THE EFFECT ON COW EMBRYOS OF COOLING TO 20, 0 AND -196°C

I. WILMUT,* C. POLGE AND L. E. A. ROWSON

*Agricultural Research Council Unit of Reproductive Physiology and Biochemistry,
307 Huntingdon Road, Cambridge*

(Received 24th March 1975)

Successful techniques of freezing and thawing have recently been developed for embryos of the mouse (Whittingham *et al.*, 1972; Wilmut, 1972a) and rabbit (Bank & Maurer, 1974; Whittingham & Adams, 1974). The proportion of embryos which survived was very high and normal young have been born for both species. Experiments on freezing and thawing the embryos of farm animals have been more limited, and although some success has been achieved with cow (Wilmut & Rowson, 1973b) and sheep (Willadsen *et al.*, 1974) embryos high survival rates have not yet been obtained.

When 8- to 16-celled cow embryos were subjected to a variety of methods of freezing and thawing similar to those which have been successful in the mouse and rabbit, only 2/111 developed normally when transferred to a rabbit oviduct for 48 to 72 hr (Wilmut & Rowson, 1973a; and unpublished observation). Since pig embryos at this stage were killed when cooled to a temperature of 10°C or below (Wilmut, 1972b), a study was made of the survival of cow embryos at early cleavage stages when cooled to 0°C .

The methods of using prostaglandin F- 2α treatment to control the oestrous cycle of the donor heifers, of inducing multiple ovulation in the donors, and of recovering and transferring the embryos have been described previously (Rowson *et al.*, 1969, 1972). The medium used throughout the experiments was Dulbecco and Vogt's phosphate-buffered salt solution enriched with energy sources and protein (Whittingham, 1971).

In Exp. 1 the survival of 8- to 16-celled embryos (early morulae) and late morulae after cooling to 0°C was compared. Embryos at these stages were recovered on Days 4 and 6 of pregnancy respectively (Day 0 = first day of oestrus) and all embryos which were collected and which were of normal appearance were used. Control embryos were maintained at room temperature ($\sim 20^{\circ}\text{C}$) while treated embryos were cooled to 0°C . Two or three embryos in 0.2 ml phosphate-buffered saline were placed in small glass test tubes (50×6 mm). The embryos were cooled to 0°C at approximately $10^{\circ}\text{C}/\text{min}$ by transferring the tubes to a bath of crushed ice. After 15 min the embryos were recovered and rinsed in fresh medium at 20°C . Viability of the embryos was assessed by transfer to ligated oviducts of oestrous rabbits for either 72 hr (early morulae) or 24 hr (late morulae). The embryos recovered from the rabbit

* Present address: Animal Breeding Research Organisation, West Mains Road, Edinburgh EH9 3JQ.

oviducts were mounted, fixed in 25% acetic ethanol and stained with aceto-orcein. Only 1/19 early morulae developed to the blastocyst stage after being cooled to 0°C, whereas 8/11 developed after being stored at 20°C ($\chi^2 = 12.06$, $P < 0.001$). In contrast, 7/9 late morulae which had been cooled to 0°C developed to blastocysts as did all three late morulae which had been left at 20°C, although there were more cells with pyknotic nuclei in the blastocysts which had developed from cooled morulae than in control blastocysts (3.3 to 19.3% versus 0.0 to 3.2%, respectively). There were cells at apparently normal metaphase in all blastocysts.

In Exp. 2, a comparison was made of the ability to develop to calves of late morulae which had and had not been cooled to 0°C. Twelve late morulae were transferred in pairs to six non-pregnant recipients whose oestrous cycles were synchronized with those of the donors. One morula transferred to each recipient had been cooled to 0°C. In three recipients the cooled morula was placed in the uterine horn adjacent to the ovary which had a CL, and in the other three the control morula was placed in that horn. Three recipients did not return to oestrus and were examined by rectal palpation during the 7th week of pregnancy to determine the site of any implantation. These heifers subsequently gave birth to four normal calves, two of which had developed from control morulae and two from cooled morulae. The origin of the calves was known with some certainty since the incidence of transuterine migration of embryos in the cow is exceedingly low (Rowson *et al.*, 1971; Scanlon, 1972).

In Exp. 3, 42 late morulae were frozen and thawed by a method developed for the low temperature preservation of mouse blastocysts (Wilmut, 1972a). The concentration of dimethylsulphoxide (DMSO) was increased to 1.5 M in three equal steps, at 10-min intervals at 20°C. The methods of inducing ice formation, freezing at 0.2°C/min and thawing at 12°C/min were as described previously (Wilmut, 1972a). Some embryos were also thawed at about 4°C/min by placing the tube in which they were frozen into a larger glass tube (16 × 125 mm) containing 15 ml ethanol at -80°C, which was then allowed to warm in air at room temperature. The DMSO was removed in either three or six steps before the embryos were transferred to ligated rabbit oviducts and in some instances the embryos were transferred directly after thawing. Twenty-six embryos were recovered 1 day later and two had developed to normal blastocysts (Table 1). Some cells were morphologically normal in three other embryos which had not developed normally.

These experiments suggest that most early morulae of cows are killed by being cooled relatively rapidly to 0°C. It is not known at which temperature the embryos are killed, or whether the extent of the damage is dependent upon the rate of cooling. By contrast, most of the late morulae used appeared to survive cooling to 0°C, although even in these embryos a small proportion of the cells had been killed. The proportion of late morulae which developed to term when transferred to non-pregnant recipients after cooling to 0°C was very low, but was the same as that of control morulae which had been stored at 20°C.

Although the number of embryos examined in these experiments was quite small, these observations suggest that there is variation between species and

Table 1. The effect on cow morulae of different methods of removing DMSO after freezing and thawing

	Dilution		
	None (direct to rabbit)	Three steps	Six steps
No. of embryos recovered	14	7	5
No. in which some cells survived	1	—	2
No. of expanded blastocysts	—	1*	1†

* Thawed at 12°C/min.

† Thawed at 4°C/min.

variation between the stages of development of the embryos within a species in their sensitivity to cooling to 0°C. These effects may be critical in the development of satisfactory methods for the storage of embryos at very low temperatures.

Further experiments are necessary to discover whether or not the proportion of morulae which survive freezing and thawing can be increased by changes in the rate of cooling or of warming, or in the cryoprotective agent and the method of its addition. Similarly, embryos at later stages of development should be studied further as two calves have been born after being frozen and stored in liquid nitrogen at the time when they were late blastocysts (Wilmot & Rowson, 1973b).

One of us (I.W.) gratefully acknowledges receipt of a Fellowship from the Milk Marketing Board of England and Wales.

REFERENCES

- BANK, H. & MAURER, R.R. (1974) Survival of frozen rabbit embryos. *Expl. Cell Res.* **89**, 188–196.
- ROWSON, L.E.A., MOOR, R.M. & LAWSON, R.A.S. (1969) Fertility following egg transfer in the cow; effect of method, medium and synchronization of oestrus. *J. Reprod. Fert.* **18**, 517–523.
- ROWSON, L.E.A., LAWSON, R.A.S. & MOOR, R.M. (1971) Production of twins in cattle by egg transfer. *J. Reprod. Fert.* **25**, 261–268.
- ROWSON, L.E.A., TERVIT, R. & BRAND, A. (1972) The use of prostaglandins for synchronization of oestrus in cattle. *J. Reprod. Fert.* **29**, 145.
- SCANLON, P.F. (1972) Frequency of transuterine migration of embryos in ewes and cows. *J. Anim. Sci.* **34**, 791–794.
- WHITTINGHAM, D.G. (1971) Survival of mouse embryos after freezing and thawing. *Nature, Lond.* **233**, 125–126.
- WHITTINGHAM, D.G. & ADAMS, C.E. (1974) Low temperature preservation of rabbit embryos. *Cryobiology* **11**, 560–561.
- WHITTINGHAM, D.G., LEIBO, S.P. & MAZUR, P. (1972) Survival of mouse embryos frozen to –196°C and –269°C. *Science, N.Y.* **178**, 411–414.
- WILLADSEN, S.M., POLGE, C., ROWSON, L.E.A. & MOOR, R.M. (1974) Preservation of sheep embryos in liquid nitrogen. *Cryobiology* **11**, 560.
- WILMUT, I. (1972a) The effect of cooling rate, warming rate, cryoprotective agent and stage of development on survival of mouse embryos during freezing and thawing. *Life Sci.* **11**, 1071–1079.
- WILMUT, I. (1972b) The low-temperature preservation of mammalian embryos. *J. Reprod. Fert.* **31**, 513–514.
- WILMUT, I. & ROWSON, L.E.A. (1973a) The successful low-temperature preservation of mouse and cow embryos. *J. Reprod. Fert.* **33**, 352–353.
- WILMUT, I. & ROWSON, L.E.A. (1973b) Experiments on the low-temperature preservation of cow embryos. *Vet. Rec.* **92**, 686–690.