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# The effect of Trimetazidine and Diazoxide on immunomodulatory activity of human embryonic stem cell-derived mesenchymal stem cell secretome

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ABSTRACT

Comprehensive proteome profiling of the factors secreted by mesenchymal stem cells (MSCs), referred to as secretome, revealed that it consists of cytokines, chemokines, growth factors, extracellular matrix proteins, and components of regeneration, vascularization, and hematopoiesis pathways. Harnessing this MSC secretome for therapeutic applications requires the optimization of production of secretary molecules. A variety of preconditioning methods have been introduced, which subject cells to stimulatory molecules to create the preferred response and stimulate persistent effects. Pharmacological preconditioning uses small molecules and drugs to increase survival of MSCs after transplantation or prolong release of effective secretary factors such as cytokines that improve immune system responses. In this study, we investigated the effect of secretome of human embryonic-derived mesenchymal stem cells (hESC-MSCs) preconditioned with Trimetazidine (TMZ) and Diazoxide (DZ) on immunomodulatory efficiency of these cells in LPS-induced peripheral blood mononuclear cells (PBMCs). PBMCs were isolated from human peripheral blood and treated with concentrated hESC-MSC-derived conditioned medium and then, the secreted levels of IL-10, TNFα and IL-1β were assessed by ELISA after induction with LPS. The results showed that TMZ and DZ-conditioned medium significantly enhanced immunomodulatory potential of hESC-MSCs by increasing the secretion of IL-10, TNFα and IL-1β from LPS- induced PBMCs. We also found that hESC-MSCs did not secrete mentioned cytokines prior to or after the preconditioning with TMZ and DZ. In conclusion, our results implied that TMZ and DZ can be used to promote the immunomodulatory effects of hESC-MSC secretome. It is obvious that for applying of these findings in clinical demands, the potency of different pre-conditioned MSCs secretome on immune response needs to be more clarified.

## 1. Introduction

The use of mesenchymal stem cells (MSCs) has been emerged as a promising therapeutic tool in clinical and pre-clinical studies of regenerative medicine and cell-based therapies (Uccelli et al., 2008). Since MSCs have the potential to differentiate into reparative cell types such as osteoblasts, chondrocytes, adipocytes, and cardiomyocytes, they are used to treat a variety of diseases (Kadivar et al., 2006). Results from the clinical and experimental application of MSCs to treat autoimmune diseases such as rheumatoid arthritis, diabetes, multiple sclerosis, Crohn's disease, and lupus erythematosus are suggestive of their efficacy to ameliorate disease pathogenesis (Uccelli et al., 2008). Moreover, several human trials investigated the effect of MSCs to reduce inflammation in disease conditions such as chronic liver failure, spinal cord injury, and knee osteoarthritis (Shi et al., 2012; Forostyak et al., 2013; Orozco et al., 2014). There are many studies indicate that MSCs not only mediate the regeneration of injured tissues by their direct differentiation into reparative cells, but also secrete an array of angiogenic and anti-apoptotic factors and cytokines that act in a paracrine manner to stimulate trophic and immunomodulatory effects (Caplan and Dennis, 2006; Paterson et al., 2014). These trophic factors inhibit apoptosis and induce mitosis, differentiation of preserved stem cells in their niches, and angiogenesis. Other factors secreted by MSCs induce immunomodulatory and anti-inflammatory effects in injured tissue (Caplan and Dennis, 2006). These paracrine properties of MSCs present the potential of improving MSC-based therapies, which avoid the current controversial issues of cell-based therapies such as massive death of transplanted cells, tumorigenicity, local inflammatory responses, and low MSC survival rates after the injection (Sze et al., 2007; Uccelli et al., 2008).

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MSCs exert their immunomodulatory effects on endogenous cells through the suppression of T-cell, B-cell, natural killer (NK) cell, and dendritic cell proliferation and activity and the induction of regulatory T cells (Djouad et al., 2003; Glennie et al., 2005; Nauta and Fibbe, 2007). Comprehensive proteome profiling of the secretome of MSCs revealed that it consists of a variety of cytokines, chemokines, growth factors, extracellular matrix (ECM) proteins, and components of pathways such as those associated with regeneration, vascularization and hematopoiesis (Sze et al., 2007). There are a number of limitations that restrict the use of MSCs in clinical demands. Among them, need for invasive procedures and the small number of cells isolated from each donor are of great importance (Pittenger et al., 1999; Rao and Mattson, 2001). A type of MSCs derived from human embryonic stem cell is known as hESC-MSCs. These cells offer an active and healthy population of stably renewing cells with greater expansion capability and enough population doublings and introduce a source with greater differentiation capability and more efficient treatment potential compared to tissue-derived MSCs (Sze et al., 2007; Yen et al., 2009; van Koppen et al., 2012). Moreover, their different origin dominates the mentioned limitations regarding to adult MSCs. Although the immunobiological and immunosuppressive features of hESC-MSCs has been confirmed, the mechanisms of their immunomodulatory effects remain largely unknown (Yen et al., 2009). Signaling pathways such as PI3 K/Akt and MAPK induce the production and release of paracrine factors from MSCs (Wang et al., 2009; Herrmann et al., 2010; Herrmann et al., 2011; Yew et al., 2011). *In vitro* administration of transforming growth factor α (TGFα) increase the secretion of human growth factor (HGF) in human MSCs (Wang et al., 2009). The function of nuclear factor NF-κB in human MSCs has been investigated under stress conditions such as TNFα, lipopolysaccharide (LPS) and hypoxia. The activity of this transcription factor leads to increased secretion of several growth factors or production of prostaglandin E2 from MSCs that increase survival and suppress inflammation (Crisostomo et al., 2008; Nemeth et al., 2009).

Different approaches have mainly concentrated on secretome manipulation and *in vitro* preconditioning of MSCs (Kamota et al., 2009; Shi et al., 2009; Afzal et al., 2010; Tang et al., 2010; van Koppen et al., 2012). Pharmacological preconditioning is an example of such approaches that takes advantage of using small molecules and drugs, leading to increased survival of transplanted MSCs and prolonged release of effective biomolecules that regulate immune responses (Yao et al., 2009; Afzal et al., 2010; Li et al., 2010). Trimetazidine (1-[2,3,4-trimethoxybenzyl]piperazine; TMZ; a common anti-ischemic drug for treating angina in cardiac patients, promotes the survival of stem cells under different conditions, such as reducing the $H_2O_2$-induced injury of endothelial progenitor cells (Wu et al., 2013), the hypoxia-induced apoptosis of bone marrow MSCs (Xu et al., 2012) and $H_2O_2$ exposed BM-MSCs (Wisel et al., 2009). Diazoxide (DZ), a pharmalogical agent to open/activate the mitochondrial ATP-sensitive potassium (mitoK(ATP)) channel, enhances cell survival of BM-MSCs upon transplantation into an ischemic environment (Afzal et al., 2010; Cui et al., 2010). According to the mentioned evidences, we aimed this study to clarify whether TMZ and DZ could improve the immunomodulatory effect of human ESC-MSC-derived secretome. For this purpose, we prepared the secretome of human ESC-MSCs preconditioned with these drugs and then we assessed their effects on secretion of immunomodulatory cytokines from PBMCs.

## 2. Materials and methods

### 2.1. Cell culture

Vials of frozen human MSCs (RH6-MSC) at passage 3 were purchased from the Royan Institute (Tehran, Iran). These cells were derived from a human embryonic stem cell line known as Royan H6 (RH6), as described previously (Lotfinia et al., 2016). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100U/mL penicillin, and 100 μg/mL streptomycin (Gibco) in T75 $cm^2$ flasks and maintained at 37 °C in a humidified incubator with 5% $CO_2$. Medium was changed every two days and the cells were passaged at a ratio of 1:3 when they reached 80–90% confluency.

### 2.2. Characterization of mesenchymal stem cells

For immunophenotyping, 80% confluent fibroblast-like cells at passage 4 were washed with warm PBS and separated by trypsin (0.05% with EDTA). After centrifuging, cell pellet was dissolved in PBS containing 2% FBS and incubated at 37 °C for 20 min. Then 2 μl of isotype control and antibodies (CD73, CD105, CD45, and CD34) were added to vials containing $2 \times 10^5$ cells and kept at 4 °C for 30 min. Cells were washed with PBS and fluorescent labeled-cells were tested using FACS Calibur flow cytometer (BD) and analyzed by the Flowing software.

### 2.3. Osteogenic and adipogenic differentiation of MSCs

Osteogenic differentiation was induced with low glucose-DMEM culture medium consisting of 200 μM ascorbic acid, 10 mM β-glycerophosphate, and 0.1 μM dexamethasone. Cells were incubated at 37 °C, 5% $Co_2$, and 20% $O_2$ for 21 days. After fixation with methanol, cells were stained with Alizarin Red (Barberi et al., 2007; Lian et al., 2007). Adipogenic differentiation was induced with DMEM/F12 medium containing 20% KOSR, 2 mM L-glutamine, 1% NEAA, 100U/mL penicillin, 100 μg/mL streptomycin (Sigma), 100 ng/mL FGF, and 1% ITS. Cells were incubated at 37 °C, 5% Co2, and 5% O2 (hypoxia) for 21 days. After fixation with paraformaldehyde, cells were stained with Oil Red (Olivier et al., 2006).

### 2.4. MTT assay

MTT assay was used to determine the optimal dose of TMZ and DZ. Cells were seeded into 96-well plates at a density of $2 \times 10^4$ cells/well and incubated for 24 h to reach 90% confluency. Then the cells were treated with different concentrations of each small molecule (0, 10, 20, 50, 100, 150, 200, 250, 500 μM) and incubated for 6 h and 30 min with TMZ and DZ respectively. Subsequently, 100 μl of MTT solution (2 mg/mL in phosphate-buffered saline) was added to each well and plates were incubated for 4 h at 37 °C in a humidified incubator. To solubilize the formazan crystals, 100 μl DMSO was added to each well and then the plates were incubated for 15 min. The absorbance was measured at 570 nm using a microplate reader. We considered the highest concentration of TMZ and DZ with no significant cytotoxicity as optimal dose for treatment of hESC-MSCs.

### 2.5. Preparation of preconditioned secretomes

hESC-MSCs were cultured in T175 $cm^2$ cell culture flask in DMEM culture medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100U/mL penicillin, and 100 μg/mL streptomycin. After 80% confluency, the cells were incubated with optimized concentrations of small molecules (50 μM TMZ for 6 h and 100 μM DZ for 30 min). Subsequent to incubation times, cells were washed three times with PBS and incubated for 24 h with 15 ml of DMEM medium containing 2 mM L-glutamine and 0.1% human serum albumin (HSA). Then the cell-free medium (preconditioned medium) was collected from each flask and centrifuged (1000*g*, 15 min, 4 °C) to remove the cell debris. To concentrate the secretome, the preconditioned medium was centrifuged (4000*g*, 40 min, 4 °C) using 3 kDa molecular mass cut-off ultrafiltration membranes (Millipore, Billerica, MA, USA). Finally, the secretomes were transferred to 1.5 ml microtubes and kept at −80 °C.