Transplantation worthiness of cryopreserved germ cells of Indian major carp rohu, *Labeo rohita*

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Cryopreservation of gametes and germ cells is an essential tool for germplasm conservation and improvement of productivity in aquaculture. Here, transplantation worthiness of isolated cryopreserved germ cells (GCs) of Indian major carp, rohu *Labeo rohita* has been tested by their viability and colonization ability in the allogenic host (*Catla catla*). GCs were cryopreserved using dimethyl sulphoxide (DMSO), ethylene glycol (EG) and glycerol. Rohu GCs were successfully cryopreserved with significantly higher viability using slow cooling rate of –1°C/min and a medium containing 1.4 M DMSO compared to EG and glycerol. It was found that more than 70% GCs were viable following this method. Transplantation experiment revealed that frozen/thawed GCs colonized and proliferated in the gonad of the recipients. Hence, this technique of transplantation of GC into adult gonads paves the way for further applications in surrogate animal development.

Keywords: Carp, cryopreservation, toxicity, transplantation, viability.

CRYOPRESERVATION of germplasm that includes male and female gametes and germ cells (GCs) offers possibilities of preserving the genome of endangered species. It is a long-term storage method that employs ultra-low temperature to preserve the structurally intact viable cells and tissues for a prolonged period of time at a relatively low cost[1]. Cells of a wide variety of organisms have been successfully cryopreserved and their banking has been a regular procedure in animal husbandry, medical practice and conservation science[2,3]. Moreover, conservation of germplasm (gametes, embryos and larvae) by cryogenic methods has become essential in the rapid climate change era[4]. Protocols for milt cryopreservation are available for many fish species[5,6], however, fish eggs and embryos have not been successfully cryopreserved till now[7,8]. In this scenario, cryopreservation of GCs offers hope for conservation of germplasm for future biotechnological application in aquaculture.

GCs are considered to be immortal since they create a link between generations and multiply through mitosis. Such lineage of GCs is called a germ line. However, involving passive and active movements, the GCs arrive at the developing gonads transformed into egg or sperm respectively. GCs (primordial germ cells, spermatogonial and oogonial cells) play a crucial role in reproduction and have the potential to undergo proliferation[9], differentiate into functional gametes and pass the genetic information to the next generation[10], making them suitable for transplantation[11]. For production of surrogate fish, a general procedure involves transplantation of GCs into the host fish. Many times the isolated GCs are in excess and at other times the host fish is not ready. Under these circumstances, it is imperative to cryopreserve isolated GCs for future use as and when required. There are reports about the cryopreservation of GCs in different fish species, viz. rainbow trout[12], Siberian sturgeon[13], *Tinca tinca*[10] and zebrafish[14].

GCs are believed to be well-suited to cryopreservation, due to their small size and a high level of sexual plasticity which allows them to differentiate into fully functional gonads of both sexes[12,15]. It has been reported that cryopreserved GCs can be transplanted between closely linked species without compromising the ability to differentiate into gametes in the host gonad[16]. In the present study, the GCs derived from male and female carps (*Labeo rohita*) have been isolated and cryopreserved with different cryoprotectants and these cryopreserved GCs have been transplanted to recipient carps (*Catla catla*) to assess the transplantation worthiness of the GCs, and colonization ability and proliferation in host fish.

For collection of gonads and GC, adult Indian major carp, *Labeo rohita* (mean body weight of males, 400.6 ± 1.44 g and females, 400.2 ± 0.86 g) were reared in 0.2 ha brood rearing earthen ponds in the aquaculture farm facility of the ICAR-Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar, India. Fish were fed daily with commercially available pellet feed ad lib.

For isolation of GC, adult male and female fish were anaesthetized prior to dissection with 1–2 phenoxyethanol. Anaesthetized fish were taken out of the water and dissected ventrally, and gonads (either testis or ovary) were taken out by cutting their basal stalk. The collected gonads were cut into pieces of 2–3 mm size using sterilized scissors. These small pieces were separated to get single cells using 2% collagenase (Sigma, St. Louis, MO, USA) in Dulbecco's modified Eagle'emdium/Ham F-12 medium (DMEM/F-12 Sigma, St. Louis, MO) at 25°C. The cell suspension was incubated with 0.25% trypsin/1mM EDTA and 0.03% DNase I for 30 min under similar conditions. An equal volume of foetal bovine serum (FBS, Gibco, Waltham, MA, USA 02451) was used to deactivate trypsin. A 45 μm mesh cell strainer was

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used to filter the cell suspension and centrifuged at 200 *g* for 10 min and re-suspended in DMEM/F-12. Thereafter, germ cell isolation was done by percoll (MP Biomedicals, LLC, France) gradient centrifugation that comprised centrifuging testicular cells for 10 min (800 *g*) at 25°C. The process resulted in the appearance of three distinctive bands. The phase containing the principal cells (GCs) was harvested from the middle layer, rinsed and subjected to trypan blue (0.4%) dye exclusion test for cell viability. The protocol described earlier by Lacerda *et al.*[17] was followed here to obtain rohu GCs.

The eventual somatic cells were removed from the pooled cell suspension by differential plating after enrichment. A total of 1.5×10^6 cells per dish were cultured in composed media with 10% FBS and 10,000 U/l ampicillin for 12 h at 28°C in an atmosphere of 5% CO_2. Satisfactory purification of GCs was possible by this procedure, since somatic cells are attached to the culture dish.

GC concentration in the gonad of each fish was estimated by counting the number of cells diluted in PBS (dilution 1 : 10) using a haemocytometer at 100× magnification and a microscope. The number of GCs was counted in 20 squares of the cell chamber with two repetitions, and GC content was expressed as the absolute number of GCs obtained from the gonad of each fish by multiplying the number of GCs by the volume of each GC sample (0.5 ml).

Red and green fluorescent cell linkers (PKH26 and PKH27; Sigma) were used to label GCs, similar to that used in domestic mammals[18]. These cell surface markers are lipophilic dyes interspaced between the cell membrane lipid bilayer and last for several days; they are reported to be non-toxic[18,19]. In order to label rohu GCs, we tested several concentrations of PKH26 and PKH27 for different time periods. Taking into account cell viability and label intensity, best conditions for labelling rohu GCs for transplantation were 14 µl of PKH26 per ml of diluent per ten million of cells for 10 min, similar to that followed by Lacerda *et al.*[17].

Chilling sensitivity of isolated GCs of rohu and toxicity of various CPAs (Cryoprotective Agents) was analysed at different concentrations to find out the appropriate concentration for obtaining best results for cryopreservation. Three CPAs, dimethyl sulphoxide (DMSO), glycerol and ethylene glycol (EG), at concentrations 0.6, 1.0, 1.4 and 1.8 M were taken for toxicity study. Cell viability was tested using trypan blue dye exclusion assay. Similarly, for chilling sensitivity, cells were kept in two refrigerators (Kirloskar, India Ltd and local manual freezer), one maintained at 0°C and the other maintained at –10°C, and they were compared with a control set of cells incubated at 28°C. The sensitivity was tested at an interval of 1 h up to 4 h. For each low-temperature regime, cells were first kept at 4°C for 30 min and then transferred to 0°C and subsequently to –10°C. Cell survivability was scored by trypan blue dye exclusion test after washing 5–6 times in 0.1X PBS to remove the CPAs. Toxicities were evaluated by assessment of cell viability of the GCs using AO/EB stain. After 5 min of incubation, the cell suspension (20 µl of aliquot) was placed on a haemocytometer and observed under a fluorescent microscope. Non-viable (dead) cells were stained with EB showing red fluorescence. The number of non-viable cells and the total number of cells were counted in each field. The viability of the cells was evaluated with triplicates in each cryoprotectant.

For cryopreservation, isolated GCs were inoculated at a cell density of approximately 10^6 cells ml^{-1}. During different treatments, CPAs were diluted accordingly with the media. GC samples from rohu male and female fish were treated individually. Before freezing, GCs 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, 50 mM D-glucose (ref. 20), and 0.6, 1, 1.4 and 1.8 M cryoprotectant to a final concentration of 10^6 cells/ml. Three cryoprotectants were studied: DMSO, glycerol, EG. Tests were conducted in triplicate for each sample. Volumes of 100 µl diluted GC were placed into cryovial (Nunc), mixed well in a step-wise manner and transferred to –10°C. They were cryopreserved using a programmable cryofreezer (KRYO 10 Programmable Freezer; Planer Products, Sudbury-on-Thames, UK) where cooling rate was maintained –1°C/min for different sets of cells up to –80°C and transferred to a deep freezer at –80°C (Haier, USA). After keeping them overnight, they were plunged into 35 litre capacity liquid nitrogen (LN_2) cryocans for storage.

Thawing of GCs was performed in a rapid warming phase. The cryovial containing cells were brought out of LN_2 and immediately immersed in a water bath at room temperature for 40 sec, diluted with 10 volumes of media after transferring into a sterilized 15 ml tube. The thawing procedure was undertaken in a laminar air flow hood and cell suspension was centrifuged at a speed of 500 *g* for 5 min.

The viability assessment of post-thawed GCs was done by trypan blue dye exclusion test and AO/EB staining. Post-thawed GCs were seeded onto gelatin-coated six-well plates in composed medium. Trypan blue stain in D-PBS (1× D-PBS prepared from 10× D-PBS stock solution) was added to make a final concentration of 0.4% to 450 µl of the cell suspension. Only dead cells were stained blue in colour. The unstained living cells were counted with the help of a compound microscope on Neubauer's glass slide. Dead and live cells were counted under a microscope using the following formula:

$$\text{Percentage of live cells} = \frac{\text{Unstained cells}}{(\text{Unstained cells} + \text{stained cells})} \times 100.$$

Assessment of cell viability of post-thawed GCs was done using the double-staining method based on the differential uptake of this florescent DNA-binding dye, acridine orange (AO) and ethidium bromide (EB; Sigma–Aldrich, USA). This differentiates viable, apoptotic and necrotic cells. AO is a green colour dye taken up by both live and dead cells, while EB is a red colour dye taken up only by dead cells. The dye mixture was prepared in PBS at pH 7.4 containing 100 µg ml^{-1} each of AO and EB. For this, 5 ml of cell suspension was taken in a T25 flask. Next 200 µl of AO/EB dye mixture was added to it and observed immediately using an inverted fluorescent microscope (Hund Wetzlar; www.hund.de).

After decapitation of fish, the gonads were excised humanely. The gonad samples were fixed by immersing in 4% paraformaldehyde (150 mM phosphate buffer, pH 7.4) for 1 and 4 h on ice. Subsequently, the gonad tissue was incubated in 25% sucrose/1× PBS overnight at 4°C for cryoprotection. Finally, the tissue was embedded in 'tissue freezing medium' (Leica Microsystems) and frozen on dry ice. Cryosections (10–12 µm) were created using a CM3050S cryostat (Leica Microsystems) and mounted onto frosted microscopic slides (Borosil). Further, the slides were stained with DAPI (4′,6 diamidino-2-phenylindole dihydrochloride), a DNA fluorescent counter stain (Roche) followed by antifade (MPS Bio; www.mpbio.com). Finally, the cover slips were fixed gently (upside down) on the slide with DPX (di-*N*-butyle phthalate in xylene) and seen under a confocal laser-scanning microscope (Leica Microsystems; www.leica-microsystems.com).

The assessed parameters were compared using one-way ANOVA with Tukey's multiple comparison tests and Graphpad prism ver. 5.0 for Windows (Graph Software, San Diego, CA, USA). Data were represented as mean (replicates) ± standard error of mean (SEM). A P value of 0.05 was considered to be statistically significant.

Figures 1–3 show the results of CPA toxicity treated with three types of cryoprotectants (DMSO, glycerol and EG) and chilling sensitivity of GCs to different immersion temperatures. The toxicity of all CPAs was maximum at 28°C, irrespective of their concentration at this temperature (Figure 1). At this temperature, most of the GCs (>50%) were non-viable. GCs immersed in DMSO, glycerol and EG at a temperature of –10°C showed maximum viability and minimum toxicity with 0.6, 1, 1.4 and 1.8 M CPA concentration (Figure 2). Among all the CPAs tested with different concentrations, 1.4 M DMSO was found to be most effective in terms of GC viability. GCs without CPAs exposed to lower temperatures of –10°C and 0°C could not sustain beyond 2 h. Their viability dropped to less than 20% (Figures 2 and 3).

The efficacy of cryopreservation was evaluated by comparing the percentage of viable GC after cryopreservation (Figure 4). The results indicate that GCs cryopreserved using DMSO (1 and 1.4 M) show significantly higher viability ($P < 0.05$) than the other two CPAs. The percentage of viable GCs after cryopreservation with three cryoprotectants having 1.4 M concentrations was: DMSO 71 ± 1.2, glycerol 61 ± 1.9 and EG 45 ± 0.8. The viability assay was based on the trypan blue dye exclusion test. Cells that took blue colour were regarded as

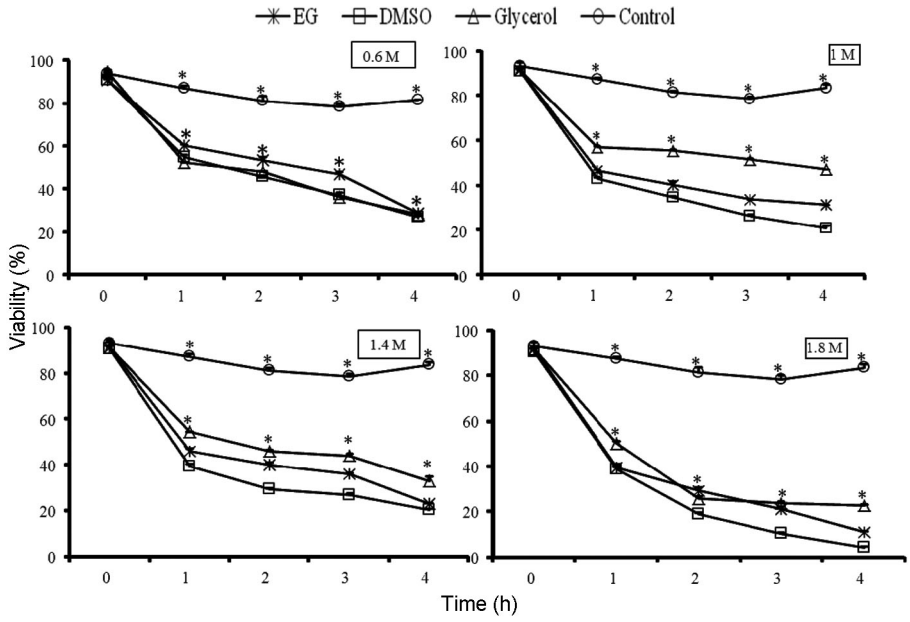

Figure 1. Chilling sensitivity of rohu germ cells stored at different concentrations of cytoprotective agents at different time intervals (1–4 h) at an incubation temperature of 28°C. Viability assessment is based on the trypan blue dye exclusion assay. Asterisks indicate significant values ($P < 0.05$). Data are shown as mean values ± SEM ($n = 4$).

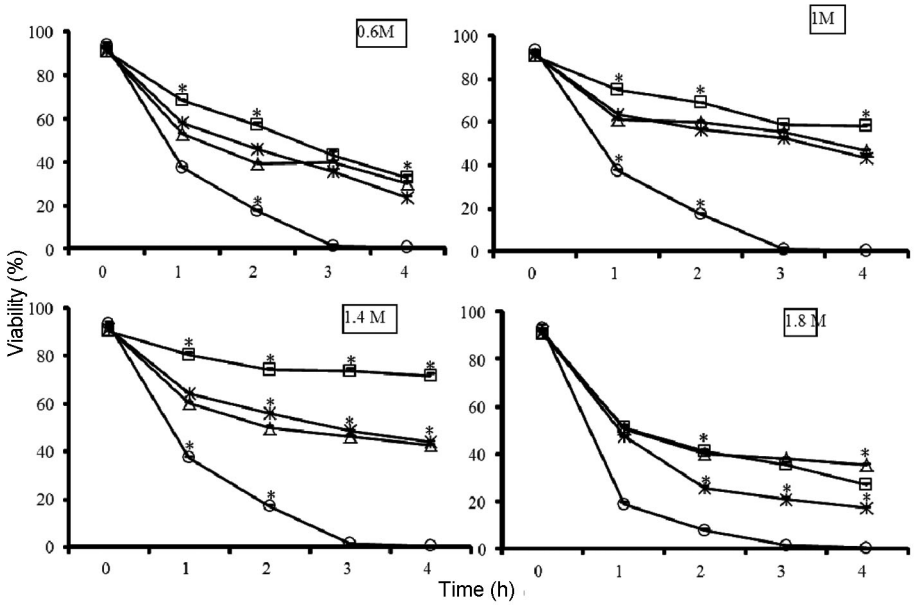

Figure 2. Chilling sensitivity of rohu germ cells stored at different concentrations of CPAs at different time intervals (1–4 h) at an incubation temperature of –10°C. Viability assessment is based on the trypan blue dye exclusion assay. Asterisks indicate significant values ($P < 0.05$). Data shown as mean values ± SEM ($n = 4$).

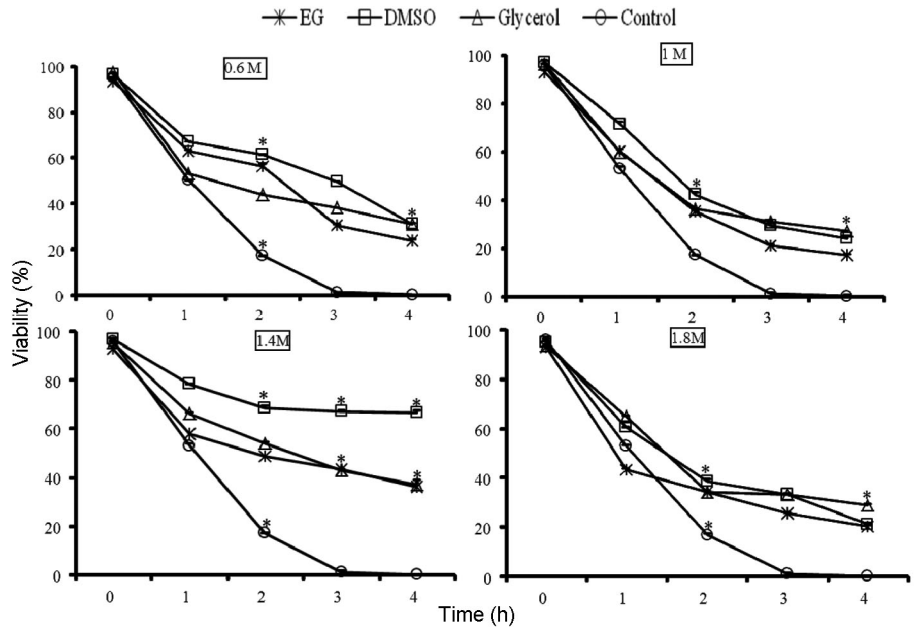

Figure 3. Chilling sensitivity of rohu germ cells stored at different concentrations of CPAs at different time intervals (1–4 h) at an incubation temperature of 0°C. Viability assessment is based on the trypan blue dye exclusion assay. Asterisks indicate significant values ($P < 0.05$). Data are shown as mean values ± SEM ($n = 4$).

dead (non-viable) and those that did not take blue colour were regarded as live (viable; Figure 5 *a*). The GCs were further tested for viability using AO/EB stains and observed under a fluorescent microscope. The viable (live) cells were visible as green and dead (non-viable) cells as red (Figure 5 *b*). After using both staining techniques, it was found that the viability rate was consistent and no significant differences were observed between them irrespective of the techniques used (Figure 6).

Before transplantation, cryopreserved GCs of *L. rohita* were tagged with two kinds of fluorescent cell linker dyes, PKH 26 and PKH 67. Dye uptake was confirmed by

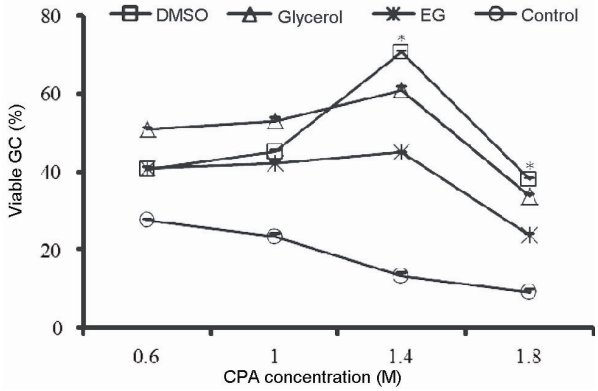

Figure 4. Effect of different cryoprotectants on germ cells (GCs) after thawing; the most effective cryoprotectant DMSO concentration is demarcated by asterisks. Data are shown as means ± SEM. ANOVA and Tukey's HSD, $P < 0.05$.

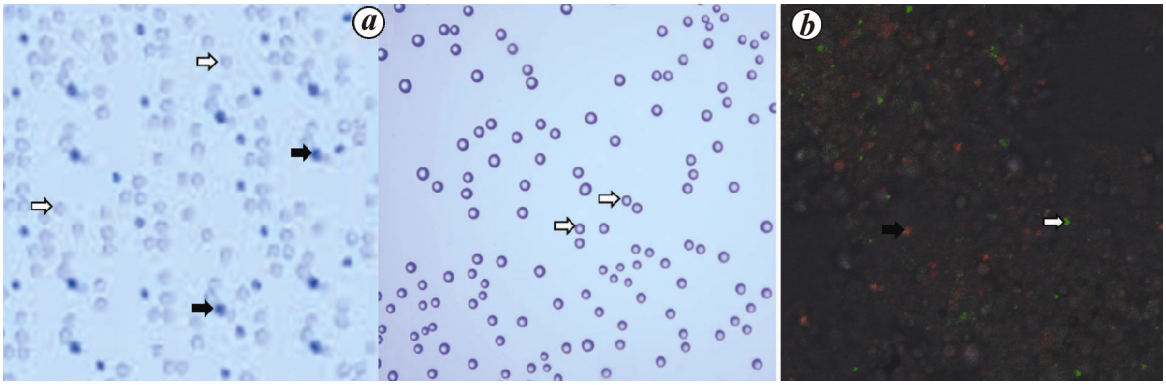

Figure 5. Viability assay of *Labeo rohita* GC by different methods. ***a***, Trypan blue exclusion test and ***b***, AO/EB. Dark arrowheads show dead (non-viable) germ cells and empty arrowheads show live (viable) germ cells.

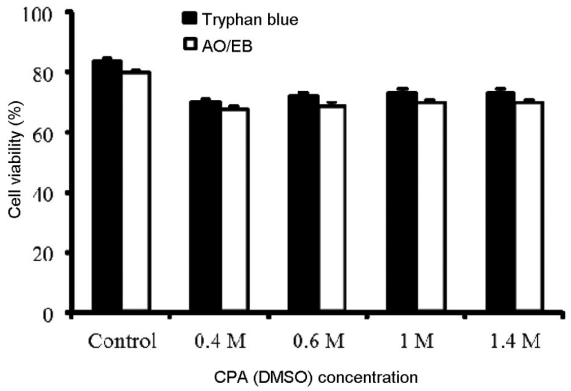

Figure 6. Comparison of viability results obtained by two cell death detection methods like AO/EB and trypan blue dye exclusion. The results are expressed as percentage. Data are shown as mean ± SEM.

visualization using a confocal microscope (Leica Microsystems; www.leica-microsystems.com; Figure 7). The transplantation worthiness was further checked by positive expression of these two dyes in the histological thin cryosections (Figure 8). It is evident from Figure 8 that the transplanted GCs got attached to the host gonad and proliferated. The results clearly indicate the transplant worthiness of cryopreserved GCs of carps as we successfully transplanted cryopreserved GCs into recipient gonads, tracked and retrieved those cells. Figure 9 provides the detailed protocol of cryopreservation and transplantation of GCs.

Cryopreservation of germplasm that includes male and female GCs is an important conservation method for any species. Cryopreservation and transplantation of primordial GCs have been reported in trout that resulted in donor-derived male and female gametes and fertile offspring from allogenic recipient[15]. Xenotransplantation has been reported in turbot fish using cryopreserved spermatogonia from Senegalese sole[21]. Here, we have shown a method of cryopreservation of GCs isolated from gonad of carps (*L. rohita*) and successfully transplanted into gonads of allogenic host *C. catla*. To check the transplant worthiness of GCs, cryopreserved GC of *L. rohita* was checked for viability and tagged using cell-linker dyes, PKH 26 and PKH 67. These tagged GCs were transplanted into the testis of an allogenic recipient carp. It was noticed that after one month of transplantation, these post-thawed cryopreserved cells colonized the recipient gonad. This was evident from the cryosectioning of the recipient gonad by confocal imaging of the red and green

Table. 1. Comparative account of transplantation of cryopreserved germ cells in different species

Donor species	Receipient Species	Germ cells	Success	Reference
Japanese quail (*Coturnix japonica*)	Japanese quail (*Coturnix japonica*)	Cryopreserved PGCs from the blood of 2-day-old embryo	Donor-derived offspring	30
Hamster	Mouse	Cryopreserved SSCs	Colonization of cryopreserved germ cells in the receipient testis	31
GFP-transgenic trout (*Oncorhynchus mykiss*)	Wild-type trout (*Oncorhynchus mykiss*)	Cryopreserved PGC	Male and female donor gametes	26
Rabbit	Mice	Cryopreserved SSCs	Colonization of Cryopreserved germ cells in the receipient testis	32
Nile tilapia (*Oreochromis niloticus*)	Strains of Nile tilapia (*Oreochromis niloticus*)	Cryopreserved SSCs	Donor-derived offspring	33
Senegalese sole (*Solea senegalensis*)	Turbot (*Scophthalmus maximus*)	Cryopreserved SSCs	Donor-derived offspring	21
Zebra fish (*Danio rerio*)	Embryo of Zebra fish (*Danio rerio*)	Cryopreserved PGCs	Donor-derived offspring	34
Rainbow trout (*Oncorhynchus mykiss*)	Whole testes (WT) triploid rainbow trout	Cryopreserved testis	Successful production of normal, frozen ASG-derived offspring	16
Rohu (*Labeo rohita*)	*Catla catla*	Cryopreserved germ cells	Colonization of cryopreserved germ cells in recipient testis	Present study

PGCs, Primodial germ cells; SSCs, Spermatogonial stem cells.

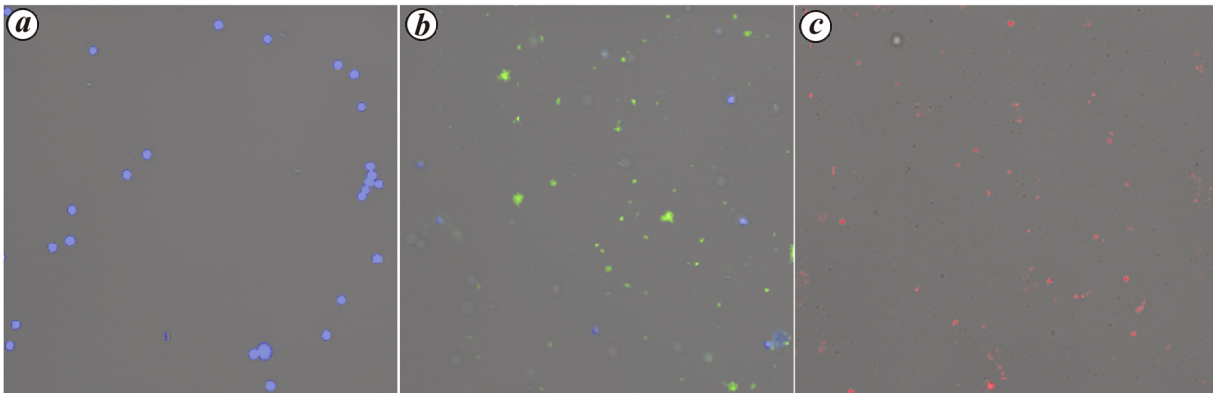

Figure 7. Post-thawed germ cells of *L. rohita* tagged with fluorescent dyes before transplantation and observed under confocal microscope. (***a***) DAPI, (***b***) PKH 67 and (***c***) PKH 26. Dye uptake shows their viable condition and transplant worthiness into recipient fish.

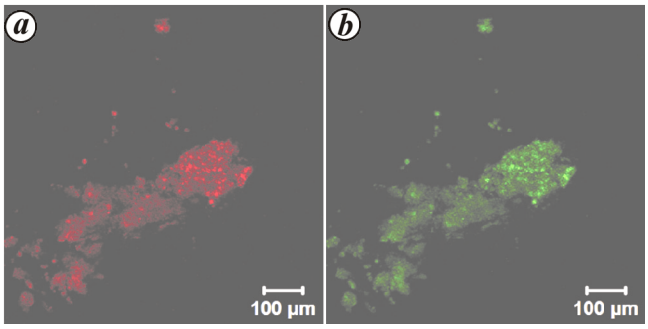

Figure 8. Confocal images of testicular GCs of *L. rohita* in the testis of recipient fish (*Catla catla*) after one month of transplantation showing proliferative condition. GCs of *L. rohita* were marked with PKH 26 and PKH 67 GC marker dyes. (***a***) PKH 26 and (***b***) PKH 67.

fluorescent cells. This technique holds promise as it may enhance the production of gametes of species that are commercially valuable, endangered or have complex reproduction, using a more readily available species as a surrogate host[12–15].

Before cryopreservation, the toxicity of CPAs and chilling sensitivity of the GCs were assessed, and DMSO was selected for further cryopreservation. DMSO is reported to be a good cryoprotectant at low temperature for several fish species and other animals[22]. It is a widely used CPA among animals as it has the highest glass-forming ability[23]. However, DMSO was found to be toxic at 28°C due to its highly permeating nature. Similarly, the other two CPAs, glycerol and EG, were effective to some

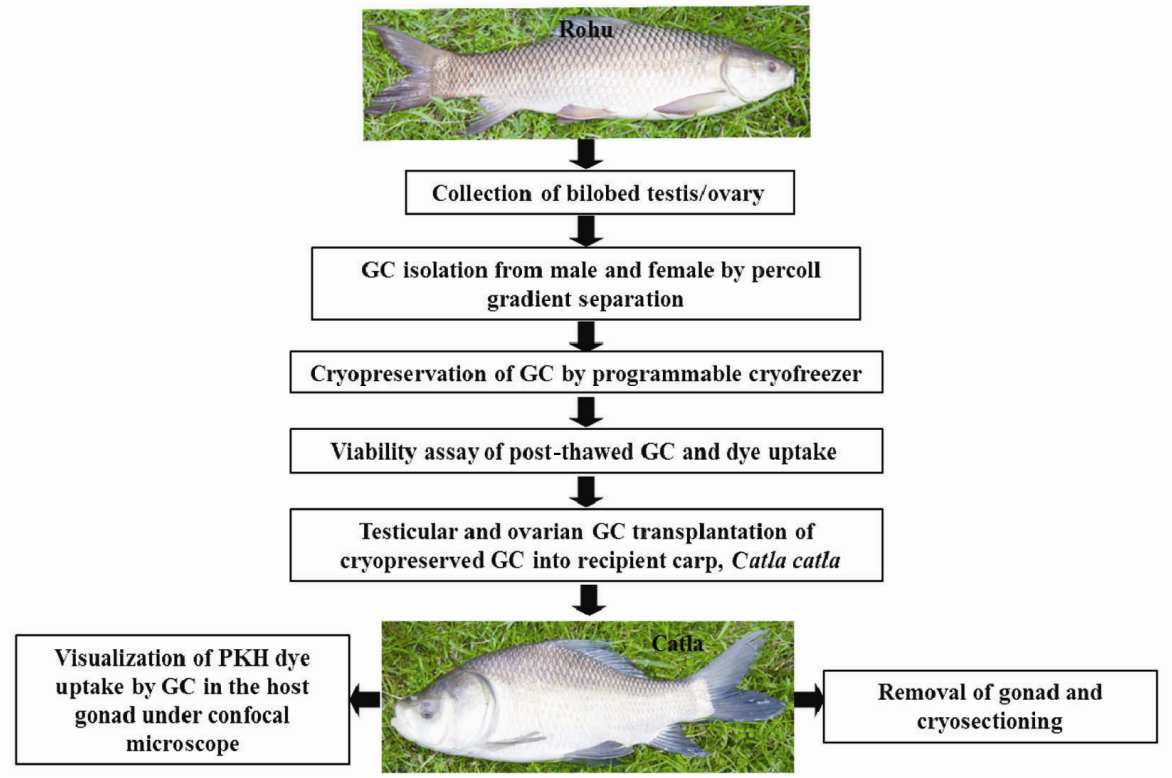

Figure 9. Process of cryopreserved GC transplantation into host gonads and its viability study.

extent but not as effective as DMSO. This may be due to their permeability status and toxicity rate. Toxicity of CPAs reported earlier[24] is similar to that in the present study: DMSO < EG = glycerol. In a comparative study on early blastocyst preservation of mouse using l CPAs (EG, PG and glycerol), EG showed better results than the other two[25]. However, DMSO was not incorporated in the study. DMSO was the cryoprotectant of choice because of low toxicity at lower temperature. Here the best concentration of permeating cryoprotectant DMSO was 1.4 M.

GCs were less sensitive to chilling temperatures of 0°C and –10°C in the presence of cryoprotectants. It was observed that with increase in concentration of CPAs, viability increased up to 70% at lower temperatures of –10°C. Similar results were also obtained at 0°C after 1 h of treatment. Cryopreservation of GCs using these post-thawed cells showed a viability rate of more than 70%. A comparable survival rate of 73% in rainbow trout primordial GCs using EG and PG has been estimated[26]. Embryonic stem cell survival of 83% in a carp species (*L. rohita*) using DMSO and trehalose as CPAs has also been reported[27].

Piscine GC transplantation into adult fish offers an alternative choice for biotechnological investigation of GCs and allows for the production of functional gametes. Furthermore, the use of adult recipients in GC transplantation has promising applications in germline transmission and conservation of valued or endangered fish species[28]. GC transplantation has allowed remarkable advances in the research on spermatogenesis itself as well as the study of interactions between sertoli and germ cells and offers potential applications in the preservation of endangered species[29]. Table 1 provides a comparative account of transplantation of cryopreserved GCs in different species. Kobayashi *et al.*[26] successfully cryopreserved genital ridges (the embryonic tissue containing PGCs (primodial germ cells)) from a transgenic rainbow trout strain using ethylene glycol (1.8 M) as the CPA. After thawing, the cryo-revived PGCs were transplanted into the peritoneal cavities of non-transgenic trout hatchlings, where they differentiated into mature gametes. Here we have used adult systems for quick understanding of GC viability and proliferation in host gonads. Our method is simple; it does not require complex laboratory equipment, except for an automatic programmable freezer. This could be a feasible method for GC isolation and cryopreservation in hatchery practice as well as in research. Hence, it may be concluded that the cryopreserved GCs derived from rohu are transplant-worthy. However, further research may be needed to study the gametes and progeny thus produced.

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