ORIGINAL ARTICLE

Human β -defensin-2 as a biochemical indicator of vaginal environment in pregnant women

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Aim: Vaginal environment is an important factor in predicting threatened abortion and premature rupture of membranes (PROM) in early pregnancy. Vaginal human β -defensin-2 (HBD-2), a main antimicrobial peptide associated with innate immunity, plays multiple roles to protect the lower genital tract from microbes. This study aimed to assess the usefulness of vaginal HBD-2 as a biochemical marker for monitoring the vaginal environment in pregnant women.

Methods: Twenty-eight pregnant women were enrolled in this study. Vaginal secretion samples were collected to determine Nugent scores, and vaginal washing fluid (4 ml saline) was used to measure HBD-2 levels by ELISA.

Results: In the first trimester, vaginal HBD-2 levels were significantly lower in patients with bacterial vaginosis (BV) (42.1 ± 19.8 pg/ml) compared to those without BV (350.1 ± 68.7 pg/ml, $P < 0.05$), and in patients with PROM (57.2 ± 31.9 pg/ml) compared to those without PROM (375.9 ± 69.4 pg/ml, $P < 0.05$).

Conclusions: Low vaginal HBD-2 levels during early pregnancy suggest a poor vaginal environment and the possibility of developing PROM.

Introduction

Bacterial vaginosis (BV) in early pregnancy is a known risk factor for preterm delivery.^{1,2)} Antibiotic prophylaxis reportedly does not improve maternal or perinatal outcomes.³⁾ Assessment of the vaginal condition in early pregnancy is important for predicting perinatal outcome, but currently, there is no biochemical marker that precisely reflects the vaginal environment. The Nugent score, which is calculated based on microscopic quantification of *Lactobacillus*,⁴⁾ is used as a standard measure to evaluate BV, although its measurement reproducibility is low.

The innate immune system plays a role in protecting the female reproductive tract from bacterial, fungal, and viral infections. Antimicrobial peptides are small cationic peptides, many of which have positively charged residues and amphipathic structures, and play multiple

roles in the innate immune system.^{5,6)} Although the precise mechanism of antimicrobial action remains to be elucidated, antimicrobial peptides are known to create pores on target membranes to exert antimicrobial activity.

Humans and other mammals have antimicrobial peptides belonging to two main families: defensins and cathelicidins.⁷⁾ Defensins are divided into three main subfamilies (alpha, beta, and theta), with varying lengths of peptide segments between cysteine residues connected by disulfide bonds.⁷⁾ Human beta-defensins (HBDs) activate the adaptive immune system through chemotaxis of T lymphocytes and dendritic cells to the infection site.⁸⁾ There are three types of HBDs (HBD-1, -2, and -3) expressed in the vaginal epithelium, placenta, amniotic epithelium, and decidua.¹¹⁾ HBD-1 is constitutively expressed in epithelial cells, whereas expressions of HBD-2 and HBD-3 are induced by stimulation from infection.^{9,10)} HBD-2 attracts memory T-cells and

immature dendritic cells, while HBD-3 is chemotactic for macrophages. (Jung S, Mysliwy J. *Antimicrob Agents Chemother*. 2011; 55(3): 954–60) High levels of HBD-2 in amniotic fluid have been reported in cases of preterm delivery.¹²⁾ In addition, a significant decrease in the level of HBD-2 was observed in patients with BV during pregnancy.¹³⁾ However, few reports have examined vaginal HBD-2 levels in cases of preterm delivery with premature rupture of membranes (PROM).

The composition of the vaginal flora is known to change in pregnancy so as to prevent uterine infection.¹⁴⁾ In this study, we examined changes in HBD-2 levels in vaginal secretions of pregnant women as a biochemical marker for evaluating the vaginal environment involved in preterm delivery and PROM.

Materials and methods

Participants and sample collection

Twenty-eight pregnant women who visited NTT West

Matsuyama Hospital were enrolled in this study in 2015. Demographic and clinical data are summarized in Table 1. Written informed consent was obtained from all participants prior to enrollment. The study protocol was approved by the ethics committee of Ehime University Hospital.

Vaginal secretion samples were collected using cotton swabs for Nugent score calculation in the first trimester (8–11 weeks of gestation) and third trimester (28–31 weeks of gestation). Vaginal washing fluid was (4 ml saline) was used for microbe analysis. A portion of each sample was centrifuged at 3,000 rpm for 10 min at 4°C, and the supernatant was stored at –80°C until the time of HBD-2 assay.

Nugent scores and microbiological analysis

Gram-stained vaginal smears of vaginal secretions were examined at a magnification of $\times 1,000$ with an oil immersion lens. The presence of large Gram-positive rods (*Lactobacillus* spp. morphotypes), small Gram-variable rods (*Gardnerella* morphotypes), and curved Gram-negative rods (*Mobiluncus* spp. morphotypes) was quantified according to the Nugent criteria. Composite scores were calculated: scores 0–3 were considered normal, and scores 4–6 and 7–10 indicated an intermediate state and BV, respectively.

The presence of *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Mycoplasma genitalium*, and *Chlamydia trachomatis* was analyzed by polymerase chain reaction (PCR). Oligonucleotide primers for PCR reaction were designed; the forward and reverse primers are listed in Table 2. PCR was carried out using Emerald Amp PCR Master Mix (TaKaRa Bio, Shiga, Japan) in a thermal cycler (Chromo4 System: Bio-Rad Laboratories, Hercules, CA, USA). The reaction profile was as follows: initial denaturation at 94°C for 120 s, followed by 35 cycles at 98°C for 10 s, 54°C for 30 s, and 72°C for 45 s, and final extension at 72°C for 420 s.

Table 1. Demographic and clinical characteristics of participants at enrollment (n = 28)

Characteristics	Value
Age (years), mean $\pm$ SD (range)	30.1 $\pm$ 4.5 (22–39)
20–29	11 (39%)
30–39	17 (61%)
Gravidity, median (range)	1 (0–4)
0	9 (32%)
≥ 1	19 (68%)
Parity, median (range)	1 (0–2)
0	13 (46%)
≥ 1	15 (54%)
Nugent score, first trimester	
0–3 (normal)	24 (85.7%)
4–6 (intermediate)	1 (3.6%)
7–10 (BV)	3 (10.7%)

Table 2. Primers used in this study

Primer	Sequence (5'–3')
<i>Ureaplasma urealyticum</i> U-5F	CAATCTGCTCGTGAAGTATTAC
<i>Ureaplasma urealyticum</i> U-4R	ACGACGTCCATAAGCAACT
<i>Mycoplasma genitalium</i> MgPaW1F	AAGTGGAGCGATCATTACTAAC
<i>Mycoplasma genitalium</i> MgPaR1R	CCGTTGTTATCATACCTTCTGA
<i>Mycoplasma hominis</i> F-P	GACACTAGCAAAGTAGAGTTAG
<i>Mycoplasma hominis</i> R	CACCATCTGTCACTCTGTTAACTC
First PCR <i>Chlamydia trachomatis</i>	TTGCGA TCCTTGACCACTT
<i>Chlamydia trachomatis</i>	GCTCGAGACCATTTAACTCC
Nested PCR <i>Chlamydia trachomatis</i>	GGTGACTTTGTTTTCGACCG
<i>Chlamydia trachomatis</i>	CTCCAATGTAGGGAGTGAAAC

Table 3. Levels of human β -defensin-2 (HBD-2) in vaginal lavage fluid from women with and without bacterial vaginosis (BV)

Nugent score	<i>n</i>	Age (years, mean $\pm$ SD)	HBD-2 (pg/ml, mean $\pm$ SE)
First trimester			
0–3 (normal)	24	30.4 $\pm$ 4.4	350.1 $\pm$ 68.7*
4–6 (intermediate)	1	22	720.9
7–10 (BV)	3	30.7 $\pm$ 4.0	42.1 $\pm$ 19.8*
Third trimester			
0–3 (normal)	28	30.1 $\pm$ 4.5	128.1 $\pm$ 22.3
4–6 (intermediate)	0		
7–10 (BV)	0		

* $P < 0.05$.

The presence of *Candida* species in the vaginal flora was determined by microbial cultivation and microscopic analysis.

HBD-2 ELISA

HBD-2 levels in vaginal fluid were determined by ELISA (HBD2 ELISA kit: Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) according to the manufacturer's instructions. Light absorbance was measured at 450 nm with a microplate reader (iMark: Bio-Rad Laboratories). All samples were measured in triplicate.

Statistical analysis

Data were expressed as mean $\pm$ SE. The parameters were analyzed by one-way ANOVA with the Bonferroni *post hoc* test. Significant differences in reverse transcriptase-PCR results were detected using the Mann–Whitney *U* test. P values < 0.05 were considered statistically significant.

Results

Participant characteristics are summarized in Table 1. In the first trimester, 85.7% of participants had normal Nugent scores (normal group), and 10.7% had BV (BV group). Vaginal HBD-2 levels were significantly higher in the normal group (350.1 $\pm$ 68.7 pg/ml) than in the BV group (42.1 $\pm$ 19.8 pg/ml, $P < 0.05$; Table 3). Vaginal HBD-2 levels were significantly increased in the presence of *Candida* ($P < 0.05$), but not affected by the presence of *U. urealyticum* (Table 4).

None of the participants had preterm birth, although five participants required hospitalization and treatment with intravenous ritodrine hydrochloride for 14–60 days to avoid preterm delivery. HBD-2 levels in vaginal secretion did not differ between participants who received treatment and those who did not (249.0 $\pm$ 125.3 and 348.0 $\pm$ 72.6 pg/ml, respectively).

Four participants developed PROM (PROM group); three were in the normal group, and one was in the BV

Table 4. Levels of human β -defensin-2 (HBD-2) in vaginal fluid in first trimester

Group	<i>n</i>	HBD-2 (pg/ml, mean $\pm$ SE)
<i>Candida</i> positive	8	598.2 $\pm$ 143.6*
negative	20	223.2 $\pm$ 52.8*
<i>Mycoplasma hominis</i> positive	0	
negative	28	330.4 $\pm$ 63.2
<i>Mycoplasma genitalium</i> positive	0	
negative	28	330.4 $\pm$ 63.2
<i>Ureaplasma urealyticum</i> positive	15	406.7 $\pm$ 105.4
negative	13	242.2 $\pm$ 56.4
<i>Chlamydia trachomatis</i> positive	1	838.7
negative	27	311.5 $\pm$ 62.6

* < 0.05 , positive vs. negative.

group. Vaginal HBD-2 levels in the first trimester were significantly lower in the PROM group (57.2 $\pm$ 31.9 pg/ml) compared to the non-PROM group (375.9 $\pm$ 69.4 pg/ml, $P < 0.05$; Figure 1). In the third trimester, no significant difference in HBD-2 levels was observed between the two groups (PROM, 78.3 $\pm$ 33.8 pg/ml; non-PROM, 141.0 $\pm$ 26.4 pg/ml).

Discussion

In pregnant women, BV confers high risks of preterm delivery and PROM. A healthy vaginal flora is important to avoid these conditions and to have a good perinatal outcome.^{1,2} In previous studies, antibiotics treatment for BV during pregnancy was not associated with perinatal outcomes.³ A useful biochemical marker that reflects changes in vaginal flora in early pregnancy is needed in order to predict preterm delivery and PROM and thereby improve perinatal outcomes.

Beta (β)-defensins, a subfamily of antimicrobial peptides, are produced by epithelial cells of the amniotic membrane, chorionic membrane, and vaginal wall. Vaginal β -defensins form the first line of defense against

Figure 1. Levels of human β -defensin-2 (HBD-2) in vaginal fluid from women with and without premature rupture of membranes (PROM).

HBD-2 levels in the first trimester were significantly decreased in patients with PROM (PROM group: 57.2 ± 31.9 pg/ml; non-PROM group: 375.9 ± 69.4 pg/ml). In the third trimester, no significant difference was observed between the two groups (PROM group: 78.3 ± 33.8 pg/ml; non-PROM group: 141.0 ± 26.4 pg/ml). Data are presented as mean $\pm$ SE. * $P < 0.05$.

vaginal infection, and play multiple roles in the innate immune system, including immunocyte chemotaxis, cytokine generation, and wound healing.^{4,6} In 2007, Soto et al.¹² reported that amniotic HBD-2 concentrations were increased in patients with intra-amniotic inflammation. *Gardnerella vaginalis* and *Candida albicans* have also been reported to induce up-regulation of HBD-2 expression in chorioamniotic membranes.^{15,16} While colonic/gingival, corneal, and respiratory HBD-2 expressions are induced by stimulation from infections with various microbes and inflammation,^{9,10,17–19} some studies have shown that vaginal HBD-2 expression is not induced in BV patients.^{13,20,21} In the present study, HBD-2 levels were clearly decreased in patients with BV in the first trimester. Moreover, vaginal HBD-2 levels were not affected by the presence of *M. hominis*, *M. genitalium*, *U. urealyticum*, or *C. trachomatis*. On the other hand, vaginal candidiasis significantly increased vaginal HBD-2 levels. The difference between *Candida* and other microbes is the ability of the former to coexist with *Lactobacillus*. In the present study, seven of eight patients with *Candida* infection had normal Nugent scores. These results suggest that vaginal HBD-2 levels might be regulated by the presence of *Lactobacillus*. In other words, the presence of not only microbes but also *Lactobacillus* might be important in maintaining the vaginal environment.

Lactobacillus levels are known to be increased in the

vaginal flora of healthy pregnant women.¹⁴ On the other hand, levels of *Atopobium*, *Prevotella*, *Sneathia*, and *Gardnerella*, which are involved in the onset of BV, are decreased in pregnant women. The composition of the vaginal flora changes during pregnancy so as to prevent reproductive tract infection. Interestingly, in the present study, vaginal HBD-2 levels were decreased during late pregnancy. Vaginal HBD-2 is suspected to play an important role against the onset of BV early in pregnancy; however, many other factors may be involved in the defense mechanism against reproductive tract infection late in pregnancy. Unique immunological conditions exist during pregnancy. Monitoring the vaginal flora and levels of antibacterial peptides is thus important to assess antibacterial activity during pregnancy.

In the present study, all Nugent scores in the third trimester were within the normal range. Although five participants required hospitalization and treatment with intravenous ritodrine hydrochloride to avoid preterm delivery, no preterm delivery or PROM was observed. Meanwhile, vaginal HBD-2 levels in the first trimester were significantly lower in participants who later developed PROM. The management of patients with low HBD-2 levels during the first trimester might help prevent preterm delivery and PROM. Yet, the effect of interventions on perinatal outcome still remains clear. A larger sample size and evaluation of severe cases of threatened premature delivery will be necessary to clarify the implication of low HBD-2 levels in early pregnancy.

In conclusion, low vaginal HBD-2 levels during early pregnancy suggest the presence of a poor vaginal environment, as well as the possibility of developing PROM. Further investigation will be needed to evaluate the effect of HBD-2 on perinatal outcomes.

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Conflict of interest

None.

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