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Investigations on the Endometrial Response to Intrauterine Administration of N-Acetylcysteine in Oestrous Mares

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Contents
In mares, mating-induced persistent endometritis contributes to low fertility. The condition is in part related to delayed clearance of mucus accumulated within the uterine lumen. The objective of this study was to investigate the endometrial response of healthy mares to intrauterine (i.u.) treatment with N-acetylcysteine (NAC). Oestrous mares (n = 12) were randomly assigned to a treatment (TM) or control (C) group and received an i.u. infusion of 5% NAC and saline (total volume 140 ml), respectively. Endometrial biopsies were collected in five of the mares 24 h after treatment, in the remaining seven mares 72 h after treatment. Endometrial biopsies were evaluated for integrity of the luminal epithelium, number of polymorphonuclear neutrophils (PMN), staining for cyclooxygenase 2 (COX2), staining with Kiel 67 antigen (Ki-67), lectins and periodic acid-Schiff (PAS). The integrity of endometrial epithelial cells was not affected by treatment (no statistical differences between groups or times). At 24 h after treatment, the mean number of PMN in endometrial biopsies from NAC- and C-mares did not differ, but at 72 h after treatment, number of PMN was significantly higher ($p < 0.05$) in C (3.9 ± 0.6 PMN/field) compared with NAC-treated mares (2.3 ± 0.2 PMN/field). At 72 h after treatment, the intensity of staining for COX2 was significantly higher after saline than after NAC treatment ($p < 0.05$). In the epithelium, no differences in staining for the proliferation marker Ki-67 were seen with respect to time and treatment. Score for the lectin wheat germ agglutinin (WGA) was slightly higher in NAC-treated mares than in C-mares 72 h after treatment ($p < 0.05$). Score for PAS staining of mucus in deep uterine glands differed significantly between groups at 24 h after treatment ($p < 0.05$). The present study demonstrates that NAC does not adversely affect the endometrial function. Moreover, an anti-inflammatory effect on the equine endometrium was observed.

Introduction

Endometritis is a major cause for infertility in mares and contributes to economic loss in horse breeding (Causey 2006; LeBlanc and Causey 2009). In the United States, uterine infection is ranked as the third most frequent medical problem in adult horses (Traub-Dargatz et al. 1991). Uterine inflammation, delayed uterine clearance and bacterial endometritis lead to increased mucus production (Freeman et al. 1990; Causey et al. 2000, 2008). Accumulation of uterine fluid and mucus during oestrus or after breeding is associated with decreased pregnancy rates (McKinnon et al. 1988; Pycock and Newcombe 1996). This may be due to inhibition of forward progression of spermatozoa in the uterine lumen (Rutllant et al. 2005). More importantly, uterine fluid accumulation is correlated with a decrease in uterine defence mechanisms such as neutrophil phagocytosis, mucociliary clearance and lymphatic drainage. Therefore, uterine fluid accumulation may lead to chronic endometrial dysfunction (Causey 2007; LeBlanc and Causey 2009) and/or uterine infection.

Current therapies of intrauterine (i.u.) fluid accumulation in oestrous mares aim to support uterine clearance mechanisms by uterine lavage and/or administration of ecbolic drugs (Causey 2006; LeBlanc and Causey 2009; LeBlanc 2010). Recently, i.u. administration of N-acetylcysteine (NAC) has been shown to support antibiotic therapy in mares with endometritis due to infection with gram-negative bacteria such as *E. coli* and *Staphylococcus spp*., or yeasts and fungi (LeBlanc and Causey 2009). These agents are able to produce biofilms – a mucoid substance produced by bacteria and yeasts facilitating colonization of the epithelial surface. Biofilm formation increases resistance to antimicrobial agents infused into the uterus and thus decreases the efficiency of i.u. antibiotics. It was suggested that NAC may dissolve the biofilm (LeBlanc 2010). The substance disrupts disulphide bonds between mucin polymers and thus exhibits mucolytic properties (Sheffner 1963; Matsuyama et al. 2006). It is effectively used for mucolytic therapy for chronic obstructive pulmonary disease of horses (Breuer and Becker 1983). Besides, it possesses antioxidant properties (Estany et al. 2007; Paintlia et al. 2008; Banerjee et al. 2009) and has been used to protect the colon mucosa against tissue damage by hypo chloric acid (Rotting et al. 2003). The protease-inhibiting property was utilized in the therapy for ulcerative keratitis (Haffner et al. 2003; Ollivier et al. 2003). Furthermore, meconium impactions and aspiration pneumonia in equine neonates can be treated by administration of NAC (Burke et al. 2002; Morresey 2008). Positive effects of i.u. treatment with NAC on fertility of mares with a history of endometritis have recently been suggested (Gores-Lindholm et al. 2009). However, information on effects of NAC on endometrial function is not available. In the present study, we have therefore investigated the endometrial response of healthy mares to i.u. treatment with NAC. We tested the hypothesis that NAC does not adversely affect uterine clearance and endometrial function in oestrous mares. For evaluation of the inflammatory response of the endometrium, the presence of polymorphonuclear neutrophils (PMN) and staining for cyclooxygenase 2 (COX2) were evaluated. For characterization of mitotic and secretory activity of the endometrium, Ki-67 (Kiel 67) as well as lectin binding was evaluated. Furthermore, periodic acid-Schiff reaction (PAS) staining was evaluated to assess

changes in mucus quality in response to NAC treatment. Intrauterine manipulation always results in an endometrial response and possible contamination with bacteria (Olsen et al. 1992); however, in the present study, intrauterine fluid accumulation was assessed, and uterine cultures were performed to evaluate for any effects of NAC on uterine clearance in comparison with a saline-induced endometrial reaction.

Materials and Methods

Animals

The study was conducted at the Clinic for Animal Reproduction, Freie Universität Berlin, Germany. Twelve mares of mixed breeds aged 2–7 years (mean 3.1 ± 1.5 years) weighing 400–500 kg (453.3 ± 36.6 kg) were included. All mares were kept outdoors in an open shelter with access to pasture. Water was freely available, and hay was provided three times daily. All experimental procedures used in this study were reviewed and approved by the Institutional Animal Care Committee of the Freie Universität, Berlin, Germany.

Management of mares

At the beginning of the experiment, a breeding soundness examination was performed. This included transrectal palpation and ultrasonography (Picker CS 9100, Physia, Neu-Isenburg, Germany) for genital health and determination of stage of the reproductive cycle. Mares without any signs of genital pathologies and negative uterine culture were included into the study as soon as they were found to be in oestrus (presence of an ovarian follicle ≥ 3.0 cm and detection of endometrial oedema). All mares had a biopsy grade of I or IIA (Kenney and Doig 1986). Information on previous susceptibility to endometritis was not available.

Experimental design

Intrauterine infusions were performed in two consecutive cycles in each mare. While half of the mares received an intrauterine infusion of NAC in the first cycle and of saline (C) in the consecutive cycle, the other half of the mares were treated in opposite order. Thus, mares served as their own control with regard to the same sampling time. Samples were collected either at 24 (n = 5 mares) or at 72 h (n = 7 mares) after treatment in two independent mare groups. Irrespective of assignment to the 24 or 72 h-group, 12 h after treatment, the genital tracts of all mares were examined via transrectal ultrasonography to assess possible effects of NAC treatment on intrauterine fluid accumulation. If an intrauterine fluid accumulation >2 cm² could be detected, it was noted and removed through a uterine flushing catheter.

Intrauterine treatment

For preparing the NAC solution, 6 g NAC (Cp Pharma, Burgdorf, Germany) and 9.5 g sodium hydrogen carbonate (Natron Pulver E 500 Otto Fischer, Saarbrücken) were diluted in 125 ml of saline to receive a 5% NAC solution (pH 7.7, final volume of 140 ml). For the saline solution, 9.5 g sodium hydrogen carbonate was diluted with 135 ml saline (pH, 7.7; final volume, 140 ml). Both solutions were prepared under sterile conditions.

Before the intrauterine infusion was administered, the tail of each mare was wrapped; the perineum and vulva were washed three times with warm water and a disinfectant soap and dried with paper towels. An insemination pipette (Minitüb, Tiefenbach, Germany) was inserted into the uterus, and a syringe filter (Corning Life Sciences, Amsterdam, the Netherlands) was placed on the end of the pipette. The prepared solution was infused through the syringe filter into the uterus.

Sample collection

At 24 or 72 h after treatment, a double-guarded uterine swab (Minitüb) and an endometrial biopsy were collected, depending on the group-assignment of the respective mare (24 h or 72 h). With a sleeved arm, the instruments were introduced through the vagina and cervix into the uterus. Material for uterine culture was collected by rotating the swab alternately to the right and to the left to obtain cellular material from the adjacent endometrium of the uterine corpus. Endometrial biopsies were taken from the base of either uterine horn using standard procedures (Ricketts 1975). In brief, the biopsy forceps was introduced through the cervix into the uterus by a sleeved arm. After the forceps was placed in the uterine lumen, the sleeved arm was withdrawn from the vagina and inserted into the rectum to guide the forceps to the desired place and to put endometrial mucosa into the forceps. Swabs were immediately transferred into Amies medium (Meus, Piove di Sacco, Italy) and examined for bacterial growth at the Institute for Microbiology, Freie Universität, Berlin. All endometrial biopsies were divided; one half was immediately transferred into 4% formaldehyde and the other half into Bouin's fixative. They were sent to the Institute for Histology, University for Veterinary Science, Vienna, Austria, for further processing. Because evaluation of samples fixed in formalin and in Bouin's solution did not reveal any differences, results from formalin-fixed tissue are presented, only.

Bacteriology

For bacteriological culture, the specimens were transferred to 5% defribinated sheep blood agar (Gassner Agar; Oxoid, Wesel, Germany) and CHROMagar (Chromagar, Paris, France) plates within 24 h of collection and incubated for 24–48 h under aerobic conditions at 36°C. Determination of bacterial species was performed according to standard procedures (Murray et al. 2007). Mixed cultures of more than three pathogens were considered as contamination. Common bacteria, such as *β-haemolytic Streptococcus*, *Escherichia coli* (*E. coli*) and *Pseudomonas spp.*, were considered as equine uterine pathogens (Bain 1966; Allen and Newcombe 1979; Digby and Ricketts 1982).

Histology and immunohistochemistry

Formalin-fixed biopsy specimens were embedded in Histocomp (Vogel, Giessen, Germany) and cut at a thickness of 5 μm. Evaluation of specimens was always performed by the same person who was blind to treatment of mares and time of collection of the biopsies. For histological evaluation, biopsies were stained with haematoxylin and eosin (H&E) as described elsewhere (Romeis 1989). Luminal epithelium was evaluated and classified as very well preserved (1), well preserved (2) and not well preserved (3). In addition, PMN were morphologically differentiated, photographed and counted in six randomly selected fields (340 μm x 260 μm) with a light microscope at 40× magnification (Reichert, Vienna, Austria) as described (Koblischke et al. 2008). Three fields were located near the uterine lumen (stratum compactum and epithelium), and the other three fields were chosen in a deeper layer (stratum spongiosum). The mean number of PMN per field was calculated. Photomicroscopy was performed with a colour camera head (DsFi1, Nikon, Tokyo, Japan) and NIS-Elements software (Nikon Instruments). For analysis of carbohydrates, sections were stained with PAS (Romeis 1989). The amount of mucus on the luminal epithelium in the superficial and in the deep glands was classified and scored as absent (0), mild (1), moderate (2) or strong (3).

Histochemical analysis with lectins *Helix pomatia* (HPA), *Ulex europaeus* I (UEA) and *Triticum vulgaris* (WGA) was performed as described (Walter and Bavdek 1997; Walter et al. 2001; Jischa et al. 2008). Paraffin sections were cut at 3 μm and mounted on poly-L-lysine-coated slides. Subsequently, they were rehydrated, and endogenous peroxidase activity was blocked by incubation in 0.6% H_2O_2 in methanol for 15 min at RT (room temperature). Afterwards, the sections were incubated in 1% BSA (bovine serum albumin; Sigma, Deisenhofen, Germany) in phosphate-buffered saline solution (PBS; Sigma) for 20 min at RT, to minimize non-specific lectin binding. Hereafter, slides were incubated for 1 h at RT with the respective biotinylated lectin (HPA, Sigma; UEA, WGA, Vector Laboratories, Burlingame, CA, USA). Lectins were used at a concentration of 10 μg/ml. After incubation with the respective lectin, an avidin–biotin–HRP (horse radish peroxidase) complex (Vector Laboratories,) was applied for 45 min at RT. Afterwards, sections were washed in PBS solution and developed in 3,3′diaminobenzidine-tetrahydrochloride (DAB, Sigma) substrate for 10 min at RT. Finally, slides were washed with distilled water, counterstained with Mayer's haemalum, dehydrated and mounted with xylene-soluble medium (DPX, Fluka Chemicals, Buchs, Switzerland). Lectin binding was classified by the intensity of staining of the epithelium (cytoplasm and glycocalyx), the stroma, the superficial and the deep uterine glands (cytoplasm and glycocalyx) as absent (0), mild (1), moderate (2) or strong (3) as described (Schönkypl et al. 2003; Jischa et al. 2008).

The expression of Ki-67 and COX2 was determined by immunohistochemical analysis according to Jischa et al. (2008) and Koblischke et al. (2008). Sections of 3 μm were deparaffinized and rehydrated. Endogenous peroxidase activity was eliminated by incubation in 0.6% H_2O in methanol for 15 min at RT. Antigen retrieval was performed by heating slides 4 × 5 min in 0.01 M citrate buffer (pH 6.0) in a microwave. Non-specific binding of proteins was blocked with goat serum (75 μl/5 ml in PBS solution; Vector Laboratories,). Afterwards, sections were incubated with the primary antibody (Ki-67: mouse monoclonal Ki67/MM1: dilution 1 : 200, Novocastra, Newcastle, England; COX-2: goat polyclonal, 1 : 500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Subsequently, the slides were incubated with the secondary antibody (anti-mouse ImmunoVision, Brisbane, CA, USA; Vector Labatories; or biotinylated anti-goat, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Afterwards, sections were washed in PBS solution, and binding sites of COX2 were detected with the avidin–biotin–horse radish peroxidase complex kit (Vector Laboratories), developed with diaminobenzidine substrate (DAB, Sigma) and counterstained with haemalum. For the Ki-67 immunostaining, slides were washed with distilled water, developed in DAB, counterstained with haemalum, dehydrated and mounted with a xylene-soluble mounting medium (DPX, Fluka, Buchs, Switzerland). Ki-67-positive cells were counted and photographed with a light microscope (40× magnification). In each slide, at least 100 cells were counted in three randomly selected fields of the superficial epithelium. COX2-positive cells were also counted at 40× magnification. In the superficial epithelium and in the superficial glandular epithelium, at least 200 cells were counted and analysed for positive staining according to Willmann et al. (2011). Furthermore, the intensity of staining of the epithelial cells was classified as absent (0), mild (1), moderate (2) or strong (3).

Statistical analysis

Statistical analysis was performed using the PASW statistic package (SPSS Inc., Chicago, IL, USA). The following parameters were evaluated as scores: HE: integrity of endometrial epithelial cells; COX2: staining of endometrial epithelial cells; PAS: mucus on endometrial epithelial cells and in superficial and deep uterine glands; lectins HPA, UEA, WGA epithelium, stroma and superficial and deep uterine glands. Therefore, the non-parametric Mann–Whitney test was used. Comparisons between groups (paired samples) were made by Wilcoxon test, comparisons between times by Mann–Whitney *U*-test. A p-value <0.05 was considered significant. Values are means ± standard error of mean (SEM).

Results

Intrauterine fluid and bacteriology

Intrauterine fluid could be detected in one (4.2%) of 24 cases (after infusion of NAC). In 3 (12.5%) of 24 endometrial swabs, uterine pathogens were detected. *Streptococcus equi ssp. zooepidemicus* was detected in two mares at 24 h (1 NAC and 1 C mare) and 1 NAC mare 72 h after treatment.

Histology

Histologic assessment of HE-stained specimens of endometrium did not show any differences in the structure of the endometrial epithelium irrespective of time and treatment. The luminal epithelium was well preserved (Fig. 1a,b; Table 1). At 24 h after treatment, the mean number of PMN in endometrial biopsies from NAC- and C-treated mares did not differ (NAC: 10.9 ± 5.4 PMN/field, C: 8.2 ± 2.1 PMN/field; no statistical differences between groups). At 72 h after treatment, the number of PMN per microscopic field was significantly higher ($p < 0.05$) in C than in NAC-treated mares (Fig. 1c,d). The number of PMN significantly decreased over time after NAC treatment, only ($p < 0.05$; Table 1).

Immunohistochemistry

Positive staining for COX2 could be detected in ciliated epithelial and glandular epithelial cells. At 72 h, the intensity of staining for COX2 in the endometrial epithelium was significantly higher ($p < 0.05$) in endometrial biopsies collected from C-treated mares (2.6 ± 0.3) than in NAC-treated mares (1.3 ± 0.4; Fig. 1e,f). At 24 h, a similar pattern was seen: after infusion of saline, the score for intensity of staining was higher (3.0 ± 0.0), than after NAC treatment (2.0 ± 0.3; $p = 0.059$). However, no differences in the number of cells stained positive for COX2 were seen between groups.

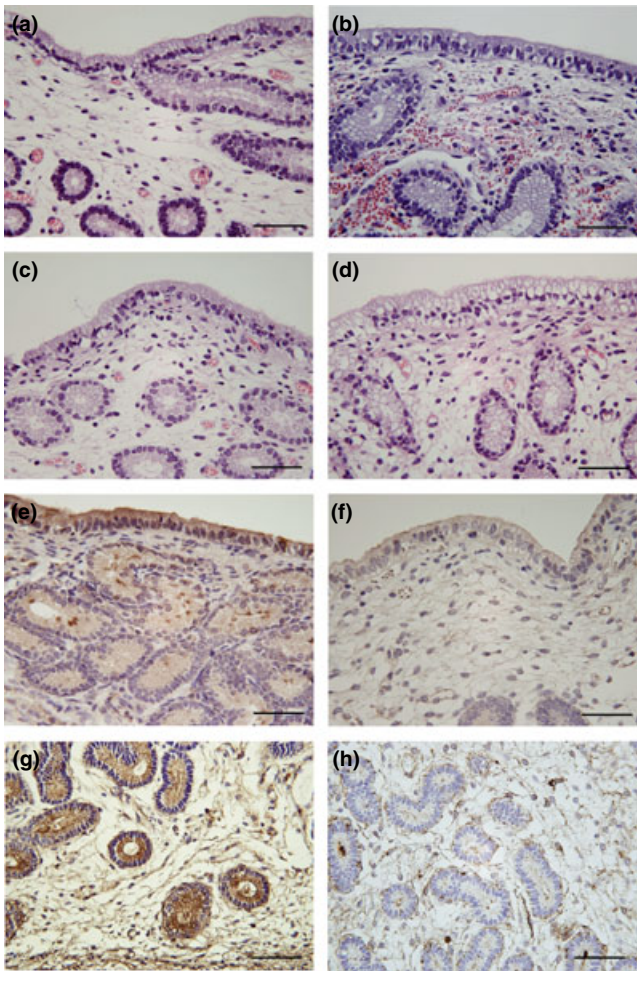

Fig. 1. Photomicrographs of endometrial biopsies collected from mares 72 h after intrauterine saline/NAC instillation. (a) Well-preserved epithelium of biopsies taken after saline instillation. (b) Well-preserved epithelium after NAC instillation. (c) Presence of PMN after saline treatment. (d) Presence of PMN after NAC treatment. (e) Positive and intense staining of COX2 after saline instillation. (f) Positive and less intense staining for COX2 after NAC instillation. (g) Intense binding of WGA in the glycocalyx of the deep endometrial glands after saline instillation. (h) Less intense binding of WGA in the glycocalix of the deep endometrial glands after NAC treatment. Scale bars = 50 μm

Table 1. Histological evaluation of endometrial biopsies collected 24 h or 72 h after intrauterine infusion of N-acetylcysteine (NAC) or physiologic saline (C). Qualitative evaluation (score from 0 to 3) for integrity of endometrial epithelial cells, COX2 and PAS staining and quantitative evaluation (%) for cells staining positive for COX2 and Ki-67 at different locations of the endometrium in mares

	24 h (n = 5 per treatment)		72 h (n = 7 per treatment)	
Treatment	NAC	C	NAC	C
Integrity of endometrial epithelial cells (score)	1.4 ± 0.3	1.6 ± 0.3	1.9 ± 0.1	1.6 ± 0.2
Number of PMN/field (n)	10.9 ± 5.4	8.3 ± 2.1	2.3 ± 0.2[a]	3.9 ± 0.6[b]
Endometrial epithelial cells staining positive for Ki-67 (%)	55.3 ± 11.8	53.6 ± 12.7	27.2 ± 5.6	33.0 ± 5.6
Epithelial cells in superficial uterine glands positive for Ki-67 (%)	11.3 ± 5.7	2.7 ± 1.1	15.5 ± 4.2	18.5 ± 7.0
COX2 staining of endometrial epithelial cells (score)	2.0 ± 0.3	3.0 ± 0.0	1.3 ± 0.4[a]	2.6 ± 0.3[b]
Endometrial epithelial cells staining positive for COX2 (%)	7.6 ± 2.0	7.6 ± 1.9	5.4 ± 1.4	7.2 ± 1.9
Epithelial cells in superficial uterine glands staining positive for COX2 (%)	21.6 ± 0.5	14.2 ± 3.1	11.3 ± 2.7	14.8 ± 2.6
PAS: mucus on endometrial epithelial cells (score)	1.2 ± 0.4	1.6 ± 0.4	1.7 ± 0.2	1.7 ± 0.2
PAS: mucus in superficial uterine glands (score)	1.2 ± 0.2	1.2 ± 0.2	1.7 ± 0.2	1.3 ± 0.2
PAS: mucus in deep uterine glands (score)	0.4 ± 0.3[a]	1.2 ± 0.2[b]	0.7 ± 0.3	0.3 ± 0.2

ab: Different superscripts mark differences between treatments ($p < 0.05$).

In the endometrial epithelium, differences in staining for the proliferation marker Ki-67 could not be revealed between groups irrespective of time and treatment (Table 1). PAS staining of deep uterine glands revealed a significant difference at 24 h after treatment ($p < 0.05$; Table 1). Lectins HPA, UEA and WGA stained the luminal epithelium, deep and superficial glands and the stroma. The binding of WGA in the glycocalyx of the deep endometrial glands was higher in treatment than in control mares at 72 h after treatment (Fig. 1g,h;

Table 2. Histological evaluation of lectin binding pattern of endometrial biopsies collected 24 h or 72 h after intrauterine N-acetylcysteine (NAC) or physiologic saline (C) infusion. Qualitative evaluation (score from 0 to 3) for staining for the lectins *Helix pomatia* agglutinin in (HPA), *Ulex europaeus* I agglutinin (UEA) and *Triticum vulgaris* agglutinin (WGA) at different locations of the endometrium in mares

	24 h (n = 5)		72 h (n = 7)	
Treatment	NAC	C	NAC	C
HPA: Epithelium cytoplasm	2.2 ± 0.2	2.6 ± 0.2	2.7 ± 0.2	2.4 ± 0.2
HPA: Epithelium glycocalyx	2.0 ± 0.3	2.2 ± 0.4	1.9 ± 0.3	1.6 ± 0.3
HPA: Stroma	0.4 ± 0.4	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
HPA: Superficial glands cytoplasm	2.2 ± 0.4	1.8 ± 0.4	2.3 ± 0.3	2.4 ± 0.2
HPA: Superficial glands glycocalyx	1.6 ± 0.4	1.4 ± 0.2	1.9 ± 0.1	2.0 ± 0.3
HPA: Deep glands cytoplasm	0.6 ± 0.2	0.4 ± 0.2	1.3 ± 0.4	1.7 ± 0.4
HPA: Deep glands glycocalyx	0.6 ± 0.2	0.4 ± 0.2	1.6 ± 0.3	1.9 ± 0.3
UEA: Epithelium cytoplasm	1.4 ± 0.5	2.9 ± 0.4	2.6 ± 0.3	2.0 ± 0.3
UEA: Epithelium glycocalyx	1.2 ± 0.5	1.8 ± 0.4	1.7 ± 0.3	1.7 ± 0.4
UEA: Stroma	0.2 ± 0.2	0.4 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
UEA: Superficial glands cytoplasm	1.4 ± 0.2	1.4 ± 0.2	1.6 ± 0.3	1.7 ± 0.2
UEA: Superficial glands glycocalyx	1.6 ± 0.4	1.2 ± 0.2	1.4 ± 0.2	1.3 ± 0.2
UEA: Deep glands cytoplasm	0.8 ± 0.2	1.0 ± 0.0	1.6 ± 0.3	1.3 ± 0.4
UEA: Deep glands glycocalyx	1.2 ± 0.2	0.8 ± 0.2	1.4 ± 0.3	1.4 ± 0.4
WGA: Epithelium cytoplasm	2.2 ± 0.4	2.2 ± 0.2	1.9 ± 0.3	2.4 ± 0.2
WGA: Epithelium glycocalyx	2.0 ± 0.4	2.0 ± 0.3	1.6 ± 0.4	1.3 ± 0.4
WGA: Stroma	1.8 ± 0.2	1.4 ± 0.4	0.9 ± 0.3	1.9 ± 0.3
WGA: Superficial glands cytoplasm	1.8 ± 0.4	1.6 ± 0.2	2.3 ± 0.3	2.0 ± 0.2
WGA: Superficial glands glycocalyx	1.2 ± 0.2	1.4 ± 0.2	1.6 ± 0.2	1.4 ± 0.3
WGA: Deep glands cytoplasm	1.0 ± 0.0	1.0 ± 0.0	1.3 ± 0.2	1.4 ± 0.2
WGA: Deep glands glycocalyx	0.8 ± 0.2	1.0 ± 0.0	1.4 ± 0.2[a]	0.9 ± 0.1[b]

ab: Different superscripts mark differences between treatments ($p < 0.05$).

$p < 0.05$). Differences in binding patterns of HPA and WGA with regard to treatment and time could not be detected (Table 2).

Discussion

Recently, the mucolytic agent NAC has been used for intrauterine treatment of mares with chronic endometritis. Beneficial effects on fertility were reported (Gores-Lindholm et al. 2009; LeBlanc 2010). However, adverse effects of this treatment on mares with intact endometrium cannot be excluded from these studies. Therefore, we investigated the endometrial response of genitally healthy mares to intrauterine treatment with NAC in comparison with a saline-induced response. With regard to the methods applied, we could not find any detrimental effects of NAC on the equine endometrium. In general, this is in agreement with the previous findings (Gores-Lindholm et al. 2009). In their study, however, the evaluation of endometrial function was restricted to the analysis of biopsy grade according to Kenney and Doig (1986) and cell height (Gores-Lindholm et al. 2009). The mares used in the present study were genitally healthy, which was proven not only by results of the breeding soundness examination performed before the study started but also by the findings assessed during the experiment itself. A mild intrauterine fluid accumulation was seen in only one mare with a negative uterine culture. Another mare was not able to clear an intrauterine contamination of Streptococcus zooepidemicus within 72 h after NAC infusion. The contamination was probably caused by intrauterine manipulation at sample collection. This mare thus may have to be classified as susceptible to endometritis (Evans et al. 1986).

Interestingly, some findings of the present study suggest that intrauterine application of NAC may reduce the response to irritation of the endometrium (e.g. intrauterine administration of fluid) in healthy oestrous mares. In general, an inflammatory endometrial reaction of healthy oestrous mares to any intrauterine infusion has to be considered physiologic (Olsen et al. 1992; Palm et al. 2008; LeBlanc 2010). In the present study, the number of uterine PMN in the 72 h-group was significantly higher in mares treated with saline compared to mares treated with NAC. An increased number of PMN demonstrates inflammation (Olsen et al. 1992). In addition, the epithelial staining for COX2 in the superficial endometrium was stronger in saline-treated mares compared to NAC-treated mares. COX2 is involved in the synthesis of PGF2α (Goff 2004) and has been shown to be a reliable marker for the presence of endometrial inflammation in oestrous (Palm et al. 2008) and luteal phase mares (Koblischke et al. 2008). A reduction of the endometrial response due to NAC treatment may be caused by anti-oxidative properties of NAC (Ollivier et al. 2003; Rotting et al. 2003).

In contrast to the intensity of epithelial COX2 staining, the number of cells staining positive for COX2 was not reduced by NAC treatment. However, the number of COX2-positive cells in the endometrium collected from oestrous mares in the present study was considerably lower than in early pregnant mares in a recent study where the same method of COX2 immunohistochemistry was used (Willmann et al. 2011). In this project as well as in the present study, it was observed that COX2 staining is always restricted to ciliated epithelial cells. However, it is feasible that the total number of ciliated endometrial cells was lower in oestrous than in luteal phase mares, and thus, the evaluation of differences between days of the cycle may have been biased.

In the present study, a pronounced mucolytic effect of intrauterine NAC treatment could not be observed. PAS is a parameter characterizing mucus production by the endometrium (Walter and Bavdek 1997; Walter et al. 2001; Jischa et al. 2008). Only in deep parts of the endometrial glands, PAS staining demonstrated a lower amount of mucus in mares 24 h after NAC treatment. However, epithelial mucus is known to be easily destroyed by collection and processing of endometrial biopsies. This may have masked the effect in superficial parts of the endometrium. In the deeper parts of the

endometrium, an effect was still detectable. It can be explained by NAC's mucolytic properties. NAC breaks up viscous mucus and reduces its viscosity (Sheffner 1963; Breuer and Becker 1983; LeBlanc 2010). Results suggest that NAC dissolved superficial mucus and thus also enabled permeation of the agent into deeper parts of the endometrium.

The lectins HPA, WGA and UEA supply information on secretory function on the endometrial epithelium and an altered glycoconjugate pattern in mares with degenerative changes in the endometrium exist (Walter et al. 2001; Jischa et al. 2008). In the present study, no effects of intrauterine NAC treatment on endometrial lectin binding patterns could be demonstrated. This finding together with the lack of alterations in endometrial proliferation as assessed by Ki-67 staining supports the conclusion that there are no adverse effects of NAC treatment on endometrial function.

Conclusion

In conclusion, our data demonstrate that NAC does not adversely affect the endometrial function of genitally healthy oestrous mares. Moreover, an anti-inflammatory effect of NAC on the equine endometrium was observed and may explain previous reports on positive effects of intrauterine NAC administration in mares with endometritis.

Conflict of interest

None of the authors have any conflicts of interest to declare.

Author contributions

All authors contributed equally to the manuscript.

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