European Review for Medical and Pharmacological Sciences 2019; 23: 2924-2934

A pilot study of human mesenchymal stem cells from visceral and sub-cutaneous fat tissue and their differentiation to osteogenic phenotype

F. MASTRANGELO[1], S. SCACCO[2], A. BALLINI[2], R. QUARESIMA[3], A. GNONI[2], D. DE VITO[2], A. SCARANO[4], G. DIPALMA[6], C. GARGIULO ISACCO[5], S. CANTORE[2], M.F. COSCIA[2], F. PETTINI[6], G. SAMMARTINO[7], M. CICCIÙ[8], P. CONTI[9], L. LO MUZIO[1]

[1]Department of Clinical and Experimental Medicine, University of Foggia, Foggia, Italy
[2]Department of Basic Medical Sciences, Neurosciences and Sense Organs, University of Bari "Aldo Moro", Bari, Italy
[3]Department of Civil Engineering, Architecture and Environment, University of L'Aquila, L'Aquila, Italy
[4]Department of Oral Science, Nano and Biotechnology and CeSi-Met, University of Chieti-Pescara, Chieti, Italy
[5]Human Stem Cells Research Center-HSC, Ho Chi Minh City, Vietnam
[6]Interdisciplinary Department of Medicine, University of Bari "Aldo Moro", Bari, Italy
[7]Department of Neurosciences, Reproductive Sciences and Dental Sciences, University Federico II of Naples, Naples, Italy
[8]Departments of Biomedical and Dental Sciences and Morphological and Functional Imaging, Messina University, Messina, Italy
[9]Immunology Division, Postgraduate Medical School, University of Chieti-Pescara, Chieti, Italy

All authors equally contributed to this work

Abstract. – OBJECTIVE: To evaluate the different behavior of two different human adult adipocytes derived stem cells (hASCs) during proliferation and osteogenic differentiation.

PATIENTS AND METHODS: Human adult adipocytes stem cells (hAT-SCs) from visceral (hAV-SCs) and subcutaneous (hAS-SCs) sites were obtained after surgery procedures of seven patients. All samples were fully investigated and the different proliferation rates were evaluated. All MSCs clusters were cultured with an osteogenic and adipogenic differentiation medium. Homogeneous pools of Mesenchymal Stem Cells (MSCs) were confirmed by Flow-Cytometry Analysis (FACS) and Spectrophotometric Assay. The differentiated cells were eventually assessed for the expression of Alkaline Phosphatase (ALP), Alizarin Red (AR) and Oil Red-O (OR-O) detection, and analyzed by the Spectrophotometric Assay. After osteogenic differentiation, the cell clusters were incubated and analyzed with Real Time-Polymerase Chain Reaction (qRT-PCR) and fluorescence microscopy.

RESULTS: The FACS analysis performed on hAT-SCs confirmed the homogenous presence of MSCs in all samples. The ALP, AR stain confirmed the osteogenic differentiation capacity of MSCs towards osteoblast-like-cells. The colorimetric cell metabolic activity (MTS) assay showed an increase in the proliferation rate with different values in both sets hAS-SCs vs. hAV-SCs.

CONCLUSIONS: These *in vitro* findings of both hAS-SCs and hAV-SCs suggested an important role of these stem cells for future clinical use in bone regeneration. Indeed, the final outcomes suggested a better performance of cells coming from subcutaneous adipose tissue vs. those from visceral fat tissue.

Key Words:
Human adult adipocytes derived stem cells (hASCs), Osteogenic proliferation and differentiation, Mesenchymal stem cells, Bone formation.

Introduction

Stem cells (SCs) represent an evolving field essential in the creation of new therapeutic strategies for tissue engineering and regeneration[1,2]. Among the different human stem cells studied until now, Mesenchymal Stem Cells (MSCs) have

Corresponding Author: Salvatore Scacco, MD; email: salvatore.scacco@uniba.it

attracted great attention basically due to their particular ability to differentiate in all three basic ectoderm, mesoderm and endoderm cell lineages, because of their capacity of exerting a specific immune-modulatory and paracrine capacity[3-9]. According to the International Society for Cellular Therapy, MSCs are morphologically defined by a spindle-like fibroblast form. They are able to adhere to plastic surfaces, they are extremely malleable and they show a fast colony formation capacity, with a reckless growth rate, proliferation and differentiation ability towards different cell phenotypes such as osteoblasts, chondrocytes, myocytes, cardiomyocytes and adipocytes; MSCs express a wide variety of pluripotent and multipotent surface markers such as CD44, CD73, CD90, CD105, CD106, CD166, STRO-1 and, lack the expression of hematopoietic stem cells markers CD14, CD34, CD45, and HLA-DR[10-12]. The adipose tissue (hAT) is one of the richest sources of MSCs[13-15]. The hAT-MSCs features, such as morphology, available quantities, colony and high expansion potential, multiple differentiation capability and immune phenotype make them very attractive tools[15-17]. Human hAT-SCs share special traits similar to those of MSCs from bone marrow (BM), umbilical cord blood (UCB), placenta or even dental-derived stem cells[18-24].

However, data from the published study confirmed that fat tissue compared to BM contains a higher presence of MSCs. One gram of fat tissue may give up to 5×10^5 MSCs which is 500-fold larger than 1 g of BM[23,24]. Yet, human adipose tissue seems to have more than a single stem cell population with similar morphological and biological characteristics. Some authors have shown two different subpopulations of stem cells derived from adipose tissue classifying them, according to the site of extraction, in subcutaneous adipose stem cells (hAS-SCs) and visceral adipose stem cells (hAV-SCs)[25-27]. The two sub-sets of AT-MSCs were analyzed by flow cytometry analysis. Initial results showed that both sub-sets express analogous surface CD markers $CD31^-$, $CD34^-$, $CD45^-$, $CD73^+$, $CD90^+$, and $CD105^+$ with equal differentiation potentials[7]. Later outcomes displayed peculiar traits which were seen as the main distinctive features exhibited by either AS-SCs or AV-SCs. The AV-SCs exhibited a significantly greater proliferative ability than that of AS-SCs. The latter revealed a better adipogenic capacity. The use *in vitro* of AS-SCs for cartilage repair revealed a higher chondrocyte activity with higher immune-modulatory performance compared to both control and AV-SCs. Intriguingly, Tang et al[27] found that hAV-SCs uncovered a higher amount of hematopoietic SCs $CD34^+$ AS-SCs feature in line with their greater proliferative ability[27-32]. The differences noted by the authors were mainly associated to the different histological features, stemness and biological asset probably in response to different microenvironmental location[27,33]. Therefore, following the previous outcomes, the main intent of the present study was to investigate and evaluate the specific physical and bio-molecular characteristics of subcutaneous and visceral adipose tissues. With this purpose, data were carefully placed together by emphasizing the differences existing between the two different adipocytes and the corresponding hAS-SCs and hAV-SCs. The *in vitro* behavior, the proliferation rate, the osteogenic differentiation mode and their respectively bone-like matrix formation ability were carefully analyzed and described.

Patients and Methods

Samples of hAS-SCs and hAV-SCs were obtained after surgery procedures of seven healthy patients (2 male and 5 female with an average age 42 years), after they signed a written informed consent in accordance with the Helsinki Declaration, for the re-use of human bio-specimens in the scientific research. The investigation was conducted in accordance with the current medical protocol as described by the Italian Government's NIH legislation. The procedure followed a precise individual medical anamnesis together with the required clinical evaluations performed at the University of Medicine of Foggia (Foggia, Italy). Procedures were conducted following our previous experience in the field, and according to manufacture specifications[14].

Human Visceral and Subcutaneous Adipocytes Cell Culture

The hAV-SCs and hAS-SCs were collected and cultured in α-MEM medium plus 10% of fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 mg/mL Amphotericin B (Gibco, Grand Island, NY, USA). Cell cultures were incubated Thermo Scientific Heracell CO_2 (5%) at 37°C (Thermo Fisher Scientific, Waltham, MA, USA), and the medium was changed twice per week. Experiments were performed only on the first six to eight cell clone generation.

Flow Cytometry for the Surface and Stromal Markers Analysis

Samples cell $5x10^5$ were treated, subsequently stained and analyzed for cell surface markers and intracellular antigens, by using specific conjugated antibodies such as fluorescein isothiocyanate (FITC) conjugated antibodies – or phycoerythrin (PE) – or 2 allophycocyanin (APC): anti-CD13 (CD13-FITC), anti-CD45 (CD45-FITC), anti-CD29 (CD29-PE), anti-CD105 (CD105-FITC) and anti-CD166 (CD166-FITC) obtained from Ancell (Ancell, Stillwater, MN, USA); anti-CD14 (CD14-FITC) was purchased by Miltenyi Biotec (Bergisch Gladbach, Germany); anti-CD90 (CD90-FITC), anti-CD73 (CD73-PE), anti-CD117 (CD117-PE), anti-CD133 (CD133-PE) and anti-CD146 (CD146-PE), anti-SSEA-4 (SSEA-4), anti-CD144 (CD144-FITC) was obtained from Acris Antibodies (Herford, Germany); anti-CD34 (CD34-PE) was purchased by Beckman Coulter (Brea, CA, USA). Cells were incubated with these antibodies for 15 min at room temperature (RT). Thereafter, the samples were gently washed with 3 ml of buffer solution, centrifuged (4°C, 400 rpm, 8 min). Cells were collected from the bottom and successively re-suspended with 1 ml of 0.5% paraformaldehyde and incubated for 5 min at RT. Cells were gently washed and centrifuged (4°C, 400 rpm, 8 min) and stored a 4°C in the dark until the achieved stability with the antibody. Finally, the antibody-bound cells were analyzed on a flow cytometer (FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA) and CellQuest™ software (BD). All antibodies were titrated under assay conditions and optimal expansions of the photomultiplier were obtained for each channel. Cells incubated with PBS solution acted as controls and all experiments were performed in a triplicate mode. Using the FlowJo™ software (TreeStar, Ashland, OR, USA), FACS data were analyzed and quantized by fluorescence intensity ratio (MFI ratio), obtained dividing positive by negative events.

Cell Proliferation

The alive cell count was achieved by the exclusion cycle using trypan blue, which involves the collection of cells after different culture periods (from 0 to 10 d) and counted by a hemocytometer (3 different fields for each sample in triplicate). The results were expressed as the total number of living cells per/ml. For the doubling time, cells at passage 5 were seeded at $1x10^4$ cells/cm^2 and cultured for different periods. Cell culture was collected by using trypsin-EDTA (Gibco Ltd., Grand Island, NY, USA) at 48, 72, and 96 h after seeding, and the alive cells were counted with the trypan blue exclusion assay. The doubling time can be computed as follows: Dt-CFU-F (colony-forming unit-fibroblast), assay: cells at P5 were plated in six-well plates at different densities (100, 250 and 500 cells/cm^2) and cultured in humidified 5% CO^2 at 37°C. The culture medium was usually changed every 3 d. After 2 weeks, the dishes were washed twice with PBS, fixed with 100% of methanol and stained with 3% of crystal violet (Sigma-Aldrich, St. Louis, MO, USA). The colonies were counted under an equipped optical microscope digital camera (Leica Microsystems, Wetzlar, Germany; Canon Inc., Tokyo, Japan), scoring as a 1 colony-forming unit-fibroblast aggregates formed by 16 to 20 or more cells. To induce adipogenesis, the culture medium was switched to the Lonza adipogenic induction medium (Cat No. PT-3004, Lonza, Italy) for 4 d and then to the Lonza adipogenic maintenance medium (Cat No. PT-3102A, Lonza, Italy) for 3 d. This procedure was repeated three times (3 weeks) based on the manufacturer's instructions.

Lipid droplets were stained with 0.5% of Oil Red O (Sigma-Aldrich, St. Louis, MO, USA) and dissolved in 100 mL of isopropanol to prepare the working solution. The Oil Red O working solution was pre-wetted with 60% of isopropanol for 15 min, and the cells were washed with PBS twice and observed under an inverted light microscope (Nikon T100).

Osteogenic Differentiation Assay in vitro

hAS-SCs and hAV-SCs were cultured in α-MEM medium supplemented with 10% FBS, 0.05 mM ascorbic acid, 10 mMb-glycerophosphate and 100 nM dexamethasone for 28 d, with medium changes every 3 d. Cultures were colored at different times with Alizarin Red Staining (ARS; Sigma-Aldrich, St. Louis, MO, USA) and the expression of calcium phosphate depositions was detected by using a Nikon Eclipse 80i Fluorescence microscope (Nikon, Melville, NY, USA) with an equipped digital camera (D200 Nikon, Japan). Fluorescence microscopic images were assessed at magnifications up to 100×. Image-Pro Plus software 6.0 was used for image analysis (Media Cybernetics, Rockville, MD, USA). Active bone formation was evaluated in relation to the presence or absence, intensity, and width of the fluorochrome markers. Briefly, 800 µl of 10% (v/v) acetic acid were added to each well; cells

were incubated for 30 min with shaking, then removed by scraping, transferred into a 1.5-mL vial and vortexed for 30 s. 500 ml of mineral oil (Sigma-Aldrich, St. Louis, MO, USA), heated at to 85°C for 10 min. Next, they were transferred to ice for 5 min, carefully avoiding the opening of the tubes until fully cooled, and centrifuged at 20,000 rpm for 15 min. The samples were acidified (pH between 4.1 and 4.5) with 200 µl of 10% (v/v) ammonium hydroxide. Aliquots (150 µl) were read in triplicate at 405 nm by a microplate spectrophotometer (Spectramax SM190, Molecular Devices, Sunnyvale, CA, USA). Cells were treated with acetoxymethyl (AM) esters and analyzed by fluorescence with a confocal scanning laser microscope (Zeiss AxioSkop and Sarastro 2000).

Evaluation of Early Osteogenic Markers

Non-tissue specific Alkaline Phosphatase (ALP) activity was measured after 1 week of cell culture. The cell monolayers were washed with PBS, lysed with 1 ml of Tris buffer (10 mM, pH 7.5, 0.1% Triton X-100) and centrifuged (2000 rpm, 1 min). Then, 20 µl of supernatant from each sample were combined with 20 µl of 1 mM p-nitrophenyl phosphate (p-NPP, Sigma, pH 10.3 solution with $MgCl^2$-diethanolamine buffer) and dispensed in 96-well plates. Samples were incubated in the dark at room temperature for 30 min and the reaction was blocked with the addition of 10 µl of 2N NaOH. The amount of p-NPP released was measured as absorbance at 405 nm. The protein content of the samples was determined through the BioRad protein test (Bio-Rad, Hercules, CA, USA). The enzymatic activity was expressed as nmol of p-NPP released per mg of protein for 30 min. Total RNA was isolated from autologous human adipocytes tissue using the RNeasy Mini kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. In detail, the cells were lysed with 350 µl of RLT lysis buffer and each sample was transferred to the Rneasy spin column after the addition of an equal volume of 70% ethanol, for a total of 700 µl. After several washes with elution buffers, 30 µl of water were added to each column to elute the RNA. NanoDrop (Agilent, Santa Clara, CA, USA) was used to quantify the extracted RNA at a concentration OD at 260 nm with purity content of OD 260/280 nm and analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). Subsequently, the RNA was transcribed in cDNA for the Omniscript Reverse Transcriptase Kit (Qiagen Inc., Valencia, CA, USA). Briefly, 7 µl of RT mix solution containing 2 µl of oligo (dt) were added to 2 µg of the total RNA solution. The reaction mixture was loaded into the PCR system Gene Amp 9700 (Applied Biosystems, Foster City, CA, USA) subjected to a 37°C cycle for 60 min. RT-PCR was developed using the ABI Prism 7900 sequential sensing system (Applied Biosystems, Foster City, CA, USA). The cells cultured in osteogenic medium were differentiated into osteoblasts. Maturation and mineralization of osteoblasts were measured by ALP activity evaluated at day 0, 3, 7, and 14. Commercially available TaqMan Gene Expression Assays (ALP, Hs01029144_m1) and TaqMan Universal PCR Master Mix (Applied Biosystems) were used according to standard protocols. The samples were normalized (DCt) against the beta-2 gene microglobulin (B2M, Hs99999907_m1, Applied Biosystems, Foster City, CA, USA) and the -DDCt value of ALP compared to the undifferentiated medium was calculated using the DDCt method.

Statistical Analysis

For the statistical analysis, GraphPad Prism 6 Software (La Jolla, CA, USA) was used. To validate ANOVA for pairwise comparisons, post-hoc-homogeneous subsets and Stenner Student-Newman-Keuls were used. Data were expressed as mean ± SE. One-way analysis of variance was used when three or more groups within one variable were compared. To analyze two groups within each cell type, the unpaired Student's *t*-test was used. To compare data of the two cell types, the multiple t-test was used and the statistical significance was determined by using the Holm-Sidak method. Values of $p<0.05$ were considered significant.

Results

Both human AT-MSCs obtained from subcutaneous and visceral adipose tissue showed a quite homogenous pattern belonging to the MSCs family; the data were confirmed by the FACS analysis. In both adipose subsets the hematopoietic markers CD34, CD13, CD14 and CD45 were not present; while the mesenchymal surface antigen markers CD90, CD166 and CD146 showed a moderate positive expression, CD73 was moderately positive and CD29 showed a high expression. Only the CD105 marker was detected with low expression (Figure 1). Cell confluence in the basal

	Antigens	hAV-SCs phenotype	hAS-SCs phenotype
Mesenchymal markers	CD5	+	+
	CD29	+++	+++
	CD73	+	++
	CD90	+	++
	CD105	+/-	+/-
	CD117	-	-
	CD133	-	-
	CD146	+	+
	CD166	+	+
LEGEND MARKERS EXPRESSION: - Negative; +/- Low; + Moderate; ++ Positive; +++ High. Cut-off MFI Ratio positivity > 2.0			

Figure 1. Flow cytometry analysis and immune phenotype profile of undifferentiated cells.

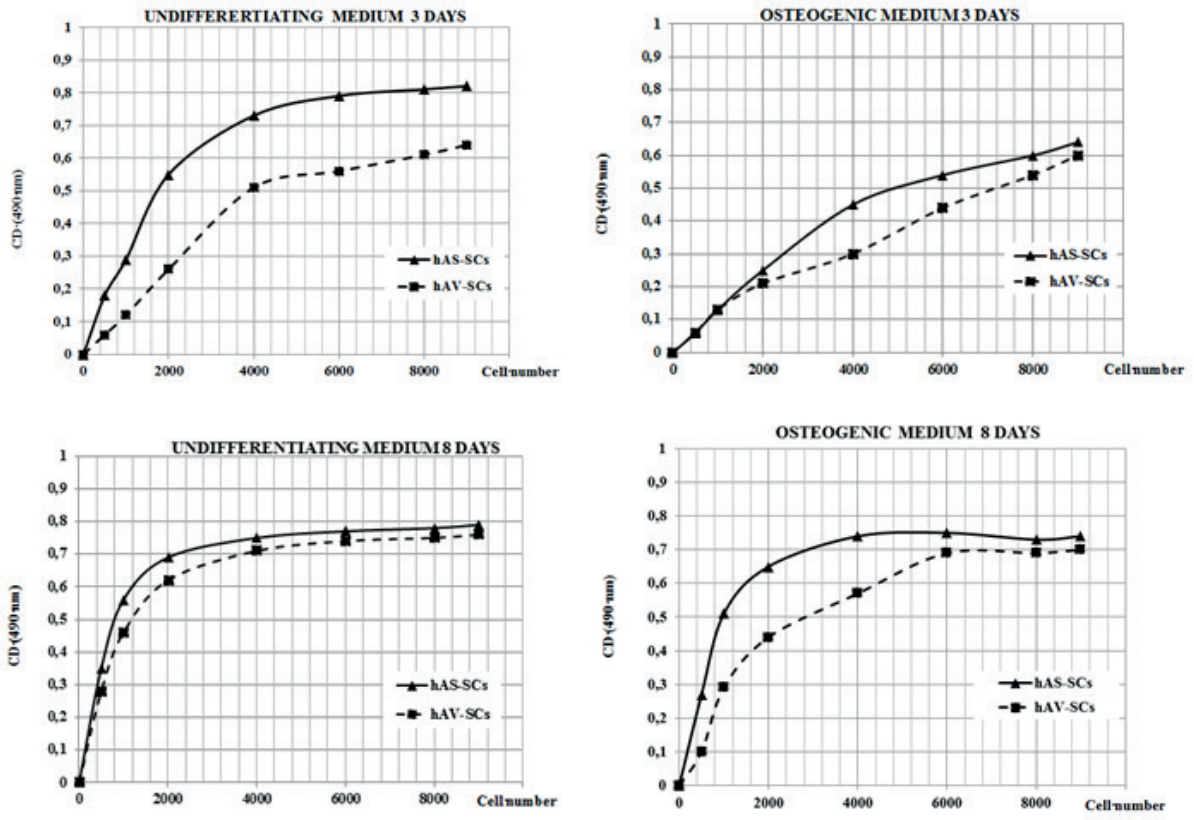

Figure 2. Different proliferative ability of MSCs from adipose tissue: cell viability – MTS assay.

medium was detected at day 10, the MTS analysis count showed a higher proliferation rate of hAS-MSCs *vs.* hAV-MSCs. Similarly, at day 8 the outcomes showed a higher osteogenic performance of hAS-MSCs *vs.* hAV-MSCs (Figure 2). The osteogenic differentiation of both cell lines was then confirmed by the positive expression of ALP, AR histochemical-staining analysis. A higher intensity of red bone aggregates in hAS-MSCs *vs.* hAV-MSCs was eventually assessed. The adipose differentiation capability of both stem cell lines was then confirmed by the OR-O assay, and

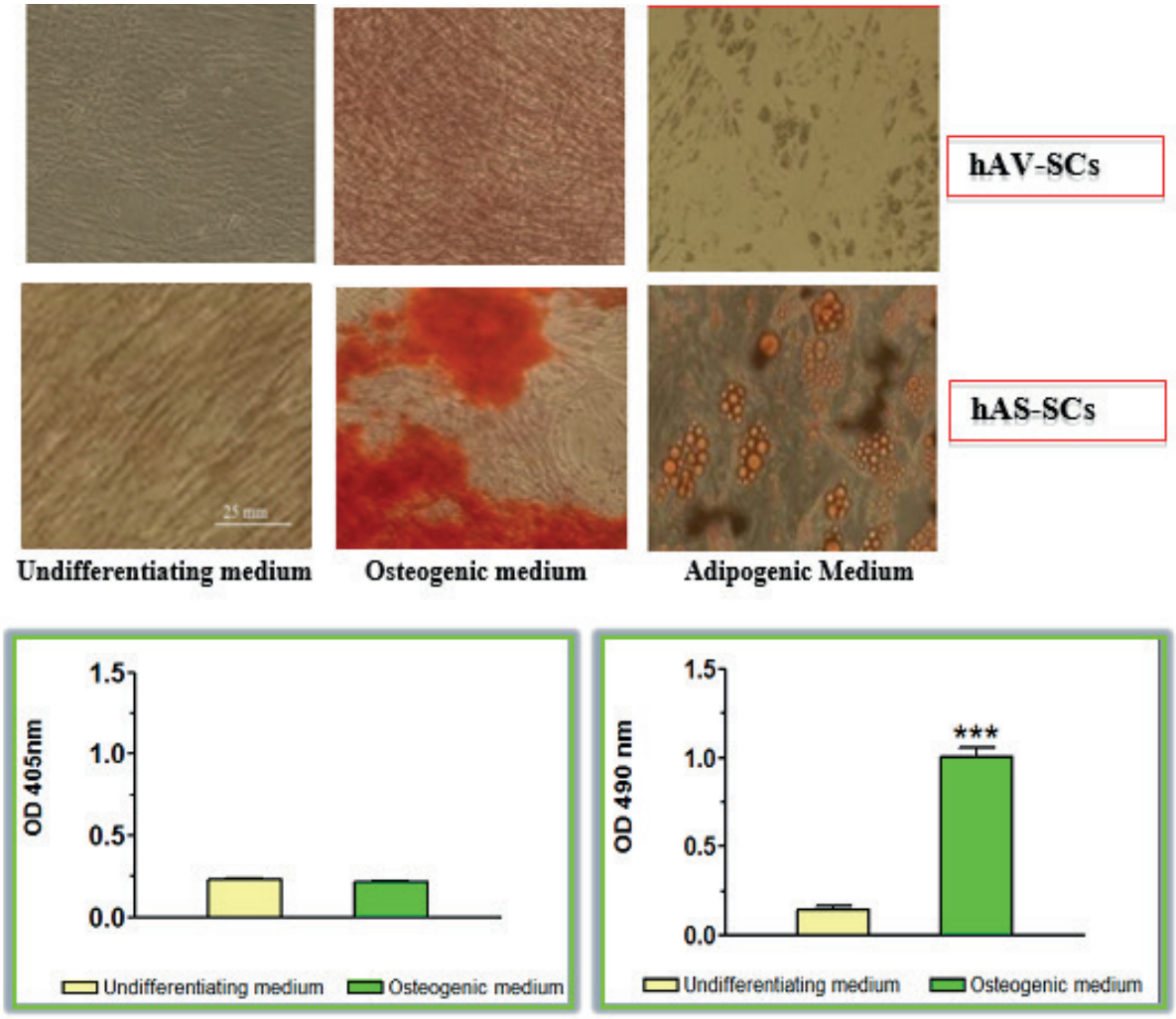

Figure 3. Alizarine Red S (central images) and Oil Red Oil assay (right images) confirmed the multipotency capability of hAV-SCs and hAS-SCs in osteogenic and adipogenic medium (Scale Bar 25 micron). Spectrophotometric Alizarine Red Assay after 21 days-Student's *t*-test (**$p<0.01$; ***$p<0.001$).

even these outcomes showed a better congruous colony formation of hAS-MSCs *vs.* hAV-MSCs (Figure 2). At 21 days the spectrophotometric ARS showed statistical significance ($p<0.001$) of hAS-MSCs colonies formation *vs.* hAV-MSCs. The ALP also confirmed the different biological behavior of hAS-SCs during osteogenesis (Figure 3). The ALP stain at day 7 revealed a higher osteogenic markers expression in hAS-MSCs compared to hAV-MSCs with statistical significance ($p<0.0001$) (Figure 4). At day 14 in osteogenic medium, the RT-PCR analysis confirmed the ALP higher expression of hAS-SCs *vs.* hAV-SCs. After 3 days, the hAV-SCs cluster showed an early lower ALP expression that continued up to 14 days (Figure 5).

Discussion

Since their first isolation attempt few decades ago, AT-MSCs have turned into a strong potential source for MSC cell-based therapy, regenerative medicine, and even drug delivery in cancer treatment.

In the present study, a typical panel consisting of MSC surface CD markers was conducted; however, the presence of unusual markers such as CD29/CD146 and CD5 among the classic MSCs CD90/CD146/CD166 was noted. These data are in line with the previously reported outcomes, where CD29 and CD146 are reported to be co-expressed in different MSC specific sub-types. The expression of CD29 could be found in embryonic stem cells (ESCs)[34] and in neural stem cells (NSCs)[35], while CD146 strong

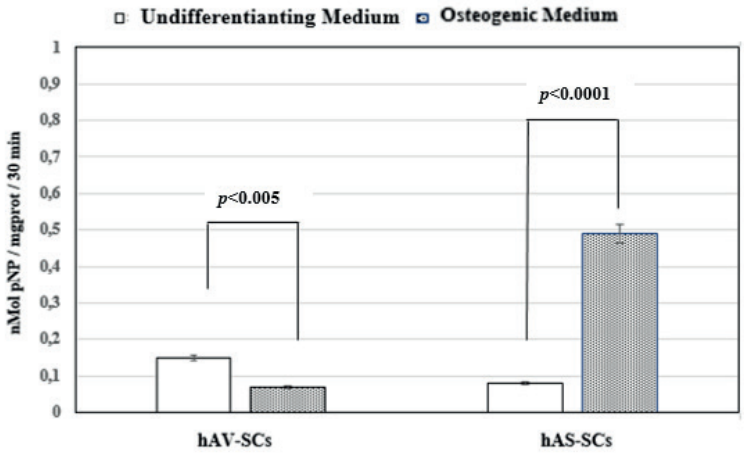

Figure 4. ALP activity statistical analysis performed with Student's *t*-test ($p<0.005$ and $p<0.0001$): as shown, the results are statistically significant marking a deep difference between the two subsets of hAT-MSCs.

expression should be found with a vascular smooth muscle cell (VSMC) phenotype together with strong calponin-1 and SM22α expression which indicate the capacity of collagen matrix recruitment[36-38]. Indeed, the presence of CD5 marker was the main intriguing event in this study, as CD5 is strictly correlated to B cell subgroup known as B-regulatory cells or Bregs and very few are the published outcomes which have reported this type of connection. Chao et al[39] mentioned the role of umbilical cord MSCs (UCB-MSCs) on Bregs as a possible molecular activity involved in reducing the inflammatory process of Crohn's disease in mice. The UCB-MSCs according to the authors, showed a beneficial effect on the up-regulation of Bregs and IL10, which, in turn, modulated the activity of pro-inflammatory Tregs/Th17 and Th1. These effects were previously explained by Qin et al[40], indicating the interventional role of SDF-1-CXCR4/CXCR7 axis secreted during the active presence of MSCs. However, the precise micro-environments, in which these CD5 progenitor stem cell precursors of B lymphocytes develop in the fetal period and in adult BM, are still largely unclear. Immunohistochemical performed by Tsuneto et al[41] revealed four expressions c-Kit(+) IL-7Rα(+) B220(low) CD19(-) SLC(-) B progenitors in contact

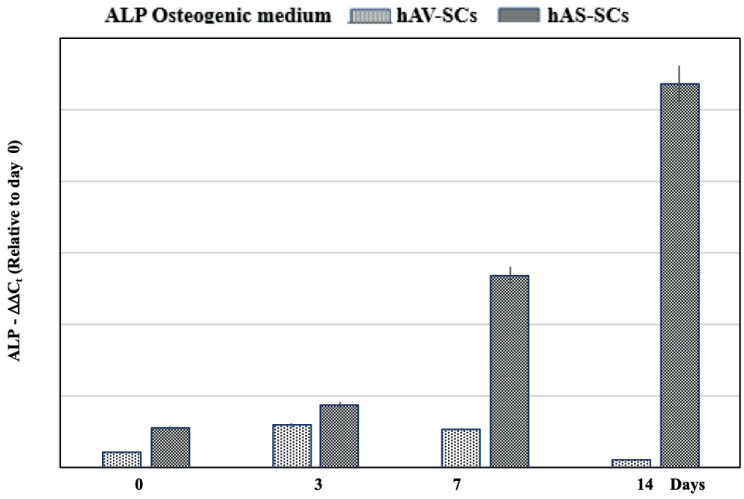

Figure 5. ALP expression results in the osteogenic medium during 14 days. It is possible to observe significant differences between the two subsets of hAT-MSCs, respectively, progressively and homogeneously expressed for hAS-MCs during the two weeks and lower and non-homogeneous, for the hAV-MCs, with the maximum at 3 days, drastically dropping on the day 14.

with vascular endothelial-type LYVE-1(high) cells on embryonic day 13.5. We may, therefore, presume that our results could be determined by the presence of very specific AT-MSCs which carried the CD5 marker from vascular pericytes of hematopoietic origin that are present within the subcutaneous and visceral adipose tissues. These data of course must be firmly validated by further and more consistent analysis and experiments. The main phenotypic differences between hAV-MSCs and hAS-MSCs were observed on CD73 and CD90 expression. The outcomes as showed in figure 1 revealed higher expression in hAS-MSCs in compare to hAV-MSCs. The two subsets also revealed consistent differences in the basal medium growth profile and in the osteogenic differentiation mode. The MTS revealed a better performance of hAS-MSCs *vs.* hAV-MSCs in both basal medium and osteogenic medium at day 3 and day 8 (Figure 2). Even the ALP and AR stain analysis showed that both hAV-MSCs and hAS-MSCs proceeded differently. In particular and partially in agreement with the outcomes from Tang et al[27], our *in vitro* hAV-MSCs showed a less significant growth rate and proliferation both in the basal and in the osteogenic medium. Human AV-MSCs revealed a lower expression rate with ALP, AR and OR-O compared to their hAS-MSCs counterparts (Figures 3-5).

Thus, in accordance with the published data, the use of hAS-SCs seems to perform better with a higher bone matrix expression revealing a more suitable therapeutic response in chondrogenic and osteogenic disorders.

The improvement of off-the-shelf MSCs, including hAS-SCs, has quickly advanced in current years with numerous clinical trials and approved products[42-44].

Many are the outcomes which have confirmed the extreme plasticity and manageability of hAS-SCs, the specific immune-modulatory features, the long-term capacity of *in vitro* cloning and, the capacity of maintaining high rate of stemness activity are all unique characteristics shared by all MSC subgroups from different sources[42-44].

Furthermore, different studies have shown the multipotent nature of hAT-MSCs and their ability to differentiate into different cell phenotypes *in vitro* under specific induction conditions such as adipocytes, osteoblasts, and chondrocytes. The marginal outcomes obtained with this pilot study allow us to endorse that there are solid arguments in the possibilities of hAT-MSCs to be used in a variety of clinical conditions, in which there are damages or injuries with a total or partial tissue loss, including traumas with avulsion wounds or in bone healing[42-44]. Due to all these qualities the hAT-MSCs may also be explored in neurodegenerative diseases clinical application. Definitely, genetically modified hAT-MSCs have been explored as possible tool to modulate the autoimmune cytotoxic responses against molecule generated by own patient's cells[45-47].

In general, the MSCs great heterogeneity makes them a valid tool also in bio-engineered genetic medicine. These MSCs could function as vectors for the transduction of corrective genes and could be used as carriers of specific anti-cancer drug molecules. The epigenetic modulation could be another venue which is currently under deep investigation, especially with regard to those diseases which require a genetic regulation and correction[48-50].

In addition, due to their general and favorable nature, these types of MSCs could be an interesting tool to be applied in medicine and dentistry for both maxillary and mandibular bone regeneration[51-58].

It can be asserted with a confident degree of certainty that inside the adipose tissue could be found a great variety of MSC subpopulations with different and ubiquitous functionality. This observation suggests functional and valuable differences among the large MSC family, essential in a prospective and large scale clinical application.

Conclusions

We suggest the potential beneficial role of adult autologous adipose tissues as MSCs source and their use in the regenerative clinical procedure. Adipose tissue-derived showed few advantages over embryonic stem cells or induced pluripotent stem cells due to their high biocompatibility, the easy access with great plasticity and non-problematic use in explanting procedures without ethical implications. Of note, the two groups, hAV-ASCs and hAS-SCs were seen capable to express both CD 5 and CD 29, features that might open the possibility of further biological clinical application.

Nevertheless, our data indicated substantial differences, the subcutaneous fat tissue showed to be a higher valuable source of MSCs used in tissue engineering and bone regeneration.

In contrast to different published results, our hAV-ASCs showed a lower viability rate with a significantly lower osteoblast differentiation capacity in osteogenic stimulation. Indeed, hAV-SCs

showed a lower ALP, AR and OR-O staining expression. Conversely, hAS-SCs have demonstrated greater qualitative and quantitative expression in ALP, AR and calcium matrix deposition. Notably, due to the superficial position of subcutaneous fat it can be easily reached and collected, it can be even found intraorally and this makes the hAS-SCs easier to be obtained and thus cultured through a minimally invasive procedure.

However, to better understand the future therapeutic appliance in clinical procedures and in tissue engineering of AT-MSCs, the specific mechanism of bone self-renewal, the interaction of AT-MSCs with other tissues, their immune-regulatory activity and their inference effects in the systemic pathways should be clearer to prevent any uncontrolled and unwanted alteration. Thus, we are well aware that additional studies and researches are still required to confirm these initial results in the perspective of the future clinical application. Regenerative medicine might become an evocative tool in reconstructive surgery whether in orthopedics, neurology and/or dentistry and will surely contribute to the accomplishment of more qualitatively results in the near future.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Author Contributions

F.M. conceived the study and collected the biological material. S.S. participated in the design of the study and in biomolecular analysis. A.G., A.S. and M.C. contributed to the isolation and expansion of mesenchymal stem cells and helped to draft the manuscript. D.D. and R.Q. gave a scientific contribution to statistical analysis. A.B. was responsible for cell culture, manuscript revision and contributed to data interpretation. S.C. contributed to data analysis, and manuscript writing.

P.C., C.G.I. and G.S. made substantial contributions to the conception of the study, experiments and coordination. G.D., M.F.C. and F.P. contributed to bibliographic research. L.L.M supervised the manuscript and gave the final approval of the version to be published. All authors read and approved the final manuscript.

References

1) Zhang XY. Biomedical engineering for health research and development. Eur Rev Med Pharmacol Sci 2015; 19: 220-224.
2) Ali F, Taresh S, Al-Nuzaily M, Mok PL, Ismail A, Ahmad S. Stem cells differentiation and probing their therapeutic applications in hematological disorders: a critical review. Eur Rev Med Pharmacol Sci 2016; 20: 4390-4400.
3) Mushahary D, Spittler A, Kasper C, Weber V, Charwat V. Isolation, cultivation, and characterization of human mesenchymal stem cells. Cytometry A 2018; 93: 19-31.
4) Di Benedetto A, Posa F, De Maria S, Ravagnan G, Ballini A, Porro C, Trotta T, Grano M, Muzio LL, Mori G. Polydatin, natural precursor of resveratrol, promotes osteogenic differentiation of mesenchymal stem cells. Int J Med Sci 2018; 13: 944-952.
5) Dong CH, Deng YS, Yang XJ, Liu J, Liu R, Hou FY, Li SS, Zhen P. The interplay of transcriptional and post-transcriptional regulation of migration of mesenchymal stem cells during early stages of bone fracture healing. Eur Rev Med Pharmacol Sci 2017; 21: 5542-5547.
6) Li JP, Zhuang HT, Xin MY, Zhou YL. MiR-214 inhibits human mesenchymal stem cells differentiating into osteoblasts through targeting β-catenin. Eur Rev Med Pharmacol Sci 2017; 21: 4777-4783.
7) Fan MP, Si M, Li BJ, Hu GH, Hou Y, Yang W, Liu L, Tang B, Nie L. Cell therapy of a knee osteoarthritis rat model using precartilaginous stem cells. Eur Rev Med Pharmacol Sci 2018; 22: 2119-2125.
8) Zhao J, Xu JJ. Experimental study on application of polypropylene hernia of fat stem cells in rats. Eur Rev Med Pharmacol Sci 2018; 22: 6156-6161.
9) Tan K, Peng YT, Guo P. MiR-29a promotes osteogenic differentiation of mesenchymal stem cells via targeting HDAC4. Eur Rev Med Pharmacol Sci 2018; 22: 3318-3326.
10) Ballini A, Scacco S, Coletti D, Pluchino S, Tatullo M. Mesenchymal stem cells as promoters, enhancers, and playmakers of the translational regenerative medicine. Stem Cells Int 2017; 2017: 3292810; doi: 10.1155/2017/3292810.
11) L Ramos T, Sánchez-Abarca LI, Muntión S, Preciado S, Puig N, López-Ruano G, Hernández-Hernández Á, Redondo A, Ortega R, Rodríguez C, Sánchez-Guijo F, del Cañizo C. MSC surface markers (CD44, CD73, and CD90) can identify human MSC-derived extracellular vesicles by conventional flow cytometry. Cell Commun Signal 2016; 14: 2; doi: 10.1186/s12964-015-0124-8.
12) Ballini A, Boccaccio A, Saini R, Van Pham P, Tatullo M. Dental-derived stem cells, their secretome and interactions with bioscaffolds/biomaterials in regenerative medicine: from the in-vitro research to translational applications. Stem Cells Int 2017; 2017: 6975251; doi: 10.1155/2017/6975251.
13) Raposio E, Bertozzi N. How to isolate a ready-to-use adipose-derived stem cells pellet for clinical application. Eur Rev Med Pharmacol Sci 2017; 21: 4252-4260.
14) Marrelli M, Gentile S, Palmieri F, Paduano F, Tatullo M. Correlation between surgeon's experience, surgery complexity and the alteration of stress related physiological parameters. PLoS One 2014; 9: e112444; doi: 10.1371/journal.pone.0112444.

15) Barbagallo I, Vanella L, Distefano A, Nicolosi D, Maravigna A, Lazzarino G, Di Rosa M, Tibullo D, Acquaviva R, Li Volti G. Moringa oleifera Lam. improves lipid metabolism during adipogenic differentiation of human stem cells. Eur Rev Med Pharmacol Sci 2016; 20: 5223-5232.

16) Liu DC, Yang XN, Huang CZ, Sun YG, Dai XM. Experimental study on co-culturing adipose-derived stem cells with osteoblasts under different conditions. Eur Rev Med Pharmacol Sci 2016; 20: 3535-3543.

17) Grygiel-Górniak B, Puszczewicz M. A review on irisin, a new protagonist that mediates muscle-adipose-bone-neuron connectivity. Eur Rev Med Pharmacol Sci 2017; 21: 4687-4693.

18) Qiao L, Liu D, Li CG, Wang YJ. MiR-203 is essential for the shift from osteogenic differentiation to adipogenic differentiation of mesenchymal stem cells in postmenopausal osteoporosis. Eur Rev Med Pharmacol Sci 2018; 22: 5804-5814.

19) Mori G, Brunetti G, Ballini A, Di Benedetto A, Tarantino U, Colucci S, Grano M. Biological characteristics of dental stem cells for tissue engineering. Key Engineering Materials 2013; 541: 51-59.

20) Di Benedetto A, Posa F, Carbone C, Cantore S, Brunetti G, Centonze M, Grano M, Lo Muzio L, Cavalcanti-Adam EA, Mori G. NURR1 downregulation favors osteoblastic differentiation of MSCs. Stem Cells Int 2017; 2017: 7617048.

21) Wang LJ, Liu LP, Gu XL, Wang M, Liu LM. Implantation of adipose-derived stem cells cures the optic nerve injury on rats through inhibiting the expression of inflammation factors in the TLR4 signaling pathway. Eur Rev Med Pharmacol Sci 2018; 22: 1196-1202.

22) Yu WY, Sun W, Yu DJ, Zhao TL, Wu LJ, Zhuang HR. Adipose-derived stem cells improve neovascularization in ischemic flaps in diabetic mellitus through HIF-1α/VEGF pathway. Eur Rev Med Pharmacol Sci 2018; 22: 10-16.

23) Tran CT, Huynh DT, Gargiulo C, Tran le BH, Huynh MH, Nguyen KH, Filgueira L, Strong DM. Adipose tissue can be generated in vitro by using adipocytes from human fat tissue mesenchymal stem cells seeded and cultured on fibrin gel sheet. Cell Tissue Bank 2013;14: 97-106.

24) Tran TC, Gargiulo C, Huynh TD, Thien Bui KH, Filgueira L, Strong DM. Adipocytes and osteoblasts from human adipose tissue mesenchymal stem cells for the production of compatible and safe biomaterial crucial in cosmetic, reconstructive, and regenerative medicine. Springer-Verlag Berlin Heidelberg 2014; 85-109.

25) Paduano F, Marrelli M, Amantea M, Rengo C, Rengo S, Goldberg M, Spagnuolo G, Tatullo M. Adipose tissue as a strategic source of mesenchymal stem cells in bone regeneration: a topical review on the most promising craniomaxillofacial applications. Int J Mol Sci 2017; 18: pii: E2140; doi: 10.3390/ijms18102140.

26) Choudhery MS, Badowski M, Muise A, Pierce J, Harris DT. Subcutaneous adipose tissue-derived stem cell utility is independent of anatomical harvest site. Biores Open Access 2015; 4: 131-145.

27) Tang Y, Pan ZY, Zou Y, He Y, Yang PY, Tang QQ, Yin F. A comparative assessment of adipose-derived stem cells from subcutaneous and visceral fat as a potential cell source for knee osteoarthritis treatment. J Cell Mol Med 2017; 21:2153-2162.

28) Tozawa K, Ono-Uruga Y, Yazawa M, Mori T, Murata M, Okamoto S, Ikeda Y, Matsubara Y. Unique megakaryocytes and platelets from novel human adipose-derived mesenchymal stem cell line. Blood 2019; 04: 842641.

29) Huang YC, Ning H, Shindel AW, Fandel TM, Lin G, Harraz AM, Lue TF, Lin CS. The effect of intracavernous injection of adipose tissue-derived stem cells on hyperlipidemia-associated erectile dysfunction in a rat model. J Sex Med 2010; 7: 1391-1400.

30) Pak J, Lee JH, Lee SH. Regenerative repair of damaged meniscus with autologous adipose tissue-derived stem cells. BioMed Res Int 2014; 2014: 436029.

31) Chau Y, Bandiera R, Serrels A, Martínez-Estrada OM, Qing W, Lee M, Slight J, Thornburn A, Berry R, McHaffie S, Stimson RH, Walker BR, Chapuli RM, Schedl A, Hastie N. Visceral and subcutaneous fat have different origins and evidence supports a mesothelial source. Nat Cell Biol 2014;16: 367-375.

32) Kim B, Lee B, Kim MK, Gong SP, Park NH, Chung HH, Kim HS, No JH, Park WY, Park AK, Lim JM, Song YS. Gene expression profiles of human subcutaneous and visceral adipose-derived stem cells. Cell Biochem Funct 2016; 34: 563-571.

33) Lennon DP, Haynesworth SE, Arm DM, Baber MA, Caplan AI. Dilution of human mesenchymal stem cells with dermal fibroblasts and the effects on in vitro and in vivo osteochondrogenesis. Dev Dyn 2000; 219: 50-62.

34) Zhao W, Ji X, Zhang F, Li L, Ma L. Embryonic stem cell markers. Molecules 2012; 17: 6196-6236.

35) Pruszak J, Ludwig W, Blak A, Alavian K, Isacson O. CD15, CD24, and CD29 define a surface biomarker code for neural lineage differentiation of stem cells. Stem Cells 2009; 27: 2928-2940.

36) Espagnolle N, Guilloton F, Deschaseaux F, Gadelorge M, Sensébé L, Bourin P. CD146 expression on mesenchymal stem cells is associated with their vascular smooth muscle commitment. J Cell Mol Med 2014; 18: 104-114.

37) Grassi FR, Pappalettere C, Di Comite M, Corsalini M, Mori G, Ballini A, Crincoli V, Pettini F, Rapone B, Boccaccio A. Effect of different irrigating solutions and endodontic sealers on bond strength of the dentin-post interface with and without defects. Int J Med Sci 2012; 9: 642-54.

38) Paduano F, Marrelli M, Palmieri F, Tatullo M. CD146 Expression influences periapical cyst mesenchymal stem cell properties. Stem Cell Rev 2016; 12: 592-603.

39) Chao K, Zhang S, Qiu Y, Chen X, Zhang X, Cai C, Peng Y, Mao R, Pevsner-Fischer M, Ben-Horin S, Elinav E,

Zeng Z, Chen B, He Y, Xiang AP, Chen M. Human umbilical cord-derived mesenchymal stem cells protect against experimental colitis via CD5(+) B regulatory cells. Stem Cell Res Ther 2016; 7: 109.

40) Qin Y, Zhou Z, Zhang F, Wang Y, Shen B, Liu Y, Guo Y, Fan Y, Qiu J. Induction of regulatory b-cells by mesenchymal stem cells is affected by SDF-1α-CXCR7. Cell Physiol Biochem 2015; 37: 117-130.

41) Tsuneto M, Tokoyoda K, Kajikhina E, Hauser AE, Hara T, Tani-Ichi S, Ikuta K, Melchers F. B-cell progenitors and precursors change their microenvironment in fetal liver during early development. Stem Cells 2013; 31: 2800-2812.

42) Truong NC, Bui KH, Van Pham P. Characterization of senescence of human adipose-derived stem cells after long-term expansion. Adv Exp Med Biol 2018; doi: 10.1007/5584_2018_235

43) Carbone A, Valente M, Annacontini L, Castellani S, Di Gioia S, Parisi D, Rucci M, Belgiovine G, Colombo C, Di Benedetto A, Mori G, Lo Muzio L, Maiorella A, Portincasa A, Conese M. Adipose-derived mesenchymal stromal (stem) cells differentiate to osteoblast and chondroblast lineages upon incubation with conditioned media from dental pulp stem cell-derived osteoblasts and auricle cartilage chondrocytes. J Biol Regul Homeost Agents 2016; 30: 111-122.

44) Cantore S, Crincoli V, Boccaccio A, Uva AE, Fiorentino M, Monno G, Bollero P, Derla C, Fabiano F, Ballini A, Santacroce L. Recent advances in endocrine, metabolic and immune disorders: mesenchymal stem cells (MSCs) and engineered scaffolds. Endocr Metab Immune Disord Drug Targets 2018; 18: 466-469.

45) Niccoli Asabella A, Simone M, Ballini A, Altini C, Ferrari C, Lavelli V, De Luca R, Inchingolo F, Rubini G. Predictive value of 18F-FDG PET/CT on survival in locally advanced rectal cancer after neoadjuvant chemoradiation. Eur Rev Med Pharmacol Sci 2018; 22: 8227-8236.

46) Pettini F, Savino M, Corsalini M, Cantore S, Ballini A. Cytogenetic genotoxic investigation in peripheral blood lymphocytes of subjects with dental composite restorative filling materials. J Biol Regul Homeost Agents 2015; 29: 229-233.

47) Lovreglio P, Bukvic N, Fustinoni S, Ballini A, Drago I, Foà V, Guanti G, Soleo L. Lack of genotoxic effect in workers exposed to very low doses of 1,3-butadiene. Arch Toxicol 2006; 80: 378-381.

48) Brunetti G, Di Benedetto A, Posa F, Colaianni G, Faienza MF, Ballini A, Colucci S, Passeri G, Lo Muzio L, Grano M, Mori G. High expression of TRAIL by osteoblastic differentiated dental pulp stem cells affects myeloma cell viability. Oncol Rep 2018; 39: 2031-2039.

49) Mori G, Brunetti G, Colucci S, Oranger A, Ciccolella F, Sardone F, Pignataro P, Mori C, Karapanou V, Ballini A, Mastrangelo F, Tete S, Grassi FR, Grano M. Osteoblast apoptosis in periodontal disease: role of TNF-related apoptosis-inducing ligand. Int J Immunopathol Pharmacol 2009; 22: 95-103.

50) Ballini A, Tetè S, Scattarella A, Cantore S, Mastrangelo F, Papa F, Nardi GM, Perillo L, Crincoli V, Gherlone E, Grassi FR. The role of anti-cyclic citrullinated peptide antibody in periodontal disease. Int J Immunopathol Pharmacol 2010; 23: 677-681.

51) Ballini A, Mastrangelo F, Gastaldi G, Tettamanti L, Bukvic N, Cantore S, Cocco T, Saini R, Desiate A, Gherlone E, Scacco S. Osteogenic differentiation and gene expression of dental pulp stem cells under low-level laser irradiation: a good promise for tissue engineering. J Biol Regul Homeost Agents 2015; 29: 813-822.

52) Ballini A, Scattarella A, Crincoli V, Carlaio RG, Papa F, Perillo L, Romanazzo T, Bux MV, Nardi GM, Dituri A, Cantore S, Pettini F, Grassi FR. Surgical treatment of gingival overgrowth with 10 years of follow-up. Head Face Med 2010 12; 6: 19; doi: 10.1186/1746-160X-6-19.

53) Cantore S, Ballini A, De Vito D, Martelli FS, Georgakopoulos I, Almasri M, Dibello V, Altini V, Farronato G, Dipalma G, Farronato D, Inchingolo F. Characterization of human apical papilla-derived stem cells. J Biol Regul Homeost Agents 2017; 31: 901-910.

54) Ballini A, Cantore S, Scacco S, Perillo L, Scarano A, Aityan SK, Contaldo M, Cd Nguyen K, Santacroce L, Syed J, De Vito D, Dipalma G, Gargiulo Isacco C, Inchingolo F. A comparative study on different stemness gene expression between dental pulp stem cells vs dental bud stem cells. Eur Rev Med Pharmacol Sci 2019; 23:1626-1633.

55) Boccaccio A, Uva AE, Fiorentino M, Monno G, Ballini A, Desiate A. Optimal load for bone tissue scaffolds with an assigned geometry. Int J Med Sci 2018; 15: 16-22.

56) Ballini A, Cantore S, Altini C, Dipalma G, Ferrari C, Niccoli Asabella A, Rubini G, Inchingolo F. 99mTc-diphosphonates bone scintigraphy for vitality evaluation in cleft palate. Eur Rev Med Pharmacol Sci 2018; 22: 8497-8501.

57) Dohan Ehrenfest DM, Del Corso M, Inchingolo F, Charrier JB. Selecting a relevant in vitro cell model for testing and comparing the effects of a Choukroun's platelet-rich fibrin (PRF) membrane and a platelet-rich plasma (PRP) gel: tricks and traps. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010; 110: 409-411.

58) Dohan Ehrenfest DM, Del Corso M, Inchingolo F, Sammartino G, Charrier JB. Platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) in human cell cultures: growth factor release and contradictory results. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010; 110: 418-421.