Journal of Fish Biology (1995) **47**, 1016–1025

Cryopreservation of leucocytes of channel catfish for subsequent cytogenetic analysis

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(*Received 28 October 1994, Accepted 7 February 1995*)

Channel catfish leucocytes cryopreserved with glycerol or dimethyl sulphoxide (DMSO) had significantly higher ($P<0{\cdot}05$) viability and recovery rates than did cells cryopreserved with methanol. After 7 days of frozen storage, a 24 to 27% reduction of viability was observed for cells cryopreserved with glycerol; a 25 to 43% reduction for cells frozen with DMSO, and a 67 to 100% reduction for cells frozen with methanol. The concentration of cryoprotectants affected the viability of cryopreserved cells significantly ($P<0{\cdot}05$). The viability reduction was 36% for cells frozen with 5% of cryoprotectants, 30% for cells frozen with 10% of cryoprotectants, and 49% for cells frozen with 15% of cryoprotectants. The viability of cells frozen at the slower rate ($-2{\cdot}7^{\circ}$ C min^{-1}) was significantly higher ($P<0{\cdot}05$) than that of cells frozen at the faster rate (-45° C min^{-1}). Best results were obtained for cells cryopreserved with 10% of glycerol or DMSO and frozen at the slower rate. The chromosomes prepared from cells cryopreserved using this procedure were identical to those prepared from fresh cells, and to those reported in the literature for channel catfish.

Key words: cryopreservation; leucocyte culture; chromosomes; *Ictalurus punctatus*.

INTRODUCTION

Cell culture is a standard procedure used for the preparation of fish chromosomes. Cultured cells have included epithelial cells (Ojima *et al.*, 1972), fibroblasts (Amemiya *et al.*, 1984), and leucocytes (Grammeltvedt, 1975). Cultured cells offer enhanced control of the cell cycle through use of treatments such as chemical synchronization of mitotic activity (Liu, 1986). Chromosomes prepared from cultured cells have minimal background debris, can be elongated, and can be prepared for banding and *in situ* hybridization studies. Cultured leucocytes have been employed as a source of chromosomes for a number of fishes including channel catfish, *Ictalurus punctatus* (Rafinesque) (e.g. Hudson, 1980; Wolters *et al.*, 1981).

Methods for the culture of channel catfish leucocytes are available, including long-term (Miller *et al.*, 1994) and short-term culture (Yammamoto & Ojima, 1973; Faulmann *et al.*, 1983). In each method, the addition of mitogens such as phytohaemagglutinin M, pokeweed, lipopolysaccharide or concanavalin A (Con A) is required to stimulate the propagation of various populations of lymphocytes. Long-term cultures of lymphocytes have been maintained continuously for more than 12 months without restimulation (Miller *et al.*, 1994). There are disadvantages, however, of using long-term culture methods for cytogenetic study. These include the labour and cost involved in maintaining cell lines, and

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0022–1112/95/121016+10 $12.00/0

the potential catastrophic loss of cultures. Also, it may be inadvisable to use long-term cultures of many passages for karyotyping because of the possibility of gain, loss or deformity of chromosomes (Sharma & Sharma, 1980).

Compared with long-term cell culture, short-term culture requires less labour, and is relatively cheap. Preparations of chromosomes from short-term cell cultures are reliable with respect to chromosome number and morphology. However, short-term cultures cannot be used repeatedly for chromosome study. The response of lymphocytes of channel catfish to mitogens has been found to be time-dependent, and to decline rapidly after 2–4 days of culture (Faulmann *et al.*, 1983). Such cultures require processing within 72 h after mitogenic stimulation to produce large numbers of metaphase cells (Wolters *et al.*, 1981; Gold *et al.*, 1990). Chemicals such as colchicine can be used to arrest cells at metaphase, but prolonged exposure to colchicine to increase the number of metaphase chromosome spreads can cause excessive contraction of chromosomes. In addition, it is not always possible to re-establish cultures by repeated sampling, especially of young or valuable fish. Therefore it would be useful to be able to store leucocytes for later study. The use of stored cells would be especially advantageous in chromosome banding studies which require exact conditions and timing of treatments. Storage would allow greater flexibility in timing of studies, and would allow direct comparison of cultures prepared from many fish at different times.

Cryopreservation has become a common method for storage of somatic cells (Penno *et al.*, 1993; Freshney, 1994), and sperm cells (Leung, 1991) of fish and higher vertebrates. In channel catfish, ovary (Bowser & Plumb, 1980) and sperm cells (Guest *et al.*, 1976; Tiersch *et al.*, 1994) were preserved successfully by freezing methods. However, there are no reports on freezing of channel catfish leucocytes. In this study we evaluated viability and cell number of channel catfish leucocytes after cryopreservation. The objectives were: (1) to evaluate the effect of three cryoprotectants at three concentrations; (2) to evaluate the effect of two freezing rates; and (3) to demonstrate preparation of chromosomes from cryopreserved leucocytes.

MATERIALS AND METHODS

LEUCOCYTE SEPARATION

Fish were maintained at the Aquaculture Research Facility, Louisiana State University Agricultural Center, Baton Rouge. Six healthy fish between 0·5 and 1·0 kg were used. Three ml of whole blood were drawn aseptically from the caudal vessels into heparinized syringes and transferred to 10 ml vacutainers. Blood was diluted 1 : 1 with RPMI-1640 incomplete medium (described below). Leucocytes were isolated by density gradient centrifugation using ficoll-hypaque solutions, following the manufacturer's procedure (Sigma Chemical Company, St Louis, MO) with minor modification. Three ml of each of two different densities of ficoll-hypaque solution were layered in a 15-ml centrifuge tube: Histopaque-1.119 was placed below a layer of Histopaque-1.083. Six ml of diluted whole blood were placed carefully on top of the Histopaque layers. The tubes were centrifuged at 450 *g* for 30 min at room temperature. After centrifugation, the leucocyte-rich layer (second from the top) was removed and washed three times with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (CMF-PBS) by centrifuging at 80 *g* for 5–7 min. After the final wash, the cell pellet was resuspended in 1 ml of RPMI-1640 incomplete medium.

CELL CULTURE

Preparation of culture medium for leucocytes was based on the procedure of Miller & Clem (1988). RPMI-160 incomplete medium was composed of 100 ml of RPMI-1640 medium (without L-glutamine) (Sigma), 2 mM L-glutamine (Gibco BRL Life Technologies, Inc., Grand Island, NY), 0·01 M Hepes buffer (Sigma), 0·01 mM sodium pyruvate (Sigma), 10 µl of non-essential amino acids (Sigma), 100 µg ml^{-1} of streptomycin (Gibco), 100 U ml^{-1} of penicillin (Gibco), and 0·1 ml of 2-mercaptoethanol (Sigma) stock solution (35 µl 10 ml^{-1} of RPMI-1640 medium). The incomplete medium was adjusted to an osmotic pressure of about 250 mosmol by addition of 10% (v/v) of deionized water (Faulmann *et al.*, 1983). Osmolality was measured with a vapour pressure osmometer (model 5500S, Wescor, Inc., Logan, UT). Complete medium was prepared in a 25-cm^2 flask immediately before use. The complete medium consisted of 7·5 ml of RPMI-1640 incomplete medium, 1·0 ml of human serum (Sigma; male, blood type AB), and 0·5 ml of channel catfish serum. Channel catfish serum was prepared by collecting blood of channel catfish into vacutainers containing no additive. The pooled serum was heated at 56° C for 30 min, and sterilized by passing through 0·22-µm syringe filter. One ml of well-suspended leucocytes was added to each flask. Cells were cultured at 26 ± 1° C with a supplementation of 5% humidified CO_2. For chromosomal studies, Concanavalin A (Sigma) was added at a final concentration of 5 µg ml^{-1}. After 48 h of culture, 5′-bromodeoxyuridine (Sigma) was added to a final concentration 50 µg ml^{-1} to produce elongated chromosomes, and cells were cultured for another 24 h before harvesting.

FREEZING AND THAWING PROCEDURES

After 68–72 h, the medium was aspirated. Cells were washed with CMF-PBS, with minimal agitation, and fresh incomplete medium was added to the flask. Cells were resuspended in the medium, first by hand-stirring, and then by pipetting. The cell suspension was centrifuged at 80 *g* for 7 min, and the pellet was resuspended in incomplete medium supplemented with 20% fetal bovine serum (Gibco), the volume of which was adjusted based on the cell concentration viability.

Study 1: comparison of cryoprotectants and concentration

In the first experiment, three cryoprotectants, glycerol, dimethyl sulphoxide (DMSO), or methanol, were added to the cells at final concentrations of 5, 10 or 15% (v/v). Cryoprotectants were added immediately before the cultures were aliquoted for freezing. A control, with medium only added, was included for each cryoprotectant. Cell suspensions were mixed and drawn into standard 0·5-ml straws (IMV International Corp., Minneapolis, MN) used for sperm cryopreservation. The equilibration period prior to loading in the freezer was ≤10 min. The straws were grouped, placed into 10-mm plastic goblets, and loaded into a computer-controlled freezer (Kryo 10 Series II, Planer Products Ltd, U.K.). Cells were frozen by the following programme: hold at chamber temperature of 5° C for 10 min; freeze at a rate of −45° C min^{-1} until a final temperature of −80° C; hold at −80° C for 20 min. At the end of the programme, cells were plunged into liquid nitrogen for storage until use. After 1 week, frozen cells were thawed by immersion for 7 s in a water bath at 45° C.

Study 2: comparison of cryoprotectants and freezing rates

The same three cryoprotectants were utilized in a second experiment at a final concentration of 10% (v/v), based on the results of the first study. Cells in 0·5-ml straws were frozen at two rates: the (fast) programme described above, and the following (slow) programme: hold at chamber temperature of 5° C for 10 min, cool at a rate of −1° C min^{-1} until 0° C, hold for 5 min; freeze at −2·7° C min^{-1} until −80° C, and hold for 5 min prior to storage in liquid nitrogen. Control treatments, with no cryoprotectants added, were included for each freezing rate. Cells were thawed as described above.

Study 3: preparation of chromosomes from cryopreserved cells

Based on results of the cryoprotectant and freezing-rate studies, 10% glycerol and 10% DMSO were chosen as cryoprotectants. Cultured cells were pooled into sterile 1·5-ml cryovials (Sigma) and were frozen by the slow programme (cooling rate of $-1°$ C min^{-1} until 0° C, and freezing rate of $-2·7°$ C min^{-1} until $-80°$ C). The equilibration time prior to loading in the freezer was ≤ 10 min. Frozen cells were thawed by placing cryovials in a 15-ml centrifuge tube, and gently shaking the tube for about 100 s in a 37° C water bath until crystals disappeared. Cell suspensions were pooled into 15-ml centrifuge tubes, diluted with RPMI-1640 incomplete medium and centrifuged at 80 ***g*** for 5 min. Cell pellets were resuspended in 1 ml of RPMI-1640 incomplete medium and cultured for 4 to 5 h using the method described above. One hour before harvesting, 0·1 ml of colchicine stock solution (200 μg ml^{-1} in CMF-PBS) was added to each culture. Hypotonic treatment using 0·075 M KCl for 25 min was followed by cold fixation in Carnoy's fixative (3 : 1 methanol–acetic acid), repeated three times, for 30 min each. Cells were dropped onto cold, wet microscope slides, dried at 40° C overnight, and stained for 30 min with 5% Giemsa (Sigma) prepared in 0·01 M phosphate buffer at pH 6·8 (Sambrook *et al.*, 1989).

ANALYSIS OF VIABILITY AND CELL CONCENTRATION

Viability and cell concentration were calculated based on counting of live and dead leucocytes using a dye-exclusion method. Cell samples from each culture (before freezing) and each treatment (after freezing) were diluted and stained with 0·1% trypan blue, and within 20 min counted in a haemacytometer at 400 × using phase-contrast microscopy (Nikon Optiphot-2). In this technique, live cells were unstained, clear, and surrounded by a refractile ring, while dead cells were stained blue and lacked the refractile ring. Calculation of cell concentration was based on numbers of cells lying within eight 1-mm^2 corner squares. Percent viability was calculated as: 100 × (the number of viable cells/the total number of viable and dead cells).

STATISTICAL ANALYSES

In the cryoprotectant study, viability and cell number were analysed by two-factor ANOVA with cryoprotectant (glycerol, DMSO, or methanol) and concentration (0, 5, 10 or 15%) as the factors. In the freezing-rate study, the same parameters were analysed by two-factor ANOVA with cryoprotectant (glycerol, DMSO, methanol, or control), and freezing rate (fast or slow) as the two factors. Duncan's multiple means comparison was used to identify differences among treatments. The percent viability data were arcsine-square root transformed prior to analysis. Treatments were considered significantly different when $P<0·05$.

RESULTS

STUDY 1: COMPARISON OF CRYOPROTECTANTS AND CONCENTRATIONS

After 7 days of frozen storage, a significant ($P<0·0001$) reduction (31 to 74%) of viable cells was observed for leucocytes cryopreserved with glycerol, DMSO, or methanol. Viability of leucocytes cryopreserved with glycerol or DMSO was significantly higher than that of cells frozen with methanol ($P<0·05$), no difference was found between glycerol and DMSO (Table I). The viabilities of cryopreserved cells were significantly different at the three concentrations ($P=0·0001$). The viability was 51 ± 15% for cells frozen with 5% cryoprotectant, 57 ± 17% for cells frozen with 10% cryoprotectant, and 38 ± 27% for cells frozen with 15% cryoprotectant. A significant ($P<0·05$) difference was observed for comparisons of any two concentrations (Table I).

TABLE I. Percent viability and total cell number (mean ± S.D.) of channel catfish leucocytes* cryopreserved with three concentrations of three cryoprotectants

Concentration (v/v)	Viability (%)				Total cell number (× 10^6)			
	Glycerol	DMSO	Methanol	Pooled (by concentration)	Glycerol	DMSO	Methanol	Pooled (by concentration)
Control (0%)	0	0	0	0^D	3·1 ± 0·3	3·5 ± 0·2	3·5 ± 0·1	$3·4 ± 0·6^C$
5%	59 ± 6	59 ± 11	34 ± 8	$51 ± 15^B$	5·2 ± 0·5	5·6 ± 1·6	3·4 ± 1·1	$4·7 ± 1·6^B$
10%	66 ± 5	65 ± 2	29 ± 13	$57 ± 17^A$	5·5 ± 0·6	3·6 ± 0·4	4·2 ± 0·1	$4·3 ± 1·2^B$
15%	56 ± 1	50 ± 6	0	$38 ± 27^C$	6·3 ± 0·2	5·9 ± 1·3	4·3 ± 0·2	$5·6 ± 1·2^A$
Pooled (by cryoprotectant)	$60 ± 6^A$	$59 ± 9^A$	$23 ± 17^B$		$5·7 ± 1·6^A$	$4·8 ± 1·5^A$	$3·9 ± 0·8^B$	

Values sharing a letter in pooled columns (cryoprotectant) or rows (concentrations), were not significantly different ($P>0·05$). Control (0%) values were not included in calculation of pooled values for each cryoprotectant.

*Initial viability=87%; initial total cell number per treatment=$6·7 \times 10^6$; sample size (number of replicates) was nine for all treatments except controls ($n=6$).

No viable cells were found in the control treatments frozen and thawed without cryoprotectant.

Total cell number of cryopreserved leucocytes treated with glycerol, DMSO or methanol was also significantly different ($P=0{\cdot}004$). Leucocytes cryopreserved with glycerol or DMSO had higher ($P<0{\cdot}05$) total cell numbers than did leucocytes treated with methanol, but no difference was found between cells treated with glycerol and DMSO (Table I). Total cell number of cells frozen with 15% of cryoprotectants was significantly higher than that of cells frozen with 5 or 10% of cryoprotectants ($P<0{\cdot}05$); no difference was found between cells cryopreserved with 5 or 10% of cryoprotectants. Total cell numbers of control treatments (with no cryoprotectants added) was significantly lower ($P<0{\cdot}05$) than that of cells frozen with cryoprotectant (Table I).

A significant interaction between cryoprotectant and concentration was observed ($P=0{\cdot}0001$). The viability of cells frozen with 10% DMSO and 10% glycerol was higher than that of cells frozen with 5% DMSO and 5% glycerol, while viability of cells frozen with 10% methanol was lower than that of cells frozen with 5% methanol (Table I).

STUDY 2: COMPARISON OF CRYOPROTECTANTS AND FREEZING RATES

After 7 days of frozen storage, a 28 to 96% reduction of viable cells was observed (Table II). The viability of cryopreserved leucocytes, treated with 10% of each cryoprotectant was significantly different ($P=0{\cdot}0001$). Percent viability (mean ± S.D.) was highest for leucocytes cryopreserved with glycerol ($49 \pm 23\%$) followed by DMSO ($41 \pm 19\%$) and methanol ($3 \pm 3\%$) (Table II). These values were each significantly different from one another ($P<0{\cdot}05$). Control cells frozen without cryoprotectant had <1% viability. Total cell numbers (mean ± S.D., expressed as $\times 10^6$) was highest for leucocytes cryopreserved with DMSO ($2{\cdot}6 \pm 1{\cdot}2$) followed by glycerol ($2{\cdot}0 \pm 0{\cdot}4$), methanol ($2{\cdot}0 \pm 0{\cdot}5$), and control ($1{\cdot}1 \pm 0{\cdot}3$). Each pairwise treatment comparison was significantly different ($P<0{\cdot}05$) except for that of glycerol and methanol, which was not different ($P>0{\cdot}05$) (Table II).

The viability of leucocytes frozen at the slow rate was significantly higher ($P=0{\cdot}006$) than that of leucocytes frozen at the fast rate (Table II). However, total cell numbers were not different between the two freezing rates. Significant interaction ($P=0{\cdot}0001$) was found between freezing rate and cryoprotectant. Under the fast freezing rate, viability of cells cryopreserved with DMSO was higher than that of cells cryopreserved with glycerol. While under the slow freezing rate, cells cryopreserved with glycerol had higher viability than cells cryopreserved with DMSO (Table II). Cells cryopreserved with methanol had significantly lower viability ($P<0{\cdot}05$) than did cells cryopreserved with DMSO or glycerol, for either freezing rate (Table II).

STUDY 3: CHROMOSOME PREPARATION

Cells were cryopreserved with 10% of glycerol or methanol and frozen at the slower rate. About 25 countable spreads of channel catfish metaphase chromosomes were prepared per slide using cryopreserved leucocytes. Chromosomes prepared by this method were identical to chromosomes prepared from fresh

TABLE II. Percent viability and total cell number (mean ± S.D.) of cultured channel catfish leucocytes* cryopreserved at two different freezing rates

Freezing rate†	Viability (%)					Total cell number (× 10^6)				
	Glycerol	DMSO	Methanol	Control	Pooled (by rate)	Glycerol	DMSO	Methanol	Control	Pooled (by rate)
Fast	31 ± 13	40 ± 23	4 ± 4	1 ± 1	19 ± 21^B	1·8 ± 0·1	2·1 ± 0·3	1·4 ± 0·3	1·0 ± 0·3	1·9 ± 0·6^A
Slow	69 ± 13	42 ± 15	2 ± 2	1 ± 1	28 ± 31^A	1·5 ± 0·4	2·2 ± 1·4	1·8 ± 0·3	0·8 ± 0·2	1·9 ± 1·1^A
Pooled (by cryoprotectant)	49 ± 23^A	41 ± 19^B	3 ± 3^C	1 ± 1^C		2·0 ± 0·4^B	2·6 ± 1·2^A	2·0 ± 0·5^B	1·1 ± 0·3^C	

Cryoprotectants were added at 10% (v/v) before freezing. Values sharing a letter in pooled columns (cryoprotectant) or rows (freezing rates), were not significantly different ($P>0·05$).

*Initial percent viability=71%; initial total cell number per treatment=4·3 × 10^6 cells. Sample size (number of replicates) was nine for all treatments and controls.

†Freezing rate of fast programme was −45° C min^{-1} until −80° C. Slow programme used a cooling rate of −1° C min^{-1} until 0° C, and a freezing rate of −2·7° C min^{-1} until −80° C.

cells (data not shown), and were not different from literature descriptions of channel catfish chromosomes (e.g. LeGrande, 1981; Wolters *et al.*, 1981).

DISCUSSION

Our study provides basic information on cryopreservation of cultured leucocytes of channel catfish, and demonstrates preparation of chromosomes from cryopreserved cells. Three main factors were found to strongly influence cell number and viability after frozen storage: cryoprotectant, cryoprotectant concentration, and rate of freezing. Overall, glycerol and DMSO provided better protection than did methanol, in terms of viability and total cell number. Cryoprotectants at concentrations of 5 and 10% (v/v) were found to be effective for cryopreservation of cultured catfish leucocytes. Glycerol and DMSO have been used widely in cryopreservation of cultured adherent cells, e.g. epithelial cells, fibroblasts, astrocytes, muscle cells, etc., from higher animals including man (Freshney, 1994). Although methanol is used less frequently in cryopreservation of cells, other studies in fish such as tilapia, *Oreochromis* (*Sarotherodon*) *mossambicus* (Peters), have shown methanol to be an effective cryoprotectant for sperm (Harvey, 1983). In sperm of channel catfish, 5% methanol was the best cryoprotectant of five studied (Tiersch *et al.*, 1994). We found in this study that methanol appeared to have toxic effects on channel catfish leucocytes at concentrations of 10 and 15% (v/v). Viable cells were not observed in samples treated with 15% methanol.

A slow freezing rate resulted in significantly higher viability for cryopreservation of cultured leucocytes. A similar rate of freezing (about -1° C min^{-1}) was reported for use in cryopreservation of marmoset B-lymphocytes (Penno *et al.*, 1993). However, freezing rate was found not critical for viability of adherent cells of the rat such as astrocytes (Gomez-Lechon *et al.*, 1992) and neurones (Jerrolyn & Barrett, 1986). Freezing of adherent cells can be accomplished by manual transfer from one temperature phase to another (Shaddock & Casciano, 1993) because these cells are apparently more tolerant to physical shock than non-adherent cells. In studies of an ovary cell line of channel catfish, Bowser and Plumb (1980) cryopreserved the adherent cells with 10% DMSO. Cells were held at 5° C for 1 h, reduced to -20° C for 1 h, and transferred to -70° C for storage, 58% of the ovary cells were viable after 7 days of frozen storage.

The interaction of cryoprotectant and freezing rate in the present study suggests that DMSO might penetrate faster than glycerol, and therefore had an advantage with the short equilibration time (<10 min). Glycerol outperformed DMSO at the slower cooling rate, perhaps due to the availability of a longer time period for entry into the cells. Freezing efficiency of leucocytes cryopreserved with glycerol might be enhanced by an increased time of equilibration prior to freezing.

Our preparation of chromosomes from cryopreserved leucocytes was based on methods used for fresh cultured cells (Gold *et al.*, 1990). We found that for best results, thawed cells should be cultured for about 24 h prior to preparation of chromosomes. Otherwise, mitotic blocking agents such as colchicine could be added before freezing. Further studies are necessary to develop procedures to establish long-term cultures of cryopreserved catfish leucocytes.

This project was supported in part by USDA Grant #93-34310-9057, and USDA-ARS Cooperative Agreement #58-6402-3-104. We thank J. Jenkins for providing information on leucocyte separation, and W. Wolters and L. Stanley for providing information on culture of channel catfish leucocytes. We thank J. Jenkins for critical review.

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