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Original Paper

Isolation and cryopreservation of early stages of germ cells of tench (*Tinca tinca*)

Z. Linhartová, M. Rodina, H. Guralp, I. Gazo, T. Saito, M. Pšenička

Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses,
Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice,
Vodňany, Czech Republic

ABSTRACT: A practical technique for isolation and cryopreservation of tench (*Tinca tinca*) (Cyprinidae, Teleostei) early stages of germ cells (GC), including spermatogonia and spermatocytes, is reported for the first time. The germ-line cells possess the ability to differentiate into functional gametes of both sexes. These early stages of germ cells are small enough to be well-suited to cryopreservation, which, together with their high level of plasticity, makes their preservation a promising tool for maintaining genetic resources. Testicular cells were distinguished and separated by Percoll gradient, with the highest proportion of GC (62.2%) obtained from the 30% layer. The concentration and viability of GC were determined, and specific staining (DDX4) for germ cells was used to distinguish GC from somatic cells. Early stages of germ cells were cryopreserved in an extender composed of phosphate buffered saline (pH 8) with 0.5% BSA, 50mM D-glucose, and containing 1.5M cryoprotectant in the pre-programmed PLANER Kryo10 series III using a cooling protocol from +10°C to –80°C at a rate of 1°C/min. The effect of six cryoprotectants – methanol, dimethyl sulfoxide, dimethyl sulfoxide + propanediol (1 : 1), glycerol, ethylene glycol, and dimethylacetamid was assessed, and the results were evaluated by comparing the percentage of viable frozen/thawed GC by ANOVA, Tukey's HSD test ($P < 0.05$). Almost the same viability rates were obtained with no significant differences among tested cryoprotectants, indicating high stability of GC in cryoprotectants. Nevertheless, glycerol at a concentration of 1.5M was associated with the highest survival rate of thawed tench GC (57.69 ± 16.85%).

Keywords: germ cells; isolation; transplantation; Percoll gradient; cryoprotectant; viability

INTRODUCTION

The germ-line cells, primordial germ cells (PGCs), and their subsequent stages, spermatogonia/oogonia (SG/OG), have the potential to undergo proliferation (Brinster 2002), differentiate into functional gametes, and transmit genetic information to the next generation (Okutsu et al. 2006b) making them suitable for xenogeneic transplantation (Yamaha et al. 2007). The major difference between manipulation of PGCs and SG is the relatively simple method of isolation of a large number of SG from testes, compared to the successful isolation of only tens of PGCs (Saito et al. 2008, 2010). A disadvantage of these early stages of germ cells (SG/OG and their subsequent stages) lies in limited migratory activity to the required position in the gonads when these cells are transplanted during embryonic stage. Therefore SG/OG must be transplanted into the peritoneal cavity near the genital ridge of hatched fry or directly into the gonads (Yoshikazi et al. 2010; Wong et al. 2011; Nobrega et al. 2012). The transplantation of PGCs/SG/OG into a closely related species,

creating a germ-line chimera, could enhance fish production for research as well as aquaculture.

In recent years, the demand for tench *Tinca tinca* fry and stock for aquaculture has increased (Podhorec et al. 2011), and the preservation of tench sperm (Rodina et al. 2007) or germ cells may benefit artificial reproduction. Therefore tench, an economically important fish tradition-ally reared in ponds of Central and East Europe (Gela et al. 2007), was chosen for this project. Tench are closely related to common carp *Cyprinus carpio*, goldfish *Carrasius auratus*, zebrafish *Danio rerio*, and the white cloud mountain minnow *Tanichthys albonubes* (Nelson 2006), species that could possibly be used as hosts for xenogeneic transplantation.

The germline stem cells, including PGCs and SG, can undergo continued spermatogenesis after transplantation (Okutsu et al. 2006a). To successfully utilize the early stages of germ cells (GC) (including SG and several spermatocytes) for cryopreservation and later transplantation, they must be separated from other testicular cell types of mature testis as spermatids and mostly the sperm. Therefore a purification step is neces-sary. Several methods of GC isolation have been developed, including cell sorting via flow cytom-etry (Kobayashi et al. 2004), magnet-activated cell sorting (Schonfeldt et al. 1999), and density gradient centrifugation-elutriation (Loir and Le Gac 1994). The step Percoll gradient is a simple and economical method that has been used in several fish species (Lacerda et al. 2006; Psenicka et al. 2012) but has not been tested on tench.

Cryopreservation and cryobanking of fish gam-etes are considered tools for artificial reproduction and genetic improvement of aquaculture species. Cryopreservation provides several benefits, such as synchronizing the availability of male and fe-male gametes, long-term storage of cells without aging, convenient transport of genetic material, and reducing the number of broodstock required in hatchery facilities and living gene banks (Suquet et al. 2000; Alavi et al. 2012). The process in fish sperm is relatively simple (Bobe and Labbe 2010) and has been used in more than 200 fish species (Billard and Zhang 2001). The disadvantage of sperm cryoconservation is that it does not provide for storing the maternal information. Oocytes and embryos have been successfully cryopreserved only in invertebrates (Labbe et al. 2013). The large size and high yolk content of fish oocytes and embryos

results in slow penetration of the extender and high sensitivity to freezing (Zhang et al. 2007; Robles et al. 2009). The most effective alternative to cryo-preservation of fish oocytes and embryos is the use of early stages of germ cells with their ability to re-colonize genital ridges upon transplantation (Yoshizaki et al. 2005, 2011; Okutsu et al. 2006b). Therefore GC are well-suited to cryopreserva-tion, due to small size and a high level of sexual plasticity which allows them to differentiate into fully functional gonads of both sexes (Okutsu et al. 2006b, 2007).

The primary aim of this study was to investigate the optimal procedure for tench GC isolation and cryopreservation, focusing on cryoprotectant solu-tion. This is the first report of tench early stages of germ cells isolation and preservation.

MATERIAL AND METHODS

The study was conducted in the aquaculture fa-cility of the Faculty of Fisheries and Protection of Waters at Vodňany (University of South Bohemia in České Budějovice, Czech Republic).

Fish and rearing conditions. Tench, 2–2.5 years of age, were obtained from outdoor ponds at the Research Institute of Fish Culture and Hydrobiology at the beginning of July 2013, transported to the hatchery, and separated by sex according to sexual dimorphism. Twenty males (30.67 ± 6.71 g average body weight and 130.94 ± 10.41 mm average body length) were selected and transferred into two 50 l aquariums. Temperature and oxygen values were monitored daily and ranged 18–22°C and 6–7 mg/l, respectively. Fish were fed daily with pelleted feed; feeding was stopped 48 h before initiation of the experiment. Four males, producing spermatozoa, were used for each trial. Testicular cells from a single immature male (did not produce any spermatozoa) were examined using an immulocytological and immunoblotting method.

Adjustment of osmolality. The osmolality of tench blood plasma was determined to optimize conditions for germ cells. Blood samples (300 µl) were collected from the caudal vein of 4 fish using a 1 ml syringe containing 10 µl of heparin (Zentiva, Prague, Czech Republic) and held at 4°C until use. Osmolality of blood samples was assessed using a Vapour Pressure Osmometer Vapro 5520 (Wescor, Logan, USA), expressed in mOsm/kg, and used for adjusting the media for isolation and separation of testicular cells.

Isolation and enzymatic dissociation of testicular cells. Four male tench (43.7–36.7 g body weight) were selected, killed by a blow to the skull, and testes (0.18 ± 0.02 g average weight) were dissected and transferred to individual Petri dishes. The lipid tissue was separated from the testes to avoid high amounts in the isolated cell suspension, and the testes were washed in phosphate buffered saline (PBS, 248 mOsm/kg, pH 8) (Sigma-Aldrich, s.r.o., Prague, Czech Republic), cut into small pieces, and transferred into 15 ml tubes with 10 ml of PBS + 0.1% each of collagenase (Life Technologies Czech Republic s.r.o., Prague, Czech Republic) and trypsin (Sigma-Aldrich) (248 mOsm/kg, pH 8) according to Psenicka et al. (2012). Testicular suspensions were incubated in a Compact Bio Shaker VBR-36 (Bionexus Inc., Oakland, USA) with gentle mixing for 1.5 h at 25°C. The enzyme activity was stopped, and DNA released from dead cells was digested by adding 1 ml of PBS containing 1% BSA (Sigma-Aldrich) and 40 μg/ml deoxyribonuclease (DNase) (AppliChem GmbH, Darmstadt, Germany), respectively. The homogeneous suspension was filtered through a 50 μm filter (Partec GmbH, Görlitz, Germany). To separate GC from other testicular (spermatids and sperm) and somatic cells, a step Percoll gradient (Sigma-Aldrich) 30 and 5% (in the order from the bottom of tube) was prepared. The testicular cell suspension was slowly transferred to the surface of the gradient and immediately centrifuged at 500 *g*, 4°C for 30 min with a slow rotor acceleration to preserve the Percoll gradient. 3 ml of cell suspension was collected from the 30% and 5% layers and the pellet (precipitate collected at the bottom of the tube), washed with 10 ml PBS (248 mOsm/kg, pH 8) and centrifuged at 500 *g*, 4°C for 30 min. Pellets with testicular cells were transferred into 1.5 ml Eppendorf tubes, diluted with PBS to 0.5 ml, and kept on ice at 4°C for analysis. The testicular cells from each layer of Percoll gradient and pellet were confirmed by light optical microscope Olympus BH2 (Olympus Corp., Tokyo, Japan) at 250× magnification and photographed by Nikon 5100 camera (Nikon, Tokyo, Japan) (Figure 1). The GC were discriminated from small testicular cells (spermatids and sperm) and components (lipid, debris) on the basis of their spherical appearance, large size, and large nucleus with small nucleolus. The number of cells was evaluated using Olympus MicroImage software (Version 4.0 for Windows 95/NT/98).

Evaluation of GC concentration. GC concentration in whole testes of each fish was determined microscopically by counting the number of cells diluted in PBS (dilution 1 : 10) using a Burker cell
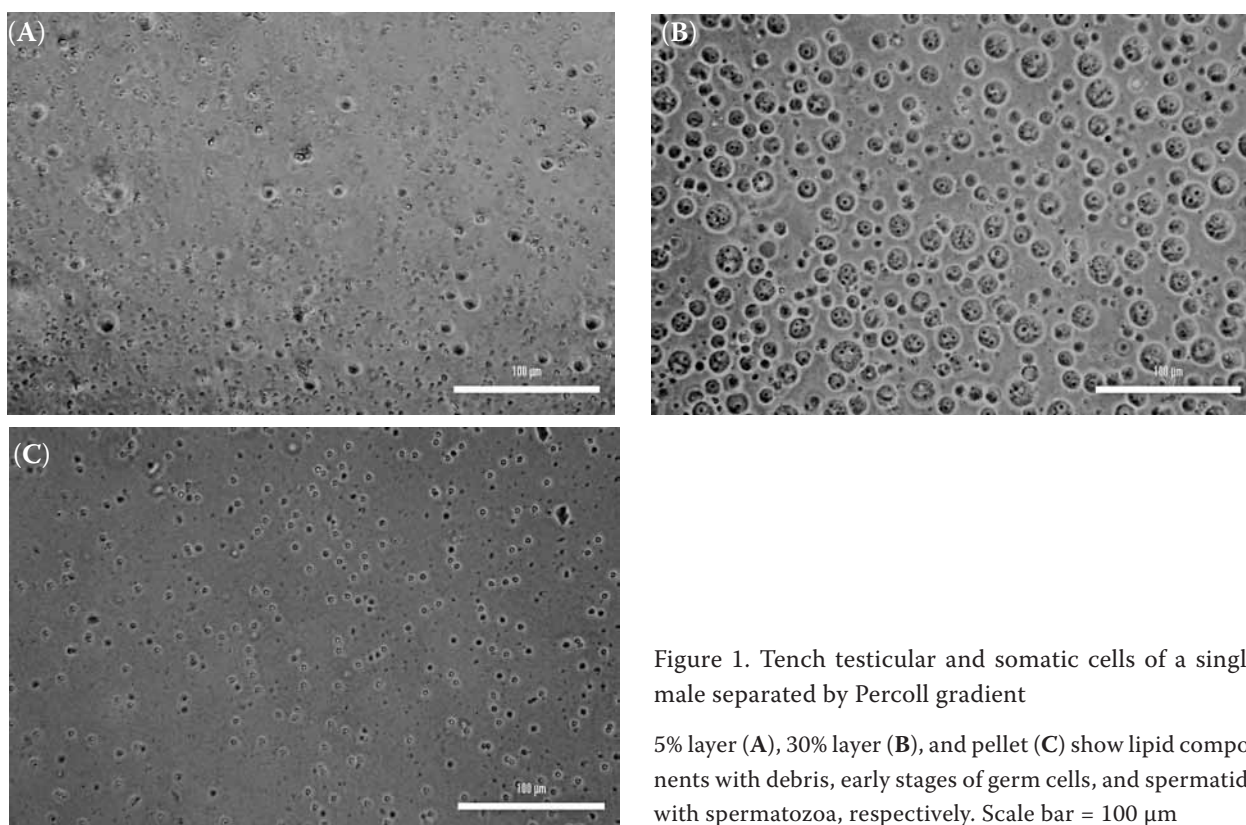

Figure 1. Tench testicular and somatic cells of a single male separated by Percoll gradient

5% layer (**A**), 30% layer (**B**), and pellet (**C**) show lipid components with debris, early stages of germ cells, and spermatids with spermatozoa, respectively. Scale bar = 100 μm

hemocytometer (Meopta-optika, s.r.o.,, Přerov, Czech Republic) at 100× magnification under an Olympus BH2 microscope. The number of GC was counted in 20 squares of the Burker cell chamber with two repetitions, and the GC content was expressed as the absolute number of GC obtained from testes of each male by multiplying the number of GC by the volume of each GC sample (0.5 ml).

Immunofluorescence labelling. A standard protocol for indirect immunofluorescence labelling was used to discriminate germ cells from somatic cells. Specific staining of germ-line cells with primary rabbit polyclonal antibody to DDX4 (DEAD (Asp-Glu-Ala-Asp) box polypeptide 4; Cat. No. GTX116575; Lot No. 40261) (GeneTex Inc., Irvine, USA) at a dilution 1 : 300 combined with secondary antibody goat anti-rabbit immunoglobulin (IgG) conjugated with fluorescein isothiocyanate (FITC) (dilution 1 : 700) (Sigma-Aldrich, s.r.o., Prague, Czech Republic) was applied. DDX4 (known as VASA) was chosen as specific marker because it is the most widely used marker of the germ cell lineage and it has been identified in the germ cells of a large number of animals (Raz 2000; Bellaiche et al. 2014). To visualize all cells, the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). As a control, somatic cells from caudal fins were examined. Slides with whole testis samples from each individual and each layer of the Percoll gradient and pellet were observed and recorded under an inverted fluorescent microscope Olympus IX 83 (Olympus Corp., Tokyo, Japan) with Hamatsu Digital camera C10600 (Hamamatsu Photonics, Hamamatsu, Japan) at 400× magnification. Percentage of GC obtained from the 30% Percoll gradient from a single immature male in which final stages of spermatogenesis did not start (no spermatids and sperm presented) was evaluated by cellSens Dimension Microscopy Imaging Software (Version 1.9, 2009–2013) and compared with data from light optical microscopy.

Immunoblotting. The Western blot was used to detect specific proteins in testis of immature tench male to confirm the specificity of DDX4 as a marker of germ cells in tench. Proteins were extracted from testis and somatic cells from fin (control) with lysis buffer (8M urea, 2M Thiourea, 4% CHAPS, 10% w/v isopropanol, 0.1% w/v Triton X-100, 100mM dithiothreithol; Sigma-Aldrich) containing phosphatase (1mM sodium orthovanadate, 50mM EDTA; Sigma-Aldrich)

and protease (100mM PMSF, 1 µl/ml pepstain A, 5 µl/ml leupeptin; Sigma-Aldrich) inhibitors. The Bradford protein assay was applied to determine the protein concentration in samples. For SDS-PAGE the samples (25 mg of proteins) were resuspended in buffer containing 65mM TRIS, 10% (vol/vol) glycerol, 2% (wt/vol) SDS, and 5% (vol/vol) beta-mercaptoethanol (Sigma-Aldrich), and denatured at 95°C for 3 min before loading onto gels. Proteins were separated on 12% gel. After electrophoresis the SDS gels were placed on polyvinyl difluoride (PVDF) membranes (Bio-Rad Laboratories, Inc., Hercules, USA) and electrically transferred. The membranes were blocked by incubation with 5% (w/v) Skim milk in TBS-T (0.1% Tween-20, 20mM Tris, 500mM NaCl at pH 7.6) (Sigma-Aldrich) at 20°C for 1 h. The membranes were washed three times with TBS-T followed by incubation for 12 h at 4°C in 5% BSA-TBST containing anti-DDX4 antibodies as the primary antibodies. Then the membranes were washed and incubated with HRP-conjugated goat anti-rabbit IgG (1 : 3000 in 3% BSA-TBST) at 20°C for 1 h. Reacted proteins were revealed with 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate (Sigma-Aldrich).

Cryopreservation and thawing procedures. GC samples from 4 tench male were treated individually. Before freezing, GC were diluted 1 : 3 (25 µl of GC diluted in PBS + 75 µl of extender) in an extender composed of PBS (pH 8) with 0.5% BSA, 50mM D-glucose (Psenicka et al. 2012), and 1.5M cryoprotectant to a final concentration of 8×10^5 of GC/ml. Six cryoprotectants were studied: (1) methanol (MET); (2) dimethyl sulfoxide (DMSO); (3) DMSO + propanediol (DMSO + P at 1 : 1); (4) glycerol (GLY); (5) ethylene glycol (EG); (6) dimethylacetamid (DMA). Tests were conducted in triplicate for each sample. Volumes of 100 µl diluted GC were placed into 1.8 ml cryotubes (Nunc, Roskilde, Denmark), transferred to a pre-programmed PLANER Kryo10 series III, and cryopreserved by cooling from +10°C to –80°C at a rate of 1°C/min. Time between GC sample treatment and initiation of freezing was 10 min. Frozen samples were transferred into liquid nitrogen (LN2) and stored for 7–14 days. To assess GC variables, samples were thawed by plunging the cryotubes into a 38°C bath until thawed up to 40 s. Finally, samples were centrifuged (500 *g*, 10 min, 4°C) for viability testing.

Evaluation of germ cell viability. The viability of GC from individual fish was tested prior to

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(in duplicate) and after cryopreservation/thawing (in triplicate). Twenty µl of GC were transferred into 1.5 ml Eppendorf tubes on ice at 4°C. Dual DNA staining to identify live and dead cells was performed using the Cellstain double staining kit (Sigma-Aldrich) according to the manufacturer's protocol. The staining was achievied by acetoxy-methyl ester of calcein (Calcein-AM) (viable cells emit strong green fluorescence), and propidium iodide (PI), a nuclei staining dye which cannot pass through a viable cell membrane and stains DNA in degenerate cells (dead cells emit red fluorescence). The staining was optimized by adding PI at 5 µl/ml because of weak red fluorescent signal of tench GC. Samples were gently mixed, and 20 µl was pipetted onto a clean microscopic slide and left for 3–5 min for sedimentation. Ten images per sample were obtained using an Olympus BX 50 microscope (Olympus Corp.) at magnification 100×, recorded with a 3CCD Sony DXC-9100P colour camera (SONY Corp., Tokyo, Japan), and processed by Olympus MicroImage software (Version 4.0 for Windows 95/NT/98) according to Flajshans et al. (2004).

Data analysis. In cryopreservation and viability trials, cryoprotectants were tested in triplicate for each male, while GC concentration and osmolality were estimated from dual measurements. Data were calculated as means ± standard deviations (SD). To compare the percentage of viable GC with different cryoprotectant treatments, ANOVA with *post hoc* comparisons using Tukey's honest significant difference (HSD) test was applied. Probability values of $P < 0.05$ were considered significant. All statistical analysis was performed using STATISTICA software (Version 12, 2013) for MS Windows.

RESULTS

Adjustment of osmolality. Osmolality of blood samples (mean ± SD; $n = 4$) was 248 ± 6 mOsm/kg. The osmolality of the media used for experiments was adjusted to this level.

Isolation of early stages of germ cells in a Percoll gradient. The GC were separated from other testicular (spermatids and sperm) and somatic cells by Percoll gradient and identified on the basis of their morphological characteristics by light microscopy (Figures 1A–C) and by immunocytochemistry with specific staining for germ cells (Figure 2). GC were identified by immunolabelling, and the mean proportion in the 5%
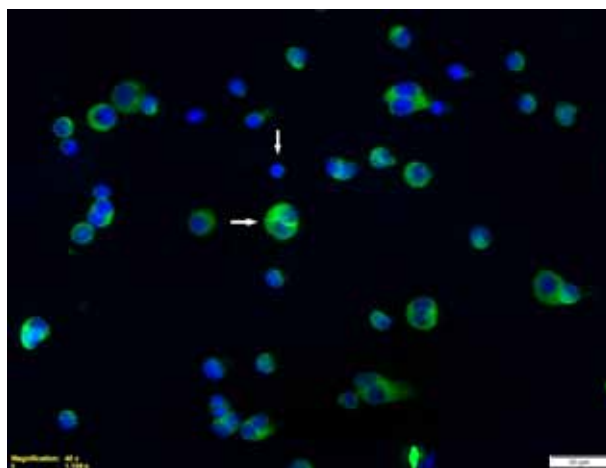

Figure 2. Fluorescent micrographs of tench testicular cells stained with DDX4 and DAPI

Specific staining with germ cell (GC) specific marker DDX4 (DEAD (Asp-Glu-Ala-Asp) box polypeptide 4) indicates a strong green signal in early stages of GC and DAPI (4', 6-diamidino-2-phenylindole) blue in early stages of GC and somatic cells. Arrows indicate labelled GC (blue and green staining, left arrow) and somatic cell (blue staining, top arrow). Scale bar = 20 µm

and 30% layer and pellet of the Percoll gradient from the four fish was calculated. Because the GC (spermatogonia and spermatocytes) are not easy to distinguish from each other by light microscopy, they were calculated together. Spermatids were counted together with sperm because of visible smaller sizes than GC. The upper layer in the Percoll gradient (5%) showed 80% lipids + debris and 20% GC. The 30% layer comprised 62.2% GC, 18% debris, 17.3% spermatids + spermatozoa, and 2.5% blood cells. The pellet consisted of 39.1% blood cells, 21.2% spermatids + spermatozoa, 20.7% debris, and 19% GC. Percentages of GC calculated from total amount of GC of 4 males in whole Percoll gradients were 25.9% in 5% layer, 50.1% in 30% layer, and 24% in pellet. Thus the highest concentration of GC was found in the 30% layer of Percoll gradient.

GC concentration. The GC concentration was defined as the number of GC measured per unit (0.5 ml PBS) from each fish to determine the yield of GC from individual testes and specify the amount used for cryopreservation. The mean number of GC isolated from 0.18 ± 0.02 g testes of 4 males in two counts ranged from 1.31 ± 0.07 to 2.06 ± 0.13 × 10^6 (Table 1) with no significant differences ($P > 0.05$) among analyzed fish. The yield of GC of 2–2.5 year old tench was consistent among individuals.

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Table 1. Yield of germ cells (GC) from testes (0.18 ± 0.02 g) of individual tench obtained from a 30% layer of Percoll gradient

Tench No.	Absolute numbers of GC ($\times 10^6$ cells) (mean ± SD)
1	1.63^a ± 0.19
2	2.06^a ± 0.13
3	1.67^a ± 0.17
4	1.31^a ± 0.07

data are shown as means ± standard deviations (SD)

[a]not significantly different values (ANOVA, Tukey's HSD test, $P < 0.05$)

***Immunocytochemistry and immunoblotting*.** Immunocytochemistry confirmed the identity of GC and distinguished them from somatic cells by staining with specific primary antibody to DEAD box protein. Germ cells were distinguished by a strong green fluorescent signal (Figure 2), while only a blue signal (DAPI) was observed in control somatic cells. The proportion of GC from the immature male isolated in the 30% Percoll gradient reached 92.9% (Figure 2) and confirmed that the 30% Percoll gradient is suitable for tench GC isolation, as shown with mature males. The Western blot technique confirmed the specificity of DDX4 as a suitable marker of tench germ cells. A bright band at 75 kDa was found at immunoblot (Figure 3) and proved that DDX4 is suitable for germ cell staining in tench.
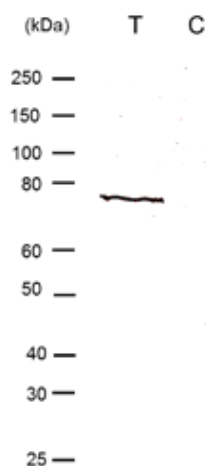

Figure 3. Immunoblot with proteins extracted from testis and caudal fin of tench male detected by DDX4

Bright band at 75 kDa indicates that DDX4 (DEAD (Asp-Glu-Ala-Asp) box polypeptide 4) is a suitable marker of tench germ cells (T) in comparison with somatic cells (control, C) where no specific staining was documented
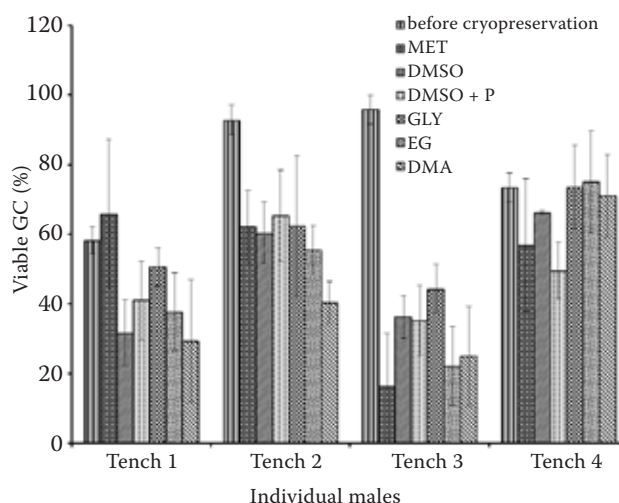

Figure 4. Percentage of viable early stages of germ cells (GC) before and after cryopreservation treated with six cryoprotectants in four fish

MET = methanol, DMSO = dimethyl sulfoxide, DMSO + P = DMSO + propanediol at 1 : 1, GLY = glycerol, EG = ethylene glycol, DMA = dimethylacetamid

Data are shown as means ± standard deviations for four individuals; viability was evaluated for six cryoprotectant treatments in duplicate before and in triplicate after cryopreservation

***Viability of GC before and after cryopreservation with the effect of cryoprotectants*.** The efficacy of cryopreservation was evaluated by comparing the percentage of viable GC before and after cryopreservation. Decreases ($P < 0.05$) in percentage of viable GC were observed in all examined samples after cryopreservation (Figure 4). The mean numbers of viable GC from 2 analyses in four males were: 58.17 ± 3.93, 92.76 ± 4.34, 95.80 ± 4.20, and 73.45 ± 4.11% before cryopreservation (Figure 4) and (mean values of 6 cryoprotectant treatments and 3 replicates) 42.70 ± 18.58, 57.69 ± 14.50, 29.85 ± 14.71, and 65.36 ± 15.54% after cryopreservation.

The effects of six cryoprotectants were tested in four males. The percentage of viable GC varied among analyzed males and those cryopreserved with DMSO, EG, and DMA showed significant differences (Figure 4). The viability of GC from individual fish was consistent across the 6 cryoprotectants, suggesting that GC are individual-dependent (Figure 4). To detect the best cryoprotectant for cryopreservation of tench GC, the analyzed data from four tench males were averaged (Figure 5). The percentage of viable GC after thawing for the six cryoprotectants was: GLY 57.69 ± 16.85%, MET
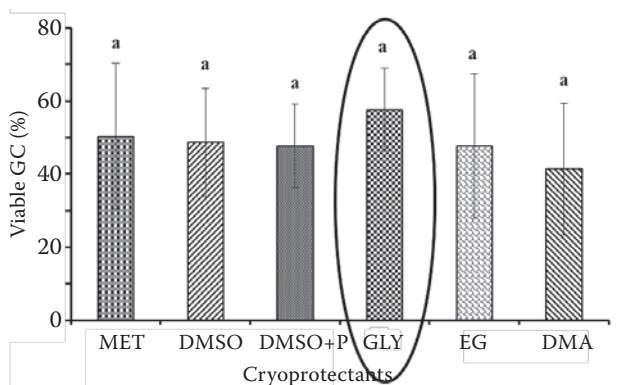
Figure 5. Percentage of viable early stages of germ cells (GC) after thawing with six cryoprotectants

MET = methanol, DMSO = dimethyl sulfoxide, DMSO + P = DMSO + propanediol at 1 : 1, GLY = glycerol, EG = ethylene glycol, DMA = dimethylacetamid

Effect of the cryoprotectants is shown as percentage of live GC after thawing of four fish with three replicates; the most effective cryoprotectant is circled. Results are shown as means ± standard deviations

[a]not significantly different values (ANOVA, Tukey's HSD test, $P < 0.05$)

50.27 ± 26.27%, DMSO 48.62 ± 16.49%, DMSO + P 47.77 ± 15.65%, EG 47.64 ± 22.76%, and DMA 41.38 ± 22.30%. ANOVA and Tukey's HSD test showed no significant differences among tested cryoprotectants. Nevertheless, glycerol was evaluated as the best cryoprotectant all over the four tested tench males for cryopreservation of GC, as it was associated with the highest viability of GC after cryopreservation (Figure 5).

DISCUSSION

Based on the results of the present study, the following can be suggested: (1) the Percoll gradient is a simple, inexpensive, and practical method of selecting testicular cells with the highest percentage of GC in the 30% layer; (2) staining with DDX4 indicates the presence of GC; and (3) addition of 1.5M glycerol to extender composition is the most suitable for freezing of tench GC.

Importance of germ cells. Germ cell transplantation to induce chimerism in fish species is an expanding focus of research. This technique can enhance the production of gametes of species that are commercially valuable, endangered, or have complex reproduction, using a more readily available species or one easily adapted to artificial reproduction as a surrogate host (Okutsu et al.

2006a; Yamaha et al. 2007). Primordial germ cell transplantation was developed (Saito et al. 2008, 2010) due to the advantage of their migration to genital ridge via chemotaxis. This characteristic was assumed to be lacking in early stages of germ cells as SG/OG (Raz and Reichman-Fried 2006), but this has been refuted by Okutsu et al. (2006a, b) and Yoshikazi et al. (2010), who argue that SG exhibit at least a low level of migration activity in response to chemotactic signals. The advantage of SG/OG compared with PGCs is in their easy isolation from testes/ovary to obtain a large number of cells which can be directly microinjected into the body cavity of recipient fry to counteract the potential migration inability of transplanted GC (Yoshikazi et al. 2010). Therefore conservation of GC in LN2 is a valuable tool for maintaining genetic resources. The early stages of germ cells also have the potential for biotechnology such as (1) shortening the reproduction period by using species with a shorter generation interval as hosts; (2) reducing the space required for culture by using small fish as hosts; (3) retaining target species without keeping adult fish; and (4) to acquire knowledge for application in other fish species.

Isolation of germ cells. The efficacy of isolation is facilitated by enzyme-induced dissolution of the germ cells from testicular tissue (Bellve et al. 1977). Lacerda et al. (2006) digested the testes of sexually mature Nile tilapia *Oreochromis niloticus* with collagenase, trypsin, and DNase and isolated the SG from the two upper bands of a Percoll gradient. Spermatogonial stem cells isolated from adult male rainbow trout *Oncorhynchus mykiss* were incubated in PBS (pH 8.2) with 0.5% trypsin (Okutsu et al. 2006b). Psenicka et al. (2012) conducted isolation and cryopreservation of early stages of germ cells of 2–4-year old Siberian sturgeon *Acipenser baerii* with a dissociation medium composed of PBS with 0.2% trypsin and sorted the testicular suspension by Percoll gradient. The highest percentage of GC was obtained from the 10–30% layer of the gradient and confirmed by staining with DDX4. The technique used for tench GC dissociation and isolation was adapted from these studies and optimized for practical use according to relevant data. We recommend using the 5–30% Percoll gradient for tench GC isolation and selection of immature males to obtain a higher concentration of GC compared to mature fish, in which the percentage of early stages of germ cells is lower because of spermatozoa production.

Cryopreservation. The importance of preserving genetic resources has increased substantially. GC cryopreservation was mainly developed based on information relating to spermatozoa preservation (Billard and Zhang 2001). Successful fish sperm cryopreservation has been achieved by optimizing the freeze/thaw conditions, dilution ratio, freezing and thawing rates, and especially the composition of an extender with cryoprotectants (Suquet et al. 2000; Alavi et al. 2012). A range of cryoprotectants has been tested for fish spermatozoa, with dimethyl sulfoxide, methanol, ethylene glycol, dimethylacetamide, and ethylene glycol being the most commonly used. Significant inter-species variations as well as significant within-species differences in cryoprotectant effectiveness have been reported in fish spermatozoa (Alavi et al. 2012). For example, 8–10% DMSO + P was associated with the highest sperm motility after freeze/thaw in tench (Rodina et al. 2007); MET was reported to be the optimal cryoprotectant for sperm of roach *Rutilus rutilus*, bream *Abramis brama*, silver bream *Blicca bjoerkna*, and barbel *Barbus barbus* (Urbanyi et al. 2006); and DMSO has been recommended for common carp (Horvath et al. 2003). The tested cryoprotectant treatments of tench GC showed no significant differences, indicating high stability of GC in cryoprotectants. Fish GC cryopreservation has been successfully established in rainbow trout (Okutsu et al. 2007) and Siberian sturgeon (Psenicka et al. 2012) under freezing conditions similar to those of the present study. Cryomedium containing 1.8M EG, 0.5% BSA, and 5.5mM D-glucose (Yoshikazi et al. 2011) resulted in a survival rate of GC of rainbow trout after thawing (45.4%) (Okutsu et al. 2007) similar to that obtained with EG in the present study, but lower than when GLY was used (57.69 ± 16.85%). In a study of cryopreservation of GC of Siberian sturgeon Psenicka et al. (2012) found the most effective (61.75% viable) cryoprotectant to be 1.5M EG in extender composed of PBS, 5.5mM D-glucose, and 0.5% BSA. For PGC preservation, an EG concentration of 1.8M resulted in the highest survival rate of thawed PGC (51.3 ± 7.25%) in rainbow trout (Kobayashi et al. 2007). We obtained differing results among individual fish; the male with GC having the highest viability before cryopreservation did not show the highest viability after cryopreservation (Figure 4). Quality of GC within individuals must be taken into account. Another possibility of cryopreservation is to freeze pieces of testes/ovary and dissociate germ cells after thawing (in process) according to Lee et al. (2013). In the future we would like to compare the viability rate of germ cells proceeded by the technique of Lee et al. (2013) with the present study and discuss their effectiveness for practical usage.

CONCLUSION

Isolation and cryopreservation of early stages of germ cells appears to be a useful and simple technique for conservation of genetic resources in tench. The effectiveness of the extender and cryoprotectant is species-specific as well as individually dependent and must be optimized for each fish species. Viability of thawed GC should be verified during each preservation procedure. Our method is simple, it does not require complex laboratory equipment, except for an automatic programmable freezer and swinging head centrifuge for high volume applications. This could be a feasible method for GC isolation and cryopreservation in hatchery practice as well as in research. These data are also considered preliminary results for application in fish bioengineering by transplanting cryopreserved GC into a sterile xenogeneic recipient.

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Corresponding Author

Mgr. Zuzana Linhartová, University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zátiší 728/II, 389 25 Vodňany, Czech Republic
Phone: +420 387 774 612, e-mail: linhartova@frov.jcu.cz