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Feasibility Studies on Vitrification of Persian Sturgeon (*Acipenser persicus*) Embryos

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Abstract

The feasibility of cryopreservation by vitrification of Persian sturgeon (*Acipenser persicus*) embryos at 48 h post-fertilization stage was investigated. Vitrification is considered the most promising option. Many factors are involved in the success of the process. The choice of a proper vitrificant solutions and temperature for thawing, were the parameters considered in the present study. Six vitrificant solutions (V1-V6) were tested using a stepwise incorporation protocol. The tested solutions contained acetamide as the main cryoprotectant +3 other permeable cryoprotectants +3 non-permeable cryoprotectants. Before loading the embryos into tubes, toxicity tested was affected with these solutions. The hatching rate of embryos that had been exposed to the vitrificant solutions was analyzed and the highest hatching rate was obtained with exposure to V1. After thawing (water bath, 0 or 20°C), embryos were incubated until hatched. The highest survival rate (69.69%) was observed in samples frozen with V1 and thawed at 20°C. These results establish that cryopreservation of Persian sturgeons embryos by vitrification is possible.

Keywords: Vitrification; Cryopreservation; Persian sturgeon; Embryo; Cryoprotectant; Vitrificant solution

Introduction

Vitrification is the solidification of a liquid brought about not by crystallization but by an extreme elevation in viscosity during rapid cooling. Vitrification has been reported as the most promising option to cryopreserve fish embryos [1]. There is one report of flounder (*Paralichthys olivaceus*) embryos surviving cryopreservation by vitrification in liquid nitrogen [2], but results have not been reproducible. Cabrita et al. [3] demonstrated that several factors must be considered in order to formulate an appropriate protocol for freezing/thawing. The toxicity of cryoprotectants is one of those factors. Solutions required for vitrification have high concentration of cryoprotectants and usually include high molecular weight cryoprotective agents [3]. Our previous studies on cryoprotectants toxicity were taken into account in the design of a stepwise protocol of cryoprotectant incorporation for the vitrification of Persian sturgeon embryos [4]. The design of a proper protocol for cryoprotectant incorporation is decisive for the success of the process, but the use of adequate freezing/thawing rates is also necessary [5]. The developmental ability in frozen/thawed Persian sturgeon embryos has not yet been reported by any author. Persian sturgeon lives is southern part of the Caspian Sea and 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, restocking and conservation of this rare and threatened species. The aim of the present study was to investigate the feasibility of cryopreservation of Persian sturgeon embryos by vitrification.

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.

Embryos

Feral Persian sturgeons were captured from the southern part of Iranian coastal waters on the Caspian Sea and were transported to the Shahid Marjani Sturgeon Propagation Center, Gorgan, Iran. Sperm 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 20°C. Embryos at the 48 h post-fertilization stage were selected to examine sensitivity of embryos to the toxicity of vitrificant solutions.

Sensitivity of embryos to the toxicity of vitrificant solutions

This study carried out with normal and permeabilized embryos. For permeabilization, embryos were treated with pronase for 5 min at 20°C (2 mg/ml diluted in Ringer Solution) befor being subjected to the vitrificant solutions. Six vitrificant solutions (V1–V6) were prepared by combining some cryoprotectants. For incorporation of the vitrificant solutions, the embryos were exposed to a six step protocol. The steps of the protocol were as follows: first-1 M acetamide (Ac) (1.5 min); second-2 M acetamide (1.5 min); third -3 M acetamide +3 other permeable cryoprotectants in minimum concentration (1 min); fourth -3 M acetamide +3 other permeable cryoprotectants in medium

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concentration (1 min); fifth -3 M acetamide +3 other permeable cryoprotectants in maximum concentration +15% sucrose and honey +5% PVP (1 min) and sixth -3 M acetamide +3 other permeable cryoprotectants in maximum concentration +20% sucrose and honey +15% PVP (1 min). The first four steps were carried out at 20°C whilst the last two were done at 0°C to reduce toxic effects on embryos (Table 1). Embryos were placed in the different solutions using a nylon mesh. After the exposure to the vitrificant solutions, embryos were carefully washed with water and incubated until hatched. Hatching rate was determined for the three replicates in each vitrificant solutions.

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

Table 1: Steps of the cryoprotectant incorporation protocol.

Vitrification trials

Embryos were exposed using the stepwise protocol for the incorporation of cryoprotectants, described before, and transferred to plastic tubes. The tubes were then plunged into liquid nitrogen. Thawing was performed in a water bath at 0 or 20°C (about 10 min). After thawing, the embryos were incubated until hatching or death. Numbers of viable embryos were determined 24 h after thawing.

Statistical analysis

Results are expressed as mean ± S.D. Hatching rates and survival rates were analyzed by one-way ANOVA and Dancan's multiple range tests. Values of $P \leq 0.05$ were considered to be statistically significant. The statically analysis was computed using SPSS software.

Results

Sensitivity of embryos to the toxixity of vitrificant solutions

Hatching rate of Persian sturgeon embryos exposed to six vtrificant solutions are shown in Figure 1. The highest hatching rate was obtained with the exposure to V1 and the lowest hatching rate was obtained with exposure to V3. Thus, vitrificant solution V1 was considered to be suitable for embryo vitrification, but V3 not.

Vitrification trials

Fortunetly, we obtained viable embryos after vitrification in some of conditions examined. Use of different vitrificant solutions yielded significant difference in embryos survival rate. After thawing, the all embryos vitrified in six vitrificant solution survived, continued to develop and hatched out (Figure 2). Better results being obtained after vitrification and thawing in water bath at 20°C. The highest survival rate (69.69%) was observed in samples that exposed to V1 and thawed at 20°C.

Discussion

Successful vitrification requires optimization of several individual steps. Factors that influence the survival of embryos by vitrification include the concentration and composition of the vitrificant solutions, the procedure used to equilibrate embryos in these solutions and the freezing and thawing conditions [6]. The vitrificant 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 temperature [4]. The non-permeable cryoprotectants act as dehydrating agents [7,8], increase medium viscosity and avoid ice crystal formation

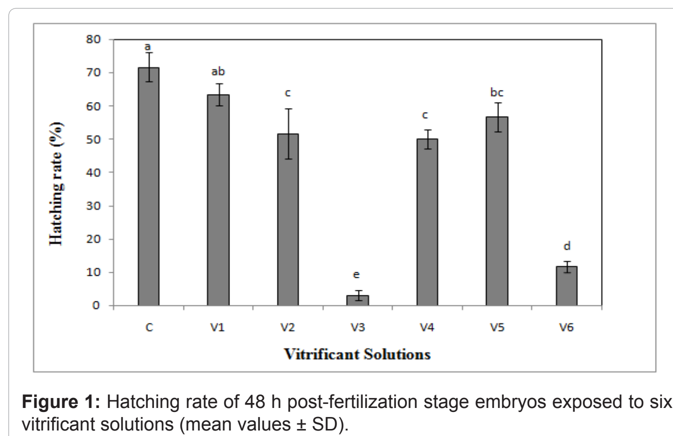

Figure 1: Hatching rate of 48 h post-fertilization stage embryos exposed to six vitrificant solutions (mean values ± SD).

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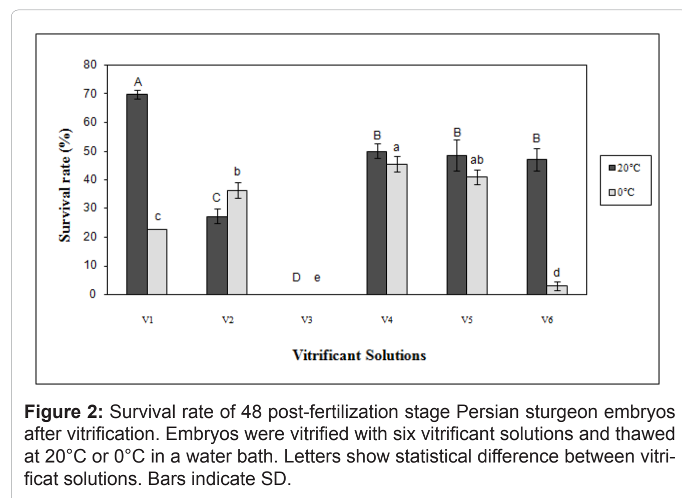

Figure 2: Survival rate of 48 post-fertilization stage Persian sturgeon embryos after vitrification. Embryos were vitrified with six vitrificant solutions and thawed at 20°C or 0°C in a water bath. Letters show statistical difference between vitrificat solutions. Bars indicate SD.

[9]. These compounds have also been demonstrated to have a substantial vitrification enhancing effect [6]. In this study, three non-permeable cryoprotectants (sucrose, honey and PVP) were added to the vitrificant solutions. To avoid excessive toxicity and osmotic stress of cryoprotectants, embryos were equilibrated in a stepwise manner, as is usually recommended [2,10]. Successful vitrification of mouse embryos was achieved using a vitrificant solution containing acetamide, DMSO and PG [11] and it was in agreement with our results. In the present study, results demonstrated that the highest survival rate was obtained in samples frozen with V1 that contained acetamide, DMSO, PG and EG.

The process of thawing is as complicated as freezing during vitrification [3]. Some authors have stated that vitrified samples should be thawed using high temperature in orderto avoid devitrification or recrystallization of ice during this process [12]. In this study, two different temperatures for thawing were tested: 0 and 20°C. Our results showed that 20°C, and therefore the fastest thawing rate, is more appropriate in this species since higher percentage of survival embryos were obtained.

The encouraging results of this study establish that cryopreservation of Persian sturgeon embryo is possible by vitrification and identify methods and procedures that promote success. Further refinements will make the technique more applicable for practicable application in aquaculture and management of rare or threatened aquatic species.

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