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Original article

Adipose tissue-derived mesenchymal stromal cells for clinical application: An efficient isolation approach

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ABSTRACT

Purpose of the study. – Mesenchymal stromal cells (MSCs) are considered a promising tool for cell therapy approaches. The translation of research-based cell culture protocols into procedures that comply with Good Manufacturing Practice (GMP) is critical. The aim of this study was to design a new method for the expansion of MSCs from Adipose Tissue (AT-MSCs) in compliance with GMP, without enzymatic tissue digestion and without the use of animal proteins as source of growth factors.
Patients and methods. – MSCs were expanded from 10 periumbilical biopsies. Our new isolation approach is based on: (1) disruption of AT with an automated, closed system; (2) use of GMP-grade medium without the addition of fetal bovine serum or platelet lysate; (3) use of human recombinant Trypsin. AT-MSCs cultured in α-MEM and minced by scalpel were used as control.
Results. – It was possible to expand MSCs from all the AT-samples for at least eight passages. MSCs displayed the typical spindle-shape morphology, a high viability, multilineage differentiation potential and high expression levels of the typical MSC-specific surface antigens and genes. Compared to standard method, MSCs obtained with the new method showed higher yield, up to passage 6, and higher purity in terms of percentage of CD34 and CD45 markers. All AT-MSCs exhibit *in vitro* immunosuppressive capacity and possess a normal karyotype.
Conclusions. – Our data clearly demonstrate that our new approach permits to generate AT-MSCs fully compliant for therapeutic use and better at least in terms of quantity and purity than those obtained with the standard method.

1. Introduction

Mesenchymal stromal cells (MSCs) are considered a promising tool for cell therapy in regenerative medicine and for the prevention or treatment of severe inflammatory and autoimmune diseases [1–4].

MSCs were first described as non-hematopoietic Bone Marrow (BM) cells, which adhere to plastic and that characteristically develop into colonies with a fibroblastic appearance [5].

Later studies better defined these cells and mainly focused on their ability to sustain hematopoiesis and their *in vitro* capacity to differentiate into various mesodermal cell types [6,7]. The International Society for Cellular Therapy (ISCT) established minimal criteria to define multipotent MSCs, as follow: (a) plastic adherence; (b) differentiation capacity; (c) surface expression of CD73, CD90, CD105, CD166, CD44, and CD29 and absence of CD14, CD34, CD31, and CD45 [8,9]. This definition has been broadly accepted by the scientific community, in order to minimize discrepancies, and to permit comparison of data generated in different laboratories. However, it is known that stem cells source, tissue treatment at the beginning of the process, culture conditions, culture medium and reagents used for cell expansion, may influence the phenotype and functions of cultured MSCs [10–12].

MSCs prepared for clinical use, are classified as advanced therapy medicinal products. The production process must respect European Regulation No. 1394/2007 and European Good

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Manufacturing Practice (GMP) rules, and satisfy quality requirements for clinical application [13,14].

The translation of research-based protocols into procedures for large-scale production of clinical-grade MSCs complying with GMP is critical and strongly depends on the safety, purity and potency of cells. This requires stringent Quality Control (QC) verification of all critical aspects of the production process, as well as the definition of strategies to reduce open manipulations during the production process and the use of appropriate reagents [15,16].

The choice of Adipose Tissue (AT) as a source of stem cells provides some advantages with respect to BM, based on the ease and standardization of collection procedures and the high frequency of stromal cell yields. The design of an AT disgregation step in accordance with GMP is critical. The "gold standard technique" for the dissociation of AT tissue fragments is the cutting with scalpel followed by enzimatic dissociation; some groups don't use enzymes, but (a) cut AT into little fragments with sterile scalpels, (b) vortex/centrifuge AT fragments many times or (c) obtain dissociation by intersirynge processing of AT [17,18]. These techniques don't represents ideal strategies in a GMP context, especially due to an extensive manipulation of the tissue; on the other hand the use of enzymes alone is a safe but not efficient method. To date, completely GMP-compliant strategies of AT disgregation are lacking.

Our protocol provides a mechanical disruption method GMP compliant, that is performed in an automated, closed system (GentleMACS^{TM} Dissociator), which minimizes inter-preparation variability, an important aspect in a GMP-setting context, and reduces the risk of contamination with considerable savings in terms of cost/production.

The use of culture media supplements is another critical aspect to consider to obtain standardized MSCs. Fetal Bovine Serum (FBS) is not recommended by regulatory agencies because of the risk of adventitious agent contamination and potentially immunogenic bovine residue in the final product. On the other hand xeno-free supplements, such as autologous serum or human platelet lysate, have been allowed for GMP compliant cell culture [19–21], but presented several obstacles such as standardization of large-scale preparation for clinical application [22]. To overcome these obstacles, in our culture protocol we utilize a chemically defined xeno- and serum-free medium. The aim of this study was to delineate and verify a new approach to isolate and expand MSCs from peri-umbilical AT in compliance with GMP rules, based on the disruption of AT with an automated, closed system, the use of GMP-grade medium without the addition of fetal bovine serum or platelet lysate, and finally the use of human recombinant Trypsin.

Cells expanded using our protocol, have been characterized in terms of morphology, growth kinetics, cells surface markers, differentiation potential, gene expression profile and genetic stability. Moreover, AT-MSCs were tested for their capability to *in vitro* modulate T cell activation. Results were compared with those obtained from AT-MSCs expanded following standard culture procedure.

2. Materials and methods

2.1. Collection of adipose tissue

The Institutional Review Board of the IRCCS Neurological Institute C. Besta Foundation approved the design of the study. AT periumbilical biopsies were collected, by tissue excision, under general anesthesia, from 10 subjects undergoing surgery for ventricularperitoneal shunt or lipofilling; all the subjects subscribed written informed consent. The mean age was 55 ± 14 years (40 min–74 max), mean weight was 82.9 ± 22.8 kg (70 min–146 max), and mean body max index was 26.7 ± 8.5 (21.1 min–50.5 max). Samples were processed within 24 h. AT samples have been equally divided into two parts and processed with both new method and standard method; the mean weight of processed samples was 3.89 ± 0.66 g (mean ± standard deviation; range from 3.15 to 4.80 g).

2.2. Processing of adipose tissue and AT-MSCs culture

2.2.1. New method

AT samples were washed once in PBS, transferred to tube type C (Miltenyi Biotec, Bergisch Gladbach, Germany), then connected to the instrument GentleMACS^{TM} Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany), and mechanically dispersed according to the manufacturer's instructions and following the method previously described by Nava et al. [23]. During the preliminary phase of the study we set a succession of suitable installed software programs for the best disgregation of AT samples, to obtain a single cell suspension; the sequence is as follow: "brain1", "brain 2", "brain 3" and "h tumor 01". After GentleMACS^{TM} disgregation, cells suspension was washed with PBS, transferred to 15 ml tube and centrifuged at 700 g for 10 min. After centrifugation, the homogenized AT forms a ring which was recovered with a sterile pipette and plated in a 25 cm^2 polypropylene culture flask, precoated with fibronectin based MSC Attachment Solution (Biological Industries, Cromwell, CT, USA). From 0.78 g to 1.5 g of AT was placed per flask. The flask was left open for 5 min, to promote adhesion of the homogenized tissue, then a small amount (2 ml) of MSC NutriStem XF Basal Medium supplemented with MSC NutriStem Supplement Mix (Biological Industries) was added, with gentle pipetting.

2.2.2. Standard method

Samples were washed in PBS, transferred to a 100 mm^2 petri dish, minced by sterile scalpel and plated in a 25 cm^2 polypropylene culture flask (up to 1.5 g/flask). After adhesion of AT, commercially available α-MEM (Euroclone, Pero, MI, Italy) supplemented with 5% fetal bovine serum (FBS) was added.

Flasks were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and checked twice weekly for cells sprouting and propagation; medium was added (1 ml) every 3–4 days. When MSCs sprouted from the tissue, AT was removed and the culture medium replaced. MSCs were harvested after reaching 80% confluence, using Tryple solution (Sigma–Aldrich), and propagated at 4000 cells/cm^2. Cells were screened on alternate days and the medium was changed at approximately 50% confluence. At 80% confluence the cells were further harvested, and at every passage a portion was used for analysis. Four out of ten cultures were maintained until senescence. The senescence phase was defined as the arrest in MSCs proliferation. MSCs in the senescence phase were closely monitored for an additional 8–12 weeks before interrupting the cultures, to look for cells that could escape from senescence and re-initiate proliferation. Senescence was also documented by staining MSCs with the senescence β-galactosidase (SA-β-gal) Staining Kit (Ozyme, Saint Quentin en Yvelines, France) following the manufacturer's instructions, and analyzed with a direct-light microscope.

2.3. Statistical analysis

Statistical analysis of proliferation and immunophenotypic characterization of MSCs obtained with the two methods was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California USA), Student t Test was applied for statistical evaluations.

2.4. Cell proliferation and post-thaw viability

Cell proliferation was evaluated by calculating the cumulative Population Doublings (cPD) for each passage using the formula: $log_{10}(N_1/N_0)log_{10}2$, where N_0 is the number of cells seeded and N_1

the number of cells harvested at the end of the passage. cPD refers to the sum of PD over passages.

Cell number and viability were assessed, using trypan blue and Burker chamber, at every detachment step and after thawing of cryopreserved cells.

2.5. Immunophenotypic characterization

AT-MSCs immunophenotypic characterization was performed by direct immunofluorescence with a flow-cytometer (Beckman Coulter) at early (P2-P4) and late (P7-P8) passages. AT-MSCs were labeled with Fluorescein Isothiocyanate (FITC)- or Phycoerythrin (PE)-conjugated monoclonal antibodies against CD90, CD44, CD31, CD45, CD34 CD73, CD105 (BD PharMingen, NJ, USA). Appropriate, isotype-matched, nonreactive fluorochrome-conjugated antibodies were used as controls. In brief, 5×10^4 cells were incubated with either conjugated specific antibodies or isotype-matched control mouse immunoglobulin G at the recommended concentrations. After staining, the cells were washed once with PBS, 0.1% BSA, and re-suspended in FACS buffer. 1×10^4 events per samples were acquired. The percentage of positive cells was calculated using Kaluza software (Beckman Coulter).

2.6. AT-MSCs differentiation in vitro

The osteogenic and adipogenic differentiation capacities of AT-MSCs were determined as previously described [20]. Osteogenic differentiation was induced at P3 by seeding AT-MSCs in two wells of a 24-well plate, in α-MEM (Euroclone) supplemented with 10% FBS (Euroclone) at 3×10^5 cells per well. After 24 h, an osteogenic-inducing cocktail composed of 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid and 100 nM dexamethasone (all from Sigma–Aldrich) was added. As a negative control, cells were seeded under the same conditions and maintained in non-inducing medium. After 21 days, one well was stained with Alizarin Red-S (AR-S) (Sigma–Aldrich,) to reveal the deposition of a calcium-rich mineralized matrix and the other well with Fast BCIP/NBT (Sigma–Aldrich) for alkaline phosphatase (AP) activity. In detail, cells were washed with PBS, fixed with 70% ethanol for 60 min at room temperature, and stained for 20 min with AR-S or 10 min with AP substrate. Adipogenic differentiation was induced at P3 by seeding 3×10^5 MSC cells/well in a 24-well plate in Dulbecco's Modified Essential Medium-low glucose (DMEM–LG, Euroclone) supplemented with 10% FBS and incubated overnight to allow cell attachment. The medium was then switched to adipogenic-induction medium composed of αMEM supplemented with 10% FBS, 10 μM bovine insulin, 200 μM indomethacin and 500 μM 3-isobutyl-1-methyl xanthine (IBMX) (all from Sigma–Aldrich). As a negative control, cells seeded under the same conditions were maintained in non-inducing medium. Medium was changed twice weekly. After 21 days, the presence of lipid droplets was visualized by Oil Red O staining. In brief, cells were washed twice with phosphate-buffered saline, fixed in 4% paraformaldehyde for 10 min, stained with 0.18% Oil Red O for 20 min, and then treated with 60% isopropilic-alchool. Adipo-, osteo- and chondrogenic differentiation capacity was also evaluated by real time PCR, analysing a panel of genes involved in the differentiation process.

2.7. Array-based gene expression analysis

Total RNA was isolated from cell pellets (200,000 cells) using the RNeasy mini kit (Qiagen), according to the manufacturer's instructions. The quantity and quality of total RNA for each preparation was determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA); 1 μg total RNA from each sample was used for reverse transcription with the RT^2 First Strand Kit (Qiagen).

Gene expression profiles of AT-MSCs stem cell-specific transcription factor at P3-P5 were analyzed by quantitative PCR (qPCR), using the Human Mesenchymal Stem Cell PCR Array (PAHS-082ZA; SABioscience, Frederick, MD, USA) following the manufacturer's instructions. Eighty-four key genes and five housekeeping genes, involved in differentiation, in maintenance of pluripotency and self-renewal status were analyzed.

qPCR was performed using an ABI7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA) and RT^2 Sybr Green qPCR Master Mix (Qiagen). The total volume of the PCR reaction was 20 μL and the thermocycler parameters were as follow: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s then 60 °C for 1 min. RT controls, and PCR controls were included in each run. The relative gene expression was calculated with the comparative threshold cycle delta Ct method, as described by Livak and Schmittgen [24]. Results were analyzed with Web-based PCR Array Data Analysis Software available at http://pcrdataanalysis/sabiosciences.com/pcr/arrayanalysis.php.

2.8. In vitro modulation of activated PBMCs

The immunomodulatory activity of the same lots expanded with new and with standard method was assessed, *in vitro*, on Phytohemagglutinin (PHA) stimulated – allogeneic – Peripheral Blood Mononuclear Cells (PBMCs) from one healthy donor. MSCs at passage P3 or P4 were used.

In detail, irradiated (30 Gy) MSCs were seeded in flat bottom 96 well plates (Corning Costar, Celbio) and allowed to attach overnight; different amounts of MSCs were plated to obtain MSCs:PBMCs ratios of 1:2, 1:20, 1:200, 1:1000. 100,000 PBMCs per well, in RPMI 1640 medium (Gibco-BRL, Life Technologies) supplemented with 10% fetal calf serum (FCS; Euroclone) were then added, with or without PHA (4 μg/ml; PHA-L; Roche, Mannheim, Germany). After a 3-day incubation at 37 °C in a humidified 5% CO_2 atmosphere, ^{3}H-thymidine (^{3}HTdR 0.5 μCi/well; Amersham, Buckinghamshire, UK) incorporation was measured during the last 21 h with a standard procedure [25]. Results were expressed as residual proliferation. The experiments were performed in triplicate per each condition.

2.9. Karyotype analysis

AT-MSCs were plated at 7000 cells/cm^2, maintained in culture medium for three days and then cultured without FBS. After 20–22 h complete medium was replaced and cells were incubated at 37 °C for 27–28 h. Then, 0.1 mM colcemid solution (Irvine Scientific, Santa Ana, CA, USA) was added for 4 h. Cells were harvested, treated with 0.56 mM KCl, and fixed in methanol/acetic acid (3:1). Cells in the metaphase were Q-banded and karyotyped in accordance with the International System for Human Cytogenetic Nomenclature recommendations. It has been reported that 20–25 valuable metaphase cells/slide can be obtained by synchronizing MSCs [26,27].

3. Results

3.1. AT-MSCs culture

We were able to isolate and expand MSCs from 10/10 processed AT samples both by new and standard method. AT-MSCs sprouted from the tissues after a median time of 11 (range: 4–25 days) grown in NutriStem XF Medium, while AT-MSCs expanded in α-MEM + 5%FBS after 10 (range: 3–23 days) days' culture. The cells were expanded *in vitro* for at least eight passages (range: 8–12 passages, P). AT-MSCs displayed the typical spindle-shaped morphology, both with the new- and standard method, as shown in Fig. 1A and B.

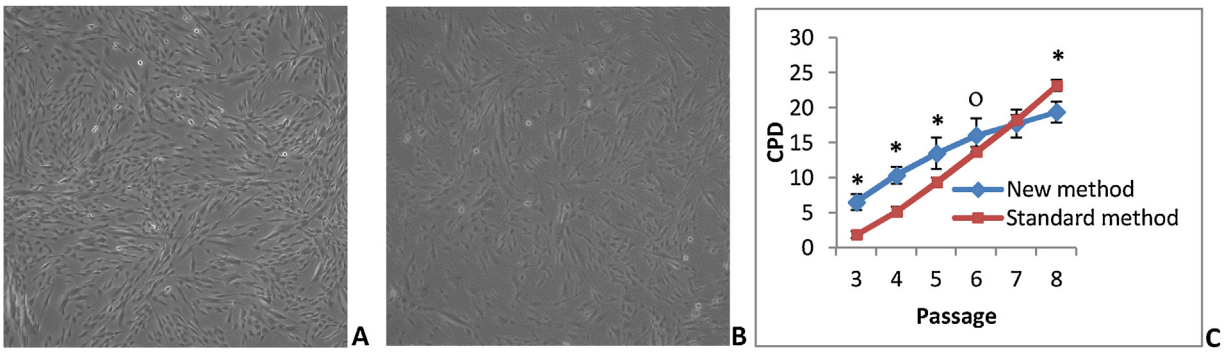

Fig. 1. AT-MSCs morphology and proliferative capacity. (A, B) Spindle-shaped morphology of AT-MSCs (P4) cultured with both new method (A) and standard method (B). Magnification 10×. (C) cumulative Population Doubling (cPD) from passage P3 to P8 of AT-MSCs obtained with both new method (blue line) and standard method (red line); results are expressed as mean ± SD of 10 different MSCs lots. Differences are significant, except at P7 (* $p < 0.01$; ° $p < 0.05$, *t* Student test).

AT-MSCs growth capacity was evaluated in terms of cPD, from P3 to P8. As shown in Fig. 1C, cPDs of MSCs obtained with the new method were significantly higher from P3 to P6, compared to the same passages of cells cultured with standard method (P3: 6.52 ± 1.12 *vs* 1.82 ± 0.48, P4: 10.36 ± 1.2 *vs* 5.1 ± 0.68, P5: 13.46 ± 2.26 *vs* 9.32 ± 0.65, P6: 16.04 ± 2.37 *vs* 13.7 ± 0.7). Passages P3–P5 showed a *p* value < 0.01, at P6 *p* value was <0.05 (*t* Student test). Conversely at P8 cPDs of MSCs obtained with standard method were significantly higher than those obtained with the new method (23.14 ± 0.77 *vs* 19.37 ± 1.53; $p < 0.01$). Results are expressed as mean ± SD of 10 cultures.

During the entire culture period, cells cultured with both methods maintained a high percentage of viability, as evaluated with trypan blue staining at every passage (new method: mean value 96.22%, range 93.25–99.36%; standard method: mean value 95.83%, range 92.25–99.51%). Cell viability was not affected by cryopreservation: the mean viability after thawing (three vials from each sample) was 93.64% (range 88.17–97.14%) for the new method and 93.23% (range 88.06–97.25%) for standard method.

3.2. Immunophenotypic characterization

AT-MSCs immunophenotypic characterization was assessed by flow cytometry at early (P2–P4) and late (P7–P8) passages. AT-MSCs cultured with new method resulted positive for the typical surface antigens and negative for hematopoietic/endothelial markers as CD31, CD34 and CD45. Cells cultured with standard method, showed same levels of the typical MSCs markers, but the percentages of CD34 and CD45 positive cells were statistically higher than those obtained with the new method (early passages: CD34 12.13 ± 13.54 *vs* 0.82 ± 0.88 $p < 0.05$; CD45 8.01 ± 4.19 *vs* 1.05 ± 0.76 $p < 0.01$; late passages: CD34 8.7 ± 7.5 *vs* 1.01 ± 0.83 $p < 0.05$; CD45 5.43 ± 1.64 *vs* 0.79 ± 0.77 $p < 0.05$). At late passages the endothelial marker CD31 appeared slightly higher in cells obtained with standard methods than in those cultured with the new method, but the difference was not statistically significant.

Results are summarized in Table 1.

3.3. Multilineage differentiation potential of AT-MSCs

AT-MSCs were able to produce calcificated matrix as demonstrated with Alkaline phosphatase and Alizarin red staining, both when grown with the new method and the standard method (Fig. 2A and C respectively).

After 21 days, AT-MSCs showed potential for adipogenic differentiation, as underlined by the accumulation of lipids in small vacuoles stained with Oil Red O (Fig. 2B and D).

AT-MSCs differentiation capacity of cells obtained with the new method was also confirmed by expression analysis of a panel of genes involved in adipo-, osteo- and chondrogenic differentiation. The genes involved in the osteogenesis process that resulted highly expressed were: *Histone deacetylase1* HDAC1 and *Runt-related transcription factor 2* RUNX2 ($\Delta Ct = 6.76 \pm 0.26$ and 7.01 ± 0.52 respectively). The genes involved in the adipogenic differentiation process that resulted highly expressed were: *Peroxisome proliferator-activated receptor gamma* PPARG and *Ras Homolog Family Member A* RHOA ($\Delta Ct = 7.83 \pm 0.86$ and 2.83 ± 0.38 respectively). The genes involved in chondrogenesis that resulted highly expressed were: *growth differentiation factor 6* GDF6 $\Delta Ct = 7.09 \pm 1.08$, *histone acetyltransferase* HAT $\Delta Ct = 5.18 \pm 0.48$, *K(Lysine) Acetyltransferase 2B* KAT2B $\Delta Ct = 8.49 \pm 0.49$ and *SRY (Sex Determining Region Y)-Box 9* SOX9 $\Delta Ct = 7.04 \pm 0.57$ (Fig. 2E).

3.4. In vitro *modulation of activated PBMC*

When we evaluated the immunomodulatory effect of AT-MSCs on the *in vitro* proliferation of PHA-activated healthy donor PBMCs,

Table 1
Percentage of AT-MSC surface markers. Immunophenotypic AT-MSCs characterization was performed by flow cytometry both at early and late passages. The percentage of positive cells is reported as the mean ± SD calculated for 10 different AT-MSCs lots, cultured both with the new method and standard method.

	New method		Standard method	
	Early passages (P2–P4)	Late passages (P7–P8)	Early passages (P2–P4)	Late passages (P7–P8)
Antigen surface markers	% of positive cells (mean ± SD)	% of positive cells (mean ± SD)	% of positive cells (mean ± SD)	% of positive cells (mean ± SD)
CD90	98.09 ± 1.07	97.82 ± 1.58	99.98 ± 0.02	97.35 ± 6.11
CD44	99.13 ± 1.08	97.66 ± 0.69	99.43 ± 0.1	98.67 ± 3.45
CD73	98.32 ± 1.2	97.4 ± 0.63	99.51 ± 0.55	96.97 ± 7.04
CD105	98.72 ± 0.89	96.83 ± 3.02	96.14 ± 4.17	96.96 ± 6.46
CD31	0.93 ± 0.19	1.06 ± 0.37	1.32 ± 1.42	5.68 ± 4.14
CD45	[a]1.05 ± 0.76	0.79 ± 0.77	[a]8.01 ± 4.19	5.43 ± 1.64
CD34	[a]0.82 ± 0.88	1.01 ± 0.83	[a]12.13 ± 13.54	8.7 ± 7.5

[a] At early passages the percentages of CD45 and CD34 positive cells cultured with standard method were statistically higher than those obtained with the new method ($p < 0.01$ for CD45; $p < 0.05$ for CD34). At late passages the percentages of CD45 and CD34 positive cells cultured with standard method were statistically higher than those obtained with the new method ($p < 0.05$ for CD34 and CD45).

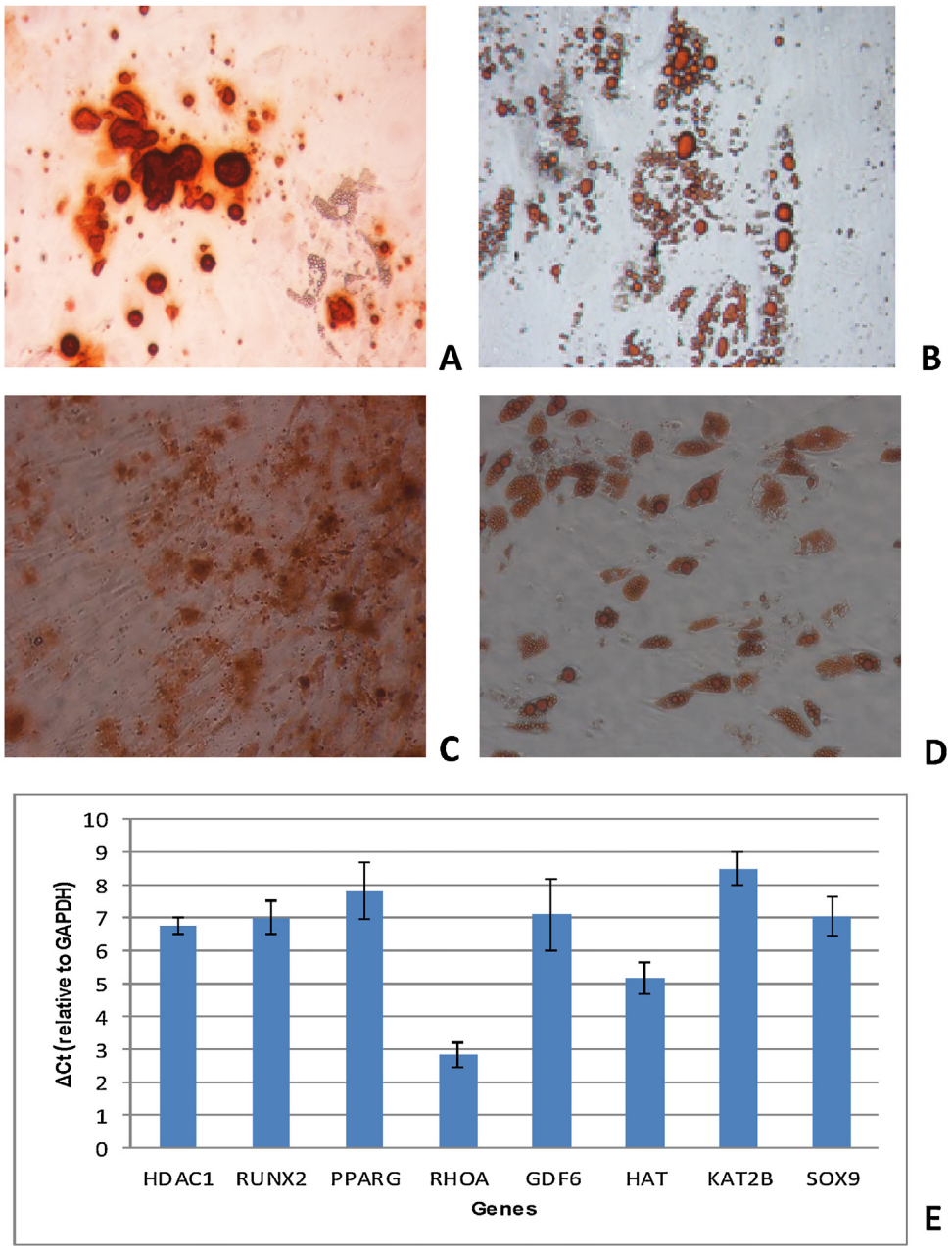

Fig. 2. AT-MSCs differentiation *in vitro*. Osteogenic and adipogenic differentiation capacity of MSCs obtained with the new method (panel A: staining with Alizarin Red; panel B: staining with Oil Red O) and standard method (panel C: staining with Alizarin Red; panel D: staining with Oil Red O). Magnification 20×. Panel E shows expression of genes involved in osteogenic, adipogenic and chondrogenic processes (new method). The expression of mRNA was normalized to GAPDH.

we observed a reduction with the addition of AT-MSCs in terms of residual proliferation, especially at 1:2 and 1:20 ratios. The degree of inhibition did not significantly change using AT-MSCs expanded with the new method (Fig. 3).

3.5. Gene expression analysis

To better characterize the nature and behavior of AT-MSCs isolated and expanded by the new protocol, we investigated gene expression levels at passages P2–P4. The analysis was performed by characterizing 84 genes involved in MSC stemness, pluripotency and self-renewal. The array also included differentiation markers involved in osteogenesis, adipogenesis, chondrogenesis, myogenesis and tenogenesis.

Eighteen out of 84 evaluated genes (21.42%) were expressed at very high levels, $\Delta Ct < 8$, 48/84 (57.14%) expressed $8 < \Delta Ct < 14$, 16/84 (19.04%) displayed median expression levels, with ΔCt between 14 and 19, while only 2 genes (2.38%) exhibited a ΔCt higher than 19 (Fig. 4A).

Among the "stemness" genes our results revealed a very high expression of *Fibroblast Growth Factor 2* (FGF2), and Leukemia Inhibitory Factor (LIF) with a $\Delta Ct = 5.08 \pm 0.47$ and 6.29 ± 0.79 respectively. Our results also indicated the consistent expression of MSC-specific markers *Activated Leukocyte Cell Adhesion Molecule* (ALCAM) $\Delta Ct = 3.34 \pm 0.26$, *Alanine Aminopeptidase* (ANPEP) $\Delta Ct = 4.09 \pm 0.81$, Caspase 3 (CASP3) $\Delta Ct = 6.79 \pm 0.83$, CD44 $\Delta Ct = 6.76 \pm 0.49$, *Endoglin*/CD105 (ENG) $\Delta Ct = 5.52 \pm 0.28$, *Integrin, Alpha V* (ITGAV) $\Delta Ct = 5.78 \pm 0.96$, *Melanoma Cell Adhesion Molecule* (MCAM) $\Delta Ct = 5.77 \pm 0.78$, *ecto-5′-Nucleotidase*/CD73 (NT5E) $\Delta Ct = 3.94 \pm 0.54$, *Platelet-Derived Growth Factor Receptor Beta* (PDGFRB) $\Delta Ct = 5.39 \pm 0.69$, *Thy-1 Cell Surface Antigen* (THY1)/CD90 $\Delta Ct = 1.72 \pm 0.25$, *Annexin A5* (ANXA5) $\Delta Ct = 1.56 \pm 0.18$,

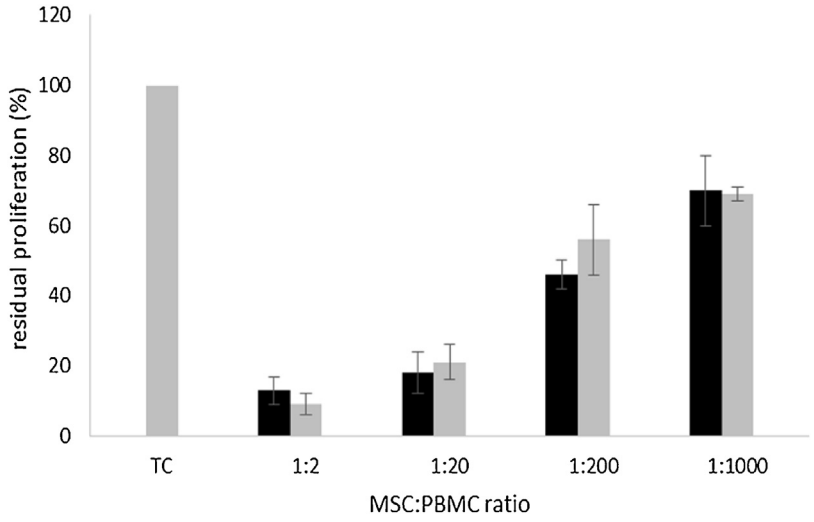

Fig. 3. *In vitro* modulation of PHA-activated PBMCs. The capability of four AT-MSCs lots, expanded with both new and standard method, to modulate the *in vitro* response to PHA of peripheral blood mononuclear cells (PBMCs) isolated from one healthy donor is reported at different MSCs/PBMCs ratios. Results are expressed as residual proliferation. Black bars: AT-MSCs expanded with new method; Gray bars: AT-MSCs expanded with standard method.

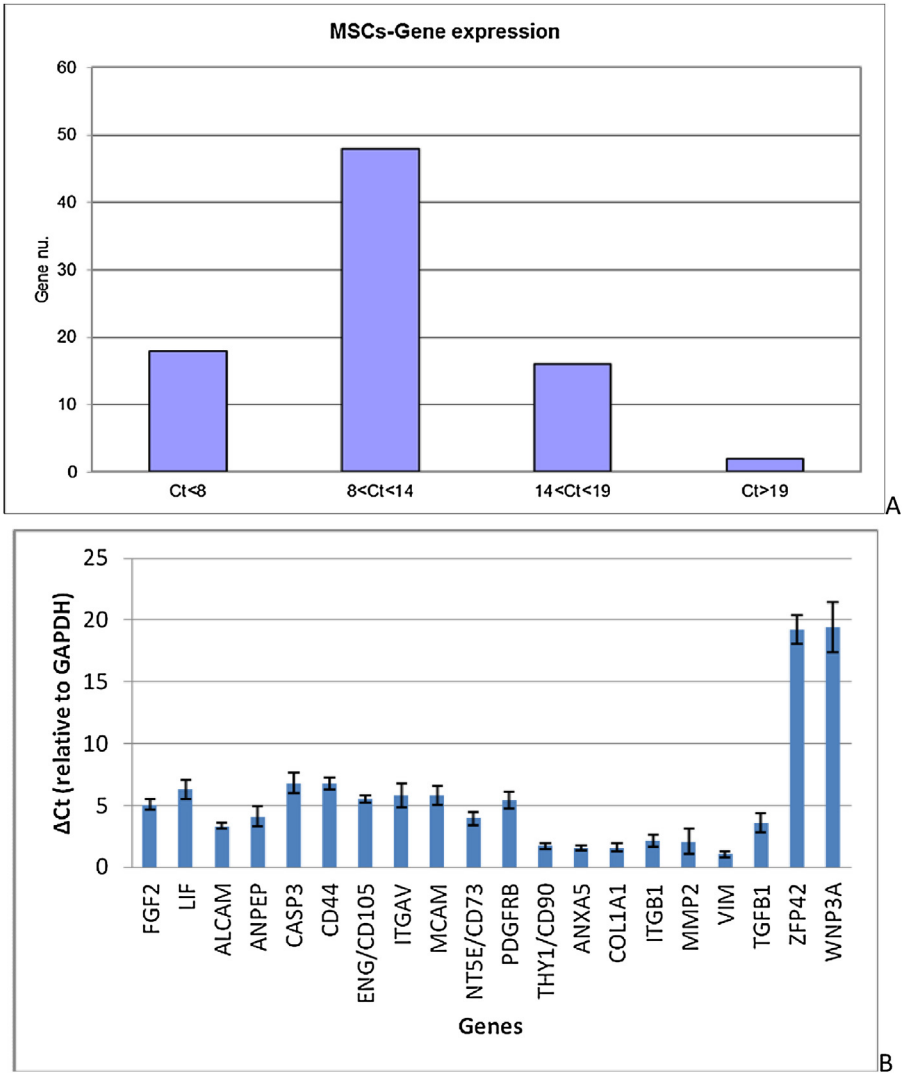

Fig. 4. AT-MSCs gene expression analysis. The gene expression levels of 84 genes involved in stemness, pluripotency and self-renewal status of MSCs were analyzed. In panel A, the number of gene expressed at different levels are reported: $\Delta Ct < 8$, $8 < \Delta Ct < 14$, $14 < \Delta Ct < 19$ and $\Delta Ct > 19$. In panel B, the genes expressed at very high levels ($\Delta Ct < 8$) and the genes (ZFP42 and WNP3A) expressed at very low levels ($\Delta Ct > 19$) are reported normalized to GAPDH.

Collagen, type I, alpha 1 (COL1A1) $\Delta Ct = 1.59 \pm 0.34$, *Integrin Beta 1* (ITGB1) $\Delta Ct = 2.16 \pm 0.49$, *Matrix Metalloproteinase-2* (MMP2) $\Delta Ct = 2.09 \pm 0.97$, *Vimentin* (VIM) $\Delta Ct = 1.05 \pm 0.23$, *Transforming Growth Factor beta-1* (TGFβ1) $\Delta Ct = 3.59 \pm 0.77$. All results are reported as the mean ± SD (Fig. 4B).

Conversely, the two genes *Zinc-Finger Protein*-42 (ZFP42) and *Washington Nuclear Project Nos 3* (WNP3A), belonging to the stem cell gene category, were expressed at very low levels (ΔCt 19.24 ± 1.17 and 19.39 ± 2.02, respectively).

3.6. Karyotype analysis

The genetic stability of *in vitro* expanded AT-MSCs was evaluated at a late passage (P7–P8) by conventional karyotyping. The analysis did not reveal any chromosomal abnormalities or submicroscopic rearrangements in the cells obtained both with the standard- and the new method.

4. Discussion

Human MSCs are attractive candidates for transplantation and regenerative therapies due to their unique therapeutic properties, the possibility of being isolated and expanded from different sources, ability to migrate to damaged tissue or toward inflammatory sites after intravenously administration and their capability to modulate different aspects of both innate and adaptive immunity, exerting regulatory functions on a wide range of immunocompetent cells. Despite these desirable features, several studies and clinical trials using MSCs have yielded conflicting results. This variability may reflect differences between individual donors, cells source and/or isolation, culture and expansion methods. In order to generate MSCs for therapeutic use, it is necessary to develop a robust expansion protocol, under GMP conditions, that enables culture and rapid proliferation of cells, maintaining the original features of stemness, multipotency and self-renewal.

In this study, we describe a new method to generate AT-MSCs in compliance with GMP.

The choice of AT, as a source of stem cells, provides some advantages based on the standardization of surgical procedures, easiness in the collection of samples and high frequency of stromal cell in the tissue. On the other hand the isolation and expansion of MSCs from AT to be used in cell therapy approaches may present many problems; first, differently from bone marrow, AT must be dissociated to be cultured as source of MSCs.

Different dissociation methods, both automatic and manual, have been described [28], but the use of AT-MSCs in clinical applications increases the need for clear and reliable information about the efficiency, the cost and safety of each method. Moreover in this context a GMP validation of the method is required. In accordance with GMP practices, tissue dissociation could be achieved using GMP-grade enzymes, with a considerable increase in production costs. In an enzyme-free context, conversely, the disgregation of AT may be difficult. Manual dissociation, by scalpel for example, may expose the product to a potential risk of microbial contamination and leads to a high inter-preparation variability.

Culture medium is another critical aspect in the generation and expansion of AT-MSCs in compliance with GMP: the optimal medium should promote cell expansion in a reasonable time frame without altering basic cellular characteristics. FBS or human platelet lysate are largely used in research culture protocols, but are not recommended by regulatory agencies for GMP compliant cell culture because of the risk of adventitious agent contamination and potentially immunogenic bovine residue in the final product, as well the problems of standardization of large-scale preparation for clinical application.

Chemically defined xeno- and serum-free media, that meet regulatory requirements, have been recently developed, ensuring advantages in the performance of expansion processes and minimizing batch-to-batch variability [29–32].

Our new approach provides the disruption of AT in an automated, closed system, without the use of enzymes: to the best of our knowledge, this is the first time in which this system has been reported for AT homogenization in order to isolate and expand MSCs. Here we demonstrate that the application of this disruption strategy, completely GMP compliant, together with the use of GMP-grade medium without the addition of FBS or platelet lysate, permits to conduct the initial culture phase with a remarkable improvement both in terms of purity and yield of cells cultured, respect to standard method. Our results showed that our protocol permits the successful isolation of MSCs in a time (11 days after the beginning of culture), that is absolutely comparable to that obtained with standard method. Interestingly, CPDs of AT-MSCs obtained with new method at early passages were significantly higher, as compared to the same passages of cells cultured with standard method. These data are particularly relevant in the context of MSCs expansion for cell therapy approaches, in which MSCs at early passages (P3–P4) are usually used to treat patients.

AT-MSCs obtained with the new methods meet the criteria required by ISCT, showing morphology, viability, differentiation potential and positivity for MSC-specific markers with no significant differences respect to cells obtained with standard method, both at early and late passages.

Cytofluorimetric analysis of AT-MSCs obtained with "standard method" were slightly positive for CD34 and CD45 both at early and late passages, unlike new-method MSCs, these data indicating that cells obtained by the standard method still contain cells other then MSCs, as already described by our group and others [33,9]. Moreover the percentage of CD31+ cells seems to be higher, at late passages, in standard MSCs cultures, but the difference is not statistically significant. These differences may be due to absence of serum or xeno-derived additives in the GMP medium that may be helpful for the proliferation of unwanted cells types (such as endothelial cells, immunological cells or hematopoietic cells). The mean fluorescence intensity (MFI) of the typical MSCs markers has also been analyzed; no significant statistical differences in MFI of CD90, CD44, CD105 and CD73 markers of MSCs obtained with new- *vs* standard method are present ($p > 0.05$ *t* Student test), this demonstrating that phenotypic properties are maintained.

Thus AT-MSCs obtained with the new approach are not only numerically greater but also qualitative better in term of purity, at least at early passages.

Cell adhesion in serum-free, xeno-free cultures still remains a major problem since most commercially media require pre-coating of culture flasks. However, the protocol used in this study, which involves the use of an attachment solution, allows an optimal adhesion of cells both at the beginning and during each culture passage. In addition, our protocol involves the use of a commercially available recombinant trypsin, free of any animal derived components, completely GMP-compliant, was used to detach the cells.

Importantly, it is known that long-term culture of cells under non-physiologic *in vitro* culture conditions may result in mutations and chromosomal aberrations [34]. We were able to confirm the genetic stability and safety of the expanded cells produced with this protocol (data not shown) thank to the conformity of karyotype analysis.

Overall, these results confirm that this reliable approach for the isolation and expansion of AT-MSCs reduces inter-preparation variability, as evidenced by the homogeneous number of expanded cells, antigen surface expression, and percentage of viable cells.

In summary, here we describe a new isolation and expansion approach to generate AT-MSCs in GMP compliance without enzymatic digestion of AT and without the use of animal or human derivates as a source of growth factors during cell culture. As request by both regulatory agencies and GMP rules, this protocol minimize inter-preparation variability, the risk of contamination, and is completely free of undefined animal components, allowing to obtain cells that maintain the original features of stemness, multipotency and self-renewal, as well as multilineage differentiation potential and completely compliant to the requirements of regulatory agencies for the production of ATMPs to be used as drugs for the treatment of patients. Moreover this protocol defines simple and basic culture conditions based on the use of a commercially available xeno- and serum-free reagent and equipment that allows inter-laboratory reproducibility of the method.

Disclosure of interest

The authors indicate no potential conflicts of interest.

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