The viability of early cleavage stages containing half the normal number of blastomeres in the sheep

S. M. Willadsen

*Agricultural Research Council Institute of Animal Physiology, Animal Research Station,
307 Huntingdon Road, Cambridge CB3 0JQ, U.K.*

Summary. The developmental capacity of embryos produced by injection of single blastomeres of 2-cell embryos, pairs of blastomeres of 4-cell embryos or 4 blastomeres of 8-cell embryos into foreign zonae pellucidae was studied. The micromanipulated embryos were embedded in tiny agar cylinders, transferred to ligated oviducts of ewes on Days 2–8 of their oestrous cycle and recovered when the total age of the embryos was 5½–6½ days. Of 78 embryos transferred, 69 (88.5%) were recovered, and of the latter 65 (94.2%) had developed at a normal rate. Thirty of these ‘half’ embryos were transferred to ewes on Day 6 of the oestrous cycle: 24 (80%) developed into lambs. There were no differences apparent between embryos derived from the 2-, 4- or 8-cell embryos with respect to developmental capacity. A further 12 ‘half’ embryos were stored by deep-freezing for 1 or 2 months. After thawing, 9 of these were selected for transfer to ewes on Day 6: 3 developed into lambs, each of which was the monozygotic twin of a lamb resulting from the first series of transfers.

Introduction

The natural incidence of monozygotic twins in the sheep has not been established but is thought to be low. Embryological evidence (Assheton, 1898; Rowson & Moor, 1964) suggests that monozygotic twins do occur. While examining 424 embryos ranging from early blastocysts collected on Day 6–7 (onset of oestrus: Day 0) to elongating gastrulae collected on Day 14, Rowson & Moor (1964) observed 4 with 2 embryonic areas. But neither the subsequent transfer of 3 of these embryos to recipient ewes (Moor, 1965) nor routine blood typing of relatively large numbers of sheep of various breeds (E. B. Tucker, personal communication) have allowed the natural occurrence of monozygotic twin sheep to be demonstrated unequivocally. The development of supernumary fetuses or the birth of supernumary lambs following embryo transplantation in sheep has not been reported.

Willadsen (1979) has described the artificial production of monozygotic twin lambs. The method not only allows pairs of genetically identical sheep of any preselected parentage to be produced virtually at will, but also implies that pregnancies with monozygotic twin conceptuses may be established with equal ease. Therefore, apart from its embryological interest, the method has the potential to become a useful tool in a wide range of physiological investigations. Technically it involves the microsurgical separation of the blastomeres of 2-cell embryos, their insertion into foreign zonae pellucidae, embedding in a protective cylinder of agar and culture in ligated sheep oviducts. The viability of late morulae and early blastocysts produced in this way was about 50%. The slightly reduced viability compared to that normally observed after embryo transplantation in the sheep (Moore & Shelton, 1964; Rowson & Moor, 1966) was attributed

primarily to mechanical damage resulting from the manipulative procedure by which the agar was removed before transplantation of the embryos to their final recipients. However, it was concluded that in the sheep each of the first 2 blastomeres has the regulatory capacity necessary for the development of an entire lamb.

In the sheep the 2-cell stage seems to be of relatively short duration. Few cleaved embryos are recovered from donor ewes earlier than 48 h after onset of oestrus, and at 60 h after onset of oestrus most embryos are at the 4-cell stage. It is virtually impossible to ensure that surgical recovery of embryos from a particular ewe takes place at a time when they are all at the 2-cell stage. This is due to variation in the interval between (a) PMSG injection and onset of oestrus, (b) onset of oestrus and onset of ovulation, and (c) first and last ovulation in prospective donor ewes. Attempts to control the time of ovulation more precisely by the use of prostaglandin and hCG have not overcome this problem, and it is therefore of interest to know whether later stages of development may be used for the production of monozygotic twins.

Normal sheep embryos can survive storage at -196°C for long periods of time (Willadsen *et al.*, 1977; Willadsen & Tischner, 1978), and such storage of twin embryos produced by the separation of blastomeres would allow the production of genetically identical sheep of differing ages.

The present paper reports experiments in which not only single blastomeres from 2-cell eggs, but also pairs of blastomeres from 4-cell eggs and quartets of blastomeres from 8-cell eggs were investigated. Firstly, the ability of pairs of blastomeres from 4-cell and quartets of blastomeres from 8-cell eggs to develop into late morulae or early blastocysts was compared with that of single blastomeres from 2-cell eggs, and the viability of such 'half' embryos was examined by transplantation to recipient ewes. Secondly, the ability of 'half' embryos to survive deep-freezing and thawing was examined.

Materials and Methods

Welsh Mountain ewes mated to a Welsh Mountain ram or inseminated with Suffolk ram semen and Suffolk ewes inseminated with Suffolk ram semen were used as embryo donors. Welsh Mountain ewes were used as recipients. Prospective donors were injected i.m. with a single dose of PMSG (Folligon: Intervet) (Welsh Mountain, 1250 i.u.; Suffolk, 1500 i.u.) between Days 9 and Day 13 of the oestrous cycle followed 48 h later by an i.m. injection of a prostaglandin analogue (0.5 ml Estrumate: I.C.I.). As soon as a ewe was observed in oestrus (generally 16–26 h after prostaglandin injection) it received an i.v. injection of 500 i.u. hCG (Chorulon: Intervet) and was penned with a fertile ram or inseminated surgically with freshly collected semen. Embryos were collected on Day 2 (2- and 4-cell embryos) or Day 3 (4- and 8-cell embryos). The methods used for induction of superovulation, insemination, embryo collection and transplantation have all been described in detail elsewhere (Willadsen, 1980; see also Hunter, Adams & Rowson, 1955). A phosphate-buffered saline medium (PBS: Whittingham, 1971) was used for recovery, storage and transfer of embryos and also for holding embryos during the various manipulations except as otherwise noted. The materials and procedures used to produce pairs of monozygotic and some single embryos were those described previously (Willadsen, 1979) with minor alterations.

The egg was held by suction with a capillary pipette while a large tear was made in the zona pellucida with a fine glass needle. The blastomeres were sucked out of the zona pellucida with the aid of a Pasteur pipette with a tip diameter sufficiently small to detain the zona while allowing the blastomeres to pass through. The blastomeres were separated by gentle suction with Pasteur pipettes of decreasing diameter for 2-, 4- and 8-cell embryos respectively. With 4- and 8-cell embryos the aim was to produce 2 groups consisting of equal numbers of blastomeres, and this did not necessarily require a complete separation of all the blastomeres. In many instances,

particularly with 8-cell embryos, at least one group contained blastomeres which had remained in close contact throughout the manipulations. Single embryos or sets of twin embryos were produced from the original 2-, 4- and 8-cell embryos by injection of separated (groups of) blastomeres into evacuated foreign zonae. The latter were prepared immediately before use from follicular oocytes which had been collected from sheep ovaries after slaughter and stored in PBS at -20°C . A 1.0% solution of Agar (Difco) in 0.9% (w/v) NaCl in distilled water was prepared. While the solution was left to cool, the embryos to be embedded were transferred to sheep serum. When the agar had cooled to below 38°C it was poured into a Petri dish, and several single embryos or a monozygotic pair of embryos were transferred to the agar solution with a fine bore Pasteur pipette, but almost immediately picked up again in a small amount of agar. The tip of the pipette containing the embryos in agar solution was quickly immersed into PBS at room temperature to hasten gelling of the agar, which was then expelled into the PBS as a solid cylinder. The ends of the cylinder were cut off with the sharp edge of a 24-gauge hypodermic needle, so that the embryos were contained in a segment measuring about 0.15×0.5 mm. This tiny cylinder was in turn embedded by a similar procedure, often together with another cylinder containing similarly treated embryos, in a larger one consisting of 1.2% agar in 0.9% NaCl and measuring about 0.7×2.5 mm.

The agar cylinders were transferred to the ligated oviducts of ewes on Days 2–8 of the oestrous cycle and recovered when the total age of the embryos was $5\frac{1}{2}$ – $6\frac{1}{2}$ days, by which age an embryo developing at the normal rate has reached the late morula–early blastocyst stage. After recovery the embryos were examined as fresh specimens under a dissecting microscope. Only those which were late morulae or early blastocysts were considered to be normal, and these were subsequently used for transplantation after removal from the agar by manipulation with a 24-gauge hypodermic needle.

Only one embryo of most of the monozygotic pairs was transferred immediately after recovery from the primary recipient; the twin embryo was deep frozen in 1.5 M-DMSO in PBS. It was cooled at $0.3^{\circ}\text{C}/\text{min}$ to -36°C , then at $0.1^{\circ}\text{C}/\text{min}$ to -60°C before being placed at -196°C . The deep-frozen embryo was thawed and transplanted only when the recipient of the other twin embryo had failed to return to oestrus. The thawing method consisted of rapid rewarming from -196 to -50°C , then at $4^{\circ}\text{C}/\text{min}$ to -10°C before rapid rewarming to room temperature. The deep freezing method has been described in detail elsewhere (Willadsen, 1977).

All embryos, whether fresh or stored, were transferred to ewes on Day 6 of the cycle. In some of these oestrus had been induced with prostaglandin, but the majority, including all recipients of frozen–thawed embryos, had come into oestrus naturally. The recipients were penned with a raddled vasectomized ram, and those not returning to oestrus were allowed to go to full term.

Results

Development of embryos from different numbers of blastomeres

As shown in Table 1, most of the embryos developed at the normal rate during the culture period and were early and expanding blastocysts when recovered from the primary recipients. The use of non-synchronous ewes for culture of the embryos exerted no apparent adverse effect on their development. In all instances when both embryos of a set of monozygotic twins were recovered both had developed at a normal rate. Among the embryos as a whole only minor differences were observed with respect to stage of development and approximate cell number, and none of these could be referred to the stage of development of the parent embryo at blastomere separation. Two blastomeres of a 4-cell embryo and 4 blastomeres of an 8-cell embryo developed into early blastocysts which were morphologically very similar to those produced from single blastomeres of 2-cell embryos, i.e. consisting of about half the number of

cells observed in ordinary Day 7 sheep embryos and with a less conspicuous inner cell mass (Pl. 1, Figs 1, 2 and 3).

Both the pregnancy rate obtained after transplantation (78.6%) and the embryo survival rate (80.0%) were as high as those obtained after transplantation of ordinary sheep embryos (Moore & Shelton, 1964; Rowson & Moor, 1966) and well above those obtained in the previously reported experiment with embryos produced from single blastomeres of 2-cell embryos. Again, there was no clear difference between those embryos produced from 4- or 8-cell embryos and those produced from 2-cell embryos. Three sets of monozygotic twins were produced, 2 sets intentionally to demonstrate that twin blastocysts produced from 8-cell embryos are both viable, the 3rd set unintentionally from what was thought to be a single embryo developing from 2 blastomeres of a 4-cell embryo.

Table 1. The development of embryos produced from single blastomeres of 2-cell embryos, pairs of blastomeres of 4-cell embryos or 4 blastomeres of 8-cell embryos during culture in ligated sheep oviducts and after transfer to final recipients

	Developmental stage at separation			
	2-cell	4-cell	8-cell	Combined
No. of recipient oviducts	9	6	7	22
No. of embryos transferred	31 [11]	21 [8]	26 [8]	78 [27]
No. of embryos recovered (%)	26 (83.9) [8]	20 (95.2) [8]	23 (88.5) [6]	69 (88.5) [22]
Degenerate (%)	1 (3.8)	1 (5.0)	1 (4.3)	3 (4.3)
Retarded (%)	1 (3.8)	0 (0)	0 (0)	1 (1.4)
Normal (%)	24 (92.3) [8]	19 (95.0) [8]	22 (95.7) [6]	65 (94.2) [22]
No. of final recipients	6*	5*	5	14*
No. pregnant	4	5	4	11
No. of embryos transferred	10	10	10 [2]	30
No. of lambs born	7	10†	8 [2]	25

Values in [brackets] refer to the no. of monozygotic pairs.

* Two recipients received 1 (Welsh) embryo produced from a single blastomere of a 2-cell embryo and 1 (Suffolk) embryo produced from a pair of blastomeres from a 4-cell embryo.

† One recipient which had received 1 Welsh and 1 Suffolk embryo gave birth to 2 Suffolk lambs which were subsequently demonstrated to be of identical blood type. These 2 lambs count as one in the estimate of viability in the text.

PLATE 1

Figs 1–3. Early sheep blastocysts of similar appearance but produced from a single blastomere of a 2-cell embryo (Fig. 1) and from 4 blastomeres of an 8-cell embryo (Fig. 2). Both blastocysts have a smaller inner cell mass than does the normal early blastocyst (Fig. 3). $\times 300$.

Fig. 4. Abnormal development of embryo produced from 4 blastomeres of an 8-cell sheep embryo. During the embedding procedure agar penetrated the perivitelline space, forcing the blastomeres to continue their development in separate niches in the agar. This has led to the formation of several blastocoelic cavities. The granular material on the surface of the zona represents one disintegrated blastomere which has been expelled via a crack in the agar in the perivitelline space due to the fluid accumulation. $\times \sim 300$.

Fig. 5. Set of monozygotic twins of which the smaller is about 2 weeks old, the other $2\frac{1}{2}$ months old. The younger lamb developed from an embryo which was stored for 2 months at -196°C before transfer, whereas its monozygotic twin embryo was transferred without storage and gave rise to the older lamb.

Fig. 6. The older twin in Fig. 5 when it was about 2 weeks old.

PLATE 1

(Facing p. 360)

Development after deep freezing

Of the blastocysts deep-frozen, 9 were thawed after storage for 2 months, 3 were thawed after 1 month and 2 were not thawed because the ewes which had received their respective co-twins returned to oestrus. One embryo was not recovered, 2 were completely degenerate. The remaining 9, of which 6 were apparently unharmed and 3 slightly damaged by freezing and thawing, were transferred to 5 recipient ewes. Of these, 2 subsequently lambled producing 3 lambs, all of which were the monozygotic twins of lambs born 1 (1 lamb) or 2 (2 lambs) months previously (Pl. 1, Figs 5 and 6). One of the sets of twins was produced from a 2-cell embryo, the other 2 sets from two 4-cell embryos.

Discussion

Due to the essentially non-random allocation of blastomeres to twin embryos in the course of the micromanipulative procedure, and the possibility that a sheep embryo has the potential to develop into more than 2 lambs, no detailed conclusion may be drawn from the present experiment with respect to the totipotency of the blastomeres of early sheep embryos, but the results do strongly suggest that up to the 8-cell stage at least the individual blastomeres are developmentally equivalent. This is in agreement with observations made by others in the mouse (see review by Wilson & Stern, 1975). Microenvironmental rather than inherent differences between blastomeres are thought to play the major part in the developmental fate of a particular blastomere with an early embryo, i.e. those blastomeres that are totally enclosed by others at the time of blastulation give rise to the inner cell mass, while the rest develop into trophoctoderm (Tarkowski & Wroblewska, 1967). The spatial position of blastomeres during this relatively brief period is therefore important, even critical, when the total number of cells in the embryo at blastulation is reduced as in the present experiment.

In preliminary experiments it was observed that if agar had entered the perivitelline space of micromanipulated eggs containing several loosely associated blastomeres, this often resulted in fixation of the blastomeres in their respective positions leading to incomplete aggregation of later generations of blastomeres so that several diminutive blastocyst-like forms developed instead of a single large one (Pl. 1, Fig. 4). In these instances few, if any, cells occupy an 'inside' position at the time of blastulation and so the cells of the resulting vesicles may not be properly differentiated into inner cell mass and trophoctoderm. Agar was therefore prevented from entering the perivitelline space by substituting relatively viscous sheep serum for PBS immediately before embedding and by cooling the agar rapidly to hasten its solidification when the inner, small cylinder was being made. However, the unexpected birth of monozygotic twins to one ewe which had received 2 unrelated embryos demonstrates that in at least one instance either 2 separate inner cell masses had formed in one blastocyst, or a separate, but fully viable, blastocyst was formed from each of 2 blastomeres of a 4-cell embryo although this was not apparent when the egg was recovered from the primary recipient.

The main conclusions to be drawn from this part of the study are that in the sheep 2 blastomeres of a 4-cell embryo and 4 blastomeres of an 8-cell embryo are able to develop into early blastocysts which do not differ morphologically from those produced from single blastomeres of 2-cell eggs, and that all 3 categories of 'half' embryos are able to give rise to a normal conceptus.

The viability of the frozen-thawed 'half' embryos was considerably below that observed after transplantation of freshly collected sheep embryos and also below that obtained when normal embryos were frozen and thawed (Willadsen *et al.*, 1977). Nevertheless, some embryos were viable and the experiment demonstrates that genetically identical sheep of differing ages may indeed be produced.

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