Safety and Efficacy of Subcutaneous Infusion of Human Adipose Tissue-Derived Mesenchymal Stem Cells in the Treatment of Mouse Atopic Dermatitis: A Pilot Study

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Abstract

Background: Atopic dermatitis (AD) is a type of inflammation of the skin. It results in itchy, swollen, red, and cracked skin. Mesenchymal Stem Cells (MSCs) could suppress allergic responses in AD. Indeed, the immunomodulatory ability of MSCs can be usefully applied for the treatment of autoimmune and inflammation – related diseases such as asthma, rhinitis and dermatitis. We aims to evaluate safety and efficacy in DNFBI – induced atopic dermatitis mouse model after a subcutaneous infusion of xenogeneic human Adipose Tissue – derived MSCs (hAT-MSCs). Therefore, hAT-MSCs have possibility as a new drug for AD.

Keywords: Mesenchymal stem cells • Atopic dermatitis • Stem cell therapy • Subcutaneous • Safety

Introduction

Mesenchymal Stem Cells (MSCs) have an inherent ability for self-renewal, proliferation, and differentiation toward mature tissues, depending on the surrounding microenvironment. Such characteristics intrinsic to stem cells make MSCs very attractive for use in the cell therapy and regenerative medicine. MSCs are found in various tissues, including Bone Marrow (BM) [1], umbilical cord blood [2], placenta [3], and fat [4]; these tissues contain a rare population of adult stem cells that have the potential to undergo multilineage differentiation into osteoblasts, adipocytes, and chondroblasts in vitro [5].

The MSCs comprise only a minor fraction of BM and other tissues, with Bone Marrow MSCs (BM-MSCs) constituting a mere 0.0001%-0.01% of all BM-nucleated cells [6]. In contrast, adipose tissues contain 1 x 10^3 MSCs in each gram of fat [7]. Furthermore, the differential capacity of Adipose Tissue-derived MSCs (AT-MSCs) is less affected by the donor age [8]. Adipose tissue is an accessible, abundant, and reliable site for the isolation of adult stem cells suitable for tissue engineering and regenerative medicine applications. In this regard, the treatment efficacy of AdMSCs for various diseases has been reported using animal models. Another interesting characteristic of MSCs is their ability to mobilize to areas of tissue injury. Localized abdomen irradiation significantly enhances MSC homing specifically to radiation-injured tissues of mice [9]. The potential for the minimally invasive delivery of MSCs via systemic infusion is of particular interest because of the easy, minimally invasive application of stem cells. Before culture-expanded stem cells can be used in the human clinic, Good Manufacturing Practices (GMP) must be developed for the production of clinical-grade human stem cells. The stem cells should be evaluated for aerobic and anaerobic bacteria, endotoxin, and ESR. In This study safety of the stem cells, including endotoxin and ESR, was confirmed. Finally, the animal's models were administered high dose (0.75 x 10^5 cells per patient) of xenogeneic hAT-MSCs subcutaneously and monitored the safety as well as the efficacy.

Materials and Methods

Isolation and culture of hADMSCs

Human adipose tissues were obtained by simple liposuction from the abdominal subcutaneous fats with an informed consent. Subcutaneous adipose tissues were digested using 1 mg/ml collagenase I (Gibco (UK)) under gentle agitation for 60 min at 37°C. The digested tissues were filtered through a 100 µm nylon sieve to remove cellular debris and were centrifuged at 470 g for 5 min to obtain a cell pellet. The cells were resuspended in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) based media containing 0.2 mM ascorbic acid and 10% Fetal Bovine Serum (FBS) (Gibco (UK)). The cell suspension was then re-centrifuged at 470 g for 5 min. The supernatant was discarded and the cell pellet was collected. The cell fraction was cultured overnight at 37°C under 5% CO₂. The cell medium was changed to Keratinocyte-SFM (Invitrogen) based media containing 0.2 mM ascorbic acid, 0.09 mM calcium, 5 ng/ml rEGF, and 5% FBS. The cells were maintained for 4–5 days until confluent (passage 0). When the cells reached 90% confluency, they were sub cultured and expanded in keratinocyte-SFM-based media containing 0.2 mM ascorbic acid, 0.09 mM calcium, 5 ng/ml rEGF, and 5% FBS. The cells were expanded up to passage 3. FBS contaminant from cultured MSCs were completely removed by several washing with PBS. Aliquots of the hADMSCs were then tested for cell viability and fungal, bacterial, endotoxin, and mycoplasma contamination as demanded by the LAI kit, PCR, before further use. The procedure for hADMSCs preparation was performed under the Skin and Stem Cell Research Center (SSRC) regulations at the Tehran University of Medical Sciences.

Flow cytometry analysis

The flow cytometric characterization of MSCs was performed using the following monoclonal antihuman antibodies including CD90, CD34, CD45 and CD44 conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE). All antibodies were purchased from the Abcam (USA) company. FACSort
(Becton Dickinson, San Jose, CA) was used for sample acquisition and FACS Flectr 6.1 software was used for the data analysis. Cells were trypsinized, after reaching 90% confluence, and re-suspended in DPBS (Invtrogen). Approximately 10^5 cells/ml were incubated in dark with labeled antibodies at 37°C for 1 h. Cells were then characterized using the following conjugated monoclonal antibody combinations; CD34/ CD45/ CD90/ CD44. Negative controls of FITC- or PE- conjugated corresponding immunoglobulins were used. Samples were then washed and acquired immediately after staining. A total 300,000 events were acquired, at low speed, after the cytometer was compensated and calibrated.

**In vitro differentiation**

For osteogenic induction, hAdMSCs cells were plated at 1 x 10^5 cells/mL in Keratinocyte-SFM-based media containing 0.2 mM ascorbic acid, 0.09 mM calcium, 5 ng/mL rEGF, and 5% FBS. The medium was replaced with osteoblast induction medium (NH Osteodiff medium) or control medium. The cells were maintained in culture for 14 days, with 90% of the media being replaced every 3 days. Osteogenic differentiation was detected by Alizarin red S staining (Stem Cell Technology Company).

**Detection of mycoplasma**

Many methods are available for the detection of mycoplasma. We used PCR in this study (Table 1). Cells for testing should be prepared fresh whenever possible, as storage or snap freezing may reduce the number of viable organism. Appropriate positive and negative controls must always be included in PCR assay.

**Polymerase chain reaction analysis**

Polymerase chain reactions (PCR) of hAdMSCs at different passages (0, 3, 6 or 9) were analyzed.

The cell cultures were obtained from hAdMSCs bank at the Skin and Stem Cell Research Center (SSCRC). Totally, 3 samples of hAdMSCs have been checked for mycoplasma contamination.

**DNA extraction and nucleotide sequences of primers**

Boiling method was used to extract DNA. Briefly, 100 µl of the cell suspension in mineral Oil was placed in boiling jar for 10 minutes. The cells were placed on ice to cool down and centrifuged at 12000 g for 10 minutes; the supernatant was then transferred to a new tube and used as template in PCR. DNA extraction and nucleotide sequences of primers

**Passaging and cell proliferation analysis**

To determine the proliferation rate, a total of 1,000 cells/cm² were seeded in T-25 flasks. Each time point and the passage had three replicates. Cells were trypsinized after reaching 90% confluency. The cell numbers were then counted in each passage and assessed for viability using trypan blue dye. Cells were re-plated for subsequent passages and a total of ten passages were performed in this study.

**Cell proliferation assay using MTT**

To correlate between the expression of cell proliferation, a colorimetric 3-(4,5-dimethylthiazol-2-y1) 2,5 dihydroxyterrazolium bromide (MTT) (Sigma-Aldrich) assay was used. The MTT assay, that replaced the radioactive tritiated thymidine incorporation assay, was the first widely accepted method to measure cell proliferation. After 4 days of culture, MTT solution was added and the cells were incubated in 5% CO₂ humidified incubator at 37°C for 4 h. Then the formazan solubilization solution was added and the absorbance was detected at 450 nm (Tables 5-8).

**Quality control test**

Contamination of the gram negative bacterial endotoxin was detected on

| Table 1. Mycoplasma detection method, sensitivity and advantage and disadvantages. |
|--------------------------------------|-----------|-----------|-----------|-----------|
| Method                          | Sensitivity | Advantage | Disadvantage |
| PCR                              | High       | Rapid     | Requires optimization |

| Table 2. Primers used for fragment amplification of the 16sRNA gene of Mycoplasma by PCR. |
|--------------------------------------|-----------|-----------|-----------|
| Species | primers sequence | fragment size |
| Mycoplasma | 5' - GGG AGC AAA CAC GAT AGA TAC CCT - 3' | 270 bp |
| Mycoplasma | 5' – TGC ACC ATC TG TAC CTC TGT AAC CTC – 3' |

| Table 3. PCR amplification with specific primers for Mycoplasma. |
|--------------------------------------|-----------|-----------|
| PCR program | Mycoplasma |
| 40 cycles | Denaturation | 94°C (4 min) |
| 40 cycles | Denaturation | 94°C (40 min) |
| 40 cycles | Annealing | 58°C (60 min) |
| 40 cycles | Extensions | 70°C (50 min) |
| Final Extensions | 72°C (7 min) |

| Table 4. Immunophenotype of culture – expanded human mesenchymal stem cells at passage 3 |
|--------------------------------------|-----------|-----------|-----------|-----------|
| Surface markers | The positive percentage (%) (n=8) |
| CD34 | 99.8 ± 0.197 |
| CD44 | 2.22 ± 97.8 |
| CD45 | 99.8 ± 0.206 |
| CD90 | 4.15 ± 95.8 |

Data are expressed as mean ± standard deviation.

| Table 5. Viability (%) of hAdMSCs after 24 hours by MTT assay |
|--------------------------------------|-----------|-----------|-----------|-----------|-----------|
| Repetition | 1 | 2 | 3 | 4 | 5 | control |
| Viability (%) | 98/66 | 88/80 | 95/55 | 93/14 | 92/14 | 98 |

| Table 6. Viability (%) of hAdMSCs after 48 hours by MTT assay |
|--------------------------------------|-----------|-----------|-----------|-----------|-----------|
| Repetition | 1 | 2 | 3 | 4 | 5 | control |
| Viability (%) | 90/5 | 89/87 | 89/69 | 89/56 | 91/05 | 96 |

| Table 7. Proliferation rate of hAdMSCs after 24 hours by MTT assay |
|--------------------------------------|-----------|-----------|-----------|-----------|-----------|
| Repetition | 1 | 2 | 3 | Control | 4 | 5 |
| Proliferation rate | 1/403 | 1/8 | 1/44 | 96 | 1/6 | 1/546 |

| Table 8. Proliferation rate of hAdMSCs after 48 hours by MTT assay |
|--------------------------------------|-----------|-----------|-----------|-----------|-----------|
| Repetition | 1 | 2 | 3 | 4 | 5 | control |
| Proliferation rate | 2/186 | 2/4 | 2/346 | 2/293 | 2/346 | 96 |
the stored hAdMSCs, using the LAL kinetic–kit (lonza). LAL test is a quantitative method to detect Gram negative bacterial endotoxin in a solution. LAL is an aqueous extract of blood cells (amebocytes) from the “horseshoe crab” Limulus Polyphemus. The endotoxin catalyzes the activation of a proenzyme in the LAL. The rate of the reaction depends on the concentration of endotoxin presence. Standard curve with 0.005 endotoxin unit EU/ml was used in this assay. Determination of detectable endotoxin was performed by the high and low points of a valid standard curve. The correlation coefficient (CC) of the calculated standard curve should be ≥0.980. In our assessment evaluation on 100 µl supernatant by incubating the hAdMSCs and the calibrators at 37°C in the presence of the LAL for 1 hour and 40 minutes in a microplate reader ELX (lonza).

**Determination of erythrocyte sedimentation rate (ESR)**

The erythrocyte sedimentation rate (ESR) is a commonly used test to screen for inflammatory conditions such as infections, autoimmune diseases, and cancers. The modified Westergen method (requires collecting 2 ml of venous blood into a tube containing 0.05 ml of sodium citrate) was used for the measurement of erythrocyte sedimentation rate. Four volumes of the blood (anticoagulated blood with EDTA) sample and 1 volume of the normal saline were mixed (Wetergen, 1957). The blood was then placed glass Westergen pipette and allowed to stand for 1 h in a vertical position. The number of millimeters the red blood cells fall during this time represented the ESR.

**Statistical analysis**

The continuous variables are presented as mean ± SD. All statistical comparisons were made using one–way ANOVA followed. P<0.05 was considered statistically significant. Analyses were performed using SPSS software.

**Results**

**Morphology of hAdMSCs during culture-expanded**

hAdMSCs were microscopically observed at every passage. We observed cell deformation up to passage 4. Cells with spindle–shaped and a fibroblast–like morphology were checked 24-48 h after isolation and kept to passages 4 (P4). These characteristics were well preserved during repeated subculture. hAdMSCs showed ability to form multilayer after being confluent. Characterization of hAdMSCs culture–expanded cells at passage 4 were prepared from 8 donors, and later on the portion of enlarged cells with changed morphology was gradually increased, the volume of the hAdMSCs increased gradually with increasing passage number, as shown in Figure 1.

**Immunophenotypic characterization of culture – expanded hAdMSCs at passage 3**

Immunophenotypic characterization of the hAdMSCs, culture–expanded cells at passage 3 was prepared from 8 donors, and surface protein expression was examined by flow cytometry. Flow cytometric analysis revealed that MSCs were identified based on several markers such as CD34, CD90, CD44 and CD45. The hAdMSCs were positive for CD90, CD44, but were negative for CD34, CD45, as shown in Figure 2. The results of FACS analysis on 8 different donor samples are listed in Table 4.

**Characterization of cell differentiation**

The ability of hAdMSCs to differentiate into various cell types was evaluated. Differentiation of hAdMSCs was confirmed using the human commercial differentiation media (Gibco, Grand Island) (Figure 3). Culture–expanded cells at passage 3 showed the ability of in vitro differentiation into osteoblasts as assessed by Alizarin red S staining (Figure 3A), and into chondroblasts as assessed by toluidine blue O staining (Figure 3B). Cells undergoing chondrogenic differentiation appeared blue when stained with toluidine blue and undergoing osteogenic differentiation appeared red when stained with Alizarin red.

**Results of the PCR**

The PCR results were interpreted by comparing the presence and size of the PCR product from test samples with those of the positive control reaction. The positive control and the inhibition control both showed a 270-bp band, whereas the negative controls showed no bands in this region (Figure 4). The test was only accepted as a valid test when the negative controls were negative and both the positive control and the inhibition control revealed appropriate bands. In this study, PCR products next to the size marker, positive and negative control using electrophoresis, on 1% mycoplasma agarose gel was used as a control. Using specific mycoplasma species primers, The PCR test was performed with applied changes and a dedicated single band was observed in the 270-bp region.

**Cell viability**

To evaluate the viability of the cultured hAdMSCs cultured different passages, the MTT assay was used. The representative absorbance of the hAdMSCs is shown in Tables 5 and 6. The cell viability at 48 hours of incubation decreased as compared with 24 hours of incubation. This result indicates that cell viability decreased with increasing the age of cells in culture.

**Proliferation of the hAd-MSCs**

Significant increase in proliferation was observed in passage 1 as compared with passage 2, indicating more rapid rate of proliferation at early stages. Based on these results, early passages, (P1) and (P2), tended to double their population in 24 hours and 48 hours as shown in Tables 7 and 8 (Figure 5).

**Endotoxin test**

The endotoxin test was performed once times, under operating conditions by the QC manager. Endotoxin concentration in 250 µl of samples, it was less than 0.43 EU/ml. Pyrogen–free water used as a negative control, had an endotoxin value less than the lowest standard according to the kit LAL protocol (lonza).

**ESR test**

The purpose of this section aimed to define treatment-associated adverse events and biological efficacy measure by ESR response criteria and
Figure 2. Immunophenotypic characterization of the in vitro cultured hAdMSCs. Representative linear plots of cytometric analysis against CD90, CD44, CD34 and CD45 for passage 3. Flow cytometric analysis was performed by gating on the CD34, CD45 negative population as shown above.

Figure 3. Multilineage differentiation of culture-expanded hAdMSCs (20x), (A) at day 14 after differentiation into osteoblasts. Calcium deposits stained in red in differentiated cells (Alizarin Red staining). (B) Image of hAdMSCs (10x), at day 21 after differentiation into chondrocytes.
injury and inflammation, where they promote functional recovery of tissue as supporting the safety of hAd-MSCs. MSCs migrate preferentially to sites of did not exhibit any embolism–related, clumped cells–related symptoms, for the first time that subcutaneous administration of xenogeneic hAd-MSCs, on the morphological, immunophenotypic, and differentiation characteristics, passaged MSCs are genetically stable. They must also obtain consistent data to the safety of culture-expanded stem cells, researchers must verify that the capacity [16], differentiation potential [17], and trophic activity [18]. With regard long – term culture can alter the quality of MSCs, including their proliferative in vivo expansion. However,

Discussion

The use of MSCs cells from adipose tissues is a new concept technology that breaks the limits of current drug and medical technologies. Adipose tissue has been demonstrated to be a viable source of MSCs in numerous species, including dogs and humans [10-16]. In regenerative medicine, stem cells can replace injured cells and tissues. They can also be used to address various incurable diseases that cannot be adequately treated with medication or surgery, including critical limb ischemia, arthritis, and atopic dermatitis. This study was designed to evaluate the clinical effects of AD-MSC therapy up to 2 weeks post infusion (Figure 6).

immunological parameters 2 weeks post infusion. In addition, to evaluate the safety and efficacy of hAdMSCs therapy up to 2 weeks post infusion (Figure 8).

Conclusion

In the present study, we demonstrated that a subcutaneous route of hAT-MSCs injection can be more effective than an intravenous route to reduce gross and histological signatures of AD in mouse model. These findings led us

![Figure 4](image-url)  
**Figure 4.** Typical gel photo from direct PCR. Evaluation of the mycoplasma PCR product in tested samples. Numbering from left to right, Lane M) marker 100- bp DNA ladder ), Lane 1) positive control (standard sample band, MS-NCTC 10124-05 in the 270- pb region), Lane 2) negative control.

![Figure 5](image-url)  
**Figure 5.** The number of hAdMSCs in two day, during incubation times 24 and 48 hours by MTT assay.

![Figure 6](image-url)  
**Figure 6.** Two weeks after infusion, the erythrocyte sedimentation rate (ESR) of the laboratory groups was measured. As shown ESR was reduced 24h in the group infused with the higher dose of hAdMSCs (0/75 × 10^6 hAdMSCs per subject) compared to other groups.

![Table](image-url)  
**Table 1:** ESR measurement of different groups.

**Model** | **Model-cell L** | **Model-cell H** | **Control**
--- | --- | --- | ---
0 | a | a,b | b
5 | b | b | b
10 | b | b | b
15 | b | b | b
20 | b | b | b
to conduct this study using subcutaneous route for the administration of hAT-MSCs. In this study, we also proved that a subcutaneous administration of hAT-MSCs can be successfully used for the treatment of AD and is well tolerated without any safety issues. Therefore, we concluded that the subcutaneous administration of 0.75, 0.375 million cells/kg hATMSCs appears to be safe and effective as a management option in mouse atopic dermatitis. The efficacy of the treatment was assessed based on the response of each patient compared with its own baseline score before administration. More experiments are needed to evaluate the safety and efficacy of hATMSCs in human tissues.

Conflict of Interest

The author has no conflict of interest to declare.

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References