Original Article:
Optimized Primary Culture and Subculture of Granulosa Cells

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Article info:
Received: 18 Jun 2021
Accepted: 07 Sep 2021

Keywords:
Cell culture techniques, Crocin, Date palm, Granulosa cells, Oocytes

ABSTRACT

Background: Primordial follicle includes an oocyte surrounded by a layer of somatic cells called Granulosa Cells (GCs). GCs, also known as nurse cells, are an important protective element for the growth and survival of oocytes. Oocytes, which lack some of the metabolic processes, require granulosa cells for their development.

Objectives: This manuscript was provided to explain the protocol of GCs primary culture extracted from NMRI mice ovaries.

Methods: For choosing the optimum protocol, we used two methods with different culture mediums to obtain more GCs and expedite the process. Hematoxylin and Eosin (H&E) staining and flow cytometry were used to analyze the type of extracted cells from ovaries. Besides, we evaluated the effect of crocin and DPP as two common natural products in Iran on the proliferation of these cells via MTT assay.

Results: Second protocol method and alpha-MEM culture medium were chosen based on the results. Our findings from HE staining and flow cytometry proved the percentage of cultured GCs in the flask. Further, MTT assessment demonstrated that crocin at high doses had a toxic effect on granulosa cells, whereas date palm pollen (DPP) stimulated them to proliferation.

Conclusion: Modifying this protocol is for the improvement of proliferation, coherence, and quality of GCs in primary culture and subculture. Regarding the effect of these two natural products on granulosa cells, we can mention the bilateral effect of crocin and DPP enhancement in proliferation.

Citation Fallah N, Paktinat M, Rasouli M, Nabiuni M, Amini E. Optimized Primary Culture and Subculture of Granulosa Cells Pharmaceutical and Biomedical Research. 2022; 8(2):155-162.

http://dx.doi.org/10

Introduction

The ovary as the main organ of the reproductive system is responsible for the secretion of progesterone, estradiol, oxytocin, inhibin, and activin [1]. The main function of the ovary is the production of the oocyte that depends on the growth of follicles. Follicles include a germ cell (oocyte) and somatic cells (granulosa cells and theca cells). The proliferation of Granulosa Cells (GCs) increases the layers that surrounded the oocyte and leads to the development of follicles [2].

GCs in the wall of the follicle are in contact with the antrum and proliferate as the follicle grows. These cells are divided into two groups (mural granulosa cells and cumulus cells) based on their function and their distance from the oocyte [3]. Mural granulosa cells are responsible for the final maturation of follicles and are sensitive to estrogen and LH. Cumulus cells remain with the oocyte during ovulation and have an important role in the resumption of oocyte meiosis [4]. Overall, granulosa cells and oocytes control the growth and development of each other reciprocally [5].

GCs cause the release of FSH and inhibit the reduction of oocyte storage in females through secretion of activin, inhibin, and Anti-Müllerian Hormone (AMH). Besides, the main function of these cells is the production of steroid hormones [6, 7].

In general, infertility term is the inability of couples to give birth to a healthy baby. The purpose of this article was to provide the optimal protocol for culturing granulosa cells, evaluating two prevalent natural products in Iran containing crocin, a bioactive component of saffron, and Date Palm Pollen (DPP) on granulosa cells as crucial cells in fertility. DPP is popular as a rich source of nutrients, such as minerals, antioxidants, sugar constituents, and amino acids. Due to these properties, from the previous decades, DPP has been known as a stimulator for fertility by improving the concentration of steroid hormones in both males and females [8]. Crocin is a carotenoid compound of saffron (*Crocus sativus* L.) and the main cause of its red color. Therefore, saffron is widely used as a food spice among Iranian people, and analyzing its impact on infertility, especially on granulosa cells is very important [9].

Materials and Methods

Animals

Animals were retained similar to the Kharazmi University guidelines of animal house. Twenty-one to twenty-five days old female NMRI mice were housed at a controlled temperature under 12 h lighting and 12 h darkness rhythm and had free access to water and food.

Make a device for the isolation of GCs (mouth pipet)

In three steps as presented below, a mouth pipette was made for the isolation of GCs (Figure 1).

1. Cutting the tube of serum set on both sides.

2. Attaching the micropipette tip to one side of the tube for use of Glass Pasteur pipette that thinned for isolation of granulosa cells by heat

3. Attaching nylon syringe filter and syringe (without plunger) on the other side of the tube for the harvest of GCs

Preparation and culture of NMRI mice GCs

In this protocol, two methods, three types of culture medium, including Alpha Minimum Essential Medium Eagle (α-MEM, Gibco-Invitrogen), Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich), Dulbecco`s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12, Sigma-Aldrich), and 10-15% Fetal Bovine Serum (FBS, Gibco-Invitrogen) were used to optimize cell culture. For the primary culture of granulosa cells, we used two methods, three culture medium types, and two different concentrations of FBS to compare and select the appropriate method. Phosphate Buffered Saline (PBS, Sigma-Aldrich) was used to wash the ovaries. Collagenase IV (Gibco-Invitrogen), Trypsin-EDTA (0.25%) (Sigma-Aldrich), and Antibiotics (penicillin at 50 units/mL and streptomycin at 50 µg/mL) (Sigma-Aldrich) were also used during the extraction of GCs (Figure 2).

Preparation of DPP

The DPP was collected from Bushehr Province Botanical garden, South of Iran. To make the stock, 0.1 g of DPP was dissolved in 10 ml PBS, then stirred for 1-2 hours, and eventually filter to remove unsolved particles by a 0.22 syringe filter [10]. DPP concentrations were obtained by diluting the stock with the culture medium. In all cases, the DPP solution was prepared 24 h before treatment.

Preparation of crocin

Crocin (digentiobiosyl 8, 8'-diapocarotene–8, 8'–oate; $C_{44}H_{64}O_{24}$) powder was purchased and was diluted with DEPC water to prepare different doses.

The first method of the establishment of primary culture

In the first method, ovaries were removed from 21-25 days NMRI mice and washed twice with PBS 1X. Drops included α-MEM/DMEM/DMEM-F12 supplemented with 1% antibiotics and 10% FBS, which were dribbled on the petri dish. Ovaries were punched in the petri dish with insulin syringes.

The GCs were collected under an inverted microscope with a mouth pipet and were transferred to a flask including α-MEM/DMEM/DMEM-F12, 1% antibiotics, 10%/15% FBS. The flask was incubated at 37°C in a humidified atmosphere with 5% CO2.

The second method of the establishment of primary culture

In the second method, ovaries were removed from NMRI mice and washed twice with PBS 1X similar to the first method. The ovaries were punched in a petri dish, which included α-MEM and 1% antibiotics, and punched ovaries and medium were transferred to a microtube. Collagenase IV was added and incubated for 20 min, and after pipetting, it was incubated for 10 min more. Then, FBS was added for neutralizing collagenase IV. The released GCs were transferred to the flask that included α-MEM, 1% antibiotics, and 15% FBS. For optimized collecting GCs, the remaining ovaries were centrifuged and transferred to the flask. The GCs were incubated in an atmosphere of 5% CO2 and 95% air at 37°C.

Establishment of subculture

The culture medium was removed from the flask and washed twice with PBS 1X. To cover the cells, trypsin-EDTA (0.25%) was added, and cells were incubated for 3-4 min at 37˚C, and then 200 µl FBS was added for neutralizing trypsin-EDTA. The cells were divided into two flasks with a fresh culture medium.

H&E staining

The GCs were stained with hematoxylin-eosin to corroborate their form and morphology studied by the inverted microscope (Zeiss, Germany).

Flow cytometry analysis

Flow cytometry analysis was conducted to confirm that the extracted cells were GCs. Inhibin alpha-subunit (Anti _inhibin a: sc-365439 FITC (Santa Cruz)) immunoreactivity was employed for GCs identification. The data were analyzed with Flowjo 7.6 software.

MTT assay

3-[4,5-dimethylthiazole-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay was used to measure the GCs viability. After 48 hours of treatment, MTT (Sigma-Aldrich, St. Louis, MO, USA) test was conducted and an ELISA reader measured the absorbance at 570 nm.

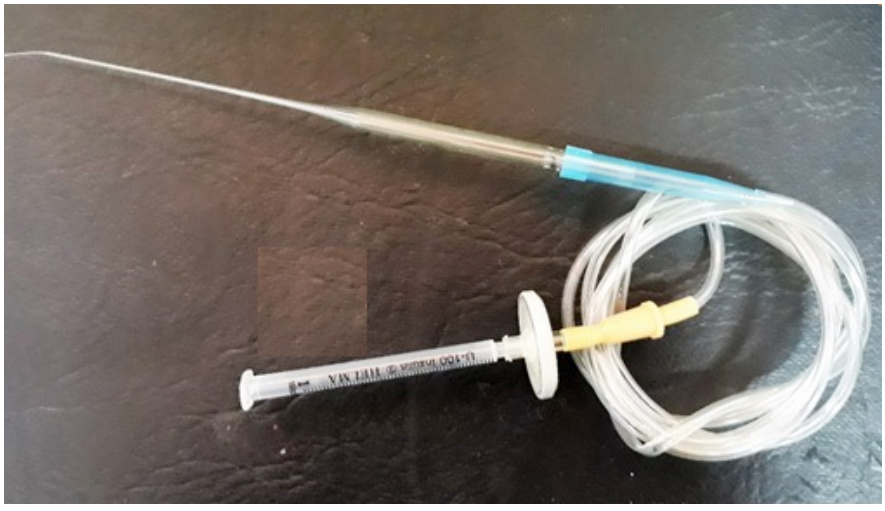

Figure 1. Mouth pipette used to harvest the granulosa cells after the ovaries punching

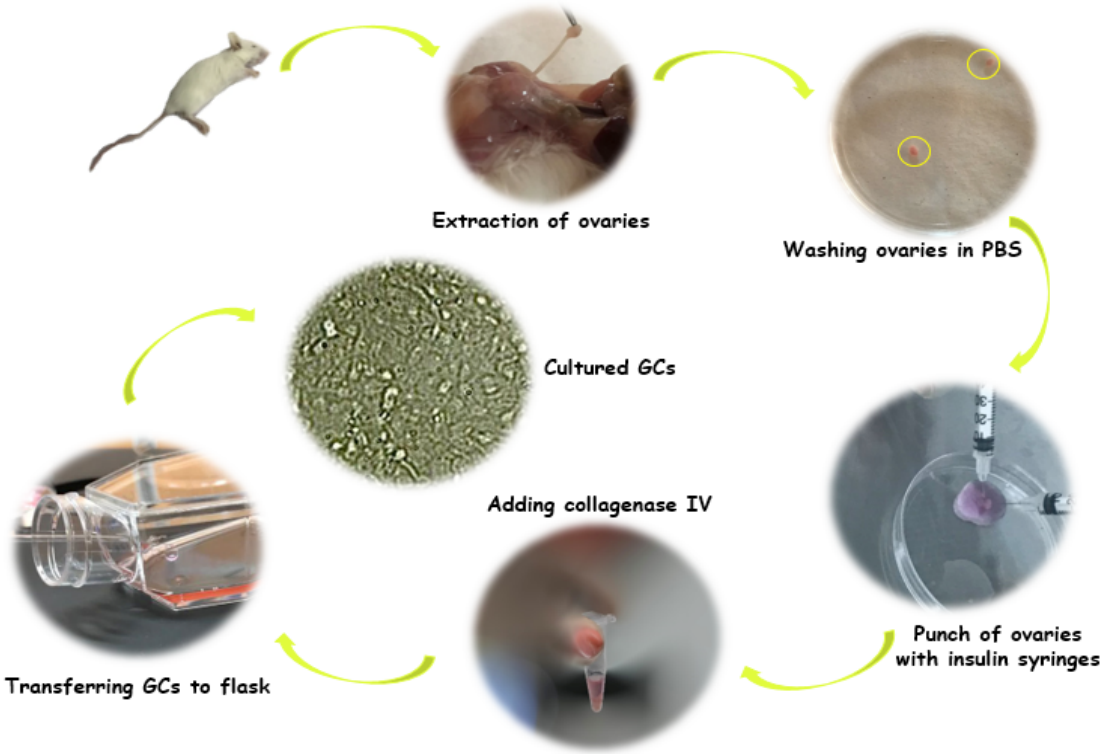

Figure 2. The steps of NMRI mice granulosa cell isolation and cultivation

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Results

According to the conducted cell culture techniques, a collection of using α-MEM, 15% FBS, mechanical and chemical digestion based on the secondary protocol, and deletion of centrifuge step in the subculture of the GCs (neutralize trypsin-EDTA with FBS; 5:1) created an optimum environment for GCs cultures. Then, HE staining and flow cytometry analysis were carried out for indicating the GCs extraction accuracy.

H&E staining

As shown in Figure 3, HE staining demonstrated the morphology of these cells with a round nucleus in the center and spindle shape, which is similar to the cells that are well known as GCs in the literature.

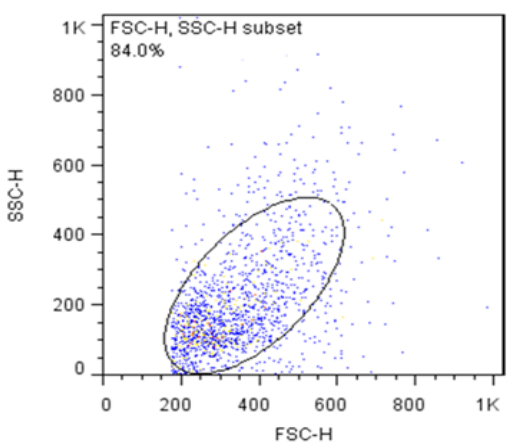

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Figure 4. Point diagram of α-inhibin expression by flow cytometry

The presence of GC cells is concentrated in the region of α-inhibin expression and 84.0% of the isolated cells are in the α-inhibin expression region.

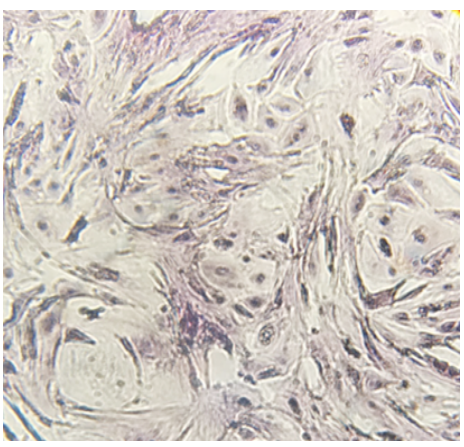

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Figure 3. HE staining was performed for the extracted mice granulosa cells

The GCs were spindle-shaped, and nuclear was in the center of the cells.

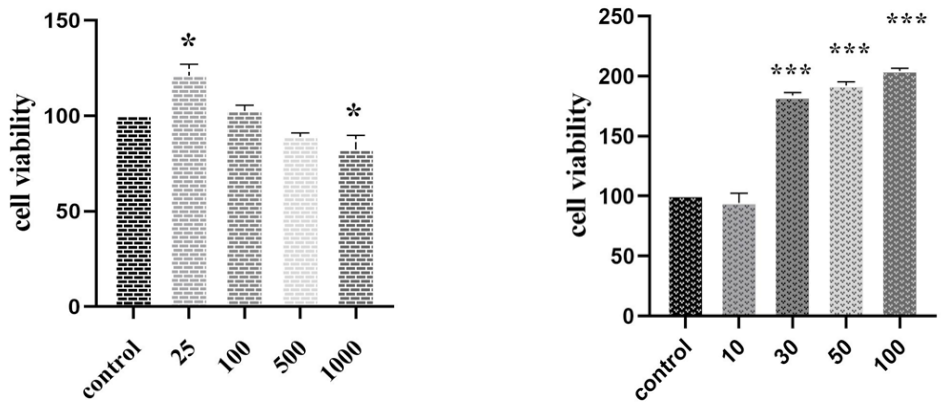

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Figure 5. The viability of GCs at 48 h after treatment with different concentrations of crocin and DPP
All experiments were replicated at least three times. Data are presented as Means±SD. (*P<0.05, **P<0.01, and ***P<0.001 versus the control).

Flow cytometry analysis for GCs identification

Flow cytometry analysis was done as a supplementary assay with an α-inhibin antibody, which is the GCs dedicated marker. The results showed that 84% of extracted cells were GCs (Figure 4).

GCs viability exposed to DPP and crocin

To analyze the effects of DPP and crocin on the viability of GCs, we performed an MTT assay on the NMRI GCs treated with DPP at 10, 30, 50, and 100 µg/ml as well as crocin at 25, 100, 500, and 1000 µg/ml. The results of these assays manifested that DPP could increase cellular viability at all doses but this increment was significant at the doses of 30, 50, and 100 µg/ml (P>0.05). In the crocin-treated group, a significant increase in GCs viability was observed at a 25 µg/ml dose, whereas the viability of GCs significantly reduced at a 1000 µg/ml dose of crocin (Figure 5).

Discussion

Granulosa somatic cells are responsible for supporting the oocyte. Tripathi et al. demonstrated that enhanced apoptosis of GCs decreased oocyte viability and the quality of oocytes [11]. The results of the GCs culture process using DMEM and 10% FBS in the first protocol indicated that cells did not have optimum proliferation and adhesion, and increment of FBS did not improve the cultivation. By change of culture medium to DMEM-F12, although proliferation and adhesion enhancement was observed for GCs, cell attachment was not observed after the subculture under both FBS concentrations. After the culture of GCs with α-MEM, albeit our results showed optimum proliferation, it took 10-12 days to reach the 80% confluence. Therefore, choosing an α-MEM culture medium containing 15% FBS brings about a suitable condition due to the increment in quality and adherence of GCs. Beyond the mentioned techniques discussed above, in pursuit of improving the GCs extraction and reducing costs, we omitted some supplements, like Insulin-Transferrin-Selenium (ITS), FSH, or implant SILASTIC capsules [12] and used α-MEM as the richest medium. In the second protocol, by using both chemical and mechanical digestion facilitates, not only the process of obtaining cells was improved but also in less time we achieved more cells of higher quality; furthermore, using collagenase IV as enzymatic digestion should be considered to reduce the damage of physical digestion.

According to the MTT assay, DPP increases the proliferation of GCs, which augment the fertility and synthesis of steroid hormones. As previous studies showed, DPP is useful for ovarian function and the growth of ovaries and eggs that are related to the enhancement of steroid hormone production [13, 14]. Next, different doses of crocin were tested on GCs to explore its impact on fertility. Our findings represented that whilst crocin at a high dose (1000 µg/ml) can disrupt fertility and cause toxicity on GCs and suppress their proliferation, at low doses, it can be suitable for fertility and stimulation of ovarian steroids production. The therapeutic effect of crocin, especially the reduction of the side effects of cyclophosphamide has been shown more recently [15]. On the other hand, similar results were also documented regarding the destructive effect of saffron as the main source of crocin on women who work on saffron farms during their pregnancy and the rate of abortion is significantly high among them [16, 17]. Apart from this natural product,

we more recently used this optimized procedure for GCs culture to evaluate the impact of gas plasma throughout normal and cancer ovarian cells [18].

Conclusion

In conclusion, two cell culture techniques, which consist of physical digestion separately and physical and enzymatic digestion concomitantly, were utilized for the cultivation of GCs to achieve an optimum environment for GCs cultures. During the cell cultures, the impact of different culture mediums and various FBS concentrations also were examined. Compared to physical digestion, we showed that combinational employing of physical and enzymatic digestion led to appropriate cultivation conditions under 15% FBS concentration and α-MEM culture medium. Regarding the effect of DPP and crocin on cultured GCs, we found that increasing DPP was consistent with GCs proliferation, whereas the viability of crocin highly depends on the crocin concentration. While at a low dose, it acts as a stimulator for GCs proliferation, at high doses, it causes toxicity and suppresses GCs proliferation. Collectively, DPP is recommended for fertility, whereas the consumption of saffron due to the existing crocin for pregnant women should be controlled.

Ethical Considerations

Compliance with ethical guidelines

The protocols were approved by the Research Ethics Committee of Kharazmi University (IR.KHU.REC.1399.011).

Funding

The paper was extracted from MSc. thesis of the first author, Department of Cell& Molecular Biology, Faculty of Biological Sciences, Kharazmi University.

Authors' contributions

Conceptualization: Elaheh Amini, Nadia Fallah, Maryam Paktinat; Methodology, Data analysis, Investigation, Writing – original draft: Nadia Fallah, Maryam Paktinat, and, Milad Rasouli. Supervision Mohammad Nabiuni. and Elaheh Amini. All authors read and approved the final version of the paper.

Conflict of interest

The authors declared no conflict of interest.

Acknowledgments

The authors wish to extend their thanks to Kharazmi University central lab.

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