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Human Mesenchymal Stem Cells Differentiation to Skeletal Muscle cells in vitro Study

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ABSTRACT

Background: To evaluate the development of skeletal muscle-like cells derived from human adipose tissue mesenchymal stem cells induced by 5-azacytidine (5-aza) and hours serum (HS) under in vitro condition.
Method: Human adipose tissue mesenchymal stem cells (ADSCs) were purified. These cells were cultured in osteogenic or adipogenic induction medium to induce osteogenic and adipogenic differentiation. On the other hand, The skeletal myogenic differentiation potential of these cells was investigated using 3µmol 5-azacytidine (5-aza) treatment for 24 hours and Hours serum(HS). In the way that the cells were put in the culture medium containing 5-aza for 24 hours and, then were put in the culture medium free of 5-aza for another 4 weeks until sampling. RT-PCR assay was performed to detect the expression of specific skeletal muscle genes including Myogenin, Myh, alpha-actin, tropomyosin and myosin at 1-4 weeks after the first induction.
Results: The adherent adipose tissue-derived mesenchymal stem cells exhibited a proliferative capacity and they showed osteogenic and adipogenic differentiation as seen in previous studies. The expression of four skeletal muscle cells genes was detected 1st to 4th week after induction in a time-dependent manner. There was a continuous increase in expression of Myogenin gene from the 1st to 4th week, whereas that of the Myh was higher after two weeks and lower after 4 weeks in comparison to the other weeks. While mRNA of alpha-actin and Myosin was at the highest level in the fourth week. Tropomyosin hasn't nan-significant expression in any samples.
Conclusion: The current study indicated that stimulating human adipose tissue-derived mesenchymal stem cells by 5-aza with the concentration of 3µmol under in vitro condition can lead to differentiating skeletal muscle-like cells. Furthermore prolonged culture duration may lead to more differentiated cells.
KEYWORDS: Adipose tissue, Mesenchymal stem cells, differentiation, skeletal muscle cell

INTRODUCTION

Skeletal muscle is one of the grown tissues which has the capability of self-renewal. The capability is dependent to peculiar cell populations which are known as satellite cells due to their anatomical position. They are located under basal lamina of each muscle fiber. Their frequencies within different muscles are different which can probably explained by their difference of performance of skeletal muscle considering the type of muscle fibers (fast oxidative, slow oxidative, fast glycolitic). Marking these cells using radio-active material shows that it is possible to stimulate those using environmental stimuli to bring them back to cell cycle. Such cell divisions form muscle maker cells which are responsible to compensate the damaged fibers via connecting to each other and forming new fibers.

However, a wide range of studies show that if a muscle is damaged the number of these cells gradually decreases when the damaged muscle is recovering. In addition, the ratio of satellite cells existing in different muscles decreases with increase of age. This explains why the capability of muscle renewal is lower in aged persons. Also various researches have indicated that satellite cells in vivo and in vitro hardly start to amplify and these cells step into aging phase quickly.

For cell-therapy and engineering the diseases which may destroy muscles we need a resource of cells with ability to build muscle fibers, including embryo stem cells or grown stem cells which are able to turn into a wide range of cells and tissues.

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Several studies in vivo and in vitro have shown differentiation of grown mesenchemycal stem cells with bone brain nature and Sinoyal membrane, umbilical cord blood and Amonion liquid to skeletal cells. It was proven that such cells have capability of surviving and differentiating into tubular cells after being transplanted in vivo. They also have ability to enter into myogenic path prior to transplant in vitro.

However, today adipose tissue has come to be a source of grown mesenchymal cells. Easy surgery, easy access to under skin tissue and simple technique for stem cell separation are all the features which turn the adipose tissue into an ideal resource for separation of mesenchyal stem cells.

5-aza and HS are the factors which stimulate mesenchymal stem cells to differentiate into skeletal muscle cells. 5-aza causes the genes' methylation and activates their expression process selectively and influences differentiation of cells. It has also proven that it can cause MSCs to differentiate to skeletal myobalst. HS serum prepared from horse blood which is commonly used in primary culture of neurons plays a distinct role in their differentiation. Experiments have already showed that short-time culture of stem cells extracted from adipose tissue and transplanting them to the damaged muscle may relatively heal the damage. However nothing is known about the mechanism which brings bout complete differentiation of adipose tissue stem cells to myogenic phenotype. Therefore, it is essential to get to know the process of differentiation and growth of skeletal muscle cells created in laboratory.

By this research we mainly intend to review how stem cells extracted from adipose tissue differentiates to skeletal muscle. For this purpose we make a comparison on the inductive effect of 5-aza and HS factors in laboratory. We hope that we can get to know the natural process of such differentiation and can analyze our results to be able to substitute the ill and atrophy and necrosis cells with our new cells.

METHOD

Preparation, purification, reproduction of mesenchymal stem cells from adipose tissue.

Having washed the sample of human adipose tissue with Phosphate Buffer Saline (PBS), the sample was cut to smaller slices. The slices were put subject to 0.075 gr of Kolagnase Type for 50 minutes under the temperature of 37 degrees and 5% CO2 and 95% moisture. When the adipose tissue was digested an amount of additional samples- twice of the volume of the former sample- was added to the sample for the purpose of neutralizing the effect of Kolagnase. The resultant liquid was transferred to a falcon 15and it was centrifuged once again for 2000 rounds within 5 minutes. The resultant cells were counted using Lam Neubar and were poured into 25cm flasks containing DMEM, FBS15% and Pens/Trep with concentration of 1200000 cells per each centimeter. Then they were incubated in incubator. For removing the adipose tissue traces, culture medium was replaced once a day. When 90% of the flask was full of cells, cells were passaged with a ratio of 1:4. The passage 4 cells were used in subsequent steps.

Verification of mesenchymal nature of stem cells using flow cytometry method:

For flow cytometry, separated cells were cultured. After being separated using Tripsin in passage 4, near ten thousands of the cells were counted and then transferred to falcon. FC blocking with concentration of %0.5 was used with BSA to avoid non-specific connection of antibodies. Then Triton – X100 with concentration of 5% was used for 4 minutes using Paformaldehid 4% in order to get the antibodies penetrated into cell. Cells were ultimately incubated with Genjougated antibodies with PE or FITC colors for 1.5 hours. Results were interpreted using Becton Dic with WinMIDI software.

Classification of the identification of mesenchymal stem cells and their differentiations into Adipogenic and Steogenic

One way to verify the status of stem cells inside mesenchymal stem cells is to distinct them into adipose mesenchymal and bone and cartilage classes. Here in this research we made two distinctions: Adipogenic and Steogenic. For this purpose, mesenchymal stem cells of adipose tissue in passage 4 were utilized. When cells achieved a concentration of 50% the inductive culture media of Adipogenic and Steogenic classes were added to 14 days and 21 days repectively. For differentiation of Adipogenic class, DMEM, FBS 10%, 0.5 micro mole of isobutyl methyl Zantin, 1micromole of Dexametazon, 60 micromole of Indometasion and 5 micrograms/mL of insulin were used. For differentiation of Steogenic class, DMEM culture medium, 10%FBS, 50micromoles of Ascorbat 2 phosphate, 10 milimoles of Beta Glicrophosphate, 0.1 micromole of Dexametazon, NaCl 9%, N HCL0.1%, 1 milimole of NgCl and 50 milimoles of Sodium bicarbonate were utilized.

Differentiation of mesenchymal stem cells to skeletal muscle cells

For initiation of myogenic differentiation process, stem cells of adipose tissue in passage 4 were used: first the cells with concentration of 8000 cells in plates of 4 boxes covered by 2% gelatin were cultivated. When cells stuck to the gelatin existing at the floor of the plate and upon the concentration of cells got to 80%, differentiation process sparked. Cells were transferred to plates of 4 boxes covered by 2% gelatin. Then one group of the cells were separated and put in a culture medium containing 3mm of 5-aza for 24 hours. Afterwards, cells were washed with PSB and incubated in the culture medium which was utterly devoid of 5-aza until the end of 4 weeks. On the other hand, other group of cells were put in a culture medium containing 2% HS, and they were incubated in the same medium up to the end of fourth week. At the end of each week, both groups were sampled. Mesenshymal stem cells of adipose tissue in passage 4 as negative control group were incubated in a culture medium containing FBS15%, and at the end of each week their gene expressions were assessed. The biopsy taken from human skeletal muscle was chosen as positive control group.

Differentiation process of cells using RT-PCR

For the purpose of comparison between the model and the degree of progress of the expression of specific genes in skeletal muscle within the differentiating cells, the differentiation indices including gene expressions of Myog, alpha-actin, Tropomyosin and Myosin were assessed at the end of the first week to the fourth week. Furthermore, GAPDH gene was taken as internal control. For this purpose, near 500.000 to 1.000.000 cells, in each case, were washed using PBS and RNx buffer. Total RNA cells were extracted through the same method using Oligo dt primer and MMULV and then the complementation string of cNDA were built. Then the PCR response was observed using primers of specific table (table 1) in a volume of 30 micro liter within 25 to 30 cycles. Ultimately the PCR products underwent electrophoresis 25 using Agares gel (see chart 1).

Statistical method: in this research, Gene Tools software was utilized to turn the gene expression into parametric quantitative data using RT-PCR technique. Then the data were alanlyzed using SPSS16 and LSD ANOVA.

Results

Appearance features of mesenchymal stem cells of adipose tissue

When studying using light microscope, mesenchymal stem cells of adipose tissue which are semi-fibroblast resemble mesenchymal stem cells morphologically at the time of separation and culture (fig 1).

Assessment of other markers specific to stem cells using flucytometery method

Assess of CD-Markers which are specific to mesenchymal cells in cells of passage 4 using flow cytometery indicated that near 98.6% of the cells expressed CD105 and near 99.6% of them expressed CD90 markers. Both of these markers have mesenchymal origin; while the same cells did not express hematopoetic stem cells makers such as CD34 and CD45. Therefore, the cells used in this research had mesenchymal nature (graph 1).

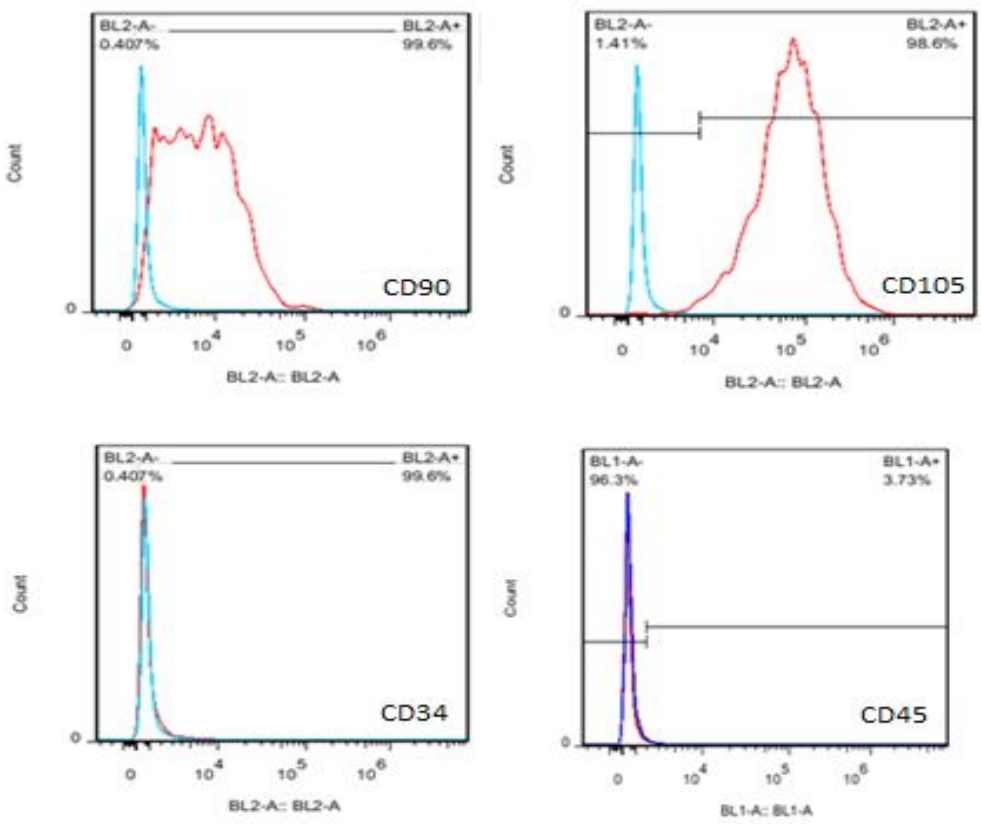
Verification of the mesenchymal nature of cells with Adipogenic and Steogenic differentiation

The differentiation potential of mesenchymal stem cells was assessed through culturing the cells in Adipotenic and Steogenic media. After two weeks of culture of cells in the former medium, adipose drops started to surface in cells cytoplasm (fig 2). On the other hand, for Steogenic medium, after three weeks the cells became layered beginning to secret mineral matrix in their surroundings which could be visible as bone colonies via Alizarin red painting technique (fig 3).

Assessment of expression of skeletal muscle genes in differentiated cells via RT-PCR technique

Results indicate that mesenchymal cells are able to express skeletal muscle specific genes when they are subject to 5-aza and HS inductions, such that the gene expression was zero or close to zero for negative control group. Statistical interpretations relating to Mean and SD, gene expressions of each group for each week was compared to that of negative control group of the same week with $P<0.05$. the results of each comparison are as follows: in 5-aza group, expression of mRNA of Myog in all samples from the first week through the fourth week showed a meaningful increase toward negative control group ($P<0.05$). expression of mRNA for a-actin and Myosin genes in the first week showed no meaningful difference than the negatice control group (p value are 0.295 and 0.07 respectively) ($P>0.05$). however, from the second week onward, the expression had a meaningful increase toward control group ($p<0.05$). expression of Tropomyosin gene showed no meaningful difference toward control group ($p>0.05$ $p<0.05$) (graph 2). in HS group, expression of Myog gene showed a meaningful increase toward control

group for all the weeks ($p<0.05$). its expression in the second week compared to the first week showed a significant increase reaching its maximal degree; while it started to decrease in the third and fourth weeks. Furthermore, the amount of expression of this gene showed a meaningful difference in all weeks toward each other ($p<0.05$). the mRNA of a-actin gene had a meaningful increase toward control group in all weeks except for the second week ($p<0.05$) , reaching its maximal amount in the third week, while starting to decrease in fourth group. Expression of Tropomyosin showed a meaningful increase toward control group in all weeks ($p<0.05$). it increased significantly during the first to third weeks but started to decrease in the fourth week. Also, the amount of expression of this gene was different meaningfully in each week toward the other week ($p<0.05$). expression of mRAN in Myosin gene had no meaningful difference toward control group in the first week ($p>0.05$), however it showed a meaningful increase during the second to fourth week ($p<0.05$) (graph 3).



Graph1: flow cytometry

Chart1: primer of genes

Genes	Primer sequences	Length (bp)
HLA-DR	F: 5'AGGCGAGTTTATGTTTGACT 3' R: 5' GGCTGTTTGTGAGCACAGTT 3'	230
Aipha-actinin	F:5' TGTTGGAGTGGATCCGCCGCACAA 3' R5' CATCCTGCCCTCAGAGGGGATGAA 3'	230
GAPDH	F 5' TATTGGGCGCCTGGTCACCAGGGCTGCT 3' R5' GGTCATGAGTCCTTCCACGACGATACC 3'	510
Tropomyosin	F:5' ATAAGAAAGCCGCTGAGGACAAG 3' R:5' CATGGCCCGGTTTTCTATC 3'	320
Myogenin	F 5'ACAGCGCCTCCTGCAGTCCAG3' R 5' GGAGGCAGCTGGATGAGGGCG 3'	400

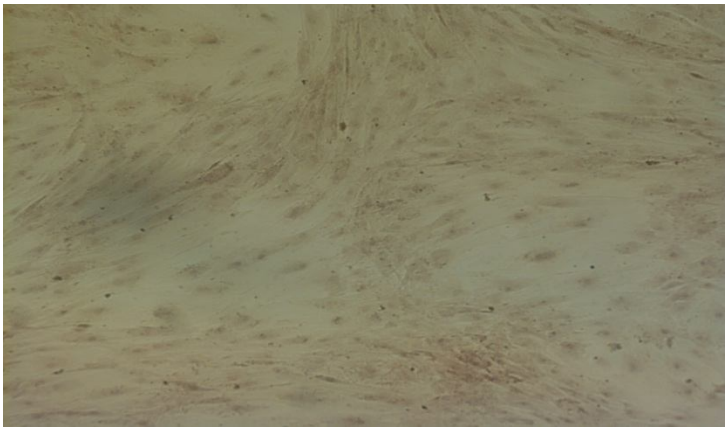


Fig1:adipose derived stem cells

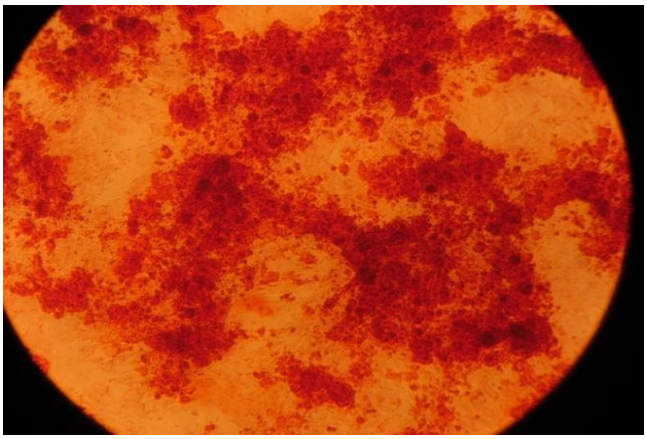


Fig2:adipogenic differentiation

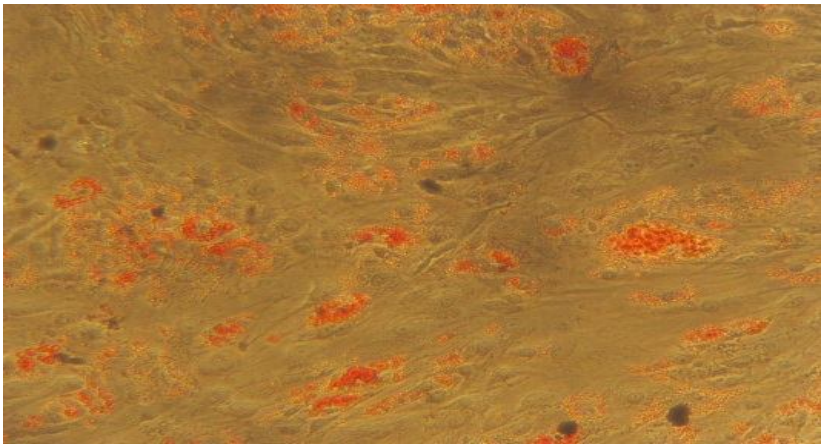
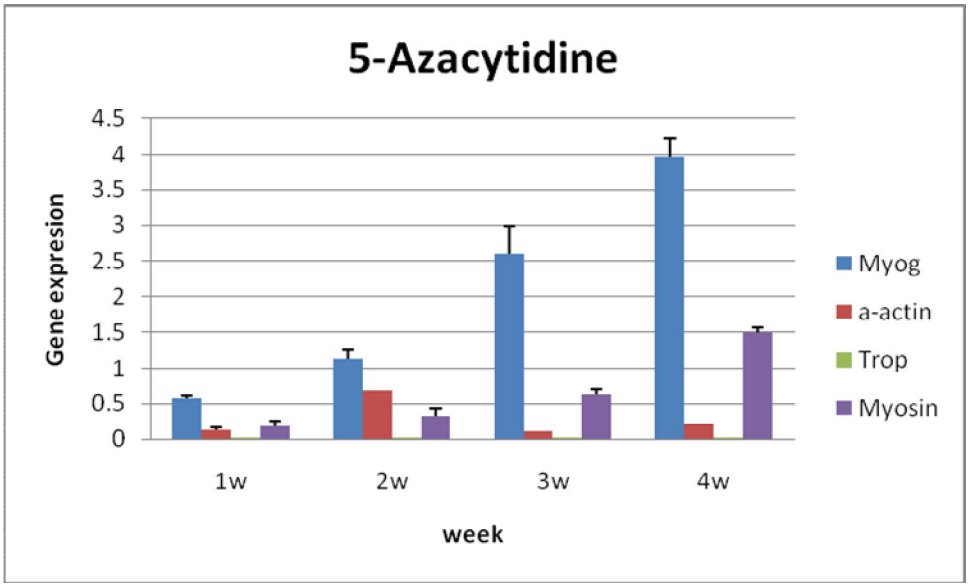
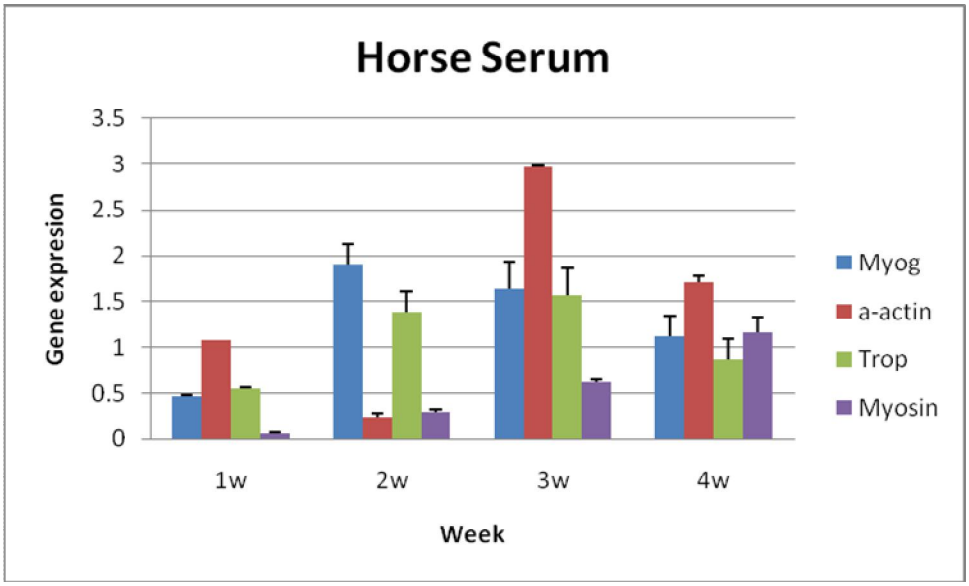


Fig3:osteogenic differentiation



Graph2: 5-aza group



Graph 3:Horse serum group

DISCUSSION

During lifetime, body muscles are continually subject to biomechanical forces and/or degenerative changes relating to a wide range of diseases. Unlike heart muscles, skeletal muscles are able to renew themselves when they are damaged. Some times when such damage is severe it may take a few years to heal completely. Primary renewal in skeletal muscle, when it faces a degenerative damage, is carried out through amplifying and differentiating myogenic stem cells. The process takes place in the muscle itself. The capability is dependent to a peculiar cell population which are known as satellite cells due to their anatomical position. They are located under basal lamina of each muscle fiber. Their frequencies within different muscles are different which can probably explained by their difference of performance of skeletal muscle considering the type of muscle fibers (fast oxidative, slow oxidative, fast glycolitic). It was long believed that the satellite cells are the sole resource of stem cells needed for renewal of skeletal muscles. However, a wide range of studies show that if a muscle is damaged the number of these cells

gradually decreases when the damaged muscle is recovering. In addition, the ratio of satellite cells existing in different muscles decreases with increase of age. This explains why the capability of muscle renewal is lower in aged persons. Also various researches have indicated that satellite cells in vivo and in vitro hardly start to amplify and these cells step into aging phase quickly. For cell-therapy and engineering the diseases which may destroy muscles we need a resource of cells with ability to build muscle fibers, including embryo stem cells or grown stem cells which are able to turn into a wide range of cells and tissues. In the recent years scientists managed to separate and culture the stem cells mainly from bone, adipose tissue and blood vessels which are able to turn into a wide range of cells and tissues. several researchers studied most of the potential myogenic factors and found that there are certain inductive factors such as 5-aza, HS, Dexametazone, Hydrocortizone, Amfotripsin B, TGF-B, mixture of insulin, Transferin and Selenium etc., play role in differentiation of stem cells to skeletal muscle.

The first part of our research (separation, identification of mesenchymal stem cells of adipose tissue) intended to review three standards proposed by Stem Cells Committee and International Association for Cell Therapy to better identification of mesenchymal stem cells of human body. The standards are:

1. Sticking to plastic culture vessels when they are put in a typical culture medium
2. Expression of surface genes of CD90, CD105 and CD73.
3. For this purpose, we need a digestion protocol which can digest adipose tissue very well without leaving any materials which remove the blood cells. For identification of mesenchymal stem cells of adipose tissue, all the above-mentioned standards were used. Therefore, the cells found a good sticking capability after one day.

Also the expression of CD34, CD105, CD90 and CD45 of surface markers was assessed through flow cytometry on the separated cells. It was found that in mesenchymal stem cells of adipose tissue, CD90 and CD105 are expressed relatively in nearly all cells but these cells are negative toward the expression of CD34 and CD45.

Until we complete the sampling phase (end of first, second, third and fourth weeks), we considered mesenchymal stem cells of adipose tissue in human body within four categories. Results of different studies show that when cells are incubated at the presence of 5-aza and HS they start to differentiate into semi-skeletal myosite cells, however determination of the precise concentration of their myogenic differentiation is already known, yet under study at this time. Taylor and Jones in 1982 reported, for the first time, that 5-aza sparks myogenic induction in embryo and grown stem cells. Considering the bulk of studies which have already been conducted, no optimal cell resource is available for myogenic differentiation and determination of precise concentration of inductive factor and the expression of specific genes of skeletal muscle in the differentiation processes with longer duration. For this purpose, expression of a number of specific genes of skeletal muscles including Myog, alpha-actin, Tropomyosin, Myosin at the end of first to fourth weeks were assessed in cells differentiated from mesenchymal stem cells of adipose tissue under the effect of 3mmol of 5-aza and HS. It was found that in both experimental groups, mRNA of the Myog gene showed a significant expression in all the weeks causing that expression of skeletal muscle genes to become up-regulate. In this research, expression of alpha-actin and Myosin showed a meaningful increase from the first week to the fourth week. Considering the positive effect of 3micromole of 5-aza and HS2% on increased gene expression, it can be concluded that such concentration can be optimal for differentiation of such cells. It should be noted that in 5-aza group, the expression of Tropomyosin as a structural gene reduced during four weeks-unlike alpha-actin and Myosin genes. Such reduction motivated us to study more on this matter. when the differentiation process of mesenchymal stem cells to skeletal muscle cell is known, we can achieve a clear solution for gaining better cells in order to use them in cell-therapy applications, in such a way that the resultant cells have more similarity to adult skeletal muscle cells and higher percentage of the stem cells are differentiated into semi-skeletal muscle cells.

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