

# Differentiation of Bone Marrow Mesenchymal Stem Cells into Chondrocytes after Short Term Culture in Alkaline Medium

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Received: 9, Feb, 2014  
Accepted: 23, Mar, 2014

## ABSTRACT

**Background:** Bone marrow mesenchymal stem cells (MSCs) are one of the undifferentiated multipotential cell sources of human body. MSCs have the capacity to form a variety of cell types, especially chondrocytes and osteocytes. Learning about responses of MSCs to external milieu and chemical factors such as pH could recommend new approaches for preparation of suitable scaffolds for bone and cartilage tissue engineering. In present study, the effect of alkaline medium on chondrogenic and osteogenic differentiation of rat MSCs was evaluated

**Methods:** MSCs were harvested from bone marrow of animals and then the response of passage 1 and 2 of MSCs (P1 MSCs & P2 MSCs) to the culture in alkaline medium (pH: 8) was evaluated. Cytochemical and immunocytochemical staining were performed to distinguish chondrocytes and osteocytes. Real-time PCR was performed to evaluate the type II collagen and osteopontin mRNA levels.

**Results:** Staining for type II collagen, a chondrocytic specific marker, revealed that after one-week culture in alkaline medium, a considerable amount of P1 MSCs had shown chondrocytic morphology. By prolonging the culture period up to 4 weeks, osteogenic cells with expanded matrix and mineralized areas around them were appeared. Results of real-time PCR showed that P1 MSCs after one week culture in alkaline medium expressed highest rate of type II collagen and osteopontin mRNA among all groups.

**Conclusion:** This study demonstrated that alkaline medium is a potent chondrogenic differentiation inducer for MSCs in their first passage.

**KEYWORDS:** Alkaline medium, Chondrocyte, Mesenchymal stem cell, Osteoblast

## INTRODUCTION

There are many disorders affecting bone and cartilage, like osteoarthritis (degenerative joint disease) and bone fractures. Recently, the new therapeutic techniques such as cell-replacement therapy and bioengineered organ grafts have

attracted many attentions. Development of cell culture techniques is necessary to find out the best method for production of adequate bone and cartilage cells for treatment of related diseases.<sup>1</sup>

Bone marrow multipotent stromal cells (BMMSCs), also known as bone marrow

mesenchymal stem cells or marrow stromal cells (MSCs), have some features such as high proliferative potentiality and safety for cell therapy. Their simple harvest from bone marrow makes them an available multipotential cell source for cell-based therapies.<sup>2,3</sup>

MSCs can differentiate into multiple cell types such as osteocytes, chondrocytes, adipocytes, neurons, and muscle cells.<sup>4,5</sup> MSCs can be isolated and expanded in culture via standard methods and appropriate conditions.<sup>4</sup> Due to this property of MSCs, they are a suitable source for repairing the tissue injuries and treatment of imperfections in cartilage, bone and tendon. There are several reports about successful application of these cells for treatment of bone and cartilage disorders in animals and humans.<sup>6-8</sup>

Accordingly, several methods with different rates of success were examined for induction of chondrogenic and osteogenic differentiation of MSCs. The effect of various scaffolds with different compositions such as hyaluronan/gelatin, photopolymerizing hydrogels, nanofibrous meshes, platelet lysate and collagen gels with or without insertion of growth factors have been investigated by many researchers.<sup>2,6,9-14</sup> Finding a rapid and easy way to generate pure chondrocytes from MSCs is a matter of interest at this time. Therefore, assessment of different growth factors and culture conditions such as pH and ionic composition along with using different scaffolds could be beneficial to find new protocols for differentiation of MSCs into chondrocytes.

Differentiation of MSCs into chondrocytes was carried out by many researchers but, at the time of production of tissue-engineered cartilage, its handling becomes difficult and needs much more amount of cells and specific culture conditions.<sup>15-17</sup> Among different protocols used for the production of chondrocytes, some of them that used growth factors such as TGF-beta had enough efficacies.<sup>18,19</sup> Furthermore, the production of chondrocytes from MSCs by using these methods is expensive and time consuming. Additionally, there are several unknown parameters in the microenvironment of bioengineered scaffolds that cause considerable cell death,<sup>20</sup> and this makes the production of bioengineered cartilage very difficult and expensive.

So, it is preferable to test the efficiency of scaffolds with less expensive cells. For this purpose, several studies such as culture in low oxygen level had already been performed.<sup>21</sup>

It has been previously reported that growth and development of osteoblasts depend on extracellular and intracellular pH. Moreover, mineralization and repairing of the bone tissue is highly affected by the microenvironment compositions such as ions and additives.<sup>22</sup> Besides, it was revealed that extracellular pH is also very important for the metabolic activities and biosynthetic ability of the chondrocytes.<sup>23</sup> Some studies indicated that extracellular pH can modify the differentiation capacity of MSCs. Few researchers evaluated this hypothesis on MSCs and different results were reported. Kohn et al. have reported that decreasing the extracellular pH reduced the amount of collagen and alkaline phosphatase activity in mesenchymal stem cells,<sup>22</sup> while Leem et al. reported that alkaline pH decreased the alkaline phosphatase activity and could delay the differentiation of human mesenchymal stem cells.<sup>24</sup> Recently, it was reported that microenvironment chemical elements such as pH and hydrostatic pressure of gases are involved in chondrogenesis of MSCs.<sup>25</sup> Therefore, the effect of pH on MSCs differentiation is a matter of debate now. Based on these findings, we tried to evaluate the effect of extracellular alkaline medium on chondrogenic differentiation of MSCs.

## MATERIALS AND METHODS

### Cell Culture

MSCs were isolated from Male Wistar rats (Pasteur Institute, Tehran, Iran) according to the previously reported studies by some small modifications.<sup>26</sup> The animals were euthanized by intra-peritoneal injection of 60 mg/kg of Ketamine-HCl (Sigma, K2753) and 10 mg/kg Xylazine-HCl (Sigma, X1251). Then, the femurs and tibiae bones were isolated carefully and adherent soft tissues were removed. Following this, epiphyses were cut and the marrow was harvested by flushing with DMEM medium (Gibco, 10566032). Harvested cells were cultured in 10 cm dishes in DMEM, 15% fetal bovine serum (Gibco, 12664025) supplemented with 100 U/ml penicillin/streptomycin (Gibco,

15140122), and the pH of medium was adjusted to 7.4. Afterwards, cells were incubated at 37°C in 95% humidity and 5% CO<sub>2</sub> for 48 hours until the attachment of stromal cells. Subsequently, non-adherent cells were removed by changing the culture medium and rinsing with phosphate buffered saline ((PBS), Gibco, 10010049). Culture of the attached cells was continued by changing the culture medium every 3-4 days until confluence appeared. After 7 days, when primary BMMCs (P0 cells) became confluent, they were detached from dish by 0.25% trypsin-EDTA (Gibco, 25200056). Afterwards, sub-culturing was carried out on passage 1 (P1) and passage 2 (P2) cells in the normal (pH=7.4) and alkaline (pH=8) media in 6-well culture plates. RNA isolation and cell staining were performed after one and four weeks of culture. All experiments were carried out in triplicate.

### Cell Staining

Chondrogenic differentiation of MSCs was evaluated by toluidine blue (Sigma, T3260) and immunofluorescence staining against collagen. Osteogenic differentiation of MSCs was determined by staining with alizarin red S (Sigma, A5533) which stained calcium deposits in adherent cells. Before staining, cells were fixed with ice-cold 4% paraformaldehyde (Sigma, P6148) for 10 minutes and after rinsing 3 times with distilled water, they were stained with toluidine blue (1mg/ml in NaOH) for 3 minutes and alizarin red (20mg/ml in distilled water) for 5 minutes at room temperature. Then, the cells were washed with distilled water several times and were examined under microscope. To determine the chondrogenic and osteogenic differentiation, toluidine blue stained cells and alizarin red stained areas were counted in 10 images captured from each group.

In order to identify the collagen type II protein, cells were stained against type II collagen, using a specific primary antibody (Abcam ab21291). For immunofluorescence staining, cells were fixed with 4% paraformaldehyde for 10 minutes, following by rinsing the samples three times with phosphate buffered saline and incubation for 30 minutes in 5% normal serum from goat (Sigma, G9023) + 0.3% bovine serum albumin (Sigma, A2153) + 0.25%

Triton X-100 (Sigma, X100) in PBS). After that, samples were incubated with primary antibody for 12 hours and rinsed three times with PBS. Then, they were incubated with secondary antibody (FITC conjugated secondary antibody, Sigma, F0382) for 2 hours and finally rinsed 5 times with PBS. The nucleus was subsequently counterstained by DNA-specific fluorochrome4, 6-diamidino-2-phenylindole dihydrochloride ((DAPI), Sigma,) and washed twice. Images were captured from stained cells with a fluorescence microscope equipped with digital camera (Olympus).

### Analysis of Gene Expression Using Quantitative Real-Time PCR

Total RNA was extracted from the cells using GF-1 total RNA extraction kit according to the manufacturer's instructions (Vivantis, Cat. No: GF-TR-025). The extracted RNA was then reverse-transcribed into single stranded cDNA, using MMLV Reverse Transcriptase and Oligo(dT) primers according to the manufacturer's instructions (Promega).

For gene expression analysis, real-time PCR was performed using SYBR-Green-based protocols in an ABI Step One system. Primers were designed by using FastPCR professional software version 6.1 (PrimerDigital Ltd). The oligonucleotide primer sequences used in this study are listed in Table1 (primers were purchased from Takapo Zist Company, Tehran, Iran). Type II collagen and osteopontin were selected for the study and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control (housekeeping gene). The real-time PCR SYBR Green Master Mix (Life Technologies, 4344463) was used for the amplification reactions. The PCR conditions were set as follow: 10 min at 95 °C, followed by 35 cycles at 95 °C for 15 seconds and 52 °C for 1 min. The expression levels of target genes in each sample were calculated by the comparative Ct method ( $2^{-\Delta\Delta Ct}$  formula) after being normalized to the Ct value of the GAPDH housekeeping gene.

### Statistical Analysis

Paired Student t-test was used for data analysis using SPSS software version17. P-values less than

0.05 were considered statistically significant. Data were presented as mean $\pm$  standard error of mean (SEM).

**Table 1:** Primer sequences used for Real-Time PCR

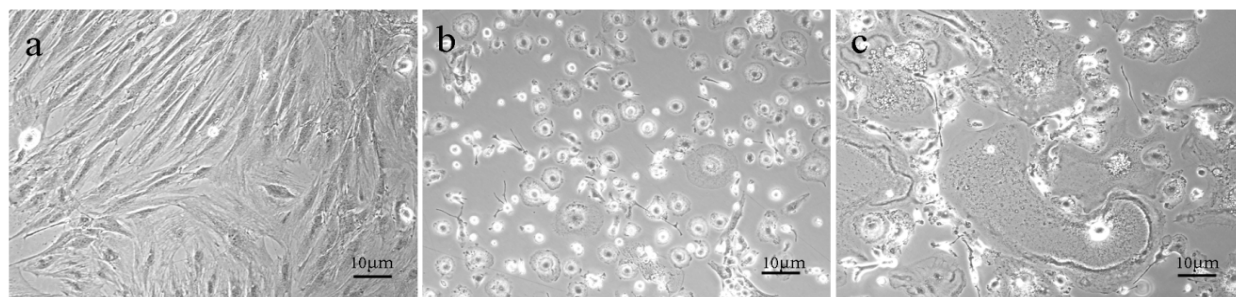
Gene name	Primer sequences	Product size
Type II collagen (GenBank ID: 25412)	5'-TCAAGTCGCTGAACAACCAG-3' 5'-GTCTCCGCTCTTCCACTCTG-3'	116bp
Osteopontin (GenBank ID: 25353)	5'-GGGAAGACCAGCCATGAGTC-3' 5'-CGACTGCTCAGTCTCTCGT-3'	114bp
GAPDH (GenBank ID:24383)	5'-GTGGTCTCCTCTGACTTCAACAG-3' 5'-CTGTAGCCAAATTCGTTGCATAC-3'	122bp

## RESULTS

### Effect of Alkaline Medium on Morphology of MSCs

During primary culture, the adherent cells have shown fibroblast-like morphology (Figures 1a and 2a). This morphology is specific for MSCs in the in-vitro culture conditions. After first passage and incubating P1 MSCs in medium with pH=8, the morphology of cells changed to circular cells with a nucleus in the middle of the cell and a shiny ring in

the cytoplasm around nucleus that is the main characteristic of typical chondrocytes (Figure 1b). Staining with toluidine blue and also with anti-type II collagen specific antibody revealed that this shiny ring is collagen, a specific protein of chondrocytes (Figure 2). Counting the number of cells stained with toluidine blue showed that 82.34 $\pm$ 3.16% of the cells were differentiated to chondrocyte that was statistically significant compared with cells cultured in normal pH medium ( $P<0.001$ , Figure 2b) (pH=7.4). Continuing the culture in alkaline medium up to four weeks led to the death of chondrocytes or changed their fate and as a result the morphology of cells changed to osteocyte-like cells and mineralized areas appeared around them (Fig 1c). As illustrated in Figure 2d, 56.19 $\pm$ 3.31% ( $P<0.001$ ) mineralized areas are apparent in images captured from cells after staining with alizarin red, while there were not any toluidine blue or alizarin red stained cells in pH=7.4 cultured cells (Figures 2a and 2c).



**Figure 1.** Fibroblast-like morphology of primary cultured MSCs at day 7 of culture (a), appearance of chondrocytic morphology (circular shape and extracellular matrix) after one-week culture in alkaline medium (b), osteoblastic morphology (expanded extracellular mineralization) after four weeks of culture in alkaline medium.

### Gene Expression

As shown in Fig. 3, real-time PCR assay on P1 MSCs showed that rising the pH of culture medium to 8 caused a large increase in type II collagen and osteopontin gene expression after one-week culture, compared with the cells cultured in medium with pH=7.4 that did not expressed these genes. Rate of the expressions in all of the pH=8 groups were significantly higher than their pH=7.4 control groups ( $P<0.0001$ ), except osteopontin expression in P2 cells after 4 weeks of culture. Expression of type II collagen in P1 MSCs after 1

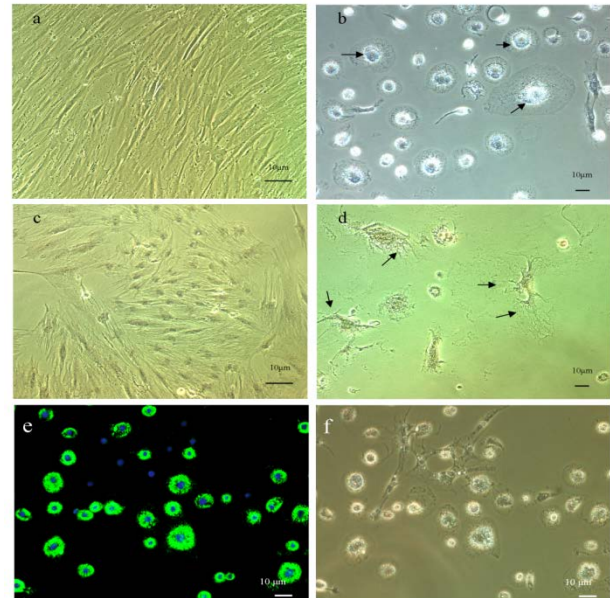
week of culture in pH=8 was significantly higher than the group cultured in same pH for 4 weeks and also from P2 MSCs that were cultured in pH=8 for 1 and 4 weeks ( $P<0.0001$ ,  $P<0.001$  and  $P<0.001$ , respectively). The expression of osteopontin in P1 MSCs for 1 week and 4 weeks was significantly higher than that of the P2 MSCs cultured in pH=8 at the same time ( $P<0.001$  for 1 week and  $P<0.0001$  for 4 weeks).

Type II collagen and osteopontin are both chondrocytic markers<sup>27,28</sup>, while osteopontin is not just specific for chondrocytes and several other cells such as osteoblasts, endothelials and smooth

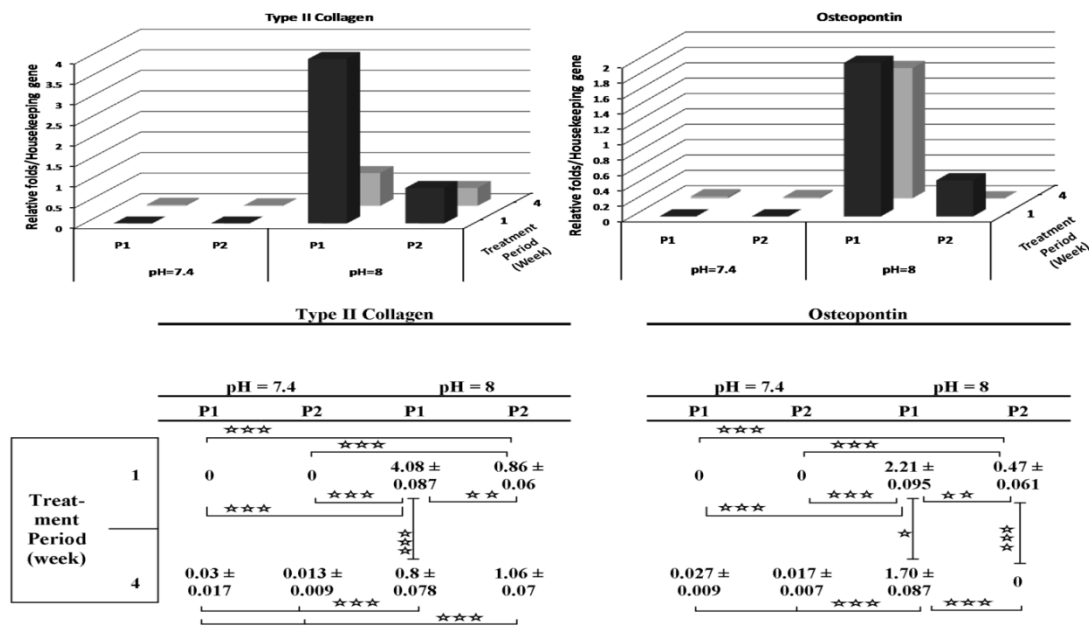
muscle cells also express it. Results of real-time PCR and cytochemical staining together with the expression of Type II collagen gene that happened in accordance with morphological changes confirmed the chondrocytic differentiation of P1 MSCs after one-week culture in alkaline media.

By continuing the culture of P1 MSCs in pH=8, the expression of type II collagen decreased, while expression of osteopontin remained partially unchanged. The expression of osteopontin gene along with the appearance of mineralized areas in culture dish indicated that the osteopontin expression in this stage was happened as a result of osteocytic differentiation. According to these findings, our study showed that culturing the P1 MSCs up to 4 weeks in alkaline medium with pH=8 could induce their differentiation toward osteocytes.

However, expression of type II collagen and osteopontin genes was significantly decreased in P2 MSCs that were cultured in pH=8 as compared with P1 cells in the same condition. This reduction indicated that the potentiality of MSCs to generate chondrocytes and osteocytes was decreased as their passage number was increased.



**Figure 2.** Toluidine blue staining of cells after one week of culture in normal (a) and alkaline medium (b), toluidine blue was used to stain collagen (blue) and nucleus (red) as seen in b, differences between a and b show dramatic changes in the morphology of cells after one-week culture in alkaline medium. Alizarin red stained mineralized areas after four weeks of culture in normal (c) and alkaline medium (d, (marked by arrows)), Immunofluorescence staining against type II collagen in P1 MSCs after one week of culture in alkaline medium (e), nucleus counterstained with DAPI, f is the phase contrast image of e.



**Figure 3.** The effect of extracellular alkaline medium on the expression of type II collagen (a) and osteopontin (b) genes using real-time PCR technique. Relative folds of expression for each gene were calculated based on the expression of housekeeping gene (GAPDH) and represented in the below table. The level of expressions in all of the pH=8 groups was significantly higher than their pH=7.4 in control groups (P<0.0001), except osteopontin expression in P2 cells after 4 weeks of culture. The level of osteopontin and type II collagen mRNA in P1 MSCs cultured for 1 week in alkaline medium was highest among all groups. Data were presented as Mean ± SEM (\*\*\*: P<0.0001, \*\*: P<0.001, \*: P<0.01).

## DISCUSSION

There are some convincing evidences indicating that acidosis alters the normal function of osteoblasts and inhibits their extracellular matrix growth and mineralization.<sup>29-31</sup> Studies on cultured human osteoblasts revealed that increasing the extracellular pH up to 7.8 enhances the collagen synthesis, alkaline phosphatase activity and thymidine incorporation in these cells,<sup>32</sup> while acidosis increases osteoclastic activity.<sup>33</sup> Therefore, the relationship between pH and function of osteoblasts and osteoclasts is unambiguous, although there is not enough evidence in the case of MSCs. Kohn et al. reported that decreasing the pH of culture medium from 7.8 to 6.6 reduces the collagen gene expression in human MSCs, while the *osteocalcin* gene expression is opposite of collagen.<sup>22</sup> Leem et al. reported that alkaline medium inhibits or delays the differentiation of human MSCs and decreases their alkaline phosphatase activity.<sup>24</sup>

It was reported already that long-term culture of human mesenchymal stem cells in normal pH can reduce the proliferation rate without changing their differentiation ability.<sup>34</sup> According to the findings of this study, it could be concluded that between P1 and P2 MSCs that were cultured in normal pH (7.4) there were not any changes in the cell morphology and expression of type II collagen and osteopontin genes, but changing the extracellular pH from 7.4 to 8.0 changed the fate of MSCs toward chondrocytic and osteocytic cells. We found that P1 MSCs have stronger ability for production of chondrocytes and osteocytes in alkaline medium. We suppose that this difference between these two groups of cells (P1 and P2) relates to the reduction of their differentiation capacity as their passage number increases. Moreover, it could be also attributed to the delayed differentiation of cells that could occur after extended culture in pH=8 medium.<sup>24</sup> The results of this study showed that MSCs in their first passage could give the best chondrocytic differentiation ratio.

Commonly, researchers have been used a variety of induction factors such as dexamethasone, ascorbic acid and growth factors for osteocytic and

chondrocytic differentiation of MSCs.<sup>10,35-37</sup> Freeman et al. in their study reported that human MSCs after 21 days priming with chondrogenic differentiation factors which was followed by 49 days of culture in growth factor-free medium could produce high level of sulfate glycosaminoglycan.<sup>38</sup> According to the results of present study, we found that increasing the extracellular pH of rat P1 MSCs modulates their differentiation capacity toward chondrogenic cells and it happened just after seven days of culture. To determine the efficacy of this method on human MSCs, application of this technique on human MSCs is required. In one study about evaluating the effect of alkaline factors on scaffolds by Zhu et al., they showed that grafting the bone marrow mesenchymal stem cells transfected with connective tissue growth factor (CTGF) gene on NaOH-treated poly lactic-co-glycolic acid (PLGA) scaffolds increased the healing rate of cartilage defects in rabbits.<sup>39</sup> Thus, the response of mesenchymal stem cells to the extracellular pH could be useful for preparation of better biodegradable scaffolds that have defined pH and could alter the differentiation property of MSCs even after transplantation in injured sites. In conclusion, the results of the present study indicate that changing the pH of culture medium from normal to alkaline medium could differentiate MSCs to chondrocytes and osteoblasts. By studying the effect of passage number on MSCs, we found that MSCs at their first passage had the highest tendency for differentiation toward chondrocytes when they cultured in alkaline medium. Also, the effect of short-term and long-term exposures to alkaline medium was different on MSCs and they were replaced by osteocytes by the prolongation of their culture in alkaline medium chondrocytes.

## CONFLICT OF INTEREST

All authors declare no conflict of interest.

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