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Uterine fluid from bitches with mating-induced endometritis reduces the attachment of spermatozoa to the uterine epithelium

S.L. Freeman, M.J. Green, G.C.W. England *

School of Veterinary Medicine and Science, Faculty of Medicine and Health Sciences, University of Nottingham, Sutton Bonington Campus, Leicestershire LE12 5RD, UK

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ABSTRACT

Persistence of free fluid in the uterine lumen of bitches with endometrial hyperplasia appears to be diagnostic for mating-induced endometritis and is associated with reduced chances of pregnancy. This study investigated the possibility that reduced fertility might be associated with an effect of uterine fluid on sperm. Uterine lavage fluid was collected pre- and post-insemination from normal bitches without ultrasonographically-detectable luminal fluid ($n = 4$), and previously non-pregnant bitches with endometrial hyperplasia and luminal fluid ($n = 4$). Concentrations of polymorphonuclear neutrophils (PMNs) were measured and the effect of the fluid on the attachment of spermatozoa to the uterine epithelium was studied using medium (M) 199 as a control. To elucidate whether any effect was accounted for by the presence of PMNs, attachment was also measured in M199 with PMNs added at the concentration found in lavage fluid.

Pre-insemination lavage fluid from both groups contained low concentrations of PMNs which increased post-insemination; the increase was larger for bitches with uterine fluid. Compared with M199 controls, lavage fluid reduced the attachment of spermatozoa; fluid from bitches with endometrial hyperplasia and uterine fluid had a greater effect than normal bitches, and post-insemination fluid had a greater effect than pre-insemination fluid. Spermatozoal attachment was reduced by a similar magnitude for M199 with added PMNs, although post-insemination fluid from bitches with endometrial hyperplasia reduced attachment more than M199 with added PMNs. Poor fertility in bitches with uterine luminal fluid might be partially associated with impaired attachment of spermatozoa to uterine epithelium, mediated principally, but not solely, by PMN influx into the uterine lumen.

Introduction

There is increasing evidence that endometritis, and particularly mating-induced endometritis, is an important clinical condition of bitches (Watts and Wright, 1995; Fontaine et al., 2009; Groppetti et al., 2010; England et al., 2012a), which results in persistence of uterine luminal fluid (England et al., 2012b); is associated with influx of increased numbers of polymorphonuclear neutrophils (PMNs) after mating or insemination (England et al., 2012a), and results in reduced pregnancy rate and litter size (England et al., 2012a,b). Interestingly, recent work has also hinted that the aetiology of the reduced fertility might be associated with bacterial contamination of the uterus, since pregnancy rates can be increased by the oral administration of antibiotics post-mating (England et al., 2012a,b). While it has been documented that uterine epithelium from bitches with endometrial hyperplasia has reduced ability to allow the attachment of spermatozoa (England et al., 2012a), little else is known about the possible aetiology of the reduced fertility. For example, understanding the role of the persistent uterine fluid associated with endometrial hyperplasia might create opportunities to develop potential therapeutic interventions.

We hypothesise that at least part of the reduced fertility seen in bitches with mating-induced endometritis is associated with an environment that is hostile to spermatozoa caused by the persistence of uterine luminal fluid containing PMNs. The aim of this study was therefore to collect uterine lavage samples before and after insemination, from bitches with endometrial hyperplasia that developed mating-induced endometritis and from normal bitches, and to measure the concentration of PMNs and the effect of the lavage fluid on the attachment of spermatozoa to the uterine epithelium in vitro. A secondary aim was to investigate whether any effect of the lavaged fluid could be attributed to the presence of PMNs.

Materials and methods

Ethical approval

Ethical approval for the procedures was granted by the School of Veterinary Medicine and Science, University of Nottingham. All animals were examined with owner consent.

* Corresponding author. Tel.: +44 115 9516411.
E-mail address: gary.england@nottingham.ac.uk (G.C.W. England).

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Study population and animal management

Bitches were identified from a group of 63 that had been monitored throughout a previous oestrous cycle, at which time they were mated and were allocated to one of two groups, either (1) normal, where a homogenous endometrium with no cystic structures had been identified by ultrasound prior to the onset of proestrus, and in which no uterine luminal free fluid had been detected prior to or after mating, and where the bitch had become pregnant at that oestrous cycle and had a normal litter size for the breed, or (2) endometrial hyperplasia and mating-induced endometritis, where 2–7 anechoic (fluid-filled) cysts of 0.5–2.5 mm in diameter had been identified by ultrasound prior to the onset of proestrus (England et al., 2012a,b), small volumes of uterine luminal free fluid had been detected both prior to mating and after mating, and where the bitch had not become pregnant at that oestrous cycle.

Throughout the oestrous cycle described in this study, and the previous cycle, detailed ultrasound examinations of the uterine body and distal uterine horns were performed trans-abdominally using a 10 MHz transducer (Pie Veterinary). Four normal bitches (one Labrador and three Golden retrievers, aged 49–61 months) and four bitches with endometrial hyperplasia that had developed mating-induced endometritis at a previous oestrus (one Labrador and three Golden retrievers, aged 66–79 months) were therefore identified. Because breeding guidelines required that bitches were not pregnant at consecutive oestrous periods, the normal bitches were examined for the present study after one rest oestrus (i.e. approximately 14 months after they were previously examined, mated and became pregnant) whilst the bitches with endometrial hyperplasia were examined at the subsequent oestrus (i.e. approximately 7 months after they were previously examined, found to have post-mating endometritis and did not become pregnant).

Approximately 5 days after the onset of proestrus of the present study, the bitches were examined with ultrasound as previously described to confirm that all normal bitches continued to have a normal endometrial appearance and absence of uterine luminal free fluid, and that all bitches with endometrial hyperplasia had obvious endometrial cysts and continued to have uterine luminal free fluid. Ultrasound examination was repeated 5 days after estimated ovulation (see below), prior to the last insemination, to confirm the presence or absence of uterine luminal free fluid.

Plasma progesterone concentrations were measured three times each week commencing 5 days after the onset of proestrus using an ELISA (Ridgeway Science) and the day of ovulation (Day 0) was estimated to be when progesterone concentrations first exceeded 5.0 ng/mL. Bitches were inseminated trans-cervically with fresh semen on Days +2, +4 and +5 as described below. Semen from six different males was used for insemination, all of which had impregnated a bitch in the previous 2 months. Semen evaluated at the time of insemination showed greater than 70% forward progressive motility and 76% morphologically normal live spermatozoa using standard techniques (England, 1999).

For uterine lavage and insemination, a 6 French gauge catheter was passed through the cervix using a rigid cysto-urethroscope (Richard Wolf Medical Instruments Corporation). On Day +2 (before the first insemination; termed 'pre-insemination') and Day +5 (after two inseminations but before the third insemination; termed 'post-insemination'), 2.0 mL medium (M) 199, pre-loaded within the catheter and syringe, was used to lavage the uterine body immediately prior to deposition of semen. Approximately 1.0 mL fluid was recovered after lavage. One drop of undiluted lavage fluid was immediately placed into a haemocytometer and the concentration of non-epithelial cells was calculated before transfer to a plain slide and Romanowsky staining (Diff Quik) to determine the concentration of PMNs/mL.

On each day that uterine lavage was performed, discarded blood samples taken for progesterone assay were used for isolation of PMNs using Percoll gradient centrifugation (Redl et al., 1983). PMNs were re-suspended in M199 at pH 7.8 supplemented with 0.632 g/L penicillin (Sigma–Aldrich) and 0.050 g/L streptomycin (Sigma–Aldrich) at a PMN concentration equivalent to that found in the fluid lavage from the relevant bitch. Samples were only used when more than 90% of PMNs were confirmed live by exclusion of Trypan blue stain following examination at ×400 with bright field microscopy (Strzemienski, 1989).

To establish binding of spermatozoa to uterine epithelium, reproductive tracts were collected from eight healthy bitches of various breeds (aged 13–19 months) presented for routine neutering at >3 months since the previous oestrus; absence of corpora lutea within the ovaries and the clinical history was used to confirm the cycle stage as anoestrus. One tract was used to investigate how lavage fluid from each bitch on each day influenced the attachment of spermatozoa. Three 1 cm sections from the uterine horns were placed in M199 supplemented with penicillin and streptomycin as described above at pH 7.8 and incubated with 5% CO_2 in air at 39 °C for ⩽6 h. The epithelial mucosa was then isolated and nine approximately 1 mm^2 explants were prepared and placed individually into culture wells (Corning) containing one of three treatments (three explants per treatment; (1) 0.5 mL M199 (control); (2) 0.5 mL uterine lavage fluid; and (3) 0.5 mL M199 + PMNs at the same concentration as in the uterine lavage fluid.

Pooled ejaculates were collected from the same three Labrador dogs on each study day. Samples were subjected to evaluation of progressive forward motility, sperm concentration and vital staining using nigrosin eosin (England, 1999). Throughout the study the percentage progressive motility and the percentage of morphologically normal spermatozoa were greater than 70% and 78%, respectively, for every sample. Pooled ejaculates were diluted in M199 at 39 °C to 35×10^6 spermatozoa/mL. Spermatozoa were loaded with fluorescent dye by adding 0.1 mg/mL Hoechst 33342 (Sigma–Aldrich) to a concentration of 3 μg H33342/mL. After 1 min, 4 mL of the labelled sperm were added to 3 mL M199 at 39 °C to a concentration of 20×10^6 spermatozoa/mL; this fluid was then added to culture wells containing explants to a final sperm concentration of 10×10^6/mL. Sperm-explant co-culture plates were incubated at 39 °C with 5% CO_2 for 30 min. The explants were washed six times by vigorous pipetting in M199 and were fixed by adding 2 mL 3% glutaraldehyde solution (BDH Laboratories) in 0.1 M phosphate buffer before they were transferred to a slide with the epithelial cell surface uppermost and mounted with Vectashield (Vector Laboratories). Explants were examined at ×400 magnification under ultraviolet illumination (excitation at 330–380 nm, emission at 420 nm). The numbers of blue-stained (attached) spermatozoa in a 0.18 mm × 0.18 mm area (0.0324 mm^2) were counted in three areas. The numbers of spermatozoa attached to 1 mm^2 of explants were calculated and the numbers of attached spermatozoa were adjusted according to the percentage of live sperm added.

Statistical analysis

The overall aim of the analysis of the data relating to the attachment of spermatozoa was to make a within-bitch comparison of the effect of treatment (M199 alone, lavage fluid, or M199 with PMNs added at the same concentration as found in lavage fluid) on sperm attachment to the uterine epithelium. Day of flushing was a variable in the analysis (+2 or +5), as was whether the bitch was previously diagnosed with cystic endometrial hyperplasia, presence or absence of uterine fluid, and bitch age. Following the initial descriptive analysis, a multilevel linear model was built with sperm attachment as a continuous outcome variable and a random effect to account for the underlying effect of bitch (Goldstein, 1995). The model took the form:

$$\text{Sperm attachment}_{ij} = \alpha + \beta_1 X_{ij} + \beta_2 X_j + u_j + e_{ij}$$

where sperm attachment$_{ij}$ was a measurement of the number of sperm bound in the ith experimental unit of the jth bitch; α was the regression intercept; X_{ij} represented covariates measured at the unit level (type of fluid); β_1 was the coefficient for X_{ij}; X_j represented covariates measured at the bitch level (age and presence of endometrial hyperplasia); β_2 was the coefficient for X_j; u_j was a random effect to account for between-bitch variation (assumed to be normally distributed), and e_{ij} was a term to reflect residual error (also assumed to be normally distributed). Models were fitted in MLwiN (version 2.26 Centre for Multilevel Modelling, University of Bristol).

Model fit was found to be good; graphical evaluations of standardised residuals revealed a normal distribution with no points exerting excessive influence on model parameters. The variable 'bitch' was investigated for inclusion as a fixed effect as well as a random term, but in neither instance did it have any influence on other model parameters. Therefore, the inclusion of bitch as a random term was used to provide estimates for the final model parameters. Model parameters were accepted as significant when $P < 0.05$.

A secondary analysis was conducted to evaluate differences in PMN concentration in fluid from bitches on Days +2 and +5, both with and without cystic endometria hyperplasia. A conventional two way analysis of variance (ANOVA) was undertaken with PMN concentration as the response variable and time period and CEH as two explanatory factors. Terms were deemed significant at $P < 0.05$ and ANOVA fit was evaluated using a visual assessment of residuals alongside tests for normality.

Results

All of the bitches had an apparently normal oestrous cycle, ovulated and were inseminated. All four of the normal bitches became pregnant with a mean litter size of 7.3 ± 0.9 (standard deviation, SD) pups, but none of the bitches with endometrial hyperplasia that had previously developed mating-induced endometritis became pregnant.

Ultrasound examination 5 days after the onset of proestrus and on Day +5 demonstrated no endometrial cysts and an absence of uterine fluid in the normal bitches, and confirmed the continued existence of endometrial cysts and the presence of uterine luminal free fluid on both days in the bitches with endometrial hyperplasia. The uterine fluid appeared as a thin anechoic line, commonly 1–2 mm in depth, confined to specific regions of the uterus rather than being identified throughout the whole of the lumen; careful examination was required to identify these small 'pockets' of fluid.

Mean (±SD) data and results of the linear model are shown in Tables 1 and 2, respectively. Compared with sperm attachment in M199 pre-insemination (Day +2), all other treatments were associated with reduced attachment of spermatozoa except for