

Production of monozygotic (identical) horse twins by embryo micromanipulation

W. R. Allen and R. L. Pashen*

*T.B.A. Equine Fertility Unit, A.R.C. Institute of Animal Physiology, Animal Research Station,
307 Huntingdon Road, Cambridge CB3 0JQ, U.K.*

Summary. The blastomeres of 19 2- to 8-cell embryos recovered surgically 1-3 days after ovulation from 23 Pony mares were mechanically separated and inserted, in various combinations, into evacuated pig zonae pellucidae to make 27 'half' and 17 'quarter' micromanipulated embryos. These were embedded in agar and cultured *in vivo* in the ligated oviducts of ewes for 3.5-5 days to allow development to the late morula/early blastocyst stage. Subsequent surgical or non-surgical transfer of 13 'half' and 17 'quarter' embryos to mares resulted in 10 established pregnancies, including 2 monozygotic pairs. Surgical transfer to mares that had not been recently used as donors of embryos was more successful (10/20) than surgical or non-surgical transfer to recently operated mares (0/10).

Introduction

Monozygotic (identical) twins are valuable research tools in many types of biological investigations. While monozygotic twins occur naturally in women, cattle and sheep, albeit at a low frequency, they have not been reported to date in equids. In a recent survey of 2673 Thoroughbred mares the spontaneous dizygotic twin conception rate was found to be 2.1% (M. W. Sanderson & W. R. Allen, unpublished data). Twin pregnancy is unwanted in the horse because of the high rate of abortion and a tendency to poor post-natal development in the few twin foals that survive to term. Therefore, most twins that are conceived are deliberately aborted early in gestation and the mare is remated in an attempt to produce a singleton pregnancy during the same breeding season (Simpson *et al.*, 1982).

Embryo micromanipulation techniques have been used to produce identical individuals in sheep, cattle and pigs (Willadsen, 1979, 1981, 1982; Willadsen & Polge, 1981). The success of the technique depends largely on the use of agar gel to seal incisions made in the zona pellucida during micromanipulation; the agar protects the blastomeres from damage by uterine secretions and leucocytes until such time as the embryo has developed sufficiently to survive *in utero* without a zona (Willadsen, 1979). This paper describes the application of a similar technique to the production of monozygotic horse twins.

Materials and Methods

Pure Welsh Mountain and crossbred Pony mares, mated one or more times during oestrus by a Welsh Mountain or a Welsh Mountain × Arab Pony stallion, were used as embryo donors. The day of ovulation (Day 0) was determined by daily palpation of the ovaries *per rectum* combined with

* Present address: Department of Reproduction, School of Veterinary Medicine, University of California, Davis, California 95616, U.S.A.

Table 1. Details of horse embryos recovered and micromanipulated to make monozygotic 'half' and 'quarter' embryos

Donor mare	Stage of embryo when recovered (no. of blastomeres)	No. of blastomeres used in each micromanipulated embryo	Days in ewe oviduct	Embryos recovered from ewe	Recipient mare (method of transfer)	Stage of recipient cycle (ovulation = Day 0)	Result
<i>Production of 'half' embryos</i>							
MT	} No embryo recovered	—	—	—	—	—	—
BN		—	—	—	—	—	—
AC	2	1	5.0	e.bl.	PD (S)	6	P
		1		e.bl.	LC (S)	7	NP
IG	2	1	4.5	Not rec.	—	—	—
		1					
DS†	3+	+1(cl.)	4.5	e.bl.	CB (S)	6	NP
CB	4	All blastomeres damaged	—	—	—	—	—
MP	4	2	4.5	e.bl.	MS (S)	5	P
		2		e.bl.	MP (NS)*	6	NP
HT	4	2	4.0	e.bl.	GH (S)	6	P
		2		l.mor.	SS (S)	6	NP
TB	4	2	4.0	e.bl.	RN (S)*	6	NP
		2		e.bl.	TB (S)*	5	NP
BA	4	2	3.5	e.bl. + e.bl.	} Not trans.	—	—
		2		e.bl. + e.bl.		—	—
LC	4	2	4.0	e.bl.	FA (S)	5	P
		2		‡e.bl. + ¼ deg.	RA (S)	5	NP
TC	4	2	5.0	‡e.bl. + ¼ deg.	TC (NS)*	6	NP
		2		‡e.bl. + ¼ deg.	WN (NS)*	5	NP
WN	4	2	5.0	‡e.bl. + ¼ deg.	} MF (NS)*	6	NP
		2		‡e.bl. + ¼ deg.		—	—
TD	5	2	4.0	ret. mor.	} Not trans.	—	—
		3		retarded		—	—
RN	5	2	4.0	e.bl.	} Not trans.	—	—
		3		degenerate		—	—
SB	7	3	3.5	e.bl.	SB (S)*	6	NP
		4		‡e.bl. + ⅓ deg.	BA (S)*	5	NP
SF	8	4	3.5	l.mor.	GG (S)	5	P
		4		l.mor.	RA (S)	4	NP
<i>Production of 'quarter' embryos</i>							
HH	No embryo recovered	—	—	—	—	—	—
DS†	3+	1	4.5	c.bl.	DS (NS)*	6	NP
		1		l.mor.	WW (S)	6	NP
BC	4	1	4.0	l.mor.	BA (S)	4	P
		1		l.mor.	TA (S)	4	P
SC	4	1	4.5	c.bl.	GM (S)	5	NP
		1		c.bl.	} TB (S)	6	NP
		1		c.bl.		—	—
SF	4	1	4.0	c.bl.	} Not trans.	—	—
		1		ret. mor.		—	—
PT	4	1	4.5	c.bl.	JR (S)	5	P
		1		c.bl.	MS (S)	4	P
		1		c.bl.	AT (S)	5	P(Res.)
		1		c.bl.	SF (S)	5	NP
SD	4	1	4.5	Not rec.	—	—	—
		1					
		1					
		1					

daily measurement of peripheral plasma progesterone concentrations. Embryos of 2–8 cells were recovered on Days 1–3 via a mid-ventral laparotomy performed under general anaesthesia.

Embryo collection

Each embryo was collected by flushing the oviduct on the side of ovulation (ipsilateral) with enriched Dulbecco's phosphate-buffered saline (PBS; Willadsen, 1979) as described by Allen (1982). A 1 cm incision was made in the antimesometrial wall of the ipsilateral uterine horn 2–3 cm from the uterotubal junction through which the wide end (8 mm diam.) of a 10 cm-long tapered glass tube was inserted into the uterine lumen. The flanged end of the tube was held over the protruding papilla of the uterotubal junction by firm manual pressure on the exterior of the uterine horn. The narrow end (2 mm diam.) of the glass tube was connected to a flexible silicone rubber tube, the distal end of which was directed into the concave glass recovery vessel. The oviduct was then flushed with 30–40 ml PBS via a rigid plastic catheter (2 mm diam.) inserted 3–5 cm into the ampulla and held in position by a second operator.

After an embryo had been located in the flushing fluid it was immediately transferred to fresh PBS in another vessel and held at room temperature (20–25°C) until processed.

Embryo micromanipulation and transfer

After removal of the zona pellucida the blastomeres of each embryo were separated into two ('half' embryos) or four ('quarter' embryos) groups, each containing an equal number of cells. The groups of cells were then injected into evacuated zonae pellucidae of pig oocytes. Each pair or group of micromanipulated embryos was briefly immersed in sheep serum and then embedded in a small cylinder of 1% agar in 0.9% (w/v) NaCl solution which was in turn embedded in a larger cylinder of 1.2% agar in 0.9% (w/v) NaCl solution as described previously (Willadsen, 1979). The agar chips, each measuring 0.8 × 2.0–2.5 mm, were then transferred to the ligated oviducts of anoestrous ewes which acted as temporary recipients to allow the micromanipulated embryos to develop to the late morula/early blastocyst stage. After 3.5–5 days the agar chips were recovered by a second laparotomy and the embryos shelled out of the agar. 'Half' and 'quarter' embryos that had developed at the normal rate during culture in the sheep oviduct were transferred to recipient mares that had ovulated within 24 h of the respective donor mares. The transfers were performed surgically or non-surgically as described by Allen (1982). Pregnancy was diagnosed by rectal palpation of the uterus and later confirmed by slaughter of the mare or birth of a live foal.

Results

Embryo collection

From the 23 mares used as donors, 20 embryos were recovered and micromanipulated. The cleavage stage of individual embryos is given in Table 1.

l.mor. = late morula; ret. mor. = retarded morula; c.bl. = contracted blastocyst; e.bl. = expanded blastocyst; deg. = degenerate; Not rec. = not recovered from ewe oviduct; Not trans. = not transferred to recipient mare; (S) = surgical transfer; (NS) = non-surgical transfer; P = pregnant; NP = not pregnant; (Res.) = conceptus resorbed.

* Recipient mare used as a surgical donor within the previous 5 days.

† Mare DS mentioned twice. One blastomere (+1 cl.) was in the process of cleaving and the micromanipulated embryo was therefore classed as a 'half' embryo. 'Quarter' embryos were made from the other two blastomeres from Mare DS (see text).

‡ These embryos were actually considered to be quarter embryos (see text).

Embryo micromanipulation

In one embryo all 4 blastomeres were visibly damaged during micromanipulation (Table 1, Mare CB). In a further three 4-cell embryos, 1 (Mare SC), 2 (Mare BC) and 2 (Mare SF) blastomeres were damaged while the rest of the cells remained intact. From these 3 embryos, and the 15 in which no blastomeres were damaged, 27 'half' embryos (including 13 monozygotic pairs) and 17 'quarter' embryos (constituting 3 monozygotic pairs, one group of 3 and 2 groups of 4 monozygotic embryos) were produced and transferred to 15 ewes. In the embryo recovered from Mare DS one of the 3 blastomeres was in the process of cleaving at the time of micromanipulation. The embryo made from this blastomere was therefore classed as a 'half' embryo whereas the two micromanipulated embryos made from the other two original blastomeres were each classed as 'quarter' embryos. For this reason Mare DS is listed in both sections of Table 1. Of the 44 agar-embedded micromanipulated embryos, 6 (one monozygotic pair of half embryos (Mare IG) and one group of 4 monozygotic embryos (Mare SD), all contained in a single agar chip) were not recovered from the recipient ewe, leaving 25 of the original 'half' embryos and 13 of the original 'quarter' embryos for further study and transfer. The composition of individual micromanipulated embryos and their development after culture in the sheep oviduct are shown in Tables 1 and 2.

'Half' embryos. By the time of removal from the sheep oviduct, 14 of the 25 'half' embryos (56%) recovered had developed normally to form early blastocysts or fully compacted late morulae. A further two 'half' embryos from Mare BA had also developed at the normal rate but each of the original 2 blastomeres remained dissociated and had formed separate, small blastocysts which were subsequently classed as 'quarter' embryos and not transferred to recipient mares. In 5 embryos (Mares LC, TC, WN) only 1 of the 2 blastomeres, and in one embryo (Mare SB) 2 of the original 4 blastomeres, had continued their development at the normal rate while their fellow blastomeres were totally degenerate. These were also subsequently classed as 'quarter' embryos. Of the remaining 3 'half' embryos, 2 were considered retarded in their development (both from Mare TD) and one was completely degenerate (Mare RN).

'Quarter' embryos. Of the 13 intended 'quarter' embryos recovered, 12 (92%) had developed at the normal rate to form small blastocysts or fully compacted late morulae. The remaining embryo (Mare SF) was considered retarded.

In all, 5 monozygotic pairs of 'half' blastocysts, 2 monozygotic pairs consisting of one 'half' and one 'quarter' blastocysts, 1 monozygotic group consisting of 1 'half' and 2 'quarter' blastocysts, 3 monozygotic pairs of 'quarter' blastocysts, 1 monozygotic group of 3 'quarter' blastocysts and 2 monozygotic groups of 4 'quarter' blastocysts were produced. The micromanipulated blastocysts were similar in general appearance to normal unmanipulated equine blastocysts except that they contained fewer total cells and were therefore noticeably smaller.

PLATE 1

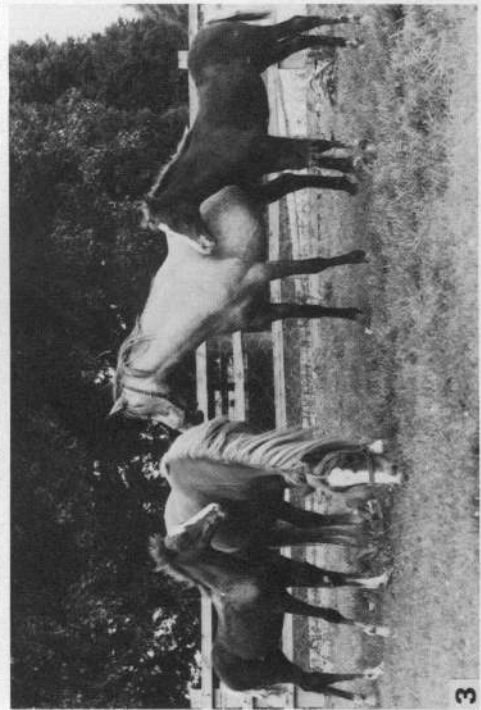
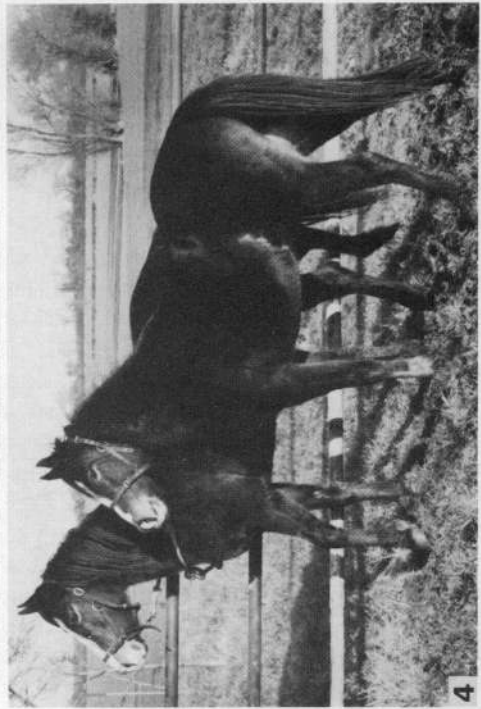
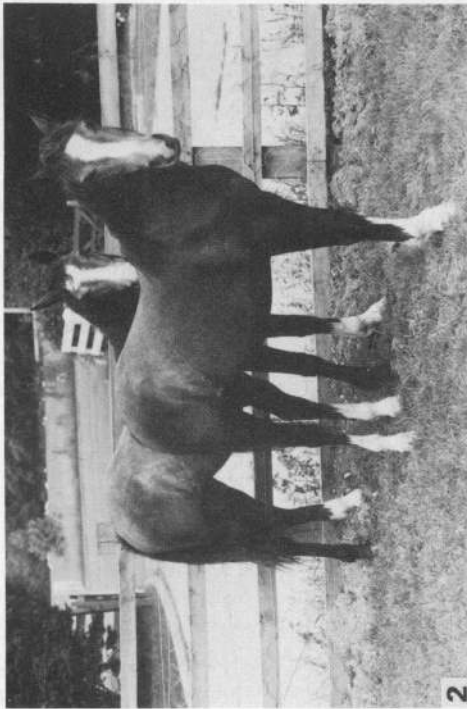
Fig. 1. Monozygotic colt foals at 5 months of age.

Fig. 2. The same colt foals at 16 months of age. Note the differences in the colour markings of the lower legs and in the outlines of the facial blazes.

Fig. 3. Monozygotic filly foals with their respective surrogate dams. The smaller foal on the left was born on Day 353 of gestation, 73 days before the photograph. The larger twin on the right was born on Day 330 of gestation.

Fig. 4. The same monozygotic fillies at 2 years 8 months. The smaller foal on the left in Fig. 3 is nearest the camera in this photograph. Note the change in leg colour markings with increased age.

PLATE 1



(Facing p. 610)

Embryo transfer

Six of the embryos (1 'half' and 5 'quarter' blastocysts from Mares MP, TC, WN, DS) were transferred non-surgically to recipient mares (MP, TC, WN, MF, DS) which had been used as surgical donors a few days previously. None of these mares became pregnant. A further 4 embryos (3 'half' and 1 'quarter' blastocysts from Mares TB and SB) were transferred surgically to recipients (Mares RN, TB, SB, BA) that had similarly acted as recent surgical donors but no pregnancies were obtained. Ten 'half' blastocysts (4 monozygotic pairs from Mares AC, HT, LC and SF and embryos from Mares DS and MP) were transferred surgically to recipients that had not recently undergone surgery. Five (50%) of these embryos (including one from each of 4 monozygotic pairs) gave rise to normal pregnancies, 2 of which were confirmed at *post mortem* of the mares on Days 49 and 67 respectively while the other 3 continued to term. Ten 'quarter' blastocysts from Mares DS, BC, SC, PT and LC were similarly transferred surgically to unoperated synchronized recipient mares of which 5 (50%) became pregnant. One conceptus, a member of a group of 4 monozygotic 'quarter' embryos from Mare PT, was resorbed between Days 50 and 55. Two other embryos from the same group and one other monozygotic pair of embryos from Mare BC continued to term (Table 2).

Birth and development of identical twins

Two monozygotic twin colt foals, the genetic progeny of a ~13 hands crossbred Pony mare (BC) mated to a 13.5 hands Welsh Pony × Arab stallion, were born 18 days apart on Days 322 and 340 of gestation respectively. One of these foals was carried by a ~13 hands Welsh Pony recipient mare (BA) while the other was carried by a much smaller (11.5–12 hands) Welsh Pony recipient (Mare TA). The foal born on Day 322 was from the smaller recipient mare and was more robust and vigorous at birth than its twin born on Day 340 from the bigger recipient. This latter foal required minor assistance to stand and suck during the first 3 h *post partum* but thereafter it grew and developed normally (Pl. 1, Fig. 1). By the time of their sale at 16 months of age, the two foals were very similar in height and general appearance (Pl. 1, Fig. 2).

The other two monozygotic twins were fillies, born 23 days apart on Days 330 and 353 of gestation. They were the genetic progeny of a ~14 hands crossbred mare (PT) and the same 13.5 hands Welsh Pony × Arab stallion that sired the colt foals. In this instance it was the foal carried by the larger (~13.5 hands) of the two recipient mares (MS) that was born robust and vigorous at the earlier stage of gestation (Day 330). Its identical twin, born on Day 353 from a smaller, thickset ~12.5 hands recipient mare (JR), showed signs of dysmaturity as described by Rosedale (1976). It was undersized, exhibited muscle wastage and mild dehydration and required assistance to stand and suck during the first 12 h *post partum*. Thereafter, however, it nursed and developed normally to weaning (Pl. 1, Fig. 3).

The size disparity between the twin fillies has persisted into adult life. At their present age of 2 years 8 months the larger foal born on Day 330 stands 13.5 hands high and weighs 352 kg. The smaller, dysmature foal born on Day 353 stands 12.5 hands and weighs only 286 kg (Pl. 1, Fig. 4).

One other feature of both sets of monozygotic twins was the marked difference in coat colour patterns of the legs exhibited by the individuals of each pair. For example, one of the colt twins had white socks (white hair from hoof to fetlock) on all 4 legs whereas the other had only 2 white socks, involving the fore and hind limbs on the near side (Pl. 1, Fig. 2). With the fillies, on the other hand, both individuals showed patchy white hairs on the lower parts of all 4 limbs during the first few weeks of life. Most of this disappeared around the time of puberty and, as adults, the larger twin had no white hair on any of its legs whereas the smaller twin had one distinct white sock on its near foreleg (Pl. 1, Fig. 4). The prominent facial blazes present in both sets of twins also showed definite differences in outline between the members of each pair.

Discussion

This study demonstrated that the techniques developed by Willadsen (1979) for the mechanical separation of blastomeres to study the developmental potential of early cleavage-stage embryos of sheep, pigs and cattle can also be used for the production of monozygotic horse twins (Table 2). The natural occurrence of identical twins in the mare is virtually precluded by her general inability to carry twin conceptuses to term. This arises from competition of the placentae for contact with the maternal endometrium which results in a nett placental insufficiency for both conceptuses. The usual outcome is for the more disadvantaged of the two fetuses to die during the second half of gestation and so initiate abortion (Jeffcott & Whitwell, 1976).

Table 2. Summary of embryo results

	'Half' embryos	'Quarter' embryos
Mares used to produce original embryos	16	7
No. of blastomeres in recovered embryos	2-8	3-4
No. of embryos produced by micromanipulation	27	17
No. of embryos which developed normally into late morulae or expanding blastocysts	14	12 (+ 10)*
No. of embryos transferred to recent donors (no. pregnant)		
Non-surgical	1 (0)	5 (0)
Surgical	3 (0)	1 (0)
No. of embryos transferred to mares not recently subjected to surgery (no. pregnant)	10 (5)	10 (5)

* These embryos (from Mares SB, WN, TC, LC and BA) were intended originally as 'half' embryos but developed as 'quarter' embryos.

Of the total of 38 potential embryos recovered from the agar chips after 3.5-5 days incubation in the oviducts of ewes, 27 (71%) were considered to have developed normally. Surgical transfers were made with 22 of these normal embryos and with two other embryos (from Mares LC and SB) which were intended originally as 'half' embryos but which had, in fact, developed into 'quarter' embryos. These transfers resulted 10 (42%) established pregnancies. Since none of the 10 embryos that were transferred, surgically or non-surgically, to recipient mares used recently as surgical donors resulted in pregnancies, it seems reasonable to assume in hindsight that the pregnancy rate would have been higher had all the embryos been transferred surgically to unoperated recipient mares. Another factor which, in retrospect, may have lowered the potential pregnancy rate was the use of some recipient mares which had ovulated up to 24 h ahead of the original donor mare. Subsequent experiments involving direct embryo transfer in equids on Days 6-8 after ovulation have demonstrated a marked fall-off in conception rates when embryos are placed in recipient mares that ovulated ahead of, rather than behind, the donor animals (Oguri & Tsutsumi, 1982; Allen, 1982).

Gestation length in the mare ranges from 315 to 360 days and is influenced by maternal size (Walton & Hammond, 1938), fetal genotype and the stage of the breeding season when conception occurs (Asdell, 1946; Howell & Rollins, 1951). Development *in utero* can also be affected by degenerative wear and tear changes of the endometrium such as cystic fibrosis and glandular atrophy (Kenney, 1978). Fetal growth will be retarded due to impaired placental function in mares exhibiting such changes and gestation may be lengthened as a consequence. The considerable differences in gestation lengths and birth weights of the twins produced in the present study is likely to have resulted from random combinations of these influences rather than from any direct effect of the micromanipulation technique. Monozygotic twins, triplets and quadruplets produced in other species by embryo micromanipulation have similarly exhibited disparities of birth weight and gestation length (Willadsen, 1982). Total cell numbers in micromanipulated embryos are certainly

reduced compared to those in normal embryos at the time when blastulation and differentiation into embryonic and placental tissues begins. However, this difference is, at most, two cell divisions (~24 h) and is readily compensated for in later development.

The differences in hair colour patterns exhibited by the monozygotic horse twins also occurs in genetically identical individuals produced by embryo micromanipulation in other species (Willadsen, 1982). The phenomenon seems likely to be related to slight random differences in clonal expansion of the hair follicle cells responsible for the white markings.

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