The physiological significance of insemination in programming pregnancy outcome

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Abstract

The cellular and molecular environment of the uterus during the pre- and peri-implantation period of early pregnancy is critical for implantation success and optimal fetal and placental development. Perturbations to this environment not only have consequences for the success of pregnancy and neonatal health and viability, but can also drive adverse health outcomes in the offspring after birth, particularly the development of metabolic disorders such as obesity, hypertension and insulin resistance.

The influence of seminal plasma on the cytokine and immune uterine environment has been previously well characterised in mice, however the effects of disruption in uterine seminal plasma exposure for pregnancy outcome have not been investigated. The studies described in this thesis employed the use of surgical seminal vesicle ablation in males and embryo transfer experiments to investigate the physiological significance of uterine seminal plasma exposure on programming fetal and neonatal outcomes, and growth and metabolic status in adult offspring.

We demonstrate that in the absence of seminal plasma, oocyte fertilisation and embryo implantation are reduced, showing that seminal plasma acts primarily to facilitate fertilisation, possibly by promoting sperm transport and survival in the reproductive tract. In addition we show that pregnancies initiated in the absence of seminal plasma give rise to offspring which display accelerated growth after birth and increased adiposity in adulthood, compared to those developed in a tract exposed to seminal plasma at the time of conception. Offspring conceived in the absence of seminal plasma also displayed alterations in serum leptin and adiponectin content, similar to those known to be associated with obesity in the mouse.

Using embryo transfer experiments, we showed that some, but not all aspects of the perturbed postnatal development are recapitulated when embryos fertilised in the presence of what semen are transferred to a recipient tract which has not been exposed to seminal plasma. More severe perturbations were seen in 2-cell transfer than in blastocyst transfer experiment. Additionally, there was a significant effect of the embryo transfer procedure, irrespective of seminal plasma exposure, on fetal and postnatal development that confounded interpretation of these experiments.

In addition, we investigated the potential mechanisms by which the influence of seminal plasma is exerted. Mediators of pre-implantation embryo development, implantation and the modulation of the maternal immune response to pregnancy were all assessed for regulation by seminal plasma using QRT-PCR. It was demonstrated that seminal plasma exposure induces the up-regulation of key embryotrophic factors, LIF, GM-CSF and IL-6, in the oviduct following insemination. Factors important in tissue remodelling required for implantation and angiogenesis, MMP-2, MMP-3 and VEGF-C, were also shown to be increased at the time of implantation after seminal plasma exposure. Additionally the generation of T-regulatory cells in uterine tissues, demonstrated by the up-regulation of the transcription factor FOXp3 was shown to be dependent on semen exposure. The influence of seminal plasma on embryonic development, implantation and modulation of the maternal immune response to pregnancy may therefore be mechanisms which contribute to the adverse outcomes seen in pregnancies initiated in the absence of seminal plasma.

Together these experiments show a role for seminal plasma signalling at the time of insemination in influencing the pre-implantation embryo to program later fetal and neonatal development, thereby impacting on the metabolic health of offspring. We conclude that seminal plasma is not simply a transport medium for sperm, but acts also as a key regulator of a female tract environment providing optimal support for the developing embryo.

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 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.

I further grant my consent to the University of Adelaide to make this thesis available for loan and photocopying once accepted for the degree.

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Publications arising from these and related studies

1. SA Robertson, JJ Bromfield, KP Tremellen.
   Seminal 'priming' for protection from pre-eclampsia – A unifying hypothesis (review).
   Journal of Reproductive Immunology. 2003 Aug; 59(2): 253-265.

2. M Johansson, JJ Bromfield, MJ Jasper, SA Robertson.
   T-Lymphocyte activation and trafficking after mating.
   Immunology. 2004 June; 112(2): 290-300.

3. SA Robertson, JJ Bromfield, DJ Glynn, DJ Sharkey, MJ Jasper.
   Actions of seminal plasma cytokines in priming female reproductive tract receptivity for embryo implantation. In Gil Mor (Ed) Immunology of Implantation 2005, Landes Bioscience, Georgetown TX.

4. JJ Bromfield and SA Robertson (in preparation)
   Seminal plasma programs uterine receptivity and pregnancy outcome in the mouse.

5. JJ Bromfield and SA Roberston (in preparation).
   Seminal plasma and the molecular and cellular events of implantation.

Abstracts arising from these studies

Presenting author underlined

2003

- JJ Bromfield, CT Roberts, SA Robertson.
  "The effect of seminal vesicle ablation on reproductive outcome in mice". Society for Reproductive Biology. 34th Annual Conference, Melbourne Australia.

- JJ Bromfield, CT Roberts, SA Robertson.
  "The effect of seminal vesicle ablation on placental development and fetal outcomes in mice". Australian Society of Medical Research. National Scientific Meeting, Adelaide Australia.

2004

- JJ Bromfield, CT Roberts, SA Robertson.
  "Seminal plasma programs uterine receptivity and pregnancy outcome". Australian Society of Medical Research. South Australian Annual Scientific Meeting, Adelaide Australia.

- JJ Bromfield, CT Roberts, SA Robertson.
  "Seminal plasma programs uterine receptivity and pregnancy outcome". Society for the Study of Reproduction. 37th Annual Meeting, Vancouver Canada.

- JJ Bromfield, CT Roberts, SA Robertson. "Semen exposure in early pregnancy impacts on fetal and neonatal growth". Society for Reproductive Biology. 35th Annual Conference, Sydney Australia.

2005

- JJ Bromfield, CT Roberts, SA Robertson.
  "Male seminal factors influence fetal and placental development, postnatal growth and adult onset obesity in mice". 19th Annual Fetal and Neonatal Physiology Workshop, Adelaide Australia.

- JJ Bromfield and SA Robertson.
  ”Significance of semen exposure in endometrial receptivity and pregnancy outcome”. Frontiers in Reproduction, 8th Annual Symposium. National Academy of Sciences, Woods Hole MA USA.

- JJ Bromfield and SA Robertson.
  “Seminal plasma impacts on pregnancy outcome through effects on both uterine receptivity and embryo development”. Society for Reproductive Biology. 36th Annual Conference, Perth Australia.

- JJ Bromfield, MJ Jasper, R Skinner, SA Robertson. ”LIF expression is induced in the mouse oviduct following activation by seminal factors”. Society for Reproductive Biology. 36th Annual Conference, Perth Australia.

- JJ Bromfield and SA Robertson. “Male seminal factors influence fetal and placental development, postnatal growth and adult onset obesity in mice”. Fetal and Neonatal Physiology Society. 32nd Annual International Conference; Jeffrey Robinson Symposium, Adelaide Australia.

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- JJ Bromfield and SA Robertson. “Seminal factors regulate molecular mediators of early pregnancy success”. Frontiers in Reproduction, 9th Annual Symposium. Woods Hole Oceanographic Institute, Woods Hole MA USA.

- JJ Bromfield and SA Robertson. “Female tract exposure to male seminal factors influences developmental programming of obesity in progeny“. Gordon Research Conference – Reproductive Tract Biology. Biannual International Conference, New London CT USA.

- JJ Bromfield and SA Robertson. “Seminal plasma regulates MMP-2, MMP-3 and VEGF-C mRNA expression in the peri-implantation mouse uterus”. Society for Reproductive Biology. 37th Annual Conference, Gold Coast Australia.

- JJ Bromfield and SA Robertson. “Male derived seminal factors contribute to the optimization of uterine receptivity toward embryo implantation”. Journal of Reproductive Immunology. 3rd International Conference on Experimental and Clinical Reproductive Immunobiology, Banff Canada.

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Abbreviations

A	Adenine
Ab	Antibody
ADCC	Antibody dependent cell cytotoxicity
APC	Antigen presenting cell
ART	Assisted reproductive technique
BDKR	Bradykinin receptor
BFGF	Basic fibroblast growth factor
Bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine
^{o}C	Degrees Celsius
CDNA	Complimentary DNA
CG	Chorionic gonadotrophin
CL	Corpus luteum
COX	Cyclooxygensase
Ct	Cycle threshold
DAB	Diaminobenzidine tetrachloride
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
ECM	Extracellular matrix
EGF	Epidermal growth factor
eNOS	Endothelial nitric oxide synthase
FASL	FAS ligand

FSH	Follicle stimulating hormone
G	Guanine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HB-EGF	Heparin binding-epidermal growth factor-like growth factor
hCG	Human chorionic gonadotrophin
HLA	Human leukocyte antigen
HPA	Hypothalamic/pituitary/adrenal
HRP	Horse radish peroxidase
ICM	Inner cell mass
ICSI	Intra-cytoplasmic sperm injection
IFN	Interferon
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IL	Interleukin
iNOS	Inducible nitric oxide synthase
ISPN	Implantation serine protease
IUGR	Intrauterine growth retardation
IVF	*In vitro* fertilisation
LAK	Lymphokine activated killer
LH	Luteinizing hormone
LIF	Leukaemia inhibitory factor
LN	Lymph node
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MLN	Mesenteric lymph node
MMP	Matrix metalloproteinase

MQ	Milli-Q
mRNA	Messenger RNA
MUC	Mucin
NK	Natural killer
NRP	Neuropilin
PALN	Para-aortic lymph node
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGE	Prostaglandin
PGF	Placental growth factor
RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulphate
T	Thymine
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumour necrosis factor
Tr	T represser
U	Uracil
uNK	Uterine natural killer
VEGF	Vascular endothelial growth factor
VEGF-R	Vascular endothelial growth factor receptor
VIA	Video image analysis