Cardiomyogenic Gene Expression Profile of 5-Azacytidine-Treated Rat Bone Marrow Mesenchymal Stem Cells

Rat Kemik İliği Mezenşimal Kök Hücrelerinde 5-Azayctidine Uygulaması Sonucu Kardiyomiyojenik Gen İfadesi Profili

Research Article

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ABSTRACT

Cell transplantation which aims to introduce healthy myogenic cells into the myocardium of the diseased heart is an important tool in myocardial regeneration therapy. The differentiation potential of bone marrow mesenchymal stem cells (BM-MSCs) can be key role for this purpose. The present study aimed to evaluate cardiomyocyte gene expression profile of 5-azacytidine treated rat BM-MSCs. 5-Aza treated BM-MSCs started to form cell colonies and especially these colonies stained with desmin and cardiac troponin-T. qRT-PCR results indicate that the expression level of cardiomyocyte specific genes especially α- cardiac actin, Mef-2b, GATA-4 were increased in 5 μM 5-Aza treated BM-MSCs. According to ELISA results, nanogram levels of cardiac troponin-I was measured especially 5 μM 5-Aza treated cells.

Key Words
Bone marrow mesenchymal stem cells, 5- Azacytidine, cardiomyocyte differentiation, cardiac troponin.

ÖZ

Miyokardiyal rejenerasyon için parlak bir gelecek sunan hücresel tedavi yöntemleri hastalıklı kalbi miyojenik hücrelerle tedavi etmeyi hedeflemektedir. Kemik iliği mezenkimal kök hücrelerinin (Kİ-MKH) farklılaşma kapasiteleri nedeniyle miyokardiyal rejenerasyon için anahtar rol oynayabileceği düşünülmektedir. Çalışmamızda *in vitro* koşullarda Kİ-MKH'den kimyasal indüklemeyle kardiyomiyositlerin gen ifadesi profilinin incelenmesi amaçlanmıştır. 5-Aza uygulanan Kİ-MKH'nin kardiyomiyositlere farklılaşma süresinde, öncelikle hücreler birbirine yaklaşmaya ve hücre kümeleri oluşturmaya başlamış ve özellikle bu hücre kümelerinin desmin ve cTn-T ile immunflorasan boyamalarının pozitif olduğu görülmüştür. RT-PCR sonuçlarına göre ise farklılaşan hücrelerde kardiyak spesifik genlerden özellikle α-kardiak aktin, Mef-2b ve GATA-4 gen ifadesinin artmış olduğu saptanmıştır. ELISA sonuçlarına göre, 5 μM 5-Aza uygulanan hücrelerde nanogram seviyesinde cTn-I ölçülmüştür.

Anahtar Kelimeler
Kemik iliği mezenkimal kök hücreleri, 5- Azacytidine, hücre farklılaşması, kardiyomiyosit, kardiyak troponin.

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INTRODUCTION

Coronary artery disease caused ischemia is a major cause of heart failure and regeneration capacity of ischemic myocardium is very limited [1,2]. Conventional treatment methods used for therapy of heart disease does not contribute to irreversible damage of cardiac muscle. The purpose of all these treatments is to prevent the formation of new damaged areas rather than the improvement of damaged cardiac muscle. All these treatments can be insufficient, if the tissue damage is extreme, a heart transplant is needed [3].

The heart is a post mitotic organ and cardiomyocytes are in a state of cell cycle arrest. Cardiomyocytes show limited and insufficient regeneration capacity during the healing process of infarcted heart muscle [4]. An experimental approach to repair myocardium by replacing the scar tissue with new healthy cells is an application of tissue engineering. In addition to muscle cells, mesenchymal stem cells (MSCs) can also be used for treatment due to their high differentiation capacity. MSCs obtained from bone marrow (BM-MSC) have been reported to differentiate into several cells types including fat, bone, cartilage, liver, nerve and muscle cells [5,6]. Transplantation of MSCs to the injured heart muscle tissue has positive impact on tissue regeneration and contractile performance of the infarcted heart [7].

5-Azacytidine (5-Aza) is a DNA demethylating chemical compound inhibits DNA methyl-transferase and causes a global demethylation of genome. Previous studies have shown that 5-Aza induces uncontrolled myogenic specification by random demethylation [8]. Also, 5-Aza was used as a therapeutic agent for cancers particularly "myelodisplastic syndrome" to induce the expression of the silenced genes that are critical for the regulation of differentiation of the proliferating cells [9,10]. Cardiac muscle differentiation from stem cells through the use of 5-Aza remains controversial; moreover, 5-Aza has not yet been proven to produce completely differentiated cells because of its toxicity effects. Thus, we evaluated which concentration is the best for cardiomyogenic gene profiling of rat BM-MSCs differentiation into cardiomyocytes in vitro.

MATERIALS and METHODS

Isolation and culture of BM-MSCs

BM-MSCs were isolated from bone marrow of rats (Wistar albino, male and six-eight weeks old). Animal procedures related to cell isolation were approved by Hacettepe University Ethical Committee (Protocol number: 2008/27-6). Following euthanasia by ethyl ether inhalation, tibias and femurs were excised and metaphyseal end were then cut off and marrow was flushed from midshaft and collected in a tube. Bone marrow samples were diluted with phosphate buffered saline (PBS) before being layered onto an equal volume of Ficoll-Hypaque density gradient. The sample was centrifuged at a speed of 2000 rpm for 30 min at room temperature. The interphase layer of mononuclear cells was transferred into new centrifugal tubes and then washed twice. The mononuclear cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (complete medium). The cells were seeded at $1x10^6$ cells per T25 culture flask and cultured in 5% CO_2 at 37°C. The MSCs were maintained and subcultured with 0.25% trypsin with EDTA; cells were passaged 4 times, morphology and growth were examined under an inverted microscope (IX70 Olympus, Japan). BM-MSCs were characterized using immunofluorescence staining for CD13 and CD29 molecules (Santa Cruz Biotechnology, Inc.) during their passage 2. For immunostaining, cells grown on a culture dish were washed in PBS (Biochrom AG, Germany) and fixed for 5 minutes in methanol at -10°C. After fixation, cells were incubated for an hour with primary antibody (in a dilution of 1:100) for CD13 and CD29 molecules and 45 minutes with a secondary antibody (donkey anti-goat Ig-TR). The cells were mounted with mounting medium and visualized under the fluorescence microscope (BH2-RFL-T3 fluorescence attachment, Olympus, Japan).

5-Azacytidine (5-Aza) Treatment

In order to select most appropriate 5-Aza concentration, passage 2 MSCs were plated in 96-well culture plates at initial density of $5x10^4$ cells/ml in six replicas and treated with either

0.1 μM, 1 μM, 3 μM, 5 μM, 10 μM, 50 μM or 100 μM 5-Aza (100 μM=0.0023 g material per 100 ml medium) for 24 hours. Untreated cells served as a control. Following the 1st, 3rd, 5th and 7th days of incubation, cell viability was determined by MTT assay. The absorbance at 570 nm was measured using an ultraviolet (UV) visible spectrophotometer (LPB Pharmacia, Bromma, Sweden).

According to the result of MTT assay, the 3 μM, 5 μM and 10 μM 5-Aza concentrations were used for differentiation of BM-MSCs during their second passage into the cardiomyocytes. The cells were treated with 3 μM, 5 μM and 10 μM 5-Aza for 24 hours. Untreated cells were served as control. After 24 hours of incubation, the medium containing 5-Aza was removed, and cells were washed with PBS. The cultures were maintained for 4 weeks to observe the process of differentiation. Culture medium was changed every 48 hours. Cell clumps as an indication of differentiation of cardiomyocytes were examined each week of incubation period for the selected 5-Aza dilutions (3 μM, 5 μM and 10 μM) under an inverted microscope (Olympus, Japan).

Immunofluorescence Staining

Immunofluorescence staining with the antibodies for desmin, cardiac troponin-T (cTn-T) and smooth muscle actin (Santa Cruz Biotechnology, Inc.), as a negative control, during their P2 for selected 5-Aza dilutions (3 μM, 5 μM and 10 μM) was done each week of incubation period. Immunofluorescence staining was done as described above. Donkey anti-goat Ig-TR was used for desmin and smooth muscle actin staining and donkey anti-goat Ig-TR was used for cTn-T as secondary antibodies. Cells were visualized under a fluorescence microscope (BH2-RFL-T3 model fluorescence attachment, Olympus, Japan). All procedures were done at room temperature.

Real-time Quantitative PCR (qRT-PCR)

qRT-PCR analysis of cardiomyocyte specific genes including alpha cardiac actin (a-cardiac actin), connexin-43 (con-43), myocyte enhancer factor-2b (Mef-2b) and GATA transcription factor-4 (GATA-4) was performed. Glyceraldehyte-3-phosphate dehydrogenase (GAPDH) was used as a house-keeping gene. Total RNA was extracted with trizol reagent. cDNA was synthesized containing 1 ng of total RNA according to the instructions of the manufacturer (Roche Transcriptor First Standart cDNA Synthesis Kit, Roche, Germany). Primers used were:

α-cardiac actin (F:5′-CCGTGAGAAGATGACACAG-3′, R:5′-GTTATGAGTCACACCGTCG-3′),
Con-43(F:5′-AAGAGCACTGACAGCCACA-3′, R:5′-CTTCAGCCTCCAAGGAGTT-3′),
Mef-2b (F:5′-CGAACTGGATATGGAAGAGG-3′, R:5′-AGCTCTGAGACCGACATTG-3′),
GATA-4 (F:5′-CAGCAGCAGTGAAGAGATG-3′, R:5′-GAGATGGATAGCCTTGTGG-3′)
GAPDH (F:5′-TATGACTCTACCCACGGCAA-3′, R: 5′-ACTCCACGACATACTCAGCA-3′).

Data analysis was performed with the $-\Delta\Delta$ CT method, where CT is cycle threshold. In brief, $-\Delta\Delta$ CT is the change in the difference of CT values between the gene of interest and the housekeeping gene over two time points or treatments [11].

ELISA Analysis

Cardiac troponin-I (cTn-I) levels of differentiated cells were also measured with an ELISA method. Differentiated passage 2 cells after the 1st, 2nd, 3rd, 4th weeks were treated with 2% Triton-X for 20 minutes and sonicated for 20 minutes. Cells were centrifuged at 1000 rpm for 10 minutes. Supernatant of cell homogenate were taken and ELISA analysis were done according to manufacturer's instructions (Life Diagnostics, Inc., West Chester, PA).

Statistical Analysis

All results reported in this study represent the mean±SEM. Differences between the groups were evaluated using unpaired Student's t test and Kruskall–Wallis nonparametric test. A p value <0.05 was considered to be statistically significant.

RESULTS

Identification of BM-MSCs

BM-MSCs were isolated based on their property of plastic adherence. After 72 hours of culture, the emergence of cells with fibroblastic

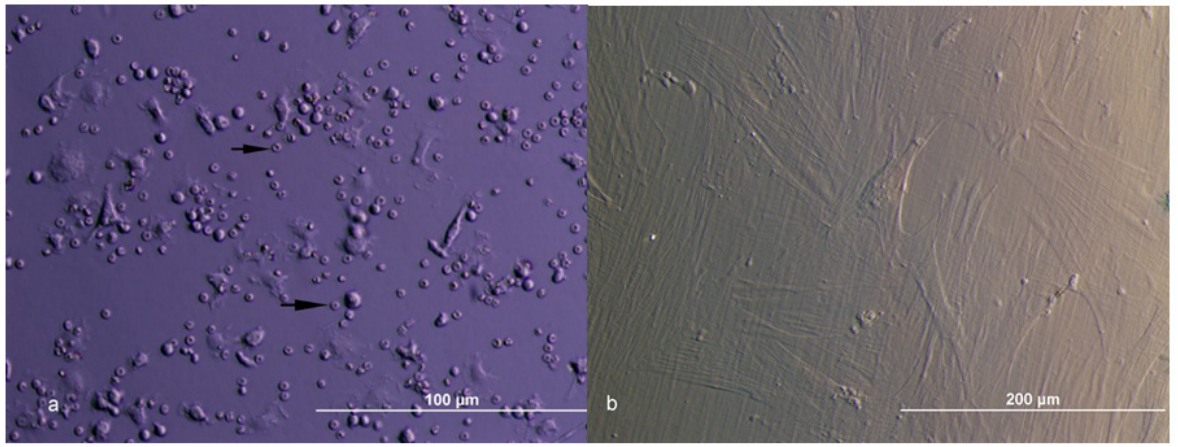

Figure 1. a. Isolated BM-MSCs after 72 hours of culture, fibroblastic cell morphology was identified among the erythrocytes (X100) **b.** P2 of BM-MSCs, cultured BM-MSCs were spindle-like morphology after passaging (X200).

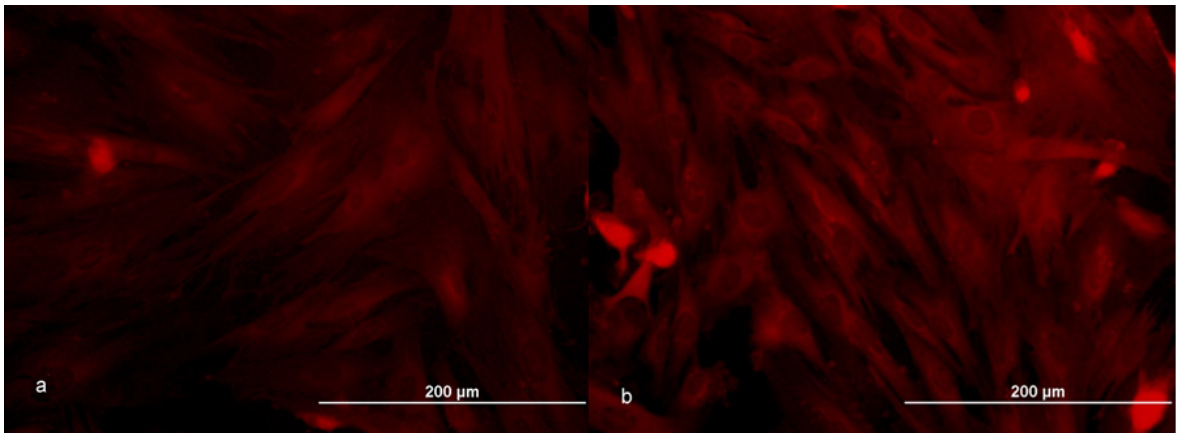

Figure 2. Immunofluorescence staining of BM-MSCs at P2, cells stained positively with mesenchymal stem cell markers **a.** CD13 **b.** CD29. (X200, Texas Red, 545-580 ηm).

cell morphology was identified among the erythrocytes and the number of the cells with fibroblastic morphology increased (Figure 1). BM-MSCs were passaged and P0, P1, P2, P3 and P4 cells were obtained. BM-MSCs starting with P0 to P4 stained positively with mesenchymal stem cell markers CD13 and CD29 (Figure 2). Passage 2 cells were used for subsequent experiments.

Aza Treatment

BM-MSCs were cultured with seven different concentration of 5-Aza. MTT cell viability assay showed that except for the 50 µM and 100 µM concentrations, all concentrations utilized (0.1 µM, 1 µM, 3 µM, 5 µM and 10 µM) demonstrated a similar proliferation compared with control groups. There was no statistically significant difference between the control group and lower concentration groups ($p>0.05$). However, cell viability was significantly decreased in the 50 and 100 µM concentrations than control group ($p<0.05$). For cardiomyogenic differentiation 3 µM, 5 µM and 10 µM concentrations were used. After the treatment with 5-Aza for 24 hours, the morphological changes over time were observed; cell morphology did not differ among the three different concentrations of the 5-Aza. Meanwhile, an increased number of cell clusters at the second week of the incubation after treatment compared to the untreated cells BM-MSCs was observed (Figure 3).

Immunofluorescence Staining

Immunofluorescence staining was performed to study the expression of a cardiac-specific protein (cTn-T), smooth muscle specific protein (smooth

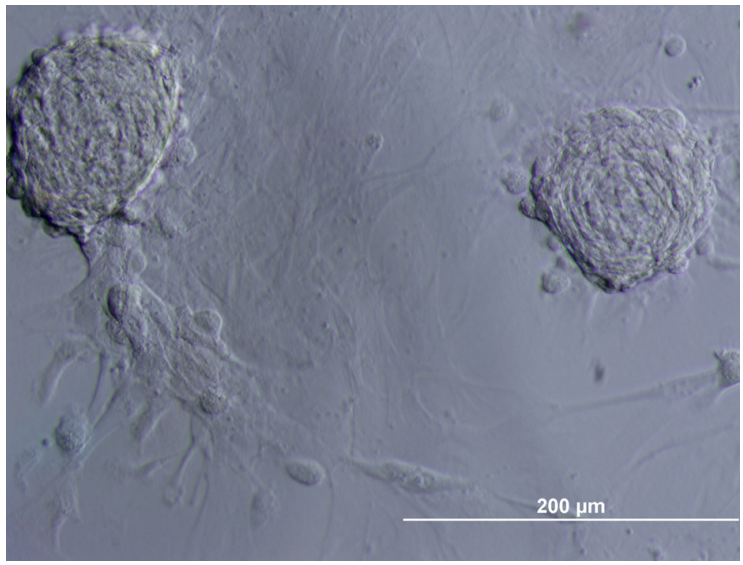

Figure 3. BM-MSCs after incubation with 5-Aza for 24 hours, cells began to form cell clusters at second weeks of incubation period.

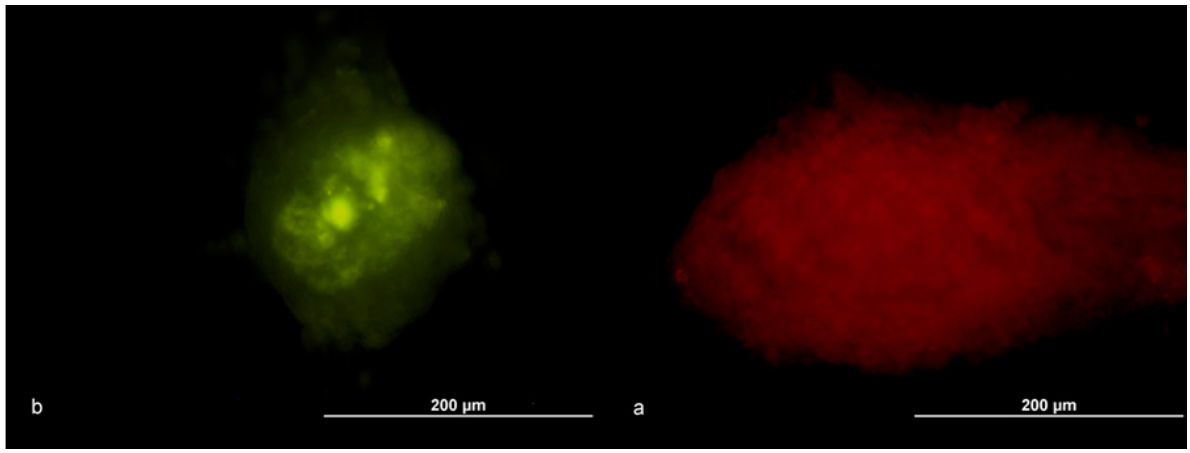

Figure 4 . Differentiated BM-MSCs after 24 hours 5 µM 5-Aza treatment at two weeks incubation period. Immunofluorescence staining of differentiated cells; a. Desmin (X200, Texas Red, 545-580 ηm) b. cTn-T (X200, FITC, 460-490 nm).

muscle actin) and the muscle intermediate filament protein (desmin). 5-Aza treated cells were positive for cTn-T and desmin (Figure 4). The highest signal intensity from immunofluorescence staining was seen in the second week with the 5µM concentration of 5-Aza. Smooth muscle actin staining was negative for all groups and control groups (5-Aza untreated).

Real-time Quantitative PCR (qRT-PCR)

Real-time quantitative PCR results indicated in 5-Aza treated cells increased expression levels of cardiac muscle-specific genes compared to the untreated cells. Four genes were expressed in 5-Aza treated BM-MSCs. The expression activity increase of cardiac-specific genes (α-cardiac actin, Con-43, Mef-2b, GATA-4) were analyzed in accordance with housekeeping gene activity (GAPDH). The α-cardiac actin and GATA-4 expression level were significantly increased in the cells that had been treated with 5 µM 5-Aza. However, Con-43 expression was very low in all of the different concentrations of 5-Aza-treated cells; and the Mef-2b expression level was higher in the 5-Aza-treated cells (Figure 5). The expression of differentiation of α-cardiac actin, GATA-4 and Mef-2b was increased during the second week and decreased in the later weeks (data not shown).

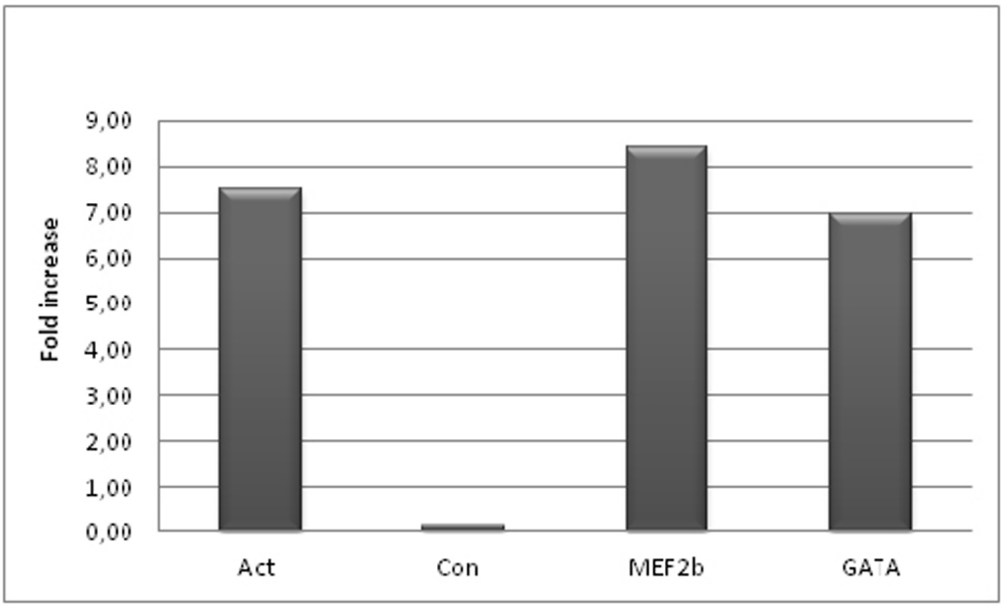

Figure 5. Expression of α-cardiac actin, Con-43, Mef-2b, GATA-4 genes 5 μM 5-Aza treated P2 BM-MSCs. Gene activity analyzed in accordance with GAPDH gene activity. Relative expression of Mef-2b revealed eight-fold increase, α-cardiac actin and GATA-4 genes had seven fold increased expression.

ELISA Analysis

According to ELISA results, cTn-I levels in the differentiated cells were measured at nanogram levels. A higher amount of cTn-I was observed in 5-Aza treated cells compared to the untreated cells. Among the different concentrations the level of cTn-I for in the 5 μM 5-Aza group, 0.022 ng at the second week and 0.019 ng third week was observed while untreated group measured 0.004 ng second week and 0.002 ng third week.

DISCUSSION

Cell based therapies for myocardium repair is a hope for heart failure. A solution for damaged myocardium could be replacing the damaged cardiomyocytes with new healthy cells. 5-Aza, a DNA demethylating agent inducing gene expression and cellular differentiation, has been demonstrated to induce the differentiation of stem cells into multiple cellular phenotypes, including cardiomyocytes [10,12-14]. This study focus on determining the best 5-Aza concentration for cardiomyogenic gene profile of differentiated BM-MSCs into cardiomyocyes.

In our study, BM-MSCs were isolated and fibroblastic cell morphology was maintained through repeated subcultures. Previous reports showed that there are many different surface markers for BM-MSCs characterization, including CD13 and CD29 expression. As expected, more than 95% of the cells stained positive for CD13 and CD29 as evaluated by immunofluorescence staining in our study. This showed that isolated BM-MSC expressed mesenchymal stem cell surface markers. Passage 2 cells were used for ongoing experiments. According to Xu et al. BM-MSCs have their highest proliferation capacities between the passage 2-passage 6 [15]. Moreover, the passage number and differentiation capacity of BM-MSCs shows variability after passage 4 [16,17].

5-Aza is a therapeutic agent for acute and chronic myelogenous leukemia, melanoma and urogenital system cancers [18,19]. This therapeutic action is connected with its effects on DNA methyltransferase. To analyze the effect of 5-Aza treatment on the proliferation of BM-MSCs, MTT assay were done. Results showed there were no statistically significant differences between the lower doses (1, 3, 5, 10 μM) of 5-Aza treated groups and control, whereas 50 μM and 100 μM treated groups an decrease was observed ($p<0,05$). Cancer treatment doses of 5-Aza were found to be 20-40 μM/kg [20,21].

In our study we assessed the effect of 24 hours 5-Aza treatment on cardiomyogenic differentiation capacity of BM-MSCs. After two weeks incubation period differentiated cardiomyocytes started to form cell clusters. Makino et al. demonstrated the cardiomyocte-like ultrastructures which include typical sarcomeres in their study [22]. The most of these cell clusters were seen in 5 μM 5-Aza concentration in our study. To confirm the specificity of the differentiation for cardiomyocytes, cells were stained with antibodies against desmin and cTn-T and all cell clusters have seen in experiments stained positive for desmin and cTn-T. In cardiomyocyte cell structure, desmin forms an interconnected network which keeps the myofibrils at Z disks and 3 μM 5-Aza treated cardiomyogenic cells showed desmin positivity as reported by Fukuda et al. [13, 23]. cTn-T and cTn-I expressed specific genes as different isomers from striated muscle [24]. In agreement with this knowledge in addition to cTn-I positivity, we measured nanogram levels of cTn-I protein in our differentiated cells. Blood levels of cTn-I has been used as a marker for heart muscle damage [24]. In previous reports, cTn-I most commonly displayed with immunostaining contrast to these reports evaluating cTn-I proteins in cells supported our data about cardiomyogenic characterization [25]. Besides 5-Aza treated BM-MSCs had differentiation capacity at range of 20% thus nano gram levels of cTn-I were not a low grade result [13,25-27].

Genetical basis of cardiogenesis in different species like mice and monkey have shown in earlier reports [28,29]. The increased gene expression of Mef-2b, α-cardiac actin and GATA-4 is similar to *in vivo* development of cardiomyoctes [29]. Even we seen eight fold increases in Mef-2b expression, Molkentin et al. also showed that Mef-2b expression is altered in cardiac muscle development [30]. Meantime α-cardiac actin expression has seven fold increase in our study and this protein is an actin isoform which only expressed in cardiomyoctes [31]. GATA-4 gene expression also altered six fold; GATA-4 is activated in the development of atrial and ventricular regions especially endocardial regions [32]. Similarly Fukuda et al. reported increased α-cardiac actin and GATA-4 gene activity in their differentiated cardiomyogenic cells [13,22]. Contrarily to Shim et al. we did not see gene expression increase in Con-43 which is an important gap junction protein in hearth development [30,33].

In summary, our study shows cardiomyogenic differentiated gene profile started at two weeks incubation period after BM-MSCs treated with 5 μM 5-Aza. Passage number and incubation period are two important factors affecting the process of cell therapy [16].

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