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 LN_2 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 LN_2 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 to expanded blastocysts and expanded blastocyst to hatched blastocysts. The ratio of live to dead cells of embryos after hatching until 72 h was determined by differential fluorochrome 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% CO_2 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.