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Post-breeding inflammation and endometrial cytology in mares

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Abstract

Endometritis has been reported to be the third most common medical condition of horses. Timely diagnosis and treatment of endometritis in mares increases the chance of pregnancy. Exfoliative endometrial cytology is often used as a clinical tool to evaluate endometrial inflammation through detection of neutrophils. There is a lack of information on the time frame for changes in endometrial cytologic parameters following breeding. The main objectives of this article are to use current information to describe systematic analysis of endometrial cytology using standardized methods for sample collection and interpretation, and discuss how these parameters change in relationship to post-breeding interval and mare susceptibility.

Keywords: Endometrial cells; Neutrophils; Cytology; Endometritis; Mare

1. Introduction

1.1. Overview of the pathogenesis of post-breeding inflammation

The acute endometrial inflammatory response after breeding is a predictable, physiologic event following the introduction of spermatozoa, bacteria, and contaminants [1]. Seminal plasma depresses the endometrial inflammatory response to spermatozoa, and is present in mares bred by live cover, or artificial insemination with fresh or cooled-transported semen [2].

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During the process of cryopreservation, seminal plasma is removed to concentrate the spermatozoa. A proportion of all mares bred develop persistent endometrial inflammation. Kotilainen et al. [3] collected intrauterine fluid 6 h post-breeding with frozen semen or extender and reported higher numbers of neutrophils in mares bred with frozen semen. Other factors that contribute to the marked persistent inflammation in mares bred with frozen semen include: removal of seminal plasma during the process of cryopreservation; allergic-type hypersensitivity reactions to components of the freezing extenders (e.g. glycerol and egg yolk); or delayed uterine clearance, as reported in mares susceptible to endometritis [4,5]. However, the marked endometrial inflammation following breeding with frozen semen is not commonly observed in young virgin mares [6].

Sperm-induced persistent endometrial inflammation has been suggested to contribute to lower fertility in mares by altering the uterine microenvironment and hindering embryonic survival. Mares with persistent inflammation typically have a number of predisposing factors, such as poor perineal conformation, a dependent uterine location, and delayed uterine clearance due to suboptimal myometrial contractility. They are termed ''susceptible'' to endometritis and experimentally are unable to clear (within 96 h) an intrauterine bacterial challenge with *Streptococcus equi zooepidemicus*. Mares that are able to clear the bacteria are termed ''resistant'' to endometritis. The susceptible mare, frequently due to poor perineal conformation, has more bacterial contamination of her reproductive tract than a resistant mare. It follows that more bacteria are available to be introduced at breeding, or are already present in the uterus at the time of insemination in a susceptible mare rather than a resistant mare. Other physical factors such as a dependent uterine location further disadvantage a susceptible mare; the myometrium contracts against gravity to remove the residual sperm and debris [7].

Peak endometrial inflammation is reached 12–24 h after breeding [3,8]. The embryo leaves its tubal phase on about day 5 post-ovulation, therefore uterine inflammation must be controlled by 96 h post-ovulation to maximize survival of the embryo. There is a lack of information regarding: changes in endometrial cytology following breeding, the decrease in inflammation following the peak, the inflammatory response in mares that are susceptible versus resistant to endometritis, and whether the lack of seminal plasma influences the inflammatory response in mares bred with frozen-thawed semen.

Early detection of non-physiologic endometrial inflammation through techniques such as transrectal palpation/ultrasound and endometrial cytology, coupled with timely intervention to manage persistent endometrial inflammation, are required to increase pregnancy rates.

2. Detection of endometrial inflammation

The detection of persistent post-mating inflammation in mares is a clinical challenge. It is usually performed by serial daily transrectal palpations and ultrasound examinations of the reproductive tract. Poor uterine tone and the presence of free intrauterine fluid are

Table 1
Quantitative methods for the interpretation of equine endometrial cytology samples

Parameter	Author	Year
>1 neutrophil in five fields (×240)	Knudsen [11]	1964
Ratio of neutrophils to epithelial cells	Digby and Ricketts [12]	1978
Ratio of epithelial cells to neutrophils >10:1	Asbury [13]	1982
>1 neutrophil per high powered field	Asbury [14]	1984
Neutrophils to epithelial cells eight fields	Couto and Hughes [15]	1984
>5 neutrophils in 10 fields	Brook [16]	1985
Ratio of neutrophils to epithelial cells	La Coeur and Sprinkle [17]	1986
<15 endometrial cells to 1 neutrophil	Ley [18]	1986
≥3–10% of cells are neutrophils	Crickman and Pugh [19]	1986
≥2% of cells are neutrophils	Ball et al [20]	1988
≥1 neutrophil per field (×400)	Purswell [21]	1989
>0.5% neutrophils	Ricketts and Mackintosh [22]	1989

associated with delayed uterine clearance and persistent inflammation [7,9]. Highly fertile mares seldom have free intrauterine fluid following breeding [6]. The relevance of the uterine fluid accumulation is evaluated by examining the depth (mm), echotexture (echodense fluid is associated with neutrophils and debris), and persistence of the fluid. Fluid present in the uterus after ovulation is often associated with mare susceptibility to endometritis [9,10].

There is a lack of agreement on guidelines for interpretation of cytology results. Table 1 shows the most common methods that have been used to interpret equine endometrial cytology samples. These methods include the use of either the number of neutrophils per high power field, neutrophils per slide, neutrophil to epithelial cell ratio, or the percentage of neutrophils. Table 1 shows the criteria for a sample being interpreted as inflamed, which is referred to as ''positive.'' Some of the authors use a positive/negative system, whereas others quantify the degree of inflammation. Categorical classification systems using positive/negative, or mild, moderate and severe endometrial inflammation mares are problematic, as there are no standardized methods for collection and interpretation of endometrial cytology and cultures [23]. There is a lack of an understanding of how these parameters change in relationship to post-breeding interval, which is relevant to the interpretation.

2.1. Standard procedure for obtaining an endometrial cytology sample

The mare is prepared as for artificial insemination; the rectum is emptied, tail wrapped and the perineum is cleansed with soap. The examiner wears a clean, inverted rectal sleeve with a sterile glove over the top and a sterile lubricant is applied to the knuckles, thumb and dorsal wrist area. The tip of the sample device is held covered in the palm of the hand and is not exposed until it is placed in the external os of the cervix. When the examiner's arm exits from the reproductive tract, the swab and the examiner's hand are checked for the presence, amount, and character of a uterine discharge. Cytology slides are dried on a warming tray and stained using a rapid Wright Giemsa stain (Diff Quik, Fischer Diagnostics, Middletown, VA, USA), and when indicated, a Gram stain.

2.2. Double-guarded swab

One technique is to use a double-guarded swab to collect an endometrial sample, which is a method that minimizes vaginal contamination. The end of the double guarded swab is kept covered and free from lubricant as it is introduced into the reproductive tract. The double-guarded swab is advanced through the cervix into the uterus and the inner sheath is pushed through the outer sheath. The sample swab is then moved forward to sample the endometrial cells, using a rolling and pushing motion in a time span of 1 min. The examiner's hand in the vagina is used to redirect the double guarded swab into different areas of the uterus. The sample swab is then retracted back into the inner sheath, which is pulled back into the outer sheath, and the entire unit is then removed. Cytology specimens are prepared by lightly rolling the side of the swab, and pushing the end on to a sterile slide. The slides are dried on a warming tray and then stained. The sample swab is then placed into a transport container (Culturette, Becton Dickinson and Co., Sparks, MD, USA) for microbiology whose swab has been discarded, so it is available for submission for culture and sensitivity if indicated.

2.3. Low-volume uterine lavage

A low-volume (60 mL) uterine lavage with phosphate-buffered saline (PBS) is performed by advancing a sterile insemination pipette into the uterus, injecting the PBS, and massaging the uterus, while moving the pipette and aspirating back to trap cells in the interior of the pipette [20,24]. Approximately 10% of the fluid is recovered, and the contents of the pipette are emptied into a 10 mL tube. A cytocentrifuge technique is used to prepare the slides. The recovered sample fluid is centrifuged at $400 \times g$ for 10 min, and the cell pellet is resuspended in 1 mL of PBS. Drops of the suspension are dropped onto slides which are dried on a warming try at 37 °C and then stained.

2.4. Technical method

The cells present in an exfoliative endometrial sample include endometrial cells, neutrophils, lymphocytes, monocytes/macrophages, erythrocytes, and squames. A differential cell counter is used to quantify the cell types. A minimum of 100 cells are counted using oil immersion ($\times$1000 magnification) with either method. There is generally a small amount of blood contamination of the sample. Endometrial cytology parameters evaluated are: percentage neutrophils, identification of the presence/type (rod, cocci, coccobaccillus) of the bacteria/yeast/fungi, and a determination of the presence/amount of debris. Bacteria and debris are frequently present in cytology specimens and should be quantified [24].

2.4.1. Neutrophils

The percentage of neutrophils present in an endometrial cytology sample is used to determine the degree of inflammation present. Guidelines for the percentage of neutrophils present (as a function of all cells in mares following breeding) are: $<$5%, non-inflammatory; 5–15%, mild inflammation; 15–30%, moderate inflammation; and $>$30%

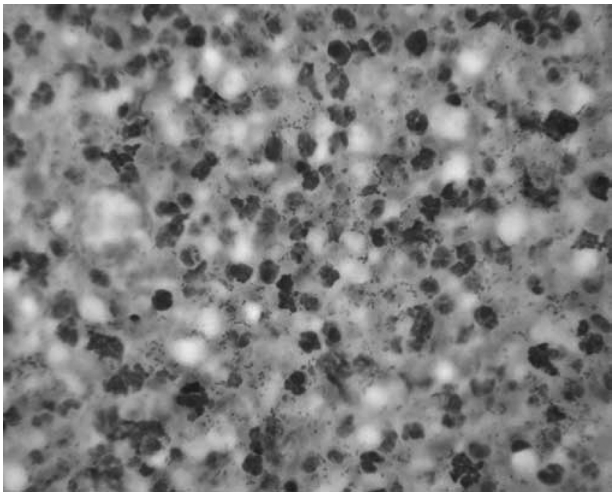

Fig. 1. Low-dose uterine lavage cytocentrifuge preparation (×400) of lavage fluid from a mare, with endometrial cells, >30% neutrophils, numerous bacteria, and <25% debris.

severe inflammation [16]. The presence of intracellular phagocytosed bacteria or other organisms should be noted. A cytocentrifuge slide with many neutrophils and bacteria is shown in Fig. 1.

2.4.2. Bacteria or other microorganisms

Bacteria are scored based on the number of bacteria per high power (×1000) field (1, no bacteria per 30 fields; 2, one bacterium per 30 fields; 3, one bacterium per 10 fields; 4, 2–10 bacteria per field; and 5, 11–50 bacteria per field). Bacteria are found free and phagocytosed within neutrophils or macrophages in the slide preparations. The same system of scoring system is applied if yeast or fungi are present. Bacteria are also classified as cocci, rods, or coccobacilli. If bacteria are identified, then another slide preparation may be made for Gram's stain. Gram negative cocci are unlikely uterine pathogens, but rods or coccobacilli should be Gram's stained to determine if they are Gram positive or Gram negative [10]. Fungal and yeast organisms are identified using a rapid Wright Giemsa stain.

2.4.3. Debris

The amount of debris on a slide is scored based (range 1–4) on the proportionate amount (1, <25%; 2, <50%; 3, <75%; and 4, >75%) of debris that covers a high-power (×1000) microscopic field. To acquaint the examiner with stain artifacts, a plain clean glass slide, and a slide smeared lightly with lubricant and dried are stained with Wright Giemsa stain and examined under oil immersion at high power (×1000). There will be little stain material visible on the plain slide, and only a small amount of stain visible under high power on the slide of the lubricant. If the cytology specimen is properly obtained, there should be no contamination of the sample with lubricant. Sample dilution may influence the outcome, hence it should be avoided [24]. A slide with 25% debris is shown in Fig. 2.

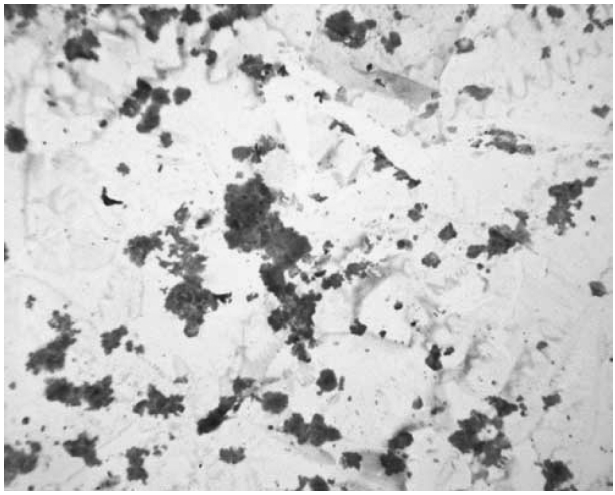

Fig. 2. Low-dose uterine lavage cytocentrifuge preparation (×400) of lavage fluid from a mare, with <5% neutrophils, no bacteria, and 25% debris.

3. Clinical interpretation of endometrial cytology samples based on day of cycle, post-breeding interval, and mare susceptibility

3.1. Non-bred mares

Endometrial cytologic parameters (neutrophil percent, bacterial score, or debris) are similar from 24 to 96 h after ovulation in non-bred mares [16]. There is a constant resident population of neutrophils (<5%) present in an exfoliative cytology [3,16] 24–96 h after ovulation. Bacterial scores are 1 bacterium per 30 fields, and <25% debris per high-power field using the cytocentrifuge technique 24–96 h post-ovulation [24].

3.2. Bred mares

Peak inflammation post-breeding is reached before 24 h. Mild inflammation, ≤5% neutrophils, is present at 24–96 h after the last breeding in mares bred once with a full insemination dose, or twice with a half-insemination dose of frozen-thawed semen. Bacterial scores are <1 bacterium per 10 high-power fields, and debris ranges from 25 to 50% per field using the cytocentrifuge technique from 24 to 96 h post-ovulation [24].

3.3. Susceptible mares

Susceptible mares that have a negative culture prior to breeding with frozen semen or infused with extender have <5% neutrophils at 96 h post-ovulation.

Persistent inflammation >10% neutrophils at 96 h post-infusion is noted only after experimental intrauterine infection with *S. equi zooepidemicus*. Persistent inflammation is

associated with increased percentages of neutrophils (>10%), higher bacterial counts (>1 bacterium per 30 fields) and increased amounts of debris (>50%) [10].

3.4. Endometrial cytology and culture

Schemes for the concurrent interpretation of endometrial cytology and culture results together have been reported [16,23]. The interpretation of an endometrial culture relies on the clinical examination findings and the results of the evaluation of endometrial cytology. Mares are characterized according to the endometrial cytology and culture results as mares with: a healthy uterus, infected mares, and questionable mares. Mares with a healthy endometrial condition, have no significant bacterial growth (a negative culture result) and an exfoliative endometrial cytology with many epithelial cells, <5% neutrophils, few bacteria, and little debris. Mares with uterine infections have a pure, heavy bacterial growth and an endometrial exfoliative cytology characterized by increased numbers of neutrophils >5%, bacteria, and debris. Questionable mares have a few colonies of bacteria, and a few neutrophils [16]. Therefore, a true positive culture result will show agreement between the cytology showing increased numbers (>5%) of neutrophils, moderate or more numbers of bacteria, moderate or more amounts of debris and the culture yielding growth. A true negative culture result will have a non-inflammatory cytology <5% neutrophils, few bacteria, little debris and a negative culture. A false-positive culture result will have a non-inflammatory cytology <5%, few bacteria, little debris, and a positive culture. A false-positive result is likely due to contamination during sample collection. False negatives, where there is no microbial growth in the presence of a cytology showing increased numbers of neutrophils, bacteria, and debris; suggests active inflammation, with or without established infection [16], infection with uterine pathogens such as *Pseudomonas* that do not alter endometrial cytologic features, or problems with sample collection, storage or culture.

4. Discussion

Persistent post-breeding endometritis is of concern to practitioners and breeders, due to its association with lower fertility. At the present time practitioners, do not have a normal range of endometrial cytologic parameters that allow them to evaluate mares post ovulation or post-breeding to determine if intervention is necessary. The presence of >5% neutrophils indicates that endometrial inflammation is present and may require further intervention. The decision to treat a mare for persistent inflammation should be based on history and clinical signs (e.g. poor uterine tone, intrauterine fluid accumulation, exfoliative endometrial cytologic evaluation, and culture/sensitivity). Clinical signs ≥48 h post-ovulation are of concern, because a non-inflammatory uterine environment is important for embryonic survival.

The most common endometrial bacterial isolate is a Gram positive cocci, *S. equi zooepidemicus*. Therefore, the presumption in endometrial samples where >5% neutrophils were identified and Gram positive cocci are present is that endometritis is likely to be caused by a Streptococcal infection. Culture is used to determine the antibiotic

sensitivity pattern of the organism, and to identify the pathogen in cases where Gram positive or negative coccobacilli or rods are identified.

Endometrial debris is recently described in a report on endometrial cytology in bred and non-bred mares. It is suggested that debris is correlated to neutrophil percent, and may be related to the effects of inflammation, such as a change in the mucus or represent an inflammatory residue.

Kotilainen et al. [3] reported that in control mares, there were no neutrophils present in specimens prepared using the low-volume uterine lavage technique, but others reported a low percentage of neutrophils in control mares [24,25]. The collective data indicated that there was a low percentage of neutrophils in the uterus, which could be attributed to a constant resident population that does not vary substantially within the first 96 h post-ovulation. The physiological inflammation described in several studies [1,2,7] was not reflected in the median values observed in samples from mares bred with frozen-thawed semen at 24–96 h [24]; perhaps a mare with >5% neutrophils in a low-volume uterine lavage, within 96 h of ovulation or breeding with frozen-thawed semen, has uterine inflammation and treatment should be considered.

There were no differences in bacterial score, debris, total or percentage neutrophils 24 h after ovulation or the last breeding using a single full dose of semen, or two breedings using half doses of semen, suggesting that inflammation was minimal using these common breeding protocols [24]. In pooled data from the same study, bacterial scores are higher in bred versus non-bred mares suggesting that semen, the insemination process, or both, introduce bacteria into the uterus and a low level of bacteria are frequently present in the uterus [24].

Troedsson et al. [2,5] suggested that persistent sperm-induced inflammation may be a more important cause of infertility in susceptible mares than infectious endometritis. This was not found by Maloufi et al. [10]; they suggested that in the absence of pre-existing problems, physiologic inflammation is negligible by 96 h post-breeding in susceptible mares, and that bacterial infection results in persistent uterine inflammation.

In conclusion, there is no day effect on endometrial cytologic parameters by day post-ovulation in non-bred mares. There is a small resident population of neutrophils, <5% in the endometrium from 24 to 96 h after ovulation Endometrial cytologic parameters were similar from 24 to 96 h after breeding, and include mild inflammation (<5% neutrophils), low bacterial numbers, and some debris.

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