Post-breeding endometritis after low dose insemination in the mare

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POST-BREEDING ENDOMETRITIS AFTER
LOW DOSE INSEMINATION
IN THE MARE

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
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ABSTRACT

Hysteroscopic insemination in mares with delayed uterine clearance (DUC mares) is controversial. While some authors proposed that insemination with a reduced volume and number of spermatozoa may reduce post-mating endometritis, others proposed that the hysteroscopic procedure is inflammatory and should not be used in DUC mares. The overall objectives of this study were to evaluate the incidence and severity of post-mating endometritis in reproductively normal and DUC mares after hysteroscopic insemination at the uterotubal junction, and to determine if hysteroscopic insemination could be used in DUC mares to reduce post-mating endometritis. The mares were classified as normal or DUC based on the presence of intrauterine fluid 24 or 48 hours after a semen challenge. In Experiment 1, the acute endometritis was evaluated 24 hours after insemination. Each mare (n=5 normal, n=5 DUC) received three treatments in three consecutive estrous cycles: UB: uterine body insemination (1 billion spermatozoa, 20 mL), HYST: hysteroscopic insemination (5 million spermatozoa, 0.5 mL) and SHAM: sham hysteroscopic insemination (semen extender, 0.5 mL). Intrauterine fluid accumulation and uterine leukocyte numbers were not influenced by treatment. In Experiment 2, residual endometritis was assessed 48 hours after insemination, and the effect of insemination method on fertility was evaluated. Each mare (n=4 normal, n=5 DUC) received four treatments in four consecutive estrous cycles: UB: uterine body insemination (1 billion spermatozoa, 20 mL), HYST: hysteroscopic insemination (5 million spermatozoa, 0.5 mL), SHAM: sham hysteroscopic insemination (semen extender, 0.5 mL) and SP: hysteroscopic infusion of seminal plasma (0.5 mL). There was no difference in intrauterine fluid accumulation between treatments in normal mares. HYST and SHAM treatments resulted in more fluid accumulation 24 hours after the procedures in DUC mares than UB and SP. However, leukocyte numbers were not different. It was concluded that the hysteroscopic procedure itself is inflammatory, so it should not be used with the intention of reducing post-mating endometritis. However, the inflammation was not greater than that induced by routine uterine body insemination and fertility was not affected. Therefore, there is no contraindication to its use in mares with delayed uterine clearance.
CHAPTER 1
INTRODUCTION

Persistent mating-induced endometritis is still a major cause of economic loss in the equine industry. In a survey involving 1149 veterinarians throughout the United States, endometritis was identified as the third most common disorder of adult horses after colic and respiratory tract diseases (Traub-Dargatz et al., 1991). The older multiparous mares that are more frequently affected by the condition are usually also the most valuable proven mares, so great effort and money are invested every year in managing and treating them.

The deficiency in myometrial contractility responsible for the delay in uterine clearance after breeding may also account for the defective sperm transport to the oviducts seen in these mares (Scott et al., 1995). Hysteroscopic insemination has been proposed as a method to increase the number of spermatozoa in the oviductal reservoir. It was also suggested that placement of semen at the uterotubal junction would minimize sperm-uterine interactions and neutrophil chemotaxis, reducing the inflammatory response compared with uterine body insemination (Lindsey et al., 2001; Squires et al., 2000). If the challenge to the endometrium were reduced, these mares would be less likely to develop persistent mating-induced endometritis and hysteroscopic insemination would become a management option for mares with delayed uterine clearance. However, the value of low dose insemination in the management of subfertile mares is still not proven.

Conversely, other authors have postulated that the hysteroscopic procedure is inflammatory by nature and should not be used on mares susceptible to develop persistent mating-induced endometritis. In one study, pregnancy rates were reduced in problem mares after low dose insemination with fresh semen (Sieme et al., 2004). However, the authors did not comment the reason for this reduction in fertility, and suggested that further studies were needed to determine the hygienic risk associated with hysteroscopic insemination in problem mares. If endoscopy induces an exaggerated inflammatory response, the method may either need to be avoided in mares with delayed uterine clearance, or the mares may need to be treated aggressively as soon after insemination as possible to maximize chances for conception.

The incidence of post-breeding endometritis after hysteroscopic insemination in reproductively normal mares is also not clear and controlled studies that compare post-breeding
endometritis after low dose insemination and uterine body insemination in reproductively normal mares as well as in mares with delayed uterine clearance are lacking.

The overall objective of this research was to evaluate the incidence and severity of post-breeding endometritis in reproductively normal mares and mares with delayed uterine clearance after hysteroscopic insemination and to determine whether hysteroscopic insemination could be used in the management of mares with delayed uterine clearance. Two studies were designed to compare the inflammatory response induced by hysteroscopic insemination at the uterotubal junction with a low dose of spermatozoa to that induced by routine uterine body insemination with a standard dose. In the first study, the acute initial inflammatory response was evaluated 24 hours after insemination. In the second study, endometritis was assessed 48 hours after the procedures and the influence of insemination method on pregnancy rates was evaluated.
2.1. Uterine Defense Mechanisms and Susceptibility to Endometritis

Uterine defense in the mare involves a number of elements that interact in a complex and timely manner. Anatomical barriers, cellular and humoral components of the immune system, and mechanical factors all play a role in defending the uterus against foreign materials. Anatomical barriers protect the uterine environment from ascending bacterial contamination. The first of these barriers is the vulvar seal. Abnormal perineal conformation results in compromise of this seal. This can lead to aspiration of air and fecal material into the vestibule and result in pneumovagina (Pascoe, 1979). Irritation from air or fecal material results in vaginitis, cervicitis and ascending endometritis. The transverse fold at the vestibulo-vaginal junction and the cervix form the second and third anatomical barriers, respectively.

Although physical barriers play an important role in protecting the uterus against ascending infection, they are bypassed during copulation as semen is deposited directly into the uterus in the horse. Endometritis is the endometrial inflammatory response to antigenic or physical stimuli, and is characterized by arrival of neutrophils into the uterine lumen. A transient endometritis normally follows mating in the mare. Uterine defense mechanisms are responsible for eliminating the bacteria and excess spermatozoa introduced during mating. Since the equine embryo reaches the uterus 5 to 6 days after ovulation (Freeman et al., 1991; Oguri and Tsutsumi, 1972), any delay in clearing bacteria and inflammatory products after mating may create a uterine environment that is incompatible with embryonic survival.

In 1969, Hughes and Loy first reported that young fertile mares had a natural resistance to establishment of infection after bacterial inoculation into the uterus during estrus. The inflammation resolved in these young mares within 96 hours. Pluriparous and barren mares, in contrast, were unable to clear the bacterial inoculum. Based on these findings, mares were classified as resistant or susceptible to bacterial endometritis if they had eliminated the uterine infection within 96 hours of inoculation or not, respectively. Subsequently, LeBlanc et al. (1994a) reported that susceptible mares had a delay in the clearance of inoculated radiocolloid compared to resistant mares. The term delayed uterine clearance was coined after this work to refer to mares that fail to remove the excess spermatozoa and bacteria introduced during mating and develop persistent mating-induced endometritis.
2.1.1. Immunoglobulins in the Equine Uterus

Immunoglobulins IgGa, IgGb, IgGc, IgGT, IgA and IgM were first isolated from the equine genital tract by Kenney and Khaleel (1975). It was later demonstrated that IgG, IgA and IgM were locally produced by endometrial epithelial cells, confirming that the uterus was part of the mucosal immune system (Widders et al., 1985).

The influence of steroid hormones on immunoglobulin concentrations is controversial. While some authors reported that total immunoglobulin concentrations were higher during diestrus due to increased IgG concentrations (Mitchell et al., 1982), others found that free immunoglobulin and immunoglobulin-containing cells did not vary throughout the estrous cycle (Watson et al., 1988).

Although immunoglobulin deficiency was suggested as a cause of increased susceptibility of some mares to uterine infection, most studies have failed to demonstrate such a deficiency (Asbury et al., 1980; Troedsson et al., 1993b). After bacterial or lipopolysaccharide infusion, there was an immediate increase in protein concentrations in uterine fluid of both resistant and susceptible mares (Williamson et al., 1987). These were found to be both uterine- and serum-derived proteins, including opsonins, that play a role in the initial non-specific response to the antigen. Total protein and IgG concentrations decreased 24 hours after bacterial infusion, while IgA concentrations were increasing, indicating that antigenic stimulation of IgA production by local plasma cells may play a role in a more specific response (Williamson et al., 1987).

2.1.2. Uterine Leukocytes and Susceptibility to Endometritis

The uterine defense mechanisms include both innate and adaptive immunity. The innate immunity consists of a non-specific response directed against common and highly preserved bacterial structures. It involves preexisting or rapidly responding chemical and cellular defense mechanisms. Macrophages, neutrophils, dendritic cells and natural killer cells are the predominant cellular type that participates in this response. The adaptive immunity consists of a specific response directed against unique antigens. Its onset is slow and the main cellular types participating in this response are B and T lymphocytes.

Neutrophils are the first inflammatory cells to arrive at an inflammatory site. Their main function is non-specific phagocytosis and destruction of invading organisms. They play an important role in uterine defense. Introduction of spermatozoa, bacteria or endotoxins into the
uterus induced an immediate influx of neutrophils within 2 hours, with maximum numbers in the uterine lumen 6 to 8 hours after the initial stimulus (Katila, 1995; Martin et al., 1988; Williamson et al., 1987). After a transient decrease in neutrophil numbers by 12 hours, a second influx was noted 24 hours after stimulation that was probably due to prolonged recruitment after accumulation of chemoattractants (Martin et al., 1988; Williamson et al., 1987).

Chemoattractants known to be produced by the equine endometrium or neutrophils include cleavage products of the complement system and metabolites from arachidonic acid such as leucotriene B\(_4\) (LTB\(_4\)), and prostaglandins E (PGE) and F\(_{2\alpha}\) (PGF\(_{2\alpha}\)) (Pycock and Allen, 1990, 1988; Watson et al., 1987a,b). Finally, the number of neutrophils decreased 96 hours after bacterial inoculation in resistant mares, while they remained high in susceptible mares (Maloufi et al., 2002).

Once migration of neutrophils to the inflammatory site has occurred in response to chemoattractants, recognition of the antigen is facilitated by opsonins that bind to the surface of the invading organisms. Opsonins are identified by receptors on the membrane of the neutrophils. Uterine opsonins include complement cleavage product C3b and IgG, both locally produced by the endometrium or derived from the serum (Watson, 1988). Once the receptor has bound to the opsonin, neutrophils are activated and phagocytosis is initiated. During the process of phagocytosis, lysosomal contents are released not only into the intracellular compartment but also into the extracellular compartment. These lysosomes contain enzymes, oxygen-derived active metabolites and metabolites from arachidonic acid that are responsible for bactericidal activity of neutrophils (Slocombe et al., 1985; Weiss and Lobuglio, 1982). Extracellular release of these contents is responsible for the tissue damage that occurs during an inflammatory reaction. Both lysozyme, a lysosomal marker, and lactate dehydrogenase, a cytoplasmic enzyme released during cell damage and death, increased during acute endometritis (Pycock and Allen, 1990). This indicated that there was lysosomal enzyme release together with death of either endometrial cells or neutrophils during endometritis.

Neutrophil function appears to be influenced by steroid hormones. Random migration of blood neutrophils to the uterus, as well as phagocytic activity of uterine neutrophils was depressed by increased progesterone concentrations (Watson et al., 1987c,d). However, neutrophils showed decreased migration after phagocytosing increasing numbers of bacteria (Watson et al., 1987c, d). Since progesterone-treated mares had delayed clearance of bacteria
compared to estradiol-treated mares (Washburn et al., 1982), it is possible that the neutrophil dysfunction was a consequence rather than the cause of persistent infection.

Watson et al. (1987a) demonstrated that neutrophils from the peripheral blood of susceptible mares were less responsive to chemoattractants and had lower migratory capacities. Similarly, uterine neutrophils recovered 12 to 18 hours after induction of endometritis from susceptible mares had depressed phagocytic and bactericidal activities. As in the case of progesterone-treated mares, it was suggested that the phagocytic capacity of neutrophils collected from susceptible mares was compromised due to persistence of infection rather than due to a primary defect. This was further supported by the findings of Asbury and Hansen (1987), who reported that uterine neutrophils from resistant and susceptible mares had similar phagocytic capacity 4 hours after induction of endometritis.

It can be concluded that the initial acute inflammatory response is similar between susceptible and resistant mares. However, susceptible mares are unable to maintain a sustained cellular and humoral response (Asbury and Hansen, 1987; LeBlanc et al., 1989; Nikolakopoulos and Watson, 1997; Williamson et al., 1987). Persistence of bacteria within the uterine lumen in both susceptible and progesterone-treated mares is probably due to impaired physical clearance (LeBlanc et al., 1989). This bacterial persistence leads to activation of neutrophils and phagocytosis. Activated neutrophils show depressed migratory and phagocytic capacities, probably due to an increase in intracellular oxygen-derived metabolites and lysozyme that shorten their lifespan. Impaired neutrophil function leads to persistence of bacteria, colonization of the endometrium, and chronic bacterial endometritis.

Although their role in endometritis is not fully understood, lymphocytes, macrophages and plasma cells have been identified in the equine endometrium. The cell-mediated immune system may also take part in resolving endometritis, as was suggested by Tunon et al. (2000). Helper T-cells were recruited to the endometrial epithelium after insemination at the same time the innate immunity was activated. The increase in the number of helper T-cells was significant only in the uterine body. Activation of lymphoid cells in this area may have been the result of prolonged fluid accumulation at the uterine body-horn junction (Tunon et al., 2000). This prolonged fluid accumulation may have been due to the anatomical shape of the uterus. The function of this specific response may include release of cytokines that promote macrophage activation or antibody formation (Tunon et al., 2000). The B-lymphocytes were also shown to
increase in number in the endometrium of mares with endometritis (Watson and Thomson, 1996). Conversely, the number of cytotoxic T-cells did not increase after antigenic stimulation (Tunon et al., 2000).

Macrophages play a role in inflammatory reactions functioning as phagocytes and in antigen presentation to the lymphocytes. Their presence has been demonstrated in the mare endometrium, but their numbers were not influenced by the stage of the estrous cycle or the presence of endometritis (Summerfield and Watson, 1998). Further studies are needed to assess type and function of equine endometrial macrophages.

2.1.3. Uterine Physical Clearance and Susceptibility to Endometritis

Once the immunological mechanisms are activated during endometritis induced by mating or bacterial inoculation, bacteria, spermatozoa, inflammatory cells and products have to be removed from the uterus. Endometritis is usually accompanied by accumulation of intrauterine fluid. As the endometritis resolves, the fluid is expelled by reproductively normal mares within 12 to 48 hours (Nikolakopoulos and Watson, 1999; Troedsson, 1997). However, fluid accumulations and inflammatory products are retained longer in susceptible mares (LeBlanc et al., 1994a; Troedsson and Liu, 1991).

The primary mechanism involved in persistence of fluid in the uterus after mating is an impaired physical clearance (Nikolakopoulos and Watson, 1999). Myometrial contractions, cervical relaxation and lymphatic drainage are known to influence the ability of the uterus to physically clear inflammatory products. Clearance to the vagina of material inoculated into the uterus started as soon as 1 to 2 hours after intrauterine inoculation (Evans et al., 1987) and persisted for 20 hours. Uterine clearance was complete in resistant mares in 20 hours (Troedsson and Liu, 1991). However, susceptible mares showed a delay in uterine clearance, which was not complete by 96 hours after inoculation of non-antigenic markers such as $^{51}$Cr labeled microspheres (Troedsson and Liu, 1991) or radiocolloid (LeBlanc et al., 1994a). Most of the inoculated material pooled in the uterine body in these mares with delayed uterine clearance (LeBlanc et al., 1994a; Troedsson and Liu, 1991). This pooling of material in the uterine body and lack of flow into the vagina in mares with delayed uterine clearance was thought to occur in part due to anatomical differences in uterine position. In older, multiparous mares the broad ligament stretches, causing the cranial reproductive tract to lose structural support and drop below the level of the cervix. Older mares with delayed uterine clearance have a more vertical
uterus that is located ventral to the cervix, hampering free flow of fluid through the cervix. Reproductively normal mares, conversely, have a more horizontal uterus located at the level of the cervix, which facilitates free flow of uterine contents into the vagina through an open cervix (LeBlanc et al., 1998).

The myometrium in reproductively normal mares responded to bacterial inoculation with an immediate increase in electrical activity (Troedsson et al., 1993a). The increase in high intensity myoelectrical activity after bacterial inoculation occurred 2 hours later in mares with delayed uterine clearance than in reproductively normal mares. While reproductively normal mares maintained increased uterine activity for at least 20 hours after bacterial inoculation, this activity declined sharply by 11 hours in mares with delayed uterine clearance (Troedsson et al., 1993a). It appears that the mechanism responsible for impaired myometrial activity in mares with delayed uterine clearance is an intrinsic contractile dysfunction of the myometrium (Rigby et al., 2001). However, the exact nature of the dysfunction is unknown. Stimulation of myometrial strips from mares with delayed uterine clearance with oxytocin and PGF$_{2\alpha}$ induced a response similar to that of reproductively normal mares, indicating that this defect was not receptor-mediated. Although age and parity are usually associated with delayed uterine clearance, Rigby et al. (2001) failed to find an effect of these parameters on the intrinsic contractility of the myometrium.

Aberrations in the uterine contractile pattern were also reported in mares with delayed uterine clearance (von Reitzenstein et al., 2002). Reproductively normal mares exhibited a normal propagation pattern, with uterine contractions starting at the uterine horn and moving towards the cervix. Mares with delayed uterine clearance exhibited an inverted propagation pattern, with contractions starting at the uterine body and moving towards the uterine horns, or a simultaneous pattern, with contractions starting simultaneously at the uterine body and horns. It was proposed that in reproductively normal mares there was a pacemaker region in the myometrium of the uterine horns. The pacemaker region in mares with delayed uterine clearance was more frequently located in the uterine body. It is possible that this ectopic location was intrinsic to mares with delayed uterine clearance, or that it developed as a consequence of stretching and hypertrophy of the myometrium from repeated pregnancies in old, multiparous mares (von Reitzenstein et al., 2002).
The depressed uterine myoelectrical activity in mares with delayed uterine clearance was also suggested to be due to accumulation of nitric oxide (NO) within the uterine lumen (Alghamdi and Troedsson, 2002). Nitric oxide is known to inhibit myometrial contractility in primates and other species (Kuenzli et al., 1998). Although the precise mechanism by which NO induces uterine relaxation, there is evidence to support that NO-induced uterine relaxation is mediated by calcium activated potassium channels in humans (Modzelewksa et al., 2003). These channels modulate smooth muscle contraction by promoting repolarization of the cell membrane (Zhou et al., 1998). Mares with delayed uterine clearance accumulated significantly more NO in uterine fluid after insemination than reproductively normal mares. This may have been because of increased expression of nitric oxide synthetase (NOS) or decreased drainage of inflammatory products due to a more pendulous position of the uterus (Alghamdi and Troedsson, 2002). Inducible NOS was localized in human endometrial epithelial cells (Telfer et al., 1997), and its expression in rat uterine smooth muscle was induced by the inflammatory mediator IL-1 (Yamamoto et al., 1997). An increased expression of NOS and increased concentrations of uterine NO during endometritis may decrease myometrial activity, resulting in further accumulation of inflammatory products and a continuous stimulus for persistent endometritis.

The myometrium in reproductively normal mares responded to bacterial inoculation with an immediate increase in electrical activity that remained high for at least 20 hours, coincident with the time when clearance was complete. Prostaglandins released by activated neutrophils have been suggested to be responsible for the increased myometrial activity after a bacterial challenge (Troedsson et al., 1993a). Maximum concentrations of PGF\(_{2\alpha}\) in uterine fluid were reached 6 hours after induction of endometritis, and the response was higher in the progesterone-dominated endometrium (Watson et al., 1988). It has been proposed that PGF\(_{2\alpha}\) is produced by activated neutrophils. However, because neutrophils have low cyclo-oxygenase (COX) activity and their numbers do not vary with the steroid environment, the endometrium is the most likely source of prostaglandins during an active inflammation (Watson, 1989). Cyclo-oxygenase enzymes are involved in prostaglandin synthesis from arachidonic acid. Although the enzyme exists in three isoforms, COX-2 is the isoform involved in inflammatory processes, and is rapidly induced by several products (Morita, 2002). Cytokines secreted by inflammatory cells, such as IL-1, induced expression of COX-2 in endometrial tissue (Huang et al., 1998) and, together with other inflammatory mediators, may be responsible for the increase in uterine prostaglandins.
Although uterine contractions play a major role in physical clearance of foreign material, adequate cervical dilation is necessary to allow fluid expulsion from the uterus (LeBlanc et al., 1994a). Steroid hormone environment is known to affect uterine clearance by influencing both uterine contractility and cervical dilation. Elimination of non-antigenic markers and bacteria occurred rapidly after inoculation into the estrogen-dominated equine uterus (Evans et al., 1987; LeBlanc et al., 1994a). Estrogens are known to decrease cervical tone and increase uterine motility, facilitating uterine drainage during the periovulatory period (Hayes and Ginther, 1986). Concurrently, under the influence of progesterone, uterine clearance was impaired (Evans et al., 1987; LeBlanc et al., 1994a). Progesterone may significantly inhibit uterine drainage by increasing the tone of the cervix and inhibiting uterine contractions (Hayes and Ginther, 1986).

Oxytocin affects uterine motility, both directly and indirectly, via release of PGF$_{2\alpha}$ (Paccamonti et al., 1999; Sharp et al., 1997). Oxytocin modulates uterine contractions by inducing intracellular increases in calcium ions and membrane depolarization (Osa et al., 1981). Prostaglandin F$_{2\alpha}$ production in response to oxytocin, is mediated by oxytocin receptors in the endometrium (Sharp et al., 1997). Oxytocin receptors vary in number during the estrous cycle under the influence of steroid hormones. Prostaglandin also stimulates myometrial contraction by inducing release of calcium ions from the sarcoplasmic reticulum (Carsten and Miller, 1977). As the number of oxytocin receptors varies during the estrous cycle, PGF$_{2\alpha}$ production in response to oxytocin also varies.

Before the study of Rigby et al. (2001) that linked delayed uterine clearance with an intrinsic myometrial defect, it was speculated that differences in production or response to oxytocin or PGF$_{2\alpha}$ were responsible for the impaired uterine contractility. However, most studies failed to show a difference in the action of uterotonic hormones between reproductively normal mares and mares with delayed uterine clearance, and the reports were not in agreement. Baseline plasma oxytocin and PGF$_{2\alpha}$ concentrations, as well as oxytocin release in response to insemination did not differ between reproductively normal mares and mares with delayed uterine clearance, and the reports were not in agreement. Baseline plasma oxytocin and PGF$_{2\alpha}$ concentrations, as well as oxytocin release in response to insemination did not differ between reproductively normal mares and mares with delayed uterine clearance (Nikolakopoulos et al., 2000a). Similarly, Cadario et al. (1999) reported that after infusion of radiocolloid during estrus, plasma PGF$_{2\alpha}$ concentrations were initially similar between reproductively normal mares and mares with delayed uterine clearance. However, PGF$_{2\alpha}$ decreased by 120 minutes in reproductively normal mares and remained low, while it started increasing again in mares with delayed uterine clearance. When oxytocin was
administered, more mares with delayed uterine clearance showed an increase in plasma PGF$_{2\alpha}$ concentrations. Those mares that responded with an increase in PGF$_{2\alpha}$ had marked inflammatory changes in their endometrial biopsies, suggesting that the different pattern in PGF$_{2\alpha}$ release was associated more with inflammation than with the susceptibility to endometritis (Cadario et al., 1999). However, another study reported that more reproductively normal mares than mares with delayed uterine clearance responded to oxytocin administration with an increase in PGF$_{2\alpha}$ (Nikolakopoulos et al., 2000a).

Other drugs are known to influence uterine activity. Administration of $\alpha$-agonists increased intrauterine pressure within 4 to 6 minutes. Xylazine induced a greater, but shorter, increase in intrauterine pressure compared to detomidine (Schatzmann et al., 1994). Reproductively normal mares and mares with delayed uterine clearance responded to xylazine with a tetanic uterine contraction that lasted for 11 minutes (De Lille et al., 2000). However, detomidine increased intrauterine pressure in reproductively normal mares but not in mares with delayed uterine clearance (von Reitzenstein, 2002). The high affinity of detomidine to $\alpha_2$ receptors and of xylazine to $\alpha_1$ receptors may have accounted for the differences in response to both drugs in mares with delayed uterine clearance. Conversely, administration of acepromazine, an $\alpha_1$ antagonist, decreased the number of uterine contractions in mares with delayed uterine clearance but not in reproductively normal mares (De Lille et al., 2000).

Uterine lymphatics are also part of the uterine physical clearance mechanism. Lymphatics drain particulate matter from the lumen and intramural fluid. Because lymphatic vessels do not contain smooth muscle, they depend on myometrial contractions to move the lymph. Accumulation of fluid in the uterine lumen and wall occurs in mares with lymphatic stasis. India ink is cleared from the uterus via lymphatic drainage. Reproductively normal mares were able to reabsorb India ink within 24 hours during estrus while mares with delayed uterine clearance had a reduced ability to reabsorb India ink, indicating that lymphatic drainage was impaired in these mares (LeBlanc et al., 1995).

In summary, myometrial contractions, cervical relaxation and lymphatic drainage are involved in uterine physical clearance. Impaired myometrial activity is the main cause of the delay in uterine clearance seen in susceptible mares. The mechanism responsible for this impaired myometrial function in mares with delayed uterine clearance is an intrinsic contractile dysfunction of the myometrium. Oxytocin and PGF$_{2\alpha}$ induce uterine contractions and are
released in response to insemination or bacterial inoculation. However, a difference in the action of uterotonic drugs between reproductively normal mares and mares with delayed uterine clearance has not been found.

2.2. Sperm-Uterine Interactions

2.2.1. Sperm Transport

Spermatozoa are carried to the tip of the uterine horn within 20 minutes of insemination into the uterine body (Katila et al., 2000) and are found in the mare’s oviduct within 2 hours of copulation. Sperm transport to the oviduct is apparently complete in 4 hours (Brinsko et al., 1991). Only a small number of the inseminated spermatozoa reach the oviduct and this number did not seem to be influenced by the insemination dose (Bader, 1982). Once spermatozoa reach the oviducts, a sperm reservoir is established. Scott et al. (2002) provided evidence of a preovulatory sperm reservoir at the uterotubal junction. Spermatozoa were present in the lumen of the papilla or the intramural segment of the uterotubal junction (UTJ) in 70% of the mares 18 to 26 hours after insemination.

The spermatozoa attach to oviduct epithelial cells, which prolong their viability by maintaining basal intracellular calcium ion concentrations and preventing capacitation (Dobrinksi et al., 1996; Thomas et al., 1996). Spermatozoa in the oviduct are mostly morphologically normal cells with intact membranes (Bader, 1982). Although differences in the number of spermatozoa between the two oviducts have not been reported, one study using color Doppler ultrasonography reported that there was an increase in blood flow to the oviduct ipsilateral to the dominant follicle. This increase was first observed 1 hour after insemination with raw semen and lasted for 12 hours. A similar response in blood flow was not seen in the contralateral ovarian artery suggesting that spermatozoa may have been selectively transported to the ipsilateral oviduct (Bollwein et al., 2003).

Uterine motility contributes to sperm transport to the oviducts. Uterine myoelectrical activity started immediately after insemination and lasted 30 minutes (Troedsson et al., 1998). These bursts of uterine contractions occurred simultaneously with oxytocin release from the pituitary in response to mating or teasing during the periovulatory period (Madill et al., 2000). Prostaglandins or oxytocin present in equine seminal plasma may also play a role in mating-induced uterine contractility, stimulating myometrial contractions responsible for transport of
spermatozoa to the oviducts (Watson et al., 1999). Oxytocin increased the number of uterine contractions after intrauterine administration (Campbell and England, 2002).

Mares released endogenous oxytocin in response to sexual stimulation, which resulted in an increase in uterine myoelectrical activity (Madill et al., 2000). However, changes in intrauterine pressure in response to exogenous and endogenous oxytocin varied during the estrous cycle (Gutjahr et al., 2000; Stecco et al., 2003). Although oxytocin was released in response to teasing both during estrus and diestrus (Nikolakopoulos et al., 2000b), stimulation of uterine activity only occurred during estrus when plasma progesterone concentration was low (Gutjahr et al., 2000; Stecco et al., 2003).

Mares with delayed uterine clearance were shown to have lower numbers of spermatozoa with lower motility in their oviducts than reproductively normal mares. They also had pathological changes in their oviductal isthmic epithelium (Scott et al., 1995). Whether the decreased numbers of spermatozoa were due to reduced transport to the oviduct or to impaired attachment to the epithelial cells is unknown, but the reduced sperm reservoir may contribute to subfertility in mares with delayed uterine clearance.

The majority of the spermatozoa ejaculated into the uterus remains in the uterus and must be removed by uterine contractions and an acute uterine inflammatory response. Spermatozoa remaining in the uterus had an increased number of morphological abnormalities and membrane disruption, especially at the level of the acrosome (Bader, 1982; Baranska and Tischner, 1995).

A second phase of uterine activity occurred 4 hours after mating and lasted for at least 12 hours. This activity may have been the result of prostaglandin production by the endometrium or activated neutrophils during the inflammatory reaction, and it was thought to contribute to physical clearance of the uterus (Troedsson et al., 1998). Evacuation of semen through the cervix into the vagina started 1.5 hours after insemination. By 2.5 hours, most of the semen was already in the vagina and little remained in the uterus. Evacuation from the vagina through the vulva was complete in 4.5 hours (Katila et al., 2000).

2.2.2. Inflammatory Response to Semen

Inflammation, characterized by an influx of neutrophils to the uterine lumen, follows mating in the mare. This reaction was initially thought to occur in response to bacterial contamination during breeding. However, the experiments of Kotilainen et al. (1994) demonstrated that spermatozoa, and not bacteria, are responsible for this transient endometritis.
The first neutrophils arrive to the uterine lumen 0.5 hours after breeding, and their numbers increase steadily to reach a maximum 6 to 8 hours after insemination (Katila, 1995). Less clear is the time at which the neutrophil numbers decrease again. Neutrophils were still present 48 hours after insemination in samples collected via uterine flushing (Nikolakopoulos and Watson, 1997, 2000). However, using a tampon to retrieve undiluted samples, Katila (1995) found that neutrophils had totally disappeared 48 hours after insemination. It is possible that the sampling technique accounted for the differing results, as the flushing technique may have been more efficient at collecting neutrophils trapped between the endometrial folds.

The chemotactic stimulus for blood neutrophils is provided by spermatozoa and involves activation of the complement system (Troedsson, 1995). The recruited neutrophils are responsible for phagocytosis of spermatozoa and bacteria introduced during mating, which is facilitated by opsonization of spermatozoa by IgG (Cohen and Werrett, 1975). Inflammatory mediators, PGE$_2$ and NO released during endometrial inflammation were probably responsible for the increase in blood flow in the uterine artery observed 1 hour after insemination (Bollwein et al., 2003).

Any introduction of foreign substances into the uterus induces an acute endometrial inflammatory response. However, the nature of the inoculum can influence the magnitude of the neutrophil response. Semen extender, phosphate buffered saline solution (PBS) or seminal plasma caused less inflammation than spermatozoa, bacteria or endotoxin infusion (Kotilainen et al., 1994; Martin et al., 1988; Williamson et al., 1987).

The volume of the inseminate also influences the magnitude of the response. Maximum inflammatory response was obtained initially when low volumes of semen were infused (Kotilainen et al., 1994). However, 48 hours after insemination, larger volumes of semen showed the lowest residual inflammation (Nikolakopoulos and Watson, 2000). It appeared that low volumes were not expelled through the cervix as efficiently as large volumes, resulting in higher residual inflammation. Sperm concentration also has an effect on inflammatory response. Concentrated semen elicited the most pronounced neutrophil migration 6 hours after insemination (Kotilainen et al., 1994). However, concentrated semen showed the least residual inflammation at 48 hours. It was concluded that a greater insult to the endometrium, represented by insemination of more concentrated semen, resulted in an intense initial inflammation. Prostaglandins released during this process probably stimulated uterine contractions, which
contributed to an early clearance of inflammatory cells and products. Therefore, it appeared that the more minor the insult, the greater the degree of residual inflammation (Nikolakopoulos and Watson, 2000). Physical manipulation of the uterus and cervix also caused an increase in neutrophil numbers (Bergman and de Kruif, 1997; Martin et al., 1988; Williamson et al., 1987).

Because uterine neutrophils are more effective at phagocitizing defective spermatozoa, it could be hypothesized that an increase in the number of dead or defective spermatozoa would increase recruitment of neutrophils. However, the inflammatory response to killed spermatozoa was found to be similar to that caused by live spermatozoa in reproductively normal mares (Katila, 1997). Uterine contamination after mating is greater than after artificial insemination. So, greater inflammation could be expected after mating. Again, reproductively normal mares showed no difference in the degree of inflammation after either type of semen deposition (Nikolakopoulos and Watson, 1997). However, it is not known if a similar response can be expected in mares with delayed uterine clearance.

2.2.3. Role of Seminal Plasma

Seminal plasma has been suggested to facilitate sperm transport to the oviducts in many species, including the horse (Mann et al., 1956). Oxytocin and prostaglandins present in stallion semen may play a role in inducing uterine contractions immediately after breeding (Watson et al., 1999).

Seminal plasma also has an immunomodulatory effect during the post-mating period. Seminal plasma caused a dose-dependent reduction of blood neutrophil chemotaxis, neutrophil phagocytosis of spermatozoa and complement-induced hemolysis in vitro (Troedsson et al., 2000). Its modulatory effect has also been demonstrated in vivo. Although the presence of seminal plasma did not decrease the number of neutrophils 6 and 12 hours after insemination of fresh or frozen semen, it shortened the duration of the breeding-induced inflammation in reproductively normal mares (Kotilainen et al., 1994; Troedsson et al., 2001b).

The immunomodulatory effect seems to be due to inhibition of complement activation (Troedsson et al., 2000), but the fractions involved in this inhibitory action have not been characterized yet. Prostaglandin E₂, present in high concentration in primate semen, has a suppressive effect on almost all the cells of the immune system. Prostaglandin E₂ reduced T-cell and B-cell proliferation and function, natural killer cell cytotoxicity, macrophage phagocytosis and neutrophil function (DeWitt, 1991; Kelly, 1995). Kelly (1995) reviewed the presence of
complement inhibitors in human semen. Clusterin has homology with complement components C7, C8 and C9, and it interacts with the C5b-7 complex preventing membrane insertion. It is present in a soluble form in human seminal plasma. Decay Accelerating Factor and Membrane Cofactor Protein, found in the inner acrosomal membrane of human spermatozoa, inhibit C3 cleavage. Another substance, CD59 inhibits the membrane attack complex and is evenly distributed along the human sperm plasma membrane. Other immunosuppressive factors have been found in human semen such as TGFβ, TNF and receptors for the Fc portion of γ-globulin, which inhibit binding of IgG to cell surfaces (Kelly, 1995). Prostasomes are extracellular secretory vesicles present in semen from several species, including the bull (Lazarevic et al., 1995), rabbit (Metz et al., 1968) and human (Skibinski et al., 1992). Human prostasomes act as reservoirs of complement activity inhibitors, such as CD59, and as inhibitors in lymphoproliferation assays in vitro (Skibinski et al., 1992). Bull prostasomes were also shown to have immunosuppressive activity (Lazarevic et al., 1995).

In summary, seminal plasma modulates the inflammatory response to semen deposition in the mare, probably by inhibiting complement activation. Several soluble and membrane-bound substances have been identified in other species, but the fractions responsible for the immunomodulatory effect in stallion semen have not been identified yet.

2.2.4. Bacterial Contamination during Breeding

Although it is recognized that spermatozoa are of greater importance in inducing a neutrophil response, bacterial contamination also occurs during breeding and becomes more important as the mare develops a chronic endometritis. The vestibulum and clitoral fossa of the mare harbor potentially pathogenic microorganisms that can be introduced into the uterus during breeding (Hinrichs et al., 1988). Stallion semen also contains a number of potentially pathogenic bacteria. Uterine bacterial growth was minimal 5 to 6 hours after insemination (Katila, 1997; Kotilainen et al., 1994). However, bacteria were present 48 hours after mating, insemination with raw semen and with extended semen, in both reproductively normal mares and mares with delayed uterine clearance. More reproductively normal mares with positive uterine cultures were found after mating compared with artificial insemination with extended semen. Whether this difference was due to the inclusion of antibiotics in the extender or a reduction in the number of bacteria inoculated is not known. It is also unknown whether the difference could be expected in mares with delayed uterine clearance (Bollwein et al., 2003; Nikolakopoulos and Watson, 1997).
2.3. Persistent Mating-Induced Endometritis

2.3.1. Definition and Clinical Significance

In 1995, Troedsson proposed the subdivision of endometritis in the mare into four categories: endometrosis or chronic degenerative endometritis, sexually transmitted disease, chronic endometritis and persistent mating-induced endometritis (PMIE). As discussed previously, intrauterine deposition of semen causes a transient endometrial inflammatory response aimed at eliminating bacteria and excess spermatozoa from the uterus. This inflammatory response is accompanied by accumulation of intrauterine fluid. Reproductively normal mares are able to clear the endometritis within 12 to 48 hours (Nikolakopoulos et al., 2000a; Troedsson, 1997). However, the condition may develop into a persistent endometritis in mares with delayed uterine clearance. Presence of intrauterine fluid 72 hours after mating was associated with decreased pregnancy rates (Pycock and Newcombe, 1996).

2.3.2. Diagnosis and Mare Classification

As the inflammatory response is usually accompanied by accumulation of intrauterine fluid, the diagnosis of delayed uterine clearance is made by observation of free intraluminal uterine fluid on ultrasonography after breeding. The difficulty in practice to identify some mares with delayed uterine clearance early has been stressed by Pycock and Newcombe (1996), since fluid is not always detected ultrasonographically in these mares, especially at the beginning of the breeding season. However, after repeated breeding and endometrial challenge, intrauterine fluid accumulations may become more evident.

The ability of the mare to remove intrauterine fluid after insemination is probably the challenge that best classifies the mare as reproductively normal (Brinsko, 2003; Nikolakopoulos and Watson, 1997). However, different authors have different opinions on when the mare should be considered to have delayed uterine clearance, ranging from 12 (Troedsson, 1997), 24 (Newcombe, 1997), 48 (Nikolakopoulos et al., 2000a) to 72 hours after breeding (Brinsko, 2003). Reproductive history, when available, can give an idea of a mare’s classification in terms of uterine clearance. Mares with delayed uterine clearance are usually pluriparous, more than 14 years old, and may conceive without difficulty for three to four breeding seasons but then start to accumulate fluid after breeding and become more difficult to get pregnant. These mares may develop chronic bacterial endometritis after repeated breedings in a breeding season. Another group of mares with delayed uterine clearance include maiden mares, particularly if they are...
above 10 years of age (Pycock, 1993). Old maiden mares usually have insufficient cervical relaxation that impedes normal uterine clearance. The nature of this defect is not known.

Uterine biopsy was used to classify mares as resistant or susceptible to chronic bacterial endometritis. Mares with grade I biopsies were found to be resistant and eliminated the bacteria inoculated into their uteri during estrus by the following estrous cycle. Mares with grade II or III biopsies were not able to eliminate inoculated bacteria by the following estrous cycle, and were classified as susceptible (Asbury et al., 1982). However, in another study, uterine biopsy was unreliable as the only method to identify mares with delayed uterine clearance. Mares with grade II biopsies could not consistently be classified as resistant or susceptible to PMIE. Mares with grade I biopsies were mostly resistant and those with grade III biopsies were mostly susceptible, so it was suggested that bacterial challenge should be used as the final criterion for classification. Resistant mares were able to clear bacteria within 96 hours of inoculation (Troedsson et al., 1993b).

LeBlanc et al. (1994a) proposed the use of scintigraphy to diagnose delayed uterine clearance. Mares with delayed uterine clearance removed <15% of infused radiocolloid within 2 hours, while reproductively normal mares were able to clear >50% of the radiocolloid within this time. Neither bacterial challenge nor scintigraphy are practical methods to be used in a clinical setting, and they have been shown to have low agreement with mare classification in terms of uterine clearance, so they are unsuitable as the sole criterion (Brinsko, 2003).

Furthermore, the presence of >2 cm of intrauterine fluid during estrus was found to be a good predictor of susceptibility of the mare to PMIE, allowing practitioners to identify mares with delayed uterine clearance before breeding. Identification of mares with delayed uterine clearance before mating would allow therapeutic interventions to be started as soon as possible after insemination (Brinsko, 2003).

2.3.3. Intrauterine Fluid Accumulation and Endometritis

The incidence of intrauterine fluid 24 hours after breeding has been reported to be 14% and 16% when artificial insemination with frozen semen or natural mating was used, respectively (Watson et al., 2001; Zent, 1998). Fluid accumulations were associated with increased age, a history of previous dystocia and being barren. A stallion effect was also identified (Adams et al., 1987; Barbacini et al., 2003; Losinno et al., 1997; Zent 1998). Mares that accumulated fluid after breeding were found to have decreased pregnancy rates and increased embryonic loss rates
(Newcombe, 1997; Pycock and Newcombe, 1996). However, pregnancy rates were improved if the mares were treated to eliminate this fluid (Watson et al., 2001; Zent, 1998). Barbacini et al. (2003) reported that if mares were treated, only those older than 16 years showed decreased fertility.

In a study involving 943 mares with unknown reproductive history, spontaneous intrauterine fluid accumulations were found in 15 to 19% of mares in anestrus, transition, estrus or diestrum (Losinno et al., 1997). Spontaneous fluid accumulation has been shown to be dynamic and usually occurs at the uterine body or base of the horns (Losinno et al., 1997; Reilas et al., 1997). The amount of fluid is maximal at the end of the luteal phase and beginning of estrus, probably due to incomplete cervical dilation. Fluid disappears within 2 days of ovulation (Adams et al., 1987; Allen and Pykock, 1988; Reilas et al., 1997).

In a study by Adams et al. (1987), the presence of fluid during diestrus was associated with reduced pregnancy rates and increased pregnancy losses. Fluid in the uterus during diestrus was also indicative of the presence of endometritis. Accumulation of large volumes of fluid was associated with release of PGF$_2$α and premature luteolysis, resulting in shorter interovulatory intervals and pregnancy loss (Adams et al., 1987).

Conversely, the nature and effect of intrauterine fluid present during estrus are less clear. Reilas et al. (1997) showed that fluid present during estrus in reproductively normal mares was a non-inflammatory transudate and, although it suppressed motility of spermatozoa in vitro, it did not affect embryo recovery rates. However, Adams et al. (1987) demonstrated that although these mares did not have reduced 14-day pregnancy rates, none of them were able to maintain the pregnancies beyond 20 days. Mares with intrauterine fluid at the time of mating may be particularly susceptible to endometritis and the transudate can quickly become an inflammatory exudate after any interference such as breeding or gynecological manipulations (Newcombe, 1998).

2.3.4. Treatment and Management Options

Treatment of PMIE is aimed at assisting the uterus to physically eliminate contaminants and inflammatory products. Uterine lavage and uterotonic agents are used for this purpose, alone or combined. Post breeding lavage with isotonic saline or lactated ringer’s solution is widely used to treat mares that accumulate fluid after breeding. Pregnancy rates are improved if lavage is performed within 24 hours of mating (Knutti et al., 2000), since removing uterine
contaminants and inflammatory products as soon as possible reduces the time available for bacteria to replicate and become established and reduces the time of contact of the endometrium with inflammatory products and chemoattractants. Uterine lavage performed 4 to 6 hours after insemination resulted in even better pregnancy rates in problem mares than when performed at 18 to 20 hours (Knutti et al., 2000). Since sperm transport to the oviducts is complete in 4 hours, lavage at this time did not have any detrimental effect on fertility in reproductively normal mares (Brinsko et al., 1991).

Uterotonic agents used in the treatment of PMIE include oxytocin and PGF$_{2\alpha}$ analogs. Both drugs increase uterine clearance by inducing myometrial contractions, although the duration of their action differs. Administration of oxytocin, 20 IU IV to mares with delayed uterine clearance effectively eliminated >90% of infused radiocolloid within 30 minutes (LeBlanc et al., 1994b). Because the oxytocin half-life is very short, only 6 minutes, and oxytocin-induced myoelectrical activity lasts for only 1 hour, frequent administration is necessary to effectively eliminate accumulated fluid (Paccamonti et al., 1999; Troedsson et al., 1995a). Prostaglandin F$_{2\alpha}$ has a longer action on uterine contractility but a slower fluid clearance rate than oxytocin. Administration of PGF$_{2\alpha}$, 10 mg IM increased myoelectrical activity for 5 hours (Troedsson et al., 1995a). The prostaglandin analog cloprostenol is preferred over dinoprost, since it induced a more rapid clearance than dinoprost. Cloprostenol cleared 65 to 75% of the radiocolloid within 60 minutes (Combs et al., 1996). There are however, some conflicting reports on the use of cloprostenol during the immediate post-ovulatory period. The administration of cloprostenol from the day of ovulation until 2 days later interfered with corpus luteum formation and function reducing plasma progesterone concentrations. However, progesterone seemed to rebound around 7 to 9 days after ovulation (Brendemuehl, 2002; Nie et al., 2002; Troedsson et al., 2001a). While some authors reported a decrease in pregnancy rates as a result of impaired early luteal function (Brendemuehl, 2002; Troedsson et al., 2001a), others failed to find a deleterious effect of cloprostenol treatment on pregnancy outcome (Nie et al., 2002). Although it has a shorter action, oxytocin induces a more rapid uterine clearance without interfering with early luteal function and it is the drug of choice for treatment of delayed uterine clearance during the pre- and post-ovulatory period.

Recently, PGE$_1$ (misoprostol) was used to induce cervical relaxation in a maiden mare that accumulated intrauterine fluid after insemination because of insufficient cervical dilation
It is possible that PGE\textsubscript{1} may become a valuable adjuvant in the treatment of PMIE in mares with impaired cervical function.

The use of post-breeding antibiotic infusions is controversial. While Troedsson et al. (1995b) showed that uterine lavage 12 hours after bacterial infusion was as effective in eliminating bacteria as prostaglandin administration or antibiotics, Pycock and Newcombe (1996) reported that addition of intrauterine antibiotics potentiated the effect of oxytocin treatment on pregnancy rates in a group of mares with unknown reproductive history when given within 72 hours after breeding. Pycock and Newcombe (1996) commented that bacteria still remained in the uterus when the mare was treated with oxytocin alone and fluid accumulated again. The difference in time from mating or bacterial infusion to treatment, as well as different degrees of susceptibility in different mares, may explain the different effects of antibiotics on pregnancy rates.

Mares with delayed uterine clearance also require more intensive breeding management with the purpose of breeding these mares only once during a given estrous cycle to minimize the uterine challenge. Frequent monitoring of ovarian activity together with pharmacologic induction of ovulation with human chorionic gonadotropin or deslorelin is usually required (Kenney et al., 1975). If semen of good quality and with good sperm longevity is used, mating may be done 48 hours instead of 24 hours before ovulation to allow more time to flush the mares more than once before ovulation if necessary (Knutti et al., 2000). Both intensive pre-breeding management and early post-breeding treatment are equally important on pregnancy outcome in mares with delayed uterine clearance.

2.4. Low Dose Insemination in the Mare

2.4.1. Endoscopic Anatomy of the Reproductive Tract of the Mare

The endoscopic appearance of the reproductive tract of the mare changes under differing hormonal influences during the different stages of the estrous cycle (Bracher and Allen, 1992). During estrus, the cervix appears edematous and lays on the floor of the vaginal fornix. The endometrium has a pink, glistening appearance and the endometrial folds are thickened due to the presence of edema. A small volume of clear viscous fluid can be seen in the uterine lumen of some mares at this stage. The increased contractility and escape of air through the relaxed cervix sometimes makes it difficult to fully distend the uterine lumen during hysteroscopy. The endometrium also appears to be very responsive to physical irritation during estrus, showing
increased hyperemia after any minor trauma. During diestrus, the cervix is tight, pale and erect in the center of the fornix. The endometrium is slightly paler and the endometrial folds look thinner due to the lack of edema. No intraluminal fluid is usually seen at this stage. Full distension is easily achieved during diestrus. In anestrus, the cervix looks pale and relaxed, the endometrium is thin and pale and distension of the uterine lumen is usually uneven.

The uterotubal junction (UTJ) has been reported to have five morphologic categories: papilla, complex, diffuse, double ridge and other. Its diameter is < 2 French (Manning et al., 1998). The UTJ also shows changes during the estrous cycle. During estrus, it is edematous, pink and relaxed; but it becomes pale, tight and protuberant during diestrus and anestrus (Bracher and Allen, 1992).

2.4.2. Insemination Techniques

In order to obtain acceptable pregnancy rates after artificial insemination in the uterine body, the recommended minimum insemination doses are 500 million motile spermatozoa for fresh semen, one billion for cooled semen and 240 million for frozen semen (Pickett et al., 2000). Since most of the ejaculate is expelled through the cervix into the vagina soon after breeding, only a small portion of the ejaculate reaches the oviducts. If the distance spermatozoa have to be transported to reach the oviduct can be reduced and retrograde flow of spermatozoa could be minimized, a lower number of spermatozoa could be inseminated without reducing pregnancy rates. This reduction in insemination dose could improve pregnancy rates when the number of spermatozoa is limited as when using sex-sorted spermatozoa. It could also increase the efficiency of use of heavily booked stallions and frozen semen when the number of straws available is limited.

Three low-dose insemination techniques have been developed. McCue et al. (2000) obtained 40% pregnancy rates after oviductal insemination via flank laparotomy with 50 thousand spermatozoa. Non-surgical intra-oviductal insemination via hysteroscopy was first reported by Manning et al. (1998) and Vazquez et al. (1998). Pregnancy rates in the first study were low and may have been due to excessive manipulation of the UTJ during cannulation attempts. However, the second group first demonstrated that pregnancies could be obtained after hysteroscopic deposition of semen onto the surface of the UTJ (Vazquez et al., 1998). Cannulation of the papilla and deposition of spermatozoa into the oviductal lumen does not seem to be necessary, since the papilla provides a sperm reservoir. Morphologically normal
spermatozoa accumulate in the folds of the uterine papilla of the UTJ and are stored bound in the epithelial crypts (Scott et al., 2002).

The minimum recommended insemination dose for hysteroscopic insemination is 1 million motile spermatozoa when semen from fertile stallions is used (Morris et al., 2000). Pregnancy rates from 40 to 75% were obtained using 5 million motile spermatozoa, and did not seem to improve when higher numbers of spermatozoa were inseminated (Brinsko et al., 2003; Lindsey et al., 2002a,b; Morris et al., 2000; Morris et al., 2003).

In 2000, Buchanan et al. first reported the use of deep horn insemination in the mare, where an insemination pipette was guided transrectally to the tip of the uterine horn and a small volume of semen was deposited close to the UTJ. Using 5 to 25 million motile spermatozoa, reported pregnancy rates ranged from 30 to 63% (Brinsko et al., 2003; Buchanan et al., 2000; Lindsey et al., 2002a; Nie et al., 2003; Woods et al., 2000). Insemination doses and pregnancy rates obtained after low dose insemination in the mare are summarized in Table 2.4.1.

Because fewer spermatozoa from subfertile stallions reached the oviducts after insemination (Scott et al., 1995), the use of low dose insemination to compensate for this deficiency in sperm transport has been proposed, but its value in stallion infertility has still not been proven. Deep horn insemination increased the number of spermatozoa recovered from the oviduct ipsilateral to the ovary containing the dominant follicle (Rigby et al., 2000) but it did not improve pregnancy rates when mares were inseminated with teratospermic ejaculates (Woods et al., 2000). Since only the subpopulation of morphologically normal spermatozoa was able to bind to oviduct epithelial cells (Thomas et al., 1994), depositing a higher number of morphologically abnormal spermatozoa did not seem to improve oviductal colonization and thus fertility (Woods et al., 2000).

Mares with delayed uterine clearance also had impaired sperm transport to their oviducts (Scott et al., 1995). The use of low dose insemination may increase the sperm reservoir and fertility. However, Rigby et al. (2000) failed to find a difference in the number of spermatozoa in the oviduct of these mares after deep horn insemination.
<table>
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<td>16</td>
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</tr>
<tr>
<td>Fresh</td>
<td>Deep horn</td>
<td>5</td>
<td>0.1</td>
<td>0</td>
<td>10</td>
<td>Lindsey et al., 2002b</td>
</tr>
<tr>
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<td>5</td>
<td>0.1</td>
<td>50</td>
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<td></td>
</tr>
<tr>
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<td>5</td>
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<td>25</td>
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<tr>
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<td>Hysteroscopic</td>
<td>20</td>
<td>0.3</td>
<td>72</td>
<td>25</td>
<td>Lindsey et al., 2002c</td>
</tr>
<tr>
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<td>20</td>
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<td>55</td>
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<td></td>
</tr>
<tr>
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<td>Deep horn</td>
<td>25</td>
<td>0.5</td>
<td>33 to 50</td>
<td>30</td>
<td>Nie et al., 2003</td>
</tr>
<tr>
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<td>Uterine body</td>
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<td>67</td>
<td>12</td>
<td>Morris et al., 2003</td>
</tr>
<tr>
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<td>Hysteroscopic</td>
<td>14</td>
<td>0.5</td>
<td>64</td>
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</tr>
<tr>
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<td>Hysteroscopic</td>
<td>3</td>
<td>0.1</td>
<td>47</td>
<td>34</td>
<td></td>
</tr>
<tr>
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<td>Uterine body</td>
<td>3</td>
<td>0.1</td>
<td>47</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Fresh cooled</td>
<td>Hysteroscopic</td>
<td>5</td>
<td>0.2</td>
<td>67</td>
<td>18</td>
<td>Brinsko et al., 2003</td>
</tr>
<tr>
<td>Fresh cooled</td>
<td>Deep horn</td>
<td>5</td>
<td>0.2</td>
<td>56</td>
<td>18</td>
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</tr>
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</table>
(Table 2.4.1. cont.)

<table>
<thead>
<tr>
<th>Type of semen</th>
<th>Technique</th>
<th>Total Motile cells (x 10^6)</th>
<th>Volume (mL)</th>
<th>Pregnancy rate (%)</th>
<th>Number of mares</th>
<th>Authors</th>
</tr>
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<tr>
<td>Fresh</td>
<td>Uterine body</td>
<td>50 to 300</td>
<td>12</td>
<td>47 to 84</td>
<td>65</td>
<td>Sieme et al., 2004</td>
</tr>
<tr>
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<td>Deep horn</td>
<td>50</td>
<td>12</td>
<td>55 to 50</td>
<td>32</td>
<td></td>
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<tr>
<td>Fresh</td>
<td>Hysteroscopic</td>
<td>50</td>
<td>2 to 12</td>
<td>71 to 33</td>
<td>55</td>
<td></td>
</tr>
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<td>Frozen</td>
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<td>0.5 to 12</td>
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<tr>
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<td>100</td>
<td>0.5</td>
<td>48 to 38</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Frozen</td>
<td>Hysteroscopic</td>
<td>100</td>
<td>0.5</td>
<td>48 to 37</td>
<td>48</td>
<td></td>
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</tbody>
</table>
2.4.3. Low Dose Insemination and Endometritis

The effect of hysteroscopic insemination on the incidence of persistent mating-induced endometritis is controversial. It was suggested that placement of semen at the UTJ would incite a reduced inflammatory response, since sperm-uterine interactions would be minimized (Lindsey et al., 2001; Squires et al., 2000). However, this hypothesis has not been tested and the value of low dose insemination in the management of subfertile mares is still not proven. The proportion of mares with intrauterine fluid accumulation after hysteroscopic insemination was as low as 2/20 and 1/30 mares in two studies (Lindsey et al., 2002b; Manning et al., 1998). Some authors however, have postulated that the hysteroscopic procedure itself is inflammatory by nature and should not be used on mares susceptible to developing persistent mating-induced endometritis (Sieme et al., 2004). In one study, 60% of the mares inseminated hysteroscopically accumulated intrauterine fluid and required treatment to evacuate uterine contents after breeding (Vazquez et al., 1998). Sieme et al. (2004) reported that the percentage of reproductively normal and problem mares with post-breeding intrauterine fluid accumulation was not different after uterine body, deep horn and hysteroscopic insemination. Although hysteroscopic insemination improved pregnancy rates in reproductively normal mares, its use in problem mares was detrimental to fertility. Mares were considered to be problem mares when they were barren or underwent embryonic loss, abortion or stillbirth during the previous breeding season. However, no mention was made in that study about the classification of these mares in terms of uterine clearance. Sieme et al. (2004) did not recommend insemination at the UTJ in problem mares.

Controlled studies that compare post-breeding endometritis after low dose insemination and uterine body insemination in reproductively normal mares, as well as mares with delayed uterine clearance, are lacking. These studies are needed to determine whether low dose hysteroscopic insemination can be used safely in mares with delayed uterine clearance, and if it has any value in improving fertility in these mares.
CHAPTER 3

ACUTE ENDOMETRIAL INFLAMMATORY RESPONSE AFTER

HYSTEROSCOPIC INSEMINATION

3.1. Introduction

As discussed earlier, a transient post-breeding endometritis occurs as a physiologic response to semen deposition. The sperm-induced response is characterized by the influx of neutrophils to the uterine lumen and it is often accompanied by accumulation of intrauterine fluid. Reproductively normal mares are able to physically clear fluid from their uteri within 12 to 48 hours after mating (Nikolakopoulos and Watson, 1997; Troedsson, 1997). However, in mares with delayed uterine clearance the condition develops into a persistent endometritis and leads to subfertility.

Abnormal myometrial activity seems to be the primary defect in mares with delayed uterine clearance (Rigby et al., 2001). However, cervical dilation, lymphatic drainage and anatomic position of the uterus also contribute to the disease (LeBlanc et al., 1998; 1995).

Treatment of persistent mating-induced endometritis is aimed at assisting the uterus in physically clearing contaminants and inflammatory products. Both uterotonic drugs, such as oxytocin and prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$), and uterine lavage after breeding are used for this purpose. In addition, breeding management to reduce the number of inseminations is essential to minimize the challenge to the uterus.

Placement of a low dose of semen at the uterotubal junction (UTJ) could minimize sperm-uterine interactions and neutrophil chemotaxis, possibly resulting in a reduced inflammatory response compared with uterine body insemination. If the challenge to the endometrium was reduced, these mares may be less likely to develop persistent mating-induced endometritis and hysteroscopic insemination could become a valuable management option for mares with delayed uterine clearance. However, the value of hysteroscopic insemination in the management of subfertile mares is still not proven.

Conversely, other authors have postulated that the hysteroscopic procedure itself is inflammatory by nature, and reported reduced pregnancy rates for problem mares inseminated with this technique (Sieme et al., 2004). If hysteroscopy induces an exaggerated inflammatory response, the method may either need to be avoided in mares with delayed uterine clearance, or
those mares may need to be treated aggressively as soon after insemination as possible to maximize chances for conception.

The incidence of post-breeding endometritis after hysteroscopic insemination in reproductively normal mares is also not clear. Controlled studies that compare post-breeding endometritis after low dose insemination with uterine body insemination in reproductively normal mares and in mares with delayed uterine clearance are still lacking.

In the present experiment, the incidence and severity of the inflammatory response induced by hysteroscopic insemination at the UTJ with low doses of spermatozoa was compared with that induced by routine uterine body insemination with a standard dose. A sham hysteroscopy was included to determine whether the hysteroscopic procedure itself would induce endometritis. Reproductively normal mares were compared with mares with delayed uterine clearance. The magnitude of the acute inflammatory response was evaluated 24 hours after insemination when the numbers of neutrophils retrieved from the uterus and the severity of intrauterine fluid accumulations are reported to be highest (Katila, 1995). It was hypothesized that hysteroscopic insemination at the UTJ with a reduced number of spermatozoa would induce a less severe acute inflammatory response after insemination in reproductively normal mares and in mares with delayed uterine clearance.

3.2. Materials and Methods

The study was conducted from June to September 2003. The mares were part of the LSU-SVM teaching and research herds and were kept in pastures at the LSU-SVM facilities for the entire length of the study. All procedures were approved and conducted following a protocol approved by the LSU Institutional Animal Care and Use Committee (Protocol 03-054).

3.2.1. Semen Challenge and Mare Classification

Twenty-nine cycling mares were classified as reproductively normal mares or mares with delayed uterine clearance based on the results of a semen challenge (Paccamonti et al., 1999). The mares were teased twice a week until estrous behavior was detected. The mares were considered to be in estrus when posturing, urinating, tail raising and clitoral eversion in response to exposure to the stallion were seen. Once in estrus, ovarian activity was monitored by palpation per rectum and transrectal B-mode, real time ultrasonography using a 5 mHz linear probe (Aloka SSD-500V, Aloka Co., Mitaka-Shi, Tokyo) every other day until a follicle \( \geq 30 \text{ mm in diameter} \) was identified. Thereafter, monitoring of ovarian activity was performed daily. When a follicle...
≥35 mm in diameter was identified during estrus, the mares were artificially inseminated with fresh semen containing one billion spermatozoa, extended to a total volume of 20 mL in a Kenney-type semen extender with polymixin B (EZ-mixin OF, Animal Reproduction Systems, Chino, CA). Ultrasonography was performed 24 and 48 hours after insemination to detect the presence of intrauterine fluid. Mares without fluid at 24 and 48 hours after insemination were considered reproductively normal while those with intrauterine fluid were considered to have delayed uterine clearance. Uterine biopsies were obtained during diestrus. Biopsy scores ranged from 2A to 3 (Kenney and Doig, 1986) in both categories of mares. Mares with evidence of chronic infiltrative endometritis were excluded from this study.

3.2.2. Experimental Design

Five reproductively normal mares and five mares with delayed uterine clearance (DUC mares) were randomly selected from the population challenged. Each mare received each of three treatments in three consecutive estrous cycles: UB: uterine body artificial insemination (n=10), HYST: hysteroscopic artificial insemination (n=10) or SHAM: sham hysteroscopic artificial insemination (n=10). The order of the treatments was randomized so that each mare received the three different treatments in different order. Teasing and monitoring of ovarian activity were performed as described for the semen challenge. When a follicle ≥35 mm in diameter and uterine edema were detected, the mares received one of the three treatments. The insemination doses and volumes for the different treatments are summarized in Table 3.2.1.

Immediately after insemination, all the mares received human chorionic gonadotropin (hCG), 2500 IU IV (Chorulon, Intervet, Millsboro, DE) to reduce the variability in time from insemination to ovulation.

Following insemination, the mares were monitored daily by palpation per rectum and transrectal ultrasonography until ovulation was detected. Six to 8 days after ovulation each mare was administered PGF$_{2\alpha}$ 10 mg IM (Lutalyse, Pharmacia & Upjohn, Kalamazoo, MI) to induce luteolysis. During the following estrus, each mare was re-assigned to a different treatment.

3.2.3. Semen Processing

To reduce variability due to stallion effects, one proven fertile stallion was used for all inseminations. Prior to the start of the study a culture was performed on the semen and no growth of pathogenic bacteria was obtained. The stallion was allowed to mount a phantom and semen was collected with a Hannover model artificial vagina. Immediately after collection, a standard
Table 3.2.1. Number of spermatozoa and volume of the inseminates received by mares in treatments UB (uterine body insemination), HYST (hysteroscopic insemination) or SHAM (sham hysteroscopy)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>UB</th>
<th>HYST</th>
<th>SHAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of spermatozoa</td>
<td>1 billion</td>
<td>5 million</td>
<td>Semen extender</td>
</tr>
<tr>
<td>Volume (mL)</td>
<td>20</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>
semen evaluation was performed and the sperm concentration was determined using a hemacytometer. A portion of the ejaculate was extended in a milk-based semen extender with polymixin B (EZ-mixin OF, Animal Reproduction Systems, Chino, CA) to obtain the desired sperm dose and volume for each inseminate. The insemination dose and volume are described in Table 3.2.1. For the uterine body inseminations, the concentration of spermatozoa in the raw sample was divided by 50 million spermatozoa/mL, which was the desired concentration in the inseminate. This provided the number of total parts of semen and extender. One unit was substracted from this number to provide the parts (n) of extender needed. The semen was then extended in semen extender using one part of semen and n parts of extender. A 20-mL aliquot of this dilution was used for the inseminations. For the hysteroscopic inseminations, calculations were done in the same way, considering the final desired concentration to be 10 million spermatozoa/mL. Calculations were done as follows:

\[
\text{Total parts of semen and extender} = \frac{\text{concentration in raw semen (million/mL)}}{\text{desired concentration (million/mL)}}
\]

\[
\text{Parts of extender} = \text{total parts of semen and extender} - 1
\]

Example: 350 million/mL/50 million/mL = 7

\[
\text{Parts of extender: } 7 - 1 = 6
\]

Final dilution: 1 part of semen and 6 parts of extender (v:v)

3.2.4. Hysteroscopic Procedure and Insemination

The mares were restrained in stocks and the perineum was cleansed with povidone iodine scrub and water. Five minutes before the hysteroscopic procedures, the mares were sedated with detomidine, 20 µg/kg IV (Dormosedan, Orion Corp., Espoo, Finland) to reduce discomfort caused by insufflation of the uterus with air. Acepromazine, 30 mg IV (PromAce, Fort Dodge Animal Health, Fort Dodge, Iowa) was administered immediately before the hysteroscopy to reduce uterine contractions, which impede the procedure.

The endoscope was cold-sterilized in a 3.4% glutaraldehyde solution for 20 minutes (Cidex Plus, Ethicon, Inc., Irvine, CA) and rinsed externally and internally with 1 L of sterile water. The biopsy channel was dried with air at high pressure to remove any chemical residues. An equine fallopian tube insemination set (V-EFIS-2-200, Cook Veterinary Products, Queensland, Australia) was used. The inner catheter had a 2- French diameter and was 200 cm in length. This catheter was loaded with semen extender (SHAM, 0.5 mL) or extended semen.
(HYST, $5 \times 10^6$ spermatozoa, 0.5 mL), and it was introduced through the biopsy channel of a 2-meter flexible videoendoscope (Series 160 Olympus, Sony, Ichinomiya, Japan). The endoscope was passed manually through the cervix into the uterus. The uterus was insufflated with non-sterilized air using the endoscope setup to allow visualization of the entire endometrial surface. The endoscope was guided to the tip of the horn ipsilateral to the ovary containing the preovulatory follicle. The UTJ was visualized, the inner catheter was exteriorized and placed close to the UTJ, and the extender (SHAM) or the semen (HYST) was deposited onto the UTJ creating froth. The endoscope was slowly removed from the uterus allowing the air to leave the uterus through the cervix.

3.2.5. Uterine Body Insemination

The mares were restrained in stocks and the perineum was cleansed as described for the hysteroscopic procedures. An equine insemination pipette, attached to a 20-mL all-plastic syringe with previously loaded extended semen, was manually passed through the cervix and the extended semen was deposited into the uterine body.

Although sedation is not usually required for insemination into the uterine body, it is required for hysteroscopy. Since both detomidine and acepromazine affect uterine motility (De Lille et al., 2000; Schatzmann et al., 1994), the mares in the UB group also received detomidine, 20 µg/kg IV (Dormosedan, Orion Corp., Espoo, Finland) and acepromazine, 30 mg IV (PromAce, Fort Dodge Animal Health, Fort Dodge, Iowa) before the procedure.

3.2.6. Evaluation of the Inflammatory Response

The inflammatory response of the endometrium to the different treatments was evaluated 24 ± 2 hours after insemination. The presence of intrauterine fluid was assessed by transrectal ultrasonography, and the amount was subjectively graded using the following classification: 0: no fluid, 1: small amount of fluid, 2: moderate amount of fluid, 3: large amount of fluid. Grading was made based on the depth and length of the pockets of free intraluminal fluid found in the uterine body or base of the uterine horns.

Uterine secretions were obtained to evaluate the number of leukocytes. To obtain the samples, the mares were restrained in stocks and the perineum was cleansed as described for the hysteroscopic procedures. A balloon-tipped catheter (V-PUF8-80, Cook Veterinary Products, Queensland, Australia), was passed manually through the cervix into the uterus, the balloon inflated with 90 mL of air, and 60 mL of sterile lactated Ringer’s solution were infused into the uterine body.
uterus. Immediately after infusion, the mares were administered oxytocin, 20 IU IV (Butler, Columbus, OH) to stimulate uterine contractions and improve fluid recovery. Fluid was recovered by gravity flow into 50-mL conical tubes for 5 minutes after oxytocin administration, in combination with transrectal massage of the uterus during the last minute.

The concentration of nucleated cells in the recovered fluid was counted in the sample using a hemacytometer and expressed in millions of cells per mL of fluid. The sample was then centrifuged at 2200 x g for 5 minutes. The supernatant was removed and the pellet was used to prepare a smear for cytologic evaluation. The smear was stained with a modified Wright’s stain (Protocol Hema 3, Fisher Diagnostics, Middletown, VA) and 100 cells were counted and classified as neutrophils, macrophages, lymphocytes, eosinophils or endometrial cells. The differential count was used to calculate the concentration of leukocytes in the fluid. Calculations were done as follows:

\[
\text{Concentration of leukocytes (million/mL)} = \text{concentration of nucleated cells (million/mL)} \times \text{percentage of leukocytes.}
\]

The severity of the endometritis was graded using the following classification (modified from Crickman and Pugh, 1986): 0 to 3% leukocytes: no inflammation, 4 to 15%: slight inflammation, 16 to 50%: moderate inflammation, >50%: severe inflammation. Uterine cytologies containing ≤3% leukocytes were considered to be indicative of the absence of endometritis (negative). Cytologies containing ≥4% leukocytes were considered to be an indicator of the presence of endometritis (positive). Intrauterine fluid was defined as absent in mares with fluid score 0, and present in mares with fluid score ≥1.

3.2.7. Statistical Analysis

The number of days from PGF$_{2\alpha}$ administration to insemination, insemination to ovulation, and insemination to subsequent insemination, and the concentration of leukocytes were tested for normality using the Shapiro-Wilk test. The effect of treatment on concentration of leukocytes, across and within mare groups, was evaluated using a mixed effect linear model that included the random variance of horse nested within groups and the fixed effect of treatments on all groups. Where there was significant interaction of groups and treatments, predetermined comparisons were made within and between groups using least squares means with a Tukey adjustment of Type I error to 0.05. Values of concentration of leukocytes are expressed as means ±SE. The number of days from PGF$_{2\alpha}$ administration to insemination,
insemination to ovulation, and insemination to subsequent insemination did not follow a normal distribution, and were analyzed using a non-parametric analysis of variance (Kruskal-Wallis test). Values of these variables are expressed as medians ± SE. Significance was determined at p<0.05.

The frequency of fluid scores and the frequency of degrees of inflammation were compared across groups and treatments using Cochran Mantel Haenszel (CMH) methods, stratifying on group. The association between the presence or absence of fluid and positive or negative cytologies was evaluated using the CMH test. The adjusted chi-square was considered significant at p<0.05.

SAS® procedures UNIVARIATE, MEANS, MIXED, MPAR1WAY and FREQ (SAS® version 9.0, SAS Institute, Cary, NC) were used to test for normality, obtain basic statistics, perform the mixed effect linear model on continuous data and non-parametric analysis of variance, and analyze categorical data, respectively.

At the beginning of the experiment, five mares (reproductively normal=3, DUC=2) were inseminated into the uterine body without receiving detomidine and acepromazine. The fluid scores, percentage and concentration of leukocytes from these mares were compared with those of the same mares after insemination into the uterine body with sedation. Comparisons were made using a Chi square test for fluid score, and a T-test for the numerical variables. Because the concentration of leukocytes did not follow a normal distribution, the data was ranked before performing the T-test.

3.3. Results

One mare initially classified as reproductively normal had cervical adhesions that worsened with repeated cervical manipulation and data from that mare were excluded from the analysis. One DUC mare developed a chronic endometritis after the first insemination and was also excluded from the analysis. Four reproductively normal mares and 4 DUC mares were included in the analysis with a total of 24 insemination procedures.

The mean number of days from PGF$_2$α injection to insemination, from insemination to ovulation, and between inseminations are shown in Table 3.3.1. These values were not different between groups or treatments (p>0.05). In all but 2 cycles, ovulation occurred within 48 hours of insemination and hCG administration.
There was no difference in fluid scores 24 hours after insemination between groups or treatments. The order of treatment did not affect the distribution of fluid scores (p>0.05) (Table 3.3.2). Fluid was present in 46% of the estrous cycles in reproductively normal mares and in 50% of the estrous cycles in DUC mares. The volume of fluid recovered from the flushings of reproductively normal and DUC mares was 41.87 ± 17.78 and 38.29 ± 14.54 mL (mean ± SD), respectively.

The concentration of leukocytes was found to follow a normal distribution. The concentration of leukocytes was not different between reproductively normal and DUC mares after any of the treatments (p>0.05). Mares with delayed uterine clearance showed no difference in leukocyte concentrations among treatments (p>0.05). In reproductively normal mares the concentration of leukocytes was not different between mares inseminated at the uterine body or at the UTJ (p>0.05). However, the sham hysteroscopies resulted in higher leukocyte numbers compared with both inseminations (p<0.05) (Figure 3.3.1). This variable was not affected by the order of treatment (p>0.05).

All the mares had cytologic signs of endometritis 24 hours after the treatments (Table 3.3.3). All but one mare had moderate to severe endometritis. The number of reproductively normal and DUC mares with different degrees of endometritis was not different, thus the data was combined for comparisons among treatments (p>0.05). There was no effect of treatment on the number of mares with different degrees of endometritis (p>0.05).

The presence of intrauterine fluid was not associated with cytological evidence of endometritis (p>0.05). Seventy five per cent of the flushings contained macrophages and/or lymphocytes in both groups of mares, and all the mares had mononuclear cells in at least one of their flushings. The fluid scores, percentage and concentration of leukocytes did not differ between mares that were inseminated into the uterine body with and without sedation (Table 3.3.4) (p>0.05).

3.4. Discussion

Uterine clearance is affected by circulating steroid hormone concentrations and differs during pre-ovulatory and post-ovulatory periods (Evans et al., 1987; LeBlanc et al., 1989). As all the treatments were performed during the pre-ovulatory period, the endocrine environment was not likely to contribute to the variability observed in this experiment.
Table 3.3.1. Number of days from PGF$_{2a}$ injection to insemination, from insemination to subsequent insemination and from insemination to ovulation in reproductively normal mares and mares with delayed uterine clearance (DUC) (median ± SE). UB: uterine body insemination with one billion spermatozoa in 20 mL, HYST: hysteroscopic insemination at the UTJ with five million spermatozoa in 0.5 mL, SHAM: sham hysteroscopic insemination with 0.5 mL of semen extender. Values are not different (p>0.05).

<table>
<thead>
<tr>
<th>Mare group</th>
<th>Treatment</th>
<th>Prostaglandin to insemination (days)</th>
<th>Insemination to insemination (days)</th>
<th>Insemination to ovulation (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>UB</td>
<td>4.5 ± 1.8</td>
<td>15.5 ± 5.6</td>
<td>2.0 ± 0.0</td>
</tr>
<tr>
<td>(n=4)</td>
<td>HYST</td>
<td>3.5 ± 1.3</td>
<td>14.5 ± 1.0</td>
<td>2.0 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>SHAM</td>
<td>3.5 ± 4.0</td>
<td>17.0 ± 6.2</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>DUC</td>
<td>UB</td>
<td>4.5 ± 1.0</td>
<td>17.5 ± 4.5</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>(n=4)</td>
<td>HYST</td>
<td>7.0 ± 0.2</td>
<td>15.5 ± 0.6</td>
<td>1.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>SHAM</td>
<td>5.0 ± 0.9</td>
<td>18.5 ± 2.4</td>
<td>2.0 ± 0.0</td>
</tr>
</tbody>
</table>

Table 3.3.2. Frequency distribution of fluid scores 24 ± 2 hours after insemination. Fluid score 0 (no fluid), 1 (small amount of fluid), 2 (moderate amount) or 3 (large amount). UB: uterine body insemination with one billion spermatozoa in 20 mL, HYST: hysteroscopic insemination at the UTJ with five million spermatozoa in 0.5 mL, SHAM: sham hysteroscopic insemination with 0.5 mL of semen extender. (p>0.05).

<table>
<thead>
<tr>
<th>Mare Group</th>
<th>Treatment</th>
<th>Fluid score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>No. mares (%)</td>
<td>No. mares (%)</td>
</tr>
<tr>
<td>Normal</td>
<td>UB</td>
<td>3 (75)</td>
</tr>
<tr>
<td>(n=4)</td>
<td>HYST</td>
<td>3 (75)</td>
</tr>
<tr>
<td></td>
<td>SHAM</td>
<td>1 (25)</td>
</tr>
<tr>
<td>DUC</td>
<td>UB</td>
<td>1 (25)</td>
</tr>
<tr>
<td>(n=4)</td>
<td>HYST</td>
<td>3 (75)</td>
</tr>
<tr>
<td></td>
<td>SHAM</td>
<td>2 (50)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>13 (54)</td>
</tr>
</tbody>
</table>
Figure 3.3.1. Concentration of leukocytes in uterine flushings 24 hours after treatments in reproductively normal mares (gray bars) and mares with delayed uterine clearance (DUC, black bars) (mean ± SE). UB: uterine body insemination with one billion spermatozoa in 20 mL, HYST: hysteroscopic insemination at the UTJ with five million spermatozoa in 0.5 mL, SHAM: sham hysteroscopic insemination with 0.5 mL of semen extender. Values with different superscript are significantly different within mare group (p<0.05).
The lack of difference in fluid scores after treatments between reproductively normal and DUC mares was unexpected, especially after uterine body inseminations, since this was the criterion used for mare classification. Had mare classification been correct, 0 and 100% reproductively normal and DUC mares respectively, should have had uterine fluid 24 hours after uterine body insemination. The fact that one mare in each group did not respond in terms of uterine clearance as expected may indicate that either mare classification for these two mares was incorrect, or that fluid accumulation does not occur consistently every cycle a mare is inseminated. Criteria for mare classification are highly variable among researchers and include reproductive history, biopsy score, clearance of radiocolloid (LeBlanc et al., 1994a), clearance of bacteria (Troedsson et al., 1993a) and presence of fluid during estrus (Brinsko, 2003). However, the ability of the mare to clear intrauterine fluid after insemination is probably the best criterion to use for classification (Nikolakopoulos and Watson, 1997).

Differences in application of this criterion exist among authors, since some have considered the mares to have delayed uterine clearance if intrauterine fluid was still present 12 (Troedsson, 1997), 24 (Newcombe, 1997), 48 (Nikolakopoulos et al., 2000a), 72 (Rigby et al., 2001) or 96 hours (Brinsko, 2003) after insemination. In the present study, mares were considered to have delayed uterine clearance if fluid was still present 24 hours or 48 hours after insemination (Paccamonti et al., 1999). Cervical tone influences the ability of the mare’s uterus to evacuate fluid. Cervical relaxation was not assessed during this study and this may have also contributed to the inconsistency in the results. It is also possible that a significant difference was not seen because of the low number of mares involved in this experiment.

The incidence of uterine fluid after the hysteroscopic procedures in both groups of mares in this study is in agreement with the results of Vazquez et al. (1998), who reported that 60% of mares hysteroscopically inseminated had significant amounts of intrauterine fluid present after the procedure and required treatment. Although hysteroscopic insemination in the present study resulted in accumulation of intrauterine fluid in 25% of mares, the incidence was not higher than after routine uterine body insemination. The lack of significant effects of insemination technique on post-breeding intrauterine fluid accumulation is in agreement with the results reported by Sieme et al. (2004).
Table 3.3.3. Frequency distribution of inflammation scores 24 hours after treatments. Values represent the number of mares within each treatment group showing no (0 to 3% leukocytes), slight (4 to 15%), moderate (16 to 50%) or severe (> 50%) endometritis. UB: uterine body insemination with one billion spermatozoa in 20 mL, HYST: hysteroscopic insemination at the UTJ with five million spermatozoa in 0.5 mL, SHAM: sham hysteroscopic insemination with 0.5 mL of semen extender. Values are not different (p>0.05)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>None</th>
<th>Slight</th>
<th>Moderate</th>
<th>Severe</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. mares (%)</td>
<td>No. mares (%)</td>
<td>No. mares (%)</td>
<td>No. mares (%)</td>
<td>(n)</td>
</tr>
<tr>
<td>UB</td>
<td>0</td>
<td>1 (12.5)</td>
<td>3 (37.5)</td>
<td>4 (50.0)</td>
<td>8</td>
</tr>
<tr>
<td>HYST</td>
<td>0</td>
<td>0</td>
<td>2 (25.0)</td>
<td>6 (75.0)</td>
<td>8</td>
</tr>
<tr>
<td>SHAM</td>
<td>0</td>
<td>0</td>
<td>1 (12.5)</td>
<td>7 (87.5)</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
<td>1 (4.2)</td>
<td>6 (25.0)</td>
<td>17 (70.8)</td>
<td>24</td>
</tr>
</tbody>
</table>
Table 3.3.4. Number and percentage of mares with intrauterine fluid, and concentration and percentage of leukocytes (mean ± SE) 24 hours after insemination into the uterine body in mares that were sedated with detomidine and acepromazine before the procedure, and mares that were not sedated. Values are not different (p>0.05).

<table>
<thead>
<tr>
<th>Sedation</th>
<th>No. mares with fluid (%)</th>
<th>Concentration of leukocytes</th>
<th>Percentage of leukocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>4 (80)</td>
<td>0.6 ± 0.5</td>
<td>50.0 ± 13.7</td>
</tr>
<tr>
<td>No</td>
<td>3 (60)</td>
<td>0.6 ± 0.5</td>
<td>29.0 ± 10.1</td>
</tr>
</tbody>
</table>
Two approaches have previously been used to retrieve samples of uterine secretions. A small volume flush technique has commonly been used to obtain samples and evaluate uterine inflammatory response (Kotilainen et al., 1994; Nikolakopoulos and Watson, 1997; Williamson et al., 1987). The flush technique improves the ability to quantitatively evaluate cytologic findings, especially in subfertile mares (Ball et al., 1988). However, fluid distribution along the endometrium may not be even and a dilution factor may be needed to calculate the volume of native secretions obtained. Radiolabeled albumin, at a known concentration, has been used for this purpose. The distribution of the labeled albumin was used to calculate the amount of fluid present in the uterus (Evans et al., 1987). Alternatively, fluid was collected by absorption into an intrauterine tampon (Katila, 1995). This allowed retrieval of undiluted samples of native secretions minimizing experimental error induced by an unknown dilution factor. In the present study, a low volume flush technique was used without incorporating any markers. Fluid distribution throughout the endometrium may not have been uniform and there may be a dilution effect on the values obtained, contributing to the variability noted.

Since the samples were obtained 24 hours after insemination, the initial acute inflammatory response was evaluated in this experiment. This acute phase comprises the period from 4 to 24 hours after breeding and is the time when maximum numbers of neutrophils are found (Katila, 1995). Because of the presence of mononuclear cells, the concentration of leukocytes was evaluated instead of the concentration of neutrophils.

Reproductively normal and DUC mares did not show any difference in their inflammatory cellular response. The high standard deviation and the small sample size may have contributed to the lack of statistical significance. However, several authors demonstrated that susceptible mares are able to mount a fully functional cellular and humoral inflammatory response, which is initially similar to that mounted by resistant mares during the first 6 hours (Asbury and Hansen, 1987; LeBlanc et al., 1989; Williamson et al., 1987).

Although hysteroscopic insemination did not reduce the severity of the endometritis compared with routine uterine body insemination, it did not induce an exaggerated response either. The endometrium of the mare in estrus was shown to be very sensitive, responding with hyperemia after any minor trauma (Bracher and Allen, 1992). Depositing the semen onto the UTJ requires air insufflation into the uterus and passage and manipulation of the endoscope up to the tip of the uterine horn, potentially increasing the trauma inflicted to the endometrium during
a highly susceptible phase of the estrous cycle. The reactive hyperemia may induce further neutrophil migration unrelated to spermatozoa chemotactic stimuli. Physical uterine and cervical manipulation, as required for uterine endoscopic procedures, induced neutrophil migration (Martin et al., 1988; Williamson et al., 1987). Although the hysteroscopic procedure is relatively quick and atraumatic, the uterine position of some mares made it both difficult and time consuming to reach the tip of the uterine horn. Unfortunately, the time required to complete each hysteroscopic procedure was not recorded, but it may have influenced the degree of inflammation.

It was surprising that reproductively normal mares infused with just semen extender and without spermatozoa at the UTJ showed the highest number of leukocytes. Seminal plasma has a damping effect on the uterine inflammatory response, suppressing neutrophil chemotaxis, phagocytosis and hemolytic complement activity in vitro, and it also shortens the duration of breeding-induced inflammation (Troedsson et al., 2001b; 2000). The absence of seminal plasma in the sham-inseminated mares may have resulted in no moderation of the inflammatory response of the endometrium to the procedure and therefore, a higher number of leukocytes.

The presence of macrophages and lymphocytes in both reproductively normal and DUC mares was also unexpected. After an initial insult, acute endometritis is characterized by the arrival of neutrophils. Macrophages arrive 3 to 4 days later to clear the debris generated by the neutrophils. If the endometrium is exposed to repeated insults, a chronic endometritis results, characterized by an increase in the numbers of helper T-cells and macrophages (Bowen et al., 1987). Although occasional macrophages and lymphocytes were found in cytologic samples from reproductively normal mares (Couto and Hughes, 1984), their presence together with neutrophils is indicative of chronic endometritis (Bowen et al., 1987; Couto and Hughes, 1984; Ley et al., 2002). An increased number of lymphocytes was also noted during the resolution phase of endometritis (Ley et al., 2002). Mares with lymphatic stasis may also have had lymphocytes in their cytologies (Couto and Hughes, 1984).

A chronic endometritis may have been present in the mares in the present study. This may have resulted from repeated breeding during consecutive estrous cycles and may have been exacerbated by the shortened interovulatory interval in the experimental design. Shortening of diestrus with dinoprost and shortening of estrus with hCG may have reduced the time allowed to clear the endometritis before the uterus was exposed to semen again. The presence of chronic
endometritis may have modified the acute response to semen deposition, possibly contributing to the lack of observed differences between treatment groups or treatments.

The number of leukocytes and fluid scores were not different after insemination into the uterine body between mares that were sedated with detomidine and acepromazine, and mares that were not sedated. Although both drugs affect uterine motility (De Lille et al., 2000; Schatzmann et al., 1994), sedation did not seem to affect uterine clearance in the mares in this experiment.

In conclusion, no differences in the severity or incidence of acute post-breeding endometritis were seen between reproductively normal mares and mares with delayed uterine clearance under these experimental conditions. The acute endometrial inflammatory response was not different 24 hours after insemination with one billion spermatozoa in the uterine body or five million spermatozoa at the uterotubal junction. These findings suggest that although hysteroscopic insemination may not be beneficial as a management tool to reduce persistent mating-induced endometritis in mares with delayed uterine clearance, it can be used in these mares without risk of inducing an exaggerated inflammatory response that may impair conception.
CHAPTER 4
RESIDUAL ENDOMETRITIS AFTER HYSTEROSCOPIC INSEMINATION

4.1. Introduction

In the first experiment, some mares responded differently in terms of the presence of uterine fluid after uterine body insemination during the semen challenge compared with after the treatment. It was concluded that either mare classification for these mares was incorrect, or presence or absence of fluid was not consistent every time a mare was inseminated. For the second experiment, the conditions of the semen challenge were changed and the mares were re-classified. The difference in fluid accumulation after bacterial inoculation between resistant and susceptible mares was greater after ovulation in a previous study and susceptible mares also had significantly more bacterial growth than resistant mares (LeBlanc et al., 1989). Therefore, in order to make differences between reproductively normal and DUC mares more evident and facilitate mare classification during the semen challenge, inseminations were done within 24 hours before anticipated ovulation and semen extender without antibiotics was used. In the first experiment, human chorionic gonadotropin (hCG) was administered immediately after the treatments, so the procedures were performed within 48 hours before anticipated ovulations.

In the first experiment, the acute inflammatory response was evaluated 24 hours after each procedure. It has been reported previously that susceptible mares were able to initially respond to an antigen as effectively as resistant mares, but were unable to sustain this response in time (Asbury and Hansen, 1987; LeBlanc et al., 1989; Williamson et al., 1987). Therefore, it is possible that differences may be more evident 48 hours after insemination when neutrophil numbers reach low values again in reproductively normal mares (Katila, 1995). Differences in neutrophil concentrations were reported between reproductively normal and mares with delayed uterine clearance (DUC mares) 48 hours after insemination in one study, although the percentage of neutrophils did not differ (Nikolakopoulos and Watson, 1997). Therefore, in the present study the incidence and severity of endometritis were evaluated 48 hours instead of 24 hours after the procedures.

As discussed previously, when uterine samples were collected using a small volume flush technique, uneven fluid distribution and dilution of the secretions may have increased the chances of experimental error. In this experiment, undiluted samples were obtained with an intrauterine tampon in order to minimize variability introduced by an unknown dilution factor.
A fourth treatment where seminal plasma was infused hysteroscopically was included to
determine whether the lack of seminal plasma was responsible for the higher leukocyte numbers
seen in the first study after the sham hysteroscopies. Since DUC mares tended to accumulate
more intrauterine fluid after ovulation (LeBlanc et al., 1989), in this experiment, the mares
received hCG the day before insemination so that ovulation would occur within 24 hours after
the procedures and the likelihood of detecting differences in fluid accumulation would increase.

Also, mare management was changed to correct the deficiencies found during
Experiment 1. Cervical dilation was included as a criterion to determine the day of treatment,
and prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) administration was postponed until 14 days after ovulation to
allow more time for the mare to eliminate any residual endometritis and avoid carry-over effects
from the previous treatment. Immediately before the procedures, endometrial cytologies were
performed to detect the presence of chronic endometritis and mares with positive cytologies were
allowed to sexually rest for one estrous cycle before being assigned to the following treatment.

Sieme et al. (2004) described a deleterious effect of low dose insemination on pregnancy
rates in problem mares. Pregnancy rates were determined in this experiment to evaluate the
effect of both insemination methods on fertility in reproductively normal and DUC mares, and to
determine whether there is any association between leukocyte numbers and pregnancy outcome.

It was hypothesized that hysteroscopic insemination at the uterotubal junction (UTJ) with
a reduced volume and number of spermatozoa would decrease the incidence of endometritis and
result in a less severe inflammatory response. The decrease in incidence of persistent mating-
induced endometritis would increase pregnancy rates in DUC mares inseminated
hysteroscopically compared with uterine body inseminations.

4.2. Materials and Methods

The study was conducted from April to September 2004. The mares were part of the
LSU-SVM teaching and research herds and were kept in pastures at the LSU-SVM facilities for
the entire length of the study. All procedures were approved and conducted following a protocol
approved by the LSU Institutional Animal Care and Use Committee (Protocol 03-054).

4.2.1. Semen Challenge and Mare Classification

Fifteen cycling mares were classified as reproductively normal or DUC mares based on
the results of a semen challenge. The mares were teased twice a week until estrous behavior was
detected. The mares were considered to be in estrus when posturing, urinating, tail raising and
clitoral eversion in response to exposure to the stallion were seen. Once in estrus, ovarian activity was monitored by palpation per rectum and transrectal B-mode, real time ultrasonography using a 5 mHz linear probe (Aloka SSD-500V, Aloka Co., Mitaka-Shi, Tokyo) every other day until a follicle ≥30 mm in diameter was identified. Thereafter, ovarian monitoring was performed daily. Uterine edema was identified as an interdigitated pattern of echogenic and anechoic areas, and was subjectively graded based on the ultrasonographic appearance of the endometrial folds. A scale from zero to three was used: 0: no edema, 1: slightly prominent endometrial folds, 2: moderately prominent endometrial folds, 3: very prominent endometrial folds (McKinnon et al., 1987).

To assess cervical relaxation, the width and length of the cervix were determined by palpation per rectum. A scale from 1 to 4 was used based on the estimated diameter of the cervix: 1: <20 mm, 2: 20 to 35 mm, 3: 35 to 45 mm, 4: 45 to 50 mm. Once a follicle ≥35 mm in diameter, uterine edema grade ≥2, and a cervical relaxation grade ≥3 were present, the mares received hCG, 2000 IU IV (Chorulon, Intervet, Millsboro, DE).

At 24 hours after hCG administration the mares were artificially inseminated with fresh semen containing one billion spermatozoa, extended to a total volume of 20 mL in a milk-based semen extender without antibiotics (EZ-mixin, Animal Reproduction Systems, Chino, CA). Ultrasonography was performed 24 and 48 hours after insemination to detect the presence of intrauterine fluid. Fluid was identified as an anechoic free intraluminal uterine content. Mares without fluid at 24 or 48 hours after insemination were considered reproductively normal while those with intrauterine fluid were considered DUC mares (Paccamonti et al., 1999). Uterine biopsies were obtained during diestrus. Reproductively normal mares had grade I to IIB uterine biopsies, and DUC mares, grade IIB to III (Kenney and Doig, 1986). Major changes detected in the uterine biopsies were periglandular fibrosis, glandular nests and lymphatic stasis. Animals with evidence of chronic infiltrative endometritis were excluded from this study.

4.2.2. Experimental Design

Four reproductively normal mares and five mares with delayed uterine clearance were selected from the population challenged and used for the study. Each mare received each of four treatments in four consecutive estrous cycles: UB: uterine body artificial insemination, HYST: hysteroscopic artificial insemination, SHAM: sham hysteroscopic artificial insemination with semen extender or SP: hysteroscopic infusion of seminal plasma. The order of the treatments was
randomized so that each mare received the four different treatments in different order. Teasing, palpation per rectum and ultrasonography were performed as described for the semen challenge. When a follicle $\geq 35$ mm in diameter, uterine edema grade $\geq 2$ and cervical relaxation grade $\geq 3$ were present, the mares received hCG, 2000 IU IV (Chorulon, Intervet, Millsboro, DE) to reduce the variability in time from insemination to ovulation. After 24 hours, the mares were assigned to one of the four treatment groups. The insemination doses and volumes are summarized in Table 4.2.1.

Following insemination, the mares were monitored daily until ovulation was detected. Pregnancy diagnosis was performed by transrectal ultrasonography 14 to 16 days after ovulation. Pregnancy was confirmed upon detection of an embryonic vesicle. At that time, pregnancy was terminated by manual reduction of the embryonic vesicle, together with injection of PGF$_{2\alpha}$ 5 mg IM (Lutalyse, Pharmacia & Upjohn, Kalamazoo, MI). An ultrasound examination was repeated 48 hours later to confirm the pregnancy termination.

Before the procedures, endometrial cytology was performed. The mares were restrained in stocks and their perineal area was cleansed with povidone iodine scrub and water. A sterile gloved hand was introduced into the vagina and the cytology samples were obtained digitally. A cytological smear was prepared and stained with a modified Wright’s stain (Protocol Hema 3, Fisher Diagnostics, Middletown, VA). Mares with cytologies containing $\geq 4\%$ leukocytes were considered to have endometritis, and mares with cytologies containing $\leq 3\%$ were considered to be free of endometritis. The mares showing cytological signs of endometritis were not inseminated during that estrus and were sexually rested for one estrous cycle before being assigned to the following treatment.

4.2.3. Semen Processing

Semen was collected and processed for inseminations as described in Experiment 1 under Section 3.2.3.

4.2.4. Preparation of Seminal Plasma

A complete ejaculate was collected as described in Experiment 1 and was centrifuged unextended at 4500 x g for 10 minutes. The supernatant was then filtered through 0.2 $\mu$m filters (Acrodisc® syringe filter, Pall Gelman Laboratory, Ann Arbor, MI) and stored frozen in 1-mL aliquots at -20°C. Seminal plasma from the same ejaculate was used for all the SP treatments and was thawed immediately before each procedure.
Table 4.2.1. Number of spermatozoa and volume of the inseminates received by mares in treatments UB (uterine body insemination), HYST (hysteroscopic insemination), SHAM (sham hysteroscopy) or SP (hysteroscopic infusion of seminal plasma)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>UB</th>
<th>HYST</th>
<th>SHAM</th>
<th>SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of spermatozoa</td>
<td>1 billion</td>
<td>5 million</td>
<td>Semen extender</td>
<td>Seminal plasma</td>
</tr>
<tr>
<td>Volume of inseminate (mL)</td>
<td>20</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>
4.2.5. Hysteroscopic Procedure and Insemination

The mares were restrained in stocks and the perineum was cleansed with povidone iodine scrub and water. Five minutes before the hysteroscopic procedures, the mares were sedated with detomidine, 20 µg/kg IV (Dormosedan, Orion Corp., Espoo, Finland) to reduce discomfort caused by insufflation of the uterus with air. Acepromazine, 40 mg IV (PromAce, Fort Dodge Animal Health, Fort Dodge, Iowa) was administered immediately before the hysteroscopy to reduce uterine contractions, which impede the procedure. The dose of acepromazine was increased compared with the first experiment. The dose used previously (30 mg) was found to be insufficient to reduce myometrial activity.

The endoscope was cold-sterilized in a 3.4% glutaraldehyde solution for 20 minutes (Cidex Plus, Ethicon, Inc., Irvine, CA) and rinsed externally and internally with 1 L of sterile water. The biopsy channel was dried with air at high pressure to remove any chemical residues. An equine fallopian tube insemination set (V-EFIS-2-200, Cook Veterinary Products, Queensland, Australia) was used. The inner catheter had a No. 2 French diameter and was 200 cm in length. The catheter was introduced through the biopsy channel of a 2-meter flexible videoendoscope (Series 160 Olympus, Sony, Ichinomiya, Japan). For the SHAM and SP treatments, the catheter was loaded with 0.5 mL of semen extender or seminal plasma, respectively, before the start of the procedure. The endoscope was passed manually through the cervix into the uterus. The uterus was insufflated with non-sterilized air using the endoscope setup to allow visualization of the entire endometrial surface. The endoscope was guided to the tip of the horn ipsilateral to the ovary containing the preovulatory follicle. To preserve sperm viability, the 0.5 mL of extended semen was loaded once the UTJ was visualized. The inner catheter was exteriorized and placed close to the UTJ, and the extender (SHAM), the semen (HYST) or the seminal plasma (SP) was deposited onto the UTJ creating froth. The endoscope was slowly removed from the uterus allowing the air to leave the uterus through the cervix. As soon as the endoscope was inserted into the uterus and air insufflation was started, time recording started. The time was recorded until the endoscope was entirely removed from the uterus.

4.2.6. Uterine Body Insemination

The mares were restrained in stocks and the perineum was cleansed as described for the hysteroscopic procedures. An equine insemination pipette, attached to a 20-ml all-plastic syringe
with previously loaded extended semen, was manually passed through the cervix and the extended semen was deposited into the uterine body.

Although sedation is not usually required for insemination into the uterine body, it is required for hysteroscopy. Since both detomidine and acepromazine affect uterine motility (De Lille et al., 2000; Schatzmann et al., 1994), the mares in the UB group also received detomidine, 20 \( \mu \)g/kg IV (Dormosedan, Orion Corp., Espoo, Finland) and acepromazine, 40 mg IV (PromAce, Fort Dodge Animal Health, Fort Dodge, Iowa) immediately before the procedure.

4.2.7. Evaluation of the Inflammatory Response

The presence of intrauterine fluid was evaluated by transrectal ultrasonography 24 and 48 hours after treatments. The amount was subjectively graded using the following classification: 0: no fluid, 1: small amount of fluid, 2: moderate amount of fluid, 3: large amount of fluid. Grading was made based on the depth and length of the pockets of free intraluminal fluid seen at the uterine body or base of the horns.

The inflammatory cellular response of the endometrium to the different treatments was evaluated 48 \( \pm \) 2 hours after the procedures. Uterine secretions were obtained with an intrauterine tampon (Tampax® Femenine Tampon-regular, Hospeco, Cleveland, OH). The tampon was placed manually into the uterus. After 10 minutes, it was removed, protected by a sterile gloved hand, and placed into a 35-mL syringe. The plunger of the syringe was compressed under a mechanical press to retrieve the fluid into 15-mL conical tubes. The volume of fluid obtained was recorded. An aliquot was first diluted 1:10 in formol buffered saline (FBS). This initial sample was further diluted 1:10 (1:100 from the original concentration) in glacial acetic acid (28.6%) to induce hemolysis of erythrocytes and facilitate the count of nucleated cells. The use of FBS avoided cell clumping that was observed when the serial dilutions were made directly with acetic acid. The concentration of nucleated cells was counted using a hemacytometer and expressed in millions of cells per mL of fluid.

Immediately after removing the tampon from the uterus, a guarded swab (Kt-3000, Kalayjian Industries, Signal Hill, CA) was introduced into the uterus. The swab was exposed so that the cap would open, and it was rolled against the endometrium for a few seconds. An endometrial cytology specimen was prepared from the cap of the swab. The smear for cytologic evaluation was stained with a modified Wright’s stain (Protocol Hema 3, Fisher Diagnostics, Middletown, VA) and 100 cells were counted and classified as neutrophils, macrophages,
lymphocytes, eosinophils or endometrial cells. The differential count was used to calculate the concentration of leukocytes in the fluid as follows:
Concentration of leukocytes (million/mL) = concentration of nucleated cells (million/mL) x percentage of leukocytes
Total leukocytes (million) = concentration of leukocytes (million/mL) x volume (mL)

The severity of the endometritis was graded based on the following classification (modified from Crickman and Pugh, 1986): 0 to 3% leukocytes: no inflammation, 4 to 15%: slight inflammation, 16 to 50%: moderate inflammation, >50%: severe inflammation. Uterine cytologies containing ≤3% leukocytes were considered to be indicative of the absence of endometritis (negative). Cytologies containing ≥4% leukocytes were considered to be indicative of the presence of endometritis (positive). Intrauterine fluid was defined as absent in mares with fluid score 0, and present in mares with fluid score ≥1.

4.2.8. Statistical Analysis

The frequency distribution of fluid scores at 24 and 48 hours, presence or absence of fluid before inseminations, number of mares that responded to hCG and frequency of degrees of inflammation were analyzed using frequency methods and the frequency distributions for the response variables of interest are reported. The frequency of responses within each treatment group were evaluated for homogeneity across treatments using Chi square test for homogeneity of strata (treatments) with the null hypothesis of homogeneity rejected at p<0.05. The adjusted Cochran-Mantel-Haenszel statistic (CMH) was considered to summarize any association of treatment with the response variable, controlling for group. A similar analysis was done for the responses within each group, stratified across treatments for presence of fluid. Significance was considered for the CMH where p<0.05.

An association between the presence of fluid and pregnancy was evaluated using Fisher’s exact test against a two-sided hypothesis with significance considered at p<0.05. A similar association was evaluated for the presence or absence of fluid and positive or negative cytology and presence of fluid and occurrence of ovulation. An association between pregnancy, presence of fluid and cytology was evaluated using the adjusted Cochran-Mantel-Haenszel statistic. Significance was considered for the CMH where p<0.05.

The number of days from PGF$_{2\alpha}$ administration to insemination, insemination to ovulation, and insemination to subsequent insemination, and the volume of fluid recovered,
concentration, percentage and total numbers of leukocytes were first tested for normality using the Kolmogorov-Smirnov test statistic with failure to reject the null hypothesis of normality at p<0.05 for all response variables analyzed. The number of days from insemination to ovulation, and insemination to subsequent insemination is expressed as median ± SE. These variables did not follow a normal distribution and were analyzed with a non-parametric analysis of variance (Kruskal-Wallis test). All the other response variables were evaluated for a fixed effect of group and treatment using a mixed effect linear model that also included the random variance of horses nested within the group*treatment factorials. Where there was significant interaction of group and treatment, least squares means were used to isolate significant difference adjusting Type I error at 0.05 using the Scheffe procedure. These variables are expressed as mean ± SE.

The correlation between the duration of the hysteroscopic procedure and concentration, percentage and total neutrophils was evaluated and Pearson’s correlation coefficients were calculated. Regression analysis was done to calculate the predicted values of time after which positive cytologies can be expected.

SAS® procedures UNIVARIATE, MEANS, MIXED, FREQ, CORR, REG and NPAR1WAY (SAS® version 9.0, SAS Institute, Cary, NC) were used to test for normality, obtain basic statistics, perform the mixed effect linear model on continuous data, analyze categorical data, obtain correlation coefficients, perform regression analysis and non-parametric analysis of variance, respectively.

4.3. Results

One of the reproductively normal mares entered the fall transition before the study could be completed, so data from the hysteroscopic insemination of that mare are missing. A total of 35 procedures were included in the analysis. The mean number of days from PGF$_2\alpha$ injection to insemination, from insemination to insemination, and from insemination to ovulation were not different across and within groups (Table 4.3.1). Ovulation occurred within 24 hours of insemination in 66% of the mares. There was no difference in the number of mares that responded to hCG between groups or treatments (p>0.05). Time of ovulation did not affect the incidence of intrauterine fluid (p>0.05). At the time of treatment, none of the reproductively normal mares and four of the DUC mares exhibited intrauterine fluid (p>0.05). Fluid accumulation at insemination in the DUC mares occurred before uterine body (n=1), sham (n=2) and seminal plasma (n=1) treatments, and was >2 cm in depth in only one these mares. The fluid
was classified as non-inflammatory in origin based on the absence of leukocytes in the cytologies performed before the procedures, and it did not have any association with the presence of intrauterine fluid or endometritis after the treatments (p>0.05).

When all the treatments were combined, more DUC (52%) than reproductively normal mares (14%) had intrauterine fluid 24 hours after the procedures (p<0.05). However, within treatments the difference between reproductively normal and DUC mares was significant only for the sham inseminations (p<0.05) (Table 4.3.2). There was no effect of treatment on intrauterine fluid accumulation in reproductively normal mares (p>0.05), but DUC mares showed more fluid after hysteroscopic and sham inseminations than after uterine body or seminal plasma treatments (p<0.05) (Table 4.3.2).

Two days after the procedures, 7 and 40% of the reproductively normal and DUC mares, respectively still had fluid (p<0.05). Again, the difference between reproductively normal and DUC mares was significant only for the sham inseminations (p<0.05) (Table 4.3.3). There was no difference in the incidence or severity of intrauterine fluid accumulations between treatments in either group of horses 48 hours after the procedures (p>0.05) (Table 4.3.3).

Pneumouterus was observed in eight mares 24 hours after the hysteroscopies. Air was visualized in the uterus of three reproductively normal mares (SHAM=1, SP=3) and five DUC mares (HYST=2, SHAM=1, SP=2). The frequency distribution of pneumouterus between groups of mares was not different (p>0.05) so the data was pooled for comparisons across treatments. Treatment did not affect the incidence of pneumouterus and the presence of air 24 hours after the procedures was not associated with the presence of endometritis (p>0.05).

The volume of fluid recovered was 2.1 ± 2.3 mL and 0.9 ± 0.8 mL from reproductively normal and DUC mares, respectively. No fluid was recovered from the tampons from 5/35 mares. Fluid was not recovered from the tampons of two reproductively normal mares after UB and SP treatments, and three DUC mares: two after HYST, and one after SP treatments. None of these mares had ultrasonographic evidence of intrauterine fluid at the moment the tampons were placed. The concentration of leukocytes was not correlated with the volume of fluid recovered (p>0.05).

There was no significant effect of treatment or group on the percentage (Figure 4.3.1) and total numbers of leukocytes (Figure 4.3.2) (p>0.05) but there was a significant interaction of group and treatment for the concentration of leukocytes (Figure 4.3.3) (p<0.05). Reproductively
Table 4.3.1. Number of days from PGF2α injection to insemination, from insemination to insemination and from insemination to ovulation in reproductively normal and DUC mares (median ± SE). UB: uterine body insemination with one billion spermatozoa in 20 mL, HYST: hysteroscopic insemination at the UTJ with five million spermatozoa in 0.5 mL, SHAM: sham hysteroscopic insemination with 0.5 mL of semen extender, SP: hysteroscopic infusion of 0.5 mL of seminal plasma. Values are not different (p>0.05).

<table>
<thead>
<tr>
<th>Mare group</th>
<th>Treatment</th>
<th>Prostaglandin to insemination (days)</th>
<th>Insemination to insemination (days)</th>
<th>Insemination to ovulation (days)</th>
<th>No. mares</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>UB</td>
<td>9.0 ± 0.4</td>
<td>29.0 ± 0.0</td>
<td>1.0 ± 0.2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>HYST</td>
<td>6.0 ± 0.4</td>
<td>28.0 ± 3.5</td>
<td>1.0 ± 0.0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>SHAM</td>
<td>7.5 ± 1.6</td>
<td>22.5 ± 2.3</td>
<td>1.0 ± 0.0</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>SP</td>
<td>7.0 ± 5.0</td>
<td>23.0 ± 6.7</td>
<td>2.0 ± 0.4</td>
<td>4</td>
</tr>
<tr>
<td>DUC</td>
<td>UB</td>
<td>6.0 ± 0.8</td>
<td>23.0 ± 4.4</td>
<td>2.0 ± 0.4</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>HYST</td>
<td>4.5 ± 1.5</td>
<td>21.0 ± 1.6</td>
<td>2.0 ± 0.9</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>SHAM</td>
<td>5.5 ± 1.5</td>
<td>20.0 ± 4.6</td>
<td>1.0 ± 0.4</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>SP</td>
<td>7.0 ± 0.0</td>
<td>23.0 ± 0.8</td>
<td>1.0 ± 0.4</td>
<td>5</td>
</tr>
</tbody>
</table>
Table 4.3.2. Frequency distribution of fluid scores 24 hours after insemination. Fluid score of 0 (no fluid), 1 (small amount of fluid), 2 (moderate amount) or 3 (large amount). UB: uterine body insemination with one billion spermatozoa in 20 mL, HYST: hysteroscopic insemination at the UTJ with five million spermatozoa in 0.5 mL, SHAM: sham hysteroscopic insemination with 0.5 mL of semen extender, SP: hysteroscopic infusion of 0.5 mL of seminal plasma. Different superscripts indicate differences between reproductively normal and DUC mares (a,b,c,d) or between insemination methods (A,B) (p<0.05).

<table>
<thead>
<tr>
<th>Mare group</th>
<th>Treatment</th>
<th>Fluid score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>UB</td>
<td>4 (100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>HYST</td>
<td>2 (67)</td>
<td>0</td>
<td>1 (33)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SHAM(^c)</td>
<td>4 (100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SP</td>
<td>3 (75)</td>
<td>1 (25)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total(^a)</td>
<td>13 (86)</td>
<td>1 (7)</td>
<td>1 (7)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DUC</td>
<td>UB(^A)</td>
<td>3 (60)</td>
<td>0</td>
<td>2 (40)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HYST(^B)</td>
<td>1 (20)</td>
<td>3 (60)</td>
<td>1 (20)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SHAM(^d, B)</td>
<td>1 (20)</td>
<td>3 (60)</td>
<td>0</td>
<td>1 (20)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SP(^A)</td>
<td>5 (100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total(^b)</td>
<td>10 (48)</td>
<td>6 (28)</td>
<td>4 (19)</td>
<td>1 (5)</td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>Total</td>
<td>23 (66)</td>
<td>7 (20)</td>
<td>4 (11)</td>
<td>1 (3)</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3.3. Frequency distribution of fluid scores 48 hours after insemination. Fluid score of 0 (no fluid), 1 (small amount of fluid), 2 (moderate amount) or 3 (large amount). UB: uterine body insemination with one billion spermatozoa in 20 mL, HYST: hysteroscopic insemination at the UTJ with five million spermatozoa in 0.5 mL, SHAM: sham hysteroscopic insemination with 0.5 mL of seminal plasma, SP: hysteroscopic infusion of 0.5 mL of seminal plasma. Values with different superscripts indicate differences between reproductively normal and DUC mares (a,b,c,d) (p<0.05).

<table>
<thead>
<tr>
<th>Mare group</th>
<th>Treatment</th>
<th>Fluid score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>UB</td>
<td>4 (100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>HYST</td>
<td>2 (67)</td>
<td>0</td>
<td>1 (33)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SHAM(^c)</td>
<td>4 (100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SP</td>
<td>4 (100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total(^a)</td>
<td>14 (93)</td>
<td>0</td>
<td>1 (7)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DUC</td>
<td>UB</td>
<td>3 (60)</td>
<td>1 (20)</td>
<td>1 (20)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HYST</td>
<td>4 (80)</td>
<td>1 (20)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SHAM(^d)</td>
<td>1 (20)</td>
<td>4 (80)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SP</td>
<td>4 (80)</td>
<td>1 (20)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total(^b)</td>
<td>12 (60)</td>
<td>7 (35)</td>
<td>1 (5)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>Total</td>
<td>26 (74)</td>
<td>7 (20)</td>
<td>2 (6)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
normal mares inseminated at the uterine body had higher concentrations of leukocytes than reproducively normal mares that underwent hysteroscopic procedures, and than DUC mares after any of their treatments.

The concentration, percentage and total number of leukocytes were positively correlated with the duration of the hysteroscopies in reproducively normal mares \((p<0.05)\) but not in the DUC mares \((p>0.05)\) (Table 4.3.4). The predicted value of duration of the hysteroscopic procedure at which positive cytologies can be expected was calculated using regression analysis. If reproducively normal mares undergo a hysteroscopy that extends beyond 9 minutes, they can be expected to have persistent endometritis after the procedure. If reproducively normal and DUC mares are included in the regression analysis, a hysteroscopy lasting 7 minutes or more is likely to induce persistent endometritis. The median durations of the procedures in this study are shown in Table 4.3.5.

The degree of inflammation was not different between reproducively normal and DUC mares so their data was combined \((p>0.05)\) (Table 4.3.6). There was no significant effect of treatment on the severity of the inflammatory response \((p>0.05)\). Overall, 46% of the mares had no signs of endometritis and had negative cytologies 48 hours after the procedures.

The presence of intrauterine fluid 24 hours or 48 hours after the procedures was not associated with the presence of endometritis 48 hours post-treatment. Mares without fluid were as likely to have cytological signs of endometritis as mares with fluid, independently of mare classification or treatment received \((p>0.05)\) (Table 4.3.7). Endometritis was associated with the presence of fluid after only 44% of the procedures. In the same way, the absence of endometritis was associated with absence of fluid after 42% of the procedures. Endometritis was defined as the presence of \(\geq 4\%\) leukocytes in the cytologic smears.

Although 3/4 reproducively normal mares conceived when inseminated at the uterine body, none of them became pregnant after hysteroscopic inseminations \((p<0.05)\). Conversely, insemination method did not affect pregnancy rates in DUC mares (Table 4.3.8) \((p>0.05)\). No association was found between the presence of intrauterine fluid or endometritis and pregnancy outcome in reproducively normal or DUC mares \((p>0.05)\).

Reproductively normal mares had lymphocytes and macrophages in five of the 15 estrous cycles \((33\%)\) and DUC mares did so in two of 20 estrous cycles \((10\%)\). The presence of
Figure 4.3.1. Percentage of uterine leukocytes 48 hours after treatment (mean ± SE). UB: uterine body insemination with one billion spermatozoa in 20 mL, HYST: hysteroscopic insemination at the UTJ with five million spermatozoa in 0.5 mL, SHAM: sham hysteroscopic insemination with 0.5 mL of semen extender, SP: hysteroscopic infusion of 0.5 mL of seminal plasma. Values are not different (p>0.05).

Figure 4.3.2. Total number of leukocytes in uterine cytologies 48 hours after treatment (mean ± SE). UB: uterine body insemination with one billion spermatozoa in 20 mL, HYST: hysteroscopic insemination at the UTJ with five million spermatozoa in 0.5 mL, SHAM: sham hysteroscopic insemination with 0.5 mL of semen extender, SP: hysteroscopic infusion of 0.5 mL of seminal plasma. Values are not different (p>0.05).
Figure 4.3.3. Concentration of uterine leukocytes 48 hours after treatment (mean ± SE). UB: uterine body insemination with one billion spermatozoa in 20 mL, HYST: hysteroscopic insemination at the UTJ with five million spermatozoa in 0.5 mL, SHAM: sham hysteroscopic insemination with 0.5 mL of semen extender, SP: hysteroscopic infusion of 0.5 mL of seminal plasma. \textsuperscript{a,b}Values with different superscript are significantly different (p<0.05).
mononuclear cells was evident only after the third procedure. These mares were sexually rested for one estrous cycle before undergoing a fourth procedure. After the period of sexual rest, the mares previously having mononuclear cells in their cytologies were able to clear the endometritis, and had no evidence of endometritis in their cytologies at the time the fourth procedure was performed.

4.4. Discussion

As discussed before, susceptible mares accumulated more fluid after ovulation and had significantly more bacterial growth than resistant mares in one study (LeBlanc et al., 1989). Therefore, in order to make differences between reproductively normal and DUC mares more evident and facilitate their classification during the semen challenge, inseminations were done 24 hours before ovulation and semen extender without antibiotics was used. This resulted in some mares that were classified as reproductively normal during the previous year being considered to have delayed uterine clearance under the conditions of this semen challenge. Whether this reflected differences in management or changes in the status of the mare is not known.

As in the first study, there was no difference in intrauterine fluid accumulations between DUC and reproductively normal mares after routine uterine body inseminations. None of the reproductively normal mares had fluid 24 hours after insemination, suggesting that the mare classification for this group was correct. However, only 40% of DUC mares accumulated fluid after breeding. If mare classification had been accurate for this group, the incidence of intrauterine fluid should have been 100%. All of these mares did accumulate fluid under the conditions of the semen challenge. Although the inflammatory response is mounted mostly against spermatozoa, addition of antibiotics to the semen extender during the treatments may have reduced bacterial growth and the incidence of endometritis in these mares during the experiment. Most likely, fluid accumulation is a dynamic condition and mares with delayed uterine clearance may not accumulate fluid consistently every estrous cycle they are bred. If this is the case, mares should be challenged over several estrous cycles in order to improve the accuracy of the classification method.

When all the treatments were included in the analysis, DUC mares had a higher incidence of uterine fluid 24 and 48 hours after the procedures. But this difference was due to more of these mares showing fluid after sham inseminations. This was the only treatment where seminal
Table 4.3.4. Pearson’s correlation coefficients for concentration, percentage and total PMNs with duration of the endoscopies. *Correlation of the parameter with the duration of the hysteroscopic procedure was significant (p<0.05).

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration of PMNs</th>
<th>Percentage of PMNs</th>
<th>Total PMNs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.97 (^a)</td>
<td>0.98 (^a)</td>
<td>0.88 (^a)</td>
</tr>
<tr>
<td>DUC</td>
<td>-0.11</td>
<td>0.13</td>
<td>0.09</td>
</tr>
<tr>
<td>Combined</td>
<td>0.7 (^a)</td>
<td>0.66 (^a)</td>
<td>0.77 (^a)</td>
</tr>
</tbody>
</table>

Table 4.3.5. Duration of the hysteroscopic procedures (minutes, median ± SE). HYST: hysteroscopic insemination at the UTJ with five million spermatozoa in 0.5 mL, SHAM: sham hysteroscopic insemination with 0.5 mL of semen extender, SP: hysteroscopic infusion of 0.5 mL of seminal plasma. Values are not different (p>0.05).

<table>
<thead>
<tr>
<th>Mare group</th>
<th>HYST</th>
<th>SHAM</th>
<th>SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>7.0 ± 2.0</td>
<td>13.0 ± 7.0</td>
<td>12.0 ± 8.8</td>
</tr>
<tr>
<td>DUC</td>
<td>4.0 ± 1.4</td>
<td>4.0 ± 1.0</td>
<td>7.5 ± 3.6</td>
</tr>
</tbody>
</table>

Table 4.3.6. Frequency distribution of inflammation scores 48 hours after treatments. Values represent the number of mares within each treatment group having no (0 to 3% leukocytes), slight (4 to 15%), moderate (16 to 50%) or severe (>50%) endometritis. UB: uterine body insemination with one billion spermatozoa in 20 mL, HYST: hysteroscopic insemination at the UTJ with five million spermatozoa in 0.5 mL, SHAM: sham hysteroscopic insemination with 0.5 mL of semen extender, SP: hysteroscopic infusion of 0.5 mL of seminal plasma. Values are not different (p>0.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>None mares (%)</th>
<th>Slight mares (%)</th>
<th>Moderate mares (%)</th>
<th>Severe mares (%)</th>
<th>Total mares</th>
</tr>
</thead>
<tbody>
<tr>
<td>UB</td>
<td>3 (33.0)</td>
<td>5 (56.0)</td>
<td>1 (11.0)</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>HYST</td>
<td>6 (75.0)</td>
<td>1 (12.5)</td>
<td>1 (12.5)</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>SHAM</td>
<td>4 (44.5)</td>
<td>4 (44.5)</td>
<td>1 (11.0)</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>SP</td>
<td>3 (33.3)</td>
<td>3 (33.3)</td>
<td>3 (33.3)</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>16 (46.0)</td>
<td>13 (37.0)</td>
<td>6 (17.0)</td>
<td>0</td>
<td>35</td>
</tr>
</tbody>
</table>
Table 4.3.7. Number and percentage of mares with or without uterine fluid 48 hours after treatment and with or without endometritis. The different groups and treatments were combined in the table. Values are not different (p>0.05).

<table>
<thead>
<tr>
<th></th>
<th>No fluid</th>
<th>Fluid</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No endometritis</td>
<td>11 (42)</td>
<td>5 (56)</td>
<td>16 (46)</td>
</tr>
<tr>
<td>Endometritis</td>
<td>15 (58)</td>
<td>4 (44)</td>
<td>19 (54)</td>
</tr>
<tr>
<td>Total</td>
<td>26</td>
<td>9</td>
<td>35</td>
</tr>
</tbody>
</table>

Table 4.3.8. Pregnancy rates in reproductively normal and DUC mares after uterine body and hysteroscopic inseminations. Different superscripts represent differences between treatments (p<0.05).

<table>
<thead>
<tr>
<th>Mare group</th>
<th>Treatment</th>
<th>Pregnant No. mares (%)</th>
<th>Non pregnant No. mares (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>UB</td>
<td>3 (75)(^a)</td>
<td>1 (25)(^a)</td>
</tr>
<tr>
<td></td>
<td>HYST</td>
<td>0(^b)</td>
<td>3 (100)(^b)</td>
</tr>
<tr>
<td>DUC</td>
<td>UB</td>
<td>1 (20)</td>
<td>4 (80)</td>
</tr>
<tr>
<td></td>
<td>HYST</td>
<td>2 (40)</td>
<td>3 (60)</td>
</tr>
</tbody>
</table>
plasma was not included in the inseminate. As discussed before, seminal plasma modulates the endometrial inflammatory response to semen deposition. It could be hypothesized that lack of seminal plasma in the sham treatments may have resulted in a more severe endometritis and fluid accumulation in DUC mares. However, the number of leukocytes after sham insemination was not higher.

The differences in post-breeding uterine fluid between hysteroscopic and uterine body inseminations in DUC mares do not agree with the results of Sieme et al. (2004) who failed to see any difference, both in reproductively normal and problem mares. However, these authors defined their group of problem mares as animals with abnormal reproductive history. No mention is made about their classification in terms of uterine clearance.

If the clinical diagnosis of persistent mating-induced endometritis is based on the presence of uterine fluid 24 hours after breeding, we have to conclude that reproductively normal mares are able to clear the endometritis independently of the insemination method used. Conversely, in DUC mares, hysteroscopic insemination induces an exacerbated endometritis. This response is generated by the procedure itself, since sham inseminations without semen deposition resulted in a similar incidence of fluid accumulation. However, by 48 hours DUC mares were able to clear any uterine fluid and the effect of treatment on fluid scores was no longer evident.

Fluid score was not influenced by the time of ovulation in this study. As steroid hormones influence uterine physical clearance, more mares that ovulate closer to insemination could be expected to have post-breeding intrauterine fluid. However, although serum progesterone concentration increases rapidly in the mare, it does not reach >1 ng/ml until 2 to 3 days after ovulation (Plotka et al., 1975). Cervical tone also does not increase suddenly but the cervix rather becomes increasingly tighter as the mare enters diestrus (Lieux, 1970). Ovulation in the mares in this study usually occurred within 24 hours after the procedures so the inflammatory response was evaluated, at the latest, 1 day after ovulation when progesterone may not have been high enough to influence physical clearance yet. Moreover, no correlation between serum concentrations of estrogen and progesterone at the time of insemination and post-insemination fluid accumulation was found in one study (Watson et al., 2001).

The results obtained in this experiment differ from those from Experiment 1. In the first experiment, no differences in fluid scores 24 hours after the procedures were observed. Because
fluid accumulation was not influenced by the time of ovulation, it is unlikely that the different results were due to the treatments being performed closer to ovulation in Experiment 2. Chronic endometritis was not associated with higher incidence of intrauterine fluid after treatment. The presence of chronic endometritis in Experiment 1 was also unlikely to contribute to the differences seen between experiments. It is probable that the accuracy of mare classification was improved in the second experiment, particularly in the group of reproductively normal mares. Reproductively normal mares accumulated fluid after 46% and 13% of the procedures in Experiments 1 and 2, respectively. Conversely, DUC mares accumulated fluid after 50% of the treatments in both experiments. The more accurate mare classification may have accounted for the differences in fluid scores between experiments.

Although uterine fluid was present in only 26% of the mares 48 hours after the treatments, cytological signs of endometritis were still present in 54% of the mares. Reproductively normal and DUC mares mounted a similar cellular inflammatory response and in contrast to the study done by Katila (1995), endometritis resolved in only half of the mares in both groups by 48 hours. Nikolakopoulos and Watson (1997) also reported the persistence of neutrophils in both reproductively normal and DUC mares 2 days after insemination. However, these authors did find a difference in the concentration of neutrophils between these groups of mares, although the percentage of neutrophils did not differ. In their work, samples were collected with a uterine flushing and the dilution factor was not calculated, so the percentage of neutrophils may be a more accurate parameter to consider than the concentration.

There was no effect of treatment on leukocyte numbers in any of the groups of mares and, although hysteroscopic insemination was associated with more DUC mares accumulating fluid post-breeding, the incidence or severity of endometritis based on cytological findings was not higher. The higher concentration of leukocytes in the group of reproductively normal mares that were inseminated at the uterine body is attributed to one mare that had an unusually high number of cells and does not reflect the true response of this group of horses. As the duration of the hysteroscopic procedure was positively correlated with neutrophil response, it is recommended to minimize the duration of the procedures as much as possible, and certainly not to extend 7 minutes to reduce the chances of inducing persistent endometritis.

It is believed that persistent mating-induced endometritis is accompanied by intrauterine fluid accumulation. However, this was true in only 44% of the mares in the present experiment.
The absence of intrauterine fluid after mating did not guarantee the absence of endometritis. Similar observations were made by Pycock and Newcombe (1996), who indicated that post-mating uterine fluid was not always present in susceptible mares. Performing endometrial cytology after breeding may help identify post-mating endometritis in some mares when fluid accumulations are not evident. Conversely, all mares with intrauterine fluid 12 to 48 hours after breeding are considered to have persistent mating-induced endometritis. This was true, however, in only 42% of the mares in this experiment. If persistent mating-induced endometritis is diagnosed based on ultrasonographic findings alone, some mares may be treated for delayed uterine clearance unnecessarily. Every time uterine lavage is performed, the anatomical barriers are bypassed and the uterine environment is disturbed. Intrauterine treatments in mares with negative cytologies represent an unnecessary risk for uterine bacterial contamination and endometritis. Endometrial cytologies may be required in order to classify the fluid as a transudate or an inflammatory exudate. Since presence of intrauterine fluid 24 hours or 48 hours after the procedures was not associated with cytologic signs of endometritis, the practice of diagnosing persistent mating-induced endometritis based only on ultrasonographic findings may need to be reconsidered.

Repeated endometrial insult in consecutive estrous cycles resulted in chronic inflammation after the third procedure. Because diestrus was not shortened with PGF$_{2\alpha}$ between treatments, its incidence was reduced in the second experiment. The impact of this finding on fertility cannot be evaluated by this study, but chronic endometritis can be expected to decrease pregnancy rates. Usually, problem mares are repeatedly bred throughout the breeding season during consecutive estrous cycles. It may be recommended that mares that do not conceive after being bred during three consecutive estrous cycles are not rebred in the following estrus or that endometrial cytology is done and appropriate treatment is performed before breeding. The presence of chronic endometritis was not associated with a higher incidence of intrauterine fluid accumulation, so identification of these mares may be difficult based on ultrasonographic findings alone.

Pregnancy rates were not influenced by mare classification and were not correlated with the presence of intrauterine fluid and/or cytological signs of endometritis at 48 hours. Only one study has previously evaluated the relation between neutrophil numbers and fertility and it was concluded that neutrophil numbers 6 hours after insemination did not correlate with pregnancy.
rates (Kotilainen et al., 1994). However, definitive conclusions cannot be made based on the results of this present experiment, since the number of mares used was limited. Although the presence of intrauterine fluid has been associated with decreased fertility, it is possible that factors other than persistence of endometritis are also responsible for the decreased fertility in DUC mares. Since these are usually older animals, age-associated changes in oviductal environment and oocyte quality, as well as endometrial degenerative changes may contribute to the lower pregnancy rates. The myometrial defect responsible for failure of uterine clearance may also be responsible for decreased embryo mobility and failure of maternal recognition of pregnancy (Carnevale and Ginther, 1992).

In conclusion, reproductively normal mares cleared intrauterine fluid within 24 hours independent of the method of insemination. Mares with delayed uterine clearance showed a higher incidence of post-breeding fluid accumulation after hysteroscopic insemination. However, the inflammatory cellular response to both methods was similar. Since the presence of uterine fluid was not correlated with pregnancy rates or with the presence of positive cytologies at 48 hours, hysteroscopic insemination does not seem to induce an inflammatory response, in either reproductively normal or DUC mares, that may impair fertility.
CHAPTER 5
CONCLUSIONS

One of the major problems found during these studies was mare classification. Intrauterine fluid accumulation after insemination was found to be inconsistent and showed variation within individual mares. Clinically, mares are considered to have delayed uterine clearance if fluid persists 12 to 48 hours after breeding. However, with the purpose of conducting controlled studies, this criterion may not be satisfactory. If a semen challenge is going to be used to classify the mares as reproductively normal mares or mares with delayed uterine clearance (DUC mares), it may need to be repeated several times to improve the accuracy. Only mares that are consistent in their uterine response should be classified.

Neutrophil percentages and concentrations recovered from uterine secretions have been used in a large number of studies to compare endometrial response to different treatments. The recovery methods have varied from a small volume uterine lavage to placement of an intrauterine tampon. Uterine lavage allows a better distribution of fluid throughout the endometrial surface and it is thought to be more effective in retrieving cells from the endometrial folds. However, if markers are not added to the flush, there may be a confounding effect of a dilution factor that may contribute to the large variability within treatments seen in most of the studies where this method was used. Conversely, the tampon method allows recovery of native secretions but has only limited contact with the endometrium and in the present study uterine samples were not recovered from all the mares. Although this method was proven to be satisfactory for evaluation of soluble factors present in endometrial secretions (Troedsson et al., 1993b), its value for evaluation of cell numbers may be questionable since some cells may remain trapped within the tampon. Nevertheless, the technique was reported for its use in assessment of cellular response after insemination in the mare (Katila, 1995).

Katila (1995) proposed that reproductively normal and DUC mares initially have a similar cellular response to semen deposition. During the first experiment in the present study, where the acute inflammatory response was evaluated, it was also evident that DUC mares were able to mount a leukocyte response similar to that of reproductively normal mares. However, Katila further suggested that, while the inflammation resolves within 48 hours in reproductively normal mares, DUC mares would have a more prolonged presence of neutrophils in their uteri. This was not supported by the results of the second experiment in the present study. The number
of reproductively normal and DUC mares that cleared the endometritis were similar, and the cellular response in terms of leukocyte numbers was not different. Further evidence of the lack of difference in the duration or magnitude of the inflammatory response between groups of mares after insemination is provided by the studies of Nikolakopoulos and Watson (1997) and Maloufi et al. (2002).

Nikolakopoulos and Watson (2000) reported that a higher number of spermatozoa resulted in a lower residual endometritis 48 hours after insemination. They suggested that the greater insult represented by infusion of 10 times more spermatozoa in the same volume resulted in a stronger acute inflammation with release of prostaglandin F$_{2\alpha}$ and concomitant uterine contractions that led to earlier clearance of the endometritis. Kotilainen et al. (1994) evaluated this acute inflammatory response and concluded that the intensity of the neutrophil reaction depended on concentration and/or volume of the inseminate. Based on those reports, the inflammatory response 24 hours after hysteroscopic insemination with five million spermatozoa in 0.5 mL (10 million/mL) compared with uterine body insemination with one billion spermatozoa in 20 mL (50 million/mL) should have been lower based on the lower concentration or the lower number of spermatozoa. However, Nikolakopoulos and Watson (2000) also suggested that a lower inseminate volume could result in a higher inflammatory response since low volumes may not be expelled from the uterus as efficiently as larger volumes. No differences in leukocyte numbers between insemination methods were found in this study at either 24 hours or 48 hours after insemination.

Sham hysteroscopies without semen deposition also induced endometritis. Neutrophil migration to the uterine lumen is induced by a large number of physical stimuli (Bergman and de Kruif, 1997; Martin et al., 1988; Williamson et al., 1987). Air insufflation during hysteroscopy together with uterine manipulation during the passage of the endoscope up to the uterotubal junction may have caused enough irritation and neutrophil migration to obscure any beneficial effect of depositing lower numbers of spermatozoa. Although this confirms that the procedure is inflammatory by nature, the endometritis induced by hysteroscopic insemination was not worse than that caused by uterine body inseminations either at 24 hours or 48 hours.

In conclusion, uterine endoscopy induces an inflammatory response that is associated with the procedure itself so it shouldn’t be used with the intention of reducing the post-breeding inflammatory response in mares with delayed uterine clearance. However, the decreased
pregnancy rate seen in problem mares by other authors cannot be explained by differences in the inflammatory response. Hysteroscopic insemination did not result in a more intense cellular response and was not detrimental to fertility in the present experiments. Based on these results there is no contraindication to its use in mares with delayed uterine clearance. Since the length of the procedure affects the severity of the inflammation, it is possible to reduce the likelihood of inducing persistent mating-induced endometritis after hysteroscopic insemination by minimizing the duration of the hysteroscopies.
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