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The Effect of TNF-α on the Expression of MMP9 in Human Mesenchymal Bone Marrow-Derived Stem Cells

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Abstract

Matrix metalloproteinase 9 (MMP9) as the enzyme of adult stem cells secreted from damage cells. In spite of low level of MMP9 enzyme in the mesenchymal stem cells, many inflammatory cytokines stimulation such as TNF-α could increase MMP9 level in cells.

Current study evaluated the expression of the MMP9 enzyme under the influence of TNF-α in human bone marrow mesenchymal stem cells.

The human bone marrow mesenchymal stem cells were classified into control and experimental groups. In the experimental groups, various concentrations of the TNF-α (1ng/ml and 10ng/ml) were administrated in different times (10 and 24 hours), whereas the control group was not treated with TNF-α. MMP9 gene expression was evaluated by Real-Time PCR. TNF-α administration in 1ng/ml and 10ng/ml dosage for 10 hours, induced the expression of MMP9 1468.3 and 1782.8 times more than the control group, respectively.

After 24h, in comparison between 1ng/ml and 10ng/ml with control groups, MMP9 expression were 442.64 and 1184.4 times more than control group, respectively.

In conclusion, the expression rate of the MMP9 gene in bone marrow mesenchymal stem cells might be effected by dosage and time of exposure to TNF-α. Furthermore, the time of exposure might have the prominent role in alteration of MMP9 gene expression induction in the mesenchymal stem cells.

Keywords: Bone Marrow, Mesenchymal Stem Cell, MMP9 Enzyme, TNF-α, Cell Migration

1. Introduction

Mesenchymal stem cells (MSCs) participate to repair of their originated tissues such as bone, cartilage, muscle, tendon, and fat. Moreover, these are supportive cells for the hematopoietic cells production (Friedenstein et al., 1966). Typical mesenchymal stem cells were specialized to differentiated cells in the same texture of originated tissues when abnormal tissue proliferation occurs, as a result, they called them adult stem cells (Ullah et al., 2015).

According to International Society of Cell Therapy (ISCT) definition Human MSCs attache to the bottom of plastic culture dishes in the normal culture conditions. They are negative for the expression of the surface markers CD105, CD73 and CD90, hematopoietic markers CD45, CD34 and other markers such as CD19, CD79, CD11b, CD14 as well as HLA-DR; and should have been able to differentiate into adipocytes, cartilage and bone in vitro (Keating, 2006).

Many studies have shown that MSCs are able to escape from immune system and inhibit the immune response which have key role in transplantation and cell therapy (Zhao et al., 2011). MSCs have been used for tissue regeneration in several clinical studies especially in wound healing (Perry et al., 2008). Also, Human and mouse MSCs have been used in the mouse models of spinal cord injury healing as well as damaged heart tissue recovery (Rojas et al., 2005).

Matrix metalloproteinases (MMPs), as a calcium-dependent zinc-containing endopeptidases, express in the most damaged tissues such as cardiovascular and liver injury. The activity of these enzymes is regulated by tissue inhibitors. Changes in the expression levels of these enzymes have been reported in liver disease and

development as well as damaged body tissue treatment such as liver transplantation or stem cell transplantation. These enzymes digest many components of the extracellular matrix (ECM) and basement membrane structure, and their role is significant in physiological and pathological processes (Duarte et al., 2015).

MMPs have been reported to be either proenzyme or enzymes in biological samples. The activity of this protein is required for the precise regulation and control of cells including keratinocytes, fibroblasts, endothelial cells, macrophages, neutrophils, mast cells, acidophilic and $CD34^+$ bone marrow, and umbilical cord. MMPs protein activity and degeneration must be controlled by the specific inhibitors. Inhibitors of this enzyme family (TIMPs) have a high concentration in the serum. (Behrendtsen & Werb, 1997).

Matrix metalloproteinase 9 (MMP9) gene family, the enzyme of adult stem cells in all tissues (especially in bone marrow, umbilical cord, and liver), is secreted from damaged cells. Kuppfer cells are important group of cells in the liver with macrophage-like behavior. Expression of MMP9 enzyme is increased with enhanced activity of Kuppfer cells. Progelatinaze B is activated by plasmin and estomelesine through protease activity, leading to a 92 kDa protein production. One of the most important members of Progelatinaze B gene family, gelatinase, is the major basement membrane composition in human cells. Gelatinase is the only member of this family with capability of binding to fibronectin and digesting collagen. Human MMP-9 gene is located on chromosome 20q and contains 13 exons which is a polymorphism C/T in the promoter region and leading to changes in the expression of this gene (Luo et al., 2004).

TNF-α belongs to the superfamily of TNF which comprises at least 19 members. TNF-α is produced by activated mononuclear phagocytic cells, antigen-stimulated T cells, NK, mast cells, and fibroblasts. TNF receptors are TNF-RI and TNF-RII that are found in most cells. TNF receptor binding strength is weaker than the other cytokines. TNF is a member of large proteins family involved in immune response and inflammation. Cytokines bind to the cytoplasmic domain of the TNF receptors induced activation of the transcription factors particularly nuclear factor κB (NF-κB) and activation protein (AP-1) which leading to caspase activation, apoptosis, and cell death (Hong et al., 2013).

The main physiological action of TNF is recruiting the monocytes and neutrophils to the site of infection. On the other hand, TNF affects the vascular endothelial cell adhesion molecules. TNF also stimulates the endothelial cells and macrophages to secrete the enzymes and cause leukocyte chemotaxis (Hong et al., 2013).

In the current study, we identified human umbilical cord mesenchymal stem cells (hUC-MSCs) by flow cytometry assay and induced differentiation assay to investigate whether the inflammatory environment could affect MSCs proliferation, migration and cytokines secretion. Also MSCs stimulation were examined by both TNF-α (20 ng/ml) and IFN-γ (50 ng/ml) as well as cell viability by MTT assay, migration by trans well assay, and cytokines expression level by real-time PCR analysis.

2. Material and Methods

2.1 Isolation and Culture of Human MSCs

Human MSCs were obtained from 5-ml iliac crest aspirates of about 15normal donors within the age range of 20-50 years who underwent bone marrow collection for a related patient after obtaining approval of the ethics committee. The code of ethics was taken from Shiraz University of Medical Sciences. Written informed consent was collected for clinical data collection and paraclinical examination. Each sample of aspirate was diluted 1:1 with Dulbecco's modified Eagle's medium (DMEM)-low glucose (1,000 mg/l glucose) (Invitrogen, Merelbeke, Belgium) and layered over about 5 ml of ficoll (Lymphoprep; Oslo, Norway). The isolation method was based on a previously reported method with slight modifications (Ayatollahi et al., 2011).briefly. after centrifugation harvested sample at 939g for 20min, the mononuclear cell layer was removed from the interface. The cells were suspended in DMEM and centrifuged again at 338g for 15 min. Then, centrifuged cells were suspended in basal DMEM medium containing 10% fetal calf serum (Invitrogen, Merelbeke, Belgium), 1% penicillin (Invitrogen, Merelbeke, Belgium), 1% streptomycin (Invitrogen, Merelbeke, Belgium), and 2 mM glutamine (Invitrogen, Merelbeke, Belgium). The cells were seeded at a density of 80.000/cm^2 in 25 cm^2 T-flasks and maintained at 37°C in an atmosphere of 5% CO2. After 4 days, the non-adherent cells were removed and the media changed every 3 days. In order to expand the MSCs cells, the adhered monolayer was detached with trypsin-EDTA (Invitrogen, Merelbeke, Belgium) for 5 min at 37°C, after 14 days for the first passage as well as every 4-5 days for successive passages sample. During *in vitro* passaging, the cells were seeded at a density of 5-10×10^3 cells/cm^2 and expanded for several passages until they no longer reached confluence.

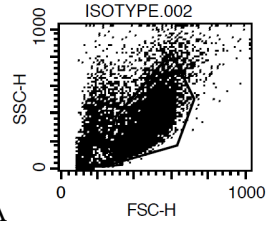
2.2 Characterization of MSCs

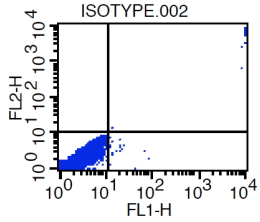
At each stage of passage, the cells were counted and analyzed for viability by trypan blue staining analysis.

Cultured MSCs have been analyzed both morphologically and surface markers examination. Functional ability of differentiation into osteocyte and adipocyte were achieved in response to specific culture conditions. Each experiment described here was replicated three times.

2.3 Flow Cytometric Analysis

A



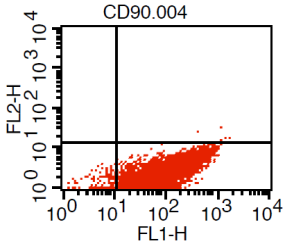


Quadrant Statistics

File: ISOTYPE.002 Log Data Units: Linear Values
Sample ID: ISOTYPE Patient ID:
Gate: G6 Gated Events: 7147
Total Events: 10000 X Parameter: FL1-H (Log)
Y Parameter: FL2-H (Log) Quad Location: 11, 11

Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	14	0.20	0.14
LL	7110	99.48	71.10
LR	23	0.32	0.23

B

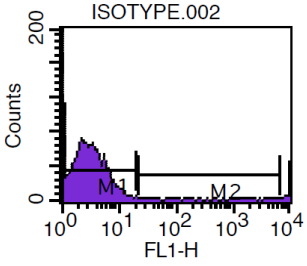


Quadrant Statistics

File: CD90.004 Log Data Units: Linear Values
Sample ID: CD90 Patient ID:
Gate: G1 Gated Events: 7073
Total Events: 10000 X Parameter: FL1-H (Log)
Y Parameter: FL2-H (Log) Quad Location: 11, 14

Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	7	0.10	0.07
LL	158	2.23	1.58
LR	6908	97.67	69.08

C

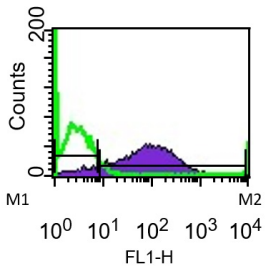


Histogram Statistics

File: ISOTYPE.002 Log Data Units: Linear Values
Sample ID: ISOTYPE Patient ID:
Gate: No Gate Gated Events: 10000
Total Events: 10000 X Parameter: FL1-H (Log)

Marker	Events	% Gated	% Total	Mean	Peak Ch
All	10000	100.00	100.00	163.74	1
M1	9740	97.40	97.40	3.30	1
M2	100	1.00	1.00	1919.57	21

D



Histogram Statistics

File: CD90.004 Log Data Units: Linear Values Sample ID: CD90 Patient ID:

Gate: No Gate Gated Events: 10000

Total Events: 10000 X Parameter: FL1-H (Log)

Marker	Events	% Gated	% Total	Mean	Peak Ch
All	10000	100.00	100.00	133.58	73
M1	670	6.70	6.70	4.39	1
M2	9255	92.55	92.55	142.88	73

Figures 1. Use Two types antibody on hMSC, graph A and histogram C ellustrate no antigen receptores for isotype 002 on hMSC but graph B and histogram D ellustrate exist antigen receptores for CD90 on hMSC

The identification of adherent cells was performed by flow cytometric analysis. At the third passage, the cells were detached from the culture flasks with trypsin-EDTA and counted. About 1×10^6 of cells were incubated on ice for 30 min with goat serum, re-suspended in phosphate-buffered saline (PBS), and pelleted by centrifugation for 4 min at 2100 rpm. Subsequently, the cells were stained for 30 min at 4°C with a fluorescent isothiocyanate (FITC)-coupled or phycoerythrin (PE)-conjugated isotype 002 (negative control), HLADR, CD45, CD90, CD80, and CD40. The labeled cells were thoroughly washed with PBS and analyzed on a flow cytometer system (FACS Calibur Becton, Dickinson, USA), by WinMidi software (Scripps Research Institute; San Diego, USA). The percentage of positive cells was calculated by the cells stained with Ig FITC/PE as a negative control.

2.4 Experimental Groups

The MSCs of the six passages were trypsinized with 0.25% trypsin-EDTA and the cell suspension was centrifuged at 1200×g for 5 min. The cells were then treated with TNF-α at different concentrations and incubated at different times. Differences in the expression levels of MMP9 were compared between TNF-α-treated and untreated MSCs. Also, differences in the expression levels of MMP9 were compared in different concentrations and times of the treatment by TNF-α. The cells were divided into the following groups: 1) without TNF- α treatment; 2) stimulated for 10hr with 1ng/ml TNF-α; 3) stimulated for 24hr with 1 ng/ml TNF-α; 4) stimulated for 10hr with 10 ng/ml TNF-α; and 5) stimulated for 24hr with 10 ng/ml TNF-α.

2.5 RNA Extraction

Total RNA was extracted from TNF-α treated and untreated MSCs groups by RNX Plus solution kit (Cinna Gene- Iran) with following protocol: 1000 µl cultured MSCs were centrifuged at 12000 rpm for 5 min. The liquid phase was removed and mixed with 100 µl phosphate buffered saline (PBS). Then, 400µl RNX Plus solution and 200µl cold chloroform were added and centrifuged at 13500 rpm for 20 min in 4°C condition which kept overnight at -20°C. Finally, the pellet was washed twice with ethanol 75% and dissolved in the 25µl DEPC water.

2.6 cDNA Synthesis

cDNA was synthesized by in-house optimized protocol in total volume of 23µl. first of all, 1µl (0.2 µg/µl) of Random hexamer (Cinna Gene-Iran), 2µl (200 U/µl) of M-MuLv reverse transcriptase (ViVantis-Indonesia), 2µl (10x) of reverse transcriptase buffer, 2µl (10 mMol) of dNTP (Cinna Gene-Iran), 1.3µl (40U/µl) RNase inhibitor (ViVantis-Indonesia), and 14.7µl DEPC water. Finally, cDNA was synthesized in 42°C for 90 minutes followed by 85°C for 5 minutes.

2.7 Real-Time Polymerase Chain Reaction

The relative quantification of MMP9 in comparison with β-actin mRNA expression was performed after the designation of the specific primers for both MMP9 and β-actin gene transcripts. The MMP9 and β-actin primer sequences were as follows respectively:

Forward primer: 5'-GGACAAGCTCTTCGGCTTCT-3`; Reverse primer: 5`-

TCGCTGGTACAGGTCGAGTA-3`and Forward primer: 5`-

GGGCGGCACCACCATGATCC-3`; Reverse primer: 5`-

GACGATGGAGGGGCCCGACT-3`.

چرا در اینجا به housekeeping gene اشاره نشده است؟

Table 1. Real-time PCR reactions for MMP9 and β-actin genes

Component	Amount (µl)	Concentration
SYBR premix Ex Taq II	10	1x
Dye	0.4	1x
Forward primer	0.4	5 pM
Reverse primer	0.4	5 pM
DEPC water	6.8	-
cDNA	2	100 ng
Total	20	-

Table 2. Real-time PCR programs for MMP9 and β-actin genes

Gene	Step	Temperature (ºC)	Time	Cycle
	Denaturation	95	2 min	1
	Denaturation	95	30 s	
MMP9	Annealing	57.5	20 s	40
	Extension	72	30 s	
	Final extension	72	5 min	1
	Denaturation	95	2 min	1
	Denaturation	95	30 s	
β-actin	Annealing	64	20 s	40
	Extension	72	30 s	
	Final extension	72	5 min	1

Upon making the PCR reaction mixes, the expression analysis was performed by the real-time PCR thermocycler (Step one plus Applied Biosystems-U.S.A) (Table 1 and 2). The fold change of relative mRNA expression was determined by using Livak method ($2^{-\Delta\Delta Ct}$).

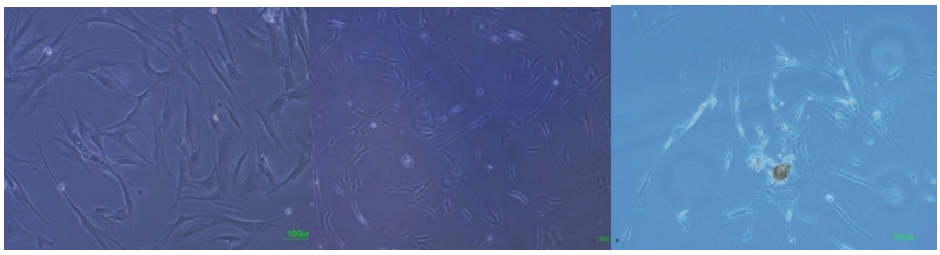
3. Results

3.1 Isolation and Expansion of Human MSCs

Adherent cells were observed in all samples after 3 days of culture and 15 days later, adherent monolayer was achieved (Figure 2A). The rapid expansion of the MSCs in the culture might be related to the presence of single-cell-derived colonies composed of a few fibroblast-like cells (Figure 2B). Bone marrow cells rapidly generated a confluent layer of cells with an elongated, fibroblastic shape. These cells contained two types: a type of cells with large and flat morphology, and a type of smaller spindle-shaped cells (Figure 2C). The cells were increased in size and showed a polygonal morphology with evident filaments in the cytoplasm, especially when early passage cells were compared with late passage cells. MSCs isolated from healthy donors were expanded for up to 10 passages.

3.2 Viability Evaluation

At each passage, the cells were counted and analyzed for viability by trypan blue staining analysis, showing a viability of 98- 100% in the samples.



A B C

Figure 2. Isolation and culture of human bone marrow derived MSCs

A) Adherent monolayer was achieved in the following 6-7 days;

B) The presence of single cell-derived colonies composed of a few fibroblast-like cells;

C) As the culture proceeded, the cells were both of small spindle, and wide-shaped morphology. Scale bar for the Figures A-C is 100 um.

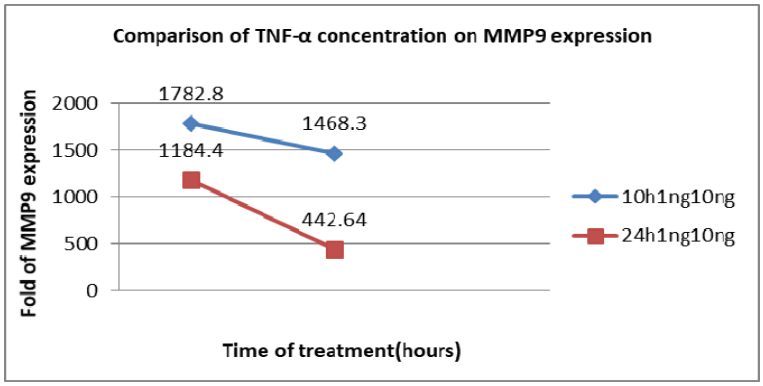
3.3 MMP9 Expression

The expression level of MMP9 gene in the untreated MSCs was lower than treatment groups (Table 3). The expression level of MMP9 gene was compared for various treatment conditions with different times and concentrations that was highly increased in the two treatments including 1ng/ml ($2^{-\Delta\Delta Ct}$=1468.3) and 10 ng/ml ($2^{-\Delta\Delta Ct}$=1782.8) TNF-α for 10 h (Table 3 and Figure 2). However, the highest level of MMP9 gene expression was found upon treatment with 10 ng/ml TNF-α for 10 h ($2^{-\Delta\Delta Ct}$ =1782.8) (Table 3 and Figure 3).

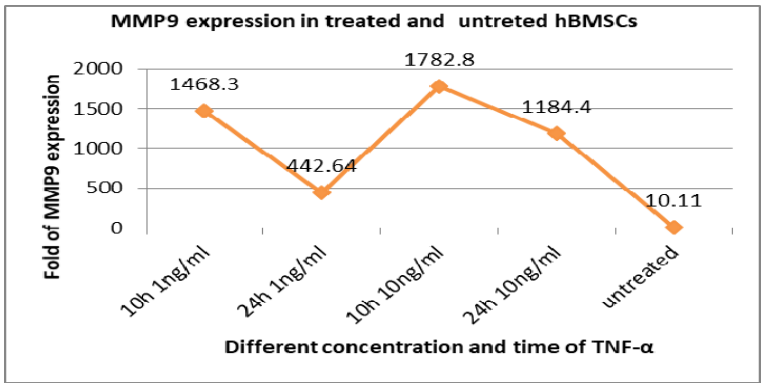
Table 3. Gene expression level of MMP9 and β-actin in MSCs after treatment with TNF-α

Conditions of treatment with TNF-α	Ct MMP9	Ct β-actin	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
1 ng/ml–10 hours	17.28	17.69	- 0.41	-52.10	1468.3
1 ng/ml–24 hours	14.89	13.57	1.32	-8.79	442.64
10 ng/ml–10 hours	19.78	20.47	- 0.69	- 10.8	1782.8
10 ng/ml–24 hours	18.14	18.97	- 0.83	- 10.21	1184.4
Negative Control (Untreated)	32.46	22.35	10.11	-	-

A) Comparison of TNF-α concentration on MMP9 expression



B) MMP9 expression in treated and untreated hBMSCs



C) Histogram chart effect of concentration TNF-α on expression mmp9 in hBMSCs

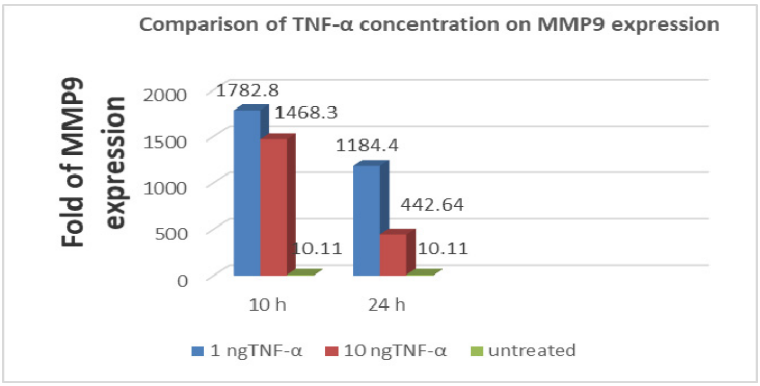


Figure 3. Comparison of TNF-α concentration on MMP9 gene expression in the treated and untreated MSCs

4. Discussion and Conclusion

Mesenchymal stem cells are nonhematopoietic stem cells with multi-proliferative and multi-differentiative potentials. The ability of MSCs to modulate the immune responses and migration to the site of inflammation makes these cells a promising source of cells for cell-based therapeutic strategies (Tsai et al., 2011). Chemokine receptors, ligands, and adhesion molecules play key roles in tissue-specific homing of the leukocytes and have also been implicated in trafficking of hematopoietic precursors into the tissues. The migration of MSCs to the sites of injury or inflammation is also mediated by chemotactic factors produced by the immune cells. It has been demonstrated that human MSCs show significant chemotaxis responses to several factors, including PDGF, VEGF, IGF-1, IL-8, bone morphogenetic protein (BMP)-4 and BMP-7 (Yorsangsukkamol et al., 2011; Kollet et al., 2003). The mechanism of MSCs migration and homing has not been fully understood. Although it seems that homing in MSCs occurs by tethering, rolling and endothelial transmigration similar to the leukocytes; however, they differ in the types of molecules involved in the migration process. For example, MSCs do not utilize E or P-selectin as a tethering mechanism since they do not express the fucosyltransferase IV or VII, and use other adhesion molecules such as CD44, VLA-4 and VCAM (Yorsangsukkamol et al., 2011). MSCs are also large cells, so they can hold up on capillary beds which allow them to transmigrate across the endothelium in response to chemokine gradient (Ullah et al., 2015).

In this study, we analyzed different gene expression patterns of chemokine receptor MMP9 on MSCs which have a critical role in migration and engraftment. It was shown that human bone marrow MSCs expressed low level of MMP9. Our observation was confirmed by other studies such as that of Wynn *et al.*; they reported that a small portion of MSCs expressed MMP9 which contributed to their migration *in vitro*. They showed that less than 1% of human MSCs express CXCR4 (Wynn et al., 2004). Ahmadian Kia *et al.* observed low or no detectable mRNA expression of MMP9 in the bone marrow MSCs, whereas Sordi *et al.* demonstrated that 26% of human bone marrow MSCs express CXCR4 (Ahmadian Kia et al., 2011; Sordi et al., 2005). There are controversial reports about expressing chemokine receptors on MSCs (Macfarlan et al., 2012; Nakagawa & Yamanaka, 2010; Meriane et al., 2006).

Considering the important role of the couple of ligand-receptor SDF-1/ MMP9 in cell migration and cell engraftment, overexpression of chemokine receptor MMP9 on MSCs increases the ability of migration and engraftment for clinical application of these cells and this can be one of the strategies for enhancing the potential of MSCs in cell-based therapies. On the other hand, there are various inflammatory cytokines and chemokines at the injured sites that can lead to movement of MSCs to the injured and inflamed tissues (Meriane et al., 2006). These cytokines can regulate the expression of genes in various cells depending on the cell type and differentiation stage (Nombela-Arrieta et al., 2011).

TNF-α is a pro-inflammatory cytokine that has been shown to affect the gene expression in cells and can influence the expression of molecules involved in the migration process (Meriane et al., 2006; Tsai et al., 2011). Recently, it has been demonstrated that MMP9 expression increase in gastric cancer cells is induced by TNF-α (Zhao et al., 2010). Croitoru-Lamoury *et al.* demonstrated that IFN-γ and IFN-1β up-regulated the chemokines and chemokine receptors in human MSCs (Croitoru-Lamoury et al., 2007). Esteve *et al.* observed that MMP9 expression in treated human astroglioma cells was enhanced by TNF-α and IL-1β (Esteve et al., 2002). However, it has also been reported that TNF-α reduces chemokine receptor expression in some types of cells. Tikhonov *et al.* have found downregulation of MMP2 expression by TNF-α in polymorphonuclear leukocytes (Tikhonov et al., 2001).

Our data suggest that TNF-α can up-regulate the MMP9 expression in human bone marrow MSCs in a time and concentration-dependent manner. We observed that the highest level of MMP9 gene expression was treated with 10 ng/ml TNF-α for 10 hours. We revealed that the optimum time of TNF-α- treatment for enhancing MMP9 expression was 10 hours.

Previously, Kulbe *et al.* treated the ovarian cancer cells with 1, 10 and 100 ng/ml TNF-α in different incubation times (Kulbe et al., 2007). They have indicated that the most rate of MMP9 expression level occurred in 10 ng/ml TNF-α between 6 to 24 hours. They also have demonstrated that there is a relationship between TNF-α/NF-κB and MMP9 expression (Cheng et al., 2008). Effect of TNF-α on MMP-9 expression in HT1376 cells, the experiment showed the expression of mmp9 in 100 ng/ml and time 24h of TNF-α (Se-Jung Lee et al., 2007).

TNF-α- induced production of matrix metalloproteinase-9 by human bronchial epithelial cells. After stimulation with TNF-a (10 ng/ml), the level of matrix metalloproteinase-9 mRNA was increased in a time-dependent manner and the expression peaked at 24h (Hozumi et al., 2001). To determine the effect of TNF-α on MMP-9

expression, MC3T3-E1 cells were incubated with various concentrations of TNF-α for the indicated time intervals. The experiment also showed expression of mmp9 in 30 ng/ml and time 48h of TNF-α(Chia-Lan Tsai et al., 2014).

The results of current study suggested that *in vitro* control of environmental factors both in the concentration and time level may be important in the stem cell migration capacity and perhaps it is crucial in stem cell transplantation therapies.

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Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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