Keivanloo and Sudagar, 2:3
http://dx.doi.org/10.4172/scientificreports674

Open Access Scientific Reports

Research Article Open Access

Preliminary Studies on the Cryopreservation of Persian Sturgeon (*Acipenser persicus*) Embryos

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Abstract

Vitrification is considered the most promising option to cryopreservation fish embryos. Since very high concentrations of cryoprotectants are needed for vitrification and fish embryos have a large volume, embryos must have low sensitivity to cryoprotectant toxicity and high permeability to water and cryoprotectants. So, in the present study, two Persian sturgeons (*Acipenser persicus*) embryonic development stage (24 and 48 h post-fertilization) were chosen and exposed to six permeable cryoprotectants: dimethyl sulfoxid (DMSO), ethylene glycol (EG), propylene glycol (PG), acetamide (Ac), methanol (MeOH) and glycerol (Gly) in concentrations ranging from 1 to 6 M; and three non-permeable cryoprotectants: sucrose and honey (10%, 15% and 20%) and polyvinyl pirolidon (PVP) (5%, 10% and 15%) for an equilibration period of 5 and 10 min. After treatment, embryos were washed and incubated until hatched. The toxicity of cryoprotectants increased with concentration and exposure time. DMSO was the best tolerated by the embryos. Embryos at 48 h post-fertilization stage exhibited greater tolerance to cryoprotectant than embryos at 24 h post-fertilization stage. Six DMSO-based cryoprotectant vitrificant solutions were designed to check their toxic effects on 48 h post-fertilization stage embryos using a stepwise incorporation protocol. DMSO-based vitrificant solutions contained 5 M DMSO + 3 other permeable cryoprotectants + 3 non-permeable cryoprotectants (sucrose, honey and PVP), which their concentrations increased gradually during a six-step protocol. The highest hatching rates for embryos were obtained with exposure to V3 and V5 that both contained MeOH. Our results suggest that Persian sturgeons can be subject to this cryoprotectant protocol without deleterious effect on hatching rate.

Keywords: Persian sturgeon; Embryo; *Acipenser persicus*; Cryoprotectant; Vitrificant solution

Introduction

The application of cryopreservation technique to store viable fish embryos could have a profound influence on aquaculture and the conservation of rare or threatened species [1]. Since the seventies, the cryopreservation of mammalian embryos has been used successfully [2]. However, application of this technique to fish embryos is still an unsolved problem. Several studies of the effects of cryoprotectants toxicity on embryos have been reported in carp [3], flounder [4] and gilthead sea bream [5]. In these studies, cryoprotectant toxicity changed during embryo development and was species-septic.

The preliminary studies on chilling sensitivity, cryoprotectant permeability, and toxicity demonstrated problems in fish embryo cryopreservation. Cold sensitivity and ice formation within the egg provided the main constraints affecting the survival of the embryos [6]. Some recent results have demonstrated that the yolk syncytial layer is a critical limiting barrier for cryoprotectant permeation throughout the embryo [7, 8]. The occurrence of chilling injury and inadequate cryoprotectant permeation suggest that vitrification could be a promising approach to fish embryo cryopreservation [6]. Embryo vitrification was first reported in 1985 by Rall and Fahy [9], who worked with mouse embryos. For fish embryos the first report concerned zebrafish [6]. Oyster and hard clam were the first marine invertebrates studied [10]. All the vitrification solutions designed incorporated permeable and non-permeable cryoprotectants. The toxicity of the extender components is very important in cryopreservation protocols, particularly when high concentrations are required for vitrification [11]. Therefore, it is very important to perform a preliminary study of the toxicity of cryoprotectants before designing specific vitrification solutions for particular species [12].

Persian sturgeon has a high commercial value, but no attempts at embryo cryopreservation have been carried out, despite the fact that this technology could improve some aspects of production and restocking.

The aim of this study was to investigate permeable and non-permeable cryoprotectant toxicity in Persian sturgeon embryos with the goal of designing optimized low-toxicity vitrification solutions for the cryopreservation of embryos from this rare species.

Materials and Methods

Chemical

Six permeable cryoprotectants, dimethyl sulfoxid (DMSO), ethylene glycol (EG), propylene glycol (PG), acetamide (Ac), methanol (MeOH), glycerol (Gly) and three non-permeable cryoprotectants, sucrose (Suc), honey (H) and polyvinyl pyrrolidone (PVP) were used in the following experiments. All the chemicals and the pronase used for chorion permeabilization (type XIV *Streptomyces griseus*), were purchased from Merck Company, Germany.

Gametes extraction and Incubation of fertilized eggs

Feral Persian sturgeons (*Acipenser persicus*) were captured from the Iranian castline on the Caspian Sea and were transported to the Shahid Marjani Sturgeon Propagation Center, Gorgan, Iran. Sperm

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Received November 16, 2012; **Published** February 13, 2013

Citation: Keivanloo S, Sudagar M (2013) Preliminary Studies on the Cryopreservation of Persian Sturgeon (*Acipenser persicus*) Embryos. 2: 674. doi:10.4172/scientificreports.674

extraction was done by abdominal massage of the male, collecting the sperm from the genital pore with a syringe. Oocytes were extracted by abdominal massage and collected in a plastic container, avoiding contamination with blood, urine and water. For fertilization, sperm was poured over the oocytes Embryos were incubated in water at 19.5°C ± 1. Two embryo development stages, 24 and 48 h post-fertilization were selected at intervals to examine the effects of cryoprotectant toxicity on embryo survival.

Assessment of permeable and non-permeable cryoprotectant toxicity

The effect of exposure to six permeable cryoprotectants, DMSO, EG, PG, Ac, MeOH and Gly, on the hatching rate of permeabilized Persian sturgeon embryo at 24 and 48 h post-fertilization was investigated. Cryoprotectants were prepared in modified Ringer solution (2.99 g/l KCl, 6.49 g/l NaCl, 0.29 g/l $CaCl_2$, and 0.202 g/l $NaHCO_3$) [12]. Before the cryoprotectant exposure, chorion permeabilization was performed using 2 mg/ml pronase (19.5°C ± 1) for 5 min. Approximately 20 eggs were exposed to 60 ml of each cryoprotectant at concentration of 1, 2, 3, 4, 5 and 6 M, 10%, 15% and 20% sucrose and honey and 5%, 10% and 15% PVP for an equilibration period of 5 and 10 min in Petri dishes at room temperature 19.5°C ± 1. After treatment, embryos were washed with water and incubated until 1 day after hatching. Control groups were incubated at same time. Each treatment group included three replicates for 20 embryos. The cryoprotectant toxicity was expressed as percentage of hatching rate, which was determined by counting the number of 1-day hatched larvae relative to the total number of incubated eggs.

Incorporation of vitrificant solutions

The vitrificant solutions were selected taking into account the results of the experiments of cryoprotectants toxicity. Six vitrificant solutions were designed to check their toxic effects on 48 h post-fertilization stage embryos. Vitrificant solutions containing DMSO as the main cryoprotectant was analyzed using 24 and 48 h post-fertilization stage embryos. For incorporation of the vitrificant solutions, approximately 20 pronase permeabilized embryos were exposed using a six step protocol. The steps of the protocol were as follows: first– 1 M DMSO (1.5 min); second– 3 M DMSO (1.5 min); third– 5 M DMSO + 3 other permeable cryoprotectants in minimum concentration (1 min); fourth– 5 M DMSO + 3 other permeable cryoprotectants in medium concentration (1 min); fifth– 5 M DMSO + 3 other permeable cryoprotectants in maximum concentration + 15% sucrose and honey + 5% PVP (1 min) and sixth– 5 M DMSO + 3 other

Treatment	Step	Cryoprotectant	Expose time	Temperature
1	1	1 M DMSO	1.5 min	19.5°C ± 1
	2	3 M DMSO	1.5 min	19.5°C ± 1
	3	5 M DMSO + 2 M EG + 2 M PG + 1 M Ac	1 min	19.5°C ± 1
	4	5 M DMSO + 4 M EG + 4 M PG + 2 M Ac	1 min	19.5°C ± 1
	5	5 M DMSO + 5 M EG + 6 M PG + 3 M Ac + 15% Suc + 15% H + 5% PVP	1 min	0°C
	6	5 M DMSO + 5 M EG + 6 M PG + 3 M Ac + 20% Suc + 20% H + 10% PVP	1 min	0°C
2	1	1 M DMSO	1.5 min	19.5°C ± 1
	2	3 M DMSO	1.5 min	19.5°C ± 1
	3	5 M DMSO + 2 M EG + 2 M PG + 1 M Gly	1 min	19.5°C ± 1
	4	5 M DMSO + 4 M EG + 4 M PG + 3 M Gly	1 min	19.5°C ± 1
	5	5 M DMSO + 5 M EG + 6 M PG + 5 M Gly + 15% Suc + 15% H + 5% PVP	1 min	0°C
	6	5 M DMSO + 5 M EG + 6 M PG + 5 M Gly + 20% Suc + 20% H +10% PVP	1 min	0°C
3	1	1 M DMSO	1.5 min	19.5°C ± 1
	2	3 M DMSO	1.5 min	19.5°C ± 1
	3	5 M DMSO + 2 M EG +2 M PG + 2 M MeOH	1 min	19.5°C ± 1
	4	5 M DMSO + 4 M EG +4 M PG + 4 M MeOH	1 min	19.5°C ± 1
	5	5 M DMSO +5 M EG + 6 M PG + 6 M MeOH + 15% Suc + 15% H + 5% PVP	1 min	0°C
	6	5 M DMSO +5 M EG + 6 M PG + 6 M MeOH + 20% Suc + 20% H +10% PVP	1 min	0°C
4	1	1 M DMSO	1.5 min	19.5°C ± 1
	2	3 M DMSO	1.5 min	19.5°C ± 1
	3	5 M DMSO + 2 M PG + 1 M Ac +1 M Gly	1 min	19.5°C ± 1
	4	5 M DMSO + 4 M PG + 2 M Ac +3 M Gly	1 min	19.5°C ± 1
	5	5 M DMSO + 6 M PG + 3 M Ac +5 M Gly + 15% Suc + 15% H + 5% PVP	1 min	0°C
	6	5 M DMSO + 6 M PG + 3 M Ac +5 M Gly + 20% Suc + 20% H +10% PVP	1 min	0°C
5	1	1 M DMSO	1.5 min	19.5°C ± 1
	2	3 M DMSO	1.5 min	19.5°C ± 1
	3	5 M DMSO + 2 M PG + 1 M Ac + 2 M MeOH	1 min	19.5°C ± 1
	4	5 M DMSO + 4 M PG + 2 M Ac + 4 M MeOH	1 min	19.5°C ± 1
	5	5 M DMSO + 6 M PG + 3 M Ac + 6 M MeOH + 15% Suc + 15% H + 5% PVP	1 min	0°C
	6	5 M DMSO + 6 M PG + 3 M Ac + 6 M MeOH + 20% Suc + 20% H +10% PVP	1 min	0°C
6	1	1 M DMSO	1.5 min	19.5°C ± 1
	2	3 M DMSO	1.5 min	19.5°C ± 1
	3	5 M DMSO + 1 M Ac +1 M Gly + 2 M MeOH	1 min	19.5°C ± 1
	4	5 M DMSO + 2 M Ac +3 M Gly + 4 M MeOH	1 min	19.5°C ± 1
	5	5 M DMSO + 3 M Ac +5 M Gly + 6 M MeOH + 15% Suc + 15% H + 5% PVP	1 min	0°C
	6	5 M DMSO + 3 M Ac +5 M Gly + 6 M MeOH + 20% Suc + 20% H +10% PVP	1 min	0°C

Table 1: Stepwise protocol of DMSO-based vitrificant solution.

permeable cryoprotectants in maximum (experimental) concentration +20% sucrose and honey +15% PVP (1 min) (Table 1). The first four steps were carried out at 19.5°C ± 1 whilst the last two were done at 0°C to reduce toxic effects on embryos. After the exposure to the vitrificant solutions, embryos were washed with water and incubated until 1–day larva. Hatching rate was determined for the three replicates in each vitrificant solutions.

Statistical analysis

Results were expressed as means ± SD and analyzed by one way ANOVA. Significant differences between the hatching rates obtained for the permeable cryoprotectants, between non-permeable cryoprotectants, embryonic development stage as well as between the vitrificant solutions were detected by the Dancan's multiple range tests, significance being set at $p<0.05$. For all cryoprotectants, differences between concentrations and between expose times were also analyzed.

Results

Toxicity of permeable cryoprotectants

All the analyzed parameters (development stage, cryoprotectant concentration and exposure time) influenced the hatching rate of Persian sturgeon embryos significantly. Increased exposure to the cryoprotectant (from 5 to 10 min) influenced the hatching rate negatively. The hatching rate decreased with concentration (except DMSO in 24 h post-fertilization stage), reducing it significantly when compared with controls. 48 h post-fertilization stage embryos were less sensitive to permeable cryoprotectant, resisting high concentration of each cryoprotectant (Table 2). DMSO was the cryoprotectant best tolerated by the embryos, since the hatching rate was significantly higher than those obtained for other cryoprotectants at the same concentration. Acetamide has promoted the highest reductions in the hatching rate in the way it shows a toxic effect at concentrations higher than 2 M (5 and 10 min exposure) in embryos at both developmental stages. The highest hatching rate for 24 h post-fertilization stage were obtained with the exposure to 3 M Gly (66.66% for 10 min exposure) and for 48 h post-fertilization stage best values were obtained with 1 M DMSO (70% for 5 min exposure) (Table 2).

Toxicity of non-permeable cryoprotectants

The results obtained with 48 h post-fertilization stage embryos demonstrated that this stage is less tolerant of exposure to non-permeable cryoprotectants than 24 h post-fertilization stage embryos (except PVP). Increased non-permeable cryoprotectants, influenced the hatching rate negatively, reducing it significantly when compared with controls (Figure 1 and Figure 2). The hatching rates decreased with exposure time (from 5 to 10 min) and were similar to those of permeable cryoprotectant (except PVP) (Figure 3).

Toxicity of vitrificant solutions

There were significant differences between the values obtained for the different treatments when compared with control. The highest hatching rates for embryos were obtained with the exposure to V3 (70%) and V5 (66.66%) without any significant difference between them ($p> 0.05$). V2 caused highest mortality because of its highly toxic characteristic (Table 3).

Discussion

Several factors must be considered in order to formulate an

CPTs exposure		control	Cryoprotectan concentrations					
			1 M	2 M	3 M	4 M	5M	6M
A								
DMSO	5 min	73.33 ± 1.66^{a}	40 ± 2.88^{c}	21.66 ± 4.40^{d}	46.66 ± 3.33^{bc}	41.66 ± 4.40^{bc}	51.66 ± 3.33^{b}	50 ± 2.88^{bc}
	10 min	71.66 ± 1.66^{a}	30 ± 2.88^{d}	28.33 ± 1.66^{d}	41.66 ± 3.33^{c}	40 ± 2.88^{c}	35 ± 2.88^{cd}	55 ± 2.88^{b}
EG	5 min	70 ± 2.88^{a}	36.66 ± 1.66^{b}	35 ± 2.88^{b}	31.66 ± 4.40^{b}	31.66 ± 1.66^{b}	21.66 ± 4.40^{c}	20 ± 0^{c}
	10 min	75 ± 2.88^{a}	53.33 ± 4.40^{b}	30 ± 0^{cd}	26.66 ± 4.40^{cde}	31.66 ± 4.40^{c}	18.33 ± 4.40^{de}	15 ± 2.88^{e}
PG	5 min	70 ± 2.88^{a}	15 ± 0^{e}	41 ± 1.66^{d}	55 ± 2.88^{b}	50 ± 0b^{c}	36.66 ± 1.66^{d}	43.33 ± 1.66^{c}
	10 min	73.33 ± 1.66^{a}	43.33 ± 1.66^{bc}	33.33 ± 4.40^{e}	36.66 ± 4.40^{cd}	45 ± 2.88^{bc}	50 ± 2.88^{b}	41.66 ± 1.66^{bcd}
Ac	5 min	75 ± 2.88^{a}	60 ± 2.88^{b}	58.33 ± 4.40^{b}	38.33 ± 1.66^{c}	25 ± 2.88^{d}	8.33 ± 4.40^{e}	0^{e}
	10 min	73.33 ± 1.66^{a}	26.66 ± 1.66^{c}	35 ± 2.88^{b}	26.66 ± 4.40^{c}	13.33 ± 1.66^{d}	0^{e}	0^{e}
MeOH	5 min	71.66 ± 1.66^{a}	15 ± 2.88^{e}	41.66 ± 1.66^{cd}	55 ± 2.88^{b}	50 ± 2.88^{bc}	36.66 ± 4.40^{d}	48.33 ± 1.66^{bc}
	10 min	71.66 ± 4.40^{a}	31.66 ± 4.40^{b}	35 ± 2.88^{b}	31.66 ± 4.40^{b}	26.66 ± 4.40^{bc}	16.66 ± 1.66^{c}	28.33 ± 3.33^{bc}
Gly	5 min	75 ± 2.88^{a}	45 ± 2.88^{c}	26.66 ± 4.40^{d}	61.66 ± 1.66^{b}	38.33 ± 1.66^{c}	40 ± 2.88^{c}	36.66 ± 3.33^{c}
	10 min	70 ± 5.77^{a}	61.66 ± 1.66^{a}	26.66 ± 4.40^{bc}	66.66 ± 4.40^{a}	31.66 ± 4.40^{b}	16.66 ± 4.40^{c}	0^{d}
B								
DMSO	5 min	75 ± 2.88^{a}	70 ± 2.88^{ab}	63.33 ± 1.66^{b}	50 ± 2.88^{c}	51.66 ± 1.66^{c}	68.33 ± 2.88^{ab}	20 ± 2.88^{d}
	10 min	75 ± 2.88^{a}	50 ± 2.88^{bc}	51.66 ± 4.40^{b}	21.66 ± 1.66^{f}	26.66 ± 2.88^{ef}	40 ± 5^{cd}	36.66 ± 4.40^{de}
EG	5 min	70 ± 2.88^{a}	38.33 ± 4.40^{bc}	35 ± 0^{c}	11.66 ± 1.66^{d}	46.66 ± 1.66^{b}	18.33 ± 1.66^{d}	28.33 ± 3.33^{c}
	10 min	73.33 ± 1.66^{a}	25 ± 2.88^{d}	36.66 ± 1.66^{c}	50 ± 2.88^{b}	40 ± 2.88^{c}	13.33 ± 1.66^{e}	0^{f}
PG	5 min	71.66 ± 1.66^{a}	60 ± 2.88^{b}	58.33 ± 1.66^{b}	40 ± 2.88^{d}	43 ± .1.66^{cd}	58.33 ± 1.66^{b}	50 ± 2.88^{c}
	10 min	73.33 ± 1.66^{a}	60 ± 0^{b}	58.33 ± 4.40^{b}	58.33 ± 1.66^{b}	55 ± 2.88^{b}	51.66 ± 1.66^{b}	40 ± 2.88^{c}
Ac	5 min	71.66 ± 1.66^{a}	25 ± 2.88^{b}	18.33 ± 1.66^{c}	3.33 ± 1.66^{d}	0^{d}	0^{d}	0^{d}
	10 min	75 ± 2.88^{a}	16.66 ± 1.66^{c}	35 ± 2.88^{b}	1.66 ± 2.88^{d}	0^{d}	0^{d}	0^{d}
MeOH	5 min	73.33 ± 3.33^{a}	18.33 ± 3.33^{bc}	10 ± 2.88^{c}	25 ± 2.88^{b}	10 ± 0^{c}	68.33 ± 4.40^{a}	21.66 ± 1.66^{b}
	10 min	76.66 ± 1.66^{a}	11.66 ± 4.40^{d}	10 ± 2.88^{d}	10 ± 2.88^{d}	25 ± 5.77^{c}	31.66 ± 4.40^{c}	48.33 ± 1.66^{d}
Gly	5 min	75 ± 2.88^{a}	25 ± 2.88^{e}	48.33 ± 1.66^{cd}	65 ± 2.88^{ab}	58.33 ± 4.40^{bc}	45 ± 5.77^{d}	46.66 ± 1.66^{d}
	10 min	73.33 ± 3.33^{a}	53.33 ± 1.66^{bc}	41.66 ± 4.40^{d}	48.33 ± 4.40^{cd}	61.66 ± 4.40^{b}	51.66 ± 1.66^{bcd}	51.66 ± 1.66^{bcd}

Values with different letters are significantly different from the control ($p<0.05$) (means ± SD).

Table 2: Hatching rate of (A) 24 h post-fertilization stage and (B) 48 h post-fertilization stage exposed to different concentration of DMSO, EG, PG, Ac, MeOH and Gly.

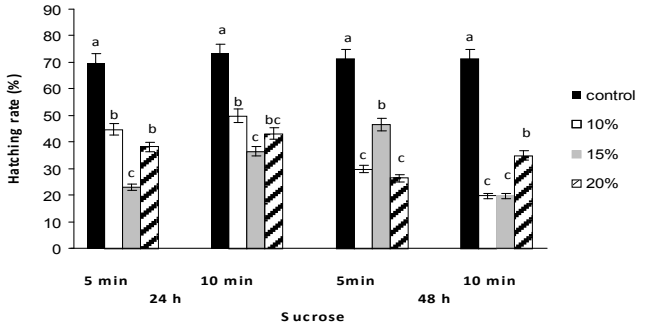

Figure 1: Hatching rate of 24 and 48 h post-fertilization stage Persian sturgeon embryos exposed to different concentration of sucrose (10%, 15% and 20%). Values with different letters are significantly different from the control ($p<0.05$) (means ± SD). Significant differences between the different volumes for each treatment are represented by different letters.

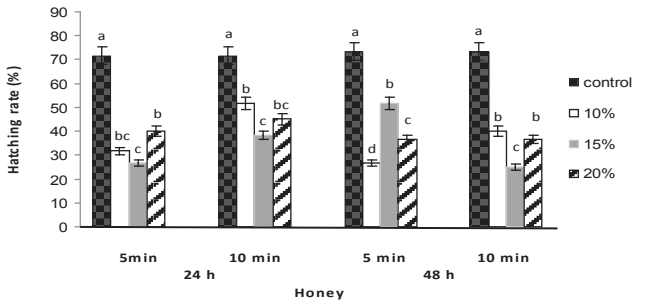

Figure 2: Hatching rate of 24 and 48 h post-fertilization stage Persian sturgeon embryos exposed to different concentration of honey (10%, 15% and 20%). Values with different letters are significantly different from the control ($p<0.05$) (means ± SD). Significant differences between the different volumes for each treatment are represented by different letters.

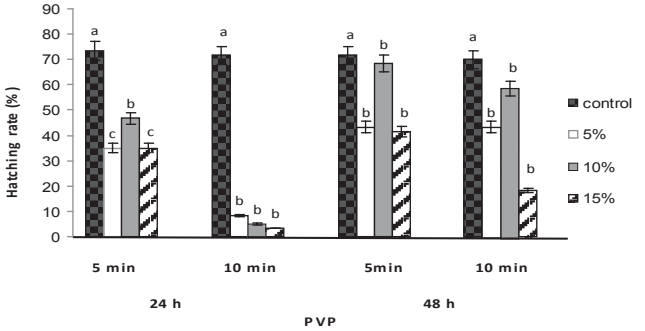

Figure 3: Hatching rate of 24 and 48 h post-fertilization stage Persian sturgeon embryos exposed to different concentration of polyvinyl pyrrolidone (PVP) (5%, 10% and 15%). Values with different letters are significantly different from the control ($p<0.05$) (means ± SD). Significant differences between the different volumes for each treatment are represented by different letters.

appropriate protocol for cryopreservation of fish embryos. Toxicity of cryoprotectants is one of those factors [5]. In this study, we investigated the toxicity of several cryoprotectants in Persian sturgeon embryos. DMSO was the least toxic permeable cryoprotectant, followed by PG, MeOH, Glycerol and EG, whereas acetamide was the most toxic. In the regard, DMSO has been most commonly used in fishes due to the good results obtained in sperm cryopreservation [13]. The hatching rate of the embryos treated with DMSO was higher than those obtained from other cryoprotectants at same concentration and exposure time. Suzuki et al. [14] reported different results of cumulative mortality for medaka, rainbow trout, pejerrey and carp embryos exposed to concentrations of DMSO up to 5 M. Interestingly, Persian sturgeon embryos were resistant to high concentration of DMSO (up to 6 M) and we have obtained high rates of hatching with some concentration of this cryoprotectant, but low or zero survival with other cryoprotectants. Dimethyl sulfoxid has been also used for cryopreservation of medaka [15], trout [16], turbot [12] and red seabream embryos [17] and was considered a good cryoprotectant for these species.

In the present study, PG, MeOH and Gly were also well tolerated by the Persian sturgeon embryos. The effect of these cryoprotectants was species-dependent in fish; PG is a commonly used cryoprotectant in cryopreservation of fish embryos. Tian and Chen [18] demonstrated that propylene glycol was better tolerated in sea perch than other cryoprotectants. This finding was also observed in flounder [4] and red seabream embryos [17].

MeOH is one of the most permeable cryoprotectants, it would be present inside embryos at a higher concentration than other cryoprotectants [7,19]. MeOH was relatively nontoxic to embryos of zebrafish [20], penaeid shrimp [21], Indian major carp [1] and flounder [4]. However, MeOH was more toxic than other cryoprotectants on the embryos of black tiger shrimp [22] and red seabream [17].

In addition to PG and MeOH, Gly was also well tolerated by the Persian sturgeon. The superior quality of glycerol as an embryo cryoprotectant has been reported for embryos of black tiger shrimp [22]. However, it was not tolerated by embryos of many aquatic species such as zebrafish [20], penaeid shrimp [23], flounder [4] and red seabream [17]. Ethylene glycol was less well tolerated by 24 and 48 h post-fertilization stage embryos than PG, methanol and glycerol. Whereas EG was better tolerated in gilthead seabream embryos [5], but not tolerated in embryos of turbot [12], sea perch [18], or flounder [4]. Ethylene glycol has few known detrimental effects on the development potential, membrane integrity, or cytoskeletal structure of oocytes [24]. But, some studies of fish enzymatic activity have demonstrated that EG is toxic and affecting G-6-PDH and LH activities [25]. It is therefore possible that in this study EG interfered with embryo metabolism, and thereby produced a decrease in the hatching rate. In the present study, acetamide was the worst tolerated by the embryos, as shown by the significantly reduced hatching rate at concentrations of higher than 2 M, and no survival was obtained when the concentration of acetamide was 6 M. Nevertheless, acetamide was considered as appropriate cryoprotectant for cryopreservation of black tiger shrimp embryos due to low toxicity [22].

The effect of each cryoprotectant does not depend only on the chemical properties but is also dependent on the exposure time. In Persian sturgeon embryos, with more prolonged exposure (from 5 to 10 min), the concentration that embryos could tolerate decreased ($P<0.05$) (DMSO in 24 h post-fertilization stage). These findings are consistent with previous reports in turbot [12], gilthead seabream [5] and red seabream embryos [17]. Inverse to other cryoprotectants we used in the present experiment, as the DMSO concentration (in 24 h post-fertilization stage) was increased the hatching rate was increased too. Similar to present observation was not found in other research.

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 [11,26-28] and are essential for reducing the toxicity of high concentrations of permeable cryoprotectants [11,12]. In the present work, we have tested the toxicity of three non-permeable

		Vitrificant solutions					
	Control	V1	V2	V3	V4	V5	V6
Hatching rate (%)	66.66 ± 4.40[a]	25 ± 2.88[c]	0[d]	70 ± 2.88[a]	55 ± 2.88[b]	66.66 ± 1.66[a]	20 ± 2.88[c]

Values with different letters are significantly different from the control ($p<0.05$) (means ± SD).

Table 3: Hatching rate of 48 h post-fertilization stage embryos exposed to different vitrificant solutions.

cryoprotectants: sucrose, honey and PVP. It has been suggested that, sugars are capable for preserving the structural and functional integrity of membranes at low water activity [24,29]. Until now there has been no information on the toxicity of honey in fish embryos. In this study, honey was used as a non-permeable cryoprotectant for first time.

Hatching rate of Persian sturgeon embryos treated with sucrose, honey and PVP decreased significantly with the increase of exposure time. Our results showed that PVP is well tolerated by embryos than sucrose and honey. Previous studies demonstrated that non-permeable cryoprotectants were less toxic than permeable one at the concentration used for gilthead seabream [5] and red seabream embryos [17]. But, it was disagreement with the results of present study. It is possible that in these studies, duration of exposure to non-permeable cryoprotectants was shorter than permeable cryoprotectants. However, in this study exposure time to the both permeable non-permeable cryoprotectants was similar.

Tolerance of fish embryos to cryoprotectant has been reported to depend on developmental stage [14,30], more mature embryos generally being more resistant than embryos at earlier development stage [31]. In this study, embryos at 48 h post-fertilization stage had more tolerance to cryoprotectant than embryos at 24 h post-fertilization stage. So, 48 h post-fertilization stage embryos were chosen for assessment of vitrificant solutions. To overcome cryoprotectant toxicity, several techniques have been adopted. A common approach is to use a mixture of these compounds in vitrification solutions. Since the concentration of each cryoprotectant in the solution is low, it is specific toxicity is reduced [32]. It has been also observed that the protective effect of combination of each cryoprotectants can be greater than would be expected if the action of each agent were simply additive [33]. The stepwise exposure of cryoprotectants is another means of reducing vitrification solution toxicity. Dimethyl sulfoxide was the most effective cryoprotectant as judged by the tolerance of embryos to high concentrations so, was chosen as based vitrificant solutions. Other permeable cryoprotectants were added based on their low toxicity at high concentrations. Sucrose, honey and PVP were incorporated at the highest concentrations necessary to vitrify the solution, in order to reduce the toxicity of high concentrations of permeable ceyoprotectants.

Chen and Tian [34] reported that five-step equilibration of flounder embryos using vitrificant solutions resulted in higher survival rates than equilibration in 4, 3, 2 or 1 step. Therefore, using a stepwise incorporation protocol designed to avoid direct exposure to high concentrations of cryoprotectants and reduce osmotic stress. Among all the vitrificant solutions, V3 and V5 showed the highest hatching rate, both of which contained MeOH. Fahy et al. [35] demonstrated that cryoprotectant could neutralize the toxicity of other cryoprotectants; hence the hatching rate increased. For flounder embryos, there was a significant reduction in toxicity of DMSO, PG, EG and Gly with the addition of methanol [4] which confirms our results.

Conclusions

Based on the response to cryoprotectant exposure, 48h post-fertilization stage embryos of Persian sturgeon were more resistant to cryoprotectants. In conclusion, the proposed vitrificant solutions can be used for the vitrification of Persian sturgeon embryos, due to their low toxic action in this species. These finding will be advantageous to development of a cryogenic protocol for Persian sturgeon embryos.

Acknowledgments

We thank Abbas Ali Hajibeglou and all the staff from Shahid Marjani Sturgeon Propagation Center (Gorgan, Iran) for their valuable practical assistance.

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