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Endometrium cytokine profiles are altered following ovarian stimulation but almost not in subsequent hormone replacement cycles

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ABSTRACT

Purpose: To investigate the impact of ovarian stimulation and hormone replacement treatment on key regulatory cytokines in endometrial secretion during endometrium implantation window.

Methods: Fifty-six patients undergoing ovarian stimulation (OS) with gonadotropin releasing hormone antagonist and frozen embryo transfer with hormone replacement treatment (HRT) were recruited. Endometrial secretion aspiration was performed repeatedly during implantation window in natural, OS and HRT cycles of every patient. The concentrations of 17 mediators, known to be involved in human embryo implantation, were assessed by multiplex immunoassay.

Results: Compared with natural cycle (NC), the concentration of IFN-γ, G-CSF and IL-8 within endometrium were almost the same following OS and HRT. Furthermore, increased MCP-1 levels were observed following HRT and OS. In addition, an increase in IL-1b, IL-7, IL-17, IL-6, TNF-a, IL-12, IL-4, IL-13, IL-10, IL-5, VEGF and MIP-1b concentrations were found in OS cycle only. The level of GM-CSF was lower in HRT cycle and higher in OS cycle, when compared with NC. Among all 17 cytokines, no correlation was found between cytokine concentrations and serum estradiol and progesterone, while only IL-7 concentration has a low correlation with serum LH level.

Conclusion: Compared to natural and hormone replacement cycle, patients' endometrium cytokine profiles present an increased inflammatory response following ovarian stimulation.

1. Introduction

Cryopreservation of supernumerary embryos has become an integral part of in vitro fertilization (IVF) treatment. Due to recent technological advances, pregnancy outcomes in frozen–thawed embryo, including clinical pregnancy rate and live birth rate, appear to be comparable (or even better) to fresh embryo transfers, and can potentially lead toward better obstetric outcomes [1–3]. Consequently, some fertility programs have only focused on providing the frozen embryo approach, which is becoming routine clinical practice across increasing number of clinical centers in China. However, before approving it as universal treatment approach, it is necessary to furthermore explore its biological plausibility.

Fresh embryo is transferred 3 or 5 days after oocytes retrieval in ovarian stimulation (OS) cycle, with the major disadvantage of supraphysiological levels of sexual hormones. Frozen embryo transfer is usually performed in natural menstruation cycle (NC) or hormone replacement cycle (HRT), with physiological sexual hormone levels. According to different histological, histochemical, and gene expression studies, the elevated concentrations of sex steroids, such as progesterone and estradiol, may impair endometrial receptivity [4]. Endometrial secretions contain a variety of molecules which presumably provide nutrition of the conceptus, and sustain embryo implantation through series of growth factors and cytokines [5–7]. Some published studies have shown that cytokines production encounter significant alteration under ovarian stimulation [8,9].

In our previous study, we have introduced a relatively non-invasive and useful method to analyze patterns of cytokines in endometrial secretion fluid [10]. The main purpose of the present study was to explore the characteristic of endometrium protein secretion in ovarian stimulation cycles and hormone replacement cycles. Accordingly, we used multiplex immunoassay to elucidate the impact of OS and HRT on key regulatory cytokines, chemokines, and growth factors in endometrial secretion during implantation window using a repeated measurement design (RMD) study.

2. Materials and methods

In this study, all patients received three times of endometrium sample aspirations in three different time point: NC, OS cycle and HRT

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cycle for frozen embryo transfer. Fifty – six patients with a regular menstrual cycle (25–32 days), who received intracytoplasmatic sperm injection (ICSI) treatment or IVF at Reproductive Medicine Center of Ruijin Hospital affiliated to Medical School, Shanghai Jiaotong University, were recruited between December 2014 and October 2015. None of the patients had hydrosalpinges or anatomic uterine abnormalities. The written informed consent was obtained from all the patients who participated in the study, and the study was approved by the Ethics Committee of Ruijin Hospital (Ethical approval NO. RJEA2014-098).

2.1. Natural cycle (NC)

First, all patients were monitored ovulation during natural menstruation cycle. Urinary LH was monitored every morning from the 10th day of menstrual cycle with a home LH ovulation predictor kit. Ovulation was defined 36–40 h after LH surge and the endometrial secretion was aspirated 5 days after the LH surge.

2.2. Ovarian stimulation (OS) and oocyte retrieval

The following menstruation cycle, all patients were placed on the regular gonadotropin-releasing hormone (GnRH) antagonist protocol for ovarian stimulation in order to obtain multiple oocytes. Briefly, daily injections of recombinant follicle-stimulating hormone (FSH; 100–300 IU/day) were given from the second or third day of a spontaneous cycle. On stimulation day 6, a GnRH antagonist (Cetrotide, Merck Serono, German) was given at a daily dose of 0.125 mg, and it was continued throughout the stimulation period. Final oocyte maturation was triggered by human chorionic gonadotropin (hCG) single subcutaneous injection of 5000 IU. Oocyte retrieval was carried out transvaginally under ultrasound guidance, 34–36 h after hCG injection. None of the patients received embryo transfer during OS cycle, while endometrial secretion was aspirated 5 days following hCG injection (hCG + 5).

2.3. Hormone replacement treatment (HRT) and frozen embryo transfer (FET)

All patients received FET one month after ovarian stimulation. Hormone replacement treatments were completed using an incremental dose of oral estradiol (4–6 mg daily for 12–20 days) followed by transvaginal ultrasonography to confirm the absence of folliculogenesis and the presence of endometrial thickness (measuring at least 8 mm). Subsequently, oil progesterone injections were started at 60 mg daily, and FET was performed on the fourth day of progesterone use. Endometrial secretion was aspirated just before embryo transfer.

2.4. Endometrial sampling and processing

First, uterine secretions were aspirated. While the patient was lying in the lithotomy position, the cervix was cleansed after the insertion of the speculum, and an embryo transfer catheter (CCD Laboratories, Paris, France) was transcervically introduced. Next, suction was gradually applied with a 2 ml syringe. To prevent contamination from the cervical mucus during catheter removal, the outer sheath of the embryo transfer catheter was advanced to a depth of 4 cm from the external cervical opening (os) following suction application. The inner catheter was then removed through the outer sheath, preventing the contact with the cervix. The outside of the catheter was cleaned to remove any potential cervical mucus. The tips of the catheter were cut off and snap frozen in liquid nitrogen and stored at −80 °C until further use. Previous studies have shown that blood contamination of the endometrial secretion can affect the measurement of some cytokines [8], so the extent of blood contamination in the endometrial secretion samples was visually graded as none, minimal, moderate, or severe. Moderately and severely contaminated samples were excluded from the analyses.

2.5. Multiplex immunoassay

The endometrial secretion samples were analyzed using a multiplex immunoassay. Key soluble implantation regulators were identified as candidate mediators for inclusion in the assay. Numerous mediators were excluded from the panel, either because appropriate antibodies were not available, or due to problems arising from cross interference. The final panel included interleukin (IL)-1b, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, IL-18, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), granulocyte macrophage-colony stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), macrophage inhibitory protein-1β (MIP-1b), and monocyte chemotactic protein-1 (MCP-1). Assays were performed as previously described [10].

2.6. Statistical analysis

Previous studies examining the ovarian stimulation on endometrial markers have obtained significant findings using sample sizes of 12–38 patients [11–13]. Through the results of pre-test, we defined a sample size of $n = 40$ was enough to get the power over 90% with alpha = 0.05 using PASS software (NCSS Inc., U.S.A). We used the log-transformation for non-normally distributed data prior to analysis and analyses were performed using SPSS software 14.0 (SPSS Inc., Chicago, IL). One way ANOVA were used to compare cycle demographics and cytokine concentrations in natural, ovarian stimulation and hormone replacement cycles. In stimulation cycles, we analyzed the relationship between hormonal levels, including estradiol (E2), luteinizing hormone (LH) progesterone (P), and cytokines using multiple linear regressions.

3. Results

From a total of 56 women who consented to participate, 16 patients were subsequently excluded since there was not enough available material to measure for the total protein content in the aspirate or blood contamination. So, there were 40 patients included in this study finally. Treatment and patients' characteristics are shown in Table 1.

Sample aspiration cycle characteristics, including sex hormone levels and endometrium thickness are shown in Table 2. All participants had a normal natural ovulation in sample aspiration cycle, while the time period before LH surge was 12.6 ± 2.3 days. The endometrium thickness in all 40 patients reached at least 8 mm in NC, OS or HRT cycle. Both serum estradiol and progesterone levels in OS cycles were significantly higher compared to other two cycles because of the ovarian stimulation. Nevertheless, there were no statistically significant differences among these two hormones in natural and HRT cycles.

The comparisons of endometrium cytokine concentrations in NC, OS

Table 1
Clinical parameters and treatment characteristics of participants.

Parameter	All participants, N = 40 mean ± SEM (range)
Age (y)	30.2 ± 3.5 (25–34)
Duration of infertility (y)	4.0 ± 1.8 (2–5)
Primary infertility (%)	60%
Primary cause of infertility (%)	
Tubapathology	60%
Andrological	12.5%
Both	22.5%
BMI	22.3 ± 2.1 (19.6–24.2)
IVF	60%
ICSI	40%
Duration of ovarian stimulation(d)	9.7 ± 1.8
Total amount of gonadotrophin (IU)	1868 ± 436
Oocyte retrieved (n)	9.2 ± 3.7

Table 2
Comparison of cycle characteristics among natural, ovarian stimulation and hormone replacement cycles in the same group of women on day of endometrial secretion aspiration, mean ± SEM (range).

	NC (n = 40)	OS (n = 40)	HRT (n = 40)	P
Endometrium thickness (mm)	8.9 ± 0.7	9.3 ± 1.2	8.3 ± 1.1	.12
LH (IU/L)	6.8 ± 5.9	6.2 ± 3.3	5.4 ± 2.2	.17
E2 (pg/ml)	105.6 ± 39.2	1039.4 ± 337.2	136.8 ± 20.7	.01[a]
P (ng/ml)	14 ± 2.7	48 ± 8.5	12.2 ± 2.2	.01[a]
Duration before LH surge (d)	12.6 ± 2.3	/	/	
Duration before HCG trigger (d)	/	10.8 ± 2.6	/	
Duration before progesterone administration (d)	/	/	15.6 ± 3.7	

Significant difference between OS and NC or HRT, $p < .05$.
[a] NC, natural cycle; OS, ovarian stimulation cycle; HRT, hormone replacement cycle.

and HRT-FET cycles are shown in Fig. 1. The concentrations of IL-1b, IL-7, IL-17, IL-6, TNF-a, IL-12, IL-4, IL-13, IL-10, IL-5, VEGF and MIP-1b were similar in both natural and HRT-FET cycles, while they were remarkably increased in OS cycle. Furthermore, IFN-γ, G-CSF and IL-8 within endometrium were not differentially expressed following both ovarian stimulation and hormone replacement treatment. Among all the cytokines, GM-CSF acted differently as it was decreased in HRT cycle and increased in OS cycle, when compared with NC. On the other hand, MCP-1 was the only mediator that displayed an increased level following both HRT cycle and OS cycle.

After being log transformed, endometrium cytokines' concentrations and serum hormone levels on sample retrieval day in OS cycles were analyzed. Through multiple linear regressions analysis, none of the cytokines revealed relationship with serum estrogen and progesterone while only IL-7 concentration was weakly correlated with LH ($r = 0.312$, $p = .032$).

In FET cycle, embryos were scored according to following criteria just before transfer into uterus. (I) Number of blastomeres (BL): 1 for 4 BL, 2 for 5 BL, 3 for 6–7 BL and 4 for 8–10 BL; (II) Degree of fragmentation (FR): 4 for none, 3 for 1–10%, 2 for 11–25% and 1 for 26–50%;(III) Equality (EQ) or variation in the sizes of BL:1 for uniform BL size and 0 for varying BL size. The total score for an embryo included the three aspects (BL, FR and EQ). Every patients received 2 embryos transferred and the average embryo score is 7.2 ± 1.6. Totally, there were 19 patients get clinical pregnant (19/40, 47.5%).

4. Discussion

In the past few years, "the freeze-all" strategy has emerged as an alternative to fresh embryo transfer during IVF cycles [13,14]. Meanwhile, numerous studies have suggested that impaired perinatal results

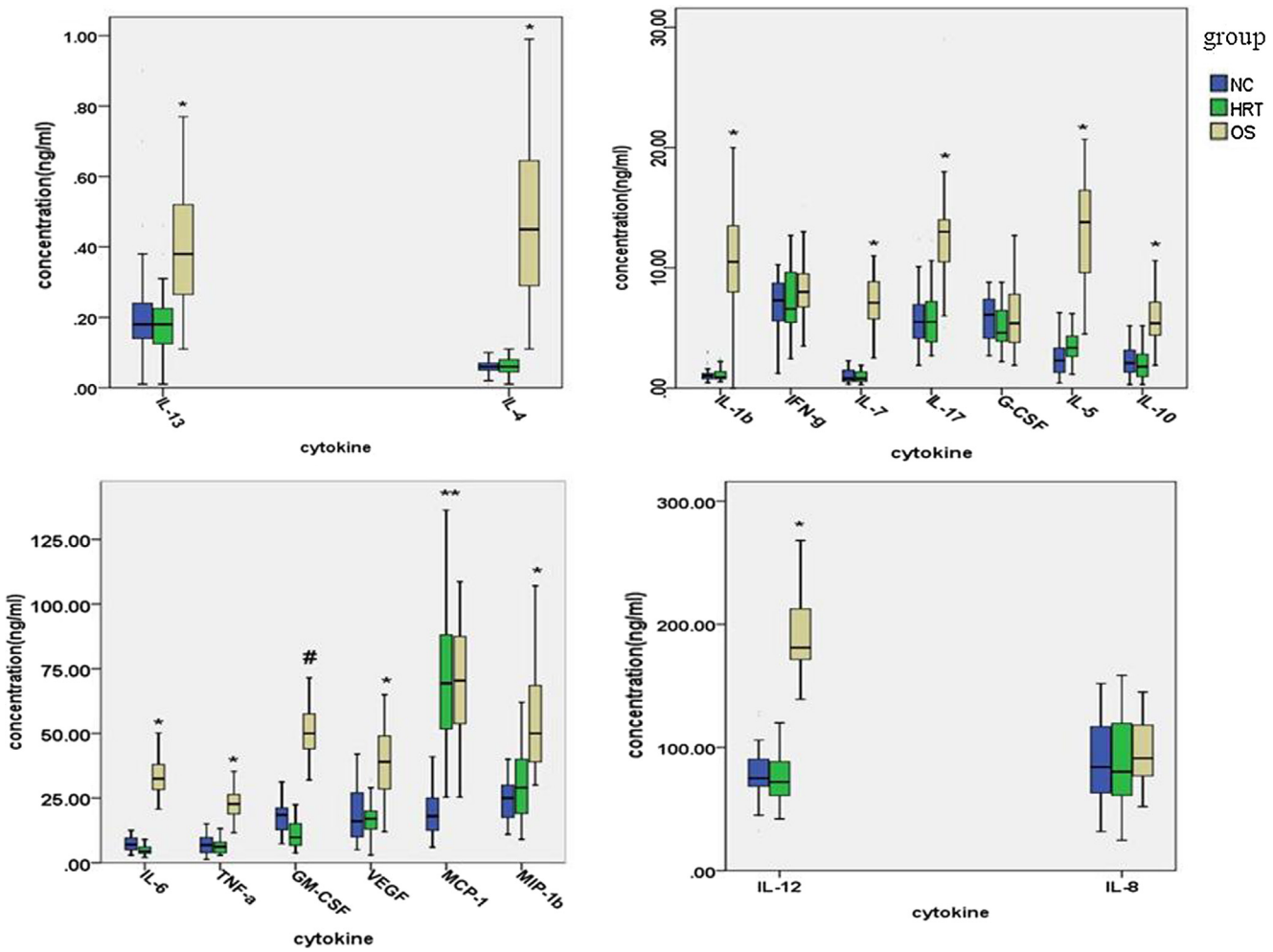

Fig. 1. Concentrations of endometrium cytokines in natural, IVF and FET cycle. Concentrations of cytokines in natural cycle (NC), ovarian stimulation cycle (OS) and hormone replacement cycle (HRT) were compared. Each box shows the median, quartiles, and extreme values within a category. *Significant difference between OS and NC or HRT cycle; $P < .05$. ** Significant difference between NC and OS or HRT cycle; $P < .05$. # Significant difference among OS, NC and HRT cycle; $P < .05$.

in the fresh embryo transfer could be explained with altered endometrial receptivity and/or placental dysfunction resulting from abnormal steroid levels that follow ovarian stimulation during the process of implantation [15–17].

Through the repeated measurement study, we found that compared with natural cycle, almost all cytokine profiles in endometrial secretions had the same concentrations in HRT cycle, except for MCP-1, which was significantly increased in both OS and HRT cycles. MCP-1 is up-regulated by the administration of progesterone, and increased levels are found in secretory rather than proliferative endometrium [18]. It is one of the key chemokines that is excreted in response to proinflammatory cytokines by regulating monocyte/macrophage migration and leukocyte activation in the endometrium during the preparation process for implantation. MCP-1 is also a strong absorber and activator of uterine natural killer cells, which were associated with abortion and infertility [19,20]. Boomsma et al. [8] showed initial embryo implantation was negatively associated with endometrial secretion concentrations of MCP-1 and Kalem et al. [21] also reported patients with recurrent pregnancy loss (RPL) and repeated implantation failure (RIF) had higher levels of MCP-1than those in the control group. Thus, the increase of MCP-1 in endometrium secretion may attribute to higher progesterone level in OS cycle or exogenous progesterone injection in HRT cycle. However, considering the negative effects of excessive MCP-1 reported by previous studies, we may recommend using as much as possible NC cycle to prepare endometrium in frozen embryo transfer. Significantly higher levels of numerous cytokines, including IL-1b, IL-5, IL-10, IL-12, IL-17, TNF-α, and heparin-binding EGF had been reported in endometrial secretions in stimulated cycles compared to natural ones [8]. To our knowledge, this is the first study to compare endometrium cytokine profile characteristics during implantation window among the three endometrium preparation protocols for embryo transfer. We found significantly higher concentrations of pro-inflammatory cytokines i.e. IL-1b, IL-7, IL-17, IL-6, TNF-a, GM-CSF and IL-12, and anti-inflammatory cytokines i.e. IL-4, IL-13, IL-10, IL-5, as well as VEGF and MIP-1b in the stimulated cycle compared to the natural cycle and HRT cycles.

During the establishment of pregnancy endometrial remodeling is characterized by three key processes: influx of uNK cells, decidualisation (differentiation) of stromal fibroblasts and remodeling of the endometrial vasculature and these processes are all based on estradiol and progesterone. There are two sources of uterine cytokines during the phase of embryo implantation: uterine natural killer (uNK) cells and endometrial epithelium/stromal cells. The uNk cells are the most abundant leukocytes in the luteal phase and its function could be regulated by estradiol and progesterone by increasing their migration, differentiation and secretion [22,23]. DeLoia et al reported significant increase in both uNK cells and macrophage populations when the women received oral estrogen, which resulted in higher serum estrogen levels [24]. As our results showed, serum estrogen and progesterone level in OS cycle were much higher than in NC or HRT cycle, which resulted in hyperproliferative endometrial epithelium/stromal cells. So, increased production of estradiol and progesterone after ovarian stimulation may be the reason of elevated secretion of growth factors and cytokines in utero. Consisting with our results, an increased number of uNK cells after ovarian stimulation has also been proved by other studies [25,26]. Except that, human chorionic gonadotropin (hCG), which is administrated in OS cycle as a “trigger” but not in natural cycle and hormone replacement cycle, can also stimulate the production of variety of cytokines by either epithelial cells or stromal cells [27].

Many of the endometrium secretion cytokines are known to exert paracrine influence on embryo implantation. For example, TNF-α recruits macrophages (monocytes) in the endometrium, which in turn causes intense endometrial inflammation and consequently interferes with embryo implantation [28]. Higher levels of TNF-α also plays a crucial role in some reproductive diseases such as endometrial infections and recurrent spontaneous abortions [29,30]. GM-CSF, a known regulator of uterine dendritic cells, is synthesized by uterine epithelial cells during induction of tolerance in early pregnancy. Insufficient GM-CSF may impair generation of T cell-mediated immune tolerance at the outset of pregnancy and may contribute to infertility and miscarriage [31]. It has also been claimed that low serum GM-CSF concentrations observed in women with recurrent miscarriages might be corrected by intravenous immunoglobulin treatment [32]. Würfel [33] reported administered human GM-CSF at the time of embryo transfer in patients with RIF, the pregnancy rate rose significantly from 19.8% to 42.9%. We found endometrium GM-CSF secretion increased in OS cycle than NC, which may be a beneficial aspect compensating for the excessive inflammatory response of endometrium in OS. An appropriate bias of T-cell immunity in different phase is critical and the disruption of the “cytokine balance” induced by exogenous or endogenous factors, can lead to pregnancy failure. It is reported that elevated concentration of IFNγ, together with TGFα and IL-1α in utero secretion during proliferative phase have been reported in idiopathic infertility women < 35 years [34]. Liang et al. [35] found higher ratio of pro- and anti-inflammatory cytokines, IFN-γ/IL-4, IFN-γ/IL-10, IL-6/IL-10, IL-6/TGF-β1, IL-1β/TGF-β1 and TNF-α/TGF-β1 in peripheral blood of RIF group in IVF cycle, suggested a shift toward a pro-inflammatory state in the patients with RIF. Kalu et al. [36] also discovered Th1 bias in unexplained recurrent failed IVF treatment and this polarization is more enhanced following ovarian stimulation. So, notably increased cytokines level after ovarian stimulation in this study indicated a more inflammatory response in utero which may lead to an undesirable pregnancy outcome. As the safety and effectiveness IVF is critical and complex issue, we should try to eliminate factors that may have adverse consequences. From this perspective, we recommend embryo transfer in natural cycle as much as possible.

Previous studies reported hormones, including E2, P and LH could regulate cytokine production in by ovarian stromal and granulosa-lutein cells [37,38]. These hormones could also affect the secretion of cytokines by endometrium cultured in vitro [39,40]. In this study, we found only IL-7 concentrations in endometrial secretions had a weak relationship with serum LH. As hCG has been proved to stimulate the production of multi-functional cytokine in endometrium in vitro [27,41], we postulate LH may also have analogous effect since the α-subunits of LH and hCG show a high degree of similarity. So, LH-regulated endometrial informational networks should further be examined.

The number and quality of embryo transferred are important determinant of initial and ongoing pregnant. In order to avoid bias caused by embryonic factors, we transferred embryos of the same number and roughly the same score for every patients. According to an up-to-date random cohort study (n = 782) [42], the pregnancy rate of frozen embryo transfer was 44.2% with two viable embryos transferred, which is consistent with our results. So, our clinical pregnancy outcome further confirmed the endometrium secretion sampling method.

Overall, the concentrations of most cytokines in endometrium during implantation window demonstrated few differences between hormone replacement cycles and natural cycles, while they were significantly increased in ovarian stimulation cycles. Significantly higher concentrations of pro-inflammation cytokines, anti-inflammation cytokines, chemokine and VEGF following ovarian stimulation represent a higher inflammatory response state and they may negatively affect embryo implantation and conspectuses development. Further investigations of the fundamental mechanisms of endometrium cytokine profiles affected by ovarian stimulation, and their respective roles within the embryo implantation, could lead to further improvements in practice and policy of embryo transfer.

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