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Original Article

The Effect of Follicular Fluid on the Proliferation and Osteoblastic Differentiation of Human Bone Marrow Mesenchymal Stem Cells

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ABSTRACT

Background and Aims: Bone marrow-derived mesenchymal stem cells (BM-MSCs) are a well-known source of multipotent adult stem cells. Despite using different methodologies of MSCs preparing for clinical applications, the top safest procedure to manipulate these cells, has not yet been determined. Recently, *ex-vivo* expansion of MSCs for their subsequent implantation, using some biological product, is suggested instead of fetal bovine serum (FBS). Previous studies have shown the effect of follicular fluid (FF) (a dynamic fluid in ovarian follicle) as an additive component in cell culture. Hence, this study aimed to decipher its role on the human BM-MSC proliferation.

Materials and Methods: In this study, BM-MSCs at 3rd passage were cultivated in the presence of 20% FF (group I), 10% FF+ 10% FBS (group II) and FBS 20% as control group. The capacity of proliferation as calculating population doubling times and gene expression levels of stem cell factor, stromal cell-derived factor 1, and transforming growth factor beta were analyzed in osteogeneic media to examine the impacts of FF on osteogenesis of MSCs.

Results: Our results corroborated an up-regulatory effect of FF on the proliferation of BM-MSCs by shorter population doubling times in the group II of treated cells and an increase in gene expression level of osteocalcin and transforming growth factor beta in the presence of higher concentrations of FF in cell culture FF 20% and 10%, respectively.

Conclusions: FF is a potent mitogen for cell proliferation. FF may be an efficient substitution of FBS in *ex-vivo* cell culture, eliminating zoonotic infections and immunological reactions.

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Introduction

Mesenchymal stem cells (MSC) have immense potential for cell-based therapy and regenerative medicine purposes, and can be isolated from different sources (e.g. bone marrow, adipose tissue) and are proposed to differentiate into various cell lineages, including chondrocytes, osteoblasts, fibroblasts and adipocytes [1, 2]. New approaches in regenerative medicine are based on the manipulation of either MSCs with multipotent differentiating potential or growth supplements, which are able to influence on obtaining efficient yields of autologous and allogeneic clinical grade MSCs for therapeutic application. [3-5] It is supposed that some human biological products can be used as growth supplements in cell expansion in order to avoid undesirable complication of fetal bovine serum (FBS) such as risk of xeno-immunization and zoonotic transmission [6, 7].

follicular fluid (FF) is a biological product which is obtained in *in-vitro* fertilization (IVF) process. It is an important component of the ovarian follicle that encompasses the growing oocyte with the enclosed follicular cells. At the pre-antral stage, FF is produced in the growing follicle by diffusion of proteins in the bloodstream through the thecal capillaries. Also, the components secreted by the cell layers that surround the follicle cells (especially the granulosa) involve a part of FF ingredients [8-10]. The basic ingredients of FF include various biological active proteins, peptides, amino-acids, hyaluronic acid, steroid hormones, polysaccharides, prostanoids, anti-apoptotic factors and also antioxidant enzymes. Based on some previous studies, it is demonstrated that FF is beneficial for the proliferation and differentiation of goat umbilical cord mesenchymal stem cells (UC-MSCs). The proteins and cytokines such epidermal growth factor and insulin-like growth factor contained in the FF affect the fate of UC-MSCs. With a precise look, higher concentrations of FF depicts an upsurge in differentiation of UC-MSCs whereas proliferation is induced by a lower concentration of FF [11, 12].

Numerous investigations have indicated that estrogen supports and promotes the osteoblastic differentiation of MSCs due to the increased expression of bone calcium and alkaline phosphatase [13-16]. To consider these findings, on the one hand, we hypothesized that FF which is rich in steroid hormones, may play an indispensable role in this process and induce the osteoblastic differentiation of MSCs. Accordingly, expression levels of osteocalcin as an osteogeneic marker has been examined. On the other hand, *SCF*/*c-KIT* signaling pathway, which has been represented to play an important role in several biologic processes such as melanogenesis, hematopoiesis and gametogenesis, can be regulated by hormonal factors [17, 18]. Hence, we studied the effect of FF on the proliferation and gene expression levels of stem cell factor (SCF) to determine if it is modulated by esterogenic micro-environment of FF.

In the present study, transforming growth factor beta *(TGF-β)* gene expression level was chosen to be evaluated, considering the presence of TGF-β superfamily members in FF and their important role in control of ovarian follicle development. From one viewpoint, some studies have indicated that, stromal cell-derived factor 1 (SDF-1) modulates the expression of cell cycle key regulators such as cyclin-dependent kinase and TGF-β-related molecules [12, 19, 20). Accordingly, we studied the effect of FF on the gene expression level of *SDF-1* within bone marrow-derived mesenchymal stem cells (BM-MSCs).

Materials and Methods

Bone marrow mesenchymal stem cell culture

BM-MSCs were kindly dedicated from Dr. M. Soleimani (Stem Cells and Tissue Engineering Department, Stem Cell Technology, Tehran, Iran.). Phenotypic characterization of the BM-MSCs were carried out by flow-cytometry (FACS Canto II, BD, USA). Cells of the third passage were detached with trypsin (Gibco, Grand Island, NY, USA) and stained with fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated antibodies against the common leukocyte antigen CD45, the cell surface expressed CD34, CD105, CD73, HLA-DR and CD90 based on manufacturer`s instructions. FITC- and PE-negative isotypes were used as control antibodies. Cells were incubated with the primary antibody at 4˚C for 30 min, then cell fluorescence was examined by flow cytometry using a FACS Calibur apparatus (BD Biosciences). Data were analyzed using Cell Quest software (Milano, Italy).

Preparation of FF

participants were pleased to come to the laboratory. FF samples were withdrawn by an authorized supervisor with sterile gauge needles. FF (~5 ml) was collected into 50 ml falcon tubes. In order to remove cellular debris, FF samples were centrifuged at 12,000×g at 4˚C for 30 min at room temperature (Eppendorf, Germany), and then supernatants were filtered (0.22 µm pore size). To deactivate complement system activity, all the samples were incubated at 56˚C (Thermo-shaker TS-100, Biosan, Russia) for 30 minutes. Subsequently, supernatant phase of fluid for all samples were transferred into labelled 1.5 ml microcentrifuge eppendorf. Later, fluid samples of all aspirated follicles were pooled and stored temporarily at -80˚C freezer until further use [11].

Treatment of the BM-MSC in non- differential condition and calculate population doubling times (PDT)

In aseptic conditions under biological safety cabinet, BM-MSCs at 3rd passage were seeded to six flat bottomed wells cell culture plates at a density of 3×10^4 cells/well (the density was set up) in the presence of DMEM (Invitrogen, Carlsbad, CA) and different concentration of FBS (Gibco, Grand Island, NY, USA) and/or FF, 20% FF (group I), 10% FF+10% FBS (group II) and FBS 20% as group control (optimal concentrations of FF were selected based on previous studies) [11, 21]. Then, ingredients of each well was gently mixed by several times aspiration and ejection. The medium in examined groups was supplemented with streptomycin (0.025 U/mL)

and penicillin (0.025 U/mL; Gibco). Thereafter, specimens were incubated at 37°C and 5% CO_2 in a 95% humidified atmosphere (the situation of incubation was set up) (Table 1&2). The capability of proliferation was evaluated by a growth curve at an interval of 24 hr. The cells from each of the growth conditions in six well-plated were trypsinized and counted for 3 consecutive days using a hemo-cytometer to calculate PDT. PDT was calculated using the following formula: $PDT=[\log_2/(\log N_t - \log N_0)]\times t]$.

Where N_t is the number of cells after t hours of culturing, and N_0 is considered as the number of cells seeded [11]. In order to investigate the levels of *SCF, SDF-1,TGF-ß, osteocalcin* expression, RNA extraction and c-DNA synthesis were performed in 2, 4, 6 days after treating the cells by additive components as shown in the table1.

Induction of differentiation into osteoblastic cells

BM-MSCs at 3rd passage were cultured in differential condition to determine gene expression of osteocalcin. To promote osteoblastic differentiation, cells were cultured for 3 weeks in DMEM culture medium supplemented with, 0.1 mM ascorbic acid 2-phosphate, 10^{-2} M β-glycerophosphate and 10^{-8} M dexamethasone by the presence of different concentration of FF and FBS as mentioned in table 2 [22]. The media were changed three times weekly. Assessment of calcium accumulation was visualized by Alizarin red staining (Bio-Optica, Milan, Italy) and monitored under inverted microscope (Leitz,Wetzlar, Germany). Then RNA extraction and c-DNA synthesis were performed in days 8, 14, 21 after treating the cells in order to analyse gene expression of osteocalcin.

Table 1. Comparison of MSCs culture in different concentrations of FF and FBS

	Day 2	Day 4	Day 6
Group I	DMEM+20% FF	DMEM+ 20% FF	DMEM+ 20% FF
Group II	DMEM+10% FF+10% FBS	DMEM+10% FF+10% FBS	DMEM+10% FF+10% FBS
Control	DMEM+ 20% FBS	DMEM+ 20% FBS	DMEM+ 20% FBS

FF= Follicular fluid; FBS= Fetal bovine serum

Table 2. Comparison of osteoblastic differentiation in different concentrations of FF and FBS

	Day 8	Day 14	Day 21
Group I	OM+ 20% FF	OM+FF 20%	OM+20% FF
Group II	OM+10% FF+10% FBS	OM+10% FF+10% FBS	OM+10% FF + 10% FBS
Control	OM+20% FBS	OM+ 20% FBS	OM+ 20% FBS

FF= Follicular fluid; FBS= Fetal bovine serum; OM= Osteogeneic media

Table 3. Specific primers were used in this study

Gene Name	Accession number	Sequence (5´-3´)	Product length (bp)
Transforming growth factor beta	NC_000019	F: TGG CGA TAC CTC AGC AAC R: ACC CGT TGA TGT CCA CTT G	181
Stromal cell-derived factor 1	NC_000010	F: TGC CCT TCA GAT TGT AGC CC R: CGA GTG GGT CTA GCG GAA AG	145
Stem cell factor	NC_000012	F: CCC AGA ACC CAG GCT CTT TA R: TGT GAC ACT GAC TCT GGA ATC TTT	154
Osteocalcin	NC_000001	F: GCA AAG GTG CAG CCT TTG TG R: GGC TCC CAG CCA TTG ATA CAG	86
Gylceraldehyde 3-phosphate dehydrogenase	NC_000012	F: ATG GGG AAG GTG AAG GTC G R: GGG GTC ATT GAT GGC AAC AAT A	108

RNA isolation and processing DNA for real-time polymerase chain reaction (PCR)

RNA was extracted from treated BM-MSCs and induced cells using Ribox (Qiagen, Beijing, China) according to the manufacturer's instructions. The mRNA was reverse transcribed to cDNA using advantage RT-for-PCR Kit (Takara, Dalian, China) based on the manufacturer's instructions. The amplification of cDNA was conducted by using a ABI GeneAmp PCR System 2400 (Takara, Dalian, China). The PCR products were resolved on 1.0% (w/v) agarose gels containing 1 mg/ml Ethidium bromide and the products were viewed and photographed under UV light.

Gene expression study using real-time PCR using cDNA for all examined group

Real-time PCR was conducted separately for each gene (*SDF-1, SCF, TGF-ß, osteocalcin*), and the data were interpreted using Pfaffl calculations. At this stage, *GAPDH* was recruited as control genes. ABI 7500 instrument was used for real-time PCR with SYBR green as the Master Mix. The primers used for real-time-PCR analyses are listed in table 3. Primers were designed using Primer 3 (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). All primer pairs were chosen. Meanwhile each primer was from a different exon to distinguish cDNA from genomic DNA products. This study accessed ethics approval from Institutional Ethics Committee of Royan Institute Tehran, Iran. (IR.ACECR.ROYAN.REC.1395.96). Ten female healthy volunteers (without any chronic inflammatory or predisposition history) undergoing IVF were recruited due to male factor infertility (20-35 years old) from Royan Institute (Tehran, Iran). First, they were ensured about moral confidence and biosafety of the study. Then, oral conscious and a written informed consent were achieved.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) version 11.5, computer software (SPSS Inc., USA) was used for the statistical analysis of the data. As a parametric statistical test One-way ANOVA was applied for statistical comparisons of the experiment. Results were expressed as mean plus minus standard errors of the mean (Mean±SEM).

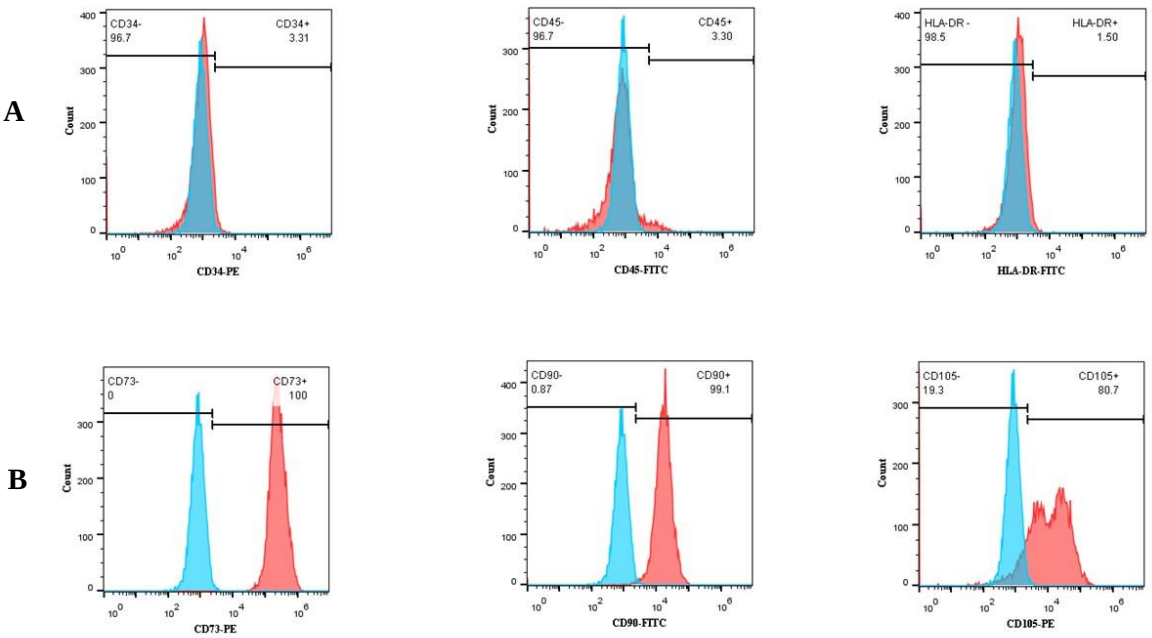

Fig. 1. Characterization of human BM-MSCs *in vitro*. (A): Flow-cytometry data shows that BM-MSCs are negative for CD34, CD45 and HLA-DR (B): and positive for the markers CD73, CD105 and CD90

It is of high significance to mention that for three independent experiments, three replicates were evaluated. Statistical significance difference and clear statistical significant difference were considered at $p<0.05$ and $p<0.01$, respectively.

Result

BM-MSC characterization

MSCs were characterized by flow-cytometry analysis using specific monoclonal antibodies against CD105, CD90, CD73, CD34, HLA-DR and CD45. MSCs that were positive for CD73, CD105, CD90 and negative for CD34, CD45 and HLA-DR are shown in figure 1.

FF promoted the proliferation of BM-MSCs *in vitro*

Two different concentrations of FF (10, 20%) were evaluated for the effects of FF on the proliferation of BM-MSCs. *In-vitro* cultivation of BM-MSCs in the presence of 20% FF for 3 consecutive days showed an almost equal proliferation index in comparison with samples treated with 20% FBS whereas those cells cultured in the presence of 10% FF+10% FBS showed an improved proliferation index (shorter PDT). The mean PDT of BM-MSCs (at passage 4) was 52.37 h versus 35.20 h cultured in the presence of 20% (group I) and 10% FF (group II), respectively (Fig. 2), ($p\leq0.01$). These results demonstrate the capability of FF to stimulate the proliferation of BM-MSCs *in vitro*. The BM-MSCs cultured in the presence of FF exhibited as typical spindle-shaped fibroblasts with uniqueness of their phenotypes as well as those cultured in FBS (Fig. 3).

Effect of FF on gene expression levels of *SCF, SDF-1, TGF-β*

A quantitative SYBR Green real-time PCR method was conducted for detection of *SCF, SDF-1* and *TGF-β* mRNA expression levels in

human BM-MSCs treated by FF (10% and 20%). Considering the estrogenic effect of FF on the expression of *SCF/c-KIT* mRNA, it was demonstrated that treatment of the BM-MSCs with 20% FF for 6 days significantly up-regulates the expression of *SCF* (1.59± 0.27 fold variation relatively to control) (Fig. 4A). Therefore, the effectivenessof FF in cell proliferation can be explained in terms of its role in *SCF* gene expression. Treatment of the BM-MSC with FF 10%+FBS 10% for 6 days significantly increased the mRNA expression of *SDF-1*(2.01±0.51 fold variation relatively to control) (Fig. 4B). According to these findings, FF can be effective in MSC survival and migration, because SDF-1/CXCR4 axis is also important for cell survival. The most crutial effect was detected in *TGF-β* gene analysis in the cells treated by 20% FF for 6 days (4.24±1.2 fold variation relatively to control) (Fig. 4C) ($p \leq 0.01$).

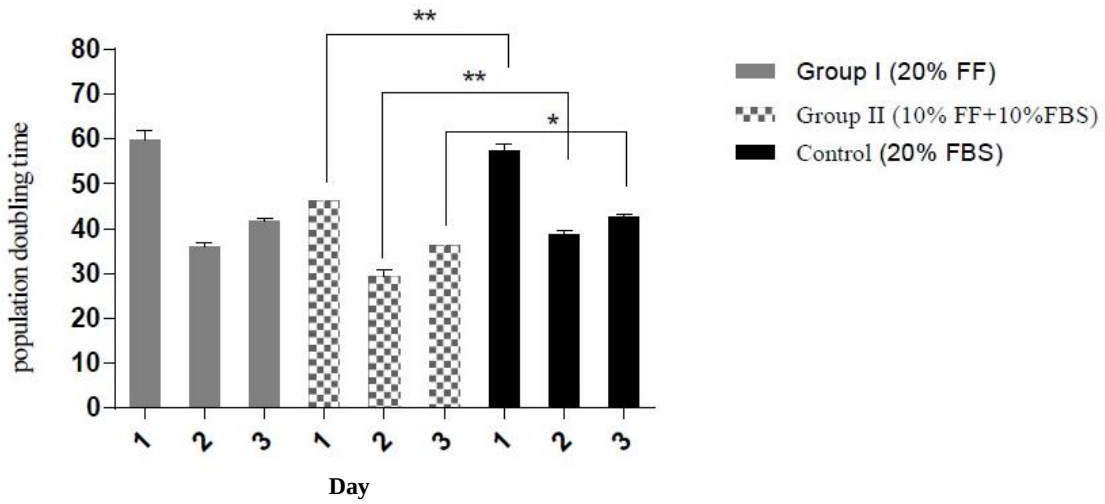

Fig. 2. Comparison of population doubling time between experimental and control groups: The mean population doubling times of bone marrow-derived mesenchymal stem cells cultured in the presence of (10 and 20%) FF for 3 consecutive days. FF promoted the proliferation of BM-MSCs in comparison with FBS. Statistical comparisons were performed using One-way ANOVA. The data are means ± SEM of three independent experiments.
FF=Follicular fluid; FBS= Fetal bovine serum; * $p \leq 0.05$; ** $p \leq 0.01$

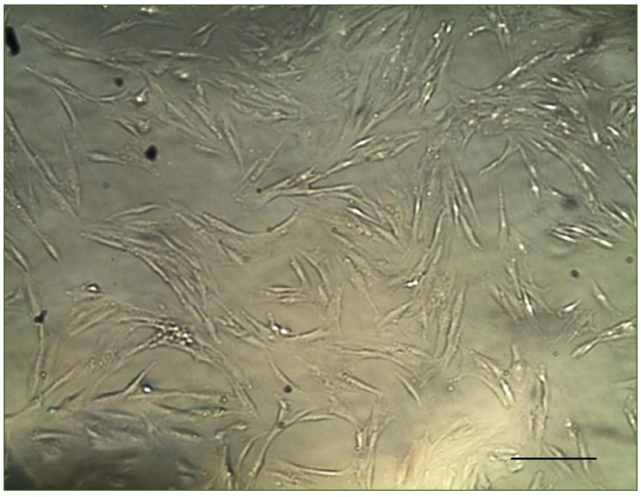

Fig. 3. Bone marrow-derived mesenchymal stem cells treated by follicular fluid. Scale bar: 50µm

Effect of FF on osteoblastic differentiation of BM-MSC

We performed alizarin red staining for those BM-MSCs that were cultured in osteogeneic media, to prove the osteoblast phenotype (Fig. 5) and also real-time PCR assays to detect the expression levels of osteoblast specific marker gene osteocalcin in the BM-MSCs treated by (10% and 20%) FF. As illustrated in Fig. 6, our results demonstrate a dose dependent function of FF on the stimulation of osteoblastic differentiation of BM-MSCs. We observed a higher level of osteocalcin mRNA expression in the cells treated by 20% FF compared to the second group (10% FF+10% FBS), (9.57±1.06 in 14th day and 5.98±0.63 in 21th day fold variation relatively to control). Fold variation in second group (10% FF+10% FBS) compared to the control was 2.54±0.27 in 14th day and 4.23±0.24 in 21th day ($p \leq 0.01$).

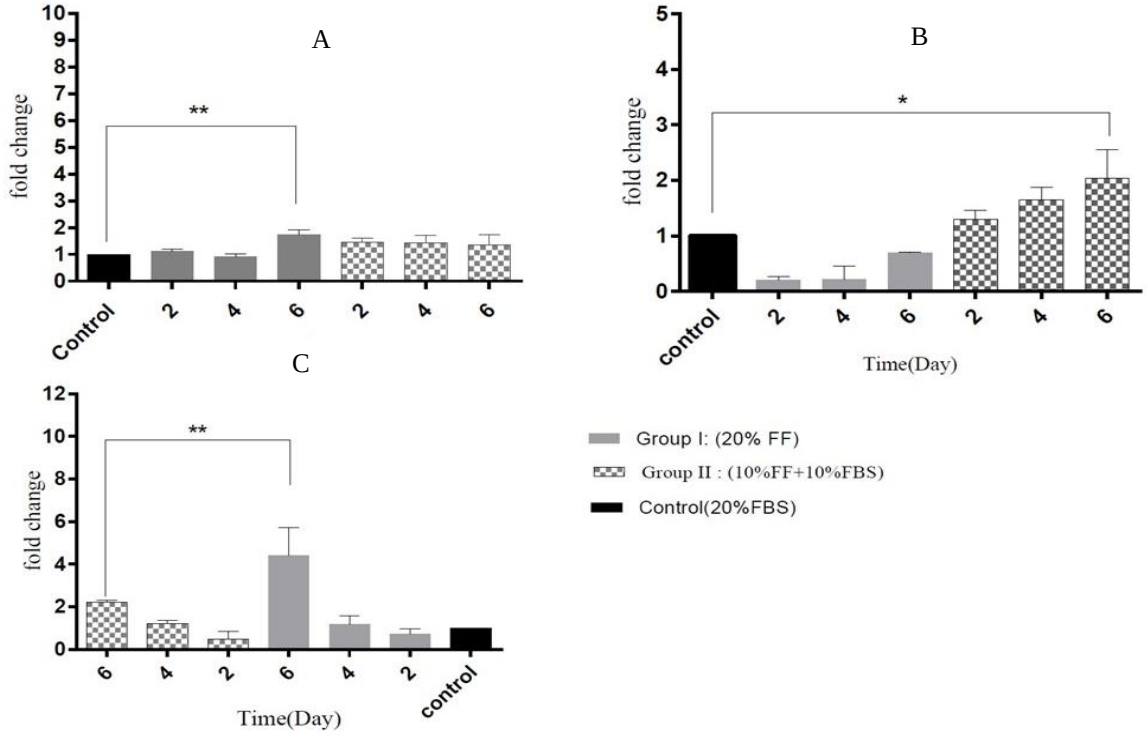

Fig. 4. A: Effect of FF on gene expression of, *SCF(A), SDF-1(B),TGF-β(C).* The data are means±SEM of three independent experiments ($^{*}p \leq 0.05$,** $p \leq 0.01$)

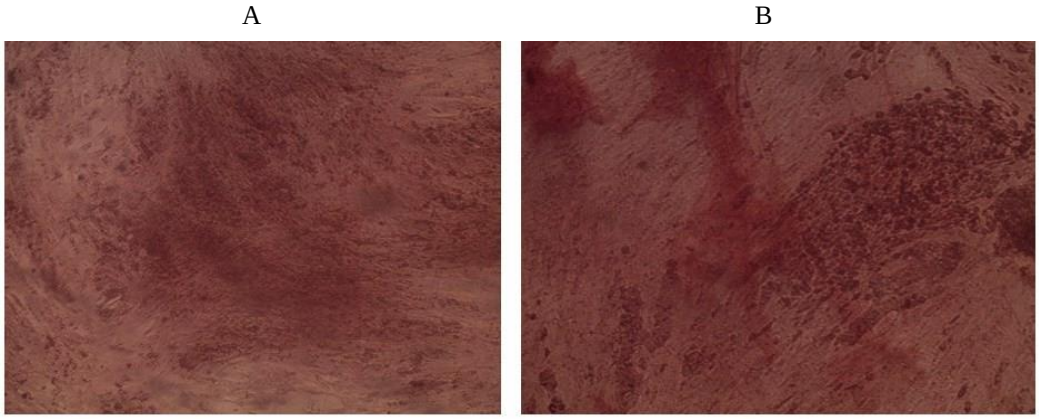

Fig. 5. *In vitro* osteogenesis differentiation of BM-MSCs cultured in osteogeneic media treated by 10% FF (A), 20% FF (B), examined by specific stain (alizarin red)

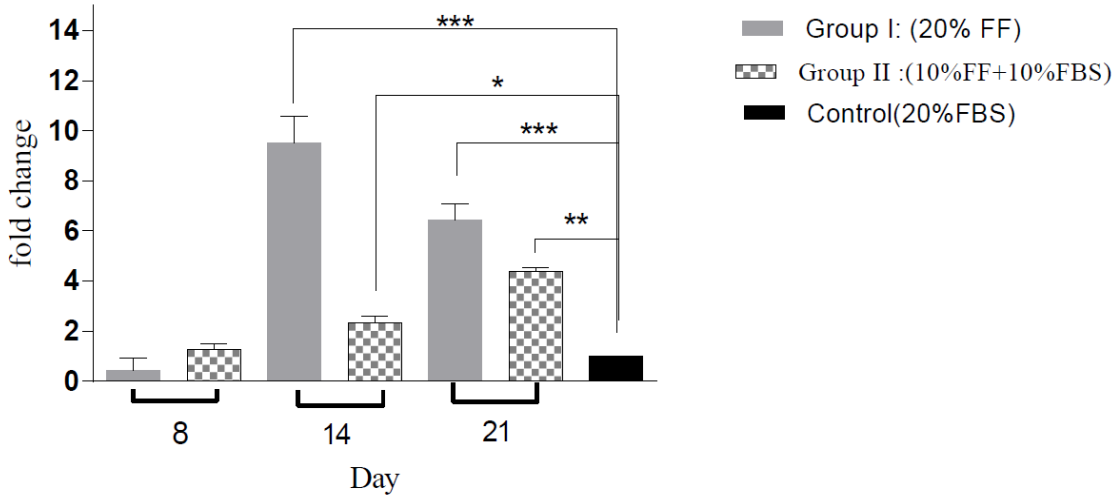

Fig. 6. Effect of FF on osteoblastic differentiation of BM-MSC (Acceleration of osteoblastic differentiation of BM-MSC by FF). Statistical comparisons were performed using One-way ANOVA. The data are means±SEM of three independent experiments. ($^{*}p\leq0.05$; $^{**}p\leq0.01$; $^{***}p\leq0.001$)

Discussion

Mesenchymal stem cells have recently been the focus of much investigations and have been successfully obtained from different sources, most commonly, the human and mouse bone marrow worldwide. Nowadays, there is a growing interest in application of BM-MSCs in regenerative medicine and tissue engineering [23, 24]. Regenerative medicine is a complex biological process to replace or repair defective or damaged tissues or organs by *in vitro* manipulation with *in vivo* use. It is a branch of tissue engineering which deals with stem cells with multipotent differentiating potential having the ability to induce the migration of stem cells to the damaged tissue thus leading to stimulation of their proliferation and tissue repair [3]. Recently some studies have been carried out on proliferation, differentiation, clonogenic capacity and gametogenesis of stem cells of different sources in the presence of FF which is not only rich in growth factors and other essential components, but is also compatible with human cells [21, 25, 26]. In this study, we, for the first time, have shown that BM-MSCs are capable of proliferation and osteoblastic differentiation in the presence of FF i.e., the fluid surrounding the ovum and granulosa cells in the ovarian follicle, containing: sex steroids, glycoprotein hormones, plasma proteins, cytokines and different enzymes. Among these substances, some have potential to either directly or indirectly influence on proliferation, self-renewability, differentiation and developmental potential of BM-MSC [27]. However, further investigation is needed to determine which factors control MSCs differentiation.

Our results revealed exacerbatory effects of FF on the proliferation of BM-MSCs. Shortened

PDT in the cells treated by FF confirmed its role in promoting cell proliferation, which has been anticipated previously, because of special components of FF such as proteins and growth factors [28, 29]. Similar results were shown by Qiu and colleagues in 2012 through evaluation the effect of bovine FF on UC-MSCs of goat [11].

In the present research, BM-MSC were also cultured in osteogeneic media for 21 days and gene expression level of osteocalcin as an osteoblast specific marker was evaluated 8, 14 and 21 days after induction with (10% and 20%) FF. Our results have shown that FF is more effective than FBS for expression of osteoblastic specific marker (osteocalcin) in BM-MSCs culture. This evidence confirmed the results of previous investigations which have shown that estrogen supports and promotes the osteoblastic differentiation of MSCs due to the increased expression of bone calcium and alkaline phosphatase [13-16].

On one hand, FF may plays an important role in promoting BM-MSCs survival rate and homing, because it has been shown in our study that higher concentration of FF in cell culture is associated with higher expression of *SCF* gene after 6 days, and on the other hand, it also plays a similar role in *SDF-1* gene expression in the presence of FBS, simultaneously.

The SCF/c-KIT system has been associated with the control of cell survival and proliferation through the regulation of several biological processes, and studies show that estrogens regulate their expression in different tissues. Accordingly, we investigated the effect of FF on *SCF* gene expression level of BM-MSCs; although significant variation has not been shown, it needs to be elucidated more [30, 31]. Furthermore, our results have shown that FF with FBS amplify the *SDF-1* gene expression level in BM-MSC, which is favorable for homing ability, according to the role of *SDF-1/CXCR4* in homing process [19]. However, our investigation offers a small step in the large area of stem cell therapy and more investigations are required to identify various aspects of FF as a supplementation in cell culture.

Conclusion

Collectively, our finding supports the stimulatory effect of higher concentration of FF on osteogeneic activity of BM-MSC and gene expression of osteocalcin whereas cell proliferation was induced by lower concentration of FF in cell culture. We recommend further investigations to define other aspects of using FF as a viable alternative to FBS for generating clinically significant numbers of explant derived MSCs for transfusion in cellular therapy.

Conflict of Interest

The authors have declared no conflicts of interest.

Acknowledgements

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