Survival of Pig Hatched Blastocysts exposed below 15°C

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Summary. Pig hatched blastocysts, especially those which had been developed *in vitro* from expanding blastocysts, had a higher tolerance to cooling compared with pre-hatching stage embryos. The blastocysts which underwent hatching *in vivo* (vivo-HB) and *in vitro* (vitro-HB) were subjected to cooling compared with control pre-hatching stage embryos: expanding blastocysts, zona-removed blastocysts, early blastocysts and morulae. The embryos suspended in Dulbecco's PBS supplemented with 16% fetal calf serum were cooled in 0.25 ml plastic straws to 6°C for 1 hr or 16 hr. Survival of the embryos were assessed by testing the ability to develop in culture for 24 hr. All of the 26 (100%) vitro-HB and 51 of 65 (78.5%) vivo-HB, which had been exposed to 6°C for 1 hr, were survived. When the length of exposure to 6°C was prolonged from 1 hr to 16 hr, a remarkable difference ($p < 0.05$) of the survival rate was found between the vitro-HB (20/21, 95.2%) and vivo-HB (22/41, 53.7%). This suggests that the vitro-HB were more tolerant of the cooling than the vivo-HB. The survival rate of the expanding blastocysts was significantly lower than that of the vitro-HB ($p < 0.001$) and vivo-HB ($p < 0.05$). None of the other control embryos showed any signs of survival, except for one small vesicle derived from one of the 15 zona-removed blastocysts.

Transfer of 18 vitro-HB, which had been cooled to 11°C, resulted in a normal fetus development, but none of the 22 morulae and 18 early blastocysts could survive this short exposure. **KEY WORDS:** PIG, EMBRYO, HATCHED BLASTOCYST, COOLING.

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After the successful freezing preservation of mouse embryos in 1972 (Whittingham *et al.*, 1972; Wilmut, 1972), this technology has been applied to many species of experimental animals (Bank & Maurer, 1974; Whittingham, 1975), domestic animals (Willadsen *et al.*, 1974, 1976; Wilmut & Rowson, 1973; Bilton & Moore, 1976) and humans (Trounson & Mohr, 1983). However, only the pig has been exceptional in this regard: pig embryos are extremely sensitive to low temperature. Although the causal reason for this intolerance is not clear, it has been thought to be associated with the lipid composition of the cell membrane (Polge, 1977). The early experimental cooling of pig embryos (Wilmut, 1972; Polge *et al.*, 1974) showed that the critical temperature

at which damage occurs is around 15°C. Attempt to cool pig embryos to 10°C employing cryoprotectant known to be effective to embryos of other species was unsuccessful (Niemann, 1985). To increase the resistance of pig embryos to the cooling, it seems necessary to induce a change of the nature of embryonic cells by some chemical treatment or other means. At present, an advanced understanding of the low temperature sensitivity of pig embryos is needed.

In the present study, we attempted to preserve pig embryos below 15°C using later-stage blastocysts, namely, hatched blastocysts that developed *in vitro* or *in vivo*. Here we report that 1) there is a remarkable difference of the tolerance to low temperature between the hatched blastocysts and

pre-hatching stage embryos, and 2) the blastocysts developed to hatching stage in culture are remarkably tolerant of the cooling and survive the exposure below 15°C.

Materials and Methods

1. Experimental animals and embryo collection

Landrace x Large White F1 gilts, aged 8 to 11 month and weighing between 90 and 130 kg, were used as embryo donors and recipients. They were tested daily for estrus with a boar. The donors were mated to Duroc boars of proven fertility, usually twice during estrus at intervals of approximately 24 hours. Embryo collection was carried out by laparotomy under general anesthesia with retamine 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 and expanding blastocysts were collected on Day 3, 4 and 5 respectively (Day 1 being the day estrus ended). Hatched blastocysts were collected on Day 5 and 6.

Embryos were evaluated for the developmental stage and morphological abnormalities by phase-contrast microscopy at $\times 100$.

2. Culture of embryos

Embryos were cultured before and after cooling as follows. The embryos were transferred 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 albumin (BSA) and PBS supplemented with 16% FCS. This medium was placed in a plastic petri dish (Lux, 5221-R), overlaid with paraffin oil, and held in a humidified atmosphere of 5% CO₂ in air at 37°C.

3. Embryos subjected to cooling

The following embryos were subjected to the cooling: 1) Hatched blastocysts which developed *in vitro* from Day 5 expanding blastocysts. These embryos were designated "*in vitro*-hatched blastocysts" (Fig. 1 & 7). 2) Hatched blastocysts collected on Day 5 and 6, which were designated "*in vivo*-hatched blastocysts" (Fig. 4 & 10). 3) Expanding blastocysts collected on Day 5 (Fig. 13). 4) Morulae collected on Day 3, and early blastocysts collected on Day 4. The zonae pellucidae of some embryos were digested by incubation in 0.5% pronase for about 1 min at room temperature, followed by thorough washing with culture medium.

Table 1. Survival of pig embryos *in vitro* after exposure to 6°C

Specimens	Period of exposure to 6°C	No. of experiments	No. of embryos cooled	No. of embryos recovered	No. of embryos survived
<i>in vitro</i> -hatched blastocyst	1 hr	n=3	26	26	26(100%) ^a
	16 hr	n=3	21	21	20(95.2%) ^b
<i>in vivo</i> -hatched blastocyst	1 hr	n=6	65	65	51(78.5%) ^c
	16 hr	n=3	41	41	22(53.7%) ^d
expanding blastocyst	1 hr	n=4	32	32	11(34.4%) ^e
zona-removed blastocyst	1 hr	n=2	15	15	1(6.7%)
morula & early blastocyst	1 hr	n=4	37	37	0(0%)

The differences between a&c ($p<0.05$) b & d ($p<0.05$), a & e ($p<0.001$) and c & e ($p<0.05$) was significant.

Fig. 1: *In vitro*-hatched blastocysts before cooling. Fig. 2: *In vitro*-hatched blastocysts at recovery from exposure to 6°C for 1 hr. Fig. 3: Specimens of the *in vitro*-hatched blastocysts showing noticeable development in culture for 24 hr after the exposure to 6°C for 1 hr. Fig. 4: *In vivo*-hatched blastocysts before cooling. Fig. 5: *In vivo*-hatched blastocysts at recovery from exposure to 6°C for 1 hr. Fig. 6: Specimens of the *in vivo*-hatched blastocysts showing noticeable expansion in culture for 24 hr after the exposure to 6°C for 1 hr. (Figs. 1-6: $\times 100$)

Fig. 7: *In vitro*-hatched blastocysts before cooling. **Fig. 8:** *In vitro*-hatched blastocysts at recovery from exposure to 6°C for 16 hr. **Fig. 9:** Specimens of the *in vitro*-hatched blastocysts showing noticeable expansion in culture for 24 hr after the exposure to 6°C for 16 hr. **Fig. 10:** *In vivo*-hatched blastocysts before cooling. **Fig. 11:** *In vivo*-hatched blastocysts at recovery from exposure to 6°C for 16 hr. **Fig. 12:** Specimens of the *in vivo*-hatched blastocysts showing signs of survival in culture for 24 hr after the exposure to 6°C for 16 hr; note the considerable cell damage. (Figs. 7-12: $\times 100$)

Fig. 13: Expanding blastocysts before cooling. **Fig. 14:** Expanding blastocysts at recovery from exposure to 6°C for 1 hr. **Fig. 15:** Specimens of the expanding blastocysts showing signs of survival after exposure to 6°C for 1 hr. **Fig. 16:** Zona-removed blastocysts at recovery from exposure to 6°C for 1 hr. **Fig. 17:** Zona-removed blastocysts that completely collapsed in culture after exposure to 6°C for 1 hr. **Fig. 18 & 19:** Morulae and early blastocysts that completely collapsed in culture after exposure to 6°C for 1 hr. (Figs. 13-19: 100×)

Fig. 20: A pig Day 27 fetus obtained by transplantation of the *in vitro*-hatched blastocysts cooled to 11°C.

4. Cooling procedures

Five to 15 embryos were aspirated into a 0.25-ml plastic straw with about 0.15 ml of PBS supplemented with 16% FCS which was kept at 30°C. The straw was sealed with gelatin and placed in a stoppered plastic test tube (Falcon, #2045) and then transferred into a low temperature incubator set at 6°C, so that samples were cooled gradually to 6°C in approximately 18 min. The cooling rate, monitored by a copper-constantan thermocouple placed in a control straw, was as follows: the samples were cooled from the initial temperature (30°C) to 20°C at 5°C/min, from 20°C to 15°C at 3.3°C/min, from 15°C to 10°C at 1.3°C/min and from 10°C to 6°C at 0.4°C/min. The samples were then held at 6°C for 1 hr or 16 hr (over night).

The cooling of embryos for transfer test was confined with short periods of exposure below 15°C. The embryos were cooled to 11°C in the same manner as the embryos employed for *in vitro* survival assay and then warmed. The exposure period below 15°C was 3 min.

5. Recovery of embryos

The straws were transferred to a 37°C incubator and held for 30 min. The contents of each straw were emptied into a plastic petri dish containing about 1 ml of PBS supplemented with

16%FCS at room temperature and the recovered embryos were washed once with the same medium. To examine the morphology, the embryos were observed under an inverted microscope ($\times 100$).

6. Survival assay

In vitro. The survival of embryos exposed to 6°C for 1 or 16 hr was assessed by testing the ability to develop in culture for 24 hr. The condition of culture was described above. Survival of the *in vitro*-hatched and *in vivo*-hatched blastocysts was defined as follows: (1) regeneration of the blastocoele of the blastocysts which had been shrinking at recovery, (2) expanding or maintaining of blastocoele of the blastocysts which had been recovered without shrinkage.

The differences in the survival rates of embryos amongst the experimental groups were analyzed by t-test after arc sin transformation.

In vivo. The viability of the *in vitro*-hatched blastocysts after the exposure below 15°C was examined by transplantation to recipients. The embryos recovered from the cooling were cultured for 1–3 hr, and the embryos showing normal appearance and/or re-expanding were transferred to the uterine horns of recipient gilts by laparotomy under general anesthesia. Gilts which had been in estrus two days later than the donors were used as recipients. The recipients were autopsied on Day 24 or 27 of gestation and the number of fetuses were recorded.

The viability of control morulae and early blastocysts, which had been cooled to 11°C in the same manner as the transferred embryos, was examined by *in vitro* culture for 24 hr.

Results

Survival of embryos *in vitro* after exposure to 6°C

The survival *in vitro* of the embryos after exposure to 6°C for 1 or 16 hr is shown in Table 1. Of 26 *in vitro*-hatched blastocysts exposed to 6°C for 1 hr and then recovered, 13 maintained almost the same morphology as they had before the cooling, and 13 were shrunk to various degrees (Fig. 1 & 2). When these recovered embryos were cultured for 24 hr, all of them developed and expanded noticeably (Fig. 3), although a number of collapsed cells were observed

in 5 specimens. All of these *in vitro*-hatched blastocysts were evaluated as to be surviving (survival rate=100%).

A total of 65 *in vivo*-hatched blastocysts were exposed to 6°C for 1 hr and recovered. Of these 65 blastocysts, 58 maintained almost the same morphology as they had before cooling and remaining 7 showed signs of shrinkage (Fig. 4 & 5). Among the recovered embryos, 51 (78.5%) were assessed as surviving judging from the noticeable expansion or maintenance of the expanded blastocoele after 24 hr's culture, although a number of damaged cells were observed in 20 of the embryos. The typical specimens of the *in vitro*-hatched and *in vivo*-hatched blastocysts that developed in culture after the recovery are shown in Fig. 3 and 6. The survival rate of the *in vitro*-hatched blastocysts was significantly higher ($p < 0.05$) than that of the *in vivo*-hatched blastocysts.

A total of 21 *in vitro*-hatched and 41 *in vivo*-hatched blastocysts were exposed to 6°C for 16 hr. All of the *in vitro*-hatched blastocysts became shrunk to various degrees (Fig. 8). When these embryos were cultured, all but one of them showed clear expansion (Fig. 9) after 24 hr, so that 20 (95.2%) of the 21 embryos were assessed as surviving.

Among the 41 *in vivo*-hatched blastocysts, 22 (53.7%) which continued expanding in culture for 24 hr after the cooling were assessed as surviving, although a considerable cell damage was observed in all of these specimens (Fig. 10–12). There was a significant difference ($p < 0.05$) between the survival rates of the *in vitro*-hatched blastocysts and *in vivo*-hatched blastocysts.

In contrast to the hatched blastocysts that survived the exposure to 6°C, the survival rate of the following pre-hatching-stage embryos was poor (Table 1).

A total of 32 expanding blastocysts were exposed to 6°C for 1 hr (Fig. 13 & 14). Culture of these embryos for 24 hr after the recovery resulted in 21 degenerated embryos and 11 (34.4%) surviving blastocysts which developed to a hatching stage or fully expanded stage, or maintained an expanded blastocoele (Fig. 15). The survival rate of the expanding blastocysts was significantly lower than that of the *in vitro*-hatched blastocysts ($p < 0.001$) and *in vivo*-hatched blastocysts ($p <$

0.05).

A total of 15 zona-removed blastocysts (Fig. 16 & 17), 18 morulae and 19 early blastocysts (Fig. 18 & 19) were exposed to 6°C for 1 hr, and no recovered embryo showed any sign of survival in culture for 24 hr except for one zona-removed blastocyst that formed a small vesicle with considerable damage.

Survival of embryos *in vivo* after exposure below 15°C

Since the above results showed that the *in vitro*-hatched blastocysts were more tolerant of low temperature than the *in vivo*-hatched blastocysts and other specimens of embryos, only the *in vitro*-hatched blastocysts were used for the transfer test, provided that the cooling of embryos was confined within the short periods (3 min) of exposure below 15°C.

A total of 18 *in vitro*-hatched blastocysts were cooled to 11°C and then recovered. When these recovered embryos were transferred to two recipients, one recipient became impregnated and a fetus with normal appearance was obtained at autopsy on Day 27 of gestation (Fig. 20).

In the control experiments, 22 morulae and 18 early blastocysts were cooled to 11°C in the same manner as the transferred embryos. The ability of these embryos to develop *in vitro* was completely lost by this short exposure below 15°C.

Discussion

Generally, to evaluate the viability of embryos preserved at low temperature, it is required to ascertain their ability to develop to conceptuses by transfer test. In the present study, the survival of the blastocysts exposed to 6°C for 1 hr or 16 hr was evaluated by the development *in vitro*, or ability to maintain or expand the blastocoele. In this survival assay, damaged embryos were also included in the category of surviving embryos, even though the viability of these embryos was obscure. However, the subject of this study was to show the differences of low temperature sensitivity of pig embryos at various developmental stages. Therefore, it seemed reasonable to choose the ability to maintain or expand the blastocoele *in vitro* as the index of survival of the cooled

blastocysts.

On the basis of this assessment of survival, the results of the present study clearly show that the pig hatched blastocysts could survive the exposure to 6°C, although under no circumstances could any morula or early blastocyst tolerate this temperature. It is unlikely that the blastocysts acquired the tolerance to low temperature due to merely being released from the zona pellucidae, because few zona-removed blastocyst survived the exposure to 6°C for 1 hr. It was thought that a qualitative change of the blastocysts' cells, accompanied with the progress of the developmental stage from pre-hatched stage to post-hatched stage, might give rise to the tolerance against cooling. This was supported by the experimental result showing that the survival rate (34.4%) of the expanding blastocysts after exposure to 6°C for 1 hr significantly increased to 78.5% ($p < 0.05$) and 100% ($p < 0.001$): these were the survival rates of the *in vivo*-hatched and *in vitro*-hatched blastocysts, respectively.

When the period of exposing to 6°C was prolonged from 1 hr to 16 hr, there was a marked difference in survival rate between the *in vitro*-hatched blastocysts and the *in vivo*-hatched blastocysts (95.2% vs 53.7%, $p < 0.05$). This indicates that the *in vitro*-hatched blastocysts were more tolerant of the exposure to low temperature than the *in vivo*-hatched blastocysts. So the difference between the *in vitro*-hatched blastocysts and the *in vivo*-hatched blastocysts is of interest. The *in vitro*-hatched blastocysts developed from Day 5 expanding blastocysts after the culture for about 24 hr. So their developmental stage corresponded to Day 6. However, the *in vitro*-hatched blastocysts were observed under the microscopical examination to consist of a smaller number of cells than the *in vivo*-hatched blastocysts collected on Day 6. Therefore, a qualitative difference between the *in vitro*-hatched blastocysts and the blastocysts that developed and hatched *in vivo* was suggested. It has been thought that the damage caused to pig embryos during cooling may be associated with lipid phase change within the membrane (Polge, 1977). On this hypothesis, it could be speculated that the tolerance of the *in vitro*-hatched blastocysts to low temperature resulted from a physicochemical change in the lipid at the peri-hatching stage. In addition,

it is tempting to speculate that the environment of the culture in which the blastocysts were led to hatching, may play a role in this change. It was reported that the sudanophilic droplets that were abundant in pig 1–8 cell stage embryos, greatly decreased at the blastocyst stage (Niimura & Ishida, 1980). This change may be associated with the difference observed between the hatched blastocysts and pre-hatching stage embryos in their tolerance to low temperature. From another point of view, the *in vitro*-hatched blastocysts could be utilized to verify the above hypothesis by examining the change of lipids in their cells during the period from pre-hatching to post-hatching stages.

On the basis of the experimental result that the survival rate of the *in vitro*-hatched blastocysts was higher than that of the *in vivo*-hatched blastocysts after the exposure to 6°C, only the *in vitro*-hatched blastocysts were subjected to the transfer testing after cooling. A normal fetus was obtained by transferring the *in vitro*-hatched blastocysts which had undergone a short exposure below 15°C, although no control morula or early blastocyst could survive this short exposure. The viability of the *in vitro*-hatched blastocysts cooled below 15°C was proved by the successful pregnancy. Furthermore, the *in vitro*-hatched blastocysts frozen at –20°C in 1.5 M DMSO/PBS+16% FCS in a preliminary experiment, showed clear expansion in culture after the thawing (unpublished data).

It seemed likely that the *in vitro*-hatched blastocyst could survive lower temperature if the cooling procedures were improved.

In conclusion, it is apparent that there are differences in the tolerance of pig embryos to low temperature between the stage of development, namely the pre-hatched stage and the post-hatched stage. There is also a considerable difference between the survival rates of *in vitro*-hatched blastocysts and *in vivo*-hatched blastocysts. This suggests that a qualitative change in cells, which resulted from the embryonic development *in vitro*, may give rise to the ability to tolerate the exposure to low temperature.

We expect to investigate the change in cells of the developing *in vitro*-hatched blastocysts in the peri-hatching stage where the tolerance to low temperature was observed. Perhaps a clue to the

freezing preservation of pig embryos at ultra low temperature can be found.

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15°C 以下に冷却された豚透明帯脱出胚盤胞の生存性

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多くの実験動物胚, 家畜胚の凍結保存が可能であるのに対して, 豚胚は15°C以下への冷却により死滅するとされてきた。著者らは, 豚の透明帯脱出胚盤胞の低温感作に対する耐性が透明帯脱出期前の胚に比して高いこと, 特に拡大期胚盤胞からの培養により透明帯脱出に至った胚盤胞 (vitro-HB) が15°C以下への冷却後も生存性を保持し得ることを見出した。

1) 上記の vitro-HB, 子宮より採取した透明帯脱出胚盤胞 (vivo-HB) および透明帯脱出期前の対照胚 (拡大期胚盤胞, 透明帯除去胚盤胞, 初期胚盤胞, 桑実胚) を16%牛胎仔血清加のダルベッコ PBS とともに0.25 ml プラスティックストローに封じ, 6°Cに冷却した。6°Cに1

時間保存後の培養により, vitro-HB の全例(26/26)および vivo-HB 65例中51例(78.5%)が生存と判定された。

一方, 対照胚では拡大期胚盤胞32例中の11例(34.4%)と, 透明帯除去胚盤胞15例中1例(6.7%)が生存徴候を示したが, 他は完全に死滅した。保存時間を16時間にした場合, vitro-HB と vivo-HB の生存性の間に明らかな差 (95.2% vs 53.7%, $p<0.05$) が認められた。

2) vitro-HB 18例を15°Cから11°Cにかけて3分間冷却した後, 2頭の雌に移植した結果, 1頭が妊娠し1匹の正常胎仔(27日齢)が得られた。この冷却条件で桑実胚22例および初期胚盤胞18例を処置したところ, 全例が死滅し, vitro-HB の低温に対する耐性の高さが示された。