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Cryopreserved semen in ecotoxicological bioassays: Sensitivity and reliability of cryopreserved *Sparus aurata* spermatozoa

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ABSTRACT

The aim of this study was to evaluate the feasibility of using cryopreserved *S. aurata* semen in spermiotoxicity tests. Cryopreservation is a biotechnology that can provide viable gametes and embryos on demand, rather than only in the spawning season, thus overcoming a limitation that has hindered the use of some species in ecotoxicological bioassays.

Firstly, the sperm motility pattern of cryopreserved semen was evaluated after thawing by means of both visual and computer-assisted analyses. Motility parameters in the cryopreserved semen did not change significantly in the first hour after thawing, meaning that they were maintained for long enough to enable their use in spermiotoxicity tests. In the second phase of the research, bioassays were performed, using cadmium as the reference toxicant, in order to evaluate the sensitivity of cryopreserved *S. aurata* semen to ecotoxicological contamination.

The sensitivity of the sperm motility parameters used as endpoints (motility percentages and velocities) proved to be comparable to what has been recorded for the fresh semen of other aquatic species (LOECs from 0.02 to 0.03 mg L^{-1}). The test showed good reliability and was found to be rapid and easy to perform, requiring only a small volume of the sample. Moreover, cryopreserved semen is easy to store and transfer and makes it possible to perform bioassays in different sites or at different times with the same batch of semen.

The proposed bioassay is therefore a promising starting point for the development of toxicity tests that are increasingly tailored to the needs of ecotoxicology and environmental quality evaluation strategies.

1. Introduction

Current strategies in monitoring programs for marine–coastal areas usually require the integration of chemical analyses and biological testing in order to better evaluate the bioavailable fraction of toxicant actually interacting with living organisms (Macova et al., 2010; Coulaud et al., 2011). In terms of biological parameters, reproductive success being a crucial factor in determining the survival of a species, ecotoxicological bioassays using gametes and embryos of aquatic species are widely employed (Losso et al., 2007; Mamindy-Pajany et al., 2010). Toxicants elicit a variety of effects depending on the species and the different synergistic and/or antagonistic effects of bioavailable substances present in combination. Therefore test batteries need to include organisms representing different phyla and different trophic levels (Macken et al., 2009). Organisms used in ecotoxicological bioassays should also be ecologically relevant and easily available all year round, and they should have well-known and above all homogeneous physiological responses. Moreover, they should come from the monitored area, or at least from similar environments, so that it is not necessary to alter the chemical and physical characteristics of the tested matrices and therefore the reactivity of the toxicants present. In addition, the relative endpoints should be selected so that they can be accurately, predictably and reliably measured (Chapman, 2002).

Although ecotoxicological tests have been standardised for gametes and embryos from a range of aquatic species, in many cases their availability (limited to the spawning season), the difficulty in collecting and storing them until the tests are carried out and above all their potentially high biological variability still limit their use (Paredes and Bellas, 2009; Schipper et al., 2008).

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Cryopreserved gametes and embryos can play a useful role in strategies to overcome these limits. Indeed, successful cryopreservation procedures provide viable gametes and embryos that can be easily transferred and stored for prolonged periods (Suquet et al., 2000; Chao and Liao, 2001). The last ten years have seen an increasing number of studies involving the cryopreservation of sperm and embryos from a range of aquatic organisms (Sansone et al., 2002; Paredes and Bellas, 2009; Chen et al., 2010). These have generated successful cryopreservation protocols, enabling the all-year-round availability of biological samples from many invertebrate and vertebrate species, whose usefulness in ecotoxicological tests can now be tested.

The sea bream *Sparus aurata* is a euryhaline species commonly found in both marine and brackish environments, intensively reared in the Mediterranean region (FAO, 2008). Due to its high commercial importance, its physiological responses to toxicant exposure have been evaluated (Cirillo et al., 2011). Its sperm morphology and motility have also been widely studied (Beirão et al., 2010; Lahnsteiner et al., 2010), and experimentally verified cryopreservation protocols have been available for some time (Fabbrocini et al., 2000; Cabrita et al., 2005).

The aim of this study was to assess the post-thawing motility patterns of cryopreserved *S. aurata* spermatozoa, and to evaluate the feasibility of their use in ecotoxicological bioassays. The effects of cadmium on the main parameters describing sperm motility were evaluated by means of both visual and computer assisted analyses, to assess their relative sensitivity with a view to their use as endpoints in spermiotoxicity bioassays.

2. Materials and methods

2.1. Experimental design

In the first phase of the research the sperm motility patterns of cryopreserved semen were evaluated immediately after thawing and in 1 h and 2 h post-thawing incubated samples. The percentage of spermatozoa with optimal motility was assessed with reference to indicators measured by visual and computer-assisted analyses, verifying whether the indicator values were maintained long enough to enable their evaluation in spermiotoxicity tests, i.e. 60–120 min of incubation, as recommended by USEPA (United States Environmental Protection Agency) (2000) and ASTM (American Society for Testing and Materials) (2004).

In the second phase of the research, spermiotoxicity tests were performed on cryopreserved semen, using cadmium as a reference toxicant, in order to evaluate the relative sensitivity of the same motility indicators as endpoints.

All the assays carried out with sea bream were performed in accordance with national and institutional guidelines for the protection of wildlife animal welfare.

2.2. Semen collection

The semen was collected by abdominal stripping from adult male sea bream bred at the Azienda Ittica Ugento and Azienda Ittica Caldoli, two fish farms situated in the Puglia Region (Southern Italy). Six collections were made at monthly intervals in two successive reproductive seasons (December–February). For each collection, 10–50 sea bream were stripped. Artificial seawater (35‰) at a ratio of 100:1 was added immediately after collection to a sample from each subject to activate the spermatozoa and evaluate the initial motility. Semen was selected by visual assessment of motility, as described in section 2.4.1. Samples found to have poor motility (with a percentage of progressive vigorously motile spermatozoa below 90%, delayed motility activation, i.e. requiring more than 30 s) or low sperm concentration, or to be contaminated by faeces or urine were discarded. Subsequently, after positive evaluation, two pools of semen, each from the five best males, were made upin each collection. Therefore, 12 pools, making a total of 60 different semen samples, were cryopreserved and used in the next phase of the research.

2.3. Cryopreservation procedure

Semen was cryopreserved in accordance with the procedure described in Fabbrocini et al. (2000). Semen was diluted at a ratio of 1:6 in 1% NaCl (motility inhibitor medium) containing 5% dimethyl sulfoxide (DMSO), inserted by Pasteur pipette into 0.25 ml plastic straws, frozen at a gradient of 10 °C min^{-1} from 0 to −150 °C, and finally immersed in liquid nitrogen. The freezing procedure was performed in a CryoMed® 8023 programmable freezer (Thermo Scientific Forma, Inc.).

2.4. Motility evaluation parameters

Straws were thawed at a rate of 15 °C sec^{-1} (7 s in a water bath at 35 °C); the thawing procedure was standardized by using straws in which a thermocouple had been inserted before freezing.

Thawed samples were randomly pooled (5 straws/pool, irrespective of the pre-freezing origin) and incubated undiluted at +4 °C for 1 h and 2 h. On thawing and at the end of the two incubation times, sperm motility was activated by diluting aliquots of semen in 35‰ ASW (artificial sea water) at 18 °C (ASTM (American Society for Testing and Materials), 2004) at a final rate of 1:100. BSA (bovine serum albumin, fraction V) at a concentration of 0.05% was used as an anti-sticking agent; the presence of anti-sticking agents in the activating medium (ASW) is believed to prevent sperm from adhering to the slide, which would alter the sperm motility recording (Kime et al., 2001).

2.4.1. Visually-assessed sperm motility

For each assessment of motility, the semen was activated as described in Section 2.4 directly on an uncoated glass slide and immediately observed by microscope (40 × 10 magnification). The percentage of progressive vigorously motile spermatozoa (PVM), calculated in relation to the total amount of cells (including immotile and poorly motile cells) observed in each field was assessed by two trained workers; a blind evaluation of the samples was also performed, in order to reduce observers' subjectivity.

For the visual evaluation of the motility patterns of cryopreserved semen six different pools were analysed ($n=6$).

2.4.2. Computer-assessed sperm motility parameters

For each assessment of motility, semen was activated in a small polyethylene vial (Eppendorf), as described in Section 2.4. 1.5 µl of semen were immediately pipetted on to a multi-chamber counting slide (10 µm thick; Leja, The Netherlands). Sperm movement was recorded using a 100 frame sec^{-1} camera (Basler, 782 × 582 resolution) attached to a microscope (Nikon Eclipse E600) with a phase-contrast objective (10 × 10 magnification) and connected to a computerised motion analysis system, the Sperm Class Analyser® (SCA, Microptic, s.l., Spain). The SCA acquisition parameters were set as follows: maximum area = 400 μm^2; minimum area = 50 μm^2; frame rate = 100 s^{-1}; total captured images = 100.

The following motion parameters were assessed:

(a) Curvilinear Velocity (VCL, µm sec^{-1}); (b) Straight-line Velocity (VSL, µm sec^{-1}); (c) Average Path Velocity (VAP, µm sec^{-1}); (d) Percentage of rapid sperm (RAP, curvilinear velocity above 100 µm sec^{-1}).

As the subpopulation of rapid sperm (in which VCL > 100 µm sec^{-1}) has been shown to be positively correlated with hatching rate in fertilisation trials with *S. aurata* cryopreserved semen (Beirão et al., 2011), additional parameters regarding this subpopulation were evaluated:

(e) ALH-r: Amplitude of lateral head displacement (µm); (f) BCF-r: Time-averaged rate at which the curvilinear sperm trajectory crosses its average trajectory (beats sec^{-1}); (g) VCL-r: (Curvilinear Velocity, µm sec^{-1}); (h) VSL-r: (Straight-line Velocity, µm sec^{-1}); (i) VAP-r: (Average Path Velocity, µm sec^{-1}).

For each semen sample, motility records were taken in 3 different microscopic fields. Each record consisted of the mean of three replicates from each field, each analysing from 250 to 500 sperm tracks. Records were carefully checked for sample drifting.

For the computer assisted evaluation of the motility pattern of cryopreserved semen ten different pools were analysed ($n=10$).

2.5. Spermiotoxicity tests

Cryopreserved straws were thawed and sperm motility after activation in ASW was rapidly assessed by microscope, as described in Section. 2.2. Successfully cryopreserved straws were pooled (5 straws/pool, irrespective of the pre-freezing origin) and used to carry out the bioassays. Thawed semen was diluted 1:6 in 1% NaCl containing scaled concentrations (0.01–100 mg L^{-1}) of cadmium as Cd(II) solution (prepared from standard solution for atomic absorption spectroscopy, Baker Italy, Milan) and incubated at +4 °C for up to 60 min. 1% NaCl was used as a negative control. Each trial was run in triplicate.

At the end of the incubation, sperm motility was triggered by dilution in ASW and the motility parameters were evaluated by visual assessment and by SCA® as described in Sections 2.4.1 and 2.4.2 respectively.

Five independent trials were performed using five different cryopreserved semen pools.

2.6. Statistical analysis

The sperm motility parameter values on thawing are expressed as mean values ± SD.

Prior to analysis, percentage data were arcsine square-root transformed. Data were tested for normality using Cochran's test and for homogeneity of variance