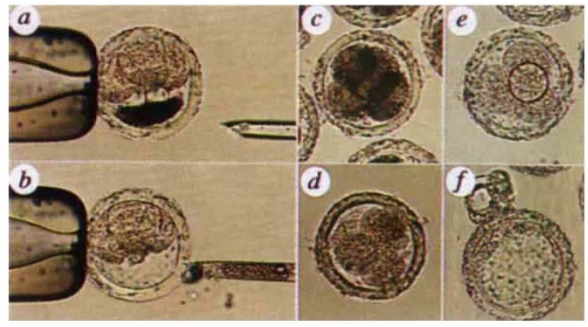
Cryopreservation of porcine embryos

SIR — Cryopreservation of mammalian embryos has important implications for the long-term storage, propagation and transport of valuable genotypes of agricultural or zoological interest, and also for *in vitro* fertilization programmes in humans. The birth of live offspring from embryos frozen in liquid nitrogen, however, is still limited[1–7] and absence of effective embryo cryopreservation technologies for major livestock species such as the pig[8] and many endangered species may result in a major loss of genetic diversity. The inability of the technique in porcine early cleavage stage embryos is related to their extreme sensitivity to low temperatures[9], and development of a method for cryopreservation may provide useful insights into some of the factors responsible for cell injury caused by freeze–thawing of mammalian embryos. Here we report that early-cleavage-stage porcine embryos survive cryopreservation in liquid nitrogen following removal of their cytoplasmic lipid droplets, and that normal offspring can be obtained from the lipid-depleted embryos after cryopreservation.

Porcine 2- to 4-cell stage embryos were centrifuged at 12,500*g* for 8 min in the presence of cytoskeletal inhibitor (7.5 μg ml^{-1} cytochalasin B) to polarize cytoplasmic lipid droplets within the cells. The resultant lipid layer was then removed (delipated) by micromanipulation using a fine suction pipette under an inverted microscope[8] (see figure). After lipid removal, delipated embryos were frozen within 2 h at the 2- to 4-cell stage or cultured *in vitro* for 14 to 18 h to the 4- to 8-cell stage and then frozen using a conventional slow cooling method in the presence of 1.5 M 1, 2-propanediol and 0.1 M sucrose. Control intact embryos at an identical stage of development were frozen concurrently. After storage in liquid nitrogen, post-thaw survival was assessed by *in vitro* culture for 96–120 h or by transferring those embryos to surrogate mothers. The developmental ability of unfrozen delipated embryos was also examined by *in vitro* culture and transfer. Procedures for the collection, micromanipulation and culture of embryos have been described in detail elsewhere[8].



Cryopreservation of low-temperature-sensitive porcine embryos after removal of cytoplasmic lipid. Four-cell stage embryos with polarized lipid droplets *a*, after centrifugation and *b*, after aspiration of the polarized lipid by micromanipulation. Note that the cytoplasm of delipated embryo (*d*) is clearer in colour compared with that of intact 4-cell stage embryo in *c*. *e*, *f*, A delipated 4-cell-stage embryo maintaining pre-freeze morphology after thawing and a blastocyst developed from a cryopreserved delipated embryo after culture for 96 h (see table).

The results show that removal of cytoplasmic lipid allows porcine embryos to be cryopreserved without compromising their developmental competence (see table). More than half of the delipated embryos survived cryopreservation, whether they had been frozen immediately after lipid removal or following *in vitro* culture. In contrast, none of the control embryos survived. Transfer of unfrozen delipated embryos resulted in the birth of piglets 113 days after transfer. Piglets were also obtained from cryopreserved delipated embryos which had been frozen immediately after lipid removal, 114 days after transfer.

Piglets born from the unfrozen and frozen–thawed delipated embryos were normal in appearance and had birth weights within the normal range for this breed (700–1,500 g). Animals grew normally as assessed by growth rates and phenotype and no anatomical abnormalities were seen by macroscopic examination after weaning at 4 weeks of age.

The present experiments clearly demonstrate that porcine early cleavage-stage embryos survive cryopreservation following removal of cytoplasmic lipid. The inability to collect sufficient embryos to carry out quantitative experiments for optimizing freeze–thawing conditions according to the species is the major factor responsible for the absence of procedures for cryopreservation of embryos of scarce and endangered species. The method we have developed for early-cleavage-stage porcine embryos represents a new approach to cryopreservation. Our experiments may present opportunities for the cryopreservation of oocytes and early-stage embryos from animals, including several endangered species that contain large amounts of cytoplasmic lipid.

IN VITRO AND *IN VIVO* DEVELOPMENT OF FROZEN–THAWED AND UNFROZEN PORCINE DELIPATED EMBRYOS

Embryos	Development *in vitro*		Development *in vivo*		
	Embryos cleaved	Embryos developed to blastocyst	Embryos transferred	Pregnant recipients total	Piglets farrowed
Delipated/unfrozen	25/27 (92.6) $P>0.001$	21/27 (77.8) $P>0.001$	66	1/2	10*
Delipated/frozen immediately at 2–4-cell stage	20/36 (55.6)	11/36 (30.6)	39	1/1	3
Control/frozen at 2–4-cell stage	0/12 (0)	0/12 (0)	–	–	–
Delipated/frozen after culture at 4–8-cell stage	58/64 (90.6) $P>0.001$	41/64 (64.1) $P>0.005$	142	2/3†	0
Control/frozen at 4–8-cell stage	0/15 (0)	0/15 (0)	–	–	–

Numbers in parentheses are percentages. *Eight live and two stillborn piglets (30.3%) were obtained from 33 embryos transferred. †Diagnosed by ultrasonography on 52 and 59 days of gestation. Unfrozen delipated embryos were cultured for 14–18 h before transfer. Cryopreserved delipated embryos were transferred to recipients within 2 h after thawing (39–52 per recipient). Embryos were transferred to recipient gilts 2 days after onset of oestrus.

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