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Microbiology of Human Follicular Fluid and the Vagina and Its Impact on in Vitro Fertilization Outcomes

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Purpose: The present study aimed to identify microorganisms in follicular fluids and to investigate their association with in vitro fertilization (IVF) outcomes.

Materials and Methods: This study was conducted as a prospective study of 49 infertile females undergoing IVF/intracytoplasmic sperm injection cycles between 2013 and 2016. Paired follicular fluid and vaginal secretions were collected on the day of ovum pick up and were cultured to detect microorganisms.

Results: Fifteen women (30.6%) had no microorganisms in follicular fluid or vaginal swabs, 23 (46.9%) had microorganisms on vaginal swab alone, 3 (6.1%) had microorganisms in follicular fluid alone, and 8 (16.3%) had microorganisms in both follicular fluid and vaginal swabs. The same microorganisms were detected in both the follicular fluid and vaginal swabs of three women, while different microorganisms were detected between follicular fluid and vaginal swabs in five women. Follicular fluid microorganisms were not associated with embryo quality or clinical pregnancy rates during IVF cycles. However, significantly decreased implantation rates (9.1% vs. 29.4%, p=0.031) and clinical pregnancy rates on embryo transfer day 5 (0% vs. 83.3%, p=0.048) were observed in the group that was positive for vaginal pathogens.

Conclusion: Follicular fluid contains microorganisms that can differ from those in the vagina of the same women; however, they do not appear to be associated with embryo quality or clinical pregnancy rates in IVF cycles. In contrast, vaginal pathogens were found to be associated with worse implantation rates and clinical pregnancy rates in IVF cycles.

Key Words: Follicular fluid, vagina, microorganisms, in vitro fertilization, pregnancy rate

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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.[1,2] 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,[3] 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.[4]

There are discrepancies among published studies regarding the impact of follicular fluid microorganisms on in vitro fertiliza-

tion (IVF) outcomes. Cottell, et al.[5] 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 endometritis[9] 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 pregnancy[11-13] and live birth rates[14] and increase miscarriage rates[15] 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/m^2 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 Follitrope; 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 (Transystem™, 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 bacteria were incubated at 37°C in 5% CO_2 for 24 h. Thioglycollate broth was used for anaerobic bacteria culture, which was incubated at 37°C without CO_2. If there was no growth after 24 h, prolonged incubation was performed for another 24 h. Cultures were considered positive when there were ≥10^3 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-

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ination, embryo quality was assessed according to morphological criteria based on the percentage of fragmentation and regularity of blastomeres: 1) grade A, 0% anucleate fragments, regular blastomeres, and no apparent morphological abnormality; 2) grade B, 50% anucleate fragments, irregular blastomeres, and apparent morphological abnormalities. On day 3 after insemination, the embryonic score was obtained by multiplying the number of blastomeres and the grade score (on a scale of 1 to 4). For example, if one embryo was grade A with eight blastomeres, the score would be 32. The cumulative embryo score (CES) was calculated as the sum of the embryonic scores in each cycle. Blastocysts were evaluated on day 5 based on developmental stage and the quality of the inner cell mass and trophectoderm. Implantation rate was defined as the number of gestational sacs confirmed by ultrasound divided by the number of embryos transferred. Clinical pregnancy was confirmed by ultrasound evidence of fetal heartbeats.

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 $p<0.05$.

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 (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.

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)	
			Vagina	Follicle
Coagulase-negative staphylococci	12 (52.2)		1 (12.5)	
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 pneumoniae	1*			
Staphylococcus aureus			1 (12.5)	
Candida albicans				1 (12.5)

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

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 3 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,[17] 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

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.

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/m^2)	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			0.894
Oocyte maturity rate (%)	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)			
Day 3	12.5 (1/8)	28.0 (7/25)	0.643
Day 5	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.

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-

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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/m^2)	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.

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.[7] 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) .[7]

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,[8] 2) biofilm formation inhibiting immune detection and reducing the effectiveness of antimicrobial treatment;[19] and 3) increased oocyte DNA fragmentation.[20]

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.[21-23] Haahr, et al.[21] 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.[24] 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-

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.[10] Kitaya, et al.[25] 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.[26] 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.[27]

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[28] and that their presence enhances IVF outcomes;[7,10,29] 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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AUTHOR CONTRIBUTIONS

Conceptualization: Jung Ryeol Lee, Byung Chul Jee, and Chang Suk Suh. Data curation: Kyu Hee Won. Formal analysis: Su Mi Kim. Funding acquisition: Chang Suk Suh. Investigation: Su Mi Kim. Methodology: Seul Ki Kim. Project administration: Jung Ryeol Lee and Chang Suk Suh. Software: Yeon Hee Hong. Supervision; Jung Ryeol Lee, Byung Chul Jee, and Chang Suk Suh. Validation: Seul Ki Kim. Visualization: Su Mi Kim. Writing—original draft: Su Mi Kim. Writing—review & editing: all authors. Approval of final manuscript: all authors.

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