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Vitamin E and hCG enhance the immunomodulatory properties of LPS-induced mesenchymal stem/stromal cells

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ABSTRACT

Mesenchymal Stem/Stromal Cells (MSCs) have been applied to modulate various immune-mediated conditions. Prolonged culture of MSCs in vitro reduces their therapeutic efficacy. Pretreatment of the cells with some chemical agents during in vitro expansion could overcome this limitation. This study intended to determine whether pretreatment of adipose-derived MSCs (ASCs) with Human chorionic gonadotrophin (hCG), a glycoprotein hormone, and Vitamin E, an antioxidant, will improve their immunomodulatory ability. In this regard, ASCs were harvested from human processed lipoaspirate. LPS-induced ASCs were preconditioned with 1 mg of hCG and 600 µM of vitamin E for 24h. TSG-6, COX-2, IL-1β, and IL-6 were assessed at the mRNA level in preconditioned and control groups. ASCs were also co-cultured with peripheral blood mononuclear cells (PBMCs) in vitro to determine the functionality of these cells. Results showed that hCG and vitamin E significantly downregulate the pro-inflammatory COX-2, IL-1β, and IL-6 gene expression, while they did not significantly increase TSG-6 expression. Besides, the co-culturing of pretreated ASCs with PBMCs demonstrated that the amount of PBMCs in treated groups (with hCG and vitamin E) was significantly lower than in control groups. These findings revealed that the preconditioning of ASCs with hCG and vitamin E might enhance their immunoregulatory capacity.

Keywords

hCG, Immune regulation, ASCs, pretreatment, stem cells, Vitamin E

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Abbreviations

hCG : human Chorionic Gonadotropin
LPS: Lipopolysaccharide
MSCs : Mesenchymal Stem/Stromal Cells
ASCs : Adipose-derived Mesenchymal Stem/Stromal Cells
PBMCs : Peripheral Blood Mononuclear Cells
TSG-6 : Tumor necrosis factor-inducible gene 6COX-2 :Cyclooxygenase-2
IL-1β : Interleukin 1 beta
IL6 : Interleukin 6
Tregs : Regulatory T cells
TLRs : Toll-Like Receptors
poly(I: C): PolyInosinic:polyCytidylic acid
LH : Luteinizing Hormone

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Introduction

Mesenchymal stem/stromal cells (MSCs), capable of self-renewal and multi-lineage differentiation, initially attracted biomedical scientists considering their reparative properties to replace damaged cells [1]. Later, the "stromalness" concept attributed most MSCs' therapeutic outcomes to the paracrine and trophic signals [2]. Recently, the role of MSCs as guardians against excessive inflammatory responses describes many of the observed favorable effects [1, 3]. Further, it became apparent that the immunomodulatory functions of MSCs are licensed by their surrounding inflammatory environment [3]. Among all human tissues which could be regarded as sources of MSCs, adipose tissue became an attractive one that was mainly due to less invasive harvesting methods, high proliferation properties, and considerable stromal function [2, 4].

To acquire sufficient cells for administration, they should be cultured for a long time, which may change their properties and make them unsuitable for clinical applications [5]. On the other hand, after systematic administration, inflammation conditions or harsh *in vivo* environments may impair cell therapeutic response, viability, homing, and biological potentials [6, 7]. The physiological features of Adipose-derived Mesenchymal Stem/Stromal Cells (ASCs) should be restored to enhance their survival rate and resolve these problems [8].

Diverse methods are developed to improve the functional properties of MSCs, such as preconditioning of the cells with bioactive molecules, genetic manipulation, and modification of the culture condition [8, 9]. In several previous studies, investigators explored the preconditioning effects of different agents on MSCs [10-13]. Moreover, the immunomodulatory ability of Naïve MSCs is low in unstimulated conditions; thus, MSCs need to be activated with proinflammatory stimuli like IFN-γ or lipopolysaccharide (LPS), or an immunosuppressive Toll-like receptor 3 (TLR3) agonist polyinosinic-polycytidylic acid (poly(I: C)), to gain their full biological potential [14-16].

For about a century, α-tocopherol (vitamin E) has been known as a potent antioxidant and a fat-soluble vitamin [17, 18]. This vitamin could trap oxygen and nitrogen radicals and protect the cell membrane by protecting polyunsaturated fatty acids (PUFAs) (19). Azzi first explained the non-antioxidant properties of this vitamin [20]. Many studies revealed that it could also modulate cell signaling and gene expression [21]. The immunomodulatory potential of vitamin E also has been reported *in vivo* [22, 23].

Accumulated evidence indicated a close relationship between inflammation and oxidative stress, one of which can promote another. Reactive species can raise proinflammatory gene expression by arousing intracellular signaling cascade. On the other hand, inflammatory cells could generate more reactive oxygen species (ROS), resulting in augmented oxidative stress at the site of inflammation. Treatment with antioxidants is thought to be a desirable approach to prevent inflammatory diseases caused by oxidative stress [24].

As the most abundant and essential free radical scavenger, Vitamin E plays its antioxidant role by decreasing overall oxidative stress. The protective role of vitamin E therapy in inflammatory diseases like atherosclerosis and diabetes mellites was established by Elbeltagy and Alshiek et al. [25, 26].

Human chorionic gonadotrophin (hCG) is a glycoprotein hormone that plays a significant role in maintaining a pregnancy [27]. Besides organs of the reproductive system, receptors for hCG shared with luteinizing hormone (LH) were represented on T and B lymphocytes and macrophages (28). The binding sites of hCG on non-reproductive tissues indicate additional roles, such as immune modulation [29]. This hormone can regulate T cells, dendritic cells, and natural killer cells and enhance vascularization [30-32]. Fuchs first evidenced the immunosuppressive effects of hCG in 1980 [33]. It was also used as a therapeutic agent in autoimmune diseases, e.g., rheumatoid arthritis [34]. The effect of hCG on stem cells may be attributed to its receptor on MSCs as they have been previously described [35].

In addition to the integral hCG molecule, linear tetrapeptide originating from the β-hCG, AQGV, exert a potential novel immune-modulatory effect [36]. This synthetic oligopeptide, currently developed under the product name EA-230 has been shown to inhibit the inflammation, disease severity, and mortality in high-dose LPS-induced inflammatory response [37]. Pramanik found that Nuclear factor-kappa B (NF-κB), the master regulator of many proinflammatory genes, was downregulated after treatment with hCG [38]. Therefore, in line with Bai results, hCG downregulated the mRNA expression of IL-1β and IL-6 and could inhibit the production of pro-inflammatory cytokines, which can help reduce inflammatory symptoms and Improvement of autoimmune diseases [39].

Abbreviations-Cont'd

ROS : Reactive Oxygen Species
NF-κB: Nuclear Factor kappa B
PBS = Phosphate-Buffered Saline
PUFAs = PolyUnsaturated Fatty Acids
DMEM = Dulbecco's Modified Eagle's Medium
RT-qPCR = quantitative Real-Time Polymerase Chain Reaction
XTT = XML Tunneling Technology.

The current study aimed to determine whether pretreatment of human ASCs with hCG and vitamin E can decrease the pro-inflammatory status of MSCs caused by LPS and enhance their immunomodulatory potential.

Results

Characterization of ASCs

Isolated cells from adipose tissues were successfully cultured and passaged *in vitro*, and they exhibited spindle-shaped morphology, as shown in Figure 1A. Osteogenic and adipogenic differentiation of the cells was determined by the appearance of calcium deposits and lipid droplets, respectively (Figures 1B, 1C, and 1D).

In addition, ASCs were characterized by positive expression of CD90 (98.7%), CD44 (99.3%), CD73 (98.9%), CD13 (99.0%) and negative expression of CD14 (1.21%), HLA-DR (1.08%), CD45 (1.11%) and CD34 (1.71%), and surface markers (Figure 1E).

Cell viability

Effects of hCG and vitamin E on ASCs viability were investigated using our established protocol, which demonstrated that preconditioning of the cells with these substances does not exert remarkable toxicity against ASCs (40, 41).

Increased secretion of proinflammatory cytokines in LPS-primed ASCs

Human ASCs were incubated with 5µg/ml of LPS for 4 hours. First, we observed the morphology of LPS-primed ASCs, which were spindle-shaped (Figure 2A).

The expression of genes related to the inflammation in LPS-primed ASCs was determined by qRT-PCR, as presented in Figure 2B. The results demonstrated that LPS increased mRNA expression of Cox-2, TSG-6, IL-1β, and IL-6 compared to unstimulated ASCs as the negative control (Figure 2B). These data confirm that LPS leads to the induction of inflammatory response in ASCs.

Treatment of LPS-primed ASCs with hCG and vitamin E

To evaluate the effects of hCG and vitamin E, we incubated LPS-primed ASCs with 10 IU hCG for 48 h and 600 µM vitamin E for 48 h. The morphology of ASCs after LPS-priming and pretreatment with hCG and vitamin E was similar to the control group and displayed a fibroblastic-like appearance (Figure 3A and 3B).

The mRNA expression of inflammatory cytokines in pretreated LPS-primed ASCs was determined by RT-qPCR. The overexpression of TSG-6, Cox-2, IL-1β, and IL-6 in LPS-primed ASCs was downregulated by pretreating cells with 10 IU hCG (Figure 3C).

Besides, vitamin E treatment of LPS-primed ASCs significantly reduced the expression level of Cox-2, TSG-6, IL-1β, and IL-6 (Figure 3D). These data demonstrated that after treatment with hCG and vitamin E, the increase of inflammatory cytokines in LPS-primed ASCs was significantly reversed so that these treatments could increase the immunomodulatory properties of LPS-primed ASCs compared to the control group.

The effect of pretreated ASCs on inhibition of PBMCs proliferation

We evaluated the proliferation of PBMCs co-cultured with treated ASCs using XTT colorimetric assay. We considered activated PBMCs proliferation as 100%, and the proliferation inhibition percentage of co-cultures was calculated using the below equation. The significance of differences among data was examined at the confidence level of 95% ($p < 0.05$) using the *t*-test. All experiments were performed in triplicate. Proliferation ratio: (Co-culture-OD / PBMCs-OD) × 100. Results demonstrated that the proliferation of activated PBMCs (stimulated with PHA) was inhibited when co-cultured with ASCs (Figure 4A). The proliferation retardation percentage is 25.2% that is statistically significant ($p < 0.05$). However, hCG-treated ASCs could reduce the proliferation ratio of PBMCs more than untreated ASCs, down to 64.09%, and that is highly significant ($p < 0.01$) (Figure 4A). Also, Ethanol treated ASCs as the control group of vitamin E pretreatment, could significantly reduce the proliferation of PBMCs (24.94%), and the inhibition activity of vitamin E treated ASCs was stronger than its control group with a retardation percentage of 50.6% and is statistically significant ($p < 0.001$) (Figure 4B).

Differences in proliferation in hCG and vitamin E treated ASCs under the co-culture conditions were significant compared to their control groups untreated and ethanol-treated ASCs, respectively ($p<0.05$) (Figure 4A (a) and 4B (b)).

Discussion

MSCs appear to be a decent choice for cellular therapy of immune-mediated disorders [42]. Over prolonged preparation passages, MSCs may lose their therapeutic efficiency [43]. Studies have shown that various surrounding microenvironments affect MSCs' paracrine signaling and polarize them to proinflammatory or anti-inflammatory phenotypes [44, 45]. Pretreatment of the cells with some chemical

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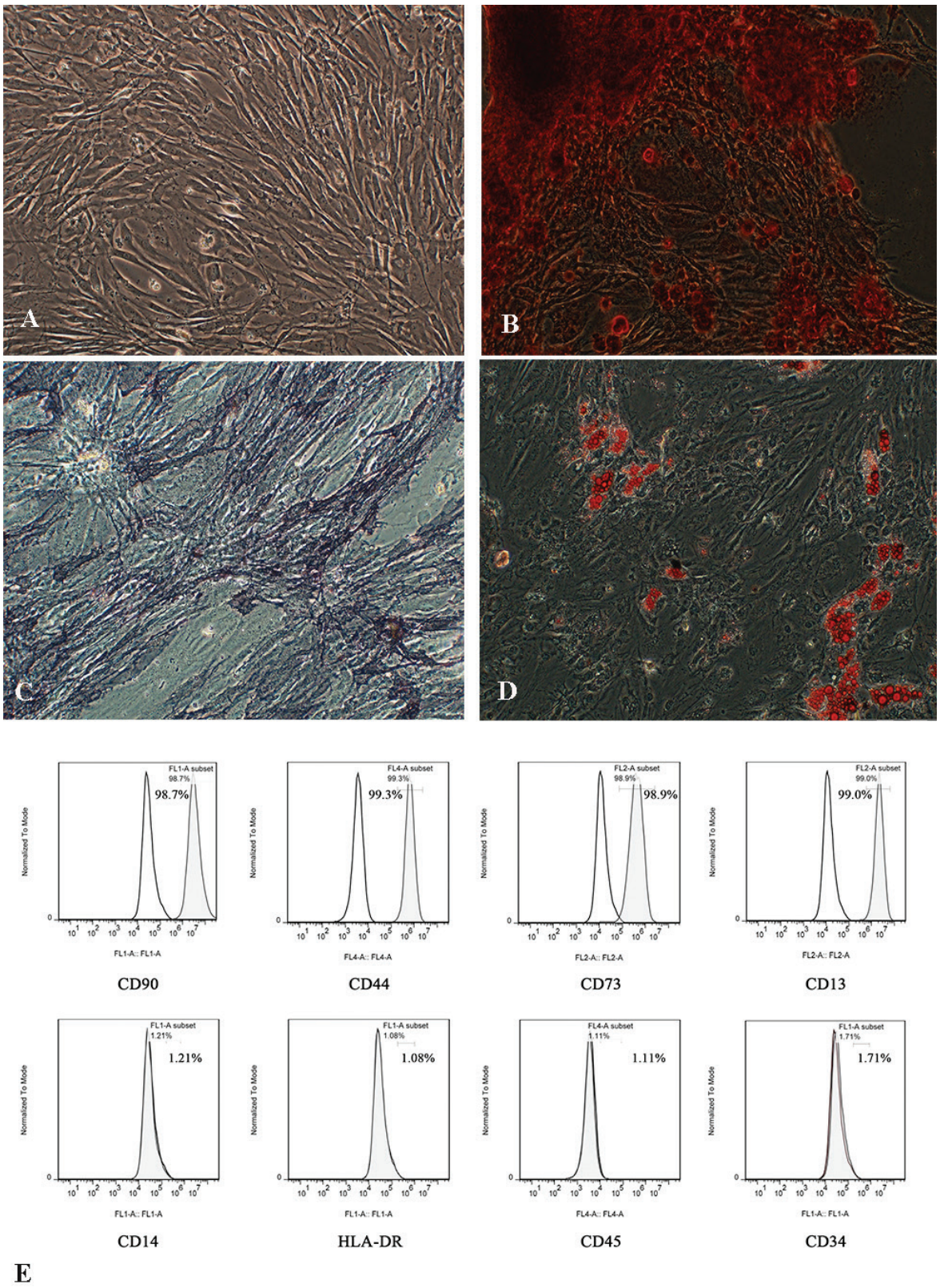

Figure 1.
Characterization of human Ad-MSCs (A) undifferentiated human Ad-MSCs represent spindle-shaped morphology (Scale bar = 200 µm). (B) Osteogenic differentiated human Ad-MSCs were stained with alizarin red (Scale bar = 100µm). (C) Alkaline phosphatase assay was used to confirm osteogenic differentiation (Scale bar = 200µm). (D) Adipogenic differentiated human Ad-MSCs were stained with oil red O (Scale bar = 100µm). (E) Flow cytometric analysis showed that almost all cultured Ad-MSCs expressed CD90 (98.7%), CD44 (99.3%), CD73 (98.9%), CD13 (99.0%), whereas a small portion of the cells expressed CD14 (1.21%), HLA-DR (1.08%), CD45 (1.11%) and CD34 (1.71%). Expressions of cell surface markers of Ad-MSCs are shown as compared with their respected isotype controls.

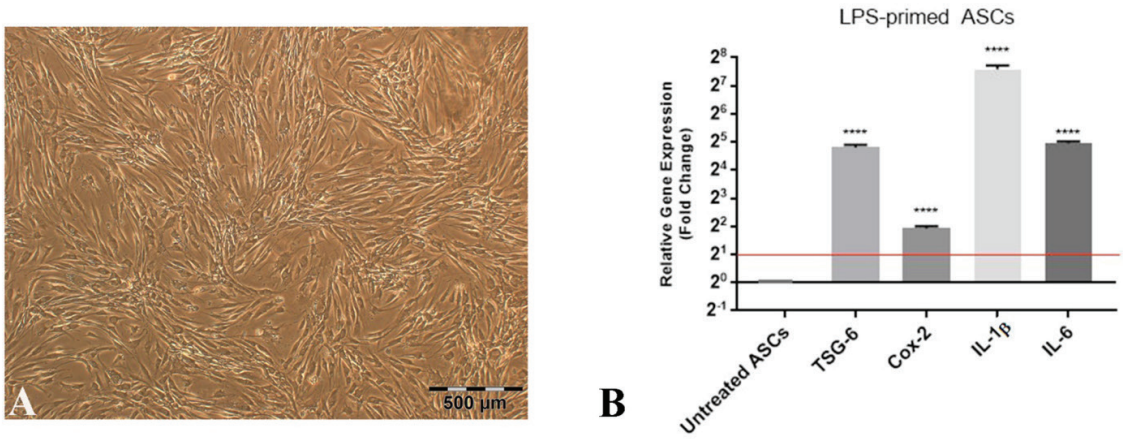

Figure 2.
Effect of LPS-primed Ad-MSCs on expressions of genes involved in inflammation. (A) Spindle-shaped morphology of LPS-primed Ad-MSCs (Scale bar = 500µm). (B) mRNA quantification of cytokines in LPS-primed Ad-MSCs: results showed that LPS pretreatment increases the inflammatory properties of Ad-MSCs. Data are illustrated as mean ± SEM (n=3) and presented as fold change (log 2) of expressions in preconditioned versus untreated cells. Four stars represent *p*-value < 0.0001.

agents during *in vitro* expansion helps overcome these limitations by restoring the physiological activities and enhancing their biological potency [46]. It is estimated that pretreatment of MSCs isolated from different sources might result in variable responses [47]. Here, we used LPS-primed ASCs. Our observations demonstrated that the licensing of ASCs by LPS and their pretreatment with hCG and vitamin E could significantly alter immunoregulatory genes' expression and enhance these cells' anti-inflammatory potential in vitro.

Toll-like receptors (TLRs) perceive danger alarms from various pathologies. Their activation recruits immune cells to the site of injury. Waterman observed that MSCs could be recruited likewise. MSCs express TLR3 and TLR4. They discovered that the engagement of some TLR-agonists could intensely regulate their migration and secretion of immune-modulating agents. They also identified that TLR4-primed MSCs, generally exert proinflammatory mediators, while TLR3-primed MSCs express mostly immunosuppressive ones [48]. It suggested that stimulation of TLR4 with Lipopolysaccharide (LPS) could mimic a proinflammatory milieu [49, 50]. In this investigation, we employed LPS-priming to provide a proinflammatory signature of ASCs. In agreement with our observations, LPS was shown to affect ASCs by overexpression of inflammatory cytokines, namely, TSG-6, COX-2, IL-1β, and IL-6 [51].

TSG-6 has been identified as a critical mediator of the anti-inflammatory effects of human MSCs. TSG-6 constricts inflammatory responses by inhibiting neutrophils' invasion into the inflammatory sites [52]. Roddy determined that the siRNA knockdown of TSG-6 impeded these cells' anti-inflammatory functions on damaged corneal epithelial cells [53].

COX-2 is a pivotal enzyme in prostaglandin E2 synthesis, a well-known participant in various autoimmune diseases like rheumatoid arthritis [54, 55], and COX inhibitors are considered therapeutic targets for inflammation-mediated disorders [56, 57]. IL-6 is considered a pleiotropic proinflammatory cytokine involved in various physiological functions, including inflammation via transcription (STAT) activators, signal transducers, and NF-κB signaling pathways. There is evidence that blockade of IL-6 prevents the progression of autoimmune diseases and tumor formation [54, 55]. IL-1β is also a pro-inflammatory cytokine regulated through the NF-κB pathway [58]. These two partners are involved in many inflammatory conditions, and they are considered therapeutic targets for inhibitory agents [59, 60]. Our data showed that pretreated LPS-primed ASCs with hCG and vitamin E exert anti-inflammatory and immunomodulatory activities. In particular, LPS-primed ASCs preconditioned with hCG, and vitamin E reduced the expression of proinflammatory COX-2, IL-1β, and IL-6 genes and maintained anti-inflammatory expression TSG-6 gene.

Our study also demonstrated that the co-culturing of pretreated ASCs with PBMCs significantly reduced proinflammatory and anti-inflammatory Gene expressions in treated groups (with hCG and vitamin E) compared with the untreated groups. Bofeng Li, in 2018, found that IL-6 has a substantial role in T cell expansion and promotes T cell proliferation [61]. It could be suggested that the amount of T cells reduced as a result of IL-6 reduction.

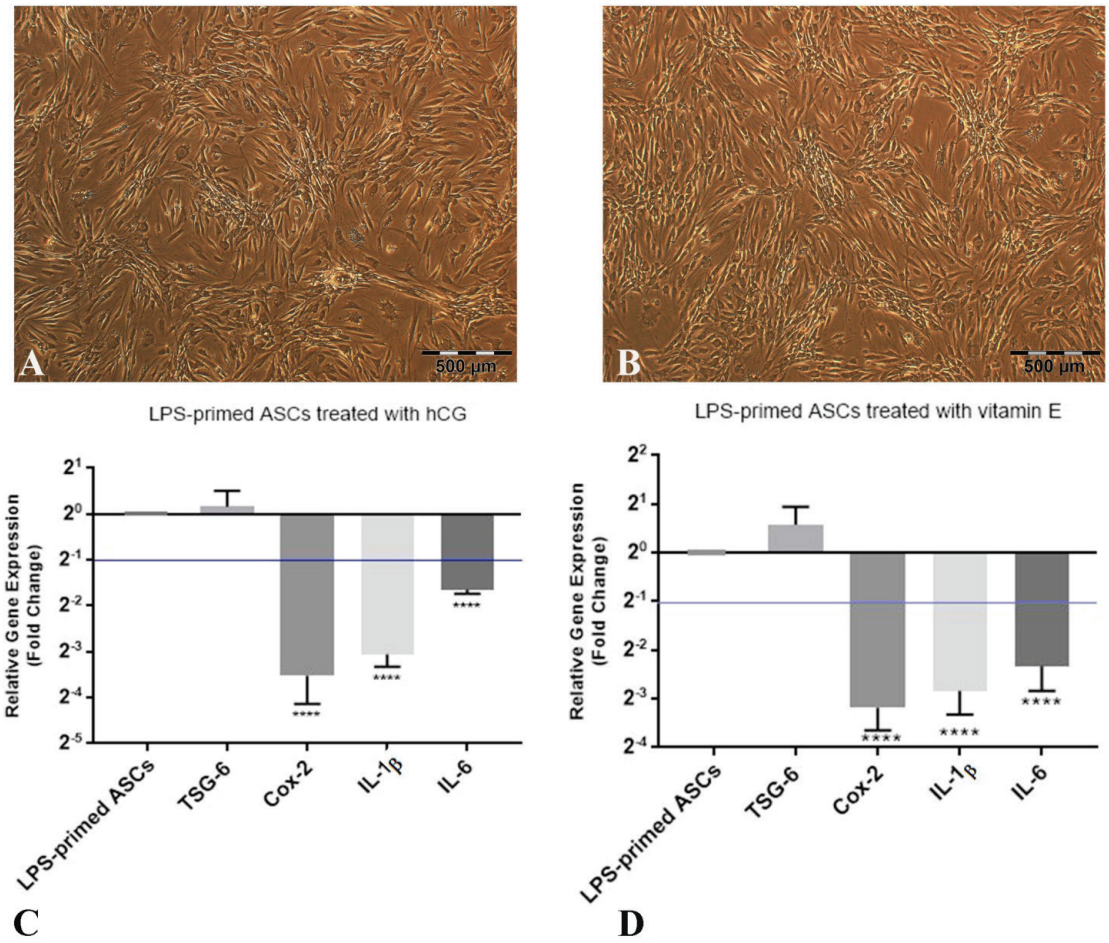

Figure 3.
Effect of hCG and vitamin E on the expression of genes involved in inflammatory .... in LPS-primed Ad-MSCs. (A and B) Spindle-shaped morphology of hCG and vitamin E treated LPS-primed Ad-MSCs, respectively (Scale bar = 500µm). (C) Treatment of LPS-primed Ad-MSCs with hCG decreases the mRNA levels of the inflammatory-related genes. (D) Vitamin E treatment reduces the expression of inflammatory cytokines in LPS-primed Ad-MSCs. Data are shown as mean ± SEM (n=3) and presented as fold change (log 2) of expressions in preconditioned versus untreated cells. Four stars representa *p*-value < 0.0001.

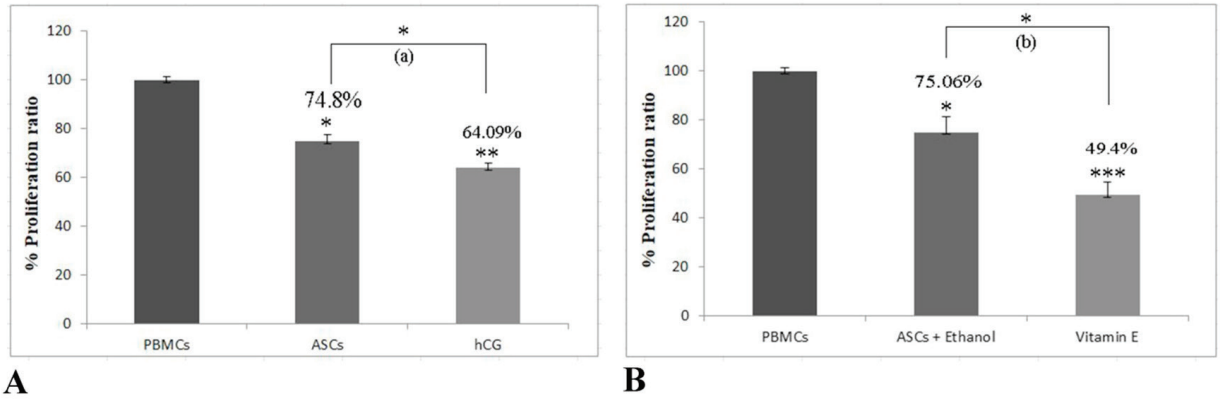

Fig 4.
Effects of treated Ad-MSCs on PBMCs proliferation. PBMCs were co-cultured with treated and untreated Ad-MSCs, and their proliferation was assessed using XTT. (A) PBMCs numbers were significantly reduced when they were co-cultured with untreated Ad-MSC (25.2%) and hCG (35.91%), and (B) Ethanol treated Ad-MSCs (24.94%), and vitamin E treated Ad-MSCs (50.6%) compared to the control group (PHA-PBMCs). Error bars display the mean ± SD, n=3, (*p < 0.05, **p < 0.01, ***p < 0.001).

In conclusion, hCG and vitamin E preconditioning were implied to improve the anti-inflammatory and immunomodulatory capacities of LPS-primed ASCs. It could be at least in part through inhibiting the expression of proinflammatory cytokines. Hence, it could be regarded as a potential therapeutic strategy.

Materials & Methods

ASCs isolation and culture

Liposuction aspirated wastes from subcutaneous adipose tissues were obtained from three healthy individuals undergoing liposuction surgery after getting informed consent at a cosmetic day clinic in Mashhad, Iran. The ACECR-Khorasan Razavi Biomedical Research Ethics Committee has approved this research (Code: IR.ACECR.JDM.REC.1398.008-009).

The aliquots of fat (200 ml) were washed 3-4 times with equal volume phosphate-buffered saline (PBS) supplemented with 0.1% penicillin-streptomycin (pen-strep) (Biosera, France), and then incubated for 1 hour at 37°C with freshly prepared 0.1% collagenase type I (Invitrogen, USA). They were shaken robustly and repeatedly for 5-10 seconds. Fetal bovine serum 10% (FBS; Gibco, USA) was applied for collagenase I inactivation. To remove adipose cell debris, we centrifuged them at room temperature (600 × g, 10 min).

Following washing the pellet with PBS and centrifugation (400 × g, 6 min), the collected cells were seeded in tissue culture flasks containing Dulbecco's Modified Eagle's Medium (DMEM, Biowest, France) supplemented by 10% FBS and 0.1% pen-strep, and kept in a 5% CO2 incubator at 37°C. ASCs were purified based on their plastic-adherent capacity, and the medium exchange was performed twice a week. Cells were trypsinized (0.025%, Gibco, USA) following reaching confluency of 85 to 90%. ASCs at the third passage were employed in all the following experiments.

Characterization of ASCs

The Flowcytometric approach was applied to identify mesenchymal lineage-specific surface markers (BD Accuri C6, USA). A suspension of 2×105 cells was 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 45 min (all from Cytognos, Spain). Data analysis was carried out via FlowJo (7.6.1) software.

The multi-lineage potential of human ASCs was confirmed by inducing their differentiation into adipogenic and osteogenic lineages using the appropriate culture conditions. Briefly, adipogenesis was induced through the culture of ASCs in the presence of DMEM supplemented by 10% FBS, 200 mM indomethacin, 1 mM dexamethasone, and 10 mM β-glycerophosphate (all from Sigma Aldrich, Germany). After 14 days, the cells were rinsed with PBS and fixed in 10% formalin solution. The differentiation induction level was evaluated by applying oil red O staining (Sigma Aldrich, Germany) to indicate intracellular lipid droplets.

Additionally, osteogenic differentiation of the cells was evaluated qualitatively based on the cytochemical analysis. To do so, ASCs were incubated with osteogenic inductive media, including 50 mM ascorbate-2- phosphate (Sigma Aldrich, Germany), 10 mM β-glycerophosphate, and 0.1 mM dexamethasone for 21 days. They were then fixed and stained with alizarin red (Sigma Aldrich, Germany) to confirm the extracellular matrix's presence of calcium mineralization, secreted by differentiated cells.

Priming ASCs with LPS

Typically, ASCs were grown to 60-70% confluence in the growth medium before the start of an experiment. Lipopolysaccharide (LPS) was added (5µg/ml) to a fresh growth medium and incubated with the cells for 4 hours (Gibco, USA). Afterward, cells were washed with PBS and used for the subsequent analysis.

Treatment of ASCs with hCG and vitamin E

LPS-primed ASCs were treated with hCG (1 mg, Homapharmed, Iran) and vitamin E (600 µM, Sigma, Germany) for 24h. Non-treated and exclusive LPS primed ASCs are used as controls. After treatment, cells and culture medium were collected for the experiment

Table 1.
Primer sequences used for RT-qPCR.

Target Gene	Sequence	Product size (bp)
RPLP0 (NM_053275.4)	F: TGGTCATCCAGCAGGTGTTCGA	119
	R: ACAGACACTGGCAACATTGCGG	
TSG-6 (NM_007115.3)	F: GCTGCTGGATGGATGGCTAAG	156
	R: CTCCTTTGCGTGTGGGTTGTAG	
COX-2 (NM_000963.3)	F: CCAGAGCAGGCAGATGAAATACC	168
	R: ACCAGAAGGGCAGGATACAGC	
IL-1β (NM_000576.2)	F: CCTCTCTCACCTCTCCTACTCAC	186
	R: CTGCTACTTCTTGCCCCCTTTG	
IL-6 (NM_000600.4)	F: ACTCACCTCTTCAGAACGAATTG	196
	R: GCAAGTCTCCTCATTGAATCCAG	
IL-10 (NM_000572.2)	F: GAGATGCCTTCAGCAGAGTGAAGA	114
	R: AGGCTTGGCAACCCAGGTAAC	

RNA extraction and quantitative PCR

Total RNA was extracted using Tripure reagent according to the protocol provided by the manufacturer (Roche, Germany).

The purity and concentration of RNA samples were detected using Nanodrop ND-1000 spectrophotometer (Bio-Tek, USA), and the integrity of RNA samples was analyzed by gel electrophoresis.

Total RNA samples were treated by one unit of DNase I (Thermo Fisher Scientific, USA) to avoid genomic DNA contamination. Afterward, 1µg of total RNA was used for first strand cDNA synthesis using PrimeScript RT reagent Kit (Cat. #RR037A, TaKaRa) according to manufacturer's protocol.

The RT-qPCR 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 duplicates. The ribosomal protein lateral stalk subunit P (RPLP0) gene was used as an internal control to normalize the expression level of the target genes. Primers were designed by AlleleID 6 software and are shown in Table 1.

To confirm PCR efficiencies for each gene we used pooled cDNA in 10-fold dilution series and used the $2^{-\Delta\Delta Ct}$ method for gene expression when the calculated slope was about ~ -3.3 equals to E = 95-105%.

Co-culture and lymphocyte proliferation assay

The pretreated ASCs effect on peripheral blood mononuclear cells (PBMCs) proliferation was investigated in the co-culture model.

Human PBMCs were isolated from healthy donors' blood using Ficoll-Paque (Biowest, Canada) density gradient centrifugation. The cells were stimulated with 205 mg/mL phytohemagglutinin for mitogenic stimulation (PHA, Sigma, Germany).

The ASCs were plated at 4×10^4 cells in a 48-well plate and left to adhere overnight. The next day, 1 mg hCG and 600 µM vitamin E were added to several wells for 48 hours.

Then, 10^5 stimulated PBMCs were added to each well and co-cultured with ASCs for 72 hours in a ratio of 1:1 of RPMI-1640: DMEM medium supplemented with 10% FBS and 0.1% penicillin-streptomycin (Biowest, Canada). Well groups are shown in Table 2.

The inhibition of PBMCs proliferation was measured using the 2H-tetrazolium salt XTT colorimetric method according to the manufacturer's instruction (Santa Cruz, USA).

Statistical analysis

The GraphPad Prism statistical program (version 7) and two samples *t*-test was used for data analysis. The values are reported as the mean of at least three independent experiments ± SD. Events with *p* values less than 0.05 were considered significant.

Table 2.
Well groups of proliferation co-culture.

Control groups	Sample groups
PHA stimulated PBMCs	-
Untreated ASCs	hCG treated ASCs
Ethanol treated ASCs	Vitamin E treated ASCs

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Authors' Contributions

Conception: MF, HRB, and HH. Planning: HH, MF, NSM, MMM, and MKN. Carrying out: SSH, SM, HH, and MKN. Writing: SM, SSH, HRB, and HH.

Conflict of interest

The authors declare no competing interest.

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