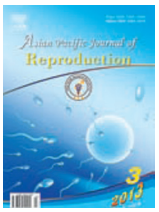
Asian Pacific Journal of Reproduction

Journal homepage: www.apjr.net



Document heading doi: 10.1016/S2305-0500(13)60144-X

Effect of different concentrations of permeable and non-permeable cryoprotectants on the hatching rate of goldfish (*Carassius auratus*) embryos

Fardin Shaluei[1*], Mohamad Reza Imanpoor[1], Ali Shabani[1], Mohamad Hossein Nasr-Esfahani[2]

[1]*Department of Fisheries, Faculty of Fisheries and Environmental Sciences, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran*

[2]*Department of Reproduction and Development, Royan Institute for Animal Biotechnology, ACECR, Isfahan, Iran*

ARTICLE INFO

Article history:
Received 21 July 2013
Received in revised form 10 August 2013
Accepted 12 August 2013
Available online 20 September 2013

Keywords:
Cryopreservation
Embryo
Cryoprotectant
Toxicity
Goldfish

ABSTRACT

Objective: To assess the toxicity of various cryoprotective agents(CPAs) to goldfish embryos. **Methods:** Heart-beat embryos were immersed in: five permeable CPAs, dimethyl sulfoxide (DMSO), methanol (MeOH), 1,2- propylene glycol (PG), glycerol (Gly), ethylene glycol (EG), and two non-permeable CPAs: polyvinylpyrrolidone (PVP) and sucrose in concentrations of 5%, 10%, 15% or 20% for 5, 15, or 30 min. The viability of the embryos after the treatments was estimated from hatching rates. **Results:** The results showed that the toxicity to goldfish embryos of the five permeable CPAs were in the following sequence: PG < DMSO < MeOH < EG ~ Gly (greater symbols mean $P < 0.05$, and approximate symbols mean $P > 0.05$). In this experiment none of the concentrations tested for sucrose reduced the hatching rate, but embryos exposed to PVP did not tolerate concentration 20%, at 30 min exposure. **Conclusion:** These finding will be advantageous for designing optimized Cryo-media for cryopreservation of goldfish embryos

1. Introduction

Successful cryostorage of fish embryos for an indefinite time could be very helpful in management of reproduction in commercial aquaculture, species conservation, developmental and cell biology and genetic management of brood stock. Although cryopreservation techniques have been largely established for the mammalian embryos, successful cryopreservation of intact fish embryos has not yet been achieved. Limiting factors affecting fish embryos cryopreservation include their complex structure, sensitivity to chilling, big size and toxic effect of the cryoprotective agents [1, 2].

Cryoprotective agents (CPAs), or cryoprotectants, are chemicals which are used to protect cells and cellular organelles during cryopreservation and long term storage in liquid nitrogen. There are two major categories of CPAs: (a) permeable CPAs, e.g. methanol (MeOH), dimethy sulfoxide (DMSO), glycerol (Gly) propylene glycol (PG) and ethylene glycol (EG) which are low-molecular weight chemicals and can penetrate the cell membrane; and (b) non- permeable CPAs, e.g. polyvinyl pyrrolidone (PVP), hydroxyethyl starch and various sugars, which are high-molecular weight agents and cannot enter cells [3]. Despite the protective potential of CPAs, the toxicity of CPAs is an obstacle to successful cryopreservation. Fish embryos tolerance to CPAs is limited and overexposure to CPAs may cause damage. The presence of a specific amount of cryoprotectant is critical to the cryopreservation process [4]. To date, several studies have been carried out on the efficacy and toxicity effects of CPAs on fish embryos to demonstrate that toxicity level depends on CPAs chemical properties, concentration and exposure period, embryo developmental stage, storage temperature, and fish species [4-10].

Garassius auratus (*C. auratus*) are a fresh water model fish easy to breed in aquaria, which can survive under a wide range of stressful conditions, from full anoxia to hyperoxia and temperature alterations from 0 to above 40 °C [11]. This species has relatively small and transparent embryos. Thus, Goldfish could be a good model for cryobiological studies of fish embryos. Goldfish also serve as useful model organisms in numerous fields such as neuroendocrine signaling, regulation of reproduction in vertebrates, cell biology, immunology, toxicology, endocrine disruption, molecular evolution and comparative genomics [12]. Therefore, the

*Corresponding author: Fardin Shaluei, Department of Fisheries, Faculty of Fisheries and Environmental Sciences, Gorgan University of Agricultural Sciences and Natural Resources, P.O. Box 45165-386 Gorgan, Iran.
Tel: +98-913-1836910
Fax: +98-171-245965
E-mail: fardin.shaluei@gmail.com

aim of the present work was to study the effect of different concentrations of CPAs and exposure times on the hatching rate of goldfish embryos for designing optimized low-toxicity cryo-media and appropriate duration of exposure for goldfish embryos cryopreservation.

2. Materials and methods

2.1. Embryos

Experiments were carried out at the aquaculture research center of Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran. Adult male and female *C. auratus*, averaging 2+ years of age, were reared in fiberglass tanks (cylindrical, 1 m in diameter and 0.5 m in depth) with aerated and dechlorinated tap water (pH 7.89 ± 0.98; dissolved oxygen (7.8 ± 0.5) mg/L; and total hardness (295 ± 10) mg/L as $CaCO_3$) at (20±1) °C on a 14L/10D photoperiod. Fish were fed twice a day at a ratio of 3% body weight with commercial goldfish pellets (Energy, Thailand). Just before experiment, brood stocks were injected intramuscularly with Ovaprim (Syndel laboratories, India) at 0.5ìL/g (estimated brood fish weight) to induce ovulation and spermiation. Twelve hours post-injection, females had ovulated and eggs could be collected by stripping. Eggs obtained from nine ovulation-induced females were pooled by mixing 3 individual samples in equal ratios and were fertilized with pooled sperm using the dry method. Thirty sec after fertilization, eggs were washed with a tannic acid solution (200 mg/L) and stirred continuously for 30 sec to remove adhesiveness. Eggs were subsequently incubated in 3 aquaria (100 L) with aerated and dechlorinated tap water. Embryonic developments were determined morphologically using a light microscope (Olympus CH_2, Japan) following Kajishima [13].

2.2. Preparations of CPAs solutions

All of the CPA solutions used in the following experiment were made with Hank's solution (8 g/L NaCl, 0.4 g/L KCl, 0.14 g/L $CaCl_2$, 0.1 g/L $MgSO_4\_7H_2O$, 0.1 g/L $MgCl_2\_6H_2O$, 0.06 g/L $Na_2HPO_4\_12H_2O$, 1 g/L glucose, and 0.35 g/L $NaHCO_3$) as the solvent [8]. Five permeable CPAs (DMSO, MeOH, PG, Gly and EG) and two non-permeable CPAs (PVP and sucrose) were used in this study. All chemicals with highest purity (99.0%) were obtained from Dae Jung (Korea), except the MeOH which was purchased from Merck (Darmstadt, Germany). Just before experiment, CPAs were diluted in Hank's solution at concentrations of 5%, 10%, 15%, and 20%. For all CPAs the percentage concentrations are expressed as volume per volume, except the sucrose as weight per volume.

2.3. Toxicity of CPAs to embryos

To evaluate the toxicity of the CPAs, heartbeat-stage of goldfish embryos (approximately 33 h after fertilization) were exposed to each CPAs at aforementioned concentrations for 5, 15 or 30 min at room temperature (20 °C). Each treatment consisted of nine replicates with approximately 100 embryos each. Control groups were exposed to Hank's solution without CPAs for 5, 15 or 30 min. After the required exposure, embryos were carefully washed with dechlorinated tap water and incubated until hatching. The CPAs toxicity was expressed as percentage of hatching rate, which was calculated as the percentage of hatched larvae in relation to the total number in each group.

2.4. Statistical analysis

All results are expressed as means ± SD. Statistical analyses were carried out using the computerized package SPSS 16 for Windows. Prior to statistical analysis, data was tested for normality and transformations were applied to normalize data where necessary. To examine the effect of CPAs on the hatching rate of goldfish embryos, data were analyzed by Tukey's *t*-test and were considered to be significant at $P < 0.05$. Moreover data obtained from the CPAs toxicity after 15 and 30min were evaluated using the Probit analysis statistical method and lethal concentrations (LC 1-50) were calculated.

3. Results

The hatching rate of embryos exposed to all permeable CPAs for 5 min were not different ($P > 0.05$) from those of the control (Figure 1A). The hatching rate of embryos treated with the five permeable CPAs for 15 min varied markedly with the type and concentration of CPA (Figure 1B). There was no significant decrease in the hatching rate of embryos exposed to 5%-15% DMSO, 5% or 10 % MeOH, 5%-15% PG, 5% Gly or EG from those of the control. However, the hatching rate of embryos exposed to 20% DMSO or PG, 15% or 20% MeOH and 10%-20% Gly or EG decreased ($P < 0.05$) compared to the control. After exposure to 5% or 10% DMSO or MeOH, 5%-15% PG and 5% Gly or EG for 30 min, the hatching rates of goldfish embryos showed no signficant difference (Figure 1C) compared to the control ($P > 0.05$). Embryos exposed to 15% or higher DMSO or MeOH, 10% or higher Gly or EG and 20% PG, for 30 min, were all damaged and showed significantly lower survival percentages compared to the control ($P < 0.05$). No embryos survived to hatching after exposure to 15% or 20% Gly and 20% EG for 30 min.

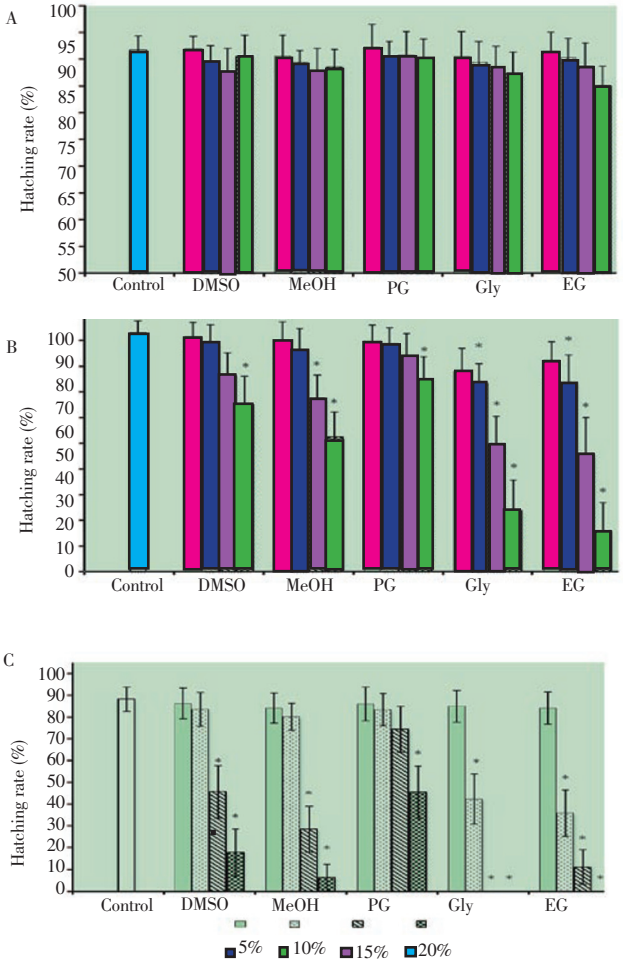


Figure 1. Hatching rates of goldfih embryos at heartbeat-stage that were exposed to different concentrations of dimethy sulfoxide (DMSO), methanol (MeOH), propylene glycol (PG), glycerol (Gly) and ethylene glycol (EG) for 5 (A), 15 (B) or 30 (C) min.
Data shown as mean and SD of nine replicates with approximately 100 embryos each. Concentrations with asterisks have significantly different hatching rates from those of the controls (Turkey's *t*-test, $P < 0.05$).

The effects of varying concentrations of non-permeable CPAs after 5, 15 and 30 min on hatching rate of embryos are shown (Table 1). No significant reduction in hatching rate compared to the control was observed between the analyzed concentrations of sucrose. The hatching rate of embryos treated with PVP varied markedly with the concentration and exposure time (Table 1). No embryos survived to hatching after exposure to 20% PVP for 30 min.

Median lethal concentrations (LC_{50}) of CPAs for goldfish embryos after 15 and 30 min exposure are presented in Table 2. Higher LC_{50} values are less toxic because greater concentrations are required to produce 50% mortality in embryos. From these results it can be concluded the toxicity to goldfish embryos of all five permeable CPAs are in the following sequence: PG < DMSO < MeOH < EG ~ Gly (greater symbols mean $P < 0.05$, and approximate symbols mean $P > 0.05$).

The least toxic non-permeable CPAs on the hatching rate of goldfish embryos were sucrose and PVP exhibited moderate toxicity.

Table 1

Hatching rates of goldfish embryos (heartbeat-stage) exposed to non-permeable cryoprotectants.

Cryoprotectants	Duration of exposure (min)	Cryoprotectants concentration (%)				
		Control	5	10	15	20
Sucrose	5	91.77 ± 2.63^{a}	94.44 ± 3.57^{a}	93.00 ± 5.17^{a}	90.55 ± 6.20^{a}	93.11 ± 5.34^{a}
	15	93.00 ± 4.63^{a}	93.55 ± 5.83^{a}	92.33 ± 5.90^{a}	89.88 ± 5.15^{a}	89.44 ± 4.92^{a}
	30	88.22 ± 5.60^{a}	92.55 ± 6.14^{a}	92.77 ± 4.99^{a}	89.66 ± 5.56^{a}	85.88 ± 7.40^{a}
PVP	5	91.77 ± 2.63^{a}	91.88 ± 5.94^{a}	88.55 ± 6.50^{ab}	85.00 ± 4.30^{ab}	83.44 ± 7.01^{b}
	15	93.00 ± 4.63^{a}	90.11 ± 6.43^{a}	62.55 ± 11.28^{b}	39.44 ± 13.78^{c}	23.55 ± 12.86^{d}
	30	88.22 ± 5.60^{a}	81.44 ± 8.91^{a}	21.77 ± 19.20^{b}	18.50 ± 8.2^{c}	0.0 ± 0.0^{d}

Data shown as mean and S.D of nine replicates with approximately 100 embryos each. Data in the same row with different superscript letters are significantly different (Turkey's *t*-test, $P < 0.05$).

Table 2

Median lethal concentrations (LC_{50}) of cryoprotectants (%) at 15 and 30 min for goldfish embryos.

Cryoprotectants	15 min		30 min	
	Concentration (%)	95% CI	Concentration (%)	95% CI
DMSO	37.30^{b}	27.8-64.0	14.42^{b}	12.7-16.1
MeOH	23.94^{c}	20.0-31.7	11.18^{c}	10.0-12.3
PG	88.08^{a}	42.3-95.3	24.20^{a}	19.3-35.9
Gly	13.25^{d}	11.7-15.2	7.90^{d}	7.3-8.5
EG	12.39^{d}	11.0-13.9	8.16^{d}	7.6-8.6
PVP	13.34^{d}	11.9-15.4	8.08^{d}	7.9-9.1

Data within a column with different superscript letters are significantly different ($P < 0.05$, Scheffe's test).

4. Discussion

One obstacle to fish embryo cryopreservation is their high chilling sensitivity [2]. It is well known that fish embryos at early cell division stages are sensitive to chilling and cryoprotectants, but this sensitivity decreases as development proceeds. Liu *et al*. reported that heartbeat-stage of goldfish embryos are less sensitive to chilling than other developmental stages [14]. Therefore embryo during this stage selected for CPAs toxicity in this study.

CPAs, used to protect biological material from damaging effect of low temperatures, can be toxic to cells themselves. Therefore, understanding the toxicity of cryoprotective agents would be important for reducing toxicity and developing optimal cryopreservation protocols. The effect of each CPA depends on their chemical properties, and is species-specific [2]. Suzuki *et al*. reported tolerance to DMSO became higher in the order of carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*), pejerrey (*Odontesthes bonariensis*), and medaka (*Oryzias latipes*) embryos [15]. Our results show that the sequence of toxicity of CPAs to goldfish embryos was: PG < DMSO < MeOH < EG ~ Gly, which is similar to the results of Rahman *et al*. [9] for Japanese whiting (*Sillago japonica*) embryos (PG < DMSO < EG < MeOH < Gly) and the results of Zhang *et al*. [6] for flounder embryos (*Paralichthys olivaceus*), (PG < MeOH < DMSO < Gly < EG). Xiao *et al*. [8] demonstrated that PG was the least toxic permeable CPAs to red sea bream (*Pagrus major*) embryos in the heartbeat-stage followed by DMSO and EG, whereas Gly and MeOH were the most toxic. In zebrafish (*Danio rerio*) embryos, DMSO had the lowest toxicity, followed by 1,2 propanediol and glycerol while ethylene glycol, methanol, and N,N-dimethylacetamide were highly toxic [10]. Tian *et al*. [16] found that sequence of toxicity of CPAs for sea perch (*Lateolabrax japonicas*) embryos was (PG < MeOH < Gly < EG < DMSO). Dinnye's *et al*. [17] reported that the toxicity to carp embryos in the heartbeat-stage increased in the order of MeOH < Gly < DMSO. These data reflect the importance of specific studies to determine optimal concentrations of CPAs for each particular species. This study did not investigate the mechanism of toxicity of CPAs, but denaturation of enzymes, disruption of trans membrane ionic pumps, producing toxic formaldehyde, disruption of the mitochondrial transport system and dissolving DNA structure have been proposed as the cause of injury to cells [18-20].

Non-permeable CPAs such as sucrose and PVP often are used in combination with permeable cryoprotectants. Cryo-media containing non-permeable and permeable CPAs seem to be more advantageous than solutions containing only a permeable CPAs [21]. The cryoprotective effect of non-permeating CPAs is mainly based on dehydration of cells prior to cooling and increase cryo-media viscosity, which results in reduced ice crystal formation during freezing [22].

Our results show that the toxicity of sucrose was very low, which is similar to the results of Xiao *et al*. [8] for red seabream embryos and the results of Lahnsteiner (2008) for zebrafish embryos [10]. Conversely, in the study of turbot embryos (*Scophthalmus maximus*) at G stage, the hatching rate of embryos treated with 20% sucrose deceased significantly [22]. In the present study, PVP exhibited moderate toxicity for gold fish embryos. Embryos exposed to PVP did not tolerate concentration 20%, at 30 min exposure. In summary, this study reveals the importance of CPAs concentration and equilibration period for cryopreservation of goldfish embryos. The present data apparently represented the first reported on the toxicity of five permeable CPAs (DMSO, MeOH, PG, Gly, EG) and two non-permeable CPAs (PVP and sucrose) to goldfish embryos. It was determined that sucrose and PG were the least toxic CPAs. These finding will be advantageous for designing optimized Cryo-media for cryopreservation of goldfish embryos.

Conflict of interest statement

We declare that we have no conflict of interest statement

References

[1] Zhang T, Rawson DM. Studies on chilling sensitivity of zebrafish (*Brachydanio rerio*) embryos. *Cryobiology*1995; **32:** 239-246.

[2] Robles V, Cabrita E, Acker JP, Herráez P. Embryo cryopreservation: what we know until now. In: Cabrita E, Robles V, Herráez MP (eds). *Methods in reproductive aquaculture: Marine and freshwater species*. London: CRC Press, Taylor & Francis Group; 2008, p. 265-295.

[3] Zhang T, Rawson DM, Pekarsky I, Blais I, Lubzens E. Low temperature preservation of fish gonad cells and oocytes. In: Babin PJ, Cerda` J, Lubzens E. (eds) *The fish Oocyte: from basic studies to biotechnological applications*. Online: Springer: 2007, p. 411-436.

[4] Beira˜o J, Robles V, Herra´ez MP, Sarasquete C, Dinis MT, Cabrita E. Cryoprotectant microinjection toxicity and chilling sensitivity in gilthead seabream (*Sparus aurata*) embryos. *Aquaculture* 2006; **261:** 897-903.

[5] Ahammad MM, Bhattacharyya D, Jana BB. Stage-dependent hatching responses of rohu (*Labeo rohita*) embryos to different concentrations of cryoprotectants and temperatures. *Cryobiology* 2003; **46:** 1-16.

[6] Zhang YZ, Zhang SC, Liu XZ, Xu YJ, Hu JH, Xu YY, et al. Toxicity and protective efficiency of cryoprotectants to flounder (*Paralichthys olivaceus*) embryos. *Theriogenology* 2005; **63:** 763-73.

[7] Cabrita E, Robles V, Wallace JC, Sarasquete MC, Herra´ez MP. Preliminary studies on the cryopreservation of gilthead seabream (*Sparus aurata*) embryos. *Aquaculture* 2006; **251:** 245-55

[8] Xiao ZZ, Zhang LL, Xu XZ, Liu QH, Li J, Ma DY, et al. Effect of cryoprotectants on hatching rate of red seabream (*Pagrus major*) embryos. *Theriogenology* 2008; **70:** 1086-1092.

[9] Rahman SM, Majhi SK, Suzuki T, Matsukawa S, Strüssmann CA, Takai R. Suitability of cryoprotectants and impregnation protocols for embryos of Japanese whiting *Sillago japonica*. *Cryobiology* 2008; **57:** 170-174.

[10] Lahnsteiner F. The effect of internal and external cryoprotectants on zebrafish (*Danio rerio*) embryos. *Theriogenology* 2008; **69:** 384 -396.

[11] Sun Y, Yin Y, Zhang J, Yu H, Wang X, Wu J, et al. Hydroxyl radical generation and oxidative stress in *Carassius auratus* liver, exposed to pyrene. *Ecotoxicol Environ Saf* 2008; **71:**446-453.

[12] Popesku JT, Martyniuk CJ, Mennigen J, Xiong H, Zhang D, Xia X, et al. The goldfish (*Carassius auratus*) as a model for neuroendocrine signaling. *Mol Cell Endocrinol* 2008; **293:** 43-56.

[13] Kajishima T. The normal development stages of the goldfish, *Carassius auratus*. *Jpn J Ichtyol* 1960; **8:** 20-28.

[14] Liu K, Chou T, Lin H. Cryosurvival of goldfish embryo after subzero freezing. *Aquat Living Resour* 1993; **6:** 63-66.

[15] Suzuki T, Komada H, Takai R, Arii R, Kozima TT. Relation between toxicity of cryoprotectant DMSO and concentration in several fish embryos. *Fish Sci* 1995; **61:** 193-197.

[16] Tian YS, Chen SL, Yan AS, Ji XS, Yu GC. Cryopreservation of the sea perch (*Lateolabrax japonicus*) embryos by vitrification. *Acta Zool Sin* 2003; **49:** 843-850.

[17] Dinnye´s A, Urba´nyi B, Baranyai B, Magyary I. Chilling sensitivity of carp (*Cyprinus carpio*) embryos at different developmental stages in the presence or absence of cryoprotectants: work in progress. *Theriogenology*1998; **50:** 1-13.

[18] Mande SC, Sobhia ME. Structural characterization of protein-denaturant interactions: crystal structures of hen egg-white lysozyme in complex with DMSO and guanidinium chloride. *Protein Eng* 2000; **13:** 133-141.

[19] Bonner G, Klibanov AM. Structural stability of DNA in nonaqueous solvents. *Biotechnol Bioeng* 2000; **68:** 339-344.

[20] Spikings E, Zampolla T, Rawson D, Wang Y, Zhang T. Effect of methanol on mitochondrial organization in zebrafish (*Danio rerio*) ovarian follicles. *Theriogenology* 2012; **77 :** 28-38.

[21] Shaw JM, Oranratnachai A, Trounson AO. Fundamental cryobiology of mammalian oocytes and ovarian tissue. *Theriogenology* 2000; **53:** 59-72.

[22] Cabrita E, Robles V, Chereguini O, Wallace JC, Herra´ez MP. Effect of different cryoprotectants and vitrificant solutions on the hatching rate of turbot embryos (*Scophthalmus maximus*). *Cryobiology* 2003; **47:** 204-213.