



Ovarian Stem Cells Differentiation into Primary Oocytes Using Follicle Stimulating Hormone, Basic Fibroblast Growth Factor, and Neurotrophin 3

Sara Tanbakooei¹, Seyed Mohammad Amin Haramshahi², Gelareh Vahabzadeh³, Mahmood Barati⁴, Majid Katebi⁵, Fereshteh Golab^{1*}, Qazal Shetabi⁶, Narges Niknam⁶, Leila Roudbari¹, Motahareh Rajabi Fomeshi^{1,2}, Soheila Amini Moghadam⁸

1- Cellular and Molecular Research Center, Iran University of Medical Sciences, Tehran, Iran

2- Department of Tissue Engineering and Regenerative Medicine, Faculty of Advanced Technologies in Medicine, Iran University of Medical Sciences, Tehran, Iran

3- Department of Pharmacology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

4- Department of Biotechnology, School of Allied Medicine, Iran University of Medical Sciences, Tehran, Iran

5- Department of Anatomy, Hormozgan University of Medical Sciences, Bandar Abbas, Iran

6- Department of Biology, Islamic Azad University, Science and Research Branch, Tehran, Iran

7- Department of Anatomy, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran

8- Department of Gynecology, Firoozgar Hospital, Iran University of Medical Sciences, Tehran, Iran

Abstract

Background: *In vitro* obtaining oocytes can be an appropriate alternative for patients with gonadal insufficiency or cancer survivors. The purpose of the current research was isolating stem cells from ovarian cortical tissue as well as evaluating the effectiveness of follicle stimulating hormone (FSH), basic fibroblast growth factor (bFGF), and neurotrophin 3 (NT3) in differentiating to oocyte-like cells.

Methods: A human ovary was dissected and cortical tissue pieces were cultured for cell isolation. Isolated cells were divided into 8 groups (3 cases in each group) of control, FSH, NT3, bFGF, FSH+NT3, FSH+bFGF, NT3+bFGF, and FSH+NT3+bFGF. Pluripotency specific gene (OCT4-A and Nanog), initial germ cells (c-KIT and VASA) and PF growth initiators (GDF-9 and Lhx-8) were evaluated by qRT-PCR. Experiments were performed in triplicate and there were 3 samples in each group. The results were analyzed using one-way ANOVA and p-value less than 0.05 was considered statistically significant.

Results: Flow cytometry results showed that cells isolated from the ovarian cortex expressed markers of pluripotency. The results showed that the expression of Nanog, OCT4, GDF-9 and VASA was significantly increased in FSH+NT3 group, while treatment with bFGF caused significant expression of c-KIT and Lhx-8 ($p < 0.05$). Also, according to the results, isolated cells treated with NT3 significantly increased c-KIT expression.

Conclusion: According to our results, the ovarian cortex cells could be differentiated into primordial follicles if treated with the proper combination of FSH, bFGF, and NT3. These findings provided a new perspective for the future of *in vitro* gamete prodest.

Keywords: Cell differentiation, Growth factors, Oogenesis, Ovarian tissue, Stem cells.

To cite this article: Tanbakooei S, Haramshahi SMA, Vahabzadeh G, Barati M, Katebi M, Golab F, et al. Ovarian Stem Cells Differentiation into Primary Oocytes Using Follicle Stimulating Hormone, Basic Fibroblast Growth Factor, and Neurotrophin 3. *J Reprod Infertil.* 2021;22(4):241-250. <http://dx.doi.org/10.18502/jri.v22i4.7649>.

* Corresponding Author:
Fereshteh Golab, Cellular
and Molecular Research
Center, Iran University of
Medical Sciences, Tehran,
Iran

E-mail:

golab.f@iums.ac.ir,
fgolab520@gmail.com

Received: Oct. 26, 2020

Accepted: May 25, 2021

Introduction

Fertility preservation is an important subject in the field of reproductive health. Cryopreservation of ovarian tissue including primordial

follicles (PFs) is a strategy for fertility preservation among women suffering from cancer (1). Reimplantation of frozen-thawed ovarian tissue has

been associated with malignancies (2), which is preventable via *in vitro* maturation of PFs. Such a mechanism can be developed by some factors activating PFs (3).

The purpose for culture of ovarian cortical tissue is the transition of PFs at resting phase into the primary and secondary follicular phase (4, 5). However, follicle development can not be supported and there is a need for nutrients or growth factors. Several intra- and extra-ovarian factors essential for activation of PFs and development of follicles in organ cultures have been investigated (6).

Developing antral follicle is associated with follicle stimulating hormone (FSH) within the mammalian ovary; however, the mechanism underlying preantral growth has not yet been identified. In genetically hypogonadotropic mice, hypophysectomy or pharmacological suppression of FSH leads to antral follicles depletion and prevents developing follicles through the late preantral phases. Likewise, in the mice with mutant FSH receptor gene, there was no improvement in follicle development over the multilayered preantral phase. Regarding human subjects, the mutations of the FSH receptor gene have been linked to the lack of mature antral follicles, estrogen, and amenorrhea (7).

It has widely been shown that FSH can induce *in vivo* development of preantral follicles and also stimulate culture of *in vitro* ovarian tissue. *In vivo* administration of FSH promoted survival of preantral follicles. FSH administration into cultures of preantral follicles resulted in the same outcomes (8). Recent research has shown that FSH binding sites are available in preantral follicles in rodents and sheep ovaries. Adult mouse ovarian surface epithelium can express FSH receptors locating on the pluripotent stem cells and ovarian germ stem cells. These stem cells are activated by pregnant mare serum gonadotropin administration leading to augmented neo-oogenesis as well as PFs accumulation (9). Parte et al. showed induction of pluripotent stem cells located in ovarian surface epithelium (OSE) by FSH in cultured human and marmoset ovarian cortical tissue (6). FSH therapy caused vast proliferation of OSE and an increase in pluripotent stem cells and germ cells (7).

Basic fibroblast growth factor (bFGF) is a crucial causative agent for PF transition, which can be expressed in the ooplasm of primordial and primary follicles, OSE, vascular smooth muscle

cells, and corpus luteum (10). In goats, development and survival of PF is enhanced by the combination of bFGF and FSH (8). bFGF has been shown to be involved in follicle recruitment and development via stimulation of granulosa cell proliferation (10). Quennell et al. (11) indicated that bFGF transcripts are available in PFs and there is a reduction in their level by growing follicles. According to Tang et al. (12), the effectiveness of FSH on PF growth and survival was increased by bFGF through culturing bovine ovarian tissue for a long period. Interestingly, FSH receptors and bFGF can be expressed in OSE (10) located on ovarian stem cells (13). Further, it has been shown that bFGF is an effective factor in the culture medium needed for maintaining the pluripotency in human embryonic stem cells (14).

Neuronal growth factors, including neurotrophins (NTs), are associated with early folliculogenesis (14). So far, five neurotrophins have been found (15). Two isoforms have been identified for neurotrophin 3 (NT3) (16). NGFs (10), containing NT3 (17), induce activation of the primordial follicle. NT3 is possibly associated with the primordial-to-primary transition in humans. It also acts via synergistic interactions with other NTs and other factors.

Accordingly, in this study, an attempt was made to investigate FSH, bFGF, and NT3 impacts on PF development focusing on ovarian stem cells within the ovarian cortical tissues in human samples. Since previous reports indicated that days 2-3 are suitable for spontaneous activation of PF *in vitro*, the third day as the time point was selected in this study (18). qRT-PCR analysis was used for different stem cells, oocytes, and granulosa cell specific markers for studying the impact of FSH, bFGF, and NT3 on ovarian stem cells as well as PF development *in vitro*. Moreover, this study was performed to investigate whether cells extracted from the ovarian cortex can be considered a promising source in obtaining oocytes *in vitro*.

Methods

Ovarian tissue preparation: The use of human ovarian tissue in this research was confirmed by the Ethics Committee of Iran University of Medical Sciences (Code: 92-03-117-24106). Ovarian tissue was collected from a perimenopausal woman under 40 without underlying diseases and cancer who had undergone total abdominal hysterectomy. The Obstetrics and Gynecology unit at Imam Khomeini Hospital of Tehran, Iran provid-

ed perimenopausal ovarian sample after obtaining the donor's consent in compliance with ethical rules.

Ovarian cortical tissue culture

Cell isolation: A human ovary was transported to the laboratory in phosphate buffered saline (PBS) including 100 IU/ml of penicillin (3%) as well as 100 µg/ml of streptomycin. Afterward, ovarian cortical tissue was dissected into 1 mm³ piece and was placed in a 25 cm² cell culture flask, and incubated with alpha-MEM (Invitrogen, USA) supplemented with sodium pyruvate (2 mM/L), L-glutamine (2 mM/L), 1% insulin, transferrin, and selenium (Sigma-Aldrich, USA), 1% penicillin (100 IU/ml)/streptomycin (100 µg/ml) (Invitrogen, USA) and 10% fetal bovine serum (Gibco, UK) and the medium was changed every 2 days. After 5 days, cells migrated from explants and attached to the cell culture flask surface and were subcultured after reaching 80% confluency; moreover, cells at passage 3 were used for subsequent experiments. The cells isolated from the explant had a variety of shapes. After three passages, the other cells were removed and most of the remaining cells had a fibroblastic morphology (Figure 1).

Flow cytometry: Cell surface markers expression in the isolated cells was evaluated using flow cytometry for CD105, CD44, and CD90, as MSCs positive markers and CD34, CD45 as negative markers. Cells were fixed using 4% V/V paraformaldehyde (Sigma Aldrich, USA). Then, the cells were incubated with primary antibodies against CD105 (1:500, Cat. N: E01423-1631, eBioscience, USA), CD73 (1:50, Cat. N: 344009, BioLegend, USA), CD90 (1:300, Cat. N: 328113, BioLegend, USA), CD34 (1:100, Cat. N: IQP-144R, IQ Products, Netherlands), and CD45 (1:50, Cat. N: 560978, BioLegend, USA) according to the manufacturer's instructions. After removing primary antibodies, PBS was used to wash the cells and incubation was done using FITC-conjugated secondary antibody (1:500/dark environment), PE-

conjugated secondary antibody (1:300/dark environment), PerCP-conjugated secondary antibody (1:200/dark environment) and marker expression was analyzed using BD FACSCalibur™ flow cytometer (BD Biosciences, USA).

Treatments: The isolated cells were divided into eight groups as follows: control group included cells without any treatment, FSH group included cells treated with 0.5 IU/ml human urinary FSH (Utrofol, Kuanart Pharmaceuticals, India), bFGFG group included cells treated with 100 ng/ml basic FGF (R&D Systems Inc, USA), NT3 group included cells treated with 100 ng/ml NT3 (R&D Systems Inc, USA), and other groups included cells treated with one of the combination of mentioned growth factors including FSH+NT3, FSH+bFGF, NT3+bFGF, and FSH+NT3+bFGF. Each group included 3 same members (10000 cells). They were kept at 37°C in 5% CO₂ followed by monitoring by inverted microscope using Hoffman modulation contrast (Eclipse TE2000-S, Nikon, Japan). Cortical tissue sections were sampled on day three and RNA extraction was done after processing.

Quantitative reverse transcription PCR (qRT-PCR) analysis: Real-time PCR was used for evaluation of transcripts rates for pluripotent stem cells, including OCT4-A and Nanog, early germ cells or oocyte specific markers of c-KIT and VASA as well as for reflecting PF transition to primary follicles (Oocyte specific GDF-9 and Lhx).

RNA extraction kit was used for total RNA extraction. RNX-Plus (Sinaclon, Iran) was resolved in RNase-free water (50 µl) based on the manufacturer's instruction. NanoDrop (Thermo scientific, USA) was applied to analyze RNA for determining its concentration as well as purity. Denaturing gradient gel electrophoresis (DGGE) was employed for measuring RNA integrity. RNA was treated using DNase I (EN0521, Sigma-Aldrich, Germany) for reducing DNA contamination. The process of converting RNA to cDNA was done through cDNA Synthesis Kit (PrimeScript RT Master Mix, Takara Bio INC Company, Japan). Synthesis of cDNAs was performed by RNA (1 µg), oligo dT (0.5 µl), and random hexamer (0.5 µl). The analyses were performed according to the recommended instruction by the producer. SYBR Green-based RT-PCR was done using the synthesized cDNA (1 µg) through 2X qPCR kit (RR820L, Tli RNaseH Plus, Takara Bio INC Company, Japan). Table 1 shows the applied primer pairs.

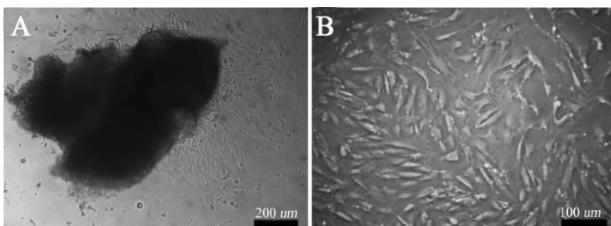


Figure 1. Cell isolation from ovarian cortical tissue. A) 5 days and, B) 20 days after human ovarian tissue culture in the culture plate

Table 1. Sequence of specific primers used for real-time quantitative reverse transcription PCR

GAPDH-F	AAGGTCGGAGTCAACGGATTTG
GAPDH-R	GCCATGGGTGGAATCATATTGG
Nanog_R	CGTCACACCATTGCTATTCTTCG
c-KIT-F	CGTCTCCACCATCCATCCATC
c-KIT-R	CCATTCAATTCTGCTTATTCTCATTCCG
VASA-F	GGCGTACTGGTCGTTGTGG
VASA-R	TAGAGGCTGTGCTAAATGGTTATCC
GDF-9-F	CTCCTGGAGACCAGGTAACAGGAAT
GDF-9-R	TGCACACACATTTGACAGCAGAGG
Lhx-8-F	AAGGACAGGCTTGAGCAGAC
Lhx-8-R	GGGTGGAGGATGAGTGATTAGG
OCT4-A-F	CCCTTCGCAAGCCCTCATTTCAC
OCT4-A-R	GCCCATCACCTCCACCACCTG

Thermocycling was done based on the following parameters: initial denaturation (95°C/ 30 s), 40 cycles at 95°C within 5 s followed by annealing and elongation (60°C/30 s). β -actin as a loading control was used for normalization of data. $\Delta\Delta C_t$ formula was employed for determining relative alteration in gene expression in the control group (19).

Statistical analysis: All data were expressed as mean \pm standard error (SEM). Oneway ANOVA was applied to clarify significant differences between groups. When a significant effect was found, the Tukey post-hoc test was performed. All anal-

yses were performed using SPSS vs. 23 (IBM, USA). The statistical significance level was set at $p \leq 0.5$.

Results

Flow cytometry analysis: The isolated mesenchymal stem cells from ovarian tissue with fibroblast like morphology expressed mesenchymal lineage specific markers including CD73, CD90, and CD105 (88.9%, 88.8%, and 81.0%, respectively) and did not express CD34 and CD45 according to flow cytometry results (Figure 2).

Expression of pluripotent stem cells markers: Levels of transcripts for pluripotent stem cells (OCT4-A, Nanog) were analyzed. Results were represented as fold change values over untreated control values expressed as 1 after normalizing with the housekeeping gene. Nanog transcripts showed increased expression treated with FSH, bFGF or NT3. Treatment with FSH+NT3 or NT3+bFGF had a synergistic effect on the expression of Nanog. OCT4-A transcripts did not show increased expression when treated with FSH, bFGF or NT3 separately. Treatment with FSH+NT3 or bFGF+FSH had an impressive effect on the expression of OCT4-A (Table 2).

Expression of germ cell markers: Levels of transcripts for germ cell markers (c-KIT, VASA) were analyzed. Results were represented as fold change values over untreated control values ex-

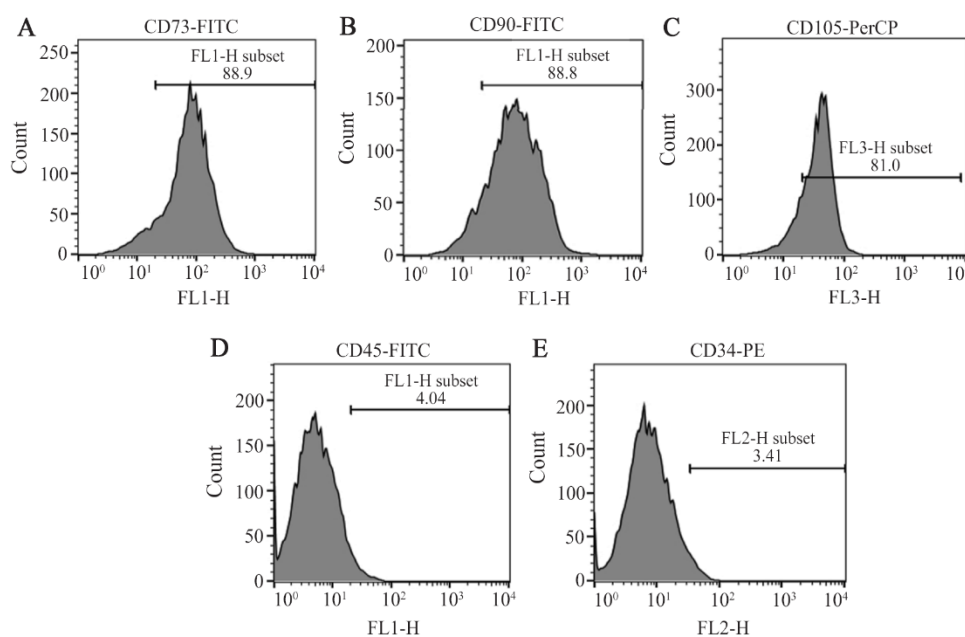


Figure 2. The isolated mesenchymal stem cells from ovarian tissue with fibroblast like morphology expressed mesenchymal lineage specific markers including CD73, CD90, and CD105 (88.9%, 88.8%, and 81.0%, respectively) and did not express CD34 and CD45 according to flow cytometry results

Table 2. Effect of bFGF, FSH and NT3 on expression of pluripotent marker by qRT-PCR

Groups	Nanog	OCT4-a
Control	1±0	1.18±0.95
FSH	31.005±4.37 *	0.14±0
NT3	114.65±8.21 *	1.001±0
bFGF	87.92±3.33 *	0.19±0.018
FSH+NT3	405.18±124.98 *,#	95.32±12.51 *,#
FSH+bFGF	74.81±9.42 *	1.5±0.33
NT3+bFGF	313.22±34.05 *,#	11.35±0.85 *
FSH+NT3+bFGF	31.304±5.96 *	0.37±0.091

OCT4-A was clearly up-regulated in response to FSH+NT3 and bFGF+FSH treatment compared to untreated control and, Nanog was clearly up-regulated in all groups especially in response to FSH+NT3 and NT3+bFGF treatment compared to untreated control. Values are mean fold changes obtained with respect to untreated controls. Data are expressed as means±SEM.

* Compared to control group. # Compared to other groups (p<0.05)

pressed as 1 after normalizing with the house-keeping gene. c-KIT transcripts showed increased expression treated with FSH, bFGF or NT3 separately. Treatment with two or three agents together had no synergistic effect on expression of this marker. VASA transcripts showed increased expression when only treated with FSH+NT3 (Table 3).

Expression of transition specific markers: Levels of transcripts for transition specific markers (GDF-9 and Lhx-8) were analyzed. Results were represented as fold change values over untreated control values expressed as 1 after normalizing with housekeeping gene. Lhx transcript showed increased expression when only treated with bFGF.

Table 3. Effect of bFGF, FSH and NT3 on expression of germ cell marker by qRT-PCR

Groups	c-KIT	VASA
Control	1±0	1.02±0.1188
FSH	3.91±1.005 *	0.001±3.96
NT3	9.83±1.823 *,#	0.02±0.008
bFGF	5.09±0.061 *,#	0.04±0.001
FSH+NT3	0.83±0.146	2.22±0.203 *,#
FSH+bFGF	1.75±0.073	0.28±0.011
NT3+bFGF	1.65±0.124	0.37±0.028
FSH+NT3+bFGF	1.72±0.103	0.003±0.0003

c-KIT was up-regulated in response to NT3 and bFGF treatment compared to untreated control and, VASA was up-regulated in response to FSH+NT3 treatment compared to untreated control. Values are mean fold changes obtained with respect to untreated controls. Data are expressed as means±SEM.

* Compared to control group. # Compared to other groups (p<0.05)

Table 4. Effect of bFGF, FSH, and NT3 on expression of transition marker by qRT-PCR

Groups	GDF	Lhx
Control	1±0	1±0
FSH	0.21±0.04	0.47±0.035
NT3	0.36±0.008	0.54±0.029
bFGF	0.22±0.023	1.43±0.051 *,#
FSH+NT3	11.81±6.68 *	--
FSH+bFGF	0.22±0.009	--
NT3+bFGF	--	--
FSH+NT3+bFGF	0.03±0.001	0.13±0.005

Lhx was up-regulated in response to bFGF treatment compared to untreated control. GDF was up-regulated in response to FSH+ NT3 treatment compared to untreated control. Values are mean fold changes obtained with respect to untreated controls. Data are expressed as means±SEM.

* Compared to control group. # Compared to other groups (p<0.05)

GDF transcript showed increased expression when only treated with FSH+NT3 (Table 4).

Discussion

Ovarian cortex culture for obtaining preantral follicles has been an appropriate technique to preserve fertility. Survival and activation of PFs can be promoted by many factors and hormones (20). Primordial to primary follicle transition has been shown to be regulated by many growth factors.

There are several factors associated with activation of PFs, like FGF-2 (21), NTs, and members of the TGF-β family, including activin and growth differentiation factor 9 (GDF-9) (22-24). Accordingly, in this study, human ovarian cortex culture was used for evaluating the effectiveness of FSH, bFGF, and NT3 in follicle growth.

Various studies have shown that PI3K pathway plays a key role in oocyte development (25). As a result, activation of this pathway by growth factors may help stem cells differentiation into oocytes *in vitro*; in fact, they are important in activation of PFs as well as transition of resting PF to primary follicles via the elevated expression of GDF-9 and Lhx-8. Various studies have shown that primary oocytes express GDF-9. Also, some studies demonstrated that GDF-9 has important role in early follicular growth and in oocyte to granulosa cell interaction (26). Activation of precursor follicles *in vivo* is a slow process in which GDF-9 and BMP-15 play role and also it involves the PI3K pathway (27, 28). In non-growing oocytes, the Lhx transcription factor blocks the activity of the PI3K pathway and keeps the oocyte

in a quiescent state (29). Indirect interaction of Lhx-8 with PI3K pathway has also been proven in some studies (30). In addition, there was an enhanced expression of pluripotent stem cells, like OCT4-A and Nanog and germ cells specific markers, such as c-KIT and VASA. Our findings indicated the direct effect of FSH, bFGF, and NT3 on ovarian stem cells and also PF development.

The growth of germ cells needs several phases including up-and down-regulating the expression of specific genes (31). According to recent investigations, primordial germ cells and oocyte like cells are produced by embryonic, differentiated pluripotent and adult stem cells *in vitro* (32). Regarding mice, pigs, and humans, somatic stem cells could be differentiated to oocyte like cells by culturing under the influence of growth factors (33), estrogenic stimuli (32), follicular fluid, and gonadotropins (34).

PF transition from primary to secondary follicles occurs through 8 to 20 days following the culture of ovarian cortical tissue (6). Parte et al. showed that stem cells within the ovary are capable of differentiation to oocyte like cells (6). Isolated ovarian stem cells can express mRNAs for OCT4 as transcription factors involving in the maintenance of cell pluripotency. OCT4 has also been reported as a regulator of Nanog expression (35). Non-coding RNA transcription can be activated by OCT4 and Nanog and they increase stem cells self-renewal as well as inhibit differentiation (36). According to our results, the expression level of OCT4 in FSH+NT3 group and bFGF+NT3 group showed a significant increase while in other groups, no increase was observed. FSH, by binding to its receptor via the PI3K pathway, can activate AKT and β -catenin and ultimately increase OCT expression (37). The results of our study showed that treatment of cells with FSH or NT3 alone could not lead to a significant increase in OCT4 expression. As a result, FSH and NT may have a synergistic effect on OCT expression. Treatment of cells with bFGF+NT3 also resulted in a significant increase in the expression of pluripotency markers, although this increase was lower than the one in the group treated with FSH+NT3.

On the other hand, the expression of Nanog in all groups showed a significant increase and the highest expression was observed in the FSH+NT3 group. As our results show, treatment of cells with any of the growth factors of FSH, bFGF, and NT3 increases the expression of Nanog, while simultaneous treatment of cells with bFGF+NT3 or FSH+

NT3 increases the expression of Nanog significantly producing a synergistic effect. Since the expression of Nanog in the two groups of FSH+NT3 and bFGF+NT3 is significantly higher than the group of FSH+bFGF and also the expression of Nanog is higher in NT3 group compared to the two groups of bFGF and FSH, it is suggested that NT3 has a greater effect on Nanog expression than bFGF and FSH.

Furthermore, treatment of cells with FSH+NT3 significantly increases the expression of VASA. VASA has been shown to be expressed in the post-migratory primordial germ cells until the post-meiotic stage of oocytes (38). Based on Dyce et al.'s results, VASA expression and differentiation of stem cells, obtained from skin, to OLCs is promoted by follicular fluid (39). VASA is a marker for germ cell differentiation among several species (40). Considering that OCT4 marker is also a marker of germ cells and its expression is significantly increased due to the treatment of cells with FSH+NT3, it can be concluded that the treatment of ovarian cortical cells with FSH+NT3 can be a good option for achieving germ cells *in vitro*. Unexpectedly, the expression of c-KIT as another germ cell marker, unlike the other two germ cell markers, did not increase in the FSH+NT3 group. Treatment of cells with FSH, NT3, and bFGF significantly increases the expression of this marker while FSH+bFGF, NT3+bFGF, and FSH+NT3+bFGF did not increase the expression of this marker compared to the control group. These results may suggest that the combination of growth factors is not a suitable method to induce the expression of germ cell markers in ovarian cortical stem cells, but other methods of cell treatment, including periodic treatment with these growth factors should be considered in next studies.

The expression of GDF-9 is specific for oocytes and is a marker for the identification of oocytes derived from stem cells (41).

Our study showed that these markers increased the influence of FSH+NT3 treatment. Silva et al. reported that five days of culture using FSH increased oocyte diameter in primary follicles (42). It has been shown that the expression of FSH receptor is developed progressively through the primordial-to-primary follicle transition (43) and also recent studies have reported the existence of FSH receptors in primary follicles (44), which indicates the essential role of FSH in oocyte development. Moreover, Roy and Albee (45) stated the crucial effect of FSH for somatic cells to early

granulosa cell differentiation through initial PF growth. Our study shows that FSH alone does not increase the expression of GDF-9, but the combination of FSH with NT3 can significantly increase the expression of GDF-9. On the other hand, our results show that Lhx expression is significantly increased only in the FSH group and has no expression in the FSH+NT3 group.

In our study, FSH alone could increase transition markers including Lhx, pluripotent stem cells marker (Nanog), and early germ cell marker (c-KIT). c-Kit, a member of tyrosine kinase receptors, together with its ligand, KIT, plays considerable role in female reproductive system (46). Consistent with the results of Parte et al., Nanog expression was indirectly representative of existence of pluripotent stem cells within the surface epithelium of ovary (47) and increase in the expression of Lhx-8 indicated PFs transition into primary follicles, which is consistent with other relevant studies (48).

The stimulation of pluripotent stem cells as well as early germ cell specific markers via bFGF is not unusual, as bFGF receptors have shown to be available in OSE (10). Based on Kezele et al.'s (49) report, bFGF can stimulate several PFs to primary and secondary follicles among rat samples. Garor et al. (50) indicated the enhanced rate of PF in the bFGF-treated cortical tissue culture of the ovary.

In our study, bFGF alone could increase transition markers including Lhx, pluripotent stem cells marker (Nanog), and early germ cells marker (c-KIT). A combination of FSH and bFGF had no such effect.

FSH and bFGF could induce an elevation in OSE proliferation and also an increase in the expression of the pluripotent markers, including OCT4-A and Nanog in perimenopausal human ovarian sample devoid of PF (6). Matos et al. (8) reported that the combination of FSH+bFGF can increase PF growth and survival in goats and bFGF-neutralizing antibody could stop the change (51). Tang et al. showed that the combination of FSH and bFGF significantly increased the rates of primary and normal follicles and also decreased the apoptotic cell count in comparison with FSH alone (12).

However, Derrar et al. (52) demonstrated that bFGF+FSH, insulin-like growth factor 1 (IGF-I), epidermal growth factor (EGF), and transforming growth factor β (TGF- β) were unable to change

PF activation or morphology of primary or secondary follicles in short-term cultures of the ovarian cortex in the cow. In our study, combination of FSH and bFGF did not have synergistic effect. NT3 is associated with the progression of rat PFs to developing follicles in culture as well as upregulating the expression of 18 genes, such as the genes involved in the plasma membrane and cytoskeleton synthesis (48). NT3 is possibly linked to the stimulation of steroid hormone formation in the antral follicle (53). By culturing hamster follicles plus NT3, a two fold increase was observed in E2 release (53). Moreover, adding NT3 into the culture medium of bovine theca cells led to elevated cell proliferation (54). According to Dissen et al.'s study, by eliminating neurotrophin genes in knockout mice, follicle production and development are influenced (55). Nilsson et al. indicated that NT3 signaling probably occurs by paracrine/autocrine mechanism in PFs.

In our study, NT3 alone could increase pluripotent stem cells maker (Nanog) and and early germ cells marker (c-KIT). NT3 concomitant with FSH or bFGF could increase pluripotent stem cells markers (Nanog and OCT4-A).

NT3 with FSH or bFGF in normal circumstances probably facilitates PF to primary follicle transition. NT3 with FSH or bFGF is capable of stimulating ovarian stem cells and it may be effective in stem cells accumulation through developed cultures. It is worth studying whether the long-term culture of perimenopausal tissue of ovary can cause PF accumulation from the stem cells with NT3, FSH, or bFGF therapy. The results can provide the context for next investigations on the effect of other hormones and growth factors on activation of PFs *in vitro*. The culture of ovarian cortical tissue seems to be a useful technique for studying ovarian stem cell biology and also PF transition.

Conclusion

The obtained findings can be used for both oncotherapy and infertility treatment. Our results are helpful for *in vitro* production of human oocytes from ovarian stem cells. The function of produced oocytes *in vitro* is considered one of the main issues for investigators. Obviously, more studies are needed to reach the final result and achieve functional oocytes *in vitro*. In the future, more research should be done to clarify the molecular pathways involved in the process studied in this

paper. It is also necessary to conduct further studies on the effect of other growth factors with different concentrations.

Acknowledgement

The authors would like to thank Dr. Mansoureh Soleimani for her sincere support and guidance.

Conflict of Interest

There is no conflict of interest.

Funding: This research was supported by a grant (No92-03-117-24106) from Iran University of Medical Sciences, Tehran, Iran.

References

1. Feigin E, Freud E, Fisch B, Orvieto R, Kravarusic D, Avrahami G, et al. Fertility preservation in female adolescents with malignancies. *Cancer Female Adol.* 2008;38:101.
2. Abir R, Feinmesser M, Yaniv I, Fisch B, Cohen IJ, Ben-Haroush A, et al. Occasional involvement of the ovary in Ewing sarcoma. *Hum Reprod.* 2010;25(7):1708-12.
3. Oron G, Ao A, Friedman O, Fisch B, Zhang XY, Ben-Haroush A, et al. Expression of neurotrophin 3 and its tropomyosin-related kinase receptor C in human preantral follicles. *Fertil Steril.* 2011;95(6):2056-62.
4. Brännström M, Milenkovic M. Advances in fertility preservation for female cancer survivors. *Nat Med.* 2008;14(11):1182-4.
5. Telfer EE, McLaughlin M, Ding C, Thong KJ. A two-step serum-free culture system supports development of human oocytes from primordial follicles in the presence of activin. *Hum Reprod.* 2008;23(5):1151-8.
6. Parte S, Bhartiya D, Manjramkar DD, Chauhan A, Joshi A. Stimulation of ovarian stem cells by follicle stimulating hormone and basic fibroblast growth factor during cortical tissue culture. *J Ovarian Res.* 2013;6(1):20.
7. Hardy K, Fenwick M, Mora J, Laird M, Thomson K, Franks S. Onset and heterogeneity of responsiveness to FSH in mouse preantral follicles in culture. *Endocrinology.* 2017;158(1):134-47.
8. Matos MH, Lima-Verde IB, Bruno JB, Lopes CA, Martins FS, Santos KD, et al. Follicle stimulating hormone and fibroblast growth factor-2 interact and promote goat primordial follicle development in vitro. *Reprod Fertil Dev.* 2007;19(5):677-84.
9. O'shaughnessy PJ, Dudley K, Rajapaksha WR. Expression of follicle stimulating hormone-receptor mRNA during gonadal development. *Mol Cell Endocrinol.* 1996;125(1-2):169-75.
10. Van Wezel IL, Umaphysivam K, Tilley WD, Rodgers RJ. Immunohistochemical localization of basic fibroblast growth factor in bovine ovarian follicles. *Mol Cell Endocrinol.* 1995;115(2):133-40.
11. Quennell JH, Stanton JA, Hurst PR. Basic fibroblast growth factor expression in isolated small human ovarian follicles. *Mol Hum Reprod.* 2004;10(9):623-8.
12. Tang K, Yang WC, Li X, Wu CJ, Sang L, Yang LG. GDF-9 and bFGF enhance the effect of FSH on the survival, activation, and growth of cattle primordial follicles. *Anim Reprod Sci.* 2012;131(3-4):129-34.
13. Bukovsky A, Svetlikova M, Caudle MR. Oogenesis in cultures derived from adult human ovaries. *Reprod Biol Endocrinol.* 2005;3:17.
14. Pereira RC, Economides AN, Canalis E. Bone morphogenetic proteins induce gremlin, a protein that limits their activity in osteoblasts. *Endocrinology.* 2000;141(12):4558-63.
15. Liu Y, Song Z, Zhao Y, Qin H, Cai J, Zhang H, et al. A novel chemical-defined medium with bFGF and N2B27 supplements supports undifferentiated growth in human embryonic stem cells. *Biochem Biophys Res Commun.* 2006;346(1):131-9.
16. Levenstein ME, Ludwig TE, Xu RH, Llanas RA, VanDenHeuvel-Kramer K, Manning D, et al. Basic fibroblast growth factor support of human embryonic stem cell self-renewal. *Stem Cells.* 2006;24(3):568-74.
17. Virant-Klun I, Stimpfel M, Skutella T. Ovarian pluripotent/multipotent stem cells and in vitro oogenesis in mammals. *Histol Histopathol.* 2011;26(8):1071-82.
18. Wandji SA, Srsen V, Nathanielsz PW, Eppig JJ, Fortune JE. Initiation of growth of baboon primordial follicles in vitro. *Hum Reprod.* 1997;12(9):1993-2001.
19. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 2001;25(4):402-8.
20. Esmaielzadeh F, Hosseini SM, Nasiri Z, Hajian M, Chamani M, Gourabi H, et al. Kit ligand and glial-derived neurotrophic factor as alternative supplements for activation and development of ovine preantral follicles in vitro. *Mol Reprod Dev.* 2013;80(1):35-47.
21. Nilsson E, Parrott JA, Skinner MK. Basic fibroblast growth factor induces primordial follicle development and initiates folliculogenesis. *Mol Cell Endocrinol.* 2001;175(1-2):123-30.

22. Eppig JJ. Oocyte control of ovarian follicular development and function in mammals. *Reproduction*. 2001;122(6):829-38.
23. Van den Hurk R, Abir R, Telfer E, Bevers M. Primate and bovine immature oocytes and follicles as sources of fertilizable oocytes. *Hum Reprod Update*. 2000;6(5):457-74.
24. Dissen GA, Romero C, Hirshfield AN, Ojeda SR. Nerve growth factor is required for early follicular development in the mammalian ovary. *Endocrinology*. 2001;142(5):2078-86.
25. Andrade GM, Da Silveira JC, Perrini C, Del Colado M, Gebremedhn S, Tesfaye D, et al. The role of the PI3K-Akt signaling pathway in the developmental competence of bovine oocytes. *PLoS One*. 2017;12(9):e0185045.
26. Bayne RA, Kinnell HL, Coutts SM, He J, Childs AJ, Anderson RA. GDF9 is transiently expressed in oocytes before follicle formation in the human fetal ovary and is regulated by a novel NOBOX transcript. *PLoS One*. 2015;10(3):e0119819.
27. Otsuka F, McTavish KJ, Shimasaki S. Integral role of GDF-9 and BMP-15 in ovarian function. *Mol Reprod Dev*. 2011;78(1):9-21.
28. Roy S, Gandra D, Seger C, Biswas A, Kushnir VA, Gleicher N, et al. Oocyte-derived factors (GDF9 and BMP15) and FSH regulate AMH expression via modulation of H3K27AC in granulosa cells. *Endocrinology*. 2018;159(9):3433-45.
29. El-Hayek S, Clarke HJ. Control of oocyte growth and development by intercellular communication within the follicular niche. *Results Probl Cell Differ*. 2016;58:191-224.
30. Ren Y, Suzuki H, Jagarlamudi K, Golnoski K, McGuire M, Lopes R, et al. Lhx8 regulates primordial follicle activation and postnatal folliculogenesis. *BMC Biol*. 2015;13:39.
31. Takai H, Smogorzewska A, de Lange T. DNA damage foci at dysfunctional telomeres. *Curr Biol*. 2003;13(17):1549-56.
32. Clark AT, Bodnar MS, Fox M, Rodriguez RT, Abeyta MJ, Firpo MT, et al. Spontaneous differentiation of germ cells from human embryonic stem cells in vitro. *Hum Mol Genet*. 2004;13(7):727-39.
33. Hübner K, Fuhrmann G, Christenson LK, Kehler J, Reinbold R, De La Fuente R, et al. Derivation of oocytes from mouse embryonic stem cells. *Science*. 2003;300(5623):1251-6.
34. Qing T, Shi Y, Qin H, Ye X, Wei W, Liu H, et al. Induction of oocyte-like cells from mouse embryonic stem cells by co-culture with ovarian granulosa cells. *Differentiation*. 2007;75(10):902-11.
35. Rodda DJ, Chew JL, Lim LH, Loh YH, Wang B, Ng HH, et al. Transcriptional regulation of nanog by OCT4 and SOX2. *J Biol Chem*. 2005;280(26):24731-7.
36. Mohamed JS, Gaughwin PM, Lim B, Robson P, Lipovich L. Conserved long noncoding RNAs transcriptionally regulated by Oct4 and Nanog modulate pluripotency in mouse embryonic stem cells. *RNA*. 2010;16(2):324-37.
37. Heng D, Wang Q, Ma X, Tian Y, Xu K, Weng X, et al. Role of OCT4 in the regulation of FSH-induced granulosa cells growth in female mice. *Front Endocrinol (Lausanne)*. 2020;10:915.
38. Castrillon DH, Miao L, Kollipara R, Horner JW, DePinho RA. Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. *Science*. 2003;301(5630):215-8.
39. Dyce PW, Shen W, Huynh E, Shao H, Villagómez DA, Kidder GM, et al. Analysis of oocyte-like cells differentiated from porcine fetal skin-derived stem cells. *Stem Cells Dev*. 2010;20(5):809-19.
40. Kee K, Angeles VT, Flores M, Nguyen HN, Pera RAR. Human DAZL, DAZ and BOULE genes modulate primordial germ-cell and haploid gamete formation. *Nature*. 2009;462(7270):222-5.
41. Linher K, Dyce P, Li J. Primordial germ cell-like cells differentiated in vitro from skin-derived stem cells. *PLoS One*. 2009;4(12):e8263.
42. Silva JR, van den Hurk R, de Matos MH, dos Santos RR, Pessoa C, de Moraes MO, et al. Influences of FSH and EGF on primordial follicles during in vitro culture of caprine ovarian cortical tissue. *Theriogenology*. 2004;61(9):1691-704.
43. Oktay K, Briggs D, Gosden RG. Ontogeny of follicle-stimulating hormone receptor gene expression in isolated human ovarian follicles. *J Clin Endocrinol Metab*. 1997;82(11):3748-51.
44. Méduri G, Charnaux N, Driancourt MA, Combettes L, Granet P, Vannier B, et al. Follicle-stimulating hormone receptors in oocytes? *J Clin Endocrinol Metab*. 2002;87(5):2266-76.
45. Roy SK, Albee L. Requirement for follicle-stimulating hormone action in the formation of primordial follicles during perinatal ovarian development in the hamster. *Endocrinology*. 2000;141(12):4449-56.
46. Packer AI, Hsu YC, Besmer P, Bachvarova RF. The ligand of the c-kit receptor promotes oocyte growth. *Dev Biol*. 1994;161(1):194-205.
47. Parte S, Bhartiya D, Telang J, Daithankar V, Salvi V, Zaveri K, et al. Detection, characterization, and spontaneous differentiation in vitro of very small

- embryonic-like putative stem cells in adult mammalian ovary. *Stem Cells Dev.* 2011;20(8):1451-64.
48. Nilsson E, Dole G, Skinner MK. Neurotrophin NT3 promotes ovarian primordial to primary follicle transition. *Reproduction.* 2009;138(4):697-707.
49. Kezele PR, Nilsson EE, Skinner MK. Insulin but not insulin-like growth factor-1 promotes the primordial to primary follicle transition. *Mol Cell Endocrinol.* 2002;192(1-2):37-43.
50. Garor R, Abir R, Erman A, Felz C, Nitke S, Fisch B. Effects of basic fibroblast growth factor on in vitro development of human ovarian primordial follicles. *Fertil Steril.* 2009;91(5 Suppl):1967-75.
51. Nilsson EE, Skinner MK. Kit ligand and basic fibroblast growth factor interactions in the induction of ovarian primordial to primary follicle transition. *Mol Cell Endocrinol.* 2004;214(1-2):19-25.
52. Derrar N, Price CA, Sirard MA. Effect of growth factors and co-culture with ovarian medulla on the activation of primordial follicles in explants of bovine ovarian cortex. *Theriogenology.* 2000;54(4):587-98.
53. Waraksa JA, Lindsay RM, Ip NY, Hutz RJ. Neurotrophin-3 augments steroid secretion by hamster ovarian follicles in vitro. *Zoolog Sci.* 1995;12(4):499-502.
54. Dissen GA, Parrott JA, Skinner MK, Hill DF, Costa ME, Ojeda SR. Direct effects of nerve growth factor on thecal cells from antral ovarian follicles. *Endocrinology.* 2000;141(12):4736-50.
55. Dissen GA, Romero C, Paredes A, Ojeda SR. Neurotrophic control of ovarian development. *Microsc Res Tech.* 2002;59(6):509-15.