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Application Note

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MSC NutriStem® XF Medium

Adipose Tissue Derived MSC Isolation Using Defined, Xeno-Free, Serum-Free Medium

Rotman Maya [1,*], **Ben-Yosef Oren** [1,]

1. Biological Industries Israel Beit Haemek Ltd., Beit Haemek, 2511500, Israel

* Correspondence
E-Mail: Maya.Rotman@Sartorius.com

Abstract

Cell therapy is a very promising therapeutic approach. The impressive progress in the field of stem cell research, due to stem cells' infinite self-renewal ability and their potential to differentiate into other cell types, has laid the foundation for cell-based therapies for diseases which cannot be cured by conventional medicines.
Mesenchymal Stromal Cells (MSCs) can be found in a variety of tissues, including Adipose tissue, Bone Marrow, Dental Pulp, etc. and are capable of differentiating mainly into connective tissue cell types – Bone (osteocytes), cartilage (chondrocytes), muscle (myocytes) and fat (adipocytes).
In this study adipose tissue mesenchymal stromal cells (AT-MSCs) were derived from human adipose tissue, expanded and later used for a clinical application, using the defined MSC NutriStem® XF Medium.
The isolation procedure and expansion protocol, including cell yield, phenotype and viability results are reviewed as followed.

Find out more: www.sartorius.com/MSC_NutriStem_XF_Medium

Introduction

Stem cell research has made impressive progress the last few years, laying the foundation for therapeutic approaches and very promising cell-based therapies, for the treatment of diseases which cannot be cured by existing conventional medicines.
Mesenchymal Stromal cells are multi potent cells that have the capability to differentiate into a variety of cell types, mainly adipose, bone, muscle and cartilage tissues. MSCs are found mostly in bone marrow, adipose tissue, cord blood, placenta and dental pulp and are known for their immunomodulatory affects. (1)
Because some sources of MSCs have relatively simple isolation techniques and MSCs in general have extensive differentiation potential and immune-modulation properties, these cells were introduced into the clinic for repairing tissue injuries, producing engineered tissues in-vitro for in-vivo transplantation, ameliorating immune-mediated diseases and other applications. (2)

Adipose tissue-derived MSCs (AT-MSCs) normally reside in the stromal vascular fraction of the adipose tissue. Subcutaneous adipose depots are abundant and easily accessible in large quantities with a minimally invasive procedure (liposuction aspiration). The AT-MSCs can easily be isolated by tissue digestion. (1)
In this experiment MSCs were extracted from adipose tissue, isolated and expanded, using MSC NutriStem® XF Medium. The MSCs were then frozen and thawed, their parameters were assessed and they were used later for tissue regeneration applications.

Materials and Methods

Materials

- MSC NutriStem® XF Medium, Sartorius Cat. # 05-200-1, 05-201-1
- MSC Attachment Solution, Sartorius Cat. # 05-752-1
- DPBS no calcium, no magnesium, Sartorius Cat. #02-023-1
- NutriFreez® D10 Cryopreservation Medium, Sartorius Cat. # 05-713-1
- MSCgo™ Adipogenic Differentiation Medium, Sartorius Cat. # 05-330-1, 05-331-1-01, 05-332-1-15
- MSCgo™ Chondrogenic Differentiation Medium, Sartorius Cat. # 05-220-1, 05-221-1
- MSCgo™ Osteogenic Differentiation Medium, Sartorius Cat. # 05-440-1
- Recombinant Trypsin-EDTA solution, Sartorius Cat. # 03-079-1
- PBS, Biological Industries
- Collagenase (NB 4 standard grade), Serva Electrophoresis
- Male AB Serum, Access Biologicals
- Aanti-human CD13-PE, + isotype control PE, eBioscience
- Anti-human CD73-PE, + isotype control PE, eBioscience
- Anti-human CD90-PE-Cy7, + isotype control PE-Cy7, eBioscience
- Anti-human CD31-PE-Cy7, + isotype control PE-Cy7, eBioscience
- Anti-human CD45-Alexa Fluor 488, + isotype control Alexa Fluor 488, R&D Systems
- Oil Red O Stock, Science Cell
- Alcian Blue, Science Cell
- Alizarin Red S, Science Cell
- Crystal Violet, Sigma-Aldrich
- Chromosome Resolution Additive (CRA),Genial Genetics
- Acetic acid glacial 100%, Merck
- Methanol abs AR, Merck

Method

AT-MSC isolation:

AT-MSCs were isolated from adipose tissue, washed with sterile phosphate buffered saline (PBS; Biological Industries) to remove debris and red blood cells, following enzymatic digestion with collagenase. The processed tissue was centrifuged, and the cellular pellet was re-suspended in MSC NutriStem® XF Medium.
Following isolation, the cells were seeded in tissue culture flasks, pre-coated with MSC attachment solution diluted 1:100 in DPBS no calcium, no magnesium, and then seeded in MSC NutriStem® XF Medium supplemented with 2.5% Male AB Serum (Access Biologicals).

AT-MSCs passaging:

AT-MSCs flasks were washed with PBS (no calcium, no magnesium), and cells were harvested from the tissue culture flask using recombinant Trypsin-EDTA solution, centrifuged (7min, 300g), resuspended in MSC NutriStem® XF Medium supplemented with Male AB serum, counted using NucleoCounter NC-200, and seeded in a new tissue culture flask at 2500-4500 cells/cm^2. Of note, for the AT-MSCs expansion period no attachment solution was applied, due to the addition of AB serum supplementing the MSC growth medium.

Cryopreservation of AT-MSCs:

P1 AT-MSCs were detached from the tissue culture flask using Recombinant Trypsin-EDTA solution, centrifuged, resuspended in MSC NutriStem® XF Medium (supplemented with AB serum), and cell count and viability were assessed by NucleoCounter NC-200 (ChemoMetec). For cryopreservation, the cells were centrifuged, and the pellet was resuspended in the cold NutriFreez® D10 Cryopreservation Medium at a cell concentration of $5 \cdot 10^6$ cells/mL, and a total of $1 \cdot 10^6$ cells (200µl volume) was transferred into a cryogenic vial (1.8mL cryogenic tube, NuncTM, Thermo Scientific), immediately placed in 2-8°C pre-cooled Mr. Frosty, and inserted into a -80°C freezer. Following 12-24 hours at -80°C, the cryogenic vials were placed in liquid nitrogen for at least 14 days.

Thawing of cryopreserved AT-MSCs:

Following a minimum cryopreservation period of two weeks, the cryogenic vials were rapidly thawed (<1min) at 37°C water bath. The cells were slowly diluted with 5mL pre-warmed MSC MutriStem® XF Medium supplemented with AB serum, centrifuged, the supernatant discarded, and the pellet resuspended with 1mL of MSC MutriStem® XF Medium supplemented with AB serum.

AT-MSC count and assessment of cell viability:

Cell concentration and viability were measured by NucleoCounter NC-200 using vial-cassette, (ChemoMetec). The cryogenic vial volume was measured with a manual pipettor, and the total cells/vial was calculated by multiplying the NucleoCounter-measured cell concentration (cells/mL) with the vial volume (mL).

Evaluation of AT-MSCs adhesion capability:

1mL of thawed AT-MSCs, originating from one cryopreserved vial ($1 \cdot 10^6$ cells), was counted by NucleoCounter NC-200, and seeded in a T-25 tissue culture flask at a seeding concentration of 2500-4500 cells/cm^2 in MSC NutriStem® XF Medium + AB serum, and incubated at 37°C (37°C, 5% CO_2 incubator) for 6-8 hours, after which the adherent cells were harvested with Recombinant Trypsin EDTA solution and counted by NucleoCounter NC-200.

AT-MSCs growth rate analysis:

AT-MSCs were thawed and seeded in a T-75 tissue culture flask, and expanded in MSC NutriStem® XF Medium to passage P3 and P4. At passage P3 and P4, respectively, the adherent cells were harvested with Recombinant Trypsin EDTA solution, counted and their viability assessed by NucleoCounter NC-200. The growth rate (µ), doubling time (g), and population doubling level (PDL) were calculated for passage P3 and P4 AT-MSCs according to the following equations (A, B and C):

$$N_t = N_0 \cdot e\hat{}\mu t \quad \text{A}$$

$$g = (\ln(2))/\mu \quad \text{B}$$

$$PDL = 3.322 \cdot (\log(N_t) - \log(N_0)) \quad \text{C}$$

Where N_t is the number of cells at time t, N_0 is the number of cells at time 0, µ is the growth rate, and g the doubling time.

AT-MSCs identity analysis by flow cytometry:

The AT-MSCs stem/stromal cell identity was analyzed, at passages P3/P4, by flow cytometry, evaluating the presence of the surface markers CD13, CD73 and CD90 and the absence of the surface markers CD31 and CD45.

AT-MSCs differentiation assay into adipocytes, chondrocytes and osteocytes:
AT-MSCs were differentiated into the adipogenic, chondrogenic and osteogenic lineages using the respective differentiation medium; adipogenic (MSCgo™ Adipogenic Basal Medium, supplemented with MSCgo™ Adipogenic SF, XF Supplement Mix I, and Mix II, chondrogenic (MSCgo™ Chondrogenic Differentiation Medium, supplemented with MSCgo™ Chondrogenic Differentiation Supplement Mix, and osteogenic differentiation medium (MSCgo™ Osteogenic SF, XF, respectively. Following differentiation, the cells were evaluated by histological analysis and stained as follows: Adipocytes were stained with Oil Red O Stock to visualize lipids and fat deposits, in chondrocytes the sulfated proteoglycan present in cartilage tissue was stained with Alcian Blue, and in osteocytes the calcium deposits in the cells were stained with Alizarin Red S.

AT-MSCs fibroblast colony-forming unit (CFU-F) assay:
50 cells/well AT-MSCs (P4) were seeded in a 6-well plate (5 cells/cm2) using 2ml/well MSC NutriStem® XF Medium on pre-coated plates (MSC attachment solution, diluted 1:100 in PBS) and were incubated for 10-14 days. Visible colonies are enumerated after fixation and staining of the plates with crystal violet dye. The % CFU-F of AT-MSCs was calculated by the following equation:

CFU-F (%)=(No.of colonies observed•100%)/(No.of seeded cells)

AT-MSCs karyotype analysis:
AT-MSCs were treated with CRA working solution (Chromosome Resolution Additive diluted 1:100 in Hank's Balance Solution and Colcemid solution. Following CRA and Colcemid incubations, the flasks were washed with PBS (without calcium and magnesium), harvested with recombinant Trypsin EDTA solution and centrifuged. The cell pellet was resuspended with hypotonic solution (Potassium chloride and Sodium citrate in water) and then washed three times with Fixer solution (1 part acetic acid with 3 parts of methanol). The karyotype analysis was performed at the Laboratory Services Division, Cytology Lab, Rambam Health Care Center, Israel.

Results

The AT-MSCs that were isolated from a donor's adipose tissue and their parameters were evaluated.

Cell viability of AT-MSCs:
The isolated AT-MSCs were cryopreserved at passage 1 (P1) at a cell concentration of $5 \cdot 10^6$ cells/mL, a total of $1 \cdot 10^6$ cells per cryogenic vial. The cryovials were immediately placed in 2-8°C pre-cooled Mr. Frosty and inserted into a -80°C freezer. The measured cell viability (by NucleoCounter NC-200) prior to cryopreservation was 96%.

Cell count and viability assessment of cryopreserved AT-MSCs following thawing:
Cryopreserved AT-MSCs were thawed (as described in the methods), counted and their cell viability assessed by NucleoCounter NC-200. Cell count was 1.0×10^6 (total cells/vial) and 94.4% of the cells were viable.

Evaluation of AT-MSCs adhesion potential:
AT-MSCs were counted and seeded in a T-25 tissue culture flask. Following 6-8 hours of incubation at 37°C, the adherent cells were harvested and counted by NucleoCounter NC-200. The total cell count was 1.0×10^6 and the Adherence (% adherent cells) was 100.0%.

AT-MSCs growth rate analysis:

AT-MSCs were counted and seeded in a T-75 tissue culture flask, at concentration of 2500-4500 cells/cm^2. At the end of passage P2, the adherent cells were harvested and their viability assessed by NucleoCounter NC-200. Cell viability was 98.0%.

The cells were seeded for passage P3 as well as for P4 at a concentration of 2500-4500 cells/cm^2. At the end of passage P3 and P4, the adherent cells were harvested, counted and their viability assessed, as well as the growth rate, doubling time, and population doubling level calculated. Results for P3 and P4 growth rate analysis are shown in table 1. In Figure 1, representative images are shown of the cryopreserved AT-MSCs following thawing (Figure 1A), at passage P3 (Figure 1B), and at passage P4 (Figure 1C).

Passage	% Viability	Growth rate - μ (1/h)	Doubling time - g (h)	PDL
P3	98.8	0.038	18.3	3.8
P4	98.1	0.0378	18.4	3.8

Table 1: AT-MSCs growth rate evaluation and viability assessment. The growth rate (μ), doubling time (g), and population doubling level (PDL) were calculated for passage P3 and P4 according to equations (A), (B), and (C), respectively (see Methods). Cell viability is presented as % viable cells; growth rate is presented as 1/h; doubling time is presented as hours.

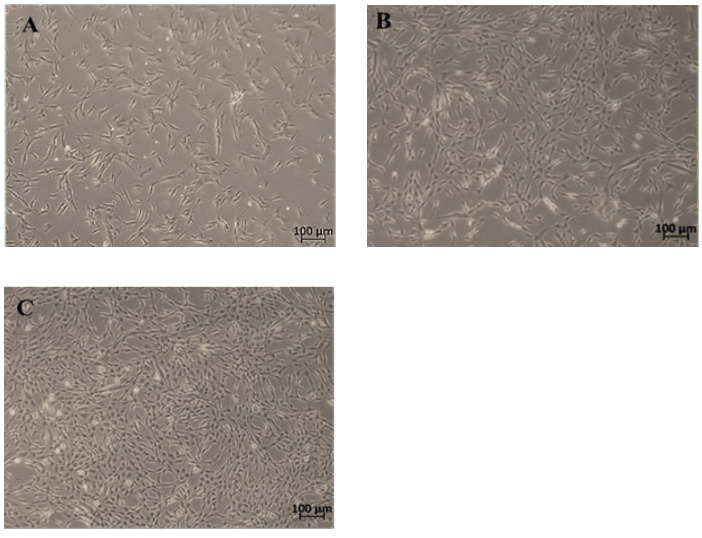

Figure 1: Representative images of AT-MSCs at passage P3 and P4. A) The cryopreserved cells (P1) were thawed and images were taken 6-8 hours after seeding (P2). For passage P3 as well as for P4 the cells were seeded at a concentration of 2500-4500 cells/cm^2 in T-75 tissue culture flasks. Representative images of AT-MSCs, taken prior to harvesting at the end of passage P3 (B), and P4 (C).

Identity analysis of the AT-MSCs:

The mesenchymal stromal cell identity of the AT-MSCs was evaluated at passage P3/P4 by flow cytometry analysis of the presence of the surface markers CD13, CD73, CD90, and the absence of the markers CD31, and CD45 (see Table 2 and Figure 2). The percentage of positive cells for the tested surface markers are presented in Table 2.

Tested surface markers (% positive cells)				
CD13	**CD73**	**CD90**	**CD31**	**CD45**
99.99	99.74	99.99	0.40	0.08

Table 2: AT-MSCs identity analysis. Data is presented as % positive cells for the respective surface marker. AT-MSCs expression level of the surface markers CD13, CD73, and CD90 should be >80% and the surface markers CD31 and CD45 <2%, according to Bourin et al., 2013.

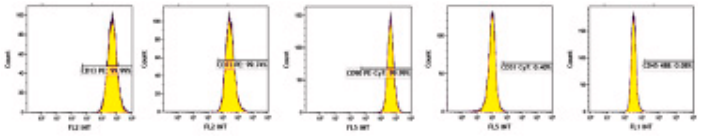

Figure 2: AT-MSCs identity analysis. Flow cytometry identity analyses are presented for AT-MSCs. The percentage of surface marker positive cells is marked.

AT-MSCs differentiation potential:

AT-MSCs at passage P3/P4 seeded and grown in adipogenic, chondrogenic and osteogenic differentiation medium, in order to evaluate the cryopreserved AT-MSCs stemness. The cells were stained as follows: Adipocytes were stained on day 10 of adipogenic differentiation with Oil Red O Stock to visualize lipids and fat deposits (Figure 3A); In osteocytes the calcium deposits in the cells were stained with Alizarin Red S on day 18-21 of osteogenic differentiation (Figure 3B); in chondrocytes the sulfated proteoglycan present in cartilage tissue was stained on day 21 of chondrogenic differentiation with Alcian Blue (Figure 3C). Histological analysis of the AT-MSCs is presented in Figure 3.

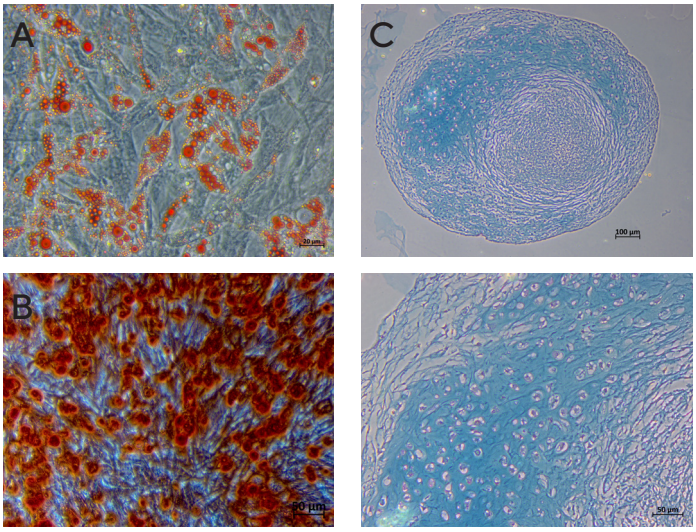

Figure 3: Differentiation potential of AT-MSCs into the adipogenic, chondrogenic and osteogenic lineages. Representative images of the differentiated AT-MSCs. The differentiated cells were stained as follows: A. Adipocytes were stained with Oil Red O Stock, B. Osteocytes were stained with Alizarin Red S, and in C. Chondrocytes were stained with Alcian Blue.

AT-MSC CFU-F assay

AT-MSCs on passage P4 were seeded at low density (5 cells/cm2) and incubated in MSC NutriStem® XF Medium for 10-14 days. CFU-F results were 30%. The percentage of CFU-F was calculated according to the equation (see Materials and Methods). The image below shows a crystal violet-stained CFU-F assay well. (Figure 4).

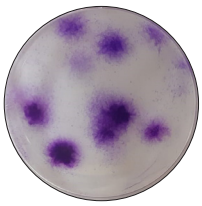

Figure 4: MSC Colonys stained with crystal violet. AT-MSC colony-forming unit (CFU-F) assay. CFU-F assay was performed. The image shows a crystal violet-stained CFU-F assay well.

Karyotype analysis

AT-MSCs were expanded to passage P6, and prepared for karyotyping, performed at Rambam Health Care Campus. The analysis results demonstrated the karyotype of the tested sample to be normal (Figure 5).

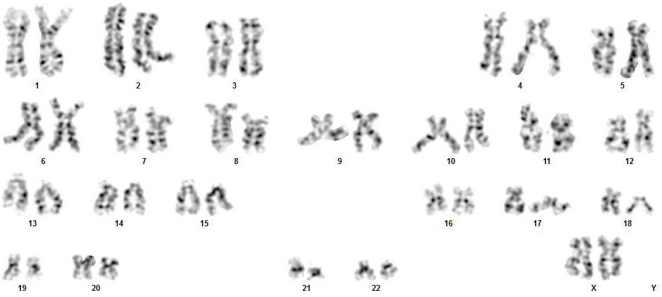

Figure 5: Karyotype analysis. Karyotype is presented for AT-MSCs.

Discussion

The study above illustrates the utility of the Sartorius MSC NutriStem® XF Medium when used to derive, isolate, and expand MSCs, as well as the MSCgo™ family of media to differentiate the aforementioned cells to the osteocyte, chondrocyte and adipocyte lineages.
MSCs are extremely sensitive cells and each stage of isolation and expansion must be adequate and sufficient. The smallest shift in cells environment and conditions might harm and influence their viability and potency.
Deriving cells for the purpose of cell therapy, regenerative medicine and other therapeutical applications has to be precise and consistent. Using the right media and creating the best conditions are crucial for achieving optimal outcomes.
MSC NutriStem® XF Medium, a defined scientifically and regulatory supported product, was developed in order to ensure optimal MSC growth parameters such as high fold expansion rate, established differentiation potential, normal karyotype and typical MSC phenotype markers.

This application note describes the extraction of highly viable adherent AT-MSCs from human adipose tissue, their growth on tissue culture flasks, expansion, detachment, freezing and thawing – followed by evaluation of viability, differentiation analysis, karyotype analysis and many other parameters. MSC NutriStem® XF Medium was used for the isolation and expansion stages and between passages. The results demonstrate excellent cell counts after derivation and post thaw, very high viability, excellent differentiation potential, high percentage of cells presenting positive MSC markers for the identity analysis and overall support evidence for normal, viable and potent cells.

Conclusion

Using the right media and solutions is crucial and critical for achieving large numbers of high quality, viable, multipotent, normal phenotype and karyotype MSCs, suitable for regenerative medicine, cell therapy and other therapeutical applications.
This application note shows that MSC NutriStem® XF Medium supports desirable outcomes when isolating, expanding and maintaining MSCs, successfully ensuring the cells fine phenotype, viability and multipotency. These results demonstrate that MSC NutriStem® XF Medium is a good reliable option for producing MSCs suitable for therapies and clinical applications.

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For further contacts, visit www.sartorius.com

Germany
Sartorius Stedim Biotech GmbH
August-Spindler-Strasse 11
37079 Goettingen
Phone +49 551 308 0

USA
Sartorius Stedim North America Inc.
565 Johnson Avenue
Bohemia, NY 11716
Toll-Free +1 800 368 7178