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Brief Communication

Vitrification of Porcine Embryos using the Open Pulled Straw (OPS) Method

Cryopreservation of porcine embryos by traditional slow-rate freezing results in limited survival as a consequence of the high amount of cytoplasmic lipid droplets, which suffer irreversible damage at temperatures between +10 and –5 °C (*Nagashima et al.* 1994, *Pollard & Leibo* 1994). Two methods may circumvent this problem: Firstly, removal of the lipid droplets by centrifugation and micromanipulation (*Nagashima et al.* 1994, 1995). This improves survival rates, but the method is rather complicated and not applicable for routine use. Secondly, certain vitrification methods have been successfully applied to cryopreserve porcine blastocysts and, with a limited efficiency, morula stage embryos (*Yoshino et al.* 1993, *Kobayashi et al.* 1994, *Kuwayama et al.* 1997).

Recently we have reported that a new vitrification technology, the Open Pulled Straw (OPS) method, would allow satisfactory survival rates of early bovine embryos (*Vajta et al.* 1997). Cryopreservation of cattle embryos of these stages has either been unresolved to date (*Leibo et al.* 1996) or required previous delipation by micromanipulation (*Ushijama et al.* 1996). The purpose of our present work was to investigate the possibility of using the OPS method to cryopreserve *in vivo* produced porcine embryos from the 4-8 cell through to the expanded blastocyst stage.

Danish Landrace sows were synchronized and inseminated according to our earlier report (*Kuwayama et al.* 1997). The day of insemination was regarded Day 0. Two, 3 and 2 sows were slaughtered on Day 2, 4 and 7, respectively. Within 30 min after slaughter, the embryos were flushed from the uterine horns using modified PBS containing 1% calf serum (Danish Veterinary Laboratory, Frederiksberg, Denmark). Embryos were cultured for 2 to 48 h in synthetic oviduct fluid (SOF) medium supplemented with non-essential and essential amino acids, sodium citrate (*Holm et al.* 1997) and 2% calf serum at 38.5°C under 5% CO_2, 5% O_2, 90% N_2 and maximum humidity.

Embryos were evaluated under stereomicroscope and those with good morphology and at appropriate stage of development were subjected to vitrification using the OPS method (*Vajta et al.* 1997). Briefly, French mini straws were heat-softened and pulled manually to reduce both the outer and inner diameter to approximately half of the original one; then they were cut at the narrowest point. All work was performed on a 39 °C heated microscope stage and at 22-23 °C room temperature. Embryos (2 to 4 in each group) were initially equilibrated in 7.5% ethylene glycol and 7.5% dimethyl sulfoxide dissolved in holding medium (TCM-199 and 20% calf serum) for 3 min, then in 18% ethylene glycol, 18% dimethyl sulfoxide and 0.6 M sucrose for approximately 30 s. Loading occurred as a result of the capillary effect by placing the narrow end of the straw into the me-

Table 1. Survival and hatching rates of porcine embryos vitrified at various developmental stages. Means within a column with different superscripts differ ($p<0.05$).

Stage of development at vitrification	Blastocyst or re-expanded after thawing and culture	Hatching
4- 8 cell stage	7/12 (58%)[a]	4/12 (33%)[a]
8-16 cell stage	1/24 (4%)[b]	0/24 (0%)[b]
Compacted morula	10/10 (100%)[c]	7/10 (70%)[c]
Early blastocyst	11/14 (79%)[a,c]	8/14 (57%)[a,c]
Blastocyst	13/15 (87%)[a,c]	11/15 (73%)[c]
Expanded blastocyst	14/14 (100%)[c]	not observed
Total (from compacted morula stage)	48/53 (91%)[c]	26/39 (67%)[c]

dium. Subsequently, the straws were immediately submerged vertically into liquid nitrogen. At warming, straws were immersed vertically into 1.2 ml holding medium containing 0.2 M sucrose. The vitrification medium became liquid within 1 s, whereupon the holding medium entered the straw. Immediately afterwards, the embryos gradually slided out of the straw and into the medium. One minute after warming, the embryos were transferred into 1.2 ml holding medium with 0.1 M sucrose, then 2×5 min into 1.2 ml holding medium. Finally, the embryos were placed into the original culture dish and cultured again *in vitro* as described earlier until Day 9 after the insemination. Evaluation of survival (blastocyst formation or re-expansion of the blastocoele) and hatching from the zona pellucida was performed under a stereomicroscope (magnification = ×50) at Days 7, 8 and 9 after the insemination. Embryos vitrified at the expanded blastocyst stage were fixed 24 h after warming and the cell number was counted under fluorescence light microscopy using Hoechst 44972 staining. Statistical analysis of survival/hatching was performed using the Chi-square test.

A total of 89 porcine embryos from 7 sows were subjected to vitrification. Their developmental stages at vitrification, survival and hatching rates are listed in Table 1. In each group, embryos originated from 2 or more sows were included. No major differences in the same developmental group between the survival rates of embryos of different sows were observed. From the 24 embryos vitrified at the 8-16 cell stage, only one developed to blastocyst; however, 58% of the embryos vitrified at the 4-8 cell stage developed to blastocysts and 57% of these hatched. Although the low survival rates of 8-16 cell stage embryos require further explanation, this is the first documented survival of pre-compacted porcine embryos after vitrification. The cumulative survival rate of embryos at or after the compacted morula stage was 91%, and the cumulative hatching rate of embryos vitrified at the compacted morula, early blastocyst and blastocyst stage was 67%. Survival and hatching rates did not differ significantly between embryos vitrified at the compacted morula and more advanced developmental stages. At 24 h after warming the surviving embryos did not show any sign of damage (Fig. 1). The average cell number of 14 embryos vitrified at the expanded blastocyst stage and fixed 24 h after warming was 114 ± 13 (mean ± SD). In conclusion, the OPS method has proven to be suitable to cryopreserve in vivo produced porcine preimplantation stage embryos. The method is simple and repeatable, it does not require special equipment or skill and it may be

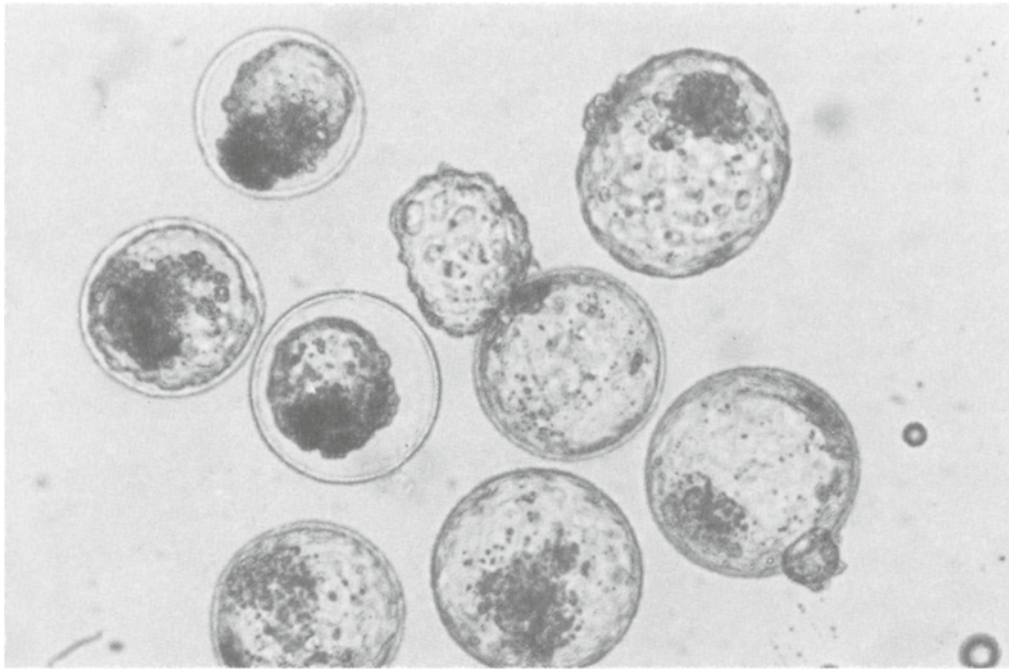

Figure 1. Porcine embryos vitrified at expanded blastocyst stage, 24 h after warming. Inverted microscopic picture, × 100.

performed also under field conditions. In contrast to the previous methods (*Kobayashi et al.* 1994, *Kuwayama et al.* 1997), survival and developmental rates did not differ between embryos cryopreserved at the compacted morula, early blastocyst, blastocyst and expanded blastocyst stages, and also 4-8 cell stage embryos were successfully vitrified. If embryo transfer will result in pregnancies and birth of piglets, the OPS method may become a decisive step to resolve the problem of porcine embryo cryopreservation.

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