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Cryopreservation of Isolated Fish Blastomeres: Effects of Cell Stage, Cryoprotectant Concentration, and Cooling Rate on Postthawing Survival

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The toxicity of the cryoprotectant dimethyl sulfoxide (Me_2SO) to isolated blastomeres was examined in three fish species representative of distinct environments: marine (whiting, *Sillago japonica*); estuarine (pejerrey, *Odontesthes bonariensis*); and freshwater (medaka, *Oryzias latipes*). The effects of embryonic stage, Me_2SO concentration, and cooling rate on the cryopreservation of blastomeres were also studied. Whiting sheds small planktonic eggs whereas the other two species shed large demersal eggs. Isolated blastomeres from the three species tolerated Me_2SO concentrations up to 9% relatively well for over 5 h but lost viability rapidly at 18%. Cells from later embryonic stages (512 or 1024 cells) were more tolerant of Me_2SO than those from earlier stages (128 or 256 cells). The three factors examined, alone or in combination, had a significant effect on the survival of blastomeres after freezing and thawing, but the extent of the effect and the optimum conditions varied with the species. In general, the highest rates of successful cryopreservation were observed with older rather than younger blastomeres, slower rather than faster cooling, and with 9–18% rather than 0% Me_2SO. Survival rates for blastomeres cryopreserved under the most effective combination of the three factors examined for each species were 19.9 ± 10.1% for whiting, 34.1 ± 8.5% for medaka, and 67.4 ± 12.8% for pejerrey.

Key Words: cryopreservation; blastomere; fish; embryo; cooling rate; cryoprotectant.

The major advantage of gamete and embryo cryopreservation in animal farming is the establishment of cryobanks, which allow the storage and dissemination of valuable genetic information and the possibility of bridging temporal and spatial gaps in the availability of gametes for assisted reproduction (3, 5, 20, 21, 24, 27). Cryobanks may also become instrumental for the conservation of endangered species (1, 27, 32). In fish, sperm can be relatively easily cryopreserved but there appear to be formidable constraints to the development of methods for the cryopreservation of eggs and embryos (9, 18, 25, 26, 29). Compared to the eggs of higher vertebrates, fish eggs are large, have large amounts of reserve substances (yolk), a thick chorion, poorly permeable membranes, and a complex structure early in development (6, 7, 9, 11, 31, 35). Thus, successful cryopreservation of fish eggs and embryos remains elusive, notwithstanding recent attempts to impregnate embryos with cryoprotectant after dechorionation (8, 12) or in the presence of mild vacuum (15).

It may be possible to cryopreserve the diploid genome of fish, and hence the maternal genome as well, by using isolated blastomeres, the early embryonic cells, instead of intact embryos. Harvey (9, 10) pioneered this idea and successfully cryopreserved blastomeres of zebra fish at −196°C, whereas attempts with whole embryos consistently failed. From the viewpoint of technical feasibility, these cells are advantageous because they are much smaller than intact embryos yet sufficiently large for easy manipulation and also because they lack the thick chorion and impermeable membranes of intact embryos. Moreover, the usefulness of these cells in animal production is justified by recent progress in blastomere transplantation techniques. Such techniques allow the integration of the blastomere germplasm into the host's genome, producing a chimera, or its insertion into an enucleated egg, producing a clone (2, 4, 17, 23, 28,

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30, 33). In addition, these cells can be easily transfected and function as vectors for the insertion of genetic modifications to the germline (4).

In a recent study, Leveroni Calvi and Maisse (16) proposed a highly efficient method for the cryopreservation of rainbow trout blastomeres. However, systematic information on the effects of factors likely to influence the outcome of blastomere cryopreservation such as the developmental stage of the donor embryos (13, 16, 19) or the optimum cryoprotectant concentration and cooling rate (14, 15) is still lacking, particularly regarding warm water and marine fish species. This paper reports the results of preliminary attempts to cryopreserve the blastomeres from three warm water species representative of marine, estuarine, and freshwater environments, respectively whiting, *Sillago japonica*, pejerrey, *Odontesthes bonariensis*, and medaka, *Oryzias latipes*. These species also represent two different types of eggs: small planktonic eggs as in *S. japonica* and large demersal eggs as in *O. bonariensis* and *O. latipes*. In preliminary experiments in these species, we were unable to successfully cryopreserve intact blastula-stage embryos or any of the blastomeres within them. Thus, our goal in this study was to determine the most suitable developmental stage of the donor embryos, the optimum cryoprotectant concentration, and the optimum cooling rate for the cryopreservation of isolated blastomeres.

MATERIALS AND METHODS

Source and Incubation of Embryos

Pairs of wild-type medaka were maintained in aquaria at 25°C and a 14-h light/10-h dark photoperiod and fed an aquarium-fish diet (Tetramin) daily to satiation. The fish spawned daily under these conditions, usually within 1 h of the onset of the light period. Because spawning substratum was not provided, the eggs remained attached by the chorionic filaments to the mother. They were collected using a wide-bored pipette while partly immobilizing the female in a net. Pejerrey eggs were obtained from natural spawnings of 2- to 4-year-old broodstock reared at 19–22°C in a recirculated-water facility at the main campus of Tokyo University of Fisheries. Pejerrey eggs were generally shed at nighttime and remained attached by their chorionic filaments to the aeration hose. Whiting eggs were obtained from wild fish held in captivity at the Banda Marine Experimental Station, Tokyo University of Fisheries. Adult fish collected just prior to the spawning season of 1995 were transferred to indoor tanks with flowing water and natural conditions of photoperiod and water temperature. Fish were fed twice daily until satiation with frozen krill. Natural spawnings were obtained almost daily during the summer months, generally between 30 min and 3 h after dawn. Fertilized, buoyant eggs were collected with a hand net near the surface of the water.

After collection, normally developing embryos were selected and transferred in groups of 15–30 to Petri dishes for incubation until the desired stage. Incubation of medaka and pejerrey embryos was conducted in 10× diluted freshwater fish Ringer solution containing traces of malachite green as a prophylactic agent whereas whiting embryos were maintained in sea water only.

Collection of Blastomeres

For collection of blastomeres, individual embryos were blotted dry and mounted in holes drilled into a plastic plate in Ca^{2+}-free, Hepes-buffered Waymouth's culture medium MB 752/1 (pH 7.4, 276 mOsm/Kg), containing 10% fetal bovine serum (FBS). The choice of this culture medium merely reflected availability and does not imply that other media may not be equally suitable. The lack of Ca^{2+} is recommended for easier dissociation of blastomeres (22, 30) whereas the presence of FBS provides protection against physical damage of the cell membrane (24). After cutting an incision in the chorion, each embryo was transferred to a well with the culture medium and repeatedly squeezed gently to expel the yolk and blastomeres through the cut in the chorion. Debris and yolk were removed as much as possible and the blastomeres were transferred to clean cul-

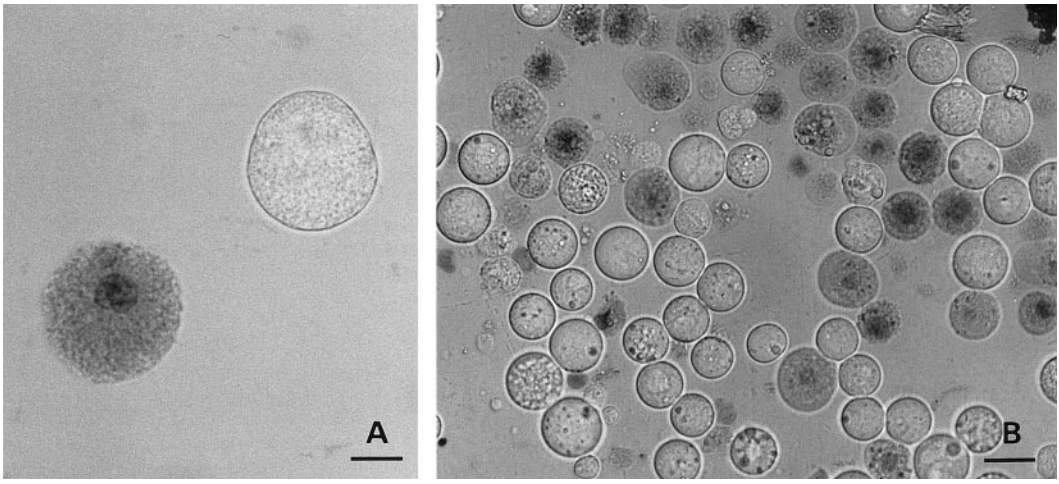

FIG. 1. (A) Typical appearance of damaged (left) and intact (right) blastomeres after incubation in 0.05% trypan blue for 10 min. (B) Spread showing approximately 65% viable pejerrey blastomeres after freezing–thawing. Bars under the letters indicate 20 μm (A) and 30 μm (B).

ture medium. If necessary, the number of cells was counted to determine the developmental stage of the embryo (by approximation to the theoretical number of cells observable with progressive cell cleavage, e.g., 128, 256, 512, 1024). Blastomeres from a number of embryos were processed in the same form and within a short time span (usually less than 10 min) and pooled for the toxicity and cryopreservation experiments.

Assessment of Blastomere Viability

Determination of blastomere viability in all cases was based on examination of the characteristics of the cell membrane and uptake of a vital stain. For this purpose, an aliquot from each treatment was mixed with an equal volume of 0.1% trypan blue in phosphate-buffered saline, incubated for about 10 min, and observed under a microscope. Blastomeres were considered viable when the cell membrane was well defined and the cytoplasm remained unstained whereas the opposite characteristics were taken as an indication of damaged/dead cells (Fig. 1).

Toxicity of Me_2SO to Isolated Blastomeres

We focused the toxicity and cryopreservation trials on the blastula stage because at this stage the cells had attained a size (diameter between 15 and 60 μm) comparable to that of the cells of successfully cryopreserved mammalian embryos. The toxicity of Me_2SO to isolated blastomeres was estimated at the stages of 1024 cells for whiting, 128, 256, and 512 cells for medaka, and 128, 256, and 1024 cells for pejerrey. Blastomeres at these stages were placed directly in wells containing 0.5 ml of 0, 9, or 18% (v/v; also 3 and 6% for whiting) Me_2SO in culture medium at room temperature. At hourly intervals up to 5 h, 0.4 ml of the supernatant was temporarily removed from each well, the remaining fluid was gently stirred, an aliquot was taken, and the supernatant was then returned. Between 70 and 500 cells per aliquot were examined for viability as described above. Between one and three trials were performed for each combination of species, embryo stage, and cryoprotectant concentration (the actual number of trials per combination is indicated in the figures).

Freezing Apparatus and Freezing and Thawing Conditions

Freezing was performed with a specially built portable apparatus because the study included trials in two different locations where neither a

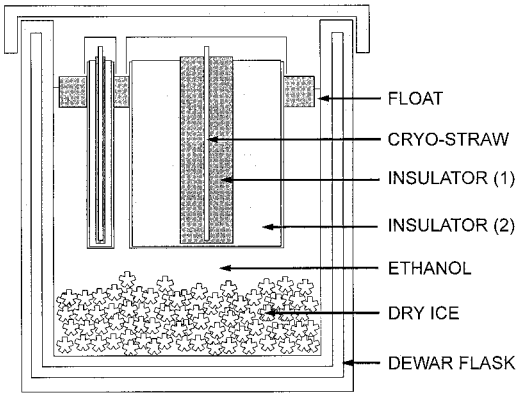

FIG. 2. Diagram of the portable apparatus used for freezing fish blastomeres at different cooling rates. Only two cylinders are shown in this transverse view.

programmable freezer nor liquid nitrogen could be employed. The apparatus consisted of a series of metal cylinders of different diameters filled with variable amounts and types of insulation materials (generally polyurethane) which allowed cooling at different rates when immersed in a dry ice/ethanol bath (Fig. 2). Near the center of each pipe, the insulation material had four to five vertical holes that permitted the tight insertion of 250-μl French cryopreservation straws. For cryopreservation, the cylinders were allowed to equilibrate at room temperature (about 25°C), the straws were inserted, and the assembly was then transferred to the dry ice/ethanol bath. The cooling rate characteristic of each cylinder was determined in advance by inserting a thermocouple into a straw containing the same amount and type of medium as for the cryopreservation trials but without sample and recording the temperature every 10 s from the start of cooling until the attainment of a plateau (usually around −75°C). The actual and nominal (−1.0, −14.2, −20.1, −34.3, −128.7°C/min) cooling rates with the five cylinders used in the cryopreservation trials are shown in Fig. 3. Seeding was not performed. All samples were maintained at the plateau temperature for a period of 1.5 h before thawing. Thawing of frozen blastomeres was by immersion of the straws in a 20°C water bath for 1 min.

Blastomere Cryopreservation: Effects of Embryonic Stage, Me_2SO Concentration, and Cooling Rate

Blastomeres from 256- and 512-cell embryos of whiting and blastomeres of the same stages that were used in the toxicity trials with medaka and pejerrey embryos were used for cryopreservation. The cells at each stage were divided

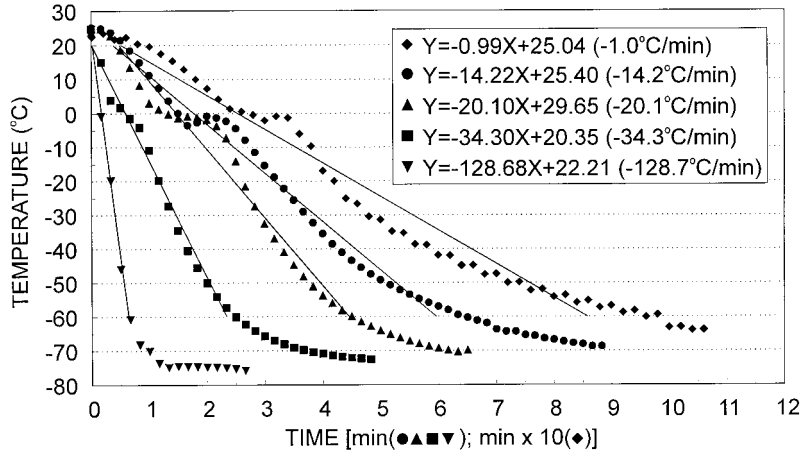

FIG. 3. Actual (dots) and nominal cooling rates obtained with five cylinders of different diameter filled with different amounts and types of insulation materials. Each dot represents the means of two recordings. The nominal cooling rates were standardized by linear regression of actual values between room temperature and −60°C.

between two and three wells with approximately the same numbers in each well and were incubated at room temperature in 0.4 ml of culture medium containing Me_2SO at concentrations of 0 and 9% for whiting and 0, 9, and 18% for medaka and pejerrey. Because the toxicity trials indicated that blastomeres lost viability rapidly in 18% Me_2SO, especially those from 128- and 258-cell stages (see below), impregnation was performed in ascending concentrations of the cryoprotectant, each increment being $\frac{1}{3}$ of the final concentration every 10 min, and then at the final concentration for 10 min. Following impregnation with cryoprotectant, blastomeres were concentrated by discarding the supernatant, suspended by agitation, and loaded in volumes of 70 μl into 250-μl cryopreservation straws. The straws were frozen as explained before using four cooling rates from -14.2 to -128.7°C/min for whiting and -1.0 and -14.2°C/min for medaka and pejerrey. The lowest cooling rate (-1.0°C/min) was not available at the time of the trials with whiting. Also, cooling rates exceeding -14.2°C/min were not tested with medaka and pejerrey because preliminary trials showed that slower rates were more effective than faster ones. After being thawed, blastomeres were divided in two groups and processed for viability determination immediately or 1 h after dilution of the blastomere/medium mixture (only available for medaka and pejerrey); in this case, sufficient clean culture medium was added to reduce the concentration of Me_2SO in the medium to 1%. Between two and four trials were conducted with each combination of species, embryo stage, cryoprotectant concentration, and cooling rate. The actual numbers of trials for each combination are indicated in the figures. Viability was determined in at least 200 cells from each straw.

Statistical Analyses

Differences between treatments and possible interactions between factors were analyzed by multiple ANOVA and statistical significance was defined as $P < 0.05$.

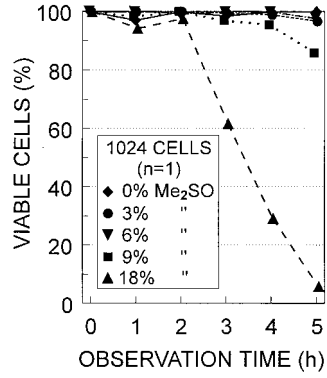

FIG. 4. Toxicity of Me_2SO to isolated whiting blastomeres at room temperature. Data are shown as the survival rates in one trial with blastomeres at the 1024-cell stage.

RESULTS

Toxicity of Me_2SO to Isolated Blastomeres

Blastomeres from 1024-cell-stage embryos of whiting tolerated concentrations of Me_2SO up to 9% for 5 h with survival generally over 90% but they lost viability rapidly after 2 h at 18% (Fig. 4). The viability of 128- and 256-cell blastomeres of medaka (Fig. 5) and 128-cell blastomeres of pejerrey (Fig. 6) incubated in culture medium without cryoprotectant was reduced to 60–80% after 3 h whereas the viability of older blastomeres of the two species remained above 90% for the duration of observation. The toxicity of 18% Me_2SO for medaka and pejerrey blastomeres was apparent sooner and more abruptly in 128- and 256-cell stages but was less marked in the 512-cell stage of medaka and the 1024-cell stage of pejerrey. A concentration of 9% Me_2SO did not impair viability as judged by comparison with incubation at 0% Me_2SO for blastomeres of medaka. In pejerrey, 9% caused considerable loss of blastomere viability at 1 h in the 256-cell stage and at 3–4 h in the 128-cell stage.

Cryopreservation of Blastomeres

The following results of blastomere viability after freezing and thawing are based on observations made immediately after thawing. Whiting blastomeres frozen without cryoprotectant had viability rates usually below 5% at all cool-

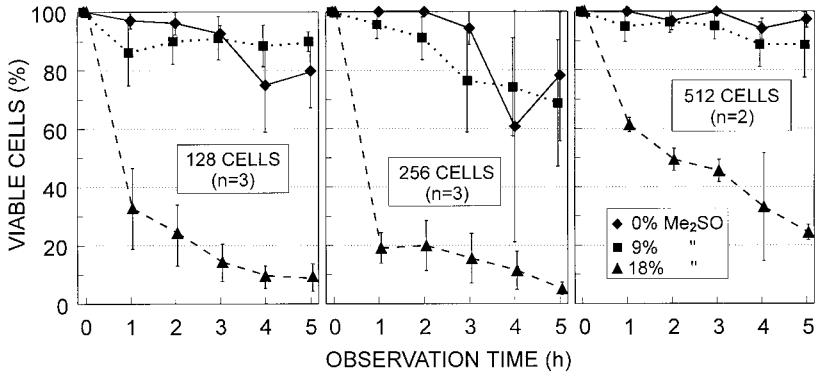

FIG. 5. Toxicity of Me_2SO to isolated medaka blastomeres at room temperature. Data are shown as mean survival rates ± SE in two to three trials with blastomeres at different embryonic stages.

ing rates whereas those incubated in 9% Me_2SO, with the exception of those frozen at −128.7°C/min, had mean viabilities between 10 and 20% (Fig. 7). The slowest cooling rate available for this species (−14.2°C/min) seemed to produce slightly better results than the other rates. No marked differences in viability after cryopreservation were observed between the blastomeres from 256- and 512-cell-stage embryos. Mean viabilities above 15% with a maximum of about 55% could be obtained only in medaka blastomeres cryopreserved at −1.0°C/min in the presence of 9% Me_2SO and seemed to increase with progressive embryonic development (Fig. 8). Viability was below 5% at all combinations of cooling rate and embryonic stage in the absence of cryoprotectant and in cells of all embryonic stages frozen at −14.2°C/min with 18% Me_2SO. Pejerrey blastomeres impregnated with 9% Me_2SO had similar viability following freezing at either of the two cooling rates, but the results with 18% Me_2SO were far better at −1.0°C/min than at −14.2°C/min (Fig. 9). Viabilities up to 95% (mean ± SE of 67.4 ± 12.8%) were obtained with 1024-cell-stage blastomeres frozen at −1.0°C/min in the presence of 18% Me_2SO. Of blastomeres cryopreserved without Me_2SO, only the 1024-cell stage frozen at −1.0°C/min had a viability above 5%. The viability of blastomeres kept in culture medium for 1 h after thawing was markedly reduced in all groups in

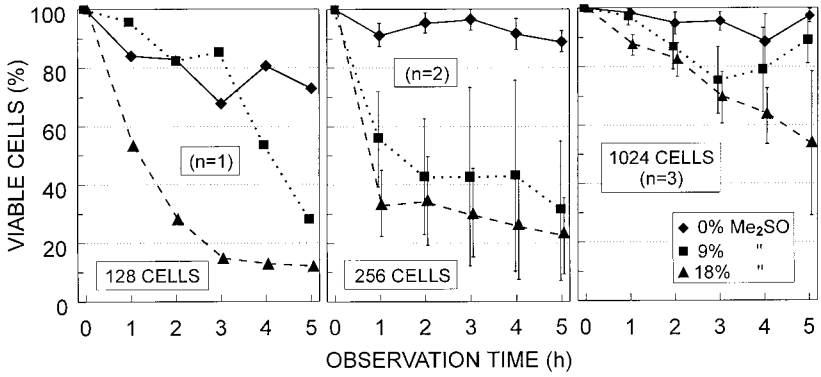

FIG. 6. Toxicity of Me_2SO to isolated pejerrey blastomeres at room temperature. Data are shown as mean survival rates ± SE in one to three trials with blastomeres at different embryonic stages.

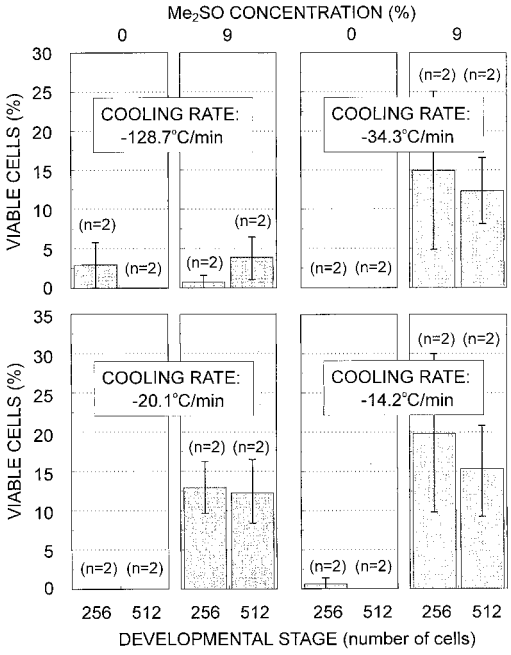

FIG. 7. Survival of isolated whiting blastomeres frozen–thawed at various combinations of embryonic stage, Me_2SO concentration, and cooling rate. Bars indicate means ± SE immediately after thawing (n = number of trials per combination).

comparison to determinations made immediately after thawing in medaka and pejerrey (Figs. 8 and 9).

Multiple ANOVA analyses revealed significant effects of cryoprotectant concentration on blastomere viability after cryopreservation for the three species, of cooling rate for medaka and pejerrey, and of embryonic stage for pejerrey (data not shown). Significant interactions were found between cryoprotectant concentration and cooling rate for medaka and pejerrey as well as between cooling rate and embryonic stage for pejerrey blastomeres.

DISCUSSION

The present trials on the cryopreservation of fish blastomeres, which to the best of our knowledge include the first reported trial with blastomeres of a marine species, yielded successful results for the three species examined. Survival rates for blastomeres cryopreserved under the most suitable combination of embryonic stage, Me_2SO concentration, and cooling rate for each species were 19.9 ± 10.1% for whiting, 34.1 ± 8.5% for medaka, and 67.4 ± 12.8% for pejerrey. These rates are considerably lower than those recently obtained by Leveroni Calvi and Maisse for rainbow trout blastomeres (16), but any comparison of these two studies must take into consideration the differences between the species examined and methodology (for example, in cryoprotectant type and concentration and cooling and thawing methods; see below). Nonetheless, this and other (16, 22) studies confirm the technical feasibility of blastomere cryopreservation, as introduced by Harvey (9, 10). Thus, blastomere cryopreservation is a useful alternative for preserving the diploid fish genome, at least as an alternative until successful methods for intact embryos are developed. In addition, a comparison of experiments with intact embryos and isolated blastomeres may help clarify the requirements for successful cryopreservation of the former (see (10) for a thorough discussion on this possibility).

Although limited in scope, the current experiments, together with published information,

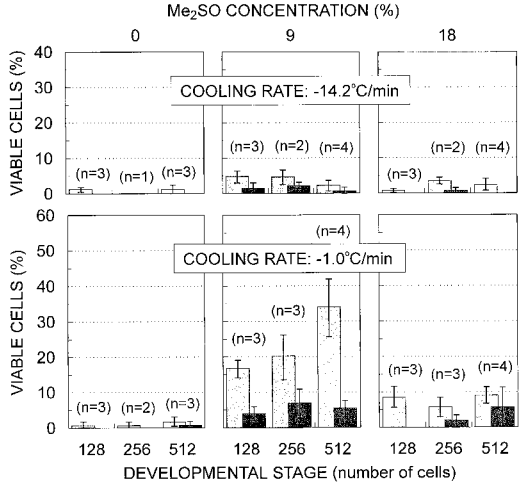

FIG. 8. Survival of isolated medaka blastomeres frozen–thawed at various combinations of embryonic stage, Me_2SO concentration, and cooling rate. Bars indicate means ± SE immediately (light) or 1 h (dark) after thawing (n = number of trials per combination).

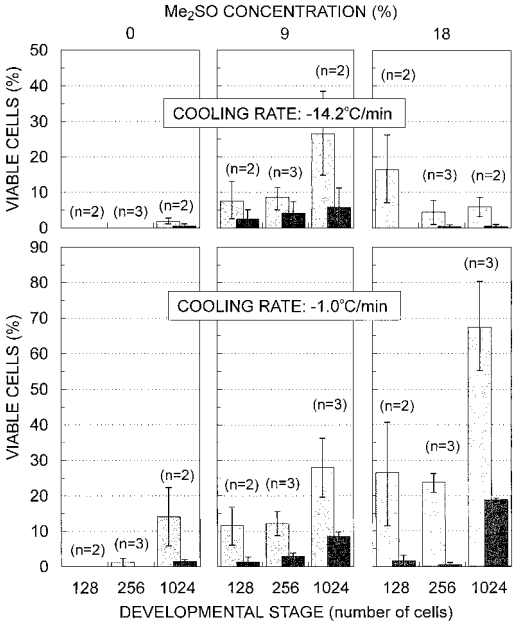

FIG. 9. Survival of isolated pejerrey blastomeres frozen–thawed at various combinations of embryonic stage, Me_2SO concentration, and cooling rate. Bars indicate means ± SE immediately (light) or 1 h (dark) after thawing (n = number of trials per combination).

permit some generalizations concerning the further development of cryopreservation methods for fish blastomeres. First, it is likely that the successful cryopreservation of fish blastomeres requires cooling rates around or slower than −1.0°C/min, the lowest rate employed in this study (see also 10, 16, 22) . Second, our results reinforce the view that the addition of a cryoprotectant is vital for successful cryopreservation (14, 15, 29) (unfortunately, neither Nilsson and Cloud (22) nor Leveroni Calvi and Maisse (16) reported attempts to cryopreserve cells without cryoprotectant). Because of the tradeoff between cryoprotection and toxicity with most permeating cryoprotectants (this study, 10, 11, 31), further experiments are required with various kinds and dosages of permeating as well as nonpermeating cryoprotectants. Moreover, future studies must also address the possibility that blastomeres could be cryopreserved by rapid cooling without cryoprotectant, as shown in *Apis mellifera* (34). Third, there seem to be species-specific variations in at least some of the factors affecting the success of cryopreservation. For instance, isolated blastomeres of pejerrey were most successfully cryopreserved with 18% Me_2SO whereas those of medaka did not tolerate this concentration. Thus, the optimum conditions for cryopreservation will have to be worked out on a species-by-species basis. Fourth, tolerance to Me_2SO and to freezing and thawing seemed to increase with developmental stage. It is unclear why blastomeres from more advanced stages tolerated higher concentrations of Me_2SO than those from younger stages. However, the fact that older blastomeres tolerated freezing better than younger ones is in agreement with observations for mammalian (13, 19) and fish (16) cells. These findings could have practical implications for the choice of the embryonic stage for cryopreservation. Thus, the older and smaller cells may be easier to cryopreserve, but the choice of stage for cryopreservation will also have to consider the increased possibility of loss of totipotency or even pluripotency in older cells (see (2, 28)).

The optimum values determined for the three factors examined in this study are only preliminary determinations; significant improvements in the success rate of blastomere cryopreservation may be achieved by examining the same parameters in further detail. For example, the use of different cooling rates for different stages of freezing (e.g., before and after seeding) and the use of seeding are recommended. Another point that deserves attention is the loss of viability observed with time after thawing. This phenomenon can be only partly attributed to cryoprotectant toxicity since it was observed also in cells frozen without Me_2SO. One possible cause could be "volume effects," or cryoinjuries due to large and sudden changes in cell volume, which are particularly common after thawing (14). Cryopreserved cells are generally hypertonic due to the addition of cryoprotectant and/or dehydration caused by ice formation in the extracellular medium. The cells therefore tend to swell upon thawing, stretching and damaging the cell membrane (14). Indeed, Leveroni Calvi and Maisse did not observe any signifi-

cant decrease in viability with time after thawing, which could be attributed to the use of a step-wise dilution method for cryoprotectant removal (16). Thus, the removal of cryoprotectant swiftly and with minor cell volume changes after thawing is an important consideration, as probably is the composition of the culture medium and the methods for manipulation of blastomeres. Finally, the loss of differentiation ability expected in older cells, as discussed above, could greatly restrict the practical application of fish blastomere cryopreservation for fish production and conservation of resources. Thus, in addition to cryopreservation methods, further studies should examine the extent of integration of the donor's and host's genomes and the limits for full-term development following insertion of blastomeres of different embryonic stages into enucleated eggs.

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