Journal of Experimental Marine Biology and Ecology 452 (2014) 91–100

Contents lists available at ScienceDirect

Journal of Experimental Marine Biology and Ecology

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

Use of JC-1 to assess mitochondrial membrane potential in sea urchin sperm

M.T. Binet [a,*], C.J. Doyle [b], J.E. Williamson [c], P. Schlegel [c]

[a] Centre for Environmental Contaminants Research, CSIRO Land and Water, Locked Bag 2007, Kirrawee, Sydney, NSW 2232, Australia
[b] Ecotox Services Australasia, 27/2 Chaplin Drive, Lane Cove, NSW 2066, Australia
[c] Marine Ecology Group, Department of Biological Sciences, Macquarie University, Sydney, NSW 2109, Australia

ARTICLE INFO

Article history:
Received 6 June 2013
Received in revised form 10 December 2013
Accepted 14 December 2013
Available online 9 January 2014

Keywords:
Acrosome
Flow cytometry
Invertebrate
Marine
Sperm motility
Stain

ABSTRACT

There is a need within marine research areas for more rapid techniques to assess the health of sperm from marine invertebrates. Originating in medical research, flow cytometry has been applied to rapidly measure a range of cellular processes within a plethora of different cell types. To date, the transfer of that knowledge to marine research has been limited. A method has been developed to assess mitochondrial membrane potential (MMP) in sea urchin (*Centrostephanus rodgersii*) sperm using the stain 5,5′,6,6′-tetrachloro-1,1′3,3′-tetrathylbenzimidazolyl-carbocyanine iodide (JC-1) and flow cytometry. MMP is a useful indicator of sperm health as the mitochondrion is the single source of ATP production, and the driver of apoptosis. The method was carefully optimised and validated with the use of positive controls. There were strong correlations between MMP measurements and sperm swimming speed and motility (R values of 0.8–0.9, $p < 0.001$). JC-1 successfully differentiated between sperm with low and high MMP. However, in sperm that were treated with the mitochondrial inhibitor, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), JC-1 fluorescence in stained sperm did not conform to that usually seen for other cell types. Using fluorescence microscopy, it was confirmed that this was due to the formation of J-aggregates in the acrosome vesicle following MMP collapse. To our best knowledge, this is the first report of J-aggregates forming in an organelle other than the mitochondria. This unexpected fluorescence response necessitated the use of a quadrant approach (% high MMP) instead of the usual ratiometric approach (FL2/FL1) to quantify MMP changes. Difficulties overcome during method development are described, many of which were likely related to the required use of seawater as a test medium. The developed method will enable rapid measurement of mitochondrial membrane potential of sea urchin sperm for application in reproductive biology, aquaculture research, and the impact of environmental stressors such as ocean acidification and pollution on sperm development and function.

1. Introduction

There is a growing need for the development of rapid techniques to increase our understanding of observed effects of physical and chemical stressors on marine invertebrate sperm function. Research areas include: aquaculture, where increased use of cryopreservation has necessitated the development of rapid tools to determine effects on gametes and embryos; ecotoxicology, where mechanisms of toxicity caused by environmental contaminants are desired; and climate change research, where researchers are eager to explain observed sperm swimming responses to climate-change stressors such as lowered pH, increased temperature and increased ultraviolet radiation (Adams et al., 2003; Akcha et al., 2012; Binet and Doyle, 2013; Havenhand et al., 2008; Lu and Wu, 2005; Paniagua-Chavez et al., 2006; Schlegel et al., 2013). While a number of techniques have already been developed for this purpose (e.g. sperm motility, morphology, viability, fertilisation success), all are based on physical observations and cannot inform researchers as to the underlying causes or mechanisms behind the observed effects. Also, such techniques are often limited by the number of sperm that can be assessed due to the time constraints of the methods.

Flow cytometry is a technique that originated in medical research and diagnosis, and enables thousands of cells to be analysed within seconds for light-scattering and fluorescence properties. This information can provide researchers with rapid, targeted knowledge on cell size, shape, structure and fluorescence. A range of fluorescent dyes (fluorochromes) have been developed for use in conjunction with flow cytometry, enabling the user to investigate particular cellular processes and

Abbreviations: MMP, mitochondrial membrane potential; JC-1, 5,5′,6,6′-tetrachloro-1,1′3,3′-tetrathylbenzimidazolyl-carbocyanine iodide; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; EV, electrical volume; SSC, side scatter; FL1, Fluorescence detector 1, 525/30 nm (green spectrum); FL2, Fluorescence detector 2, 575/30 nm (orange spectrum); FL3, Fluorescence detector 3, >670 nm (red spectrum).

* Corresponding author. Tel.: +61 2 9710 6812; fax: +61 2 9710 6800.
E-mail addresses: monique.binet@csiro.au (M.T. Binet), cdoyle@ecotox.com.au (C.J. Doyle), jane.williamson@mq.edu.au (J.E. Williamson), peter.schlegel@mq.edu.au (P. Schlegel).

0022-0981/$ – see front matter.
http://dx.doi.org/10.1016/j.jembe.2013.12.008

functional status. For spermatozoa, flow cytometry research and application have mostly been within the areas of cell biology, reproductive biology, pathology and fertility assessment (Spano and Evenson, 1993). Applications of flow cytometry to mammalian sperm include enumeration, viability, acrosomal integrity, mitochondrial function, chromatin intactness, capacitation status, apoptotic changes, oxidative stress and sorting of sperm by chromosomal sex (Hossain et al., 2011). However, only a select few of these methods have been adapted for use in sperm from marine invertebrates to assess viability, acrosomal integrity and mitochondrial function (e.g. Adams et al., 2003; Akcha et al., 2012; Favret and Lynn, 2010).

Sea urchins have been the model species used in developmental biology since the 1970s, from which much of our current knowledge on generalized sperm physiology has stemmed (Lewis and Ford, 2012). Sea urchins play an important ecological role in coastal ecosystems as they are a key food for higher animals and through their consumption of macroalgae are important regulators of seagrass and rocky reef communities (Shears and Babcock, 2002; Valentine and Duffy, 2006; Wright et al., 2005). They have also become an important aquaculture species, as over-fishing of sea urchins for human consumption in several Asian and South, Central and North American countries has reduced natural populations of edible urchins (Lawrence, 2007). Like all echinoderms, and many other marine invertebrates, sea urchins are broadcast spawners, releasing their gametes into the water column, where fertilisation takes place externally. Sea urchin fertilisation success and larval development are highly sensitive to slight changes in their environment, and for this reason they are often used to study the effects of environmental stressors such as contaminants (e.g. Dinnel et al., 1989) and climate change (Wicks and Roberts, 2012). While both eggs and sperm of broadcast spawners are vulnerable to environmental stressors, sperm lack the variety of proteins, antioxidants and DNA-repair enzymes that somatic cells and eggs have, rendering them almost defenceless against environmentally induced damage (Aitken et al., 2004; Lewis and Ford, 2012). Sperm are therefore often more sensitive to environmental perturbation than eggs (e.g. Fitzpatrick et al., 2008; Nahon et al., 2009; Rahman et al., 2009).

Unlike mammalian sperm, which have a variety of available glycolic and respiratory substrates in the vaginal tract for ATP production, and around 100 mitochondria (Hossain et al., 2011; Ruiz-Pesini et al., 2007), sea urchin sperm do not undergo glycolysis and rely entirely on mitochondrial respiration in their one mitochondrion for ATP production (Christen et al., 1983; Mita and Nakamura, 1998). The energy required for motility is derived from ATP, and, therefore, sperm motility is highly correlated with sperm mitochondrial activity (Ruiz-Pesini et al., 2007). Mitochondria are also the drivers for apoptosis and are involved in sperm maturation (Hossain et al., 2011). A functioning mitochondrion is therefore critical to the viability of sea urchin sperm.

One of the ways that has previously been used to measure mitochondrial activity in mammalian sperm is to assess for changes in the MMP. In healthy cells, the proton pump of the electron transport chain (ETC) establishes the proton motive force (Δp), which forces protons across the inner mitochondrial membrane to the inter-membrane space, creating a significant pH and electrical gradient across the inner mitochondrial membrane (Cottet-Rousselle et al., 2011). The protons eventually flow back into the mitochondria via ATP-ase, driving ATP synthesis (Perry et al., 2011). The total force driving protons (i.e. Δp) is typically around 180–220 mV, and is a combination of the MMP, which accounts for around 150–180 mV, and the mitochondrial pH gradient of around 0.5–1.0 units, which contributes to the remaining 30–60 mV (Cottet-Rousselle et al., 2011; Perry et al., 2011). Therefore it is useful to measure the MMP component of Δp, as any change in the MMP will cause a change in ATP synthesis, and thus affect sperm swimming behaviour and overall health of the sperm. In fact, for human sperm, the detection of changes in MMP is said to be one of the most efficient tests to evaluate sperm quality in the preliminary steps of in-vitro fertilisation (IVF) for humans (Marchetti et al., 2011).

To date, flow cytometric measurement of MMP in marine invertebrate sperm has been conducted using Rhodamine 123 (R123; Adams et al., 2003; Lu and Wu, 2005; Paniagua-Chavez et al., 2006) or Mitotracker red (Akcha et al., 2012; Favret and Lynn, 2010). R123 is not retained well by cells, has high non-specific binding in mitochondria, and cannot distinguish between cells with high and low levels of MMP (Cottet-Rousselle et al., 2011; Salvioli et al., 1997). Mito-tracker red is superior to R123 for MMP assessment, but has been suggested to detect both MMP-dependent and MMP-independent processes, making it less useful to assess for changes in MMP (Marchetti et al., 2004). The carbocyanine fluorescent probe, 5,5′,6,6′-tetrachloro-1,1′3,3′-tetrathylbenzimidazolyl-carbocyanine iodide (JC-1) is a popular fluorochrome for assessing changes in MMP in mammalian sperm. The main advantage of JC-1 over its predecessors is its ability to differentiate between cells with varying levels of functioning mitochondria. JC-1 exists as a monomeric green-emitting form when MMP is low (<80–100 mV), an aggregate, orange/green-emitting form when MMP is high (>80–100 mV to ≈180 mV) and has also been observed as a red-emitting aggregate when MMP is very high (>190 mV) (Cossarizza and Salvioli, 2000; Reers et al., 1995). The aim of the current study was to develop a flow cytometric method for assessing MMP in sperm from the sea urchin *Centrostephanus rodgersii* using JC-1. Our JC-1 stain uptake method was validated by comparing it with measurements of sperm motility and speed.

The temperate urchin *C. rodgersii* was used as a model species due to its ecological importance. *C. rodgersii*, commonly found on sub-tidal rocky reefs in South-eastern Australia, Northern New Zealand and the Kermadec Islands (Andrew and Byrne, 2001), has experienced a significant range shift south due to the climate change-induced poleward extension of the East Australian Current (Johnson et al., 2011). As a result, *C. rodgersii* has been the focus of several studies assessing effects of stressors associated with climate change impacts on embryonic development, fertilisation success and sperm health (e.g. motility, speed; Foo et al., 2012).

2. Methods

2.1. Animal collection and gamete preparation

C. rodgersii were collected in July and August 2012 at the peak of their spawning season from subtidal areas at Fairlight (33°48′1″S, 151°16′3″E), Sydney, Australia, and were kept in a recirculating seawater system, maintained at ambient conditions at Macquarie University for a maximum of one week. Animals were fed ad libitum the kelp *Ecklonia radiata* during holding. Spawning was induced by a non-lethal injection of 1–2 mL of 0.5 M KCl through the peristomal membrane and sperm were collected "dry" on ice to extend their lifespan. Each male was spawned once only. Sperm were activated immediately prior to use in experiments by diluting an aliquot of dry sperm in test treatment solution such that the final sperm density was approximately 10^7 sperm mL^{-1}. The density of the activated sperm was confirmed using a Neubauer haemocytometer and microscope under 100× magnification (Olympus BX53, Olympus, Japan). Test treatments were prepared in filtered (0.22-μm) seawater in 10-mL glass vials, 5-mL Teflon tubes or 3-mL polyethylene tubes depending on the volumes of sperm suspensions required. All experiments were conducted at a temperature of 18 ± 1 °C.

2.2. Seawater and reagents

Experimental seawater (salinity = 35 ± 1, pH = 8.1 ± 0.1) was collected from the coast of Sydney and filtered (0.22 μm) prior to use.

A 2.5 mM stock (w/v) of JC-1 (Sigma-Aldrich, St Louis, MO, USA) was prepared in dimethyl sulfoxide (DMSO, 97% purity, Sigma) and divided into 25-μL aliquots in 5-mL Teflon tubes. These were capped and frozen