THE ROLE OF PRE-EQUILIBRATION FOR VITRIFICATION OF IN VITRO PRODUCED (IVP) BOVINE EMBRYOS

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The role of equilibration for successful vitrification was investigated by one-step or three-step addition and one-step removal of the cryoprotective agents (CPAs) in vitrification solution. The blastocysts and expanded blastocysts produced in vitro on day 7, 8 or 9 (IVF = day 0) were used for these experiments.

Firstly, in one-step addition, embryos were placed directly in a precooled (4°C) vitrification solution (VS) containing 40% ethylene glycol (EG; Wako Pure Chemical Industries Ltd., Osaka, Japan), 0.3 M trehalose (Wako) and 12% polyvinylpyrrolidone (PVP; Denka Pharmaceutical Co., Kawasaki, Japan) in Dulbecco's phosphate buffered saline (DPBS; Gibco, Grand Island, NY, USA) supplemented with 0.3% bovine serum albumin (BSA; Sigma Chemical, St Louis, MO, USA) for 2 min. Then, the embryos put in 0.25 ml plastic straws and submerged in LN₂ for storage. Secondly, in three-step addition, embryos were placed in 10% EG in mPBS (10% calf serum and 0.6% BSA in DPBS) for 5 min and in 10% EG plus 0.3M trehalose in mPBS for 5 min at room temperature (22-25°C) and then in precooled VS at 4°C for 1 min before loading in 0.25 ml plastic straws and submerging in LN₂ for storage. Warming was performed in a waterbath at 30°C and embryos were placed directly in mPBS and examined under a light microscope. Then the embryos were cultured and rates of development were recorded visually at 24-, 48- and 72-h intervals by monitoring the re-expansion of the blastocoel cavity and expansion of blastocysts. Expanded blastocysts were subjected to hatching in culture. The ratio of live to dead cells of embryos after hatching until 72 h was determined by differential fluorescence staining. In brief, hatched blastocysts were rinsed in fresh culture medium and incubated in DPBS containing propidium iodide (Wako) and bisbenzimide (Hoechst 33342; Sigma) at a final concentration of 10 µg/ml for 30 min at 38.5°C in 5% CO₂ in air. Embryos were washed in DPBS with 0.3% BSA and mounted on a glass slide. Cells were separated by applying gentle pressure to the cover slip and examined under a fluorescence microscope (filter 330-380 nm). Nuclei of the live cells fluoresced blue (bisbenzimide positive) and dead cells nuclei fluoresced pink (propidium iodide-positive). The total number of cells and ratio of live to dead cells in embryos were determined for each day of culture. Embryos that had not hatched even after 72 h in culture were treated with 0.25% pronase in TCM 199 for 4-6 min to dissolve the zona pellucida.

Frequency of development and hatching rates were 73/98 (75%) and 42/98 (43%) for day 7, 48/83 (58%) and 20/83 (24%) for day 8, 25/75 (33%) and 6/75 (8%) for day 9 blastocysts and expanded blastocysts using one-step addition of CPAs and 73/85 (86%) and 66/85 (78%), 66/77 (73%) and 30/77 (39%) or 34/73 (47%) and 8/73 (11%) for day 7, 8 or 9 blastocysts and expanded blastocysts, after three-step additions of CPAs, respectively. The results in all cases varied significantly (P<0.05). The ratio of live to dead cells and the percentage of live cells on days 7, 8 or 9 were 129 to 8 and 95%, 113 to 11 and 91% or 97 to 14 and 88% for one-step addition; as compared to, 134 to 4 and 98%, 124 to 5 and 95% or 109 to 9 and 93% for three-step addition of CPAs, respectively. The percentage of live cells for each day of embryos differed significantly (P<0.05) in case of one-step addition of CPAs but in three-step addition that of day 9 embryos differed significantly (P<0.05) from day 7.

The development rates of day 7 embryos for the one-step (73/98, 75%) and three-step (73/85, 86%) were similar; however, the hatching rates for one-step (42/98, 43%) and three-step (66/85, 78%) differed significantly (P<0.01). Similarly, there was a difference (P<0.05) in the percentage of live cells between one-step (95%) and three-step (97%) vitrification.

In control embryos, the total cell numbers on day 7, 8 and 9 were 141±22, 126±23 and 113±16. There was a significant difference (P<0.05) between the total cell number of day 7 and day 9 embryos. The live to dead cell ratio were 139:2, 124:2 and 111:2 for day 7, 8 and 9 embryos. But there was no significant difference was found among the percent of live cells of day 7 (99%), 8 (99%) and 9 (98%) embryos (P>0.05).

The results indicate that three-step addition of cryoprotectants improves in vitro survival of in vitro produced bovine blastocysts and expanded blastocysts.