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cartilage and bone surfaces, although they increased in number. Liberated osteoclasts had well-developed cytoplasmic processes and showed normal ACPase activity. Osteoclasts remaining on the cartilage and bone surfaces, although they lacked a ruffled border, were seen phagocytizing bone fragments by cytoplasmic invagination and ingesting spherical calcified nodules by pinocytosis. This finding may suggest an alternative mechanism for bone resorption. ACPase-positive products in these osteoclasts were of greater abundance than those in normal cells. Osteoblasts in rachitic chicks had well-developed RER with dilated cisternae, and the distribution of ALPase- and ACPase-positive products in these cells were the same as in

normal osteoblasts. The increased undifferentiated mononuclear cells were divided into two types, round- and spindle-cell types. The former cells were identified as preosteoclasts and the latter ones as preosteoblasts. In the rachitic chicks, hypertrophy and hyperplasia of the parathyroid glandular cells were observed. These cells showed the characteristic morphology of cells at the synthesizing and secretory phases. These findings indicate that hyperparathyroidism may be essential to initiate the proliferation of precursor osteoclastic and osteoblastic cells, which subsequently followed by a corresponding proliferation of osteoclasts and osteoblasts in the metaphysis of rachitic bone.

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Vitrification of bovine blastocysts derived from *in vitro* maturation,  
*in vitro* fertilization and *in vitro* culture

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Since the first report of successful cryopreservation of mouse embryos by vitrification (Rall & Fahy, 1985), many studies have focused on the cryopreservation of embryos from other species (Niemann, 1991). However, there has been no report about the vitrification of bovine blastocysts derived from *in vitro* maturation, *in vitro* fertilization and *in vitro* culture (IVMFC). Therefore, the following studies have been carried out to establish efficient procedures for cryopreservation of bovine blastocyst derived from IVMFC by vitrification.

1. Vitrification of IVMFC bovine blastocysts using glycerol and propylene glycol

Four experiments were conducted to deter-

mine the optimal conditions for the vitrification of IVMFC bovine blastocysts and to investigate the relationship between survival rates and ultrastructural appearance of the blastocysts after vitrification using the freeze-replica technique. In Exp. 1, the optimal concentrations of glycerol and propylene glycol in the basic medium (modified TCM199) for cooling and warming without formation of ice crystals were determined. A straw containing 0.25 ml of the solution was plunged into liquid nitrogen (LN<sub>2</sub>), and then warmed. Vitrification of the medium was observed when both glycerol and propylene glycol were present at the concentrations higher than 45% (v/v) in the solution. In Exp. 2,

blastocysts were equilibrated in a solution containing 22.5% glycerol and 22.5% propylene glycol in stepwise manners (1, 2, 4, 8, or 16 steps). After the removal of the cryoprotectants, the blastocysts were cultured for 24 h *in vitro*. The survival rates for the embryos equilibrated in 1, 2, 4, 8 and 16 step (s) were 56, 89, 100, 100 and 100%, respectively. Blastocysts equilibrated as above were vitrified in LN<sub>2</sub>, then warmed and cultured *in vitro*. Higher survival rates were obtained for blastocysts equilibrated in 4, 8 and 16 steps (79, 82 and 87%, respectively) than for those in 1 (0%) or 2 (10%) steps. In Exp. 3, blastocysts were equilibrated in vitrification solution (VS: 22.5% glycerol + 22.5% propylene glycol) using either the 16-step method (18 min in total) or the 2-step method (10% glycerol + 20% propylene glycol for 10 min and then exposure to VS). The blastocysts were then vitrified in LN<sub>2</sub>, and cultured for 24 h. While 83% of blastocysts survived following the 16-step method, no blastocysts survived by the 2-step method. In Exp. 4, freeze-replica observations were carried out on blastocysts vitrified by the 16-step and the 2-step methods. No ice crystals were observed in the cytoplasm, blastocoelic cavity or extracellular areas of blastocysts, confirming complete vitrification. Little ultrastructural change was observed in the plasma membrane of the blastocysts equilibrated by the 16-step method. In contrast, blastocysts equilibrated by the 2-step method exhibited distinct aggregation of intramembrane particles and small vesicles in the plasma membranes. Ten blastocysts that survived after vitrification were transferred to ten recipients. Six of them became pregnant and 5 normal calves were obtained.

## 2. Vitrification of IVMFC bovine blastocysts using ethylene glycol and sucrose

Four experiments were conducted to develop a simple and efficient vitrification method and an in-straw dilution method after vitrification

for IVMFC bovine blastocysts. In Exp. 1, blastocysts were equilibrated in 10% (v/v) glycerol and 20% calf serum (CS) in modified phosphate buffered saline (mPBS) for 5 min, and exposed to a vitrification solution (20% CS in mPBS + 1M sucrose) containing 30% glycerol or 30% ethylene glycol. The embryos were then diluted out of the cryoprotectants, and cultured for 24 h. The survival rates for the blastocysts exposed to glycerol or to ethylene glycol were 87 and 100%, respectively. Blastocysts equilibrated as described above were vitrified in LN<sub>2</sub>, warmed and cultured for 24 h. High survival rates were obtained from both treatments (70% by glycerol, 73% by ethylene glycol). In Exp. 2, blastocysts were vitrified by 30% ethylene glycol + 1M sucrose solution as described above. After warming, blastocysts were exposed to diluents containing 0.5M sucrose and 20% CS in mPBS supplemented with either 0, 5, 10, 15 or 20% egg yolk. The highest survival rate (97%) was obtained when the diluent contained 10 or 15% egg yolk. In Exp. 3, blastocysts were equilibrated in mPBS containing 10% glycerol and 20% CS (5 min), exposed to VS (30% ethylene glycol, 1M sucrose and 20% CS in mPBS), and then loaded into a straw by one of three methods. In group 1, blastocysts were aspirated with 25  $\mu$ l of VS into a straw. In group 2, 25  $\mu$ l of VS containing a blastocyst was aspirated into the bottom of a straw and 150  $\mu$ l of diluent (10% egg yolk, 0.5M sucrose and 20% CS in mPBS) was injected onto the VS column without air partition. In group 3, blastocysts were introduced to the VS column (25  $\mu$ l) which had been loaded under a column of 150  $\mu$ l of diluent (10% egg yolk, 0.5M sucrose and 20% CS in mPBS). All straws were then sealed, plunged into LN<sub>2</sub> and stored for 3 to 5 days. After warming, VS and diluent were mixed in the straw for 30 sec, the blastocysts were then washed and cultured for 24 h. The highest survival rate (93%) was obtained in Group 3. In Exp. 4, 20 vitrified-warmed and

in-straw diluted blastocysts were transferred to 20 recipients. Eleven of them became pregnant and 9 normal calves were obtained.

These results demonstrate for the first time that by modification of the vitrification procedure of *in vivo* derived bovine embryos, IVMFC bovine blastocysts can be successfully cryopreserved.

To achieve high survival rates of blasto-

cysts, both the cells and their extracellular environment must be completely vitrified, and ultrastructural damage to the cell membrane must be minimized. The method developed in this study for in-straw dilution of vitrified blastocysts is simple and efficient. Consequently, vitrification of bovine blastocysts has great practical and commercial application.

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Original papers of this thesis appeared in "Journal of Reproduction & Fertility", Vol. 96, 187-193 (1992), and "Cryobiology", Vol. 31, 415-422 (1994).