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Comparative analysis of mesenchymal stromal cells from different tissue sources in respect to articular cartilage tissue engineering

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Abstract. The main goal of this study was a comparison of biological properties of mesenchymal stromal cells (MSCs) obtained from bone marrow, adipose tissue and umbilical cord with respect to articular cartilage regeneration. MSCs were isolated and expanded *in vitro* up to the third passage. The kinetics of proliferation was analyzed by cell analyzer CEDEX XS and expression of selected markers was assessed by flow cytometry. The morphology was analyzed by inverted microscope and TEM. Pellet culture system and chondrogenic medium containing TGF-β1 was used to induce chondrogenic differentiation. Chondrogenesis was analyzed by real-time PCR; the expression of collagen type I and type II was compared. MSCs from all sources showed similar kinetics of proliferation and shared expression of CD73, CD90 and CD105; and were negative for CD14, CD20, CD34 and CD45. Observation under inverted microscope and TEM showed similar morphology of all analyzed MSCs. Cells from all sources underwent chondrogenic differentiation – they expressed collagen type II and acid mucopolysaccharides typical for hyaline cartilage. On the basis of obtained results it should be emphasized that MSCs from bone marrow, adipose tissue and umbilical cord share biological properties. They possess the chondrogenic potential and may be utilized in cartilage tissue engineering.

Key words: Mesenchymal stromal cells — Bone marrow — Adipose tissue — Umbilical cord — Cartilage tissue engineering

Introduction

Despite the advances in modern medicine, there are still many conditions due to injuries or pathological processes that affect articular cartilage. They are usually accompanied by retarded or restricted healing and significant pain. Moreover, they may lead into total immobilization of affected individuals. Unfortunately, many conventional therapies, including conservative and surgical approaches fail and do not produce expected results. Thus, research of stem cells, which have ability to promote and accelerate the healing process, has attracted the attention of many clinicians and researchers in field of regenerative medicine (Mardones et al. 2015; Pastides et al. 2015).

Stem cells possess unique biological characteristics which make them promising tool of regenerative medicine. These

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are mainly capability of long-term self-renewing, multilineage differentiation potential (plasticity) and active ability of migration to diseased tissues (Shanti et al. 2007). They are generally divided into embryonic and adult stem cells. The utilization of embryonic stem cells is restricted by ethical considerations in many countries. Therefore, the research is focused on more acceptable adult stem cells – termed as mesenchymal stromal (stem) cells (MSCs) (Heslop et al. 2015).

MSCs can be easily obtained from different tissue sources, including bone marrow, adipose tissue, umbilical cord, placenta, skin, etc. These adherent cells are characterized according to the expression of several surface antigens. They have to be positive at least for CD73, CD90 and CD105; and negative for CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR. Moreover, MSCs must differentiate to osteoblasts, adipocytes and chondroblasts *in vitro* (Dominici et al. 2006). More recently, genomic studies performed on MSCs provided evidence of pluripotent marker's (Oct-4, Nanog and Sox2) expression. These findings clarified their undifferentiated state (Patterson et al. 2012). Moreover, Kestendjieva et al. (2008) showed that MSCs expressed survivin, which is also widely expressed in most human cancers (Halasova et al. 2009, 2013).

The main goal of this study was to perform comprehensive characterization and comparison of selected biological properties of MSCs isolated from bone marrow, adipose tissue and umbilical cord in respect to their potential clinical utilization in cartilage regeneration.

Materials and Methods

The sampling protocols and experimental protocols were approved by local Ethical committee of University hospital in Bratislava. All sampling procedures were performed always after obtaining patient's written and verbal informed consent in compliance with the Helsinki Declaration during planed surgeries or physiological deliveries. All chemical reagents were purchased from Sigma-Aldrich (USA) unless otherwise noted.

Cell isolation and in vitro expansion

Bone marrow-derived MSCs (BMSCs) were isolated from fresh samples (n = 3), and supplemented with 20 U/ml heparin in a sterile physiological solution. To obtain nucleated cells, gradient centrifugation using Ficoll® PM400 at 1800 rpm for 30 min was performed. The obtained cells were carefully washed in phosphate-buffered saline (PBS) then centrifuged at 1200 rpm for 6 min. Pellets were resuspended in a complete culture medium consisting of α-MEM, 10% fetal bovine serum (FBS, PAA, Austria), 100 U/ml Penicillin and 100 µg/ml Streptomycin. The cells (200 000 cells/ml) were plated in 100 mm Petri dishes and were cultured at 37°C in a humidified atmosphere containing 5% CO_2. After 24 h, non-adherent cells were removed by aspiration the culture medium. During subsequent cultivation, the medium was refreshed every three days. After 8–12 days, cells were detached by 0.25% trypsin (Gibco, USA) and sub-cultured up to the third passage.

Adipose tissue-derived MSCs (ATSCs) were obtained from fresh lipoaspirates (n = 6) by standard protocol. Briefly, lipoaspirates were roughly rinsed with sterile PBS to remove contaminating erythrocytes. This procedure was repeated several times until the colour of PBS became light pink. After that, adipose tissue fragments were digested with 0.1% collagenase type I (PAA, Austria) at 37°C for 45 min with constant shaking, followed by centrifugation at 1200 rpm for 10 min. Supernatant containing fat and floating mature adipocytes was aspired and cell pellet was resuspended in D-MEM/F12 containing 10% FBS and centrifuged at 1200 rpm for 5 min. Final pellet was resuspended in complete culture medium consisted of D-MEM/F12 supplemented with 10% FBS, 100 U/ml Penicillin and 100 µg/ml Streptomycin; and filtered through a 40 µm cell strainer (Becton Dickinson, USA). Obtained cells were plated at density of 1×10^6 cells/ml into100 mm Petri dishes and were 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 refreshed every three days. After 7–10 days, cells were detached by 0.25% trypsin and sub-cultured up to the third passage.

Umbilical cord-derived MSCs (UCSCs) were isolated from fresh samples (n = 6) by explant technique. Briefly, cords were rinsed with PBS to remove contaminating blood. The cords were cut into small pieces (approximately 3 cm) and blood vessels were removed from each piece after incising the cord lengthwise. Afterwards, cord was cut into small fragments with an approximate diameter of 4 mm which were transferred into100 mm Petri dishes. After 5 min, when fragments adhered to surface of Petri dish, the complete culture medium consisted of D-MEM/F12 supplemented with 10% FBS, 100 U/ml Penicillin and 100 µg/ml Streptomycin was carefully added. During subsequent cultivation, the medium was refreshed every three days. After 10 days, tissue fragments were removed and UCSCs were cultured until confluence. Afterwards they were detached by 0.25% trypsin and sub-cultured up to the third passage.

Kinetics of proliferation

The evaluation of the growth characteristics of MSCs was performed by generating a growth curve prepared

according to a standard method. Briefly, suspension of MSCs (5×10^4 cells/ml) at passage 3 was seeded into 60 mm Petri dishes (TPP, Switzerland). During the next 7 days of cultivation, three dishes were monitored on a daily basis for density. Growth curves were plotted and population doubling time (PDT) was calculated by means of the formula: PDT = days in exponential phase/(log N2 – log N1)/log 2, where N1 was the number of cells at the beginning of the exponential growing phase, and N2 was the number of cells at the end of the exponential growing phase.

Flow cytometry

MSCs assigned for flow cytometry were detached by 0.25% trypsin digestion and then resuspended in blocking buffer consisting of PBS with 0.5% bovine serum albumin (PAA, Austria). The MSC Phenotyping kit (Miltenyi Biotec) containing antibodies against human CD14, CD20, CD34, CD45, CD73, CD90 and CD105 were used in all experiments. Cell suspensions processed according to protocol provided by manufacturer. The samples were analyzed by MACSQuant® Analyzer (Miltenyi Biotec).

Morphological analysis

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

MSCs asigned for transmission electron microscopy (TEM) were fixed in 2.5% glutaraldehyde (Sigma-Aldrich), pH 7.2, at 4°C for four hours. After fixation, cells were carefully rinsed by PBS and post-fixed with 2% osmium tetraoxide for 2 hours, then rinsed in distilled water and dehydrated in a graduated series of ethanol. Subsequently, the samples were embedded in Durcupan and cut into semi-thin sections. The obtained sections were stained by toluidine blue for 10 minutes, 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).

Chondrogenic differentiation

For chondrogenic differentiation, a three-dimensional pellet culture system was used. Pellets were formed by centrifugation of 5×10^5 MSCs and control chondrocytes from first passage at 1500 rpm for 10 min in 15 ml polypropylene tubes (TPP, Switzerland). The chondrogenic medium consisted of DMEM/F12 1:1, 10% FBS, 100 U/ml Penicillin and 100 µg/ml Streptomycin and 5 ng TGF-β1 (STEMCELL Technologies, USA). All tubes were maintained in the incubator at 37°C with humidified atmosphere of 5% CO_2. The culture medium was carefully refreshed every third day during 21 days. After termination of the experiment, histological evaluation of pellets was performed by Hematoxilin and Eosin (HE) as well as Alcian blue staining (AB).

Gene expression analysis

Total RNA was extracted from MSCs using GeneJET RNA Purification Kit (Thermo Scientific, USA) according to the manufacturer's recommendations. First-strand cDNA was synthesized from total RNA with Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific). The reaction was performed according to protocol recommended by manufacturer. The thermal cycling conditions were composed of 25°C for 10 min, 50°C for 15 min and 85°C for 5 min. Obtained cDNA was used as a template for quantitative PCR to determine the expression level of the selected genes (for collagen type I and II). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. All primers used in this study are listed in Table 1. The PCR reactions were performed on Eco Real-Time PCR System (Illumina). The reaction was performed in 5 µl mixture consisted of 2.5 µl Maxima Probe/ROX qPCR Master Mix (2x) (Thermo Scientific), 0.25 µl of each primer – TaqMan®Gene Expression Assay (Applied Biosystems), 0.5 µl of cDNA and the rest of the reaction volume was adjusted with water. The thermal cycling conditions were composed of 50°C for 2 min followed by an initial denaturation step at 95°C for 10 min, 45 cycles at 95°C for 15 s, 60°C for 1 min. Expression of all analyzed genes was normalized to GAPDH.

Statistical analysis

Data were analyzed using SPSS 14.0 (SPSS Inc., USA) and subjected to one-way analysis of variance with a Tukey post hoc test to determine significant difference ($p < 0.05$) in PDT and gene level expression between BMSCs, ATSCs and UCSCs. Data are presented as the mean ± standard deviation (SD).

Table 1. Primers used in Quantitative real-time polymerase chain reaction

Target gene	Accession Number of TaqMan® Gene Expression Assay
COL1A1	Hs00164004_m1
COL2A1	Hs00156568_m1
GAPDH	Hs03929097_g1

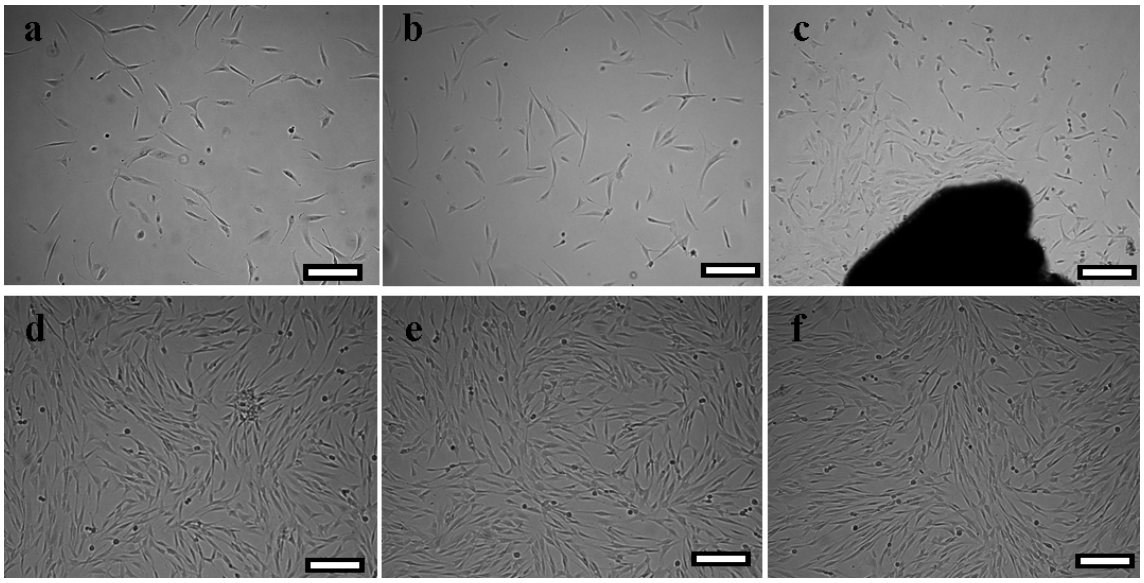

Figure 1. Morphological features of isolated human mesenchymal stromal cells (MSCs): primary isolated BMSCs (**a**), primary isolated ATSCs (**b**), UCSCs outgrowth from tissue fragment (**c**), confluent layer of BMSCs in third passage (**d**), confluent layer of ATSCs in third passage (**e**), confluent layer of UCSCs in third passage (**f**). Scale bar = 20 μm. BMSCs, bone marrow-derived MSCs; ATSCs, adipose tissue-derived MSCs; UCSCs, umbilical cord-derived MSCs.

Results

Cell isolation and in vitro expansion

In case of BMSCs and ATSCs, the cells adhered to substrate and began to proliferate. They display spindle-shaped appearance (Figures 1a,b). After several days they started to form colonies and later on they reached 80% confluence. In that time they displayed typical fibroblast-like morphology and were sub-cultured until third passage. In case of UCSCs, fragment of tissues quickly adhered to substrate and after several days the migration of cells were recorded (Figure 1c). On the 10th day of culture, the fragments were removed; cells with typical fibroblast morphology reached confluence and were sub-cultured until third passage. All analyzed cells displayed typical prolonged fibroblast-like morphology during further cultivation (Figures 1d,e,f).

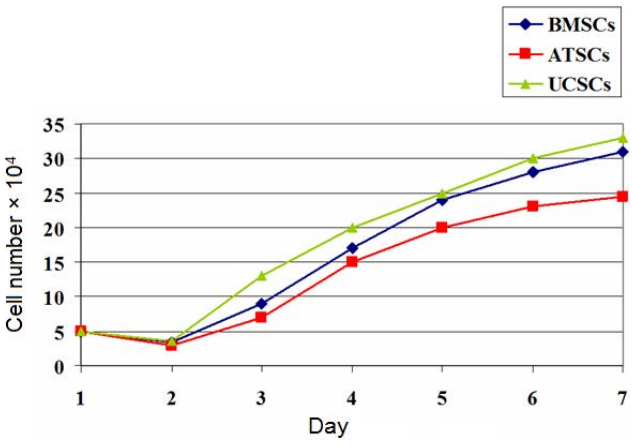

Figure 2. Representative growth curves of mesenchymal stromal cells derived from bone marrow (BMSCs), adipose tissue (ATSCs) and umbilical cord (UCSCs) in third passage.

Kinetics of proliferation

All analyzed MSCs from third passage shared similar kinetics of proliferation (Figure 2). They had very short lag phase, which was followed by an exponential growth phase in the duration of 5 days. Population doubling time was significantly higher in case of ATSCs (Figure 3) but they have lower expansion capacity when compared to BMSCs and UCSCs.

Expression of surface antigens

Flow cytometric analysis revealed that all MSCs were positive for expression of CD73, CD90 and CD105, but negative for CD14, CD20, CD34 and CD4 (Table 2).

Morphology of MSCs

Light microscopy analysis showed that MSCs from third passage display typical fibroblast-like morphology (Figure 1).

TEM analysis showed normal ultrastructure of protheosynthetically active cells (Figure 4). They had irregular shape with noticeable nuclei (often eccentrically located) with huge amount of euchromatine. Every nucleus contained 1 to 3 nucleoli. All cells had prominent rough endoplasmic reticulum. ATSCs also contained residual lipid droplets.

Histological evaluation of pellets

When BMSCs, ATSCs and UCSCs were centrifuged, the condensation of the pellets into single aggregates was observed after 24 hours. The condensed pellets grew continually during further 21 days. They gradually became white and opaque. Photomicrographs of HE stained samples (Figure 5a,b,c) showed a homogenous cell distribution in all samples. Cells were mostly round with darkly stained nuclei what indicate their excessive proliferation. In case of pellets consisted of BMSCs we recorded higher compactness in comparison with ATSCs and UCSCs. However the lacuna formation, typical for hyaline cartilage, was not recorded. On other hand, Alcian blue staining revealed presence of acid mucopolysaccharides in all analyzed samples (Figure 5d,e,f).

Expression of cartilage-specific genes

The cartilage-specific genes expression analysis revealed increased production of collagen type II typical for articular cartilage in all types of MSCs (Figure 6). However, the expression of collagen type II was lower than in control group; the highest chondrogenic potential was recorded in case of BMSCs. On the other hand the expression of collagen type I, typical for fibrillar cartilage, was significantly lower in all aggregates which provide evidence of hyaline cartilage production.

Discussion

Recently, regenerative medicine and tissue engineering provides novel promising approach which should be used for treatment patients with damaged articular cartilage. It combines different types of cells, including primitive undifferentiated stem cells, different biomaterials and appropriate growth factors to prepare artificial cartilage tissue with suitable biological, biochemical and biomechanical properties (Makris et al. 2015). Selection of suitable cells and thorough knowledge of their biological properties belongs to most important prerequisite prior to their clinical application. In order to prepare the cartilage, chondrocytes appeared to be most suitable cell type. However, they undergo dedifferentiation process when expanded *in vitro.* They gradually change their morphology and the expression of collagen type II is replaced by production of collagen type I typical for fibrillar cartilage. These events may be overlapped by adding the specific growth factors (e.g. BMP-2 and TGF-β1) (Claus et al. 2012; McNary et al. 2014). More recently, the interest of scientists has been focused on utilization of multipotent MSCs, which may

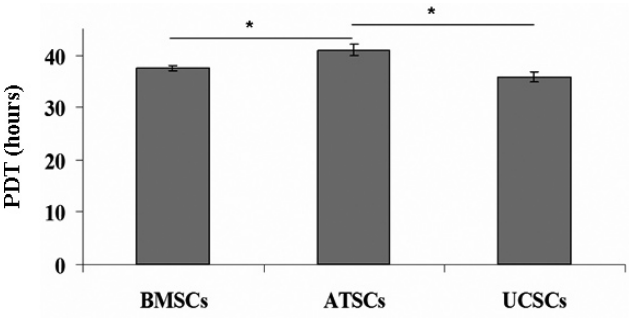

Figure 3. Analysis of PDT in third passage. PDT in case of ATSCs was significantly higher then in BMSCs and UCSCs. Data are means ± SD, $n = 3$, * $p < 0.01$. PDT, population doubling time. For more abbreviations see Fig. 1.

Table 2. Analysis of surface markers expression in BMSCs, ATSCs and UCSCs

	CD14	CD20	CD34	CD45	CD73	CD90	CD105
BMSCs	–	–	–	–	+	+	+
ATSCs	–	–	–	–	+	+	+
UCSCs	–	–	–	–	+	+	+

All analyzed cells expressed markers of mesenchymal stromal cells and were negative for endothelial and hematopoietic markers (+ more than 95%; – less than 5%). BMSCs, bone marrow-derived MSCs; ATSCs, adipose tissue-derived MSCs; UCSCs, umbilical cord-derived MSCs.

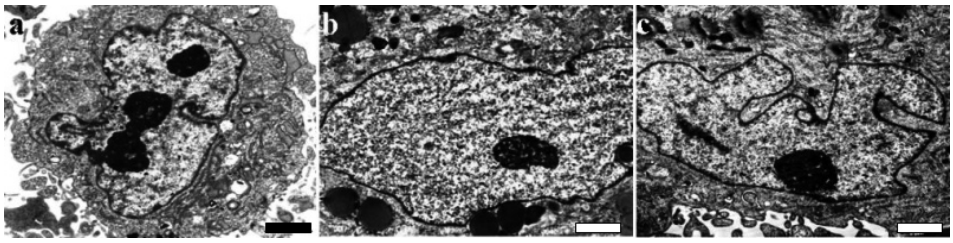

Figure 4. Representative electron micrographs of mesenchymal stromal cells: BMSCs (**a**), ATSCs (**b**), UCSCs (**c**). Scale bar = 2 μm. For abbreviations see Fig. 1.

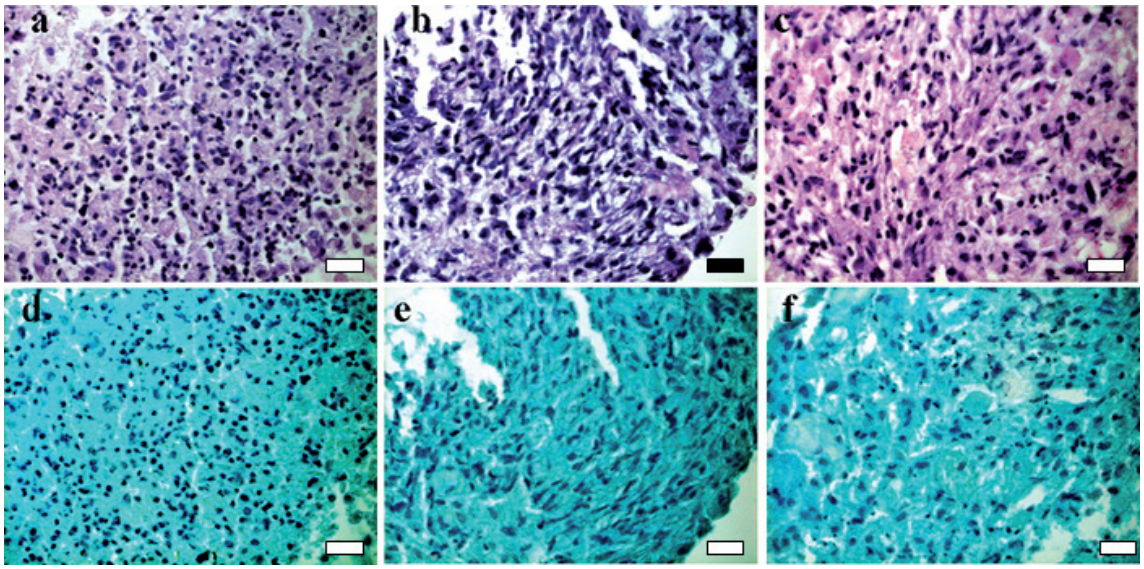

Figure 5. Representative micrographs of mesenchymal stromal cells: BMSCs (**a,** HE), ATSCs (**b,** HE), UCSCs (**c,** HE), BMSCs (**d,** AB), ATSCs (**e,** AB,), UCSCs (**f,** AB) cultured in pellet. All cell produced its own extracellular matrix which contained predominantly acid mucopolysaccharides typical for hyaline cartilage. Scale bar = 20 μm HE, Hematoxilin and Eosin staining; AB, Alcian blue staining. For more abbreviations see Fig. 1.

be easily obtained from different tissues, including bone marrow, adipose tissue, umbilical cord etc. It was shown that these MSCs undergo chondrogenic differentiation *in vitro*. The best results were obtained by using 3D (micromass) culture technique, which mimic condition *in vivo*. It provides 3D architecture of tissue in which the cells are in deep contact and may influence each other by paracrine signaling (Mara et al. 2010). This effect is also additively enhanced by supplementation of growth factors such as TGF-β1, TGF-β3, BMP-2, BMP-6 and GDF-5 (Murphy et al. 2015; Shademan et al. 2015).

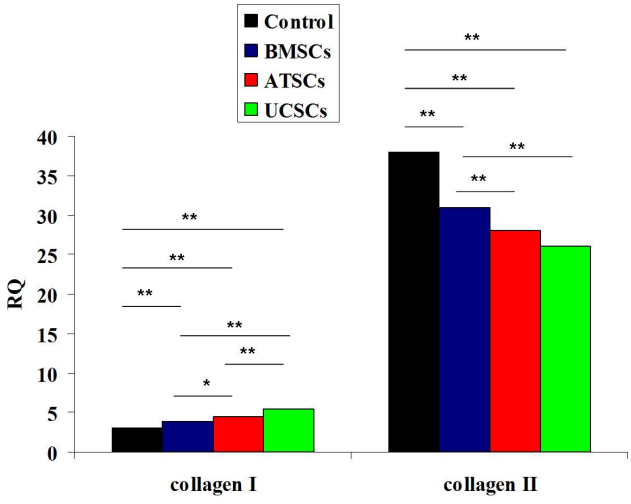

Figure 6. Chondrogenic differentiation of *BMSCs*, *ATSCs* and *UCSCs* gene level expression was significantly different when compared with control – chondrocytes from first passage ($n = 3$, ** $p < 0.01$, * $p < 0.05$). RQ, relative quantitative expression. For more abbreviations see Fig. 1.

In this study, we performed analysis of selected biological characteristics of MSCs from bone marrow, adipose tissue and umbilical cord in respect to potential application in articular cartilage healing. All experiments were performed on the MSCs in third passage on the basis of generally accepted premise of minimal effect of short-term cultivation on biological properties. The cultures were established according to standard protocols. All studied MSCs displayed similar proliferation kinetics and morphological features as documented by light and electron microscopy. This observation was fully consistent with results of other authors (Hsiao et al. 2012; Vishwanath et al. 2013; Wang et al. 2014). After reaching the confluence they were sub-cultured up to third passage and were characterized according to expression of surface antigens. We found similar phenotype of BMSCs, ATSCs and UCSCs, they shared expression of CD73, CD90 and CD105, but they did not express CD14, CD20, CD34 and CD4 typical for endothelial and hematopoietic cells (Pittenger et al. 1999). On the other hand, this argument, mainly in case of CD34 is not strictly accurate. Some authors provide evidence for CD34 expression in tissue resident MSCs. They attribute negativity for CD34 as a consequence of cell cultivation (Lin et al. 2012; Braun et al. 2013; Zimmerlin et al. 2013).

Afterwards, we analyzed the chondrogenic differentiation potential of all MSCs. We used generally accepted micromass (pellet) culture system with chondrogenic medium contained TGF-β1 to induce chondrogenesis. Our findings fully support the use of BMSCs, ATSCs and UCSCs for purposes of articular cartilage healing. The chondrogenic nature in our micromass cultures was supported mainly by the expression of acid mucopolysaccharides and by the expression of collagen type II mRNA, as confirmed by real time RT-PCR analysis. However, we found that the best chondrogenic potential was in case BMSCs but in case of ATSCs and UCSCs this potential should be enhanced by optimization of culture techniques as it was indicated by other authors (Shih et al. 2011; Alimperti et al. 2014; Shang et al. 2014). More recently, interesting results were obtained by Subramanian et al. (2015), who suggested different biological properties, including chondrogenic differentiation potential of MSCs derived from various compartments of the human umbilical cord. In addition, we did not observe the lacuna formation typical for hyaline cartilage. This finding was in contrast to the results documented by Shademan et al. (2015), who recorded lacunae in their pellets. They used pellet culture system with more complex chondrogenic medium consisted of dexamethasone, ascorbic acid, ITS, TGF-β3 and BMP-6. Some authors suggest not only the utilization of growth factors but also other physical and mechanical factors, including oxygen tension and hydrostatic pressure (Wagner et al. 2008; Markway et al. 2010; Puetzer et al. 2013).

In summary, MSCs from bone marrow, adipose tissue and umbilical cord shared biological properties such as morphological features, expression of surface antigens, and kinetics of proliferation, but slightly differ in chondrogenic potential. According to these findings we can emphasize that after performing further experiments focused mainly on optimization of culture techniques which may lead into enhancement of chondrogenic differentiation all analyzed MSCs may play important role in cartilage tissue engineering.

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