Experiences in the application of embryo bisection in sheep MOET programmes.

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ABSTRACT

Bisected embryos not only create identical twins, but can also improve the efficiency of embryo transfer. We report here our experiences in applying the technique to sheep MOET programmes.

Our basic technique involves holding a Day 5-6 embryo by negative pressure on a holding pipette and tearing the *zona pellucida* using 2 needles. A razor blade fragment is used to bisect the embryo and if required, a suction pipette manipulates demi-embryos into suitably prepared host zonae.

In a preliminary study bisected embryos were examined for development during 24-48 h culture. Bisection in Ca^{2+}, Mg^{2+}-free media had no effect on viability. Naked demi-embryos blastulated more readily than those which were zona-enclosed and later stages tended to be the most viable, with morulae showing particularly poor development. This was supported by transfer data, where 11% of bisected late morulae, 50% of early blastocysts and 74% of blastocysts resulted in lambs. In comparison, 40% of control (unbisected) embryos lambed. Transfer of zona-enclosed demi-embryos tended to yield more lambs per original embryo (67%) than naked demi-embryos (43%). When the technique was applied to early blastocysts and blastocysts during 3 exotic sheep multiplication programmes in 1988, 47% and 55%, 100% and 39%, 73% and 47% of bisected and control embryos resulted in lambs respectively. Survival to lambing of dehydrated late morulae, bisected and subsequently enclosed in zonae, was similar to untreated control embryos (73% vs. 59%) and greater than for naked embryos (36%).

Our experiences indicate that bisected early blastocysts and blastocysts can increase the number of offspring in a MOET programme. However, since few blastocysts are available for bisection, and survival of demi-embryos is variable, we do not routinely use the technique.

Keywords Sheep, embryo, bisection, splitting, MOET.

INTRODUCTION

The capacity for early preimplantation embryos of domestic animals to develop following division into two halves was first demonstrated using sheep embryos (Willadsen, 1979). Since then, simplified procedures using later stage embryos have been developed and applied to a number of species: cattle (Ozil, 1983), horses (Allen and Pashen, 1984), goats (Tsunoda *et al.*, 1985), sheep (Willadsen and Godke, 1984) and pigs (Rorie *et al.*, 1985). The value of embryo bisection to animal breeding includes the production of identical twins for experimental comparisons, and the potential to increase numbers of liveborn offspring from embryo transfer programmes. The latter increase has been reported for cattle (Leibo and Rall, 1987), but variable results have been observed in both sheep and goats (Chesne *et al.*, 1987; Maurer, 1988; Tsunoda *et al.*, 1985; Udy, 1987). For this reason, a number of investigations were performed in our laboratory to develop and assess a technique and to then apply it to MAF Technology's exotic sheep multiple ovulation and embryo transfer (MOET) programme, at Hopuhopu Quarantine Station. In this report, we document our results and discuss the application of the technique.

TECHNIQUES AND RESULTS

Embryo production and recovery

Embryos for bisection were recovered surgically from superovulated ewes on Day 5-6 after oestrus detection (oestrus = day 0). The experimental trials were conducted early in the 1988-1989 breeding seasons (February-March) using mature, mixed-age Coopworth ewes. At Hopuhopu embryos were recovered from mixed-age Texel and Oxford Down ewes during April to June 1988. Methods of superovulation and recovery have

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been described in detail elsewhere (Thompson *et al.*, 1990; Tervit and Havik, 1976). Embryo recovery was on Day 5-6 and usually resulted in embryo stages ranging from morulae to hatched blastocysts.

Equipment and techniques for embryo bisection

The equipment and techniques used to bisect embryos by our group have already been detailed elsewhere (Udy, 1987). Briefly, the technique involves visualising embryos with a video inverted microscope (Ensor Scientific Instruments, Auckland) and manipulating with 5 microtools. During manipulation, embryos are held at room temperature in enriched Dulbecco's phosphate buffered saline (PB1; Tervit *et al.*, 1972). An embryo is selected, orientated and held in position by negative pressure on a holding pipette. The *zona pellucida* is then torn with two finely drawn glass needles. A fragment from a razor blade is manouvered through the torn *zona pellucida* and bisects the embryonic cell mass into two approximately equal halves. If required, a suction pipette is used to manipulate each half into an empty *zona pellucida*.

Preliminary studies

These experiments investigated the effects of embryo bisection on viability by examining development *in vitro* following culture for 24-48 h. Formation of a blastocyst or reblastulation was considered to indicate demi-embryo survival. Following bisection, naked (i.e. without a *zona pellucida*) or zona-enclosed demi-embryos and control (unsplit) embryos were placed in 1ml volumes of SOF medium (Tervit *et al.*, 1972) supplemented with 10% sheep serum and incubated at 38 C under 5% CO2 in air (Thompson *et al.*, 1989). Data were subjected to Chi-square analyses.

Experiment 1

Assessed the effect of embryo stage at bisection. Eighty-nine morula to blastocyst stage embryos were bisected and cultured for 48 h. In addition, 61 morula to blastocyst stage embryos were cultured as comparative controls. Results are presented in Table 1. Overall, more control than demi-embryos developed in culture ($P<0.001$) and later stages tended to blastulate most readily ($P<0.1$). Of the bisected embryos, naked embryos blastulated more readily than zona-enclosed ($P<0.05$) and later stages tended to be the most viable ($P<0.1$) with morulae showing particularly poor development.

TABLE 1 Effect of embryonic stage on blastulation following culture of bisected sheep embryos.

Embryo stage	No. blastulating/No. cultured (%) control embryos	for demi-embryos naked	in zona
Morula	3/6 (50)	2/6 (33)	0/8 (0)
Late morula	11/14 (79)	10/26 (38)	7/16 (44)
Early blastocyst	14/20 (70)	16/26 (62)	9/28 (32)
Blastocyst	15/21 (71)	23/38 (61)	13/30 (43)

Experiment 2

This assessed the effect on viability of holding and splitting embryos in Ca^{2+}, Mg^{2+}-free media (which weakens intercellular contact). Twenty seven early blastocyst and blastocyst stage embryos were held in Ca^{2+}, Mg^{2+}-free PB1 (CMF-PB1) for approximately 1h. Nineteen of these embryos were then bisected in CMF-PB1. The remaining 8 embryos served as unsplit controls. Fifteen embryos were also held in PB1 and subsequently 8 were bisected while 7 served as controls. All embryos and demi-embryos were then incubated for 24 h in SOF medium. The proportions of demi-embryos blastulating following splitting in CMF-PB1 or PB1 were similar at 71% (27/38) and 81% (13/16) respectively. Results for comparative controls were 88% (7/8) and 71% (5/7) respectively. Furthermore, there was no advantage in the mechanical aspects of bisecting embryos in CMF-PB1 as compared to normal PB1.

Embryo and demi-embryo transfer to recipient ewes

Seventy-nine late morulae, early blastocysts and blastocysts were collected in March 1988. Of these, 35 were selected at random as controls and were held in PB1 and then transferred to the uterine horns of 18

synchronous recipient mature Coopworth ewes. The remaining 44 embryos were bisected in PB1 using our standard procedures and then transferred to 44 recipient ewes. Forty-two of the 88 demi-embryos (21 bisected embryos) were each manipulated into an empty *zona pellucida* prior to transfer. The remaining demi-embryos were transferred naked. The results of this trial are presented in Table 2. Numbers are low, and the tendancy for bisected embryos to produce more lambs than controls did not reach significance. The survival of bisected embryos was affected by stage of development ($P<0.05$) with late morulae surviving poorly. Transfer of bisected embryos within zonae tended to improve survival rates, with zona-enclosed blastocysts and early blastocysts producing 38% and 17% more lambs than their respective controls.

TABLE 2 Embryo survival to lambing following bisection and subsequent transfer.

	Number embryos	Number lambs born	% Embryo survival
Control	35	14	40
Split			
late morula	9	1 (0)*	11
early blastocyst	12	6 (2)	50
blastocyst	23	17 (6)	74
In zonae	21	14	67
Naked	23	10	43

()* denotes sets of identical twins.

Embryo bisection at Hopuhopu Quarantine Station.

The 1988 exotic sheep MOET programme involved subjecting donors to three rounds of embryo recovery at monthly intervals beginning in early April. Early blastocysts and blastocyts were bisected in each round. Pregnancy scanning data from the preliminary experiments described above, were not available prior to the first round of transfers at Hopuhopu. Thus all demi-embryos were transferred naked to synchronised recipients. In rounds 2 and 3, all demi-embryos were enclosed in zonae and transferred to recipients which had shown oestrus one day after the donors. Embryo survival for the demi-embryos and for control unsplit embryos from the same donors are shown in Table 3.

Embryo bisection significantly increased the number of offspring born in rounds 2 and 3. Overall, survival of bisected early blastocysts was 91% and bisected blastocysts 65% ($p<0.05$)

TABLE 3 Embryo survival to lambing at Hopuhopu

Round no.	Embryo type	No. embryos transferred	No. and (%) lambs born	No. sets identicals
1	Bisected	19	9 (47)	1
	Control	105	58 (55)	
2	Bisected	19	19 (100)	5
	Control	122	48 (39)	
3	Bisected	30	22 (73)	6
	Control	103	48 (47)	

Dehydration of late morulae

This experiment, conducted early in the 1989 breeding season (Feb-Mar), assessed the effect of dehydrating late morulae prior to and during bisection on subsequent survival following transfer. Dehydration was achieved by incubating embryos in a modified PB1 (m-PB1) supplemented with 500 mM sucrose and a total Ca^{2+} concentration of 2.21 mM (Herr *et al.*, 1988). A total of 29 late morulae were incubated for approximately 30 min prior to bisection in m-PB1. After bisection, demi-embryos were placed into PB1 prior to transfer, to rehydrate. Thirty demi-embryos, from the bisection of 15 embryos, were manipulated into empty zonae prior to transfer. The remaining demi-embryos were transferred naked. Forty-four late morulae and early blastocysts, not subject to dehydration, were also transferred to serve as unsplit controls.

Overall, embryo bisection after dehydration did not increase the number of lambs born compared to control transfers (55% v. 59%). However, within bisected embryos, those transferred within zonae produced more lambs than those transferred naked (73% v. 36%, $p<0.05$).

DISCUSSION

In a successful embryo bisection programme, it is generally found that about 35% more offspring are produced from bisected compared to control embryos (e.g. Williams *et al.*, 1984). In the present studies a clear advantage of bisected early blastocyst and blastocyst demi-embryos, transferred in zonae, was demonstrated in rounds 2 and 3 at Hopuhopu and in the preliminary transfer trial.

Our preliminary studies highlighted a number of factors affecting the efficiency of ovine embryo bisection. Firstly, in common with studies in sheep, goats and cattle (Maurer, 1988; Tsunoda *et al.*, 1985; Williams *et al.*, 1984), later stage embryos survived bisection better than early stages. This was also reflected in a low incidence of blastulation in cultured morulae and late morulae. Similarly, late morulae transferred to recipients survived poorly. The latter result was surprising since, unlike precompaction morulae, post-compaction morulae with a cracked zona are generally expected to survive readily *in utero* (Williams *et al.*, 1984). The sheep late morula appears an exception as Maurer (1988) also reported poor survival of Day 6 embryos and Herr *et al.*, (1988) found that late morulae are easily damaged during bisection. The latter workers inferred from culture results that bisecting dehydrated morulae in sucrose-containing medium might improve post-bisection survival rates. Our results showed that this is so, since 73% of zona-enclosed dehydrated and bisected late morulae produced lambs compared to 33% of zona-enclosed late morulae in unmodified PB1. The result, however, has not convinced us to routinely bisect late morulae since the extra lambs produced over control embryos was low.

We obtained satisfactory results with transferred zona-enclosed early blastocysts and blastocysts. Interestingly, the few expanded blastocysts we recovered and bisected developed readily in culture but survived poorly when transferred (data not presented). We have little experience bisecting hatched blastocysts but it appears that these embryos can give high survival rates (Chesne *et al.*, 1987).

Unlike Baker and Shea (1985), we found that naked demi-embryos survived better during culture than zona-enclosed demi-embryos. This may reflect the reduced manipulation trauma experienced by naked embryos prior to being placed in culture.

With our technique, demi-embryos survive best in recipients when enclosed within a zona. This conflicts with the theory that the zona does not play a role in embryonic development from the compact morula stage onward (Williams *et al.*, 1984; Chesne *et al.*, 1987). Perhaps the zona is needed for a short time after transfer to protect the damaged embryo.

Despite the fact that our embryo bisection technique can give satisfactory results, we are not routinely using it in our MOET programmes. There are a number of reasons for this: our technique is complex, and takes many hours of practice for an operator to become competent; it only gives satisfactory results with a limited range of embryos (i.e. good quality early blastocysts and blastocysts); donors average only one suitable embryo per flush, thus the technician's time is not efficiently utilised; embryo survival is variable. These problems are being addressed partially, by assessing a simplified bisecting technique (Wrightson Breeding Services, Hamilton, N.Z.). However, since we prefer not to work with hatched blastocysts (as they are difficult to identify and cannot be frozen for storage and exportation), we need to develop or obtain access to a proven and reliable technique which utilises a wider range of zona-enclosed embryo stages.

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