Reducing early embryonic loss in the pig

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Report prepared for the
Co-operative Research Centre for High Integrity Australian Pork

By

Associate Professor Mark Nottle[1] & Dr Pieter Langendijk[2]

[1]School of Paediatrics and Reproductive Health, University of Adelaide
Phone 08 8303 4087 Fax: 08 8303 4099 mark.nottle@adelaide.edu.au

[2]Research Scientist, SARDI
Phone: 8303 7716 Fax: 8303 7689 Pieter.langendijk@sa.gov.au

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1. Executive Summary

The profitability of pig production is dependent on the number of pigs weaned per sow per year. The Australian sow ovulates on average 22 oocytes per cycle yet the number of pigs born live is around 11. Recent work by us in multiparous sows from the Roseworthy herd has shown that 33% of embryos (7 potential piglets) are lost by day 35 of gestation, with 23% (5 potential piglets) lost by day 21 (Langendijk *et al.*, Pork CRC project 5A-105). In contrast studies by the Foxcroft group suggest that much of this loss in North American herds now occurs post day 30 (Oliver *et al.*,2011; Patterson *et al.*, 2011), suggesting that there are inherent differences in embryo and fetal loss between Australian and overseas herds. The mechanisms governing early embryonic loss in the Australian herd have not been widely studied. Previous studies in mice have demonstrated that the number of cells in the inner cell mass of blastocysts is correlated with implantation rate and that this can be influenced by nutrition (Lane and Gardener 1997). These are the cells in the embryo which give rise to the fetus. Whether a similar relationship between inner cell mass number and implantation rate exists in the pig has not been determined. As a first step in understanding the reasons for early embryonic loss in the Australian herd the present study examined the relationship between nutrition and inner cell mass number in gilts and sows using the known effect of feed restriction to increase early embryonic loss. In the gilt study animals were assigned to two groups and fed at 2.5 times maintenance (control) or maintenance (restricted) from 5 days after puberty until their second ovulation (approximately 16 days). In the sow experiment animals were be fed at their normal feed intake level (control), or pair-fed at 60% of that level (restricted), during the last week of lactation). After weaning, sows in control and restricted groups were treated the same in terms of feeding, insemination etc. In both studies embryos were recovered at day 5 post ovulation as determined by ultrasound, and ovulation rate, the number of embryos and the number of these that were blastocysts was recorded. Blastocyst stage embryos were differentially stained to determine, total cell number, trophectoderm (cells which give rise to the placenta) and inner cell mass cell number. Restricting feed intake in gilts significantly reduced ovulation rate and the number of embryos collected compared with controls. Interstingly blastocysts from the control group tended to have lower total, trophectoderm inner cell mass cell numbers compared with the restricted group. Similar trends in blastocyst cell numbers were also observed in control sows compared with feed restricted animals. This was an unexpected finding as we expected blastocysts from control animals to have higher total, trophectoderm and inner cell mass cell numbers. We have interpreted this to mean that that under normal managment conditions gilts and sows produce a proportion of embryos which are less viable (as indicated by cell number) which are not ovulated under conditions of feed restriction and which are a major contrbutor to early embryonic loss. Given the potential significance of such a finding the present study needs to be extended to confirm this hypothesis. If demonstrated the current approach will not only have identifed a major source of early embryonic loss but also provide a model whereby strategies designed to reduce this loss (potentially five piglets) can be evaluated by determining whether or not they increase inner cell mass number.

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2. Introduction

The profitability of pig production is dependent on the number of pigs weaned per sow per year. The Australian sow ovulates on average 22 oocytes per cycle yet the number of pigs born live is around 11. A recent study by us has shown that 33% of embryos (7 potential piglets) were lost by day 35 of gestation, with 23% (5 potential piglets) lost by day 21 (Langendijk *et al.*, Pork CRC project 5A-105). In contrast studies by the Foxcroft group suggest that much of this loss occurs in in a subpopulation of animals resulting in a skewing of the sex ratio towards males Vinsky *et al.*, 2006a,b) and that the majority of embryos are now lost post day 30 (Oliver *et al.*, 2011; Patterson *et al.*, 2011). Together these findings suggest that there are inherent differences in embryo and fetal loss between Australian and overseas sow herds. This is perhaps not surprising given that the Australian herd has been closed for more than 40 years which has seen reproductive performance remain relatively unchanged compared with the increases seen in overseas herds. The mechanisms governing embryonic loss in the Australian herd have not been widely studied. However it has long been known that embryos vary considerably in their size by the start of elongation and attachment around day 12. And it has been suggested that smaller embryos tend to be lost at this stage while those embryos that manage to implant continue to be retarded in their development contributing to the variation seen in piglet birth weight and subsequent growth performance resulting in further production losses (Pope *et al.*, 1990; Giesert and Schmitt 2002). This variation is still evident in the Australian Herd today (Figure 1).

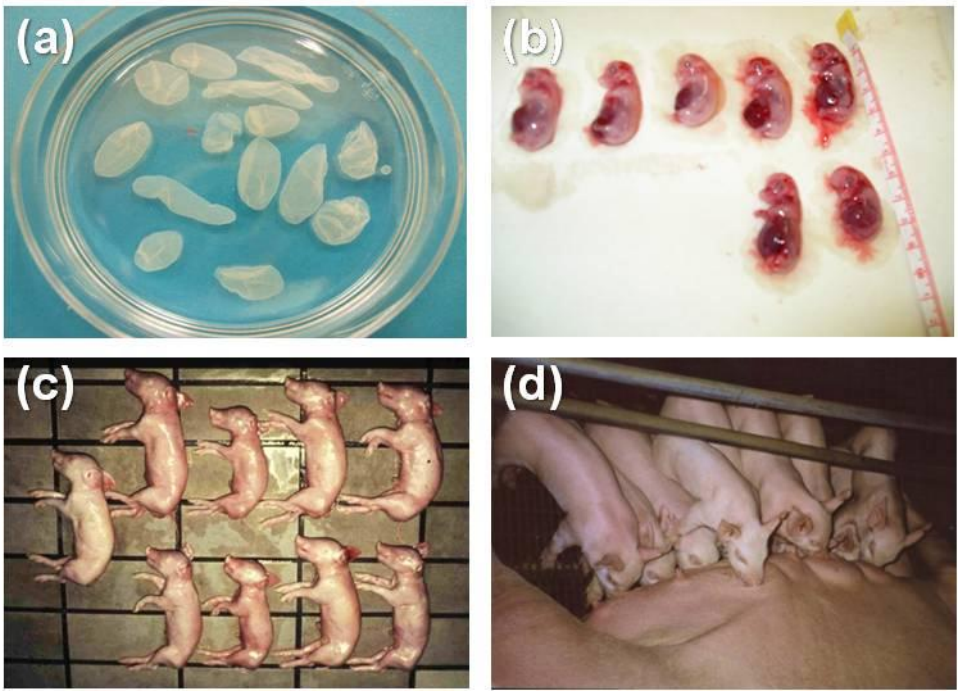

Figure 1. Variability within litter mates during embryonic, fetal and pre-weaning development. Embryo diversity can be seen as early as day 11 of gestation (a) continuing through pregnancy at day 35 (b) and day 70 (c) of gestation. This variation also is seen in littermates after birth (d) and influence growth and development throughout life (Photos courtesy Ms Emmy Bouwman, SARDI)

The variation in embryonic size seen at the start of attachment suggests that early embryonic loss is determined prior to this stage. In particular it has been suggested that this is due to effects on the final stages of oocyte maturation

resulting in epigenetic changes in gene expression in the developing embryo (Vinsky *et al.*, 2007). Previous studies in mice have demonstrated that the number of cells in the inner cell mass of blastocysts is correlated with implantation rate and fetal development (fetuses present on day 15 per blastocysts transferred) that this can be influenced by nutrition (Figure 2 Lane and Gardener 1997). These are the cells which give rise to the fetus. The aim of the present study was to determine whether a similar relationship exits in the pig between inner cell mass number and the number of foetuses present after the embryo elongates and becomes attached. In particular we hypothesise that the external environment influences oocyte maturation and early embryo development via changes in epigenetic gene expression. This is reflected in the number of inner cell mass cells in the developing embryo and that there are a minimum number of these below which embryo survival is compromised. As a first step in examining this hypothesis, the present study examined the relationship between nutrition and inner cell mass number in gilts and sows using feed restriction models used previously to study early embryonic loss.

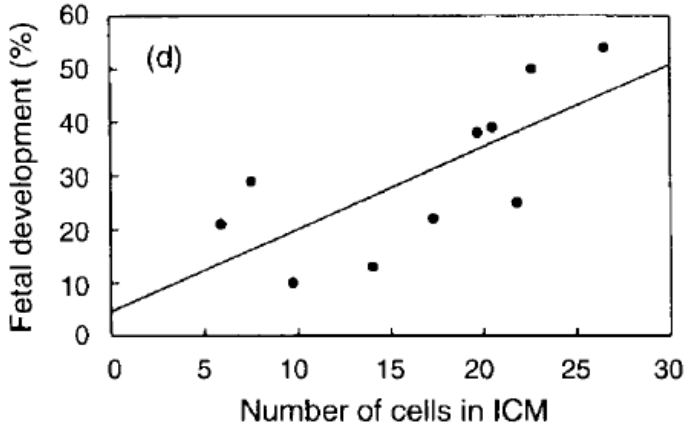

Figure 2 - Relationship between inner cell mass number and fetal development (number of fetuses present day 15 per blastocyst transferred) in mice (from Lane and Gardener 1997)

3. Methodology

The effect of nutritional status on inner cell mass number was examined using feed restriction models previously used to study embryonic loss (Chen *et al.*, 2012; Vinsky *et al.*, 2006a,b).

Experiment 1 - Effect of nutrition on blastocyst development in gilts
Fifty-five prepubertal gilts (average BW 106 kg) were injected with PG600 at 24 wks of age to induce puberty. Those gilts that showed oestrus within one week (n=41) were assigned to two groups and fed at maintenance (restricted) or 2.5 times maintenance (control) from 5 days after puberty until their second ovulation (approximately 16 days). Following the first luteal phase, gilts were injected with prostaglandin F2α at 12 days after their first ovulation, to synchronise the start of the (second) follicular phase and time of ovulation was monitored by ultrasound every 12h. Gilts were inseminated every day of oestrus. Animals were then slaughtered at a local abattoir at 5 days after ovulation as determined by repeated ultrasound by and the reproductive tracts collected. Embryos were recovered by flushing the whole reproductive tract and recovering these under low magnification. Ovulation rate, number of embryos and the number of these that were blastocysts was recorded. Approximately half of these were differentially stained to determine blastocyst total cell number trophectoderm and inner cell mass cell number. The remaining embryos were

frozen to determine gene expression in the advent that a difference in cell number was demonstrated.

Experiment 2 - Effect of nutrition on blastocyst development in sows
In the sow model 15 sows were be fed at their normal feed intake level (control), and 15 sows were pair-fed at 60% of that level (restricted), during the last week of lactation. Mixed parity (average parity 2) sows were allocated to a treatment based on their average feed intake during three days before the start of the treatments, calculated for each sow. Based on that feed intake sows were assigned to pairs of similar feed intake, which were then split between the ad lib fed treatment and the restricted (60% of paired ad lib) sows. The resulting feed intake in the last week of lactation was 6.7 kg for ad lib sows and 4.2 kg/d for restrict fed sows. Litter size was standardised to 11 piglets at the start of lactation and sows were fed according to a step-up regime until allocation to treatments at one week before weaning. Sows with a feed intake lower than 4.5 kg before allocation were excluded from the trial. After weaning sows in control and restricted treatments were treated the same in terms of feeding, boar exposure, heat detection and insemination. Embryos were recovered at day 5 post ovulation as determined by repeated ultrasound by flushing tracts and recovering these under low magnification. Ovulation rate, number of embryos and the number of these that were blastocysts was recorded. Approximately half of these were differentially stained to determine, total cell number trophectoderm and inner cell mass cell number. The remaining embryos were frozen to determine gene expression in the advent that difference in cell number was demonstrated.

Statistical analyses
Differences between treatments were tested using ANOVA, except for binomially distributed variables (incidence of oestrus, pregnancy) which were tested using a Chi-square-test. Variables that were not normally distributed such as cell number embryo stage were log-transformed or arc-sine transformed and tested again with ANOVA. However, this did not yield any different outcome in terms of statistical significance.

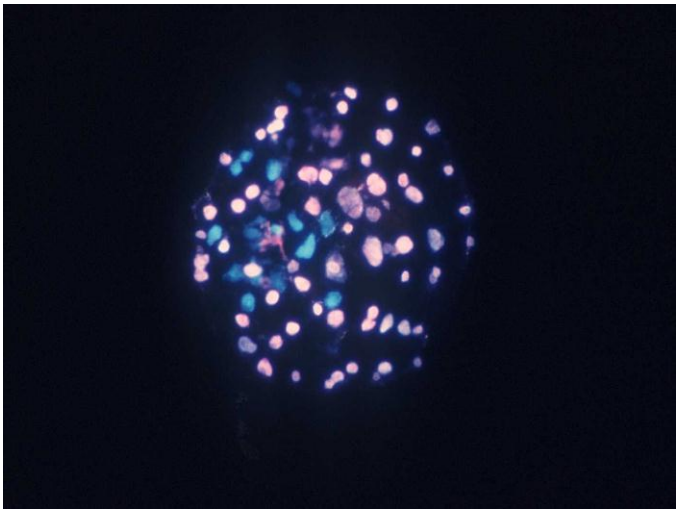

Figure 3 - Differentially stained pig blastocyst. Pink cells are the trophectoderm cells which form the placenta. Blue cells are the inner cell mass cells which give rise to the fetus

4. Outcomes

Experiment 1 - Effect of nutrition on blastocyst development in gilts

Restricted fed gilts lost 235 g/d and gilts on the high feed level had an average growth rate of 594 g/d. Of the gilts assigned to either feed level, 17/22 from the control group a showed a second heat and 12/19 in the restricted fed showed a second heat (overall 70%), which was not significantly different. The average gestational age at slaughter was similar for the two treatments (5.0 ± 0.08 vs. 4.9 ± 0.11 d). The number of embryos at various developmental stages and the results from the cell counts are shown in Table 1. Ovulation rate was 2.8 ovulations higher in the control group which resulted in a trend for more embryos to be recovered in this treatment (P<0.1). The proportion of early, non-expanded, expanded and hatched blastocysts was not different between treatments. There was a trend (P<0.1), for feed restricted gilts to have blastocysts with a higher total, trophectoderm and inner cell mass cell number.

Table 1 - Effect of nutrition on blastocyst development in gilts

	control	restricted
n	14	9
liveweight gain g/d	594 ± 61	-235 ± 69
ovulation rate	14.8±1.28[b]	12.0 ± 0.2[a]
embryos recovered (%)	12.0+1.7 (81)	9.1±1.1(75)
morula (%)	0.8+0.4	0.5±0.8 (0.5)
early blastocysts (%)	1.6+0.7 (11.3)	3.0±0.4 (10.0)
blastocysts (%)	2.1+0.8 (17.1)	2.1±0.8 (22.1)
expanded blastocyst (%)	6.5+1.3 (52.8)	4.5±1.5 (52.1)
hatched blastocysts (%)	1.0+0.6 (10.9)	0.8±0.8 (10.4)
total cell number	59.9+5.2	73.4±11.7
trophectoderm cell number	48.5+4.3	59.8±9.0
inner cell mass number	11.1+1.3	13.5±3.0

Within rows values with different superscripts are significantly different (P< 0.05)

Experiment 2 - Effect of nutrition on blastocyst development in sows

In the first part of lactation before allocation to treatments, sows lost 12 kg body weight on average. During the last wk of lactation, restrict fed sows had an energy balance of -38 MJ ME/d compared to -15 MJ ME/d in the ad lib fed sows. As a consequence feed restricted sows lost 17 kg in the last week of lactation compared to 4 kg in the ad lib fed sows. Feed restricted sows had a delayed weaning to ovulation (6.7 ± 0.1 d vs. 6.2 ± 0.2d; P<0.05)) compared to the ad lib fed sows. Ovulation rate and the number of embryos were lower in feed restricted sows but this was not significant. Similar to the gilt experiment, blastocysts from feed restricted sows tended to have a higher number of total, trophectoderm and inner cell mass cells.

Table 2 - Effect of nutrition on blastocyst development in sows

	control	restricted
n	14	15
liveweight loss in last wk of lactation , kg	4.4 ± 1.5[a]	16.6 ± 1.1[b]
energy balance last wk of lac, MJ ME/d	-14.5 ± 3.2[a]	-39.2 ± 2.7[b]
ovulation rate	19.9+ 1.0	18.4+0.7
embryos recovered (%)	15.9 + 1.5 (80)	14.7 + 1.0 (80)
morula (%)	1.7 + 0.9 (8.4)	0.7 + 0.4 (4.3)
early blastocysts (%)	0.5 + 0.2 (3.1)	0.5 + 0.3 (4.1)
blastocysts (%)	0.8 + 0.4 (4.7)	1.8 + 1.0 (11.4)
expanded blastocyst (%)	10.4 + 1.7 (62.2)	10.3 + 1.3 (72.9)
hatched blastocysts (%)	2.6 + 1.2 (21.5)	1.1 + 0.4 (7.3)
total cell number	71.2 + 9.8	80.5 + 6.4
trophectoderm cell number	56.7 + 7.7	62.2 + 5.3
inner cell mass number	14.5 + 2.4	18.4 + 1.4

These apparent differences in inner cell mass number did not appear to be related to energy balance in gilts and sows as shown in Figure 4.

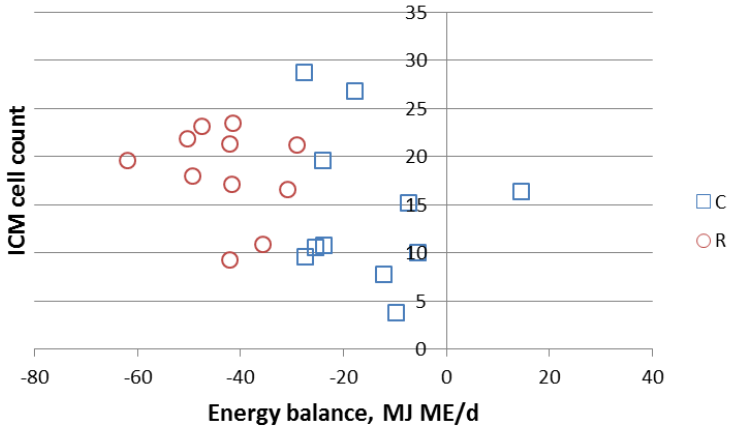

Figure 4 - Relationship between inner cell mass number and energy balance in sows. Control sows open circles. Restrict fed sows open squares.

5. Application of Research

The Australian sow ovulates on average 20 oocytes per cycle yet the number of pigs born live is around 10. A recent study by us showed that 33% of embryos (6 potential piglets) were lost by day 35 of gestation, with 23% (4 potential piglets) lost by day 21 (Langendijk *et al.*, Pork CRC project 5A-105). Apparent differences in embryo survival between Australian and overseas herds suggest that further studies in this area are required. Previous studies in mice have demonstrated that the number of cells in the inner cell mass of blastocysts is correlated with fetal

development (fetal number divided by number of blastocysts transferred) and that this can be influenced by nutrition (Lane and Gardener 1997). As a first step in understanding the reasons for this loss in Australian Herds the present study examined the relationship between nutrition and inner cell mass number in gilts and sows using the known effect of feed restriction to increase early embryonic loss.

6. Conclusion

The present study confirmed previous studies that gilts fed at recommended levels for at least two weeks prior to ovulation (control) have a higher ovulation rate and as a consequence numbers of embryos, compared with those fed at maintenance (restricted). Interestingly blastocysts from the restricted group tended to have higher total, trophectoderm and inner cell mass cell numbers. Similar trends albeit non significant in ovulation rate and blastocyst total, trophectoderm and inner cell mass cell number were also seen in the sow study. In both studies all animals had blastocyst with fewer cells. In other words we did not identify a subset of animals which had blastocysts with lower cell numbers. In contrast Vinsky *et al.*, (2006 a,b) have suggested that early embryonic loss occurs in a subset of animals undergoing significant catabolic loss, further highlighting potential differences between Australian and overseas herds.

7. Limitations/Risks

This is the first time to our knowledge that the effect to feed restriction on blastocyst inner cell number has been examined in gilts and sows. The apparent increase in cell number with restricted feeding was an unexpected finding which we have interpreted to mean that under normal management conditions gilts and sows produce a proportion of embryos which are less viable (as indicatd by inner cell mass cell number) which is a major contrbutor to early embryonic loss. Given the potential significance of such a finding the present study needs to be extended to confirm this hypothesis. If demonstrated the current approach will not only have identifed a major source of early embryonic loss but also provide a model whereby strategies designed to reduce this loss can be evaluated by determining whether or not they increase inner cell mass number.

8. Recommendations

We recommend that the gilt experiment be extended to test the hypothesis that under normal management conditions animals produce a proportion of embryos which are less viable. To do this we propose to:

1. Undertake a second replicate to confirm (statistically) that total, trophectoderm and inner cell mass cell number are higher in feed restricted animals compared with controls. As well we will determine the sex of these embryos to rule out the possibility that the apparent increase in cell number is due to the skewing towards male embryos (which grow faster) as a consequence of feed restriction as described by Vinsky *et al.* (2006a,b) to occur in North American herds.

2. Examine embryo survival by including a second group for each treatment where animals are slaughtered at day 21 and the number of fetuses determined. If our hypothesis is correct we would expect early embryonic loss (the number of fetuses present divided by ovulation rate) to be higher in the control group compared with the feed restricted group.

3. In collaboration with the Foxcroft group, correlate changes in inner cell mass number with changes in blastocyst gene expression

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