THE IMPACT OF STIMULATION ON VAGINAL MICROBIOTA OF WOMEN UNDERGOING IVF TREATMENT

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Abstract: A total of 210 patients with recurrent implantation failure (RIF) were included in the present study. 123 microbiological vaginal samples were taken from unstimulated women in regular menstrual cycle (control group) and 87 samples were taken from women during hormonal stimulation treatment (studied group). All samples were taken during the early follicular phase of the menstrual cycle. The applied culture methods for determination of dominant microorganisms in vaginal microbiota involve cultivation on sheep blood agar, MacConkey II agar, Sabouraud agar with chloramphenicol, Gardnerella selective agar, MRS agar and additional identification of gram-negative bacilli with BBL CRYSTAL™ Enteric/ Nonfermenter (E/NF) Identification (ID) System. The applied ovarian stimulation did not lead to a significant change in the frequency of occurrence of *Lactobacillus* as a dominant species (53% vs. 50%; stimulation group vs. control group, respectively). However, we observed a dramatic increase in the percentage of cases with *Gardnerella sp.* (28%) in the stimulation group, which were classified as a transition to bacterial vaginosis. These findings suggest that stimulation have a negative effect on vaginal microbiota that could be a matter of concern in the consecutive IVF treatment. For this reason ovarian stimulation should be followed by vaginal microbiota recovering procedures. The use of probiotics to populate the vagina and to treat the observed infection should be considered as an optional treatment before the next IVF cycle.

INTRODUCTION

The human microbiome affects all stages of reproduction: gametogenesis, fertilization, embryo migration, implantation, pregnancy and childbirth (Haahr *et al.,* 2016; Moreno *et al.* 2016). It has been shown that the female reproductive tract has an active microbiome and its composition varies in response to infections and ageing processes (Huang *et al.,* 2014). Although the normal vaginal microbiota in healthy

women is dominated by lactobacilli (Ravel *et al., 2011*), there are some noticeable variations due to age and hormonal balance, infections and sexual behavior (Gajer *et al., 2012*). For example, vaginal flora during infancy is a mixture of aerobic and anaerobic bacterial populations, including *Prevotella sp., Enterobacteria sp., Streptococcus sp.,* and *Staphylococcus sp.* (Huang et al., 2014), whereas the puberty-induced estrogenic environment results in glycogen rise and subsequent dominance of lactobacilli species, responsible for decrease in pH.

The pathological alterations of vaginal microbiota profile are associated with poor reproductive outcomes for in vitro fertilization patients (Oakeshott *et al., 2002*; Leitich *et al., 2007*; Jason *et al., 2015*; Moreno *et al., 2016*). The most common vaginal microbiota disorder in women is the bacterial vaginosis (BV), characterized by a reduced number of hydrogen peroxide (H_2O_2)–producing *Lactobacillus spp.* It is caused by displacement of the dominant lactobacilli by pathogenic microorganisms such as gram-negative and/or anaerobic bacteria such as *Bacteroides spp., Gardnerella vaginalis, Mobiluncus spp., Mycoplasma hominis,* and *Ureaplasma urealyticum* (Spiegel *et al., 1980*; Forsum *et al., 2005*; Larsson *et al., 2005*; Srinivasan *et al., 2008*; Livengood, 2009). A series of studies have suggested the negative impact of BV on the female fertility (Oakeshott *et al., 2002*; Leitich *et al., 2007*; Haahr *et al., 2016*). The abnormal microbiota characterizing BV is associated with fewer pregnancies after IVF (Haahr *et al., 2016*) and a high risk of miscarriages (Oakeshott *et al., 2002*; Leitich *et al., 2007*). Therefore the regulation of the microbiome balance during IVF treatment should be considered in order to avoid poor ART outcomes.

It has been reported that the bacterial species associated with the vaginal epithelium are affected by estrogen treatment in rats (Savage *et al., 1974*; Larsen *et al., 1978*; Bezirtzoglou *et al., 2008*). Given the fact that vaginal microbiome is controlled by the hormonal levels in the physiologic range (Hyman *et al., 2005*), the applied controlled ovarian hyperstimulation during IVF treatment could also cause shifts in the vaginal bacterial composition. Recently Hyman *et al.* (2012) found a relation between the hormone-dependent microbiotal shifts to pregnancy outcomes. Therefore, it is important to note the impact of the hormonal stimulation on the vaginal microbial balance during in vitro procedures (Garcia-Velasco *et al., 2017*; Franasiak *et al., 2016*).

The objective of our study was to evaluate the impact of conventional hormonal stimulation on vaginal microbiota of infertile women with recurrent implantation failure (RIF).

MATERIALS AND METHODS

Patients
This is a prospective pilot study that included 210 patients undergoing long ovarian stimulation and IVF treatment at Nadezhda Women's Health Hospital from May to November 2017. The selected group of patients included women of varying age and body mass index (BMI). Inclusion criteria were established based on previous

cases of vaginal microbiota disorders and at least 2 failed pregnancy experiences. The ovarian stimulation protocols remained constant during the study period. The study was conducted after the approval of the local institutional ethics committee.

Culture collection and microbial isolation

Vaginal samples were collected from each patient with dry, sterile, cotton-tipped swabs and cultured on selective agar plates (MacConkey II Agar / Blood Agar, Blood Agar with 5% Sheep Blood; MacConkey II Agar / Blood Agar, Sabouraud Agar with chloramphenicol, MacConkey II Agar; and Gardnerella selective agar with 5% human blood).

Culture plates for aerobic microorganisms were incubated at 37°C in 5%–10% CO_2 and examined after 24–48 hours. Culture plates for anaerobic microorganisms were incubated within an anaerobic glove box at 37°C for 5 days.

Bacterial identification was carried by conventional Gram's staining methodology (Murray *et al.,* 1995) and biochemical tests (BBL Crystal TM Identification Systems Enteric and BBL Crystal TM Identification Systems, Gram-positive ID Kit (BD Diagnostic Systems, Sparks, MD). Single colonies of the culture were suspended in a tube of BBL Crystal ANR, GP, RGP, N/H ID inoculums fluid and incubated at 37°C for 24 hours. The resulting profile numbers were entered to the BBL Crystal Mind Software (BD Diagnostic Systems, Sparks, MD) to identify each of the tested bacteria.

Statistical analysis

All statistical analyses were performed by SPSS v.21. The Chi-square test of significance was conducted to compare the percentage of the observed microorganisms. P value of $P < 0.05$ was considered statistically significant. All values are presented as means ± standard error unless otherwise stated.

RESULTS AND DISCUSSION

A total of 15 different dominant microorganisms were identified in the analysis of 210 patients with recurrent implantation failure (RIF). Most frequently - 45% of the patient group expressed a massive growth of Lactobacillus sp. In a relatively high percentage of the studied women the dominant microorganisms were *Gardnerella sp.* (14%), *Candida albicans* (11%) and *Streptococcus agalactiae* (11%). (**Figure 1**)
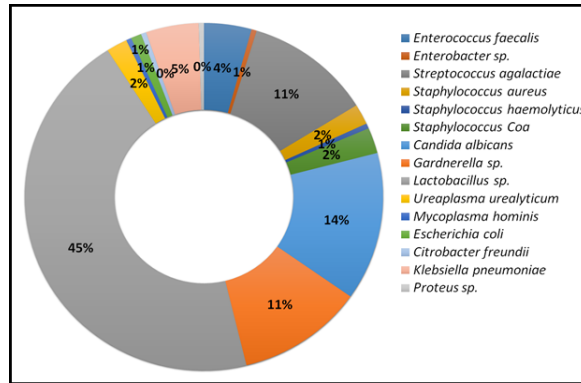
Fig. 1. Frequency of occurrence of dominant vaginal microorganism species in the studied patient group.

Totally, more than 50% of the enrolled women had dominant microorganism different from *Lactobacillus sp.,* which could be regarded as a prerequisite for the development of bacterial vaginoses, candidiases and bacterial infections, caused by other pathogens. Thorsen (1998) and Hellberg (2001) have conducted similar studies and they found a much higher percentage of vaginal lactobacilli (73.7% / 74.3%) in women without bacterial vaginosis and a lower incidence rate (29.8% / 38,4%) in BV women.
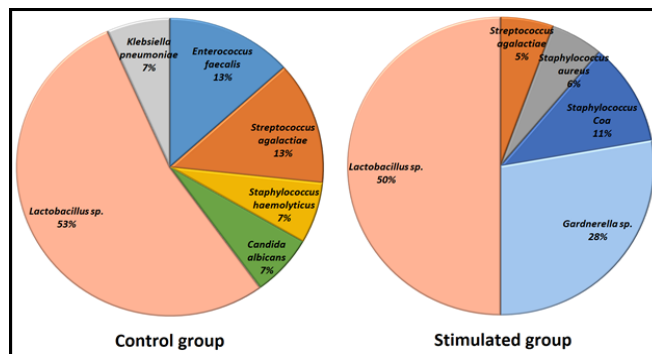

Fig. 2. Pie chart representation of the distribution of dominant vaginal bacterial species in (a) the control patient group and (b) the patient group with hormonal stimulation.

The performed comparison of women in natural cycles (control group) and patients during hormonal stimulation treatment (studied group) revealed several significant differences in the dominant groups of vaginal microorganisms. The main differences between the two patient groups were in the percentage of the cases having *Gardnerella sp., Enterococcus faecalis* and *Candida albicans* (**Figure 2**). We observed a dramatic increase in the percentage of cases with Gardnerella sp.

(28%), which were classified as a transition to bacterial vaginosis. The stimulation procedure has led to an increase in the number of anaerobic microorganisms that are considered responsible for BV. On the other hand, *Lactobacillus sp.* was observed in a similar percentage of women in both groups (53% vs. 50%). It could be suggested that the applied ovarian stimulation did not lead to a significant change in the frequency of occurrence of *Lactobacillus* as a dominant species. This result is confirmed by previous studies that defined dominant Lactobacillus flora during ovarian stimulation before IVF treatment (Jakobsson *et al.,* 2007). Hyman *et al.* (2014) also observed that ovarian stimulation for IVF altered vaginal microbiome, but not the diversity of the microbial species.

The hormonal regulation on the microbial communities is not widely studied. Nevertheless, it is known that ovarian hormonal dysfunction causes alterations of the vaginal microbial composition (Nelson *et al.,* 1995; Robinson *et al.,* 2003) and loss of the protective effect of *Lactobacillus* in the vaginal flora (Devillard *et al.,* 2004; Heinemann *et al.,* 2005).

Comparing the two groups, 28% of the stimulated women had *Gardnerella sp.* as a dominant vaginal microorganism. *Gardnerella vaginalis* forms dense, highly structured polymicrobial biofilm, strongly adhered to the vaginal epithelium (Swidsinski *et al.,* 2013). This condition is proven to lead to BV (Mikamo *et al.,* 2000; Zariffard *et al.,* 2002) that is related to failure of IVF therapy (Hyman *et al.,* 2012). The effectiveness of lactobacillus-containing vaginal tablets has been discussed recently in treating the BV (Mastromarino *et al.,* 2009). This could be also applied as therapeutic intervention for improving IVF outcomes.

CONCLUSION

The stimulation procedure has a negative effect on vaginal microbiota that could be a matter of concern in the consecutive IVF treatment. It could be suggested that ovarian stimulation should be followed by an adequate vaginal microbiota recovering procedures. The application of probiotics to populate the vagina and to treat the observed infection should be considered as an optional treatment before the future IVF cycles.

AUTHOR CONTRIBUTIONS
B.H., E.T., D.P, G.S. and T.C. conceived the experiment; B.H., E.T. and D.P. conducted the experiment; D.P. and E.T. analysed the results. E.T., R.G. and D.P. wrote the main manuscript text and prepared tables and figures. T.C., G.S. and B.H edited the manuscript and made its final revision. All authors critically reviewed and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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