Effects of Epidermal Growth Factor, Glial Cell Line-Derived Neurotrophic and Leukemia Inhibitory Factor on the Proliferation and Differentiation Potential of Adipose Tissue-Derived Mesenchymal Stem Cells
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Abstract

Background: There is a great deal of interest in using adipose tissue-derived mesenchymal stem cells (AT-MSCs) for clinical applications. However, the important limitations of clinical application of stem cells are the small number of cells and their differentiation into undesirable lineage in vitro. To overcome this problem, various growth factors are studied extensively.

Objectives: The current study aimed at using 3 different doses of epidermal growth factor (EGF), glial cell line-derived neurotrophic (GDNF), and leukemia inhibitory factor (LIF) to culture AT-MSCs and evaluating their effects on proliferation, viability, differentiation potential, and maintenance of the stemness state of cells.

Methods: The current experimental study was conducted on 8 - 10 male NMRI (Naval medical research institute) mice provided from research center and experimental animal house of Jundishapur University of Ahvaz, Iran, from September 2016 to April 2017. AT-MSCs were isolated from mice adipose tissue. The cells were cultured with three different doses of EGF, LIF, and GDNF. The morphology and cell proliferation of the AT-MSCs were studied on the days 5, 7, and 11 by an inverted microscope and MTT assay, respectively. To evaluate the stemness state of the cells, Oct4 expression was measured using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Also, differentiation potential of AT-MSCs toward adipogenic and osteogenic lineages was assessed. All tests were done in triplicate.

Results: Proliferation and viability of the AT-MSCs cultured in 10 µg/mL EGF, 5 µg/mL LIF, 5 µg/mL GDNF (b2 group) and 20 µg/mL EGF, 5 µg/mL LIF, 5 µg/mL GDNF (b3 group) increased significantly in the days 7 and 11 (170.27 (13.94), 174.39 (18.85) versus 100 (12.08) P < 0.001 (the day 7) and 152.45 (15.75) P < 0.001, 131.53 (19.17) versus 97.64 (13.43) P < 0.022 (the day 11). And differentiation potential of the cells was sustained, but Oct4 overexpressed in treatment groups on the days 7 and 11.

Conclusions: EGF, LIF, and GDNF enhanced proliferation and viability of the AT-MSCs, but for clinical purposes, the growth factors should be applied cautiously.

Keywords: Stem Cells, Cell Differentiation, Epidermal Growth Factor, Leukemia Inhibitory Factor, Glial Cell Line-Derived Neurotrophic Factor

1. Background

The appropriate candidates for cell therapy and regenerative medicine are stem cells (1-3). Three main characteristics of stem cells are high proliferation, self-renewal, and differentiation (4). Adipose tissue-derived mesenchymal stem cells (AT-MSCs) are easy-to-obtain and safe. They are one of the suitable options for cell therapy for many diseases such as infertility (5). In recent years, researchers developed a new method to treat infertility by differentiating stem cells into male and female germ cells in vitro and in vivo; they took promising steps toward treatment of infertility (6, 7). The important limitations of clinical applications of stem cells are the small number of cells and their differentiation into undesirable lineage in vitro (8, 9). To obtain large numbers of these cells, preventing
the senescence process and differentiation in vitro, various growth factors and nutrient supplements are studied extensively (10-12). Epidermal growth factor (EGF) is a 53-amino acid protein (13), which increases spermatogonial proliferation and modifies spermiogenesis and steroidogenesis in mammalian testes (14, 15). It is reported that EGF signaling pathway stimulates proliferation and maintenance of MSCs without differentiation into specific lineages (16, 17). EGF increases proliferation of bone marrow mesenchymal stem cells (BMSCs), while sustains differentiation potential of the cells (16). Also, EGF and basic fibroblast growth factor (bFGF) enhance proliferation of human AT-MSCs (hAT-MSCs), but modify differentiation potential of the cells (18), and in combination with bFGF and insulin-like growth factor 1 (IGF-I) can significantly increase the growth and viability of MSC-derived NPCs (12). Evidence implies that treatment of MSCs with EGF is useful for the maintenance of stem cells in the stemness state (19). After binding the receptor, LIF activates the JAK-STAT signaling pathway (20). This signaling pathway is reported to play a role in stem cells self-renewal (21). LIF activates the STAT3 and preserves the undifferentiated state of embryonic stem cells and MSCs, and allows effective proliferation in vitro (22, 23). Also, LIF plays a role in proliferation of primordial germ cells (24). GDNF is a neurotropic factor produced by brain glial cell (25, 26). Also, GDNF is expressed in ovary and testis during development. In the testis, GDNF is responsible for self-renewal of spermatogonial stem cells in vitro and in vivo (27).

Generally, EGF, LIF, and GDNF are secreted in testicular niche and play a role in the process of proliferation and maintenance of spermatogonial stem cells (15, 22). Thus, these factors can be appropriate candidates for culturing stem cells for cell therapy and treatment of some problems such as male infertility.

2. Objectives

The current study aimed at determining the appropriate doses of 3 growth factors (EGF, GDNF, LIF) for culturing AT-MSCs in order to enhance the proliferation rate and sustain the stemness state and differentiation potential of the cells for clinical applications. The concomitant effects of EGF, LIF, and GDNF on culturing AT-MSCs are not reported so far. The combination of these testicular niche factors for AT-MSCs culturing can be appropriate to cell therapy of infertility treatment purposes.

3. Methods

3.1. Experimental Animals

The current experimental study was conducted from September 2016 to April 2017 at Cellular and Molecular Research Center of Jundishapur University of Ahvaz, and Shahid Chamran University of Ahvaz, Iran. All experiments were done in triplicate by calibrated instruments.

All tests on animals were in accordance with the guide for the care and use of experimental animals and approved by the animal care and use committees of Jundishapur University of Ahvaz (IR.AJUMS.REC.2015.739).

Ten male NMRI (Naval medical research institute) mice, 4-6-week-old were provided by research center and experimental animal house of Jundishapur University of Ahvaz, Iran as cell donors. Animals were maintained in temperature-controlled rooms (20 - 22°C) at 20% - 50% moisture content, under 12:12 hour light/dark cycle conditions. Mice were fed with nutritionally adequate diet.

3.2. Isolation and Culturing Mouse Adipose Tissue-Derived Mesenchymal Stem Cells

AT-MSCs were isolated from preperitoneal and the surrounding epididymal adipose tissue of 4-6-week-old mice according to the previously described method with some modifications (28). Adipose tissue was washed 3 times with PBS (phosphate-buffered saline) (Gibco, Life Technologies, USA) supplemented with 0.03 penicillin/streptomycin antibiotic (Gibco, Life Technologies, USA) and amphotrysin B (Sigma Aldrich, USA). Tissue samples were physically sliced into 1-2-cm³ pieces and incubated with the Dulbecco modified eagle's medium (DMEM) (DMEM; Gibco, USA) containing 1% collagenase type I at 37°C for 25 - 30 minutes. To neutralize the collagenase, 10% FBS (fetal bovine serum) (Gibco, Life Technologies, USA) was added to the culture.

The suspension of cells was centrifuged at 1200 rpm for 6 minutes to pellet the cells. Then, the cells were cultured in 25-cm² culture flasks in either a, Basal medium composed of DMEM, 2% FBS, and 0.01 penicillin/streptomycin; or b, Basal medium supplemented with 3 different doses of growth factors 1, 10 µg/mL EGF (Sigma, USA), 1 µg/mL LIF (Millipore, USA), and 1 µg/mL GDNF (b1 group) 2, 10 µg/mL EGF, 5 µg/mL LIF, and 5 µg/mL GDNF (b2 group) or 3) 20 µg/mL EGF, 5 µg/mL LIF, and 5 µg/mL GDNF (b3 group). Then, the cells were incubated at 37°C in 0.05% CO₂.

3.3. Flow Cytometry

MSCs have an adhesive nature, differentiation potential, and fibroblast like morphology (29, 30). However, the type of cells extracted from animals should be approved. It is routinely studied by CD markers on the cell surface.
using flow cytometry analysis. In the current study, the lack of expression of CD45 (abcam, USA) and CD31 (abcam, USA) markers (specific to endothelial and hematopoietic stem cells, respectively) and the expression of the CD44 (abcam, USA) and CD90 (abcam, USA) markers (specific to mesenchymal stem cells) were assessed (31).

3.4. Analysis of AT-MSCs Morphology and Proliferation

The AT-MSCs cultured in described mediums were incubated with 0.25 trypsin-EDTA (ethylenediaminetetraacetic acid) and cultured in a 12-well plate at 10^4 cell/mL concentration; the morphology of AT-MSCs was assessed by an inverted microscope (Olympus, IX71, Japan) on the days 3, 7, and 11.

Cell proliferation and viability were assessed using MTT assay. In the current study, the cells were cultured with 3 doses of growth factors in a 96-well plate at 5000 cell/mL concentration on the days 3, 6, and 11. After washing the cells with PBS, cells were incubated with MTT (0.5 mg/mL) at 37°C for 4 hours. Then, 130 µL of DMSO was added to the wells and the plate was shaken for 30 minutes; afterward, absorbance or optical density (OD) was read at 570 nm on an ELISA (enzyme-linked immunosorbent assay) reader (Biorad, 680 series, USA). The cells cultured in DMEM containing 2% FBS, without growth factors, were used as negative control.

3.5. RNA Extraction, cDNA Synthesis, and Real-Time PCR

Overall RNA was extracted using RNeasy Mini Kit (QIagen, Germany) according to manufacturer’s protocol as follows: 10^6 cells seeded in a culture medium containing growth factors, as well as a basal culture medium without growth factors as control group, were collected on the days 3, 7, and 11. After washing the cells using PBS, the Buffer RLT was added to the cells and centrifuged at maximum speed. Supernatant was extracted and used in the next steps; then, 70% ethanol was added to lysate and the sample was transferred to RNeasy Mini spin column and centrifuged at 8000 g. Then, Buffer RQI and Buffer RPE were respectively added and recentrifuged. At the final stage, RNase Free water was added to the column and the obtained RNAs were collected.

cDNA synthesis was carried out using QuantiNova reverse transcription kit (Qiagen, Germany) according to the manufacturer’s instructions.

Real-time polymerase chain reaction (PCR) was conducted based on a standard method according to the manufacturer’s instructions. Primers for Oct4 and Gapdh genes were exclusively designed using GeneBank sequences. Sequences of Oct4 and Gapdh primers are as follows:

Oct4 primers:
Forward 5’-AGCTGCTGAAGCAGAAGG-3’
Reverse 5’-TCATTGTGTCGGCTCCT-3’

Gapdh primers:
Forward 5’-AAGGTATCCACAGCTGGA-3’
Reverse 5’-CTGTCACCACCTCTCTGTA-3’

The quantitative reverse transcription (qRT)-PCR was carried out using Applied Biosystems 7500 Sequence Biosystem. In summary, 100 µg of cDNA and 100 µM of each of the primers was added to Syber Green PCR Master Mix (Syber Premix Ex Taq™II; Takara, Korea) in a final volume of 10 µL and the reaction was amplified at 45 cycles of 95°C for 15 seconds and 58°C for 1 minute. Expression of the gene in each sample was normalized with Gapdh gene and the data were studied using 2^(-∆∆Ct) method.

3.6. Analysis of Differentiation Potential of the Cells

To assess and compare the differentiation potential of the cells cultured in a medium with and without growth factors, osteogenic and adipogenic differentiation were done. For this purpose, AT-MSCs of passage 3 were cultured in a 12-well plate at 2 x 10^4 cell/mL concentration in 6 groups. The cells were cultured in a basal culture medium containing osteogenic and adipogenic differentiation medium with and without growth factors. When the cells reached 80%-90% confluence, culture medium was replaced with adipogenic and osteogenic differentiation medium. During a 21-day period, once every 4 days, cells culture medium was changed and cells morphological changes in the experimental groups were studied. Adipogenic and osteogenic differentiation medium contained IBMX (3-isobutyl-1-methylxanthine) 0.5 mM, dexamethasone 10^-7 M, insulin 66 µM, indomethacin 0.2 mM, beta-glycerol-phosphate 10 mM, ascorbic acid bisphosphate 50 µg/mL (Sigma-Aldrich, St. Louis, Mo, USA)

Osteogenic differentiation of the cells was approved by alizarin red staining at the end of the day 21. For this purpose, first, cells were washed 3 times with PBS. Then, cells were fixed in 10% formalin for an hour and after washing, alizarin red was added to the cells for 45 minutes. To study the differentiation of the cells into adipocytes, after 14 days, the oil red staining was used.

3.7. Statistical Analysis

All data were expressed as the standard deviation (SD). Variations were evaluated using one-way analysis of variance (ANOVA), as well as the Dunnett, and Tukey HSD tests. P values < 0.05 were considered statistically significant.
4. Results

4.1. Isolated Stem Cells Expressed the Markers of Mesenchymal Stem Cells

AT-MSCs were removed from preperitoneal and the surrounding epididymal adipose tissue of male NMRI mice by the previously described method (Figure 1), expression of CD44 and CD90 (specific to mesenchymal stem cells) and lack of the expression of CD31 (specific to endothelial cells) and CD45 (specific to blood cells) were evaluated. Isolated cells showed high expression of CD44 (99.4%) and CD90 (72.4%) markers, and low expression of CD45 (2.02%) and CD31 (2.16%). These results showed the high purity of isolated AT-MSCs (Figure 2).

Figure 1. A, Morphology of the Isolated AT-MSCs on the Day 0; B, Day 3; C, Day 5; D, Days 1st-11th After Isolation

The isolated cells are fibroblast-like with fusiform morphology. Bar indicate 100 µm.

4.2. Growth Factors Increase the Proliferation and Viability of the Cells

The simple morphological observations of the cells in experimental groups over 11 days were assessed and compared with the control group. It was observed that the 3 cell groups were morphologically fibroblast-like and fusiform and had not changed compared with the control group. AT-MSCs proliferated continuously. Proliferation of the cells on the days 7 and 11 was higher in b2 and b3 groups compare with the control group (Figure 3A and B).

Viability of the cells cultured with 3 different doses of hormones on the days 3, 7, and 11 was assessed using MTT assay. On the day 3, viability of the cells in b1 and b2 groups was higher than that of the controls (P < 0.001) and there was no significant difference between the b2 and b3 (Figure 4B, C) (Tables 2 and 3, respectively). Due to the more economical dose of hormones in the b2 group, it was selected for the next experiments.

4.3. EGF, GDNF, and LIF Increased Oct4 Expression in the Treated Cells

To assess the expression of Oct4 gene in the cells cultured in the medium containing growth factors and control group on the days 3, 7, and 11, the real-time PCR was employed. The Oct4 gene expression was measured using Gapdh reference gene. On the day 3, expression of Oct4 in the group containing growth factors showed no significant difference compared with that of the control group, but on the days 7 and 11, about 6.9 and 4.5 folds increase were observed in expression of Oct4, respectively (P < 0.05) (Figure 5). On the whole, these factors increased Oct4 expression in the cells.

4.4. The Cells Cultured with Growth Factors Maintained Their Differentiation Potential

Differentiation potential of the cultured cells in the b2 group was assessed in terms of differentiation into adipocytes and osteocytes. On the day 10, tiny lipid particles appeared in the control group and the group containing growth factors. On the day 14, oil red staining was used to verify the existence of lipid particles, and differentiation into adipocytes was also proved in both the assessed groups (Figure 6 B - E).

Also, osteogenic differentiation in the 2 groups was proved using alizarin red staining (Figure 6F, G).

5. Discussion

Since AT-MSCs are easy-to-obtain and safe with immunomodulatory and anti-inflammatory effects, and are capable of differentiation into various lineages, they are suitable candidates for cell therapy and therapeutic applications (32). The limitations of the use of these cells are their low number, difficulty of maintaining their viability, and differentiation into undesirable lineages (8, 9). A solution for overcoming this problem is to use growth factors. In the present study, 3 different doses of EGF, GDNF, and LIF were used for culturing AT-MSCs. These factors are expressed in testicular niche and are responsible for spermatogonial stem cell self-renewal (22, 33, 34). The combination of these growth factors is not used for culturing AT-MSC so far. These cells can be used for cell therapy of male infertility. It was found out that the proliferation rate of cultured cells in 10 µg/mL EGF, 5 µg/mL LIF, and 5 µg/mL
Figure 2. Flow Cytometry Analysis

Isolated cells show high expression of CD44 (99.4%) and CD90 (72.4%), and low expression of CD45 (2.02%) and CD31 (2.16%) markers.

| Day 3 | Mean (SD) | Statistical Test | P Value |
|-------|-----------|------------------|---------|
| Control | 91.32 (13.15) | The Dunnet test | 0.003 |
| B1 group | 119.207 (11.56) | The Dunnet test | 0.049 |
| B2 group | 108.8 (18.63) | The Dunnet test | 0.064 |

Table 2. Data Analysis of MTT Results in the Day 7

| Day 7 | Mean (SD) | Statistical Test | P Value |
|-------|-----------|------------------|---------|
| Control | 100 (12.08) | The Dunnet test | 0.994 |
| b1 group | 95.86 (14.64) | The Dunnet test | < 0.001 |
| b2 group | 170.27 (13.94) | The Dunnet test | < 0.001 |
| b3 group | 174.39 (38.85) | The Dunnet test | < 0.001 |
| b1 and b2 groups | - | The Tukey HSD | < 0.001 |
| b1 and b3 groups | - | The Tukey HSD | < 0.001 |
| b2 and b3 groups | - | The Tukey HSD | 0.982 |

GDNF increased significantly. In previous studies, it was observed that in lower doses of EGF (5 µg/mL) and bFGF (10 µg/mL), the AT-MSCs proliferation increased (10); hence, EGF and PDGF increased the proliferation of BMSCs, and the use of EGF for the proliferation of BM-MSC and AT-MSCs are recommended (16, 35).

Due to the small number of cells, maintenance of differentiation potential was also important. The cultured cells maintained their differentiation potential in the presence of these growth factors. Similarly, Tamama et al., reported that EGF and PDGF did not interfere with the differentiation of BMSCs into adipogenic and osteogenic lineages, although PDGF delayed subsequent differentiations (16). Also, EGF enhanced the proliferation of AT-MSCs and
Figure 3. The Impact of EGF, LIF, and GDNF on Proliferation of AT-MSCs

The morphology of the cells was assessed by an inverted microscope. A (36) The cell groups are morphologically fibroblast-like and fusiform on the day 7; and B (36) on the day 11. Proliferation of cells are higher in b2 and b3 groups, compared with the controls. A, Control; B, b1 group; C, b2 group; D, b3 group. Bar indicate 100 µm.

Table 3. Data Analysis of MTT Results in the Day 11

| Day 11 Mean | Statistical Test | P Value |
|-------------|-----------------|---------|
| Control     | 97.64 (13.43)   |         |
| b1 group    | 103.92 (7.53)   | The Dunnet test | 0.806 |
| b2 group    | 152.45 (15.75)  | The Dunnet test < 0.001 |
| b3 group    | 131.53 (19.17)  | The Dunnet test 0.022 |
| b1 and b2 groups | -    | Tukey test < 0.001 |
| b2 and b3 groups | -    | Tukey test 0.132 |

BMSCs, but did not interfere with the differentiation potential of the cells (16, 17, 35). Conversely, it was reported that in lower doses of EGF (5 µg/mL) and bFGF (10 µg/mL), neural differentiation increased and differentiation into mesoderm lineage decreased (10). Also, EGF increased osteogenic differentiation and morphological change of dental pulp stem cells (36).

In the current study, Oct4, a stemness marker, overexpressed in the b2 group. Hu et al., also reported that EGF and bFGF altered oct4 gene expression as well as the pluripotency of ASCs (10).

The Oct4 is a major initial regulator and maintains pluripotent cells during embryonic development and its exact expression is a key determinant of ESC fate (37, 38).
A. Viability of the cells cultured in control and 3 experimental groups (b1, b2 and b3) on the day 3, 7, and 11; and B, day 3, 7, and 11. The viability of the cells in the b2 and b3 groups was higher (P < 0.001). Significant differences between experimental and control groups are shown by asterisk (*P < 0.05).

![Figure 4. MTT Assay](image)

B. Viability of the cells cultured in control and b2 group on the days 3, 7, and 11 by real-time-PCR. Gapdh was used as a reference gene. Oct4 overexpressed on the days 7 and 11. Significant difference between experimental and control groups are shown by asterisk (*P < 0.05).

![Figure 5. EGF, LIF, and GDNF Increase the Oct4 Expression](image)

C. Only when expression of Oct4 is at a normal level, the pluripotent capability of cells is maintained and the level of Oct4 expression plays a major role in switch between pluripotency and differentiation that should be carefully controlled (39).

In the present study, Oct4 expression increased in the intervention groups, compared with that of the control group, which can have 2 consequences: 1, Increases the ability of AT-MSCs to maintain their stemness potential; and 2, The cells may differentiate into a specific lineage, which is an issue that requires further researches.

Two drawbacks of the current study are limitations in evaluating the cell differentiation into other lineages, and expression of other stemness markers such as Sox2 and Lin28 in order to confirm or reject these possibilities. How-
ever, over expression of Oct4 is most probably due to an increase in the proliferation and viability of the cells in the presence of these growth factors in the intervention groups, compared with the controls. In general, for cell therapy and clinical purposes, caution should be taken before using hormones for proliferation of stem cells.

5.1. Conclusion

Use of 10 µg/mL EGF, 5 µg/mL LIF, and 5 µg/mL GDNF in culture media of AT-MSCs increased the proliferation and viability of the cells; they can be supplemented with media culture of these cells, but due to over expression of oct4 and the possibility of differentiation of the cells into other specific lineages, for cell therapy and clinical purposes, caution should be taken before using hormones for the proliferation of stem cells.

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Footnotes

Authors’ Contribution: Masoumeh Eliyasi Dashtaki, study design, performing the experiments, and preparing and drafting the manuscript; Seyed Reza Kazemi Nezhad, study design and project conduct; Masoud Hemadi and Ghasem Saki, cell culture preparation; drafting the manuscript, and performing the statistical analyses; Javad Mohammadiasl, cooperation with molecular genetic studies.

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