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Cryopreservation of Persian sturgeon (*Acipenser persicus*) embryos by DMSO-based vitrificant solutions

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ABSTRACT

Vitrification could provide a promising tool for the cryopreservation of fish embryos. To achieve successful cryopreservation, several parameters should be taken into account in the design of a vitrification protocol. In the present study, some relevant factors were investigated (choice of a proper vitrificant solutions and temperature for thawing) using neurulation-stage embryos. Six DMSO-based vitrificant solutions (V1–V6) were tested using a 6-step incorporation protocol. DMSO-based vitrificant solutions contained DMSO + permeable cryoprotectants + nonpermeable cryoprotectants. Embryos were immersed in vitrificant solutions for 7 minutes and directly plunged into liquid nitrogen. After vitrification (−196 °C for 10 minutes), the thawing was performed in a water bath at 0 or 20 °C and then embryos incubated until hatched. Our results demonstrated that some embryos vitrified in 5 of 6 vitrification solutions survived and hatched out, but none survived after vitrification in V2. The highest survival rate (45.45%) was observed in samples frozen with the best vitrificant solution (V6) and thawing combination (20 °C). These results establish that cryopreservation of Persian sturgeon (*Acipenser persicus*) embryos by DMSO-based vitrificant solutions is possible.

1. Introduction

Vitrification, as ice-free cryopreservation technique using high cryoprotectant concentration and rapid freezing rates, offers some advantages to traditional cryopreservation methods [1]. This technique has been successfully applied to embryos of mammals [2] and other marine invertebrates [3]. Recently, Chen and Tian [1] reported the successful cryopreservation of Japanese flounder (*Paralichthys olivaceus*) embryos by vitrification.

Before their report, there had been only 1 article on the deep cryopreservation of fish embryos; in 1989, Zhang et al. [4] reported that common carp (*Cyprinus carpio*) embryos survived cryopreservation after slow freezing, but this success has since to be reproduced [5].

Among all factors in the vitrification process, the cryoprotectant is the most important as it facilitates the transition to a vitreous state and reduces cryo-damage [6–8]. However, cryoprotectant toxicity is a substantial concern [9,10]; it is dependent on embryo developmental stage and the temperature at which embryos are exposed. As cryoprotectant toxicity is species dependent [11], specific cryoprotectant solutions must be tested for each species.

Previous studies on cryoprotectant toxicity [12] were taken into account in the design of a stepwise protocol of cryoprotectant incorporation for the vitrification of Persian sturgeon (*Acipenser persicus*) embryos. Our recent report demonstrated that 48-hour post-fertilization stage (neurulation stage) [13] embryos of Persian sturgeon were more resistant to cryoprotectants, and DMSO was better tolerated than the other cryoprotectants [14].

Caspian Sea is the habitat for the 4 commercial species of sturgeon [15]. A major part of the world's sturgeon catch (90%–92%) originates from the Caspian Sea [16]. Caviar is

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one of the most exclusive and expensive fishery product and overfishing is the main reason for sturgeon stock deficiencies. Since 1998, the trade of all kinds of sturgeon caviar has been controlled by customs, and special permission has become necessary including exact species declaration of the traded caviar [17]. However, stocks of sturgeons are decreasing dramatically [18], the total sturgeon catches and caviar production in Iran in 1993 were 1710 and 106 t, whereas in 2006 it decreased to 330 and 31.3 t, respectively [19].

Persian sturgeon (*A persicus*) live in southern part of the Caspian Sea and has a high commercial value, but few attempts at embryo cryopreservation have been carried out, despite that this technology could improve some aspects of production, restocking, and conservation of this rare and threatened species.

The overall objective of the present study was to develop an effective protocol for cryopreservation of Persian sturgeon embryos, a fish with high commercial value in world.

2. Materials and methods

2.1. Embryos

For the study of embryos cryopreservation by DMSO-based vitrificant solutions, fertilized Persian sturgeon embryos at neurulation stage (48 hours after fertilization) were used. Embryos were obtained from the Shahid Marjani Sturgeon Propagation Center (Gorgan, Iran) and transferred to incubators (Russian incubator, $39 \times 29 \times 18.5$ cm^3) equipped with recirculating water system. Water temperature, oxygen content, and pH were maintained at 20 ± 1 °C, greater than 5.6 mg/L, and 8.1, respectively, during incubation.

Embryos were incubated under these conditions until neurulation stage as described by Shafizadeh [13] for Persian sturgeon (*A persicus*) embryos (~2.5 mm in diameter) that corresponds to approximately 48 hours after fertilization. Persian sturgeon embryos required 2.5 to 3 days for development to hatching in Russian incubator at 20 ± 1 °C.

Our recent report [14] demonstrated that Persian sturgeon embryos at neurulation stage (48 hours after fertilization) had more tolerance to cryoprotectant than gastrulation stage (24 hours after fertilization). So, neurulation-stage embryos were chosen for assessment of vitrificant solutions. DMSO also was better tolerated than the other cryoprotectants [14].

2.2. Chemical

Six permeable cryoprotectants, DMSO, ethylene glycol (EG), propylene glycol (PG), methanol (MeOH), acetamide, and glycerol, and 3 nonpermeable cryoprotectants, sucrose (Suc), honey (H), and polyvinyl pyrrolidone (PVP), were used in the following experiments.

For permeable and nonpermeable cryoprotectants, the concentrations used were 1, 3, and 5 M for DMSO; 2, 4, and 5 M for EG; 2, 4, and 6 M for PG; 2, 4, and 6 M for MeOH; 1, 2, and 3 M for acetamide; 1, 3, and 5 M for glycerol; 10%, 15%, and 20% for sucrose and honey; and 5% and 10% for PVP. Cryoprotectant concentrations were prepared in Ringer solution (2.99 g/L KCl, 6.49 g/L NaCl, 0.29 g/L $CaCl_2$, and 0.202 g/L $NaHCO_3$).

All the chemicals and the pronase used for chorion permeabilization (type XIV *Streptomyces griseus*) were purchased from Merck (Darmstadt, Germany).

2.3. Vitrificant solutions

As preliminary experiments, we examined the formation of ice in vitrificant solutions during cooling and warming. Six DMSO-based vitrificant solutions (V1–V6) were designed. DMSO-based vitrificant solutions contained DMSO + permeable cryoprotectants + nonpermeable cryoprotectants (Fig. 1). Each solution was loaded into 15-mL transparent plastic tubes that were immersed in liquid nitrogen (LN_2). After 10 minutes, the plastic tubes were warmed in water at 0 or 20 °C. It was observed whether the vitrificant solutions became opaque during the cooling or warming.

2.4. Vitrification methods

Only good-quality embryos were used, and the adequate developmental stages were selected under stereoscopic microscope to perform the experiments. This study was carried out with permeabilized embryos. For permeabilization, embryos were treated with pronase for 5 minutes at 20 °C (2 mg/mL diluted in Ringer solution) before being subjected to the protocol of cryoprotectant incorporation.

For incorporation of cryoprotectants, embryos were exposed first to 1 M DMSO, then to 3 M DMSO, next to 5 M DMSO + 3 other permeable cryoprotectants in minimum concentration, then to 5 M DMSO + 3 other permeable cryoprotectants in medium concentration, and finally to 5 M DMSO + 3 other permeable cryoprotectants in maximum concentration + 15% Suc and 15% H + 5% PVP. The final vitrification solution was to 5 M DMSO + 3 other permeable cryoprotectants in maximum concentration + 20% Suc and 15% H + 5% PVP.

First, embryos were exposed for 1.5 minutes at room temperature (20 °C), before being immersed in the second solution for 1.5 minutes at room temperature. Embryos were exposed to the third and fourth solution for 1 minute at room temperature, and finally, embryos were exposed to fifth and sixth solution for 1 minute at 0 °C (Fig. 1).

Embryos were placed in the different solutions and placed in plastic tubes (15-mL plastic test tubes). The tubes were then plunged directly into LN_2 (−196 °C). After storage for 10 minutes in LN_2, tubes containing frozen embryos were quickly removed from LN_2 and immersed in water bath at 0 or 20 °C for 10 seconds.

Immediately after thawing, the embryos were transferred to hatchery water in the Russian incubator until hatching or death. Physicochemical conditions for the thawed embryos were 20 ± 1 °C, 2 ± 0.5 ppt salinity, greater than 5.6 mg/L O_2, and 8.1 ± 0.2 pH (mean ± SD).

Embryos were evaluated as viable when they revealed a normal development without signs of malformations, spontaneous movement, and a functional heartbeat and blood circulation. Evaluations were made in a dissecting microscope at 4- to 10-fold magnification.