Reprod. Nutr. Dev. 44 (2004) 243–250

DOI: 10.1051/rnd:2004028

Original article

Effect of frozen semen on the uterus of mares with pathological uterine changes

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(Received 4 December 2003; accepted 11 March 2004)

Abstract – Pregnancy rates after frozen semen inseminations (AI), particularly in older and problem mares, are lower than after fresh semen AI. Uterine contractility and the inflammatory reaction after frozen semen insemination were studied in two groups of mares: the abnormal group comprised of 6 old barren mares categorized in biopsy category IIB or III, and the control group including 6 reproductively normal young maiden mares in biopsy category I or IIA. All 12 mares were inseminated in the first cycle with 2 mL of phosphate-buffered saline (PBS) and in their second cycle with 2 mL of frozen semen containing 800×10^6 spermatozoa. Before and 1, 2, 4, 8, and 20 to 24 h after this treatment, all mares were examined by ultrasonography for intrauterine fluid accumulations (IUFA). The examinations were videotaped to count the number of uterine contractions later. Uterine fluid was obtained by tampon before treatment, and by the tampon method followed by uterine lavage after the last examination. Fluids were cultured bacteriologically, and polymorphonuclear leukocytes (PMN) were counted. Trypsin-inhibitor capacity (TIC), lysozyme concentration, and β-glucuronidase (BGase) and N-acetyl-β-D-glucosaminidase (NAGase) activities were determined in frozen-thawed tampon and lavage fluids. Both treatments induced significant neutrophilia in the uterine lumen. Although PMN concentrations were numerically higher after frozen semen AI than after PBS-treatment, the difference was not significant. There was not any difference between the mare groups either. The amount of IUFA differed only in the normal group between frozen semen AI and PBS treatment, and between 0- and 24-h samples for frozen semen AI. Although abnormal mares showed consistently more fluid than normal mares, this difference was not significant. Uterine contractions and enzyme concentrations between groups did not differ. None of the variables showed significant differences between the normal and abnormal mares in their reaction to frozen semen AI.

horse / insemination / frozen semen / uterine fluid / inflammation / leukocyte

1. INTRODUCTION

The pregnancy rates of mares inseminated with frozen semen are lower than with fresh semen. Although a wide variation between the stallions is generally recognized, practical experience has shown that the mares also affect the outcome. Older mares [1, 2], and particularly old maiden mares (> 8 years of age), have a poor chance

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of conceiving with frozen semen [3]. The diagnosis most frequently encountered in uterine biopsies of older barren mares is widespread chronic degenerative endometrosis characterized by periglandular fibrosis and cystic distension of the glands [4]. These glands may have altered secretion, possibly resulting in a uterine luminal environment not optimal for spermatozoa.

The inflammatory reaction of the normal estrous uterus is stronger after AI (artificial insemination) with concentrated semen, e.g., frozen semen, than with extended semen [5]. Whether a strong inflammatory reaction is good or bad for fertility is under debate: frozen semen may be harmful for mares susceptible to endometritis because of the subsequent intensive inflammation.

Because mostly normal mares have been studied, whether the inflammatory reaction after insemination differs in problem mares is unclear. Maloufi et al. [6], inseminated mares defined as resistant or as susceptible to endometritis with frozen semen or with freezing extender and collecting uterine lavage fluid 96 h after insemination. They discovered no differences in the polymorphonuclear leukocyte (PMN) response between these two groups [6]. In another study, mares susceptible to endometritis had significantly more PMN in uterine lavage fluid 48 h after AI with chilled extended semen than did resistant mares [7]. Only one resistant mare showed a small amount of uterine fluid 48 h after AI, whereas 4 of 6 susceptible mares exhibited $\geq$ 100 mL of intrauterine fluid. Naturally, the presence of intrauterine fluid accumulations depends also on the timing of the examination [8].

Endometrial contractions are important in sperm transport and uterine clearance [9]. Insemination of mares induces uterine contractions [10, 11] which carry spermatozoa into the tips of the horns and at the same time help to remove excessive spermatozoa and inflammatory by-products via the cervix. However, mares susceptible to endometritis show deficient myometrial contractility [12]. These mares presumably have an intrinsic contractile defect of the myometrium resulting in delayed uterine clearance [13].

The purpose of our study was to investigate whether old mares with widespread chronic degenerative changes in their endometrial glands differ after frozen semen insemination from young normal mares in the intensity of the inflammatory reaction, in the capability to eliminate fluid or in uterine contractility. The most common methods to evaluate inflammation in the equine uterus are measuring intrauterine fluid accumulations with ultrasonography or counting PMN in uterine swabs or fluid, but other variables may also be sensitive indicators of inflammation. Lysozyme and trypsin-inhibitor capacity (TIC) reflect uterine inflammation in experimental endometritis [14] and in post-partum mares [15]. N-acetyl-β-D-glucosaminidase (NAGase) and β-glucuronidase (BGase) may provide additional information on uterine secretions and their accumulation in the uterine lumen [16]. The variables chosen were the presence of ultrasonically detectable intrauterine fluid, PMN numbers in the uterine fluid, and concentrations of TIC, lysozyme, NAGase, and BGase in the uterine fluid.

2. MATERIALS AND METHODS

2.1. Animals

The study was carried out during April and May 2002 at MTT Agrifood Research Finland, Equine Research. Twelve mares were selected as abnormal and control groups based on reproductive tract examinations by rectal palpation, ultrasonography, vaginoscopy, uterine swabbing, evaluation of endometrial biopsies according to Kenney and Doig [17], and reproductive history. The abnormal mares ($n = 6$) were on average 15 years old (from 13 to 17 years) and belonged to biopsy category IIB or III, mostly exhibiting widespread and severe degenerative glandular changes. All mares had been barren for one to several years;

two mares with occasional intrauterine fluid accumulations (IUFA) had poorly dilating cervices, and one mare had almost constantly shown IUFA. The normal mares ($n = 6$) were on average 6-year-old maiden mares (from 4 to 7 years), clinically normal, regularly cycling, and classed as biopsy category I or IIA.

2.2. Examinations and treatments

The estrous cycles of the mares were synchronized with 7.5 mg of luprostiol (Prosolvin®, Intervet International B.V., Boxmeer, The Netherlands) intramuscularly. When a follicle of ≥ 3.5 cm in diameter and endometrial edema were detected ultrasonically, 1500 IU of hCG (Chorulon®, Intervet International B.V., Boxmeer, The Netherlands) were administered intravenously.

Twenty-four hours after hCG-administration, the mares were examined by ultrasonography (Aloka SSD-500, 5 MHz probe) for the degree of edema and number and size (length and height, cm) of intrauterine fluid accumulations in the uterine body and in the left and right horns. For analysis of data, the length and height were multiplied and the values of the uterine body, and left and right horns were combined into one value. Ultrasonography of the uterus for uterine contractions was carried out for > 1 min and videotaped. The number of uterine contractions were counted later in the videotapes. After that, the tail was encased in a plastic sleeve and the perineal area was washed 3 times and dried. A sterilized minisize tampon (Tampax Inc., Palmer, MA, USA) equipped with a long cotton string and inserted within a metal tube was introduced into the uterus by manual guidance through the cervix. After 30 min, the tampon was removed through a sterilized speculum. Fluid absorbed into the tampon was squeezed out into a sterile plastic tube with a sterile garlic press.

During the first estrus, 2 mL of phosphate-buffered saline (PBS) was infused into the uterus after withdrawal of the tampon. Ultrasonographic examinations including videotaping were carried out 1, 2, 4, and 8 h after the treatment. The next morning, 20 to 24 h after the PBS treatment, the mares were examined by ultrasound and the sessions were videotaped. In addition, the uterine fluid was collected by the tampon technique as described above. Then 50 mL of PBS were infused into the uterus through a modified 22 Ch Foley catheter (Rüsch-Gold®, Willy Rüsch AG, Kernen, Germany). After 5 min of equilibration, uterine lavage fluid was allowed to flow into sterile plastic tubes. When required, the recovery of fluid was assisted by manipulation of the uterus via the rectum.

During the next cycle, the mares were inseminated with 2 mL of semen containing approximately 800×10^6 spermatozoa with 30% progressive motility. Semen had been frozen by the Hanoverian method [18] in 2.5-mL straws. Only one stallion was used, but the straws used for AI had been processed from different ejaculates. All examinations and samplings before and after insemination were performed in the same manner as during the first cycle. The mares were checked for pregnancy 15 to 17 days after AI by ultrasonography.

2.3. Laboratory analyses

The volumes of tampon fluid and lavage fluid were measured and the samples were taken for cytology and bacteriology. The remaining fluid was frozen for enzyme assays at –70 °C.

Tampon and lavage fluids (10 µL) were plated onto blood agar, and microbial growth after a culture of 48 h at 37 °C was scored as 0 (no growth), 1 (1–10 colonies), 2 (10–100 colonies), and 3 (>100 colonies). PMN numbers in tampon and lavage fluids were counted with a Bürker chamber. The detection limit was 1×10^4 PMNmL^{-1}. A drop of tampon fluid and lavage fluid concentrated by centrifugation was used to prepare a smear for a differential cell count. The slides were air-dried, fixed by methanol,

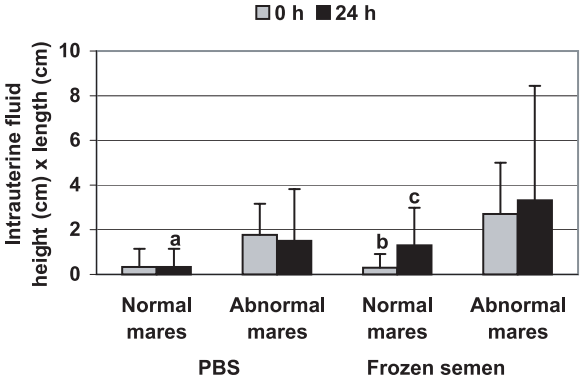

Figure 1. Amount of ultrasonically detected intrauterine fluid in normal and abnormal mares 0 and 24 h after insemination with phosphate-buffered saline (PBS) and frozen semen. The values are means ± SD. Differences between a and c, and b and c are significant ($P < 0.05$).

and stained with May-Grünewald-Giemsa. A total of 100 cells/slide were counted.

TIC was measured by a colorimetric assay [19]. Human α1-antitrypsin (Sigma A-6150) served as a standard. Lysozyme was measured as described previously [14], but on microtitration trays and with a Multiskan MS photometer (Labsystems, Helsinki, Finland). This method is a modification of the turbidimetric method described by Mörsky [20]. The analysis of NAGase was carried out by a method developed for microtitration plate fluorometry [21]. The method for determining BGase, a modification of the procedure described by Nagahata et al. [22], was similar to that used for determining NAGase [14]. The fluorescence of released 4-methyl-umbelliferone was measured with a Fluoroskan Ascent fluorometer (Labsystems, Helsinki, Finland).

2.4. Statistical analysis

The effect of treatments on the number and percentage of PMN, enzyme level, and size of IUFA (combined size of all fluid accumulations detected) in the abnormal and the control group were analyzed with the Wilcoxon signed rank test using the statistical program SPSS for Windows 10 (SPSS Inc. Microsoft cooperation). The Wilcoxon rank sum test was used to compare 0- and 24-h samples of both treatments in normal and abnormal mares. Repeated measures analysis of variance was used to test the differences in the number of uterine contractions and the amount of IUFA during the 24-h follow-up.

3. RESULTS

Both treatments induced an influx of PMN, as indicated by 0-h sample-difference from 24-h samples. After frozen semen insemination, the numbers of PMN in tampon fluid and percentages of PMN in smears were significantly higher than prior to the AI in both groups of mares ($P < 0.05$). After PBS treatment, only the percentage of PMNs in smears differed significantly from the pre-treatment values in both groups. The difference between 0 and 24 h in IUFA was significant only in normal mares inseminated with frozen semen ($P < 0.05$) (Fig. 1).

Although the numbers and percentages of PMN in tampon and lavage fluid were numerically higher after frozen semen treatment than after PBS treatment in both groups of mares, the difference was not significant (Fig. 2). Instead, significantly more

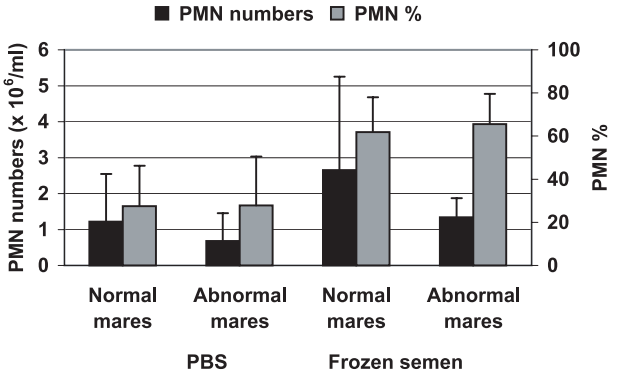

Figure 2. Concentration and percentage of polymorphonuclear leukocytes (PMN) in uterine fluid collected by tampon from normal and abnormal mares 24 h after phosphate-buffered saline (PBS) and frozen semen treatments. The values are means ± SD.

IUFA was detected after frozen semen AI than after PBS treatment in normal mares at 24 h ($P < 0.05$) (Fig. 1).

Between normal and abnormal mares there appeared no significant differences in 0- or 24-h samples after PBS or frozen semen treatments for the concentration or percentage of PMN (Fig. 2) or for IUFA (Fig. 1). The amount of IUFA did not change significantly within time after the treatments, but abnormal mares showed consistently higher levels of IUFA than did normal mares. Normal mares exhibited very little IUFA: one mare after the PBS treatment and the same mare and another one after frozen semen AI had > 1 cm^2 of IUFA. The majority of abnormal mares showed IUFA: only one mare had no fluid after the PBS treatment and another mare did not show fluid after frozen semen AI.

BGase values were below the detection limit in the lavage fluid of most mares, but tampon fluid yielded measurable concentrations. On the contrary, pre-treatment tampons did not always yield sufficient fluid for enzyme analysis. BGase, NAGase, lysozyme, and TIC values showed no significant differences between 0- and 24-h samples, between the treatments or between the mare groups. Numerical values 24 h after frozen semen AI were higher in abnormal than in normal mares, but with a large variation between the mares (Fig. 3).

The total number of uterine contractions during follow-up was 6.2 after the PBS treatment and 7.0 after frozen semen AI in normal mares, and in abnormal mares the number of contractions were 5.5 and 9.8. No differences appeared within that time period or between mares or treatments.

Bacteriological cultures of lavage fluids did not show significant growth; only 3 samples of 23 yielded 2 to 3 colonies. Most of the tampon fluid obtained before or after the treatments yielded mixed growth (scores 1 to 2). None of the mares were diagnosed as pregnant.

4. DISCUSSION

None of the variables were able to demonstrate significant differences between the group of young normal mares and the group comprising older mares with biopsy categories IIB or III and with an abnormal reproductive history. The abnormal mares were not a homogenous group. Although they all had chronic degenerative endometrosis, some of them accumulated intrauterine fluid, and

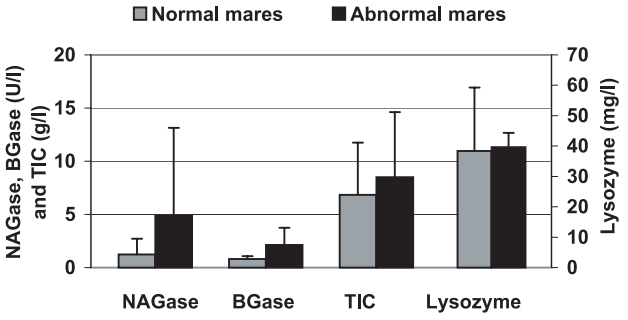

Figure 3. N-acetyl-β-D-glucosaminidase (NAGase), β-glucuronidase (BGase), trypsin-inhibitor capacity (TIC), and lysozyme concentration of uterine fluid collected by tampon from normal and abnormal mares 24 h after insemination with frozen semen. The values are means ± SD. (U = μmol product·min^{-1}).

they may have had other problems as well. Of course, limiting factors in the statistical analysis were the small number of mares per group and the large standard deviations within groups. Tampon fluid of normal mares contained numerically, but not significantly, higher amounts of PMN than that of abnormal mares. However, the total number of PMN in the uterine lumen might have been equally high in abnormal mares, since they had more intrauterine fluid. In the lavage fluid of normal and abnormal mares, concentrations of PMN were equal.

All mares had higher numbers of PMN after frozen semen treatment than after PBS treatment as also shown by Kotilainen et al. [5]. Any uterine treatment is bound to cause some degree of PMN influx [23], but spermatozoa cause massive PMN chemotaxis by activating the complement [24]. It is obvious that the concentrated frozen sperm induced neutrophilia. There has been some concern that the components of freezing extenders (milk, egg yolk, glycerol) might cause uterine inflammation, but we have shown earlier [5] that they elicit only a minor reaction, comparable to PBS.

After frozen semen AI, the occurrence of IUFA was more likely than after PBS treatment. Whether intense neutrophilia and increased amounts of intrauterine fluid are initially connected is questionable. Semen concentration and volume did not correlate with IUFA 6 h after AI [5]. Intrauterine fluid tended to diminish with time after AI, since more mares exhibited IUFA 12 h after frozen semen AI than 18 to 24 h afterwards (35 vs. 14%) [8]. It is clear that in prolonged post-breeding endometritis, neutrophilia and intrauterine fluid occur together, as shown by Nikolokopaulos and Watson [7] 48 h after AI of susceptible mares with fresh extended semen.

The presence of IUFA could not be explained by deficient uterine contractions, at least not during these time-points and with these techniques. The mares were not examined during the first hour after AI, so any initially defective uterine contractions would have remained unnoticed. Scintigraphy has shown that uterine contractions are frequent and strong during the first 30 min after AI [10]. Further, we examined no mares between 8 and 20 h after AI, although susceptible mares have demonstrated weaker myometrial contractility 10 to 20 h after bacterial inoculation, as measured by electromyography [12]. Transrectal ultrasonography never shows the direction of contractions, and only strong contractions can be detected. Campbell and England [25] suggest using M-mode ultrasonography, which allows numerical recording of contractions and their amplitude, duration, and frequency.

Lysozyme levels and TIC were determined in uterine fluid because they are sensitive indicators of inflammation in the post-partum equine uterus [15]. In mares with IUFA, activities of TIC and BGase decrease, whereas NAGase levels are independent of the presence of IUFA. Although both NAGase and B-Gase are lysosomal enzymes, their secretion or inactivation rates may be different [16]. However, none of these variables appeared to give additional information as to the composition of the uterine fluid in abnormal mares or as to their reaction to frozen semen.

Because there were a low number of mares per group and because the group of mares had low fertility, pregnancy rates were not considered meaningful. The frozen semen doses used, despite reasonable progressive motility and adequate sperm numbers, were obviously not of very good quality.

The low pregnancy rates after frozen semen AI in the older mares showing chronic degenerative glandular changes cannot be explained by the sperm-induced inflammatory reaction, because the rates did not differ from the reaction in normal mares. The abnormal mares did not show fewer uterine contractions and did not accumulate more fluid after insemination than did the normal mares. The abnormal mares did not seem to react to frozen semen AI differently than the normal mares, but the low number of animals did not allow us to make definite conclusions. The majority of abnormal mares had IUFA already before the treatments, and the presence of fluid may decrease sperm viability or their transport. In vitro, incubation of sperm in uterine fluid reduces spermatozoal motility [16]. It is also possible that mares with degenerative uterine changes have pathological changes in the oviducts as well, which may affect the transport and survival of sperm in the oviduct. Frozen-thawed spermatozoa may require better conditions in the uterine and oviductal environment to insure survival. Dobrinski et al. demonstrated the better ability of fresh spermatozoa to attach to equine oviductal epithelial cells as compared to frozen-thawed spermatozoa [26]. The combination of frozen-thawed spermatozoa and mares with compromised oviductal function may result in the loss of spermatozoa and their function.

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