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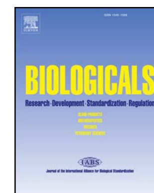

Distinct effect of fetal bovine serum versus follicular fluid on multipotentiality of human granulosa cells in *in vitro* condition

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A R T I C L E I N F O

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A B S T R A C T

This study aimed to develop an appropriate medium for preservation of multipotentiality in human granulosa cells. To compare the possible effect of different media supplemented with follicular fluid or fetal bovine serum, granulosa cells were cultured *in vitro* over a period of 14 days. Stemness feature and any alteration in the cell phenotype were monitored using colony count assay and flow cytometry analysis by monitoring the expression of Oct3/4 and GATA-4 factors. Transcript expression level of Sox-2, Klf-4, and Nanog were investigated using quantitative real-time PCR analysis. Cells were cultured in the medium supplement with follicular fluid showed normal cell morphology and epithelial-like appearance, however, cells treated with fetal bovine serum, exhibited the clonogenic potential of granulosa cells which was increased after exposure to follicular fluid after 14 days ($p < 0.05$). Flow cytometry analysis revealed a significant reduction in the protein level of GATA-4 in cells cultured in presence of follicular fluid compared with cells received fetal bovine serum ($p < 0.001$). Quantitative real-time PCR analysis disclosed reduction of Sox-2, Klf-4 and Nanog levels in cells exposed to fetal bovine serum. Our experiment showed the exposure of human granulosa cells to follicular fluid efficiently preserves the stemness characteristics of the cells.

1. Introduction

A sophisticated strategy in regenerative medicine is the targeted differentiation of somatic cells to the other cell types, particularly pluripotent cells [1]. It has been demonstrated that introduction of four factors, Oct3/4, Sox2, Klf, and c-Myc, to fibroblasts could induce the stemness feature [2]. Differentiation of the granulosa cells (GCs) into extra-ovarian cells, including neurons, chondrocytes, and osteoblasts, was noted in previous studies [3]. This feature predisposes GCs to open a new avenue in the infertility medicine. Along with this statement, various investigators endeavor to establish GC differentiation into various cell lineages and implement their application in the treatment of infertility [4]. Two distinct types of GCs have been reported within the follicles; mural GCs, surrounding the antrum, located on the wall of the follicle and cumulus GCs juxtapose to oocyte [5]. Recent studies have found that GCs play an important role in the growth and maturation of the oocyte because they potentially create a relationship between oocyte and hormones implementing hormone receptors. Studies on GCs were performed to understand the ability of an oocyte for fertilization and implantation [6–8]. The antrum of follicles is filled with a unique biological fluid known as follicular fluid (FF), which is derived both from the bloodstream and components secreted by somatic cells inside the follicle [9]. It encompasses a variety of molecules such as steroids and protein hormones, anticoagulants, enzymes and electrolytes [10]. This fluid has a substantial function in regulating the proliferation and maturation of oocytes also the establishment of communication between differentiated cells and germ cells inside follicles [11]. There are a large number of bioactive factors in the FF, which are pivotal for the growth and development of oocytes and acquiring needful competence for fertilization and implantation. For

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instance, insulin-like growth factor-1 and -2 (IGF-1 and IGF-2), vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), as well as other factors such as bone morphogenic protein (BMP15) and differentiation growth factor (GDF9) are identified abundantly in FF [12–19]. It was demonstrated that multipotent stem cells can form germ-like cells and mature up to gametes using numerous differentiation strategies, including exogenous factors such as leukemia inhibitory factors (LIF) or FF, however, co-culturing of multipotent stem cells with ovarian granulosa cells were also reported [20–23]. LIF, as a member of the IL-6 family, is a glycosylated protein with a molecular weight of 37–62 kDa. This factor is secreted from the outside of the fetus, as well as many mature cells notably endometrial cells, fibroblast, bone cells, monocytes, macrophages, T cells and exert its functional effects by binding to receptors called LIFR and gp130 [23–25]. It has been thought that LIF is a key cytokine in maintaining the pluripotency of stem cells by preserving self-renewability [21]. Therefore, in this study, we aimed to develop an appropriate medium for inducing the expression of the stem cell-specific factors in human cumulus granulosa cells.

2. Material and methods

2.1. Ethical issues

In the current experiment, the institutional review board of ethics committee of Tabriz University of Medical Sciences (TBZMED.REC.1394.100) approved all phases used. All volunteers signed informed consent sheets.

2.2. Subjects

The healthy women aged between 20 and 35 years old with the body mass index (BMI) of 19–25 kg/m^2 underwent for *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI), receiving standard dose of gonadotropin (GnRH), recombinant follicular stimulation hormone (FSH) and human chronic gonadotropin (HCG) about 36 h prior to puncture, were enrolled to this study. We considered inclusion scale for the selection of patients with male factor infertility including partners with total sperm number $< 1 \times 10^6$/ml, motility rate of < 5% and abnormality index > 95%, according to World Health Organization guideline [26] also partners with tubular factor infertility. The exclusion criteria included polycystic ovarian syndrome (PCOS), a patient with human immunodeficiency virus, hepatitis C, and B virus, and cytomegalovirus infectivity.

Cumulus oocyte-complexes (COCs) were obtained from healthy women underwent the *in-vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) procedures. To compare the possible effect of different media supplemented with FF or FBS, colony count assay, flow cytometry evaluation for Oct3/4 and GATA-4, as well as quantitative real-time PCR analysis of Sox-2, Klf-4 and Nanog were performed.

2.3. Collection of human follicular fluid

During IVF or ICSI program, FF was retrieved from the ruptured follicle and then emptied into a petri dish. COCs were collected from FF followed by centrifugation of FF in 3000 RPM at 10 min aiming to remove blood cells and cell debris. The supernatant was filtered by 0.22 μm pore size micro-filter to eliminate the cell debris. The filtered fluid was inactivated at 56 °C for 20 min and stored at −20 °C until used for the experiments.

2.4. Cell culture and expansion protocols

COCs were obtained via transvaginal ultrasound-guided suction system from ovary by an expert gynecologist and then GC masses was isolated from COCs by using a fine and sterile pipet in a pre-warmed medium (37 °C) containing Hyaluronidase (Cat No: H1136, Sigma). GC samples were mixed with an enzymatic solution containing Hyaluronidase for 3 h and washed three times with phosphate-buffered saline (PBS). To compare the possible effect of different media supplemented with FF or FBS, GCs samples were classified into two groups; Group I: medium DMEM/F12 (Sigma) containing 10% FBS and Group II: medium DMEM/F12 with 10% FF and 2% FBS. On day 7, 1000 IU LIF was added to both groups and maintained the cells for the next 8 days.

2.5. Characterization of GCs by immunofluorescence imaging

Prior to the experimental procedure, cells were plated on each well of Cell Chamber Slides (SPL). After 24 h, the cells were fixed with 4% paraformaldehyde solution for 10 min and washed twice with phosphate buffer saline (PBS). After cell exposure to permeabilization buffer (eBioscience) for 20 min, we incubated cells with anti-DDX4 (Cat no: ab13840, Abcam) for 1 h at room temperature. Following twice washing with PBS, FITC–conjugated goat anti-rabbit secondary antibody (Cat no: ab6717, Abcam) was used. For background staining, cells were incubated with 1 μg/ml DAPI solution (Sigma) for 30 s. Slides were visualized by using Fluorescent microscopy (Olympus).

2.6. Colony count assay

To investigate the effect of FBS and FF on the stemness feature of GCs, the number of GCs colonies was counted after 14 days. For this propose, the number of colonies was monitored in seven random high power fields.

2.7. Flow cytometry analysis

Using flow cytometry analysis, the percent of Oct 3/4$^+$ cells (Stem cell specific marker) and GATA-4$^+$ (GC cell specific marker) were evaluated. After 14-day incubation of GCs with different culture media, cells were detached using 0.25% Trypsin-EDTA solution (Gibco). The permeabilization step was done by the treatment of cell suspension with 0.1% TritonX-100 (Sigma) for 3 min. After twice washing with PBS, a panel of antibodies OCT3/4 and GATA-4 (eBioscience) were implemented followed by incubation for 30 min at RT. The BD FACSCalibur™ system and FlowJo software (ver. 7.6.1) were used to perform flow cytometry analysis.

2.8. Real-time PCR assay

The mRNA expression levels of Nanog, Sox-2 and Klf-4 were measured using quantitative real-time PCR. On the end stage of the experiment, total cellular RNA was extracted with an RNX PLUS Kit according to the manufacturer's protocol (Cinnagen, Iran). The quality and quantity of isolated RNA from all the samples were evaluated by a NANODROP 2000c spectrophotometer (Bioneer). The RNA samples then reverse-transcribed into cDNA (Bioneer) and real-time PCR analysis was performed using Corbett Rotor-Gene 6000 system (Corbett Life Science, Australia). PCR reaction was carried out in a final volume of 14 μl, consist of 0.8 μl of each primer (outlined in Table 1), 7 μl of SYBR green reagent (Takara Bio, Japan), 0.8 μl of cDNA template and nuclease-free water. Housekeeping gene GAPDH has been used for normalization of the expression levels.

2.9. Statistical analysis

The data were expressed as mean ± SD in this study. The significant differences between FBS- and FF-treated groups were analyzed by Student t-test via GraphPad InStat software version 2.02. $p < 0.05$ was considered statistically significant.

Table 1
The sequence of primers used for real-time PCR.

Gene	Primer sequence	Accession No	Annealing (°C)
GAPDH	F:5′AAGCTCATTTCCTGGTATGACAACG-3′ R:5′′TCTTCCTCTTGTGCTCTTGCTGG-3′	NM_002046.3	58
SOX2	F: CATGCACCGCTACGACGTGA R: CTTGACCACCGAACCCATGGA	NM_003106.3	58
Nanog	F:CTGTGATTTGTGGGCCTGAA R:TGTTTGCCTTTGGGACTGGT	NM_024865	59
Klf-4	F:GGCACTACCGTAAACACACG R:CTGGCAGTGTGGGTCATATC	NM_001314052.1	59

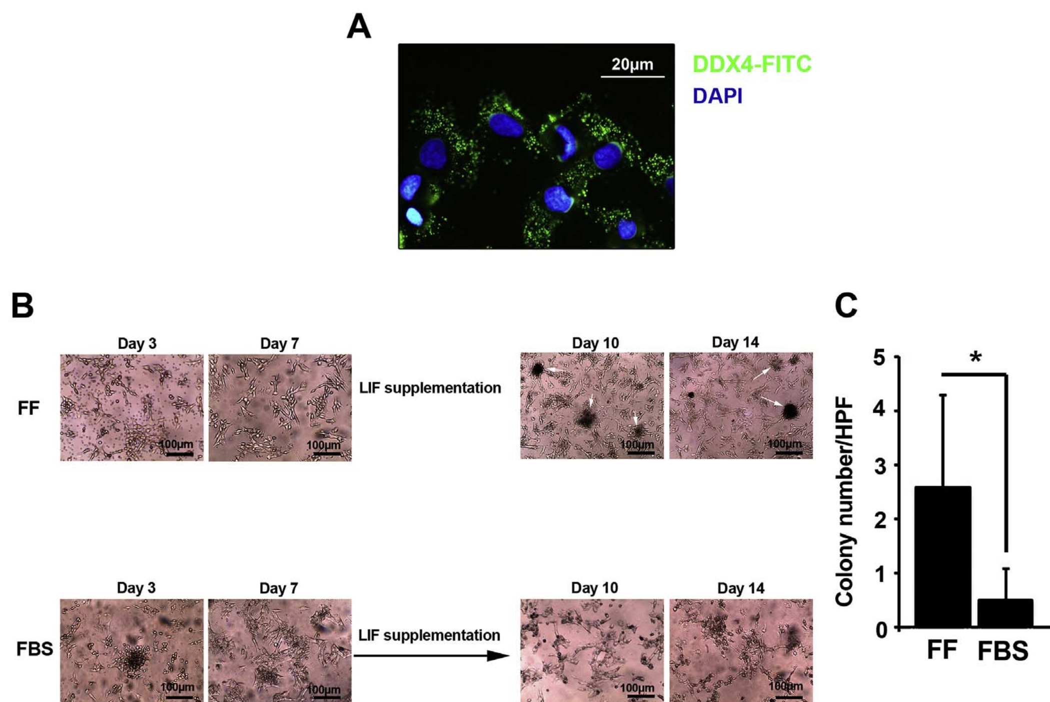

Fig. 1. Fluorescence microscopy imaging for DDX4 detection in primary GCs (A). Photomicrograph of cultured GCs exposed to the follicular fluid and fetal bovine serum over a period of 14 days (**B**). GCs exposed to a combined regime of FF and LIF appropriately preserved their morphological features. Mean colony number 14-day incubation *in* vitro (**C**). Data analysis revealed a significant difference in the number of colonies exposed to FF after 14 days ($p < 0.05$). Student t-test, $*p < 0.05$ ($n = 3$). HPF = High Power Field.

3. Result

3.1. Isolated GCs expressed DDX4

Immunofluorescence detection of DDX4 showed cytoplasmic staining in cultured GCs. The existence of intracellular DDX4 in isolated cells confirmed germ cell phenotype (Fig. 1A).

3.2. FF preserved the normal morphology of cultured GCs

We observed GCs cultured in medium supplement with FF preserved their normal cell morphology and showed epithelial-like appearance over a period of 14 days while cell exposure to FBS found to initiate emergence of GCs with non-epithelial like appearance (Fig. 1B). These data possibly support the notion the efficient role of FF in preserving and maintaining cell morphology in the *in-vitro* condition.

3.3. FF supplementation of primary GCs increased clonogenic capacity

Colony formation is a prominent feature of stem cells and progenitors. In this experiment, the effect of FF and FBS was determined in clonogenic properties of human GCs over a period of 14 days. According to our data, we recorded generating GC colonies and micro-aggregates, 3 days after initial plating in both groups. After 14 days, a significant difference in the number of GC colonies was evident in FF cells as compared with FBS group ($p < 0.05$; Fig. 1C).

3.4. Flow cytometry analysis revealed the decrease of stemness-related factors in GCs under FBS treatment

It has been identified that cells expressing Oct3/4 show stemness properties. In our experiment, flow cytometric analysis confirmed the increase in the number of GCs expressing Oct3/4 in both FF- and FBS-treated GCs over a period of 14 days, indicating the successful induction of stemness-related factor in response to LIF (Fig. 2). The percent of Oct3/4 positive cells was found to significantly increase in the FF-treated medium as compared with FBS group ($p < 0.01$) (Fig. 2A). Commensurate with these comments, FF had potential to preserve GCs stemness 14 days after *in-vitro* culture. Monitoring the expression of GATA-4 revealed the decrease in the number of GCs expressing GATA-4 after exposure to the combined regime of LIF with FBS or FF (Fig. 2B). Our data showed a higher level of decrease in GATA-4 levels from 74% to 18% in FF-treated cells, demonstrating the efficient induction of multipotentiality in these cells. In FBS-treated cells a slight decreased in the level of GATA-4 was evident in comparison to FF group after 14 days ($p < 0.001$) (Fig. 2).

Fig. 2. Flow cytometric analysis of Oct3/4 (**A**) and GATA-4 (**B**) expression in different media over a period of 14 days. Student *t*-test. $^{**}p < 0.01$; $^{***}p < 0.001$ ($n = 3$).

3.5. Real-time PCR analysis showed down-regulation of Sox-2, Klf-4, and Nanog in FBS-treated GCs

Our finding showed that the transcript level of Sox-2, Klf-4, and Nanog were decreased in FBS-treated GCs as compared to FF group (Fig. 3). We notified the significant inhibitory effect of FBS on the expression of Klf-4 ($p < 0.5$) rather than Nanog and Sox-2 expression. Although no significant difference was recorded for Sox-2 and Nanog, the expression was less than in GCs from FBS group (Fig. 3). These data support a notion that FF could be more efficient for preserving the potentiality and stemness as compared to medium containing FBS.

4. Discussion

This fact that GCs consist of a subpopulation of pluripotent and self-renewal capabilities is an important subject for ongoing investigations and helpful in the development of a promising therapeutic strategy in the regenerative medicine [27]. Detection of the pluripotent gene transcripts such as Oct3/4, Nanog, Sox-2, TERT, and STAT-3 was first reported in ovarian surface epithelium by the Parte et al. [28]. Virant-

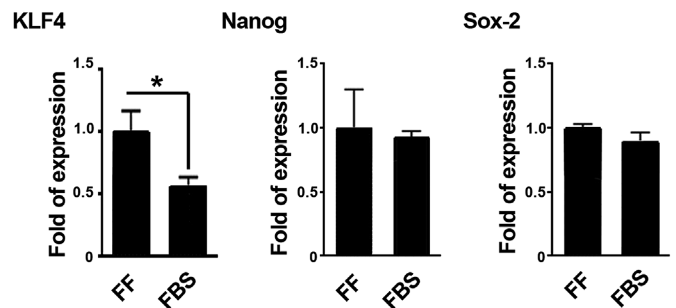

Fig. 3. Real-time PCR analysis of representative stemness factors; Klf-4, Sox-2, and Nanog at the end of the experiment (Day 14). The expression of Klf-4, Sox-2, and Nanog was found to decrease after exposure of GCs to FBS-contained medium. Student t-test, $^{*}p < 0.05$ ($n = 3$).

Klun et al. also showed the presence of stem cells in adult human ovaries with the potential to develop into oocyte-like and parthenote-like structures [29]. One of the most important studies in this field was done by Kossowska-Tomaszczuk et al. in which the presence of

multipotent GCs was detected in the context of ovarian tissue [3]. To exclude the impact of infertility on the function of GCs, the authors pooled GCs from all the different volunteers and cultured in FF- or FBS enriched media containing LIF. LIF is the only known pro-pluripotency factor, which supports the self-renewal of stem cells by activating various signaling pathways such as STAT3, and BMP-4, contributing to the suppression of MAP kinase pathways [4]. It is revealed that substantial subpopulations of GCs had potential to express POU5F1, CD29, CD44, CD90, CD105, CD117, and CD166 factors [4]. Prolonged culture of GCs in medium supplemented with LIF resulted in less differentiated GCs with a high degree of plasticity. Along with this issue, the similar finding reported by Varras et al. which showed the expression of Oct-4 in luteinized GCs in women underwent IVF or ICSI [30]. In contrast, Mattioli et al. [31] did not detect the existence of Oct-4 expression in swine GCs. However, the authors reported the expression of other three typical pluripotent stem cell markers including Nanog, Sox-2, and TERT in GCs [31].

The most important issue in the trans-differentiation of the GCs into other cell lineages is the maintenance of GCs stemness [32]. In the current experiment, we noted a potent ability of FF in inducing stemness capacity in human GCs over a period of 14 days. It was previously identified that FF encompasses various factors secreted from GCs, theca cells, and oocytes, including GDF9, GDF9b, stem cell factor (SCF), basic fibroblast growth factor and estrogen [33]. These factors are thought to involve in the regulation of follicular development [33,34]. In the study of Virant-Klun and co-workers, the culture of the epithelial cells in a medium containing FF resulted in successful detection of primitive oocyte-like cells and typical round-shaped cell clusters positively stained with alkaline phosphatase, SOX-2 and SSEA-4 [27]. Single oocyte-like cells expressed genes OCT4A, SOX-2, NANOG, NANOS, STELLA, CD9, LIN28, KLF4, GDF3, and MYC, characteristic of pluripotent stem cells (27). In another study done by Dyce et al., several culture systems were examined to identify conditions favoring the induction of germ-cell formation from porcine skin-derived sphere cells [22]. In the combined regime of FF and BMP-4, the expression of markers consistent with germ-cell differentiation was previously determined in *in-vitro* condition [22]. Consistent with these studies, GCs exposure to FF caused to preserve normal cell morphology and epithelial-like appearance. We also noted an increase in the clonogenic potential of human GCs after 14 days. FF promoted the expression of stemness-related factors, especially Klf-4. These data support a notion that FF could efficiently preserve the stemness characteristics as compared to medium containing FBS. Consistent with results from the current experiment, FBS had less ability to preserve or induce pluripotency in cultured human GCs due to the high rate of GATA-4 and low rate of Oct3/4.

In conclusion, our study showed that the exposure of the human GCs to FF efficiently preserves the stemness characteristics of these cells, offering appropriate strategy in infertility and regenerative medicine and the treatment of gynecological disorders.

Conflicts of interest

Authors declare no conflict of interest regarding the current experiment.

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