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SHORT COMMUNICATION

The pro-inflammatory and anti-inflammatory cytokine profile in peripheral blood of women with recurrent implantation failure

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Abstract Limited information is available on the balance state of pro- and anti-inflammatory cytokines in patients with recurrent implantation failure (RIF). This study assessed the pro- and anti-inflammatory cytokines in plasma of 34 patients with RIF, compared with those of 25 women with a successful pregnancy in the first IVF/intracytoplasmic sperm injection-embryo transfer (IVF/ICSI–ET) cycle. The IFN-γ, IL-1β, IL-6 and IL-4 concentrations were higher, whereas the TGF-β1 concentration was lower in the RIF group compared with the control group. Furthermore, the ratios of pro-inflammatory and anti-inflammatory cytokines IFN-γ/IL-4, IFN-γ/IL-10, IFN-γ/TGF-β1, IL-6/IL-10, IL-6/TGF-β1, IL-1β/TGF-β1 and TNF-α/TGF-β1 were higher in the RIF group (all $P < 0.01$). The results suggested a shift toward a pro-inflammatory state in peripheral blood of the patients with RIF.

KEYWORDS: anti-inflammatory cytokines, IVF/ICSI–ET, pro-inflammatory cytokines, recurrent implantation failure

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Introduction

With the improvements in ovarian stimulation protocols and laboratory culture conditions, IVF/intracytoplasmic sperm injection–embryo transfer (IVF/ICSI–ET) has evolved greatly since the first successful pregnancy. However, recurrent implantation failure (RIF) is still a challenging step for IVF/ICSI–ET. The aetiology of RIF mainly includes three categories: decreased endometrial receptivity, embryonic defects and other combined effects (Margalioth et al., 2006).

The immunological causes, especially the cytokine network, may be one of the most important factors that influence endometrial receptivity and embryo implantation (Orsi and Tribe, 2008). Cytokines are divided into pro-inflammatory and anti-inflammatory cytokines according to their functions. The pro-inflammatory cytokines include Th1-type cytokines (such as IFN-γ and TNF-α) and other cytokines (such as IL-1β and IL-6), whereas the anti-inflammatory cytokines are Th2-type cytokines (such as IL-4 and IL-10) and other cytokines (such as TGF-β1). Cumulative evidence indicates that the pro- and anti-inflammatory cytokines form an intricate signalling network and that the physiological balance between them is critical for embryo implantation and pregnancy maintenance (Wilczynski, 2005).

The intracellular Th1 cytokine polarization of the T helper cell in peripheral blood was present in patients with RIF (Kwak-Kim et al., 2003); nevertheless, a series of pro- and anti-inflammatory cytokines in plasma and their balance state in RIF patients have not been sufficiently studied. Although cytokines are produced mainly by T helper cell subsets, the antigen-presenting cell and other immune cells can also participate in cytokine secretion. Therefore, the cytokine in plasma can probably better reflect the whole immunological status in peripheral blood. Moreover, in terms of the co-operative and antagonistic action between pro-inflammatory and anti-inflammatory cytokines, it is unlikely that the defect of one single cytokine in the network would affect the overall balance status of the systemic immunity.

This prospective study assessed the pro-inflammatory and anti-inflammatory cytokine profile in plasma of the RIF patients in the mid-luteal phase, i.e. the implantation window, compared with that of successful pregnancy women in the first IVF/ICSI–ET cycle at the same point prior to IVF/ICSI–ET treatment, aiming to evaluate the balance status of pro-inflammatory and anti-inflammatory cytokines in the peripheral blood of the RIF patients.

Materials and methods

The study was performed at the Fertility Centre, Shenzhen Zhongshan Urology Hospital and was approved by the Research Ethics Committee of Shenzhen Zhongshan Urology Hospital on 28 February 2013 (reference no. SZZSRPHU-201300012). The informed consent was obtained from each patient before the study.

The inclusion criteria of the RIF group include: (i) failure to achieve a pregnancy following two to six IVF/ICSI–ET cycles, in which more than 10 high-grade embryos were transferred; (ii) ≤ 38 years old; and (iii) basal FSH < 10 mIU/ml.

The inclusion criteria of the control group include: (i) became pregnant following fresh ET in the first IVF/ICSI–ET cycle; (ii) ≤38 years old; (iii) basal FSH < 10 mIU/ml and antral follicle counts in both ovaries ≥8; and (iv) the ovarian stimulation protocol was a standard long protocol.

The exclusion criteria of all subjects include: (i) uterine abnormality; (ii) a history of polycystic ovary syndrome (PCOS), spontaneous abortions and autoimmune diseases; (iii) abnormal chromosome karyotype of either wife or husband; and (iv) the white cell count and C-reactive protein were abnormal on the blood sampling day.

In total 34 women were enrolled in the RIF group and 25 women were enrolled in the control group. All the blood samples were drawn in the mid-luteal phase, the very specific time of uterine receptivity, prior to IVF/ICSI–ET treatment. This can also minimize the effects of ovarian stimulation drugs on the cytokines. Whole blood samples (5 ml) were drawn in venous blood collection tubes with anticoagulant from the mid-luteal phase. The whole blood samples were incubated with 25 ng/ml phorbol myristate acetate (PMA) and 1 mmol/l ionomycin (Sigma, USA) for 4 h. After culture, the supernatant was drawn and centrifuged at 2500*g* for 20 min at 4°C to obtain the plasma samples. Aliquots were stored at 70°C until use by flow cytometry measurements. The pro-inflammatory cytokines IFN-γ, TNF-α, IL-6 and IL-1β, anti-inflammatory cytokines IL-4, IL-10 and TGF-β1 in plasma were detected using BD Cytometric Bead Array (CBA) (BD Biosciences Pharmingen, USA). The CBA immunoassay was carried out according to the manufacturer's instructions. The latent TGF-β1 was measured in the immunoreactive form using a single-plex assay, while other cytokines were measured in a multiplex assay.

Statistical analyses

Statistical analyses were performed using the Statistical Package for Social Sciences version 18 (IBM, Armonk, NY, USA). The data were shown as mean ± standard error. Unpaired *t*-tests were applied for comparisons of cytokine expression in plasma and pro-inflammatory/anti-inflammatory cytokine ratios between the RIF and control groups ($P < 0.05$ was considered significant).

Results

The mean age of the RIF group was 34.35 ± 2.53 (range: 31–38) years, and the mean age of the control group was 33.12 ± 2.29 (range: 29–38) years. No significant differences were observed in the age, basal sex hormones and body mass index (BMI) between the control and RIF groups (data not shown).

The unit for all cytokines measured in the present study was pg/ml. Concentrations of pro-inflammatory cytokines IFN-γ (1323.2 ± 900.35 versus 352.58 ± 60.53, $P < 0.001$), IL-1β (3411 ± 360.57 versus 1924 ± 357.51, $P = 0.006$), IL-6 (7959.8 ± 385.79 versus 4243.9 ± 427.31, $P < 0.001$) and anti-inflammatory cytokines IL-4 (58.55 ± 5.97 versus 29.43 ± 4.19, $P < 0.001$) were significantly higher in the RIF group compared with the control group. The concentration of anti-inflammatory cytokine TGF-β1 was significantly lower in the RIF group compared with the control group (1608.95 ± 89.90 versus 2334.28 ± 105.20, $P < 0.001$). There were no differences seen in the concentrations of cytokines TNF-α (4074.12 ± 385.96 versus 3393.52

Table 1 The pro-inflammatory and anti-inflammatory cytokine concentrations and ratios in the RIF and Control groups.

Cytokine	*RIF group (n = 34)*	*Control group (n = 25)*
Cytokine (pg/ml)		
IFN-γ	1323.20 ± 154.41***	352.58 ± 60.53
IL-6	7959.80 ± 385.79***	4243.90 ± 427.31
IL-1β	3411.00 ± 360.57**	1924.00 ± 357.51
TNF-α	4074.10 ± 385.96	3393.5 ± 486.56
IL-4	58.55 ± 5.97***	29.43 ± 4.19
IL-10	58.07 ± 6.18	51.60 ± 6.84
TGF-β1	1608.95 ± 89.90***	2334.28 ± 105.20
Cytokine ratio		
IFN-γ/IL-4	26.43 ± 3.00**	12.82 ± 2.15
IFN-γ/IL-10	30.11 ± 4.52***	7.00 ± 1.11
IFN-γ/TGF-β1	0.88 ± 0.11***	0.16 ± 0.03
IL-6/IL-4	189.50 ± 23.04	244.76 ± 66.21
IL-6/IL-10	202.65 ± 29.42*	113.18 ± 16.60
IL-6/TGF-β1	5.35 ± 0.34***	1.80 ± 0.16
IL-1β/IL-4	78.83 ± 9.87	102.38 ± 30.57
IL-1β/IL-10	78.48 ± 9.78	48.75 ± 12.03
IL-1β/TGF-β1	2.26 ± 0.24***	0.82 ± 0.15
TNF-α/IL-4	196.65 ± 12.60	180.60 ± 45.09
TNF-α/IL-10	94.83 ± 13.45	88.70 ± 17.13
TNF-α/TGF-β1	2.74 ± 0.28**	1.44 ± 0.21

Values are mean ± SE
RIF = recurrent implantation failure.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus Control group.

± 486.56) and IL-10 (58.07 ± 6.18 versus 51.60 ± 6.84) between the RIF and control groups. The ratios of pro- and anti-inflammatory cytokines IFN-γ/IL-4 (26.43 ± 3.00 versus 12.82 ± 0.15, $P = 0.001$), IFN-γ/IL-10 (30.11 ± 4.52 versus 7.00 ± 1.11, $P < 0.001$), IFN-γ/TGF-β1 (0.88 ± 0.11 versus 0.16 ± 0.03, $P < 0.001$), IL-6/IL-10 (202.65 ± 29.42 versus 113.18 ± 16.60, $P = 0.019$), IL-6/TGF-β1 (5.35 ± 0.34 versus 1.80 ± 0.16, $P < 0.001$), IL-1β/TGF-β1 (2.26 ± 0.24 versus 0.82 ± 0.15, $P < 0.001$) and TNF-α/TGF-β1 (2.74 ± 0.28 versus 1.44 ± 0.21, $P = 0.001$) were significantly higher in the RIF group compared with the control group (**Table 1**).

Discussion

A wide and evolving literature exists on the Th-1/Th-2 equilibrium in pregnancy. Although a certain local pro-inflammatory environment is necessary for embryo implantation and angiogenesis (Mor et al., 2011), the dominant Th-1 cytokine or pro-inflammatory cytokine is seen to be harmful in pregnancy and the Th-2 or anti-inflammatory cytokine regulates and ameliorates the Th-1 response (Saito et al., 2010). The data from this study show that a pro-inflammatory state was presented in RIF patients: increased pro-inflammatory cytokines IFN-γ, IL-1β and IL-6, decreased anti-inflammatory cytokine TGF-β1 and raised pro-inflammatory/anti-inflammatory cytokine ratios. The disturbance of the pro- and anti-inflammatory cytokine balance in peripheral blood was probably associated with the RIF.

The findings are in line with the several published studies that have shown Th-1 cytokine or pro-inflammatory cytokine polarization of T helper intracellular cytokines in RIF patients (Kalu et al., 2008; Kwak-Kim et al., 2003). A previous study also demonstrated the increased ratios of Th-1/Th-2 in the IVF/ICSI–ET failure patients (Liang et al., 2015). However, due to the method restriction, the number of intracellular cytokines of T helper cells that can be detected simultaneously was limited, and the results were presented in percentages. In contrast, a variety of cytokines in plasma can be measured and analysed quantitatively using the Cytometric Bead Array in this study, as this is an accurate and precise method to measure cytokines in plasma. From the literature, it has been seen that not only do the T cells play an important role in pregnancy, but the innate immune cells are also essential for embryo implantation and angiogenesis (Lee et al., 2011). The maternal immune tolerance is likely to be modulated by both adaptive and innate immunity during pregnancy. The natural killer cells – macrophage, dendritic cell and Treg cell – migrate and increase in the endometrium during the implantation window. Deletion of these cells has deleterious effects on implantation and placental development (Hanna et al., 2006; Sargent et al., 2006). Previous studies have shown that the circulating and local immune cells communicate with each other, and the systematic immune state may influence local immune cell mobilization and activation (Fujiwara, 2009). Therefore, it is indeed very important that the whole cytokine contents in peripheral blood be analysed at the putative time of embryo implantation.

The lower concentration of TGF-β1 in the RIF group was another result consistent with the imbalance of pro-inflammatory and anti-inflammatory cytokines. Recent studies have reported that TGF-β1 is an essential immune-regulatory

and anti-inflammatory cytokine during embryo implantation and it is also implicated in the generation and differentiation of Treg cells, which play a vital role in immune tolerance in pregnancy (Guerin et al., 2009). A previous study reported that the Treg cell decreased in peripheral blood of RIF patients (Chen et al., 2009). It is hypothesized that decreased expression of TGF-β1 could be a contributing factor to the elevated pro-inflammatory cytokines and the decrease of Treg cells, which could be detrimental to embryo implantation.

Although the concentration of anti-inflammatory cytokine IL-4 in the RIF group was increased, which maybe a regulated expression of the higher pro-inflammatory cytokine polarization, the ratio of IFN-γ/IL-4 in the RIF group was still twofold higher than those of the control group. This indicated that the IL-4 increase in concentration probably did not change the status of pro-inflammatory cytokine polarization in the RIF group. These results are in line with the previously published literature on patients with demyelinating diseases (Hohnoki et al., 1998).

Although the mechanism of pro-inflammatory polarization is unknown, it may affect embryo implantation through a number of mechanisms. Firstly, excess pro-inflammatory cytokine (especially IFN-γ) could activate macrophage and induce its differentiation, which may express nitric oxide synthase (iNOS) and mediate directly the damage to trophoblasts (Haddad et al., 1997). Secondly, it was reported that both extravillous trophoblast proliferation and invasion were inhibited *in vitro* by the combined pro-inflammatory cytokines (Otun et al., 2011). Therefore, the dominance of pro-inflammatory cytokines was probably one of the important factors affecting embryo implantation.

Although the results suggested that a pro-inflammatory state was present in RIF patients, it should be noted that whether the circulating immunological status in the peripheral blood could reflect the cytokine status at the local site in the uterus during embryo implantation was not determined in the present study. As it is extremely difficult to study embryo implantation *in vivo* in women, the evaluation of immunological status in the peripheral blood may be a feasible way to investigate the immunological cause of RIF and can easily be put into application.

In conclusion, the results from this study showed the pro-inflammatory and anti-inflammatory cytokine profile shifted toward a pro-inflammatory state in peripheral blood of RIF patients. Further study will be needed to explore the exact mechanism.

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