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Cryopreservation of rainbow trout (*Oncorhynchus mykiss*) blastomeres

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Abstract

Cryopreservation of sperm is an important component of genetic conservation programs for fish. However, since fish eggs or embryos cannot be successfully cryopreserved and since the mitochondrial DNA is maternally inherited, the mitochondrial genomes of fish populations are presently not being preserved in these programs. One method of obtaining eggs from stored materials would be to cryopreserve embryonic cells that could later be transplanted into female recipient embryos, forming chimeras; a proportion of these transplanted cells would be expected to enter the germ line and subsequently develop into eggs. The hypothesis of this study is that isolated blastomeres of rainbow trout can be successfully cryopreserved and thawed. The objective of this investigation was to determine if the rate of cooling or the type of container alters cryopreservation success. Isolated blastomeres in a freezing solution containing 8.7% DMSO were subjected to one of two different cooling rates in either 0.5 ml French semen straws or cryovials. Thawed samples were examined using phase contrast microscopy, with cells scored as being either intact or non-intact. In the nonfrozen control group, 85.4% ± 7.6 ($\bar{x}$ ± S.D.) of the blastomeres were intact. There was no significant difference between the cooling rates used in this investigation based on the proportion of intact cells, but there was a significant ($p < 0.002$) difference in the proportion of intact cells between samples frozen in French straws (35.9% ± 16.5) and those frozen in cryovials (19.4% ± 4.9).

Keywords: Cryoconservation, blastomere, trout, *Oncorhynchus mykiss*.

Cryoconservation de blastomères de la truite arc-en-ciel (Oncorhynchus mykiss).

Résumé

La cryoconservation de sperme est un composant important des programmes de conservation génétique pour les poissons. Cependant, alors que les œufs ou les embryons de poisson ne peuvent être conservés avec succès et que l'ADN mitochondrial est un héritage maternel, les génomes mitochondriaux des populations de poissons sont conservés dans le cadre de ces programmes. Une méthode d'obtention d'œufs à partir de matériel conservé serait de conserver des cellules embryonnaires qui pourraient être plus tard transplantées dans des embryons, formant des chimères; une proportion de ces cellules transplantées devrait ainsi faire partie de la lignée germinale et par la suite se développer en œufs. L'hypothèse de cette étude est que des blastomères isolés de truite arc-en-ciel peuvent être successivement cryoconservés et décongelés. L'objectif de ces recherches était de déterminer si la vitesse de refroidissement ou si le type de récipient altère le succès de la cryoconservation. Des blastomères isolés dans une solution réfrigérante contenant 8,7% DMSO ont été soumis à une ou deux vitesses de refroidissement différentes dans des paillettes françaises de 0,5 ml ou dans des cryotubes. Les échantillons décongelés ont été examinés sous microscope à contraste de phases, en comptant les cellules intactes ou non. Dans le groupe témoin non congelé, 85,7 ± 7,6% ($\bar{x}$ + S.D.) des blastomères étaient intacts. Il n'y avait de différence significative entre les vitesses de refroidissement utilisées dans cette étude basée sur la proportion de cellules intactes, mais il y avait une différence significative ($p < 0,002$)

dans la proportion de cellules intactes entre les échantillons congelés dans les paillettes françaises (35,9 + 16,5 %) et ceux congelés dans les cryotubes (19,4 ± 4,9 %).

Mots-clés : Cryoconservation, blastomère, truite, *Oncorhynchus mykiss*.

INTRODUCTION

Cryopreservation of gametes or embryos is an important component of numerous conservation programs designed to maintain the genetic diversity of species. Cryopreservation of salmonid sperm is successful (Stoss, 1983; review) and is presently being used in Norway to preserve the genetic structure of native Atlantic salmon (Bergen *et al.*, 1991), but there are no successful protocols for the cryopreservation of salmonid eggs or embryos. Therefore, although the nuclear genome of threatened and endangered salmonid populations can be cryopreserved and stored in liquid nitrogen for extended time periods, the mitochondrial DNA inherited maternally through the oocytes is lost. An alternative or additional approach is to cryopreserve embryonic cells that could be eventually transplanted into female, recipient embryos in order to re-establish the individual cells into a germ line and which would subsequently develop into eggs.

The successful transplantation of isolated embryonic cells into recipient blastulae has been recently demonstrated for both rainbow trout (Nilsson and Cloud, 1989; Nilsson and Cloud, 1992) and zebrafish (Lin *et al.*, 1992). For both species, the investigators have shown that the donor cells participated in the development of the recipient embryo, and the data from these investigations are consistent with the hypothesis that blastomeres of fish blastulae are pluripotent. Additionally, because the life cycle of zebrafish is relatively short, Lin *et al.* (1992) were able to show that the transplanted blastomeres could also contribute to the germ line of the resultant chimeras.

Harvey (1983) demonstrated that individual fish blastomeres could survive freezing and thawing using isolated embryonic cells derived from zebrafish blastoderms at half epiboly. Therefore, we hypothesized that a proportion of the much larger blastula cells of rainbow trout would also withstand cryopreservation and thawing. Two factors which are known to affect how well cells withstand cryopreservation are the rate at which cells are cooled (Mazur, 1970) and the shape of the container in which cells are cooled (Massip *et al.*, 1979). The objective of this study was to determine if rate of cooling or sample container shape affects the proportion of rainbow trout blastomeres which survive cryopreservation.

MATERIALS AND METHODS

Gametes and fertilization

Rainbow trout gametes were obtained from Mt. Lassen Trout Farms, Redding, CA. Eggs were fertilized by the methods previously described (Nilsson and Cloud, 1992), and incubated in a Heath-Tecna incubator (Heath Tecna Corp., Kent, WA) at 11.5°C.

Harvest of blastomeres from embryos

Following sixty to seventy-two hours incubation, Ballard stage seven trout embryos (Ballard, 1973) were placed in Ca^{+2}-Mg^{+2} free Niu Twitty's solution (King, 1966) and manually de-chorionated. For each embryo, the blastoderm was dissected from the yolk sac, placed into a well of a twenty-four well plate with 1 ml of Ca^{+2}-Mg^{+2} free Niu Twitty's solution and allowed to dissociate for 20-30 min.

Addition of cryoprotectant to blastomeres

One half ml of supernatant was removed from each well, leaving the cells settled on the bottom. DMSO was added in a step-wise fashion in Ca^{2+}-Mg^{2+} free Niu Twitty's solution. The cells were allowed to equilibrate for ten minutes at each of five sequential steps with a final concentration equal to 8.7% DMSO.

Freezing protocols and containers

Blastomeres suspended in 0.5 ml freezing solution were placed into either 0.5 ml French semen straws (Cassou, France) or 1.8 ml cryovials (NUNC), before being subjected to one of the following two freezing protocols (Cryomed model 1010 operating instructions, 1992) using a temperature controlled freezing chamber (Cryomed model 1010; New Baltimore, MI, U.S.A.):

1. Faster cooling rate:
   a) Cool at 1°/min from 4° to −4°C
   b) Induce ice formation
   c) Cool at 1°/min to −40°C
   d) Cool at 10°/min to −90°C
   e) Plunge into liquid nitrogen

2. Slower cooling rate:
a) Cool at 1°/min from 4° to −4°C
b) Cool at 0.5°/min from −4° to −7°C
c) Induce ice formation
d) Cool at 0.3°/min to −40°C
e) Cool at 2°/min to −80°C
f) Plunge into liquid nitrogen.

Samples were stored in liquid nitrogen for forty-eight hours or more before thawing.

Thawing procedure and viability evaluation

Samples were removed from liquid nitrogen and placed into ice water (0°) to thaw. After five minutes, the thawed cell suspensions were transferred to wells in a 24 well culture plate and held for five minutes at 4° before being examined using an inverted phase contrast-microscope (Nikon Diaphot). Between 100 and 200 blastomeres in each sample were examined and categorized as either intact or non-intact, based primarily on whether the cell borders were smooth, the nucleus was prominent and the cytoplasm was intact and fine-textured (*fig.* 1). The percentage of intact cells was calculated for each sample.

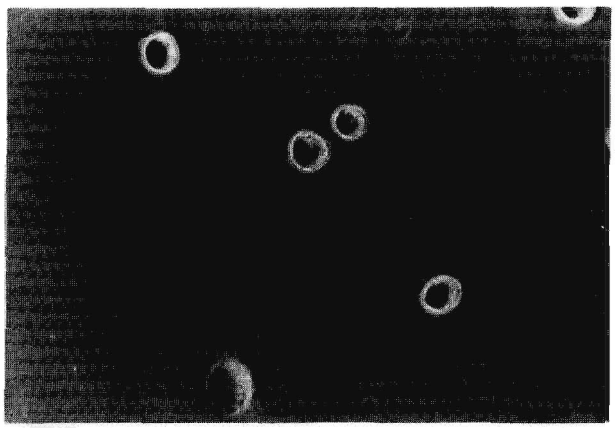

Figure 1. – Photomicrograph of blastomeres viewed with phase contrast microscopy. A: Intact blastomeres. B: Typical non-intact blastomere.

Statistical analysis

A two way analysis of variance was performed to examine the effects of container type and cooling rate on what proportion of blastomeres are intact after thawing. Data were arcsin transformed prior to analysis. The analysis was run using SAS° release 6.07 with the GLM subroutine and the following model:

$$y_{ij} = \mu + C_i + R_j + e_{ij} \quad (1)$$

where μ is the population mean, C_i is the effect of container type i (straw or cryovial), R_j is the effect of freezing rate (faster or slower), and e_{ij} is random error.

RESULTS

For the nonfrozen control group isolated cells were subjected to the same concentrations of cryoprotectant as were those samples which were frozen and thawed. In the nonfrozen control group 85% of the cells were intact. In contrast, cell integrity of cryopreserved and thawed groups of blastomeres ranged from 23% to 42% of the control mean (*table* 1).

Table 1. – Effect of cryocontainer and rate of cooling on the proportion of intact cells after thawing.

Treatment groups	Percent intact cells (Mean ± S.D.)
Non frozen control	85.4 ± 7.6 (12)*
French straws: Faster cooling rate	32.0 ± 20.5 (10)
French strasws: Slower cooling rate	39.4 ± 11.6 (11)
Cryovials: Faster cooling rate	16.2 ± 11.6 (12)
Cryovials: Slower cooling rate	23.4 ± 17.9 (10)

* The number in parenthesis equals the number of samples evaluated.

Statistical analysis showed no significant difference ($p > 0.149$), between the faster and slower protocols on cell integrity after thawing, but the samples cooled more slowly had higher mean values of intact cells in both the French straw and cryovial treatment groups.

There was a significant statistical difference ($p < 0.002$) in cell integrity between cells frozen in French straws and cells frozen in cryovials. An average of 36% of the blastomeres cryopreserved in straws were intact after thawing, compared with 19% intact with cryovials. The highest proportion of intact cells was seen in cells that were frozen in straws at the slower cooling rate (39.4%, see *table* 1).

DISCUSSION

The improved cell integrity seen in samples cryopreserved in straws as compared to cryovials might be attributed to the larger surface area of French straws allowing more even cooling of the cell suspension and quicker equilibration with temperatures in the freezing chamber.

The two freezing protocols used in this study differ primarily in the rate at which the samples are cooled after ice crystal formation has been initiated. Freezing protocol #1 is derived from protocols recommended for cryopreserving cells from *in vitro* culture, and

cools samples at 1°/min. Freezing protocol #2 is derived from protocols designed for cryopreserving mammalian embryos, which are somewhat larger (approximately 100 µm) than the individual trout blastomeres used in this study (40-50 µm). This second protocol cools samples at 0.3°/min.

The endpoint of these experiments, the morphological appearance of the blastomeres, was based on similar methods of visual evaluation of mammalian embryos (Shea, 1981; Lindner and Wright, 1983). Although this visual inspection is rapid and non-invasive, other tests used to evaluate viability of mammalian embryos (Butler and Biggers, 1989) may be more appropriate and are being examined presently as to there usefulness to evaluate the developmental potential of isolated trout blastomeres.

Harvey in 1983 demonstrated that isolated blastomeres of zebrafish could be frozen and thawed with an 85% survival rate. This is a considerably higher survival rate than exhibited by the trout blastomeres in this study (*table* 1). It is possible that the larger size of trout blastomeres (40-50 µm vs. 10-15 µm for zebrafish blastomeres) resulted in a difference in the diffusion of cryoprotectant into the cells or the movement of water out of the cells during freeze/thawing, leading to increased cellular damage for the trout cells. Alternatively the zebrafish cells were derived from embryos at a different developmental stage than the rainbow trout which may also affect their ability to withstand chilling.

Since only about 15% of the cells derived from one donor embryo are transplanted into any one recipient during the production of chimeras (Nilsson and Cloud, 1989; Nilsson and Cloud, 1992), the rate of survival of the blastomeres in this investigation (39.4%) is acceptable, because enough viable cells are available for transplantation. However, whether these thawed blastomeres still retain the capability to participate in development following transplantation into a recipient embryo still remains to be tested.

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