Research Article

Platelet Rich in Growth Factors (PRGF): A Suitable Replacement for Fetal Bovine Serum (FBS) in Mesenchymal Stem Cell Culture

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Abstract

Introduction: Due to high differentiation potential and self-renewality, Mesenchymal stem cells are now widely considered by researchers in several diseases. FBS is used as a supplement in culture media for proliferation, differentiation, and other culture processes of MSCs, which is associated with transmission risk of a variety of infections as well as immune responses. PRGF derived from platelets contains growth factors causing the growth and proliferation of MSCs. This study was conducted to compare the effect of PRGF in comparison to FBS on the expression of h-TERT gene, in umbilical cord-derived MSCs.

Materials and Methods: This study is an experimental research. Four expired platelet concentrate bags were obtained from Kerman blood transfusion center, and PRGF was prepared by multiple centrifugation rounds of the platelet bag. Calcium chloride was added as an anticoagulant to PRGF in order to prevent gelatinization of the culture medium. On the other hand, mesenchymal stem cells were isolated from the umbilical cord as a primary culture. The phenotype of the cells was confirmed by flow cytometry, and the cells were randomly cultured as control (using FBS) and experimental (using PRGF) groups. The expression of the gene involved in increasing cell longevity (h-TERT) was investigated by real-time PCR technique after six days.

Results: Mesenchymal stem cells were successfully isolated from the umbilical cord. Morphologically, the mesenchymal cells cultured in the experimental group (using PRGF) were similar to the cells in the control medium. The cells exhibited a high expression level of CD73, CD90, and CD105, while the surface markers of hematopoietic cells such as CD45 and CD34 were slightly expressed. Therefore, there was no significant difference in the expression of cell surface markers between control and experimental groups. In this study, using the real-time PCR technique, it was shown that PRGF derived from the platelet could increase the expression of h-TERT gene in the umbilical cord mesenchymal stem cells compared with the control. ($P = 0.034$).
**Conclusion:** PRGF have been shown to be effective in increasing expression of h-TERT gene in the umbilical cord mesenchymal stem cells and may also be an appropriate substitute for FBS in cell culture media.

**Keywords:** FBS, h-TERT, Mesenchymal stem cell, PRGF

1. Introduction

Mesenchymal stem cells (MSCs) are described as a type of non-hematopoietic cell, which are present in many tissues, including bone marrow, umbilical cord, adipose tissue, etc [1]. According to International Society for Cellular Therapy (ISCT) in 2007, MSCs have three unique features:

1. They are non-hematopoietic multi-potential cells with self-renewal and can differentiate into different types of mesodermal, ectodermal, and endodermal tissues, especially bone, fat, and cartilage.
2. They are fibroblastic or spindle-shaped in culture media and adhere to the culture vessel.
3. CD73, CD90, and CD105 are expressed in more than 95% of MSCs, while CD34, CD45, CD11b, CD14, CD19, CD79a, HLA-DR, CD80, and CD81 are expressed in less than 2% of MSCs [2].

Findings of in vitro and in vivo studies, animal models, and clinical practices have suggested different applications for MSCs [3, 4]. There are a large number of clues about the efficiency of MSCs in regenerative medicine, tissue engineering, and immunotherapy [5, 6]. Genetic modifications in these cells lead to increased expressions of anti-tumor genes, which pave the way for successful application of them as anticancer therapies in clinical practices [4, 7]. Simple in vitro cultivation is an advantage for MSCs, so that their maintenance, cultivation, and proliferation are cost effective, they are easily extracted from various sources, and their handling is a convenient task [1, 8].

Despite all the mentioned benefits and features, these cells are not immortal and they have limited life span in vitro [9, 10], and exhibit aging processes, which is accompanied by lower proliferation potential and morphological changes after a certain number of proliferations (about 6-12) [11, 12]. So far, various methods have been proposed to resolve the above-mentioned problems, each of which has its pluses and minuses. Most of the studies conducted to increase proliferation, life span, and even immortality of MSCs have supported the transfection of genes involved in increasing proliferation and life span of these cells [13]. For instance, different studies have used viral methods to induce h-TERT gene expression and inactivate P16 (cell proliferation inhibitor) gene, which are involved in the immortalization process and increasing life span. These methods contribute to the survival of the virus inside the cell; also, they are costly and time consuming [14]; therefore, studies have focused on non-viral methods.
The conventional method for the cultivation of mesenchymal stem cells is the use of LG-DMEM (Low Glucose-Dulbecco’s Modified Eagle Medium) containing 10% FBS (fetal Bovine Serum) and 1% Penicillin-Streptomycin [15]. The use of FBS leads to the transfer of infectious agents to humans [16]. Platelet Rich In Growth Factors (PRGF) and platelet lysate prepared by centrifugation of platelet products can provide good conditions for cell growth and affect the expression of genes involved in proliferation and longevity because it contains many growth factors and proteins [17]. Growth factors such as TGF-b, b-FGF, PDGF-A, B, and EGF in platelet granules play a role in stimulating the proliferation of MSCs and epithelial cells [18].

Therefore, considering the problems and costs of FBS, as well as reports on positive effects of PRGF-containing medium on growth, multiplication, and differentiation of mesenchymal stem cells, this study aimed to compare the effect of PRGF and FBS on the expression of the h-TERT gene, which is a major gene involved in proliferation and longevity of the mesenchymal stem cells isolated from the umbilical cord. This research was conducted in the Department of Hematology and Laboratory Sciences, Faculty of Paramedical Sciences, Kerman University of Medical Sciences in 2017.

2. Materials and Methods

The present study was carried out experimentally.

2.1. Collection and isolation of MSCs

The umbilical cord sample from obstetrics and gynecology department of Afzalipour Hospital was collected, stored in PBS, and immediately transferred to the laboratory. Separation steps were performed under the laminar flow hood under sterile conditions. The umbilical cord was washed to remove the residual cord blood. Then, it was cut into 4-5 cm long pieces. The pieces were placed in a microbial culture plate and minced into 1-2 mm fragments with a surgical blade. The fragments were placed inside a 50-ml Falcon tube (Maxwell, South Korea) containing PBS, and two centrifuge rounds were carried out at a rate of 1750 RPM for 7 minutes. Then, tissue fragments were cultured in a microbial culture plate containing DMEM medium (Gibco, England), 15% fetal bovine serum (FBS; Gibco, England), and 1% penicillin/streptomycin (Gibco, England), which was incubated at 37 °C in a humidified atmosphere with 5% CO2. The culture medium was changed 48 hours later, and it was then changed once every 2-3 days. Approximately two weeks later, when fibroblast-like cells had appeared, these tissue fragments were removed, the cells were harvested using 0.25% Trypsin/EDTA (Gibco, England), and then seeded in the T25 flask (SPL, South Korea) for further expansion. At 80-90% confluence, the cells were passed into low glucose DMEM containing 10% FBS and 1% penicillin/streptomycin. The cells at passag 3 were used for the experiment.
2.2. Characteristics of MSCs

In order to confirm the MSCs isolated from the umbilical cord, after the growth of cells in a specific culture medium, the morphological characteristics of them were examined using a phase-contrast microscope (Nikon, Japan). In the next step, the presence of specific markers on the cells was examined. For this purpose, the cell culture medium was first removed. After washing the cells with PBS, the cells were floated using 0.25% Trypsin/EDTA. The added trypsin was neutralized with FBS medium; the cells were transferred to a 15-ml Falcon tube (Maxwell, South Korea) and centrifuged at about 2000 rpm for 5 minutes. After discarding the supernatant, the cell suspension was washed twice with phosphate buffer. After counting the number of cells with a hemocytometer, 100,000 cells were transferred to each test tube as well as the isotype control tube. 5 μl of monoclonal antibodies, including CD73-PE, CD105-PE, CD45-FITC, CD90-FITC, CD34-PE (BD Bioscience, USA) were added to the test tubes, and 5 μl of IgG- FITC/PE (isotype control, BD Bioscience, USA) was added to negative control tube. The tubes were placed at room temperature for 20 minutes. Finally, the samples were examined by Flow Cytometry (BD FACSCalibur, USA) and were analyzed by Flomax software.

2.3. PRGF preparation

Expired platelet bags were prepared from Kerman Blood Transfusion Center. The contents of the bags were poured into a 50-ml Falcon tube and centrifuged at room temperature at 5000 rpm for 10 minutes, and the supernatant was gently transfer to another Falcon. This step was repeated, and after transfer of the supernatant to a new Falcon tube, the suspension was centrifuged at room temperature at 12,000 rpm for 20 minutes to separate the PRGF. Finally, the supernatant was poured into a Falcon, exposed to 1:10 calcium chloride at 15 mM, and placed for 1 hour in a 37 °C water bath. The formed clot was removed; the remaining product was filtered, and finally maintained at -80 °C until used.

2.4. Exposure of MSCs to PRGF and FBS

At this stage, the culture medium was supplemented with 10% FBS and 10% PRGF separately. Thus, when MSCs reached 80% confluency, they were trypsinized and added to each flask so that the final cell density of 30% was achieved. The final volume of each flask was 4 ml. For this purpose, the DMEM-low glucose medium with 10% FBS and 1% antibiotic was added to the control group, and DMEM-LG medium with 10% PRGF and 1% antibiotic was added to the test group. The flasks were then incubated for 6 days in a 37 °C incubator with CO2. After that, the morphology of cells was examined by phase-contrast microscope.
2.5. Investigation of h-TERT gene expression with Real-time PCR

The cells were evaluated for the expression of h-TERT gene. For this purpose, the RNA of the cell was extracted in two groups of 10% FBS and 10% PRGF using the unique TRizol solution (Yektajihiz, Iran) according to the manufacturer’s instructions. The quantity and quality of the extracted RNA were evaluated by a Nanodrop device (thermo scientific, USA) and 1.5% agarose gel electrophoresis, respectively. Then, 80 ng of extracted RNA was used to synthesize cDNA in accordance with Takara Bio kit instructions (Primescript RT reagent) in a final volume of 10 μL.

Real-Time 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. In this method, in order to normalize the data, the GAPDH gene, was used as reference gene or internal control. Specific primers for the reference gene and the h-TERT gene are listed in Table 1. The expression level of target gene was shown as Relative Gene Expression using the formula \(2^{-\Delta\Delta C_t} \).

2.6. Statistical analysis

All statistical analyses were performed using SPSS 22 software. The data were presented as mean ± SD, and analysed by independent t test, for comparison between control and test groups. \( P < 0.05 \) was considered to indicate a statistically significant difference.

3. Results

3.1. Morphological features/characteristics

Following the separation of umbilical cord tissue and its initial culture in a microbial culture plate after 15 days, human MSCs began to isolate around the fragments and stick to the bottom of the plate. After 18 days, a large number of stem cells were isolated from the primary tissue fragment. At the end of day 18, after removing the tissue fragment and seeding the cells in the T25 flask, spindle-like cells similar to fibroblasts began to grow and proliferate (Figure 1).
3.2. Immunophenotype analysis by flow cytometry

In order to confirm the phenotypic identity of the umbilical cord-derived MSCs, flow cytometric analysis was performed on cells of passage 3 in this study. The results showed a high expression of CD90, CD105, and CD73 markers but a low expression of CD34 and CD45 markers (Figure 2), (Table 2).

<table>
<thead>
<tr>
<th>Cell surface marker</th>
<th>CD90</th>
<th>CD73</th>
<th>CD105</th>
<th>CD34</th>
<th>CD45</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marker expression percentage</td>
<td>97.91%</td>
<td>95.31%</td>
<td>99.07%</td>
<td>0.06%</td>
<td>0.15%</td>
</tr>
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</table>

3.3. Expression of h-TERT gene

When cell confluency was about 30%, treatment of MSCs was performed in both control and test groups, and when their confluency reached 90% (day 6), extraction of RNA and cDNA synthesis were done and Real-Time PCR reaction was performed. Real-Time PCR reaction was shown that PRGF derived from the platelet could increase the expression of h-TERT gene in the umbilical cord mesenchymal stem cells (Chart 1).

4. Discussion

Mesenchymal stem cells (MSCs) are described as a type of non-hematopoietic cell present in multiple tissues such as adipose tissue, periosteum, trabecular bone, synovial fluid, skeletal muscle, primary tooth, embryonic pancreas, lung, liver, amniotic fluid, umbilical cord blood, Wharton’s Jelly, and the entire umbilical cord [19]. Today,
Figure 2: Immunophenotypic characteristics of the umbilical cord MSCs. A: Distribution of cell size and granularity, B: Control isotype of PE (fl1), C: Isotype of FITC (fl2) control, D: Expression of the CD105, E: CD73, and F: CD90 antigens. G: Extremely low expression of CD45, and H: CD34 antigens confirming MSCs phenotype. In the graphs above, the horizontal axis represents the intensity of conjugate color (FITC, PE) associated with antibodies of interest. The vertical axis represents the number of cells. The RN1 and RN2 lines represent the marker-labeled cells identified by antibodies indicated with different fluorescent materials.

Umbilical cord is of great interest because of easy access, non-invasive sampling, painless procedures, simplicity, and higher ethical acceptability considering that the isolation is performed on a discarded tissue [20]. Umbilical Cord-MSC is a more appropriate option for research on differentiation and eventual clinical use in cell therapy due to easy access, non-invasive withdrawal method for donors, lower risk of viral infections, higher proliferation capacity compared to other mesenchymal stem cells as well as unique characteristics such as self-renewal and plasticity compared with other mesenchymal stem cells. Also, since umbilical cord is commonly considered as clinical waste matter, it does not involve ethical problems and has not the limitation of sample preparation [21, 22].

Therefore, in this study, the umbilical cord source has been used for preparation of mesenchymal stem cells with spindle fibroblast-like morphology. Flow cytometry
Gene expression in the FBS and PRGF groups. The expression of h-TERT gene in the PRGF group was significantly (*P=0.034) higher than the FBS group.

Recent researches have shown that mesenchymal stem cells are capable of differentiation into mesoderm-derived cells (including bone, fat, and cartilage). They are also capable of differentiation into cells with ectodermal (e.g., neuronal) and endodermal features (e.g., liver cells) in vitro [3, 23]. Mesenchymal cells are committed to give rise to a specific cell lineage or specific series of cell lineages, but are capable of producing other cell lineages through in vitro artificial stimulation and signaling. Therefore, this ability has increased their use in research and clinical education [24].

MSCs partly contribute to the repair of damaged tissues in the body, but because of the small number of these cells in various tissues of the body, they cannot play an effective role in tissue repair. Therefore, it is necessary to cultivate them in the laboratory and increase their number in order to be used in the treatment. Nevertheless, the problem is that MSCs have a limited lifespan in the laboratory and enter the aging phase after an average of 6-12 passages [11, 12].

For laboratory culture of mesenchymal stem cells, the DMEM-LG medium is usually enriched with 10% FBS, because it is easily produced (as a substitute in the process of slaughtering pregnant cows) and is also rich in growth factors that can support the growth and multiplication of many cell lines [25]. However, along with these benefits, the FBS has some drawbacks, including the fact that many of its compounds have not yet been identified and their effects are still unknown [26]. On the other hand, high levels of endotoxin in the FBS have undermined its safety [27]. FBS may also stimulate immunological response against animal-derived serum antigens [28].

Spees et al. (2004) showed that FBS could provoke immune response even with the first cell inoculation in patients [29]. Some studies have reported anaphylactic shock and anaphylaxis reactions in patients undergoing cell proliferation in presence
of FBS [30]. FBS is also a potential source of microbial, fungal, bacterial, viral, and prion infections [16]. In addition to the unaffordability of FBS and scientific concerns associated with it, acceptable methods for FBS preparation, including drawing blood from a fetal bovine animal, endanger the animal and cause pain and discomfort [31], while modern studies should be minimize animal suffering.

For the above reasons, researchers have focused over recent years on various human protein combinations that can be used as an alternative to FBS for cell proliferation and differentiation. It is preferable to provide the supplements to human culture media from human sources since they are to be used in human subjects. One of the human products used in the culture medium is called PRGF, which is obtained by centrifugation of platelets. Due to the presence of growth factors such as PDGF, FGF, EGF and so forth, this product plays an effective role in the growth, proliferation, and differentiation of cells [32, 33].

In 2009, Eduardo Anitua and colleagues in a study on dental implants in an animal model concluded that PRGF accelerated bone regeneration and improved bone attachment of a titanium dental implant [34]. In addition, comparing the effects of PRGF and PRF (Plasma Rich in Fibrin) on gingival fibroblasts, Surena Vahabi et al. in 2015 found that the addition of PRGF to the gingival fibroblastic cell culture medium had a much stronger stimulatory effect on the proliferation and survival of these cells [35]. Paknejad et al. also conducted a study in 2012 on the repair and bone graft of rabbits. In this study, they added PRGF together with deproteinized bovine bone mineral (DBBM) for transplantation, compared it with the control group (DBBM without PRGF), and observed that the addition of PRGF increased the rate of bone formation and repair [36].

Therefore, in our study, PRGF was used in cell culture of umbilical cord mesenchymal stem cells instead of FBS, and the effects of these two growth supplements on h-TERT gene expression were investigated. The h-TERT gene encodes an enzymatic unit of telomerase, an enzyme responsible for maintaining the length of telomeres and a main factor in preventing aging and apoptosis in the cell. The h-TERT gene is not expressed in normal somatic cells of adult tissues; however, it is reactivated in more than 85% of cancers and is considered as a main factor in the process of cellular immortalization and tumorigenesis [37]. This gene is not expressed in MSCs or has a very low expression in these cells [13]. In this study, the expression of h-TERT gene increased 14 fold under the influence of PRGF compared with FBS.

5. Conclusion

PRGF derived from expired platelet bags can be used in cell culture media similar to FBS due to its high growth factors. Also, since PRGF has been able to significantly increase the expression of the h-TERT gene in this study, it may be possible to use this product to increase the MSC’s lifespan.
Acknowledgments

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Conflict of Interest

The authors have not announced any conflicts of interest

References


