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Note

Cryopreservation of carp (*Cyprinus carpio*) blastomeres

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Abstract – The technique already adopted by the authors for the cryopreservation of rainbow trout blastomeres was applied to carp. Three stages were utilised for cell freezing: the morula stage, the early blastula stage and the late blastula stage. Survival rates were 89 ± 1.6 % for morula cells, 94 ± 0.6 % for early blastula cells and 96 ± 0.4 % for late blastula cells. Early blastula and late blastula cells reaggregated into morula-like bodies within 2 h in culture medium. Results suggest that the freezing technique adopted for rainbow trout may be successfully applied to carp. The morula stage blastomeres were more sensitive to freezing than blastula stage blastomeres, as already found in rainbow trout. © Ifremer/Elsevier, Paris

Embryos / blastomeres / cryopreservation / carp / *Cyprinus carpio*

1. INTRODUCTION

The unsuccessful results obtained so far with the freezing [5, 13] or vitrification of fish embryos [12], due to the complexity of their structure [4], have raised interest in the cryopreservation of isolated blastomeres [5, 6, 9] as genetic stock both of paternal and maternal origin.

As was recently pointed out by Maisse et al. [8], the successful results obtained with the cryopreservation of blastomeres may provide a useful tool for establishing gene banks in the context of genetic selection in aquaculture or in the wider perspective of ichthyodiversity preservation.

In this view, the technique adopted by the authors for the cryopreservation of rainbow trout (*Oncorhynchus mykiss*) blastomeres [6] was applied to carp (*Cyprinus carpio*) in order to verify the adaptability of the technique to another species, and the same experimental approach of evaluating the influence of the embryo stage on freezing tolerance of the blastomeres was developed.

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2. MATERIALS AND METHODS

2.1. Broodstock management

Broodstock came from a population of carp bred at the Agricultural University Station in Carmagnola (Turin, Italy).

For the purpose of the experiment, a group of 4–5-year-old breeders (50 females and 50 males) was selected and kept in running water tanks at 15 °C for the whole duration of the study (April–August). One week before each planned experiment, four females were conditioned by thermal treatment in a warm water aquarium programmed for a gradual increase in temperature from 16 °C to 22 °C. After one week, ovulation was induced by a single injection of fresh pituitary extract [3]. Males were conditioned for 3 d and spermiation was induced by warm water treatment only.

2.2. Embryos

Ten replicates were made for each stage. Artificial fertilisation was performed following the technique reported by Billard et al. [1] and eggs were left undisturbed at room temperature (22 °C) for development.

After 4 h, embryos were selected on the basis of morphological criteria (regular cell shape, synchronous mitosis and yolk integrity) [7, 11], in order to provide the stages required by the experimental freezing protocol. Around 100 embryos per stage were pooled in tap water in 10-cm Petri dishes. After half-an-hour, dechorionation of around 40 embryos per stage was performed by pronase treatment (1 mg pronase/mL of fish embryo medium (FEM) for 30 min; pronase was from *Streptomyces griseus*, Fluka; FEM was: 106 mM NaCl, 3 mM KCL, 3 mM $CaCl_2$, 1 mM $MgSO_4$, 100 mM sucrose, 3 $g \cdot L^{-1}$ BSA, 5 mM Hepes sodium salt, 15 mM Bicine, pH 7.5, 320 mOsm). The dechorionated embryos were at the following stages: morula (256–512 cells), after 5 h development at 22 °C; early blastula (516–1 000 cells), after 5.5 h development at 22 °C and late blastula (> 1 000 cells), after 6 h development at 22 °C. After having been washed three times in FEM, the dechorionated embryos were mechanically detached from the yolk by suction with glass capillary pipettes (0.8-mm internal diameter). Around 20 embryos per stage were finally utilised for the purpose of the experiment and placed in Ca^{2+}- and Mg^{2+}-free FEM where cells were left to dissociate.

2.3. Cells and freezing

After complete dissociation (20 min), the calcium concentration of the medium was adjusted to 20 µM. The whole freezing and thawing procedures were performed as described for rainbow trout blastomeres [6]. Briefly, cryoprotection was provided by the step-wise addition of 1.4 M 1,2 propanediol into FEM and cells loaded into 250-µL straws and slowly frozen to –80 °C (seeding at –6.6 °C) before being plunged into liquid nitrogen. A low thawing rate was adopted by thawing the straws on melting ice for 10 min, followed by the step-wise removal of the cryoprotectant.

Fresh cell controls were left in FEM with 1.4 M 1,2 propanediol at 22 °C for 3 h.

2.4. Viability assays

The viability of fresh, cryoprotectant conditioned, and frozen-thawed cells was evaluated by morphological, metabolic and mechanical activity.

Morphological integrity of the cells was evaluated by light microscopy (200×, 400×) and video recording was performed in order to detect their pseudopodial activity. Cell metabolism and overall survival rate were evaluated by viable staining with fluorescein diacetate (FDA) [2] and by counting the fluorescent cells over the total number of cells on at least eight fields per slide sample. Cell mechanical activity was evaluated by transferring samples of thawed cells into FEM enriched with 10 % calf serum (Cool calf 2, Sigma) in order to detect their response to the presence of calcium and their ability to reassociate and form morula-like bodies.

2.5. Statistical analysis

The Wilcoxon T-paired ranks nonparametric test was used to compare survival rates for each stage.

3. RESULTS

3.1. Survival rates

Overall results in survival rates of the frozen-thawed blastomeres for the 3 stages are reported in *table I*. The cryoprotectant 1,2-propanediol showed very low toxicity on fresh blastomeres at all stages within 3 h at 22 °C with control values exceeding 95 % intact cells. The mean survival rate of morula cells was 89 ± 1.6 % and it differs significantly from the values of early blastula and late blastula cells (94 ± 0.6 and 96 ± 0.4 % respectively), which were not significantly different. A statistically significant difference was found between morula cells and both early blastula and late blastula controls, as well as between the survival rates of the frozen blastomeres and their unfrozen controls for the three stages. As shown in *figure 1*, morula cells showed the highest, however small, variability, as compared to early blastula and late blastula cells.

Table I. Mean survival rates (%), ± mean standard error, of isolated carp blastomeres before (control) and after freezing and thawing (frozen) for each embryonic stage considered in the experiment (n = 10). Means not marked by the same letter are significantly different, $P \leq 0.05$.

Stage	Morula	Early blastula	Late blastula
Control	95 ± 0.6^a	97 ± 0.4^b	98 ± 0.2^b
Frozen	89 ± 1.6^c	94 ± 0.6^a	$96 \pm 0.4^{a,b}$

3.2. Reaggregation

Frozen-thawed early blastula and late blastula cells reaggregated into morula-like bodies within 2 h culture in FEM enriched with 10 % calf serum, while morula cells did not show such a prompt adhesion to each other, although a pseudopodial activity in situ was detectable.

4. DISCUSSION

The accurate selection of the embryos before freezing might have contributed to the high survival rates of the frozen cells at all stages.

The difference between morula controls and early blastula and late blastula controls might stem from

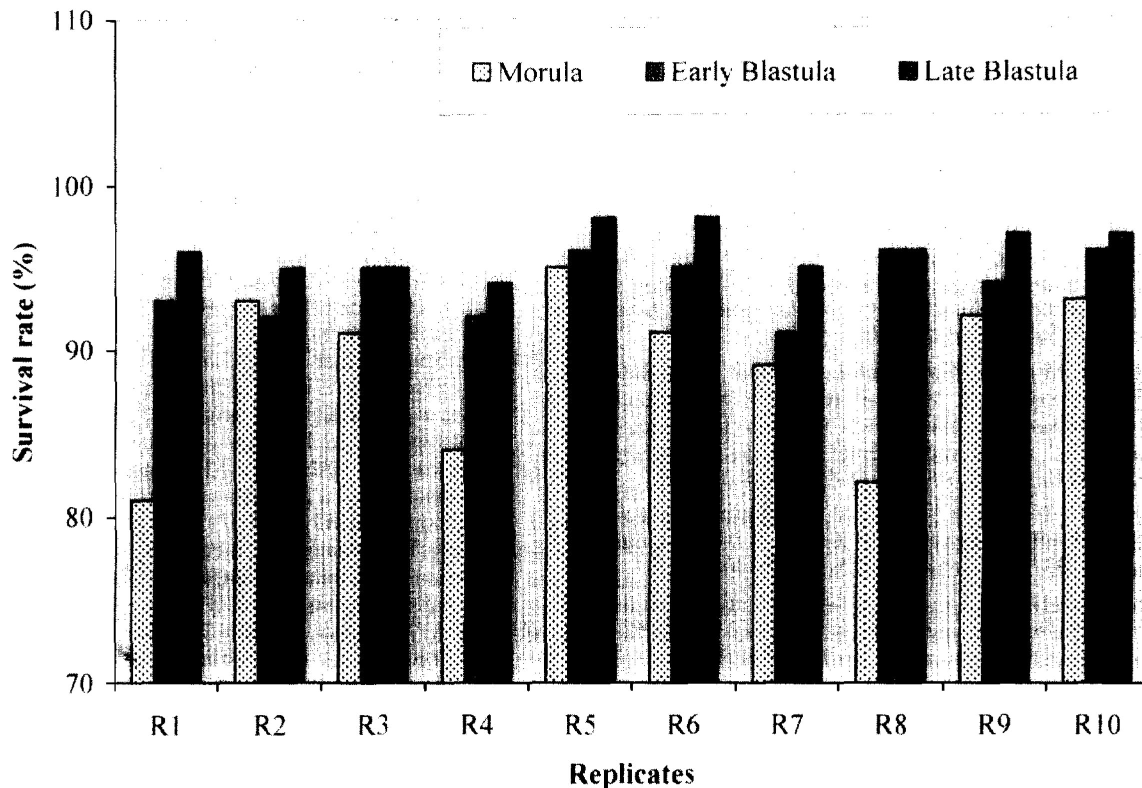

Figure 1. Scatter diagram showing the survival rate of isolated carp blastomeres after freezing and thawing for each embryonic developmental stage in each replicate.

mechanical damage caused by repeated mechanical pipetting applied to morula cells when the embryos were detached from the yolk. The procedure is much easier and quicker when a blastocoele is already formed, which is the case for early blastula and late blastula but not for morula embryos [7]. The better survival rates and reduction in variability with advancing stages, already observed in rainbow trout, would provide further evidence for the relationship between cell cycle and cold tolerance as suggested by Roubaud et al. [10]: the progressive desynchronisation of mitosis and the lengthening of cells' cycle with advancing stages, would reduce the probability for cells to be in their mitotic phase, therefore in their most sensitive phase, at the time of cooling and freezing. Active pseudopodial activity in frozen-thawed morula cells and quick reaggregation and formation of morula-like bodies for early blastula and late blastula cells suggest that their reaggregation ability is maintained after freeze-thawing.

5. CONCLUSION

Results obtained with carp blastomeres are consistent with those already reported for rainbow trout and suggest that the same freezing technique may be applied to other species. In this view, our preliminary results obtained with medaka (*Oryzias latipes*) are satisfying (survival rate after thawing above 85 %, unpubl. data). Further evidence has been provided on the relationship between freezing tolerance of the blastomeres and stage of development of the embryo.

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