Microbial colonization of follicular fluid: alterations in cytokine expression and adverse assisted reproduction technology outcomes

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BACKGROUND: Previous studies have measured cytokines expressed within follicular fluid and compared the profiles with the aetiology of infertility and/or successful or unsuccessful assisted reproduction technology (ART) outcomes.

METHODS: In this study, 71 paired follicular fluid and vaginal secretions collected from ART patients were cultured to detect microorganisms and tested for the presence of cytokines. Patient specimens were selected for assay based on two criteria: whether the follicular fluid specimen was colonized (with microorganisms prior to oocyte retrieval) or contaminated by vaginal flora and; the aetiology of infertility. Patients included fertile women (with infertile male partners; n = 18), women with endometriosis (n = 16) or polycystic ovary syndrome (PCOS, n = 14), or couples with a history of genital tract infection (n = 9) or idiopathic infertility (n = 14).

RESULTS: Microorganisms and cytokines were detected within all tested specimens. Colonizing microorganisms in follicular fluid were associated with: decreased fertilization rates for fertile women (P = 0.005), women with endometriosis (P = 0.0002) or PCOS (P = 0.002) compared with women whose follicular fluid was contaminated at the time of oocyte retrieval and with decreased pregnancy rates for couples with idiopathic infertility (P = 0.001). A single cytokine was discriminatory for women with an idiopathic aetiology of infertility (follicular fluid interleukin (IL)-18). Unique cytokine profiles were also associated with successful fertilization (IL-1α, IL-1β, IL-18 and vascular endothelial growth factor).

CONCLUSIONS: Follicular fluid is not sterile. Microorganisms colonizing follicular fluid and the ensuing cytokine response could be a further as yet unrecognized cause and/or predictor of adverse ART outcomes and infertility.

Key words: cytokines / follicular fluid / bacteria / assisted reproduction technology / infertility

Introduction

Cytokines, chemokines and growth factors are produced by cells following activation of the innate or adaptive immune response (Husband et al., 1999). Previous studies have shown that cytokines are essential for ovarian function (Richards et al., 2002), modulating the secretion of ovarian steroid hormones (Richards et al., 2002) and the development and regression of corpus luteum function in humans (Sunderkotter et al., 1994; Chen and Peng, 2000). Cytokines also have an essential function in embryonic development and implantation, promoting cellular differentiation, vascularization and finally trophoblast invasion of the endometrium (Vujisic et al., 2006). In recent years, a number of studies have investigated the presence of cytokines in follicular fluid and correlated these with assisted reproduction technology (ART) outcomes: these studies demonstrated that cytokines interleukin (IL)-1, IL-1α, IL-1β, IL-12/23 (p40) and vascular endothelial growth factor (VEGF) were associated with successful outcomes including increased fertilization rates, successful embryo transfer and clinical pregnancy (Karagouni et al., 1998; Vujisic et al., 2006), whilst IL-12, VEGF and IL-15 were associated with poor fertilization and failed conception (Gazvani et al., 2000; Asimakopoulou et al., 2006; Vujisic et al., 2006). Cytokine profiles within follicular fluid also varied depending on...
the reproductive pathology or the cause of infertility (Hill et al., 1990; Fasciani et al., 2000; Amato et al., 2003). To our knowledge, no previous study has tested follicular fluid for microorganisms and correlated the presence of microorganisms with the cytokine profile within follicular fluid and ultimate outcomes of ART cycles.

Spandorfer et al. (2001) investigated abnormal vaginal flora and vaginal pro-inflammatory cytokines in women with idiopathic infertility undergoing ART cycles. A correlation between bacterial vaginosis, elevated IL-1β and IL-8, and idiopathic infertility was demonstrated. However, they found no correlation between the presence of bacterial vaginosis and ART pregnancy outcomes. This finding is in contrast to previous reports, which have found an association between bacterial vaginosis and second trimester miscarriage (Oakeshott et al., 2002); Spandorfer et al. (2001) suggested that administration of antimicrobial prophylaxis following oocyte retrieval may explain this anomaly. In a prospective study of 262 women undergoing ART treatment, we have detected the presence of bacteria within the follicular fluid of 99.5% of these women: the follicular fluid of only one woman was sterile.

We hypothesize that microorganisms present in follicular fluid adversely affect oocyte quality and, in addition, microorganisms themselves or the products of their metabolism elicit an immune response that results in adverse reproductive health outcomes. The aim of this study was to determine if cytokine profile(s) were correlated with (or could predict) (i) the presence of bacteria within follicular fluid and (ii) if distinct cytokine profiles were associated with the aetiology of infertility or altered ART outcomes, including rates of oocyte retrieval, ART fertilization and/or ART embryo transfer. In the current study, a paired vaginal swab and follicular fluid specimen(s), collected at the time of transvaginal oocyte retrieval, were tested to detect and identify microorganisms present at these anatomical sites. Aliquots of these clinical specimens then were tested for the presence of microorganisms and 18 cytokines to compare the cytokines present in the lower and upper genital tract specimens collected from each woman and identify if any cytokines are associated with adverse ART outcomes.

Materials and Methods

Specimen collection

From September 2007 to November 2008, consenting patients commencing fully stimulated ART cycles at Wesley-Monash IVF (Brisbane, QLD) were enrolled in this study. Ethical approval for this study was obtained from the review boards of Uniting Care Health, Human Research Ethics Committee and Queensland University of Technology Human Research Ethics Committee. All patients gave permission for researchers to access medical records to obtain their reproductive history and ART outcomes. Results of the testing of 71 follicular fluid and vaginal specimens are reported in this paper.

The IVF unit used a ‘boost’ protocol for controlled ovarian hyperstimulation. A GnRH agonist was administered from day two of the menstrual cycle and FSH was administered from Day 4. Follicle size was monitored by transvaginal ultrasound scans and once multiple follicles reached 17–19 mm diameter, the patient received an hCG injection. Oocytes were collected 36 h later by transvaginal oocyte retrieval. In addition to the follicular fluid collected at the time of oocyte retrieval, two vaginal swabs were taken from each patient. One vaginal swab was collected prior to cleansing of the vagina and was tested for cytokines, and a second swab (after cleansing of the vagina with sterile saline) was reserved for microbiological culture. Briefly, the ultrasound probe was wiped with sterile distilled water and finally an isowipe. The ultrasound probe was covered with a disposable sheath. Transvaginal oocyte retrieval was performed using a sterile needle (K-OPS-1032-WMC Cook Medical Single Lumen Aspiration Needle, Brisbane, QLD, Australia) attached to a needle holder on a vaginal ultrasound probe. The vaginal wall was prepared using sterile saline to remove excess mucous and cellular debris. Immediately prior to oocyte retrieval, the needle was flushed with follicle flush buffer (K-SIFB-100 Cook Medical—Follicle Flush Buffer). For each study participant, the follicular fluid from the largest, most accessible follicle (17–20 mm) in either the left or the right ovary was aspirated first. Follicular fluid was aspirated directly into sterile test tubes in the theatre. This follicular fluid specimen was aseptically transferred into a sterile culture dish to determine if there was an oocyte present, the oocyte was transferred to a sterile culture dish and the follicular fluid was divided into aliquots: 1 ml was added to 80% glycerol; 1 ml was added to 20 μl protease inhibitor cocktail (Sigma-Aldrich, Castle Hill, NSW, Australia); and the remaining follicular fluid was transferred into a sterile 15 ml falcon tube. All specimens were stored at −80°C. The oocytes were then transferred to fresh culture media and pooled prior to IVF insemination or hyaluronidase treatment and ICSI.

Follicular fluid culture and colony identification

Follicular fluid specimens in glycerol were thawed prior to culture. For a subset of the women in this study (n = 6), the follicular fluid from both the left and right ovary was cultured. Using a sterile 1 ml calibrated inoculating loop (Becton Dickinson, North Ryde, NSW, Australia) a range of culture media/agar plates (horse blood agar, chocolate agar, MacConkey agar, Sabouraud Dextrose agar, de Man Rogosa Sharpe agar, anaerobic blood agar; Oxoid, Adelaide, SA, Australia) was inoculated using a 16-streak technique. An aliquot of follicular fluid was also inoculated into thioglycollate broth (Oxoid). Plate media were incubated either aerobically in 5% carbon dioxide or anaerobically in anaerobic jars (Oxoid anaerogen, 2.5L, Oxoid) at 37°C. Colony forming units (CFUs) on aerobic plates were quantified after 24 h, whilst those on facultative plates were counted after 24 h and again at 48 h. The anaerobic plates and thioglycollate broths were examined and CFUs enumerated at 48 h and then every second day up to 14 days. Each different CFU from all plates was Gram stained and subcultured for biochemical identification.

Gram-negative rods were identified using API20E identification strips (Biomerieux, Baulkham Hills, NSW, Australia), catalase positive Gram-positive cocci were identified by Staphylase (Oxoid) and rabbit plasma for tube coagulase testing (Becton Dickinson). Identification of pigmented catalase positive, Gram-positive cocci suspected to be of the Micrococcaeeae family were identified by 16S rRNA PCR assay followed by sequencing of the amplicon at the Australian Genome Research Facility (AGRF, University of Queensland, QLD). Catalase negative Gram-positive cocci from the Streptococcus family were identified by Lancefield grouping (Oxoid) and bacitracin (Oxoid) or optochin (Oxoid) where appropriate. Anaerobic isolates were identified by API20A (Biomerieux) identification strips followed by a 16S rRNA PCR assay and sequencing (AGRF, QLD) if isolates scored a poor API T-value, which is an estimate of how closely the profile corresponds to the most typical set of reactions for the stated species or percentage identification.

Prior to the 16S rRNA PCR assay each colony was first resuspended in 100 μl of 100 μg/ml proteinase K (Sigma-Aldrich) and 100 μl of sterile distilled water. The suspension was vortexed and then incubated at 37°C for 1 h. DNA was extracted using a QIAamp mini DNA extraction kit (Qiagen, Doncaster, VIC, Australia) as per the manufacturer’s
instruction for extraction from blood and body fluids. The 16S rRNA PCR assay was performed using the previously published primers DG74 and RW01 (Greisen et al., 1994) with a degeneracy built into the 3’ end of the upstream RW01 primer. The PCR master mix included: 1 X Buffer; 200 µM of dNTPs (Roche, Castle Hill, NSW, Australia); 0.5 µM of each primer (Sigma-Aldrich); 5 U Taq polymerase (Roche) and 8 µl of the extracted bacterial DNA, to a final volume of 50 µl. PCR cycling conditions included an initial denaturation at 95°C for 15 min followed by 25 cycles of denaturation at 94°C for 20 s, primer annealing at 55°C for 20 s, extension at 72°C for 20 s and a final extension step at 72°C for 10 min (PTC-200, Peltier Thermal Cycler, BioRad, Gladesville, NSW, Australia). PCR amplicons were electrophoresed in a 2% agarose gel containing ethidium bromide at 100 V for 60 min and visualized under ultraviolet light. Prior to sequencing at AGRF the PCR product was purified using a Roche High Pure PCR clean up kit. The sequence obtained was entered into the Basic Local Alignment Search Tool (BLAST, NCBI) for identification of clinical isolates.

Criteria for the selection of specimens for cytokine screening

Follicular fluids were classified as ‘colonized’ if microorganisms were detected within the follicular fluid but not in the vagina (at the time of oocyte retrieval) or ‘contaminated’ if microorganisms present in the vagina were also detected within the follicular fluid. Clinical specimens then were selected based on the couples’ aetiology of infertility (Table I): from 37 women with colonized follicular fluid and from 34 women with contaminated follicular fluid. These women had a history of infertility related to endometriosis, polycystic ovary syndrome (PCOS), genital tract infection or idiopathic infertility. Specimens from ‘fertile’ women, i.e. from couples with male factor infertility were selected as the control group.

Cytokine testing of follicular fluid and vaginal secretions

Follicular fluid cytokine levels were measured by flow fluorimetry in 96-well microtiter plates using an automated dual laser flow cytometer (Bio-Plex Cytokine Assay System, Bio-Rad, NSW, Australia). The multiplex bead working solution was prepared for the two sets of cytokines (Bio-Rad) to be assayed: Human Group II cytokine 6-plex panel: IL-1α, IL-12 (p40), IL-18, leukaemia inhibitory factor (LIF), macrophage colony stimulating factor (M-CSF), tumour necrosis factor (TNF)β; and Human Group I cytokine 12-plex panel: IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17, granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), interferon gamma (IFNγ), TNFα and VEGF.

The follicular fluids, previously aliquotted into the cryovials containing 20 µl protease inhibitor cocktail (Sigma), were thawed on ice and prepared for testing by centrifuging at 2000g for 20 min at 4°C to remove any cell debris. Blood-stained follicular fluid specimens were not included in this study. The vaginal swabs were thawed on ice and rotated 20 times in 500 µl sterile phosphate-buffered saline containing 10 µl protease inhibitor cocktail. All swab preparations were performed by a single operator. Swab suspensions were then centrifuged at 2000g for 20 min at 4°C immediately prior to assay.

The standards were prepared and cytokine assays performed as per the manufacturer’s instructions. Briefly, the wells in the microtiter plate were pre-wet with 100 µl assay buffer, which was removed by vacuum filtration. Then 50 µl of bead working solution was added to each plate, the excess solution was removed by vacuum filtration and the plates were washed twice in 100 µl of wash buffer. Standards and samples were added to the plate and incubated at room temperature, agitating at 300 rpm for 30 min. Standards and samples were assayed in duplicate. The limit of detection for all cytokines in this assay was determined to be 2–32 000 pg/ml. The plates were washed and 25 µl of detection antibody was added to each well, incubated and agitated at 300 rpm for 30 min. Excess antibody was removed by vacuum filtration and 50 µl of streptavidin-PE was added to each well, incubated at room temperature, and agitated at 300 rpm for 10 min. After a final wash, the beads were resuspended in 125 µl of assay buffer, read on the plate reader (Bio-Plex 200, Bio-Rad) and analysed with Bio-Plex Manager 4.0 software.

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences version 17 for Windows XP. Multinomial logistic regression was used to model the relationship between categorical outcomes and explanatory variables because all variables were not dichotomous. The dependent variables that were considered were colonization (yes/no) and contamination (yes/no). Discriminant analyses were used to identify cytokines associated with: (i) the aetiology of infertility; (ii) either follicular fluid ‘colonized’ prior to oocyte retrieval or ‘contaminated’ during the retrieval process; and (iii) the ART outcomes for the treatment cycle.

In the regression analyses, a P-value of P < 0.05 was considered statistically significant (Gelman and Hill, 2007). All variables were entered into the model. No variables were transformed. In the discriminant analyses, Wilk’s lambda was used to assess overall significance of the discriminant functions. Wilk’s lambda is used for multivariate hypothesis testing and is an extension of the familiar F-test used in univariate hypothesis testing. A Wilk’s lambda value close to zero indicates that group means differ (Sokal and Rohlf, 1995). The authors note that the statistical analyses are based on a small sample size and therefore, these analyses provide indicative, not definitive results.

**Table I** Colonized and contaminated* follicular fluids from women with different aetiologies of infertility.

<table>
<thead>
<tr>
<th>Aetiology</th>
<th>Endometriosis, n = 16</th>
<th>PCOS, n = 14</th>
<th>Genital tract infection, n = 9</th>
<th>Male factor*, n = 18</th>
<th>Idiopathic, n = 14</th>
<th>Total, n = 71</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colonized follicular fluid</td>
<td>6 (38%)</td>
<td>7 (50%)</td>
<td>5 (55%)</td>
<td>12 (67%)</td>
<td>7 (50%)</td>
<td>37</td>
</tr>
<tr>
<td>Contaminated follicular fluid</td>
<td>10 (62%)</td>
<td>7 (50%)</td>
<td>4 (45%)</td>
<td>6 (33%)</td>
<td>7 (50%)</td>
<td>34</td>
</tr>
</tbody>
</table>

*Follicular fluids were classified as ‘colonized’ if microorganisms were detected within the follicular fluid but not in the vagina (at the time of oocyte retrieval) or ‘contaminated’ if microorganisms present in the vagina were also detected within the follicular fluid.

*Specimens from ‘fertile’ women, i.e. from couples with male factor infertility were selected as the control group.
Results

Patients

Bacteria were detected in all 71 follicular fluids and vaginal secretions, as well as colonized and contaminated follicular fluids for each classification of infertility that was tested for this study (Tables I–III).

The mean (±SD) age of all women in this study was 37 ± 4 years. The mean age of fertile women was 35 (± 4 years) compared with 38 (± 4 years) for infertile women (P = 0.007). However, the age of women was not associated with any adverse ART outcomes (P > 0.05). For fertile and infertile women there was no difference in number of prior ART treatment cycles. Fertile women had participated in an average of 1 (range 0–3, ± 1 cycle) previous transvaginal oocyte retrieval procedures compared with infertile women who had participated in 1 (range 0–5, ± 1 cycle, P > 0.05). Furthermore, there was no difference in the number of oocytes retrieved for fertile (10 ± 5) or infertile women (11 ± 6, P > 0.05).

Culture and colony identification

The bacteria colonizing follicular fluid included Lactobacillus iners (detected only in follicular fluid from the right ovary) Actinomyces spp., Corynebacterium aurumcosum, Fusobacterium spp., Peptipholus ascorarolylticus; Peptastreptococcus spp., members from the genera Propionibacterium and Prevotella; Staphylococcus spp., and the yeast Candida parapsilosis. In accordance with our definition, these same species were not isolated from the paired vaginal secretion (Supplementary data, Table SI). For all women, regardless of their aetiology of infertility, a greater number of different microorganisms were detected in the left ovarian follicle (n = 14 for colonized follicular fluid; n = 33 for contaminated follicular fluid) compared with the right ovarian follicle (n = 7 and 21, respectively; Supplementary data, Table SI).

The lowest fertilization rates occurred in women with colonized follicular fluid and an aetiology of infertility of endometriosis (49%), PCOS (58%) and fertile women (male factor infertility cohort) (43%) compared with women with contaminated follicular fluid with fertilization rates of 71, 81 and 61%, respectively (P < 0.005; Table II). Increased fresh embryo discard rates were observed for fertile women whose follicular fluid was contaminated at the time of oocyte retrieval (57%) and women with PCOS (75%) compared with women with colonized follicular fluid (25 and 57%, respectively, P = < 0.0001). In women with colonized follicular fluid a lower pregnancy rate was observed (for all cohorts except for women with endometriosis) compared with women with contaminated follicular fluid.

For couples with idiopathic infertility and colonized follicular fluid no pregnancies were achieved after embryo transfer: in contrast, 5 of the 7 (71%) women with contaminated follicular fluid achieved a pregnancy after fresh embryo transfer (P = 0.001; Table II). For fertile women, a decreased pregnancy rate for freeze-thawed embryos was associated with colonized follicular fluid (44%) compared with women with contaminated follicular fluids (100%) (P = 0.0001; Fig. 1).

Outcomes for all fertile (male infertility cohort) and all infertile women with ‘colonized follicular fluid’ or ‘contaminated follicular fluid’ were compared (Table III). Fertile and infertile women with colonized follicular fluid had decreased fertilization rates (43 and 36%, respectively) compared with women with contaminated follicular fluid.
Microbes in follicular fluid, cytokines and pregnancy

Table III

<table>
<thead>
<tr>
<th>Aetiology</th>
<th>Fertilization rate</th>
<th>Embryo discard rate</th>
<th>Pregnancy rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infertile</td>
<td>219/345 (63%)</td>
<td>199/257 (77%)</td>
<td>137/219 (63%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>137/199 (69%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;0.05</td>
</tr>
<tr>
<td>&lt;0.001</td>
</tr>
<tr>
<td>0.002</td>
</tr>
<tr>
<td>0.0001</td>
</tr>
<tr>
<td>&gt;0.05</td>
</tr>
<tr>
<td>0.005</td>
</tr>
<tr>
<td>0.01</td>
</tr>
<tr>
<td>0.05</td>
</tr>
</tbody>
</table>

**Cytokines**

The logistic regression analyses (Supplementary data, Table SII) demonstrated that the presence of colonizing bacteria in follicular fluid was associated with a decreased fertilization rate and an aetiology of endometriosis. Decreased embryo transfer rates were also observed in women with a diagnosis of PCOS. The regression model found that no follicular fluid or vaginal secretion cytokine profiles could differentiate between infertile women with a diagnosis of endometriosis or PCOS, or a history of upper genital tract infection when normalized against the control group of ‘fertile’ women (who had partners with male factor infertility). The cytokines IL-6, IL-8, IL-10 and/or G-CSF in follicular fluid of women with endometriosis, PCOS, previous genital tract infection and idiopathic aetiologies of infertility were the most significant cytokines present when normalized against the follicular fluid from ‘fertile’ women for each aetiology (Supplementary data, Table SII). Women with idiopathic infertility could be differentiated from all other groups by the presence of elevated levels of IL-1B in follicular fluid. We also found that the presence of IL-12p40 and IFNγ in follicular fluid was a distinctive markers of successful embryo transfer. Successful fertilization was predicted by the presence of IL-1α and VEGF in vaginal secretions. Seventeen of the 18 cytokines tested were detected within follicular fluid, only IL-17 was not detected (Supplementary data, Table SII). Testing of vaginal secretions demonstrated the presence of 13 cytokines, but LIF, M-CSF, GM-CSF, IFNγ and TNFs were not detected (Table IV).

CART analysis (Fig. 2) revealed that six follicular fluid cytokines (IL-6, IL-8, IL-12, IL-18, IL-10 and VEGF) could be used to predict a relationship between cytokines and the aetiology of infertility. This analysis was also useful for selecting cytokines (IL-1B, VEGF, IL-10, IL-8, IL-6 and IL-18), which could be markers for embryo transfer (Fig. 3). For example, CART analysis revealed that the following combinations of variables: IL-6 (<22.45 pg/ml), IL-8 (<10.60 pg/ml) and VEGF (>66.40 pg/ml), or IL-6 (<22.45 pg/ml), IL-8 (<10.60 pg/ml), IL-10 (>14.10 pg/ml) and VEGF (>749.10 pg/ml) were predictive of an idiopathic aetiology [Fig. 2 (12—idiopathic infertility)] of infertility. Successful embryo transfer was indicated by IL-1B (>0.191 pg/ml), VEGF (>219.90 pg/ml) and IL-10 (>3.10 pg/ml), or IL-1B (<0.20 pg/ml), VEGF (>219.90 pg/ml but <801.20 pg/ml) and IL-8, where IL-1B and VEGF are the most important cytokines (indicated by the long tree branches). The length of the tree branch (Fig. 3) indicates the relative importance of each cytokine. There appears to be substantial interactive effects between the cytokines in differentiating both the aetiology of infertility and embryo transfer, as shown by the network of branches, which differ by combinations of variables.

Discriminant analyses (Table V) were performed to compare the aetiology of infertility, fertilization rate and embryo transfer rates using both follicular fluid cytokine levels and vaginal secretion cytokine...
levels. No results presented reached significance however; the first discriminatory function for follicular fluid indicates that >80% of variation in aetiology is coming from between group variation. Discriminant analysis of follicular fluid found two major discriminatory functions (groups of cytokines), which classified 83.7% of the groups for infertility aetiology (Table V). These were not significant by Wilk’s lambda but were highly weighted (by the Eigenvalue) suggesting that there could be physiological interactions occurring between: M-CSF, IL-4, IL-10 (first function aetiology) and LIF, IL-10, TNFβ, VEGF and M-CSF (second function aetiology). Only a single function was required for discriminating between 100% of successful and unsuccessful outcomes for the fertilization rate and embryo transfer rate groups: M-CSF, TNFβ, GM-CSF, IL-10, LIF (first function fertilization rate); and IL-8 (first function embryo transfer). Whilst these were again not significant by Wilk’s lambda they were also highly weighted. A similar trend was observed with the vaginal secretion discriminant analysis. The analysis found two major discriminatory functions which classified 80% of aetiology groups: IL-1β and IL-8 (first function) and IL-1β, IL-8 and G-CSF (second function) (Table V). Fertilization rate and embryo transfer rate groups again required only a single function to discriminate between 100% of the group: IL-1β (first function fertilization rate) and M-CSF, TNFβ and IL-4 (first function embryo transfer). Wilk’s lambda was not significant for any discriminant functions of vaginal secretions cytokines. The functions at group centroid indicate that follicular fluid and vaginal secretion cytokine profiles for fertilization (Fig. 4A and B) and embryo transfer (Fig. 5A and B) both group in the same manner and samples of either would be suitable markers to predict these ART outcomes albeit using different cytokine profiles. In contrast, follicular fluid and vaginal secretion

Table IV Mean cytokine concentrations detected in follicular fluid and vaginal secretions.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Cytokines in follicular fluid (pg/ml)</th>
<th>Cytokines in vaginal secretion (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>0.16 ± 0.69a</td>
<td>0.75 ± 1.43</td>
</tr>
<tr>
<td>IL-12 (p40)</td>
<td>9.08 ± 26.31</td>
<td>4.92 ± 28.60</td>
</tr>
<tr>
<td>IL-18</td>
<td>1.48 ± 3.12</td>
<td>1.05 ± 5.86</td>
</tr>
<tr>
<td>LIF</td>
<td>0.01 ± 0.05</td>
<td>0</td>
</tr>
<tr>
<td>M-CSF</td>
<td>0.44 ± 2.32</td>
<td>0</td>
</tr>
<tr>
<td>TNF-β</td>
<td>0.14 ± 1.10</td>
<td>1.41 ± 7.53</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.25 ± 0.81</td>
<td>7.18 ± 24.80</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.53 ± 2.62</td>
<td>0.10 ± 0.87</td>
</tr>
<tr>
<td>IL-6</td>
<td>20.70 ± 14.58</td>
<td>3.51 ± 6.71</td>
</tr>
<tr>
<td>IL-8</td>
<td>101.68 ± 209.53</td>
<td>391.41 ± 1090.58</td>
</tr>
<tr>
<td>IL-10</td>
<td>28.82 ± 36.11</td>
<td>3.85 ± 9.96</td>
</tr>
<tr>
<td>IL-12 (p70)</td>
<td>5.06 ± 13.58</td>
<td>0.48 ± 3.16</td>
</tr>
<tr>
<td>IL-17</td>
<td>0</td>
<td>0.86 ± 6.97</td>
</tr>
<tr>
<td>G-CSF</td>
<td>5.46 ± 18.69</td>
<td>16.11 ± 42.59</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>0.10 ± 0.59</td>
<td>0</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>880.66 ± 2308.55</td>
<td>0</td>
</tr>
<tr>
<td>TNF-α</td>
<td>4.33 ± 27.52</td>
<td>0</td>
</tr>
<tr>
<td>VEGF</td>
<td>1364.93 ± 1621.35</td>
<td>234.04 ± 829.52</td>
</tr>
</tbody>
</table>

a Mean ± SD.

LIF, leukaemia inhibitory factor; M-CSF, macrophage colony stimulating factor; TNF, tumour necrosis factor; GM-CSF, granulocyte macrophage colony stimulating factor.

Figure 1 Overall thaw cycle pregnancy rate for fertile versus infertile women with colonised follicular fluid. Fertile women with colonized follicular fluid had a significantly decreased pregnancy rate following frozen embryo transfer when compared with fertile women with contaminated follicular fluid (P = 0.0001).
cytokine group aetiologies of infertility are very different (Fig. 6A and B). Follicular fluid cytokines readily differentiate women with a history of upper genital tract infection from all other groups (Fig. 6A). This may represent the overt inflammatory response associated with long-term sequelae as a result of upper genital tract infection. Vaginal fluid cytokines distinguish women from male factor (control group) couples from women with all other causes of infertility (Fig. 6B). This could be a useful non-invasive early screening test for women from partnerships where the males are infertile.

Discussion

In this study, we have demonstrated the presence of bacteria (either as colonizers or contaminants) within follicular fluid collected from women undergoing ART cycles. We have also demonstrated that the follicular fluid from the left ovary is more prevalently and heavily colonized/contaminated than the right ovary, often with different microbial species. This was observed for all women despite the aetiology of infertility. Clinicians performing transvaginal oocyte retrieval randomly selected which follicle to approach first. Therefore, we concluded that the differences identified between the microflora of the left and right ovaries was the result of a physiological process. This evidence further supports our hypothesis that even though the follicular fluid of women is colonized the women are asymptomatic. We propose that this may be related to the separate vasculature of the two ovaries; an ovary with a better blood supply could be a more suitable niche for microbial persistence and survival allowing for continual waste removal and nutrient supply.

Cottell et al. (1996), investigating the microorganisms present in embryo culture systems, collected follicular fluids at the time of oocyte retrieval and reported similar findings, isolating bacteria from 40% of left side ovarian follicles and 33% of right sides ovarian follicular fluids. Monteleone et al. (2008) found that vascularized follicles were associated with increased fertilization rates (90%), embryo quality (64.7%) and pregnancy rates (37.5%) when compared with follicles with less vasculature (73.6, 41 and 21.1%, respectively). These findings further support a relationship between vasculature and the quality of oocytes and demonstrate the significance of independent blood supply to each ovarian follicle, particularly if these microorganisms are spread haematogenously.

Women undergoing ART cycles are stimulated to produce multiple maturing follicles. Ultrasound studies have demonstrated that there is a significant increase in ovarian blood supply from the proliferative period of one menstrual cycle to the proliferative phase of following cycle (Gaytan et al., 1999; Jokubkiene et al., 2006). The presence of microorganisms within ovarian follicles may be a consequence of prior infertility treatment protocols, with bacteria being introduced into the ovary at the time of oocyte retrieval. Alternatively, bacteria from other sites, such as the oral cavity (Cohen et al., 2003) or the respiratory tract (Goulet et al., 1995), could access the ovary after
haematogenous dissemination and subsequently colonize the ovary. Previous studies have reported cytokine profiles for ovarian conditions including endometriosis and PCOS (Hill et al., 1990; Fasciani et al., 2000; Amato et al., 2003). In this study, we have confirmed previous findings but in addition we have compared cytokine profiles of women with microbial colonization or contamination of follicular fluid and different causes of infertility with their ART outcomes.

We have shown that the presence of colonizing bacteria within follicular fluid adversely affected fertilization rates for all fertile (male factor) and infertile women, and in particular for women with infertility due to endometriosis and women with PCOS (when compared with women from the same groups who had contaminated follicular fluid specimens). This could be a result of damage to the oocyte in vivo after exposure to the bacteria or bacterial metabolic products.

We have also demonstrated that the clinical pregnancy rates were decreased in women with idiopathic infertility and follicular fluid colonization when compared with women with idiopathic infertility whose follicular fluid was contaminated by vaginal bacteria at the time of oocyte retrieval. The spontaneous conception rate is only around 20% per month in fertile couples (Te Velde and Cohlen, 1999). Patients in our ‘fertile women’ cohort achieved a pregnancy rate of 30% with colonized follicular fluid and 60% with contaminated fluid, placing their fecundity rate above the average level, but suggesting that the presence of colonizing species in follicular fluid was detrimental in both fertile and infertile women.

It is interesting to note that both fertile and some infertile women had increased fresh embryo discard rates in the presence of contaminating bacteria, however, this same significant effect was not present following embryo freezing (results not shown). We have found that the freeze-thaw process destroys up to 10% of microorganisms frozen and this would include those frozen and then thawed with the embryo. Taken together these results demonstrate that the presence of colonizing bacteria in follicular fluid adversely affects fertilization rates thereby reducing the number of embryos available from each ART treatment cycle and, furthermore, bacteria that contaminate the oocytes at the time of retrieval increase the embryo discard rate for all ART patients. It is not surprising that there is no difference in the ART pregnancy rate for fertile and most infertile women with colonized follicular fluid compared with those with contaminated follicular fluid, as the oocytes and embryos that were affected by bacteria were eliminated either because of failure to fertilize or failure to develop, as demonstrated by the increased fresh embryo discard rate in this cohort.

A shortcoming of this study is that only the follicular fluid from the largest most accessible follicles in both the left and/or right ovary were cultured to detect microorganisms. However, more recently in our laboratory, we have cultured the pooled follicular fluids collected from all follicles in the left and right ovaries, and analysed the follicular fluids separately, and pooled, and we have demonstrated that for 35% of women the microorganisms present within the largest follicle are the same as those collected from other follicles within the same ovary (J. Harris, Unpublished data.). In future studies, it would be useful to collect follicular fluid from women undergoing laparoscopic surgery to remove all risk of lower genital tract contamination of the follicular fluid.

**Figure 3** CART analysis of the cytokines associated with embryo transfer. CART analysis revealed cytokines (IL-1β, VEGF, IL-10, IL-8, IL-6, IL-18), which could be markers for failed, or successful (single or double) embryo transfer.
We have identified specific follicular fluid and vaginal secretion cytokine levels in women with idiopathic infertility. In a study of women with idiopathic infertility, Spandorfer et al. (2001) reported elevated levels of IL-1β and IL-8 in cervical secretions from women with bacterial vaginosis but they found no correlation between altered vaginal flora and IVF outcomes: this may be because women received prophylactic antimicrobial treatment following oocyte retrieval. We detected these same cytokines (IL-1β and IL-8) and also IL-6 and IL-10 in vaginal secretions as well as IL-6, IL-10 and IL-18 within follicular fluid from women with idiopathic infertility: IL-18 (previously known as interferon inducing factor) was a discriminating cytokine detected only in the follicular fluid of women with idiopathic infertility in this study. This suggests that there may be a pathological cause for this infertility. Hook et al. (2005) demonstrated that elevated levels of IL-12 and IL-18 acted synergistically to produce the necessary IFN-γ response required to clear genital tract infections caused by Chlamydia trachomatis and, in addition, bacterial vaginosis in women with idiopathic infertility was also associated with significantly higher levels of cervical IFN-γ. IL-18 has also previously been reported within the semen of infertile men with a history of genital tract infection, however, it was not associated with any alterations in semen andrology parameters (Matalliotakis et al., 2006). Together, these reports suggest that IL-18 could be a marker of asymptomatic genital tract infection, which in infertile couples may have been classified as idiopathic infertility. Cytokine screening of vaginal secretions and follicular fluid specimens during an ART treatment cycle could therefore offer rapid, valuable information on immune modulation, in addition to the standard morphological assessment of embryos. Cytokine profiles related to successful ART outcomes could further inform the selection of good quality embryos for transfer back to the uterus.

The inactive precursor of IL-18 is reportedly expressed in various cell types including macrophages. The inactive pro-IL18 is cleaved by caspase-1 or proteinase-3 to form the biologically active mature IL-18. In addition to IFN-γ induction, the pro-inflammatory characteristics of IL-18 also result in enhancement of T and natural killer (NK) cell maturation, cytokine production and cytotoxicity. The chemotactic activity of IL-18 could be a reason for the large number of lymphoid cells within the ovary: cells from the white cell lineage including monocytes, macrophages, lymphocytes, neutrophils and eosinophils have been described within ovarian tissues (Norman and Brannstrom, 1996). Of these, macrophages and monocytes account for 5–15% of the cell types within follicular fluid (Loukides et al., 1990) and as a result many studies have investigated the role of follicular fluid cytokines on the physiology of reproduction and ART outcomes. Macrophages are present in ovarian tissues and are activated during an immune response to pathogens. These immune effectors also play a significant role in homeostasis via cytokine production and secretion (Gordon, 1999). During an endometrial cycle, the secretion of inflammatory (IL-1, IL-6, TNFα) and anti-inflammatory (IL-10) cytokines...
Figure 4 Functions at group centroid for (A) follicular fluid cytokines and (B) vaginal secretion cytokines and fertilization. Investigations of the relationship between cytokines identified in the follicular fluid and vaginal secretions indicated that for fertilization, either specimen would be potentially useful in predicting ART outcomes if the site-specific cytokine profile was used.

Figure 5 Functions at group centroid for (A) follicular fluid cytokines and (B) vaginal secretion cytokines and embryo transfer. Investigations of the relationship between cytokines identified in the follicular fluid and vaginal secretions indicated that for embryo transfer, either specimen would be potentially useful in predicting failed or successful ART outcomes if the site-specific cytokine profile were used.
cytokines and growth factors in endometrial tissues facilitate cellular proliferation and angiogenesis (IL-6, IL-8, VEGF) necessary for the cyclical changes from the proliferative (follicular) through to the secretory (luteal) phase, in preparation for implantation and ongoing pregnancy (Wu et al., 2004). Similarly, macrophages control events within the ovarian follicles and have been detected within the ovary in fluctuating numbers throughout the menstrual cycle (Brannstrom et al., 1994). During follicle growth, macrophage numbers within the ovary increase and localize to dominant (maturing) follicles (Wu et al., 2004), secreting cytokines to stimulate cellular proliferation (IL-1α, IL-1β), follicular growth, steroid hormone production and secretion (IL-2, IL-6, LIF, TNFα) and the suppression of apoptosis (IL-1β) (Richards et al., 2002). An increase in macrophage numbers within the ovarian theca just prior to ovulation also results in the secretion of proteases and cytokines thought to be essential for ovulation to occur (Brannstrom et al., 1995; Tadros et al., 2001). Furthermore, the formation and regression of the corpus luteum is modulated by macrophage-secreted cytokines, those associated with angiogenesis (Sunderkotter et al., 1994) and increased production of progesterone (Chen and Peng, 2000).

NK cells are also present in high numbers within follicular fluid collected at the time of transvaginal oocyte retrieval and are capable of modulating cytokine expression (Krızan et al., 2009). An alteration in the level of immune-regulatory follicular fluid NK cells and NK-T cells has been correlated with IVF outcome (Krızan et al., 2009; Fainaru et al., 2010). This could suggest that women with impaired NK cell function could have an altered follicular fluid cytokine profile and also an impaired ability to respond to viral challenges, which may further contribute to their infertility.

Alterations in cytokine levels and profiles have been reported in follicular fluid and ovarian tissue for women with endometriosis (Fasciani et al., 2000), PCOS (Amato et al., 2003), premature ovarian failure (Hill et al., 1990) and ovarian cancer (Pisa et al., 1992). In this study, we confirmed the association between four follicular fluid cytokines (IL-6, IL-8, IL-10 and G-CSF) and three vaginal fluid cytokines (IL-1β, IL-8 and IL-10) (Table IV) previously reported in women with an aetiology of infertility of endometriosis (Van Blerkom et al., 1997; Fasciani et al., 2000; Salmassi et al., 2005). Endometriosis is an inflammatory condition involving the ectopic development of transplanted endometrial tissue (Speroff and Fritz, 2005). This transplanted endometrial tissue has potent angiogenic properties with the ability to progress and infiltrate many ectopic sites, and this is modulated by altered functions in immune response cells (Ho et al., 1997). In women with endometriosis there is also an increased expression of the cytokines IL-1β, IL-6, IL-8 and IL-10 in peritoneal fluid (Ho et al., 1996; Wu and Ho, 2003) and IL-6, IL-10, G-CSF and VEGF in follicular fluid (Pellicer et al., 1999; Kilic et al., 2007; Falconer et al., 2009). IL-6 (Motro et al., 1990) and IL-8 (Koch et al., 1992) have also been reported in association with angiogenesis, when cytokine expression levels were correlated with the growth of new vasculature, endometrial cells, ectopic endometrium (Arici et al., 1998) and disease severity (Gazvani et al., 1998). High levels of angiogenic factors [VEGF (Fig. 1), IL-6 and IL-8 (Fig. 1 and Table V)] reported within follicular fluid in the current study are further evidence of the angiogenic activity surrounding the ovarian follicles.
In this study, women with PCOS had increased expression of follicular fluid and vaginal fluid IL-8 and G-CSF. Whilst these same patterns of cytokine expression have previously been reported for women with PCOS (Amato et al., 2003), others found no detectable differences between cytokines when comparing women with PCOS to those without polycystic ovaries (Jasper and Norman, 1995; Pellicer et al., 1999).

Ge and You (2008) demonstrated expression of an IL-17 receptor in oocytes, but the function of this cytokine in the ovary is not known and in this current study IL-17 was not detected within follicular fluid. We have reported cytokine profiles which were associated with adverse ART outcomes, including decreased fertilization and embryo transfer rates, and propose that the cytokine profiles were generated in response to the colonizing or contaminating bacteria present within follicular fluid. The cytokines we detected within follicular fluid and vaginal secretions, which were associated with fertilization and embryo transfer outcomes were from both the pro-inflammatory (IL-6, IL-8, IL-12p40, IL-10, G-CSF) and anti-inflammatory (IL-4, IL-10, G-CSF) cytokine groups (Tables IV and V, Fig. 1). However, it is interesting to note that none of the follicular fluid specimens tested in this study appeared to be infected on gross examination. Similarly, women with bacterial vaginosis and idioopathic infertility most frequently have no symptoms of infection but demonstrate an altered cytokine profile in their cervical secretions (Cherpes et al., 2008). However, the overall pro-inflammatory to anti-inflammatory cytokine balance in women with asymptomatic bacterial vaginosis remains unchanged and it has been proposed that the balanced immune response could be responsible for the lack of symptoms (Cherpes et al., 2008). Therefore, in this study we have demonstrated that infertile women with no pathological symptoms have altered follicular fluid cytokine profiles, which were associated with adverse ART outcomes.

We propose that the alteration in the immune response in women with colonized follicular fluid could be a previously undescribed cause of infertility. Furthermore, we have demonstrated site-specific (follicle/vagina) cytokine profiles, which could be used as screening tools to determine the aetiology of infertility or to help predict which women are likely to have successful or unsuccessful ART cycles. The identification of a cytokine profile, which predicts adverse ART outcomes, including failed fertilization, failed embryo transfer, failed conception or early pregnancy loss, could lead to the development of targeted therapy to modulate the immune response within the upper genital tract of these women, which could potentially improve ART pregnancy outcomes.

**Authors’ roles**

E.S.P.: Wrote the manuscript; contributed to experimental design and research plan; performed all experimental work. J.A.A.: Assisted with organization and collection of clinical specimens by gynaecologists and IVF scientists. Approved nurse recruitment of participants. Critically reviewed manuscript and approved final version of manuscript. K.C.: Immunology expertise; provided feedback and assistance on experimental design and executions; critically revised manuscript and approved final version of manuscript. K.M.: Statistical expertise; provided feedback and assistance on statistical analysis and interpretation; critically revised statistical section of the manuscript and approved final version of manuscript. J.M.A.: Critically reviewed manuscript and approved final version of manuscript. T.L.: IVF liaison for specimen collection. Critically reviewed manuscript and approved final version of manuscript. K.B.: Involved in experimental planning and design; critically reviewed manuscript proofs, contributed to the intellectual input of the manuscript and approved final version of manuscript. C.L.K.: Involved in experimental planning and design; critically reviewed manuscript proofs, contributed to the intellectual input of the manuscript and approved final version of manuscript.

**Supplementary data**

Supplementary data are available at http://humrep.oxfordjournals.org/.

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