Comparative Analysis of Somatic Stem Cells With Emphasis on Osteochondral Tissue Regeneration

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Summary

Congenital anomalies, diseases, and injuries may result in osteochondral damage. Recently, a big hope has been given to somatic stem cells (SSCs) which are characterized as undifferentiated cells with an ability of long-term self-renewing and plasticity. They are adherent with a fibroblast-like morphology *in vitro* and express various surface markers (e.g. CD29, CD73, CD90, and CD105), but they are negative for CD31, CD34, CD45, and HLA-DR. SSCs secrete various bioactive molecules, which are involved in processes of regeneration. The main goal of the present study was the characterization and comparison of biological properties of SSCs obtained from adipose tissue, dental pulp, and urine concerning osteochondral regeneration. SSCs were maintained in an appropriate growth medium up to the third passage and were analyzed by light and electron microscope. The immunophenotype was analyzed by flow cytometry. The kinetics of proliferation was measured by MTT assay. Human Cytokine/Chemokine Multiplex Assay was used, and SSCs secretory profile was measured by Luminex MAGPIX® Instrument. Pellet cultures and a chondrogenic medium were used to induce chondrogenic differentiation. Osteogenic differentiation was induced by the osteogenic medium. Chondrogenic and osteogenic differentiation was analyzed by real-time PCR. SSCs had similar fibroblast-like morphology. They have similar kinetics of proliferation. SSCs shared the expression CD29, CD44, CD73, CD90, and CD105. They lack expression of CD29 and CD34. SSCs secerned similar levels of IL10 and IL18 while differing in IFN-gamma, IL6, IL8, MCP-1, and RANTES production. SSCs possess a similar capacity for chondrogenic differentiation but slightly differ in osteogenic differentiation. In conclusion, it can be emphasized that SSCs from adipose tissue, dental pulp, and urine share the majority of cellular characteristics typical for SSCs and have great potential to be used in osteochondral tissue regeneration.

Key words

Stem cells • Immunophenotype • Secretory profile • Differentiation • Osteochondral regeneration

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Introduction

Damage to osteochondral tissues refers to the trauma or impairment of the complex structure that includes the overlying cartilage and the underlying bone in a joint. Osteochondral damage can result from various factors, such as injury, degenerative conditions, repetitive

stress, or underlying medical conditions. The integrity and function of osteochondral tissues are crucial for proper joint function and movement. When damaged, the smooth cartilage surface that covers the bone can become compromised, leading to pain, inflammation, and limited mobility. The underlying bone may also be affected, resulting in structural changes and potential bone loss [1].

Treatment options for osteochondral damage depend on the extent and severity of the injury. They can include non-surgical approaches such as rest, physical therapy, pain management, and assistive devices. In more severe cases, surgical interventions such as arthroscopy, microfracture, mosaicplasty, or cartilage transplantation may be considered to promote healing and restore joint function [2]. However, in many cases, they do not bring the expected effect. That is why, efforts in research and regenerative medicine are focused on developing innovative approaches to repair and regenerate damaged osteochondral tissues, including stem cells and their derivates, such as extracellular vesicles [3].

Somatic stem cells (SSCs), also known as adult stem cells, are a specialized type of cells found in various tissues and organs of the body. Unlike embryonic stem cells derived from early-stage embryos, somatic stem cells exist throughout a person's life and play a crucial role in tissue repair, regeneration, and maintenance [4]. These cells can self-renew, generate more stem cells, and differentiate into specialized cell types to replenish damaged or aging tissues. SSCs are adherent and have a fibroblast-like morphology. They are positive for various surface markers, including CD29, CD44, CD73, CD90, and CD105. On the other hand, SSCs do not express markers of hematopoietic and endothelial cells, such as CD31, CD34, CD45, and HLA-DR. SSCs are also characterized by the ability of multilineage differentiation [5,6]. More recently, the most important property of SSCs is considered to be their paracrine potential, through which they can affect neighboring cells, and thus promote a variety of biological processes, such as cell proliferation, differentiation, migration, and angiogenesis. The secretome of somatic stem cells consists of a diverse array of proteins, growth factors, cytokines, extracellular vesicles (including exosomes), and other signaling molecules. These components are released into the surrounding microenvironment and can have profound effects on tissue repair, regeneration, and modulation of the local cellular environment [3,7].

Based on the above, it can be summarized that SSCs have garnered significant attention in medical research and regenerative medicine due to their potential for treating a wide range of diseases and injuries, offering promising avenues for personalized therapies and improved healthcare outcomes. Regeneration of damaged cartilage and bone is not an exception. The main goal of the present study was the characterization and comparison of biological properties of SSCs obtained from adipose tissue (ATSCs), dental pulp (DPSCs), and urine (USCs) concerning bone and cartilage tissue engineering.

Methods

Procurement of samples

All sampling procedures were performed after obtaining the patient's written and verbal informed consent in compliance with the Helsinki Declaration during planned surgeries in case of obtaining lipoaspirates (n=6) and teeth (n=6). Urine samples (n=7) were obtained from healthy adult volunteers *via* spontaneous voiding. Obtained biological material was immediately transported to the laboratory of cell cultures for further processing.

Cell isolation and cultivation

ATSCs were obtained from fresh lipoaspirates. Briefly, lipoaspirates were rinsed with sterile phosphate-buffered saline (PBS). After that, adipose tissue fragments were digested with 0.1 % collagenase type I (Gibco, USA) at 37 °C for 45 min with constant shaking, followed by centrifugation at 300× g for 10 min. The obtained supernatant was aspired, and the cell pellet was resuspended in a complete growth medium consisting of D-MEM low glucose, 10 % fetal bovine serum FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma Aldrich, USA) and centrifuged at 300× g for 10 min. The final pellet was resuspended in a complete culture medium and filtered through a 40 µm cell strainer (Becton Dickinson, USA). ATSCs were plated at a density of 1×10^6 cells/ml into 75 cm^2 culture flasks and cultured at 37 °C in a humidified atmosphere containing 5 % CO_2. After 24 h, non-adherent cells were removed by aspiration medium. During subsequent cultivation, the medium was changed every three days. After 7-10 days, cells were detached by 0.05 % trypsin (Sigma Aldrich, USA) and sub-cultured up to the third passage.

DPSCs were isolated from the pulp of extracted teeth, which was carefully rinsed with sterile PBS. Dental pulp was treated with 0.1 % collagenase type I for 60 min

at 37 °C. The cell suspension was resuspended in a complete growth medium and centrifuged at 300× g for 10 min. The obtained sediment was resuspended in a complete growth medium, plated in 100 mm Petri dishes, and cultured at 37 °C in a humidified atmosphere. After 48 h, unattached cells were removed by changing the medium. During subsequent cultivation, the medium was refreshed three times a week. After ten days, when the culture became almost confluent, DPSCs were detached by 0.05 % trypsin and sub-cultured up to the third passage.

USCs were obtained from fresh urine samples. Briefly, samples were centrifuged at 500× g for 10 min. The supernatants were carefully discarded, leaving approximately 1 ml of the pellet in the centrifugation tubes. Next, PBS was added to wash the cell pellets, followed by the second centrifugation at 300× g for 10 min, and supernatants were gently removed leaving approximately 1 ml of the pellets in the tubes which were subsequently resuspended in the complete culture medium consisting of embryonic fibroblast medium and keratinocyte serum-free medium at the ratio 1:1, supplemented with 5 % FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell suspensions were plated in a 24-well plate and incubated at 37 °C in a humidified atmosphere with 5 % CO_2. Renal Epithelial Growth Medium SingleQuotsTM Kit (Lonza, Swiss) and ROCK-Inhibitor (Sigma Aldrich, USA) in a final concentration of 10 μM was also applied. The culture medium was refreshed after 72 h, and then, the changing of the medium was performed every 3 days. After reaching 80-90 % confluency, cells were passaged using 0.05 % trypsin.

Morphological analysis

The morphology of SSCs was continually analyzed during cultivation using an inverted microscope Zeiss Axiovert 100 (Carl Zeiss, Germany).

SSCs appointed for transmission electron microscopy (TEM) were fixed in 2.5 % glutaraldehyde (Sigma Aldrich, USA), pH 7.2, at 4 °C for four hours. After fixation, cells were carefully rinsed with PBS and postfixed with 2 % osmium tetroxide (Sigma Aldrich, USA) for 2 h, then rinsed in distilled water and dehydrated in a graduated series of ethanol. The samples were embedded in Durcupan and cut into semi-thin sections. The obtained sections were stained by toluidine blue for 10 min, and cut into ultra-thin sections. Then, they were mounted on 200 mesh copper grids, double stained using uranyl acetate and lead citrate, and examined using a TEM FEI Morgagni 268D (FEI, USA).

Kinetics of proliferation

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (The CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, USA) was used to analyze the kinetics of SSCs proliferation. Briefly, SSCs with a density of 5×10^4 cells per well were seeded into a 24-well plate. followed by incubating at 37 °C for 48 h. Afterward, 10 μl of MTT solution was applied, followed by 4 h incubation. Absorbance was recorded at 490 nm using a plate reader BioTek EL800 (BioTek, USA). All experiments were performed in triplicate.

Flow cytometry

Immunophenotypic analyses with flow cytometry were performed according to the manufacturer's recommendations. Briefly, 1×10^6 cells per sample were centrifuged at 300× g and resuspended in 100 μl of buffer. Afterward, 10 μl of the respective antibodies, CD29, CD44, CD56, CD73, CD90, CD105, CD146, HLA-DR, and SSEA-4 (Miltenyi Biotec, Germany) and a cocktail of CD14/CD20/CD34/CD45 (Miltenyi Biotec, Germany), was added to cell suspension and incubated for 10 min in the dark in the refrigerator. Then, cells were washed with 2 ml of buffer and centrifuged. The supernatant was aspirated, and the final sediment was resuspended in a buffer for flow cytometry analysis. Similarly, respective isotype controls were used to assess background fluorescence and non-specific binding of antibodies to cells. All data were acquired using a MACSQuant Analyzer 10 (Miltenyi Biotec, Germany) and analysed by MACS Quantify software (Miltenyi Biotec, Germany).

Assessment of the cytokine and chemokine profile

The secretory function of SSCs was estimated by using MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel-Immunology Multiplex Assay (Merck, Germany), and the measurement was performed on a Luminex MAGPIX® Instrument (Luminex, USA). For the determination of cytokine concentrations, triplicates of cell-free supernatants were collected 48 h after stem cells were seeded at the density of 1×10^5 cells in 6-well plates. Cells were kept in a starving medium (D-MEM low glucose in case of ATSCs and DPSCs and embryonic fibroblast medium and keratinocyte serum-

free medium in case of USCs). Further, sample processing and measurement were performed according to the manufacturer´s instructions. Data were analyzed and processed using BelysaTM software (Merck, Germany).

Chondrogenic and osteogenic differentiation

SSCs at passage 3 were induced to differentiate into chondrogenic and osteogenic cell lineages.

For the chondrogenic induction, 3D cell cultures were established. Briefly, 2.5×10^5 cells in a sterile conical 15 ml tube were centrifuged at 300× g for 10 min and left overnight to form an aggregate in a complete culture medium. After 24 h, the medium was replaced with 0.5 ml of the chondrogenic differentiation medium (Miltenyi Biotec, Germany). The medium was refreshed every 72 h, and the experiment lasted for 28 days. RT-PCR was subsequently performed in order to assess the successful differentiation. Chondrocytes in the second passage were used as a control group.

For the osteogenic induction, 50×10^3 of SSCs were seeded in 12-well plates. After the initial seeding, the standard proliferation medium was replaced with an osteogenic differentiation medium (Miltenyi Biotec, Germany). The medium was changed every 72 h. The cells were cultured for 28 days, and after this time period, the results were evaluated by RT-PCR analysis. mRNA from primary osteoblasts culture was used as a positive control.

RNA isolation and quantitative real-time PCR

RNA from the samples was isolated using the GeneJET RNA Purification Kit (Thermo Fischer Scientific, USA) according to the manufacturer's guidelines. Obtained RNA was further used to synthesize complementary DNA with Maxima First Strand cDNA Synthesis Kit (Thermo Fischer Scientific, USA). Subsequently, RT-PCR was performed using Eco Real-Time PCR System (Illumina, USA). Reactions were carried out as follows: 50 °C for 2 min, 40 cycles at 95 °C for 10 min, 95 °C for 15 s and finally 60 °C for 1 min. Quantification of the following proteins was estimated: aggrecan, collagen type I and II in case of chondrogenic differentiation, and alkaline phosphatase and osteopontin for osteogenic differentiation. Human glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. Relative expression levels of the selected genes were measured by the $^{\Delta\Delta}$CT method.

Statistical analysis

Quantitative data were presented as mean ± standard deviation (SD). One-way ANOVA followed by Bonferroni and Holm *post hoc* tests for multiple comparisons were used when appropriate, and $p<0.01$ with $p<0.05$ were considered as statistically significant. All experiments were performed in triplicate.

Results and Discussion

Hyaline cartilage has limited self-healing capabilities because it lacks a direct blood supply. Traditional treatments, such as pain management and joint replacement, have limitations in restoring cartilage function and longevity. Tissue engineering offers a potential solution to these challenges [8]. Somatic stem cells from different tissue sources belong to the most promising pillars of cartilage tissue engineering [9]. In the present study, we performed an analysis of the biological characteristics of ATSCs, DPSCs, and USCs with an emphasis on potential application in articular cartilage regeneration. All experiments were performed on the SSCs in the third passage on the basis of the generally accepted premise of the minimal effect of short-term cultivation on biological characteristics [10].

ATSCs, DPSCs, and USCs displayed similar morphological features under *in vitro* conditions as documented by light and electron microscopy (Fig. 1). All cells had spindle-shaped morphology. Ultrastructural morphology reflects proteosynthetic and metabolic activity. Each cell contained an irregularly shaped large pale nucleus with a large amount of euchromatin. Abundant cisterns of rough endoplasmic reticulum and numerous coated matrix vesicles, abundant mitochondria, and lysosomes were present in the cytoplasm. The secretion of extracellular vesicles was also observed. These observations were fully consistent with the results of other authors [11-13].

MTT assay, which is widely applied to measure cellular metabolic activity as an indicator of cell viability and proliferation was used to evaluate the kinetics of SSCs proliferation. All analyzed SSCs showed similar course and kinetics of proliferation (Fig. 2). However, the highest rates were recorded in the case of USCs which may be related to their increased mitotic activity [14].

Further, the immunophenotype of ATSCs, DPSCs, and USCs was evaluated by flow cytometric analysis. Overall, all SSCs ubiquitously expressed CD29, CD44, CD73, CD90, and CD105. On the other hand, they

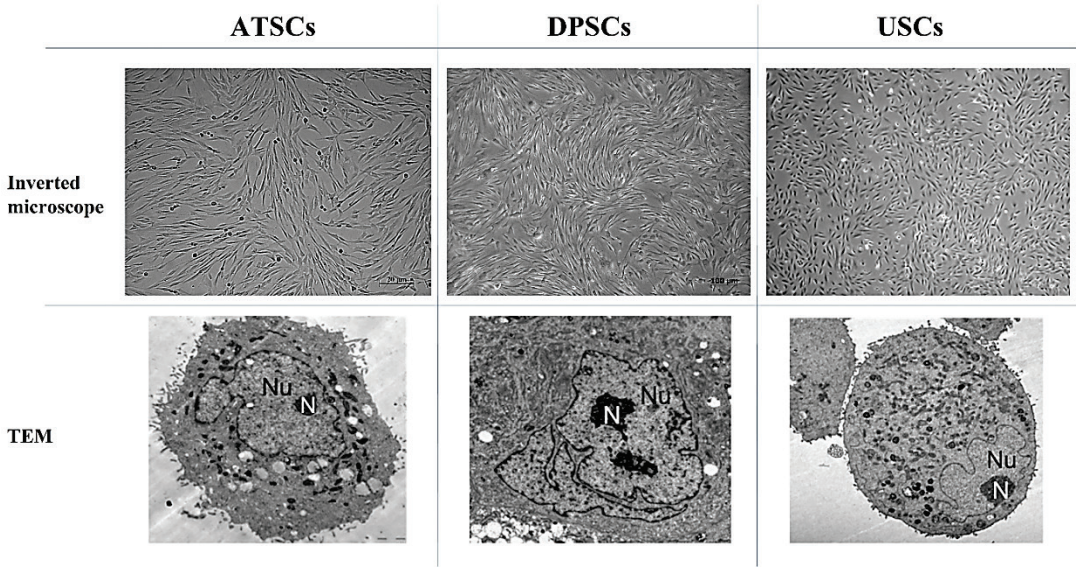

Fig. 1. Morphological analysis of different MSCs (ATSCs, DPSCs, and USCs) acquired with inverted and TEM.

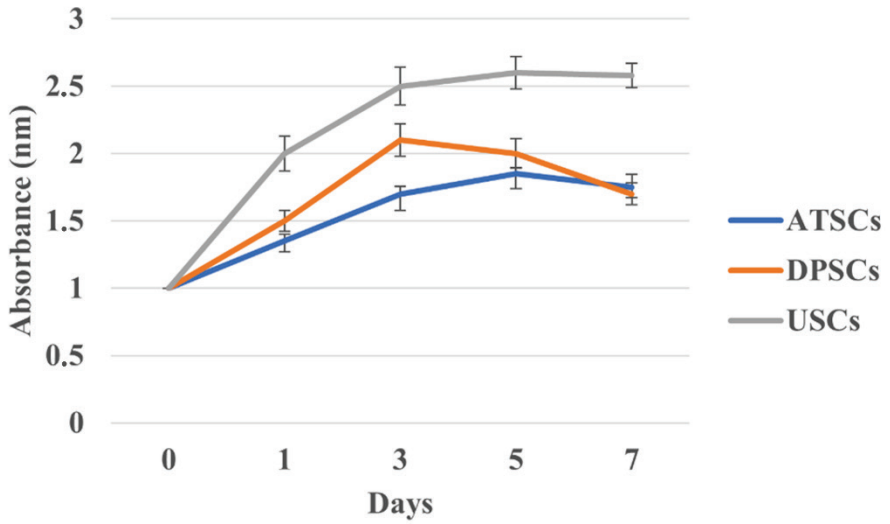

Fig. 2. Kinetics of proliferation in MSCs obtained from different tissues – ATSCs, DPSCs, and USCs. The highest rates were recorded in the case of USCs. ATCSs and DPSCs displayed a similar course of proliferation.

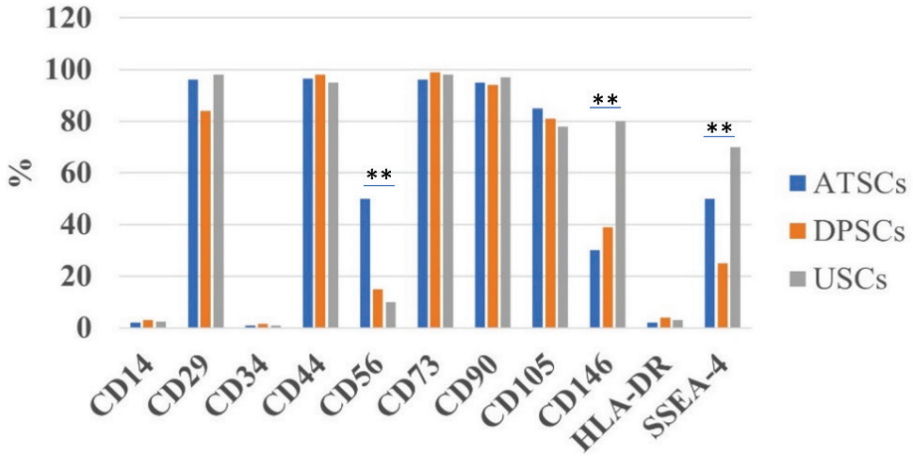

Fig. 3. Expression of surface antigens in MSCs obtained from different tissues - ATSCs, DPSCs, and USCs. MSCs shared the expression of typical markers, such as CD29, CD44, CD73, CD90, and CD105. They lack expression of hematopoietic and endothelial markers. In the case of USCs, interestingly the highest expression of SSEA was recorded (** $p<0.01$).

lack expression of CD14, CD34, and HLA-DR which are considered typical hematopoietic and endothelial cell markers. Moreover, USCs also display significantly higher expression of CD146 and SSEA-4 (maker of embryonic stem cells associated with the pluripotent status) (Fig. 3). This is consistent with the reported cell surface marker profile of different human SSCs [14-16] as well as the definition of human stem cells by the International Society for Cellular Therapy (ISCT) [5]. Our data also demonstrated that all analyzed SSCs represent cell populations with therapeutic potential because of their lack of immunological reactivity. In addition, the expression of CD105 seems to be a good predictive marker of chondrogenic differentiation [17].

The therapeutic effect of the MSCs is conventionally assigned to their paracrine activity [18]. To assess the secretion profile of ATSCs, DPSCs, and USCs, analysis of the conditioned media for selected cytokines and chemokines was performed (Fig. 4). Results revealed similar secretion of IL10 and IL18. On the other hand, all SSCs display significantly different productions of IFN-gamma, IL6, IL8, MCP-1, and RANTES. Interestingly, USCs were characterized by the strongest production of IL6 and IL8. IL6 plays an important role in the anti-inflammatory effects of SSCs by stimulating local lymphocytes. Moreover, IL-6-stimulated SSCs can activate Treg cells, which are known for their immunosuppressive capacity and immune system modulation [19,20]. IL8 seems to be important in the context of SSCs utilization in tissue engineering because of its ability to promote angiogenesis through increased secretion of vascular endothelial growth factor [21].

We also analyzed the potential of chondrogenic differentiation of ATSCs, DPSCs, and USCs. We applied a generally accepted micro mass (pellet) culture system with a chondrogenic medium containing TGF-β1 to induce chondrogenesis. The obtained results (Fig. 5) fully support the use of all SSCs for purposes of articular cartilage regeneration due to the expression of aggrecan and collagen type II genes. However, we found that ATSCs had the best chondrogenic potential but in the case of DPSCs and USCs, this potential should be enhanced by optimization of culture conditions (cocktail of growth factors, physical and mechanical factors), as was indicated by other authors [22-24].

Finally, we studied the osteogenic differentiation potential of ATSCs, DPSCs, and USCs. We used a 2D culture system with an osteogenic medium (StemMACS OsteoDiff Medium) to induce osteogenic differentiation of SSCs. The obtained findings (Fig. 6), according to the expression of alkaline phosphatase and osteopontin, proved osteogenic differentiation in all SSCs. The best osteogenic potential was recorded in the case of ATSCs followed by DPSCs, while USCs showed the lowest potential. However, several authors provided evidence that a more complex cultivation medium and 3D culture environment may enhance the osteogenic activity of USCs, as well [25,26]. Moreover, in the most recent study, it was demonstrated that the osteogenic potential of USCs may be significantly increased by 30Kc19α-Lin28A treatment and thus make USCs more attractive for bone regeneration [27].

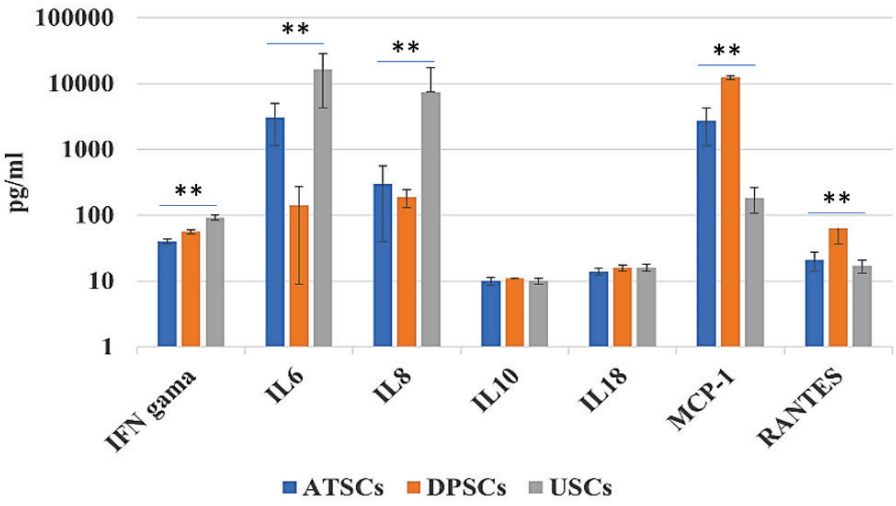

Fig. 4. Cytokine and chemokine profile of MSCs obtained from different tissues – ATSCs, DPSCs, and USCs. MSCs secerned similar levels of IL10 and IL18 while differing in the production of IFN-gamma, IL6, IL8, MCP-1, and RANTES (** $p<0.01$).

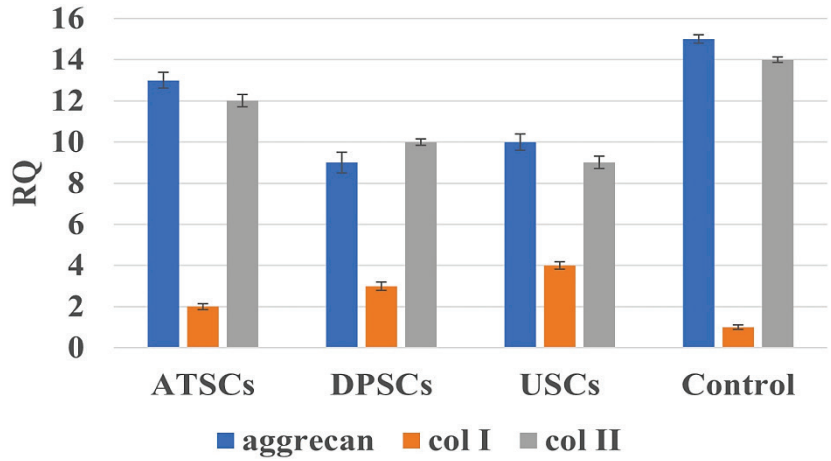

Fig. 5. Chondrogenic differentiation of SSCs obtained from different tissues (ATSCs, DPSCs, and USCs).

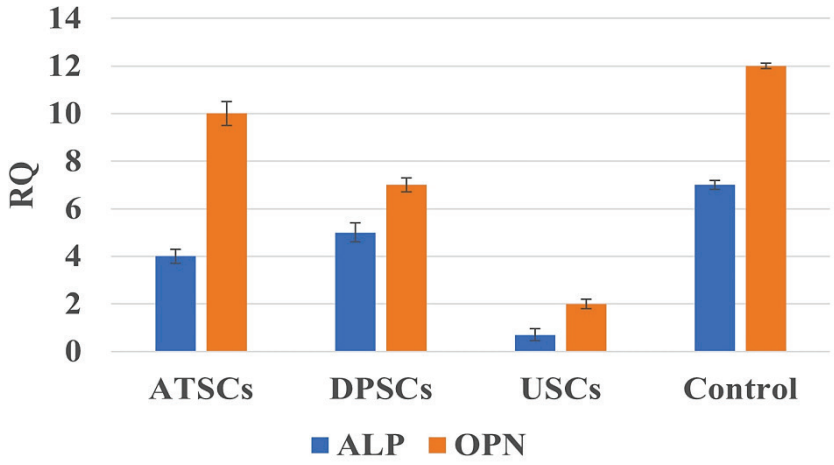

Fig. 6. Osteogenic differentiation of SSCs obtained from different tissues (ATSCs, DPSCs, and USCs).

Conclusions

In conclusion, based on the obtained findings, it can be emphasized that SSCs from adipose tissue, dental pulp, and urine share the majority of cellular characteristics typical for SSCs and have great potential to be used in osteochondral tissue regeneration. Further research will be focused mainly on the enhancement of their chondrogenic and osteogenic differentiation potential by introducing new biomaterials and technologies that allow mimic 3D environments with different differentiation factors and well-defined architecture that resembles the structure of hyaline cartilage and bone tissues.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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