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Article Type: RECEARCH ARTICLE

Cytokine Profile in the Endometrium of Normal Fertile and Women with Repeated Implantation Failure

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ABSTRACT

Background: Repeated Implantation Failure (RIF) is one of the most intricate obstacles in assisted reproduction. The cytokine and chemokine composition of uterine cavity seem to play important roles in the implantation process. **Objective:** To compare the cytokine profile in the endometrium of normal fertile women and those with repeated implantation failure. **Methods:** After enzymatic digestion of endometrial tissues, whole endometrial cells and endometrial stromal cells from RIF and normal fertile women were cultivated and stimulated for cytokine secretion. The levels of IL-10, TGF-β, IFN-γ, IL-6, IL-8 and IL-17 in culture supernatants of the two groups were assayed by ELISA and compared together. **Results:** Endometrial stromal cells and whole endometrial cells of normal fertile women produced higher levels of IL-6, IL-8 and TGF-β compared to RIF group, although this difference was statistically significant only in endometrial stromal cells (p=0.005, 0.002 and 0.001, respectively). In addition, endometrial stromal cells of normal fertile women produced lower levels of IL-10 in comparison with RIF group (p=0.005). **Conclusion:** Disturbances in cytokine production at the feto-maternal interface could be a cause of implantation failure. A pro-inflammatory cytokine milieu seems to be pivotal for successful implantation.

Keywords: Cytokine, Endometrium, Implantation, Stromal Cells

INTRODUCTION

Repeated Implantation Failure (RIF) is one of the most intricate obstacles in assisted reproduction (1). RIF is defined as three or more failed attempts of assisted reproductive techniques (ART) or failed pregnancy following ten or more embryo transfers in multiple transfers (2). Recent studies show that under optimum conditions, chances of

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attaining successful implantation per cycle is around 40% (3,4). Causes of RIF could be categorized empirically into three groups: embryonic development defects, decreased endometrial receptivity and imperfect interaction between these two components (5).

Successful implantation requires an orchestrated interaction between receptive endometrium and developing blastocyst. This coordination is mediated by hormones, adhesive molecules, cytokines and growth factors at the feto-maternal interface (6).

The cytokine and chemokine composition of uterine cavity seem to play a pivotal role in the development of newly-formed embryo and its attachment to receptive endometrium. Cytokines such as Leukemia Inhibitory Factor (LIF), Interleukin 6 (IL-6), Interleukin 11 (IL-11) and Transforming Growth Factor β (TGF-β) have been determined as key cytokines for embryo implantation (6,7). However, due to redundancy and pleiotropy in cytokine network, crucial cytokines for implantation and their absolute contribution during this step should be determined by knock-out models (1).

Although Kwak-Kim et al. reported that infertile women with multiple implantation failures have significantly elevated Th1/Th2 cytokine-producing cell ratios in the peripheral blood when compared to normal controls (8), Chaouat et al. explained that "the T helper cell type 1/2 paradigm, as useful as it has been to explain pregnancy, is no longer sufficient in view of the emerging complexity of the cytokine network at the materno-fetal interface" (9). Kalu et al. investigated the changes in the systemic Th1/Th2 cytokine profiles in women with repeated implantation failure and indicated Th1 polarization in these subjects (10).

In this study, secretion of IL-6, IL-10, TGF-β, IL-8, IFN-γ and IL-17 by whole endometrial cells and endometrial stromal cells of RIF women and normal fertile subjects was compared in vitro.

MATERIALS AND METHODS

Subjects. A total of 22 women participated in this study. For control group, 12 volunteers with proven fertility and age under 40 years were recruited. All of the control group had at least one live birth with no history of abortion, infertility or ART trials in previous pregnancies. They had regular menstrual cycles and referred to clinic for tubal ligation. After it was confirmed that the participants were not pregnant (by determining serum levels of Human β-Chorionic Gonadotropin), endometrial samples were taken through biopsy curette. All the normal fertile group signed informed consent before participating in the study.

RIF group were chosen from women under 40 years of age with three failed attempts of in vitro fertilization (IVF) or intra cytoplasmic sperm injection (ICSI) followed by transfer of grade A embryos. Women with any known etiology of RIF such as anatomic abnormalities (judged by hysterosalpingography), hormonal disorders, immunological disturbances (judged by evaluation of anti-thyroglobulin, anti-thyroid peroxidase, anti-nuclear antibody, anti-double stranded DNA), thrombophilia (judged by evaluation of protein-C), protein-S, anti-phospholipid Abs (lupus anti-coagulant and anti-cardiolipin), mutations in factor V leiden, prothrombin, methylene tetrahydrofolate reductase (MTHFR) and plasminogen activator inhibitor-1 (PAI-1), chromosomal abnormality (judged by karyotyping) and male factor infertility were excluded. Endometrial samples were obtained during their routine diagnostic evaluation after signing informed consent.

The patient group had not received any medication or treatment at least three months before the time of sample collection. This study was approved by the institutional review board and ethics committee of Avicenna Research Institute.

Sample Collection. Endometrial samples were obtained through biopsy curette in the secretory phase, according to women's menstruation history and under sterile conditions. The samples were transferred immediately in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM-F12) (Sigma; St Louis, MO, USA) containing 100 U/ml penicillin (Sigma; St Louis, MO, USA) and 100 μg/ml streptomycin (USB; Cleveland, OH, USA) in cold chain condition to the lab.

The samples were frozen in DMEM-F12 culture medium containing 10% Fetal Bovine Serum (FBS) (Gibco, Eggenstein, Germany) and 20% dimethyl sulphoxide (DMSO) (Sigma; St Louis, MO, USA) in cryotubes and kept at -80°C (11). After 24 hours, the samples were transferred to liquid nitrogen until the pathology results were obtained. Also a small fragment of the specimens was used for pathologic evaluation.

Culture of Whole Endometrial Cells (WECs) and Endometrial Stromal Cells (ESCs). Endometrial samples with no pathology were thawed and washed twice with culture medium until the complete removal of DMSO. Endometrial tissues were finely chopped with scissors under sterile conditions and digested enzymatically in DMEM-F12 containing 0.3% collagenase D (Roche, Penzberg, Germany) and 30 μg/ml DNase I (Roche, Mannheim, Germany) for 90 minutes at 37°C. During this period, minced tissues were vortexed every 15 minutes to disperse the cells. The endometrial cell suspensions were then washed twice with the culture medium. The resultant whole endometrial cells (WECs) were cultivated in 24-well plates (5×10^5 cells/well) in a final volume of 1 ml DMEM culture medium containing 10% fetal bovine serum (FBS) (Sigma, St Louis, MO, USA) and non-essential amino acids (NEAAs) (Gibco, Paisley, UK). The plates were incubated in a humified incubator with 5% CO_2 at 37°C. After 6 hours, the whole endometrial cells which reached to 70% confluency, were stimulated with 0.1 ng/ml IL-1 (Sigma, St. Louis, MO, USA).

For purification of endometrial stromal cells (ESCs), a part of WECs was transferred to 75-ml culture flasks and cultivated in a CO_2 incubator. Culture medium was removed after 2 hours to remove non-adherent and dead cells. Adherent cells were cultured and passaged using Trypsin-EDTA (Gibco, Paisley, UK) after reaching to a 70% confluency. The ESCs obtained after three passages were cultured in 24-well plates for cytokine assay. The identity of purified ESCs was confirmed as cytokeratin-/vimentin+ by immunofluorescent staining and CD10+ by flowcytometry. WECs and ESCs were stimulated with 0.1 ng/ml IL-1 (Sigma, St. Louis, MO, USA). After 48 hours, the supernatants were collected and centrifuged at 200×g. This interval was the same for all cytokines. The supernatants were aliquated and stored at -70°C. Each test for RIF or fertile group was done in triplicate and each plate consisted of both RIF and fertile WECs or ESCs. All the conditions were made similar as much as possible.

Measurement of Cytokine Production. The levels of cytokines in culture supernatants were assayed using specific capture ELISA sets. IL-8, IFN-γ, IL-10 and TGF-β1 were quantified using BD OptEIA ELISA sets (BD; San Diego, CA, USA), while IL-6 and IL-17 were quantified by eBioscience ELISA sets (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. Concentration of the cytokines in culture supernatants was determined in reference to standard curves. Minimal detection limits for IL-6, IL-17, IL-8, IFN-γ, IL-10 and TGF-β1 were 2, 4, 3.1, 4.7, 7.8 and 125 pg/ml, respectively. Cytokine levels below minimal detection limits were considered as zero.

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Statistical Analyses. To compare the cytokine levels in cell culture supernatants between RIF and normal groups, Mann-Whitney U test was used. Data entry and analysis was done using SPSS software (version 13; SPSS Inc, Chicago, IL). Results were presented as Mean ± SEM. P Values less than 0.05 were considered as statistically significant.

RESULTS

Pro-Inflammatory Cytokine Production by Endometrial Cells of RIF and Normal Groups. WECs and ESCs of both groups failed to produce detectable amounts of IFN-γ and IL-17; hence no comparison could be made in this regard. WECs of the control group produced higher levels of IL-6 compared to RIF group, although the difference was not statistically significant. ESCs of normal fertile group, however, produced significantly higher levels of IL-6 compared to the RIF group (p=0.005) (Figure1).

Anti-Inflammatory Cytokine Production by Endometrial Cells of RIF and Normal Groups. ESCs obtained from RIF group secreted significantly higher levels of IL-10 compared to the normal fertile group (p=0.005), while no statistically significant difference was observed in IL-10 production by WECs in RIF as compared with the control group. Although, WECs and ESCs from the normal fertile group produced more TGF-β compared to the RIF group, this difference was only statistically significant in ESCs (p=0.001) (Figure 1).

IL-8 Production by Endometrial Cells of RIF and the Normal Groups. As with IL-6, WECs and ESCs of the control group produced higher levels of IL-8 compared to the RIF group, but this difference was only statistically significant in the ESCs (p=0.002) (Figure 1).

DISCUSSION

Implantation is a crucial step for establishing the pregnancy. This process depends on mutual communication between the developing blastocyst and the receptive endometrium and a part of this process is mediated by cytokines and chemokines. These soluble proteins were produced not only by endometrial cells but also by immune cells in the implantation area (12).

Our results showed that endometrial stromal cells of normal fertile group produce higher levels of pro-inflammatory cytokines, IL-6 and IL-8; while produce lower levels of IL-10 compared to RIF group. The pattern of cytokine production by WECs was similar to ESCs for IL-6 and IL-8; while it was different for IL-10. However, these differences were not statistically significant in WECs. WECs are composed of endometrial cells and the immune cells. Uterine NK cells and monocytes, as two main subpopulations of immune cells, produce high levels of IL-10 in the endometrial milieu (13).
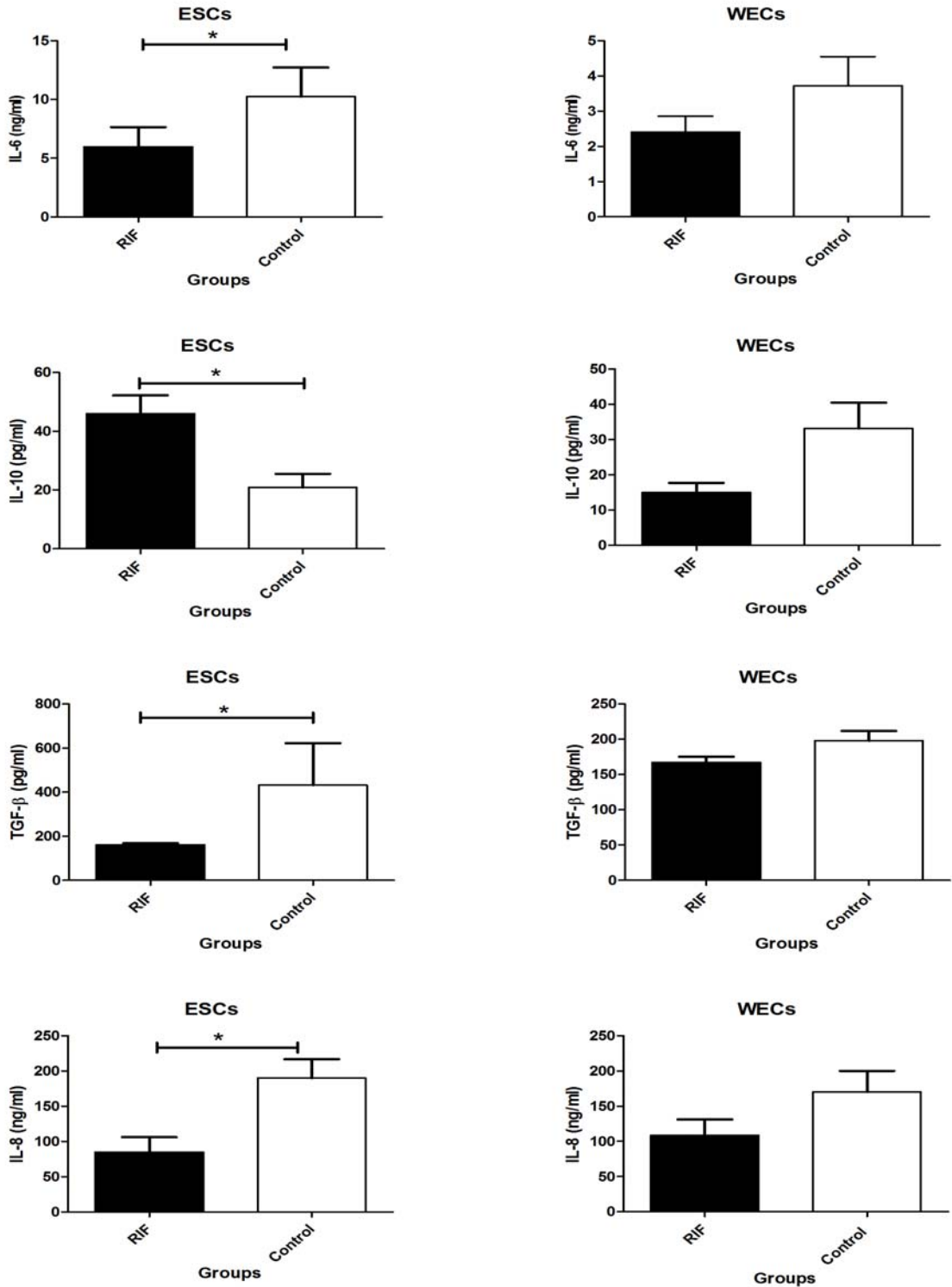
* p<0.05

Figure 1. Cytokine profile in the whole endometrial cells and endometrial stromal cells from normal fertile women and women with repeated implantation failure. Whole endometrial cells (WECs) and endometrial stromal cells (ESCs) from women with repeated implantation failure (RIF) and normal subjects were stimulated with IL-1. Levels of IL-6, IL-10, TGF-β, and IL-8 production were measured in culture supernatants by sandwich ELISA. Each bar represents the Mean ± SEM data from 12 fertile or 10 RIF subjects.

According to our results, it seems that in the WEC of normal fertile group, the interaction of endometrial cells and immune cells resulted in a higher level of IL-10 as compared to the RIF group; although this difference was not statistically significant. In concordance with our study, Ingaki et al. showed a decreased IL-10 level in endometrial flushings of women with recurrent failed embryo transfer compared to fertile women (14). It seems that cytokines secreted by WECs in vitro are more similar to cytokines within endometrial flushings. Also, our results showed that endometrial cells of fertile women produce higher levels of IL-6 and IL-8 compared to the RIF subjects. Previous studies indicate that IL-6 is mostly produced by endometrial cells at the time of implantation and has an essential role in this process. IL-6 is also involved in decidualization, tissue remodeling and the development of placenta (12,15). IL-8 is produced by endometrial cells and NK cells at the feto-maternal interface. This chemokine with pro-angiogenic activity and the ability to induce Fas-ligand production on endometrial cells, prepares the endometrium for implantation, a process which is accompanied by the formation of new vessels (16,17).

As a key cytokine with a profound effect on the process of implantation (12), the levels of TGF-β production by the endometrial cells of RIF and the control groups were also measured and compared. Our results confirmed this notion and showed that ESCs and WECs of normal fertile women produce higher levels of TGF-β compared to the RIF group. TGF-β is another cytokine with a positive immunoregulatory effect on implantation which regulates the attachment of trophoblast cells to extra-cellular matrix as well as the production of degrading enzymes to facilitate the penetration of trophoblast cells in to the prepared endometrium (12,18).

Although there are a considerable number of studies on cytokine production in recurrent spontaneous abortion (RSA) (19,20), data on the role of these cytokines in repeated implantation failure are limited. Moreover the majority of these studies has been done on serum or PBMCs of women with recurrent miscarriage or repeated implantation failures.

A great proportion of the above mentioned studies indicated systemic propensity toward Th1 in RSA and implantation failures. For instance, Wu et al. investigated serum levels of IL-10, IL-11, LIF and TGF-β in patients undergoing IVF/ET and indicated that a rise in the serum level of IL-10 from day 14 to 21 after embryo transfer is associated with a normal pregnancy outcome (21). This was supported by another study which showed that circulatory IL-10 producing CD8+ T-cell counts were significantly lower and TNF-α producing CD4+ T-cell count were significantly higher in patients with RSA and implantation failures when compared to those of normal fertile women and concluded that dominant Th1 response may be the cause of reproduction failure (22). Another study by Kwak-kim et al. declared that Th1/Th2 cytokine-producing cell ratio was elevated in women with repeated implantation failure in comparison with fertile women (8). In contradiction to our study, It has been shown in another investigation that there is an association between day 11 IFN-γ concentration and poor outcome of ART trial (23), while another study did not support this finding (24). Investigation on the serial changes of Th1:Th2 profile of the peripheral blood of patients undergoing IVF trials revealed that women with repeated implantation failure had a Th1 polarization in the periphery (10).

Although great proportion of these studies supported a systemic pro-inflammatory response in implantation failure, it should be emphasized that tendency toward Th1 responses which existed in the periphery could not mimic the endometrial milieu. In

concordance with this finding (9), Thum et al. showed that there was no association between the systemic levels of cytokines and the outcomes of ART trials (25). More-over, there are two studies which investigated cytokine levels in endometrial flushings (14,26). Boomsma et al. studied the association of cytokines in endometrial secretions and the pregnancy outcome in women undergoing IVF and indicated that there was an association between a higher level of TNF-α and IL-1β with clinical pregnancy (26). This finding indicated that inflammatory milieu could be beneficial for implantation which is in agreement with our findings. Our results on the lower levels of IL-10 within WECs supernatants of RIF group supports the findings of Ingaki on the level of IL-10 in the endometrial flushings of women with implantation failure (14).

Although numerous studies on cytokine levels in the periphery exist, it seems that Th1 tendency existing in serum or the PBMCs of RIF subjects could not correspond to the cytokine situation in the endometrial milieu (9,27). Indeed our study for the first time compares the cytokine profile of endometrial milieu between RIF subjects and the nor-mal fertile women in vitro.

Former general paradigm about pregnancy was predominance of anti-inflammatory cy-tokine profile during pregnancy (28,29), a premise that has been revised on the basis of differential expression of cytokines in a timely-dependent manner during entire period of pregnancy. Now, it is well-established that the first and third trimesters of pregnancy are associated with pro-inflammatory milieu, and it is the second trimester which is ac-companied with a shift toward T helper 2 (TH2) cytokine profiles (16). Inflammation is an indispensable component of implantation (15,16) but excessive inflammation may be damaging for the developing embryo. Regulatory T cells (Treg) are the principal cell types which modulate excessive inflammation in the uterus at the implantation site and their frequency increases instantaneously after successful implantation (18).

Barash et al. were the first to demonstrate the beneficial effect of local injury and subse-quent inflammatory responses on the process of implantation (30). They showed that this manipulation is associated with the induction of decidualization in endometrial stromal cells which predisposes the endometrium for implantation. These findings were confirmed later in an elegant study conducted by Raziel et al. (31).

Collectively, it seems that the cytokine profile in the periphery could not mimic the cy-tokine network which exists in the endometrial milieu. Our results emphasize that dis-turbances in the regulation of cytokine network at the feto-maternal interface could po-tentially associate with an implantation failure. In this context, medications with anti-inflammatory action such as prednisolone would be avoided during the implantation window in women with recurrent implantation failure. Similarly, maneuvers with in-flammatory consequences such as coitus prior to embryo transfer could conceivably im-prove the implantation rate.

ACKNOWLEDGMENTS

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