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Gamete cryobanks for laboratory research: Developing a rapid and easy-to-perform protocol for the cryopreservation of the sea urchin *Paracentrotus lividus* (Lmk, 1816) spermatozoa ☆

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ABSTRACT

Gamete cryopreservation is a biotechnology that can guarantee a continuous supply of gametes, regardless of the seasonal reproductive cycle. In this study we developed a protocol for the cryopreservation of the sea urchin *Paracentrotus lividus* spermatozoa, with a view to the creation of cryobanks of semen to be used as a model system in laboratory research and ecotoxicological tests. All the key phases of the procedure were separately considered and the effect on sperm motility was evaluated by means of computer assisted analysis. The best results were obtained using 7% dimethylsulfoxide in 1% NaCl plus 0.04 M trehalose as the extender, at a freezing rate of −20 °C/min. On thawing, in semen samples cryopreserved in accordance with this protocol the velocity parameters of the sub-population of rapid sperm (best performing spermatozoa) did not significantly differ from semen on collection; in addition also the fertilization ability was restored, and about 50% of normal developed plutei larvae were obtained by thawed semen. The developed protocol is rapid and easy-to-perform; moreover, the use of gametes from reared urchins makes it unnecessary to continuously collect specimens from natural populations, making this procedure a promising starting point for the creation of alternative and more sustainable methodologies in laboratory research on sea urchin gametes and embryos.

Introduction

The sea urchin *Paracentrotus lividus* (Lmk, 1816) is a regular echinoid inhabiting rocky shores in the Mediterranean sea and the eastern Atlantic from Ireland to Southern Morocco [19]. Besides its value as a luxury food [19], it is widely used as a model organism for laboratory research, mainly molecular and developmental biology [27,41]. Sea urchin spermatozoa are also excellent models for the study of the physiology of sperm motility, and there have been many studies of flagellar movement and sperm swimming behavior both in the past [14] and in recent years [39,51,52]. In addition, ecotoxicological tests with sea urchin sperm and embryos are used worldwide for the evaluation of marine ecosystem quality in biomonitoring programs [13,30]. Therefore, there is a need for continuous supply, regardless of the seasonal reproductive cycle, of gametes characterized by high quality and low biological variability. Production for this supply needs to be carried out using laboratory-scale systems that can be easily managed even in the absence of specifically equipped facilities [47]. To overcome this limitation, off-season broodstock conditioning has been successfully attempted [31,47], and gamete cryopreservation may also be a useful strategy [13]. The feasibility of using cryopreserved sperm in ecotoxicological tests has already been demonstrated for the sea bream *Sparus aurata* [32,33]. A challenging solution may be a combined use of these two biotechnologies: indeed, the cryopreservation of gametes from conditioned broodstock may provide gametes on demand, as well as increasing the yield of the rearing procedure, as it would be possible to collect

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gametes and to store, even for prolonged periods, those not immediately used.

Cryopreservation protocols have been successfully developed for the gametes and embryos of a wide range of aquatic species, mainly fish and to a lesser extent bivalves [15,17,49]; conversely, relatively few data are available for echinoderm species [1,3,4,10]; reviewed in [35]. Early trials were performed by Dunn and McLachlan [24] on *Strongylocentrotus droebachiensis* and by Asahina and Takahashi on *Strongylocentrotus intermedius*, *Strongylocentrotus nudus* and *Hemicentrous pulcherrimus* [3,4]; although satisfactory fertilization rates were achieved, no detailed data on the effects of different cryoprotectants or freezing rates were reported. More recently, a successful cryopreservation protocol was developed for the *Evechinus chloroticus* sperm by Adams et al. [1], who obtained fertilization rates higher than 85% with thawed semen, observing different results not only as a function of the cryoprotectant concentration but also among different semen samples. On the contrary, to our knowledge no information on *P. lividus* sperm cryopreservation trials can be found in the current literature. As sperm cells from different species may require different conditions or cryoprotectant types, so that protocols cannot simply be transferred from one species to another [54], in order to develop a protocol for the *P. lividus* sperm cryopreservation, each phase needs to be purposely optimized.

It is well known that freezing/thawing procedures affect cells to be cryopreserved. Ice crystal formation and osmotic stress can impair sperm cells, damaging their structural integrity [11,22] and increasing the reactive oxygen species (ROS) that in turn lead to lipid and protein alterations and DNA/RNA lesions [16,38]. Also, sperm motility activation and duration rely on a complex series of mechanisms which depend on the integrity of cellular structures such as the plasma membrane, flagellum and mitochondria [21,51,55], as well as the metabolic pathways governing the balance between ATP storage and production [21,52]. Therefore, the choice of extender solution (i.e. cryoprotectant and its concentration), as well as the cooling and freezing rates, need to be carefully investigated [15,35], as the achievement of good quality samples on thawing depends on the interaction of all these factors, and the damage caused during each step necessarily accumulates, leading to a considerable loss of viability by the end of the procedure [50].

Another issue that still limits the widespread use of sperm cryobanks for many aquatic species lies in the complexity and cost of the procedures. Indeed, cryopreservation protocols need to be as simple, rapid and low-cost as possible, in order to be easily upscaled and applied even in the field [2,37,44].

The aim of this study was to develop a rapid and easy-to-perform protocol for the cryopreservation of spermatozoa from the sea urchin *P. lividus* with a view to the creation of cryobanks of semen to be used as a model system in laboratory research and ecotoxicological tests. To this end, all the key phases of the procedure (i.e. formulation of the extender, equilibration conditions, freezing rates) were separately considered. Gametes were collected from purposely reared urchins instead of from field collected specimens. For a rapid assessment of the effects of the tested procedures on sperm viability, the sperm motility parameters on activation were evaluated by means of computer-assisted analysis.

The biology of sperm motility in sea urchin has been widely studied and the mechanisms of the motility and of the flagellar beating are actually well-known; sperm cells are immotile in the gonads and the increase in environmental osmolarity occurring on spawning activates membrane Ca^{2+} channels that in turn, by means of Ca^{2+} and cAMP dependent messengers, induce the flagellar beating [18,20,34,52]. It has been described as sea urchin spermatozoa swim with circular trajectories of about 50 μm diameter [52]; moreover, the percentage of motile sperm and their relative velocities have been evaluated by computer assisted analysis [6,7,29] and, unlike what observed for most fish species [21] they were found to not significantly decrease also 1 h after activation in sea water [23], making easier the management of semen before freezing and its quality assessment.

Materials and methods

Experimental design

As the success of a cryopreservation protocol depends on the interaction of many variables, an experimental design involving multiple factors allows an easier and quicker screening of the best performing conditions. In this study, the experimental design involved two successive phases. In the first phase the combined effect of three cryoprotectant solutions (extenders) and three equilibration conditions were evaluated. At the end of this phase, the three equilibration protocol-cryoprotectant couples that gave the best results in terms of overall sperm motility were selected. In the second phase two freezing rates were tested on semen previously equilibrated in accordance with the three previously selected protocols. The sperm motility pattern assessed by computer assisted analysis was used as the evaluation parameter. Fertilization ability of thawed semen was also evaluated.

All reagents were purchased from Sigma–Aldrich (Milan, Italy).

Animals

Adult sea urchins were collected from an artificial rock ledge near Termoli (41°54′ N, 16°10′ E), on the southern Adriatic coast of Italy, during the resting period; they were reared for about 14 weeks in a 200 L recirculating aquarium (temperature 18 °C, salinity 35‰, pH 8.00–8.20, natural photoperiod), fed *ad libitum* on NIFA feed (NOFIMA, Tromsø, Norway), a pelletized feed specifically formulated for sea urchins [53], in order to induce gonad maturation as described in [31].

Semen collection

The sea urchins were induced to spawn by injection of 1 ml of KCl 0.5 M. Semen was dry-collected; artificial seawater (ASW) 35‰ [5] was immediately added to a drop of semen from each male subject to activate the spermatozoa and evaluate their quality by direct observation by microscope. The best samples in terms of sperm concentration and motility were selected for the pools (three individuals per pool), which were dry-stored at the relative equilibration temperature (4 °C and 18 °C) and used within 30 min.

Motility evaluation

For evaluation of sperm motility parameters, aliquots of dry sperm samples were diluted in ASW at a rate of 1:1000. BSA (bovine serum albumin, fraction V) at a final concentration of 0.05% was added to ASW as an anti-sticking agent [40]. Sperm movement was recorded using a 100 frame/s camera (Basler A602f-2, 782 × 582 resolution) attached to a Nikon Eclipse E600 microscope (Nikon Instruments, Florence, Italy) with a phase-contrast objective (10 × 10 magnification) connected to a computerized motion analysis system, the Sperm Class Analyzer® (SCA®, Microptic, s.l., Barcelona, Spain). The SCA acquisition parameters were set as follows: max area = 400 μm^2, min area = 50 μm^2; frame rate = 100/sec; total captured images = 100. Each recorded field consisted of a mean of three replicates, each one analyzing from 250 to 500 sperm tracks. Records were carefully checked for sample drifting.