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Investigation the Effect of Serum Rich in Growth Factors on Proliferation, Growth and Expression of Genes Involved in Cell Longevity by Mesenchymal Stem Cells

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Abstract

Background: The limited lifespan of Mesenchymal Stem Cells (MSCs) has highly restricted their application. The Serum Rich in Growth Factors (SRGF) contains growth factors that are involved in processes of the growth and proliferation of MSCs. The present study was aimed to examine the regulatory effects of SRGF on the expression of some genes which effect both proliferation and lifespan of MSCs.

Methods: SRGF was obtained from platelets and MSCs were isolated from umbilical cord. The MSCs morphology and phenotype have been analyzed using phase-contrast microscope and flow cytometry, respectively. Cells were cultured either in presence of FBS 10% (as control) or SRGF 5% plus FBS 5% and FBS 10% alone (as tests). The cell Population Doubling Time (PDT) was measured hourly. The expression of related genes was analyzed employing real-time PCR technique.

Findings: Finding of the present study showed that the experimental groups were morphologically and phenotypically as similar as to control group. We observed that the PDT in the experimental group was shorter than that was found in the control. We have also found that the expression of hTERT and c-MYC genes was increased, while, and P16 and P53 genes were down- regulated. These results were superior in the 10%SRGF group than in the 5% SRGF + 5% FBS group.

Conclusion: According to the finding of this study, SRGF could possibly serve as an effective proliferative and lifespan inducing factor for MSCs. These results also indicated that SRGF has the tendency to be employed as an appropriated alternative for FBS in cell culture.

Keywords: Mesenchymal stem cells; SRGF; hTERT; c-MYC; P16

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Introduction

Mesenchymal Stem Cells (MSCs) are amongst non-hematopoietic cell types present in various tissues, varied from bone marrow and umbilical cord to adipose tissues [1]. The bone marrow mesenchymal stem cells were initially isolated by Friedenstein during mid-1970 decade [2]. MSCs are a heterogeneous subgroup of stromal stem cells presenting behaviours of non-hematopoietic multi-potential cells including the capacity of self-renewality which are able to differentiate into different types of mesodermal, ectodermal, and endodermal tissues, especially bone, fat, and cartilage. They phenotypically are fibroblastic or spindle-shape in culture media and adhere to the culture flask. Approximately, over 90% of MSCs express CD73, CD90, and CD105 whilst CD34, CD45, CD11b, CD14, CD19, CD79a, HLA-DR, CD80, and CD81 are expressed on less than 2% of MSCs membrane [3,4].

Several various applications have been demonstrated for MSCs, based on *in vitro* and *in vivo* studies, animal models, and clinical practices [5,6]. A large body of evidence exists regarding the efficiency of MSCs in regenerative medicine, tissue engineering, and immunotherapy [7,8]. The increased expression of anti-tumor genes is achieved by MSCs genetic modification and this in turn paves the path for successful application of MSCs as

anticancer therapies in clinical practice [9]. Simple *in vitro* culture is an advantage of MSCs, so that their seeding, maintenance, and proliferation becomes cost-effective, they are easily extracted from various sources, and their handling is a convenient task [10].

Despite all of the mentioned benefits and features, MSCs are not immortal and have limited life span *in vitro* [11,12], exhibiting aging processes accompanied by lower proliferation potential and morphological changes following a certain number of passage (about 6-12) [13,14]. To date, multiple various methods have been proposed for resolve in above-mentioned problems; however, they have their own advantages and disadvantages. Most studies have been undertaken to increase proliferation rate, life span, in addition to immortality of MSCs. These investigations have supported the transfection of genes involved in increasing proliferation and lifetime of MSCs [15]. As an instance, different studies have used viral methods to induce hTERT gene expression and inactivate P16 (cell proliferation inhibitor) gene, which are involved in the immortalization process and higher lifespan of MSCs. These methods contribute to the survival of virus inside the cell but are costly and time consuming [16]; therefore, studies have focused on non-viral methods.

LG-DMEM (Low Glucose-Dulbecco's Modified Eagle Medium) containing 10% FBS (fetal Bovine serum) and 1% Penicillin-Streptomycin is known as a conventional culture medium for mesenchymal stem cells for long time [17]. The Serum Rich in Growth Factors (SRGF) and platelet lysate (prepared by centrifugation of platelet products) are both able to provide good conditions for cell growth and affect the expression of genes involved in MSCs proliferation and longevity due to the fact that SRGF contains many growth factors and proteins [18,19]. Growth factors such as TGF-b, b-FGF, PDGF-A, B, and EGF that are all present in platelet granules play pivotal parts in stimulating of MSCs and epithelial cells proliferation [20].

Considering the paramount importance and prominent parts played by MSCs in cell therapy, hematopoietic stem cell transplantation, improvement of clinical symptom of various diseases, and their limited lifespan properties and benefits of SRGF, this study was aimed to investigate the SRGF impacts on the expression of c-Myc, P16, P53 and hTERT genes in MSCs, as major genes involved in proliferation and longevity of the MSCs isolated from the umbilical cord.

Materials and Methods

The present study was an experimental type which was performed at the Kerman University of Medical Sciences during year 2017.

Isolation of MSCs

The umbilical cord samples were collected from obstetrics and gynecology department of Afzalipour university Hospital Kerman. Iran and stored in PBS supplement with 1% antibiotics, specimenent were immediately transferred to the laboratory and cells where then separated under a septic condition complying a laminar flow hood. The harvested umbilical cords were gently rinsed, in order to remove the residual cord blood and further were cut into 1-2 mm pieces. Following washing steps, then supernatant was discarded and tissue fragments were cultured in a microbial culture plate containing DMEM medium (Gibco, England) supplemented with 15% fetal bovine serum (FBS) (Gibco, England), 1% penicillin/streptomycin (Gibco, England) and incubation at 37°C in a humidified atmosphere with 5% CO_2 was continued. The culture medium was replaced by freshly prepared medium following 48 hours and then the replacing procedure was performed every 2-3 days. Approximately two weeks later, when fibroblast-like cells had appeared, the tissue fragments were removed, cells were harvested using 0.25% Trypsin/EDTA (Gibco, England) and seeded on to a T25 flask (SPL, South Korea) for further expansion. At 70-80% confluence, cells were again passed into low-glucose DMEM containing 10% FBS and 1% penicillin/streptomycin. The resultant cells were used for experimented procedure at the passage-3. Following isolation and initial culture of cord blood and further 15 days, human MSCs began to aggregate around the fragments as well as attach to the bottom of the culture plates. A variety of stem cells was isolated from the primary tissue fragment, after 18 days. At the end of the day 18th and further removing of the tissue fragment and seeding of cells on to T25 flask, spindle-like cells homologue to fibroblasts started to grow and proliferate.

Characteristics of MSCs

To confirm the presence of MSCs isolated from the umbilical cord in culture, after growing of cells in a specific culture medium, the cellular morphological characteristics were examined by a phase-contrast microscope (Nikon, Japan). Within the next step, expression of specific markers on the cells membranes was examined. To examine this, initially the culture medium was removed and cells were subjected to after washing with PBS and were finally floated using 0.25% Trypsin/EDTA. The added trypsin was neutralized by FBS containing medium, cells were transferred to a 15 mL Falcon tube (Maxwell, South Korea) and centrifuged at 2000 rpm for 5 minutes. The resultant cell suspension was rinsed twice by PBS after discarding the supernatant. Cells were counted with a hemocytometer using light microscope and 100,000 cells were transferred either to the test or isotype control tubes for further examination. A volume of 5 µL monoclonal antibody from CD73-PE, CD105-PE, CD45-FITC, CD90-FITC, CD34-PE (BD Bioscience, USA) was added to the relevant test tubes, and 5 µL of IgG- FITC/PE (isotype control, BD Bioscience, USA) was also added to the negative control tube. Tubes were incubated at room temperature for 20 minutes. Finally, samples were examined by Flow Cytometery (BD FACS Calibur, USA) and were analyzed by Flomax software. To confirm the phenotypic identity of umbilical cord-derived MSCs, flow cytometric analysis was performed on cells at the third passage.

SRGF preparation

Four expired platelet bags were prepared from Kerman Blood Transfusion Center. The contents of bags were transfer into a 50 mL Falcon tube and subjected to centrifugation at room temperature whit 5000 rpm for 10 minutes. Following isolation the resultant supernatant was again gently transferred to another Falcon tube. The later step was repeated, and after transferring of supernatant to a fresh Falcon tube, the suspension was

centrifuged at room temperature with 12,000 rpm for 20 minutes to separate the PRGF. The supernatant was finally poured into a new Falcon tube and exposed to 1:10 calcium chloride at 15 mM, and was kept in a 37°C water bath for one hour. The formed clot was removed, the remaining yielded product was filtered and labeled as SRGF and was kept at -80°C for being used in further experiments.

Treatment of MSCs with SRGF and FBS

The culture medium was supplemented either with FBS 10%, SRGF 10%, or combination of SRGF 5%+FBS 5%. The MSCs were trypsinized and added to each flask, at a final cell density of 30%, where ever a confluency of 80% was achieved, The DMEM-low glucose medium supplemented with FBS 10% and antibiotic 1% was used as the control group. The DMEM-LG medium contained SRGF 10% and antibiotics 1%, in addition to SRGF 5%+FBS 5% and antibiotic 1% were used as the three different test groups. The first group of flasks was then incubated for 6 days in a humidified 37°C incubator with CO_2 5%. The second group was incubated for 6 days and was then freezed and thawed one month later. Finally, the morphology of cells in the two groups was further examined by phase-contrast microscope.

Population doubling time survey

To determine the PDT, the cells were counted by a hemocytometer and a number of 50,000 cells was added either to the control or test flasks. MSCs were digested by trypsin and the number of cells was calculated via counting, when the cellular density was closed to 80% of confluency. The initial number of cells (which was 50,000), their final number, and the time spent by the cells to reach the desired density were recorded and inserted in the following formula:

Doubling Time=duration × log(2)/log(Final Concentration)-log(Initial Concentration)

Accordingly, the relevant time of cell doubling for each group was thus separately calculated, and the results were statistically analyzed.

Gene expression analysis by quantitative real time PCR (Q RT-PCR)

The cellular expression of hTERT, c-Myc, P16, and P53 genes by MSCs was evaluated using QRT-PCR. To achieve this, the RNA content of cells was extracted in treated groups of FBS 10%, SRGF 10%, and SRGF 5% +FBS 5% guidelines using the unique TRizol solution (Yektatajhiz, Iran) according to the manufacturer's instructions. The quantity and quality of the extracted RNAs were evaluated by a Nanodrop device (thermo scientific, USA) and agarose 1.5% gel electrophoresis, respectively. The cDNA was synthesized by 80 ng of extracted RNA with Takara Bio kit (Primescript RT reagent) (TAKARA, Japan) in a final volume of 10 µL.

QRT-PCR was performed using the RealQ Plus 2x Master Mix Green (AMPLIQON Company) and the Rotor Gene 6000 Real-Time PCR Machine (Qiagen) in standard PCR conditions. The GAPDH gene was used as the reference gene or internal control for normalization of the data. Specific primers for the genes are listed. The expression level of the target genes was calculated as Relative Gene Expression using (2ΔΔ- CT) formula.

Statistical analysis

The SPSS 22-software was used for statistical analysis of the data. Data were presented as mean ± SD and analyzed by one way ANOVA (post HOC) for comparison between control and test groups. A $P<0.05$ was considered to indicate a statistically significant difference.

Results

The morphology of MSCs

MSCs morphological changes in response to SRGF treatment was examined by phase-contrast microscope. We have observed that the Morphology of MSCs was similar in control and test groups. MSCs were all spindle-shaped and fibroblast-like, which adhered to the plastic flask.

Immunophenotype analysis by flow cytometry

In this investigation our results indicate that the expression of CD90, CD105, and CD73 was elevated while hematopoietic markers such as CD34 and CD45 were down expressed on MSCs.

Population doubling time

Our findings showed that MSCs which were treated with SRGF 10% had shorter PDT than these MSCs that were treated with either SRGF 5% +FBS 5% or FBS 10%, alone.

Expression of hTERT, c-MYC, P16 and P53 genes

Treatment of MSCs was performed, when the cellular confluency has reached to 30%. RNA extraction and cDNA synthesis has been undertaken and followed by QRT-PCR. Our QRT-PCR- based findings demonstrated that SRGF was able to up-regulated the expression of hTERT and c-MYc gene while down-regulated the expression of P16 and P53 genes in an inverse fashion where by compared with FBS in MSCs.

Discussion

The MSCs are defined as a type of non-hematopoietic cell which presents in several tissues, including bone marrow, umbilical cord, adipose tissue and etc... [1]. Bone marrow is the most commonly used source of MSCs because these samples are autologous and contain a larger number of cells. However, harvesting of bone marrow is an invasive method posing the patient at severe pain and increase possibility of viral infections transmission [21]. Furthermore, the numbers of MSCs alongside with their differentiation potential are decreased with increasing of the donors age [3]. Therefore, finding of an alternative source for MSCs is highly deserved.

Evidence are in favor of the fact that MSCs could be isolated from various tissues such as adipose, periosteum, trabecular bone, synovial fluid, skeletal muscle, primary tooth, embryonic pancreas, lung, liver, peripheral blood, amniotic fluid, umbilical cord blood, Wharton's Jelly, and umbilical cord [22]. These days, umbilical cord is of the great interest, due to being easily available

non-invasive sampling, painless procedures, simplicity, and higher ethical acceptability considering MSCs isolation from a discarded tissue [23]. Umbilical Cord-MSC is an appropriate cellular source for research on differentiation and eventual clinical application in cell therapy due to easy access, non-invasive withdrawal method for donors, lower risk of viral infections, higher proliferation rate of MSCs as well as unique characteristics such as self-renewality and plasticity. Additionally umbilical cord is commonly considered as a clinical waste matter and does not involve ethical problems and is not restricted by sample preparation [24,25].

Thus, in the present study, the umbilical cord was employed as a source of MSCs with spindle fibroblast-like morphology. Flow cytometry analysis has demonstrated that these cells were positive for surface markers such as CD73, CD90, and CD105 but had poorly been expressed by HSC markers like CD34 and CD45, which is away confirming their mesenchymal phenotype.

Researchers have shown that mesenchymal stem cells are able to differentiate into mesoderm-derived cells (including bone, fat, and cartilage). Additionally, they have also the ability to differentiation toward cells with ectodermal (e.g. neuronal) and endodermal features (e.g. liver cells) *in vitro* [5,26]. Furthermore, mesenchymal cells are not only committed to produce a specific cell lineage, they also generate other cell lineages in response to environmental stimulation and signaling *in vitro*. In fact, this ability has increased their application in both research field and clinical state [27].

MSCs can partly contribute to the repair of the body's damaged tissues; however, due to the presence of small number of these cells in various tissues these cells don't play a pivotal part in processes of tissue repair. Regardingly, it is highly deserved to cultivate MSCs in the laboratory to increase their number for therapeutic demands. The problem that is associated with MSCs is their limited lifespan in the laboratory and entering into the aging phase after an average of 6-12 passages [13,14]. Julia Franzen and co-workers indicated that MSCs have limited *in vitro* lifespan and enter to the aging phase only after a few divisions and this in turn not only reduces the reproductive capacity, but it also causes changes in morphology, size, and potential for differentiation, thereby affecting their potential for being applied use in different therapies. However, the extraordinary features of MSCs should not be ignored, so different ways have been used to mask the imperfection of MSCs and increase their longevity [28].

The gene of hTERT encodes an enzymatic unit of telomerase (responsible for maintaining the length of telomeres) that serves as a preventing factor for aging and apoptosis of cells. The hTERT gene is not generally expressed in normal somatic cells of adult tissues; however, it is reactivated in more than 85% of cancers, which is considered as a main factor of cellular immortalization and tumor genesis [29]. The hTERT gene is not either expressed by MSCs or has a very low intensity on these cells [15]. Therefore, researchers have used viral methods to increase the expression of this gene. To overcome the limitation of MSCs lifespan within the culture medium, David S and co-workers have introduced the hTERT gene to these cells through lentiviruses vectors. Their results indicated that untransfected cells proliferated at a slower rate and ceased growth at passage 16 with a maximum Population-Doubling Level (PDL) of 14 but the cells transfected with the virus had a PDL of 70, while phenotype of transfected and untransfected cells and the differentiation power to fat and bone was maintained similar. They showed that transfected cells were able to preserve their own features; however, these cells have exhibited over expression of hTERT gene [30].

Other genes that are involved in the aging processes of MSCs include P16 and P53. These markers fit within the cyclic kinase inhibitors of cell cycle regulation and are widely considered as aging markers. These proteins are important components of the p53/p21WAF1 and p16INK4A/pRb tumor suppressor pathways, which play fundamental role in controlling of the growth halt of the old cells [28,31]. Izadpanah and colleagues have studied the effect of age on the features of adipose-derived MSCs and reported that aging has weakened the features while increased P16 and P53 gene expression by early MSCS and down-regulated the hTERT gene. Therefore, whereby these cells are applied in the clinic this important issue needs to be considered [32]. Similarly, Liu Tong Ming and colleagues have also surveyed the problem of limited number alongside with early aging of MSCs, and demonstrated that the down-regulation of P53 gene and simultaneous up-regulation of hTERT gene through si-RNA and lentivirus vectors contribute to evasion from the critical phase of cell division and prevent cell apoptosis and aging [14].

In addition to the pivotal parts of P53 and hTERT in processes of MSCs aging, the over expression of c-MYc as an oncogene also leading to elevated cellular proliferation and that in turn accelerates entering of MSCs into the S phase of cell cycle from the G1 [33].

In the present study we have examined the expression of hTERT, c-MYC, P16, and P53 genes by MSCs in response to FBS and SRGF separately and/or in combination. To overcome the MSCs' longevity problem various techniques have been introduced and each of them has its own disadvantages. In this study, we have used an expired platelet product called SRGF, which is obtained from platelets via centrifugation of their bags. Due to the presence of multiple growth factors and other mediators such as PDGF, FGF, EGF, VEGF, ECGF, TGFα, TGβ, this product plays pivotal parts in growth, proliferation, and differentiation of various cell types [34].

It has also been confirmed that SRGF is pivotally involved in processes of wound healing, tissue regeneration, and various medical fields, including dentistry, dental implants, orthopedics, and ophthalmology [35]. For example, Eduardo Anitua and colleagues in a study on dental implants in an animal model indicated that plasma Rich in Growth Factors (PRGF) was able to accelerate bone regeneration and improve bone attachment of a titanium dental implant [36]. Additionally, whereby the effects of PRGF and PRF (Plasma Rich in Fibrin) on gingival fibroblasts was compared, Surena Vahabi et al. it was found that addition of PRGF to the culture medium of gingival fibroblastic cells had exhibited a much stronger stimulatory effect on the proliferation and survival of cells [37]. Paknejad et al. have also conducted

a study on the repair and bone graft of rabbits, in process of PRGF and Deproteinized Bovine Bone Mineral (DBBM) or its absence (with DBBM but without PRGF as control). Paknejad and colleagues demonstrated that PRGF supplementation because both bone formation as well as its repair [38].

Accumulating evidences revealed that SRGF was able to appropriately provide good conditions for growth of cells due to richness in growth factors and proteins, which can affect the expression of genes involved in proliferation and longevity. One of the valuable advantages of SRGF over viral methods for prolonging lifespan and proliferation of cells is that it is inexpensive, does not cause other forms of viral infection, and is much faster than the vectors. The important point of this investigation is the fact that the expired platelet bags are used and they are economically feasible and cost-effective.

In this study, we have used SRGF alone (instead of FBS 10%) and in combination with FBS (FBS 5%+SRGF 5%) in two steps post 6 days (short-term culture) and following a month of freezing. Our results which were obtained from different groups showed that SRGF has significantly up-regulated the expression of hTERT and c-Myc genes and inversely down-regulated the expression of P53 and P16 genes. SRGF alone had a greater effect on the expression of studied genes after 6 days where by was compared with the FBS combination group. In other words the processes of freezing/thawing have not inhibited the effect of SRGF, and in all of genes, the impact of SRGF 10% was much higher than those of the combined groups. These results were also confirmed at cell culture stage, so that in the SRGF 10% group the doubling time of cells was remarkably attenuated compared to FBS combination group. Overall, PDT was decreased in both test groups compared to the control group, which confirms the effectiveness of SRGF. However, positive effects of SRGF on growth, proliferation, and development of MSCs should be further investigated. According to the results, it is worthy to note that either elevation or suppressing of the expression of genes involved in the proliferation and life span of mesenchymal stem cells can lead to carcinogenesis and uncontrolled cellular division and proliferation [39]. Therefore, more studies on controlling their growth are highly deserved. This issue was not investigated in this project but it should be investigated prior performing clinical trials.

Conclusion

SRGF which is derived from expired platelet bags could be used in cell culture similar to FBS due to its highly growth factor contents. Also, since SRGF has been able to significantly influence on the expression of longevity genes in this study, it may be possible to use this product to increase the lifespan of MSC.

Conflict of Interest

The authors declare that they have no conflict of interest. This article does not contain any studies with human participant or animals performed by any of the authors.

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Author's Contributions

R.M contributed to conception and design. F.H and M.S contributed to all experimental work, data and statistical analysis, and interpretation of data. R.M, A.F, A.F and G.H; were responsible for overall supervision and revision. All authors read and approved the final manuscript.

Ethical Statement

Collecting informed consent and conducting all the experimental processes were on the basis of the Ethical Committee of Kerman University of Medical Sciences' guidelines, Kerman, Iran; No: 95000420.

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