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Evaluating the suitability of cryopreservation solutions for common carp (*Cyprinus carpio*) embryos stored at -2 °C

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Abstract

In the present study, cryopreservation of common carp (*Cyprinus carpio*) embryos was studied using a stepwise cooling protocol. Embryos at heartbeat stage were selected, placed in 15- ml plastic tubes and stored at -2 °C for 1, 7, 14, and 21 days in 2 different cryopreservation solutions: S1 (methanol + dimethyl sulfoxide + sucrose) and S2 (methanol + propylene glycol + sucrose). Embryo viability was assessed by survival rate, counting live larvae and number of failed eggs under a stereomicroscope. The results showed that storage of common carp embryos was possible for up to 7 days at -2 °C, while the survival did not occur when storage period were 14 and 21 days. The combination of methanol with propylene glycol (S2) gave higher survival rate after 1 and 7 days stored at -2 °C. Further studies are needed in order to extend the storage time and to improve the survival rate for this species.

Keywords: Cryopreservation, Common carp, Cryoprotectant

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Introduction

Cryopreservation can play an important role in sustainable aquaculture, genetic management and conservation of threatened and endangered fish species (Rall, 1993; Gwo, 2000; Chen and Tian, 2005). Furthermore, it can be integrated in gamete and embryo cryopreservation in any selective breeding program for stock improvement and for transfer of fish among hatcheries. It permits the storage of genetic material of important species for future application (Sharifuddin and Siti Azizah, 2014).

While there is growing interest in sperm cryopreservation for teleosts such as salmonid (Erdahl *et al*., 1984; Baynes and Scott, 1987), rainbow trout (Billard, 1992), whitefish (Piironen, 1987), barramundi (Leung, 1987), mahasheer (Ponniah *et al*., 1999), and common carp (Linhart *et al*., 2000), little is known about the cryopreservation of fish embryos in general and of carp in particular.

It has been reported that common carp embryos have been successfully cryopreserved (Zhang *et al*., 1989), but these results have not been duplicated elsewhere (Hagedorn *et al*., 1997a).

The successful cryopreservation of fish embryos requires procedures that incorporate information on cryoprotectant toxicity, chilling sensitivity, embryo permeability to cryoprotectant and the development stages of embryos for optimum tolerance of cryoprotectant (Rall, 1993; Zhang and Rawson, 1995).

A pilot study on toxicity of several cryoprotectants to common carp embryos showed that methanol, dimethyl sulfoxide, propylene glycol and sucrose would be good options for cryopreservation of this species. In the same study, it has been noticed that embryos at heartbeat stage exhibited greater tolerance to cryoprotectant than embryos at half-epiboly stage.

The development of effective cryopreservation protocols for fish embryos has great potential not only to conserve endangered populations of fish but also to reduce the cost of production. It could also ensure the continuous supply of seed stocks year-round and simplify the exchange of germplasm between hatcheries (Leung and Jamieson, 1991).

The use of cryopreservation technology is quite new to aquaculture in Iran. The common carp species that are farm bred only once a year (April to Jun) which results in stocking materials being unavailable throughout much of the year. This causes economic loss to the aquaculture industry.

In the present study, two different cryopreservation solutions were investigated in order to develop a cooling protocol for common carp (*Cyprinus carpio*) embryos stored at -2 °C for 1, 7, 14, and 21 days.

Materials and methods

Location of experiments and chemicals

All experiments were carried out in the aquaculture research center of Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran.

Three permeable cryoprotectants, 1 and 4 M methanol (MeOH), 3 M for dimethyl sulfoxide (DMSO) and 6 M propylene glycol (PG) and one non-

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permeable cryoprotectant, 20 % sucrose were prepared in Ringer solution (2.99 g L^{-1} KCl, 6.49 g L^{-1} NaCl, 0.29 g L^{-1} $CaCl_2$, and 0.202 g L^{-1} $NaHCO_3$) as the solvent (Cabrita *et al*., 2003). The concentration of cryoprotectants was chosen based on their tolerance by the common carp embryos in the pilot study. All chemicals used were of high purity and were purchased from Merck (Darmstadt, Germany), except pronase enzyme (Type XIV of *Streptomyces griseus*) which was purchased from Sigma Co, Madrid, Spain.

Embryos

Embryos were obtained from the Golestan Bony Fish Stock Assessment Center (Gorgan, Iran) during the peak carp breeding season (May– June 2016). The fertilized eggs were collected in a bucket with purified aerated water immediately after mixing with milt in the tray and were transferred to the laboratory hatcheries. Water temperature, dissolved oxygen and pH were maintained at 23±1 °C, 6.4±0.5 mg L^{-1} and 7.8±0.2, respectively during incubation.

Developmental stages of embryos were determined morphologically under a stereomicroscope. Embryos developed to the heartbeat stage were used for experiments which correspond to approximately 32 h after fertilization (Okada, 1960).

Cryopreservation solutions and storage procedures

Cryopreservation solutions were composed of a mixture of permeable and non-permeable cryoprotectants (Table 1). Before the exposure to cryopreservation solutions, chorion permeabilization was performed using 2 mg ml^{-1} pronase at 20 °C for 5 min (Cabrita *et al*., 2003; Keivanloo and Sudagar, 2016).

For incorporation of cryoprotectants, embryos at the heartbeat stage were exposed first to 1 M MeOH (2 min, 23 °C), then to 4 M MeOH (2 min, 23 °C), next to 4 M MeOH+DMSO or PG (4 min, 23 °C), and finally to 4 M MeOH+ DMSO or PG+20 % sucrose (4 min, 8 °C) (Table 1).

Table 1: Steps of the cryoprotectant incorporation protocol.

Treatment	Step	Cryoprotectant	Expose time	Temperature
S1	1	1 M MeOH	2 min	23 °C
	2	4 M MeOH	2 min	23 °C
	3	4 M MeOH + 3 M DMSO	2 min	23 °C
	4	4 M MeOH + 3 M DMSO + 20 % Suc	4 min	8 °C
S2	1	1 M MeOH	2 min	23 °C
	2	4 M MeOH	2 min	23 °C
	3	4 M MeOH + 2 M PG	2 min	23 °C
	4	4 M MeOH + 2 M PG + 20 % Suc	4 min	8 °C
Control	1	Ringer solution	2 min	23 °C
	2	Ringer solution	2 min	23 °C
	3	Ringer solution	2 min	23 °C
	4	Ringer solution	4 min	8 °C

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After treatment with the cryopreservation solutions according to the four-step protocol as described, embryos were loaded into plastic tubes (15 ml) along with the cryopreservation solutions whose total volume was up to 2/3 tubes (10 ml). Control groups were exposed to Ringer solution in a similar manner. Then sealed tubes containing embryos and cryopreservation solutions were stored in a refrigerator set at -2 °C for 1, 7, 14 and 21 days.

Evaluation of viability

At the end of the storage period, the sealed tubes were removed from the refrigerator. The treated embryos were thoroughly rinsed with water (23–24°C) and transferred to the hatcheries until they hatched or died.

Some hatched larvae (the larvae that had ruptured the chorion) showed no difference with normally cultured embryos; while the other hatched larvae stopped development before developing into live larvae. The vigorous mobility of the hatchlings after one day was the standard characteristics for viability assessment. The experiment was repeated three times, and each replicate utilized approximately 35 embryos.

Results

Table 2 presents the data on survival rates of common carp embryos stored at -2 °C. The viability of embryos cooled in the control group (cryoprotectant free treatment) was not preserved and the embryos indicated 100 % mortality after storage for 1, 7, 14 or 21 days at -2 °C.

In S1, the survival rate of embryos at -2 °C after 1 and 7 days were 12.38 % and 1.90 % respectively (Table 2). The survival rate of embryos preserved with S2 at -2 °C was 14.28 % and 9.52 % when storage periods were 1 and 7 days, respectively (Table 2). Embryos stored with S1 did not survive after storage at -2 °C for 14 or 21 days duration. The same was true for S2 treated embryos after 14 or 21 days storage (Table 2).

Of the total 73 hatched larvae with cryopreservation solutions after storage at -2 °C, only 40 embryos developing into the normal larvae and survived. No abnormalities of hatched larvae were observed in any cryopreservation solutions tested.

Table 2: Survival rate of common carp (*Cyprinus carpio*) embryos at the heartbeat stage stored at -2 °C using different cryopreservation solutions.

Cryopreservation solutions	Storage duration (day)	Number of frozen embryo (number of embryo in each tube)	Number of hatched larvae	Number of live larvae	Survival rate (%)
S1	1	105 (35, 35, 35)	20 (9, 4, 7)	13 (8, 2, 3)	12.38± 9.18[a]
	7	105 (35, 35, 35)	5 (1, 2, 2)	2 (1, 0, 1)	1.90± 1.64[b]
	14	105 (35, 35, 35)	0	0	0 [b]
	21	105 (35, 35, 35)	0	0	0 [b]

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Table 2 continued:

S2	1	105 (35, 35, 35)	30 (10, 12, 8)	15 (5, 7, 3)	14.28± 5.71[a]
	7	105 (35, 35, 35)	12 (5, 2, 5)	10 (5, 1, 4)	9.52 ± 5.94[a]
	14	105 (35, 35, 35)	6 (1, 2, 3)	0	0[b]
	21	105 (35, 35, 35)	0	0	0[b]
Control	1	105 (35, 35, 35)	0	0	0
	7	105 (35, 35, 35)	0	0	0
	14	105 (35, 35, 35)	0	0	0
	21	105 (35, 35, 35)	0	0	0

Data are presented as mean ± standard deviation (SD); data within a column for each solution separately with different superscript letters are significantly different (p<0.05).

Discussion

The successful cryopreservation of fish embryos depends on the selection of suitable cryoprotectant treatments, taking into account both its toxicity and its ability to avoid or minimize the formation of internal and external ice crystals, i.e. its efficiency (Rahman *et al*., 2008; Roux *et al*., 2008). Cryoprotectants can suppress most cryoinjuries but, when used at higher (more effective) concentrations, most of them become toxic (Paredes and Bellas, 2009).

A pilot study on the toxicity of cryoprotectants on common carp embryos revealed that MeOH was the least detrimental followed by PG and DMSO. The cryopreservation solutions used in this study were designed taking into account the toxicity of each individual cryoprotectant. None of the cryoprotectants used were toxic at these concentrations and the specified exposure time and temperatures.

In this study, we tested the efficiency of the two cryopreservation solutions to common carp embryo. Methanol was used in both combinations, because of its low toxicity and because it is one of the most permeable cryoprotectants (Hagedorn *et al*., 1996; Hagedorn *et al*., 1997b), it would be present inside embryos at a higher concentration than other cryoprotectants. Hagedorn *et al*. (1996) demonstrated that methanol is more permeable to both DMSO and propylene glycol in zebrafish embryos. According to Dinnyes *et al*. (1998), Zhang and Rawson (1998) and Zhang *et al*. (1993), methanol would be more efficient for the protection of zebrafish embryos against low temperatures (Ninhaus-Silveira *et al*., 2009).

The efficiency of methanol as a permeable cryoprotectant qualifies its use in the development of this cooling protocol. Only the groups of embryos treated with cryopreservation solutions that contained methanol, DMSO and PG (S1 and S2) presented some degree of viability after the cooling protocol, while the use of Ringer solution resulted in no survival rate at all. It was found that the survival rate of common carp embryos was higher when MeOH and PG were combined as one storage solution in S2. This result indicated that combination of these two cryoprotectants had a relatively lower toxicity level and a stronger ability for preventing ice crystals formation, which was consistent with the study by observations of several previous studies (Hua and Ren, 1994; Chen and Tian,

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2005; Wang *et al*., 2007; Shaluei *et al*., 2014; Keivanloo and Sudagar, 2016). Although the mechanism of protection by large polymers is unclear, the addition of non-permeable cryoprotectants has been adopted in embryo freezing. Non-permeable cryoprotectants are good inhibiters of ice crystal formation (Leibo and Oda, 1993; Kuleshova *et al*., 1999; Nowshari and Brem, 2000; Kuleshova *et al*., 2001) and are essential for reducing the toxicity of high concentrations of permeable cryoprotectants (Kuleshova *et al*., 1999; Cabrita *et al*., 2003). In the present study, we tested the effect of sucrose for common carp embryos.

Studies have shown that sugars protected membranes from the consequences of dehydration in mammalian embryos (Anchordoguy *et al*., 1987). The addition of sucrose with methanol was inevitable for the survival of mrigal (*Cirrhinus mrigala*) embryos (Ahammad *et al*., 1998), and it enhanced the survival rate in catla (*Catla catla*) and rohu (*Labeo rohita*) embryos (Ahammad *et al*., 1998). This implied that toxicity of cryoprotectants was reduced by the presence of sucrose. According to Dinnyes *et al*. (1998), the beneficial effect of sucrose may be related to a moderate level of dehydration that helped to protect the cell membrane at low temperature.

The survival was significantly decreased following 21 days exposure at -2 °C. In this study, when the refrigeration time increased from 1 to 7 days, the mean survival rates of common carp decreased, while the survival rate was zero after 14 and 21 days of refrigeration. This result is in agreement with the study by Ahammad *et al*. (2003), which reports that the survival rate of the common carp (*C. carpio*) decreases as the refrigeration time increases.

According to Dinnyes *et al*. (1998) such sensitivity is influenced by cell and tissue types, number of cells, effectiveness of repair mechanisms, and enzymatic reactions. Changes in the size and structure of the yolk compartment and membrane structure might also be among the other key factors. Low temperature can cause direct or indirect chilling injuries on embryo cell during cooling (Dinnyes *et al*., 1998; Zhang *et al*., 2003; Delgado *et al*., 2005). This has been attributed to depolymerization of microtubules and irreversible cellular processes. Furthermore, the plasma membrane may undergo lateral phase separation; protein denatured due to the destabilization of hydrophobic bound; cell membrane may shrink relative to the intracellular spaces which ultimately result in stress damage to the membrane (Martino *et al*., 1996; Hagedorn *et al*., 1997a; Dinnyes *et al*., 1998; Delgado *et al*., 2005).

Stepwise increments in cryoprotectant concentrations during cryoprotectant loading are widely used to reduce the cryoprotectant toxicity and osmotic stress on the embryos (Gwo, 2000; Cabrita *et al*., 2003; Chen and Tian, 2005). As the cryoprotectant concentration outside the cells rises it leads to the loss of water from the cells. This dehydration of cells can be lethal if it occurs too rapidly and therefore,

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cryoprotectant solutions should be added to the medium in a series of increasing concentrations (Leibo *et al*., 1974; Fahning and Garcia, 1992; Denniston *et al*., 2000; Rahman *et al*., 2008). Therefore, common carp embryos were equilibrated in a stepwise manner, as is usually recommended.

It can be concluded that the protocols tested were able to prevent the formation of intracellular ice crystals, which led to achieving live larvae after storage at -2 °C Further studies are needed in order to extend the storage time and to improve the survival rate for this species.

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