Department of Clinical Veterinary Sciences
Faculty of Veterinary Medicine
University of Helsinki,
Helsinki, Finland
and
MTT Agrifood Research Finland
Animal Production Research
Equines
Ypäjä, Finland

UTERINE LUMINAL ENVIRONMENT OF THE MARE

by

Tiina Reilas

ACADEMIC DISSERTATION

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To my family

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ORIGINAL ARTICLES

ABSTRACT

The uterine luminal environment, determined primarily by uterine secretions, must offer optimal conditions first for the fertilizing spermatozoa and later for the development of the embryo. Relatively few studies exist, however, concerning the biochemical composition of equine uterine secretions. This thesis includes investigations on centrifuged uterine lavage fluids (ULFs) collected after hormone treatments and bacterial inoculations of ovariectomized (OVX) mares. Uterine lavage fluids were also collected from non-parturient cyclic mares and from post-partum (p.p.) mares within 4 weeks of parturition, after the first p.p. ovulation and before embryo recovery 7 to 8 days later. Polymorphonuclear neutrophils (PMNs) were compared with total protein and lysozyme concentrations, trypsin-inhibitor capacity (TIC), and activities of plasmin, N-acetyl-ß-D-glucosaminidase (NAGase), ß-glucuronidase (B-Gase) and acid phosphatase to determine whether more sensitive inflammatory markers could be found to diagnose endometritis. In p.p. mares, these parameters were used to determine the restoration of secretory activity after foaling and to investigate their relation to foal heat fertility. Undiluted uterine fluids were collected by tampon from oestrous mares to study whether composition of uterine fluid differs in mares with and without ultrasonically detectable intrauterine fluid accumulations (IUFA). The intensity of inflammatory reaction 6 h after different types of insemination was studied by analysing PMNs from lavage fluid.

Seven days' treatment of OVX-mares with progestin increased accumulation of NAGase, B-Gase and acid phosphatase ($P<0.05$) in ULF. PMNs, lysozyme concentration, TIC and plasmin activity were mostly below detection limits. After bacterial inoculation, PMN numbers remained significantly elevated until 24 h in control mares and in mares treated with oestradiol, and until day 21 in mares treated with progestin plus oestradiol or progestin alone. Lysozyme activities were elevated in both acute and persistent endometritis. TIC was a better indicator of acute than of persistent inflammation. Despite acute inflammation, total protein concentration, TIC and plasmin activity did not increase significantly in oestradiol-treated mares. Progestin, not infection, was the main reason for increased activities of NAGase, B-Gase and acid phosphatase.

In non-parturient mares, activities of NAGase, B-Gase and acid phosphatase in ULFs were significantly higher in dioestrus than in oestrus ($P<0.05$). Lysozyme concentration, TIC and plasmin activity were below detection limits. In p.p. mares, levels of total protein, NAGase, B-Gase and acid phosphatase were high soon after foaling but thereafter declined rapidly. In most mares, lysozyme and plasmin activities decreased after foaling. TIC peaked around day 6 p.p.. Total protein and lysozyme concentrations, TIC, and B-Gase ($P<0.01$) and acid phosphatase ($P<0.05$) activities were significantly higher in parturient mares during p.p. oestrus than in oestrous non-parturient mares. During dioestrus, differences between p.p. and non-parturient mares were not statistically significant.

After the first p.p. ovulation but prior to insemination, PMNs, TIC and lysozyme levels in ULF were elevated in 3/4 mares not producing embryos. However, only 1/5, 1/5 and

0/5 embryo-producing mares had elevated levels of PMNs, TIC and lysozyme, respectively. None of the measured parameters was significantly related to embryo recovery 7 days later ($P \leq 0.10$). At embryo recovery, NAGase was higher in mares not producing embryos ($P < 0.05$). The relation of this finding to embryo recovery is unclear.

IUFA were detected by ultrasonography in 39% of oestrous mares. None of the mares exhibited cytological or bacteriological evidence of acute endometritis. Total protein concentration, TIC and B-Gase activity in undiluted uterine fluid were significantly lower in mares with than in those without IUFA ($P < 0.01$), whereas NAGase and plasmin activity did not differ between these groups. The presence of small fluid accumulations at the time of insemination did not seem to affect fertility.

In the post-breeding study, mares infused with phosphate-buffered saline, seminal extenders or supernatant from centrifuged frozen-thawed semen exhibited only a mild neutrophil response. Insemination with frozen semen resulted in higher concentrations of PMNs than insemination with extended fresh semen ($P < 0.05$). Highest PMN counts were found after insemination with frozen semen or concentrated fresh semen. Bacterial contamination of uteri was negligible 6 h after breeding.

In conclusion, none of the examined parameters was superior to PMN counts in diagnosing endometritis. High total protein concentration, TIC and detectable lysozyme and plasmin activities during p.p. oestrus were associated with uterine inflammation. The endometrium of p.p. mares had presumably resumed normal secretory capacity by the time of the first p.p. dioestrus. Endometrial inflammation at the time of insemination seems to be the best explanation for lowered foal heat fertility. Non-echogenic fluid accumulations during oestrus were associated with compositional changes in uterine secretions. Neutrophilia appears to be induced by spermatozoa rather than bacteria. Intensity of the neutrophil reaction apparently depends on concentration and/or volume of inseminate.

LIST OF ORIGINAL ARTICLES

This thesis is based on the following original articles which are referred to in the text by their Roman numerals:

I. Reilas, T., Ristiniemi, M. & Katila, T. Influence of hormone replacement therapy and bacterial inoculation on proteins and enzymes in uterine lavage fluid of ovariectomized mares. Reprod. Dom. Anim. 33, 1998: 11-19.

II. Kotilainen, T., Huhtinen, M. & Katila, T. Sperm-induced leukocytosis in the equine uterus. Theriogenology 41, 1994: 629-636.

III. Reilas, T., Katila, T., Mäkelä, O., Huhtinen, M. & Koskinen, E. Intrauterine fluid accumulation in oestrous mares. Acta vet. scand. 38, 1997: 69-78.

IV. Reilas, T. & Katila, T. Proteins and enzymes in uterine lavage fluid of postpartum and nonparturient mares. Reprod. Dom. Anim. (accepted).

V. Reilas, T., Huhtinen, M., Oksanen, M. & Katila, T. Relationship between embryo recovery rate and uterine lavage fluid composition in postpartum mares. Reprod. Nutr. Dev. 40, 2000: 383-391.

ABBREVIATIONS

AI	artificial insemination
B-Gase	β-glucuronidase
BSA	bovine serum albumin
CV	coefficient of variation
DPBS	Dulbecco's phosphate-buffered saline
E	oestrogen/oestradiol
E. coli	*Escherichia coli*
FCS	foetal calf serum
GAGs	glycosaminoglycans
i	inoculation
Ig	immunoglobulin (A, G, M, T)
i.m.	intramuscular
i.u.	intrauterine
IUF	intrauterine fluid
IUFA	intrauterine fluid accumulation (detected by ultrasonography)
i.v.	intravenous
MHC	major histocompatability complex
MOT	total motility
4-MU	4-methylumbelliferone
NAGase	N-acetyl-β-D-glucosaminidase
OVX	ovariectomized
P	progesterone/progestin
PBS	phosphate-buffered saline
PG	prostaglandin
p.o.	per os
p.p.	post-partum
α_1-PI	alpha-1-proteinase inhibitor
PMN	polymorphonuclear neutrophil
PMOT	progressive motility
RT	room temperature
S. aureus	*Staphylococcus aureus*
SD	standard deviation
SEM	standard error of mean
spp.	species
Strep.	*Streptococcus*
TIC	trypsin-inhibitor capacity
U	μmol product/min
ULF	uterine lavage fluid
VAP	average path velocity

INTRODUCTION

The uterus has many roles: it is the site of semen deposit in the horse and contributes to the travel of spermatozoa on their way to the oviduct, it houses and nourishes the rapidly growing foetal foal, and finally, the myometrium is essential in the expulsion of the foal during parturition. The pregnancy causes enormous changes in the myometrium, which has to adapt and stretch from dimensions of centimetres to metres during the gestation of 11 months (Rossdale 1997). As amazing is the rapid return of the organ to its original size within 2 to 3 weeks and the ability of the mare to conceive in the first post-partum (p.p.) oestrus (known as foal heat) as early as only a week after parturition. However, the pregnancy rates in foal heat are usually lower than in subsequent oestruses. Unfortunately, we have no reliable way of diagnosing which mares are ready to be bred in foal heat and which ones are not.

The composition and quality of the uterine environment determine reproductive readiness and performance of the female. The uterus is part of the endocrine system: a target organ for steroids, but also the site of prostaglandin production. Hormones control quantitative and qualitative secretory activity of the uterus. Synchrony between maternal hormones and embryonic development is obligatory to establish pregnancy (Fischer & Beier 1986; Hinrichs & Kenney 1987). Before implantation, the conceptus is supported solely by secretions that accumulate in the uterine lumen; a phenomenon called histotrophic nutrition (Ashworth 1995). In the mare, implantation and placentation take place considerably later than in other species, and therefore, histotrophe is particularly important in the horse. An inadequate uterine milieu causes embryonic mortality, which has a high incidence in horses. Changes in uterine environment during puerperium, inflammation and stages of the cycle are not very well known in horses.

The genitalia of stallions and mares harbour a microflora consisting of many bacterial species, the most frequently occurring of which are streptococci (Dimock & Snyder 1924; Dimock & Edwards 1928). Dimock & Edwards (1928) recognized that stallions may infect mares and that infection of mares at the time of service is probably dependent upon some predisposition. Mares who are unable to clear bacteria after breeding continue to present a problem in studfarm practice. In mares, endometritis is often not very obvious. Discharge from the vulva may alert the owner, but sometimes the only sign is a failure to conceive. A cytological examination to identify polymorphonuclear neutrophils (PMNs) is an easy and quick way to diagnose acute endometritis and distinguish between bacterial contamination and significant endometritis (Knudsen 1964a; Wingfield Digby 1978); however, it does not detect chronic endometritis. In contrast, while histopathology of uterine biopsies reveals acute and chronic inflammation in addition to degenerative glandular changes (Kenney 1978), it is not a quick test. Detection of excessive intrauterine fluid is easy with ultrasonography (Ginther & Pierson 1984), but it gives little information on the composition of the fluid. Thus, there is a need to find new tests for diagnosis of acute and persistent endometritis which are sensitive, rapid and easy to perform.

REVIEW OF LITERATURE

1. Uterine luminal environment

The uterine environment has to offer optimal conditions first for the fertilizing spermatozoa and later for the development of the embryo. Uterine fluid consists of hormones, prostaglandins, enzymes, energy substrates, ions, vitamins, amino acids, peptides, serum proteins and uterine proteins (Fischer & Beier 1986). The blood-uterine lumen barrier determines the rate at which matter is exchanged between the vascular and extravascular fluids of the uterus. Extracellular uterine fluid can be divided into the following compartments: vascular, endometrial extracellular and luminal extracellular (McRae 1988).

1.1. Effect of steroid hormones

According to McRae (1988), the uterine luminal milieu is dynamic since the composition of the fluid varies between stages of the oestrous cycle and during the course of pregnancy. The secretion and release of proteins and enzymes are regulated by steroids. Tight junctions between epithelial cells change with hormone status. The normal uterus contains small amounts of fluid that is produced locally by uterine glands or represents transudate from blood vessels. Uterine capillaries and venules are relatively impermeable to plasma proteins except during transient responses to oestrogen. Epithelial cells are selectively permeable to substances primarily on the basis of molecular size and lipid solubility. Uterine epithelium functions as a rate-limiting boundary between blood and uterine lumen even when vascular permeability is increased (McRae 1988).

Ovarian steroids cause structural and functional alterations to the endometrium. The effects of steroids can be studied in cyclic mares and in hormone-supplemented ovariectomized (OVX) or seasonally acyclic mares. The hormone supplementations are oestrogen (E), progesterone (P) and combinations of oestrogen and progesterone (E + P).

It is commonly agreed that intrauterine fluid accumulation (IUFA) during dioestrus indicates an inflammatory process with negative effects on pregnancy (Adams et al. 1987). In contrast, the significance of IUFA during oestrus is unclear. In anovulatory mares, IUFA was often detected, but the presence of fluid changed dynamically, and the fluid was not associated with uterine or oviductal inflammation (Losinno et al. 1997). Significant differences have been shown for uterine fluid concentrations of total protein (Zavy et al. 1979, 1982), acid phosphatase (Zavy et al. 1979, 1982; McDowell et al. 1987; LeBlanc et al. 1988) and prostaglandins (PGF and PGE_2) (Watson et al. 1988a), all being higher during progesterone dominance. Total protein concentrations in uterine lavage fluids peaked at day 12 of dioestrus and decreased thereafter (Zavy et al. 1979, 1982). Similar findings have been reported by others, but the differences between oestrus and dioestrus have not been statistically significant (Strzemienski & Kenney 1984; LeBlanc et al. 1988). Three progesterone-dependent proteins have been separated from uterine secretions of mares by Beier-Hellwig et al. (1995): uteroglobin

(<6.5 kDa), equine phospholipase A2 (17 kDa) and a third unidentified protein migrating at 22 kDa.

Asbury et al. (1980) reported that IgA levels were highest during oestrus, whereas IgG was detected only from dioestrous samples of uterine fluid. However, Widders et al. (1985) did not notice cyclic changes in free Ig staining or plasma cell numbers using immunoperoxidase staining techniques. Similarly, hormone treatment (E or P) of OVX-mares did not significantly affect the numbers of cells secreting IgA, IgG or IgM (Watson & Stokes 1988). More major histocompatability complex (MHC) Class II-positive cells (macrophages, monocytes, dendritic cells, epithelial cells and endothelial cells) were present in the epithelium and stratum compactum during oestrus than dioestrus, but numbers of T cells were unaffected by cycle stage (Watson & Dickson 1993).

The pattern and activity of myometrial contractions have been shown to change during the cycle (Taverne et al. 1979; Cross & Ginther 1987; Griffin & Ginther 1990; Troedsson et al. 1993b). Synchronization of uterine electrical activity among different sites in the uterus was more marked during oestrus than during dioestrus. This suggests a hormone-dependent mechanism for the regulation of cell communication (Troedsson et al. 1993b). Progesterone inhibits and oestrogen stimulates formation of endometrial gap junctions in the myometrium (Garfield et al. 1980). Using transrectal ultrasonography, the lowest uterine activity scores were associated with low steroid concentrations (Cross & Ginther 1987; Griffin & Ginther 1990). The daily administration of oestradiol to anoestrous mares resulted in a gradual increase in uterine activity over 5 days (Cross & Ginther 1987). Griffin & Ginther (1990) observed in cyclic mares a progressive increase in activity between days 2 and 4 of dioestrus and again between days 11 and 12, the activity being maximal on days 13 and 14.

Bacterial adherence to epithelial cells *in vitro* has been shown to be higher with P and lower with E (Watson et al. 1988b). Freeman et al. (1990) found mucins in the luminal epithelial cells of the equine endometrium. The secretion, elicited by hormonal or irritative/inflammatory stimuli, may be a protective response (Freeman et al. 1990). Tunón et al. (1995) demonstrated that endometrial epithelial cells on the uterine surface and in the glands showed intense cellular vacuolation during oestrus. This indicates high secretory activity. Mucins synthesized actively during oestrus may render adherence of bacteria to the epithelium difficult.

It is common knowledge that acyclic mares treated with progesterone remain infected after bacterial challenge for an extended period, persisting as long as the treatment continues (Washburn et al. 1982; Evans et al. 1986; Hinrichs et al. 1992). P-treatment of acyclic mares has even been suggested as a model to study endometritis in mares (Colbern et al. 1987). Fluid accumulation in the uterine lumen has been shown to occur after uterine inoculation of bacteria when mares are treated with P (Evans et al. 1986; Hinrichs et al. 1992). The cervix is relaxed during E-dominance and tight during P-dominance (Evans et al. 1986).

1.2. Introduction of micro-organisms into the uterus

The genitalia of the mare have a microflora that consists of harmless species and opportunistic organisms that can be pathogenic in susceptible animals (Dimock & Snyder 1924). Bacterial numbers decrease from the vagina to the uterus, and the few organisms found in the cervix and uterus are probably transient (Scott et al. 1971; Hinrichs et al. 1988). Four organisms are responsible for the vast majority of endometritis in the mare: *Streptococcus zooepidemicus*, *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Alpha-haemolytic streptococci, *Staphylococcus spp*. and other bacteria may also be recovered but are usually contaminants. Yeast and fungi may cause endometritis as well; *Candida spp*., *Aspergillus spp*. and *Mucor spp*. are most common. *Taylorella equigenitalis* is the cause of venereally transmitted contagious equine metritis (Blanchard et al. 1998).

Micro-organisms enter the uterus by ascending from the vagina. Breeding (Dimock & Snyder 1924) and foaling (Dimock & Edwards 1928; Dimock 1935), the two important events that are repeated annually in broodmares, are the most important ways of introducing bacteria into the uterus. Some procedures performed by veterinarians may also carry bacteria into the uterus, e.g. insemination, embryo transfer, uterine biopsies or swabs, and infusion of medications (Hinrichs et al. 1992; McDonnell & Watson 1992). Conformational abnormalities, such as pneumovagina (Caslick 1937a), urovagina and cervical lesions, facilitate the entrance of bacteria into the uterus (Van Camp 1986). Hinrichs et al. (1988) emphasized the importance of a properly functioning vulvovaginal fold as barrier to ascending bacterial contamination. They also found that clitoral fossa swab specimens had more bacterial growth (94% of the specimens) than any other parts of the genital tract. This finding suggests the clitoris as a source of contamination during intrauterine (i.u.) manipulations (Hinrichs et al. 1988).

Breeding

Breeding is commonly advocated as a cause for endometritis. All stallions harbour several species of bacteria on their external genitalia and in their semen (Dimock & Snyder 1924; Hughes et al. 1967; Millar & Francis 1974; Simpson et al. 1975). A negative uterine pressure during coitus draws semen directly into the mare's uterus (Millar 1952). As various bacteria are commonly cultured also from normal mares, and most frequently from the caudal parts of the tract (Hinrichs et al. 1988), it is natural that representatives of the microflora move to the more cranial portion of the reproductive tract during breeding. More than 80% of mares showed bacteriologically positive cervical cultures 24 h after being bred (Bryans 1962). Today, the use of artificial insemination (AI) and, particularly, semen collection by the open-ended artificial vagina has diminished bacterial contamination associated with breeding (Tischner et al. 1974).

Foaling

Management of foaling mares and events during foaling affect the degree of bacterial contamination (Knudsen 1983). The incidence of bacteriologically positive cultures has often been lower 1 to 2 days p.p. (around 20-40% of mares) compared with 3 to 6 days p.p. (70-90%) (König 1975; Gygax et al. 1979; Saltiel et al. 1987; Katila et al. 1988b). Mares with puerperal disturbances – dystocia or retained placenta – had more bacterial growth 3, 6 and 9 days p.p. than mares having normal deliveries (Glatzel & Belz 1995). In all these studies, same mares had been sampled on several consecutive days. Therefore, uterine swabbing may have increased contamination of the uterus.

1.3. Endometrial inflammation

In acute inflammation, fluid moves from the blood into the tissues, causing oedema and swelling. Increased permeability of dilated small blood vessels also allows large molecules, including antibodies and complement components, to escape into tissues. PMNs adhere to the endothelial cells lining the vessels and migrate between the cells into the tissue. The migration of PMN is directed by chemotaxis. At the inflammatory site, PMNs phagocytose and kill invading organisms. During phagocytosis lysosomal contents are released. These include proteins and enzymes, as well as free oxygen radicals. Their main task is destruction of bacteria, but they may also be destructive to the tissues (Tizard 1996). Endogenous neutral proteases are derived principally from PMNs and macrophages and they hydrolyse proteins. The peptide products have a number of physiological functions, including macrophage chemotaxis, and they are, in part, responsible for perpetuating the inflammatory response (Matthews 1994).

Enzymes discharged upon cell death or during phagocytosis have been detected in uterine fluid of mares after experimental infection. Lactate dehydrogenase, a cytoplasmic enzyme, has been detected in uterine flushings of oestrous mares. After the inoculation of *Streptococcus zooepidemicus*, the concentrations increased significantly within 4 h indicating death of PMNs, endometrial cells, or both (Pycock & Allen 1990). Alkaline phosphatase has been shown to be present in uterine fluid of cyclic mares collected by tampons, but the concentrations did not differ during the days of the cycle. After i.u. inoculation of streptococci, the alkaline phosphatase levels increased drastically and peaked at 12 h (Katila et al. 1990). Williamson et al. (1983) identified alkaline phosphatase in uterine lavage fluid and found no difference between infected and non-infected mares. Pycock & Allen (1990) and Katila et al. (1990) have reported elevated lysozyme activities in uterine fluid of mares after bacterial challenge.

Inflammation associated with breeding

PMNs are known to be present in the endometrium of mares post-service (Brook 1985). High numbers of leucocytes were detected in uterine lavage fluid of mares after insemination, and phagocytosis of spermatozoa was frequently observed (Bader & Krause 1980). It has been commonly believed – based on the study of Bryans (1962) - that bacteria cause the uterine inflammation associated with breeding. However, in rabbits (Tyler 1977) and in humans (Cohen 1984), leucocytosis occurs in the cervix as

a normal physiological response to spermatozoa. It is very likely that the PMN response in the mare's uterus is provoked by sperm.

Inflammation associated with foaling

Uterine swabs taken from p.p. mares commonly show PMNs in the cytological examination (Knudsen 1964a; Gygax et al. 1979; Saltiel et al. 1987; Katila et al. 1988b). Neutrophils are needed to clear bacterial infections introduced during and immediately after parturition (Brook 1985) and also to destroy microcaruncles (Gygax et al. 1979; Katila 1988). Mares with puerperal disturbances, particularly with retained placenta, showed high numbers of PMNs in lochial secretions 3 to 6 days p.p. (Belz & Glatzel 1995). During foal heat approximately one-third of the mares showed no PMNs on cytological examination, one-third had only a few PMNs and one-third had moderate to high numbers of PMNs (Shideler et al. 1987; Katila et al. 1988b; Huhtinen et al. 1996).

Inflammation associated with intrauterine manipulations

Any manipulation of the equine uterus easily causes increased numbers of neutrophils (Bennett et al. 1980). Intrauterine antibiotic treatments are irritant (Asbury 1982), but even the infusion of physiological saline and the passing of an instrument transcervically into the uterus induce neutrophilia (Knudsen & Nydahl 1983). Stimulation of the cervix and uterus, but not of the vagina, provoke a PMN response (Williamson et al. 1987). Since collection of uterine fluid and other types of uterine sampling always involve the passing of a catheter into the uterus and other manipulations, frequent samplings of the same mare cannot be done without causing erroneous results.

Elimination of uterine inflammation and infection

The ability of mares to eliminate uterine infections has been intensively studied. A frequently used method is to infuse the uterus of mares with an irritating substance or with bacteria and take uterine samples before infusion and at certain time intervals after the infusion. Experimental bacterial inoculations have commonly involved intrauterine infusion of *Streptococcus zooepidemicus*. This has been the traditional method of studying the course of endometritis and uterine defence mechanisms since the first reports in 1969 by Hughes & Loy and Peterson et al. These studies showed – and several subsequent ones have confirmed this – that there is an intense and early (<6h) PMN response in the endometrium, which starts to subside after 12 h. In young healthy mares, the reproductive tract returns to normal appearance within 96 h, whereas persistently infected mares require more time to eliminate bacteria (Hughes & Loy 1969; Peterson et al. 1969).

Although Hughes & Loy (1969) and Peterson et al. (1969) noticed that mares are different in their susceptibility to uterine infections, it is not clear how they differ. Numerous studies have attacked this problem using an intrauterine bacterial challenge.

A problem in these studies is the selection of mares into the susceptible vs resistant groups. The most commonly used criteria have been reproductive history and/or uterine biopsy categories. Troedsson et al. (1993a) found that a normal endometrium was resistant to uterine infections, while severe histopathological changes were associated with susceptibility. Mild to moderate lesions did not correlate with susceptibility or resistance. Another study (Williamson et al. 1989) came to a somewhat different conclusion. A history of recurrent endometritis provided a more sensitive (0.9) and specific (0.95) indication of susceptibility to uterine infection than a uterine biopsy with a significant degree of endometrial degeneration (sensitivity 0.5, specificity 0.75). The best test is to infuse bacteria into the mare's uterus. If the mare is able to clear the infection within 96 h on her own, she should be considered resistant to uterine infections (Troedsson 1991). Another proposed method is the infusion of charcoal into the uterus of oestrous mares. Mares that retain charcoal in the uterus for 48 h are susceptible to uterine infections (LeBlanc 1994).

Polymorphonuclear neutrophils

The role of PMNs in the equine uterine defence has been studied thoroughly since at one time it was widely held that delayed or impaired PMN function is why some mares are unable to eliminate uterine infections. To kill invading organisms, neutrophils have to be able to migrate to the site of infection and phagocytize bacteria. Migration of blood and uterine PMNs of normal and infected mares has been studied *in vitro* using chemotactic chambers analysis (Blue et al. 1984; Strzemienski et al. 1984; Liu et al. 1985, 1986), agarose plates (Watson et al. 1987b, c) and cell elastometry (Liu et al. 1985). Opsonization of bacteria by IgG or complement enhances phagocytosis and bacterial killing by PMNs. Opsonic activity of the uterus has been studied in normal and infected mares (Asbury et al. 1982; Brown et al. 1985; Hansen & Asbury 1987; Watson et al. 1987c; Watson & Stokes 1990). The conflicting results of these studies can be partly explained by the findings of Magnusson & Jonsson (1991), who showed that opsonic activity in uterine flushings does not accurately reflect the corresponding activity in native secretions. However, it can be concluded that 1) one aspect of acute inflammatory response is the accumulation of opsonins in the uterine lumen (Brown et al. 1985), 2) serum-derived complement contributes considerably to opsonic activity in uterine secretions (Asbury et al. 1984; Watson 1988; Håkansson et al. 1993), and 3) opsonic activity is present in the normal endometrium (Håkansson et al. 1993).

The phagocytic activity of blood and uterine PMNs of mares has been investigated by yeast phagocytosis (Watson et al. 1987b) or by measuring peak chemiluminescence (Washburn et al. 1982; Cheung et al. 1985). Studies on bacterial killing have been carried out by Washburn et al. (1982), Strzemienski et al. (1984, 1987) and Watson et al. (1987c). While PMN functions are vital in uterine defence of the mare, the above-mentioned studies have yielded conflicting results. It is questionable how applicable *in vitro* assays are for studying uterine PMNs. Uterine PMNs are not a homogenous population of cells because they are continuously recruited from the peripheral circulation in persistent infection (Liu et al. 1986). Uterine PMNs have been exposed to bacterial stimulation and they have phagocytized. This may have changed their ability to migrate and kill bacteria (Watson et al. 1987b). The above-mentioned studies have not convincingly shown differences in any aspects of neutrophil function. The

initial neutrophil response is neither delayed nor decreased in mares susceptible to uterine infections (Couto & Hughes 1985; Williamson et al. 1987; Katila et al. 1990), but these mares continue to have PMNs in the uterus because of persistent infection. Mares with persistent endometritis had significantly elevated concentrations of PGF and total protein as well as higher percentage of PMNs and mononuclear cells in uterine washings than normal mares (Watson et al. 1987a).

Cellular and humoral immunity

Immunoglobulins IgG, IgT, IgA and IgM have been found in uterine secretions of mares (Kenney & Khaleel 1975). Higher concentrations of immunoglobulins have been measured in uterine secretions than in serum, confirming the potential of the endometrium for local immunoglobulin production (Liu et al. 1981; Widders et al. 1984). Immunoglobulin levels were elevated in the uterus in the presence of active (Asbury et al. 1980; Williamson et al. 1983) or chronic (Widders et al. 1984) infection. The presence of endometritis greatly increased MHC Class II expression and T lymphocyte numbers (Watson & Dixon 1993). The densities of CD4+ (helper T-cells), CD8+ (cytotoxic T-cells) and B-cells were also significantly increased in mares with endometritis (Watson & Thomson 1996). However, no evidence exists that any disturbances or deficiencies in either humoral or cellular immunity are present in mares that are susceptible to uterine infections.

Mechanical clearance

The importance of physical clearance was for long overlooked despite the results of the elegant study carried out by Evans et al. (1986). Susceptible mares tend to accumulate fluid and retain bacteria or infused material in the uterus (Evans et al. 1986; Allen & Pycock 1988; LeBlanc et al. 1989, 1994, 1995; Troedsson & Liu 1991). Lymphatic drainage from the uterine lumen is slower in susceptible than in resistant mares (LeBlanc et al. 1995). Myometrial contractions probably play a major role since significant differences have been detected in electromyography between normal and susceptible mares (Troedsson et al. 1993c), and since inhibition of uterine contractions in normal mares decreases mechanical fluid clearance (Cadario et al. 1995; Nikolakopoulos & Watson 1999). The degree of cervical relaxation is also important in physical clearance (Evans et al. 1986; LeBlanc et al. 1994).

1.4. Uterine involution

Involution is the restoration of the endometrium p.p. to a condition where conception can take place again and new embryonic development can be carried out. Bearing in mind the great uterine changes during pregnancy, parturition and after parturition, it is amazing that some mares are able to conceive as early as one week after foaling.

When the villous epitheliochorial placenta separates at the maternal-foetal interface, it leaves the uterine epithelium relatively undamaged. During involution, degenerating or detached epithelial cells above the maternal crypts are replaced and the uterine epithelium redifferentiates into ciliated and secretory cell types (Steven et al. 1979).

The numerous tiny microcaruncles disappear rapidly by degeneration and resorption. The major feature by days 6 and 7 p.p. are small focal remnants consisting of condensed stroma and crypts lined with contracted epithelial cells. In biopsy specimens taken 10 and 15 days p.p., the former microcaruncles are detectable only by the occurrence of siderophages and lymphocytes (Gygax et al. 1979; Bailey & Bristol 1983; Katila 1988). Uterine glands are numerous, evenly distributed and dilated, and their lumen contains various amounts of cellular debris for 2 days p.p.. By day 4-5 p.p., the glands in the upper layers of the stratum spongiosum have regenerated. Siderophages are present in large numbers around the glands and in the glandular epithelium. By day 15, almost all glands have returned to the pregravid size, but mitotic figures are still common in the epithelium (Bailey & Bristol 1983; Katila 1988).

Biopsy specimens obtained 1 to 2 days p.p. show PMNs concentrated in the stratum compactum, particularly around the microcaruncles. By day 5, lymphocytes appear and replace PMNs. Lymphocytes are seen in the stratum compactum, particularly in the cell-dense areas, and also appear diffusely through the stratum spongiosum. Periglandular and –vascular lymphocytic infiltrations are a common finding in biopsy specimens obtained 10 days p.p. (Shideler et al. 1987; Katila 1988).

Uterine size, tone and contents

A tremendous change in uterine size takes place after foaling, particularly during the first week after foaling (Gygax et al. 1979; Vandeplassche et al. 1983; Katila et al. 1988a). Use of ultrasonography has shown that by 3 weeks the uterus has returned to its pregravid size (McKinnon et al. 1988b; Sertich & Watson 1992).

For several days following parturition, the uterus is extremely firm upon transrectal palpation, particularly during the first week (Saltiel et al. 1987; Katila et al. 1988a; Griffin & Ginther 1991). This is probably due to extensive p.p. oedema. In humans, uteri 14 days p.p. show an increase in water content and this elevation is observed until 5 weeks p.p. (Morrione & Seifter 1962). Prostaglandin $F_{2\alpha}$ release, as measured by its plasma metabolite concentration, is high immediately after foaling but returns to baseline within 3 days (Sertich & Watson 1992). Very low levels of ultrasonically detectable uterine contractility have been observed between the day of parturition and the first ovulation (Griffin & Ginther 1991). Both of these findings are somewhat surprising since one would expect high contractile activity to facilitate emptying and cleansing of the uterus and involution.

Intrauterine fluid accumulations are commonly seen in the transrectal ultrasonography of p.p. mares. During the first 2 days p.p. the number of mares with IUFA and the diameter of the collections were smaller than on days 3 to 5 p.p. This pattern of fluid accumulation suggests that the collections were not residual placental fluids but instead developed in association with involution (Griffin & Ginther 1991). After this the number of mares with IUFA and the diameter of accumulations decreased over time (McKinnon et al. 1988b; Griffin & Ginther 1991; Arrott et al. 1994).

Foal heat

The first post-partum oestrus, commonly called foal heat, has some unique features as compared with other species. The onset is very early, the heat is visible despite the absence of a preceding progesterone phase and the heat culminates in ovulation. The majority of mares (>90%) begin oestrus within 5 to 12 days p.p. (Ginther 1992). The first ovulation usually takes place 9 to 12 days p.p. but is dependent on the time of the year. For thoroughbreds in Kentucky, the average interval to first ovulation was 10.2 ± 2.4 days; 43% of mares had ovulated by day 9, 93% by day 15 and 97% by day 20 p.p. (Loy 1980). In Finland, mares ovulated on average on 11.7 ± 3.4 days p.p. (Koskinen 1991).

Because of the long gestation of 11 months, a mare would need to conceive within a month after the previous parturition in order to foal every year. During this time she can have only two oestrous periods. The short breeding season and the long gestation impose pressures to breed in foal heat. The lowered pregnancy rate, the tendency of increased pregnancy loss and the risks in the transport of a young foal are the most important reasons for postponing breeding. It is important to note that the time from foaling to conception is on average 18.5 days shorter in mares bred in foal heat than in those bred in the 2nd p.p. oestrus (Loy 1980). Clearly, if any complications have occurred during or after parturition, breeding in foal heat should not be attempted. Breeding in foal heat does not diminish the mare's chances of conceiving in the next oestrus (Lieux 1980).

Pregnancy rates of mares bred in foal heat have been reported to be 10 to 20% lower than pregnancy rates at subsequent oestrous periods (Caslick 1937b: 39% vs 58%; Lieux 1973: 40% vs 51%; Sullivan et al. 1975: 53% vs 66%; Lieux 1980: 39% vs 55%; Fiolka et al. 1985: 52% vs 66%). Mares ovulating later in foal heat naturally have higher pregnancy rates than mares ovulating earlier. The pregnancy rate of mares ovulating on day 11 p.p. or later was 59% as compared with the rate of 45% for mares ovulating <11 days (Loy 1980). Similarly, the pregnancy rate of mares that ovulated <15 days p.p. was 50%, whereas mares that ovulated >15 days p.p. had a significantly higher pregnancy rate of 82% (McKinnon et al. 1988b). On different stud farms, pregnancy rates vary markedly, and this can be explained by managemental factors (Loy 1980; Lenz 1986). Because of improved management and veterinary skills, and the use of A.I. and ultrasonography, the pregnancy rates of all mares have increased considerably. The same increase can be seen in the pregnancy rates of mares bred in foal heat (Arrott et al. 1994: 82%; Camillo et al. 1997: 72%).

Merkt & Günzel (1979) reported a higher pregnancy loss rate for lactating mares (17%) than for non-lactating mares (6%). In many other studies, however, the pregnancy loss rates of mares conceiving in foal heat as compared with foaling mares conceiving in subsequent oestruses have not been significantly different (Lieux 1980: 16% vs 14%; Loy 1980: 13% vs 12%; Fiolka et al. 1985: 12% vs 7%; Woods et al. 1987: 11% vs 12%). On the other hand, when the risk of early embryonic loss was evaluated using the logistic regression model, mares bred during foal heat were 1.9 times more likely to experience early embryonic mortality (Meyers et al. 1990). Futhermore, abortion rate has been reported to be higher for foaling mares bred 16

days p.p. or earlier than for mares bred >16 days p.p. (11.1% vs 7.6%, P<0.001) (Chevalier-Clément 1989).

2. Examination methods of uterine luminal environment

2.1. Transrectal palpation and ultrasonography

Rectal palpation is the oldest and still most commonly used method to evaluate the reproductive tract of the mare. The uterus is palpated for its size, symmetry, tone and endometrial folds. However, rectal palpation is somewhat subjective and requires a lot of experience to be reliable. While it is not very accurate in the diagnosis of endometritis, large amounts of fluid in ventral dilatations of the uterus can be detected (Knudsen 1964b), and pyometra is easy to diagnose.

Transrectal ultrasonography allows the detection of even small fluid accumulations (Ginther & Pierson 1984), sometimes causing unnecessary worrying among practitioners. According to McKinnon et al. (1988a), prominent oedema of endometrial folds and small (<3 mm in diameter) fluid collections are normal findings during oestrus. The degree of echogenicity of fluid accumulations is correlated with the amount of debris or white blood cells. Clear fluid is non-echogenic (black) and exudate is echogenic (grey-white) (McKinnon et al. 1988a).

2.2. Endoscopy

Visualization of the uterine lumen is possible by endoscopy. The appearance of the mucosal surface, fluid, pus, cysts, adhesions, etc. can be detected. Minor surgical procedures and small biopsies can be performed (Tunón et al. 1995). The relatively high cost of the instrument has limited its widespread use.

2.3. Sampling of uterine luminal contents

Cells - PMNs, eosinophils, bacteria and fungi – are detached from the luminal wall by the swabbing method. When uterine fluid is collected, the numbers of PMNs and bacteria can be counted, and various constituents of uterine fluid can be determined. Luminal contents, mucus, blood, PMNs and other cells are also seen in uterine biopsy specimens, but the technique is most useful in examining the histology of the endometrium.

Swabs

Cervical swabs were originally used in diagnosing endometritis, but the more specific and less contaminated uterine swabs have replaced them. Over the last few decades, the simple unguarded swabbing rods have developed into double-guarded instruments, which prevent contamination of the swab during sampling. Contamination of the swab before, during or after passage through the reproductive tract may cause erroneous culture results (Blanchard et al. 1981). Swabs are most commonly taken manually by

guiding the rod through the cervix with the fingers. Another possibility is to introduce the rod through a vaginal speculum into the cervix and uterus. This is, however, only possible if the cervix is open. The swabs are routinely used for bacteriological cultures and for cytological examinations (Brook 1984). Uterine swabbing is a quick, easy method to detect bacteria and neutrophils the uterus, but it is not a quantitative technique.

The swabs are usually cultured on blood agar and incubated for 24 to 48 h at 37°C in aerobic conditions. The use of enrichment media is not advised because they may stimulate overgrowth of certain bacteria. It is important to plate the swab soon after sampling onto the appropriate agar. If this is not possible, a transport medium should be used, although transport media may modify the number of bacteria (Brook 1984).

Cellular integrity may be lost in two hours on a cotton swab, so the swab should be plated on a slide immediately after it has been obtained (Brook 1984). The slide is air-dried and can be stained by a variety of methods. Epithelial cells should be seen in correctly sampled and prepared smears. Some people evaluate the number of PMNs in relation to epithelial cells, others use simple scoring systems. Sometimes also red blood cells, macrophages or eosinophils are detected (Brook 1984).

Methods for uterine fluid collection

The most commonly practised method is the low-volume uterine lavage, where a small amount (often 50 ml) of physiological or phosphate-buffered saline (PBS) is injected into the uterus through a Foley catheter, and after some minutes fluid is aspirated by syringe (Zavy et al. 1978). This method does not take into consideration the diluting effect but allows the determination of the total amount of certain constituents, if all the fluid is recovered.

Undiluted uterine fluid can be absorbed into a tampon placed in the uterus for some minutes (Katila et al. 1990). Accurate determinations of concentrations of uterine fluid components are possible, but the tampon method does not give any information on the total amount present in the uterus. Troedsson & Liu (1992) have combined the tampon and lavage methods to obtain more accurate assessments on uterine fluid composition.

If there is a sufficient amount of fluid in the uterus, fluid can be aspirated using a catheter and a syringe (Pycock & Allen 1990). A normal uterus is often too dry, and therefore, the aspiration technique is most commonly applied after experimental or spontaneous infections.

3. Diagnostic and prognostic value of uterine samples

3.1. Uterine cytology

Cytological examination of uterine samples is focused primarily on the presence or absence of neutrophils. PMNs are not present in normal mares at any stage of the

cycle, except for p.p. and post-service mares, and therefore, their presence indicates endometritis.

Cytological examination of uterine swabs is a quick method to assess the presence and degree of acute and persistent inflammation in uterine lumen and on the surface of the walls. It can be considered a semi-quantitative method.

PMNs and other cells can be counted from a certain volume of uterine fluid in counting chambers or by cell counters. Native fluid obtained by tampons is used as such, but uterine lavage fluid (ULF) may need centrifuging to lower the detection limit. According to Ball et al. (1988), the uterine lavage technique allows the detection of smaller numbers of PMNs than the swabbing method.

3.2. Bacterial cultures

The relationship of endometritis with bacterial infection in mares, first reported in 1924 by Dimock & Snyder, has continued to interest clinicians and researchers world-wide. In 1928, one-third of mares had bacteriologically positive cervical or uterine cultures (Dimock & Edwards 1928), and similar incidences of positive bacteriological cultures have been reported in several surveys conducted in different countries over many decades. The recovery of organisms from uterine swabs was considered to be a uterine infection, leading to a practice of culturing more and more mares and treating the positive ones with antibiotics. Gradually, practitioners and researchers began to realize that contamination of samples is possible and that bacteriological examination comprises only part of the examination of the entire genital tract of the mare (Bryans 1962). Since bacterial contamination of the swab is possible, the significance of any bacteria isolated should be assessed by cytological examination. An agreement of 86% between the presence of bacteria and PMNs in samples of p.p. mares has been reported by Katila et al. (1988b). The occurrence of bacteria without PMNs is easily explained by contamination during sampling. The detection of PMNs without bacteria probably shows that bacteria have already been eliminated, but signs of the inflammatory process remain (Brook 1984).

In post-partum mares, streptococci and coliforms are the most frequently found organisms; in some studies, they have been equally common in the early p.p. equine uterus (König 1975; Gygax et al. 1979; Bailey & Bristol 1983; Grammer 1989). *E. coli* is the most typically isolated micro-organism found in early p.p., being replaced by streptococci during the advancing involution (König 1975; Gygax et al. 1979; Katila et al. 1988b). Anaerobic bacteria do not appear to be a problem in the p.p. mare (Purswell et al. 1989). Although uterine swabs obtained from mares in foal heat are often bacteriologically positive, the incidence of positive samples is lower than shortly after foaling (König 1975; Gygax et al. 1979; Bailey & Bristol 1983; Saltiel et al. 1987). If only samples yielding heavy or moderate bacterial growth are considered to be meaningful, the number of positive mares in foal heat is small (Katila et al. 1988b; Purswell et al. 1989; Sertich & Watson 1992). However, it is obvious that the process of bacterial elimination is not completed in all mares by the end of the foal heat.

The clearance of bacteria has been evaluated by sampling of the uterus at certain intervals. In some earlier studies, frequent uterine sampling of the same mares may have caused further bacterial contamination, particularly during the time of progesterone dominance. Hinrichs et al. (1992) and McDonnell & Watson (1992) showed that the population of bacteria changed during progesterone treatment, which was attributable to the introduction of organisms during transcervical manipulations.

Culturing of ULF allows the quantification of bacterial growth. A fixed volume of uterine fluid is transferred with a loop onto an agar plate and spread evenly throughout the surface. After the incubation the colonies are counted. It is also possible to centrifuge the lavage fluid before culturing, if bacterial numbers are low. The low-volume uterine flush technique has been shown to be more sensitive than uterine swabbing in detecting bacteria (Ball et al. 1988). It is also possible to culture uterine fluid obtained by tampons, but bacterial contamination during the introduction and removal of the tampon is likely to occur, and therefore, the results are not very dependable.

3.3. Uterine histology

Histology of the endometrium is examined from uterine biopsy specimens, which are obtained by an endometrial biopsy punch. Acute and chronic inflammatory changes are distinguished and their severity and spread can be assessed. Degenerative glandular changes, such as periglandular fibrosis, cystic distention, can be diagnosed only by a histological specimen. Lymphatic lacunae and vascular changes can also be seen (Kenney 1978).

On the basis of histological findings, Kenney (1978) divided mares into three biopsy categories. Mares placed in category I have a normal endometrium and a good chance of conceiving. Mares in category III show widespread severe changes that are not correctable and lead to very low foaling rates (<10%). The initial category II, which turned out to be quite large, was later divided into IIA (predicted foaling rate 50-80%) and to IIB, with a forecasted foaling rate of 10-50% (Kenney & Doig 1986).

Uterine biopsy specimens provide a more comprehensive picture than swabs. In 69% of p.p. samples, similar results for PMNs were given by cytology and histology (Katila et al. 1988b).

3.4. Biochemical analysis of uterine fluid

Various constituents of uterine fluid (proteins, lysosomal enzymes, inflammatory mediators etc.) can be analysed from either native fluid or ULF. Lysosomal enzyme activity has been found in the endometria and in uterine flushings of cows (Roberts & Parker 1974; Linford & Iosson 1975; Hussain et al. 1989), ewes (Roberts et al. 1976a; Hansen et al. 1985), gilts (Roberts et al. 1976b; Hansen et al. 1985) and mares (Hansen et al. 1985). These studies have shown that progesterone induces accumulation of

lysosomal enzymes in uterine lumen. Therefore, these enzymes might indicate restoration of secretory activity of the endometrium in p.p. mares. In equine leucocytes, N-acetyl-β-D-glucosaminidase (NAGase) and acid phosphatase activities are high both in mononuclear cells and in granulocytes, whereas β-glucuronidase (B-Gase) activity is higher in mononuclear cells (Healy 1982). Measuring concentrations of lysosomal enzymes may provide a more accurate means of quantifying inflammation than by counting leucocyte numbers.

In acute inflammation, the volume of uterine fluid increases mostly because of increased vascular permeability. All researchers agree that total protein levels in uterine lavage fluid are higher in spontaneously or experimentally infected mares than in mares with healthy genitalia (Williamson et al. 1983; Blue et al. 1984; Strzemienski & Kenney 1984; Pycock & Allen 1990). In addition to protein, plasminogen and proteinase inhibitors leak into tissues during inflammatory reaction (Tizard 1996), possibly reflecting permeability changes.

Lysosomal enzymes

Lysozyme

Lysozyme is an enzyme that is present in all body fluids and in neutrophils. It splits peptidoglycans in the cell wall of Gram-positive bacteria. Lysozyme is a potent opsonin, facilitating phagocytosis in the absence of specific antibodies. Lysozyme is found in high concentrations in the lysosomes of PMNs, and therefore, it is present in high concentrations in areas of acute inflammation. Optimal pH for lysozyme activity (pH 3 to pH 6) is easily achieved in inflammatory sites as well as within phagosomes (Tizard 1996).

In cyclic mares, lysozyme concentrations in uterine fluid have varied from 0 to 47 μg/ml, being on average 11 μg/ml. No significant changes have been observed during the oestrous cycle of mares (Katila et al. 1990), but in pigs, lysozyme activity appears in uterine secretions in response to progesterone treatment (Roberts & Bazer 1988). While it is unclear which cells produce lysozyme, epithelial cells or migratory cells of the immune system have been suggested (Roberts & Bazer 1988).

Lysozyme levels have been reported to be very high 12 h after experimental bacterial infection, in parallel with PMN numbers (Katila et al. 1990). Lysozyme concentration in uterine lavage fluids of oestrous mares was in average 1.2 μg/ml (Pycock & Allen 1990). After i.u. inoculation of streptococci, the highest lysozyme levels were obtained at 4 h. The average concentration in uterine exudate was 71 μg/ml, and in uterine lavage fluid 6.3 μg/ml. The pH of the exudate decreased from 7.0 at 30 min post-inoculation to 6.5 at 4 h (Pycock & Allen 1990).

N-acetyl-β-D-glucosaminidase

NAGase hydrolyses glycosaminoglycans (GAGs). It attacks the non-reducing terminals of N-acetylglucosaminyl and N-acetylgalactosaminyl residues under acidic conditions. Transformation of GAGs is essential for cervical softening and dilatation

during parturition. In women, NAGase activity in the plasma gradually increases as gestation advances to reach a maximum 3-4 d before the onset of labour. NAGase is released to the maternal circulation mainly from the decidua and partly from the amnion (Takenaka et al. 1991).

Analysis of milk NAGase can be used to monitor inflammation within the mammary gland (Kitchen et al. 1978). In bovine mastitis, the somatic cell count of milk increases mainly because of PMN influx from blood. The high activity of NAGase in bovine granulocytes (Healy 1982; Dulin et al. 1985) and the finding that much of milk NAGase originates from the cellular components of milk (Kaartinen et al. 1988) explain the reported high correlation between NAGase and somatic cell count.

In cows, both uterine fluid and serum NAGase activities have been reported to be elevated after calving, declining gradually until the 32nd day p.p.. NAGase levels were higher in uterine fluid than in serum, which suggests that the enzyme present in uterine fluid comes mainly from within the uterus. The highest levels of NAGase were detected during the early p.p. period. The origin of the enzyme is mainly lochia, which consists of the remains of foetal membranes and fluids, blood leaking from ruptured umbilical and endometrial vessels, and sloughed surfaces of caruncles. Uterine leucocytes and bacterial infection probably contribute only marginally to the total uterine activity of NAGase. Thus, NAGase levels have been suggested for use in monitoring uterine involution (Hussain et al. 1989).

In the endometrium of cows, NAGase values were significantly higher in the epithelial layers (composed of pseudostratified epithelial cells and secretory cells) than in subepithelial layers consisting of connective tissue. Further, the mean NAGase values for the epithelial layer were significantly higher during the luteal phase and pregnancy than during the non-luteal phase. The authors thus concluded that the bovine endometrial epithelial tissue is active in the release of NAGase, that this is related to increased progesterone concentration, and that the phagocytes are not the main contributors to the total activity of NAGase in the uterine tissues (Hussain et al. 1992b).

When NAGase was measured in uterine flushings of OVX-mares, simultaneous administration of E and P stimulated accumulation of the enzyme. However, treatment of mares with P or E alone did not significantly affect enzyme accumulation (Hansen et al. 1985).

β-glucuronidase

B-Gase has been analysed in uterine and oviductal fluids of hamsters. The concentration did not vary during the oestrous cycle (Tulsiani et al. 1996). In the uterus of pregnant rats, B-Gase activity was high in stromal tissues, glandular epithelial cells and their secretions, and luminal epithelial cells. However, luminal epithelial cells in contact with the blastocyst showed marked depletion of B-Gase activity, suggesting that in these regions the release of B-Gase was initited by the blastocyst (Roy et al. 1983). B-Gase has also been used as a marker enzyme for detection of abnormal udder

secretions in cows (Nagahata et al. 1987). B-Gase has been found in equine leucocytes (Healy 1982) but it has not been analysed in the mare's uterus.

Acid phosphatase

Acid phosphatase levels were higher in uterine lavage fluid of dioestrous mares than in the fluid of oestrous mares (LeBlanc et al. 1988). The concentrations peaked 12-14 d after ovulation and decreased to almost undetectable levels during oestrus, but were higher in pregnant mares than in non-pregnant dioestrous mares (Zavy et al. 1979, 1982). Treatment of OVX-mares with progesterone, and particularly with progesterone and oestradiol simultaneously, increased acid phosphatase concentration and total amounts in uterine flushes (McDowell et al. 1987). Acid phosphatase levels were very high 4 days p.p. On day 8 p.p., the level was lower but was still elevated as compared with the values in cyclic mares (LeBlanc et al. 1988).

Uteroferrin, a cationic phosphatase containing two iron atoms, is a major component in acid phosphatase activity of progesterone-induced uterine secretions of mares (Zavy et al. 1982; McDowell et al. 1987; LeBlanc et al. 1988). Uteroferrin has an important role as a transplacental iron transporter in pigs, and probably also in horses, which have a similar placenta to pigs. Uteroferrin is synthesized and secreted by epithelial cells of the uterine glands for subsequent transport of iron to the foetal placental unit (Roberts & Bazer 1988).

Proteolytic and antiproteolytic activity

When fluid exudes from the bloodstream into the tissues, three enzyme cascades are activated: the complement system, the coagulation system and the fibrinolytic system. Activation of the coagulation cascade also initiates the fibrinolytic system. This leads to activation of plasminogen activator, which in turn generates plasmin (Tizard 1996). Activators of plasminogen are present in blood, vessel walls, body fluids and most tissues (Kaneko et al. 1997). Plasmin is a serine protease with trypsin-like specificity and a potent fibrinolytic enzyme. In destroying fibrin, plasmin releases peptide fragments that are chemotactic for neutrophils (Tizard 1996). In contrast to plasminogen, plasmin is normally absent from blood and body fluids because a group of circulating antiplasmins rapidly inactivates free plasmin (Kaneko et al. 1997). Plasminogen is present in uterine secretions, presumably as a serum transudate (Roberts & Bazer 1988). Proteolytic activity in the form of plasmin has not been measured in the uterine fluid of mares.

Since acute inflammation can be destructive, it must be controlled. Plasma contains several molecules that either inactivate inflammatory mediators directly or inhibit the enzymes that generate the mediators (Tizard 1996). In horses, plasma antiprotease activity resides in two major electrophoretic fractions. The slower of these is the α-2 macroglobulin, the faster being the α-1-protease inhibitor (α_1-PI) (Matthews 1994). This is also called the α_1-antitrypsin and its activity can be measured as trypsin-inhibitor capacity (TIC) (Berninger 1986). α_1-PI or α_1-antitrypsin inhibits proteolytic

action of serine proteinase elastase produced by neutrophils, thereby protecting tissues from excessive proteolytic damage (Berninger 1986).

Scudamore et al. (1994) showed that α_1-PI is not produced in the equine endometrium but leaks from blood into the uterine lumen. A significant increase occurred in the concentrations of α_1-PI and albumin relative to total protein in uterine flushings recovered from mares during oestrus as compared with dioestrus. After inoculation of bacteria, concentrations of α_1-PI, albumin and total protein increased in uterine flushings. Thus, no difference was observed in the concentrations of α_1-PI and albumin relative to total protein before and after the induction of endometritis.

3.5. Predicting foal heat fertility

With the increased use of modern but more expensive breeding techniques, such as frozen semen, imported fresh semen or embryo transfers, it has become more important to know which mares are ready to be bred in foal heat and which ones are not. It seems natural to expect the size of the uterus to indicate the rate of uterine involution, and thus, the probability of the mare conceiving. Experienced practitioners have, however, shown that this is not the case (Loy 1980; Lenz 1986). None of the following parameters examined – uterine size, tone and contents; palpability of endometrial folds; amount and character of vaginal contents; oedema and colour of portio vaginalis – were correlated with the outcome of foal heat insemination (Katila et al. 1988a).

The presence of bacteria or PMNs in uterine swabs had no correlation with foal heat pregnancy rate (Katila et al. 1988b) or embryo recovery rate (Huhtinen et al. 1996). Mares who had large amounts of PMNs in uterine biopsies on the 5th day p.p. showed significantly ($P<0.05$) decreased foal heat pregnancy rates (Katila et al. 1988b), but processing of biopsy samples takes too long for routine use in practice.

Transrectal ultrasonography is useful in the evaluation of foal heat mares, as shown by McKinnon et al. (1988b). Fewer ($P<0.005$) mares became pregnant if they were bred when uterine fluid was detected during foal heat (33%), as compared with mares bred when fluid was not detected (84%). Mares with i.u. fluid accumulations during breeding did not have larger uteri than mares with no fluid. No correlation was found between change in uterine size (from ovulation to 29-31 days p.p.) and pregnancy rates (McKinnon et al.1988b). In conclusion, traditional methods for examining mares (rectal palpation, vaginoscopy) have not provided any solutions for decision-making with regard to foal heat. Ultrasonography appears promising, but more sensitive indicators are needed.

AIMS OF THE STUDY

Uterine lavage fluids or undiluted uterine fluids were collected from ovariectomized, cyclic or post-partum mares to:

1. determine whether proteolytic and antiproteolytic activities or various lysosomal enzymes could be used to diagnose uterine inflammation and monitor recovery from endometritis, and whether these inflammatory markers were more sensitive than PMNs,

2. study the inflammatory reaction of the uterus following insemination and compare the effects of fresh and frozen semen,

3. determine whether accumulation of uterine fluid during oestrus is associated with compositional changes in uterine secretions,

4. establish the time at which the secretory function of the endometrium is restored after parturition, and

5. determine the prognostic value of proteolytic and antiproteolytic activities and lysozyme concentrations, in particular, for predicting fertility in foal heat.

MATERIALS AND METHODS

Data were collected at the Agricultural Research Centre, Equine Research Station, Ypäjä, Finland (Studies II, III, IV and V), and at the College of Veterinary Medicine, Department of Obstetrics and Gynaecology, Hautjärvi, Finland (Studies I, II, IV). A summary of materials is presented in Table 1.

1. Animals

One hundred and twelve different mares were used in 5 studies. The mares, weighing 400 to 750 kg, were either Finnhorses (60), standardbreds (33), warmblood riding horses (18) or ponies (1). Their ages ranged from 3 to 18 years. They had foaled 0 to 8 times, had no history of reproductive failure and were clinically normal. All 4 stallions used for inseminations were known to be fertile.

2. Experimental design

2.1. Hormone treatments and bacterial inoculation (I)

Four OVX mares were treated with oestradiol benzoate on Days 1 to 7 (treatment E), oestradiol plus synthetic progestin on Days 8 to 14 (treatment EP) and progestin on Days 22 to 28 (treatment P). Mares were examined and sampled on Day 1 (C, control samples) and on Days 8, 15 and 29 (samples E, EP and P, respectively). In Experiment 2, the mares were either treated or not treated (C) with hormones as in Experiment 1, but the hormone treatments continued for 4 wks (Days -7 to 21). On Day 0, the uterus was swabbed and acute endometritis was induced by inoculation of bacteria. The mares were examined and sampled at 6 and 24 h, and at 3, 7, 14 and 21 d after inoculation.

2.2. Post-breeding study (II)

Groups of 6 to 8 oestrous mares received the following treatments: 1) no treatment (control); 2) 30 ml of skim-milk extender; 3) 2 ml of egg yolk-glycerol extender (Martin et al. 1979); 4) 10 ml of frozen-thawed seminal plasma; 5) 10 ml of raw semen; 6) 10 ml of raw semen diluted with 30 ml of skim-milk extender; 7) 2 ml of frozen semen; 8) 2 ml of frozen semen mixed with 10 ml of frozen-thawed seminal plasma; 9) 30 ml of phosphate-buffered saline (PBS); 10) raw semen concentrated by centrifugation and resuspended in 2 ml of skim-milk extender; 11) 2 ml of frozen semen diluted with 30 ml of skim-milk extender; 12) 2 ml of supernatant from frozen semen centrifuged after thawing; 13) 2 ml of frozen semen centrifuged after thawing and resuspended in egg yolk-glycerol extender; and 14) natural breeding. The number of spermatozoa was 800 million in treatments containing frozen semen or centrifuged raw semen, and 1100 to 2400 million in treatments containing raw semen. Insemination with fresh semen or natural breeding was repeated every second day and insemination with frozen semen once daily until ovulation. Treatments which did not include spermatozoa were undertaken only once. Six hours after the first treatment, ultrasonography was used to estimate uterine fluid quantity and the mares were sampled.

Table 1. A summary of animals, treatments, samplings and laboratory analyses.

Study	Number of mares	Treatments	Timing of uterine samplings	Uterine samples	Examinations on uterine fluids	Other examinations
I	4 OVX	C, E, EP, P; C, E, EP, P + bacterial inoculation	after treatments; before inoculation; 6 sampling d/3 wks after inoculation	swab, lavage swab swab, lavage	PMNs, bacteria, protein, TIC, plasmin, NAGase, B-Gase, acid phosphatase, lysozyme (n=112)	biopsy
II	47	8 types of AI/breeding, 5 other types of infusions, controls	in oestrus, 6 h after treatments	lavage	PMNs, bacteria (n=104)	endometrial oedema, IUFA
III	57	AI (35 mares)	in mid-oestrus (before AI) (57 mares)	tampon	PMNs, bacteria (n=57); protein, TIC, plasmin, NAGase, B-Gase (n=27)	oedema, IUFA; embryo recovery (n=35); biopsy (n=53); sperm motility *in vitro* (n=9)
IV	16 12 p.p. 3 OVX p.p.		5 sampling days/cycle; 12 sampling days/4 wks p.p.; 4 sampling days/4 wks p.p.	lavage swab, lavage lavage	PMNs, bacteria, protein, TIC, plasmin, NAGase, B-Gase, acid phosphatase, lysozyme (n=95)	 progesterone progesterone
V	15 p.p.	AI	after the first p.p. ovulation (before AI and before embryo recovery	lavage	PMNs, bacteria, protein, TIC, plasmin, NAGase, B-Gase, lysozyme (n=24)	embryo recovery (n=15)

For abbreviations, see the list on page 9.

2.3. Fluid accumulation study (III)

The presence of fluid accumulation in the uterine lumen was determined by ultrasonography every second day at the beginning of oestrus, daily after detection of a follicle ≥35 mm, and on the second day after ovulation. Mares were sampled when a mature follicle was detected. Thirty-five of the mares were inseminated immediately after or within 2 to 5 d of sampling. Embryos were recovered 6 d after detection of ovulation. Ultrasonography was performed and an endometrial biopsy specimen taken during oestrus, after completion of the embryo transfer programme.

2.4. Involution study (IV)

After parturition, 3 groups of intact mares (4 mares/group) were sampled on days 1, 6, 12 and 20 (group A), on days 2, 8, 14 and 24 (group B) and on days 4, 10, 16 and 28 (group C). OVX mares were sampled on post-partum days 4, 10, 16 and 28. Sixteen non-parturient mares were sampled once or twice per cycle, either on the 2nd or 4th (or 5th) day of oestrus, and on day 3, 8 or 14 after ovulation (7 mares/day).

2.5. Foal heat fertility study (V)

The first p.p. ovulation was determined by ultrasonographic examination every 12 h. Mares were inseminated within one hour of detection of ovulation. Collection of ULFs was attempted from 12 out of 15 mares immediately after ovulation detection but before insemination and from all 15 mares before embryo recovery 7 to 8 d later.

3. Ovulation control and ultrasonography

Before beginning studies II and III, blood samples for progesterone determination were collected weekly to establish cyclicity and oestrus. Serum progesterone was measured by direct radioimmunoassay (Farmos Diagnostica, Turku, Finland). Progesterone was measured 3 times per wk to confirm the physiological status of foaling mares (IV, V).

Ultrasonography (Aloka SSD-210 Dx, 5 MHz probe) was used to monitor follicular growth and to detect ovulation, endometrial oedema and free fluid within uterine lumen. In foaling mares, rectal palpation and ultrasonography were started 5 d after parturition (IV, V). Examinations were performed every second day at the beginning of oestrus (II-V), daily (II, III, IV) or every 12 h (V) after detection of a follicle ≥35 mm, and on the second day after ovulation (III). In study I, ultrasonography was used to detect any intrauterine fluid accumulations (IUFA) before hormone treatments, bacterial inoculation and samplings of uterus. Endometrial oedema was graded from 0 (no oedema) to 3 (marked endometrial oedema) (II, III). The maximum depth of free fluid was measured in one (I, II) or three places (III). IUFA scores (II) (no intrauterine fluid detected, score 0; maximum height ≤10 mm, score 1; height 11 to 39 mm, score 2; height ≥40 mm, score 3) or sums of the three measurements (III) were used in the

analyses. The appearance of the fluid was also evaluated: non-echogenic (black), hypoechogenic (dark fluid with white spots) and hyperechogenic (light grey to white).

The degree of cervical opening was scored from 0 to 3 during sampling according to the number of fingers that could easily be passed through the cervix (I, III). Examination for pregnancy was undertaken using ultrasonography 15 to 16 days after ovulation (II).

4. Inseminations (II, III, V)

Artificial inseminations with fresh semen were undertaken every second day until ovulation (II, III) or once within one hour after detection of ovulation (V). All semen was collected using an open-ended artificial vagina. The insemination dose was ≥ 500 x 10^6 progressively motile spermatozoa extended in skim milk-glucose extender (Kenney et al. 1975) (III, V) or 1100 to 2400 million spermatozoa (II). Natural breeding was repeated every second day until ovulation (II).

Insemination with frozen semen was repeated once daily until ovulation (II). Semen was frozen using the procedure developed at the University of Hannover, West Germany (Martin et al. 1979), but instead of 4-ml straws, 2-ml straws were used. Centrifugation was used to concentrate raw semen (1000 g, 10 min), to obtain seminal plasma from raw semen (2000 g, 15 min), to remove extender from frozen-thawed semen (800 g, 10 min) and to obtain supernatant from frozen-thawed semen (1500 g, 15 min) (II).

5. Hormone replacement therapy (I)

In Experiment 1, 4 OVX mares were treated daily with 5 mg of oestradiol benzoate (Estrogen, Orion-Farmos, Turku, Finland) i.m. on Days 1 to 7 (treatment E), with 5 mg of oestradiol benzoate i.m. plus 27.5 mg of allyltrenbolone (Regumate, Hoechst-Roussel, Paris, France) p.o. on Days 8 to 14 (treatment EP) and with 27.5 mg of allyltrenbolone on Days 22 to 28 (treatment P). In Experiment 2, the mares received either no hormones or E, EP or P treatment over a period of 4 wks (Days -7 to 21; treatment groups E + i, EP + i, and P + i). The day of bacterial inoculation (i) was designated Day 0. Treatments were allocated by randomization to each of the 4 mares.

6. Intrauterine streptococcal inoculation (I)

Streptococcus zooepidemicus, cultured from the uterus of a mare clinically affected with endometritis, had been stored in liquid nitrogen. Bacteria were thawed, streaked onto a blood agar plate and incubated overnight at 37°C. Ten colonies were suspended in 2 ml of Todd-Hewitt broth (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD, USA) and incubated for 6 h at 37°C. The suspension was diluted (1:10) with Todd-Hewitt broth, divided into 1-ml doses and frozen at -20°C. The inoculum was prepared by rapidly thawing 1 dose in a water bath at 37°C and incubating it for 4 to 5 h at 37°C. This protocol consistently resulted in 10^8 to 10^9

colony-forming units per ml. Bacteria were suspended in 10 ml of PBS and injected into the uterus with an insemination pipette.

7. Embryo recovery (III, V)

Embryos were recovered non-surgically 6 (III) or 7 to 8 d (V) after detection of ovulation by flushing the uterus three times with 1 l PBS (III) or three times with Dulbecco's PBS (DPBS; Gibco BRL, Life Technologies, Paisley, Scotland) (1 l, 2 l and 2 l, successively, V). PBS was produced from laboratory quality salts and distilled water, and supplemented with bovine serum albumin (BSA) (2 g/l) and kanamycin (25 mg/l). DPBS was supplemented with 1% heat-inactivated foetal calf serum (FCS; Gibco BRL, Life Technologies, Paisley, Scotland), 0.1% glucose and 25 mg/l kanamycin. Flushing of the uterus was done via a two-way equine embryo flushing catheter (Embryonen-Spühlkatheter Ch 26, Klinika Medical-Vertriebs GmbH, Hannover, Germany) and the fluid collected in 1-l cylinders. The recovered fluid was either passed through an embryo filter (Emcon - Immuno Systems, Spring Valley, WI, USA) (III) or all but 50 ml of the fluid was siphoned from the top of each cylinder after a 20-min equilibration time (V). The fluid in the filter cup, and retained fluids and rinse fluids from glassware were examined for the presence of embryos using a stereomicroscope. Once an embryo was located, it was transferred to flush medium with 5% FCS, photographed, classified according to McKinnon & Squires (1988) and measured with an eyepiece craticule (V). Immediately after embryo recovery, the donor mares were given 7.5 mg of luprostiol i.m..

8. Sampling of the uterus

8.1. Endometrial swabs (I, IV)

The endometrium was swabbed with a guarded culture swab (Guarded Culture Instrument; Kalayijian Industries, Inc., Long Beach, CA, USA) using a proven technique (Blanchard et al. 1981). The same swab was used for bacteriological and cytological studies.

8.2. Uterine lavage (I, II, IV, V)

Fifty millilitres of warm (37°C) saline (0.9% wt/vol NaCl) (I, IV), PBS (II) or DPBS (DPBS; Gibco BRL, Life Technologies, Paisley, Scotland) (V) was infused into the uterus via a modified 22 G Foley catheter (Bard Ltd., Sunderland, UK), and the uterus was briefly massaged per rectum. On post-partum days 1, 2, and 4, a rubber tube was used for uterine lavage (IV). After 5 min of equilibration, the lavage fluid was allowed to flow into sterile centrifuge tubes. When required, the recovery of fluid was assisted by manipulation of the uterus via the rectum. Before embryo recovery (V), ULF was collected using an embryo flushing catheter (Embryonen-Spühlkatheter Ch 26; Klinika Medical-Vertriebs GmbH, Hannover, Germany) by flushing the uterus with 50 ml of DPBS. Recovered fluid was examined for the presence of embryos before further handling.

Microbiological and cytological analyses were performed on fresh samples. Twenty millilitres of each lavage fluid was centrifuged at 2000 g for 15 min (at 4°C) (II), and portions of the supernatant were stored at -20°C until analysis.

8.3. Tampon technique (III)

Intrauterine fluid (IUF) was collected using a regular size Tampax tampon (Tampax Inc., Palmer, MA, USA). The technique was a modification of the procedure described by Katila et al. (1990). A 24-inch piece of umbilical tape was attached to the tampon, which was then passed through the cervix into the uterus using a modified double-glove technique. The distal part of a rectal glove was cut off from the wrist to make a plastic tube. The gloved hand with the tampon was placed inside the tube, and the tube was closed by gathering its end with one's fingers. The gloved hand was set free from the plastic tube before reaching the cervix. The tampon was allowed to absorb uterine fluid for 15 min and was then withdrawn through a sterile vaginal speculum. After removal, each tampon was placed inside a 20-ml syringe, and the fluid absorbed by the tampon was squeezed out into a sterile plastic tube. Microbiological and cytological analyses were performed on fresh samples. The remaining fluid was stored at -20°C for later protein and enzyme analyses and *in vitro* semen motility studies.

8.4. Endometrial biopsy (I, III)

A uterine biopsy was taken according to the technique described by Kenney (1978), using an instrument with alligator-type jaws. The specimens were fixed in Bouin's solution for 24 h and then transferred to 70% alcohol. The fixed tissues were embedded in paraffin, sectioned at a thickness of 6 µm and stained with haematoxylin and eosin.

9. Counting of neutrophils

Cytological smears were made by rotating the side of the swab (I, IV) or the tampon (III) on a slide. Smears were also prepared from ULFs (IV). The slides were air-dried, fixed with methanol and stained with 0.25% methyl violet or May-Grünewald-Giemsa. Numbers of PMNs per 10 fields at a magnification of 400 were scored as follows: none (no PMNs), +1 (1-10 PMNs), +2 (>10 but mostly isolated PMNs) and +3 (large clumps of PMNs) (I, IV). In the fluid accumulation study (III), cytological examination results were considered indicative of inflammation when more than 1 neutrophil was seen per high-power field (400X).

Neutrophils in ULF (I, II, IV, V) and in IUF (III) were counted using a haemocytometer (25 µl sample + 475 µl 1% acetic acid or 0.8% NaCl). The detection limit was 100 000 PMNs/ml. To prevent inaccurate counts as a result of coagulation, hypotonic saline (0.3%) was used to dilute viscous samples (II). For samples with none or only a few neutrophils, the detection limit was lowered to 10 000/ml by centrifuging 10 ml of ULF and resuspending the pellet in 1 ml of PBS (II, IV) or in 1 ml of saline (0.9% wt/vol NaCl) (V).

10. Bacteriological examination

The tip of the swab was streaked onto a blood agar plate (I, IV). Uterine lavage fluid (10 µl) was plated onto blood agar using a loop (I, II, IV, V). Microbial growth after 48 h incubation at 37ºC was scored as none, +1 (1-10 colonies), +2 (11-100 colonies), and +3 (>100 colonies). Bacterial growth was considered significant when more than 10 colonies were recovered per plate.

One drop of uterine fluid (50 µl) absorbed by the tampon was squeezed out onto blood agar (III). If no fluid was obtained from the tampon, blood agar was touched with the tip of the tampon and further streaking with a loop undertaken. Bacterial growth was considered significant when more than 10 colonies of potential pathogens (ß-haemolytic streptococcus, *Staphylococcus aureus*, haemolytic *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) were recovered per plate. Identification was based on colony morphology, haemolysis, the catalase test and Gram-stain. Eosin methylene blue agar was used when necessary.

11. Histological examination

The biopsies in study I were evaluated according to the principles outlined by Kenney & Doig (1986). In the fluid accumulation study (III), inflammatory (acute and chronic) and degenerative changes and the number of lymphatic lacunae were individually evaluated for each sample. Quantities of PMNs and mononuclear cells were scored as none, slight, moderate or severe. Foci of gland nests or cystically distended glands surrounded by periglandular fibrosis were counted and fibrosis scored from 0 to 4. Lymphatic lacunae were rated for size and frequency from 0 to 4.

12. Biochemical analyses

12.1. Total protein (I, III-V)

Protein concentrations (g/l) in ULF and in IUF were measured with the dye-binding Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA, USA) (Bradford 1976) and a microtitration plate reader (Titertek Multiskan Photometer, Labsystems, Finland) using BSA as a standard.

12.2. Trypsin-inhibitor capacity (TIC) (I, III-V)

TIC was measured by means of a colorimetric assay, as described by Mattila et al. (1985). Analyses were performed on microtitration trays. The assay expresses antitrypsin activity in relative units, where a value of 1.0 represents the TIC of a standard bovine milk sample. The α_1-proteinase inhibitor (α_1-PI) content of this standard was 9 mg/l. The detection limit of the assay is 0.1. Intra- and inter-assay coefficient of variation (CV) is 6% (Mattila et al. 1985).

12.3. Plasmin (I, III-V)

A fluorogenic coumaryl peptide substrate was used for the analysis of plasmin (Richardson & Pearce 1981). The determinations were performed on microtitration trays at room temperature (RT) (Mattila & Sandholm 1986). The sensitivity of the assay, when translated to enzyme activity/min, was approximately 0.03 µmol product/min/l. The intra-assay CV was about 2%.

12.4. Lysozyme (I, IV, V)

A modification of the turbidimetric method described by Mörsky (1983) was used to determine lysozyme. Hen-egg-white lysozyme (Sigma L-6876; Sigma Chemical Co., St. Louis, MO, USA) working standards were prepared in 0.9% saline with 1 g/l BSA and 0.1 g/l sodium azide. The substrate suspension contained 210 mg of *Micrococcus lysodeikticus* cells (Sigma M-3770) in 100 ml of working buffer (67 mM phosphate buffer, pH 6.2 with 0.1 g/l BSA). Determinations were performed at 37°C. Sixty microlitres of standards and samples were mixed with 440 µl of working buffer in disposable FP-901 plastic cuvettes (Labsystems, Helsinki, Finland). The reaction was started by adding 100 µl of bacterial suspension. The cuvette block was shaken briefly in a mixer, and absorbance was measured at 620 nm by a FP-901 Chemistry Analyser (Labsystems), which measures 9 cuvettes simultaneously. Decreases in absorbance were monitored for 2 min. The detection limit of the assay was 0.3 mg/l. The intra- and inter-assay CV were about 6% and 12%, respectively.

12.5. N-acetyl-ß-D-glucosaminidase (NAGase) (I, III-V)

Analysis of NAGase was carried out using a method developed for microtitration plate fluorometry (Linko-Löppönen & Mäkinen 1985). Ten microlitres of lavage fluid or IUF was mixed with 50 µl of substrate solution [2.25 mM 4-methyl-umbelliferyl-N-acetyl-ß-D-glucosaminide (Sigma M-2133; Sigma Chemical Co., St. Louis, MO, USA) in 0.25 M citrate buffer, pH 4.6]. After 15 min of incubation in the dark at RT, the reaction was stopped by adding 100 µl of 0.2 M glycine-NaOH buffer, pH 10.7. The fluorescence of the released 4-methyl-umbelliferone (4-MU) was measured (excitation 355 nm, emission 480 nm) using a Fluoroskan-1 fluorometer (Labsystems, Helsinki, Finland). NAGase activities of samples (µmol product/min/l at RT) were interpolated from a standard curve obtained using serial dilutions of 4-MU (Sigma M-1381) in 0.2 M glycine-NaOH buffer (pH 10.7). The sensitivity of the assay was 0.02 µmol product/min/l. The intra- and inter-assay CV for NAGase were about 2% and 5%, respectively.

12.6. ß-glucuronidase (B-Gase) (I, III-V)

The method for determining B-Gase activity, a modification of the procedure described by Nagahata et al. (1987), was essentially the same as that used for determining NAGase activity. Four-methyl-umbelliferyl-ß-glucuronide (Sigma M-9130; Sigma Chemical Co., St. Louis, MO, USA) was used as substrate, and the incubation period

was increased to 1 h. The sensitivity of the assay was 0.005 µmol product/min/l. The intra- and inter-assay CV for B-Gase were about 2% and 5%, respectively.

12.7. Acid phosphatase (I, IV)

Determination of acid phosphatase was similar to that used for determining NAGase. Four-methyl-umbelliferyl-phosphate (Sigma M-8883; Sigma Chemical Co., St. Louis, MO, USA) was used as substrate, and the incubation period was 15 min. The sensitivity of the assay was 0.02 µmol product/min/l. The intra- and inter-assay CV for acid phosphatase were about 2% and 5%, respectively.

13. *In vitro* motility studies of spermatozoa (III)

Intrauterine fluid collected from 9 mares in mid-oestrus and semen from 1 stallion were used to investigate the effects of IUF on spermatozoal motion. Semen was collected using an open-ended artificial vagina. Spermatozoal concentration was determined, and the semen was extended 1:1 with Kenney's extender (Kenney et al. 1975). The extended semen was added to 9 test tubes containing a) 0.4 ml of IUF, b) 0.2 ml of IUF + 0.2 ml of Kenney's extender and c) 0.4 ml of Kenney's extender. The final sperm concentration in each tube was 40 x 10^6/ml. The tubes were transferred into a water bath (37^{o}C). Spermatozoal motility was measured at 15 min, 2 h 15 min and 5 h 15 min. The motion characteristics of spermatozoa were measured using a Hamilton-Thorn motility analyser (HTM-S version 7.2) with Makler chambers. The basic parameter settings had been determined earlier (Andersson & Katila 1992).

14. Statistics

Because of right-skewed distributions and heterogeneity in variances, the values were first transformed logarithmically. Statistical analyses were performed with the Statgraphics program (STSC 1986) (II, IV, V) and with the SAS/STAT (SAS Institute Inc. 1988) (I, III). Significance was set at $P<0.05$. Effects of hormone replacement therapy, bacterial inoculation and time on proteins, enzymes and PMNs were tested using repeated measures analyses of variance (I). Effects of oestrous cycle on total protein concentration and lysosomal enzyme activities were tested using one-way analysis of variance followed by Tukey's test (IV).

Differences in total protein concentration, TIC and enzyme activities in IUF between mares with or without IUFA were analysed using general linear model procedures for analysis of variance (III). The Mann-Whitney test was used to compare medians of proteins and enzymes in lavage fluids between post-partum and non-parturient mares in oestrus or in dioestrus, and between ovariectomized and intact p.p. mares sampled on the same days (IV). T-tests for paired samples were used to compare ULFs collected within 13 h after the first p.p. ovulation with those collected 7 to 8 days later, and t-tests for two independent samples were used to compare lavage fluids from mares producing an embryo with those from mares not producing an embryo (V). Associations between total protein, NAGase, B-Gase and plasmin in lavage fluid

collected 7 to 8 days after the first p.p. ovulation were subjected to a Pearson correlation analysis. In the post-breeding study (II), PMNs in lavage fluid were analysed using one-way analysis of variance, employing the least significant difference (LSD) statistic for mean separation. A paired-sample t-test was used when appropriate.

Associations between age, numbers of foalings, IUFA scores, degrees of endometrial oedema and PMNs in lavage fluid were subjected to Spearman rank correlation analysis (II). Chi-square analysis or Fisher's exact test was used to assess significance of associations between IUFA and endometrial oedema, degree of cervical opening, age of mares and endometrial biopsies (III). One-way analysis of variance was used to determine the significances of effects of IUF on spermatozoal motility (III).

RESULTS

The effects of exogenous hormones and the stage of the cycle on the composition of ULF are presented in studies I and IV, respectively. The effects of experimental infection on the composition of lavage fluid are also described (I). Numbers of PMNs after AI (II) and composition of IUF in oestrus (III) are described. The results of analyses of uterine lavage fluids collected during uterine involution (IV) and after the first p.p. ovulation and during the subsequent dioestrus (V) are presented. Results will be shown in connection with the examination method used.

1. IUFA, endometrial oedema and biopsies

Most mares had echogenic fluid accumulations in the uterus 6 and 24 h after inoculation of bacteria (I). In treatments EP+i and P+i, the amount of free fluid increased with time from inoculation. In the post-breeding study (II), IUFA were detected before treatments in 21% (22/104) of oestrous cycles. All fluid accumulations appeared completely black (non-echogenic). Pretreatment fluid scores correlated with the degrees of endometrial oedema ($r=0.29$; $P<0.01$). Positive correlations were found between age and free uterine fluid before ($r=0.36$; $P<0.001$) and 6 h after treatment ($r=0.24$; $P<0.05$). Age also correlated positively with degree of oedema ($r=0.22$; $P<0.05$).

IUFA were detected during oestrus on one or more occasions in 22 of 57 mares (39%) (III). Fifteen mares (26%) exhibited accumulation of non-echogenic fluid on the sampling day. Fluid accumulations were detected in 11/33 (33%) mares aged >7 years and in 4/24 (17%) mares ≤7 years ($P=0.16$). In these mares, the sums of maximal depths of fluid at three locations were 1 to 15 mm (n=9), 16 to 30 mm (n=4) and >30 mm (n=2). At the time of sampling, 6/15 (40%) mares with IUFA and 14/42 (33%) mares without IUFA had moderate to marked endometrial oedema (scores 2 to 3). On endometrial biopsy, fibrosis was more prominent ($P<0.05$) in mares with IUFA (n=9) than in mares without IUFA (n=44), and in mares >7 years than in mares ≤7 years of age. Neutrophils, mononuclear cells and lymphatic lacunae were not associated with fluid accumulation ($P>0.05$).

At the time of ovulation detection, non-echogenic intrauterine fluid accumulations of less than 20 mm in maximum depth were detected by ultrasonography in 2/15 p.p. mares (V).

2. PMN numbers

After bacterial inoculation (I), PMN numbers in ULF rose sharply within 6 h ($P<0.01$) in all treatments (Figure 1). Numbers decreased significantly between 6 and 24 h in treatments C+i and E+i ($P<0.05$). PMN numbers were significantly elevated until 24 h in treatments C+i and E+i ($P<0.05$), and until Day 21 in treatments EP+i and P+i ($P<0.05$).

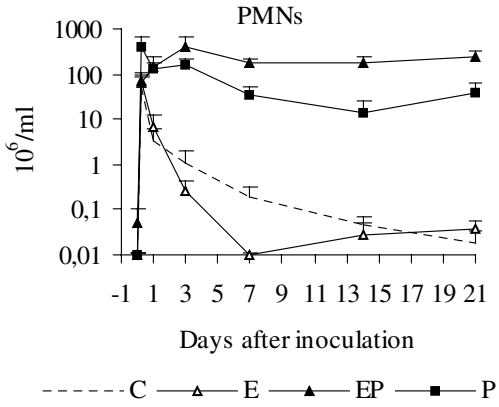


Figure 1. Effects of exogenous hormones and intrauterine inoculation of streptococci on neutrophil numbers in uterine lavage fluid (I). Values are means ± SEM of four ovariectomized mares. The mares received either no hormones (C), oestradiol (E), oestradiol and progestin (EP) or progestin alone (P).

Table 2. Number of polymorphonuclear leucocytes (PMNs) in uterine lavage fluids 6 h after treatment (II).

	Treatment group	PMNs 10^6/ml (mean ± SEM)	Multiple-range analysis for PMN^x by group (LSD)
1	No treatment	0.0 ± 0.0	a
9	PBS	1.4 ± 1.3	b
12	Supernatant	0.3 ± 0.2	b
3	Egg yolk extender	0.3 ± 0.1	bc
2	Skim milk extender	0.3 ± 0.1	bc
4	Frozen seminal plasma	9.5 ± 5.9	cd
6	Extended fresh semen	5.0 ± 4.4	cde
14	Natural breeding	19.4 ± 12.0	cdef
11	Extended frozen semen	8.7 ± 4.4	def
5	Raw semen	7.4 ± 2.5	defg
13	"Washed" frozen semen	29.2 ± 13.5	defg
10	Concentrated fresh semen	38.3 ± 23.9	efg
8	Frozen semen + seminal plasma	53.7 ± 34.6	fg
7	Frozen semen	58.6 ± 19.8	g

x: Log-transformed data; $^{a-g}$ means with different letters are significantly different ($P<0.05$).

In the post-breeding study (II), all lavage fluids collected from control mares were cytologically negative. All infusions elicited PMN influx into the uterus (Table 2). Insemination with frozen semen resulted in a greater neutrophil response than insemination with fresh extended semen ($P<0.01$) or with raw semen ($P<0.05$). However, when all groups were compared, no significant difference ($P>0.05$) was found between frozen and raw semen (Table 2). Neutrophil concentrations did not differ between mares inseminated with frozen semen and those inseminated with concentrated fresh semen ($P>0.05$). Significantly more neutrophils were recovered from ULFs after insemination with frozen semen than after infusion of egg yolk-glycerol extender ($P<0.001$) or supernatant from centrifuged frozen semen ($P<0.01$). A negative correlation existed between PMNs in ULF and volumes of inseminate (r = -

0.38; P<0.01). Age and numbers of foalings had no correlation with neutrophil numbers in the lavage fluid. In mares inseminated with fresh semen, PMN numbers did not correlate with pregnancy rates.

In intact post-partum mares, 15/24 (63%) and 5/23 (22%) of ULFs collected 1 to 10 days and 12 to 28 days p.p., respectively, were cytologically positive (IV). All ULFs from non-parturient mares (n=35) were cytologically negative (IV). At the time of the first p.p. ovulation, PMN numbers were elevated in 3/4 nonembryo-producing mares and in 1/5 embryo-producing mares (P=0.10; Table 5) (V). At embryo recovery 7 to 8 days after ovulation detection, PMN numbers exceeded the detection limit in 1 out of 15 samples.

3. Bacteriological cultures

Six hours after bacterial inoculation (I), all mares had streptococcal endometritis based on results of uterine swabs and/or flushings. On Day 3 post-inoculation, 2/4, 1/4, 3/4 and 4/4 of the mares in treatments C+i, E+i, EP+i and P+i, respectively, had streptococcal endometritis. On Day 21, 1/4, 1/4, 3/4 and 4/4 of the mares in treatments C+i, E+i, EP+i and P+i, respectively, exhibited pathogenic organisms and PMNs in their uterine swabs/lavage fluids. Pure growths of *Strep. zooepidemicus*, *E. coli* and *Klebsiella spp*. were cultured on 3, 3 and 1 occasion, respectively; on 2 occasions, the mares established a mixed bacterial growth (*Strep. zooepidemicus* with *Klebsiella spp*. or *Staphylococcus aureus*). In progestin treatments (EP+i and P+i), proportions of samples with invading organisms increased with time from inoculation of *Strep. zooepidemicus* (to 1/8, 3/8, 4/8, 4/8 and 5/8 on Days 1, 3, 7, 14 and 21, respectively).

In the post-breeding study (II), 17/104 samples (16%) were bacteriologically positive, but the growth was considered insignificant (score in all cases +1). Positive samples occurred sporadically among the groups.

Bacteria were a common finding in lavage fluids collected 1 to 4 days p.p. (9/12, 75%) (IV). Two out of nine lavage fluids collected after detection of the first p.p. ovulation were bacteriologically positive, but the growth was considered insignificant (<10 colonies or +2 growth of mixed bacteria) (V).

4. Biochemical analyses

4.1. Total protein

Hormone replacement therapy of OVX-mares (I) had no statistically significant effect on total protein concentrations in ULFs (Figure 2). However, in non-parturient cyclic mares (IV), total protein concentration was significantly lower (P<0.05) during early oestrus than during mid- and late dioestrus (Figure 2). Total protein concentration in IUF was significantly lower in mares with fluid accumulation than in those without fluid accumulation (P<0.001) (Table 3) (III).

After bacterial inoculation (I), total protein concentration increased significantly within 6 h in treatments C+i, EP+i and P+i ($P<0.05$), but not in treatment E+i (Figure 2). In treatments EP+i and P+i, values remained significantly elevated until Day 14 and Day 21, respectively ($P<0.05$).

After foaling (IV), total protein concentrations in ULFs were high during the first 4 days p.p. and declined steadily thereafter (Figure 2). Differences between intact and OVX p.p. mares were not statistically significant. The mean concentration during foal heat was significantly higher than during subsequent dioestrus ($P<0.001$) or during oestrus of non-parturient mares ($P<0.01$) (Table 4). During dioestrus, differences between lavage fluids from p.p. and non-parturient mares were statistically insignificant. No significant differences were detected between post-ovulatory samples from embryo-producing and nonembryo-producing mares (Table 5) (V). During dioestrus, total protein concentration in lavage fluid tended to be higher in nonembryo-producing mares ($P<0.10$) (Table 6).

Table 3. Protein concentrations and enzyme activities in uterine fluid in mares with and without intrauterine fluid accumulation detected using ultrasonography (III).

	No fluid detected (n=13)	Fluid detected (n=14)	P value
Total protein (g/l)	25.2 ± 12.9	8.2 ± 8.7	0.0005
TIC (relative units)	71.4 ± 72.8	9.4 ± 14.7	0.0045
Plasmin (U/l)	0.89 ± 0.84	1.39 ± 1.40	0.2701
NAGase (U/l)	57.4 ± 43.5	98.8 ± 147.4	0.3405
B-Gase (U/l)	4.66 ± 2.94	1.89 ± 0.99	0.0047

Values are expressed as means ± SD. TIC: trypsin-inhibitor capacity; NAGase: N-acetyl-ß-D-glucosaminidase; B-Gase: β-glucuronidase; U: µmol product/min.

Table 4. Composition of uterine lavage fluids from post-partum and non-parturient mares (IV).

	Oestrus		Dioestrus	
	Post-partum mares (n=8)	Non-parturient mares (n=14)	Post-partum mares (n=8)	Non-parturient mares (n=14)
Total protein (g/l)	4.500 ± 4.140	0.113 ± 0.056	0.298 ± 0.086	0.253 ± 0.106
TIC (relative units)	17.48 ± 21.52	ND	0.20 ± 0.14	0.140 ± 0.113
Plasmin (U/l)	0.378 ± 0.699	0.012 ± 0.006	ND	ND
Lysozyme (mg/l)	1.26 ± 2.36	ND	ND	ND
NAGase (U/l)	3.74 ± 3.63	2.48 ± 3.09	23.60 ± 15.53	35.27 ± 25.46
B-Gase (U/l)	0.046 ± 0.032	0.009 ± 0.018	0.088 ± 0.052	0.060 ± 0.055
Acid phosphatase (U/l)	146.5 ± 244.8	27.0 ± 36.6	78.6 ± 37.7	153.7 ± 245.6

Values are expressed as means ± SD. TIC: trypsin-inhibitor capacity; NAGase: N-acetyl-β-D-glucosaminidase; B-Gase: β-glucuronidase; U:µmol product/min; ND: not detected.

Table 5. Comparison of protein concentrations, enzyme activities and PMN numbers in uterine lavage fluids collected after detection of the first p.p. ovulation from mares producing or not producing embryos 7 to 8 days later (V).

	Embryo recovered (n=5)	No embryo recovered (n=4)	P value
Total protein (g/l)	0.496 ± 0.081	0.892 ± 0.609	0.234
TIC (relative units)	0.096 ± 0.192	0.730 ± 0.787	0.097
Plasmin (U/l)	0.058 ± 0.018	0.104 ± 0.064	0.305
Lysozyme (mg/l)	ND	0.89 ± 0.91	0.069
NAGase (U/l)	2.28 ± 1.17	2.90 ± 1.46	0.515
B-Gase (U/l)	0.002 ± 0.001	0.015 ± 0.016	0.168
PMNs (10^6/ml)	0.04 ± 0.08	0.85 ± 1.43	0.101

Values are expressed as means ± SD. TIC: trypsin-inhibitor capacity; NAGase: N-acetyl-β-D-glucosaminidase; B-Gase: β-glucuronidase; PMNs: neutrophils; U: µmol product/min; ND: not detected.

Table 6. Comparison of protein concentrations, enzyme activities and PMN numbers in uterine lavage fluids collected before embryo recovery 7 to 8 days after ovulation detection from mares producing or not producing embryos (V).

	Embryo recovered (n=8)	No embryo recovered (n=7)	P value
Total protein (g/l)	0.390 ± 0.173	0.617 ± 0.244	0.070
TIC (relative units)	ND	0.07 ± 0.15	0.350
Plasmin (U/l)	0.114 ± 0.094	0.164 ± 0.134	0.874
Lysozyme (mg/l)	ND	0.11 ± 0.26	0.350
NAGase (U/l)	11.57 ± 4.51	17.78 ± 5.42	0.035
B-Gase (U/l)	0.074 ± 0.093	0.226 ± 0.301	0.054
PMNs (10^6/ml)	ND	0.005 ± 0.011	0.350

Values are expressed as means ± SD. TIC: trypsin-inhibitor capacity; NAGase: N-acetyl-β-D-glucosaminidase; B-Gase: β-glucuronidase; PMNs: neutrophils; U: µmol product/min; ND: not detected.

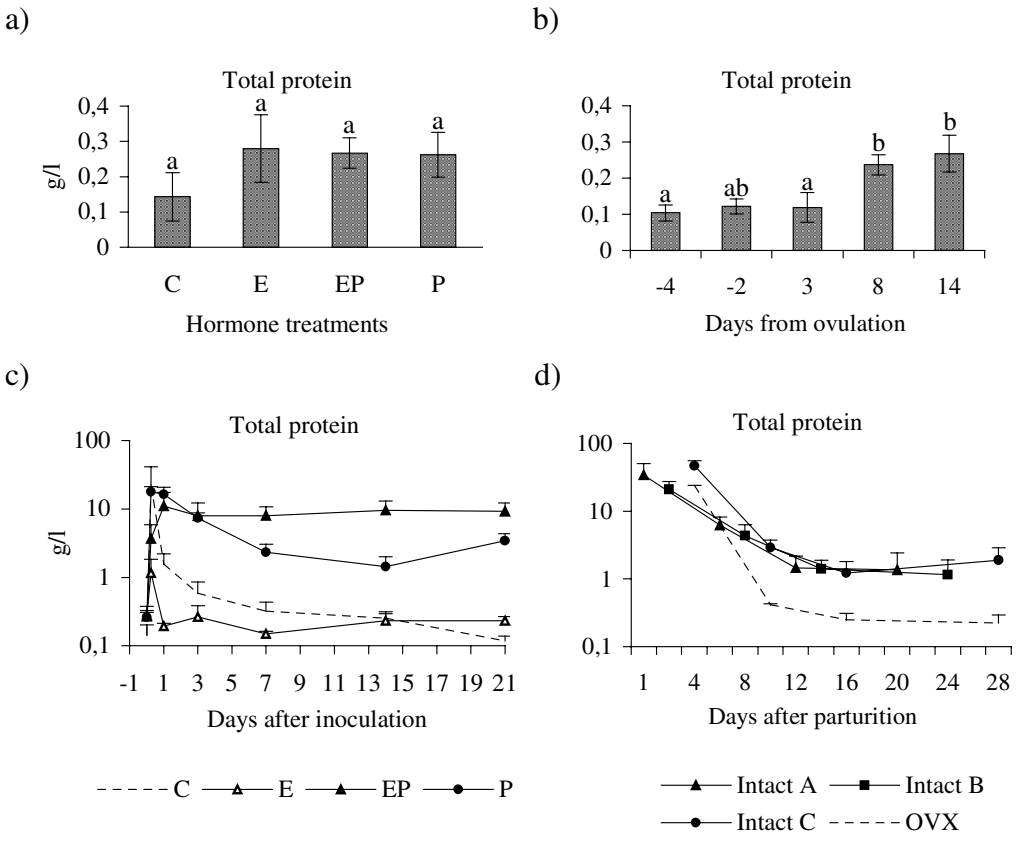


Figure 2. Total protein concentration (mean ± SEM) in centrifuged uterine lavage fluid collected from a) OVX-mares before and after hormone treatments (I), b) cyclic mares on different days of the oestrous cycle (IV), c) hormone-treated OVX-mares after intrauterine inoculation of streptococci (I) and d) intact and OVX-mares on different days post-partum (IV). OVX-mares received no hormones (C, p.p. mares), oestradiol (E), oestradiol and progestin (EP) or progestin alone (P). Different letters above the columns indicate statistical differences ($P<0.05$).

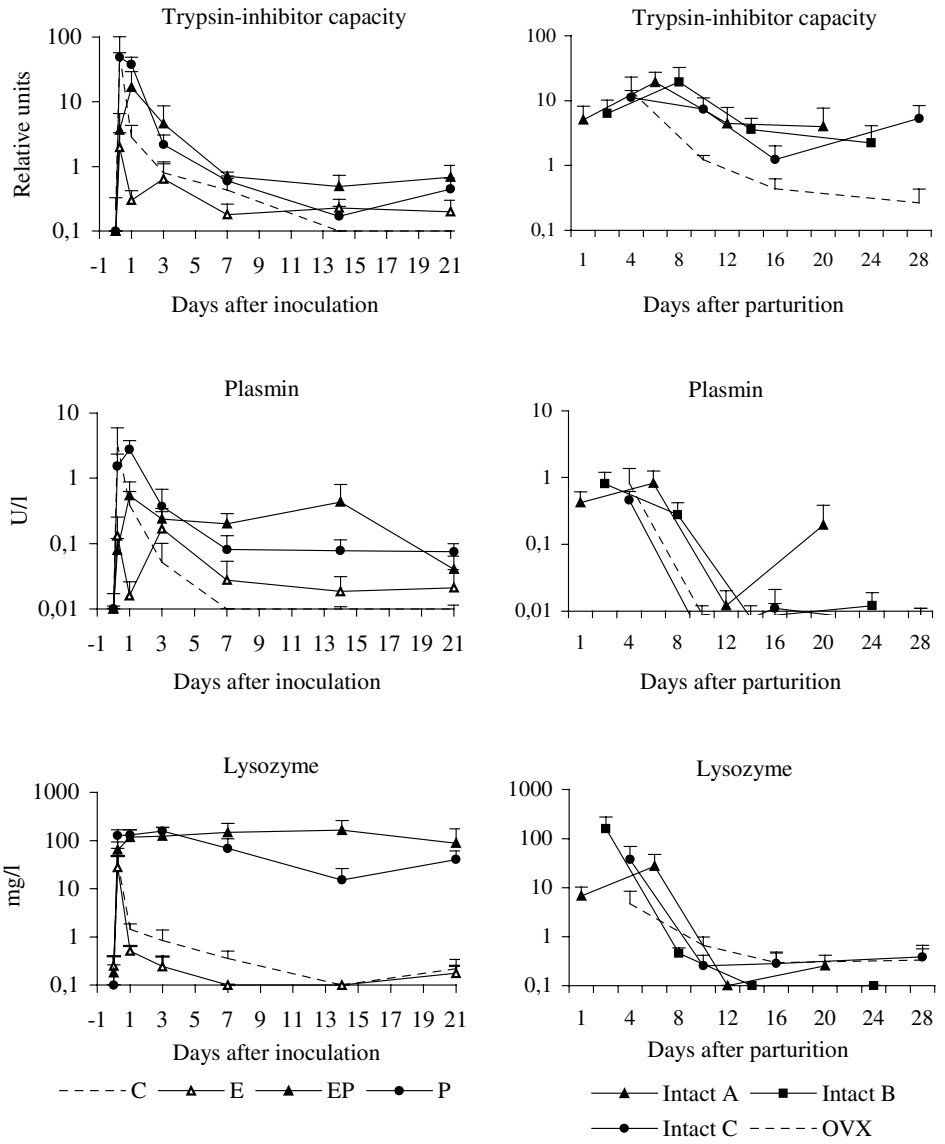


Figure 3. TIC, plasmin activity and lysozyme concentration in centrifuged uterine lavage fluid collected from hormone-treated OVX-mares after intrauterine inoculation of streptococci (I), and from intact and OVX-mares on different days post-partum (IV). OVX-mares received no hormones (C, p.p. mares), oestradiol (E), oestradiol and progestin (EP) or progestin alone (P). Values are means ± SEM. U = µmol product/min.

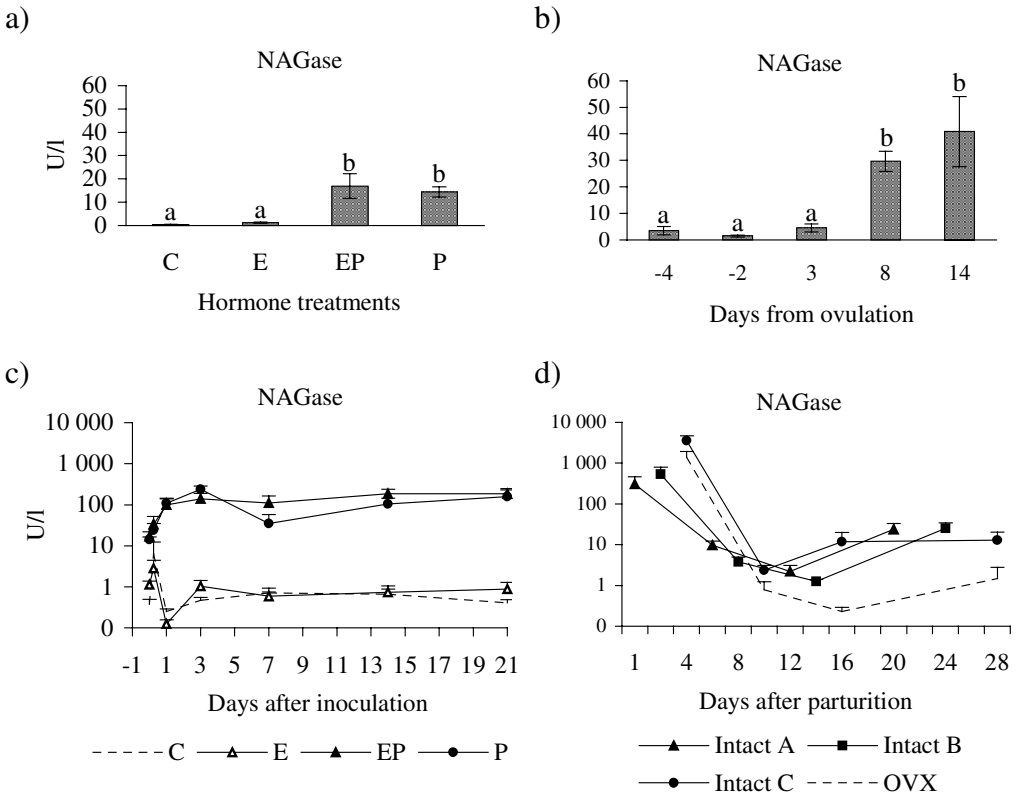


Figure 4. NAGase activity (mean ± SEM) in centrifuged uterine lavage fluid collected from a) OVX-mares before and after hormone treatments (I), b) cyclic mares on different days of the oestrous cycle (IV), c) hormone-treated OVX-mares after intrauterine inoculation of streptococci (I) and d) intact and OVX-mares on different days post-partum (IV). OVX-mares received no hormones (C, p.p. mares), oestradiol (E), oestradiol and progestin (EP) or progestin alone (P). Different letters above the columns indicate statistical differences ($P<0.05$). U = µmol product/min.

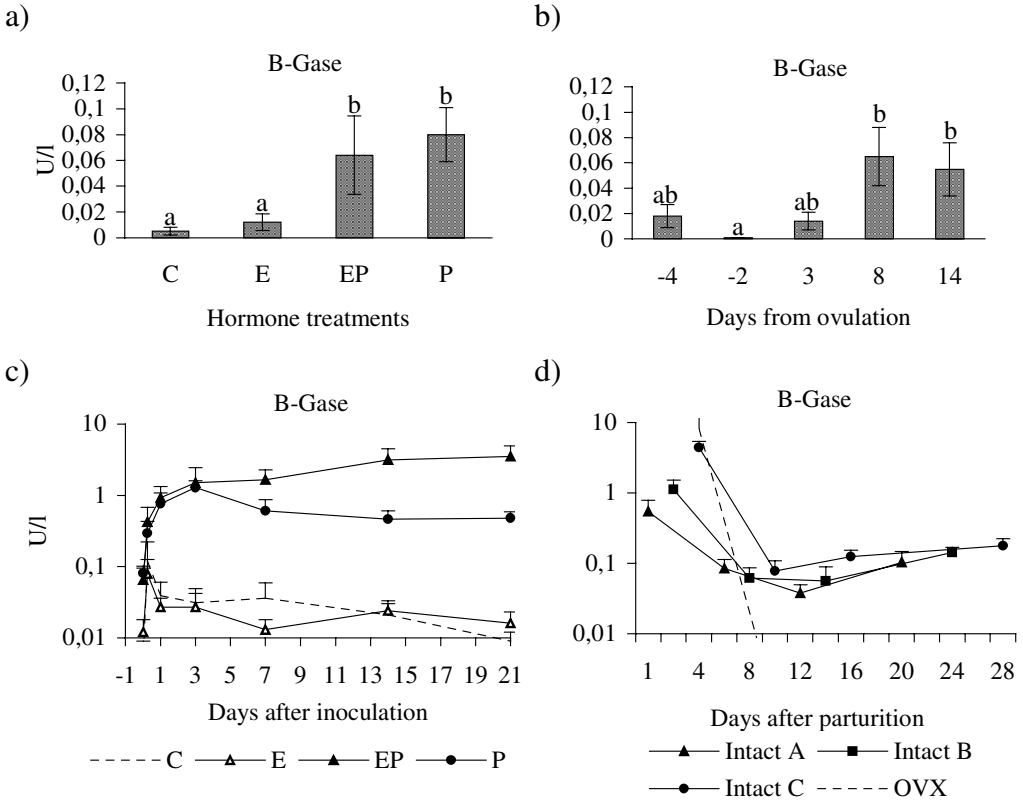


Figure 5. B-Gase activity (mean ± SEM) in centrifuged uterine lavage fluid collected from a) OVX-mares before and after hormone treatments (I), b) cyclic mares on different days of the oestrous cycle (IV), c) hormone-treated OVX-mares after intrauterine inoculation of streptococci (I) and d) intact and OVX-mares on different days post-partum (IV). OVX-mares received no hormones (C, p.p. mares), oestradiol (E), oestradiol and progestin (EP) or progestin alone (P). Different letters above the columns indicate statistical differences ($P<0.05$). U = µmol product/min.

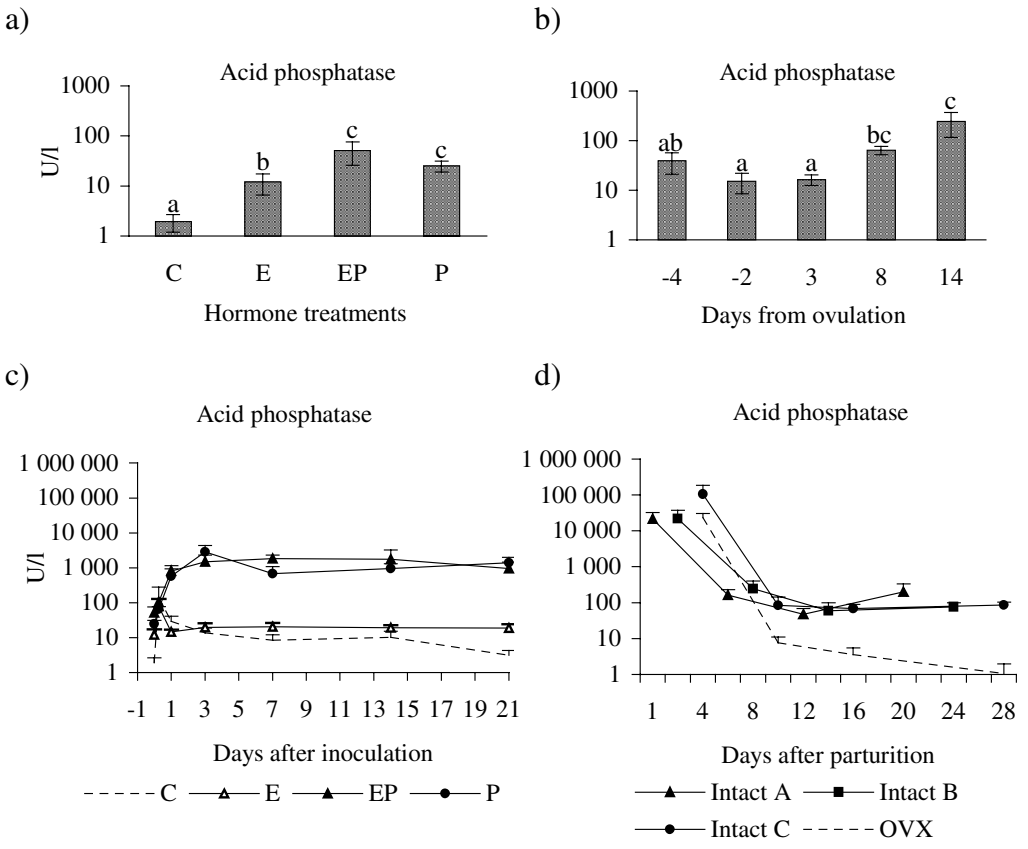


Figure 6. Acid phosphatase activity (mean ± SEM) in centrifuged uterine lavage fluid collected from a) OVX-mares before and after hormone treatments (I), b) cyclic mares on different days of the oestrous cycle (IV), c) hormone-treated OVX-mares after intrauterine inoculation of streptococci (I) and d) intact and OVX-mares on different days post-partum (IV). OVX-mares received no hormones (C, p.p. mares), oestradiol (E), oestradiol and progestin (EP) or progestin alone (P). Different letters above the columns indicate statistical differences ($P<0.05$). U = µmol product/min.

4.2. TIC

In hormone-treated OVX-mares (I) and in non-parturient cyclic mares (IV), TIC was below the detection limit in all but 1 and 2 lavage fluids, respectively. TIC in IUF was significantly lower ($P<0.01$) in mares with fluid accumulation than in those without accumulation (Table 3) (III).

After bacterial inoculation (I), TIC rose sharply within 6 h in all treatments ($P<0.05$) except treatment E+i (Figure 3). TIC exceeded the pre-inoculation value until 24 h in treatment C+i, and until Day 7 in treatments EP+i and P+i ($P<0.05$).

In post-partum mares (IV), TIC in lavage fluid increased gradually, reaching peak values within approximately 1 wk of foaling. Thereafter, TIC declined steadily to low values by day 16 p.p. (Figure 3). Differences between intact and OVX p.p. mares sampled on the same days were not statistically significant. Mean TIC in lavage fluid was significantly higher during foal heat ($P<0.01$) than during oestrus of non-parturient mares (Table 4). Immediately after the first p.p. ovulation (V), TIC was slightly elevated in 1/5 embryo-producing mares and in 3/4 nonembryo-producing mares ($P<0.10$; Table 5).

4.3. Plasmin

In hormone-treated OVX-mares (I) and in non-parturient cyclic mares (IV), plasmin activity was below the detection limit in all but 1 lavage fluid. Plasmin activity in IUF did not differ statistically between mares with fluid accumulation and mares without fluid accumulation (Table 3) (III).

After bacterial inoculation (I), plasmin activity in ULF rose sharply within 6 h in all treatments ($P<0.01$), except treatment E+i (Figure 3). Plasmin exceeded the pre-inoculation value until 24 h in treatments C+i and P+i, and until Day 7 in treatment EP+i ($P<0.01$).

In post-partum mares (IV), plasmin activities in lavage fluid were high during the first week after foaling but declined to low levels thereafter (Figure 3). No significant differences were detected between intact and OVX-mares sampled on the same days ($P>0.05$). No significant differences ($P>0.05$) were detected between post-ovulatory samples, or dioestrous samples, from embryo-producing and nonembryo-producing mares (Tables 5 and 6) (V). Nor did post-ovulatory samples differ significantly from dioestrous samples in these two groups.

4.4. Lysozyme

In hormone-treated OVX-mares (I), lysozyme concentrations exceeded the detection limit in 2 lavage fluids. In non-parturient mares (IV), lysozyme concentration was below the detection limit in all lavage fluids.

After bacterial inoculation (I), lysozyme concentrations increased significantly within 6 h in all treatments ($P<0.05$) (Figure 3). Lysozyme concentrations were significantly higher

than pre-inoculation values until 6 h in treatment E+i, until 24 h in treatment C+i, until Day 7 in treatment EP+i and until Day 21 in treatment P+i ($P<0.05$).

In post-partum mares (IV), the highest lysozyme concentrations were found on day 2 p.p. (Figure 3). After high values on days 1 to 6 p.p., lysozyme concentration declined rapidly. During foal heat, lysozyme concentration in lavage fluid was significantly higher ($P<0.01$) than during oestrus of non-parturient mares (Table 4). During dioestrus 16 to 28 d p.p., lysozyme concentration remained below the detection limit in all samples. At the time of the first p.p. ovulation (V), lysozyme concentrations were elevated in 3/4 nonembryo-producing mares. None of the 5 embryo-producing mares had elevated levels of lysozyme (Table 5). At embryo recovery 7 to 8 days after ovulation detection, lysozyme concentration exceeded the detection limit in 1 out of 15 samples (Table 6).

4.5. NAGase

Treatment of OVX-mares (I) with progestin significantly increased NAGase activity in ULF ($P<0.01$) (Figure 4); mean activity was 37 times higher in treatment P than in treatment C. A slight interaction effect (P*E) was detected for NAGase ($P<0.05$). In non-parturient cyclic mares (IV), NAGase activities in lavage fluids were significantly higher ($P<0.05$) during mid- to late dioestrus than during oestrus (Figure 4). No difference in NAGase activity of IUF was found between oestrous mares with fluid accumulation and mares without fluid accumulation (Table 3) (III).

Bacterial inoculation (I) did not increase NAGase activity in lavage fluid in treatments C+i and E+i (Figure 4). In treatments EP+i and P+i, activities increased gradually up to Day 3. From Day 3 to Day 21, NAGase values were for the most part significantly higher ($P<0.05$) than pre-inoculation values.

In post-partum mares (IV), peak values in lavage fluid occurred on day 4 p.p. (Figure 4). Thereafter, activities declined rapidly to low values. During foal heat, NAGase activity was significantly lower ($P<0.001$) than during subsequent dioestrus, and did not differ from activities of oestrous non-parturient mares (Table 4). On day 16 p.p., the median value of NAGase was 18 times as high in intact dioestrous mares as in OVX-mares ($P<0.10$) (Figure 4). NAGase activities in post-ovulatory samples (V) from embryo- and nonembryo-producing mares did not differ ($P>0.05$) (Table 5). At embryo recovery 7 to 8 days after ovulation detection, NAGase activity was lower ($P<0.05$) in embryo-producing mares (Table 6).

4.6. B-Gase

In hormone-treated OVX-mares (I), progestin significantly increased activities of B-Gase ($P<0.05$) in ULF (Figure 5); mean activity was 15 times higher in treatment P than in treatment C. In non-parturient cyclic mares (IV), B-Gase activity was significantly higher ($P<0.05$) during mid- and late dioestrus than in mid- to late oestrus (Figure 5). B-Gase activity in IUF was significantly lower ($P<0.01$) in oestrous mares with IUFA than in mares without IUFA (Table 3) (III).

Bacterial inoculation (I) did not increase B-Gase activity in lavage fluid in treatments C+i and E+i (Figure 5). In treatments EP+i and P+i, activities were significantly higher ($P<0.05$) than pre-inoculation values on Days 7 and 21, and on Days 1, 3 and 21, respectively.

In post-partum mares (IV), activities in lavage fluid declined rapidly after peak values on day 4 p.p. (Figure 5). However, during the first p.p. oestrus, B-Gase activity was significantly higher ($P<0.01$) than in non-parturient mares during oestrus (Table 4). During dioestrus, differences between p.p. and non-parturient mares were not statistically significant (Table 4).

Both in embryo- and nonembryo-producing mares, B-Gase activities were significantly lower ($P<0.05$) soon after the first p.p. ovulation than in dioestrus (V). No significant differences were detected between post-ovulatory samples from these two groups ($P>0.05$) (Table 5). At the time of embryo recovery, B-Gase activities tended to be higher ($P<0.10$) in mares not producing embryos (Table 6).

4.7. Acid phosphatase

Treatment with progestin significantly increased activities of acid phosphatase ($P<0.01$) in ULF (Figure 6); mean activity was 13 times higher in treatment P than in treatment C (I). In non-parturient mares (IV), acid phosphatase activity was significantly higher ($P<0.05$) during mid- and late dioestrus than in mid- to late oestrus (Figure 6).

Bacterial inoculation (I) did not increase acid phosphatase activity in treatments C+i and E+i (Figure 6). In treatments EP+i and P+i, activities exceeded the pre-inoculation values only on Day 7, and on Days 3 and 21, respectively ($P<0.05$).

In post-partum mares (IV), peak activities occurred on day 4 p.p. (Figure 6). Thereafter, activities declined rapidly, but during foal heat, they remained significantly higher ($P<0.05$) than in oestrous non-parturient mares (Table 4). During dioestrus, differences between post-partum and non-parturient mares were not statistically significant (Table 4). Differences detected between ovariectomized and intact p.p. mares were not statistically significant (Figure 6).

5. Associations between PMNs, proteins and enzymes (I,IV)

PMN numbers, total protein concentration and lysozyme activity in uterine lavage fluid parallelled each other after bacterial challenge (Figure 7). TIC relative to total protein was higher in acute inflammation than in persistent endometritis.

NAGase, B-Gase and acid phosphatase activities in lavage fluid followed a similar pattern of change after parturition (Figure 8). After day 14 p.p., NAGase activities increased because of rising serum progesterone concentrations.

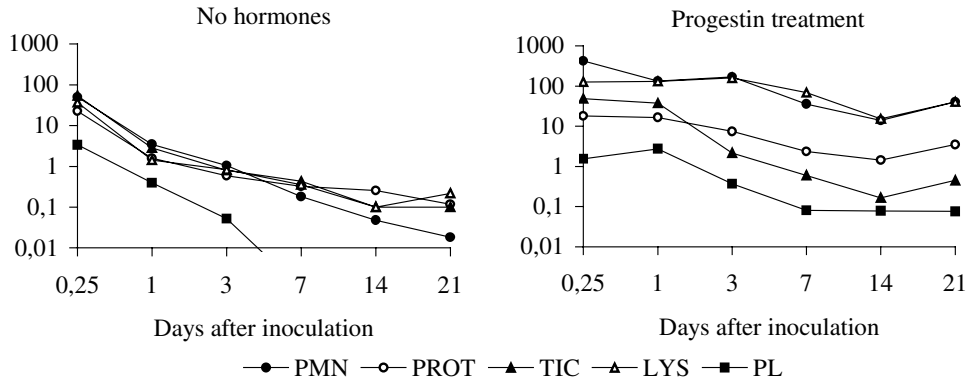


Figure 7. Parallelism of PMN numbers (billions/l), total protein concentration (g/l), TIC (relative units), lysozyme concentration (mg/l), and plasmin activity (U/l) in uterine lavage fluid after bacterial inoculation (I). Values are means of four OVX-mares.

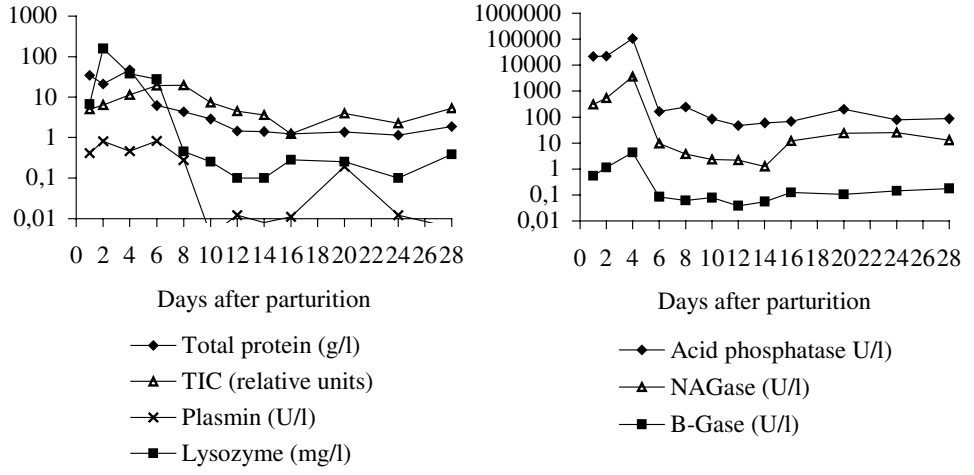


Figure 8. Combined data of intact post-partum mares (IV). Values are means of four mares.

6. *In vitro* motility studies of spermatozoa

Addition of undiluted IUF to extended semen significantly reduced progressive motility (PMOT), average path velocity (VAP) and percentage of rapid spermatozoa ($P<0.01$; Figure 1. in III). The effect on total motility (MOT) was less pronounced ($P<0.05$). Addition of diluted uterine fluid to extended semen had no effect on MOT, PMOT, VAP or percentage of rapid spermatozoa.

7. Embryo recovery and pregnancy results

In the post-breeding study (II), pregnancy rates were 86% (6/7) for raw semen, 83% (5/6) for extended fresh semen, 50% (4/8) for concentrated raw semen, 50% (3/6) for natural breeding, 25% (2/8) for frozen semen, 38% (3/8) for frozen semen plus seminal plasma, 0% (0/8) for diluted frozen semen and 25% (2/8) for centrifuged resuspended frozen semen.

In non-parturient mares (III), the embryo recovery rate was 66% (23/35). Twenty-five embryos (including 2 sets of twins) were recovered. Embryo recovery rate in mares inseminated after the first p.p. ovulation was 53% (8/15) (V). All embryos were considered normal and of excellent or good quality.

DISCUSSION

1. Collection methods of uterine fluid

In endometritis (I, II) and post-partum studies (IV, V), uterine lavage was chosen as the collection method of uterine secretions for several reasons. Firstly, lavage technique enables sufficient fluid recovery also from non-infected mares with small volumes of uterine secretions. Secondly, uterine flush technique has been shown to be superior to the swab technique for the diagnosis of endometritis, on the basis of quantitative cytological and microbiological findings (Ball et al. 1988). Thirdly, uterine lavage is the only way to collect uterine secretions at embryo recovery without compromising embryo recovery results. Accordingly, uterine lavage technique allowed comparison between the different studies. However, it should be pointed out that this technique does result in dilution of native secretions to an unknown extent. Consequently, the flushing technique does not distinguish mares with small amounts of concentrated uterine fluid from mares with larger amounts of less concentrated uterine fluid. Despite the drawbacks of uterine lavage, this technique has diagnostic value. In the present studies, steroid hormones, bacterial inoculation, different types of breeding, and foaling had significant effects on ULF composition. Troedsson & Liu (1992) emphasized the importance of using undiluted uterine secretions or dilution markers in washings because of the different concentrations of uterine secretions in washings from susceptible and resistant mares. Looking at this from a different point of view, while their analysis of undiluted uterine secretions obtained after bacterial challenge failed to show differences in protein concentration between susceptible and resistant mares, the different course of endometritis in these two groups of mares was detected using uterine lavage (Troedsson & Liu 1992).

In the post-breeding study (II), the use of uterine lavage enabled better quantitation of bacteria and PMNs than the swab or the tampon. The flush technique allows a larger area of the endometrium to be sampled. This is an advantage, particularly when numbers of bacteria are low. In our experience, this technique also results in more positive and fewer false-negative diagnoses than the swab technique in diagnosing acute endometritis. Bacteriological examination of uterine secretions obtained by tampon yields unreliable results. A non-guarded tampon is likely to become contaminated especially during withdrawal through a vaginal speculum. This was clearly shown in the fluid accumulation study (III), where 54% of the tampons collected from normal mares without cytological evidence of endometrial inflammation yielded some bacteria. However, in that study, bacteriological examination was of secondary importance; more importantly, the tampon technique allowed comparison of uterine secretions in oestrous mares with and without ultrasonically visible fluid accumulations. Analysis of undiluted uterine fluid provided an opportunity to speculate about the causes of uterine fluid accumulations. In addition, use of native secretions permitted detection of those parameters that fell below the detection limits of assays when lavage fluids from non-infected oestrous mares were analysed.

The collection of IUF with a tampon proved satisfactory in mares with fluid accumulation (93%). Good recovery rates have also been reported by others after

bacterial challenge (Katila et al. 1990; Troedsson & Liu 1992). However, in mares without fluid accumulation, our fluid recovery rate of 31% was much poorer than the 96% reported by Tunón et al. (2000). Moreover, the average volume of IUF recovered was remarkably lower (0.5 ml) than the amount of fluid (3.5 ml) obtained by Tunón et al. (1998). The 20-ml syringe used in our study for squeezing the fluid from the tampon may have had a weaker pressure system than the manual press used by Tunón et al. (2000). Another possible reason for the low recovery rate in our study may have been an inadequate absorption time. Instead of 30 min, the tampon was only allowed to absorb fluid for 15 min to minimize irritation of the endometrial mucosa. The speed of inflammatory response to physical stimulation is unknown. Leucocyte and protein concentrations in uterine flushings were significantly elevated 6 h after uterine and cervical stimulation, but the inflammatory response was not studied earlier (Williamson et al. 1987). The first neutrophils have been shown to enter the uterus within an hour after intrauterine infusion of oyster glycogen (Watson et al. 1987a), and within half an hour after AI (Katila 1995) and bacterial infusion (Pycock & Allen 1990). Presumably, an excessive tampon absorption time will distort findings.

2. Composition of uterine lavage fluid in non-infected mares (I, IV)

The dosage of oestradiol benzoate (5 mg/day) used in OVX-mares was sufficiently high to produce an endometrium typical of an oestrous mare. Doses as high as 10 mg/day have also been used (McDowell et al. 1987). However, 1 mg/day (Watson et al. 1988b) or 1.5 mg/day (Hamer et al. 1985) have been reported to induce biopsy changes consistent with oestrus. To produce the endometrial changes characteristic of dioestrus, synthetic progestin was used at the recommended dose for suppression of oestrus.

Seven days' treatment of ovariectomized mares with oestradiol, oestradiol plus progestin or progestin alone had no significant effect on total protein concentrations of ULF. Total protein concentration in ULF was significantly higher during mid- and late dioestrus than during early oestrus. In some studies, no significant differences have been observed in total protein concentration between dioestrus and oestrus (Blue et al. 1984, Strzemienski & Kenney 1984). Others have reported that progression of dioestrus (Zavy et al. 1978) and increasing dosage and duration of progesterone treatment increased total recoverable protein (McDowell et al. 1987).

Lavage fluids diluted the uterine fluid so much that lysozyme concentration, TIC and plasmin activity fell below the detection limits of the assays. Scudamore et al. (1994) measured α_1-PI, also known as α_1-antitrypsin, in equine uterine flushings using enzyme linked immunosorbent assay. In their study, α_1-PI concentrations relative to total protein were higher during oestrus than during dioestrus. Immunological analyses measure the concentration of total α_1-PI (complexed α_1-PI and the native α_1-PI isoforms), which probably differs from that of functionally active α_1-PI determined in our study. Although lysozyme activity in porcine uterine flushings appeared to be induced by progesterone (Roberts et al. 1976b), its activity in undiluted equine uterine fluid did not differ statistically between dioestrus and oestrus (Katila et al. 1990). This may represent either a difference between species or between sample collection techniques. The results indicate

that with the methods used no TIC, lysozyme or plasmin activity should be detected in ULF from non-inflamed mares at any stage of the cycle. To detect differences caused by steroid hormones, undiluted uterine fluids would need to be sampled.

Treatment with progestin significantly increased activities of NAGase, B-Gase and acid phosphatase in ULF. A slight interaction effect (progestin*oestradiol) was detected only for NAGase. In contrast to an earlier study (McDowell et al. 1987), a synergistic effect of oestradiol and progesterone on acid phosphatase was not observed. Hansen et al. (1985) reported 10 times higher NAGase activities in 3 mares treated with progesterone for 28 days than in mares treated with oestradiol benzoate or sesame oil. Accumulation of NAGase was significantly stimulated only by a combination of progesterone and oestrogen. In study IV, activities of acid phosphatase, B-Gase and NAGase were significantly higher during dioestrus than during oestrus. Acid phosphatase activities in uterine flushings of the mare have been shown to reach a maximum at days 12-14 of the luteal phase and then to decline to almost undetectable levels by day 20 (Zavy et al. 1982).

3. Composition of uterine lavage fluid after bacterial inoculation (I)

The well-known problems associated with endometritis studies using progesterone-treated mares (Hinrichs et al. 1992; McDonnell & Watson 1992) were also encountered in the present study. Increasingly more progestin-treated mares became infected with contaminant bacteria during the 3 weeks' treatment period. Re-infection with coliforms is likely to have affected the composition of uterine fluid. The different duration of hormone treatments at each sampling time should also be noted. Concentrations obtained after bacterial challenge may have been a combination of the effects of hormones and uterine inflammation.

Neutrophil numbers in ULF rose sharply within 6 h in all treatment groups, and total protein concentrations increased in all treatment groups other than E. Total protein concentrations in uterine washings have been shown to peak within 6 h after bacterial infusion (Williamson et al. 1987). In our study, protein concentration was approximately 20 times higher in progestin-treated mares than in oestrogen-treated mares 6 h after bacterial challenge. A high concentration of protein in lavage fluid may indicate a severe inflammatory reaction with large amounts of exudate and/or prevention of drainage.

During oestrus or oestrogen dominance, lowered adhesion of bacteria to endometrial cells (Watson et al. 1988b) and effective uterine drainage through an open cervix (Evans et al. 1986) facilitated by myometrial contractions (Troedsson et al. 1993b) assist removal of bacteria, semen, inflammatory by-products and accumulated uterine fluid from the mare. Removal of material may be so effective during oestrogen treatment that the rapidly decreasing numbers of bacteria only provoke a mild inflammatory reaction. This may indicate adjustment of inflammatory reaction to fulfil a need, while avoiding becoming excessively pronounced since inflammation itself is destructive.

TIC was measured to determine whether it reflects permeability changes in the uterus, as it does in bovine mastitis (Honkanen-Buzalski & Sandholm 1981). In the mare, α_1-PI is not produced in the endometrium but leaks from the blood into the uterine lumen (Scudamore et al. 1994). Thus, an enhanced accumulation of α_1-PI is expected when vascular permeability is increased. TIC and plasmin activity rose sharply within 6 h in all other treatments except treatment E. When infection persisted, TIC and plasmin activities remained elevated but were markedly lower than during the initial phases of inflammation. Reasons for the lowered activities may have been reduced vasopermeability during persistent infection, proteolytic inactivation or inactivation because of antiproteinase-proteinase complex formation. Purified equine α_1-PI and neutrophil elastase have been shown to form a complex *in vitro* (Scudamore et al. 1993). In our study, TIC relative to total protein was higher in the 6- and 24-h samples than in other samples in progestin-treated mares. Vascular leakage, which occurs during inflammation, is probably greatest in the early stages of inflammation. Part of the elevated TIC relative to total protein may have been due to this high permeability, and the blood-uterine lumen barrier actively selecting certain substances over others (McRae 1988).

Lysosomal enzymes seem to be normal secretory products of the endometrium. Progesterone, not infection, is the main reason for increased activities. In our study, acid phosphatase, NAGase and B-Gase activities parallelled each other after bacterial challenge. Although these enzymes can be found in equine leucocytes (Healy 1982), their activities in uterine flushings were not significantly elevated in acute endometritis. It should be noted that in our studies analyses were performed on centrifuged samples. Studies on experimental mastitis have shown that much of the milk NAGase is within the cells (Kaartinen et al. 1988, 1990). The degree of cellular NAGase sequestration was related to different mastitis pathogens (Kaartinen et al. 1990). *Streptococcus agalactiae*, in particular, was able to block the release of NAGase from phagocytes. Thus, non-centrifuged frozen-thawed samples are perhaps more suitable for analysis of these lysosomal enzymes. Future research could investigate the effects of acute endometritis-producing organisms on NAGase release. Lysozyme, on the other hand, turned out to be a reliable indicator of both acute and persistent endometritis. Activities closely correlated with neutrophil numbers and total protein concentrations. Another study has reported that neutrophil numbers and lysozyme followed the same pattern of change after bacterial inoculation in oestrous mares (Katila et al. 1990).

The functions of lysosomal enzymes are unclear. They are believed to play important roles in several processes of reproduction. The *in vitro* study by Hussain et al. (1992) demonstrated that NAGase has bactericidal activity on some Gram-positive pathogens of the cow. NAGase treatment significantly decreased numbers of viable *Actinomyces pyogenes*, *S. aureus* and *Strep. agalactiae*, but had no effect on *E. coli* and *Enterobacter aerogenes*. Consequently, the blockage of NAGase release from PMNs seems to be a counter-strategy of some bacteria against antibacterial effects of NAGase. In our study, streptococci were often replaced by coliforms in mares receiving progestin. Resistance to NAGase or to other bactericidal enzymes like lysozyme may have facilitated colonization of coliforms.

4. Intrauterine fluid accumulations during oestrus (II, III)

Intrauterine fluid was detected by ultrasonography during oestrus in 21% of cycles in the post-breeding study (II) and in 39% of mares in the fluid accumulation study (III). This reflects the incidence of IUFA in normal oestrous mares. Small amounts of fluid are not an unusual finding (McKinnon et al. 1988a). Secretions from the uterine glands and transudate from the blood are generally believed to be sources of uterine fluid in the mammalian uterus. Accumulation of fluid in the uterus can be caused by increased production or decreased elimination. Slower lymphatic drainage caused by deficient myometrial contractions has been suggested as one of the factors impairing drainage (LeBlanc et al. 1995). An inadequate relaxation of the cervix has been proposed to predispose a mare to fluid accumulation (Evans et al. 1986; LeBlanc et al. 1994). If impaired mechanical clearance was the only cause of fluid accumulation, the composition of uterine fluid would probably be the same in mares with and without IUFA. However, in our study (III), compositional changes in IUF suggest different levels of fluid production.

The mean total protein concentration in undiluted uterine fluid of mares with IUFA was one-third of that found in mares without IUFA. The low protein content resembled that of a transudate. The mean concentration in mares without IUFA (25.2 g/l) was somewhat lower than in a subsequent study (34.5 g/l; Tunón et al. 1998). In both studies, variation among mares was high. High albumin concentration has been found in uterine secretions from healthy oestrous mares (Tunón et al. 1998). Since albumin is not produced by the endometrium, the authors concluded that it must have resulted from transudation. The high similarity between protein profiles of uterine fluid and those of serum further supported their theory that serum transudation contributes to the accumulation of intrauterine fluid during oestrus. The diluting effect of the transudate upon the specific secretion from the uterine glands has also been proposed by Tunón et al. (2000).

An increased TIC was expected in IUF of mares with IUFA. However, the mean TIC of uterine fluid was lower in mares with fluid accumulations than in mares without. One possible explanation is that TIC was derived from selective instead of passive transudation. The proteolytic activity of the uterine fluid in the form of plasmin did not differ between mares with and without IUFA. Plasmin, formed from blood-derived plasminogen, is inhibited by α_1-PI. Because of possible antiproteinase-proteinase complex formation, analyses of plasmin activity and TIC are not good measures of transudation.

Mean NAGase activities did not differ in mares with and without fluid accumulation. NAGase is produced by the endometrium, the secretory activity of which is highly dependent on oestrogen and progesterone stimulation. Non-ciliated cells on the uterine surface and in the mid-deep glandular section present numerous secretory vesicles, indicating a high secretory activity (Tunón et al. 1995). Uterine fluid NAGase activity of our mares was about ten times higher than equine plasma NAGase activity found by Raulo et al. (1996). If a transudate had constituted a substantial portion of the fluid accumulations, secretions of uterine glands would have been diluted. Our results suggest that secretory activity of the endometrium is high in mares with fluid

accumulations. Owing to larger fluid volumes, the total NAGase activity in the uterus is likely to be higher in mares with than without IUFA. Unlike NAGase, the mean B-Gase activity was significantly lower in mares with fluid accumulation. Although NAGase and B-Gase are both lysosomal enzymes, their origin, secretion rates or inactivation rates may differ. However, since the results show the activities not the concentrations, it is difficult to draw conclusions about their secretion rates.

In study II, IUFA scores correlated with the degree of endometrial oedema. In study III, 40% of mares with and 33% of mares without IUFA had moderate to marked oedema of endometrial folds, but this difference was not significant. It would seem logical that mares with marked oedema might also have more fluid in the uterine lumen. Tunón et al. (2000) have suggested that transudation is a major mechanism for the formation of intraluminal uterine fluid in the mare. Causey et al. (2000) observed that the extracellular mucus blanket stained less intensely in biopsy samples taken during oestrus than during dioestrus. Increased hydration due to greater endometrial oedema at oestrus was suspected as the cause of less intense staining.

In study II, positive correlations were found between age and occurrence of free uterine fluid, but in study III, the incidence of IUFA did not differ in mares ≤7 years and >7 years. The cut-off point in study III was probably too low. Different results might have been obtained if mares older than 12 or 14 years would have been categorized as old. Carnevale & Ginther (1992) reported that endometrial biopsies of old mares (≥15 years) had more inflammatory cell infiltrations, more fibrotic changes and less dense glands than biopsies of young mares. In their study, IUF collections were more abundant in old than in young mares, and pregnancy rate on day 12 was lower in old mares. In study III, fluid accumulation was associated with increased fibrosis of the endometrium, but not with lymphatic lacunae in endometrial biopsy specimens. Endometrial fibrosis is known to increase with age (Kenney 1978; Carnevale & Ginther 1992). Endometrial fibrosis has been suspected of blocking uterine drainage through the lymph vessels (LeBlanc 1994). Fibrosis may also be involved in increased mucus production of the endometrium. Causey et al. (2000) observed hypersecretion of mucus in fibrotic nests and inflamed endometrial samples.

Neither of our studies showed any negative effects of IUFA during oestrus on pregnancy rates. Pycock & Newcombe (1996) reported higher pregnancy rates for mares with no fluid (62%) than for mares with a small amount of fluid (27%) or those with >20 mm of fluid (13%). In McKinnon et al. (1987), embryo recovery rates were unaffected when the estimated fluid quantity was <100 ml. Fluid quality may be more important for fertility than quantity (Squires et al. 1989). In our study, the fluid was non-echogenic and contained neither PMNs nor significant numbers of bacteria. It was considered not to be of inflammatory origin, and therefore, negative effects on fertility were unlikely. The addition of undiluted IUF to extended semen did significantly reduce sperm motility *in vitro*, but no effect was observed when uterine fluid was diluted. Similar results have been published by Squires et al. (1989) and recently by Alghamdi et al. (2001). Further studies are needed to clarify the relationship of intraluminal fluid at oestrus to fertility.

5. Uterine involution and secretory function of the endometrium after parturition (IV)

During the post-partum oestrus, 5 out of 8 mares showed cytological evidence of inflammation. In most cases, neutrophils were found only in the lavage fluid. In previous studies, 31% (Ricketts & Mackintosh 1987) and 23% (Purswell et al. 1989) of foal heat mares yielded cytologically positive swabs. The high number of cytologically positive mares in our study was probably related to the sampling technique. The flush technique has been shown to be superior to the swab technique for the diagnosis of endometritis (Ball et al. 1988). Mares cytologically positive during foal heat were free from neutrophils during the first post-partum dioestrus.

One to four days post-partum, total protein concentration in ULF was high but declined rapidly thereafter. As expected, total protein concentration during oestrus was significantly higher in samples from post-partum mares than in those from non-parturient mares. The concentration in p.p. mares was similar to that obtained by LeBlanc et al. (1988) at 8 days post-partum. The highest protein concentrations and TIC, as well as increased activities of lysozyme and plasmin, were associated with uterine inflammation. In mares with clear cytologic evidence of uterine inflammation, TIC and plasmin were higher than in samples collected 6 days earlier. In cytologically negative mares and in mares with only a few neutrophils, a downward trend was evident in TIC and plasmin. It seems that both proteolytic and antiproteolytic activities of ULF decrease during the normal involutionary process but increase in mares with acute inflammation. These results suggest that TIC and plasmin activity in ULF may have some diagnostic value in assessing the course of endometrial inflammation in post-partum mares.

Lysozyme concentration in ULF was high soon after foaling but decreased rapidly thereafter. During the p.p. oestrus, lysozyme was detectable in 4 of the 5 mares with cytological evidence of inflammation. In contrast to TIC and plasmin, lysozyme followed a downward trend also in these mares. Our results (I), like the results of Katila et al. (1990), show that lysozyme concentrations correlate closely with PMN numbers. Factors other than neutrophils may have contributed to the elevated lysozyme activity immediately post-partum. Lysozyme has been shown to accumulate in allantoic fluid in the pig (Roberts et al. 1976b) and in amniotic fluid in humans (Barling et al. 1985). Whether this applies to the horse remains to be seen.

One to four days p.p., acid phosphatase, B-Gase and NAGase activities in ULF were high but thereafter declined rapidly both in intact and ovariectomized mares. The high activities were most likely due to residual placental fluids. Cell death resulting in enzyme release may have contributed to enzyme activity. Karyorrhexis and cytoplasmic vacuolization with subsequent cellular lysis have been shown to be involved in removal of some microcaruncles (Gygax et al. 1979). Acid phosphatase and B-Gase activities were still significantly higher in parturient mares during the p.p. oestrus than in oestrous non-parturient mares. Our results confirm the findings of LeBlanc et al. (1988), who observed a decrease in the acid phosphatase activity of uterine lavage fluid between days 4 and 8 after parturition.

After the first post-partum ovulation, activities of acid phosphatase, B-Gase and NAGase increased in individual mares because of rising serum progesterone. This increase was most obvious for NAGase, the activity of which had already returned to normal levels by foal heat. As expected, this rise in enzyme activities was not observed in ovariectomized post-partum mares. In cows, NAGase activity in undiluted uterine fluid peaked at day 5 p.p. and then gradually declined, reaching its lowest value at day 32 (Hussain et al. 1989). The more rapid return of NAGase activities in the mare was anticipated because of a different involution process in this species. Extensive changes take place in the mare's uterus during the first week p.p.. Changes have been reported in uterine size (Gygax et al. 1979; Katila et al. 1988a; McKinnon et al. 1988b; Sertich & Watson 1992; Arrott et al. 1994), uterine histology (Gygax et al. 1979; Bailey & Bristol 1983; Katila 1988), elimination of bacteria and inflammation (König 1975; Gygax et al. 1979; Katila et al. 1988b; McCue & Hughes 1990) and secretory activity (LeBlanc et al. 1988). Ultrasonographic studies on uterine size have shown that uterine involution is completed in a mean of 23 days (McKinnon et al. 1988b) or by 27 to 31 days (Griffin & Ginther 1991). Repair of uterine tissues occurs even faster. Resorption of microcaruncles was completed by 7 days, dilated glands had returned to normal size by 11 days and the endometrium had resumed its pregravid appearance by 14 days (Gygax et al. 1979). Sertich et al. (1988) have shown that uterine involution is independent of ovarian function.

The completion of uterine involution in the present study, as determined by biochemical evaluation, was consistent with the results reported by others using ultrasonographic and histological evaluation. After day 16, protein and enzyme levels in ULF of post-partum mares had decreased to normal levels, showing that the secretory function of the endometrium had presumably been restored to its pregravid state.

6. Predicting fertility in foal heat (V)

None of the parameters measured at the time of AI were significantly related to the embryo recovery rate 7 days later, possibly because the number of mares was so small. Embryo recovery rates were 1/4 and 4/5 in mares with and without PMNs in their uterine lavage fluid at the time of AI, respectively. Neutrophils in uterine biopsy specimens obtained at 5 days p.p. have been shown to correlate significantly with foal heat pregnancy rate (Katila et al. 1988b), whereas those obtained by uterine swabs showed no similar correlation (Gygax et al. 1979; Katila et al. 1988b; McCue & Hughes 1990). Embryo recovery rates were 0/3 and 5/6 in mares with lysozyme present or absent, and 1/4 and 4/5 in mares having TIC present or absent in ULF at the time of AI, respectively. Determinations of lysozyme and TIC in ULF appear to be sensitive methods for quantifying uterine inflammation in p.p. mares. Further studies are needed to establish whether PMNs, lysozyme or TIC in ULF can be used in predicting conception. The good foal heat pregnancy rates reported during 1990's (Arrott et al. 1994; Camillo et al. 1997) challenge the traditional belief of low foal heat fertility. Consequently, the need for prognostic indicators of foal heat fertility has diminished.

At the time of embryo recovery, NAGase activities in ULF were significantly higher, and B-Gase activities tended to be higher in mares not producing embryos than in embryo-producing mares. In mares not producing embryos, B-Gase activity showed no correlation with total protein concentration, as it did in embryo-producing mares, but instead correlated negatively with plasmin activity. The relation of these findings to embryo recovery is unclear. Although these lysosomal enzymes accumulate in the uterine lumen in response to progesterone (I, IV), other factors seem to have been responsible for higher activities in mares not producing embryos. Whether endometrial inflammation during oestrus is expressed as increased NAGase or B-Gase activity during the subsequent dioestrus has yet to be determined.

7. Neutrophilia after insemination (II)

The reason for initiating the post-breeding study was an observation made on mares that had been inseminated with frozen semen: purulent discharge from the vulva was frequently seen 6 to 12 h after insemination. One of our hypotheses was that glycerol and egg yolk in the freezing extender cause the strong neutrophilia. It turned out that this was not the case. All extenders used (PBS, egg yolk-glyserol extender, skim milk) caused a very mild neutrophil response, which was significantly different from the reaction caused by frozen semen or by concentrated fresh semen. These results have later been substantiated by others (Bergman & deKruif 1997; Parlevliet et al. 1997).

Another hypothesis was that the inflammatory reaction was due to destructive or irritating enzymes released from damaged or dead spermatozoa that are always present in high numbers in frozen-thawed stallion semen. However, supernatant obtained by centrifuging frozen-thawed semen elicited a very mild neutrophilia, of the same magnitude as that obtained using the extenders. When frozen semen was centrifuged and the spermatozoa resuspended in new egg yolk-glycerol extender (washed frozen semen), a strong neutrophilia followed the insemination. This shows that spermatozoa themselves provoke neutrophilia. Later, it was shown that the PMN numbers in uterine fluid 5 h after AI were not different for mares inseminated with live or dead spermatozoa (Katila 1997).

The third hypothesis was that marked neutrophilia was the result of high numbers of spermatozoa being present in a small volume of frozen semen. Our findings support this assumption. The highest neutrophil counts were found after insemination with frozen semen or concentrated fresh semen. Inflammatory reaction after natural mating and AI with fresh semen was not significantly different, which was later confirmed by Nikolakopoulos & Watson (1997). A negative correlation was observed between PMN numbers and volumes of inseminate ($P<0.01$). Nikolakopoulos & Watson (2000) also concluded that large inseminate volumes decrease the inflammatory response. This could be related to improved mechanical drainage when large inseminate volumes are used.

Bacterial contamination of the uterus 6 h after breeding or insemination was insignificant. For more than 30 years - since the study of Bryans (1962) - it was believed that massive numbers of bacteria are introduced into the uterus of the mare

during breeding (Asbury 1986). At the same time, it was well known that PMNs rapidly invade the uterus after breeding, but this was considered to be caused by bacterial insult. In our study, the samples were taken only once, at 6 h, so it remained unclear whether bacteria had already been eliminated or whether they had had insufficient time to multiply. In a subsequent study, mares were sampled from 0.5 to 48 h after insemination (Katila 1995). At 1-h post-insemination, uterine fluid of all mares exhibited growth of corynebacteria after a 48-h incubation. The same organism was cultured from all ejaculates of the stallion. In subsequent samplings, the number of positive mares decreased rapidly, showing that the bacteria had not been established in the uterus.

One explanation for the discrepancy between the study of Bryans (1962) and ours could be the difference between natural cover and AI. AI, particularly when semen is diluted with extenders that contain antibiotics, is believed to be a more hygienic way of breeding mares than natural service (Kenney et al. 1975). In the semen collection, we used an open-ended artificial vagina, which results in semen with very low numbers of bacteria as compared with the traditional Missouri model artificial vagina (Lindeberg et al. 1999). However, the mares that were bred naturally did not show more bacterial growth in uterine samples than inseminated mares. It seems, therefore, that bacterial contamination during breeding and AI is much smaller than has been expected and is not the cause for the massive neutrophilia.

Our conclusion was that spermatozoa, not bacteria, induce the strong, transient neutrophilia in the equine uterus in association with breeding. The leucocyte response after insemination with frozen semen was of the same magnitude as after bacterial inoculation in study I. Troedsson (1995) reported that the inflammatory response to 700 x 10^6 spermatozoa freed from seminal plasma and bacteria was comparable with uterine response to *Streptococcus zooepidemicus* observed in a previous study (Troedsson et al. 1993d). Sperm-induced leucocytosis has also been shown in many other species, e.g. in rats and mice (Austin 1957), goat and cattle (Mattner 1968), rabbits (Tyler 1977), women (Cohen 1984) and swine (Rozeboom et al. 1999). Troedsson et al. (2001) suggested that spermatozoa initiate chemotaxis of PMN by activation of complement, which results in cleavage of factors C5a and C3b. C5a is chemotactic for PMN, causing it to migrate from the blood to the uterine lumen. C3b opsonises spermatozoa, thus facilitating their phagocytosis by PMN.

Seminal plasma has been shown to have a dose-dependent suppressive effect on PMN chemotaxis, phagocytosis of spermatozoa and complement-induced cytolysis *in vitro* (Troedsson et al. 2000). In rabbits (Tyler 1977) and in women (Cohen 1984), seminal plasma without spermatozoa did not induce neutrophilia. In our study, addition of frozen-thawed seminal plasma to frozen-thawed semen did not reduce the inflammatory reaction of the uterus 6 h after insemination. Contrary to expectations, seminal plasma also induced neutrophilia. Presumably, the centrifugation was not sufficiently effective to remove spermatozoa. Instead, semen should have been filtrated (Magistrini et al. 2000). The role of seminal plasma, especially in the use of frozen semen, requires further studies. Seminal plasma is removed before semen processing and storage because it is detrimental to the survival of sperm (Pruitt et al. 1993).

However, it may be important in the female genital tract in the regulation of breeding-induced endometritis with regard to duration and magnitude of inflammation (Rozeboom et al. 1999; Troedsson et al. 2001) and in facilitating sperm transport into the oviducts (Einarsson & Viring 1973; Overstreet & Tom 1982).

After our results had been published, further discussion has taken place on the possible detrimental effects of marked neutrophilia. Since frozen semen induced the strongest neutrophilia, this has been interpreted as one reason for the low pregnancy rates after AI with frozen semen. Because insemination with frozen semen is usually performed at the time of ovulation, time left for uterine clearance before the cervix closes is fairly short. Mares that are susceptible to endometritis may fail to clear the post-breeding endometritis. Generally, the inflammatory response should be considered a normal physiological phenomenon that is necessary to eliminate excessive spermatozoa and any contaminating bacteria.

Another aspect is the possible negative effect of the inflammatory response to sperm. It has been shown that uterine secretions obtained from mares after insemination decreased sperm motility *in vitro* (Alghamdi et al. 2001). On the other hand, a double-mating study in rabbits has shown that spermatozoa destined to fertilize can pass undamaged through large numbers of PMNs that had resulted from an earlier mating (Taylor 1982). Daily inseminations of normal mares with frozen semen seemed to have no negative effects, with the pregnancy rate per cycle being 50% (Love et al. 1989).

CONCLUSIONS

1. NAGase, B-Gase and acid phosphatase accumulated in the uterine lumen in response to progestin treatment and during natural dioestrus. During acute inflammation, measuring activities of these lysosomal enzymes from centrifuged uterine lavage fluid apparently has no diagnostic value. Lysozyme activities were elevated in ULF in both acute and persistent endometritis. TIC and plasmin activity seemed to be better indicators of acute than persistent inflammation. None of the examined parameters was superior to PMN counts in diagnosing endometritis.

2. The occurrence of intrauterine fluid accumulations in clinically normal mares was not accompanied by cytological, histological or bacteriological evidence of acute endometritis. Accumulation of uterine fluid during oestrus was associated with compositional changes in the uterine secretions. This presumably indicates that the fluid is composed both of glandular secretions and of a transudate.

3. Protein and enzyme levels were high in ULFs of early p.p. mares. During oestrus, total protein and lysozyme concentrations, TIC, and B-Gase and acid phosphatase activities in ULF were significantly higher in parturient mares than in non-parturient mares. During dioestrus, differences were not statistically significant. The results suggest that the endometrial inflammation present at foal heat subsides, and the endometrium resumes normal secretory capacity by the time of the first p.p. dioestrus.

4. None of the parameters examined could reliably predict conception in foal heat. However, detection of PMNs, TIC and lysozyme activity in ULF of most mares not producing embryos suggests that endometrial inflammation at the time of insemination is probably the best explanation for lowered pregnancy rates in foal heat.

5. Intrauterine post-breeding neutrophilia is induced by spermatozoa rather than bacteria. The high neutrophil counts found after insemination with frozen semen or concentrated fresh semen suggest that the intensity of the neutrophil reaction depends on concentration and/or volume of the inseminate.

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