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ScienceDirect
Theriogenology 72 (2009) 1251–1261
Theriogenology
www.theriojournal.com

Sperm quality evaluation in *Solea senegalensis* during the reproductive season at cellular level

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Received 11 January 2009; received in revised form 13 July 2009; accepted 29 July 2009

Abstract

Sperm quality seems to be one of the reasons for the reproduction constraints faced by Senegalese sole (*Solea senegalensis*) aquaculturists. Previous studies in this species indicated that the sperm quality of individuals kept in culture varies throughout the year and that different sperm subpopulations can be identified in ejaculates according to the motility pattern of spermatozoa. Aiming to better understand factors affecting sole sperm quality in captivity, sperm of 11 males was assessed during the reproductive season using different parameters: motility characteristics using CASA analysis; cell plasma membrane resistance to seawater hyperosmolarity; DNA fragmentation with single-cell gel electrophoresis; and early apoptosis, labeled with Annexin-V FITC. Computer-assisted sperm analyses motility data were treated using multivariate analysis to identify the presence of different spermatozoa subpopulations according to their motility pattern. Four distinct sperm subpopulations were obtained: Subpop1, which includes fast linear spermatozoa; Subpop2, made up of fast nonlinear spermatozoa; Subpop3, which includes slow linear spermatozoa; and Subpop4, which contains slow nonlinear spermatozoa. The sperm subpopulation structure varied with time after activation and with male. Low cell resistance to the seawater hyperosmotic conditions was noticed. The Annexin-V assay allowed the identification of an apoptotic population ranging from 6% to 20%. A high percentage of cells (64.1%) showed a DNA fragmentation level below 30%, but these values varied significantly between males. DNA fragmentation appears to be related to cell membrane resistance to hyperosmotic conditions faced by the cells when in contact with seawater. This condition seems to modulate the composition of the motile sperm population and performance after activation. This phenomenon could be related to the spermatozoa maturation process.

Keywords: Annexin-V; DNA fragmentation; Hoechst 33258; Senegalese sole; Sperm subpopulations

1. Introduction

The massive production of the Senegalese sole (*Solea senegalensis*) is mainly impaired by the nonexistence of control over the reproduction, by poor egg quality, and by low fertilization rates [1]. Moreover spawns are obtained naturally in the tank, and few experiments were able to collect eggs by stripping and performing artificial fertilization, controlling the quality of oocytes and sperm [2]. Recent studies have shown that low-quality spawns (low rate of fertilization) in Senegalese sole broodstocks might be related to poor and highly variable semen quality [1,3,4]. Limited information on sole gamete quality leads to difficulties in controlling the reproductive cycle of this species. Similar to its relative *Solea solea*, *S. senegalensis* has a

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0093-691X/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2009.07.021

very low gonadosomatic index when compared with that of other fish species, with very small variations during spermatogenesis [5]. They present asynchronous germ cell maturation and low sperm concentration, volume, and production throughout the year. All these facts might be related to reproductive behavior, as the males only release sperm when in contact with the eggs and the female [1].

Sperm quality monitoring is an important step in the selection of male breeders and might provide some clues to sperm problems. Any quantifiable parameter that directly correlates with the fertilization capacity of sperm could potentially be used as a measure of sperm quality. Rurangwa et al. [6] and Cabrita et al. [7] have reviewed in fish the different parameters that may be related to sperm fertilization ability such as motility, spermatocrit, sperm density, osmolarity, pH and seminal plasma constituents, spermatozoa morphology and ultrastructure, and sperm viability and membrane integrity. In a previous work, Cabrita et al. [1] measured some of these parameters in sole throughout the year and characterized sperm quality and production in this species. Moreover, Martinez-Pastor et al. [3] examined sperm motility in Senegalese sole using computer-assisted sperm analyses (CASA) and noticed the existence of different sperm subpopulations within the same sample. Computer-assisted sperm analysis allows the quantification of different motility-associated parameters in a simple and rapid way and could allow sperm fertilizing ability to be predicted [6–8]. In recent years, several authors have used these programs to assess fish sperm quality in different species [9–11].

Beside sperm motility, membrane integrity and its resilience to the external conditions has been used to assay fish sperm quality. In a study of sole sperm motility, Martinez-Pastor et al. [3] found a low cell resistance of sperm to seawater in some cell populations after motility activation. This fact could be related to seawater hyperosmolarity relatively to the seminal plasma, indicating the importance of designing a test to evaluate sperm cell membrane resistance to this type of damage. Cells presenting a certain degree of damage might not be able to achieve fertilization due to the effects of osmotic stress.

Cell apoptosis is a necessary process during spermatogenesis; however, the presence of apoptotic cells in the ejaculate could indicate dysregulation of the normal sperm maturation process. In human spermatozoa, cell apoptosis is negatively correlated with sperm motility [12]. This parameter can be measured using Annexin-V, a calcium-dependent binding protein that has high affinity to phosphatidylserine (PS) in the presence of Ca^{2+}. This phospholipid is translocated to the outer leaflet of the cell plasma membrane during early apoptosis [13].

DNA stability is an index of sperm quality [7]. Although DNA fragmentation is part of the apoptotic process, it can also be promoted by other factors, leading to apoptosis by itself. DNA integrity can be analyzed using a single-cell gel electrophoresis, commonly known as comet assay, permitting the identification of DNA strand breaks and alkali labile sites by measuring the migration of DNA from the immobilized nucleus [14]. Both techniques proved to be useful in the analysis of sperm quality in *Solea senegalensis* [4] and could help in better understanding which factors affect sole sperm quality.

Only a more complete understanding of the semen features responsible for its low quality observed during the reproductive season can help us to find better solutions. Hence, bearing in mind the knowledge obtained to date in previous studies, and using the above-mentioned assays, we decided to study Senegalese sole semen quality during the reproductive season and to analyze how these parameters are correlated.

2. Materials and methods

2.1. Chemicals

All the chemicals were obtained from Sigma-Aldrich (Madrid, Spain), unless otherwise indicated. Chemicals were reagent grade or higher.

2.2. Broodstock husbandry conditions and sampling

The individuals used in the experiments were captured in the wild and maintained for 2 yr at the Ramalhete Experimental Station (37°00′22″N, 7°58′03″W), Faro, Portugal. Fish (1:2, female:male) were kept in 3000 L, round fiberglass tanks with sand substrate and aeration. Photoperiod simulates the environmental conditions in the area. Temperature (10.9 to 26.4 °C) and salinity (36.6 ± 0.8 ppt) were natural for the season. Water exchange was 500 L/h.

Sperm samples (n = 11) were obtained from fluent males during the reproductive season, from March to May. Fishes were anesthetized in a seawater tank with 300 ppm 2-phenoxyethanol. To obtain the semen, the urogenital pore was cleaned from mucus, feces, and water, and a syringe without needle was used to collect the semen by gently pressing the testes in the fish blind side. The sperm was placed in microcentrifuge tubes