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Embryo and recipient contributions to pregnancy loss following the transfer of cloned embryos derived from foetal and adult somatic cells

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ABSTRACT

The purpose of this paper is to report on the reproductive outcomes following the transfer of cloned bovine embryos produced following somatic cell nuclear transfer. All recipients received two embryos. In Expt 1 (19 recipients), donor cells were derived from a 34 day old female foetus, while adult cows were used as the genetic donors in both Expt 2 ('Lady' from Enderby Island, 38 recipients) and Expt 3 (Elizabeth, a high genetic merit Friesian, 56 recipients). Overall, embryo survival rate to term was 12%, with little affect of experiment. This compares unfavourably with the reported 30-35% survival rate to term in embryos generated using in vitro production procedures without nuclear transfer. Of all transferred embryos, 36% were competent to survive to term. Furthermore, only 36% of the recipients were competent enough to support a pregnancy to term. We conclude that actual embryo survival is lower with nuclear transfer and IVP technology because both embryo and recipient competence is compromised.

Keywords: cloning; embryo survival; recipient cattle; embryo transfer.

INTRODUCTION

There are a variety of methods available to artificially produce cloned, or genetically identical, animals. The simplest of these methods involves manipulating the early embryo over the first few days of development following fertilisation, before the cells (or blastomeres) of the embryo have begun to differentiate or become specialised. Thus blastomeres up to the third cell division, the 8-cell-stage, may be individually isolated and some may on their own develop into viable offspring (Moore *et al*., 1968). Early embryos may also be bisected into two equal halves up to the blastocyst-stage to generate identical twins. This form of cloning has been integrated into commercial MOET schemes aimed at increasing the rate of multiplication of valuable animals (Wells *et al*., 1990).

A potentially much more powerful means of producing larger numbers of cloned animals, however, is through the use of an embryo manipulation procedure termed "nuclear transfer". Technically, nuclear transfer involves the enucleation (removal of chromosomal material) from the recipient oocyte to generate what is called a cytoplast (a cell without a nucleus). A donor cell, containing the new genetic material, is then electrically fused with the cytoplast and the reconstructed 1-cell embryo artificially activated to commence development. Typically, nuclear transfer-derived embryos are cultured in vitro using standard procedures before transfer at a suitable stage to the reproductive tract of recipient females. As a technique, nuclear transfer is not in itself novel as fifty years ago researchers developed the procedure for studying developmental biology in amphibians. It was used to test the hypothesis of 'nuclear equivalence' of cells as they become increasingly more specialised and restricted in their fate as cellular differentiation proceeds throughout embryo and foetal development, culminating in an adult animal. On the basis of these early studies and in subsequent experiments in mice, it was concluded in the journal *Science* that beyond early embryonic cleavage-stages "the cloning of mammals by simple nuclear transfer was biologically impossible" (McGrath and Solter, 1982). Given this (now false) belief, early studies with nuclear transfer in farm animal species focused on cloning using undifferentiated embryonic blastomeres in sheep (Willadsen, 1986) and cattle (Robl *et al*., 1987). The motivation for these studies lay in the potential of producing more offspring (clones) from embryos originally obtained from selected matings between genetically superior livestock (Bondioli *et al*., 1990). However, the number of offspring that could be generated from any one embryo was limited (both by the small number of donor blastomeres available for cloning and technical inefficiencies), even despite efforts to serially reclone nuclear transfer-derived embryos (Stice and Keefer, 1993).

Recent advances in cloning technology have resulted in the ability to produce offspring from cultured differentiated cells grown in the laboratory. These cells have been obtained from either embryos, foetuses or adults in both sheep (Campbell *et al*., 1996; Wilmut *et al*., 1997; Wells *et al*., 1997) and cattle (Cibelli *et al*., 1998; Wells *et al*., 1999a, b). Research in this area has been initially fuelled by the desire to practically apply these techniques for the ultimate benefit of both agriculture and biomedicine. Because of the ready access to millions of cells growing in culture, there are potentially two primary applications of this technology, described in more detail elsewhere (Wells *et al*., 1998b). In brief, they are: 1) the ability to rapidly multiply desirable livestock; and 2) the ability to introduce very precise genetic modifications to the cells growing in culture and to select those cells having undergone the desired alteration before using them for nuclear transfer to produce transgenic animals. Because it has now been demonstrated that it is in fact biologically possible to clone animals from differentiated somatic cells, it is the wide-ranging transgenic opportunities that currently form the focus of much of ex-

perimentation in this area at AgResearch. However, it is acknowledged that there are many technical issues that remain to be resolved to improve the efficiency of nuclear transfer before the technique will ever find widespread, practical and cost-effective used in simply multiplying valuable livestock in agriculture. In particular, a major limiting factor is the low survival of transferred cloned embryos to term compared to that expected from embryos derived from either in vitro procedures or following donor stimulation and embryo recovery.

The purpose of this paper is to report on the reproductive outcomes at Ruakura following the transfer of cloned cattle embryos, and to determine the contribution of embryos and recipients to embryos survival rate.

MATERIAL AND METHODS

The nuclear transfer and embryo culture methods used to produce the embryos described here have been reported elsewhere (Wells *et al*., 1998; 1999a, 1999b). Day 7 in vitro produced (IVP) embryos were transferred in pairs into the same uterine horn of recipients, using standard transrectal transfer procedures.

Experiment 1

Two treatments were applied during the nuclear transfer procedure, AFS (denoting activated and fused simultaneously) and FBA (fused before activation). The transfer of embryos took place during May 1997 in 4 batches. Of the 19 recipients used, 10 received embryos from the AFS treatment and 9 received embryos from the FBA treatment. Pregnancy assessments were made using ultrasound on Day 40 and Day 90 of pregnancy (Day of oestrus = Day 0), and the number of calves born at term was recorded.

Experiment 2

Transfer into recipients took place in September/October 1997 in 3 batches (Early group, n=12 recipients) and in March 1998 in 2 batches (Late group, n=26 recipients). All embryos were produced from the FBA treatment. Pregnancy assessments were carried out on Day 55 and 85, and the number of calves born at term was recorded. Differences between the Early and Late groups were analysed in order to determine the consistency with which foetuses and calves could be produced.

Experiment 3

The transfer of embryos took place during November/December 1997 in 8 batches (n=56 recipients). While the majority of transferred embryos were produced from the standard FBA treatment, some 16 embryos were produced by recloning the first generation nuclear transfer embryos (RC treatment). Pregnancy assessments were on Day 60 and 100 and the number of calves born at term was recorded.

Estimating embryo and recipient contributions to embryo survival

The distribution of recipients with either 0, 1 or 2 foetuses or calves present was used to estimate embryo (e) and recipient (r) contributions to embryo survival using a recently published model (McMillan, 1998). Briefly, the model assumes that embryo survival can be partitioned into independent binomial effects of embryos and recipients, and expected embryo survival is the product of e and r. The maximum likelihood values determined for e may be interpreted as the proportion of embryos which are competent to survive to a given time point, independent of the effect of the recipient. Similarly, the values determined for r may be interpreted as the proportion of recipients which are competent to maintain a pregnancy to a given time point, independent of the effect of the embryo.

The model was applied to the data in two ways. Firstly, values for e and r were estimated using individual sets of data. As noted previously, the degrees of freedom associated with using data from only twin transfers is 0, and the observed and expected data (generally) match perfectly (McMillan, 1998). The results from applying the model to individual sets of data are shown in Tables 1-3. The second application was to test whether the apparent differences between groups (Table 1-3) were real or not. This was achieved by simultaneously fitting either common or different values for e and r to more than one set of data, as previously described (McMillan, 1998). If a common estimate could be fitted to more than one set of data, no real differences exist between the sets of data. Conversely, if different estimates could be fitted, then real differences exist between sets of data. Using the model in this way enables some assessment to be made of the consistency with which competent embryos and recipients were produced in the three experiments. As discussed previously, using observed embryo survival rates to assess embryo competence might be misleading. Comparisons based on e and r are suggested as more meaningful (McMillan, 1998).

RESULTS

Reproductive performance data from Experiment 1 is shown in Table 1. Overall pregnancy rate and embryo survival to Day 40 was 63 ± 11% and 50 ± 8%, respectively, with no difference between treatments ($\chi^2 = 0.09$ and 0.42, respectively, NS). Although the proportion of competent embryos appeared higher in the AFS treatment when fitting values to individual sets of data (e = 0.91 vs. 0.50, Table 1), this difference was not significant, since a common value also fitted the observed distributions (0.71 ± 0.27 vs. 0.71 ± 0.14). Similarly, although the proportion of competent recipients appeared lower in the AFS treatment (r = 0.61 vs. 0.89, Table 1) recipient competence was in fact similar in the two treatment groups (0.72 ± 0.20 vs. 0.72 ± 0.18). Of the recipients that were single or twin pregnant on Day 40, embryo survival to term was similar (20 ± 18% vs. 14 ± 9%, $\chi^2 = 0.09$, NS).

TABLE 1: Number of recipients with 0, 1 or 2 calves present, pregnancy rate (± s.d.), embryo survival (± s.d.) and embryo (e) and recipient (r) competence (± se) at different stages of pregnancy and with either AFS[1] or FBA[1] treatment (Trt) ([1]see text)

Stage of pregnancy	Trt	No. recipients with 0-2 calves 0	1	2	% pregnant	Embryo survival (%)	e	r
Day 40	AFS	4	1	5	60 ± 16	55 ± 11	0.91 ± 0.09	0.61 ± 0.16
	FBA	3	4	2	67 ± 16	44 ± 12	0.50 ± 0.22	0.89 ± 0.33
Day 90	AFS	10	0	0	0	0	-	-
	FBA	7	1	1	22 ± 14	17 ± 9	0.67 ± 0.31	0.25 ± 0.17
Term	AFS	10	0	0	0	0	-	-
	FBA	7	1	1	22 ± 14	17 ± 9	0.67 ± 0.31	0.25 ± 0.17

By Day 90, no pregnancies were evident in the AFS treatment group compared with two in the FBA group (χ^2 = 3.24, NS) (Table 1). These two pregnancies were maintained to term. Although the proportion of competent FBA embryos appeared lower at Day 40 compared with Day 90 or term (e = 0.50 vs. 0.67 vs. 0.67, Table 1), embryo competence was in fact similar at all three stages of pregnancy (0.52 ± 0.17 vs. 0.52 ± 0.22 vs. 0.52 ± 0.22). In contrast, a common recipient competence at all stages of pregnancy did not adequately fit the FBA data. Instead, recipient competence was estimated to be lower to Day 90 and term compared with Day 40 (0.29 ± 0.22 vs. 0.29 ± 0.22 vs. 0.84 ± 0.27, P<0.05).

Reproductive performance data from Experiment 2 is shown in Table 2. Overall pregnancy rate and embryo survival to Day 55 was 42 ± 8% and 29 ± 5%, respectively, with no difference between Early and Late groups (χ^2 = 0.55 and 1.12, respectively, NS). Although a model containing different e and r-values was initially fitted to the data (Table 2), subsequent analysis indicated a simpler model involving a common value for e and r also explained the data. Estimates for embryo and recipient competence were, respectively, 0.54 ± 0.46 and 0.54 ± 0.40 for the Early group and 0.54 ± 0.14 and 0.54 ± 0.14 for the Late group. Of the recipients that were single or twin pregnant on Day 55, embryo survival to term was the same 50 ± 16 vs. 50 ± 14%, respectively.

Overall pregnancy rate and embryo survival to Day 85 was 34 ± 8% and 24 ± 5%, respectively, with no difference between Early and Late groups (χ^2 = 0.06 and 0.16, respectively, NS). Although a model containing different e and r-values was initially fitted to the data (Table 2), subsequent analysis indicated a simpler model involving a common value for e and r also explained the data. Estimates for embryo and recipient competence were, respectively, 0.49 ± 0.31 and 0.49 ± 0.30 for the Early group and 0.49 ± 0.19 and 0.49 ± 0.19 for the Late group.

Overall pregnancy rate and embryo survival to term was 21 ± 7% and 15 ± 5%, respectively, with no difference between Early and Late groups (χ^2 = 0.20 and 0.11, respectively, NS). Although a model containing different e and r-values was initially fitted to the data (Table 2), subsequent analysis indicated a simpler model involving a common value for e and r also explained the data. Estimates for embryo and recipient competence were, respectively, 0.39 ± 0.32 and 0.39 ± 0.31 for the Early group and 0.39 ± 0.24 and 0.39 ± 0.23 for the Late group.

TABLE 2: Number of recipients with 0, 1 or 2 calves present, pregnancy rate (± s.d.), embryo survival (± s.d.) and embryo (e) and recipient (r) competence (± se) at different stages of pregnancy in Early[1] and Late[1] treatment groups ([1]see text)

Stage of pregnancy	Trt	No. recipients with 0-2 calves 0	1	2	% pregnant	Embryo survival (%)	e	r
Day 55	Early	8	3	1	33 ± 14	21 ± 8	0.40 ± 0.28	0.52 ± 0.34
	Late	14	7	5	46 ± 10	33 ± 7	0.59 ± 0.14	0.56 ± 0.14
Day 85	Early	8	3	1	33 ± 14	21 ± 8	0.40 ± 0.28	0.52 ± 0.34
	Late	17	5	4	35 ± 9	25 ± 6	0.62 ± 0.16	0.41 ± 0.12
Term	Early	10	1	1	17 ± 11	13 ± 7	0.67 ± 0.31	0.19 ± 0.13
	Late	20	4	2	23 ± 8	15 ± 5	0.50 ± 0.22	0.31 ± 0.14

Reproductive performance data from Experiment 3 is shown in Table 3. Overall pregnancy rate and embryo survival to Day 60 was 70 ± 6% and 44 ± 5%, respectively, with no difference between FBA and RC treatment groups (c^2 = 0.23 and 0.30, respectively, NS). Although a model containing different e and r-values was initially fitted to the data (Table 3), subsequent analysis indicated a simpler model involving a common value for e and a common but different value for r also explained the data. Estimates for embryo and recipient competence were, respectively, 0.44 ± 0.08 and 1.00 ± 0.17 for the FBA group and 0.44 ± 0.57 and 1.00 ± 1.04 for the RC group. Of the recipients that were single or twin pregnant on Day 60, embryo survival to term was 21 ± 8 vs. 20 ± 13%, respectively (χ^2 = 0.002, NS).

Table 3: Number of recipients with 0, 1 or 2 calves present, pregnancy rate (± s.d.), embryo survival (± s.d.) and embryo (e) and recipient (r) competence (± se) at different stages of pregnancy and with FBA[1] or RC[1] nuclear transfer treatment ([1]see text)

Stage of pregnancy	Trt	No. recipients with 0-2 calves 0	1	2	% pregnant	Embryo survival (%)	e	r
Day 60	FBA	14	25	9	71 ± 7	45 ± 5	0.45 ± 0.09	1.00 ± 0.17
	RC	3	4	1	63 ± 17	38 ± 12	0.38 ± 0.23	1.00 ± 0.54
Day 100	FBA	34	7	7	29 ± 7	22 ± 4	0.67 ± 0.12	0.33 ± 0.08
	RC	8	0	0	0	0	-	-
Term	FBA	40	6	2	14 ± 5	10 ± 3	0.40 ± 0.20	0.26 ± 0.13
	RC	8	0	0	0	0	-	-

Overall pregnancy rate and embryo survival to Day 100 was 25 ± 6% and 19 ± 4%, respectively, with no difference between FBA and RC groups in pregnancy rate (χ^2 = 3.11), but a difference in embryo survival rate (χ^2 = 4.31, P<0.05). No embryos from the RC group survived to Day 100. Overall pregnancy rate and embryo survival to term was 14 ± 5% and 9 ± 3%, respectively, with no difference between FBA and RC groups (χ^2 = 1.56 and 1.83, NS).

Overall embryo and recipient contributions to embryo survival

The results of fitting common or different e and r-values to the embryo survival data in the 3 experiments are shown in Table 4. Different e and r-values were required in each experiment to describe embryo survival to about 7 weeks. However, a common e and r vale adequately described embryo survival to about Day 90 (0.48) and to term (0.36).

Table 4: Fitting common or different e and r-values for embryo survival in Experiment 1-3

	Day 40-60		Day 85-100		Term	
	e	r	e	r	e	r
Expt 1	0.74 ± 0.11	0.68 ± 0.13	0.48 ± 0.30	0.48 ± 0.31	0.36 ± 0.32	0.36 ± 0.33
Expt 2	0.55 ± 0.13	0.53 ± 0.13	0.48 ± 0.15	0.48 ± 0.15	0.36 ± 0.19	0.36 ± 0.19
Expt 3	0.44 ± 0.08	1.00 ± 0.16	0.48 ± 0.20	0.48 ± 0.20	0.36 ± 0.20	0.36 ± 0.21

DISCUSSION

The key finding in this study was that embryo survival to term following the transfer of nuclear transfer embryos derived from differentiated somatic cells is lower by 20-30 percentage units than expected following the transfer of either in vivo or in vitro embryos derived from germ cells (McMillan, 1998). Interestingly, these results are similar to what has been reported previously with undifferentiated embryonic blastomeres (Kimura *et al*., 1997; Peura *et al*.,1 998; Stice and Keefer, 1993). While the analysis did not provide insight into the physiological causes of this greater embryonic and foetal wastage, it did attribute it about equally to both lowered embryo and recipient competence. Early embryo survival, as well as embryo and recipient competence differed amongst the three experiments that used different cell lines and different groups of recipients. However, we found consistency amongst all three experiments in embryo and recipient competence, and therefore embryo survival, to either about 90 days or to term.

Cloned embryos in the current study had a competence to survive to term of about 60% of that achieved with IVP embryos derived from germ cells (McMillan, 1998). Only some of the additional loss of embryo competence was evident at about 7 weeks of pregnancy, since the e values that were obtained are slightly lower than achieved to the same stage of pregnancy in other studies using IVP embryos (McMillan *et al*., 1997). Most of the additional loss appears to take place subsequent to this period. Importantly, the loss rate from about Day 60 to term appears twice as high in this study with cloned embryos compared with previous reports relating to IVP embryos (McMillan *et al*., 1996). It was highlighted in this previous report that embryo competence alone accounted for foetal wastage form Day 60 to term, and that recipients were fully competent to support pregnancies to term after two months. This is apparently not the case following the transfer of cloned cattle embryos.

This study did not provide any direct clues as to the physiology of reduced embryo competence. Studies have recently commenced to investigate this further. Our working hypothesis, based on data from IVP pregnancies, is that aberrations in allantoic development are likely to be higher when cloned embryos are transferred. The pattern of foetal wastage reported in the current study is consistent with the condition referred to as allantoic aplasia (Peterson and McMillan, 1998). This condition may be exacerbated during the micromanipulation procedures necessary during nuclear transfer. Since other placental pathologies have been identified with nuclear transfer cloned foetuses and calves (Kruip and den Daas, 1997), it is possible that these may also contribute to the additional foetal loss.

The level of recipient competence capable of supporting cloned pregnancies to term in the current study is at the lower extreme observed in over 40 sets of data with IVP and in vitro-derived embryos (McMillan, 1998). However, the higher and variable competence seen at about 7 weeks is comparable with recipient performance with IVP embryos (McMillan *et al*., 1997). The finding of a common r value for each of the 3 experiments which adequately explains the data at about Day 90 and term, differs from previous findings where r has been shown to be highly variable amongst studies (McMillan, 1998). It is difficult to explain in physiological terms why recipient competence to term is very low in the current study. However, it is known that in the statistical model used here, changes in pregnancy rate are usually reflected in changes in r more so than e (McMillan, 1998). This also explains why r-values are typically lower when IVP rather than in vivo-derived embryos are used (McMillan, 1998).

Overall, there was no difference between single and twin pregnancies (27 vs. 26%) in the embryo survival rate from about 7 weeks to term. This result is consistent with published findings (e.g., McMillan, 1996), and supports the practice of using twin transfers to save on recipient costs. Amongst experiments, embryo and recipient competence were most variable when estimated at about 7 weeks. In addition, embryo survival at this early stage was not a good indicator of embryo survival to term. However, at about 90 days, embryo survival, as well as e and r, were similar in all experiments. In all 3 experiments, about half of the foetuses present at about Day 90 were not represented by calves at term. This is about twice the loss rate expected from IVP-derived foetuses (McMillan *et al*., 1996).

An intriguing result in this study was the lack of single births in the 23 recipients that were twin pregnant at about 7 weeks. These recipients delivered either zero or twins calves at term. We are not aware of this all-or-none phenomenon in any other sets of data and conclude that it may be specific to nuclear transfer somatic cell pregnancies. The survival rate of foetuses from about Day 60 to term is known to be an independent rather than all-or-none phenomenon (McMillan *et al*., 1996, Sinclair *et al*., 1995). Possible explanations for this phenomenon could include major recipient effects or foetus-to-foetus transfer of some deleterious 'agent'. Despite this apparent all-or-none loss rate in twin pregnant recipients, the survival rate from about 7 weeks to term was similar in single and twin pregnancies. This similarity in single and twin survival rates is in agreement with studies using IVP-derived conceptuses (McMillan *et al*., 1996, Sinclair *et al*., 1995).

In conclusion, this study has identified both lowered embryo and recipient competence as contributing about equally to the lowered embryo survival to term when using nuclear transfer somatic cell cloned embryos, when compared with IVP embryo survival. The embryo and foetal loss pattern is consistent with that observed in IVP embryos with compromised allantoic development. The all-or-none calving outcome in previously twin pregnant recipients has not been observed previously, and highlights the different

biology associated with somatic cell cloned embryos. Studies are underway to identify the causes of the additional embryo loss. Finally, we have demonstrated that our laboratory can consistently produce cloned embryos and recipients of similar competence to produce calves.

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