INTRODUCTION

Although the vagina contains various microbes, the upper female genital tract is generally considered sterile, and some authors have observed antimicrobial activity for human follicular fluids. However, molecular investigations of the human microbiome have revealed that tissue or body fluids considered highly sterile, such as peritoneal fluids, have their own microbiome, and recently, it has been revealed that the microbiota of the female reproductive tract account for approximately 9% of the total bacterial load in humans. There are discrepancies among published studies regarding the impact of follicular fluid microorganisms on in vitro fertili-
tion (IVF) outcomes. Cottell, et al.6 first reported the presence of microorganisms in follicular fluid, but found no significant impact on IVF cycles. These findings are consistent with those reported by Usman, et al.6 However, multiple microorganisms have been detected in follicular fluid, and their presence has been found to be related to adverse IVF outcomes, including decreases in embryo transfer (ET) and pregnancy rates and an increase in embryo discard rates.7,8

Vaginal-cervical microorganisms are speculated to affect IVF outcomes. IVF procedures involve needle puncture of the vaginal wall and transfer of embryos using a catheter through the cervix, with risks of microbial inoculation. This can induce chronic endometritis9 or alter the biochemical or ultrastructural characteristics of the endometrium.10 Previous studies have reported that pathogenic microorganisms in the vagina or cervix reduce clinical pregnancy11-13 and live birth rates14 and increase miscarriage rates15 after IVF cycles. A healthy intra-follicular environment supports the acquisition of developmental competence in oocytes.16 Therefore, there is the potential for disruption of the intrafollicular environment by microorganisms, and this can have an adverse effect on oocyte competence and embryo quality. Vaginal-cervical microorganisms may affect uterine receptivity. Subsequently, all these factors may, in turn, influence the clinical outcomes of IVF.

To date, the effect of follicular fluid microorganisms on IVF outcomes has not been widely investigated. To the best of our knowledge, our study is the first to evaluate the association of follicular fluid microorganisms with embryo quality. The present study aimed to identify microorganisms in follicular fluids and to investigate their association with IVF outcomes.

**MATERIALS AND METHODS**

**Study subjects**

Between December 2013 and February 2016, 49 infertile females commencing fully stimulated IVF/intracytoplasmic sperm injection (ICSI) cycles at a university-based hospital were enrolled in this study. Female patients aged <40 years seeking infertility treatment at our facility whose follicle-stimulating hormone (FSH) levels on cycle day 3 were <10 mIU/mL and whose body mass index (BMI) was <30 kg/m² were included in this study. The exclusion criteria were an anti-Müllerian hormone (AMH) level of <0.5 ng/mL and greater than three retrieved oocytes in a previous treatment cycle. The median age of the women was 36.0 years. The indications for IVF were unexplained (n=19), endometriotic (n=6), tubal (n=10), male sex-related (n=7), ovulatory (n=5), and uterine (n=2) factors. Informed consent was obtained from all subjects, and the use of human semen for this study was approved by the Institutional Review Board of Seoul National University Bundang Hospital (B-1310-223-004).

**Controlled ovarian stimulation protocols**

Recombinant FSH (Gonal-F; Merck-Serono, Darmstadt, Germany, or follitrop; LG Chem, Seoul, South Korea) or highly purified human menopausal gonadotropin (Menopur; Ferring, Saint-Prex, Switzerland) was administered from day 3 of the menstrual cycle. Pituitary downregulation was achieved with a mid-luteal long protocol of gonadotropin-releasing hormone (GnRH) agonist (0.1 mg/d of Decapeptyl; Ferring) (n=4) or a GnRH antagonist protocol (0.25 mg/d of Cetrotide, Merck-Serono) (n=43). Pituitary downregulation was not performed in two cycles. Follicle size was monitored by transvaginal ultrasound scan. Once the leading follicle reached a mean diameter of ≥18 mm or two follicles reached a mean diameter of ≥17 mm, the patient received a subcutaneous injection of 250 µg of recombinant human chorionic gonadotropin (Ovidrel; Merck-Serono). Oocytes were collected 35–36 h after the triggering.

**Specimen and oocyte collection**

Before transvaginal oocyte retrieval, a vaginal swab using Amies agar gel transport media (TransystemTM, Copan, Italy) was taken from the posterior fornix after the vaginal wall was irrigated using 50 mL of sterile saline to remove excess mucus and cellular debris. The ultrasound probe was covered with a sterile condom and a disposable sheath. Transvaginal oocyte retrieval was performed using a sterile needle (Cook Medical Single Lumen Aspiration Needle; Brisbane, QLD, Australia) attached to a needle holder on a vaginal ultrasound probe. The follicular fluid from the largest and most available follicles of each ovary was aspirated first. The follicular fluid was aspirated directly into sterile test tubes and transferred to a sterile culture dish to obtain oocytes. After retrieving the oocytes, the remaining follicular fluid was transferred to a sterile Falcon tube to detect the microorganisms.

**Detection of microorganisms from follicular fluid and vaginal swabs**

All specimens including follicular fluid and the vaginal swabs were examined for aerobic and anaerobic bacteria simultaneously. Follicular fluid specimens were inoculated aerobically onto a blood agar plate and MacConkey agar. The vaginal swabs were cultured aerobically on blood, chocolate, and MacConkey agars. All plates for aerobic and anaerobic bacteria were incubated at 37°C for 24 h. Thioglycollate broth was used for anaerobic bacteria culture, which was incubated at 37°C without CO₂. If there was no growth after 24 h, prolonged incubation was performed for another 24 h. Cultures were considered positive when there were ≥10⁶ colony-forming units/mL.

**Fertilization, ET, embryo quality assessment, and confirmation of pregnancy**

Mature oocytes were inseminated using conventional methods (27 cycles) and ICSI (20 cycles). Normal fertilization was defined as the presence of two pronuclei. On day 3 after insem-
Follicular fluid and vaginal swabs (Table 1). Among women who only had vaginal swab positivity, coagulase-negative staphylococci were the most prevalent species (n=12, 52.2%), followed by Streptococcus agalactiae (group B) (n=4, 17.4%), Escherichia coli (n=3, 13.0%), alpha-streptococcus (n=3, 13.0%), and Enterococcus faecalis and Klebsiella pneumonia (n=1, 4.3%, detected in the same woman). Alpha-streptococcus (n=2, 66.7%) and Kocuria kristinae (n=1, 33.3%) were detected in the group with only follicular fluid positivity. The same microorganisms were detected in both the follicular fluid and vaginal swabs of three women (Table 2), while different microorganisms were detected in follicular fluid and vaginal swabs in five women.

Follicular fluid microorganisms and IVF outcomes
Data on patient characteristics, controlled ovarian hyperstimulation (COH), and IVF outcomes in positive and negative follicular fluid culture groups are summarized in Table 3. Both groups were comparable in regards to age, BMI, causes of infertility, ovarian reserve markers (serum AMH and serum FSH), dose of gonadotropin administration, endometrial thickness, and serum estradiol level at triggering day. Also, there were no significant differences in IVF outcomes, such as the number of total oocytes retrieved, number of mature oocytes retrieved, oocyte maturity rate, and CES. Fertilization rates (60.0% vs. 75.0%) and percentages of grade A embryos at day 3 (14.3% vs. 31.0%), implantation rates (11.8% vs. 23.9%), clinical pregnancy rates at day 5 ET (12.5% vs. 28.0%), and clinical pregnancy rates at day 5 ET (0% vs. 62.5%) were higher in the negative culture group, but the differences did not reach statistical significance.

Vaginal microorganisms and IVF outcomes
Data on patient characteristics and COH and IVF outcomes in the positive and negative vaginal culture groups or positive normal flora group are outlined in Table 4. Coagulase-negative staphylococci were considered normal flora, therefore, we excluded cases with coagulase-negative staphylococci (n=13) in the positive vaginal culture group (n=31) and defined them newly as a positive pathogen group (n=18). Both groups were similar in age, BMI, causes of infertility, ovarian reserve markers (serum AMH and serum FSH), gonadotropin dose, endometrial

**RESULTS**

Species of microorganisms from follicular fluid and vaginal swabs
Fifteen women (30.6%) had no microorganisms in their follicular fluid or vaginal swabs, 23 (46.9%) had microorganisms in vaginal swabs alone, 3 (6.1%) had microorganisms in follicular fluid alone, and 8 (16.3%) had microorganisms in both follicular fluid and vaginal swabs. Statistical analysis
Statistical analyses were performed using SPSS version 22 software (IBM Corp., Armonk, NY, USA). Proportions were compared using the chi-square test or Fisher's exact test. Continuous variables were compared across groups using the Mann-Whitney U test. Differences were considered statistically significant at p<0.05.

**Table 1. Microorganism Species Detected in Vaginal Swabs and Follicular Fluid**

| Genus and species | Only Vagina (+) (n=23) | Only FF (+) (n=3) | Both (+) (n=8) |
|------------------|-----------------------|------------------|----------------|
| Coagulase-negative staphylococci | 12 (52.2%) | 1 (12.5%) | 2 (25.0%) |
| Streptococcus agalactiae (group B) | 4 (17.4%) | 1 (12.5%) | 2 (25.0%) |
| Escherichia coli | 3 (13.0%) | 2 (25.0%) | | |
| Alpha-streptococcus | 3 (13.0%) | 2 (66.7%) | 3 (37.5%) | 2 (25.0%) |
| Kocuria kristinae | 1 (33.3%) | | | |
| Enterococcus faecalis | 1 (4.3%)* | 3 (37.5%) | | |
| Klebsiella pneumonia | | 1* | | |
| Staphylococcus aureus | | 1 (12.5%) | | |
| Candida albicans | | 1 (12.5%) | | |

FF, follicular fluid. Data presented as n (%).
*Detected in the same patient.

**Table 2. Microorganism Species Detected in Both Vaginal Swabs and Follicular Fluid from Simultaneous Culture**

| Vagina (+) | FF (+) |
|------------|-------|
| 1 Streptococcus agalactiae (group B) | S. agalactiae (group B) |
| 2 Alpha-streptococcus | Alpha-streptococcus |
| 3 Alpha-streptococcus | Alpha-streptococcus |
| 4 Escherichia coli | Enterococcus faecalis |
| 5 Alpha-streptococcus | E. faecalis |
| 6 Staphylococcus aureus | S. agalactiae (group B) |
| 7 E. coli | Candida albicans |
| 8 Coagulase-negative staphylococci | E. coli |

FF, follicular fluid.
thickness, and serum estradiol level on the triggering day. There were no significant differences between the two groups in IVF outcomes, such as the number of total oocytes retrieved, number of mature oocytes retrieved, oocyte maturity rate, fertilization rates, CES, and percentages of grade A embryos on day 3. However, implantation rates were significantly lower in the pathogen-positive group (9.1% vs. 29.4%, p=0.031). Clinical pregnancy rates on day 3 ET were decreased (14.3% vs. 31.6%), albeit non-significantly, in the pathogen-positive group. However, significantly lower clinical pregnancy rates on day 5 ET were observed in the pathogen-positive group (0% vs. 83.3%, p=0.048).

**DISCUSSION**

In the present study, although several microorganisms were detected in follicular fluid, they were not associated with embryo quality or clinical pregnancy rates during IVF cycles. However, significantly lower implantation rates and clinical pregnancy rates on day 5 ET were observed in women who were positive for vaginal pathogens.

It is still debated whether microorganisms exist in follicular fluid or are inoculated during the IVF procedure. Of the eight patients who had both follicular fluid and vaginal swab microorganisms, only three had the same strains in both follicular fluid and vaginal swab (Table 1). These three patients were likely to have been “contaminated” with vaginal-cervical strains at the time of puncture for transvaginal oocyte aspiration. However, the five patients with different strains suggests that follicular fluids had microorganisms independent of the IVF procedure (“colonization”). Spence, et al.18 reported discordant results when investigating microorganisms detected in the lower and upper genital tracts of asymptomatic women. Anaer-

### Table 3. Comparison of Characteristics and IVF Outcomes according to Results of Follicular Fluid Culture

| Variables                              | FF (+) (n=11) | FF (-) (n=38) | p value |
|----------------------------------------|---------------|---------------|---------|
| Age of female (yr)                     | 37.0 [34.0, 39.0] | 35.5 [33.0, 37.3] | 0.129   |
| BMI of female (kg/m²)                  | 23.5 [19.5, 25.8] | 21.7 [20.2, 23.5] | 0.181   |
| Causes of infertility (%)              |               |               |         |
| Unexplained                            | 3 [27.3]       | 16 [42.1]     |         |
| Endometriosis                          | 0 [0]          | 6 [15.8]      |         |
| Tubal                                   | 3 [27.3]       | 7 [18.4]      |         |
| Male                                    | 2 [18.2]       | 5 [13.2]      |         |
| Ovulatory                               | 2 [18.2]       | 3 [7.9]       |         |
| Uterine                                 | 1 [9.1]        | 1 [2.6]       |         |
| Serum anti-Müllerian hormone (ng/mL)   | 2.56 [1.38, 5.47] | 3.23 [1.23, 4.78] | 0.990   |
| Serum FSH (mIU/mL)                     | 5.6 [4.6, 8.8]  | 5.7 [3.7, 7.0] | 0.463   |
| Dose of gonadotropin (IU)              | 1800 [1500, 1800] | 1800 [1575, 2250]| 0.443   |
| EMT at triggering day (mm)             | 8.4 [7.2, 10.2] | 9.2 [8.0, 11.1] | 0.125   |
| Serum estradiol at triggering day (pg/mL)| 949 [804, 3200] | 1531 [764, 2740] | 0.771   |
| No. of total oocytes retrieved         | 10.0 [2.0, 13.0] | 7.5 [4.0, 12.0] | 0.943   |
| No. of mature oocytes retrieved        | 4.0 [2.0, 9.0]  | 4.0 [3.0, 6.3] | 0.631   |
| Fertilization rate (%)                 | 60.0 [40.0, 80.0] | 66.7 [41.4, 80.1] | 0.150   |
| No. of ET cycle, n                     | 60.0 [40.0, 100.0] | 75.0 [66.7, 100.0] | 0.655   |
| Day 3                                  | 8              | 25            |         |
| Day 5                                  | 1              | 8             |         |
| Transferred embryos                    | 2.0 [1.0, 2.0]  | 2.0 [1.8, 2.0] | 0.435   |
| Percentages of grade A embryo at day 3 (%)| 14.3 [5.6, 40.0] | 31.0 [14.9, 50.0] | 0.230   |
| CES                                    | 114.0 [51.0, 190.0] | 96.5 [46.5, 151.5] | 0.610   |
| CES of transferred embryo (day 3)      | 55.0 [39.0, 70.0] | 56.0 [44.0, 63.0] | 0.757   |
| Implantation rate                      | 11.8 [2/17]    | 23.9 [16/67]  | 0.343   |
| Clinical pregnancy rate per ET, % (n)  | 12.5 [1/8]     | 28.0 [7/25]   | 0.643   |
| Day 3                                  | 0 [0/1]        | 62.5 [5/8]    | 0.444   |

IVF, in vitro fertilization; FF, follicular fluid; BMI, body mass index; FSH, follicular stimulating hormone; EMT, endometrial thickness; CES, cumulative embryo score; ET, embryo transfer.

Data presented as a median [interquartile range]. Medians were compared using the Mann-Whitney U test. Proportions were compared using the chi-squared test or Fisher’s exact test.
obic bacteria were detected in the peritoneal fluid obtained by laparoscopic aspiration, but not in the vagina or cervix in 25% of the subjects. These results suggested that the peritoneal cavity of normal healthy women is not always sterile and that bacteria might colonize the upper genital tract without evidence of infection. The natural flora of other organs has been suggested as a possible source of follicular fluid microorganisms. Microorganisms may spread into follicular fluid via body fluid or hematogenous dissemination. Pelzer, et al. reported that isolated species found to colonize follicular fluid were part of the body’s natural microflora, including the gastrointestinal tract (enteric bacteria, S. agalactiae), skin (Staphylococcus), and oral mucosa (Streptococcus).  

Although the current study did not show an association between follicular fluid microorganisms and IVF outcomes, previous studies have reported negative effects on IVF outcomes. The suggested mechanisms of the association of follicular fluid microorganisms with IVF outcomes are 1) alteration in the immune response, especially increased cytokine, interleukin-18, biofilm formation inhibiting immune detection and reducing the effectiveness of antimicrobial treatment; and 3) increased oocyte DNA fragmentation.  

In the present study, pathogenic vaginal microorganisms were not associated with embryo quality, but they were associated with worse implantation rates and clinical pregnancy rates in IVF cycles. Our results are in accordance with the results of previous reports that showed that the presence of pathogenic bacteria in the vagina reduces pregnancy rates after IVF. Haahr, et al. reported that women with bacterial vaginosis were significantly less likely to obtain a clinical pregnancy (9%) in comparison with overall pregnancy (35%, p=0.004). Ricci, et al. showed that the presence of genital tract pathogens, obtained from vaginal/endocervical swabs, was predictive of a negative IVF outcome. These results suggest that microorgan-

### Table 4. Comparison of Characteristics and IVF Outcomes according to Results of Vaginal Culture

| Variables                        | Pathogen (n=18) | Normal flora or negative (n=31) | p value |
|----------------------------------|----------------|-------------------------------|---------|
| Age of female (yr)               | 37.5 [34.0, 39.0] | 36.0 [33.0, 38.5] | 0.103 |
| BMI of female (kg/m²)            | 22.7 [20.5, 24.8] | 21.7 [20.3, 23.7] | 0.457 |
| Causes of infertility (%)        |                |                               |         |
| Unexplained                      | 2 (11.1)       | 17 (50.0)                     |         |
| Endometriosis                    | 2 (11.1)       | 4 (3.6)                       |         |
| Tubal                            | 7 (38.9)       | 3 (21.4)                      |         |
| Male                             | 4 (22.2)       | 3 (10.7)                      |         |
| Ovulatory                        | 1 (5.6)        | 4 (10.7)                      |         |
| Uterine                          | 2 (11.1)       | 0 (0)                         |         |
| Serum anti-Müllerian hormone (ng/mL) | 2.62 [1.14, 4.17] | 3.38 [1.46, 4.96] | 0.553 |
| Serum FSH (mIU/mL)               | 5.4 [3.8, 7.7]  | 5.9 [3.9, 7.2]               | 0.753 |
| Dose of gonadotropin (IU)        | 1800 [1575, 2287.5] | 1800 [1575, 1950] | 0.555 |
| EMT at triggering day (mm)       | 8.6 [7.5, 10.2] | 9.5 [8.2, 11.0]              | 0.136 |
| Serum estradiol at triggering day (pg/mL) | 1531 [871, 3094] | 1474 [718, 2605.5] | 0.460 |
| No. of total oocytes retrieved   | 9.0 [4.0, 14.3] | 7.0 [3.0, 12.0]              | 0.388 |
| No. of mature oocytes retrieved  | 5.0 [3.0, 9.0]  | 4.0 [2.0, 6.0]               | 0.164 |
| Oocyte maturity rate (%)         | 72.1 [53.3, 80.8] | 63.4 [33.3, 79.1] | 0.271 |
| Fertilization rate (%)           | 79.2 [60.0, 100.0] | 75.0 [62.5, 100.0] | 0.921 |
| No. of ET cycle, n               |                |                               | 0.259 |
| Day 3                            | 14             | 19                            |         |
| Day 5                            | 9              | 6                             |         |
| Transferred embryos              | 2.0 [1.8, 2.0]  | 2.0 [1.0, 2.0]                | 0.627 |
| Percentages of grade A embryo at day 3 (%) | 25.0 [12.2, 50.0] | 28.6 [14.3, 50.0] | 0.889 |
| CES                              | 115.0 [89.8, 187.0] | 78.0 [42.0, 138.0] | 0.148 |
| CES of transferred embryo        | 56.0 [52.0, 66.0] | 56.0 [36.0, 62.0] | 0.217 |
| Implantation rate                | 9.1 [3/33]     | 29.4 [15/51]                  | 0.031 |
| Clinical pregnancy rate per ET, % (n) |            |                               |         |
| Day 3                            | 14.3 [2/14]    | 31.6 [6/19]                   | 0.416 |
| Day 5                            | 0 [0/3]        | 83.3 [5/6]                    | 0.048 |

IVF, in vitro fertilization; FF, follicular fluid; BMI, body mass index; FSH, follicular stimulating hormone; EMT, endometrial thickness; CES, cumulative embryo score; ET, embryo transfer.
Data presented as a median [interquartile range]. Medians were compared using the Mann-Whitney U test. Proportions were compared using the chi-squared test or Fisher's exact test.
isms might have an effect on the endometrium rather than on embryo quality. Pathogenic vaginal microorganisms may be associated with subclinical chronic endometritis, which causes poor uterine receptivity. Kitaya et al. reported that 33.7% of infertile women with repeated implantation failure were diagnosed with chronic endometritis. In this group, microorganisms, such as Corynebacterium and Mycoplasma hominis, were frequently detected in the endometrium. Although the exact mechanisms are still unknown, vaginal-cervical microorganisms may modulate immune reactions in the uterus and cause morphological changes in the endometrium. Uterine immune cells detect E. coli via Toll-like receptor 4 binding its pathogenic ligand, lipopolysaccharide, and stimulate the endometrium to produce prostaglandin F and E2. 

Our study has several limitations. First, there was no relationship between follicular fluid microorganisms and IVF outcomes in the present study, with the small sample size being a possible reason for the lack of statistical significance. However, potentially harmful effects cannot be completely excluded; therefore, a larger-scale study is required to confirm our conclusions. Second, in the culture-based approaches, the results could have been underestimated compared to sequencing-based molecular diagnosis. Recently, microbiome research has revealed differences in the distributions of a large number of strains through 16S rRNA sequencing or next-generation sequencing. Nevertheless, it is difficult to conclude whether such differences in distributions demonstrated by these advanced techniques elicit a practically meaningful bacterial load. Therefore, positive results from culture-based techniques might have more definite clinical significance. Generally, sequencing-based molecular diagnosis cannot be performed in clinical practice; as such, culture-based data are useful and informative. Third, there were no data on Lactobacillus spp. in our study. When reporting vaginal swab results in our hospital, lactobacilli are not reported. Meanwhile, studies have indicated that the Lactobacillus spp. are dominant in the vagina and that their presence enhances IVF outcomes; therefore, we considered it imprudent to prove this again in the current study.

In conclusion, we found that follicular fluid contained microorganisms that differed from those in the vagina, but they were not associated with embryo quality or clinical pregnancy rate in IVF cycles. In contrast, vaginal pathogens were associated with worse implantation rates and pregnancy rates in IVF cycles. Larger-scale studies, however, are needed to elucidate the exact mechanisms underlying these findings. Understanding how follicular fluid or vaginal microorganisms affect IVF outcomes could lead to the establishment of new therapeutic interventions, including antibiotic therapy, and the improvement of IVF outcomes.

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