Plastic Adherence Method for Isolation of Stem Cells Derived from Infrapatellar Fat Pad

MIHAI HURMUZ[1], FLORINA BOJIN[2,3]*, MIHAI IONAC[4], FABIAN TATU[1], DANIELA PUSCASIU[5], CARMEN TATU[2,3]

[1] Victor Babes University of Medicine and Pharmacy Timisoara, Department of Orthopaedics, Urology and Medical Imaging, 2A Eftimie Murgu Sq., 300041, Timisoara, Romania

[2] Victor Babes University of Medicine and Pharmacy Timisoara, Department of Functional Sciences, 2A Eftimie Murgu Sq., 300041, Timisoara, Romania

[3] Pius Brinzeu Emergency Clinical County Hospital Timisoara, Regional Center of Transplant Immunology, 156 Liviu Rebreanu Str., 300723, Timisoara, Romania

[4] Victor Babes University of Medicine and Pharmacy Timisoara, 2nd Department of Surgery, 2A Eftimie Murgu Sq., 300041, Timisoara, Romania

[5] Victor Babes University of Medicine and Pharmacy Timisoara, Department of Microscopic Morphology, 2A Eftimie Murgu Sq., 300041, Timisoara, Romania

Stem cells are nowadays isolated from multiple sources and used in biotechnological application. Our study presents a new method for isolation and expansion of stem cells derived from infrapatellar fat pad (IPFP) by simple adherence to plastic surface of culture flasks. This method yielded adipose-derived stromal cells (ASC) with morphological and phenotypical characteristics of mesenchymal stem cells, without using any enzymatic digestion, making these cells more suitable for medical application, in treatment of different human pathologies.

Keywords: stem cells, infrapatellar fat pad (IPFP), adipose stem cells (ASC), plastic adherence

Stem cells represent cells capable of self-renewal and cells that have the capacity to differentiate into multiple lineages [1-3]. Multipotent stem cells can be harvested from several sources like bone marrow, muscle, trabecular bone, umbilical cord, amniotic fluid, and adipose tissue [4-7]. Mesenchymal stem cells (MSCs) are adult stem cells that were originally identified in bone marrow as multipotent cells. Bone marrow-derived mesenchymal stem cells have been studied extensively, but adipose-derived stromal cells (ASC) represent an easily accessible cell type that may substitute for BM-derived MSCs [8]. The abundance of tissue and the simplified harvesting process have made the adipose tissue a new source for providing cells for regenerative medicine [9]. The minimal criteria that define the mesenchymal stem cells (MSCs) set by the International Society for Cellular Therapy include the presence of CD105, CD73 and CD90 surface marker, the lack of CD45, CD34, CD14, CD31, HLA-DR markers, their capacity to adhere to plastic and to differentiate into osteogenic, chondrogenic and adipogenic lineage [10]. Plastic adherence is a property of MSC, and even unique subsets of MSC that have been described maintain this property [11,12, 19].

The plastic used in Petri dishes used for cell culture is made of polystyrene, a very clear and with appearance much like glass, which have good heat, oil and chemical resistance [13, 14, 19].

The need to find new sources of fat tissue for the treatment of different pathologies has opened a perspective for the infrapatellar fat pad (IPFP). This mass of fat is standardly excised during total knee arthroplasties and can also be exploit during knee arthroscopy.

Experimental part

Harvesting of infrapattelar fat pad

We have harvested, with the written consent of the patients, the infrapatellar fat pad from 4 patients admitted to the Orthopaedic Department from the Military Emergency Hospital Timisoara. Two of the patients have undergone total knee arthroplasty and two of them arthroscopic anterior cruciate ligament reconstruction.

The tissue harvested was then placed into PBS mixed with Streptomycin/Penicillin 1%. All the samples were transferred to the Immunology Laboratory from Emergency County Hospital Timisoara where all the specimens were analyzed. All tissue samples were obtained after signing the written informed consent elaborated under an approved protocol by the Ethics Committee of Victor Babes University of Medicine and Pharmacy Timisoara, according to the World Medical Association Declaration of Helsinki.

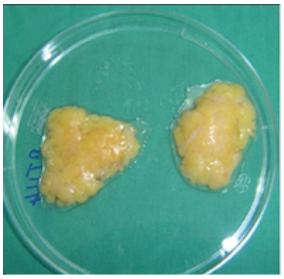

Fig.1. Samples from the harvested infrapattelar fat pad

Isolation of adipose-derived stem cells

The infrapattelar fat was minced using a sterile scalpel, and adipose-derived cells were isolated by explants method, based on plastic-adherence properties of the cells. The Petri dishes used for this experiment weight about 8 grams and are made from Polystyrene material free of heavy metal. The material is able to resist a wide range of temperatures from -20 to even +60. The Petri dishes are aseptically manufactured under clean conditions ensuring a free of detectable DNase/RNase environment with a probability of detecting a living organism less or equal to $1 \cdot 10^{-3}$ (1 colony per 1000 products). For superior clarity for microscopic examination are optically clear and also the

* email: florinabojin@umft.ro; Tel: 0729580609

	Sample 1	Sample 2	Sample 3	Sample 4
Sex	Male	Female	Male	Male
Age (years)	23	65	67	70
Surgical procedure	Arthroscopic	Opened	Opened	Opened
Fat pad weight (g)	10.30	12.20	11.35	10.47
Number of ASCs isolation (P1*) (x10^6 cells)	6.5	7.2	5.8	6.75

*1st passage

Table 1
CHARACTERISTICS OF THE SAMPLES USED FOR OBTAINING ASCs

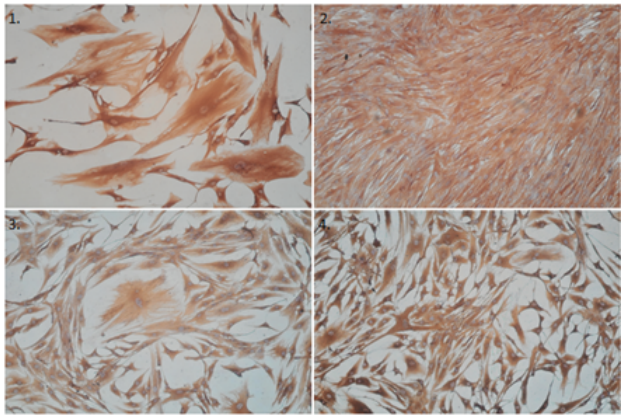

Fig. 2. Vimentin expression in IPFP-derived ASCs obtained from all samples harvested. The ASCs grown *in vitro*, in plastic culture flasks show homogenous expression of this cytoskeleton marker

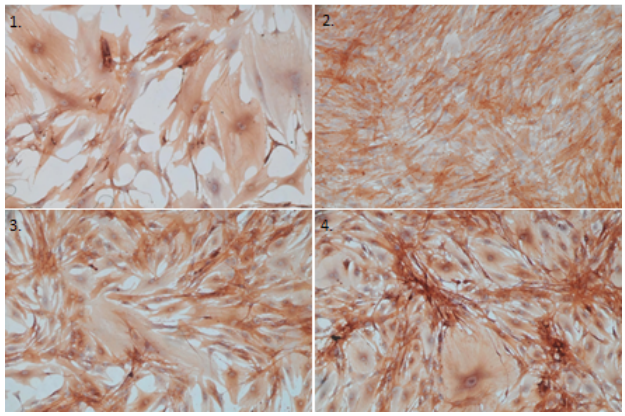

Fig. 3. CD105 (endoglin) expression of IPFP-derived ASCs. Cells obtained from all samples were positive for this stem cell marker

outstanding flatness of the growth surface allos uniform cell growth without clumping [15].

The culture and expansion media was alpha-minimum essential medium (MEM; Gibco BRL, Carlsbad, CA, USA), supplemented with 10% Fetal Calf Serum (FCS; PromoCell, Heidelberg, Germany) and 1% Penicillin/Streptomycin solution (10,000 IU/mL; PromoCell, Heidelberg, Germany). Cells were used in the following experiments at passages 2-5.

Immunocytochemical analyses

Infrapatellar fat-derived stem cells prepared for these analyses were fixed with methanol and investigated for expression of interest markers, employing mouse anti-human antibodies for vimentin (clone V9), endoglin, CD105 (clone SN6h), Ki-67 antigen (clone MIB-1), and cytokeratin (clone MNF116). All primary antibodies were provided by DakoCytomation (Glostrup, Denmark), and tested for human specificity and cross-reactivity. Staining protocol continued with secondary biotinylated antibody binding, substrate addition, and hematoxylin counterstaining of the nuclei (DakoEnVision Systems-HRP) following the manufacturer procedures.

Flowcytometry analysis

Cultured cells reaching 80-90% confluence were detached from the culture plate, washed and stained with mouse anti-human conjugated antibodies following the manufacturer instructions (BD Pharmingen™, Heidelberg, Germany). PE-conjugated - CD 29 and CD 73, and FITC-conjugated - CD44 and CD90 antibodies were used for data acquisition on FACSCalibur flowcytometer (BD Biosciences), while analyses were performed using Flowing Software 2.5.1 dedicated software.

Results and discussions

The baseline characteristics of the IPFP-ASCs are presented in table 1. Patients were of both genders, various ages, and suffered different surgical procedures for harvesting the IPFP. The weight was relatively homogenous and the samples yielded weight-independent number of ASCs.

In optic microscopy, the cells obtained from IPFP samples shared similar morphological aspect, being adherent to plastic culture flasks, with fibroblast-like morphology and variation in cells size. Immuno-cytochemical staining showed that the cells are positive for Vimentin and CD105 (fig. 2 and 3), while being negative for CD117 and Cytokeratin (fig. 4 and 5), which is accordant with the stem cells definition criteria.

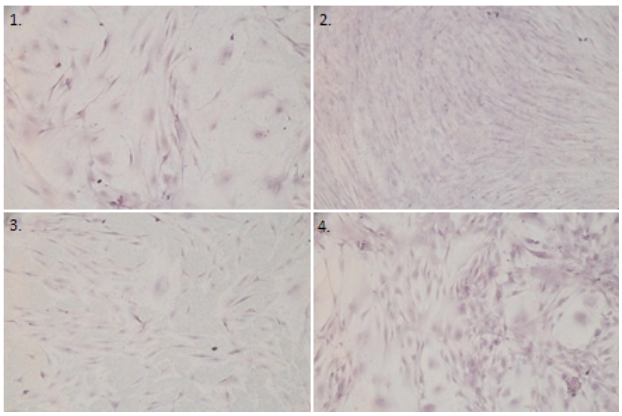

Fig. 4. CD117 expression of IPFP-derived ASCs. Cells obtained from all samples were negative for this marker

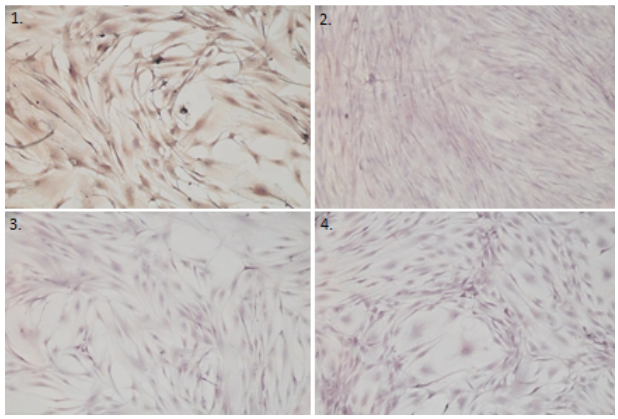

Fig. 5. Expression of cytokeratin in IPFP-derived ASC. Cells obtained from sample 1 showed slightly positive expression of this marker on the corners of the culture flasks at passage 1; this expression disappeared on further passages

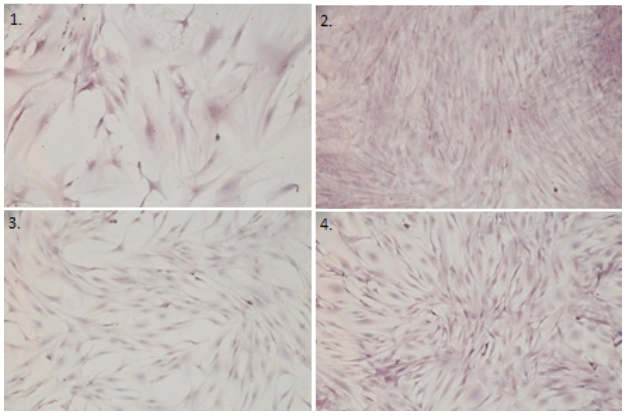

Fig. 6. Growth rate evaluated by Ki-67 index in IPFP-derived ASCs. All cell cultures presented similar proliferation index at all studied passages (P2-P5)

The proliferation rate was estimated by the Ki-67 staining, cells were counted on 5 different microscopic fields, and the fraction of Ki-67-positive ASCs (the Ki-67 labelling index) was determined. The results showed that ASCs obtained from different IPFP samples have similar proliferation rate, not dependent on gender or age (Ki-67% = 10-12%) (fig. 6).

To further characterize the IPFP-derived ASCs, we assessed the presence of cell surface markers by flowcytometry. Flowcytometric analysis indicated that the ASCs maintain their cell surface markers at different periods of cell culture (P2-P5), being positive for CD90 (95.75%±1.5%),CD44 (97.34%±1.2%), CD29 (92.44%± 0.9%), and CD73 (90.23%±1.24%) (fig. 7). The cells isolated from all samples were negative for CD45, CD34, and CD31 markers (data not shown), which is in accordance with stem cells definition criteria. Other studies showed similar values of markers determined by flowcytometry in humans and other species [16,17].

The use of stem cells in bone and cartilage regeneration holds great promises, and ASCs could be principal progenitors of several musculoskeletal tissues. Adipose tissue is more accessible than bone marrow for obtaining stem cells, and our isolation method using plastic adherence properties of these cells makes them more suitable for clinical application than the use of enzymatic digestion method employed in most of the studies [18].

Conclusions

The results of this study showed that ASCs derived from IPFP, isolated using the plastic adherence method, have all the morphological and phenotypical characteristics of stem cells. These characteristics are maintained during different cellular passages, while the cells also retain their proliferative capability. Although the amount of infrapatellar fat and the harvesting method employed were different between samples, the number of isolated ASCs was similar, thus suggesting that there is no correlation between these parameters and large amounts of ASCs can be obtained using this non-enzymatic procedure. To conclude, IPFP-derived ASCs can be an alternative source of stem cells which can be used in various procedures of tissue engineering.

Acknowledgement: This work was supported by UEFISCDI, PNII–Idei, Project Nos. 318/2011 and 259/2011.

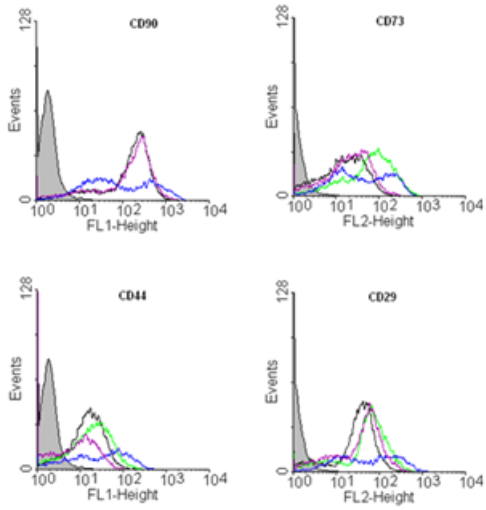

Fig. 7. Histogram representation of cell surface markers expression obtained by flowcytometric analysis of IPFP-derived ASCs

References

1.HWANG, J.H., KIM, I.G., PIAO, S., JUNG, A,R., LEE, J.Y., PARK, K.D., LEE, J.Y., Combination therapy of human adipose-derived stem cells and basic fibroblast growth factor hydrogel in muscle regeneration. Biomaterials. 2013;**b**: p 6037-45.

2.LIN, Y.C., GRAHOVAC, T., OH, S.J., IERACI, M., RUBIN, J.P., MARRA, K.G., Evaluation of a multi-layer adipose-derived stem cell sheet in a full-thickness wound healing model. Acta Biomater. 2013;**9(2)**: p 5243-50.

3.ROUSSEAU, A., FRADETTE, J., BERNARD, G., GAUVIN, R., LATERREUR, V., BOLDUC, S., Adipose-derived stromal cells for the reconstruction of a human vesical equivalent. J Tissue Eng Regen Med. 2015;**9(11)**:E p 135-43.

4.ZHANG, R., JACK, G.S., RAO, N., ZUK, P., IGNARRO, L.J., WU, B., RODRÍGUEZ, L.V., Nuclear fusion-independent smooth muscle differentiation of human adipose-derived stem cells induced by a smooth muscle environment. Stem Cells. 2012;**30(3)**: p 481-90.

5.ZUK, P.A., ZHU, M., MIZUNO, H., HUANG, J., FUTRELL, J.W., KATZ, A.J., BENHAIM, P., LORENZ, H.P., HEDRICK, M.H., Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. 2001;**7(2)**: p 211-28.

6.GIMBLE, J.M., Stem cells bleed into brown fat. Cell Metab. 2012;**16(3)**: p 288-9.

7.HEYDARKHAN-HAGVALL, S., SCHENKE-LAYLAND, K., YANG, J.Q., HEYDARKHAN, S., XU, Y., ZUK, P.A., MACLELLAN, W.R., BEYGUI, R.E., Human adipose stem cells: a potential cell source for cardiovascular tissue engineering. Cells Tissues Organs. 2008;**187(4)**:p263-74.

8.GRONTHOS, S., FRANKLIN, D.M., LEDDY, H.A., ROBEY, P.G., STORMS, R.W., GIMBLE, J.M., Surface protein characterization of human adipose tissue-derived stromal cells. J Cell Physiol. 2001;**189(1)**:p54-63.

9.KUANG-HUNG, C., TZU-LEI, K., KUNG-KAI, K., CHANG-CHUN, H., Human adipose-derived stem cells: Isolation, characterization and current application in regeneration medicine. Genomic Med Biomarkers Health Sci 2011;**3**:p53-62.

10.DOMINICI, M., LE BLANC, K., MUELLER, I., SLAPER-CORTENBACH, I., MARINI, F., KRAUSE, D., DEANS, R., KEATING, A., PROCKOP, D.J., HORWITZ, E., Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;**8(4)**:p315-7.

11.COLTER, D.C., CLASS, R., DIGIROLAMO, C.M., PROCKOP, D.J., Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. Proc Natl Acad Sci U S A. 2000;**97(7)**:p3213-8.

12.JIANG, Y., JAHAGIRDAR, B.N., REINHARDT, R.L., SCHWARTZ, R.E., KEENE, C.D., ORTIZ-GONZALEZ, X.R., REYES, M., LENVIK, T., LUND, T., BLACKSTAD, M., DU, J., ALDRICH, S., LISBERG, A., LOW, W.C., LARGAESPADA, D.A., VERFAILLIE, C.M., Pluripotency of mesenchymal

stem cells derived from adult marrow. Nature. 2002;**418(6893)**:p41-9.
13.WILLIAM, A., General purpose polystyrene resins. In Handbook of Plastics Joining: A Practical Guide, 1997, Ed. Technology & Engineering.
14.NAVARRETE, L., HERMANSON, N., The changes in ethylene oxide sterilization and their effect on thermoplastics, Medical Plastics, 2004, **1996** p 51-64.
15.TERUO O., NORIKO Y., MINAKO O., HIDEAKI S., YASUHISA S., Mechanism of cell detachment from temperature-modulated, hydrophilic-hydrophobic polymer surfaces. Biomaterials. 1995; **16(4)**, p297-303
16.SKALSKA, U., KONTNY, E., Adipose-derived mesenchymal stem cells from infrapatellar fat pad of patients with rheumatoid arthritis and osteoarthritis have comparable immunomodulatory properties. Autoimmunity. 2016;**49(2)**:p124-31.
17.VAHEDI, P., SOLEIMANIRAD, J., ROSHANGAR, L., SHAFAEI, H., JAROLMASJED, S., NOZAD CHAROUDEH, H., Advantages of sheep infrapatellar fat pad adipose tissue derived stem cells in tissue engineering. Adv Pharm Bull. 2016;**6(1)**:105-10.
18.TANGCHITPHISUT, P., SRIKAEW, N., NUMHOM, S., TANGPRASITTIPAP, A., WORATANARAT, P., WONGSAK, S., KIJKUNASATHIAN, C., HONGENG, S., MURRAY, I.R., TAWONSAWATRUK, T., Infrapatellar fat pad: an alternative source of adipose-derived mesenchymal stem cells. Arthritis. 2016;**2016**:4019873.
19.GHIBAN,N., BORTUN,M., BORDEASU, I., GHIBAN, B., FAUR,N., CERNESCU, A., Evaluation of mechanical properties by stereo-and scanning electron microscopy of some heat curing dental resins, Mat. Plast., **47**, no.2, 2010, p.240

Manuscript received: 9.01.2016