THE STORAGE OF FERTILIZED SHEEP OVA AT 5°C

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Abstract

Fertilized day-2 (1–4 cells) and day-5 (morulae) sheep ova were cooled at a rate of 0·25 or 1·0 degC/min to 5°C then stored at 5°C for intervals between 10 min and 6 hr, after which they were cultured *in vitro* for two days at 37·5°C. Storage had no effect upon subsequent development in culture. Of 103 day-2 ova, 75 (73%) developed to eight cells and 57 of 88 (65%) day-5 ova developed to blastocysts. There were no effects of age of ovum, cooling rate, or duration of storage on development in culture.

A total of 30 day-5 ova stored for from 10 min to 6 hr and seven day-5 ova stored for 2 days then cultured *in vitro* were transferred to 20 recipient ewes of which 10 subsequently lambed, producing 11 lambs. There were no effects of duration of storage or cooling rate on the proportion of ova that developed to lambs.

I. INTRODUCTION

The development of successful methods for long-term storage of fertilized ova would have many applications, particularly in the breeding and improvement of farm animals as well as for the storage of genetic material for future study and use. The most obvious method of storage involves the use of low temperatures. To date some success has been achieved with storage at temperatures above freezing point, but until recently no success has been achieved using storage at sub-zero temperatures.

Several attempts at the storage of fertilized sheep ova at temperatures above 0°C have been reported, but the results have been poor and often conflicting (Averill and Rowson 1959; Harper and Rowson 1963; Kardymowicz *et al.* 1963). Far too few ova were used in these studies to enable a critical assessment to be made of either the procedures involved or the results obtained. In one experiment Averill and Rowson (1959) reported that 4 of 10 sheep ova stored in sheep serum at 4·5–7·6°C for 24 hr developed into lambs when transferred to recipient ewes. An attempt to extend this initial study met with no success (Harper and Rowson 1963). No ova stored at 7°C for 3 or more days developed into lambs after transfer to recipient ewes.

The ultimate test of the viability of stored ova lies in their subsequent development when transferred to recipient females, but a much more rapid, if only partial, assessment of the effects of storage on viability can be obtained by observations on the subsequent development of ova when cultured *in vitro*. In the present study the effects on subsequent viability of age of ova, rate of cooling, and duration of storage at 5°C were examined. Viability was assessed by observing the subsequent development of stored ova on incubation *in vitro*, and *in vivo* in recipient ewes.

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II. Materials and Methods

(a) General

In a preliminary experiment designed to study the effects of rate of cooling and storage temperature, 36 fertilized ova of 1–4 cells, collected from donor ewes two days after they were first observed in oestrus, were stored at 5 or 15°C for 10 min or 1 hr. The ova were cooled from 37·5°C at rates of 1·0 or 0·25 degC/min and after storage they were incubated for two days at 37·5°C.

The preliminary study showed no effect of storage temperature (see Table 1) and in a subsequent major experiment all ova were stored at 5°C after which they were incubated *in vitro* for two days at 37·5°C. Fertilized ova were collected from donor ewes two or five days after the onset of oestrus (hereafter designated day-2 and day-5 ova respectively) and cooled to 5°C at a rate of 0·25 or 1·0 degC/min, then stored for 10 min, 1 hr, or 6 hr at 5°C before incubation (see Tables 2 and 3).

(b) Collection of Fertilized Ova

Fertilized ova were collected from mature medium-wool Merino ewes following treatment with an equine anterior pituitary extract to induce multiple ovulation (Moore and Shelton 1964*a*). Two or five days after mating to fertile rams ova were recovered by flushing *in vivo* the fallopian tubes and uterine horns with Dulbecco phosphate buffer* (D.P.B.) containing 10% heterologous sheep serum. On each day of collection ova were recovered from several donor ewes. Immediately after collection the ova were washed twice in D.P.B. + 10% serum, and prior to cooling ova of each age group were pooled in D.P.B. + 20% serum.

(c) Cooling, Storage, and Culture

After collections were completed ova were transferred to Pyrex glass culture tubes (7·5 by 1·0 cm) containing 1·5–2 ml D.P.B. + 20% serum and covered with a layer of 3–4 mm of lightweight paraffin oil. Three to six ova were placed in each tube. The tubes were then partially immersed in water-baths pre-warmed to 37·5°C where they were allowed to equilibrate for 30 min. After equilibration the water-baths and tubes were transferred to cold rooms maintained at 4°C (cooling rate 0·25 degC/min) or −20°C (cooling rate 1·0 degC/min). During cooling the water-baths were stirred constantly and any departure from the predetermined cooling rates was corrected by the addition of warm or cold water. After cooling the tubes were transferred to vacuum flasks and placed in a refrigerated cabinet maintained at 4·5–5·0°C. After storage the tubes were placed in racks in incubators maintained at 37·5°C where they remained for two days. No attempt was made to control the rate of warming, but in practice equilibration to 37·5°C was obtained within 40–60 min. After incubation for two days the ova were examined as fresh specimens and then either transferred to recipient ewes or again examined after staining with 1% orcein.

Subsequent to the main experiment nine day-5 ova were cooled at a rate of 1 degC/min, stored at 5°C for two days, incubated for two days, then transferred to recipient ewes.

(d) Transfer Procedures

A total of 30 day-5 ova from the main investigation and a further seven of the nine ova stored for two days were transferred, after storage and culture, to the uterine horns of 20 recipient ewes. Potential recipients were run with harnessed vasectomized rams and inspected for oestrus once daily.

(e) Preparation of Media, Paraffin Oil, and Culture Tubes

The media used for flushing, storage, and culture of ova were prepared fresh each day. Penicillin (100 i.u./ml) and streptomycin sulphate (50 i.u./ml) were added to the media, which were sterilized by passage through Millipore filters of pore size 0·45 μm. Paraffin oil was sterilized by heating to 120°C for 30 min, and the glass culture tubes were sterilized by heating to 180°C for 1 hr.

* Commonwealth Serum Laboratories, Melbourne. The buffer had the following composition (g/l): NaCl 8·0, KCl 0·2, Na_2HPO_4 1·15, KH_2PO_4 0·2, $CaCl_2$ 0·1, $MgCl_2$ 0·1.

III. Results

(*a*) *Preliminary Study*

Of the 36 ova stored at 5 or 15°C, 27 developed to eight cells during culture (Table 1). There was no effect of storage temperature on subsequent development in culture and in the major study that followed all ova were stored at 5°C.

TABLE 1

DEVELOPMENT OF DAY-2 OVA IN CULTURE FOLLOWING STORAGE AT 15 AND 5°C—PRELIMINARY STUDY

Storage times are given in parentheses. After storage all ova were cultured at 37·5°C for two days

Cell stage	No. of ova at cell stage (10 min)	(60 min)	Total No. of ova
Cooling rate 0·25 degC/min; stored at 15°C			
Pre-storage			
One cell	1	2	3
Two cell	3	4	7
Post-culture			
Four cell	1	1	2
Eight cell	3	5	8
Cooling rate 1·0 degC/min; stored at 15°C			
Pre-storage			
One cell	2	0	2
Two cell	6	0	6
Four cell	0	0	0
Post-culture			
Two cell	0	0	0
Four cell	2	0	2
Eight cell	6	0	6
Cooling rate 0·25 degC/min; stored at 5°C			
Pre-storage			
One cell	0	0	0
Two cell	5	5	10
Post-culture			
Four cell	2	1	3
Eight cell	3	4	7
Cooling rate 1·0 degC/min; stored at 5°C			
Pre-storage			
One cell	0	2	2
Two cell	0	2	2
Four cell	4	0	4
Post-culture			
Two cell	0	1	1
Four cell	1	0	1
Eight cell	3	3	6

(*b*) *Main Study*

(i) *Development during Culture*

Studies in this laboratory have shown that day-2 ova of 1–4 cells should develop to eight cells during two days culture *in vitro*, while day-5 ova should develop in two days from morulae of about 20 cells to blastocysts, some having expanded and a few having hatched from the zona pellucida (Figs. 1 and 2). The majority of day-2 ova were of two cells at the time of collection and after storage and culture 75 of the 103 ova stored and cultured were found to be of eight cells (Table 2). Of 21 ova cultured but not stored, 15 developed to eight cells and thus storage had little effect upon subsequent development in culture—75 of 103 (73%) *v.* 15 of 21 (71%) ova developed to eight cells. Similarly, with day-5 ova 57 of 88 stored ova (65%) *v.* 11 of 16 ova that had not been stored (69%) developed to blastocysts in culture (Table 3).

An analysis of variance carried out after angular transformation of the raw data on the proportion of stored ova that developed to eight cells or blastocysts showed no significant main effects or interactions.

TABLE 2

DEVELOPMENT OF DAY-2 OVA IN CULTURE FOLLOWING STORAGE AT 5°C—MAIN STUDY

Storage times are given in parentheses. After storage all ova were cultured at 37·5°C for two days. Control ova were incubated without storage

Cell stage of ova	No. of ova: (10 min)	(1 hr)	(6 hr)	Total	No. of ova: (10 min)	(1 hr)	(6 hr)	Total	Overall total*	Control values
	Cooling rate 0·25 degC/min				Cooling rate 1·0 degC/min					
Pre-storage										
One cell	0	1	0	1	1	2	0	3	4	5
Two cell	19	16	18	53	5	2	6	13	66	13
Four cell	0	3	1	4	10	12	7	29	33	3
Post-culture										
Two cell	0	2	0	2	2	1	0	3	5	0
Four cell	4	5	5	14	4	2	3	9	23	6
Eight cell	15	13	14	42	10	13	10	33	75	15
Total No. of ova†	19	20	19	58	16	16	13	45	103	21

* For both cooling rates. † All stages.

TABLE 3

DEVELOPMENT OF DAY-5 OVA IN CULTURE FOLLOWING STORAGE AT 5°C—MAIN STUDY

Storage times are given in parentheses. Culture details as for Table 2. M = morula, B = unexpanded blastocyst, EB = expanded blastocyst, HB = hatched blastocyst

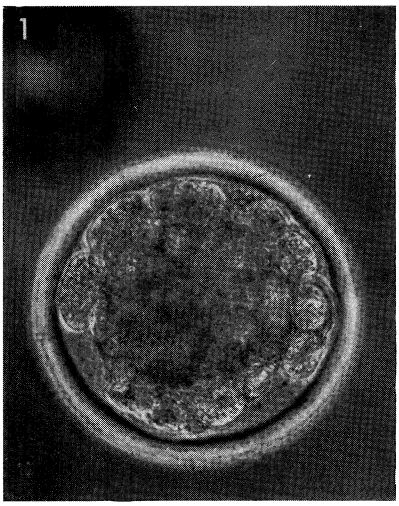
Stage of development	No. of ova: (10 min)	(1 hr)	(6 hr)	Total	No. of ova: (10 min)	(1 hr)	(6 hr)	Total	Overall total*	Control values
	Cooling rate 0·25 degC/min				Cooling rate 1·0 degC/min					
Pre-storage										
M	11	15	15	41	15	15	17	47	88	16
Post-culture										
M	3	6	5	14	8	4	5	17	31	5
B	7	4	3	14	2	2	3	7	21	0
EB	1	5	6	12	1	6	9	16	28	9
HB	0	0	1	1	4	3	0	7	8	2
Total No. of ova†	11	15	15	41	15	15	17	47	88	16

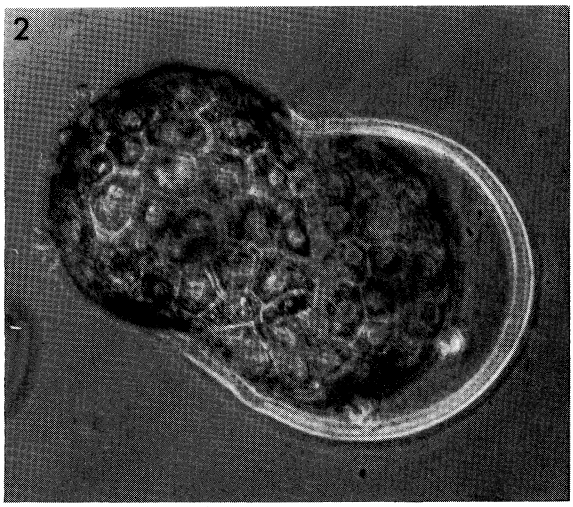
* For both cooling rates. † All stages.

The microscopic examination of fresh ova and of orcein-stained preparations after culture revealed no gross cellular or nuclear abnormalities except in several ova that showed no development during culture. In these ova the nucleii of one or more cells were either irregular in shape or poorly defined (Fig. 3).

(ii) *Development of Ova transferred to Recipient Ewes*

In all, 30 day-5 ova from the main experiment were transferred to 16 recipient ewes. The ova had been stored for between 10 min and 6 hr and all had developed, in





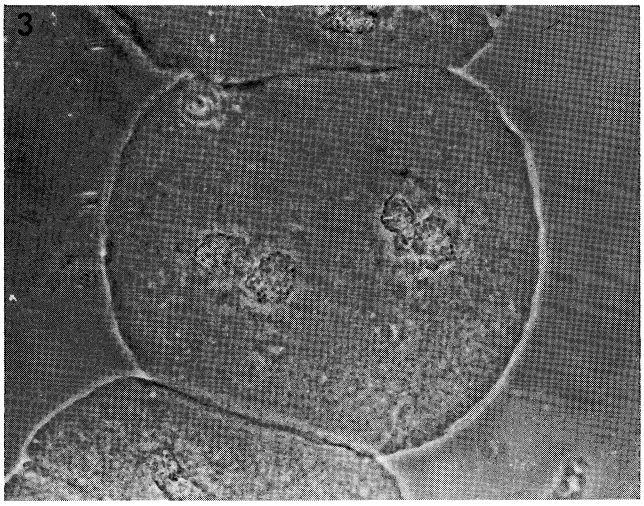


Fig. 1.—Day-5 morula before storage and culture (unstained, × 260).
Fig. 2.—Hatching blastocyst: day-5 ovum after storage and culture (unstained, × 170).
Fig. 3.—Day-2 two-cell ovum which showed no development after storage. Note irregular nuclei. (Orcein-stained, × 335.)

culture, to blastocysts prior to transfer (Table 4). A further seven of the nine ova stored for two days at 5°C then cultured for two days were transferred to four ewes. All seven ova had developed to blastocysts; the remaining two ova had showed limited development and were not transferred. In order to estimate the degree of asynchrony between age of ova and age of reproductive tract it was assumed that ova did not develop during storage and that development in culture proceeded at the rate which occurs *in vivo*. Thus, when ova were stored for two days synchronous transfers (degree of asynchronization = 0) were those in which ova were transferred to recipients that had been first observed in oestrus two days after their respective donors.

Of the 20 recipients 10 subsequently lambed, producing 11 lambs. The length of gestation varied from 149 to 153 days. There was no evidence to suggest any marked effect of duration of storage or rate of cooling on the survival and development of transferred ova, nor was there any major effect of degree of asynchrony.

TABLE 4

DEVELOPMENT OF DAY-5 OVA TRANSFERRED TO RECIPIENT EWES FOLLOWING STORAGE AT 5°C AND *IN VITRO* CULTURE FOR TWO DAYS

Storage time	Cooling rate (degC/min)	Recipient No.	D.A.* (days)	Ova transferred†	Lambs born‡
10 min	1·0	1	−2	1HB	Nil
10 min	1·0	2	−2	1EB, 1HB	Nil
1 hr	1·0	3	−2	2EB, 2HB	F
1 hr	1·0	4	−2	1EB, 1HB	Nil
6 hr	0·25	5	0	2EB	FF
6 hr	0·25	6	−1	2EB	F
6 hr	0·25	7	−1	2B	Nil
6 hr	0·25	8	0	1EB	Nil
6 hr	0·25	9	+1	1HB	M
6 hr	0·25	10	+1	1B, 1EB	Nil
6 hr	1·0	11	−1	2EB	Nil
6 hr	1·0	12	−1	2EB	M
6 hr	1·0	13	−1	2B	F
6 hr	1·0	14	−1	2EB	F
6 hr	1·0	15	−1	1EB	M
6 hr	1·0	16	−1	2EB	F
2 days	1·0	17	0	1B, 1EB	F
2 days	1·0	18	0	2EB	Nil
2 days	1·0	19	0	1EB	Nil
2 days	1·0	20	0	2EB	Nil

* Degree of asynchronization corrected for duration of storage; + indicates recipient served before donor, − indicates recipient served after donor.

† B = blastocyst; EB = expanded blastocyst; HB = hatched blastocyst.

‡ F, female; M, male.

IV. DISCUSSION

Storage of day-2 and day-5 ova at 5°C had little or no effect upon their subsequent development in culture. Furthermore, the birth of lambs following transfer of stored and cultured day-5 ova to recipients clearly illustrates that viability was retained in an appreciable proportion of ova. The proportion of ova that developed to lambs (11 of 37, i.e. 30%) was low, but most recipients received more than one ovum and it is unlikely that many of the recipients were capable of supporting more than one foetus to term. Further, 14 of the 20 transfers were asynchronous by as much as two days and it is well documented that maximum survival rates can only be expected when the degree of asynchrony between donors and recipients is no more than 12 hr (Moore and Shelton 1964*b*). It is conceivable that the subsequent rate of development might have been temporarily decreased by storage and perhaps by culture, but if there was any decrease in rate of development then it was probably not of any great magnitude, nor

permanent, as the length of gestation in ewes that lambed was well within the expected range for Merinos.

Development in culture showed that day-2 and day-5 ova survived storage equally well, but due to lack of potential recipients no day-2 ova were transferred and their viability after storage and culture must remain in doubt. However, their appearance after culture suggested that they were viable. It is apparent that further studies are required, particularly on the effects of duration of storage and of age of ovum on subsequent viability of stored ova. The technique of *in vitro* culture would be a most useful adjunct to the studies as it does provide a rapid indication of viability. It is equally apparent that storage at temperatures below freezing should be examined, and encouragement for such studies comes from the recent reports of successful freezing and thawing of mouse ova. In an initial report Whittingham (1971) found that over 50% of eight-cell mouse ova and early blastocysts survived freezing to −79°C when polyvinylpyrrolidone was used as a protective agent. However, ova could not be stored for more than 30 min without complete loss of viability. Subsequently, Whittingham *et al.* (1972) used slower and better controlled rates of cooling and thawing, and with the addition of dimethyl sulphoxide or glycerol to the storage medium they found that some 40% of mouse ova of one to eight cells survived storage for up to eight days at −196°C and subsequently developed into living young when transferred to recipient females.

V. Acknowledgment

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VI. References

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