PRELIMINARY STUDIES ON *IN VITRO* CULTURE OF FERTILIZED SHEEP OVA*

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Continued development *in vitro* of recently fertilized ova has been recorded in a number of laboratory species and Brinster (1965*a*, 1965*b*, 1965*c*, 1965*d*) has achieved a sufficient degree of success with mouse ova to allow critical studies of the nutritional requirements during development from two cells to blastocysts. Fertilized sheep ova have been successfully stored for short periods between collection and transfer to recipient ewes but there are no reports of continued development of sheep ova during longer periods of *in vitro* culture.

Experimental

A total of 307 fertilized ova collected from Merino ewes following treatment with an equine anterior pituitary extract to induce multiple ovulation (see Moore and Shelton 1964) were incubated at 37°C for 48 hr in one of the following media: (1) basal salt solution (BSS); (2) BSS with 1 mg bovine serum albumin per millilitre (Commonwealth Serum Laboratories); (3) BSS with 15% heterologous sheep serum; (4) heterologous sheep serum. The composition of the BSS was as follows:

Compound	Concn. (g/l)	Concn. (mM)	Compound	Concn. (g/l)	Concn. (mM)
NaCl	5·214	89·21	$MgSO_4.7H_2O$	0·294	1·19
KCl	0·356	4·77	$NaHCO_3$	0·400	4·76
$CaCl_2$	0·189	1·70	Sodium lactate	5·603	50·00
KH_2PO_4	0·162	1·19	Sodium pyruvate	0·055	0·50

It was similar to that described by Brinster (1963) for the culture of mouse ova. All media contained 100 i.u. potassium penicillin and 50 i.u. streptomycin sulphate per ml. The pH of all media apart from sheep serum, on which no measurements were made, fell within the range 6·9–7·1.

The ova were collected either 48–60 hr or 72–84 hr after the donors were first served by fertile Merino rams. Collection at 48–60 hr provided ova mostly of two and four cells, whilst the majority of 72- to 84-hr ova were of eight cells. Culturing was carried out in small droplets of media (0·1–0·2 ml) under lightweight paraffin oil in small Petri dishes in an atmosphere of 5% CO_2 in air. Each droplet contained 3–5 ova. After culturing, 194 ova were stained with 1% orcein and examined, and 113 ova were transferred to recipient ewes. Transfers were made to the fallopian tubes of recipients that had been first served by vasectomized rams within ±12 hr of their respective donors; each ewe received one ovum.

Results and Discussion

Of the 194 ova stained and examined, 108 had cleaved in culture, while 9 of the 113 ova transferred to recipients developed into lambs (Table 1). There was an effect of age of ovum at the time of collection on the subsequent proportion of ova which

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cleaved in culture. Whilst there was no overall effect of type of medium, the addition of 15% serum to the BSS tended to increase the proportion of ova which cleaved (38 of 57 *v.* 20 of 42; $\chi^2 = 3{\cdot}59$; $0{\cdot}05 < P < 0{\cdot}10$); of the nine ova which developed into lambs eight had been cultured in serum or BSS plus serum.

Only 11 of the 194 ova examined after culture cleaved more than once during culture. Eight eggs collected 48–60 hr after detection of oestrus developed from two or four cells to eight or more cells and three 72–84-hr ova developed to 16–20 cells. Ova which cleaved more than once were randomly distributed throughout the various media.

TABLE 1

DEVELOPMENT OF FERTILIZED SHEEP OVA AFTER 48 HR IN CULTURE MEDIA

Medium	Age of Ova (hr)*	Stained Ova		Transferred Ova	
		Number Cultured	No. (%) Cleaved in Culture†	Number Transferred	No. Developed into Lambs
BSS‡	48–60	30	16 (53)	14	0
	72–84	12	4 (33)	13	0
	Total	42	20 (48)	27	0
BSS+albumin	48–60	26	11 (42)	15	0
	72–84	8	5 (63)	11	1
	Total	34	16 (47)	26	1
BSS+sheep serum	48–60	30	27 (90)	15	2
	72–84	27	11 (41)	15	2
	Total	57	38 (67)	30	4
Sheep serum	48–60	32	19 (59)	15	2
	72–84	29	15 (52)	15	2
	Total	61	34 (56)	30	4
Total	48–60	118	73 (62)	59	4
	72–84	76	35 (46)	54	5
Grand total		194	108 (56)	113	9

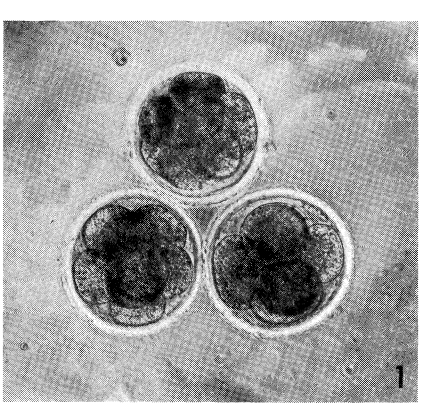
* Time elapsing between onset of oestrus and collection of ova.

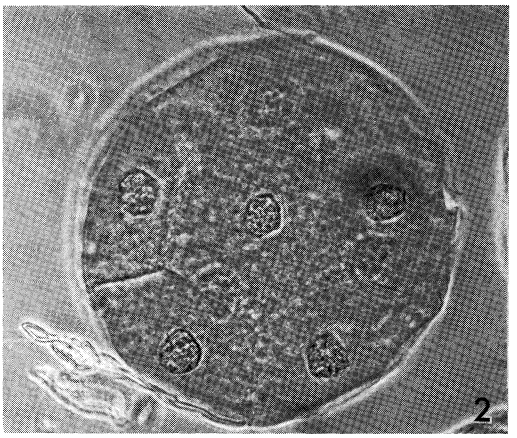
† χ^2 tests for proportion of ova which cleaved in culture: age of ovum, $\chi^2_1 = 4{\cdot}68$, $P < 0{\cdot}05$; type of medium, $\chi^2_3 = 4{\cdot}93$, n.s.

‡ Basal salt solution.

Of the eggs which cleaved in culture, 20 showed gross nuclear abnormalities. In 15 the nuclei of all or the majority of cells were irregular in shape, and in 5 the nuclei appeared normal but one or more blastomeres contained two nuclei (Fig. 3). Similar abnormalities were observed in 24 of the 86 eggs which did not cleave (21 with irregular nuclei and 3 with two nuclei in individual blastomeres). The incidence of nuclear abnormalities was not influenced by either age of ovum or type of medium and only 1 of the 11 ova which cleaved more than once showed nuclear abnormalities (irregularly shaped nuclei).

Overall, cleavage occurred more frequently in the younger ova, but even in these very few cleaved more than once and none reached a stage of development comparable with their chronological age. Had the ova remained *in vivo* for a further 2 days, 48- to 60-hr ova would have advanced to 20 or more cells and 72- to 84-hr ova would have developed into late morulae or early blastocysts (Moore, unpublished data).





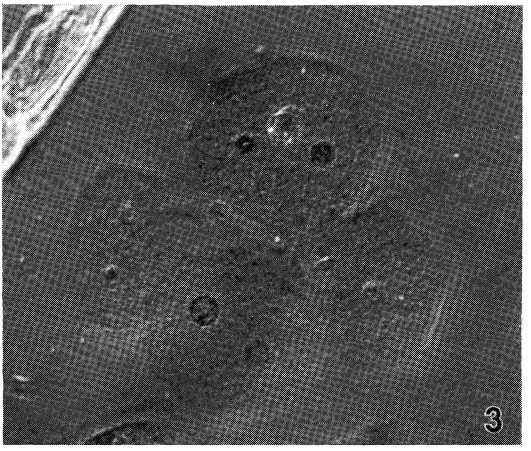


Fig. 1.—Three four-cell eggs after culture in BSS with 15% sheep serum. Two eggs developed to eight cells and one to six cells. Unstained. ×75.

Fig. 2.—One of the eight-cell eggs shown in Figure 1. Eight well-defined nuclei visible. Orcein stained. ×150.

Fig. 3.—One four-cell egg after culture in BSS. No development in culture. Note two nuclei in two cells. Orcein stained. ×150.

The rate of cleavage may not have been depressed. First cleavage may have proceeded at a normal rate, but thereafter further development in the majority of eggs ceased. It may be that cellular division in a number of ova which cleaved during culture had been initiated at the time of collection. However, the low incidence of actively dividing cells observed in ova stained immediately after collection (Moore, unpublished data) would suggest that cellular division in at least some eggs was initiated in culture.

The major components of tubal fluid of the ewe were included in the basal salt solution, but the concentration of lactate was somewhat greater and that of bicarbonate somewhat lower than found by Restall and Wales (1968) in tubal fluid during the immediate post-oestrous period. Detailed studies on *in vitro* culture of mouse ova have shown the importance of pH. Brinster (1965*a*), using a medium similar to that employed in the present study, found that the optimum pH for development of mouse ova was about 6·8. Hadek (1953) found the tubal washings of the ewe during oestrus and immediately post-oestrus to have a pH of 6·8–7·0. Hence, a pH of 6·9–7·1 was employed in the present study. However, more recent observations suggest that a somewhat higher pH may be required for *in vitro* development of the sheep egg (Moore, unpublished data).

Although development in culture was retarded, nine ova did develop into lambs and it is interesting to note that three of them had shown no development in culture. These three ova were of eight cells before and after culture. All ova at the time of transfer were, as far as cell stage is concerned, 1–2 days less advanced than the tracts of their respective recipients. Should cell stage be more important than chronological age in determining the fate of transferred ova, then it could be expected that more ova would have survived and developed had transfer been made to recipients one or two days "younger" than their respective donors.

Acknowledgments

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