www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 17: 681-687 (2017) DOI: 10.4194/1303-2712-v17_4_04

RESEARCH PAPER

Effect of Vitrification by Propylene Glycol-Based Solutions on the Survival Rate of the Persian Sturgeon (Acipenser Persicus) Embryos

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Received 14 November 2016
Accepted 02 January 2017

Abstract

The effects of vitrification on the embryo viability of the Persian sturgeon (*Acipenser persicus*) were investigated in this study. Vitrificant solutions (VS1-VS6) were prepared by combining propylene glycol (PG) as basic cryoprotectant plus other permeable and non-permeable cryoprotectants in different concentration. Embryos at neurulation stage were selected and incubated in six vitrificant solutions for 7 minutes using four-step incorporation protocol before transferring into liquid nitrogen. Following these treatments, the embryos were washed well and incubated until hatching. The results showed that PG-based vitrificant solutions seemed to be suitable for the vitrification of Persian sturgeon embryos except VS4. After vitrification (-196 °C for 10 min), the embryos were thawed (water bath, 0 or 20 °C), rehydrated and incubated to determine survival rate. Results demonstrated that the embryos treated with solutions VS1, VS2, VS3, VS5 and VS6 presented some degrees of viability after vitrification, whereas none survived after vitrification in VS4. There were no differences in the results obtained using either 0 or 20 °C thawing temperatures. Among six vitrificant solutions, the combination of 6 M PG + 5 M dimethyl sulfoxide + 6 M ethylene glycol + 3 M acetamide (VS1) had the highest survival rate (59.09 %).

Keywords: Vitrification, Persian sturgeon, Embryo, Cryopreservation, Cryoprotectant.

Introduction

Embryo cryopreservation is a method used to preserve embryos by cooling them to a very low temperature and storing them in liquid nitrogen (-196 °C). Cryopreservation of fish embryos has been widely studied since 1978 (Whittingham & Rosenthal, 1978) for its advantages in strain management, research, and applications in aquaculture. Vitrification has been reported as the most promising option to cryopreserve fish embryos, due to their high chilling sensitivity (Zhang & Rowson, 1996). This technique has been successfully applied to flounder (*Paralichthys olivaceus*) embryos (Chen & Tian, 2005) but results have not been reproducible (Edashige *et al*., 2006).

High sensitivity to chilling, the inevitable risk of intracellular ice formation during cooling, a large amount of yolk and the membrane complexity of fish embryos are believed to be the major obstacles in fish embryo cryopreservation (Cabrita *et al*., 2003a; Zhang & Rawson, 1995). To overcome these obstacles, knowledge of the cryoprotectant toxicity, suitable concentrations and combinations of cryoprotectants, membrane permeability together with appropriate cooling protocols are essential (Zhang *et al*., 2005).

The toxicity of the cryoprotectants is very important in cryopreservation protocols, particularly when high concentrations are required for vitrification (Kuleshova *et al*., 1999). Cryoprotectant tolerance by embryos is also stage-dependent (Robertson & Lawrence, 1988; Urbanyi *et al*., 1997). Therefore, it is very important to perform a preliminary study of the toxicity of cryoprotectants before designing specific vitrification solutions for particular species. Recent report demonstrated that Persian sturgeon embryos at neurulation stage (48 h after fertilization) had more tolerance to cryoprotectant than gastrulation stage (24 h after fertilization) and also different concentrations of propylene glycol, which is a commonly used cryoprotectant in cryopreservation of fish embryos, was well tolerated by Persian sturgeon embryos at this stage (Keivanloo & Sudagar, 2013).

The purpose of the present study was to evaluate the efficiency of propylene glycol-based vitrificant solutions for vitrification of Persian sturgeon (*Acipenser persicus*) embryo. This species inhabits in the southern part of the Caspian Sea, being one of the most important components of the Caspian Sea ichthyofauna. In the mid-1980s, this sturgeon species constituted 85% of the standing stock of the world

sturgeon population (Abhari & Tavakkoli, 1999), although the population collapsed in the 1970s (Moghim *et al*., 2006). Persian sturgeon is among the most vulnerable fish species because of their overfishing for meat and caviar production, destruction of their spawning grounds and water pollution; this species is currently included in the IUCN Red Data List. For these reasons, most research in recent years has been focused on the culture of Persian sturgeon for restocking and commercial aquaculture programs. The purpose of the present study was to explore the vitrification method for cryopreservation of the Persian sturgeon embryos, which would provide technological approaches for improving some aspects of production, restocking and conservation of the rare and threatened species.

Materials and Methods

Fish Breeding and Embryo Collection

Persian sturgeon embryos culture was performed at Shahid Marjani Sturgeon Propagation Center (Gorgan, Golestan, Iran). Broodstock was selected from wild breeders originating from the Caspian Sea and stocked in a 75.4 m^3 tank with a freshwater supply. Persian sturgeon embryos were obtained from artificial propagation in April–May 2016. The type and dose of hormone administration for artificial propagation were LH-RH-A2 and 5 μg kg^{-1}, respectively. Sperm extraction was done by abdominal massage of the male, collecting the sperm from the genital pore with a syringe and then quality of sperm was estimated using naked eyes. 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. Eggs were de-adhesived with fuller's earth and then transferred to incubators (Russian incubator, 39×29×18.5 cm^3) equipped with recirculating water system. Egg incubation was performed at 20 ± 1 °C (pH 8.2 ± 0.2 and dissolved oxygen 5.4 ± 0.5 mg/l) (mean ± SD).

Developmental stages of embryos were determined morphologically under a stereomicroscope. Embryos developed to the neurulation stage were used for experiment which correspond to approximately 48 h after fertilization (Shafizadeh, 1999). Persian sturgeon embryos required 2.5 to 3 day for development to hatching in Russian incubator at 20 ± 1 °C.

Materials

Six permeable cryoprotectants, 2, 4 and 6 M propylene glycol (PG), 3 and 5 M dimethyl sulfoxide (DMSO), 4 and 6 M ethylene glycol (EG), 2 and 3 M acetamide (Ac), 4 and 6 M methanol (MeOH), 3 and 5 M glycerol (Gly) and three non-permeable cryoprotectants, 20% sucrose (Suc) and honey (H) and 10% polyvinyl pyrrolidone (PVP) were used in the following experiments. All the cryoprotectants were combined with Ringer solution (2.99 g/l KCl, 6.49 g/l NaCl, 0.29 g/l $CaCl_2$, and 0.202 g/l $NaHCO_3$) for the preparation of the experimental vitrification solutions (Cabrita *et al*., 2003b). Those concentrations were selected on the basis of previous studies carried with Persian sturgeon embryos (Keivanloo & Sudagar, 2013). All the chemicals and the pronase used for chorion permeabilization (type XIV *Streptomyces griseus*), were purchased from Merck Company, Germany.

Vitrificant Solutions

Based on past results on cryoprotectants toxicity (Keivanloo & Sudagar, 2013), Six vitrificant solutions (VS1-VS6) were prepared by combining propylene glycol (PG) as basic cryoprotectant + permeable cryoprotectants + non-permeable cryoprotectants. The composition of these vitrificant solutions is shown in Figure 1.

As preliminary experiments, we examined the formation of ice in vitrificant solutions during cooling and warming. Each solution was loaded into 15 mL transparent polyethylene plastic tubes that were immersed in liquid nitrogen (LN_2) and then the plastic tubes were warmed in water at 20 °C for 10 min. It was observed whether the vitrificant solutions became opaque during the cooling or warming.

Toxicity of Vitrificant Solutions

The toxicity of six vitrificant solutions (VS1–VS6) designed containing PG as the main cryoprotectant was analyzed using Persian sturgeon embryos at neurulation stage.

Before the vitrificant solutions exposure, chorion permeabilization was performed using 2 mg/ml pronase at 20 °C for 5 min (Keivanloo & Sudagar 2013, 2016). For incorporation of the vitrificant solutions, the embryos were exposed to a four-step protocol. The steps of the protocol were as follows: first- 2 M PG (2 min); second- 4 M PG (2 min); third- 6 M PG + 3 other permeable cryoprotectants (1.5 min); fourth- 6 M PG + 3 other permeable cryoprotectants + 20% sucrose and honey + 10% PVP (1.5 min). The first two steps were carried out at 20 °C whilst the last two were done at 0 °C to reduce toxic effects on embryos (Figure 1). After the exposure to the solutions, embryos were carefully washed with water and incubated until hatched. Control embryos were exposed to Ringer solution in a similar manner and then transported to the incubator. The experiment was repeated three times, and each replicate utilized approximately 22 embryos. The toxicity of the vitrificant solutions was determined by the hatching rate (number of hatched larvae relative to the total number of embryos in each treatment).

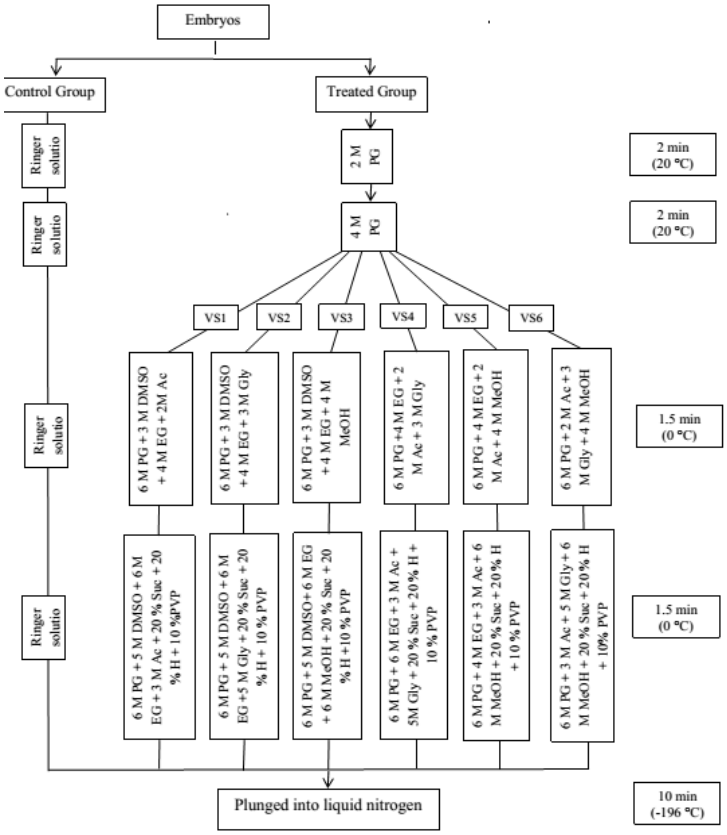

Figure 1. Steps of the cryoprotectant incorporation protocol. In the flow chart, time and temperature of incubation are showed next to each step of the protocol.

Vitrification of Embryos in Different Vitrificant Solutions

For vitrification, embryos at the neurulation stage were collected. After treated with the vitrification solutions according to the four-step protocol as described above (Figure 1), embryos were loaded into plastic tubes (15 mL) along with the vitrificant 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 were immediately immersed into liquid nitrogen (LN_2) for at least 10 min. The sealed tubes were then removed from LN_2 and thawed using a water bath at 0 or 20 °C for 30 s. The embryos were removed from the tubes, washed with incubator water and transferred to incubators until hatching or death. Physicochemical conditions for the thawed embryos were 20 ± 1 °C, 2 ± 0.5 ppt salinity, 5.4 ± 0.5 mg/L O_2, and 8.2 ± 0.2 pH (mean ± SD).

When embryonic development was completed (72 h after fertilization), both control and treated groups were carefully removed from incubators. Hatched larvae, the larvae that had ruptured the chorion, whether completed their embryonic development or not; and live larvae, the larvae with vigorous mobility and swimming capability were counted under a stereomicroscope (4- to 10-fold magnification). Survival rate (number of live larvae / total number of embryos in each treatment) were determined 1 day after normal hatching for each treatment group.

For all experiments, 3 replicates (~924 embryos in total) were used for each treatment and each replicate utilized approximately 22 embryos.

Statistical Analysis

Statistical analysis was carried out using SPSS 16.0 (SPSS Inc. Chicago, Illinois, USA). All results were expressed as means ± SD. The statistical significance of the differences between means was analyzed by one-way ANOVA followed by the Duncan's multiple range tests. Differences were considered as statistically significant at a probability value of $P<0.05$.

Results

As preliminary experiments, we cooled and warmed vitrificant solutions in plastic tubes without embryos to see the formation of ice (Table 1). When the solutions in tubes were cooled by being plunged

into LN_2, VS4 became opaque but other vitrificant solutions (VS1, VS2, VS3, VS5 and V6) remained transparent during cooling, suggesting that ice crystals formed not in VS1, VS2, VS3, VS5 and V6 but in VS4 during cooling. During warming at 20 °C, all the vitrificant solutions remained transparent suggesting that they all remained uncrystallized, except for VS4 (Table 1).

Toxicity of Vitrificant Solutions

Effects of vitrificant solutions on hatching rate of Persian sturgeon embryos at the neurulation stage are shown in Table 2. Among the six vitrificant solutions, the hatching rate of embryos was highest in VS1 (not significantly different from VS5 and control) and lowest in VS4. Thus, solution VS1 was considered to be suitable for embryo vitrification, but VS4 was not.

Survival of Embryos after Vitrification

The embryos vitrified without vitrificant solutions (control group) indicated 100% mortality after thawing. Table 3 presents the results of the survival rate after vitrification and thawed at 20 °C. The results showed that some embryos continued to develop and survived after cryopreservation when using the vitrification solutions VS1, VS2, VS3, VS5 and VS6, while none survived after vitrification in VS4. The similar results also observed after thawing at 0 °C (Table 4).

In total, 792 embryos at the neurulation stage cryopreserved with vitrificant solutions, 318 hatched larvae were obtained after thawing. Some showed no difference with normally cultured embryos; while the other hatched larvae stopped development before developing to live larvae with vigorous mobility and swimming capability. With 318 hatched larvae, 251 developed to larval fish with normal development without signs of malformations.

There were no differences in the results obtained using either 0 or 20 °C thawing temperatures. The highest survival rate (59.09 %) was observed in samples exposed to VS1 and also VS5 and thawed at 20 °C (Table 3).

During the development in the embryos surviving vitification both deformity and abnormity were not observed. Survived embryos were reared for 30 days. Fingerlings were then released in Gorganrood River by the Shahid Marjani Sturgeon Propagation Center (Gorgan, Iran).

Discussion

Vitrification is the rapid cooling of Liquid medium in the absence of ice crystal formation (Rall & Fahy, 1985). The medium forms an amorphous glass as a result of rapid cooling by direct submersion of the embryo into liquid nitrogen. The glass retains the normal molecular/ionic distributions of a liquid but remains in an extremely viscous, super-cooled form (Rall, 1987). The glass is devoid of ice crystals, and embryos are not subjected to the physical damage that is associated with ice crystal formation (Rall & Fahy, 1985). During cryopreservation, cryoprotectants are used to protect intracellular organelles during preservation in liquid nitrogen (Dobrinsky, 2001).

Cryoprotectants exert both protective and toxic effects on fish embryos. The higher cryoprotectant concentration in vitrificant solution is, the more successful the vitrification is. However, a cryoprotectant at high concentration exerts greater toxicity than the same cryoprotectant at a lower concentration (Chen & Tian, 2005). To overcome cryoprotectant toxicity, several techniques have been adopted. A common approach is to use a mixture of these compounds in vitrificant solutions. Since the concentration of each cryoprotectant in the solution is low, it is specific toxicity is reduced (Mazure, 1990). It has been also observed that the protective effect of combination of cryoprotectants can be greater than would be expected if the action of each agent were simply additive (Chian *et al*., 2004).

The vitrificant solutions used in this study was 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 (Keivanloo & Sudagar, 2013).

In this study, we tested the efficiency of the various vitrificant solutions to Persian sturgeon embryo. Propylene glycol was used in all combinations, because of its low toxicity (Keivanloo & Sudagar, 2013) and because it forms glassy solids at relatively low concentrations (Hagedorn *et al*., 1996). The result indicated that PG-based vitrificant solutions had a relatively lower toxicity level and a stronger ability for vitrification, which was consistent with the study by Hua and Ren (1994) and Tian *et al*. (2015).

There were differences in viability and survival among the embryos that were exposed to different vitrification solutions. It was found the highest survival rate was obtained in samples frozen with VS1 that contained PG, DMSO, EG and acetamide as compared with those treated with other solutions. Similarly, Ding *et al*. (2007) reported that high percentages of morphologically intact embryos (50.6%) was obtained when number 1 cryoprotectant mixture was used for vitrification red sea bream (*Pagrus major*) embryos that contained DMSO + acetamide + PG. Successful vitrification of mouse embryos also was achieved using a vitrificant solution containing PG, DMSO and acetamide (Rall & Fahy, 1985).

This analysis revealed that the embryos treated with solutions that contained glycerol (VS2, VS4 and VS6) presented low degree of viability after the vitrification. Permeating cryoprotectants, such as

Table 1. Formation of ice crystals in vitrificant solutions during cooling and warming in tubes

Vitrificant solutions	Cooling	Warming	Vitrificant solutions	Cooling	Warming
VS1	-	-	VS4	+	+
VS2	-	-	VS5	-	-
VS3	-	-	VS6	-	-

- Ice crystal did not form.
+ Ice crystal formed in tubes.

Table 2. Hatching rate of Persian sturgeon embryos at the neurulation stage exposed to six propylene glycol-based vitrificant solutions

	Vitrificant solutions						
	Control	VS1	VS2	VS3	VS4	VS5	VS6
Hatching rate (%)	70 ± 2.88[a]	65 ± 4.40 [a]	41.66 ± 4.40[b]	35 ± 2.88[b]	6.66 ± 2.88[c]	61.66 ± 1.66[a]	38.33 ± 1.66[b]

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

Table 3. Survival rate of Persian sturgeon embryos at the neurulation stage after vitrification with six propylene glycol-based vitrificant solutions and thawed at 20 °C

Treatment	Number of embryo frozen (number of embryo in each tube)	Number of hatched larvae	Number of live larvae	Survival rate (%) *
Control	66 (22, 22, 22)	0	0	0 [e]
VS1	66 (22, 22, 22)	45 (13, 17, 15)	39 (12, 14, 13)	59.09 ± 4.54 [a]
VS2	66 (22, 22, 22)	40 (15, 12, 13)	30 (11, 10, 9)	45.45 ± 4.55 [b]
VS3	66 (22, 22, 22)	25 (9, 8, 8)	16 (6, 6, 4)	24.24 ± 5.24 [d]
VS4	66 (22, 22, 22)	0	0	0 [e]
VS5	66 (22, 22, 22)	45 (17, 13,15)	39 (14, 12, 13)	59.09 ± 4.54 [a]
VS6	66 (22, 22, 22)	25 (10, 7, 8)	22 (9, 6, 7)	33.33 ± 6.94 [c]

*Survival rate (number of live larvae / total number of embryos in each treatment) Data are presented as mean ± standard deviation (SD); data within a column with different superscript letters are significantly different (P< 0.05).

Table 4. Survival rate of Persian sturgeon embryos at the neurulation stage after vitrification with six propylene glycol-based vitrificant solutions and thawed at 0 °C.

Treatment	Number of embryo frozen (number of embryo in each tube)	Number of hatched larvae	Number of live larvae	Survival rate (%) *
Control	66 (22, 22, 22)	0	0	0 [e]
VS1	66 (22, 22, 22)	35 (11, 13, 11)	31 (10, 11, 10)	46.96 ± 2.62 [a]
VS2	66 (22, 22, 22)	9 (3, 2, 4)	7 (2, 2, 3)	10.60 ± 2.62 [d]
VS3	66 (22, 22, 22)	40 (12, 15, 13)	28 (11, 8, 9)	42.42 ± 6.94 [ab]
VS4	66 (22, 22, 22)	0	0	0 [e]
VS5	66 (22, 22, 22)	24 (9, 7, 8)	24 (9, 7, 8)	36.36 ± 4.54 [b]
VS6	66 (22, 22, 22)	30 (9, 11, 10)	15 (4, 6, 5)	22.71 ± 5.24 [c]

*Survival rate (number of live larvae / total number of embryos in each treatment) Data are presented as mean ± standard deviation (SD); data within a column with different superscript letters are significantly different (P<0.05).

glycerol, among other properties, act to depolymerize microfilaments and microtubules. Depolymerization may be beneficial towards protecting these cytoskeletal components during osmotic stresses induced by exposure to or removal of cryoprotectants. When coupled with vitrification, however, disruption to the microfilaments and/or microtubules by high concentrations of cryoprotectants can be irreversible and lethal to embryos (Dobrinsky, 2001).

The stepwise exposure of cryoprotectants is another means of reducing vitrificant solution toxicity (Gwo, 2000). The addition of cryoprotectants causes the release of water from the cells and the resulting dehydration can be lethal if it occurs too rapidly (Leibo *et al*., 1974). Therefore in the present study, as is usually recommended (Gwo, 2000; Cabrita *et al*., 2003b; Chen & Tian, 2005; Cabrita *et al*., 2006), using a stepwise incorporation protocol was designed to avoid direct exposure to high concentrations of cryoprotectants and reduce osmotic stress.

The addition of high molecular weight compounds such as sucrose polymer, polyvinyl alcohol (Nowshari & Brem, 2000) and polyvinyl pyrrolidone (PVP) (Leibo & Oda, 1993) has successful in embryo freezing, apparently because they are good inhibitors of ice crystal formation. The mechanism of protection by large polymers is unclear. Meryman (1966) first proposed that these compounds would prevent the osmotic injury caused by rapid removal of extracellular water, and also that they had the ability to coat the cells and protect the cell membrane from denaturation. These compounds have also been demonstrated to have a substantial vitrification enhancing effect (Rall *et al*., 1987). In this study, three non-permeable cryoprotectants (sucrose, honey and PVP) were added to the vitrificant solutions. The addition of carbohydrates to permeable cryoprotectants aids the dehydration of embryos (Kuleshova *et al*., 1999). It has been suggested that sugars are capable of preserving the structural and functional integrity of membranes at low water activity (Crowe *et al*., 1990; Hotamisligil *et al*., 1996).

The design of proper protocol for cryoprotectant incorporation is decisive for the success of cryopreservation, but adequate freezing/thawing rates are also necessary (Cabrita *et al*., 2006). The process of thawing is as complicated as freezing during vitrification (Cabrita *et al*., 2006). Some authors have stated that vitrified samples should be thawed using high temperatures in order to avoid devitrification or recrystallization of ice during this process (Wusterman *et al*., 2002). In the present study, there were no differences in the results obtained using either 0 or 20 °C thawing temperatures, this finding is in accordance with results obtained by Cabrita *et al*. (2006) when studying gilthead seabream (*Sparus aurata*) embryos. Nevertheless, previous report (Keivanloo & Sudagar, 2016) showed better results using 20 °C for thawing Persian sturgeon embryos. The present results cannot be compared directly with those of Keivanloo and Sudagar (2016), because there were several differences in the used methods, including substance that was used as basic cryoprotectant in vitrificant solutions, combinations of cryoprotectants and cryopreservation protocol.

In conclusion, surviving embryos were obtained in the present study when using vitrification cryopreservation with PG-based vitrificant solutions and four-step incorporation protocol, which will provide a strong basis for subsequent studies. However, further investigations and experiments are necessary to extend the storage time and to improve the survival rate.

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