How to Support the *In Vitro* Culture of Human Ovarian Cells and Follicles? A Preliminary Step for Assembling an Artificial Ovary

Mojgan Moshrefi
medical nanotechnology and Tissue engineering reserach center, Yazd reproductive sciences institute, Shahid Sadoughi university of medical sciences, Research and clinical center for infertility, Yazd reproductive sciences institute, Shahid sadoughi university of medical sceinces

Abbas Aflatoonian
Research and clinical center for infertility, Yazd reproductive sciences institute, Shahdi sadoughi university of medical sciences

Saeed Ghasemi-Esmailabad
Clinical Research Center

Mojgan Karimi-Zarchi
Iran University of Medical Sciences

Fatemeh Sadeghian-Nodoushan
medical nanotechnology and tissue engineering research center, Yazd reproductive sciences institute, Shahid Sadoghi university of medical sceinces, Yazd, Iran

Sajad Shahmohamadi
Medical Nanotechnology and tissue engineering reserahc center, Yazd reproductive sciences institute, Shahid sadoughi university of medical sciences, Yazd, Iran

Habib nikukar (habibnik@gmail.com)
Yazd University of Medical Science: Shahid Sadoughi University of Medical Sciences and Health Services

Research

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Abstract

Background

The first step in assembling an artificial ovary is supporting the in vitro growth of ovarian cells/follicles. Therefore, proliferation of granulosa or cumulus cells (CCs) is important and due to limited proliferation, improving the mediums for *in vitro* propagation of these cells is helpful.

Objective

This study aimed to characterized the appropriate media and supplements for *in vitro* culture of ovarian cells (OCs) and CCs.

Material & Methods

Cortical, medullar and hilar cells of ovary were cultured and their conditioned medium (CMs) collected. The expression of GDF9, as a key factor for ovarian follicular growth, was evaluated. CCs were collected from healthy women, who referred due to male factor infertility. To choose the optimum basal medium, a mixture of OCs was cultured with basal mediums, supplemented with two concentrations of fetal bovine serum (FBS) and human serum albumin (HSA). The cocktails were as follows: [Serum free mediums], [mediums+10%FBS], [mediums+20%FBS], [mediums+1%Alb)], [mediums+2%Alb], [mediums+10%FBS+1%Alb], [mediums+10%FBS+2%Alb], [mediums+20%FBS+1%Alb] & [mediums+20%FBS+2%Alb]. The same process was repeated for CCs. Also, the effect of various concentrations of L-Glutamine, bovine serum albumin (BSA), HSA, insulin transferrin selenium (ITS), Follitropin alfa® and Pregnyl® was evaluated on the growth of CCs. As well, CCs were treated with various concentrations of follicular fluids (FFs) and CMs. CMs were collected from ovarian, testicular, adipose & amniotic derived and ovarian carcinoma cells. FFs were collected from women with poor responders, endometriosis, advanced age, polycystic ovarian syndrome, male factor and unknown infertility. Then, CCs morphology, proliferation capacity and culture duration were evaluated.

Results

All the ovarian cells expressed GDF9. αMEM+20%FBS and DMEMF12+20%FBS were the most suitable cocktails for OCs and CCs, respectively. 20%FBS was superior to 10% for both OCs and CCs. HSA could not support the growth of OCs and CCs, alone. The cocktail of mediums with 20%FBS superior to the others. The CMs of cortical and (hillar+medullar) cells and FFs from male factor and unknown infertility patients 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 proliferation of CCs.

Conclusion

CMs, serums and FFs can support the CCs growth alongside with basal mediums, supplemented with hormones, ITS and L-Glutamine, which are cheaper and more accessible.

Background

Inability to getting pregnancy refers to infertility. The women are considered infertile after one year of trying to have child with normal sexual functions and usual intercourse. In females, infertility is more common than males (1). Conventionally, infertility treatment consists of an ovarian hyper stimulation cycle, followed by assisted reproductive technology (ART). But, due to various reasons, the conventional treatments sometime encounter unfortunate results. Therefore, gradually new methods have been developed to improve the results of infertility treatment such as follicular *in vitro* growth (FIVG). The ability to develop human oocytes by FIVG has many potential applications which are explained in the following.

1. Two and three dimensional FIVG can be used for the following aims: A) For cryopreserved tissue of recovered cancerous patients, as fertility preservation (2), B) For poor responder patients, C) For patients with premature ovarian failure (POF) (3) and D) For avoiding the follicles from Hippo signalling pathway (4).
2. FIVG, followed by *in vitro* maturation (IVM), can be used to achieve competence oocyte from primary stages follicles (alone or in co-culture with other cells like cumulus cells (CCs)) (5).

So, FIVG is the main process of ovarian tissue engineering, which tries to return the ovarian functions by means of the fabrication of an artificial ovary and engineering the ovarian cycle.

In all the upper-mentioned approaches, the main process is FIVG. The above options are explained in the following, by detail.

FIVG is a method for higher follicular recovery rate, especially in women who undergo cancer treatment and ovarian cryopreservation (2). During cryopreservation, the ovarian segments are cryopreserved as cortical strips. Re-implantation of cryopreserved segments has been the only option for using stored tissue (6) but it has the risk of reintroducing the probable malignant cells to body. Also, the rate of success in implanted tissue is largely low. Therefore, recently 3D ovarian follicle growth by reconstruction of an artificial ovary is in the spotlight (6). So, FIVG can be helpful by the culture of the follicles, isolated from the frozen cortical strips.

Another approach of FIVG is for the patients with poor response to ovarian hyperstimulation (poor responders). Recently it was suggested to activate the ovarian dormant follicles with *in-vitro* activation. It is a new treatment for poor responders and premature ovarian failure patients (3).

Also, one of the stimulators of ovarian follicle growth is the suppression of Hippo signalling pathway which consists of several negative regulators. The process of ovarian drilling and ovarian wedge resection are for suppress of this pathway. So, FIVG can be another method for keeping the ovarian follicle away from these inhibitory signals, which can improve the rate of *in vitro* folliclar growth (4).

Expansion of GCs and CCs is a key point in FIVG and oocyte competence. Also, some studies proposed that IVM results have been improved by co-culture with CCs of a mature oocyte. In the other word, *in vitro* expansion of CCs can improve the IVM results (5).

Although all of the upper-mentioned tactics have been developed, FIVG still needs to be improved due to many unknown factors, which are involved in GCs and CCs proliferation, ovarian follicle expansion and oocyte maturation (7). Therefore, optimisation of culture medium can be helpful.

The pre-antral follicle growth is gonadotropin independent and is based on a crosstalk between oocyte and GCs. But after antrum formation the gonadotropin dependant stages is started (8) and GCs are divided into two groups including 1) corona radiate and 2) CCs which are defined as the oocyte closely associated granulosa cells (GCs) that surround the oocyte. The second are the cells that line the inside of the antrum cavity. Indeed, antrum is characterized by two GCs subpopulations including: 1) the CCs surrounding the oocyte, and 2) the mural GCs that line the follicle wall. CCs participate directly in oocyte maturation and fertilization (9). When ovulation occurs, the oocyte throws into the fallopian tubes along with the CCs. Therefore, naturally during final stages of ovarian follicle development, the growth of CCs is the key point and all the FIVG based methods, need the support of CCs growth. Many materials and factors are proposed for optimising the medium for the *in vitro* culture of CCs. Taken together, the FIVG and tissues need the growth of ovarian cells (OCs) especially GCs and CCs. Therefore, the present study focuses on the efficacy of different cell culture mediums, hormones and supplements over the growth of human OCs and CCs.

Results

Ovarian Cells Isolation, Characterization and GDF9 Expression

In the current study, the cells of three parts of ovary were isolated including cortex & ovarian surface epithelium (OSE), medulla and hilum. The mixture of the cells of cortex and OSE exhibited the spindle form and epithelia like cells. Two types of cells morphology were identified (Fig. 4, A). Gradually after the first passage, some cells detached from the plate and proliferate while they were floating. In fact, they make the ovarian spheroid bodies (SBs) or ovarian germ cell nest (Fig. 4, B). The suspended SBs were isolated, cultured, expanded and they created colonies (Fig. 4, C). The cortical cells, left in culture, continued their proliferation (Fig. 4, D) and finally the cortical colony forming cells created the typical colonies (Fig. 5, E & F).

Regarding the ovarian medulla, during the first passage the cells showed fibroblastic like shape (Fig. 5, medulla A). Through the second passage the smaller cells with epithelial like shape, namely polygonal cells with regular dimensions were appeared (Fig. 5, medulla B & C) and they finally build ovarian medullar cells colonies (Fig. 5, medulla D). The majority of the cells of ovarian hilum, exhibited fibroblast like shape (Fig. 5, hilus A) which became more uniform in the next passages (Fig. 5, hilus B). They make colonies from the first passage onwards (Fig. 5, hilus C).

Also, immunocytochemistry analysis showed that the cells of cortex (17.72%), medulla (13.33%), hilum (18.10%) and SB (16.56%) expressed GDF9 marker. It was visualized green under fluorescent microscope (Fig. 6). It was interesting that its expression in the cells of medulla and hilus was higher than that of the cortex and SB cells.

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

The CCs were isolated from punctured dominant follicles. In the early days of culture, they were small cells with no elongation, appendages or branching (Fig. 7, A). Then, they started to become flat (Fig. 7, B) and exhibited short distance contact communications with the other cells by branched cytoplasmic extensions or protrusion, similar to dendritic cells arborisation (dendritic branching) (Fig. 7, C & D). Then they became large

cells with more cytoplasmic organelles like probably but not definitely “rough endoplasmic reticulum” (RER) (Fig. 7, E). The cells started to become round (Fig. 7, F & G), the cytoplasmic filaments increased; meanwhile the nucleus migrates from the margin to the center (Fig. 7, H & I). The cells organelles increased, especially a lot of granules were appeared which may probably attach and move on RER that surround the centrally located nucleus (Fig. 7J, K) (Fig. 8, A). Then the cells occupied with a lot of vesicles (Fig. 7, L) and vacuoles (Fig. 7, M), which are the characteristics of secretory cells. At this stage the communication of cells was increased with the extra cellular environment (Fig. 7, N & O). For example, in movie 1 some particles (probably vesicles) attached to cells, pass through the cells and exit, which probably caused some material exchange or macromolecule modifications. Also, since the cell size was increased, cytoplasmic streaming or protoplasmic streaming or cyclosis help the material exchange into and out of the cells. It helps to speed the transport of organelles and molecules around the cell, which is especial movement for large cells (unlike the small cells that diffusion is more rapid). For example, in movie 1 the organelles around the nucleus that seems to be granules on RER, are moving around the nucleus, while some material or macro molecules are entering the cells from left side and exit the cell from right side (Movie 1) (Fig. 8, B & C). Gradually the secretory vesicles were appeared in cells and filled the cytoplasm (Fig. 7, M) and the cytoplasmic space looked like a lot of cytoplasmic holes or cytoplasmic sacs (secretory vacuoles) (Fig. 7, N). However, the cells still had contact communication with the surrounding cells, which were at different stages of structural and functional development (Fig. 7, N & O).

Compared to other OCs, the CCs grow slowly and they do not have considerable *in vitro* proliferation in basal mediums, even over time. In a basal medium without any supplements, they divided very slowly and their maximum passage number is 3–4. If no supplements, the CCs have no considerable growth or for go for apoptosis, while culturing them in a medium enriched with the mentioned supplements, cause the CCs to be viable even after a month

In movie 2 an apoptotic cell is shown. The cell begins to shrink and membrane blebbing and bubbling is observed (Movie 2). While in movie 3, it seems that the early events of a necrotic cell is happening. Here the cell volume is increased and no sign of shrinkage is seen (Movie 3).

Effect of Different Mediums on Ovarian Cells Proliferation and Viability

To evaluate which basal cell culture medium is more efficient for *in vitro* proliferation of human OCs, five mediums were compared including α-MEM, DMEM HG, DMEM LG, DMEMF12 and RPMI. FBS 10 & 20% and Alb 1& 2% were used as additives. Since many studies used α-MEM + 10%FBS for ovarian follicular and tissue culture, at the first step all mediums + 10%FBS were compared with it (α-MEM + 10%FBS). The results showed that when cells are cultured with α-MEM + 10%FBS, the cell proliferation and percentages of viable cells are higher than other mediums, followed by DMEMF12 and DMEM HG. Compared to α-MEM, DMEM LG and RPMI reduced cell proliferation and viability, significantly ($P < 0.01$). So, α-MEM + 10%FBS, was chosen as the medium of high performance for OCs culture (Fig. 2).

Then 9 cocktails of each medium were prepared and the results of OCs proliferation were assessed by MTT assay after 72h cultivation in every media. Finally, the results compared with α-MEM + 10%FBS. The results

show that 20%FBS (α-MEM + 20%FBS) ($P < 0.0001$), DMEM LG + 20%FBS ($P < 0.01$) and DMEMF12 + 20%FBS ($P < 0.01$), caused more cell viability than α-MEM + 10%FBS. Also, in 10%FBS + 1%Alb group, DMEMF12 ($P < 0.0001$), and α-MEM (not significant) resulted to more cell viability. As well, in 20%FBS + 2%Alb group, DMEMF12 ($P < 0.0001$), and α-MEM ($P < 0.0001$) had higher cell proliferation. In the 10%FBS + 2%Alb group, DMEMF12 ($P < 0.001$) and α-MEM (not significant) showed higher cell proliferation and viability. Finally, the 20%FBS + 1%Alb group, DMEMF12 ($P < 0.0001$), and α-MEM ($P < 0.0001$) resulted to higher cell viability and proliferation, compared to the control (α-MEM + 10%FBS) (Figs. 9 & 11).

Altogether we concluded that for the culture of OCs, DMEMF12 and α-MEM are the choice mediums. They can be used with 20% FBS alone or with a combination of FBS and Alb. The comparison of all the groups showed that α-MEM + 20%FBS, followed by (DMEMF12 + 10%FBS + 1%Alb), (α-MEM or DMEMF12 + 20%FBS + 2%Alb) and (α-MEM or DMEMF12 + 20%FBS + 1%Alb) are the best combinations of mediums for ovarian cell culture (Figs. 2 and 9 and 11).

Effect of Different Mediums on Cumulus Cells Proliferation and Viability

The results of culture mediums on a mixture of OCs (cortex + medulla + hilus) demonstrated that (α-MEM + 20%FBS) was the best medium. So, to evaluate which combination of basal mediums is more efficient for *in vitro* proliferation of human CCs, the combinations of upper mentioned mediums + (FBS 10 & 20% and Alb 1& 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 72h culture.

The results showed that in the 20%FBS group (DMEMF12 + 20%FBS) ($P < 0.0001$) and (DMEM HG + 20%FBS) (not significant) resulted in more cell viability and proliferation. Also, in (20%FBS + 2%Alb) group, DMEMF12 ($P < 0.01$), and DMEM HG ($P < 0.05$) caused more cell proliferation and viability. In (10%FBS + 2%Alb) group, DMEMF12 (not significant) caused more cell proliferation and viability, than control. Finally, in (20%FBS + 1%Alb) group, DMEMF12 (not significant), and DMEM HG (not significant) caused more cell proliferation and viability, than control group (Figs. 10 & 11).

Altogether we concluded that for the culture of CCs, DMEMF12 and DMEM HG, followed by α-MEM are the choice mediums (Fig. 11). They can be used with 20% FBS alone or with a combination of FBS and Alb. The comparison of all the groups showed that (DMEMF12 + 20%FBS), followed by (DMEM HG + 20%FBS), (DMEMF12 & DMEM HG + 20%FBS + 2%Alb), (DMEMF12 + 10%FBS + 2%Alb) and (DMEMF12 & DMEM HG + 20%FBS + 1%Alb) are the best combination of mediums for ovarian cell culture (Figs. 2, 10 & 11).

The Effect of Conditioned Mediums on Cumulus Cells Proliferation

The culture of CCs, without the mentioned supplements, used in current study, leads to cell death, through the time. So, to evaluate which OCs can better support the CCs growth, we exposed the CCs to CMs obtained from OCs. Four CMs were collected from the OCs environment. All of CMs lead to greater than or equal to control group viability and growth. All of CMs from cortex [(OSE + Cortex), (floating SBs), (cortex after SB creation) and (colony forming cortex) ($P < 0.0001$) and (cultures SBs) ($P < 0.001$)] significantly increased the

CCs growth and viability, while the cells of medulla and hilum showed viability ≤ that of the control group. Therefore, the cells of cortex (OSE, cortical & SBs) are more important for the support of CCs proliferation and viability. Also, the combination of [50% medullar and 50% hilum CMs] ($P < 0.001$)) and the combination of all ovarian CMs ($P < 0.001$) caused the same results as the cortical cells. It means that these two combinations are as effective as the cortical cells. Overall, it seems that the CMs of cortex and (medulla + hilum) are more efficient to support the growth and viability of CCs.

Also, we used two CMs, collected from adipose tissue derived mesenchymal stem cells (ATDCs) and amniotic fluid derived mesenchymal stem cells (AFDCs). They just caused slightly higher growth of CCs (not significant), compare to untreated cells, whereas the combination of these two CMs lead to significant increase of cells viability and growth ($P < 0.05$).

Finally, the result of TESE derived CM showed the same result as the control group and the CM from the OVCAR3 cells resulted significantly increased growth of CCs ($P < 0.001$). OVCAR3 cells are serous epithelial ovarian cancer but not ovarian surface epithelium cancer. They originate from the epithelium and endometrium of the (distal) fallopian tube (10) (Fig. 12).

Effect of Hormones on Cumulus Cells Viability & Growth

The growth of CCs becomes follicle stimulating hormone (FSH) dependent from the stage of growing follicles. Routinely, for women who referred for infertility treatment, the FSH-based drugs are prescribed. Two FSH-based drugs Gonal-F and pregnyl were used in this study. The results showed that 300mIU/ml of Gonal-F was the best concentration for proliferation and viability maintenance of CCs ($P < 0.001$) followed by 400mIU/ml of Gonal-F ($P < 0.01$). The concentrations of 100 & 200 mIU/ml Gonal-F were not so efficient, compared to control and 500mIU/ml increased the 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 of CCs viability ($P < 0.0001$). Among them 3.5 IU/ml pregnyl followed by 2.5 IU/ml pregnyl caused the most significant increase of cell viability and growth (Fig. 13). We concluded that pregnyl (FSH and luteinizing (LH) like drug) was more efficient than Gonal-F (FSH like drug) for CCs *in vitro* proliferation.

Effect of Follicular Fluid on Cumulus Cells Viability & Growth

FFs were collected from six groups of patients including: unknown infertility, poor responders, advanced age, male factors infertility, polycystic ovarian syndrome (PCO) and endometriosis. 50% & 75% of FF were added to basal culture mediums. Generally, the results showed that addition of 50% FF is more efficient than 75%. Also, 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 the CCs proliferation and viability, they had no significant increasing effect on cell proliferation and viability (Fig. 13).

Effect of Serum (FBS, BSA, HSA)

Regarding the FBS concentration, our results showed that 20%FBS significantly increased the growth of even OCs or CCs. Also, supplementation of culture medium with both FBS and Alb can be more efficient than10%FBS, 1%Alb and 2%Alb, alone (Fig. 11).

About HSA, although all the concentrations from 3–25 mg/ml increased the CCs growth, just the concentration 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 the cell proliferation and viability (Fig. 13).

Regarding the use of BSA, all the used concentrations including 3mg/ml ($P < 0.01$), 6 mg/ml ($P < 0.01$), 12 mg/ml ($P < 0.01$) and 24 mg/ml ($P < 0.001$) showed significantly increase of cells proliferation and viability (Fig. 13).

Effect of L-Glutamine and ITS

L-Glutamine is recommended to be used as 2-2.8 mM/ml in basal medium. Therefore, we compared several concentrations of L-Glutamine with DMEM + (2 mM/ml L-Glutamine), as control. Various concentrations of L-Glutamine including 2, 4, 12, 17, 22, 27 & 37 mM/ml were used. The concentrations of 12 mM/ml ($P < 0.05$) and 17mM/ml ($P < 0.05$) of L-Glutamine showed higher cell proliferation and viability. 17mM/ml ($P < 0.05$) had the most considerable increasing effect on cells growth and viability (Fig. 13).

Regarding ITS, the concentrations of 5ng/ml, 10ng/ml and 5µg/ml had higher viability rate, compared to control. But just two concentrations of ITS [10 ng/ml ($P < 0.01$) and 5 µg/ml ($P < 0.05$)] caused significant increases of CCs growth. The concentration of 10 ng/ml ($P < 0.01$) had greater effect on CCs growth.

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

CCs cells had slower proliferating rate than other OCs. Without supplements, they did not show considerable incoherent contact interaction and no cellular aggregation was observed. But instead, in the presence of some materials like human chorionic gonadotropin (HCG), Gonal-F, FFs, ITS and L-Glutamine, we observed that the CCs formed some aggregates similar to the colony forming unit. The mentioned structures were very similar to the natural spatial shape of a growing follicle. It means in the presence of the FSH, FF, ITS and L-Glutamine the cells can rearranged and demonstrated their potential 3-dimensional morphology. These colony-like structures were more compact and uniform in the presence of HCG and FF (from male factor infertility patients) (Fig. 14).

Conclusion

The current study confirmed this knowledge that ovarian and CCs require different growth needs and dissimilar mediums can support their in vitro growth. Also, we concluded that ovarian cells in vitro growth in a basal medium can progress even to higher passages, while CCs demand some other requirements like hormone, serums, ITS, L-Glutamine and growth factors. Moreover, addition of ovarian CMS and FFs from healthy women and ovarian cortical, medullar and hilum cells secretums can be replaced with synthetic

growth factors, which are more economical and closer to normal body condition. So, a cocktail of basal mediums supplemented with other complements is essential for in vitro culture of CCs and finally the follicles. We suggested that these supplements assess directly on follicular growth as well.

Discussion

Supplementation of culture medium with nutrients, growth factors and hormones has crucial aspect for the culture of ovarian follicle. The medium considerably support both of the cell survival and proliferation. FIVG needs an optimized culture condition which can support the growth of CCs and finally oocyte maturation and follicle growth. In the present study we compared the effect of different basal and conditioned mediums, supplements, FFs and hormones on the *in vitro* growth of CCs. We tried to examine the majority of required components.

In the present study, different basal mediums with FBS (10% & 20%) and Alb (2% & 4%) on OCs and CCs, were compared. Regarding the OCs, our results showed that FBS is more efficient than Alb for *in vitro* proliferation of OCs and CCs. Also, FBS 20% was more superior to FBS 10%, while increases in Alb concentration could not support the growth of ovarian and CCs, alone. For OCs, in the presence of 10% or 20% FBS, α-MEM was the best medium. Whereas for CCs, if FBS 10% was used, α-MEM was the best medium, but if FBS 20% was applied, so DMEMF12 was superior. Also, the combination of both FBS and Alb had positive effect on OCs and CCs growth.

The mediums for FIVG are two kinds; basal medium and maturation medium. These mediums should support cell survival, proliferation and function. It should support both oocyte and CCs requirements. The applied media in FIVG are classified to: 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 our study we concluded that (DMEMF12 + 20%FBS) or (DMEMF12 + 20%FBS + 2%Alb) and (DMEM HG + 20%FBS + 2%Alb) showed higher cell viability and proliferation for CCs than α-MEM. We proposed (α-MEM + 20%FBS), DMEMF12 and DMEM LG for OCs and DMEMF12 and DMEM HG (+ FBS + Alb) for CCs. Regarding the DMEM medium, for OCs low glucose is superior but for CCS high glucose is better.

The culture mediums have some basic component 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, co2 bicarbonate], trace elements and antibiotic. The details of five used mediums are presented in Table 3 (Table 3). α-MEM has the minimum essential material for cell culture, while DMEM and DMEMF12 have more components. We concluded that α-MEM + 20% FBS and DMEMF12 + 20%FBS can be used for the culture of ovarian and CCs, respectively. So, it seems that OCs can grow with the minimum essential materials but CCs need more constituents. Our results are parallel with some previous studies, that used DMEMF12 as the best media for OCs and CCs (14) (15). As well, in line with our results, Bukovsky et al. used DMEM HG and DMEMF12 in the presence of 20% FBS for *in*

vitro oogenesis (16). Also, Yang et all used DMEMF12 supplied with 15% FBS for *in vitro* culture of porcine CCs (17). But unlike our study some other studies used α-MEM for the culture of ovarian follicles (18).

It is proposed that phenol red free DMEMF12 medium is superior, because phenol red is a weak estrogen with obvious biological effects (19). Also, it has shown detrimental effect on cat ovarian cryopreserved tissue (16). So, some studies proposed RPMI media for *in vitro* culture of CCs, because it is mostly free of phenol red (16). But our results did not show any beneficial effect with RPMI on CCs growth. RPMI1640 is the most enriched medium, followed by DMEMF12. Many studies suggested RPMI for the culture of normal and tumor cells. But in our study, we concluded that this enriched medium is not suitable for ovarian and CCs culture. Compare to DMEM, it has lower concentration of calcium (0.8 mM) and higher concentration of phosphate (5 mM) (20) and mostly it is phenol red free. Altogether, the main difference between RPMI and the other media is the absence of phenol red and calcium chloride (CaCl2). In cat, Brito et all reported that phenol red may led to follicular degeneration, especially in vitrification process (21). The domestic cats and dogs are good models for studying *in vitro* human ovarian follicular development. From this side, RPMI is a good medium but our results on CCs growth showed no advantages for this medium.

One of the differences between the cell culture mediums is in the pyruvate and glucose concentration. All mediums have pyruvate and were categorized to high and low glucose mediums. The amount of these components is important because the oocyte of growing follicle preferentially metabolises pyruvate over glucose, but the somatic compartments of ovarian follicles are more glycolytic. Glucose metabolism in cumulus/oocyte complex is very complicated during hormone-stimulated stages (22). The cells need glucose or pyruvate for their growth. It has been confirmed that most of the mammalian oocytes prefer pyruvate as energy substrate and have a low glycolytic activity. While, CCs prefer glucose over pyruvate. Primordial follicles consume 2-fold more pyruvate than glucose, probably due to the lowest numbers of surrounding CCs. In growing follicles (from primary to secondary stage), the CCs proliferated and increased in numbers, so glucose consumption and lactate production increase, as well. This pattern change around the time of antrum formation, with antral follicles become predominantly glycolytic (23). This may be the reason that in our study, DMEM HG was superior than LG for CCs culture.

Beside the basal medium component, CCs growth needs some other ingredients. They are categorized to gonadotropins, serums as the protein source, survival factors like ITS and growth/paracrine factors (Table 4) (11). So, in the present study we compared different kinds of serum (FBS, HSA and BSA), ITS, L-Glutamine and hormones. Since, the growth factors are expensive; we tried to compensate the absence of growth factors with FF and serums, which are enriched of various growth factors.

Another part of our study was on FSH like drugs. We concluded that 300mIU/ml Gonal-F, as a FSH like drug and 3.5IU/ml pregnyl (a FSH and LH like drug) have the most increasing effect on CCs growth. The growing follicles are FSH dependant and supplementation of FSH can improve both the follicle growth and 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 early antral follicles. Also, it is essential for the steroidogenesis and regulates the connection between oocyte and GCs (24). All the pervious researches increased the rate of follicle survival with addition of FSH to culture medium (11). The controversy is on the FSH dosages. A minimal concentration of 10 mUI/ml of FSH is essential for IVG of intact preantral follicles (24). Javed et all

added 10–200 mIU/ml FSH to culture medium. They observed by addition of 100 mIU/ml FSH to culture medium, follicles survival, their diameters, germinal vesicles breakdown and oocyte maturation rates were increased (25). Silvia et al, reported that 10-µg/mL insulin and 100-µg/mL FSH can improve the *in vitro* meiotic resumption rate of caprine preantral follicles. Barros et al. used FSH with fixed or sequential concentrations. They showed that a sequential concentration of 750 ng/mL recombinant human FSH (compared to control 1000 ng/mL) improved oocyte and follicle growth and maturation (26). We compared 100-500mIU/ml Gonal-f and concluded that just 300 and 400mIU/ml concentrations increased CCs growth and viability, significantly.

Also, we compared several dosages of pregnyl. It is composed of alpha and beta subunits. The alpha is identical to LH, FSH and alpha subunit of thyroid stimulating hormone (TSH). So, its function is similar to both LH and FSH. LH plays an important role in the maturation of follicles, supports follicle development and induces ovulation. But it was not consider as necessary component of FIVG culture medium, but recently its positive effect on follicle growth is being actively debated (27). HCG and LH bind to the same receptor as FSH but activate different signalling pathway (28). Mediums supplemented with HCG stimulate *in vitro* maturation of oocyte. HCG has a more significant luteinizing effect compare to LH (18), while LH shows larger follicular diameters than HCG (29). Most studies used 1.5 IU/ml HCG (27), but our results showed that 3.5 IU/ml of HCG has more growing effect on CCs. It was shown that FSH and LH supplementation in a serum free medium caused the decrease of DNA fragmentation in GCs and increase of DNA fragmentation in theca cells (30). We concluded that HCG can lead to higher CCs proliferation than FSH, alone. The reason may be due to its slight LH like effect.

Serum is another component of the culture media. It causes the decrease of estradiol secretion with an unknown reason. It has been shown that during FIVG of *in vitro* preantral follicle, FSH steroidogenic and mitogenic effects changed (31). In the present study various kinds of serums were evaluated. They containe various growth factors and hormones. They help cell attachement to other cells and surfaces, so they act as spreading factor. They could be as a buffering agent and/or a binding protein. Finally they decrease the mechanical damages to the cells. Also, they have some disadvatages including different compositions in various samples. It may induces some inhibitory factors and the risk of contamination (31).

We evaluated three kinds of serums for CCs growth (FBS, HSA and BSA). FBS has some advantages in cell culture including stimulatory factors and low concentration of immunoglobulin (32). BSA is shown to improve *in vitro* development of follicles (33). In simplified medium, replacement of FBS with BSA creates a defined medium that provides better conditions for oocyte *in vitro* maturation (24). Regarding HSA, it was shown that HSA + ITS cause the decrease of atretic follicles and increase of healthy follicles rate and follicle size. Also, HSA promotes cell proliferation and act as free-radical and reactive oxygen species (ROS) scavengers (24). We added some concentrations of HSA and BSA from 3–25 mg/ml. Compared to control, all the concentrations increase CCs proliferation and viability. Unlike HSA, 3 and 6 mg/ml of BSA increased the CCs growth, significantly. Also, we concluded that the HSA alone had no beneficial effect on the growth of ovarian and CCs, whereas in combination with FBS it caused considerable growth.

Also, the CCs need amino acids. L-Glutamine is an essential amino acid for both ovarian and CCs. Generally, its amount is adjusted to be around 2 mM, but it can vary from 0.5 to 10mM, depending on the cell and

media types (vary from 0.68 mM in medium 199 to 4mM in DMEM). It was shown that arginine, glutamine and leucine, used in the ovarian tissue culture, can accelerates *in vitro* activation of primordial follicles in 1-day-old mouse ovary (34). Some combinations of glutamine are available including: L-glutamine, GlutaMax or GlutaGro. L-glutamine is nearly unstable and degrades over time in refrigerator and more rapidly in incubator, but GlutaMax is more stable. So, addition of an extra amount of L-glutamine to culture medium wouldn't harm the cells, even it may be necessary component especially for media close to its expire date (35). Glutamine supports the growth of cells which have high energy requirements. Also, it synthesizes large amounts of nucleic acids and proteins. It acts as an alternative energy source for the cells that use glucose inefficiently or for rapidly dividing cells. So, when glucose level is low but energy demand is high, cells can metabolize amino acids like glutamine, as the most readily available amino acids for use as energy (https://www.sigmaaldrich.com/life-science/cell-culture/learning-center/media-expert/glutamine.html). Most of the mediums are supplemented with 2mM/ml L-Glutamine (36) (37). In the current study we used 4-37mM/ml L-Glutamine. The results showed that 12 and 17 mM/ml L-Glutamine caused higher CCs growth compare to 2mM/ml. But more than 22mM/ml was detrimental for CCs growth. Altogether, L-Glutamine accelerate *in vitro* growth of CCs and *in vitro* activation of primordial follicles (37).

ITS or insulin, transferrin and selenium are other components that 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 together with selenium and transferrin act as survival factor. But, high amount of insulin, mimics insulin resistance model, encouraged apoptosis in GCs (38).

Selenium is another essential trace mineral which is relevant to various patho-physiological processes (38). It is added to IVG medium, as well. But high doses of selenium reduce the rate of proliferating primordial follicles. It regulate the 17β-estradiol bio-synthesis and the growth of the GCs in adult ovaries (39). Heat stress induces apoptosis in various cells. Selenium has effects on maintaining the cellular physiologic functions and protects the cells against chronic heat stress induced apoptosis in GCs (40).

Transferrin is the carrier of iron, a mandatory requirement of the cells. These cells should have transferrin receptor for intracellular transport of iron. It was expressed in a subpopulation of human granulosa lutein cells which are isolated from follicular puncture. Normally, transferrin and iron concentration increase in FF with advance in follicular maturation. Transferrin insufficiency causes iron overload and leads to oocyte dysmaturity. Small growing follicles with 1–2 layers of GCs have cytoplasmic transferrin in their cuboidal GCs (41).

Previous studies used various dosages of ITS from 10 ng/ml to 10µg/ml. We used four concentrations of 5 & 10 ng/ml to 5 & 10µg/ml. Our results showed that 10 ng/ml followed by 5µg/ml caused to higher GCs viability and growth.

Also, we compared the effect of FF on CCs growth. FFs were isolated from 6 groups of patients. The results showed that 50% and 75% FFs, isolated from male factor infertility patients (healthy women) increased CCs growth significantly, followed by 50% FF of unknown cause infertility patients. Also, 50% FF was superior to

75%. FF has estradiol, progesterone and testosterone. The level of steroids is correlated with follicular diameter. According to Wed et al. study, FF progesterone level is 6100 times more than estradiol and 16900 times higher than testosterone. FF has two kinds of vesicles, [exosomes (50–150 nm) and microvesicles (100–1000 nm)]. The content of these vesicles is bioactive components such as proteins, mRNAs, lipids and miRNAs (42). FF is a plasma filtrate with a large dynamic range of proteins. These proteins are involved in metabolic processes, cellular processes, cellular communication and immune responses (43). Also, FF has some kinds of GCs, called immortalized GCs (44). Although the FFs from PCO patients, endometriosis, advanced aged patients and poor responders did not have positive effect of CCs growth and viability, they did not cause negative effect, compared to control group, as well.

In the present study, the CMs, collected from ovarian cortical cells significantly improve the CCs growth. It is not far from mind, because cortex is the natural place of ovarian follicles which makes its micro environment. But it is interesting that the mixture of medullar and hilus CMs also caused the CCs growth as much as the cortical CMs. This data proposes this idea that probably ovarian medulla and hilum are as important as ovarian cortex for follicular growth.

Also, our results demonstrated that the isolated cells of SB, cortex, medulla and hilum expressed GDF9. It is essential factor for follicular symphony and acts as a regulators of ovulation, folliculogenesis and oocyte quality (45). Interestingly, its expression was higher in medulla and hilum. Regarding the poor responder patients, it was shown that the expression of GDF9 is decreased by age in FF and GCs which results to decrease in assisted reproductive outcomes (46). Also, GDF9 enhances the proliferation and metabolism of CCs and GCs and acts as a CCs expansion factor (47). In mouse, GDF9 promotes the development of CCs by help of oestrogen (48). In our study the addition of ovarian cells CMs could enhance the in vitro proliferation of CCs. One of the suggested reasons may be related to GDF9. In our studies the cells of cortex, medulla and even hilum expressed GDF9 and the results of MTT of CMs on CCs demonstrated that both cortex and a combination of (hilum and medulla) CMs gave the same results.

Also, CMs, collected from ADMSCs + AFDCs has positive effect on CCs growth, but this effect was not as much as that of the OCs. In parallel to our study, the previous studies used CM, collected from mesenchymal stem cells, for *in vitro* maturation and subsequent development of oocyte (49). It was shown that ATDCs can promote the early stages of follicles survival, growth and maturation. They secret factors which promote the early stages of follicle growth (50). Also, AFDCs have shown to preserve ovarian follicle after chemotherapy (51) and they have potential to differentiate into primordial follicle (52). In a study on the expression of ovarian cells mesenchymal markers (still not published data), we concluded that the cells of all part of ovary expressed mesenchymal markers. So, probably another reason that CM of OCs could support CCs growth is the mesenchymal nature of these cells.

They are many unknown factors which are involved in ovarian follicular development. By applying FFs and CMs, which are enriched of growth factors, hormones and the other efficient elements, we can better support the follicular growth, with the lower cost.

Methods

Materials

All the culture mediums, 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®).

Human Ovarian Tissue Collection

The project was approved by ethical committee of “Yazd Reproductive Sciences Institute”, Shahid Sadoughi University of Medical Sciences, Yazd, Iran (Ethic 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. Also, it was based on “The Code of Ethics of the World Medical Association (Declaration of Helsinki)”.

Human ovarian tissues were retrieved after informed signed consent. Ovarian biopsies were collected from ovariectomized patients after surgery due to benign problems. The samples have been transferred into PBS (4^0C), containing 5% pen/strep & 5% amphotericin B and immediately transferred to cell culture laboratory.

Ovarian Cells Isolation

The excised ovaries were cut by sterile surgical scalpel. Anatomically and macroscopically three parts were considered for ovarian biopsies including: 1) the outer part or ovarian surface epithelium (OSE) (Fig. 1, A, blue arrow) and its underlying part was ovarian cortex (Fig. 1, A, yellow arrow). The OSE and ovarian cortex were removed and scraped from the inner part by a bistoury (Fig. 1, B). 2) The second inner part was medulla (Fig. 1, A, pink arrow). It was the intermediate part between cortex and hilum where no follicular structures were seen. 3) The ovarian hilum was the last part that connects the ovary to the ovarian pedicle (Fig. 1, A, green arrow). Therefore, the segments were collected from these three parts, rinsed with PBS + 3% pen/strep + 3% amphotericin B, chopped with a sterile scissor (Fig. 1, C) and then transferred into three separate 15ml 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 (Fig. 1, D). Then, the samples were incubated at standard condition, 37 ^{0}C, 5% CO2 and 95% humidity with frequent shaking till partial digestion. Depending on the tissue consistency, the time of enzymatic digestion was variable from 2 h to 8 h. The collagenase activity was blocked with αMEM + 10% FBS. The samples were centrifuged (8 min at 1400 rpm (300g)) 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 fresh medium every 2–3 days and the culture continued to get confluence. The cells behaviour and morphology were investigated by an inverted microscope.

Immunocytochemistry

Isolated cells from cortex, medulla and hillum were stained for “Growth differentiation factor 9” (GDF9), as a key factor in ovarian follicle development. The cells were fixed in 4% paraformaldehyde for 20 min at 4^0C, Then the samples were washed with 2 normal HCl for 20 min at room temperature followed by incubation for 30 min with 0.3% Triton X-100 for permeabilizion. Then 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 polyclonal rabbit primary antibodies anti GDF9 in humid chamber at a dark room in refrigerator. Following twice washing with PBS, polyclonal goat anti-rabbit secondary antibodies were added for 60 min at room temperature in a dark place. After three times washing with phosphate buffered saline (PBS), the cells were counterstained with DAPI and were monitored under the fluorescence microscope. Positively stained cells expressed green fluorescence for GDF9 under fluorescence microscope.

Cumulus Cells Collection & 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 females were healthy and under the age of 35 with acceptable anti Mullerian hormone (AMH) level (Over 1 ng/ml). CCs were collected after puncturing the follicles and denudation. The denudated droplets were collected, centrifuged, washed with medium and finally the cells pellet was collected and cultured in αMEM + 10% FBS, 2% pen/strep and 2% amphotericin B.

Effects of Different Mediums on Ovarian & Cumulus Cells

To evaluate which basal cell culture medium is better for growth of human OCs and human CCs, we compare the following mediums including:

1. **α MEM**: It is a modification of Minimum Essential Medium (MEM).
2. **DMEM (high & low glucose)**: Dulbecco's Modified Eagle Medium is based on 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 concentration of glucose and lower concentration of calcium and phosphate, compare to DMEM.

So, α MEM medium, DMEM (high & low glucose) and DMEM/F-12 as basal media and RPMI 1640 as a complex medium were compared. These mediums were supplemented with 10% & 20% FBS and 1% & 2% HSA with various combinations. We choose 1 and 2% albumin (Alb), because previously we evaluated various concentrations of Alb on CCs, to choose the best dosages (1 and 2%). Therefore, several formulations of upper mentioned mediums were prepared including: (Serum free mediums), (mediums + 10%FBS), (mediums + 20%FBS), (mediums + 1%Alb), (mediums + 2%Alb), (mediums + 10%FBS + 1%Alb), (mediums + 10%FBS + 2%Alb), (mediums + 20%FBS + 1%Alb) & (mediums + 20%FBS + 2%Alb).

Also, since many studies have suggested α MEM for ovarian and follicular culture, so (α MEM + 10%FBS) was considered as control and the other medium combination were compared with it. A mixture of OCs (cortex, medulla and hillus) were seeded at the density of 1×10^4 in 96 wells plates and cultured with the prepared mediums for 72h. Then, the percentage of viable cells was determined with MTT assay. For this assay, MTT reagent was added for 3h in a dark chamber at the incubator. Following 3 h, formazan crystals formed and give purple color with various powers according to the cell population growth. Formazan and cells were solubilized with DMSO and formazan crystals were quantified by ELISA. The results of above tests showed that (α MEM + 20%FBS) could support the ovarian cell growth better than other mediums. So, in comparison with (α MEM + 10%FBS), it was concluded that (α MEM + 20%FBS) was the best medium for culture of OCs.

At the next step, we aimed to evaluate which medium was better for CCs growth. Therefore, all the above medium formulations were prepared, and the results of CCs culture in every medium were compared with (α MEM + 20%FBS), as the best medium for culture of ovarian cells (Fig. 2).

Effect of Different Conditioned Mediums on Cumulus Cells Growth

To evaluate which group of ovarian cells (OCs) can provide more support for the growth of CCs, OCs were cultured and their CMs were collected. The mediums were collected from these cells: 1) Ovarian cortical cells, 2) Cells, isolated from spheroid bodies (originated from ovarian cortical cells), 3) Ovarian medullar cells and 4) Ovarian hilus cells. All these cells expressed the markers of germ cells and pluripotent stem cells [DDX4, DAZL, GCNA, SSEA4, OCT4] and showed Osteogenic & adipogenic differentiation ability (still not published data). The detailed criteria are summarized in Table 1 (Table 1). Also, some other cells were cultured and their CMs were collected. Among them ATDCs and AFDCs were cultured and their secretum was collected (the data showed osteogenic & adipogenic differentiation ability and flow cytometry expression of mesenchymal markers have not been presented in the current study). Besides, the CMs of human ovarian carcinoma cell line (OVCAR-3) and CMs of isolated cells from “testicular sperm extraction (TESE)” surgery were collected. The CMs were centrifuged at 5000 rpm for 10 minutes, filtered and frozen for future use. The control medium incubated alone without cells for the time equal to CMs collection time. 40% CMs was added to basal culture medium.

Effect of Hormones

To evaluate the effect of hormones on CCs growth, several concentrations of Gonal-F (FSH hormones, used in ovarian hyper stimulation) and Pregnyl® (ORGANON Holland), (Human chorionic gonadotropin (HCG) 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). So, both of the used drugs are FSH like. The majority of previous studies used 100 mIU/ml of FSH and 1.5 IU/ml of HCG (25) (53). So, according to the previous studies we tested 100–500 mIU/ml concentrations of Gonal-F and 1.5–7.5 IU/ml concentrations of Pregnyl.

Follicular Fluid Collection & Preparation

To analyse the effect of follicular fluid (FF) on 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 (Table 2). The collected FF quickly transferred to a specialized laboratory, centrifuged two times, the supernatant was de-complemented in 56^0C for 30 min in a water bath and after cooling the samples were stored at 4^0C for further use. To be the representative of the community, from each group, 10 samples were collected, aliquoted and before using they 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 CCs growth, we analyzed two concentrations of FBS (10 & 20%) on CCs growth. Also, two kinds of albumin were compared including bovine serum albumin (BSA) and human serum albumin (HSA). The previous studies usually applied 3 mg/ml BSA and HSA (18) (33). In this study, we compared 3, 6, 12 and 25 mg/ml of BSA and 3, 6, 10, 12, 20 and 25 mg/ml of HSA.

Effect of L-Glutamine and ITS

L-Glutamine is recommended to be used as 2- 2.8 mM/ml in basal medium (24). In the present study we compared different concentrations of L-Glutamine including: 2, 4, 12, 17, 22, 27 & 37 mM/ml. DMEM+ (2 mM/ml) L-Glutamine was used as control, because (2 mM/ml) L-Glutamine is routinely used in most of the culture medium.

About the ITS, the previous studies used a wide ranges of ITS from 5–10 ng/ml (54) to 5–10 µg/ml (53). So, to understand which concentration of ITS is better, we compared 5–10 ng/ml and 5–10 µg/ml of ITS. The details of study are summarized in Fig. 3 (Fig. 3).

MTT Assay Test & Statistical Analysis

Cells were seeded in 96 wells plates at the density of 1×10^4. When cells get 60% in confluency, the treatments were done. After treatment with various basal medium formulation + [(FBS and/or Alb), hormones, CMs and supplements], the cells culture continued for 72 h. Then, cell viability was evaluated with MTT assay test. The untreated cells were cultured in DMEM + 10%FBS + 2mM/ml L-Glutamine and considered as the control group. After incubation with MTT dye for 3 h, Dimethyl sulfoxide (DMSO) was added for 15 min with subsequent shaking. Then every 96 wells plate was read with enzyme-linked immunosorbent assay (ELISA) reader (test wave length 540 and reference wavelength 630nm). Triplicated samples were treated. The means of data was normalized and analyzed with GraphPad Prism 8 (GraphPad Software Company, US, California, San Diego). One-way analysis of variance (ANOVA) was applied to determine the significant differences between the means of treated groups and control, followed by post hoc Dunnett's test.

The figures were prepared and grouped with Microsoft office picture manager 2010, publisher 2010 and resized with Photoshop.

Abbreviations

Cumulus cells (CCs)

Ovarian cells (OCs)

Conditioned medium (CMs)

Fetal bovine serum (FBS)

Growth and differentiation factor 9 (GDF9)

Human serum albumin (HSA)

Bovine serum albumin (BSA)

Insulin transferrin selenium (ITS)

Follicular fluids (FFs)

Assisted reproductive technology (ART)

Follicular *in vitro* growth (FIVG)

Premature ovarian failure (POF)

Amniotic fluid derived mesenchymal stem cells (AFDCs)

Adipose tissue derived mesenchymal stem cells (ATDCs)

In vitro maturation (IVM)

Ovarian surface epithelium (OSE)

Spheroid bodies (SBs)

Rough endoplasmic reticulum (RER)

Follicle stimulating hormone (FSH)

Polycystic ovarian syndrome (PCO)

Earle's balanced salt solutions (EBSS)

N-2-hydroxyethylpiperazine-N-ethanesulfonic acid (HEPES)

calcium chloride (CaCl2)

human chorionic gonadotropin (HCG)

thyroid stimulating hormone (TSH)

luteinizing hormone (LH)

reactive oxygen species (ROS)

Dimethyl sulfoxide (DMSO)

One-way analysis of variance (ANOVA)

enzyme-linked immunosorbent assay (ELISA)

human ovarian carcinoma cell line (OVCAR-3)

Declarations

Ethical approval: The project was approved by ethical committee of “Yazd Reproductive Sciences Institute”, Shahid Sadoughi University of Medical Sciences, Yazd, Iran (Ethic 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 informed signed consent.

D**ata availabilitystatement**: 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.

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Author Contribution:

Moshrefi Mojgan: Conceptualization, methodology, design, formal analysis and investigation, material preparation, acquisition of data, data analysis, literatures collection, original draft preparation

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

Ghasemi-Esmailabad Saeed: Design, methodology, material preparation, acquisition of data

Mojgan Karimi-Zarchi: Ovarian tissue providing & resources

Fatemeh Sadeghian-Nodoushan: Scientific consulting, material preparation

Shahmohamadi Sajad: Acquisition of data

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

All authors approve the final manuscript, reviewed the manuscript and confirmed submission to “Biological Procedure Online”.

Author Contribution:

Moshrefi Mojgan: Conceptualization, methodology, design, formal analysis and investigation, material preparation, acquisition of data, data analysis, literatures collection, original draft preparation

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Shahmohamadi Sajad: Acquisition of data

Habib Nikukar: Supervision, scientific consulting, review and editing, English revising the manuscript & final approvalAll authors approve the final manuscript.

Author information:

Mojgan Moshrefi: DVM, PhD candidate in reproductive biology

Abbas Aflatoonian: Professor of obstetrics and Gynecology, The father of IVF in Iran

Ghasemi-Esmailabad Saeed: Master of molecular biology

Mojgan Karimi-Zarchi: Professor of obstetrics and Gynecology

Fatemeh Sadeghian-Nodoushan: PhD candidate in cell therapy

Shahmohamadi Sajad: Master of molecular biology

Habib Nikukar: Dr Habib Nikukar, pediatrician, PhD of nanobiotechnology

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Tables

Table 1
The used cells for collection of conditioned mediums

Cells	Origine	Conditioned Medium Collection
Ovarian surface epithelial cells + Cortical cells	Isolated from the OSE and underlying cortex cultured cells	The conditioned medium collected before spheroid bodies appearance
Floating pheroid bodies (SBs)	Through the 1th, 2nd and sometime 3th passages of ovarian surfacial and corticel cells culture, SBs were appeared	The conditioned medium collected from the medium of cells when floating SBs were apeared
Cultured spheroid bodies (SBs)	The floating SBs were cultured, attached to dish, expanded and maked colony	The conditioned medium collected after expansion and culture of SBs
Cortical cells, after colony formation	Isolated from the culture of ovarian cortex alone (without OSE and SBs), after colony formation	The conditioned medium collected after 4th passages that SBs were not appeared never again, but colony formation happen
Medulla cells	Isolated from the culture of ovarian medulla	The conditioned medium collected after 3th passages that two group of cells were apreaed
Medulla cells, after colony formation	Isolated from the culture of ovarian medulla cells after colony formation	The conditioned medium collected after 3th passages of medulla cells that maked colony
Hillus cells	Isolated from the culture of ovarian hillus cells	The conditioned medium collected after 3th passages of hillus cells
Hillus cells, after colony formation	Isolated from the culture of ovarian hillus cells after colony formation	The conditioned medium collected after 3th passages of medulla cells that maked colony

Table 2
The used ceriteria for collection of the follicullar fluids

Eitiology	Difinition	Age
Male factor infertility	1) A healthy women under infertily treatment who reffered 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 hirsotism 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	≤ 35
Advanced age But with good ART results	1) The patients who were older than 35 2) AMH ≥ 1.1 ng/mL 3) Follicole ≥ 5 4) With good ART results	≥ 35 ≤ 45

Table 3
Cell culture media comparison

Mediums	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 2mM/L is added	Phenol Red 10mg/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 4mM/L	Phenol Red 15mg/ml	Fourfold concentrations of amino acids and vitamins, compare 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 4mM/L	Phenol Red 15mg/ml	Fourfold concentrations of amino acids and vitamins, compare 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 2.5mM/L	Phenol Red 1.2 mg/ml	Fourfold concentrations of amino acids and vitamins, compare to MEM + Ham's F-12 medium	Sodium bicarbonat	• All 21 amino acids • w/wo Sodium pyruvate • Salt • 10 vitamins

Mediums	D-Glucose Concentration	L-Glutamine	Phenol Red	Comparison to MEM Medium	Buffering system	Other Ingredients
RPMI 1640	Almost high glucose 4g/L	w/wo almost with 2mM/L	w/wo but almost without phenol red. If any Phenol Red 5mg/ml		Low sodium bicarbonat HEPES	• All 21 amino acids • Sodium pyruvate • Salt • 11 vitamins

Table 4
The essential ingredients of ovarian in vitro follicular growth (IVG) medium

Factors Group	Subgroups
Gonadotropins	FSH & LH
Serums as protein source	FCS, BSA, FBS, Hypogonadal mouse serum
Survival Factors	ITS, IGFI
Growth/Paracrine Factors	cGMP, TNFα, EGF, ActivineA, Inhibin B, GDF9, Kit ligand, AMH, EGF Leptin

Figures

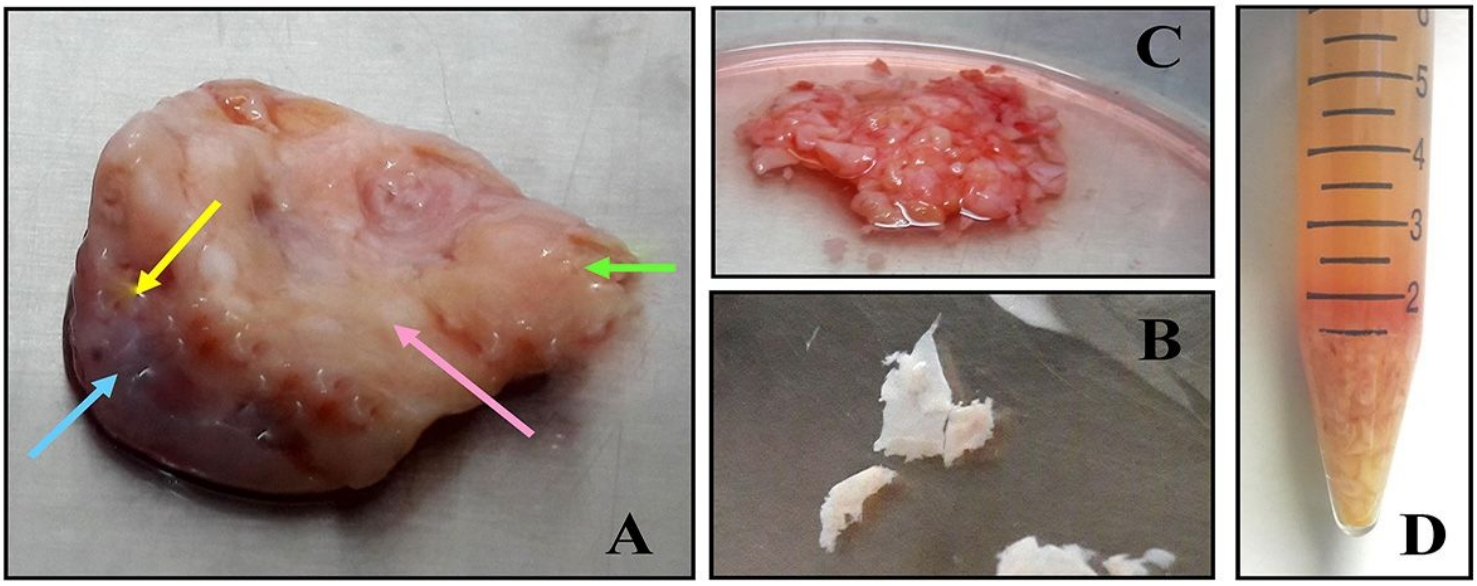

Figure 1

The steps of ovarian cells isolation A) A 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 & 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 15ml tubes.

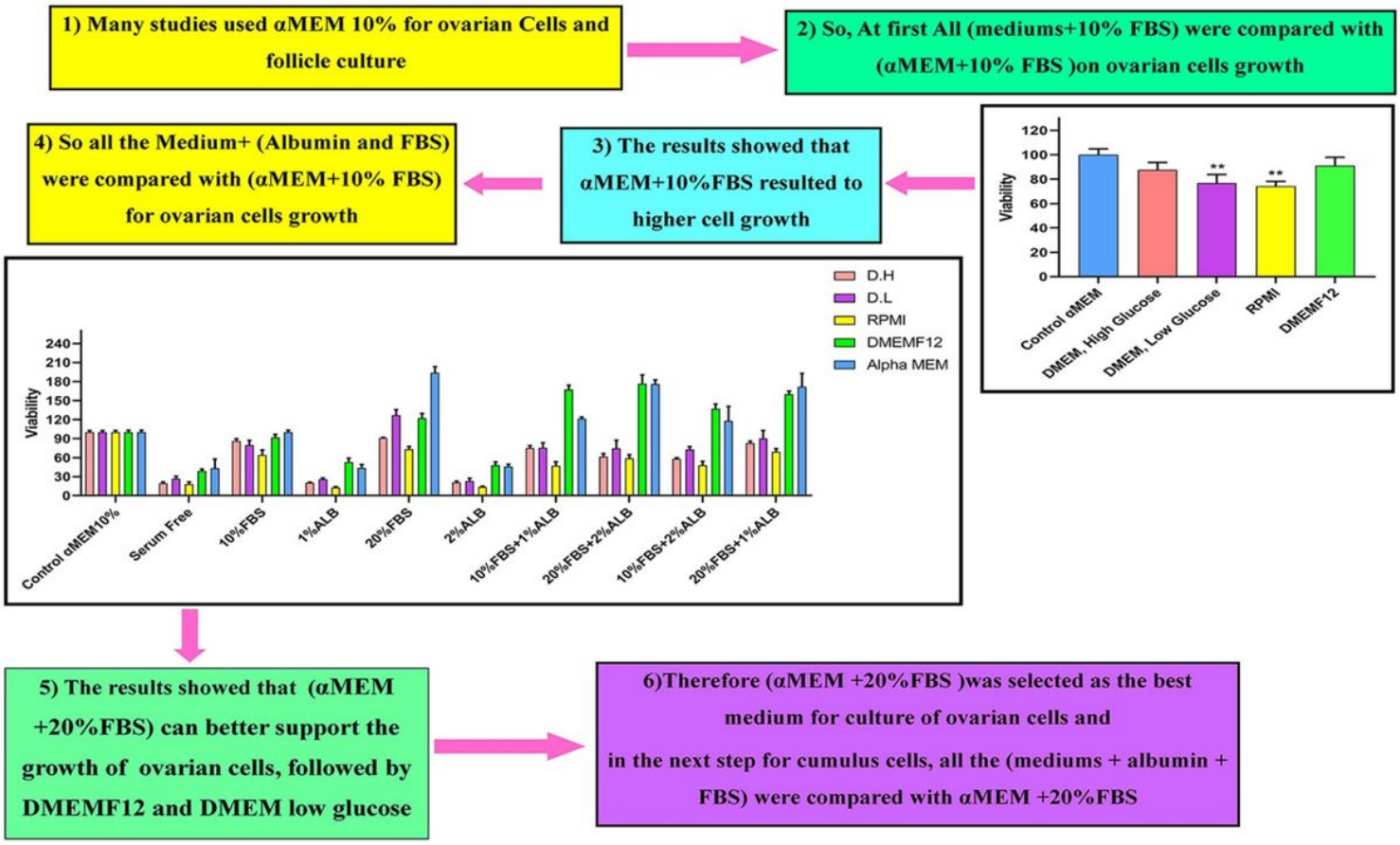

Figure 2

A graphical abstract on the steps for selecting α-MEM, as a control medium In the present study, several mediums were prepared and the OCs were cultured. The results were compared with (α-MEM+10%FBS). Also, the CCs were cultured with the same cocktails and the results were compared with (α-MEM+20%FBS).

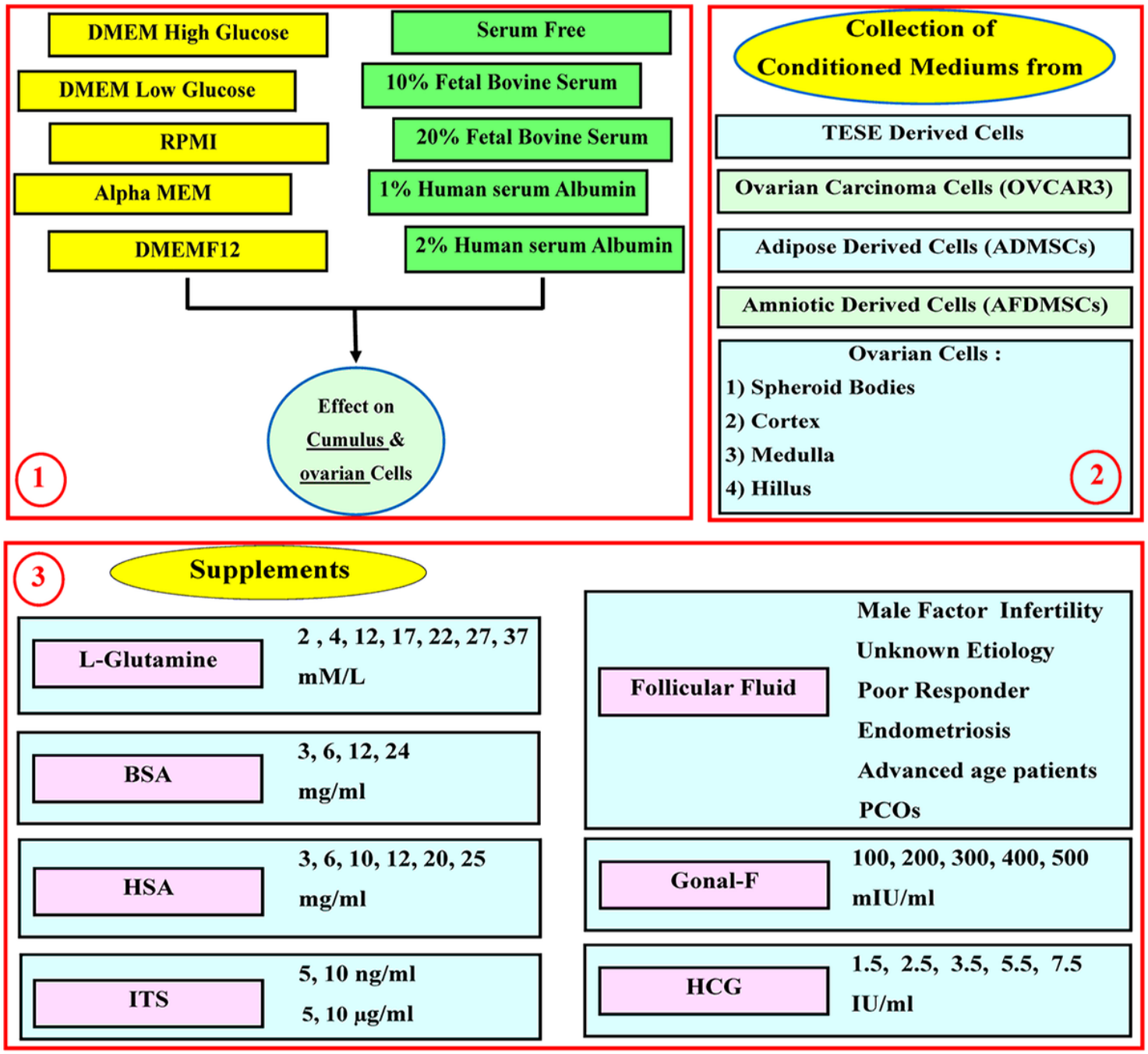

Figure 3

A summary of different treatments on ovarian and CCs 1) The ovarian and cumulus cells were cultured with five kinds of mediums +five combinations of serums (FBS and/or Albumin). 2) Eight kinds of conditioned mediums were collected and their effect on cumulus cells growth was evaluated. 3) Some supplements including: L-Glutamine, BSA, HAS, ITS, follicular fluids, Gonal-F and Pregnyl were exposed to cumulus cells with different concentrations and the cell growth was assessed.

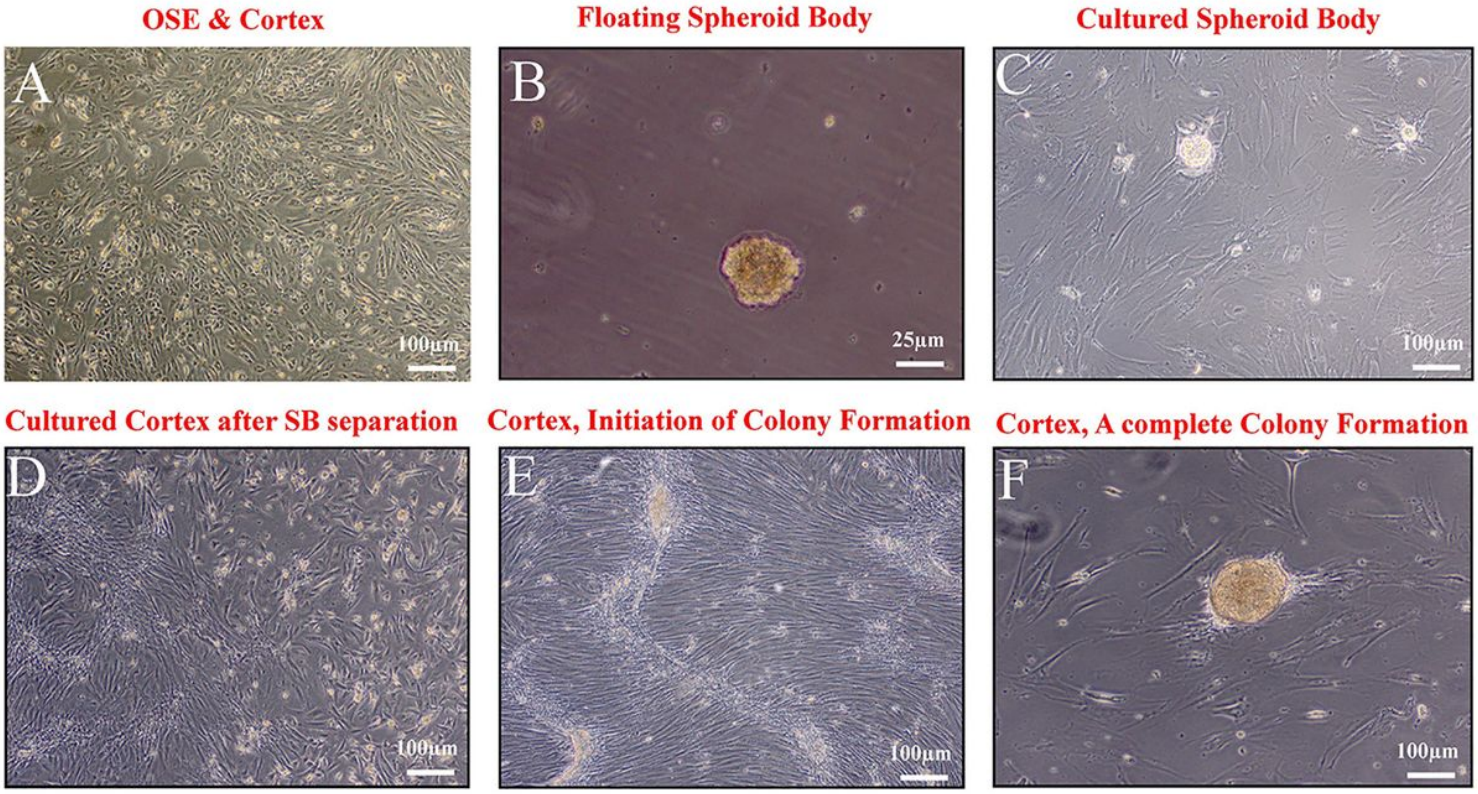

Figure 4

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

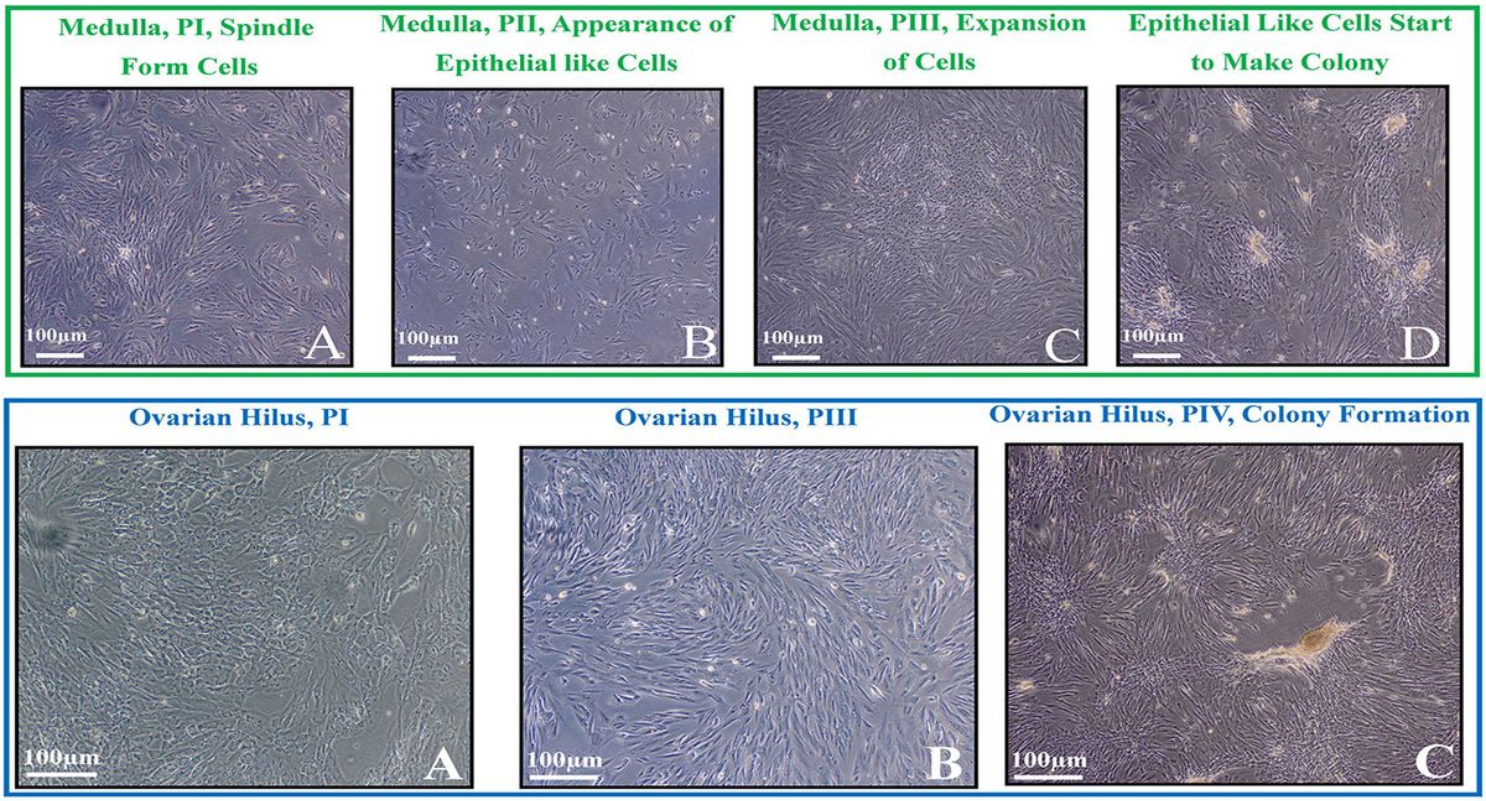

Figure 5

The morphology of ovarian medullar & hilum cells The medulla cells: A) The primary culture of ovarian medulla cells (first passage). The cells showed fibroblast like architecture. B) The secound passage of medullar cells, the smaller cells with epithelial like shapewere appeared. C & D) The cells expanded, proliferated and made colonies (magnification × 100). The hilus 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).

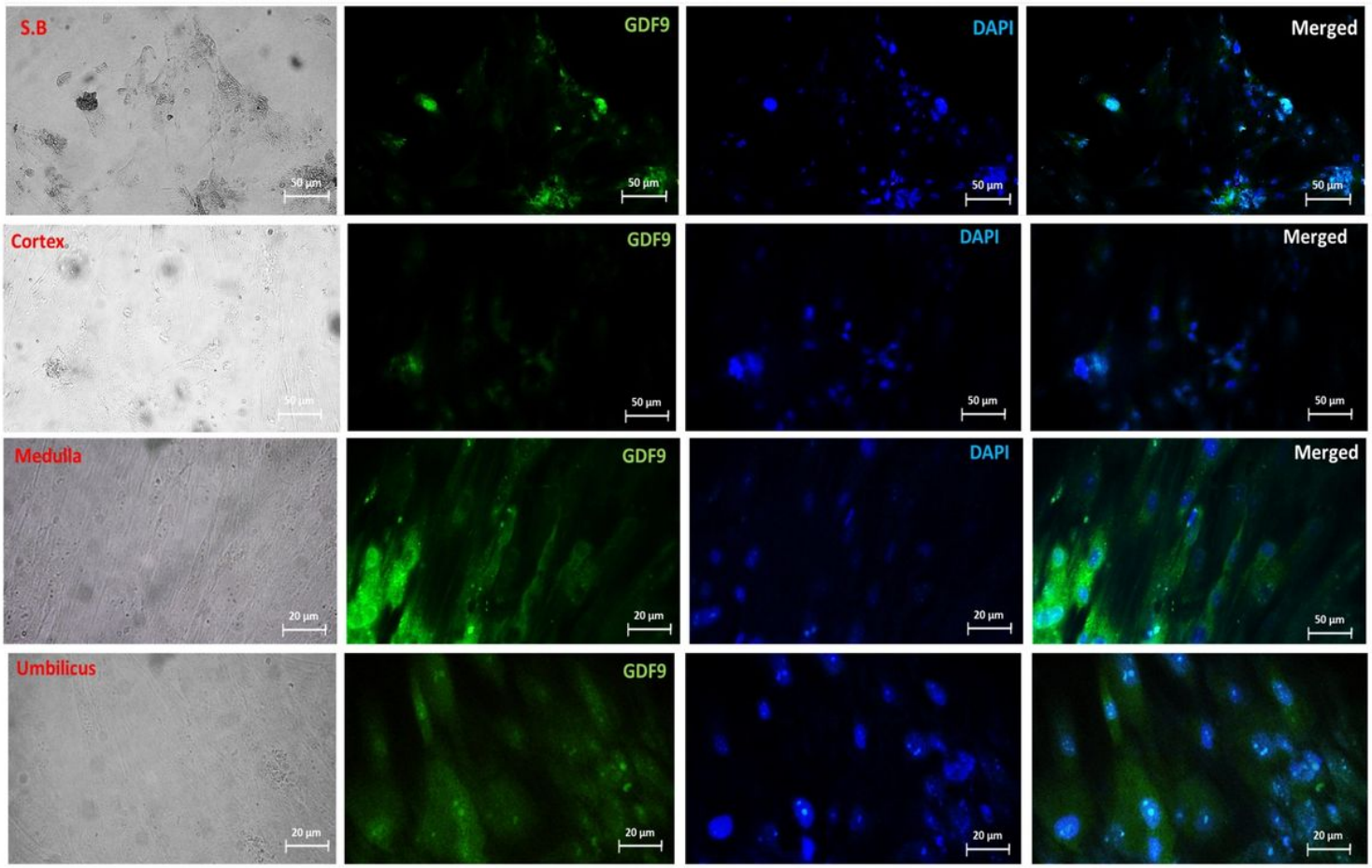

Figure 6

GDF9 expression in ovarian cortex, medulla, hilum and SB cells by immuno-staining under fluorescent microscope The green color shows positive GDF9 cells (magnification × 200 & 400). immunocytochemistry analysis showed that the cells of cortex (17.72%), medulla (13.33%), hilum (18.10%) and SB (16.56%) expressed GDF9 marker.

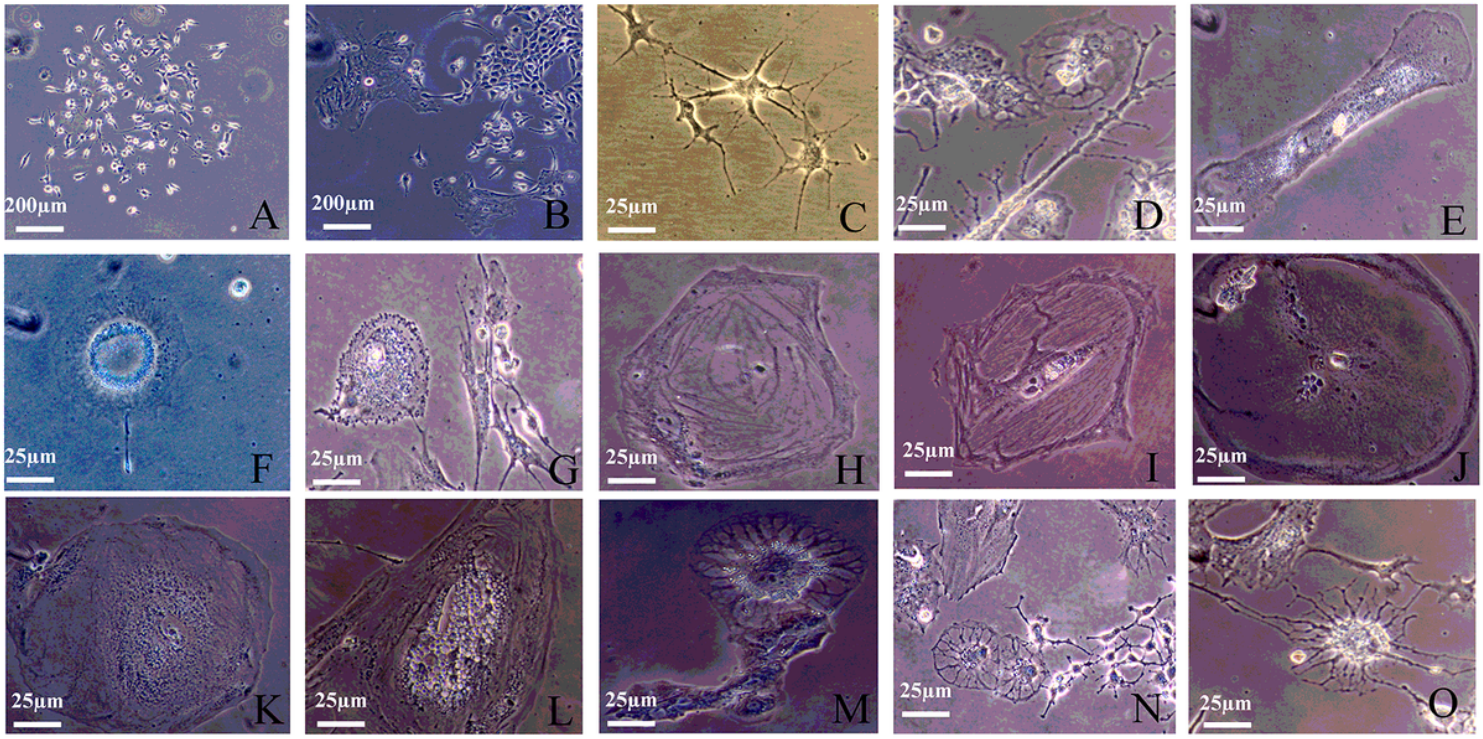

Figure 7

changes in Cumulus cells morphology A) The primary culture of cumulus cells. The cells were small cells with no appendages or branching. B) Then, the CCs started to became flat. C & D) gradually, the cells protrusion and cytoplasmic extensions were appeared. E) The cells size increased. F & G) finally the cells became round. H) The cytoplasmic filaments increased. I) The cell nucleus migrated from the margin to the center. J) The cells organelles increased, especially the RER, which surrounded the centrally located nucleus. K) The cells completely became round with a centrally located nucleus and two nucleoli. L) A large cumulus cells with developed RER that surrounded the nucleus. M) A lot of the secretory vesicles were appeared in cells and filled the cytoplasm. N) The cytoplasm space filled with cytoplasmic sacs or secretory vacuoles. O) The cells at different stages had contact communication with the surrounding cells (magnification × 40 & 400).

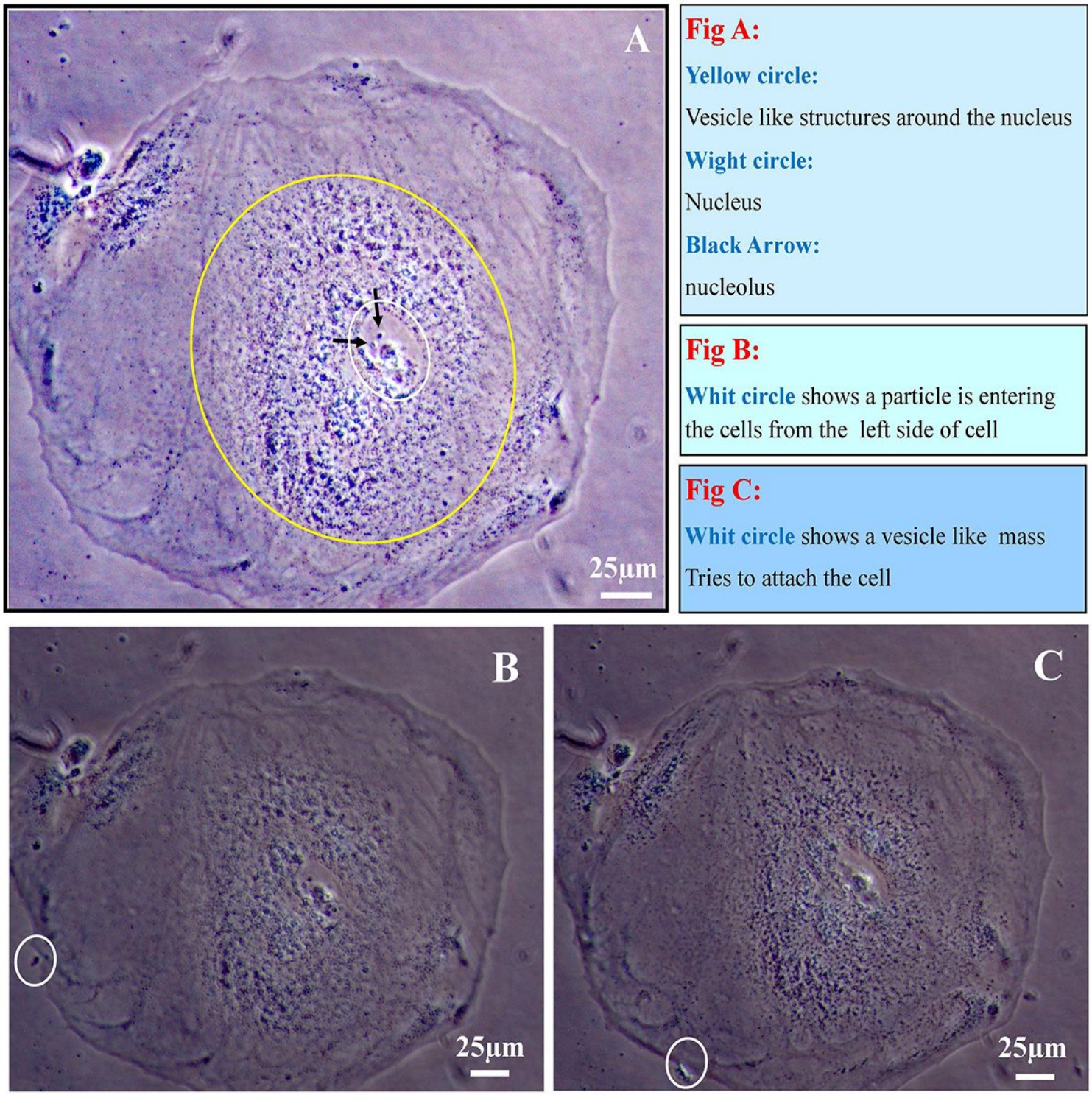

Figure 8

A large round cumulus cells which is shown in movie 1 A) Yellow circle showed the RER. The white circle marked the nucleus. The black arrows showed the nucleoli. B) The white circle showed a particle entering the cells. C) The white circle demonstrated a vesicle like mass, tried to attach the cell membrane.

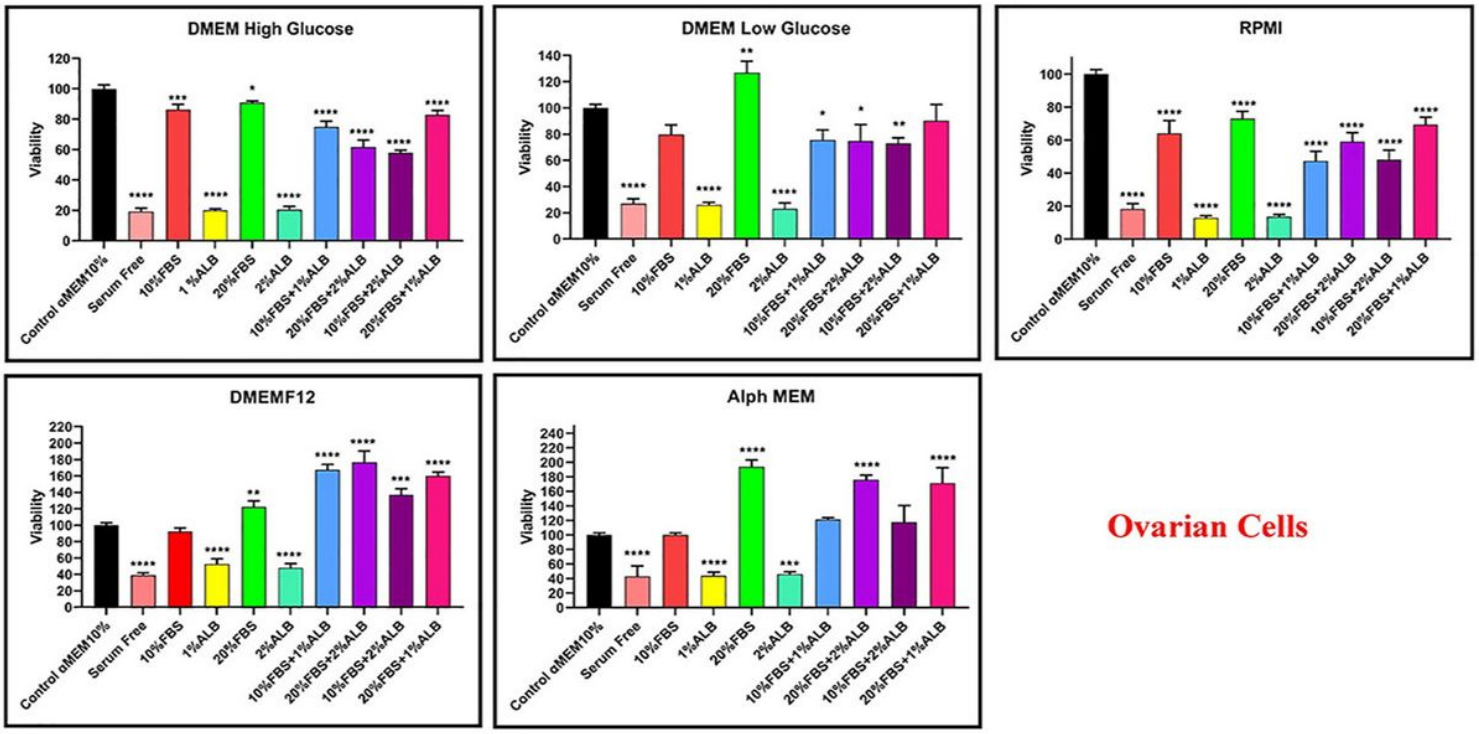

Figure 9

The comparison of five basal mediums with (α-MEM+10%FBS) for ovarian cells culture To assess which basal medium can better support the in vitro proliferation of human OCs, five mediums were compared including α-MEM, DMEM HG, DMEM LG, DMEMF12 and RPMI. FBS 10 & 20% and Alb 1& 2% were used as serum with various combinations. According to fig 2, all the combinations were compared with (α-MEM+10%FBS). * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

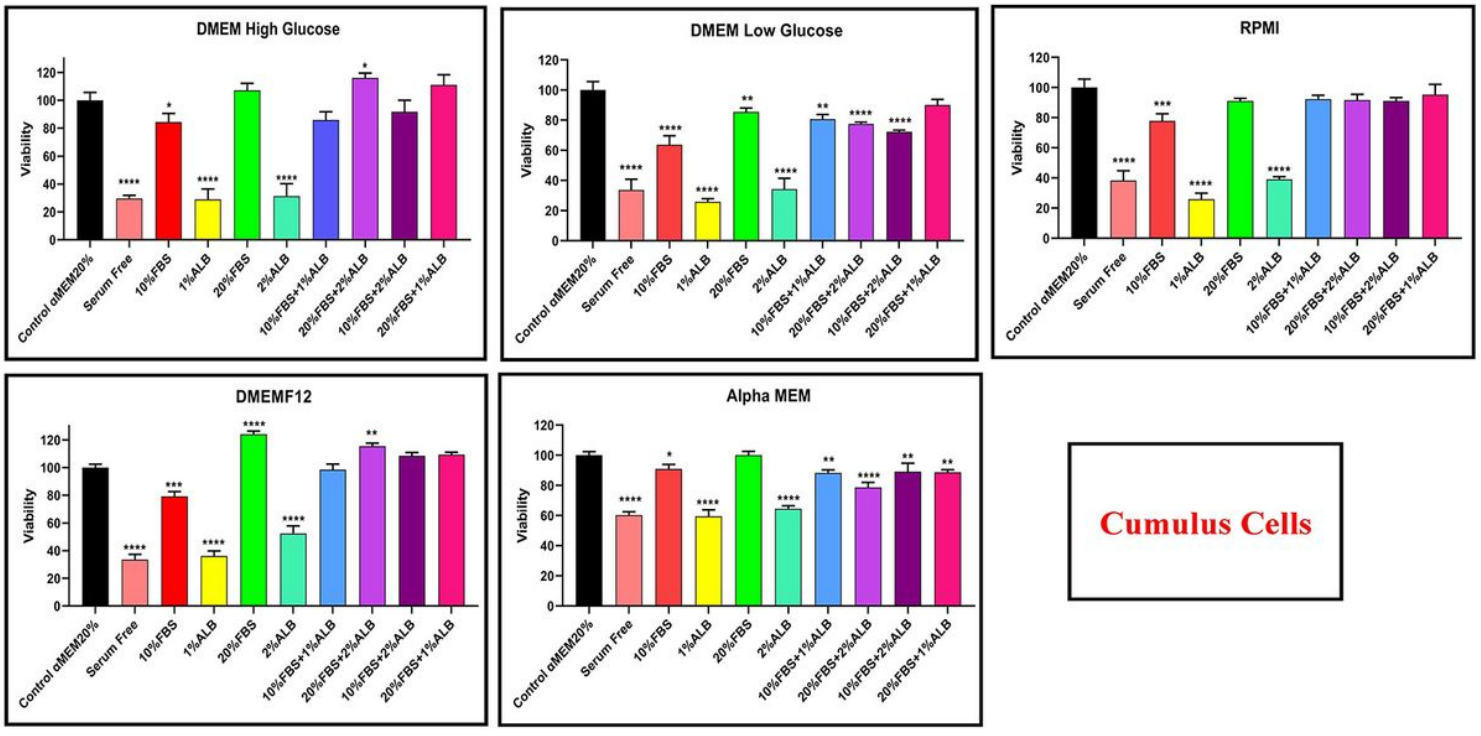

Figure 10

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

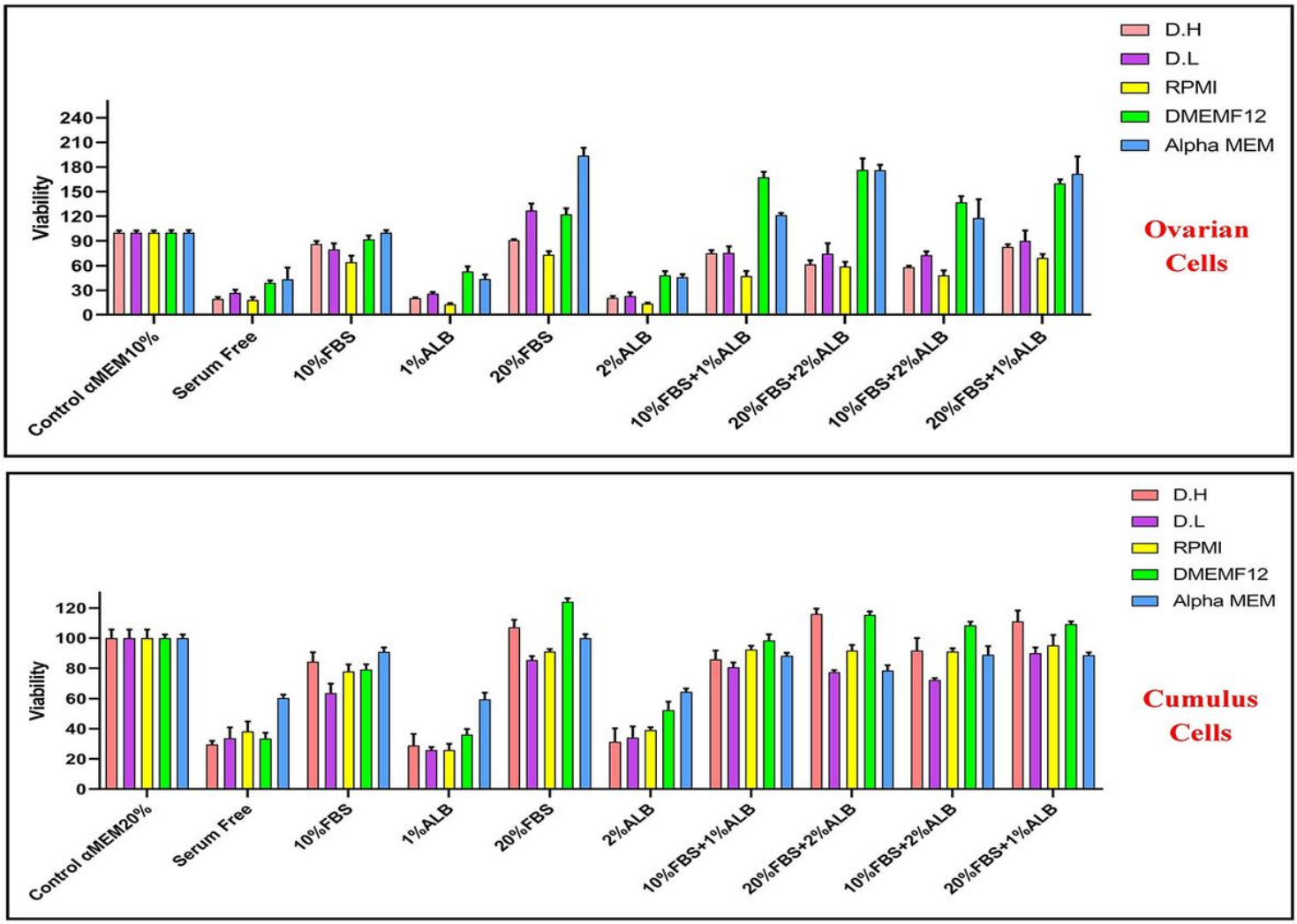

Figure 11

The final comparison of basal mediums/FBS/Albumin combinations on proliferation of ovarian cells and cumulus cells. According to this diagram, α-MEM+20%FBS cocktail was the choice medium for ovarian cell culture. But for cumulus cells in vitro culture, DMEMF12 +20%FBS was the best medium. Also, 20% FBS concentration was more efficient than 10% FBS even for OCs or for CCs. Albumin cannot support the in vitro growth of ovarian and cumulus cells alone.

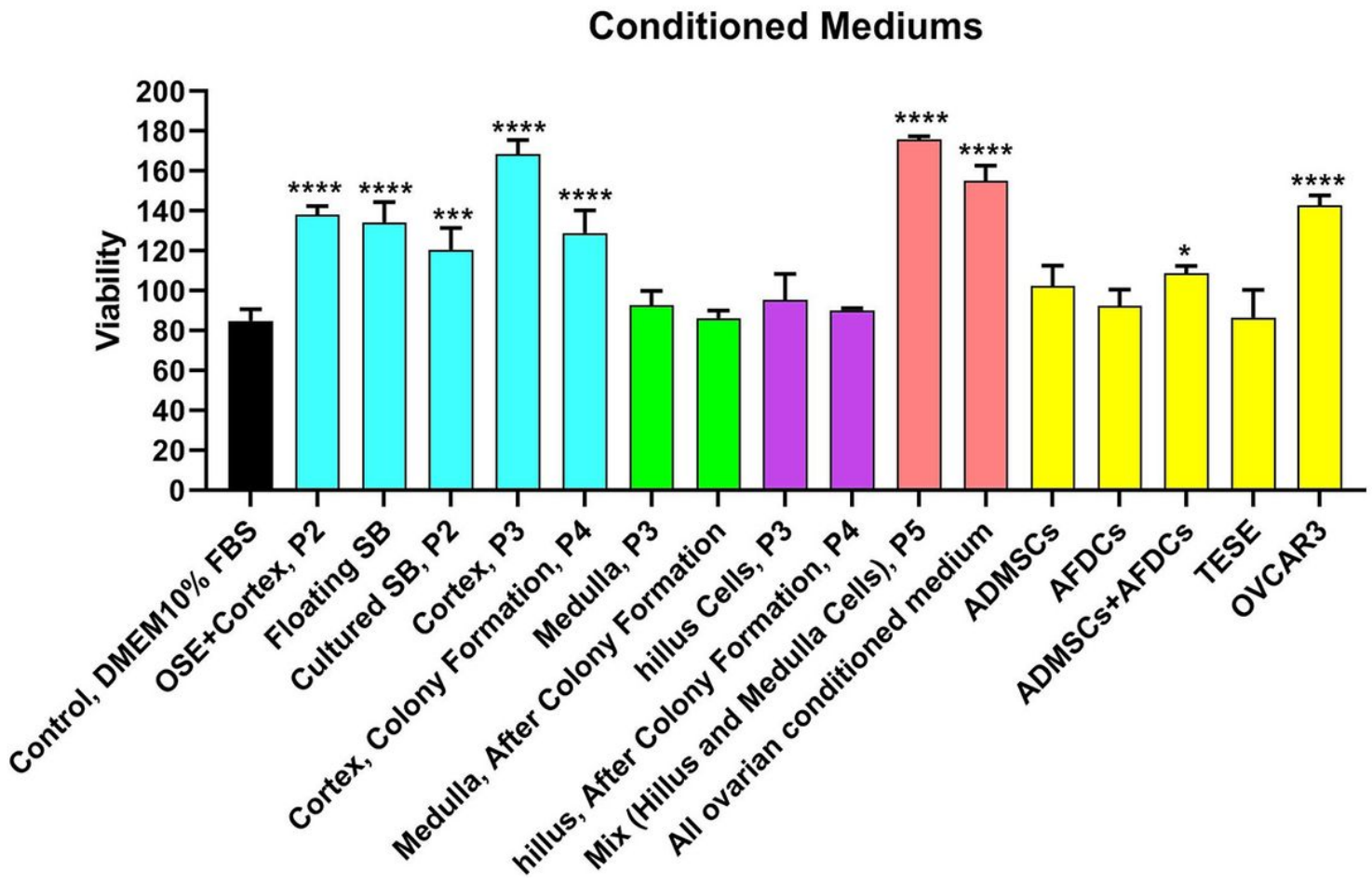

Figure 12

The effect of various conditioned mediums on cumulus cells proliferation CMs were isolated from the ovarian cortex, medulla and hilum. Also, the CMs of mesenchymal cells (ADMSCs & AFDCs), TESE derived cells and OVCAR3 cell lines. The CMs from ovarian cortex at early passages and from the mixture of medulla and hilus were the most effective mediums for supporting the growth of CCs. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

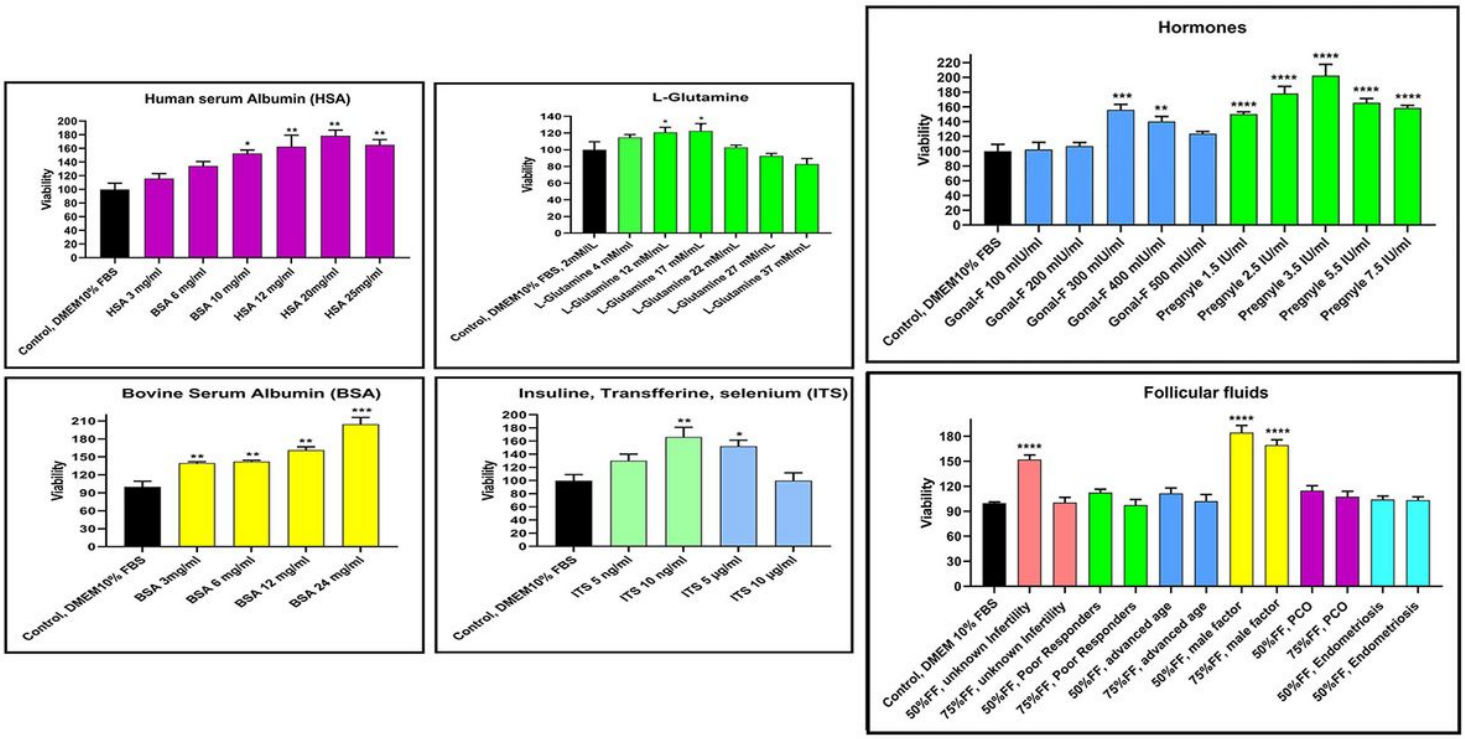

Figure 13

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

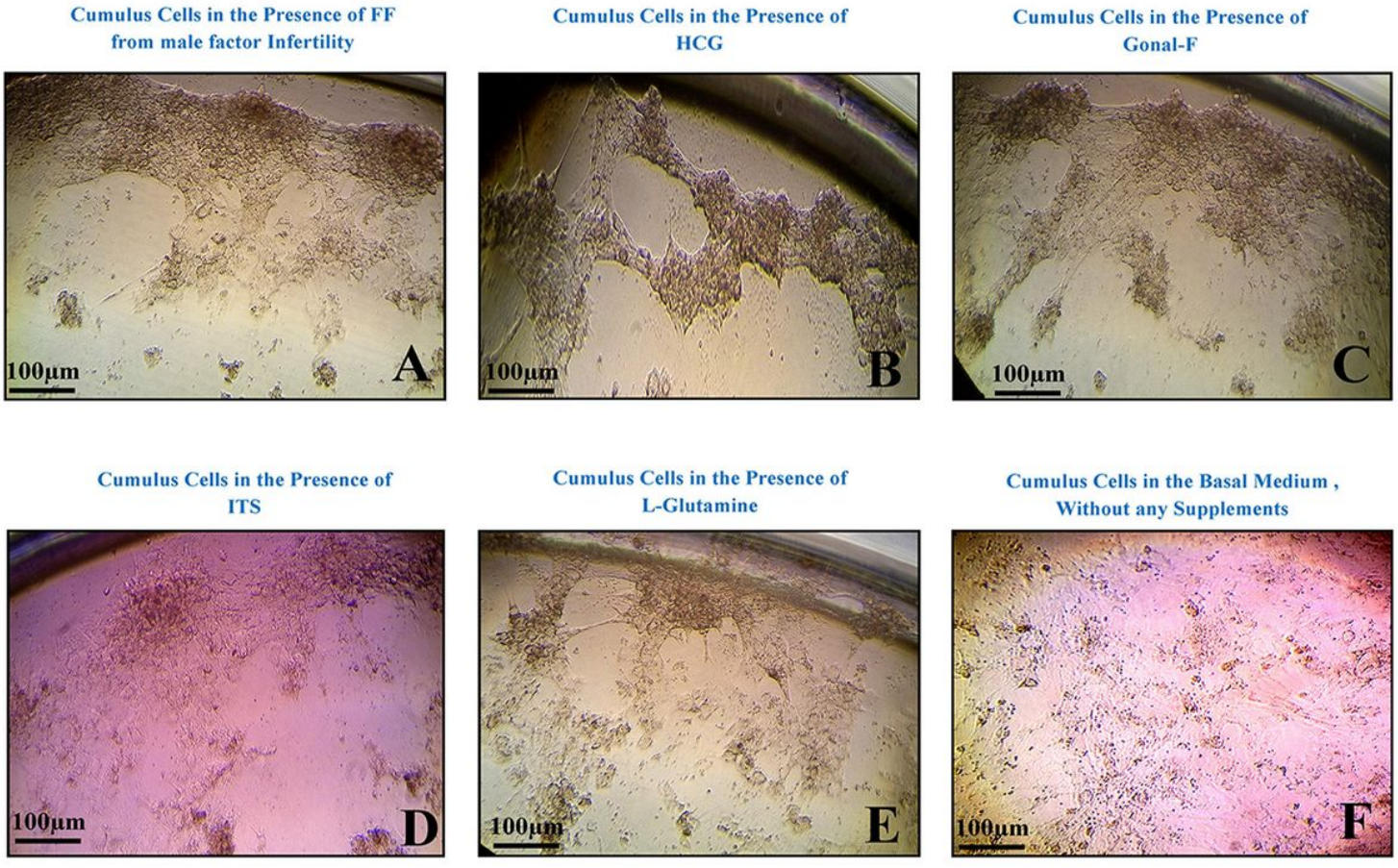

Figure 14

The Morphology of Cumulus Cells in the Presence of Various Supplements, FFs and Hormones In the presence of some materials like HCG, Gonal-F, FFs, ITS and L-Glutamine, the CCs formed some aggregations similar colony forming unit. The cells rearranged and demonstrated their potential morphology. These colony-like structures were more compact and uniform in the presence of HCG and FF (from male factor infertility patients).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Movie1.mp4
- Movie2.mp4
- Movie3.mp4