Uterine Inflammatory Response to Simultaneous Treatment With Frozen Semen and Bacteria in Resistant Mares

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There is no synergistic or additive effect of simultaneous treatment with frozen semen and bacteria on uterine inflammation in resistant mares at 72 h after infusion. Authors’ addresses: Department of Large Animals Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK S7N 5B4, Canada (Hunter, Raz, Grey, Card); and Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK S7N 5B4, Canada (Chirino-Trejo); e-mail: claire.card@usask.edu (Card). © 2006 AAEP.

1. Introduction

Stallions, during natural mating, ejaculate into the mare’s vagina, and they may deposit spermatozoa, contaminants, and bacteria into the uterus by pelvic/penile thrusting and belling of the glans penis during coitus. Buchi et al. recovered bacteria from post-coital swabs in 72.5% of mares cultured 4–69 h after natural breeding. They reported that the bacteria isolated from post-coital swabs did not correlate with the pre-breeding examination findings, which suggests that the post-coital bacteria recovered were introduced together with spermatozoa at the time of breeding. Artificial insemination involves the passage of a pipette through the estrous mare’s cervix, providing the opportunity for bacteria to be introduced into the uterus. Mares with poor external and internal anatomic barriers have increased bacterial contamination of the genital tract, which increases the probability of bacteria ascending, colonizing the uterus, and causing endometritis. Despite the fact that bacteria may opportunistically ascend into the uterus or may be introduced into the mare’s uterus by natural breeding or artificial insemination, the combined effects of simultaneous introduction of spermatozoa and bacteria on uterine inflammation have not been characterized under experimental conditions.

In the mare, the mild or acute uterine inflammatory response that follows breeding is normal and necessary to clear the uterus of debris, bacteria, and excess spermatozoa. Uterine inflammation should be completely resolved within 48 h of breeding. Persistent acute post-breeding uterine inflammation is a leading cause of subfertility in mares. On one hand, older mares bred with frozen semen have been reported to develop this condition more commonly than young mares or mares bred naturally or with fresh/cooled semen. On the other hand, persistent uterine inflammation is not commonly seen in young mares bred with frozen semen.
A proportion of all mares bred with frozen semen develop marked persistent inflammation that has been attributed to (1) removal of seminal plasma during the process of cryopreservation, (2) allergic-type hypersensitivity reactions to components of the freezing extenders such as glycerol and egg yolk, (3) delayed uterine clearance because of poor myometrial contractility, and (4) dependent uterine location, as reported in mares susceptible to endometritis.2,3 In vitro and in vivo studies suggest conflicting roles for seminal plasma in uterine inflammation.3–7 Persistent inflammation has been reported to be a cause of lower pregnancy rates in mares bred with frozen semen.3,8

Maloufi et al.9 reported that susceptible mares had persistent uterine inflammation only as a result of bacterial challenge but were similar to resistant control mares in terms of neutrophil numbers and percentages when treated with frozen semen or tender alone. The possibility of additive or synergistic effects of both frozen semen and bacteria together were not examined.

Frozen semen is the most efficient means of preserving genetic material, and it has become widely accepted by most breed associations. It is important to understand the causes of prolonged and marked post-breeding inflammation so that veterinarians can intervene appropriately when necessary to maximize pregnancy rates and minimize breeding costs to clients.

The objective of this study was to treat mares with frozen semen, bacteria, or both on the day ovulation was detected and determine the uterine inflammatory response at 72 h post-breeding in young, reproductively sound mares. Our goal was to determine if significant differences existed in uterine cytologic parameters and bacterial cultures between the three treatment groups. We hypothesized that mares infused with both frozen semen and bacteria would show evidence of prolonged uterine inflammation because of either a synergistic or additive effect of frozen semen and bacteria, whereas those bred with only frozen semen or only bacteria would clear these contaminants during the normal acute uterine inflammatory response time of within 72 h after breeding.

2. Materials and Methods

The experimental protocol was approved by the University of Saskatchewan’s Institutional Animal Care and Use Committee. Semen was collected from a fertile Quarter Horse stallion using a Missouri-type artificial vagina. Semen was filtered and extended 1:1 with commercial skim-milk extender. The seminal plasma was 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 200 million sperm/ml. Extended semen was packaged in 0.5-ml straws at room temperature and passively cooled to 4°C over 2 h. It was then suspended 5 cm above liquid nitrogen for 10 min and then plunged. Insemination doses of 1×10⁹ total spermatozoa were selected from frozen ejaculates. An isolate of Streptococcus equi subspecies zoonedimicnicus was obtained from the uterus of a mare with endometritis. Fresh, actively growing cultures of this isolate were used to make aliquots of 5 × 10⁶ S. equi ssp. zoonedimivicus bacteria. Aliquots were preserved in liquid nitrogen until immediately before treatment.

Mares were between 2 and 6 yr of age (mean age = 3 yr) and were defined as resistant to endometritis based on their history, age, good perineal conformation, and normal utero-ovarian patterns as observed on daily ultrasonogaphy and palpation of the reproductive tract before the start of the study. Each mare was identified by either a brand or unique color marking. The mares were housed in a dry lot and fed grass, hay, oats, mineral, salt, and free-choice water.

Mares were monitored daily in estrus using rectal palpation and transrectal ultrasound examination with a GE Ausonics machine and a variable-frequency 6-MHz linear-array probe. When a 35-mm follicle was detected, mares were treated with 2000 IU hCG IM to induce ovulation. On the day ovulation was detected, mares (n = 13) were randomly assigned to one of three treatment groups. The treatments were 1 × 10⁹ frozen-thawed spermatozoa in lactose EDTA extender for group 1 (FS; n = 8), 5 × 10⁶ frozen-thawed S. equi ssp. zoonedimivicus for group 2 (FB; n = 6), and the aforementioned doses of frozen-thawed semen and bacteria together for group 3 (FSB; n = 8). Five mares received one treatment, seven mares received two treatments, and one mare received all three treatments. Mares that were used in more than one treatment group received at least one rest cycle between treatments. The straws/aliquots were thawed in a water bath at 37°C for 45 s and then extended to a total volume of 10 ml with Kenney extender.5 Mares were prepared using aseptic technique, and the treatment was delivered into the uterus using a syringe and an insemination pipette. Mares were monitored daily until 72 h post-treatment with rectal ultrasound/palpation for the presence of intrauterine fluid. If intrauterine fluid was present, the amount (millimeters) was measured ultrasonographically, and the character of the fluid was assigned a grade from 0–4 (0 is non-echogenic, and 4 is maximally echogenic).

At 72 h post-treatment, a low-volume uterine lavage was performed to obtain samples for cytology and bacterial culture. The mares were prepared for artificial insemination, and a sterile uterine-flushing catheter was aseptically inserted transcervically into the uterus. Sixty milliliters of phosphate-buffered saline (PBS) was infused into the uterus followed by 60 ml of air to clear the catheter of fluid. The fluid was agitated in the uterus through rectal massage and then allowed to drain back out the catheter into sterile tubes. The
cuff of the catheter was inflated; the mare was flushed with 2 l of sterile saline, and 2 ml of oxytocin was administered, IV or IM. Mares were monitored for two days following insemination for accumulation of uterine fluid and were treated as needed with oxytocin and/or uterine lavage. At 5 days post-ovulation, mares were short-cycled with 5 mg PGF2α, SC.

The total volume of the fluid collected was measured, and a portion was submitted for bacterial culture and quantitative colony-forming unit (CFU) counts. Bacterial growth was noted as yes/no, and cultures were classified as pure or mixed. Total CFU was calculated by adding all CFU of pure or mixed cultures together.

The remainder of the recovered fluid was centrifuged at 350 g for 15 min, and the supernatant was removed, leaving 1 ml for resuspension of the pellet. Total cells were determined by multiplying the cell concentrations, as determined by cell counts on a hemocytometer, by the volume originally infused into the mare (60 ml). Total numbers of neutrophils were determined by multiplying the percent neutrophils by the total cells in a sample. Percent neutrophils were ascertained from differential counts of 300 cells done at 1000× from a cytology slide stained with a rapid Wright Giemsa stain. All differential counts were done in duplicate by two investigators blinded to sample identity.

Intergroup comparisons were made using a non-parametric Kruskal-Wallis one-way analysis of variance (ANOVA) or χ² test at p < 0.05. Post-hoc evaluations of median ranks were used to evaluate differences between groups. Data were analyzed using StatistiX version 8.0 software. Data below are shown are medians (first and third quartiles).

3. Results

There were no significant differences (p > 0.05) between treatment groups in percent neutrophils (FS, n = 8, 4.9% [3.1, 10.3]; FB, n = 6, 11.0% [2.9, 23.9]; FSB, n = 8, 4.9% [1.7, 11.6]) or total neutrophils (all measurements are ×10⁶; FS, 1.8 [0.02, 7.8]; FB, 1.6 [0.2, 5.3]; FSB, 0.4 [0.2, 2.7]) in uterine lavage fluid collected at 72 h post-treatment. A significant difference was noted in intrauterine fluid (milliliters) at 24 h post-ovulation between FS (0 mm [0, 0]) and FB (1.0 mm [0, 1.3]; p = 0.0035), but intrauterine fluid in FS was similar to FSB (0 mm [0, 1]). Both FB (6 of 6 cycles) and FSB (8 of 8 cycles) showed significantly more bacterial growth (yes, no) on culture than FS (4 of 8 cycles; FB vs. FS, p = 0.0426; FSB vs. FS, p = 0.0192). There were significantly more pure cultures grown from FB (4 of 6) than FS (0 of 8; p = 0.0353), and a similar trend was found in FSB (3 of 8) versus FS (0 of 8; p = 0.0596). The total CFU from FB (1.5 × 10⁶ CFU [1.8 × 10⁵, 6.9 × 10⁴]) and FSB (7.5 × 10⁴ CFU [1 × 10⁴, 8.1 × 10²]) were significantly greater than FS (35 CFU [0, 87.5]; p = 0.0596; FB vs. FS, p = 0.0008; FSB vs. FS, p = 0.0008).

4. Discussion

Spermatozoa have been reported to be the main mediators of acute uterine inflammation. It has been reported that uterine inflammation peaks <24 h from insemination, and inflammation is cleared from the uterus by 24–48 h. The role of seminal plasma in uterine inflammation is still controversial. The amount of seminal plasma in frozen semen is reduced during the process of cryopreservation, because centrifugation is commonly used to concentrate the spermatozoa. In vitro seminal plasma has been found to decrease neutrophil function in response to spermatozoa. Recent work by Portus et al. showed that seminal plasma in vivo increased uterine inflammation and decreased contractility. Guvenc et al. showed that there were no significant differences between mare groups in uterine fluid accumulation, contractility, or production of enzymes associated with inflammation in young and old mares bred with frozen semen or PBS. We chose to use frozen semen because of the reports of excessive inflammation associated with its application. Chronic uterine bacterial infection has been proposed as another cause of subfertility. The combined effects of inflammation from chronic bacterial infection and spermatozoa are problematic in clinical cases. The data in this study show that resistant mares readily clear combined uterine and bacterial challenges by 72 h post-treatment.

The increased detection of free intrauterine fluid in the FB group compared with the FS group at 24 h post-treatment was consistent with findings by Maloufi et al. In our study, the only experimental group where fluid was not detected was the FS group. The lack of differences between the other groups in the presence and depth of free intrauterine fluid may be caused by the small number of mares per group. It is possible that the presence of spermatozoa in the FS and FSB groups increased uterine clearance compared with FB through the stimulation of prostaglandin secretion, cytokine production, or another mechanism-modulating uterine tissue like contractility, fluid secretion, or drainage. The growth of significantly more bacteria and more pure cultures of bacteria from FSB- and FB-treated mares was expected because the uterus had been deliberately inoculated with bacteria in these two groups. It also showed that the frozen semen used in the study was not a significant source of pure or large numbers of bacteria, because few CFU of bacteria were recovered from mares treated with FS alone.

Although these inflammatory agents may be found together in the uterus and both contribute to the inflammatory response, no experimental model to date has deliberately placed both bacteria and spermatozoa into the uterus at the same time. We believed that if both FS and FB were present in the uterus simultaneously, a synergistic or additive reaction might occur, resulting in both more pro-
nounced and prolonged uterine inflammation. We thought that it was important to establish the effects of bacteria and sperm together on uterine inflammation in resistant mares before evaluating susceptible mares, because the results in susceptible mares were expected to be more variable. The number of spermatozoa chosen is commonly used for insemination of frozen semen, and the number of CFU of *S. equi* sspp. *zooepidemicus* has been used experimentally in the past to cause endometritis. The data indicate that the resistant mare is able to rapidly clear significant amounts of bacteria and spermatozoa from the uterus. It is possible that synergistic or additive effects may occur earlier or only in susceptible mares where delayed uterine clearance may lead to increased replication of bacteria, prolonged endometrial contact, or changes in the microenvironment that lead to increased inflammation.

We chose our sample collection time as 72 h after treatment, because young resistant mares should have cleared a bacterial challenge by this time-point. However, it was believed that the uterine clearance process might be prolonged if the inflammatory agents were synergistic or additive. We were concerned with the persistence of inflammatory processes in resistant mares before evaluating the uterus on day 5 post-ovulation. The FS and FSB groups had a median value of ~5% neutrophils at 72 h after treatment, which is consistent with a non-inflamed uterus. The FB group had 11% neutrophils, indicating mild inflammation, and although not statistically different from FB and FSB, a larger sample size may show a difference. Therefore, considering this result, moving the sampling time closer to the treatment might show acute differences in uterine inflammation in response to these treatments. It would be possible to determine the number of bacteria at 24 h post-infusion through quantitative culture techniques. If more inflammation is present at 24 h in mares treated with bacteria but is resolved by uterine defense mechanisms by 72 h, the early differences in inflammation may not interfere with the mare’s ability to support embryonic development or the maternal recognition of pregnancy. Thus, it may not be clinically relevant.

In conclusion, the finding of non-inflammatory uterine cytology in all three groups at 72 h after treatment suggested that no synergistic or additive relationship between bacteria and frozen semen existed. Mares with a healthy uterine condition rapidly clear uterine challenges with spermatozoa, bacteria, or both simultaneously.

References and Footnotes


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