Mares Susceptible or Resistant to Endometritis Have Similar Endometrial Echographic and Inflammatory Cell Reactions at 96 Hours After Infusion with Frozen Semen and Extender

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Mares susceptible or resistant to endometritis have a similar mild endometrial inflammatory cell reaction after breeding with frozen semen or extender. Uterine ultrasonographic features post-treatment with frozen semen or extender were similar in resistant and susceptible mares, but fluid at 24 and 96 h was a risk factor for susceptibility after bacterial infection. After artificial insemination with frozen semen, mares that develop significant post-breeding inflammation are likely to have bacterial endometritis. 

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1. Introduction

The acute post-breeding uterine inflammatory response in mares is believed to be caused by the introduction of spermatozoa and bacterial contaminants. Seminal plasma has been shown to have a modulating depressant effect on the endometrial inflammatory response. Marked persistent inflammation has been observed clinically in some mares bred with frozen semen, and it has been attributed to the removal of seminal plasma during the process of cryopreservation; components of the freezing extenders, such as glycerol and egg yolk; allergic-type hypersensitivity reactions; or delayed uterine clearance as is reported in mares susceptible to endometritis.1,2 However, the marked uterine inflammatory cell reaction after breeding with frozen semen is not commonly observed in young virgin mares.3 Sperm-induced persistent inflammation has been described as contributing to lower fertility in susceptible mares by creating a uterine environment that is incapable of supporting a pregnancy. 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. After breeding in susceptible mares, delayed uterine clearance of spermatozoa and contaminants has been identified as the underlying cause of persistent inflammation. Mares susceptible to endometritis have been reported to accumulate fluid post-breeding. A num-

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number of breed associations have recently approved the use of frozen semen, which has resulted in an increase in its use. It has become increasingly important to determine the underlying cause of the aggressive post-breeding endometrial inflammation seen in some mares.

Our objectives were to characterize endometrial edema scores and uterine fluid accumulations in susceptible and resistant mares during estrus before and after experimentally induced uterine inflammation using conventional ultrasonography and to evaluate the relationship between mare susceptibility and the persistence of inflammation at 96-h post-treatment with bacteria, frozen semen, and extender. It was hypothesized that mares susceptible to endometritis would have significantly more post-treatment fluid accumulation and higher inflammatory cell counts.

2. Materials and Methods

Semen was collected from a fertile Quarter Horse stallion using a Missouri-type artificial vagina. Semen was filtered, extended 1:1 with commercial skim milk extender, the seminal plasma removed by centrifugation at 400 g for 12 min, and the sperm pellet was aspirated and resuspended in lactose-EDTA extender at a concentration of 300 million sperm/ml. Extended semen was packaged in 0.5-ml straws at room temperature, passively cooled to 4°C over 2 h, suspended 5 cm above liquid nitrogen for 10 min, and then plunged. Insedation doses of 1 × 10^9 total spermatozoa were selected from frozen ejaculates that had a mean ± SD of 47 ± 8% morphologically normal spermatozoa and 25.5 ± 6% average post-thaw motility for the study.

An isolate of *Streptococcus equi* zoonoepidemicus was obtained from the uterus of a mare with endometritis. Fresh, actively growing cultures of this isolate were used to make aliquots of 5 × 10^6 *Streptococcus equi* zoonoepidemicus bacteria. Aliquots were kept frozen in liquid nitrogen until immediately before endometrial bacterial challenge.

An Aloka ultrasound machine (SSD 500)^a^ and 5-MHz linear array probe were used for all ultrasound examinations, and the settings that affect image attributes such as gain and focal length were held constant throughout the experiment. Daily ultrasound examinations were performed during estrus, and for 4 days after infusion, endometrial edema score^4^ and presence/depth of free intratuterine fluid were recorded.

Twenty-two mares ranging in age from 4 to 22 yr were used for the study. The mares were short cycled by an injection of PGF$_{2\alpha}$^a^ SC in mid-diestrus. During the first estrus after PGF$_{2\alpha}$ treatment, mares were monitored (palpation, ultrasonography, vaginoscopy, and cytology/culture) to ensure they were free of residual endometrial inflammation. Mares with cytologic evidence of inflammation or significant endometrial culture results were treated until free of inflammation/infecion. Mares were followed using transrectal ultrasonography for one spontaneous cycle prior to any challenge with bacteria, frozen semen or extender. Mares were then infused with 5 × 10^6 S. equi zoonoepidemicus in the periovulatory period to establish their status of susceptibility to endometritis and the magnitude of their endometrial inflammatory cell reaction. Frozen aliquots of 5 × 10^6 of *S. equi zoonoepidemicus* were thawed and diluted in 30 ml of sterile saline. The perineal and clitoral regions of the mares were thoroughly washed and cleansed. Sterile gloves, pippettes, and lubricant were used to infuse the solution containing *S. equi zoonoepidemicus* into the uterus. Ninety-six hours after each of the three uterine challenges, a double-guarded swab was obtained from the uterus using aseptic technique, and a low-volume uterine lavage of 60 ml of phosphate buffered saline was performed. The recovered uterine flush was divided into three 10-ml aliquots. One aliquot of fluid was submitted for aerobic culture. The fluid was used to determine the number of colony forming units (CFU) of bacteria, and the culture swab was plated and used to identify the bacterial organism(s). A culture result was considered significant if >50 CFUs of a known endometrial pathogen were isolated, and the neutrophil count was over 5%. During a challenge treatment with frozen semen or extender, if the culture results were significant, the data were excluded from further data analyses. The second aliquot was centrifuged at 200 g for 10 min, and the cell pellet was resuspended in 1 ml PBS. Cells were counted using a hemocytometer and counts reported in numbers per milliliter of flushed solution. The third aliquot of the uterine lavage sample was submitted for cytology, and slides were prepared using the cytocentrifuge technique. Differential cell counts (300 cells) were performed on stained slides (Diff-Quik), and the percentage of neutrophils was reported. The differential neutrophil count was multiplied by total cell count per milliliter to determine the total neutrophil number per milliliter of flushed media.

At 96 h, mares that had between 5% and 15% neutrophils were classified as having mild inflammation, mares with 15–30% were classified as moderate inflammation, and >30% were classified as severe inflammation. Mares with positive culture results for *S. equi zoonoepidemicus* with or without >5% neutrophils at 96 h were classified as susceptible to endometritis. Mares with negative culture results for *S. equi zoonoepidemicus* and less than 5% neutrophils were classified as resistant to endometritis. Mares were given a rest cycle between each challenge and were evaluated before each challenge to be sure they were free of infection/inflammation. Using a randomized cross-over design, mares were then challenged with 1 × 10^9 frozen thawed spermatozoa in 4–6 ml of extender or 6 ml of lactose-EDTA extender. Uterine infusions were performed during the periovulatory stage when the dominant follicle was ≈38 mm in diameter.
uterine infusion was counted as the zero point to adjust data for comparisons between and within categories for susceptibility to endometritis.

A significance level of $p < 0.05$ was used for all statistical tests. A Mann-Whitney U test was used to examine the relationship between status of susceptibility to endometritis and endometrial edema scores in 24-h interval post-uterine challenges. Differences within categories for susceptibility to endometritis were evaluated using the Wilcoxon sign rank test. A $\chi^2$ analysis was used to determine the frequency of free intrauterine fluid accumulation, and an odds ratio was calculated. The percentage of neutrophils and total neutrophil counts of low volume lavage samples collected at 96-h post-uterine challenges with *S. equi* zooepidemicus, frozen semen, or extender were analyzed using a Kruskal-Wallis test. Data were analyzed using a statistical computer software program.

3. Results

Cytological and culture results at 96 h after uterine infusion with *S. equi* zooepidemicus showed that 10 mares, with a mean age of 8.9 $\pm$ 3.7 yr, were susceptible (S) to endometritis. The lavage fluid from these mares had a median of 14% neutrophils with inter-quartile range of 7–23% neutrophils. *S. equi* zooepidemicus was isolated from all but one mare who had 20% neutrophils in her lavage sample. The average age of the 12 mares resistant (R) to endometritis was 7.9 $\pm$ 3.4 yr. These mares had $\leq$5% neutrophil (median 2%, inter-quartiles 2–5%), in their 96-h post-uterine lavage samples.

Bacterial Culture

The majority (9/10) of S mares had a significant *S. equi* zooepidemicus culture, and no R mares had a significant culture during the bacterial challenge. The number of mares that had significant bacterial growth as assessed by plating and a determination of the CFU per milliliter of lavage fluid in the extender and frozen semen challenge cycles are listed in Table 1. One S mare bred with frozen semen and three S and one R mare treated with extender had significant cultures. These mares had very high neutrophil counts (>70%) in their lavage samples. In total, there were more cycles where mares were treated with extender (n = 4) that had significant cultures than mares bred with frozen semen (n = 1); however, of the five mare cycles with significant cultures, four of them were from S mares. Data from these five mare cycles were not included in the analysis of the inflammatory cell responses during frozen semen or extender challenges.

Conventional Ultrasonography

Post-challenge uterine fluid was detected in a number of mares in both the S and R groups (Table 1). A comparison of the depth of free intrauterine fluid data recorded in 24-h intervals after uterine challenges using a Mann-Whitney U test demonstrated no difference in the depth of free intrauterine fluid between S and R mares. However, the frequency that fluid was detected was greater using $\chi^2$ analysis at 24 and 96 h in the *S. equi* zooepidemicus challenge. The odds ratio and relative risk of free intrauterine challenge in the *S. equi* zooepidemicus challenge are presented in Table 2. At 24- and 96-h post-uterine challenge with *S. equi* zooepidemicus, S mares were 1.25 (95% CI of 1.01 and 1.55) and 5.4 (95% CI of 5.15 and 19.12) times more likely than R mares to develop detectable amounts of free intrauterine fluid, respectively. Evaluation of endometrial edema scores data recorded in 24-h intervals during the 96-h period post-uterine infusions with *S. equi* zooepidemicus, frozen semen or extender, demonstrated no effect of status of susceptibility on edema scores between or within categories for susceptibility to endometritis.

Endometrial Inflammatory Cells

The lowest median percentage of neutrophils was recorded from R mares after uterine challenges with *S. equi* zooepidemicus and extender (median 2%, inter-quartiles 2–5%), and the highest percentages were observed in S mares after uterine infusion with *S. equi* zooepidemicus (median 14%, inter-quartiles 9–23%). After uterine challenges with extender and frozen semen in both S and R mares, medians of <4% neutrophils were recorded (Fig. 1). After uterine challenge with *S. equi* zooepidemicus, S mares had the highest median value for total neutrophil counts ($2.6 \times 10^2$/ml). The values for median total number of neutrophils between S and R mares after uterine challenges with frozen semen or extender are similar; however, examination of the inter-quartile range (the middle half of data) demonstrated a wider dispersion in data from S mares (see Fig. 2).

Comparisons of median neutrophil percentages ($p = 0.0000$) and total neutrophil counts ($p = 0.0012$) were significantly different between S and R mares during the *S. equi* zooepidemicus challenge. There were no differences between S and R mares in the extender or frozen semen challenge. There were no differences when the comparisons were made across treatment groups by mare status.

### Table 1. Frequency of Significant Bacterial Growth in Lavage Fluid Obtained at 96h Post-Infusion with Frozen Semen or Extender in Mares Susceptible or Resistant to Endometritis

<table>
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<th>Challenge</th>
<th>Susceptible</th>
<th>Resistant</th>
<th>Total Cycles</th>
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<tr>
<td>Frozen Semen</td>
<td>1/10</td>
<td>0/10</td>
<td>1/20</td>
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<tr>
<td>Extender</td>
<td>3/10</td>
<td>1/10</td>
<td>4/20</td>
</tr>
<tr>
<td>Total</td>
<td>4/20</td>
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4. Discussion

Associations between detection of intrauterine fluid in various intervals post-breeding or ovulation with

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lower pregnancy rates have been reported. We are the first to report in an experimental trial that mares susceptible to endometritis are 5.5 times more likely to develop detectable free intrauterine fluid compared with R mares at 96-h post-uterine bacterial challenge. The larger number of S mares with intrauterine fluid at 96 h suggests that delayed uterine clearance may allow bacteria to persist and multiply. This finding is consistent with LeBlanc et al., who suggested that chronic uterine infection may lead to persistent post-mating endometritis. Thus, in mares with delayed uterine clearance, the bacteria may create ongoing endometrial damage, which has been speculated to contribute to intrauterine fluid accumulation. Bacterial infection with *S. equi zooepidemicus* may cause excess uterine fluid production, an altered tissue distribution of fluid, or a dysfunction in the removal of fluid. Therefore, the parameters we measured regarding free intrauterine fluid accumulation suggests that small amounts of intrauterine fluid might be detected in short interval post-uterine challenges in both S and R mares; however, the detection of intrauterine fluid at 96 h post-insemination indicates a problem, such as delayed uterine clearance or persistent endometritis, which would require further investigation or treatment in a clinical setting. Our failure to detect a significant difference in development of free intrauterine fluid after uterine challenges with frozen semen or extender between categories of susceptibility to endometritis in mares suggests that these substances are less inflammatory and less persistent than live bacteria. It is possible that mares with pre-existing endometrial infection have a different response to the introduction of frozen semen or extender. Delayed uterine clearance in susceptible mares treated with these challenges did not seem to influence uterine fluid accumulation, suggesting that a bacterial component is necessary for most S mares to accumulate fluid. We expected to detect significantly higher percentages and total neutrophil numbers in S mares compared with R mares after uterine challenges with frozen semen. However at 96-h post-treatment, we were not able to demonstrate a difference between S and R mares using either fro-

<table>
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<th>Time (H)</th>
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<th>Risk of FIUF</th>
<th>RR for FIUF</th>
<th>χ² p value</th>
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<td></td>
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<td>R Mares</td>
<td>S Mares</td>
<td>R Mares</td>
</tr>
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<td>1/12</td>
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Fig. 1. Box and Whisker plots for percent neutrophils in uterine lavage fluid at 96-h post-uterine challenges with *S. equi zooepidemicus* (Strep), Lactose-EDTA extender (Extender) or frozen semen (Frozen semen) in mares susceptible (S) or resistant (R) to endometritis. Outliers are not shown. Different capital letters denote significant differences between S or R mares across groups. Different lower case letters denote significant differences within S or R mares in a group.
The majority of studies performed on susceptibility to endometritis in mares are designed to evaluate the mare’s acute inflammatory response, and therefore, most uterine samples are obtained less than 24 h after treatment. In lavage samples obtained at 96 h post-uterine challenge, the persistence of the endometrial inflammatory reaction is evaluated. Prolonged contact of spermatozoa and contaminants with the endometrium and defective uterine clearance have been proposed as the etiology for persistent mating–induced endometritis in susceptible mares.\textsuperscript{10–13} We believed that delayed uterine clearance and low volume of concentrated frozen sperm would result in more continuous endometrial contact and result in more inflammation.

The presence of greater than 5% neutrophils at 96 h after bacterial challenge from the uterus has been reported to be an indicator of endometritis.\textsuperscript{6} At 96-h post-uterine challenge with frozen semen, both resistant and susceptible mares had median neutrophil percentages of less than 5% (3% and 4%, respectively). In these mare groups, the interquartile ranges were similar and ranged from 2% to 11% neutrophils, suggesting there was no or mild inflammation present, regardless of their status of susceptibility to endometritis. Kotilainen et al\textsuperscript{12} collected intrauterine fluid samples at 6-h post-infusion with frozen semen or extender. They reported higher numbers of neutrophils in mares treated with frozen semen compared to extender, and suggested that frozen semen induced more acute inflammation. In contrast we failed to find a difference in neutrophil counts or percentages at 96-h post infusion with frozen semen or extender in the present study. These differences may be explained by true differences in acute versus persistent inflammation, a smaller sample size (six to eight mares), unknown status of susceptibility to endometritis in their mares, potential for pre-existing uterine conditions, biologic variability, factors associated with the semen preparations, or error.

We observed positive cultures and high neutrophil counts in one S mare treated with frozen semen and four mares (3 S, 1 R) treated with extender. In these mares, the neutrophil counts were >70%, which we believe was a reflection of the mare’s combined response to treatment and bacteria. We noted that more mares had significant bacterial growth when bred with extender than frozen semen, and there was a 4:1 ratio of S to R mare cycles where significant growth was obtained. We suggest that there is no inherent feature of the frozen semen or extender that induces excess inflammation, rather that the conditions in the uterus of the susceptible mares makes them more prone to bacteria infection after any uterine manipulation.

Our observations raised the question as to the critical time frame for evaluation of the endometrial inflammatory response to identify susceptibility of a mare to endometritis, because data collected in acute phase of inflammation may not be extrapolated to the persistent phase of inflammation. With the recent recognition of a transient-physiological inflammatory response of endometrium after breeding or insemination with spermatozoa, the evaluation of uterine clearance in a time frame as short as few hours from uterine challenge may be potentially misleading. We suggest that the persistence of the inflammatory response is more important than the acute response in....

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the detection of mares with defective uterine clearance. From the stand point of fertility, if a mare is bred with frozen semen, which potentially induces more acute inflammation than fresh semen but subsequently engages uterine clearance mechanisms that overcome the breeding induced endometritis, she is likely to become pregnant. The earlier acute inflammation would carry little significance. Experimentally, we need to continue to evaluate the relationship between acute and persistent inflammation if we are to understand their etiopathogenesis and their impact on mare fertility.

Our data also emphasize the importance of the absence of pre-existing inflammation and infection on the inflammatory outcome of insemination with frozen semen. It seems that after induction of a non-infectious endometritis either with concentrated spermatozoa such as frozen semen or with extender, in the absence of pre-existing endometrial inflammation or infection, mares known to be susceptible to endometritis exhibit an endometrial inflammatory response similar to their R counterparts, even without any therapeutic intervention.

Mares that are susceptible to chronic or recurrent uterine infections have been reported to have impaired physical clearance of uterine luminal contents. These mares often develop persistent mating induced endometritis (PMIE). Persistent mating induced endometritis begins with a physiologic inflammation associated with the introduction of spermatozoa, seminal plasma and debris into the uterus. The proposed pathogenesis of PMIE includes a physiologic inflammation associated with breeding, contamination of the uterus with bacteria from poor perineal conformation, a dependent uterine location, and a myoelectrical disturbance that leads to decreased physical clearance. The decrease in physical clearance from the abnormal myoelectrical activity results in an increase in intraluminal and interstitial fluid due to poor lymphatic drainage, and because spermatozoa and contaminants persist in the uterus there is an increased influx of neutrophils resulting in persistent inflammation. The information on abnormal myoelectrical activity was obtained by recording the electromyographic patterns of mares following bacterial infection, hence the same myoelectrical reaction may not occur in response to insemination unless there is significant bacteria already present or introduced at breeding. A delay in physical emptying was shown through the use of radiolabeled microspheres and bacteria and radiocolloid alone. The reaction to bacteria was believed to have been the underlying cause of the delayed uterine emptying in the radiolabeled microsphere study. In the radiocolloid studies the data were collected in a short time frame (4h) following infusion. The reaction of the mare's endometrium to the colloid is not likely to be the same as bacteria or spermatozoa, hence these studies may address intrinsic emptying rather than emptying associated with inflammation.

The data present in this study are the first to provide direct evidence that spermatozoa do not induce persistent inflammation at 96h, even in susceptible mares. Future studies will be required to determine if abnormal myoelectrical activity accompanies infusion with frozen semen or extender in susceptible mares free of preexisting conditions, and whether differences in acute phase uterine clearance results in persistent inflammation. As others have suggested, we believe that most mares with PMIE have bacteria resident within the uterus, or have a uterine environment that supports the proliferation and/or adhesion of bacteria that are incidentally introduced at breeding.

In conclusion, the presence of free intrauterine fluid at 96 h from a uterine challenge with pathogenic bacteria suggested susceptibility to endometritis. Endometrial edema scores, levels (mm) of free intrauterine fluid, or an analysis of endometrial echotexture (npv) through serial ultrasound examinations were not useful in identifying mares susceptible to endometritis following uterine challenge with frozen semen or extender. Despite reports of more acute endometrial inflammation induced by frozen semen caused by high concentrations of sperm cells and low amounts of seminal plasma, at 96-h post-uterine challenge, S and R mares in our study have similar neutrophil numbers and percentages. We believe that aggressive post-breeding inflammation in mares after breeding with frozen semen suggests the presence of bacterial infection. Bacterial infection may be the result of pre-existing conditions such as chronic infectious endometritis. There may be an additional or synergistic effect on endometrial inflammation when bacteria and frozen semen are concurrently present in the uterus. Pre-breeding treatments to ameliorate pre-existing conditions such as chronic infectious endometritis, may be important in minimizing the post-breeding endometrial inflammatory response in susceptible mares.

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References and Footnotes


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