Females with impaired ovarian function could be vulnerable to environmental pollutants: identification via next-generation sequencing of the vaginal microbiome

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\textbf{ABSTRACT}

The vaginal microbiome has been widely investigated. However, its relationship with impaired ovarian function has not been evaluated. We conducted a next-generation sequencing (NGS) study of the vaginal microbiome in females with normal and decreased ovarian function and analysed its sensitivity to environmental pollutants. Vaginal swabs were collected from 92 individuals (22 with impaired ovarian function). The 16S rDNA sequences were assembled by FLASH and clustered in OTUs. Diversity analysis was performed using QIIME. The impaired function group showed lower AMH ($p<.01$) and higher FSH ($p=.04$). Only two species showed significant differences: \textit{Propionibacterium acnes} and \textit{Prevotella copri}. Moreover, more environmental pollutants were related to changes in the vaginal microbiome in the impaired ovarian function group than in the normal group. Vaginal microbiomes in young women with decreased ovarian function tended to be more sensitive to environmental pollutants, especially volatile organic compounds.

\textbf{IMPACT STATEMENT}

\begin{itemize}
  \item \textbf{What is already known on this subject?} In this study, the possible influence of environmental pollutants, especially volatile organic compounds to ovarian function were identified via next-generation sequencing.
  \item \textbf{What do the results of this study add?} This is the first study that shows vaginal microbiomes in young women with decreased ovarian function to be more sensitive to environmental pollutants.
  \item \textbf{What are the implications of these findings for clinical practice and/or further research?} The association between impaired ovarian function and environmental pollutants from this study could be helpful when counselling patients with POI.
\end{itemize}

\textbf{Introduction}

The microbiome, the collective genetic material from a microbial community in a particular environment, varies according to body site and individual characteristics (Group et al. \textit{2009}). Increasing evidence has demonstrated that the microbiome has a considerable effect on host metabolism and the response to various diseases (Turnbaugh et al. \textit{2007}). Although the techniques used for analysis of microbiomes are continuously improving, from culture-dependent methods to DNA and RNA sequencing (Relman \textit{2015}), our knowledge of the role of the microbiome in human health and disease is still limited.

The vaginal microbiome has been widely investigated because of its value in the diagnosis of vaginitis and its association with other conditions (Thies et al. \textit{2007}; Shi et al. \textit{2009}; Lambert et al. \textit{2013}; Muzny et al. \textit{2013}; Chaban et al. \textit{2014}; Di Paola et al. \textit{2017}; Wang et al. \textit{2017}). Our understanding of the vaginal microbiota has been extended by the development of molecular approaches based on 16S rRNA gene cloning and sequencing, which has allowed the identification of taxa that were not previously detected by conventional culture methods. A distinct bacterial community, with a low abundance of \textit{Lactobacillus}, has been shown to be associated with vaginal atrophy in menopausal women (Brotman et al. \textit{2014}), implying that the composition of the vaginal microbiome could be related to menopausal conditions. Premature ovarian insufficiency (POI), which is the depletion or dysfunction of ovaries with menopause or ovarian failure before the age of 40, is a relatively poorly understood condition with various aetiologies (Torrealday et al. \textit{2017}). Despite ongoing efforts, advancements in our understanding and treatment of POI have been minimal, and...
clinicians remain frustrated when managing patients with POI. The proposed aetiologies of POI are thought to be genetic, iatrogenic, metabolic, infectious or autoimmune (Vabre et al. 2017). Recent evidence suggests that environmental factors contribute to the onset of POI by acting as major determinants of ovarian reserve (Richardson et al. 2014). However, the relationship between environmental factors and POI has not been evaluated.

In this study, we investigated the association of the vaginal microbiome with environmental factors and ovarian damage. We evaluated the vaginal microbiome in patients with impaired ovarian function using next-generation sequencing (NGS) and its association with environmental pollutants.

Materials and methods

Study population

The individuals enrolled in this study were part of the ‘Environmental Health Action Program’ of the Korean Ministry of Environment. This program was designed to build a cohort of patients with POI and to determine the mechanism of POI using animal models. The study was approved by the ethical committee of Korea University Medical Center Anam Hospital (no. 2016AN0251) and was performed in accordance with relevant guidelines and regulations of the committee. The participants were recruited voluntarily via an online survey and at an outpatient clinic in multiple institutions from March 2016 to January 2017. Informed consent forms were obtained from all participants for the use of human-derived materials and their personal information. All women were 30–45 years old and had not taken any antibiotics in the past 30 days.

The populations were divided into two groups according to hormonal status: ‘normal group’ with no menstrual problem and with normal hormone levels, and ‘impaired ovarian function (IO) group’ who have an amenorrhea for more than a year and FSH level ≥40 or who have menstruations but show decreased AMH value as high as 2 years older than actual age. The appropriate sample size for study could not be calculated before initiation of the study due to the rarity of the POI.

Specimen acquisition and cohort composition

The following samples were obtained from all participants: serum (10 mL), urine (40 mL) and vaginal swabs. The serum analysis included hormone levels, thyroid function, prolactin, lipid profiles and other basic laboratory findings (Table 1). All participants were administered human papilloma virus (HPV) and underwent Pap tests, and vaginal swabs were obtained for NGS of the vaginal microbiome. Urine samples were sent to the Department of Preventive Medicine, where the concentrations of various environmental pollutants were determined.

Analysis of toxic chemical metabolites in urine

Urine was stored at −70°C until analysis, and during the analysis, contact with plastic was avoided. Fifteen metabolites were analysed by the Smartive Corporation (Seoul, Republic of Korea). Polycyclic aromatic hydrocarbons (PAHs; 1-hydroxypyrene and 2-hydroxynaphthalene) and cotinine (COT) metabolites were analysed using a gas chromatography-mass spectrometer (GC-MS, Clarus 680T, PerkinElmer, Waltham, MA). The following compounds were analysed using a high-performance liquid chromatography-triple tandem mass detector (HPLC-MS/MS, 6410B, Agilent, Santa Clara, CA): phthalate metabolites (mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP)), environmental phenols (bisphenol A (BPA), methyl paraben (MP), ethyl paraben (EP), propyl paraben (PP) and 2,4-dichlorophenol (24DCP)), volatile organic compounds (VOCs: trans-muconic acid (ttMA), benzyl mercapturic acid (BMA), phenyl glyoxylic acid (PGA) and o.m.p-methyl hippuric acid (MHA), mandelic acid (MA)).

MiSeq-metagenomic sequencing

Genomic DNA was extracted from the vaginal swabs using the Power Soil DNA Isolation Kit (MOBIO, Carlsbad, CA). The 16S rRNA genes were amplified according to the Illumina 16S Metagenomic Sequencing Library protocols as follows. Universal primers (16S Amplicon PCR forward 5′-TCGTCGGC AGCGTCAATGATATTAACACGCCTACGGGNGGCWGGCAG and 16S Amplicon PCR reverse 5′-GTCTTGAGGCTGTGAGAGA TGTGATAAGAGACAGCCTACGGGNGGCWGGCAG and 16S Amplicon PCR reverse 5′-GTCTTGAGGCTGTGAGAGA TGTGATAAGAGACAGCCTACGGGNGGCWGGCAG) were used for PCR amplification of the V3–V4 hypervariable regions of the 16S rRNA gene from 12.5 ng of the genomic DNA prepared from each sample. A subsequent limited-cycle amplification step was performed to add multiplexing indices and Illumina sequencing adapters. The final products were normalised and pooled using PicoGreen, and the size of the libraries was verified using the LabChip GX HT DNA High

| Specimen         | Analysed components                                                                 |
|------------------|--------------------------------------------------------------------------------------|
| Serum            | CBC, electrolytes, liver function tests (AST, ALT, bilirubin, ALP, protein and albumin), lipid profile (TG, HDL, LDL, TC), thyroid functional tests (TSH and free T4) and hormones (FSH, E2, progesterone, AMH and prolactin) |
| Urine            | Phthalates (MEHHP and MEOHP), phenols (BPA, MP, EP, PP and 24DCP), cotinine, VOCs (ttMA, BMA, BGA, PA, MA and MHA), 1-OHP and 2-NAP |
| Vaginal smear     | Next generation sequencing of the vaginal microbiome                                   |

1-OHP: 1-hydroxypyrene; 2-NAP: 2-naphthol; 24DCP: 2,4-dichlorophenol; ALT: alanine amino-transferase; AMH: anti-Müllerian hormone; AST: aspartate aminotransferase; BMA: benzyl mercapturic acid; BPA: 2,2-bis(4-hydroxyphenyl)propane(also known as bisphenol A); CBC: complete blood cell count; E2: oestradiol; EP: ethyl phenol; FSH: follicular stimulating hormone; HDL: high-density lipoprotein; LDL: low-density lipoprotein; MA: mandelic acid; MEHHP: mono 2-ethyl-5-hydroxyhexyl phthalate; MEOHP: mono 2-ethyl-5-oxohexyl phthalate; MHA: methyl hippuric acid; MP: methyl phenol; PGA: phenyl glyoxylic acid; PP: propyl phenol; TC: total cholesterol; TG: triglyceride; TSH: thyroid stimulating hormone; ttMA: trans,trans-muconic acid; VOCs: volatile organic compounds.
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Sensitivity Kit (PerkinElmer, Waltham, MA). We used the MiSeq™ platform (Illumina, San Diego, CA) at Macrogen, Inc. (Seoul, Korea), according to the manufacturer’s instructions.

Operational taxonomic unit analysis
The sequence of each operational taxonomic unit (OTU) was used as a query against the NCBI reference 16S Microbial database with BLASTN (v2.4.0) to obtain a taxonomic assignment by identifying the most similar species. Microbial community comparisons were performed using QIIME (v1.8).

Canonical correlation analysis
The results of the urine analysis of 15 metabolites and the vaginal microbial population were subjected to canonical correlation analysis, which was conducted at the species level using R (v 3.1.2R).

Results

Characteristics of the study participants
A total of 92 samples were obtained from the study participants. Analysis of hormonal levels revealed that of the 22 patients in the IO group, five had definite POI, and the other 17 patients had impaired ovarian function with low AMH levels. The clinical characteristics and blood analysis results are shown in Table 2. Most variables were similar between the two groups, except for follicular stimulating hormone (FSH) and AMH levels.

Table 2. Clinical characteristics of the study participants.

|                  | Normal group | IO group | p Value |
|------------------|--------------|----------|---------|
| (n = 70)         | (n = 22)     |          |
| Age              | 33.8         | 33.9     | .87     |
| BMI              | 23.15        | 23.07    | .47     |
| Parity           |              |          | .79     |
| 0                | 1            | 2        |         |
| 1                | 34           | 12       |         |
| 2 or more        | 10           | 2        |         |
| History of ovarian surgery | 8       | 2       | .76 |
| AMH              | 5.07         | 0.71     | <.01    |
| FSH              | 3.96         | 15.28    | .01     |
| E2               | 77.81        | 76.81    | .58     |
| Prolactin        | 10.82        | 8.85     | .20     |
| Progesterone     | 5.11         | 7.55     | .26     |
| TSH              | 2.61         | 2.67     | .75     |
| Free T4          | 1.28         | 1.21     | .08     |
| Protein          | 7.31         | 7.33     | .86     |
| Albumin          | 4.51         | 4.44     | .39     |
| Total cholesterol| 178.7        | 179.9    | .86     |
| Direct bilirubin | 0.17         | 0.11     | .33     |
| AST              | 20.62        | 23.20    | .58     |
| ALT              | 16.42        | 19.02    | .23     |
| Haemoglobin      | 12.96        | 12.91    | .25     |
| Platelets        | 266.4        | 270.0    | .81     |
| WBC              | 6.13         | 6.43     | .41     |

ALT: alanine amino-transferase; AMH: anti-Müllerian hormone; AST: aspartate aminotransferase; E2: oestradiol; FSH: follicle stimulating hormone; HDL: high-density lipoprotein; LDL: low-density lipoprotein; TSH: thyroid stimulating hormone; WBC: white blood cell.

Next-generation sequencing of the vaginal microbiome
A total of 937 species were identified in the analysis of the vaginal microbiome, and 24 dominant species were selected for further analysis. The samples had 56,338–147,588 read counts, and >89.06% had a PHRED (Q) score above 30, indicating a 1:1000 probability of an incorrect base call. Taxonomic abundance showed that lactobacilli and enterococci were the main taxa in the normal group, but that the species tended to be more heterogeneous in the IO group (Figure 1). A heat map was drawn using the taxonomy information, which showed that the two groups were not different from each other (Figure 2). The proportions of each species were compared between the two groups, and only two species (Propionibacterium acnes and Prevotella copri) were significantly more abundant in the POI group (p = .005 and p = .002, respectively).

Correlations with environmental pollutants
Urine samples were analysed for 15 pollutants. The average amount of each pollutant and its correlation with the composition of the vaginal microbiome as determined using canonical correlation analysis are shown in Table 3. In the normal group, MEOHP and EP levels were significantly correlated with vaginal microbiome status, with a low $R^2$ value, while four other pollutants were related to species composition, showing a relatively higher $R^2$ value in the POI group (Figure 3). MA and 2-NAP were not significantly related to the composition of the vaginal microbiome, but they showed a tendency towards sensitivity in the IO group, with a higher $R^2$ value than in the normal group.

Discussion
The vaginal microbiome reflects not only the bacterial composition of the vagina but also the general condition of the host, including hormonal and immunity status (Moncla et al. 2016; Jespers et al. 2017). Although most previous studies have investigated the relationship between vaginal microbiome and health using animal models, a few human studies have examined the epidemiology of the vaginal microbiome and its relationship with general health. Only one study observed that the concentration of phthalate in human urine is associated with a lower antral follicle count (Messerlian et al. 2016).

In the present study, the change in the composition of the vaginal microbiome in young females with impaired ovarian function was more related to environmental pollutants than the change observed in the healthy group. To our knowledge, this is the first study to investigate the influence of environmental pollutants on the vaginal microbiome in young women with decreased ovarian function. Although the square correlation was low for the majority of tested pollutants, the relevance was higher in the IO group than in the normal healthy group, and a larger number of pollutants showed a significant relationship in the IO group. Although this does not prove that these pollutants are a causative factor of ovarian dysfunction, it implies that such pollutants
may have a greater effect on the composition of the vaginal microbiome in this population; thus, young women with impaired ovarian function could be more vulnerable to these pollutants. This may be an important finding, especially given the prevalence of pollutants nowadays.

As mentioned above, canonical analysis showed that two environmental pollutants were correlated with the vaginal microbiome in the normal group, and four were correlated in the IO group. MEOHP and MEEHP are phthalates that are used as plasticisers to increase the flexibility and transparency of plastics; they are also used to make polyvinyl chloride (PVC) softer (Wallner et al. 2016). EP and MP are phenols that are often included in sterilisers or deodorisers (Aker et al. 2016). People are frequently exposed to these chemicals in everyday life during their daily activities, and these pollutants were related to the vaginal microbiome in both groups.

BMA, MHA and MA showed a tendency to affect the vaginal microbiome only in the IO group. These substances are VOCs, which are a diverse group of carbon-based chemicals that are volatile at room temperature. Sources of VOCs
include vehicles, gas evaporation and industrial processes (Okada et al. 2012); thus, VOCs are a considerable cause of air pollution, especially fine dust (Jones 2016). Although we cannot conclude that these VOCs cause ovarian damage, the results of the canonical correlation analysis imply that VOCs could alter the vaginal condition in young females with impaired ovarian function. This finding should not be overlooked because it could be an important issue given the current environment in South Korea, as urbanisation, industrialisation and the activities of neighbouring countries are worsening air pollution, which is an important risk factor for human diseases (Leem et al. 2015). Moreover, VOCs are identified in the components of disposable sanitary pads (Bae et al. 2018). This is also a significant issue in South Korea. All the participants in this study were at the age of users of the pads, so using disposable pads could have harmful effects on vaginal condition in individuals with impaired ovarian function. More robust studies may confirm the relationship between VOCs and impaired ovarian function, and this information could be useful for counselling females with decreased function.

Figure 3. Canonical correlation analysis of the species in the normal and impaired ovarian function groups.

The most abundant species in the vaginal microbiome was *Lactobacillus*, which is consistent with previous studies (Zhou et al. 2004; Gajer et al. 2012; Huang et al. 2014). A healthy vagina is usually dominated by *Lactobacillus* species, as there are several mechanisms that inhibit its colonisation by pathogens (Ravel et al. 2011). In the present study, no remarkable difference in the composition of the vaginal microbiome between the normal and impaired ovarian function groups was observed. Only two species showed a significant difference in abundance in the IO group; however, their role remains unclear. Brotman et al. evaluated the association of the vaginal microbiome with menopausal status and vaginal atrophy (Brotman et al. 2014). They revealed the existence of a distinct bacterial community with a low abundance of *Lactobacillus* in patients with vaginal atrophy, but there was no relationship in menopausal women without atrophy. The role of oestrogen therapy is thought to be important. Hormone replacement therapy is effective for vaginal atrophy in menopausal women and helps maintain the abundance and dominance of *Lactobacillus* (Heinemann and Reid 2005; Ozkinay et al. 2005). The change of vaginal pH because of menopause could be normalised after hormone replacement therapy (Panda et al. 2014). In our study, all patients with POI were receiving oestrogen supplementation; therefore, the vaginal conditions may be not that different from those of the normal healthy group. Accordingly, the difference in the microbiome in IO group would not be present because of decreased ovarian function. However, further studies are warranted to confirm the influence of environmental pollutants on ovarian function.

There are a few limitations to this study. First, we were not able to obtain any information about the presence of vaginitis or any other underlying disease. The existence of vaginitis or vaginal atrophy could be related to the differences observed in the vaginal microbiome (Hong et al. 2016). However, we retrospectively reviewed the medical records of all participants, and identified only three women with suspected vulvovaginitis on physical examination (two in the normal group and one in the IO group). This low incidence should not have caused a difference in the entire study population. Second, the number of patients with POI in this study was small, as most of the participants in the IO group were still menstruating and had some reproductive function, although their AMH levels were low. Therefore, additional investigations including more patients with POI are necessary to definitively determine the influence of pollutants on the vaginal microbiome between women with normal ovarian function and patients with POI.
Nevertheless, this study has important implications in terms of the unique analytical methods used to infer the effects of environmental pollutants on the vaginal microbiome depending on ovarian function. To our knowledge, this is the first evaluation of this association using NGS. Previous studies have investigated the association of the vaginal microbiome with menopause (Muhleisen and Herbst-Kralovetz 2016), perinatal outcome (Prince et al. 2015), preterm birth (Stout et al. 2017), sexually transmitted diseases (Lewis et al. 2017), vaginitis (Hong et al. 2016), HPV (Mitra et al. 2016) and urinary tract infections (Stapleton 2016). No study has evaluated the relationship between the vaginal microbiome and environmental pollutants in young females with impaired ovarian function. A possible susceptibility of this group to VOCs was revealed in our study. Therefore, additional studies should be conducted to confirm the underlying mechanism.

In conclusion, the vaginal microbiome in young women with decreased ovarian function showed a tendency to be more sensitive to various environmental pollutants, especially VOCs, when compared to the microbiome of females with normal ovarian function. This implied a considerable association between impaired ovarian function and air pollution, and VOCs might be a hazard to females with diminished ovarian function. This finding could be helpful when counseling patients with POI.

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Consent for publication: All participants provided written informed consent for biological studies.

Disclosure statement

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Data availability statement

The datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

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