Original Article

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Conditioned medium of lipopolysaccharide-treated embryonic stem cell-derived mesenchymal stem cells modulates *in vitro* secretion of inflammatory cytokines

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ABSTRACT

Background: The immunomodulatory properties of mesenchymal stem cells (MSCs) secretome have been considered by many investigators, and several preconditioning strategies such as pharmaceutical preconditioning have shown to strengthen the immunomodulatory outcomes of MSCs. **Objectives:** Current research aimed to scrutinize the release of some main inflammatory related cytokines in peripheral blood mononuclear cells (PBMCs) induced by lipopolysaccharide (LPS), following the treatment with secretome of LPS-preconditioned human embryonic stem cell-derived MSCs (hESC-MSCs). **Materials and Methods:** First, hESC-MSCs were cultivated and characterized, then they were preconditioned with 1 μg/mL of LPS. The conditioned medium was collected and concentrated 15-folds. In the next step, PBMCs were separated from human peripheral blood and processed by concentrated LPS-preconditioned hESC-MSC secretome. Subsequently, PBMCs were induced by 1 μg/mL of LPS. Finally, the secreted amounts of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-10 were investigated through ELISA assay. Results: LPS-preconditioned hESC-MSC secretome considerably enhanced the secretion of IL-10 and IL-1β in PBMCs ($P > 0.0001$). The change of secreted level in TNF-α was not significant. **Conclusion:** The results implied that LPS could support the immunomodulatory outcomes of hESC-MSC secretome.

Keywords: Inflammation, lipopolysaccharide, mesenchymal stem cell, preconditioning, secretome

INTRODUCTION

Dysregulated wound healing represents an important problem in inflammatory diseases and is characterized by over accumulation of macrophages and aberrant secretion of pro-inflammatory cytokines.[1,2] Secretome is referred to the total molecules secreted or surface dropped by stem cells including bioactive peptides such as cytokines, chemokines, and growth factors.[3] However, the origin of stem cell can influence the secretome. During the last decades, different researchers have focused on the stem cell application and tried to design strategies for expanding high efficient stem cell therapy. Stem cell transplantation improves regeneration of injured tissues and restores cellular function.[4,5] Notably, it has been indicated that cell-free conditioned media of stem cells are contributed to these beneficial effects through complex paracrine actions.[6] Hence, these findings have

attracted the scientific attentions toward stem cell-derived secretome.

Macrophages are the main Sources of pro-inflammatory and anti-inflammatory ligands involved in the modulation of inflammatory reactions. Interleukin (IL)-1β, as a pro-inflammatory cytokine, is secreted mostly by monocytes and macrophages during cell injury, invasion, infection, and inflammation. Tumor necrosis factor (TNF)-α is another pro-inflammatory ligand with a vital importance during inflammatory states, by regulating NF-κB activation and apoptotic pathways through two cell surface receptors, TNFR1 and TNFR2, associated with several signaling pathways. IL-10 is a major anti-inflammatory cytokine with sharp features. This cytokine downregulates the production of inflammatory ligands (such as TNF-α, IL-6, and IL-1) and their receptors in activated macrophages. Since it can modulate the secretion and activity of pro-inflammatory cytokines,[7] restoring the inflammatory response to the normal state could be a useful method for accelerating the wound healing.[8]

Mesenchymal stem cell (MSC) therapy could be a useful strategy in regenerative Medicine of various inflammatory conditions.[9] Indicated by several studies, MSCs can support skin regeneration, promote cell proliferation and relieve inflammation.[10] Transplanting stem cells into injured tissues of lung, kidney, and liver failure has shown to be accompanied by expression of numerous paracrine signaling factors such as anti-inflammatory ligands, angiogenic growth factors, and anti-apoptotic factors, which are contributed in stem cell-mediated wound healing.[11-15] Accordingly, secretory components of MSCs might exert immunomodulatory effects on inflammatory immune cells.

Several studies on different organ injuries models have indicated that conditioned medium of MSCs inhibits apoptosis of cardiomyocytes, reduces the number of apoptotic cardiomyocytes, preserves heart function, reduces the number of infiltrating leukocytes, enhances liver regeneration, and improves the new vessel formation by generating bioactive components.[16-18]

However, different preconditioning strategies have been evaluated to enhance the regenerative effects of MSCs such as hypoxia, and pharmaceutical priming.[19-23] Besides, preconditioning of MSCs with lipopolysaccharide (LPS) has shown to be a promising remedy for treatment of inflammatory maladies and tissue damages.[24] Although many studies have indicated that LPS preconditioning perceptibly promotes the protective and regenerative features of MSCs,[25,26] there is not enough evidence about the paracrine effects of LPS preconditioned ESC-MSCs secretome.

Regarding these facts, we hypothesize that LPS preconditioning of ESC-MSCs may result in producing a more potent secretome. In this study, LPS- induced mononuclear cells were exposed to the LPS-pretreated MSC secretome for obtaining a high-yield secretome and their anti-inflammatory properties were evaluated in comparison with untreated MSC secretome.

MATERIALS AND METHODS

Cells and Reagents

At the current research, third passage human MSCs (RH6-MSC) were supplied by Royan Institute (Tehran, Iran). All cell culture reagents (Dulbecco's Modified Eagle's Medium (DMEM) – low glucose, fetal bovine serum (FBS), L-glutamine, trypsin-ethylenediaminetetraacetic acid (EDTA) %0.05, penicillin and streptomycin) were obtained from Gibco (Germany). Dimethyl sulfoxide (DMSO) and 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Company (Germany). All regents for adipogenic and osteogenic differentiation, including ascorbic acid, β-glycerophosphate, dexamethasone, knockout serum (KOSR), fibroblast growth factor (FGF), Insulin-Transferrin-Selenium (ITS), and National Environment Appellate Authority (NEAA), were purchased from Sigma-Aldrich Company (Germany). Phycoerythrin-labeled mouse anti-human CD105 and CD73 antibodies, FITC-labeled mouse anti-human CD34 and CD45 antibodies, and IgG1 antibodies were purchased from the eBioscience Corporation (United States).

Cell Culture

Third passage RH6-MSCs were cultivated in DMEM-low glucose culture medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin and incubated in 5% CO_2 at 37°C. Media were refreshed daily and cells with 80–90% confluency were passaged at a ratio of 1:3.

Characterization of RH6-MSCs

For Immunophenotyping, fibroblast-like RH6-MSCs with 80% confluency at fifth passage were washed with phosphate-buffered saline (PBS) and isolated using 0.05% trypsin-EDTA. Upon centrifuging and washing, cell sediment was suspended in PBS buffer supplied with 2% FBS and placed into incubator at 37°C for 20 min. Next, 2 μl of IgG1 isotype control and antibodies (CD105, CD73, CD45, and CD34) were added to the cells in separate tubes and incubated at 4°C for 30 min. Fluorescent labeled-cells were washed with PBS. The cell surface expression of mentioned CD markers was quantified through FACS Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany) and results were interpreted through Flow Jo software (Tree Star, Ashland, OR, USA).

Osteogenic and Adipogenic Differentiation of RH6-MSCs

Differentiating into bone tissue was triggered using DMEM cultivating media containing 200 μM ascorbic acid, 10 mM β-glycerophosphate, and 0.1 μM dexamethasone. Afterward, the cells were cultivated at 37°C, 5% CO_2, and 20% O_2 for 21 days. Following methanol fixation, the cells were dyed using Alizarin Red and analyzed through microscopic observation. Adipogenic differentiation was further induced by DMEM/F12 media supplemented with 20% KOSR, 2 mM L-glutamine, 1% NEAA, 100 U/mL penicillin, 100 μg/mL streptomycin, 100 ng/ml FGF, and 1% ITS. Subsequently, cells were cultivated at 37°C, 5% CO_2, and 5% O_2 (hypoxia condition) for 21 days. Following the paraformaldehyde fixation, cells were

dyed with oil red and were analyzed through microscopic observation.

MTT Test

MTT test was applied for determination of the LPS optimal dose. RH6-MSC cells were cultivated into a 96-well plate at a population of 2×10^4 cells, then they were placed into incubator for an overnight to adhere. Afterward, cells were incubated for further 2 h with 0, 1, 2.5, 5, 7.5, 10, 12.5, and 15 μg/mL of LPS. Each test was at least performed 3 times. After specified incubation time, 100 μl of MTT solution (1 mg/mL in PBS) was added into the wells, then plates were placed in the 37°C humidified incubator for 4 h. The supernatant was carefully aspirated, and 100 μL of DMSO was added into the well to solubilize formazan blue crystals. Following 15 min incubation, 570 nm absorbance was investigated through Absorbance Reader (Biotech, South Korea) in accordance with the supplier's manual. Cell viability was evaluated according to following equation: Viability (percentage of control) = [(Absorbance sample-absorbance blank)/ [Absorbance Control-Absorbance blank)] ×100.

Treating RH6-MSCs with LPS and Assemblage of Preconditioned Secretome

RH6-MSCs were cultivated in T175 cultivation flasks (SPL, South Korea) in DMEM medium to achieve 90–95% confluency. Then, the cells were incubated with 1 μg/mL of LPS for 2 h, washed 3 times using warm PBS and treated with 15 mL of DMEM-low glucose medium containing 2 mM L-glutamine and 0.1% human serum albumin (HSA) without FBS and cultivated for 24 h. Cell-free medium was collected from supernatant derived from treated cells, then centrifuged at 1000 g for 15 min at 4°C for removal of the cell debris. 3kDa molecular mass cutoff ultrafiltration membranes (Millipore, Billerica, MA, USA) were applied for ultracentrifugation at 4000 g for 40 min at 4°C to concentrate 15-fold. The secretomes were aliquoted and stored at −20°C.

Peripheral blood mononuclear cells (PBMCs) Separation and Processing

PBMCs were separated from freshly-collected whole peripheral human blood through Ficoll gradient (density =1.077 g/cc; GE Healthcare) and plated into 96 well plates with 1×10^5 cell/well density in 50 μL of RPMI-1640 culture medium enriched with 10% FBS, 1% L-glutamine, 1% NEAA, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.1 mM β-mercaptoethanol. PBMCs were subsequently processed using 50 μl LPS preconditioned RH6-MSCs medium (LPS-MSC-CM) for 18 h and induced with 30 μg/mL of LPS for 5 h. As negative control, one group of PBMCs was only induced with LPS, but not subjected to any other treatment. Moreover, one group of PBMCs was treated with MSC-CM (RH6-MSC secretome not preconditioned with LPS) to investigate the influence of LPS preconditioning on immunomodulatory properties of RH6-MSC secretome. The cell culture supernatants were isolated and centrifuged for 5 min at 1000 g to remove the cell debris.

ELISA

The collected cell culture supernatants from PBMCs were used for evaluating the cytokines secretion levels by ELISA kits (Thermo Scientific - United States) in accordance with the supplier's manual. In this study, TNF-α and IL-1β were presenting pro-inflammatory ligands, and IL-10 was regarded an anti-inflammatory ligand. Optical density was investigated at 450 nm by a microplate reader. Results were expressed as pg/mL according to the standard curve and were compared with the negative control group.

Statistical Data Interpretation

GraphPad Prism 6.0 was used for analyzing the acquired statistics. Obtained data were provided as mean ± standard deviation of three independent experiments. $P < 0.05$ was considered statistically meaningful. Statistical differences among groups were determined through one-way analysis of variance. Dunnett's adjustment was used for multiple comparisons.

RESULTS

RH6-MSCs Characterization

RH6-MSCs showed a fibroblast-like morphology at third passage [Figure S1, part A]. Microscopic evaluation of RH6-MSCs indicated that these cells are enable to differentiate to adipogenic and osteogenic lineages as orange lipid droplets in the cytoplasm and red points within extracellular matrix were observed respectively following oil red O and alizarin red dyes [Figure S1, parts B and C]. Osteogenic and adipogenic differentiation of MSCs were presented in phase contrast field to focus on the red points showing calcium deposits in the extracellular matrix in alizarin red staining, and adipogenic differentiation of ESC-MSCs and oil red staining, showing positively stained cells with red fat granules in the cytoplasm.

Furthermore, flow cytometry analysis showed that RH6-MSCs express CD73 and CD105 (as mesenchymal markers) in high levels. In contrast, approximately no cell surface expression of CD45 and CD34 (as hematopoietic and endothelial markers, respectively) was observed [Figure S2].

Effect of LPS on RH6-MSC Cell Viability

Cell viability was analyzed through MTT assay. RH6-MSCs were treated with different LPS concentrations. Data analysis demonstrated that cell viability is decreased in a dose dependent manner, as concentrations higher than 2.5 μg/ml of LPS significantly decrease the cell survival in comparison with negative control groups (untreated RH6-MSCs) [Figure 1]. Therefore, we considered 1 μg/mL of LPS as treatment concentration to precondition RH6-MSCs.

PBMCs Treated with LPS- MSC-CM Secreted High Levels of IL-10 and IL-1β

ELISA assay of PBMCs supernatant on stimulation with LPS showed that treating LPS-stimulated PBMCs with LPS-MSC-CM significantly promoted the IL-10 secretion, as an anti-inflammatory ligand and IL-1β, as a pro-inflammatory cytokine (IL-10 = 723.4 ± 80.73, IL-1β =303 ± 25.95, $P < 0.0001$), but not TNF-α (358.4 ± 14.26, $P = 0.7924$) compared to negative control group (IL-10 = 47.14 ± 3.48, IL-1β =12.13 ± 6.44, TNF-α =386.7 ± 17.93). In contrast,

MSC-CM did not change IL-10, IL-1β, and TNF-α secretion levels from LPS stimulated PBMCs (IL-10 = 176.2 ± 11.27, IL-1β =65.32 ± 6.31, and TNF-α =472.4 ± 59.3) [Figure 2]. Furthermore, ELISA assessment showed no secretion of IL-10, IL-1β, and TNF-α in MSC-CM and LPS-MSC-CM (data not shown).

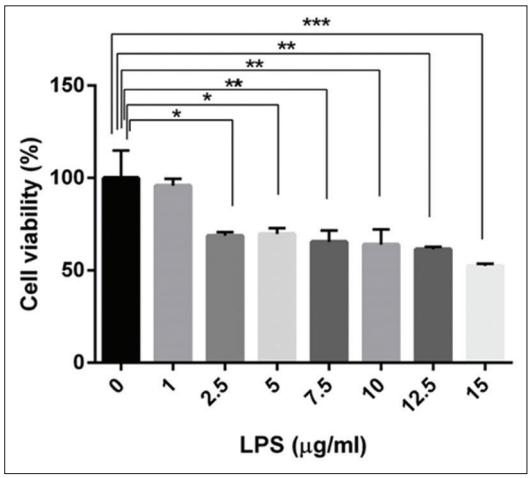

Figure 1: MTT assay of different lipopolysaccharide (LPS) concentrations (0, 1, 2.5, 5, 7.5, 10, 12.5, 15, and 20 μg/ml) on the RH6- mesenchymal stem cells (MSCs) viability after 2 h. The results present the means ± SEM. from six independent experiments. Significant difference was compared with negative control (untreated RH6-MSCs with LPS): *P <0.05, **P <0.01, ***P <0.001

DISCUSSION

The present study was performed with the aim of evaluating the anti-inflammatory properties of LPS-MSC-CM on PBMCs, in comparison with untreated MSC secretome. As mentioned in material and method section, RH6-MSCs were washed 3 times, using warm PBS after incubation with 1 μg/ml of LPS. Then, the cells were treated with 15 mL of DMEM-low glucose medium containing 2 mM L-glutamine and 0.1% HSA without FBS and cultivated 24 h. Therefore, LPS-MSC-CM cells were free from LPS.

This study indicated that LPS-MSC-CM could enhance the secretion of IL-10 and IL-1β cytokines from PBMCs. PI3K/Akt signaling is capable of modulating IL-10 responses and upregulation of pAkt, but LPS preconditioning possibly can modulate the Akt pathway for increasing the IL-10 secretion. Previously, it has been indicated that transplantation of LPS preconditioned-MSCs into an acute myocardial infarction rat model could raise the survival of grafted cells, activate the PI3k/Akt pathway and induce cardiac protection[27]. Even though, unfortunately it is not yet clear that how LPS-MSC-CM is capable of stimulating the PBMCs to produce inflammatory and anti-inflammatory cytokines.

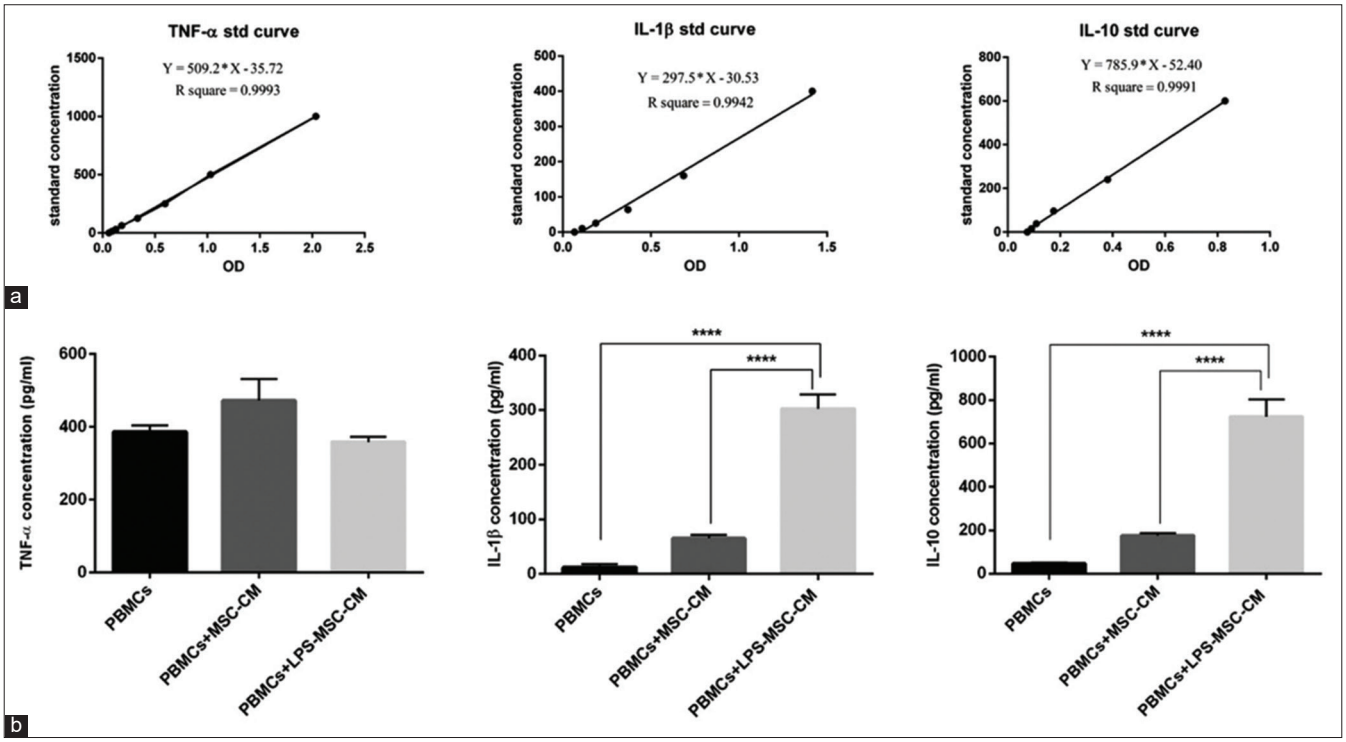

Figure 2: (a) ELISA standard curves were plotted by making serial dilutions of standard with known concentrations from the standard solution. (b) Secretion of pro- and anti-inflammatory cytokines, interleukin (IL)-10, tumor necrosis factor-α, and IL-1β from lipopolysaccharide (LPS)-induced peripheral blood mononuclear cells (PBMCs). The results indicate the mean ± SEM from three-independent experiments. Significant difference was compared with negative control (untreated RH6- mesenchymal stem cells [MSCs] with LPS): *P <0.05, **P <0.01, ***P <0.001, ****P <0.0001. NC: Negative Control, PBMC+ LPS-MSC-CM: LPS-induced PBMCs treated with LPS preconditioned MSC secretome, PBMC+ MSC-CM: LPS-induced PBMCs treated with non-preconditioned MSC secretome, LPS-MSC-CM: LPS preconditioned MSC secretome (concentrated 15-fold via 3KD cutoff ultra-centrifugal tubes), MSC-CM: non-preconditioned MSC secretome (concentrated 15-fold through 3KD cutoff ultra-centrifugal tubes)

Although stem cell paracrine influences have been achieved from hematopoietic and MSCs (HSCs and MSCs respectively), MSCs have more benefit than HSCs including their availability, ease of culturing and proliferation, simple isolation, and their ability to circumvent rejection[28,29]. Moreover, using ESC-MSCs is more feasible than bone marrow MSCs because of not requiring invasive methods. Accordingly, in this report, we decided to choose MSCs**-**derived from embryonic stem cells for investigation. Following several experiments, researchers supposed that preconditioning MSCs with some particular small molecules might enhance regenerative effects of these cells and change the diversity and concentration of secreted bioactive components. Previously, several studies have been performed on determining how pretreating and priming MSCs with TNF-α, IFN-β, and LPS can optimize MSCs activities[24,30,31].

MSCs express some TLRs such as TLR4 on their surface and can be stimulated through TLR agonists[32,33]. On the other hand, LPS is a TLR4 agonist and polarizes macrophages toward pro-inflammatory properties [34,35]. Previously, it has been indicated that LPS imposes MSCs to exert pro-inflammatory ligands, such as IL-1β, IL-6, IL-8, IL-12, type I IFN, and TNF-α [36,37]. According to these findings, we considered LPS as a factor to stimulate MSCs and enrich their paracrine factors. It has been reported that MSCs pre-treatment with low and non-toxic LPS loads before transplantation could decrease tissue damage in several organs[37-39]. Moreover, it has been realized that 1.0 μg/mL of LPS can protect MSCs against apoptosis by oxidative stress and increases their expansion[40]. Our results are in agreement with these findings, as we have shown that concentrations higher than 1.0 μg/mL of LPS significantly decrease cell survival of RH6-MSCs, while treating these cells by 1.0 μg/mL of LPS for 2 h, do not change cell viability in comparison with untreated cells. Therefore, we considered 1.0 μg/mL of LPS as non-toxic dose to pretreat MSCs.

At the next step, we concentrated conditioned media, acquired from MSCs, 15-fold through ultrafiltration entities, using a 3-kDa-molecular-weight cutoff. By earlier studies, it has been indicated that transplantation of LPS preconditioned-MSCs into an acute myocardial infarction rat model could increase survival of grafted cells, activated the PI3k/Akt pathway, and induced cardiac protection[24]. In the current study, cytokine production analysis from PBMCs following treating with LPS-MSC-CM revealed considerable increase of IL-10 and IL-1β, but not TNF-α. Moreover, a new paradigm for MSCs has been proposed that suggests human MSCs, such as monocytes, are polarized into two phenotypes including MSC1 and MSC2 which depend on the downstream TLR signaling. TLR4 agonists polarize human bone marrow-derived MSCs toward a pro-inflammatory phenotype (MSC1), while TLR3 agonists polarize the MSCs toward an immunosuppressive phenotype (MSC2)[41]. Accordingly, released cytokines from LPS-preconditioned MSCs might be involved in modulating cytokine secretion from PBMCs through binding to their receptors on the cell surface and triggering related signaling pathways and regulating inflammatory transcription factors.

It has been reported that treating bone marrow-derived MSCs with LPS causes the release of cytokines, including IL-1 β, IFN-γ, and IL-6, and the activation of NF-κB through the TLR4-MyD88-relying pathway[42]. Furthermore, there are reports that LPS-pretreated adipose-derived stem cells conditioned media could induce p-STAT3 expression[25]. The results by the current study demonstrated that LPS-MSC-CM induces the release of IL-1β, TNF-α, although the increased secretion level of TNF-α was not significant. However, these effects might be different in experimental *in vivo* models and could be modified using multiple factors, such as the concentration and duration of incubation with LPS, MSC sources, and expression levels of TLR4.

CONCLUSION

Pharmacological preconditioning is a promising strategy for preventing the detrimental impacts of MSCs and promotes their paracrine effects. This study indicated that LPS preconditioning significantly enhances the secretion of IL-10 and IL-1β in peripheral blood MSCs induced with LPS, which highlights its ability to enhance immunomodulatory outcomes of human embryonic stem cell-MSC secretome.

ETHICAL CONSIDERATION

Helsinki protocol was followed accordingly in this research. Informed written consent was obtained from participants based on the Research Ethics committee Guidelines at Pasteur Institute of Iran (Tehran, Iran). This moral committee has accepted the current study and agreed the devotion of an institutional grant from Pasteur Institute of Iran for this research (grant No. 802).

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CONFLICTS OF INTEREST

The authors state no conflicts of interest that could negatively influence the study.

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SUPPLEMENTARY FIGURES

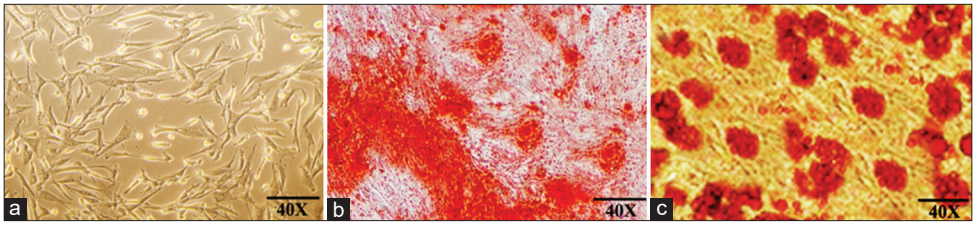

Figure S1: (a) Fibroblast-like morphology of RH6-MSCs at passage 3 (40X) (b) Osteogenic differentiation of ESC-MSCs and staining with Alizarin Red (40X). Red points show calcium deposits in the extracellular matrix (40X). (c) Adipogenic differentiation of ESC-MSCs and Oil Red staining, showing positively stained cells that with red fat granules in the cytoplasm (40X).

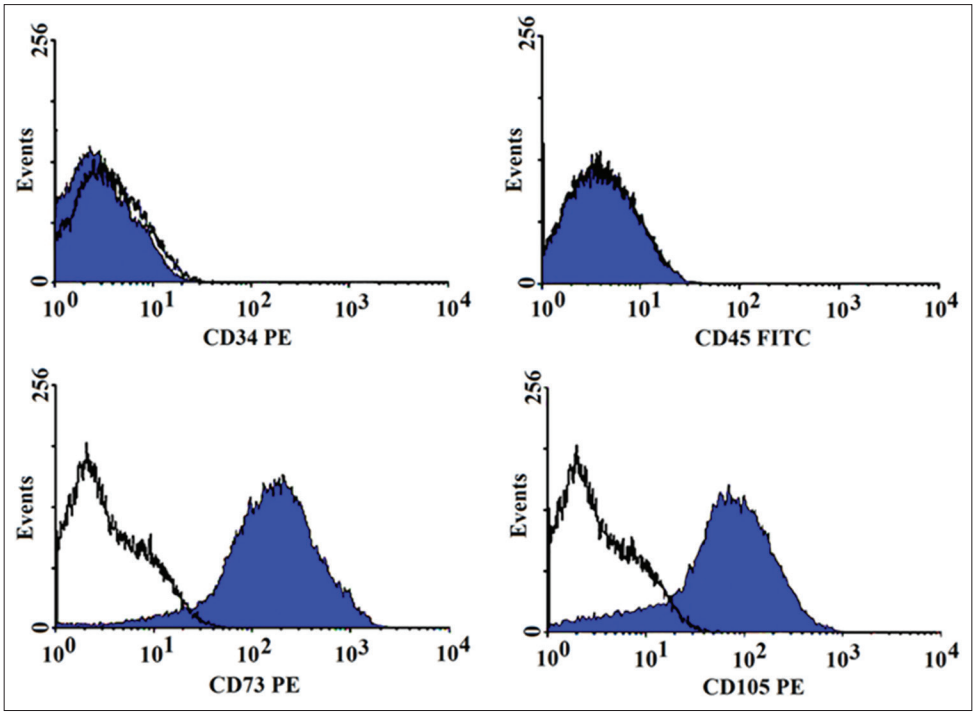

Figure S2: Flow cytometry analysis for mesenchymal markers CD73 and CD105, hematopoietic marker CD45 and endothelial marker showed the expression of mesenchymal markers on the cells.