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Supporting the in vitro Expansion of Human Cumulus Cells as an Initial Step for Culturing the Ovarian Follicles and Assembling an Artificial Ovary

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ABSTRACT

Background: Assembling an artificial ovary needs supporting the in vitro growth of cumulus cells, and finally, follicles. This study aimed to determine the appropriate cocktail for culture of cumulus cells (CCs).

Methods: CCs were collected from healthy women and cultured with 9 cocktails of basal media, supplemented with 10% and 20% fetal bovine serum (FBS) and 1% and 2% human serum albumin (HSA). Ovarian cells were isolated from cortex, medulla, and hilum, and their conditioned media (CM) were collected. Expression of GDF9 in ovarian cells was evaluated. CCs were treated with various concentrations of CMs from ovarian cells and mesenchymal stem cells. Also, they were cultured with various concentrations of supplements including L-Glutamine, bovine serum albumin (BSA), HSA, insulin transferrin selenium (ITS), Follitropin alfa, and Pregnyl. Also, they were treated with various concentrations of follicular fluids (FFs), collected from patients with different infertility etiologies. Finally, CCs proliferation and culture stability were evaluated.

Results: All the ovarian cells expressed GDF9. DMEMF12 + 20% FBS was the most suitable cocktail for CCs. 20% FBS was superior to 10% FBS. HSA alone could not support the growth of CCs. The CMs of (cortical + hilar + medullar) cells and FFs from healthy women caused higher CCs proliferation. 17 mM/l L-Glutamine, 24 mg/ml BSA, 20 mg/ml HSA, 10 ng/ml ITS, 300 mIU/ml Follitropinα, and 3.5 IU/ml Pregnyl led to higher CCs proliferation.

Conclusion: Supplementation of the basal medium with CMs, serums, FFs, hormones, ITS and L-Glutamine, can better support the culture of CCs.

Keywords: Cumulus Cells, In vitro Culture, Follicles, Culture Medium, Supplements

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Introduction

Inability to conceive refers to infertility. The women are considered infertile after one year of trying to have child with normal sexual functions and usual intercourse. Conventionally, infertility treatment consists of an ovarian hyper stimulation cycle, followed by assisted reproductive technology (ART). However, due to various reasons, the conventional treatments sometimes encounter unfortunate consequences. Therefore, new methods gradually have been developed to improve infertility treatment outcomes using ovarian tissue engineering. One of the first step for *in vitro* reconstruction of the ovary is *in vitro* culture of ovarian cells and follicles. In vitro expansion of cumulus cells (CCs) is a primitive step for culturing the ovarian follicles, which helps providing a suitable condition for follicular *in vitro* growth (FIVG). Successful FIVG (two- and three-dimensional) is the final goal in reconstruction of an artificial ovary and its applications are explained in the following:

1) For fertility preservation in cryopreserved tissue of recovered patients with cancer (1).
2) For poor responder patients
3) For patients with premature ovarian failure (POF) (2).
4) FIVG, followed by *in vitro* maturation (IVM), can be used to achieve competence oocyte from primary stage follicles (alone or in co-culture with other cells like CCs) (2).

In all the above-mentioned issues, ovarian follicles culture is the key factor, so FIVG is the main process of ovarian tissue engineering, which tries to return the ovarian functions using fabrication of an artificial ovary and engineering the ovarian cycle. Expansion of granulosa cells (GCs) and CCs is a key point in FIVG and oocyte competence. Although all of the above-mentioned tactics have been developed, FIVG still needs to be improved due to many unknown factors, which are involved in limited *in vitro* proliferation of GCs and CCs (3). Therefore, culture medium optimization for GCs and CCs is essential, because they are the cells with different growth needs. The pre-antral follicle growth is gonadotropin-independent and is based on a crosstalk between oocyte and GCs. But after antrum formation, the gonadotropin-dependent stages are started and GCs are divided into two groups including: 1) corona radiate and CCs that are defined as the oocyte closely associated GCs, which surround the oocyte. 2) Mural GCs are the cells that line inside of the antrum cavity. Therefore, CCs participate directly in oocyte maturation and fertilization (4). When ovulation occurs, oocyte is thrown into the fallopian tubes along with the CCs. Therefore, naturally during the final stages of ovarian follicle development, the growth of CCs is the key point and all the FIVG-based methods need support of CCs growth. Many materials and factors are proposed for optimizing the medium for the *in vitro* culture of CCs. No study has been reported on the optimized medium for *in vitro* culture of CCs. This study aimed to mimic the ovarian natural microenvironment for culturing the ovarian follicles, but since obtaining a lot of human ovarian follicles for assessment of the different factors is limited ethically, therefore, for mimicking the ovarian follicles chemical micro environment, the present study was designed on ovarian CCs, as the most important cells in ovarian follicle growth. Therefore, the present study focused on the efficacy of different cell culture media, hormones, supplements, and conditioned media (CMs) on the growth of human CCs. Briefly, the impact of different concentrations of serums including fetal bovine serum (FBS), human serum albumin (HSA), and bovine serum albumin (BSA) was evaluated. The effect of various follicular fluids (FF) and supplements including L-glutamine, insulin transferrin selenium (ITS), and hormones on the *in vitro* growth of CCs was investigated, too. Moreover, cells from different parts of the ovary (cortex, medulla, and hilum) were cultured separately. Adipose tissue-derived mesenchymal stem cells (AMSCs), amniotic fluid-derived mesenchymal stem cells (AFMSCs), testicular sperm extraction (TESE)-derived cells, and human ovarian carcinoma cell line (NIH: OVCAR-3) were also cultured. Finally, the CMs

of all these cells were added to CCs culture in order to determine the cells that can better support the CCs growth. Ovarian follicles growth needs many materials like growth factors that are so expensive. Therefore, CMs and some other supplements can be more cost-effective than growth factors. So, the results of this study can support the follicular growth, with the best cost benefit, compared to growth factor. The details of the study are summarized in supplementary Figure 1.

Material and Methods

All the culture media, collagenase type II, ITS, and FBS were purchased from Gibco (Gibco™, Thermo Fisher Scientific Company). Antibiotics penicillin/streptomycin (pen/strep) and Amphotericin B (FUNGIZONE®) were purchased from Biowest Company (Biowest, the serum specialist). Phosphate-buffered saline (PBS) tablets, L-Glutamine, and BSA were obtained from Sigma (Sigma-Aldrich Company). HSA 20% was purchased from Biotest Company (Biotest®). Antibodies were purchased from Abcam (Abcam Company, US).

Human Ovarian Tissue Collection

In the present study, no cell lines were used. The applied cells in this study were isolated from human adult ovary. The project was approved by the Ethics Committee of Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran (Ethical code: IR.SSU.RSI.REC.1396.21). This study was conducted in accordance to the ethical principles and the national norms and standards for conducting medical research in Iran. Also, it was based on the ethical code of the World Medical Association (Declaration of Helsinki).

Human ovarian tissues were retrieved after obtaining informed signed consent. Ovarian biopsies were collected from ovariectomized patients after surgery due to benign problems. The samples were transferred into PBS (4°C), containing 5% penicillin-streptomycin (pen/strep) and 5% amphotericin B, and immediately, transferred to cell culture laboratory.

Ovarian Cells Isolation

The excised ovaries were dissected by a sterile surgical scalpel. Anatomically and macroscopically, three parts were considered for ovarian biopsies including: 1) the outer part or ovarian surface epithelium (OSE) (Figure 1A, blue arrow) and its underlying part was ovarian cortex (Figure 1A, yellow arrow). The OSE and ovarian cortex were removed and scraped from the inner part by a bistoury (Figure 1B). 2) The second inner part was medulla (Figure 1A, pink arrow). It was the intermediumste part between cortex and hilum where no follicular structures were observed. 3) The ovarian hilum was the last part that connects the ovary to the ovarian pedicle (Figure 1A, green arrow). Therefore, the segments were collected from these three parts, rinsed with PBS + 3% pen/strep + 3% amphotericin B, chopped with sterile scissors (Figure 1C), and then, transferred into three separate 15 ml conical tubes. After subsequent washing, the segments were transferred into another tube and 0.5-1 mg/mL collagenase type II (10%) was added to samples for enzymatic digestion (Figure 1D). Then, the samples were incubated at standard conditions, temperature of 37°C, 5% CO_2, and 95% humidity with frequent shaking till partial digestion. Depending on the tissue consistency, the time of enzymatic digestion was variable from 2 to 8 h. The collagenase activity was blocked with αMEM + 10% FBS. The samples were centrifuged [for 8 min at 1400 rpm (300 g)] and washed with pre-equilibrated αMEM + 10% FBS medium. Lastly, after centrifugation, the supernatants were discarded and the pellets were cultured in αMEM + 10% FBS, 2% pen/strep, and 2% amphotericin B. During next days, the culture plates were checked to investigate the attached cells. The medium was replaced with a fresh medium every 2-3 days and the culture continued to get confluence. The cells behavior and morphology were investigated by an inverted microscope.

Immunocytochemistry

Ovarian cells isolated from the cortex, medulla, and hilum were stained for growth differentiation factor 9 (GDF9), as a key factor in CCs expansion and ovarian follicle development. The cells were fixed in 4% paraformaldehyde for 20 minutes at 4°C. Then, the samples were washed with 2 normal HCl for 20 minutes at room temperature followed by incubation for 30 minutes with 0.3% Triton X-100 for permeabilization. Afterwards, 20% normal goat serum was added for 10 minutes for blocking non-specific epitopes for one hour at room temperature. The cells were incubated overnight with primary anti-GDF9 antibody in a humid chamber at a dark room in the refrigerator. Following twice washing with PBS, polyclonal secondary antibodies were added for

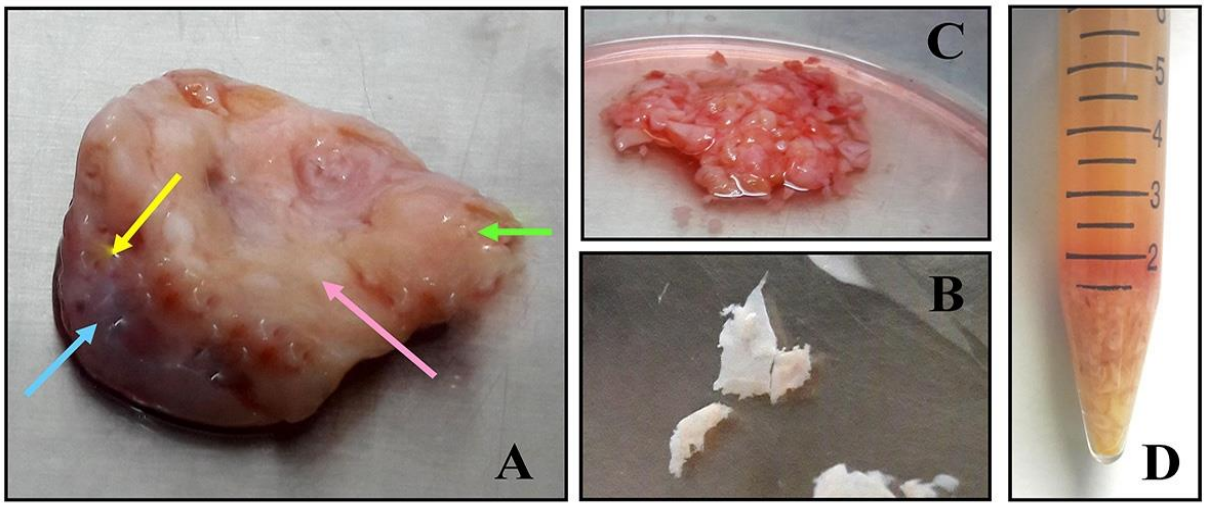

Figure 1. The steps of ovarian cells isolation. piece of the ovarian biopsies. Macroscopically three parts were considered for ovarian biopsy. The outer part or ovarian surface epithelium is OSE (Blue arrow). The underlying part is ovarian cortex (Yellow arrow). The medulla and hilus are marked with pink and green arrows, respectively (Pink *and* Green arrows). B) The OSE and ovarian cortex were removed and scraped from the inner part by a bistoury. C) Some segments were collected from these three parts, rinsed with PBS + 20% pen/strep + 20% amphotericin B, chopped with sterile scissors. D) The cut segments transferred into conical 15 ml tubes.

60 minutes at room temperature in a dark place. After washing three times with PBS, the cells were counterstained with DAPI and monitored under the fluorescence microscope. Positively stained cells expressed green fluorescence for GDF9 under a fluorescence microscope.

Cumulus Cells Collection and Culture

The CCs were collected through the puncture of the follicles from normal women who referred to our center for infertility workup due to male factor infertility. The selected women were healthy and under the age of 35 years with acceptable anti-Mullerian hormone (AMH) level (over 1 ng/ml). The CCs were collected after puncturing the follicles and denudation. The denudated droplets were collected, centrifuged,

washed with medium, and finally, the cell pellet was collected and cultured in αMEM + 10% FBS, 2% penicillin/streptomycin, and 2% amphotericin B.

Effects of Different Media on Cumulus Cells

To evaluate the best basal cell culture medium for growth of human CCs, the following basal media were compared:

1) **α MEM:** It is a modification of Minimum Essential Medium (MEM).

2) **DMEM (high and low glucose):** Dulbecco's Modified Eagle Medium (DMEM) is based on the Eagle's minimal essential medium with a fourfold concentration of vitamins and amino acids.

3) **DMEM/F-12:** It is a mixture of DMEM and Ham's F-12 (ratio 1:1). It combines DMEM + high concentrations of glucose, vitamins, and amino acids. Also, Ham's F-12 has a wide variety of components.

4) **RPMI 1640:** It contains higher concentrations of glucose and lower concentrations of calcium and phosphate, compared to DMEM. The details of upper-mentioned media are presented in table 1.

Table 1. Comparative evaluation of cell culture media

Media	D-Glucose Concentration	L-Glutamine	Phenol Red	Comparison to MEM Medium	Buffering System	Other Ingredients
α MEM	Low glucose 1 g/L	w/wo Almost without but if any, 2 mM/L is added	Phenol Red 10 mg/ml		Sodium bicarbonat	13 essential amino acids Non-essential amino acids 8 vitamins Sodium pyruvate salt
DMEM Low glucose	Low glucose 1 g/L	w/wo Almost with 4 mM/L	Phenol Red 15 mg/ml	Four-fold concentrations of amino acids and vitamins, compared to MEM	Sodium bicarbonat	Amino acids Vitamins w/wo Sodium pyruvate Salts Additional upplementary components
DMEM High Glucose	High glucose 4.5 g/L	w/wo Almost with 4 mM/L	Phenol Red 15 mg/ml	Four-fold concentrations of amino acids and vitamins, compared to MEM	Sodium bicarbonat	Amino acids Vitamins w/wo Sodium pyruvate Salts Additional upplementary components
DMEM/F12	High glucose 4.5 g/L	w/wo almost with 2.5 mM/L	Phenol Red 1.2 mg/ml	Four-fold concentrations of amino acids and vitamins, compared to MEM + Ham's F-12 medium	Sodium bicarbonat	All 21 amino acids w/wo Sodium pyruvate Salt 10 vitamins
RPMI 1640	Almost high glucose 4 g/L	w/wo Almost with 2 mM/L	w/wo Almost without phenol red. If any, Phenol Red 5 mg/ml		Low sodium bicarbonat HEPES	All 21 amino acids Sodium pyruvate Salt 11 vitamins

Many studies have applied αMEM for ovarian and follicular culture. But there is no published quantitative report regarding the advantages of αMEM for ovarian cells. So, in the first step, the effect of basal media, supplemented with 10% FBS, on the ovarian stromal cells was compared. The results showed that αMEM + 10% FBS led to more cell proliferation (Figure 2A). In the second step, the effect of basal media, supplemented with 20% FBS, on the ovarian stromal cells was compared to understand the effect of FBS concentration. The results showed that αMEM + 20% FBS was the best medium for culture of a mixture of ovarian cells (Figure 2B) (Figure 2). Then, in order to find which medium is better for culture of CCs and whether the combination of FBS with HSA can cause higher cumulus cell proliferation, several cocktails were provided and their effects on CCs proliferation were compared with (αMEM + 20% FBS), as the control.

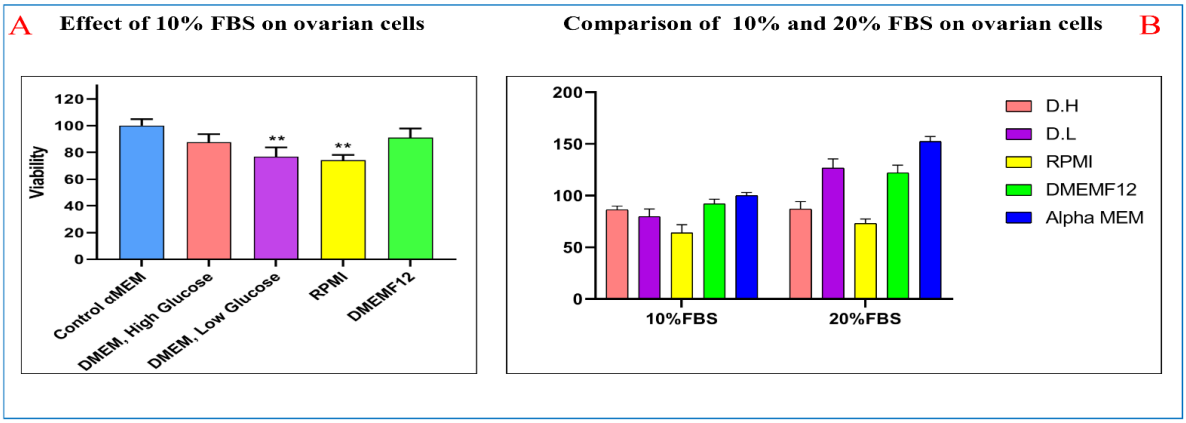

Figure 2. The effect of basal medium, supplemented with 10% and 20% FBS on ovarian cells proliferation
Alpha MEM + 20% FBS was better than the others

So, αMEM medium, DMEM (high- and low-glucose) and DMEM/F-12 as basal media and RPMI 1640 as a complex medium were compared. These media were supplemented with 10% and 20% FBS and 1% and 2% HSA with various combinations. 1% and 2% HSA were chosen, because previously in another study various concentrations of HSA on the CCs were evaluated to choose the best dosages (1% and 2% were the best ones). So, several formulations of the above-mentioned media were prepared including (Serum free media), (media + 10% FBS), (media + 20% FBS), (media + 1% HSA), (media + 2% HSA), (media + 10% FBS + 1% HSA), (media + 10% FBS + 2% HSA), (media + 20% FBS + 1% HSA), and (media + 20% FBS + 2% HSA).

Effect of Different Conditioned Media on Cumulus Cells Growth

To understand that which cells of ovary are more supportive for CCs growth, ovarian cells were cultured and their CMs were collected. The media were collected from the following cells: 1) Ovarian cortical cells before formation of spheroid bodies (SBs). Spheroid bodies are the suspended ball-shaped colonies that originated from the ovarian cortical cells and they are the ovarian germ cell nest. 2) Cells isolated from SBs. 3) Ovarian cortical cells after formation of SBs. 4) Ovarian medullar cells, and 5) Ovarian hilar cells. Also, some other cells were cultured and their CMs were collected such as ADMSCs and AFDMSCs (the data that showed osteogenic and adipogenic differentiation potential and flow cytometry expression of mesenchymal markers, have been presented in the other under review papers and due to duplication are not presented here). Besides, the CM of OVCAR-3 and CM of isolated cells from testis (obtained by TESE surgery) were collected. CMs were collected from 2-3 passages of cells, after 1 weak culture, centrifuged at 5000 rpm for 10 minutes, filtered and frozen for future use. For collecting CMs, all the cells were cultured in DMEM + 10% FBS for 5 days. The control medium (DMEM + 10% FBS) was incubated alone without cells, for the time equal to the CMs collection time. 40% CMs was added to basal culture medium and after 72 hours of culture, the cell proliferation was assessed by MTT assay.

Effect of Hormones

In order to evaluate the effect of hormones on CCs growth, several concentrations of Gonal-F (FSH like drug, used in ovarian hyper stimulation), Pregnyl® (ORGANON Holland), [Human chorionic gonadotropin (HCG) like drug which is used in ovarian hyper stimulation], were used. HCG is composed of alpha and beta subunits. The alpha is identical to human gonadotropins (LH and FSH) and alpha subunit of human thyroid stimulating hormone (TSH). Therefore, both of the used drugs are FSH-like, but Pregnyl also has some LH Like effect. The majority of previous studies used 100 mIU/ml of FSH and 1.5 IU/ml of HCG (5). Therefore, according to the previous studies, 100-500 mIU/ml

concentrations of Gonal-F and 1.5-7.5 IU/ml concentrations of Pregnyl were tested in this study.

Follicular Fluid Collection and Preparation

To analyze the effect of FF on the CCs growth, the FF samples were collected from 5 groups of patients, who referred to our infertility treatment center. The detailed criteria are summarized in Table 2. The collected FF was quickly transferred to a specialized laboratory, centrifuged two times, the supernatant was de-complemented at 56°C for 30 minutes in a water bath. After cooling, the samples were stored at 4°C for further use. To be the representative of the community, from each group, 10 samples were collected and aliquoted, and before using them, they were mixed together and filtered. 50% and 75% of these FFs were added to basal medium.

Effect of Serum (FBS, BSA, HSA)

To evaluate the effect of serum on the CCs growth, two concentrations of FBS (10 and 20%) on the CCs growth were analyzed. Two types of albumin, BSA and HSA, were compared. The previous studies usually applied 3 mg/ml BSA and HSA (6, 7). In this study, 3, 6, 12 and 25 mg/ml of BSA and 3, 6, 10, 12, 20, and 25 mg/ml of HSA were compared.

Table 2. The ceriteria used for collection of the follicullar fluids

Eitiology	Difinition	Age (year)
Male factor infertility	1) The healthy women under infertily treatment who referred due to male factor infertility 2) AMH ≥ 1.1 ng/mL	≤ 35
Polycyctic ovarian syndrom (PCOS)	Based on Revised 2003 of Rotterdam consensus criteria (2 out of 3): 1) Oligo ovulation or anovulation 2) Biochemical and clinical signs of hyperandrogenism like hirsutism and obesity 3) Polycystic ovaries feature in ultrasound 4) Exclusion of other etiologies like Cushing's syndrome and congenital adrenal hyperplasia	≤ 35
Endometriosis	The patients who diognosed for endometrioma, a type of cyst formed in ovary	≤ 35
Poor responders	The patients who have at least two of these criteria: 1) A previous history of poor ovarian response (≤3 oocytes) 2) An abnormal ovarian reserve AMH (<0.5-1.1 ng/mL) 3) Women older than 40 years	≤ 35
Advanced age but with good ART results	1) The patients who were older than 35 years 2) AMH ≥ 1.1 ng/mL 3) Follicole number ≥ 5 4) Good ART results	≥ 35 ≤ 45

Effect of L-Glutamine and ITS

Using L-Glutamine is recommended as 2-2.8 mM/ml in basal medium (8). In the present study, different concentrations of L-Glutamine (2, 4, 12, 17, 22, 27, and 37 mM/ml) were compared. [DMEM+ (2 mM/ml) L-Glutamine] was used as control medium, because (2 mM/ml) L-Glutamine is regularly used in most of culture media.

Regarding the ITS, in the previous studies, a wide range of ITS from 5-10 ng/ml (9) to 5-10 µg/ml was used (10). Hence, 5-10 ng/ml and 5-10 mg/ml of ITS were compared in order to determine the best concentration of ITS.

MTT Assay Test and Statistical Analysis

Cells were seeded in 96-well plates at the density of 1×10^4. When cells get 60% confluency, the treatments were done. After various treatment, cell culture continued for 72 h. Then, cell viability was evaluated with MTT assay test. The cells of control group were cultured in DMEM + 10% FBS + 2 mM/ml L-Glutamine. After incubation with MTT dye for 3 h, Dimethyl sulfoxide (DMSO) was added for 15 minutes with subsequent shaking. Then, every 96 wells of plate was read using enzyme-linked immunosorbent assay (ELISA) reader (test wavelength: 540 nm, reference wavelength: 630 nm). Triplicated samples were

treated. The mean of data was normalized and analyzed using GraphPad Prism 8. One-way analysis of variance (ANOVA) was applied to determine the significant differences between the mean of treated and control groups, followed by post-hoc Dunnett's test.

The figures were prepared and grouped using Microsoft publisher 2010 and resized by Photoshop.

Results

Ovarian Cells Isolation, Characterization and GDF9 Expression

In the present study, cells from three parts of the ovary, including (cortex + ovarian surface epithelium), medulla and hilum, were isolated. The mixture of cortical and ovarian surface epithelium cells exhibited spindle-shaped and epithelial-like shape (Figure 3A). Gradually after the first passage, some cells detached from the plate and proliferated while they were floating. In fact, they made the ovarian SBs or ovarian germ cell nest (Figure 3B). The suspended SBs were isolated, cultured, expanded, and they created colonies (Figure 3C). The cortical cells, left in culture, continued to proliferate (Figure 3D), and eventually, the cortical colony forming cells created the typical colonies (Figure 3E and F).

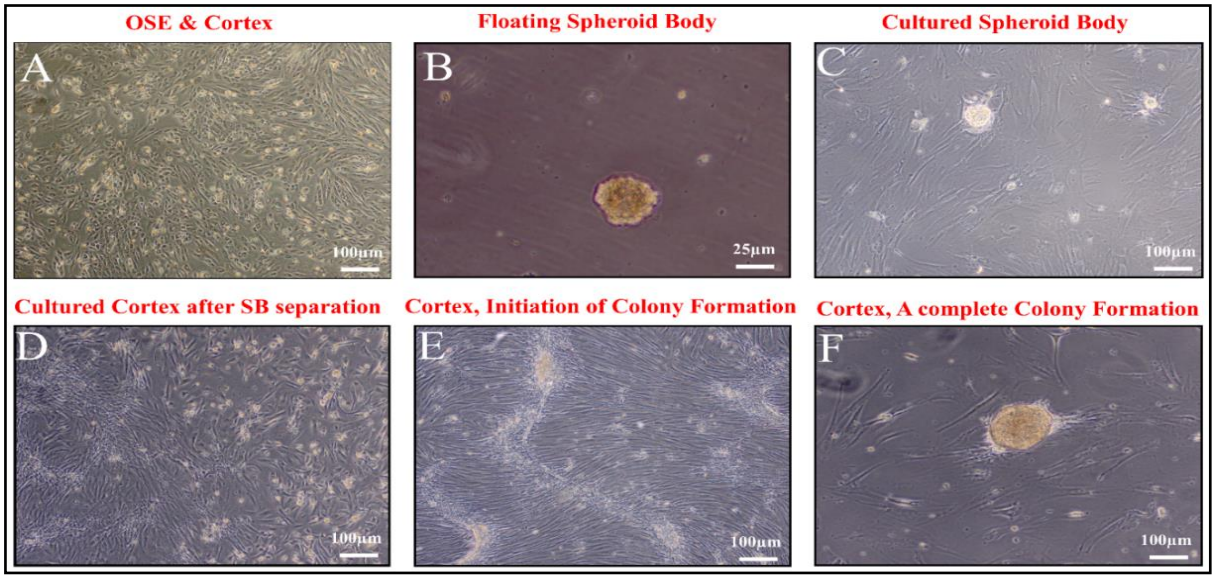

Figure 3. The morphology of ovarian cortical.
A) A primary culture of cortex and OSE with two morphology including spindle-like cells and epithelial-like cells (magnification × 100 and scale bar = 100 µm). B) The detached cells from the ovarian cortex culture, which grow to make a giant floating spheroid (magnification × 400 and scale bar = 25 µm). C) The suspended SBs were isolated, cultured, expanded, and left to make colonies (magnification × 100 and scale bar = 100 µm). D) The remaining cortical cells continued their proliferation (magnification × 100 and scale bar = 100 µm). E) Finally, the ovarian cortical cells started to make colonies (magnification × 100 and scale bar = 100 µm). F) A complete colony of ovarian cortex (magnification × 100 and scale bar = 100 µm).

Regarding the ovarian medulla, the cells showed even fibroblastic-like shape (Figure 4, medulla A) or the smaller cells (epithelial like shape) namely polygonal cells with regular dimensions (Figure 4, medulla B and C), and finally, they formed ovarian medullar cell colonies (Figure 4, medulla D). Most ovarian hilar cells exhibited a fibroblastic-like shape (Figure 4, hilus A), which became more uniform in the next passages (Figure 4, hilus B). They form colonies from the first passage onwards (Figure 4, hilus C).

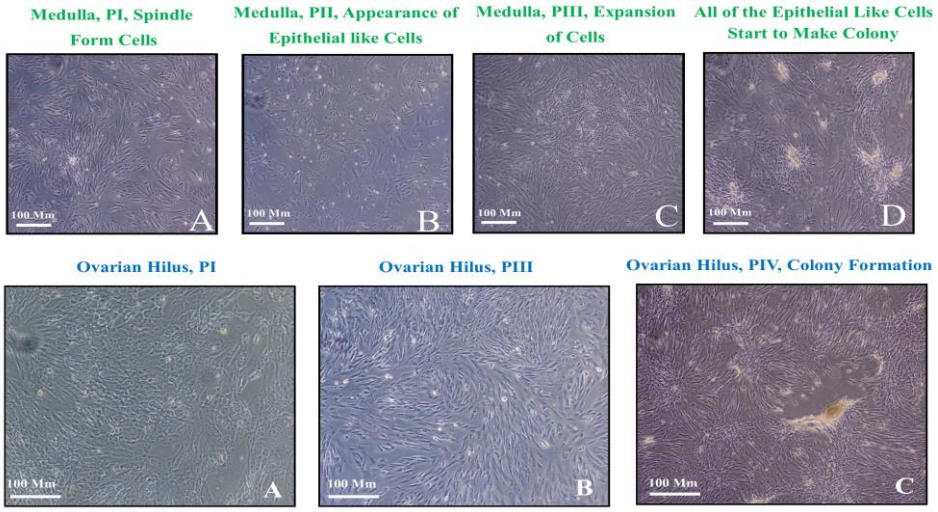

Figure 4. The medullar cells: A) the primary culture of ovarian medulla cells (first passage). The cells showed fibroblast like architecture. B) The second passage of medullar cells, the smaller cells with epithelial like shape were appeared. C & D) the cells expanded, proliferated and made colonies (magnification × 100 and scale bar = 100 µm).
The hilar cells: A) the primary culture of ovarian hilus cells. The majority of cells exhibited fibroblast like shape. B) The cells became more uniform in the next passages. C) The colonies of ovarian hilus cells (magnification × 100 and scale bar = 100 µm).

Also, immunocytochemistry analysis showed that the cells of cortex, medulla, hilum, and SB expressed GDF9 marker. It was visualized green under fluorescent microscope (Figure 5). Interestingly, its expression was higher in medulla and hilum cells than in the cortex and SB cells.

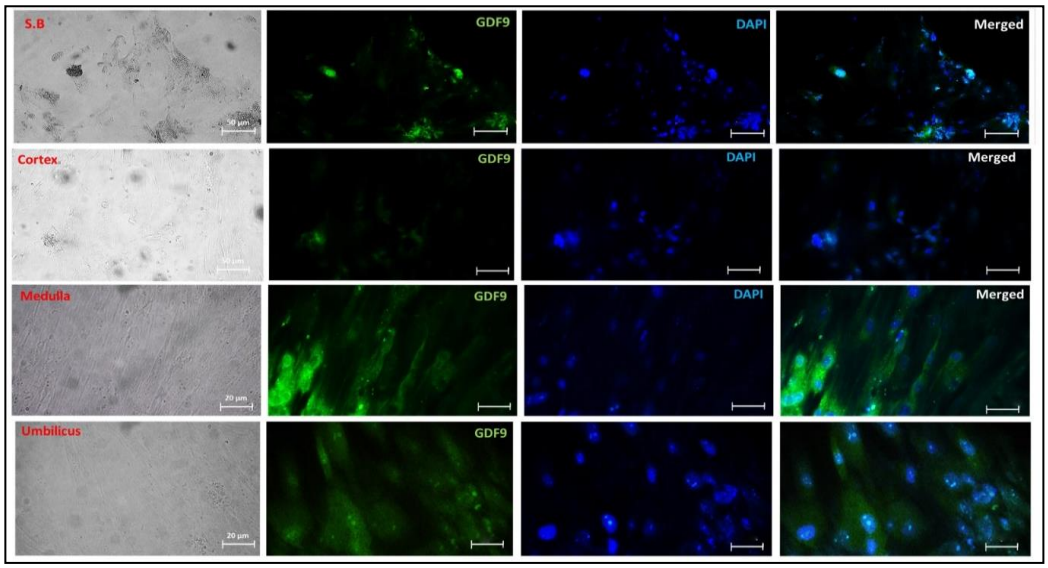

Figure 5. GDF9 expression in ovarian cortex, medulla, hilum, and SB cells by immuno-staining under fluorescent microscope.
The green color shows positive GDF9 cells in OSE and cortex (magnification × 200 and scale bar = 50 µm), and medulla and hilum (magnification × 400 and scale bar = 20 µm). Immunocytochemistry analysis showed that the cells of cortex, medulla, hilum, and SB expressed GDF9 marker.

Cumulus Cells Isolation, Shape, *In vitro* Growth and Morphology

CCs were isolated from punctured dominant follicles. In the early days of culture, they were small cells with no elongation, appendages or branching (Supplementary Figure 2A). Then, they started to become flat (Supplementary Figure 2B) and exhibited short-distance contact communications with other cells using branched cytoplasmic extensions or protrusion, similar to dendritic cells arborisation (dendritic branching) (Supplementary Figure 2C and D). Then, they became large cells with more cytoplasmic organelles, which probably

resemble a rough endoplasmic reticulum (RER) (Supplementary Figure 2E). The cells started to become round (Supplementary Figure 2F and G), the cytoplasmic filaments increased; meanwhile, the nucleus migrates from the margin to the center (Supplementary Figure 2H and I). Cell organelles increased, especially a lot of granules appeared, which may probably attach to and move on RER that surrounds the centrally located nucleus (Supplementary Figure 2J, 2K and 2L). Then, the cells were filled with a lot of vesicles (Supplementary Figure 2M) and vacuoles (Supplementary Figure 3N), which are the characteristics of secretory cells. It means that the secretory vesicles were gradually appeared in cells and filled the cytoplasm (Supplementary Figure 2N) and the cytoplasmic space looked like a lot of cytoplasmic cavities or cytoplasmic sacs (secretory vacuoles) (Supplementary Figure 2N and 2O). However, the cells still had contact communication with the surrounding cells, which were at different stages of structural and functional development (Supplementary Figure 2N and O).

CCs have extensive chemical communication with adjacent cells, for example, in movie 1, some particles (probably vesicles) attached to cell from the left, pass through the cells and exit from the right, which probably caused some material exchange or modifications. Also, as the cell size increases, cytoplasmic streaming or protoplasmic streaming or cyclosis helps the material exchange substances in and out of the cells. It helps organelles and molecules transfer in the cell rapidly, which is special movement for large cells (unlike the small cells that their diffusion is more rapid). In movie 1, the organelles around the nucleus, which seem to be RER granules, move around the nucleus, while a cargo enter the cells from the left side and exit the cell from the right side (Movie 1) (Supplementary Figure 3B and C).

Compared to other OCs, CCs grow slowly and are not significant in terms of *in vitro* proliferation in basal medium even over time. In basal medium without any supplements, they are divided very slowly and their maximum passage number is 3-4. If any supplements are added, CCs have no considerable growth and apoptosis will occur, while culturing them in a medium enriched with the mentioned supplements allows CCs to survive even after more than a month.

Movie 2 presents a cell which shows the sign of apoptosis and it is probably an apoptotic cell. The cell begins to shrink, and membrane blebbing and bubbling occur, which are the signs of apoptotic cells (Movie 2). While in movie 3, early events of a necrotic cell seem to be happening. Here, the cell volume is not decreased and no sign of shrinkage is seen (Movie 3).

Effect of Different Media on Cumulus Cells Proliferation and Viability

The results of basal media on a mixture of OCs (cortex + medulla + hilus) demonstrated that (α-MEM + 20% FBS) was the best medium (Figure 2B). Thus, in order to evaluate the most efficient combination of basal media for *in vitro* proliferation of human CCs, the combinations of the above-mentioned media + (FBS 10 and 20% and HSA 1 and 2%) were compared with (α-MEM + 20% FBS). Then, 9 cocktails of each medium were prepared and the results of CCs proliferation were assessed by MTT assay after 72 h culture.

The results showed that in 20% FBS group, (DMEMF12 + 20% FBS) ($P<0.0001$) and (DMEM HG + 20% FBS) (not significant) resulted in higher cell viability and proliferation rates. Also, in (20% FBS + 2% HSA) group, DMEMF12 ($P<0.01$) and DMEM HG ($P<0.05$) caused higher cell proliferation and viability rates. In (10% FBS + 2% HSA) group, DMEMF12 (not significant) caused higher cell proliferation and viability rates than control. Finally, in (20% FBS+1% HSA) group, DMEMF12 (not significant) and DMEM HG (not significant) caused higher cell proliferation and viability rates than control group (Supplementary Figures 4 and Figure 6).

In general, it is concluded that for culturing CCs, DMEMF12 and DMEM HG, followed by α-MEM are the best media. They can be used with 20% FBS alone or in combination with FBS and HSA.

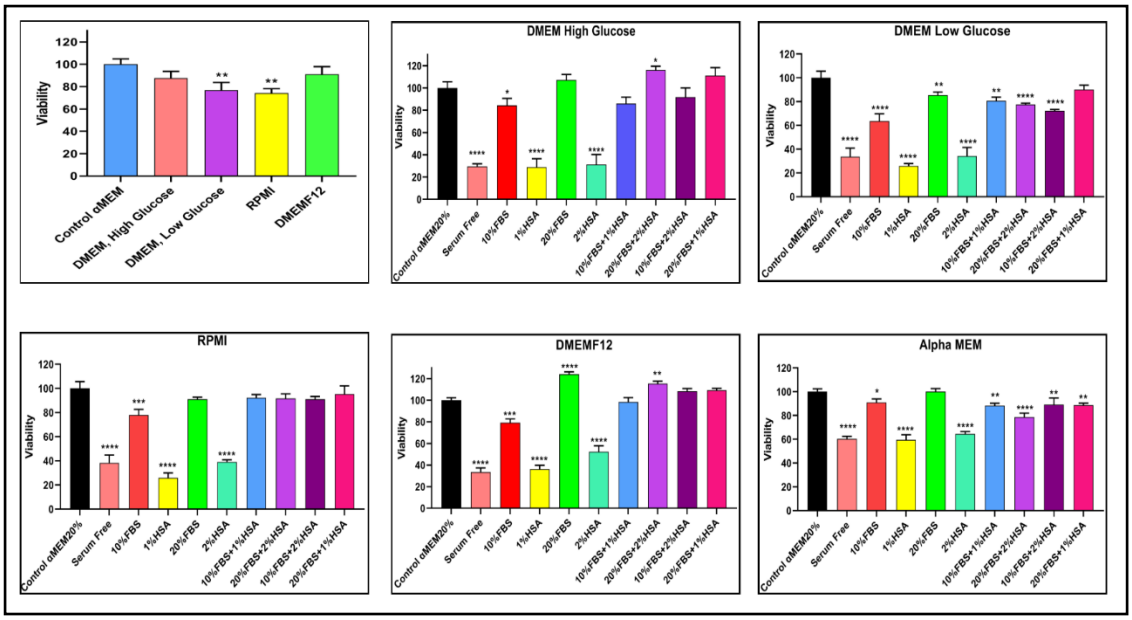

Figure 6. The comparison of five basal media with (α-MEM + 20% FBS) for ovarian cells culture.
The first diagram shows that (α-MEM + 20% FBS) can better support the CCs growth. So, to assess which basal medium can better support the in vitro proliferation of human cumulus cells, five media including α-MEM, DMEM HG, DMEM LG, DMEMF12, and RPMI were compared. FBS 10% and 20% and Alb 1% and 2% were used as serum with various combinations. According to Figure 2, all the combinations were compared with (α-MEM + 20% FBS). * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

The Effect of Conditioned Media on Cumulus Cells Proliferation

Culture of CCs without the above-mentioned supplements, was used in the current study, which leads to cell death over time. Thus, in order to determine the most effective ovarian cells that can better support CCs growth, CCs was exposed to CMs obtained from ovarian cells. The CMs were collected from the cultured ovarian cells. All of ovarian cells CMs led to greater than or equal to, viability and growth of the control group. All of CMs from cortex before SBs formation, [(OSE + Cortex), (cultured SBs), and (cortex after SBs formation)] (P<0.0001) significantly increased CCs growth and viability, while the cells of medulla and hilum showed viability ≤ that of the control group. Therefore, cortical cells (OSE, cortical, and SBs) are more important for supporting CCs proliferation and survival. Also, the combination of [50% medullar and 50% hilum CMs], (P<0.001) and combination of all ovarian cells CMs (P<0.001) showed viability ≤ than that of the control group. Overall, CMs of cortex and (medulla + hilum) seem to be more effective in supporting the growth and viability of CCs.

Two CMs collected from adipose tissue (AMSCS) and amniotic fluid (AFMSCs) were used as well. They only caused slightly larger growth of CCs (not significant), compared to untreated cells.

Finally, the result of CM from TESE-derived cells showed the same result as the control group and CM from OVCAR3 cells resulted in significantly increased growth of CCs (P<0.001). OVCAR3 cells are serous epithelial ovarian cancers but not ovarian epithelium cancers. They originate from the epithelium and endometrium of the distal fallopian tube (Figure 7).

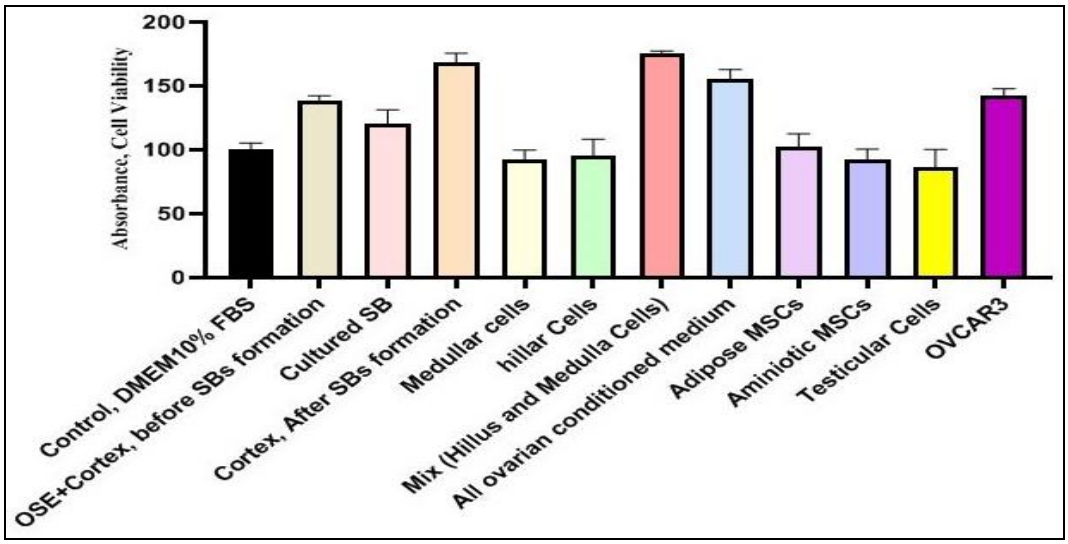

Figure 7. The effect of CMs on CCs after 72-h culture. The CMs from ovarian cortex and a mixture of (medulla + hilum) were the most effective media for supporting the growth of CCs. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

Effect of Serum (FBS, BSA, HSA)

Regarding FBS concentration, the results showed that 20% FBS significantly increased the growth of CCs. Also, culture medium supplementation with both FBS and HSA can be more efficient than 10% FBS, 1% HSA, and 2% HSA, alone (Supplementary Figures 4 and Fig 6). Although all concentrations of 3-25 mg/ml of HSA increased CCs growth, only the concentrations of 10 mg/ml (P<0.05), 12 mg/ml (P<0.01), 20 mg/ml (P<0.01), and 25 mg/ml (P<0.01) significantly increased cell proliferation and viability (Figure 8).

Regarding the use of BSA, all the used concentrations including 3 mg/ml (P<0.01), 6 mg/ml (P<0.01), 12 mg/ml (P<0.01) and 24 mg/ml (P<0.001) showed a significant increase in cell proliferation and viability (Figure 8).

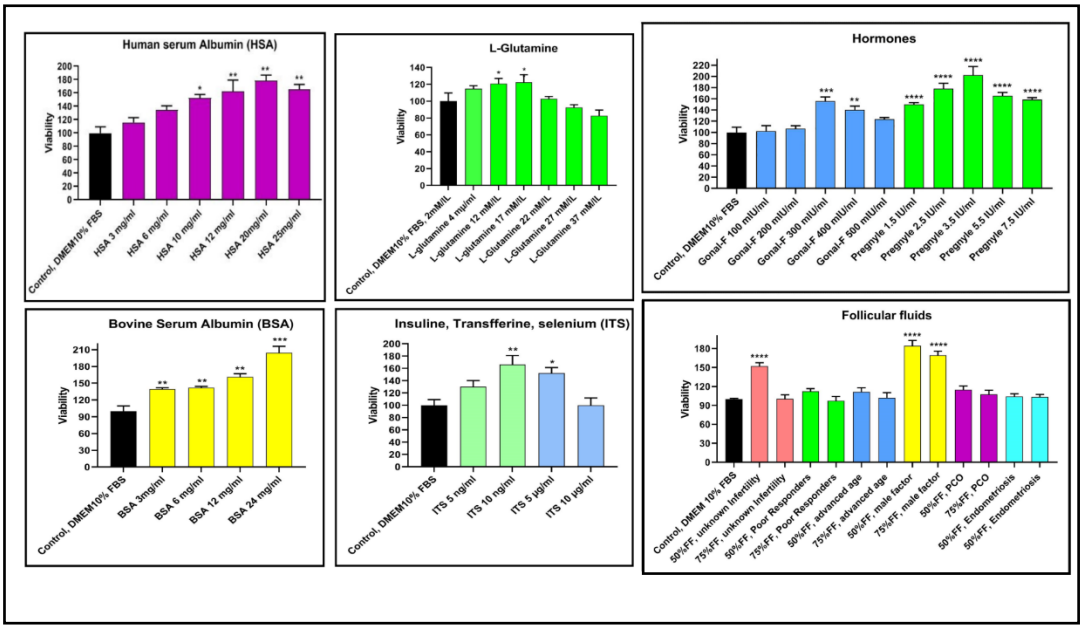

Figure 8. The effect of hormones, FFs, serums, ITS, and L-Glutamine on CCs after 72-h culture.
* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

Effect of L-Glutamine and ITS

Using L-Glutamine is recommended as 2-2.8 mM/ml in basal medium. Therefore, several concentrations of L-Glutamine were compared with DMEM + (2 mM/ml L-Glutamine) as control. Various concentrations of L-Glutamine including 2, 4, 12, 17, 22, 27, and 37 mM/ml were used. Among them, the concentrations of 12 mM/ml (P<0.05) and 17 mM/ml (P<0.05) of L-Glutamine showed higher cell proliferation

and viability rates. 17 mM/ml ($P<0.05$) had the most considerable increasing effect on cell growth and viability (Figure 8).

Regarding ITS, the concentrations of 5, 10, and 5 µg/ml had higher viability rates than the control. But only two concentrations of ITS [10 ng/ml ($P<0.01$) and 5 µg/ml ($P<0.05$)] led to a significant increase in the CCs growth. The concentration of 10 ng/ml ($P<0.01$) had greater effect on the CCs growth (Figure 8).

Effect of Hormones on Cumulus Cells Viability and Growth

Regularly, FSH-based drugs are prescribed for women who referred for infertility treatments. In this study, two FSH-based drugs, Gonal-F and Pregnyl, were used. The results showed that 300 mIU/ml of Gonal-F was the best concentration for proliferation and viability maintenance of CCs ($P<0.001$) followed by 400 mIU/ml of Gonal-F ($P<0.01$). The concentrations of 100 and 200 mIU/ml Gonal-F were not so efficient, compared to the control and 500 mIU/ml increased cell viability and proliferation but it was not significant. On the other hand, all the concentrations of Pregnyl (1.5-7.5 IU/ml) caused a significant increase in CCs viability ($P<0.0001$). Among them, 3.5 IU/ml Pregnyl, followed by 2.5 IU/ml Pregnyl significantly increased cell viability and growth. The results showed that Pregnyl (FSH and LH like drug) was more efficient than Gonal-F (FSH-like drug) for CCs in vitro proliferation (Figure 8).

Effect of Follicular Fluid on Cumulus Cells Viability and Growth

FFs were collected from six groups of patients including unknown infertility, poor responders, advanced age, male factor infertility, PCOS, and endometriosis (Table 2). 50% and 75% of FF were added to basal culture media. Generally, the results showed that adding 50% FF is more efficient than 75%. So, 50% and 75% of FF collected from the patients with male factor infertility significantly increased cells growth and viability ($P<0.0001$), followed by 50% FF from patients with unknown infertility ($P<0.0001$). Although the other FFs did not decrease CCs proliferation and viability, they had no significant increasing effect on increasing cell proliferation and viability (Figure 8).

The Morphology of Cumulus Cells in the Presence of Various Supplements, FFs, and Hormones

Based on our previous experience (not published data), CCs cells had slower proliferation rate than other ovarian cells. Without supplements, they did not show considerable incoherent interactions and no cellular aggregation was observed. Instead, in the presence of some materials such as Pregnyl, Gonal-F, FFs, ITS, and L-Glutamine, it was observed that CCs formed some aggregates similar to the colony-forming unit (CFU) or follicle unity. The listed structures were very similar to the natural spatial shape of growing follicles. It means that in the presence of Pregnyl, Gonal-F, FF, ITS, and L-Glutamine, cells can rearrange and demonstrate their potential 3-dimensional morphology. These colony-like structures were more compact and uniform in the presence of HCG and FF, collected from patients with male factor infertility (Supplementary Figure 5).

Discussion

In the present study, the effects of different basal and CMs, supplements, FFs and hormones on in vitro growth of CCs were compared. It was tried to examine the most required components for follicle in vitro culture, and the effect of different basal media with FBS (10% and 20%) and HSA (1% and 2%) on CCs were compared. The results showed that FBS was more efficient than HSA for in vitro proliferation of CCs. Also, 20% FBS was more superior to 10% FBS, while increasing the concentration of HSA could not support the growth of CCs, alone. For CCs, α-MEM was the best medium if 10% FBS was used, and DMEMF12 was superior if 20% FBS was applied. Also, the combination of both FBS and HSA had a positive impact on CCs growth.

There are two types of media for FIVGs, namely, basal and maturation media. These media are supposed to support cell survival, proliferation, and function. They also support both oocyte and CCs requirements. The applied media in FIVG are classified into: 1) basal media like MEM, Waymouth's medium, DMEM and McCoy's 5A medium, 2) balanced salt solutions like Earle's balanced salt solutions (EBSS), and 3) mixed media like DMEM + F12 and α-MEM + Glutamax (11).

Many studies on FIVG used α-MEM medium for short- and long-term culture in

human and different animals (12, 13). While in the present study, it was revealed that (DMEMF12 + 20% FBS) or (DMEMF12 + 20% FBS + 2% HSA) and (DMEM HG + 20% FBS + 2% HSA) showed higher cell viability and proliferation for CCs than α-MEM. DMEMF12 and DMEM HG (+ FBS + HSA) which are suggested for CCs.

Culture media have some basic components including amino acids, proteins and peptides, carbohydrates, fatty acids and lipids, vitamins, inorganic salt, serum, buffering systems [N-2-hydroxyethylpiperazine-N-ethanesulfonic acid (HEPES), phenol red, CO_2 bicarbonate], trace elements, and antibiotics. α-MEM has the minimum essential materials for cell culture, while DMEM and DMEMF12 have more components. It was concluded that α-MEM + 20% FBS and DMEMF12 + 20% FBS can be used for culture of ovarian stromal cells (OSCs) and CCs, respectively. So, it seems that OSCs can grow with the minimum essential materials but CCs need more constituents. According to the results of the present study, DMEMF12 is the best media for OSCs and CCs, which is consistent with the results of previous studies (14, 15). Inconsistent with the results of the present study, Bukovsky *et al.* (2005) used DMEM HG and DMEMF12 in the presence of 20% FBS for in vitro folliculogenesis and oogenesis (16) and DMEMF12 supplied with 15% FBS for in vitro culture of porcine CCs was used (17). Contrary to this study, some other studies used α-MEM to culture ovarian follicles (6).

It was proposed that phenol red-free DMEMF12 medium is superior, because phenol red is a weak estrogen with obvious biological effects (18). Therefore, some studies have suggested RPMI media for in vitro culture of CCs, because it is mostly free from phenol red (16). But the results of this study did not show any beneficial effect with RPMI on the CCs growth. RPMI 1640 is the most enriched medium followed by DMEMF12. Many studies have suggested RPMI for the culture of normal and tumor cells. But in this study, this enriched medium was reported to be not suitable for CCs culture.

One of the differences between the cell culture media is the concentration of pyruvate and glucose. All media have pyruvate and were categorized as high- and low-glucose media. The amount of these components is important because the oocyte of growing follicle preferentially metabolizes pyruvate to glucose, but the somatic compartments of ovarian follicles are more glycolytic. Glucose metabolism in cumulus/oocyte complex is very complicated during hormone-stimulated stages (19). Cells need glucose or pyruvate for their growth. It has been confirmed that most of the mammalian oocytes prefer pyruvate as the energy substrate and have a low glycolytic activity. While, CCs prefer glucose to pyruvate. Primordial follicles consume 2-fold more pyruvate than glucose, probably due to the lowest number of surrounding CCs. In growing follicles (from primary to secondary stage), the CCs proliferated and increased in numbers, so glucose consumption and lactate production also increased. This pattern changes approximately at the time of antrum formation, since antral follicles become predominantly glycolytic (20). This may be because DMEM HG was superior to LG in terms of CCs culture in the present study.

In addition to basal medium components, CCs growth requires some other materials. They are categorized into gonadotropins, serums as the protein source, and survival factors like ITS and growth/paracrine factors (11). So, in the present study, different kinds of serum (FBS, has, and BSA), ITS, L-Glutamine, and hormones, were compared. Since the growth factors are expensive, it was tried to compensate the lack of growth factors with FF and serums, which are enriched with various growth factors.

Another part of the present research was on FSH-like drugs. It was concluded that 300 mIU/ml Gonal-F as a FSH-like drug and 3.5 IU/ml Pregnyl as a FSH- and LH-like drug have the most increasing effect on CCs growth. The growing follicles are FSH-dependent, and FSH supplementation can improve the follicle growth as well as oocyte maturation. So, FSH is not only necessary for CCs growth, but also, essential for oocyte nucleus maturation. It causes more growth and differentiation in primary antral follicles. It is also essential for the steroidogenesis and regulates the relationship between oocyte and GCs (8). All pervious researches increased the rate of follicle survival by adding FSH to the culture medium (11). The controversy is over the FSH dosages. A minimal concentration of 10 mUI/ml of FSH is essential for FIVG of intact preantral follicles (8). Javed *et al.* added 10-200 mIU/ml FSH to the culture medium. They observed that

because of adding 100 mIU/ml FSH to the culture medium, follicles survival, diameters, germinal vesicles breakdown, and oocyte maturation rates in mice were increased (12). It was reported that 10-µg/mL insulin and 100-µg/mL FSH could improve the in vitro meiotic resumption rate for caprine preantral follicles. Barros *et al.* used fixed or sequential concentrations of FSH. They showed that a sequential concentration (750 ng/mL) of recombinant human FSH (compared to control 1000 ng/mL) improved oocyte and follicle growth and maturation (22). In the present study, different concentrations (100-500 mIU/ml) of Gonal-f were compared and it was revealed that only 300 and 400 mIU/ml concentrations increased CCs growth and viability, significantly.

Several dosages of Pregnyl were also compared. It is composed of alpha and beta subunits. The alpha is identical to LH, FSH, and alpha subunit of thyroid stimulating hormone. So, its function is similar to both LH and FSH. LH plays an important role in follicle maturation, supports follicle growth, and induces ovulation. But it has not been considered as an essential component of FIVG culture medium. However, its positive effect on follicle growth has been actively discussed (23). HCG and LH bind to the same FSH receptor but activate different signaling pathways (24). Media were supplemented with HCG stimulate in vitro maturation of oocyte. HCG has a more significant luteinizing effect than LH (6), while LH shows larger follicular diameters than HCG (25). Most studies used 1.5 IU/ml HCG (23), but the results of the present study showed that 3.5 IU/ml of Pregnyl has more significant effect on growth of CCs. It was shown that FSH and LH supplementation in a serum-free medium caused a decrease in DNA fragmentation in GCs and an increase in DNA fragmentation in theca cells (26). It is concluded that HCG alone can lead to higher CCs proliferation rates than FSH, which can be due to its slight LH-like effect.

Serum is another component of culture medium. It reduces estradiol secretion for an unknown reason. It has been shown that during FIVG of in vitro preantral follicle, the effects of FSH steroidogenic and mitogenic changed (27). In the present study, various kinds of serums were evaluated. They contain various growth factors and hormones. They help cells attach to other cells and surfaces, so they act as a spreading agent. They could be as a buffering agent and/or a binding protein. Finally, they decrease the mechanical damages to cells. Also, they have some disadvantages including different compounds in various samples, which may cause some inhibitors and the risk of contamination (27).

Three kinds of serums for CCs growth (FBS, HSA, and BSA) were evaluated. FBS has some advantages in cell culture including stimulatory factors and a low concentration of immunoglobulin (28). BSA has been shown to improve in vitro follicular development (7). In simplified medium, replacement of FBS with BSA creates a defined medium that provides better conditions for oocyte in vitro maturation (8). Regarding HSA, the combination of (HSA + ITS) has been shown to reduce atretic follicles and increase healthy follicles rate and follicle size. HSA promotes cell proliferation as well, and acts as a free-radical and reactive oxygen species (ROS) scavenger (8). Some concentrations of HSA and BSA from 3-25 mg/ml were added in this study. Compared to control, all the concentrations increase CCs proliferation and viability. Unlike HSA, 3 and 6 mg/ml of BSA significantly increased CCs growth. It was also concluded that HSA alone had no beneficial effect on ovarian and CCs growth, whereas in combination with FBS, it caused considerable growth.

Also, CCs need amino acids. L-Glutamine is an essential amino acid for both ovarian and CCs. Generally, its amount is set at about 2 mM, but it can vary from 0.5 to 10 mM, depending on the cell and media types (from 0.68 mM in medium 199 to 4 mM in DMEM). Arginine, glutamine, and leucine used in the ovarian tissue culture, have been shown to accelerate in vitro activation of primordial follicles in the ovaries of a 1-day-old mouse (29). Some combinations of glutamine are available including L-glutamine, GlutaMax or GlutaGro. L-glutamine is almost unstable and degrades over time in the refrigerator and faster in the incubator, but GlutaMax is more stable. Therefore, adding an extra amount of L-glutamine to culture medium does not harm the cells, it may even be a necessary component especially for media nearing their expiration date (30). Glutamine supports the growth of cells that have high energy requirements. It also synthesizes large amounts of nucleic acids and proteins. It acts as an alternative energy source for the cells that use glucose inefficiently or

rapidly dividing cells. Thus, when glucose level is low but energy demand is high, cells can metabolize amino acids such as glutamine, which are the most readily available to use as energy (https://www.sigmaaldrich.com/life-science/cell-culture/learning-center/media-expert/glutamine.html). Most of the media are supplemented with 2 mM/ml L-Glutamine (31). In the present study, 4-37 mM/ml L-Glutamine was used. The results showed that 12 and 17 mM/ml L-Glutamine caused higher CCs growth compared to 2 mM/ml. But L-Glutamine with a concentration higher than 22 mM/ml was detrimental for CCs growth. Altogether, L-Glutamine accelerates in vitro growth of CCs and in vitro activation of primordial follicles (31).

ITS or insulin, transferrin and selenium are other components used in FIVG. Glucose is an essential factor for CCs growth. Its metabolism is influenced by ovarian growth factors and insulin. Insulin also increases the uptake of metabolic precursors like amino acids. So, for FIVG, insulin is added to culture medium at the dosage of 5 mg/ml (supra physiological concentration). Insulin along with selenium and transferrin, act as survival factors. But large amounts of insulin, mimic insulin-resistant model, exacerbated apoptosis in GCs (32).

Selenium is another essential trace mineral that is relevant to various pathophysiological processes (32). It is also added to IVG medium. But high doses of selenium reduce the rate of proliferating primordial follicles. It regulates the 17 β-estradiol biosynthesis and the growth of GCs in adult ovaries (33). Heat stress induces apoptosis in various cells. Selenium is effective in maintaining the cellular physiologic functions and protects cells against chronic heat stress- induced apoptosis in GCs (34).

Transferrin is the carrier of iron, a mandatory requirement of cells. These cells should have transferrin receptor for intracellular transport of iron. It was expressed in a subpopulation of human granulosa lutein cells isolated from the follicular puncture. Normally, the concentration of transferrin and iron increases in FF with the development of follicular maturation. Transferrin deficiency causes iron overload and leads to oocyte dysmaturity. Small growing follicles with 1-2 layers of GCs contain cytoplasmic transferrin in their cuboidal GCs (35).

Previous studies used various dosages of ITS from 10 ng/ml to 10 µg/ml. In this study, four concentrations (from 5 and 10 ng/ml to 5 and 10 µg/ml) were used. The results showed that 10 ng/ml followed by 5 µg/ml caused higher viability and growth of GCs.

Also, the effect of FF on CCs growth was compared. FFs were isolated from 6 groups of patients. The results showed that 50% and 75% FFs, isolated from patients with male factor infertility (healthy women) significantly increased CCs growth, followed by 50% FF of infertile patients with unknown causes. Also, 50% FF was superior to 75%. FF contains estradiol, progesterone, and testosterone. The level of steroids is correlated with follicular diameter. According to a study by da Silveira et al., FF progesterone level is 6100 times higher than estradiol and 16,900 times higher than testosterone. FF has two types of vesicles, exosomes (50-150 nm) and micro-vesicles (100-1000 nm). The content of these vesicles are bioactive components such as proteins, mRNAs, lipids, and miRNAs (36). FF is a plasma filtration with a wide dynamic range of proteins. These proteins are involved in metabolic processes, cellular processes, cellular communication, and immune responses (37). Also, FF has some types of GCs called immortalized GCs (38). Although the FF of patients with PCO, endometriosis, advanced aged patients, and poor responders did not have a positive effect on the CCs growth and viability, compared to control group, they did not have a negative effect either.

In this study, CMs collected from the ovarian cortical cells significantly improved CCs growth. It is not implausible, because cortex is the natural place of ovarian follicles that makes the microenvironment. Interestingly, the mixture of CMs from medullar and hilar cells also caused the growth of CCs as much as cortical CM. This data suggest that ovarian medulla and hilum may be as important as the ovarian cortex for follicular growth.

The results also demonstrated that the isolated cells of SB, cortex, medulla, and hilum expressed GDF9. It is an essential factor for follicular symphony and acts as a regulator of ovulation, folliculogenesis, and oocyte quality (39). Interestingly, its expression was higher in medulla and hilum. In the poor responder patients, it was shown that the expression of GDF9 decreased with age in FF and GCs, leading to reduced assisted reproductive outcome (40). Also, GDF9 enhances the proliferation and metabolism of CCs and GCs

and acts as a CCs expansion factor (41). In mice, GDF9 promotes the development of CCs with the help of oestrogen (42). In the present study, the addition of ovarian cells CMs could enhance in vitro proliferation of CCs. One of the suggested reasons may be related to GDF9. In our studies, cortical cells, medulla and even hilum expressed GDF9, and MTT results of CMs on CCs demonstrated that both cortex and the combination of (hilum and medulla) CMs presented the same results.

Also, CMs collected from ADMSCs and AFMSCs have a positive effect on CCs growth, but this effect was not as much as the effect of ovarian cells CMs. In parallel to the present study, previous studies used CM, collected from mesenchymal stem cells for in vitro maturation and subsequent development of oocyte (43). It was shown that ADMSCs can promote the early stages of follicles survival, growth, and maturation. They secrete factors that promote the early stages of follicle growth (44). Also, ADMSCs have shown to preserve ovarian follicle after chemotherapy (10) and they have the ability to differentiate into primordial follicle (45). In a study on the expression of ovarian cells mesenchymal markers (still not published data), it was concluded that cells in all parts of the ovary express mesenchymal markers. Thus, another reason that CM of ovarian cells could support CCs growth is probably the mesenchymal nature of these cells.

They are many unknown factors which are involved in ovarian follicular development and CCs proliferation. By applying FFs and CMs which are enriched with growth factors, hormones, and other efficient elements, follicular growth can be supported at a lower cost.

Conclusion

The present study confirmed that OCs and CCs require different growth needs, and media with different materials can support in vitro growth. It was also concluded that in vitro growth of ovarian cells in a basal medium can progress to even higher passages, while CCs meet some other requirements such as hormone, serums, ITS, L-Glutamine, and growth factors for further growth. Moreover, synthetic growth factors can be replaced by the addition of CMs, isolated from ovarian cells and FFs, collected from healthy women, which are more economical and closer to normal body conditions. Thus, a cocktail of basal media supplemented with other complements, is essential for in vitro culture of CCs, and finally, follicles.

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Author's contributions

Moshrefi Mojgan: Conceptualization, methodology, design, formal analysis and investigation, material preparation, data collection and analysis, literatures collection, original draft preparation, and final approval of the manuscript.

Aflatoonian Abbas: Scientific consulting, scientific review and editing and final approval of the manuscript.

Ghasemi-Esmailabad Saeed: Design, methodology, material preparation, data collection, and final approval of the manuscript.

Mojgan Karimi-Zarchi: Ovarian tissue providing and resources and final approval of the manuscript.

Fatemeh Sadeghian-Nodoushan: Scientific consulting, data collection, material preparation, and final approval of the manuscript.

Shahmohamadi Sajad: Data collection and final approval of the manuscript.

Habib Nikukar: Supervision, scientific consulting, review and editing, English revising and final approval of the manuscript.

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Declarations

Ethical approval

The project was approved by the Ethics Committee of Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran (Ethical code: IR.SSU.RSI.REC.1396.21). It was found to be in accordance to the ethical principles and the national norms and standard for conducting medical research in Iran.

Consent to participate
Human ovarian tissues were retrieved after obtaining informed signed consent.

Data availability statement
The data that support the findings of this study are not publicly available, but they are available on request from the corresponding author, if required.

Conflict of interests
The authors declare that they have no conflict of interests.

Software application
The figures were prepared and grouped with publisher 2010 and resized via Photoshop.

References

1. Rivas Leonel EC, Lucci CM, Amorim CA. Cryopreservation of human ovarian tissue: A review. Transfus Med Hemother. 2019; 46(3):173-81. doi: 10.1159/000499054.
2. Zhai J, Yao G, Dong F, Bu Z, Cheng Y, Sato Y, et al. *In vitro* activation of follicles and fresh tissue auto-transplantation in primary ovarian insufficiency patients. J Clin Endocrinol Metab. 2016; 101(11):4405-12. doi: 10.1210/jc.2016-1589.
3. Telfer EE. Future developments: *In vitro* Growth (IVG) of human ovarian follicles. Acta Obstet Gynecol Scand. 2019; 98(5):653-58. doi: 10.1111/aogs.13592.
4. Zhou CJ, Wu SN, Shen JP, Wang DH, Kong XW, Lu A, et al. The beneficial effects of cumulus cells and o20ocyte-cumulus cell gap junctions depends on oocyte maturation and fertilization methods in mice. Peer J. 2016; 4:1761. doi: 10.7717/peerj.1761.
5. Xiao GY, Cheng CC, Chiang YS, Cheng WT, Liu IH, Wu SC. Exosomal miR-10a derived from amniotic fluid stem cells preserves ovarian follicles after chemotherapy. Sci Rep. 2016; 6:23120. doi: 10.1038/srep23120.
6. Skory RM, Xu Y, Shea LD, Woodruff TK. Engineering the ovarian cycle using *in vitro* follicle culture. Hum Reprod. 2015; 30(6):1386-95. doi: 10.1093/humrep/dev052.
7. Rodrigues GQ, Silva CMG, Faustino LR, Bruno JB, Magalhaes DM, Campello CC, et al. Bovine serum albumin improves *in vitro* development of caprine preantral follicles. Anim Reprod. 2010; 7(4):382-88.
8. Bahrami M, Morris MB, Day ML. Amino acid supplementation of a simple inorganic salt solution supports efficient *in vitro* maturation (IVM) of bovine oocytes. Sci Rep. 2019; 9:11739. doi: 10.1038/s41598-019-48038-y.
9. Abedelahi A, Salehnia M, Allameh AA. The effects of different concentrations of sodium selenite on the *in vitro* maturation of preantral follicles in serum-free and serum supplemented media. J Assist Reprod Genet. 2008; 25(9-10):483-8. doi: 10.1007/s10815-008-9252-z.
10. Xiao S, Zhang J, Romero MM, Smith KN, Shea LD, Woodruff TK. *In vitro* follicle growth supports human oocyte meiotic maturation. Sci Rep. 2015; 5:17323. doi: 10.1038/srep17323.
11. Simon LE, Kumar TR, Duncan FE. *In vitro* ovarian follicle growth: A comprehensive analysis of key protocol variables†. Biol Reprod. 2020; 103(3):455-70. doi: 10.1093/biolre/ioaa073.
12. Magalhães-Padilha DM, Fonseca GR, Haag KT, Wischral A, Gastal MO, Jones KL, et al. Long-term *in vitro* culture of ovarian cortical tissue in goats: Effects of FSH and IGF-I on preantral follicular development and FSH and IGF-I receptor mRNA expression. Cell Tissue Res. 2012; 350(3):503-11. doi: 10.1007/s00441-012-1498-1.
13. Higuchi CM, Maeda Y, Horiuchi T, Yamazaki Y. A Simplified method for three-dimensional (3-d) ovarian tissue culture yielding oocytes competent to produce full-term offspring in Mice. PLoS One. 2015; 10(11):e0143114. doi: 10.1371/journal.pone.0143114.
14. Tagler D, Makanji Y, Anderson NR, Woodruff TK, Shea LD. Supplemented αMEM/F12-based medium enables the

survival and growth of primary ovarian follicles encapsulated in alginate hydrogels. Biotechnol Bioeng. 2013; 110(12):3258-68. doi: 10.1002/bit.24986.

15. Asaduzzman M, Cui X, Zhang H, Young F. Three dimensional *in vitro* culture of murine secondary follicles in a defined synthetic matrix. Journal of Biomaterials and Nanobiotechnology 2018; 9:244-62. doi: 10.4236/jbnb.2018.93014.
16. Bukovsky A, Svetlikova M, Caudle MR. Oogenesis in cultures derived from adult human ovaries. Reprod Biol Endocrinol. 2005; 3:17. doi: 10.1186/1477-7827-3-17.
17. Yang L, Du X, Liu L, Cao Q, Pan Z, Li Q. miR-1306 mediumstes the feedback regulation of the TGF-β/SMAD signaling pathway in granulosa cells. Cells. 2019; 8(4):298. doi: 10.3390/cells8040298.
18. Guzel Y, Bildik G, Dilege E, Oktem O. Sphingosine-1-phosphate reduces atresia of primordial follicles occurring during slow-freezing and thawing of human ovarian cortical strips. Mol Reprod Dev. 2018; 85(11):858-64. doi: 10.1002/mrd.23043.
19. Collado-Fernandez E, Picton HM, Dumollard R. Metabolism throughout follicle and oocyte development in mammals. Int J Dev Biol. 2012; 56(10-12):799-808. doi: 10.1387/ijdb.120140ec.
20. Guzel Y, Oktem O. Understanding follicle growth *in vitro*: Are we getting closer to obtaining mature oocytes from in vitro-grown follicles in human? Mol Reprod Dev. 2017; 84(7):544-59. doi: 10.1002/mrd.22822.
21. Javed A, Rezaei-Zarchi S, Anvari M, Ghani MJ, Barzegari Firouzabadi F, Jamil A, et al. Determination of effective dosage of FSH and hCG in the maturation of preantral follicles and enclosed oocytes in mice. Iranian Journal of Reproductive Medicine. 2008; 6(5):199-204.
22. Barros VRP, Monte APO, Lins TLBG, Santos JM, Menezes VG, Cavalcante AYP, et al. In vitro survival, growth, and maturation of sheep oocytes from secondary follicles cultured in serum-free conditions: Impact of a constant or a sequential medium containing recombinant human FSH. Domest Anim Endocrinol. 2019; 67:71-9. doi: 10.1016/j.domaniend.2018.12.003.
23. Filatov M, Khramova Y, Parshina E, Bagaeva T, Semenova M. Influence of gonadotropins on ovarian follicle growth and development in vivo and *in vitro*. Zygote. 2017; 25(3):235-43. doi: 10.1017/S0967199417000168.
24. Choi J, Smitz J. Luteinizing hormone and human chorionic gonadotropin: Origins of difference. Mol Cell Endocrinol. 2014; 383(1-2):203-13. doi: 10.1016/j.mce.2013.12.009.
25. Park KE, Ku SY, Jung KC, Liu HC, Kim YY, Kim YJ, et al. Effects of urinary and recombinant gonadotropins on *in vitro* maturation outcomes of mouse preantral follicles. Reprod Sci. 2013; 20(8):909-16. doi: 10.1177/1933719112468948.
26. Monga R, Sharma I, Datta TK, Singh D. Characterization of serum-free buffalo granulosa cell culture and analysis of genes involved in terminal differentiation from FSH- to LH-responsive phenotype. Domest Anim Endocrinol. 2011; 41(4):195-206. doi: 10.1016/j.domaniend.2011.07.001.
27. Adriaens I, Cortvrindt R, Smitz J. Differential FSH exposure in preantral follicle culture has marked effects on folliculogenesis and oocyte developmental competence. Hum Reprod. 2004; 19(2):398-408. doi: 10.1093/humrep/deh074.
28. Pazoki H, Eimani H, Farokhi F, Shahverdi A, Yazdi RS, Tahaei LS. Comparing the growth and the development of mouse pre-antral follicle in medium with PL (Platelet Layset) and with FBS. Middle East Fertility Society Journal. 2015; 20(4):231-6. doi: 10.1016/j.mefs.2015.01.006.
29. Alborzi P, Jafari Atrabi M, Akbarinejad V, Khanbabaei R, Fathi R. Incorporation of arginine, glutamine or leucine in culture medium accelerates *in vitro* activation of primordial follicles in 1-day-old mouse ovary. Zygote. 2020; 1-8. doi: 10.1017/S096719942000026X.

30. Arora M. Cell culture mediums: A review. Mater Methods. 2013; 3:175. doi: 10.13070/mm.en.3.175.

31. Campen KA, Lavallee M, Combelles C. The impact of bisphenol S on bovine granulosa and theca cells. Reprod Domest Anim. 2018; 53(2):450-7. doi: 10.1111/rda.13130.

32. Ni XR, Sun ZJ, Hu GH, Wang RH. High concentration of insulin promotes apoptosis of primary cultured rat ovarian granulosa cells via its increase in extracellular HMGB1. Reprod Sci. 2015; 22(3):271-7. doi: 10.1177/1933719114549852.

33. Qazi IH, Angel C, Yang H, Pan B, Zoidis E, Zeng CJ, et al. Selenium, selenoproteins, and female Reproduction: A review. Molecules. 2018; 23(12):3053. doi: 10.3390/molecules23123053.

34. Xiong Y, Yin Q, Jin E, Chen H, He S. Selenium attenuates chronic heat stress-induced apoptosis via the inhibition of endoplasmic reticulum stress in mouse granulosa cells. Molecules. 2020; 25(3):557. doi: 10.3390/molecules25030557.

35. Li A, Ni Z, Zhang J, Cai Z, Kuang Y, Yu C. Transferrin insufficiency and iron overload in follicular fluid contribute to oocyte dysmaturity in infertile women with advanced endometriosis. Front Endocrinol (Lausanne). 2020; 11:391. doi: 10.3389/fendo.2020.00391.

36. da Silveira JC, Andrade GM, Del Collado M, Sampaio RV, Sangalli JR, Silva LA, et al. Supplementation with small-extracellular vesicles from ovarian follicular fluid during *in vitro* production modulates bovine embryo development. PLoS One. 2017; 12(6):e0179451. doi: 10.1371/journal.pone.0179451.

37. Zamah AM, Hassis ME, Albertolle ME, Williams KE. Proteomic analysis of human follicular fluid from fertile women. Clin Proteomics. 2015; 12(1):5. doi: 10.1186/s12014-015-9077-6.

38. Ai A, Tang Z, Liu Y, Yu S, Li B, Huang H, et al. Characterization and identification of human immortalized granulosa cells derived from ovarian follicular fluid. Exp Ther Med. 2019; 18(3):2167-2177. doi: 10.3892/etm.2019.7802.

39. Sanfins A, Rodrigues P, Albertini DF. GDF-9 and BMP-15 direct the follicle symphony. J Assist Reprod Genet. 2018; 35(10):1741-50. doi: 10.1007/s10815-018-1268-4.

40. Gong Y, Li-Ling J, Xiong D, Wei J, Zhong T, Tan H. Age-related decline in the expression of GDF9 and BMP15 genes in follicle fluid and granulosa cells derived from poor ovarian responders. J Ovarian Res. 2021; 14(1):1-10. doi: 10.1186/s13048-020-00757-x.

41. Chang HM, Qiao J, Leung PC. Oocyte-somatic cell interactions in the human ovary-novel role of bone morphogenetic proteins and growth differentiation factors. Hum Reprod Update. 2016; 23(1):1-18. doi: 10.1093/humupd/dmw039.

42. Sugiura K, Su YQ, Li Q, Wigglesworth K, Matzuk MM, Eppig JJ. Estrogen promotes the development of mouse cumulus cells in coordination with oocyte-derived GDF9 and BMP15. Mol Endocrinol. 2010; 24(12):2303-14. doi: 10.1210/me.2010-0260.

43. Ling B, Feng DQ, Zhou Y, Gao T, Wei HM, Tian ZG. Effect of conditioned medium of mesenchymal stem cells on the *in vitro* maturation and subsequent development of mouse oocyte. Braz J Med Biol Res. 2008; 41(11):978-85. doi: 10.1590/s0100-879x2008005000053.

44. Green LJ, Zhou H, Padmanabhan V, Shikanov A. Adipose-derived stem cells promote survival, growth, and maturation of early-stage murine follicles. Stem Cell Res Ther. 2019; 10:102. doi: 10.1186/s13287-019-1199-8.

45. Yu X, Wang N, Qiang R, Wan Q, Qin M, Chen S, et al. Human amniotic fluid stem cells possess the potential to differentiate into primordial follicle oocytes *in vitro*. Biol Reprod. 2014; 90(4):73. doi: 10.1095/biolreprod.113.112920.