

Strategies for the Conservation of Female Genetic Traits in Common Carp

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Abstract

Common carp is one of the most cultured fish species. However, there is lack of protocols for preservation of its maternal genetic resources. In this study, several experiments were conducted in order to develop an optimal protocol for slow-rate freezing and short-term storage (-80 or 4 °C) of common carp oögonia. Dimethyl sulfoxide with concentration of 1.5 M was identified as the best cryoprotectant in comparison with propylene glycol and methanol. When comparing supplementation of sugars (glucose, trehalose, sucrose) in different concentrations (0.1, 0.3, 0.5M), glucose and trehalose in 0.3 M were identified as optimal. Finally, different equilibration times revealed a positive impact of prolonged equilibration on post thaw viability. Short-term storage options for ovarian tissue pieces at -80 °C and 4 °C were tested as an alternative to cryopreservation and liquid nitrogen storage. Oögonia presence was confirmed by immunocytochemistry and viability after storage was determined by trypan blue exclusion. This study identified the best protocol for oögonia cryopreservation using slow rate freezing. Short-term storage in -80 °C freezer or on dry ice and in +4 °C can be applied when liquid nitrogen is not available. The frozen/thawed oögonia were labelled by PKH-26 and transplanted into goldfish recipients. The success of the transplantation was confirmed by presence of fluorescent cells and later on by RT-PCR with carp vasa specific primers.

Keywords: Cryopreservation, slow rate freezing, gene banking, oögonia, common carp, surrogate reproduction.

Highlights

- Protocol developed for common carp oögonia cryopreservation yielded ~65% post thaw survival
- Cryopreserved and fresh oögonia were recovered in surrogate host
- Identity of donor derived cells in recipients was confirmed by membrane labelling and RT-PCR

1 Introduction

Carp aquaculture uses many different breeds and strains and their F1 hybrids. Therefore, cryogenic storage of valuable genetic material is necessary to preserve purebred strains. Preservation of common carp germplasm has been developed only in males through sperm cryopreservation [1–4]. Preservation of oocytes in fish is limited generally due to different properties in comparison with the spermatozoa. Large cellular volume and presence of large quantities of yolk material hamper successful cryopreservation of mature oocytes. Some attempts of early-stage oocyte [5–7] as well later oocyte stage [8] cryopreservation were conducted in zebrafish, however, ovarian follicles are severely damaged after cryopreservation resulting in failure during attempts to achieve their growth *in vitro* culture [9,10]. Thus, practical application of follicles manipulation is limited at this time. However, future progress in oocyte cryopreservation in fish could facilitate surrogate reproduction using intraovarian transplantation as it was already described with non-cryopreserved follicles [11].

Manipulations of female early-stage germ stem cells (oogonia) seems to be a more favourable approach for cryopreservation as well as for transplantation in fish. Oogonia are able to survive cryopreservation [12–14] and colonize the recipient's genital ridge after transplantation [15], and undergo gametogenesis resulting in production of functional donor-derived gametes of both sexes since they are bipotential [16,17]. Surrogate reproduction has an immense potential for several fish species including endangered ones having long maturation time, big body size or problematic rearing and reproduction in captivity. All these pitfalls can be overcome by surrogacy when a convenient species is used as a recipient [15,18,19].

Several steps are necessary in order to succeed in surrogate reproduction technology. Availability of donor cells and synchronization with recipients is crucial because germ cell transplantation should be carried out in relatively short time window after hatching [20,21]. Germ cells can be cryopreserved for virtually infinite time and then recovered by

transplantation[20,22,23], giving possibility to conduct cryopreservation and schedule transplantation procedure according to the recipient availability indeed . Successful cryopreservation by slow-rate freezing (~ 1 °C/min) was so far performed in several salmonids [14,24], Siberian sturgeon [12], Nile tilapia [25] or cyprinid fishes such as tench [26,27] and goldfish [26]. This approach is convenient from point of long-term storage for gene banking, however, viability decrease is inevitable. As an alternative, germ cells can be stored hypothermically for a restricted time [28,29]. This alternative can be convenient when gonadal tissue is excised and/or during transfer when needed. Similarly, short-term storage would be needed when a large-scale transplantation is carried out, or recipient's size and age is not ready yet for intraperitoneal transplantation.

The aim of this study was to develop optimal protocol for carp oogonia freezing as a tool to preserve valuable maternal genotypes and ensure highest post-thaw viability, with an alternative strategy for short-term storage of ovarian tissue at 4 °C or -80 °C. Finally, the functionality of cryopreserved cells was assessed through inter-specific transplantation into goldfish surrogate host. Common carp, having a big size of ovary, can be considered as a good model species representing cyprinids in this kind of study.

2 Material and methods

The study was conducted in the aquaculture facility of the Genetic Fisheries Centre and Laboratory of Germ Cells at the Faculty of Fisheries and Protection of Waters (FFPW), University of South Bohemia in České Budějovice, in Vodňany, Czech Republic. Experiments conducted in Czech Republic were approved Ministry of Agriculture of the Czech Republic (reference number: 53100/2013-MZE-17214). The study did not involve endangered or protected species. All experiments were carried out in accordance with the Animal Research Committee of the FFPW. Fish were maintained according to the principles based on the EU harmonized animal welfare act of the Czech Republic and principles of laboratory animal care

in compliance with the national law (Act No. 246/1992 on the protection of animals against cruelty).

2.1 Animal husbandry and tissue collection

Common carp were produced by artificial propagation of Ropsha strain described elsewhere [30]. Produced offspring (BW: 7.3 ± 1.8 g) were held in a recirculation system at constant temperature of 22 °C, fed once per day with a low-fat diet. Fish were euthanized by a 2-phenoxyethanol overdose. The body was disinfected by 70% ethanol and decapitated. Ovarian tissue was excised aseptically and washed in phosphate buffered saline (PBS) with 100 U/mL penicillin and 0.1 mg /ml streptomycin. Ovaries were cut and the weight of each piece was recorded. One part (fresh control) was immediately minced with spring scissors and digested in the following conditions: each tissue piece was dissociated in 1 ml of digestion medium containing PBS with 0.15% trypsin, 0.05% DNase grade II (Roche) at 22 °C, on a laboratory shaker (30 rpm). Digestion was terminated after 1.5 h by addition of 10% (v/v) Fetal bovine serum (FBS) and 500 µl L-15 medium. The suspension was filtered through CellTrics® 30 µm (Sysmex, Germany) filters. The suspension was centrifuged for 10 min at 500 ×g, the supernatant was removed and the pellet was resuspended by gentle pipetting with addition of an appropriate volume of L-15 medium (30-100 µl). Resuspended cells were stained 1:1 with 0.4% Trypan blue solution dissolved in PBS. Oogonia viability was counted using Burker cell haemocytometer in 30 squares under 40× magnification (Nikon Eclipse Ci). Cell viability was assessed as the percentage of live cells isolated from cryopreserved tissue compared to the number of live cells isolated from the fresh tissue while correcting for the tissue size according to Lujic et al. [13].

$Viability (\%) = (N_{cryopreserved} / N_{fresh}) \times CF \times 100$ where $CF = Weight_{fresh\ tissue} / Weight_{cryopreserved\ tissue}$. All samples were assessed in this manner unless otherwise stated.

2.2 *Histology*

Ovarian fragments from 3 individuals used in the first experiment were collected and fixed overnight in the Bouin solution. Fixed samples were washed in 80% ethanol, dehydrated and cleared in an ethanol–xylene series, embedded into paraffin blocks and cut into 4- μ m-thick sections using a rotary microtome Diapath (Diapath Galileo, Italy). Paraffin slides were stained with hematoxylin and eosin by using a staining machine (Tissue-Tek DRS 2000; Sakura, Torrance, CA, USA) according to standard procedures. Histological sections were photographed and evaluated.

2.3 *Immunolabelling*

Cell suspension obtained from fresh and cryopreserved ovary, as well as control somatic cells (obtained from dissociated fin clips) were processed as described in Linhartová et al. [27]. Briefly, cells dissociated from ovarian tissue were allowed to stick on a poly-L-lysine slide, excess of the suspension was removed and adherent cells were subsequently fixed in 4% paraformaldehyde. Cells were permeabilized by 0.3% Triton in PBS and washed 3 times in PBS containing 1% BSA and 0.05% Tween. Slides were incubated with a primary rabbit polyclonal antibody to DDX4 (DEAD (Asp-Glu-Ala-Asp, also known as VASA) box polypeptide 4; Cat. No. GTX116575; Lot No. 40261) (GeneTex Inc., Irvine, USA) at a dilution of 1:300 overnight at 4 °C. Slides were then washed and subsequently incubated with a secondary goat anti-rabbit immunoglobulin antibody conjugated with fluorescein isothiocyanate (dilution 1:800) for 1 h at room temperature. Slides were washed and nuclei were stained by VECTASHIELD HardSet Antifade Mounting Medium containing DAPI. Samples were observed and photographed under an inverted fluorescent microscope at 40x magnification (Olympus IX83, Japan) and processed using cellSense software (Olympus, Japan).

2.4 *Cryopreservation*

Cryomedium was composed of 1.5 M cryoprotectant (dimethyl sulfoxide – Me₂SO, methanol – MeOH or propylene glycol – PG), 1.5% Bovine Serum Albumin (BSA), 25 mM Hepes and 0.1 M Glucose diluted in PBS. Weighted tissue pieces were loaded into 1.8 ml cryotubes (Nunc®) with 1 ml of cryomedium. Samples were placed in a freezing container CoolCell® FTS30 (Biocision) and equilibrated for 30 min on ice, then transferred into a -80 °C freezer (Sanyo). After 4 h, samples were plunged into liquid nitrogen and remained there until the viability analysis. Samples were thawed in a 26 °C water bath, tissue pieces were rehydrated and washed 3 times in L-15. Digestion and counting procedures were conducted as it is described for control samples.

Protocol optimization was conducted in five sequential cryopreservation trials where in each experiment one cryopreservation parameter was changed, and the best outcome was used in the subsequent experiment. Firstly, the addition of 1.5 M of Me₂SO, MeOH or PG to the cryomedium was tested. To find the optimal cryoprotectant concentration, 1, 1.5, 2, 2.5, 3 M (first trial) and 1.25, 1.5, 1.75, 2 M (second trial) of Me₂SO were tested. During these trials, the extender consisted of 1.5% BSA, 25 mM Hepes and 0.1 M glucose diluted in PBS. In the subsequent experiment, glucose, trehalose and sucrose in 0.1, 0.3, 0.5 M concentrations were tested as a supplement to the cryomedium containing 1.5 M Me₂SO, 1.5% BSA and 25 mM Hepes. Further, incubation times in the cryoprotectant before freezing of 15, 30, 60 or 120 min were tested. Lastly, ovarian fragments of 25, 50 or 100 mg were equilibrated for 1 h to assess whether fragment size affects the oögonia viability.

2.5 *Short-term storage*

Samples for liquid nitrogen and -80 °C storage were loaded into cryotubes filled with cryomedium containing 1.5M Me₂SO, 1.5% BSA, 25 mM Hepes and 0.1 M trehalose and equilibrated for 60 min on ice, then frozen to -80 °C in a container. Part of the samples remained

till analysis in $-80\text{ }^{\circ}\text{C}$ freezer for different time (1-7d). Tissue fragments in size of $21.3\pm 5.4\text{ mg}$ for $+4\text{ }^{\circ}\text{C}$ storage were held in 1.5 ml PBS or L-15 with 100 units penicillin and 0.1 mg streptomycin/mL supplemented with 25mM Hepes and 0.1M glucose in 24 well plates. The medium was replaced daily. All samples were analyzed for viability at 0,1,2,3,5,7 day of the storage.

2.6 Transplantation

Fertilized goldfish eggs were injected under the blastodisc at the 2-cell stage with 100 mM solution of antisense *dead end* morpholino (*dnd*-MO) oligonucleotide according to Goto et al. [31] (target sequence CATCACAGGTGGACAGCGGCATGGA) using a M-152 micromanipulator (Narishige, Japan) and CellTram Vatio microinjector (Eppendorf, Germany) in order to disrupt endogenous PGCs migration and produce sterile recipients for transplantation.

Eight days post fertilization, goldfish larvae were anesthetized in 0.05% tricaine and divided into three groups with 80 individuals per group: 1) only *dnd*-MO treated controls, 2) larvae transplanted intraperitoneally with cryopreserved/thawed oogonia stained by PKH-26, 3) larvae transplanted intraperitoneally with fresh oogonia stained by PKH-26. Group 4 consisted intact control larvae. Thirty individuals from each group were sacrificed and dissected to evaluate presence of PKH-26 positive cells in gonadal region one month post-fertilization. Goldfish were examined again at 2 months post transplantation by RT-PCR.

The excised gonads were sampled and total RNA was isolated using TRIzol and then RNA was transcribed into cDNA using Transcriptor High Fidelity cDNA Synthesis Kit (Roche). Primers for RT-PCR were designed for goldfish and carp vasa gene and tested for specificity and to find suitable annealing temperature. Carp forward primer CGGCCGGCCGGAGAGATGAG, reverse primer GATCTGGATAACCCCATACA, expected amplicon size 200bp. Primers were diluted according to the manufacturer's instruction. The reaction mixture for PCR contained 1

µl template cDNA, 0.5 µl forward and 0.5 µl reverse primer, 5 µl PPP Master Mix (Top-Bio) and 3 µl PCR H₂O (Top-Bio). Reaction conditions were 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. Products were analyzed on gel electrophoresis on 2% agarose gel on a UV illuminator.

2.7 Statistical analysis

All performed analysis were conducted in triplicates, the percentage of viability was set as a dependent variable; therefore, arcsine transformation was used. Normality and homoscedasticity of transformed data were tested by using Shapiro–Wilk test and Levene’s test. If data were normally distributed with equal variances, analysis of variance (Anova) was used for testing of significance of experimental factors. Otherwise, Kruskal–Wallis test was used. Anova analysis was followed by Turkey’s HSD test for obtaining significant differences among levels of each factor and their interactions. All tests were performed in R v. 3.4.2.

3 Results

3.1 Histological analyses

Gonads of all individuals used in this study were immature as demonstrated by the presence of solely OSCs and primary oocytes within the ovaries (Fig. 1A). Stereological analysis displayed that the OSCs were the predominant cell type within the ovaries, followed by the early-stage POs (Fig. 1B). After dissociation, only OSCs and 1A POs were present in the cell suspensions due to size-exclusive filtration (approximately 85% OSCs). Additionally, the germline origin of these cells was demonstrated by the expression of the vasa protein (Fig. 1C).

3.2 Cryopreservation

Comparisons of different cryoprotectants identified significantly higher oogonia viability rates when using Me₂SO in comparison with ME and PG (Tukey’s HSD; $p < 0.05$) (Fig. 2 A). Thus, subsequent trials were performed with Me₂SO-based cryomedia. When testing different Me₂SO

concentrations, the highest post-thaw oogonia viability was repeatedly recorded with 1.5 M (Fig. 2 B and C). The following trial tested impact of different equilibration time before freezing initiation. No significant differences were observed between the tested equilibration times, however, oogonia post-thaw viability was more favourable with prolonged equilibration times (Fig. 2 D). Equilibration time 60 min was chosen for following trial because negligible difference in post thaw viability in comparison with 120 min (Kruskal-Wallis test; $p > 0.05$). When testing different sugars, only sucrose displayed significantly lower post-thaw viability (Tukey's HSD; $p < 0.05$). Additionally, no significant differences were observed between varying sugar concentrations. (Fig. 2 E). Similarly, freezing 25, 50 or 100 mg ovarian fragments did not affect the oogonia post-thaw viability (Fig. 2 F). Identity of germ cells was confirmed by immunolabeling with germ cell-specific *vasa* antibody (Fig. 3). For the transplantation assays, we frozen 100 mg ovarian fragments, equilibrated 60 min in a cryomedium containing 1.5 M Me₂SO supplemented with 0.3 M glucose.

3.3 Short-term storage

Two different strategies were tested for short-term storage: (1) storage at $-80\text{ }^{\circ}\text{C}$ in the cryomedium prepared for cryopreservation, or (2) hypothermic storage at $4\text{ }^{\circ}\text{C}$ in a PBS or L-15 based storage solution. Results showed that both hypothermic and $-80\text{ }^{\circ}\text{C}$ storage had a negative impact on oogonia viability even after the first 24 h. Hypothermic storage was found to be more suitable as it displayed significantly highly viability rates throughout the duration of the test (Tukey's HSD; $p < 0.05$). Oogonia viability in hypothermically stored tissue retained above 50 % viability for both media (based on L15 or PBS) until day 3 of storage, however, it then decreased below 35 % for both media on day 7 of the storage (Fig. 2 G).

3.4 Transplantation

In order to test the physiological activity of oogonia after freezing, both fresh and frozen/thawed oogonia were transplanted into goldfish surrogate hosts. One month after transplantation,

dissection of the recipients revealed that both freshly isolated and cryopreserved carp oogonia had a similar colonizing ability as PKH-26 positive cells were detected in recipient genital ridges (Fig. 4; Table 1). Unfortunately, PKH-26 positive cells were not detected at 2 months post-transplantation. However, RT-PCR analysis (Fig. 5) confirmed the presence of carp germ cells expressing carp *vasa* in goldfish gonads.

group	Transplanted	Survival		No PKH-26		Carp <i>vasa</i> positive no /%
		no/% 24h pt	no/% 1 month pt	No/% with PKH-26 positive cells 1 month pt*	positive cells per germ line chimera*#	
Cryopreserved OOG	80	80/100%	69/86.2%	22/73.3%	13.4±3.1	19/63.3%
Fresh OOG	80	79/98.7%	72/90%	23/76.6%	11.7±6.9	18/60%
MO treated	80 [†]	80/100%	78/97.5%	0	0	0
Control	80 [†]	80/100%	79/98.7%	0	0	0

[†] no operation was conducted prior to transplantation in MO treated and control groups, *30 individuals analysed per group, # data are presented as a mean ±SD;

Table 1. Overall results from transplantation

4 Discussion

In the present study, we have developed for a first time a cryopreservation methodology for common carp oogonia through ovarian tissue freezing. The physiological activity of oogonia after cryopreservation was confirmed after interspecific transplantation into sterile goldfish recipients. Additionally, different short-term storage strategies were attempted as an alternative to cryopreservation. Results of this study can serve as an alternative way for long-term preservation of common carp germplasm which can be recovered in a surrogate recipient through interspecific germ cell transplantation.

4.1 *Cryopreservation*

Protocol for ovarian tissue cryopreservation using slow rate freezing was developed and subsequently improved by several trials. Me₂SO was identified as the most suitable cryoprotectant for freezing common carp ovarian tissue similarly to the studies conducted on rainbow trout [22], tench [27], goldfish [26] and Manchurian trout [24]. On the other hand, Pšenicka et al. described higher level of sturgeon spermatozoa [32] and spermatogonia and oogonia damage using cryopreservation with Me₂SO [12]. In the second study, ethylene glycol was found as the most suitable cryoprotectant, which indicates an inter-subclasses differences between chondrosteian and teleostean fishes [12].

Different equilibration times from several minutes to few hours have been used for slow-rate freezing across different species and tissues or cells. Therefore, no clear consensus or recommendation exists for gonadal tissue preservation in fish. Equilibration times from 10 min [12,27] to 60 min [24] were reported previously. Our results showed that oogonia post-thaw viability had an increased trend with prolonged equilibration time up to 1 hour before the freezing. However, differences were not statistically significant. Samples equilibrated for 2 h showed a negligible increase in viability, thus for practical reasons, 1 h lasting equilibration can be recommended.

Viability rates in different sugars and their concentration were comparable between glucose and trehalose and statistically different in comparison with sucrose irrespectively of the concentrations used. Therefore, with respect to practical use, inexpensive glucose was used in further trials and can be recommended as an alternative to the costly trehalose.

4.2 *Short-term storage*

The present study demonstrates that storage at 4 °C is a suitable alternative to freezing for up to 3 days of storage in case of tissue fragments in the L-15 medium. Oogonia viability decrease was not statistically significant in PBS and L-15 media over storage duration. This could be

caused by a decreased cellular metabolism supporting only basic requirements. This finding was also described for human pancreatic cells where no differences among 3 different media were observed [33]. Also, surrounding cells might provide a more convenient environment for oogonia resulting in high viability over days in comparison with isolated cells storage. On the other hand, almost 50% loss of viability after first 6 h of hypothermically stored ovarian tissue in common carp was reported, while oogonia viability further decreased to 20% after 24 h of hypothermic storage [29]. Obvious differences between our results and result presented by Lujčić et al. [29] are most likely influenced by the ovarian tissue developmental stage. In the present study, we used immature fish containing only oogonia and stage I oocytes (not larger than 150 μm in diameter; Fig. 1) indicating ovary developmental stage I. and early beginning of stage II. according to Gupta [34], while the study of Lujčić et al. [29] utilized fully mature individuals. This indicates that the carp ovarian tissue containing later-stage oocytes is more susceptible to hypothermia and lower temperatures as it was described on zebrafish oocytes of I-III stage when smaller oocytes were more tolerant to chilling [35]. Sensitivity of larger oocytes is attributed to due to lower membrane permeability [36], subsequently causing homeostasis failure as ATP production is insufficient to maintain ionic and osmotic equilibrium [37], resulting in early mortality of large oocytes and their subsequent deteriorating effect on the surrounding cells. According to our results, immature gonads can be stored as small fragments, however, mature gonads should be firstly dissociated and then stored as isolated cells ensuring that large oocytes are removed during isolation procedure [29]. High sensitivity of hypothermically stored isolated germ cells was reported by Falahatkar et al [28] in rainbow trout. Significant viability decrease was reported after 24 h even when the medium was supplemented with 10% FBS and 1.95% L-glutamine. Moreover, total number of cells decreased to around 25% after 24 h in comparison with fresh cells [28]. Most likely, cellular sensitivity to hypothermic storage is affected by their origin, size and composition.

In the case of -80 °C storage, oogonia viability decreased rapidly after 24 h of storage and it was below 10% after 7 days of storage. Similar decrease in viability of spermatogonia to 20.7% in comparison with control was reported in sterlet (*Acipenser ruthenus*) was reported after 48 h of storage at -80 °C, while the viability of spermatogonia immediately stored in liquid nitrogen was 31.8%. Sturgeon spermatogonia viability further decreased below 7% after 7 days of -80 °C storage [38]. Thus, it is obvious that immediate transfer into liquid nitrogen after the slow-rate freezing to -80 °C, or alternative storage of no longer than 24 h at -80 °C is recommended. Short-term viability at -80 °C can be used for slow-rate freezing performed on dry ice enabling certain portability with subsequent samples transport into liquid nitrogen within 24 h. However, it is necessary to follow up on the recommendation given by the freezing container manufacturer.

Similarly, prior to germ cell transplantation, the age of the recipients has an influence on the transplantation success in terms of successful colonization of the genital ridge [20]. Thus, hypothermic storage can be used to synchronize recipients originating from different batches with no need for cryopreservation when transplantation from a single donor is intended. The only issue is potential contamination with bacteria or fungi during gonad dissection and storage caused by medium exchange or others manipulations. Contamination by bacteria can result in decreased viability or cell quality as it was shown for sperm during its cryopreservation [39]. But more importantly, transplantation of contaminated cells could result in total mortality of recipients after transplantation (data not shown). Therefore, maximum efforts for aseptic dissection and use of antibiotics are crucial to avoid and control potential contamination.

4.3 Transplantation

Interspecific transplantation of carp oogonia revealed goldfish as a convenient host from several points of view. Primordial germ cell depletion achieved by morpholino injection against the *dead end* gene resulted in 100% sterility of morphants [31]. Sterilized recipients showed almost

no mortality after the transplantation (comparable to the control groups). Similar sturdiness of larvae to manipulation was reported for masu salmon [40]. Colonization rate assessed one month post-transplantation revealed that 22 from 30 recipients contained PKH-26 labelled cells in case of cryopreserved and 23 from 30 in case of fresh oogonia. Average number of positive cells per individual was 13.4 ± 3.1 and 11.7 ± 6.9 for cryopreserved and fresh oogonia respectively. We presume that a high number of PKH-26 positive cells found at 1 month post transplantation is indicative of the onset of their proliferation in the recipient's gonads rather than the number of cells which migrated and colonized the genital ridge successfully. Since reported numbers of donor cells after the transplantation are usually lower, up to 4 cells in rainbow trout transplanted by brown trout and grayling germ cells 60 days post transplantation [41]. Similarly, Lee et al. [22] reported up to 3 donor derived cells 20 days post transplantation, with observable proliferation at 31 days post transplantation of rainbow trout germ cells transplanted in triploid recipients. Donor derived cells with fluorescent signal were not detected at 2 months post transplantation in this study. However, RT-PCR showed carp *vasa* expression as a marker of donor-derived germ cells in recipients gonads, which clearly indicated that donor derived cells were present and undergoing gametogenesis. Reason for no PKH-26 signal could be photo-bleaching or poor staining efficiency in combination with excessive dye dilution due to proliferation of transplanted cells. Thus, higher volume of PKH-26 dye is probably necessary as it is recommended by Lujić et al. [41].

Conclusion

This is a first report about cryopreservation of common carp oogonia. Cryopreservation protocol using slow rate freezing was developed using cryoprotectant based on Me_2SO . Protocol was optimized through several trials when the final post-thaw oogonia viability was 67.5%. Field or local conditions can give rise to several limitations in availability of cryopreservation equipment. Therefore we demonstrated a possibility for short-term storage of

ovarian tissue in +4 °C simulating storage on ice with subsequent slow rate freezing and storage in liquid nitrogen. Similarly, short-term storage in -80 °C freezer was performed in order to simulate availability of dry ice only, or liquid nitrogen unavailability, but applicability of this approach is limited to progressively decreasing viability.

Results of this study can be adopted as a complete strategy for practical use when ovarian tissue from immature specimens can be cryopreserved efficiently or stored short-term on ice or in a common refrigerator up to 3 days. Transplantation assay demonstrated that cryopreserved oogonia can be recovered in goldfish recipients with comparable success to fresh oogonia. We intend to produce more germline chimeras between carp and goldfish and obtain progeny after recipient maturation. To succeed, focus transplantation optimization is necessary. Further steps will be also taken to develop vitrification procedure for ovarian tissue as it is rapid way to preserve gene resources.

Conflict of interest

Authors declare that no conflicts of interest exist.

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Figures

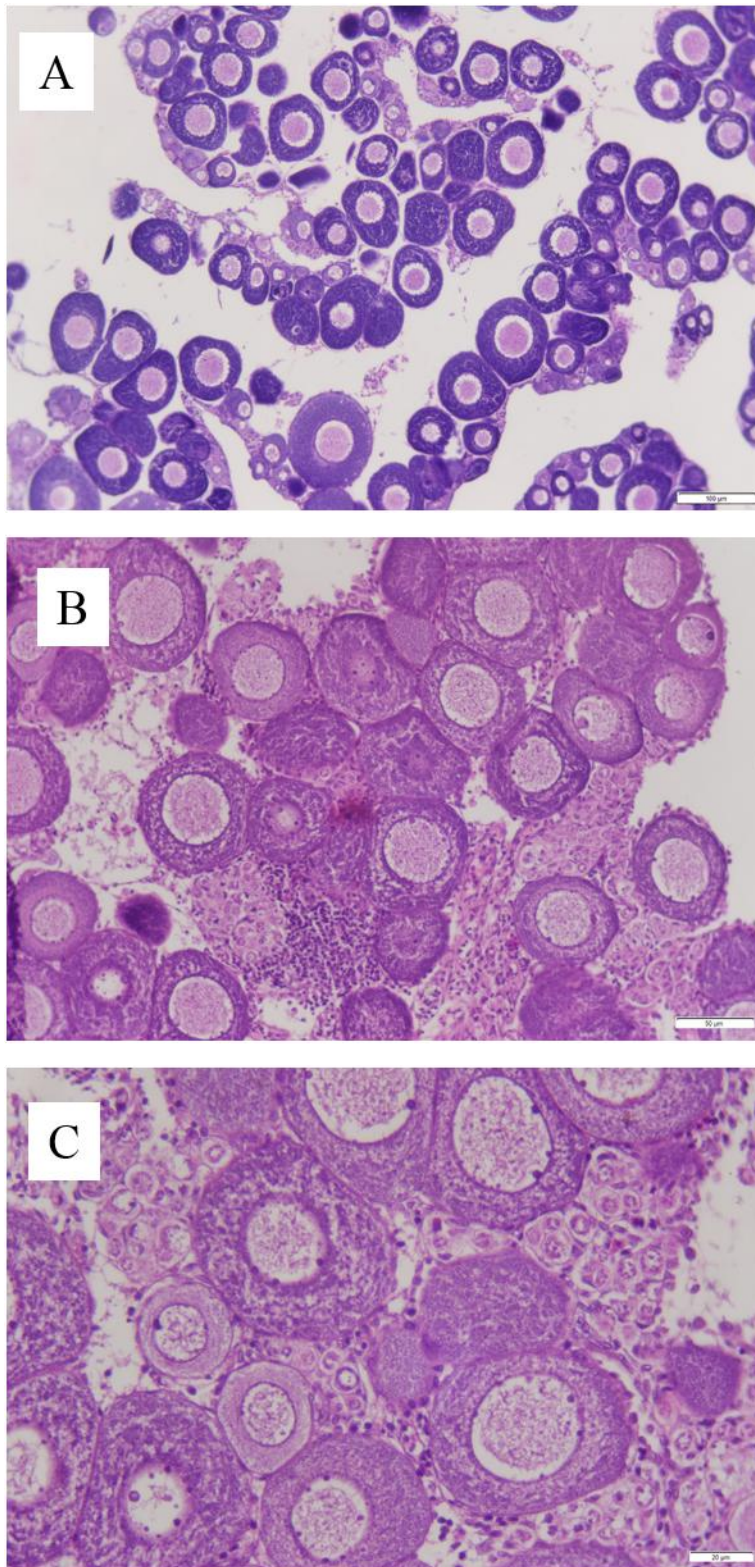


Fig 1. Histological section from ovaries used in experiments

Juvenile ovaries contained mostly primary oocytes, having diameter less than 150 µm. Scale bars: A – 100 µm, B - 50 µm, C – 20 µm

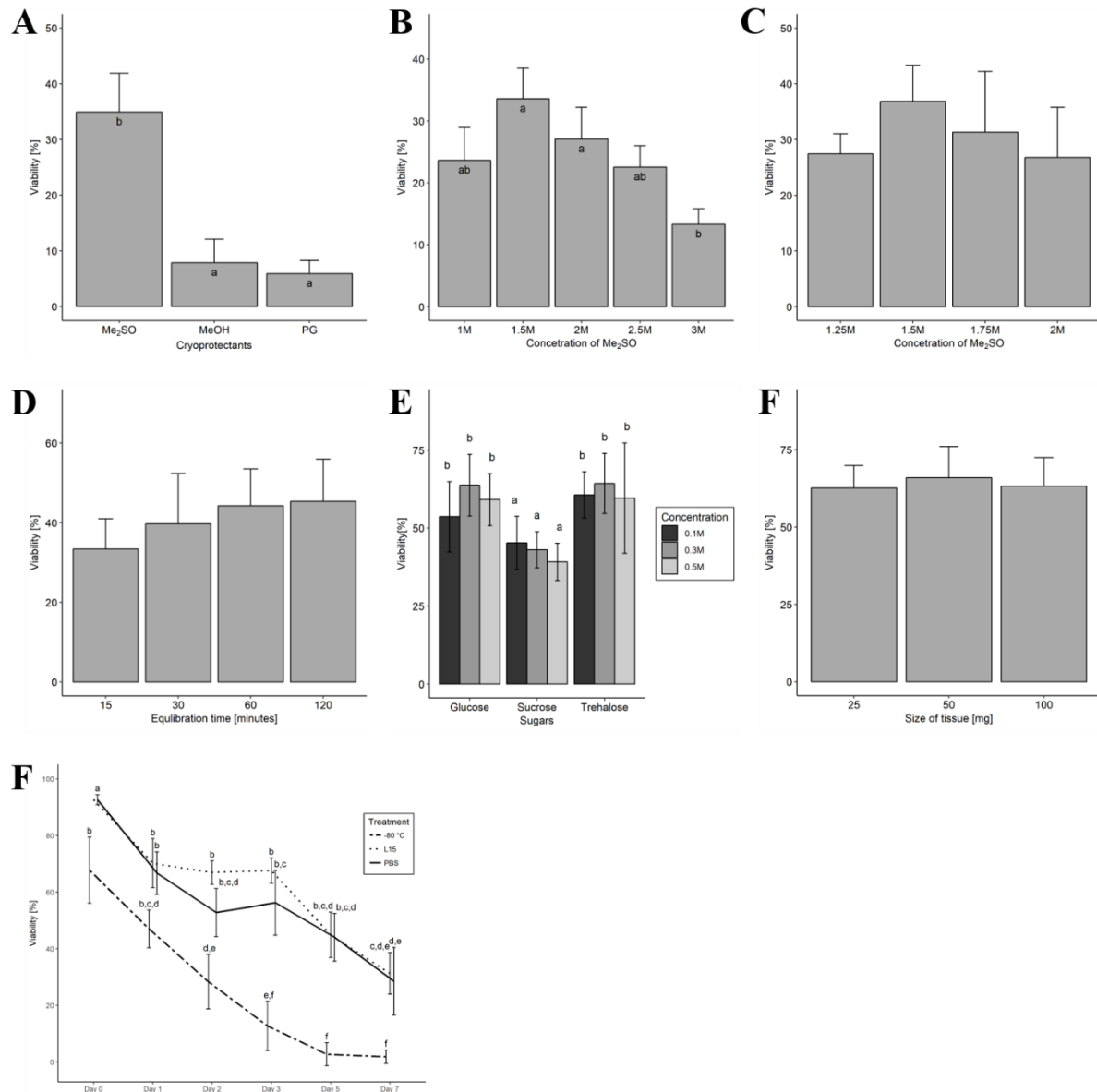


Fig. 2. A-F Oogonia post-thaw viability in % cryopreserved by slow rate freezing and stored in liquid nitrogen, all data are presented as mean ± SD.

A) Viability in different cryoprotectants (Me₂SO – dimethylsulfoxide, MeOH – methanol, PG – propylene glycol), and B,C) Me₂SO concentration. A,B) - different letters above the SD bars indicate significant difference (Tukey’s HSD; p < 0.05). C) Viability in % cryopreserved in 1.5M Me₂SO based cryoprotectant with different equilibration times before freezing initiation. No significant difference (Kruskal-Wallis test; p > 0.05) Results are presented as mean ± SD.

E) Cryopreservation with different sugars supplementation (glucose, sucrose, trehalose) and concentrations (0.1M, 0.3M, 0.5M) (Tukey's HSD; $p < 0.05$). F) Influence of differently sized ovarian tissue fragments (25, 50, 100 mg) on viability. No significant difference (Shapiro-Wilk test; $p > 0.05$). G) Oogonia viability in % obtained after short-term storage(0-7 days) period of hypothermic (L15 or PBS medium) and -80 °C storage after slow rate freezing (cryopreserved in 1.5M Me2SO based cryoprotectant). Different letters above the SD bars indicate significant difference (Tukey's HSD; $p < 0.05$).

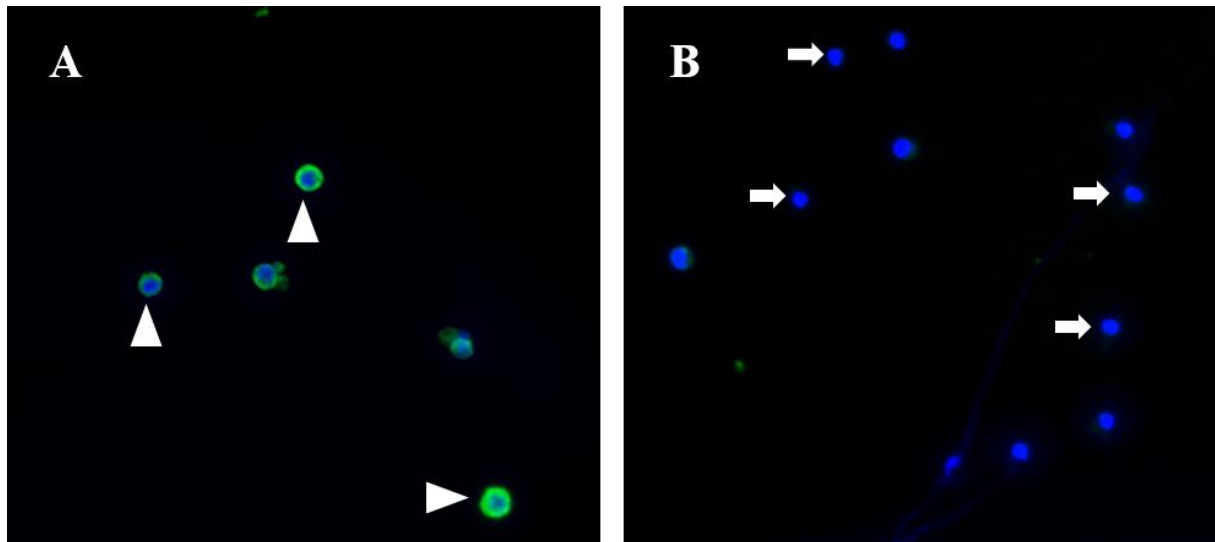


Fig. 3

Immunolabelling of carp oogonia and somatic cells with *vasa* antibody and DAPI. A- cryopreserved cells with green (*vasa* antibody) signal and blue (DAPI) signal pointed by arrowheads. B- control somatic cells with blue (DAPI) signal pointed by arrows .

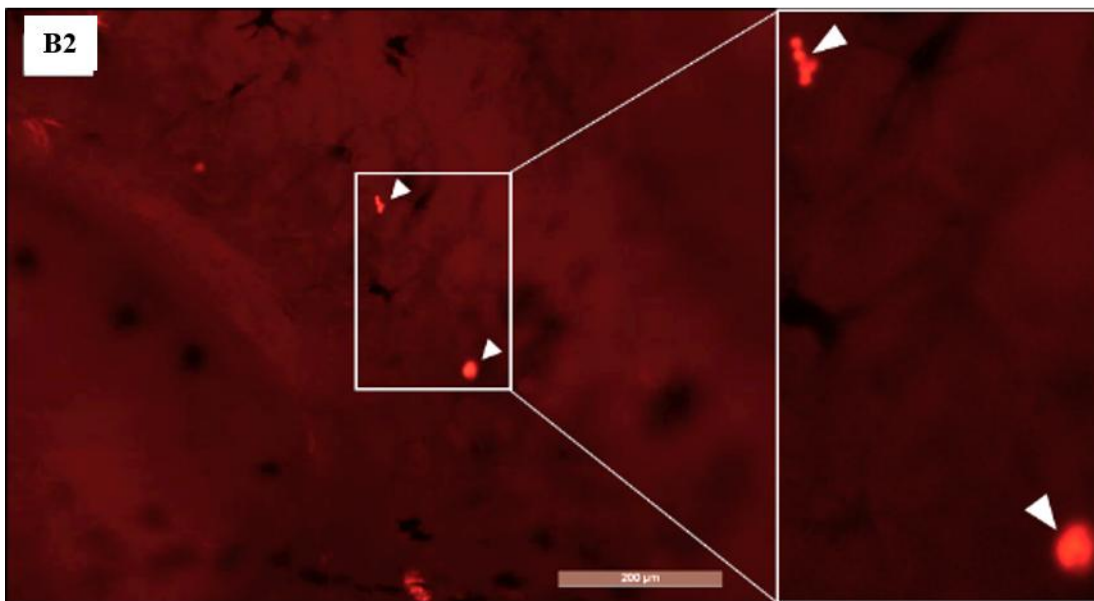
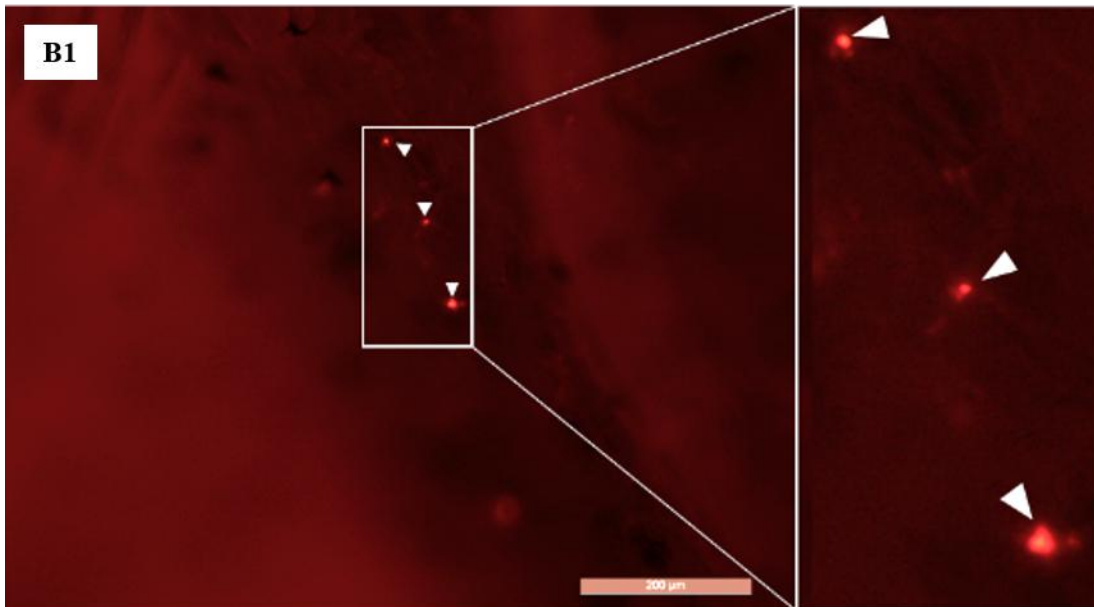
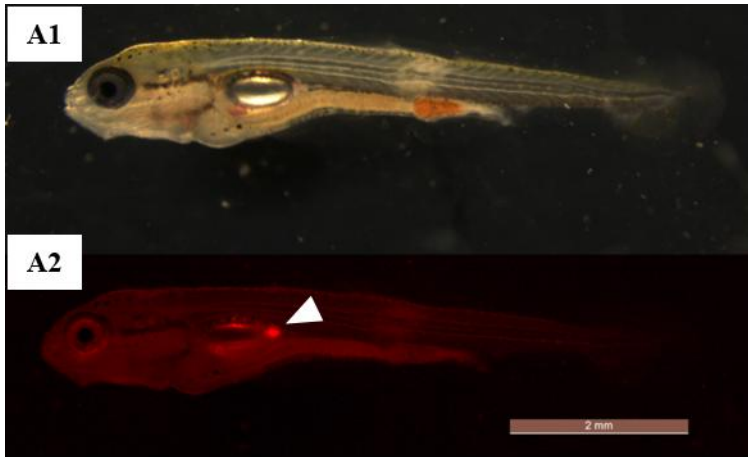


Fig. 4

Goldfish germ line chimera transplanted with cryopreserved PKH-26 stained carp oogonia. A- Goldfish larvae just after the transplantation of PKH-26 labelled cells, A1 – bright field, A2- DsRed channel, PKH-26 positive cells are depicted by arrowhead, scale bar 200 μm . B – Dissected goldfish larvae at 1 month post transplantation, B1 – Goldfish transplanted with cryopreserved cells, B2 – Goldfish transplanted with fresh cells, PKH-26 positive cells are depicted by white arrowheads.

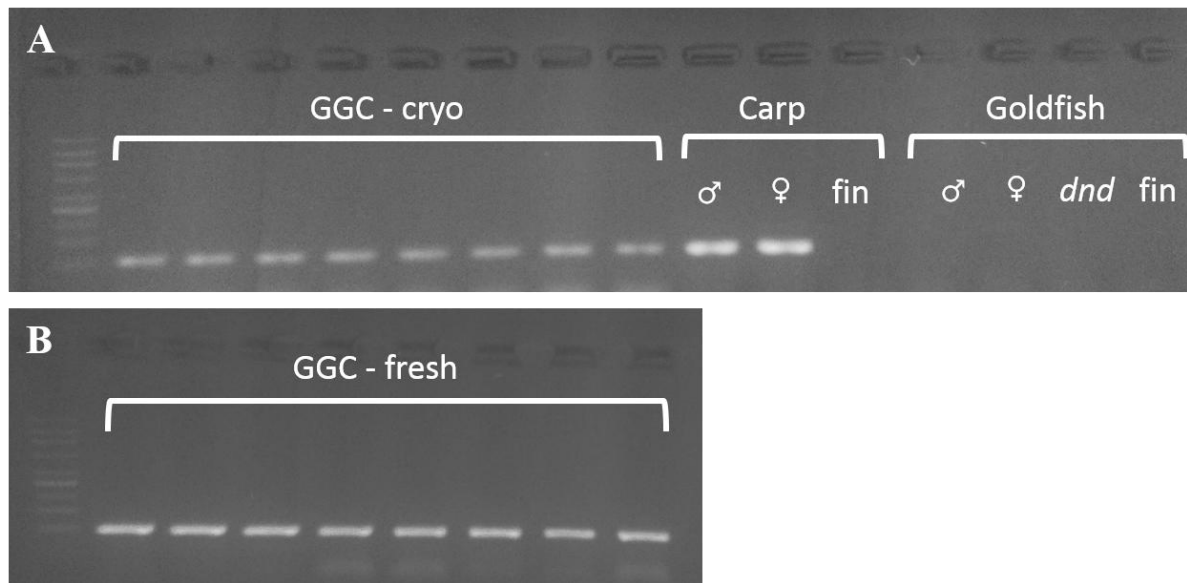


Fig. 5

Gel electrophoresis of amplicons from RT-PCR with carp *vasa* positive primers. RNA isolated and transcribed to cDNA from gonads of goldfish germline chimeras transplanted with cryopreserved/thawed oogonia (GGC - cryo), control carp ovary (♀), testes (♂) and fin (Carp control), from goldfish control ovary (♀), testes (♂), fin and *dnd* Morpholino injected fish (*dnd* MO) (A), goldfish germline chimeras transplanted with fresh oogonia (GGC – fresh) (B).

Graphical abstract

