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

Thesis submitted by

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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 Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.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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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

PUBLICATIONS

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CONFERENCE PROCEEDINGS

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