DEVELOPMENT OF *IN VITRO* EMBRYO PRODUCTION SYSTEMS AS A BREEDING TOOL FOR THE PIG
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Final report prepared for the Co-operative Research Centre for an Internationally Competitive Pork Industry

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Executive Summary

The ovary of the sow contains some 300,000 eggs (oocytes) at the time of sexual maturity. The majority of these are lost as a result of atresia resulting in less than 1% being ovulated during the sow's lifetime. We and others have developed systems for the *in vitro* maturation, fertilisation and culture (*in vitro* embryo production or IVP) of preovulatory oocytes from antral follicles and demonstrated that embryos produced using these can result in the birth of live animals. These systems have improved dramatically over recent years and are used commercially in cattle as a means of genetic improvement. In this species oocytes are repeatedly aspirated non surgically over several cycles from live animals and fertilised *in vitro* using (sexed) semen from superior animals, frozen and then transferred non surgically. In contrast, pig IVP together with methods for the non surgical collection of oocytes and transfer of embryos need to be developed further before a similar system can be used commercially in the pig. The aim of the present study was to further develop this system for the pig system and ultimately methods all the oocytes can be collected from the ovary, grown and fertilised *in vitro* with (sexed) semen, frozen and then transferred non surgically. As a first step original aims of the project were modified to demonstrate proof of concept in 18 months namely that oocytes could be collected non surgically from live animals and then fertilised and cultured *in vitro* before being transferred to recipient animals. To date we have:

1. Develop non surgical embryo transfer catheter - 9 months.

A catheter was developed and trialled with *in vivo* derived embryos (obtained surgically from live animals) as well as *in vitro* produced embryos (IVP, ooytes derived abattoir ovaries matured fertilised and cultured for 6 days) as described below.

2. Demonstrate acceptable pregnancy rates using IVP embryos produced using abattoir ovaries and non surgical embryo transfer - 12 months

In vitro produced embryos. Six transfers were performed . Of the 4 transfers performed successfully (correct insertion of catheter) and which used good quality embryos, 1 transfer (25%) resulted in a pregnancy . This pregnancy was not maintained and the sow returned by d28.

In vivo derived embryos. Twelve transfers were performed. Of the 5 transfers performed successfully (correct insertion of the catheter) and which used good embryos, 2 transfers (40%) resulted in pregnancies which resulted in litters of 3 piglets each.

3. Develop non surgical oocyte collection using laparoscopic aspiration -15 months

Ongoing

4. Demonstrate proof of concept ie piglets born using oocytes collected from live animals using laparoscopic aspiration, *in vitro* embryo production and non surgical embryo transfer - 18 months.

Not commenced.

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1. Introduction

The ovary of the sow contains some 300,000 eggs (oocytes) at the time of sexual maturity. The majority of these are lost as a result of atresia resulting in less than 1% of being ovulated during the sow's lifetime. We and others have developed systems for the *in vitro* maturation, fertilisation and culture (*in vitro* embryo production or IVP) of preovulatory oocytes from antral follicles and demonstrated that embryos produced using these can result in the birth of live animals (Table 1 Beebe et al 2007). These systems have improved dramatically over recent years and are used commercially in cattle as a means of genetic improvement (De Roover et al 2008) . In this species oocytes are repeatedly aspirated non surgically over several cycles from live animals and fertilised *in vitro* using (sexed) semen from superior animals, frozen and then transferred non surgically. In contrast, pig IVP together with methods for the non surgical collection of oocytes and transfer of embryos need to be developed further before a similar system can be used commercially in the pig.

Table 1 - Surgical transfer of *in vitro* produced blastocyts to recipient gilts (Beebe et al 2007)

Recipients	Embryos transferred	Pregnant	Total liveborn
6	23-35 per recipient	4/6 (67%)	21 (5,5,7,4)

Current IVP systems use oocytes obtained from large (antral) follicles visible on the surface of the ovaries. However the majority (99%) of oocytes reside in the preantral and primordial follicles below the surface of the ovary. Systems for the *in vitro* growth of oocytes from these follicles have been developed for mice and viable offspring produced. However similar systems have are yet to be developed for the pig which has a much longer growing period (approx 4 months compared with 3 weeks in mice). Nevertheless recent improvements in *in vitro* growth systems for humans (growing period of 12 months) would suggest this is now possible for the pig. The aim of the present study is to develop a system whereby ultimately all the oocytes are collected from the ovary, grown and fertilised *in vitro* with (sexed) semen, frozen and then transferred non surgically.

2. Methodology

The original aims of the project were to:

1. Develop a method for the repeated collection of oocytes from live animals.
2. Develop a method for the non surgical transfer of IVP embryos.
3. Develop maturation, fertilisation and culture media to maximise the production of embryos from gilts and sows.
4. Develop methods for freezing IVP embryos. IVP produced embryos differ from *in vivo* derived embryos in their freezability. As such we need to modify current protocols for use with these.
5. Demonstrate proof of concept namely that acceptable pregnancy rates and litter sizes can be produced using oocytes collected non surgically from live animals, matured, fertilised and cultured *in vitro*, frozen and transferred non surgically.
6. Develop systems for the *in vitro* growth of immature oocytes from preantral and primordial follicles.

Revised project milestones

However these were modified by the R&D committee. In particular a stop/go point incorporated was incorporated at 18 months. As such the project was modified and the following milestones developed/agreed

1. Develop non-surgical embryo transfer catheter 9 months
2. Demonstrate acceptable pregnancy rates using IVP embryos produced using abattoir ovaries and non-surgical embryo transfer 12 months.
3. Develop non-surgical oocyte collection using laparoscopic aspiration - 15 months.
4. Demonstrate proof of concept ie piglets born using oocytes collected from live animals using laparoscopic aspiration, *in vitro* embryo production and non-surgical embryo transfer -18 months.

3. Outcomes

Progress against milestones

1. Develop non-surgical embryo transfer catheter

Dr Langendijk and Ms Bouwman have developed and tested various catheter prototypes based on the one they developed in the Netherlands. The protype is shown in Figure 1. The catheter used for the non-surgical embryro transfer is based on a model developed by Kemp and Hazeleger (1994). A PVC guiding rod (3 mm OD) with a bended end of 1 cm, and a guiding tube attached to it is guided through the cervical folds, and upon passing the cervix, the catheter containing the embryos is passed through the guiding tube, and the embryos are deposited in a 0.1 ml volume into the uterine bifurcation. The embryos are inserted in the transfer catheter by aspiration prior to transfer, so they sit in the tip of the catheter. Of crucial importance is that the PVC guiding rod is of the right flexibility and rigidness at the same time. This prevents folding of the rod when manipulating through the cervical folds, and at the same time allows enough flexibility to navigate through the cervical foldings. Most non-surgical transfer devices tested so far do not combine these two properties, either resulting in the device folding during the insertion procedure because of too much flexibility, or being too rigid to navigate through the cervical folds, resulting in perforating the cervical canal or uterine wall.

The non-surgical embryo transfer technique was first tested using *in vivo* derived embryos that were collected at a local abattoir from synchronised peri-pubertal gilts. In 5 cohorts, 10 F2 gilts per cohort were induced to puberty using PG600. First oestrus was checked daily in a DMA. Following the first luteal phase, gilts were injected with 1000 IU eCG at the start of the follicular phase and ovulation was induced with 750 iu hCG 72 h later. Gilts then received two AIs, approximately 12 h prior to expected ovulation and subsequently around ovulation. Gilts were sacrificed at a local abattoir at day 5 after ovulation and reproductive tracts were flushed with PBS containing 1% lamb serum. Subsequently, embryos were transported at 37 °C to the lab for quality assessment and selection for transfer. Generally, around 30 embryos of good quality (expanded blastocysts) were transferred per recipient. For recipients, weaned multiparous sows were used that received 800 IU eCG 24 h after weaning and 750 IU hCG 72 h after eCG. Both donor gilts and recipient sows were heat checked in a DMA daily after injection with hCG, and time of ovulation was estimated using ultrasound at 12 h intervals around the time of expected ovulation (9 am). At the time of transfer, embryos were 5 days of age and recipients were at 5 days after ovulation.

Table 1 - Ovulation rate and embryo recovery and transfer rate from prepubertal gilts

Number of donors	Ovulation rate	Recovered embryos	Transferable embryos
50	19 ± 1.7	13.4 ± 1.7	80-90 %

The above table shows the average ovulation rate and number of recovered embryos per donor gilt. Reproductive tracts of donor gilts were flushed after killing and scalding. In some donor gilts this resulted in urine accumulating in the uterus, affecting embryo quality, explaining the

poor quality of some of the embryo cohorts in Table 2. For future *in vivo* embryo collection, it would be advisable to collect embryos from reproductive tracts before scalding or from skinned sows, as these are hanged up before emptying of the bladder. It is also advisable to not supply donors with drinking water at least 12 h prior to slaughter.

2. Demonstrate acceptable pregnancy rates using *in vivo* embryos and IVP embryos produced using abattoir ovaries and non-surgical embryo transfer.

Using this prototype transfers with *in vivo* derived and *in vitro* produced embryos were undertaken . The results are shown in Table 1.

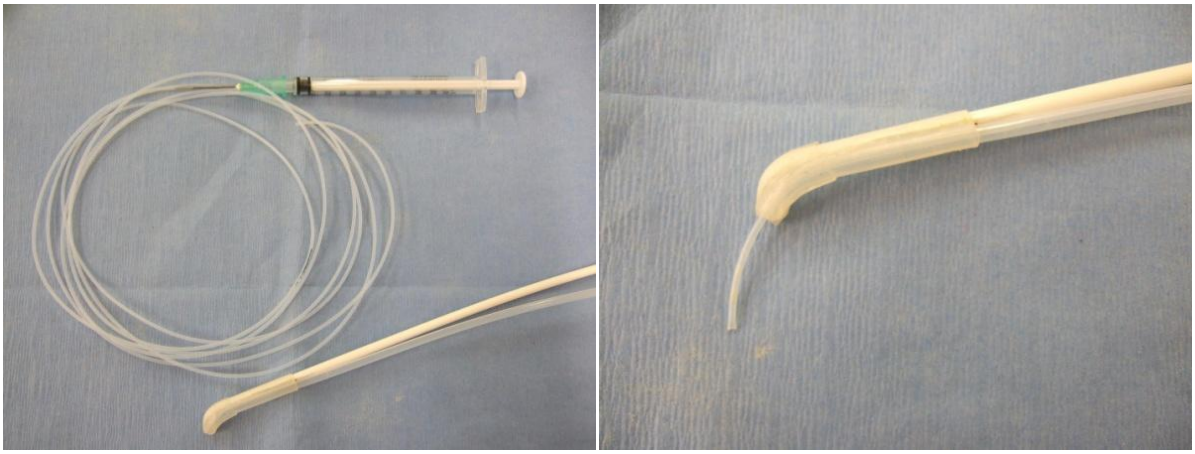

Fig 1 – Non-surgical embryo transfer catheter.

The transfer catheter is passed through a second tube attached to a stiffening rod . Embryos are introduced into the transfer catheter using a 1ml syringe.

Table 2 –Non-surgical transfer of *in vitro* produced (IVP) and *in vivo* derived embryos

Source of embryos	Quality of embryos	Score for transfer	Pregnant	Outcome/ remarks
IVP	poor	difficult	no	
IVP	good	difficult	no	
IVP	good	good	no	
IVP	good	good	no	
IVP	good	good	no	
IVP	good	good	yes	positive ultrasound d22, returned d28
in vivo	good	difficult	no	practice
in vivo	good	difficult	no	practice
in vivo	good	difficult	no	practice
in vivo	poor	failed	no	catheter modified
in vivo	poor	failed	no	
in vivo	poor	difficult	no	
in vivo	poor	failed	no	
in vivo	good	good	no	
in vivo	good	good	no	
in vivo	good	good	no	
in vivo	good	good	yes	3 piglets
in vivo	good	good	yes	3 piglets

Failed = perforation of uterus or cervix, difficult= location of catheter in uterus uncertain, good= certain of location.

Summary

IVP embryos. Six transfers were performed . Of the 4 transfers performed successfully (correct insertion of catheter) and which used good quality embryos, 1 transfer (25%) resulted in a pregnancy . This pregnancy was not maintained and the sow returned by d28.

In vivo derived embryos. Twelve transfers were performed. Of the 5 transfers performed successfully (correct insertion of the catheter) and which used good embryos, 2 transfers (40%) resulted in pregnancies which resulted in litters of 3 piglets each. These litters are the first reported litters born in Australia from a non-surgical embryo transfer.

Figure 2 - Piglets born following the non surgical embryo transfer.

3. Develop non surgical oocyte collection using laparoscopic aspiration

An oocyte aspiration and collection pump was developed. Discussions were held with Dr Brussow who pioneered laparoscopic oocyte pickup in pigs (Brussow and Ratky 1994) and with various colleagues who have had experience with ultrasound guided transvaginal oocyte pickup. On the basis of these discussions and a recent publication in this area (Rodriguez et al 2009) it

was decided that the feasibility of performing transabdominal ultrasound guided pick up in the pig needs to be investigated because it is less invasive (reduced adhesions etc) , has a reduced infection risk and a reduced recovery period compared with laparoscopic aspiration and as such is more suitable for repeated oocyte collections from the same animal.

4. Demonstrate proof of concept ie piglets born using oocytes collected from live animals using laparoscopic aspiration, *in vitro* embryo production and non surgical embryo transfer.

This milestone is yet to be completed because of delays in the preceding milestones. In particular the development of the catheter delayed the project by an estimated 6 months because twice as many transfers than originally planned had to be undertaken because of the need to modify the catheter etc.

As part of this study we have also made improvements to our *in vitro* embryo production system. In particular we have developed a two stage culture media similar to that used in human IVF and examined a range of energy substrates as well as the addition of non essential as well as essential amino acids. These modifications have resulted result in significant increases in the number and viability of *in vitro* produced embryos (Beebe et al 2009 , Table 2).

Table 3 *In vitro* development of IVP embryos cultured in standard versus improved media

Media	No. fertilised	No. cleaved	No. blastocyst (%)	Cell number
Standard	317	222 (70)[a]	129 (41)[a]	63.5 + 3.0[a]
Improved	316	249 (79)[b]	185 (59)[b]	75.6 + 1.9[b]

Values with different superscripts in the same column are significantly different (P<0.05)

4. Application of Research

The aim of the present study is to develop a system whereby ultimately all the oocytes are collected from the ovary, grown and fertilised *in vitro* with (sexed) semen, frozen and then transferred non surgically.

5. Conclusion

In summary we believe we have made good progress in a relatively short period (it is perhaps worth noting in this context that similar systems have taken upwards of a decade to develop in cattle). Given that this method is now widely used in the cattle industry, it should find similar acceptance in the Pork Industry. And as such would provide an alternative to for example cloning. Furthermore there is in the potential in the medium to long term to develop methods whereby all 300,000 oocytes in the ovary can be harvested as has already been demonstrated in mice .

6. Limitations/Risks

The limitations/risks are as outlined above namely that the project is yet to demonstrate proof of concept due to delays arising from technical issues.

7. Recommendations

That this work, or alternatively cloning, be funded to develop a reproductive tool for increasing productivity. At present this is restricted to natural mating and the (limited) use of artificial insemination. The project was originally designed to be undertaken over three years. This was revised and a stop/go decision point at 18 months incorporated. We believe we have made good progress in the first 18 months of the project. However because the development and testing of the catheter took longer than anticipated (together with Ms Bouwmann taking maternity leave) we are yet to complete milestones 3 and 4. As such we wish to request a 12 month extension to the project to complete the remaining milestones as well as compare aspiration methods . Specifically we will:

1. Compare laparoscopic collection with ultrasound guided oocyte pick up -10 months.
2. Demonstrate proof of concept *ie* birth of piglets using oocytes collected from live animals using laparoscopic aspiration, *in vitro* embryo production and non-surgical embryo transfer - 15months.

This extension will also allow us to investigate the commercialisation of the embryo transfer catheter. Although not a milestone there was an expectation that we could use sexed semen for this study. Unfortunately the equipment for doing was sold by University of Sydney to Total Livestock Genetics in Victoria who are yet to set this up. However we will discuss this possibility with them as part of the extension

8. References

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