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Research Article

Platelet-Derived Microparticles Increase Expression of hTERT in Umbilical Cord Mesenchymal Stem Cells

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Abstract

Introduction: Mesenchymal stem cells (MSCs) are widely studied due to their self-renewal potential and capacity to differentiate into multiple tissues. However, they have a limited life span of several divisions *in vitro*, which alters various cellular characteristics and reduces their application.

Aim: We evaluated the effect of platelet-derived microparticles on gene expression of hTERT, one of the main factors involved in aging and cell longevity.

Materials and methods: Umbilical cord MSCs were used for this study. Cells were characterized by evaluating morphology via inverted microscope and identifying associated surface markers using flow cytometry. Platelet-derived microparticles were prepared by centrifuging platelet bags at varying speeds, and their concentrations were determined by Bradford assay. At 30% confluency, MSCs were treated with 50 µg/mL of microparticles for five days. Then, RNA was extracted and cDNA was synthesized. Quantitative expression of hTERT was assessed using real-time polymerase chain reaction (PCR).

Results: Fibroblast-like cells were isolated from umbilical cord tissue and MSCs were identified by the presence of mesenchymal surface markers via flow cytometry. Real-time PCR showed that gene expression of hTERT increased by more than three times when treated with platelet-derived microparticles, in comparison to expression of the control group.

Conclusion: We concluded that platelet-derived microparticles may be a potentially safe and effective method to increase hTERT gene expression in MSCs, ultimately prolonging their life span *in vitro*.

Keywords: Cell-Derived Microparticle, Mesenchymal Stem Cell, Platelets

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1. Introduction

Mesenchymal stem cells (MSCs) are non-hematopoietic, multipotent cells with self-renewal potential and the capability to differentiate into mesodermal cells, such as

bone, cartilage, and fat [1–3]. In addition, recent studies indicate that MSCs are capable of differentiating into ectodermal and endodermal layers, such as liver and neurons [4]. These characteristics make MSCs a promising tool to study regenerative medicine and tissue engineering [5, 6]. However, isolating and obtaining adequate numbers of cells from various sources of tissues is difficult; therefore, *in vitro* tissue culture of MSCs is indispensable [7].

MSCs are not immortalized cell lines and are subject to aging after several passages) approximately 6-12) in tissue culture. This phenomenon is accompanied by reduced proliferation and differentiation potential, alteration in morphology, and significant changes in the expression of genes involved in proliferation and miRNA [2, 4]. Consequently, these changes reduce the efficacy of MSCs and their applications in research and clinical use. While there are many factors that induce aging in MSCs, telomerase reverse transcriptase (hTERT) in humans is the most important one [8]. Expression of hTERT is typically silenced in differentiated, normal cells, whereas it is hyperactivated in many cancers, contributing to cell immortality and carcinogenesis [9]. It has been determined that the short life span of MSCs is caused by loss of telomerase activity due to low expression of hTERT; thus, telomere lengths in MSCs decrease with each successive passage [8]. Shortening of telomere length is a characteristic event of a finite life span [5]. The clinical applications of MSCs in regenerative medicine and cell therapy is limited due to the dramatic changes in the morphology that arise from aging of MSCs [9, 10]. Therefore, it is essential to develop new methods of tissue culture that will overcome the aging of MSCs *in vitro*. Recent studies employed the use of lentiviruses and retroviruses to transfer genetic factors involved in proliferation, such as hTERT, into MSCs [5, 11]. However, pathogenicity, toxicity, and survival of the virus in living cells, as well as expensive costs associated with these methods, have been identified as substantial problems [2, 12]. Consequently, in this study, we evaluated the effect of platelet-derived microparticles as a non-viral, safe, and effective method of enhancing gene expression of hTERT in MSCs to prolong cell longevity.

Cell-derived microparticles are plasma membrane vesicles, ranging from 0.05-1 μm in size, that are released by many cells, including platelets, white blood cells, endothelial cells, and red blood cells. Approximately 70-90% of cell-derived microparticles are released by platelets into biological fluids, such as the blood stream. The release of microparticles (MPs) from cell membranes increases upon activation by apoptosis or other stimuli, such as stress [13–15]. Platelet-derived microparticles (PMPs) contain NADP, growth factors, oxidases, chemotactic factors, and other essential cellular components [13]. In addition, they mediate shuttling of active molecules, such as lipids, enzymes, and growth factors, to receptor cells. Furthermore, it has been postulated that these PMPs may contain mRNA and miRNA [16]. Essentially, PMPs play many roles in cell-cell interactions, including immunomodulation, inflammation, vascularization, vascular repair, and most importantly, cell survival and death [17–19]. This study aimed to investigate the effect of platelet-derived microparticles, as carriers of growth factors and miRNAs, on gene expression of hTERT and assess the potential of platelet-derived microparticles to increase the longevity of MSCs.

2. Materials and Methods

This experimental study was carried out in Paramedical School of Kerman University of Medical Sciences.

2.1. Isolation of MSCs

Umbilical cord tissue sample was randomly collected from the Obstetrics and Gynecology Department at the Afzalipour Hospital of Kerman, with written consent from donor. Under sterile conditions, the umbilical cord sample (20 cm) was cut into small pieces (1-2 cm) with a surgical scissor and blade. Umbilical cord segments were washed with phosphate buffered saline (PBS) two or three times to remove red blood cells. Umbilical cord segments were added to 10 cm plates and incubated with 15 mL of low-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, England), supplemented with 15% fetal bovine serum (FBS; Gibco, England) and 1% penicillin-streptomycin (Gibco, England). Tissue samples were stored at 37°C with 5% CO_2. After four days, non-adherent cells were removed by replacing culture media with fresh DMEM, supplemented with 10% FBS. Thereafter, culture media was changed every two days until cells reached 80-90% confluency. After approximately two weeks when fibroblast-like cells appeared, umbilical cord tissue fragments were removed from the plates. Cells were harvested using 0.25% Trypsin/EDTA (Gibco, England), and then, seeded in T25 flask (SPL, South Korea) for further expansion. At 80-90% confluency, cells were passaged into low-glucose DMEM, containing 10% FBS and 1% penicillin/streptomycin. Cells harvested at passage 3 that displayed a homogenous MSC phenotype were used for the study.

2.2. Morphological analysis of MSCs

Cells isolated from umbilical cord tissue were examined using a phase-contrast microscope at each passage (Nikon, Japan). The morphological characteristics were assessed to identify whether the cells were MSCs.

2.3. Flow cytometry of MSCs

Cells at the third passage were harvested for flow cytometry to validate their mesenchymal immunophenotype. After trypsinization, cells were suspended in PBS. Cell suspensions (100 µL) was added to solutions (10 µL) of mesenchymal markers, CD73-FITC, CD105-PE, and CD90-PE, and hematopoietic markers, CD34-PE and CD45-FITC (BD Bioscience, USA). In addition, an isotype control against murine cells was included to identify non-specific binding. Tubes were incubated at room temperature for 20 minutes. BD FACSCalibur (USA) and Flomax software were used to measure and analyze flow cytometry data, respectively.

2.4. Preparation of microparticles

Expired platelet concentrate bags were obtained from the Kerman Blood Transfusion Center, the contents were transferred to falcon tubes under sterile conditions. Tubes were centrifuged at 5,000 rpm for 10 min to separate and remove red and white blood cells. The supernatant was transferred to new tubes and the previous step was repeated to remove any remaining cells. Next, the supernatant was centrifuged at 18,000 rpm for 30 min to sediment platelet-derived microparticles. Pellets were dissolved in DMEM (1 mL) and stored at -80°C until experimental use.

2.5. Determination of microparticle concentration by Bradford assay

Protein concentration of platelet-derived microparticles was determined by Bradford assay, using a NanoDrop spectrophotometer (WPA, UK). Briefly, varying dilutions of bovine serum albumin (BSA) were prepared (312.5 µg/mL, 625 µg/mL, 1250 µg/mL, 2500 µg/mL, 5000 µg/mL, 10000 µg/mL). BSA (10 µL) or microparticle samples were mixed with Bradford reagent (200 µL) and then, absorption was measured at 595 nm. A standard curve was drawn using the BSA samples and used to determine the concentration of microparticles.

2.6. Treatment of MSCs with platelet-derived microparticles

At 80% confluency, MSCs were trypsinized. The number of cells was counted by Neubauer slide, and then, cells (50,000) were seeded into 25 cm^2 flasks and incubated with low-glucose DMEM, supplemented with 10% FBS and 1% penicillin/streptomycin.

MSCs in the treatment group were treated with 50 µg/mL platelet-derived microparticles, while the control group was treated with complete media. Culture flasks were incubated at 37°C and 5% CO_2 for five days. Morphology of the cells was assessed using an inverted microscope. After five days, RNA was extracted from cells and cDNA was synthesized. The gene expression of hTERT was evaluated by real-time PCR.

2.7. Quantitative analysis of hTERT gene expression using real-time PCR

RNA extraction was performed using Trizol (Yektatajhiz, Iran). Quantitative and qualitative assessments of the extracted RNA samples were conducted via NanoDrops and electrophoresis on agarose gel (1.5%), respectively. Then, cDNA was synthesized from RNA samples (300 ng), according to the Primescript RT reagent (TaKaRa) protocol. The synthesis temperature program comprised of two steps: 37°C for 15 min and 85°C for 5 s. The primers used in the real-time PCR reactions for the amplification of hTERT and GAPDH (control) are outlined in Table 1. Real Q Plus 2x Master Mix Green kit and Qiagen thermocycler were used. Gene expression data was analyzed using the $2^{-\Delta\Delta CT}$ formula.

Table 1: Primer sequences and thermal profile of hTERT and GAPDH genes utilized for real-time PCR.

Thermal profile	Reverse primer	Forward primer	Gene
95 °C/30s 60 °C/60s	5′-CACTGTCTTCGACAAGTTCAC-3′	5′- TGACACCTCACCTCACCCAC-3′	hTERT
	5′- GAAGATGGTGATGGGATTTC-3′	5-′GAAGGTGAAGGTCGGAGTC -3′	GAPDH

2.8. Statistical analysis

Statistical analysis was performed using SPSS 20 software. Comparison of gene expression between the two groups was performed using the paired sample *t*-test, and P value < 0.05 was considered statistically significant.

3. Results

3.1. Morphological characterization of MSCs derived from umbilical cord

MSCs derived from umbilical cord samples demonstrated the capacity to adhere to culture flasks and were fibroblast-like in appearance during the initial days of incubation. After three passages, red blood cells were seldom observed under a light microscope, and cells gradually grew into small colonies (Figure 1).

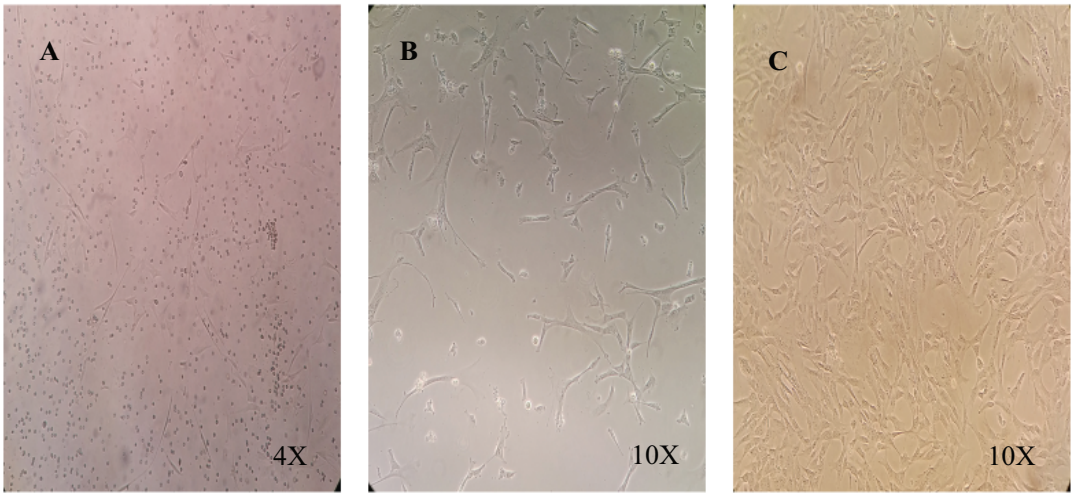

Figure 1: Morphological analysis of mesenchymal stem cells isolated from umbilical cord tissue. (A) Initial detachment of cells, (B) Proliferation and expansion of mesenchymal stem cells, (C) Density of cells with fibroblast-like morphology.

Table 2: Expression percentages of mesenchymal stem cell surface markers.

CD45	CD34	CD105	CD73	CD90	Cell surface marker
1.47%	1.51%	99.5%	99.72%	99.80%	% expression

3.2. Immunophenotypic characterization of MSCs derived from umbilical cord

MSCs characteristically express mesenchymal surface markers, such as CD105, CD73, and CD90, while they lack expression of hematopoietic markers, such as CD34 and CD45. Consequently, cells isolated from umbilical cord samples were validated as MSCs by their expression or non-expression of specific surface markers. Flow cytometry using BD FACSCalibur was performed to confirm the immunophenotype of the MSCs. As illustrated in Table 2 and Figure 2, more than 99% of the cells were positive for CD105, CD90, and CD73, whereas there was no apparent expression of CD34 and CD45. Therefore, the cells isolated from umbilical cord samples were confirmed as MSCs.

3.3. Evaluating gene expression of hTERT in treated MSCs

MSCs were treated with 50 µg/mL platelet-derived microparticles for five days. RNA samples were extracted from the treatment and control groups, and real-time PCR was conducted to analyze gene expression of hTERT. In cells treated with platelet-derived microparticles, gene expression of hTERT was three-fold higher than expression in the untreated, control group (Figure 3).

4. Discussion

MSCs can be extracted from various tissues, such as bone marrow, fat, umbilical cord blood (UCB), umbilical cord, cartilage, and dental pulp [1, 2]. Amongst these sources, the umbilical cord is often used in research studies due to easy accessibility, non-invasiveness, and painless sampling from donors [20]. Moreover, the ethical concerns are less controversial because umbilical cord samples are discarded tissues [21, 22]. In addition, multiple reports show that cells extracted from UCB demonstrate longer life spans, shorter population doubling times (PDT), and higher proliferation potential [22, 23]. Thus, in this study, we chose to use umbilical cord as the source from which to extract MSCs.

It has been reported that MSCs show a decline in differentiation potential and proliferation rate with increasing passage *in vitro*. Leonard Hayflick first postulated in 1960 the limited life span and aging phase of MSCs, which has since been demonstrated to pose limitations in their clinical applications [24]. Molecular events, such as shortening of telomere length and altered TERT expression, are associated with cell senescence. Studies show a correlation between the proliferative capacity of MSCs and telomere length, both in *in vitro* culture and aged donor cells [23]. Furthermore, it has been

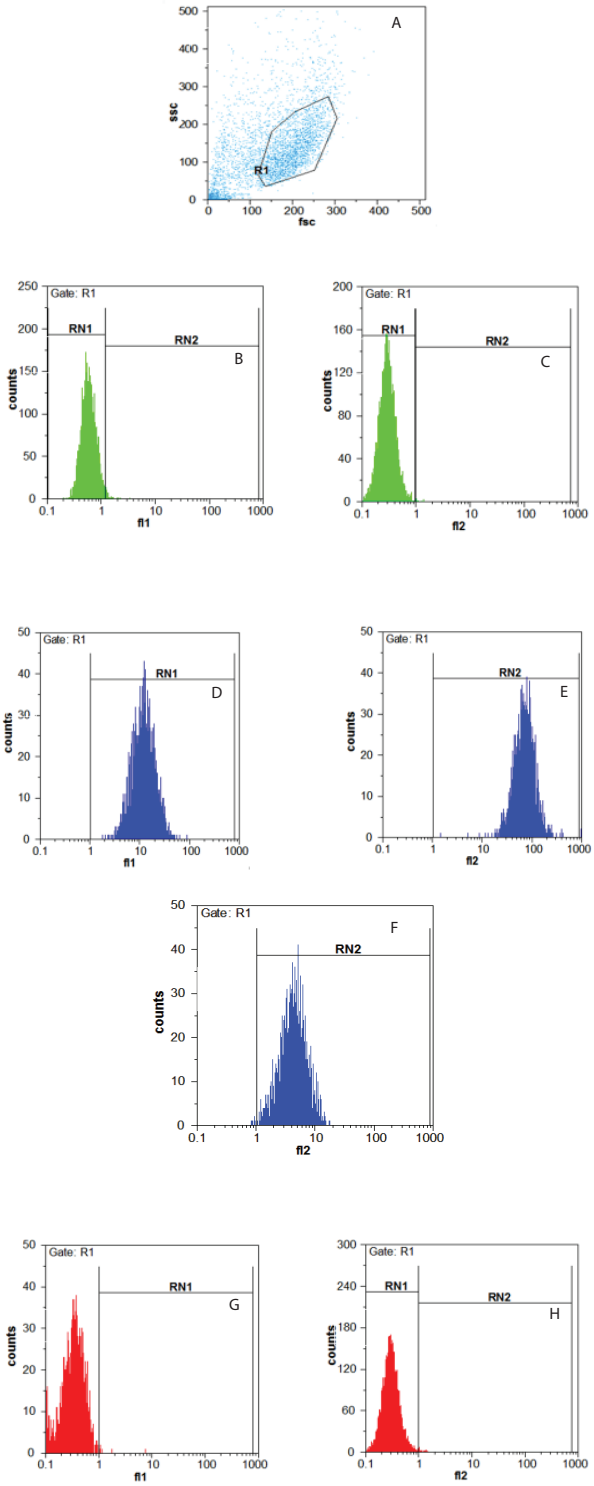

Figure 2: Flow cytometry analysis of mesenchymal stem cells isolated from the umbilical cord. (A) Scatter of cell size and granularity. (B) FITC isotype control (fl1), and (C) PE isotype control (fl2). Absolute expression of (D) CD73, E) CD90, and (F) CD105 antigens. Extremely low expression of (G) CD45, and (H) CD34 antigens. Vertical axis represents the number of cells and horizontal axis represents the intensity of conjugate color (FITC, PE) associated with antibodies of interest. RN1 and RN2 lines represent the cells with the targeted marker identified by fluorescently labeled-antibodies.

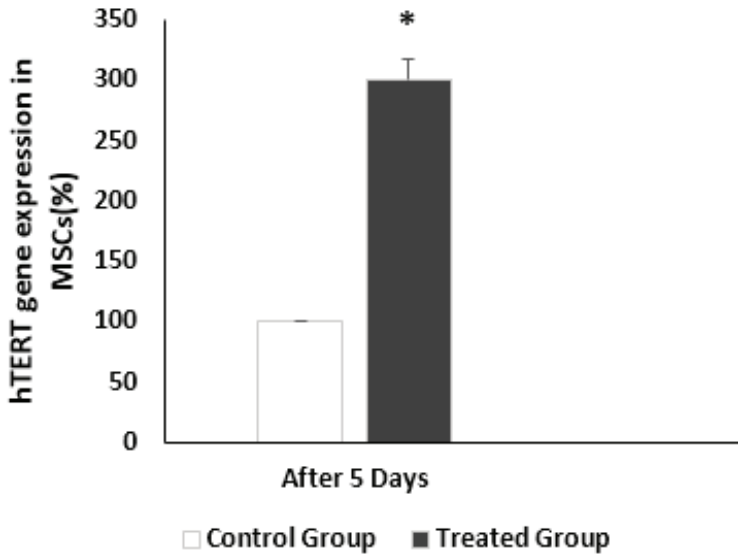

Figure 3: Gene expression of hTERT in treated vs. control (non-treated) cells. The expression of hTERT was significantly higher in the treated group than the control group (P = 0.048). * P < 0.05 denotes statistical significance.

demonstrated that the erosion of telomeres of MSCs at each cell division until a length of approximately 10 kb leads to cell apoptosis [24]. Interestingly, some studies reported that adult stem cells derived from the hematopoietic system exhibited reduced levels of telomerase activity [25]. Many reports show that there is no telomerase activity detectable in most normal somatic cells [2, 25]. Consequently, identifying and developing novel methods to reduce replicative senescence in MSCs has become a focus in cell transplantation research. Böcker W and Huang G (2004, 2008) conducted experiments that demonstrated the shortening of telomere length was a prominent factor in cell aging. To overcome this issue, they exogenously delivered hTERT into MSCs using viral vectors, improving the life span of MSCs [11, 25]. However, many attempts have been made to increase cell longevity of MSCs using non-viral methods, because the use of viral vectors pose problems, such as pathogenesis, toxicity, and persistence of the virus in host cells. Farahzadi R *et al*. (2016) investigated the effect of L-carnitine on gene expression of telomerase and telomere length, proposing that treatment with antioxidants may potentially serve as a non-viral method of increasing the life span of MSCs. The study showed that L-carnitine effectively increased expression of hTERT in MSCs derived from adipose tissue [9].

In the search for non-viral approaches to culturing MSCs *in vitro*, platelet-derived microparticles were identified as carriers of growth factors, proteins, miRNAs, enzymes, lipids, and other biologically active compounds and became a focus of study. Baj-Krzyworzeka M *et al*. (2002) showed that PMPs induced the release of cytokines, expression of growth factors, and inhibition of apoptosis in hematopoietic cells [26]. Furthermore, Dashevsky O (2008) demonstrated that PMPs mediated the proliferation and survival of prostate cancer cells *in vitro* [27].

Therefore, we hypothesized that PMPs presented the potential to improve the life span of MSCs cultured *in vitro*. In this study, we demonstrated that treatment of MSCs

with 50 µg/mL of PMPs significantly increased hTERT gene expression by three-fold, as compared to untreated cells in the control group. In addition, we observed that the use of expired platelet concentrate bags as a source of PMPs provided a cost-effective and accessible method of harvesting high concentrations of PMPs. However, DNA damage, accumulation of p16, an inhibitor of cell proliferation, and oxidative stress are factors that contribute to the aging process in cells, which were not evaluated in the present study [3, 4]. Therefore, further studies are necessary to determine the full extent of the effect of PMP treatment on MSCs *in vitro*.

5. Conclusion

Platelet-derived microparticles are abundantly available and can be easily isolated from human body fluids. Although further investigation is necessary, we demonstrated that treatment of MSCs with platelet-derived microparticles effectively increased gene expression of hTERT. This poses a potentially safer alternative to prolonging the life span of MSCs *in vitro*.

Acknowledgments

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Conflict of Interest

The authors declare no conflicts of interest.

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