Research Article

Evaluating the Effect of Eugenol on the Expression of Genes Involved in the Immunomodulatoty Potency of Mouse Mesenchymal Stem Cells In Vitro

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Abstract

The immunomodulatory ability of mesenchymal stem cells (MSCs) has attracted interest as a unique property that makes them interesting tools for the treatment of inflammatory and autoimmune diseases. Eugenol is a volatile compound from the phenylpropanoids class of chemical compounds. Despite extensive investigations on the biological and pharmacological properties of Eugenol, its effect on stem cells, especially, on MSCs remains to be clarified. Therefore, this study was designed to evaluate the effect of Eugenol on the expression of genes (*Tlr3*, *Tlr4*, *Ccl2,* and *Ccl3*) involved in immunomodulatory potency of mouse bone-marrow derived MSCs by quantitative real-time PCR (qRT-PCR). To do so, MSCs were isolated from 4-8 weeks old mouse bone marrow (BM). The effect of Eugenol on the viability of BM-MSCs was evaluated by MTT assay at 24, 48, and 72h after treatment. The results showed that Eugenol reduced the number of BM-MSCs in a dose- and time-dependent manner. In addition, the half maximum inhibitory concentration of Eugenol on MSCs was 400µg/ml at 24 and 48h and 200µg/ml at 72h after treatment. Moreover, about 90% of MSCs were alive at the concentration of 12.5µg/ml 24h after treatment. The qRT-PCR results indicated that *Tlr3*, *Tlr4*, and *Ccl3* genes were up-regulated 1.6-, 1.8-, and 2.2-fold, respectively, in Eugenol-treated BM-MSCs compared to untreated controls (Fold change > 1.5; P ≤0.05). In conclusion, we suggest that Eugenol may somewhat regulate the immunomodulatory potency of MSCs and thus this study provides a background for further studies on the effect of Eugenol on MSCs characteristics and functions, which may finally improve their potency for cell-based therapy applications.

Keywords: Eugenol, Mesenchymal stem cells, Immunomodulatory potency, Mouse

Introduction

Mesenchymal stem cells, or MSCs, are multipotent stromal cells, which have received considerable attention in clinical cell-based therapies during the past decade. MSCs are easily isolated from various fetal and adult tissues (Hass et al., 2011; Huang et al., 2014; Savickiene et al., 2015; Wang et al., 2015). They have also remarkable self-renewal, migratory and immunomodulatory potentials and can differentiate into a variety of cell types (Zhao et al., 2016; Sisakhtnezhad et al., 2017). In addition to producing and secreting various factors, increasing evidence has also suggested that the main therapeutic potential of MSCs results from their immunomodulatory activity. Interestingly, MSCs show flexible immunomodulatory functions and they have two immunomodulatory phenotypes, pre-inflammatory (MSC1) or anti-inflammatory (MSC2) phenotypes, which have the immunoactivatory and immunosuppressive ability, respectively. MSCs can also switch from immunosuppressive to immunoactivatory phenotype (Zhao et al., 2016). Recent studies have been indicated that the immunomodulatory potency of MSCs is controlled by inflammatory factors in vivo (Ma et al., 2014). It has been shown that inflammatory environment induces or suppresses the expression of a large number of chemokines, cytokines, adhesion molecules and their correspondence receptors such as CXCRs ligands, CCRs ligands, intercellular adhesion molecule -1 (ICAM-1) and vascular cell adhesion molecule -1 (VCAM-1) (Ren et al., 2008; Ren et al., 2010). Renner et al., reported that low levels of inflammatory factors induce immunosuppressive properties of MSCs, while high levels of inflammation promote the immunoactivatory potential of MSCs (Renner et al., 2009). This conceptual change has crucial implications for the clinical applications of MSCs. Therefore, there is attention to new strategies to induce pre-inflammatory (MSC1) or anti-inflammatory phenotypes (MSC2) of MSCs to affect the immune system for treating human diseases, such as cancer

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and autoimmune diseases. It also suggests that the immunomodulatory potency of MSCs may purposefully alter by adding the influential external factors in the cell culture medium.

Today, natural products, especially herbal active ingredients, as an alternative to chemical drugs have received considerable attention to prevent and treat a range of human diseases. Eugenol is a volatile compound in the essential oils of some plants. Some in vitro and in vivo studies have demonstrated the widespread pharmacological effects of Eugenol. In this regard, it was reported that Eugenol has a wide range of anti-microbial, anti-oxidant, anti-inflammatory, immunomodulatory, anti-nociceptive, anti-tumourigenic, anti-genotoxic and neuroprotective effects (Ito et al., 2005; Raghavenra et al., 2006; Singh et al., 2007; Bachiega et al., 2012; Kamatou et al., 2012). Despite the well-known biological and pharmacological capabilities of Eugenol, its effect on the characteristics and function of stem cells, especially MSCs, remains to be studied and clarified. Given the importance of immunomodulatory potency of MSCs in cell-based therapy applications and extensive biological and pharmaceutical effects of Eugenol, this study, for the first time, was aimed to evaluate the effect of Eugenol on the expression of genes involved in the immunomodulatory potency of the mouse BM-derived MSCs in vitro.

Materials and Methods

Isolation and culture of mouse BM-MSCs

For the derivation of mouse MSCs, 4-8 weeks old NMRI mice were obtained from the Laboratory of Cell & Developmental Biology at Razi University (Kermanshah, Iran). Isolation of MSCs from mouse bone marrow (BM) was carried out under the approval (permit number 395-1-111) and the bioethics guidelines of the Deputy Research of Razi University. In the current study, cervical dislocation method was used to kill mice. Consequently, BM was isolated and collected from the tibias and femurs of mice. The BM from one animal were cultured in a T25 flask in the presence of Dulbecco's modified Eagle's medium (DMEM) (Gibco, Scotland) containing 15% (v/v) fetal bovine serum (FBS) (Gibco, Scotland), 2mM L-glutamine, and 1x penicillin-streptomycin (Pen-Strep) (Bioidea, Iran) at 37ºC and 5% CO_2. After 72h, the cell culture medium was replaced to remove unattached cells. The cultured cells of BM were reached to confluency after 7-10 days. The confluent primary culture of BM cells was then trypsinised by trypsin-EDTA (0.25%-1mM) (Bioidea, Iran) and the sub-cultured cells were incubated under previous conditions. Finally, the obtained cells in passage 3 were used for following studies.

Characterization of BM-MSCs

Phenotypic characterisation of MSCs by Flow-cytometry

The BM-MSCs were detached by 0.25% trypsin-1mM EDTA. After centrifugation, result pellet was solved in phosphate-buffered saline (PBS) and then 1×10^6 cells were used for flow-cytometry analysis. The phenotypic characterization of cells was carried out on a FACS Caliber for the presence of mesenchymal stem cell markers CD44, CD90, CD105 and hematopoietic marker CD45 and CD34.

Differentiation of MSCs to adipocytes in vitro

MSCs at third passage were cultured in an adipogenic medium composed of DMEM supplemented with 10% FBS, vitamin C (50 µg/ml) (Sigma, Germany), dexamethasone (1×10^{-3} µmol/ml) (Osvah, Iran), indomethacin (50 µg/ml) (Sigma, Germany) and 0.1 x Pen-Strep. After 21 days, the differentiation of MSCs into adipocytes was confirmed by Oil-red O (Acros, USA) staining.

Differentiation of MSCs to osteoblasts in vitro

To induce differentiation of BM-MSCs to osteoblasts, cells were cultured in an osteogenic medium containing DMEM supplemented with 10% FBS, vitamin C (50 µg/ml), dexamethasone (1×10^{-3} µmol/ml), β-sodium glycerolphosphate (10 mM) (Sigma, Germany) and 0.1 x Pen-Strep for 21 days. Consequently, the differentiation of MSCs into osteoblasts was investigated by Alizarin-red S (Acros, USA) staining.

The effect of Eugenol on BM-MSCs viability

BM-MSCs were cultured in 96-well plates at 8×10^3 cells per well and incubated at 37ºC and 5% CO_2 overnight. Consequently, Eugenol at concentrations of 12.5, 25, 50, 100, 200, 400, 800, 1600, 3200 µg/ml was added to each well. To evaluate the cytotoxicity effect of Eugenol, the tetrazolium-based colourimetric assay was used at 24, 48, and 72h after treatments. To do so, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, Germany) solution at the concentration of 5 mg/ml was added to each well and incubated at 37ºC and 5% CO_2 for 4h. The MTT salt was cleaved by mitochondrial enzymes of metabolically active cells and produced purple formazan crystals. The resulting crystals were

solubilised with 150 µl of dimethyl sulfoxide (DMSO) and the absorption of the solution was then measured at 570 nm in an ELISA reader. MTT assay was carried out at least in triplicate and the percentages of living cells against the controls were calculated by the following equation for each treatment:

Cell viability (%) = The mean absorbance of Eugenol-treated cells in each well / The mean absorbance of control cells × 100

Morphological evaluation

BM-MSCs in passage 3 were cultured in 6-well plates at 0.5×10^6 cells per well and incubated at 37ºC and 5% CO_2 overnight. Cells were then treated with different concentrations of Eugenol and morphological changes were evaluated under an inverted microscope 24h after treatments.

Gene expression analysis

The effect of Eugenol on the immunomodulatory potential of MSCs was investigated by evaluating the expression of *Tlr3*, *Tlr4*, *Ccl2,* and *Ccl3* genes using real-time PCR. To evaluate the effect of Eugenol on the expression of the genes in BM-MSCs, first, the half maximal inhibitory concentration (IC_{50}) of Eugenol was determined 24h after treatment. BM-MSCs were then treated with Eugenol at IC_{50} for 24h. Consequently, total RNA was extracted from the Eugenol-treated and control BM-MSCs using RNX-Plus reagent (SinaClon, Iran). The RNA samples were then treated with RNase-free DNase I (Fermentas, Germany) according to the manufactures' instruction. Finally, 1 µg of the DNase-treated total RNA was converted into cDNA using reverse transcription (RT)-enzyme (Fermentas, Germany) at 42ºC for 60 min. In this study, Allele ID (version 6) software was used to design primers (Table 1) for the gene expression analysis by quantitative RT-PCR. To assess and compare the expression of genes in the Eugenol-treated BM-MSCs relative to untreated control cells, quantitative gene expression analysis was performed in duplicates using specific primers and SYBR Green PCR master kit (Takara, Japan) in a real-time PCR instrument. The real-time PCR reactions were carried out in a 10 µl reaction volume using the two-step time and temperature program, including 10 sec at 95ºC and 30 sec at 60ºC for 40 cycles. The beta-actin (*β-actin*) gene was considered as housekeeping gene and the expression of interesting genes (*Tlr3*, *Tlr4*, *Ccl2,* and *Ccl3*) were normalised to the *β-actin* expression. Quantitative real-time PCR (qRT-PCR) reactions were performed in duplicates and each experiment was repeated three times. The standard deviations were calculated for the expression of each gene. The comparative C_t method was used to calculate the relative quantification of the gene expression in the Eugenol-treated BM-MSCs relative to untreated control cells (Livak & Schmittgen 2001). Finally, the fold-change more than or equal to 1.5 was considered as significant to identify differentially expressed genes in the Eugenol-treated BM-MSCs in comparison to untreated control cells.

Table 1: Nucleotide sequences of the primers used for the gene expression analysis by qRT-PCR.

Gene	Accession number	Primer	Sequence
Tlr3	NM_126166	Forward	5′-GCCACCAGCGAGAGCACTTTC-3′
		Reverse	5′-GAGAAGGAACCGTTGCCGACATC-3′
Tlr4	NM_021297	Forward	5′-TGGGAGGGAAGAGGCAGGTG-3′
		Reverse	5′-TGGTGTCAGGCAGGAGAAGAAC-3′
Ccl2	NM_011333	Forward	5′-AGAGAGCCAGACGGGAGGAAG-3′
		Reverse	5′-TGAATGAGTAGCAGCAGGTGAGTG-3′
Ccl3	NM_011337	Forward	5′-ACACCAGAAGGATACAAGCAGCAG-3′
		Reverse	5′-GTAGGAGAAGCAGCAGGCAGTC-3′
Β-actin	NM_007393	Forward	5′-GGCTGTATTCCCCTCCATCG-3′
		Reverse	5′-CCAGTTGGTAACAATGCCATGT-3′

Statistical analysis

Data analysis was carried out using the SPSS software package (version 21.0) for Windows through a Student's *t*-test. Data were also expressed as mean value ± standard deviation and differences were also considered significant when the P-value was 0.05 or less.

Results

Culture of BM-MSCs

The mouse bone marrow tissue was cultured and cells from the BM tissue were started to attach on the surface of the flask. After 72h, the cell culture medium was replaced and, thereby unattached cells

were removed from the primary culture. The primary culture was reached to confluency after 7-10 days and, therefore, it trypsinised and sub-cultured to new flasks. Finally, the purified BM-MSCs in passage 3 were used for following studies. The mouse bone marrow-derived primary cell culture and the purified BM-MSCs are shown in Figure 1A & B, respectively.

Flow-cytometry analysis

Phenotypic characterization of cells was achieved using flow-cytometry analysis. Results indicated that although cells derived from mouse BM were positive for the MSCs markers CD44, CD90, CD105, they were also negative for hematopoietic markers CD45 and CD34 (Figure 2).

Adipogenesis potential of BM-MSCs

Results of differentiation assay showed that the adipogenic medium induced the conversion of BM-MSCs into adipocytes. Twenty-one days after the culture of BM-MSCs with the osteogenic medium, the Oil-red O staining and microscopic observations of cells demonstrated distinguishable primary lipid droplets in cells (Figure 3A). Results also indicated that lipid droplets were not detectable in untreated BM-MSCs (Figure 3B).

Osteogenesis potential of BM-MSCs

Twenty-one days after treatment of BM-MSCs with the osteogenic medium, Alizarin-red S staining was used to evaluate osteogenesis in the cells. The differentiated cells displayed a red colour

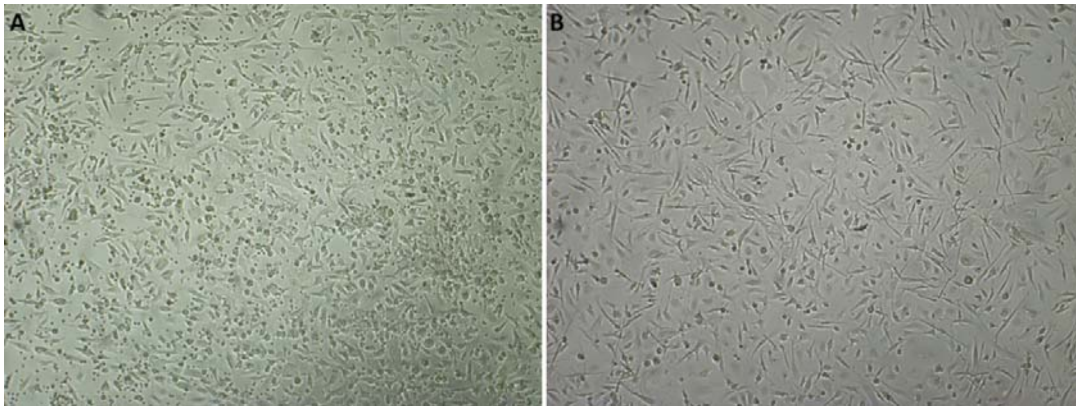

Figure 1. Mouse bone marrow-derived cell cultures. The primary cell culture derived from BM tissue (A) and the BM-derived MSCs in passage 3 (B).

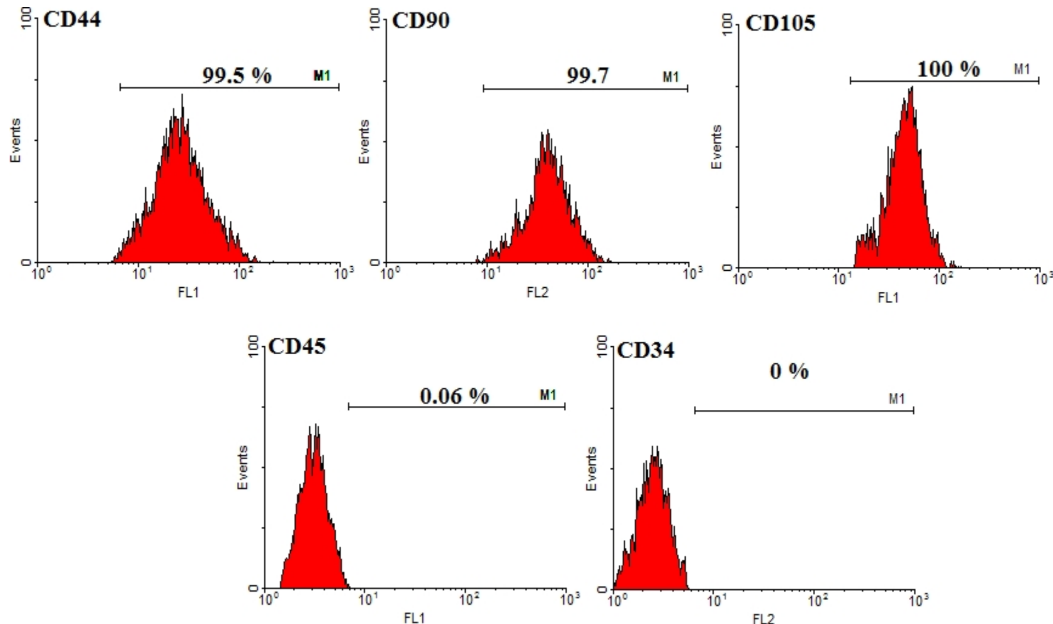

Figure 2. Phenotypic characterization of BM-MSCs by Flow-cytometry. Results showed that the mouse BM-MSCs were positive for CD44, CD90 and CD105, but they were negative for hematopoietic cell markers CD45 and CD34.

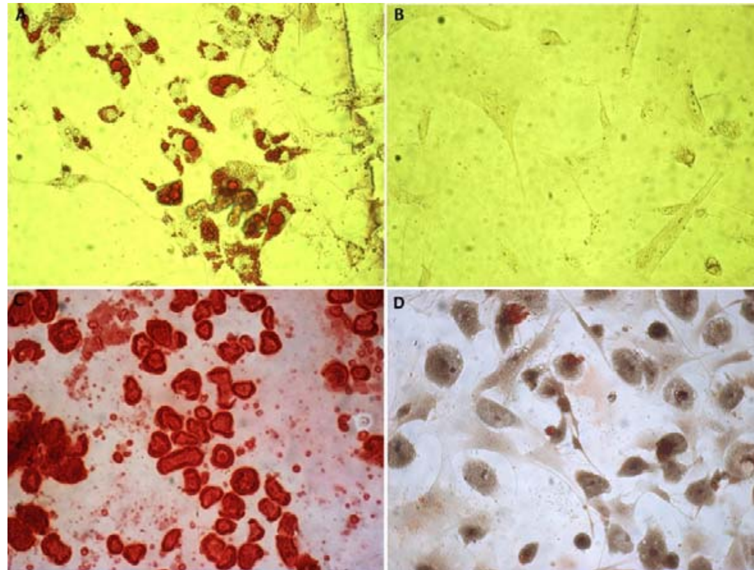

Figure 3. Differentiation potency of BM-MSCs. Oil-red O staining demonstrated that lipid-rich vacuoles formed in the BM-MSCs after 3 weeks of adipogenic induction (A); untreated BM-MSCs were considered as negative control (B). Alizarin-red S staining showed that mineralised nodules formed in the BM-MSCs after 3 weeks under the osteogenic induction. After 3 weeks of osteogenic induction, alizarin-red S staining indicated the osteocyte formation (C), while untreated BM-MSCs were negative for osteogenesis (D).

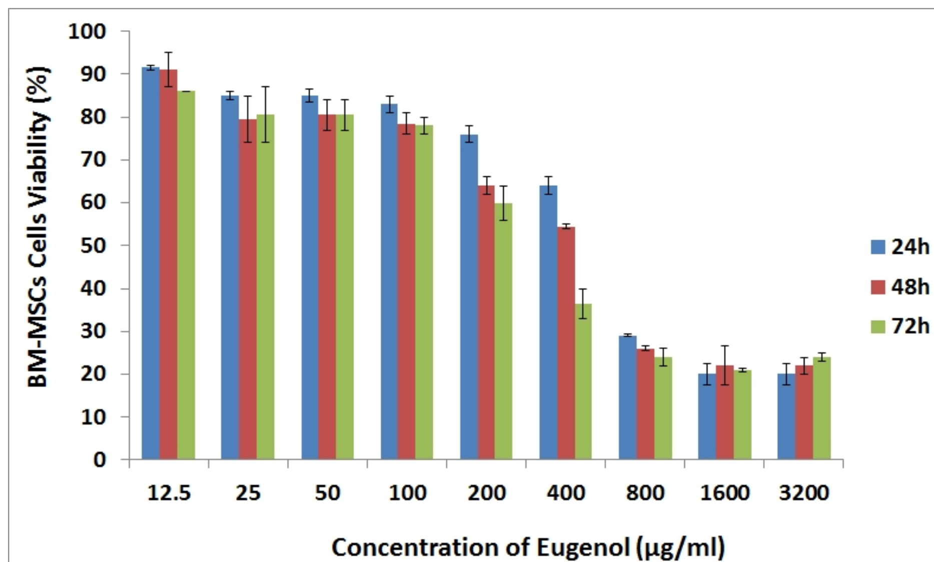

Figure 4. The cytotoxic effect of Eugenol was evaluated on BM-MSCs by MTT assay at 24, 48, and 72h after treatment.

after staining (Figure 3C), while this phenomenon was not observed in untreated BM-MSCs (Figure 3D).

Cytotoxic effect of Eugenol on MSCs

The cytotoxic effect of Eugenol was evaluated on BM-MSCs by MTT assay at 24, 48, and 72h after treatment. Results demonstrated that Eugenol reduced the viability of BM-MSCs in a dose- and time-dependent manner and the IC_{50} values for Eugenol were 400 µg/ml after 24 and 48h, and 200 µg/ml after 72h. Moreover, the results of the MTT assay showed about 90% cell viability at the concentration of 12.5 µg/ml 24h after treatment (Figure 4).

Morphological changes

Microscopic observations demonstrated that Eugenol affects the shape of the BM-MSCs in a dose-dependent manner and prominent morphological alterations were common 24h after treatments. The morphological changes of BM-

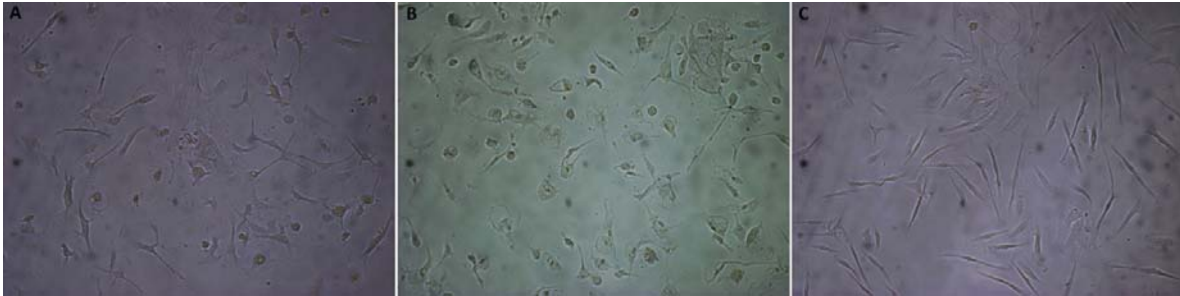

Figure 5. The effect of Eugenol on the morphology of BM-MSCs. Microscopic observations demonstrated the morphological alterations in the mouse BM-MSCs treated with 100 (A) and 300 μg/ml (B) of Eugenol, while untreated BM-MSCs were not showing the morphological changes (C).

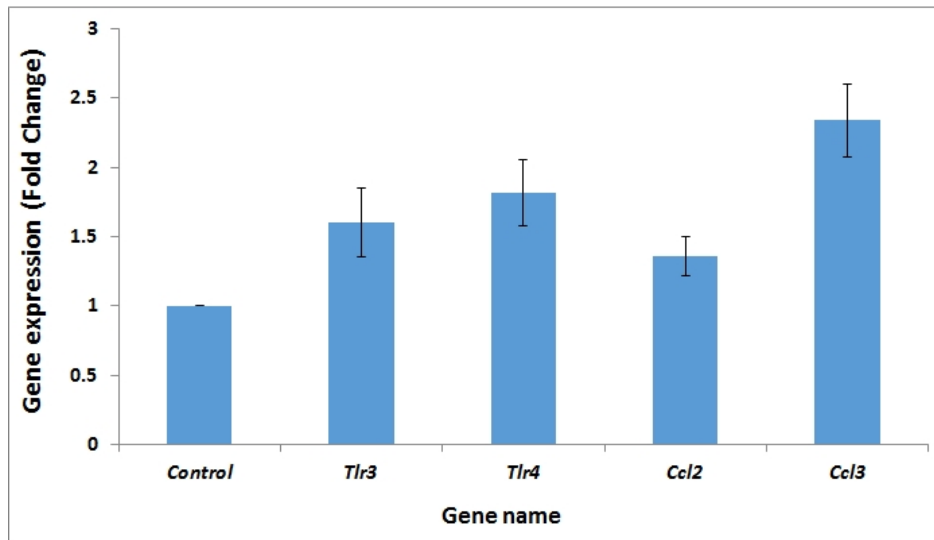

Figure 6. qRT-PCR for *Tlr3*, *Tlr4*, *Ccl2,* and *Ccl3*. The gene expression analysis showed that *Tlr3*, *Tlr4*, *Ccl2,* and *Ccl3* genes were over-expressed in the Eugenol-treated BM-MSCs in comparison to untreated control cells. However, results indicated that *Tlr3*, *Tlr4*, and *Ccl3* have 1.6, 1.8 and 2.2-fold change of expression, respectively, in Eugenol-treated mouse BM-MSCs relative to untreated cells.

MSCs treated with 100 and 300 μg/ml of Eugenol are shown in Figure 5A & B. To note, the morphological alterations were not detectable in untreated BM-MSCs (Figure 5C).

Gene expression analysis of the Eugenol-treated BM-MSCs

The effect of Eugenol on the expression of genes involved in the immunomodulatory potency of BM-MSCs, including *Tlr3*, *Tlr4*, *Ccl2,* and *Ccl3*, was evaluated at transcript level by qRT-PCR method. The results of the gene expression analysis showed that *Tlr3*, *Tlr4*, *Ccl2,* and *Ccl3* genes were up-regulated 1.6-, 1.8-, 1.3-, 2.2-fold, respectively, in the Eugenol-treated MSCs compared to untreated control cells (Figure 6). These findings indicated that *Tlr3*, *Tlr4*, and *Ccl3* have the fold change of expression more than 1.5 in Eugenol-treated BM-MSCs relative to untreated cells. Moreover, *Ccl3* and *Ccl2* had the highest and the lowest levels of expression in the Eugenol-treated cells.

Discussion

Immunomodulatory potency is a unique property of MSCs and, therefore, it makes them interesting tools for the treatment of inflammatory and autoimmune diseases. Scientists have tried to find the influential factors that influence the immunomodulatory properties of MSCs in vitro. Eugenol is a volatile compound from the phenylpropanoids class of chemical compounds, which has widespread biological and pharmacological properties. Despite extensive investigations on the biological and pharmacological effects of Eugenol (Ito et al., 2005; Raghavenra et al., 2006; Singh et al., 2007;

Bachiega et al., 2012; Kamatou et al., 2012), its effect on MSCs remains to be clarified. Therefore, in the current study, we investigated the effect of Eugenol on the expression of genes, including *Tlr3*, *Tlr4*, *Ccl2,* and *Ccl3*, involved in the MSCs immunomodulatory potency. The results of qRT-PCR showed that Eugenol influence the expression of *Tlr3*, *Tlr4*, and *Ccl3* genes in BM-MSCs. Interestingly, MSCs can be polarised into two anti-inflammatory (MSC1) or a pro-inflammatory (MSC2) phenotype through activation of different Toll-like receptors (TLRs) (Waterman et al., 2010). It has been found that MSCs express different types of TLRs, including TLRs-1, 2, 3, 4, 5, 6, 7, 8 and 9 (Najar et al., 2017). The role of TLR3 and TLR4 in regulating the function of MSCs has been shown in different studies. Waterman et al. demonstrated that MSCs polarisation process toward MSC1 or MSC2 phenotype may occur depending on the type of TLR activated. They found that MSC1 and MSC2 phenotype express TLR4 and TLR3, respectively (Waterman et al., 2010). Interestingly, TLRs signalling pathways induced MSC migration and immunomodulatory factor secretion (Tomchuck et al., 2008; Hwang et al., 2014). For example, activation of TLR3 amplifies MSCs multifunctional trophic factors and enhances therapeutic potency (Mastri et al., 2012). Activation of TLR3 enhances the therapeutic potential of MSCs by stimulating the expression and secretion of interleukin-6 (IL-6), IL-8 IFNγ, STO-1, hepatic growth factor (HGF), and vascular endothelial growth factor (VEGF) (Tomchuck et al., 2008; Mastri et al., 2012; O'Neill et al., 2013). Activation of TLR4 increases the production and secretion of IL-6 and IL-8 by of MSCs, as well (Tomchuck et al., 2008). The therapeutic advantages of TLR3 and TLR4 activation are not only limited to secretion of trophic factors but also to its immunoregulatory response (Delarosa et al., 2012). A recent study revealed that stimulation of TLR3 signalling by RNA mimetic polyinosinic-polycytidylic acid [Poly(I:C)], enhanced the immunosuppressive ability of human umbilical cord-derived MSCs (UC-MSCs) through microRNA-143 inhibition (Zhao et al., 2014). Moreover, Zhang et al., demonstrated that the activation of TLR3 and TLR4 in human UC-MSCs stimulates the expression of inflammatory markers (Zhang et al., 2015). A study by Rashedi et al., also showed that activation of TLR3 or TLR4 in MSCs increases Treg induction via the Notch pathway (Rashedi et al., 2017). Mechanistically, it has been found that stimulation of TLR3 and TLR4 by lipopolysaccharide and Poly-IC, respectively, resulted in enhanced phospho-IKKα/β and phospho-MAPK and thus activation of NF-kB and/or MAPK signalling in MSCs. Significantly, TLR3 induced the expression of stem cell markers and inhibited the differentiation potential of UC-MSCs into osteocytes. On the other hand, TLR4 inhibited the expression of stem cell markers and, therefore, increased the osteogenic differentiation of UC-MSCs (Zhang et al., 2015). Eugenol exhibits anti-inflammatory and immunomodulatory activities through inhibiting the activity of enzymes such as COX-1/2, 15-LOX, 5-LOX manganese prostaglandin H synthase (Mn-PHS) and NF-KB signalling pathway. It induces the production of free radicals, like tyrosyl, and lipid peroxidation, and also preventing the production of inflammatory mediators such as prostaglandin (PGE2) and leukotrienes (LTC-4) (Ito et al., 2005; Raghavenra et al., 2006; Singh et al., 2007; Bachiega et al., 2012). Generally, it has been found that the anti-oxidant and anti-inflammatory activity of Eugenol is achieved at low concentrations, while at higher concentrations it acts as a pro-oxidant, which leading to tissue damage by increasing the production of free radicals (Kabuto & Yamanushi 2011; Kamatou et al., 2012). The protective effects of Eugenol on immune cells have also been proven in a few studies. Our quantitative gene expression analysis also showed that Eugenol at 10 μg/ml somewhat enhanced the expression of *Tlr3* and *Tlr4* genes in BM-MSCs. Therefore, we propose that Eugenol can also influence on MSCs characteristics in vitro. Moreover, although Eugenol slightly over-expressed *Tlr3* or *Tlr4* in BM-MSCs, more experimental studies will be necessary to confirm the effect of Eugenol on the immunomodulatoty potency of MSCs.

In addition to TLRs, the pro-inflammatory chemokine (C-C motif) ligands (CCLs) are important regulators of the immunomodulatory properties of MSCs. Collectively, different studies revealed that MSCs exhibit changes in gene expression indicative of a pro-inflammatory phenotype, with increased expression of CCL2, CCL3, CCL4, CCL5, CCL7, CCL20, CXCL6, CXCL10, CXCL12 and IL-8 mRNA (Anton et al., 2012; Pyo et al., 2015; Lee et al., 2017). Functionally, the secretion of pro-inflammatory chemokine (C-C motif) ligands, including CCL2, CCL3 and CCL12 by MSCs stimulates the migration of immune cells such as monocytes and T cells and recruits them into inflamed tissues to induce wound repair at the site of injury (Le Blanc & Davies 2015; Lee et al., 2017). Mechanistically, it was also elucidated that CCL2 increases the

duration of the MSC–T cell contacts through enhancing the expression of VCAM-1 in T cells (Pyo et al., 2015; Lee et al., 2017). In the present study, we also found that Eugenol significantly up-regulated the expression of *Ccl3* in BM-MSCs. Therefore, we suggest that Eugenol may enhance the immunomodulatory potency of BM-MSCs through activation of *Ccl3* expression. However, the exact mechanisms and the roles of this regulation are needed to be elucidated in further study.

In this study, we investigated the effect of Eugenol on the mouse BM-MSCs through evaluation of the expression of *Tlr3*, *Tlr4*, *Ccl2,* and *Ccl3*, which are involved in the immunomodulatory potency of MSCs. Our findings showed that Eugenol somewhat affects the expression of *Tlr3*, *Tlr4*, and *Ccl3* in BM-MSCs. Therefore, we declare that Eugenol can influence on MSCs characteristics. This study also provides a background for further studies on the effect of Eugenol on MSCs characteristics and functions, especially their immunomodulatory potency, which may finally improve their potency for cell-based therapy of the human inflammatory and autoimmune diseases.

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