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Cryopreservation of Sperm in Brazilian Migratory Freshwater Fish

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1. Introduction

The Brazilian freshwater fish diversity is the richest in the world. The majority this fish species migrate during the spawning season (a phenomenon known as piracema). Urbanization, pollution, hydroelectric dams and deforestation are some of the causes of stock depletion or even local extinction of some of these species [5].

The disappearance of native fish species and the decrease of genetic stocks undertake the aquatic ecosystem and commercial food production. Cryopreservation of semen is a biotechnology that has the ability to minimize these problems, increasing flexibility and operationalization of the reproductive period and improving assisted reproduction programs in native fish [1,2]. This also enables the biotechnology application of new techniques such as transplantation of gametes to form chimeras [3].

May be cited as advantages of semen cryopreservation: (i) sincronization of the gametes release from both sexes, (ii) semen economy, (iii) easeness of the management with breeders, (iv) transport of gametes from different locations and (v) genebanks for genetic selection programs and species conservation [4] and decreased need for males breeders from nature.

However, the heterogeneity of semen responses after freezing has hampered the standardization of a protocol for the different species of fish [5]. Protocols have been tested for *Salminus*

maxilosus and *Salminus brasiliensis* [6,7], *Colossoma macropomum* [8], *Brycon orbignyanus* [6,9], *Brycon nattereri* [2], *Brycon amazonicus* [10], *Leporinus elongatus* [11], *Prochilodus lineatus* [12,13,14], obtaining different results.

2. Sperm characteristics essential for freezing

Due to the great variation in semen quality is extremely important to make a preliminary evaluation of fresh semen to be cryopreserved by determining a set of qualitative and quantitative variables. Traditionally, the quality parameters evaluated are sperm motility (percentage of motile cells, duration and speed), sperm concentration and sperm morphology [15].

In most teleost fish immobility sperm are present in the male genital tract and their motility is acquired from the moment that there is contact with water [7], being the difference in osmolarity between the semen and the solution responsible for activating.

The seminal plasma is the substance responsible for the suppression of testicular sperm motility, being a component of the isotonic semen [16]. The seminal fluid is rich in nutrients and ions, and for the activation, external environmental factors such as pH, osmolarity, temperature and ions can affect the quality and motility of sperm [17].

For the activation of the sperm and motility two fundamental changes must occur, the first is related to the fluidity of the plasma membrane [18], while the second is related to the activation of ion channels, increasing calcium intracellular generation of cyclic adenosine monophosphate (cAMP) [19].

The activation of ion channels and the passage of water, whether for water channels or aquaporins mediated the activation of second messengers, particularly the cAMP pathway that are essential for signal transduction. The activation of the cAMP pathway determines the activation of flagellar proteins by phosphorylation / dephosphorylation [20,21,22].

These phosphorylation and dephosphorylation activities are mainly related with a primer motion phosphoproteins of 15 kDa (kilodalton) and dynein light chain of 22 kDa that are associated with microtubules of the sperm tail [23,24].

Sperm motility is one of the most important factors when you want to analyze fish semen quality and evaluate the effect of biotechnologies such as cryopreservation [25]. According to [14] the minimum rate of sperm motility necessary for the semen processing for cryopreservation is 80%, therefore, should be considered some factors such as temperature, nutritional status and health, activating solutions employed, studied species and reproductive season in which the breeder is [26].

The sperm concentration is also an indicator that should be considered to evaluate the quality of semen. However, despite being traditionally used, sperm concentration is not as very sensitive and specific, since shows great variability among species. For example, see [27] observed a negative correlation between sperm concentration and sperm cryopreservation

potential of *Gadus morhua*, probably related to variations in the composition of the extender and seminal plasma among evaluated males.

Indirectly, the fertilization capacity can also be used as a parameter of sperm quality. However, this analysis may not be reliable since the quality of the oocytes is variable and may affect the success of fertilization. Another important aspect is that eggs for fertilization sometimes are not available, which can limit this procedure and sometimes the posterior development cannot occur [28]. Along with the fertilization rate, embryo survival is the parameter used to evaluate the ability of the fertilized egg to develop successfully and thus assess the quality of gametes [29].

The morphological study of the sperm cell and its relationship with semen quality is also extremely important and increasingly has become an integral part of the routine semen analysis. The morphology evaluation permits inferences about the potential fertilization and assists in the characterization of samples of semen cryopreservation [30].

3. Extenders used in freezing semen of fish

Cryopreservation involves the dilution of semen in a cryoprotectant solution, which must provide appropriate nutritional and osmolarity, avoiding damages of the semen [2] and activation of sperm. If the semen is activated, the energy sources of the cell will be scarce, thus shortening their activity [31]. Typically the extenders present in the composition carbohydrates or salts, isotonic stable and high thermal conductivity [32].

Some media have been tested as extenders, where we can cite from simple solutions such as distilled water [33] with 3% glucose [34] and 5% glucose [35] by diluting synthetic and complex commercially available as Betsville Thawing Solution (BTS-Minitub™) [12], M IIITM (Merck III) or Androstar™ (Minitub™) [34]. In addition, they should exhibit pH and osmolality similar to plasma and after freezing, must support the sperm while maintaining viability and the ability of these cells to fertilization [36].

The proportions of semen dilution may vary according to the species of fish, may influence the quality sperm after thawing. For some species such as *Piaractus mesopotamicus*, *Salminus brasiliensis*, *Leporinus obtusidens* and *Brycon orbignyanus* dilutions can be made in the ratio of 1:3 (semen / extender) [6]. For *Prochilodus lineatus*, 1:4 [12,14] and *Clarias gariepinus* 1:10 [37].

4. Cryoprotectant solutions and packages of semen doses

Cryoprotectants prevents formation of crystal ice within the spermatozooids, which are considered lethal, but in high concentrations can be toxic, even during the input or output of cells [38]. There are intern and extern cryoprotectants, the first reduce intracellular cryoscope point, reducing the formation of ice microcrystals and external cryoprotectants stabilize the outer membrane, preventing cell disruption [39].

The association between intracellular and extracellular cryoprotectant is indicated by [40] and [41] and have been tested for the native species rheophilic, of which we can mention the *Prochilodus lineatus* [12,13] and *Brycon orbignyanus* [9].

Among the most commonly used internal cryoprotectants can be cited glycerol [5], ethylene glycol [42], methanol [13,9,12] or dimethylsulfoxide DMSO [10,12] and methyl cellulose [43,44]. Among external cryoprotectants are used coconut water [13], egg yolk [45], glucose [46] and milk powder [6].

The semen storage can be accomplished in different types of straw which vary the volume conditioning (0.25 ml, 0.5 ml, 1.0 ml, 1.2 ml, 2.5 ml and 5 ml.) According [47] the fertilization rate using 1.2 mL straw was similar to 0.5 ml straw for salmonids by using low freeze temperature and high thawing temperature. The 5 mL straw resulted in successful fertilization of only 40% comparing to fresh semen control. Straws of 0.5 ml are the most commonly used among researchers in the freezing of fish semen, since higher caliber straws do not provide a uniform thawing, because the surface thawed more quickly than the central portion [40].

5. Freezing and thawing semen

The success of freezing semen with liquid nitrogen requires cooling rates between 10°C and 50°C min⁻¹ [48]. [49] froze semen of *P. lineatus* using temperatures that range from 17.9 to 52.9°C min⁻¹. The use of cylinders containing only liquid nitrogen vapor, such as those used by [40], provides cooling rates within this interval.

Most of cells support a rapid thaw, even if she does not fully hydrate. The speed after thawing is necessary to prevent recrystallization, which forms small ice crystals that regroup to form large crystals that are lethal to the cell [50]. The frozen semen straw must be removed from the liquid nitrogen and gently shaken in a water bath for a few seconds to thaw uniform [49].

6. Sperm activators

The use of appropriate activating solutions, which mimic the seminal plasma and do not compromise the quality of semen, can increase the duration and intensity of sperm motility, both contributing to higher survival and fertilizing capacity of the sperm, thus counteracting the deleterious effects of exposure to the aquatic environment or the activator sperm [51,52]. Moreover, it can help to improve semen cryopreservation.

The ionic composition of the activator, although important, has less influence on the sperm compared osmolality, since nonionic solutions are also capable of activating sperm motility. However, the addition of ions brings additive effects on motility parameters [53].

The sperm activation can be performed with different solutions, influencing the rate of semen motility. Can be include as solutions that can activate sperm motility distilled water, calcium bicarbonate (NaHCO₃) [54] and potassium chloride (KCl) [55], and these solutions may

promote increased time rate and motility in various tropical species [56,14]. According [57] the osmolarity and pH of the activating solution were the main factors that affected the rate and duration of sperm motility after semen activation of *P. lineatus*. For this same species [58], verify that besides the osmolarity the dilution ratio (semen: activator) can also influence the rate and duration of motility.

For *Brycon orbignyanus* water was considered a good activator after thawing, causing high rates of hatch compared to a solution of NaCl (50 mM) at a ratio of 1:5 [9]. In *P. lineatus* was observed that different solutions can influence the activating sperm motility and fertilization rate of cryopreserved semen, being considered the best activator solution was sodium bicarbonate (60 mM), providing higher motility duration rates than in the activated semen with distilled water. However, this solution significantly reduced the fertilization rate, suggesting a deleterious effect to spermatogenic cell [30].

7. Cryopreservation effect on semen quality

The success of cryopreservation may be affected by the high variability in semen quality to be used. This variability is mainly associated with intrinsic factors that determine cryoresistance of the sperm cell, which is nothing more than the ability of sperm to preserve their morpho-functional characteristics after cryopreservation. The cryoresistance is a function of the resistance of cell membrane and the ability to maintain their intracellular structures and basic functions [15].

In the cryopreservation process, the sperm cells are exposed to external conditions that are not physiologically appropriate and this ultimately affect many parameters of sperm activity, such as motility, morphology, and composition of seminal plasma antioxidant activity [59].

Among the causes of this deleterious effect, the exposure of sperm to the cryoprotectant medium can be highlight, which causes cell osmotic stress and consequently damage to sperm function. The formation of internal ice microcrystals during freezing is also deleterious to sperm which can cause dehydration of cells and osmoconcentration [60]. Metabolic and oxidative damage caused by ROS (reactive oxygen species) are also highly detrimental to sperm events [61].

Among the parameters affected by cryopreservation, sperm motility can be citing, which is significantly reduced when the cell is exposed to low temperatures. As an example, see [12] showed that cryopreservation of *P. lineatus* semen progressively decreased their motility. According [20], the reduction of the motility parameters can be associated with damage to the flagellum, reduced mitochondrial function and degradation of proteins.

Sperm morphology can also be affected by the cryopreservation process. Ultrastructural alterations in sperm may occur due to changes in the osmolarity of the medium surrounding them. These changes may be due to membrane disruption, reduced mitochondrial function, spiralization, breakage or adhesion of the axoneme, and functional abnormalities which are responsible for reduced motility and fertilization ability [62].

Species	Solution*	Dilution (semen: solution)	Motility (%)	Motility duration (s)	Abnormal sperm post-freezing (%)	Thawing rate (°C) - time (s)	Reference
<i>P. lineatus</i>	Propylene glycol 10% + BTS5%	1:4	64.3 ± 1.6	29.7 ± 21.3	27.71 ± 1.87	40 – 8	Andrade et al. (UD)
	Methanol 10%+ BTS 5%	1:4	74.6 ± 5.6	50.1 ± 6.9	30.57 ± 3.90	40 – 8	
	Glycerol 10%+ BTS 5%	1:4	71.8 ± 3.6	52.2 ± 5.4	15.28 ± 6.90	40 – 8	
	DMSO 10%+ BTS 5%	1:4	52.1 ± 10.2	127.3 ± 47.7	25.85 ± 0.56	40 – 8	
	Ethylene glycol 10%+ BTS 5%	1:4	70.3 ± 2.6	39.7 ± 14.2	25.85 ± 0.56	40 – 8	
	DMSO 8% + egg yolk 5% +BTS 5%	1:5	60.8 ± 24	58.2 ± 49	20.6 ± 6	60 - 8	Felizardo et al. [12]
	DMSO 8% + lactose 5% +BTS 5%	1:5	64.1 ± 26	70.8 ± 53	30.8 ± 10	60 – 8	
	Methanol 8% + egg yolk 5% +BTS 5%	1:5	67.8 ± 25	74.3 ± 59	21.4 ± 6	60 – 8	
	Methanol 8% + lactose 5% +BTS 5%	1:5	58.9 ± 26	75.3 ± 44	27.3 ± 6	60 – 8	
	Methanol 5% + BTS 5%	1:4	63	40	41.3	60 – 8	
	Methanol 7.5% + BTS 5%	1:4	43	43	30.4	60 – 8	Miliorini [30]
	Methanol 10% + BTS 5%	1:4	47	47	25.6	60 – 8	
	Methanol 12.5% + BTS 5%	1:4	53	53	30.2	60 – 8	
	DMSO 5% + BTS 5%	1:4	76	48	31.2	60 – 8	
	DMSO 7.5% + BTS 5%	1:4	78	54	27	60 – 8	
	DMSO 10% + BTS 5%	1:4	85	62	26.1	60 – 8	
	DMSO 12.5% + BTS 5%	1:4	69	83	24.3	60 – 8	
<i>B. orbignyanus</i>	Propylene glycol 10% + BTS 5%	1:4	21.4 ± 9.1	34.4 ± 16.6		60 - 5	Andrade et al. (UD)
	Methanol 10% + BTS 5%	1:4	0	0		60 – 5	
	Glycerol 10%+ BTS 5%	1:4	0	0		60 – 5	
	DMSO 10%+ BTS 5%	1:4	0	0		60 – 5	
	Ethylene glycol 10% + BTS 5%	1:4	13.6 ± 3.5	15.6 ± 3.3		60 – 5	
	DMSO 8% + egg yolk 5% +BTS 5% DMSO	1:5	28	63.2	22.8	60 - 8	Felizardo et al. [12]
	8% + lactose 5% +BTS 5%	1:5	28	69.6	18.6	60 – 8	
	Methanol 8% + egg yolk 5% +BTS 5%	1:5	22.5	50.5	26	60 – 8	
	Methanol 8% + lactose 5% +BTS 5%	1:5	30	66.2	23.4	60 - 8	
<i>P. mesopotamicus</i>	Propylene glycol 10%+ BTS 5%	1:4	67.1 ± 12.6	71 ± 5.8		60 - 8	Andrade et al. (UD)
	Methanol 10%+ BTS 5%	1:4	61.6 ± 8.8	91.4 ± 20.2		60 - 8	
	Glycerol 10%+ BTS 5%	1:4	22.5 ± 18.8	33.9 ± 20.4		60 - 8	
	DMSO 10%+ BTS 5%	1:4	52.1 ± 2.1	71.7 ± 6.3		60 - 8	
	Ethylene glycol 10% + BTS 5%	1:4	42.5 ± 4.7	46 ± 11.9		60 - 8	

*DMSO: dimethyl sulfoxide, BTS: Beltsville Thawing Solution.
UD=unpublished data

Table 1. Cryopreservation protocols and parameters found in various fish species.

The sperm abnormalities can be divided into two groups, the minor or larger ones. Minor abnormalities occur during spermatogenesis due to factors that affect breeding. The largest abnormalities are related to handling procedures during collection [30]. For fish, the acceptable limits of sperm abnormalities have not yet been established, however, during cryopreservation of *P. lineatus* semen abnormalities observed was 20-30%, which was associated with decreased motility rate post-thaw [12].

The exposure of sperm cells to cryoprotectant medium is responsible for causing sperm damage. In addition to osmotic shock, dilution in extenders alters the constitution of seminal plasma, diluting major components such as proteins and antioxidants enzymes. Therefore, when seminal plasma constituents present in suboptimal conditions, may impair the protective function of the spermatozoa and thus the potential for freezability [63].

Although these observed changes in semen quality after cryopreservation, this is a safe method to be used for the preservation of genetic resources, since, after the process of freezing and thawing sperm motility and fertilization capacity can be recovered. However, for the maintain of the sperm in low temperature is necessary to use a suitable cryopreservation protocol which depends on the quantity and quality of the extender, type and concentration of internal cryoprotectants and the sample volume and sperm cell characteristics [64].

8. Semen vitrification

Vitrification is a process of ultra-fast freezing characterized by the use of high concentrations of cryoprotectant (40-60%) and high cooling rates (up to 1000 °C min⁻¹) by immersion of semen directly into liquid nitrogen. This method inhibits the formation of hexagonal ice crystals and induces a glassy state, which differs from traditional methods in which the permeability of cryoprotectants and dehydration occur before starting the freezing [65].

A number of advantages of ultra-fast freezing can be listed, among them, does not require expensive equipment, is a quick and simple technique allows preservation of samples in the field. Despite these advantages, the addition of cryoprotectants in high concentrations can be toxic and cause osmotic damage sperm cell, being necessary find alternatives that overlap this limitation.

The possibility to obtaining a glassy state result in improved survival of sperm is the main starting point for using the vitrification technique [66]. Although it represents a viable alternative to semen preservation of fish native species of Brazil, there are no reports on the use of this technique to date.

In conclusion we believe that there are protocols defined for the different stages of cryopreservation in different species of native fish in Brazil. Moreover, vitrification semen offers a new option for conservation of native species. However, it is still necessary to improve the studies in this area, to find favorable results.

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