Seminal plasma cytokines as determinants of ovulation, embryo development and pregnancy success in the pig

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A thesis submitted to The University of Adelaide in fulfilment of the requirements for admission to the degree of Doctor of Philosophy
“Somewhere, something incredible is waiting to be known”

Carl Sagan 1934 - 1996
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ABSTRACT

Determinants of litter size in the pig are ovulation rate, fertilisation rate and embryo and fetal mortality. In practice, litter size is normally about half the ovulation rate with 40% or more of potential piglets being lost before day 30 of pregnancy. Successful embryo development depends on optimal timing of events beginning with ovulation, fertilisation and preparation of the uterine environment for the attachment of the developing embryo. In the pig these processes are tightly controlled and are highly sensitive to disruption. The determinants of optimal early embryo development remain to be fully elucidated but evidence provided in mouse and human studies indicate that constituents of seminal plasma may provide a beneficial ‘priming’ stimulus acting at natural mating to synchronise and enhance early reproductive events. The cytokine transforming growth factor beta (TGFβ) is present in large quantities in mouse and human seminal plasma and is a principal active constituent in mediating seminal fluid signalling in the female reproductive tract.

Experiments described in this thesis were designed to investigate whether boar seminal plasma can exert changes in the female reproductive tract during the pre-attachment period in the pig that are comparable to those described in mouse and human. Studies in this thesis demonstrate that seminal plasma causes a transient inflammatory response in the uterus characterised by induction of cytokine gene expression and immune cell changes that occur during the critical period in which the pig embryo is most vulnerable to demise. Seminal factors were also observed to enhance ovarian function, promoting synthesis of progesterone and influenced the rate of embryo development. The effect of these early changes due to seminal plasma was investigated in a large-scale field trial. However, this failed to demonstrate an effect of frozen-thawed seminal plasma on reproductive outcome in gilts. Moreover, the presumed active constituent of seminal plasma, TGFβ, was identified at high levels in boar semen but did not correlate with boar fertility.

The information from these experiments provide a comprehensive understanding of mechanisms underlying the potentially beneficial actions of seminal plasma in early pregnancy. Ongoing studies will assist in the strategic design of (1) novel ‘surrogate seminal plasma’ or ‘semen extender’ products incorporating active constituents of seminal plasma, and (2) assays for measuring cytokine / immunostimulants of seminal plasma that are predictive of boar fertility.
DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Sean O’Leary and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Sean O’Leary

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PUBLICATIONS ARISING FROM THESE STUDIES

Invited seminars (Australia)

1999  Seminal plasma induces a local inflammatory response in the reproductive tract of gilts. Australian Pig Science Association Biannual Meeting. Adelaide, South Australia

2000  Seminal factors influences serum progesterone levels during early pregnancy in the pig. 11th International conference of Endocrinology (Satellite symposia) Adelaide, South Australia

Intrauterine seminal plasma induces endometrial inflammatory response in gilts. Fertility Society of Australia and Australian Society for Reproductive Biologists. Canberra, Australia

Presence of transforming growth factor beta in boar seminal plasma. Australian Society for Medical Research. Adelaide, Australia

2001  Seminal plasma treatment stimulates progesterone synthesis during early pregnancy in the pig. Endocrine Society of Australia and Society for Reproductive Biology Annual Scientific Meeting. QLD, Australia.

Intrauterine seminal plasma increases ovarian steroidogenesis during early pregnancy in the pig. Australian Pig Science Association Biannual Meeting. Adelaide, South Australia
Invited Seminars (International)

2001  *Seminal plasma cytokines as determinants of ovulation, embryo development and pregnancy success in the pig.*  Division of Animal Physiology, School of Biological Sciences (Prof. Morag Hunter), University of Nottingham, 18th June. Loughborough, UK.

*Seminal plasma cytokines, implications for pre-natal mortality in pigs.*  Department of Obstetrics, Gynaecology and Reproductive Biology, Brigham and Women’s Hospital, Harvard Medical School, (Assoc. Prof. Deborah Anderson) 24th June, Boston, USA.

Intrauterine seminal plasma increases ovarian steroidogenesis in the pig. Society for Study of Reproduction, 34th Annual meeting, Ottawa, Canada.

Seminal plasma cytokines as determinants of ovulation, embryo development and pregnancy success in the pig. Department of Animal Science, Ontario Veterinary College (Prof. Anne Croy) 7th July, Guelph, Canada.

*Seminal plasma influences on ovarian function, implications for fertility enhancement in pigs.*  Department of Agriculture, University of Alberta (Prof. George Foxcroft) 11th June, Edmonton, Canada.

Seminal plasma cytokines as determinants of ovulation, embryo development and pregnancy success in the pig. Department of Animal Science, University of Missouri (Prof. Billy Day) 23rd July, Columbia, USA.

Enhancing reproduction in pigs: Transforming growth factor beta in boar seminal plasma, a potential candidate as a surrogate treatment for seminal plasma. Department of Animal Sciences, University of Idaho (Dr. Troy Ott) 28th July, Moscow, Idaho, USA.


O'Leary S, Armstrong DT, Robertson SA. Intrauterine seminal plasma increases ovarian steroidogenesis during early pregnancy in the pig. In: Australasian Pig Science Association (APSA); 2001; Adelaide, South Australia. 190.


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O'Leary S, Armstrong DT, Robertson SA. Seminal factors influence serum progesterone levels during early pregnancy in the pig. In: Satellite Symposia to the 11th International Congress of Endocrinology; 2000; South Australia. 68.


O'Leary S, Robertson SA and Armstrong DT. Seminal plasma treatment stimulates progesterone synthesis during early pregnancy in the pig. In: Endocrine Society of Australia & Society Reproductive Biology Annual Scientific Meeting; 2001; QLD, Australia.


ABBREVIATIONS

Ab  Antibody
AI  Artificial insemination
BMP Bone morphogenic protein
Bp  Base pairs
BPE Bovine pituitary extract
BSA Bovine serum albumin
cAMP Cyclic adenosine monophosphate
cDNA Complimentary DNA
CL  Corpora lutea or corpus luteum
Ct  Cycle threshold
cv  Coefficient of variation
DAB Diaminobenzidine tetrachloride
DMEM Dulbecco’s modified minimal essential medium
DNA Deoxyribonucleic acid
DNAse Deoxyribonuclease
DPBS Dulbecco’s PBS
DTH Delayed-type hypersensitivity
ECM Extracellular matrix
EDTA Ethylenediaminetetraacetic acid
EGF Epidermal growth factor
ELISA Enzyme-linked immunosorbancy assay
EU Equivalent units
FACS Fluorescence-activated cell scanning
FCS Fetal calf serum
FSH Follicle stimulating hormone

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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GIFT</td>
<td>Gamete intrafallopian transfer</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotrophin</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>h</td>
<td>Hour</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>ID</td>
<td>Identification</td>
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<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
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<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
</tr>
<tr>
<td>IUGR</td>
<td>Intrauterine growth retardation</td>
</tr>
<tr>
<td>IVF</td>
<td>In vitro fertilisation</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobase pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo-dalton</td>
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<tr>
<td>L</td>
<td>Litre</td>
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<tr>
<td>LAL</td>
<td>Limulus amebocyte lysate assay</td>
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<tr>
<td>LAP</td>
<td>Latency associated protein</td>
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<td>LCA</td>
<td>Leukocyte common antigen</td>
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<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
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<tr>
<td>LIF</td>
<td>Leukaemia inhibitory factor</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LTBP</td>
<td>Latent transforming growth factor binding protein</td>
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<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
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<tr>
<td>MCP</td>
<td>Monocyte chemotactic protein</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>Abbreviation</td>
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</tr>
<tr>
<td>Min</td>
<td>Minute</td>
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<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
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<tr>
<td>Ms</td>
<td>Millilitres</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<td>MQ</td>
<td>Milli-Q</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>NHS</td>
<td>Normal human serum</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
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<tr>
<td>°C</td>
<td>Degrees Celcius</td>
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<tr>
<td>OCT</td>
<td>Optimal Cutting Temperature Compound</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PGE</td>
<td>Prostaglandin E</td>
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<tr>
<td>PGF</td>
<td>Prostaglandin F</td>
</tr>
<tr>
<td>PSP</td>
<td>Pig seminal protein</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RNAse</td>
<td>Ribonuclease</td>
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<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SABOR</td>
<td>South Australian Boar</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SLA</td>
<td>Swine leukocyte antigen</td>
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<td>TGF</td>
<td>Transforming growth factor</td>
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<tbody>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>VIA</td>
<td>Video image analysis</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
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Chapter 1

Literature Review
1.1 GENERAL INTRODUCTION

Successful pregnancy outcome in mammals is dependent on the synchronous cellular and functional development of ovarian, uterine and subsequent embryonic and fetal tissues during the period from mating through pregnancy and parturition. Emerging evidence is highlighting the role of the maternal immune system in preparing for and participating after pregnancy is initiated. Pregnancy itself represents an immunological paradox in which the female tolerates an antigenically foreign fetus. This condition has been acknowledged since 1924 when Little noted the need for a mechanism to allow the female to tolerate her foreign embryo (cited by Billington, 1989). However, it wasn't until Peter Medawar initiated the study of transplantation immunology that a feasible hypothesis was put forward (Medawar 1953). Incorporating Macfarlane Burnet's concept of tolerance to self antigens as an essential prerequisite to a functioning immune system (Tizard 1995) and adding observations that mouse embryos inoculated with foreign tissue became tolerant to them, led Medawar to propose three explanations for this paradox: “(1) the anatomical separation of fetus from mother; (2) the antigenic immaturity of the fetus; and (3) the immunological indoleness or inertness of the mother” (Medawar 1953). This experimental evidence for tolerance led to Medawar and Burnet jointly being awarded the Nobel Prize in 1960 (Tizard 1995). Since this time there has been a great deal of experimentation investigating these hypotheses, with the aim being to unravel the mystery of embryonic survival in utero, and increase knowledge of the biology of early pregnancy in relation to maternal immune responses to the semi-allogeneic embryo.

The maternal immune responses to mating and the developing embryo have been widely discussed in relation to potential roles in embryo survival and pregnancy success. This review is concerned with: (1) the current literature regarding proposed causes of early pregnancy loss in the pig with specific emphasis on the maternal immune response and its influence on embryo survival; (2) early pregnancy in the pig, with special reference to comparing mechanisms for alleviation of immune rejection in this species with those in other mammals; (3) immunological strategies ensuring reproductive success, and (4) implications for developing treatments to increase reproductive outcome in commercial pig production. These strategies in part include the role of cytokines and other immune modulating moieties present in seminal plasma, which appear to influence the maternal immune response and thus affect reproductive outcome in the pig.
1.2 IMMUNE SYSTEM: OVERVIEW

1.2.1 Function of the mammalian immune system

Immunology is the study of cells and molecules that are primarily involved in the recognition and elimination of potential pathogens. To achieve this it is imperative that immune cells are capable of distinguishing between ‘self’ and ‘non-self’, or perhaps more precisely ‘non-threatening’ and ‘dangerous’ stimuli, to eliminate or inactivate potentially hostile challenges. The cell populations that are principally responsible for this discrimination are antigen presenting cells (APC) which include macrophages and dendritic cells. APCs act to elicit activation and expansion of specific populations of T lymphocytes, including helper T (Th) cells, cytotoxic T (Tc) cells and regulatory T (Treg) cells. The balance between these populations is then the key determinant of the quality and strength of the ensuing immune response.

1.2.2 Helper T cells

An immune response can be defined as the net outcome of the interaction between antigen and the variety of host cells that can respond (Tizard 1995). In an existing immune response, responding cells are either T or B lymphocytes. B lymphocytes can respond to intact soluble antigen primarily by producing antibodies. T cells however, respond only to antigen (non-self peptides) complexed to cell surface proteins encoded by the major histocompatibility complex (MHC). There are two types of MHC proteins; MHC class I molecules which are present on the surface of most nucleated cells and MHC class II molecules which are normally expressed on APC, as well as activated B cells and endothelial cells. Once stimulated by the interaction with an APC, the Th cell produces proteins called cytokines, which regulate the responses of other lymphocytes to that antigen. Various alternative outcomes exist, depending on the type of Th cells that become activated. Tc cells, which recognise non-self peptides complexed to MHC class I molecules, typically those expressed by virally infected cells, are activated by T helper type 1 (Th1) cells. Alternatively, when T helper type 2 (Th2) cells predominate, the ensuing immune response is skewed towards synthesis of antibodies by B lymphocytes. Immune responses are initiated when the antigen-specific T cell receptor of resting CD4+ T cells are stimulated by MHC II-peptide complexes and co-stimulatory molecules expressed on APCs (Romagnani 2000). This induces T cells to proliferate, produce cytokines and express the cell surface molecules: IL-2 receptor, CTLA4 and the CD40 ligand necessary for T cell function. The functionality of T cells is dependent on the affinity and duration of binding of the T cell receptors with MHC II-peptide complexes (Tizard 1995).
1.2.3 Th1 and Th2 cytokines

The current model to explain immune responses generated by Th cells was first proposed by Mosmann et al in 1986 using mouse CD4+ T cells and later with human CD4+ T cells (Cherwinski et al. 1987, Mosmann et al. 1986). The basis of this model was the different patterns of cytokine production by activated T cell clones in vitro (Mosmann et al. 1996). Cytokines are paracrine, autocrine and at times endocrine intercellular peptide signals produced by virtually all stimulated or injured nucleated cells, and they have pleiotrophic effects on immune, inflammatory and other receptor bearing cell types (Oppenheim et al. 1992). T helper 1 (Th1) cells are defined by their cytokine production of interleukin 2 (IL-2), interferon gamma (IFNγ) and tumour necrosis factor alpha (TNFα) and Th2 cells are defined by their production of IL-4, IL-5, IL-6, IL-10 and IL-13. After this model was put forward it was found that there are also murine T cell clones that produce the Th1 cytokines IL-2 and IFNγ and the Th2 cytokines IL-4 and IL-5. A diagram (Figure 1) illustrates that cytokines are produced by the Th1 and Th2 cells and the general assumption that the majority of naïve T helper cells (Th0) can be induced to vary in their cytokine pattern according to the type of APC stimulation they experience. The functions of Th1 and Th2 cells are the result of their distinctive cytokines and these characteristic cytokines are mutually inhibitory. Thus IFNγ selectively inhibits proliferation of Th2 cells and IL-10 inhibits cytokine production of Th1 cells. In vivo support for this model came from mice infected with Leishmania major (Mosmann et al. 1996). Recovery from L. major infection is associated with Th1 cell synthesis of IFNγ and disease progression is associated with Th2 cell synthesis of IL-4. The role of these two subsets of T cells was confirmed by the treatment with neutralising antibodies to IFNγ and to IL-4 (Locksley et al. 1991). Thus Th1 and Th2 cells can be defined by the cytokine profile they produce.

1.2.4 Th1 and Th2 type responses in pregnancy

Although there has been little work on the Th response in pregnancy in the pig, data from other mammalian species indicate that the Th1/Th2 model is an oversimplification of immunoregulation in vivo (Kelso 1995). However this Th1/Th2 model (Figure 1) does provide a useful conceptual framework to allow better understanding of the immune response in pregnancy. Experiments supporting the Th1/Th2 model operating in early pregnancy involve the administration of either Th1 or Th2 cytokines to pregnant mice. Administration of the Th2 cytokine IL-10, and neutralising anti-IL-2 antibody, have been shown in mice to be associated with successful pregnancy, whereas a single administration of a dose of IL-2, IFNγ or TNFα (Th1 cytokines) during mid-gestation has been shown to be deleterious (Chaouat et al. 1990, Lala 1990, Tezabwala et al. 1989). This response is indicative of the cross-regulation in cytokine production between Th1 and Th2 subset of T cells; for instance, IFNγ secreted by Th1 subset of T cells preferentially inhibits proliferation of the Th2 subset and IL -10 secreted by Th2 cells down-regulates
secretion of IFN$\gamma$ and IL-2 by the Th$_1$ subset (Figure 1). In studies investigating the role of T helper cytokines in human pregnancies a similar pattern was found. In women who experience recurrent spontaneous abortions, type 1 cytokines were found to predominate in endometrial biopsies (Hill 1995, Hill et al. 1995, Lim et al. 2000), and conversely type 2 cytokines were highly expressed with low expression of type 1 cytokines in endometrial samples from women with healthy pregnancies. The predominance of Th$_2$ cytokines in pregnancy and the harm that is attributed to Th$_1$ cytokines in pregnancy (Anderson et al. 1988) has led to the proposal that successful pregnancy can be considered a Th$_2$ phenomenon (Hill 1992, Wegmann et al. 1993).

1.2.5 Other T Helper phenotypes

Transforming growth factor beta (TGF$\beta$) is a cytokine normally associated with the Th$_2$ subset by virtue of its reciprocal relationship with both IFN$\gamma$ and IL-12 (Zhang et al. 1995), but it has also been shown to be able inhibit the production of and response to cytokines associated with each subset (Coffman et al. 1997). T regulatory (Treg) cells are a subset of CD4$^+$ T cells with a suppressive phenotype, associated with the secretion of TGF$\beta$ and IL-10. These cells have essential roles in the maintenance of pregnancy (Aluvihare et al. 2004, Guerin et al. 2009). The production of TGF$\beta$ by another subset of Th cells that also express IL-4 and IL-10 but not IL-5 and IL-6 and rely on the secretion of TGF$\beta$ to suppress an inflammatory response may mark a third unique Th subset referred to as Th$_3$ cells (Bridoux et al. 1997, Letterio et al. 1998, Letterio et al. 1997). This characteristic production of TGF$\beta$ by Th$_3$ cells (Figure 1) is an important factor in the development of oral tolerance, but the potential role of these cells in the immune response after mating or during pregnancy has not yet been investigated.
Figure 1.1 Schematic representation of the activation of resting CD4\(^+\) T cells by antigen and cytokine profile of T helper (Th) cells.

Resting CD4\(^+\) T cells recognise antigens complexed with MHC on the surface of antigen presenting cells (APC) and differentiate after activation into T helper 1 (Th\(_1\)) cells in a cytokine environment containing IL-12 and IFN\(_\gamma\) or into T helper 2 (Th\(_2\)) cells in a cytokine environment containing a predominance of IL-4. Successful pregnancy outcome may depend on the balance between Th\(_1\) and Th\(_2\) subsets and also the presence of T regulatory (Treg) and T helper 3 (Th\(_3\)) cells producing cytokines IL-10 and TGF\(\beta\) inducing tolerance to the semi-allogeneic embryo. The red lines represent the inhibitory effect that Th\(_1\) cytokine IFN\(_\gamma\) has on activating the Th\(_2\) subset of CD4\(^+\) T cells and conversely the inhibitory effect of Th\(_2\) cytokine IL-10 on Th\(_1\) subset of CD4\(^+\) T cells. In addition Treg/Th\(_3\) cytokines have an inhibitory effect on activation of the Th\(_1\) subset of CD4\(^+\) cells. Key; TCR: T cell receptor; MHC: major histocompatibility complex.
1.3 REPRODUCTION IN THE PIG

Pigs are unique compared to other mammals with regard to reproductive physiology, and have evolved several strategies to maintain pregnancy and reproduce successfully. Indeed the domestic sow is predominantly either pregnant or lactating and feeding piglets. In essence, as soon as lactation finishes the inhibition for the production of reproductive hormones follicle stimulating hormone (FSH) and luteinizing hormone (LH) is lifted and the sow resumes cycling and can be bred again within 7 to 10 days after weaning. The oestrous cycle in the sow lasts for approximately 21 days and consists of a relatively short follicular phase involving the recruitment and selection of the oestrogenic preovulatory follicles followed by a much longer luteal phase after ovulation when the follicle differentiates into progesterone producing luteal tissue (Foxcroft et al. 1994). Behavioural changes in the sow exhibiting the “standing reflex” are associated with female receptivity to mating and is generally taken as the beginning of oestrus and the start of physiological events leading to ovulation and fertilisation (Claus 1990).

Pigs are multiparous animals with long reproductive tracts that can accommodate 17 or more piglets per pregnancy. At least four implanted embryos in one uterine horn are necessary for the maintenance of pregnancy in pigs (Polge et al. 1966) and this is due to a requirement for a minimum level of oestradiol synthesized by the embryos to support maintenance of the corpora lutea (Bazer et al. 1977). Ovulation in the pig occurs over a 1-3 hour period as measured by Pope et al. (1989) and in naturally cycling gilts ovulation occurs approximately 34 hours after the onset of oestrus, while induction of oestrus with human chorionic gonadotrophin (hCG) delays ovulation to between 44-46 hours after the onset of oestrus (Webel et al. 1982). Other studies have reported ovulation to take place 44 h from the onset of the LH surge (Soede et al. 1992, Soede et al. 1991) and in equine chorionic gonadotrophin/human chorionic gonadotrophin (eCG/hCG) treated gilts the mean interval ranges from 34 to 48 h (Hunter 1972). The variation in the period between LH surge and ovulation most likely reflects differences in genotypes of the sows within each study. In both naturally cycling and induced sows, it was noted that the majority of follicles ovulate over a short period of time, while the remaining minority of follicles ovulate over a more protracted period, which potentially yields oocytes with different levels of maturity. This is likely to result in fertilised oocytes with asynchronous development and has implications for subsequent embryo survival and implantation.
1.3.1 Determinants of litter size in the pig

1.3.2 Embryonic diversity

Litter size is a function of ovulation rate, fertilisation rate and embryonic and fetal mortality. Ovulation rates in the domestic pig are consistent and high and within the last 30 years have increased from an average of 17 (Scofield 1972) to about 25 in the modern domestic sow (Foxcroft 2009). Fertilisation rates are also high: between 95-100% (Short 1979). In Australia, the average litter size is about 10 piglets live born (Australian_Pork_Limited 2006-2008) but in European countries such as Denmark and France the average litter size is 12.7 and 12.5 respectively (Lawlor et al. 2007). Therefore, implantation failure and embryo and fetal loss during gestation are major determinants of the number of viable piglets produced and profitability of the commercial Australian pig herd. Using the average ovulation rate of sows, the reported periods of conceptus loss during gestation which determine the litter size in pigs can be demonstrated (Figure 2). In the pig there is also considerable loss in the perinatal and pre-weaning period (Foxcroft 2009), but a discussion of the factors influencing this is beyond the scope of this thesis.

Diversity amongst littermate embryos in pigs is evident from day 4 of development, once embryos pass into the uterus from the oviduct. Approximately 20% of embryos are smaller and contain less total protein (Pope et al. 1990). The causes of diversity amongst embryos are not fully understood but potential factors include follicular/oocyte maturation, ovulation, SLA (swine leukocyte antigen) genotype, genetic and epigenetic influences, and uterine location (Pope et al. 1990). Xie et al. (1990) examined the fate of particular oocytes in relation to when they were ovulated (i.e. first/middle/last) by selectively eliminating follicles (by electrocauterisation) and concluded that oocytes of follicles that were ovulated first became the more developed embryos, while late ovulating oocytes yielded embryos with retarded development. These results suggest that the diversity amongst embryos in early gestation could be predetermined by factors associated with oogenesis and follicular development.

Also, genetic variation in SLA genotype has an effect on early embryo development. Ford et al. (1988) measured the DNA content in blastocysts flushed at days 6, 9 and 11 from homozygous females (SLA^a, SLA^c and SLA^d) mated to homozygous males of the same genotypes. Significant differences existed between SLA^{aa} or SLA^{cc} and SLA^{dd} embryos in DNA content especially between days 9 and 11, demonstrating that genetic influences may influence the rate of embryonic development (Ford et al. 1988). In the same study Ford et al. also noted that Chinese Meishan gilts showed more uniformity in blastocyst development than Large White gilts. This was interpreted as genetic variation between breeds accounting for the variation in early embryo size.
The consequences of the variability in development amongst littermate embryos in early pregnancy have been investigated by a number of groups (Pope et al. 1988, Scofield 1972, Stone 1985, Ford et al. 1988). There is a general consensus that morphologically advanced embryos are more likely to survive. This increase in survival has been linked to the finding that the more morphologically mature blastocyst produces more oestradiol from days 10 to 11 (Stone et al. 1986). Geisert et al. (1982) demonstrated that uterine exposure to oestradiol could stimulate uterine secretions (Geisert et al. 1990). This suggests that variation in microenvironments may exist in the gravid uterus and after intrauterine migration has finished, advanced microenvironments would be spatially associated with the more developed blastocysts. This leads to the suggestion that the more morphologically mature embryo may be preferentially advantaged by the increased uterine secretions (Pope et al. 1990), and grow more rapidly. Whether or not the larger embryos in the more advanced microenvironments cause the demise of the less developed embryos remains unknown but could be a limiting factor in litter size. Thus diversity due to oocyte maturation, SLA haplotype and breed, may have lasting effects past day 10-12 of pregnancy and may determine the variation in birth weight and size of the litter at term.

Variation in size and growth can be observed macroscopically in litter mate embryos from day 10 of gestation (Figure 2a) and in fetuses at day 35 and day 70 (Figure 2b and 2c). This diversity in prenatal development amongst litter mates is likely to explain diversity that is present in piglets after birth (Figure 2d) and may dictate development and growth performance during the life of the pig. This observation also supports the fetal origins of adult development hypothesis that has been put forward to explain why low birth weight babies are predisposed to growth changes after birth and adult onset diseases including diabetes mellitus and heart disease (Barker et al. 1990).
Figure 1.2 Macroscopic variability within litter mates during embryo, fetal and pre-weaning development.

Embryo diversity can be seen as early as day 11 of gestation (a) and this diversity within embryo litter mates may continue through pregnancy as comparable variation was evident in these litters at day 35 (b) and day 70 (c) of gestation. Diversity of embryos and fetuses during gestation may also determine the diversity seen in littermates after birth (d) and may influence growth performance throughout the life of the pig (Photographs kindly donated by E.G. Bouwman, University of Adelaide School of Paediatrics and Reproductive Health, 2009).
It is of interest to note that the uterine location of embryos has been found to have no relationship to the protein content or morphology of early embryos in the pig (Anderson et al. 1976, Anderson 1978). This seems to be a peculiarity of the pig, since in other polytocous mammals, including mice and rabbits, the position of embryos in the uterine horn has an influence on the development of the embryo. However, after implantation and with a relatively larger number of fetuses present in the uterus, fetuses that have implanted at or around the bifurcation of the uterine horns are likely to fail to survive due to insufficient placental attachment to the uterine tract (G.R. Foxcroft, personal communication, 2009). Moreover and in contrast with the literature reviewed above, some studies have shown that individual gilts can have 100% survival of conceptuses at day 28 of gestation even though the final average litter size is 10 to 12 piglets, suggesting that for these gilts uterine capacity may be the limiting factor for prenatal survival (Foxcroft et al. 2006).

1.3.3 Uterine capacity

Genetic selection based on ovulation rate and embryo survival has been used to increase litter size in pigs. In a study using 11 generations of composite Landrace and Large White gilts and boars ovulation rate was increased by 7.4 ova and litter size was increased by 3.4 fetuses at day 50 of gestation (Johnson et al. 1999). This was associated with a modest increase in live born of 1.1 piglets due to increased fetal loss and increased still born pigs. In addition, the increased litter size was associated with a decreased birth weight of pigs born alive leading to lower pre-weaning viability (Johnson et al. 1999). This response may in part be due to physical limitations of uterine capacity imparts on the number of fetuses surviving to term.

Uterine capacity is based on the concept that intrauterine crowding becomes a limiting factor for conceptus survival when the number of embryos exceeds 14 after day 30 of pregnancy (Dziuk 1968, Wu et al. 1987, Wu et al. 1988). The likely physiological mechanism leading to embryo/fetal demise is the intrauterine competition between fetuses for adequate space for placental attachment, with excessive crowding leading to smaller placentas and inadequate nutrient and metabolite exchange between the fetal and maternal circulations (Foxcroft et al. 2006). Not only can uterine crowding lead to fetal demise, but it also explains variability in weight and survival of post natal piglets (Town et al. 2004). In a study using unilateral oviductal ligation to reduce the number of embryos and provide a non-crowded uterine environment, embryo survival at day 30 of gestation was increased from 79% in non-ligated controls to 91% due to the non-crowded uterine environment (Town et al. 2004). In addition, unilateral surgical ligation to reduce uterine crowding results in higher placental weights, increased numbers of secondary muscle fibres at day 90 gestation and increased the male to female ratio of fetuses (Town et al. 2004, Tse et al. 2008).
1.3.4 Endocrinological processes leading to pregnancy loss in the pig

Other factors that affect embryo survival and the resulting litter size in the pig include progesterone levels, especially in the first few days of pregnancy, which can vary as the result of nutritionally-mediated effects (Foxcroft 1997) and embryogenic oestradiol production in which threshold levels are implicated in the minimum number of embryos required for the persistence of the corpus luteum (Polge et al. 1966). Low levels of progesterone have been implicated in decreasing embryo survival after a high plane of nutrition was supplied to gilts 3-4 days after mating. The effect of high-plane nutrition on embryo survival was reversed by treatment with exogenous progesterone (Ashworth 1991) demonstrating a strong link between pre-mating nutrition and peripheral plasma progesterone levels (Ashworth et al. 1999a). This might result from increased hepatic function due to high plane nutrition and a resulting increase in the rate of metabolic clearance of progesterone in the liver (Ashworth et al. 1999b). Also, increases in hepatic blood flow due to high nutrition may divert blood away from the ovarian circulation (Jindal et al. 1997) leading to detrimental effects on the developing embryo and uterine preparation for pregnancy.

Progesterone levels in early pregnancy in the pig are also implicated in seasonal effects on pregnancy success. The wild pig is a seasonal breeder and predominantly breeds during the months of the year with shorter day length in Europe (Tast et al. 2001a). Although the modern domestic pig breeds all year round, it still displays seasonal effects that are more apparent in its wild progenitor. These seasonal effects manifest as reduced farrowing rates, delayed puberty in gilts, extended intervals from weaning to estrus, early disruption of pregnancy and a reduction in litter sizes during the summer-autumn period (Peltoniemi et al. 2000). Sows affected by seasonal effects conceive after mating but lose embryos and quite often the whole litter before day 30 of pregnancy (Love et al. 1993). It is known that long days in summer alter the level and rhythmicity of melatonin secretion controlled by the suprachiasmatic nucleus (SCN) in the hypothalamus (Tast et al. 2001b). Altered melatonin secretion affects GnRH production which leads to the reduced LH secretion by the anterior pituitary and thereby reduced progesterone production in the ovary (Tast et al. 2001b). Decreased progesterone production leads to a reduction in embryo development and embryo secretion of oestradiol and signalling to the ovary (Ashworth et al. 2009). This results in a self-reinforcing cycle of low ovarian progesterone production causing a lowered oestradiol response of the embryo, which in turn hampers the support to the CL and reducing progesterone even further, eventually ending in embryo demise and pregnancy loss.

In summary, factors affecting litter size in the pig are multifactorial and likely involve interactions between the environment, systemic endocrine responses and the maternal immune system. Periods of conceptus loss are described in Figure 3 based on studies over the last three decades (Foxcroft et al.
2006). However, with the ever evolving ‘domestic sow’ selected for the number of piglets produced in a lifetime (Serenius et al. 2006) uterine capacity is becoming increasingly more important in limiting litter size (Foxcroft 2009). Devising and implementing strategies to address the influence of these factors have met limited success in reaching and sustaining litter sizes near the capacity of the modern domestic pig. Studies reported in this thesis also explore strategies to increase pregnancy success and litter size in pigs with specific emphasis given to the role of the maternal immune response in early pregnancy.
Figure 1.3 A time line representing the loss of viable conceptuses over the course of gestation in the pig.

In this example, an ovulation rate of 25 oocytes with 100% fertilisation is likely to produce a total born (live born plus stillborns and mummies) of 12 piglets after attrition due to embryo and fetal mortality. The shaded bar indicates the period of conceptus loss due to uterine capacity which peaks between day 30 to day 40 of gestation (modified from Foxcroft, 2006).
1.3.5 Early embryo development in the pig

Porcine embryos arrive in the uterus approximately 2.5-3 days after conception, but it is not until approximately 14 days later that attachment to the uterine endometrium takes place (Perry & Rowlands 1962). Within this relatively long period of free existence in the oviductal and uterine luminal fluid, the embryos undergo cleavage, morulation, blastulation and hatching from the zona pelucida. Around day 11-12, there is rapid elongation of the blastocyst up to 1 meter in length (Geisert et al. 1982), and the early blastocyst takes up a thread-like appearance for a period of 8-10 days before contraction takes place and the embryos become spaced evenly throughout the uterine horns (Dzuik 1977). The pig shows epithelio-chorial placentation with the epithelium of the uterus remaining intact during the oestrus cycle and pregnancy. This type of placentation is commonly referred to as ‘attachment’ as opposed to ‘implantation’, as the trophoblast cells do not invade the maternal tissue after attachment to the epithelium as is seen in rodents, rabbits and primates (Carlson 1996).

In summary, there are many factors during pregnancy in the pig that can lead to embryo demise (Straw et al. 2006). If pregnancy is lost before embryo attachment or before day 35 of gestation, often the clinical manifestation is a return to oestrus or a delay in the return to oestrus for the sow (Figure 4). This is often not noticed in large production units and causes of sub fertility due to early pregnancy loss can go unnoticed. After day 35 of gestation, usually the whole pregnancy is not lost and the clinical manifestation of embryo or fetal loss is the present of mummified fetuses and still born piglets at farrowing (Figure 4) leading to smaller than optimal litter sizes or perhaps more low weight piglets with smaller than optimal growth performance before and after weaning (Foxcroft 2009).
Figure 1.4 Diagrammatical representation of pregnancy associated events determining the litter size in pigs.

Using the day of mating as day 0 of pregnancy the resultant loss of potential piglets during gestation with probable clinical manifestation during each period of pregnancy are given. Key: mating: first artificial insemination or day of natural mating; Attachment: period during which the elongating blastocyst is attaching to the uterine wall; Skeletal calcification: the period in which the fetal period begins; Farrowing: the end of pregnancy, birth of piglets. (Adapted from Straw et al., 2006).
1.4 SEMINAL PLASMA AND REPRODUCTION

In human seminal plasma there are over 100 protein and peptide components including cytokines, growth factors, hormones, antibodies and enzymes that have been identified (Fung et al. 2004). This provides a plethora of substances capable of influencing the maternal immune system after mating to provide ‘immunosuppression’ to protect sperm viability and at the same time ‘immunocompetence’ to prevent possible bacterial and viral contamination (Christopher-Hennings et al. 2008). The boar provides an excellent model for immune and reproductive studies for humans. Pigs share similar immunological pathways involving Th1 IFNγ-dominated and Th2 IL-4-dominated immune responses (Dawson et al. 2005). From a reproductive perspective, boars and men have a number of similarities with regard to seminal plasma. The ejaculate from both species consist of secretions from homologous accessory sex glands (seminal vesicles, prostate and bulbourethral gland) and the seminal vesicles contribute to the bulk volume and total protein content of the liquid portion of the ejaculate (Christopher-Hennings et al. 2008). Moreover, both species produce an alkaline ejaculate and produce a gelatinous coagulum produced from the interactions between prostatic and seminal vesicle secretions (Christopher-Hennings et al. 2008).

1.4.1 Responses to mating in the pig

Natural mating in pigs involves between 150 and 500 mls of semen being deposited directly into the uterus. Coitus initiates uterine contractions, which evenly distributes the semen within the tract and transports sperm to the isthmus region of the oviduct approximately 15 minutes after ejaculation has taken place. During the protracted period of coitus (approx 5-15 minutes) the ejaculate is released as a heterogenous fluid, fractions of which originate from the various accessory sex glands of the boar. The series of fractions of the ejaculate consist of watery and gel pre-sperm secretions, a sperm rich fraction, followed by a post sperm and gelatinous fraction (Hunter 1982). Such a sequence can be considered as one full wave of ejaculation and can last between 3-5 minutes. A second and third wave may follow. Viable spermatozoa first appear in the oviduct approximately 15-30 minutes after artificial insemination (AI) and remain in the lower part of the isthmus for about 6-24 hours in relatively constant numbers (First et al. 1968). The rapid transport of sperm and storage in the upper part of the reproductive tract has been proposed to be necessary to protect against immunological attack on sperm by the post-coital invasion of leukocytes into the uterine lumen. These leukocytes are not found to be present in the isthmus of the oviduct (Claus 1990). Thus the sperm seem to be relatively protected from immune attack once they have passed through the utero-tubal junction.

The ejaculate contains seminal plasma originating mostly from the seminal vesicles and to a lesser extent the prostate and the bulbourethral gland in the boar (Mann 1964). Originally seminal plasma
was thought to simply provide nutritive and transport support for the spermatozoa (Mann 1964). However, in more recent years the importance of seminal plasma and its components has been the impetus for research into the effects of seminal plasma on the porcine uterus (Engelhardt et al. 1996, Bischof et al. 1994) ovary (Waberski 1997) and the conceptus (Murray et al. 1983).

The gel fraction originates from the bulbourethral glands and the proposed function of the gel fraction is to act as a cervical plug to reduce the amount of semen lost as backflow (Mann 1964). This function has been verified by laparotomy, demonstrating that in uterine horns fully distended with semen by the time natural mating has finished, up to a third of the ejaculate is lost due to backflow through the cervix (Hunter 1982). The remainder of the ejaculate is largely absorbed by the uterine mucosa by about 2 hours after insemination (Hunter 1982). This absorption by the mucosa may be implicated in initiating a series of events that initially prepare the uterus for subsequent implantation but may also activate mechanisms for maintenance of immune tolerance of conceptus antigens derived from the male (Robertson 2005).

1.4.2 Seminal oestrogens effect on fertilisation rates in the pig

One function of seminal plasma is to assist sperm motility and transport of sperm along the tract by increasing uterine contractions. In a number of species seminal prostaglandins including prostaglandin F2α (PGF2α) appear to play a role in stimulating uterine contractions at the time of mating (Hunter 1973). Although seminal plasma has been shown to increase uterine contractions in pigs, the low concentration of PGF2α in boar semen suggests that it is unlikely to have a major physiological effect in the female genital tract. For example in humans and rabbits the seminal prostaglandins PGF2α and PGE reach total amounts of several micrograms (Mann 1964), but the average concentrations in boar semen is less than 0.5 ng/ml for both of these prostaglandins (Hunter 1973). Also, oxytocin concentrations in boar semen are reported to be below detectable levels (less than 0.5 pg/ml). Thus, even taking into account the high volume of boar ejaculates, these hormones are unlikely to play a physiological role in the female genital tract.

Oestrogens are found in high concentrations in boar semen with amounts for total oestrogen per ejaculate of 11.5 µg. This high level is likely to exert a physiological response and Claus (1990) has shown that intrauterine administration of 10 µg of oestrogen (oestrone, oestradiol or oestrone sulphate: to simulate seminal oestrogens) leads to a rapid rise of the PGF2α concentrations in the uterine vein within 1 minute after infusion. This rise in PGF2α was also observed to be coincident with uterine contractions in the direction of the oviduct (Claus 1989). Thus, seminal oestrogens, via PGF2α, have the potential to improve fertilisation rates by promoting sperm transport along the uterine tract.
Mechanical stimulation of the cervix may also have a role in promoting sperm transport along the tract, as transcervical infusion of saline has also been shown to also increase uterine contraction frequency, although to a lesser extent than instillation of oestrogen (Claus 1990).

1.4.3 Immunomodulatory proteins in seminal plasma

1.4.3.1 Pig seminal proteins

In addition to oestrogens, boar semen contains many proteins that may play a role in modulating the maternal immune response after mating. Both high and low molecular weight proteins in boar semen have been found to have immunomodulating qualities. Major protein components of boar seminal plasma are the pig seminal proteins PSP-I and PSP-II. They exist in a soluble form in seminal plasma as heterodimers. Their potential involvement with immune function was suggested when it was demonstrated that PSP-I binds to IgA and IgG antibodies (Kwok et al. 1993). Leshin et al. (1998) demonstrated that PSP-I and PSP-II (and their heterodimer) enhance mitogen-induced pig lymphocyte proliferation in vitro. In response to pokeweed mitogen (PWM), a stimulator of B and T lymphoblasts, PSP-I and PSP-II enhanced pig lymphocyte proliferation. The use of the more specific T-cell stimulators phytohaemagglutinin (PHA) or concanavalin A (Con A) resulted in only a weak response or no response at all. Therefore, it is likely that these pig seminal proteins are primarily B lymphocyte stimulators. The in vivo effect of PSP remains to be determined but it is reasonable to suggest that these proteins may enhance the proliferation of B lymphocytes and, thereby, promote the production of antibodies and cytokines necessary to defend the tract from infections.

1.4.3.2 TGFβ in seminal plasma

The presence of cytokines in seminal plasma is also likely to have an immune modulating effect on the female reproductive tract, and has been implicated in skewing the maternal immune response after mating away from a type 1 response (Robertson 2000). The inflammatory response generated after mating has been shown to involve changes in uterine leukocyte populations in many species including mice, pigs, rabbits and humans (Hunt et al. 1996, Bischof et al. 1994, Pandya et al. 1985, Phillips et al. 1977). In mice, where the characterisation of the inflammatory response is most clearly characterised, it has been shown that an active constituent in seminal plasma is TGFβ (Tremellen et al. 1998). High concentrations of the two isoforms TGFβ1 and TGFβ2 have been found in human semen (Srivastava et al. 1996) in both active and latent forms.

TGFβ proteins have pleiotrophic biological activities which can be broadly characterised in three areas: (1) they are inhibitory to the growth of most cells and virtually all immune haemopoietic functions (Roberts et al. 1990), (2) they are immunosuppressive (Letterio et al. 1997), partly from their anti-
proliferative properties and capacity to induce Treg cells (Fu et al. 2004), and (3) they stimulate connective tissue growth and enhance the deposition of extracellular matrix (Lawrence 1996). Female mice, mated to males from which seminal vesicles are surgically excised, have a reduction in uterine epithelial cell production of granulocyte-macrophage colony-stimulating factor (GM-CSF) and reduced infiltration of inflammatory leukocytes (Treme llen et al. 1998). This reduction was likely due to the absence of TGF\(\beta\) in seminal vesicle fluid because instillation of recombinant TGF\(\beta\) protein replicated the physiological inflammatory response (i.e. infiltration of leukocytes into the uterus) and increases in luminal fluid content of GM-CSF similar to that seen on day 1 of pregnancy. This finding was supported by in vitro studies where addition of recombinant TGF\(\beta_1\) stimulates the production of GM-CSF by uterine epithelial cells in vitro, and the GM-CSF stimulating activity of seminal vesicle fluid was neutralised by antibodies specific for TGF\(\beta\) (Robertson et al. 1997). Thus cytokines in seminal plasma, specifically TGF\(\beta\), may initiate the cellular and molecular events that are characteristic of the classical inflammatory response seen in the female reproductive tract after mating. A working model for the induction of the post mating inflammatory response initiated by seminal plasma and with TGF\(\beta\) as the proposed active constituent is shown (Figure 5). Also this model suggests that there may be indirect effects on the ovary and embryo, which will be discussed in detail in chapter 4.

A study by Koshaka et al. (2000), using a similar approach to that used by Robertson et al. (1997) in mice, cultured uterine epithelial cells from the pig and reported comparable effects. GM-CSF expression was shown to be produced in significant quantities (121.4 ± 21.8 pg/ml) in response to boar seminal plasma (Kohsaka et al. 2000). The precise role of seminal TGF\(\beta\) in pigs is yet to be determined. However, as the deposition of semen in pigs and mice is directly into the uterus, it is likely that a similar mechanism is operating in the pig and TGF\(\beta\) in seminal plasma is responsible for initiating a maternal immune response after mating.

1.4.4 Immunological priming of the uterus for pregnancy

The postulate that immunological stimuli may influence reproductive performance in the pig has generated a great deal of interest. Several groups have reported that litter size in pigs can be increased by enhancing the immunological response to mating by infusion of either non-viable (dead) semen (specific paternal antigenic stimulation) at oestrus before mating (Stone et al. 1987, Murray et al. 1986, Murray et al. 1983) or peripheral blood leukocytes (non-specific paternal antigenic stimulation) at the time of insemination (Almlid 1981). However others have been unable to reproduce these effects (Van der Lende et al. 1986, Giles et al. 1990). To some extent these contradictory results may be due to differences in experimental design. For instance the age of gilts varied between studies. Giles et al.
(1990) mated gilts at their third oestrus, which may have masked any benefits of increased litter size due seminal plasma because of the increased rate of ovulation expected for gilts in their third cycle [Foxcroft, 1982 #285]. Also the volume and therefore perhaps the antigenicity of the treatments varied between the studies; Murray and Grifo (1983) used a larger dose of inseminate than that used by Giles et al. (1990). Furthermore, litter sizes were also determined at different time points, ranging at from day 8-10 of pregnancy (ie before the critical attachment period) to 2 weeks post partum. Of the two studies that showed an effect of enhancement of the immune response on litter size, it is not clear if the two approaches (ie. specific vs non specific paternal antigens) acted through the same mechanism.

Studies in mice have highlighted the role of components of seminal plasma in generating a favourable type-2 immune response in the mother and in the cellular remodelling of the uterine tract in preparation for the implanting embryo (Robertson et al. 1997). More recently, components of seminal plasma have been found to induce maternal tolerance to the semi-allogeneic embryo by inducing the expansion of Treg cells before implantation of the embryo takes place (Robertson et al. 2009). Factors in seminal plasma as opposed to sperm themselves generate the expansion and activation of Treg cells with spermatozoa predominantly providing the paternal antigens for presentation (Robertson et al. 2009). Studies also from our laboratory have highlighted the role of maternal dendritic cells in the draining lymph nodes of the uterus presenting male antigens and initiating T cell activation after mating (Moldenhauer et al. 2009).

Similarly in the pig, insemination induces the recruitment and activation of immune cells into the uterus and endometrium (Bischof et al. 1994, Engelhardt et al. 1996). This semen-induced inflammatory response diminishes as pregnancy ensues and by the time embryos are attaching to the endometrium when maternal exposure of paternal antigens present on the embryo occurs, it is possible that tolerance to paternal antigens due to semen-induced induction and expansion of Treg cells has already occurred. In addition to paternal antigens present in semen (Moldenhauer et al. 2009), cytokines including TGFβ present in boar seminal plasma (Robertson et al. 2002) are also likely to drive this tolerogenic response mediated by Treg cells.

In humans, studies have reported improvements in implantation rates after insemination in in-vitro fertilisation (IVF) (Bellinge et al. 1986) and gamete intrafallopian transfer (GIFT) following prior uterine exposure to seminal antigens via sexual intercourse (Marconi et al. 1989). Whether uterine priming in gilts with dead semen or seminal plasma represents a viable option for increasing embryo survival still remains to be elucidated.
1.5 EFFECT OF SEMINAL PLASMA ON OVULATION AND EMBRYOGENESIS

1.5.1 Oestrous cycle in the pig

The oestrous cycle in sexually mature female pigs lasts for a period of 18 to 21 days continually throughout the year with seasonal variation contributing to a slightly longer oestrus in the summer period (see section 1.3). Behavioural oestrus in the sow is manifested by the standing response which is usually initiated by odour and the courting grunts of the boar (Signoret 1972). This behaviour can last between 24-72 hours and the time of ovulation is variable and can occur from 10 hours to 60 hours after onset of oestrus (Almeida et al. 2000a, Soede et al. 1995, Weitze et al. 1994). The duration of ovulation is usually between 3-4 hours and ovulations occur about 4 hours earlier in mated than in unmated sows (Anderson 1993). Corpora lutea (CL) are well formed by day 4 or 5 of the oestrous cycle and progesterone secretion increases from that time to maximum production between days 12 and 14 of the oestrous cycle. Luteal regression begins on about day 15 in non-pregnant females and plasma progesterone concentrations decline rapidly to basal levels (1 ng/ml or less) by days 17 to 18 (Foxcroft et al. 1982), leading to recurrent oestrous cycles unless interrupted by pregnancy or events leading to endocrine dysfunction. There is an interdependence between the uterine endometrium and the CL in the pig: the secretory function of the uterine endometrium is dependent on a functional CL and the lifespan of the CL is in turn, dependent on release of the uterine luteolytic factor PGF2α (Claus 1990).

Release of PGF2α from the non-pregnant uterine endometrium results in morphological regression of the CL, progesterone secretion ceases and, therefore, progesterone dependent endometrial secretory activity is terminated in the late luteal phase of the oestrous cycle. In the pregnant pig, endometrial blastocyst interactions begin to occur by day 11 of pregnancy. Blastocysts produce oestrogen, which results in CL maintenance and continued endometrial development and secretory activity.

Follicles also respond to the preovulatory LH surge with an increased prostaglandin synthesis. These prostaglandins stimulate the activity of enzymes, collagenase and elastase which are involved in follicular rupture (Evans et al. 1983). A parallel preovulatory rise in PGF2α has been measured in the follicular fluid (Ainsworth et al. 1975, Armstrong 1981, Evans et al. 1983) suggesting that uterine-derived PGF2α may reach the ovary by counter current exchange to induce luteolysis.

1.5.2 Seminal plasma effects on ovulation

The rich source of oestrogen in boar semen could provide an explanation for the variable oestrous related events seen in mated as compared to unmated sows. Studies designed to show that PGF2α may influence the onset and/or the duration of ovulation include the addition of PGF2α and its analogues to the seminal dose used for AI (Flowers et al. 1993, Niwa et al. 1982). Although in these
studies there were increases in farrowing rate and litter sizes in sows, the result did not reach statistical
significance (reviewed by Flowers and Ebenshade, 1993). However, a more recent study showed that
intravulval injection of PGF$_{2\alpha}$ at the time of AI can significantly increase litter size and fertility (Pena et
al. 1998), with the authors suggesting that the increase in fertility was possibly attained due to reducing
the time between insemination and ovulation.

Intrauterine administration of the 1-10kDa fraction of seminal plasma in gilts has been shown to effect
ovarian function. Waberski et al. (1995) using the Mariensee model (Jungblut et al. 1991) showed that
seminal plasma infusion in one side of the uterine horn advanced ovulation in the ipsilateral ovary by
10.7 hours compared to the contralateral ovary. It was interesting to note that in the same study PGF$_{2\alpha}$
in PBS had no effect on advancing ovulation and oestradiol in PBS shortened the time interval between
the ovaries to only 3.3 hours (Waberski et al. 1995). This study supports a role for a non steroidal
component of seminal plasma advancing ipsilateral ovulation, perhaps diminishing the mechanism of
seminal plasma put forward by Claus (1990) since the unilateral detection of ovulation time by
transcutaneous sonography was clearly established.

Effects of seminal plasma on ovarian function have been extensively demonstrated in pigs. The
introduction of transrectal and transcutaneous ultrasonography made it possible to relate the timing of
oestrus to ovulation and hormonal changes around ovulation in conscious sows (Soede et al. 1995,
findings of Waberski et al. described above, a Dutch study using Meishan and Landrace boar seminal
plasma before mating in sows, failed to show any advancement of ovulation due to seminal plasma
(Soede et al. 1998). In contrast to Waberski et al., oestrus was synchronised in these sows with hCG
and timing of ovulation was determined using trans-rectal ultrasonography. Although it is difficult to
directly compare these studies, the failure of seminal plasma to advance ovulation may be due to a lack
of ovulation-inducing moieties in the boars seminal plasma or the different genetic background of the
boars and sows used between these studies.
Figure 1.5 Schematic diagram illustrating the proposed role of TGFβ in seminal plasma during early pregnancy in the pig.

Based primarily on data from studies with mice, at mating TGFβ in seminal plasma binds to receptors on uterine epithelial cells to induce expression of proinflammatory cytokines including GM-CSF, IL-6 and chemokines (Tremellen, 1998). This causes the recruitment and activation of immune cells within the endometrial stroma leading to uterine tissue remodelling and establishing the cytokine and growth factor profile for preparedness of the uterine environment for pregnancy. Studies in pigs have highlighted the role of seminal plasma on ovulation and corpora lutea function (Waberski, 1995). In this model, active factors in seminal plasma initiate changes in uterine function, maternal immune response to mating and ovarian function. These seminal plasma-directed events establish a cytokine and growth factor milieu to facilitate optimal embryo growth and development.
1.6 BREEDING PRACTICES IN THE PIG INDUSTRY

1.6.1 Artificial insemination

The pig industry in Australia relies heavily on artificial insemination (AI) technology with most farms utilising AI mating in more than 75% of matings as part of their breeding strategy (Meo et al. 1999). The reason for developing and implementing AI programs are numerous but relate mainly to improving the profitability of the commercial pig herd. The benefits of AI to the pig industry include: (1) genetic improvement of livestock, providing a wider variety of new genes for a herd; (2) reducing the risk of introducing disease to a herd by the introduction of new boars; (3) reduction of service costs, as AI centres are quite often able to offer a service at a lower cost than for natural service, especially when housing and maintenance of a large number of boars is considered and (4) adoption of batch farrowing procedures, where use of AI can prevent overuse of boars and can facilitate breeding programs where oestrous synchronisation is utilised (Reed 1982).

AI involves the dilution of the sperm rich fraction of semen (Reed 1982). The sperm rich fraction is collected and diluted in a diluent (usually containing egg albumin, glucose and antibiotics) to a sperm concentration of $5 \times 10^8$ ml (Reed 1982). An insemination dose of 100 mls was recommended (containing $5 \times 10^9$ spermatozoa/inseminate) for each service to achieve reasonable fertility under field conditions (Reed 1982). In the last 2 decades there has been considerable advances made with regard to semen diluents and AI technology (Singleton 2001). More recently, insemination doses of 100 ml can contain between $2 \times 10^9$ to $3 \times 10^9$ spermatozoa per inseminate and may contain semen from more than one boar (Singleton 2001). An average sperm rich fraction from a mature, fertile boar will provide more than 10 doses which are usually sent from AI centres to farms and stored between 18-21°C until required for up to a period of 5 days.

1.6.2 AI vs natural mating

Reports on fertility rates using AI compared to natural mating vary and are difficult to analyse due to variations in breeding practices including, the inseminator’s experience, the method of oestrous detection and the number of inseminations used. However, it is generally accepted that natural mating compared to AI in the pig leads to higher conception rates and increased litter sizes (Claus 1990, Skjervold 1975). In a retrospective analysis of breeding records from a large commercial pig facility in Queensland Australia, natural mating compared with AI in over 51,000 observations resulted in an
average increase in total born piglets per litter by 0.43 (11.53 ± 0.02 vs 11.96 ± 0.03; p < 0.0001; O'Leary and Robertson, unpublished data). The increase litter size due to natural mating was still evident after correcting for parity and season. The volume of inseminate and sperm density used cannot explain this difference (Baker et al. 1968) but regulating stimuli provided by the boar and dilution of key constituents of seminal plasma are likely to be contributing factors. Thus, identification of active constituents in seminal plasma responsible for reducing embryo loss due to immune modulating effects and enhancing ovarian function may improve reproductive outcome in AI breeding programs. There is a lack of scientific studies in the literature investigating the differences in herd fertility, including farrowing rates and litter sizes, using AI compared with natural mating. The reason for this is multifactorial but is mainly due to commercial factors. The number of sows required to formally define the differences in fertility between the two breeding regimens is estimated to be in excess of 300 per group to show a statistical difference of 10% in litter size and would also require a large number of fertile boars to be kept.

### 1.7 SUMMARY AND THESIS AIMS

The precise mechanism responsible for the paradoxical success of the semi-allogeneic fetus in mammalian pregnancy is presently unknown. Studies described in this review from several species suggest that seminal plasma not only provides a local response in the uterus but might also have implications for ovarian function and embryo development. The response to seminal plasma is thought to facilitate pregnancy progression through the pre-attachment period and also may be involved in initiating longer term immune protective mechanisms ensuring that adverse Th1 responses are inhibited throughout gestation.

In mice, seminal plasma cytokines appear to have a major role in regulating the post mating inflammatory response that is associated with the activation of epithelial cells to increase cytokine production. One active constituent in seminal plasma in mice has been shown to be TGFβ which initiates a series of cellular and molecular events leading to a tolerogenic immune response in the mother.

Similar studies investigating the mechanism of seminal cytokines and the active factors in the pig are generally lacking. While there are some studies suggesting a benefit of using seminal plasma or other antigenic treatments at mating to improve reproductive outcome, there have been few studies to investigate the local cellular and molecular consequence of seminal plasma action in the female reproductive tract of the pig.
1.7.1 Hypothesis

The hypothesis underpinning the studies described in this thesis is that seminal plasma regulates the induction of an inflammatory response in the uterine endometrium after mating in pigs. This post-mating inflammatory response (1) advances ovulation, (2) promotes embryo growth and development and (3) facilitates the induction of a ‘receptive’ uterine environment. Thus, seminal plasma is potentially a primary determinant of embryonic mortality, implantation and pregnancy success in the pig.

1.7.2 Aims

The studies reported in this thesis aim to increase our understanding of the role of seminal plasma cytokines in reproduction in pigs. The specific aims of this study were (1) to characterise the endometrial inflammatory response induced by seminal plasma; (2) to investigate the effect of seminal plasma on embryonic viability and its role in synchronisation of embryo development; (3) to characterise the effect of seminal plasma on the ovary and the temporal relationship between ovulation and fertilisation, and (4) to determine whether administration of seminal plasma to gilts at AI can improve reproductive outcomes.
1.8 PUBLICATION: THE INFLUENCE OF SEMINAL PLASMA ON OVARIAN FUNCTION IN PIGS – A NOVEL INFLAMMATORY MECHANISM?

NOTE: This publication is included in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://dx.doi.org/10.1016/S0165-0378(02)00042-6
Chapter 2

Materials and Methods
2.1 ANIMAL HUSBANDRY

The small scale animal experiments described in this thesis were conducted at The University of Adelaide, Pig and Poultry Production Institute (PPPI) at Roseworthy, S.A. The field trial involving 282 gilts and 19 boars was conducted at a commercial piggery, Bunge Meat Industries (BMI) PTY. LTD. at Corowa, NSW, Australia. All treatments, housing and retrieval of tissues were approved by The University of Adelaide Animal Ethics Committee and when appropriate the Animal Ethics Committee at BMI. Semen and seminal plasma used for this study were provided by SABOR Pty. Ltd. (Clare, Australia) and from BMI in Corowa.

All experimental animals were under the supervised care of veterinarians associated with the piggery and decisions to exclude animals from any experiments were made in consultation with them. All housing, feeding, detection of oestrus and AI procedures were carried out as per Australian pig industry and farm standards. Deviations from standard procedures are acknowledged in the thesis where appropriate. Animals in which the reproductive tracts were retrieved were killed at commercial abattoirs; Port Wakefield Abattoir, Port Wakefield, S.A., or Marks Meats Abattoir in Balaklava, S.A. by electrocution and by captive bolt respectively.

2.2 EXPERIMENTAL DESIGN: ACUTE EFFECTS OF SEMINAL PLASMA

The design of experiments to investigate acute uterine and ovarian responses to intrauterine seminal plasma treatment is described diagrammatically in figure 2.3. A cohort of 33 F1 (Landrace x Large White) pre-pubertal gilts were used in these experiments. Age of gilts at selection was 22 weeks (mean ± SEM = 157 ± 0.25 days) sourced from the breeding herd at PPPI piggery, Roseworthy, S.A. where monthly batch farrowing is practiced as part of standard farm procedures. The weight of the selected animals was between 80.0 and 111.0 kg (mean ± SEM = 92.5 ± 1.47).

Gilts were randomly allocated into four groups employing three oestrous synchronisation strategies and retrieval of reproductive tissue at three time points post intrauterine infusion of PBS or seminal plasma. To minimise stress, the gilts were housed in pens in groups of eight and housed together for the period of the experiment.

First oestrus was induced and synchronised at 24 weeks of age in groups I, II and III by intramuscular injection of 750 iu of equine chorionic gonadotrophin (eCG) (Folligon™, Intervet, Holland) followed 72 h later by 500 iu of human chorionic gonadotrophin (hCG) (Chorulon™, Intervet). Group II gilts were given two intramuscular injections of 0.5mg of prostaglandin F2α (Estrumate™, Intervet, Holland) on day 16 and 17 post hCG, in order to evaluate whether this protocol would influence ovulation and provide tighter synchronisation for entry into second oestrus. Group IV gilts did not receive any
exogenous treatment to induce or synchronise oestrus, however it was the practice at the piggery to provide twice daily boar stimulation to induce oestrus in gilts and groups II, III and IV all received equal amounts of boar stimulation. Gilts were then randomly assigned to a 2 x 3 factorial arrangement in which they received a 100 ml intrauterine infusion by transcervical catheter of seminal plasma or filter sterilised PBS. Treatments were delivered 1-2 h after hCG and reproductive tracts were collected from n = 4-6 gilts at each of three time points for each treatment; at 34 h (‘pre-ovulatory’) in group I, and on day 5 and day 9 after treatment with seminal plasma or PBS (after onset of second estrous, groups II, III, and IV). Gilts in the day 5 and day 9 time points received two standard artificial inseminations of 3 x 10^9 spermatozoa per dose each; the first given 2 h after intrauterine infusion and the second the following afternoon. Alternatively, if treatment and first AI were given in the afternoon, the second dose was administered the following morning. A 2 hour interval was chosen between infusion of seminal plasma or PBS and the first AI since fluid after natural mating is reported to be lost from the uterus within 2 h (Lovell et al. 1968). This was to ensure that there would be no further dilution of the AI dose due to treatment.

Four gilts were excluded from experimental analysis, one from each of the 34 h and day 5 time points and two from the day 9 time point, all in the PBS infused control groups, due to endometritis with cystic ovaries (34 h post treatment), uterine infection indicated by increased volume and acidic luminal fluid (day 5), failure to conceive and lameness (one each at day 9).

2.3 SEMINAL PLASMA AND PBS TREATMENT

The full ejaculate minus the gel fraction was collected from boars of known fertility by the glove-in-hand method used as a standard collection technique at SABOR and BMI piggery. Collections took place following at least 5 days abstinence from a previous collection from as many boars required to yield the volume of seminal plasma needed for each experiment. For small experiments the number of boars required was usually 3 to 5 at SABOR and for the field trial (section 2.19) the ejaculate from 8 boars was routinely collected until the required volume of seminal plasma required was reached. The semen was pooled, placed on ice and seminal plasma was collected after centrifugation at 1200 g at 4°C for 20 min. Microscopic evaluation (100X magnification) of the supernatant was performed to ensure that the cellular content of semen was removed. Aliquots of seminal plasma were placed in 100 ml artificial insemination bottles and stored at -20°C until required.

Semen for artificial insemination was prepared, stored and administered according to standard protocols at SABOR Pty. Ltd., PPPI and BMI piggery. Briefly, the sperm-rich component of the ejaculate was collected and diluted to produce 80 ml doses of extended semen containing 3 x 10^9
spermatozoa. Semen was extended in Androhep™ (Minitub, Tiefenbach, Germany) containing BSA and neomycin sulphate (0.1% w/v). Disposable plastic insemination catheters (spirettes) (Minitub, Tiefenbach, Germany) were used for AI and for intra uterine infusions of seminal plasma and PBS. Aliquots (approximately 5 ml) of semen collected for AI at BMI piggery for the field trial study (section 2.19) were removed before extension with Androhep™ and centrifuged for 20 minutes at 1200g to recover seminal plasma for storage at -20°C prior to later analysis for cytokine content. The semen used for artificial insemination and the seminal plasma for intrauterine infusions were not necessarily from the same boar.

2.4 BLOOD SAMPLES

Blood samples were taken for steroid analysis immediately following slaughter and at various time points throughout the course of experiments. Blood was collected in heparinised tubes and placed on ice before transport to the laboratory where plasma was collected after centrifugation at 200g for 20 minutes. Plasma was then stored at -20°C until analysis.

2.5 EAR VEIN CATHETERISATION

In order to evaluate the effect of uterine exposure of seminal plasma on ovarian function, serial blood samples were taken via ear vein catheters. Gilts from the day 9 time point group were immobilised by a soft cotton rope looped around the upper jaw (lasso) and tied to the front side rail of a stall. The ear was disinfected with hibitane solution and one of the auricular veins was occluded by digital pressure. 14 guage catheter placement units (Optiva, CA, USA code # 1014, 14 guage x 45mm) were inserted into the vein and approximately 100 cm polyvinyl catheter (Chritchley Electrical Products, NSW, Australia, code # 111070; 1.5 mm OD, 1.0 mm ID) was introduced into the ear vein till a depth of approximately 50 cm. After testing for patency with a syringe, the catheter was filled with sterile saline containing heparin (100 iu/ml) and plugged with an 18 guage blunt needle and cap (Becton Dickinson, Singapore). The catheter was held in place by adhesive bandage (Elastoplast™, Smith and Nephew, London, UK) wrapped around the entire ear, enclosing the catheter in a loop so that the catheter emerged proximally. Adhesive bandage was wrapped around the neck of the gilt, enclosing a pouch on the dorsal side of the neck and the ear containing the catheter on the lateral surface. The plugged end of the catheter was placed completely in a denim pouch between blood collections so that no part of the catheter was exposed. Diluted Hibitane 1:10 v/v in water was used to sterilise catheters before use. Catheters were kept patent by daily flushing with approximately 5 ml of 100 iu/ml heparin solution in sterile saline (0.9% w/v Baxter Healthcare, QLD, Australia code # AHF7123). This flush was repeated before and after each blood collection and the first 2 ml of blood withdrawn was discarded to avoid dilution of the sample with the flush.
Figure 2.1 Photograph of blood sampling via an ear vein catheter illustrating the bandage around the gilt’s neck.

This procedure allowed for multiple blood sampling without observable discomfort for the pig. The catheter was kept clean using a demin pouch incorporated into the bandage on top of the pig.
2.6 REPRODUCTIVE TRACT COLLECTION AND PROCESSING

Gilts were transported to a commercial abattoir (section 2.1.1) the night before scheduled slaughter. As soon as possible after slaughter (10 to 20 min) the reproductive tracts were retrieved, the uterine horns were trimmed of mesentery and weighed. In the 34 h post treatment group, luminal fluid was drained from the tract, collected in 50 ml Falcon tubes, the volume was recorded and tubes were placed on ice prior to processing for cell counts. In the day 5 and day 9 group the uterine tract was ligated at the cervix and 20 ml of filtered-sterilised 1x PBS was flushed through the tract in order to collect embryos from each uterine horn. Gross measures of tissue inflammation were recorded including (a) volume of uterine luminal fluid (ml), (b) uterine tissue mass (g), and (c) uterine vascular index (subjective score for ‘redness’, + → ++++).

For immunohistochemistry, full thickness biopsies of uterine tissue were collected at two sites, approximately 10 cm proximal to the oviduct and 10 cm distal to the cervix, and placed in Optimal Cutting Temperature Compound (OCT Compound, Tissue-Tek®, Sakura Finetek USA, Inc., Torrance, CA), immersion-frozen in liquid nitrogen-cooled isopentane and stored at -70°C until further processing. Approximately 200 mg of endometrial tissue was collected at the same sites for quantitative RT-PCR analysis, snap frozen in liquid nitrogen and stored at -70°C.

Ovaries were weighed and approximately 100 mg of ovarian tissue (follicle in group I gilts, CL tissue in group II, III and IV) was collected for quantitative RT-PCR analysis, snap frozen in liquid nitrogen and stored at -70°C. Representative ovarian tissue was also collected for immunohistochemistry as described above. Ovaries were then placed in media HEPES-buffered tissue culture media 199 (HTCM) and transported to the laboratory on ice. Follicles and CLs were dissected and recovery of follicular fluid and isolation of ovarian cells is described in more detail in section 2.12.

2.7 EMBRYO RETRIEVAL AND STAINING

Embryos were retrieved at autopsy on day 5 and day 9 post treatment, for assessment of cell number (day 5) or diameter (day 9), by three consecutive 20 ml flushes of DMEM (Dulbecco’s modified Eagle medium) into each uterine horn.

Blastocysts collected on day 5 were washed twice in culture media and the zona pellucida was removed by incubation at room temperature for 5 min in Acid Tyrodes solution (Sigma) followed by incubation at 37°C with 0.5% pronase solution (Sigma) for approximately 3 minutes. After washing in culture media the blastocysts were stained using 0.1 mM Hoechst 33342 (bisbenzamide, Sigma) for 15
min and then prepared for microscopic evaluation by fixing under a cover slip in 10% glycerol. The number of blastomeres were counted under 100x magnification using UV illumination with an Olympus BH-2 microscope and the mean number of blastomeres per cohort of embryos for individual gilts was calculated.

Day 9 embryos were washed in PBS, fixed in 1% paraformaldehyde solution and evaluated directly under light microscopy (Olympus BH-2) using a 2.5x video lens and 4x objective lens. Using Video Pro software, the horizontal and vertical axis of the approximately spherical embryos were measured and the mean blastocyst diameter per cohort of embryos for individual gilts was calculated.

2.8 IMMUNOHISTOCHEMISTRY

Immunohistochemical analysis was performed on OCT-embedded uterine tissues collected from gilts slaughtered at 34 h, day 5 and day 9 after seminal plasma or PBS treatment. Tissue was serially cut into 7 µm thick cryostat sections, placed on poly-L-lysine-coated slides (PolysineTM Microslides, Menzel-Glaser, Germany) and allowed to air-dry. Dried sections were fixed in 96% ethanol for 10 min at 4°C then washed 3 times in 1 x PBS. Sections were blocked with 1% bovine serum albumin (BSA) in PBS for 2 min and then incubated with primary antibody diluted to 10 µg ml⁻¹ in 10% normal pig serum in PBS (PBS-NPS) for 2 h at 4°C. Murine monoclonal antibodies reactive with porcine MHC class II (HB142), CD45 (MCA1222) and macrophage antigen (MSA3) were purchased from Serotec (Oxford, UK). Sections were washed in PBS and incubated with biotinylated rabbit anti mouse Ig (Dakopatts, Glostrup, Denmark) in PBS-NPS for 60 minutes at room temperature. Sections were then washed in PBS and incubated in horse radish peroxidase-conjugated strepavidin (Dakopatts, Glostrup, Denmark) in PBS-NPS (30 min, room temperature). Bound antibody was visualised by incubating slides with 0.05% diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) in Tris buffered saline containing 0.1% hydrogen peroxide (10 min, room temperature). Slides were counterstained with haematoxylin, dehydrated, cleared in Safsolvent and mounted in DPX.

The number of positively stained leukocytes were quantified in ten randomly selected fields from each of two sections collected from the proximal and distal uterine horn of each gilt. Video image analysis was employed using Video Pro software (Leading Edge Software, Adelaide, Australia) with a 10x objective and 3.3x photo eyepiece as previously described (Robertson et al. 1996b). Data are expressed as ‘percent positivity’, calculated as the mean area of DAB stain as percentage of the area of total stain in the ten fields (average of proximal and distal sections). Leukocytes were quantified separately for three distinct areas of uterine tissue, including the superficial endometrium (immediately subadjacent to the luminal epithelium), endometrial stroma (at least 500 µm from the uterine
epithelium), and the myometrium. Repeated measurements of a single test field validated the precision of this method (less than 10% within assay variation).

2.9 REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

cDNA was prepared from endometrial tissue collected from gilts slaughtered at 34 h, day 5 and day 9 after seminal plasma or PBS treatment. Total cellular RNA was extracted using RNA Bee solution (Tel-Test, Friendswood, Texas) and following treatment with RNase-free DNase I (500 IU ml⁻¹; 60 min at 37°C) (Roche, Basel, Switzerland), then first strand cDNA was reverse transcribed from 1 µg RNA employing an Expand Reverse Transcriptase kit (10 min at 30°C then 45 min at 42°C) (Roche). The cDNA solution was diluted to 100 µl and stored at -20°C. Primer pairs specific for published Genbank cytokine cDNA sequences were designed using Primer Designer software (Scientific and Educational Software, State Line, PA) or Primer Express software (Applied Biosystems, Foster City, CA).

The PCR amplification employed reagents supplied in a 2x SYBR Green PCR master mix (Applied Biosystems), and each reaction volume (20 µl total) contained 0.5-1.0 µM 5' and 3' primer and 3 µl of cDNA. The negative control included in each reaction consisted of H2O substituted for cDNA. PCR amplification was performed in an ABI Prism 5700 Sequence Detection System (Applied Biosystems) according to the manufacturer’s specifications to allow amplicon quantification. PCR primers and optimised PCR reaction conditions for each primer pair are listed in Table 2.1. Reaction products were analysed by dissociation curve profile. Validation experiments were initially performed to examine the efficiencies of each primer pair in the PCR. Cycle threshold values (Ct), defined as the cycle number at which the detected fluorescence exceeds the threshold value, were determined for serial 8-fold dilutions of cDNA in a range of 0.06-30 ng total RNA. For each primer pair the linearity of detection was confirmed to have a correlation coefficient of > 0.94 over the detection range, when plotted as Ct versus log of cDNA dilution. Specificity of the PCR was confirmed by detection of a single distinct peak on examination of the dissociation curve profile of the reaction product. In addition, reaction products were analysed by electrophoresis in 2% agarose gel containing 0.5 µg ml⁻¹ ethidium bromide and visualised over an ultra-violet light box (figure 2.1).

mRNA expression data from experimental tissues was normalised to β-actin mRNA expression and given as percent of the mean value for the PBS-treated 34 h group using the arithmetic equation \(2^{-\Delta Ct} \times 100K^{-1}\) (Applied Biosystems User Bulletin #2) where K is the normalising constant. A single ‘Relative mRNA Expression’ value for each cytokine mRNA for each individual gilt was calculated as the mean of duplicate cDNA samples from proximal and distal endometrial tissue.
<table>
<thead>
<tr>
<th>Primer</th>
<th>PCR conditions</th>
<th>5'/3' bp position</th>
<th>Genbank #</th>
<th>Size bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>95˚-15s, 60˚-1m</td>
<td>80-99/474-455</td>
<td>U07786</td>
<td>395</td>
</tr>
<tr>
<td>IL-1α</td>
<td>95˚-20s, 59˚-20s, 72˚-1m</td>
<td>493-510/711-692</td>
<td>X52731</td>
<td>219</td>
</tr>
<tr>
<td>IL-1β</td>
<td>95˚-15s, 57.5˚-15s, 72˚-1m</td>
<td>225-242/473-456</td>
<td>M86725</td>
<td>249</td>
</tr>
<tr>
<td>IL-6</td>
<td>95˚-15s, 60˚-1m</td>
<td>598-618/698-679</td>
<td>M80258</td>
<td>101</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>95˚-20s, 62˚-20s, 72˚-1m</td>
<td>137-157/316-299</td>
<td>U67175</td>
<td>180</td>
</tr>
<tr>
<td>MCP-1</td>
<td>95˚-20s, 57.5˚-20s, 72˚-1m</td>
<td>93-111/279-261</td>
<td>X79416</td>
<td>187</td>
</tr>
<tr>
<td>TNFα</td>
<td>95˚-20s, 59˚-20s, 72˚-1m</td>
<td>441-459/661-644</td>
<td>X57321</td>
<td>221</td>
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<tr>
<td>COX-2</td>
<td>95˚-15s, 60˚-1m</td>
<td>951-971/1062-1043</td>
<td>AF207824</td>
<td>112</td>
</tr>
</tbody>
</table>

Table 2.1 PCR primer sequence, PCR product size and primer binding sites on Genebank sequence.

Figure 2.2 Agarose gel electrophoresis of RT-PCR amplification products using primer sets for β-actin, MCP-1, IL-6, TNFα, COX-2, IL-1β and GM-CSF mRNAs.
2.10 OVARIAN MEASUREMENTS

Section 2.2 describes the experimental strategy regarding synchronisation and treatment protocols. Following sections describe the methods for analysis of steroid hormone content of blood samples and the isolation and culture of ovarian cells and analysis of their responses in vitro.

Ovarian responses in vivo were assessed by gross measurements including CL number, individual CL weights and ovary weights. In addition, surface follicles in ovaries retrieved from gilts at the 34 h time point were counted, their diameters measured with callipers and recorded.

2.10.1 Ovarian steroid hormone assays

Ovarian steroid hormone content was assessed in plasma samples prepared from blood either taken through the course of experiments or immediately after the animals were sacrificed. Serial blood samples were collected by means of ear vein catheterisation (section 2.5) prior to expected ovulation and for the first 9 days post treatment. The progesterone content of plasma or cell culture supernatants were measured using Diagnostic Systems Laboratories, Inc. (DSL) radioimmunoassay kit (DSL, Texas, USA. Code # 3400) according to the manufacturer’s instructions.

2.10.2 Collection of follicular fluid

Pre-ovulatory size (>8 mm diameter) follicles (34 h time point) were aspirated to recover follicular fluid (FF) and oocyte-cumulus complexes (OCC), using dry 1 ml syringes with 20 gauge 1-inch needles. Pooled FF containing OCCs was delivered into 35 mm petri dishes for retrieval of OCCs. All OCCs were transferred to 1.7 ml Eppendorf tubes for fixation prior to assessment of meiotic status. After recovery of OCCs pooled FF from each gilt were transferred to 1.7 ml Eppendorf tubes, weighed to determine FF volume, and frozen without centrifugation for FF steroid analyses.
2.10.3 Isolation of ovarian cells

Eight follicles from each gilt were placed in 35 mm petri dishes in HTCM and each follicle was split in half. Mural granulosa cells (MGC) were scraped from the follicle wall, and cells were pooled from 8 follicles for processing for culture. After scraping of the follicle walls, the remaining thecal tissue pieces were cut into quarters, washed through two washes of HTCM and one of BTCM and pieces were distributed into wells of 4-well culture plates, with 4 x ¼ theca from each of 4 follicles per well, for in vitro treatments.

Pooled MGCs from 4-6 follicles from each ovary were rinsed as cell aggregates without dissociation, by centrifugation (500 rpm for 5 min) through 2 washes of HTCM and 1 of BTCM. The final HTCM pellet was resuspended in 4 ml for cell counting. Cell suspensions were then diluted with BTCM to provide the desired final cell density and 0.1 ml aliquots pipetted into duplicate or triplicate wells of 96-well tissue culture plates. An aliquot of each cell suspension was centrifuged and the pellet was weighed, providing a second independent method of granulosa cell quantification.

2.10.4 Granulosa cell culture

Aliquots of pooled granulosa cells from each ovary or each pair of ovaries per gilt were plated into wells of a 96-well culture plate and cultured for 24 h in BTCM containing various hormones and growth factors. Cultures were carried out in a humidified atmosphere of 5% CO₂ in air, at 39°C for 24 h. Cultures were pulse-labelled with ³H-thymidine during the last 6-8 h of culture. Granulosa cell densities ranged from 2 x 10⁵ to 1 x 10⁶ cells per well, or the equivalent of 0.05 to 0.25 follicle equivalents (FE) per well. In addition to counting cells, an estimate of cell mass (mg/well) was made by gravimetric determination of the wet weight of an aliquot of each cell suspension. Based on this estimate, the mean mass of cells per well ranged from 0.01 to 0.3 mg wet weight/well.

At the end of culture, aliquots of media were removed and stored at –20°C for determination of steroid content by RIA. The cells were then harvested onto glass fibre filters with a Tomtec Cell Harvester 96 (Tomtec, Hamden, CT) and washed to remove soluble radioactivity. Incorporated radioactivity was determined by liquid scintillation counting of the cells collected on glass fibre filters.

2.10.5 Thecal cell culture

Thecal quarters, prepared from follicles by scraping off the granulosa layers, were pooled and cultured in 0.5 ml BTCM in 4-well culture plates, with 4 quarters per well (1FE/well). Cultures were carried out in humidified atmosphere of 5% CO₂ in air, at 39°C for 24 h with various hormones and growth factors.
Culture media were removed and frozen for steroid assay, and thecal tissue blotted on filter paper and weighed to determine wet weight of tissue per well.

2.11 TGFβ ELISA

The content of latent and active forms of TGFβ in boar seminal plasma was assayed in commercial Promega TGFβ1 and TGFβ2 Emax® Immunoassay kits (Promega, Madison, USA). The assay was validated by demonstrating (a) parallelism over dilutions of fresh seminal plasma from 1/3 to 1/500, (b) lack of inhibition in samples spiked with recombinant TGFβ, and (c) the capacity to distinguish between active and latent (material released after acid-activation) forms of the molecule. Using samples from four boars, an optimal seminal plasma dilution was established as 1/256.

The effects of storage at 4°C, -20°C and at -70°C on the TGFβ1 content of seminal plasma were investigated, including the effect of repeated rounds of freeze/thawing with liquid nitrogen. In addition the stability of TGFβ in seminal plasma at room temperature was evaluated to ensure that minimal loss could be expected with the experimental protocols employed. Seminal TGFβ1 and TGFβ2 content was determined for 46 boars at BMI piggery in which fertility data was available including, but not restricted to, the results of matings in the field trial (section 2.19).

2.12 IFNγ ELISA

Seminal plasma samples were diluted 1:10 in PBS and assayed for IFNγ content using commercially available Porcine IFNγ ELISA kit from Pierce Endogen (Illinois, USA code #EPIFNG).

2.13 ENDOTOXIN ASSAY

To determine the content of bacterial endotoxin in boar semen a commercially available kit based on Limulus Amebocyte Lysate (LAL) and a colour producing substrate to detect endotoxin chromogenically (Bio Whittaker, Maine, USA code # 50-647) was used. Seminal plasma was diluted in LAL Reagent Water 1:10 for the assay.

2.14 EXPERIMENTAL DESIGN: FIELD TRIAL

To establish whether seminal plasma treatment of gilts prior to AI can significantly increase conception rate and/or litter size, or reduce variability in birth weight at parturition, a large-scale field experiment at BMI piggery was undertaken from October 2000 until mid April 2001. Based on litter size and live-born birth weight variability in first parity sows at BMI piggery (seasonal variation was also taken into account), statistical power analysis was performed to determine the minimum number of gilts required per treatment group to detect 15% changes in birth weight and litter size (returns to oestrus and other
measures were not included). The sample size (n) was determined by Power Analysis Calculator provided by University of California, Los Angeles (UCLA) Department of Statistics on the internet at http://calculators.stat.ucla.edu/powercalc/. The Power calculation model involved 2 samples (ie. Treatment (SP-treated gilts) and Control (PBS-treated gilts) with equal variances, normal distribution and with mean of population$_1$ > mean of population$_2$ by 15%, which equates in this model to an extra 1.5 piglets due to seminal plasma treatment. The common standard deviations for both populations was ± 2.0, significance level (reject null hypothesis (H$_0$: $\mu_1 = \mu_2$) given it is true) was 0.05 and the Power described to test probability was 1.0. On the basis of this analysis a minimum sample size of 270 gilts was determined with 135 gilts in each treatment group. The final experiment comprised a total of 282 gilts randomly selected from BMI piggery’s breeding herd.

Genetic strain of gilts used for this study were BMI line 1 and 2 and these lines of boars and sows were developed from F1 Landrace X Large White based on growth rates and high fertility. The fertility of line 1 and 2 in this field study were not significantly different (p>0.05). Line 1 and 2 gilts were evenly distributed between treatments. Gilts at 28 weeks of age without exogenous synchronisation treatment apart from boar stimulation twice daily (standard farm practice for oestrous induction and detection) were randomly allocated into two treatment groups. Detection of oestrus was determined if the standing response (lordosis) was observed in the presence of a boar or in response to pressure on the back of the gilt applied by the AI technician. Once oestrus was detected the following afternoon or the following morning (if detection was in the afternoon), gilts received a 150 ml intrauterine infusion by transcervical catheter of either frozen and thawed seminal plasma or filter sterilised PBS (section 2.3). Approximately 90 min after intrauterine treatment the first of two AI doses were given containing 3 x 10$^9$ spermatozoa each. The second AI dose was given the following morning if treatment and first AI was the previous afternoon, or in the late afternoon of the same day if treatment and first AI were given in the early morning. After treatment and AI gilts were group housed and remained together within group pens until transfer to farrowing stalls at approximately 110 days following AI.

Pregnancy was assessed by ultrasound on day 30 after AI. Returns, abortions, not-in- pig, illnesses and deaths were recorded and provided data for success of matings and farrowings, littersize and individual birthweights were also recorded as soon as practicable after farrowing. In addition, 14 day weights were also recorded for indication of piglet viability and growth.

2.15 STATISTICAL ANALYSES

Values are given as mean ± SEM. The effect of treatment on uterine weights, leukocyte abundance and mean embryo size or mean blastomere number was analysed using Student’s t test for
independent samples. Effect of treatment on cytokine mRNA expression data was analysed using Kruskal Wallis one way ANOVA and Mann Whitney Rank Sum test. Effect of treatment on progesterone content in serial blood samples collected for 9 days post treatment was compared using ANOVA repeated measures. Statistical significance was inferred when \( P < 0.05 \). All statistical analyses were performed using SPSS 11.0 for Windows.
Figure 2.3 Oestrous synchronisation and treatment protocol in short term experiments.
Chapter 3

Uterine and embryo responses to seminal plasma
3.1 INTRODUCTION

Successful pregnancy in the pig is reliant on sequential processes of oocyte fertilisation, embryo development, implantation and fetal growth. To occur optimally, these processes require the synchronous function and development of key components of the reproductive system including the hypothalamic pituitary axis (HPA), ovary, oviduct, and uterine tract. However, there is also a male contribution consisting not only of sperm for fertilisation, but also seminal plasma originating from the secretions of accessory sex organs; prostate gland, bulbo-urethral gland and the seminiferous vesicles. Each of these organs provide components to make up the seminal plasma, which was originally believed to simply provide nutritive and transport support for spermatozoa (Mann 1964). Studies in rodents have highlighted the contribution of seminal plasma in remodelling the cellular framework of the endometrium including the recruitment of key immune cells and in the formation of new vasculature (Robertson et al. 1996c). In the pig, these processes are crucial for preparing the uterine tract for the critical period of elongation and attachment of the developing embryo.

In rodents, and many other mammals including pigs, the response to insemination is akin to a classical inflammatory response involving the recruitment and infiltration of immune cells including neutrophils, eosinophils and macrophages into the endometrial stroma and uterine lumen (Bischof et al. 1994, Engelhardt et al. 1996, Lessard et al. 2003, Robertson et al. 1998a, Rozeboom et al. 1999). The duration of this inflammatory response to insemination in the rat is reported to last for 2-3 days and begins to resolve due to increasing levels of serum progesterone (Kachkache et al. 1991). The infiltration of immune cells into the endometrium appears to be predominantly due to the seminal plasma component of semen, as the infiltration of leukocytes is still present after vasectomy of male mice (Choudhuri et al. 1993) but is not detectable after mating with males that are seminal vesicle deficient (Tremellen et al. 1998). Further research in our laboratory investigating the active components of seminal plasma found that pro-inflammatory cytokine transforming growth factor β (TGFβ) was largely responsible for triggering the post-mating inflammatory response in mice (Tremellen et al. 1998). In pigs, Bischoff et al. (1994) used a similar strategy to investigate the immune responses after insemination comparing uterine post-mating immune responses of vasectomised matings with unmated controls (Bischof et al. 1994). Although these studies provided information implicating seminal plasma in driving the post-mating inflammatory response in the uterus, they fell short in demonstrating the role of seminal plasma alone in initiating and sustaining changes in the endometrium and the possible effects this may have on porcine embryo development and survival.
The studies described in this chapter investigated the influence of seminal plasma in eliciting changes in immune parameters in the reproductive tract and the possible consequences of seminal plasma constituents on early embryo development.

### 3.2 STUDY DESIGN

A cohort of 32 F1 (Landrace x Large White) pre-pubertal gilts at 22 weeks of age were selected and randomly allocated into 4 groups of 8 (Section 2.2). Briefly, oestrus was induced and synchronised in gilts by giving intramuscular injections of eCG/hCG at 24 weeks of age (Figure 2.3). Pooled seminal plasma or PBS was administered directly into the uterus via a transcervical AI catheter to gilts within 2 hours post hCG and for Group I reproductive tracts were retrieved 34 hours later, before expected ovulation. Treatments for gilts in Groups II, III, and IV were given in the following spontaneous oestrus and reproductive tracts were retrieved on either 5 or 9 days after intrauterine treatment. Gilts in the day 5 and day 9 groups in addition received two standard artificial inseminations containing $3 \times 10^9$ spermatozoa each, the first 2 h after intrauterine infusion of seminal plasma or PBS and the second the following morning. Four gilts were excluded from the trial, one from each of the 34 h and day 5 time points and two from the day 9 time point, all in the PBS infused control groups, due to endometritis with cystic ovaries (34 h post treatment), uterine infection indicated by increased volume and acidic luminal fluid (day 5), failure to conceive and lameness (one each at day 9).

As soon as possible after slaughter (10 to 20 min) reproductive tracts were retrieved. Uteri were trimmed of connective tissue and dissected from the vagina, leaving the cervix intact and oviduct and then weighed. Each uterine horn was flushed twice with 20 mls of filter-sterilised Dulbecco’s modified Eagle’s medium (DMEM) and the flushings were collected for quantification of cellular content and cytokine analysis.

### 3.3 EFFECT OF INTRAUTERINE SEMINAL PLASMA ON UTERINE WEIGHT, LUMINAL FLUID LEUKOCYTE CONTENT, LUMINAL FLUID VOLUME AND VASCULARITY

Uterine tracts were evaluated initially for gross measurements of an inflammatory response (Table 3.1). Compared with tracts retrieved from PBS-treated gilts, the tracts from seminal plasma-treated gilts at 34 h post treatment were on average 68% heavier ($P = 0.03$) and contained 3.5-fold more luminal fluid ($P = 0.19$). This effect was short lived, since uterine weights and luminal fluid content were not different in gilts at day 5 and day 9 post-treatment. In addition, treatment with seminal plasma influenced the number of leukocytes, principally granulocytes, infiltrating into the uterine lumen with a 5.4-fold increase in the mean leukocyte content of luminal fluid from seminal plasma treated gilts ($P = 0.01$). Visual
inspection of the colour of the uterine tract suggested increased uterine vascularity after exposure to seminal plasma in tracts retrieved from pre-ovulatory gilts (Table 3.1 and Figure 3.1).
Figure 3.1 Photograph of representative uterine horns retrieved from gilts 34 h after intrauterine infusion of saline (PBS) and seminal plasma (SP) demonstrating the difference in vascularity at this time point.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>34 h (n=3)</th>
<th>Day 5 (n=4)</th>
<th>Day 9 (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uterine weight</td>
<td>79 ± 5</td>
<td>116 ± 8*</td>
<td>110 ± 14</td>
</tr>
<tr>
<td>(g, mean ± SEM)</td>
<td></td>
<td>92 ± 7</td>
<td>112 ± 9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>112 ± 9</td>
<td>109 ± 9</td>
</tr>
<tr>
<td>Luminal fluid volume</td>
<td>6.7 ± 1.2</td>
<td>23.2 ± 9.2</td>
<td>ND</td>
</tr>
<tr>
<td>(ml, mean ± SEM)</td>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Luminal fluid leukocytes</td>
<td>1220 ± 330</td>
<td>6540 ± 2300**</td>
<td>ND</td>
</tr>
<tr>
<td>(per µl)</td>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Vascular indexa</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>(median)</td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 3.1 The effect of seminal plasma treatment on uterine weight, luminal fluid volume, total luminal fluid, leukocyte number and vascularity in gilts.

Data are expressed as mean ± SEM. Key: *P = 0.034, **P = 0.012

*aVascularity index: + pale appearance; ++++ dark red appearance

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3.4 EFFECT OF SEMINAL PLASMA ON UTERINE LEUKOCYTES

Uterine tissue samples consisting of luminal epithelial, endometrial and myometrial layers were taken from the proximal and distal portions of one uterine horn for immunohistochemical analysis at 34 hours, day 5 and day 9 after infusion with PBS or seminal plasma. The proximal section was taken between 5 and 8 cm from the bifurcation of the uterine horn and the distal section was taken from approximately 10 cm from the utero-tubal junction. Each section was embedded in OCT (Section 2.6) and later examined for the abundance and distribution of leukocytes using specific monoclonal antibodies reactive with all leukocytes (CD45; MCA1222), macrophages (HB142) and swine leukocyte antigen (SLA) class II positive cells expressed by monocytes, activated macrophages and/or dendritic cells (Figure 3.2). There was similar numbers of cells of each phenotype in the proximal and distal sections of uterine tissue at each time period and the data reported here consists of the mean of these two sections. Therefore each value for leukocyte numbers represents the mean of proximal uterine sections and the mean of distal uterine section. There was spatial distribution of leukocytes at all time points within the uterine tissue, with mean leukocyte positivity approximately 2-fold greater in the superficial endometrium and endometrial stroma than in the myometrium as shown within the PBS infused group (Figure 3.3).

In uterine tissue from the pre-ovulatory group (34 h), endometrial exposure to seminal plasma led to 31%, 47% and 77% increases in percent positivity of CD45+ staining in the superficial endometrium, endometrial stroma and myometrial tissue respectively compared with tissues from the PBS treatment group ($P < 0.01$) (Table 3.2). The infiltrating leukocytes within each uterine section comprised predominantly of macrophages showing 62-85% increases due to seminal plasma treatment, and to a lesser extent of monocytes including activated macrophages and dendritic cells (SLA class II positive cells) which increased by 30-45% due to seminal plasma treatment in each compartment (Figure 3.3).

The increase in leukocytes due to treatment with seminal plasma persisted until 9 days after treatment in the superficial endometrium and endometrial stroma compartments with a general decline in the myometrial compartment for all cell types investigated ($P<0.05$; Table 3.2). Moreover, treatment with seminal plasma resulted in longer term retention of leukocytes of all phenotypes ($P <0.05$; Table 3.2 ).

Table 3.2 highlights the change in leukocyte densities over the time period comparing the trajectory for each cell type from the preovulatory (34 h) to the day 5 and day 9 groups. In PBS-treated gilts, the abundance of uterine CD45+ cells (leukocytes) was reduced 3.5-fold and 2.3-fold in the day 5 and day
group respectively compared with those measured from the pre-ovulatory group ($P <0.05$). A similar trend was seen in the seminal plasma-treatment groups but with a greater density of macrophages and SLA II$^+$ cells present at the day 5 compared to the pre-ovulatory tissue ($P <0.001$). The effect of seminal plasma was generally diminished for all cell types by day 9 post treatment, but still had a significant effect in increasing the abundance of leukocytes by 31% compared to PBS-treated gilts ($P <0.05$). In addition, this study demonstrated a gradual shift in the HB142 positive macrophages to the SLA class II-activated macrophage and dendritic phenotype during this time course in early pregnancy regardless of seminal plasma exposure, with approximately equal proportions in the 34 h and day 5 group to an 2.3-fold increase in the activated phenotypes by day 9. This trend of lineage distribution was also evident in the deeper endometrial stroma and myometrial tissue (Figure 3.2).
Figure 3.2 Photomicrographs illustrating immunohistochemical analysis of CD45+ cells in representative sections of endometrial tissue.

Reproductive tracts were retrieved 34 h after intrauterine infusion of (a) PBS or (b) seminal plasma. Arrows indicate CD45+ cells in superficial endometrium (SE) and endometrial stroma (St). Bar = 50 µm.

Figure 3.3 The effect of intrauterine seminal plasma infusion on the abundance and distribution of leukocytes in the (a) superficial endometrium, (b) endometrial stroma and (c) myometrium in gilts at 34 hours, day 5 and day 9 after treatment.

Data were compared using ANOVA followed by Bonferroni t-test.

Key: *P <0.05, **P <0.001 compared with PBS-treated group for the same time period.
(a) Superficial endometrium

(b) Endometrial stroma

(c) Myometrium

Percent positivity

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53
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Antigen</th>
<th>34 h</th>
<th>Day 5</th>
<th>Day 9</th>
</tr>
</thead>
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<tr>
<td></td>
<td>PBS</td>
<td>76.4</td>
<td>± 17.2</td>
<td>± 54.3</td>
</tr>
<tr>
<td></td>
<td>(n=3)</td>
<td>(n=4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superficial</td>
<td>CD45</td>
<td>59.5</td>
<td>± 2.3**</td>
<td>± 4.1†</td>
</tr>
<tr>
<td>Endometrium</td>
<td>HB142</td>
<td>37.8</td>
<td>± 7.4 ± 1.6‡</td>
<td>± 27.1</td>
</tr>
<tr>
<td></td>
<td>3.4</td>
<td>7.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SLA II</td>
<td>36.9</td>
<td>± 18.2</td>
<td>± 49.57</td>
</tr>
<tr>
<td></td>
<td>7.1</td>
<td>11.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endometrial</td>
<td>CD45</td>
<td>50.2</td>
<td>± 11.9</td>
<td>± 49.7</td>
</tr>
<tr>
<td>Stroma</td>
<td>1.8</td>
<td>1.6†</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HB142</td>
<td>35.1</td>
<td>± 4.2</td>
<td>± 21.4</td>
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<td>4.7</td>
<td>4.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SLA II</td>
<td>44.5</td>
<td>± 16.7</td>
<td>± 45.1</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>1.8†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myometrium</td>
<td>CD45</td>
<td>27.6</td>
<td>± 12.1</td>
<td>± 53.8</td>
</tr>
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<td></td>
<td>1.4</td>
<td>1.5†</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HB142</td>
<td>24.5</td>
<td>± 5.29</td>
<td>± 24.7</td>
</tr>
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<td></td>
<td>4.3</td>
<td>2.2†</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SLA II</td>
<td>27.6</td>
<td>± 13.6</td>
<td>± 49.4</td>
</tr>
<tr>
<td></td>
<td>4.3</td>
<td>2.3†</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2 The effect of seminal plasma treatment on the abundance of leukocytes in uterine tissue from gilts at 34 h, day 5 and day 9 after treatment.
The data were compared using ANOVA followed by Bonferroni t-test. Key: *$P<0.05$, **$P<0.01$ compared with the PBS-treated group for the same time period; † $P<0.05$ compared with the 34 h time period.

### 3.4.1 Effect of seminal plasma on cytokine expression

Immediately after the flushing of the uterine tracts with the same cohort of animals as described above, uterine biopsies were taken from proximal and distal portions of uterine horns in gilts at 34 h and day 5 and day 9 after treatment with PBS or seminal plasma. Quantitative RT-PCR analysis was performed on these tissues to evaluate the effect of seminal plasma treatment on the level of expression of key pro-inflammatory cytokines. PCR primers were designed and synthesised for granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-6 (IL-6), tumour necrosis factor α (TNFα), cyclooxygenase-2 (COX-2), interleukin-1β (IL-1β) and monocyte chemoattractant protein-1 (MCP-1) (Section 2.9; Table 2.1). The primers produced amplicons of expected size (Figure 2.2). Intra-uterine infusion of seminal plasma induced expression of GM-CSF, IL-6, and MCP-1 mRNA 34 hours after treatment with a 5-fold, 9.3-fold and 2.1-fold increase over mean values for PBS-treated animals respectively (Figure 3.4 a, b and f). However, unlike the seminal plasma-induced leukocyte infiltration, the increase in expression was short lived, with the expression of GM-CSF mRNA declining more than 5-fold by day 5 and remaining at this level at day 9 after treatment (Figure 3.4a). The expression profile for MCP-1 was similar with a significant reduction in expression in the seminal plasma treatment compared to the values for PBS-treated animals (Figure 3.4f). Levels of mRNA expression for TNFα and IL-1β were not significantly different between treatment groups, however like GM-CSF and MCP-1 expression profiles, were significantly reduced compared to the mean values of the pre-ovulatory 34 h tissues on day 5 and day 9 after treatment ($P<0.05$) (Figure 3.4c and e). In contrast, IL-6 mRNA expression remained high in 34 h and day 5 tissue and significantly decreased 9.8-fold to low levels by day 9 ($P<0.05$) (Figure 3.4b).

Endometrial exposure to seminal plasma generally resulted in an expression profile higher for cytokines in the pre-ovulatory period with expression reducing dramatically as pregnancy ensued. However, GM-CSF and COX-2 mRNA expression in the PBS-treated animals deviated from this trend having the highest level of expression at day 5 and lowest levels at 34 h and day 9 after treatment ($P<0.05$) (Figure 3.4a and 3.4d). The level of GM-CSF mRNA expression in the PBS-treated animals was 3.2-fold higher than the seminal plasma-treated counterparts ($P=0.01$) at day 5 and appeared to remain at this level until day 9 (Figure 3.4a). Interleukin-6 had a similar profile for the PBS-treated
tissue and was still expressed at relatively high levels at day 9 after treatment (Figure 3.4b). Monocyte chemotactic protein-1 expression was also significantly higher in the PBS-treated tissue compared with the seminal plasma-treated tissue at day 9 after treatment ($P <0.05$) (Figure 3.4f).

Cyclo-oxygenase-2 mRNA expression profile for the seminal plasma-treated tissue followed the same trend as GM-CSF mRNA with a 4.5-fold increase in expression over the mean values for the PBS at 34 h post treatment ($P <0.05$) and reducing by 7.8-fold to low levels by day 5 and remaining low until day 9 after treatment ($P <0.01$). The expression was distinctly different in the PBS-treated tissue with the highest level of COX-2 mRNA expression at day 5 relative to the 34 h time period and mean relative mRNA expression 6-fold greater than that observed in the seminal plasma-treated tissue ($P =0.04$).

![Figure 3.4](image_url)  
Figure 3.4 The effect of seminal plasma treatment on endometrial cytokine mRNA expression in gilts at 34 h, 5 days (d5), 9 days (d9) after treatment.

Data are mean ± SEM relative mRNA expression of (a) GM-CSF, (b) IL-6, (c) TNF-α, (d) COX-2, (e) IL-1β and (f) MCP-1. Relative mRNA expression is calculated by normalising data to β-Actin mRNA expression and expressed as percent of the mean value in the 34 h PBS-treated group. Data were analysed using Kruskal-Wallis followed by Mann-Whitney U-test.

Key:  ● PBS-treated group (n=4-6 animals per time point);  ■ Seminal plasma-treated group (n=4-6 animals per time point. *$P <0.05$ compared to the same time point in the PBS-treated group; †$P <0.05$ compared with the 34 h time point for data within the PBS-treated group; ‡$P < 0.05$ compared with the 34 h time-point for data within the seminal plasma-treated group.
3.4.2 The effect of seminal plasma on embryo development

To investigate the effect of seminal plasma on embryo development, uteri from gilts treated with intrauterine PBS or pooled seminal plasma were flushed with 3 consecutive 20 ml flushes of Dulbecco's modified Eagle's medium into each uterine horn (Section 2.7). Comparisons were made on the number of embryos retrieved, blastomere number at day 5, and embryo size at day 9 (Table 3.2) since the time of ovulation and fertilisation was not precisely known to definitively determine the effect on embryo development between treatments. The numbers of blastomeres were quantified after the blastocysts were analysed using Hoechst stain (Section 2.7). Intrauterine infusion of seminal plasma resulted in more embryos being flushed from the tracts at day 5 and day 9, reaching statistical significance when values from both days were combined with a 33% increase in embryos retrieved in gilts treated with seminal plasma ($P = 0.03$) (Table 3.2). Viability of embryos was assessed as those with a blastocoel cavity at day 5 or a spherical shape at day 9 (Figure 3.6). In this study there were no observable differences in the viability of embryos using these criteria for both day 5 and day 9 time points or the embryo survival rate (ESR) based on average ovulations for gilts within both groups (Table 3.2). Seminal plasma treatment did not have an effect on the number of blastomeres in embryos present at day 5 but did have a significant effect on embryo size at day 9 post treatment with a reduction of 40% in diameter compared with the PBS-treated counterparts ($P < 0.001$) (Table 3.2).
<table>
<thead>
<tr>
<th></th>
<th>PBSa</th>
<th>SPa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryos on day 5 (n)</td>
<td>8.7 ± 1.0 (4)</td>
<td>12.1 ± 1.3 (7)</td>
</tr>
<tr>
<td>Embryos on day 9 (n)</td>
<td>9.2 ± 0.9 (5)</td>
<td>11.8 ± 1.6 (6)</td>
</tr>
<tr>
<td>Total embryos (day 5 + day 9) (n)</td>
<td>9.0 ± 0.6 (9)</td>
<td>12.0 ± 1.0 (13)*</td>
</tr>
<tr>
<td>Viable embryos on day 5 (%)</td>
<td>6.5 ± 2.3 (75%)</td>
<td>10.5 ± 1.6 (87%)</td>
</tr>
<tr>
<td>Viable embryos on day 9 (%)</td>
<td>7.2 ± 2.0 (78%)</td>
<td>11.3 ± 1.4 (96%)</td>
</tr>
<tr>
<td>Total viable embryos (day 5 + day 9)</td>
<td>6.9 ± 1.4 (77%)</td>
<td>10.9 ± 1.0 (91)*</td>
</tr>
<tr>
<td>Blastomere number on day 5 (n)</td>
<td>38 ± 3 (36)</td>
<td>34 ± 4 (95)</td>
</tr>
<tr>
<td>Embryo diameter (µm) on day 9 (n)</td>
<td>497 ± 40 (46)</td>
<td>284 ± 23** (71)</td>
</tr>
<tr>
<td>Embryo survival rateb</td>
<td>62.5%</td>
<td>83.3%</td>
</tr>
</tbody>
</table>

Table 3.2 The effect of intra uterine seminal plasma infusion on retrieved embryos on day 5 and day 9 post treatment.

Key: a Data are means ± S.E.M. number of embryos flushed per gilt, with viable embryo defined as those with a blastocoel cavity at day 5 or spherical blastocyst at day 9. * P<0.05, ** P<0.001 compared with PBS-treated group (Student’s t-test).

b Embryo survival rate is the number of total embryos retrieved expressed as a percentage of mean ovulation for all gilts. The mean ovulation rate for all gilts was 14.4 ± 0.6.
Figure 3.4 The effect of seminal plasma on embryo development. Representative embryos are shown.

Embryos were retrieved on (a) day 5 after treatment and incubated with Hoescht stain to enable blastomere quantification (100 X magnification) or (b) on day 9 after treatment to enable measurement of embryo diameters in PBS-treated gilts (b) and (c) in seminal plasma-treated gilts. Scale bar = 50µm.

3.5 DISCUSSION

The abundance of neutrophils and eosinophils at different stages of the oestrous cycle in the porcine endometrium has been noted since the early 1920’s (Corner 1921). More recently, immune responses to mating and in early pregnancy have been investigated identifying endometrial plasma cells, uterine intra epithelial lymphocytes and lymphocyte subpopulations (Bischof et al. 1994, Hussein et al. 1983, King 1988). However, information regarding the nature and magnitude of immune response to mating and in early pregnancy and its physiological significance is still lacking.

The experiments described in this chapter have provided new information on the characteristics of the post-mating immune response elicited by constituents of seminal plasma. Within a 9 day period, seminal plasma initiated and sustained cellular and molecular changes within the uterus and the cytokine and growth factor milieu that would be experienced by the early embryo. In the pre-ovulatory period the most prevalent amongst the infiltrating cells were macrophages and SLA class II+ cells in the endometrium and granulocytes in the luminal fluid. Macrophages and activated cells with the SLA class II phenotype can secrete cytokines that are reported to be embryotrophic including TGFβ, GM-CSF and IL-6 (Robertson 2000, Robertson et al. 1997).

The changes observed in the cellular and molecular events induced by seminal plasma are characteristic of a typical inflammatory response and include the recruitment of leukocytes and the increased expression of pro-inflammatory cytokines. Most prevalent amongst the infiltrating cells were macrophages and SLA class II positive cells in the endometrium and granulocytes in the luminal fluid. Previous studies in pigs have also described an increase in these cell types after mating with intact or vasectomised boars including endometrial hypertrophy (Bischof et al. 1994, Engelhardt et al. 1996) consistent with our observation on uterine vascularity, luminal fluid content and increased uterine weight. Our experiments using pooled seminal plasma further delineates the seminal plasma effect in the initiation and extent of the inflammatory response by eliminating the effect of mating with the boar and by further defining the cytokines involved in this response. Moreover, by quantifying the cellular
changes, we have confirmed that both macrophages and SLA class II positive cells increase in response to seminal plasma.

Studies from our laboratory suggest that in mice, MHC class II+ cells (SLA class II is the phenotypic equivalent cell type in pigs) mature from precursor MHC class II− monocytes as they are recruited into the endometrium from the blood (Hudson-Keenihan et al. 2004). This observation reflects the ratio of SLA class II+ cells to macrophages over the course of early pregnancy reported in this chapter and implies that there is similar differentiation of these cells at this time in pigs. These overt inflammatory indices subside significantly within days after seminal plasma exposure to the endometrium and as pregnancy progresses. This dissolution of the inflammatory response is likely to be due to increased levels of progesterone present in early pregnancy that has been shown to reduce expression of pro-inflammatory mediators during early pregnancy in the mouse (Robertson et al. 1996a) and human (Critchley et al. 1999).

Studies in mice have also highlighted the role of key molecular mediators that drive the cellular infiltrate in response to seminal plasma and have identified pro-inflammatory cytokines GM-CSF and IL-6 production by uterine epithelial cells increase after seminal plasma exposure 20-fold and 200-fold respectively (Robertson et al. 1992b). Together with several chemokines that are upregulated within hours of mating (Pollard et al. 1998, Robertson et al. 1992a, Wood et al. 1997), they are likely to function synergistically to orchestrate further leukocyte recruitment and the activation of precursor inflammatory cells. The observations reported in this chapter suggest that similar processes take place in the pig after endometrial exposure to seminal plasma with increased expression of GM-CSF, IL-6 and MCP-1 including the temporal pattern of the rise and fall in expression during the pre-implantation period of pregnancy.

Of particular note is the increased expression of cytokines GM-CSF and IL-6. These two cytokines were investigated due to their proposed roles during the pre-implantation period in mice (Robertson 2000). Granulocyte macrophage–colony stimulating factor has been shown to have direct effects on the developing embryo and was first identified as having growth-promoting effects in mouse blastocysts (Robertson et al. 1991) and later shown to prevent apoptosis and increase the number of inner cell mass cells (Robertson et al. 2001b). In other species, including the cow and in humans, GM-CSF has been shown to increase in vitro blastocyst development (de Moraes et al. 1997, Sjoblom et al. 2002) and to stimulate maternal recognition of pregnancy signal interferon-τ in sheep (Imakawa et al. 1987, Imakawa et al. 1993).
In addition to the embryotrophic properties of GM-CSF, this cytokine is also recognised for its role in the recruitment and regulation of uterine macrophages along with IL-6 (Robertson et al. 1992b). Furthermore, when IL-6 was infused into the uterine lumen of rats, antigen presentation by epithelial and mixed stromal cells was increased (Prabhala et al. 1995). The role in leukocyte recruitment and in antigen presentation by GM-CSF and IL-6 respectively implicates these cytokines in local mucosal immunity within the uterus and also probable key functions in paternal antigen presentation of antigens contained within semen and involved in induction of immune tolerance to the embryo when paternal antigens begin to be expressed around the time of implantation. In mice, uterine antigen presenting cells traffic to the lymph nodes draining the uterus causing hypertrophy (Johansson et al. 2004), where immune tolerance to paternal antigens occurs (Moldenhauer et al. 2009). In addition, studies from our laboratory have further delineated the pathways leading to tolerance of the semi-allogeneic embryos during pregnancy in the mouse. After mating, factors in seminal plasma drive the expansion of Treg cells within the uterus (Robertson et al. 2009) conferring immune tolerance to the embryos (Aluvihare et al. 2004). The expansion of uterine Treg cells is not seen in vasectomised or seminal vesicle-deficient mice (Robertson et al. 2009) and is likely to underpin the mechanism behind impaired embryo development, implantation and fetal growth trajectories in pregnancies initiated in mice in the absence of seminal plasma (Bromfield 2006).

The significant increase in GM-CSF and IL-6 endometrial expression due to seminal plasma treatment is likely to impact on the growth and development of porcine embryos by increasing the level of embryotrophic cytokines in the uterine-embryo milieu and also enhancing immune tolerance to the semi-allogeneic embryo. MCP-1 is a cytokine implicated in regulating local macrophage/monocyte and natural killer cell chemotaxis (Drake et al. 2001, Kyaw et al. 1998) which is also involved in ovine implantation and placentation (Asselin et al. 2001) and macrophage accumulation in mice (Wood et al. 1997) but whether this cytokine has any effects on pre-implantation embryos has not been described. The approximate 2-fold increase of MCP-1 due to seminal plasma observed in this study in the pre-ovulatory 34 h time point is likely to contribute to recruiting macrophages and other leukocytes to establish the immune environment before the developing embryo reaches the uterine tract. The significant decrease in MCP-1 expression in the seminal plasma treated gilts by day 9 after treatment may indicate that seminal plasma results in the more rapid resolution of the inflammatory response in the pre-implantation period.

The uterine environment is a well-recognised determinant of embryo development and survival in the pig (Pope 1988, Vallet et al. 2002) and embryo survival during the crucial early stages is dependent on the complex interactions between maternal tract-derived cytokines and growth factors and conceptus...
signalling (Jaeger et al. 2001). Evidence presented here of seminal plasma influencing the uterine environment via altering maternal expression of key mediators, adds to an interesting mix of signalling cascades of maternal and embryo origin that ensure successful placentation and future development of the conceptus (Robertson et al. 1997). The physiological effect of seminal plasma described above is likely to have been a contributing mechanism in the observation of increased numbers of viable embryos and altered embryo growth over the 9-day period after exposure to seminal plasma. Surprisingly, this was associated with a significant reduction in the size of embryos retrieved at day 9 post treatment. Whether seminal plasma-induced maternal factors act to restrict embryo growth remains to be determined. However, cytokines and growth factors can act to promote and control embryo growth and to co-ordinate synchronous development with the maternal tract (Jaeger et al. 2001). For example, in the prolific Chinese Meishan pig, embryos are significantly smaller than their European-derived commercially-bred counterparts (Bazer et al. 1988) with fewer cells present during the blastocyst stage (Rivera et al. 1996). Unique uterine-embryo signalling in Meishan pigs is believed to underpin a maternal ability to control embryo growth resulting in the prolificacy of this breed (Bazer et al. 1988, Christenson et al. 1993).

Cyclooxygenase-2 is often referred to as a pro-inflammatory enzyme due to its role in the generation of immune responses (Critchley et al. 1999) and catalyses the formation of prostaglandin (PG) F₂α and PGE₂. Prostaglandins have pleiotrophic effects in pregnancy and have the potential to influence maternal immune responses (Kelly et al. 1997), embryo development (Claus et al. 1987) and ovarian function (Armstrong 1981, Christenson et al. 1994). Most notable is the role PGs play in the maternal recognition of pregnancy with studies indicating that in non-mated gilts, uterine-derived PGF₂α (Bazer et al. 1977) is transferred to the ovary via a counter-current mechanism facilitated by close apposition of the uterine venous net to the ovarian artery (Krzymowski et al. 1990). In mated gilts, PGE₂ pre-dominates and negates the luteolytic effects of PGF₂α (Christenson et al. 1994). The increased expression of COX-2 mRNA in this study extends the possible role of seminal factors amplifying uterine synthesis of anti-luteotrophic prostaglandins enhancing ovarian support of early embryo development. However, further experimentation would be required to determine specific prostaglandin responses to seminal plasma.

In summary, seminal plasma induces a cascade of cellular and molecular events in early pregnancy that contribute to conditioning the uterine environment for the developing embryo during the period of greatest conceptus loss in the pig. The effect of seminal plasma on ovarian function and the identity of active constituents in seminal plasma are the subject of chapter 4 and 5 respectively.
3.6 PUBLICATION: SEMINAL PLASMA REGULATES ENDOMETRIAL CYTOKINE EXPRESSION, LEUKOCYTE RECRUITMENT AND EMBRYO DEVELOPMENT IN THE PIG

NOTE: This publication is included in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

[http://dx.doi.org/10.1530/rep.1.00160](http://dx.doi.org/10.1530/rep.1.00160)
Chapter 4

Ovarian responses to seminal plasma
4.1 INTRODUCTION

The previous chapter describes studies which show that seminal plasma induces an inflammatory response in the endometrium characterised by induction of mRNA expression encoding proinflammatory cytokines and COX-2 and recruitment and activation of leukocyte populations. In addition, intrauterine seminal plasma infusion resulted in increased numbers of viable embryos during the critical pre-implantation period implicating a possible role for seminal factors in increasing embryo survival and supporting the proposal that seminal plasma conditions the reproductive tract for embryo implantation and establishing pregnancy.

Several lines of evidence suggest that the effects of seminal plasma may not be restricted to the uterus, and may extend to the ovary. Studies with Bactrian camels, alpacas and koalas show that seminal plasma can influence ovarian function, potentially through the action of ovulating-inducing factors present in seminal plasma acting directly on the pituitary gland (Chen 1985, Pan et al. 2001, Paolicchi 1999, Ratto et al. 2005, Johnston et al. 2004). Seminal plasma has been implicated in causing ovarian leukocyte recruitment in mice, likely due to an indirect mechanism via intermediary molecules produced in the uterus (Gangnuss et al. 2004). The role of leukocytes in the ovary has yet to be fully elucidated, but studies in rodents suggest that leukocytes and cytokines are involved in follicle development, ovulation, and corpora lutea development and regression (Brannstrom et al. 1993b, Brannstrom et al. 1994b, Van der Hoek et al. 2000). Moreover the process of ovulation exhibits characteristics similar to an acute inflammatory response, in both the participating cell types and the chemical mediators involved (Espey 1980, Espey 1994).

Data from pigs also provides evidence for a role of seminal plasma on ovarian function. Uterine exposure to seminal plasma from natural mating as well as transcervical administration of seminal plasma at the beginning of oestrus caused advancement of ovulation by reducing the interval between oestrus and ovulation by up to 14 h in gilts and sows (Signoret et al. 1972, Weitze et al. 1990a, Waberski et al. 1995). Using unilateral uterine horn infusions (the Mariensee model), Waberski et al. (1995), demonstrated that the advancement of ovulation elicited by seminal plasma was the result of local communication between the uterine horn and the adjacent ovary, and activity was mainly attributed to a non-steroidal component (Waberski et al. 1995). However these studies did not examine the effects of seminal plasma exposure on the luteal function of the ovary, which would be expected to be instrumental in successful progression of early pregnancy. Furthermore the underlying mechanisms by which seminal factors may exert their effects were not determined.
The experiments described in this chapter investigated the effect of seminal plasma on ovarian responses in gilts, from the time immediately prior to expected ovulation until day 9 before embryo elongation and attachment begins. In addition, a series of in vitro experiments were carried out on retrieved ovarian tissue to examine whether or not prior seminal plasma exposure of the tract influences the responsiveness of steroidogenic cells to gonadotrophins and growth factors in vitro.

4.2 STUDY DESIGN

Two in vivo and a number of in vitro experiments were designed to investigate ovarian responses to seminal plasma. Experiment 1 investigated ovarian responses in gilts at three time points including one before expected ovulation and two before expected embryo elongation and attachment. Two different oestrous synchronisation protocols were used (Chapter 2, Fig. 2.1). Experiment 2 investigated the ovarian steroidogenic response to intrauterine seminal plasma in gilts, with indwelling ear vein catheters enabling serial blood collections before ovulation and for nine days post treatment (Chapter 2, 2.2 and 2.5).

4.2.1 Experiment 1: Effect of seminal plasma on ovarian parameters at 34 h, day 5 and day 9 post treatment

A cohort of 32 F1 (Landrace x Large White) pre-pubertal gilts at 22 weeks of age were selected and randomly allocated into 4 groups of 8 (Chapter 2, 2.2). Groups underwent different oestrus synchronisation protocols and were killed at different time points to examine the effect of seminal plasma at different stages of early pregnancy. The different oestrus synchronisation protocols were designed to allow evaluation of any interactions between seminal plasma treatment and cycle-regulation. Gilts in Groups I, II and III received intramuscular injections of eCG/hCG at 24 weeks of age and Group IV gilts were left to cycle naturally (Chapter 2, 2.1). Intrauterine seminal plasma or PBS was given to Group I gilts within 2 hours post hCG and reproductive tracts were retrieved 34 hours later before expected ovulation. Two additional replicates (4-6 gilts per treatment) were killed at 34 h post treatment to provide additional pre-ovulatory data, for a total of 12 gilts per treatment group at that time point. To assist synchrony for the second cycle, Group II gilts were given intramuscular injections of prostaglandin F2α on day 16 and 17 post hCG. Intrauterine seminal plasma or PBS was given to gilts in Groups II, III, and IV once standing oestrus was observed in the following spontaneous oestrous cycle. Reproductive tracts were retrieved at either day 5 or day 9 post intrauterine treatment for the gilts in Groups II, III and IV. Gilts in the day 5 and day 9 groups in addition received two standard artificial inseminations of 3 x 10⁹ spermatozoa each, the first 2 h after intrauterine infusion of seminal plasma or PBS and the second the following morning. Four gilts were excluded from the trial, one from each of the 34 h and day 5 time points and two from the day 9 time point, all in the PBS infused control groups,
due to endometritis with cystic ovaries (34 h post treatment), uterine infection indicated by increased volume and acidic luminal fluid (day 5), failure to conceive and lameness (one each at day 9).

4.2.2 Experiment 2: Effect of intrauterine seminal plasma on ovarian function in vivo

To investigate the effect of intrauterine infusion of seminal plasma on ovarian steroidogenesis in vivo, a separate experiment involving 10 large white x Landrace gilts at 24 weeks of age was performed using the eCG/hCG protocol of oestrus synchronisation and seminal plasma and PBS infusions as detailed above, except the treatment and AI doses were applied at the induced oestrous cycle and these gilts did not receive boar exposure. All gilts were housed in single stalls immediately after ear vein catheterisation (Chapter 2, 2.6), which took place between 2-3 days before oestrus synchronisation began.

4.2.3 Tissue collection

As soon as possible after slaughter (10 to 20 min) the reproductive tracts were retrieved and ovaries were dissected and weighed and the number of large follicles or corpora lutea were counted. For immunohistochemistry, a sample of each ovary (comprising approximately one quarter and containing at least one preovulatory follicle) was dissected off and placed in OCT, then immersion-frozen in liquid nitrogen-cooled isopentane and stored at -70°C until further processing.

4.2.4 Isolation and culture of ovarian cells

An in vitro culture system was developed to investigate the effects of seminal plasma exposure on ovarian cell responsiveness to growth factors and gonadotrophins. DNA synthesis and progesterone secretion was examined in granulosa and theca cells retrieved from gilts exposed to intrauterine seminal plasma or PBS and cultured in the presence of hCG, FSH and/or IGF-1. Granulosa cells were isolated from whole follicles from in vivo treated pre-ovulatory gilts in experiment 1 (Chapter 2, 2.14) and cells were pooled from 8 follicles per gilt prior to processing for culture. After scraping of the follicle walls, the remaining pieces of thecal tissue were cut into quarters (one each of 4 follicles allocated per well). Thecal quarters were placed in 4-well culture plates and cultured in 0.5 ml BTCM, with 4 quarters per well (1 follicular equivalent). Cultures were carried out in a humidified atmosphere of 5% CO₂ in air at 39°C for 24 h, after which supernatants were removed and frozen for progesterone assay, and thecal tissue was blotted on filter paper and weighed to determine the wet weight of tissue per well.
4.2.5 Immunohistochemistry

Immunohistochemical analysis was performed on ovarian tissues collected from gilts slaughtered at 34 h after intrauterine infusion of seminal plasma or PBS. Murine monoclonal antibodies reactive with porcine CD45 (MCA1222) and swine leukocyte antigen (SLA) Class II antigen (MSA3) were used to identify total leukocytes, and macrophages and dendritic cells respectively. Bound antibody was visualised by diaminobenzidine tetrahydrochloride (DAB) staining and quantified using Video Image Analysis (Chapter 2, 2.8). Data were expressed as ‘percent positivity’, calculated as the mean area of DAB stain expressed as a percentage of the total stained area in the ten fields. Repeated measurements of a single test field indicated the precision of this method (< 10% intra-assay variation).

4.3 EFFECT OF OESTROUS SYNCHRONISATION ON OVARIAN FUNCTION, BODY WEIGHT, BACK FAT, AGE AND OVULATION RATE AT SECOND OESTRUS (EXPERIMENT 1)

4.3.1 Luteolysis and return to oestrus after eCG/hCG-induced first oestrus

Intramuscular injections of PGF$_{2\alpha}$ were given to investigate whether PGF$_{2\alpha}$ would either advance second oestrus or provide tighter synchrony amongst the cohort of gilts. PGF$_{2\alpha}$ did induce luteolysis during the luteal stage of the eCG/hCG-induced first cycle (Group II) reflected by the sharp decline in plasma progesterone levels immediately following treatment (Table 4.1). However, the interval between the eCG/hCG-induced oestrus and the following spontaneous oestrus was not influenced by PGF$_{2\alpha}$ nor was there an anabolic effect on body weight due to PGF$_{2\alpha}$ (Table 4.1).
Table 4.1  The effect of PGF$_{2\alpha}$ as a luteolytic treatment in gilts after eCG/hCG-induced first oestrus.

Oestrous synchrony in gilts after first oestrus was induced with eCG/hCG was investigated using PGF$_{2\alpha}$ given to gilts on day 16 and day 17 after hCG injection. First day of natural oestrus was recorded when the gilt would display standing reflex in the presence of a boar. Data are expressed as mean ± SEM. Differences between treatments detected using Student’s t-test. †post-induced oestrus, *$P = 0.05$, **$P <0.01$. 

<table>
<thead>
<tr>
<th>Induction</th>
<th>Plasma P4 (nmol/L)</th>
<th>First natural oestrus after eCG/hCG</th>
<th>Body weight (Kg) at oestrus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days post PGF$_{2\alpha}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>2</td>
<td>Age (days)</td>
</tr>
<tr>
<td>eCG/hCG + PGF$_{2\alpha}$ (n=8)</td>
<td>70 ±14 8 ± 2** 3 ± 1** 204 ± 4</td>
<td>22.2 ± 0.8</td>
<td>129 ± 5</td>
</tr>
<tr>
<td>eCG/hCG (n=8)</td>
<td>51 ± 9 36 ±14 22 ± 11 195 ± 2</td>
<td>20.5 ± 0.8</td>
<td>127 ± 6</td>
</tr>
</tbody>
</table>
4.4 THE EFFECT OF INDUCED OESTRUS VERSUS NATURALLY CYCLING GILTS ON BODY WEIGHT AND TIME TO SECOND OESTRUS

PGF$_{2\alpha}$ was shown to have a luteolytic effect when applied in the mid luteal phase in gilts. However, as there were no significant effects due to PGF$_{2\alpha}$ on the time to next oestrus, the data from Groups II and III were combined and compared with those of gilts undergoing spontaneous oestrus (Group IV). Prior oestrus induction with eCG/hCG increased body weight by more than 10 per cent in gilts measured just before onset of their spontaneous second oestrus. This effect was likely due to the anabolic effect of follicular steroids secreted at the first oestrus. Naturally cycling gilts (Group IV) did not begin to cycle until approximately 20 days later than the induced group and there was a trend for decreased ovulation rate in spontaneously ovulating gilts to the oestrus–induced gilts (Table 4.2).
Table 4.2 The effect of first oestrus induced by eCG/hCG on body weight and age and ovulation rate at second spontaneous oestrus.

Data are expressed as mean ± SEM. Differences between treatments detected using Student’s t-test. a vs b P<0.05, c vs d P<0.001, e vs f P<0.07.
4.5 EFFECT OF INTRAUTERINE SEMINAL PLASMA ON OVARIAN WEIGHT, CL WEIGHT, CL NUMBER, FOLLICULAR FLUID VOLUME AND PROGESTERONE CONTENT

Gilts in Group I were killed 34 h after intrauterine treatment (36-38 h post hCG). Some had commenced ovulation and some were near ovulation at the time of sacrifice. There was no significant difference in the proportion of gilts treated with seminal plasma (4/12, 33%) ovulating at 34 h after treatment, compared with PBS controls (2/10, 20%). Preovulatory follicles (>8 mm diameter) were present in the majority of gilts and the mean volume of these follicles, as determined by weighing the aspirated FF, was 30% greater in seminal plasma treated gilts but this was not statistically significant (Table 4.3). The majority of these follicles contained oocytes that had either begun or completed meiotic maturation at the time of collection, with no significant difference between groups in the percentage of recovered oocytes at either germinal vesicle breakdown (GVBD) or meiosis II (MII) stages of maturation by observation of the chromosomal dyads. However, there was a significant effect of seminal plasma treatment on follicular steroidogenesis, with mean follicular fluid progesterone content 5-fold higher in seminal plasma-treated gilts compared to the content of fluids from PBS-treated gilts ($P = 0.05$) (Table 4.3).
Table 4.3 Effect of seminal plasma (SP) (n=12) or PBS (n=10) infusion on ovarian parameters 34 hours after treatment.

Data are expressed as mean ± SEM. †Number (%) of gilts that had commenced ovulating at 34 hours after treatment. *P <0.05.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence of ovulation†</th>
<th>Oocyte maturation</th>
<th>FFV (ml)</th>
<th>FF P4 (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%GVBD</td>
<td>%MII</td>
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<tr>
<td>PBS</td>
<td>2/10 (20%)</td>
<td>93 ± 5</td>
<td>84 ± 10</td>
<td>127 ± 15</td>
</tr>
<tr>
<td>SP</td>
<td>4/12 (33%)</td>
<td>97 ± 2</td>
<td>97 ± 2</td>
<td>168 ± 24</td>
</tr>
</tbody>
</table>
4.6  EFFECT OF INTRAUTERINE SEMINAL PLASMA ON OVARY WEIGHT, CL WEIGHT AND CL NUMBER AT DAY 5 AND DAY 9 POST TREATMENT

In the day 5 and day 9 post treatment groups, the total corpus luteum weight was increased by approximately 30% in seminal plasma-treated gilts compared to PBS-treated controls ($P = 0.05$) but there was no corresponding increase in ovary weights (Fig. 4.4).

Figure 4.4 Effect of intrauterine seminal plasma infusion on the weight of ovaries and corpora lutea (A) and number of corpora lutea (B) after treatment.

Data are mean ± SEM; *$P \leq 0.05$. Data are combined for gilts sacrificed at day 5 and day 9 (PBS-treated, n=10; SP-treated, n=11).
4.7 THE EFFECT OF INTRAUTERINE SEMINAL PLASMA ON OVARIAN LEUKOCYTES AT 34 HOURS POST TREATMENT

To examine the effect of intrauterine seminal plasma infusion on ovarian immune cells, ovaries were recovered at 34 hours post treatment. Sections containing stroma and thecal tissues were assessed for the presence of total leukocytes and SLA class II positive cells (activated macrophages and dendritic cells). Staining patterns were comparable for both cell types, with labeled cells distributed throughout the sections and more positive cells surrounding the follicles (Fig. 4.5). Compared to PBS-treated control gilts, ovarian sections from seminal plasma-treated gilts had 4-fold more leukocytes and more than 3-fold increases in SLA class II positive cells \( (P < 0.05) \) (Fig. 4.5).

Figure 4.5 Effect of intrauterine seminal plasma (SP) infusion or PBS on the abundance of leukocytes in ovarian tissue retrieved from gilts 34 h after treatment \( (n = 8, \text{ PBS}; n = 7, \text{ SP}) \).

The number of cells expressing CD45+ (all leukocytes) and SLA class II (activated macrophages and dendritic cells) were quantified by video image analysis. Data are mean ± SEM; * \( P < 0.05 \). SLA: Swine Leukocyte Antigen

O’Leary Chapter 4
4.8 THE EFFECT OF INTRAUTERINE SEMINAL PLASMA ON OVARIAN CYTOKINE EXPRESSION AT 34 HOURS POST TREATMENT

To investigate further the effect of uterine exposure to seminal plasma on ovarian function, and in particular the influence this may have on ovarian immune parameters, ovarian tissue was analysed for cytokine and COX-2 mRNA expression at 34 hours, day 5 and day 9 post treatment. The cytokines examined included GM-CSF, IL-1α, IL-1β, IL-6, MCP-1 and TNFα. No significant difference in expression was seen for any of the cytokines and COX-2 mRNA between the seminal plasma treated gilts and the PBS treated controls (Fig. 4.6).

Figure 4.6 The effect of seminal plasma treatment on ovarian cytokine mRNA expression in gilts at 34 h post intrauterine seminal plasma (SP) or PBS treatment.

Data are expressed as mean ± SEM and are also shown as symbols representing data from individual gilts. Relative mRNA expression of (A) COX-2; (B) GM-CSF; (C) IL-1α; (D) IL-1β; (E) IL-6; (F) TNFα, and (G) MCP-1 in seminal plasma-treated groups (hatched bars) (n = 4) or PBS-treated group (open bars) (n = 4). Relative mRNA expression is calculated by normalising data to β-actin mRNA expression and is expressed as percent of the mean value in the 34 h PBS-treated group. Data were compared by Kruskal Wallis followed by Mann Whitney U-test.
Relative mRNA expression

- **COX-2**
- **IL-1α**
- **MCP-1**
- **IL-6**
- **GM-CSF**
- **IL-1β**
- **TNFα**

Comparing PBS and SP conditions.
4.9 THE EFFECT OF INTRAUTERINE SEMINAL PLASMA ON OVARIAN FOLLICLE RESPONSES IN VITRO

To investigate whether intrauterine exposure to seminal plasma modulates follicular cell responses, in vitro assays were established using granulosa cells and theca tissues retrieved from gilts 34 h after intrauterine infusion of seminal plasma or PBS.

4.9.1 The effect of intrauterine seminal plasma on granulosa DNA synthesis in vitro

DNA synthesis in granulosa cells isolated from preovulatory follicles obtained 36-38 h after hCG treatment was measured by pulse labeling with $^3$H-thymidine for the last 6 h of 24 h culture in the presence of various hormones and growth factors. In vitro treatments of granulosa cells with either hCG or IGF-1 significantly increased $^3$H-thymidine incorporation when compared to in vitro controls (Fig. 4.4). Cells from PBS-infused gilts exhibited no further increase of DNA synthesis when cultured with hCG and IGF-1 in combination, but the stimulatory effects of combined treatment with the two agonists produced an additive response in cells from seminal plasma infused gilts.

4.9.2 The effect of intrauterine seminal plasma on progesterone secretion in vitro

Progesterone secretion during culture of granulosa and thecal cells was measured by radioimmunoassay of culture media at the termination of the cultures. Progesterone secretion by granulosa cells from PBS-infused gilts was increased 2-fold by in vitro addition of either hCG or IGF-1, but not FSH (Fig. 4.4 B and C). A significantly greater stimulatory effect of hCG, but not IGF-1, occurred in cells from seminal plasma infused gilts ($P<0.01$), and the effect of combined treatment with hCG and IGF-1 was similar to that of hCG alone. Mean secretion of progesterone by theca tissue was several fold less than that observed in granulosa cell cultures, expressed on a per follicle basis (Fig. 4.4 C). A trend for increased secretion of progesterone in the theca tissue was evident from seminal plasma-treated gilts following in vitro treatment of hCG alone, with a further enhancement in the presence of hCG plus IGF-1, but statistical significance was not met.
Figure 4.7. DNA synthesis (A) and progesterone secretion (B) in cultured granulosa cells and theca tissue (C) retrieved from gilts 34 hours after intrauterine infusion of seminal plasma (SP) or PBS.

Granulosa cells and theca tissue were cultured for 24 h under serum-free conditions in medium alone (Control) or in the presence or combination of 50 IU/ml of FSH, 5 IU/ml of hCG or 50 ng/ml IGF-1. Data are presented as mean ± SEM of raw data in which each treatment were performed in triplicate. The effect of treatment were evaluated by Student's t-test (SPSS 13.0); *P 0.05 SP versus PBS with same growth factor or gonadotrophin treatment; † P < 0.05, effect of in vitro treatment versus control with medium alone. (PBS: n=3; SP n=3) † FE: Follicular Equivalent
4.10 EFFECT OF INTRAUTERINE SEMINAL PLASMA INFUSION ON PROGESTERONE SECRETION IN VIVO (EXPERIMENT 2)

To investigate the effect of seminal plasma on ovarian function in early pregnancy, progesterone content was measured in serial blood samples taken daily from gilts (PBS, n=10; SP, n=11) by means of a chronic indwelling ear vein catheter (Chapter 2, 2.5). Three days after treatment progesterone profiles began to diverge between treatment groups (Fig. 4.6) and blood plasma progesterone levels from seminal plasma-treated gilts remained higher until the experiment was terminated at day 9 post-treatment (Fig. 4.5).
Figure 4.8 Effect of intrauterine infusion of seminal plasma (SP) or PBS on plasma progesterone concentration from 24 h until 9 days after treatment.

Data are mean ± SEM of natural log transformed data. The effect of treatment was evaluated using growth curve analysis (SAS 9.1) * $P = 0.0006$. 
4.11 DISCUSSION

The results of experiments described in this chapter extend earlier observations (Waberski et al. 1997b, Waberski et al. 1997a, Waberski et al. 1999) to provide evidence for a previously undocumented effect of seminal plasma on ovarian luteal function in pig. These experiments show that effects of seminal plasma interaction with the female tract are evident in the ovary where they influence the cellular composition and function during the pre-ovulatory and luteal period. Specifically, seminal plasma treatment was found to result in enhanced follicular steroidogenesis, more rapid growth and development of corpora lutea, and increased progesterone production by luteal cells extending to at least day 9 of pregnancy.

Pre-pubertal gilts were used in this study to determine whether gilts, which normally have lower conception rates and smaller litter sizes (Brooks 1982), would respond favourably to intrauterine seminal plasma treatment after induced oestrus. Due to the high variation in onset and duration of oestrus in gilts we employed two oestrus induction regimens and compared these to natural cycles. In this model prostaglandin F2α, was effective in causing luteolysis 14 days post hCG but was not effective in synchronising or advancing the following spontaneous cycle. Apart from synchronising oestrus for Group I gilts, eCG/hCG given at 24 weeks of age caused a significant increase in body weight compared to naturally cycling gilts evident by approximately three weeks after treatment. The effect is likely to be due to the anabolic effects of the follicular steroids (Velle 1976) produced during the induced oestrus. An increase in growth trajectory of this scale could potentially have application in increasing sow productivity for the pig industry as growth rate and backfat thickness are significantly related to onset of puberty and pregnancy success in pigs (Eliasson et al. 1991). The effect of increased body weight at first mating and the consequences for the lifelong reproductive productivity of the sow warrants further investigation, but was not within the scope of this thesis.

Previous studies have provided evidence that seminal plasma can influence pre-ovulatory function in pigs by reducing the interval between the LH peak or insemination and ovulation (Weitze et al. 1990c, Waberski et al. 1994, Waberski et al. 1997b). In addition a corresponding increase in day 3 fertilisation rate was also observed (Weitze et al. 1990a). The present study was unable to confirm an effect of intrauterine seminal plasma on the incidence or timing of ovulation due to the large between-gilt variability, suggesting a considerably larger number of gilts would be required to formally address this. Nonetheless, several indices of follicle maturation showed a trend towards more advanced development when gilts had been treated 34 h earlier with seminal plasma. While seminal plasma-
treated gilts tended to yield oocytes at more advanced stages of maturation, the effect was inconclusive because a high proportion of oocytes from pre-ovulatory follicles had resumed meiotic maturation at the time of collection, indicating an earlier time point of treatment may have been more informative. Furthermore while the mean follicular fluid volume was increased by 32% after seminal plasma treatment, there was wide variance across groups and this failed to reach statistical significance. However, a 4.8-fold increase in follicular fluid progesterone concentration was seen in seminal plasma-treated ovaries, which is consistent with a more mature developmental status (Ainsworth et al. 1980).

The effect of uterine exposure to seminal plasma on pre-ovulatory ovarian immune parameters has not been previously shown. However, studies in our laboratory have shown a significant decrease in activated macrophages in corpora lutea in mice mated to seminal plasma-deficient males (Gangnuss et al. 2004). In the present study, activated macrophages and dendritic cells detected on the basis of their expression of SLA class II, were increased approximately 4-fold in ovarian follicular and stromal tissues after uterine exposure to seminal plasma during the pre- and peri-ovulatory period. This elevated abundance of activated macrophages in the thecal and peri-follicular stromal tissue suggests a role for these cells in mediating the effects of seminal plasma in the ovary. It is likely that these cells and their secretory products influence the architecture and functionality of the vascular stroma and theca tissues of the ovary with direct or indirect effects on granulosa cells. Leukocytes, in particular macrophages, are abundant in the ovary during all stages of the oestrous cycle in pigs (Standaert et al. 1991) where, as in other species, they have been reported to have roles in ovulation and corpus luteum formation through their tissue remodelling and cytokine synthesis capabilities. In particular, macrophages have been recognised for their role in inducing progesterone production in granulosa cells in co-culture experiments in mice (Kirsch et al. 1981), humans (Halme et al. 1985) and in pigs (Standaert et al. 1990) without concomitant granulosa cell proliferation. Two potent cytokines secreted by macrophages, interleukin-1 and tumor necrosis factor-α, have been shown to stimulate both progesterone and prostaglandin synthesis (Kokia et al. 1992, Watanabe et al. 1993, Roby et al. 1999, Nakamura et al. 1990, Brannstrom et al. 1995, Brannstrom et al. 1994a) and these are likely to interact with other macrophage-derived factors to fine-tune gonadotrophin regulation of corpus luteum function and demise (Wuttke et al. 1997). In vitro experiments from human granulosa cells further implicate cytokines originating from ovarian cells in promoting proliferation and/or functional activation of steroidogenic cells (Jasper et al. 1996).

Increased expression of proinflammatory cytokines and COX-2 seen in uterine tissue after exposure to seminal plasma (Chapter 3) was not mirrored in the ovary even though similar increases in leukocyte recruitment were seen. Subtle effects of seminal plasma on cytokine expression may have
been masked by the large between-gilt variability or the result of poor quality RNA due to possible RNA degradation because the time taken to retrieve tissue after slaughter at the commercial abattoir was greater than optimal. Indeed, many of the mRNA transcripts were barely detectable. Moreover, the variable nature of cytokine expression and the relatively small number of animals involved in these experiments may have also contributed to the result.

The observations reported in this chapter and by Waberski et al. (1997) indicate that the effects of intrauterine seminal fluid on the pre- and peri-ovulatory ovary manifest as clear changes in the development and responsiveness to endogenous hormones including LH (Waberski et al. 1997a). Indeed, the significant increase in follicular progesterone due to seminal plasma as reported in this chapter may indicate a greater responsiveness to LH in vivo, likely responsible for the advancement in ovulation seen by others (Waberski et al. 1997b, Waberski et al. 1995, Weitze et al. 1990c, Weitze et al. 1990b). Consistent with these in vivo responses, prior seminal plasma treatment also altered ovarian cell behaviour ex vivo. Cultured granulosa cells from pre-ovulatory follicles showed increases in progesterone secretion, and were also more responsive to IGF-1 induced proliferation after recovery from seminal plasma-stimulated gilts. Cultured thecal tissue also showed greater steroidogenic responsiveness to IGF-1. Collectively, the in vitro results indicate that intrauterine seminal plasma induces functional changes to follicle cells that are maintained ex vivo and in isolation from other ovarian cells and immune cells present within the ovary. While the mechanisms underpinning this requires further dissection, it seems reasonable to speculate that uterine exposure to seminal plasma results in increased sensitivity to gonadotrophin hormones via changes in LH receptor or steroidogenic enzyme expression. Real time PCR analysis of LH receptor expression or expression of key enzymes involved in the steroidogenic pathway on cultured granulosa cells prior to and after in vitro treatments of hCG and growth factors may help to elucidate changes that occur in vivo due to seminal plasma treatment.

Combined day 5 and day 9 data indicated that CL from gilts treated with seminal plasma attained a greater mass without a corresponding increase in ovary weight or ovulation rate as determined by CL number. This response may be the result of increased maturation of the pre-ovulatory follicles, as reflected by increased steroidogenesis at the 34 h time point. Histochemical analysis of the CL would be one strategy to evaluate whether the increase in weight was due to greater vascularity or increased numbers of luteal cells.

Synthesis of progesterone by the corpus luteum in early pregnancy is essential for implantation and embryo survival. The kinetics of the rise in progesterone is an important factor in determining embryo
viability and differences in embryo survival have been related to changes in plasma progesterone levels in early pregnancy in sheep (Ashworth et al. 1989) and in gilts (Jindal et al. 1996).

Nutritionally-induced upregulation of progesterone early in pregnancy results in improved embryonic survival (Jindal et al. 1997, Almeida et al. 2000b). The effect is likely to be due to the influence of progesterone in uterine tissues, and the induction of endometrial receptivity via direct and indirect effects on uterine-derived embryotrophic growth factors (Robertson 2000). Furthermore, progesterone may have a direct effect on the ovary, indicated by experiments showing that exogenous progesterone injected intramuscularly 24 h before expected ovulation reduces the duration of ovulation by approximately 5 h in pigs (Duanyai et al. 1998). This presumably increases synchrony within the cohort of developing embryos, thereby potentially improving implantation success (Dzuik 1987).

In this chapter, the most significant result that implicates the effect of seminal plasma on improved embryo survival in the pig is the increased plasma progesterone levels extending past day 7 of pregnancy. An effect of seminal plasma on ovarian progesterone synthesis has not been previously reported in any species. In Chapter 3, intrauterine seminal plasma treatment was shown to be associated with increased numbers of viable embryos and a change in embryo development trajectory at day 5 and day 9. This was linked with altered patterns of uterine cytokine expression and an increased abundance of endometrial leukocytes that occurred not just during the acute response to insemination, but persisted for at least nine days into the pre-implantation phase. Others have also reported that seminal plasma treatment can reduce embryo mortality and increase synchrony of embryo development in pigs (Waberski et al. 1997b). The increase in embryo survival is likely due to elevated local progesterone production, acting to differentially regulate several progesterone-responsive uterine parameters including the altered cytokine expression which we observed.

The previous chapter discussed the role of seminal plasma in modulation of the uterine environment by inducing an inflammatory response characterised by the recruitment of leukocytes and upregulation of cytokines and COX-2 expression; the present chapter describes a similar response to seminal plasma in the ovary, reflected by the recruitment of leukocytes leading to enhanced steroidogenesis during a critical period for embryo development in the pig. Both support the working hypothesis that factors in seminal plasma enhance embryo survival by conditioning or priming the reproductive tract for the developing embryos before elongation and attachment occurs.

It is clear that the ovarian response to uterine exposure to seminal plasma involves an intermediary factor or factors that are generated in the uterus and transported to the ovary. What is less clear is the identity of the specific seminal factor(s) which generate the intermediary factor(s) and whether the
responses seen in this chapter and Chapter 3 result in increased embryo survival to term. However, the results reported here for the pig, and elsewhere in rodents (Tremellen et al. 1998) have implicated likely key candidate molecules involved in the mechanism behind the ovarian responses to seminal plasma.

TGFβ has been shown in mice to be a key active constituent in seminal plasma initiating an upregulation of pro-inflammatory cytokines and recruitment of leukocytes into the uterus (Tremellen et al. 1998). TGFβ is also present in abundance in boar semen (Chapter 5) and is likely to be involved in the seminal plasma-generated uterine inflammatory response. While it is difficult to envisage how seminal proteins could directly access the ovary, it seems reasonable to postulate that inflammatory mediators induced in the uterus after insemination might be transmitted in an indirect pathway.

Counter-current transfer may be defined as the transfer of solutes through closely connected vessels and viewed as means of local regulation of organ function (Einer-Jensen et al. 2005). In particular, ovarian function is maintained by the counter-current transfer that allows for the local transfer of solutes from the ovary to the uterus (Hunter et al. 1983, Cicinelli et al. 2004) and also transfer of solutes of uterine origin including prostaglandins to the ovary (Krzymowski et al. 1989). The nature of the counter-current transfer enables significant increases in the local concentration of solutes compared to that, which would be delivered by the systemic circulation. In most mammalian species, main arterial blood supply to the uterus is via the uterine artery, whereas the venous blood leaves only partly through the uterine vein with a significant fraction leaving through the uterine-ovarian vein thus facilitates a means for uterine substances to influence ovarian function (Ginther 1981, Einer-Jensen et al. 2005).

Inflammatory mediators induced in the uterus include the pro-inflammatory cytokines GM-CSF and IL-6 synthesized in the endometrium after seminal plasma infusion (Chapter 3). However, based on the molecular size and lipophilic properties of cytokines, it seems unlikely that these could be transported and exert their effects distally in the ovary.

A more likely candidate for a uterine-derived ovarian signal is prostaglandin E₂ (PGE₂). COX-2, the enzyme that catalyses the formation of PGE₂ (Jones et al. 1997) is upregulated in the uterus in response to uterine exposure to seminal plasma (Chapter 3). Prostaglandins produced in the uterus have been shown previously to be transported to the ovary via the uterine-ovarian counter current mechanism (Krzymowski et al. 1989), where their availability to the theca cells would be enhanced by increased vascularity of the thecal layer in the preovulatory follicle. Experiments to evaluate whether PGE₂ can mimic the effect of seminal plasma, or alternatively whether COX-2 inhibitors depress the response to seminal plasma, would be of value to investigate this postulate.
The role of prostaglandins in pre-ovulatory follicular function has been also been described in pre-pubertal gilts synchronised with eCG and hCG (Tsang et al. 1979, Ainsworth et al. 1980, Evans et al. 1983). Levels of PGE\(_2\) in follicular fluid were observed to peak at around 36 h post hCG in the follicle (Tsang et al. 1979). An inverse relationship of progesterone to estrogen was observed at the time of the PGE\(_2\) peak (Ainsworth et al. 1980), and theca cells were found to be the predominant follicular source of PGE\(_2\) compared with granulosa cells (Evans et al. 1983). These observations, together with up-regulation of endometrial COX-2 by seminal plasma, support the proposal that uterine-derived PGE\(_2\) may be the active intermediary in accelerating the follicular events which culminate in ovulation followed by corpus luteum development. In addition, the increased proliferative responses and progesterone production in granulosa cells retrieved from seminal plasma-treated gilts observed in the present study are consistent with a conditioning effect of higher levels of PGE\(_2\) leading to enhanced responses of these cells to hCG and IGF treatments in vitro compared to their PBS-treated counterparts. Through the capacity of PGE\(_2\) to induce chemokine expression and modulate the behaviour of myeloid leukocytes (Jones et al. 1997, Harizi et al. 2005), elevated PGE\(_2\) in the ovary could also contribute to the increased macrophage recruitment seen after seminal plasma treatment, so an indirect role of PGE\(_2\) acting via macrophages is also consistent with this model.

In conclusion, the results in this chapter show that factors in seminal plasma influence ovarian function most likely via a semen-uterine-ovarian communication axis. Key molecules behind this response potentially include seminal plasma-derived TGF\(\beta\) and PGE\(_2\) synthesised in the uterus in response to seminal plasma. Further investigations are required to fully understand the processes involved in the ovarian responses to seminal plasma and the effects on early embryo development in the pig.

4.12 PUBLICATION: SEMINAL PLASMA REGULATES OVARIAN PROGESTERONE PRODUCTION, LEUKOCYTE RECRUITMENT AND FOLLICULAR CELL RESPONSES IN THE PIG

NOTE: This publication is included in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

[http://dx.doi.org/10.1530/rep.1.01119](http://dx.doi.org/10.1530/rep.1.01119)
Chapter 5

The measurement and quantification of TGFβ₁ and TGFβ₂ in boar seminal plasma
5.1 INTRODUCTION

Pregnancy success in pigs is dependent on the tight control of events during each stage of the reproductive cycle. The previous chapters have described experiments that show how components of seminal plasma contribute to these events by influencing the cytokine, growth factor and steroid hormone milieu for the developing embryo. Experiments described in Chapter 3 show that intrauterine infusion of seminal plasma in gilts induces marked changes in endometrial cytokine expression and in resident leukocyte populations within the endometrium and luminal fluid. In addition, these local changes within the tract are accompanied by changes in ovarian leukocyte populations and elevated ovarian progesterone production from day 5 of pregnancy (Chapter 4).

The boar ejaculate varies from 150 to 500 ml and has a spermatozoa density ranging from 25,000 – 300,000 per µl (Mann et al. 1953). The large volume of ejaculate and the relative ease of collection have enabled boar semen to be studied extensively for several decades. As early as 1938, McKenzie et al. determined the relative volumetric contributions to the ejaculate as follows: 15-25% by the seminal vesicles, 10-20% by the Cowper's glands, 55-70% by the prostate and the urethral glands and only 2-5% by sperm and the epididymal fluid (cited by Mann & Leone 1953). Up to half of the ejaculate volume can consist of the gelatinous material which is produced in the bulbo-urethral glands (Mann 1954).

During the manual collection of the boar ejaculate the 'split-ejaculate method' is applied to distinguish 3 fractions of the semen. According to McKenzie et al., (1938) (cited by Mann, 1954) the pre-sperm fraction comprises 5-20%, the sperm-rich fraction 30-50%, and the post-sperm fraction 40-60% of the total ejaculate (Mann 1954). The pre-sperm fraction is watery and is likely to be of urethral origin, the sperm-rich fraction follows this and may also contain some gel, however the gel fraction usually follows the sperm-rich fraction. These 3 fractions, which complete an ejaculation 'wave' may be followed by a second ejaculation 'wave' which is also fractionated (Mann 1954). The 2 ejaculatory waves may last up to 30 minutes. For artificial insemination programs in commercial piggeries, only the sperm-rich portion of the ejaculate is collected and is normally diluted in diluent to a concentration of 3 x 10^9 spermatozoa per 100 ml. This equates to a dilution factor of the sperm-rich portion of the ejaculate ranging from 10-fold to 50-fold depending on volume and sperm density of the collected fraction.

The diluent used to make the semen doses can be regarded as a seminal plasma surrogate containing nutrient sources needed for the metabolic requirements of the sperm cell. These nutrients normally include glucose as an energy source to provide for the high metabolic demands of sperm in order to remain viable during transport to the site of fertilisation, bovine serum albumin (BSA) to protect the
sperm against cold shock, bicarbonate, tris buffer and HEPES for control of pH, and NaCl and KCl to regulate the osmotic potential (Althouse 1997, Althouse et al. 2000). In addition most semen extenders include antibiotics to inhibit bacterial growth. However, components including growth factors, steroid hormones and many seminal fluid proteins are not present in the commercially available semen extenders.

Seminal plasma contains many immuno-modulatory molecules and has paradoxically been shown to be immunosuppressive and immuno-stimulatory in a variety of species and under different assay conditions (Skibinski et al. 1992b, Kelly et al. 1994, Dostal et al. 1995, Cechova et al. 1989, Veselsky et al. 1992, Veselsky et al. 1997, Skibinski et al. 1992a, Stanek et al. 1985, Kelly et al. 1997). Of particular interest, and a focus of this chapter, is transforming growth factor beta (TGFβ), a potent cytokine present in the seminal fluid of most mammalian species studied to date (Robertson et al. 2001a). Seminal TGFβ has been identified in our laboratory as being a principal active factor that drives the post-mating inflammatory response characterised by the recruitment of leukocytes and up-regulation of pro-inflammatory cytokine expression in the uterus in mice (Tremellen et al. 1998).

TGFβ is present in murine seminal plasma at approximately 100 – 200 ng/ml in the inactive or latent form, and is subsequently activated in the female reproductive tract. TGFβ isoforms play key roles in the initiation, progression and resolution of the mammalian immune response via the capacity to influence T cell proliferation, inflammatory cell recruitment and activation and dendritic cell differentiation (reviewed by Robertson 2002). The actions of TGFβ might also be described as paradoxical with immune-suppressive and pro-inflammatory roles depending on the microenvironment. For example, TGFβ is instrumental in providing immune privilege in the anterior chamber of the eye in mice via skewing the immune response away from Type 1 dominated response towards a favourable Type 2 mediated immune response (D’Orazio et al. 1998). However, in a different environment such as in sites of inflammation and injury, TGFβ isoforms act as proinflammatory agents in the recruitment and activation of monocytes (Ashcroft 1999). The multifunctionality of TGFβ, therefore, rests on the cells and tissues including other cytokines present that influence TGFβ action (Sporn 1999). For example, IFNγ is known to impede TGFβ action by inhibiting TGFβ signalling (Massague 2000) and bacterial lipopolysaccharide (LPS) elicits TGFβ production by monocytes (Reed 1999).

In this chapter, the optimisation and validation protocols for determining levels of TGFβ1 and TGFβ2 in porcine seminal plasma are described. In addition, levels of IFNγ and endotoxin (LPS) were determined in a subset of boars from the Bunge Meat Industries (BMI) herd in NSW.
5.2 STUDY DESIGN

Semen was collected from stud boars at South Australian Boar Stud (SABOR) and BMI. The ejaculate minus the gel fraction was collected by AI technicians using the glove-in-hand method and seminal plasma was prepared and stored as previously described (Chapter 2.4). Semen was also collected using the split ejaculate method (Section 2.3.1) from boars at SABOR to measure the levels of TGFβ₁ and TGFβ₂ within the different fractions that make up the complete ejaculate minus the gel fraction. The gel fraction was not included in our analysis for TGFβ content for practical reasons. All the seminal plasma samples used to optimise and validate the measurement of seminal TGFβ in boar seminal plasma were obtained from boars at SABOR.

In total, semen was collected from 53 boars (12 from SABOR and 41 from BMI piggeries) of known fertility. Fertility in this context was determined by the mating outcomes and litter sizes of at least 5 matings for each boar. The breeds of boars were Large White, Landrace or F1 (Large White X Landrace). The Pearson correlation test (SPSS v13) was used to investigate the relationship between concentration of TGFβ₁ and TGFβ₂ and levels of IFNγ and bacterial endotoxin in boar seminal plasma are investigated. In addition to the measurements of these cytokines and endotoxin in boar seminal plasma made in this chapter, the relationships between these and boar fertility are described in Chapter 6.

5.3 PRINCIPLE AND METHOD OF TGFβ ASSAY

Initially, ELISA systems from R&D (Quantikine : R&D Systems, Minneapolis, MN) and Promega were compared for cost and sensitivity using porcine colostrum and boar seminal plasma. The Promega Emax® ImmunoAssay System (Madison, WI) was chosen as being more cost effective, whilst having equivalent sensitivity to the R&D ELISA system.

TGFβ₁ and TGFβ₂ content in boar seminal plasma were measured using the Emax® ImmunoAssay System by Promega which is sensitive and specific for the detection of biologically active TGFβ₁ and TGFβ₂ in an antibody ELISA sandwich format. Flat bottom 96 well plates were coated with TGFβ mAb, which binds soluble TGFβ₁ or TGFβ₂. After overnight incubation, standards, controls and samples were aliquoted using a transferpipette into the wells and any TGFβ present was bound to the immobilised coating antibody. The captured TGFβ was then bound by a second specific polyclonal antibody conjugated to horseradish peroxidase (TGFβ HRP conjugate). Any unbound conjugate was removed.
by washing and following incubation with a chromogenic substrate, the colour change of which was measured using Bio-Rad Laboratories Benchmark Microplate reader. The amount of TGFβ in the samples is proportional to the colour generated in the oxidation-reduction reaction. Using this assay, biologically active TGFβ was quantified in diluted boar seminal plasma in the range of 32-1000 pg/ml.

Total TGFβ was measured by transient acidification of the sample before addition to wells, and to measure the amount of naturally processed or active TGFβ in the sample, the acidification step was bypassed. Thus, total TGFβ refers to the amount of TGFβ measured in seminal plasma after transient acidification. Active TGFβ refers to the amount of TGFβ determined in seminal plasma without transient acidification.

Samples were analysed in duplicate but also in triplicate and quadruplicate in optimisation and validation assays. A standard curve was produced by plotting the log absorbance (Y axis) versus log dilution of recombinant standard (X axis) for each plate. Dilutions of samples were prepared to ensure that the absorbance of samples fell within the linear range of the standard curve. Regression analyses using the standard curve were performed to determine the concentration of TGFβ in the diluted boar seminal plasma samples and the value calculated was multiplied by the dilution factor to yield the concentration of TGFβ in pg/ml.

5.4 VALIDATION AND OPTIMISATION OF ELISA ASSAYS FOR MEASURING TOTAL TGFβ1 AND TGFβ2 CONTENT IN BOAR SEMINAL PLASMA

The boar seminal plasma samples used for validating ELISA assays, stability analysis of seminal TGFβ and effect of semen collection frequency on seminal TGFβ content, were from any one or a combination of three Large White boars (CW198, CW213 and CW318) and one Landrace boar (CL790) located at SABOR boar stud at Clare in South Australia. These boars were owned by The University of Adelaide and semen was frequently collected from these boars for the AI program at the PPPI piggery at Roseworthy in South Australia. Aliquots of acid activated seminal plasma samples of single ejaculates were stored at -20°C for use as positive controls for each ELISA plate.

5.4.1 Activation of TGFβ1 and TGFβ2 in boar seminal plasma

TGFβ is secreted in vivo by most cells as a biologically inactive complex, called the large latent TGFβ complex (Lawrence 1996). The large latent TGFβ complex is comprised of the TGFβ homodimer non-covalently associated with a latency-associated peptide (LAP), which is covalently bound to a larger
peptide called latent TGFβ binding protein (LTBP). TGFβ can be released and activated from the large latent complex in vivo by a number of factors including oxidants, cell associated plasmin, and via interactions with integrins or matrix glycoproteins (Gleizes et al. 1997). The latency proteins contribute to TGFβ stability. In serum, free TGFβ has a half-life of about 2 minutes whereas latent TGFβ has a half-life of 90 minutes (Lawrence 1996). In vitro, TGFβ can be released to its biologically active form by physiochemical means that denature the LTBP and allow the release of the biologically active TGFβ from the LAP (Lawrence 1996).

Using R&D Systems Quantikine TGFβ1 ELISA kit (Cat. No. DB100) the effectiveness of heat (80°C for 10 min), acid (pH of 3.0 for 10 min) and alkaline (pH of 8.9 for 10 min) treatments to release biologically active TGFβ from its LAP were investigated. The manufacturers’ (Promega and R&D) instructions to measure total TGFβ1 and TGFβ2 in biological samples are to acidify the sample to pH 3.0 or below. This procedure was used to transiently acidify seminal plasma and porcine colostrum (used for comparison purposes) diluted 1:5 in PBS to approximately pH 2.6 using 1M HCl for 20 minutes. The sample was then neutralised to approximately pH 7.6 by adding the equivalent volume of 1M NaOH. The final dilution was 1:5.26 after checking that the required pH was met. For activation of latent TGFβ using the alkaline activation method, 25 µl of 2.7M NaOH in 1M HEPES was added (Table 5.1) to attain a pH of between 8.8 and 9.1 for a period of 20 mins (R&D protocol) followed by 13 µl of 1M HCl, resulting in a dilution of seminal plasma of 1:5.38. The components involved in the activation of latent TGFβ1 and TGFβ2 by acid and alkaline means are listed in Table 5.1.

The activation protocol was evaluated using seminal plasma samples from two boars and a colostrum sample from a post-partum sow. These samples were activated, diluted 1:1052 in 1x sample buffer and assayed using the Promega Immunoassay® System. The levels of TGFβ1 in these samples under different activation protocols are shown (Table 5.2).
### Acid Activation

- **Aliquot 100 µl seminal plasma or colostrum**
- **Add 400 µl PBS**
- **Add 13 µl HCl (sample acidified to pH 2.52 and incubate at RT for 20 min)**
- **13 µl NaOH (sample neutralised to pH 7.7)**

### Basic Activation

- **Aliquot 100 µl seminal plasma or colostrum**
- **Add 400 µl PBS**
- **Add 25 µl 2.7N NaOH/1M HEPES (sample alkalised to pH 8.9 and incubate at RT for 20 min)**
- **13 µl HCl (sample neutralised to pH 7.8)**

Table 5.1 The activation protocol to measure total TGFβ content in seminal plasma and porcine colostrum.

Colostrum, which is naturally high in TGFβ content, was used for comparison.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Acid activation (ng/ml)</th>
<th>Alkaline activation (ng/ml)</th>
<th>Heat activation (ng/ml)</th>
<th>No activation (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colostrum</td>
<td>233.6</td>
<td>203.2</td>
<td>196.5</td>
<td>87.3</td>
</tr>
<tr>
<td>SP Boar 1</td>
<td>438.5</td>
<td>411.2</td>
<td>359.2</td>
<td>343.8</td>
</tr>
<tr>
<td>SP Boar 2</td>
<td>172.1</td>
<td>134.2</td>
<td>148.7</td>
<td>135.7</td>
</tr>
</tbody>
</table>

Table 5.2 Comparison of activation methods used to measure biologically active TGFβ1 in seminal plasma (SP) and colostrum.

All values above represent the mean of samples assayed in triplicate and all values have a CV of less than 14%.
After investigating activation protocols for the measurement of total TGFβ₁ in seminal plasma, it was decided to activate all seminal plasma samples using the standard acid activation protocol detailed above. This was due to the simplicity of this activation protocol and also the relatively low levels of assay variation observed (Section 5.5). Samples of seminal plasma were analysed from boars from SABOR boar stud to determine TGFβ₁ and TGFβ₂ content present in both active and total states. These samples were acidified, diluted and analysed and concentrations of TGFβ₁ and TGFβ₂ are shown (Figure 5.1).
Figure 5.1 The concentration of total TGFβ₁ and TGFβ₂ compared with active TGFβ₁ and TGFβ₂ in boar seminal plasma.

Seminal plasma from 5 boars was analysed for TGFβ content before acid activation (active TGFβ) and after transient acidification for 20 minutes (total TGFβ). Following transient acidification, samples were further diluted to a final dilution of 1:1052 in 1x Sample Buffer immediately prior to assay. All seminal plasma samples were measured in triplicate for this experiment. The boars in this study are from SABOR piggery. Data from individual boars are displayed as black dots and the mean + SEM are represented as the column plot.
The content of total compared with active TGFβ₁ and TGFβ₂ in boar seminal plasma was not significantly different in seminal plasma collected from the boars for this analysis. Thus, almost all of the TGFβ₁ and TGFβ₂ present in the boar ejaculate was present in the active form, free of latency associated peptide. In addition, the TGFβ₂ content for this cohort of boars from SABOR in South Australia is approximately equal to the level measured for the TGFβ₁ isoform. The acid activation produced slightly higher levels of TGFβ compared with the basic activation of seminal plasma (Table 5.2). It can be noted however, that full evaluation of the activation protocols was not carried out and the intra-assay and inter-assay variation in measuring total TGFβ₁ and TGFβ₂ using the basic activation and heat activation method was not compared.

5.4.2 Validation of TGFβ₁ Immunoassay – Linearity-of-dilution and Spike-and-recovery experiments

Seminal plasma is a biological fluid that may contain binding proteins including antibodies that could inhibit or interfere with detection of TGFβ using ELISA. To investigate the precision and accuracy of the Promega TGFβ₁ Emax® Immunoassay System, linearity-of-dilution and spike-and-recovery experiments were performed. In the first experiment the optimal dilution of seminal plasma was determined by serially diluting activated seminal plasma from 3 boars and measuring TGFβ₁ content in 3 replicate assays. The coefficient of variation (CV) was determined for each sample within each assay and was plotted against the dilution of each sample to determine the optimal dilution producing the lowest CV values (Figure 5.2). The CV was greatest in the more concentrated seminal plasma samples ranging from 1:10 to 1:400 of activated seminal plasma in sample buffer. By comparison, dilutions between 1:400 and 1:1000 were found to have levels of variation within an acceptable level for determining protein content using immunoassays (Reid 2000).
Figure 5.2 The effect of dilution of activated boar seminal plasma on coefficient of variation using Promega TGFβ1 Emax® Immunoassay System.

Seminal plasma samples from 3 boars were acid activated and serially diluted from 1:10 to 1:2000 for the determination of total TGFβ1 content. This procedure was repeated for 3 separate assays and the amount of variation in total TGFβ1 concentration between each assay was determined for each boar and each dilution. Data are mean coefficient of variation (%) ± SD versus each of the dilutions tested.
For the spike and recovery experiments, known amounts of recombinant human TGFβ1 (rhTGFβ1) protein (R&D Systems Cat. No. 100-B-001) was added to seminal plasma from 2 boars and one colostrum sample which were diluted (1:1052) in 1x sample buffer after transient acidification and immediately assayed for TGFβ1 content. The content of recovered TGFβ1 was determined based on the values calculated from the standard curve using the rTGFβ1 contained within the kit (Table 5.3). The average recovery of TGFβ1 was 98% in seminal plasma for boar 1 (range 96-102) and 97% for boar 2 (range 89-105). Recovery rates were lower for colostrum, but still within the acceptable range with an average recovery rate of 92% (range 86-97).
<table>
<thead>
<tr>
<th>Sample</th>
<th>Endogenous TGFβ1 (ng/ml)</th>
<th>TGFβ1 Spiked Amount (ng/ml)</th>
<th>Expected TGFβ1 (ng/ml)</th>
<th>Measured TGFβ1 (ng/ml)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP Boar 1</td>
<td>425</td>
<td>500</td>
<td>925</td>
<td>944</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>425</td>
<td>250</td>
<td>672</td>
<td>645</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>425</td>
<td>125</td>
<td>550</td>
<td>533</td>
<td>97</td>
</tr>
<tr>
<td>SP Boar 2</td>
<td>98</td>
<td>500</td>
<td>598</td>
<td>628</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>98</td>
<td>250</td>
<td>348</td>
<td>341</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>98</td>
<td>125</td>
<td>223</td>
<td>198</td>
<td>89</td>
</tr>
<tr>
<td>Colostrum</td>
<td>212</td>
<td>500</td>
<td>712</td>
<td>690</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>212</td>
<td>250</td>
<td>462</td>
<td>434</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>212</td>
<td>125</td>
<td>337</td>
<td>290</td>
<td>86</td>
</tr>
</tbody>
</table>

Table 5.3 Spike and recovery experiments for the validation of measuring TGFβ1 in boar seminal plasma.

Seminal plasma (SP) from two boars and colostrum from one sow were transiently acidified for activation of TGFβ1, neutralised, diluted 1:1052 and spiked (see text) with recombinant human TGFβ1. Samples were then assayed using Promega Immunoassay for the detection of total seminal TGFβ1 including the spiked rhTGFβ1 level.
5.4.3 Inter-assay and intra-assay variation using the Promega Emax® Immunoassay System for measurement of TGFβ₁ and TGFβ₂.

Inter-assay and intra-assay variation was determined using activated seminal plasma samples from 3 boars and one colostrum sample from one sow. Variation in detected levels of TGFβ₁ and TGFβ₂ content was determined by calculating the coefficient of variation as described previously (Section 5.3.2). Intra-assay variation for the detection of TGFβ was similar between the two isoforms investigated (Table 5.4 and 5.5).
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>N (# of replicate wells)</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Mean (ng/ml)</td>
<td>363.1</td>
<td>479.9</td>
<td>215.5</td>
<td>219.0</td>
<td>315.9</td>
<td>417.7</td>
<td>195.6</td>
<td>83.9</td>
</tr>
<tr>
<td>Std Dev</td>
<td>29.4</td>
<td>21.8</td>
<td>12.5</td>
<td>19.7</td>
<td>35.7</td>
<td>39.7</td>
<td>24.0</td>
<td>7.9</td>
</tr>
<tr>
<td>Intra-assay CV (%)</td>
<td>8.1</td>
<td>4.5</td>
<td>5.8</td>
<td>9.0</td>
<td>11.3</td>
<td>9.5</td>
<td>12.3</td>
<td>9.4</td>
</tr>
</tbody>
</table>

Table 5.4 Intra-assay variation in detection of TGFβ₁ and TGFβ₂ content in boar seminal plasma and colostrum using Promega Emax® Immunoassay System.

Seminal plasma and colostrum (Colost) samples were transiently acidified and diluted to 1:1052 in 1x Sample Buffer.
Table 5.5 Inter-assay variation observed in the detection of TGFβ₁ and TGFβ₂ content in boar seminal plasma and colostrum using Promega Emax® Immunoassay System.

Seminal plasma and colostrum (Colost) samples were transiently acidified and diluted to 1:1052 in 1x Sample Buffer. Each sample was analysed in triplicate each of 4-7 individual assays stated.
5.4.4 The effect of collection frequency on TGFβ content in boar seminal plasma

In the pig industry, fertile boars are utilised extensively to provide semen for AI programs to commercial piggeries. In Australia, there seems to be little consistency with regard to how often semen is collected from boars. Generally, young boars (1-2 years of age) may be collected 3 to 4 times per week and mature boars (>3 years of age) can be collected on average 1-2 times per week (personal communication D. Tonkin; SABOR, S.A.). To investigate the possibility that frequency of semen collection may influence seminal TGFβ₁ and TGFβ₂ content, the full ejaculate from 3 boars was collected 3 times within a 7 day period (day 0, day 3 and day 5) and assayed for TGFβ₁ and TGFβ₂. In addition, the full ejaculate was collected using the split-ejaculate method to investigate the levels of TGFβ₁ and TGFβ₂ within each fraction and determine whether these levels vary across the series of collection. The boars used in this experiment had no semen collections for at least 7 days before the first semen sample was collected.

Seminal TGFβ₁ and TGFβ₂ were present in all 3 fractions of the boar ejaculate with the vast majority, due to the higher volume, contained within the second fraction (Table 5.6 and 5.7). Within each fraction of the ejaculate, the concentration of TGFβ₁ and TGFβ₂ dropped by 40% - 52% for the third collection for TGFβ₁ and 34% - 40% for TGFβ₂ (Table 5.6 and 5.7). In addition, there was a reduction in total TGFβ₁ and TGFβ₂ in progressive collections for each of these three boars. Using the total TGFβ per ejaculate to compare TGFβ content of sequential samples, there was a 43% to 97% drop in total TGFβ₁ (µg) from first collection to third collection seven days later for these 3 boars (Table 5.8). Similarly, total TGFβ₂ levels per ejaculate were depleted with an approximate 60% drop in levels from the first to the last collection. In this small study, seminal TGFβ₁ concentrations were more variable between boars and the levels of depletion of TGFβ₁ after repeated collection were also variable, with one boar having a 97% reduction in total TGFβ₁ per ejaculate between the first and third collection within seven days (Table 5.8). The volume of ejaculate collected also varied due to timing of collection and between boars. The volume of ejaculate dropped on average 35% between the first and second collection. Changes in the ejaculate volume between the second and third collection were smaller. However, ejaculate volume was correlated with total TGFβ₁ (r² = 0.86, P = 0.003) but did not correlate as strongly with total TGFβ₂ (r² = 0.60, P = 0.09).
Table 5.6 The effect of repeated collection on TGFβ1 concentration within fractions of boar ejaculate.

Semen was collected by the split ejaculate method (see text). Fraction 1: pre-sperm rich fraction; fraction 2: sperm rich fraction; fraction 3: post-sperm rich fraction. Data are presented as mean ± SEM of three boars.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Collection day 1</th>
<th>Collection day 4</th>
<th>Collection day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TGFβ1 ng/ml</td>
<td>Vol. (mls)</td>
<td>TGFβ1 ng/ml</td>
</tr>
<tr>
<td>1</td>
<td>34.7 ± 5.1</td>
<td>7.0 ± 0.6</td>
<td>28.4 ± 1.8</td>
</tr>
<tr>
<td>2</td>
<td>33.2 ± 3.4</td>
<td>177.3 ± 27.4</td>
<td>27.3 ± 7.0</td>
</tr>
<tr>
<td>3</td>
<td>31.1 ± 2.3</td>
<td>17.3 ± 5.4</td>
<td>25.1 ± 5.0</td>
</tr>
</tbody>
</table>

Table 5.7 The effect of repeated collection on TGFβ2 concentration within fractions of boar ejaculates.

Semen was collected by the split ejaculate method (see text). Fraction 1: pre-sperm rich fraction; fraction 2: sperm rich fraction; fraction 3: post-sperm rich fraction. Data are presented as mean ± SEM of three boars.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Collection day 1</th>
<th>Collection day 4</th>
<th>Collection day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TGFβ2 (ng/ml)</td>
<td>Vol. (mls)</td>
<td>TGFβ2 (ng/ml)</td>
</tr>
<tr>
<td>1</td>
<td>64.0 ± 5.7</td>
<td>7.0 ± 0.6</td>
<td>39.9 ± 1.4</td>
</tr>
<tr>
<td>2</td>
<td>53.6 ± 8.8</td>
<td>177.3 ± 27.4</td>
<td>49.9 ± 8.9</td>
</tr>
<tr>
<td>3</td>
<td>25.3 ± 14.7</td>
<td>17.3 ± 5.4</td>
<td>25.3 ± 16.6</td>
</tr>
</tbody>
</table>
### Table 5.8 The effect of repeated collection on TGFβ1 concentration and total TGFβ1 content per ejaculate.

TGFβ1 content was determined by ELISA assay with each data point representing 3 replicates with a cv < 14%. Total TGFβ1 was calculated using the volume (minus the gel fraction) recorded for each ejaculate collected. Percent reduction from first collection is given in parentheses on collection day 7.

<table>
<thead>
<tr>
<th>Boar ID</th>
<th>Collection day 1</th>
<th>Collection day 4</th>
<th>Collection day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TGFβ1 (ng/ml)</td>
<td>TGFβ1 total (µg)</td>
<td>TGFβ1 (ng/ml)</td>
</tr>
<tr>
<td>107W</td>
<td>38.9</td>
<td>9.8</td>
<td>27.6</td>
</tr>
<tr>
<td></td>
<td>19.3 (50%)</td>
<td></td>
<td>3.2</td>
</tr>
<tr>
<td>108W</td>
<td>31.4</td>
<td>6.6</td>
<td>38.7</td>
</tr>
<tr>
<td></td>
<td>33.0 (0%)</td>
<td></td>
<td>3.8</td>
</tr>
<tr>
<td>CW1180</td>
<td>28.7</td>
<td>4.1</td>
<td>14.7</td>
</tr>
<tr>
<td></td>
<td>1.7 (94%)</td>
<td></td>
<td>0.2</td>
</tr>
</tbody>
</table>

### Table 5.9 The effect repeated collection on TGFβ2 concentration and total TGFβ2 content per ejaculate.

TGFβ2 content was determined by ELISA assay with each data point representing 3 replicates with a cv < 14%. Total TGFβ2 was calculated using the volume (minus the gel fraction) recorded for each ejaculate collected. Percent change from first collection is given in parentheses on collection day 7.

<table>
<thead>
<tr>
<th>Boar ID</th>
<th>Collection day 1</th>
<th>Collection day 4</th>
<th>Collection day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TGFβ2 (ng/ml)</td>
<td>TGFβ2 total (µg)</td>
<td>TGFβ2 (ng/ml)</td>
</tr>
<tr>
<td>107W</td>
<td>35.7</td>
<td>9.0</td>
<td>32.3</td>
</tr>
<tr>
<td></td>
<td>22.3 (37%)</td>
<td></td>
<td>3.7</td>
</tr>
<tr>
<td>108W</td>
<td>56.6</td>
<td>11.9</td>
<td>62.9</td>
</tr>
<tr>
<td></td>
<td>41.2 (27%)</td>
<td></td>
<td>4.7</td>
</tr>
<tr>
<td>CW1180</td>
<td>64.3</td>
<td>9.2</td>
<td>48.9</td>
</tr>
<tr>
<td></td>
<td>31.1 (52%)</td>
<td></td>
<td>3.7</td>
</tr>
</tbody>
</table>
5.5 THE EFFECT OF DIFFERENT STORAGE TIMES AT 4°C ON DETECTION AND MEASUREMENT OF TGFβ1 AND TGFβ2 IN BOAR SEMINAL PLASMA

To investigate the stability of TGFβ1 and TGFβ2 in the sperm-rich portion (fraction 2) of the boar ejaculate at 4°C, the ejaculate from one boar was collected and the sperm-rich fraction was put on ice and transported from SABOR to the laboratory arriving approximately one hour later. The sperm-rich portion was centrifuged at 1200 g for 20 minutes to recover seminal plasma and divided into 5 ml aliquots. Aliquots of seminal plasma were then stored at 4°C for periods ranging from 1 hour to seven days (Table 5.10) before being snap frozen in liquid nitrogen and stored at -80°C until assay for TGFβ1 and TGFβ2 content.

The concentrations of TGFβ1 measured by immunoassay remained relatively static for at least 24 h after collection with storage at 4°C (Table 5.10). Thereafter, detection of TGFβ1 dropped 18% after storage seminal plasma at 4°C for 2 days, to a 62% reduction in detectable levels by 7 days of storage at 4°C. A similar pattern was observed for TGFβ2 with a significant reduction of 21% from original levels by day 4 to a 57% reduction by day 7 (Table 5.10).
<table>
<thead>
<tr>
<th>Time of storage of SP at 4°C post collection (h/days)</th>
<th>TGFβ₁ (ng/ml)</th>
<th>TGFβ₂ (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h</td>
<td>28.1</td>
<td>43.9</td>
</tr>
<tr>
<td>4 h</td>
<td>28.1</td>
<td>47.8</td>
</tr>
<tr>
<td>1 d</td>
<td>27.6</td>
<td>46.5</td>
</tr>
<tr>
<td>2 d</td>
<td>23.1 (18%)</td>
<td>44.9</td>
</tr>
<tr>
<td>3 d</td>
<td>21.4 (24%)</td>
<td>46.7</td>
</tr>
<tr>
<td>4 d</td>
<td>21.2 (25%)</td>
<td>34.7 (21%)</td>
</tr>
<tr>
<td>5 d</td>
<td>20.4 (28%)</td>
<td>31.9 (27%)</td>
</tr>
<tr>
<td>6 d</td>
<td>19.3 (32%)</td>
<td>25.6 (42%)</td>
</tr>
<tr>
<td>7 d</td>
<td>10.7 (62%)</td>
<td>19.2 (57%)</td>
</tr>
</tbody>
</table>

Table 5.10 The effect of storage at 4°C on the detection and measurement of TGFβ₁ and TGFβ₂ in seminal plasma derived from the sperm-rich portion of the boar ejaculate. The percent reduction in measured TGFβ₁ and TGFβ₂ is given in parentheses.
5.5.1 The stability of TGFβ₁ and TGFβ₂ at -20°C

To investigate the stability of the immunoactive TGFβ protein in boar seminal plasma at -20°C, the sperm-rich portion of fresh ejaculates from two boars were collected and transported to the laboratory on ice arriving within an hour of collection. Seminal plasma was collected by centrifugation at 4°C and 2 ml aliquots were stored at -20°C. These aliquots were also used as positive controls for future ELISA analyses. An aliquot of the fresh seminal plasma from each boar was processed on the same day of collection for measurement of TGFβ₁ and TGFβ₂ by ELISA. There was no appreciable reduction in TGFβ content from the fresh seminal plasma sample for at least 6 months storage at -20°C and interestingly 15% more TGFβ₂ was detected in boar 1 seminal plasma, which may reflect variation between assays for this sample.

5.5.2 The stability of TGFβ₁ after repeated freeze/thaw cycles in liquid nitrogen

The ejaculate from one boar was collected to investigate the stability of TGFβ₁ in seminal plasma. A dewar of liquid N₂ and a portable centrifuge was taken to the boar semen collection laboratory at SABOR in South Australia. The sperm-rich fraction of a boar ejaculate was collected and centrifuged to collect seminal plasma. Several aliquots were made and were immediately placed in ice (4°C; fresh seminal plasma) and into liquid N₂. At the University laboratory, two aliquots at a time were taken out of liquid N₂ and allowed to thaw to room temperature before being returned to the liquid N₂. This cycle was repeated for 4 and 10 times for the 2 aliquots. After 10 freeze/thaw cycles there was no appreciable change above intra-assay variation for the immune-active TGFβ₁ by ELISA. The concentrations of TGFβ₂ were not investigated but are likely to have shown a similar response to TGFβ₁.
### Table 5.11: The stability of TGFβ₁ and TGFβ₂ content in boar seminal plasma after storage at -20°C.

<table>
<thead>
<tr>
<th>Storage at -20°C</th>
<th>TGFβ₁ (ng/ml)</th>
<th>TGFβ₂ (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Boar 1</td>
<td>Boar 2</td>
</tr>
<tr>
<td>Fresh seminal plasma</td>
<td>189.6</td>
<td>274.3</td>
</tr>
<tr>
<td>1 week</td>
<td>200.4</td>
<td>285.2</td>
</tr>
<tr>
<td>4 months</td>
<td>192.7</td>
<td>287.9</td>
</tr>
<tr>
<td>3 months</td>
<td>204.1</td>
<td>310.3</td>
</tr>
<tr>
<td>6 months</td>
<td>195.1 (8%)</td>
<td>309.1 (11%)</td>
</tr>
</tbody>
</table>

Immuno activity of TGFβ₁ and TGFβ₂ was measured in seminal plasma aliquots from 2 boars using Promega ELISA ImmunoAssay System. These seminal plasma aliquots were kept at -20°C for use as positive controls for ELISA detection of TGFβ in seminal plasma for the boars involved in experiments described here and in chapter 6. Fresh seminal plasma refers to an aliquot of seminal plasma that was immediately stored at 4°C and processed for ELISA the same day it was collected. The percent change from the fresh seminal plasma sample is shown in parentheses. Seminal plasma was activated for the detection of TGFβ immediately before ELISA analysis took place and was stored at -20°C undiluted.

### Table 5.12: The stability of TGFβ₁ content in seminal plasma after repeated freeze/thaw cycles in liquid nitrogen.

<table>
<thead>
<tr>
<th>Freeze/Thaw cycles</th>
<th>TGFβ₁ (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 – Fresh seminal plasma</td>
<td>162.9</td>
</tr>
<tr>
<td>1 x Freeze/thaw</td>
<td>178.2</td>
</tr>
<tr>
<td>4 x Freeze/thaw</td>
<td>193.7</td>
</tr>
<tr>
<td>10 x Freeze/thaw</td>
<td>185.2 (11%)</td>
</tr>
</tbody>
</table>

Table 5.12: The stability of TGFβ₁ content in seminal plasma after repeated freeze/thaw cycles in liquid nitrogen.
TGFβ1 was measured in seminal plasma aliquots from one boar (110W) after aliquots were subjected to repeated freeze/thaw cycles in liquid N₂. Immuno-activity was detected and quantified using R&D Quantikine kit after activation of TGFβ using transient acidification.
5.5.3 Variation in TGFβ₁ and TGFβ₂ in boar seminal plasma over time

One of the aims of this thesis is to explore whether TGFβ might be useful as diagnostic marker of boar fertility for application in the pig industry. An ideal fertility indicator is one in which the concentrations do not fluctuate within an individual significantly over time. To determine if the content of seminal TGFβ is relatively constant within individual boars, 6 seminal plasma samples were taken from 5 boars over a period of 6 months with at least one week of abstinence prior to each collection. The 5 boars were selected on the basis of the concentrations of TGFβ₁ measured in their semen to represent boars with high, middle and low seminal TGFβ₁ content. Representative data from 5 of these boars is shown below for TGFβ₁ and TGFβ₂ (Figure 5.3 and 5.4).

Both the TGFβ₁ and TGFβ₂ content of these samples were found to vary less than 20% from the mean value for each boar across the entire collection period.

---

**Figure 5.3** The concentrations of TGFβ₁ in individual boars for 6 collections.

Six collections of seminal plasma were collected from 5 boars over a period of 6 months with at least 7 days between collections. Total TGFβ₁ concentration was measured by ELISA after transient acidification. Each symbol represents a single collection from an individual boar.

**Figure 5.4** The concentrations of TGFβ₂ in individual boars for 6 collections.

Six collections of seminal plasma were collected from 5 boars over a period of 6 months with at least 7 days between collections. TGFβ₂ was measured by ELISA after transient acidification. Each symbol represents a single collection from an individual boar.
5.5.4 The content of TGFβ₁ and TGFβ₂ content in boar seminal plasma

Seminal plasma collected from a larger cohort of boars from SABOR and BMI Industries were analysed after transient acid activation by immunoassay for total TGFβ₁ and TGFβ₂ concentration to obtain a measure of the variation in boar TGFβ content in a larger population. Seminal plasma from 36 boars consisting of Landrace (n=8), Large White (n=4) and F1 (Landrace x Large White; n= 24) strains were assessed. TGFβ₁ was the predominant isoform in boar seminal plasma (Figure 5.5) and was approximately 4-fold higher than the TGFβ₂ isoform (mean ± SEM; 200.7 ± 21.8 vs 51.4 ± 2.6, p < 0.01). However, some boars had increased concentrations of TGFβ₂ compared with TGFβ₁ (108W and CW1180, Section 5.4.4). There were not sufficient numbers of pure bred boars involved in this study to determine whether different strains of boars had significantly different levels of TGFβ in their seminal plasma.
Figure 5.5 The level of total TGF$\beta_1$ and TGF$\beta_2$ in boar seminal plasma. Seminal plasma from 36 boars was analysed by immunoassay after transient acidification (total TGF$\beta$).

All seminal plasma samples were measured in duplicate and the mean ± SEM are represented as column plots. The stud boars of proven fertility were from SABOR (n = 5) and BMI (n= 31) piggeries and measured 200.7 ± 21 ng/ml (range 49.9 – 737.4) for TGF$\beta_1$ and 51.4 ± 3 ng/ml (range 25.1 – 102.4) for TGF$\beta_2$ content in seminal plasma. a:b p < 0.01
5.5.4.1 The relationship between TGFβ₁ and TGFβ₂ in boar seminal plasma

Pearson's correlation coefficient's ($r^2$) were calculated to investigate the relationship between TGFβ₁ and TGFβ₂ content in boar seminal plasma. There was a weak, non-significant linear positive relationship between the concentrations of the two isoforms ($r^2 = 0.28$, $p = 0.09$; Figure 5.6). In addition, there were similar concentrations of TGFβ₁ and TGFβ₂ in seminal plasma from boars at SABOR in South Australia and boars from BMI in NSW.
Figure 5.6 The relationship between total TGFβ1 and TGFβ2 concentration in boar seminal plasma.

Pearson correlations ($r^2$) were made between total TGFβ1 and TGFβ2 concentrations in seminal plasma samples from 36 boars. Each dot point represents the mean value of TGFβ1 and TGFβ2 for one boar. Data was analysed using SPSS statistical software. $r^2 = 0.29$, $p = 0.089$. 
5.5.5 The presence of interferon gamma and bacterial endotoxin in boar seminal plasma

Seminal plasma contains many proteins that may synergise or inhibit the action of TGFβ. Two known modulators of TGFβ action are interferon gamma (IFNγ) and bacterial endotoxin. The presence of IFNγ in boar seminal plasma was investigated using Endogen Porcine Interferon Gamma Colorimetric ELISA kit. Diluted seminal plasma in sterile PBS (1:10; SP:PBS) from 42 boars was analysed in duplicate. For determining the amount of bacterial endotoxin present in boar seminal plasma, a Limulus Amebocyte Lysate (LAL) test from BioWhittaker, Inc. was used. Only two boars out of 42 tested positive for IFNγ in their seminal plasma with similar levels of IFNγ detected (Table 5.11). The level of IFNγ in these two positive seminal plasma samples were 39 and 40 pg/ml, significantly above the threshold of 2.2 pg/ml for this assay. The presence of relatively high levels of IFNγ in seminal plasma from these two boars is surprising. Whether an infection or another cause was responsible could not be determined as these two boars had normal fertility and were not identified as showing signs of infection during this trial. In addition, these boars were part of the field trial and matings (A.I.) with these boars produced live born offspring with similar pregnancy outcome parameters to the other boars used in the trial (Chapter 6).

The bacterial LAL assay was used to detect and quantify the gram-negative bacterial endotoxin level in boar seminal plasma. The outer membrane of gram-negative bacteria consists of lipopolysaccharide (LPS) which acts as an endotoxin in binding toll-like receptor 4 and eliciting cytokine responses in mammalian cells (Tizard 1995). The presence of LPS in semen has the potential to activate cytokine expression in the female after mating but also could influence the action of other signalling agents such as TGFβ present in seminal plasma (Robertson et al. 2001a). All of the seminal plasma samples from 30 boars were positive for the presence of bacterial endotoxin with a mean value of 21.5 EU/ml (Table 5.14). There was large variation in the concentration of bacterial endotoxin between boars and the range was 11 to 193 EU/ml. The relationship between seminal endotoxin level and boar fertility was investigated in the field trial (Chapter 6).
Seminal component measured | Content (positive samples/total number)
---|---
Interferon gamma (n=42) | 39 and 40 pg/ml (2/42)†
all others < 2 pg/ml
Bacterial endotoxin (n=30) | 21.5 ± 19.2 EU (30/30)†

Table 5.13 The presence and content of interferon gamma and bacterial endotoxin in boar seminal plasma.

Interferon gamma was measured by diluting boar seminal plasma 1:10 in sterile PBS and analysed in duplicate using Endogen pig ELISA IFN-γ kit. Bacterial endotoxin was measured by diluting boar seminal plasma 1:10 in pyrogen-free water and analysed in duplicate using BioWhittaker Quantitative Chromogenic Limulus Amebocyte Lysate kit. †The mean ± SEM for samples in which activity was detected, and the number of positive samples out of total individual boar samples measured is given in parentheses.
5.6 DISCUSSION

Detection and quantification of TGFβ in boar seminal plasma using ELISA assays for the major TGFβ isoforms TGFβ1 and TGFβ2 was validated and optimised using the Promega Emax® ImmunoAssay System. The immuno-detection of these isoforms of TGFβ displayed reasonable parallelism over dilutions from 1:400 to over 1:1000 dilution of the sperm rich portion (fraction 2) of the boar ejaculate. Using transient acidification of seminal plasma both active and latent forms of TGFβ were quantified and, uniquely in the boar when compared to mouse and human semen (Nocera et al. 1995, Robertson et al. 2002), approximately 95% of the TGFβ1 and TGFβ2 in seminal plasma was seen to exist in the active form.

Transforming growth factor beta is present in boar seminal plasma in considerable quantities with a mean of 200 ng/ml for TGFβ1 (range 32-737) and 51 ng/ml (range 28-102) for TGFβ2 and similar values were obtained in samples from a cohort of boars from piggeries in South Australia and NSW. The vast majority of the TGFβ present in boar seminal plasma is already in its active state ready to bind with its cognate receptor. Since TGFβ is not synthesised in an active form but must be activated after contact with activating agents after secretion from cells, it seems reasonable to hypothesise that the TGFβ is activated during the mixing of fluids from accessory sex glands during ejaculation in the boar since it would seem potentially hazardous to generate active TGFβ in high levels within the accessory glands.

The concentrations of total and active TGFβ1 and TGFβ2 have not been previously reported for boar seminal plasma and are similar in concentration for TGFβ1 to human seminal plasma (Nocera et al. 1995). However, total content of TGFβ1 in a boar ejaculate is much higher due to the much larger volume of ejaculate produced. There was considerable variation in the concentrations of TGFβ between individual boars with an approximate 24-fold range, but less variation in the TGFβ2 isoform with a 3.6-fold range between boars. In addition, in some boars the predominant isoform was often TGFβ2 and this contrasts to both human and mouse seminal plasma where TGFβ2 is usually less than 10% of the content of seminal TGFβ1 (Nocera et al. 1995, Robertson et al. 2002).

However, minimal variability in concentrations of the two major TGFβ isoforms was seen in consecutive samples taken from individual boars. The content of both TGFβ1 and TGFβ2 within a cohort of 12 boars from BMI fluctuated less than 20% from the mean value for each boar of 6 collections over a period of six months. This stability in the content of TGFβ over time supports the value of measuring TGFβ in
seminal plasma as a possible fertility indicator (Chapter 5). However, the frequent ejaculate collection within individual boars had a dramatic effect on the detection of TGFβ in boar seminal plasma with a loss of between 43 to 97% for TGFβ₁ and a loss of approximately 60% for TGFβ₂ after 3 collections within the same week.

Due to the relatively short half-life of TGFβ in biological fluids (Lawrence 1996), the effect of transport and storage conditions of seminal plasma on the immuno-detection of TGFβ₁ and TGFβ₂ was investigated. Samples of seminal plasma stored at 4°C for 24 h had little effect on the levels of TGFβ₁, however by 4 days at 4°C immuno-detection in seminal TGFβ₁ dropped by 18% and by 7 days there was a decline of more than 62%. A similar pattern was seen with TGFβ₂ with an initial stability for the first few days and then a dramatic drop in recovery from day 4 to day 7. The effect of long term storage at -20°C, -70°C or repeated freeze/thawing cycles on the quantification of seminal TGFβ₁ and TGFβ₂ were also investigated. There was no significant difference in immuno-activity in both isoforms of TGFβ when seminal plasma was stored for weeks or many months at -20°C or -70°C. Also, up to 10 freeze/thaw cycles in liquid Nitrogen had no effect on the detection of TGFβ₁ or TGFβ₂. This result provided confidence in the storage conditions used for investigating the effect of intrauterine seminal plasma treatment in the field trial (Chapter 6).

Transforming growth factor beta is present in biological fluids along with several of other proteins and substances that can influence TGFβ bioactivity and its ability to bind to its cognate receptor. Studies using cell culture of human and mouse endometrial and cervical cells have shown that bacterial lipopolysaccharides (LPS) induces cytokine synthesis through binding to Toll-like receptors TLR2 and TLR4 and potentially acts in an additive manner with TGFβ function to influence uterine cytokine expression (Robertson 2005, Schaefer et al. 2004). Conversely, other proteins such as the Type 1 cytokine interferon gamma (IFNγ) act as a potent inhibitor of TGFβ signalling (Glynn et al. 2004, Robertson 2005). To investigate whether these two possible modulators of TGFβ function are present in boar seminal plasma, seminal plasma from 42 boars were assayed for interferon gamma (IFNγ) content and seminal plasma from 30 boars for presence of bacterial endotoxin. Although optimisation and validation protocols were not performed for the utility of these assays with seminal plasma, 2 out of 42 boars tested positive for IFNγ in their semen and all samples from 30 boars were positive for bacterial endotoxin. The presence of IFNγ in seminal plasma in humans may indicate the presence of an infection in the reproductive tract and is rarely detected in normal healthy men (Politch et al. 2007). It may be possible that the 2 boars that tested positive for IFNγ had a current or recent urinary tract infection, however other symptoms of infection were not overtly evident and the possibility was not
investigated further. The collection of boar ejaculate is not performed in a sterile environment and is normally collected with a view to minimising any potential bacterial contamination. The finding that all samples contained bacterial endotoxin suggests that gram negative bacteria that normally inhabit the reproductive tract of the boar are largely responsible for the endotoxin levels measured. However, due to time and cost limitations this could not be fully investigated. The significance of these immune modulators present in boar seminal plasma remains to be elucidated but the results here do provide the first evidence in the boar that immunostimulatory molecules are present and are likely to influence endometrial gene expression and, therefore, possibly pregnancy outcome in the sow. Moreover, extensive dilution of the sperm-rich fraction of the boar ejaculate in AI regimens without regard to the depletion of the immune-regulatory molecules contained within may explain lower pregnancy outcomes due to AI matings compared with natural mating in the pig (Skjervold 1975). Further investigations into the effect of cytokines contained within seminal plasma may provide support for surrogate seminal plasma treatments that can be incorporated in AI diluents to increase embryo survival and litter size in pigs.

In summary, TGFβ1 and TGFβ2 are present in boar seminal plasma in concentrations similar to those seen in human and mouse semen. There is large variation in seminal plasma TGFβ content between boars but low variation within individual boars over time, and both isoforms are predominantly present in the active state. This TGFβ together with other immune regulatory factors in seminal plasma such as LPS likely act to induce cytokine gene expression in the sow after mating. Other factors such as IFNγ present in seminal plasma of some boars could modulate the effects of TGFβ and LPS.
Chapter 6

Effect of intrauterine seminal plasma infusion on litter size in gilts
6.1 INTRODUCTION

The previous chapters showed that components of boar seminal plasma influence early events within the female reproductive tract after mating resulting in changes in uterine and ovarian function which are likely to influence embryo development and survival. In addition, it was shown that seminal plasma contains immune-regulatory molecules that have the potential to initiate these events and improve reproductive outcome in the pig.

The observed responses to seminal plasma during early pregnancy in the pig uterus are similar to those seen for mouse models and show that exposure to seminal plasma initiates an inflammatory response in the endometrium characterised by increased production of cytokines and the recruitment of leukocytes (Robertson 2005). More recently, studies from our laboratory have demonstrated that factors in seminal plasma induce the expansion of T cell populations, including T regulatory cells, in early pregnancy in mice and induce immune tolerance to paternal antigens that may be expressed on the semi-allogeneic embryos (Guerin et al. 2009, Moldenhauer et al. 2009, Robertson et al. 2009). These studies suggest that tolerogenic processes may also contribute during early pregnancy in the pig, especially during the embryonic attachment period when the maternal immune system would presumably have closer contact with paternal antigens expressed on the implanting embryo. The effects of seminal plasma on ovarian function in the pig (Chapter 4) are also reflected in mouse studies with increases in leukocytes within stromal tissue and increases in CL weight (Gangnuss et al. 2004), although in the mouse there was no concomitant rise in progesterone production.

The responses to seminal plasma in the early stages of pregnancy have the potential to significantly improve embryo survival during the critical pre-attachment period in the pig. Cytokines play a major role in the immunology of the reproductive tract with respect to immune cell function, recruitment, paternal antigen processing, endometrial function and maternal immune-permissiveness to the developing embryo (Robertson et al. 1997). The initial maternal response to seminal fluid may influence the success of many downstream processes necessary for pregnancy success in mammals. One key driver of the seminal plasma-induced immune response is TGFβ which is present in considerable quantities in boar seminal plasma along with other key modulators of TGFβ action including IFNγ and LPS (Chapter 5).

This chapter reports a field study that aimed to investigate whether intrauterine administration of seminal plasma applied before the first AI can improve reproductive outcome in the pig. In addition, seminal plasma levels of TGFβ1 and TGFβ2 were measured and correlations were made to indices of pregnancy success in pigs.
6.2 STUDY DESIGN

The field trial to investigate the effect of intrauterine seminal plasma on reproductive outcome was carried out at Bunge Meat Industries Piggery (BMI) in Corowa NSW. The trial took place over a period of approximately eleven months from November 1999 through to August 2001 to investigate whether the changes elicited by seminal plasma treatment result in increased pregnancy rates and larger litter sizes. The protocol for the field trial was described in Chapter 2.16. Briefly, conception rate, litter size, birth weight and day 14 piglet weight were compared in 282 gilts treated at 30 weeks of age during oestrus with either 150 ml seminal plasma or saline (sterile PBS), delivered transcervically to the uterus approximately 90 min prior to the first AI. Each 150 ml of seminal plasma used for treatment was provided from seminal plasma pooled from the ejaculates of 6 boars and stored frozen at -20°C. The 6 boars that provided the semen for the seminal plasma treatments were selected from a cohort of 19 proven fertile boars that were required to provide enough seminal plasma for 141 gilts for this treatment arm of the trial. There was at least 7 days of abstinence between collections with 112 semen collections used in total. All gilts received a second AI within 24 h of the first insemination as per standard piggery practice. All semen used in AI doses and seminal plasma was assayed for TGFβ₁ and TGFβ₂ content, such that total TGFβ content was determined for every mating in this study.
6.3  THE EFFECT OF INTRAUTERINE SEMINAL PLASMA INFUSION ON REPRODUCTIVE OUTCOME IN GILTS

6.3.1  The effect of intrauterine seminal plasma on conception parameters in gilts

All mated gilts and sows at BMI underwent ultrasound scanning to determine pregnancy approximately 30 days after mating. Conception data based on the number of gilts that returned to oestrus after mating (returns), number of gilts that had a positive pregnancy ultrasound assessment but did not farrow (not in pig, NIP), the number of gilts that aborted their pregnancy as defined by observing a vaginal discharge after receiving a positive pregnancy diagnosis by ultrasound (abortions) and number of gilts that died for usually unknown reasons (deaths) were recorded (Table 6.1).
### Table 6.6.1 The effect of intrauterine infusion of seminal plasma or PBS on conception data in gilts.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Farrowings</th>
<th>Returns</th>
<th>NIPs</th>
<th>Abortions</th>
<th>Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP (n=141)</td>
<td>93 (66%)</td>
<td>20 (14%)</td>
<td>15 (11%)</td>
<td>7 (5%)</td>
<td>6 (4%)</td>
</tr>
<tr>
<td>PBS (n=141)</td>
<td>101 (72%)</td>
<td>21 (15%)</td>
<td>12 (8%)</td>
<td>4 (3%)</td>
<td>3 (2%)</td>
</tr>
</tbody>
</table>

Intrauterine infusion of pooled seminal plasma (SP) or filtered-sterile phosphate buffered saline (PBS) was given 90 min prior to first AI in gilts at approximately 30 weeks of age. At approximately 30 days after the first AI all mated gilts received ultrasound scanning to determine pregnancy status. Data are recorded values and the proportion of gilts within each category is represented as a percent of total in parentheses.
Intrauterine insemination of pooled seminal plasma in gilts did not have any effect on farrowing rate or pregnancy state in gilts when compared with the PBS control counterparts (Table 6.1). The effect of intrauterine infusion of seminal plasma on fertility parameters in gilts resulted in a 66% farrowing rate that was slightly lower than that for gilts infused with PBS, (however this did not reach statistical significance). Seminal plasma treatment also had no effect when compared to PBS-treated controls to the rates of returns, NIPs, abortions and deaths. The values for all these fertility measures are below industry targets, however were not significantly different from the overall on-farm conception results that commercial piggery experienced for this period (data not shown).

### 6.3.2 The effect of intrauterine seminal plasma on pregnancy outcome in gilts

Approximately 115 days after AI and seminal plasma and PBS treatment, the gilts farrowed and the number of piglets born alive, stillborn and mummified were recorded. In addition, piglets were weighed on their day of birth and 14 days later to provide a measure of piglet viability and growth. Intrauterine seminal plasma treatment did not increase the litter size of gilts nor did it influence numbers of stillborn or mummified piglets (Table 6.2). In addition, there were no significant differences in piglet weight or rate of weight gain to day 14.
Table 6.6.2 The effect of intrauterine infusion of seminal plasma or PBS on pregnancy outcome and weight gain in piglets.

A the onset of standing oestrus, gilts at approximately 30 weeks of age received intrauterine infusions of pooled seminal plasma or sterile PBS. Litter size data and birth weights and piglet weight at day 14 were collected. Data are mean ± SEM and treatment effects were analysed by Student’s t-test (SPSS), no significant differences in any parameter were found between gilts treated with intrauterine seminal plasma or sterile PBS.
The total number of piglets born (data not shown) and the number of piglets born alive to gilts within this experiment was not different to the average on-farm pregnancy results for the same period. The still born and mummified fetus rates are consistent with industry and on-farm targets as are the birth weights of piglets within this study. However, the litter sizes for gilt matings recorded for this study (Table 6.2, Col 2) and for the piggery are significantly below industry targets (Meo et al. 1999).

### 6.4 THE EFFECT OF TGF\(\beta\) CONTENT IN SEMINAL PLASMA ON CONCEPTION AND PREGNANCY OUTCOMES IN GILTS

The relationship between content of total TGF\(\beta_1\), TGF\(\beta_2\) and TGF\(\beta_1\) plus TFG\(\beta_2\) in seminal plasma collected from 44 boars used in this field experiment and pregnancy outcome was examined in several hundred matings across different parities from 10/2/2000 to 30/4/2001. All the matings consisted of two standard AI procedures. Even though the boars were involved in the field trial described above, the analysis of correlations with fertility did not include those matings involved in this study. TGF\(\beta\) content was evaluated against fertility parameters including proportion of (1) live born, (2) still births, and (3) mummified births per successful pregnancy, and ‘conception’ parameters eg. (1) returns and (2) farrowings from all services.

#### 6.4.1 The relationship between seminal plasma TGF\(\beta\) content and boar fertility

A single sample of seminal plasma was collected from 44 boars at BMI, with at least 7 days of abstinence prior to sample collection. The total TGF\(\beta_1\) and TGF\(\beta_2\) content was individually determined. The median (range) values for TGF\(\beta_1\) were 150 (28-495) ng/ml and 123 (96-144) ng/ml for TGF\(\beta_2\). There was a considerable variation between boars for both TGF\(\beta\) isoforms with a 16-fold range seen for TGF\(\beta_1\) content and a less than 2-fold range for TGF\(\beta_2\) content. No significant correlation was found between TGF\(\beta\) content and any parameters of conception rate (Table 6.3) or fertility (Table 6.4).

The content of TGF\(\beta_1\) and TGF\(\beta_2\) either individually or combined in seminal plasma did not correlate with the rate of farrowing, the rate of returns, or the number of gilts that did not farrow after mating (Table 6.3).
Table 6.3 The relationship between TGFβ content in boar seminal plasma and boar fertility.

<table>
<thead>
<tr>
<th></th>
<th>% gilts farrowing(^1)</th>
<th>% return to oestrus</th>
<th>% gilts not farrowing(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFβ1 Pearson correlation</td>
<td>-0.23</td>
<td>0.12</td>
<td>0.20</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>0.26</td>
<td>0.60</td>
<td>0.55</td>
</tr>
<tr>
<td>N</td>
<td>44</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td>TGFβ2 Pearson correlation</td>
<td>-0.19</td>
<td>-0.11</td>
<td>-0.21</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>0.33</td>
<td>0.64</td>
<td>0.54</td>
</tr>
<tr>
<td>N</td>
<td>44</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td>TGFβ1&amp;2 Pearson correlation</td>
<td>-0.25</td>
<td>0.14</td>
<td>0.14</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>0.20</td>
<td>0.68</td>
<td>0.68</td>
</tr>
<tr>
<td>N</td>
<td>44</td>
<td>21</td>
<td>11</td>
</tr>
</tbody>
</table>

Table 6.3 The relationship between TGFβ content in boar seminal plasma and boar fertility.

Boar seminal plasma was collected from 44 individual boars and assayed for TGFβ\(_1\) and TGFβ\(_2\) content. Relationship between TGFβ content and fertility indices were compared using Pearson correlation analysis (SPSS). N: number of boars, \(^1\)Data are pooled from gilts mated with individual boars in BMI trial (n=6-12 matings per boar) \(^2\)Not farrowing includes gilts yielding negative pregnancy test, not in pig or aborting.
<table>
<thead>
<tr>
<th></th>
<th>Liveborn¹</th>
<th>%still born</th>
<th>%mummified</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFβ1 Pearson correlation</td>
<td>-0.19</td>
<td>-0.12</td>
<td>-0.11</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>0.24</td>
<td>0.44</td>
<td>0.50</td>
</tr>
<tr>
<td>N</td>
<td>44</td>
<td>44</td>
<td>40</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Liveborn¹</th>
<th>%still born</th>
<th>%mummified</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFβ2 Pearson correlation</td>
<td>-0.25</td>
<td>-0.11</td>
<td>-0.14</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>0.11</td>
<td>0.50</td>
<td>0.40</td>
</tr>
<tr>
<td>N</td>
<td>44</td>
<td>44</td>
<td>40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Liveborn¹</th>
<th>%still born</th>
<th>%mummified</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFβ1&amp;2 Pearson correlation</td>
<td>-0.23</td>
<td>-0.13</td>
<td>-0.13</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>0.15</td>
<td>0.40</td>
<td>0.41</td>
</tr>
<tr>
<td>N</td>
<td>41</td>
<td>44</td>
<td>40</td>
</tr>
</tbody>
</table>

Table 6.4 The relationship between TGFβ content in boar seminal plasma and pregnancy outcome in gilts.

TGFβ content of seminal plasma was determined by ELISA ¹ Data are pooled from gilts mated with individual boars (N=6-12 matings per boar).
In addition to the analysis above, the TGFβ1 and TGFβ2 content of seminal plasma collected from boars used for the pooled seminal plasma treatment arm of the BMI trial were analysed (Table 6.5). In total there were 22 collections for the pooled seminal plasma treatments and gilts that received seminal plasma treatment received on average 225.6 ng/ml of TGFβ1 and 48.4 ng/ml of TGFβ2 approximately 90 minutes before the first A.I. Moreover, the level of TGFβ1 and TGFβ2 in the seminal plasma treatments were analysed against 'conception' parameters eg. (1) returns and (2) farrowings within 74 gilts from the SP treatment arm of the trial. No significant correlation was found between TGFβ content in seminal plasma and measures of conception rates (Table 6.6).
Table 6.5 TGFβ₁ and TGFβ₂ content in pooled seminal plasma.

Seminal plasma was collected from 4 – 6 boars on 22 separate occasions to provide enough seminal plasma for the treatment arm of the field trial. Each pooled seminal plasma collection was analysed for TGFβ₁ and TGFβ₂ content by ELISA analysis.

<table>
<thead>
<tr>
<th>Fate</th>
<th>TGFβ₁ (ng/ml)</th>
<th>TGFβ₂ (ng/ml)</th>
<th>TGFβ 1&amp;2 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farrowings (n=53)</td>
<td>191 (50-283)</td>
<td>46.9 (30-81)</td>
<td>238 (80-334)</td>
</tr>
<tr>
<td>Returns (n=10)</td>
<td>148 (50-291)</td>
<td>42.1 (30-81)</td>
<td>190 (80-325)</td>
</tr>
<tr>
<td>'Not in pig' (n=2)</td>
<td>251 (211-291)</td>
<td>55 (34-77)</td>
<td>306 (288-325)</td>
</tr>
<tr>
<td>Neg. preg. (n=4)</td>
<td>261 (211-287)</td>
<td>44 (31-77)</td>
<td>305 (288-321)</td>
</tr>
<tr>
<td>Aborts (n=5)</td>
<td>212 (50-287)</td>
<td>50 (30-81)</td>
<td>263 (80-334)</td>
</tr>
</tbody>
</table>

Table 6.6 The concentration of TGFβ₁ & TGFβ₂ in pooled intrauterine seminal plasma treatments delivered to gilts before AI in relation to conception parameters.

N: number of gilts, Data are from gilts in the seminal plasma arm of the BMI trial. Neg Preg: gilts that did not return to oestrus but tested negative for pregnancy (Neg Preg) 21 days after AI.
In addition, sperm density and ejaculate volume were recorded on all samples. There was a positive correlation between TGFβ levels and sperm density (Table 6.7). There were no correlations between TGFβ content and ejaculate volume.
<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Sperm density</th>
<th>Ejaculate volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFGβ1 Pearson correlation</td>
<td>0.30</td>
<td>0.14</td>
</tr>
<tr>
<td>Sig. (2 tailed)</td>
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<td>0.24</td>
</tr>
<tr>
<td>N</td>
<td>69</td>
<td>69</td>
</tr>
<tr>
<td>TFGβ2 Pearson correlation</td>
<td>0.29</td>
<td>0.04</td>
</tr>
<tr>
<td>Sig. (2 tailed)</td>
<td>0.02*</td>
<td>0.73</td>
</tr>
<tr>
<td>N</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>TGFβ1&amp;β2 Pearson correlation</td>
<td>0.32</td>
<td>0.14</td>
</tr>
<tr>
<td>Sig. (2 tailed)</td>
<td>0.007*</td>
<td>0.26</td>
</tr>
<tr>
<td>N</td>
<td>65</td>
<td>65</td>
</tr>
</tbody>
</table>

Table 6.7 The relationship between TGFβ1 and TGFβ2 and sperm density and ejaculate volume in BMI line 1 and line 2 boars.

1Sperm density was determined by Computer-Aided Sperm Assessment Spectrophotometry. Relationship between TGFβ content and sperm density and ejaculate volume were compared using Pearson correlation analysis (SPSS). *P<0.05.
6.5 DISCUSSION

Litter size in the pig is a major limitation to the profitability of the pig industry. The potential litter size determined by ovulation rate is rarely realised and ovulation rate is estimated to be 35-45% higher than actual litter size (Dzuik 1987). Since the advent of efficient and cost effective artificial insemination procedures in the pig industry, strategies have been developed to replicate actions of seminal plasma to be used in conjunction with AI to improve litter sizes without negating the benefits that AI affords to the industry. The extensive dilution of seminal plasma in AI regimens could influence reproductive performance in sows by substantially diminishing the exposure of the female tract to steroid hormones and immune regulatory factors contained within seminal plasma. Two studies from South America infused oestrogen at the onset of oestrus before AI and compared pregnancy outcome with seminal plasma treatment similar to the protocol used within this study (Bortolozzo et al. 2000, Stahlberg et al. 2001); the researchers did not see an effect with oestrogen. However, in their first study comparing the effect of season, seminal plasma infusion increased litter size by an average of 1.34 piglets in 570 sows during summer but there was no effect of seminal plasma seen during the winter period in 449 sows (Bortolozzo et al. 2000). Approximately a year later, a repeat experiment comparing intrauterine infusions of seminal plasma and oestrogen to a non-infused group showed no effect on litter size in 211 sows (Stahlberg et al. 2001). The differences in outcome may lie in the differences in collection, storage and preparation of seminal plasma which may influence bioavailability and half life of active components.

The previous chapter described the quantification of immunoreactive TGFβ in boar seminal plasma. TGFβ is present in high levels in boar seminal plasma and is likely to be one of the active factors in driving the maternal immune responses after mating in the pig, as reported for the mouse (Robertson et al. 2002, Tremellen et al. 1998). Intrauterine seminal plasma treatment in gilts influences the maternal immune response, characterised by the increase in recruitment of leukocytes into the endometrium and also into ovarian tissue within nine days post treatment (Chapter 3 and 4). The aim of the experiments described in this chapter was to investigate in a field trial at a commercial piggery whether the events influenced by seminal plasma result in an increase in embryo survival leading to increased successful pregnancy rates and litter sizes.

Intrauterine seminal plasma treatment given approximately 90 minutes prior to AI did not result in an increase in pregnancy rate or litter size in gilts. Compared to the PBS-treated control gilts, there were no differences detected in farrowing rates or the number of gilts returning to oestrus, not in pig or the
number of abortions. Likewise, the number of piglets born alive, stillborn and mummified fetuses was similar between treatment groups.

Although the observations of this fertility trial may at first sight argue against the central hypotheses, there are several confounding factors that need to be considered before the hypothesis is rejected. Firstly, before starting the study, statistical power analysis was performed (Chapter 2, 2.19) using litter size data based on the previous year’s (1997-1998) average litter size data (personal communication P. Smith, research manager BMI) to determine sample size (n). The standard deviation was 2.0 and the mean litter size was 10 for first parity sows. Within the period of 12 months on this farm, the litter size for first parity sows dropped to below 9 and the standard deviation rose to 2.6. This change in litter size was also reflected in this study. The increase in variation in litter size in itself would not prevent a detection of a difference of one piglet per litter due to treatment, but it does indicate that there are other confounding factors present at the piggery that could influence or mask the effect of seminal plasma treatment. Secondly, TGFβ bioactivity (in contrast to TGFβ immunoactivity) is very labile in biological tissues with a half life of approximately 3-4 minutes (Wakefield et al. 1992). Normally in biological fluids and tissues TGFβ is complexed to its latency associated peptide extending the half life to beyond 100 minutes (Wakefield et al. 1992). Thus, it remains possible that the pooled, frozen and thawed seminal plasma preparations used in the BMI trial may have been largely depleted of bioactive TGFβ. Considering the commercial farm setting, an extended period between thawing the seminal plasma and its administration to gilts is likely to have adversely influenced the amount of bioactive TGFβ in the intrauterine treatment.

Additionally, it is possible that artificial administration of seminal plasma does not fully replicate the physiological effects of natural mating. Clearly there are differences in the timing of delivery with respect to sperm delivery and ovulation compared with natural mating. Also the female receives neuroendocrine signals from boar exposure and the physical events of mating and intromission that are not replicated by artificial insemination. Furthermore the sequential delivery of different component fluids of the ejaculate (especially the gel fraction) does not occur. These may interact with the cellular and molecular changes to influence the net effect on reproductive outcome.

Using the data of the TGFβ1 and TGFβ2 concentrations in seminal plasma from 44 boars part of the seminal plasma trial and correlating these levels with the results of matings with mixed parity sows that were not part of this trial, we were unable to show an association between TGFβ content in seminal plasma and the fertility or fecundity of boars. The TGFβ concentrations were the mean values of several seminal plasma collections and the fertility data was retrospective analysis from the BMI
piggery records from several AI matings with each of these boars. The rationale for this study was to investigate whether TGFβ content of seminal plasma, knowing that it is relatively stable between collections greater than 7 days (Chapter 5), could provide a predictive fertility measure even though the seminal plasma is extensively diluted in providing extended semen doses for AI. However, this did not prove to be the case. It is reasonable to expect that quantification of TGFβ may not on its own be a sufficient measure of seminal plasma activity. Studies in other species have identified additional factors including bacterial endotoxin and other cytokines that synergise or inhibit the action of TGFβ, altering its capacity to elicit the desired response in female reproductive tract tissues (Glynn et al. 2004, Robertson et al. 2002, Sivaramakrishnan et al. 2004). We have also shown that at least some of these additional moieties are present in porcine seminal plasma (Chapter 5). For example, boar semen clearly contains abundant gram negative bacteria. Thus it may be expected that any assay for boar fertility would need to include measures of each of these other active contributing moieties, and that a net ‘cytokine index’ or even ‘index of bacterial load’ is required. Other TGFβ family members are present in human seminal plasma and have been shown to be active in human cervical cells in vitro assays (Robertson and Sharkey, unpublished data).

Nevertheless, on the basis of these data we cannot refute the alternative null hypothesis and it may be that seminal plasma treatment does not influence reproductive outcome in gilts. This would be consistent with the view that the role of the post-mating inflammatory response is limited to preventing infection after mating, and/or removal of seminal fluid debris, and has no bearing on uterine receptivity to pregnancy or embryo development. An interesting finding from the study investigating the content of TGFβ in seminal plasma and boar fertility was the positive correlation between TGFβ and sperm density. This might be due to TGFβ protein binding to boar sperm as immunocytochemical studies with human sperm have shown positive staining for TGFβ predominantly in the post-acrosomal region of the sperm after incubation with anti-TGFβ1 antibodies (Chu et al. 1996). Alternatively, the two parameters may not be directly associated but instead reflect common regulatory control, for example both are ultimately regulated by testosterone (de Kretser 1979, Ingman et al. 2009).
Chapter 7

General discussion
One major limitation to profitability in the pig industry is the relatively small litter sizes born compared to the potential litter size based on ovulation rate. The potential litter size is believed to be 50% greater than what is usually realised in many Australian herds (Australian_Pork_Limited 2006-2008). By far the greatest obstacle in reaching optimal litter sizes is the extent of embryonic mortality before day 30 of pregnancy. Successful pregnancy in the pig as in other species is dependent on the tight control of a series of reproductive events that act in concert to produce changes in ovarian, uterine and embryo function. Emerging evidence is beginning to challenge our traditional understanding of the influence of each of these compartments on the developing embryo to determine its viability and survival during the critical pre-implantation period.

The studies described in this thesis add to the current knowledge of the events in early pregnancy that contribute to embryo development and pregnancy success in the pig. In particular, the results add to a growing bank of evidence suggesting that seminal factors deposited directly into the uterus at mating have a direct effect on the functionality not only on the endometrium it contacts, but also on the ovary and the embryo for some time after insemination. In addition, maternal immune responses are likely to be modified and primed to tolerate paternal antigens present on the cells of the embryo. The initial response to semen in the female tract in mammals is the rapid and extensive influx of inflammatory cells to the site of semen deposition. This post-mating inflammatory response has been characterised mostly in mice (De et al. 1991, McMaster et al. 1993, Robertson et al. 1996c) and to a lesser degree in humans (Sharkey et al. 2007), horses (Fiala et al. 2007), pigs (Bischof et al. 1994, Rozeboom et al. 1999) and insects (Kapelnikov et al. 2008). In all species, the influx of immune cells due to exposure of the female reproductive tract to semen is associated with a concomitant rise in tissue mRNA expression of cytokines and chemokine sustaining the recruitment and/or activation of the inflammatory cells.

The experiments contained herein have expanded our current knowledge regarding the influence of seminal plasma during early pregnancy in the pig and begin to define a molecular and cellular pathway that may explain why AI has less than optimal reproductive outcomes compared with natural mating. The results demonstrate the physiological consequences of seminal plasma deposition in the reproductive tract of the female pig. These include (1) the characterisation of the post-mating inflammatory response beginning with uterine inflammation and oedema, and maintained for some time on a molecular level represented by the increases in endometrial mRNA expression of pro-
inflammatory cytokines and COX-2 and on a histological level the recruitment of leukocytes, (2) effects in the ovary in terms of leukocyte recruitment, corpora lutea development and increased production of progesterone and (3) possible effects on the development of the embryo during the critical period of embryo loss in the pig.

The results reported in this thesis provide a substantial advance in our understanding of the molecular and cellular events occurring in response to seminal fluid in the pig. In chapter 3, the inflammatory response to seminal plasma was characterised in uterine tissue using immunohistochemical analysis for the detection and quantification of infiltrating leukocytes. Intrauterine seminal plasma treatment increased the abundance of uterine endometrial, stromal and myometrial leukocytes between 30 and 77% higher than in PBS treated gilts in the pre-ovulatory time point and this increase was maintained until at least 9 days after treatment. The leukocytes were predominantly macrophages with at least one third of them being professional antigen presenting cells of the activated macrophage and dendritic cell phenotypes. Activated macrophages along with other leukocytes secrete potent signalling molecules including vascular endothelial growth factor (VEGF) involved in tissue restructuring and angiogenesis (Sunderkotter et al. 1994). The initial observation once the uterine tracts were recovered from gilts 34 h after treatment was the increased vascularisation and weight of the tracts (Chapter 3). This observation may be in part due to the increased recruitment of activated immune cells increasing vascularisation and oedema at the tissue level after exposure to seminal plasma. The significance of these changes for early embryo development in the pig remains to be fully determined, but is likely to involve the extensive tissue remodelling that takes place before day 30 of pregnancy in the uterus and in producing ‘messengers’ in the crosstalk between the uterine tissue and the developing embryo purported to be crucial for the successful attachment of the embryo to the edometrium and for programming its subsequent developmental competence (discussed later and described in Figure 7.2).

Quantitative real-time PCR analysis identified proinflammatory cytokines expressed in uterine tissue that are implicated in controlling the inflammatory response to seminal plasma. In the pre-ovulatory period in the pig, seminal factors dramatically increased uterine mRNA expression of GM-CSF, IL-6 and MCP-1. The increase in expression of these cytokines was dissipated by day 5 post treatment when compared to PBS-treated gilts. GM-CSF is known not only as a proinflammatory cytokine (Robertson et al. 2000) but also has embryotrophic qualities (Robertson et al. 1998b, Robertson et al. 2001b). Although not measured in these studies, the increase in mRNA expression of GM-CSF and IL-6 before fertilisation in the pig is likely to be maintained during the period before day 5 post seminal plasma exposure as the developing embryo is moving from the site of fertilisation into the uterine horn. This increase in GM-CSF and IL-6 would be expected to have beneficial effects on
blastoect formation (Diaz-Cueto et al. 2001, Ishiwata et al. 2000, Robertson et al. 1997) and could have a significant role in mediating the crosstalk between the conceptus and maternal cells crucial for establishing the precise co-ordination of maternal and embryo signalling events for optimal development and later embryo elongation and attachment to the endometrium (Jaeger et al. 2001, Robertson et al. 2006). In the pig, murine GM-CSF increases porcine blastocyst development in vitro and increased expression of LIF receptor (Cui et al. 2004) implicating a role for GM-CSF in porcine embryo development and possibly implantation.

Together the influx of leukocytes and the increased expression of pro-inflammatory and embryotrophic cytokines in response to uterine exposure to seminal plasma is likely to have ramifications that extend beyond the site of deposition and influence viability of embryo, fetus and health of the piglet after birth. Although the time of fertilisation was not recorded to make a definitive assessment on embryo development after seminal plasma treatment, there were some significant differences in embryos at the day 5 and day 9 post treatment time points compared with embryos retrieved from PBS-treated gilts. More embryos were retrieved from the reproductive tract after intrauterine treatment with seminal plasma, and embryos retrieved 9 days after treatment were smaller than those retrieved from PBS-treated gilts. Comparisons were made in chapter 3 to embryo development in the prolific Meishan breed of pigs with smaller embryos than the European-derived breeds that are typical in Australia. The Meishan embryo has fewer cells present in the blastocyst stage (Rivera et al. 1996) which may explain the reduced embryo mortality that is experienced with this breed. Although not investigated in this thesis, seminal plasma-induced uterine cytokines may mediate this effect on the pre-implantation embryo, controlling the size/growth of the blastocyst and reducing the variability in development within the cohort of embryos. This level of control in embryo development could provide some advantage to the embryo during this stage of pregnancy with regard to synchrony with uterine development. It is also interesting to note that during early gestation in the Meishan breed of pigs there is a higher concentration of PGE2 in uterine fluid compared with European Yorkshire-Landrace sows (Matte et al. 2008). Prostaglandins including PGE2 are critical for uterine decidualization and implantation in mice (Tranguch et al. 2005) and changes in prostaglandin synthesis leads to delayed implantation, poor feto-placental development and reduced litter sizes (Song et al. 2002). In the pig, the importance of PGE2 in decidualization and embryo attachment has not been fully investigated but is implicated in increasing uterine vascular permeability required for embryo attachment (Kaczmarek et al. 2009).

The extent to which seminal plasma deposited in the uterus during oestrus influences events in the ovary which is remote to the site of deposition was explored in Chapter 4. Intrauterine infusion of
In the pre-ovulatory period, follicular fluid progesterone concentration was 5-fold greater due to seminal plasma treatment. The increase in follicular fluid progesterone is likely to influence oocyte maturation due to improved follicle growth and development associated with increased follicular fluid steroid content (Ainsworth et al. 1980, Grupen et al. 2003). This may have downstream effects and improve early development of the blastocyst post fertilisation due to higher progesterone production from the corpora lutea. Furthermore, the leukocyte infiltration due to seminal plasma observed in uterine tissue similarly occurred in the ovarian stroma with a 4-fold increase in leukocytes and a 3-fold increase in activated macrophages and dendritic cells. The increase in these cell populations may enhance tissue remodelling associated with ovulation (Brannstrom et al. 1993a, Espey 1980) and corpora lutea formation to contribute to the increased corpora lutea weight seen on day 5 and day 9 post treatment. However, the most striking response in relation to how uterine seminal plasma exposure influences ovarian function with implications for increased embryo survival was the observation of increased progesterone production until day 9 post treatment. This increase in progesterone production over the course of early pregnancy is likely to improve embryo survival and litter size in the pig (Ashworth 1991, Athorn et al. 2009) presumably through the effects on reproductive tract secretions. Moreover, differences between treatments were also observed ex vivo with cultured granulosa cells from seminal plasma treated gilts producing more progesterone after culture with hCG and IGF-1. The increased responsiveness of granulosa and theca cells due to seminal plasma treatment indicates that factors in seminal plasma induce functional changes that are maintained after culture ex vivo, independent of their source of origin and cellular milieu in the ovary.

Collectively, these early responses to uterine exposure to seminal plasma influence each compartment of the reproductive tract of the female pig which collectively would be expected to influence embryo development and embryo survival (summarised in Figure 7.1).
Figure 7.1 Schematic diagram summarising the action of seminal plasma signalling in the female reproductive tract in the pig described in chapter 3 and 4.

Seminal plasma acts on the uterine epithelia and increases endometrial expression of GM-CSF, MCP-1, IL-6 and COX-2 mRNA. This response leads to (1) an increase in endometrial leukocyte recruitment and activation (SLA class II+ cells), (2) an increase in recruitment and activation of ovarian leukocytes, (3) an increase in ovarian progesterone production and (4) an influence on CL development. In addition, the effects uterine exposure to seminal plasma is postulated to influence early embryo development and survival.
The active moiety in seminal plasma that drives the post-mating immune response has yet to be determined in the pig. Using protein chromatography and neutralising antibodies, the initiating factor for the post-mating inflammatory response in mice has been identified as TGFβ1 (Tremellen et al. 1998). In the mouse the seminal vesicle glands provides the bulk of the seminal plasma and is also the major source of the TGFβ (Tremellen et al. 1998). Furthermore, TGFβ is present in a relatively high concentration, predominantly in its latent form (Tremellen et al. 1998), and needs to be activated in the female tract to become biologically active (Robertson et al. 2002). In mouse seminal plasma, TGFβ content consists mainly of the TGFβ1 isoform with less than 10% present in the TGFβ2 isoform (Robertson et al. 2002). There is a similar pattern of TGFβ content present in human seminal plasma with relation to concentration and the relative proportions of the two isoforms (Robertson et al. 2002). Isoforms of TGFβ1 and TGFβ2 are similarly present in boar seminal plasma in considerable quantities and these isoforms are present in a ratio of approximately 4:1 (TGFβ1: TGFβ2) and are present in their biologically active form (Chapter 5).

The biological significance of the activation state of TGFβ in boar seminal plasma may lie in the fact that (1) due to the large quantity of ejaculate produced and the pH of boar semen being between pH 7.5 - pH 8.0 (King and McPherson 1966) would be likely to inhibit the activation of TGFβ in the female tract and (2) in comparison to the mouse there is minimal mixing or dilution of seminal components with uterine fluid and therefore less opportunity for mixing with female-derived activating factors. In the human, activation of TGFβ in seminal fluid may take place in the acidic environment of the vagina or due to the activity of plasmin or other enzymes present in the female tract known to activate TGFβ (Chu et al. 1996, Robertson et al. 2002, Khalil 1999). It therefore makes biological sense for TGFβ in boar seminal plasma to be present in its biologically active form, enabling TGFβ signalling to take place immediately once the semen is deposited in the female tract. Moreover, the relatively short half-life of active TGFβ is less likely to be a limiting factor of overall TGFβ action in the female tract as the volume of the boar ejaculate allows for total uterine tract contact of TGFβ in seminal plasma. This is not necessarily the case in the mouse or in humans. In the mouse the ejaculate is diluted up to 2-fold by uterine fluid present in the uterus during oestrus therefore the presence of TGFβ in its latent form allows activation over a comparably extended time period. This presumably would allow the initiation of the post-mating inflammatory response to take place as the TGFβ in the mouse seminal plasma is being activated in the female tract due to activating factors in the uterine fluid. A similar situation may exist in the human, whereby TGFβ in seminal plasma is activated over a period of time either due to the low pH in the vagina or secretion of activating factors in vaginal fluid. In addition, if TGFβ was
present in boar seminal plasma predominantly in its latent form necessitating activation over an extended period of time, then it would be possible that the uterine inflammatory response would also be extended providing a hostile environment at the time when the early embryo is traversing into the uterus from the oviduct.

Apart from the activation state of TGF-β and the exposure to activation factors either present in seminal plasma or the female reproductive tract, the action of TGF-β is also determined by the presence of IFN-γ and LPS (Glynn et al. 2004). In chapter 6, the relationship between TGF-β, IFN-γ and LPS in seminal plasma was compared to measures of fertility and fecundity in boars. No association was found between content of TGFβ1, TGFβ2, total TGFβ (TGFβ1 + TGFβ2), IFNγ and LPS in seminal plasma and boar fertility and fecundity (Chapter 6).

The intrauterine infusion of pooled seminal plasma given 90 minutes before first AI in gilts failed to increase pregnancy rates or litter size compared with intrauterine infusion of sterile PBS. The failure to show that effects of seminal plasma during early pregnancy leads to an increase in embryo survival and resultant increase in litter size in pigs may not however disprove the central hypothesis. Several factors need to be considered with regard to negating the hypothesis based on the findings of the field and TGFβ-fertility study. These were discussed in chapter 6 and include (1) the bio activity of TGFβ in seminal plasma may be different to the immunoreactivity of TGFβ (2) other unknown active moieties may be present in seminal plasma that may synergise or inhibit the action of TGFβ (3) the on-farm collection and storage of seminal plasma in this commercial setting may have inhibited biological activity of TGFβ in the pooled seminal plasma treatment and (4) on-farm unidentified problems with fertility that may have masked any potential benefit of treatment on fertility.

However the alternate view cannot be disproved. In the pig, extensive dilution of seminal factors, as is common in AI regimens, may have no adverse influence on fertility. The differences in fertility between AI and natural mating may be due to other factors including the difficulty in the detection of oestrus in sows, performing AI after ovulation and poor insemination technique in administering AI. If this were so, the post-mating inflammatory response in the pig might affect the potential to respond to bacterial and other contaminants that may be introduced at mating but may not have an effect on embryo survival. Although possible, this alternate view is unlikely to be true and is not supported by previous fertility studies in the pig. It is more likely that technical or procedural limitations in this study led to failure to demonstrate an effect of seminal plasma treatment on litter size in pigs. The average farrowing rates and litter sizes in this study for both treatments and throughout the farm were significantly lower than industry standards, indicating in itself that there existed problems in the commercial piggery production.
systems likely to add confounding variables and mask any increases in fertility that seminal plasma treatment could offer.

Even though seminal plasma has been shown to influence litter size in the pig, to date, no single component of seminal plasma that correlates directly with fertility has been identified. This is also the case for humans where there is significant evidence to support the importance of seminal factors in pregnancy outcome. There has been small studies showing that the presence of IFN\(\gamma\) in semen is negatively correlated with healthy sperm parameters (Paradisi et al. 1996) in men and is elevated in the seminal plasma of partners of women experiencing recurrent miscarriage (Sharkey, D.J. and Robertson S.A., unpublished data), but to date there have been no studies investigating fertility taking into account the cytokine interaction within biological fluids and tissues (Kelso 1998). We were also unsuccessful in showing that TGF\(\beta\) content, together with two known modulators of TGF\(\beta\) action IFN\(\gamma\) and LPS, is a useful fertility measure for boars. Apart from the possible limitations discussed above, it is likely an assay that takes into account all of the active moieties in seminal plasma, consisting of several cytokines, bacterial components and other still undefined signalling agents will be required. Future research taking into account the net effect of cytokines, bacterial species and other signalling agents present in boar seminal plasma on early embryo survival has the potential to provide seminal plasma surrogates for use in conjunction with AI regimens and also as a diagnostic assay for boar fertility.

The net effect of signalling factors present in seminal plasma on the maternal reproductive tract and their influence in downstream events during early pregnancy in the pig are becoming more defined. It is now clear that molecular messengers induced by the post-mating inflammatory response are part of the ‘uterine milk’ (Solymosi et al. 1994) needed to provide the optimal amount and balance of nutrients, growth factors, and hormones for optimal embryo development (Matte et al. 2008, Robertson et al. 1994) during the critical period of embryo elongation and attachment to the endometrium. IL-6, TGF\(\beta\)2, IFN\(\gamma\) and PGE\(_2\) have been reported to be present in the uterine fluid and expressed by uterine endometrium at day 15 of pregnancy in the pig (Chabot et al. 2004). These mediators of the immune system are likely to be key components of the ‘crosstalk’ between maternal and embryo tissues important in the regulation of cell differentiation and maturation including the prevention of maternal rejection of the embryo (Hunt et al. 1996, Robertson et al. 1994) (a schematic of the ‘fetomaternal’ dialogue is represented in Figure 7.2).
In this diagram the expanding pig embryo is represented as a yellow disc between the uterine endometrium. Immunoregulation involves the infiltration of leukocytes (purple cells), activated macrophages (green cells with concave nuclei) and dendritic cells (yellow cells) along with cytokines (IL-6, GM-CSF, TGFβ, IL-10), growth factors (GFs), prostaglandins (PGE) and hormones including estrogens (E₂) produced by uterine cells and embryonic tissue. The molecules represented in this diagram have been implicated in the success of implantation and gestation (Robertson et al. 1994). Those represented in bold font have been shown to be present in uterine fluid during the time of embryo elongation and attachment in the pig (Chabot et al. 2004) and those shown in red are factors shown in this thesis to be induced by, or be present in, seminal plasma.
Consistent with other mammalian species, TGFβ in seminal plasma is likely to be the active factor driving the responses to seminal plasma described in this thesis. TGFβ is present in boar seminal plasma in large quantities and what appears to be unique in the pig is present predominantly in its biologically active form ready to bind with its cognate receptor, rather than needing to be activated once deposited in the female reproductive tract. This might result from the mixing of latent TGFβ together with other as yet unidentified activating agents potentially including proteases and integrins at the time of ejaculation. These could be made in other accessory sex organs, in order to prevent precocious activation of TGFβ prior to ejaculation. Further studies are needed to investigate the source and presence of TGFβ activating agents in semen and their role in controlling the effect of TGFβ in the female tract. There is considerable variation in TGFβ1 and TGFβ2 content between boars, however the content remains relatively stable between collections except when there are frequent collections within the period of a week. In addition, there are factors in boar seminal plasma including IFNγ and LPS that act as agonists to, or synergise with, TGFβ action present in boar seminal plasma.

The potential positive effects of seminal plasma treatment given during oestrus in gilts on embryo development and uterine and ovarian function did not result in an increase in litter sizes in gilts in a field trial within a commercial setting. We were also unable to demonstrate a relationship between TGFβ content in seminal plasma and boar fertility. However, these studies do provide interesting insights into the role of immunoregulatory moieties in seminal plasma and their influence on the cells and tissues of the female reproductive tract. Furthermore, continuing research is warranted in determining the role of these molecules on embryo survival in the pig and will facilitate development of products, including surrogate seminal plasma treatments, to improve reproductive output and profitability in the pig industry.


Chemokine and cyclooxygenase-2 expression in human endometrium coincides with leukocyte accumulation.


