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Inactive Platelet Rich Plasma in Culture Conditions Increases the Proliferation and Decreases the Apoptosis and Senescence of Human Adipose Derived Mesenchymal Stem Cells

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Abstract

BACKGROUND/AIMS: The aim of this study was to evaluate the effects of 10% platelet rich plasma (PRP) on the biological activity of human adipose-derived mesenchymal stem cells (hADSCs).

MATERIALS AND METHODS: hADSCs were obtained from Erciyes University Gevher Nesibe Genom and Stem Cell Institute. The cells were transferred into plates at a density of 5,000 cells/cm^2 in DMEM supplemented with 10% fetal bovine serum. Immunophenotyping was performed by means of primary antibodies selected against CD44, CD90, CD105, CD34, CD45 and CD73 antigens. The experimental groups were composed of 10% PRP treated cells (the *study group*) and untreated cells (the *study group*). 2,5-diphenyltetrazolium bromidetest was performed to demonstrate proliferation. Viability and apoptosis were evaluated in the experimental groups on days 1, 3 and 7. Senescent cells were determined by β-galactosidase staining in the experimental groups on day 7.

RESULTS: hADSCs were positive for the immunophenotype of mesenchymal stem cells. The viability and proliferation parameters were statistically significant and higher in the *study group* at days 1, 3 and 7. Apoptosis and senescence were lower in the 10% PRP group ($p<0.05$).

CONCLUSION: 10% PRP increases proliferation and viability, and prevents the senescence and total apoptosis in hADSCs. In regenerative medical studies, 10% PRP can be used to increase the biological characteristics of hADSCs.

Keywords: Platelet rich plasma, adipose-derived mesenchymal stem cell, apoptosis, proliferation, cellular senescence

INTRODUCTION

Stem cells are the main cells which can proliferate, self-renew and differentiate. One of the stem cells in adult tissues are mesenchymal stem cells (MSCs). Bone marrow, adipose tissue, synovial fluid, umbilical cord and dental pulp are important sources of MSCs.[1] MSCs express a high level of surface markers such as CD44, CD73, CD90 and CD105. Under appropriate conditions, they are differentiated into adiogenic, osteogenic and chondrogenic cell lines. Therefore, they are multipotent stem cells.[2] Adipose tissue is one of the richest sources of MSCs. The stem cells obtained here are anti-inflammatory and exhibit multi-lineage differentiation properties very well.[3] Adipose-derived stem cells (ADSCs) are similar to other MSCs in terms of their regenerative potential.[4] In terms of orthopedics, regenerative medicine and tissue engineering, platelet rich plasma (PRP) is an important biological material which contains plenty of growth factors. Recently, it has been used as a cell scaffold.[5] PRP is also an autologous biological product which can be

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used in existing cellular and regenerative medicine treatments. PRP induces proliferation and differentiation of MSCs. As it can be used alone, the use of stem cells together may increase the effectiveness of treatment.[6] PRP refers to the plasma in which the platelet concentration is increased. Platelets contain key factors in key pathways such as vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β). Increases in platelet concentration provide more growth factors to the environment. These factors play a role as a potent potentiator in cells.[7] The purpose of this study was to evaluate the effects of 10% non-activated PRP on the biological activities of human adipose-derived mesenchymal stem cells (hADSCs) such as viability, apoptosis, proliferation and senescence.

MATERIALS AND METHODS

Culture and Characterization of hADSCs

hADSCs were obtained from the Erciyes University Genom and Stem Cell Center. The cells were thawed rapidly in a 37-degree water bath and were transferred onto plates at a density of 5,000 cells/cm^2 in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Beit-Haemek, Israel). Phenotyping was carried out by flow cytometry antibodies specific for CD markers (CD34, CD45, CD73, CD90, CD44, and CD105) and analyzed by flow cytometry Navios (BeckmanCoulter, USA). The cells were first gated on the basis of light-scatter properties to screen out debris and cell surface phenotypes were verified via antibodies. The data were analyzed with KALUZA software (BeckmanCoulter, USA). More than 50% staining was regarded as positive. In addition, immunocytochemistry was performed for CD44, CD105 and CD34 markers (BD Bioscience, Heidelberg, Germany).

Preparation of PRP

PRP was isolated from a healthy donor with an optimized method using a PROSYS PRP Kit. Approximately 20 mL of venous blood was collected and centrifuged by means of a blood cell separation kit (PROSYS PRP). After separating the platelet poor plasma formed on the kit, a PRP was injected. The resulting PRP was brought to optimum pH with 8.4% sodium bicarbonate and applied at a 10% concentration to the experimental groups. After a third passage, the cells were treated with 10% PRP (the *study group*) and they were seeded into 6-well plates (3x10^4/well). Those cells without treatment served as the *control group*.

MTT Proliferation Assay

For the proliferation of the *control* and the *study groups*, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted. The cells were seeded at 5,000 cells/cm^2 in a 96-well plate in a standard culture condition. Every day for 7 days of culturing, the culture medium was replaced with 500 µg/mL MTT in medium. Following 4 hours of incubation, the MTT solution was removed and dimethylsulfoxide was added to dissolve the formazan. The absorbance was measured at a wavelength of 560-750 nm with a Glomax Multi Detection System microplate reader (Promega, USA).

Cell Viability Assay

Viability percentages of the cells in the *control* and *study groups* were determined according to the product protocol with the Muse Cell Analyzer (Merck Millipore) cell count and viability kit (Muse Count & Viability Kit MCH100102) on the first, third and seventh days.

Apoptosis (Annexin V) Assay

Rates of total apoptosis in the *control* and *study groups* were determined according to the product procedure by fluorescence-labeled Annexin V using Muse EasyCyte flow cytometry on the first, third and seventh days (Muse EasyCyte, Merck Millipore, Germany).

Senescence-Associated Beta-Galactosidase Assay

X-gal staining for β-galactosidase activity was performed on the *control* and *study groups* on the seventh day. The samples were rinsed twice with PBS and fixed in 2.5% glutaraldehyde for 15 minutes. The cells were washed with PBS, and then stained overnight at 37 °C in X-gal solution with a pH of 6.0 containing 1 mg/mL X-gal (Sigma-Aldric, US.), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$. X-gal positive cells were examined under an inverted microscope (Nikon Eclipse-Ti, Netherlands).

Statistical Analysis

Statistical analyses were performed using the GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego California USA). A two tailed, unpaired Student's t-test was applied to analyze the difference between the average responses of the *control* and *study groups*. Three levels of significance were considered: $^*p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$.

RESULTS

hADSCs Positively Expressed the MSC Markers

hADSCs were expanded in DMEM supplemented with 10% FBS (Figure 1). At the end of passage 3, the phenotype characterization of the hADSCs demonstrated a homogeneous population of cells negative for CD34, CD45 and positive for CD44, CD73, CD90, and CD105 (Figure 2). In addition, the results of immunoflorescent stain showed positive for CD44 and CD105 and negative for CD45 (Figure 3).

PRP Increased the Proliferation of hADSCs

According to MTT assay results, proliferations were statistically significant and higher in the *study groups* at all times (on day 1: p=0.0002, on day 2: p<0.0001, on day 3: p<0.0001, on day 4: p<0.0001, on day 5: p<0.0001, on day 6: p<0.0001, on day 7: p=0.0037) (Figure 4).

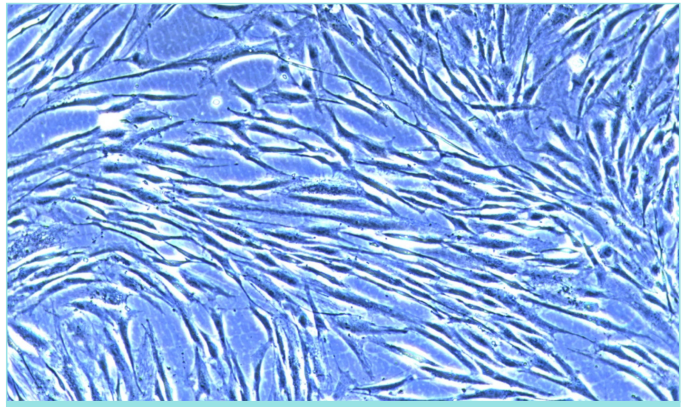

Figure 1. The morphology of hADSCs in culture. Passage 3, microscope magnification 10x.

hADSCs: Human adipose-derived mesenchymal stem cells.

Viability Assay

At the end of the viability tests performed by using the Muse® Cell Analyzer count and viability, first day viability was found to be 91.2±1.39% in the *control group* and 95.76±0.71% in the *study group* (p=0.0833). Third day viability was found to be 91.55±1.14% in the *control group* and 95.15±0.92% in the *study group* (*p*=0.1628). Seventh day viability was found to be 82±0.87% in the *control group* and 86.23±1.65% in the *study group* (*p*=0.1042). There was no statistically significant difference, although there was some increase in viability on days 1, 3 and 7 in the *study group* (Figure 5).

PRP Decreased the Apoptosis of hADSCs

At the end of apoptosis tests performed by using the Muse® Cell Analyzer Annexin V, first day total apoptosis was found to be 8.05±1.48% in the *control group* and 3.21±0.45% in the *study group* (p=0.0071). Third day total apoptosis was found to be 7.5±1.13% in the *control group* and 3.18±0.98% in the *study group* (p=0.0108). Seventh day total apoptosis was found to be 14.95±0.45% in the *control group* and 3.23±0.43% in the *study group* (p=0.0002) (Figure 6).

Senescence-Associated Beta-Galactosidase Assay

Senescence were statistically significant and lower in the *study group* (p<0.05) (Figure 7). PRP decreased the cellular senescence of hADSCs. The number of beta-galactosidase positive cells were found to be 93±5.86 in the *control group* and 14±3.18 in the *study group* (p=0.0003).

DISCUSSION

PRP is a blood plasma containing a high platelet concentration. Platelets contain large amounts of natural growth factors. The concentration of circulating platelets in one microliter is 200,000, while the concentration of platelets in PRP is over two million per microliter.[5] Platelets contain growth factors which regulate many processes associated with healing and tissue repair at high concentrations.[6] These processes include cell migration, proliferation, angiogenesis, inflammation mediator and collagen synthesis.[7] PRP is an important tool used in orthopedic surgery and regenerative medicine. PRP, which can also be administered allogeneically due to the immunosuppressive properties of MSCs, contains TGF-β1, BMP2, platelet-derived growth factors, VEGF and many other factors. Another study previously performed by us showed that it improves the potentials of MSCs of TGF-β1 overexpression.[8] PRP is

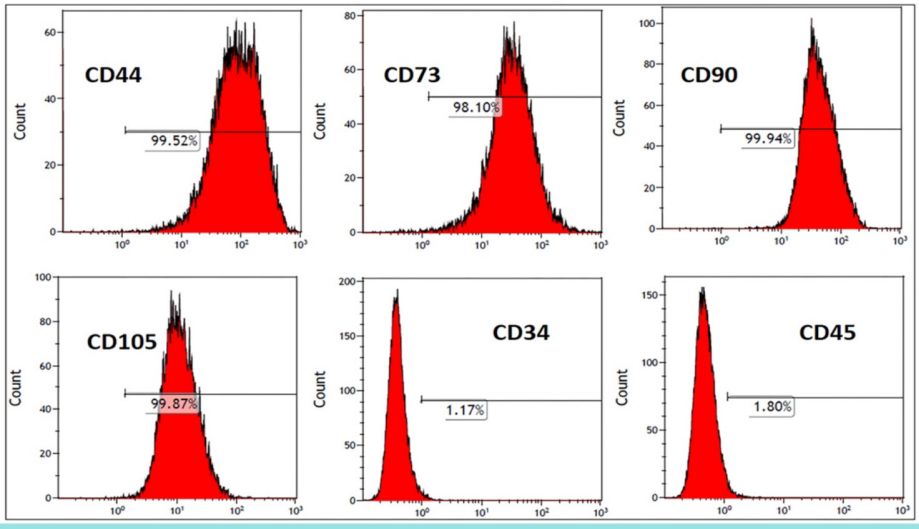

Figure 2. Analyses of flow cytometry for hADSCs. The results of flow cytometry show the following: 99.52% of CD44, 98.10% of CD73, 99.94% of CD90, 99.87% CD105, 1.17% of CD34 and 1.80% of CD45.

hADSCs: Human adipose-derived mesenchymal stem cells.

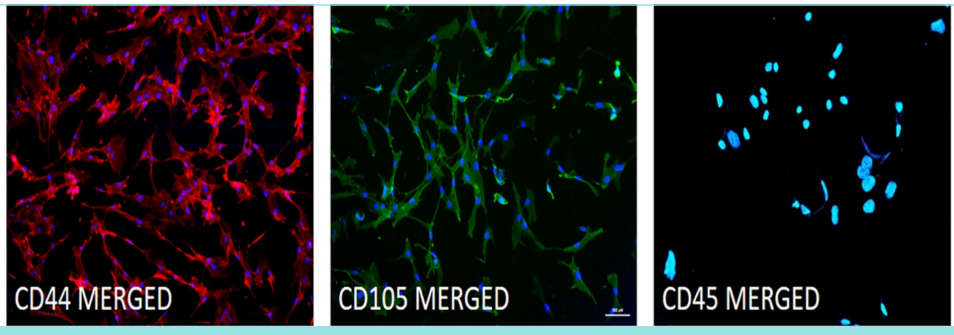

Figure 3. The immunocytochemistry of hADSCs. CD44 positive, Texas Red, CD105 positive, FITC and CD45 negative, FITC. Microscope magnification is 20x for the CD44 and CD105 merged images, and it is 40x for the CD45 merged image.

hADSCs: Human adipose-derived mesenchymal stem cells.

a biological source which is rich in growth factor TGF-β1 and it can be used in studies such as cellular therapy, tissue engineering and regenerative medicine to improve the biological potential of cells. In our study, we employed PRP at 10% concentration, with the aim of determining if PRP could stimulate the proliferation of hADSCs. There are several stem cell studies in the literature with various percentages for PRP applications. Lucarelli et al.[9] found that PRP was an inducer at 10% concentration for the proliferation of marked bone marrow cells. Other investigations have also shown the effects of PRP on the proliferation of MSC.[10,11] The effects of PRP on the proliferation and differentiation of bone marrow-derived MSCs were assessed in 2005 by Doucet et al.[12]. Lee et al.[13] investigated the effects of PRP obtained from human umbilical cord blood on the proliferation and osteogenic differentiation of dental stem cells.Various concentrations of PRP were tried and found to induce the proliferation and osteogenic differentiation of dental stem cells. Stessuk et al.[14] reported that 10% PRP increased the proliferation of keratinocytes and fibroblasts and that wound healing and chronic wounds could achieve re-epithelization with ADSC and PRP. In 2015, Seyhan et al.[15] reported that the combination of PRP + ADSC reduced fat resorption and improved fat grafts in adipose tissue transplantations. Also, Shen et al.[16] reported that autologous PRP application enhanced the proliferation and chondrogenic differentiation of ADSCs. At the same time, the success of the implantation of ADSC with PRP in human articular joints was reported in the literature by Pak et al.[17]. Activated PRP has been proposed as a 3D scaffold and as being effective in the repair of articular cartilage damage with ADSCs.[18] PRP increases angiogenic and osteogenic differentiation, induces cartilage regeneration, and is used as a scaffold in tissue engineering.[19-21] In the literature, the effects of PRP on the proliferation and differentiation of ADSCs have been evaluated in general, but its effects on biological mechanisms such as apoptosis and senescence have not been investigated in detail. In our study, the effects of PRP were evaluated on proliferation, viability, apoptosis and senescence in hADSCs. Our findings showed that PRP significantly increases proliferation in hADSCs, reducing total apoptosis and senescence. In 2017, Felthaus et al.[22] reported that PRP concentrations above 20% showed an inhibitory effect on ADSCs.

CONCLUSION

As a result of this, a 10% PRP concentration was preferred in our study. Using a biological, readily available, inexpensive and reliable source, such as PRP, which naturally contains many of these factors in its own right, instead of giving exogenous recombinant proteins or growth factors for the proliferation and differentiation of cells, can greatly enhance the potential of stem cells. Thus, PRP may be used safely on hADSCs.

MAIN POINTS

- 10% PRP increases the vitality of ADSCs.
- 10% PRP increases the proliferation of ADSCs.
- Inactive PRP application reduces apoptosis in ADSCs.
- Inactive PRP application reduces cellular senescence in ADSCs.
- 10% inactive PRP can be used safely to strengthen the biological characteristics and therapeutic effects of ADSCs.

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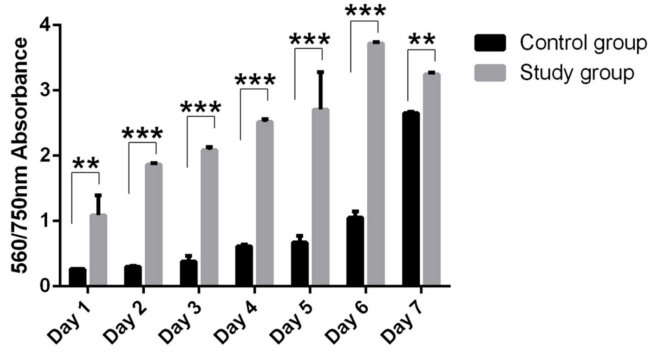

Figure 4. The results of MTT measurements show induced proliferation at 7 days of 10% PRP in hADSCs.

MTT: 2,5-diphenyltetrazolium bromide, PRP: Platelet rich plasma, hADSCs: Human adipose-derived mesenchymal stem cells.

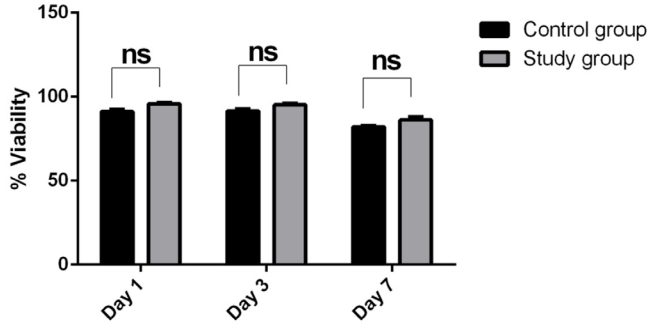

Figure 5. Muse Count and Viability (Millipore, USA) test. There was no statistically significant difference, although there was some increase in viability on days 1, 3 and 7 in the *study group*.

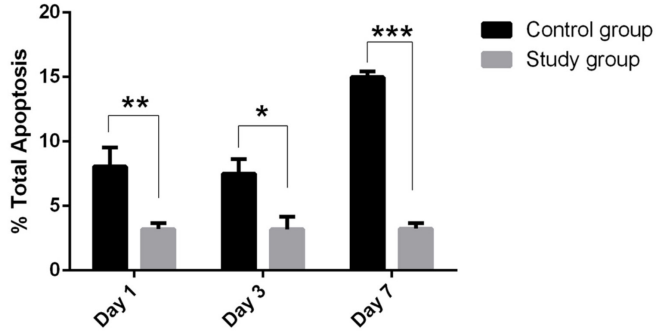

Figure 6. Total apoptosis percentages are shown. According to these results, the total apoptosis in the first, third and seventh days decreased significantly in the *study group*.

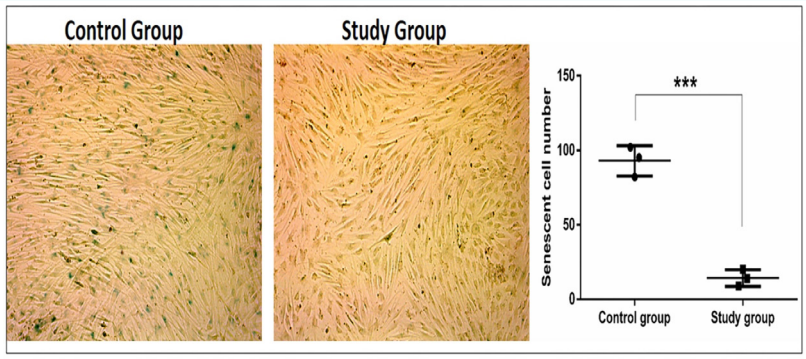

Figure 7. β-galactosidase staining on the 7th day after PRP treatment.
PRP: Platelet rich plasma.

ETHICS

Ethics Committee Approval: Since human adipose-derived stem cells were obtained from Erciyes University Genome and Stem Cell Center as a ready-made commercial cell line in our study, an ethics committee document was not needed.

Informed Consent: Cells were obtained as commercial cell line, not human.

Peer-review: Externally peer-reviewed.

DISCLOSURE

Financial Disclosure: The author declared that this study had received no financial support.

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