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Improvement of the cryopreservation protocols for the dusky grouper, *Epinephelus marginatus*

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ABSTRACT

The dusky grouper, *Epinephelus marginatus*, is a potential species for aquaculture production although the limited number of males kept in captivity has been the cause of some constraints in its production. Sperm cryopreservation emerged as a solution for this problem. However, spermatozoa can be severely affected by freezing/thawing processes and poor sperm quality is a limiting factor in reproduction success. The present study aimed at evaluating two main aspects in the design of a cryopreservation protocol-extender additives (taurine, glucose, cholesterol, BSA) and sperm containers (0.5 mL straws, 2 mL cryovials and 5 mL macrotubes). Sperm quality was assessed through the evaluation of the percentage of motile cells, viable cells, DNA fragmentation, lipid peroxidation and apoptosis. Some specific techniques, such as Caspase 3/7 detection, which provides information on membrane integrity and cell death, detecting early and late apoptotic and necrotic events, were required to establish an optimized cryopreservation protocol for this species. Taurine was the most suitable cryopreservation additive in terms of viable cells and cholesterol presented the highest percentage of necrotic cells in this study. Caspase 3/7 assay enabled us to detect necrotic damage induced by cryopreservation.

Statement of relevance: The development of reproductive tools in dusky grouper, a potential species for aquaculture production, emerges as important tool to decrease the number of wild males maintained in captivity. A cryopreservation protocol was previously described for this species although several constraints in terms of sperm quality were detected. Our work provided new evidences that cryopreservation protocols can be improved through the addition of certain additives and use of appropriate sperm containers. Specific sperm analysis was crucial to identify and establish the most suitable conditions for breeders management and species conservation purposes.

1. Introduction

Epinephelus marginatus (Lowe 1834) (Pisces: Serranidae, subfamily Epinephelinae), commonly known as the dusky grouper, is an important and valuable demersal species in coastal fisheries (Schunter et al., 2011). In recent decades, grouper aquaculture has developed rapidly and many grouper species have been widely cultivated in China and South-East Asian countries (Hong and Zhang, 2003; Pierre et al., 2008). However, the sustainable development of the grouper industry is being threatened by the degradation of germplasm resources and low availability of fry hatcheries. In the Mediterranean and on the Southeast Asian coast, the dusky grouper (*Epinephelus marginatus*) and red-spotted grouper (*Epinephelus akaara*) have been classified as two endangered species (IUCN Red List, 2016). This is mainly due its reproductive strategy and to overfishing. The dusky grouper is a protogynous monandric hermaphrodite, meaning that individuals first mature as females and only much later (10–17 years of age) change into males, resulting in a sex ratio of 1 male: 5 females in the wild and making capture and maintenance of captive males difficult due to their large size and older age (Cabrita et al., 2009). These reproductive characteristics are the main constraints for the reproduction of the species in captivity because of low availability of males.

Cryopreservation of gametes, especially sperm, emerged as a viable solution to overcome some of the obstacles found in grouper reproduction, being useful for a wide range of studies such as breeding programmes, fertilization techniques and conservation strategies (Cabrita et al., 2014; Robles et al., 2016). The success of this process depends on several factors, such as cryoprotectants, which prevent intracellular ice crystal formation and excessive dehydration. DMSO was previously established as the most suitable sperm cryoprotectant in different grouper species including *Epinephelus marginatus*, *E. akaara* and *E. lanceolatus* (Cabrita et al., 2009; He et al., 2011; Liu et al., 2016; Tian et al., 2015). However, in previous studies performed by our group,

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cell viability was severely affected by cryopreservation (Cabrita et al., 2009) thus underlining the need to design strategies to reduce this damage.

Oxidative stress can be avoided by adding several compounds with specific properties to cryopreservation solutions. Membrane stabilizers, such as egg yolk or bovine serum albumin (BSA) are also known to help stabilize cell membrane components (Cabrita et al., 2009; Fan et al., 2014). Considering that reactive oxygen species (ROS) attack is one of the causes of cryodamage (Ball, 2008; Pérez-Cerezales et al., 2009; Wang et al., 1997), antioxidants are usually used to counteract this effect (Cabrita et al., 2011; Martínez-Páramo et al., 2012a, 2012b). Direct supplementation of the extender media with amino acids (taurine-antioxidant), lipids (cholesterol) and sugars (glucose, trehalose) has been tested also to improve cell resistance to the freezing/thawing process (Cabrita et al., 2011; Horváth et al., 2003). Studies in mammalian and fish sperm demonstrated the beneficial effects of introducing antioxidants as additives in the extender media of several species (Bucak et al., 2007; Osipova et al., 2014; Thuwanut et al., 2008). However, a different pattern of protection was observed in different species and should be studied in each particular case.

Although sperm cryopreservation is a powerful technique benefiting the aquaculture industry, during this process spermatozoa are exposed to multiple stress, causing cell damage due to ice crystal formation, osmotic stress, cryoprotectant toxicity and others. Traditional techniques such as viability and motility have been employed to evaluate post-thaw sperm quality in fish, including the dusky grouper (Cabrita et al., 2009). As in other species, many efforts have recently been made in the study of DNA damage using different approaches such as the comet assay (single cell gel electrophoresis), TUNEL (terminal deoxynucleotidyl transferase-nick-end-labelling) or SCSA (sperm chromatin structure assay) (Cabrita et al., 2010). Previous studies in the dusky grouper highlight the possibility of DNA damage and apoptotic events as a cause of embryo abortion after fertilization, leading to a decrease in hatching rates (Cabrita et al., 2009). However, none of these techniques have been applied in dusky grouper sperm until now and this information may be useful in the design and improvement of cryopreservation protocols. Although specific parameters such as DNA fragmentation and lipid peroxidation provide considerable information on cell status, other novel techniques have been proven to be useful in checking sperm quality after cryopreservation. The detection of apoptotic spermatozoa is particularly relevant in the prevention of failed embryonic development (Grunewald et al., 2005; Said et al., 2007; Weng et al., 2002). The importance of detecting apoptotic activity in fish sperm was revealed in a recent study in Senegalese sole, where authors implemented a sperm selection method for the recovery of a non-apoptotic sperm subpopulation (Valcarce et al., 2016). This study demonstrated that caspase detection in the identification of apoptotic cells in seminal samples was more specific than other fluorescent dyes such as YO-PRO-1 (Valcarce et al., 2016). In this respect, novel assays based on Caspase 3 and 7 activity detection has been described. These assays detect Caspase-3/7 activity and a cell death dye, 7-aminoactinomycin D (7-ADD) provide information on membrane integrity and cell death, detecting early and late apoptotic events (Gray et al., 2010).

This work aimed to test the effect of four different extender additives (BSA, taurine, glucose and cholesterol) in order to improve post-thaw sperm quality and three different cryopreservation containers (straws, cryovials and macrotubes) in order to select the best container for cryopreservation. An exhaustive post-thaw sperm analysis in *Epinephelus marginatus* was used for the first time.

2. Material and methods

2.1. Broodstock management and sperm collection

Dusky grouper, *E. marginatus*, broodstock was acclimatized and kept for 5 years at the facilities of IPMA (Olhão, Portugal). Both males (7.9–9.3 kg BW) and females (3.9–6.1 kg BW) were maintained indoors in three 10 m^3 tanks with densities between 4 and 5 kg/m^3. Aeration and water exchange at 15–25%/h were maintained in the tanks. The photoperiod was natural for the season and the number of hours of light was adapted simulating environmental conditions in the area. Temperature was controlled from March to September and kept between 21 and 23 °C. Out of this period, the temperature was that expected for the season (winter months: 12° to 16.5 °C). All the individuals were tagged with PIT-Tags (Trovan, NL) to identify each one. The broodstock was fed daily *ad libitum* with squid, sardines and dry pellets.

Sperm samples were collected in two consecutive years between the 1st of June and the 1st of July from males induced hormonally with 25 μg/kg GnRHa implants.

The experiments were carried out in compliance with the Guidelines of the European Union Council (2010/63/EU).

2.2. Sperm processing and experimental design

Forty-one cryopreserved samples were processed from twelve individual sperm samples from different *Epinephelus marginatus* males ($n > 3$). They were stored in a LN_2 container and used in two different experiments to test two different factors:

In Experiment 1 (Exp. 1), the following containers were tested: 2 mL-cryovials, 0.5 mL-straws and 5 mL-macrotubes. The cryoprotectant solution was 1% NaCl +10% DMSO + 1% BSA (Cabrita et al., 2009).

In Experiment 2 (Exp. 2), the best container selected for *Epinephelus marginatus* sperm established in the first experiment was used and different additives in cryoprotectant solutions were tested according to the best concentrations previously described for each additive: 1% NaCl + 10% DMSO (control), 1% NaCl + 10% DMSO + 50 mM Taurine (taurine) (Bucak et al., 2007; Martins-Bessa et al., 2009), 1% NaCl + 10% DMSO + 1% BSA (BSA) (Cabrita et al., 2009), 1% NaCl + 10% DMSO + 300 mM glucose (glucose) (Figueroa et al., 2015; Tian et al., 2013) and 1% NaCl + 10% DMSO + 1.5 mg/mL cholesterol (cholesterol) (Amidi et al., 2010).

Taking into account that the main objective of this work is to check the effect of the additive on cryopreserved sperm, this work does not report data for fresh sperm and we included a cryopreserved control without additives to demonstrate this effect.

Shortly after sperm collection, each sperm sample was separated in 3 aliquots for Exp. 1 and in 5 aliquots for Exp. 2. In Exp. 1 sperm was diluted 1:9 (sperm: extender) in 1% NaCl + 10% DMSO + 1% BSA..In Exp. 2, the same dilution rate was used, and several additives were included, as mentioned before. The straws, macrotubes and cryovials were placed in a horizontal rack 3 cm above liquid nitrogen (N_2) in a covered Styrofoam box. The sperm were frozen in liquid nitrogen vapors for 10 min. The straws, macrotubes and cryovials were then immersed in liquid nitrogen and stored in a N_2 container (Cabrita et al., 2009). Thawing was performed immediately prior to analysis. A water bath set at 25 °C for 30 s was used for the straws. For the cryovials and macrotubes, the thawing temperature was set at 40 °C for 150 s and 40 °C for 50 s, respectively.

2.3. Cholesterol-loaded cyclodextrin and incorporation on sperm

Methyl-β-cyclodextrin (MβCD) was preloaded with cholesterol to deliver this compound into the sperm plasma membrane. Two solutions were prepared separately: a cholesterol solution was prepared from a stock solution (100 mM cholesterol) diluted 1:1 in chloroform, and a MβCD solution was prepared from a stock solution (500 mM) diluted 1:9 in chloroform. Both solutions (50 mM) were mixed 1:1 and vortexed vigorously before evaporation at 60 °C for 30 min. Before being used, the resulting powder was diluted in 1 mL of 1% NaCl (stock concentration: 9.5 mg/mL). For sperm incubation with MβCD-cholesterol, 1 volume of sperm (160 μL) was diluted in 1 volume of MβCD-cholesterol (160 μL) and then diluted 3× in 1% NaCl (final