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Approaches for in vitro culture of granulosa cells and ovarian follicles

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Abstract

The in vitro culture of ovarian follicles or cumulus-oocyte complexes (COC) is used to study the factors that regulate follicular development and may have potential use in artificial reproductive technology (ART). Before ovulation, the follicle is formed by oocyte and cell populations known as granulosa cells (GCs). These cells build the internal and external mass of the follicular wall. Oocyte growth and proliferation of the surrounding cells depend on the gap junctions between the oocyte and the GCs. Maintenance of the optimal in vitro culture system allowing for preservation of follicle architecture and granulosa-oocyte interaction may be critical for success in vitro maturation of follicles. Recently many studies have focused on a culture of GCs, which have important functions related to steroidogenesis. Granulosa cells maintained in in vitro conditions exhibit stem cell properties making it important to consider in vitro culture (IVC) methods of the GC population.

Running title: Granulosa cells and ovarian follicles

Keywords: granulosa cells (GCs), cumulus-oocyte complexes (COC), ovarian follicle, ovarian follicle, in vitro culture systems

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Introduction

The ovarian follicle is the ovary's basic functional unit. It consists of an oocyte surrounded by GCs, which play a major role in a primordial follicles activation to further development. Activation of the primordial follicles is the most important initial step of oocyte development, involving the signalling pathways and interactions of inhibitory, stimulating and growth factors [1]. During the maturation and development of the follicle, changes in the shape and division of GCs are observed, including an increase in the number of GC layers [2]. Folliculogenesis is a complex and multi-stage process requiring interaction between somatic cell components and the oocyte, like the follicular wall. At first, primary germ cells migrate to the developing gonad in early embryogenesis and multiply and differentiate into an oogony during fetal development. At birth, the human ovary contains 1-2 million primordial follicles. Each follicle contains an oocyte in the meiotic arrested in the prophase I – dictyotene [3–5]. Follicles at this stage remain for a long time, resulting in the formation of the stock of resting follicles (RF) located in the stromal cortex until the female becomes sexually mature. Under the appropriate hormonal stimulation, several primordial follicles transform into the primary follicles, where the oocyte grows and is surrounded by a layer of granulosa cubic-shaped cells [6]. Between the oocyte and the cumulus cells, a thick transparent layer of glycoproteins (zona pellucida) is formed [7]. During the growth of the follicle, the gap junctions are created and through the transparent layer can occur cytoplasmic processes [1,8]. Only one of the primary follicles, the dominant follicle, completes the maturation process during the menstrual cycle each month. The remaining primary follicles degenerate into atretic follicles [9,10]. Follicular maturation takes about three months. Simultaneously the dominant primary follicle develops to the secondary stage, where the oocyte grows and acquires its final size (around 70-120 μm – depending on the species) [11]. Here, the number of GC layers increases to about six layers and cells surrounding the follicle differentiate into two types: theca externa (the outer layer) and interna (the inner layer) [4]. Subsequently, the theca externa becomes vascularised and due to exposure to gonadotrophins - FSH stimulation, the secondary follicle develops into an antral preovulatory follicle [12,13]. The antrum enlarges and the GCs surrounding the oocyte differentiate into cumulus cells (CCs), building the *cumulus oophorus* from the surrounding GCs, which is characteristic for the preovulatory follicle. In its intercellular spaces, a small amount of the alveolar fluid appears and fills the antrum. Fluid is probably derived from blood flowing through the thecal capillaries and crosses the potential barriers of the endothelium, subendothelial basal lamina, interstitium, follicular basal lamina and gets to membrana granulosa to be secreted by granulosa theca interna cells [14]. FF has nutritive properties for the oocyte [15]. At this stage, the first meiotic division is completed and the oocyte begins its second division. The complex of an oocyte, zona pellucida, and corona radiata is excreted during ovulation. After being released into the fallopian tube, the oocyte resumes its second meiotic division up to metaphase II [16]. This division is completed only when the gamete is fertilized [16,17].

Moreover, recent studies show that the population of GCs and other ovarian follicle cells have exceptional plasticity and can show stem cell properties through the expression of stem cell markers (such as OCT-4, NANOG and SOX-2) [18,19], suggesting their potential use in programs involving fertility preservation, *in vitro* feralization (IVF) or in regenerative medicine [19,20].

Ovarian follicle cells culture supplementation

Supplementation of culture medium with nutrients, growth factors, hormones play a key role in follicle and GCs IVC. Cells retrieved from animals or human tissues can be cultured either using artificial media or a completely natural medium. Natural medium (physiological medium) consists of naturally occurring biological fluids (e.g. for ovarian cells could be follicular fluid obtained in IVF procedure) [21,22]. However, the use of an artificial medium requires the addition of appropriate supplements.

The most common media (artificial) used in *in vitro* cultures of GCs are:

- Minimal Essential medium (MEM) [23];
- Dulbecco Modified Eagle medium (DMEM) [24,25];
- Waymouth medium [26];
- McCoy 5a medium [27];
- mixed media, e.g. Dulbecco's modified Eagle's medium/Ham's nutrient F12 – (DMEM/F12) [22,27,28];

These artificial media are grouped into categories:

- serum-containing media, e.g. Fetal Bovine Serum (FBS) or Fetal Calf Serum (FCS) or bovine serum albumin (BSA), common protein supplements essential for *in vitro* follicular development [19,28];
- serum-free media [29–31];
- chemically defined media, which commonly consist of a minimal/basal medium supplemented with animal serum (such as FBS) and other specific compositions such as glucose, growth factors, ions, amino acids, etc. [32];
- protein-free defined media;

The most frequently supplements added to artificial media during GCs, CCs *in vitro* culture are:

- **glucose** as a source of carbon energy - we can distinguish media with high or with low glucose concentration;

- **L-glutamine** as amino acid, required for cell proliferation and growth [32];
- **fetuin,** which is a glycoprotein and component of the fetal blood and body fluids produced by hepatocytes. It has been proved that human follicular fluid contains significant quantities of fetuin [36]. Research has shown that the combination of VEGFA 165 with fetuin leads to activation and development of resting follicles [33];
- **antibiotics** (generally a mix of them), e.g. penicillin/streptomycin;
- **growth factors** such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) [30,33–35];
- **leukemia inhibitor factor (LIF)** - a cytokine, glycoprotein with a wide range of functions in various tissues [36–40].

In vitro ovarian follicular cells cultivation techniques

Two-dimensional (2D) IVC of GCs

Many studies are focused on the processes occuring in the ovarian follicles and characteristics of ovarian cells. Cells from tissues collected from primates [41,42], e.g. rodents [36,43–46], porcine [24,29,35,47], bovine [48–54], ovine [55–57], caprine [58,59] have been employed in studies aimed at understanding ovarian functions.

Currently, assisted reproductive technology procedures have become a source of the follicular aspirate, which is usually wasted. The aspirate is a complex biological fluid that surrounds the developing oocyte. Follicular fluid (FF) contains GCs, CCs, thecal and ovarian surface epithelial cells, and also a variety of molecules, such as proteins, steroid hormones, polysaccharides, metabolites, etc. Scientific studies have confirmed that FF can be used for further research or testing as a potential source of cells with stemness potency [60,61].

Serum-free medium

There have been elaborated new cell culture protocol, which allows to culture primary human GCs using serum-free conditions while expressing GCs markers, e.g. FSHR, at least for two weeks. Isolated and purified cells were counted and live ones were plated onto Matrigel-covered 6-well plates in DMEM/F12 growth medium with 20% Knock Out Serum Replacement (KSR), 1% penicillin/streptomycin, and Primocin (100μg/ml). Growth factors - IGF2 (50 ng/ml) and FGF2 (8 ng/ml) both, or none were added to the defined basal growth medium to find optimal culture conditions supporting proliferation and expression of common granulosa cell markers, such as FSHR, AMHR2, LHR and CYP19A1 [30].

The serum-free method can be beneficial for luteal-GCs in long-term culture because it provides reliable conditions for luteal function [31] and will ensure new standards in human GCs to study reproductive biology [30].

Granulosa cells culture with FF

It has been suggested that the proliferation of human GCs is maintained in culture with the FF addition. It is used as a natural element providing a follicular environment in the medium.

Modern methods of proteomic analysis, such as mass spectrometry coupled with liquid chromatography enable the detection of many endogenous molecules (e.g. proteins, peptides) [62] in body fluids, including follicular fluid. Using reversed-phase nanoliquid chromatography coupled to matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (nano-LC MALDI TOF/TOF MS), a certain number of proteins were identified in FF. They are essential for communication of the oocyte with the surrounding environment during follicular development and oocyte maturation. Among all the findings, two proteins,- sex hormone-binding globulin (SHBG) and inhibin A (INHA), involved in hormone secretion during folliculogenesis were identified [63].

Moreover, GCs were cultivated in DMEM/F12 containing a low amount of serum (2% FCS) and supported by FF as well as FSH and growth factors (bFGF and EGF). The FF contributes to the cell adhesion process. GCs retained their morphology, developed intercellular junctions, and were strongly attached to the culture dish. The results suggest that the nutrients and FF in the culture medium are essential to maintain the proliferative potential of the primary culture of GCs [22].

Exposure of the human GCs to FF during the IVC may efficiently preserve the stemness characteristics of these cells as compared to a medium containing FBS. FBS may have a lower ability to preserve or induce pluripotency of GCs because of GATA-4 (GCs specific marker) high expression but lower rate of Oct 3/4 (stem cells specific marker), suggesting that FF is essential to conduct experiments concerning on GC's ability to differentiate to other types cells [64].

GC culture with the addition of LIF

LIF is a cytokine commonly used in culture to inhibit stem cell differentiation [65]. FCS or FBS supplemented with LIF has become the golden standard for mESC cultivation. Other studies indicate that LIF is important in the self-renewal process of stem cells, such as neural stem cells [37]. The analysis performed using reverse-transcription polymerase chain reaction (RT-PCR) techniques showed that LIF might also be involved in the initiation of growth of human primordial follicles. As LIF RNA and LIF-R RNA transcripts have been detected in previously collected primary follicles from human fetal ovaries and adolescent and adult ovaries [66].

In 2009, Kossowska-Tomaszczuk et al. [19] conducted an experiment comparing two approaches on GC cultures to evaluate the effect of LIF on the prolonged survival of GCs *in vitro*. The luteinizing GCs were split into two groups and cultured separately. One group was cultured in DMEM containing a high concentration of glucose supplemented with 15% FCS, penicillin/streptomycin L-glutamine, β-mercaptoethanol, recombinant FSH, and 1,000 IU/ml LIF, and the second group was cultured without LIF. The results show that supplementation of LIF permits the prolonged survival of luteinizing GCs. The luteinizing GCs cultured without LIF became apoptotic and consistently died within two weeks. In contrast, those cultured in medium supplemented with LIF remained viable for up to 4 months and could be passaged. Although LIF permitted the prolonged IVC of luteinizing GCs, they progressively lost their major characteristics, such as the FSHR and aromatase [19].

A simple conditioned medium for the primary adherent culture of CCs

In a recent study, which aim was to isolate, culture, and investigate the biological characteristics of human CCs, researchers used a simplified method of culture conditions. After oocyte isolation, the cumulus mass was carefully separated from COCs using hypodermic needles and treated with 300 µl/ml hyaluronidase for the 30 s. To inactivate the enzyme, Dulbecco's Modified Eagle Medium (DMEM) was added, and cells were washed using centrifugation at 200 × *g* for ١٠ min. The pellet was separated into -٢٥cm^2 tissue culture flasks containing DMEM supplemented with ٪٢٠ FBS and ١ µl/ml mix of antibiotics (penicillin-streptomycin) and incubated at 37°C and 6% CO_2. Those conditions were suitable because they allowed for the proliferation and dispersal of human CCs from the attached cumulus clusters over subsequent days of culture [25].

Similar culture conditions were used to culture CCs isolated from *in vitro* -matured COCs aspirated from buffalo ovaries. The CCs were seeded into 25-cm^2 tissue culture flasks with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and seeded and with 50 µg mL^{-1} gentamicin sulfate [67].

Three-dimensional (3D) IVC of GCs

3D system of GCs in conical microtubes containing rat collagen (type I)

Kossowska-Tomaszczuk et al. [19] suggest that the presence of LIF in a medium is crucial to receive a pro-longed GCs culture. Subsequently, the authors combined the two important factors and created a 3D culture system containing rat type I collagen and LIF [68]. GCs were cultured in complete medium made of DMEM-high glucose, supplemented with 15% FCS, penicillin/streptomycin, L-glutamine, β-mercaptoethanol, 100 ng/ml recombinant FSH, 200 ng/ml recombinant human LH and 1,000 IU/ml LIF. For the 3D culture, GCs were suspended in complete medium containing type I rat collagen in 1.5 ml conical microtubes incubated for 15 min at 37°C. Subsequently, cells were centrifuged for 3 min at 390 × g, to obtain a 3D-pellet. The supernatant was discarded and 1 ml of fresh complete medium was added. Subsequently, these microtubes were placed on an orbital shaker, to avoid the adherence of the pellet at the bottom of the tube and incubated at 37°C, 5% CO_2 conditions. A 3D model allows for the survival and growth of a subpopulation of GCs isolated from mature ovarian follicles and their proliferation into spherical structures showing steroidogenic characteristics. The proliferation during cultivation with LIF supplementation of GCs in a 3D colony was proven by the expression of the FSH-R receptor - a specific marker for GCs [68].

Thanks to the optimization of cultivation conditions, a follicular microenvironment was maintained, allowing the development of early ovarian follicles *in vitro.*

GCs spheroid culture systems

GC spheroids provide a model for *in vitro* tests without limiting aggregate yield. Spheroids can be easily obtained in rotary culture, where there is direct access to the cell-conditioned medium. Rotary culture is a commonly used approach to culture the GCs collected from different species allowing the study of the correlation between autophagy and apoptosis *in vitro*, steroidogenesis, *in vitro* toxicity testing, etc. [45,69,70].

Recently, the new protocol for the formation of ovarian rat cell spheroids using *AggreWell™ 400* plates has been constructed. The aim of the study was to compare three types of ovarian spheroids (the non-aggregated ovarian cells, nonlayered and multi-layered ovarian cell spheroids), which were encapsulated with collagen gel. To generate spheroids, an equal amount of rat's granulosa and theca cells were mixed and seeded in the *AggreWell* 400 µm plates, which were then centrifuged at 2300 rpm for 6 min in a swinging bucket rotor to pellet cells in each V-bottom microwell. After culturing for four days, the aggregates were embedded into a collagen gel (containing rat tail collagen I). Volumes of 150 µL of each cell-collagen mixture containing 1 × 10^5 granulosa cells and the same amount of theca cells were added in wells of 48-well plates and allowed to gel at 37°C for 20 min. After gelation, 400 µL of serum-free McCoy's 5A medium supplemented with L-glutamine, BSA, penicillin, streptomycin amphotericin B, ITS (10 µg/mL insulin, 5.5 µg/mL transferrin, 5 ng/mL selenium), FSH, LH, and IGF-I were added in each well. The spheroids were incubated for up to 30 days at 37°C in an atmosphere of 5% CO_2 in humidified air [45].

In 2007, Becker's team developed a method of forming human GCs spheroids using endothelial cell basal medium and methocel. The methocel was produced by dissolving 6 g carboxymethylcellulose in 500 ml endothelial cell basal medium.

A total of 400 human GCs were suspended in each well of non-adherent round-bottom 96-well plates and cultured for 24 hours at 37°C with 5% CO2 and 100% humidity to establish spheroids. Subsequently, the methocel (4.5 ml) and FCS (4:1) were put onto 400 spheroids and mixed with 4.5 ml of collagen gel containing (4 ml acidic collagen type 1) and 0.5 ml Medium 199 10x and NaOH to adjust the pH = 7.4. In this study, a 24-well plate was prewarmed and 1 ml of the prepared mixture containing 50 spheroids was given into each well into each of the eight middle wells. The gels were left to polymerize for 30 min, after which the corresponding test substance (e.g. human chorionic gonadotropin) in endothelial cell basal medium (100 ml/ml) was pipetted onto the gels [70].

3D hanging drop system of buffalo GCs

The hanging drop method is an excellent model for culturing GCs to mimic the intrafollicular environment, e.g. *in vivo* preovulatory follicular environment [71].

The GCs collected from aspirated FF (from buffalo ovaries) were pelleted by centrifugation at 1000 × g for 5 min. The cell pellet was suspended in 2 ml serum-free media (DMEM) supplemented with L-glutamine, BSA, sodium selenite, transferrin, androstenedione, bovine insulin, 100X MEM nonessential amino acid, ovine FSH, human recombinant insulin growth factor-1 (rIGF1), penicillin and streptomycin A. In this study, the GCs were cultured with a density of 20,000 cells/10 µl in a six-well culture plate. The 3 µl of media regularly added to each HD nutritional losses replenish losses due to evaporation and cell's metabolism (after every 48 h) [72].

2D follicle and oocyte IVC systems

The 2D culture systems may be more successful for culturing follicles retrieved from smaller animals like mice and rats in short-term conditions than 3-D models [73]. However, the follicles tend to be flattened and GCs surrounding and nurturing the growing oocyte can migrate away on the culture surface and leave a naked oocyte unable to complete the maturation process [74].

Droplet and hanging drop culture systems

The droplet method has been one of the first techniques for cultivating follicles and COCs at various stages from multiple animal species [56,75]. It is a process of suspension individual follicles in a drop of culture media in a culture dish, which is often overlaid with mineral or paraffin oil [76].

2D systems with covered plate surfaces

Follicles can be cultured either directly on the surface of a culture dish or well or on surfaces coated with ECM components such as collagen, fibronectin, laminin, or Matrigel [73,76]. Studies have proven that ECM has important roles in the ovary, including regulating cell adhesion, morphology, differentiation, endocrine communication, and steroidogenesis [77,78].

3D IVC systems for follicles and oocyte maturation

Maintenance of the 3D culture can preserve sustainable follicle architecture with the oocyte surrounded by GCs [79,80]. It is critical for cell-cell interactions such as granulosa-oocyte interactions and cell-matrix signaling. These two factors can be lost in conventional 2D adherent tissue culture systems. However, long-term cultivation methods for ovarian follicle cells have already been developed and improved during the last few years [81,82].

Suspension follicle culture system

Suspension culture is a type of 3D system maintaining follicle morphology by various non–scaffold-based approaches, including continuous floating in test tubes or glass bottles, (rotating/roller system), inversion, or levitation with magnetic beads [74].

A new approach for cultivating bovine follicles using magnetic levitation with nanoparticles assembly in a 3D system has recently appeared. The study aimed to observe the changes in the in vitro development of bovine secondary follicles by maintaining the 3D levitation culture system. The effects showed greater viability of follicles and antrum formation and lower extrusion and degeneration rates than a traditional 2D system. The follicles also produced viable oocytes that resumed meiosis after *in vitro* maturation. The basic culture medium TCM199 was used and supplemented with BSA, insulin-transferrin-selenium (10 µg/ml insulin, 5.5 µg/ml transferrin, 5.0 ng/ml selenium), glutamine, hypoxanthine, ascorbic acid, gentamycin and streptomycin. This technique should be considered a potential alternative method to follicle IVC in several species, including humans [50].

Follicle IVC systems: outlook on gel matrices and hydrogels

The aim of testing the novel biomaterials such as matrices and hydrogels for 3D culture of follicles is to ultimately create the culture model that will support the maturation of animal and human ovarian follicles and preserve their functions [82–84].

Alginate is a natural, non-toxic polysaccharide isolated from brown algae widely used as hydrogel and matrice for follicle cultivation. Alginate hy-

drogels are usually the first choice in planning 3D culture due to their well-defined constitution and ability to provide a stable and reproducible culture system [84]. It has been described that using alginate as a 3D porous scaffold for the GCs influences the proliferative rate and function of GCs [44].

The hydrogel-based system has been improved by combining alginate and fibrin hydrogel to create a dynamic interpenetrating fibrin–alginate network [73]. Fibrin is a non-globular protein involved in the blood-clotting process. Earlier, fibrin and alginate were often used individually as scaffolds, but combining the components may provide a benefit. Interpenetrating fibrin–alginate network promotes follicle growth and upregulates the meiotic competence of oocytes relative to either fibrin or alginate used alone [85]. It is assumed that the addition of fibrin to the alginate matrix may better mimic the natural ovarian follicular environment. For example, follicles in macaques interpenetrating fibrin–alginate network grew to the primary stage but did not reach secondary one [41]. It was also documented that encapsulated fibrin-alginate hydrogel culture system supports the development and steroidogenesis of caprine preantral follicles and improves oocyte maturation [58].

3D culture systems using various concentrations of type I collagen hydrogels ranging from 1% to 7% (weight/volume) affect ovarian follicle survivability, development and sex hormone production. For example, 17β- estradiol and progesterone were detected in the conditioned media collected from cultured follicles within collagen hydrogels [86].

Hyaluronan, a ubiquitous glycosaminoglycan, has been tested for its biocompatibility with ovarian follicles [87]LH, ITS and 5% FBS. Maturation was triggered by addition of hCG and EGF after in vitro culture (IVC in tyramine-based hyaluronan hydrogel with and without adding extracellular matrix proteins (ECM).

Generally, ECM plays a distinct role in the maturation of follicles *in vitro*. Still, surprisingly in this study, it was indicated that ECM modified- hyaluronan gel did not meaningly affect the follicle's parameters, which were measured. The outcomes relating to hyaluronan and ECM modified- hyaluronan gels were similar comparing the parameters like the follicle survival ability, resumption of meiosis (GVBD) and oocyte maturation to the metaphase II stage. However, the ECM modified- hyaluronan scaffold supported greater estradiol production [87].

Because of the recent interest in using synthetic matrices as hydrogels for 3D culture, the number of available systems employing poly-ethylene glycol (PEG) has increased. The significant advantage of the PEG is the possibility of crosslinking PEG with peptides, such as RGD motif (tripeptide Arg-Gly-Asp) [88]. The modifications are performed *in situ* in physiological conditions and harmless to follicles. A new method for co-encapsulation of small ovarian follicles with mouse embryonic fibroblasts within PEG *in vitro* 3D culture system. It appears that the maturation rate of the follicles was improved by including PEG in the system compared with the control *in vitro* culture of follicles individually or without the feeder cells [89].

New perspective for follicle IVC

The latest breakthrough in *in-vitro* follicle growth shows that matrix-free culture with the support of stage-specific anti-Müllerian hormone (AMH) modulation can continue follicular development of the early-stage follicle and oocyte maturation. AMH is a glycoprotein hormone and belongs to the transforming growth factorbeta superfamily. It is produced by GCs of primary, preantral and small antral follicles maintaining the right follicle growth [90].

In a latest study [91] researchers observed that follicles grew to the secondary stage during three weeks of culture with ovarian cortical tissue. *In-vitro*-developed follicles expressed AMH and levels of secreted AMH increased in the culture media over time. Secondary follicles isolated from cultured ovarian tissue survived and grew to the antral stage during six weeks of individual follicle culture. *In-vitro*-developed antral follicles produced granulosa and theca cell-derived steroid hormones and paracrine factors, which were detectable in the culture media. This protocol could be applied to the *in-vitro* development of follicles from cryopreserved ovarian tissue to obtain mature oocytes [91].

Conclusions

To summarize, the research in the primary culture of human GCs has provided important knowledge about giving optimal conditions for the stable *in vitro* culture. These studies have been challenging for a long time because the primary cells usually proliferate very slowly in culture and tend to lose their characteristic marker genes expression during *in vitro* culture. It should be noted that human GCs are usually are obtained during IVF procedure, meaning that they are generally in the luteinized state [30,92].

Maintaining the 3D culture system can also meet many obstacles. The suitable conditions for preserving the proper structure and morphology of ovarian follicles must be determined. 3D culture systems using mentioned hydrogels effectively improve the *in vitro* ovarian follicle culture, supporting follicle morphology, growth, steroidogenesis, expression of characteristic markers and enhancing oocyte maturation.

Ethical approval
The conducted research is not related to either human or animal use.

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Conflict of interest
The authors declare they have no conflicts of interest.

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