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ORIGINAL ARTICLE

ROLE OF BACTERIAL AND VIRAL INFECTIONS AND CO-INFECTIONS IN MISCARRIAGES

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Abstract. Aim: *To investigate the potential role of the following bacterial/viral panel (Chlamydia trachomatis, Ureaplasma urealyticum/parvum, Mycomplasma hominis/genitalium, Gardnerella vaginalis, HSV1/2, EBV, CMV, VZV, HHV6, HHV7, HHV8) as causative factors for miscarriages in women by testing endometrial biopsies. Anaerobic and aerobic microorganisms causing dysbiosis and endometrial bacterial colonization by unbalanced growth were additionally tested.* ***Materials and methods:*** *In total, 65 patients with a history of early and late miscarriages were analyzed. DNA extractions, real-time qPCR, agarose gel-electrophoresis were applied. Comparative analysis of the current with previously obtained data on the described panel in menstrual tissue samples was performed.* ***Results:*** *In 64,6% of all tested endometrial biopsies bacterial and/or viral pathogens were detected. In 49,23% of all tested samples we found bacterial, while in 15,3% – viral pathogens. These results are similar to our previous data on menstrual tissue samples of infertile women – 61,1% infected, as 48,8% had bacterial and 22,2% had viral pathogens. Gardnerella vaginalis and Ureaplasma parvum were detected in 31,25% and 3,12% of all bacterial infected endometrial biopsies, significantly lower in comparison to the estimated rate of 69,31% and 61,36% on menstrual tissue. Anaerobic and aerobic dysbiosis were detected in 53,33% and 27% of the bacterial infected endometrial samples. In 13,33% a dysbiosis with a mixed etiology was found, while in 7% a dysbiotic condition with a totally absent findings of targeted bacteria and Lactobacillus was observed. EBV, CMV, HHV6 and HHV7 were detected in 30%, 30%, 20% and 20% of the positive for viral factors endometrial biopsies and in 40%, 7,5%, 10% and 42,5% in menstrual tissue samples. In the current study 62,5% bacterial co-infection and 12,5% bacterial/viral co-infection variants were found. Infections with the rest of the target pathogens were not detected in the endometrial biopsies. In contrast to the endometrial biopsy results, Mycomplasma hominis, Ureaplasma urealyticum and HSV2 were detected in our previous research on menstrual tissue samples.* ***Conclusions:*** *Our research suggests a possible dysbiosis as a consequence of bacterial/viral endometrial colonization, associated with miscarriages. We prove that menstrual tissue, containing parts of the functional endometrial layer, is a reliable and accurate noninvasive sample for infectious screening of the upper genital tract.*

Key words: *endometrium; inflammation; HHVs; infertility; menstrual tissue*

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INTRODUCTION

Miscarriage represents one of the most common and still unclear adverse pregnancy outcomes. Miscarriage is the spontaneous loss of a pregnancy before 12th gestational week (early miscarriage) or between 12th and 24th gestational week (late miscarriage). Unfortunately, a miscarriage occurs in one of five pregnancies and could cause serious physiological and psychological implications for the suffering patient and couples [1]. The reasons for a miscarriage are often unknown but in 50% of early miscarriages the fetus exhibits chromosomal aberrations, as morphological and structural alteration or abnormal chromosomal numbers [2]. Besides genetic causes, other factors are associated with increased risk for miscarriage as well: advanced age of both parents, especially for woman, ethnic origin, mother's psychological condition, very low or very high pre-pregnancy body mass index (BMI), stress, non-steroidal anti-inflammatory drugs, smoking, alcohol consumption [3-4].

It is known, that a number of infections are associated with miscarriage and other unfavorable outcomes such as stillbirth, preterm delivery [5-6]. Different infections are the cause of approximately 15% of early and 66% of late miscarriages [7-8].

The diagnostics of pathogenic microorganisms in the upper genital tract resulting in endometrial colonization, dysbiosis and inflammation is of a great significance. Some bacterial communities are able to multiply and colonize the female endometrium through mechanisms of virulence, such as mucin degradation, biofilm formation and antimicrobial resistance, leading to a state of dysbiosis [9]. The population of commensal microorganisms also plays an essential role in maintaining an eubiotic equilibrium, including Lactobacillus. Unbalanced bacterial fractions may also affect the intrauterine metabolic composition and thus, the growth of pathogenic bacteria in that imbalanced and unregulated, even toxic microenvironment is stimulated.

AIM

To investigate the potential role of the following bacterial/viral panel (*Chlamydia trachomatis, Ureaplasma urealyticum/parvum, Mycomplasma hominis/genitalium, Gardnerella vaginalis, HSV1/2, EBV, CMV, VZV, HHV6, HHV7, HHV8*) as causative factors for miscarriages in women by testing endometrial biopsies. Anaerobic and aerobic microorganisms causing dysbiosis and endometrial bacterial colonization by unbalanced growth were also tested.

MATERIALS AND METHODS

Patients and samples: In total, 65 females at the average age of 31,2 years were selected, mainly on the basis of their history of early (5th-12th gestation week) – 85% and/or late recurrent miscarriages (13th-27th gestation week) – 15%. No autoimmune disorders or other acquired diseases were present. The clean status in regard to the target bacterial/viral panel in cervical-vaginal swabs was the leading criteria for the patient selection. Anaerobic and aerobic microorganisms causing inflammation, dysbiosis and endometrial bacterial colonization by unbalanced growth were also tested. All selected women were tested for the described bacterial/viral pathogens in the upper genital tract by analyzing endometrial biopsies. The examination was performed at least 2 months after the miscarriage. Comparative analysis of the current with previously obtained data on the same pathogens in menstrual tissue samples was made.

Endometrial biopsies were performed using endometrial suction catheter following the established standards in the gynecological practice. As a transport medium for DNA preservation 2 ml 0.5M EDTA with pH 8.0 was used. Protein digestion with proteinase K (15 µl) for 24 hours at 65oC was performed and sediment was collected after centrifugation for 15 min at 8000 rpm. Total DNA was extracted from the 120 µl sediment using AmpliSens DNA isolation kit (Ecoli s.r.o, Slovak Republic). As a DNA carrier, 2 µl (15 mg/µl) glycogen was added after the preparation with a lysis buffer, simultaneously with the addition of the DNA-sorbent component. An additional washing step with 75% ethanol was included, resulting in a higher yield and purity of the obtained genomic viral/bacterial DNA. The amplification of the target viral and bacterial DNA fragments was performed, using AmpliSens commercial amplification kits (Ecoli s.r.o, Slovak Republic) and DNA Technology commercial amplification kits (Moscow, Russia) based on Real-Time qPCR.

RESULTS

The current research does not include healthy control group, because the procedure for obtaining endometrial biopsy is invasive.

In 49,23% of all tested endometrial biopsies we found bacterial, while in 15,3% viral pathogens. These results are similar to our previous data on menstrual tissue samples of infertile women – 48, 8% had bacterial and 22,2% had viral pathogens.

Gardnerella vaginalis and *Ureaplasma parvum* were detected in 31,25% and 3,12% of all bacterial infect-

ed endometrial biopsies, which are significantly lower percentages in comparison to the estimated rates on menstrual tissue – 69,31% and 61,36%, respectively. Anaerobic and aerobic dysbiosis were detected in 53,33% and 27% in all endometrial samples with bacterial invasion. Dysbiosis with a mixed etiology was reported in 13,33%, while in 7% a dysbiotic condition without the target bacteria or *Lactobacillus* was found (Fig. 1).

Active infection of *EBV, CMV, HHV6* and *HHV7* was detected in 30%, 30%, 20% and 20% of all infected with viral factors endometrial biopsies (Table 1) and in 40%, 7,5%, 10% and 42,5% of the menstrual tissue samples, respectively.

In the current study 62,5% bacterial and 12,5% bacterial/viral co-infection variants were found. Infections with the rest of the target pathogens were not detected in the endometrial biopsies, namely *Chlamydia trachomatis, Ureaplasma urealyticum, Mycomplasma hominis/genitalium, HSV1/2, VZV*, and *HHV8*. In contrast to the endometrial biopsies results, *Mycomplasma hominis, Ureaplasma urealyticum* and HSV2 were detected in our previous research on menstrual tissue samples with frequencies of 2,27%, 2,27% and 2,5%, respectively. All patients included in the current study had molecular-genetic testing for thrombophilia and in a high proportion (67,69%, 44/65) a genetically risk profile was detected.

DISCUSSION

Our study revealed positive active infection for the targeted bacterial and/or viral pathogens in 64,6% of the endometrial biopsies of patients with a history of recurrent early and/or late miscarriages. In 49,23% of all tested samples we found bacterial, while in 15,3% viral pathogens, which is consistent with our previously unpublished data on menstrual tissue of infertile women, where 48,8% had bacterial and 22,2% had viral pathogens [10].

Active infection with *Gardnerella vaginalis* was detected in 31,25% of all endometrial biopsies with bacterial invasion, which is two times lower in comparison to our previously reported results (in 69,31% of the menstrual tissue samples). Approximately one half of the cases with *Gardnerella vaginalis* dominant active infection were presented in co-infection variant with other anaerobic bacteria predominantly (*Peptostreptococcus spp., Eubacterium spp., Atopodium vaginae, Megasphaera spp., Veilonella spp., Dialister spp.*). The cases of Gardnerella vaginalis dominant active co-infection with mixed etiology, including anaerobic/aerobic bacteria (*Staphylococcus spp.*) were much rarer.

The presence of an abundant reservoir of *Gardnerella vaginalis*, combined with other potentially pathogenic bacteria configured in a polymicrobial biofilm, attached to the endometrium closely to the myometrium and amniotic compartment was reported by other researchers, which is sustained by our work [11-12]. Our data confirms the hypothesis that the endometrial bacterial colonization, resulting in dysbiosis and inflammation has a key role in the pathogenesis of non-viable pregnancy and adverse outcome as miscarriage. The implication of bacterial endotoxins, macrophages, *IL-1, TNF*, etc. in pro-inflammatory response against infection was already proved. We sustain the hypothesis that endometrial infection leads to pathogenic intrauterine inflammatory-immunological changes in the host, which disrupt the endometrial functions by decreasing its receptivity to embryo implantation and development [13-14].

The low frequency of *Gardnerella vaginalis* in the endometrial biopsies in comparison to menstrual tissue (31,25%) is considered as a normal finding, because all tested women were negative for the targeted pathogens in the cervical-vaginal swabs. The number of the currently tested endometrial biopsies (65) is relatively small, compared with the number of the previously tested menstrual tissue samples (180). It is so, because the endometrial biopsies were selected only on the basis of a positive history for recurrent miscarriages, while the menstrual tissue samples selection included a great variety of reproductive and healthy problems. The current study included testing of about 50% of the sexual partners of the selected women for the targeted bacterial pathogens by microbial culture (data not provided). Their negative status further explains lower frequency of *Gardnerella vaginalis* and *Ureaplasma parvum* in the present study and is interpreted as a limited possibility for sexual pathogens transmission. It can explain also partially the lower *Gardnerella vaginalis* frequency in endometrial biopsies, compared with examined menstrual tissue. We do not exclude also a variant of bacterial re-activation and uncontrolled growth in the upper genital tract due to unbalanced and negatively changed environment particularly in the cases of opportunistic pathogens.

Ureaplasma parvum was found in only 3,12% of all bacterial infected endometrial biopsies, which is extremely low percentage compared to our data from menstrual tissue samples – 61,36%. Our results do not comply with data in the literature, according to which *Ureaplasma parvum* infection is associated with recurrent miscarriages [15]. Our explanation of the data regarding the low frequency of *Ureaplasma parvum* is similar with the interpretation of *Gardnerel-*

la vaginalis results – negative results in the cervical-vaginal swabs, negative status among up to 50 % of the tested sexual partners and very strict criteria for inclusion in the study.

Anaerobic and aerobic dysbiosis were detected in 53,33% and in 27% from all endometrial samples with bacterial invasion. Dysbiosis with a mixed etiology was reported in 13,33%, while in 7% a dysbiosis without the targeted bacteria or Lactobacillus was found. Our data on the detected anaerobic and aerobic bacteria in the endometrial biopsies (Fig. 1) correlates with the results from high-tech studies on endometrial microbiome [16-17]. The most prevalent variant of *Gardnerella vaginalis* dominant anaerobic dysbiosis was in combination with *Prevotella bivia, Porphyromonas spp.* and the second one, *Mobiluncus spp., Corynebacterium spp., Eubacterium* and *Atopobium vaginae. Staphylococcus spp.* was detected predominantly in the cases of aerobic dysbiosis.

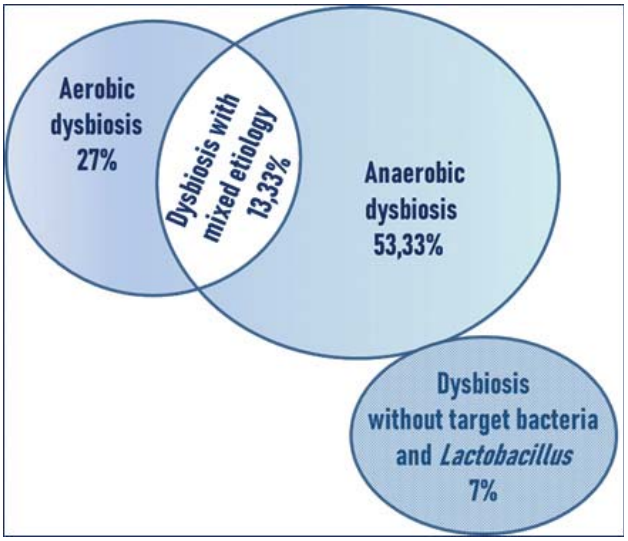

Fig. 1. Types of dysbiosis, as a consequence of unbalanced bacterial growth, endometrial colonization and inflammation. **Anaerobic dysbiosis** /positive bacterial findings/: *Mobiluncus spp., Corynebacterium spp. Eubacterium spp., Megasphaera spp., Veillonella spp., Dialister spp. Eubacterium spp., Gardnerella vaginalis, Prevotella bivia, Porphyromonas spp., Peptostreptococcus spp., Atopobium vaginae, Lachnobacterium spp., Clostridium spp.*; **Aerobic dysbiosis** (positive bacterial findings): *Streptococcus spp., Enterobacteriaceae spp., Staphylococcus spp.*

As a common feature of dysbiotic conditions, we accept the decreasing proportion of *Lactobacillus* to different degree, as was also reported in the literature [18-19]. Some scientific groups establish bacterial interactions (networks) and prove that *Lactobacillus* is negatively associated with *Gardnerella, Bifidobacterium and Atopobium* [17], as we also observed regarding *Gardnerella* and *Atopobium*.

It is worth mentioning that in the lower genital tract (cervical-vaginal swabs) all patients studied represented normocenosis and conventional normocenosis, but in the endometrium we found moderate and strong dysbiosis and co-infection. These differences are not a surprising finding, because the physiological nucleus of vaginal and endometrial microbiome was not clarified and determined. This ambitious task requires numerous future investigations. Some scientists report coinciding results concerning the vaginal and endometrial microbiome [20-21], but others describe significant differences, as it was established in our work [22]. The endometrial microbiome is significantly affected by hormonal changes and evidence for that is the exogenous progestin, which significantly alters the endometrial microbiome by reducing the phylotype diversity of *Lactobacillus spp.* [23]. Probably that could explain the differences which we found in our study.

According to our results *Chlamydia trachomatis*, *Mycoplasma hominis/genitalium* and *Ureaplasma urealyticum* were not detected, which does not comply completely with data in the published literature [24]. Only *Mycoplasma hominis* and *Ureaplasma urealyticum* were detected with a very low frequency (2,27%) in noninvasive menstrual tissue samples. *Mycoplasma hominis/genitalium* were reported with negligibly low frequency (2,9%) in the published data [25].

Active infection with *EBV, CMV, HHV6* and *HHV7* was detected in 30%, 30%, 20% and 20% of the positive for viral factors endometrial (Table 1), and in 40%, 7,5%, 10% and 42,5% of the menstrual tissue samples [10]. We suppose that these differences are due to the smaller number of the tested endometrial biopsies (65) compared to the menstrual tissue samples (180). If the endometrial biopsies are increased, then we expect to obtain more similar to the menstrual tissue samples results. Infections with *Herpesviridae* family have a direct negative, even cytotoxic effect on male spermatozoa. By vertical viral transmission during fertilization *HHVs* affects negatively the tissues of the female upper genital tract and the implanted newly formed embryo [26]. A common cause of failure in vitro fertilization attempts or miscarriages is the reactivation of *HHVs* as a result of hormonal stimulation by pregnancy hormones. Most probably a complex cascade of immunological rejection of the embryo was triggered in our cases with positive HHVs findings and recurrent miscarriages. Generally the unfavorable scenario includes increased NK cells level, disturbed Th1/Th2 balance, increased cytotoxicity, increased fibrosis and reduced levels of Tregs resulting from viral active infections [27-28].

Table.1. Positive results in the group of viral infected probands and co-infection variants.

Viral factor	Viral frequency	Description of viral-bacterial co-infection variant
*EBV**	30%	–
*CMV**	30%	● *CMV* + moderate aerobic dysbiosis with dominant *Streptococcus spp*.
		● *CMV* + dysbiosis with a mixed etiology: *Lachnobacterium spp*. + *Clostridium spp., Enterobacteriaceae* and *Streptococcus spp*.
*HHV6**	20%	● *HHV6* + strong anaerobic dysbiosis with a dominant *Eubacterium spp*.
*HHV7**	20%	–

*After applied individualized therapy (1,5-3 months), an entirely bacterial and/or viral cleaning of the upper genital tract was observed. Re-examination was performed on endometrial biopsies again.

The viral pathogens *HSV1/2, VZV* and *HHV8* were not detected in the tested endometrial biopsies, which is in agreement with the published data for negligibly low frequencies of these viral factors [29]. In the menstrual tissue samples we detected only **HSV2** with very low frequency (2,5%), which again supports the literature data. There is a controversial data for the impact of *HHV7* in contrast to the categorically proven association of *EBV* and *CMV* to the reproductive failure, including miscarriages, primary unexplained infertility, and even pathogenesis of preeclampsia in association to *HHV6* [30]. Our data assumes a potential association between the active asymptomatic infection with *HHV7* in the female endometrium with recurrent miscarriages and points *HHV7* as a risk co-factor implicated in the fertility loss. Further investigations are needed and they would be of a great importance, because *HHV7* turned out to be morphologically similar to *HHV6* and even more it participates simultaneously with *HHV6* in other diseases [31-32].

In 67,69% of all patients included in the current study a genetic risk profile for thrombophilia was detected. The high proportion of a risk molecular profile to thrombophilia in the current study determines its role as an independent risk factor or co-factor for the complex nature of miscarriages. Genetic risk profile to thrombophilia, combined with positive bacterial/viral status in endometrium could increase significantly the risk of a pregnancy loss.

In 15% of the patients included in the study both menstrual tissue and endometrial biopsy samples were tested. The observed negative or positive bacterial/viral findings were absolutely identical in both samples. The reported data confirms once again that the noninvasive menstrual tissue sample, containing parts of the functional endometrium is representative for assessment of the infectious status in the upper genital tract.

Finally, an individualized therapy (1,5-3 months) was applied to all bacterial/viral infected patients. A total bacterial and viral cleaning of the upper genital tract was observed by retesting new endometrial biopsies. Simultaneously a maintaining therapy for minimizing the risk of thrombophilia (during future pregnancy) was applied where necessary. It is worth mentioning that in 97% of the patients where an adequate therapy was applied, a natural conception was achieved. In the remaining 3% of the cases an endometrial polyp of hyperplastic type was found and in these patients the treatment still goes on.

CONCLUSIONS

Our pilot data shows that an individualized approach for treatment of fertility loss can gain positive impact in the fight against recurrent miscarriages.

Powerful tools of the modern medicine and molecular biology contribute to clarify easily reproductive problems and to perform adequate and successful therapy in the affected couples.

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