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Human Neutrophil Peptides in Vaginitis/Cervicitis of Different Etiology

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Abstract

Development of female genito-urinary infections depends on many factors, such as immune system activity, virulence of microorganism and production of factors inhibiting the growth of microorganisms. Taking into account the possibility of relapses or severe complications, it is very important to appropriately diagnose and treat infections. Because of recently observed increase of microbial resistance to antibiotics, researchers are looking for alternatives. In our study we evaluated and compared the concentration of human neutrophil peptides (HNP 1–3) in cervico-vaginal lavages (CVL), obtained from women with vaginitis/cervicitis. Swabs from the posterior vaginal fornix and from the endocervical canal as well as CVL samples were obtained from 32 patients with vaginitis/cervicitis and 29 healthy women (control group). Supernatants of CVL were used for determination of concentration of HNP by ELISA. The difference between concentrations of HNP 1–3 in studied and control groups was statistically significant ($p = 0.018$). The maximal concentration was determined in patients with mixed infections (28.41 ng/ml), and Group B Streptococci, GBS, (28.06 ng/ml), the minimal concentrations in cases of *C. trachomatis* (mean concentrations did not differ from those in the control group: 16.93 ng/ml and 16.39 ng/ml, respectively). Maximal correlation was determined for control-studied group with isolation of GBS ($r = 0.79$), and very high negative correlation for group of GBS – *C. trachomatis* ($r = -0.98$).

Key words: CVL, genito-urinary infections, HNP 1–3

Introduction

The female birth canal is rich in microorganisms. Infection starts when a pathogenic microorganism enters into the birth canal and dominates the physiologic microflora, breaking protective barriers and starting an inflammatory process. Vaginal environment (stable temperature, humidity, presence of nutrients, and low level of oxygen) is appropriate for the growth of microorganisms. Development of infection depends on many factors, such as virulence of microbes, immune system activity, production of different factors inhibiting growth of microorganisms and others.

In Poland about 35% of infections of female genito-urinary tract are caused by yeasts (Gołąb-Lipińska and Kurnatowska, 2001), especially *Candida albicans* (80–95%), urogenital mycoplasmas; *Ureaplasma urealyticum* – 20% of cases of NGU (Denys, 2006), and *Chlamydia trachomatis* – 20–40% (Choroszy-Król *et al.*, 2000; Zbroch *et al.*, 2004). Although group B streptococci (GBS) is a part of the physiologic microflora of vagina (colonization – 34%) the frequency of infection by this bacterium increases during inflammatory processes in the genito-urinary tract (Dybaś *et al.*, 2005).

Taking into account the possibility of relapses or severe complications as infertility, and possibility of transmission of infectious microorganisms to newborns, it is very important to appropriate diagnose and treat such infections. Because of recently observed increase of microbial resistance to antibiotics, researchers are looking for alternative treatment methods. Many investigators worked on AMPs (antimicrobial peptides) – small cationic peptides that have antimicrobial activity. Probably, AMPs can be used in the future as alternatives to antibiotics. For this reason it is very important to study which peptides and in which concentrations are produced locally as a response to microbial inflammation. The purpose of basic protective mechanisms of the human body is the

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localization and elimination of pathogenic microorganisms mainly by nonspecific mechanisms. Human neutrophil peptides, HNPs, are α-defensins produced in female birth canal against infectious agents. HNPs are localized in the azurophilic granules of neutrophils as the main proteins, participating in oxygen – independent phagocytosis of microorganisms (Ganz *et al.*, 1985). α-Defensins are part of host's natural antimicrobial immunity, responsible for the first line of defense against pathogenic microorganisms. Additionally they play an important role in acquired antimicrobial immunity through the production of specific antigens and promote maturation of dendritic cells (Yang *et al.*, 2002). *In vitro* HNPs demonstrate a cytolytic effect against bacterial strains, yeasts and viruses. This causes increasing permeability of microbial membrane, pore formation and outflow of ions and bigger molecules. HNPs can also competitively substitute divalent cations, which form bridges between lipopolysaccharide molecules (Hancock, 1997; Zasloff, 2002). It is possible that development of different mechanisms of co-influence with bacterial cell wall plays an important role among the properties of α-defensins against different microorganisms. Mechanisms of α-defensin actions are similar, but effects are different, depending on specific construction of target cell wall (Lynn *et al.*, 2004).

The aim of this study was to evaluate and compare the concentration of HNP 1–3 in cervico-vaginal lavages, obtained from women with vaginitis and cervicitis, caused by different etiological agents.

Experimental

Materials and Methods

Samples source. Samples taken from 61 non-pregnant women aged 19–40 (mean age 28.5) attending the Department of Medical Microbiology at the Medical University of Silesia, Katowice for diagnostic purposes were studied. The study group includes 33 patients (mean age 28.6) with symptoms of vaginitis/cervicitis (redness of vaginal and cervical epithelium and/or mucopurulent endocervical discharge and/or pain and contact bleeding) before antibiotic treatment. The control group includes 29 healthy women (mean age 28.4). All patients gave informed consent for this study.

Sampling procedure. Swabs from the posterior vaginal fornix and from the endocervical canal and also cervico-vaginal lavage samples were obtained from each patient for this study (Fig. 1). Gram stained microscopic slides were studied for bacterial vagi-

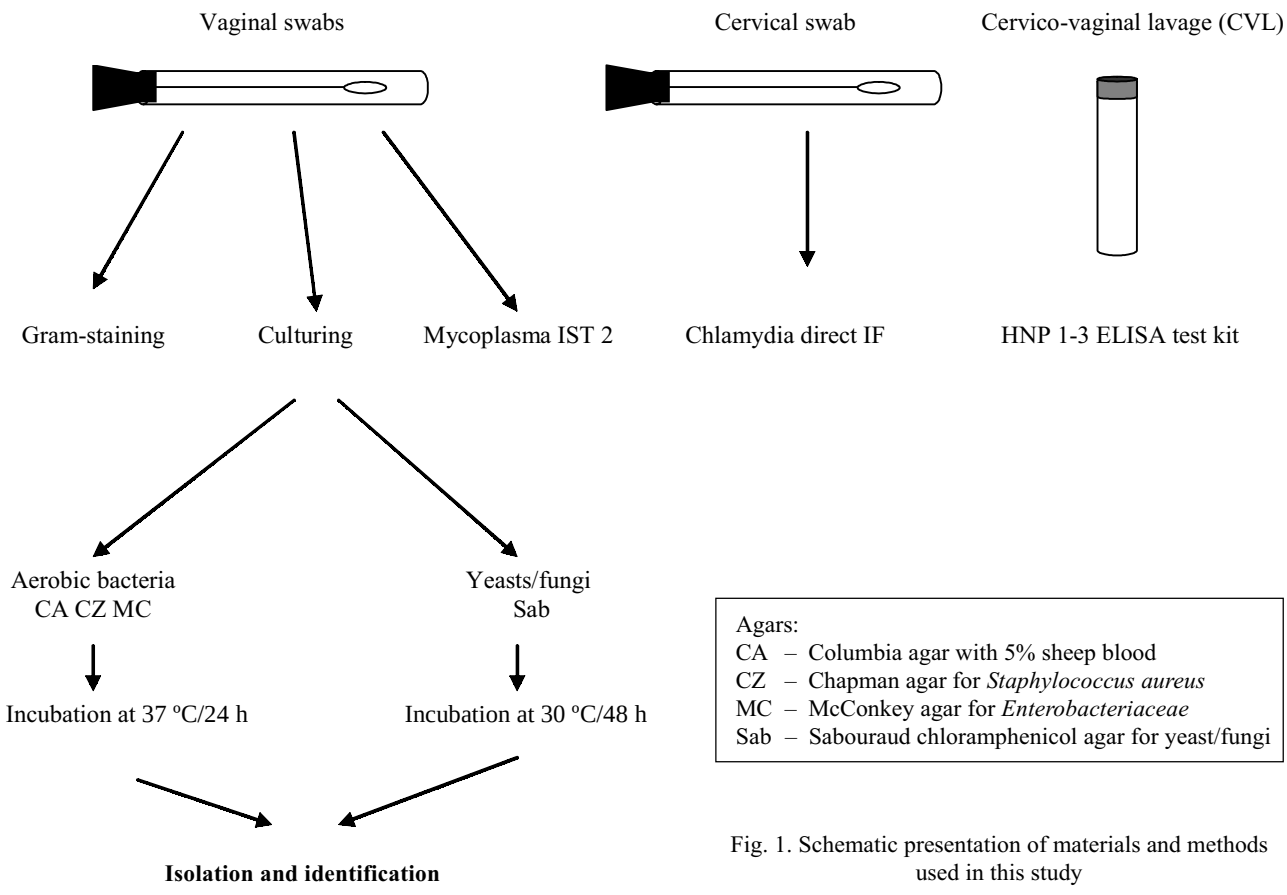

Fig. 1. Schematic presentation of materials and methods used in this study

nosis (BV) using Amsel and Nugent criteria and patients with BV were excluded from this study (Zbroch *et al.*, 2004).

Microorganisms culturing and identification. Vaginal swabs were used for Gram staining and microorganisms culturing. Culturing was performed according to routine microbiological practice. Typing of β-hemolytic streptococci was performed by Microscreen Strep (Microgen Bioproduct Ltd., UK). Yeasts and other fungi were identified by ID 32C test (bioMérieux, France). Identification of mycoplasmas was performed by Mycoplasma IST 2 (bioMérieux, France), and cervical Dacron swabs were used for Chlamydia Direct IF (bioMérieux, France) determining antigens of *Chlamydia trachomatis* according to the manufacturer's instructions.

HNP assay. Cervico-vaginal lavage samples were obtained by introducing 5 ml of PBS by sterile syringe followed by aspiration. Lavage-samples were centrifuged for 10 min at 1000 × g at 4°C. Supernatants were used for determination of concentration of HNP 1–3 by ELISA test kit (Cell Sciences, Inc., USA) according to the manufacturer's instructions by using spectrophotometer mQuant (Biotek Instruments Inc., USA) at λ 450 nm.
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Results

No statistically significant difference was observed between mean ages of women in the studied and control groups (28.6 and 28.4 respectively), also socio-economic conditions in both groups were comparable.

The studied group was divided into subgroups according to detected etiological agents: GBS (n = 5), *C. albicans* (n = 7), *C. trachomatis* (n = 6), *U. urealyticum* (n = 7) and mixed infections (n = 7). Etiologic agents of mixed infection are presented in Table I. In control group only microorganisms of physiological microflora were detected. In the studied group the maximal concentration of HNP 1–3 was found in patients with mixed infections (28.41 ng/ml) and in cases of GBS (28.06 ng/ml), but the minimal concentrations of HNPs were determined in cases of *C. trachomatis*, mean concentrations did not differ from those in the control group (16.93 ng/ml and 16.39 ng/ml, respectively) (Table II). The difference between concentrations of HNP 1–3 in studied and control groups was statistically significant (p = 0.018).

Maximal Pearson correlation index was determined for control group-studied group with isolation of GBS ($r = 0.79$), and very high negative dependence was determined for studied group with GBS and with *C. trachomatis* ($r = -0.98$). We demonstrated high correlation between number of neutrophils observed in microscopic smears and mean concentration of HNP 1–3, especially in control group (no statistically significant correlation) – Table III.

Table I
Etiologic agents and concentrations of HNP 1–3 in mixed infection subgroup

Etiologic agents	n	Concentration of HNP 1–3 (ng/ml)
GBS + *C. albicans*	1	35.84
GBS + *C. trachomatis* + *U. urealyticum*	1	31.24
C. albicans + *U. urealyticum*	1	28.71
C. albicans + *C. trachomatis* + *U. urealyticum*	1	26.42
C. albicans + *C. trachomatis*	3	25.56

Table II
Concentrations of HNP 1–3 in cervico-vaginal lavage of studied women

	n	Median ± SD (ng/ml)
Studied group	33	25.35 ± 13.55*
GBS	5	28.06 ± 14.59
Candida albicans	7	25.96 ± 11.63
Chlamydia trachomatis	6	16.93 ± 15.12
Ureaplasma urealyticum	7	24.66 ± 16.56
Mixed infection	8	28.41 ± 10.33
Control group	29	16.39 ± 13.53*

* statistically significant difference (p = 0.018)

Table III
Correlation between number of neutrophils observed in microscopic smear and mean concentrations of HNP 1–3 in cervico-vaginal lavage

Number of neutrophils	Studied group		Control group	
	Number	Median concentr. of HNP 1–3 (ng/ml)	Number	Median concentr. of HNP 1–3 (ng/ml)
>5	n = 10	29.23	n = 13	19.98
4–5	n = 11	24.11	n = 9	16.52
0–3	n = 12	23.24	n = 7	9.55

Neurophiles were counted at the magnification of 400 x

Discussion

In the beginning phase of infection neutrophils adhere to the surface of epithelial cells and their proteins determine the first defense against infection.

* **Statistics**. For statistical analysis the program Statistica 6.1 (Statsoft, USA) was used. This study was approved by the Bioethical Committee of Medical University of Silesia (NN-6501-113/04).

Infection process triggers morphological changes in epithelial cells (mainly in nucleus and cytoplasma). These changes usually depend on the type of infectious agent.

Our study demonstrated differences in expression of HNPs depending on isolated etiological agent, although further genetic studies are required for confirmation of these results. In the medical literature we did not find any data regarding the expression of HNP 1–3 in GBS infection, as well as mixed-infection. In cases of mixed infection concentrations of HNP 1–3 determined in our study evidenced increasing immune response, only in one case the level of HNP 1–3 was low – 6.45 ng/ml (Table I). Such high immune response to GBS infection may be connected with the fact, that GBS is also a part of vaginal physiological microflora. It was found that saprophytic microorganisms can induce defensin expression, but they also demonstrate relative tolerance against them. On the other hand, pathogenic microorganisms do not induce defensin expression and also can evade innate immune mechanisms and cause disease (Yeaman and Yount, 2003). Low concentration of defensins in women infected with *C. trachomatis*, observed in our study, confirms the results described by Wiesenfeld *et al.* (2002) and Porter *et al.* (2005), who described a lower concentration of HNP 1–3 in women with chlamydiasis compared with women infected by *Trichomonas vaginalis* or *Neisseria gonorrhoeae*. Studies of Yasin *et al.* (1996) demonstrated minimal role of α-defensins against *C. trachomatis.* It is also a well known fact that HNP 1–3 with other antimicrobial peptides, like LL-37, and HBD-1, acts as a synergistic barrier and kills pathogenic microorganisms (Tollin *et al.*, 2003). This mean that even low expression of HNP 1–3 by epithelial cells (lower than active concentration) also promotes development of immune mechanisms. A very active response was detected during *C. albicans* infection. Lehrer *et al.* (1988) and Raj *et al.* (2000) demonstrated that HNP-1 has a high antimycotic activity, but HNP-3 – very low. Structures of HNP-1 and HNP-3 differ by one amino-acid in N-terminus of peptide, which determines defensin properties (Oren and Shai, 1997). This was confirmed also in for other yeasts; *Cryptococcus neoformans* is inhibited by HNP-1 and HNP-2 (Ganz *et al.*, 1985), but intracellular growth of *Histoplasma capsulatum* in murein of macrophages is inhibited by xenogenic expression of HNP-1 (Couto *et al.*, 1994; Salzman *et al*., 2003).

There are no reports on the effect of HNP 1–3 on urogenital mycoplasmas. In our study we demonstrated similar results for *U. urealyticum* as in the case of yeasts. We didn't observe correlation between defensins level and age of woman. Similar results were obtained by Wiesenfeld *et al.* (2002). They demonstrated lack of correlation between level of HNP and hormonal anticonception, phase of menstrual cycle and use of condoms. Increasing of NHP 1–3 concentration is a response to inflammation by neutrophil and epithelial cells. Valore *et al.* (2002) demonstrated that because antimicrobial effect of vaginal proteins depends on concentrations, increasing of neutrophil defensisns concentration contributes to host defense. It is also very important that these peptides can act synergistically with other factors, such as LL-37 (Nagaoka *et al*., 2000). On the other hand many microorganisms developed mechanisms to evade bacteriocidal antimicrobial molecules (Ganz, 2001). In the opinion of Lynn *et al.* (2004) probability, that these mechanisms stimulate adaptive evolution of α-defensins is very high. For example, pathogens *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Streptococcus pyogenes* produce sulfur compounds, which bind and neutralize HNP-1 (Schmidtchen *et al.*, 2001), but protein SIC (streptococcal inhibitor of complement) inhibits antibacterial activity of several antimicrobial peptides, like lizozyme, SLPI, LL-37, HNP-1 and β-defensins 1, 2 and 3 (Fernie-King *et al.*, 2006).

We have shown that the ability of interaction between AMPs and microorganisms changes host response to infection depending mainly on structure and properties of etiological agent. Further *in vivo* and *in vitro* studies of interactions of AMPs with different microorganisms are required to shed light on the possibility of using them against antibiotic-resistant microorganisms.

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