CELLULAR AND MOLECULAR GENESIS OF THE CERVICAL-UTERINE POST-INSEMINATION INFLAMMATORY RESPONSE IN THE EWE

Thesis submitted by

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James Cook University

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DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institute of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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STATEMENT ON THE CONTRIBUTION OF OTHERS

Professor Phillip Summers and Associate Professor Natkunam Ketheesan provided supervision for the research carried out in this thesis and were co-authors on all papers resulting from this thesis.

A stipend was provided by the School of Veterinary and Biomedical Sciences for the duration of the research candidature (3.5 years). Project costs were met from a research expenditure allocation associated with the stipend and IRA and Reproduction Service accounts held by Professor Summers.

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DECLARATION ON ETHICS & BIOSAFETY

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *Joint NHMRC/AVCC Statement and Guidelines on Research Practice* and the *James Cook University Statement and Guidelines on Research Practice* (2001). All research procedures reported in this thesis received the approval of the James Cook University Ethics Review Committee (Animal Ethics Number A 846_03) and the James Cook University Biosafety Committee (Biosafety Number PPR11_06).

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ABSTRACT

Insemination induces an inflammatory response in the cervix and endometrium, and there is increasing evidence that it plays an important role in the establishment of successful pregnancy. Several different leukocytes and cytokines are involved in the response, but the range of mediators involved, the progression of events and their significance in terms of reproductive success are uncertain. This study examined the temporal development of the inflammatory response in the reproductive tract of the ewe following mating, investigated the components of ram semen responsible and compared the reaction in the oestrogen and progesterone dominated reproductive tract. The central hypothesis of the study was that components of semen induce an inflammatory reaction in the female reproductive tract via the synthesis and secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-8 (IL-8) from endometrial and cervical epithelial cells.

In initial studies, reproductive tracts were collected from ewes at three, six, 18, 24 and 48 hours following mating or the onset of oestrus without mating. Leukocytes in the vagina, cervix and uterus were identified and quantified. In non-mated ewes, numbers of neutrophils and mast cells in the uterus were highest at three hours then declined by 48 hours following the detection of oestrus, whereas the number of macrophages increased in most tissues. Luminal macrophages were highest at 18-24 hours but had declined by 48 hours after oestrus. Neutrophil and macrophage numbers increased in the posterior cervical and uterine tissues following mating and neutrophils also increased in the cervical and uterine lumen. In uterine tissues numbers of neutrophils peaked at six hours and macrophages at 18-24 hours after mating. The number of mast cells initially decreased after mating but then increased by 48 hours, whereas the number of eosinophils remained constant. It was concluded that leukocyte populations in the reproductive tract of the ewe are influenced by ovarian steroid hormones, and changes after mating vary between different sites. Numbers of neutrophils and macrophages increased in response to mating whereas mast cells decreased and the number of eosinophils did not change.

Tissues and luminal fluid from the reproductive tract of mated and non-mated ewes were also examined for the presence of GM-CSF and IL-8 using monoclonal and polyclonal sheep-specific antibodies. Both GM-CSF and IL-8 were detected in luminal and glandular endometrial epithelium, to a lesser extent in cervical epithelium and neither in vaginal epithelium. There were higher luminal concentrations of GM-CSF at all sites in the reproductive tract of mated compared with control ewes, and the vaginal lumen contained the highest concentration of IL-8 compared with all other sites irrespective of mating status. These findings suggested that an increase in GM-CSF following mating may contribute to the influx of leukocytes which occurs at this time, but the changes in IL-8 following mating were not clear.

Semen was collected from each of seven rams on three separate occasions by electro-ejaculation and examined for the presence of cytokines. Transforming growth factor-beta 1 (TGF-β1) was present in all samples of ram seminal plasma, but neither GM-CSF nor IL-8 were found. Concentrations of seminal TGF-β1 ranged between 0.12 and 1.5 ng/ml and approximately 90% was present in a latent form. It is still not certain what role TGF-β1 has in contributing to the inflammatory reaction to semen.

Oestrous and luteal stage ewes were anaesthetised and their uterus surgically ligated into five sections. Whole semen, washed spermatozoa, seminal plasma, modified Tyrode's albumin-lactate-pyruvate (TALP) and normal saline were injected into the ligated uterine sections and the reproductive tracts collected 22 hours later. Selected ewes had antibiotics added to the treatments. Whole semen, seminal plasma and spermatozoa caused an increase in neutrophil numbers in uterine tissues and increased luminal IL-8, but including antibiotics in treatments reduced this response. An increase in luminal GM-CSF occurred in response to spermatozoa and whole semen but only when antibiotics were not used. Eosinophils increased in the mid- and deep endometrial stroma when antibiotics were not used, whereas fewer mast cells were present in the deep endometrial stroma after all treatments and numbers were reduced further in the presence of antibiotics. More macrophages were present in uterine tissues in response to whole semen, spermatozoa and seminal plasma than other treatments and antibiotics reduced this response. These results indicate that spermatozoa, seminal plasma and possibly bacteria or bacterial products such as

lipopolysaccharide (LPS) all contribute to leukocyte and cytokine changes during the post-insemination inflammatory response in the uterus of the ewe.

Neutrophils, GM-CSF and IL-8 underwent greater increases in response to insemination at oestrus compared to during the luteal phase, whereas numbers of eosinophils were higher at oestrus but unaffected by insemination. Total macrophage numbers were not influenced by the stage of the oestrous cycle, however their distribution within uterine tissues was affected, with more located in the superficial endometrial stroma at oestrus. These results suggest that leukocytes, GM-CSF and IL-8 in the ovine uterus are under the influence of ovarian hormones and oestrogen enhances and/or progesterone suppresses aspects of the post-insemination inflammatory response in the ewe.

It was concluded that the post-insemination inflammatory response in the reproductive tract of the ewe involves an increase in numbers of neutrophils and macrophages and a reduction or degranulation of mast cells. These changes are likely to be driven, at least in part, by the concurrent increase in GM-CSF and IL-8 which occurs in response to a combination of spermatozoa, seminal plasma and bacteria or bacterial products. These leukocyte and cytokine changes may be involved in preparing the ovine endometrium for pregnancy.

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Figure 7.11 Ranked staining intensity of IL-8 in uterine tissues from oestrous and luteal ewes 22 hours after injection of various treatments. Data are presented as mean + SEM; oestrus (n=9), luteal (n=7). Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride. **161**

Figure 7.12 IL-8 in luminal fluid of luteal ewes 22 hours after injection of various treatments into the lumen. Data are presented as mean ± SEM; no antibiotics (n=4), antibiotics added to treatments (n=3). [a] P <0.01 compared to TALP; [b] P <0.01 compared to saline. Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride. **162**

Figure 7.13 IL-8 in luminal fluid of oestrous and luteal ewes 22 hours after injection of various treatments without antibiotics into the lumen. Data are presented as mean + SEM; oestrus (n=6), luteal (n=4). [a] P <0.01 compared to luteal ewes. Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride. **162**

LIST OF ABBREVIATIONS

AC	Anterior cervix
AEC	3-amino-9-ethylcarbazole
ANOVA	Analysis of variance
BSA	Bovine serum albumin
BU	Body of the uterus
C	Celcius
CA	Contralateral anterior uterine horn
CD	Cluster differentiation
CIDR	Controlled intravaginal drug releasing device
CM	Contralateral mid-uterine horn
CSF	Colony-stimulating factor
D	Deep endometrial stroma
DAB	3-3 diaminobenzadine tetrahydrochloride
DPBS	Dubecco's phosphate buffered saline
DPX	Dibutylphthalate polystyrene xylene
ELISA	Enzyme linked immunosorbent assay
g	Gig (reciprocal centrifugal force)
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H&E	Haematoxylin & Eosin
HPF	High power field
HRP	Horse-radish peroxidase
IA	Ipsilateral anterior uterine horn
IFN	Interferon
IL	Interleukin
IM	Ipsilateral mid-uterine horn
IVF	*in vitro* fertilisation
KC	Cytokine induced neutrophil chemoattractant
LA	Left anterior uterine horn
LAP	Latency associated peptide
LIF	Leukaemia inhibitory factor
LM	Left mid-uterine horn
M	Mid-endometrial stroma
MC	Mid-cervix
MCAF	Monocyte chemotactic and activating factor
MCP	Monocyte chemotactic protein
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
OC	Cervical ostium
OCT	Optimum cutting temperature
OD	Optical density
OS	Ostium
PBS	Phosphate buffered saline
PC	Posterior cervix
PCR	Polymerase chain reaction
PGE	Prostaglandin of the E series
RA	Right anterior uterine horn
RANTES	Regulated upon activation normal T-cell expressed and secreted

RM	Right mid-uterine horn
RT	Room temperature
S	Superficial endometrial stroma
SEM	Standard error of the mean
TALP	Tyrode's albumin-lactate-pyruvate
TBS	Tris buffered saline
TGF	Transforming growth factor
TMB	3,3',5,5'tetramethylbenzidine
TNF	Tumor necrosis factor
V	Vagina

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CHAPTER 1
GENERAL INTRODUCTION

1.1 Background

Insemination induces a rapid and transient inflammatory reaction in the female reproductive tract. This reaction involves an accumulation of leukocytes, mainly neutrophils and macrophages, in the endometrium, and the migration of these cells into the uterine and cervical lumen (De *et al.*. 1991; McMaster *et al.* 1992; Sanford *et al.* 1992). Aspects of the inflammatory reaction have been investigated in a number of species, but much remains unclear. Several different leukocytes and an array of associated cytokines appear to play important roles in the progression of the inflammatory response. It is likely that a complex network of intercellular communication is involved. However, the range of mediators involved, the sequence of events that occurs and the significance of the reaction in terms of reproductive success remain to be elucidated.

The inflammatory reaction to insemination is regarded as a normal physiological response, and there is increasing evidence that it plays an important role in the establishment of successful pregnancy. It has been postulated that the inflammatory reaction contributes to the development of the unique state of immunotolerance arising between mother and conceptus during pregnancy (Barratt *et al.* 1990; Robertson 2002). Possible mechanisms involved in this process are a focus for current research, but many questions remain unanswered. Conflicting evidence in the literature confuses the situation, and often more questions are generated than are answered. Species differences may account for some discrepancies in results, but further research is required for clarification.

Questions that need further investigation include the following: What triggers the post-insemination inflammatory reaction? What mechanisms are involved in the progression of the inflammatory reaction? Does the reaction differ between species? What is the purpose of the inflammatory reaction? Is it simply a mechanism to remove by phagocytosis excess or defective spermatozoa and micro-organisms in

semen? Does it aid the survival and improve the fertilising capacity of those spermatozoa not phagocytosed? Does it prime the uterus to receive the conceptus? Does it set up systems for immunological communication between the maternal immune system and the conceptus? Does it play a role in preventing an immunological response to sperm antigens and subsequent development of anti-sperm antibodies? Several studies have attempted to answer some of these questions, but the detailed events that occur and the implications for successful reproduction remain unclear.

Successful reproduction is not only necessary for species survival, but can be extremely important for individuals or groups within a species. For instance, humans highly value the ability to have their own children, and will often undergo prolonged programs of *in vitro* fertilisation in order to conceive. Farmers are continually searching for ways to improve reproductive performance of production animals such as cattle, sheep (Figure 1.1) and pigs, in order to maximise profits. Conservationists run captive breeding programs for endangered wildlife, in an attempt to preserve biodiversity. Reproductive dysfunction therefore has social, ethical, commercial and ecological repercussions.

Figure 1.1 A Merino ewe with her 3 day old lamb.

The cause of reproductive dysfunction is often unknown. Many cases of infertility may be due to aberrations in maternal immune function. It has been postulated that dysfunction in the cytokine/leukocyte networks involved in the post-insemination inflammatory reaction may lead to a breakdown in maternal immunotolerance to the conceptus, and thereby contribute to disorders such as implantation failure or recurrent spontaneous abortion (Bellinge *et al.* 1986; Thaler 1989; Robertson *et al.* 1997). Our current knowledge regarding the complex intercellular and immunological communication occurring after insemination and during the pre-implantation stage is insufficient. If we can improve our understanding of the processes that occur during this period, and the underlying mechanisms involved, we will be better able to formulate therapeutic strategies to overcome reproductive disorders that arise during this time.

Specific areas that need further clarification include the cell types involved, the changes in cell type and cellular distribution as the reaction progresses over time, and the cytokines involved and their specific functions. In addition, a causal link needs to be identified between the component(s) of semen and the mechanism(s) responsible for attracting leukocytes from the vasculature into interstitial tissue and the cervical and uterine lumen. Sheep were chosen as a model to investigate the post-insemination inflammatory response as they have a number of features that make them useful for these studies including:

(a) It is a species in which semen is deposited in the cranial vagina, similar to the site of semen deposition in humans.
(b) The adult female is large enough to perform surgical preparations of the uterus. Previous work in this laboratory has developed a surgical system for ligation of sections of the uterus for installation of components of semen (Hilla, 1999).
(c) Semen can be readily collected from rams for separation into various components.
(d) Well-defined systems are in place for the husbandry of sheep at James Cook University, and for manipulation of the oestrous cycle.

1.2 Working Hypothesis

The general working hypothesis of this research was: Components of ram semen induce an inflammatory reaction in the reproductive tract of the ewe via the synthesis and secretion of cytokines such as interleukin-8 (IL-8) and granulocyte-macrophage colony-stimulating factor (GM-CSF) from endometrial and cervical epithelial cells. This inflammatory reaction involves the accumulation of leukocytes in reproductive tissues and the migration of these cells across the epithelium into the lumen (Figure 1.2).

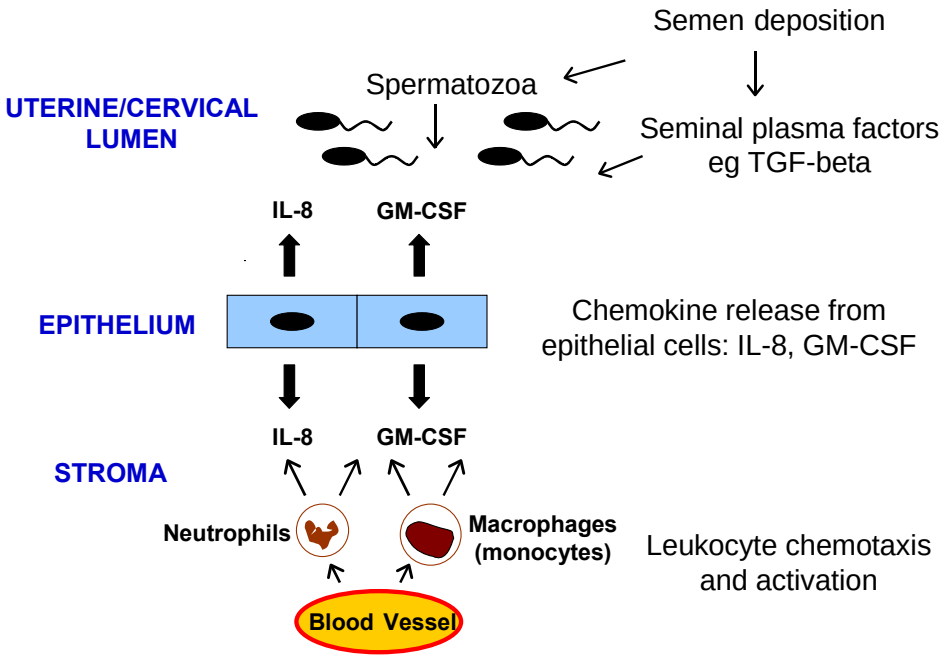

Figure 1.2 Hypothetical cytokine/leukocyte interactions following insemination in the ewe.

1.3 Aims and Objectives of the Research Project

The specific aims of the project were as follows:

1. Define the temporal development of the post-insemination inflammatory reaction in the cervix and uterus, the types of leukocytes involved and their distribution within tissues.
2. Identify and quantify cytokines (IL-8 and GM-CSF) in vaginal, cervical and uterine tissues and lumens.
3. Identify the component(s) of ram semen that induces the inflammatory reaction.
4. Identify the chemical link(s) between semen components and the mechanism for attracting and transferring leukocytes from blood vessels to the interstitial tissue and lumen (eg cytokines in ram seminal plasma).
5. Compare the inflammatory response in the oestrogen-dominated and progesterone-dominated uterus.

CHAPTER 2
LITERATURE REVIEW

2.1 Anatomy and Histology of the Ewe Reproductive Tract

2.1.1 Anatomy

The main structures of the female reproductive tract are the ovaries, oviducts, uterus, cervix, vagina and vulva. The ovaries are generally ovoid in shape and consist of an outer cortex and inner medulla. The cortex contains ovarian follicles at various stages of development and may also contain a corpus haemorrhagicum (recently ruptured follicle) or a corpus luteum, depending on the stage of the oestrous cycle. The oviduct consists of the infundibulum (a funnel-like opening near the ovary that collects the newly ovulated oocyte), the ampulla (the middle, thicker area where fertilisation occurs) and the isthmus (the thick-walled portion that leads into the uterine horn). The uterus of the ewe is bicornuate with a small uterine body and two medium length uterine horns (Figure 2.1). The inner surface of the uterus contains many caruncles, which serve as attachment sites for the foetal placenta during pregnancy. The ovine cervix is thick walled and highly convoluted with many crypts and folds on its inner surface. It provides a barrier to sperm transport and also isolates the uterus from the external environment during pregnancy. The cervical ostium (os) is the posterior portion of the cervix that protrudes into the anterior vagina. Semen is deposited in the anterior vagina of the ewe (Senger 2003).

2.1.2 Histology

The oviduct is a muscular tube with an outer serosal surface and an inner lining of simple columnar epithelium which is thrown into numerous longitudinal folds. The uterus also has three layers, an outer perimetrium (serosa), a middle myometrium (muscular layer) and an inner endometrium (mucosa). The endometrium consists of simple columnar epithelium and branched endometrial glands extending into the stromal tissue. The caruncles are easily recognised as non-glandular areas that protrude outwards from the surrounding endometrium. The cervix of ewes is lined by

a simple columnar epithelium with mucus secreting goblet cells. The surface is thrown up into numerous folds and crypts, creating a tortuous, convoluted pathway from the vagina to the uterus. The cervical ostium is the posterior portion of the cervix which protrudes into the vagina, and is lined by stratified squamous epithelium. The vagina is also lined by stratified squamous epithelium (Bacha and Wood 1990).

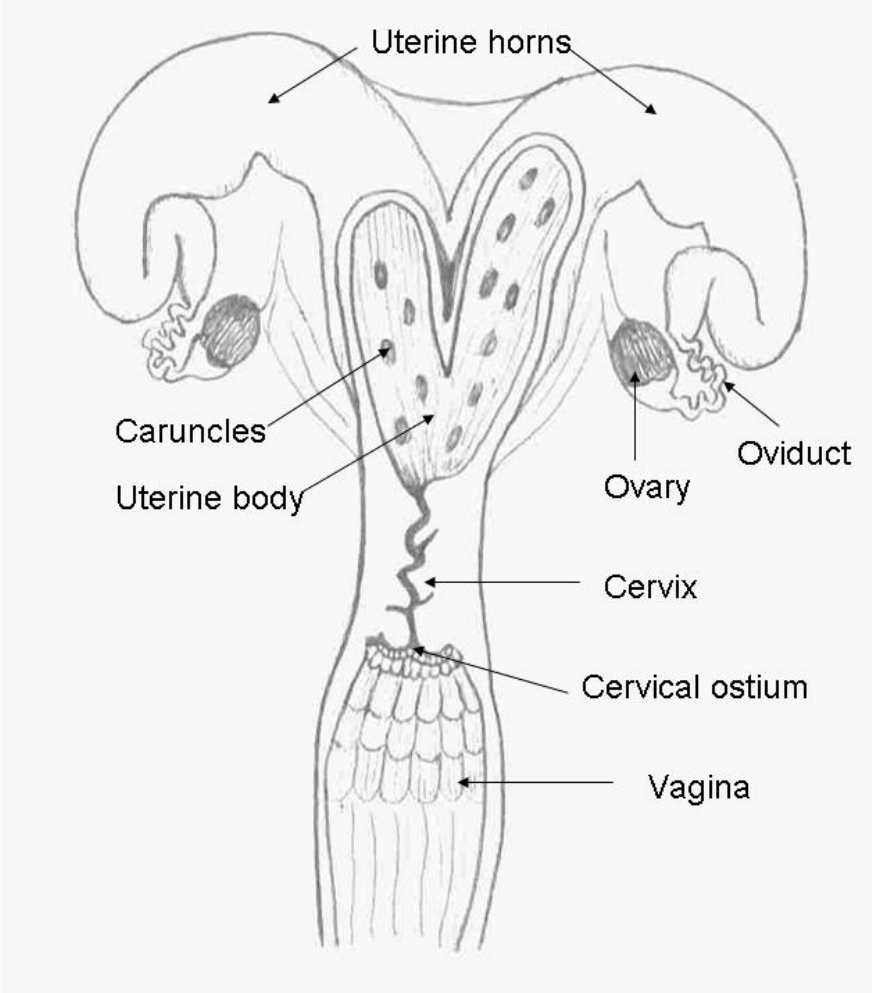

Figure 2.1 Diagram of the reproductive tract of the ewe (modified from Miller 1991).

2.2 Cyclical Changes in the Endometrium and Cervical Mucosa

2.2.1 Endometrial leukocyte populations

The distribution and migration of leukocytes in the endometrium and cervical mucosa and their egress into the lumen has been investigated in several species. Populations of leukocytes within the endometrium were significantly altered by cyclical changes in the concentrations of circulating oestrogens and progesterone (Starkey *et al.* 1991; Bischof *et al.* 1994a; Kaeoket *et al.* 2002b). In most species oestrogen caused an increase in leukocyte numbers in the endometrium (Tibbetts *et al.* 1999; DeLoia *et al.* 2002). Subsequently there were significantly more leukocytes present during oestrus than during the luteal phase of the reproductive cycle (Bischof *et al.* 1994a; Kaushic *et al.* 1998). However, cyclical changes in endometrial leukocyte populations appear to vary between species.

2.2.1.1 Rodents

Several studies using monoclonal antibodies against specific leukocyte cell markers have been undertaken in rodents. There was a marked infiltration of leukocytes in the rat endometrium during oestrus, compared to few leukocytes present during the luteal phase. Neutrophils, eosinophils, macrophages, dendritic cells and lymphocytes all increased in numbers when there was a high concentration of circulating oestrogens (Ross and Klebanoff 1966; Kaushic *et al.* 1998). There was an increase in antigen presentation by endometrial epithelial cells in response to oestrogens, but antigen presentation by vaginal cells was reduced when circulating oestrogen concentrations were high. Leukocytes were present in the vagina throughout the oestrous cycle, but increased during the luteal phase, the opposite to that which occured in the uterus (Kaushic *et al.* 1998).

Immunohistochemical analysis of uterine tissue from mice found that macrophages were the predominant leukocyte in the endometrium, and their numbers remained relatively constant throughout the oestrous cycle. However, macrophage distribution was influenced by the oestrous cycle, with a subepithelial accumulation during

oestrus compared to a relatively even distribution throughout the endometrial stroma during the luteal phase. Additionally, administration of exogenous oestrogen and progesterone alone or in combination increased the number of macrophages in the endometrium of ovariectomised mice (De and Wood 1990). Similarly, a more recent immunohistochemical analysis demonstrated that neutrophil and macrophage numbers both increased in response to exogenous oestrogen administration. However, treatment with oestrogen and progesterone together appeared to reduce the ability of oestrogen to attract macrophages and neutrophils into the uterus, suggesting that oestrogen facilitates leukocyte infiltration into the endometrium and progesterone antagonises this effect. Lymphocyte numbers were unaffected by changes in oestrogen and progesterone levels (Tibbetts *et al.* 1999).

2.2.1.2 Rabbits

Histological examination of rabbit uterine tissues during oestrus revealed an infiltration of neutrophils and mononuclear leukocytes into the endometrium. This was accompanied by physiological oedema and hyperaemia. Infiltration by leukocytes was not evident when the uterus was under the influence of progesterone, such as during pseudopregnancy (McDonald *et al.* 1952).

2.2.1.3 Pigs

In the pig, immunohistochemical studies found that lymphocytes were the predominant leukocyte in the endometrium throughout the oestrous cycle. Of these, $CD8^+$ lymphocytes occurred more frequently in the surface epithelium, whereas $CD4^+$ lymphocytes were more common in the endometrial stroma (Bischof *et al.* 1994a; Kaeoket *et al.* 2002a). During oestrus there was a marked increase in the number of neutrophils in the subepithelial stroma of the endometrium, along with an increase in lymphocytes in the epithelium. Macrophages also increased in the epithelium towards the end of oestrus and were also common in the subepithelial and glandular layers of the endometrium (Bischof *et al.* 1994a; Kaeoket *et al.* 2001; Kaeoket *et al.* 2002b).

2.2.1.4 Horses

There are limited data regarding cyclical changes in endometrial leukocyte populations in horses as few studies have focussed on leukocyte changes in response to fluctuating hormone levels. Most investigations of leukocytes in the mare endometrium have explored the response to insemination or bacterial infection. However, larger numbers of MHC Class II positive cells and T cells were demonstrated in the mare endometrium at oestrus compared to during the luteal phase of the reproductive cycle and the MHC Class II positive cells had the morphological characteristics of macrophages and dendritic cells (Frayne and Stokes 1994). In contrast to these findings, macrophage numbers in the equine endometrium were found to be similar at oestrus and during the luteal phase of the oestrous cycle (Summerfield and Watson 1998).

2.2.1.5 Humans

Several immunohistochemical studies found that endometrial leukocyte populations in humans underwent obvious cyclical changes. Leukocytes comprised 10-15% of the stromal cell population during the follicular phase of the menstrual cycle, and increased to 20-25% in the late luteal phase (Kamat and Isaacson 1987). Hence, an increase in leukocyte numbers occured when circulating concentrations of oestrogens were falling, differing to what occurs in other species. However, administration of exogenous oestrogen actually increased leukocyte numbers in the human endometrium (DeLoia *et al.* 2002).

The major leukocytes present in the human endometrium are T lymphocytes and macrophages (Wira and Kaushic 1996). Macrophages tended to be localised in the subepithelial stromal region of the endometrium, increasing in response to administration of exogenous oestrogen (Bonatz *et al.* 1992; DeLoia *et al.* 2002). This is in contrast to results from earlier work that found macrophages remain at fairly constant numbers throughout the menstrual cycle (Starkey *et al.* 1991).
T lymphocytes were present in the endometrium in fairly constant numbers throughout the menstrual cycle and were located mainly in clusters within the stromal tissue (Starkey *et al.* 1991; Bonatz *et al.* 1992; DeLoia *et al.* 2002). Natural

killer cells were also distributed throughout the endometrial stroma (Wira and Kaushic 1996). During the late luteal phase, when oestrogen and progesterone concentrations were falling, large granular lymphocytes markedly increased in numbers (Starkey *et al.* 1991; Stewart *et al.* 1998). Because endometrial leukocyte populations have no receptors for oestrogens or progesterone, the changing hormonal levels must regulate cell numbers indirectly, possibly via cytokines (Stewart *et al.* 1998; DeLoia *et al.* 2002).

2.2.1.6 Ruminants

Information regarding cyclical changes in endometrial leukocyte populations in ruminants is limited, as few studies have focussed entirely on the response to fluctuating hormone levels. Some studies have investigated endometrial cell populations during pregnancy (Leung *et al.* 2000). Others have focused on the number of leukocytes found within the lumen, using flushing procedures to recover cells in uterine luminal fluid (Anderson *et al.* 1985). Several studies have examined the response to experimental bacterial infection (Hawk *et al.* 1961; Brinsfield *et al.* 1963; Brinsfield *et al.* 1967) or to insemination (Mahajan and Menge 1967; Mattner 1968; Mattner 1969; Hilla 1999). Some of these studies incorporated the different responses seen at various stages of the oestrous cycle. For instance, there was a greater infiltration of neutrophils into the endometrium both in response to bacteria (Hawk *et al.* 1961; Brinsfield *et al.* 1963) and to insemination (Hilla 1999) during oestrus than during the luteal phase in sheep.

Histological examination of the sheep endometrium demonstrated greater numbers of neutrophils at oestrus than during the luteal phase (Hilla 1999). These results were obtained from surgically ligated sections of uterus, where adjacent sections were injected with components of semen. Neutrophil numbers may have been influenced by treatments on adjacent sections, as the control (untreated) sections showed similar, but less marked increases in neutrophils to sections that were inseminated.

Immunohistochemical studies in cattle indicated that macrophage numbers in the endometrium do not vary between different stages of the reproductive cycle. Macrophages and lymphocytes appeared to be the main cell types expressing MHC

class II molecules, and there was an increased expression of MHC class II antigens during oestrus. Marked changes in lymphocyte subpopulations occured in response to hormonal fluctuations. Cytotoxic T lymphocytes increased during oestrus, whereas helper T lymphocytes increased during the luteal phase (Cobb and Watson 1995). An earlier study using histological examination of intraepithelial lymphocytes in the bovine uterus found no changes in total lymphocyte numbers between different stages of the oestrous cycle (Vander Wielen and King 1984). This indicates that total lymphocyte numbers in the bovine endometrium remain relatively constant, but lymphocyte subtypes vary in response to hormonal fluctuations. Recent immunohistochemical analysis of goat endometrium also found that lymphocyte populations are under hormonal influence. However, in this study, lymphocyte numbers significantly increased during the luteal phase when oestrogen concentrations were low (Perez-Martinez *et al.* 2002).

2.2.1.7 Origin of endometrial leukocytes

Fluctuations in leukocyte numbers may either result from alterations in the number of cells migrating from the circulation, or by proliferation of locally derived cell populations. It has been suggested that monocytes and lymphocytes enter the endometrium from the peripheral vasculature, and then further differentiate under the influence of the local uterine environment (Bonatz *et al.* 1992; DeLoia *et al.* 2002). This is supported by the observation of monocytes near blood vessels, accompanied by an increase in differentiated macrophages in the endometrium of pregnant rats (reviewed by Hunt 1994). Some leukocytes already present in the endometrium are capable of replicating, probably in response to local alterations in cytokine production. Large granular lymphocytes show proliferative activity in the human endometrium, but not enough to fully account for the large numbers that appear during cyclical changes (Pace *et al.* 1989; Habiba *et al.* 1999; DeLoia *et al.* 2002). Neutrophils are terminally differentiated and incapable of proliferation (Cassatella 1995; Roitt *et al.* 2001) so an increase in neutrophil numbers in the uterus must be due to migration from the circulatory system. It is likely that a combination of both migration from the circulation and some local cell proliferation contribute to increases in leukocyte numbers occurring in the endometrium.

2.2.2 Resistance to infection

The female reproductive tract forms part of the mucosal immune system, which defends the body against invasion by pathogenic organisms. Immune function in reproductive tissue changes in response to hormonal fluctuations during the oestrous cycle. There is greater resistance to infection during oestrus, when oestrogen concentrations in the uterine vasculature are high, than during the luteal phase, when progesterone concentration is high (McDonald *et al.* 1952; Hawk *et al.* 1961; Brinsfield *et al.* 1967; Mahajan and Menge 1967; Ramadan *et al.* 1997).

Several factors contribute to altered resistance to infection at different stages of the oestrous cycle. The female reproductive tract secretes immunoglobulins of the IgA and IgG classes. Concentrations of these immunoglobulins rise in response to oestrogens, providing greater defence against pathogens during oestrus (Parr and Parr 1990; Wira and Kaushic 1996). Antigen presenting cells also play an important role in immune protection in the female reproductive tract. During oestrus, antigen presentation by uterine epithelial cells is increased, and there is a simultaneous reduction in antigen presentation by stromal cells. The reverse situation occurs during the luteal phase, with uterine epithelial cells showing a lesser degree of antigen presentation than stromal cells and it is likely that these changes contribute to variations in resistance to infection (Wira and Rossoll 1995).

Immunosuppressive factors in uterine secretions may also contribute to reduced resistance when progesterone concentration in the uterine vasculature is high. Uterine secretions caused a greater suppression of lymphocytes *in vitro* during the luteal phase and during early and late pregnancy, than during oestrus (Segerson *et al.* 1984; Segerson *et al.* 1986; Hansen *et al.* 1987; Chacin *et al.* 1990). It is also possible that variations in uterine motility at different stages of the cycle cause altered rates of contaminant clearance from the uterus. There was a greater degree of spontaneous uterine motility during oestrus than during the rest of the reproductive cycle, which corresponds with the period of enhanced resistance to uterine infection (Rodriguez-Martinez *et al.* 1987; Chacin *et al.* 1990).

Uterine epithelial cells are thought to play an important role in the defence against infection by releasing chemokines and pro-inflammatory cytokines in response to stimulation of Toll-like receptors on their surface by pathogenic organisms (Schaefer *et al.* 2004). The subsequent changes in endometrial leukocyte populations may contribute to altered resistance to infection at different stages of the oestrous cycle (McDonald *et al.* 1952; Hawk *et al.* 1961; Brinsfield *et al.* 1963), however there are conflicting reports from different species regarding the significance of such changes.

Studies in sheep have indicated that neutrophils are important contributors to variability in uterine defence at different stages of the reproductive cycle. There was a greater influx of neutrophils into the endometrium and uterine lumen in response to bacteria during oestrus than during the luteal phase (Hawk *et al.* 1961; Brinsfield *et al.* 1963). In addition, phagocytosis of bacteria was proportional to the number of neutrophils present, leading to a more rapid clearance of bacteria during oestrus (Hawk *et al.* 1961). Neutrophil infiltration in response to bacteria was also more rapid in the absence of both oestrogen and progesterone (ovariectomised ewes), suggesting that reduced resistance during the luteal phase may be due to suppression of uterine defences by progesterone rather than stimulation by oestrogen (Hawk *et al.* 1961; Brinsfield *et al.* 1963). Distribution of neutrophils in reproductive tissues in response to bacterial infection also varied between stages of the cycle. During the luteal phase, and in ovariectomised ewes, small numbers of neutrophils were scattered diffusely throughout the endometrium, whereas at oestrus, large numbers accumulated in the subepithelial stroma (Brinsfield *et al.* 1963), coinciding with a greater resistance to infection at this stage of the reproductive cycle.

Studies in rabbits also suggest that alterations in neutrophil activity play a role in variable resistance to infection at different stages of the oestrous cycle. More neutrophils infiltrated the rabbit endometrium during oestrus, compared to when progesterone was predominant, as in pseudopregnancy. Furthermore, at oestrus, bacteria induced a mild increase in neutrophil numbers and infection was easily eliminated. However, when progesterone predominated, a greater infiltration of neutrophils was observed in response to bacteria and a severe infection developed (McDonald *et al.* 1952). So in rabbits, as occurs in sheep, more neutrophils were present in the endometrium at oestrus, along with an increased resistance to

infection. However, more neutrophils entered the rabbit uterus in response to bacteria when progesterone levels were high, but infection persisted. These results could be explained by several different mechanisms. It is possible that during oestrus, the neutrophils present in the endometrium are primed and ready to rapidly defend the uterus against pathogens. Immunosuppressive factors may also be present in the uterus due to high levels of progesterone. Another factor to consider is that this study compared rabbits during oestrus with pseudopregnant rabbits, and although progesterone is the dominant hormone during pseudopregnancy, these results may not completely reflect the changes that occur during the luteal phase of the oestrous cycle.

In contrast to results in sheep and rabbits, studies in cattle have suggested that neutrophil activity and migration is not involved in variable resistance to infection at different stages of the reproductive cycle. No difference in neutrophil numbers in response to bacteria was observed in the bovine uterus at oestrus compared to during the luteal phase (Chacin *et al.* 1990). In addition, there was no difference in phagocytosis by uterine neutrophils between oestrus and the luteal phase (Anderson *et al.* 1985). This evidence suggests that mechanisms other than neutrophil activity are involved in the decreased resistance that occurs during the luteal phase of the oestrous cycle.

Neutrophils are a major component of the innate immune system, and are largely responsible for the initial reaction to pathogens, by rapid phagocytosis of invading micro-organisms (Roitt *et al.* 2001). It is likely that in most species, increased neutrophils in the endometrium during oestrus contribute to greater resistance to infection during this stage of the reproductive cycle. Copulation is more likely to occur during oestrus, exposing the female reproductive tract to potentially pathogenic organisms and an enhanced resistance to infection at oestrus is an important factor in preventing transmission of disease during coitus.

2.3 Components of Semen

2.3.1 Spermatozoa

Spermatozoa are the main cellular component of semen and contain the paternal genetic material. A typical ram ejaculate has a volume of 0.5 - 2.0 ml and contains approximately 1.5-5.0 x 10^9 spermatozoa per ml. Freshly ejaculated spermatozoa have a low fertilising ability, and need to undergo the process of capacitation before they are capable of fertilisation (Miller 1991).

Spermatozoa possess a number of different surface antigens that are capable of eliciting an immune response in the female reproductive tract. Some of these antigens are inherent in the plasma membrane, developing during spermatogenesis, some appear during epididymal transit, and others are glycoproteins from seminal plasma that adhere to the surface of spermatozoa (Alexander and Anderson 1987; Fichorova *et al.* 1995; Iborra *et al.* 1996). Interactions between spermatozoa and surrounding fluid alter their antigenicity. The changing nature of the surface membrane of spermatozoa during transport may result in the removal or masking of antigenic compounds, or the unmasking of previously existing compounds (Dacheux *et al.* 1989).

Production of anti-sperm antibodies occasionally occurs, and can result in infertility, however immunoregulatory mechanisms are normally at play to prevent this from occurring (Anderson and Tarter 1982; Alexander and Anderson 1987; Thaler 1989; Stern *et al.* 1994; Kim *et al.* 1999). It has been documented in several species that seminal plasma contains immunosuppressive components. This is believed to be vital for successful reproduction, by protecting spermatozoa from immunological damage in the female reproductive tract, and preventing female sensitisation to sperm antigens after mating (Anderson and Tarter 1982; James and Hargreave 1984).

2.3.2 Seminal plasma

Seminal plasma is the acellular fluid component of semen, and is derived mainly from the seminal vesicles, prostate and bulbourethral glands. The complete range of constituents and their functions are not fully understood (James and Hargreave 1984; Kelly *et al.* 1997; Robertson and Sharkey 2001). However, it is generally believed that seminal plasma facilitates transport and survival of spermatozoa (Kelly *et al.* 1997; Maxwell *et al.* 1999; Maxwell and Johnson 1999), with fructose supplying an energy source for spermatozoal motility (Miller 1991). Prostaglandins and oxytocin stimulate smooth muscle contractions in the female reproductive tract, and may facilitate passive sperm transport (Watson *et al.* 1999; Katila 2001). In addition, the pH of seminal plasma acts as a buffer for spermatozoa, neutralising the acidity encountered in the vagina (Miller 1991). The presence of seminal plasma improves implantation rate and enhances embryo development *in vitro*, and is thought to contribute to optimal pregnancy success *in vivo* (Kelly 1999; Robertson and Sharkey 2001). Additionally, exposure to semen advances ovulation in pigs by 8 to 10 hours, and insemination early in oestrus is likely to enhance fertilisation success. This is mediated by local mechanisms in the female reproductive tract, since there is no change in the level of circulating luteinising hormone (Waberski *et al.* 1995; Waberski *et al.* 1997) which differs to mechanisms in animals that are induced ovulators, such as the Bacitran camel, where factors in seminal plasma increase luteinising hormone and subsequently induce ovulation (Zhao *et al.* 2001).

There are conflicting reports regarding beneficial versus detrimental effects that seminal plasma has on spermatozoa. In cattle, pigs and sheep, seminal plasma is reported to maintain membrane integrity and viability of spermatozoa (Ashworth *et al.* 1994; Maxwell *et al.* 1996; Garner *et al.* 2001). However, it has also been reported that seminal plasma has deleterious effects on the survival of spermatozoa in sheep, cattle, rabbits (Dott *et al.* 1979), and horses (Varner *et al.* 1987). Furthermore, the presence of seminal plasma improved the fertilising capacity of spermatozoa in sheep (Maxwell *et al.* 1999) and cattle (Henault *et al.* 1995), but reduced fertility in mice (Tecirlioglu *et al.* 2002) and had no effect on fertility of rabbit spermatozoa (Castellini *et al.* 2000). In addition, seminal plasma enhanced sperm motility in sheep (Dott *et al.* 1979; Graham 1994) and rabbits (Castellini *et al.*

2000) and facilitated cervical mucus penetration by spermatozoa in the ewe (Maxwell *et al.* 1999), whereas it inhibited sperm motility in mice (Tecirlioglu *et al.* 2002), horses (Varner *et al.* 1987) and humans (Iwamoto and Gagnon 1988).

Seminal plasma contains important immunomodulatory factors that are capable of influencing the environment in the female reproductive tract (James and Hargreave 1984; Alexander and Anderson 1987; Robertson 2002). Seminal plasma of mice contains an array of cytokines including transforming growth factor–beta (TGF-β) (Robertson *et al.* 1996a; Tremellen *et al.* 1998), eotaxin, regulated upon activation normal T-cell expressed and secreted (RANTES), keratinocyte-derived cytokine (KC), granulocyte colony-stimulating factor (G-CSF), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), macrophage inflammatory protein (MIP)-1α, and MIP-1β (Gopichandran *et al.* 2006). Cytokines such as IL-6 and IL-8 have also been detected in human seminal plasma (Shimoya *et al.* 1995; Srivastava *et al.* 1996), TGF-β has been detected in humans (Nocera and Chu 1993; Loras *et al.* 1999) and pigs (O'Leary *et al.* 2002) and monocyte chemotactic protein-1 (MCP-1) is a major component of bovine seminal plasma (Wempe *et al.* 1991).

2.3.2.1 Immunosuppressive effects of seminal plasma

Seminal plasma has potent immunosuppressive effects, and contains constituents able to inhibit both humoral immunity and cell-mediated immunity (Anderson and Tarter 1982; James and Hargreave 1984; Alexander and Anderson 1987; Thaler 1989; Veselsky *et al.* 1997; Kelly 1999). *In vitro,* human seminal plasma inhibits B and T lymphocytes, natural killer cells, macrophages and neutrophils (Alexander and Anderson 1987) and also inhibits the up-regulation of adhesion molecules involved in neutrophil phagocytosis of spermatozoa (D'Cruz and Haas 1995). Boar seminal plasma inhibits mouse lymphocyte proliferation and antibody production *in vitro* (Veselsky *et al.* 1997). Furthermore, seminal plasma from the mouse prevents activation of lymphocytes, suppresses antibody responses to spermatozoa and inhibits complement-mediated lysis of cell membranes *in vivo* (Anderson and Tarter 1982). Whilst the immunosuppressive nature of seminal plasma is widely accepted, the components responsible for causing these effects are still under investigation.

A variety of factors contribute to the immunosuppressive effects of seminal plasma. These include TGF-β, prostaglandins, uteroglobulin, transglutaminase, polyamines, lactoferrin, zinc, spermine and prostasomes (James and Hargreave 1984; Kelly 1999). In the mouse, components of prostatic, epididymal and seminal vesicle fluids all have immunosuppressive effects on lymphocytes (Anderson and Tarter 1982). In rats, a major protein from the seminal vesicles (SV-IV) reduces immunogenicity of spermatozoa, and it is suggested that this protein plays an important role in preventing an immune response to sperm by the female (Peluso *et al.* 1994). Secretory vesicles found in bovine seminal plasma, similar to prostasomes found in human semen, have immunosuppressive qualities, inhibiting activity and proliferation of immune cells (Lazarevic *et al.* 1995; Kelly 1999). It has also been proposed that immunosuppressive factors in bovine seminal plasma may promote sperm survival in the female reproductive tract (Lazarevic *et al.* 1995).

High concentrations of prostaglandins of the E series (PGE) are present in human seminal plasma. The effects of PGE include a pro-inflammatory effect, mainly involving vasoactive properties that contribute to the formation of oedema, and an immunosuppressive effect on leukocytes, either directly or via the induction of cytokine changes (James and Hargreave 1984; Kelly 1999). Prostaglandins from seminal plasma induce increased synthesis of IL-10 and TGF-β and a decreased synthesis of IL-12 in the female reproductive tract. These changes in cytokine production inhibit the cytotoxic effects of T lymphocytes and natural killer cells, and contribute to the creation of an immunosuppressive environment (Kelly *et al.* 1997; Kelly 1999).

Transforming growth factor-beta is present in seminal plasma of many species and is well known for its immunosuppressive effects, but it shows a diverse range of effects depending on the circumstances (Clemens 1991; Nocera and Chu 1995; Robertson *et al.* 1997; O'Leary *et al.* 2002). The immunosuppressive nature of TGF-β may immunologically protect spermatozoa in the female reproductive tract (Nocera and Chu 1993; Nocera and Chu 1995) and may play a role in creating a hypo-responsiveness to paternal antigens (Robertson *et al.* 1997; Robertson *et al.* 2002). However, TGF-β induces GM-CSF release from uterine epithelial cells, leading to an

inflammatory response in the endometrium (Robertson *et al.* 1992; Robertson *et al.* 1996a; Tremellen *et al.* 1998).

The pro-inflammatory and immunosuppressive effects that TGF-β is capable of eliciting in the female reproductive tract may seem contradictory. However, the network of interactions taking place after insemination is complex, and the post-insemination inflammatory response has been postulated to contribute to the generation of an immunosuppressive environment, increasing the likelihood of pregnancy success (Robertson 2002). The multiple effects of TGF-β may be controlled by its ability to exist in either a dormant or active form, and by the regulation of target cell receptors at different stages of cell maturation and activation (Wahl 1992). The pro-inflammatory and immune deviating effects shown by TGF-β are antagonised by IFNγ. Studies in humans suggest that high levels of interferon-gamma (IFNγ) in semen may reduce pregnancy success by reducing the effects of TGF-β (Sharkey *et al.* 2002).

2.3.2.2 Immunostimulatory effects of seminal plasma

Immunosuppressive effects of semen are widely documented, but more recently its immunostimulatory properties have also been investigated. A decrease in the number of lymphocytes has been observed in the endometrium of pigs in response to insemination, however simultaneous activation of lymphocytes in uterine lymph nodes occurred. There appeared to be a lack of adaptive immunity involved, suggestive of a non-specific response (Bischof *et al.* 1994b). Recent studies in pigs demonstrated a slight increase in lymphocytes in the endometrium after insemination rather than a decrease. It is possible that these different results in lymphocyte activity were related to the use of gilts in earlier studies, compared to the use of sows in more recent studies. Cytotoxic T lymphocytes were more numerous in the epithelium, and helper T lymphocytes were more numerous in stromal tissue, similar to the situation prior to mating (Kaeoket *et al.* 2003b).

Spermadhesins are proteins secreted by the seminal vesicle epithelium and account for greater than 80% of proteins in boar seminal plasma (Assreuy *et al.* 2003). Lymphocyte proliferation is enhanced by the presence of spermadhesins and it is

possible that lymphocyte populations induced by boar semen have an immunosuppressive role, protecting against immunorejection of spermatozoa and the developing conceptus (Leshin *et al.* 1998). In mice, exposure to semen is linked to the proliferation and activation of immunosuppressive lymphocyte populations, which are recruited into the implantation site early in pregnancy. This is thought to play a role in promoting pregnancy success, by mediating maternal immune tolerance (Robertson and Sharkey 2001; Bromfield and Robertson 2002; Robertson 2002).

2.3.2.3 Pro- and anti-inflammatory effects of seminal plasma

Seminal plasma can have both pro-inflammatory and anti-inflammatory effects. Human seminal plasma and its associated prostaglandins stimulate the release of IL-8, which has pro-inflammatory and neutrophil chemotactic properties, and IL-10, which has an anti-inflammatory effect, from cervical explants. This is proposed to initially promote survival of spermatozoa in the female reproductive tract, and later facilitate the removal of excess spermatozoa (Denison *et al.* 1999) and the immunosuppressive nature of seminal plasma may provide temporary protection for spermatozoa in transit to the site of fertilisation (Anderson and Tarter 1982; James and Hargreave 1984; Troedsson *et al.* 1998). The distribution of seminal plasma coincides with that of spermatozoa in the vagina and uterus, but only spermatozoa, and not seminal plasma, enter the oviducts (Carballada and Esponda 1997). Additionally, there is no inflammatory response in the oviducts of mice following insemination (Dalton *et al.* 1994). The inflammatory response elicited in the uterus may mop up debris and excess spermatozoa, along with contributing to the generation of immunotolerance to paternal antigens, whilst spermatozoa that have reached the oviduct are safe from phagocytosis, and can proceed with the process of fertilisation.

Seminal plasma from cattle, pigs and horses can inhibit an inflammatory response by suppressing chemotaxis and migration of neutrophils, and by reducing their capacity for phagocytosis (Clark and Klebanoff 1976; Strzemienski 1989; Troedsson *et al.* 1998; Rozeboom *et al.* 1999; Rozeboom *et al.* 2001). In pigs, the presence of seminal plasma protects spermatozoa from an already inflamed uterine environment,

increasing the likelihood of fertilisation and subsequent pregnancy success (Rozeboom *et al.* 2000). However, converse to the anti-inflammatory effects of seminal plasma in several species, components of seminal plasma in mice elicit the post-insemination inflammatory response (McMaster *et al.* 1992; Sanford *et al.* 1992; Robertson *et al.* 1996a; Robertson *et al.* 1997; Tremellen *et al.* 1998). These discrepancies may simply reflect different mechanisms occurring in different species. It is also possible that results *in vitro* may not adequately represent what occurs *in vivo*, and misleading results may ensue from differing laboratory techniques.

As well as immunostimulatory effects, spermadhesins also have pro-inflammatory effects, contributing to the post-insemination inflammatory reaction in pigs by chemotaxis of neutrophils, which appears to be at least partially mediated by cytokines from activated macrophages (Assreuy *et al.* 2002; Assreuy *et al.* 2003). The first fraction of boar ejaculate contains spermatozoa but lacks spermadhesins. The initial absence of these pro-inflammatory mediators may allow spermatozoa to reach the sperm reservoir in the oviduct prior to the onset of inflammation in the uterus (Assreuy *et al.* 2002). This may protect the initial spermatozoa population from phagocytosis, enhancing fertilisation success. Additionally, cellular changes in the uterus induced by spermadhesins may contribute to uterine immunomodulation, thereby enhancing the likelihood of reproductive success (Assreuy *et al.* 2003).

2.4 General Features of Inflammation and Leukocyte Migration

Inflammation is the tissue response to injury. It is a protective mechanism that acts to remove or wall off the injurious agent, neutralise noxious substances and begin the process of repair. During the acute stage of inflammation, vascular dilation and increased permeability of vessel walls lead to slowed blood flow and an accumulation of protein rich fluid in interstitial tissue. Leukocytes (mainly neutrophils, but also monocytes, lymphocytes, eosinophils and basophils) also move into interstitial tissue during inflammation (Cotran *et al.* 1999; Muller 2003). During the first 24 hours, neutrophils are the main leukocyte to migrate to the site of inflammation. Monocytes follow within 24 to 48 hours, replacing neutrophils as the predominant leukocytes present (Cotran *et al.* 1999; Kaplanski *et al.* 2003).

The vascular and cellular responses that occur during inflammation are modulated by chemical mediators (Cotran *et al.* 1999). Of these mediators, chemokines produced by resident cell populations provide the main mechanism to recruit leukocytes into the area of inflammation (Garcia-Ramallo *et al.* 2002). Chemokines are cytokines that have the ability to stimulate directional movement of leukocytes. They attract specific types of leukocytes to the inflammatory site by binding to receptors on the surface of target cells (Rottman 1999). Chemokines are divided into four classes (CXC, C-C, C and CX_3C) according to the arrangement of their conserved cysteine residues, which is important for their tertiary shape and hence activity (Wuyts *et al.* 1998; Cotran *et al.* 1999). Several different chemokine receptors have been identified for each chemokine class, chemokines often bind to more than one receptor and most receptors recognise more than one chemokine. Leukocytes express a variety of receptors and can potentially utilise more than one pathway to gain access to the site of inflammation (Baggiolini 1998; Rottman 1999). Important chemokines involved in the inflammatory response include interleukin-8 (IL-8) (a CXC chemokine) which attracts neutrophils via receptors such as CXCR1 and CXCR2, and macrophage chemotactic protein-1 (MCP-1) (a C-C chemokine) which attracts monocytes via receptors such as CCR2 (Baggiolini 1998; Cotran *et al.* 1999; Rottman 1999).

Chemotactic agents, including chemokines from endothelial cells and from the site of inflammation, cause activation of leukocytes within blood vessels. This results in an increased binding affinity of adhesion molecules (selectins and integrins) on the surface of leukocytes for corresponding adhesion molecules on endothelial cells. In addition, cytokines, particularly interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α), and chemokines induce the expression of adhesion molecules (mainly intercellular adhesion molecules and vascular cell adhesion molecules) on endothelial cells. Combined, the increased avidity of binding and the increased number of adhesion molecules enhances adhesion of leukocytes to the endothelium and their transmigration into interstitial tissue (Cotran *et al.* 1999; Muller 2003).

Extravasation of leukocytes from the blood vessel lumen into interstitial tissue involves several steps. Initially, leukocytes gather peripherally within the blood vessel (margination) and begin rolling along the endothelial surface, via loose binding between leukocyte and endothelial surfaces. Leukocytes then firmly adhere

to the surface (adhesion) and transmigrate through the endothelium (diapedesis). Leukocyte activation induces the disassembly and then reassembly of contractile elements within the cell, which enhances the cell's ability to move out of the vasculature and through tissue. Once within the interstitial tissue, leukocytes migrate toward the site of inflammation along a chemical concentration gradient (chemotaxis), localising at higher chemokine concentrations (Cotran *et al.* 1999; Van Miert 2002; Kaplanski *et al.* 2003). The chemical gradient is established and maintained by the chemoattractant binding to extracellular matrix glycoproteins (Rottman 1999).

2.5 The Post-insemination Inflammatory Reaction

Insemination induces a rapid and transient inflammatory reaction within the cervix and uterus (Pandya and Cohen 1985; De *et al.* 1991; McMaster *et al.* 1992). This response has been observed in a number of species including rodents (De *et al.* 1991; McMaster *et al.* 1992), rabbits (McDonald *et al.* 1952), pigs (Bischof *et al.* 1994b), horses (Kotilainen *et al.* 1994; Troedsson *et al.* 1998), ruminants (Mahajan and Menge 1967; Mattner 1968) and humans (Pandya and Cohen 1985). The inflammatory reaction is characterised by an accumulation of leukocytes, mainly neutrophils and macrophages, in the subepithelial stroma of the cervix and uterus, and the egress of these cells into the lumen of the cervix and uterus (De *et al.* 1991; McMaster *et al.* 1992; Sanford *et al.* 1992; Robertson *et al.* 1996a; Tremellen *et al.* 1998). Vascular changes are also involved, with an increase in vascular permeability causing subsequent accumulation of interstitial fluid (McDonald *et al.* 1952; Tyler 1977; Bischof *et al.* 1994; Troedsson *et al.* 2001). The inflammatory response is short-lived, and generally subsides within 24 to 48 hours after insemination (Mahajan and Menge 1967; De *et al.* 1991; Sanford *et al.* 1992).

There is a more rapid post-insemination inflammatory response during oestrus than during the luteal phase of the reproductive cycle. In rabbits, a leukocyte infiltration is already obvious in the endometrium during oestrus, and insemination stimulates a greater degree of inflammation to be superimposed over this (McDonald *et al.* 1952). Similarly, in the sheep cervix there is a greater expression of the pro-inflammatory

cytokine IL-8 mRNA during oestrus than during the luteal phase, and this increases further in response to insemination (Mitchell *et al.* 2002). In cattle, there is a more rapid migration of neutrophils from the endometrium into the uterine lumen in response to insemination during oestrus than during the luteal phase. This response appears to be due to delayed migration of leukocytes into the lumen during the luteal phase, as leukocyte numbers in endometrial tissue following insemination were similar at oestrus and the luteal phase (Mahajan and Menge 1967).

The location of the post-insemination inflammatory reaction may vary between species and between natural mating and artificial insemination. It is likely that the site of semen deposition influences the location of the subsequent inflammatory reaction. For instance, some species experience primarily vaginal deposition of semen (ruminants, humans, rabbits), whereas other species undergo intra-uterine deposition of semen (rodents, pigs, horses) (First *et al.* 1968; Mattner 1969; Tyler 1977; McMaster *et al.* 1992; Kotilainen *et al.* 1994; Denison *et al.* 1999). It seems logical to surmise that the inflammatory response will be more intense in the vicinity of semen deposition. It follows that ruminants, rabbits and humans are likely to have a more intense reaction in the vagina and cervix, whereas rodents, pigs and horses will have a greater inflammatory response in the uterus. However, there are many conflicting reports regarding details of the inflammatory response, including location, stimulus and cell types involved. For instance studies in sheep carried out in the 1960's showed that deposition of spermatozoa into a ligated cervix induced no inflammation in the cervix (Mattner 1969). It has also been suggested that in sheep, the cervix acts as a reservoir of spermatozoa, where they accumulate in cervical mucus and this protects them from phagocytosis by leukocytes (Mattner 1963; Mattner 1966; Mattner 1969). However, more recent studies indicate that an inflammatory reaction does occur in the cervix of sheep in response to insemination, as demonstrated by an increased expression of the pro-inflammatory cytokine IL-8 mRNA (Mitchell *et al.* 2002).

2.5.1 Cellular changes during the inflammatory reaction

2.5.1.1 Cell types involved

The post-insemination inflammatory reaction in the endometrium has been described as a typical acute inflammatory response, with granulocytes, macrophages and lymphocytes involved (De *et al.* 1991; Sanford *et al.* 1992). It has also been reported that the leukocytic reaction in the uterine lumen is an atypical inflammatory response, as lymphocytes do not enter the lumen (Rozeboom *et al.* 1999). The most numerous cells to infiltrate the endometrium during the reaction are neutrophils, and these are the first to appear. Closely following this is a longer lasting infiltration by mononuclear cells, mainly macrophages, but also lymphocytes and some mast cells (De *et al.* 1991; Sanford *et al.* 1992). Not all leukocytes that appear in the endometrium after insemination cross the epithelium into the uterine lumen. Studies in several different species have found that the predominant leukocyte migrating into the uterine and cervical lumen post-insemination is the neutrophil (Lovell and Getty 1968; Mattner 1969; Sanford *et al.* 1992; Thompson *et al.* 1992; Kotilainen *et al.* 1994). Neutrophils and macrophages have been observed in the uterine lumen of sheep following insemination (Hilla 1999).

2.5.1.2 Cellular changes within uterine and cervical tissues

In humans, insemination induces cervical leukocytosis within 15 minutes (Pandya and Cohen 1985). A rapid leukocytosis has also been observed in the cervix of rabbits, with large numbers of neutrophils appearing in the epithelium and subepithelial stroma within 30 minutes of mating (Tyler 1977). Neutrophils are the first leukocyte to increase in the endometrial stroma of the mouse post-insemination, appearing within hours of mating (De *et al.* 1991). As the inflammatory response progresses, neutrophil infiltration is closely followed by a mononuclear response, which peaks within 24 hours (De *et al.* 1991; McMaster *et al.* 1992; Sanford *et al.* 1992). Neutrophils and macrophages both increase in the endometrium following insemination, but the lymphocytic response varies between different studies.

In horses and humans, lymphocytes increase in the endometrium after insemination, with the predominant subtype being helper T lymphocytes (Thompson *et al.* 1992; Tunon *et al.* 2000). Lymphocytes also increase in the mouse endometrium following insemination (De *et al.* 1991) but studies in pigs have produced conflicting results. A marked reduction in lymphocytes in the gilt endometrium has been observed following insemination (Bischof *et al.* 1994b) but recent researchers found an increase rather than a reduction in lymphocytes in response to insemination (Kaeoket *et al.* 2003a).

Information regarding the cell types involved in the post-insemination inflammatory response in the ruminant endometrium is limited. Neutrophils increase in the endometrium of sheep and goats after insemination (Mattner 1968; Hilla 1999) and IL-8, a major neutrophil chemoattractant, has been demonstrated in ovine cervical cells (Mitchell *et al.* 2002). There do not appear to have been any studies carried out to assess macrophage and lymphocyte populations in the endometrium of ruminants in response to insemination.

2.5.1.3 Cellular changes in the uterine and cervical lumen

Neutrophils are present in the lamina propria subjacent to the endometrial epithelium in sows at oestrus, but only move into the lumen after insemination (Lovell and Getty 1968). Neutrophils appear to be specifically attracted across the epithelium into the uterine lumen of the mouse, suggesting that a specific neutrophil chemoattractant is involved (Sanford *et al.* 1992). The predominant leukocyte entering the uterine lumen post-insemination in horses and pigs is also the neutrophil, with the greatest response occurring within 6 hours (Kotilainen *et al.* 1994; Rozeboom *et al.* 1999). This time frame is consistent with results seen in sheep and cattle, with large numbers of neutrophils evident in the uterine lumen within 6 hours of insemination (Mahajan and Menge 1967; Mattner 1969; Hilla 1999). In humans, neutrophils are the predominant leukocyte found in the cervical lumen before and after insemination, with numbers increasing significantly within 4 hours of semen introduction (Thompson *et al.* 1992).

In ruminants, large numbers of neutrophils enter the cervical lumen following insemination. It has been suggested that these drain through from the uterine lumen rather than by migrating across the cervical epithelium, as inflammation of the cervical mucosa was not detected after insemination (Mattner 1968; Mattner 1969) In contrast to this, the recent demonstration of IL-8 in the ovine cervix suggests there is a marked cervical inflammatory response to semen (Mitchell *et al.* 2002). Macrophages reportedly increase in the uterine lumen post-insemination in sheep (Hilla 1999) however the study was based on cellular morphology rather than using specific macrophage markers and numbers may have been overestimated due to their similar appearance to sloughed epithelial cells. Further investigation is required in order to clarify the presence or absence of an inflammatory reaction in the ovine cervix and uterus, and the origin of leukocytes found in the cervical lumen.

2.5.2 Cytokines involved in the inflammatory reaction

Cytokines are regulatory proteins produced by many cell types, particularly activated lymphocytes and macrophages. They act as intercellular messengers and influence the behaviour of other cells (Cotran *et al.* 1999). The ways in which cytokines exert their effects are numerous and varied, often creating a very complex network of communication. A particular cytokine often has multiple functions, and may have different effects on different cell types. Several cytokines may have similar and overlapping effects on a particular cell type (Clemens 1991; Sanchez-Cuenca *et al.* 1999; Parkin and Cohen 2001). Cytokines play major roles in inflammation and immune responses. For instance, TNF-α and IL-1 play key roles in inflammation, by direct pro-inflammatory actions and via the induction of other cytokines and chemokines (Van Miert 2002). Chemokines such as IL-8 and MCP-1 are important for attracting leukocytes to inflammatory sites (Cotran *et al.* 1999), and are likely to be involved in the post-insemination inflammatory reaction. Cytokines are also important for intercellular signalling during the reproductive cycle and are involved in communication between the conceptus and endometrial cells during early pregnancy (Robertson *et al.* 1994).

Several cytokines have been implicated in contributing to the post-insemination inflammatory reaction in the female reproductive tract. Cytokines may play a role by

their presence in semen causing a direct reaction in the female reproductive tract, or via their release from female reproductive tissue in response to components of semen. Transforming growth factor–beta from seminal plasma elicits an inflammatory reaction in the mouse uterus (Robertson *et al.* 1996a; Robertson *et al.* 1997) and other cytokines in mouse seminal plasma such as eotaxin, RANTES, KC, G-CSF and IL-4 may also contribute to the inflammatory reaction (Gopichandran *et al.* 2006). Similarly, cytokines such as IL-6 and IL-8, which have been detected in human seminal plasma (Shimoya *et al.* 1995; Srivastava *et al.* 1996) and MCP-1, a major component of bovine seminal plasma (Wempe *et al.* 1991), may also contribute to the post-insemination inflammatory response.

A variety of cytokines associated with the inflammatory response have been demonstrated in the rodent uterus post-insemination. These include macrophage colony-stimulating factor (CSF-1), GM-CSF, IL-1 α, IL-1 β, IL-6 and TNF-α (Sanford *et al.* 1992; Gutsche *et al.* 2003). Of these, GM-CSF plays a major role in inducing post-insemination inflammation in the mouse uterus (Robertson *et al.* 1996a; Robertson and Sharkey 2001). Granulocyte-macrophage colony-stimulating factor expression is also induced in the human cervix following insemination (Sharkey *et al.* 2004). Interleukin-8 has been detected in cervical tissue of sheep (Mitchell *et al.* 2002) and humans (Denison *et al.* 1999; Sharkey *et al.* 2003) following exposure to semen. Interleukin-6 expression also increases in the human cervix (Sharkey *et al.* 2003; Sharkey and Robertson 2004) and endometrium (Gutsche *et al.* 2003) after semen exposure. Leukaemia inhibitory factor (LIF) is essential for successful implantation in mice and its expression is increased in the human endometrium in response to seminal plasma. This response extends into the oviducts in mice (Jasper *et al.* 2005). It has also been suggested that LIF may play a role in macrophage chemotaxis following insemination (Gutsche *et al.* 2003; Kimber 2005). Human seminal plasma also induces the release of IL-10 from monocytes (Denison *et al.* 1999), which may play a role in immunomodulation of the female reproductive tract post-insemination. Several of these cytokines are involved in implantation, placental development, and growth and differentiation of the conceptus (Crainie *et al.* 1990; Martal *et al.* 1997).

Prostaglandins are chemical mediators of inflammation which also appear to play a role in the post-insemination inflammatory reaction. Prostaglandins of the E series (PGE) from seminal plasma of humans have been implicated in contributing to inflammation and immunomodulation via changes in cytokine production (Denison *et al.* 1999; Kelly 1999).

2.5.2.1 Transforming growth factor beta

Transforming growth factor beta (TGF-β) belongs to a large superfamily of structurally similar cytokines. Three different isoforms (TGF-β1, TGF-β2 and TGF-β3) occur in mammals (Derynck and Choy 1998). Transforming growth factor beta is produced by many cell types and has many diverse effects. It may either inhibit or stimulate cell proliferation depending on the circumstances (Clemens 1991), but it is generally immunosuppressive and anti-inflammatory (Roitt *et al.* 2001). It exists in either a latent or an active form (Wahl 1992). Latent TGF-β is associated with a latency-associated peptide (LAP) which is cleaved on activation by factors such as proteases, thrombospondin-1, integrins or an acid environment (Derynck and Choy 1998; Annes *et al.* 2003).

Transforming growth factor beta is produced in seminal vesicles of mice and in the human prostate. It has been identified in seminal plasma from several species including mice (Robertson *et al.* 1996a; Tremellen *et al.* 1998), humans (Nocera and Chu 1993; Loras *et al.* 1999) and pigs (O'Leary *et al.* 2002). In human seminal plasma, approximately 80% of TGF-β is present in the latent form, which is subsequently activated by an acid environment, such as encountered in the female reproductive tract (Nocera and Chu 1995). Similarly, in mice, at least 70% of TGF- β is present in a latent form (Tremellen *et al.* 1998). In contrast to humans and mice, most TGF- β in pig seminal plasma is present in its active form (Robertson *et al.* 2002).

The role that TGF-β plays in semen and the female reproductive tract is not entirely clear. It has well known immunosuppressive effects, and functions in a similar manner to the immunosuppressive actions of PGE in human seminal plasma (Kelly 1999). Transforming growth factor beta from seminal vesicles of mice stimulates the

release of the pro-inflammatory cytokine GM-CSF from uterine epithelial cells, inducing leukocyte infiltration into the endometrium (Robertson *et al.* 1992; Robertson *et al.* 1996a; Tremellen *et al.* 1998). Transforming growth factor beta also stimulates the release of GM-CSF and IL-6 from human cervical epithelial cells (Sharkey and Robertson 2004). The inflammatory response to TGF-β is stronger at oestrus than during the luteal phase, suggesting oestrogen enhances induction of GM-CSF by TGF-β (Tremellen *et al.* 1998).

2.5.2.2 Granulocyte-macrophage-colony stimulating factor

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is produced by a variety of cells including lymphocytes, fibroblasts, monocytes and endothelial cells (Clemens 1991; Quesniaux and Jones 1998). Endometrial epithelial cells are the predominant source of GM-CSF in the uterus of mice (Robertson *et al.* 1992), sheep (Imakawa *et al.* 1993), cattle (de Moraes *et al.* 1999) and humans (Giacomini *et al.* 1995). Granulocyte-macrophage colony-stimulating factor is a potent pro-inflammatory cytokine, and plays a vital role in various functions of the immune system. It stimulates the proliferation and differentiation of progenitors of granulocytes and macrophages, as well as activating mature granulocytes and macrophages. It has numerous biologic effects, including the enhancement of phagocytosis, antigen presentation and other immune functions by cells such as macrophages, monocytes, dendritic cells and neutrophils (Fleischmann *et al.* 1986; Fischer *et al.* 1988; Clemens 1991; Armitage 1998; Quesniaux and Jones 1998).

Granulocyte-macrophage colony-stimulating factor is produced in the mouse (Robertson and Seamark 1990; Sanford *et al.* 1992) and pig (O'Leary *et al.* 2004) uterus in response to seminal components and GM-CSF expression is increased in the human cervix in response to insemination (Sharkey *et al.* 2004; Sharkey and Robertson 2004). Production of GM-CSF is regulated by ovarian steroid hormones, with expression increasing in response to oestrogens (Robertson *et al.* 1996b; McGuire *et al.* 2002). In mice, release of GM-CSF from glandular and luminal epithelial cells of the endometrium is stimulated by TGF-β following mating (Robertson and Seamark 1990; Robertson *et al.* 1992; Robertson *et al.* 1996a;

Tremellen *et al.* 1998). Similarly, TGF-β stimulates the release of GM-CSF from human cervical epithelial cells (Sharkey and Robertson 2004).

Increased GM-CSF in the mouse uterus following insemination induces an inflammatory response in the endometrium which involves a marked increase in neutrophils and macrophages, and the migration of neutrophils into the uterine lumen (Robertson *et al.* 1996a). However, GM-CSF is not wholly responsible for the inflammatory response, as GM-CSF deficient mice still show a comparable inflammatory response post-mating (Robertson *et al.* 1998b). While GM-CSF stimulates an increase in phagocytosis and cytotoxic activity, and prolongs the life of granulocytes, there are mixed reports regarding its chemotactic abilities. It has previously been reported that GM-CSF does not attract leukocytes into inflammatory sites, and therefore other factors must be involved as chemoattractants (Lopez *et al.* 1986). However, more recent studies have found that GM-CSF does have chemotactic properties, enhancing adhesion to vascular endothelium and inducing the migration of both monocytes and neutrophils (Wang *et al.* 1987; Quesniaux and Jones 1998). Furthermore, neutrophils, macrophages and dendritic cells that are attracted into mouse endometrial tissue following insemination, express binding sites specific for GM-CSF, and their increase in numbers in the endometrium parallels an increase in GM-CSF concentration (Robertson *et al.* 2000).

2.5.2.3 Interleukin-1

Interleukins act primarily as intercellular messengers between lymphocytes, but may also be produced by, or act upon other types of cell. Interleukin-1 is produced mainly by monocytes, but also by many other cell types (Clemens 1991). It is a multifunctional cytokine and has effects on most types of cell. Interleukin-1 occurs in two forms: IL-1α and IL-1β. The biological effects of both forms of IL-1 are very similar and they are often considered together. Interleukin-1 has potent pro-inflammatory and immunostimulatory effects (Dinarello 1998). It was previously known as lymphocyte activating factor or endogenous pyrogen due to its ability to induce fever (Clemens 1991). Both forms of IL-1 are induced in the mouse uterus following insemination (Sanford *et al.* 1992). It is also found in placental trophoblasts and appears to play a role in embryonic development (Dinarello 1998).

2.5.2.4 Interleukin-6

Interleukin 6 (IL-6) is produced by a variety of cells including lymphocytes, macrophages, fibroblasts and endothelial cells, and has a wide variety of actions on an array of different target cells. Its effects include transformation of B cells into antibody-secreting plasma cells, induction of acute phase proteins (Clemens 1991) and the transition from neutrophil to macrophage recruitment during inflammation (Bartoccioni *et al.* 2003; Kaplanski *et al.* 2003). Interleukin-6 is present in human seminal plasma (Shimoya *et al.* 1995), is induced in the mouse (Sanford *et al.* 1992) and pig (O'Leary *et al.* 2004) uterus and the human cervix (Sharkey and Robertson 2004) in response to semen, and is likely to play a role in the inflammatory reaction that ensues. Endometrial epithelial cells are the main source of IL-6 in the mouse uterus, but leukocytes and fibroblasts also contribute, particularly post-insemination (Robertson *et al.* 1992).

2.5.2.5 Interleukin-8

Interleukin 8 (IL-8) is a C-X-C chemokine produced by a variety of cell types, including monocytes, macrophages and lymphocytes (Wuyts *et al.* 1998; Cotran *et al.* 1999). It has pro-inflammatory effects and is a powerful neutrophil chemoattractant (Clemens 1991). As well as attraction and activation of neutrophils, it has limited stimulatory effects on many other cell types including monocytes and eosinophils (Wuyts *et al.* 1998; Cotran *et al.* 1999). Many different factors can stimulate the release of IL-8 including bacterial lipopolysaccharide (LPS) and cytokines such as GM-CSF and IL-1 (Wuyts *et al.* 1998).

Interleukin-8 is produced by epithelial and glandular cells in the female reproductive tract of humans, under the regulation of oestrogen and progesterone (Arici *et al.* 1998; Palter *et al.* 2001) and may contribute to the post-insemination inflammatory response. Insemination in sheep increases the expression of IL-8 mRNA in luminal epithelial and fibroblastic cells of the cervix (Mitchell *et al.* 2002) and human seminal plasma can also stimulate the secretion of IL-8 from cervical tissue (Denison *et al.* 1999). However, unlike the case in GM-CSF and IL-6 secretion, IL-8 production by human cervical cells is not stimulated by TGF-β (Sharkey and

Robertson 2004), so other factors in semen are likely to be responsible. Interleukin-8 has also been detected in human seminal plasma (Srivastava *et al.* 1996).

2.5.2.6 Interleukin-10

Interleukin 10 (IL-10) is produced by many cell types, particularly monocytes, macrophages and lymphocytes, and it has potent anti-inflammatory and immunosuppressive effects. It inhibits the activation of monocytes, macrophages, neutrophils and dendritic cells, and prevents the expression of major histocompatibility complex (MHC) class II molecules. It also causes a shift from the production of pro-inflammatory to anti-inflammatory cytokines (de Waal Malefyt and Moore 1998).

Interleukin-10 may have an important role in limiting the post-insemination inflammatory reaction. In mice, a lack of IL-10 increases the post-insemination inflammatory response in the uterus and impairs its resolution. Additionally, IL-10 plays a role in normal foetal growth (White *et al.* 2004). Components of human seminal plasma have also been shown to stimulate the release IL-10 from monocytes *in vitro* (Denison *et al.* 1999). If this also occurs *in vivo*, it may contribute to the immunosuppressive effects seminal plasma has in the female reproductive tract.

2.5.2.7 Monocyte chemotactic protein-1

Monocyte chemotactic protein-1 (MCP-1) is a C-C chemokine produced by activated macrophages, lymphocytes and other cell types. It is a powerful chemoattractant for monocytes (Cotran *et al.* 1999). Monocyte chemotactic protein-1 is reported to be a major component of bovine seminal plasma (Wempe *et al.* 1991). A similar chemokine (its genetic sequence is 80% identical to MCP-1) known as monocyte chemotactic and activating factor (MCAF) has also been detected in human seminal plasma. It has been postulated that MCAF in semen attracts monocytes into the human cervix (Shimoya *et al.* 1995). Monocyte chemotactic protein-1 is also induced in the pig uterus in response to insemination (O'Leary *et al.* 2004). It is probable that MCP-1 plays a role in attracting leukocytes into female reproductive tract tissues

during the post-insemination inflammatory reaction, by its presence in seminal plasma, and/or via its induction in reproductive tissues post-insemination.

2.5.2.8 Tumor necrosis factor alpha

Tumor necrosis factor-alpha (TNF-α) is mainly produced by macrophages, but may also be produced by a wide variety of other cell types including lymphocytes and fibroblasts (Clemens 1991). It has a diverse range of biological activities, with mainly pro-inflammatory and cytotoxic effects. The net effects of TNF-α depend on a complex array of interacting factors (Zhang and Tracey 1998). Tumor necrosis factor-alpha is also known as cachectin due to its role in tissue wasting (Clemens 1991). It is induced in the mouse uterus following insemination (Sanford *et al.* 1992) and has recently been implicated in contributing to neutrophil chemotaxis secondary to stimulation of macrophages by spermadhesins in boar seminal plasma *in vitro* (Assreuy *et al.* 2003).

2.5.2.9 Prostaglandins

Prostaglandins are chemical mediators of inflammation derived from arachidonic acid in cell membranes (Cotran *et al.* 1999). Like TGF-β, prostaglandins can have both pro-inflammatory and immunosuppressive effects. Some species, including humans, have high levels of prostaglandins of the E series (PGE) in seminal plasma, whereas other species, such as mice, have low levels. The role played by PGE in semen is still under investigation, but it appears to have important immunomodulatory effects (Kelly 1999).

Prostaglandins in human seminal plasma stimulate the release of several cytokines in the female reproductive tract. These include IL-8, which has pro-inflammatory and neutrophil chemotactic properties, IL-10, which has an anti-inflammatory effect (Denison *et al.* 1999), and TGF-β that has pro-inflammatory and immunosuppressive effects (Kelly 1999). It has also been shown that PGE regulates IL-2 and GM-CSF expression in bovine lymphocytes (Emond *et al.* 1998), and it may play a similar role in the reproductive tract. Prostaglandins in seminal plasma also lead to reduced synthesis of IL-12 in the female reproductive tract. These changes in cytokine

production inhibit the cytotoxic effects of T lymphocytes and natural killer cells, and contribute to the creation of an immunosuppressive environment (Kelly *et al.* 1997; Kelly 1999).

2.6 Induction of the Post-insemination Inflammatory Reaction

It is generally accepted that component(s) of semen, and not just mechanical stimulation, are responsible for inducing the inflammatory reaction seen in the uterus and cervix post-insemination (McMaster *et al.* 1992; Tremellen *et al.* 1998). However, there are conflicting reports in regard to which components of semen actually trigger this response. Some studies indicate that spermatozoa are responsible for the inflammatory reaction, whereas others implicate components of seminal plasma. Species differences may explain some of these discrepancies, but there are also differing reports from studies undertaken on the same species of animal. A recent study in pigs led to the conclusion that the physical presence of fluid in the uterus is responsible for the inflammatory reaction, and not any specific components of the fluid (Matthijs *et al.* 2003). However, this seems unlikely, as many studies have shown an absence of any noticeable inflammatory reaction after instillation of various control fluids into the uterus.

2.6.1 Rodents

Relatively consistent results have been produced from separate morphological and immunohistochemical studies in rodents. Antigenicity of spermatozoa does not appear to be involved in eliciting the post-insemination inflammatory reaction in the mouse (Robertson and Seamark 1990). Mating with vasectomised males produces the same inflammatory reaction in the mouse uterus as that produced when spermatozoa are present in the ejaculate. This suggests that a factor in the ejaculate other than spermatozoa initiates the inflammatory response (McMaster *et al.* 1992; Sanford *et al.* 1992). Transforming growth factor-beta from seminal plasma elicits inflammation in the mouse uterus, via the stimulation of GM-CSF production (Robertson *et al.* 1996a; Robertson *et al.* 1997; Tremellen *et al.* 1998). Transforming growth factor-beta is produced in the seminal vesicles of mice, and semen from

seminal vesicle deficient males fails to induce GM-CSF or the post-insemination inflammatory response (Robertson *et al.* 1996). However, GM-CSF is not wholly responsible for the inflammatory response, as GM-CSF deficient mice still show a comparable inflammatory response post-mating (Robertson *et al.* 1998b) so mechanisms other than GM-CSF must also be involved. One study demonstrated that spermatozoa are capable of eliciting anti-sperm antibodies after being inserted into the mouse uterus, but the study involved the use of human rather than mouse spermatozoa (Stern *et al.* 1994).

2.6.2 Rabbits

Studies in rabbits have produced conflicting results in regard to which components of semen are responsible for the post-insemination inflammatory reaction. One study using histological examination of cervical tissue concluded that spermatozoa are responsible for inducing inflammation. Mating with a vasectomised buck or insemination with sperm-free seminal plasma induced very little inflammatory response in the cervix, compared to a marked reaction to whole semen (Tyler 1977). The direct opposite was concluded following a study involving insemination with spermatozoa lacking accessory gland secretions. Histological examination found that spermatozoa did not induce significant inflammation in the rabbit uterus (McDonald *et al.* 1952). In contrast to both of these studies, an inflammatory reaction has been observed in the rabbit vagina in response to both sperm-free seminal plasma and to spermatozoa (Phillips and Mahler 1977). Similarly, injection of either concentrated spermatozoa or diluted semen into ligated sections of the rabbit vagina, uterus and oviduct, induced similar levels of inflammation, suggesting it is not only spermatozoa that contributes to the reaction (Howe 1967).

2.6.3 Pigs

Studies conducted in pigs have also produced conflicting results. Uterine lavage following insemination demonstrated that spermatozoa induced a greater degree of leukocytosis in the uterine lumen of gilts than did seminal plasma (Rozeboom *et al.* 1999; Rozeboom *et al.* 2000). *In vitro* studies of neutrophils have also indicated that seminal plasma suppresses the post-insemination inflammatory response by reducing

spermatozoa-induced chemotaxis (Rozeboom *et al.* 2001). In contrast to these findings, spermadhesins in seminal plasma cause chemotaxis of neutrophils (Assreuy *et al.* 2002) and immunohistochemical staining of gilt endometrium following insemination by vasectomised boars demonstrated that seminal plasma induces an inflammatory response. The inflammatory reaction involved an increase in macrophages and dendritic-like cells in the uterus, but a marked reduction in lymphocytes (Bischof *et al.* 1994b). In contrast to this lymphocyte effect, *in vitro* studies have demonstrated that spermadhesins in boar seminal plasma cause proliferation of lymphocytes rather than a reduction in their number (Leshin *et al.* 1998). Recent immunohistochemical studies of sow endometrium also found an increase in lymphocytes, along with infiltration by other leukocytes following insemination (Kaeoket *et al.* 2003a). An increase in pro-inflammatory cytokines and leukocytes (mainly macrophages) was found in the gilt uterus after exposure to seminal plasma (O'Leary *et al.* 2004), which lends further support for seminal plasma being responsible for the post-insemination inflammatory response in pigs.

Contrary to other studies of the post-insemination inflammatory reaction in pigs, recent findings indicated that the physical presence of fluid in the uterus was responsible for inflammation rather than any specific component of semen. Similar reactions were observed following the introduction of several different types of fluid into the sow uterus, including seminal plasma and spermatozoa (Matthijs *et al.* 2003).

2.6.4 Horses

Spermatozoa induce uterine inflammation in horses and seminal plasma has an immunosuppressive effect (Kotilainen *et al.* 1994; Troedsson *et al.* 1998; Troedsson *et al.* 2001; Troedsson *et al.* 2005). Highly concentrated semen provokes a greater inflammatory response than less concentrated semen and seminal plasma, indicating spermatozoa are responsible (Kotilainen *et al.* 1994). Equine spermatozoa cause chemotaxis of neutrophils via complement activation, whereas seminal plasma contains immunosuppressive components including DNase that inhibits this chemotactic effect, reducing phagocytosis by neutrophils and protecting viable but

not dead spermatozoa (Troedsson *et al.* 2001; Alghamdi *et al.* 2004; Alghamdi and Foster 2005; Troedsson *et al.* 2005).

2.6.5 Humans

It is not clear which components of semen are responsible for inducing the post-insemination inflammatory reaction in humans. A leukocyte reaction in the uterus of humans occurs in response to insemination, but the number of spermatozoa introduced had no effect on the intensity of reaction (Williams *et al.* 1993). Previous studies demonstrated that spermatozoa induced inflammation in the human cervix, but sperm-free seminal plasma caused no inflammatory reaction (Pandya and Cohen 1985; Thompson *et al.* 1992). However, it has been postulated that the inflammatory effect which spermatozoa has on the human cervix is possibly due to the washing process involved in sperm preparation, which alters the surface antigenicity of sperm, rather than the spermatozoa themselves. Recent *in vitro* findings indicate that seminal plasma contributes to the inflammatory response by inducing pro-inflammatory cytokine expression in the human uterus (Denison *et al.* 1999).

The human cervix produces mucus which is thought to prevent high concentrations of spermatozoa and seminal plasma from entering the uterus and uterine peristaltic waves are believed to facilitate passive sperm transport from the cervix into the uterus (Kunz *et al.* 1998). However, the amount of spermatozoa and seminal plasma reaching the uterus is uncertain. If significant amounts of seminal plasma reach the human uterus, seminal components such as IL-8 and TGF-β may play a role in priming the human endometrium for implantation (Gutsche *et al.* 2003).

2.6.6 Ruminants

It is not clear which factors in semen induce the post-insemination inflammatory reaction in ruminants. An inflammatory reaction to semen has been demonstrated in cattle (Mahajan and Menge 1967), goats (Mattner 1968) and sheep (Mattner 1969) but these studies did not separate spermatozoa from seminal plasma. The available literature suggests that in cattle, spermatozoa may be responsible for eliciting inflammation and seminal plasma may play an inhibitory role. Bovine spermatozoa

are chemotactic to neutrophils *in vitro* via complement activation, whereas bovine seminal plasma inhibits both chemotaxis of neutrophils (Clark and Klebanoff 1976) and phagocytosis of spermatozoa (Strzemienski 1989). Recent work in sheep demonstrated a greater degree of leukocytosis in the endometrium and uterine lumen in response to spermatozoa than to seminal plasma. However both induced some degree of inflammation and the effects appeared to be additive (Hilla 1999). Further investigation is required to clarify what factor(s) induce the post-insemination inflammatory reaction in ruminants.

2.6.7 Discrepancies in induction of the inflammatory reaction

It is still not clear which components of semen are responsible for eliciting the post-insemination inflammatory reaction and different mechanisms may be involved in different species. Another factor to consider is that some studies have focused on the inflammatory reaction within the endometrium, whilst others have looked mainly at inflammatory cells within the lumen of the uterus and cervix. This could be partly responsible for differing results, as cell populations infiltrating the endometrium may not be entirely consistent with those appearing in the lumen.

It is also possible that misleading results could arise due to various techniques used in the processing of semen, as certain procedures may affect the antigenicity of spermatozoa or components of seminal plasma. For instance, frozen equine semen provokes a stronger inflammatory reaction than fresh semen (Kotilainen *et al.* 1994), suggesting that the freezing process may increase the antigenicity of semen. There have also been reports indicating that centrifugation, a commonly used procedure in semen preparation, is damaging to spermatozoa (Graham 1994; Garcia-Lopez *et al.* 1996). Additionally, extensive dilution of semen, such as occurs during flow cytometric sorting, reduces sperm motility and viability in several species and may have detrimental effects on membrane integrity, altering spermatozoa antigenicity and subsequently affecting the inflammatory response (Maxwell *et al.* 1996; Maxwell and Johnson 1999). Ingredients in semen extenders such as yolk and glycerol may also contribute to inflammation in the female reproductive tract (Troedsson *et al.* 1998).

Identifying a specific stimulus for the post-insemination inflammatory response is not simple. There may be various pro-inflammatory and anti-inflammatory stimuli involved, producing a complicated network of effects. Further research is necessary to obtain a greater understanding of the mechanisms involved in induction of the post-insemination inflammatory reaction in different species.

2.7 Purpose of the Post-insemination Inflammatory Reaction

Leukocytosis is a normal physiological reaction to insemination (Pandya and Cohen 1985; Barratt *et al.* 1990). There are several postulated reasons for the post-insemination inflammatory reaction. Removal of excess, dead and defective spermatozoa is thought to be an important function, and leukocytes have been observed to actively phagocytose spermatozoa within the lumen of the cervix and uterus (First *et al.* 1968; Lovell and Getty 1968; Hilla 1999). Other possible functions include removal of introduced microorganisms to protect the female reproductive tract and selection of sperm with superior fertilising capability (Pandya and Cohen 1985; Thompson *et al.* 1992; Troedsson *et al.* 2001). It has also been suggested that in sheep, cervical inflammation induced by insemination plays a role in facilitating transport of spermatozoa across the cervix and into the uterus, by inducing cervical dilation (Mitchell *et al.* 2002).

The post-insemination inflammatory reaction is thought to "prime" the female reproductive tract for implantation. It has been proposed that the inflammatory reaction may play a role in the development of maternal immunotolerance to the conceptus during pregnancy (Barratt *et al.* 1990; Robertson *et al.* 1997; Mellor and Munn 2000; Robertson 2002; Robertson 2005).

2.7.1 Maternal immunomodulation post-insemination

Successful reproduction is an immunological challenge to the female. Tolerance to foreign paternal antigens must develop, but at the same time, adequate defence against micro-organisms must be maintained. It has been proposed that such a balance may be achieved by suppression of adaptive (specific) immunity and

activation of innate (non-specific) immunity (Sacks *et al.* 1999). Changes in maternal endometrial leukocyte function and cytokine expression, along with altered expression of MHC antigens on trophoblast cells contribute to maternal acceptance of the conceptus (Hansen and Liu 1996). However, the exact sequence of events leading to an environment where the maternal immune system accepts the presence of foreign antigens in the uterus is unknown. A complex series of immunological mechanisms work together to accommodate the conceptus and allow the establishment of successful pregnancy (Murray *et al.* 1983).

The maternal immune system needs to respond appropriately to antigens encountered initially in spermatozoa, and then in the developing conceptus (Hansen and Liu 1996), and the cellular and molecular components necessary for an immune response to occur are all operational in the endometrium at the time of insemination (Robertson and Sharkey 2001). It has been postulated that the post-insemination inflammatory reaction in the uterus contributes to the generation of an appropriate maternal immune response to paternal antigens, and is therefore an important factor in the early stages of pregnancy establishment (Thaler 1989; Robertson *et al.* 1997; Robertson and Sharkey 2001).

Studies in several species suggest that exposure to components of semen prior to fertilisation may enhance the likelihood of pregnancy success (Murray *et al.* 1983; Bellinge *et al.* 1986; Bischof *et al.* 1994b). In pigs, exposing the uterus to semen up to three weeks prior to breeding, significantly enhanced reproductive performance. An increase in litter size of 10-15% was observed in gilts following pre-exposure to semen (Murray *et al.* 1983; Murray and Grifo 1986). Increased implantation rates and improved pregnancy success have also been demonstrated in human *in vitro* fertilisation and embryo transfer programs after exposure of the female to semen (Bellinge *et al.* 1986; Tremellen *et al.* 2000). In mice, exposure of the female reproductive tract to seminal vesicle fluid prior to embryo transfer increased both foetal and neonatal weights (Bromfield *et al.* 2004a; Bromfield *et al.* 2004b) and these effects have been postulated to be largely orchestrated by the presence of TGF-β in seminal plasma (Robertson 2002; Robertson 2005). However, addition of TGF-β to boar spermatozoa prior to artificial insemination had no effect on implantation

rate, litter size or foetal survival in gilts, suggesting that TGF-β in semen is not an important factor in foetal growth and survival in pigs (Rhodes *et al.* 2006).

A major redistribution of leukocytes occurs in the endometrium during implantation and pregnancy, along with marked alterations in the production of cytokines (Hunt 1994; Robertson *et al.* 1997). Following insemination there is a transient accumulation of neutrophils and then macrophages in the subepithelial area of the endometrium. A progressive disappearance of these cells then leads to an almost complete absence of leukocytes around the site of implantation during the early implantation period. It has been speculated that this may reduce the likelihood of immune conflict between the conceptus and the maternal immune system in the peri-implantation period (Kachkache *et al.* 1991). Within five days of insemination there is an increase in macrophage numbers in the deeper layers of the endometrium along with the marked proliferation of endometrial stromal cells known as the decidual reaction, which is seen during early pregnancy (De *et al.* 1991; Hunt 1994).

Endometrial lymphocyte populations also undergo marked changes during pregnancy, but alterations in specific subpopulations are not completely understood (Hansen and Liu 1996). It is, however, thought that lymphocytes activated following insemination may play a role in facilitating embryo implantation. Activated lymphocytes recovered from lymph nodes draining the murine uterus following insemination infiltrate tissue at the site of implantation when transferred into pregnant mice (Johansson *et al.* 2004).

It has been postulated that the leukocyte and cytokine network induced by insemination directly contributes to the changes observed in the mouse endometrium during implantation and pregnancy (Robertson *et al.* 1997; Robertson *et al.* 1998b; Robertson 2005). It is also possible that the post-insemination inflammatory response induces local changes in the maternal immune system well before implantation of the conceptus (Mellor and Munn 2000). Therefore, aberrations in leukocyte/cytokine interactions during the post-insemination inflammatory reaction may adversely affect implantation and the progression of pregnancy.

2.7.1.1 Role of GM-CSF in maternal immunomodulation

Granulocyte-macrophage colony-stimulating factor appears to play an important role in ensuring successful pregnancy in the mouse. The inflammatory reaction GM-CSF induced in the murine uterus post-insemination involved local antigen presenting cells, such as macrophages and dendritic cells, that process and present paternal MHC antigens to the female immune system (Robertson *et al.* 1997; Robertson 1999; Robertson and Sharkey 2001). Along with other cytokines, including gamma interferon (IFN-γ), GM-CSF increased the number of MHC class II molecules on antigen presenting cells, enhancing their activity (Fischer *et al.* 1988; Clemens 1991). Furthermore, GM-CSF deficient mice had a reduced number of antigen presenting cells recruited into the uterus after mating, and underwent compromised placental and foetal development (Roberts *et al.* 1998; Robertson *et al.* 1998a; Hudson and Robertson 1999; Robertson *et al.* 1999). It has been suggested that in the environment created by seminal plasma and an array of associated cytokines, the increase in antigen presentation of paternal antigens to the female immune system leads to a state of maternal immunological hypo-responsiveness (Robertson *et al.* 1997; Robertson 1999; Robertson and Sharkey 2001).

Granulocyte-macrophage colony-stimulating factor is thought to play an important role in tissue remodelling and immunomodulation during the pre-implantation period in mice (Robertson *et al.* 1994) and it has been reported that GM-CSF not only contributes to priming the uterus for implantation, but plays an important role in embryo development and the formation and maintenance of placental tissues (Robertson and Seamark 1990; Robertson *et al.* 1992; Robertson *et al.* 1998). In support of this, GM-CSF enhances *in vivo* development of mouse embryos (Robertson *et al.* 1998; Robertson *et al.* 1999) and *in vitro* development of bovine (de Moraes and Hansen 1997) and human embryos (Sjoblom *et al.* 1999; Sjoblom *et al.* 2005).

A similar immunomodulating role for GM-CSF in sheep and goats has been suggested by reports that GM-CSF production by ovine uterine endometrial cells was doubled during pregnancy (McGuire *et al.* 2002). Furthermore, GM-CSF increased the production of ovine trophoblast interferon or interferon-tau (IFNτ) by the ovine

conceptus, a factor which plays a major role in maternal recognition of pregnancy and is necessary for the establishment of successful pregnancy (Imakawa *et al.* 1993; Imakawa *et al.* 1997; Rooke *et al.* 2005). Ovine trophoblast protein stimulated expression of the chemokine IFN-γ-inducible protein 10 kDa (IP-10) by the ovine endometrium (Nagaoka *et al.* 2003). In goats, IP-10 was reported to regulate lymphocyte populations (particularly natural killer cells) which were recruited into the endometrium in early pregnancy, causing increased IL-10 production and contributing to maternal immunomodulation towards the conceptus (Imakawa *et al.* 2005).

2.7.1.2 Immunological role of neutrophils post-insemination

Along with increased MHC class II expression in the epithelium and subepithelial layer of the endometrium following insemination, neutrophil numbers greatly increased (Bischof *et al.* 1994b; Kaeoket *et al.* 2003b). Both neutrophil numbers and antigen presentation by endometrial cells are under the influence of ovarian hormones, and oestrogens caused an increase in the number of endometrial neutrophils (McDonald *et al.* 1952; Tibbetts *et al.* 1999). Oestrogens also caused an increase in antigen presentation by epithelial cells, with progesterone causing a reduction, whereas the opposite occured in stromal cells (Wira and Rossoll 1995). Neutrophils, which are the predominant leukocyte involved in the post-insemination inflammatory reaction, produce a variety of cytokines involved in immune function, including TGF-β, TNF-α, IL-1, IL-8 and IFN-γ (Cassatella 1995; Yeaman *et al.* 1998). Additionally, expression of MHC class II antigens depends on the presence of cytokines such as IFN-γ (Clemens 1991). Taken together, this suggests that under the influence of oestrogens, neutrophils play a significant role in contributing to modulation of the immune response in the female reproductive tract (Cassatella 1995; Yeaman *et al.* 1998). Neutrophils also produce matrix metalloproteinases in the mouse endometrium in response to seminal plasma. From this, it has been postulated that neutrophils contribute to the degradation of extracellular matrix in the endometrium which is necessary for decidualisation and successful implantation of the murine embryo (Daimon and Wada 2005).

2.7.1.3 Immunological role of macrophages post-insemination

Closely following neutrophils, macrophages infiltrate reproductive tissues during the post-insemination inflammatory reaction (De *et al.* 1991; McMaster *et al.* 1992; Sanford *et al.* 1992). Macrophages present paternal antigens to maternal lymphocytes in conjunction with MHC class II molecules (Parkin and Cohen 2001). Macrophage distribution in the subepithelial and stromal tissue of the porcine endometrium had a similar pattern to the distribution of MHC class II molecules, suggesting they play a significant role in antigen presentation in this area. However, there were more MHC class II expressing cells than there are macrophages, indicating that other cell types are also involved (Kaeoket *et al.* 2002a; Kaeoket *et al.* 2002b; Kaeoket *et al.* 2003b). At least three different populations of antigen presenting cells have been identified in the murine uterus, including undifferentiated macrophages, mature macrophages and myeloid dendritic cells (Hudson Keenihan and Robertson 2004).

Macrophages increase markedly in the pregnant mouse uterus, and are capable of inhibiting the proliferation of maternal lymphocytes (Hunt *et al.* 1984; Hunt *et al.* 1985). However, antigen presenting cells such as macrophages and dendritic cells transport components of semen to draining lymph nodes following insemination, resulting in the activation of lymphocytes. Rather than this immune response causing rejection of paternal antigens, the presence of immunoregulatory molecules such as TGF-β in semen, may result in a state of maternal hypo-responsiveness in Type I immunity. This is believed to contribute to the creation of an immuno-tolerant environment, protecting the conceptus from a maternal immune response (Robertson *et al.* 1997; Johansson *et al.* 2004; Robertson 2005). Macrophages that are recruited into murine reproductive tissues following insemination are also likely to be involved in tissue remodelling and angiogenesis in the endometrium, thereby promoting embryo implantation and placental development (Robertson 2005).

2.8 Summary and Conclusion

The post-insemination inflammatory reaction in the female reproductive tract is a well recognised phenomenon and has been investigated in a number of different

species. However, the precise chain of events involved and the physiological significance in terms of reproductive success are not well understood. There is considerable disagreement between researchers regarding several aspects of the reaction and it is possible that the underlying mechanisms involved vary between species, as different components of semen have been implicated in eliciting the reaction in different species. However, it is not always clear whether these differences are representative of what occurs naturally, or are experimentally induced. There are also discrepancies between results from separate studies undertaken on the same species.

The post-insemination inflammatory reaction may have an important role in priming female reproductive tissues for pregnancy, and changes in the cytokine/leukocyte networks in the endometrium are thought to facilitate the development of maternal immunotolerance to the conceptus. If this is the case, aberrations in the cellular and molecular mechanisms involved are likely to contribute to reproductive failure. The mechanisms involved in the post-insemination inflammatory reaction need to be investigated further in order to fully define the processes involved. Recent research has attempted to identify causal links between insemination and the events occurring during the establishment of pregnancy, however current knowledge is incomplete. Areas that need further clarification include the cell types and cytokines involved, including any anatomical specific events, and their changes as the reaction progresses over time. Additionally, a causal link needs to be identified between the component(s) of semen and the mechanism(s) responsible for attracting leukocytes into reproductive tissues and the lumen.

CHAPTER 3
GENERAL MATERIALS AND METHODS

3.1 Animals

The animals utilised during this project were 46 non-parous Merino ewes 12-36 months of age, two teaser (androgenised) Merino wethers for oestrus detection, and eight Merino rams aged between 12 and 48 months. Ewes were purchased as required from graziers in North Queensland. The wethers and rams used in this project were resident within the large animal facilities at the School of Veterinary and Biomedical Sciences, James Cook University. Animal ethics approval was granted by the James Cook University Ethics Review Committee (Animal Ethics Number A 846_03).

All animals were vaccinated against clostridial diseases (Ultravac 5 in 1, CSL Ltd, Parkville, VIC Australia) and regularly treated for gastrointestinal parasites (Cydectin, Fort Dodge Australia Pty Ltd, Baulkham Hills, NSW Australia). Rams were grazed on irrigated natural pastures with *ad libitum* access to water. In addition, they received supplementary feed with 400 g per ram per day, of a pelleted protein and energy supplement (Barastoc Graze-R 30, Ridley Corporation Ltd, Sydney, NSW Australia). Ewes and wethers were grazed on natural pastures and had access to a protein and mineral supplement (Weangro, Coleman Stock Foods, Charters Towers, QLD Australia) between May and December. Wethers were androgenised by subcutaneous injections of 375 mg testosterone proprionate (Teprohormone, Virbac Animal Health, Peakhurst, NSW Australia) every 18 days.

3.2 Oestrus Synchronisation

Four ewes at a time were synchronised for oestrus by intravaginal insertion of progesterone containing devices (Eazi-breed CIDR sheep and goat device, Pharmacia & Upjohn Pty Ltd, Rydalmere, NSW Australia) for 13 days. Upon removal of the CIDR, oestrus was expected to commence within 36-48 hours. Twenty-four hours after removal of the CIDR, two ewes were placed in a yard with one androgenised

wether for oestrus detection. Wethers were fitted with a marker harness and crayon (Donaghys Ram Crayon, Donaghys Industries Ltd, Christchurch, New Zealand), with an apron hanging anterior to the prepuce to prevent intromission of seminal fluid (Figure 3.1). Once ewes were detected in oestrus, the time and date was noted and they were returned to pasture. Sixteen days later (the day prior to their expected return to oestrus) ewes were again placed with a harnessed wether for oestrus detection.

Figure 3.1 Detection of oestrus in two ewes by an androgenised wether. The wether is wearing a crayon harness with an apron attached to prevent intromission.

3.3 Mating

Fifteen ewes were mated naturally with the same ram (ram number 12). Mating occurred at the first return to oestrus following initial oestrus synchronisation. Ewes were checked for oestrus between 7:00 and 8:00 am and between 5:00 and 6:00 pm. Once detected in oestrus, ewes were mated immediately. For mating purposes, one ewe was placed in a pen with the ram for one hour. Intromission was observed to occur two to four times within the one hour session. The interval between repeat use of the ram was two to 14 days. Immediately after the mating period ended, a vaginal sample was taken from the ewe using a speculum and a syringe with a plastic 30 cm

artificial insemination tube attached to extend its length. The vaginal sample was examined under a light microscope for the presence of spermatozoa to confirm a successful mating.

3.4 Collection of Ram Semen

Semen was collected from seven rams (ram numbers 5, 9, 10, 15, 16, 17 and 18) by electro-ejaculation using standard procedures (Evans and Maxwell 1987). The ram was restrained in lateral recumbency and the penis was extruded. Cotton gauze was placed around the penis posterior to the glans to hold the penis extruded and direct it into a sterile plastic 15 ml screw top tube (Sarstedt Cat No 62.554.502, Germany). Ejaculation was achieved by placement of a rectal probe connected to a mobile electrical stimulator (Electrojec, Ratex Instruments, Mitcham, VIC Australia) which allowed stimulation of the male accessory glands and nerves to the penis. Electrical stimuli were applied for three seconds with three second breaks between each stimulation. Voltage was gradually increased from zero to five volts returning to zero between stimuli. A maximum of 16 stimulations were applied. On completion of semen collection, a small amount of antiseptic cream was applied to the penis before retraction into the prepuce, and the area was gently massaged for a minute.

3.5 Euthanasia

Fifteen mated ewes were killed for collection of reproductive tracts at three, six, 18, 24 or 48 hours after the end of the one hour mating session. Fifteen non-mated ewes were killed at similar time periods (plus one hour) after the onset of oestrus for use as controls. A further 12 ewes were killed 22 hours after surgical insemination. All ewes were killed humanely with an intravenous injection of 8ml of phenobarbitone (Lethabarb, Virbac Animal Health, Peakhurst, NSW Australia).

3.6 Collection of Samples

The entire reproductive tract was removed from each ewe immediately following euthanasia. Both ovaries were examined for the presence of a pre-ovulatory follicle,

corpus haemorrhagicum or corpus luteum, and the side it was on (left or right) was noted. For the purposes of investigating the inflammatory response in the uterine horns, rather than referring to left and right uterine horns, the uterine horn on the same side as the pre-ovulatory follicle or corpus haemorrhagicum was termed the ipsilateral horn. The other uterine horn was termed the contralateral horn.

3.6.1 Luminal secretion collection

Luminal secretions were collected from the vagina, mid-cervix, uterine body, left and right mid-uterine horns and left and right anterior uterine horns of all naturally mated (n = 15) and non-mated oestrus ewes (n = 15) and the volume recorded. Secretions were collected from the lumen of four ligated sections of the uterine horns and from the uterine body in surgically inseminated sheep (n = 12) and the volume recorded. The vagina, cervix, uterine body, mid-uterine horns (2.5 cm length) and anterior uterine horns (3 cm to compensate for differences in diameter) were incised using a scalpel to expose the lumen. Tissues were held in an everted position and two impression smears were made from each site, one onto a plain glass slide (Superfrost, Menzel-Glaser, Braunschweig, Germany) and the second onto a similar glass slide which had previously been silanised. Remaining luminal secretions were collected by application of 200 µl (naturally mated and control ewes) or 100 µl (surgically inseminated ewes) of sterile 0.9% saline per site and repeated aspiration of the fluid using Pasteur pipettes. Initially 50-100 µl of saline was applied to the tissue. This fluid was then aspirated back into the pipette and the process repeated 3-4 times to assist secretions to be flushed from the tissue. The samples were collected into plastic microtubes (Sarstedt Cat No 73.690, Germany) and kept on ice until further processing (up to one hour).

3.6.2 Tissue samples

From all naturally mated ewes (n = 15) and non-mated oestrous ewes (n = 15), two adjacent 15 x 2 mm tissue samples were collected using a scalpel blade from the anterior vagina, posterior cervix, mid-cervix, anterior cervix, uterine body, left and right mid-uterine horns, and left and right anterior uterine horns (Figure 3.2). From surgically inseminated ewes (n = 12), two adjacent 15 x 2 mm tissue samples were

collected from the central region (away from sutures) in the four ligated sections of the uterine horns and from the uterine body. At this time, ligatures were examined to ensure the lumen had been adequately sealed at each site.

The first tissue sample from each site was collected from fresh reproductive tracts and immersed in Tissue-Tek Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Torrance, California USA) in 24 x 24 x 5 mm plastic moulds (Pangalark Pty Ltd, Brisbane, QLD Australia). These moulds were placed on ice during collection. The remainder of the reproductive tract was then placed in a jar containing 4% neutral buffered formaldehyde. The second tissue sample from each site was collected from the fixed reproductive tract 48 hours later. In addition to the nine sites from which tissues were collected to be frozen, another formaldehyde fixed sample was collected from the cervical ostium (os).

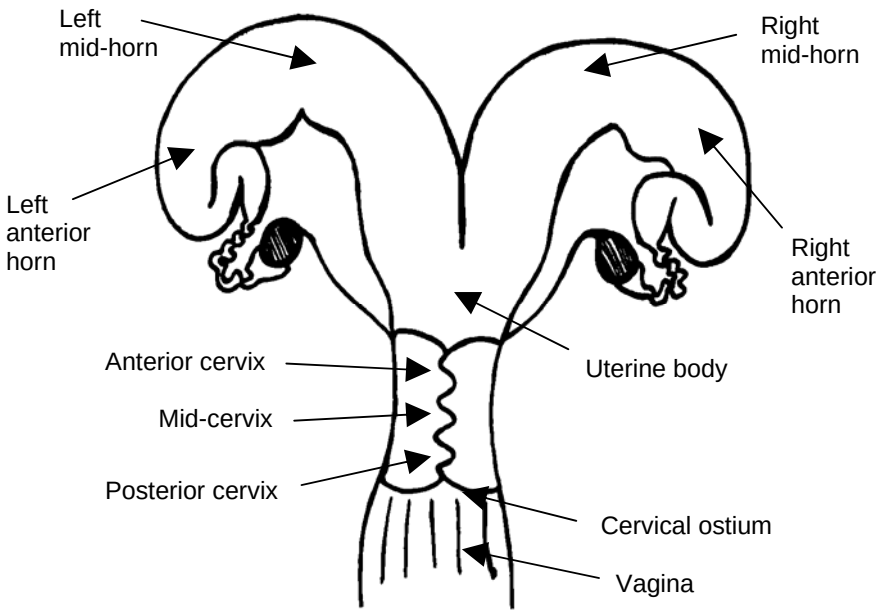

Figure 3.2 Sites of tissue sample collection from ewe reproductive tracts.

3.6.2.1 Additional tissue samples from preliminary work

A preliminary study was undertaken using formaldehyde-fixed reproductive tracts from 12 ewes used in previous work. Three ewes had been mated three hours prior to collection, four had been mated six hours prior to collection and five had been mated

24 hours prior to collection. Tissue samples were taken from 15 sites (the anterior vagina, posterior cervix, mid-cervix, anterior cervix, uterine body, left and right mid-uterine horns, left and right anterior uterine horns, left and right oviductal ampulla, left and right oviductal isthmus and left and right oviductal infundibulum).

3.7 Silanised Slides

Plain glass slides had a positive charge applied to their surface to aid adherence of mucus and tissue during immunohistochemistry. To do this, slides were placed in racks of thirty, soaked in tap water containing detergent (Sunshine GP, Sun-Chem, Gympie, QLD Australia) for two hours, rinsed in running tap water for half an hour and then sequentially rinsed in two baths of deionised water and two baths of absolute ethanol for five minutes each. Slides were dried at 37^{o}C for 10 minutes then submerged in a solution of 8ml silane (3-aminopropyltriethoxysilane; Sigma, Castle Hill, NSW Australia) in 400ml acetone for 45 seconds. The slides had a final rinse in deionised water for 30 seconds and were then dried overnight at 37^{o}C. One hundred and eighty slides (six racks) were silanised at a time, and then stored in cardboard slide boxes until required.

3.8 Initial Processing and Fixation of Samples

3.8.1 Luminal secretions

Luminal smears on plain glass slides were air dried, fixed in methanol and stained with a modified Wright's stain (Diff Quik, Harleco AHS/Australia Pty Ltd, Epping, NSW Australia). Once dry, glass coverslips were applied using dibutylphthalate polystyrene xylene (DPX)(BDH Laboratory Supplies, Poole England). Luminal smears on silanised slides were air dried and then fixed in acetone for 10 minutes at 4^{o}C. These slides were then placed in plastic storage boxes, sealed in plastic bags and frozen at -80^{o}C for later use in immunohistochemistry.

Microtubes containing luminal fluid were kept on ice until all samples were collected. Sample collection took approximately 45 minutes. The samples were then

mixed thoroughly using a vortex mixer and separated into three aliquots of approximately 50 µl each. Samples were then stored at -80^{o}C until required. Samples were thawed at room temperature immediately prior to use in ELISA.

3.8.2 Tissue samples

3.8.2.1 Frozen tissues

Tissue samples in plastic moulds containing OCT compound were snap frozen in liquid nitrogen. Once frozen, the blocks were removed from the moulds, wrapped in aluminium foil and stored at -80^{o}C until required. Before use, frozen blocks were brought up to -20^{o}C in a cryostat chamber. Frozen tissues were cut into 5 µm sections using a cryostat. Tissue sections were affixed to silanised glass slides and allowed to dry overnight at room temperature (RT). Tissues were fixed immediately prior to their use in immunohistochemistry.

The method of fixation used on frozen tissue sections was dependent on the particular antibody to be used. Several different chemicals, temperatures and times of fixation were tried. Chemicals tried included methanol, ethanol, acetone, standard 4% neutral buffered formaldehyde and 4% methanol-free formaldehyde (16% paraformaldehyde, Electron Microscopy Services, Hatfield, Pennsylvania USA) in PBS. Temperatures tried were RT, 4^{o}C and on ice. Times of fixation tried ranged from two minutes to 20 minutes. Many different combinations of fixative, temperature and time were tried. For cytokine identification, the optimal method of fixation determined was 4% methanol-free formaldehyde in PBS on ice for 20 minutes. For macrophage identification, the method of fixation used was 4% methanol-free formaldehyde in PBS at 4^{o}C for 10 minutes.

3.8.2.2 Formaldehyde fixed tissues

Formaldehyde-fixed tissue samples from each site in the reproductive tract were fixed in 4% neutral buffered formaldehyde for at least 48 hours. The samples were then processed overnight in an automatic tissue processor, through graduated ethanol and xylene, and then embedded in blocks of paraffin wax. The paraffin blocks were

placed on ice and allowed to harden (30 minutes) and tissue samples were then cut into 5 μm sections using a microtome. Two duplicate tissue sections were cut for each site. Tissue sections were floated on a 46^{o}C water bath and collected onto plain glass slides, which were then dried in racks at 60^{o}C for 30 minutes prior to staining.

3.9 Histology

Paraffin embedded tissue sections were deparaffinised and rehydrated by placing racks of slides sequentially in two xylene baths for two minutes each, three absolute ethanol baths for one minute each and then a five minute water bath. One copy of each tissue section was then stained with Haematoxylin and Eosin (H&E) and the second with Toluidine blue. After staining, the slides were rinsed briefly in water (approximately 30 seconds) and were then dehydrated through three absolute ethanol baths of one minute duration and three xylene baths of two minutes duration. Glass coverslips were applied to slides using DPX mounting media and slides were placed in a 37^{o}C oven to dry overnight.

3.9.1 Haematoxylin and Eosin stain

Once sections were deparaffinised and had been rinsed in water, one section from each site was stained with Mayer's Haematoxylin (Carson 1990) for eight minutes. Slides were then rinsed in water for five minutes and blued in Scott's tap water substitute (Drury and Wallington 1967) for 30 seconds, rinsed in water for five minutes and then stained with Young's Eosin (eosin [yellowish] 15 g, erythrosin 5 g, calcium chloride 5 g, water 2 L) for four minutes. Slides were used to identify and quantify neutrophils and eosinophils in reproductive tissues.

3.9.2 Toluidine Blue stain

Once sections were deparaffinised and had been rinsed in water, the second section from each site was stained with Toluidine blue O (ProScitech, Thuringowa, QLD, Australia) 0.2% in 60% ethanol for 5 minutes. Slides were used to identify and quantify mast cells in reproductive tissues.

3.10 Histological Examination

All tissue sections were examined at high power (400X) with a light microscope (Leitz Laborlux K). The same microscope was used for all microscopy. A 100 square Leitz eyepiece graticule was used to assist counting of cells. The area of the high power field of view (HPF) was determined using a stage micrometer (Olympus). Ten high power fields, each with an area of 0.15 mm^2 were examined on each tissue section, to give a total cell count for 1.5 mm^2 per site. Each of the 10 HPFs examined included the surface epithelium. The first high power field examined on each slide was chosen close to one edge of the tissue. Subsequent fields were chosen by sequentially skipping over 2-3 high power fields. Areas with missing epithelium or artefactual damage were avoided. When examining uterine tissues, cell counts were performed in areas of glandular endometrium and caruncles were avoided.

Vaginal and cervical tissues only required the superficial mucosa (including the surface epithelium) to be examined. However, due to the thicker nature of the mucosa in the uterus, deeper layers of the endometrium in all uterine tissues were also examined. In addition to the examination of 10 HPFs in the superficial endometrium, a further 10 HPFs in the mid-stroma and 10 HPFs in the deep stroma were examined. Mid-stromal tissue was examined approximately half way between the surface epithelium and the myometrium. Deep stromal tissue was examined adjacent to the myometrium (Figure 3.3). Leukocytes were identified and quantified in all tissue sections, and their distribution in tissues was examined and described.

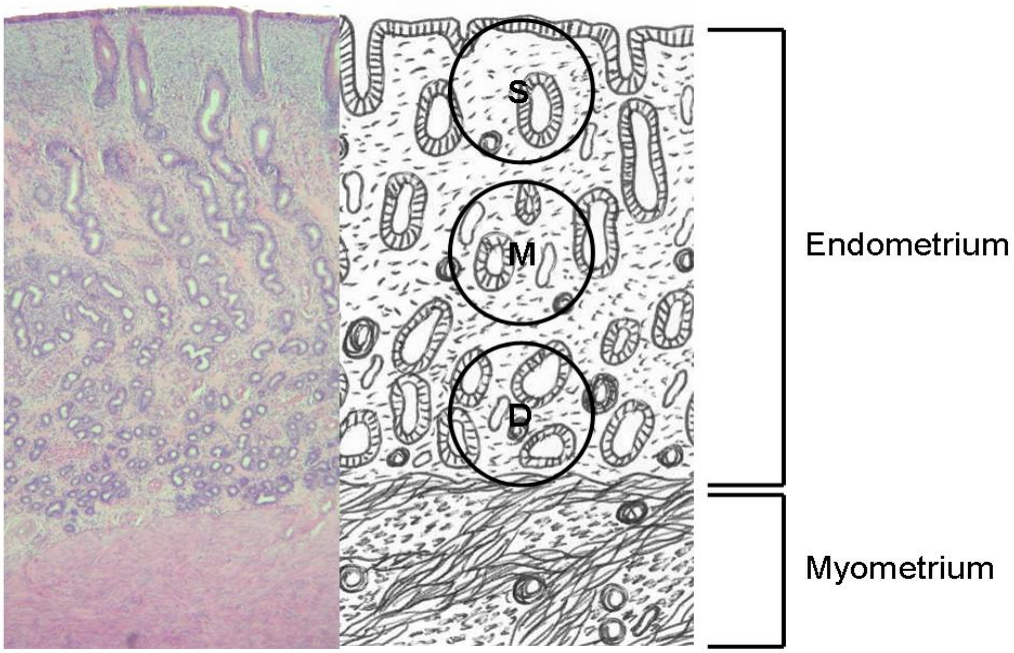

Figure 3.3 Photomicrograph (H&E stained) and diagram of endometrium showing positions of microscopic examination of high power fields of view. S = superficial endometrium, M = mid-stromal endometrium, D = deep stromal endometrium.

3.11 Buffer Solutions

3.11.1 Immunohistochemistry buffers

Phosphate buffered saline (PBS) at a pH of 7.4 was the standard buffer used for most immunohistochemical procedures. It was used as a wash buffer and as an antibody diluent. Analytical grade reagents were used in all buffer solutions. One litre of PBS was prepared by adding 8.0 g NaCl (Ajax Chemicals Pty Ltd, Auburn, NSW Australia), 0.2 g KCl (Fluka, Buchs, Switzerland), 1.75 g Na_2HPO_4 (Ajax Chemicals Pty Ltd, Auburn, NSW Australia) and 0.2 g KH_2PO_4 (Fluka, Buchs, Switzerland) to 800 ml of deionised water, whilst stirring. Once dissolved, deionised water was added to make a final volume of one litre. The pH was adjusted if necessary with HCl or NaOH.

Tris buffered saline (TBS) at a pH of 7.4 was tried in several immunohistochemical techniques. Tris buffered saline was prepared with 6.05 g

tris(hydroxymethyl)methylamine (Ajax Chemicals Pty Ltd, Auburn, NSW Australia) and 8.77 g NaCl made up to one litre with deionised water.

3.11.2 ELISA buffers

The ELISA coating buffer (used to coat ELISA plates with capture antibodies) for IL-8 and GM-CSF was a 0.05 M carbonate/bicarbonate buffer. It was made with 0.8 g Na_2CO_3 (Fluka, Buchs, Switzerland) and 1.46 g $NaHCO_3$ (Fluka, Buchs, Switzerland) made up to 500 ml with deionised water, at a pH of 9.6. The ELISA coating buffer for TGF-β was a 0.025 M carbonate/bicarbonate buffer. It was prepared by diluting 0.05 M coating buffer 50:50 in deionised water, at a pH of 9.7.

The ELISA blocking buffer (used to block non-specific binding of antibodies to ELISA plates) was made using PBS plus 0.1% casein (Sigma, Castle Hill, NSW Australia). The ELISA diluting buffer (used to dilute recombinant cytokines and antibodies) was made using PBS plus 0.5% tween 20 (Sigma, Castle Hill, NSW Australia) and 1% casein (Sigma, Castle Hill, NSW Australia).
The ELISA wash buffer for IL-8 and GM-CSF was made using PBS (see3.11.1) with 0.5% tween 20 (Sigma, Castle Hill, NSW Australia) included. The ELISA wash buffer for TGF-β was 20 mM Tris-HCl (pH 7.6), 150 mM NaCl with 0.05% tween 20. It was made using 20 ml of 1M Tris-HCl (121.14 g tris(hydroxymethyl)methylamine with 6 M HCl added to a pH of 7.6 in 1 L deionised water), 50 ml of 3 M NaCl (175.3g NaCl in 1 L deionised water) and 0.5 ml Tween 20 made up to 1 L with deionised water.

Dulbecco's phosphate buffered saline (DPBS) was used to dilute samples of ram semen for acidification prior to analysis for TGF-β content via ELISA. One litre of DPBS was prepared by adding deionised water to 8.0 g NaCl (Ajax Chemicals Pty Ltd, Auburn, NSW Australia), 0.2 g KCl (Fluka, Buchs, Switzerland), 1.15 g Na_2HPO_4 (Ajax Chemicals Pty Ltd, Auburn, NSW Australia) and 0.2 g KH_2PO_4 (Fluka, Buchs, Switzerland) to a final volume of 1000 ml deionised water whilst stirring. The pH was adjusted to 7.35 using 1 N HCl or 1 N NaOH if necessary. Once dissolved, 100 mg 1M $MgCl_2.6H_20$ (Ajax Chemicals Pty Ltd, Auburn, NSW

Australia) and 130 mg 1M $CaCl_2.2H_20$ (Merck, Darmstadt, Germany) was added and the solution was mixed thoroughly.

3.12 Immunohistochemistry

Once fixed, frozen tissue sections were immediately placed on a horizontal draining surface and covered in PBS. Immunohistochemical techniques were used to detect macrophages, GM-CSF and IL-8. Several different antibodies were tested, using numerous different techniques. Concentration of antibodies and solutions, buffers used, incubation times and temperatures, and sequence of steps were tried in many different combinations in order to optimise results.

3.12.1 Primary antibodies

3.12.1.1 Macrophages

1. Monoclonal mouse anti-sheep macrophage (42/44KD) antibody (clone VPM63, Cat No MCA918, Serotec, Oxford, England)
2. Monoclonal mouse anti-human macrophage antibody (clone MAC387, Cat No MCA874G, Serotec, Oxford, England)
3. Monoclonal mouse anti-CD14 antibody (clone CAM36A, VMRD, Pullman, Washington State USA)
4. Monoclonal mouse anti-human CD68 macrophage antibody (clone PG-M1, Cat No M0876, DakoCytomation Pty Ltd, Botany, NSW Australia)
5. Monoclonal mouse anti-human CD68 macrophage antibody (clone EBM11, Cat No M0719, DakoCytomation Pty Ltd, Botany, NSW Australia)

3.12.1.2 GM-CSF

1. Monoclonal mouse anti-sheep GM-CSF (clone 3C2, Cat No MCA1924, Serotec, Oxford England)
2. Biotinylated monoclonal mouse anti-sheep GM-CSF (clone 8D8, Cat No MCA1923B, Serotec, Oxford England)

3.12.1.3 IL-8

1. Monoclonal mouse anti-sheep IL-8 (clone 8M6, Cat No MAB1044, Chemicon, Temecula, California USA)
2. Polyclonal rabbit anti-sheep IL-8 (Cat No AB1840, Chemicon, Temecula, California USA & Cat No 1139, Epitope Technologies, Southbank, VIC Australia)

3.12.2 Secondary antibodies

When monoclonal primary antibodies were used, the preferred secondary antibody was a horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (STAR13B, Serotec, Oxford, England). When the primary antibody used was a polyclonal antibody raised in a rabbit, an HRP-conjugated pig anti-rabbit IgG antibody was used (P0217, DakoCytomation Pty Ltd, Botany, NSW Australia). A biotinylated goat anti-mouse/rabbit IgG antibody in conjunction with streptavidin-HRP (LSAB kit, DakoCytomation Pty Ltd, Botany, NSW Australia) was also tried with both monoclonal and polyclonal primary antibodies.

3.12.3 General immunohistochemical techniques

Most steps of the immunohistochemical process were carried out with the slides placed horizontally on a supporting framework over a sink. Incubation periods with primary and secondary antibodies were carried out in a humidified container (sealed plastic box with moist paper towel in the bottom). Generally 10-20 slides were processed simultaneously. Solutions were placed directly onto slides individually, either directly from a bottle as in the case of PBS, or via a pipette. Phosphate buffered saline was applied liberally and other solutions generally had 50 to 100 µl applied per slide. Apart from wash steps where PBS was tipped off and then immediately replaced with fresh PBS, between each step the preceding solution was tipped off and the slides were blotted dry (avoiding the area with tissue affixed).

3.12.3.1 Macrophage immunohistochemistry protocol for tissues

Frozen tissues were cut into 5 μm sections, affixed to silanised glass slide, placed in a slide rack and dried overnight at room temperature (RT). Tissues were fixed in 4% methanol-free formaldehyde for 10 minutes at 4^{o}C, rinsed in PBS for five minutes and 10% normal rabbit serum (TropBio, Douglas, QLD Australia) in PBS was applied for 10 minutes at RT. Excess fluid around tissue sections was blotted and the primary antibody: mouse anti-human CD68 macrophage antibody (clone EBM11, cat No M0719, DakoCytomation Pty Ltd, Botany, NSW Australia) was applied at 1/100 in PBS. Slides were placed in a humidified box and incubated for two hours at RT.

Slides were rinsed three times in PBS for five minutes each, then H_2O_2 0.6% (vol/vol) in PBS:methanol (50:50) was applied for 30 minutes at RT. Slides were rinsed in PBS for five minutes, then excess fluid around tissue sections was blotted and the secondary antibody (HRP-conjugated rabbit anti-mouse IgG (STAR13B, Serotec, Oxford, England) was applied at 1/50 in 10% normal sheep serum (collected from Merino wethers at James Cook University) in PBS. Slides were placed in a humidified box and incubated for one hour at 37^{o}C.

Slides were rinsed three times in PBS for five minutes each, then 3-3 diaminobenzadine tetrahydrochloride (DAB) (K3468; DakoCytomation Pty Ltd, Botany, NSW Australia) was applied for five minutes at RT. Slides were rinsed in water for five minutes and Mayer's haematoxylin was applied for five minutes. Slides were rinsed in water for 30 seconds, blued in Scott's tap water substitute for 30 seconds and rinsed in water for two minutes. Slides were dehydrated through absolute ethanol (three baths of 10 dips, 10 dips and one minute) and xylene (three baths for two minutes each) then mounted with coverslips using DPX.

3.12.3.2 Macrophage immunohistochemistry protocol for smears

Luminal smears on silanised glass slides were fixed in 4% methanol-free formaldehyde for one minute at 4^{o}C, rinsed in PBS for one minute, then 10% normal rabbit serum in PBS was applied for five minutes at RT. The primary antibody: mouse anti-human CD68 macrophage antibody (clone EBM11, cat No M0719,

DakoCytomation Pty Ltd, Botany, NSW Australia) was applied at 1/50 in PBS for 30 minutes at RT, then slides were rinsed three times in PBS for one minute each. The secondary antibody (HRP-conjugated rabbit anti-mouse IgG (STAR13B, Serotec, Oxford, England) was applied at 1/50 in 10% normal sheep serum in PBS for 30 minutes at RT. Slides were rinsed three times in PBS for one minute each, then DAB was applied for five minutes at RT. Slides were rinsed in water for five minutes and Mayer's haematoxylin was applied for two minutes. Slides were rinsed in water for 30 seconds, blued in Scott's tap water substitute for 30 seconds, and rinsed in water for two minutes. Slides were dehydrated through absolute ethanol (three baths of 10 dips, 10 dips and one minute) and xylene (three baths for two minutes each) then mounted with coverslips using DPX.

3.12.3.3 GM-CSF immunohistochemistry protocol

Frozen tissues were cut into 5 μm sections, affixed to silanised glass slides, placed in a slide rack and dried overnight at RT. Tissues were fixed in 4% methanol-free formaldehyde for 20 minutes on ice, rinsed in PBS for five minutes and 10% normal rabbit serum in PBS was applied for 30 minutes at RT. Excess fluid around tissue sections was blotted and the primary antibody: mouse anti-sheep GM-CSF antibody (clone 3C2, Cat No MCA1924, Serotec, Oxford England) was applied at 1/50 in PBS. Slides were placed in a humidified box and incubated for 2 ½ hours at RT.

Slides were rinsed three times in PBS for five minutes each, then H_2O_2 0.6% in PBS:methanol (50:50) was applied for 30 minutes at RT. Slides were rinsed in PBS for five minutes, then excess fluid around tissue sections was blotted and the secondary antibody: HRP-conjugated rabbit anti-mouse IgG (STAR13B, Serotec, Oxford England) was applied at 1/50 in 10% normal sheep serum in PBS. Slides were placed in a humidified box, incubated for one hour at 37^{o}C.

Slides were rinsed three times in PBS for five minutes each, then DAB was applied for five minutes at RT. Slides were rinsed in water for five minutes and Mayer's haematoxylin was applied for two minutes. Slides were rinsed in water for 30 seconds, blued in Scott's tap water substitute for 30 seconds, and rinsed in water for

two minutes. Slides were dehydrated through absolute ethanol (three baths of 10 dips, 10 dips and one minute) and xylene (three baths for two minutes each) then mounted with coverslips using DPX.

3.12.3.4 IL-8 immunohistochemistry protocol

Frozen tissues were cut into 5 μm sections, affixed to silanised glass slides, placed in a slide rack and dried overnight at RT. Tissues were fixed in 4% methanol-free formaldehyde for 20 minutes on ice, rinsed in PBS for five minutes, then 10% normal pig serum (collected from pigs at James Cook University) in PBS was applied for 30 minutes at RT. Excess fluid around tissue sections was blotted and the primary antibody: polyclonal rabbit anti-sheep IL-8 antibody (Cat No AB1840, Chemicon, Temecula, California USA) was applied at 1/500 in PBS. Slides were placed in a humidified box and incubated overnight at 4^{o}C.

Slides were rinsed three times in PBS for five minutes, H_2O_2 0.6% in PBS:methanol (50:50) was applied for 30 minutes at RT, then slides were rinsed in PBS for five minutes. Excess fluid around tissue sections was blotted and the secondary antibody: HRP-conjugated pig anti-rabbit IgG (P0217, DakoCytomation Pty Ltd, Botany, NSW Australia) was applied at 1/100 in 10% normal sheep serum and 10% normal pig serum in PBS. Slides were placed in a humidified box and incubated for one hour at RT.

Slides were rinsed three times in PBS for five minutes and DAB was applied for five minutes at RT. Slides were rinsed in water for five minutes and Mayer's haematoxylin was applied for two minutes. Slides were rinsed in water for 30 seconds, blued in Scott's tap water substitute for 30 seconds, and rinsed in water for two minutes. Slides were dehydrated through absolute ethanol (three baths of 10 dips, 10 dips and one minute) and xylene (three baths for two minutes each) and mounted with coverslips using DPX.

3.12.4 Controls

Each batch of tissues that underwent immunohistochemical staining was accompanied by three control slides. Control slides contained sections of tissue that were known to be positive for the antigen in question. The first control was a positive control slide. It was treated in exactly the same manner as the tissues being tested. It needed to show a positive result for comparison against test tissues. The second control slide had the primary antibody omitted and replaced with PBS. It needed to be negative to show there was no non-specific binding to tissue by the secondary antibody. The third control slide had both the primary and the secondary antibodies omitted and replaced with PBS. This slide needed to be negative to show that the chromagen (DAB) was not binding to endogenous peroxidase in the tissue. All three control slides needed to show the expected result in order for the batch to be deemed successful. If any of the slides were not adequate, the entire batch was repeated.

3.12.4.1 Macrophage positive control

For macrophage identification, the positive control sections were cut from a granuloma known to contain many macrophages. A granulomatous reaction was induced in the subcutaneous tissue behind the right shoulder of a ewe by injection of 1ml of Freunds Complete Adjuvant (Sigma, Castle Hill, NSW Australia) subcutaneously. One week later, the area of reaction was surgically removed after subcutaneous injection of local anaesthetic (Lignocaine 20, Troy Laboratories Pty Ltd, Smithfield, NSW Australia). The skin was sutured using 3-0 silk (Ethicon, Somerville, New Jersey USA) and the sutures were removed 14 days later. The tissue collected from the surgical site was cut into 2 mm x 15 mm sections, placed in OCT compound, frozen in liquid nitrogen and stored at -80^{o}C until required.

3.12.4.2 Cytokine positive controls

For cytokine immunohistochemistry, the positive control sections were cut from uterine tissue that had previously stained positive for the particular cytokine. Other sheep tissues (lymph nodes, tonsils and Peyer's patches from the ileum) were initially analysed for the presence of GM-CSF and IL-8, but showed no positive

staining. The positive control tissues used were from the uterine body and uterine horns of mated ewes. The tissues used were changed during the course of the work in order to retain some tissue from each site.

3.13 Immunohistochemical Examination

3.13.1 Macrophage identification

Similar to examination of tissue sections for other cell types, 10 HPFs that included the surface epithelium were examined under a light microscope for each tissue section. In uterine tissues, 10 additional HPFs were examined in both the mid- and deep stromal tissue. Macrophages were identified and quantified, and their distribution in tissue sections was examined.

3.13.2 Cytokine identification

Slides that underwent immunohistochemistry for the presence of cytokines were examined at high power, compared against control tissues and scored for intensity of staining: none (-), mild (+), moderate (++) or strong (+++) staining (Figure 3.4)

3.14 Cytology

3.14.1 Smears stained by Diff Quik

Luminal smears that were stained with Diff Quik were used to identify and quantify neutrophils. Where possible, a total of 500 cells, including leukocytes and epithelial cells were enumerated. Only individual cells were counted, and cells clumped together were not included. If there were less than 500 cells on a slide, all cells present were counted. This occurred in several of the cervical smears. The number of neutrophils was expressed as a percentage of total cells present on the slide.

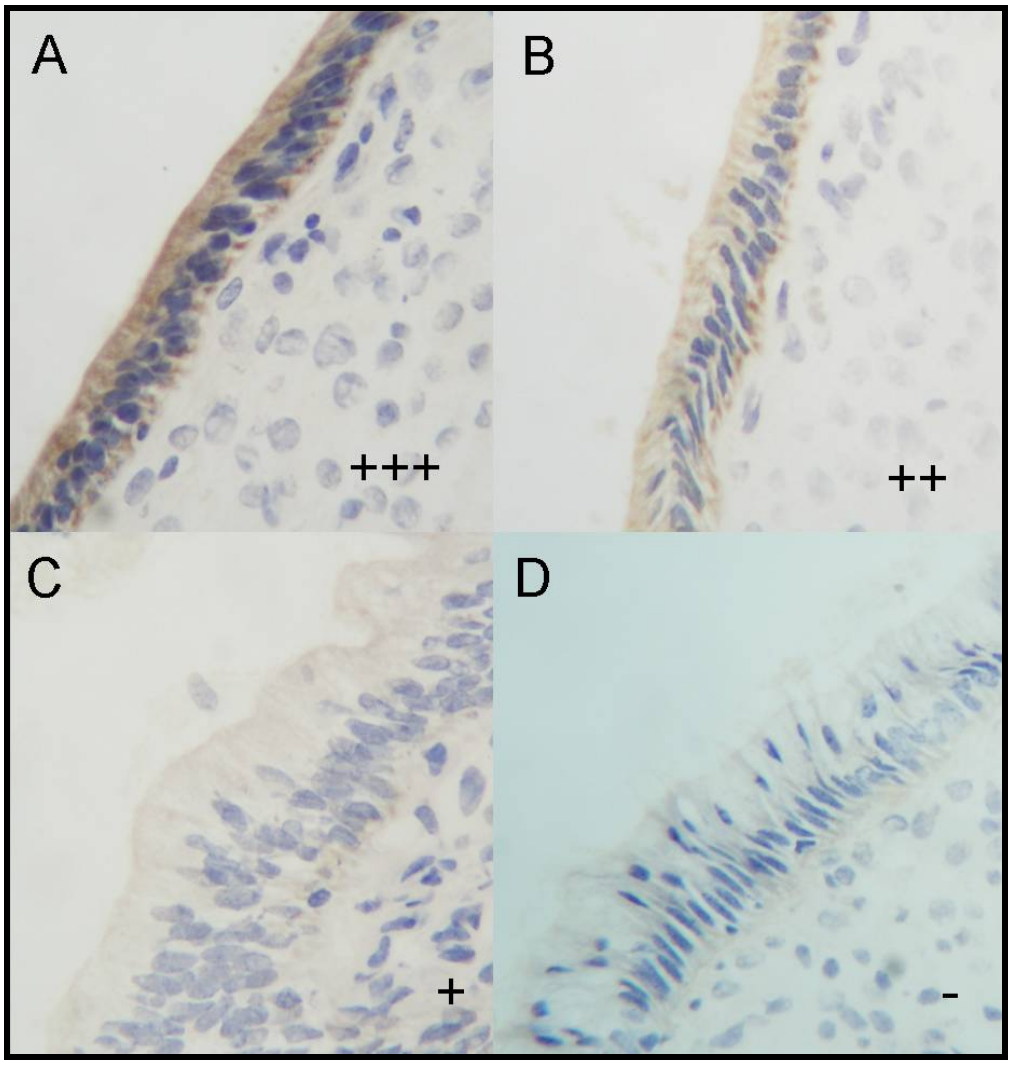

Figure 3.4 Staining intensity scoring for cytokines: (A) strong (+++), (B) moderate (++), (C) mild (+) and (D) negative (-).

3.14.2 Smears stained by immunohistochemistry

Frozen smears were thawed and immunohistochemistry was used to identify and quantify macrophages. The primary antibody used was the same as that used on tissue sections (mouse anti-human CD68 antibody). The secondary antibody used was the peroxidase-conjugated rabbit anti-mouse antibody. The procedure performed was a shortened version of that used to stain macrophages by immunohistochemistry in tissue sections. Rinses in PBS were brief (less than 2 minutes) and both the primary and secondary antibody incubations were for 30 minutes at RT. The hydrogen peroxide step was omitted. The haematoxylin counterstain step was for 5 minutes.

Where possible, a total of 500 cells were counted. If there were less than 500 cells on a slide (as in some cervical smears), all cells present were counted. The number of macrophages present was expressed as a percentage of total cells.

3.15 Detection of Cytokines in Luminal Fluid

Luminal fluid collected from each site in each ewe reproductive tract was analysed by enzyme linked immunosorbent assay (ELISA), using antibodies specific for ovine GM-CSF and IL-8. Before use, fluids were removed from the -80^{o}C freezer and thawed at room temperature.

3.15.1 GM-CSF ELISA protocol

The ELISA procedure used to detect GM-CSF in ovine tissues (Entrican *et al.* 1996) was based on a standard protocol available from Serotec (Serotec, Oxford England). Mouse anti-sheep GM-CSF antibody (clone 3C2, Cat No MCA1924, Serotec, Oxford England) was diluted to 10 µg/ml (1/100 dilution ie 100 µl in 10ml for whole plate) in coating buffer and 100 µl was added to each well of the ELISA plate (Maxisorp, Nunc, Roskilde, Denmark). Plates were incubated overnight at 4^{o}C. Solution was flicked from the wells and 200 µl of blocking buffer was added to all wells. Plates were incubated at RT for one hour then washed once.

A standard curve was set up in duplicate in rows A and B. Firstly, 2.56 µl of recombinant sheep GM-CSF (courtesy of Dr Gary Entrican, Moredun Research Institute, Edinburgh Scotland) was diluted in 400 µl of diluting buffer, and 200 µl was added to the first well of rows A and B. Then 100 µl of diluting buffer was added to the remaining wells in rows A and B, and 100 µl was transferred from the first well in row A to the second well in row A. The contents of the second well in row A were mixed five times with a pipette and 100 µl was transferred to the third well. The 1/2 dilution was continued across the plate to the second last well (column 11). The 100 µl remaining from the second last well was discarded once mixed. The the last well (column 12) was left as as a buffer only control. The process was

repeated for row B. This gave standard concentrations of GM-CSF (in ng/ml) in rows A and B of: 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 and 0.

The samples to be tested were set up by adding 66 µl of diluting buffer and 33 µl of each sample to the remaining wells (1/3 dilution). Plates were incubated at RT for one hour then washed four times. The biotinylated mouse anti-sheep GM-CSF antibody (clone 8D8, Cat No MCA1923B, Serotec, Oxford England) was diluted to 5 ng/ml (1/200000: ie 0.2 µl in 400 µl, then 100 µl of this into 10 ml for whole plate) in diluting buffer and 100 µl was added to each well. Plates were incubated at RT for one hour then washed four times. Streptavidin-HRP (BD Pharmingen, North Ryde, NSW Australia) was diluted to 1/1000 (10 µl in 10 ml for whole plate) in diluting buffer and 100 µl was added to each well. Plates were incubated at RT for one hour, washed five times, then 100 µl of 3,3',5,5'tetramethylbenzidine (TMB) (T0440, Sigma, Castle Hill, NSW Australia) was added to each well and plates were incubated at RT for 10 minutes. The reaction was stopped by adding 100 µl of 0.5M H_2SO_4 to each well, and the plates were read on a micro-plate reader at 450 nm.

3.15.2 IL-8 ELISA protocol

The ELISA procedure used to detect IL-8 in ovine tissue (Caswell *et al.* 1998) was based on that supplied by Dr Peter McWaters (CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, VIC Australia). Mouse anti-sheep IL-8 antibody (clone 8M6, Cat No MAB1044, Chemicon, Temecula, California USA) was diluted to 5 µg/ml (1/200 dilution ie 50 µl in 10ml for whole plate) in coating buffer and 100 µl was added to all wells of the ELISA plate (Maxisorp, Nunc, Roskilde, Denmark). Plates were incubated overnight at 4^{o}C. Solution was flicked from the wells and 200 µl of blocking buffer was added to all wells. Plates were incubated at RT for one hour then washed once.

A standard curve was set up in duplicate in rows A and B. Firstly, 0.2 µl of recombinant sheep IL-8 (courtesy of Dr Peter McWaters, CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, VIC Australia) was diluted in 300 µl of diluting buffer (1/1500 dilution) and 100 µl was added to the first well of rows A and B. Then 50 µl of diluted IL-8 standard and 100 µl of diluting

buffer was added to the second well of rows A and B and 100 µl of diluting buffer was added to the remaining wells in rows A and B. The contents of the second well in row A were mixed five times with a pipette, and 50 µl was transferred to the third well. The 1/3 dilution was continued across the plate to the second last well (column 11). The 50 µl remaining from the second last well was discarded once mixed. The last well (column 12) was left as a buffer only control. The process was repeated for row B. This gave standard concentrations of IL-8 (in ng/ml) in rows A and B of: 110, 36.7, 12.2, 4.07, 1.36, 0.45, 0.15, 0.05, 0.017, 0.0056, 0.0019 and 0.

The samples to be tested were set up by adding 66 µl of diluting buffer and 33 µl of each sample to the remaining wells (1/3 dilution). Plates were incubated at RT for one hour then washed four times. The rabbit anti-sheep IL-8 antibody (Cat No AB1840, Chemicon, Temecula, California USA or Cat No 1139, Epitope Technologies Pty Ltd, Southbank, Vic Australia) was diluted to 1/2000 (5 µl in 10 ml for whole plate) in diluting buffer and 100 µl was added to each well. Plates were incubated at RT for one hour then washed four times. The HRP-conjugated pig anti-rabbit IgG antibody (PO217, DakoCytomation Pty Ltd, Botany, NSW Australia) was diluted to1/1000 (10 µl in 10 ml for whole plate) in diluting buffer and 100 µl was added to each well. Plates were incubated at RT for one hour, washed five times, then 100 µl of TMB was added to each well and plates were incubated at RT for 10 minutes. The reaction was stopped by adding 100 µl of 0.5M H_2SO_4 to each well. Plates were read on a micro-plate reader at 450 nm.

3.16 Preparation of Samples for Surgical Insemination

3.16.1 Spermatozoa diluent (TALP) preparation

A modified version of Tyrode's albumin-lactate-pyruvate (TALP) medium (Parrish *et al.* 1988) was used as a diluent for spermatozoa for surgical insemination of ewes. It was prepared using 584.4 mg NaCl (Sigma, USA), 23.11 mg KCl (BDH Chemicals), 29.4 mg $CaCl_2$ (Ajax Chemicals, Australia), 3.81 mg $MgCl_2$ (Sigma, USA), 210.1 mg $NaHCO_3$ (Ajax Chemicals, Australia), 4.68 mg $NaH_2PO_4.2H_2O$ (BDH Chemicals, Australia), 242.14 mg L-Lactic acid (Sigma, USA), 238.3 mg

HEPES (Sigma, USA), 11 mg sodium pyruvate (Sigma, USA), 600mg BSA-V (Sigma, USA) made up to 100 ml in deionised water. It was sterilised by filtration and stored in a sterile container at 4^{o}C for up to two weeks. Prior to surgery, a 250 µl sample of TALP was placed in a sterile microtube and kept at 37 ^{o}C until required.

3.16.2 Whole semen preparation

Ten µl of ram semen was added to 190 µl of TALP (1/20 dilution) in a sterile plastic microtube and gently mixed. Two µl was placed into a semen analyser, spermatozoa concentration was determined in three fields of view and the average was calculated. The concentration of spermatozoa in a 250 µl sample was then adjusted to 1.5 x 10^{9} per ml, which is the approximate minimal concentration of spermatozoa found in normal ram semen (Miller 1991), by adding seminal plasma (see Appendix 23). The final whole semen sample prepared contained 375 x 10^{6} spermatozoa in 250 µl of seminal plasma. It was placed in a sterile microtube kept at 37^{o}C until required.

3.16.3 Seminal plasma preparation

Seminal plasma was prepared using methods previously described for rams (Graham 1994). Semen was spun at 300 ***g*** for 10 minutes at 23^{o}C in a sterile 15 ml plastic tube. Seminal plasma was removed using a sterile pipette, placed in a new sterile tube and respun at 1400 ***g*** for 10 minutes at 23^{o}C. The supernatant was removed, placed in another sterile tube and spun again at 1400 ***g*** for 10 minutes at 23^{o}C. A drop was placed on a glass slide with a coverslip and examined under a phase-contrast microscope for the presence of spermatozoa. If spermatozoa were present, the supernatant was respun in a new tube and re-examined. Once sperm-free, 250 µl of seminal plasma was placed in a sterile microtube and kept at 37^{o}C until required.

3.16.4 Washed spermatozoa preparation

Washed spermatozoa were prepared using methods previously described for rams (Graham 1994). The pellet remaining after initial centrifugation and removal of seminal plasma (see 3.16.3) was resuspended in 10 ml of TALP and spun at 300 ***g*** for eight minutes at 23^{o}C. The supernatant was removed and the spermatozoa were

resuspended in another 10 ml of TALP and respun for eight minutes at 23^{o}C. The supernatant was removed and spermatozoa were resuspended in 1 ml of TALP. The concentration of spermatozoa was determined using the semen analyser and the concentration was adjusted to 1.5 x 10^{9} per ml. (see Appendix 23). The final washed spermatozoa sample prepared contained 375 x 10^{6} spermatozoa in 250 µl of TALP. It was placed in a sterile microtube and kept at 37^{o}C until required.

3.16.5 Antibiotic preparation for surgical treatments

Penicillin (Penicillin G: sodium salt; Sigma, Castle Hill, NSW Australia) and streptomycin (Streptomycin sulphate; Sigma, Castle Hill, NSW Australia) were added to all five treatments in selected oestrous and luteal ewes. The recommended antibiotic dose for intrauterine insemination of ewes is 1000 IU of sodium penicillin and 1 mg of streptomycin per ml of diluent, which is subsequently added to semen at a 1:1 to 1:3 dilution (Evans and Maxwell 1987). This results in final amounts of 500-750 IU penicillin and 500-750 µg streptomycin per ml. For this study, penicillin was used at 700 IU per ml and streptomycin at 750 µg/ml.

3.17 Surgical Insemination of Ewes

Two groups of ewes were used for surgical insemination. The first group were nine ewes that were in oestrus at 8:00 am on the morning of scheduled surgery. The second group were seven ewes in the mid-luteal phase of the oestrous cycle (8 days following the onset of oestrus).

3.17.1 Anaesthesia of ewes

Food and water were withheld from ewes overnight. Each ewe selected for surgery was kept with another ewe for company in order to reduce psychological stress. Anaesthesia was induced by an intravenous injection of 10 mg/kg thiopentone sodium (Thiobarb powder: Jurox Pty Ltd, Rutherford, NSW Australia) into the jugular vein. Ewes were placed in dorsal recumbency on an operating table with a slight decline toward the head, and intubated with an endotracheal tube (9.0 mm

tracheal tube: SIMS Portex Ltd, Kent UK) (Figure 3.5). Anaesthesia was maintained using halothane (Halothane BP: Laser Animal Health, Salisbury, QLD Australia) delivered via a closed circle anaesthetic machine. Depth of anaesthesia was monitored continuously by auscultation of heart rate and observation of respiratory rate, eye position and palpebral reflexes.

Figure 3.5 An anaesthetised ewe positioned in dorsal recumbency on an operating table. The ewe has an endotracheal tube in place which is connected to a closed circle anaesthetic machine supplying gaseous halothane.

3.17.2 Surgical technique

The technique used for surgical insemination of ewes was based on a model developed previously at James Cook University (Hilla 1999). Once anaesthetised, ewes were placed in dorsal recumbency and the ventral abdomen was clipped and cleaned thoroughly with antiseptic solution. Throughout surgery, aseptic techniques were adhered to. A ventral midline incision was made and the uterine horns were exteriorised. Ovaries were examined for the presence of a pre-ovulatory follicle

(oestrous ewes) or a corpus luteum (luteal phase ewes). The uterine horns were ligated with 4-0 PDS 11 monofilament Polydioxanone suture material (Johnson and Johnson Medical PTY LTD, North Ryde NSW, Australia) using purse-string sutures that were carefully placed in the myometrium. A second suture was placed around the site of each purse-string suture to ensure the lumen was completely closed. Each uterine horn had three sutures placed so as to be divided into two adjacent sections. The posterior sections were approximately 2 cm in length and the anterior sections 3 cm long to compensate for the smaller diameter.

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Each ligated section of the uterine horns had one treatment fluid inserted using a blunt 23G needle and a 1ml tuberculin syringe (Terumo Philippines Corporation, Laguna, Phillipines). Ligated sections were selected at random to receive each treatment. The treatments were:

1. 0.1 ml of whole semen containing 150 x 10^6 spermatozoa
2. 0.1 ml of seminal plasma
3. 0.1 ml of washed spermatozoa in TALP containing 150 x 10^6 spermatozoa
4. 0.1 ml TALP

The uterine body received 0.1 ml of physiological saline (0.9% sodium chloride intravenous infusion BP, Baxter Healthcare Pty Ltd, Toongabbie, NSW Australia). Once all five treatments had been inserted into the ligated sections of uterine horns, the uterus was replaced and the abdominal wall was closed using 4-0 metric chromic catgut (Ethicon Catgut: Ethicon 2000, Norderstedt, Germany) in a simple continuous suture pattern. Subcutaneous tissue was sutured with catgut in a simple interrupted suture pattern and the skin was closed with mattress sutures using 0.4 mm Vetafil (Vetafil Bengen: Clements Stanson Medical, North Ryde NSW Australia). An intramuscular injection of 400 mg oxytetracycline (Engemycin 100: Intervet Australia Pty Ltd, Bendigo, VIC Australia) was administered in the hind leg before returning the ewe to a pen.

3.18 Detection of Transforming Growth Factor-beta in Semen

Transforming growth factor beta-1 concentration in ram semen was measured using a TGF-β1 ELISA kit (Promega, Cat G7591, Annandale, NSW Australia) following the manufacturers instructions.

Immediatedly following collection of ram seminal plasma, a portion of each sample was acid treated. Samples were diluted by adding 130 µl of DPBS to 130 µl of seminal plasma (1/2 dilution). Then 5 µl of 1N HCl was added to each sample, and samples were mixed. It was verified that the pH was 3.0 or lower using pH indicator strips (non-bleeding pH 0-14: Merck, Darmstadt Germany) and samples were incubated for 15 minutes at RT. Samples were then neutralised by adding 5 µl of 1N NaOH, and the pH was verified to be approximately 7.6. Untreated and acid treated seminal plasma samples were stored at -20^{o}C until required.

The monoclonal anti-TGFβ1 antibody was diluted to 1 µg/ml (1/1000 dilution: ie 10 µl in 10 ml for whole plate) in coating buffer and 100 µl was added to all wells of the ELISA plate (Maxisorp, Nunc, Roskilde, Denmark). Plates were incubated overnight at 4^{o}C. The TGFβ 1X blocking buffer was prepared by adding 5.6 ml of TGFβ 5X blocking buffer to 22.4 ml deionised water. Plates were warmed to RT (10-15 minutes), solution was flicked from the wells and 270 µl of TGFβ 1X blocking buffer was added to each well. Plates were incubated for 35 minutes at 37^{o}C then washed once. The 1X sample buffer was prepared by adding 3.3 ml of TGFβ sample 10X buffer to 29.7 ml of deionised water.

A standard curve was set up in duplicate in columns 1 and 2. The TGFβ1 standard was diluted to 1000 pg/ml (1/1000 dilution: ie 5 µl in 245 µl, then 25 µl of this into 475 µl) in 1X sample buffer, then 200 µl of diluted TGFβ1 standard was added to the first well in columns one and two. One hundred µl of 1X sample buffer was added to wells B through to H in columns one and two, then 100 µl was transferred from well A in columns one and two to wells B in these columns. The contents were mixed five times with a pipette and 100 µl was transferred to wells C. The 1/2 dilution was continued down the plate to wells G. The 100 µl remaining from wells G were discarded once mixed. No TGFβ1 standard was added to wells H. This gave standard concentrations of TGFβ (in pg/ml) in the first two columns of: 1000, 500, 250, 125, 62.5, 31.2, 15.6 and 0.

Acidified and natural ram seminal plasma samples were thawed at RT prior to analysis. Serial dilutions of acidified ram seminal plasma were performed in duplicate. Two hundred µl of each acidified sample was mixed with 200 µl of 1X

sample buffer (1/2 dilution) and 200 µl of this was placed into wells A in columns three and four. Then 100 µl of 1X sample buffer was added to wells B through to H in columns three to 12, and 100 µl from wells A in columns three and four was transferred to wells B in these columns. The contents of wells B were mixed five times with a pipette and 100µl was transferred to wells C. The 1/2 dilution was continued down the plate to wells H. The 100 µl remaining from wells H were discarded once mixed. This gave final dilutions for each sample of 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256 and 1/512.

Serial dilutions of natural ram seminal plasma samples were performed in duplicate. One hundred µl of seminal plasma was mixed with 300 µl of 1X sample buffer (1/4 dilution) and 200 µl was placed into wells A in columns five and six, then 100 µl from wells A in columns five and six was transferred to wells B in these columns. The contents were mixed and 100 µl was transferred to wells C. The 1/2 dilution was continued down the plate to wells H. The 100 µl remaining from wells H were discarded once mixed. This gave final dilutions of 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256 and 1/512. Steps 8 and 9 were repeated for two more ram semen samples in columns seven to ten. As another control, serial dilutions of natural boar semen (Premier Pig Genetics, Wacol, QLD Australia) were performed in column 11 and acidified boar semen in column 12 as per step 9.

Plates were incubated for 90 minutes at 37^{o}C then washed five times. The polyclonal anti-TGFβ1 antibody was diluted to 1/1000 (10 µl in 10 ml for whole plate) in 1X sample buffer, and 100 µl was added to each well. Plates were incubated at RT for two hours then washed five times. One hundred µl of stock TGFβ HRP conjugate was diluted in 9.9 ml of 1X sample buffer and 100 µl was added to each well. Plates were incubated at RT for two hours, washed five times, then 100 µl of TMB was added to each well. Plates were incubated at RT for 15 minutes. The reaction was stopped by adding 100 µl 1N HCl to each well. Plates were read on a micro-plate reader at 450 nm.

CHAPTER 4
LEUKOCYTE CHANGES IN RESPONSE TO INSEMINATION

4.1 Introduction

Insemination causes an accumulation of leukocytes in the subepithelial stroma of the female reproductive tract of many species. This cellular infiltrate is comprised of mostly neutrophils and macrophages, although other cell types including lymphocytes and eosinophils are also involved (De *et al.* 1991; McMaster *et al.* 1992; Sanford *et al.* 1992). The post-insemination inflammatory reaction has been extensively investigated in the rodent, with a lesser amount of research carried out in domestic animals and humans.

The aims of this study were to: 1) determine the cell types involved in the post-insemination inflammatory reaction in the reproductive tract of the ewe, 2) describe the distribution of these cells in tissue from various sites in the reproductive tract, and 3) describe the changes in cell types, numbers and distribution over the 48 hour time period following mating and following oestrus without mating.

4.2 Materials and Methods

A preliminary investigation was undertaken using 12 formaldehyde-fixed reproductive tracts collected from mated ewes at three, six or 24 hours following mating (see 3.6.2.1). Ewes were killed (see 3.5) and tissue samples and luminal smears were collected from the reproductive tracts of a further 30 ewes (see 3.6.1 & 3.6.2). Half were collected at various times (three, six, 18, 24 & 48 hours) following mating (see 3.3) and the rest were collected from non-mated ewes at similar times following the onset of oestrus (plus one hour to parallel the mating period).

4.2.1 Neutrophils and eosinophils

Formalin fixed tissue samples from 15 sites (see 3.6.2.1) in each of 12 reproductive tracts from the preliminary study and from 10 sites (see 3.6.2) in the reproductive

tracts of a further 30 ewes were processed (see 3.8.2.2), stained with H&E (see 3.9.1) and examined for the presence of neutrophils and eosinophils (see 3.10). Luminal smears from seven sites (see 3.6.1) in 30 ewe reproductive tracts were stained with Diff Quik (see 3.8.1) and examined for the presence of neutrophils and eosinophils.

4.2.2 Mast Cells

Formalin fixed tissue samples from 10 sites (see 3.6.2) in the reproductive tracts of 30 ewes were processed (see 3.8.2.2), stained with Toluidine blue (see 3.9.2) and examined for the presence of mast cells (see 3.10).

4.2.3 Macrophages

Tissue from nine sites (see 3.6.2) in the reproductive tracts of 30 ewes were frozen (see 3.8.2.1) and stained immunohistochemically (see 3.12.3.1) using a mouse anti-human CD68 macrophage antibody (clone EBM11, Cat No. M0719, DakoCytomation Pty Ltd, Botany, NSW Australia). Tissue sections were examined for the presence of macrophages (see 3.10). Luminal smears from seven sites (see 3.6.1) in 30 ewe reproductive tracts were fixed in acetone (see 3.8.1) and stained immunohistochemically (see 3.12.3.2) using the same antibodies as used in tissue sections. Macrophage numbers were expressed as a percentage of total cells counted.

4.2.3.1 Macrophage antibody optimisation

Several anti-macrophage antibodies were evaluated in attempts to stain macrophages in ovine tissue. During initial antibody trials, a positive control tissue (subcutaneous granuloma) was used, as it was known to contain numerous macrophages. For each antibody, many different techniques were tried in an attempt to optimise staining. A variety of concentrations of antibodies, different buffer solutions, variable incubation times and temperatures, and different sequences of steps were tried in many different combinations. In addition to the variety of techniques tried for other anti-macrophage antibodies, when using the second anti-macrophage antibody listed below (MAC387), different nuclear stains and different chromagens were also tried. In addition to Mayer's haematoxylin, nuclear stains tried were methyl green

(Proscitech, Thuringowa, QLD Australia), neutral red (Difco Laboratories, Surrey, UK) and nuclear fast red (British Drug Houses, London, UK). In addition to DAB, the chromagen AEC (3-amino-9-ethylcarbazole) (Zymed, S. San Francisco, California USA) was tried. This required the use of an aqueous mounting medium (GVA mount: Zymed, S. San Francisco, California USA).

Anti-macrophage antibodies tried were:

1. Mouse anti-sheep macrophage (42/44KD) antibody (clone VPM63, Cat No MCA918, Serotec, Oxford, England)
2. Mouse anti-human macrophage antibody (clone MAC387, Cat No MCA874G, Serotec, Oxford, England)
3. Mouse anti-CD14 antibody (clone CAM36A, VMRD, Pullman, Washington State USA)
4. Mouse anti-human CD68 macrophage antibody (clone PG-M1, Cat No M0876, DakoCytomation Pty Ltd, Botany, NSW Australia)
5. Mouse anti-human CD68 macrophage antibody (clone EBM11, cat No M0719, DakoCytomation Pty Ltd, Botany, NSW Australia)

The optimum technique for staining macrophages in ovine tissues and luminal smears used the mouse anti-human CD68 macrophage antibody (clone EBM11, cat No M0719, DakoCytomation Pty Ltd, Botany, NSW Australia) and a HRP-conjugated rabbit anti-mouse IgG (STAR13B, Serotec, Oxford, England) secondary antibody (see 3.12.3.1 and 3.12.3.2).

4.3 Statistical Analyses

4.3.1 Statistical analyses of cell counts in tissues

Leukocyte counts from tissue sections were not normally distributed, therefore Log_{10} transformation was used to convert data into normal distributions (see Appendix 1). Parametric tests were used to analyse the transformed data. Three way ANOVA were used to compare mean cell counts for mated versus control ewes, different time periods and different sites in the reproductive tract (see Appendix 2). When

significant differences were detected, *post hoc* tests (Fisher LSD: least significant difference) were used to locate these differences (see Appendix 3). Student's *t*-tests were used to compare cell counts in mated and control ewes for each site in the reproductive tract (see Appendix 4) and each time period (see Appendix 5). Pearson's correlation coefficients were used to identify correlations between mast cell numbers and eosinophil numbers in tissues. Additionally, cell count data were simplified by combining the 10 sites in the reproductive tract into three main sites based on histological structure. These sites were the vagina (vagina and cervical ostium, both lined by stratified squamous epithelium), the cervix (posterior, mid- and anterior cervix, all lined by simple columnar epithelium with goblet cells) and the uterus (uterine body and uterine horns, all with a glandular endometrium lined by simple columnar epithelium). The simplified data were analysed using one way ANOVA to compare cell counts in each of the three sites (see Appendix 6).

4.3.2 Statistical analyses of cell counts in luminal smears

Cell counts from smears were not normally distributed, and could not be converted into normal distributions by transformation. Therefore non-parametric tests were used to analyse the data. Mann-Whitney U tests were used to compare cell counts between mated and non-mated ewes for each site (see Appendix 7) and for each time period (see Appendix 8). Kruskal-Wallis tests were used to compare cell counts at different time periods for each site in the reproductive tract for mated and for control ewes separately (see Appendix 9). For all statistical analyses performed, a *P* value of less than 0.05 was considered significant.

4.4 Results

4.4.1 Neutrophils

4.4.1.1 Neutrophil infiltration of tissues

A preliminary investigation of 12 reproductive tracts collected from mated ewes used in previous work indicated an increase in neutrophil numbers in reproductive tissues

following mating. Non-mated reproductive tracts were not available as controls, so comparisons were made between the different time periods post-mating. In the vagina and cervix there were more neutrophils present at six hours post-mating than at either three or 24 hours post-mating. Uterine tissues showed an earlier response, with more neutrophils present at three hours than at six or 24 hours post-insemination (Figure 4.1). Neutrophils were mostly distributed in the subepithelial stroma, with some in an intraepithelial location. They were rarely observed in the oviducts (isthmus, ampulla or infundibulum). Neutrophil infiltration following mating appeared to occur only in the vagina, cervix and uterus and did not extend into the oviducts. Further investigations therefore focused on the vagina, cervix and uterus.

Neutrophil counts in tissues from a further 30 ewes demonstrated a significant increase (P <0.01) in neutrophil numbers in the female reproductive tract following mating, with numbers in mated ewes higher than non-mated ewes at all sites examined (Table 4.1). Significantly more neutrophils were present in the cervical os (P <0.01), posterior cervix (P <0.01) and the superficial endometrial stroma of the uterine body (P <0.05) of mated than control ewes. There were also significantly more neutrophils in tissues from mated compared to control ewes at six hours (P <0.05), 18 hours (P <0.01) and 24 hours (P <0.05) post-mating (Figure 4.2).

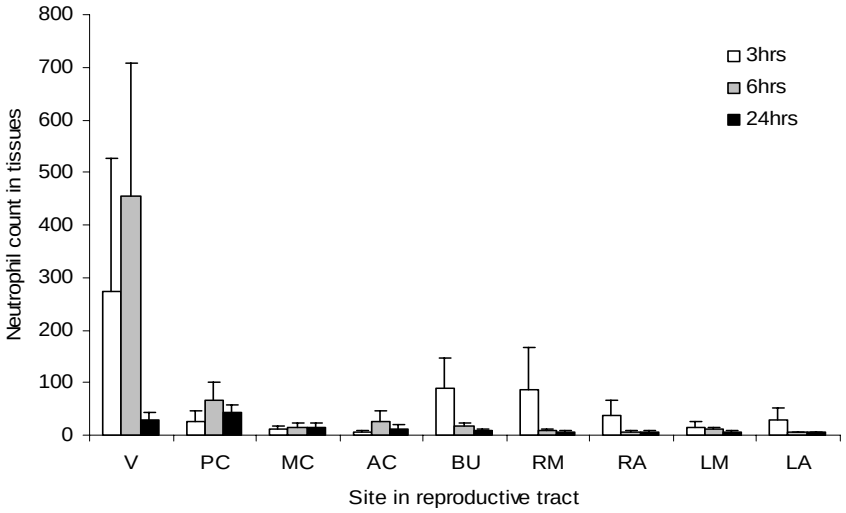


Figure 4.1 Neutrophil numbers in reproductive tissues of ewes at 3hrs (n=3), 6hrs (n=4) and 24hrs (n=5) post-mating. Data are presented as the mean + SEM per 1.5mm^2 tissue. Sites in the reproductive tract were: V, vagina; PC, posterior cervix; MC, mid-cervix; AC, anterior cervix; BU, uterine body; RM, right mid-uterine horn; RA, right anterior uterine horn; LM, left mid-uterine horn; LA, left anterior uterine horn. Mean values for uterine tissues were from cell counts in the superficial endometrium.

Table 4.1 Neutrophil numbers in reproductive tissues of mated and non-mated ewes.

	Control ewes (n=15)			Mated ewes (n=15)		
Site	Superficial	Mid-	Deep	Superficial	Mid-	Deep
V	60.7±28.4 [c]			80.0±28.6 [c]		
OC	48.7±35.9 [c]			135.1±52.6 [ac]		
PC	4.1±1.3			23.7±7.9 [a]		
MC	1.9±0.8			3.5±0.8		
AC	1.7±0.4			3.0±0.8		
BU	11.5±4.1	2.5±1.0	0.3±0.2	34.5±13.0 [b]	13.6±8.5	5.3±4.7
IM	8.7±3.6	1.0±0.5	0.3±0.3	17.8±6.2	8.3±4.2	6.0±5.3
IA	11.2±3.8	1.2±0.4	0.5±0.3	18.1±6.1	4.9±2.8	15.8±12.6
CM	14.0±6.5	1.5±0.8	0.5±0.3	22.6±7.4	8.7±5.6	13.3±9.5
CA	9.5±3.0	1.6±0.8	1.1±0.5	19.7±7.4	17.7±11.1	26.1±17.7

Data are presented as the mean ± SEM cell counts in 1.5mm^2 tissue. V, vagina; OC, cervical os; PC, posterior cervix; MC, mid-cervix; AC, anterior cervix; BU, uterine body; IM, ipsilateral mid-uterine horn; IA, ipsilateral anterior uterine horn; CM, contralateral mid-uterine horn; CA, contralateral anterior uterine horn. Superficial, mid- and deep refer to depth in endometrium. [a] P <0.01, [b] P <0.05 compared with same site in control ewes; [c] P <0.01 compared with other sites in the reproductive tract.

Table 4.2 Neutrophil numbers in luminal smears of mated and non-mated ewes.

	Site in reproductive tract						
	V	C	BU	IM	IA	CM	CA
Controls (n=15)	13.4±4.2 [c]	0.8±0.3	0.5±0.2	0.4±0.1	0.3±0.1	1.1±0.5	0.7±0.3
Mated (n=15)	24.6±5.9 [c]	15.4±6.1 [a]	3.1±1.7 [b]	2.1±1.3	1.0±0.5	3.0±1.6	1.1±0.3

Data are presented as the mean ± SEM percentage of neutrophils in luminal smears. V, vagina; C, cervix; BU, uterine body; IM, ipsilateral mid-uterine horn; IA, ipsilateral anterior uterine horn; CM, contralateral mid-uterine horn; CA, contralateral anterior uterine horn. [a] P <0.01, [b] P <0.05 compared with same site in control ewes; [c] P <0.01 compared with other sites in the reproductive tract.

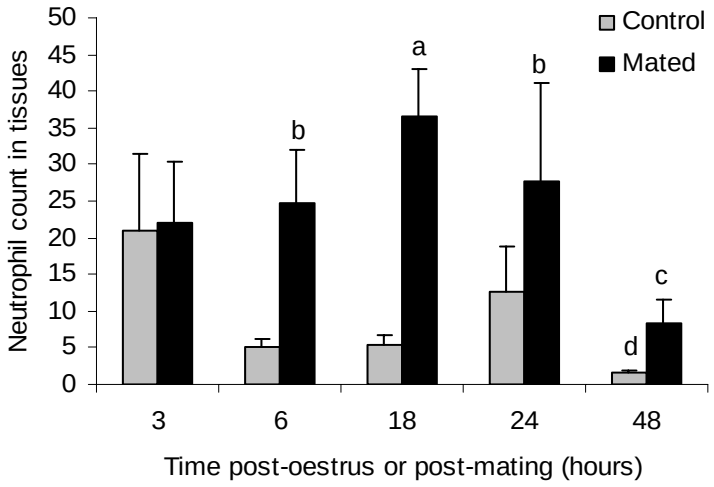


Figure 4.2 Neutrophil numbers in reproductive tissues of control and mated ewes at various times post-oestrus (controls) and post-mating. Data are presented as the mean + SEM (n=60) in 1.5mm^2 tissue. [a] P <0.01, [b] P <0.05 compared with control ewes; [c] P <0.01 compared with all other times post-mating; [d] P <0.05 compared with 3 hours post-oestrus.

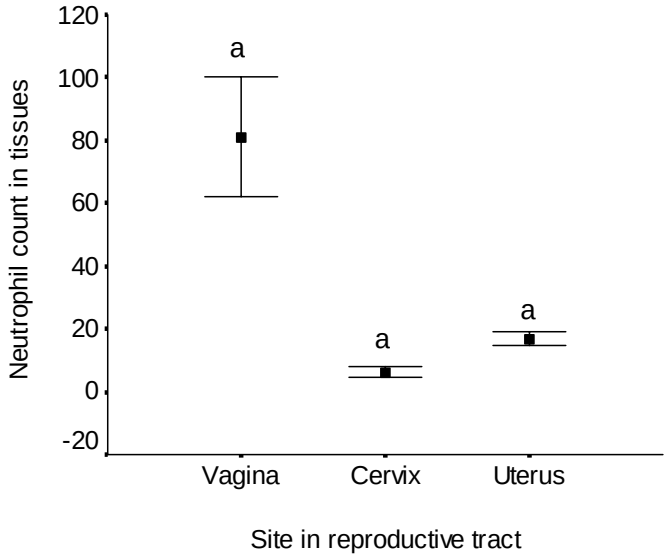


Figure 4.3 Neutrophil numbers in different sites in the reproductive tract of ewes when sites were combined into vagina (n = 60), cervix (n = 90) or uterus (n = 150). Data are presented as the mean ± SEM in 1.5mm^2 tissue. Data from different time periods and from both mated and control ewes were pooled. Values for uterine tissues were from cell counts in the superficial endometrium. [a] P <0.01 compared with other sites.

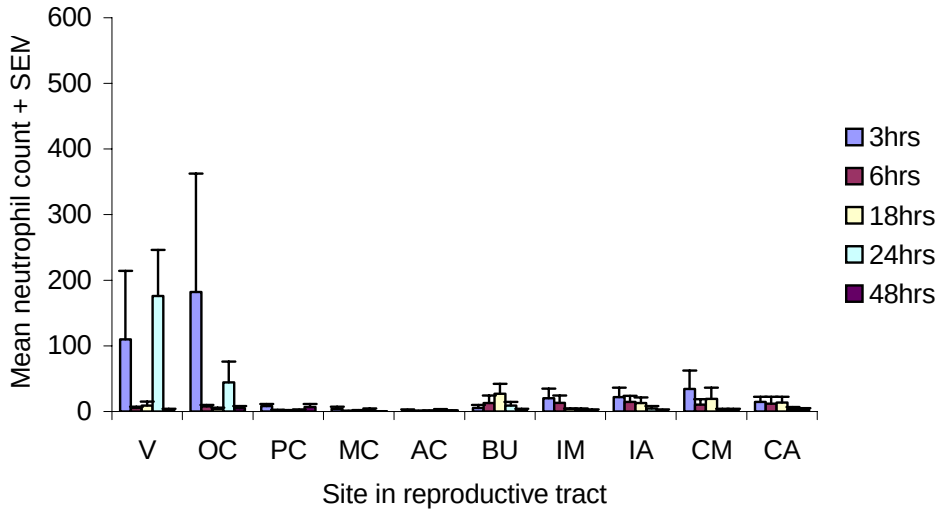
The number of neutrophils in the uterine body and horns peaked at six hours post-mating and then declined. In the posterior cervix, the number of neutrophils steadily increased over time to peak at 48 hours (Figure 4.4B) whereas in the cervical os, numbers increased by six hours, declined somewhat, then peaked at 24 hours. Neutrophils in the vagina peaked at three hours, fluctuated slightly then declined by 48 hours. The number of neutrophils was significantly lower (P <0.01) by 48 hours post-mating than at any other time measured (Figure 4.2). In control ewes, the number of neutrophils in the uterine body peaked at 24 hours post-oestrus, whereas in the uterine horns numbers peaked by three hours and then steadily declined (Figure 4.4A). By 48 hours neutrophil numbers had declined to their lowest levels in all sites, and were significantly less (P <0.05) than at three hours following the onset of oestrus (Figure 4.2).

The number of neutrophils in the vagina and cervical os of both mated and non-mated ewes was significantly higher (P <0.01) than in all other sites in the reproductive tract (Table 4.1). The next highest number of neutrophils occurred in tissue from the uterine body, which had significantly higher (P >0.01) numbers than the mid- and anterior cervix, which had the lowest numbers of all sites examined. The number of neutrophils in the posterior cervix, uterine body and uterine horns were not significantly different from one another. When data were combined into vagina, cervix or uterus, the vagina had the highest number of neutrophils, followed by the uterus and then the cervix, all of which were significantly different (P <0.01) from one another (Figure 4.3).

The distribution of neutrophils within tissues varied between different sites in the reproductive tract. In vaginal tissue, the cervical os and the endometrium, neutrophils accumulated in the subepithelial stroma, immediately subjacent to the base of the epithelium (Figure 4.5A), whereas most neutrophils in the posterior cervix were clustered in occasional subepithelial foci in the tips of villi, with very few neutrophils in the crypts. In uterine tissues, neutrophil distribution varied according to depth in the endometrium. Unlike the uniform subepithelial distribution in the superficial stroma, neutrophils in the mid- and deep endometrial stroma were generally sparse or scattered diffusely. There were also some foci of intense neutrophil accumulation, often near the base of the glandular epithelium (Figure 4.5B). By 24 hours post-

mating, most neutrophils in the vagina and cervical os were in an intraepithelial location, with fewer neutrophils in the subepithelial stroma.

A



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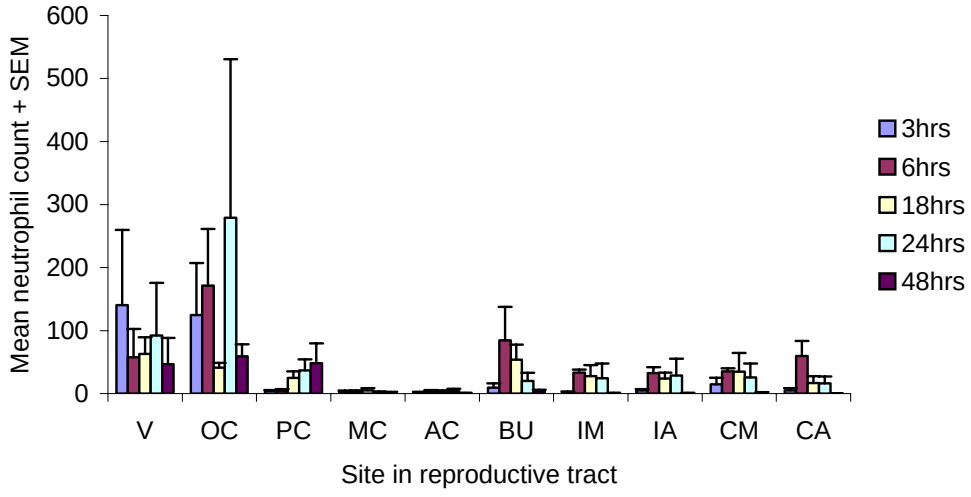


Figure 4.4 Neutrophil numbers in reproductive tissues of (A) control ewes and (B) mated ewes at different sites and different time periods following oestrus or mating. Data are presented as the mean neutrophil percentage of total cells + SEM (n = 3). V, vagina; OC, cervical os; PC, posterior cervix; MC, mid-cervix; AC, anterior cervix; BU, uterine body; IM, ipsilateral mid-uterine horn; IA, ipsilateral anterior uterine horn; CM, contralateral mid-uterine horn; CA, contralateral anterior uterine horn. Values for uterine tissues are from cell counts in the superficial endometrium.

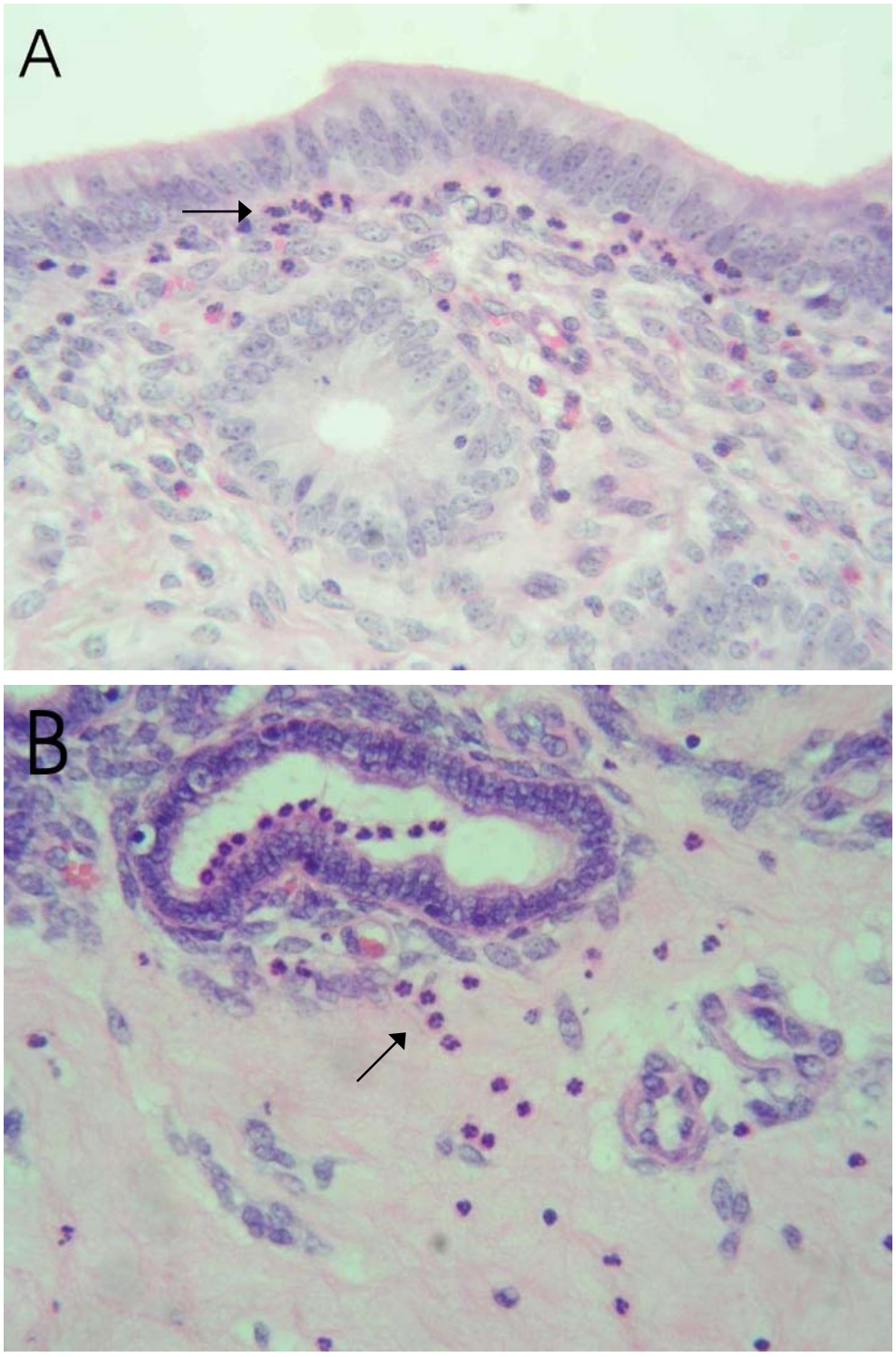


Figure 4.5 Photomicrographs of neutrophils in reproductive tissues from a ewe 18 hours post-mating: (A) evenly distributed in a subepithelial location in the superficial endometrium (arrow) and (B) in a deep stromal cluster near a gland in the endometrium (arrow).

4.4.1.2 Neutrophils in the lumen

Neutrophils in luminal smears were expressed as a percentage of total cells counted. Other cells present were mostly epithelial cells, with occasional erythrocytes, macrophages, eosinophils and lymphocytes. Numbers of neutrophils were significantly higher in smears from the cervix ($P < 0.01$) and the uterine body ($P < 0.05$) of mated ewes than control ewes (Table 4.2). Smears from the vagina and uterine horns of mated ewes also had higher neutrophil counts when compared to the same sites in non-mated ewes, but the differences were not statistically significant.

Neutrophils were present in the lumen of all sites of the reproductive tract examined for both mated and non-mated ewes. Numbers of neutrophils were significantly higher ($P < 0.01$) in smears collected from the vagina than from any other site in the reproductive tract, both when sites were examined individually and when they were combined into vagina, cervix or uterus (Figure 4.6). Smears from the cervix had the second highest number of neutrophils recorded, but this was not significantly different from the number of neutrophils present in uterine smears.

There were significantly more ($P < 0.01$) neutrophils in smears from mated than control ewes at three, 18 and 48 hours post-mating (Figure 4.7). In smears from the cervix of mated ewes, the highest number of neutrophils occurred at three hours post-mating, with reduced numbers at six hours and then another increase at 18 hours post-mating (Figure 4.8B). After 18 hours the number of neutrophils declined, but by 48 hours, numbers were still higher than in cervical smears of non-mated ewes. In vaginal smears from mated ewes, the number of neutrophils at six hours was less than at three hours post-insemination but numbers then rose again to peak at 24 hours post-mating. In the uterus, the highest number of neutrophils in smears was seen at 18 hours, except for in the right mid-horn which showed a peak at three hours, then another smaller peak at 18 hours post-mating. Vaginal smears from control ewes had a decline in neutrophils from three hours through to 18 hours, then a rise in numbers at 24 hours, before declining to very low numbers at 48 hours post-oestrus (Figure 4.8A). Other sites had very few neutrophils present.

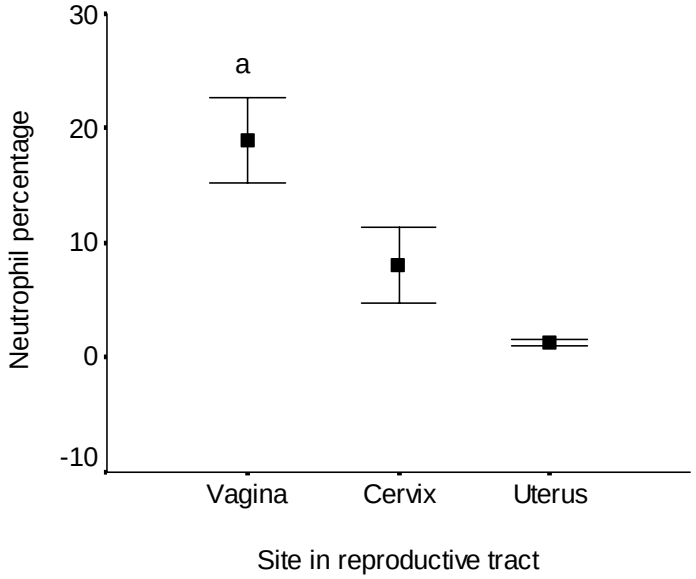


Figure 4.6 Neutrophil percentages in smears from different sites in the reproductive tract of ewes when sites were combined into vagina (n = 30), cervix (n = 30) or uterus (n = 150). Data are presented as the mean ± SEM. Data from different time periods and from both mated and control ewes were pooled. [a] P <0.01 compared to other sites.

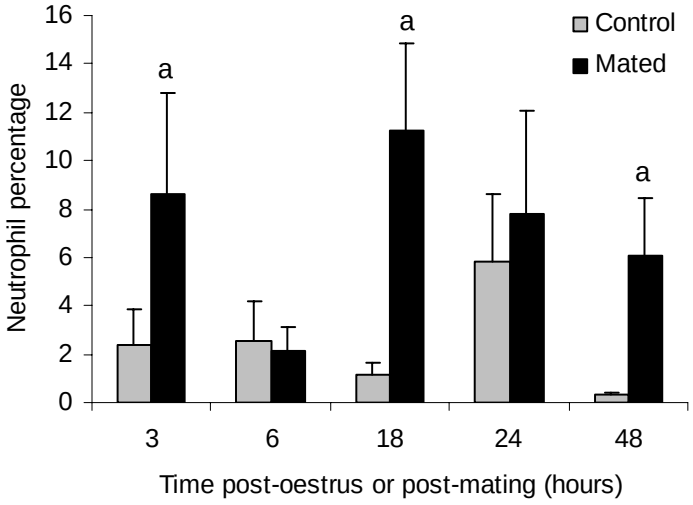
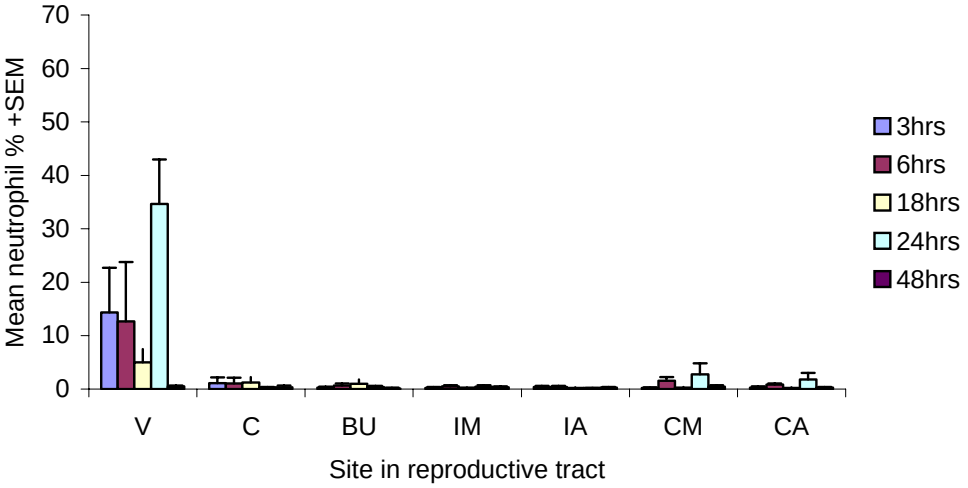


Figure 4.7 Neutrophil percentages in smears from the reproductive tract lumen of control and mated ewes at various times post-oestrus (controls) and post-mating. Data are presented as the mean + SEM (n=21) percentage of total cells in smears. [a] P <0.01 compared with control ewes.

A



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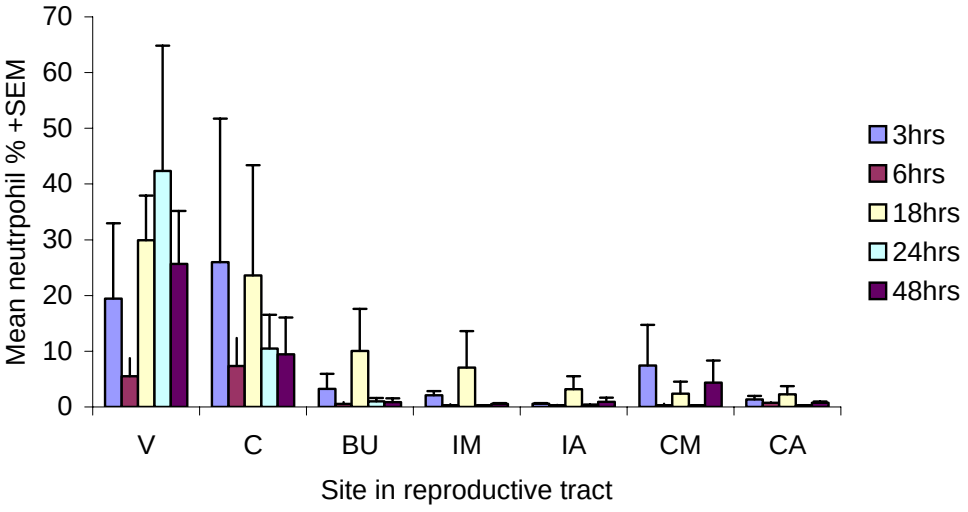

Figure 4.8 Neutrophil percentages in mucus smears of (A) control ewes and (B) mated ewes at different sites and different time periods following oestrus or mating. Data are presented as the mean neutrophil percentage of total cells + SEM (n = 3). V, vagina; PC, posterior cervix; MC, mid-cervix; AC, anterior cervix; BU, uterine body; IM, ipsilateral mid-uterine horn; IA, ipsilateral anterior uterine horn; CM, contralateral mid-uterine horn; CA, contralateral anterior uterine horn.

4.2.1 Eosinophils

There was no significant difference in the number of eosinophils between control and mated ewes. However, there were significantly more (P <0.01) eosinophils in the superficial endometrial stroma of the uterine body of both control and mated ewes than in any other site in the reproductive tract (Table 4.3). Numbers of eosinophils in the vagina, cervical os and uterine horns were not significantly different from one another, but were significantly higher than eosinophil counts from the posterior, mid- and anterior cervix (P <0.05). When eosinophil data were simplified by being combined into vagina, cervix or uterus, uterine tissues contained the highest number of eosinophils, followed by the vagina and then the cervix, all of which were significantly different (P <0.01) from one another (Figure 4.9).

The number of eosinophils present and their distribution were highly variable, being abundant in the reproductive tissues of some ewes but scarce in others. Distribution of eosinophils also varied between tissues and within a single piece of tissue. They were distributed diffusely in the stroma or located in intense foci with up to 70 counted per high power field (Figure 4.10A). An occasional eosinophil was observed in smears from the cervix and uterine body and horns, but there was no difference in the number between mated and control ewes.

4.4.3 Mast cells

Mast cells were generally easily distinguishable by their metachromatic cytoplasmic granules, however some had fewer granules, were paler staining and were less readily identified. The number of mast cells present in tissues varied at different time periods following oestrus or insemination. In control ewes, significantly more (P <0.05) mast cells were present at three, six and 18 hours than at 24 or 48 hours post-oestrus. Following mating, significantly more (P <0.05) mast cells were present at three hours and at 48 hours than at six, 18 or 24 hours. Fewer (P <0.01) mast cells were found in tissues from mated ewes compared with control ewes at six and 18 hours post-mating, and more (P <0.01) by 48 hours post mating (Figure 4.11).

Table 4.3 Eosinophil numbers in reproductive tissues of mated and non-mated ewes.

	Control ewes (n=15)			Mated ewes (n=15)		
Site	Superficial	Mid-	Deep	Superficial	Mid-	Deep
V	17.7±7.4			21.2±7.5		
OC	11.3±3.5			16.8±11.4		
PC	4.2±2.5			1.1±0.4		
MC	0.7±0.4			0.5±0.2		
AC	1.1±0.4			2.7±2.2		
BU	105.0±38.5 [ab]	17.9±6.8	3.5±1.5	84.3±23.3 [ab]	19.7±8.9	6.5±2.3
IM	21.6±7.6	8.5±3.0	7.3±2.9	7.8±2.4	4.7±1.5	5.9±2.1
IA	18.9±5.4	8.0±2.7	7.2±2.9	39.7±14.2 [b]	17.7±6.9	11.8±4.7
CM	41.2±24.2	8.9±3.7	7.8±3.6	13.9±3.8 [a]	3.1±1.1	8.6±2.3
CA	27.1±12.3	11.3±3.2	7.3±1.9	35.9±20.3 [b]	13.1±5.3	11.4±8.1

Data are presented as the mean ± SEM cell counts in 1.5mm^2 tissue. V, vagina; OC, cervical os; PC, posterior cervix; MC, mid-cervix; AC, anterior cervix; BU, uterine body; IM, ipsilateral mid-uterine horn; IA, ipsilateral anterior uterine horn; CM, contralateral mid-uterine horn; CA, contralateral anterior uterine horn. Superficial, mid- and deep refer to depth in endometrium. [a] P <0.05 compared with mid-endometrium; [b] P <0.05 compared with deep endometrium.

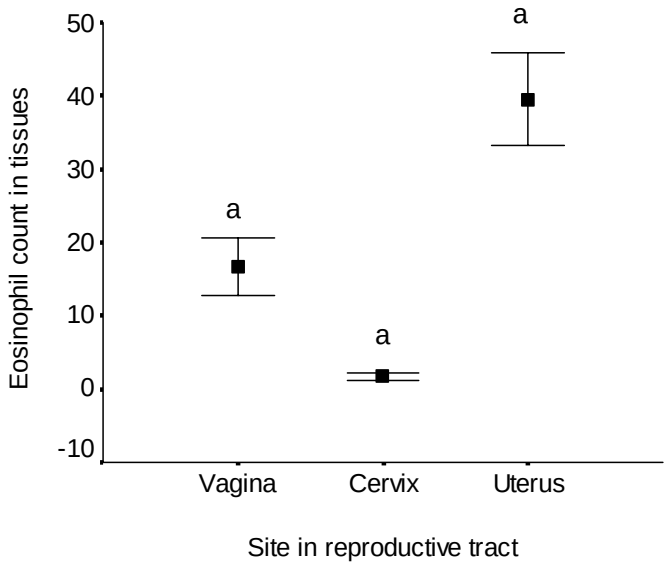

Figure 4.9 Eosinophil numbers in different sites in the reproductive tract of ewes when sites were combined into vagina (n = 60), cervix (n = 90) or uterus (n = 150). Data are presented as the mean ± SEM in 1.5mm^2 tissue. Data from different time periods and from both mated and control ewes were pooled. Values for uterine tissues were from cell counts in the superficial endometrium. [a] P <0.01 compared with other sites.

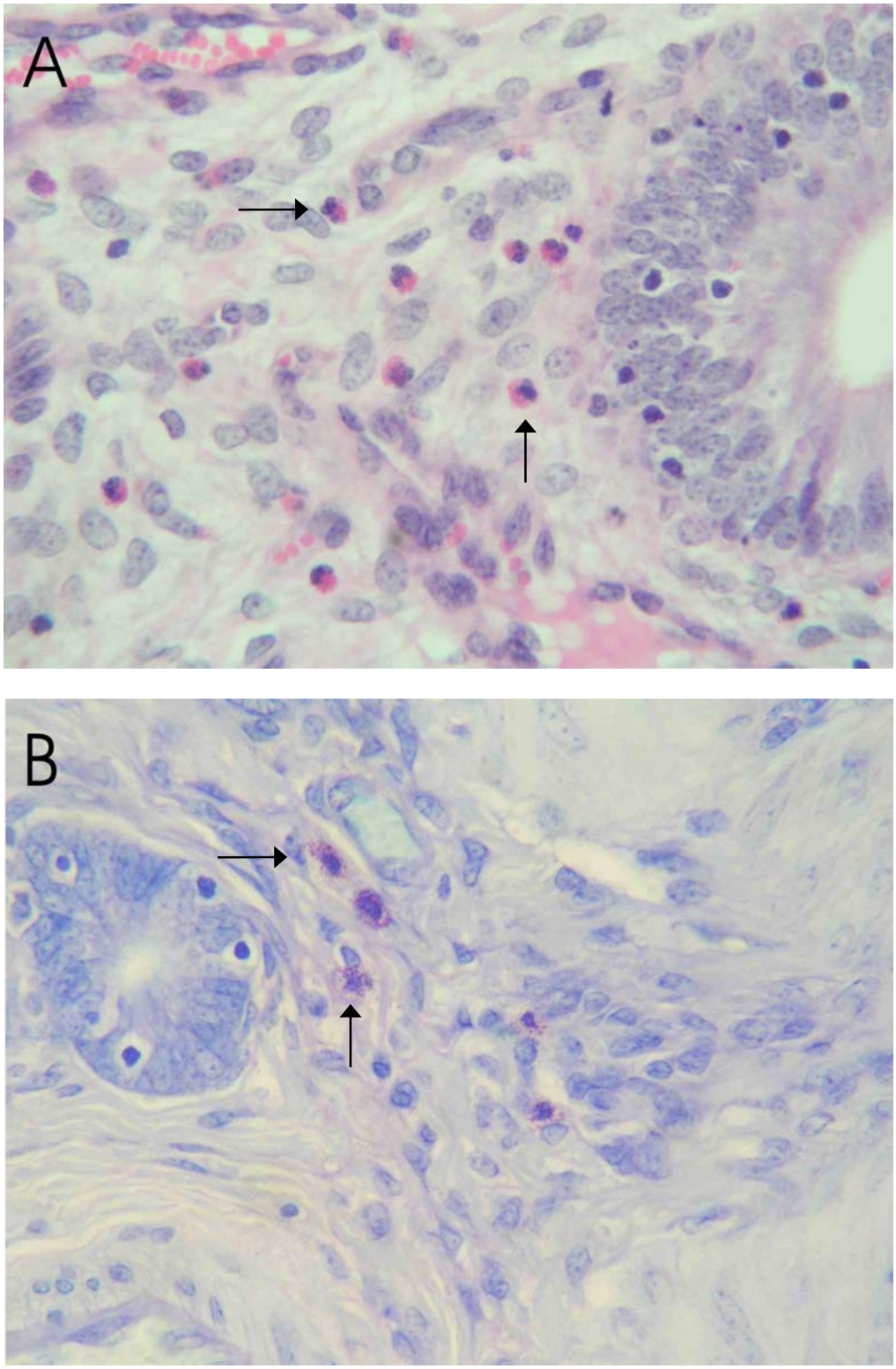

Figure 4.10 Photomicrographs of (A) eosinophils (H&E stained) in the superficial endometrium (arrows) and (B) a cluster of mast cells (Toluidine blue stained) in the deep endometrial stroma (arrows).

Mast cell distribution in uterine tissues varied between different depths in the endometrium. Most were in the deep and mid-stroma of the anterior uterine horns (Table 4.4). The superficial stroma of the anterior uterine horns had significantly fewer (P <0.05) mast cells. Furthermore, there were significantly more (P <0.05) mast cells in the mid- and/or deep endometrial stroma than in the superficial endometrial stroma at all other uterine sites for both mated and control ewes. There were significantly fewer (P <0.01) mast cells in cervical sites than in other sites in the reproductive tract. When all sites in the reproductive tract of both mated and control ewes were combined into the vagina, uterus or cervix, there were significantly higher numbers of mast cells in the vagina (P <0.01) and uterus (P <0.01) than in the cervix (Figure 4.12).

Mast cells were generally distributed singly, but also occurred in clusters of three to six, both in the mid-stromal endometrium and close to the endometrial-myometrial junction (Figure 4.10B). There was no correlation between mast cell and eosinophil numbers or distribution within tissues.

Table 4.4 Comparison of mast cell counts in reproductive tissues of mated and non-mated ewes.

	Control ewes (n=15)			Mated ewes (n=15)		
Site	Superficial	Mid-	Deep	Superficial	Mid-	Deep
V	1.6±1.1			1.1±0.4		
OC	0.6±0.4			0.6±0.3		
PC	0.2±0.1			0.6±0.2		
MC	0.0±0.0			0.1±0.1		
AC	0.1±0.1			0.2±0.1		
BU	0.4±0.2 [a]	1.1±0.4	2.7±0.9	0.3±0.2 [a]	1.6 ±1.0	2.5±1.0
IM	1.9±1.2 [ab]	4.0±1.4	4.3±1.1	0.5±0.3 [a]	1.3±0.4	2.7±0.7
IA	0.9±0.5 [ab]	3.1±0.9	4.9±1.5	1.3±0.4 [b]	2.3±0.8	3.5±1.0
CM	1.3±0.6 [a]	4.1±1.3	3.7±1.3	0.4±0.2 [ab]	1.7±0.4	2.2±0.7
CA	0.9±0.6 [ab]	4.7±1.4	4.0±1.1	1.7±0.5 [b]	2.1±0.7	3.5±1.0

Data are presented as the mean ±SEM cell counts in 1.5mm^2 tissue. V, vagina; OC, cervical os; PC, posterior cervix; MC, mid-cervix; AC, anterior cervix; BU, uterine body; IM, ipsilateral mid-uterine horn; IA, ipsilateral anterior uterine horn; CM, contralateral mid-uterine horn; CA, contralateral anterior uterine horn. Superficial, mid- and deep refer to depth in endometrium. [a] P <0.05 compared with mid-stroma; [b] P <0.05 compared with deep stroma.

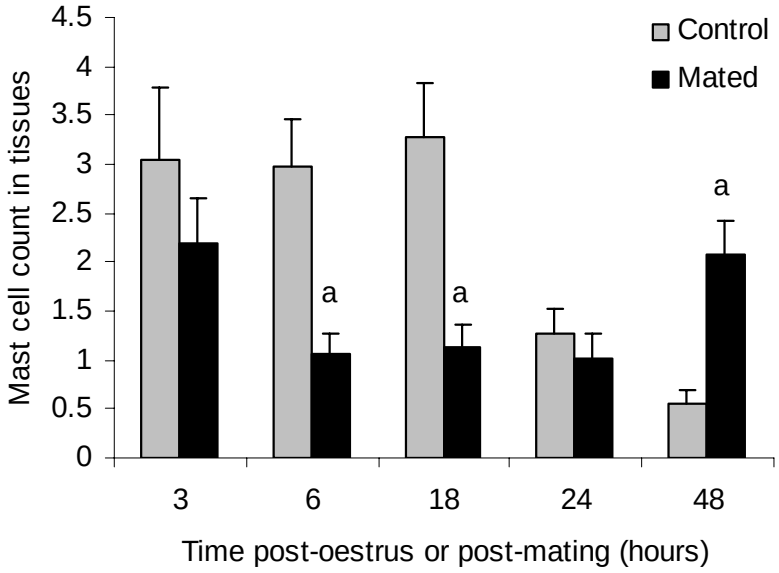

Figure 4.11 Mast cells in reproductive tissues of control and mated ewes at various times post-oestrus (controls) and post-mating. Data are presented as the mean + SEM (n=60) in 1.5mm^2 tissue. [a] P <0.01 compared with control ewes.

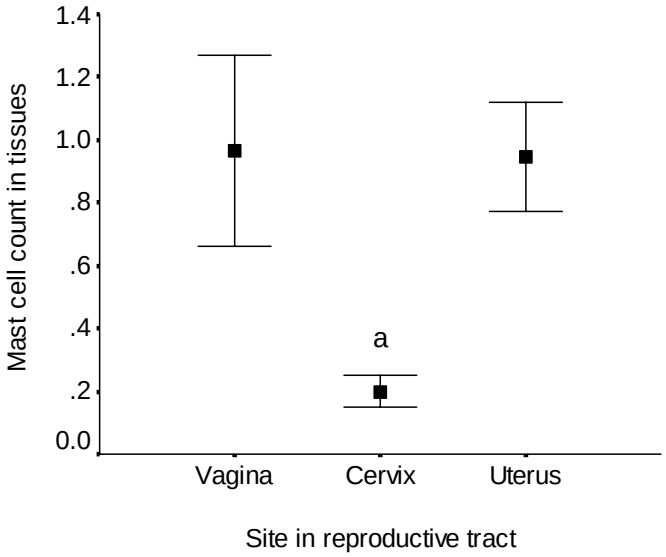

Figure 4.12 Mast cells in different sites in the reproductive tract of ewes when sites were combined into vagina (n = 60), cervix (n = 90) or uterus (n = 150). Data are presented as the mean ± SEM in 1.5mm^2 tissue. Data from different time periods and from both mated and control ewes were pooled. Values for uterine tissues were from cell counts in the superficial endometrium. [a] P <0.01 compared to other sites.

4.4.4 Macrophages

4.4.4.1. Anti-macrophage antibody optimisation

The first anti-macrophage antibody tried was a mouse anti-sheep macrophage (42/44KD) antibody (clone VPM63, Cat No MCA918, Serotec, Oxford, England). Despite numerous attempts using a variety of different techniques, no staining of macrophages was evident. After many trials, the use of this antibody was ceased.

The second anti-macrophage antibody tried was the mouse anti-human macrophage antibody (clone MAC387, Cat No MCA874G, Serotec, Oxford, England). This antibody resulted in very intense intracytoplasmic staining of macrophages. However, it also stained polymorphonuclear leukocytes very intensely, and as the chromagen completely concealed the nuclear morphology, it was not possible to distinguish between the different cell types (Figure 4.13A). Several different techniques were used in an attempt to overcome this problem, but none were successful. For instance, reduction of antibody concentration and reduction of incubation times did not reduce the staining sufficiently to be able to identify the nuclear shape. The use of different nuclear stains and the use of a less intensely staining chromagen (AEC) also did not overcome the problem. It was decided that while this antibody worked under most conditions, it was not possible to distinguish between macrophages and neutrophils therefore it was not adequate for the requirements of this study.

The third anti-macrophage antibody tried was a mouse anti-CD14 antibody (clone CAM36A, VMRD, Pullman, Washington State USA). This antibody demonstrated positive staining of macrophages, however the staining was quite diffuse (Figure 4.13B), and it was impossible to accurately count cell numbers in tissue. Several techniques were tried, including various fixatives and staining protocols, but staining was not distinct enough for quantification of macrophages in ovine tissues.

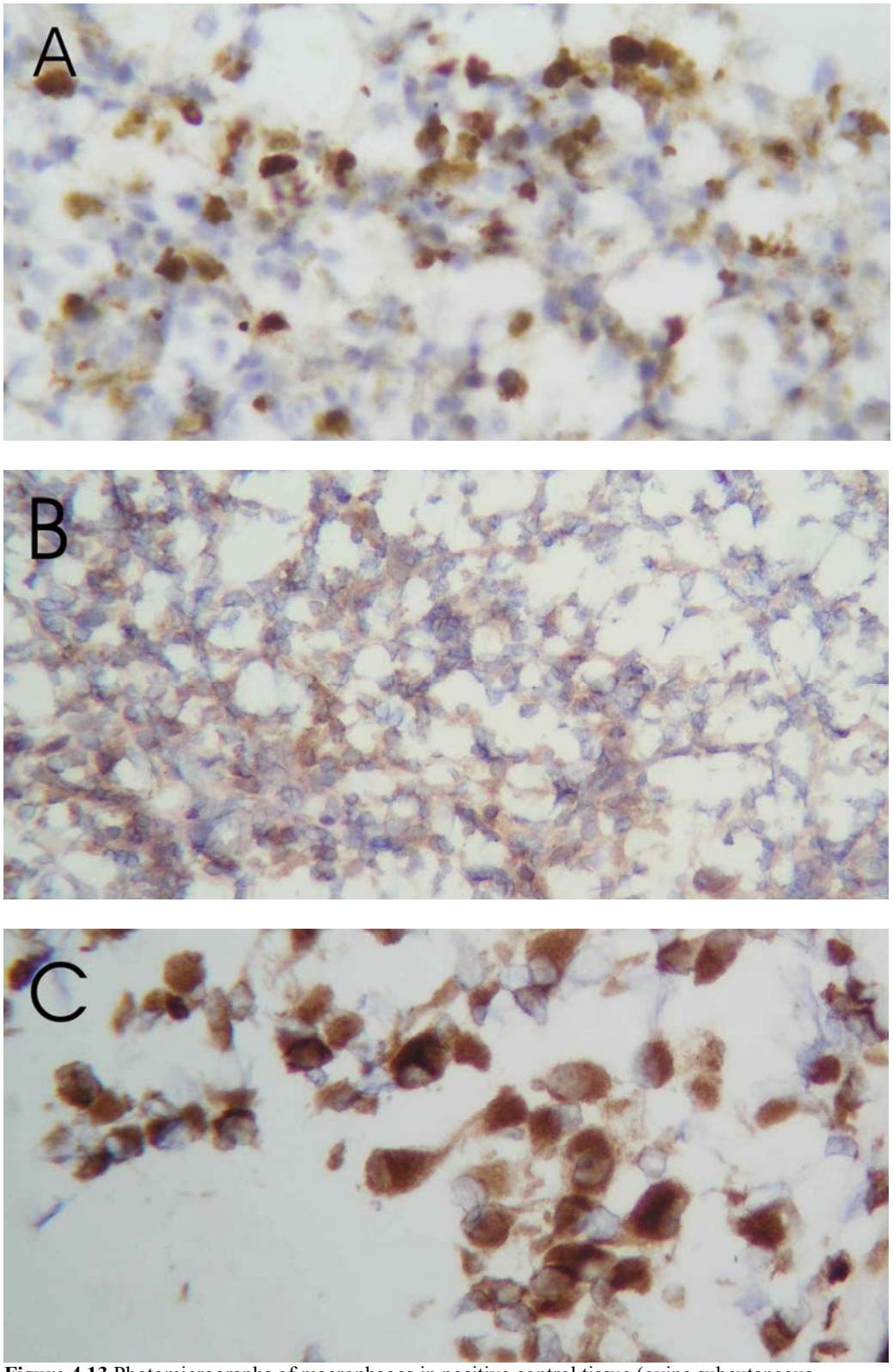

Figure 4.13 Photomicrographs of macrophages in positive control tissue (ovine subcutaneous granuloma) with various antibodies: (A) Serotec: MCA874G, (B) VMRD: CAM36A and (C) DakoCytomation: EBM-11.

The fourth anti-macrophage antibody tried was a mouse anti-human CD68 macrophage antibody (clone PG-M1, Cat No M0876, DakoCytomation Pty Ltd, Botany, NSW Australia). Despite several attempts using various different techniques, staining of macrophages in ovine tissue with this antibody was not achieved.

The fifth anti-macrophage antibody tried was a mouse anti-human CD68 macrophage antibody (clone EBM11, cat No M0719, DakoCytomation Pty Ltd, Botany, NSW Australia). This antibody demonstrated strong, obvious staining of macrophages in ovine tissue (Figure 4.13C). The staining was cytoplasmic and often appeared granular. Cells were of variable size, and the shape varied from very round to irregular and almost stellate. It is likely that the latter were dendritic cells stationed within tissues. The antibody was very robust, working well under many different conditions. Fixation in acetone was the method recommended by the manufacturer. However, the fixative used for this study was 4% methanol-free formaldehyde for 10 minutes at 4^{o}C. Whilst fixation in acetone for 10 minutes at 4^{o}C gave a stronger chromagen stain of macrophages, it also resulted in poor morphology of the surrounding tissue. The superior morphology achieved with the use of a 4% methanol-free formaldehyde fixative outweighed the mild reduction in staining intensity of macrophages. As well as staining macrophages, this antibody lightly stained polymorphonuclear leukocytes. However, this was not a problem as macrophages were easily differentiated due to their larger size and different nuclear morphology (Figure 4.14A). Due to its superior staining ability, the mouse anti-human CD68 macrophage antibody (clone EBM11, cat No M0719, DakoCytomation Pty Ltd, Botany, NSW Australia) was used throughout the remainder of the studies.

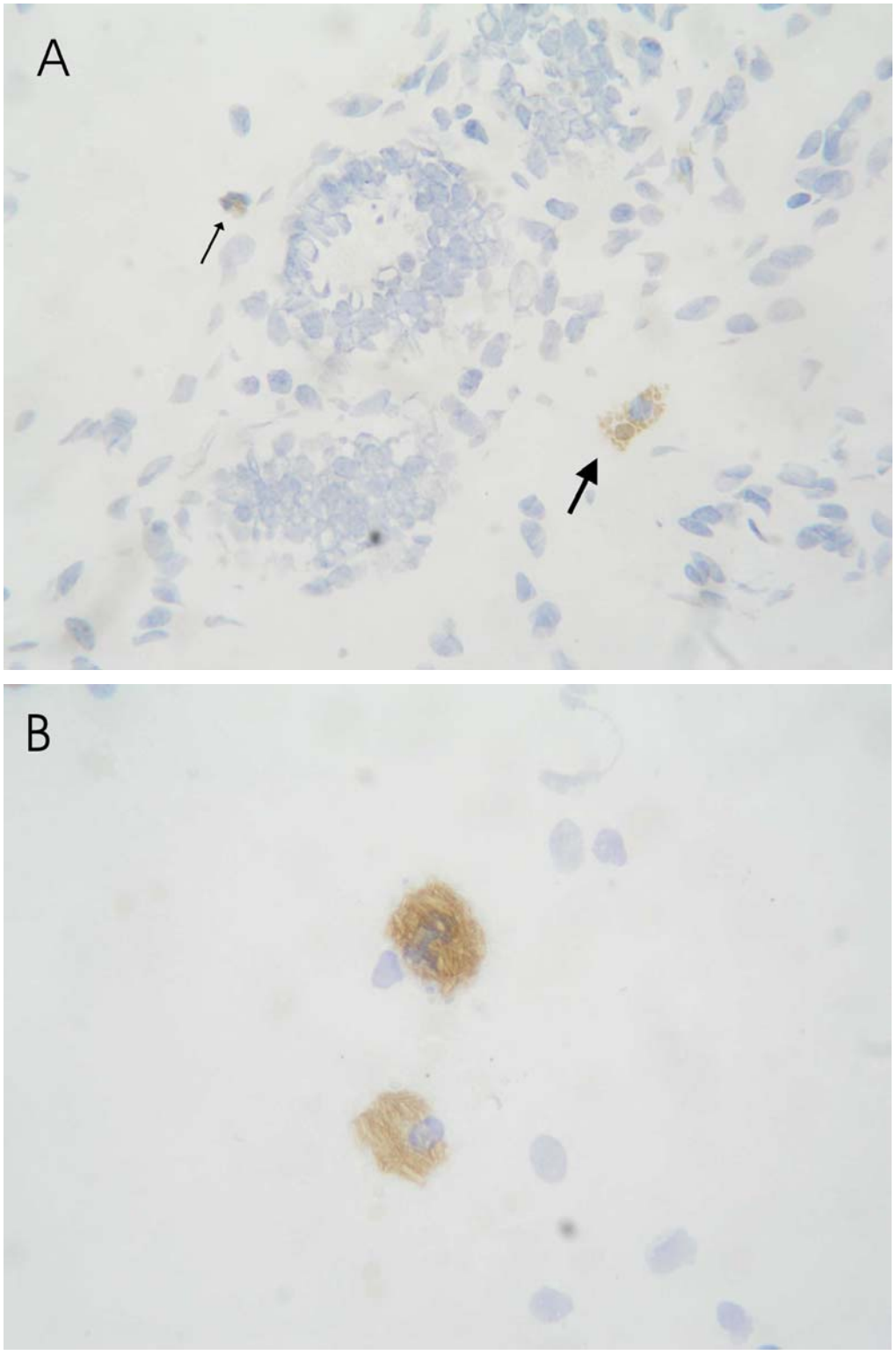

Figure 4.14 Photomicrographs of macrophages stained with EBM-11 (DakoCytomation) and haematoxylin counterstain (A) in the endometrium and (B) in a cervical mucus smear. Large arrow points to a macrophage, small arrow points to a neutrophil.

4.4.4.2 Macrophage infiltration of tissues

Macrophages increased following mating in all tissues except the mid-stromal region of the ipsilateral mid-uterine horn. The differences between mated and non-mated ewes were significant in the vagina ($P < 0.05$), posterior ($P < 0.01$) and mid-cervix ($P < 0.05$), superficial stroma of the ipsilateral mid-uterine horn ($P < 0.05$), mid-stroma of the uterine body ($P < 0.05$), contralateral mid- ($P < 0.05$) and anterior ($P < 0.05$) uterine horns and the deep stroma of the ipsilateral anterior uterine horn ($P < 0.05$) (Table 4.5). Significantly more ($P < 0.01$) macrophages were found in tissues from mated compared with control ewes at all times except 48 hours (Figure 4.15).

The number of macrophages in the vagina, posterior and anterior cervix and uterine body of mated ewes peaked at 18 hours. Numbers of macrophages in the mid-cervix and contralateral mid-uterine horn were highest at three hours post-insemination. The remaining sites of the uterine horns had the highest number of macrophages at 24 hours post-insemination. A smaller increase in macrophages was observed in tissues from control ewes, with numbers peaking between 18 and 48 hours post-oestrus.

Most macrophages were found in the uterine body, followed by the uterine horns, but these were not significantly different from one another. The uterine body had a significantly higher number of macrophages than the vagina ($P < 0.05$) and cervical sites ($P < 0.01$). The cervix had the lowest number of macrophages recorded. Similarly, when data were combined into three main sites (vagina, cervix and uterus), the uterus had significantly more macrophages than either the vagina ($P < 0.05$) or the cervix ($P < 0.01$), and the vagina and cervix were not significantly different from one another (Figure 4.16). Distribution of macrophages in uterine tissues varied between different depths in the endometrium. In most uterine tissues from both mated and control ewes, there were more macrophages in the superficial endometrium than either the mid- or deep endometrium but the differences were not statistically significant (Table 4.5).

Table 4.5 Macrophage numbers in reproductive tissues of mated and non-mated ewes.

	Control ewes (n=15)			Mated ewes (n=15)		
	Superficial	Mid-	Deep	Superficial	Mid-	Deep
V	4.6±1.1			13.4±3.4 [b]		
PC	2.7±0.6			10.4±3.8 [a]		
MC	3.0±0.7			8.5±1.9[b]		
AC	5.3±1.4			12.5±4.9		
BU	10.7±2.4	6.3±1.2	6.2±2.0	19.7±3.9	10.9±2.8 [b]	9.6±2.1
IM	7.9±1.9	9.2±1.9	6.9±1.7	14.2±2.0 [b]	8.9±1.4	8.5±1.4
IA	10.5±2.5	5.5±0.8	3.9±0.7	11.6±1.9	10.1±3.6	8.9±1.4 [b]
CM	9.3±2.4	6.9±2.6	7.3±2.7	16.7±7.0	11.9±2.8 [b]	9.7±2.2
CA	10.3±3.0	6.7±0.6	5.5±1.0	12.3±2.5	10.1±1.3 [b]	6.4±1.3

Data are presented as the mean ±SEM cell counts in 1.5mm^2 tissue. V, vagina; OC, cervical os; PC, posterior cervix; MC, mid-cervix; AC, anterior cervix; BU, uterine body; IM, ipsilateral mid-uterine horn; IA, ipsilateral anterior uterine horn; CM, contralateral mid-uterine horn; CA, contralateral anterior uterine horn. Superficial, mid- and deep refer to depth in endometrium. [a] P <0.01, [b] P <0.05 compared with same site in control ewes.

Table 4.6 Macrophage counts in luminal smears of mated and non-mated ewes.

	Site in reproductive tract						
	V	C	BU	IM	IA	CM	CA
Controls (n=15)	0.0±0.0 [a]	0.01±0.01 [a]	0.33±0.10	0.51±0.19	0.13±0.06	0.28±0.19	0.27±0.16
Mated (n=15)	0.12±0.09 [a]	0.02±0.02 [a]	0.39±0.10	0.28±0.09	0.31±0.10	0.32±0.16	0.48±0.17

Data are presented as the mean ± SEM percentage of macrophages in luminal smears. V, vagina; C, cervix; BU, uterine body; IM, ipsilateral mid-uterine horn; IA, ipsilateral anterior uterine horn; CM, contralateral mid-uterine horn; CA, contralateral anterior uterine horn. [a] P <0.01 compared with uterine sites.

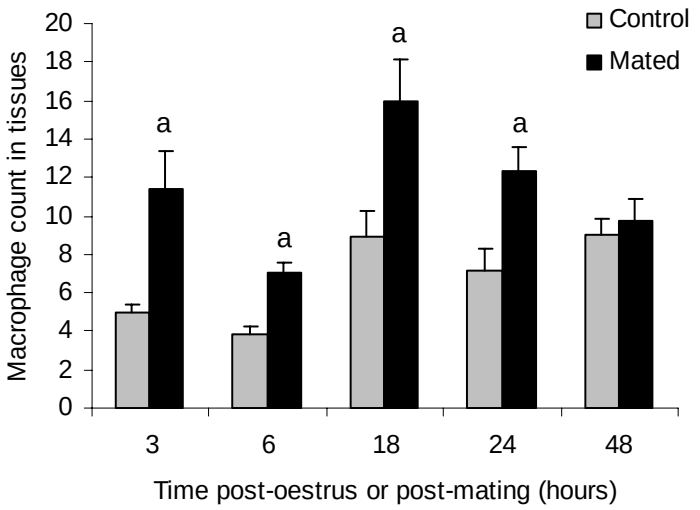

Figure 4.15 Macrophage numbers in reproductive tissues of control and mated ewes at various times post-oestrus (controls) and post-mating. Data are presented as the mean + SEM (n=57) in 1.5mm^2 tissue. [a] P <0.01 compared with control ewes.

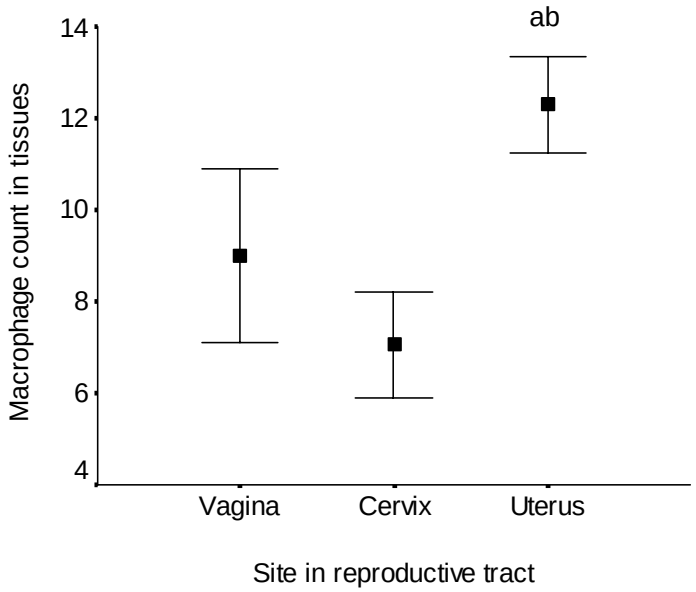

Figure 4.16 Macrophage numbers in different sites in the reproductive tract of ewes when sites were combined into vagina (n = 60), cervix (n = 90) or uterus (n = 150). Data are presented as the mean ± SEM in 1.5mm^2 tissue. Data from different time periods and from both mated and control ewes were pooled. Values for uterine tissues were from cell counts in the superficial endometrium. [a] P <0.01 compared to cervix, [b] P <0.05 compared to vagina.

4.4.4.3 Macrophages in the lumen

Macrophages were identified in luminal smears from both mated and non-mated control ewes (Figure 4.14B). The number of macrophages in smears from control ewes peaked at 18 hours post-oestrus and then declined, whereas in mated ewes the number of macrophages continued to steadily increase over time to peak at 48 hours post-mating, by which time there were significantly more (P <0.05) macrophages in smears from mated ewes than control ewes (Figure 4.17). Very few macrophages were observed in smears from the vagina and cervix, with significantly more (P <0.01) in smears from the uterine body and horns (Table 4.6). Similarly, when data were combined into vagina, cervix or uterus, smears from the uterus had significantly more (P <0.01) macrophages than either the vagina or cervix (Figure 4.18).

The staining intensity of macrophages on smears was reduced in smears that had been stored for prolonged periods (six to 12 months) at -80^{o}C, when compared to slides that had been stored for a shorter time (less than six months) prior to being stained. However, macrophages were still readily distinguished from other cells types.

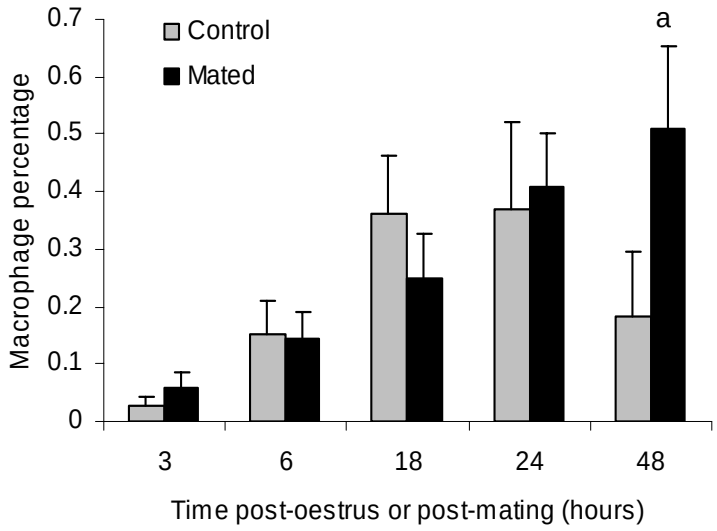

Figure 4.17 Macrophage percentages in smears from the reproductive tract lumen of control and mated ewes at various times post-oestrus (controls) and post-mating. Data are presented as the mean ± SEM (n=21) percentage of total cells in smears. [a] P <0.05 compared with control ewes.

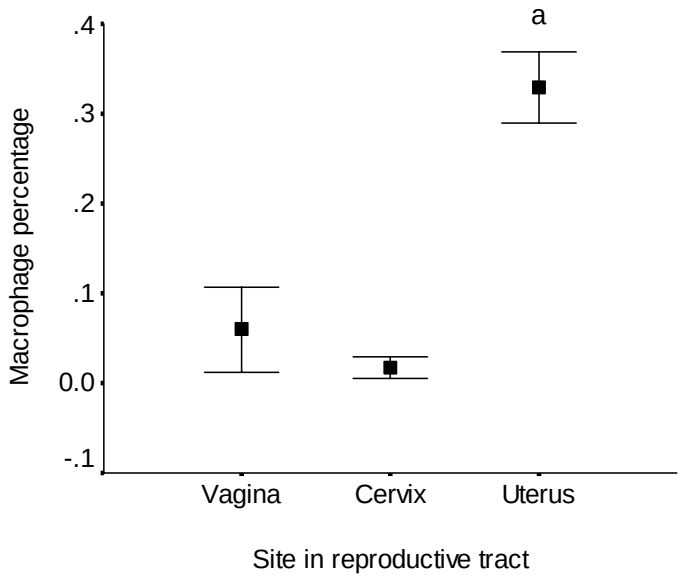

Figure 4.18 Macrophage percentages in smears from different sites in the reproductive tract of ewes when sites were combined into vagina (n = 30), cervix (n = 30) or uterus (n = 150). Data are presented as the mean ± SEM. Data from different time periods and from both mated and control ewes were pooled. [a] $P < 0.01$ compared to other sites.

4.5 Discussion

In other species, leukocytes in reproductive tissues are under the influence of ovarian steroid hormones (McDonald *et al.* 1952; De and Wood 1990; Bischof *et al.* 1994a; Frayne and Stokes 1994; Kaushic *et al.* 1998; Tibbetts *et al.* 1999; Kaeoket *et al.* 2001; DeLoia *et al.* 2002; Kaeoket *et al.* 2002b). In the current study, leukocyte populations in ewe reproductive tissues underwent significant changes during the 48 hour period following the onset of oestrus. The number of neutrophils in the uterus of control ewes was significantly reduced by 48 hours following oestrus, suggesting that neutrophil infiltration may be influenced by ovarian steroid hormones, as occurs in rats (Kaushic *et al.* 1998) and pigs (Bischof *et al.* 1994a). The number of mast cells was also reduced by 48 hours following oestrus whereas the number of macrophages in most tissues was higher at 48 hours than at three hours after the onset of oestrus, suggesting a reverse influence by ovarian hormones. Macrophage migration into the reproductive tract lumen also appeared to be under the influence of ovarian hormones, with a rise in the number of luminal macrophages by 18-24 hours and then a decline by 48 hours post-oestrus. In humans (DeLoia *et al.* 2002), horses

(Frayne and Stokes 1994), rodents (De and Wood 1990; Tibbetts *et al.* 1999) and pigs (Kaeoket *et al.* 2001; Kaeoket *et al.* 2002b), macrophages in reproductive tissues are believed to be under the influence of ovarian steroid hormones although other authors have reported that the number of macrophages in the endometrium remain constant (Starkey *et al.* 1991; Cobb and Watson 1995; Summerfield and Watson 1998). Interestingly, there were no changes in the number of eosinophils in the 48 hours following oestrus. Further investigations comparing leukocyte numbers in the ewe reproductive tract at oestrus and during the luteal phase are necessary to confirm a hormonal influence.

Numbers of neutrophils and macrophages both significantly increased in the reproductive tract of the ewe in response to mating whereas the number of mast cells decreased and the number of eosinophils did not change. In uterine tissues, a peak in neutrophils was evident within six hours of mating, whereas macrophages did not peak until 18 to 24 hours after mating. This follows the pattern expected in a general inflammatory reaction, where neutrophils are the first to arrive at sites of inflammation, followed within 24 to 48 hours by macrophages (Cotran *et al.* 1999; Kaplanski *et al.* 2003). These results are consistent with the inflammatory reaction to insemination seen in other species (Pandya and Cohen 1985; De *et al.* 1991; McMaster *et al.* 1992; Bischof *et al.* 1994b; Kotilainen *et al.* 1994). The effect of insemination on neutrophil infiltration had ceased by 48 hours except in the posterior cervix, where the number of neutrophils was highest at 48 hours post-insemination. The reason neutrophils remain at this site may be due to the continued presence of a reservoir of semen within the cervix (Mattner 1966).

Following insemination neutrophils accumulated in the subepithelial stroma, and by 24 hours most neutrophils in the vagina and cervical os were intraepithelial, suggesting that most had migrated or were in the process of migrating into the lumen. Neutrophils in the reproductive tract lumen are believed to remove micro-organisms, phagocytose dead and excess spermatozoa and may be involved in sperm selection (Lovell and Getty 1968; Pandya and Cohen 1985; Thompson *et al.* 1992; Troedsson *et al.* 2001). The effect of insemination on neutrophil migration was most pronounced in the cervical lumen but the mid- and anterior cervix showed minimal neutrophil infiltration of tissues. Therefore, at least some neutrophils in the cervical

lumen must have originated in the uterus or vagina, rather than migrating across the cervical epithelium. This observation agrees with the finding that neutrophils drain into the ovine cervix from the uterus (Mattner 1969). Additionally, neutrophils in posterior cervical tissue were located in small clusters in the tips of villi with very few in the crypts. This is compatible with the presence of a sperm reservoir in cervical crypts (Mattner 1963; Mattner 1966; Mattner 1969).

In contrast to the subepithelial distribution of neutrophils, macrophages were distributed diffusely in the endometrial stroma. Following mating, large numbers of macrophages were situated in the superficial stroma of the uterine body and mid-uterine horns. This may be associated with preparation for implantation of the ovine conceptus, as macrophages are believed to have a role in inducing immunotolerance to paternal antigens, thereby facilitating implantation (Robertson *et al.* 1997; Robertson and Sharkey 2001).

Of the five anti-macrophage antibodies tried during this study, the mouse anti-human CD68 antibody (clone EBM11, cat No M0719, DakoCytomation Pty Ltd, Botany, NSW Australia) produced the best staining of macrophages in sheep reproductive tissues. Macrophages were clearly visible in tissues and mucus smears, were readily differentiated from granulocytes and were easily quantified. Positive staining for CD68 in sheep tissue was generally cytoplasmic and granular in appearance, which is consistent with the findings of other researchers (Tekin and Hansen 2004). Additionally, smaller, irregular, somewhat stellate cells stained positively for CD68. These were presumably dendritic cells or immature macrophages, and were counted collectively with the larger, positively stained macrophages. Not all dendritic cells stain positively for CD68 but those that did had less intense staining than observed for macrophages (Hart 1997). Additionally, reactivity to the EBM11 antibody in dendritic cells is reported to be clustered more closely around the nucleus rather than throughout the cytoplasm as in macrophages (Betjes *et al.* 1991). This is consistent with the staining pattern observed in smaller CD68 positive cells in the current study.

The mouse anti-human macrophage antibody (clone MAC387, Cat No MCA874G, Serotec, Oxford, England) produced strong, readily visible staining but macrophages were not readily differentiated from polymorphonuclear leukocytes. The mouse anti-

CD14 antibody (clone CAM36A, VMRD, Pullman, Washington State USA) also stained macrophages, but the staining was paler and more diffuse than that observed with the two previously mentioned antibodies. This finding is consistent with that of previous researchers (Tekin and Hansen 2004) who suggested that this antibody may stain diffusely due to the release of CD14 from cells and its subsequent absorption to the extracellular matrix.

Interestingly, the number of mast cells in tissues decreased by 6-18 hours post-mating, but by 48 hours the number had increased and was significantly higher in mated than control ewes. The reduction in the number of mast cells following insemination may have been due to degranulation making their detection difficult, as occurs in bovine endometritis (Corbeil *et al.* 2005). The release of chemical mediators such as histamine could play a role in modulating contractions of the reproductive tract following insemination as mast cell degranulation is involved in contraction of the cervix in the guinea pig (Bytautiene *et al.* 2002) and in uterine contractility in humans and rodents during pregnancy (Garfield *et al.* 2000; Bytautiene *et al.* 2004). Additionally, mast cell degranulation has been linked with an increase in the number of macrophages in the rat cervix (Bosquiazzo *et al.* 2005) and it is possible that a similar effect occurs in the ewe. The increase in mast cells by 48 hours post-mating may indicate a role for mast cells during the pre-implantation period. There was no difference in the number of eosinophils in reproductive tissues between mated and control ewes, no correlation with the number of mast cells or their distribution, and their function in the ewe reproductive tract remains unclear.

In conclusion, the post-insemination inflammatory response in the ewe involves an increase in the number of neutrophils and macrophages present and a reduction or degranulation of mast cells in the vagina, posterior cervix and uterus. The leukocyte population changes occurring in the ovine reproductive tract in response to insemination may be involved in preparation for implantation of the ovine conceptus.

CHAPTER 5
CHEMOKINES IN REPRODUCTIVE TISSUES AND SECRETIONS

5.1 Introduction

Several cytokines are thought to contribute to the post-insemination inflammatory reaction in the female reproductive tract. These include granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (CSF-1), interleukin 1 alpha (IL-1 α), interleukin 1 beta (IL-1 β), interleukin 6 (IL-6), interleukin 8 (IL-8) and tumour necrosis factor alpha (TNF-α) (Sanford *et al.* 1992; Denison *et al.* 1999; Mitchell *et al.* 2002). In this study GM-CSF and IL-8 in the ewe reproductive tract were investigated due to their well known chemotactic properties in other inflammatory reactions. Neutrophil and macrophages both increased in reproductive tissues of the ewe post-insemination, and it was postulated that this leukocyte recruitment was directed by an increase in the synthesis and release of GM-CSF and IL-8 from epithelial cells in the reproductive tract following insemination.

The aims of this study were to demonstrate the presence of GM-CSF and IL-8 in cervical and uterine epithelial cells post-insemination and determine if these cytokines were present in luminal secretions from the reproductive tract.

5.2 Materials and Methods

Tissue samples and luminal secretions were collected from the reproductive tracts of 30 ewes (see 3.6.1 & 3.6.2). Half were collected at various times (three, six, 18, 24 & 48 hours) following mating (see 3.3) and the rest were collected from non-mated ewes at similar times following the onset of oestrus.

5.2.1 GM-CSF

5.2.1.1 Tissue sections and antibody optimisation

Tissues from nine sites (see 3.6.2) in the reproductive tracts of 30 ewes were frozen (see 3.8.2.1) and stained immunohistochemically (see 3.12.3.3) using a mouse anti-sheep GM-CSF antibody (clone 3C2, Cat No MCA1924, Serotec, Oxford, England). Several antibody combinations were tried in an attempt to stain GM-CSF in tissues from ewes. Tissue sections were stained with a monoclonal mouse anti-sheep GM-CSF antibody (clone 3C2, Cat No MCA1924, Serotec, Oxford, England). The secondary antibody used was HRP-conjugated rabbit anti-mouse IgG (STAR13B, Serotec, Oxford England). Multiple combinations of different fixation methods, antibody concentrations, incubation times and temperatures and buffer solutions were tried. Staining of GM-CSF in tissues was also attempted using a biotinylated monoclonal mouse anti-sheep GM-CSF antibody (clone 8D8, Cat No MCA1923B, Serotec, Oxford, England) in combination with streptavidin-HRP (BD Pharmingen, North Ryde, NSW Australia).

The optimum technique for staining GM-CSF in ovine reproductive tissues used the mouse anti-sheep GM-CSF antibody (clone 3C2) and the HRP-conjugated rabbit anti-mouse antibody (see 3.12.3.3).

5.2.1.2 ELISA for GM-CSF in luminal fluid

Luminal secretions from seven sites (see 3.6.1) in the reproductive tracts of 30 ewes were analysed with an ELISA for the presence of GM-CSF (see 3.15.1). The capture antibody used was a monoclonal mouse anti-sheep GM-CSF antibody (clone 3C2, Cat No MCA1924, Serotec, Oxford, England) and the detection antibody was a biotinylated monoclonal mouse anti-sheep GM-CSF antibody (clone 8D8, Cat No MCA1923B, Serotec, Oxford, England). Streptavidin-HRP (BD Pharmingen, North Ryde, NSW Australia) was used in place of a secondary antibody.

5.2.2 IL-8

5.2.2.1 Tissue sections and antibody optimisation

Tissues from nine sites (see 3.6.2) in the reproductive tracts of 30 ewes were frozen (see 3.8.2.1) and stained immunohistochemically (see 3.12.3.4) using a polyclonal rabbit anti-sheep IL-8 antibody (Cat No AB1840, Chemicon, Temecula, California USA). Several different antibody combinations were tried in an attempt to stain IL-8 in tissues from ewes. A polyclonal rabbit anti-sheep IL-8 antibody (Cat No AB1840, Chemicon, Temecula, California USA) was used. Two secondary antibodies were tried with this primary antibody. The first was a biotinylated goat anti-mouse/rabbit IgG antibody, along with streptavidin-HRP (LSAB kit, DakoCytomation Pty Ltd, Botany, NSW Australia). The next secondary antibody tried was HRP-conjugated pig anti-rabbit IgG (P0217, DakoCytomation Pty Ltd, Botany, NSW Australia). Staining of IL-8 in tissues was also attempted using a monoclonal mouse anti-sheep IL-8 antibody (clone 8M6, Cat No MAB1044, Chemicon, Temecula, California USA). The secondary antibody used was HRP-conjugated rabbit anti-mouse IgG (STAR13B, Serotec, Oxford, England).

The optimum technique for staining IL-8 in ovine reproductive tissues used a polyclonal rabbit anti-sheep IL-8 antibody and the HRP-conjugated pig anti-rabbit IgG (see 3.12.3.4).

5.2.2.2 ELISA for IL-8 in luminal fluid

Luminal secretions from seven sites (see 3.6.1) in the reproductive tracts of 30 ewes were analysed with an ELISA for the presence of IL-8 (see 3.15.2). The capture antibody used was a monoclonal mouse anti-sheep IL-8 antibody (clone 8M6, Cat No MAB1044, Chemicon, Temecula, California USA) and the detection antibody was a polyclonal rabbit anti-sheep IL-8 (Cat No AB1840, Chemicon, Temecula, California USA). The secondary antibody used was an HRP-conjugated pig anti-rabbit IgG antibody (P0217, DakoCytomation Pty Ltd, Botany, NSW Australia).

5.3 Statistical Analyses

5.3.1 Statistical analyses of cytokines in tissues

Data for cytokine staining intensity of tissues from the reproductive tract of ewes were semi-quantitative non-parametric ranked data. Staining intensity was ranked as none, mild, moderate or strong (see 3.13.2). Initially, non-parametric tests were used to analyse all data. Mann-Whitney tests were performed to determine if there was a significant difference in cytokine staining intensity of tissues between mated and non-mated ewes (see Appendix 10). Kruskal-Wallace tests were used to compare staining intensity of tissues from different sites in the reproductive tract both for original sites sampled and when sites were combined into the three main sites of vagina, cervix and uterus (see Appendix 11). Additionally, data were ranked prior to parametric analyses (Conover and Iman 1981) using three way ANOVA, (see Appendix 12), *post hoc* tests (Fisher's LSD) (see Appendix 13) and Student's *t*-tests (see Appendix 14). Where no significant differences were detected in a particular category (mated versus control ewes, anatomical sites or time periods) data in that category were analyzed collectively.

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5.3.2 Statistical analyses of cytokines in luminal fluid

Data for cytokine concentrations in luminal fluid from the reproductive tract of ewes were not normally distributed and could not be normalised by transformation. Initially, non-parametric tests were used to analyse all data. Mann-Whitney tests were performed to determine if there was a significant difference in the cytokine concentration in fluid between mated and non-mated ewes (see Appendix 15). Kruskal-Wallace tests were used to compare cytokine concentrations in fluid from different sites in the reproductive tract both for original sites sampled and when sites were combined into the three main sites of vagina, cervix and uterus (see Appendix 16). Kruskal-Wallace tests were also used to compare cytokine concentrations in fluid at different times for mated and control ewes separately (see Appendix 17). Additionally, data were ranked prior to parametric analyses (Conover and Iman 1981) using three way ANOVA (see Appendix 18), *post hoc* tests (Fisher's LSD)

(see Appendix 19) and Student's *t*-tests (see Appendix 20). Where no significant differences were detected in a particular category (mated versus control ewes, anatomical sites or time periods) data in that category were analyzed collectively.

5.3.3 Statistical correlation between cytokines and cell counts

Correlation between ranked staining intensity and ranked luminal cytokine concentration was determined using Pearson's correlation coefficient and scatter plots. Non-parametric (Spearman's) analysis of correlation and scatter plots were used to identify correlations between cell counts (Chapter 4) and cytokines both in tissues and in luminal fluid samples (see Appendix 21). For all statistical analyses performed, a *P* value of less than 0.05 was considered significant.

5.4 Results

5.4.1 GM-CSF

5.4.1.1 Immunohistochemistry antibody optimisation

Two different antibody combinations were tried in order to identify GM-CSF in the reproductive tissues of ewes. Staining for GM-CSF was initially attempted using a biotinylated monoclonal anti-sheep GM-CSF primary antibody in combination with streptavidin-HRP. However, tissues demonstrated heavy background staining even when streptavidin-HRP was used alone (the primary antibody step was omitted), possibly due to endogenous biotin in the tissue. Several blocking techniques were tried including the use of bovine serum albumin, 10% normal sheep serum or 10% normal goat serum as a pre-incubation step, or the addition of these to the streptavidin-HRP. However, background staining remained intense even when any of these blocking steps were used. It was therefore decided to cease use of this antibody for immunostaining of ovine reproductive tissue sections. A monoclonal mouse anti-sheep GM-CSF antibody in conjunction with HRP-conjugated rabbit anti-mouse IgG was then tried. Initial attempts to stain GM-CSF with this antibody combination were not successful. However, different combinations of fixative, incubation times,

temperatures and buffer solutions were tried, and an optimal technique for staining GM-CSF in tissues was identified (see 3.12.3.3).

5.4.1.2 GM-CSF in tissues

Granulocyte-macrophage colony-stimulating factor was detected by immunohistochemistry in the apical and basal cytoplasm of endometrial and cervical epithelial cells of both mated and non-mated ewes. There were no significant differences in staining intensity of GM-CSF between mated and control ewes in any site or at any time period. However, there were significant differences (P <0.01) in GM-CSF staining intensity between different sites in the reproductive tract (Table 5.1). Uterine luminal epithelium (Figures 5.1A, 5.1B and 5.1C) stained more intensely than cervical epithelium (Figure 5.1D) and vaginal tissues did not stain (Figure 5.1E). Furthermore, luminal endometrial and superficial glandular epithelial cells stained more intensely than deep glandular epithelial cells. Similarly, when data were combined into three main sites, there were significant differences (P <0.01) in GM-CSF staining intensity between the vagina, cervix and uterus (Figure 5.3). In both control and mated ewes the staining intensity of GM-CSF peaked at 24 hours. In mated ewes there was a significant reduction (P <0.01) in mean staining intensity by 48 hours post-mating (Table 5.1).

5.4.2.3 GM-CSF in the lumen

The ELISA standard curve generated using recombinant ovine GM-CSF gave an excellent correlation between optical density (OD) and GM-CSF concentration (see Appendix 22).

Granulocyte-macrophage colony-stimulating factor was detected more consistently in luminal fluid from reproductive tracts of mated than control ewes (Table 5.2). The concentration of GM-CSF detected in luminal flushings ranged from 0 to 1.6 ng/ml. In both mated and control ewes, highest concentrations were found in the ipsilateral anterior uterine horns (Figure 5.4), and in control ewes this was significantly higher (P <0.05) than in the cervix and contralateral mid-uterine horn. Luminal GM-CSF concentration was higher in mated than control ewes at all sites of the reproductive

tract (Figure 5.4) but the differences were not significant. However, when all uterine sites were pooled, the difference in luminal GM-CSF between mated (M) and control (C) ewes (C = 0.09 ± 0.02; M = 0.22 ± 0.04 ng/ml) approached statistical significance (P <0.06).

Similar concentrations of GM-CSF were detected in luminal flushings from mated and control ewes at three hours post-oestrus, however from six hours until 48 hours post-mating, GM-CSF concentration was higher in mated than control ewes. In control ewes there were higher GM-CSF concentrations in luminal fluid at three and six hours post-oestrus than by 18 post-oestrus and GM-CSF concentration remained low at 24 and 48 hours post-oestrus (Table 5.2). However, there appeared to be an individual ewe effect, as GM-CSF was present in several or all anatomical sites in the reproductive tract of some ewes (eg. 3rd ewe, 24 h mated, Table 5.2), whereas in other ewes no GM-CSF was found in any site (eg. 1st ewe, 24 h mated, Table 5.2). There was no correlation between luminal GM-CSF concentration and tissue staining intensity.

5.4.2 IL-8

5.4.2.1 Immunohistochemistry antibody optimisation

Initial attempts to identify IL-8 in tissues were undertaken using a polyclonal rabbit anti-sheep IL-8 antibody in conjunction with a biotinylated goat anti-mouse/rabbit IgG antibody and streptavidin-HRP. However, use of this secondary antibody produced a large amount of background staining, even when several different protein blocking steps were used. This background staining may have been due to the presence of endogenous biotin. Alternatively, it may have been due to cross-reactivity between goat IgG and sheep tissues due to the close genetic relationship between the two species. A monoclonal mouse anti-sheep IL-8 antibody in combination with HRP-conjugated pig anti-rabbit IgG was also tried. Despite trying several combinations of different techniques it was not possible to get this antibody to stain IL-8 in tissues.

A polyclonal rabbit anti-sheep IL-8 antibody was also tried in conjunction with HRP-conjugated pig anti-rabbit IgG antibody. This combination produced some mild background staining which was blocked with the use of pig serum blocking steps. The primary antibody also demonstrated some non-specific binding to muscle tissue in the deep layers of the reproductive tract, however this was not evident in the stromal tissue. Positive staining of luminal and glandular epithelium was clearly distinguishable in contrast to the lack of staining in adjacent stromal tissue.

Table 5.1 Staining intensity of GM-CSF and IL-8 in tissues from reproductive tracts of ewes at various times post-oestrus (controls) or post-mating.

	Control ewes					**Mated ewes**				
Site/time	3 hrs	6 hrs	18 hrs	24 hrs	48 hrs	3 hrs	6 hrs	18 hrs	24 hrs	48 hrs [b]
GM-CSF										
V [a]	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
C [a]	0,0,0	1,0,1	1,1,1	1,0,1	0,0,0	0,1,0	0,0,0	0,2,1	1,1,0	1,0,1
BU	1,2,1	1,0,2	1,1,0	2,1,2	2,1,2	2,3,3	2,0,3	1,1,2	2,1,2	0,0,1
IM	1,1,0	1,2,2	1,1,0	2,1,2	1,2,2	2,1,1	1,2,1	2,1,2	1,2,2	0,1,1
IA	1,1,0	2,2,2	2,1,1	2,1,2	1,1,2	1,1,1	0,1,1	0,2,2	2,1,2	1,1,0
CM	1,1,1	2,1,1	0,1,0	2,2,2	0,1,2	0,0,1	1,2,3	1,0,2	3,1,2	1,1,1
CA	1,1,0	1,0,1	1,1,1	2,0,2	1,0,1	1,1,3	1,1,1	2,2,1	2,2,2	1,0,0
IL-8										
V [a]	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
C [a]	0,1,2	1,0,1	1,1,1	1,1,1	1,2,0	1,2,0	1,0,1	2,1,1	1,0,0	1,0,1
BU	1,2,2	2,2,1	1,1,0	2,2,2	1,1,1	2,2,1	2,1,0	2,2,2	2,2,1	1,1,2
IM	2,1,2	0,1,3	2,1,2	2,1,2	2,1,2	2,2,1	2,3,1	2,2,2	2,1,2	1,2,3
IA	1,2,2	2,1,1	1,1,1	2,2,2	2,1,0	0,2,2	2,0,1	2,2,2	2,1,2	1,3,3
CM	1,1,2	1,2,1	1,2,2	2,2,2	2,2,1	1,2,1	2,2,2	2,2,1	2,0,2	0,2,2
CA	1,2,2	2,2,0	1,2,1	1,2,0	2,2,2	2,1,1	1,0,2	2,2,0	2,2,2	1,3,3

Data are presented as 0 (no staining), 1 (light staining), 2 (moderate staining) or 3 (strong staining). Data are observations from individual ewes (n=3 per time period). V, vagina; C, cervix; BU, uterine body; IM, ipsilateral mid-uterine horn; IA, ipsilateral anterior uterine horn; CM, contralateral mid-uterine horn; CA, contralateral anterior uterine horn. [a] $P < 0.01$ compared to other sites (collective time periods). [b] $P < 0.01$ compared to other time periods (collective sites).

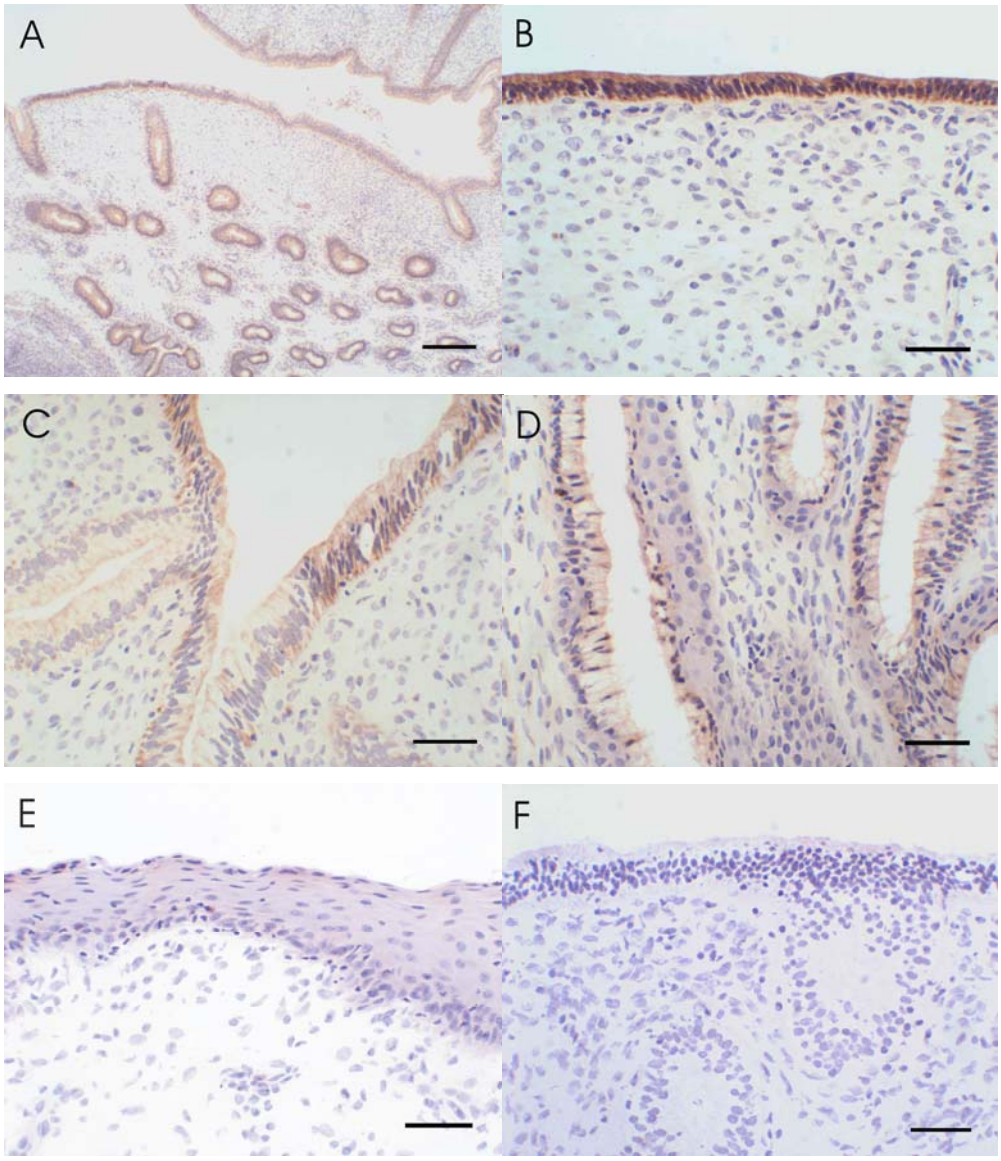

Figure 5.1 Photomicrographs of GM-CSF immunohistochemical staining: (A) distribution of GM-CSF in uterine luminal and glandular epithelium, (B) GM-CSF +++ in uterine body epithelium, (C) GM-CSF ++ in uterine horn epithelium, (D) GM-CSF + in cervical epithelium, (E) GM-CSF - in vaginal epithelium, (F) GM-CSF negative control in uterine body. Scale bars in (A) = 200 μm, scale bars in (B)-(F) = 50 μm.

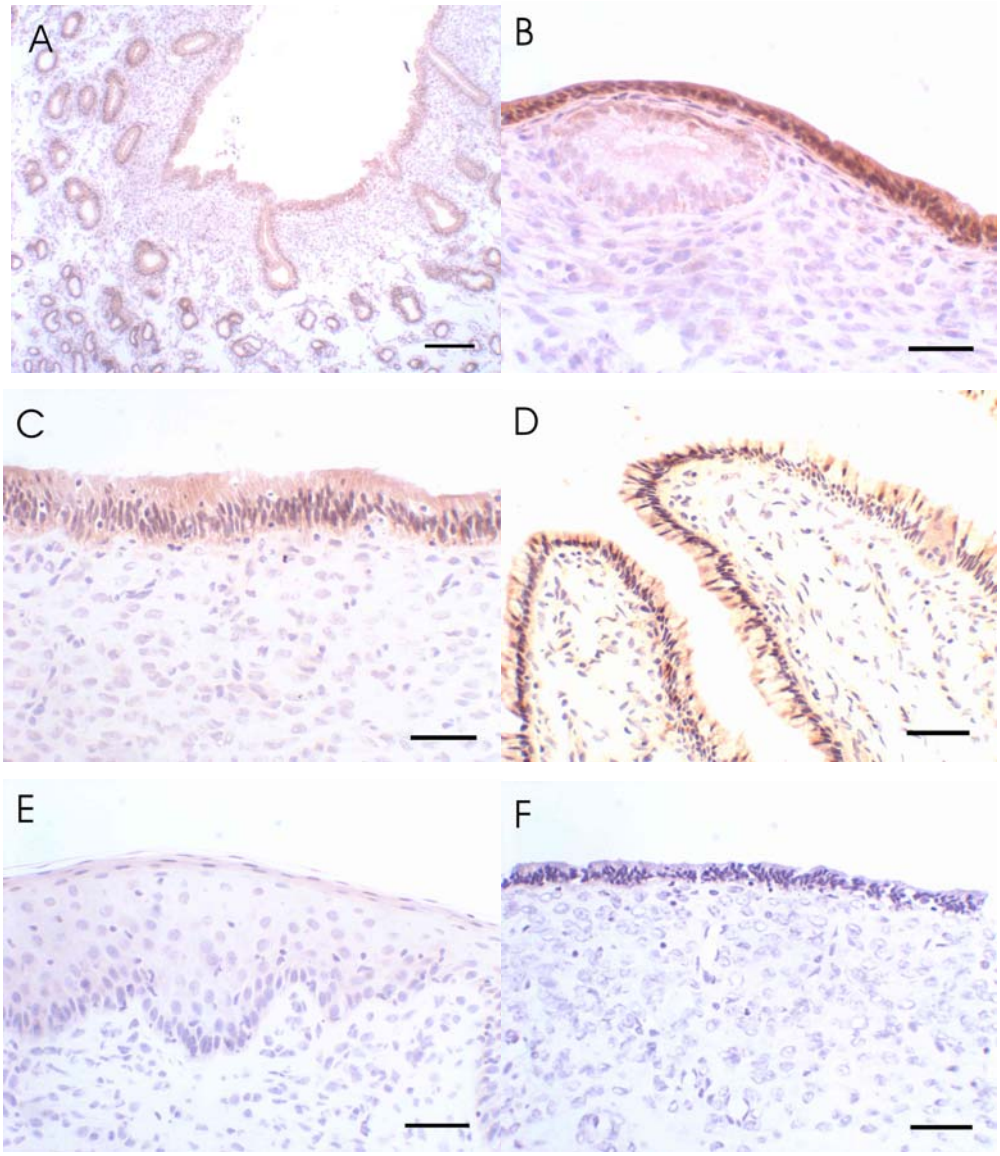

Figure 5.2 Photomicrographs of IL-8 immunohistochemical staining (A) distribution of IL-8 in uterine luminal and glandular epithelium, (B) IL-8 +++ in uterine body epithelium, (C) IL-8 ++ in uterine horn epithelium, (D) IL-8 + in cervical epithelium, (E) IL-8 - in vaginal epithelium, (F) IL-8 negative control in uterine body. Scale bars in (A) = 200 μm, scale bars in (B)-(F) = 50 μm.

Table 5.2 GM-CSF and IL-8 concentrations in luminal fluid from reproductive tracts of ewes at various times post-oestrus (controls) or post-mating.

	Control ewes					**Mated ewes**				
Site/time	3 hours	6 hours	18 hours	24 hours	48 hours	3 hours	6 hours	18 hours	24 hours	48 hours
GM-CSF										
V	0,0,0	0,0,0.4	0,0,0	0,0,0	0,0,0	0,0.4,0	1.1,0,0.5	0,0,0	0,0,0.4	0,0,0
C	0,0,0	0,0,0	0,0,0	0.2,0,0	0,0,0	0,0.4,0	0,0.6,0	0,0,0	0,0,0.8	0,0.3,0
BU	0,0,0.2	0,0,0.8	0,0,0	0,0,0	0,0,0.1	0,0.7,0	0,0.8,0	0,0.5,0	0,0.5,0.3	0,0.4,0
IM	0,0,0.6	0,0,0.5	0,0,0	0,0,0.1	0,0,0	0,0,0.9	0,0.4,0	0,0,0	0,0,0.6	0,0.4,0
IA	0.4,0,0.5	0,0.4,0.5	0,0,0	0,0.3,0	0,0,0	0,0,0	0,0.4,1.6	0,1.6,0	0,0,0.4	0,0,0.4
CM	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0.1	0,0,0	0,0,1.0	0,0,0	0,0,1.3	0,0.6,0
CA	0.4,0,0.7	0,0,0.5	0,0,0	0,0,0	0,0,0	0,0,0	0,0.5,0.5	0,0,0	0,0,0.6	0,0.5,0.6
IL-8										
V	30.2,0.8,3.6[a]	2.4,0.1,62.3[a]	0.6,3.8,0[a]	7.2,1.9,23.7[a]	6.7,62.3,26.3[a]	72.5,11.4,1.0[a]	2.3,12.9,24.6[a]	7.2,2.5,0.9[a]	13.8,3.8,20.4[a]	51.8,13.1,32.3[a]
C	0,0,0.3	0.4,0.1,0	0,0,0	0.4,0,0	0.1,0,0	0,0.5,0	0.3,0,0	0.2,0,0	0,0,0	0,0,0
BU	0,0,0.1	0.6,0.6,0	0,0,0	0.4,0,0	0,0,0	0.9,0.25,0	0.1,0,0	0.2,0.2,0	0,0,0	0.1,0,0
IM	0.4,0,0	0.1,0.1,0	0,0,0	0,0,0	0,0,0	0,0,0	0.1,0,0	2.4,0,0	0,0,0	0.2,0.1,0
IA	0,0,0	0,0,0.1	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
CM	0,0,0	0.2,0.1,0	0,0,0	0,0,0	0,0,0	0,0.1,0	0,0,0	0.4,0.1,0	0,0,0	0,0,0
CA	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0.2,0,0	0,0,0	0,0,0

Data are presented as concentration (ng/ml). Data are observations from individual ewes (n=3 per time period). V, vagina; C, cervix; BU, uterine body; IM, ipsilateral mid-uterine horn; IA, ipsilateral anterior uterine horn; CM, contralateral mid-uterine horn; CA, contralateral anterior uterine horn. [a] $P < 0.01$ compared to other sites.

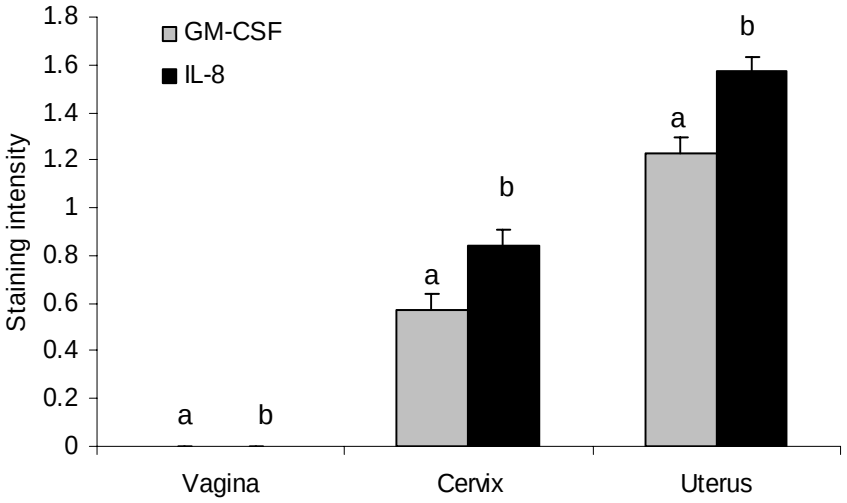

Figure 5.3 Staining intensity of GM-CSF and IL-8 in tissues at different sites in the reproductive tracts of ewes. Data are presented as the mean + SEM: vagina (n=30), cervix (n=90), uterus (n=150). [a] GM-CSF P <0.01 compared with other sites; [b] IL-8 P <0.01 compared with other sites.

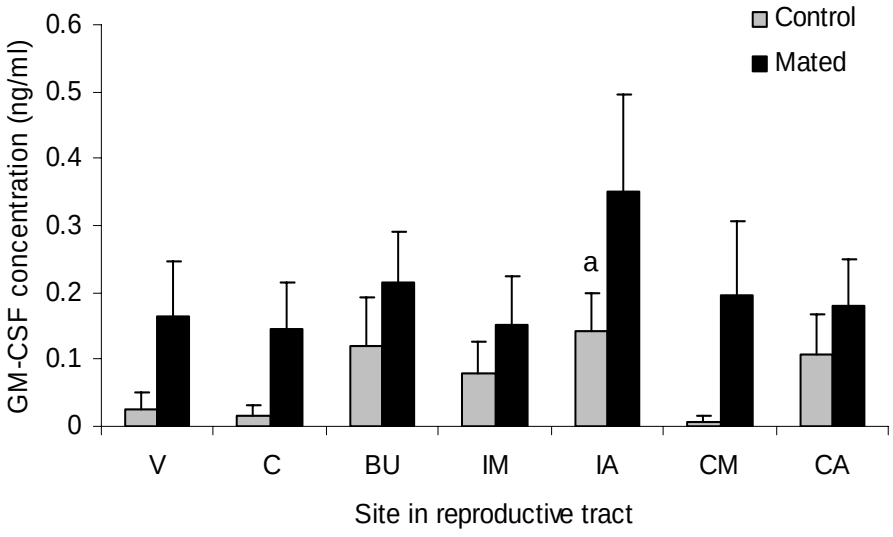

Figure 5.4 Concentration of GM-CSF in reproductive tract luminal fluid at various sites in control and mated ewes. Data are mean concentration (ng/ml) + SEM (n=15). [a] P <0.05 compared with cervix and contralateral mid-uterine horn. V, vagina; C, cervix; BU, uterine body; IM, ipsilateral mid-uterine horn; IA, ipsilateral anterior uterine horn; CM, contralateral mid-uterine horn; CA, contralateral anterior uterine horn.

5.4.2.2 IL-8 in tissues

Interleukin-8 was detected in the apical and basal cytoplasm of luminal and glandular endometrial epithelial cells (Figure 5.2A) and in cervical epithelium (Figure 5.2D) of both mated and non-mated ewes. There was no significant difference in staining intensity between mated and control ewes at any site or for any time period. However, there were significant differences (P <0.01) in IL-8 staining intensity between different sites in the reproductive tract (Table 5.1). The uterine body (Figure 5.2B) and uterine horns (Figure 5.2C) had the most intense IL-8 staining, cervical tissue had light to moderate staining (Figure 5.2D) and vaginal tissue did not stain (Figure 5.2E). Staining was less intense in glandular endometrial epithelial cells than luminal endometrial epithelium. Similarly, when data were combined into three main sites, there were significant differences (P <0.01) in IL-8 staining intensity between the vagina, cervix and uterus (Figure 5.3).

5.4.2.3 IL-8 in the lumen

The ELISA standard curve generated using recombinant ovine IL-8 gave an excellent correlation between OD reading and IL-8 concentration (see Appendix 22).

The concentration of IL-8 detected in luminal fluid from the reproductive tract of ewes ranged from 0 to 72.5 ng/ml. Significantly higher (P <0.01) concentrations of IL-8 were detected in the vagina than in any other site and vaginal samples from 29 out of 30 ewes contained IL-8 (Table 5.2). There appeared to be an individual ewe effect regarding the presence of IL-8 in other sites. Some ewes had IL-8 present in several sites other than the vagina (eg. 1st ewe 18 h mated, Table 5.2), but many had IL-8 present only in the vagina (eg. 3rd ewe 18 h mated, Table 5.2). Slightly higher mean concentrations (ng/ml) of IL-8 were detected in mated compared to control ewes in the vagina (C = 15.5 ± 5.5; M = 18.0 ± 5.3), ipsilateral (C = 0.04 ± 0.3; M = 0.18 ± 0.15) and contralateral mid-uterine horns (C = 0.02 ± 0.01; M = 0.04 ± 0.03), but there was variability between ewes and the differences between mated and control ewes were not statistically significant. Similar concentrations of IL-8 were detected in control and mated ewes in the cervix (C = 0.08 ± 0.04; M = 0.07 ± 0.04) and uterine body (C = 0.11 ± 0.06; M = 0.11 ± 0.06). Both anterior uterine horns

from mated and control ewes had less than 0.01ng/ml of IL-8 detected in luminal fluid. There were no significant differences in luminal IL-8 between different time periods following oestrus or mating. There was a significant (P <0.01) negative correlation (Pearson -0.412) between staining intensity and luminal IL-8 concentration that was most evident in the vagina, which had highest luminal IL-8 and least tissue staining (Table 5.1).

5.4.3 Correlation between cytokines and cell counts

5.4.3.1 Correlation between IL-8 and cell counts

Correlation of ranked IL-8 concentration in luminal fluid with neutrophil counts in luminal smears indicated a statistical significance (P <0.01), with a Spearman's correlation coefficient of 0.48. Similarly, when data from the vagina were excluded, correlation of IL-8 concentration in luminal fluid with neutrophils in smears indicated a statistical significance (P <0.01) with a Spearman's correlation coefficient of 0.231. However, upon examination of scatter plots, no discernable pattern to the correlation was evident. It was therefore concluded that no biologically significant correlation was demonstrable between IL-8 concentration in luminal fluid and number of the neutrophils in luminal fluid. Furthermore, no correlation was detected between IL-8 staining intensity of tissues and neutrophil counts in tissues.

5.4.3.2 Correlation between GM-CSF and cell counts

Correlation of GM-CSF concentration in luminal fluid with macrophage counts in luminal smears indicated a statistical significance (P <0.05) with a Spearman's correlation coefficient of 0.167. However, upon examination of scatter plots, no discernable pattern to the correlation was evident. It was concluded that no biologically significant correlation was demonstrable between GM-CSF concentration and the number of macrophages present. Similarly, no correlation was detected between GM-CSF staining intensity of tissues and macrophage counts in tissues. Additionally, no correlation was detected between GM-CSF and neutrophil counts for either luminal fluid or tissues.

5.5 Discussion

In mice (Robertson and Seamark 1990; Sanford *et al.* 1992), humans (Sharkey *et al.* 2004) and pigs (O'Leary *et al.* 2004), GM-CSF increases in the reproductive tract in response to insemination. Our results suggest that luminal GM-CSF also increases slightly in the reproductive tract of the ewe following mating. In contrast to previous work which found no GM-CSF in the reproductive tract lumen of non-mated mice (Robertson and Seamark 1990), this study found small amounts of GM-CSF in some luminal secretions from non-mated ewes. In cows, GM-CSF is synthesised in a uniform manner throughout the oestrous cycle (de Moraes *et al.* 1999) and no difference was found in the amount of GM-CSF produced in the reproductive tract of the ewe in response to exogenous oestrogen and progesterone administration, even though there was an increase in GM-CSF mRNA expression in oestrogen treated and in pregnant ewes (McGuire *et al.* 2002). However, we found a reduction in GM-CSF concentration in luminal fluid over the 48 hour period following the onset of oestrus. There was a lower GM-CSF concentration in luminal fluid by 18 hours post-oestrus than at three and six hours and GM-CSF concentration remained low at 24 and 48 hours post-oestrus. It is likely that in the ewe, GM-CSF production is increased at oestrus, and there is a further increase in GM-CSF synthesis and release in response to insemination. To confirm the pattern of GM-CSF synthesis and secretion during the oestrous cycle in ewes, samples from different stages of the cycle, including the luteal phase would need to be examined.

The ipsilateral anterior uterine horn had the highest mean luminal GM-CSF concentration following mating, which may be related to preparation for pregnancy, although there appeared to be individual ewe variations. It is likely that during mating, variable volumes of semen are deposited into the vagina and cervix of different ewes, and this may in turn affect the increase in cytokines and leukocytes involved in the subsequent inflammatory response. If this is the case, the variability encountered during natural mating in a flock may affect fertility rates. In order to clarify this effect, comparisons would need to be made between natural mating and artificial insemination of ewes using a known volume and concentration of inseminate.

Granulocyte-macrophage colony-stimulating factor was present in the apical and basal cytoplasm of endometrial and cervical epithelial cells of mated and non-mated ewes. Although staining intensity of GM-CSF was most intense in uterine epithelium, followed by cervical epithelium, there was no difference in the concentration of GM-CSF in the lumen between these sites. This may indicate that GM-CSF is synthesised in endometrial (and to a lesser extent cervical) epithelial cells, released into the lumen and subsequently dispersed throughout the reproductive tract. The reduction in staining intensity of GM-CSF by 48 hours after mating could indicate it had been secreted into the lumen and less was present within epithelial cells. Less intense staining was observed in deep glandular epithelium. This could be explained by semen having greater contact with surface epithelium than deep glandular epithelium, resulting in greater stimulation of GM-CSF synthesis and secretion in epithelial cells near the endometrial surface. The distribution of GM-CSF in endometrial tissue in this study is consistent with previous reports in mice (Robertson *et al.* 1992), cows (de Moraes *et al.* 1997; de Moraes *et al.* 1999) and sheep (McGuire *et al.* 2002). Additionally, GM-CSF has been detected in leukocytes in the endometrial stroma and in oviductal epithelium of cows (de Moraes *et al.* 1997; de Moraes *et al.* 1999).

Increased expression of IL-8 has been found in the cervix of humans (Sharkey *et al.* 2003; Sharkey and Robertson 2004) and sheep (Mitchell *et al.* 2002) following insemination. However, the study in sheep compared non-mated progesterone synchronised ewes with similarly synchronised mated ewes. It was found that while mated ewes had a significantly higher level of IL-8 expression in the cervix than non-mated ewes, both groups had a lower IL-8 expression than control ewes during natural oestrus. It was concluded that the use of progesterone to synchronise oestrus caused a reduction in the amount of IL-8 in the ovine cervix compared to a ewe in natural oestrus. It was proposed that increased IL-8 would also be observed in response to insemination of ewes following a natural oestrus but this was not confirmed (Mitchell *et al.* 2002). In view of this research, IL-8 was expected to increase in ovine reproductive tissues and luminal fluid in response to insemination. Our study found a similar staining intensity of IL-8 in cervical tissue from mated and control ewes, but higher IL-8 concentration in the lumen of mated compared to non-mated ewes in the vagina and mid-uterine horns although the differences were not

statistically significant. There was variability in luminal IL-8 between individual ewes which may be related to the volume of semen deposited during natural mating, and it remains unclear whether IL-8 production increases in response to insemination in the ewe.

The distribution of IL-8 in reproductive tissues of ewes is consistent with that observed by other researchers. Interleukin-8 has been demonstrated in luminal and glandular endometrial epithelial cells and oviductal epithelium in humans (Arici *et al.* 1998; Palter *et al.* 2001; Young *et al.* 2002) and in cervical epithelium of sheep (Mitchell *et al.* 2002). However, IL-8 mRNA was not expressed in the endometrium of non-pregnant ewes, but was detectable by 26 days following mating (Nasar *et al.* 2004). The study examined the expression of IL-8 in endometrial caruncles, whereas our work focused on immunohistochemical staining for IL-8 in the intercaruncular region. Positive staining for IL-8 was, however, observed in epithelial cells overlying caruncles as well as over intercaruncular areas in both mated and non-mated ewes.

The concentration of IL-8 in luminal fluid had a different distribution to the staining intensity of IL-8 in tissues. For instance, IL-8 was not detected in vaginal tissue but luminal fluid from the vagina contained the highest IL-8 concentration. Furthermore, staining intensity for IL-8 was strongest in uterine epithelium, whereas the concentration of IL-8 detected in the uterine lumen was much lower than in the vagina. One explanation for this apparent discrepancy is that IL-8 may have been secreted by vaginal epithelium into the lumen but was no longer present in these cells. This seems logical, as IL-8 in the vagina is likely to function as part of the mucosal defence system by attracting neutrophils into the area to phagocytose micro-organisms. Alternatively, IL-8 may have flowed or been transported into the vaginal lumen from the uterus and/or cervix. Due to the lack of any appreciable contribution to such a flow by gravity as the ewe reproductive tract is positioned horizontally in the abdomen, this would rely on active smooth muscle contractions causing retrograde flow of secretions within the reproductive tract. Regular contractile activity has previously been demonstrated in the cervix and uterus of ewes around the time of ovulation (Garcia-Villar *et al.* 1983). The direction of contractions in both ewes and cows are mostly away from the cervix and towards the oviducts. These contractions are believed to aid the transport of spermatozoa from the cervix into the

oviducts (Hawk 1975; Hawk and Conley 1975; Rodriguez-Martinez *et al.* 1987). However, uterine contractions do occur in both directions and the cervix undergoes its own spontaneous motility independent of the rest of the reproductive tract (Garcia-Villar *et al.* 1982). By 48 hours after oestrus, the direction of contractions in the ewe reproductive tract is reversed, with 75% originating in the oviducts and moving towards the cervix (Hawk 1975).

In conclusion, GM-CSF and IL-8 were detected both in reproductive tissues and luminal fluid from mated and non-mated ewes. Granulocyte-macrophage colony-stimulating factor increased slightly in the reproductive tract of ewes after natural mating but the effect of insemination on IL-8 remains unclear. The high concentration of IL-8 detected in the vagina of both mated and non-mated ewes is likely to function as a defence against micro-organisms by attracting neutrophils to the area. The significance of any increase in GM-CSF following insemination in the ewe is not clear, but it is likely to contribute to the influx of leukocytes observed after mating and thereby facilitate preparation of the uterus to accept the conceptus. Comparison of the cytokine and leukocyte response between natural mating and artificial insemination with a known volume of inseminate is warranted, along with investigation of other cytokines with chemotactic properties, such as MCP-1 and MIP1-α.

CHAPTER 6
COMPONENTS OF SEMEN THAT CAUSE INFLAMMATION

6.1 Introduction

Insemination induces an inflammatory reaction in the uterus and cervix, but there are conflicting reports regarding which component(s) of semen trigger this response. Species differences may contribute to some of these discrepancies. For instance, in mice, seminal plasma is consistently reported to cause the reaction (Robertson and Seamark 1990; McMaster *et al.* 1992; Sanford *et al.* 1992; Tremellen *et al.* 1998). More specifically, TGF-β from mouse seminal vesicles stimulates the synthesis and secretion of GM-CSF from uterine epithelial cells, inducing leukocyte infiltration into the endometrial stroma (Robertson *et al.* 1992; Robertson *et al.* 1996a; Tremellen *et al.* 1998). In contrast to mice, spermatozoa rather than seminal plasma are consistently reported to cause uterine inflammation in horses (Kotilainen *et al.* 1994; Troedsson *et al.* 1998; Troedsson *et al.* 2001).

In some species, it remains unclear which components of semen cause inflammation in the female reproductive tract. Both seminal plasma (Gutsche *et al.* 2003) and spermatozoa (Pandya and Cohen 1985; Thompson *et al.* 1992) have been implicated in humans, and TGF-β stimulates release of GM-CSF and IL-6 from human cervical epithelial cells (Sharkey and Robertson 2004). The chemoattractant IL-8 has also been detected in human seminal plasma (Srivastava *et al.* 1996). Seminal plasma (McDonald *et al.* 1952), spermatozoa (Tyler 1977) and a combination of both (Howe 1967; Phillips and Mahler 1977) will cause the inflammatory reaction in rabbits. Similarly, seminal plasma (Bischof *et al.* 1994b; Assreuy *et al.* 2002; O'Leary *et al.* 2004), spermatozoa (Rozeboom *et al.* 1999; Rozeboom *et al.* 2000; Rozeboom *et al.* 2001) and the physical presence of fluid (Matthijs *et al.* 2003) reportedly cause the reaction in pigs.

An inflammatory reaction to semen has been demonstrated in cattle (Mahajan and Menge 1967), goats (Mattner 1968) and sheep (Mattner 1969) but no differentiation has been made between spermatozoa and seminal plasma, although *in vitro* studies

suggest spermatozoa may be responsible for the reaction in cattle (Clark and Klebanoff 1976). It is not known which component(s) of semen cause the post-insemination inflammatory reaction in the ewe, nor is it known if TGF-β, GM-CSF or IL-8 are present in ram semen.

The aims of this study were to compare the response in the ovine uterus to whole semen, spermatozoa and seminal plasma and to determine if TGF-β1, GM-CSF and IL-8 are present in ram semen.

6.2 Materials and Methods

6.2.1 Ewes

Ewes were synchronised for oestrus (see 3.2) and nine ewes that were in oestrus at 8:00 am on the morning of scheduled surgery were selected for surgical insemination. Ewes had whole semen, spermatozoa, seminal plasma, TALP or normal saline injected into the lumen of ligated uterine sections and their reproductive tracts collected 22 hours later. Three of these ewes had antibiotics added to treatments prior to injection.

6.2.2 Semen collection from rams

Approximately one hour prior to surgical insemination, semen was collected by electro-ejaculation (see 3.4) from each of three rams. Semen from the same three rams (ram numbers 5, 9 and 16) were used for all surgical inseminations. The semen samples were placed in a styrofoam box with bottles containing warm water at approximately 37^{o}C and taken to the laboratory within 20 minutes of collection. Semen was pooled and placed in a 37^{o}C incubator.

Additionally, semen from seven rams (ram numbers 5, 9, 10, 15, 16, 17 and 18) was analysed with an ELISA for the presence of TGF-β1, GM-CSF and IL-8. Semen was collected by electro-ejaculation from each ram on three occasions (day 1, day 10 and

day 30). Semen samples were placed in a styrofoam box at ambient temperature and taken to the laboratory within 20 minutes of collection.

6.2.3 Preparation of samples for surgical insemination of ewes

Three treatments and two controls were prepared for surgical insemination into the uterus of each ewe. The treatments were whole semen (see 3.16.2), sperm-free seminal plasma (see 3.16.3) and 150 x 10^6 washed spermatozoa in TALP medium (see 3.16.4). The controls were TALP medium (see 3.16.1) and physiological saline (0.9% NaCl). All treatments for three of the nine ewes contained 700 IU/ml of penicillin and 700 µg/ml of streptomycin (see 3.16.5).

6.2.4 Anaesthesia of ewes

Anaesthesia was induced via an intravenous injection of thiopentone, ewes were intubated and anaesthesia was maintained with halothane (see 3.17.1).

6.2.5 Surgical technique

The uterus was ligated into five sections (Figure 6.1A) prior to injection of treatments (Figure 6.1B) (see 3.17.2). The technique used was based on a model developed previously at James Cook University (Hilla 1999).

6.2.6 Uterine sample collection and analysis

Ewes were killed (see 3.5) 22 hours following surgical insemination and the location and diameter of the pre-ovulatory follicle was recorded. Luminal fluid, luminal smears and tissue samples were collected from five uterine sites (right and left anterior horns, right and left mid-horns and the uterine body) in each ewe (see 3.6.1 & 3.6.2). Luminal fluid was processed and stained (see 3.8.1 & 3.12.3.2) for the presence of leukocytes (neutrophils, eosinophils and macrophages) and analysed with an ELISA (see 3.15) for the presence of GM-CSF and IL-8. Tissues were processed and stained for the presence of leukocytes (neutrophils, eosinophils, mast cells and macrophages) and GM-CSF and IL-8 (see 3.8.2, 3.9 & 3.12).

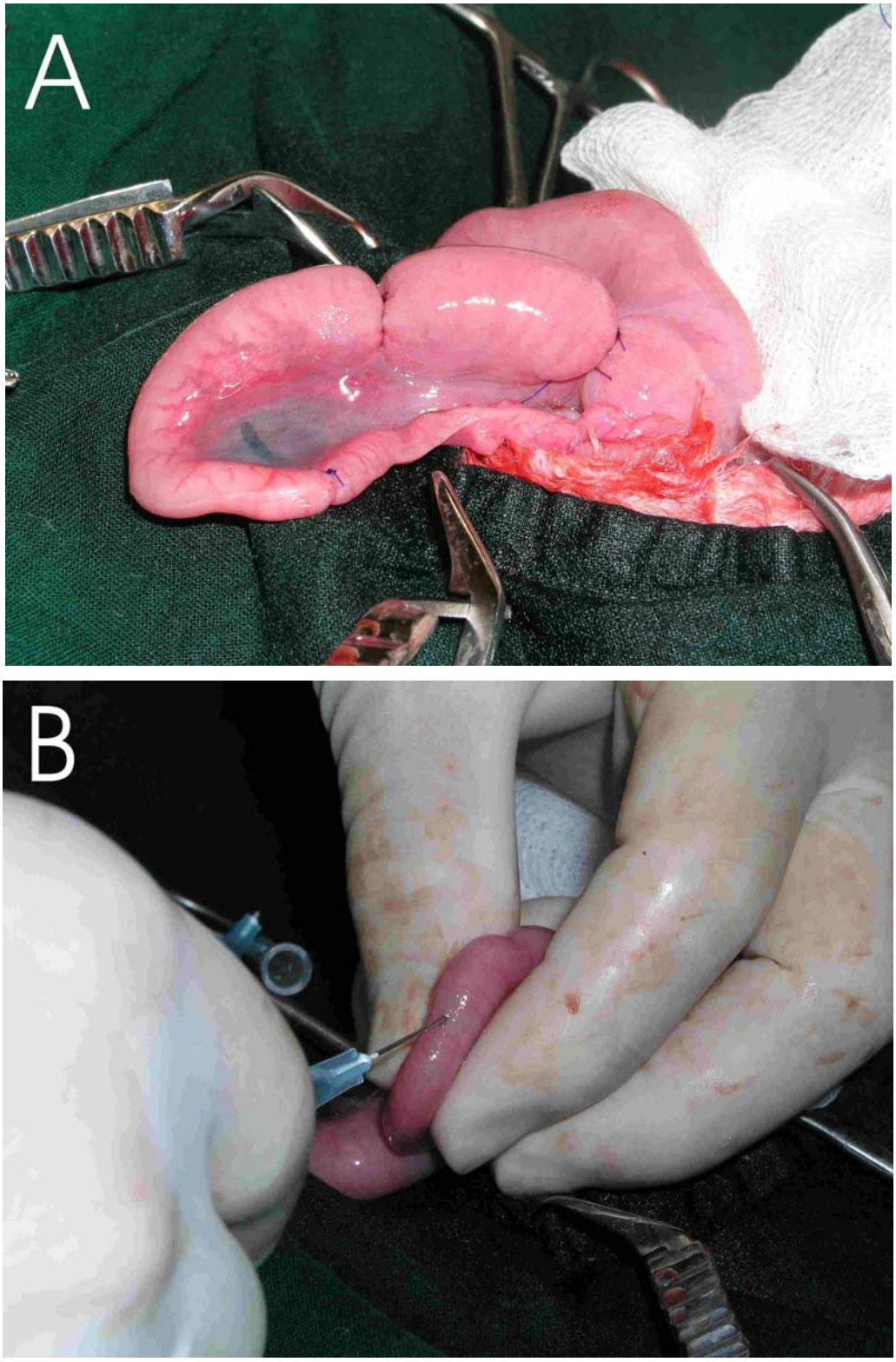

Figure 6.1 (A) Ligated uterine horns and (B) injecting a treatment into a ligated section of an anterior uterine horn.

6.2.7 Calculation of total cytokine content in luminal fluid

Total cytokine content in each luminal fluid sample was determined by multiplying the concentration of cytokine (ng/ml) detected by ELISA by the volume of fluid collected from the lumen (ml) (Table 6.3).

6.2.8 Preparation of seminal plasma for cytokine detection

Semen was separated into spermatozoa and seminal plasma by centrifugation at 1400 ***g*** for 15 minutes at 10^{o}C. The supernatant was removed into a fresh tube using a Pasteur pipette and one drop was placed on a glass slide with a coverslip and examined with a phase-contrast microscope for the presence of spermatozoa. If spermatozoa were present, the sample was re-spun and rechecked. Once the supernatant was free of spermatozoa, 200 µl was placed into a plastic microtube (Sarstedt Cat No 73.690, Germany) and frozen at -20^{o}C. Another 130 µl was placed into a second plastic microtube and was acid treated (see 3.18) before being frozen at -20^{o}C until required.

6.2.9 ELISA protocols for cytokine detection in ram semen

Transforming growth factor beta-1 concentration in ram semen was measured using a TGF-β1 ELISA kit (Promega, Cat G7591, Annandale, NSW Australia) following the manufacturers instructions (see 3.18). Granulocyte-macrophage colony-stimulating factor and IL-8 were measured with an ELISA (see 3.15.1 and 3.15.2).

6.3 Statistical Analyses

6.3.1 Statistical analyses of leukocytes in response to semen

Data for leukocyte counts in tissues were converted to normal distributions by Log_{10} transformation and analysed by parametric tests. Data for leukocyte counts from smears were ranked prior to parametric analyses (Conover and Iman 1981). Three way ANOVA (see Appendix 24), *post hoc* tests (Fisher's LSD) (see Appendix 25)

and Student's *t*-tests (see Appendix 26) were used to compare the response of each cell type to the different treatments, the response between use of antibiotics or no antibiotics in treatments and the response in different depths of the endometrium.

6.3.2 Statistical analyses of cytokines in response to semen

Data for cytokine staining in tissues were semi-quantitative (see 3.13.2) and data for cytokine content in luminal fluid were not normally distributed. Data were ranked prior to parametric analyses (Conover and Iman 1981). Two way ANOVA (see Appendix 27), *post hoc* tests (Fisher's LSD) (see Appendix 28) and Student's *t*-tests were used to compare the cytokine response to the five different treatments and the response between use of antibiotics compared to no antibiotics in treatments.

6.3.3 Statistical analyses of TGF-β1 in ram semen

Data for TGF-β1 concentration in ram semen were not normally distributed and could not be normalised by transformation. Data were ranked prior to parametric analyses (Conover and Iman 1981). One way ANOVA (see Appendix 29) and *post hoc* tests (Fisher's LSD) were used to determine whether there was any difference in total, active and latent TGF-β1 concentration between different rams. For all statistical analyses performed, a *P* value of less than 0.05 was considered significant.

6.4 Results

6.4.1 Neutrophil response to different components of semen

6.4.1.1 Neutrophil infiltration of tissues

More neutrophils were present in uterine tissues of oestrous ewes when no antibiotics were added to treatments, compared to when penicillin and streptomycin were included (Table 6.1), but the differences were not statistically significant. There were significantly more ($P < 0.01$) neutrophils in tissues following treatment with whole semen (Figure 6.2A), seminal plasma (Figure 6.2B) and washed spermatozoa

compared to either TALP (Figure 6.2C) or saline (Figure 6.2D). Highest neutrophil numbers were recorded in tissues after treatment with whole semen, followed by seminal plasma and then washed spermatozoa. Smaller numbers of neutrophils accumulated in tissues after treatment with TALP, and fewest neutrophils were present following treatment with saline (Figure 6.3). Most neutrophils accumulated in the subepithelial stroma subjacent to the endometrial epithelium (Figure 6.2). They were also scattered diffusely throughout the mid- and deep stroma, although there were significantly fewer (P <0.01) in these regions than in the superficial stroma (Table 6.1).

6.4.1.2 Neutrophils in the lumen

There were significantly more (P <0.01) neutrophils in uterine luminal smears from oestrous ewes when no antibiotics were included with the treatments, compared to when penicillin and streptomycin were added, except for saline which induced a minimal response with or without antibiotics. Highest neutrophil numbers were present in smears after treatment with whole semen, followed by seminal plasma, washed spermatozoa, TALP then saline and there were significantly fewer (P <0.01) neutrophils in smears after treatment with saline compared to all other treatments (Figure 6.4).

6.4.2 Eosinophil response to different components of semen

Eosinophil numbers were highly variable, and there were no significant differences in the number of eosinophils present in tissues following different treatments. The use of antibiotics did not alter the number of eosinophils in the superficial endometrium, but there were significantly fewer (P <0.05) eosinophils in the mid- and deep stroma when antibiotics were added to treatments. Without antibiotics, the number of eosinophils in the superficial, mid- and deep stroma were similar to one another, but when antibiotics were added, there were fewer eosinophils in the mid- and deep stroma than the superficial stroma, although the differences were not statistically significant (Table 6.1). Eosinophils were rarely found in luminal smears.

Table 6.1 Leukocyte numbers in uterine tissues of oestrous ewes 22 hours after injection of various treatments into the lumen.

	No antibiotics added			Antibiotics added		
	Superficial	Mid-	Deep	Superficial	Mid-	Deep
Neutrophils						
Semen	932±230 [ab]	104±44.4 [d]	47.2±24.0 [d]	540 ±371	10±10 [d]	0 [d]
SP	895±296 [ab]	65.8±44.5 [d]	30.5±20.9 [d]	545±473	0	2.3±1.2
Sperm	527±204 [ab]	70.7±70.9 [d]	85.2±85.0 [d]	432±162 [c]	6.3±6.3 [d]	0.7±0.7 [d]
TALP	91.3±52.1	1.2±1.2 [d]	0.17±0.17 [d]	66.3±51.3	0 [e]	0.7±0.7 [e]
Saline	15.5±9.5	0	0.5±0.3	10.3±6.4	0.3±0.3	0.3±0.3
Eosinophils						
Semen	10.8±9.9	13.7±12.3	24.0±18.6	1.3±1.3	1.0±0.6	1.7±1.2
SP	16.0±15.0	11.7±8.9	20.2±18.4	13.0±11.1	0	2.0±1.5
Sperm	6.7±6.3	11.7±10.3	15.0±13.6	39.3±39.3	21.3±21.0	8.3±6.4
TALP	17.0±12.0	7.7±6.9	8.2±4.7	9.0±8.5	1.0±1.0	3.0±2.0
Saline	31±29.2	7.2±6.2	19.0±15.9	28.0±28.0	1.0±1.0	0.7±0.7
Mast Cells						
Semen	0.17±0.16	1.0±0.4	2.0±0.9	0	0.3±0.3	0.7±0.7
SP	0	0.5±0.2	2.7±1.4 [d]	0	1.0±1.0	1.0±0.6
Sperm	0.17±0.16	0.8±0.5	2.5±1.4	0	1.0±0.6	1.3±0.3 [e]
TALP	0	0.2±0.2	2.7±0.7 [df]	0	0.3±0.3	0.3±0.3 [g]
Saline	0	0.5±0.5	1.8±0.9 [e]	0	1.0±0.6	1.0±1.0
Macrophages						
Semen	13.7±3.4	11.3±3.1	9.0±3.29	7.3±2.9	7.0±2.6	2.7±0.3
SP	15.3±3.9	10.8±3.4	8.5±1.8	8.0±2.3	11.0±5.1	4.7±2.7
Sperm	14.7±3.8	13.8±3.6	6.5±1.7	12.0±2.6	7.3±3.3	5.3±2.6
TALP	13.0±3.2	11.3±3.3	5.3±1.8	5.3±0.9	6.7±1.7	4.0±1.2
Saline	9.7±2.2	10.5±2.8	6.2±1.8	9.7±2.7	9.0±4.0	7.7±3.3

Data are presented as the mean ± SEM cell counts in 1.5mm^2 uterine tissue. Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride. No antibiotics (n=6), antibiotics added to treatments (n=3). Superficial, mid- and deep refer to depth in endometrium. [a] P <0.01compared with TALP; [b] P <0.01, [c] P <0.05 compared with saline; [d] P <0.01, [e] P <0.05 compared with superficial stroma; [f] P <0.05 compared with mid-stroma; [g] P <0.05 compared with no antibiotics.

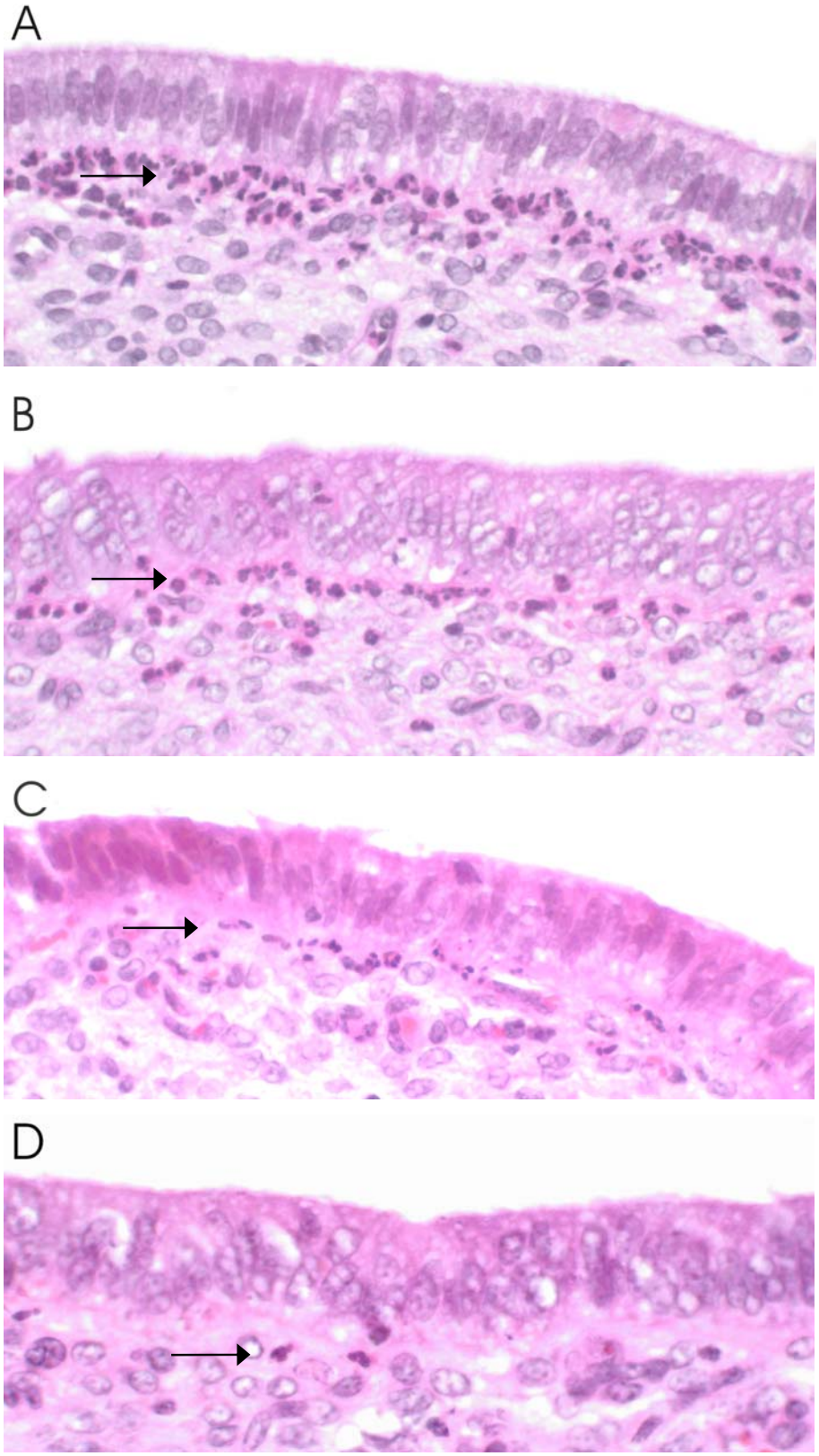

Figure 6.2 Photomicrographs of neutrophils in the subepithelial stroma of the uterus from an oestrous ewe following treatment with (A) whole semen, (B) seminal plasma, (C) TALP and (D) saline.

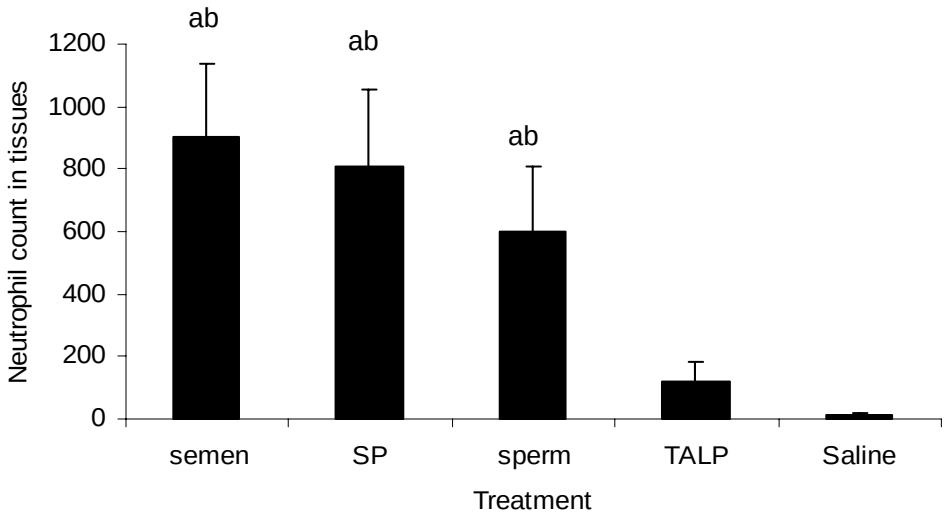

Figure 6.3 Neutrophils in uterine tissues of oestrous ewes 22 hours after injection of various treatments into the lumen. Data are presented as mean + SEM (n=9) in 4.5mm^2 tissue (superficial, mid- and deep stroma). [a] P <0.01 compared to TALP; [b] P <0.01 compared to saline. Treatments were: Semen, whole semen; SP, seminal plasma; sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride.

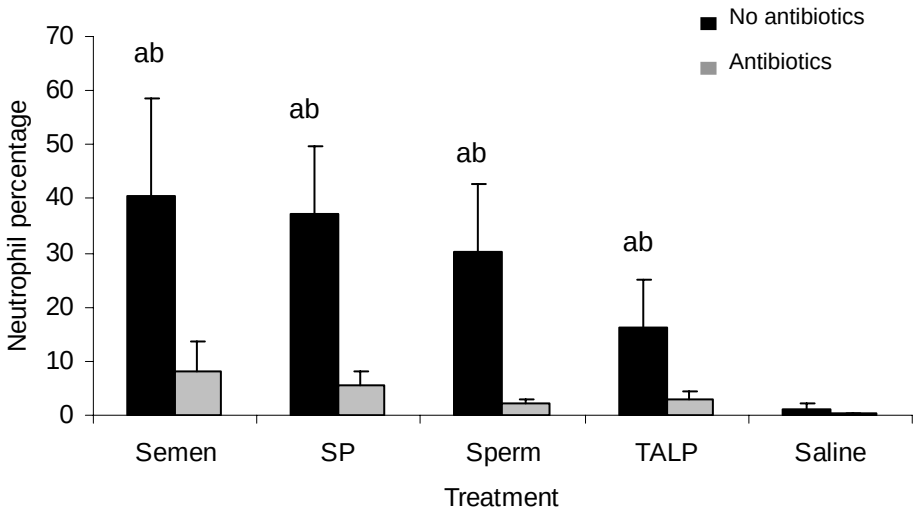

Figure 6.4 Neutrophils in uterine luminal smears from oestrous ewes 22 hours after injection of various treatments with or without the addition of antibiotics into the lumen. Data are presented as mean + SEM percentage of total cells in smears; no antibiotics (n=6), antibiotics added to treatments (n=3). [a] P <0.01 compared to treatment containing antibiotics; [b] P <0.01 compared to saline. Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride.

6.4.3 Mast cell response to different components of semen

There were no significant differences in the number of mast cells present in tissues following different treatments. However, there were significantly more (P <0.01) mast cells in the deep endometrial stroma than in superficial or mid- stromal tissue (Table 6.1). Additionally, there were significantly more (P <0.05) mast cells present in the deep endometrial stroma when no antibiotics were used compared to when penicillin and streptomycin were included with treatments (Figure 6.5) and this pattern was evident for all treatments (Figure 6.6) (Table 6.1).

6.4.4 Macrophage response to different components of semen

The greatest numbers of macrophages were present in the superficial endometrial stroma after injection of seminal plasma, followed by washed spermatozoa, whole semen then TALP, with the fewest following the injection of saline, but the differences were not statistically significant (Table 6.1). Similarly, there were no significant differences in the number of macrophages between different treatments in the mid- or deep endometrial stroma. However, there were significantly more (P <0.05) macrophages in uterine tissues when no antibiotics were used compared to when penicillin and streptomycin were included with treatments, except for saline (Figure 6.7). There were also significantly fewer (P <0.05) macrophages in the deep endometrial stroma compared with the superficial stroma (Table 6.1).

Very few macrophages were present in uterine luminal smears, and there was no difference between the numbers present after treatments with or without antibiotics. However, there were significantly more (P <0.05) macrophages in smears following treatment with washed spermatozoa (0.07 ± 0.03%) or seminal plasma (0.05 ± 0.03%) compared with whole semen, TALP or saline, all of which had no macrophages present.

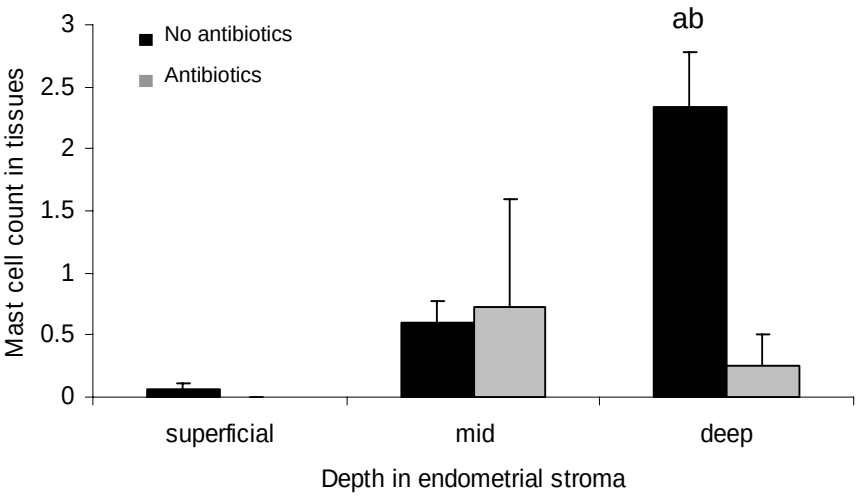

Figure 6.5 Mast cells in different depths of the endometrial stroma of oestrous ewes 22 hour after injection of treatments with or without the addition of antibiotics into the lumen. Data are presented as mean + SEM in 1.5mm^2 tissue; no antibiotics (n=30), antibiotics added to treatments (n=15). [a] P <0.01 compared to mid- and superficial stroma; [b] P <0.05 compared to treatment containing antibiotics.

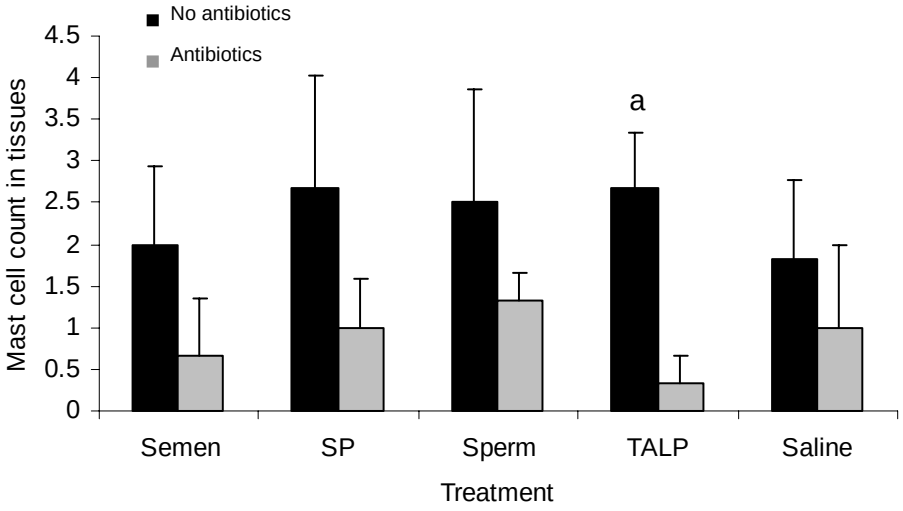

Figure 6.6 Mast cells in the deep endometrial stroma of oestrous ewes 22 hours after injection of various treatments with or without the addition of antibiotics into the lumen. Data are presented as mean + SEM in 1.5mm^2 tissue; no antibiotics (n=6), antibiotics added (n=3). [a] P <0.05 compared to treatment containing antibiotics. Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride.

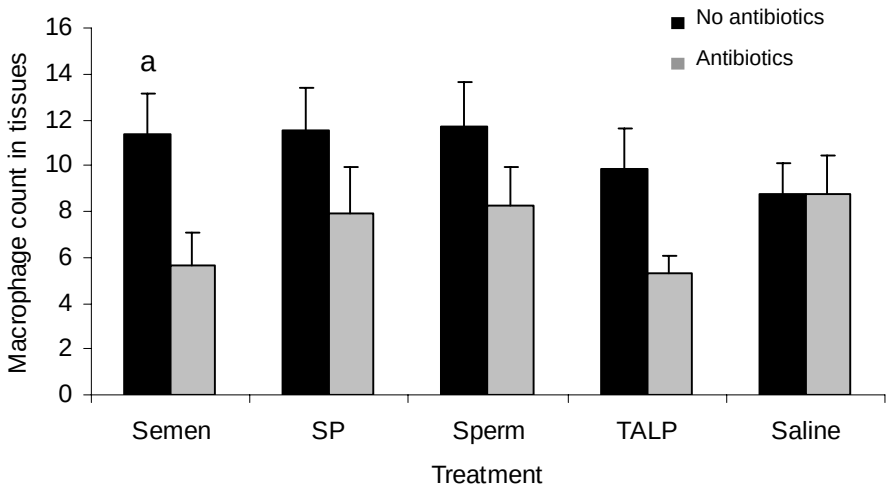

Figure 6.7 Macrophages in uterine tissues of oestrous ewes 22 hours after injection of various treatments with or without the addition of antibiotics into the lumen. Data are presented as mean + SEM in 1.5mm^2 tissue; no antibiotics (n=18), antibiotics added to treatments (n=9). [a] P <0.05 compared to treatment containing antibiotics. Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride.

6.4.5 GM-CSF response to different components of semen

The apical and basal cytoplasm of luminal and glandular endometrial epithelial cells stained positively for GM-CSF. Staining intensity of GM-CSF following treatments without antibiotics included was strongest after injection of whole semen or spermatozoa compared to injection of seminal plasma, TALP or saline (Figure 6.8), however the differences were not statistically significant. There were no significant differences in staining intensity of GM-CSF between different treatments, or between treatments with or without antibiotics (Table 6.2).

Granulocyte-macrophage colony-stimulating factor was not detected in any sample of uterine luminal fluid from oestrous ewes when antibiotics were added to the treatments (Table 6.3). Additionally, GM-CSF was not detected in luminal fluid following treatment with saline or seminal plasma when no antibiotics were included. However, significantly more (P <0.01) GM-CSF was detected in luminal fluid following treatment with whole semen or washed spermatozoa without the addition of antibiotics compared to other treatments. Whole semen induced the

greatest response, washed spermatozoa induced a slightly less response, and there was a minimal response to TALP (Figure 6.9).

6.4.6 IL-8 response to different components of semen

Interleukin-8 was detected in luminal and glandular endometrial epithelial cells of oestrous ewes. There were no significant differences in staining intensity of IL-8 in uterine tissues between treatments with or without antibiotics (Table 6.2). However, there was a significantly stronger (P <0.05) staining intensity in uterine tissues after injection with whole semen, compared to injection of TALP or saline. Staining intensity of IL-8 after injection of seminal plasma and spermatozoa were similar to one another, and intermediate between TALP and saline (Figure 6.10).

Lesser amounts of IL-8 were detected in uterine luminal fluid after injection of treatments containing antibiotics than those without, although the difference was not statistically significant (Table 6.3). Whole semen induced the greatest response, with significantly more (P <0.01) IL-8 detected than in response to saline or TALP. Seminal plasma and washed spermatozoa both induced approximately half as much IL-8 as whole semen. Less IL-8 was produced following treatment with TALP, and the response to saline was less (P <0.01) than any other treatment (Figure 6.11).

Table 6.2 Staining intensity of GM-CSF and IL-8 in uterine epithelium from oestrous ewes 22 hours after injection of various treatments into the lumen.

	GM-CSF		**IL-8**	
	No antibiotics	Antibiotics	No antibiotics	Antibiotics
Semen	2,2,1,0,1,2	1,0,2	1,2,2,1,1,3	2,1,2
SP	2,2,1,1,1,0	1,0,2	1,2,1,2,1,1	2,0,2
Sperm	2,2,2,1,1,1	0,0,1	2,1,1,1,0,2	2,1,1
TALP	2,1,1,1,0,1	0,0,2	1,2,1,1,0,1	1,0,2
Saline	2,0,1,1,1,1	1,0,0	2,1,1,0,0,1	1,0,2

Data are presented as 0 (no staining), 1 (light staining), 2 (moderate staining) or 3 (strong staining). Data are observations from individual ewes; no antibiotics (n=6), antibiotics added to treatments (n=3). Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride.

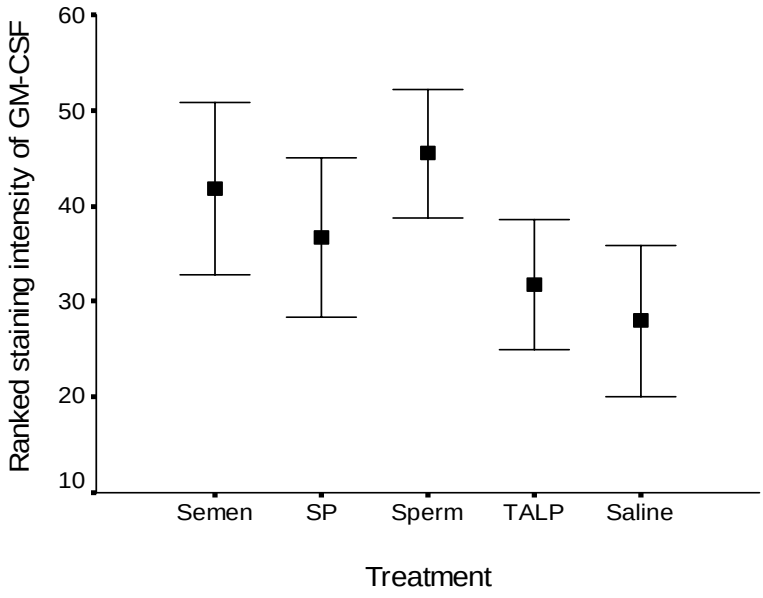

Figure 6.8 Ranked staining intensity of GM-CSF in uterine tissues from oestrous ewes 22 hours after injection of various treatments without the addition of antibiotics into the lumen. Data are presented as mean ± SEM (n=6). Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride.

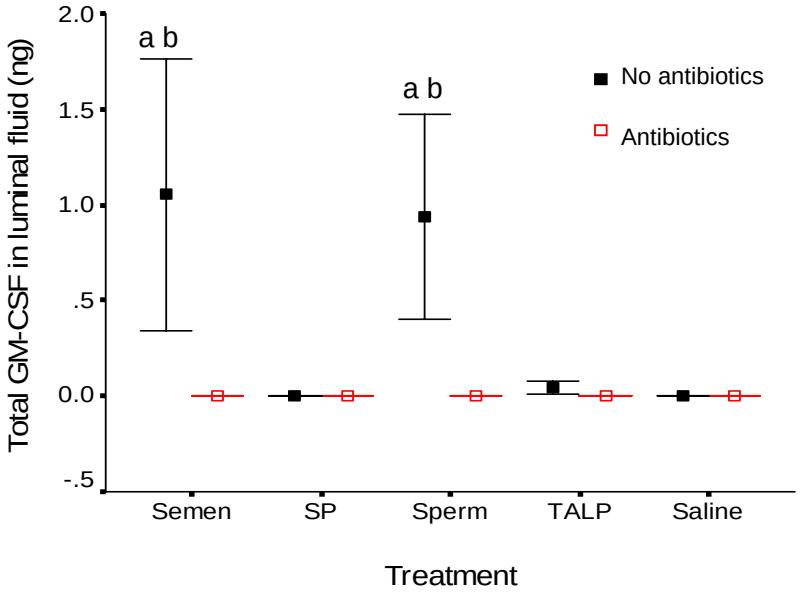

Figure 6.9 GM-CSF in luminal fluid of oestrous ewes 22 hours after injection of various treatments into the lumen. Data are presented as mean ± SEM; no antibiotics (n=6), antibiotics added to treatments (n=3). [a] P <0.01 compared to treatment containing antibiotics; [b] P <0.01 compared to SP, TALP and saline. Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride.

Table 6.3 Fluid volumes (µl) and cytokine concentrations (ng/ml) in uterine luminal fluid from oestrous ewes 22 hours after injection of various treatments.

		Treatment				
Ewe Number		Whole semen	Seminal plasma	Washed sperm	TALP medium	0.9% NaCl
161	**Volume**	1500	100	800	120	1000
	GM-CSF	0	0	3.92	0	0
	IL-8	36.40	80.34	19.85	30.56	37.67
165	**Volume**	1300	750	400	450	70
	GM-CSF	1.29	0	4.93	0	0
	IL-8	28.68	0	4.08	1.51	0
174	**Volume**	3000	2300	900	1900	200
	GM-CSF	1.45	0	0	0	0
	IL-8	30.06	34.48	30.93	15.89	0.48
180	**Volume**	1400 [bc]	1000 [bc]	1200 [bc]	400 [b]	100
	GM-CSF	0	0	0.29	0	0
	IL-8	62.64	78.54	76.17	10.70	0.20
177	**Volume**	400	1300	400	300	160
	GM-CSF	0.75	0	0.13	0.70	0
	IL-8	3.61	5.22	2.77	0.36	0.77
181	**Volume**	2000 [c]	700	1000	100	100
	GM-CSF	0	0	0.13	0.32	0
	IL-8	24.90	7.10	13.71	1.31	0
186 [a]	**Volume**	1800	1000	300	750	100
	GM-CSF	0	0	0	0	0
	IL-8	8.25	9.31	3.30	2.04	0.72
188 [a]	**Volume**	1200	500	500	1500	100
	GM-CSF	0	0	0	0	0
	IL-8	2.69	0.080	3.02	1.28	0.008
166 [a]	**Volume**	1300	1800 [b]	600	600	100
	GM-CSF	0	0	0	0	0
	IL-8	4.65	3.29	24.35	0.74	0

[a] antibiotics added to treatments; [b] blood tinged fluid; [c] cloudy fluid.

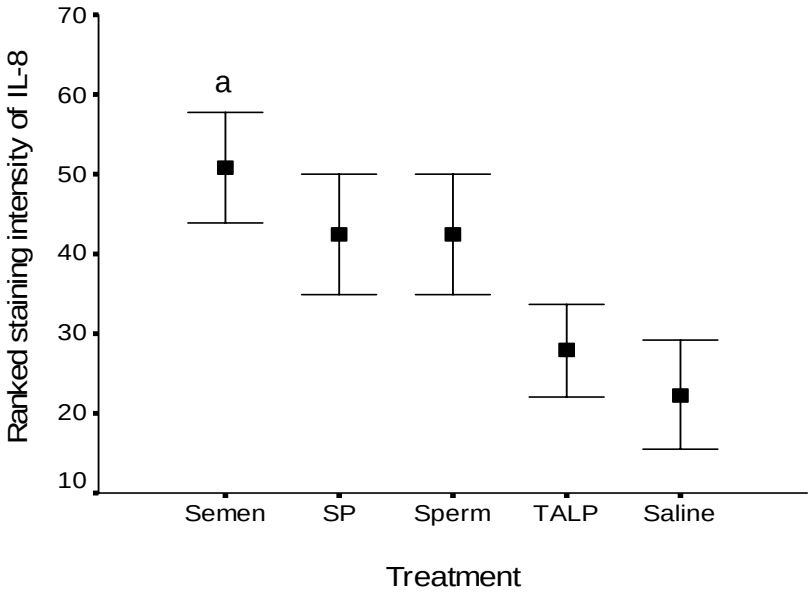

Figure 6.10 Ranked staining intensity of IL-8 in uterine tissues from oestrous ewes 22 hours after injection of various treatments into the lumen. Data are presented as mean ± SEM (n=9). [a] *P* <0.05 compared to TALP and saline. Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride

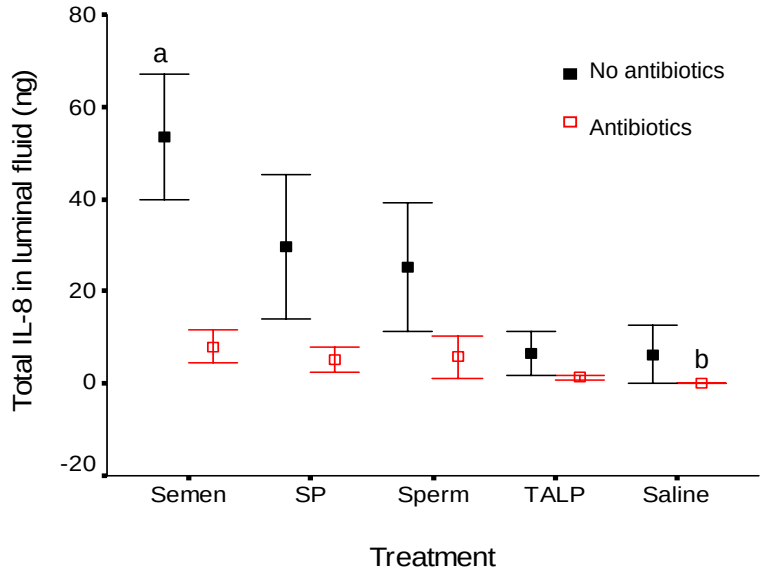

Figure 6.11 IL-8 in luminal fluid of oestrous ewes 22 hours after injection of various treatments into the lumen. Data are presented as mean ± SEM; no antibiotics (n=6), antibiotics added to treatments (n=3). [a] *P* <0.01 compared to TALP and saline; [b] *P* <0.01 compared to all other treatments. Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride.

6.4.7 Cytokines in ram semen

The standard curve generated from recombinant TGF-β1 gave excellent correlation between OD and TGF-β1 concentration (see Appendix 22).

Transforming growth factor-beta was detected in the semen of all seven rams on all three days of collection (Table 6.4). The mean total TGF-β1 concentration was 400 ± 67 pg/ml. Of this, 90 % (363 ± 68 pg/ml) was in a latent form and the rest (37 ± 14pg/ml) was in an active form. There were no significant differences in latent or active TGF-β1 concentration between different rams. However, there were significant differences (P <0.05) in total TGF-β1 between different rams, with rams 16 and 18 having the highest TGF-β1 concentration in their semen, and rams 9,10 and 18 having the lowest (Figure 6.12). Repeat measures of seminal plasma from one boar, which was used as a control, contained 7907 ± 774 pg/ml TGF-β1, with 93 % in an active form.

Neither GM-CSF nor IL-8 were found in any of the ram seminal plasma samples.

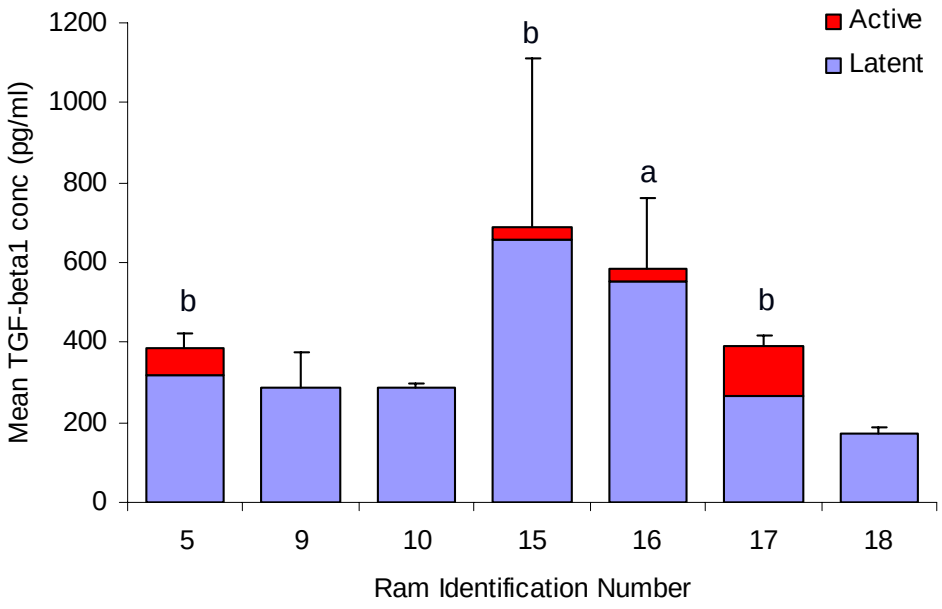

Figure 6.12 Active and latent TGF-beta 1 in semen from seven rams. Data are presented as the mean + SEM (total) TGF-beta concentration (pg/ml) in semen samples (n=3) for each ram. [a] P <0.05 compared to rams 9,10 and 18; [b] P <0.05 compared to ram 18.

Table 6.4 Active, latent and total TGF-β1 concentration in ram semen.

		TGF-β1 concentration (pg/ml)			
		Active TGF-β1	Latent TGF-β1	Total TGF-β1	% Active
Ram 5	Day 1	214	103	317	67%
	Day 10	0	418	418	0%
	Day 30	0	426	426	0%
Ram 9	Day 1	0	404	404	0%
	Day 10	0	339	339	0%
	Day 30	0	121	121	0%
Ram 10	Day 1	0	297	297	0%
	Day 10	0	295	295	0%
	Day 30	0	266	266	0%
Ram 15	Day 1	88	214	302	29%
	Day 10	0	228	228	0%
	Day 30	0	1533	1533	0%
Ram 16	Day 1	0	358	358	0%
	Day 10	0	469	469	0%
	Day 30	96	837	933	10%
Ram 17	Day 1	127	296	423	30%
	Day 10	174	239	413	42%
	Day 30	76	265	341	22%
Ram 18	Day 1	0	139	139	0%
	Day 10	0	179	179	0%
	Day 30	0	192	192	0%

6.5 Discussion

Spermatozoa and seminal plasma both induced neutrophil infiltration of the endometrial stroma and uterine lumen of the ewe. Seminal plasma induced a greater response than spermatozoa, and some, but not all of the response to spermatozoa was due to its suspension in TALP, possibly caused by bovine serum albumin (BSA) in the TALP medium. Antibiotics reduced the neutrophil response but did not eliminate it, suggesting bacteria or bacterial products may have contributed to the reaction. Alternatively, it is possible that the presence of antibiotics contributed to the reduced neutrophil response via inherent anti-inflammatory properties in penicillin and/or streptomycin, rather than by anti-bacterial actions. Some classes of antibiotics,

including macrolides and quinolones, are known to have anti-inflammatory and immunomodulating effects, affecting both neutrophil numbers and function and cytokine production (Jaffe and Bush 2001; Gogos *et al.* 2004). However, it is not certain whether the classes of antibiotics used in our study (penicillins and aminoglycosides) have such effects on inflammatory responses.

Bacterial lipopolysaccharide (LPS) introduced at mating may enhance neutrophil infiltration into the ovine uterus by induction of chemoattractants, as occurs in other species, such as IL-8 in humans (Watari *et al.* 2003) or cytokine induced neutrophil chemoattractant (KC) in mice (Glynn and Robertson 2005). Previous research in horses has indicated that although bacteria are introduced at the time of mating, contamination of the equine uterus is minimal six hours later, and the use of antibiotics in semen for artificial insemination does not eliminate the post-insemination influx of neutrophils into the uterus. It was concluded from this that spermatozoa rather than bacteria induce the inflammatory response in the equine uterus (Kotilainen *et al.* 1994). In contrast, sterile spermatozoa induce no inflammation in the rabbit uterus, whereas bacteria in semen cause significant uterine inflammation (McDonald *et al.* 1952). It is likely that species differences exist, and bacteria or their products, along with seminal components, may contribute to uterine inflammation even if bacteria are rapidly removed by the uterine defence mechanisms after insemination. However, it is not clear how much or which components of semen penetrate the ovine cervix into the uterus during natural mating.

The reason for an increase in neutrophils following insemination is believed to be in part, to phagocytose excess and dead spermatozoa and micro-organisms (First *et al.* 1968; Lovell and Getty 1968; Pandya and Cohen 1985; Thompson *et al.* 1992; Troedsson *et al.* 2001). However, the neutrophil increase in response to seminal plasma suggests a further role of neutrophils in the ovine uterus post-insemination, possibly contributing to the establishment of successful pregnancy via release of cytokines. Neutrophils produce a variety of cytokines involved in immune function including TGF-β, TNF-α, IL-1, IL-8 and IFN-γ (Cassatella 1995; Yeaman *et al.* 1998), and expression of MHC class II antigens depends on the presence of cytokines such as IFN-γ (Clemens 1991) suggesting that neutrophils play a

significant role in contributing to modulation of the immune response in the female reproductive tract (Cassatella 1995; Yeaman *et al.* 1998). It has also been postulated in mice, that neutrophils contribute to the degradation of extracellular matrix in the endometrium which is necessary for decidualisation and successful embryo implantation (Daimon and Wada 2005).

Eosinophil populations and distribution in uterine tissues were not significantly different following injection of different components of semen or control medium into the uterine lumen, however, the use of antibiotics decreased the number of eosinophils in the mid- and deep endometrial stroma. Macrolide antibiotics are thought to reduce recruitment of eosinophils (Jaffe and Bush 2001), but it is uncertain whether penicillin or streptomycin have a similar effect. Alternatively, the reduction in eosinophil numbers observed with antibiotic treatments may indicate that bacteria or bacterial products such as LPS contributed to an increase in eosinophils in these regions. However, due to the high variability of eosinophils in ewe reproductive tissues, a larger sample size and challenge with bacterial components such as LPS would be required to confirm this. In mice, eosinophils migrate from the deep endometrial stroma to a subepithelial location in response to insemination (Robertson *et al.* 1996a; Robertson *et al.* 1998b), but this pattern was not evident in the ewe.

More mast cells were detected in the deep endometrial stroma of control ewes at oestrus than ewes that were naturally mated (Table 4.4) or surgically inseminated. There were no differences in mast cell numbers between different surgical treatments, but fewer mast cells were present in the deep endometrial stromal when antibiotics were included with treatments. Mast cell degranulation has a role in myometrial contractions in other species (Garfield *et al.* 2000; Bytautiene *et al.* 2004) and the physical presence of fluid in the ovine uterus in this study may have induced the release of chemical mediators to assist in fluid expulsion. Mast cell degranulation may also have been associated with an IgE response to antigens in semen, as occurs during bovine endometritis (Corbeil *et al.* 2005). It is not clear why antibiotics reduced the number mast cells detected in uterine tissues, but it is possible that penicillin or streptomycin caused increased mast cell degranulation additional to that which occurred after natural mating or the injection of fluid into the uterus.

More macrophages were present in uterine tissues after injection of components of semen and TALP medium than saline, and the addition of antibiotics reduced the macrophage response for all treatments except saline. When antibiotics were used, most macrophages were present in the superficial and mid- endometrial stroma after injection of washed spermatozoa and seminal plasma respectively. These results indicate that spermatozoa, seminal plasma and BSA induced macrophage infiltration of uterine tissues. This reaction was either enhanced by bacteria or bacterial products such as LPS, or reduced by anti-inflammatory effects of the antibiotics. Components of seminal plasma may have a role in induction of maternal immunotolerance to paternal antigens in the ovine conceptus during pregnancy as has been reported in other species (Barratt *et al.* 1990; Robertson *et al.* 1997; Mellor and Munn 2000; Robertson 2002; Robertson 2005). Macrophages are also known to be activated by LPS and may have a role in uterine contractility (Mackler *et al.* 2003).

Granulocyte-macrophage colony-stimulating factor increased in endometrial epithelial cells and the uterine lumen in response to whole semen and washed spermatozoa, but only when antibiotics were not included with the treatments. This suggests that either bacteria or bacterial products such as LPS induced the response, or the presence of penicillin or streptomycin suppressed the response. Seminal plasma, saline and TALP did not induce GM-CSF in the presence or absence of antibiotics. The reason seminal plasma did not elicit a response might be due to either inherent antibacterial components, or to contaminating bacteria from semen collection being forced to the bottom of samples during centrifugation, therefore when seminal plasma was removed, bacteria stayed amongst the spermatozoa. It is possible that bacteria or bacterial products contribute an increase in GM-CSF in other species post-mating, as many previous studies involved natural mating, which has the potential to introduce bacteria into the female reproductive tract. In mice, TGF-β induces GM-CSF and leukocyte infiltration of the uterus, and significantly less GM-CSF is produced after mating with seminal vesicle deficient males compared to intact males (Robertson *et al.* 1992; Robertson *et al.* 1996a; Tremellen *et al.* 1998), although bacterial products could also contribute to the reaction.

Interleukin-8 was synthesised by uterine epithelial cells and secreted into the lumen in response to whole semen, with approximately half due to seminal plasma and half

to spermatozoa. Antibiotics reduced the amount of IL-8 produced, although the pattern remained the same. Reduced IL-8 in the presence of antibiotics could be due to an anti-inflammatory effect by penicillin or streptomycin, similar to the reduction in IL-8 production that occurs during macrolide antibiotic therapy (Jaffe and Bush 2001). Alternatively, IL-8 may have been further increased above the response to semen due to the presence of bacteria or bacterial products such as LPS, which is known to induce IL-8 production (Wuyts *et al.* 1998). In humans, IL-8 production by cervical cells is stimulated by components in seminal plasma other than TGF-β (Sharkey and Robertson 2004), possibly LPS, which has been detected in semen of humans (Sharkey *et al.* 2002) and boars (O'Leary *et al.* 2002). During mating, LPS could be introduced into the uterus from the vagina and/or cervix and/or from semen.

Transforming growth factor-β1 was detected in all ram semen samples, consistent with findings in mice (Robertson *et al.* 1996a; Tremellen *et al.* 1998), humans (Nocera and Chu 1993; Loras *et al.* 1999) and pigs (O'Leary *et al.* 2002). However, the concentration of TGF-β1 detected in ram semen was lower than has been reported in other species. In this study, TGF-β1 concentration in ram semen ranged between 0.12 and 1.5 ng/ml, with an average of 0.4 ng/ml. The concentration of TGF-β1 in seminal vesicles of mice is approximately 70 ng/ml, which is diluted to approximately 30ng/ml in seminal plasma (Tremellen *et al.* 1998; Robertson *et al.* 2002). The concentration of TGF-β1 previously detected in human seminal plasma ranges between 60 and 230 ng/ml (Nocera and Chu 1995; Robertson *et al.* 2002). In pigs, 150-185 ng/ml of TGF-β1 has been detected in seminal plasma (O'Leary *et al.* 2002; Robertson *et al.* 2002).

Approximately 90% of TGF-β1 found in ram semen occurred in a latent form, which was activated by treatment with acid for 15 minutes. This is consistent with findings for human semen, where approximately 80% of TGF-β1 is present in its latent form, and it is subsequently activated by an acid environment, such as encountered in the vagina (Nocera and Chu 1995). Similarly, at least 70% of TGF- β1 in mouse semen is present in a latent form (Tremellen *et al.* 1998). In contrast, nearly all TGF- β1 present in pig semen is in an active form (Robertson *et al.* 2002). It is likely that the different proportions of active and latent TGF-β found in different species are related to site of semen deposition. Vaginal deposition of semen allows latent TGF-β to be

activated after insemination, whereas direct deposition of the majority of semen into the non-acidic uterine environment, such as occurs in pigs, requires TGF-β to already be in its active form.

The concentration of both latent and active TGF-β1 in ram semen varied between rams and between collections from the same ram. This variability may be partly due to the method of semen collection (electro-ejaculation). Factors including the exact positioning of the rectal probe and the number of stimulations required to produce semen may have affected the semen quality. Some collections only produced accessory gland fluid whereas others produced both accessory gland fluid and spermatozoa. The relative contributions by the prostate, bulbourethral glands and seminal vesicles may have varied between collections. In mice, TGF-β1 is produced in the seminal vesicles. This may also be the case in rams, and variability in the concentration of TGF-β1 detected may reflect different proportions of seminal vesicle fluid contributing to the sample collected. The three rams with the lowest TGF-β1 concentration in semen had no active TGF-β1 present. It would be interesting to investigate whether TGF-β1 content in ram semen is correlated with ram fertility and what effects TGF-β1 has on the function of ram spermatozoa.

In conclusion, seminal plasma and spermatozoa both contributed to increased synthesis and secretion of IL-8 by endometrial epithelial cells, and this probably contributed to the concurrent infiltration of uterine tissues by neutrophils. An increase in GM-CSF occurred in resposne to whole semen and spermatozoa only when antibiotics were not used. Eosinophils were increased in the mid- and deep endometrial stroma when antibiotics were not included in treatments, whereas fewer mast cells were present in the deep endometrial stroma after all treatments including antibiotics, which may indicate their degranulation. More macrophages were present in uterine tissues in response to spermatozoa and seminal plasma than other treatments and antibiotics reduced this response. These results indicate that spermatozoa and seminal plasma contribute to the post-insemination inflammatory response in the ovine uterus, and the use of antibiotics significantly reduces this response, possibly by antibacterial or anti-inflammatory effects. It is still not certain what role seminal TGF-β1 has in contributing to the influx of leukocytes that occurs in the reproductive tract of the ewe following insemination.

CHAPTER 7
HORMONAL INFLUENCES ON THE INFLAMMATORY RESPONSE IN THE UTERUS

7.1 Introduction

Leukocyte populations within the endometrium are influenced by cyclical changes in the concentrations of circulating oestrogens and progesterone (Starkey *et al.* 1991; Bischof *et al.* 1994a; Kaeoket *et al.* 2002b) with oestrogens causing an increase in leukocyte numbers in the endometrium of most species (Bischof *et al.* 1994a; Kaushic *et al.* 1998; Tibbetts *et al.* 1999; DeLoia *et al.* 2002). Additionally, a more rapid post-insemination inflammatory response occurs during oestrus than during the luteal phase of the reproductive cycle. Previous research has demonstrated a greater expression of the pro-inflammatory cytokine IL-8 mRNA in the ovine cervix during oestrus than during the luteal phase, which increased further in response to insemination (Mitchell *et al.* 2002). In cattle, leukocyte numbers in the endometrial stroma after insemination are similar at oestrus and during the luteal phase, although there is a more rapid migration of neutrophils into the uterine lumen in response to insemination during oestrus than during the luteal phase (Mahajan and Menge 1967).

The aims of this study were to compare the inflammatory response to insemination in the oestrogen-dominated and progesterone-dominated ovine uterus.

7.2 Materials and Methods

Ewes were synchronised for oestrus (see 3.2) and seven ewes in the mid-luteal phase of the oestrous cycle (8 days following the onset of oestrus) underwent surgical insemination. Semen was collected from three rams and prepared for surgical insemination (see 6.2.2 and 6.2.3). Ewes were anaesthetised (see 3.17.1) and had whole semen, spermatozoa, seminal plasma, TALP and normal saline injected into the lumen of ligated uterine sections (see 3.17.2). Their reproductive tracts were collected 22 hours later (see 6.2.6). Three of these ewes had antibiotics added to the

treatments prior to injection. The results from these ewes were compared with the results from nine ewes in oestrus (see Chapter 6).

7.3 Statistical Analyses

Statistical analyses of leukocyte and cytokine changes in response to surgical insemination was undertaken (see 6.3.1 and 6.3.2). Additionally, three way ANOVA (see Appendix 30) and Student's *t*-tests (see Appendix 31) were used to compare the leukocyte response to different treatments between oestrous and luteal ewes. Three way ANOVA (see Appendix 32) and Student's *t*-tests (see Appendix 33) were also used to compare the cytokine response to different treatments between oestrous and luteal ewes. For all statistical analyses performed, a *P* value of less than 0.05 was considered significant.

7.4 Results

7.4.1 Neutrophil response to different components of semen

There were no significant differences in the number of neutrophils in uterine tissues of luteal ewes when no antibiotics were added to treatments, compared to when penicillin and streptomycin were included (Table 7.1). However, there were significantly more neutrophils in tissues following treatment with whole semen ($P < 0.05$) compared to washed spermatozoa, TALP and saline (Figure 7.1) and significantly more ($P < 0.05$) in the superficial than the mid- or deep stroma (Table 7.1). Oestrous ewes had significantly more ($P < 0.01$) neutrophils in uterine tissues in response to all treatments when compared to luteal ewes (Table 7.2).

There were significantly more ($P < 0.01$) neutrophils in uterine luminal smears from luteal ewes when no antibiotics were included with whole semen, seminal plasma and washed spermatozoa, compared to when penicillin and streptomycin were added (Table 7.3). However, there was no difference between the response with or without antibiotics for TALP or saline. Highest neutrophil numbers were present in smears after treatment with seminal plasma, followed by washed spermatozoa, TALP and

then whole semen and there were significantly fewer (P <0.01) neutrophils in smears after treatment with saline compared to all other treatments (Figure 7.2). More neutrophils were present in uterine luminal smears in response to whole semen, seminal plasma and washed spermatozoa in oestrous ewes compared to luteal ewes, but the differences were not significant (Table 7.3).

7.4.2 Eosinophil response to different components of semen

There were no significant differences in the number of eosinophils present in uterine tissues of luteal ewes following different treatments. Similarly, the use of antibiotics did not significantly alter the number of eosinophils in tissues, although there were slightly fewer when antibiotics were included with treatments. The number of eosinophils in the superficial, mid- and deep stroma in luteal ewes were not significantly different. However, there were significantly more (P <0.01) eosinophils in uterine tissues of oestrous than luteal ewes (Table 7.2) which was evident in the superficial, mid- and deep endometrial stroma (Tables 6.1 and 7.1).
Eosinophils were rarely detected in uterine luminal smears from luteal ewes.

7.4.3 Mast cell response to different components of semen

There were no significant differences in the number of mast cells present in tissues from luteal ewes following different treatments. However, significantly more (P <0.01) mast cells were present in the deep endometrial stroma than in the superficial or mid- stromal tissue (Table 7.1). Additionally, there were significantly more (P <0.05) mast cells present in the deep endometrial stroma when penicillin and streptomycin were included with treatments compared to when no antibiotics were used (Figure 7.3) and this pattern was evident for all treatments (Figure 7.4) (Table 7.1). When antibiotics were not included with treatments, there were significantly more (P <0.05) mast cells in the deep endometrial stroma of oestrous ewes compared to luteal ewes. However, when penicillin and streptomycin were added to treatments, the reverse occurred, with significantly more (P <0.05) mast cells in the deep endometrial stroma of luteal ewes compared to oestrous ewes (Figure 7.5).

Table 7.1 Leukocyte numbers in uterine tissues of luteal ewes 22 hours after injection of various treatments into the lumen.

	No antibiotics added			Antibiotics added		
	Superficial	Mid-	Deep	Superficial	Mid-	Deep
Neutrophils						
Semen	8.3±7.8 [ab]	0	0	1.0±1.0	0.3±0.3	0
SP	2.8±1.4	0	0	0.7±0.7	0.3±0.3	0
Sperm	0.25±0.25	0	0	0	0	0
TALP	0	0	0	0.7±0.7	0	0
Saline	0	0	0	0	0.7±0.7	0
Eosinophils						
Semen	1.25±1.25	1.5±1.5	2.0±1.7	0	1.0±1.0	0
SP	0.25±0.25	1.8±0.8	2.0±2.0	0.3±0.3	0	0.7±0.7
Sperm	0	0.5±0.5	1.5±1.5	0	0.7±0.3	0
TALP	0.5±0.5	0.5±0.5	1.25±1.25	0	0.7±0.7	0.3±0.3
Saline	0.25±0.25	0	0	0	1.3±1.3	0
Mast Cells						
Semen	0	0.25±0.25	1.0±0.4	0.7±0.7	0.3±0.3	3.0±2.0
SP	0	0.25±0.25	0	0.7±0.7	2.0±0.6	1.3±0.7
Sperm	0	0	1.5±1.2	0.7±0.7	1.3±0.9	3.3±2.0
TALP	0	0	1.0±0.4	0.3±0.3	0.3±0.3	1.3±0.3
Saline	0.25±0.25	0.75±0.75	0.5±0.3	0	0.7±0.7	2.7±0.7
Macrophages						
Semen	8.0±2.1	6.8±1.3	5.5±1.3	8.0±2.1	8.0±3.6	7.0±3.1
SP	7.5±3.3	9.5±3.4	8.3±1.2	9.7±2.0	13.7±0.3	5.7±2.6
Sperm	10.0±2.2	11.8±2.5	7.3±1.7	8.3±2.4	10.7±3.7	10.7±2.3
TALP	11.8±3.5	13.25±3.0	9.8±1.3	10.3±2.4	13.0±5.5	12.3±4.5
Saline	7.3±2.0	11.25±2.6	8.8±0.5	8.0±1.2	9.3±2.6	10.0±1.5

Data are presented as the mean ± SEM cell counts in 1.5mm^2 uterine tissue. Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride. No antibiotics (n=6), antibiotics added to treatments (n=3). Superficial, mid- and deep refer to depth in endometrium. [a] $P < 0.05$ compared to washed spermatozoa, TALP and saline; [b] $P < 0.05$ compared to the mid- and deep stroma.

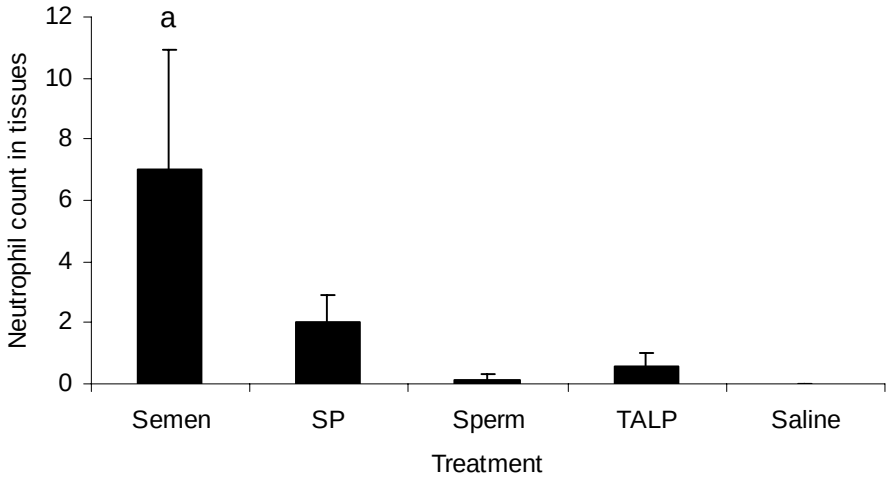

Figure 7.1 Neutrophils in uterine tissues of luteal ewes 22 hours after injection of various treatments into the lumen. Data are presented as mean + SEM (n=7) in 4.5mm^2 tissue (superficial, mid- and deep stroma). [a] P <0.05 compared to spermatozoa, TALP and saline. Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride.

Table 7.2 Leukocyte numbers in uterine tissues of oestrous and luteal ewes 22 hours after injection of various treatments into the lumen.

	Treatment				
	Semen	SP	Sperm	TALP	Saline
Neutrophils					
Oestrus (n=9)	902±235 [a]	811±246 [a]	601±207 [a]	120±61 [a]	14±6 [a]
Luteal (n=7)	7.0±3.9	2.0±0.9	0.1±0.1	0.6±0.4	0
Eosinophils					
Oestrus (n=9)	33.3±27.3 [b]	37.0±28.0 [a]	45.2±28.1 [a]	26.4±15.8 [a]	48.0±34.5 [a]
Luteal (n=7)	3.1±1.8	2.7±1.5	1.4±1.1	1.7±0.6	0.7±0.6
Macrophages					
Oestrus (n=9)	28.3±6.8	30.6±6.4	31.6±5.3	25.6±5.2	26.3±4.7
Luteal (n=7)	21.4±3.8	26.9±3.7	29.3±3.9	35.1±5.5	27.3±3.1

Data are presented as the mean ± SEM cell counts in the superficial, mid- and deep stroma (4.5mm^2 of uterine tissue). Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride. [a] P <0.01, [b] P <0.05 compared with luteal ewes.

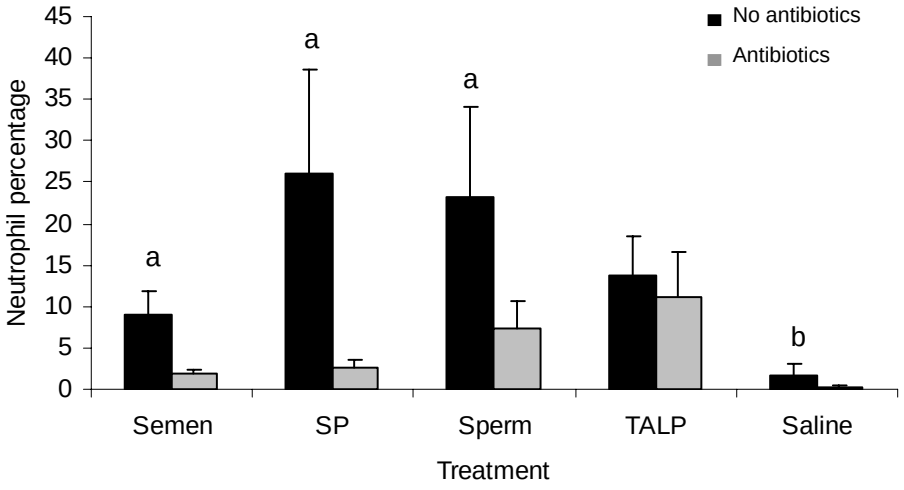

Figure 7.2 Neutrophils in uterine luminal smears from luteal ewes 22 hours after injection of various treatments with or without the addition of antibiotics into the lumen. Data are presented as mean + SEM percentage of total cells in smears; no antibiotics (n=4), antibiotics added to treatments (n=3). [a] P <0.01 compared to treatment containing antibiotics; [b] P <0.01 compared to all other treatments. Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride.

Table 7.3 Neutrophil numbers in luminal smears from oestrous and luteal ewes 22 hours after injection of various treatments into the lumen.

	Treatment				
	Semen	SP	Sperm	TALP	NaCl
Oestrus (n=6)					
No antibiotics	40.7±17.9 [ab]	37.1±12.7 [ab]	30.1±12.6 [ab]	16.3±8.8 [ab]	1.2±1.0
Antibiotics	8.1±5.5	5.5±2.7	2.3±0.7	3.1±1.2	0.3±0.2
Luteal (n=4)					
No antibiotics	8.9±2.3 [a]	26.0±12.7 [a]	23.2±10.9 [a]	13.8±4.6	1.6±1.5 [c]
Antibiotics	1.9±0.6	2.6±0.9	7.2±3.5	11.2±5.4	0.3±0.1

Data are presented as the mean ± SEM percentage of total cells in smears. Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride. [a] P <0.01 compared to treatment containing antibiotics; [b] P <0.01 compared to saline; [c] P <0.01 compared to other treatments.

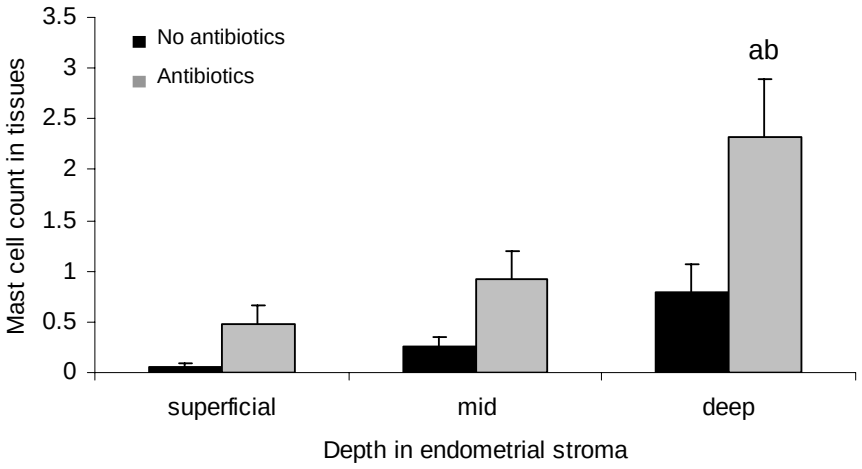

Figure 7.3 Mast cells in different depths of the endometrial stroma of luteal ewes 22 hour after injection of treatments with or without the addition of antibiotics into the lumen. Data are presented as mean + SEM in 1.5mm^2 tissue; no antibiotics (n=20), antibiotics added to treatments (n=15). [a] P <0.01 compared to mid- and superficial stroma; [b] P <0.05 compared to treatment containing antibiotics.

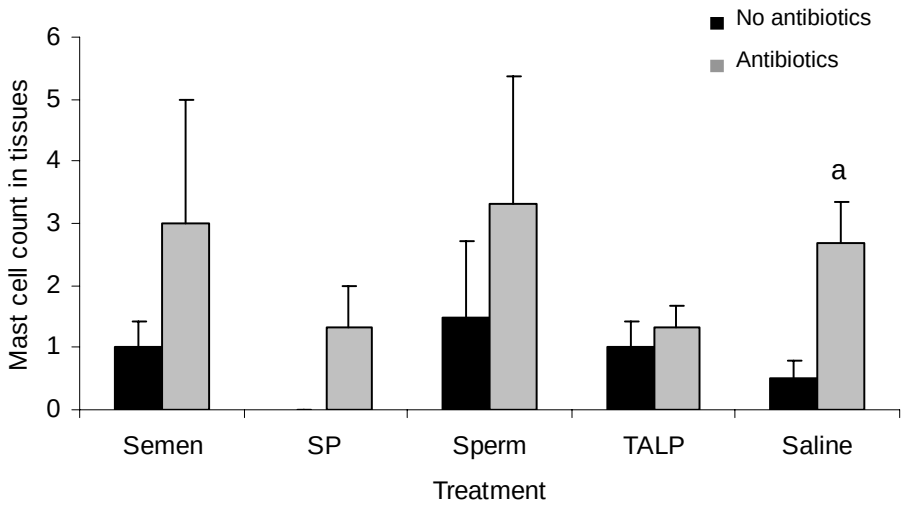

Figure 7.4 Mast cells in the deep endometrial stroma of luteal ewes 22 hours after injection of various treatments with or without the addition of antibiotics into the lumen. Data are presented as mean + SEM in 1.5mm^2 tissue; no antibiotics (n=4), antibiotics added to treatments (n=3). [a] P <0.05 compared to treatment containing antibiotics. Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride.

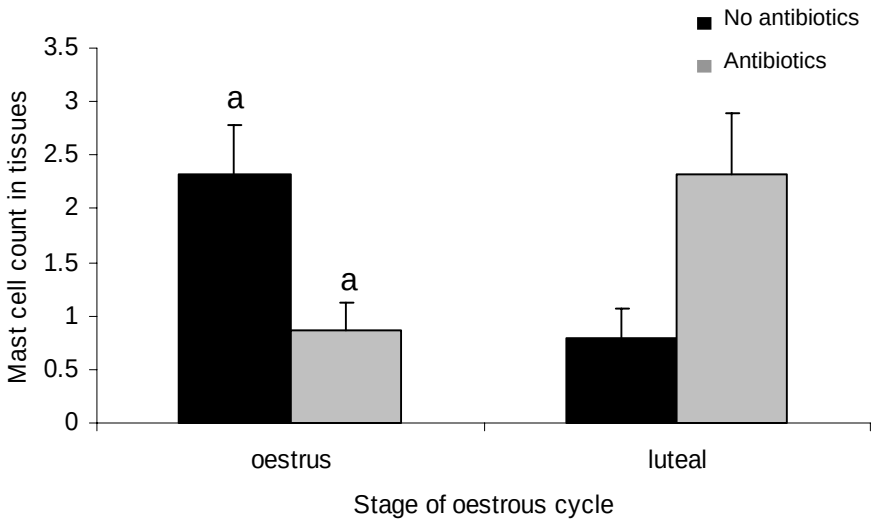

Figure 7.5 Mast cells in the deep endometrial stroma of oestrous and luteal ewes 22 hours after injection of treatments with or without the addition of antibiotics into the lumen. Data are presented as mean + SEM in 1.5mm^2 tissue; oestrus no antibiotics (n=6), oestrus antibiotics added (n=3), luteal no antibiotics (n=4), luteal antibiotics added (n=3). [a] P <0.05 compared to luteal ewes.

7.4.4 Macrophage response to different components of semen

Slightly more macrophages were found in uterine tissues of luteal ewes when penicillin and streptomycin were added to treatments compared to when no antibiotics were used, but the differences were not statistically significant. More macrophages were present in tissues after injection of TALP, which was significantly more (P <0.01) compared to whole semen (Figure 7.6). There were no significant differences in the number of macrophages between different depths in the endometrium (Table 7.1). There were more macrophages in uterine tissues of oestrous ewes compared to luteal ewes after injection of whole semen, seminal plasma and washed spermatozoa, fewer after TALP and similar numbers after saline, but the differences were not statistically significant (Table 7.2).

The presence or absence of antibiotics in the treatments did not influence the number of macrophages present in uterine luminal smears of luteal ewes. However, there were significantly more (P <0.01) macrophages in smears following treatment with whole semen (0.26 ± 0.1%), seminal plasma (0.34 ± 0.15%) or washed spermatozoa

(0.14 ± 0.06%) compared with TALP or saline, both which had no macrophages present. Significantly more (P <0.01) macrophages were present in luminal smears from luteal compared with oestrous ewes after injection with whole semen and seminal plasma (Table 7.4).

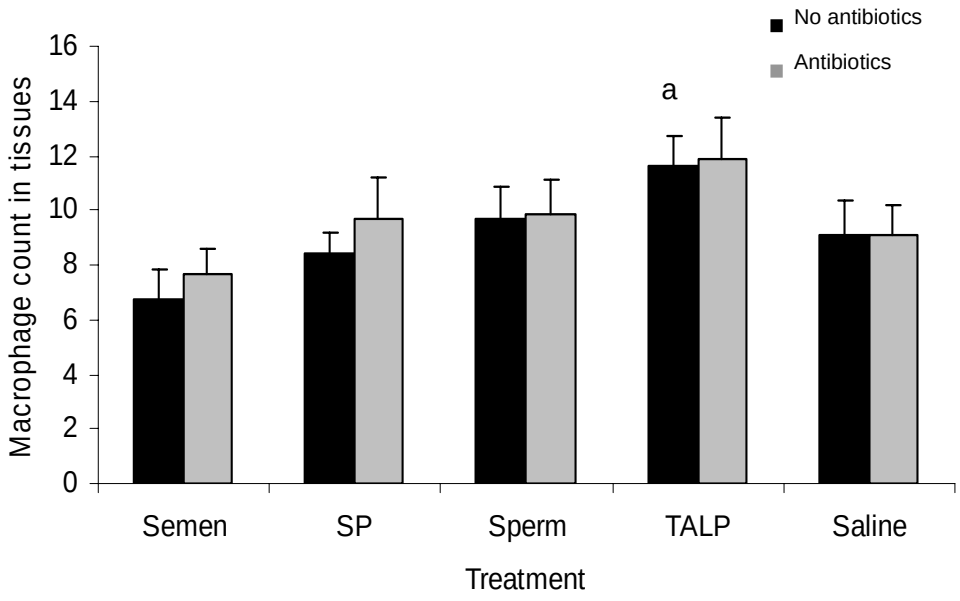

Figure 7.6 Macrophages in uterine tissues of luteal ewes 22 hours after injection of various treatments with or without the addition of antibiotics into the lumen. Data are presented as mean + SEM in 1.5mm^2 tissue; no antibiotics (n=12), antibiotics added to treatments (n=9). [a] P <0.01 compared to whole semen. Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride.

Table 7.4 Macrophage numbers in luminal smears from oestrous and luteal ewes 22 hours after injection of various treatments into the lumen.

	Treatment				
	Semen	SP	Sperm	TALP	NaCl
Oestrus (n=9)	0	0.05±0.03	0.07±0.03	0	0
Luteal (n=7)	0.26±0 [a]	0.34±0.15 [a]	0.14±0.06	0	0

Data are presented as the mean ± SEM percentage of total cells in smears. [a] P <0.01 compared with oestrous ewes. Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride.

7.4.5 GM-CSF response to different components of semen

7.4.5.1 GM-CSF in tissues

Uterine luminal and glandular epithelial cells of luteal ewes stained positively for GM-CSF. There were no significant differences in staining intensity of GM-CSF between different treatments, or between treatments with or without antibiotics (Table 7.5). However, there was significantly stronger (P <0.01) staining of GM-CSF in uterine tissues during the luteal stage of the oestrous cycle compared with during oestrus, and this was evident for all treatments (Figure 7.7).

7.4.5.2 GM-CSF in the lumen

Granulocyte-macrophage colony-stimulating factor was not detected in any sample of uterine luminal fluid from luteal ewes when antibiotics were added to the treatments (Table 7.6). Additionally, GM-CSF was not detected in luminal fluid following treatment with saline when no antibiotics were added. The highest amounts of GM-CSF detected in luminal fluid of luteal ewes followed treatment with seminal plasma, followed by whole semen, TALP and washed spermatozoa, although the differences were not statistically significant (Figure 7.8). When antibiotics were not included with treatments, more GM-CSF was present in uterine luminal fluid of oestrous compared to luteal ewes after injection of whole semen and washed spermatozoa (Figure 7.9), and this approached statistical significance for washed spermatozoa (P <0.06).

Table 7.5 Staining intensity of GM-CSF and IL-8 in uterine epithelium from luteal ewes 22 hours after injection of various treatments into the lumen.

	GM-CSF		**IL-8**	
	No antibiotics	Antibiotics	No antibiotics	Antibiotics
Semen	2,2,2,0	2,2,1	1,1,1,1	1,2,2
SP	1,3,2,1	2,2,2	1,2,1,2	2,2,2
Sperm	1,2,1,0	2,2,2	1,1,1,2	2,1,2
TALP	1,2,3,1	3,2,1	2,1,1,1	1,1,2
Saline	1,2,3,0	1,3,2	1,2,1,1	1,2,1

Data are presented as 0 (no staining), 1 (light staining), 2 (moderate staining) or 3 (strong staining). Data are observations from individual ewes; no antibiotics (n=4), antibiotics added to treatments (n=3). Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride.

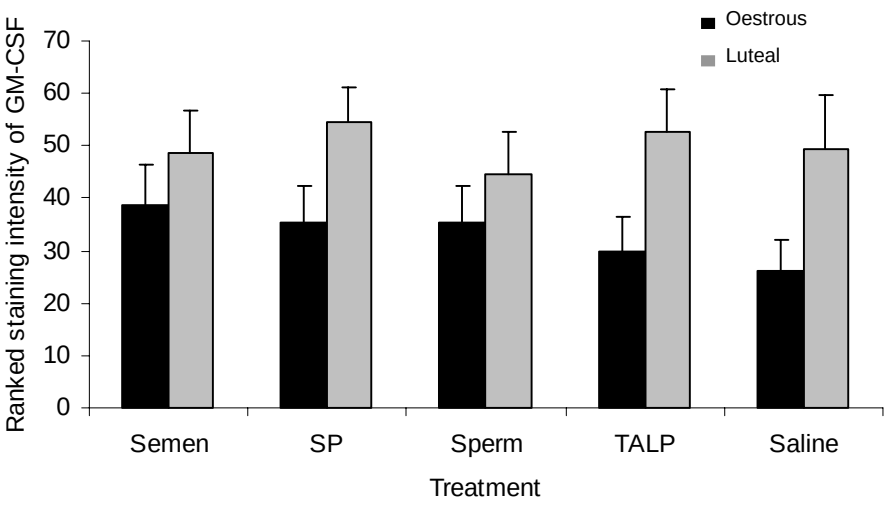

Figure 7.7 Ranked staining intensity of GM-CSF in uterine tissues from oestrous and luteal ewes 22 hours after injection of various treatments. Data are presented as mean + SEM; oestrus (n=9), luteal (n=7). Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride.

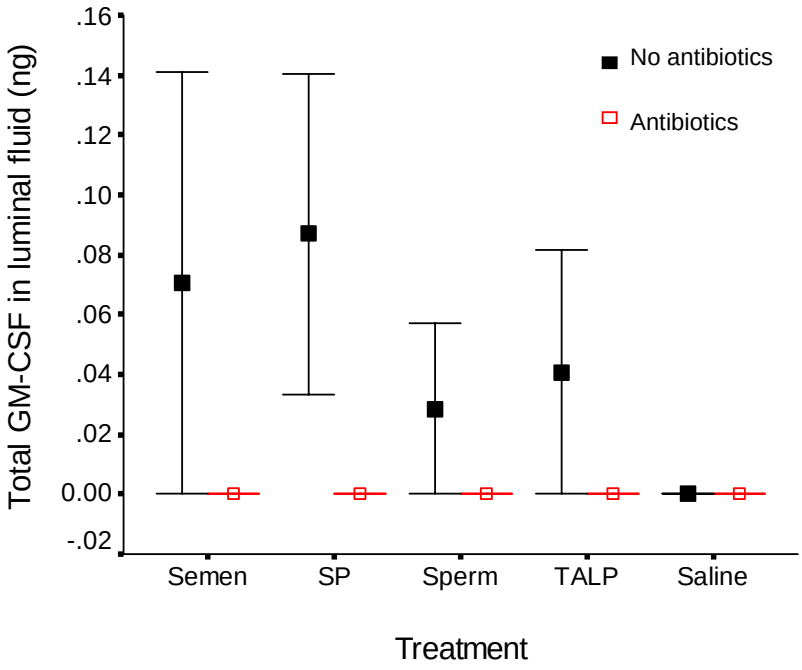

Figure 7.8 GM-CSF in luminal fluid of luteal ewes 22 hours after injection of various treatments into the lumen. Data are presented as mean ± SEM; no antibiotics (n=4), antibiotics added to treatments (n=3). Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride.

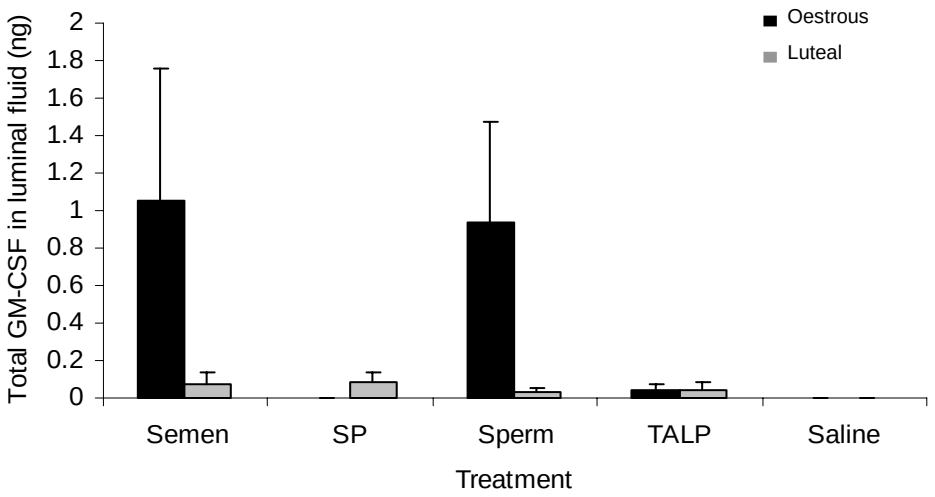

Figure 7.9 GM-CSF in luminal fluid of oestrous and luteal ewes 22 hours after injection of various treatments without antibiotics into the lumen. Data are presented as mean ± SEM; oestrus (n=6), luteal (n=4). Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride.

7.4.6 IL-8 response to components of semen

7.4.6.1 IL-8 in tissues

Interleukin-8 was detected in luminal and glandular endometrial epithelial cells of luteal ewes. The staining intensity of IL-8 in uterine tissues was significantly stronger (P <0.05) after treatments that included antibiotics (Table 7.5). Seminal plasma induced the strongest staining both with and without antibiotics, although the difference was not significant compared to other treatments (Figure 7.10). There was no significant difference in the staining intensity of IL-8 in uterine tissues between ewes in the luteal stage compared with oestrous ewes (Figure 7.11).

7.4.6.2 IL-8 in the lumen

Lesser amounts of IL-8 were found in uterine luminal fluid after injection of treatments containing antibiotics than those without, although the difference was not statistically significant (Table 7.6). Seminal plasma induced the greatest response, followed by whole semen, both with significantly more (P <0.01) luminal IL-8 than

in response to saline or TALP. Washed spermatozoa induced significantly more (P <0.05) IL-8 than saline (Figure 7.12). Significantly more (P <0.01) IL-8 was present in the uterine lumen of oestrous compared to luteal ewes after injection of whole semen, seminal plasma, washed spermatozoa and TALP (Figure 7.13).

Table 7.6 Fluid volumes (µl) and cytokine concentrations (ng/ml) in uterine luminal fluid from luteal ewes 22 hours after injection of various treatments.

		Treatment				
Ewe Number		Whole semen	Seminal plasma	Washed sperm	TALP medium	0.9% NaCl
163	**Volume**	200	150	250	180	100
	GM-CSF	0	0	0	0	0
	IL-8	1.84	1.53	0.075	0.018	0.16
167	**Volume**	80	100	100	100	150
	GM-CSF	0	0	0	0	0
	IL-8	3.51	31.09	9.51	0.23	0.47
170	**Volume**	100 [b]	100	100	100	100
	GM-CSF	2.82	1.29	1.14	0	0
	IL-8	0.76	0.39	0	0	0.062
171	**Volume**	300 [c]	300	100	300	100
	GM-CSF	0	0.73	0	0.54	0
	IL-8	22.60	19.22	2.91	0	0
173 [a]	**Volume**	100	100	100	100	100
	GM-CSF	0	0	0	0	0
	IL-8	0.74	0.40	2.67	0.11	0
176 [a]	**Volume**	100	100	100	100	100
	GM-CSF	0	0	0	0	0
	IL-8	1.21	0.66	0.58	3.47	0
178 [a]	**Volume**	100	100	100	100	100
	GM-CSF	0	0	0	0	0
	IL-8	0.43	3.26	0.72	0.094	0

[a] antibiotics added to treatments; [b] blood tinged fluid; [c] cloudy fluid.

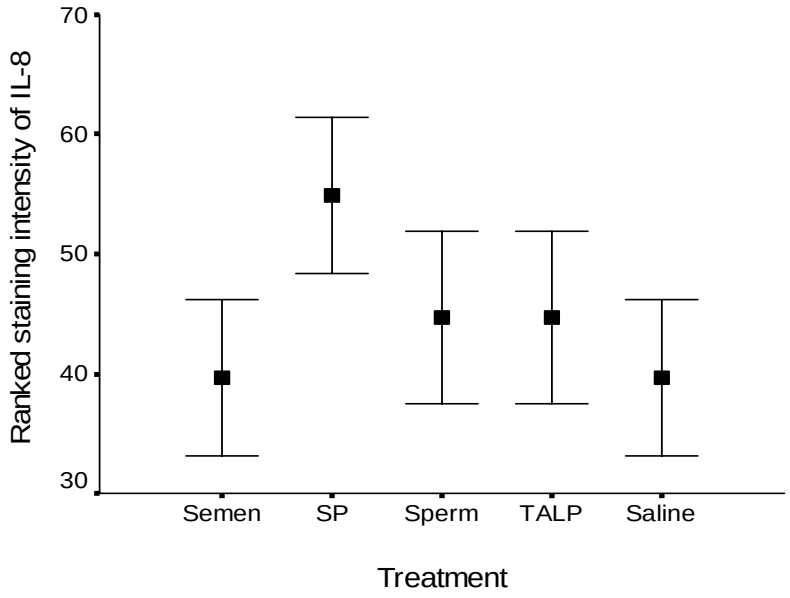

Figure 7.10 Ranked staining intensity of IL-8 in uterine tissues from luteal ewes 22 hours after injection of various treatments into the lumen. Data are presented as mean ± SEM (n=7). Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride.

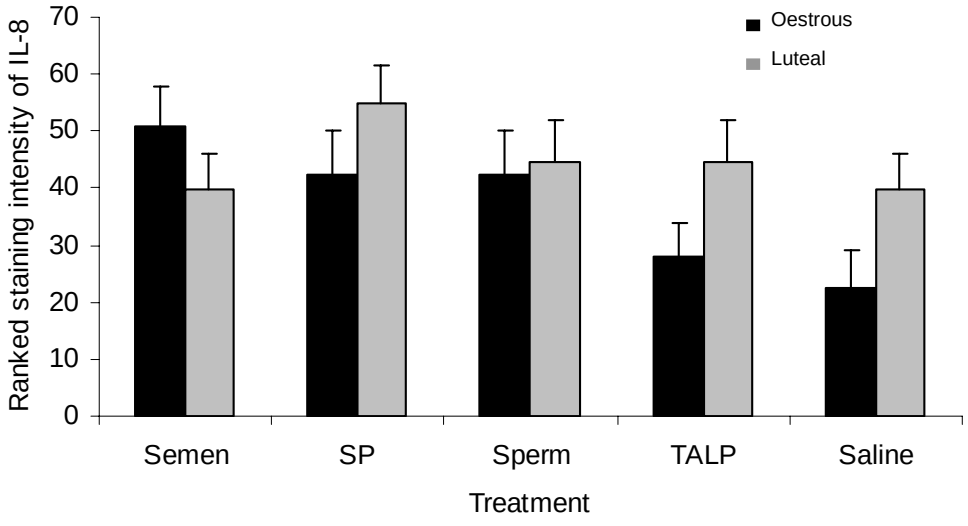

Figure 7.11 Ranked staining intensity of IL-8 in uterine tissues from oestrous and luteal ewes 22 hours after injection of various treatments. Data are presented as mean + SEM; oestrus (n=9), luteal (n=7). Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride.

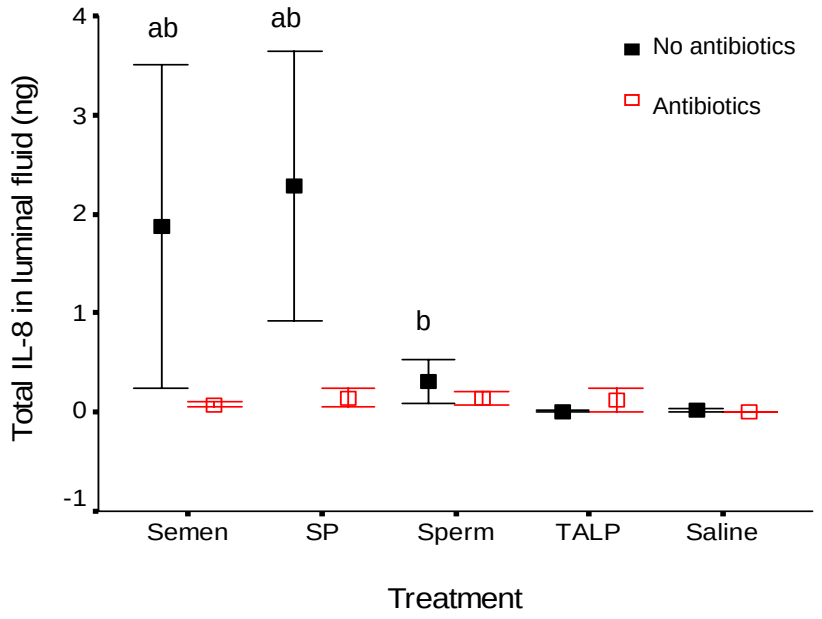

Figure 7.12 IL-8 in luminal fluid of luteal ewes 22 hours after injection of various treatments into the lumen. Data are presented as mean ± SEM; no antibiotics (n=4), antibiotics added to treatments (n=3). [a] P <0.01 compared to TALP; [b] P <0.01 compared to saline. Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride.

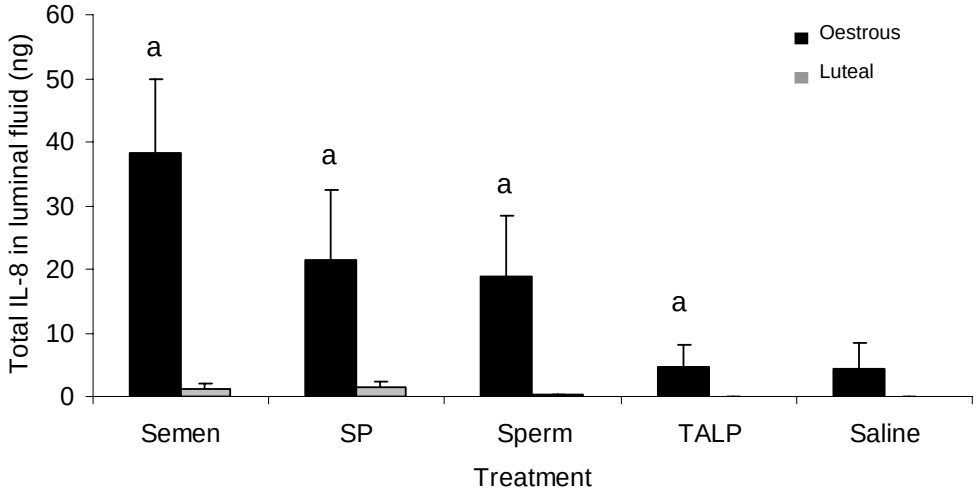

Figure 7.13 IL-8 in luminal fluid of oestrous and luteal ewes 22 hours after injection of various treatments without antibiotics into the lumen. Data are presented as mean + SEM; oestrus (n=6), luteal (n=4). [a] P <0.01 compared to luteal ewes. Treatments were: Semen, whole semen; SP, seminal plasma; Sperm, washed spermatozoa; TALP, modified Tyrode's medium; Saline, 0.9% sodium chloride.

7.5 Discussion

Insemination induced a much greater infiltration of neutrophils into uterine tissues of the ewe at oestrus compared to during the luteal phase of the oestrous cycle. Whole semen, followed by seminal plasma, then washed spermatozoa induced the greatest neutrophil response during both stages of the oestrous cycle, but the response was several hundred times greater during oestrus. This result differs to previous research in cattle that found a similar infiltration of tissues in response to insemination at oestrus and during the luteal phase of the oestrous cycle (Mahajan and Menge 1967). The numbers of neutrophils in uterine tissues of oestrous ewes following injection of saline were similar to numbers in oestrous ewes without any treatment (see Table 4.1), indicating that injection of saline had no effect on neutrophil infiltration of tissues. Fewer neutrophils were present in uterine tissues of luteal ewes after saline injection, suggesting that an underlying increase in neutrophil numbers occurs in uterine tissues of the ewe at oestrus in response to circulating ovarian hormone concentrations. This is further supported by the changes in neutrophil numbers that occurred during the 48 hour period following oestrus (see 4.4.1.1), and is consistent with the increase in neutrophil numbers that occurs in the uterus of rats (Kaushic *et al.* 1998), mice (Tibbetts *et al.* 1999) and pigs (Bischof *et al.* 1994a) when blood concentrations of oestrogens are high. Similar to the tissue response, more neutrophils migrated into the uterine lumen after injection of whole semen, seminal plasma and washed spermatozoa at oestrus compared to during the luteal phase, which may be related to the increased uterine blood flow that occurs at oestrus (Ford 1995), but the luminal response was less marked than in tissues. This differs to findings in cattle, which have a slower migration of neutrophils into the uterine lumen in the luteal phase compared to oestrus, but by 24 hours post-insemination, numbers of luminal neutrophils were similar in luteal and oestrous heifers (Mahajan and Menge 1967).

Eosinophils were up to fifty times more abundant in the superficial and deep endometrial stroma of ewes at oestrus compared to during the luteal phase. Their numbers were minimally affected by insemination and appear to be primarily under the influence of circulating ovarian hormones. Eosinophils in rats are also under the

influence of the oestrous cycle, with large numbers infiltrating the endometrial stroma at oestrus (Ross and Klebanoff 1966). Mast cells were more abundant in the deep endometrial stroma of oestrous ewes compared to luteal ewes when no antibiotics were used, but surprisingly, when antibiotics were added to treatments the reverse occurred, with more mast cells in luteal compared to oestrous ewes. The reason for this is not certain, but may indicate an interaction between the influence of ovarian hormones and the effects of penicillin and/or streptomycin on mast cell degranulation and their subsequent ease of detection.

There were slightly more macrophages in uterine tissues of ewes at oestrus compared to the luteal phase of the oestrous cycle following treatment with components of semen, but the differences were not statistically significant. There are conflicting reports in the literature regarding the influence of the oestrous cycle on endometrial macrophage populations. The number of macrophages have alternatively been reported to remain relatively constant throughout the oestrous cycle of mice (De and Wood 1990), horses (Summerfield and Watson 1998) and humans (Starkey *et al.* 1991) or to increase at oestrus or in response to oestrogen administration in mice (Tibbetts *et al.* 1999), horses (Frayne and Stokes 1994), humans (Bonatz *et al.* 1992; DeLoia *et al.* 2002) and pigs (Kaeoket *et al.* 2001; Kaeoket *et al.* 2002b). In our study, there was no apparent difference in the number of macrophages in the endometrial stroma of ewes between oestrus and the luteal phase of the oestrous cycle. Small fluctuations in the number of macrophages in the lumen did occur during the 48 hour period immediately following the onset of oestrus (see 4.4.4.3) but more macrophages migrated into the lumen during the luteal phase compared with oestrus. Unlike the number of macrophages, the distribution of macrophages within uterine tissues of the ewe was significantly influenced by the stage of the oestrous cycle, with more macrophages in the superficial than the deep endometrial stroma at oestrus compared to a relatively even distribution throughout the endometrial stroma during the luteal phase. This is consistent with previous findings in mice (De and Wood 1990).

Staining intensity of GM-CSF was stronger in uterine epithelia during the luteal phase than at oestrus, although there was an opposite pattern for luminal GM-CSF, with greater amounts in the uterine lumen of oestrous ewes than luteal ewes, in

response to whole semen and washed spermatozoa without antibiotics. The opposite pattern between staining intensity and luminal GM-CSF at oestrus compared to the luteal phase may reflect greater release of GM-CSF into the lumen at oestrus, and a subsequent reduction in staining intensity of epithelial cells. A greater amount of uterine luminal GM-CSF in ewes at oestrus compared to the luteal phase is consistent with previous research that indicates GM-CSF in the reproductive tract is regulated by ovarian steroid hormones, with mRNA expression increasing in response to oestrogen in mice (Robertson *et al.* 1996b) and sheep (McGuire *et al.* 2002).

The staining intensity of IL-8 in the uterine epithelium of ewes was similar at oestrus and during the luteal phase of the oestrous cycle. However, approximately 20 times more IL-8 was present in the lumen of oestrus ewes compared to luteal ewes in response to whole semen, and approximately 10 times more in response to seminal plasma and washed spermatozoa. This is consistent with previous reports of a greater expression of IL-8 mRNA in the ovine cervix during oestrus than during the luteal phase, and a further increase in response to insemination (Mitchell *et al.* 2002). The reason that IL-8 staining intensity in uterine epithelial cells was similar at oestrus and the luteal phase of the reproductive cycle even though luminal IL-8 was higher at oestrus, may be due to similar amounts of IL-8 being present in the epithelial cells at different stages, despite continued production and release of IL-8 from the epithelium into the lumen of oestrous ewes.

In conclusion, neutrophils, GM-CSF and IL-8 underwent greater increases in response to insemination at oestrus compared to during the luteal phase of the oestrous cycle in the ewe, whereas the number of eosinophils was markedly influenced by the oestrous cycle but was less affected by insemination. The number of macrophages increased following insemination without antibiotics, and their distribution within uterine tissues was affected by the oestrous cycle with more located in the superficial endometrial stroma at oestrus, however total numbers were similar at oestrus and the luteal stage. These results suggest that leukocytes, GM-CSF and IL-8 in the ovine uterus are under the influence of ovarian hormones, and oestrogen enhances and/or progesterone suppresses aspects of the post-insemination inflammatory response in the ewe.

CHAPTER 8
GENERAL DISCUSSION

8.1 Scope of this Research Project

The main theme of this research project was to understand the inflammatory response in the reproductive tract of the ewe following insemination. The aims were to identify and quantify leukocytes and selected cytokines in the reproductive tract and describe their distribution and temporal changes during the 48 hour period following mating, to determine which component(s) of semen are responsible for the response, and to compare the response between oestrogen and progesterone dominated reproductive tracts. The aims of this project were achieved and the hypothesis that components of ram semen induce an inflammatory response which involves the synthesis and secretion of cytokines and the accumulation of leukocytes in the reproductive tract of the ewe was accepted.

Routine histological processing and staining techniques were used to identify neutrophils, eosinophils and mast cells in reproductive tissues and smears. However, macrophages were less readily identified and immunohistochemistry was used to differentiate them from other cell types. As immunohistochemical techniques were being developed and optimised for macrophage identification, and several of the reagents including secondary antibodies and chromagens could serve multiple purposes, immunohistochemisty was also employed for cytokine identification in tissues. Although several cytokines have been implicated in the post-insemination inflammatory response, IL-8 and GM-CSF were selected for investigation in this project due to their pro-inflammatory and chemotactic effects, and the availability of suitable reagents. Few anti-sheep antibodies and even fewer recombinant ovine cytokines are available commercially, which limits the ability to detect cytokines in ovine tissues and secretions. Also considered in this project was investigation into the presence of MCP-1 in tissues and secretions as it has macrophage chemotactic properties. A polyclonal anti-sheep MCP-1 antibody is available commercially, but this antibody cross reacts with ovine IL-8. Because this cross-reactivity would hinder MCP-1 detection, and a source of recombinant ovine MCP-1 (which would be

necessary as a standard for ELISA) was not found, it was decided not to pursue this line of enquiry.

The inflammatory reaction that occurs in the reproductive tract following mating involves the uterus, cervix and vagina, but the response varies between different sites. In uterine tissues, the number of neutrophils and macrophages increased in the superficial stroma and to a lesser extent the mid- and deep stroma after mating. Neutrophils, but not macrophages increased in the uterine lumen. Little change was observed in the anterior cervix, but neutrophils increased in the posterior cervix and cervical ostium, and macrophages increased in the mid- and posterior cervix, with both cell types increasing in the cervical lumen. Neutrophil and macrophage numbers both increased in vaginal tissues and the vaginal lumen. The number of eosinophils present in ewe reproductive tissues was highly variable, with no significant changes following mating, whereas mast cell numbers were reduced in the deep endometrial stroma after mating.

In uterine tissues, the number of neutrophils peaked at six hours and macrophages at 18-24 hours post-mating, with numbers of both cell types increasing in response to spermatozoa and seminal plasma, with a reduced response in the presence of antibiotics. In humans and rodents, increased neutrophils and macrophages in uterine tissues are likely to promote implantation of the conceptus, by modulation of the maternal immune response and by contributing to tissue remodelling in the endometrium (Cassatella 1995; Yeaman *et al.* 1998; Johansson *et al.* 2004; Daimon and Wada 2005; Robertson 2005). Increased leukocytes in the ovine endometrium following mating may also facilitate maternal immunomodulation towards the ovine conceptus, via mechanisms such as paternal antigen presentation and the production of immuno-regulatory cytokines, as is thought to occur in other species (Robertson 2005). However, unlike haemochorial placentation that occurs in rodents and humans, where trophoblast cells penetrate the endometrial epithelium and stroma into the maternal circulation, ruminants undergo epitheliochorial placentation, which involves the non-invasive attachment of trophoblast cells to the luminal endometrial epithelium over caruncles, and the formation of hybrid syncytial cells between trophoblast binucleate cells and luminal epithelial cells. Therefore minimal remodelling of endometrial tissue occurs during implantation of the ruminant

conceptus. Furthermore, the preimplantation period is prolonged in ruminants compared to humans and rodents, with the ovine conceptus not implanting until 16 to 22 days following mating (Bowen and Burghardt 2000; Spencer *et al.* 2004). This suggests that the function of endometrial leukocytes in implantation and development of the ruminant conceptus may differ somewhat to other species, but the downstream or cascade effects following endometrial leukocytic infiltration remain unclear.

Neutrophils in the uterine lumen phagocytose excess and dead spermatozoa (First *et al.* 1968; Lovell and Getty 1968) and introduced microorganisms. They may also be involved in sperm selection (Pandya and Cohen 1985; Thompson *et al.* 1992) whereby defective or suboptimal spermatozoa are removed and fertilising spermatozoa are protected. In horses, seminal plasma contains immunosuppressive components that inhibit neutrophil chemotaxis, reducing phagocytosis by neutrophils and protecting viable but not dead spermatozoa (Troedsson *et al.* 2001; Alghamdi *et al.* 2004; Alghamdi and Foster 2005; Troedsson *et al.* 2005). Similar mechanisms may occur in the uterus of other species including sheep.

The number of eosinophils in the ovine uterus was not significantly affected by the injection of different components of semen, although numbers were highly variable, and the addition of antibiotics to treatments reduced the number of eosinophils in the mid- and deep endometrial stroma of oestrous ewes. It is possible that bacteria or bacterial products such as LPS contributed to an increase in eosinophils in this region, or that antibiotics reduced eosinophil numbers via an anti-inflammatory effect. However, further investigation would be required to confirm this. In mice, it is postulated that the migration of eosinophils from the deep endometrial stroma to a subepithelial location in response to cytokines in seminal plasma has an important role in maternal immunomodulation (McMaster *et al.* 1992; Robertson *et al.* 1996a; Robertson *et al.* 1998b; Gopichandran *et al.* 2006) but this was not evident in the ovine uterus.

A reduction in numbers or degranulation of mast cells in the deep endometrial stroma was evident between 6 and 24 hours after natural mating, and similar numbers were present 22 hours after all surgical treatments, suggesting this effect may be partly

induced by the physical presence of fluid in the uterine lumen. This probably serves to expel excess fluid from the uterus following insemination, as mast cell degranulation is associated with myometrial contractions in other species (Garfield *et al.* 2000; Bytautiene *et al.* 2004). Mast cell degranulation is also associated with IgE responses to antigens and clearance of bacterial infection during bovine endometritis (Corbeil *et al.* 2005) and mast cells are likely to have a similar role in the ovine uterus. The reverse effect by antibiotics on mast cell numbers in the deep endometrial stroma between oestrus and the luteal phase is unexplained and needs further clarification.

Both GM-CSF and IL-8 were present in luminal and glandular endometrial epithelial cells, to a lesser extent in cervical epithelial cells, and neither were detected in vaginal epithelium. Endometrial epithelial cells secreted GM-CSF into the uterine lumen in response to whole semen and washed spermatozoa, but this effect was not evident when antibiotics were used. This differs to reports in mice (Robertson and Seamark 1990) and pigs (O'Leary *et al.* 2004), where GM-CSF is secreted in response to components of seminal plasma. Large amounts of IL-8 were present in the vaginal lumen irrespective of mating status, and IL-8 increased in the uterine lumen in response to whole semen, seminal plasma and spermatozoa. Antibiotics reduced this response, possibly by antibacterial or anti-inflammatory effects. Secretion of IL-8 from has previously been reported to increase in response to seminal plasma and LPS (Wuyts *et al.* 1998; Denison *et al.* 1999), and to be reduced in response to macrolide antibiotic therapy (Jaffe and Bush 2001).

The findings from this study indicate that uterine inflammation in the ewe is induced by a combination of spermatozoa and component(s) of seminal plasma. Bacteria or bacterial products may also contribute to the reaction. However, it is not certain how much or which components of ram semen make it through the cervix into the uterus, or whether bacteria or bacterial products enter the uterus of the ewe following natural mating. Both spermatozoa and seminal plasma have been implicated in contributing to the post-insemination inflammatory reaction in humans (Pandya and Cohen 1985; Thompson *et al.* 1992; Gutsche *et al.* 2003), pigs (Bischof *et al.* 1994b; Rozeboom *et al.* 1999; Rozeboom *et al.* 2000; Rozeboom *et al.* 2001; Assreuy *et al.* 2002; O'Leary *et al.* 2004) and rabbits (McDonald *et al.* 1952; Howe 1967; Phillips and Mahler

1977; Tyler 1977) whereas spermatozoa are reportedly responsible for the reaction in horses (Kotilainen *et al.* 1994; Troedsson *et al.* 1998; Troedsson *et al.* 2001) and seminal plasma causes the reaction in mice (McMaster *et al.* 1992; Sanford *et al.* 1992; Robertson *et al.* 1996a; Robertson *et al.* 1997; Tremellen *et al.* 1998). It is likely that bacteria or bacterial products also contribute to the inflammatory reaction in the female reproductive tract after natural mating, both due to the presence of LPS in semen (O'Leary *et al.* 2002; Sharkey *et al.* 2002) and because male and female genitalia are non-sterile environments which contain large numbers of microflora. Cytokine expression is upregulated in the mouse uterus in response to LPS (De *et al.* 1990), and it has previously been suggested that the post-insemination inflammatory response may have developed to protect against microbial invasion (De *et al.* 1991).

In this study, TGF-β1 was detected in ram semen, but it is still not clear what role it has in contributing to the influx of leukocytes that occurs in the ovine reproductive tract following insemination. In mice, TGF-β in seminal plasma induces GM-CSF and leukocyte infiltration of the uterus (Robertson *et al.* 1992; Robertson *et al.* 1996a; Tremellen *et al.* 1998). Similarly, TGF-β in human semen (Nocera and Chu 1993) stimulates secretion of GM-CSF from human cervical epithelial cells (Sharkey and Robertson 2004). Seminal plasma also stimulates IL-8 secretion from human cervical tissue (Denison *et al.* 1999). However, unlike GM-CSF, IL-8 production by human cervical cells is not stimulated by TGF-β (Sharkey and Robertson 2004), so other factors in semen must be responsible. It is possible that TGF-β1 or other factors in ram semen contribute to leukocyte infiltration in the ovine reproductive tract.

The leukocytic response to insemination was much greater at oestrus than during the luteal phase of the oestrous cycle, indicating that circulating concentrations of ovarian steroid hormones influence the post-insemination inflammatory response. Neutrophils and eosinophils were more abundant in uterine tissues at oestrus compared to during the luteal phase, consistent with findings in rats, mice and pigs (Ross and Klebanoff 1966; Bischof *et al.* 1994; Kaushic *et al.* 1998; Tibbetts *et al.* 1999) and neutrophils but not eosinophils increased significantly following mating. Whilst the distribution of macrophages within uterine tissues was affected by the stage of the oestrous cycle, with more in the superficial endometrial stroma at oestrus, total endometrial macrophage numbers were unchanged, similar to findings

in mice (De and Wood 1990). More macrophages infiltrated the endometrium after treatment with whole semen, seminal plasma or spermatozoa at oestrus compared to during the luteal phase, although the differences were not significant. More macrophages migrated into the lumen during the luteal phase, possibly due to reduction in other uterine defence mechanisms at this time compared to during oestrus.

The cytokine response to insemination was greater at oestrus than during the luteal phase, indicating an influence by circulating concentrations of oestrogens and progesterone. More IL-8 was present in the uterine lumen of oestrus ewes than luteal ewes, which is consistent with reports of greater expression of IL-8 mRNA in the ovine cervix during oestrus (Mitchell *et al.* 2002). Whilst GM-CSF was not detected in the uterine lumen of oestrus or luteal ewes 22 hours after surgical treatments containing antibiotics, larger amounts of GM-CSF were detected in non-mated ewes at three and six hours than at 18, 24 or 48 hours following oestrus. The absence of GM-CSF in the uterine lumen of surgically treated oestrous ewes may reflect reduced secretion of GM-CSF by 22 hours following oestrus, but may also be partly due to the sensitivity of the ELISA, as the concentrations detected in luminal fluid were low. It has been reported that GM-CSF is synthesised in a uniform manner throughout the bovine oestrous cycle (de Moraes *et al.* 1999) and similarly, there was no difference in the amount of GM-CSF produced in the reproductive tract in response to exogenous oestrogen and progesterone administration, although there was increased GM-CSF mRNA expression in oestrogen treated and pregnant ewes (McGuire *et al.* 2002). Our initial investigations suggested that GM-CSF is increased up to six hours following the onset of oestrus but further investigation using more sensitive tests may be required to clarify the effect of ovarian hormones on GM-CSF production in the ewe.

8.2 Future Research Directions

During mating, semen is deposited into the cranial vagina and around the cervical os of ewes, rather than into the uterus as occurs in some species including pigs and horses. Additionally, the ovine cervix is highly tortuous and presents a formidable

barrier to spermatozoa penetration. Therefore, determination of how much seminal plasma, if any, actually enters the uterus of ewes following insemination is an important line of investigation. This will help to determine if constituents of seminal plasma contribute to uterine inflammation after mating in the ewe. This could be carried out by labelling constituents specific to seminal plasma, with markers such as fluorescent dyes or radio-isotopes, artificially inseminating ewes with semen containing these labelled compounds, and then attempting to recover or recognise the markers at various sites in the reproductive tract of ewes. The marker used would need to form a stable bond with the target constituent of seminal plasma to ensure it remained securely attached during transit through the reproductive tract.

Further investigation into the constituents of ram seminal plasma would assist in identifying potential mediators of the post-insemination inflammatory response. Of particular interest are bacterial LPS, chemokines such as MCP-1, CSF-1 and RANTES, and cytokines with pro-inflammatory effects including TNF-α, Il-1 and IL-6. Further to this, seminal plasma composition may vary between samples collected by different techniques, depending on relative contributions by different accessory sex glands. Therefore, comparison of seminal components between samples obtained using an artificial vagina and by electro-ejaculation is warranted. Additionally, comparison of the inflammatory reaction after natural mating and artificial insemination is necessary, as it is possible that variations in the response may have an impact on pregnancy success and flock fertility.

Challenge to the ewe reproductive tract by TGF-β and other ram seminal constituents, such as LPS and chemokines/cytokines, is necessary to determine if they contribute to inflammation in the ovine uterus. Further investigation into the roles of specific leukocytes in the ovine reproductive tract, such as mast cells, are also needed. Additionally, the synthesis of IL-8 in vaginal epithelium needs to be investigated to determine if the high concentrations of luminal IL-8 originate locally or from elsewhere in the reproductive tract. The expression of IL-8 mRNA in vaginal epithelial cells could be investigated with techniques such as *in situ* hybridisation. Luminal secretions and tissue samples from the vagina and elsewhere in the reproductive tract would need to be taken simuletaneously to compare the presence and distribution of IL-8 protein with the expression of IL-8 mRNA. Examination of

samples taken at various stages of the oestrous cycle would facilitate our understanding of the influence ovarian steroid hormones have on IL-8 synthesis and secretion.

Increased knowledge of the effects that different constituents of ram seminal plasma, including TGF-β, have on spermatozoa motility, viability and fertilising capacity could lead to improved semen preparation and storage techniques, thereby improving pregnancy success rates in artificial insemination programs. Comparisons could also be made between artificial insemination success rates using different seminal plasma constituents and anti-inflammatory factors along with spermatozoa, to determine if the inflammatory reaction is necessary for optimal reproductive efficiency in sheep.

Analysis of different accessory sex glands is also of interest, to determine the site of TGF-β synthesis and secretion in the ram, as species differences may occur. In mice, TGF-β is produced in the seminal vesicles, and in humans the prostate is a rich source but it is unknown where seminal TGF-β is produced in the ram. Investigation of the fertilising capacity of different rams with variable concentrations of active and latent TGF-β in their semen is also of importance. If significant correlation exists, seminal TGF-β may be a useful indicator to predict ram fertility. Such investigations could be carried out by mating rams with known seminal composition with ewes, and monitoring the subsequent pregnancy rate.

Monitoring the effects of seminal cytokines on development of the pre-implantation ovine embryo *in vitro* would aid our understanding of the complex chemical communication network that occurs during this period. Furthermore, downstream or cascade effects following the initial inflammatory reaction needs to be investigated, such as leukocyte function and trafficking, and cytokine synthesis and secretion in the endometrium during the pre- and peri-implantation periods. Detection and localisation of cytokine and chemokine receptors in endometrial tissues would also further our understanding of the links between insemination and the generation of a uterine environment conducive to successful pregnancy.

Further research is also required to determine how ovarian steroid hormones exert their effects on leukocytes and cytokines in the ovine reproductive tract both

following insemination and throughout the oestrous cycle. This would require the examination of reproductive tracts at several different stages during the oestrous cycle, and the identification of hormone receptors on leukocytes and endometrial epithelial and stromal cells. Techniques such as *in situ* hybridisation would be useful to confirm the distribution and pattern of cytokine synthesis in ovine reproductive tissues. Exposure of cell cultures to oestrogens and progesterone and monitoring the effects on leukocyte populations and cytokine production would also generate valuable information regarding hormonal influences on ovine reproductive tissues.

8.3 Conclusion

In conclusion, the post-insemination inflammatory response in the reproductive tract of the ewe involves an increase in numbers of neutrophils and macrophages and a reduction or degranulation of mast cells, whilst the effect on eosinophils remains unclear. These changes in leukocyte populations are likely to be driven, at least in part, by a concurrent increase in GM-CSF and IL-8 which occurs in response to a combination of spermatozoa, seminal plasma and possibly bacteria or bacterial products. The leukocyte and cytokine changes occurring in the reproductive tract of the ewe in response to insemination are likely to both clear contaminants and facilitate preparation for implantation of the ovine conceptus.

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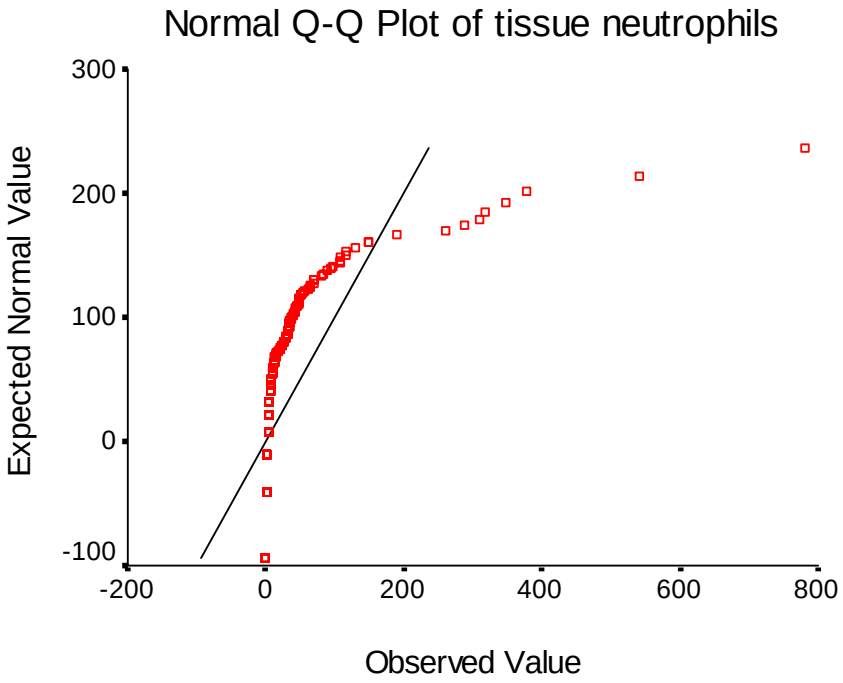
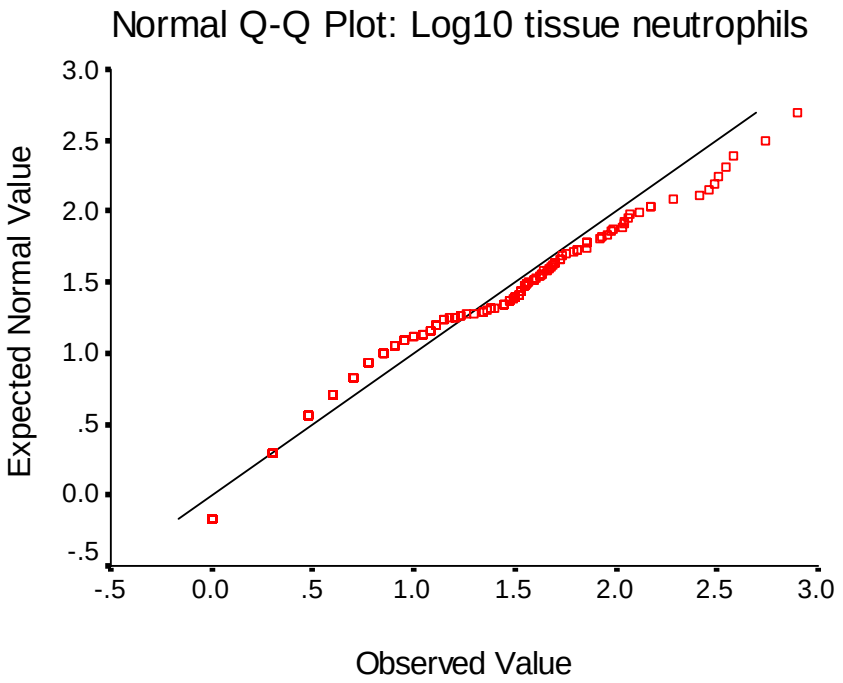
APPENDICES

Appendix 1.





Appendix 2.

Univariate Analysis of Variance (3 way ANOVA) for log10 neutrophil counts in tissues for mated vs controls, different time periods and different sites

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
MATED	1	Control ewes	300
	2	Mated ewes	300
HOURS	3		120
	6		120
	18		120
	24		120
	48		120
SITEOVUL	1	vagina	30
	2	posterior cervix	30
	3	mid cervix	30
	4	anterior cervix	30
	5	uterine body	30
	6	ip midhorn	30
	7	ip anterior horn	30
	8	co midhorn	30
	9	co anterior horn	30
	10	cervical os	30
	11	mid BU	30
	12	deep BU	30
	13	mid ip midhorn	30
	14	deep ip midhorn	30
	15	mid ip anthorn	30
	16	deep ip anthorn	30
	17	mid co midhorn	30
	18	deep co midhorn	30
	19	mid co anthorn	30
	20	deep co anthorn	30

Appendix 2 (cont.)

Tests of Between-Subjects Effects

Dependent Variable: LGTNEUTS

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	149.034[a]	199	.749	3.207	.000
Intercept	211.355	1	211.355	905.042	.000
MATED	12.930	1	12.930	55.370	.000
HOURS	14.857	4	3.714	15.905	.000
SITEOVUL	78.724	19	4.143	17.742	.000
MATED * HOURS	5.721	4	1.430	6.124	.000
MATED * SITEOVUL	4.583	19	.241	1.033	.421
HOURS * SITEOVUL	17.750	76	.234	1.000	.484
MATED * HOURS * SITEOVUL	14.468	76	.190	.815	.861
Error	93.412	400	.234		
Total	453.802	600			
Corrected Total	242.446	599			

a. R Squared = .615 (Adjusted R Squared = .423)

Appendix 3.

Post hoc test (Fisher's LSD) to detect differences in log10 neutrophil counts in tissues between different time periods (hours)

Multiple Comparisons

Dependent Variable: LGTNEUTS

LSD

(I) HOURS	(J) HOURS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
3	6	.0421	.06239	.501	-.0806	.1647
	18	-.1260*	.06239	.044	-.2486	-.0033
	24	.0980	.06239	.117	-.0247	.2206
	48	.3510*	.06239	.000	.2284	.4737
6	3	-.0421	.06239	.501	-.1647	.0806
	18	-.1680*	.06239	.007	-.2907	-.0454
	24	.0559	.06239	.371	-.0667	.1786
	48	.3090*	.06239	.000	.1863	.4316
18	3	.1260*	.06239	.044	.0033	.2486
	6	.1680*	.06239	.007	.0454	.2907
	24	.2240*	.06239	.000	.1013	.3466
	48	.4770*	.06239	.000	.3544	.5997
24	3	-.0980	.06239	.117	-.2206	.0247
	6	-.0559	.06239	.371	-.1786	.0667
	18	-.2240*	.06239	.000	-.3466	-.1013
	48	.2531*	.06239	.000	.1304	.3757
48	3	-.3510*	.06239	.000	-.4737	-.2284
	6	-.3090*	.06239	.000	-.4316	-.1863
	18	-.4770*	.06239	.000	-.5997	-.3544
	24	-.2531*	.06239	.000	-.3757	-.1304

Based on observed means.

*. The mean difference is significant at the .05 level.

Appendix 4.

T-Test to compare log10 neutrophil counts in tissues from mated vs control ewes in the posterior cervix.

T-Test

Group Statistics

	MATED	N	Mean	Std. Deviation	Std. Error Mean
LGTNEUTS	Control ewes	15	.5320	.39907	.10304
	Mated ewes	15	1.0607	.57169	.14761

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
LGTNEUTS	Equal variances assumed	4.644	.040	-2.937	28	.007	-.5288	.18002	-.89750	-.16001
	Equal variances not assumed			-2.937	25.026	.007	-.5288	.18002	-.89949	-.15803

Appendix 5.

T-Test to compare log10 neutrophil counts in tissues from mated vs control ewes for 6 hour time period.

T-Test

Group Statistics

	MATED	N	Mean	Std. Deviation	Std. Error Mean
LGTNEUTS	Control ewes	60	.4780	.46998	.06067
	Mated ewes	60	.7709	.73869	.09536

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
LGTNEUTS	Equal variances assumed	20.688	.000	-2.591	118	.011	-.2929	.11303	-.51672	-.06906
	Equal variances not assumed			-2.591	100.041	.011	-.2929	.11303	-.51714	-.06865

Appendix 6.

Univariate Analysis of Variance (one way ANOVA) to compare log10 neutrophil counts in tissues between pooled sites (vagina vs cervix vs uterus)

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
POOLSITE	1	Vagina	60
	2	Cervix	90
	3	Uterus	450

Tests of Between-Subjects Effects

Dependent Variable: LGTNEUTS

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	39.628[a]	2	19.814	58.323	.000
Intercept	194.804	1	194.804	573.410	.000
POOLSITE	39.628	2	19.814	58.323	.000
Error	202.818	597	.340		
Total	453.802	600			
Corrected Total	242.446	599			

a. R Squared = .163 (Adjusted R Squared = .161)

Appendix 7.

Mann-Whitney Test to compare neutrophil counts between mated and control ewes in smears from the cervix

NPar Tests
Mann-Whitney Test

Ranks

	MATED	N	Mean Rank	Sum of Ranks
SNEUTS	Control ewes	15	11.30	169.50
	Mated ewes	15	19.70	295.50
	Total	30		

Test Statistics[b]

	SNEUTS
Mann-Whitney U	49.500
Wilcoxon W	169.500
Z	-2.664
Asymp. Sig. (2-tailed)	.008
Exact Sig. [2*(1-tailed Sig.)]	.008[a]

a. Not corrected for ties.

b. Grouping Variable: MATED

Appendix 8.

Mann-Whitney Test to compare neutrophil counts between mated and control ewes in smears at the 18 hour time period.

NPar Tests
Mann-Whitney Test

Ranks

	MATED	N	Mean Rank	Sum of Ranks
SNEUTS	Control ewes	21	15.05	316.00
	Mated ewes	21	27.95	587.00
	Total	42		

Test Statistics[a]

	SNEUTS
Mann-Whitney U	85.000
Wilcoxon W	316.000
Z	-3.430
Asymp. Sig. (2-tailed)	.001

a. Grouping Variable: MATED

Appendix 9.

Kruskal-Wallis Test to compare neutrophil counts in smears between different time periods for mated ewes

NPar Tests
Kruskal-Wallis Test

Ranks

	HOURS	N	Mean Rank
SNEUTS	3	21	58.52
	6	21	43.90
	18	21	66.88
	24	21	42.98
	48	21	52.71
	Total	105	

Test Statistics[a,b]

	SNEUTS
Chi-Square	9.279
df	4
Asymp. Sig.	.054

a. Kruskal Wallis Test

b. Grouping Variable: HOURS

Appendix 10.

Mann-Whitney Test to compare GM-CSF staining intensity of tissues between mated and control ewes

NPar Tests
Mann-Whitney Test

Ranks

	MATED	N	Mean Rank	Sum of Ranks
TGMCSF	Control ewes	135	131.27	17721.50
	Mated ewes	135	139.73	18863.50
	Total	270		

Test Statistics[a]

	TGMCSF
Mann-Whitney U	8541.500
Wilcoxon W	17721.500
Z	-.950
Asymp. Sig. (2-tailed)	.342

a. Grouping Variable: MATED

Appendix 11.

Kruskal-Wallis Test to compare GM-CSF staining intensity of tissues at different sites in the reproductive tract both when (a) original sites were compared and (b) when sites were combined into three main sites

(a) NPar Tests
Kruskal-Wallis Test

Ranks

	SITEOVUL	N	Mean Rank
TGMCSF	vagina	30	51.50
	posterior cervix	30	82.55
	mid cervix	30	102.52
	anterior cervix	30	139.52
	uterine body	30	178.93
	ip midhorn	30	177.25
	ip anterior horn	30	167.63
	co midhorn	30	163.57
	co anterior horn	30	156.03
	Total	270	

Test Statistics[a,b]

	TGMCSF
Chi-Square	94.408
df	8
Asymp. Sig.	.000

a. Kruskal Wallis Test

b. Grouping Variable: SITEOVUL

(b) NPar Tests
Kruskal-Wallis Test

Ranks

	POOLSITE	N	Mean Rank
TGMCSF	Vagina	30	51.50
	Cervix	90	108.19
	Uterus	150	168.68
	Total	270	

Test Statistics[a,b]

	TGMCSF
Chi-Square	82.986
df	2
Asymp. Sig.	.000

a. Kruskal Wallis Test

b. Grouping Variable: POOLSITE

Appendix 12.

Univariate Analysis of Variance (3 way ANOVA) of ranked GM-CSF staining in tissues for mated vs controls, different time periods and different sites

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
MATED	1	Control ewes	135
	2	Mated ewes	135
HOURS	3		54
	6		54
	18		54
	24		54
	48		54
SITEOVUL	1	vagina	30
	2	posterior cervix	30
	3	mid cervix	30
	4	anterior cervix	30
	5	uterine body	30
	6	ip midhorn	30
	7	ip anterior horn	30
	8	co midhorn	30
	9	co anterior horn	30

Tests of Between-Subjects Effects

Dependent Variable: RANK of TGMCSF

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	918605.000[a]	89	10321.404	3.570	.000
Intercept	4957267.500	1	4957267.500	1714.419	.000
MATED	4830.237	1	4830.237	1.670	.198
HOURS	57819.593	4	14454.898	4.999	.001
SITEOVUL	505054.800	8	63131.850	21.833	.000
MATED * HOURS	49786.096	4	12446.524	4.305	.002
MATED * SITEOVUL	26416.963	8	3302.120	1.142	.337
HOURS * SITEOVUL	164828.441	32	5150.889	1.781	.010
MATED * HOURS * SITEOVUL	109868.870	32	3433.402	1.187	.240
Error	520472.500	180	2891.514		
Total	6396345.000	270			
Corrected Total	1439077.500	269			

a. R Squared = .638 (Adjusted R Squared = .460)

Appendix 13.

Post hoc test (Fisher's LSD) to detect differences in ranked GM-CSF staining in tissues between different time periods (hours)

Post Hoc Tests
HOURS

Multiple Comparisons

Dependent Variable: RANK of TGMCSF

LSD

(I) HOURS	(J) HOURS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
3	6	-15.90741	10.348580	.126	-36.32754	4.51273
	18	-15.96296	10.348580	.125	-36.38310	4.45717
	24	-36.98148*	10.348580	.000	-57.40162	-16.56134
	48	4.59259	10.348580	.658	-15.82754	25.01273
6	3	15.90741	10.348580	.126	-4.51273	36.32754
	18	-.05556	10.348580	.996	-20.47569	20.36458
	24	-21.07407*	10.348580	.043	-41.49421	-.65394
	48	20.50000*	10.348580	.049	.07986	40.92014
18	3	15.96296	10.348580	.125	-4.45717	36.38310
	6	.05556	10.348580	.996	-20.36458	20.47569
	24	-21.01852*	10.348580	.044	-41.43866	-.59838
	48	20.55556*	10.348580	.049	.13542	40.97569
24	3	36.98148*	10.348580	.000	16.56134	57.40162
	6	21.07407*	10.348580	.043	.65394	41.49421
	18	21.01852*	10.348580	.044	.59838	41.43866
	48	41.57407*	10.348580	.000	21.15394	61.99421
48	3	-4.59259	10.348580	.658	-25.01273	15.82754
	6	-20.50000*	10.348580	.049	-40.92014	-.07986
	18	-20.55556*	10.348580	.049	-40.97569	-.13542
	24	-41.57407*	10.348580	.000	-61.99421	-21.15394

Based on observed means.

*. The mean difference is significant at the .05 level.

Appendix 14.

Student's t-test to detect difference in ranked GM-CSF staining intensity between mated and control ewes at 24 hours T-Test

Group Statistics

	MATED	N	Mean	Std. Deviation	Std. Error Mean
RANK of TGMCSF	Control ewes	27	153.70370	80.724991	15.535532
	Mated ewes	27	165.55556	77.908024	14.993406

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
RANK of TGMCSF	Equal variances assumed	.061	.806	-.549	52	.585	-11.85185	21.590623	-55.1766	31.472902
	Equal variances not assumed			-.549	51.935	.585	-11.85185	21.590623	-55.1779	31.474202

Appendix 15.

Mann-Whitney Test to compare GM-CSF concentration in luminal fluid between mated and control ewes

NPar Tests
Mann-Whitney Test

Ranks

	MATED	N	Mean Rank	Sum of Ranks
FGMCSF	Control ewes	105	99.14	10409.50
	Mated ewes	105	111.86	11745.50
	Total	210		

Test Statistics[a]

	FGMCSF
Mann-Whitney U	4844.500
Wilcoxon W	10409.500
Z	-1.926
Asymp. Sig. (2-tailed)	.054

a. Grouping Variable: MATED

Appendix 16.

Kruskal-Wallis Test to compare GM-CSF concentration in luminal fluid at different sites in the reproductive tract (a) when original sites were compared and (b) when sites were combined into three main sites

(a) NPar Tests
Kruskal-Wallis Test

Ranks

	SITEOVUL	N	Mean Rank
FGMCSF	vagina	30	101.28
	mid cervix	30	97.23
	uterine body	30	112.52
	ip midhorn	30	103.53
	ip anterior horn	30	117.40
	co midhorn	30	95.15
	co anterior horn	30	111.38
	Total	210	

Test Statistics[a,b]

	FGMCSF
Chi-Square	5.531
df	6
Asymp. Sig.	.478

a. Kruskal Wallis Test

b. Grouping Variable: SITEOVUL

(b) NPar Tests
Kruskal-Wallis Test

Ranks

	POOLSITE	N	Mean Rank
FGMCSF	Vagina	30	101.28
	Cervix	30	97.23
	Uterus	150	108.00
	Total	210	

Test Statistics[a,b]

	FGMCSF
Chi-Square	1.535
df	2
Asymp. Sig.	.464

a. Kruskal Wallis Test

b. Grouping Variable: POOLSITE

Appendix 17.

Kruskal-Wallis Tests to compare GM-CSF concentration in luminal fluid between different time periods for (a) mated ewes and (b) control ewes

(a) NPar Tests

Kruskal-Wallis Test mated ewes

Ranks

	HOURS	N	Mean Rank
FGMCSF	3	21	51.02
	6	21	61.48
	18	21	41.05
	24	21	54.74
	48	21	56.71
	Total	105	

Test Statistics[a,b]

	FGMCSF
Chi-Square	7.577
df	4
Asymp. Sig.	.108

a. Kruskal Wallis Test

b. Grouping Variable: HOURS

(b) NPar Tests
Kruskal-Wallis Test controls

Ranks

	HOURS	N	Mean Rank
FGMCSF	3	21	59.69
	6	21	67.00
	18	21	44.43
	24	21	47.69
	48	21	46.19
	Total	105	

Test Statistics[a,b]

	FGMCSF
Chi-Square	16.809
df	4
Asymp. Sig.	.002

a. Kruskal Wallis Test

b. Grouping Variable: HOURS

Appendix 18.

Univariate Analysis of Variance (3 way ANOVA) of ranked GM-CSF concentration in fluid for mated vs controls, different time periods and different sites

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
MATED	1	Control ewes	105
	2	Mated ewes	105
HOURS	3		42
	6		42
	18		42
	24		42
	48		42
SITEOVUL	1	vagina	30
	3	mid cervix	30
	5	uterine body	30
	6	ip midhorn	30
	7	ip anterior horn	30
	8	co midhorn	30
	9	co anterior horn	30

Tests of Between-Subjects Effects

Dependent Variable: RANK of FGMCSF

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	116328.833[a]	69	1685.925	.767	.890
Intercept	2337352.500	1	2337352.500	1063.982	.000
MATED	15189.505	1	15189.505	6.914	.010
HOURS	28119.893	4	7029.973	3.200	.015
SITEOVUL	14033.933	6	2338.989	1.065	.387
MATED * HOURS	6940.793	4	1735.198	.790	.534
MATED * SITEOVUL	1288.729	6	214.788	.098	.997
HOURS * SITEOVUL	22599.674	24	941.653	.429	.991
MATED * HOURS * SITEOVUL	28156.307	24	1173.179	.534	.963
Error	307551.667	140	2196.798		
Total	2761233.000	210			
Corrected Total	423880.500	209			

a. R Squared = .274 (Adjusted R Squared = -.083)

Appendix 19.

Post hoc test (Fisher's LSD) to detect differences in ranked GM-CSF concentration in fluid in control ewes at different time periods (hours)

Post Hoc Tests
HOURS

Multiple Comparisons

Dependent Variable: RANK of FGMCSF

LSD

(I) HOURS	(J) HOURS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
3	6	-.80952	11.182996	.942	-23.11331	21.49426
	18	28.83333*	11.182996	.012	6.52955	51.13712
	24	16.90476	11.182996	.135	-5.39902	39.20855
	48	19.26190	11.182996	.089	-3.04188	41.56569
6	3	.80952	11.182996	.942	-21.49426	23.11331
	18	29.64286*	11.182996	.010	7.33907	51.94664
	24	17.71429	11.182996	.118	-4.58950	40.01807
	48	20.07143	11.182996	.077	-2.23235	42.37521
18	3	-28.83333*	11.182996	.012	-51.13712	-6.52955
	6	-29.64286*	11.182996	.010	-51.94664	-7.33907
	24	-11.92857	11.182996	.290	-34.23235	10.37521
	48	-9.57143	11.182996	.395	-31.87521	12.73235
24	3	-16.90476	11.182996	.135	-39.20855	5.39902
	6	-17.71429	11.182996	.118	-40.01807	4.58950
	18	11.92857	11.182996	.290	-10.37521	34.23235
	48	2.35714	11.182996	.834	-19.94664	24.66093
48	3	-19.26190	11.182996	.089	-41.56569	3.04188
	6	-20.07143	11.182996	.077	-42.37521	2.23235
	18	9.57143	11.182996	.395	-12.73235	31.87521
	24	-2.35714	11.182996	.834	-24.66093	19.94664

Based on observed means.

*. The mean difference is significant at the .05 level.

Appendix 20.

Student's t-test to detect difference in ranked GM-CSF concentration in fluid between mated and control ewes at 24 hours

T-Test

Group Statistics

	MATED	N	Mean	Std. Deviation	Std. Error Mean
RANK of FGMCSF	Control ewes	21	92.92857	29.945904	6.534732
	Mated ewes	21	122.21429	54.408541	11.872917

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
RANK of FGMCSF	Equal variances assumed	28.706	.000	-2.161	40	.037	-29.28571	13.552449	-56.6762	-1.895193
	Equal variances not assumed			-2.161	31.099	.039	-29.28571	13.552449	-56.9226	-1.648866

Appendix 21.

(a) Pearson's correlation between ranked IL-8 luminal concentration and ranked tissue staining intensity

Correlations

		RANK of FIL8	RANK of TIL8
RANK of FIL8	Pearson Correlation	1	-.412**
	Sig. (2-tailed)	.	.000
	N	210	210
RANK of TIL8	Pearson Correlation	-.412**	1
	Sig. (2-tailed)	.000	.
	N	210	270

**. Correlation is significant at the 0.01 level (2-tailed).

(b) Spearman's correlation between ranked IL-8 concentration and neutrophil percentage in luminal mucus

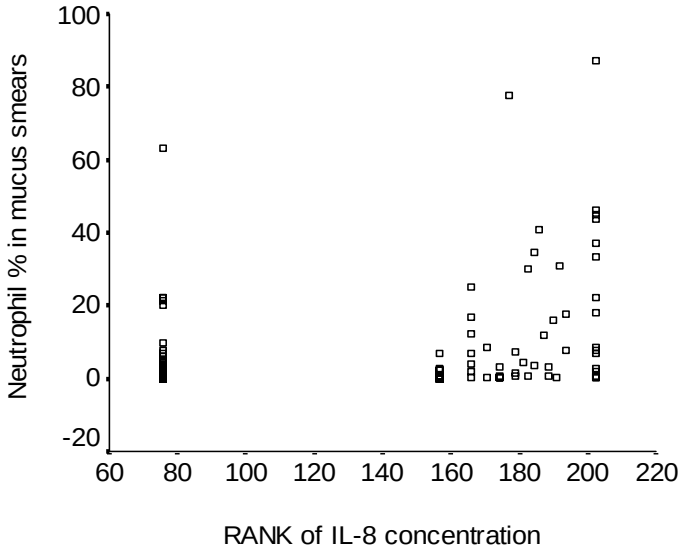
Nonparametric Correlations

Correlations

			RANK of FIL8	SNEUTS
Spearman's rho	RANK of FIL8	Correlation Coefficient	1.000	.480**
		Sig. (2-tailed)	.	.000
		N	210	210
	SNEUTS	Correlation Coefficient	.480**	1.000
		Sig. (2-tailed)	.000	.
		N	210	210

**. Correlation is significant at the .01 level (2-tailed).

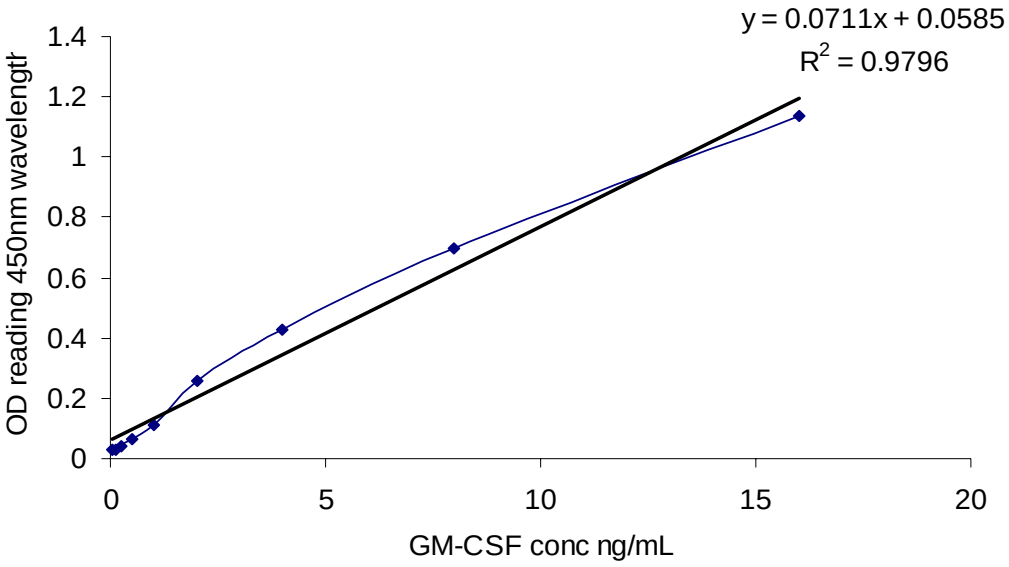
(c) Scatter plot for ranked IL-8 concentration and neutrophil percentage in luminal mucus



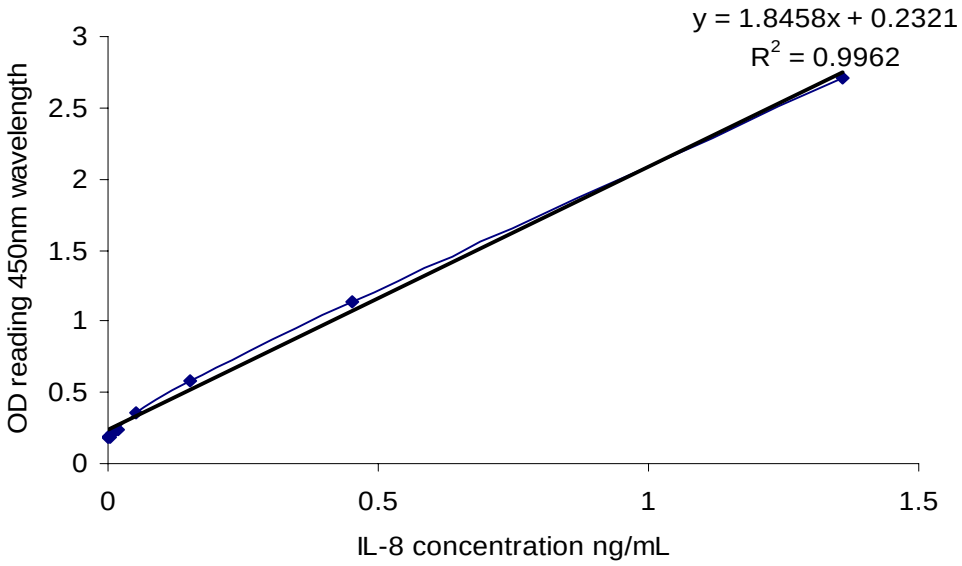
Appendix 22.

Sample standard curves from ELISAs for (a) GM-CSF, (b) IL-8 and (c) TGF-beta

(a) GM-CSF

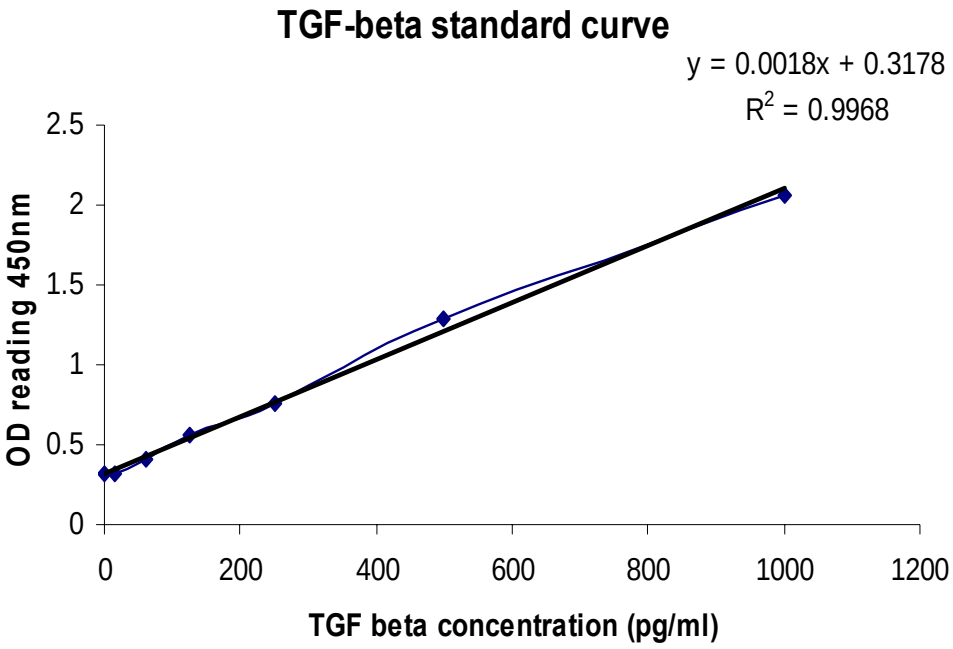


(b) IL-8



Appendix 22 (cont.)

(c) TGF-beta



Appendix 23.

Sperm concentration adjustment to 300 x 10^6 in 200µl TALP (equivalent to 1.5 x 10^9 per ml)

C_1 = Concentration of sperm required in final sample (1500 x 10^6/ml)

V_1 = Volume required in final sample (0.250ml)

C_2 = Concentration of sperm in original sample (from semen analysis machine)

V_2 = Volume required of original sample

$V_2 = C_1V_1/C_2$

$V_2 = (1500 \times 10^6) \times 0.25/C_2$

V_2 is then made up to 250µl with seminal plasma (for whole semen) or TALP (for washed spermatozoa)

Appendix 24.

Three way ANOVA comparing log10 neutrophils in tissues of oestrus ewes between different treatments, the use of antibiotics versus no antibiotics and different depths in the endometrium

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
ANTIBIOT	1	no antibiotics	90
	2	pen/strep	45
TREAT	1	Semen	27
	2	SP	27
	3	Sperm	27
	4	TALP	27
	5	NaCl	27
DEPTH	1	superficial	45
	2	mid	45
	3	deep	45

Tests of Between-Subjects Effects

Dependent Variable: LOGTNEUT

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	114.959[a]	29	3.964	7.205	.000
Intercept	93.481	1	93.481	169.915	.000
ANTIBIOT	3.162	1	3.162	5.748	.018
TREAT	18.218	4	4.555	8.278	.000
DEPTH	64.100	2	32.050	58.255	.000
ANTIBIOT * TREAT	4.835	4	1.209	2.197	.074
ANTIBIOT * DEPTH	.105	2	5.254E-02	.096	.909
TREAT * DEPTH	7.367	8	.921	1.674	.113
ANTIBIOT * TREAT * DEPTH	1.575	8	.197	.358	.940
Error	57.767	105	.550		
Total	291.183	135			
Corrected Total	172.726	134			

a. R Squared = .666 (Adjusted R Squared = .573)

Appendix 25.

Post Hoc test to compare log10 neutrophils in tissues between different treatments

Post Hoc Tests
TREAT

Multiple Comparisons

Dependent Variable: LOGTNEUT

LSD

(I) TREAT	(J) TREAT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Whole	SP	.3762	.32538	.252	-.2742	1.0266
	Washed	.2583	.34082	.451	-.4230	.9396
	TALP	1.0829*	.37229	.005	.3387	1.8271
	NaCl	1.6846*	.37229	.000	.9404	2.4288
SP	Whole	-.3762	.32538	.252	-1.0266	.2742
	Washed	-.1179	.33635	.727	-.7903	.5545
	TALP	.7067	.36820	.060	-.0294	1.4427
	NaCl	1.3084*	.36820	.001	.5724	2.0444
Washed	Whole	-.2583	.34082	.451	-.9396	.4230
	SP	.1179	.33635	.727	-.5545	.7903
	TALP	.8246*	.38191	.035	.0611	1.5880
	NaCl	1.4263*	.38191	.000	.6629	2.1897
TALP	Whole	-1.0829*	.37229	.005	-1.8271	-.3387
	SP	-.7067	.36820	.060	-1.4427	.0294
	Washed	-.8246*	.38191	.035	-1.5880	-.0611
	NaCl	.6017	.41024	.147	-.2183	1.4218
NaCl	Whole	-1.6846*	.37229	.000	-2.4288	-.9404
	SP	-1.3084*	.36820	.001	-2.0444	-.5724
	Washed	-1.4263*	.38191	.000	-2.1897	-.6629
	TALP	-.6017	.41024	.147	-1.4218	.2183

Based on observed means.

*. The mean difference is significant at the .05 level.

Appendix 26.

T-test to compare log10 macrophage counts between no antibiotics and use of penicillin/streptomycin

T-Test

Group Statistics

	ANTIBIOT	N	Mean	Std. Deviation	Std. Error Mean
LOGTMACR	no antibiotics	30	.7089	.31507	.05752
	pen/strep	15	.3794	.29796	.07693

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
LOGTMACR	Equal variances assumed	.061	.807	3.365	43	.002	.3294	.09791	.13198	.52688
	Equal variances not assumed			3.429	29.569	.002	.3294	.09606	.13313	.52573

Appendix 27.

Two way ANOVA comparing ranked GM-CSF in luminal fluid between different treatments and the use of antibiotics versus no antibiotics

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
ANTIBIOT	1	no antibiotics	30
	2	pen/strep	15
TREAT	1	Whole	9
	2	SP	9
	3	Washed	9
	4	TALP	9
	5	NaCl	9

Tests of Between-Subjects Effects

Dependent Variable: RANK of GMCSFNG

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	7080.778[a]	9	786.753	4.429	.001
Intercept	63467.778	1	63467.778	357.268	.000
ANTIBIOT	1867.778	1	1867.778	10.514	.003
TREAT	1737.667	4	434.417	2.445	.065
ANTIBIOT * TREAT	1737.667	4	434.417	2.445	.065
Error	6217.667	35	177.648		
Total	93099.000	45			
Corrected Total	13298.444	44			

a. R Squared = .532 (Adjusted R Squared = .412)

Appendix 28.

Post Hoc test to compare ranked GM-CSF in luminal fluid between different treatments
Post Hoc Tests

Multiple Comparisons

Dependent Variable: RANK of GMCSFNG

LSD

(I) TREAT	(J) TREAT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Whole	SP	14.77778*	6.283092	.024	2.02242	27.53313
	Washed	-8.00000	6.283092	.211	-20.75536	4.75536
	TALP	6.77778	6.283092	.288	-5.97758	19.53313
	NaCl	14.77778*	6.283092	.024	2.02242	27.53313
SP	Whole	-14.77778*	6.283092	.024	-27.53313	-2.02242
	Washed	-22.77778*	6.283092	.001	-35.53313	-10.02242
	TALP	-8.00000	6.283092	.211	-20.75536	4.75536
	NaCl	.00000	6.283092	1.000	-12.75536	12.75536
Washed	Whole	8.00000	6.283092	.211	-4.75536	20.75536
	SP	22.77778*	6.283092	.001	10.02242	35.53313
	TALP	14.77778*	6.283092	.024	2.02242	27.53313
	NaCl	22.77778*	6.283092	.001	10.02242	35.53313
TALP	Whole	-6.77778	6.283092	.288	-19.53313	5.97758
	SP	8.00000	6.283092	.211	-4.75536	20.75536
	Washed	-14.77778*	6.283092	.024	-27.53313	-2.02242
	NaCl	8.00000	6.283092	.211	-4.75536	20.75536
NaCl	Whole	-14.77778*	6.283092	.024	-27.53313	-2.02242
	SP	.00000	6.283092	1.000	-12.75536	12.75536
	Washed	-22.77778*	6.283092	.001	-35.53313	-10.02242
	TALP	-8.00000	6.283092	.211	-20.75536	4.75536

Based on observed means.

*. The mean difference is significant at the .05 level.

Appendix 29.

One way ANOVA for comparison of ranked TGF-beta concentration in semen between different rams

Oneway

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
RANK of TOTAL	Between Groups	458.667	6	76.444	3.438	.027
	Within Groups	311.333	14	22.238		
	Total	770.000	20			
RANK of ACTIVE	Between Groups	248.000	6	41.333	2.391	.084
	Within Groups	242.000	14	17.286		
	Total	490.000	20			
RANK of LATENT	Between Groups	303.333	6	50.556	1.517	.243
	Within Groups	466.667	14	33.333		
	Total	770.000	20			

Appendix 30.

Three way ANOVA comparing log10 neutrophils in tissues between different treatments, the use of antibiotics versus no antibiotics, and oestrus versus luteal ewes

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
TREAT	1	Semen	48
	2	SP	48
	3	Sperm	48
	4	TALP	48
	5	NaCl	48
ANTIBIOT	1	no antibiotics	150
	2	pen/strep	90
STAGE	1	oestrus	135
	2	luteal	105

Tests of Between-Subjects Effects

Dependent Variable: LOGTNEUT

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	78.379[a]	19	4.125	6.012	.000
Intercept	52.436	1	52.436	76.424	.000
TREAT	11.553	4	2.888	4.209	.003
ANTIBIOT	2.029	1	2.029	2.957	.087
STAGE	34.760	1	34.760	50.661	.000
TREAT * ANTIBIOT	3.995	4	.999	1.456	.217
TREAT * STAGE	6.252	4	1.563	2.278	.062
ANTIBIOT * STAGE	.984	1	.984	1.434	.232
TREAT * ANTIBIOT * STAGE	1.043	4	.261	.380	.823
Error	150.946	220	.686		
Total	306.829	240			
Corrected Total	229.325	239			

a. R Squared = .342 (Adjusted R Squared = .285)

Appendix 31.

Student's T-test to compare Log10 neutrophils in tissues in response to whole semen between oestrus and luteal ewes

T-Test whole semen

Group Statistics

	STAGE	N	Mean	Std. Deviation	Std. Error Mean
LOGTNEUT	oestrus	27	1.4366	1.27077	.24456
	luteal	21	.2821	.77019	.16807

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
LOGTNEUT	Equal variances assumed	20.308	.000	3.667	46	.001	1.1545	.31481	.52084	1.78818
	Equal variances not assumed			3.891	43.690	.000	1.1545	.29674	.55635	1.75268

Appendix 32.

Three way ANOVA comparing ranked GM-CSF in luminal fluid between different treatments, the use of antibiotics versus no antibiotics, and oestrus versus luteal ewes

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
TREAT	1	Semen	16
	2	SP	16
	3	Sperm	16
	4	TALP	16
	5	NaCl	16
ANTIBIOT	1	no antibiotics	50
	2	pen/strep	30
STAGE	1	oestrus	45
	2	luteal	35

Tests of Between-Subjects Effects

Dependent Variable: RANK of GMCSFNG

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	8847.833[a]	19	465.675	2.556	.003
Intercept	111123.205	1	111123.205	609.888	.000
TREAT	953.038	4	238.260	1.308	.277
ANTIBIOT	2477.051	1	2477.051	13.595	.000
STAGE	80.128	1	80.128	.440	.510
TREAT * ANTIBIOT	953.038	4	238.260	1.308	.277
TREAT * STAGE	986.269	4	246.567	1.353	.261
ANTIBIOT * STAGE	80.128	1	80.128	.440	.510
TREAT * ANTIBIOT * STAGE	986.269	4	246.567	1.353	.261
Error	10932.167	60	182.203		
Total	151000.000	80			
Corrected Total	19780.000	79			

a. R Squared = .447 (Adjusted R Squared = .272)

Appendix 33.

Student's T-test to compare ranked GM-CSF in luminal fluid in response to washed spermatozoa without antibiotics between oestrus and luteal ewes

T-Test

Group Statistics

	STAGE	N	Mean	Std. Deviation	Std. Error Mean
RANK of GMCSFNG	oestrus	6	67.16667	17.382942	7.096556
	luteal	4	41.75000	17.500000	8.750000

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
RANK of GMCSFNG	Equal variances assumed	.057	.818	2.259	8	.054	25.41667	11.249035	-.523656	51.356989
	Equal variances not assumed			2.256	6.545	.061	25.41667	11.266038	-1.602930	52.436264