Morphometric determination of endometrial leukocyte migration
during different stages of the equine oestrous cycle

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I, David Gerber, do hereby declare that the experiments presented in this dissertation, entitled "Morphometric determination of endometrial leukocyte migration during different stages of the equine oestrous cycle" were conceived, planned and executed by myself and, apart from the normal guidance from my supervisor, I have received no assistance.

Neither the substance, nor any part of this dissertation has been submitted in the past, or is to be submitted in the future for a degree at this University.

This dissertation is presented in partial fulfilment of the requirements for the degree MMedVet (Gyn) in animal reproduction.

I hereby grant the University of Pretoria free license to reproduce this dissertation in part or as a whole, for the purpose of research or continuing education.

Signed .....................................................

Place .....................................................

Date .....................................................
ABSTRACT

Uterine defences against bacterial challenge are more efficient during oestrus than during dioestrus. The exact reasons and mechanisms responsible for this difference are, however, still incompletely understood. The leukocyte reaction is one of the defence mechanisms that has been cited as being able to respond better to a bacterial challenge during oestrus than during dioestrus. The aim of the present study was to test the hypothesis that the magnitude of endometrial leukocyte migration following the instillation of semen into the uterine lumen is greater during oestrus than during dioestrus.

Eight Nooitgedacht mares of normal fertility, aged between 8 and 16 years (11.5 ± 2.7; mean ± SD), were used in the study. Each mare received a different treatment during each of four oestrous cycles, with a rest cycle after each treatment. Two treatments were performed during dioestrus and two during oestrus. One treatment for each stage of the cycle was a control treatment without challenge to the endometrium. At time zero of challenged cycles a single aliquot of 13 ml raw semen, frozen-thawed without addition of any cryoprotectant or extender, was instilled into the uterus. An endometrial biopsy was taken 6 and 48 h after time zero and a swab for cytology and culture (if cytology was positive) was collected 48 and 120 h after time zero. An image analyzer was used to record the total number of cells, round cells, neutrophils and eosinophils per unit surface area of epithelium, stratum compactum (SC) and stratum spongiosum (SS). The relative number of round cells, neutrophils and eosinophils were expressed as proportions of the number of each cell type to the total number of cells. The use of an image analyser made the collection of quantitative data from histologic sections possible. However, the operator still had to make some critical decisions, namely to choose the field of the section for analysis and to assign individual cells to a chosen category. The total numbers of cells in the epithelium and the SS were greater during dioestrus than during oestrus, while no such difference could be demonstrated for the SC. The stage of the oestrous cycle had no meaningful influence on any other (measured or calculated) variable. During challenged cycles, absolute and relative numbers of neutrophils were significantly greater in the epithelium, SC and SS than during control cycles. There was an interaction (not always reaching significance) between treatment and time with regard to the absolute and relative numbers of neutrophils in epithelium and SS and round cells in the epithelium. Numbers of neutrophils and round cells were significantly higher 6 h after treatment than 48 h.
after treatment in challenged cycles, but did not differ during control cycles. During challenged cycles, the stage of the oestrous cycle when treatment occurred had no effect on the duration of the induced endometritis, the occurrence of positive cytology or culture results, or the type of bacteria that were cultured. Regardless of the stage of their cycles when they were challenged, all mares rid themselves of the opportunistic pathogens placed into the uterine lumen within one oestrous cycle.

The hypothesis was rejected and it is therefore concluded that the stage of the oestrous cycle did not influence the magnitude of the endometrial leukocyte response to a standardized challenge with semen in these reproductively sound mares. A similar study will be required to test whether this conclusion also holds true for mares that are susceptible to endometritis.
SAMEVATTING

Die baarmoeder se vermoë tot weerstand teen bakteriële uitdaging is beter tydens estrus as tydens diestrus. Die redes en mekanismes wat hierdie verskil onderlê word nog nie volledig begryp nie. Dit is aangetoon dat die leukosietreaksie een verdedigingsmeganisme is wat beter reageer teen bakteriële uitdaging tydens estrus as tydens diestrus. Die doel van hierdie studie was om die hipotese te teets dat endometriale leukosietmigrasie nadat semen in die baarmoederholte geplaas is groter is tydens estrus as tydens diestrus.

Agt normaal-vrugbare Nooitgedachtmerries van 8 tot 16 jaar (gemiddeld 11.5, SD 2.7 jaar) is gebruik. Elke merrie is gedurende elk van vier estrussiklusse aan 'n ander behandeling onderwerp met 'n siklus van rus na elke behandeling. Twee behandелings is tydens diestrus en twee gedurende estrus uitgevoer. Een behandeling tydens elke stadium van die siklus was 'n kontrolebehandeling sonder enige uitdaging tot die endometrium. Op tyd nul van uitgedaagde siklusse was 13 ml rou semen wat sonder enige verdunner of kriobeskermer bevries en ontndooi was in die baarmoederholte geplaas. Endometriale biopsies was 6 en 48 uur na tyd nul geneem, terwyl deppers vir sitologie en kweking (indien die sitologie positief was) 48 en 120 uur na tyd nul versamel is. Die aantal selle, rondeselle, neutrofiele en eosinofiele per eenheidsoppervlakte van die epiteel, stratum compactum (SC) en stratum spongiosum (SS) is m.b.v. 'n beeldanaliseerder bepaal. Die relatiewe aantal rondeselle, neutrofiele en eosinofiele is gedefinieer as die verhouding van die aantal van elke onderskeie selsoort tot die totale aantal selle. Alhoewel die beeldanaliseerder dit moontlik gemaak het om kwantitatiewe inligting van histologiese snitte te versamel moes die operator steeds deurslaggewende besluite neem, naamlik om die veld van die snit wat geanaliseer moes word te kies en om individuele selle aan die onderskeie kategorieë toe te wys.

Daar was meer selle in die epiteel en SS tydens diestrus as tydens estrus, maar geen verskil in die SC nie. Die stadium van die siklus het geen betekenisvolle effek op enige gemete of berekenende veranderlike gehad nie. Tydens uitgedaagde siklusse was die aantal en relatiewe aantal neutrofiele beduidend hoër in die epiteel, SC en SS as gedurende kontrolesiklusse. Daar was 'n interaksie (nie altyd beduidend nie) tussen behandeling en tyd m.b.t. die aantal en relatiewe aantal neutrofiele in die epiteel, SS en SC, asook rondeselle in die epiteel. Tydens uitgedaagde siklusse was die aantal neutrofiele en rondeselle 6 ure na behandeling beduidend hoër as 48 uur daarna, terwyl geen verskil tydens kontrolesiklusse voorgekom het nie. Tydens
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ABBREVIATIONS

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<tr>
<td>AI</td>
<td>artificial insemination</td>
</tr>
<tr>
<td>BM</td>
<td>basal membrane</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming units</td>
</tr>
<tr>
<td>Co</td>
<td>control cycles, i.e. oestrous cycles without challenging the endometrium</td>
</tr>
<tr>
<td>Di</td>
<td>dioestrus</td>
</tr>
<tr>
<td>Ec_mm</td>
<td>number of epithelial cells per mm epithelium counted on a histological section of 2 μm thickness</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>eosinophil granulocytes</td>
</tr>
<tr>
<td>Er_mm</td>
<td>number of round cells per mm epithelium counted on a histological section of 2 μm thickness</td>
</tr>
<tr>
<td>En_mm</td>
<td>number of neutrophils per mm epithelium counted on a histological section of 2 μm thickness</td>
</tr>
<tr>
<td>Er_100</td>
<td>number of round cells in the epithelium, expressed as percentage of the total number of cells, calculated from counts made on a histological section of 2 μm thickness. In the text the term &quot;percentage of round cells&quot; is used.</td>
</tr>
<tr>
<td>En_100</td>
<td>number of neutrophils in the epithelium, expressed as percentage of the total number of cells, calculated from counts made on a histological section of 2 μm thickness. In the text the term &quot;percentage of neutrophils&quot; is used.</td>
</tr>
<tr>
<td>HE</td>
<td>haematoxylin and eosin</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>neutrophil granulocytes / polymorphonuclear neutrophil leukocytes (PMN)</td>
</tr>
<tr>
<td>Oe</td>
<td>oestrus</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>Round cells</td>
<td>lymphocytes, plasma cells and macrophages</td>
</tr>
<tr>
<td>Rx</td>
<td>treatment cycles, i.e. oestrous cycles with irritation of the endometrium</td>
</tr>
</tbody>
</table>
SC  stratum compactum

SCfb_mm  number of cells that were not recognised as round cells, neutrophils or eosinophils, per mm² of stratum compactum counted on a histological section of 2 μm thickness. The vast majority of these cells are fibroblasts.

SCr_mm  number of round cells per mm² of stratum compactum counted on a histological section of 2 μm thickness.

SCn_mm  number of neutrophils per mm² of stratum compactum counted on a histological section of 2 μm thickness.

SCeo_mm  number of eosinophils per mm² of stratum compactum counted on a histological section of 2 μm thickness.

SCr_100  number of round cells per area of stratum compactum expressed as percentage of the total number of cells, calculated from counts made on a histological section of 2 μm thickness. In the text the term "percentage of round cells" is used.

SCn_100  number of neutrophils per area of stratum compactum expressed as percentage of the total number of cells, calculated from counts made on a histological section of 2 μm thickness. In the text the term "percentage of neutrophils" is used.

SCeo_100  number of eosinophils per area of stratum compactum expressed as percentage of the total number of cells, calculated from counts made on a histological section of 2 μm thickness. In the text the term "percentage of eosinophils" is used.

SD  standard deviation

SS  stratum spongiosum

SSfb_mm  number of cells that were not recognised as round cells, neutrophils or eosinophils, per mm² of stratum spongiosum counted on a histological section of 2 μm thickness. The vast majority of these cells are fibroblasts.

SSr_mm  number of round cells per mm² of stratum spongiosum counted on a histological section of 2 μm thickness.
SSn_mm number of neutrophils per mm$^2$ of stratum spongiosum counted on a histological section of 2 μm thickness.

SSeo_mm number of eosinophils per mm$^2$ of stratum spongiosum counted on a histological section of 2 μm thickness.

SSr_100 number of round cells per area of stratum spongiosum expressed as percentage of the total number of cells, calculated from counts made on a histological section of 2 μm thickness. In the text the term "percentage of round cells" is used.

SSn_100 number of neutrophils per area of stratum spongiosum expressed as percentage of the total number of cells, calculated from counts made on a histological section of 2 μm thickness. In the text the term "percentage of neutrophils" is used.

SSeo_100 number of eosinophils per area of stratum spongiosum expressed as percentage of the total number of cells, calculated from counts made on a histological section of 2 μm thickness. In the text the term "percentage of eosinophils" is used.

Time sampling time after challenge of the endometrium.
1. INTRODUCTION

Endometritis is the most common cause of infertility in the mare (Gerber and Volkmann 1995; Brendemuehl 2002). In spite of intensive research, the mare’s natural defence mechanisms against endometritis are still not fully understood. It is generally assumed that the uterine defence mechanisms are more efficient during oestrus than during dioestrus. The exact mechanisms responsible for this difference are, however, still unknown; and results of in vitro trials can often not be confirmed by in vivo studies. This may be one of the reasons for some contradictory results published in terms of uterine defence mechanisms in the mare. Different mechanisms are involved in uterine defence against microbial challenge. The three main defence mechanisms are mechanical clearance of the uterine lumen, cellular response to bacterial challenge and humoral response to antigenic challenge. These three mechanisms combined are very efficient in curing endometritis in normally fertile mares, but are unable to eliminate introduced bacteria in mares susceptible to endometritis.

A problem regarding much of the published information on cellular defence mechanisms is its lack of objectivity. Computerised image analysis is described as a means of collecting objective histological data (Gerstenberg 1994; Marchevsky et al. 1987; Vecht-Lifshitz and Ison 1992). Cell numbers counted on histological sections can provide absolute measurements of the cellular defence mechanisms in the endometrium of the mare. Sections analysed from biopsies taken at different stages of the oestrous cycle and before or after an endometrial challenge should show how these variables change in response to changes in hormonal status.

The aim of this study was to test the hypothesis that the magnitude of the leukocyte response (migration through the equine endometrium) of fertile mares after intra-uterine challenge with raw semen is greater during oestrus than during dioestrus.
2. **LITERATURE REVIEW**

2.1 **HISTOLOGY OF THE ENDOMETRIUM**

The technique of taking endometrial biopsies has been described some three decades ago (Doig 1980; Kenney 1978; Ricketts 1975a; Ricketts 1975b; Witherspoon et al. 1972), and has since become a routine procedure in equine practice.

2.1.1 **General endometrial histology**

The equine endometrium is non-deciduous. The endometrium forms 12-15 longitudinal folds. These folds have a core of gland-free connective tissue and are covered by the glandular endometrial mucosa. In a cross section through the uterus these endometrial folds form a series of radially arranged capillary spaces. A biopsy specimen usually consists exclusively of a portion of an endometrial fold. If the biopsy consists of the entire thickness of the fold one can see two endometrial layers separated by the glandless core of the fold. Occasionally a part of the inner circular muscle layer may be visible on a biopsy specimen (Kenney 1978).

The endometrium is the inner of 3 layers of the uterine wall (Figure 2.1), followed by the myometrium and the perimetrium. The endometrium does not contain any muscular tissue. The endometrium is made up of the luminal epithelium and the lamina propria. Less than half of the cuboidal to tall columnar epithelial cells are ciliated. The lamina propria contains numerous uterine glands and is composed of the stratum compactum (SC) and the stratum spongiosum (SS). The glands are derived from and connected to the luminal epithelium. The two strata are not distinctly separated, but their stromal cell density is different. The SC has a high cell density, contains numerous capillaries and the excretory ducts of the uterine glands. The SS is characterised by a low cell density, numerous blood vessels, capillaries and lymphatic vessels. It harbours the numerous branched tubular uterine glands (Kenney 1978).

During anoestrus the endometrium undergoes some degree of atrophy. The luminal epithelium is cuboidal (about 10 µm thick) and the cytoplasm of the epithelial cells is basophilic. The endometrium shows a high density of uterine glands and their ducts appear relatively straight with a low cuboidal epithelium. The glandular lumen often contains inspissated amorphous hyaline or granular eosinophilic material (Kenney 1978).
Figure 2.1: Diagram of the normal equine uterine wall, consisting of inner endometrium, myometrium and outer perimetrium. The myometrium has been reduced for illustrative purposes (from Kenney, 1978).

Lymphocytes are found in small numbers in normal SC and SS but are greatly increased in sites of chronic inflammation (Fawcett 1986a). Plasma cells can occur at any level of the endometrium but are most common in the upper layers of the SC. They indicate continuing presence of an antigen, which is usually presumed to be of microbial origin (Kenney 1978). Neutrophils are scarce in the normal endometrium. In the case of an acute inflammation neutrophils accumulate in large numbers at the site of irritation and marginate in venules of the SS towards the uterine lumen (Kenney 1978). Neutrophils are much more common in the uterine lumen than lymphocytes; even over areas of the epithelium or the SC which are infiltrated by lymphocytes. Lymphocytes appear to degenerate while migrating through the epithelium before reaching the uterine lumen (Kenney 1978). This was confirmed in a later study, where leukocyte migration into the uterine lumen was induced by intrauterine injection of recombinant human interleukin 8. More than 98% of cells flushed from the uterus were neutrophils (Engelke et al. 1999). Few eosinophils can be seen scattered in the endometrium, but they do appear most commonly in the SC. According to Kenney (1978) there is no specific event associated with the presence of eosinophils, except that they are more frequent in an inflamed endometrium during oestrus (compare chapter “2.2.2.2 Eosinophil granulocytes” on page 9). The most common macrophages in the endometrium are siderocytes (macrophages which have phagocytosed red blood cells). They are normally seen in great numbers during the first month after parturition (Kenney and Doig 1986).
2.1.2 Histology of the endometrium under the influence of ovarian steroids

In late oestrus and early dioestrus the luminal epithelium is cuboidal to low columnar and is ≤15-20 µm thick and then grows continuously to reach a thickness of 30-40 µm just before the onset of oestrus. The density of uterine glands increases during dioestrus due to a decrease in stromal oedema and due to an increase in tortuosity of the gland ducts. It is not known if there is a real increase in the number of gland branches (Kenney 1978). Under the influence of oestrogens the epithelium changes from cuboidal to high columnar. It reaches an average height of 20-30 µm during the oestrogen surge, but can reach up to 50 µm at the time of the oestrogen peak. Vacuolation in the basal third of the epithelial cells tends to correlate with oestrus, while it is a common feature in the rest of the cells for any stage of the cycle (Kenney 1978). In a controlled study basal vacuolation of epithelial cells was found throughout the oestrous cycle in mares after taking repeated biopsies and after uterine flushes with either saline or iodine (Olsen et al. 1992).

Under the influence of oestradiol the endometrium becomes oedematous. The oedema develops more rapidly in the stroma between glands than in the stroma between the branches of one particular gland. Therefore, gland nesting observed in pro-oestrus must be differentiated from the nesting as a consequence of endometrial fibrosis (Kenney 1978). Due to oedema cell and gland density per surface area is lower during oestrus than during dioestrus. Oedema without other changes can be caused by the collection of biopsies (Kenney 1978). The glandular lumen is greater during oestrus than during dioestrus (Hammond and Wodzicki 1941). The change in oedema during the oestrous period was accurately described in a study using repeated ultrasonography (Pelehach et al. 2002).

During oestrus neutrophils tend to marginate in venules of the SS towards the uterine lumen, but they do not normally migrate in large numbers through the epithelium or across the stroma. According to Kenney (1978) lymphocytes only rarely migrate through the luminal epithelium; this is contradictory to earlier work, where increased lymphocyte migration through the epithelium was described two days after the end of oestrus (Hammond and Wodzicki 1941).

Using immuno-histological techniques it was shown that oestrogen and progesterone receptor expression is synchronously induced by increasing levels of 17β-oestradiol (Aupperle et al. 1992).
2000; Watson et al. 1992). In contrast to these findings another study, measuring receptor DNA, showed that progesterone, but not oestrogen receptors were induced in the endometrium of ovariectomised pony mares within 12 hours after oestradiol injection (Pelehach et al. 2002). Oestrogen receptor expression was low in luminal epithelial cells and in the epithelium of gland ducts (Aupperle et al. 2000). Maximal oestrogen receptor expression in this study was reached in early dioestrus in glands and on the day of ovulation in stromal cells. Glandular progesterone receptors were maximally expressed in early dioestrus, declined in middle dioestrus and increased again slightly until oestrus. In stromal cells and vessels progesterone receptor expression was low in early dioestrus and maximal in late dioestrus (Aupperle et al. 2000).

2.2 UTERINE DEFENCE MECHANISMS

Hinrichs et al. (1992) injected 12 mares daily with 250 mg progesterone and infected them with *Pseudomonas aeruginosa*. All mares developed endometritis, but pseudomonas bacteria could only be isolated from 5 of the mares one week after inoculation. The other seven mares were infected with a variety of different bacteria. The continued presence of free intra-uterine fluid was deemed not to be a useful indication of inflammation, because fluid could have been retained after resolution of inflammation solely because of the persistence of progesterone induced suppression of the mechanisms needed for uterine clearance (Hinrichs et al. 1992).

Uterine defence mechanisms must restore a favourable environment in the uterine lumen, (which is contaminated during mating) before five to six days after ovulation when the embryo arrives in the uterus (LeBlanc et al. 2003). Any particles present in the uterine lumen have to be removed and any bacteria in the lumen or the endometrium must be destroyed. The three major components of the uterine defence mechanisms are mechanical clearance and cellular and humoral defence mechanisms. All three of these mechanisms function in conjunction with each other.

The literature review in this chapter focuses on the cellular defence mechanisms. Mechanical clearance and humoral defence mechanisms are only mentioned where they have a direct influence on cellular defence mechanisms.
2.2.1 Mechanical clearance of the uterus

During dioestrus myometrial and cervical musculature is contracted; the cervical lumen is closed and the mucus produced by the cervical mucosa is tenacious (Hammond and Wodzicki 1941). During oestrus myometrial and cervical musculature is relaxed; the cervical lumen is open and the mucus is thin, allowing sperm to pass through the cervix into the uterus. Leukocytes are numerous in cervical mucosa, epithelium and lumen (Hammond and Wodzicki 1941).

Numerous studies have shown that mechanical clearance of the uterus is an essential part of the uterine defences against infection. Mares susceptible to bacterial endometritis often have a reduced ability to mechanically clear the uterus (Pascoe 1992; Varner and Blanchard 1990).

Bacteria, charcoal and microspheres, the latter two as non-antigenic markers, were inoculated into the uteri of healthy mares. Mechanical clearance of charcoal and microspheres was slower in mares treated with progesterone than in mares treated with oestradiol and in untreated controls. In mares under the influence of oestradiol clearance started within one or two hours after inoculation (Evans et al. 1986). Radio-labelled semen was deposited in the uteri of oestrous mares and its subsequent movement followed by scintigraphy. Uterine contractions moved the semen from the uterine body to the horn tips within seconds and back again into the uterine body. The semen stayed significantly longer in the uterine body than in the uterine horns (Katila et al. 1998). Similar findings were described by Troedsson et al. (1998) who measured uterine myoelectrical activity by electromyography. Myoelectrical activity increased immediately following insemination and lasted for 0.5 h. Myometrial activity was directed from the cervix towards the oviduct and from the horn tips to the cervix. A second surge in myoelectrical activity, similar to activity following a bacterial inoculation in normal mares, was observed between 4 and 12 h after insemination. This activity was assumed to have been caused by a semen induced inflammatory reaction (Troedsson et al. 1998). It is likely that the physiological function of this second phase of myometrial activity is to remove any remaining semen from the uterus. Myoelectrical activity differed between reproductively sound mares and mares susceptible to endometritis after bacterial challenge. Healthy mares showed an immediate increase in myoelectrical activity, while susceptible mares responded with a 3 h delay (Troedsson et al. 1998). Assuming that this delay in myometrial response is also present after insemination it could explain the reduced number of
spermatozoa present in the oviducts of susceptible mares when compared to normal mares (Scott et al. 1995). A recent study tested the in vitro contractility of the myometrium in response to different stimuli in young, older healthy and older susceptible mares. An intrinsic contractile defect, downstream of intracellular Ca$$^{++}$$ release, was deemed to contribute to the reduced post breeding uterine contractility in mares with delayed uterine clearance (Rigby et al. 2001).

Alghamdi and Troedsson (2002) determined the nitric oxide levels in uterine flushes 13 hours after insemination. Mares susceptible to endometritis had significantly higher levels of nitric oxide in uterine flushes than normal mares. This was confirmed in another study, where uterine flushes and biopsies were taken 13 hours after insemination (Alghamdi et al. 2005). Nitric oxide levels in uterine flushes and expression of nitric oxide synthase in uterine biopsies were both higher in susceptible mares than in normal mares. Nitric oxide mediates smooth muscle relaxation; it might therefore reduce uterine contractility and clearance that are essential in resolving breeding induced endometritis within 36 hours after breeding. Such an effect could be either direct or through a nitric oxide associated pathway (Alghamdi et al. 2005).

2.2.2 Cellular defence mechanisms

An acute reaction or inflammation of the endometrium is characterised by a neutrophil infiltration, whereas lymphocytes dominate in chronic endometritis. Less often there may be a chronic change with a superimposed acute exacerbation. The numbers and locations of plasma cells, macrophages, eosinophils and mast cells probably depend on the nature, distribution and persistence of the stimulus (Kenney 1978).

2.2.2.1 Neutrophil granulocytes

When irritated, the uterus responds with an immediate and massive neutrophil influx. After a challenge of the endometrium with Streptococcus equi subsp. zooepidemicus intrauterine neutrophil concentration peaks around four hours after inoculation (Munyua et al. 1991). After an instillation of PBS or a solution of Escherichia coli derived lipopolysaccharides, neutrophil infiltration showed a biphasic influx, peaking at six and 24 hours after inoculation (Martin et al. 1988). The immediate neutrophil response was massive and independent of the
kind of irritation, but depending on the insult, the duration of neutrophil persistence was very different. If the irritation was not of bacterial origin, mast cells and round cells usually predominated after a few days (Olsen et al. 1992; Pascoe 1992). Neutrophils are fully differentiated and have no synthetic capacity; therefore they no longer need a nucleolus for assembly of ribosomal RNA. They are the first line of defence against invading microorganisms, which they avidly phagocytose. At the site of inflammation they migrate in great numbers through the walls of post-capillary venules and into the surrounding tissue (Fawcett 1986b). After instillation of 500 ml saline on day zero and two, mares showed an initial acute inflammatory response, followed by a more chronic reaction (Olsen et al. 1992). After intra-uterine instillations of 500 ml of a 1 % povidone iodine solution on days zero and two, mares developed such a severe reaction that the SC and the SS became indistinguishable. In some areas the reaction reached even the myometrium. The reaction was acute for 7-10 days, followed by a subsequent mononuclear reaction, predominated by macrophages, plasma cells and mast cells. After day 15 the number of eosinophils throughout the lamina propria increased (Olsen et al. 1992). This is in accordance with the described interaction of mast cells and neutrophils (Fawcett 1986a).

Bacteria, charcoal and microspheres were inoculated into the uteri of healthy mares. Mares treated with progesterone had higher numbers of bacteria and leukocytes than mares treated with oestradiol. A possible explanation for this finding is that the leukocyte mobilisation rate is lower in mares under the influence of progesterone than in mares under the influence of oestradiol. Bacteria would therefore have more time to multiply and in the end cause a higher neutrophil reaction than in oestradiol treated mares, which get rid of bacteria within a short time (Evans et al. 1986). In a second trial the same material was inoculated into mares’ uteri. A correlation between number of white blood cells and age of mares was found under the influence of oestradiol, but not under the influence of progesterone (Evans et al. 1987).

In flushes recovered six hours after breeding phagocytosed spermatozoa were occasionally seen inside neutrophils (Kotilainen et al. 1994).

Taking a uterine biopsy does not cause a massive inflammation. After collecting endometrial biopsies from control mares on days 0, 3, 5, 7, 10, 15, 20 and 30, the mean number of
neutrophils per surface area remained relatively stable over a trial period of 30 days (Olsen et al. 1992).

**2.2.2.2 Eosinophil granulocytes**

Eosinophils characterise the acute inflammation associated with pneumo-uterus (Kenney and Doig 1986; Slusher et al. 1984) and are inconsistently frequent in cases of fungal endometritis (Kenney and Doig 1986). About 2 weeks after iodine treatment, the number of eosinophils was increased throughout the lamina propria; they were still present 30 days after treatment at the end of the study (Olsen et al. 1992).

Conditions predisposing to pneumo-vagina were found in 22 out of 27 mares that had eosinophils in a uterine cytology and/or in a biopsy specimen. However, about as many mares, which also had conditions predisposing them to pneumo-vagina, did not have eosinophils in either of their cytology or biopsy specimens. An eosinophil reaction could be demonstrated within 24 hours after the induction of pneumo-uterus in 3 of 3 mares. Eosinophils were always accompanied by neutrophils (Slusher et al. 1984). This finding agrees with the description that acute inflammation of mares suffering from pneumo-uterus is associated with a simultaneous influx of eosinophils (Kenney and Doig 1986).

The role of eosinophils is not well understood. They do selectively ingest and destroy antigen-antibody complexes (Fawcett 1986a; Fawcett 1986b). Eosinophils appear to be attracted to sites where basophil granulocytes and mast cells abound, because these cells release chemotactic factors (Fawcett 1986b; Fawcett 1986a). The exact interaction between basophils and mast cells and eosinophils is not known; eosinophils may counteract inflammatory effects produced by histamine and other mediators secreted by basophils and mast cells (Fawcett 1986a; Fawcett 1986b).

**2.2.2.3 Round cells: Mast cells, Lymphocytes, Plasma cells and Macrophages**

The presence of round cells (lymphocytes, monocytes and plasma cells) in tissues indicates a chronic inflammatory process (Kenney 1978; Kenney and Doig 1986; Pascoe 1992).
Mast cells are sensitive sentinels, detecting foreign substances, and have thousands of IgE’s, produced by plasma cells, on their surface (Fawcett 1986b). Contact between an antigen and these IgE’s triggers the release of heparin, histamine, serotonin and other substances, which will initiate a local inflammatory reaction (Fawcett 1986b). Mast cells have a very low turnover rate; they can be detected for several months (Fawcett 1986b). Mast cells can be observed throughout the lamina propria, but are most commonly found in the sub-epithelial SC or around blood vessels (Kenney 1978). They cannot be reliably recognised in H&E-stained sections, but are easily visible in sections stained with metachromatic stains such as toluidine blue (Kenney 1978).

Lymphocytes are involved in the immuno-surveillance against bacteria and other antigenic foreign substances (Fawcett 1986a). The cell-mediated immune response was intensely researched over the last few years. Helper T-lymphocytes direct a specific immune response by reacting to and releasing cytokines and thereby promoting macrophage activation and antibody production (Rook and Blackwell 1998). There are more T-lymphocytes and MHC (major histocompatibility complex) Class II expressing cells in the SS than in the SC (Watson and Dixon 1993). Healthy mares in oestrus have more helper T-lymphocytes and cytotoxic T-lymphocytes in the uterine body than in the uterine horns (Tunón et al. 1999b). This T-cell distribution makes sense, because semen is deposited in the uterine body of mares (Tunón et al. 2000). Mares inseminated during oestrus have a significantly higher number of helper T-lymphocytes 6 h after insemination than before, indicating an early response to semen deposition not only by neutrophils, but also by cells of the lymphoid lineage (Tunón et al. 1999a; Tunón et al. 2000). While in one study helper T-lymphocyte numbers were still elevated 48 h after insemination (Tunón et al. 2000), this finding was in discrepancy with that of an earlier study (Tunón et al. 1999a).

Plasma cells are the principal producers of immunoglobulins. They arise from B-lymphocytes that have migrated from the blood stream into the tissues (Fawcett 1986a). Treatment of ovariectomised mares with oestradiol or progesterone did not influence the number of cells secreting IgA, IgG or IgM. Furthermore, mares with persistent endometritis did not have higher numbers of endometrial plasma cells than normal mares (Watson and Stokes 1988).
After the instillation of 500 ml saline on days zero and two, mares showed an initial acute inflammatory response, followed by a prolonged, chronic reaction. The concentration of round cells per surface area, demonstrated in sections of biopsies collected on days 0, 3, 5, 7, 10, 15, 20 and 30, was significantly higher than in controls (Olsen et al. 1992).

Macrophages are monocytes that left the blood stream and play a major role in the defence against invading microorganisms (Fawcett 1986b). Macrophages in a non-active state in connective tissue are called histiocytes. If activated, they phagocytose endogenous breakdown products, microorganisms and foreign materials, such as residues of drugs. Large numbers of macrophages indicate the presence of foreign materials, such as drugs, rather than the presence of microorganisms (Kenney and Doig 1986). Normal mares had more macrophages in the SS, especially in peri-glandular areas, than in the SC. Macrophages were absent in luminal and glandular epithelia. The number of endometrial macrophages did not differ between mares with endometritis and normal mares (Summerfield and Watson 1998).

2.2.2.4 Response to irritation with semen and semen diluents

The effects of natural breeding, artificial insemination with fresh and frozen semen, seminal plasma and different semen diluents on endometrial fluid of healthy mares were determined by Kotilainen et al. (1994). Concentrations of colony forming bacteria and of neutrophils were measured in uterine flushes that were performed 6 h after instillation. The number of colony forming bacteria did not differ between the control group (no uterine instillation at all) and the different treatment groups (p < 0.05). Sixteen percent of samples were bacteriologically positive, but growth was considered to be insignificant in all samples (fewer than 10 colony forming units (CFU) per 10 μl). In contrast, high numbers of neutrophils were counted in flushing fluids. The highest neutrophil counts were found in mares inseminated with frozen or concentrated fresh semen (38.3 ± 23.9 to 58.6 ± 19.8 x 10⁶/ml [mean ± SEM]). Supernatant from frozen semen induced a significantly milder neutrophil reaction (0.3 ± 0.2 10⁶/ml). The high neutrophil numbers after insemination with frozen semen could thus not be ascribed to enzyme release from spermatozoa during freezing and thawing, because supernatants from frozen semen elicited hardly any inflammatory reaction. An egg yolk and a skim milk extender provoked a very mild reaction of about the same level as the one from the supernatant of frozen semen. The severity of the reaction to frozen-thawed
seminal plasma (9.5 ± 5.9) was between that caused by semen extender and that from semen. Small volumes (10 ml) of highly concentrated semen provoked the greatest neutrophil response. The authors gave two possible explanations for this observation. Highly concentrated semen results in more contact between spermatozoa and endometrium, and inseminations with small volumes may result in less effective mechanical clearance of the uterus than inseminations with large volumes (Kotilainen et al. 1994). In a review Troedsson et al. (2001) state that semen itself has complement mediated chemotactic properties, and that a transient inflammation after breeding is therefore physiological and necessary to clear the uterus after mating. The post breeding endometritis is both, up- and down-regulated by seminal components.

2.2.3 Humoral defence mechanisms
Transient, mating induced uterine inflammation could be modulated by the presence of seminal plasma (Troedsson et al. 1998). Considering the low pregnancy rate with frozen-thawed semen (from which the seminal plasma had been removed before freezing) this could have some important implications (Troedsson et al. 1998). Both, chemotaxis and random migration of neutrophils are suppressed by seminal plasma. Spermatozoa do not have a direct chemotactic effect on neutrophils, but they appear to induce chemotaxis via complement activation (Troedsson et al. 1998). Cellular and humoral defence mechanisms of the uterus appear to be active in both, healthy and susceptible mares, but cannot be sustained in susceptible mares (LeBlanc et al. 1989).

2.2.4 Influence of ovarian steroids
The frequency of spontaneous myometrial activity bursts, measured by electromyography, was the same during oestrus and dioestrus, but the duration of each burst was longer and the intensity higher during oestrus than during dioestrus (Troedsson et al. 1993). In contrast, total time of uterine activity was higher during dioestrus than during oestrus. Synchronisation of electrical uterine activity at different sites of the uterus was more marked during oestrus than during dioestrus (Troedsson et al. 1993). Nikolakopoulos and Watson (2002) described similar findings based on ultrasonographic examinations. It appeared that direction and speed of uterine contractions rapidly changed during oestrus, but not during dioestrus. In dioestrus,
waves of contractions were not well recognisable; it was a uniform contraction extending over the entire uterus rather than peristaltic waves progressing along the uterus.

During oestrus *Streptococcus equi* subsp. *zooepidemicus* adhered significantly less to uterine epithelial cells than during dioestrus (Watson *et al.* 1988). The distribution of T-lymphocytes in the endometrium was similar in oestrus and in dioestrus, but the number of MHC Class II bearing cells (macrophages, lymphocytes, monocytes, dendritic cells, epithelial cells and endothelial cells) increased significantly in the luminal epithelium and tended to increase in the sub-epithelial layers during oestrus (Watson and Dixon 1993). The number of macrophage-like cells in the lamina propria of the endometrium did not differ between oestrous and dioestrous mares (Summerfield and Watson 1998).

The intrauterine pH of healthy mares is higher during oestrus (pH 7.2) than during dioestrus (pH 6.6). This could be important since *in vitro* phagocytic function was lower at pH 5.5, 6.5 and 8.5 than at pH 7.5 (Lyle 1991). Compared to oestradiol treated or control mares, ovariectomised mares on progesterone treatment tended to have lower titres of IgG and IgA in uterine secretions. The supernatant of endometrial cell cultures from ovariectomised mares on progesterone treatment tended to have lower titres of IgG and IgM and was less effective at opsonising *Streptococcus equi* subsp. *zooepidemicus in vitro* (Watson 1987). However, the rate of antibody synthesis was not affected by steroid injections. In contrast, Johnson *et al.* (1994) reported that neither immunoglobulin concentration nor hormone treatment had a direct effect on streptocidal activity of uterine fluid.

Oestrogens stimulate the reticulo-endothelial activity, in particular by increasing the phagocytic activity. Progesterone has a weaker stimulatory effect at low doses, and no effect at higher doses (Nicol *et al.* 1965). Progesterone treatment reduced the random migration of blood neutrophils and the phagocytic activity of uterine neutrophils (Asbury and Hansen 1987; Washburn *et al.* 1982; Watson *et al.* 1986). In contrast to the effect on peripheral neutrophils, oestradiol treatment inhibited the migration of uterine derived neutrophils after intra-uterine infusion of bacteria, while progesterone treatment stimulated their migration (Watson 1988). The phagocytic activity of uterine neutrophils of susceptible mares is not affected by the stage of the oestrous cycle (Asbury and Hansen 1987; LeBlanc *et al.* 1989).
2.3 HISTOLOGICAL ASSESSMENT OF EQUINE ENDOMETRIAL BIOPSY SPECIMENS

2.3.1 Subjective assessment

In most cases a single biopsy is representative of the entire endometrium (Brandt 1970; Bergman and Kenney 1975; Kenney 1975). Only in exceptional cases the classification according to the Kenney and Doig system (Kenney and Doig 1986) varies by one category between biopsies collected from the same mares (Blanchard et al. 1987; Waelchli and Winder 1989). In contrast to these findings Slusher et al. (1984) described a single biopsy as not being representative in cases with acute inflammatory processes. This was substantiated by the finding that in some cases where eosinophils were found on cytological examination they were absent in biopsies, a finding that led to the conclusion that eosinophils could be absent in one biopsy specimen, but be present in another of the same uterus (Slusher et al. 1984). In one study the histological category differed by 2 or 3 classes (as classified by Kenney and Doig; 1986) in 15% of cases if post mortem samples from the two horns and the uterine body were compared (Dybdal et al. 1990).

The description of endometrial abnormalities proposed by Kenney (Kenney 1978) is widely used (Table 2.1). In a first step the pattern of the lesion is described in respect of the overall distribution and in relation to the different anatomical structures. Then the type of cellular reaction is described. Finally the degree of inflammation or fibrosis is categorised.

Inflammatory foci occur most frequently in the SC, often involving the overlying epithelium, indicating that the route of bacterial invasion was from the lumen (Kenney 1978). Chronic inflammatory reactions can involve the SS as well as the SC or be restricted to the SS. There is no specific pattern of inflammation for any aerobic bacterium (Kenney 1978).

Histological findings were first classified into three categories (Kenney 1978), but later into four (Kenney and Doig 1986) to better correlate histological findings with the mares’ predicted ability to carry a foal to term. Mares in category I are considered to have an endometrium with a normal ability to carry a foal to term. The endometrium is neither hypoplastic nor atrophic. Any pathological changes are only slight and widely scattered. Category II mares have mild to moderate changes of the endometrium. This category is further subdivided into categories IIA and IIB which show mild or moderate endometrial
changes, respectively. Category III mares are those with severe pathological changes of the endometrium and only a small chance of carrying a foal to term. The breeding history of mares should be taken into account when assigning them to one of the described categories. A mare with scattered, mild fibrosis, for example, would qualify for group IIA if she had a foal during the last season. The same mare being barren for 2 years would, however, be classified as IIB (Kenney and Doig 1986).

Table 2.1: Description of endometrial pathological changes.

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>• Overall pattern of frequency</td>
<td>• Cell type</td>
<td>• Inflammation</td>
</tr>
<tr>
<td>Widespread</td>
<td>Polymorphonuclear (acute)</td>
<td>Small &lt; 120 µm</td>
</tr>
<tr>
<td>Scattered</td>
<td>Lymphocyte (chronic)</td>
<td>Moderate = 120-300 µm</td>
</tr>
<tr>
<td>Frequent</td>
<td>Macrophage</td>
<td>Large &gt; 300 µm</td>
</tr>
<tr>
<td>Moderately frequent</td>
<td>Siderocyte</td>
<td>Periglandular fibrosis</td>
</tr>
<tr>
<td>Infrequent (widely)</td>
<td>Plasmacyte</td>
<td>Mild = 1-3 layers</td>
</tr>
<tr>
<td>• Anatomic pattern</td>
<td>Eosinophil</td>
<td>Moderate = 4-10 layers</td>
</tr>
<tr>
<td>Stratum compactum</td>
<td>Mast cell</td>
<td>Severe ≥ 11 layers</td>
</tr>
<tr>
<td>Stratum spongiosum</td>
<td></td>
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<tr>
<td>Perivascular</td>
<td></td>
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<tr>
<td>Periglandular</td>
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<tr>
<td>Region of ducts</td>
<td></td>
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<tr>
<td>Midgland region</td>
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<tr>
<td>Basal gland region</td>
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Adapted from Kenney (1978).

This evaluation system is widely accepted and commonly used in practice and for research. Generally, there is a good correlation between each category and mares’ future foaling rates. The expected foaling rate for mares belonging to the categories I, IIA, IIB and III are 80-90 %, 50-80 %, 10-50 % or below 10 %, respectively (Kenney and Doig 1986). The prognostic value of an endometrial biopsy for the prediction of the future fertility of a mare was confirmed in numerous studies (de la Concha-Bermejillo and Kennedy 1982; Gordon and Sartin 1978; Held and Rohrbach 1991; Held and Rohrbach 1992; Kenney 1975; Schoon et al. 1992; Waelchli 1990).
2.3.2 Objective assessment of a biopsy specimen; quantitative microscopy

The need for a quantitative evaluation of histopathological findings has been expressed for many years; but it is unlikely that quantitative pathology or histology will replace the human being making a diagnosis. A computer will not usually be able to make a diagnosis on its own, but it is necessary to reach objectivity and reproducibility (Mariuzzi and Tosi 1986). It is well known that in subjective histological diagnoses the levels of intra- and inter-observer agreement are truly low (Gerstenberg 1994; Montironi et al. 1986; Ooms et al. 1983). Gerstenberg found that computerised interactive morphometry is a valid system to evaluate endometrial features in mares and that it can be used to describe the nature of changes and to monitor such changes over a period of time. A subjective evaluator tends to miss minor changes occurring concurrent with obvious pathological changes. In contrast, computerised interactive morphometry frequently identified and quantified more than one type of pathological change in a single histological section (Gerstenberg 1994).

Most objective systems used in histology to date are interactive morphometric systems that are connected to a computer that performs counting, measurement and processing tasks, but leaves the recognition of cells to a trained observer. Morphometric analysis in histology is applied in human pathology (Marchevsky et al. 1987), has been used to analyse the human endometrium (Johannisson et al. 1982) and has been applied in the evaluation of the uterus of cattle (Gonzalez et al. 1985). Strankmeyer (1993) described a method to objectively determine the stage of the oestrous cycle of mares by morphometric measurements of epithelial and glandular parameters.
3. MATERIALS AND METHODS

3.1 EXPERIMENTAL DESIGN

The trial was completed over a period of seven consecutive oestrous cycles of eight Nooitgedacht mares (description of mares in section 1.1 on page 21). Mares were identified by freeze-brand numbers on their necks; numbers were 4, 54, 69, 71, 89, 90, 131 and 138. Cycles 2, 4 and 6 were designated rest cycles, while the remaining 4 cycles (1, 3, 5 and 7) were used as challenge and control cycles. Each mare was exposed to four different treatments, of which two were control treatments without any challenge to the endometrium. Two treatments were performed during dioestrus and two during oestrus. One of the treatments during dioestrus and one during oestrus were control treatments, without any challenge to the endometrium. After each treatment the mares went through a rest-cycle before they were exposed to the next treatment (Table 3.1). The sequence of treatments was as follows: dioestrous challenge (Di/Rx), dioestrous control (Di/Co), oestrous challenge (Oe/Rx), oestrous control (Oe/Co). The starting treatment for each mare was assigned randomly; two mares started with each treatment (Table 3.1). Dioestrous treatments started between day three and five after ovulation and oestrous treatments between day two and four of behavioural oestrus.

Table 3.1: Sequence of the four treatments and allocation of mares to these treatments.

<table>
<thead>
<tr>
<th>Cycle number</th>
<th>dioestrous Rx (challenge-cycle)</th>
<th>dioestrous Co (control-cycle)</th>
<th>oestrous Rx (challenge-cycle)</th>
<th>oestrous Co (control-cycle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>69 &amp; 138</td>
<td>71 &amp; 4</td>
<td>54 &amp; 131</td>
<td>89 &amp; 90</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>rest-cycle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>89 &amp; 90</td>
<td>69 &amp; 138</td>
<td>71 &amp; 4</td>
<td>54 &amp; 131</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>rest-cycle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>54 &amp; 131</td>
<td>89 &amp; 90</td>
<td>69 &amp; 138</td>
<td>71 &amp; 4</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>rest-cycle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>71 &amp; 4</td>
<td>54 &amp; 131</td>
<td>89 &amp; 90</td>
<td>69 &amp; 138</td>
</tr>
</tbody>
</table>
During challenge-cycles, at time zero, an aliquot of 13 ml semen (semen used to challenge endometrium is described in section 3.4 on page 22) was instilled trans-cervically into the uterus with an AI pipette connected to a 20 ml syringe. For the two control treatments no procedure was performed at time zero. Six and 48 h after challenge an endometrial biopsy was taken from each mare (Kenney and Doig 1986) (Figure 3.1).

Prior to collecting a biopsy 48 h after time zero two guarded endometrial swabs were taken (Blanchard and et al. 1981) from the uterine horn contra-lateral to the proposed biopsy site. If the smear made from the second swab yielded any cytological suggestion of an endometritis (Lon et al. 1993) the first swab was submitted for aerobic and micro-aerophilic culture; if there was no evidence of an endometritis on the second swab the first one was discarded. A second set of two swabs was taken 120 hours after time zero and handled in the same way. If any bacteria were cultured another set of two swabs was taken during the dioestrous period of the mare’s subsequent rest-cycle and the animal was only exposed to the next treatment if there was no cytological evidence of endometritis (Figure 3.1).

![Figure 3.1 Treatments carried out on mares during challenge- (Rx) and control- (Co) cycles. The treatments were the same during dioestrus and during oestrus.](image)

<table>
<thead>
<tr>
<th>time (h)</th>
<th>treatment</th>
<th>control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Rx B</td>
<td>Co B</td>
</tr>
<tr>
<td>6</td>
<td>S (C) &amp; B</td>
<td>S (C) &amp; B</td>
</tr>
<tr>
<td>48</td>
<td>S (C)</td>
<td>S if C120 +</td>
</tr>
<tr>
<td>120</td>
<td>S if C120 +</td>
<td>S if C120 +</td>
</tr>
</tbody>
</table>

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Preparation of histological sections

Biopsies were spread out on a piece of paper with the myometrial side on the paper, exposing the endometrial surface. The paper was cut around the biopsy and placed with the biopsy facing downwards into a container containing 10% buffered (pH 7.4) formaldehyde solution. After fixation for at least 7 days biopsies were embedded in a plastic embedding medium,
Immuno-Bed™ (Electron Microscopy Sciences, Fort Washington, PA 19034, USA). Two \( \mu m \) sections were cut and stained with haematoxylin and eosin (Gerstenberg 1994).

### 3.2.2 Measured and calculated variables

In order to reduce bias, biopsies were marked randomly with numbers 1-64. The operator analysing the biopsy sections did not know which one of these numbers belonged to which treatment. Slides were first screened under 200 x magnification to control if the quality of each section was acceptable and to check if there were any sections with focal changes and/or artefacts. Areas with focal changes and areas with artefacts were marked with a permanent marker on the slide and excluded from the analysis.

Sections were then analysed under 400 x magnification, using a microscope equipped with a video camera that was connected to a computer. Parameters were captured and extruded to an Excel™ data sheet with the image analysis software Optimas™ 6.0 [Carl Zeiss (Pty) Ltd, PO Box 3003, Randburg 2125, South Africa]. A haemocytometer was used to calibrate the system in \( \mu m \) in both, the x- and the y-axis. The visible frame had a width of 297 \( \mu m \) and a height of 230 \( \mu m \). Cells were identified on the projected image on the computer screen and not directly through the microscope.

In order to minimize evaluator bias the epithelium was first analysed on all sections, then the stratum compactum (SC) and finally the stratum spongiosum (SS). Ten fields of each one of the three layers of the endometrium were analysed. The 10 fields were selected randomly, but more or less evenly distributed over the length of the biopsy section. To this end the microscope table was moved in steps of approximately one tenth of the length of the section without looking at the section under the microscope or on the monitor. If necessary, the position microscope table was adjusted a little to show a maximal possible area of the tissue layer that was to be analysed on the monitor. The image was then frozen and analysed (Figure 3.2).
3.2.2.1 Epithelium

Visible length of epithelium on the monitor and number of epithelial cells, as well as number of round cells and neutrophils migrating through the epithelium were counted and recorded from each one of ten fields.

Using the measured variables, the number of epithelial cells (Ec_mm) and the number of migrating round cells (Er_mm) and neutrophils (En_mm) per mm epithelium were calculated. Number of round cells and neutrophils were further expressed as percentage of total number of cells counted per unit of epithelial length (Er_100 and En_100).

3.2.2.2 Stratum compactum (SC) and stratum spongiosum (SS)

The same variables were measured for the SC and for the SS. The surface area of the region of interest (ROI) excluded blood vessels and uterine glands (Figure 3.3). Within each ROI the following variables were counted: round cells, neutrophils, eosinophils and other cells. The other cells were mainly fibroblasts, and a few cells that were not recognisable. The area of each ROI, measured in mm², and cell numbers in the ROI were exported into an Excel data sheet.
Figure 3.3: Image frozen in Optimas™ 6.0 of the stratum spongiosum (SS) of mare 138 during her dioestrus control cycle, 48 h after time zero (Di/Co/48h); magnification: 400x.

Top: PC image before analysis.

Bottom: same PC image with measured region of interest (ROI) surrounded by a red line; note that glands and blood vessels are excluded form the ROI; round cells are marked with a red +; neutrophils are marked with a red ×.

Using the measured variables the following parameters were calculated: number of round cells (SCr_mm, SSR_mm), neutrophils (SCn_mm, SSn_mm), eosinophils (SCeo_mm, SSEo_mm) and other cells (SCfb_mm, SSfb_mm) per mm² tissue, excluding glands and blood vessels; percentage of round cells (SCn_100, SSn_100), neutrophils (SCn_100, SSn_100) and eosinophils (SCeo_100, SSEo_100) of the total number of cells.
3.3 Mares used in the study

Eight Nooitgedacht horses (freeze brand no. 4, 54, 69, 71, 89, 90, 131 and 138) of the Faculty's teaching herd were used in the trial. Mares were between 8 and 16 years old (11.5 ± 2.7; mean ± SD) and their body mass varied between 346 and 482 kg (mean 418.0 ± 49.61). All mares were considered sound for breeding purposes, rectal palpation and ultrasonographic examination revealing no uterine pathology. Each mare became pregnant at least twice following the last five breeding attempts (by either natural mating or AI with fresh semen) that preceded the onset of the study. Mares no. 69, 90, 131 and 138 were lactating. Before the beginning of the study an endometrial swab for cytology was collected and evaluated from each mare to confirm that she was free of endometritis the onset of the study (Blanchard et al. 1981).

Mares were kept as a group in one of the Faculty's paddocks during the entire duration of the trial. They were fed *Eragrositis tef* hay and water *ad libitum*. Mares were teased with one of the Faculty's Nooitgedacht stallions (Hearn 2000) every morning for the entire duration of the study.

3.4 Collection and processing of semen used to challenge the endometrium

Three Nooitgedacht stallions, a breed originating from the Basotho pony, of the Faculty's teaching herd were used in the trial; they were 5, 9 and 14 years old and weighed 443, 459 and 439 kg respectively. They were housed individually in paddocks during the day and in stables at night. Each stallion received 1-2 kg of pelleted concentrate ration to maintain condition, and water and hay (*Eragrositis tef*) *ad libitum*.

Semen was collected into an artificial vagina three times per week, using an oestrous mare as a mount. After removing the gel fraction, semen was poured into a one litre glass bottle and stored at -18°C without addition of any semen diluent, antibiotic or cryoprotectant. Ejaculates were added to the bottle until a total volume of one litre of gel-free semen had been collected. Frozen semen was then thawed at room temperature, mixed well, divided into 13 ml aliquots and again stored at -18°C until used in the trial.
One of these aliquots was thawed and submitted for bacterial culture under aerobic and micro-aerophilic conditions. All bacteria were identified and colony counts were performed.

3.5 STATISTICAL ANALYSIS

The effect of the stage of the oestrous cycle (Di or Oe), treatment (Co or Rx) and time after time zero (6 h or 48 h) were tested by repeated measures ANOVA with stage of the oestrous cycle, treatment and sampling time as main effects and with mares as subjects. All interactions were included. The Geisser-Greenhouse $\varepsilon$-corrections were applied to determine the level of significance.

All statistical analyses were performed with the computer program NCSS 2001 (NCSS, 329 North 1000 East, Kazsville, Utah 84037, USA).
4. RESULTS

4.1 EPITHELIUM

Results of the cellular reaction of the epithelium to irritation of the endometrium are summarised in Table 4.1 and graphically presented in the Appendix, Figure 7.1 on page 48 to Figure 7.55 on page 57.

Table 4.1: Mean number and percentage of cells in the epithelium (E) depending on stage of the cycle (dioestrus or oestrus), treatment (control or challenge) and sampling time after treatment (6 or 48 hours); standard deviations (SD) of all values are written in brackets under the corresponding means.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cycle</th>
<th>Treatment</th>
<th>Time</th>
<th>Significant Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Di</td>
<td>Oe</td>
<td>Co</td>
<td>Rx</td>
</tr>
<tr>
<td>Ec_mm</td>
<td>194.2</td>
<td>147.4</td>
<td>173.6</td>
<td>168.0</td>
</tr>
<tr>
<td></td>
<td>(27.77)</td>
<td>(25.34)</td>
<td>(39.87)</td>
<td>(30.61)</td>
</tr>
<tr>
<td></td>
<td>***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Er_mm</td>
<td>7.7</td>
<td>6.6</td>
<td>6.8</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>(5.00)</td>
<td>(3.97)</td>
<td>(3.52)</td>
<td>(5.36)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>En_mm</td>
<td>12.3</td>
<td>11.7</td>
<td>4.2</td>
<td>19.8</td>
</tr>
<tr>
<td></td>
<td>(18.75)</td>
<td>(19.51)</td>
<td>(11.11)</td>
<td>(21.97)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Er_100</td>
<td>3.6</td>
<td>4.0</td>
<td>3.7</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>(2.13)</td>
<td>(2.25)</td>
<td>(1.82)</td>
<td>(2.53)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>En_100</td>
<td>5.3</td>
<td>6.1</td>
<td>2.1</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td>(7.67)</td>
<td>(8.99)</td>
<td>(3.99)</td>
<td>(9.81)</td>
</tr>
<tr>
<td></td>
<td>***</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Di dioestrus; Oe oestrus; Co control; Rx challenge; 6h 6 hours after treatment; 48h 48 hours after treatment; Ec epithelial cells; Er round cells migrating through the epithelium; En neutrophils migrating through the epithelium; _mm number of cells per mm length of the epithelium; _100 number of cells expressed as a proportion of the total number of cells per mm length of the epithelium. *** p<0.001; ** p<0.01; * p<0.05.
Epithelial cell numbers per mm were higher (p<0.001) during dioestrus (194.2 ± 27.77; mean ± SD) than during oestrus (147.4 ± 25.34) (Figure 7.1). Stage of the oestrous cycle had no influence on round cell (Figure 7.12 and Figure 7.34) and neutrophil (Figure 7.23 and Figure 7.45) migration through the epithelium. There was no significant interaction between stage of cycle and treatment or sampling time after treatment.

Neither stage of the cycle (Figure 7.12 and Figure 7.34), treatment (Figure 7.13 and Figure 7.35) nor sampling time after treatment (Figure 7.14 and Figure 7.36) on their own had an influence on the number of round cells migrating through the epithelium or on the number of these cells expressed as a percentage of the total number of cells. There was, however, a significant interaction between treatment and sampling time (p<0.05) on the number of migrating round cells (Figure 7.17). During control cycles the number of round cells was not different 6 h (6.3 ± 3.57) and 48 h (7.2 ± 3.53) after treatment. In treated cycles the number of round cells was higher 6 h (9.4 ± 4.62) after treatment than 48 h (5.7 ± 5.56) after treatment. The same effect was seen for the round cells expressed as a percentage of the total number of cells, but this interaction only tended towards significance (p=0.06; Figure 7.39).

During control cycles the total number of neutrophils migrating through the epithelium (4.2±11.11; Figure 7.24; p<0.01) and their percentage of the total number of cells (2.1 ± 3.99; Figure 7.46; p<0.001) was lower than during treated cycles (19.8 ± 21.97 and 9.4 ± 9.81). The sampling time after treatment also influenced the number and the percentage of neutrophils migrating through the epithelium (p<0.05). Six hours after treatment the total number (15.7 ± 22.27) and the percentage (7.4 ± 10.07) of neutrophils was higher than 48 h after treatment (8.3 ± 14.45 and 4.1 ± 5.73). Treatment and sampling time combined tended to have an interactive influence (p=0.08) on both, the number and the percentage of neutrophils migrating through the epithelium (Figure 7.27 and Figure 7.50). In control cycles number and percentage of neutrophils did not differ 6 h and 48 h after treatment, whereas in treated cycles they tended to be higher at 6 h after treatment than 48 h after treatment.
4.2 Stratum Compactum

Results of the cellular reaction of the stratum compactum to irritation of the endometrium are summarised in Table 4.2 and graphically presented in the Appendix, Figure 7.56 on page 58 to Figure 7.132 on page 71.

The stage of the oestrous cycle on its own had no influence on any one of the measured or calculated variables. There was, however, a significant interaction (p<0.05) between the stage of the oestrous cycle and the sampling time after irritation on the number of round cells per mm$^2$ of stratum compactum (SCR$_{\text{mm}}$). During dioestrus SCR$_{\text{mm}}$ was similar 6 h (712.1 ± 228.67) and 48 h (753.0 ± 445.87) after treatment, but during oestrus it was higher 6 h (716.8 ± 282.38) after treatment than 48 h (523.5 ± 240.11) after treatment (Figure 7.71).

Treatment had a significant influence on the number of neutrophils per mm$^2$ of stratum compactum (SCN$_{\text{mm}}$; p<0.01; Figure 7.79) and on the percentage of neutrophils (SCN$_{\text{100}}$; p<0.001; Figure 7.112). In control cycles SCN$_{\text{mm}}$ and SCN$_{\text{100}}$ were lower (143.9 ± 246.33 and 2.6 ± 3.47) than in treated cycles (638.3 ± 687.15 and 10.7 ± 9.20).

The time after treatment had no influence on any of the measured neutrophil variables.

While SCEO$_{\text{mm}}$ was almost twice as high during Oe than during Di, after treatment than during control cycles and at 48 h than at 6 h after treatment, these differences in absolute eosinophil numbers or eosinophil percentages of total cells were not statistically significant.
Table 4.2: Mean number and percentage of cells in the stratum compactum (SC) depending on stage of the cycle (dioestrus or oestrus), treatment (control or challenge) and sampling time after treatment (6 or 48 hours); standard deviations (SD) of all values are written in brackets under the corresponding means.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cycle</th>
<th>Treatment</th>
<th>Time</th>
<th>Significant interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Di</td>
<td>Oe</td>
<td>Co</td>
<td>Rx</td>
</tr>
<tr>
<td>SCfb_mm</td>
<td>4293.0 (1335.96)</td>
<td>4403.1 (1677.35)</td>
<td>4160.7 (1259.38)</td>
<td>4535.4 (1716.40)</td>
</tr>
<tr>
<td>SCr_mm</td>
<td>732.6 (349.18)</td>
<td>620.1 (275.91)</td>
<td>695.4 (357.39)</td>
<td>657.3 (275.87)</td>
</tr>
<tr>
<td>SCn_mm</td>
<td>339.7 (431.38)</td>
<td>442.5 (683.72)</td>
<td>143.9 (246.33)</td>
<td>638.3 (687.15)</td>
</tr>
<tr>
<td>SCEo_mm</td>
<td>9.8 (26.23)</td>
<td>18.5 (35.40)</td>
<td>9.3 (24.54)</td>
<td>19.0 (36.46)</td>
</tr>
<tr>
<td>SCr_100</td>
<td>13.9 (5.21)</td>
<td>12.0 (5.40)</td>
<td>13.9 (5.03)</td>
<td>12.0 (5.57)</td>
</tr>
<tr>
<td>SCn_100</td>
<td>5.9 (6.78)</td>
<td>7.4 (9.13)</td>
<td>2.6 (3.47)</td>
<td>10.7 (9.20)</td>
</tr>
<tr>
<td>SCEo_100</td>
<td>0.2 (0.46)</td>
<td>0.3 (0.62)</td>
<td>0.2 (0.48)</td>
<td>0.3 (0.61)</td>
</tr>
</tbody>
</table>

Di dioestrus; Oe oestrus; Co control; Rx challenge; 6h 6 hours after treatment; 48h 48 hours after treatment; SCfb cells not recognised as round cells, neutrophils or eosinophils, i.e. mainly fibroblasts; SCr round cells; SCn neutrophils; SCEo eosinophils; _mm number of cells per mm² of stratum compactum; _100 number of cells expressed as a proportion of the total number of cells per mm² of stratum compactum. *** p<0.001; ** p<0.01; * p<0.05.
4.3 **Stratum spongiosum**

Results of the cellular reaction of the stratum spongiosum to irritation of the endometrium are summarised in Table 4.3 and graphically presented in the Appendix, Figure 7.133 on page 72 to Figure 7.209 on page 85.

The stage of the oestrous cycle influenced the number of fibroblasts (includes all cells not recognised as round cells, neutrophils or eosinophils) per mm$^2$ of stratum spongiosum ($SS_{fb\_mm}$; $p<0.001$; Figure 7.133) and the number of round cells per mm$^2$ of stratum spongiosum ($SS_{r\_mm}$; $p<0.001$; Figure 7.144). $SS_{fb\_mm}$ and $SS_{r\_mm}$ were higher during dioestrus ($3572.2\pm1136.33$ and $268.8\pm86.43$) than during oestrus ($2387.8\pm1158.89$ and $182.0\pm82.98$). The percentage of these cells did not differ between Di and Oe.

The treatment had an effect on the total ($p<0.001$) and relative ($p<0.01$) number of neutrophils ($SS_{n\_mm}$ and $SS_{n\_100}$). In Co cycles number and percentage of neutrophils ($16.4\pm38.17$ and $0.5\pm1.16$) were lower than in Rx cycles ($118.9\pm137.55$ and $4.4\pm5.68$; Figure 7.156 and Figure 7.189).

The sampling time after treatment also influenced $SS_{n\_mm}$ ($p<0.05$; Figure 7.157) and $SS_{n\_100}$ ($p<0.05$; Figure 7.190). $SS_{n\_mm}$ and $SS_{n\_100}$ were higher 6 h after treatment ($95.8\pm147.04$ and $3.7\pm5.97$) than 48 h after treatment ($39.6\pm50.47$ and $1.2\pm1.64$).

Treatment and sampling time tended to interact on $SS_{n\_mm}$ ($p=0.08$; Figure 7.160) and on $SS_{n\_100}$ ($p=0.06$; Figure 7.193). $SS_{n\_mm}$ and $SS_{n\_100}$ during Co cycles were $4.5\pm6.14$ and $0.2\pm0.26$ 6 h after treatment and $28.4\pm51.66$ and $0.8\pm1.58$ 48 h after treatment. In Rx cycles $SS_{n\_mm}$ and $SS_{n\_100}$ were $187.1\pm163.88$ and $7.2\pm6.89$ 6 h after treatment and $50.8\pm48.24$ and $1.6\pm1.67$ 48 h after treatment.

Table 4.3: Mean number and percentage of cells in the stratum spongiosum (SS) depending on stage of the cycle (dioestrus or oestrus), treatment (control or challenge) and sampling time after treatment (6 or 48 hours); standard deviations (SD) of all values are written in brackets under the corresponding means.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cycle</th>
<th>Treatment</th>
<th>Time</th>
<th>Significant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Di</td>
<td>Oe</td>
<td>Co</td>
<td>Rx</td>
</tr>
<tr>
<td>----------------</td>
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<td>-----</td>
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<td>----</td>
</tr>
<tr>
<td><strong>SSfb_mm</strong></td>
<td>3572.2</td>
<td>2387.8</td>
<td>2982.8</td>
<td>2977.2</td>
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<tr>
<td><strong>SSr_mm</strong></td>
<td>268.8</td>
<td>182.0</td>
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<td>226.2</td>
</tr>
<tr>
<td><strong>SSn_mm</strong></td>
<td>83.8</td>
<td>51.6</td>
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<td><strong>SSn_100</strong></td>
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<td>7.3</td>
<td>7.3</td>
<td>7.1</td>
</tr>
<tr>
<td><strong>SSr_100</strong></td>
<td>2.6</td>
<td>2.3</td>
<td>0.5</td>
<td>4.4</td>
</tr>
<tr>
<td><strong>SSn_100</strong></td>
<td>0.3</td>
<td>0.8</td>
<td>0.7</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>SSn_100</strong></td>
<td>0.7</td>
<td>0.4</td>
<td>1.25</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Di dioestrus; Oe oestrus; Co control; Rx challenge; 6h 6 hours after treatment; 48h 48 hours after treatment; SSfb cells not recognised as round cells, neutrophils or eosinophils, i.e. mainly fibroblasts; SSr round cells; SSn neutrophils; SSeo eosinophils; _mm number of cells per mm² of stratum spongiosum; _100 number of cells expressed as a proportion of the total number of cells per mm² of stratum spongiosum. *** p<0.001; ** p<0.01; * p<0.05.

### 4.4 CYTOLOGY AND CULTURE RESULTS

#### 4.4.1 Bacterial culture and colony counts on semen

Six different bacteria were cultured from the pooled semen that was used to challenge the endometrium (Table 4.4). Five of these bacteria were staphylococci and one was
*Streptococcus equisimilis.* The former differed in colony morphology and were all coagulase negative. None of these bacteria were isolated from the endometrium of the mares during the study.

**Table 4.4: Bacteria types and colony forming units in semen used to challenge the endometrium.**

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Colony forming units per ml of semen</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcus equisimilis</em></td>
<td>$1 \times 10^4$</td>
</tr>
<tr>
<td>Staphylococcus spp</td>
<td>$4 \times 10^3$</td>
</tr>
<tr>
<td>Staphylococcus spp</td>
<td>$6 \times 10^3$</td>
</tr>
<tr>
<td>Staphylococcus spp</td>
<td>$6 \times 10^3$</td>
</tr>
<tr>
<td>Staphylococcus spp</td>
<td>$5 \times 10^4$</td>
</tr>
<tr>
<td>Staphylococcus spp</td>
<td>$7 \times 10^3$</td>
</tr>
</tbody>
</table>

**4.4.2 Cytology and culture results of the mares after the treatments**

In 27 of the 64 cases (8 mares, each swabbed twice in four cycles), where the cytology was indicative of an endometritis, an endometrial swab was submitted for culture. Culture results are listed in Table 4.5. None of the mares was treated. Thirteen of the 27 cultures yielded no bacterial growth, while 14 were positive. Of the positive cultures, 11 yielded *Streptococcus equi* subsp. *zooepidemicus*, in one case concurrent with *Pasteurella multocida*. This was the only case where a mixed culture was obtained.

Of the 27 samples in which the cytology was indicative of endometritis, 19 were taken during dioestrus and eight during oestrus; 12 of the 19 dioestrous and 2 of the 8 oestrous samples yielded bacterial growth. Ten of the samples were taken during control cycles and 17 during challenged cycles. Five of the ten samples taken during control cycles and 9 of the 17 taken during challenged cycles yielded positive culture results. Sixteen samples were taken 48 h after challenge (9 of which yielded positive cultures) and 11 were taken 120 h after challenge (5 of which yielded positive cultures).
Table 4.5: Culture results from guarded endometrial swabs of mares of which the endometrial cytology was indicative of endometritis.

<table>
<thead>
<tr>
<th>Mare</th>
<th>Di/Oe</th>
<th>Co/Rx</th>
<th>Time</th>
<th>Culture results</th>
</tr>
</thead>
<tbody>
<tr>
<td>54</td>
<td>Co</td>
<td>120</td>
<td>Pasteurella multocida</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rx</td>
<td>48</td>
<td>Streptococcus equi subsp. zooepidemicus and Pasteurella multocida</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Co</td>
<td>48</td>
<td>sterile</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rx</td>
<td>48</td>
<td>sterile</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rx</td>
<td>120</td>
<td>sterile</td>
<td></td>
</tr>
<tr>
<td>69</td>
<td>Co</td>
<td>48</td>
<td>Streptococcus equi subsp. zooepidemicus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rx</td>
<td>48</td>
<td>Actinobacillus equuli</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rx</td>
<td>120</td>
<td>sterile</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oe</td>
<td>Co</td>
<td>48 sterile</td>
<td></td>
</tr>
<tr>
<td>71</td>
<td>Di</td>
<td>Rx</td>
<td>120 sterile</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oe</td>
<td>Rx</td>
<td>48 Bacillus spp</td>
<td></td>
</tr>
<tr>
<td>81</td>
<td>Co</td>
<td>48</td>
<td>sterile</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rx</td>
<td>48</td>
<td>sterile</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rx</td>
<td>120</td>
<td>sterile</td>
<td></td>
</tr>
<tr>
<td>89</td>
<td>Di</td>
<td>Rx</td>
<td>120 Streptococcus equi subsp. zooepidemicus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oe</td>
<td>Co</td>
<td>120 sterile</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>Di</td>
<td>Co</td>
<td>48 Streptococcus equi subsp. zooepidemicus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rx</td>
<td>48 Streptococcus equi subsp. zooepidemicus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rx</td>
<td>120 Streptococcus equi subsp. zooepidemicus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oe</td>
<td>Rx</td>
<td>48 sterile</td>
<td></td>
</tr>
<tr>
<td>131</td>
<td>Di</td>
<td>Co</td>
<td>120 Streptococcus equi subsp. zooepidemicus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rx</td>
<td>48 Streptococcus equi subsp. zooepidemicus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oe</td>
<td>Rx</td>
<td>120 Streptococcus equi subsp. zooepidemicus</td>
<td></td>
</tr>
<tr>
<td>138</td>
<td>Co</td>
<td>48</td>
<td>Streptococcus equi subsp. zooepidemicus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rx</td>
<td>48</td>
<td>Streptococcus equi subsp. zooepidemicus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rx</td>
<td>120</td>
<td>sterile</td>
<td></td>
</tr>
</tbody>
</table>

Five of the 11 samples where cytology was indicative of endometritis 120 h after challenge were collected from mares in which the cytology was not indicative of endometritis at 48 h after challenge during the same cycle. In six cycles cytology was positive 48 h and 120 h after challenge. Only one of these cycles was a control cycle (mare 131, Di, Co). The same cycle was also the only one during which the culture was negative 48 h after challenge, but positive 120 h after challenge. In one case both cultures yielded no growth (mare 81, Di, Rx), and in
two cases the 48 h sample was positive, while the 120 h sample was negative (mare 69, Di, Rx and mare 138, Di, Rx).

In all 11 cases where the cytology was indicative of endometritis 120 h after challenge, another set of guarded endometrial swabs was taken from the mares during the dioestrous period of their subsequent rest cycles. In all of these cases the subsequent cytologies were not indicative of endometritis.
5. **DISCUSSION**

Without specific staining it is impossible to recognise the type of each and every cell on a histological section. Especially in sections of only 2 μm thickness (as they were used in the present study) there are numerous cells that cannot be identified with absolute certainty. It was impossible to differentiate consistently between different types of round cells. The ability of the evaluator to differentiate between cell types was further reduced, because cells were classified on a computer screen having a distinctly lower resolution than the microscope. The main goal of this study was to test whether or not there is a difference in the acute cellular response of the equine endometrium after challenge with semen between oestrus and dioestrus. Failure to differentiate between different round cell types was therefore not considered a major problem during the study. After challenging the endometrium, as it was done in this study, one would expect more of a neutrophil than a round cell reaction (Büchi *et al.* 1991; Kotilainen *et al.* 1994; Martin *et al.* 1988; Munyua *et al.* 1991; Pascoe 1992).

5.1 **CELLULAR RESPONSE OF THE ENDOMETRIUM TO A CHALLENGE WITH SEMEN**

Considering the oedema during oestrus it can be expected that the number of cells per surface area, or per length in the case of the epithelium, is smaller during oestrus than during dioestrus. This was the case for epithelium and SS only, whereas no difference in cell density was evident in the SC. This absence of a lower cell density in the SC during oestrus can be real or an artefact. If it were real it would confirm that the SC is less sensitive to oestradiol than the epithelium or the SS (Kenney 1978). Even if the cell density in the SC of individual mares is evaluated (Figure 7.62), there is not even a trend towards a significant difference between dioestrus and oestrus. Therefore, it seems unlikely that this finding is an artefact caused by a large SD. Previous reports also state that the epithelium and the area between glands are most sensitive to the effects of oestrogen (Kenney 1978; Kenney and Doig 1986). The complete lack of sensitivity of the stratum compactum to oestrogen as observed in the present study has, to the best of the author’s knowledge, not been demonstrated before and thus constitutes a new finding.

Contrary to our expectations, the stage of the oestrous cycle had no effect on the neutrophil response of the endometrium challenged by semen. For many years it has been accepted that oestrus in cows, just as in mares, is associated with greater efficiency of the uterine defence...
mechanisms than can be expected during dioestrus (Black et al. 1953; Rowson et al. 1953). Subandrio and Noakes (1997) found that, contrary to their expectations, the number of neutrophils migrating into the uterine lumen of cattle after irritation was higher during dioestrus than during oestrus. They suggested that a higher neutrophil migration rate during dioestrus might compensate for the previously described reduced phagocytic efficiency of neutrophils under the influence of progesterone.

Also, contrary to our expectations, the stage of the oestrous cycle had no effect on the number of round cells. There were, however, two exceptions that deserve further discussion. During dioestrus there were significantly more round cells in the stratum compactum than during oestrus. Since the total cell numbers during dioestrus were also higher than during oestrus, the round cell numbers relative to the total number of cells are therefore more meaningful than the absolute numbers counted per unit of surface area of tissue. The percentage of round cells (SSr_100) was similar during dioestrus and during oestrus. The only significant difference in cell numbers found in the SC was between the total number of round cells which was higher 6 h after challenge than at 48 h after challenge during oestrus (Figure 7.71). This observation is unlikely to be of any significance, because, once again, the relative number or round cells (Figure 7.104) did not differ between dioestrus and oestrus. The latter finding agrees with published data. Watson and Stokes (1998) reported that the number of plasma cells secreting immunoglobulins does not differ between progesterone and oestradiol treated mares. In normal, fertile mares the stage of the cycle had no influence on the activity of T- or B-lymphocytes (Watson and Dixon 1993; Watson and Thompson 1996). Similarly, the number of macrophage like cells in the lamina propria of the equine endometrium is not dependent on the stage of the oestrous cycle (Summerfield and Watson 1998).

Immunohistochemical studies on the equine endometrium showed that the concentration of free immunoglobulins and the number of immunoglobulin containing cells remained at a constant level throughout the oestrous cycle (Waelchli and Winder 1987). Furthermore, mares with persistent endometritis had increased numbers of immunoglobulin containing cells when compared to mares with a healthy genital tract (Waelchli and Winder 1991). From these and other findings it was concluded that antibody mediated uterine defence mechanisms are functional in susceptible mares and that the pathophysiology of susceptibility to persistent endometritis is caused by other factors (Troedsson 1999).
Further evidence for the absence of a significant difference in cellular response between dioestrus and oestrus can be drawn from the figures in which absolute or relative cell numbers are plotted against the three variables cycle, treatment and time. In all of these figures the first four series of data points reflect cell numbers during dioestrus and the second four series of data points reflect cell numbers during oestrus. In most cases the general patterns in dioestrus and oestrus measurements are very similar. The absolute (Figure 7.159) and the relative (Figure 7.192) number of neutrophils in the SS can be used as an example. Both figures show a massive increase of neutrophils during both, dioestrus and oestrus, 6 h after treatment.

Neutrophils migrating through the epithelium (Figure 7.23 and Figure 7.45) or into the SC (Figure 7.78 and Figure 7.111) were also not influenced by the stage of the oestrous cycle. It appears logical that the number of neutrophils 48 h after time zero was higher during dioestrus than during oestrus, because the closed cervix during dioestrus will have trapped the instilled semen inside the uterine lumen for a longer period of time, while the open cervix during oestrus allowed the antigenic material to escape much faster. In the present study the initial neutrophil response (at 6 h) was similar during dioestrus and oestrus. The figures suggest that the high number of neutrophils persisted longer during dioestrus than during oestrus, but none of the differences reached statistical significance. Whether or not the persistence is, indeed longer during dioestrus than during oestrus, needs to be tested on a larger group of mares. Should the difference prove to be statistically significant in a larger group of mares, it will not be due to a direct hormonal effect on neutrophil migration, but rather due to an indirect mechanical effect (during dioestrus the closed cervix traps the semen inside the uterus for longer than the open cervix does during oestrus; Katila 1996).

The finding derived from in vitro experiments, where progesterone significantly reduces migration of neutrophils (Blue et al. 1982; Watson et al. 1986) could not be confirmed with the present in vivo study. The difference between in vitro migratory activity and in vivo susceptibility to infection was shown before (Watson 1988). There appears to be a difference between in vitro experiments with neutrophils harvested from normal mares and neutrophils derived from susceptible mares. The stage of the cycle had no effect on the neutrophil migration in susceptible mares, whereas neutrophils from resistant mares showed higher migratory activity during oestrus than during dioestrus (Asbury and Hansen 1987).
According to Kenney (1978) there are more eosinophils in an inflamed, oestrous endometrium than in a normal uterus. A similar trend was found in the present study but, most likely due to the huge variation between mares, it was not statistically significant. It is unlikely that the tiny volume of air that was probably infused into the uteri together with semen was the cause of the increase in eosinophils. For such a response, as described by Slusher et al. (1984), a larger volume of air appears to be necessary.

The higher number of neutrophils in challenged cycles compared to control cycles is not surprising. Total and relative numbers of neutrophils in the SS were lower 6 h than 48 h after time zero in control cycles, but higher 6 h than 48 h after time zero in challenged cycles. In the control cycles the increase in neutrophil numbers was probably caused by the irritation of the endometrium by sampling (swabs and biopsies), while in treated cycles the initial irritation caused by the challenge was high initially and decreased over time. This is in agreement with the finding that the endometrial neutrophil concentration peaks 6 and 24 hours after bacterial challenge (Martin et al. 1988).

Based on the discussion presented in the preceding paragraphs it is therefore appropriate to reject the hypothesis that the magnitude of the endometrial leukocyte response to a challenge with raw semen is greater during oestrus than during dioestrus.

5.2 CYTOLOGY AND CULTURE AFTER IRRITATION

All the bacteria that were cultured in the semen that was used to challenge the endometrium have been described as normal, non-pathogenic bacteria that can be found on the penis, prepuce and in the smegma of healthy, fertile stallions (Braun 1986; Picket 1993; Slusher 1997). A normal flora of commensal bacteria on the penis and prepuce of stallions is necessary to prevent overgrowth of possible pathogenic bacteria, such as Pseudomonas aeruginosa, Klebsiella pneumoniae or Taylorella equigenitalis (Varner et al. 1991).

The bacteria isolated from mares in this study were neither, primary pathogens (Ricketts et al. 1993) nor introduced with the semen. All of the isolated bacteria can be part of the normal, commensal bacterial population of the caudal reproductive tract of mares. There were no
significant differences in the number of mares that were cytologically or bacteriologically positive between any of the evaluated criteria (stage of the cycle, treatment or time after challenge). The isolated bacteria were probably all part of the normal vaginal flora of the experimental mares and were most likely introduced during the transcervical procedures (instillation of semen, collection of swabs and/or biopsies). This interpretation is supported by the finding that some of the mares tested negative 48 h after challenge but positive 120 h after challenge. In these mares bacteria were most likely introduced during the harvesting of the endometrial biopsy 48 h after challenge. While all biopsy punches used in the trial were sterile, they were not guarded. It was therefore possible, even likely, that bacteria from the caudal reproductive tract were introduced into the uterus of at least some mares when the biopsy instruments were passed through their cervixes.

The fact that none of the mares showed any signs of endometritis during their rest cycles confirms the previously published statement that bacteria cannot easily invade the uterine lumen and the endometrium of healthy mares. Endometrial swabs obtained three days after ovulation from resistant mares that were inoculated with \( \text{Streptococcus equi} \) subsp. \( \text{zooepidemicus} \) before ovulation yielded no bacterial growth, while \( \text{Streptococcus equi} \) subsp. \( \text{zooepidemicus} \) was cultured from 67% and \( \text{E. coli} \) from 20% of susceptible mares treated in the same manner (LeBlanc et al. 1989).

The present findings are in agreement with results of 368 uterine swabs where no differences in cytology or bacterial culture could be ascribed to the stage of the cycle (oestrus vs. dioestrus) when the swabs were taken (Waelchli et al. 1993). Ferreira-Dias et al. (1994) reported that \( \text{Streptococcus equi} \) subsp. \( \text{zooepidemicus} \) was equally likely to adhere to the endometrium of healthy dioestrous and oestrous mares. While their findings agree with those of the present study, the results of the two studies should not be compared directly, because the bacteria infused into the uterus with the semen used in this study were all non-pathogenic.

### 5.3 Quantitative Image Analysis

There is a huge potential for the application of image analysis in biotechnology. For image analysis to become effective, it is, however, necessary that a system is found where computers recognise desired features automatically. Only this will allow for the objective analysis of
large areas of tissue within a reasonable amount of time, while removing most of the influence of an operator. The system used in the current study is impractical for general use, because not all cell types can be recognised and because it takes approximately eight hours to analyse a single biopsy section. The main applications of image analysis, such as DNA-sequencing, asbestos monitoring, cell counting and gunshot residue analysis, fulfil the above-mentioned criteria, where a computer recognises and measures automatically (Vecht-Lifshitz and Ison 1992). Gerstenberg concluded that computerised interactive morphometry is more consistent than subjective evaluation and that it can be used to quantify results (Gerstenberg 1994). In the author’s opinion the validity of quantitative morphometry of the endometrium still needs to be verified in a trial where two operators analyse the same biopsy specimens with the same analysis software. In all probability, a specific stain would be necessary for each cell type to reliably analyse and quantify large areas of biopsy specimens. At this stage stereological methods with linkage to a computer are used for the analysis of histological sections, and much effort is still required to combine this with an image analyser for semi-automatic measuring of dimensions and counting of particles (Schleicher and Zilles 1990; Vecht-Lifshitz and Ison 1992).

The failure of the stratum compactum to respond to changing oestrogen concentrations has not previously been mentioned in the literature, either because there were no changes observed during the oestrous cycle or, because the more outspoken effects on the luminal epithelium and stratum spongiosum caused observers to overlook the lack of changes in the stratum compactum. Either way, this new hitherto unrecognised feature lends support to Gerstenberg’s theory, stating that computer assisted analysis is more objective than subjective evaluation of tissue, because it is more likely to result in the detection of any minor features (Gerstenberg 1994).

Inter-observer variation can be substantial in interactive computerised morphometry. It is therefore suggested that special sampling rules should be tested to determine a method with minimal inter-observer variation (Collan et al. 1986). In addition to the formulation of sampling rules it appears to be essential that a system be developed that allows for a more reliable distinction between cell types in the section.
Gland density, height of surface epithelium and numbers of leukocytes are highly variable in
the normal equine endometrium (Kenney 1978; Kenney and Doig 1986; Leishman et al.
1982). Findings of the current study support the report by Leishman et al. (1982) that minor
pathological changes can be masked by this normal variation. Leishman’s conclusion, that
quantitative morphometry of the equine endometrium offers a technique to evaluate and
interpret endometrial biopsies by technicians without extensive interpretive skills can,
however, not be supported. Firstly, it takes a tremendous amount of time to assess a biopsy
morphometrically (approximately 8 hours per section in this study). Secondly, substantial
skills are essential to decide when to exclude or include specific areas of a section for
morphometric analysis. Such decisions can substantially influence the quantitative results and
hence the conclusions that are derived from the results. A fast interpretation by technicians
might be possible if specific staining techniques were used for each cell type of interest and if
the luminescence could be measured on an entire biopsy specimen. Such staining techniques
have, for example, been described for equine endometrial macrophages (Summerfield and
Watson 1998).

Even though it still takes a skilled person to analyse equine endometrial biopsies by
interactive morphometry, the technique may be very useful for the objective determination of
normal variation in this tissue. This approach has been applied successfully to the human
endometrium (Johannisson et al. 1982).

5.4 GENERAL CONCLUSIONS

The stage of the oestrous cycle does not influence the magnitude of leukocyte migration
through the endometrium after the latter has been challenged with frozen-thawed, raw semen.

Mares not susceptible to endometritis could rid themselves of opportunistic bacterial
pathogens that were placed into the uterine lumen within one oestrous cycle, regardless of the
stage of the cycle when the bacteria were introduced.

To evaluate equine endometrial biopsy sections it is, at this stage, not possible to replace a
well-trained operator by a technician using interactive morphometry. An operator using an
image analyser still has to make essential decisions by selecting or excluding fields for
analysis and it is not possible to reliably distinguish between all cells types on conventionally stained sections. The use of image analysis is thus likely to remain limited to research applications where the procurement of objective data is essential when searching for answers to specific questions. Even for such applications it would be desirable to confirm the objectivity by testing for the repeatability and inter-observer variation of data obtained by image analysis.
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7. APPENDIX

Figure 7.1: Number of epithelial cells per mm of epithelium (Ec_mm) during dioestrus (Di) and oestrus (Oe).

Figure 7.2: Number of epithelial cells per mm of epithelium (Ec_mm) in control (Co) and treatment (Rx) cycles.

Figure 7.3: Number of epithelial cells per mm of epithelium (Ec_mm) 6 and 48 h after treatment.

Figure 7.4: Number of epithelial cells per mm of epithelium (Ec_mm) during dioestrus (Di) and oestrus (Oe) in control (Co) and treatment (Rx) cycles.

Figure 7.5: Number of epithelial cells per mm of epithelium (Ec_mm) during dioestrus (Di) and oestrus (Oe) 6 and 48 h after treatment.

Figure 7.6: Number of epithelial cells per mm of epithelium (Ec_mm) during control (Co) and treatment (Rx) cycles 6 and 48 h after treatment.
Figure 7.7: Number of epithelial cells per mm of epithelium (Ec_mm) for individual mares during dioestrus (Di) and oestrus (Oe).

Figure 7.8: Number of epithelial cells per mm of epithelium (Ec_mm) for individual mares during control (Co) and treatment (Rx) cycles.

Figure 7.9: Number of epithelial cells per mm of epithelium (Ec_mm) for individual mares 6 and 48 h after treatment.

Figure 7.10: Number of epithelial cells per mm of epithelium (Ec_mm) for 8 mares.

Figure 7.11: Influence of stage of cycle (Di or oe), treatment (Co or Rx) and time after treatment (6 or 48 h) on the number of epithelial cells per mm of epithelium (Ec_mm) of individual mares.
Figure 7.12: Number of round cells per mm of epithelium (Er_mm) during dioestrus (Di) and oestrus (Oe).

Figure 7.13: Number of round cells per mm of epithelium (Er_mm) in control (Co) and treatment (Rx) cycles.

Figure 7.14: Number of round cells per mm of epithelium (Er_mm) 6 and 48 h after treatment.

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Figure 7.198: Influence of stage of cycle (Di or oe), treatment (Co or Rx) and time after treatment (6 or 48 h) on the number of neutrophils per mm² of stratum spongiosum (SSn_100) of individual mares.
Figure 7.199: Number of eosinophils as a percentage of total cells per mm² of stratum spongiosum (SSeo_100) during dioestrus (Di) and oestrus (Oe).

Figure 7.200: Number of eosinophils as a percentage of total cells per mm² of stratum spongiosum (SSeo_100) in control (Co) and treatment (Rx) cycles.

Figure 7.201: Number of eosinophils as a percentage of total cells per mm² of stratum spongiosum (SSeo_100) 6 and 48 h after treatment.

Figure 7.202: Number of eosinophils as a percentage of total cells per mm² of stratum spongiosum (SSeo_100) during dioestrus (Di) and oestrus (Oe) in control (Co) and treatment (Rx) cycles.

Figure 7.203: Number of eosinophils as a percentage of total cells per mm² of stratum spongiosum (SSeo_100) during dioestrus (Di) and oestrus (Oe) 6 and 48 h after treatment.

Figure 7.204: Number of eosinophils as a percentage of total cells per mm² of stratum spongiosum (SSeo_100) during control (Co) and treatment (Rx) cycles 6 and 48 h after treatment.
Figure 7.205: Number of eosinophils as a percentage of total cells per mm² of stratum spongiosum (SSeo_100) for individual mares during dioestrus (Di) and oestrus (Oe).

Figure 7.206: Number of eosinophils as a percentage of total cells per mm² of stratum spongiosum (SSeo_100) for individual mares during control (Co) and treatment (Rx) cycles.

Figure 7.207: Number of eosinophils as a percentage of total cells per mm² of stratum spongiosum (SSeo_100) for individual mares 6 and 48 h after treatment.

Figure 7.208: Number of eosinophils as a percentage of total cells per mm² of stratum spongiosum (SSeo_100) for 8 mares.

Figure 7.209: Influence of stage of cycle (Di or oe), treatment (Co or Rx) and time after treatment (6 or 48 h) on the number of eosinophils as a percentage of total cells per mm² of stratum spongiosum (SSeo_mm) of individual mares.