Effect of stage of cycle, sampling frequency and recovery of micro-organisms on total protein content of mare uterine flushings

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Summary. Mares with sound reproductive tracts were divided into two groups. In Group I (N = 12), uteri were flushed once per oestrous cycle during alternate cycles while in Group II (N = 8) mares were flushed twice in a cycle for 2 contiguous cycles. Total protein concentrations and total recoverable protein of uterine flushings taken on Day 3 of oestrus and Day 8 after ovulation in each of the 2 groups and between the 2 groups did not differ significantly. The length of oestrus and dioestrus was not affected by the flushing procedures. Total recoverable protein and total protein concentrations of flushings were higher at Day 3 of oestrus and Day 8 and 15 after ovulation ($P < 0.01$) when a micro-organism was isolated from the uterus before flushing.

Introduction

During oestrus, the mare cervix is relaxed, providing a direct route for micro-organisms to be carried into the uterus at coitus (Millar, 1952). The uterus at this time not only must move spermatozoa towards the oviduct but must also support sperm survival and eliminate micro-organisms so that the environment is suitable for the spermatozoa as well as for the developing embryo.

Information is scanty concerning mare uterine control of microbial growth or the concomitant uterine secretory changes induced by uterine micro-organisms. Six of the 10 known equine immunoglobulins have been found in mare uterine flushings (Kenney & Khaleel, 1975) and levels of some of these immunoglobulins appear to be higher in fertile than in sub-fertile mares (Asbury, Halliwell, Foster & Longino, 1980; Mitchell, Liu, Perryman, Stabenfeldt & Hughes, 1982). Flushings from infected mare uteri also have significantly higher total protein concentrations than do those from uteri not harbouring bacteria (Blue, Brady, Davidson, & Kenney, 1982). The influence of micro-organisms that do not infect the uterus on uterine protein levels has not been determined.

Reports of mare uterine secretory activity in relation to the oestrous cycle are at variance: secretory rates are higher at dioestrus (Zavy, Bazer & Sharp, 1978) or are not different in horse mares (Blue *et al.*, 1982) or pony mares (Zavy *et al.*, 1982). One difference is the frequency of collection: flushings were taken by Blue *et al.* (1982) twice in a cycle whereas Zavy *et al.* (1978) obtained one flushing in a cycle.

The objective of the present studies of mare uterine resistance to bacteria was to determine whether protein content of uterine fluid is affected by frequency of flushing, presence of micro-organisms in the uterus before flushing or stage of cycle.

Materials and Methods

Mixed-breed mares with an average age of 10 years were used. The 20 mares were maintained on pasture with a daily grain ration and were selected on the basis of having a palpably normal genital tract, a Category I or II endometrium (Kenney, 1978) and an endometrial swab culture at the time of examination which gave no bacterial growth. The mares were individually teased with a stallion daily for signs of oestrus. Ovulation was confirmed by palpation *per rectum*. The day of ovulation was termed Day 1 after ovulation.

Flushing procedure

Mares were confined in stocks with their tails enclosed in plastic bags. The vulva and perineal area were alternately scrubbed and rinsed three times using a povidone-iodine solution (Betadine: Purdue Frederick Co. CT 06856, U.S.A.). A double-guarded swab was passed into the uterine lumen (Blanchard, Garcia, Hurtgen & Kenney, 1981) and subsequently streaked on a 5% sheep blood agar plate. Microbial growth was assessed after aerobic incubation for 48 h at 37°C. An endometrial swabbing was considered positive if 2 or more similar colonies were isolated on the streak. When an organism was isolated, the endometrium was reswabbed 3–6 days later and results compared. After swabbing the endometrium, the tip of a modified Foley catheter was immediately placed into the uterine lumen. The cuff was filled with approximately 100 cc of air to seal the internal os of the cervix. Sterile saline, 50 ml 0.9% (w/v) NaCl, was then infused into the uterine lumen and allowed to equilibrate for 2 min. Fluid was collected aseptically by gravity into a sterile 50-ml plastic centrifuge tube (Falcon). Procaine penicillin (3×10^6 units, Squibb) or neomycin sulphate (2 g, Professional Veterinary Laboratories) in 60 ml sterile saline were then infused into the uterine lumen. The flushings were centrifuged initially for 20 min at 600–1000 *g*. The supernatant was centrifuged again at 4°C for 50 min at 10 000 *g*. The flushings were frozen in aliquots at –20°C until assay.

Flushing intervals

All mares were allowed to undergo one oestrous cycle to establish that they had normal cyclic behaviour. Flushings were taken either once (Group I) or twice (Group II) within an oestrous cycle. For mares flushed once in a cycle, flushings were collected during alternate oestrous cycles on Day 3 of oestrus or Day 4, 6, 8, 10 or 15 after ovulation. When two flushings were taken within an oestrous cycle, one flushing was taken on Day 3 of oestrus and the other on Day 8 after ovulation during contiguous cycles. Flushings were also taken from anoestrous mares in January and early February (Group A). The presence of small, inactive ovaries, a flaccid cervix and lack of oestrous behaviour for 3 weeks were the criteria used to identify anoestrus.

Protein analysis

Total protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin (Sigma, St. Louis, MO, U.S.A.) as a standard. Differences in total protein concentration and total recoverable protein (total protein concentration $\times$ volume of flushing fluid recovered) due to sampling frequency were analysed as 2×2 factorials using two levels of frequency and two stages of cycle. Total recoverable protein and total protein concentration of flushings recovered at Day 3 of oestrus, Days 8 and 15 after ovulation and during anoestrus were analysed by one-way analysis of variance. The effect of micro-organisms on total protein concentration and total recoverable protein was analysed as 2×3 factorials with a no-growth and a positive growth status of the uterus as the first factor and three stages of the cycle as the second factor (Steel & Torrie, 1960).

Results

Recovery of fluid infused into the uterine lumen averaged 76% (Table 1). No statistical difference was found for recovery at different stages of the cycle, or in relation to the presence of micro-organisms or frequency of flushing. Considerable variation in recovery occurred for flushings taken at oestrus. Part of this variation was due to a failure of the catheter cuff to prevent fluid leakage through a relaxed cervix. On the other hand, recoveries have also reached 150% at oestrus.

Table 1. Comparison of (%) uterine fluid recovery from mares according to whether uterine swab cultures were positive or negative

Stage of cycle	Positive/negative swab	No. of samples	% recovery* (mean $\pm$ s.e.m.)
Day 3 of oestrus	—	18	68.8 $\pm$ 8.0
	+	7	86.3 $\pm$ 13.5
Dioestrus	—	24	83.1 $\pm$ 3.4
	+	5	80.8 $\pm$ 4.5
Anoestrus	—	4	58.0 $\pm$ 17.7
	+	1	32.0
Total		59	76.4 $\pm$ 3.6

*(Volume collected/volume infused) $\times$ 100.

Flushing did not affect the length of oestrus or dioestrus. The length of dioestrus in cycles before flushing ($n = 28$) was 16.25 ± 0.56 (s.d.) days while the length of oestrus was 5.57 ± 0.39 days. For mares in Groups I and II dioestrus continued for 17.00 ± 0.72 days ($n = 22$) and 17.44 ± 1.26 days ($n = 9$) respectively while oestrus lasted for 5.86 ± 0.45 and 6.22 ± 0.46 days respectively.

Total recoverable protein and total protein concentration of flushings taken from uteri with no bacterial growth before flushing are shown in Table 2. The levels of total protein concentrations and of total recoverable protein in flushings taken on Day 8 after ovulation from Group I mares, although higher, were not significantly different from values at Day 3 of oestrus or at Day 8 after ovulation in Group II mares. Pooled values for Day 3 of oestrus and for Day 8 after ovulation were

Table 2. Total protein concentrations and total recoverable protein in uterine flushings from mares which had negative or positive uterine swabs

Group	Stage of cycle	Negative swabs			Positive swabs		
		No. of samples	Total protein (μ g/ml)	Total recoverable protein (mg)	No. of samples	Total protein (μ g/ml)	Total recoverable protein (mg)
I	Day 3 of oestrus	8	458.74 $\pm$ 47.66	15.40 $\pm$ 0.99			
	Day 4†	1	219.65	10.54			
	Day 6†	1	446.05	21.41			
	Day 8†	7	889.80 $\pm$ 303.94	32.00 $\pm$ 14.84			
	Day 10†	1	427.92	17.54			
	Day 15†	4	629.34 $\pm$ 86.83	29.08 $\pm$ 3.98	2	2982.30 $\pm$ 109.50*	120.18 $\pm$ 11.97*
II	Day 3 of oestrus	9	487.36 $\pm$ 66.89	17.91 $\pm$ 3.52			
	Day 8†	12	720.32 $\pm$ 156.06	27.63 $\pm$ 7.50			
A	Anoestrus	4	838.41 $\pm$ 383.36	15.28 $\pm$ 4.08	1	1859.00	29.74
I + II	Day 3 of oestrus	17	473.89 $\pm$ 40.80	16.73 $\pm$ 1.89	7	1840.38 $\pm$ 1042.86*	63.46 $\pm$ 27.14*
I + II	Day 8†	19	782.76 $\pm$ 145.31	28.91 $\pm$ 6.62	3	1323.38 $\pm$ 413.29*	52.74 $\pm$ 17.05*

Values are mean $\pm$ s.e.m.

* Significantly different from values for negative mares, $P < 0.01$.

† After ovulation.

therefore used for further statistical comparisons. The values for Group I mares on Day 15 after ovulation and Group A mares were not significantly different from those for Day 3 of oestrus and Day 8 after ovulation (Groups I & II, Table 2). The flushings taken at Day 4, 6 or 10 after ovulation in Group I mares also had total protein values within the range found for Day 3 of oestrus and Day 8 after ovulation.

When micro-organisms were recovered from the uterus before flushing, total protein concentration and total recoverable protein levels increased (Table 2). Mean values at Day 3 of oestrus and Days 8 and 15 after ovulation were significantly higher ($P < 0.01$) than mean values when the uterus was not harbouring an organism.

Most flushings appeared clear at recovery without a noticeable difference in pellet size after centrifugation. However, considerable variation in total protein concentrations was noticed, especially in flushings taken on Day 3 of oestrus. When the individual total protein concentrations were examined and compared with those in Table 2, values were not always elevated when an organism had been isolated before flushing (Table 3). For example, *Aspergillus* sp. was not associated with a dramatic increase in total protein concentration or total recoverable protein levels, as was seen with *Pseudomonas aeruginosa* or *Staphylococcus* sp. With the exception of *Escherichia coli*, uterine residence of these micro-organisms was short-lived because they were not recovered when the uterus was re-swabbed 3–6 days later. The uterus from which *E. coli* was isolated was treated with antibiotics to ensure immediate recovery for another series of experiments. A vulvar discharge was not detected with any of the isolations.

Table 3. Total recoverable protein and total protein concentration in uterine flushings after recovery of specific micro-organisms from the uteri of mares

Stage of cycle when flushed	Micro-organism	Total protein (µg/ml)	Total recoverable protein (mg)
Day 3 of oestrus	<i>Aspergillus</i> sp.	1073.6	30.06
		680.4	29.26
	<i>Staphylococcus</i> sp.	574.0	28.70
	<i>Pseudomonas aeruginosa</i>	1446.8	108.51
		508.5	10.17
	β-Haemolytic streptococcus	550.1	19.80
Day 8 after ovulation	<i>Mucor</i> sp.	8049.0	402.45
	<i>Aspergillus</i> sp.	519.0	22.31
	<i>Pseudomonas aeruginosa</i>	1561.0	54.64
	<i>Escherichia coli</i>	1890.20	81.28
Day 15 after ovulation	<i>Staphylococcus</i> sp.	2872.8	132.15
	Not identified	3091.8	108.21
Anoestrus	β-Haemolytic streptococcus	1859.0	29.74

Discussion

Total protein concentration and total recoverable protein levels in mare uterine flushings taken singly on Day 8 after ovulation in alternate cycles were elevated but not significantly higher than values obtained on Day 8 after ovulation for flushings taken as one of two flushings in successive cycles. Evidently the interval chosen for flushing in successive cycles is ample for the maintenance of uterine protein levels. A significant rise of protein levels in flushings taken during alternate cycles would indicate a slow replacement of uterine luminal proteins by the mare.

The technique of flushing, by its limitations, can only estimate uterine fluid content (Heap, 1962). The rise in protein content of uterine flushings at mid- and late dioestrus found by Zavy *et al.* (1978) indicates increased uterine secretory activity during dioestrus. However, in this study and in

the work of Blue *et al.* (1982), no significant rise in uterine fluid protein was found during dioestrus. This discrepancy may be resolved by examination of flushing constituents. An increase in uterine total protein concentration would be expected if there was an inflammatory reaction to a micro-organism due to mobilization of white blood cells and increased vascular permeability (Hawk, Brinsfield & Righter, 1963). Low protein values associated with some positive swab cultures in this study may be due to uterine drainage through a relaxed cervix under the influence of oestrogen (Black, Simon, Kidder & Wiltbank, 1954). Low values may also indicate a contaminant picked up from the lower reproductive tract. The use of a double guarded swab greatly reduces this possibility (Blanchard *et al.*, 1981) but does not abolish it. The micro-organisms recovered in this study may also have been transitory in nature. The uterus is prone to invasion of bacteria from the lower reproductive tract. Observations of women (Bollinger, 1964) and mares (Scott, Daley, Baird, Sturgess & Frost, 1971; Newcombe, 1978) have indicated a transient population of microbes in the uterus. Positive swab cultures of the uterus have been recovered with an absence of uterine inflammation viewed histologically (Williamson, Dunning, O'Connor & Penhale, 1983). Therefore, low total protein values, may indicate little reaction to the isolated organism while micro-organisms penetrating the mucosal barrier may activate the second line of defence (Brandtzaeg, 1973) with a resultant increase in total protein concentration of the flushings.

At no time in this study was a cycle significantly shortened due to flushing. This is in agreement with the work of others (Zavy *et al.*, 1978) in that trauma to the uterus is minimal compared to infusion of large volumes of saline (Arthur, 1975). Uterine biopsy or cervical dilatation has been shown to shorten the mare oestrous cycle (Hurtgen & Ganjam, 1979). To investigate whether the corpus luteum may be influenced by flushing, plasma samples were taken from two mares after flushing on Day 8 after ovulation. Progesterone levels from these samples were compared to progesterone levels 24 h later: on Day 8 the values were 14.5 and 13.3 ng/ml and on Day 9 they were 15.5 and 15.4 ng/ml respectively. These results suggest that corpus luteum function has not been altered by the flushing procedure. Failure to shorten the cycle in this study may have been due to lack of cervical and uterine stimulation during flushing or removal of the responsible mediator (e.g. prostaglandin) with the flushing.

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