisions form the parenchymal tissue of the ovary, forming the ovarian cortex that comprises fibroblasts, lutein cells, granulosa and theca cells, and collagen connective tissue (17, 18). This work describes the co-culture of whole ovaries containing preantral follicles, with the ovarian parenchyma cell suspension, a method which can support the development of follicles, after which their oocytes may be able to undergo fertilization *in vitro*. On The other hand, it is well known that ovarian folliculogenesis and differentiation depend on coordinated interactions and expression of multiple growth marker genes. Thus, the maturity levels and development of cultured follicles were evaluated through expression of known growth marker genes such as the growth differentiation factor 9 (*Gdf9*), the bone morphogenetic protein 15 (*Bmp15*), and the bone morphogenetic protein 6 (*Bmp6*) (19, 20). This major objective of this study was to simulate an *in vivo* environment in the lab, and in order to do so, the effects of ovarian parenchyma cell suspension on the survival and development of preantral follicles were assessed.

Materials and Methods

Animals

This experimental study was approved by the Ethics Committee of Royan Institute (IR.ACECR.ROYAN.REC.1399.035). The NMRI mice used in this study were obtained from the laboratory animal science unit of Royan Research Institute and kept in suitable conditions with 12 hours of light and 12 hours of darkness at 20-25°C temperature and free access to food and water. At the initial steps of the study, female NMRI mice were sacrificed by cervical dislocation and their ovaries were removed, placed in modified minimum essential medium (α-MEM, 11900073, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, 10270106, Gibco, USA). Parenchyma cell suspension was prepared from the ovaries of adult 6 to 8 weeks (42-56 days) old female mice, while preantral follicles were isolated from ovaries of 2 weeks (12-14 days) old female mice.

Follicle isolation

First, 12-14 day-old mouse ovaries were removed and transferred into 100 µl droplets of α-MEM culture medium containing 10% FBS. 120 to 140 µm follicles were mechanically removed by sterile fine 29G syringe needle under stereo microscope. The isolated follicles were collected in a 30 µl droplet of α-MEM culture medium and individually transferred to experimental groups (21).

Vitrification and thawing of the follicles

To freeze the follicles, equilibrium and vitrified solutions were used. Briefly, the follicles were first placed in equilibrium solution at room temperature for 3 minutes. For preparation of the equilibrium solution, α-MEM culture medium was supplemented with 7.5% ethylene glycol (EG, 293237, Sigma-Aldrich, Germany), 7.5% dimethyl sulfoxide (DMSO, D2650, Sigma, USA) and 20% FBS.

The follicles were then vitrified at room temperature for one minute. To prepare the vitrification solution, 15% EG, 15% DMSO, 0.5 M sucrose (S7903, Sigma, USA), and 20% FBS were added to α-MEM medium. Afterwards, the follicles were removed with an appropriate pipette and loaded on the thin end of the Cryotop strip, which was immediately submerged into liquid nitrogen vertically with rapid horizontal movements to obtain the maximum cooling rate.

Thawing the follicles was performed by removing the samples from liquid nitrogen, and immersing them directly into the primary melt solution containing α-MEM medium supplemented with 20% FBS and 1 M sucrose. The samples were rinsed for 1 minute in the melt solution at 37°C. Subsequently, follicles were washed in the second thaw solution, which was α-MEM medium containing 20% FBS and 0.5 M sucrose. The second wash was done at room temperature for 3 minutes (14).

Isolation of ovarian parenchyma cell suspension

Adult (6-8 weeks old) female mice were sacrificed by cervical dislocation, and their ovaries were removed by creating a transverse abdominal incision. The ovaries were placed in 200 µl droplets of α-MEM culture medium containing 10% FBS, and cut into very small pieces using a 29G syringe needle under a stereomicroscope. The minced tissues were pipetted gently 5 to 10 times using 1000-µl, and 100-µl micropipettes and 2-ml syringes with 29G needles. After the ovarian tissues became uniformly shredded, they were centrifuged at 261 g for 5 minutes. The viable cells in the sediment were counted under an inverted microscope, and the samples with a specified number of 5000-10000 cells per drop were placed into the culture medium.

Freezing and thawing of ovarian parenchyma cell suspension

Parenchyma cell suspension was centrifugated at 261 g for 5 minute to separate the cells from the medium. The supernatant was removed, the remaining cell pallet was resuspended into a mixture of 90% FBS and 10% DMSO. The cell mixture was then transferred to cryovials and stored at -20°C for 1 hour to give the cell sample the opportunity to absorb DMSO. The long-term freezing process was initiated by transferring the cryovial to the -80°C freezer for 5 to 12 hours, followed by a final storage in a liquid nitrogen tank.

For thawing the cell suspension, the frozen parenchyma cells were removed from the nitrogen tank and left in bain-marie set to 37°C. The α-MEM medium was slowly added up to as much as 2 to 4 times the volume of the base culture medium solution to stabilize the suspension, and then it was centrifuged at 261 g for 5 minutes to completely separate the DMSO residues from the cell suspension (22).

Experimental groups

The preantral follicles were randomly divided into five experimental groups and cultured for 12 days:

G1: Untreated follicles that were cultured as controls (Ctrl).

G2: Fresh follicles co-cultured with fresh ovarian parenchyma cell suspension (FF+FPCS).

G3: Vitrified-warmed follicles co-cultured with fresh ovarian parenchyma cell suspension (VF+FPCS).

G4: Fresh follicles co-cultured with frozen-thawed ovarian parenchyma cell suspension (FF+FTPCS).

G5: Vitrified-warmed follicles co-cultured with frozen-thawed ovarian parenchyma cell suspension (VF+FTPCS).

In vitro culture of follicles

As much as 15 µl of fresh and frozen-thawed ovarian parenchyma cell suspension containing 5000-10000 cells were added to 45-µl droplets of α-MEM culture medium and placed in a petri dish covered with mineral oil and incubated at 37°C and 5% CO_2 for 3 to 5 hours. Then, fresh and vitrified-warmed follicles in 60-µl droplets of α-MEM culture medium supplemented with 10% FBS, 10 Iu/ml of human follicle-stimulating hormone (FSH, Gonal-f, Merck, Germany), and 1% insulin-transferrin-selenium (ITS 100X, 41400045, Gibco, USA) for the Ctrl group (23, 24), and the cell suspension with culture medium mentioned for the following groups were cultured for 12 days in an incubator at 37°C, 96% humidity and 5% CO_2. To feed the cells half of the culture medium was replaced with an equal volume of fresh medium every other day. Through the culture period, the progress of follicle growth, diameter, and morphological changes were evaluated.

Measuring the diameter of follicles and oocytes

Using an inverted microscope (Nikon, ECLIPSE TS100), the follicles were imaged after 3 hours of culture and also on days 4, 8, and 12 of the culture periods. Additionally, the diameter of the follicles and their oocytes were measured using the Image-J application during the aforementioned culture periods.

Assessment of morphological changes

The survival rate of the follicles was evaluated on culture days 0, 4, 8, and 12, and the dark follicles without oocytes were considered as degenerated follicles. Formation of clear spaces between the follicles' granulosa cells was considered the antrum cavity during the culturing period. Then, to evaluate the growth of the follicles and the healthy oocytes, the number of antral follicles, cumulus-oocyte complex (COC) formation, germinal vesicle breakdown (GVBD), and metaphase II (MII) were examined. Developed and enlarged antrum cavity was observed on day 12 of the culture period, at which time the culture medium was replaced with fresh medium supplemented with 1.5 Iu/ml human chorionic gonadotropin (hCG), for induction of ovulation (Pregnyl, Organon, USA). Subsequently, the ovulation rate was evaluated under an inverted microscope after 18 to 24 hours. Accordingly, the released oocytes were classified into MI and MII based on their detected morphologies, where MI represented when the germinal nucleus of the vesicle disappeared, and MII indicated when the first polar body (1PN) was developed (24).

In vitro fertilization

To evaluate the development competence of the oocytes, IVF was conducted on the developed oocytes. Thus, sperms collected from the 12-week-old male NMRI mice, were used for fertilization of the oocytes. First, the testis epididymal tail was removed and placed in a droplet containing T6 culture medium and 15% bovine serum albumin (BSA, a.3311, Sigma-Aldrich, Germany). Afterwards, drops containing sperm, were incubated in 5% CO_2 at 37°C for 1-2 hours. To conduct development, the fertilization process 6 or 8 MII oocytes were transferred to each fertilization drop, followed by the addition of 10-12 sperms for each oocyte and incubated for 4 hours. Oocytes that released their 2PN were successfully fertilized so they were transferred to the droplet containing 4% BSA+T6 to complete the development process (25).

Evaluation of expressed growth marker genes

The maturity levels and development of cultured follicles were evaluated through expression of known growth factor marker genes *Gdf9*, *Bmp15*, and *Bmp6*. Real-time polymerase ciain reaction (PCR) was employed to estimate the expression level of each gene in the cultured follicles. Initially, extraction of RNA was performed from the cultured follicles (20 healthy follicles/replicate for each time point) using RNeasy Micro Kit (Qiagen, Germany) according to the manufacturer's protocol. Three replicates were performed for each group to estimate the total RNA extraction. Total RNA from the cultured follicles was reverse transcribed into first-strand cDNA with cDNA Synthesis Kit (SMO Bio primer, Taiwan) and random hexamers based on the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as an endogenous control, and primers were designed using PrimerExpress (Applied Biosystems, USA). Finally, to reach the final reaction volume (10000 µl), each PCR reaction consisted of 2500 µl Power SYBR ampliqon (realQ plus 2x master mix green), 2000 µl of cDNA, 1000 µl sense primer, 1000 µl antisense primer with a concentration of 5 pmol and 3500 µl dH2O. Analysis of expression level was performed by an ABI StepOne plus thermocycler and StepOne Software version 2.3 with the following protocol: 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Each reaction was run in duplicates. Relative gene expression between all follicle groups was calculated by the ΔΔCT comparison method, in which -2ΔΔCT values were estimated for each expressed gene on both 3 hours and 4 days culture periods for the five experimental groups (13).

Statistical analysis

The results were presented as mean ± SEM, and a $P \leq 0.05$ was considered to be statistically significant. At least three separate repeats were performed for each experiment, and average values were used for data analysis. All data were analyzed by GraphPad Prism (Insightful Science, USA) and SPSS software version 26 (IBM, USA). The Kolmogorov-Smirnov test was used to determine the normality of all tests. ANOVA test was used for statistical assessment of the follicle and oocyte diameters and evaluation of oocyte and embryo development after IVF. The Kruskal-Wallis test was used to assess statistically significant differences in survival, antrum formation, and related gene expression results.

Results

In the present study, follicle growth rate and morphology were evaluated in 5 distinct experimental groups to identify the viable cells in each condition. We also assessed survival rate, percentage of antrum formation, resumption of meiosis, as well as COC compared to antral follicle for further growth analysis. The rate of oocyte maturation was examined in 7 replications, but oocyte fertilization and embryo development were performed in 4 replications.

For culture analysis, any naturally occurring, light-colored follicles containing healthy oocytes with clear zona pellucida were considered as healthy, whereas any changes, such as spontaneous release of an oocyte, termination of growth, or the darkening of the follicles, placed them in the category of degenerative follicles.

Evaluation of follicle and oocyte diameter during culture

The diameter of the follicles and oocytes were examined on days 0, 4, and 8 of culture. The results obtained for each of the experimental groups indicate a robust growth of fresh follicles in G2 and G4 groups, cultured from fresh and frozen-thawed ovarian parenchymal cell suspension, respectively, compared to the control (G1) group. Also, the growth rate in these groups was significantly different in comparison to the other two groups on day 8 of culture (Ctrl: 266.33 ± 20.59, G2: 373.49 ± 27.007, G3: 333.12 ± 15.63, G4: 409.55 ±16.45, G5: 356.87 ± 18.58 - P: G2: 0.0160- G4:0.020) (Fig.1A). Analysis of the oocyte diameters from follicles on different days among the groups, demonstrated that the G2 and G4 groups were significantly different from the control and G3 groups (Fig.1B) (day 8: Ctrl: 67.15 ± 1.18, G2: 73.02 ± 1.21, G3: 68.61 ± 1.05, G4: 72.75 ± 1.93, G5: 70.62 ± 1.89) (P: G2-Ctrl: 0.0004- G4-Ctrl:0.0006- G2-G3: 0.0002- G4-G3:0.0001). On the other hand, images taken on different days of culture showed that groups treated with fresh and frozen ovarian parenchyma cell suspension had higher growth rates (Fig.2).

Survival rate, formation of antral follicles and cumulus-oocyte complex

A noteworthy issue in assessing the survival rate of the follicles up to day 12 among the five experimental groups was that in the G2 and G4 groups, we found

significant differences between fresh preantral follicles co-cultured with fresh and frozen-thawed ovarian parenchyma cell suspension, respectively. [(survival rate %) Ctrl: 73.66 ± 4.56, G2:87.99 ± 3.39, G3: 82.70 ± 3.34, G4: 94.37 ± 2.80, and G5:78.59 ± 5.55] [P: (G2:0.006) (G4:0.0004)] (Table 1, Fig.3A). Although the experimental groups treated with fresh and frozen-thawed ovarian parenchyma cell suspension were better in the evaluation of antrum cavity formation, no significant differences were found among of the groups (P>0.05, Table 1, Fig.3B). The rate of COC formation in the experimental groups showed that fresh preantral follicles treated with fresh and frozen-thawed ovarian parenchyma cell suspension had a successful culture in COC formation compared to the other groups, especially the control and G3 groups (Ctrl: 81.66 ± 4.96, G2: 94.28 ± 2.97, G3: 80.95 ± 5.36, G4: 97.95 ± 2.04, and G5: 89.38 ± 5.80) [P: (G2:0.004) (G4:0.005)] (Fig.3C).

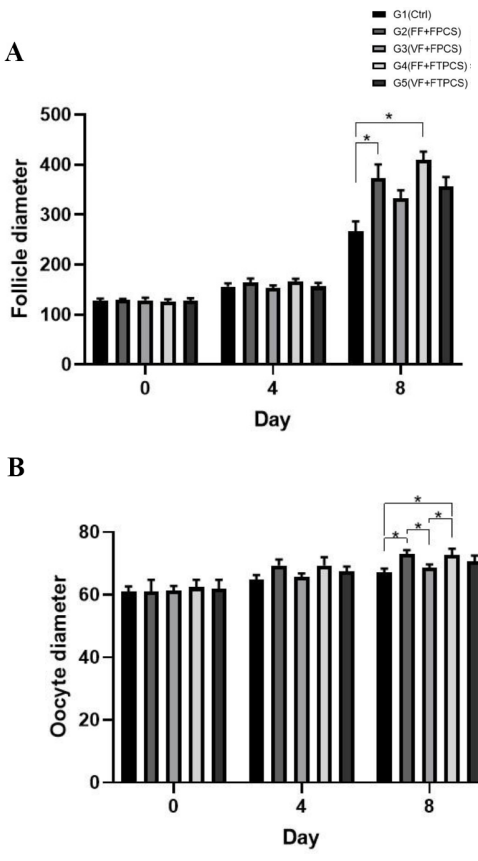

Fig.1: The effect of ovarian parenchyma cell suspension on follicle and oocyte diameter. **A.** Preantral follicle diameter increased notably in the presence of ovarian parenchyma cell suspension, and this increase was significantly higher when co-cultured with fresh follicles, especially at the end of the growth phase groups (P: G2: 0.016- G4:0.020). **B.** The diameter of the oocytes in the preantral follicles was examined on different days of culture and co-culture of fresh follicles with fresh and frozen-thawed ovarian parenchyma cell suspension was significantly higher than other groups (P: G2-Ctrl: 0.0004- G4-Ctrl:0.0006- G2-G3: 0.0002- G4-G3:0.0001. The Kolmogorov-Smirnov and ANOVA tests were used for statistical assessment, G1; Control, G2; Follicle with fresh parenchyma cell suspension, G3; Vitrified-warmed follicle with fresh parenchyma cell suspension, G4; Follicle with frozen-thawed parenchyma cell suspension, G5; Vitrified-warmed follicle with frozen-thawed parenchyma cell suspension, and *; Significant difference (P≤0.05).

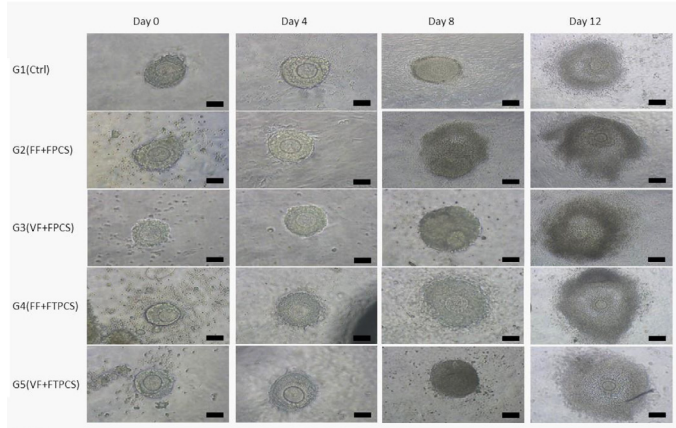

Fig.2: Images of preantral follicles on different days of culture (scale bar: day 0 and 4: 10 µm , day 8 and 12: 16 µm). G1; Control, G2; Follicle with fresh parenchyma cell suspension, G3; Vitrified-warmed follicle with fresh parenchyma cell suspension, G4; Follicle with frozen-thawed parenchyma cell suspension, and G5; Vitrified-warmed follicle with frozen-thawed parenchyma cell suspension.

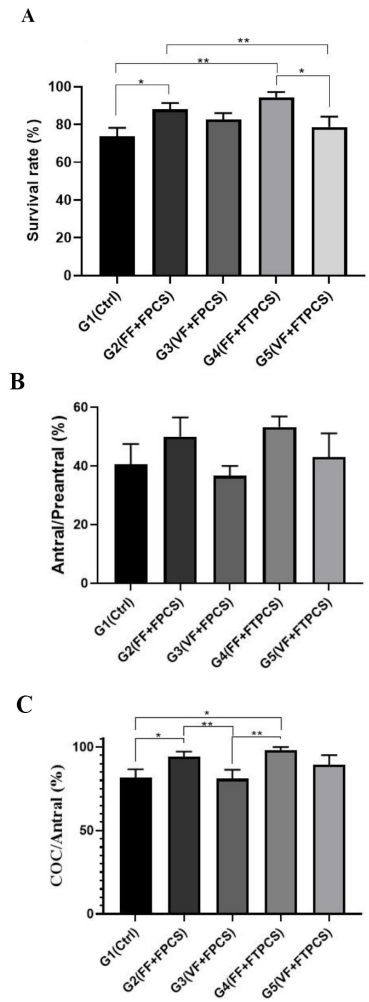

Fig.3: Graphs comparing the growth and survival rates of the follicles in 12 days of culture. A. Percentage of survival rate during in vitro culture. Data are expressed as mean ± SEM [P: (G2:0.006) (G4:0.0004)]. B. Percentage of antral/pre-antral follicles during in vitro culture. C. Percentage of antral follicles/COC during in vitro culture [P: (G2:0.004) (G4:0.005)]. The Kruskal-Wallis and Kolmogorov-Smirnov tests were used for statistical assessment. G1; Control, G2; Follicle with fresh parenchyma cell suspension, G3; Vitrified-warmed follicle with fresh parenchyma cell suspension, G4; Follicle with frozen-thawed parenchyma cell suspension, G5; Vitrified-warmed follicle with frozen-thawed parenchyma cell suspension, COC; Cumulus-oocyte complex, *, and **; Significant difference only compared to the G2 and G4 groups (P<0.05).

Table 1: The development of cultured mouse preantral follicles in five different experimental groups after 12 days of IVC in 7 replications (group A) and oocyte maturation and evaluation of embryo development after *in vitro* fertilization in 4 replications (group B)

Group A	Survival rate n (%)		Antrum formation n (%)	Developmental stage of oocyte n (%)	
				COC	GVBD+MII
G1(Ctrl)	70/95 (73.66)		39//95 (40.52)	31/39 (81.66)	24/31 (77.77)
G2(FF+FPCS)	89/100 (87.99)		50/100 (50)	47/50 (94.28)	40/47 (86.44)
G3(VF+FPCS)	61/74 (82.70)		27/74 (36.63)	22/27 (80.95)	15/22 (64.99)
G4(FF+FTPCS)	87/92 (94.37)		49/92 (53.29)	48/49 (97.95)	40/48 (83.69)
G5(VF+FTPCS)	52/68 (78.59)		28/68 (43.10)	24/28 (89.38)	17/24 (84.52)
Group B	**COCs (n)**	**MII (n)**	**2PN n (%)**	**2-Cell n (%)**	**4-Cell n (%)**
G1(Ctrl)	16	9/16	7/9 (79.16)	3/9 (58.33)	1/9 (8.33)
G2(FF+FPCS)	20	14/20	12/14 (81.25)	7/14 (48.33)	1/14 (5)
G3(VF+FPCS)	12	5/12	3/5 (66.66)	2/5 (66.66)	0
G4(FF+FTPCS)	23	14/23	12/14 (82.5)	7/14 (66.66)	3/14 (20.83)
G5(VF+FTPCS)	10	6/10	4/6 (83.33)	3/6 (55.55)	1/6 (11.11)

The Kolmogorov-Smirnov and Kruskal-Wallis tests were used for statistical assessment for group A. The Kolmogorov-Smirnov and ANOVA tests were used for statistical assessment for group B (mean ± SEM, P<0.05). G1; Control, G2; Follicle with fresh parenchyma cell suspension, G3; Vitrified-warmed follicle with fresh parenchyma cell suspension, G4; Follicle with frozen-thawed parenchyma cell suspension, G5; Vitrified-warmed follicle with frozen-thawed parenchyma cell suspension, COC; Cumulus-oocyte complex, GVBD; Germinal vesicle breakdown, MII; Metaphase II, and 2PN; Second polar body.

In vitro maturation

The present study we evaluated follicle maturation and fertilization up to the 4-cell stage. The G2 and G4 groups with cultured fresh follicles plus fresh and frozen-thawed ovarian parenchyma cell suspension showed a better maturation rate (Ctrl: 77.77 ± 3.92, G2: 86.44 ± 5.23, G3: 64.92 ± 8.45, G4: 83.69 ± 5.26, and G5: 84.52 ± 7.80) than the control and other groups. However, significant difference was not observed in maturation process (Table 1, Fig.4).

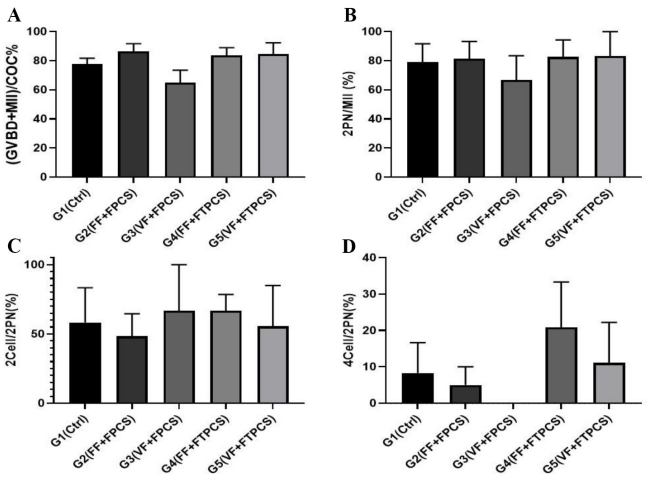

Fig.4: Evaluation of oocyte and embryo development after *in vitro* fertilization. Percentage of **A.** (GVBD+MII)/COC. **B.** 2PN/MII. **C.** 2Cell/2PN. **D.** 4Cell/2PN. No significant difference was observed in the above experiments (P<0.05). The Kolmogorov-Smirnov and One-way ANOVA tests were used for statistical assessment. G1; Control, G2; Follicle with fresh parenchyma cell suspension, G3; Vitrified-warmed follicle with fresh parenchyma cell suspension, G4; Follicle with frozen-thawed parenchyma cell suspension, and G5; Vitrified-warmed follicle with frozen-thawed parenchyma cell suspension, GVBD; Germinal vesicle breakdown, MII; Metaphase II, COC; Cumulus-oocyte complex, and 2PN; Second polar body.

Embryo development

Four experimental replications were done in the examination of 2PN, 2-cell, and 4-cell. Analysis of the 2PN formation rate (Ctrl: 79.16 ± 12.50, G2: 81.25 ± 11.96, G3: 66.66 ± 16.66, G4: 82.5 ± 11.81 and, G5: 83.33 ± 16.66) did not reach statistically significant difference. Also, the results for 2-cell and 4-cell did not reach statistically significant difference, despite the fact that Table 1 indicates that there are higher percentages of 4-cells in the G4 and G5 groups compared to the Ctrl group (P>0.05, Table 1 , Fig.4).

Expression pattern of *Gdf9*, *Bmp6*, *Bmp15* genes

The estimation of expression of maturity genes in the first 3 hours and on day 4 of culture in the 5 experimental groups showed that the highest expression in cultured groups occurred in the first few hours, but the level of expression was declined 4 days into the culture period.

In the present study, although the expression level of the *Gdf9* transcript was not significantly different in the first 3 hours of culture within the experimental groups, but it was significantly higher on day 4 of culture in the follicles of the G5 group compared to the other groups [P: (*Gdf9*: G5:0.01) (*Bmp15*: G5:0.019)].

The expression level of the *Bmp6* gene in the G2 group increased on day 4 compared to the first 3 hours of incubation in culture medium. Nevertheless, no significant difference was found among the experimental groups.

Furthermore, in the study of *Bmp15* gene transcript expression in the experimental groups during the first 3 hours of culture, the expression level in the G5 group increased compared to the other groups and was significantly different (Fig.5).

Expression of growth factor genes in cryopreserved groups was significantly higher than the other groups, which may indicate that the protective effects of parenchyma cell suspension on follicles is damaged by vitrification solutions.

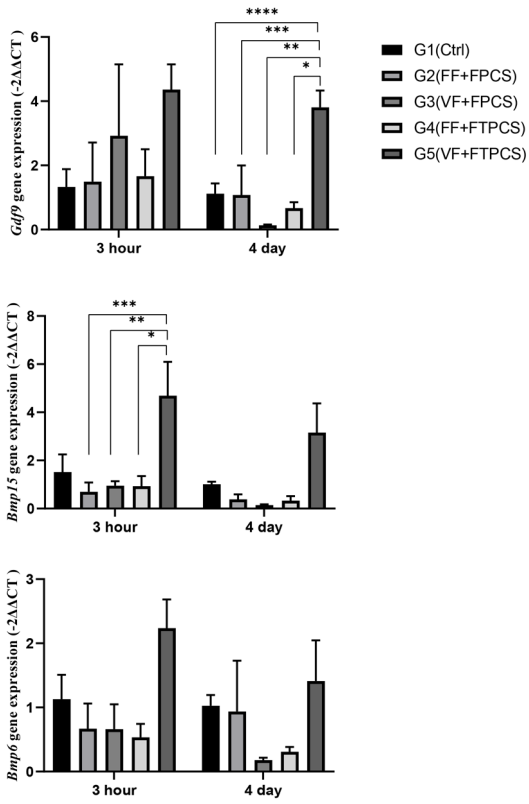

Fig.5: Relative expression of total mRNA for maturation genes in the follicles on different days of in vitro culture. Data are expressed as mean ± SEM. The Kolmogorov-Smirnov and Kruskal-Wallis were used for statistical assessment. Expression of *Gdf9* was higher in the G3 and G5 groups during the first 3 hours of culture than in the other groups. Expression of *Bmp6* and, *Bmp15* was higher in the G5 group during the first 3 hours of culture than in the other groups and in the fourth day of culture the expression level was decreased in all groups, but in the G3 and G5 group was higher than the other groups. This indicates the positive effects of cell suspension on follicles damaged by vitrification solutions (*Gdf9*: G5: P: 0.01) (*Bmp15*: G5: P: 0.019). *, **, ***, and ****; Significant difference only compared to the G5 group (P<0.05).

Discussion

Understanding follicle-oocyte interactions has important implications for identifying biological markers that influence the ability of an oocyte to be fertilized and develop into a healthy embryo in both natural pregnancy and pregnancy by assisted reproduction technology (ART). Ovarian follicles, the functional units of the ovary, comprise the ovarian parenchymal tissue that consists of embryonic stem cells and other cells including fibroblasts, lutein cells, granulosa and theca cells, as well as collagen connective tissue. Searching new ways for follicles to grow and develop efficiently may improve the pregnancy rate in ART.

Bi-directional communication between the oocyte and follicle somatic cells is the key element in regulating follicle and oocyte development. Follicle diameter as well as hormone levels are used as the primary markers for determining the maturity of the follicle and oocyte prior to oocyte retrieval in IVF (26). Prolonged culturing frequently results in atresia because the typical culture systems are unable to supply the metabolic needs of the developing follicle and because there is no paracrine communication with the stromal tissue around it. The complicated process of folliculogenesis is one of the main reasons why normal culture conditions are unable to support or stimulate the development of small follicles (3). One of the essential issues in ovarian follicle culture is the lack of complete knowledge of the influential growth factors with regard to the surrounding cells in parenchyma. Therefore, improving follicle culture systems and follicle cryopreservation is highly studied today. Based on the obtained information, no studies have been performed on the co-culture of ovarian parenchyma cells with follicles. In the present study, we investigated the effects of ovarian cell culture with preantral follicles on survival, follicle development, maturation, and fertilization of the resulting oocytes and the expression of their genes on different days *in vitro*. In terms of oocyte and follicle diameter, all treatment groups had better growth than the control group. However, G2 and G4 groups showed a significant follicle growth compared to the control and G3 groups, indicating that co-culturing with ovarian parenchyma cell suspension indeed stimulated the growth rate in the follicles. It has been demonstrated that growth and differentiation in a range of species' reproductive tissues are altered by fibroblast growth factors (FGFs). The effects of FGFs seem to be concentrated on the ovary in the female reproductive tract, and FGF2, which promotes granulosa cell proliferation and inhibits differentiation, has received the most attention. FGF2 acts on granulosa cells to promote cell proliferation and decrease apoptosis and steroidogenesis, which has been known for more than 20 years (27). In a 2014 study by Malekshah et al. (12) و the follicles that were co-cultured with fibroblast cells developed and survived better than the control group.

According to previous research on follicle survival, stress from mechanical separation, a high concentration of cryoprotectants, and abrupt temperature changes brought on by freezing are some of the main reasons of follicle mortality (23). In the present study, the survival rate of the follicles in both fresh and cryopreservation groups treated with ovarian parenchyma was higher than in the control group. However, statistically, G2 and G4 groups showed better survival rates than the control and G5 groups, in comparison to other studies conducted in the field of co-culturing with fibroblasts and enzyme-derived ovarian cells (11, 15). In a study conducted by Jamalzaei et al. (13, 16), the effects of different alginate concentrations along with enzyme-derived ovarian cells on the growth of preantral follicles was investigated. Their result has shown that the presence of ovarian cells in the culture medium may affect the survival of follicles and positively affect their structure.

On day 12 of the culture, the follicles that formed the

complete antrum cavity were examined. The number of follicles that formed antrum cavities was higher in both G2 and G4 groups than in the control group, with no significant difference among the groups. Studies conducted on fibroblasts and mesenchymal cells extracted from menstrual blood, by Kim et al. (11) and Rajabi et al. (14) illustrated that utilization of stem cells in culture with preantral follicles improve survival as well as development of antrum cavities. While only one cell line has been utilized in earlier investigations, all of the ovarian cortex cells were used in this study, and cell-cell interaction can be maintained to a large extent in this method. It has been proven that using FGFs, along with one cell line in the ovarian parenchymal tissue, are influential in the growth of follicles.

The transforming growth factor beta (TGF-β) superfamily has received additional attention due to its crucial role in the process of folliculogenesis, growth, and development of both follicles and oocytes. In the present study, the expression levels of its family members, including *Gdf9*, *Bmp15*, and *Bmp6* genes were determined to evaluate the growth and differentiation of the follicles. Many studies have been conducted on the mechanisms of the ovaries and their role in growth and development of follicles and oocytes. Moreover, it has been suggested that the rate of early follicular growth is a reliable factor for predicting pregnancy potential for both natural and ART-mediated pregnancies, with slow initial growth of the follicles being an indicative of negative pregnancy results. Although numerous studies indicated *Gdf9* and *Bmp15*, as essential maturation genes that are specifically expressed in oocytes, (28), but, in a previous study, Chen et al. (29) demonstrated that *Bmp15* is expressed in human cumulus cells in addition to oocytes. These two genes are also expressed in all follicular stages except the early one and play an important role in the proliferation of granulosa cells and subsequent growth of follicles, especially in the early stages of their development (30-32). *Bmp6* is other gene that expressed in the early stage of follicle development, different stages of oocyte development and granulosa cells (33, 34). Cook-Andersen et al. (35) and de-Castro et al. (28), studies on mice ovary illustrated that *Gdf9* stimulates the growth of primary follicles. Dong et al. (36) showed that follicles in mice with *Gdf9* mutations, did not grow after the primary stage. Therefore, the expression of *Gdf9* is essential for folliculogenesis and the fertility process and involved in the mitosis and steroidogenesis of granulosa cells and the proliferation of cumulus cells (37, 38).

So far, many studies have been performed on the structure, biological function, expression pattern, and how growth factors may affect the process of folliculogenesis. In the present study, the expression levels of *Gdf9*, *Bmp15*, and *Bmp6* genes showed a relatively similar pattern both after 3 hours and the fourth day of culture. In a study conducted by Jamalzaei et al. (13) on fresh and vitrified follicles, the highest expression of these genes was seen in frozen follicles 3 hours after culture; however, it decreased as the final days of the culture approached. These results showed that high mRNA levels are produced in the oocyte at the early stages of growth, but they gradually decreased as the growth continued. In the present study, the expression levels of the three growth factor genes were significantly higher in the groups with vitrified follicles compared to the other groups. This could indicate the protective effect of co-culture of preantral follicles with ovarian parenchyma cell suspension. As it was mentioned in previous studies, gene expression was specifically higher in the first 3 hours of culture and significantly decreased by the fourth day of the culture, with similar functions found in other studies.

Conclusion

Overall, this study provided evidence for the advantages of using ovarian parenchyma tissue for preantral follicle growth and development. The primary purpose of this research was to try to bring suitable *in vivo* environment for the effective growth of the follicles through a co-culture system that permits mice preantral follicles to develop into a 2D culture system. The co-culture of ovarian parenchyma cells with preantral follicles improved the growth, survival, and maturation rates of the follicles. In addition, higher expression of maturity genes in cryopreserved follicles indicates the compensation role and protective effects of ovarian parenchyma on the cryopreservation process. We demonstrated that parenchyma tissue could be used as a supportive tool for the growth and maturation of preantral follicles. This result may help clinical applications of the process in ART to improve fertility preservation and IVC-IVM approaches.

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Authors' Contributions

J.N.S.; Writing-Original Draft, Review & Editing, Investigation, Resources, Methodology, Conceptualization, Software, and analysis. H.E.; Project administration and Supervision. A.H.Sh., M.T.; Validation and Methodology. R.F., L.S.T.; Conceptualization and Methodology. S.A.M.; Conceptualization-Resources - Review & Editing. S.M.J.T.-M.; Methodology and Investigation. All authors read and approved the final manuscript.

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