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Cryobanking of fish somatic cells: Optimizations of fin explant culture and fin cell cryopreservation

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Abstract

When gametes or embryos are not available, somatic cells should be considered for fish genome cryobanking of valuable or endangered fish. The objective of this work was to develop a method for fin explant culture with an assessed reliability, and to assess fin cells ability to cryopreservation. Anal fins from goldfish (*Carassius auratus*) were minced and gently loosened with collagenase before explants were plated at 20 °C in L-15 medium supplemented with fetal bovine serum and pH buffering additives. Quantification of cell-donor explants per fin rated the culture success. Cells were successfully obtained from every cultured anal fin (mean = 65% cell-donor explant per fin). All other fin types were suitable except the dorsal fin. Explant plating could be deferred 3 days from fin collecting. Fins from seven other fish species were successfully cultured with the method. After 2–3 weeks, sub-confluent fin cells from goldfish were cryopreserved. Cryopreservation with dimethyl sulfoxide and sucrose at a slow freezing rate allowed the recovery of half the goldfish fin cells. Cells displayed the same viability as fresh ones. 1,2-propanediol was unsuitable when a fast freezing rate was used. The procedure could now be considered for cryobanking with only minimal adaptation to each new species.

Keywords: *Carassius auratus*; Cyprinidae; Goldfish; Primary culture; DMSO; Freezing; Anal fin; Cryobanking

1. Introduction

The use of somatic cells as nucleus carrier for fish genome cryobanking must be strongly considered when gametes and embryos are not available. This can be the case either in the wild, when the maturational state of the caught fish is uncertain, or in aquaculture for species whose sexual maturation takes several years, or when a dramatic epizooticy requires the genome to be collected quickly before the stock is slaughtered. Somatic cells cryopreservation and fish reconstruction with cloning technology could also be considered for fish bearing a valuable phenotype or genotype, in order to obtain large numbers of progeny from this single animal. In most cases, it is essential to recover the somatic cells from the fish body without sacrificing the animal, and fin explants are good candidates for this purpose. They are easy to sample and they have natural regenerative capacities (reviewed by Akimenko et al., 2003). This prevents long-term disabling of the fish and it should provide a good proliferation of cells from the fin in a culture system.

When dealing with valuable fish, the highest quality and reliability of a fin culture method is an absolute requirement in order to dependably obtain somatic cells which will be used for cryobanking. Previous works have described methods for fin cells culture, either to study cell proliferation and ageing (*Carassius auratus*; Shima et al., 1980), or to karyotype the cells (*C. auratus, Esox lucius*, *Sparus aurata*, and *Apogon imberbis*, Alvarez et al., 1991; Wang et al., 2003). Cells cultured from fin explants of bighead carp (*Aristichthys nobilis*) and medaka (*Oryzias latipes*) were also recently used for cloning (Liu et al., 2002; Ju et al., 2003). Some successful attempts to improve the cell culture method have compared culture media such as MEM and L-15, tested fish serum supplementation and culture temperature, and have compared cell line versus primary cells, or normal versus regenerated fin (*Oncorhynchus mykiss*, Mothersill et al., 1995; *C. auratus*Hashimoto et al., 1997; *Tor putitora*, Prasanna et al., 2000). Most of these experiments were intended to establish cell lines, and were therefore focused on the optimization of cell growth and doubling time of cell number. As

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a consequence, little attention was paid to the explants themselves and to the ability of fin explants from a given precious fish to unfailingly outgrow somatic cells.

Cryopreservation of fish cultured cells, mainly cell lines, was described for several fish species. It is the case in Chou et al. (1989) on the goldfish (*C. auratus*), in Zhang et al. (1998) on the channel catfish (*Ictalurus punctatus*), in Zhang and Rawson (2002) on bluegill sunfish (*Lepomis macrochirus*), and in Wang et al. (2003) on the sturgeon (*Acipenser transmontanus*). Good viability and efficient cell attachment after cryopreservation were often stated, but little documented information on cell losses after thawing was available. Again, if cell losses are not a problem when restarting a cell line after cryopreservation of large batches of cells, cell recovery after cryopreservation of small cell samples from rare fish is an important issue. Besides, only Chou et al. (1989) and Zhang and Rawson (2002) tested several cryopreservation conditions, and to our knowledge, no data are available on the optimization of a cryopreservation method for fin cells from primary culture.

The present work on fin cell culture and cryopreservation was set up with the perspective of cryobanking fish somatic cells from fin explants of highly valuable fish with an assessed and optimal success rate. We thereby focused our strategy on the improvement of the existing methods, on the assessment of factors susceptible to induce some variability in the results, and on the careful estimation of the cell recovery success. The model species that was chosen here was goldfish (*C. auratus*), a species belonging to the large Cyprinidae family. This family includes more than 2000 species belonging to 210 genera (Nelson, 1994), and most European freshwater species belong to it. Furthermore, many works on cloning have been conducted on Cyprinidae (reviewed by Zhu and Sun, 2000), including zebrafish (Lee et al., 2002) and goldfish (Sun et al., 2005). The application of the culture protocol was tested on several other species, and a cryopreservation procedure for cells grown out of fin explants was developed.

2. Materials and methods

2.1. Animals

Goldfish (*Carassius auratus*) were used to set up the fin explant culture and the fin cell cryopreservation. Goldfish were raised in outdoor ponds at the INRA U3E experimental farm at Le Rheu. Several weeks before sampling, they were transferred in 0.3 m^3 recycled water tanks and acclimated at 14 °C under a natural photoperiod, with a trout diet supplemented with tubifex. Fish from several other species were tested for fin explant culture: Cyprinidae fish species other than goldfish were ide (*Leuciscus idus*), bream (*Abramis brama*), roach (*Rutilus rutilus*), gudgeon (*Gobio gobio*) and zebrafish (*Danio rerio*). Fish belonging to 2 other families were also tested: European perch (*Perca fluviatilis*) belonging to Percidae, and rainbow trout (*O. mykiss*) belonging to Salmonidae. Rainbow trout and zebrafish were reared in recycled water tanks at 13 and 27 °C, respectively, under a natural photoperiod. The other species were caught in the Flume River (France) and kept in recycled water tanks at 12 and 15 °C under natural photoperiod. All fish were reared and manipulated by people with official authorization for fish manipulation. The sampling was conducted according to French regulations on laboratory animals.

2.2. Fin sampling and medium characteristics

The whole fin was wiped to remove mucus and cut from the fish with sterile scissors. It was washed 3 times for 3–10 min in 5 mL sterile medium A (culture medium L-15, pH 7.3 and osmolality 290 mOsm/kg, supplemented with HEPES 25 mM, $NaHCO_3$ 5 mM, gentamycin 100 μg/mL and amphotericin B 2.5 μg/mL). This medium was adapted to culture under air atmosphere. We observed that contrarily to medium A, the use of DMEM/F12 culture medium induced a rapid alkalinisation of the explant culture (pH > 10 within 2 days) although DMEM/F12 was successfully used by Alvarez et al. (1991) for several freshwater species, including goldfish. In our case, the explants had difficulties in adhering and cells did not proliferate. This problem was overcome by changing the type of the medium, increasing the pH buffering capacity with HEPES and keeping the bicarbonate content low (see composition medium A). All the reagents used were purchased from Sigma-Aldrich (Saint Louis, MO, USA) unless otherwise specified.

2.3. Cell culture and success rating

The next steps were performed under sterile conditions. After the largest fin rays were removed with a razor blade, the fins were chopped into 1 mm^2 explants with a tissue chopper (McIlwain, The Mickle Laboratory Engineering Co Ltd.). Unless specified, the explants were gently digested for 30 min in 10 mL of medium B (medium A with gentamycin reduced to 50 μg/mL, L-glutamine 2 mM-GIBCO/Invitrogen, Grand Island, USA, fetal bovine serum 10% v/v, GIBCO/Invitrogen, Grand Island, USA and collagenase 0.2 mg/mL). The explants were then rinsed with 10 mL medium C (medium B without collagenase) and distributed into 12-well plates (Costar®, Corning, New York, USA) at a density of about 10–15 explants per well. Explants were half covered by a layer of medium C (200 μL per well) and allowed to adhere for 3 days at 20 °C under air atmosphere.

Three days after explant plating, explants surrounded by adhering cells (Fig. 1a) were counted as adhering explants. The number of these successful explants was expressed as a percentage of total explants plated from a given fish, and the results were expressed as the percentage of cell-donor explants (one value for one fish, whatever the explant number). Medium C was then removed and 1 mL of medium D (medium C without fetal bovine serum and with 2% v/v Ultroser® SF, Biosepra, Cergy Saint Christophe, France) was added to each well. The medium was changed every 3 days. Confluence was reached after 3–4 weeks at 20 °C.

2.4. Experimental designs for fin explant culture

2.4.1. Collagenase pre-treatment of fin explants

The relevance of a collagenase pre-treatment of the explants on culture outcome was tested on goldfish anal fins. On each of 6