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Fish sperm subpopulations: Changes after cryopreservation process and relationship with fertilization success in tambaqui (*Colossoma macropomum*)

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ABSTRACT

Fish tambaqui (*Colossoma macropomum*) is the native Brazilian fish with the highest agricultural production under intensive aquaculture in South America. However, the decrease in the genetic variability in fish farms has become necessary the improvement of cryopreservation process through new statistical studies of spermatozoa (like subpopulation studies). The evaluation of the kinetic data obtained with a computer-assisted sperm analysis system, applying a two-step cluster analysis, yielded in tambaqui three different subpopulations in fresh sperm: SP1, considered as a slow nonlinear subpopulation; SP2, considered as a fast nonlinear subpopulation, and finally; SP3, considered as a fast linear subpopulation. For cryopreserved sperm, the cluster analysis yielded only two sperm subpopulations: SP1′, considered as a slow nonlinear subpopulation and SP2′, which seemed to be an intermediate subpopulation (showing medium motility and velocity values) merged from SP2 and SP3 obtained from fresh sperm. Coefficients of correlation (r) and determination (r^2) between the sperm subpopulations from fresh sperm and the fertilization rates were calculated, and SP2 and SP3 (the fast-spermatozoa subpopulations) showed a high-positive correlation with the fertilization rates ($r = 0.93$ and 0.79, respectively). In addition, the positive significant correlations found in curvilinear velocity ($r = 0.78$), straight line velocity ($r = 0.57$), and average velocity ($r = 0.75$) indicate that sperm kinetic features seem to be a key factor in the fertilization process in tambaqui, as occur in other fish species.

1. Introduction

The presence of various sperm types within a species is a widespread phenomenon both in invertebrates and vertebrates, implying that spermatozoa with different physiological and/or morphological characteristics coexist in the same ejaculate [1]. These spermatozoa can be grouped by clusters based on different biological features and classified in different sperm subpopulations. Although this topic was initially reported in many species of mammals [2–4], recently it has received considerable attention in fish studies [5–7].

The first step for the sperm subpopulations study is the choice of a classification criterion (head or flagella morphology, swimming pattern, and so forth), and it is reasonable to assume that criterion chosen should be related with the sperm quality. In this respect, the kinetic features of spermatozoa represent a suitable approach for

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making subpopulations, and the gradual appearance of computer-assisted sperm analysis (CASA) systems represents a useful tool for working on this topic. Nevertheless, although most of these CASA's parameters have been independently correlated with the fertilization ability in some species [8,9], there are scarce reports linking sperm subpopulations and the fertilization success both in mammals and fish.

Analysis of sperm subpopulations has been successfully applied in several scientific matters, among them we can emphasize the assessment and improvement of cryopreservation protocols [5]. In this respect, sperm cryopreservation has led to transcendental changes in the reproductive biotechnology in fish, and this technique has provided important advantages such as (i) the simplification of broodstock management, (ii) the synchronization of gamete availability of both sexes, (iii) the transport of gametes from different fish farms, and (iv) the germplasm storage for conservation of species [10]. However, cryopreservation protocols also generate damage to cell structure and physiology, altering sperm motion patterns of spermatozoa [11]. In this respect, the task of assessing the influence of the freezing process in the ability of spermatozoa to fertilize the oocyte becomes essential for improving the cryopreservation field.

The choice of working with the Amazonian fish tambaqui (*Colossoma macropomum*) is related to the great economic and ecological importance of this species in South America, being the native Brazilian fish with the highest agricultural production under intensive aquaculture [12]. However, a decrease in the genetic variability of broodstocks used in fish farms has been recently observed, triggering deterioration in the genetic traits of the produced fingerlings. Therefore, both the analysis of sperm subpopulations based on motility characteristics and the improvement of cryopreservation process could minimize these obstacles, helping to assess the status of the sperm sample and its fertility potential.

In summary, the main goals of this study were (i) to identify and characterize sperm subpopulations through kinetic parameters in fresh sperm of tambaqui, assessing the changes caused by the cryopreservation process, and (ii) evaluate the correlation of these subpopulations with the fertilization rates, trying to find sperm quality biomarkers (subpopulations) for its application in the aquaculture sector.

2. Materials and methods

2.1. Fish handling and gamete collection

All the trials were carried out in accordance with the animal guidelines of the Principles of Laboratory Animal Science and performed at the Santa Clara Aquaculture Farm (Propriá, Sergipe, Brazil) and the Animal Reproduction and Biotechnology Laboratory of Embrapa Coastal Tablelands (Aracaju, Sergipe, Brazil).

Sexually mature males and females of tambaqui were transferred from earthen pond to 5-m^3 running freshwater tanks at 27 °C to 29 °C. Males were induced to maturation by a single dose of carp pituitary extract (CPE) of 2.0 mg kg^{-1} and females by an initial dose of 0.5 and a final dose, 12 hours later, of 5 mg kg^{-1} of CPE. Approximately 10 hours after the last CPE injection, the genital area of both males and females was cleaned and thoroughly dried to avoid the contamination (feces, urine, or freshwater), and gentle abdomen pressure was applied to obtain the gametes. Sperm from each male was collected into a glass test tube and maintained at 4 °C until motility analysis. Oocytes were collected just before the fertilization assay.

The number of fish used for each trial was different: (i) for the characterization of fish sperm subpopulation of fresh and cryopreserved sperm, 61 males were used; for the characterization of sperm kinetics parameters of fresh and cryopreserved sperm, eight males were used; and finally, for the fertilization trail, 15 males and one female were used.

2.2. Assessment of sperm motility parameters

Sperm samples were evaluated mixing an aliquot of 2 or 20 μL of fresh or cryopreserved sperm, respectively, with 500 μL of $NaHCO_3$ (230 mOsm, pH adjusted to 8.2). A 3-μL drop of activated sperm was placed and observed on the microscope (Nikon H550S, ECLIPSE 50i, Japan) using a Makler chamber (10-μm deep; Sefi Medical Instruments, Haifa, Israel). Video sequences were recorded at 100 fps using a high-sensitivity video camera (Basler Vision Technologies A-602fc-2, Germany). Ten 1-second videos were automatically captured every 3 seconds for each sample starting at 10 seconds after sperm activation. All the motility analyses were performed in triplicate using the motility module of Sperm Class Analyzer (SCA, Microptics S.L., Barcelona, Spain).

The parameters assessed in this study were total motility (TM, %), defined as the percentage of motile cells; progressive motility (PM, %), defined as the percentage of spermatozoa which swim in an essentially straight line; curvilinear velocity (VCL, μm/s), defined as time-averaged velocity of a sperm head along its actual curvilinear path, as perceived in two dimensions in the microscope; straight line velocity (VSL, μm/s), defined as the time-averaged velocity of a sperm head along the straight line between its first detected position and its last; average path velocity (VAP, μm/s), defined as time-averaged velocity of a sperm head along its average path; straightness (STR, %), defined as the linearity of the average path (VSL/VAP); linearity (LIN, %), defined as the linearity of a curvilinear path (VSL/VCL); wobble (%), defined as a measure of oscillation of the actual path about the average path (VAP/VCL); the amplitude of lateral head displacement (μm), defined as magnitude of lateral displacement of a sperm head about its average path. It can be expressed as a maximum or an average of such displacements; and beat cross frequency (BCF, beats/s), defined average rate at which the curvilinear path crosses the average path. Spermatozoa were considered immotile if their VCL was lower than 20 μm/s.

2.3. Sperm cryopreservation

Sperm samples were cryopreserved according to the protocol proposed by Carneiro et al. [13]. Sperm was