

## Immunosuppressive Effects of Human Chorionic Gonadotropin (hCG) on Mesenchymal Stromal Cells

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### Abstract

Mesenchymal stem/stromal cells (MSCs) as one of the most important types of adult stem cells secrete a variety of immunomodulatory cytokines. However, their immunomodulatory features strongly depend on the molecular cross-talk between cells and the surrounding microenvironment. Hence, some strategies were proposed to empower their beneficial effects during cell-therapeutic procedures to avoid confusing results. Licensing the cells with chemical compounds could be considered as one of the most applicable methods for induction of anti-inflammatory status in the cells. Human chorionic gonadotropin (hCG) is a pregnancy related hormone which has been shown to be essential for the establishment of a successful pregnancy. HCG supports the implantation of fetus in the maternal endometrium, due to its immunomodulatory effects. Moreover, the regulatory role of hCG has been previously mentioned in case of some autoimmune-based diseases. In the present study, the capacity of this hormone for induction of different immune-encountered genes expression was examined in primary cultures of human adipose tissue derived mesenchymal stem cells (Ad-MSCs). In this regard, Ad-MSCs were exposed to 10 IU of hCG for 72 hours. Molecular studies via quantitative Real-time PCR (qRT-PCR) experiments were performed to detect gene expression modifications based on the application of SYBR Green as the fluorescent dye and in comparison to the *RPLP0* as the housekeeping gene. Results confirmed that hCG significantly upregulated *TSG-6*, *TGF-β1*, *IL-1β* and *IL-6* expression levels comparing with the control group, while it downregulates *COX-2* expression, and had no statistically significant effects on *IL-10* and *TDO2*. In conclusion, priming Ad-MSCs with hCG may enhance the proliferation and immunoregulatory potential of these cells, although it needs further investigations to reveal involved molecular pathways.

**Keywords:** Mesenchymal stem cells, Human Chorionic Gonadotropin, Pregnancy, Immunomodulation, Pretreatment

### Introduction

The human immune system employs a complicated network of organs, tissues, cells and molecules to defend the host against a wide range of pathogens (Netea et al., 2016). Both innate and adaptive immune responses are playing role in the recognition and eradication of infections. During innate immune responses some biologically active small molecules such as different cytokines and chemokines are secreted by activated cells. Pregnancy is a physiological exception during which the fetal allograft is well tolerated by the

maternal immune system (Chaplin, 2010). Decidual stromal cells normally produce chemokine gradients, which in turns recruited maternal leukocytes.

On the other hand, trophoblastic cells can induce the differentiation of immune cells into a trophoblast-supporting phenotype (Ander et al., 2019). While the majority of the decidual leukocytes are natural killer cells (NK) with the capacity for production of a vast array of growth factors and cytokines, and decidual anti-inflammatory M2-like cells, T cell subsets are also playing functional roles (Liu et al., 2017). Although exact molecular events which are involved in the fetal tolerance have still remained unknown, it is clear that *i)* the uterus/placenta displays a distinguished immune site and that *ii)* there are multiple mechanisms for maintenance of local inflammation and hemostasis of T-cell specific responses (Pazos et al., 2012). Pregnancy is a

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combination of pro-inflammatory and anti-inflammatory situations, depending on the stage of gestation. Hence, understanding the mechanisms which are engaged with tolerance induction and immune system modulation during gestation may provide new insights into the regulatory systems which may be disturbed during autoimmune diseases (Mor et al., 2011).

Human chorionic gonadotropin (hCG) is a specific hormone that has been demonstrated to have immunoregulatory properties and is essential for the establishment of a successful pregnancy (Schumacher et al., 2009). hCG has a necessary role in modulating the phenotype of B cells and their ability to produce IL-10 (Fettke et al., 2016). Moreover, other functions have been reported for hCG, including regulating the phenotype of macrophages in the benefit of M2-like cells, suppressing Th1 cytokine production and *in vivo* induction of T regulatory suppressive activities (Schumacher et al., 2014). It has been demonstrated that hCG potentially induces attraction of neutrophils, monocytes, and lymphocytes. Also, stimulation of monocytes with hCG increased the production of IL-8, as a leukocyte attractors (Schumacher et al., 2009, Ushigoe et al., 2000). It has been confirmed that hCG-treated decidual dendritic cells had a high capacity for induction of regulatory T cells (Schumacher et al., 2013). In addition, the application of hCG in a murine model of autoimmune diabetes could prevent disease progression (Khil et al., 2007). Finally, a great potential of hCG, as an anti-rejection agent following solid organ transplantation, has been clearly demonstrated (Theofanakis et al., 2017). The remission of several autoimmune-based diseases during pregnancy recommends the roles of hCG as an immunosuppressive agent in solid organ or allogeneic transplant settings (Koldehoff et al., 2011).

Regenerative properties of MSCs resulted in their therapeutic applications in a majority of disorders, such as orthopedic injuries (Volarevic et al., 2017). Also, it has been demonstrated that MSCs modulate the functions of immune cells, including T cells, B cells, natural killer (NK) cells, antigen-presenting cells, macrophages and neutrophils, which makes them promising candidates for cell therapies in a variety of auto-immune and inflammation-related diseases (Wang et al., 2016). It has been possible to extend the release of effective biomolecules and immune response effectors by pre-conditioning of MSCs with small molecules (Hu and Li, 2018). For

example, it was shown that pre-treatment of Ad-MSCs with progesterone enhances the expression of some immunomodulatory factors such as Human Leukocyte Antigen G (HLA-G), Prostaglandin E2 (PGE2) and Interleukin 6 (IL-6) (Susheelamma et al., 2018). Also, treatment of Ad-MSCs with melatonin, increased the release of mitogenic factors, such as basic Fibroblast Growth Factor (b-FGF) and Hepatocyte Growth Factor (HGF); while reduced the production of harmful inflammatory cytokines like TNF- $\alpha$  (Han et al., 2016a). Moreover, aspirin treatment could significantly enhance the immunoregulatory properties of MSCs via up-regulation of regulatory T cells (Tregs) and down-regulation of T helper 17 (Th17) cells (Tang et al., 2014).

Taken together, considering outstanding properties of MSCs in cell-based therapeutics and pivotal immunoregulative features of hCG, we conducted this *in vitro* study to shed light on the consequences of hCG priming on immunomodulatory properties of MSCs at the molecular level.

## Materials and Methods

### Primary cells derivation and expansion

Freshly prepared adipose tissues obtained from subcutaneous sites were acquired from healthy individuals with informed consent. All procedures were performed under internationally accepted ethical guidelines of working with human tissues, as approved by the ACECR Biomedical Research Ethics Committee (Mashhad, Iran, Code: IR.ACECR.JDM.REC.1398.008).

Adipose tissues were washed three times with phosphate-buffered saline (PBS1X) supplemented with penicillin-streptomycin (Biosera, France) and then incubated for 1 hour at 37°C with freshly prepared 0.1% collagenase solution (Invitrogen, USA), 0.1% of BSA (Biowest, France) and 2mM CaCl<sub>2</sub> which were prepared in PBS. They were shaken vigorously for 5 to 10 seconds periodically to avoid phase dissociation. After that, fetal bovine serum (FBS) was added to the reaction as the inhibitor of collagenase activity. Digested adipose tissues were then centrifuged at room temperature (600 g, 10 minutes). Pellets were re-suspended in freshly prepared media and following another round of centrifugation (400 g, 6 minutes) they were seeded in culture vessels containing Dulbecco's Modified Eagle's Medium (DMEM, Biowest, France), supplemented by 100 mg/mL streptomycin and 100 U/mL penicillin (Invitrogen,

USA). Mesenchymal stromal cells were purified based on their plastic-adherent capacity. Cells of passage number 3 were applied for downstream applications.

The medium was exchanged every 2–3 days. Upon reaching proper confluency (85 to 90%), cells were detached via trypsin/EDTA (0.025%, initial seeding density 5000–10000 cells/cm<sup>2</sup>).

### Characterization of human adipose tissue derived mesenchymal stromal cells

To evaluate the expression levels of mesenchymal lineage-specific cell surface markers, Ad-MSCs (2x10<sup>5</sup> cells) were trypsinized following two rounds of washing with PBS 1X and were precipitated by centrifugation.

Cell pellets were then re-suspended in 100 µl of cold PBS, containing 5% FBS and stained with 2 µg/ml of PE-conjugated CD73, CD13, FITC-conjugated CD90, CD34, CD14 and HLA-DR, and APC-conjugated CD44, CD45 antibodies for 1 hour (all from Cytognos, Spain). Data were obtained via FACScalibur Cytometer equipped with 488-nm argon laser; the analysis was performed with FlowJo software (version 7.6.1).

The capacity of the cells for adipogenic differentiation was evaluated following exposing the cells with adipogenic differentiation media for a period of two weeks. Low glucose DMEM supplemented by 10% FBS was applied as the basic media. It was complemented by 200 mM indomethacin, 1 mM dexamethasone, and 10 mM β-glycerophosphate (all from Sigma Aldrich, Germany). The differentiation medium was changed every 3 days. The level of differentiation induction was evaluated by the application of Oil Red O staining (Sigma Aldrich, Germany) as the indicator of intracellular lipid droplets.

In addition, osteogenic differentiation of the cells was assessed qualitatively based on the cytochemical analysis (alizarin red, Sigma Aldrich, Germany). To do so, Ad-MSCs were treated with osteogenic inductive media containing 50 mM ascorbate-2-phosphate (Sigma Aldrich, Germany), 0.1 mM dexamethasone, and 10 mM β-glycerophosphate. Three weeks later, osteogenesis was explored based on staining procedures which reveals the calcium mineralization of extracellular matrix and alkaline phosphatase (AP) activity, respectively (Sigma Aldrich, Germany).

### Treatment of MSCs with hCG

Fully characterized cultures of human Ad-MSCs with proper cell densities were treated with hCG

(10 IU/ml, Homapharmed, Iran) for 72h. The concentration of hCG and incubation time was selected based on our initial screening experiments. Untreated cells were applied as controls.

### MTT assay

Cell survival was determined using 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay (5 mg/ml, Sigma Aldrich, USA). Briefly, cells were seeded in 96-well plates at a density of 5000 cells per well and then MSCs were treated with various concentrations of hCG (2, 4, 6, 8, 10, 12 IU/ml). After 24 to 72 hours incubation, proper amount of MTT dye was added per well. 4 hours later, the media removed from all replicates and formazan crystals were solved in Dimethyl sulfoxide (DMSO, Sigma Aldrich, Germany). Optical densities (ODs) were read at 540 nm wavelength.

### RNA extraction and RT-PCR

Cells, from different treatments, were homogenized in TriPure reagent (Roche Diagnostics, Germany) for extraction of total RNA according to the manufacturer instructions. Quantity and purity of all RNA samples were determined using the Nanodrop ND-1000 spectrophotometer (Bio-Tek, USA). The integrity of RNA samples was also evaluated by gel electrophoresis to detect 28s and 18s ribosomal RNAs.

Total RNAs were treated with 1 unit of *DNase I* (Thermo Fisher Scientific, USA), to avoid amplification of contaminating genomic DNA. cDNA was synthesized using oligo(dT) primer and *M-MuLV* reverse transcriptase (Thermo Fisher Scientific, USA) according to the manufacturer protocols.

### Quantitative Real-time PCR (qRT-PCR)

The qRT-PCR was carried out using the Bio-Rad CFX-96 system (Bio-Rad, USA). Each reaction mixture contained 2 µl cDNA (0.1 diluted), 10 µl SYBR Green PCR Master Mix (Takara, Japan) and 1 µl of 10 pmol/ml mixture of forward and reverse primers in a final volume of 20 µl. Experiments were performed in duplicate. Specific primers applied for gene amplifications are listed in Table 1. The expression levels of target genes were normalized to *RPLP0*. Relative quantification of gene expression modifications was carried out based on the application of log 2-ΔΔCt formula.

**Table 1.** Primer sequences applied for qRT-PCR experiments.

Target Gene	Sequence	Product size (bp)
<i>RPLP0</i>	F: TGGTCATCCAGCAGGTGTTTCGA R: ACAGACACTGGCAACATTGCGG	119
<i>TGF-β1</i>	F: GTTCAAGCAGAGTACACACAGC R: GTATTCTGGTACAGCTCCACG	154
<i>TSG-6</i>	F: GCTGCTGGATGGATGGCTAAG R: CTCCTTTGCGTGTGGGTTGTAG	156
<i>COX-2</i>	F: CCAGAGCAGGCAGATGAAATACC R: ACCAGAAGGGCAGGATACAGC	168
<i>IL-1β</i>	F: CCTCTCTCACCTCTCTACTCAC R: CTGCTACTTCTTGCCCCCTTTG	186
<i>IL-6</i>	F: ACTCACCTTTCAGAACGAATTG R: GCAAGTCTCCTCATTGAATCCAG	196
<i>IL-10</i>	F:GAGATGCCTTCAGCAGAGTGAAGA R:AGGCTTGCCAACCCAGGTAAC	114
<i>TDO2</i>	F: ACCTCCGTGCTTCTCAGACAG R:GACCTCCTTTGCTGGCTCTATTC	151

**Statistical analysis**

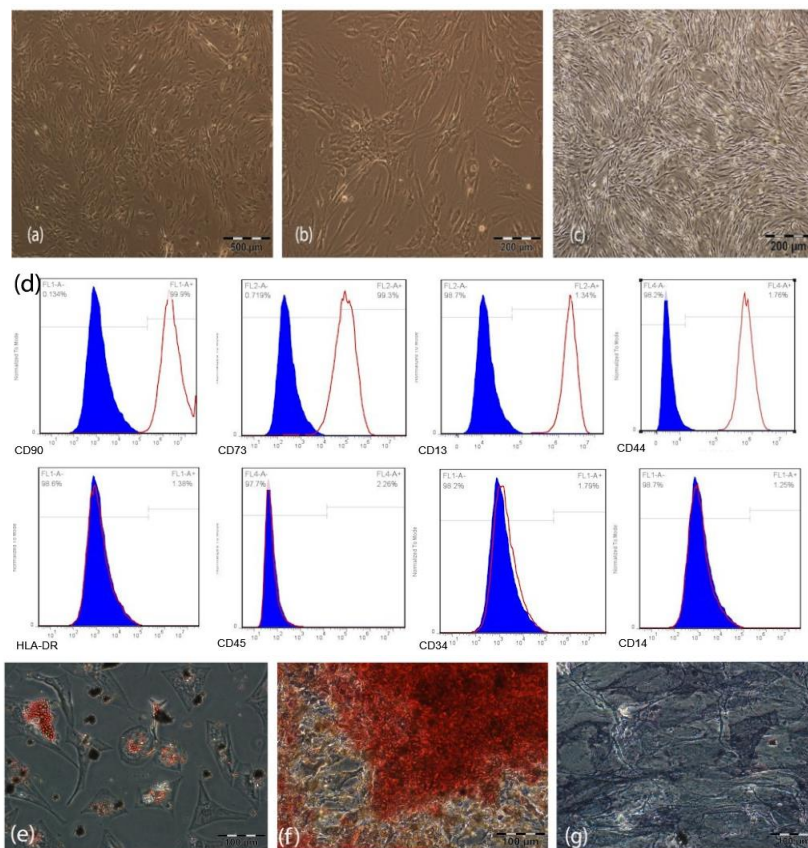
The GraphPad Prism statistical program (version 7) was used for data analysis. The values are reported as mean of at least three independent experiments ± SD. The significance of differences among data was examined at the confidence level of 95% ( $p < 0.05$ ) using the T-test.

**Results**

**Characterization of human Ad-MSCs**

The authentication of Ad-MSCs was performed based on surface antigen characterization. Cells were demonstrated a spindle-like morphology (Figure 1, a-c). The expression levels for positive markers including CD73, CD90, CD13 and CD44 were equal to 99.3%, 99.9%, 98.7% and 98.2%, respectively.

Ad-MSCs indicated the expression of non-

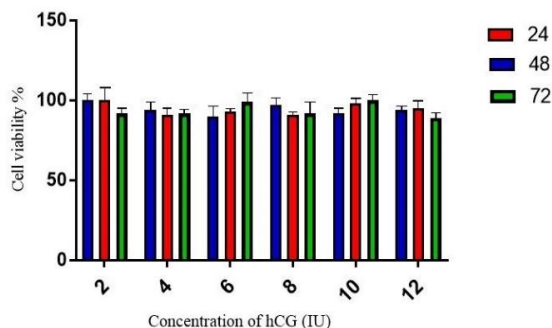


**Figure 1.** Characterization of Human Ad-MSCs, (a, b) Undifferentiated human Ad-MSCs represent spindle-shaped morphology (magnification 4x and 10x, respectively). (c) Morphological characteristics of MSC exposed to 10 IU/ml of hCG for 72 hours. (d) Flow cytometric analysis showed that almost all cultured Ad-MSCs expressed CD73 (99.3%), CD13 (98.7%), CD90 (99.9%), and CD44 (98.2%), whereas a small portion of the cells were expressed CD34 (1.79%), CD45 (2.26%), CD14 (1.25%) and HLA-DR (1.38%). Expressions of cell surface markers of Ad-MSCs are shown as compared with their respected isotype controls. (e) Adipogenic differentiated human Ad-MSCs were stained with Oil Red O. (f) Osteogenic differentiated human Ad-MSCs were stained with alizarin red. (g) Osteogenic differentiation as evidenced by alkaline phosphatase activity assay.

mesenchymal cell-specific markers for less than 3%: CD45 (2.26%), CD34 (1.79%), CD14 (1.25%) and HLA-DR (1.38%) (Figure 1d).

### Effects of hCG on cell viability

MSCs viability assessment was performed using MTT assay. Ad-MSCs were treated with a range of hCG concentrations (2, 4, 6, 8, 10, 12 IU/ml). Although minor decrease was observed in cell viability percent following treatment with different concentrations of hCG in comparison to the control group, these changes were not statistically significant. Thus, our results demonstrated that preconditioning of the cells with hCG does not exert remarkable toxicity against Ad-MSCs (Figure 2).



**Figure 2.** Cell viability (MTT) assay. Results indicated that pretreatment of Ad-MSCs with different concentrations of hCG does not affect their survival significantly.

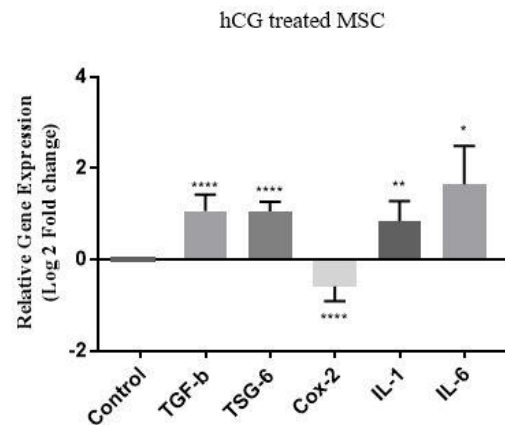
### Effects of hCG on the expression of genes involved in MSC-mediated Immuno-regulation

We evaluated the expression levels of some pro- and anti-inflammatory cytokines ( $TGF-\beta 1$ ,  $COX-2$ ,  $TSG-6$ ,  $IL-1\beta$  and  $IL-6$ ) in hCG-stimulated Ad-MSCs, in comparison with untreated cells from passage number 3, via qRT-PCR. Results showed that hCG markedly enhanced the mRNA levels of  $TGF-\beta$ ,  $TSG-6$ ,  $IL-1\beta$  and  $IL-6$  and reduced the expression of  $COX-2$  compared with the control group (Figure 3).

### Discussion

As a specific soluble factor produced by the placenta, hCG supports pregnancy by inhibiting adverse immune responses, facilitating trophoblast invasion, promoting angiogenesis and ensuring the nourishment of the fetus. It has been shown that hCG immunoregulatory properties is through binding to special LH/CG receptors expressed by immune cells (Schumacher et al., 2014). hCG

exerts anti-inflammatory effects in decidual tissues through different mechanisms: 1) increasing the proportion of Tregs; 2) alleviating the proportion of macrophages and neutrophils; 3) inducing an M1→M2 macrophage polarization and also 4) increasing the percentage of decidual Th17 cells (Schumacher et al., 2009).



**Figure 3.** mRNA quantification of cytokines in Ad-MSCs treated with 10 IU hCG after 72hrs: Results showed that hCG preconditioning decreases the inflammatory properties of MSCs. Data are shown as mean  $\pm$  SEM (n=3) and presented as fold change (log 2) of expressions in preconditioned versus untreated cells. One star represents  $p < 0.05$ , two stars represent  $p < 0.01$ , three stars represent  $p < 0.001$  and four stars represent  $p < 0.0001$ .

hCG increases the secretion of macrophage inhibitory factor in human endometrial and granulosa cells. This hormone also enhances the secretion of NO, ROS, IL6, IL12p40 in bone marrow-derived macrophages (Schumacher et al., 2009). Yoshimura and colleagues found that hCG upregulates maturation markers on peripheral blood DCs, stimulates the secretion of inflammatory cytokines and increases T cells activity (Yoshimura et al., 2003).

Moreover, Poloski showed that in addition to its function as a Treg cell attractor, hCG has the potential to stimulate the conversion of non-Treg cells into Treg cells (Poloski et al., 2016). In a previous study, it has been shown that hCG inhibits the upregulation of MHC class II molecules on DCs, diminishes their T cell stimulatory capacity, and induces the secretion of IL-10 and  $TGF-\beta$  *in vitro* (Segerer et al., 2009, Tang et al., 2014). Other studies indicated the effects of hCG on Regulatory B cells (Breg cells). It was shown that hCG not



only increases the number of Breg cells but also enhances the production of IL-10 (Fettke et al., 2016). Another study confirmed the effects of 10 IU/ml of hCG on murine T and B cells. This concentration of hCG could activate B cells, but failed to affect T cells' functionality *in vivo* (Schumacher, 2017). It was already demonstrated that hCG is a strong attractor of neutrophils, monocytes, and lymphocytes at very low doses, and stimulation of monocytes with hCG reinforced the production of IL-8 (Schumacher et al., 2009). hCG anti-inflammatory effects makes it a proper candidate for development of inventive biomimetic strategies toward a pro-regenerative phenotype in case of tissue injuries or inflammations (Han et al., 2016b). Also, during pregnancy, hCG is responsible for the amelioration in the symptoms of rheumatoid arthritis, induction the shift of Th1 mediated cellular immunity to a pre-pregnancy Th2 immunity and increased T cell regulatory activities (Ostensen et al., 2012). In addition, other hormones such as estrogen and testosterone affect immune cells differentiation and function leading to either a pro-inflammatory or an anti-inflammatory phenotype. Testosterone regulates the immune system activities by enhancing Th1 responses, down-regulating NK cells activities and increasing the secretion of anti-inflammatory IL-10. Estrogen modulates B cell functions and switches Th1 responses to Th2 ones (Taneja, 2018).

As we mentioned before, stem cells are extensively used in regenerative medicine applications because of their considerable advantages, especially their ability to release bioactive molecules that were known to have an immunosuppressive capability (Caplan, 2007). The substantial immunosuppressive effects of MSCs are due to their ability for T cell proliferation inhibition and reprogramming of M1 macrophages to M2 cells (Regmi et al., 2019). Furthermore, inflammatory cascades are influenced by active MSCs. They take part in tissue homeostasis through the secretion of trophic factors with anti-inflammatory functions (Lange-Consiglio et al., 2016). It was proposed that various cytokines, growth factors, anti-inflammatory agents, and extracellular vesicles (EVs) are responsible for immunomodulatory features of MSCs (English et al., 2009).

This study was conducted to quantitatively analyze the expression levels of important immunoregulatory genes (including *TGF-β1*, *TSG-6*, *COX-2*, *IL-1β*, and *IL-6*) in hCG primed Ad-MSCs. The secretion of *TGF-β1* by MSCs leads to

Treg generation and confining lymphocyte activation (Regmi et al., 2019). *TGF-β* and interleukin 6 (IL-6) are pleiotropic cytokines that play crucial roles in immune suppression (Basu et al., 2017). hCG-mediated production of IL-6 by DCs affected by different stimulation conditions (Wan et al., 2008). It has been shown that IL-6 is responsible for differentiation of pro-inflammatory M1 cells into the anti-inflammatory M2 macrophages (Wan et al., 2008). IL-1β is a central inflammatory cytokine, which could modulate the inflammatory cytokines through IL-1 receptor/MYD88 and also could be affected by NF-κB signal transduction pathways (Hoesel and Schmid, 2013). TSG-6 is synthesized in response to pro-inflammatory mediators. It has been identified as a critical mediator of anti-inflammatory effects of human MSCs. TSG-6 attenuates inflammatory events through the inhibition of neutrophils invasion into the inflammatory sites (Dyer et al., 2016). It has been shown that IL-1β induces the expression of *TSG6* in multiple cell types, whereas its suppression enhances the level of inflammation. *TSG6* can reduce inflammation by the inhibition of NF-κB signaling (Mittal et al., 2016). *COX-2* is directly responsible for the induction of prostaglandin-mediated inflammation. In line with these studies, targeting *COX-2* by hCG could be proposed as a logical therapeutic strategy for management of inflammatory situations (Basu et al., 2017). *TGF-β* has serine/threonine kinases receptors that directly phosphorylate Smad2/3, and with co-stimulation of IL-1β can induce expression of IL-6 in pericytes (Rustenhoven et al., 2016, Travis and Sheppard, 2014). According to our results, we hypothesized that *IL-6* over expression is a direct or indirect consequence of to the *TGF-β* and/or *IL-1* over expression.

To our knowledge, this is the first study which reports the effects of hCG on immunoregulatory and anti-inflammatory properties of MSCs by evaluating the expression levels of some critical genes. In conclusion, hCG could enhance the anti-inflammatory ability of MSCs accompanied by increased *TGF-β*, *TSG-6*, *IL-1β*, and *IL-6* expressions and decreased *COX-2* expression. Also, it was confirmed that hCG treatment of MSCs had no significant effects on *IL-10* and *TDO2* expression levels (Data not shown). Further studies should be performed to unravel the mechanistic functions of hCG treatment on MSCs transcriptome and proteome networks.

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## Conflict of Interest

Authors declare that there is no conflict of interest regarding the publication of this article.

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