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ATTEMPTED STORAGE OF SHEEP OVA AT 7° CENTIGRADE

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Summary. Sixty fertilized sheep ova were stored at 7° C in sterile sheep serum for either 72, 96 or 120 hr. Thirty of these ova were between two and six cells and after storage were transferred to the Fallopian tubes of fifteen recipient ewes. The remaining thirty ova were at the eight-cell stage or older and after storage were transferred to the uteri of fifteen recipient ewes. In each case two ova were transferred to each ewe. None of the ova survived these procedures and no lambs were born. It was observed that in the groups of ewes receiving ova which had been stored for the shorter periods, four ewes failed to return to oestrus within the normal cycle limits. It was suggested that this might indicate that some of the ova had 'implanted' but failed to survive to term.

Data are also presented on the ovulatory response of Welsh mountain ewes to a standard dose of pregnant mares' serum gonadotrophin (PMS) when given on the 12th or 13th day of the oestrous cycle. Some observations on the stage of development of the ova in relation to the time elapsing between the onset of oestrus and recovery are included.

INTRODUCTION

Many experiments have been carried out on the culture of mammalian ova *in vitro* (*see* Austin, 1961, for review), but relatively few attempts have been made to store ova at low temperatures. Chang (1947, 1948a, b, c, 1952) has shown that unfertilized and fertilized rabbit ova are still capable of further development after storage in pure rabbit serum between 0 and 10° C for varying periods of time. More recently Hafez (1961) has found that rabbit ova will survive storage at 10° C in a gelled medium of 1 : 1 serum : saline solution containing 1 % gelatine for up to 144 hr. Unfertilized mouse ova will survive storage up to 3½ hr in a modified Locke's solution containing 5 % glycerol at −10° C (Sherman & Lin, 1958), and up to 6 hr at 0° C (Sherman & Lin, 1959). Smith (1953) reported unsuccessful attempts to deep freeze rabbit ova. Averill & Rowson (1959) recorded that after storage in blood serum at 5·0 to 8·0° C for 6 to 9 hr 75·0 % of ova were developing normally 17 days later, and that after 24 hr storage 46·4 % survived. However no ova survived after storage for 48 or

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72 hr. In a second experiment they found that 66·7 % of ova developed into lambs after storage for 24 hr at 4·5 to 7·0° C and 40·0 % after 72 hr. In this experiment no ova survived if the temperature fell below 4·5° C. It should be pointed out that in this experiment (Averill & Rowson, 1959) all the ova, whatever their stage of development, were transferred to the uteri of the recipient ewes. In another paper (Averill & Rowson, 1958), however, it was shown that no two-cell ova and only 15·8 % of four-cell ova survived when transferred to the uterus, even when the oestrous stages of the donor and recipient ewes were correctly synchronized. Consideration of these data suggests that of the five ova stored for 70 to 72 hr only two, both eight-cell, were likely to survive after transfer to the uterus. In fact both these ova did produce lambs, while neither the two four-cell ova nor the one two-cell ovum did so.

On the basis of this very encouraging result, it was decided to repeat the experiment of Averill & Rowson (1959) under optimum conditions, and with a larger number of ova. It was hoped to repeat their success after 72 hr storage and to extend, if possible, the period of storage, and also to examine the effect of stage of development of the ova on their ability to withstand the storage procedures.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

The animals used were Welsh Mountain ewes, aged between 4 and 6 years, and drawn from a flock of fifty running under field conditions at the Animal Research Station, Cambridge. These experiments were conducted during the breeding season of 1958–59. Donor and recipient ewes were run together with raddled vasectomized rams and the occurrence of oestrus observed daily.

The donor animals were injected with 800 i.u. PMS on the 12th or 13th day of the oestrous cycle, and then placed with active fertile rams of the same breed. These ewes were examined every 8 hr subsequently for the onset of the next oestrous period.

Since the ova were to be stored for 3, 4 or 5 days, the number of donors superovulated at any one time was determined by the number of recipient ewes that were expected to come into oestrus 3, 4 or 5 days after the donor animals. In defining the age of the stored ova in relation to the selection of suitable recipients, the duration of storage was ignored on the assumption that development would not take place during storage at the chosen temperature. The age of any ovum was considered to be its age at the time of recovery from the donor ewe in relation to the onset of oestrus in that ewe. The recipient ewes were allowed to mate with the vasectomized rams without the treatment described by Hunter, Adams & Rowson (1955) for inducing synchronization of oestrus. In these animals also the occurrence of the onset of the next oestrous period was checked at 8-hr intervals.

RECOVERY OF OVA

The method used for the recovery of ova was similar to that used by Hunter *et al.* (1955) and Averill (1958), as modified by Moore, Rowson & Short (1960). For

recovery of ova which had developed to the two- to six-cell stage, eight donor ewes were subjected to laparotomy between 57 and 68 hr after the onset of oestrus. Eight further donor animals were laparotomized between 74 and 84 hr after the onset of oestrus for recovery of ova which had developed to the eight-cell stage or beyond. In all cases the ova were recovered by flushing from the tip of the uterus through the utero-tubal junction and out of the fimbriated end of the Fallopian tube. The medium used for recovery, storage and transfer of the ova was homologous sheep serum, made sterile by Seitz filtration, to which was added enough penicillin and streptomycin to give final concentrations of 1000 units/ml and 500 units/ml, respectively.

STORAGE OF OVA

As soon after recovery as possible the ova were transferred with 0·5 ml serum to dialysis chambers similar to those described by Averill & Rowson (1958). These chambers were kept at a temperature of 30 to 35° C for periods of up to 45 min while recovery of ova from more than one donor animal was carried out.

Up to six ova were placed in each dialysis tube which was then closed with a small rubber cork. In the outer nylon tube 12 ml serum were placed. The whole chamber was cooled to room temperature in 30 min (a fall of 0·3° C/min) (Averill & Rowson, 1959). The chambers were then suspended in a beaker of water at room temperature (25° C) and placed in an insulated refrigerator running at a constant temperature of 7° C. The beaker of water containing the dialysis chambers reached a temperature equilibrium in a further 150 min. This gave a rate of cooling from 25 to 7° C of 0·12° C/min. Every 12 hr the serum in the outer tube was replaced with serum which had been stored at −20° C until 12 hr before use, and which had then been placed in the refrigerator running at 7° C and allowed to reach this temperature gradually.

After storage periods lasting for 72, 96 or 120 hr, the beaker containing the dialysis chambers was removed from the refrigerator and allowed to stand at room temperature, while the recipient ewes were subjected to laparotomy. The ova were then removed from the tubes and, without prior warming, transferred either to the Fallopian tube or to the uterus, depending on their stage of development.

Three groups of ten ova, between the two- and six-cell stages, were stored for 72, 96 or 120 hr and transferred to fifteen recipient ewes, one ovum being placed in each Fallopian tube. A further three groups of ten ova, of eight cells or more, were stored for similar periods of time and then transferred to the uteri of fifteen recipient ewes, both ova being placed in one uterine cornu. The design of the experiment is shown in Table 1.

TRANSFER OF OVA

The procedure adopted for the transfer of the ova, after storage, to the reproductive tracts of the recipient animals was the same as that used by Moore *et al.* (1960). In no case were ova transferred to a recipient that had not a corpus luteum in its ovary, and moreover no recipient ewes were used in which the time of onset of oestrus varied from that of the donor ewe by more than 25 hr,

after making due allowance for the number of hours of storage. The importance of correct synchronization of donor and recipient ewes has been stressed by Averill & Rowson (1958). It will be apparent that no control transfers were performed in this series of experiments. However, at the same time as these

TABLE 1

DESIGN OF THE EXPERIMENT

Group	*Stage of development of stored ova*	*Storage time (hr)*	*Site of transfer*	*No. ova transferred*	*No. recipient ewes*
1 A	Two to six cells	72	Fallopian	10	5
B		96	tube	10	5
C		120		10	5
2 A	Eight or more cells	72	Uterus	10	5
B		96		10	5
C		120		10	5
Total				60	30

experiments were being performed, Moore *et al.* (1960) were carrying out experiments on ovum transfer in the sheep at the same place and using identical techniques. Their results were felt to constitute adequate controls.

After the ova had been transferred to the recipient ewes, the recipients were kept in for 2 to 3 days post-operatively, and then allowed to run with vasectomized rams. They were checked daily for the occurrence of oestrus. The viability of the ova was intended to be shown by the number of lambs born. This would have made no allowance for any prenatal losses, but Moore *et al.* (1960) had shown that when two ova are transferred to each ewe 56 % of all ova transferred will develop into lambs.

TABLE 2

EFFECT OF GONADOTROPHIN TREATMENT IN RELATION TO STAGE OF THE OESTROUS CYCLE

Day of oestrous cycle 800 *i.u.* PMS *injected*	*No. ewes*	*Corpora lutea*		*Large follicles*		*Ova recovered*		
		Total	*Mean*	*Total*	*Mean*	*Total*	*Mean*	%
12	8	73	9·1	20	2·5	52	6·5	71·2
13	8	59	7·4	13	1·6	48	6·0	81·4
Total	16	132	8·3	33	2·1	100	6·3	75·8

RESULTS

RESULTS OF THE SUPEROVULATION TREATMENT

A total of sixteen ewes were subjected to superovulation treatment, of which eight were injected with 800 i.u. PMS on Day 12, and the remaining eight injected with 800 i.u. PMS on Day 13 of the oestrous cycle. In Table 2 are set out

the mean numbers of ova shed, large follicles that failed to rupture, and ova recovered. It appears that injection with PMS on the 12th day of the cycle produces a larger number of ovulations (9·1 versus 7·4), but the mean number of ova recovered is only slightly greater (6·5 versus 6·0) than with injections on the 13th day. The mean number of ova recovered per ewe was 6·3 which meant that 75·8 % of the ova shed were recovered.

A total of one hundred ova was recovered, but only sixty were included in the experiment. The remainder of the ova recovered were excluded for the following reasons: eighteen were unfertilized, two were developing abnormally, one was lost during transfer to the dialysis chamber, and for nineteen normal ova no recipient ewes were available.

A classification of the stage of development of these ova is shown in relation to the time of recovery in Table 3. Those ova recovered between 50 and 60 hr

TABLE 3

STAGE OF DEVELOPMENT OF OVA IN RELATION TO THE TIME OF RECOVERY AFTER THE ONSET OF OESTRUS

Time after onset of oestrus (hr)	*No. ewes*	*Ova*				*Stage of development*					
		No. shed	*Recovered No.*	*(%)*	*No. unfertilized*	*No. of cells* 1	2	4	6	8	8+
50 to 59	5	30	27	90·0	1	8	4	9	5	–	–
60 to 69	3	29	21	72·4	6	–	3	6	6	–	–
70 to 79	5	52	39	75·0	8	–	1	–	–	22	8
80+	3	21	13	61·9	3	–	–	–	–	3	7
Total	16	132	100	75·8	18	8	8	15	11	25	15

after the onset of oestrus were fairly evenly distributed in development between one-, two-, four- and six-cell stages. This situation was not markedly changed in those ova recovered between 60 and 70 hr, but after 70 hr from the onset of oestrus the majority of the ova had reached the eight-cell stage, and some had even progressed further. After 80 hr the majority of the ova had more than eight blastomeres. It is noticeable that the percentage recovery of ova falls markedly throughout this time range. The low percentage recovery of ova in the three ewes subjected to laparotomy 80 hr or later after the onset of oestrus probably indicates that some of the ova had already passed into the body of the uterus.

RESULTS OF THE STORAGE EXPERIMENT

The results of the storage experiment are summarized in Table 4. It can be seen that a number of ova representing nearly all stages of cleavage were stored for either 72, 96 or 120 hr at 7° C. Attempts were made to synchronize as nearly as possible the stage of development of the ovum and the endometrial development of the recipient ewe. For this purpose the age of the ovum was considered to be its post-oestrous age at the time of recovery from the donor animal. The number of hours of storage were ignored in this calculation, since it was assumed

that development of the ovum during storage was at the minimal level compatible with continued viability. In fact the post-oestrous age of the ova stored for 72 hr ranged from 12 hr less to 16 hr more than the post-oestrous stage of the recipient animals at the time of laparotomy. Similarly for the ova stored for 96 hr their stage of development ranged from 15 hr less to 7 hr more, and

TABLE 4

SURVIVAL OF SHEEP OVA AFTER STORAGE AT 7° C AND RETRANSFER TO RECIPIENT EWES

Experimental group	*Storage period (hr)*	*No. and cell stage of ova stored*	*No. recipient ewes*	*Ova surviving storage and transfer*	*Days elapsing between pre-transfer oestrus and the first post-transfer oestrus*	
					No.	*Mean*
1A	72	2(2) 3(4) 5(6)	5	0	17, 18, 20, 33, 68	26·1
2A		5(8) 5(8+)	5	0	17, 17, 17, 19, 35	
1B	96	2(1) 3(2) 3(4) 2(6)	5	0	17, 17, 17, 17, 18	18·6
2B		5(8) 5(8+)	5	0	16, 17, 18, 18, 31	
1C	120	1(2) 5(4) 4(6)	5	0	17, 17, 17, 18, 18	17·2
2C		5(8) 5(8+)	5	0	15, 16, 17, 18, 19	
Total		60	30	–		

for the ova stored for 120 hr from 25 hr less to 24 hr more than the number of hours post-oestrus at which the recipients were subjected to laparotomy. Irrespective of the length of storage time, the age of the ova stored, the degree of synchronization of donor and recipient animals, or the site to which the ova were transferred, no ova survived to produce lambs.

The majority of the recipient ewes returned to oestrus after the normal interval between 15 and 20 days after the pre-transfer oestrus, and most of these ewes returned within 16 to 18 days. However, it is interesting to note that three of the recipient ewes receiving ova stored for only 72 hr and one ewe which received ova that had been stored for 96 hr did not return to oestrus within these time limits. The three ewes receiving ova stored for 72 hr returned to oestrus after 33, 35 and 68 days, respectively, and the other ewe receiving ova stored for 96 hr returned after 31 days. It should be pointed out that these figures represent approximately two or four dioestrous periods, and it is possible that these ewes were experiencing silent heats. However, since these experiments were carried out during November and December, which is still within the normal breeding season, this may not be the explanation.

The fact that the ewes which failed to return to oestrus at the normal time are all concentrated in those groups in which the ova were stored for the shortest times suggests that some of these ova may still have been partially viable. It is possible that they started to develop normally, but that the storage period had in some way impaired their capacity for development, and they therefore failed to survive to term.

It should also be noted that even after 120 hr storage all the ova that were transferred looked normal on gross examination. The outlines of the individual

blastomeres were not blurred and the zonae pellucidae appeared perfectly spherical and not distorted. These ova looked in every way as normal as others examined immediately after recovery from donor ewes. None of these ova underwent cleavage during storage at this temperature.

DISCUSSION

It has been shown that in the mouse (McLaren & Michie, 1956), in the rat (Noyes & Dickmann, 1960), in the rabbit (Chang, 1950) and in the sheep (Averill & Rowson, 1958) the stage of development of the transferred ovum must be synchronized with the stage of development of the uterine endometrium for the ovum to continue to develop normally. Synchronization in this context means that the ovum should be chronologically and physiologically the same age as, or older than the endometrium in relation to the preceding oestrus. An ovum 'older' than the uterine environment may have a better chance of survival, since Tarkowski (1959) has shown that the transfer procedures can cause the development of the mouse ovum to be retarded by as much as 1 day. In the present experiment only one ovum in each case was more than 24 hr younger or more than 24 hr older than the corresponding post-oestrous stage of the recipient ewe. This calculation does not include the period of time during which the ovum was stored, since it was assumed that during this time development was suspended. No ovum was observed to cleave during storage and Chang (1948a) has recorded that no rabbit ovum stored below 15° C cleaved during storage.

In their experiments on ovum transfer in sheep Averill & Rowson (1958) have clearly shown that two- and four-cell ova should always be transferred to the Fallopian tube, while eight-cell ova should be transferred to the uterus. However it was found by Averill (1956) that only four (40 %) of ten two- and four-cell ova transferred to the tubes developed into lambs, compared to thirty-eight (83 %) of forty-six six- to sixteen-cell ova developing into lambs following transfer into the uterine horns. Moore *et al.* (1960) also observed that while the percentage of ewes becoming pregnant after transfer of ova to the tubes or uteri was similar (70 versus 80 %), only nineteen (48 %) out of forty ova transferred to the tubes, compared to twenty-seven (68 %) out of forty ova transferred to the uterus, survived. This comparative failure of ova transferred to the Fallopian tubes may have been due to the lack of cumulus oophorus cells. Bennett & Rowson (1961) and Wintenberger-Torres (1961) have shown that a cumulus coating is necessary to ensure normal transport of the ovum through the ampullar region of the Fallopian tube of the ewe.

In the present experiment, no ova were transferred without storage but it was felt that such control experiments were unnecessary in view of the good results that were obtained by Moore *et al.* (1960) at the same time, in the same place and by the same techniques. Despite their success with ova transferred without storage and the success of Averill & Rowson (1959) with ova transferred after storage periods of up to 72 hr, none of the ova in the present experiments survived storage and re-transfer. It has already been shown from consideration of the results of Moore *et al.* (1960) that it is unlikely that these failures could have been entirely due to faulty transfer techniques.

In general the procedures adopted for the storage of the ova were identical to those used by Averill & Rowson (1959). One important difference was that Averill & Rowson (1959) cooled their ova from 25° C to 8° C at a rate of 0·5° C/min, whereas in the present experiment cooling through this temperature range proceeded at a rate of 0·12° C/min. There is a substantial difference between these rates of cooling and this may have caused the ova to lose viability. However, Chang (1947, 1948a) has shown that rapid cooling from 25° C to 0° C or to 5° C is more detrimental to rabbit ova than is slow cooling, but that the viability of rabbit ova is not affected by rapid or slow cooling between 25° C and 10° C. Chang (1948a) also found that 10° C was the optimum temperature for the storage of rabbit ova. At this temperature some ova (23·6 %) survived for 144 hr. Chang (1948b, c) also stated that the younger the ova the better their chance of survival after storage.

In the present experiments the temperature described as optimum by Averill & Rowson (1959) was used, ova of all stages being subjected to storage for periods of 72 to 120 hr, with slow cooling down to the storage temperature, and yet apparently no ova survived. It has been pointed out in the results that three ewes and one ewe to which ova stored for 72 and 96 hr, respectively, had been transferred, failed to return to oestrus at the normal time. If these failures to return to oestrus had been randomly distributed throughout the three experimental groups, then it would have been clear that the ewes were experiencing silent heats. However the fact that the great preponderance of these failures occurred in the groups in which the ova were stored for the shortest period—a storage period after which Averill & Rowson (1959) had reported success—would suggest that in some of these ewes the ova started to develop normally. This development did not continue for very long and the observation must suggest that if the ova did implant then their capacity for continued normal growth had been destroyed. Another indication suggesting that these ova may have developed, is provided by the fact that all the ova looked perfectly normal on gross examination after storage. Chang (1948c) has recorded that after storage some rabbit ova implanted but resorbed during pregnancy leaving no trace. He also noted that the percentage of ova degenerating after implantation increased from the control level of 8·6 % to 23·3 % after storage under optimum conditions. Chang (1948a) postulated that, for the rabbit ovum, 72 hr storage was the critical time for the differential effect of various storage temperatures to be exerted.

Averill (1958) observed that as the time from the onset of oestrus to recovery of ova increased from 50 hr the percentage of ova recovered dropped markedly. On a much smaller sample of ewes this trend has been confirmed, but in the present experiments percentage recovery in each time group is higher than those reported by Averill (1958). Clark (1934) and Green & Winters (1945) have made observations on the stage of cleavage of the ova in relation to the time elapsing after mating at the end of oestrus. In the present experiments, all timings were related to the onset of oestrus and could in fact be in error by as much as 8 hr either way, though such a large difference would be exceptional. As far as a comparison is possible it would seem that the rate of ovum cleavage recorded in these ewes agrees reasonably with those recorded by Clark (1934)

and Green & Winters (1945), and also with that by Amoroso, Griffiths & Hamilton (1942) in the goat. It is also in agreement with the findings of Hunter *et al.* (1955) who based their timing on the onset of oestrus.

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