Aquaculture 498 (2019) 223–229

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Comparative study on cellular and molecular responses in oyster sperm revealed different susceptibilities to cryopreservation

Marta F. Riesco[a], Francisca Félix[a], Domitilia Matias[b], Sandra Joaquim[b], Marc Suquet[c], Elsa Cabrita[a,*]

[a] CCMAR, University of Algarve, Campus of Gambelas, 8005-139 Faro, Portugal
[b] IPMA, IP, Av.5 de Outubro s/n, 8700-305 Olhão, Portugal
[c] IFREMER, UMR 6539 LEMAR, Departement PFOM/Laboratoire ARN Station Expérimentale d'Argenton, Presqu'île du Vivier, 29840 Argenton, (France)

ARTICLE INFO

Keywords:
Crassostrea angulata
Crassostrea gigas
Sperm cryopreservation
Standardization
Sperm post-thaw analysis

ABSTRACT

The Portuguese (*Crassostrea angulata*) and Pacific oyster (*Crassostrea gigas*), both from the genus *Crassostrea*, are two important species for production and conservation. Although they have common characteristics, different susceptibilities to rearing conditions have been described in these species. Overall, in the case of *C. angulata*, only a few remaining populations are present in the south of Portugal and Spain. The preservation of genetic material from improved stocks or from the original population is crucial in oysters to prevent the potential impacts of epidemic diseases and natural disasters. Sperm cryopreservation in oysters has progressed in recent years. However many issues, such as protocol standardization, are still unsolved for the application of research results. In the present study a sperm cryopreservation protocol, previously published in *C. angulata* by our group, were analyzed in terms of cellular and molecular damage, in an effort to determine the most sensible parameters to standardize the cryopreservation protocols for both species. Different approaches in the analysis of sperm quality were performed for the first time in this genus to detect different susceptibilities between the two valuable species. Our results revealed that our previously published protocol containing 10% DMSO as cryoprotectant is more suitable comparing to 10% EG in both species. In addition, an integrative analysis was performed in both oyster species comparing all cellular parameters and *C.gigas* showed a higher susceptibility to cryopreservation using this optimized protocol. Moreover, higher susceptibility to transcript degradation was detected in *C. gigas* using this optimized cryopreservation protocol. This study highlights the importance of using different techniques and exhaustive analyses for selecting the most suitable cryopreservation protocol and its standardization, thus ensuring the total safety of the technique.

1. Introduction

The establishment of genebanks for the conservation of natural *Crassostrea angulata* populations and improved protocols for *Crassostrea gigas* is an important tool in population management, restocking programs and in the preservation of genetic material from improved stocks. It implies the use of standardized cryopreservation techniques and the control assessment of gamete quality. Sperm cryopreservation in oysters has progressed in the last decade by developing protocols for a number of species. Special efforts have been made in the case of the Pacific oyster (*C. gigas*) (Dong et al., 2007, 2005; Erickson et al., 2014; Ieropoli et al., 2004) and more recently in the Portuguese oyster, *C. angulata* (Riesco et al., 2017). Although protocols have been developed for these closely related species, different susceptibilities have been detected concerning sperm cryopreservation depending on oyster species or genetic material composition (Dong et al., 2011). Protocols of sperm cryopreservation can vary because of species-specific differences in sperm size, shape, and biochemical characteristics and therefore different susceptibilities can be observed (Dong et al., 2011). Specifically in oysters, there has been a general lack of standardization among sperm cryopreservation studies, rendering some difficulties in the reproducibility of results (Hassan et al., 2015). Pacific and Portuguese oysters, both from the *Crassostrea* genus, have been described as two different species due to significant genetic and phenotypic differences (Leitão et al., 2007; Soletchnik et al., 2002).

Moreover many issues remain unsolved with regard to the application of research results to conservation and production programs. The variation in fresh sperm quality compared to cryopreserved leads to

* Corresponding author.
E-mail address: ecabrita@ualg.pt (E. Cabrita).

https://doi.org/10.1016/j.aquaculture.2018.08.049
Received 23 April 2018; Received in revised form 15 July 2018; Accepted 21 August 2018
Available online 23 August 2018

inconsistencies in the outcome of cryopreservation studies. Therefore, future research is required to explain the mechanisms controlling post-thaw sperm quality in oysters. As in other species, the freezing/thawing process is known to damage spermatozoa at several levels (Cabrita et al., 2014). This damage can ultimately affect cell viability and fertilization capacity. The development of techniques for the detection and characterization of these effects could provide significant improvements in the safety and efficiency of storage procedures (Cabrita et al., 2010; Martínez-Páramo et al., 2012; Robles et al., 2016). Moreover, the application of new specific methods of sperm quality evaluation should contribute to standardizing, as much as possible, procedures within the same genus, identifying the main damage mechanisms affecting sperm quality after cryopreservation.

Lipid peroxidation and DNA damage are two parameters widely studied in fish sperm cryopreservation (Cabrita et al., 2010; Martínez-Páramo et al., 2012). Reactive oxygen species (ROS) are known to cause different injuries in sperm, such as membrane damage, decreased motility and in the last instance, causing infertility (Beirão et al., 2015, 2011; Lahnsteiner et al., 2010; Martínez-Páramo et al., 2012). Quantification of lipid peroxidation is essential to assess oxidative stress after cryopreservation. In this process free radicals take electrons from the lipids (generally in cell membranes), resulting in cell damage (Martínez-Páramo et al., 2012). A simple tool to evaluate the effect of lipid peroxidation on spermatozoa is the determination of malondialdehyde (MDA) levels in sperm, which is a stable lipid peroxidation product. Measuring the end products of lipid peroxidation is one of the most widely accepted assays for oxidative damage analysis.

The comet assay is commonly employed in the analysis of fish sperm DNA (Cabrita et al., 2005; Cartón-García et al., 2013; Riesco et al., 2012) measuring deoxyribonucleic acid (DNA) strand breaks. Using fluorescent dyes, fragmented DNA can be visualized in the tail of the comet whereas intact DNA remains in the nucleus. The comet assay has been employed to evaluate DNA damage caused by cryopreservation in *C. gigas* thawed sperm (Gwo et al., 2003). However until now, it has not been applied in *C. angulata* spermatozoa.

Methods based on qPCR analysis were recently developed for the detection and quantification of sperm mRNA transcripts in fish, important in fertilization and offspring development (Cartón-García et al., 2013; González-Rojo et al., 2014; Guerra et al., 2013; Robles et al., 2016). Cryopreservation induces a decrease in the levels of certain RNAs in sperm, and is a useful biomarker of post-thaw quality (Guerra et al., 2013; Meseguer et al., 2004; Valcarce et al., 2013). This assay can be especially important in conservation programs where sperm storage should not interfere with progeny quality afterwards.

The main goal of our study was the inclusion of these new molecular approaches in the analysis of sperm quality in oyster thawed sperm to establish the best cryopreservation methodology for use in the conservation of the genetic material of these species. To demonstrate the importance of these new analyses, a comparative study on cellular and molecular responses in oyster sperm was performed showing putative susceptibilities between species to cryopreservation.

2. Materials and methods

2.1. Animals

Oyster broodstocks (*C. angulata* and *C.gigas*) with a commercial weight > 70 g were acquired from the Ria de Alvor and Mira river bivalve farms during the natural spawning season and kept in the Experimental Shellfish Aquaculture Station (IPMA), Portugal. During this period the oysters were fed daily with a mixture of two microalgae (*Chaetoceros calcitrans* and *Skeletonema costatum*: 50/50 cells/μL) and kept in open systems. Temperature was maintained between 20 °C and 22 °C. All procedures were conducted with appropriate concern for animal welfare.

2.2. Sperm collection

The oysters were opened and a small sample of gonad tissue was examined microscopically to determine sex. Sperm was collected by the dry method, extracting it directly from the gonad using a micropipette, as previously described by our group (Riesco et al., 2017). The gonadal area was previously wiped to remove any contamination. The sperm was pre-diluted 1:10 (v/v) in artificial sea water (Asw) (pH = 8.1; osmolarity: 1150 mOsm/Kg). The diluted sperm was filtered using two different sieves: 100 μm to retain larger impurities such as gonad clumps and 20 μm for the smaller debris, enabling only the spermatozoa to drop into the beaker. Finally, the diluted and filtered sperm was maintained at 4 °C until used. In all experiments sperm from 6 pools from 3 individual males each was used.

2.3. Cryopreservation assays

Oyster sperm of both species was cryopreserved according to the previous published protocol (Riesco et al., 2017) using 10% DMSO (dimethylsulphoxide). Moreover, in *C. gigas* sperm cryopreservation, 10% EG (ethylene glycol) was included as cryoprotectant taking into account that this cryoprotectant (CPA) has been described as the most suitable for this species (Ieropoli et al., 2004).

The pre-diluted sperm was re-diluted in DMSO and EG at the same concentration (10%), both diluted in Asw. The freezing rates performed were those reported previously in our protocol in *C. angulata* (Riesco et al., 2017), (6 °C/min from 0 to −70 °C and then samples were plunged directly into liquid nitrogen). A portable programmed biofreezer (Asymptote Grant EF600, UK) was used to design the rates. All samples were cryopreserved in 0.5 mL French straws (IMV, France).

For thawing, the straws were removed from the liquid nitrogen and placed in a water bath at 37 °C for 10 s and the samples were immediately used to evaluate sperm quality.

2.4. Sperm quality assays

2.4.1. Membrane integrity

Propidium Iodide (PI-Sigma, Spain) was added at 1 μg/mL (final concentration) to detect dead cells. Immediately after this, the samples were acquired in a flow cytometer (FACS Calibur, BD Biosciences, CA, USA) adjusted for the blue excitation (488 nm) line for the detection of PI (670/30). Flow cytometer settings were previously adjusted using a positive (100% dead cells) and a negative control (fresh sperm). Data analysis was performed applying Weasel 3.1 free software. A total of 75,000 events were counted for each sample. 3–6 pools containing sperm from 3 individuals were analyzed for each species and CPA. The percentage of viable cells was recorded in both fresh and cryopreserved samples.

2.4.2. Comet assay

The comet assay was performed according to the method described by Cabrita et al. (2005) with slight modifications for oyster sperm. Briefly, 10 μL of semen was diluted in Asw to obtain a final concentration of approximately 1 million spermatozoa per mL. Cells were embedded in 0.5% agarose prepared in 0.1 M PBS and placed in agarose pre-coated slides. The slides were placed in a coplin jar containing the lysis solution (2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris, 1% Triton X-100, 1% Lauril sarcosine, pH 10) at 4 °C for 1 h. For electrophoresis, the slides were placed in an electrophoresis cube (sub-Cell GT, Bio-Rad, Portugal) filled with approximately 1.5 L of electrophoresis solution (0.3 M NaOH, 1 mM Na_2-EDTA, pH 12). Electrophoresis was conducted at 25 V and 300 mA at 4 °C for 10 min. After electrophoresis, the slides were drained and washed in the neutralizing solution (0.4 M Tris, pH 7.5) at 4 °C for 5 min (this step was performed twice). The slides were drained, fixed in pure methanol and stored at 4 °C until further observation. Slide observation was conducted in a fluorescent