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High serum AMH inhibits pathological growth of the low biomass endometrial microbiome

S. ERSAHIN[1], A. ERSAHIN[2], N.D. GUNGOR[2], K. GUNGOR[3], D. YALÇIN[4], C. ERSAHIN[2], N. CELIK[5]

[1]Department of Obstetrics and Gynecology, School of Medicine, Altinbas University, Istanbul, Turkey
[2]Department of Obstetrics and Gynecology, School of Medicine, Bahcesehir University, Istanbul, Turkey
[3]Department of Endocrinology, Istanbul Medeniyet University, Istanbul, Turkey
[4]Medicalpark Goztepe Hospital Microbiology, Istanbul, Turkey
[5]Department of Biochemistry, Behcet Uz Children's Hospital, Izmir, Turkey

Abstract. – OBJECTIVE: Although host microbiome play a role in both hormonal status and fertility, this issue has not yet been clarified. Since the endometrium is a sterile tissue, it is accepted that microbiota does not grow under normal conditions. The aim of the study was to reveal the characteristics of endometrial microbiota according to serum AMH levels in women with implantation failure.

PATIENTS AND METHODS: Forty-five women aged 20-30 years with two or more implantation failures were included in the study. They were divided into 3 groups according to their serum AMH values: Group 1 -AMH <1.3 ng/ml; Group 2 - AMH between 1.3-2.6 ng/ml; Group 3 - AMH >2.6 ng/ml. Twenty-two healthy fertile women who were the same age as the infertile group and applied for cervical smear screening were accepted as the control group. Following the embryo transfer, the tip of the catheter was inserted into the transport medium under sterile conditions. Sowing was carried out by touching the tips of the catheter to the blood agar medium. After the evaluation of the petri dishes at the end of 48 hours of incubation incubation, colonies were stained with Gram stain. Microorganisms in the colonies were identified with the Vitek-2 device according to their gram-staining characteristics and their antibiograms were made.

RESULTS: A negative correlation was detected between low AMH values and the microbiome detection rates in endometrial cultures. In patients with low serum AMH levels, the chance of endometrial microbiota growth was higher in the endometrial culture medium. The most common bacteria were found to be *MSSA, MRKNS* and *lactobacillus*. Clinical pregnancy rates were found to be significantly higher in the group with high AMH levels. As AMH levels increased, positive flora detection rates decreased, while clinical pregnancy rates increased.

CONCLUSIONS: Low serum AMH level increases the rate of positive endometrial microbiome in culture and decreases clinical pregnancy rates.

Key Words:
Endometrial microbiome, AMH, Implantation failure.

Introduction

The endometrium was initially considered to be a sterile environment like other upper genital tract organs. The isolation of some pathogenic bacteria, especially *lactobacillus,* in endometrial samples has questioned whether the endometrium is a sterile tissue[1,2]. Subsequently, there was criticism that vaginal contamination caused bacterial growth during transcervical collection of endometrial samples. Critics[1-3] claimed that the endometrium was actually a sterile organ, and bacteria grew as a result of the catheter coming into contact with the vagina or cervix while taking a sample. However, the detection of endometrial microbiome in samples taken from hysterectomy materials under sterile conditions led to the idea that the endometrium is not a sterile tissue[3]. The fact that the majority of bacteria grown in endometrial samples are similar to the vaginal flora has led to the idea that these bacteria reach the endometrium through the transcervical canal from the vagina. Although it is thought that the gastrointestinal bacteria can reach the endometrium through the neighborhood and the pathogens in the oral flora can reach the endometrium by the hematogenous way, vaginal spread is still the most accepted view[4]. Detection of bacterial DNA by standard microbial culture methods and next generation sequencing are the most commonly used methods in the diagnosis of endometrial microbiome[5]. The 16S rRNA-targeted polymerase chain reaction is the most commonly used method. While the risk of contamination is high in conventional culture,

Corresponding Author: Suphan Ersahin, MD; e-mail: suphanersahin@hotmail.com

the presence of dead bacterial DNA is also an important risk in next generation sequencing[6].

Endometrial microbiome tests have been used for two decades in patients with subfertile, infertile or recurrent implantation failure. Microbial culture of the distal end of the transfer catheter during embryo transfer has been the most widely used method for detecting the endometrial microbiome. Pathogen growth in cultures made from the catheter tip significantly reduced clinical pregnancy rates[7]. Interestingly, *lactobacillus* growth in culture increased clinical pregnancy rates[8]. Certain conditions must be met for pathogenic bacteria from the vaginal flora to pass through the cervical canal and reach the endometrium. Increased vaginal colonization of bacteria, deterioration of vaginal pH, weakening of the cervical mucus plug, and impaired immunity of the patient may facilitate bacterial invasion of the endometrium. Since the functions of the cervical mucus plug are regulated by estrogen and progesterone, the biggest obstacle for the colonization of the endometrium should be a healthy follicular development. It has long been thought that there is a positive relationship between infertility and the endometrial microbiome. A relationship has been shown between recurrent implantation defects and the presence of endometrial microbiome[9]. Anti-Mullerian hormone (AMH) is a glycoprotein[10] secreted from the granulosa cells of primary and early-stage antral follicles. Since AMH is an important indicator of ovarian reserve, it is also an indirect indicator of the synthesis of ovarian sex steroids. Low AMH levels due to age or underlying disease may lead to changes in cervical mucus plug and vaginal Ph, as it will impair follicular development and sex steroid synthesis. In addition to impaired vaginal pH, failed sex steroid synthesis and weak cervical mucus may facilitate the passage of bacteria from the vaginal flora into the endometrium. This study was planned to determine the relationship between serum AMH levels and endometrial microbiome in infertile patients with a history of two or more implantation failures. Secondary aim was to determine whether the endometrial microbiota affects fertility outcome.

Patients and Methods

Forty-five women aged 20-30 years with two or more implantation failures were included in the study. The patients were divided into 3 groups according to their serum AMH values. The number of patients in each group was 15, divided as follows: Group 1 – AMH < 1.3 ng/ml; Group 2 – AMH between 1.3-2.6 ng/ml; Group 3 – AMH > 2.6 ng/ml. Serum AMH levels were measured with Gen II Beckman Coulter AMH ELISA kit (Abbott Laboratories, IL, USA), according to the manufacturer's instructions. Twenty-two healthy fertile women who were the same age as the infertile group and who applied for cervical smear screening were accepted as the control group. The endometrial microbiome was investigated by microbial culture of the distal end of the transfer catheter. Following the embryo transfer, the tip of the catheter was inserted into the transport medium under sterile conditions and the upper part of the catheter was removed. Sowing was carried out by touching the tips of the catheter to the blood agar medium. After the evaluation of the petri dishes at the end of 48 hours incubation, colonies were stained with Gram stain. Microorganisms growing in the colonies were identified with the Vitek-2 device (bioMérieux, Marcy-l'Étoile, France), according to their gram staining characteristics, then their antibiograms were made. In the control group, the embryo transfer catheter was inserted and removed before the cervical smear, and the catheter tip was transferred to the transport medium and exposed to the same scatter and Gram staining procedures. This study was conducted after obtaining local ethical approval from Bahcesehir University (Approval No: 18551/2022) and patient consent.

Statistical Analysis

Data analyzes were performed using SPSS 19 (SPSS Inc., Armonk, NY, USA). Whether the data distributions were normal or not was determined using the Shapiro-Wilk test. Normally distributed variables were calculated with *t*-test and abnormal variables were calculated with Mann-Whitney U test. The relationship between the variables was calculated with the Spearman correlation analysis test. Data were reported as mean±SD. $p < 0.05$ was considered statistically significant.

Results

We found a negative correlation between the decrease in AMH values, and the microbiome detection rates in endometrial cultures. In patients with low serum AMH levels, the chance of endometrial microbiota growth was higher in the medium. The most common bacteria were found to be *MSSA, MRKNS* and *Lactobacillus.* Clinical pregnancy rates were found to be significantly higher in

Table I. Distribution of microbiome according to AMH values.

	Group 1 AMH <1.3 ng/ml	Group 2 AMH 1.3-2.6 ng/ml	Group 3 AMH >2.6 ng/ml	Control Group
No propagation	4	7	12	12
Contamination	3	3	-	6
MRSA	-	1	-	-
MSSA	2	-	-	-
MRCNS	-	3	-	-
MSSE	1	-	-	-
MRSE	-	-	2	2
Lactobacillus spp.	-	1	1	1
Candida spp	1	-	-	-
Proteas dispensa	1	-	-	-
Enterococcus faecalis	1	-	-	1
Pseudomonas aeruginosa	1	-	-	-
Streptococcus agalactia	1	-	-	-
Total	**15**	**15**	**15**	**22**

MRSA: Methicillin-resistant Staphylococcus aureus, MSSA: Methicillin-susceptible Staphylococcus aureus, MRCNS: Methicillin-resistant coagulase-negative Staphylococcus, MSSE: Methicillin-susceptible Staphylococcus epidermidis, MRSE: Methicillin-resistantStaphylococcusepidermidis.

the group with high AMH levels. As AMH levels increased, positive flora detection rates decreased, while clinical pregnancy rates increased.

While the rate of detection of microbiota in the endometrium was 20% in those with AMH value >2.6 ng/mL, it was 33.3% in those with AMH 1.3-2.6 ng/ml. In patients with AMH <1.3 ng/ml, the rate of positive microbiota was 53.3%. The group with the highest microbiota was the group with the lowest AMH values (Table I). Positive microbiota culture rates of patients in the control group and patients with AMH >2.6 ng/ml were similar (20% *vs.* 18.1%, $p<0.01$). *MSSA* was the most commonly grown bacteria in Group 1, while *MRCNS* was found in Group 2. The most common bacteria in group 3 was *Lactobacillus*, while it was *MRSE* in the control group. Bacterial growth was not detected in 35 of 67 participants. Contamination was detected in 12 of all participants. Bacterial growth was observed in only 20 of 67 patients. No characteristic was found in the distribution of bacteria between groups. A specific relationship was not detected between AMH values and growing bacterial species. It was remarkable that the frequency of *Lactobacillus* was low in all groups. In patients with endometrial microbiome in culture, clinical pregnancy rates were correlated with AMH values. Even if microbiome was detected in cases with high AMH values, pregnancy rates were significantly higher than in groups with low AMH values (Table II).

Table II. The relationship between AMH values and clinical pregnancy and microbiome types.

	Group 1 AMH <1.3 ng/ml	Group 2 AMH 1.3-2.6 ng/ml	Group 3 AMH >2.6 ng/ml	Control Group
N	15	15	15	22
Positive endometrial microbiota, n (%)	8 (53.3%)	5 (33.3%)	3 (20%)	4 (18.1%)
Most common bacteria	MSSA	MRCNS	Lactobacillus MRSE	MRSE
Clinical pregnancy n (%)	3 (20%)	4 (26.6%)	6 (40%)	NA
CPR in microbiota positive cases	1 (12.5%)	1 (20%)	1 (33.3%)	NA

MSSA: Methicillin-susceptible Staphylococcus aureus, MRCNS: Methicillin-resistant coagulase-negative Staphylococcus, MRSE: Methicillin-resistant Staphylococcusepidermidis.

Discussion

The endometrial microbiome plays an important role in both the health of the endometrium and the emergence of diseases. There are studies[11,12] showing that the profile of the endometrial microbiome changes in patients presenting with implantation failure. Consistent with this, Moreno et al[11] reported that the dominant bacteria in the endometrium of healthy individuals is *Lactobacillus spp.* The high rate of *Lactobacillus* species in the endometrial flora compared to other pathogens significantly increases implantation and clinical pregnancy rates in IVF/ICSI patients[12].

Current study reports for the first time the relationship between serum AMH levels, endometrial microbiome and reproductive outcome. We found *Lactobacillus* and *MRSE* as the most growing endometrial bacteria in the group with serum AMH level of >2.6 ng/ml. Interestingly, the clinical pregnancy rates of this group were significantly higher than the other two groups with low AMH values. In general, the growth of pathogenic bacteria other than *Lactobacillus* in the endometrium reduces implantation and clinical pregnancy rates. For example, detection of pathogens such as *Bifidobacterium, Gardnerella* or *Streptococcus* along with *Lactobacillus* in the endometrium reduces implantation, clinical pregnancy, and live birth rates, while causing a significant increase in abortion rates[11,13]. Although *MRSE* is detected with *Lactobacilli*, the reason for the high pregnancy rates may be related to the colonization rates of bacteria in the endometrium. If *Lactobacillus* constitutes more than 90% of the bacteria colonizing the endometrium, the growth of other pathogens in culture does not adversely affect the reproductive outcome[11]. In addition, the high AMH values of this group may contribute to the preservation of the integrity of the cervical mucus plug by ensuring the continuation of healthy follicular development. High AMH values are critical to the maintenance of cervical mucus, as they increase healthy follicular growth and sex steroid synthesis. For this reason, the growth of other pathogens in addition to *Lactobacillus* in culture may be neutralized indirectly by high AMH values.

Lactobacillus, which is the main host of the vaginal flora, limits the vaginal colonization of other pathogen bacteria by producing lactic acid and short-chain fatty acids. The number of bacteria in the vagina in a normal healthy woman varies between 10^{1^0}-10^{11}. The endometrial microbiome, on the other hand, is formed by bacteria at a rate of about one quarter of the flora in the vagina[13]. One of the greatest barriers to bacterial colonization of the endometrium is the presence of the cervical mucus plaque. Serum AMH levels are a strong indicator of ovarian reserve. In the presence of high AMH, increased estrogen and progesterone synthesis prevents the passage of bacteria by maintaining the mucus plug. The high clinical pregnancy rates in the group with high AMH is a finding that supports this idea. Conversely, in patients with low AMH values, the weakened mucus plaque facilitates pathogenic bacteria to reach the endometrium, thus reducing pregnancy rates. High AMH not only makes *Lactobacillus* a dominant bacterium, but also increases pregnancy rates by preventing pathological inflammation in the endometrium.

In the group with AMH <1.3 ng/ml, *Lactobacillus* was not detected in the endometrial microbiome, but mostly *MSSA* and *MSS* type bacteria grew. The clinical pregnancy rates of this group were recorded as the lowest. The significant decrease in fertility outcome may be due to both low AMH levels and growth of pathogenic bacteria. Pathogenic bacteria may prevent implantation by causing inflammation in the endometrium and impairing immunomodulation. Dysbiosis between increasing pathogenic microorganisms and *Lactobacillus* may impair decidua formation and expression of receptivity modulators, leading to decreased clinical pregnancy rates[14,15].

Conclusions

Despite the small number of cases, it is the first study to compare AMH values with endometrial microbiome and clinical pregnancy rates. High AMH prevents colonization of the endometrium with bacterial pathogens. When the AMH value decreases, the endometrium becomes open to pathogens. The increase in the rate of pathogenic bacteria in the endometrium reduces clinical pregnancy rates by disrupting *Lactobacillus* colonization and decidualization.

Conflict of Interest

All authors have no conflicts of interest.

Informed Consent

Informed consent was obtained from all participants.

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