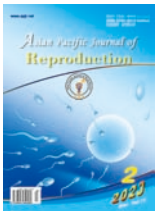

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Cytokine changes and embryo attachment in mouse endometrial cells following treated with peripheral blood mononuclear cells (PBMCs) expressing ectopic hCG, and hCG-activated PBMCs

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ABSTRACT

Objective: To compare the effect of human chorionic gonadotropin (hCG)-producing peripheral blood mononuclear cells (PBMCs) and PBMCs activated by hCG *in vitro* and expressions of related immune genes in mouse implantation.

Methods: hCG-producing PBMCs (transfected PBMC) and PBMCs activated by hCG *in vitro* were introduced into isolated mouse endometrial cells, while cell cultures were divided into four groups: the control, PBMC, transfected, and activated PBMC groups. The expression of studied genes (*IL-1β*, *IL-6*, *Lif*, and *Vegf*) was evaluated and blastocyst attachment on the cocultured cells (isolated endometrial cells and PBMC cells) was monitored in all four groups.

Results: Data showed that expression decreased in the PBMC group compared to the treated PBMC (transfected and activated PBMCs) and increased in transfected PBMC compared to the activated PBMC. Attachment and migration of blastocysts were dramatically enhanced in the transfected PBMC group compared to the activated PBMC group ($P<0.05$).

Conclusions: Use of hCG-producing PBMCs (transfected PBMC) has more influence on endometrial receptivity.

KEYWORDS: Peripheral blood mononuclear cells; hCG; Embryo attachment; Immune response; Immune cells; *In vitro*; Expression gene

1. Introduction

The use of infertility methods creates the cost of infertility drugs and monitoring. *In-vitro* fertilization (IVF) and other artificial reproductive technologies cause stress for sick women under treatment[1,2]. The use of the best method to reduce the rate of embryo implantation failure can be of great help to patients stricken by infertility. One of the factors that are involved in pregnancy and embryo implantation is the presence of maternal immune cells at the site of embryo implantation[3,4]. Cell-based therapy by the creation of a dynamic and individual interaction can be an efficient method for patients with implantation failure which may be the reason of pathophysiological conditions of these patients[5]. During the maintenance of the embryo in the uterus, the immune microenvironment of the uterus plays an important role, while immune cells and cytokines are the main regulators. Mammalian peripheral blood mononuclear cells (PBMCs) are

Significance

Repeated embryo implantation failure are an extremely frustrating condition for both patients and clinicians, which significantly hampers *in vitro* fertilization success. Immune cell therapy with peripheral blood mononuclear cells (PBMCs) can be an effective approach for the enhancement of endometrial receptivity. The study reveals that the embryo attachment showed a significant increase in presence of PBMCs and hCG, and the expression of endometrial genes changed following therapy with PBMCs and hCG. Use of PBMCs that have the ability to produce hCG can be more effective in increasing embryo implantation than activated PBMCs with hCG.

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made up of lymphocytes (B and T cells) and monocytes. PBMCs are responsible for production of inflammatory cytokines such as interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α), which positively impacts endometrium and endometrial receptivity. These cells regulate invasion for the establishment of the hemochorial placentation and provide suitable immune tolerance for embryo implantation[3,4]. The effects of immune responses on embryo implantation could be like endocrine hormones such as human chorionic gonadotropin (hCG) which have an important role in pregnancy. Yoshioka *et al* found that intrauterine injection of hCG-exposed and activated PBMCs significantly increased pregnancy rate and live birth in patients with recurrent implantation failure[6]. Yu *et al* showed that PBMC activated by hCG remarkably enhanced the number of invasive JAR cells in an invasion assay without affecting proliferation. Also, hCG-activated PBMC upregualted matrix metalloproteinase-2 (MMP-2), MMP-9, and vascular endothelial growth factor (Vegf) and downregulated tissue inhibitor of metalloproteinase-1 (TIMP-1) and TIMP-2 in JAR cells in a dose-dependent manner[7]. Therefore, the use of immune cell therapy using maternal PBMCs to improve the outcome of IVF has been suggested. The hCG stimulates the production of cytokines by the PBMCs[1], and according to various studies, PBMCs can regulate embryo implantation by controlling the balance of Th1/Th2 cytokines through the production of cytokines such as IL-1 and IL-8[1,8]. In this study, we compared the effect of the hCG-producing PBMCs and activated PBMC with hCG *in vitro* on endometrial receptivity and expressions of related immune genes [*IL–6*, *IL–1β*, leukemia inhibitory factor (*Lif*), *Vegf*] in mice implantation.

2. Materials and methods

2.1. Materials

All chemicals were acquired from Sigma Chemical Corporation (St. Louis, MO), if not otherwise specified. Figure 1 demonstrates the schematic representation of procedures used in this study design. The experimental and animal care methods performed in this study were in accordance with the guidelines provided by the CPCSEA and World Medical Association Declaration of Helsinki on Ethical Principles for Medical Research. We have received the code of ethics for the ethics committee permission and ethical practices from our respective university.

2.2. Animals

A total of 50 (15 male and 35 female) B6D2F1 (C57BL/6 DBA/2) mice were obtained from Pasteur institute (Tehran, Iran). The average weight was 15.5 g and 14.5 g for female and male, respectively. The prepared mice were kept in controlled room for standard temperature (22℃–28℃) and light conditions (12 h light: 12 h dark) for more than a week, and the standard diet of food and water was followed for them.

2.3. Cloning of protein–coding hCG gene into an expression vector

The *hCG* gene fragment was cloned into plasmid expressing green fluorescent protein-N1 (pEGFP-N1) expression vector (Bioneer,

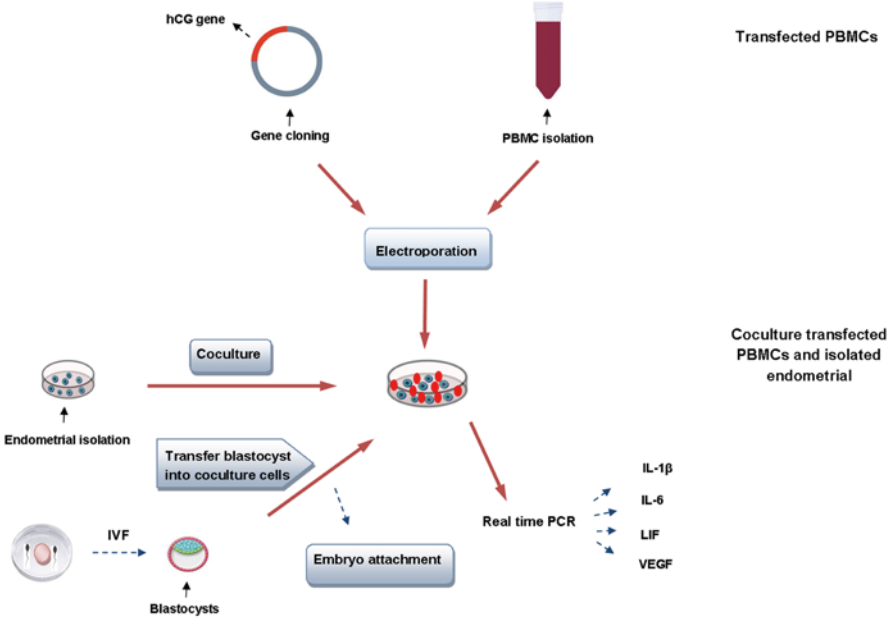

Figure 1. Our schematic plan used in this study. The recombinant vector is transfected to isolated PBMC cells. This study has two main groups: transfected PBMC with recombinant vector group and activated PBMCs created by incubation PBMCs with hCG *in vitro*. The embryos obtained from the IVF procedure are used in embryo attachment assay. Endometrial cells are isolated from the mouse uterus. Embryo attachment in cocultured cells (PBMCs and endometrial cells) is investigated in the studied groups. After the treatment of endometrial cells with PBMC and HCG, the expression of studied genes is assessed by real-time PCR. PBMC: peripheral blood mononuclear cell; hCG: human chorionic gonadotropin; IVF: *in–vitro* fertilization; Lif: leukemia inhibitory factor; Vegf: vascular endothelial growth factor; IL-6: interleukin 6; IL-1β: interleukin 1β.

Korea). Digestion of pEGFP-N1 vector was done by the BamHI and EcoRI enzymes (Takara, Dalian, China). DNA extraction and purification were done with a DNA extraction kit (Qiagen, Hilden, Germany). The CaC_{12} transformation method was used to transform the ligated recombinant vector (containing *hCG* gene) into competent cells. Finally, confirmation of successful cloning was explained using restriction enzyme digestion and sequencing methods (Sanger Sequencing, ABI3500 Genetic Analyzer, CA, USA).

2.4. Isolation of PBMC, transfection with an expression vector containing hCG gene and produced hCG by transfected PBMCs

Peripheral blood samples were obtained from female mice (ethics code: IR.SBMU.RETECH.REC.1396.1297) and PBMC isolation was performed using the Ficol density gradient according to the standard protocol. Next, PBMCs containing both adherent and non-adherent cells were cultured in RPMI-1640 (Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS). For PBMCs transfection, approximately 1×10^6 cells/mL PBMCs were centrifuged and diluted in an electroporation buffer (pH 7.2). Expression vector (pEGFP-N1-hCG) at a final concentration of 10 μg was added to buffer-diluted PBMC cells. The mixture was placed into a 0.2 cm cold electroporation cuvette, and electroporation was performed at 500 us/pulse 2/500 V using an Eppendorf AG 22331 electroporation instrument (Eppendorf, Humburg, Germany). The concentration of produced hCG was assessed every 24 h for a period of 72 h, and measured by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Biocompare, USA). hCG protein content was measured in the 1×10^6 cells/mL suspension.

2.5. Activated PBMCs with hCG

To prepare activated PBMCs, 1×10^6 cells/mL PBMCs were incubated with 10 IU/mL of hCG in the RPMI-1640 medium.

2.6. Oocyte collection

To obtain a large number of oocytes from 6-8 week female mice, we superovulated female mice with peritoneal peritoneum with 10 IU pregnant mare serum gonadotropin (PMSG) by intraperitoneal injection. After 48 h, 10 IU of hCG was injected in the same way. 16 h after the last injection, mice were killed *via* cervical dislocation and the cumulus-oocyte complex was collected from the oviduct ampulla and immediately transferred into a flushing holding medium and were released in droplets of a tubal fluid medium and coated with mineral oil.

2.7. Sperm collection

To obtain mature sperm, 7-week-old BDF1 male mice were killed by cervical dislocation. Mature spermatozoa were removed from the cauda epididymis and vasa deferentia, and sperm suspension was transferred to the dish that contained human tubal fluid medium supplemented with 4 mg/mL bovine serum albumin. The dish containing the sperm was kept in an incubator at 37℃ with 5% CO_2 for 45 min.

2.8. IVF

For IVF, 1×10^6 sperm/mL was inoculated to 100 μL of the tubal fluid medium that contained the matured MⅡ oocytes and incubated for 6 h under the same conditions. After this time, the hypothetically fertilized oocytes were washed well in flushing holding medium and then moved to potassium simplex optimized medium supplemented with 4% bovine serum albumin. 24 h later, the fertilization rate was checked through counting the number of two-cell embryos. Finally, 98 h after IVF, the oocytes were observed under an optical microscope (LW Scientific Z4 Embryo-GLO Stereo Microscope, 7× to 45× magnification) for blastocyst formation rate[9].

2.9. Mouse embryo attachment assay

2.9.1. Endometrial cell preparation

To prepare cell cultures from endometrial cells, uterine tissues were isolated from day-8 pseudopregnant mice. For this purpose, the removed tissues were diced and then placed in a 0.25% collagenase (Type Ⅳ) solution and digested for 60 min at 37℃. To stop the enzymatic digestion reaction by collagenase, Dulbecco's modified Eagle's medium (DMEM)/Fl2 (Gibco-BRL) was enriched with 10% FBS. The cells were washed by centrifugation (at 470×*g*, for 5 min at room temperature) in DMEM/F12 containing 105 FBS three times.

2.9.2. Coculture of endometrial cells and PBMC cells

For the coculture, PBMC cells (1×10^6 cells/mL) were transferred on a layer of epithelial and stromal cells in a single well of a four-well plate and were incubated at 37℃ and 5% CO_2 for 72 h. The medium used for the coculture of cells was DMEM/F12 medium with 10 nmol/L of estradiol, 100 nmol/L of progesterone, and 10% FBS. The experimental groups were divided into four groups: endometrial cell (the control group), endometrial cell with PBMC cells (the PBMC group), endometrial cell with hCG-producing PBMC (the transfected PBMC group), and endometrial cell incubated with hCG (the activated PBMC group).

2.9.3. Assessment of blastocyst attachment

To evaluate the blastocyst attachment, the zona pellucidae of the blastocysts were eliminated by acidic Tyrode's solution. Then, the blastocysts were added to the coculture cells and kept for 72 h under the same conditions as above. Every 24 h, blastocyst adhesion and migration on the cells was checked by microscopic technique.

Table 1. List of primers employed in a quantitative real-time polymerase chain reaction (RT-PCR).

Primer name	Sequence (5'-3')
Lif	F: CCCATCACCCCTGTAAATGCC R:CGCACATAGCTTTTCCACGTTG
Vegf	F: TCATGCGGATCAAACCTCACC R:CGGGATTTCTTGCGCTTTCG
IL-6	F: CTTCCATCCAGTTGCCTTCTTG R:AATTAAGCCTCCGACTTGTTGC
IL-1β	F: GGCTCCGAGATGAACAACAAAA R: CACTTTGCTCTTGACTTCTATCT
Beta-2-microglobulin	F: GCTATCCAGAAAACCCCTC R: CCCGTTCTTCAGCATTTG

Lif: leukemia inhibitory factor, Vegf: vascular endothelial growth factor, IL-6: interleukin 6, IL-1β: interleukin 1β.

2.10. RNA extraction and cDNA synthesis for target genes

Total RNA was isolated from cocultured-PBMCs and endometrial mouse cells according to the instructions of the commercial kit (GeneAll Hybrid-R RNA Purification Kit, Seoul, South Korea). Next, while the microtubes were on the ice, for complementary DNA (cDNA) synthesis 5 µL of nuclease-free water, 3 µL random hexamer and 4 µL of extracted RNA, were added to each microtube and then placed in a Bio-Rad thermocycler for 5 min at 75℃. cDNA synthesizing was done based on a protocol reported in the Rezaee *et al*'s study[10].

2.11. Quantitative real–time polymerase chain reaction (qRT–PCR) analysis

qRT-PCR was carried out to assess the expressions of IL-6, IL-1β, Lif, Vegf, using ABI StepOne Plus (Applied Biosystems) in studied groups: the control, PBMC, transfected PBMC and activated PBMC groups. Total samples were run in duplicate and each test was done in triplicate. Reverse transcription and quantitative PCR were used to assess the expressions of the studied genes as previously described by Rezaee *et al*[11]. Normalization of target genes was done with beta-2-microglobulin as the reference gene. The $2^{-\Delta\Delta CT}$ method was applied for the examination of relative expression. Table 1 lists the primer sequences and related information.

2.12. Statistical analysis

Statistical analysis was performed on data sets that consisted of at least three independent experiments and analyzed with the software SPSS 17.0 (IMB, Chicago, IL, USA). REST®2009 software was also used to analyze the relative expression of target genes. Blastocyst attachment was analyzed by one-way analysis of variance (ANOVA, Tukey test). The Mann-Whitney test was used to compare the means for oocyte maturation and development. Data were expressed as mean±standard deviation (mean±SD). $P<0.05$ was considered statistically significant.

2.13. Ethics statement

The experimental and animal care methods performed in this study were in accordance with the Institutional Animal Care and Utilization Committee at Shahid Beheshti University of Medical Sciences, Tehran, Iran, with the IR.SBMU.RETECH.REC.1396.1297 design code.

3. Results

3.1. Cloned hCG into expression vector

The synthetic hCG gene was successfully cloned into pEGFP-N1 expression vector. Cloning was confirmed by colony PCR, restriction enzyme digestion with BamHI and EcoRI enzymes and sequencing. Colony PCR was done with universal PE primers F: 5' CGCAAATGGGCGGTAGGCGTG 3' and R: 5' GGCCCGTTTACGTCGCCGTCC 3'.

A PCR product of approximately 670 bp confirmed the successful cloning of hCG gene into a vector (Figure 2A). The target insert band (560 bp) was shown in the enzyme digestion (Figure 2B).

3.2. Transfection of PBMC with recombinant vector and production of hCG in transfected PBMCs

To obtain the transfected PBMCs, the electroporation strategy was performed according to the procedure described in the material and method section. The protein content of hCG in transfected PBMCs was 465 pg/10^6 cells (Figure 2C).

3.3. Treated endometrial cells with PBMC and hCG increased blastocysts attachment

The results of the developmental competence rate in embryos derived by IVF were as follows: fertilization rate (95.8%), cleavage rate from the four-cell to the morula stages (91.6%, 90.0% and 87.5%, respectively) and blastulation rate (84.1%). We cultured the blastocysts on the cocultured endometrial cells to investigate the effect of PBMC and hCG on the attachment of the blastocyst. Blastocyst attachment and migration at 24 and 72 h after transfer to the cocultured cells are shown in Figure 3. We observed an increased blastocyst attachment rate in transfected PBMCs [(72.5±4.8)%] onto the endometrial cell compared to the activated PBMC [(61.5±7.1)%, P=0.049], PBMC [(59.5±1.48)%, P=0.023] and control [(31.11±2.8)%, P=0.007] groups (Figure 3). Our data showed that the rate of attachment in the transfected PBMC group was higher than other groups.

3.4. Enhancement of target genes expression level in the endometrial cells containing transfected PBMCs compared with activated PBMCs

We performed qRT-PCR to assess the expression levels of target genes in different groups of the study. The results of real-time PCR confirmed that in endometrial cells with transfected PBMCs, the expression level of all of *Lif, Vegf, IL–1β*, and *IL–6* genes significantly increased compared to endometrial cells with activated PBMCs. In the endometrial cells with transfected PBMCs, the highest expression of *Lif, Vegf, IL–1β*, and *IL–6* detected was 12.4-fold, 8.91-fold, 15.9-fold, and 9.96-fold, respectively. Whereas in endometrial cells with activated PBMCs, the expression level of *Lif, Vegf, IL–1β*, and *IL–6* was 10.54-fold, 7.54-fold, 12.7-fold, and 8.5-fold, respectively. As shown in Figure 4, a significantly increased expression was shown in genes in the PBMC group compared to the control group.

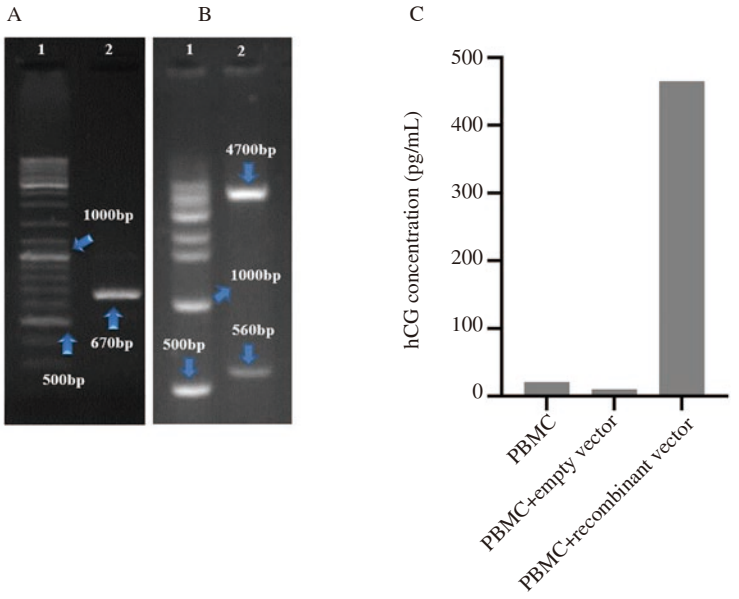

Figure 2. (A) Colony PCR with universal PE primers: Lane 1: DNA ladder (1 kb). Lane 2: positive clone (670 bp). (B) Digestion of recombinant vector by restriction enzymes. Lane 1: DNA ladder (500 bp) and Lane 2: lower band (560 bp) is the hCG gene, and the upper band (4 700 bp) is the PEGFP-N1 vector. (C) Concentrations of produced hCG in the different groups (measured by ELISA). PCR: polymerase chain reaction; pEGFP-N1: plasmid expressing green fluorescent protein-N1; PBMC-pE: transfected PBMC with empty pEGFP-N1 vector; PBMC-pE-hCG: transfected PBMC with pEGFP-N1-hCG vector.

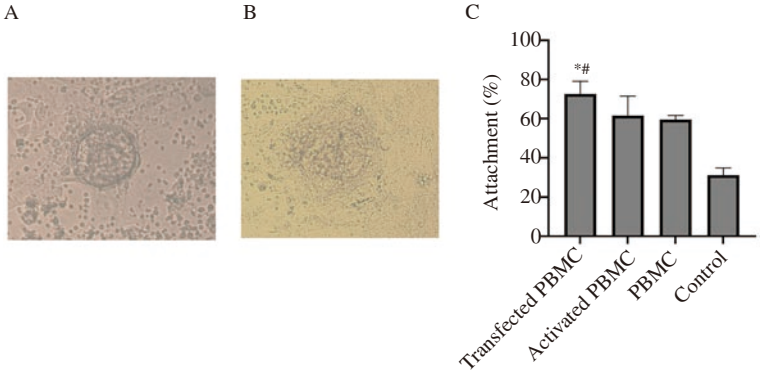

Figure 3. Blastocysts attached to the mouse cocultured cells (endometrial cells with PBMC cells). (A-B) Progression of the blastocyst attachment steps. (C) The percentage of attached blastocysts to the mouse cocultured cells. $^{*}P<0.05$: compared to the activated PBMC and PBMC groups; $^{\#}P<0.01$: compared to the control group.

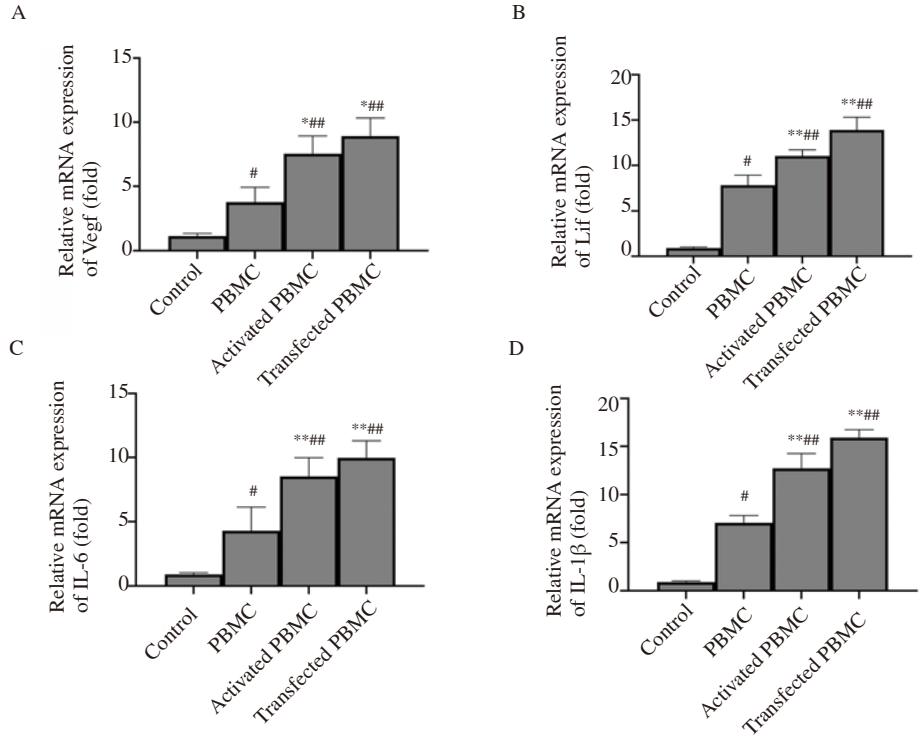

Figure 4. Relative expression of Vegf (A), Lif (B), IL-6 (C), and IL-1β (D) in the four groups. $^{\#}P<0.05$, $^{\#\#}P<0.01$: compared to the control group; $^{*}P<0.05$, $^{**}P<0.01$: compared to the PBMC group.

4. Discussion

Embryo implantation is a complex process that involves many factors such as immune response and requires harmonized interaction among hatched blastocysts and receptive uterus. The immune response to pregnancy may be one of the key regulators of pregnancy maintenance, and the deregulation of the immune response may be responsible, at least in part, for the majority of pregnancy loss that can occur during pregnancy[12,13].

The current study was designed to compare the impact of activated PBMC with hCG *in vitro*, and hCG-producing PBMC on embryo attachment and immune response genes in pregnancy. We found that PBMC and hCG have a significant effect on endometrial cell receptivity through alteration in immune molecules expression levels. The use of PBMCs in increasing uterine receptivity has been previously confirmed.

A study showed that transmission of activated PBMCs, incubation with hCG for 48 h, into the uterine cavity of patients with infertility has noticeable results on uterine receptivity[6]. Intrauterine injection of PBMCs without hCG incubation has a positive effect on embryo implantation[14]. Our qRT-PCR results confirmed that expression of the studied genes (*Lif, Vegf, IL–1β*, and *IL–6*) increased in the endometrial cells containing transfected PBMCs and activated PBMCs. We observed that a higher level of target genes was expressed in transfected PBMCs compared to the activated PBMCs. Also, our results indicated that hCG-producing PBMCs (transfected PBMCs) significantly enhanced endometrium receptivity and embryo attachment. We propose the production of hCG by transfected PBMC should continue for some time after transfer to endometrial cells. Also, hCG in addition to its role is effective in PBMC activation and activated PBMC could promote the secretion of inflammatory cytokines (IL-6, IL-1β and TNF-α), and this increased secretion may stimulate positively the embryo invasion[3]. PBMCs target numerous genes that are involved in embryo attachment, such as *Lif, Vegf*, and inflammatory cytokines genes. These genes play an important role in endometrial attachment and our results also confirmed it[3,10]. In a study by Yu *et al*, incubated PBMCs with hCG increased the expression of inflammatory cytokines such as IL-1β and TNF-α. Cytokine production may improve blastocyst invasion and blastocyst implantation in mice with embryo implantation dysfunction[3].

In addition to the expression of cytokines that are effective in implantation, PBMCs can stimulate progesterone production by luteal cells and progesterone plays an important role in embryo implantation and endometrial receptivity[7]. One of the glycoprotein hormones that are essential for pregnancy is hCG. The initial production of this hormone occurs 6-8 days after fertilization by the blastocyst[15,16]. Kosaka *et al* found that human monocytes respond to hCG and secrete IL-8, which proposes that this hormone can react with not only endocrine cells but also immune cells early in pregnancy[17]. hCG invokes regulatory T cells at the fetal-maternal interface and subsequently temporarily regulates immune tolerance obvious at the site of embryo implantation in pregnancy[18]. Incubation of isolated PBMCs from non-pregnant women with hCG *in vitro* could stimulate embryo endometrial and BeWo cell invasion[19]. In pregnancy, the immune environment in the uterus is essential for the stability of the embryo in the uterus, and cytokines and immune molecules are the main regulators in the uterus[20]. Failure in embryo implantation in patients may be due to the maternal immune system. It has been shown that bovine PBMCs modify the endometrium environment and help to improve the development of pre-attachment embryos to the wall of the uterus[21]. Hashii *et al* also stated that the culture of luteal cells with isolated PBMCs from pregnant women stimulates the immune response of Th-2 and increases the expression of cytokines such as IL-4 and IL-10. These molecules are attributed to the induction of embryo–maternal cross-talk, endometrial differentiation and implantation[22]. In this study, we reported that hCG could stimulate PBMC to secrete IL-1β, and IL-6. However, a study by Kosaka *et al* demonstrated that in the endometrial cell culture derivated from late proliferative and early luteal phases which synchronized with autologous PBMCs, the rate of embryo attachment significantly increased. These PBMCs were not incubated with hCG which indicates that PBMCs alone also stabilize the endometrial wall for embryo attachment[19].

Despite the useful results of this study, we also had some limitations. The limitations of this study are: the immunological dysfunction of the uterine cavity environment may differ in the studied mice, therefore not following the embryos in order to evaluate the live birth rate in mice was a limitation of our study. Also, various immune cells and other factors like progesterone are involved in endometrial receptivity, which needs to investigate. The use of steroid hormones is important for preparing the endometrium receptivity, but their overuse interferes with the endometrial receptivity process. Therefore, in infertility centers, the correct usage of steroid hormones or replacement with other methods should be considered.

In conclusion, the study results show that expression of immune cells is involved in embryo attachment, and we conclude from this study that the use of hCG-producing PBMCs (transfected PBMC) has more influence on endometrial receptivity compared to the activated PBMCs with hCG.

Conflict of interest statement

The authors declare no conflicts of interest.

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Authors' contributions

Mohammad Salehi, Mojgan Bandehpour and Delsuz Rezaee contributed to project administration, study conceptualization and methodology. Bahram Kazemi, Saiyad Bastaminejad and Sajad Najafi performed the review, and editing of manuscript and analysis. Delsuz Rezaee performed the experiment, original manuscript writing, and submitted the data for analysis.

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