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Chilling sensitivity of *Steindachneridion parahybae* (Siluriformes: Pimelodidae) oocytes in different cryoprotectants

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ABSTRACT

The viability of post-thaw fish oocytes can be affected by different stages of the freezing process, such as cryoprotectant toxicity, cold sensitivity, freezing curves and thawing. Therefore, these steps need to be investigated for the development of a protocol. In the present study, the aim was to investigate chilling sensitivity at different oocyte stages of *Steindachneridion parahybae*. Immature and mature oocytes were incubated in Hanks' or 90% L15 solutions containing different CPAs (cryoprotectant solutions) per experiment: (1) 0.1–0.4 M sucrose + 1–2 M methanol and (2) 1–4 M methanol X 1–4 M propylene glycol X 1–4 M DMSO for mature oocytes; (3) 0.5 M sucrose or fructose + 2 M methanol or PG or DMSO and (4) 0.25–1 M fructose + 1–4 M DMSO for immature oocytes. All treatments were kept for 120 min at −5.9 ± 2.8°C. For the control treatment, only Hanks' or 90% L15 solutions were carried out. Evaluations were made by viability tests: membrane integrity staining in 0.4% Trypan blue (TB) and fertilization rate (%F) sole for mature oocytes. Results presented that mature oocytes were the most sensitive to lower temperatures, because there was no %F. All cryoprotectants tested in the different concentrations can be used for immature oocytes, however the statistically superior cryoprotectant was CPA with fructose and DMSO, with the low concentration of this CPA being was the best statistically. This may indicate that for this species the immature stages have presented a lower chilling sensitivity than the mature stages.

1. Introduction

The ability to successfully preserve the oocytes at low temperatures has numerous practical and economical benefits and will positively impact the cryobanks of endangered species (Martinez-Páramo et al., 2016), animal breeding programs and assisted conception in human beings (Wang, Naib, Sun, & Lonergan, 2010). There are millions of animals from several species which are descendant of cryopreserved material, as well as thousands of children who were born after *in vitro* fertilization carried out after oocyte cryopreservation. Nonetheless, there are still deficiencies in the methods used to cryopreserve mammalian oocytes, in addition to unsolved problems to establish this technique in fish, even using a variety of study protocols (Guan, Rawson, & Zhang, 2008; Isayeva, Zhang, & Rawson, 2004; Tsai, Rawson, & Zhang, 2009a; Tsai, Rawson, & Zhang, 2009b; Zhang, Isayeva, Adams, & Rawson, 2005; Zhang, Rawson, Tosti, & Carnevali, 2008; Godoy, Streit, Zampolla, Bos-Mikich, & Zhang, 2013).

Designing a cryopreservation protocol should take into account the sequence of events that occur during cryopreservation. The current protocols of cell cryopreservation involve several steps, including the addition of cryoprotectants, the process of chilling and thawing, and finally, cryoprotectant removal (Mazur, 2004). On the other hand, dramatic changes occur in cell structure during oocyte maturation, as well as in the distribution of cytoplasmic organelles and elements of the cytoskeleton as the oocyte completes meiosis, so that the characteristics of membrane permeability also change (Wang, Naib, Sun, & Lonergan, 2010).

Studies on cryopreservation may vary with the use of rapid cooling or slow cooling. In both methods, the effect of the cryoprotectant solution (CPA) arising from the exposure of the cells to a hypertonic extracellular environment for a long period of time is the main cause of cell damage (Solocinski et al., 2017). The combination between CPAs

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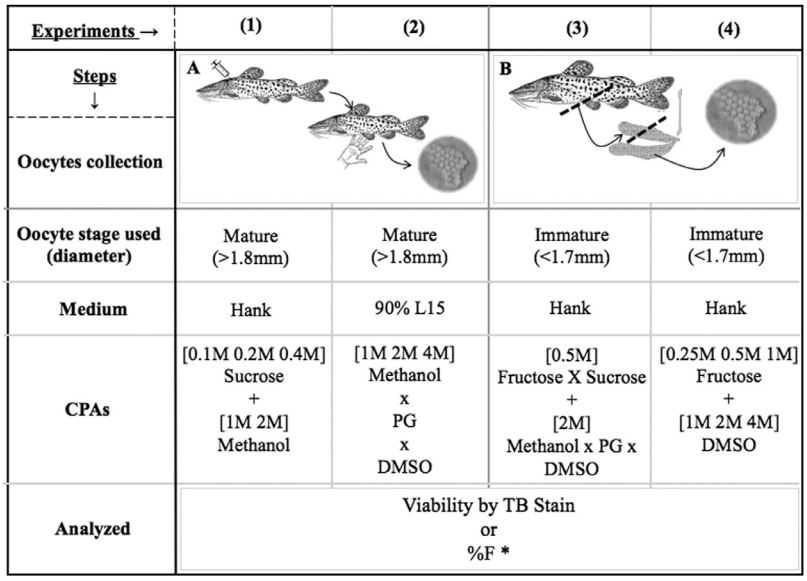

Fig. 1. Flow chart with the steps performed in each of the four experiments conducted to test the chilling sensitivity of surubim-do-Paraíba, *Steindachneridion parahybae*, oocytes. CPAs- Cryoprotectant solutions; PG- Propylene glycol; DMSO- Dymetil sulfoxide; TB- Trypan blue; %F- Fertilization rates.

and the chilling rate to low subzero temperatures is one of the most important factors for cell survival, and this optimum chilling rate can vary over a broad range in different cell types. Cryoprotectants tend to protect the cells during slow chilling, and this occurs according (proportionally) to the concentrations of cryoprotectant used, considering that in the absence of these solutions cell damage caused by chilling would occur (Wang et al., 2010).

The mechanism of action of cryoprotectants during slow chilling is ascribed first by the protective effects of low-molecular-weight substances such as alcohols by their nonspecific colligative ability to reduce the concentration of damaging solutes, to increase the unfrozen fraction or to reduce volume excursions during chilling and thawing; second by the additional protection of sugars, which are able to protect the cell external surface, on which extracellular ice formation lead to osmotic dehydration, that may result in irreversible membrane change and cell death (Pegg, 2002).

In fish, cryopreservation of semen from several species has been extensively studied, and the cryopreserved material has been efficiently used in the reproduction of many species (Asturiano, Cabrita, & Horváth, 2016; Cabrita, Robles, & Herráez, 2009; Cabrita et al., 2014), including *S. parahybae* (Sanches et al., 2015). However, many attempts on cryopreservation fish embryos and oocytes have been carried out over the last three decades, but so far the results have been contradictory and discouraging, and make it impossible to define a long-term cryopreservation protocol, besides the species-specific characteristics involved.

Steindachneridion parahybae (Steindachner, 1877) (Siluriformes: Pimelodidae), an endemic gray catfish from Paraíba do Sul river basin, known as surubim-do-Paraíba (Garavello, 2005), is currently on the red list of Brazilian fauna threatened with extinction (MMA - Ministério do Meio Ambiente, 2008; IBGE – Instituto Brasileiro de Geografia e Estatística, 2014) due to over-fishing and environmental pollution. In order to preserve their genetic material, the aim of this research was to study the chilling sensitivity of oocytes prior to freezing.

2. Material and methods

The experiment was conducted at the Hydrobiology and Aquaculture Station of CESP (São Paulo Energy Company), in the city of Paraibuna, SP, Brazil (23◦24′54''S; 45◦35′52''W), using broodstock originating from induced reproduction performed with wild specimens at the same station (F1). The fish were kept in two earthen ponds (200 m^2) with concrete walls and a sandy bottom and received extruded commercial feed for carnivorous fish with 40% crude protein at a rate of 5% biomass/week, offered twice daily, at 8a.m. and 4p.m., three days per week.

Broodstock were selected in the pond during the reproductive period (Dec/2016, and Jan–Feb/2017). The selected broodstock (three females per experiment) were transferred to the laboratory, weighed and kept in aquaria (500-L) equipped with aeration. Afterwards, they were either sacrificed (so that immature oocytes could be collected) or in order to collect mature oocytes, the females were hormonally induced by injections of crude carp pituitary extract (CCPE) diluted in saline solution (0.9% NaCl), in two dosages (0.5 and 5.0 mg CCPE.kg^{-1}) at an interval of 12 h.

Oocytes at different stages (diameter of initial stage until vitellogenic stage < 1.7 mm) were obtained by removing the ovaries before decapitation (female fish sacrificed by overdose of benzocaine), or by mature oocytes (non-fertilized eggs, diameter > 1.8 mm), that were collected only when they could be easily released after gentle abdominal pressure. The gametes were collected after abdominal massage performed from head to tail (stripping). To estimate the fertilization rates, males not hormonally induced were used. So, the males were stripped 15 min before the female was stripped to obtain the sperm and verify fertilization (Sanches et al., 2013).

The CPAs were prepared in maintenance solutions (to which the oocytes were also submitted immediately after collection): Hanks' (Ref. H9269, Sigma-Aldrich, São Paulo, SP, Brazil) or 90% Leibovitz (L15 - Ref. L1518, Sigma-Aldrich, São Paulo, SP, Brazil). Thirty to fifty oocytes were put into each well of the six-well culture plates. The excessive maintenance solution was removed, and 1.0 mL CPA solution was added. Thus, based on the results obtained by Lopes, Sanches, Caneppele, and Romagosa (2018) on oocyte toxicity in different cryoprotectants, four experiments were carried out: (1) [0.1 M, 0.2 M, 0.4 M] Sucrose + [1 M, 2 M] Methanol; (2) [1 M 2 M 4 M] Methanol or Propylene glycol (PG) or Dimethyl sulfoxide (DMSO); (3) [0.5 M] Fructose or Sucrose + [2 M] Methanol or PG or DMSO; and (4) [0.25 M, 0.5 M, 1 M] Fructose + [1 M, 2 M, 4 M] DMSO; using mature (1 and 2) or immature (3 and 4) oocytes (Fig. 1). The oocytes with diameter lower than 1.7 mm were considered immature for presenting characteristics such as peripheral nucleus and cortical or vitellogenic alveoli; whereas the mature oocytes (diameter higher than 1.8 mm)

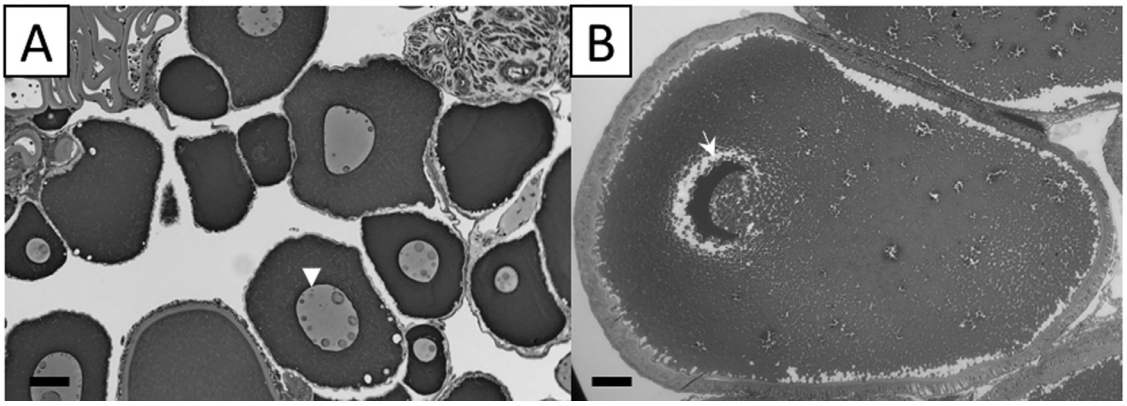

Fig. 2. Oocyte stages collected for experimentation. A) Immature oocytes (diameter < 1.7 mm); B) Mature oocytes (diameter > 1.8 mm).

presented peripheral or migrating nucleus, according to Fig. 2.

The chilling curve was controlled in 1°C/min using a digital thermometer: for these, we used three different thermal boxes contain water and ice to keep the temperature in 15, 5 and −5°C, respectively. The samples spent 10 min in each of these boxes / temperatures (decreasing the temperature exactly 1°C/min) until they were taken directly to the freezer.

All treatments were kept for 120 min at −5.9 ± 1.8°C, temperature range in which no formation of ice crystals occurs in the presence of the cryoprotectant solutions, thus enabling the evaluation of the sensitivity of the cells exclusively to cold, without being affected by injuries caused by ice. Afterwards, the oocytes were rinsed twice with maintenance solutions, and oocyte viability was assessed by membrane integrity using 0.4% Trypan Blue Staining (Gibco, Life Technologies, São Paulo, Brazil) for five minutes (Fig. 3A1-2).

Mature oocytes were also analyzed according to their fertilization rate (Fig. 3B1-3). For this, after bath in the cryoprotectant solutions the oocytes were washed twice with the respective maintenance solution (Hanks' or 90% L15), subsequently fresh semen was added (86.0% motility rate and 98.3 μm s^{-1} curvilinear velocity), and water was also added to start the hydration and fertilization processes at room temperature (24.1 ± 1.1°C). To certify that the eggs were fertilized, the embryo development was assessed until about 10 h after fertilization. At this moment blastoporous is closed, and only fertilized eggs are able to continue their development, being possible to visualize and count the number of live and unhatched (white) embryos. This methodology was approved by the Ethical Committee on Animal Experimentation from Fishery Institute of São Paulo (CEEAIP) under protocol number 05/2017.

The results were submitted to a one-factor analysis of variance (one-way ANOVA) at the 5% significance level. The combination of CPAs and concentration was considered a factor. Three replicates for each treatment, negative control (maintenance solutions: Hank or 90%-L15 at chilling temperature) and positive control (only water at room temperature) were used. In the case of a significant effect, t-test was applied at the same level of significance for the comparison of the means. The statistical analysis was performed by the software Statistica 9.0.

3. Results

We first submitted mature oocytes of *S. parahybae* to a variety of CPAs in two media and evaluated their viabilities at approximately −6 ºC. All CPAs in Hank's medium caused a 60–80% drop in viability compared to the positive control (Fig. 4A), which was the only condition to keep a high fertilization rate (94.8 ± 1.6%). When prepared in 90% L15 solution, the CPAs provided even lower viabilities, below 10% (Fig. 4B), and zero fertilization rate. Both experiments demonstrate how sensitive to cold temperatures the mature *S. parahybae* oocytes are, being more pronounced in 90% L15 medium.

Using immature oocytes, with diameter < 1.7 mm, we observed a higher susceptibility to cold in the absence of CPAs (Fig. 5, negative control). All CPAs, especially 0.5 M fructose + 2 M DMSO, provided increased viability for these oocytes, evidencing the need for cryoprotectants.

We next used varying concentrations of DMSO and fructose hoping to fine-tune cryoprotectant conditions for immature oocytes. The results shown in Fig. 6 indicate that lower concentrations of both the internal and external CPAs (1 M DMSO + 0.25 M Fructose) provided the best outcome, with 60% viability.

4. Discussion

Fish oocytes undergo a dynamic and complex process of oogenesis with continuous changes in their structures, besides the influence of numerous surrounding components responsible for oocyte development and maturation (Menn, Cerda, & Babin, 2007). Therefore, to know the relation of the cryoprotective effect with the chilling sensitivity in

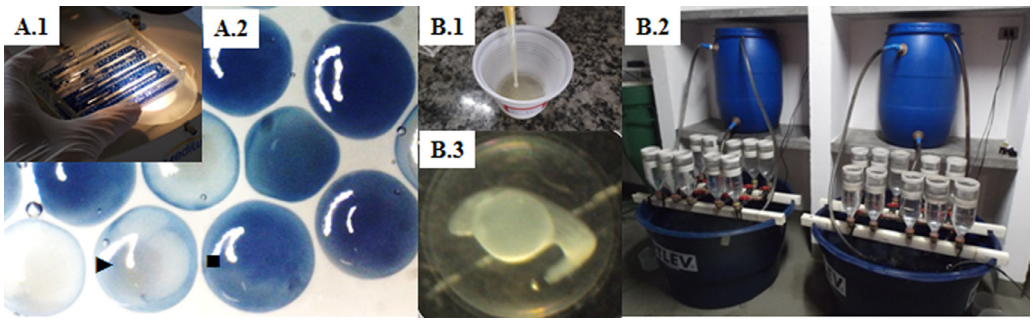

Fig. 3. Analysis of the effect of chilling by membrane integrity (Trypan Blue staining); and Fertilization Test. A1-2) Immature oocytes after Trypan Blue stain: ► uncolored oocyte (whole membranes); ■ oocyte stained (ruptured membranes). B1-2) Fertilization processes; B3) Embryo post-fertilization.

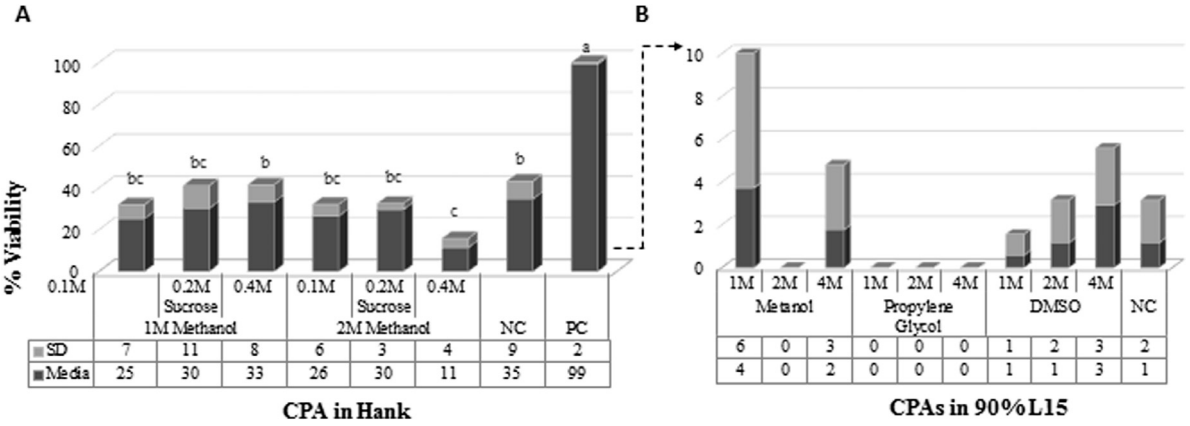

Fig. 4. Viability of mature oocytes (diameter > 1.8 mm) of surubim-do-Paraíba, *Steindachneridion parahybae* in different cryoprotectant solutions (CPAs). A) Experiment 1- solutions prepared in Hank´s medium; B) Experiment 2- solutions prepared in 90% Leibovtz 15 (L15) medium. NC- negative control; PC- Positive control; SD- Standard deviation.

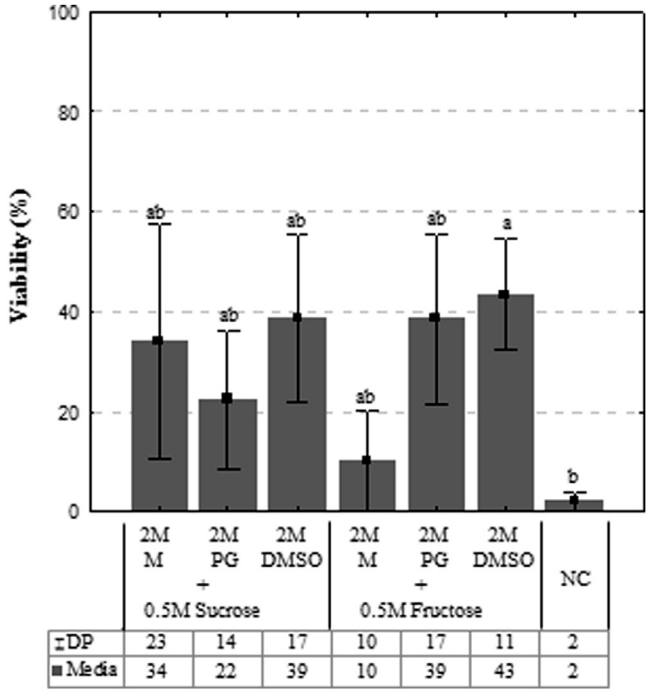

Fig. 5. Viability of immature oocytes (diameter < 1.7 mm) of surubim-do-Paraíba using different cryoprotectant solutions (CPAs): sucrose, fructose, methanol (M), propylene glycol (PG), and dimethyl sulfoxide (DMSO). NC- negative control.

distinct moments of the development can bring relevant information for the advancement of studies in cryopreservation of fish oocytes.

Lopes et al. (2018) studied the toxicological effects of the different cryoprotectants in Hank and 50% L15 medium and they found no difference between the media or cryoprotectants (sucrose, fructose, methanol, propylene glycol and DMSO). Thus, for these reasons we tested CPAs containing the substances mentioned in the previous experiments to better understand not only the toxicity of these cells to cryoprotectants, but which is the relation between the toxic effect with the chilling resistance provided by these substances. In this context, Pegg (2002) affirms that chilling causes concentration of the solution around the cells, and as a consequence the cells reduce the rhythm of ice formation when permeable substances (alcohols) are used. Furthermore, this behavior is dependent of the concentration of the cryoprotectants used. The present study used relatively low concentrations, which were sufficient to act as cryoprotectant at temperatures below zero (~ −15 °C).

According to the results found, mature oocytes cannot be a good option for the cryopreservation because they have high sensitivity to temperature below zero, even if in a low concentration of cryoprotectant solutions, mainly when in Leibovitz (L15) medium. However, studies with this stage of development are important for many reasons, such as easy manipulation, not requiring animals sacrifice, and no need for *in vitro* incubation, which is a problem to be solved for each species.

On the other hand, immature oocytes seem less susceptible to cold, and the most efficient medium was the one with Hanks'. All groups involving L15 medium had 0% Fertilization, including the Negative Control. It was not due to exposure to low temperatures, but simply due to exposure of oocytes to L15. This occurs because the oocytes were probably activated by L15 and lost their capacity to be fertilized long before the 120-min exposure time expired, which did not happen in Hank's medium. So, this stage of development could be better for cryopreservation studies, but for the fertilization *in vitro* maturation is necessary, although this technique has not been resolved yet. In this context, the first positive results were showed by Silva et al. (2018) using different hormones for vitelogenic oocytes (Stage III) maturation in zebrafish, *Danio rerio*, which is very important for future studies in this way.

Experiments with stage III oocytes of zebrafish were done by Isayeva et al. (2004), who showed that survival declined as temperature decreased (manly in zero and subzero temperatures) and as duration of exposure time increased, because the oocytes that survived fell by half in the 15 to 60 min of exposure. These authors justify that the sensitivity to chilling showed by fish oocytes were due to highly specialized cells, mainly because of the larger size than any other type of cell. Additionally, it undergoes structural damage. These reasons also explain the survival of *S. parahybae* immature oocytes (with better results than the mature ones) around no more than 40%, with exception to one treatment (60 ± 19% of viability to 0.25 M fructose + 1 M DMSO).

On the first studies on fish oocyte cryopreservation, Plachinta, Zhang, and Rawson (2004) and Isayeva, Zhang, and Rawson (2004) believe that early stages of oocyte development are more susceptible to chilling injury. In the same group of research (Tsai, Rawson, & Zhang, 2009a; Tsai, Rawson, & Zhang, 2009b) aimed to know the oocyte stage for chilling sensitivity and discovered that the early stages of zebrafish oocytes are less sensitive to chilling, as well as what we observed for *S. parahybae* oocytes. In general, stage III is the most used in studies (Guan et al., 2008; Zhang et al., 2008; Godoy et al., 2013; Guan, Rawson, & Zhang, 2010; Seki et al., 2011). It is known that specific mechanisms of chilling damage are stage dependent, but the specific reason for this, is not yet completely understood.

The CPAs offer thus a protection mechanism to prevent cell injuries during the chilling process, and their ability to permeate cell

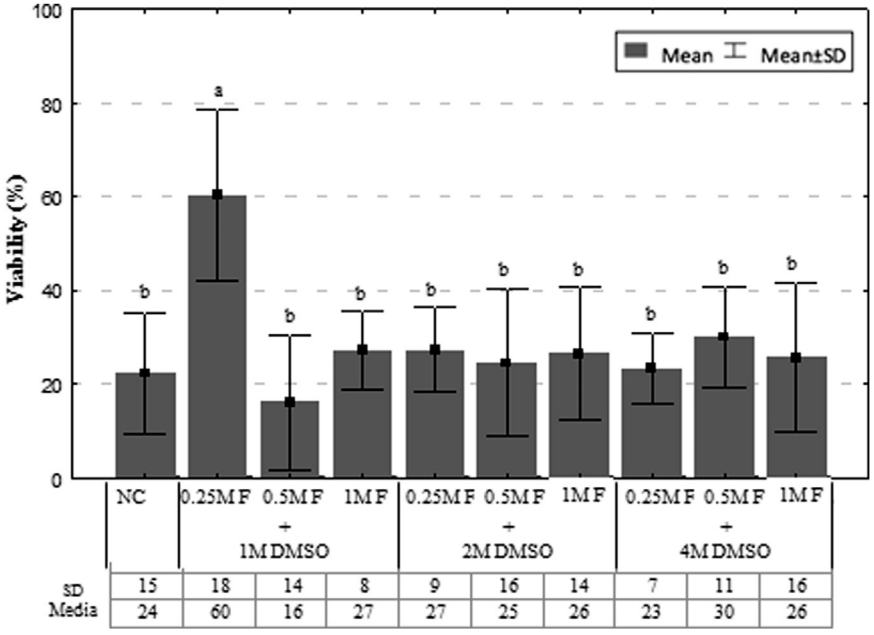

Fig. 6. Viability of immature oocytes (diameter < 1.7 mm) of surubim-do-Paraíba using different concentrations of Fructose (F) + Dymetil sulfoxide (DMSO). NC-negative control.

membranes has a fundamental role in the prevention of intracellular ice formation, while the non-permeable CPAs modulate the extracellular ice formation. However, the biggest problem of CPAs is their cytotoxic effect, which is more pronounced at high concentrations. One way to avoid this problem is the association of more than one substance with cryoprotectant properties in the composition of a CPA (Solocinski et al., 2017). In this context, the cryoprotectant properties found in the present work for cryoprotectant solutions containing DMSO (permeable) and fructose (non-permeable) are a strong evidence that their use, even associated to other substances, can increase resistance to chilling in immature oocytes.

We can conclude that oocyte immature stages have shown a lower chilling sensitivity than the mature ones. The CPA with fructose and DMSO in a low concentration appears to be the best CPA to protect surubim-do-Paraíba immature oocytes from chilling at −5.9 ^{0}C. Therefore, this information will contribute to progress in studies, so that the other steps of the freezing process can be carried out.

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