

**Effects of 5-Azacytidine on Differentiation of Ovine Mesenchymal Stem Cells**

Research Article

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**Abstract**

**Background:** Mesenchymal stem cells (MSCs) have potential of self-renewal and differentiation into many cell types and can be used for cells therapy. Addition of epigenetic drugs can also convert these cells into cardiomyocytes.

**Objective:** The aim of this study was to evaluate the effect of 5-azacytidine on differentiation of sheep fetal bone marrow MSCs into cardiomyocytes.

**Materials and Methods:** Isolated bone sheep fetal marrow MSCs was cultured in DMEM: F12 medium. Passaged-3 cells were examined for their differentiation capacity into osteocytes and adipose cells. Surface antigens of isolated cells were analyzed using flow cytometry. Cells after third passage treated with 5 μM 5-azacytidine for 48h followed by medium without 5-azacytidine for twenty-one days. Expression of cardiac alpha-actinin and Troponin I (cTnI) were analyzed by immunohistochemistry.

**Results:** MSCs expressed CD44, CD166 and weak expression of CD34 and CD45, apart from this expression a very weak expression of CD105 was also observed. Oil red for adipose cells and alizarin red staining for osteocytes indicated that the cells from all studied groups maintained the differentiation potential into adipose and osteocytes cells. Expression of Gata4, connexin 43, Atrial Natriuretic Peptide (ANP) was also detected by reverse transcriptase polymerase chain reaction and weak expression of troponinI. Sarcomeric alpha-actinin and cTnI were also partially detected by immunohistochemistry on these cells.

**Conclusion:** This result indicated that addition of 5-azacytidine into culture medium could be effective on MSCs differentiation into cardiomyocytes.

**Keywords:** 5-Azacytidine; Mesenchymal Stem Cells; Sheep Fetal; Cardiomyocytes Differentiation.

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**Introduction**

Myocardial infarction among the major diseases strongly influenced the Longevity and quality of life. Human adult cardiomyocytes has a limited capacity for regeneration after myocardial

infarction, the irreversible loss of cardiomyocytes, and loss of contraction muscle and increase proliferation and replacement of fibroblast cells can lead to progressive ventricular remodeling of nonischemic myocardium, and the ventricular remodeling can result in progressive ventricular dilatation and heart failure [1]. Clinically there is no treatment to regeneration of infarcted myocardium. On this basis, cell therapy is ideal treatment to regenerate the damaged cardiac area.

A lot of studies tried to improve cardiac function after myocardial infarction with cell therapy; namely: using embryonic stem cells [2] and mesenchymal stem cells (MSCs) [3, 4]. MSCs have some advantages than others because they do not form teratoma and will not raise any ethical problems as embryonic stem cells do. Using of MSCs provides basic fundamental of mechanism of fate and function of cell improvement.

Different strategies for differentiation of MSCs into cardiomyocytes can be used. Small molecules such as ascorbic acid, dexamethasone, all-trans retinoic acid and 5-azacytidine can induce differentiation of stem cells into cardiomyocytes [5]. Demethylating epigenetic drugs have gained interest because of their reprogramming effects during stem cell differentiation [6].

Makino et al., for the first time used 5-azacytidine in order that differentiation of mouse MSCs, they showed that mouse MSCs can differentiate into beating cells [7]. Addition of 10  $\mu$ M 5-azacytidine in culture of hMSCs for 28 days could induce differentiation towards cardiomyogenic lineage, whereas same cultural condition for a period of 24h lead to cardiac-like muscle cells [8, 9]. Fukuda et al. showed that eight weeks after treatment with 3 mM 5-azacytidine cardiomyogenic cell line from murine bone marrow stromal [10].

The better model for studying human pathology than rodent models are domestic animals because immunologically and physiologically they are similar to human [11].

On the basis of research reports there is little information available about the effects of 5-azacytidine on ruminant bone marrow MSCs, we therefore designed our experiment to evaluate the effect of 5-azacytidine (small molecule) on differentiation of sheep fetal MSCs into cardiomyocytes.

## Materials and Methods

### Chemicals

Except where otherwise indicated, all chemicals were obtained from Sigma-Aldrich (USA).

### Isolation, cell culture, and phenotypic characterization

Ovine fetus (30-35 days) obtained from slaughtered animal at Rock slaughterhouse, Karaj. Fetuses were transported to laboratory in Dulbecco's PBS on ice. The Bone marrow MSCs were collected from ovine fetus by aspirating femurs and tibias with DMEM: F12 (3:1) culture media supplemented with 100 IU/ml penicillin, 100 IU/ml streptomycin. Bone marrow aspiration sample was placed on top of ficoll (equal volume) and centrifuged at a speed of 4000 rpm for 30 min at room temperature. Cloudy layer collected and transferred into new tube and washed twice with same medium. The mononuclear cells with concentration of  $5 \times 10^6$  cells/ml were cultured in DMEM: F12 supplemented with 5% fetal calf serum (FCS), L-glutamin and penicillin/streptomycin. Cells were incubated at 37°C with 5% CO<sub>2</sub>. The medium was changed every 3 days after initial plating. After 80% confluency, cells were subcultured using 0.25% trypsin/1 mM EDTA.

Undifferentiated sheep fetal MSCs surface markers were examined by flow cytometry. Sheep fetal MSCs were cultured as described above, cells were centrifuged and pellet was resuspended in cold DPBS with 108 cells/ml. Cells were incubated with primary antibodies (CD34-FITC; CD44-FITC; CD45-FITC; CD90-FITC and CD166-FITC) followed by secondary conjugated antibody. Isotype-matched antibody (mouse anti-human IgG) was used as negative control, treated cells were incubated at 4°C for 30 min, and the complex was analyzed by flow cytometry.

### Differentiation of cardiomyocyte-like cells

After three passages of MSCs, cells were cultured in 6-well plates. When cells reached at 80% confluency, they were treated with 5  $\mu$ M 5-azacytidine for 48h, then 5-azacytidine was deleted from culture medium and again cultured for 21 days, medium was changed every three days. MSCs cultured in complete medium without 5-azacytidine was used as control group.

Total RNA was extracted with AccuZol (Bioneer) from induced and uninduced MSCs. Transcriptional expression of myocardium-specific Connexin 43, ANP (Atrial Natriuretic Peptide), GATA4, Troponin I was determined by semi-quantitative RT-PCR using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, USA) and PCR master mix (Fermentas, USA) according to the manufacturer's instructions. Transcript levels were standardized to the corresponding sheep GAPDH level. The forward and reverse primers for each PCR set were designed to be located by the Oligo6 software [Connexin 43: For: GTCGTGTCGTTG-GTGTCCTCTTG, Rve: GCCAGTGGTAGTGTGGTAAGG (114 bp), Troponin I: For: CCGCTCGTCAGCCAACTAC, Rev: GAGTTCCTGCTTCGCAATCTG (124 bp), Gata4: For: GGGCGAGCCAGTGTGTAATG, Rev: GGACCTGCTG-GTGTCCTTGATTG (141bp), ANP: For: ACCGTTTG-GAGGACAAGATGC, Rev: TCTCTCTGGGCTGCGTTGAC (147bp), GAPDH: For: ATCGTGGAGGGACTTATGACC; Rev: CGCCAGTAGAAGCAGGGATG (130bp). The thermal profile for PCR was 95 °C for 3 min, followed by 40 cycles of 45 sec at 95 °C, with 1 min annealing intervals (57.8 for GAPDH, Troponin I, Gata4, ANP and 58.2 for Connexin 43) followed by 1 min extension at 72 °C. Additional 10-min incubation at 72°C was included after completion of the last cycle. Following the PCR, 5  $\mu$ l of the PCR product was electrophoresed on a 1% agarose gel and photographed under UV light.

### Immunohistochemistry analysis

Induced and uninduced cells were fixed in 4% paraformaldehyde in PBS for 15 minutes, after rinsing with PBS cells were permeabilised in PBS containing 0.2% Triton X-100 for 30 minutes and blocked in blocking buffer containing PBS supplemented with 0.1% BSA and 0.1% Triton X-100. Primary antibodies were alpha-actinin (A7811, Sigma) and troponin I (cTnI) (Abcam, ab47003). Antibodies were diluted 1:500 for alpha-actinin and 1:300 for troponin I in PBS containing 1% BSA. Cells were incubated in the appropriate antibody solution at 4°C overnight. They were rinsed three times with PBS for 15 minutes each. Cells were incubated with FITC (F9137 Sigma) conjugated secondary antibody diluted 1:400 with PBS for 2 hours at room temperature. Cells were then rinsed three times with PBS. Nuclear staining was performed by 5 min incubation with DAPI (4,6-diamidino-2-phenylindole). Images were analyzed with a fluorescent inverted microscope (TE2000; Nikon).

## Results

Cultured MSCs grew rapidly and expanded in primary cultures media, non adherent cells were removed while changing the media after 48h. Cells after 5 days incubation showed 80% confluency (Figure 1).

Ovine BMSCs at third passage were evaluated for potential differentiate into adipogenic and osteogenic lineages. Twenty one days after osteogenic induction, BMSCs began to mineralize their matrix. Cultured cells were stained positively with Alizarin Red. BMSCs differentiated cell to adipocytes formed the lipid droplets were also stained with Oil red (Figure 2).

Flow cytometric analysis (Figure 2) showed that the isolated cells at passage 3 expressed the surface markers CD44 (93.48%), CD166 (96.28%) in contrary to the results reported in the human, we also did not get positive expression of CD105 (6.30%). Cells

were negative for surface markers of hematopoietic stem cells such as CD34 (6.52%) and CD45 (16.80%).

RT-PCR analysis for the expression of cardiomyocyte genes represented that both induced and uninduced cells expressed connexin 43 and GAPDH (Figure 3), whereas other cardiomyocyte-specific genes such as ANP, Gata4 and Troponin-I were only detected in induced cells. Induced cells treated for 21 days without 5-azacytidine also expressed ANP, Gata4 and Connexin 43. We could detect weak expression of troponin-I (Figure 4).

Immunocytochemistry examination clearly detected the localizations of cardiac alpha-actinin and cardiac troponin I on cardiomyocytes like cells (Figure 5A and B).

## Discussion

MSCs isolated from animals such as mice, pigs, sheep, goats, horses, and dogs are used in preclinical trials and in the veterinarian clinic for the treatment of conditions such as osteoarthritis in dogs and musculoskeletal conditions in horses.

One of the difficulties for characterization of MSCs isolated from ruminants is lack of accessibility of specific monoclonal antibodies [12]. Godoy et al., found that sheep MSCs isolated from bone marrow, synovial membrane and adipose tissue are positive for CD44 and MHC class I [13]. In our study, flow cytometry analysis for surface markers showed that bone marrow sheep MSCs were positive for CD44 and CD166 but the expression of CD105 was

Figure 1. Passage 2 sheep fetus mesenchymal stem cells (magnification×100).

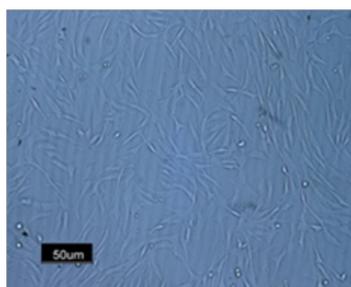


Figure 2. Multi-lineage differentiation of the isolated mesenchymal cells from ovine bone marrow. A) Adipogenic culture stained by Oil red; B) Osteogenic culture stained by alizarin red (magnification×200).

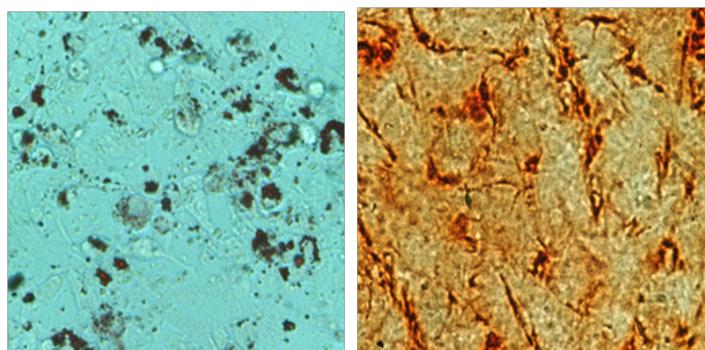
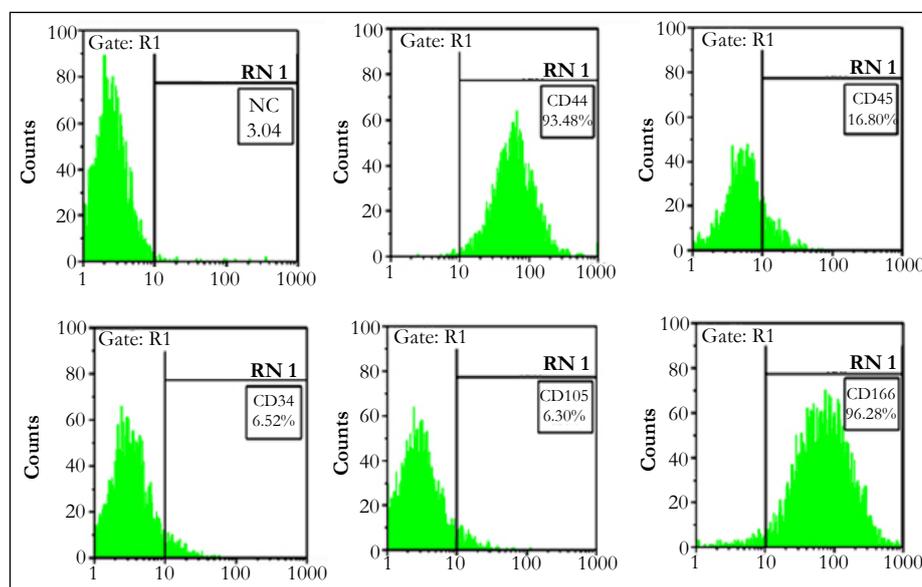
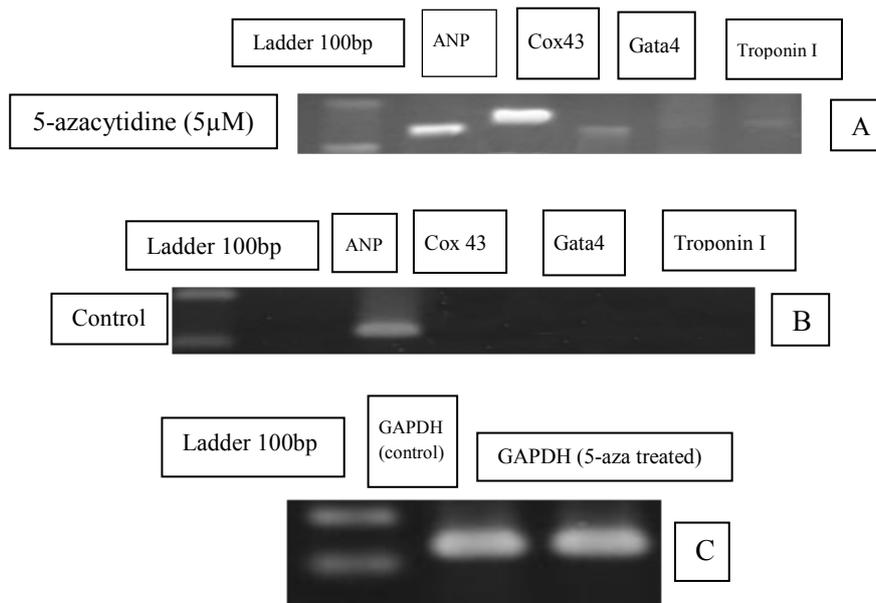


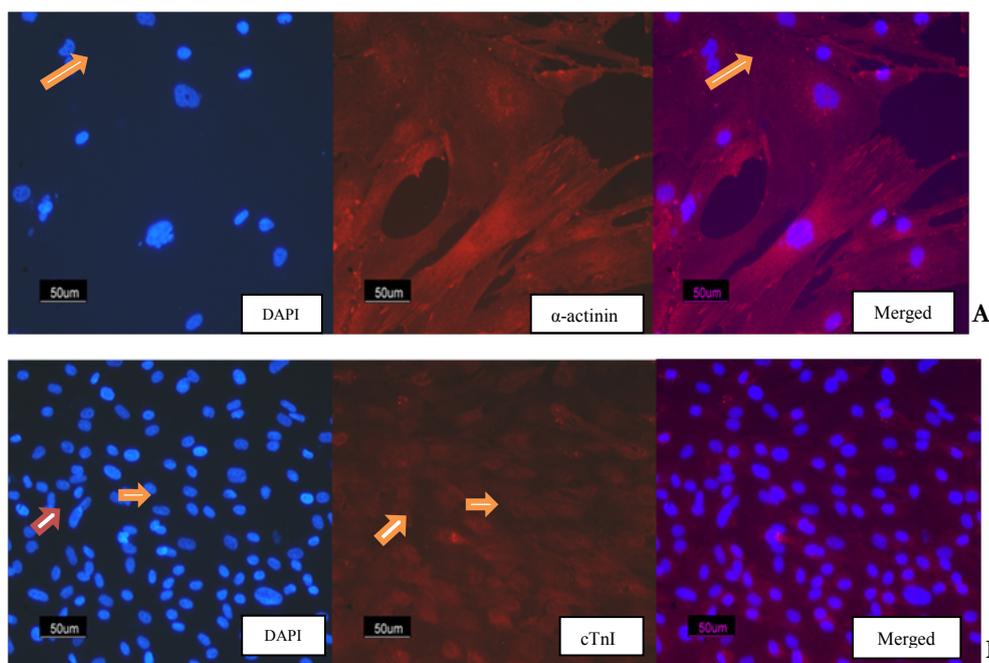
Figure 3. Sheep fetus bone-marrow derived mesenchymal stem cells. Majority of the isolated cells expressed mesenchymal markers including CD44, CD166 CD105. Hematopoietic (CD45 and CD34) markers were expressed on the isolated cells.



**Figure 4. RT-PCR analysis of specific expression of cardiomyogenic markers. (A) Induced cells with 5-azacytidine for 48h, (B) uninduced cells and (C) GAPDH expression for induced cells with 5-azacytidine and uninduced cells.**



**Figure 5. Expression of alpha-actinin (A) and troponin I (B), in day 21 in cardiomyocytes like cells of sheep fetal. multinucleated cardiac-like cells were formed in vitro induced ovine bone-marrow MSCs. Arrows point to multinucleated cells(magnification×200).**



weak. Despite the widespread use of MSCs from non-humans, there are no established minimal criteria for the identification of MSCs in non-humans. While all MSCs isolated from non-human display tri-lineage differentiation and plastic adherence, not all express the same panel of surface antigens that has been described for human MSCs. The expression of CD29 and CD44 in most non-human MSCs observed but the expression of some of the CD markers such as CD90, and CD105 varies depending on species and strain [14].

Most of studies have examined differentiation of MSCs into cardiomyocytes that have been treated with specific growth factors [15] and histone modifiers [16]. 5-azacytidine as a DNA methyltransferase inhibitor can affect DNA and histone methylation and causes DNA hypomethylation. Epigenetics drugs, such as 5-aza-

cytidine can manage gene silencing activity [17], the activity of this drug may cause upregulating the differentiation-promoting factors of stem cells. The accurate function of the 5-azacytidine on cardiomyocytes differentiation from MSCs remains controversial.

The results of previous studies showed that after treatment with 5-azacytidine, differentiation rates of adult and fetal MSCs from different species is varies. The study of Zhang et al., showed that supplementation of 10 μmol/l of 5-azacytidine in culture medium of human fetal MSCs for 21 days decrease proliferation of but increases differentiation rate of human fetal MSCs into cardiomyocyte-like cells compared with 24-48h induction [18]. Antonitsisa et al., studied the addition of 5-azacytidine to the culture medium for 24h could be differentiated adult mesenchymal stem cells to

cardiomyocytes [8]. Liu et al., found that, presence of 5-azacytidine in the culture medium of rat MSCs, neither the spontaneously beating cells nor the formation of myotubes were found in the primary and first passaged [1]. The presence of 5-azacytidine in the culture medium of adipose-derived stem cells (ASCs) toward cardiomyocytes differentiation was not enough [19]. Supokawej et al., proved that the addition of 15  $\mu$ M 5-azacytidine could promote the expression of skeletal and cardiac muscle-specific genes to human bone marrow MSCs in culture medium [20].

In our study, the addition of 5-azacytidine to the culture medium for 48h days and then 21 days culture without this drug was caused expression of Gata4 a myocardial transcript factor in the early stage of cardiac differentiation, connexin 43 and ANP can expression in secondary stage of cardiac differentiation but we could see weak expression of troponin I. We saw the expression connexin 43 in the uninduced cells, on the basis of literature connexin 43 during embryogenesis expressed and was maintained in many different cell types [21]. Connexin 43 for multipotency and cellular migration of the skin-derived stem cell was required [21]. Although the mechanisms of 5-azacytidine toward the cardiomyocytes differentiation are still unclear, it has been proposed that this epigenetic drug may activate some silent genes by inhibiting DNA methylation [22] or induced in some genes of signaling pathways related to differentiation [23]. We think that the time of cardiac differentiation (21 days) for expression of cardiac-specific genes when 5-azacytidine for 48h added in the culture medium of sheep fetal bone marrow mesenchymal stem cells was enough. Further study is needed to elucidate the best conditions to differentiate the sheep fetal MSCs into mature cardiogenic cells.

## Conclusion

In conclusion, taken together we indicated that 5-azacytidine at 5 $\mu$ M could enhance sheep fetal bone marrow MSC differentiation into like cardiomyocytes.

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