Changes in Freezing Tolerance of Pig Blastocysts in Peri-Hatching Stage

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(Accepted for publication April 25, 1989)

Summary. Freezing tolerance of pig embryos at various peri-hatching stages was investigated. Survival of expanded blastocysts, hatching blastocysts and hatched blastocysts, as well as earlier staged embryos, i.e. morulae and blastocysts as control, was evaluated by culturing after they were frozen at −20°C with 1.5 M DMSO. Although none of the early staged embryos survived after freezing, 16 (48.5%) of 33 expanded blastocysts, 13 (56.5%) of 23 hatching blastocysts and 26 (47.3%) of 55 hatched blastocysts showed development after freezing and thawing. The highest survival rate (25/30 , 83.3%) was obtained by the freezing of the hatched blastocysts which had developed in vitro from the expanded blastocysts. These results indicate that the expanded blastocysts of pig is at a critical stage where the freezing tolerance increases, and that the hatched blastocyst is tolerant of freezing.

KEY WORDS: PIG, BLASTOCYST, FREEZING TOLERANCE.


There have been no documented reports on freezing preservation of pig embryos at ultra-low temperature, i.e. −80 or −196°C. Although information on freezing of pig embryos reported so far was limited (Wilmot, 1972; Polge et al., 1974; Polge, 1977), differences in the stages of embryonic development that tolerate exposure to low temperature have been suggested (Polge, 1977; Nagashima et al., 1988). To develop a method for freezing preservation of pig embryos, the low temperature sensitivity of pig embryos or embryonic cells must be investigated in detail. If an embryonic stage of high tolerance to freezing could be detected, it would lead us to a clue of this subject.

In the present study, freezing of pig embryos at various peri-hatching stages was carried out, on the basis of our previous report that demonstrated the superior tolerance of hatched blastocysts to low temperature (Nagashima et al., 1988). Survival of the pig blastocysts after freezing at −20°C was examined by in vitro culture, aiming at exploring a tolerant stage to freezing, and the differences between embryonic stages in the freezing tolerance was also examined.

Materials and Methods

Experimental animals and embryo collection

Landrace x Large White F₁ gilts, aged 8 to 11 months and weighing between 90 and 130 kg, were used as embryo donors. The donors were mated to Duroc boars usually twice during an estrous period at an interval of approximately 24 hr. Embryo collection was carried out by laparotomy under general anesthesia with ketamine HCl (Ketalar, Sankyo) and halothane (FlOUTHANE, Takeda). Embryos were collected by flushing the exposed uterine horns with Dulbecco’s phosphate buffered saline (PBS) supplemented with 1%
heat-inactivated fetal calf serum (FCS). Morulae, early blastocysts, expanded blastocysts and hatched blastocysts were collected on Day 4 to 7 (Day 0 = onset of estrus). The embryos collected were evaluated for the developmental stage and morphological normalities by phase-contrast microscopy at ×100.

**Culture of embryos**

*In vitro* culture of embryos before and after freezing was performed as follows. The embryos were cultured in droplets of the mixed medium composed of an equivalent volume of Whittingham's medium (M-16, Whittingham, 1971) supplemented with 12 mg/ml bovine serum albumine (BSA) and PBS supplemented with 16% FCS. This medium was overlaid with paraffin oil, and held in a humidified atmosphere of 5% CO₂, in air at 37°C.

**Embryos subjected to freezing**

Blastocysts at various peri-hatching stages were subjected to freezing with the control earlier staged embryos, namely blastocysts and morulae. Hatched blastocysts (HB) collected on Day 6 or 7, expanded blastocysts (EXB; Day 6), blastocysts (B; Day 5) and morulae (M; Day 4) were frozen within 1 hr after the collection. In addition to these embryos, peri-hatching staged blastocysts were obtained by culturing the collected embryos for 20 to 24 hr and they were also subjected to freezing. The embryos obtained after cultivation were as follows: Largely expanded hatched blastocysts were designated "cultured-HB". Hatched blastocysts and hatching blastocysts, which were designated "vitro-HB" and "vitro-HgB", were obtained by culturing the expanded blastocysts. Expanded blastocysts developed *in vitro* from the late morulae or early blastocysts, and blastocysts developed from morulae were also produced. These two kinds of embryos were designated "vitro-EXB" and "vitro-B", respectively.

**Procedures for freezing and thawing**

Addition of dimethylsulfoxide (DMSO) was achieved by transferring the embryos stepwise at an interval of 10 min through a gradient of 0.5 M, 1 M and 1.5 M DMSO diluted with PBS+16% FCS at room temperature (22–25°C). Groups of 5–20 embryos were aspirated into 0.25 ml plastic straws with 1.5 M DMSO solution and equilibrated for 10 min at 20°C. The embryos were then cooled by a programmable freezer (Hoxan, Cryoembryo HP) from 20°C to −5°C at a rate of 1°C/min and seeded at −5°C, followed by a keeping time for 7 min at −5°C. After the ice seeding, the embryos were cooled to −20°C at a rate of 0.5°C/min and kept at −20°C for 3 min. The embryos were thawed rapidly by immersing the straws in a water bath kept at 25°C for 30 sec. The cryoprotectant was removed from the recovered embryos by transferring the embryos stepwise at intervals of 5 min through a gradient of 1 M, 0.5 M and 0.25 M sucrose solution (in PBS+16%FCS) at room temperature. The embryos were then washed through three changes of culture medium.

**Survival assay**

The survival of embryos frozen at −20°C was assessed by testing the ability to develop in culture for up to 48 hr. The blastocysts which expanded or maintained the blastocoel cavity in culture were evaluated as surviving. The differences in the survival rates of embryos were analysed by χ²-test.

**Results and Discussion**

As the category of post-hatching staged blastocysts, a total of 59 cultured-HB, 55 HB, 32 vitro-HB, 38 vitro-EXB and 38 vitro-B were evaluated. The survival rate of embryos frozen at −20°C and thawed was significantly lower than that of non-frozen embryos. The differences between a & b (P<0.05), a & c (P<0.001), a & d (P<0.05), a & e (P<0.05), a & f (P<0.001) and b & f (P<0.01) were significant. * Refer to text for the abbreviations.

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Figs. 1–6. Specimens of the frozen-thawed peri-hatching staged blastocysts after culture for 24 hr. Fig. 1. cultured-HB; Fig. 2. HB; Fig. 3. vitro-HB; Fig. 4. vitro-HgB; Fig. 5. EXB; Fig. 6. B & M (Refer to Materials and Methods for the abbreviations)
HB and 23 vitro-HgB were frozen and thawed. Recovery rates of the frozen-thawed embryos were 93%–100% (Table 1) in these specimens. At recovery, all of the frozen-thawed blastocysts were shrunk to various degrees. Survival of the recovered embryos was summarized in Table 1. Thirty-six (62.1%) of 58 cultured-HB, 26 (47.3%) of 55 HB, 25 (83.3%) of 30 vitro-HB and 13 (56.5%) of 23 vitro-HgB were assessed as surviving, on the basis of noticeable expansion or maintenance of their blastocoele cavity in culture for 48 hr. Figs. 1–4 show the specimens of the surviving embryos. As shown in the figures, signs of damage, e.g. debris of degenerated cells and/or cell darkening were observed in a number of embryos which had been assessed as surviving.

As the category of pre-hatching staged embryos, 33 EXB, 57 vitro-EXB, 33 vitro-B, 25 B and 21 M were subjected to freezing. Of the EXB and vitro-EXB recovered after freezing and thawing, 16 (48.5%) EXB (Fig. 5) and 21 (36.8%) vitro-EXB were evaluated as surviving, respectively. On the other hand, none of the vitro-B, B and M survived the freezing at −20°C (Table 1).

These results clearly demonstrate that the expanded blastocyst stage is a critical stage at which the freezing tolerance of pig embryos arises. Compared with the expanded blastocysts, the hatched blastocysts seemed to be equally or more tolerant of freezing. The vitro-HB, which obtained by culturing the expanded blastocysts for about 24 hr, showed higher tolerance than the other specimens of the post-hatching staged blastocysts, i.e. vitro-HgB (P<0.05), cultured-HB (P<0.05) and HB (P<0.001). The vitro-HB thus showed superior tolerance, although it is generally known that a long period of in vitro culture prior to freezing results in decreasing the viability of embryos. This fact suggested that the blastocysts in this case progressed in a further tolerant stage during the culture period before freezing, in other words, the hatched blastocyst stage is an embryonic stage with high freezing tolerance. It might be speculated that transition of the state of development or differentiation of embryonic cells accompany with the change of the low temperature sensitivity of pig embryos.

In the practical aspect of pig embryo transfer, to choose the hatched blastocyst as a material of freezing preservation involves a disadvantage resulting from lack of the zona pellucida which is a barrier to disease pathogens (Singh, 1987). In this regard, the embryos in pre-hatching stages should be used for freezing. However, the early staged blastocysts and morulae were extremely sensitive to freezing, while the hatched blastocysts were tolerant. This data might indicate that the freezing tolerance of pig embryos depends on the embryonic development in the early development of pig embryos. If there was a stage dependent increasing of the freezing tolerance of pig embryos in the peri-hatching stage, the expanded blastocysts immediately before hatching, namely the blastocysts at the most advanced pre-hatching stage, might be one of the stage to be chosen.

As for the freezing tolerance, however, the expanded blastocyst is not at the best stage for freezing. For freezing preservation of pig embryos, it requires to choose more strictly the embryonic stage than other species in which the technique for freezing embryos has been well established. To overcome this, a method to increase the tolerance of pig embryos prior to freezing must be devised. The result of this study: the vitro-HB that is the pre-cultured blastocysts has eminent freezing tolerance, seems to imply a strategy that effect a change of freezing tolerance of pig embryonic cells.

References


透明帯脱出期前後における豚胚発生の凍結耐性の変化

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透明帯脱出期前後の発達段階にある豚胚発生を用い、豚胚の凍結に対する耐性について調べた。拡張胚
発生、透明帯脱出後の胚発生、透明帯脱出胚発生および対照の検査胚、胚発生を1.5 M DMSO 存在で-20℃
に凍結し、融解後の生存性を培養により判定した。凍結
・融解の結果、拡張胚発生33例中16例(48.5%)、透明帯
脱出以後胚発生23例中13例(56.5%)および透明帯脱出胚
発生55例中26例(47.3%)の生存例が得られた。これに対
し、対照胚すなわち、より初期の胚では全く生存例が得
られなかった。さらに、拡張胚発生を培養して得られた
透明帯脱出胚発生32例を凍結・融解したところ、高い生
存率(83.3%)が認められた。以上の結果より、豚胚の凍
結に対する耐性は、透明帯脱出期前後において拡張胚発
生を境として生じる事、および透明帯脱出胚発生が凍
結耐性に優れていることが示された。