Pferdeheilkunde 33 (2017) 3 (May/June) 216-222 DOI 10.21836/PEM20170301

Expression of NOD1 and NOD2 transcripts in the healthy and diseased equine endometrium

Sandra Schöniger[1], Hilke Gräfe[1], Franziska Richter[2], Christin Helmschrodt[2] and Heinz-Adolf Schoon[1]

[1] Institute of Pathology, Faculty of Veterinary Medicine, Leipzig University, Leipzig, Germany
[2] Institute of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Leipzig University, Leipzig, Germany

Summary: In mares, endometrial diseases are the main cause of reduced fertility and have a high financial impact on equine breeding. Of particular importance are subclinical diseases, since they can only be diagnosed by the histological examination of an endometrial biopsy. Moreover, the precise pathogenesis of some frequent subclinical diseases, i.e. nonsuppurative endometritis and endometrosis (periglandular fibrosis), is unknown. The reason for the increased susceptibility of some mares to develop a persistent postbreeding endometritis (PPBE) is also still unsolved. The nucleotide-binding oligomerization domain proteins 1 and 2 (NOD1 and NOD2) are innate immunity receptors of epithelial and antigen presenting cells. On the one hand, they defend against intracellular bacteria and thus help to prevent and clear tissue infections. On the other hand, their altered expression can contribute to tissue inflammation and fibrosis. It has been shown that NOD1 and NOD2 are also involved in the pathogenesis of uterine diseases in women and other species. To the best of the authors' knowledge, no data are published in regard to the expression of NOD1 and NOD2 in the equine endometrium. The aim of this study was to examine if NOD1 and NOD2 transcripts are expressed in the healthy and diseased equine endometrium. Endometrial samples of 31 adult mares were investigated by PCR and histology. The former was performed in comparison on native and formalin-fixed paraffin-embedded (FFPE) samples. By PCR, transcripts of NOD1 and NOD2 were detected in native and FFPE endometrial samples of all 31 mares. The morphology of endometrial glands was secretory (n = 19), proliferative (n = 6) or in transition between both stages (n = 6). Five tissue samples had no pathological alterations; two of these were from a gravid mare. The remaining showed one or several endometrial diseases including different forms of endometritis (n = 12), endometrosis (n = 20), angiosclerosis (n = 20), perivasculitis and vasculitis (n = 1), infiltration with neoplastic lymphocytes (n = 1) and placental retention (n = 1). Results of this study are consistent with a constitutive expression of NOD1 and NOD2 transcripts during all stages of the endometrial cycle within the healthy and diseased equine endometrium. They imply a functional importance of NOD1 and NOD2 in the equine endometrium for the regulation of physiological processes as well as the pathogenesis of endometrial diseases. In human beings, certain polymorphisms and mutations of NOD genes have been identified as predisposing factors for chronic inflammation. Thus, it appears possible that NOD gene polymorphisms may also contribute to the resistance or susceptibility of mares to develop PPBE. This study provides the basis for future investigation into the contribution of NOD1 and NOD2 to the pathogenesis of equine endometrial diseases. The results of those future studies may reveal information that is beneficial for the prevention and treatment of equine endometrial diseases.

Keywords: endometrium, equine, mare, NOD1, NOD2, PCR, transcripts, Reproduktion

Citation: Schöniger S., Gräfe H., Richter F., Helmschrodt C., Schoon H. A. (2017) Expression of NOD1 and NOD2 transcripts in the healthy and diseased equine endometrium. Pferdeheilkunde 33, 216-222; DOI 10.21836/PEM20170301

Correspondence: Dr. Sandra Schöniger, Institute of Pathology, Faculty of Veterinary Medicine, University Leipzig, An den Tierkliniken 33, 04103 Leipzig, Germany; E-Mail: sandra.schoeniger@vetmed.uni-leipzig.de

Introduction

In mares, endometrial diseases are the main cause of reduced fertility and have a high financial impact on equine breeding. Of particular importance are subclinical diseases, since they can only be diagnosed by the histological examination of an endometrial biopsy (*Schoon* et al. 1997). The precise pathogenesis of most of these subclinical diseases, i.e. nonsuppurative endometritis, endometritis eosinophilica as well as endometrosis (periglandular fibrosis) is unknown (*Schoon* et al. 1997). The reason for the increased susceptibility of some mares ("susceptible mares") to develop a persistent endometritis after breeding or insemination (PPBE) is also still unknown (*Katila* 2012).

The nucleotide-binding oligomerization domain-containing proteins 1 and 2 (NOD1 and NOD2) are receptors that are part of the afferent arm of the innate immunity and are located in the cytoplasm of epithelial cells and antigen presenting cells (*Inohara* and *Nuňez* 2003, *Strober* et al. 2006, *Rosenstiel* et al. 2008). Their main functions are the prevention and clearance of intracellular bacterial infections (*Inohara* and *Nuňez* 2003). They bind degradation products of bacterial cell wall peptidoglycans (*Inohara* and *Nuňez* 2003, *Strober* et al. 2006) and initiate an inflammatory response (*Inohara* and *Nuňez* 2003, *Strober* et al. 2006) or pyroptosis (*Inohara* and *Nuňez* 2003). NOD1 is activated by g-D-glutamyl-meso-diaminopimelic acid (iE-DAP) of mostly Gram-negative bacteria (*Strober* et al. 2006, *Rosenstiel* et al. 2008). The ligand of NOD2 is muramyl dipeptide (MPD), a cell wall component of Gram-positive and Gram-negative bacteria (*Inohara* and *Nuňez* 2003, *Strober* et al. 2006). NOD1 and NOD2 also recognize parts of molecules released during tissue damage, i.e. danger associated molecular patterns (DAMPS, *Tang* et al. 2012). The altered expression or continuous stimulation of NOD1 and NOD2 may lead to persistent inflammation or tissue fibrosis (*Lafyatis* and *Farina* 2012).

NOD1 and NOD2 transcripts were identified in the non-pregnant endometrium of women (*Hart* et al. 2009) and NOD1 transcripts were detected in the bovine endometrium (*Herath* et al. 2000). Further, in the human placenta, these receptors are expressed as transcripts and proteins in tro-

phoblast cells (*Costello* et al. 2007). Besides mediating the protection of the embryo against bacterial infections, NOD receptors are also involved in the pathogenesis of pregnancy complications such as preterm labour and intrauterine growth restriction (*Abrahams* 2008).

To the best of the authors' knowledge, no published data exist so far on the presence of NOD1 and NOD2 in the equine endometrium. The hypothesis of this investigation is that in the equine endometrium NOD1 and NOD2 also contribute to physiological parameters and the pathogenesis of diseases. The aim of this study was to examine the expression of NOD1 and NOD2 transcripts in the healthy and diseased equine endometrium. Results of this investigation will increase the basic knowledge on the innate immunity of the equine endometrium. Further, they are a requirement for further studies into the possible contribution of innate immune mechanisms to endometrial health and disease in the mare.

Materials and methods

Endometrial tissue samples used for PCR and histology

Endometrial tissue samples were collected from 31 mares that were euthanized due to other reasons than a clinically evident reproductive disorder 1 hour to 3 hours after their death. Mares were between 1.5 and 21 years of age. The average age was 12 years. Mares were of different breeds including 10 Warmblood horses, 6 Haflinger, 2 Thoroughbred horses, 2 draft horses and 1 Shetland pony. The age of 6 mares and the breed of 10 mares were not mentioned in the clinical history. From each mare, two tissue samples were collected. One of these was native frozen in liquid nitrogen and stored at -80 °C until processing for RNA isolation. The other tissue sample was fixed in 10% buffered formalin and embedded in paraffin wax.

RT-PCR for the detection of NOD1 and NOD2

The predicted mRNA sequences were received from NCBI Genbank (www.ncbi.nlm.nih.gov/Genbank). Primers for NOD1 and NOD2 were designed by the use of the program primer 3 (Table 1). The housekeeping gene glycerinaldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as well. The primer for the detection of GAPDH was obtained from the literature (*Klein* et al. 2011). RNA isolation from native and FFPE tissues, reverse transcription, thermocycling as well as the electrophoretic separation of PCR products and their visualisation were performed as described by *Schöniger* et al. (2013). For cDNA amplification (GAPDH, NOD1 and NOD2) 25 µl of the reaction mixture and the PTC 200 Thermocycler (MJ Research, St. Bruno, Canada) were used. The reaction was composed of 1 µl of the generated cDNA, 0.2 mM dNTP mix (Roche Diagnostics GmbH, Mannheim, Germany), 0.4 µM sense and antisense primers (Eurofins MWG Operon, Ebersberg, Germany), PCR buffer with 1.5 mM MgCl (Roche Diagnostics GmbH) and 0.5 U Taq DNA polymerase (Roche Diagnostics GmbH). In the negative controls, the cDNA was substituted by diethyl dicarbonat treated water. All primer pairs were applied to native tissue. For FFPE tissue, the primer pair NOD1-1 (Table 1) was omitted, since in FFPE material the mRNA is degraded into smaller fragments (*Klopfleisch* et al. 2011). The mRNA quality of representative RNA isolates from 9 native endometrial tissue samples (Nos. 1, 6, 14, 16, 18, 19, 24 and 27) was measured by the use of a bioanalyzer (Agilent). The obtained RNA integrity numbers ranged from 7.9-9.3. Photometric measurements (260/280 nm) ensured purity of RNA samples prior to cDNA synthesis.

Processing of tissue samples for histology

Tissue samples were processed routinely, sectioned at 2 µm, placed on glass slides and stained with haematoxylin-eosin (H.E.) and a Picrosirius red stain. The H.E.-stained sections were evaluated for the functional morphology of endometrial glands and the presence of endometritis, endometrosis and angiosclerosis (*Kenney* and *Doig* 1986, *Schoon* et al. 1992, 1997, *Schöniger* et al. 2013). Endometritis was further subclassified into acute and subacute suppurative inflammation, nonsuppurative forms as well as endometritis eosinophilica (*Schoon* et al. 1997, *Schöniger* et al. 2013). If the endometritis was restricted to the stratum compactum, it was diagnosed as superficial endometritis. Endometrosis was subdivided into destructive and nondestructive forms (*Schoon* et al. 1997). Picrosirius red stained sections were used to confirm the severity of endometrosis as well as the degree of arterio- and phlebosclerosis.

Table 1 Primer pairs for the detection of NOD1, NOD2 und GAPDH in the equine endometrium

Gene	Primer designation	Primer sequence	Amplicon size (bp)	Annealing temp	Genbank accession
NOD1	NOD1-1	F: ATGTGGGAGCCAGGTACATC R: CACATCCCACCTCAAAGAT	151	58 °C	XM_005609279.1
NOD1	NOD1-2	F: TCCACACATCCGGAACACT R: TACCAGGTCCAGGATTTTGC	134	58 °C	XM_005609279.1
NOD2	NOD2-1	F: ATTCACCCTCAAGGGCTTCT R: CTCTGAGCAGGGAGTGAGG	101	58 °C	XM_001915288.2
NOD2	NOD2-2	F: AGCCTGTCGTATCACCCATC R: GCAACAAACTTAGGGTGGA	105	60 °C	XM_001915288.2
GAPDH	GAPDH*	F: AGAAGGAGAAAGGCCCTCAG R: GGAAACTGTGGAGGTCAGGA	87	54 °C	NM_001163856

NOD1: nucleotide-binding oligomerization domain-containing protein 1; NOD 2: nucleotide-binding oligomerization domain-containing protein 2
GAPDH: glycerinaldehyde 3-phosphat dehydrogenase; F: forward primer; R: reverse primer; bp: base pairs temp: temperature; **Klein* et al. (2011)

Results

Detection of NOD1 and NOD2 transcripts in the healthy and diseased equine endometrium: The results of the PCR and histology are listed in Table 2. Representative PCR data and histological findings are illustrated in Figures 1 and 2, respectively.

By the use of all applied primer pairs, transcripts of NOD1 and NOD2 were retrieved from native and FFPE endometrial tissue samples of all 31 mares. False positive results were ruled out by the absence of amplicons in the negative controls (replacement of cDNA by diethyl dicarbonat treated water). In parallel, the housekeeping gene GADPH was amplified from all examined native and FFPE tissue samples as well.

The functional morphology of endometrial glands was secretory (n = 19), proliferative (n = 6), proliferative-secretory (n = 5) and secretory-proliferative (n = 1). The endometrium of five mares had no evidence of endometrial diseases; two of these mares were gravid. One mare had a retained placenta. Twelve mares displayed endometritis. It was nonsuppurative in seven mares, acute suppurative in two mares and subacute suppurative in two mares; in these mares the inflammation was mostly superficial (n = 7) and mild (n = 9). The remaining mare with endometrial inflammation showed a superficial diffuse endometritis eosinophilica. Endometrosis was diagnosed in 20 mares; it was mild in 10 mares and moderate in the other 10 mares. The destructive form was observed in 4 mares, whereas the remaining mares with

Table 2 Expression of NOD1 and NOD2 transcripts in the healthy and diseased equine endometrium (31 mares).

Nos	GAPDH	NOD1	NOD2	Glands	E-metritis	E-metrosis	A-sclerosis	P-sclerosis	Additional F
1	X	X (N, F)	X (N, F)	prolif	-	X (++)	-	-	-
2	X	X (N, F)	X (N, F)	prolif-secr	-	-	-	-	-
3	X	X (N, F)	X (N, F)	prolif-secr	-	-	-	-	-
4	X	X (N, F)	X (N, F)	secr	-	-	-	-	-
5	X	X (N, F)	X (N, F)	secr	-	-	-	-	X (G)
6	X	X (N, F)	X (N, F)	secr	-	X (+)	X (+++)	X (+++)	
7	X	X (N, F)	X (N, F)	secr	X (LP, s, +)	-	X (+)	X (++)	-
8	X	X (N, F)	X (N, F)	prolif	-	X (++)	-	X (+)	X (LL)
9	X	X (N, F)	X (N, F)	secr	-	X (++)	X (++)	X (++)	-
10	X	X (N, F)	X (N, F)	prolif	X (AS, s, +)	X (+)	X (+)	-	-
11	X	X (N, F)	X (N, F)	secr	-	-	-	-	X (G)
12	X	X (N, F)	X (N, F)	prolif-secr	-	X (d, ++)	X (++)	X (++)	-
13	X	X (N, F)	X (N, F)	proli	X (SAS, +)	X (+)	X (+++)	X (++)	-
14	X	X (N, F)	X (N, F)	proli	X (LP, s,+)	X (+)	-	X (++)	-
15	X	X (N, F)	X (N, F)	secr	X (E)	X (d, ++)	X (+++)	X (+++)	-
16	X	X (N, F)	X (N, F)	secr	-	-	-	-	X (PVAS)
17	X	X (N, F)	X (N, F)	secr	-	X (++)	X (++)	-	-
18	X	X (N, F)	X (N, F)	secr	X (LP, +)	X (d, +)	-	-	-
19	X	X (N, F)	X (N, F)	secr	X (LP, s, +)	X (+)	X (+)	-	-
20	X	X (N, F)	X (N, F)	secr	-	X (+)	X (++)	X (+++)	-
21	X	X (N, F)	X (N, F)	secr	-	X (+)	-	-	-
22	X	X (N, F)	X (N, F)	secr	X (LP, s, +)	-	-	X (+)	-
23	X	X (N, F)	X (N, F)	secr	-	-	X (++)	X (++)	X (RP)
24	X	X (N, F)	X (N, F)	prolif	X (LP, s, +)	-	X (++)	X (+)	-
25	X	X (N, F)	X (N, F)	prolif-secr	-	X (d, ++)	X (++)	X (+)	-
26	X	X (N, F)	X (N, F)	prolif-secr	X (AS, s, ++)	-	-	-	-
27	X	X (N, F)	X (N, F)	secr-prolif	X (LP, +)	X (++)	X (++)	X (++)	-
28	X	X (N, F)	X (N, F)	secr	-	X (++)	-	-	-
29	X	X (N, F)	X (N, F)	secr	-	X (+)	X (+)	-	-
30	X	X (N, F)	X (N, F)	secr	-	X (++)	X (+++)	X (++)	-
31	X	X (N, F)	X (N, F)	secr	X (SAS, +++)	X (+)	X (+++)	X (+++)	-

Nos: numbers of mares; GAPDH: glycerinaldehyde 3-phosphat-dehydrogenase; NOD1: nucleotide-binding oligomerization domain-containing protein 1; NOD2: nucleotide-binding oligomerization domain-containing protein 2; E-metritis: endometritis; E-metrosis: endometrosis A-sclerosis: arteriosclerosis; P-sclerosis: phlebosclerosis; Additional F: additional findings; X: present; -: absent; N: native tissue ; F: formalin-fixed paraffin-embedded tissue; prolif: proliferative; secr: secretory; secr-prolif: transition from a secretory to a proliferative glandular function; prolif-secr: transition from a proliferative to a secretory glandular function; +: mild; ++: moderate; +++: marked ; LP: lymphoplasmacellular; AS: acute suppurative; SAS: subacute suppurative; s: superficial; d: destructive; G: gravidity; LL: lymphocytic leukaemia; PVAS: perivasculitis and vasculitis; RP: retained placenta

endometrosis had the nondestructive type. Mild, moderate or marked arterio- and/or phlebosclerosis were diagnosed in 20 mares. The endometrium of one mare showed a lymphohistiocytic vasculitis and perivasculitis, and the endometrium of another mare was infiltrated by neoplastic lymphocytes due to a lymphocytic leukaemia.

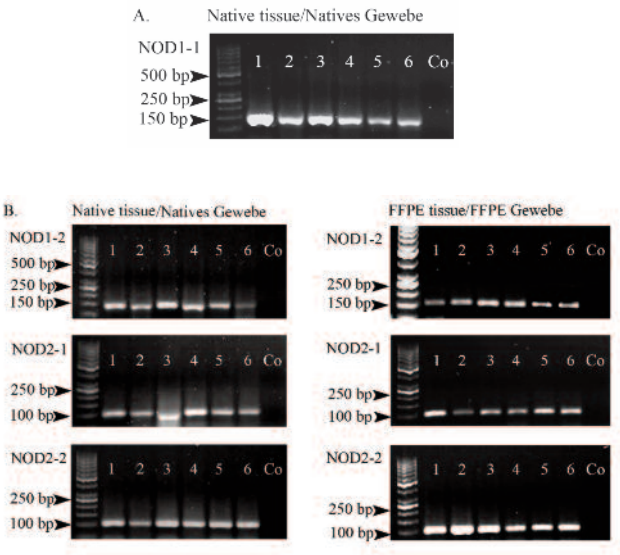

Fig. 1 PCR investigations into the expression of nucleotide-binding oligomerization domain containing proteins 1 and 2 (NOD1 and NOD2) in the equine endometrium. Native tissue and formalin-fixed paraffin-embedded (FFPE) samples were used. For the detection of NOD1 and NOD2 different primer pairs were applied designated as NOD1-1, NOD1-2, NOD2-1 and NOD2-2. **A**. The primer pair NOD1-1 was only applied to native tissue. **B.** The remaining primer pairs (NOD1-2, NOD2-1 and NOD2-2) were applied to native and FFPE tissue. By the use of the applied primer pairs, transcripts of NOD1 and NOD2 were detected within all examined tissue samples of the healthy and diseased endometrium from different stages of the endometrial cycle. Depicted are representative results from the endometria of 6 mares with different histologic findings (Nos. 1–6). In the negative controls (Co), the cDNA was replaced by diethyl dicarbonat treated water. Mare 1: Endometrium with secretory glands and a lymphohistiocytic perivasculitis and vasculitis. Mare 2: Endometrium with secretory glands and moderate endometrosis. Mare 3: Secretory morphology of endometrial glands, mild superficial lymphoplasmacellular endometritis, mild arteriosclerosis and moderate phlebosclerosis. Mare 4: Endometrial glands in transition from the proliferative to the secretory stage and moderate acute suppurative superficial endometritis. Mare 5: Endometrium with secretory glands, marked subacute suppurative endometritis, mild endometrosis as well as marked arteriosclerosis and phlebosclerosis. Mare 6: Secretory endometrial glands, moderate arteriosclerosis and phlebosclerosis as well as retention of the placenta.

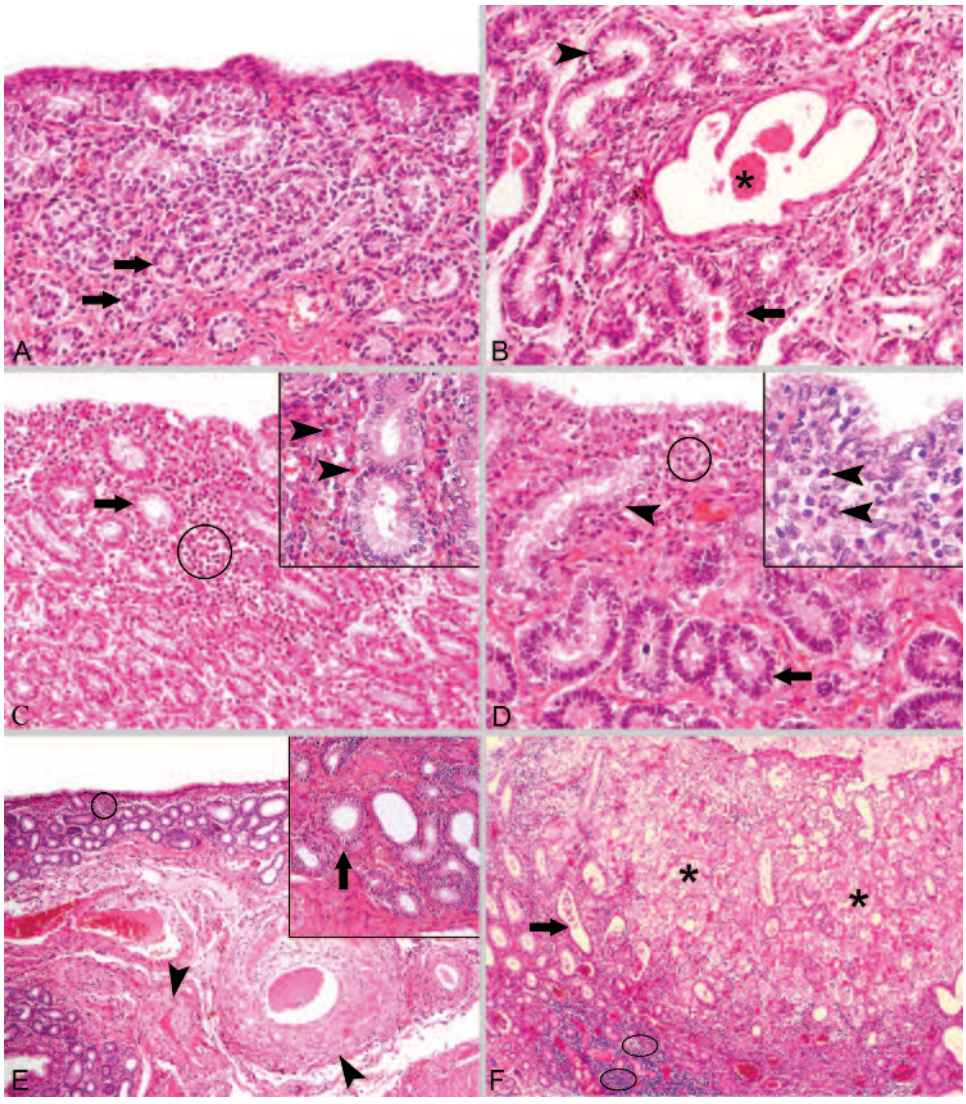

Fig. 2 Histological findings of equine endometrial tissue samples with the detection of NOD1 and NOD2 transcripts. Haematoxylin and eosin stain. **A)** Healthy endometrium with secretory glands (arrows). **B)** Endometrium with glands in transition from a proliferative (arrow) to a secretory stage (arrowhead) and moderate destructive endometrosis (asterisk). Depicted is a moderately dilated endometrial gland (asterisk) with periglandular fibrosis and multifocal loss of the lining epithelium (destructive endometrosis). **C)** This endometrium has secretory glands (arrow) and multifocal to coalescing moderate nonsuppurative endometritis; the circle marks the lymphoplasmacellular infiltrates. Inset: Endometrial sample of another mare with endometritis eosinophilica characterized by the presence of numerous eosinophils (arrowheads) within the stroma. **D)** Moderate superficial acute suppurative endometritis (circle). Endometrial glands show a transition from a proliferative (arrow) to a secretory (arrowhead) functional morphology. Inset: The infiltrating neutrophils are depicted at a higher magnification (arrowheads). **E)** Within the endometrium, there is marked angiosclerosis (arrowheads), marked subacute suppurative endometritis (circle) as well as mild nondestructive endometrosis (inset, arrow). **F**) Depicted is an endometrial cup (asterisks) of a gravid uterus. Densely packed trophoblast cells are surrounded by dilated glands (arrow) and densely packed lymphocytes and fewer plasma cells, macrophages as well as neutrophils and eosinophils (circle).

Discussion

This study demonstrates that the equine endometrium is equipped with transcripts of NOD1 and NOD2 receptors as innate defence mechanisms that specifically recognize cytosolic PAMPs of Gram-positive and Gram-negative bacteria. The obtained results indicate a constitutive expression of NOD1 and NOD2 transcripts in the healthy and diseased equine endometrium over the entire endometrial cycle. Examined endometria were considered as representative (qualitatively and quantitatively) for the types of inflammatory and degenerative diseases that are predominantly observed in endometrial biopsies submitted for diagnostic evaluation (*Schoon* et al. 1997, *Ebert* et al. 2014); they even included a case of the rare endometritis eosinophilica (*Schoon* et al. 1997). Similar to endometrial biopsies submitted for routine microscopic evaluation and prognostication of fertility, they often showed the concurrent presence of two or more endometrial diseases (*Schoon* et al. 1997). In addition, it was confirmed that NOD1 and NOD2 transcripts are also expressed in the placenta.

In the endometrium of mares, there is evidence for a constitutive expression of transcripts of other NLR family members (*Marth* et al. 2015) and additional components of the innate immunity, i.e. TLRs (*Atli* et al. 2010, *Schöniger* et al. 2016) and beta-defensin (*Schöniger* et al. 2013). Transcript levels of these other NLR family members (*Marth* et al. 2015) and different TLRs, however, are influenced by the stage of the endometrial cycle (*Atli* et al. 2010, *Marth* et al. 2015), bacterial infections (*Marth* et al. 2015) and/or the presence of endometritis (*Siemieniuch* et al. 2016). Based on these findings, it appears likely that in the equine endometrium the expression of NOD1 and NOD2 is also modulated by hormonal factors and/or bacterial infections. RNA input quantity and quality were similar for all native tissue samples in the present study, but gel electrophoresis indicated varying band widths and densities (Figs. 1A and B), suggesting different quantities of PCR products and thus transcript expression. Quantitative PCR analyses in healthy versus diseased conditions will confirm this qualitative impression in future studies. It has further considered that cellular functions of these molecules are dependent on their translation into proteins and transcript and protein levels do not necessarily correlate (*Silva* et al. 2012). Thus, NOD1 and NOD2 protein expression has to be subsequently investigated as well.

Common causes of endometritis in mares are Streptococcus species and E. coli (*Wittenbrink* et al. 2008). These are facultative intracellular pathogens of epithelial cells and/or macrophages (*Molinari* and *Chhatwal* 1999, *Sheldon* et al. 2010, *Mayer* et al. 2011, *Xu* et al. 2016). In comparison, Chlamydia species represent obligate intracellular pathogens of these cell populations (*Darville* and *Hiltke* 2009) and possible causative agents of infertility in mares (*Hülsey* 2001). These intracellular pathogens activate NOD receptors and their associated signal transduction (*Mukura* et al. 2012, *Lapaquette* et al. 2012, *Kavathas* et al. 2013, *Liu* et al. 2014). Ligand binding to NOD and TLRs promotes bacterial clearance by initiating a proinflammatory response and the secretion of defensins (*Kannaki* et al. 2011, *Philpott* et al. 2014). This response, however, can cause tissue damage and the formation of DAMPS as endogenous ligands of NOD and TLRs (*Kannaki* et al. 2011, *Tang* et al. 2012). Thus, a vicious cycle of inflammation and further tissue damage may be established (*Tang* et al. 2012). This may finally lead to chronic inflammation and the induction of repair mechanisms in form of fibrosis (*Lafyatis* and *Farina* 2012). In regard to the equine endometrium, alterations of the glandular basal membrane are regarded as likely triggering factor of endometrosis (*Klose* and *Schoon* 2016). These lesions may be initiated by inflammatory cells (*Buczkowska* et al. 2014, *Klose* and *Schoon* 2016) and/or could represent a primary degenerative alteration (*Buczkowska* et al. 2014). They could possibly lead to the release of endogenous ligands of TLRs and/or NOD receptors.

It is known that certain genetic polymorphisms or mutations of NOD receptors, TLRs and defensins are associated with an increased susceptibility to infection and/or chronic inflammation (*Bagnicka* et al. 2010, *Lafyatis* and *Farina* 2012, *Strober* et al. 2006, *Philpott* et al. 2014, *Branković* et al. 2015). For example, women with particular NOD1 polymorphisms are predisposed to genital chlamydial infections (*Branković* et al. 2015). Further, human beings with certain NOD1 and NOD2 mutations have an increased susceptibility to inflammatory bowel disease and Crohn's disease, respectively (*McGovern* et al. 2005, *Strober* et al. 2006, *Philpott* et al. 2014). Thus, it appears possible that polymorphisms of NOD genes may also contribute to the susceptibility or resistance of mares to develop PPBE. Sequencing of NOD genes retrospectively from archived material will further investigate this hypothesis. In mares, chronic endometritis including PPBE and endometrosis (periglandular fibrosis) are important causes of reduced pregnancy rates (*Bracher* et al. 1997, *Schoon* et al. 1997). Their clinical importance is further increased by the facts that chronic endometritis shows a poor response to antibiotic treatment and endometrosis even represents an irreversible condition (*Schoon* et al. 1997). As a consequence, the characterization of the immune mechanisms of the equine endometrium is an important prerequisite to decipher the pathogenesis of endometrial diseases and to find appropriate prophylactic regimes as well specific treatment options.

In conclusion, this study provides the basis for future investigation into the contribution of NOD1 and NOD2 to the pathogenesis of equine endometrial diseases. The detection of NOD transcripts in FFPE samples allows the examination of identical sample by PCR and histology. The suitability of FFPE tissue is also a prerequisite for retrospective studies on archived material.

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Erweiterte Zusammenfassung

Nachweis von NOD1 und NOD2 Transkripten im gesunden und erkrankten equinen Endometrium

Bei Stuten stellen endometriale Erkrankungen die Hauptursache einer Subfertilität dar und können deshalb zu wirtschaftlichen Verlusten für die Pferdezucht führen. Diesbezüglich von besonderer Bedeutung sind subklinische Erkrankungen, weil diese nur durch die histologische Untersuchung eines Endometriumbioptates aufgedeckt werden können. Die genaue Pathogenese einiger der häufigsten subklinischen Erkrankun-

gen ist zudem unbekannt, dabei handelt es sich um die nicht-eitrige Endometritis und die Endometrose (periglanduläre Fibrose). Weiterhin ist unklar warum die nach einer Bedekkung oder Besamung auftretende Endometritis bei bestimmten Stuten in eine persistierende chronische Endometritis übergeht („susceptible mares"), während diese bei anderen Stuten physiologischerweise nach 48 Stunden wieder abgeklungen ist („resistant mares"). Diese Bedeckungs- und Besamungs-assoziierten persistierenden Endometritiden vermindern erheblich die Aussichten der Entstehung und Aufrechterhaltung einer Gravidität. Bei den Nukleotid-bindenden Oligomerisierungsdomänen-enthaltenden Proteinen 1 und 2 (NOD1 und NOD2) handelt es sich um im Zytoplasma gelegene Rezeptoren, die zu der angeborenen Immunität gehören und von Epithelzellen und Antigen-präsentierenden Zellen exprimiert werden. Deren Liganden sind überwiegend Peptidoglykanbestandteile der Zellwand Gram-positiver (NOD1) und Gram-negativer Bakterien (NOD1 und NOD2). Diese Rezeptoren können auch durch die bei einer Zellschädigung freigesetzten körpereigenen Moleküle stimuliert werden. NOD1 und NOD2 dienen der immunologischen Überwachung und der Abtötung intrazellulärer Bakterien. Deren Aktivierung kann jedoch auch zu Entzündungsreaktionen und Gewebefibrosen führen. Zudem wurde gezeigt, dass NOD1- und NOD2-Rezeptoren an der Manifestation uteriner Erkrankungen bei Frauen und anderen Tierarten beteiligt sind. Nach Kenntnis der Autoren liegen bezüglich der Expression von NOD1 und NOD2 im equinen Endometrium keine publizierten Daten vor. Ziel dieser Studie war zu überprüfen, ob NOD1 und NOD2 im gesunden und erkrankten equinen Endometrium exprimiert werden.

Dafür standen endometriale Gewebeproben von 31 euthanasierten Stuten zur Verfügung, die mittels PCR und Histologie untersucht wurden. Von jeder Stute lagen 2 Gewebeproben vor. Eine wurde nativ in flüssigem Stickstoff eingefroren, die andere mit 10% gepuffertem Formalin fixiert und in Paraffin eingebettet. Die PCR-Untersuchungen wurde vergleichend an Nativgewebe und Formalin-fixierten Paraffin-eingebetteten (FFPE) Gewebeproben durchgeführt. Die verwendeten Primer wurden unter Anwendung des Programmes „Primer 3" zusammengestellt. FFPE-Gewebe wurde zudem routinemäßig für die histologische Untersuchung aufgearbeitet. Diese diente der Feststellung des Funktionszustands der endometrialen Drüsen und der Diagnose entzündlicher und degenerativer endometrialer Erkrankungen. Bei 25 Stuten lag eine Altersangabe vor. Das Alter lag zwischen 1,5 und 21 Jahren (Durchschnittsalter: 12 Jahre). Mittels PCR konnte bei allen 31 Stuten mit jedem der eingesetzten Primerpaare das Vorliegen von NOD1- and NOD2- sowie GAPDH-Transkripten in den nativen und FFPE-Gewebeproben des Endometriums nachgewiesen werden.

Die RNA-Integritäts-Nummern der repräsentativ untersuchten Nativgewebeproben lagen zwischen 7,9 und 9,3 und bestätigten somit das Vorliegen einer guten bis sehr guten Qualität der isolierten RNA. Bei der histologischen Untersuchung zeigten die Gewebeproben der meisten Stuten eine sekretorische Funktionsmorphologie der uterinen Drüsen (19 Stuten). Proliferative Drüsen lagen bei 6 Stuten vor. Bei den übrigen 6 Stuten befanden sich die endometrialen Drüsen im Übergang zwischen beiden Funktionsstadien. Fünf Stuten wiesen keine endometrialen Veränderungen auf; zwei von diesen waren trächtig. Die übrigen Stuten (n = 26) zeigten eine oder mehrere (n = 21) der nachfolgenden endometrialen Erkrankungen: unterschiedliche Formen der Endometritis (n = 12), Endometrose (n = 20), Angiosklerose (n = 20), Perivaskulitis und Vaskulitis (n = 1), Infiltration mit neoplastischen Lymphozyten (n = 1) und Retention der Plazenta (n = 1). Bei 7 Stuten war die Endometritis nicht-eitrig und bei je 2 Stuten akut bzw. subakut eitrig. Die eitrigen und nicht-eitrigen Endometritiden (n = 11) waren überwiegend oberflächlich (n = 7) und geringgradig (n = 9). Eine Stute hatte eine diffuse oberflächliche Endometritis eosinophilica. Je 10 Stuten wiesen eine geringgradige bzw. mittelgradige Endometrose auf.

Diese Studie zeigt eine konstitutive Expression von NOD1- und NOD2-Transkripten im gesunden und erkrankten equinen Endometrium während aller Phasen des endometrialen Zyklus. Weiterhin konnte das Vorliegen von NOD1-und NOD2-mRNA im graviden Uterus von Stuten bestätigt werden. Die Ergebnisse dieser Studie zusammen mit den nachfolgenden Literaturdaten deuten mit hoher Wahrscheinlichkeit darauf hin, dass NOD1- und NOD2-Rezeptoren auch im equinen Endometrium sowohl an der Regulation physiologischer Prozesse beteiligt sind als auch zu der Pathogenese uteriner Erkrankungen beitragen können. Die häufigsten Endometritiserreger von Stuten sind Streptokokken und E. coli. Diese stellen fakultativ intrazelluläre Pathogene dar und aktivieren somit NOD-Rezeptoren und die assoziierten Signaltransduktionswege. Die Stimulierung von NOD-Rezeptoren durch bakterielle Erreger ruft häufig nicht nur eine Entzündungsreaktion, sondern auch eine Gewebeschädigung hervor. Diese wiederum führt zur Freisetzung endogener Liganden dieser Rezeptoren und nachfolgend deren verstärkte Aktivität. Daraus kann sich eine chronische Entzündungsreaktion mit der Einleitung von Reparaturmechanismen in Form von Gewebefibrosen entwickeln. Das Auftreten chronischer Entzündungsreaktionen kann durch genetische Polymorphismen oder Mutationen von NOD-Rezeptoren begünstigt werden. Somit könnten genetische Polymporphismen von NOD1- oder NOD2-Rezeptoren möglicherweise auch zu der Prädisposition bestimmter Stuten („susceptible mares") für persistierende Bedeckungs- und/oder Besamungs-induzierte Endometritiden beitragen. Beim Menschen ist zudem bekannt, dass Polymorphismen oder Mutationen von NOD-Rezeptoren mit einer erhöhten Empfindlichkeit gegenüber Infektionen assoziiert sind. Zum Beispiel prädisponieren bestimmte NOD2-Polymorphismen zu einer genitalen Infektion mit Chlamydien. Diese obligaten intrazellulären Erreger stellen auch eine mögliche Ursache für Endometritiden und Aborte bei Stuten dar.

Zusammenfassend bildet diese Studie die Grundlage für zukünftige Untersuchungen über die mögliche Beteiligung einer veränderten Expression von NOD1 und NOD2 an der Pathogenese endometrialer Erkrankungen von Stuten. Der Nachweis von NOD1- und NOD2-Transkripten auch in FFPE-Endometriumproben ermöglicht vergleichende molekularbiologische und histologische Untersuchungen an derselben Gewebeprobe. Zukünftige Untersuchungen werden wahrscheinlich zu Ergebnissen führen, die für die Prävention und Therapie endometrialer Erkrankungen von Stuten von Vorteil sind.

Schlüsselwörter: Endometrium, equine, NOD1, NOD2, PCR, Stute, Transkripte, Reproduktion