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Freezing injuries in the embryos of *Piaractus mesopotamicus*

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Summary

Cryopreservation of mammal embryos has been technically feasible for many years, but morphological injuries still persist in fish embryos during cryopreservation. Thus, the objective of the present study was to describe these freezing injuries in *Piaractus mesopotamicus* embryos. Two hundred and twenty-five embryos were collected at the post-gastrula stage and assigned into four treatments of sucrose at 8.5, 17.0, 25.0 or 34.0% plus 9.0% methanol. The control was prepared with distilled water only. The gradual decrease in the temperature was 0.5°C/min. After the seeding stage, the fish embryos were stored in liquid nitrogen at −33°C. Thereafter, they were thawed for evaluating per cent hatching, and the samples collected from every treatment were submitted to scanning electron microscopy for morphological analysis. The micrographic images showed that there was substantial alterations in embryo morphology under the highest concentrations of sucrose. These solutions did not prevent the formation of ice crystals, which lead to deformities and killed the embryos, but the observed reduced level of morphological structure in these embryos when treated with 17.0% sucrose plus 9.0% methanol is a compelling argument for additional studies.

Keywords: Cryoprotectant, Methanol, Morphological, Pacu, Reproduction, Sucrose

Introduction

Cryopreservation of mammal embryos has been technically feasible for many years, but morphological injuries still persist in fish embryos during cryopreservation (Ahammad *et al.*, 1988; Ninhaus *et al.*, 2008). The impediments to develop a safety protocol have been: the lower permeability of the embryonic membranes; embryo size with low surface-to-volume ratio; large-sized cells; and the sensitivity of the eggs to the subfreezing temperatures (Hagedorn *et al.*, 1997; Zhang & Rawson, 1998).

Cryopreservation consists of storing quiescent embryos in liquid nitrogen for long periods (Neves, 2008). Consequently, a successful protocol can have profound effects on farming and fishery management, preservation of rare species (Wildt, 1993), offspring production of rheophilic species during all the year and the safe storage of diverse gene pools in minimal space (Janik *et al.*, 2000).

Cryoprotectants are the substances required to submit fish embryos to subzero temperatures (Harvey, 1983). These chemicals are classified according to their site of action. Methanol, ethylene-glycol and dimethyl sulphoxide (DMSO), for example, have intracellular activities (Reichenbach *et al.*, 2001), and sucrose, glucose and trehalose have extracellular activities (Niemann, 1991; Denniston *et al.*, 2000). Thus, Streit Jr *et al.* (2007) tested several combinations of cryoprotectants, and suggested cooling the embryos of *P. mesopotamicus* with 17.0% sucrose plus 9.0% methanol.

The methods for preserving bovine embryos are based on the standard freezing curve developed by Willadsen *et al.* (1976, 1978), who recommended

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decreases in thermal rates of between 3 and 1°C/min. Thus, the embryos are induced to seeding after stabilization at −7°C, which is followed by freezing at −33 or −35°C, they are then stored in liquid nitrogen. However, these methods have often been detrimental to the embryo viability because cryopreservation is still resulting in morphological injuries to these embryos (Neves, 2008; Ninhaus-Silveira, 2008).

The investigation of many morphological modifications by using the stereo- or light microscopy to describe and evaluate the morphological structures of fish embryos is more cost-effective. Nevertheless, electron micrographic images have been the best way to characterize abnormalities, deformities and injuries (Galman & Avtalion, 1989; Shardo, 1995; Ninhaus-Silveira *et al*., 2007; Neves, 2008).

In Brazil, researchers have been developing technological on-farm systems to raise *P. mesopotamicus* since the mid-1970s (Urbinati & Gonçalves, 2005) because the pacu has excellent farming characteristics for large acreages (Castagnolli & Zuim, 1985; Lima *et al*., 1991; Silva *et al*., 1997).

The pacu is also an excellent model for the present investigation because of its rheophilic behaviour. Thus, the objective of the present experiment was to investigate the effects of different concentrations of sucrose with 9.0% methanol on the morphological structures of *P. mesopotamicus* embryos.

Materials and methods

The experiment was conducted in the Estação de Hidrologia e Aqüicultura of the DUKE ENERGY INTERNATIONAL–Geração Paranapanema, Salto Grande (SP) in collaboration with the PeixeGen/UEM and Aquam/UFRGS research groups.

Parental fish were randomly selected at the gonad stage, which was visually detected through the enlarged abdomen and the reddish urogenital pore in the females, and the presence of milting after a gentle pressure along the male body (Woynarovich & Horváth 1983). Thereafter, they were transferred to an aquarium with 1000 l of clean-flowing water.

The females received injections of carp pituitary at 5.5 mg kg^{-1} of body weight divided into 10% at the first application, and the remained 90% at the second application 12 h later. When this second injection was applied to the females, one dose of carp pituitary at 2.5 mg kg^{-1} of body weight was also applied to the males. Thereafter, the water temperature and the reproductive signals from the females were monitored at 1-h intervals because the pacu requires about 240 h to spawn. The gametes and semen were both expelled into the same beaker after applying a gentle pressure along the fish body. After the addition of water for the fertilizing, the eggs were transferred into 7-l capacity incubators.

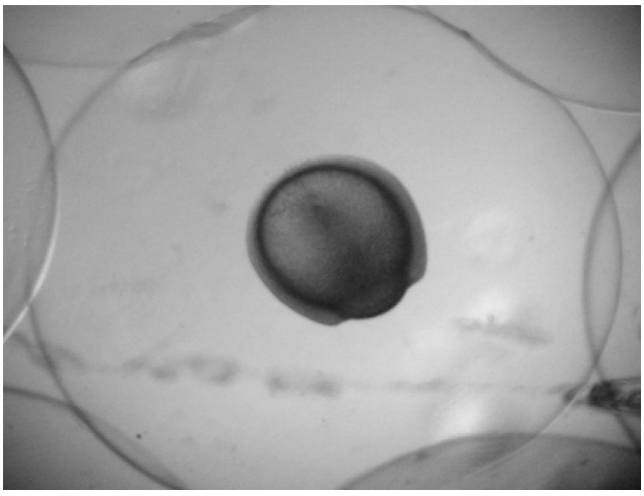


Figure 1 Fish embryo image of *Piaractus mesopotamicus* at the post-gastrula stage, 95% epiboly–blastopore closure (digital image at 12 megapixels taken with a stereomicroscope, ×25).

The fertilization rate was evaluated by counting the number of live embryos and dead eggs from three samples randomly collected soon after blastopore closure. The hatching rate of the live embryos that were incubated without any cryoprotectant (control 1) was evaluated from three replicates of 100 individuals.

Fish embryos developed under 27.4 ± 0.2°C for 6 h were selected according to their viability, health and normal chorion, which was visualized at blastopore closure (Fig. 1). Groups of 15 individuals collected using Pasteur pipettes were submitted to the treatments described in Table 1.

In the 0.5 ml palettes, 15 fish embryos were submitted to the freezing curve regime using a programmable cooler Biocom, model Dominum K, in which the decrease in temperature was 0.5°C/min until stabilization at −7°C for manual seeding.

Table 1 Sucrose solutions for freezing the embryos of *Piaractus mesopotamicus*.

Treatments	Sucrose[a]	Cryoprotectants (%)		Water (ml)
		Sucrose (g)[b]	Methanol (ml)[c]	
1	0.25	8.50	9.00	82.50
2	0.50	17.00	9.00	73.90
3	0.75	25.50	9.00	65.35
4	1.00	34.00	9.00	56.80
Control 2	0.00	00.00	0.00	100.00

[a]Molar concentration of sucrose; [b]Per cent sucrose; [c]Per cent methanol.

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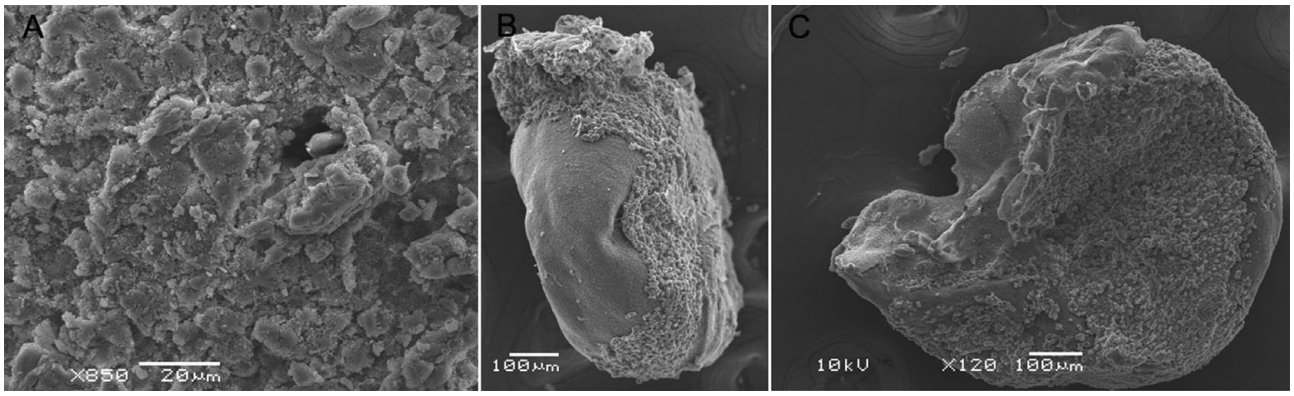


Figure 2 Scanning electron micrographic images after the freezing/thawing process, and taken from fish embryos treated with no cryoprotectant. (*A*) The injuries in the blastoderm cells. (*B*) Fish embryo with a disfigured vitello and completely damaged blastoderm. (*C*) The destroyed vitello and the damaged blastoderm.

Thereafter, the temperature was reduced to −33°C and the embryos were stabilized for 6 min. Next, the embryos were stored in liquid nitrogen.

The frozen embryos were thawed at 45°C for 15 s according to the protocol of Streit Jr *et al.* (2006). Soon after thawing, these embryos were immersed in 10% sucrose to reduce osmotic pressure. Next, they were transferred ito incubators with 1.5 l of flowing water. Samples were randomly taken before fixing these embryos with 2% glutaraldehyde in 0.2 M phosphate buffer.

The fixed samples were first rinsed once with alcohol at 30, 50 and 70% for 30 min, twice in 90% alcohol and then in 100 ethanol for 15 min. Next, the samples were submitted to the Critical Point Dryer (BAL-TEC CDP 030), which uses liquid and gaseous CO_2 for complete dehydration. Finally, they were coated with a gold–palladium alloy in the metal stubs of the Metal Desk II Denton vacuum, and scanning electron micrographic images were used to detect normal development or injuries, photographed using a JEOL (JSM-540) scanning electron microscope at the UFRGS, Porto Alegre, Rio Grande do Sul State, Brazil.

Results

In the control 1 group, the fertilization rate was $88.60 \pm 8.2\%$ and the hatching rate was $93.40 \pm 2.14\%$. These responses indicated the use of high quality biological material. All the thawed embryos, however, died during the first few hours in the incubators, although some were well rehydrated and presented good morphological conformation.

Despite the apparent normality of some morphological components treated with sucrose at 8.50, 17.00 and 25.50%, the chorion was only visualized after rehydration of those embryos submitted to 17.0% sucrose plus 9% methanol.

In the control 2 group, the images showed fish embryos with damaged blastoderm cells and disfigured vitello. The blastoderm was incapable of overcoming the freezing process, and the micropore, which is the chorion microstructure that controls fluid exchanges (Rawson *et al.*, 2000), was completely damaged (Fig. 2).

The use of 8.5% sucrose led to some injuries, but in most of the embryos there were no vitello destruction (Fig. 3). The embryos treated with 17.0% sucrose had no apparent injuries in the chorion, and the vitelline membrane had no structural damage. Furthermore, the blastoderm had damage, and the syncytial vitelline layer showed deformities (Fig. 4).

Under 25.6% sucrose the fish embryos presented structural modifications that characterized the presence of injuries, and a reduced and damaged syncytial layer. The chorion was disrupted and all the vitellos presented structural modifications (Fig. 5). In 34.0% sucrose, the vitellos of all fish embryos were destroyed and the blastoderms were injured (Fig. 6).

These scanning electron micrographic images, therefore, showed some of the inherent difficulties in maintaining the integrity of the fish embryos after use of sucrose and methanol.

Discussion

In the control 1 group, the fertilization rate reflected the healthy conditions of the embryos and, consequently, they were perfectly capable of resisting cryopreservation treatments (Lahnsteiner, 2008); the high viability (Bobe & Labbé, 2009) was important when comparing the efficiency of these cryoprotectants.

The presence of whitish embryos after the thawing process indicated the formation of ice crystals (Zhang & Rawson, 1996). These whitish embryos were different to the translucent embryos, which had good

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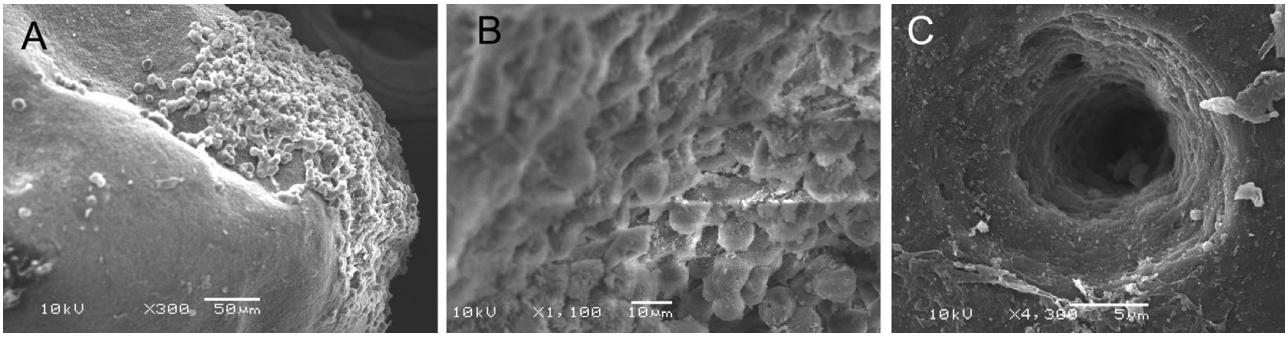


Figure 3 Scanning electron micrographic images of fish embryos (*Piaractus mesopotamicus*) after the freezing process using 8.5% sucrose plus 9.0% methanol. (*A*) Injuries in the blastoderms cells and the modifications in the vitello. (*B*) The embryonic cells with structural modifications in the blastoderm. (*C*) The chorion micropore with injuries caused by the flux/influx of cryoprotectant and water.

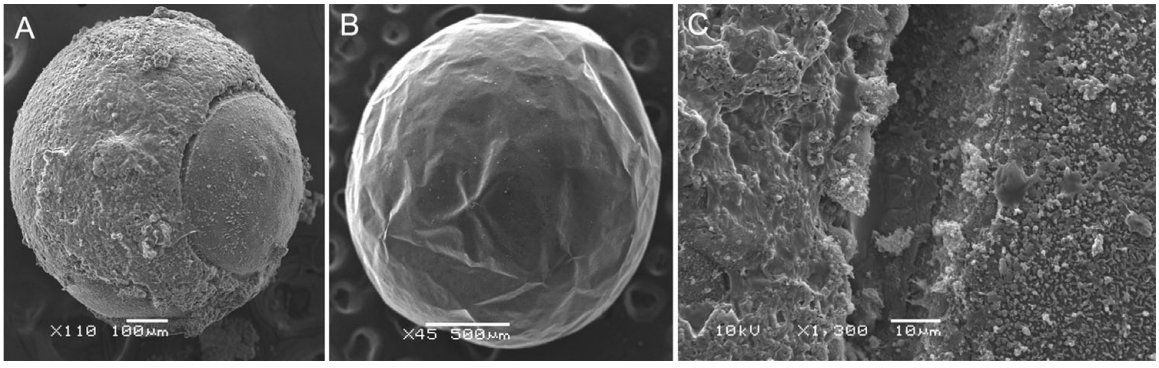


Figure 4 Scanning electron micrographic images of fish embryos (*Piaractus mesopotamicus*) after the freezing process using 17.5% sucrose plus 9.0% methanol. (*A*) Fish embryo with intact vitello and injuries in the blastoderm. (*B*) The hydrated fish embryo showing the intact chorion. (*C*) Apparent damages in the syncytial vitelline layer.

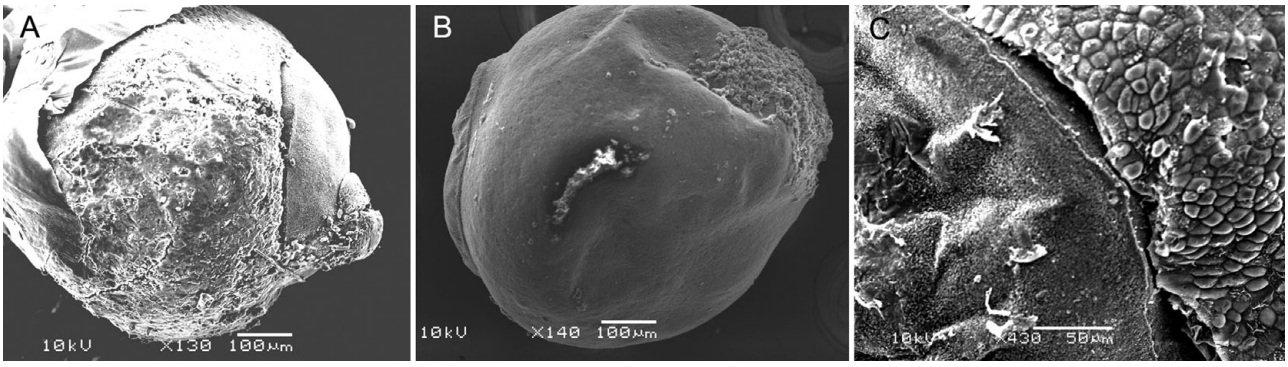


Figure 5 Scanning electron micrographic images of fish embryos after the freezing process using 25.6% sucrose plus 9.0% methanol. (*A*) The damaged blastoderm and the destroyed chorion. (*B*) The disfigured vitello and the destroyed blastoderm. (*C*) Apparent damages in the syncytial vitelline layer.

conformation. Ice crystals were also reported by Neves (2008) who also studied *P. mesopotamicus* and Ninhaus-Silveira *et al*. (2008) who studied the corimbatá (*P. lineatus*). Injuries in the blastoderm cells and the disfigured vitello were probably caused by these ice crystals, as reported by Ninhaus-Silveira *et al*. (2007). These ice crystals may have also been responsible for chorion absence and the destruction of the embryonic and vitelline membranes, as reported by Neves (2008).

Although most of the fish embryos were severely injured by these cryoprotectant solutions, the responses from every concentration showed different levels of intensity. Sucrose at 8.5%, for example, induced injuries, but the vitello was not destroyed in most embryos (Fig. 3). The blastoderm, however,

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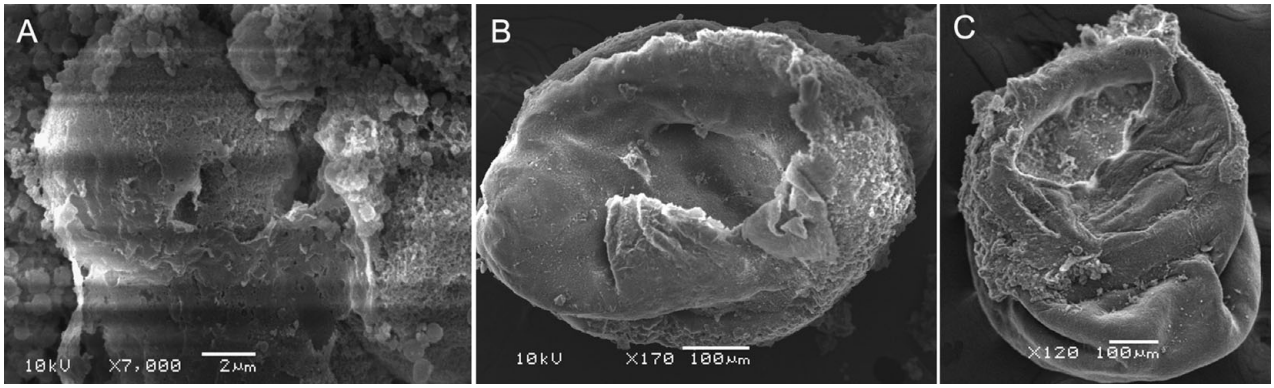


Figure 6 Scanning electron micrographic images of fish embryos (*Piaractus mesopotamicus*) after freezing using 34.2% sucrose plus 9.0% methanol. (*A*) The destruction of the blastoderm cells. (*B*) The destroyed vitello and the remains of the blastoderm. (*C*) Vitellos completely destroyed.

was incapable of overcoming the freezing process, and the micropore was completely damaged (Fig. 2C). These injuries, which should have been prevented by the use of sucrose solutions (Woelders, 1997), may have been caused by the flux/influx of water under a sucrose concentration of 8.5%. Sucrose at 25.5 or 34.0% can disrupt the vitello because of osmotic pressure (Ahammad *et al.*, 2003). This fact can be associated with the present treatments because, under 34.0% sucrose all vitellos were destroyed and the blastoderms were injured (Fig. 6).

The successful cryopreservation of oocytes and fish embryos has numerous constraints associated with injuries throughout the freezing process. The failure may have been due to one of the following four causes: first, most fish eggs have a diameter more than 1 mm, which represents a low area-to-volume ratio that reduces flux of both water and cryoprotectant during the cryopreservation; the second cause is the quantity of vitello; third, the numerous morphological structures of fish embryos; and, finally, the complex system of membranes, which is characterized by low levels of permeability and high sensitiveness to freezing conditions (Billard & Zhang, 2000).

Therefore, in conclusion, in the cryopreservation of *P. mesopotamicus*, the different concentration levels of sucrose plus 9.0% methanol were unable to avoid the formation of ice crystals during the freezing and thawing processes. Ice crystals were responsible for these injuries, but the reduced level of injury observed in the morphological structures of fish embryos treated with 17.0% sucrose plus 9.0% methanol is a compelling argument for additional studies.

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