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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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ABSTRACT

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.