Original Article

# The Effect of Normal Follicular Fluid on the Differentiation of PCOS Ovarian Stem Cells into Oocyte-Like Cells

**Mahin Taheri Moghadam[1,2], Hanan Nazayer[2], Saeed Azandeh[1,2], Ali R. Eftekhari Moghadam[3], Roshan Nikbakht[4]**

[1]Cellular and Molecular Research Center, Medical Basic Sciences Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, [2]Department of Anatomical Science, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, [3]Department of Anatomical Science, Faculty of Medicine, Baqiyatallah University of Medical Sciences, Tehran, Iran, [4]Fertility, Infertility and Perinatology Center, Imam Khomeini Hospital, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

## Abstract

**Background:** Polycystic ovarian syndrome (PCOS) is one of the causes of infertility for which treatment methods do not have a high rate of pregnancy. In this study, the stem cells in the follicular fluid (FF) of patients were grown in the normal FF, and their differentiation into oocytes was evaluated.

**Materials and Methods:** The FF of PCOS patients was centrifuged, and their cells were cultured with and without 20% normal FF for 2 weeks. The cells were evaluated for their morphology by inverted microscope and for markers of stem cells (NANOG and OCT4) and oocytes (zona pellucida (ZP) 2 and ZP3) by RT-PCR and immunocytochemistry. The amount of steroids was measured by enzyme-linked immunosorbent assay (ELISA).

**Results:** The cells were all round on day 0. After that, they had a heterogeneous morphology (fibroblast-like cells, epithelial-like cells, and round oocyte-like cells). In the first week, NANOG and OCT4 genes in the study group were less expressed than those in the control group ($P < 0.0001$) (~0.5-fold), while ZP2 and Z3 genes were more expressed ($P < 0.0001$) (~2-fold). In the second week, stem cell genes were more expressed in the control group (~2 fold), and oocyte genes were more expressed in the study group ($P < 0.0001$) (~2.5–3.11 fold). These results were also confirmed by immunocytochemistry. The amount of steroids was much higher in the study group (three times and five times in two weeks) ($P < 0.0001$).

**Conclusions:** Stem cells can be obtained from the FF of PCOS, and normal FF has a positive effect on the growth and maturation of oocyte-like cells *in vitro*.

**Keywords:** Follicular fluid, oocyte retrieval, PCOS, stem cell, zona pellucida

**Address for correspondence:** Dr. Mahin Taheri Moghadam, Cellular and Molecular Research Center, Medical Basic Sciences Research Institute; Department of Anatomical Science, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.
E-mail: taherimoghadam-M@Ajums.ac.ir
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## Introduction

Today, infertility affects about 48 million couples worldwide and has become a major challenge for health systems and society.[1] In this regard, one of the most common endocrine disorders and infertility due to lack of ovulation is polycystic ovarian syndrome (PCOS).[2]

The name of this syndrome is due to the presence of large ovaries containing a large number of small cysts in the ovarian cortex. The symptoms of this syndrome include menstrual disorders, especially oligomenorrhea, hyperandrogenism such as hairiness or hirsutism, acne, hair loss, and infertility.[3] Also, these patients can be exposed to serious complications

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such as increased risk of endometrial and breast cancer with dyslipidemia and hypertension, heart disease, diabetes, and obesity.[4]

Today, extensive research has been conducted in the field of infertility treatment by assisted reproductive technology (ART), and its use is increasing. In this method, the oocyte in the ovarian follicle is removed from the ovary along with the follicular fluid (FF) and given to the laboratory to perform assisted reproductive procedures. After picking up the oocyte from the follicle, the FF is discarded.

Studies have shown that aspirated FF contains different cell populations, such as granulosa cells, leukocytes, large epithelial cells, and stem cells,[5] as well as various factors affecting oocyte growth.[6] Stem cells in FF originate from the superficial lining of the ovary.[7] Also, in 2014, researchers were able to obtain mesenchymal stem cells (MSCs) from the FF of mothers who underwent ART.[8] Another study showed that MSC can express the specific CD markers of oocyte cells such as zona pellucida (ZP) 1, ZP2, and ZP3 and differentiate to the germinal vesicle stage of the oocyte.[9] They said these cells probably have the ability to transform into mature oocytes.[10] For this purpose, researchers have decided to obtain the conditions for the transformation of oocyte-like cells into mature oocytes.[8]

One of the important factors in the growth and maturation of the oocyte is the substance in the follicle fluid. Part of this liquid is leaked from the blood serum and contains glucose, urea, cholesterol, triglycerides, and some hormones. Another special part of FF compounds is microRNA (mRNA), which are related to the metabolic activity of the follicle. It affects the diagnosis and prognosis of many diseases and infertility problems in women.[11] Also, this liquid contains leptin, inhibin, and activin that have an effect on oocyte growth.[12]

In this project, PCOS ovarian stem cells were taken for the first time and cultured in normal FF, and their oocyte-like cells were examined in terms of differentiation. For this, expression of stem cell markers such as NANOG and OCT4 and oocyte markers such as ZP2 and ZP3 was evaluated. Therefore, in this study, the effect of normal FF on the differentiation process of oocyte-like cells in PCOS was investigated.

## Materials and Methods

### Human subjects

The study protocol was approved by the Ethics Committee of the School of Medicine of Ahvaz University of Medical Sciences (IR.AJUMS.MEDICINE.REC.1400.005). To prepare the stem cells of PCOS patients, women with clinical symptoms of hyperandrogenism, polycystic ovaries, oligoovulation, and anovulation were included in this study. Patients with androgen-secreting tumors, congenital adrenal hyperplasia, and hyperprolactinemia were excluded from the study. Also, for the preparation of normal FF, women who were referred to the infertility center and whose husbands were the cause of their infertility were selected.

### Ovarian FF collection

After obtaining written informed consent from patients, the ovarian FFs were collected from 40 infertile PCOS and 20 non-PCOS (normal patients with male factor infertility) subjects. They were treated with controlled ovarian hyperstimulation for *in vitro* fertilization (IVF) at the center of reproductive medicine at AJUMS. For the removal of red blood cells, FF was pooled in a tapered 50-ml Falcon tube consisting of two drops of heparin (10–30 IU/ml), and the hypoosmotic lysis technique was applied to them.[13] In this technique, FF was centrifuged at 300 g for 6 min, the supernatant was discarded, and the cell slurry was transferred into a 15-ml Falcon tube. Then, 9 ml of the sterile distilled water was spilled into the cell pellet, and the tube was mixed carefully. After 20 seconds, 1 ml of concentrated phosphate-buffered saline (PBS) 10 × (pH = 7.2–7.3) was added and the mixing was continued. The tubes were then centrifuged at 150 g for 3 minutes. Ultimately, the cell pellet was resuspended in 0.5 ml of Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich). Cell viability and counting assessments were done in 0.2% trypan blue by a hemocytometer.

To prepare normal FF, after picking up the oocytes, the FF was centrifuged at 1400 rpm for 6 minutes. Then, under the hood, the supernatant was removed with a sterile Pasteur pipette and filtered by a sterile 0.45-μm filter for removing granulosa cells, theca cells, blood cells, and immunological cells. Then, FF was placed at 56°C for 45 minutes to inactivate immunological factors. After cooling, it was aliquoted and stored at -20°C until use.[14] During use, the FF was thawed at room temperature and added to the culture medium.

### Cell culture of FF cells

Cells in PCOS FF were divided into a control group and a study group. The cells of the groups were plated in triplicate at 100,000–150,000 viable cells per well in 4-well culture dishes (BD Biosciences). The culture medium comprised DMEM supplemented with L-glutamine (2 mM), 1% penicillin/streptomycin (Gibco), 1.25 μg/mL Fungizone (Gibco, Grand Island, NY), and 20% fetal bovine serum (FBS) in the control group and 20% normal FF in the study group. The cells were allowed to attach for 48 hours and then washed with culture media to remove unattached cells and debris. The cells were incubated for 2 weeks at 37°C and 5% CO2 and monitored daily under an inverted microscope (Nikon, Japan) for assessment of morphology. Every two days, the cell culture medium is changed.

### RNA extraction and quantitative real-time polymerase chain reaction (RT-PCR)

The RT-PCR assessment was done on FF-harvested cells on days 7 and 14 in the control and study groups. This protocol was based on our previous studies.[5,15] The extraction of total RNA was conducted from $10^5$ to $10^6$ cells using RNeasy Mini Kit (Qiagen). RNA samples were eluted in 40–60 ml of RNase-free water. Later, according to the manufacturer's directions, 500 ng of total RNAs underwent reverse transcription into complementary DNA through Superscript II Reverse

Transcriptase Kit (Fermentas Life Sciences). A relative real-time RT-PCR test was performed with an ABI 7900 Thermocycler System using Power SYBR Green PCR Master Mix (Applied Biosystems). The total volume of the PCR mix was 25 ml, and thermocycling conditions were 40 cycles: 95°C for 15 s, 56–62°C for 30 s (depending on primer), 72°C for 30 s, and 75°C for 30 s for the final cycle. The annealing temperatures, accession number, and primer sequences for OCT4, NANOG, ZP2, ZP3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are presented in Table 1. The amplicon size was verified by electrophoresis in 2% agarose gels, and the specificity of the PCR products was evaluated by melting curve analyses. All the gene expression levels were normalized to GAPDH. The relative expression values for each gene were measured by adopting the $2^{-\Delta\Delta pt}$ method with efficiency correction and applying one control sample as a calibrator. Each test was run in duplicate.

### *Immunocytochemistry staining*

Immunocytochemistry was performed to confirm RT-PCR findings and for the detection of mesenchymal protein and phenotypic evaluation of FF-derived MSCs. On day 14 of culture, the adherent cells with and without 20% normal FF-enriched medium were initially washed with PBS 1 × (pH = 7.2–7.4) and fixed with 4% ice-cold paraformaldehyde for 15 minutes at room temperature (RT). Then, permeabilization with 0.1% Triton X-100 was done at RT for 10 minutes after three washes with PBS. Blocking of nonspecific antigens was performed with blocking solutions that included 1% bovine serum albumin (BSA) and 1X PBS for 45 minutes. Afterward, the incubation of primary antibodies was performed with anti-NANOG (rabbit antihuman, 1:200; Bio Legend), anti-OCT4 (rabbit antihuman, 1:200; S Bio Legend), anti-ZP2, and anti-ZP3 (mouse monoclonal, 1:100; Santa Cruz Biotechnology) antibodies at 4°C overnight. Later, cells were washed three times with PBS and incubated with fluorescein isothiocyanate conjugated (FITC) goat anti-rabbit or rabbit anti-mouse antibodies (Sc2012; Santa Cruz Biotechnology, Inc.) diluted at 1:500 in PBS 1X at RT for 1 h. In the final step, after three washes with PBS 1X, cell nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI). The samples were observed under a fluorescence microscope (Leica M205 FA; Leica Microsystems).[15,16]

### *Estradiol assays by enzyme-linked immunosorbent assay (ELISA)*

The culture medium was collected from the cells on days 0, 7, and 14. Each was centrifuged separately and stored at −80°C until the test. In the ELISA test (Catalog No. 1920; Alpha Diagnostic International), culture media were diluted with enzyme-linked immunoassay buffer (1:4) according to the manufacturer's instructions. Then, it was incubated with estradiol enzyme conjugate for one hour. It was rinsed three times with diluted enzyme-linked immunoassay buffer and added to the kit with a substrate solution. After that, the absorbance of the sample was read at 450 nm using a microplate reader (Synergy Two Multi-Mode Microplate Reader).

## Results

### *Morphological studies of cells*

About 24 hours after cultivation, small round cells and some red blood cells were seen in the culture medium [Figure 1a].

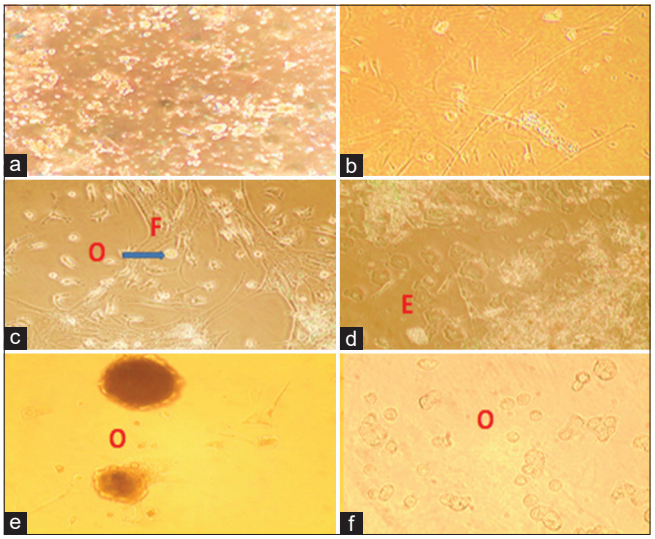


**Figure 1:** Morphology of the cultured cells of the follicular fluid of PCOS (a) cells on the first day, (b) spindle-shaped cells on the second day, (c) fibroblast-like cells (F) and oocyte-like cells (O) in the third and fourth days, (d) epithelial-like cells (E) in the third and fourth days, (e) oocyte-like cells (O) in the control group in two weeks, and (f) oocyte-like cells (O) in the study group (with normal follicular fluid) in two weeks. (20X)

**Table 1: List of primers used in RT-PCR**

| Genes | Primers | Amplified size (bp) | Accession number | Annealing temperature |
|---|---|---|---|---|
| OCT4 | GGCCCGAAAGAGAAAGCGAACC<br>ACCCAGCAGCCTCAAAATCCTCTC | 224 | NM_203289.5 | 64 |
| NANOG | GGGCCTGAAGAAAACTATCCATCC<br>TGCTATTCTTCGGCCAGTTGTTTT | 400 | NM_001355281.2 | 59 |
| ZP2 | CAGAGGTGTCGGCTCATCTGA<br>GCAGTCTTGTGCCCTTTGGT | 110 | NM_001376233.1 | 61 |
| ZP3 | GACCCGGGCCAGATACACT<br>CATCTGGGTCCTGCTCAGCTA | 110 | NM_007155.6 | 61 |
| GAPDH | GGGAGCCAAAAGGGTCATCA<br>TGATGGCATGGACTGTGGTC | 203 | NM_001357943.2 | 60 |

About 48 hours after culture, by changing the culture medium, the red blood cells were removed from the medium, and there were small cells attached to the bottom of the plate, which had a spindle-shaped appearance similar to mesenchymal cells [Figure 1b]. During the third and fourth days of culture, cell accumulations with different forms of fibroblastic and epithelial cells were seen in the culture medium, and during the first week, small and large round cells were gradually more visible [Figure 1c and d]. Also, in the second week of cultivation, the mesenchymal cells changed their shape and size and spontaneously transformed into oocyte-like cells, which were mostly located between and close to fibroblastic cells. These round and large oocyte-like cells in the study group with normal FF were more numerous and also larger in volume than those in the control group [Figure 1e and f].

### Expression of stem cells and oocyte genes

RT-PCR was performed to evaluate the stem cell (OCT4, NANOG) and oocyte (ZP2, ZP3) genes in the culture medium. In this method, the internal control GAPDH was used, and to compare the expression of genes between the control and study groups on different days, relative analysis and the ΔΔCT method were used.

As shown in Figure 2, all genes of stem cells and oocytes are well expressed during two weeks, but in comparison, when the cells grown in a culture medium containing normal FF (the study group) are compared to the cells of the control group (containing FBS), changes in the expression of these genes can be seen. In the first week, NANOG and OCT4 genes in the study group were less expressed than those in the control group ($P < 0.0001$) (~0.5-fold), but ZP2 and Z3 genes were more expressed in the medium containing FF ($P < 0.0001$) (~2-fold). Also, as in the first week, in the second week, stem cell genes were more expressed in the control group (~2-fold), and oocyte genes were more expressed in the study group ($P < 0.0001$) (~2.5–3.11-fold).

In the comparison of these genes in the second week compared with the first week, NANOG ($P < 0.0001$), ZP2 ($P = 0.009$), and ZP3 ($P < 0.0001$) genes were more expressed, but the OCT4 gene was less expressed ($P < 0.0001$) in the control group. Also in the study group, these genes were more expressed ($P < 0.0001$), but the expression of the OCT4 gene did not show a significant difference ($P = 0.0922$) in the second week.

### Immunofluorescence staining for markers of stem cell and oocyte

To confirm the results obtained by RT-PCR, an immunocytochemical test was performed. The pluripotent index of mesenchymal cells was investigated by OCT4 and NANOG markers. These two markers were expressed in both control and study groups in the first and second weeks. Also, in this study, the index of oocyte cells was examined by ZP2 and ZP3 markers, and these two markers were also expressed in both groups in the first and second weeks. In all stages of the experiment, a group without a primary antibody was used as a negative control, and no light was obtained from these samples [Figure 3].

### Estradiol in the culture medium

As seen in Figure 4, the fluid around the cells on day 0 has little estradiol, but with the cultivation of cells and their growth and differentiation in the culture medium containing FF (study group), this amount increases to 3.3 times in the first week. In the second week, it increases to 5.3 times, and these increases are all statistically significant ($P < 0.0001$). However, estradiol in the control group had a slight increase compared with day 0, but there was no significant difference ($P > 0.5$). Also, the amount of estradiol in the study group increased significantly compared with the control group, so that in the first week its amount reached 2.55 times and in the second week it reached 3.58 times that of the control group ($P < 0.0001$).

## Discussion

PCOS is one type of infertility that affects 6–10% of women. Unfortunately, the main cause of this syndrome is still unknown. Today, to solve this problem, many treatments are performed, including assisted reproductive methods. In these methods, the oocyte is taken from the ovary along with FF, and after fertilization with sperm, it is again transferred to the uterus, but the same methods, in addition to causing hormonal problems for the person, do not have a high rate of pregnancy.[17] For this reason, researchers are looking for other treatment solutions today. Since it has been stated in studies that the FF has stem cells that can spontaneously differentiate into oocyte-like cells,[5] in this study, it was decided to isolate FF stem cells from PCOS patients and investigate their differentiation into oocytes in a suitable culture medium. Also, studies have shown that some factors, such as retinoic acid and BMP4, help better differentiate these cells.[18,19] Therefore, in this study, normal FF was used as the culture medium, and the degree of differentiation of stem cells into oocytes was investigated.

In this study, the isolated cells from FF were all around on 0 days. In the first week, they had a heterogeneous morphology (fibroblast-like cells and epithelial forms) in the culture medium. This morphological state was consistent with our previous research and other studies.[15,20] In previous studies, it was shown that the origin of these cells is from granulosa cells and the ovarian surface epithelium (OSE).[5] It was also indicated that the granulosa cells have the properties of stem cell potential multipotency and can differentiate into chondrocytes, osteoblasts, and neuronal cells.[21] Also, the researcher isolated epithelial cells from the culture medium and showed that, like granulosa cells, they have stem cell and differentiating properties from other cells.[5] In the present study, it was also shown that these cells have NANOG and OCT4 markers, and in our previous studies, we stated that these cells can differentiate into osteocyte and chondrocyte cells and express the surface marker CD105,[20] so we concluded that these cells are MSCs.

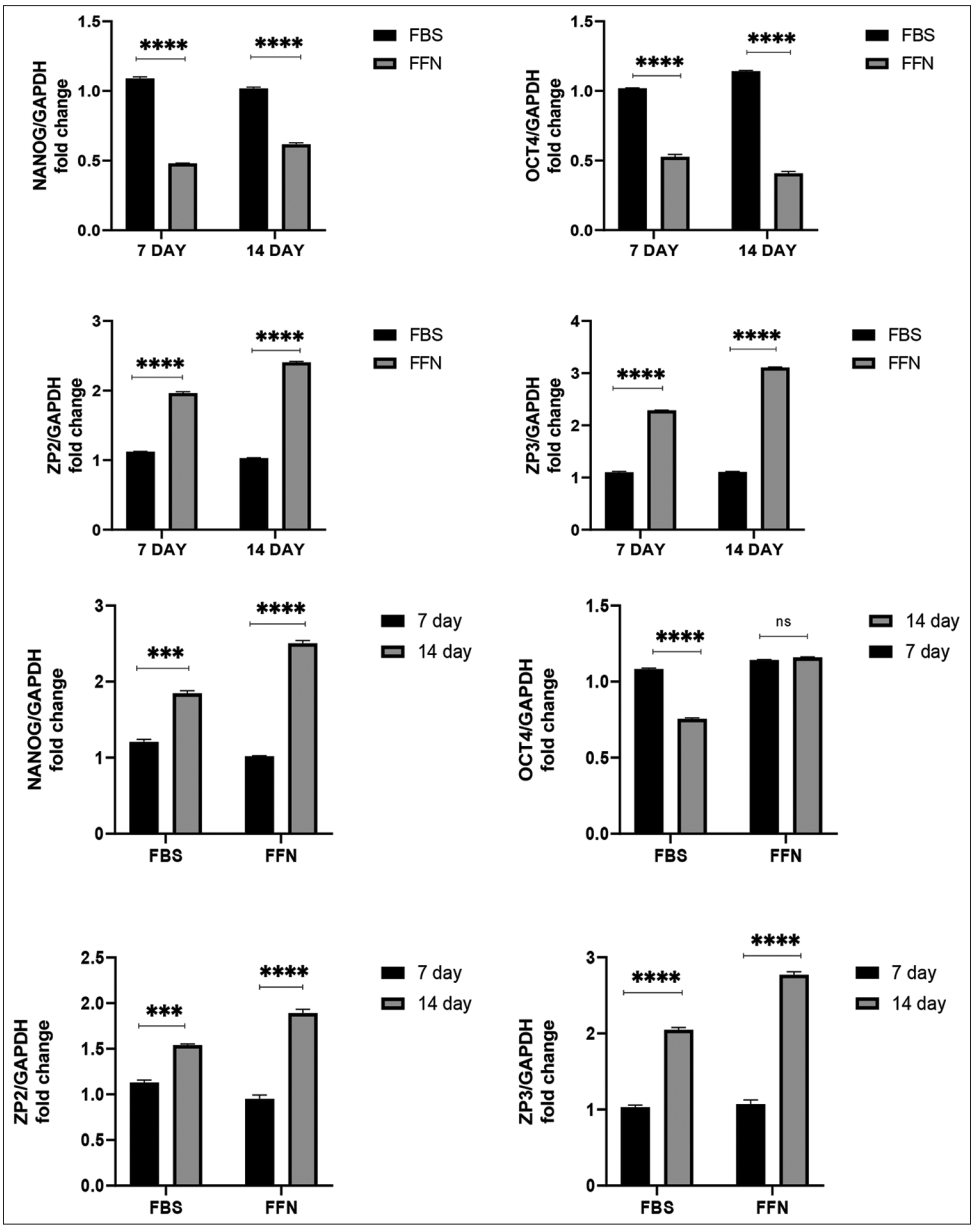


**Figure 2:** Expression of NANOG and OCT4 genes in stem cells and ZP2 and ZP3 in oocyte cells in the control group (with FBS) and study group (with follicular fluid) in the first and second weeks of culture. ****: $P < 0.0001$, ***: $P = 0.009$, ns = not significant

Also, these cells were differentiated into small and large round cells within two weeks. In this study, it was found that these cells have ZP2 and ZP3 oocyte markers, so these cells are oocyte-like cells. As we know, the ZP surrounds oocytes and blastocysts and is made of four types of proteins (ZP1-ZP4). Some studies showed the important role of ZP in the quality of the oocyte,[22] blastocyst formation,[23] and pregnancy.[24] Another study showed that the expression of ZDP 1, ZDP 2, and ZDP 4 genes decreases in mature oocytes, but ZP3 expression does not show a significant decrease. Also, it stated the ratios of the four ZP mRNAs (1/4/14/2 and 1/3/14/1 for ZP1/2/3/4 in immature and mature oocytes, respectively). It was confirmed that ZP2 and ZP3 are the main structural components of the ZP.[25] Therefore, according to this issue, it can be said that the oocyte-like cells in the present study have good differentiation because they have good expression of ZP2 and ZP3 markers.

Also, in the current study, the effect of normal FF on the differentiation of stem cells was investigated. In this study, fibroblastic and epithelial-like cells were seen in the culture medium during the first days of cell culture. Then, in the first and second weeks, round oocyte-like cells were also seen. Also, by examining the NANOG and OCT4 genes for the presence of stem cells and checking the ZP2 and ZP3 genes, the presence of oocyte-like cells in this medium was confirmed. By comparing the values of these genes with the control group (without FF), the results showed that NANOG and OCT4 genes are expressed much less (about half-fold) in the medium containing FF, while ZP2 and ZP3 genes are much

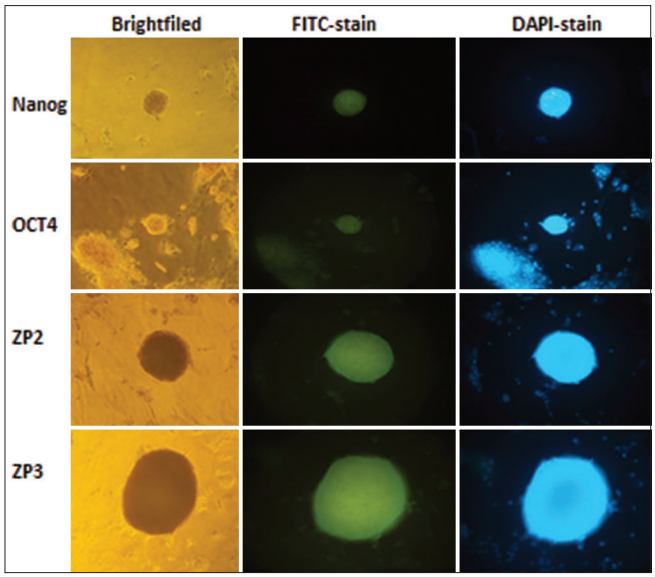


**Figure 3:** Immunofluorescence staining of stem cell markers (NANOG and OCT4) and oocyte-like cells (ZP2 and ZP3)

more expressed (about 2–3 times). These results were also confirmed by immunocytochemistry. Also, by examining the amount of steroids in the culture medium, the results showed that this hormone was secreted much more in the culture medium containing FF (about three times in the first week and five times in the second week). These results showed more differentiation of oocyte-like cells in the medium containing FF. Of course, to determine the similarity of oocyte-like cells to oocytes, other genes in oocytes such as VASA, DAZL, and FIGLA should be investigated in oocyte-like cells. For a better evaluation, these results were compared with other studies.

In 2020, the researchers extracted stem cells from Wharton's jelly and differentiated them into oocyte-like cells in two mediums containing FF and granulosa cell fluid. In this study, they concluded that the FF performs better in the differentiation of oocyte-like cells.[26] In another study, the researchers showed that mRNAs in bovine FF can cause oocyte maturation and protect it from oxidative stress.[27] Also, another researcher stated that the addition of FF and granulosa cell supernatant to the oocyte of a PCOS patient improves oocyte growth in the culture medium.[28] In a study in 2013, the researcher scraped the ovarian epithelial cells of POF patients and cultured them in the presence of FF. They concluded that the FF causes the transformation of stem cells into oocyte-like cells.[7] In 2018, it was suggested that the mRNA of normal FF and PCOS are different, and this difference can be a factor in the cause of PCOS.[29] Also, in 2015, a study showed that normal FF is an important biological fluid that plays a very important role in oocyte growth and follicle development.[30] Therefore, according to these studies, it can be said that in the present study, normal FF, through its proteins, metabolites, ionic and hormonal compounds, and mRNAs, has a significant effect on the differentiation of stem cells into oocyte-like cells and can be used for further differentiation of stem cells into oocytes.

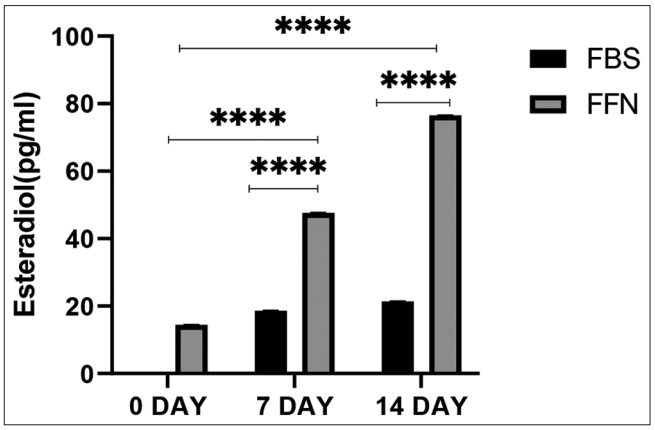


**Figure 4:** Determining the amount of estradiol in culture media containing normal follicular fluid (FFN) and without follicular fluid (FBS) in the first and second weeks ****: $P < 0.0001$

## Conclusion

This study shows that stem cells can be obtained from the FF of PCOS patients, and these cells can differentiate into oocyte-like cells in the presence of normal FF. This study can be the basis for obtaining oocytes and embryos from PCOS patients. Of course, more research is needed to have a completely differentiated oocyte.

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### *Data availability statement*

The data that support this study will be shared upon reasonable request with the corresponding author.

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### *Conflicts of interest*

There are no conflicts of interest.

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