Cryopreservation of rainbow trout blastoderm

by

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ABSTRACT. - In the present study, we successfully cryopreserved entire blastoderms of rainbow trout, Oncorhynchus mykiss, embryos being at around midblastula stage of development. The method is a potential alternative to cryopreserving the whole fish embryo, not successful so far.

Key words. - Blastoderm - Blastomeres - Cryopreservation - Embryonic Cells - Trout.

Introduction

No method exists for successful cryopreservation of fish embryo due to a number of reasons related mostly to the big size of embryos and insufficient permeation of cryoprotective substances to cells (Leveroni Calvi and Maisse, 1998). However, isolated blastomeres, that is embryonic cells being at early developmental stages before differentiation, can be successfully cryopreserved, retaining high viability (Harvey, 1983; Leveroni Calvi and Maisse, 1998) and capacity to undergo further development after transplantation to a recipient embryo, including germline transmission (e.g. Kusuda et al., 2004). The major limitations of blastomere cryopreservation include low donor rates, random transplant sites, and low, if any, germline transmission. Regarding the latter aspect, Goro Yoshizaki’s group has recently developed a technology for manipulations of primordial germ cells, including their harvesting, cryopreservation and germline transmission (Kobayashi et al., 2007). Cryopreservation of the entire blastoderm would be an interesting alternative method giving control on donor transmission rate as well as on transplant size and site. Methods for transplanting fish blastoderms have been developed in cyprinids (e.g. Yamaha et al., 1997) and, recently, in salmonids (Babiak et al., in preparation). To date, cryopreservation of entire zebrafish blastoderm showed very limited success (Harvey, 1983) and no other attempts to cryopreserve fish blastoderms are known to us.

The goal of the present study was to determine a suitable protocol for cryopreserving blastoderms of an aquaculture fish species, rainbow trout, Oncorhynchus mykiss. We tested two commercially available cryopreservation media and two published research extenders, used previously to cryopreserve salmonid free embryonic cells. Also, we compared two freezing-thawing protocols, and two developmental stages at which the blastoderms were harvested. We also cryopreserved dispersed blastomeres in order to have positive controls for blastoderms.

Methods

Gametes were collected from rainbow trout breeders (4 females and 4 males) kept at the Department of Salmonid Research, Inland Fisheries Institute in Rutki, Poland and then transported to the Department of Animal Biochemistry and Biotechnology, Olsztyn University, Poland. After fertilization, eggs were incubated at 12°C or at 2-4°C to prolong availability of embryos at early to late blastula stage during experiments (Babiak and Dabrowski, 2003).

In experiment 1, four extenders were tested for suitability to cryopreserve both dispersed blastomeres and whole blastoderms. Extenders tested were: 1, medium-DMSO, containing Minimal Essential Medium, Fetal Bovine Serum and Calf Serum Albumin (Sigma Aldrich C6164); 2, medium-glycerol (the same as previous but containing glycerol, Sigma Aldrich C6039); 3, medium by Leveroni Calvi and Maisse, containing 1.4 M 1,2-propanediol as a cryoprotectant (1998); and 4, medium by Kobayashi et al. (2003), containing 1.8 M ethylene glycol as a cryoprotectant. The whole blastoderms were isolated from embryos being at approximately mid-late blastula stage (c. 25 days) after microsurgical cutting a chorion. Modified Hanks’ Balanced Salt Solution (HBSS; Sigma Aldrich H 8264), containing Ca²⁺ and Mg²⁺, was an operation medium. Isolated blastoderms were kept intact in HBSS for approximately 30 min. Their structure and cell integrity was not altered during the incubation. Freezing and thawing were proceeded according to Leveroni Calvi and Maisse (1998) with some modifications. Two blastoderms or dispersed blastomeres from three blastoderms per one 0.25 ml French straw were used. Four straws per treatment were used. Blastoderms were put into 0.3 ml of HBBS + 0.3% Bovine Serum Albumine (BSA) or 0.3 ml of PBS + 0.3% BSA, respectively, to cryopreserve whole blastoderms or dispersed blastomeres. In the latter case, the samples were gently shaken to disperse blastomeres. Extenders were added stepwise, according to Leveroni Calvi and Maisse (1998) method. Loaded straws were kept at 10°C for 2 h before freezing. Thawing procedure was according to Leveroni Calvi and Maisse (1998) except that thawing media were HBBS+BSA and PBS+BSA, for blastoderms and blastomeres, respectively. The structure of frozen-thawed blastoderms

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They were incubated in PBS for 5 min than gently shaken. After thawing and stabilizing was completed, Eppendors with dispersed blastomeres were centrifuged (300 rpm, 3 min) to facilitate collection of cells. Survival of cryopreserved cells was estimated using Live/Dead viability kit (Invitrogen), which method was subsequently standardized in our lab to trypan blue staining as a reference. For each straw, counting of random 100 cells in triplicate was performed under a microscope with epifluorescence light. Cells incorporating any propidium iodide (red colour) were considered as dead whereas cells incorporating exclusively Sybr 14 (green colour) were considered as live.

In experiment 2, the purpose was to test the suitability of fast freezing protocol for blastoderm cryopreservation. Blastoderms or dispersed blastomeres were derived from a single batch of embryos being approximately at mid blastula stage (c. 21 days). Two extenders (medium-DMSO and Leveroni Calvi and Maisee, 1998) were tested. An extender was added stepwise as in experiment 1. Straws were frozen according to the method by Kusuda et al. (2002). Two blastoderms or dispersed blastomeres from 3 blastoderms were loaded per straw. Straws were kept in liquid nitrogen overnight. Thawing was as in Kusuda et al. (2002). Straws were submerged in 15°C water bath for 20 s, and then the content was released to an Eppendorf tube. Further procedure was as in experiment 1.

In experiment 3, the purpose was to determine whether blastoderms being in early blastula stage of development could be cryopreserved as successfully as blastoderms being more advanced in the development (experiments 1 and 2). Whole blastoderms, blastoderm fragments (10-50%), or dispersed blastomeres were frozen in Leveroni Calvi and Maisee (1998) medium following Leveroni Calvi and Maisee freezing protocol. Embryos were derived from a single batch of eggs, being at early-mid blastula stage (16 days). Cells were prepared and equilibrated in extender like in experiment 1. They were thawed after 1 h of keeping in liquid nitrogen and check for viability as in experiment 1.

For statistical analyses, percentage data were transformed (arc sine square roots transformation) and ANOVA was performed. Tukey’s post hoc test was used to determine significance of differences between treatments (values from experiments 1 and 2 were not included because only single live cells were found).

Results and discussion

In experiments 1 and 2, the type of freezing medium used was the important factor (F = 225, p < 0.001 and F = 794, p < 0.001, respectively) whereas the status of material cryopreserved (dispersed blastomeres versus the whole blastoderm) showed no effect (F = 0.2, p > 0.05 and F = 3.4, p > 0.05, respectively). Leveroni Calvi and Maisee (1998) medium was the best of tested, securing survival of cryopreserved cells at the level of control, with except for survival of dispersed blastomeres in experiment 2 (Tab. 1). Slower freezing-thawing protocol (Leveroni Calvi and Maisee, experiment 1, and Kusuda et al., experiment 2) respectively showed no considerable differences in the efficiency, when comparing survival of cryopreserved cells to controls (Tab. 1). In experiment 3, survival of cryopreserved dispersed blastomeres was significantly higher than survival of cells frozen in whole blastoderms or blastoderm fragments (Tab. 1).

The results of the study clearly demonstrate that isolated entire blastoderm of rainbow trout can be successfully cryopreserved. The smaller rainbow trout blastomeres are, the higher survival rate after cryopreservation is (Leveroni Calvi and Maisee, 1998). In experiments 1 and 2 of the present study, blastoderms and dispersed blastomeres were derived from embryos being at approximately midblastula stage, corresponding to Ballard’s 6C stage (Ballard, 1973). At this developmental stage, Leveroni Calvi and Maisee (1998) obtained the highest survival rates of cryopreserved blastomeres. In our study, survival of cells cryopreserved in the whole blastoderm or dispersed before freezing was similar, indicating that penetration of deep layer cells with cryoprotective components of the extender was adequate. Indeed, at this stage, blastomeres are not firmly attached to each other and they can be easily dissected, as well as the whole blastoderm can be easily dissected from the yolk cell (own observations, unpublished). In experiment 3, survival rates of cells frozen in blastoderms or blastoderm fragments were significantly lower than in the case of dispersed blastomeres. This developmental stage corresponded approximately to Ballard’s 6B stage (Ballard, 1973). At this stage, the cryoprotective action to the deeper layer cells in the blastoderm was likely insufficient. Blastomeres at this stage are bigger, firm-
ly attached to each other and their mechanical dissection is difficult (own observations, unpublished).

It has been demonstrated that cryopreserved blastomeres can proliferate (Leveroni Calvi and Maisse, 1998) and contribute to the somatic and germ lines of the recipient after transplantation (Kusuda et al., 2004). Therefore, there is a chance that cryopreserved blastoderm can incorporate into the host, including germline transmission. Aside of technology for cryopreserving isolated primordial germ cells (Kobayashi et al., 2007), cryopreservation of blastoderms is a potent alternative method to overcome technical problems of fish embryo cryopreservation, especially that the technique for blastoderm transplantation in salmonids does exist (Babiak et al., in preparation).

Conclusions

Isolated whole blastoderms of rainbow trout can be successfully cryopreserved. Survival rates are comparable with those obtained with dispersed blastomeres when embryos are approximately at mid-late blastula developmental stage, but lower than in the case of dispersed blastomeres, when embryos are at earlier developmental stage.

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