Vaginal Microbiome of Women with Premature Ovarian Insufficiency - Descriptive cross-sectional study

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Abstract

Objective: To describe the vaginal microbiome of women with premature ovarian insufficiency (POI) receiving systemic hormone therapy (HT). **Study design:** Descriptive cross-sectional study. **Methods:** Forty women with POI receiving systemic HT for at least 6 months, were included in the study. Vaginal secretion was collected for DNA extraction followed by Pyrosequencing of the 16S rRNA. The samples were pooled into phylogenetic groups (Ravel – I, II, III, IV, V). **Results:** Women had mean age of 37.13 (± 7.27) years and POI diagnosis at 27.90 (± 8.68) years, a mean HT duration of 8.20 (± 8.73) years. It was observed that 33.4% of the women presented group I flora, with a predominance of *L. crispatus*; 9% group II flora, with a predominance of *L. gasseri*; 33.4% group III flora, with predominance of *L. iners*; 15.2% group IV flora, with a predominance of anaerobic bacteria; and 9% group V flora, with a predominance of *L. jensenii*. **Conclusion:** Women with POI receiving HT presented a vaginal microbiome with a predominance of lactobacilli in the composition of the vaginal flora, specifically *L. crispatus* and *L. iners* when evaluated by molecular biology through the pyrosequencing of 16S rRNA.

Introduction

Women with Premature Ovarian Insufficiency (POI) present a premature reduction in ovarian activity, with serum concentration of sex hormones reduction, especially estrogen, establishing body repercussions. POI affects approximately 1% of the sexually active female population, but it increases the risk of various diseases. (1)

Studies have shown psychological repercussions, impairment of cardiovascular health; bone mass; urogenital system; reproduction; sexual function; quality of life and even a reduction of life expectancy when POI is not adequately treated. One of the pillars for POI treatment is systemic hormonal therapy (HT). (1, 2-6)

Prolonged hypoestrogenemia also lead to vulvovaginal atrophy. Hypoestrogenism leads to macroscopic, histological and functional changes, which are progressive over time (7,8). The balance of the vaginal ecosystem is crucial to maintain vaginal health, especially in women with preserved sexual activity. Women with POI are young and expect to have sexual activity like any other woman without estrogen deficiency.

Estrogen promotes the preservation of a homeostatic (eubiotic) vaginal microenvironment. It is a key part of promoting accumulation of glycogen in the cells of the vaginal mucosa, by the action of α-amylase. A glycogen-rich vaginal milieu is propitious of *Lactobacilli* proliferation, facilitated by lactic acid production and decreased pH. Therefore, the predominant presence of Lactobacillus (LB) in the vaginal ecosystem is fundamental. Lactic acid, produced by LBs, promotes an increase in the TH17 immune response and inhibits the proliferation of harmful anaerobic bacteria in the extracellular matrix of the tissue (9). However, recently, identification techniques for bacteria have revolutionized the study of microorganisms. The use of amplification, cloning techniques and subsequent analysis of sequences of bacterial genes (genes that codify

for bacterial rRNA 16 S) in samples of vaginal secretion have identified the majority of species of Lactobacilli and other microorganisms. These techniques demonstrated that Lactobacilli sp don't always correspond to the dominant species in the vagina of healthy women (10). Vaginal ecosystem of POI women studies were carried out focusing on the maturation of the epithelial cells (vaginal mucosa) and the lubrication that is obtained with HT. There is a scarce data of microbiota and the vaginal microbiome. In one of the rare literature studies with such a focus, were studied women with POI receiving HT and evaluated the vaginal microenvironment through amine testing, bacterioscopy, and fungal specific culture. The vaginal flora of these women was similar to women with same age and with preserved gonadal function (11). This flora was made up predominantly of Lactobacillus sp.

Lactobacilli and their antimicrobial and anti-inflammatory products along with components of the epithelial mucosal barrier provide an effective first line defense against invading pathogens including bacterial vaginosis, aerobic vaginitis-associated bacteria, viruses, fungi and protozoa(9), So, the human vaginal microbiota appears to play an important role in preventing numerous urogenital diseases. . General consensus attributes this to lactic acid producing bacteria, mainly Lactobacillus sp, (12).

However, the methodology usually used for this identification has limitations in terms of the specificity for the identification of microorganisms that inhabit the human body.. A feature that has been recently studied, and has proven to be quite efficient, is the study of the microbiome. Microbiome is the totality of microorganisms, their genetic elements (genome), and interactions in a specific environment (13). Modern DNA tests have made it possible to identify most of these microorganisms, since they are not easily grown in the laboratory (14). DNA-based Microbiome studies can be categorized as amplicon specific studies or, more recently, metagenomic studies. It is a challenge not to include host DNA in the study (13). After the Amplicons are sequenced, phylogenetic molecular methods are used to infer the composition of the microbial community. To determine the identity of a phylogenetic sequence, it is compared to the complete genomic sequences available using methods such as the BLAST programs (13).

The vaginal microbiome of women with IOP has not been described. Thus, this study was developed with the objective of evaluating the vaginal flora of women with POI receiving HT verifying the microbiome by bacterial (16S rRNA) DNA extraction methods.

Materials and Methods

Study design

Cross-sectional study developed at Department of Obstetrics and Gynecology of the School of Medical Sciences, University of Campinas - UNICAMP. This study was approved by the Ethics Committee of the institution under number CAAE 06124512,6,2001,5404. Participants were recruited at the institution's Endocrinology Gynecology Outpatient Clinic, a tertiary center. This study is a descriptive analysis of the population studied in a larger study comprising a clinical trial with interventions REBEC number RBR-74zbms.

Participants

We included 40 women with POI diagnoses who had been using oral systemic HT for at least 6 months. POI diagnosis was based on hypergonadotrophic amenorrhea with at least two FSH dosages greater than 25 IU/mL (1). All participants signed an Informed Consent Term. Women who had a specific acute infectious process involving vulvovaginitis or ulcers in the genital region, or who had used medications related to mucositis (long-term antibiotics, immunomodulators, chemotherapeutics), and women with systemic or localized allergic conditions, were excluded.

Methodology

All women included in the study collected vaginal secretion with a swab (Eswab Liquid-based Multipurpose Collection and Transport System). The collected samples were vigorously shaken and centrifuged at 800g for 10 minutes and the pellets obtained were submitted to DNA extraction using the MiSeq Reagent Kit (600-

cycle), Illumina Brasil Produtos de Biotecnologia Ltda, São Paulo, Brazil, according to the recommended protocol by the manufacturer for DNA extraction from bacteria. The samples were stored as DNA in a biorepository in specific freezers (Freezers at -20*C) and used only for this purpose in the specialized clinical laboratory. After thawing, the obtained DNA were quantified and evaluated for purity and quality and were submitted to the technique of Pirsequencing 16S rRNA gene amplicons in the Laboratory of Insulin Resistance of the School of Medical Sciences of the University of Campinas - UNICAMP.

To carry out the 16S rRNA extraction through pyrosequencing, the 16S library preparation protocol was performed. The samples, after reaching room temperature, were submitted to the first stage of PCR (Polymerase Chain Reaction) and then PCR cleaning through the reagents Ampure XP Beads, EtOH 80% fresh, RSB and then placed in PCR postplates. The second PCR stage was performed and the post-PCR cleaning procedure was repeated with the same reagents. Data was then placed into the quantification and normalization library and sequentially in the MiSeq denaturing and loading library of samples. The entire methodology followed the KIT protocol (MiSeq Reagent Kit (600-cycle), Illumina Brasil Produtos de Biotecnologia LTDA, Sao Paulo, Brazil).

The data of the microbiome, obtained from the pyrosequencing, were sent for analysis to the Scylla bioinformatics service, Campinas, Brazil, for organization and phylogenetic classification to divide the groups according to the cited classification in literature by Ravel (4) as follows: Roman numerals indicate five groups of bacterial vaginal communities. Groups of communities I, II, III and V have as predominant bacterial Lactobacillus crispatus, L. gasseri, L. iners and L. Jensenii, respectively, while group IV contains a diversity of strict or facultative anaerobic bacteria (Prevotella, Dialister, Atopobium, Gardnerella, Megasphaera, Peptoniphilus, Sneathia, Eggerthella, Aerococcus, Finegoldia, and Mobiluncus).

Ravel et al., in a study with healthy young women at reproductive age revealed that in this population, group I communities, which occurred in 26.2%, were dominated by L. crispatus, while groups II (6.3%) , III (34.1%) and V (5.3%) were dominated by L. gasseri, L iners and L. jensenii, respectively (12).

Sample Size Calculation

This descriptive study is a secondary analysis of a clinical trial. The sample size was calculated based on the main objective, which was calculated in 30 patients, divided into two treatment groups. However, in view of possible loss of follow-up, it was decided to include 40 women with POI. The current study represents a descriptive analysis of the vaginal microbiome of the women at the inclusion time of the study.

Data Analysis

To describe the profile of the sample, tables with absolute frequency (n) and percentage (%) values and descriptive statistics of the numerical variables with mean values, standard deviation were made. To classify the samples into their phylogenetic groups according to Ravel, tables were also made with absolute (n) and percentage (%) frequency values. For statistical analysis SAS software was used for Windows version 9.2.

Results

Two hundred medical records of women with POI were reviewed, and 85, who met the inclusion criteria according to the medical record, were selected. After the interviews, 30 women were excluded due to incorrect adherence to HT; HT used for less than 6 months or absence of sexual activity at the time (criterion for inclusion in the main study). The other women were then invited to participate in the study. Fifteen women refused to participate. Forty women were then examined and samples of vaginal secretion were collected. At the time of pyrosequencing, seven samples were excluded because of insufficient amount of genetic material resulting from DNA extraction (Figure 1).

The mean age of the participants was 37.13 (+- 7.27) years, with a diagnosis of POI at 27.90 (+- 8.68) years. The time elapsed between the date of diagnosis and the inclusion in the study was 9.23 (+- 8.45) years, duration of treatment with systemic hormonal therapy was 8.20 (+- 8.73) years (Table 1). Out of the total number of women, 77.5% were using oral conjugated estrogen (EE) or 17 beta estradiol (E2)

associated with a progestogen (medroxyprogesterone acetate or norethisterone acetate), 15% used combined oral contraceptives, and 7.5% tibolone, as a systemic hormonal therapy. Fifty-seven percent were nulliparous and 42.5% had 1 or more children.

The results obtained from the vaginal secretion samples and analyzed for microbiome description (taxonomic plot for distribution of bacteria for each subject is presented in Figure 2) showed that 33.4% (N = 11) of the women with POI and using HT had a group I compatible vaginal microbiome, with a predominance of *L. crispatus* ; 9% (N = 3) of the women had a group II flora microbiome with a predominance of *L. gasseri* ; 33.4% (N = 11) of the women, had a group III flora microbiome, with predominance of *L. iners* ; 15.2% (N = 5) of the women had group IV flora, with a predominance of anaerobic bacteria; and 9% (N = 3) of the women had group V flora, with a predominance of *L. jensenii* (Graph 1).

Discussion

This is the first study performed to characterize the vaginal microbiome in women with POI. The study revealed that, for women with POI using systemic HT, and by means of analysis by DNA extraction, the vaginal flora is predominantly Lactobacillus sp., which is the flora considered healthy and suitable for estrogenized women. There was a predominance of *L. cripatus*in the same proportion of *L. iners* , followed at a smaller proportion by *L. gasseri* and *L. jensenii* . It should also be considered that Ravel group IV bacteria were present in 15.2% of the cases, even though these women were adequately estrogenized by systemic HT (15-19).

Studies with evaluation of the vaginal flora, through different methods, reveal that to maintain a healthy vaginal ecosystem, it is necessary to have superior counting of Lactobacillus sp colonies in order to maintain adequate pH through the production of acids, especially lactic acid, to prevent pathogenic bacterial proliferation (20-22). A study in which the authors compared the vaginal flora (but not the microbiome) of women with POI using HT, with women of the same age with preserved gonadal function by means of bacterioscopy and culture for fungi, showed similar floras, with no difference between groups. Thus, it can be assumed that systemic HT was able to provide sufficient estrogenic levels to restore the vaginal flora, despite loss of ovarian function (23).

Considering that common evaluation methods of the vaginal ecosystem have no specificity for which species of lactobacilli are present and that different species of lactobacilli produces different types of acids and concentrations, the present study used up-to-date techniques (DNA study of the vaginal flora). The specific phylogenetic classification of women with POI may provide evidence of HT action on the vaginal flora restoration (24).

Molecular biology has become a great ally in the search for vaginal flora identification. Efforts to characterize vaginal microbial communities using culture methods undoubtedly led to significant improvements in understanding the role of microorganisms in vaginal health, but they were limited because of the biases inherent in culturing methods. It is now known that most environmental microorganisms associated with the host are not identified in the laboratory using traditional cultivation techniques (25).

Culture of microorganisms is fundamental for the understanding of their physiological and phenotypic characteristics, and it continues being a very useful tool in studies of microbial ecology. Promising developments in the cultivation of fastidious bacteria using state-of-the-art techniques are likely to enable the cultivation of many previously inaccessible microorganisms (26-29). However, studies aimed at evaluating fine-scale variation in host-associated microbial communities within and between individuals or exploring ecological relationships within those communities require methods that provide detailed information on microbial diversity and, at the same time, be scalable for processing samples and also cost-effective. In response to this need, independent culture methods have become, in recent years, the standard approach to characterize the diversity of microorganisms residing in the human body (30-34).

Ravel et al. found that the group I communities in healthy women of reproductive age, which occurred in 26.2% of the women that they have studied, were dominated by L. crispatus, while groups II (6.3%),

III (34, 1%) and V (5.3%) were dominated by *L. gasseri, L iners and L. jensenii,* respectively. Curiously, communities dominated by Lactobacillus species other than *L. crispatus* have slightly higher pH, ranging from 4.4 (group III) to 5.0 (group II), indicating that these communities as a whole may not produce as much acid as group I (4). These results are in agreement with the findings in the present study, where there was also a predominance of groups I (*L. crispatus*) and III (*L. iners*), indicating a healthy vaginal flora and a percentage expected for estrogenized women. Thus, it is possible to infer that HT in women with POI may be able to maintain adequate vaginal flora.

Two important points will have to be clarified in the future. Intriguingly, we found 15.2% of bacteria associated with bacterial vaginosis in adequately estrogenized women. Second, there is no predominance of L. crispatius on L. iners. These two points may point out that the use of systemic estrogens is not sufficient to determine vaginal colonization with L crispatus, producers of lactic acid. Probably, other factors such as frequency of sexual intercourse, type of sexual practices, feeding, individual immune response, among others, may interfere with vaginal colonization.

Although this study is one of the first in literature to evaluate the vaginal flora of young women, with POI undergoing systemic hormone therapy, by using advanced and reliable technique such as the evaluation of vaginal microbiology through molecular biology with the 16S rRNA pyrosequencing, we understand that the fact of having been a descriptive study only limits our conclusions or future inferences, as is already known in the descriptive studies. The small sample size also makes us understand that the findings are limited to this group studied and that further studies are needed with larger cases where there is comparison with women using topical replacement therapy and/or with women at menacme with preserved ovarian function. On the other hand, we have to consider that the studies with POI, do not have large case-by-case studies due to the difficulties inherent in this nosological entity. Work with large case-by-case studies about POI often come from databases involving several institutions.

Conclusion

Women with POI treated with systemic hormonal therapy have a vaginal flora with a predominance of lactobacilli specifically L. crispatus and L. iners, analyzed through molecular biology and with microbiome obtained through the pyrosequencing of 16S rRNA, which is similar to women with preserved gonadal function.

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