Original Article

Utilizing Calcium Alginate for the Assessment of Bone Morphogenetic Protein 15 Induction Effect on the Differentiation of Mesenchymal Stem Cell Derived from Human Follicular Fluid to Oocyte-Like Structure

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Abstract

Background: Follicular fluid (FF)-derived mesenchymal stem cells (MSCs) are possible new source of cells in the study of oogenesis and regenerative medicine. Several biomaterials have been used as scaffolds to mimic ovarian tissue stroma. Using good matrix is essential for increasing the cell survival rate, proliferation, and differentiation. However, no study has been performed to investigate the effects of BMP15 and calcium alginate hydrogel on the differentiation potential of FF-derived MSCs to oocyte-like structures (OLSs). **Materials and Methods:** In this work, FF MSCs, which were collected from women in routine *in vitro* fertilization procedure, were capsulated with 0.5% calcium alginate, and then the encapsulated cells were cultured in medium containing BMP15 for 2 weeks. Trypan blue staining was carried out to determine cell viability. Real-time polymerase chain reaction (PCR) and immunofluorescence (ICC) staining method were performed to characterize the expression of OCT4, Nanog, ZP2, and ZP3 genes and protein. The encapsulation process did not change the morphology and viability of the encapsulated cells. **Results:** Reverse-transcription-PCR and ICC showed that MSCs expressed germ line stem cell markers such as OCT4 and Nanog. After 4 days of culture, OLSs formed and expressed zona pellucida markers. OLSs at least reached 180–230 μm in diameter in the control and BMP15-treated groups. Finally, a reduction in the expression pattern of pluripotency and ZP markers was detected in the encapsulated cells cultured in the BMP15-supplemented medium. **Conclusion:** The three-dimensional alginate culture system seems to be a promising method of getting *in vitro* differentiation and development of ovarian cells, which could mimic the native ovarian condition.

Keywords: *Bone morphogenetic protein 15, calcium alginate, follicular fluid, mesenchymal stem cells, oocytes*

Introduction

Today, infertility becomes a serious health and social relevance and affects 48.5 million couples worldwide.[1] To study the exact molecular mechanisms of infertility, germline stem cells (GSCs) are the best choice. It has been accepted that, in most mammals, the number of oocytes existing throughout life is controlled during fetal life.[2] Studies, however, declared that there is ovarian regenerative activity in postnatal mammalian ovaries.[3,4] Large number of evidence illustrated that female germline stem cells could be obtained from mammalian ovary involving mice, rats, and humans.[3,5] Previous studies on the multipotency in follicular antrum cells have shown that some of these cells have the characteristics of mesenchymal stem cells (MSCs).[6] In an experiment, Wood and Tilly showed the active germ cells, both *in vitro* and *in vivo*, in mature mice and human ovary, which can differentiate into oocytes.[2,7] Therefore, many researchers are engaged in whether animal or human embryonic stem (ES) cells have the potential for *in vitro* differentiating into primordial germ cells or oocyte-like cells (OLCs).[8] Today, scientists are looking for a way to identify the characteristics of OLCs and more differentiation of these cells into mature oocytes. Ovarian stem cells have opened a new horizon to better realize human oogenesis and infertility preservation and intervention. However, because of ethical problems, the shortage of human ovarian samples prohibited these applications. Follicular

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fluid (FF) aspiration produced during routine assisted reproductive technology (ART) methods could be a reasonable alternative for ovarian tissue samples. Evidence suggests that FF-derived cells could provide a new source of GSCs.[9] Hence, these results led us to choose FF as a source of MSCs, which discarded after cumulus oocyte complex (COC) removal at the infertility centers worldwide.

During the process of growth and differentiation of ovarian follicles, there is a bilateral communication in cytokines and growth factors between the oocyte and its surrounding granulosa cells.[10] In this way, members of the transforming growth factor-beta superfamily, especially BMP15, play an essential role in regulating the proliferation, growth, and differentiation of ovarian stem cells.[11] Some of these proteins promote the growth and differentiation of germ cells, including their specificity, migration, formation of ovarian follicles, and maintenance of this important ovarian structure.[12] However, the precise role of BMP signaling molecules in the activation and growth of ovarian somatic cells has yet to be investigated.[12] Among the various signaling molecules present in ovarian tissue, BMP15 and its homolog (GDF9) are secreted at different stages of ovarian follicular development, controlling the growth and proliferation of granulosa cells and the steroidogenesis process of these cells.[13] It contributes to controlling the growth and proliferation of granulosa cells, the steroidogenesis process (in a follicle–stimulating hormone [FSH]-independent fashion), and the prevention of apoptosis in these cells.[14,15] Hence, BMP15 can be an important factor in the simultaneous proliferation of granulosa cells and a promoter of natural reproductive physiology.[16]

As mentioned above, the growth, proliferation, and differentiation of the cells that form the ovarian follicles are mediated by an intricate process, and require precise relation through junctional and transcellular communications.[17] In the *in vitro* culture system of ovarian follicles, the maintenance of a natural conformational structure of interest is pivotal for acquisition of the development of competence.[18,19] Conventional two-dimensional (2D) culture systems have disadvantages, despite their facility. These include the dissociation and migration of proliferating granulosa cells from the oocyte onto the 2D substrate. These culture approaches have not been able to support normal follicle development in human and other mammalian systems,[19] while three-dimensional (3D) ovarian follicle culture systems provide the native architecture of the follicle and the oocyte–granulosa and cell–matrix interactions within the tissue.[18] Several matrices, such as polyethylene glycol, collagen, agarose, and alginate, have been used so far for *in vitro* 3D encapsulation of follicles.[18] Among these matrices, alginate hydrogel produced from brown algae, is one of the most frequently used biomaterial.[20] Alginate is an inert polymer and does not interact with integrins of animal cells,[21] so it permits the introduction of well-defined signals. Because of its nature, it must be perfect to support 3D follicle structure and adhesion should improve cell survival and development.[22] *In vitro* evidence suggests that granulosa and theca cells encapsulated by alginate could secrete sex hormones and other steroids in response to ovarian gonadotropins.[23] The aim of the present study was to evaluate the effect of BMP15 on the differentiation ability of MSCs derived from human FF into OLCs and the relative expression of oocyte gene and protein markers in alginate 3D culture system.

Materials and Methods

Chemical reagents

Sigma Chemical Co., St. Louis, MO (USA) was selected to purchase each chemical reagent. Moreover, Santa Cruz Biotechnology, Inc. 1.800.457.3801 Europe, and Bio Legend Inc., 9727 Pacific Heights Blvd, San Diego, CA 92121, USA, were chosen to buy the antibody.

Ethical statement

This protocol was approved by the IRCCS Bioethics Committee (Protocol number IR.AJUMS. REC.1396.433, approval date July 15, 2017). A written consent form was employed to apply the derived cells and surplus follicular liquids.

Follicular fluid sampling

In this work, ~20–60 mL of FF was acquired from 78 women who underwent controlled ovarian hyperstimulation for *in vitro* fertilization (IVF) program at the Infertility and Perinatology Center of Jundishapur, the Jundishapur University of Medical Sciences. Briefly, human ovarian FF s were collected by transvaginal aspiration needles under direct ultrasound monitoring from women who were undergoing IVF/intracytoplasmic sperm injection procedures.[24,25] The oocyte retrieval process was conducted by two operators under an inverted microscope to achieve better quality control. After recognizing COC by the first operator, when no more oocytes were detected, the FF was pooled in a heparin (10 IU/mL)-coated 50-mL conical Falcon tube. To enrich the follicular cells and omitting red blood cells from the FFs, the hypo-osmotic lysis technique was conducted.[26] Thus, FF aspirates were centrifuged at 800 × *g* for 6 min. Then, the aspirating supernatant was discarded, and gently cell slurry was conveyed into a 15-mL Falcon tube. In the next phase, 9.0 mL of sterile distilled H_2O was spilled into the cell pellet, and the tube was shaken. After 30 s, 1.0 mL of 10x concentrated phosphate-buffered saline (PBS, pH = 7.2) was added, and the mixing in the tube continued, the tubes were centrifuged at 150g for 3 min again. Finally, the cell pellet was resuspended in 0.5 mL of Dulbecco's Modified Eagle's Medium (DMEM) (Sigma–Aldrich, USA). To

count the number of cells and viability, 1 μL of the cell suspension was separated and mixed with 0.2% trypan blue on a hemocytometer lame.[26]

Preparation of alginate hydrogel

Lyophilized sodium alginate (55%–65% guluronic acid) was produced by Sigma Aldrich (Cat. No. 71238, Mn: 100–200 kDa, Sigma–Aldrich, USA). The alginate was dissolved in deionized water to a concentration of 0.5% (W/V). To eliminate impurities, the alginate was sterilized by using 0.22-μm membrane filters (Millipore, Merck KGaA, Darmstadt, Germany) and ultraviolet light, then aliquoted sterilely.[27]

Calcium alginate microencapsulation and oocyte-like structure differentiation

FF-derived cells at 1 × 10^6 concentration were transferred to 4-well plates (BD Biosciences; Two Oak Park Bedford, MA 01730 USA) and growing was done in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% fetal bovine serum (FBS), L-glutamine 2 mM, and 1% penicillin/streptomycin, (Gibco, Grand Island, NY, USA). After 48-h floating, cells accompanied with exhausted medium were discarded. Then, the adherent cells were dissociated with 0.25% trypsin solution, washed with PBS, and suspended at a density of 2 × 10^5–5 × 10^5 cells/mL. The cell suspension was centrifuged at 160 g for 5 min. In the next step, the supernatant was discarded and the cell slurry was resuspended in 0.5% sodium alginate solution. The mixture of FF-harvested cells in sodium alginate was slowly released through a 25G needle as droplets falling into a beaker containing 0.1 M $CaCl_2$ solution. The droplets quickly gelled to form alginate scaffolds (beads). Beads containing individual cells were later transferred from the beaker by glass pipettes and transferred to culture media with and without human recombinant BMP15 (100 ng/mL) (R and D Systems, Minneapolis, MN, catalog no. 5096-BM; derived from Chinese hamster ovarian cells) for 2 weeks.[28-30] The alginate scaffolds containing FF cells were transferred to a T25 cell culture flask (Jet Biofil ; Guangzhou Jet Bio-Filtration Co., Ltd.) and then cultured for 14 days in DMEM supplemented with 15% FBS, 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA), and L-glutamine 2 mM, at 37 C, 100% humidity, and 5% CO_2.[29] Every 2 days, the culture media were replaced.[28]

Viability assessment of encapsulated cells

The viability of cultured FF-derived cells 2, 7, and 14 days after encapsulation was evaluated based on morphological criteria and 0.4% trypan blue staining test. Follicle-like structures classified as viable were spherical with an even thickness, homogeneous cytoplasm, as observed by an inverted microscope (ZEISS Axio Vert. A1–Carl Zeiss, Germany).

Estradiol assays

For evaluation of estradiol secretion, the daily-spent culture medium was collected, centrifuged to dispose of any cellular debris, and stored at −80°C for analyzing the estradiol concentration. A special commercially available estradiol enzyme-linked immunosorbent assay kit was utilized to analyze estradiol concentrations in the culture supernatant (Catalog No. 1920) (Alpha Diagnostic International, San Antonio, TX, USA). The analysis was adjusted corresponding to the manufacturers' directions, adopting a method explained in an early study.[31] The culture media were diluted with enzyme-linked immunoassay buffer (1:4), located in microplate wells, blended with estradiol enzyme conjugate, and incubated for 1 h. After incubation, the unbound conjugate was washed three times with diluted enzyme-linked immunoassay buffer, and substrate solution was added to provide expansion of color. After 30 min, the absorbance of the plate was read at 450 nm utilizing a microplate reader (Synergy™ 2 Multi-Mode Microplate Reader, Winooski, VT, USA). The results were taken using the four-parameter logistic curve with the Reader fit program (Hitachi Solutions America, Ltd., 2012, Irvine, CA 92618 United States). The intra-assay coefficient of variation and sensitivity of the assay were 3.2% and 0.02 ng/mL, respectively.

Immunocytochemistry

For the evaluation of different markers of FF MSCs, and to prevent alginate degradation, the beads were rinsed with washing buffer (10 mM TRIS-HCl, 0.14 M NaCl, and 5 mM $CaCl_2$, pH 7.4), and again fixed in 4% paraformaldehyde comprising 10 mM $CaCl_2$ at 37ºC for 1 h. Following fixation, the alginate beads were washed three times in PBS and Following fixation, the alginate beads were washed three times in PBS, then rinsed constructs with blocking solution containing 2% (w/v) PBS and 0.1% (w/v) Triton X-100 at room temperature for 45 min. In the next step, the beads were rinsed three times with PBS containing washing buffer, and then incubation was done with anti-OCT4 (rabbit antihuman, 1:100; Bio Legend), anti-NANOG (rabbit antihuman, 1:100; Bio Legend), and anti-ZP2 and anti-ZP3 (mouse monoclonal, 1:50; Santa Cruz) antibodies at 4°C overnight. Again, the beads were washed three times with PBS, transferred to a 1.5-mL conical tube, and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit or rabbit anti-mouse antibodies (Sc2012; Santa Cruz Biotechnology, Inc.) diluted at 1:200 in PBS at 37°C for 2 h. Then, 0.4-μL/mL DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride) in PBS was used for staining cellular nuclei for 20 min. Finally, the beads were washed three times with PBS containing wash buffer and transferred to a 96-well plate, and visualization was performed under a fluorescence microscope (Leica M205 FA; Leica Microsystems; Mannheim, Germany). For negative controls, the primary

antibody was removed by the PBS buffer 1 × and secondary antibody alone.[32]

Gene expression analysis by quantitative real-time polymerase chain reaction

For gene expression assessment, FF-derived MSCs from the control and BMP15-treated groups were collected on days 0, 7, and 14 of culture. For recovery of alginate-encapsulated cells, the culture medium was removed by using an aspiration pipette and beads were washed twice with PBS. Then, 1-mL sodium citrate (pH.4) solution was added to the culture dish and mixed for 5–15 min at room temperature to dissolve the alginate beads. The cell pellet was obtained by centrifuging at 400 × *g* for 10 min and supernatant was removed, released cells carried to RNeasy purification system for total RNA extraction. Then, 500 ng of the total RNA was used for reverse transcription into cDNA through Superscript II Reverse Transcriptase kit (Invitrogen; Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008) and random hexamers (Fermentas Life Sciences, Schwerte, Germany) according to the manufacturer's guidelines. The quantitative polymerase chain reaction (PCR) process was carried out in duplicate for each sample in a final volume of 10 μL by SYBR Green master mix kit by ABI Prism 7900 sequence detector (both from Applied Biosystems, Foster City, CA, USA) by ABI Prism 7900 sequence detector (Applied Biosystems, Foster City, CA 94404 USA). Next, the synthesized cDNA (2.0 μL) was added into SYBR Green mixture (5 μL) at 0.3 μM concentration of forward and reverse primers, mixed by water to reach 10-μL final volume. The set program for forty cycles was done under the following conditions: 95°C for 15 s, 56°C–62°C for 30 s (depending on primer), 72°C for 30 s, and 75°C for 30 s for the final cycle. Table 1 describes the primer sequences for OCT4, NANOG, ZP2, ZP3, and GAPDH. It reported the data as a time-fold change corresponding to the internal control gene expression. The data were then normalized to transcription levels of day 0 culture using ΔCT and ΔΔCT methods. The formula of $2^{-\Delta\Delta Ct}$ (comparative threshold cycle method) was used to assign the Ct values to normalized relative gene expression levels for all PCR products.[33]

Statistical analysis

Data were statistically analyzed by the software package SPSS (IBM® SPSS® Statistics v24.0 Student Version) and GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). Differences in the mRNA expression of the markers and estradiol concentrations in the culture supernatant between the cells induced to differentiate and the undifferentiated cells were analyzed using the one-way analysis of variance. If significance was found in the analysis, the data underwent post-hoc comparisons. All statistical tests were two sided, and $P < 0.05$ was considered statistically significant.

Results

Morphological analysis

On the 2nd day after encapsulation of FF-derived mesenchymal cells, cell clusters were observed at different points of the beads. During the 3rd to 7th days after encapsulation, the cell aggregates continued to grow, OLCs appeared and remarkably developed, and most of them were seen in the margin of the beads. The average size of OLSs in the control and BMP15 groups increased from day 4 to day 14 and reached approximately 180 and 230 μm, respectively [Figures 1 and 2].

Viability evaluation and estradiol production

FF-encapsulated cells, analyzed by trypan blue staining, were evaluated in both BMP15-treated and control groups. On the 2nd day of encapsulation, 84% of the cells were viable; after 7 days of culture, 79% of cells and 74% of oocyte-like cells were viable; and other cells were viable after 14 days of culture. No difference in viability was observed among the control and BMP15-treated groups. Estradiol production in BMP15-treated and control groups revealed decrease on day 14. However, there was no significant difference between the two groups during the 1st week of cell culture, but in the 2nd week, there was a meaningful decrease in estradiol production in the treated groups (14.02 ± 1.03) compared to controls (15.63 ± 0.89) ($P < 0.001$) [Figure 3].

Table 1: List of primer sequences used in real-time polymerase chain reaction

Gene	Primers	Accession number	Annealing temperature
OCT4	GGCCCGAAAGAGAAAGCGAACC	NM_203289.5	64
	ACCCAGCAGCCTCAAAATCCTCTC		
NANOG	GGGCCTGAAGAAAACTATCCATCC	NM_001355281.2	59
	TGCTATTCTTCGGCCAGTTGTTTT		
ZP2	CAGAGGTGTCGGCTCATCTGA	NM_001376233.1	61
	GCAGTCTTGTGCCCTTTGGT		
ZP3	GACCCGGGCCAGATACACT	NM_007155.6	61
	CATCTGGGTCCTGCTCAGCTA		
GAPDH	GGGAGCCAAAAGGGTCATCA	NM_001357943.2	60
	TGATGGCATGGACTGTGGTC		

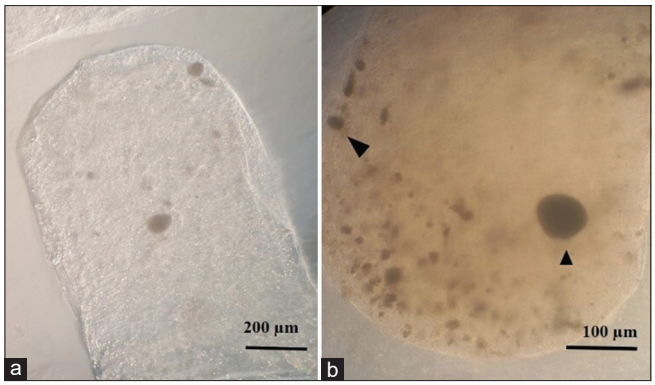

Figure 1: Alginate capsules containing follicular fluid-derived mesenchymal stem cells. Alginate bead-encapsulated stem cells representing a spherical shape (a). (b) The cell clusters in the alginate matrix. The average diameter of the beads is 810 µm

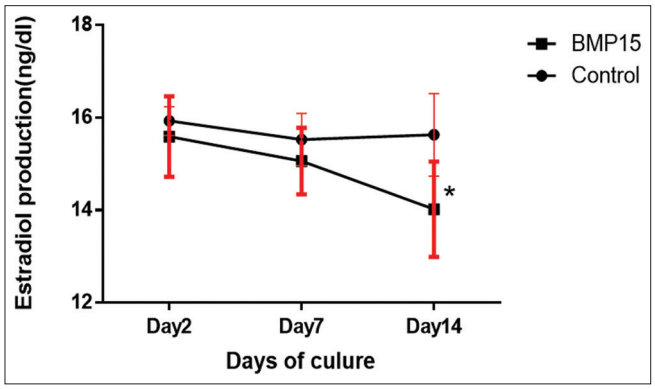

Figure 3: Determination of estradiol production in medium within the process of differentiation; a difference exists in the levels of estradiol between days 7 and 14 in BMP15-induced group and control ($P < 0.001$)

Immunofluorescence detection for pluripotency markers and oocyte maturation

The cell clusters showed positivity for OCT4 and Nanog in both BMP15-treated and control groups [Figure 4]. To investigate the possibility and differentiation of FF-derived mesenchymal cells to OLCs in alginate culture, oocyte-specific markers of ZP2 and ZP3 were evaluated in both groups. During the 7th and 14th days of culture, immunochemistry showed positivity for ZP2 and ZP3, and these proteins were detected in the cytoplasm of OLSs. However, the fluorescent light intensity and immuno-localization of these proteins were not different between the control and treated groups [Figure 5].

Gene expression analysis of specific markers of stemness and oocytes

To determine the differentiation of FF-derived MSCs into OLCs and also the effect of 3D culture medium on the stem cell ability of these structures, the OCT4, Nanog, ZP2, and ZP3 genes were evaluated on days 7 and 14, compared to day 0 [Figure 6]. However, fold changes in BMP15-treated cells in comparison with the control groups for each marker showed specific dynamic changes during

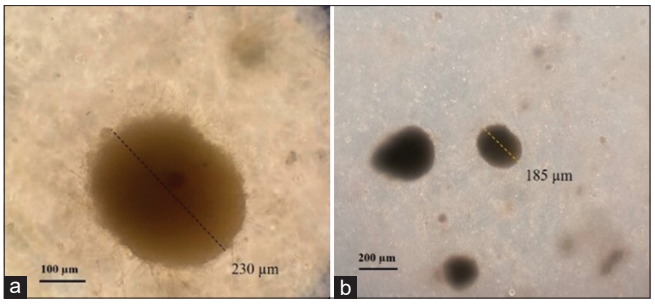

Figure 2: Morphology of oocyte-like structures in BMP15 and the control groups. (a and b) The spontaneously developed oocyte-like cells at the center of alginate beads with different diameters, while not attached to the plate stratum

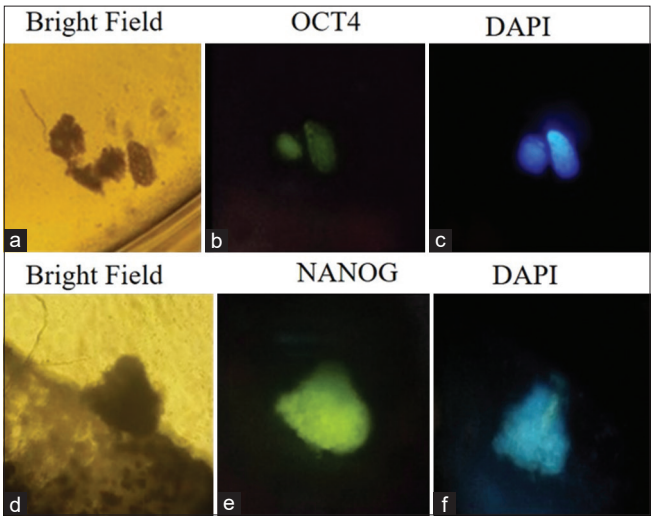

Figure 4: Immunofluorescent (fluorescein isothiocyanate) staining for pluripotent markers of OCT4 and Nanog. (a-d) The expression of OCT4 and Nanog proteins in cell clusters of alginate-encapsulated follicular fluid-derived mesenchymal stem cells. Nuclear deoxyribonucleic acid was stained with DAPI,4′,6-diamidino-2-phenylindole. (c and f) Blue fluorescence. Scale bar = 200 µm

the encapsulation process ($P < 0.05$). Based on the results, in the BMP15-treated group, ZP2 expression on day 7 showed a meaningful increase (33.4-fold) compared to day 0 (before encapsulation) ($P < 0.001$), whereas in other genes, there was no significant difference in comparison with day 0. Pluripotent gene expression for OCT4 and NANOG was higher in day 7, which showed upregulation at approximately 20.01- and 30.38-fold change, respectively ($P < 0.000$). However, there was no significant difference in ZP2 and ZP3 expression during this period. Statistically significant increase in OCT4, Nanog, and ZP3 gene expression level was observed during the 7–14th days of cell culture (5.5, 5.38, and 231.88 fold, respectively) when compared to day 0 ($P < 0.001$), but no significant difference in ZP2 expression was observed. During the same time in the control group, OCT4 and Nanog expression decreased considerably and showed downregulation ($P < 0.001$), whereas the expression of ZP3 remained nonsignificant from days 7–14 in the control group. The relative expression fold changes of OCT4, Nanog, ZP2, and ZP3 are shown in Figure 6, individually.

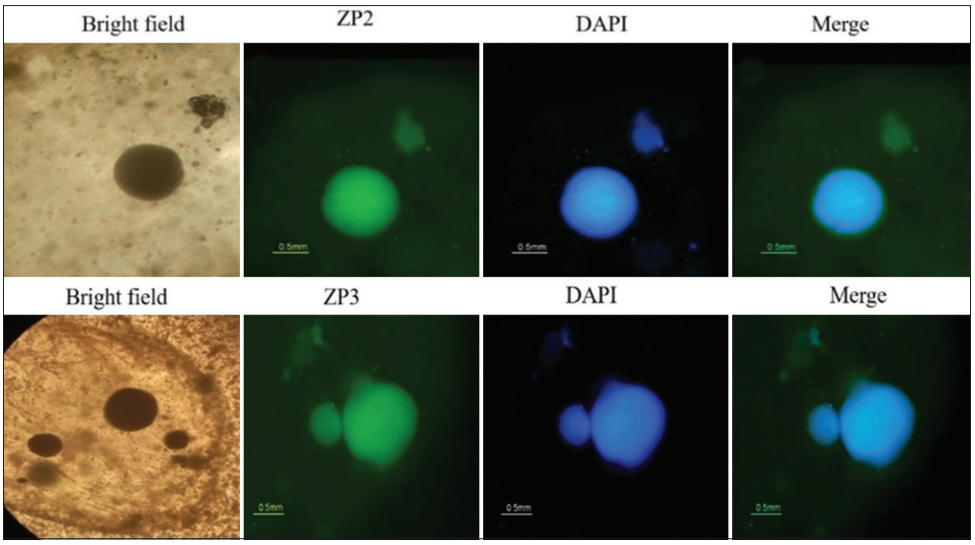

Figure 5: Immunofluorescent assessment of oocyte-specific markers ZP2 and ZP3 of oocyte-like structures encapsulated with calcium alginate. The oocyte-like structures stained for ZP2 (a-d) and ZP3 (e-h) following 14 days' cultivation; the nuclei were counterstained by 4′,6-Diamidino-2-phenylindole

Discussion

In vitro generation and development of human organs will be one of the major challenges of future researches and will need the co-operation of biologists, biotechnologists, and physicians. Much of our understanding about oogenesis and its related mechanism was based on a 2D culture system because of its facility. So far, among the different evaluated human organs, the ovary has certain characteristics because each ovarian follicle has unique anatomy and histological feature and its blood supply can be considered a separate small organ. The main challenge is the maintenance of the 3D structure throughout the culturing time with preserving physical and chemical interactions between the oocyte and its surrounding cells. Ovarian cells and follicles can be cultured by using 3D natural scaffolds that mimic ovarian stromal tissue. Choosing a suitable scaffold can improve the survival and growth of encapsulated ovarian cells.[18] In the study ahead, we decided to used calcium alginate 0.5% as 3D scaffold for encapsulation of human FF-derived MSCs which was discarded in conventional ART procedures. Our results showed that alginate is a suitable and nontoxic combination for the encapsulation of FF-derived MSCs. Molecular studies also showed that cells encapsulated in this 3D medium in the presence of BMP15 differentiation factor could form oocyte-like structures. Yu *et al*.[34] evaluated the *in vivo* effect of BMP15 on amniotic fluid-derived SCs to OLCs, and deduced that BMP15 is effective in stimulating the differentiation of these cells, which may give an *in vivo* model to examine human germ cell development. It was observed that the 3D scaffold increased the number of OLCs compared to monolayer cultivation (three to four cells per each bead). The number of these cells in the control and BMP15 treatment groups was not different. In the present study, as the first survey to encapsulate FF-derived MSCs, alginate-encapsulated MSCs displayed round cells and formed separate cell clusters. The results here are in line with those of Vigo *et al*.'s[35] observation, which showed that bovine ovarian follicular cells are prone to form cell aggregates after encapsulation with calcium alginate.

The viability of MSCs on different days of culture (86% on the 2nd day after encapsulation) indicates that the cell encapsulation process adopted in this survey did not affect cell survival. In fact, viability evaluation revealed that alginate provided an appropriate biocompatible environment for FF-derived MSCs. Yin *et al*.[36] showed that secondary human follicles can develop into small antral follicles and remain hormonally active in an alginate encapsulation culture system. They demonstrated that follicles that underwent encapsulation remained viable *in vitro* and became steroidogenically active after culturing in 3D alginate matrix.[36]

The estradiol production by encapsulated cells in calcium alginate without addition of androgen could suggest the functional activity of MSCs in these scaffolds. Despite our findings, however, Kreeger *et al*. noted that it is impossible to detect estradiol and progesterone from cultured ovarian follicles without addition of exogenous FSH.[37] In addition, identification of estradiol validates the functional activity of OLSs and its surrounding cells. Reduction of estradiol concentration over the BMP15-induced groups on the 2nd week, possibly maybe due to the effect of BMP15 on these cells. Our findings are compatible with those of Prapa *et al*.'s[13] results, who demonstrated that BMP15 could reduce basal estradiol levels in human granulosa cells.

After the assessment of cell viability and estradiol production of FF-derived MSCs in calcium alginate, the effects of BMP15 were evaluated on gene and protein expression pattern between experimental groups. In

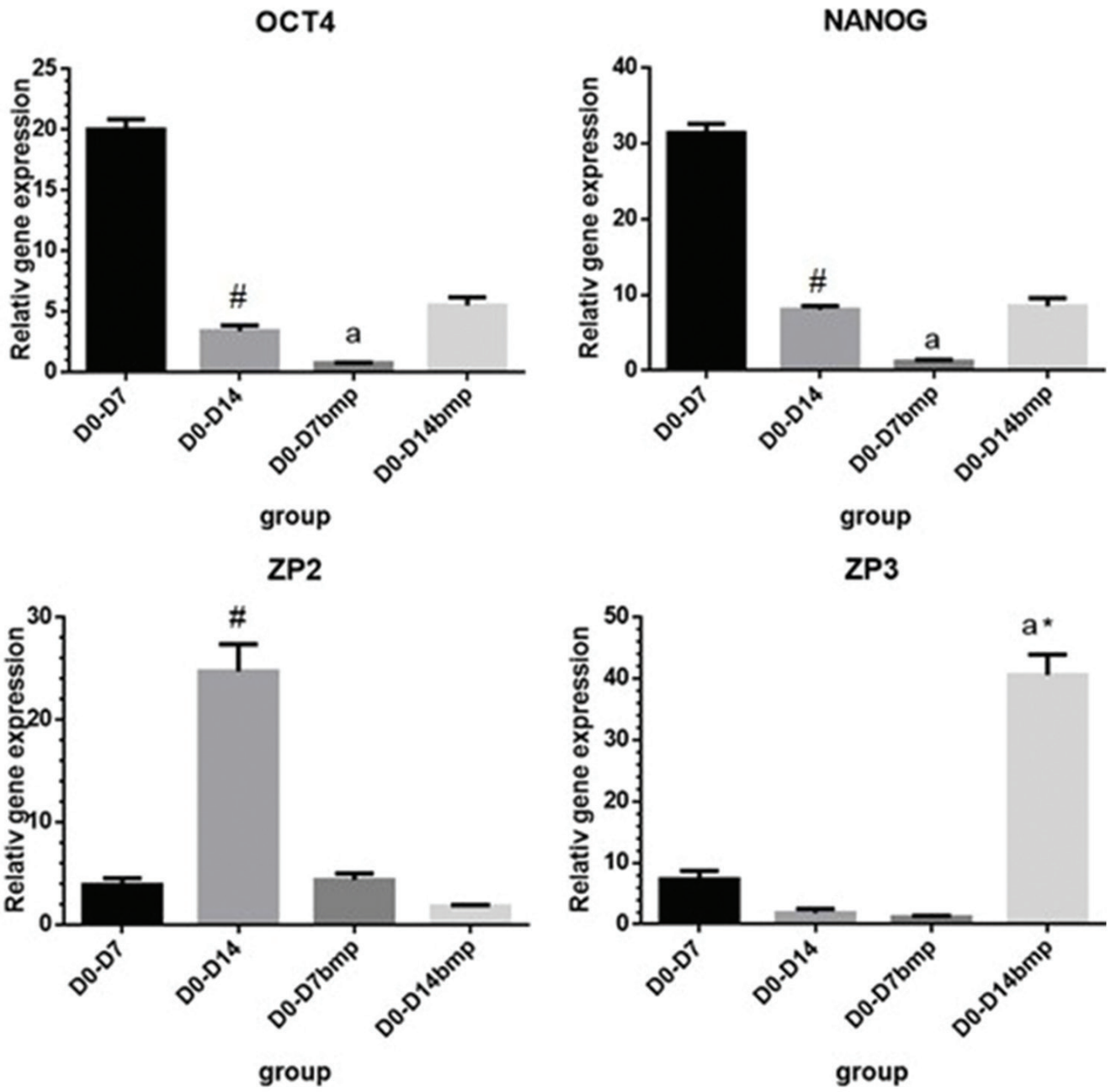

Figure 6: Relative fold expression of pluripotency and oocyte specific markers of human FF derived MSCs cultured for 2 weeks and comparison after treatment with BMP15. (A, B) gene expression markers showed relative expression of OCT4 and NANOG in earlier days and reduction after 14 days of culturing. C, D exhibit the gene expression of ZP2 and ZP3 in MSCs. ZP2 increased on day 14 and showed reduction after BMP15 treatment, However, gene expression of ZP3 increased on the 7th day after treatment.Data show mean ± standard error of three independent experiments. * = significant comparison between treatment groups. # = Significant comparison between control groups, and a indicates significant comparison between the treatment group and the control group.

this step, we evaluated our experimental groups at the molecular level to attain further understanding of the *in vitro* MSC differentiation process and OLS formation. OCT4 and Nanog are essential genes implicated in the pluripotency of MSCs. In our study, the fold change expression pattern of these genes was found to increase during the 1st week of cultivation process in both groups, although, in the 2nd week, their expression level decreased and followed a downregulating trend. These evidences are consistent with those of Wei *et al*.'s study,[38] which concluded that scaffolds promote stemness maintenance of ES cells, and this could give a hopeful platform for ES cell study. ZP2 and ZP3, oocyte-specific genes, which are necessary for mammalian fertilization, were analyzed. In the control group, ZP2 expression pattern was found to increase during the 1st week of culture, then

showed a reduction in the 2nd week compared to day 0, before encapsulation. In the BMP15-treated group, ZP2 decreased and showed downregulation in the expression pattern. It could be concluded that alginate integration and BMP15 lead to differentiation of OLSs; such alterations in gene expression pattern have been reported in earlier researches.[37] Whereas, high expression of ZP3 was observed in the 2nd week of BMP15 induction group, which could be due to ZP3 importance in development competence and fertilization activity of oocyte.[38] As it is known, cell clustering and morphology changes of 3D scaffold on oocyte-like structures help them to maintain pluripotency and self-renewal; this phenomenon is proven for human ES cells.[38]

Immunocytochemical study of the present inquiry confirmed the reverse-transcription-PCR findings. The expression of OCT4 and Nanog proteins was observed in the cytoplasm of MSC clusters derived from FF. The pluripotency markers had then diminished after 14 days' treatment by BMP15, which showed that most of the cell aggregates underwent differentiation. It is worth mentioning that, the mechanism underlying desirable stemness ability in 3D scaffolds is still obscure.[39] The expression of oocyte-specific proteins, ZP2 and ZP3, was obviously seen in the cytoplasm of OLSs through immunofluorescence staining. This indicates a good evidence for the observed reduction of ZP2 and ZP3 expressions on day 14 of culture in both groups, which could illustrate the translation of transcripts to proteins.

Conclusion

The calcium alginate encapsulation of MSCs derived from FF would prepare an applied model for *in vitro* maturation of human ovarian follicles, and may be used in assisted reproduction and regenerative medicine. The results disclosed that this fluid can be another source for MSCs, and encapsulation of these cells provides a promising technical device in the study of an intricate process of oogenesis. Our findings illustrated that BMP15 along with 3D alginate matrix increases the developmental ability of OLSs, and supplies an additional *in vitro* platform to investigate the mechanisms, by which germ cells are formed and differentiated.

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Conflicts of interest

There are no conflicts of interest.

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