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International Journal of Molecular and Immuno Oncology

Original Article

Isolation and characterization of mesenchymal stem cells derived from amniotic fluid: A prospective study

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Received : 30 November 2019
Accepted : 13 December 2019
Published : 13 May 2020

DOI
10.25259/IJMIO_22_2019

Quick Response Code:

ABSTRACT

Objectives: The study aims to isolate, expand, and check the feasibility and differentiation potential of amniotic fluid mesenchymal stem cell (AF-MSC) from the minimum amount of amniotic fluid.

Materials and Methods: Amniotic fluid samples were collected from women undergoing 2nd trimester amniocentesis between 16 and 24 weeks of period of gestation. MSCs were isolated and characterized by MSCs surface marker profiling and were expanded in specific growth media to assess their differentiation capability into osteocytes, chondrocytes, and adipocytes. The differentiation was confirmed using specific staining.

Results: The isolated AF-MSCs showed successful stem cell population for 18 samples out of 23. All the isolated AF-MSCs showed positivity for MSCs surface markers. For osteocyte differentiation, cells were cultured in osteogenic induction media for 4 weeks, and the differentiation was confirmed by staining with Alizarin Red S stain, which showed extracellular matrix mineralization. For adipocytes differentiation, the induction media exhibited lipid droplets and positive staining with Oil Red O stain. Similarly, cells cultured in chondrocytes differentiation media, showed positive staining with Alcian Blue.

Conclusion: AF-MSCs have the capacity to differentiate into common mesodermal cell types. Considering their easy accessibility, amniotic fluid could be a good source for MSCs with a greater potential for cellular therapy in various chronic disabling diseases, for example, spinal cord injuries, massive bone and cartilage damage, and demyelinating diseases.

Keywords: Amniotic fluid, Mesenchymal stem cells, Trilineage differentiation

INTRODUCTION

Stem cell biology is one of the most upcoming and promising research areas having applications in regenerative medicine and cell therapy. Stem cells can be isolated from both fetal and adult tissue sources. Embryonic stem cells (ESCs) are the stem cells derived from an embryo. ESCs were first isolated from the blastocyst and they were able to propagate readily and generate a variety of specialized cells including neural, cardiac, and pancreatic cells.[1,2]

ESCs are the most reliable source of stem cells, but retrieval of ESC lines through destroying a human embryo raises ethical issues. Furthermore, ESCs have a potential of teratoma formations.[3] Other alternative sources studied till now are bone marrow mesenchymal stem cells (BM-MSCs), adipose tissue, and umbilical cord.[4-6] However, tissue-specific MSCs may exhibit lower differentiation potential.[2] For BM-MSCs, tissue samples are obtained from donors

and a significant decrease in both quality and differentiation potential of BM-MSCs is found with the age of the donor. Several authors have demonstrated a negative correlation between donor age, number, and proliferative capacity of MSCs isolated from young or old donors.[7-12] Thus, the disadvantages of ESCs, and BM-MSCs, have encouraged scientist to search for alternative sources of stem cells.

Human amniotic fluid has been used for more than 70 years for the diagnosis of various fetal disorders and chromosome abnormalities. Since 1980, amniotic fluid is being considered more than simple diagnostic tool.[13] Amniotic fluid contains heterogeneous cell population derived from placental membranes, fetal skin, and digestive, respiratory, and urinary tract. Amniotic fluid is an important source of undifferentiated and partially differentiated cells due to its direct contact with the fetus.[14-16] The possibility of using amniotic fluid-derived pluripotent stem cells has been found appealing due to the relative safety of procedure required to retrieve the cells. In various studies till now, amniotic fluid stem cell lines have showed ability to differentiate into adipogenic, osteogenic, myogenic, endothelial, neurogenic, and hepatic cells. Importantly, unlike ESCs amniotic fluid-derived MSCs have not been found to induce tumor formation in the studies.[17-20] Research related to human stem cells is associated with the hope that these cells can be used for stem cell therapy for a wide spectrum of human diseases. Research on stem cell therapy is under rapid progress but so far, no therapeutic approach based on stem cell therapy has reached the level of routine clinical application. However, a variety of new research results provides strong evidence that amniotic fluid MSCs (AF-MSCs) can serve as a useful tool in regenerative medicine. These cells might play a key role in regenerative medicine in future as more knowledge is gained about them.

Although there are studies on amniotic fluid, only few human studies are available on isolation, characterization, and differentiation potential of MSCs derived from amniotic fluid. The aim of the present study was to isolate, culture, and characterize MSCs derived from human amniotic fluid during the 2nd trimester amniocentesis and further investigates their multilineage differentiation potential.

MATERIALS AND METHODS

Sample collection

The study was started after taking ethical clearance from the Institutional Committee for Stem Cell Research (IC-SCR-229/21-06-2014). An informed consent was taken from the patients. A total of 23 pregnant women of age 20–40 years, undergoing ultrasound-guided amniocentesis for genetic study after 16 weeks of gestation were recruited in the study. Other inclusion criteria were women undergoing amnioreduction in view of gross polyhydramnios. Pregnancy with a known gross fetal congenital malformation and cytogenetic study suggestive of abnormal karyotype and meconium or blood-stained liquor were excluded from the study.

After recruitment, women underwent amniocentesis. Approximately 5 mL (2–7 mL) of amniotic fluid was taken for this study in addition to 20 mL of amniotic fluid for fetal karyotyping. The samples were transported to stem cell facility within ½ h of the procedure in sterile container.

Processing, isolation, and establishment of AF-MSCs culture

All samples without visible contamination with blood were immediately processed for the establishment of primary culture. Samples were centrifuged at 1200 rpm and pellet was seeded into two Petri dishes with Dulbecco's Modified Eagle's Medium-low glucose (DMEM-LG) containing 10% fetal bovine serum (FBS) (HyClone, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL) in a humidified atmosphere at 37°C with 5% CO_2. After 3 days of incubation, the media were changed and the subsequent media changes were done at every 3rd day. When grown to confluence, cells were passaged using trypsin/ethylenediaminetetraacetic acid (Life Technologies, USA).

Characterization of AF-MSCs

Morphological analysis

Cultures of AF-MSCs were regularly observed for any morphological changes along with signs of contamination. The results were documented and cells were photographed with phase-contrast inverted microscope (Nikon, Japan).

Immunophenotyping

Single-cell suspensions were prepared using triple E solution and fluorochrome-conjugated antibodies were added to it, followed by incubation at room temperature for 1 h 20 min in the dark. The following anti-human antibodies were used: CD73-phycoerythrin (PE), CD90-PE conjugated with cyanine dye derivative (PECy5), HLA Class I-allophycocyanin (APC), HLA Class II-fluorescein isothiocyanate (FITC) (Becton Dickinson, USA), and CD29-FITC and CD105-APC (eBioscience, USA). Unlabeled cells were taken as controls. The cells were finally acquired on a BD-LSR II flow cytometer (Becton Dickinson, USA) and analyzed with FACs DIVA software (version 6.1.2).

Proliferation assay

To study the doubling time, AF-MSCs at the 3rd passage were seeded in 35 mm dishes. Ten thousand cells were seeded and

were kept in an incubator for 48 h. Cells were then trypsinized and counted. The mean of the counts was calculated and population doubling time (PDT) was calculated using the formula:

$$PDT = \frac{(T - T0)\ Log\ 2}{(Log\ N\ -\ Log\ N0)}$$

Where,
T: Time of harvesting
T0: Time of seeding
N: Number of cells harvested
N0: Number of cells seeded.

Trilineage differentiation of AF-MSCs

The differentiation potential of MSCs was examined using the 3rd passage cells.

Osteogenic differentiation

AF-MSCs were induced using DMEM-LG (Life Technologies, USA), 10% FBS (Life Technologies, USA), 50 µM ascorbic acid-2-phosphate, 0.1 µM dexamethasone, and 10 mM ß-glycerophosphate (Sigma-Aldrich, USA) for 28 days with intermittent change in media every 3rd day. Uninduced AF-MSCs were considered as experimental controls. The differentiation was confirmed by Alizarin Red S staining (HiMedia, India). After incubation, staining solution was removed and cells were washed at least 5 times with distilled water to get rid of the excessive color. Cells were then observed under a microscope.

Adipogenic differentiation

AF-MSCs were cultured in DMEM-LG with 10% FBS supplemented with 100 µM indomethacin, 1 µmol/L dexamethasone, 500 µM 3-isobutyl-1-methylxanthine, and 1 µg/mL insulin (Sigma-Aldrich, USA) for 21 days. Uninduced AF-MSCs were considered as experimental controls. The medium was changed every 3rd day. The differentiation was confirmed by Oil Red O staining (HiMedia, India).

Chondrogenic differentiation

Commercially available kit (Life Technologies, USA) was used for chondrogenic differentiation. Single-cell suspensions were made. Micromass culture was generated by seeding 10 µL droplets (consisting about 60,000–80,000 cells) of cell solution in 35 mm tissue culture plate and incubated for 2 h at 37°C with 5% CO_2 followed by addition of chondrogenic differentiation media. The cultures were referred every 3rd day for 14 days. Uninduced AF-MSCs were considered as experimental controls. Alcian Blue staining (Sigma-Aldrich, USA) was used for confirmation of differentiation.

Cryopreservation of expanded AF-MSCs

The expanded cultures of AF-MSCs were cryopreserved for further experimental uses. For this, the monolayer was detached using trypsinization method. The cells were centrifuged at 1200 rpm for 5 min at 20°C. The cell pellets were broken for calculation of cell number and viability. The cell pellets were suspended in cold cryopreservation media containing dimethyl sulfoxide (Sigma-Aldrich, USA), 70% FBS, and ×1 DMEM-LG, in a dropwise manner with the regular tapping of the cells at a concentration of 1×10^6 cells/mL. The cells with the cryomedia were put in pre-labeled (name, date, and cell concentration) cryovials (1.8 mL) and shifted to Mr. Frosty (Thermo Scientific, USA) which enabled a gradual drop in temperature at an average rate of 1°C/min. After 12 h, the vials were shifted to −196°C (LN2) storage container for long-term storage.

Statistical analysis

All data were presented in mean, standard deviation, median, frequency, and percentage. Correlation was assessed by Pearson's correlation coefficient. The continuous variable was compared in two groups by Wilcoxon rank-sum (Mann–Whitney) test. The data were entered into MS Excel sheet and statistical analysis was done using SPSS version 20.0. $P < 0.05$ was taken as statistically significant.

RESULTS

Isolation, culture, and morphological observation of AF-MSCs

Amniotic fluid sample from 23 women was obtained. On average, we obtained 4.48 ± 1.2 mL/sample (range 2–7 mL). AF-MSCs were successfully isolated from 18 out of 23 (culture efficiency is 78.3%) samples. Five samples showed bacterial contamination and no cell growth was found in those samples.

In culture, cells could be observed after 3–5 days. These were initially polygonal in shape then after 10–12 days became globular or strip shaped. With successive culture, the cell body resumed typical spindle-shaped morphology. AF-MSCs were cultured up to eight passages. [Figure 1].

Growth kinetics

Comparison between the period of gestation and total number of cells obtained at the 3rd passage and doubling time at the 3rd passage was done using median values. Wilcoxon rank-sum test was applied. Statistically, a significant difference was found between periods of gestation with doubling time. AF-MSCs from a period of gestation <20 weeks showed average doubling time of 37.29 h, whereas cells from POG ≥20 weeks doubled in population every 37.88 h.

A significant difference was found between periods of gestation with total number of cells. AF-MSCs from POG <20 weeks were on average 5 million while cells from POG ≥20 weeks showed cell growth only up to 4.8 million at the 3rd passage ($P < 0.05$).

Flow cytometry

Flow cytometry analysis showed that isolated AF-MSCs were positive for all surface markers CD29, CD73, CD90, and CD105, but negative for CD34/45 (hematopoietic markers), representing characteristic phenotypes of MSCs. The results also showed that the AF-MSCs expressed HLA-ABC (MHC Class I), but not HLA-DR (MHC class II) manifesting the low immunity of AF-MSCs [Figure 2].

Trilineage differentiation

Isolated AF-MSCs were studied for their trilineage differentiation potential.

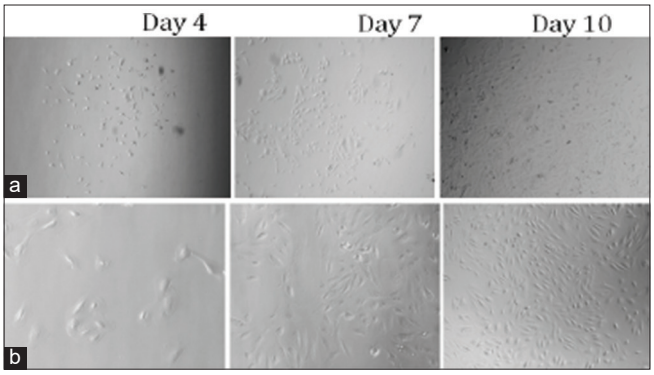

Figure 1: Representative phase-contrast images of amniotic fluid mesenchymal stem cells from day 4 to 10. (a) Images in ×4, (b) Images in ×10.

For osteogenic differentiation, cells cultured in osteogenic induction media for 4 weeks. Cells from regular culture were taken as controls. Cells from osteogenic differentiation media showed extracellular matrix mineralization and positive staining with Alizarin Red S stain. [Figure 3].

Similarly, cells cultured in adipogenic induction media exhibited lipid droplets and showed positive staining with Oil Red O stain after 3 weeks of culture. Cells cultured in chondrogenic differentiation media showed positive staining with Alcian Blue after 2 weeks of culture.

DISCUSSION

Stem cell attracts both biologist and clinician alike due to their self-renewal capacity and multipotency. We found that even 2–5 mL of amniotic fluid is enough for growing 4.8–5 million cells at the 3rd passage. Culture efficiency was 78.34% in the present study. Culture efficiency varied from 31.25% to 100% in other studies.[17,20,21] The use of different culture media and growth supplements or variation in the period of gestation of sampling could be a reason for the difference in culture efficiency. Studies showing lesser culture efficiency were from a higher period of gestation, for example, in Kunisaki *et al.*[22] who studied a wide range of period of gestation (20–37 weeks), including the 3rd trimester as compared to our study where only the 2nd trimester women were enrolled and isolated for AF-MSC. On the other hand, You *et al.*[20] included amniotic fluid samples during the cesarean section in his study. Even in another study by Spitzhorn *et al.*,[23] stem cells were isolated during cesarean sections, and they showed high similarity with MSC as evidenced by the expression of CD133 and various pluripotency-associated markers such as SSEA4, c-Kit, TRA-1-60, and TRA-1-81.

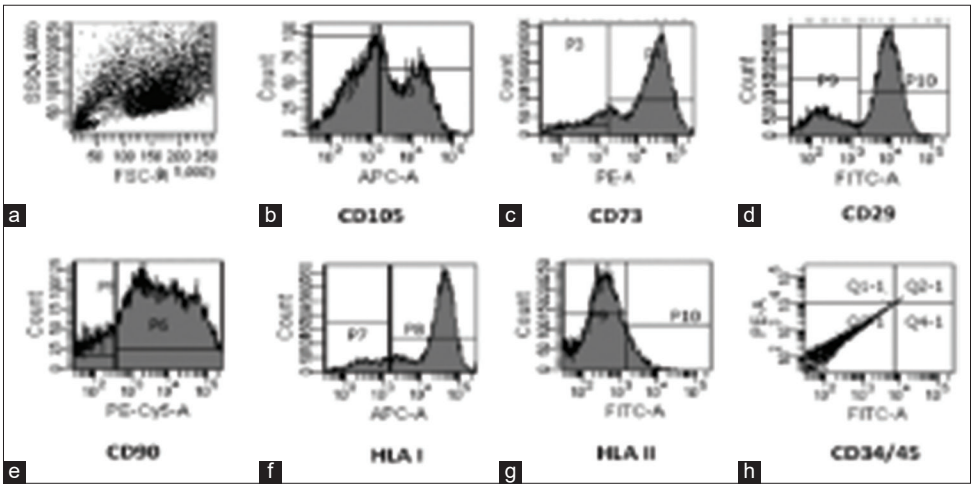

Figure 2: Quantitative flow cytometry analysis. (a) Representative flow cytometry histogram plot of unlabeled amniotic fluid mesenchymal stem cells (MSCs). (b-h) Representative flow cytometry histogram plots representing the expression of MSC-specific surface markers.

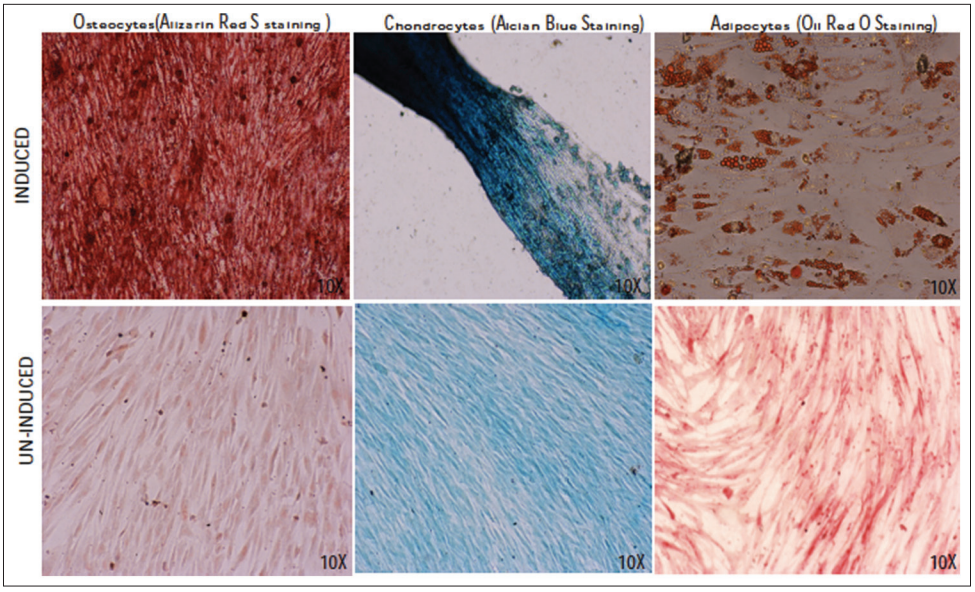

Figure 3: Representative images of differentiation of amniotic fluid mesenchymal stem cells into osteocytes, chondrocytes, and adipocytes.

In the present study, we succeeded in achieving our objective to isolate the MSCs from the amniotic fluid collected during the 2nd trimester amniocentesis between 6 and 24 weeks period of gestation. These cells were also positive for typical MSC surface markers and were able to differentiate into osteocytes, chondrocytes, and adipocytes. However, we could culture these cells only up to 7–8 passages and after that, these cells started showing morphological changes senescence. Interestingly, plethora of studies showed that AF-MSCs could be cultured up to 30 passages.[23] The reason for low passage expansion in the present study may be the differential culture conditions. However, the time constraint limited our potential to explore the reasons for early senescence of these MSCs for this study.

The differentiation of AF-MSC into osteocytes, chondrocytes, and adipocytes predicts its use in the treatment and management of various chronic disabling diseases, for example, spinal cord injuries, massive bone and cartilage damage, and demyelinating diseases. However, the major limitation is the source of MSC and the time of gestation at which it should be isolated. Sepsis remains a major constricting factor after the isolation of MSC from women in the developing countries.[24] However, this factor is being recently explored in animals and it is found that there are very slight differences between MSC capacities depending on tissue source such as their impact on regulatory T cells but none justifying the use of a source over the other in terms of potency. However, it was concluded that sepsis marginally depends on their source, with at least as potent profile conferred by Wharton jelly-MSCs.[24]

CONCLUSION

Thus, it can be concluded that the flexible potential of MSC to differentiate into several tissues and their ability to expand for extended periods of time without losing their original characteristics highlights them for use in stem cell-based therapies and translational research. Although it is obvious that even small variations in the isolation and culture protocol, such as centrifugation speed, media composition, and serum concentration can significantly influence the yield, quality, and composition of the isolated cell population, their ability to differentiate into specialized cells makes them distinct from other stem cells. Still, their self-renewal and differentiation mechanisms still remain to be fairly understood. This stresses the need for having a standard protocol of isolation and characterization of MSC for a proper yield. In our study, 1–4 vials/samples are cryopreserved for future use. Future plans are to study the effect of other culture media on cell growth and differentiation of MSCs into other cell lines and to do comparative studies.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Authors' contributions

All authors have equally contributed to this manuscript.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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How to cite this article: Deedwania P, Deka D, Mohanty S, Dadhwal V, Sharma A. Isolation and characterization of mesenchymal stem cells derived from amniotic fluid: A prospective study. Int J Mol Immuno Oncol 2020;5(2):67-72.