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Increased mRNA expression of selected antimicrobial peptides around ovulation and during inflammatory processes in the bovine endometrium postpartum

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ABSTRACT

In the uterus, the first pathogen confrontations take place at the luminal endometrial epithelium. Therefore, it is required that these cells have the potential to recognize and respond to a bacterial infection. Antimicrobial peptides (AMP), part of the innate immune system in addition to cytokines, are principal effector molecules of mucosal immunity against pathogens. One important family of AMP that can permeabilize bacterial membranes is the beta-defensin (DEFB) family, which includes the following members: DEFB1, DEFB4A, and DEFB5, lingual AMP, and tracheal AMP. The bactericidal/permeability-increasing protein is also a cationic AMP that results in the death of bacteria. Another AMP family is the S100 calcium-binding protein (S100A) family including the following members: S100A8, S100A9, S100A11, and S100A12. These AMP exert their antimicrobial action through chelation of several ions. The aim of the present study was to evaluate mRNA expression patterns of selected AMP in bovine endometrial cells collected (1) at different stages of the estrous cycle (postovulatory, early-to-mid luteal, late luteal, and pre-ovulatory phase); (2) during the puerperium depending on uterine health status (healthy, subclinical, or clinical endometritis) starting on Day 24 to 30 postpartum for 3 weeks on a weekly basis; and (3) *in vitro* after co-culturing with *Bacillus pumilus* at three different multiplicities of infection (MOI 1, 5, and 10) up to 6 hours. The results reported that the mRNA expression of all candidate AMP, except *DEFB1*, *S100A8,* and *S100A9*, was estrous cycle dependent. In particular, around the time of ovulation, the transcription level of most AMP was higher ($P < 0.05$) compared with the luteal phase. Almost all candidate AMP mRNA expression was dependent on uterine health status, with a higher transcription level ($P < 0.05$) in inflamed endometrial tissues, especially during the late stage of the puerperium (Day 45–51 postpartum). Members of the DEFB family were nearly unaffected in their mRNA expression in primary endometrial cells co-incubated with *B. pumilus*. However, *S100A8* and *S100A9* mRNA contents were higher after 4 and 6 hours of co-incubation with *B. pumilus* compared with untreated controls. In conclusion, higher mRNA expression of the candidate AMP around ovulation or in inflamed endometrial tissue during the puerperium suggests their crucial role in uterine innate immunity in the defense against invading bacteria.

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1. Introduction

The puerperium is a crucial period for dairy cows to sustain their fertility. After calving, up to 85% of uteri become contaminated by a wide range of bacterial strains including pathogenic bacteria, such as *Escherichia coli* and *Trueperella pyogenes* [1]. During the ongoing postpartum (PP) involution process, some cows show an appropriate immune response. These cows eliminate the bacteria and are able to conceive again. However, about 40% of cows develop metritis within the first week after parturition, which persists as endometritis for several weeks in about 25% of cows [2]. The effect of *E. coli* and *T. pyogenes* on bovine endometrial epithelial cells has been studied extensively to reveal mechanisms of bacterial infection and inflammation [3–5]. Nevertheless, the role of potentially pathogenic uterine bacteria like *Bacillus* spp. is still unclear regarding the development of uterine disease and their influence and interaction within a healthy uterus. *Bacillus* spp. were isolated from healthy and diseased bovine uteri with a frequency of 10% and half of the strains belonged to the species *B. pumilus* [6]. It has been reported that *B. pumilus* caused cell death and increased the mRNA expression of pro-inflammatory factors in bovine endometrial cells *in vitro* [7]. Potentially pathogenic bacterial species may be useful for examining immunologic and clearance processes in the endometrium because they do not cause cell death in co-culture as rapidly as pathogenic bacteria.

The luminal endometrial epithelium is considered the first line of defense against invading bacteria by providing a physical barrier with a mucus lining. In addition, endometrial epithelial cells can initiate an immune response to a bacterial infection through Toll-like receptors [8]. This leads to the increased production of pro-inflammatory cytokines, prostaglandins, and antimicrobial peptides (AMP).

As part of the innate immune system, AMP are widely expressed in various tissues and cell types in almost all species, which is evidence for their important role as effective weapons against bacteria [9]. Antimicrobial peptides prevent and/or reduce infection by killing microorganisms or inhibiting their growth [10,11].

On the basis of the structure and mechanism of action, AMP can be divided into diverse classes. Defensins represent one of the most important classes of AMP. Because of their cationic and hydrophobic clusters, they cause the permeabilization of bacterial membranes resulting in bacterial cell death [10]. The bovine beta-defensin (DEFB) family includes DEFB1, DEFB4A, and DEFB5, lingual antimicrobial peptide (LAP), and tracheal antimicrobial peptide (TAP) [12].

In addition, bactericidal/permeability-increasing protein (BPI) is also a cationic AMP with high affinity for lipopolysaccharide (LPS) in the outer membrane of bacterial cells. Binding of BPI to living bacteria results in growth arrest and damage of the bacterial membrane [13].

Another class of AMP is the S100A protein family, whose members exert their antimicrobial action through chelation of several ions required for microbial growth [14]. Moreover, they are calcium-binding proteins and are involved in different physiological calcium-dependent cellular processes, such as contraction, motility, cell growth, differentiation, and structural organization of membranes [15].

Antimicrobial peptides were also described as part of the bovine endometrial innate immune system. Several AMP were expressed in healthy bovine endometrial tissue and in inflamed endometrial tissue during the early puerperium and in endometrial cells *in vitro* [16,17]. Furthermore, primary bovine endometrial cells reported an upregulation of gene transcription for *LAP*, *TAP*, *S100A8*, *S100A9,* and *S100A12* in response to LPS or *E. coli* stimulation [5,18].

This leads to the hypothesis that AMP play an important role in the bovine uterus in protection against bacterial infections, which likely occurs during the puerperium. In addition, AMP may be involved in the immune response during estrus, when the cervix is open and bacteria can invade during mating or artificial insemination.

Therefore, the aim of this study was to elucidate processes within the bovine endometrium for a better understanding of the innate immune response. The objectives were to evaluate the mRNA expression patterns of candidate AMP in bovine endometrial epithelium samples collected (1) at different stages of the estrous cycle, (2) during the postpartum period in cows with and without a uterine infection, and (3) during *in vitro* short-time co-culturing with the Gram-positive strain *B. pumilus* to reveal defense mechanisms against a bacterial infection.

2. Materials and methods

2.1. Collection of endometrial epithelium samples

Bovine endometrial epithelial cells for the study of estrous cycle-dependent mRNA expression were collected as previously described [19]. Briefly, uteri were harvested from cows at a local abattoir within 20 to 30 minutes after slaughtering. Uteri showing any signs of pathologic conditions or pregnancy were excluded. Obtained uteri were classified according to the utero-ovarian appearance into one of the following 4 groups ($n = 8$ for each group) [20,21]: postovulatory (Day 1–5), early-to-mid luteal (Day 6–12), late luteal (Day 13–18), and pre-ovulatory phase (Day 19–21). Luminal endometrial epithelial cells were harvested from three different regions of dissected uteri (corpus, ipsilateral, and contralateral horn) using a cytobrush (Celltip; Mediware, Wesel, Germany). Each region was brushed at two different sites, and collected cells were preserved in RNAlater (Sigma, Deisenhofen, Germany) until extraction of total RNA. In addition, one cytobrush from the corpus of each uterus was used to prepare a cell smear on a glass microscope slide for cytologic analysis to determine the percentage of polymorphonuclear neutrophils (PMN); 29 samples did not reveal any presence of PMN and only three samples reported a countable number of PMN (<2%). Therefore, all uteri were considered to be healthy.

For the study of health status–dependent mRNA expression, luminal endometrial epithelial cells were collected *in vivo* by the cytobrush technique as previously described [19]. One sample served for RNA analysis and was transported to the laboratory in liquid nitrogen. The second