STUDIES ON THE CRYOPRESERVATION OF BOAR SPERMATOZOA AND ITS INTEGRATION INTO ASSISTED REPRODUCTIVE TECHNOLOGIES

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DECLARATION

Apart from the assistance mentioned in the acknowledgments and where due reference is made in the text, this thesis represents original research of the author and has not been previously submitted for a degree to any other institute.

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LIST OF ABBREVIATIONS

AAAO	aromatic amino acid oxidase
AI	artificial insemination
ANOVA	analysis of variance
ATP	adenosine triphosphate
BTS	Beltsville Thawing Solution
BSA	bovine serum albumin
CASA	computer-assisted sperm analysis
COC	cumulus-oocyte complex
CTC	chlortetracycline
DABCO	1,4-diazabicyclo[2,2,2]octane
DIU	deep intrauterine
DMSO	dimethyl sulphoxide
DNA	deoxyribose nucleic acid
DTT	dithiolreitol
EDTA	ethylenediaminetetraacetic acid
ET	embryo transfer
FAA	fertility-associated antigen
FITC-PNA	fluorescein-conjugated peanut agglutinin
FCS	foetal calf serum
FM	fertilisation medium
FR	farrowing rate
GSH	reduced glutathione

hCG	human chorionic gonadotrophin
hpi	hours post insemination
HSPM	human sperm preservation medium
IMV	Instruments de Médicine Vétérinaire
IU	intrauterine
IVF	in vitro fertilisation
IVP	in vitro production
LDL	low density lipoproteins
LPC	lysophosphotidylcholine
LPE	lysophosphotidylethanolamine
MM	maturation medium
MUFA	monounsaturated fatty acids
NRR	non-return rate
PAF	platelet activating factor
PAF:AH	platelet activating factor: acetylhydrolase
PBS	phosphate buffered saline
PC	phosphotidylcholine
PCR	polymerase chain reaction
PE	phosphotidylethanolamine
PET	polyethylene terephthalate
PI	phosphotidylinositol
PMSG	pregnant mare serum gonadotrophin
PS	phosphotidylserine
PUFA	polyunsaturated fatty acids
PVA	polyvinyl alcohol

PVC	polyvinyl carbonate
PVP	polyvinyl pyrollidone
RO	reverse osmosis
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SFA	saturated fatty acids
SOD	superoxide dismutase
TLC	thin layer chromotography
UTJ	uterotubal junction
UV	ultraviolet
ZF	zinc finger

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PUBLICATIONS ARISING FROM THIS WORK

R. Bathgate, B. Eriksson, W.M.C. Maxwell and G.Evans (2001). Comparison of boar semen freezing methods. *Faculty of Veterinary Science Postgraduate Research Conference* – Conference paper, p.15

R. Bathgate, B. Eriksson, W.M.C. Maxwell, G. Evans (2001). Comparison of boar semen freezing methods. *Australasian Pig Science Association* – Conference paper, p.191

R. Bathgate, B.M. Eriksson, W.M.C. Maxwell, G. Evans (2002). Effect of seminal plasma on frozen-thawed boar semen. *Society for Reproductive Biology* – Conference paper, p.19

R. Bathgate (2002) The effect of seminal plasma on frozen-thawed boar sperm. *Faculty of Veterinary Science Postgraduate Research Conference* – Winner, best conference paper, p.13

R. Bathgate, B. Eriksson, W.M.C. Maxwell, G. Evans (2003). Potential damage to the uterine lining after non-surgical deep intrauterine insemination of sows. *Australasian Pig Science Association* – Conference paper, p.57

B.M. Eriksson, **R. Bathgate**, W.M.C. Maxwell and G. Evans (2003). Effect of seminal plasma protein fractions on boar spermatozoa motility and acrosome integrity. *5th International Conference on Boar Semen Preservation* – Conference paper, III-P33

R. Bathgate, B.M. Eriksson, W.M.C. Maxwell and G. Evans (2003). Low dose deep intrauterine insemination of sows with fresh and frozen-thawed spermatozoa. *5th International Conference on Boar Semen Preservation* – Conference paper, IV-P49

R. Bathgate, B.M. Eriksson, W.M.C. Maxwell and G. Evans (2003). Observational study on the effect of bleeding from the reproductive tract on the fertility and fecundity of sows after deep intrauterine insemination. *Faculty of Veterinary Science Postgraduate Research Conference* – Winner, best conference paper, p.10

B.M. Eriksson, **R. Bathgate**, W.M.C. Maxwell, and G. Evans (2004). Developing techniques for pig AI. *Australian Association of Pig Veterinarians* – Review paper, pp61-69

Maxwell WMC, Evans G, Hollinshead FK, **Bathgate R**, de Graaf SP, Eriksson BM, Gillan L, Morton KM and O'Brien JK (2004). Integration of sperm sexing technology into the ART toolbox. *Animal Reproduction Science* 82-83:79-95 – Invited review paper

R Bathgate, KM Morton, BM Eriksson, D. Rath, B Seig, O Chami, T Stojanov, WMC Maxwell and G Evans (2005) Production of porcine embryos of a predetermined sex after in vitro fertilisation of in vitro matured oocytes with sex-sorted frozen-thawed boar sperm *Reproduction, Fertility and Development* 17:303

R Bathgate, BM Eriksson, WMC Maxwell and G Evans (2005) Effect of pre-freeze addition of platelet-activating factor and platelet-activating factor:acetylhydrolase on the post thaw integrity of frozen-thawed boar sperm *Reproduction, Fertility and Development* 17:189

SYNOPSIS

The aim of this thesis was to investigate the possibility of integrating frozen-thawed boar semen into reproductive technologies and into commercial production of pigs in Australia. This was to be achieved by establishing a semen freezing and AI regime that was of a standard acceptable to industry, and integrating the resultant frozen-thawed sperm into other reproductive technologies, such as flow cytometric sperm sorting and IVF.

Initially, a protocol for freezing and thawing boar semen was established, based on the method described by Westendorf *et al.* (1975) and attempts were made to modify this protocol to improve the post-thaw sperm quality, as determined by in vitro assessment of motility, acrosome integrity and longevity. First, the egg yolk used in the freezing extenders was investigated, and the chicken yolk was replaced with either duck or quail yolk. It was shown that there was no benefit in substituting yolk from duck or quail for the chicken yolk traditionally used in freezing extender.

Second, the effect of seminal plasma addition to the freezing extender, or seminal plasma addition to resuspension medium post-thaw was tested. Incorporating whole seminal plasma into the freezing extender at levels above 50% was found to be detrimental to post-thaw sperm quality. Reducing levels to 20% of the final volume improved acrosome integrity, but adversely affected motility of sperm. However, adding 20% seminal plasma to the resuspension medium used after thawing of boar semen had no significant influence on sperm quality compared with resuspension in medium without seminal plasma.

The antioxidant catalase, and the iron chelator desferal added to the freezing extender, did not improve post-thaw sperm quality, nor was any benefit seen with addition of these substrates to the resuspension medium post-thaw. However, the bioactive phospholipid PAF and its regulating enzyme PAF:AH appeared to enhance post-thaw motility and acrosome integrity of sperm, respectively, when added to the semen pre-freezing. Unfortunately, due to the restrictions imposed on rPAF:AH as a research drug, it was not possible to test the in vivo effects at this time.

After the in vitro experiments were completed, the in vivo fertility of frozen-thawed sperm was tested using the optimal freezing protocol and a novel technology, enabling non-surgical deep intrauterine insemination of sows. The aim was to establish the lowest possible dose of frozen-thawed sperm that could be used, without compromising fertility. Successful pregnancies were achieved with doses as low as 62.5 x 10^6 frozen-thawed sperm but the farrowing rates were too low to be practicable on a commercial scale. This is the first report of litters born after insemination of such a low dose of frozen-thawed sperm and using the novel DIU insemination technique. However, it was concluded that a double dose of 250 x 10^6 frozen-thawed sperm was the minimum dose required for maintaining acceptable fertility.

Reduction in sperm numbers to such an extent made it possible to consider non-surgical insemination of sex-sorted, frozen-thawed semen. Previously, pregnancies had been achieved only after surgical insemination of sex-sorted boar sperm, or with DIU insemination of unfrozen sperm, immediately after sex-sorting. The low numbers of sex-sorted sperm available restricted the inseminate dose used here to 50 x10^6 motile

sperm. A litter of 5 piglets was born after a low-dose, DIU insemination of sex-sorted, frozen-thawed sperm. This is the first report of piglets born after insemination with sex-sorted frozen-thawed sperm and non-surgical insemination.

The low farrowing rate achieved in this experiment prompted the investigation of integrating sex-sorted, frozen-thawed boar sperm into IVF. Morulae were produced after IVF with sex-sorted, frozen-thawed sperm and successfully transferred using non-surgical techniques. This is the first report of pregnancy achieved with non-surgical transfer of embryos produced after IVF and IVC of IVM oocytes with sex-sorted, frozen-thawed boar sperm. Unfortunately, the pregnancy did not hold, and the embryos were lost prior to Day 32, but PCR of non-transferred embryos confirmed successful pre-selection of sex.

Overall, this thesis demonstrated that it is still not economically feasible to incorporate frozen-thawed boar semen into the commercial production of pigs although it has considerable application in breeding programmes. However, the development of novel techniques enabling reduction in sperm dose, and for non-surgical transfer of embryos into recipient sows and incorporation of frozen-thawed semen into these technologies means that progress is being made with the integration of reproductive technologies and frozen-thawed semen into the pig industry.