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Attempts to Produce Identical Offspring in the Sheep by Mechanical Division of the Ovum

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Abstract

In an attempt to produce identical offspring artificially, the inner cell mass of ova, collected on days 4, 6 and 7 after oestrus (day 0 = day of oestrus), was mechanically divided into two equal sections. The sections were then either cultured *in vitro* for 2 days and the resulting apparently normal blastocysts transferred to recipient ewes or, in the case of day-7 ova, transferred to recipient ewes without a period of *in vitro* culture.

Half sections of day-4 ova showed little further development in culture and in most the individual cells separated and dispersed. Those of day 6 and 7 showed no dispersion and 24 apparently normal blastocysts were recovered following the culture of 82 half sections. Fourteen of the normal blastocysts developed from each of both half sections of seven day-6 ova.

Nineteen normal blastocysts were transferred individually to recipients following culture, but only two developed into lambs and both lambs developed from blastocysts of different ova. A further 18 half sections of day-7 ova were transferred without an intervening period of culture but none developed into lambs.

Although a substantial proportion of the half sections of day-6 and day-7 ova showed apparently normal development in culture, very few showed continued normal development in recipients. It is suggested that cellular specialization within the inner cell mass may have occurred before the inner cell mass was divided.

Introduction

It has been shown that single blastomeres of early-cleavage-stage ova of the mouse, rat, rabbit and pig are capable of continued cleavage (Nicholas and Hall 1942; Seidel 1952; Tarkowski 1959*a*, 1959*b*; Tarkowski and Wróblewska 1967; Moore *et al.* 1968, 1969) and single blastomeres of two-, four- and eight-cell rabbit ova have developed into apparently normal young following transfer to recipient does (Seidel 1952; Moore *et al.* 1968). Similar results have been achieved in sheep (Moore, unpublished data). However, survival and development of the single blastomeres in recipients appears to be dependent upon their enclosure in a relatively intact zona pellucida. Naked blastomeres were rapidly destroyed. Moore *et al.* (1969) suggested that each of the blastomeres of these early-cleavage-stage ova may well possess the potential for full development, thus raising the possibility of artificially producing identical offspring. However, attempts at producing identicals in the rabbit and pig failed (Moore *et al.* 1968, 1969).

There are two approaches that might be successful in attempts to produce identical offspring: (1) protecting the blastomeres from direct exposure to the uterine environment, and (2) culturing *in vitro* denuded blastomeres to the stage of development at which the zonae are normally shed before transferring them to recipients. In the present study the latter alternative was investigated.

Materials and Methods

Fertilized ova were collected from mature Merino ewes that had been treated with an equine anterior pituitary extract (HAP) to induce multiple ovulation. Ova were recovered on days 4, 6 or 7 after oestrus (day 0 = day of oestrus) by flushing the uteri and oviducts with sterile Dulbecco phosphate buffer (DB; obtained from Commonwealth Serum Laboratories, Melbourne) enriched with 10% heterologous sheep serum (S).

Division of Ova

Ova were removed from the flushings and washed in DB + 20% S and incubated (1½–8 min) in a 5% (w/v) solution of a proteolytic enzyme [Protease from *Streptomyces griseus* (Sigma)] which reduced the zona pellucida to a thin shapeless membrane. They were then washed three times in DB + 20% S to remove any remaining Protease. The washed ova together with 0·5–1·0 ml of fresh DB + 20% S were transferred to small concave glass containers and placed under micromanipulators (Leitz Wetzlar). By means of sterile glass needles, remnants of the zonae were removed and the inner cell mass of each ovum was divided equally into two portions (Fig. 1). In a few ova one or more blastomeres were ruptured during manipulation, but in most there appeared to be no damage to any blastomeres. During the latter stages of the experiment it was found that intact zonae could be removed without damage to the inner cell mass (Fig. 2) and the use of Protease to digest the zonae was omitted from the procedures.

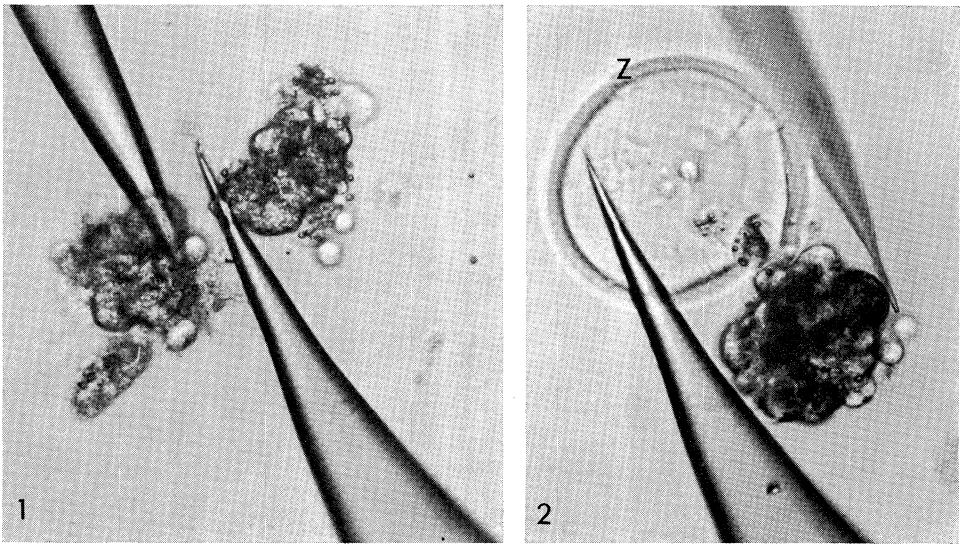


Fig. 1. Completed mechanical division of a day-6 morula into two approximately equal sections using finely drawn glass needles. × 360.

Fig. 2. Evacuation of the inner cell mass of a day-6 morula from the zona pellucida (*Z*) by mechanical methods. × 360.

Incubation with Protease and manipulation of ova were carried out in a constant-temperature cabinet maintained at 35°C.

Culture in vitro

Immediately after division, day-6 and day-7 ova were washed in DB + 20% S and each section was incubated, individually, for 48 h at 37·5°C in polystyrene test tubes (12 by 75 mm; Falcon Plastics) containing 2–3 ml of DB + 20% S and held under atmospheric conditions in sterile anaerobic jars. In this laboratory these conditions and medium regularly support the development of some 80% of Protease-treated and untreated day-6 sheep ova (morulae) to expanded blastocysts (Figs 3 and 4) which shed their zonae after culture for 3 days (Trounson and Moore, unpublished data). Day-4 ova

were washed in a balanced salt solution buffered with bicarbonate (composition 107·7 mM NaCl, 4·77 mM KCl, 1·07 mM $CaCl_2$, 1·19 mM KH_2PO_4, 0·10 mM $MgCl_2$, 25·07 mM $NaHCO_3$, 100 i.u/ml penicillin potassium salt, 50 i.u./ml streptomycin sulphate). The solution had a total osmolarity of 309 m-osmol. Heterologous sheep serum was added to give a final concentration of 20% serum. A constant pH of 7·6 was maintained by gassing the culture medium prior to and during culture with a gaseous mixture composed of 5% CO_2, 5% oxygen and 90% nitrogen. The divided portions of ova were then cultured individually for 2 days in 2–3 ml of the bicarbonate buffer + 20% S. Preliminary tests had shown that untreated day-4 sheep ova cultured under these conditions developed from 8 to 16 cells to late morulae or early blastocysts during 2–3 days culture.

Following culture the divided ova were either transferred to recipient ewes or examined as fresh specimens and then again after staining with 1% orcein.

Transfer of Divided Ova

The majority of day-6 ova that had developed during culture to become apparently normal blastocysts, together with a number of day-7 ova that had been divided but not cultured, were transferred to the uterine horns of recipient ewes. Each recipient received only one portion of a divided ovum. Blastocysts resulting from the culture of divided day-6 ova were transferred to recipients that had been detected in oestrus 12 or 48 h after their respective donors. In the case of day-7 ova, respective donors and recipients had been in oestrus at the same time. With day-6 ova asynchronous transfers were carried out in an attempt to compensate for any retardation in development that may have occurred during culture (12 h asynchrony) or have resulted from division of ova (48 h asynchrony).

Following transfer, the recipients were run with harnessed vasectomized rams and inspected daily for oestrus for at least 40 days after transfer. Those that were not marked by the rams or had doubtful marks were kept under surveillance in confined areas at the expected time of lambing.

Table 1. Development in culture of half sections of day-6 and day-7 sheep ova

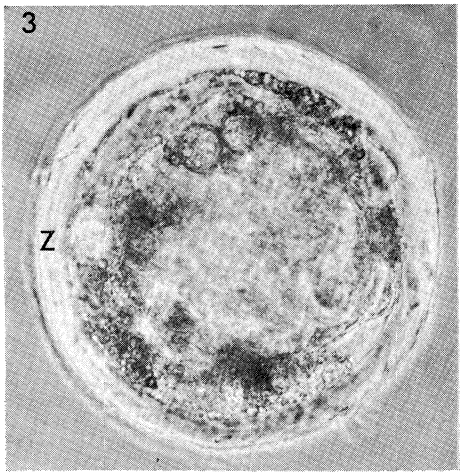
Development during culture[A]	Number of ova cultured: Day-6 ova	Day-7 ova
No development (both halves)	15	1
False blastocysts from one half	5	1
Two false blastocysts	2	—
Normal blastocyst from one half	7	2
Normal and false blastocysts	1	—
Two normal blastocysts	7	—
Total	37	4

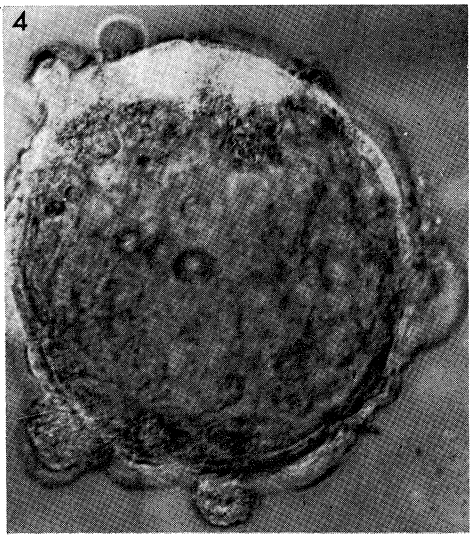
[A] Ova cultured for 2 days.

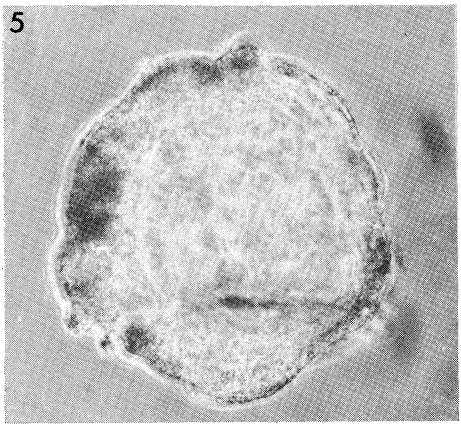
Results

Day-4 ova were of 8 to 20 cells at the time of collection and division. A total of 26 were divided without rupture of more than one or two blastomeres and the resulting 52 separated halves were cultured for 2 days. During culture, separation and dispersion of the individual blastomeres occurred in 34 half sections (65%) and it was not possible to determine if cellular cleavage had occurred during culture. Fourteen half sections (27%) maintained their cellular aggregation, but showed only one or two cleavage divisions. A further four failed to show cleavage in culture. Following staining with orcein after culture it was found that nuclei of most cells were dispersed or grossly irregular in shape. They were considered to be non-viable and none were transferred to recipients.

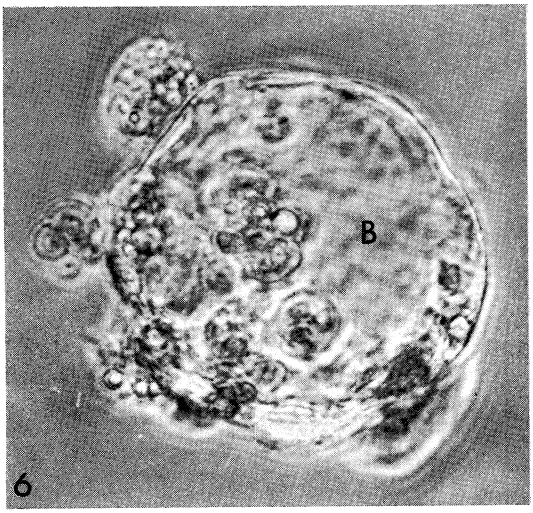
At the time of collection, day-6 and day-7 ova were morulae of more than 20 cells. Thirty-seven day-6 and four day-7 ova were successfully divided and the resulting 82 halves were cultured for two days (Table 1). Of the 82 halves, 47 showed no

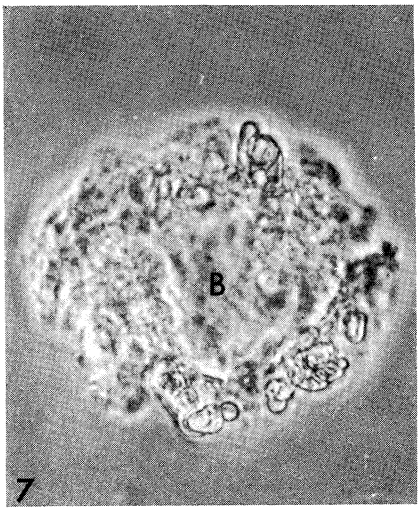






Figs 3–5. Expanded blastocysts recovered after culture of day-6 morulae in Dulbecco phosphate buffer + 20% heterologous sheep serum for 2 days. ×650. **3**, Untreated ovum with intact zona pellucida (*Z*). **4**, Ovum treated with 5% Protease to remove the zona prior to culture. **5**, Ovum mechanically divided in half prior to culture. Note the marked similarity of all blastocysts, suggesting apparently normal development *in vitro*.





Figs 6 and 7. False blastocysts obtained following culture of mechanically divided half sections of day-6 morulae in Dulbecco phosphate buffer + 20% heterologous sheep serum. Note well developed blastocoele (*B*) and irregular nature of peripheral cells. **6**, ×1130. **7**, ×900.

development in culture, 11 developed into 'false' blastocysts and 24 were recovered from culture as well-formed, apparently normal blastocysts (Fig. 5). 'False' blastocysts showed a well-developed blastocoele but the cells lining their periphery were irregular in size and frequently quite large spaces were observed between adjacent peripheral cells (Figs 6 and 7). Blastocysts classed as normal were somewhat smaller than those which have, in this laboratory, developed from day-6 and day-7 ova cultured for 2 days in a similar medium (Figs 3 and 4).

Of the 24 normal blastocysts recovered after culture, 14 had developed from each half of seven divided ova, all seven having been collected from donors on day 6. Nineteen of the 24 were transferred individually, to recipient ewes (Table 2). A further 18 half

Table 2. Returns to service and lambing data following transfer of half sections of day-6 and day-7 ova to recipient ewes

Age and treatment of ova	Asynchrony of recipients[A]	Number of recipients: Returned to service[B] 16–20	25–30	>35	Lambed	Total
Day 6, cultured	−12 h	9	2	1	2	14
Day 6, cultured	−48 h	2	2	1	0	5
Day 7, not cultured	0 h	18	0	0	0	18

[A] Recipients in oestrus 12 or 48 h after their respective donors.
[B] At indicated number of days after the oestrus immediately preceding transfer.

sections from nine day-7 ova that were not cultured were transferred. Following transfer 29 recipients returned to service within 20 days of the immediate pre-transfer oestrus, four returned 25–30 days after oestrus and one returned on day 38. Of the three recipients that did not return to oestrus during the observation period of 40 days after transfer, two lambed, each producing one apparently normal lamb. The lambs had developed from individual halves of different day-6 ova and both recipients that lambed had been in oestrus 12 h after their respective donors.

Discussion

The development *in vitro* of some 20% of both sections of mechanically divided day-6 morulae to apparently normal blastocysts shows that continued development was not completely dependent upon the retention of the entire cell mass of the morula. However, very few of the apparently normal blastocysts that developed from the halves during culture showed the capacity to develop into lambs *in vivo*. There are two possible explanations for failure of divided ova to show full development to lambs. Firstly, the treatment procedures and the culture conditions may have been appropriate for continued growth, but not for the retention of full viability. Secondly, cellular specialization may have already occured and, if this is so, then there would be little chance of each half possessing the potential for full development. There is little evidence to support the former explanation. Normal day-25 embryos have developed from ova transferred to recipient ewes following removal of the zona and culture for 2 days in a medium similar to that used in this study (Trounson and Moore, unpublished data). Further, Moore and Bilton (1973) obtained lambs following storage of ova at 5°C and culture for 2 days in Dulbecco phosphate buffer + 20% heterologous

sheep serum. Development of a number of false blastocysts from divided portions of day-6 and day-7 ova tends to suggest that cellular specialization had already occurred at the time the ova were divided. In most of the apparently normal blastocysts it was difficult to identify with any degree of certainty an embryonic disk and it may be that many were, in fact, trophoblastic vesicles. In several recipients the time elapsing between the transfer oestrus snd return to service was extended beyond the length of a normal oestrous cycle. This may indicate that some of the blastocysts were capable of limited development *in vivo*.

The potential for full development of single blastomeres of two- to eight-cell ova of a number of species has been well documented, but there is no evidence that single blastomeres of more advanced ova possess this potential. The results of Tarkowski and Wróblewska (1967) would suggest that, at least in the mouse, the potential for full development decreases as the age of the ova increases. They found that when portions of divided four- to eight-cell ova were cultured, there was a marked increase in false blastocysts and trophoblastic vesicles in developing portions of eight-cell ova when compared to those of four-cell ova.

It is possible that the use of early-cleavage-stage ova may provide a greater chance of obtaining full development of divided portions of the cell mass, but a major problem would seem to be the separation and dispersion of cells of these ova following mechanical division of the cells.

Acknowledgments

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