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Cryopreservation and storage of mussel (*Mytilus* spp.) haemocytes for latent analysis by the Comet assay

Anthony Kwok[a,b], Brett P. Lyons[a], Nikolas J. Hodges[b], Tim P. Bean[a,*]

[a] Centre for Environment, Fisheries and Aquaculture Science, Cefas Weymouth Laboratory, Weymouth, DT4 8UB, United Kingdom
[b] School of Biosciences, The University of Birmingham, Edgbaston, Birmingham, B15 2TT, United Kingdom

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ABSTRACT

Estuarine and coastal habitats are known to be polluted by a range of chemical contaminants from both industrial and domestic sources. Blue mussels (*Mytilus* spp.), which inhabit these areas, are widely used as bio-indicators in eco-toxicological studies, because of their sedentary nature and their ability to bio-accumulate contaminants. The analysis of DNA damage in mussel haemocytes is a valuable tool for biomonitoring but sampling issues related to storage, handling and transportation have often limited its application in large-scale monitoring programmes. This study uses a trial and error method to evaluate and validate a suitable protocol for cryopreservation of mussel haemocytes, thereby allowing material collected in the field to be analysed later under controlled laboratory conditions. Three different cell-culture media, i.e. Leibovitz-15, Hank's balanced salt solution and mussel physiological saline, along with four different cryoprotectants, i.e. dimethyl sulphoxide (10% and 20%), 1,2-propanediol (10%), ethylene glycol (10%) and glycerol (10%) were tested to assess their suitability for cryopreservation of mussel haemocytes for analysis in the comet assay. Experimental studies where mussel haemocytes were also exposed to UV radiation or benzo(*a*)pyrene were conducted in order to mimic environmental stresses and to verify the effectiveness of newly defined cryopreservation protocols. The comet assay was used to demonstrate that mussel haemocytes could be preserved at cryogenic temperatures for a month without altering levels of DNA damage, which could possibly be used for lab or field studies where time constraints or facilities do not allow instant analysis.

1. Introduction

Estuaries and the coastal environment are natural geographical repositories of mud and sand sediment that are often contaminated with chemicals and oil from urban run-off, factories, tankers and pipeline discharges [1,2]. In Europe a number of legislative initiatives are now in place to protect the marine environment, such as the Marine Strategy Framework Directive (MSFD), a recently adopted European Union (EU) framework directive (2008/56/EC) policy, which aims to achieve good environmental status (GES) in European seas by 2020 [3]. Other EU initiatives such as the Council Directive (96/61/EC) on Integrated Pollution Prevention and Control (IPPC), which was adopted in 1996, also aim to achieve high-level control of industrial pollution and emissions to air, land and water [4]. Although these and other EU directives have been enforced, the intertidal zones of many coastal areas in Europe are still prone to pollution. For example, field surveys have shown that industrialised estuaries are impacted with a range of contaminants, some of which are known to display genotoxic activity [5,6]. Accidental discharges also contribute to the contamination of the marine environment as exemplified by the Deepwater Horizon accident resulting in the release of 600,000 tonnes of crude oil into the Gulf of Mexico [7] and the sinking of the tanker 'Prestige' in November 2002, which led to 60,000 tonnes of fuel oil polluting the coastline of Galicia with polycyclic aromatic hydrocarbons (PAHs) and heavy metals such as nickel [8].

Mussels (*Mytilus* spp.) are sentinel species in estuaries [9] and they are widely used as bio-indicators due to their sessile nature, high filtering capacity and their ability to bioaccumulate many environmental contaminants. Their ubiquitous distribution and efficient contaminant cycling ability in estuarine ecosystems make them valuable for environmental and ecotoxicological research [9]. Mussel haemocytes are sensitive to environmental stress, and chronic exposure to environmental contaminants is known to have an adverse effect on their immune system [10]. The sensitivity of *Mytilus* spp. to genotoxicants has also been demonstrated through the detection of DNA strand-breaks and DNA adducts in a range of tissues including haemocytes, the digestive gland, and gill cells [11,12]. For biomonitoring studies, the measurement of DNA damage is used as a genotoxicity end-point to assess the impact of

* Corresponding author. Tel.: +44 01305 206600; fax: +44 0 1305 206601.
E-mail address: tim.bean@cefas.co.uk (T.P. Bean).

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contaminant mixtures [5,13]. However, as discussed by a panel of experts in 2007, the study of DNA damage in field situations has been hampered by a combination of problems with sensitivity to environmental stressors and difficulty in transferring samples to laboratories with suitable facilities for conducting genotoxicity assays [14].

Genotoxicity end-points can be analysed by the comet assay (also known as the single-cell gel electrophoresis assay), which detects DNA single- or double-strand breakage and alkali-labile sites (ALS) within the DNA, induced by chemical or biological sources [15]. It has been widely adopted in applications of biomonitoring and ecological testing [16,17] with tail moment (a function of tail length and fraction of DNA in tail) or % DNA in tail (also known as tail intensity) used as a direct measures of DNA damage [18].

Studies have previously shown that haemocytes from unexposed mussels can be stored for a week at 4 °C without the induction of DNA damage [19]. However, the study did not demonstrate that haemocytes from exposed mussels could be stored in a similar fashion, and this raises concern; mussels containing a reactive genotoxicant in the hemolymph at the time of sampling would continue to be damaged over the duration of post-sampling storage. Cryopreservation is frequently used in conservation and long-term storage of biological tissues, cell cultures, embryos and gametes at sub-zero temperature [20] and cryopreservation of the bivalve gametes such as Pacific oysters, *Crassostrea gigas* and the sperm from Common carp, *Cyprinus carpio* has been proven a success and has brought huge benefits to aquaculture [21,22]. Sampling in the marine environment may also require mussels to be collected while at sea, which for practical reasons (e.g. ship movement affecting electrophoresis) inhibits the immediate analysis of tissue samples via the comet assay. Hence the need to develop a reliable protocol for cryopreservation of haemocytes, which would allow samples to be stored and transported from field to laboratory for subsequent analysis under controlled conditions with minimal or no issues of latent DNA damage.

This study evaluates protocols for the cryopreservation of mussel haemocytes for subsequent screening of DNA damage with the comet assay. Protocol development was done through four stages of experimental design, the first to determine suitable culture media for haemocytes that did not increase the background levels of DNA damage, the second to compare cryoprotective agents, the third stage to test different cold-storage facilities and the last to test the complete cryopreservation method after haemocytes had been exposed to physical and chemical stress (UV radiation and benzo(*a*)pyrene). After each stage the comet assay was used to detect DNA damage in haemocytes and to assess the outcome of each protocol. In addition, this study provides further evidence that mussel haemocytes are a useful model for studying mechanisms of genotoxicity in bivalves through *in vitro* exposures.

2. Material and methods

2.1. Chemicals and materials

Sodium chloride, Trizma base, ethylenediaminetetraacetic acid disodium salt (Na_2-EDTA), Triton X-100, n-Lauroyl-Sarcosine, dimethyl sulphoxide (DMSO), 1,2-propanediol (PROH), ethylene glycol (EG), glycerol (G), sodium hydroxide, ethylenediaminetetraacetic acid, HEPES, magnesium sulphate ($MgSO_4$), potassium chloride, calcium chloride, hydrochloric acid, hydrogen peroxide, benzo(*a*)pyrene (B*a*P), low-melting-point (LMP) agarose, Leibovitz medium (L-15), Hank's balanced salt solution (HBSS) and ethidium bromide were obtained from Sigma-Aldrich (Dorset, U.K.). Comet-assay slides were from Trevigen (Gaithersburg, MO). Mussel physiological saline (MPS) was freshly prepared (20 mM HEPES, 0.436 M NaCl, 53 mM $MgSO_4$, 10 mM KCl, 13 mM $CaCl_2$, pH 7.4) and L-15 and HBSS media were osmolarity-modified as previously described with addition of 22 g L^{-1} of NaCl to match mussel hemolymph at 990 mOsm L^{-1} [19]. To achieve a constant rate of cooling the CoolCell® (BioCision LLC) device was used.

2.2. Collection of mussel haemocytes

Mytilus edulis with average shell length of 40 mm were obtained from a local mussel farm in Portland harbour, Dorset, U.K. and maintained in aquaria with a constant flow of ambient temperature seawater. Haemolymph was aspirated from the adductor muscle using a 1 mL syringe with 30 mm/23-gauge needles. Haemocytes were collected from randomly chosen mussels, and maintained independently in an equal volume of culture medium such as modified L-15, modified HBSS or MPS. If required, haemocytes and cell-culture medium were maintained for short periods in the dark at 4 °C prior to further procedures.

2.3. Haemocyte treatment protocols

Treatment 1: Haemocytes were treated with 100 μM H_2O_2 for 20 min or exposed to UV light at 302 nm using High Performance UV Transilluminator (UVP, U.K.) for 5 min (UVB intensity, 500–800 μW cm^{-2}; UVA intensity,1800–2000 μW cm^{-2}).

Treatment 2: Haemocytes were exposed to benzo(*a*)pyrene (100 μM B*a*P) for 1 h, or to UV radiation (UVB intensity, 2800–3000 μW cm^{-2}; background UVA intensity, 330–360 μW cm^{-2}) for 0, 45, 90, 135, 180 and 225s (using Gel Doc™ XR™ Image System, BioRad) either separately or in combination (UV exposure time in 'B*a*P + UV combined' was 40s).

2.4. Development protocols for cryopreservation

Protocol 1: Haemocytes were suspended in L-15, HBSS or MPS medium followed by treatment 1 prior to the comet assay. Haemocytes suspended in media without treatment were set as negative control.

Protocol 2: Haemocytes were suspended in HBSS with individual cryoprotectants: DMSO (10% or 20%, v/v), EG (10%), PROH (10%) or G (10%). Haemocytes then underwent treatment 1 followed by the comet assay. A control without cryoprotectant was also included to compare studies and to assess genotoxicity associated with the cryoprotectant.

Protocol 3: Haemocyte samples suspended in HBSS and processed through treatment 1 were placed in CoolCell® (BioCision LLC) and incubated with cryoprotectant G (10%) at 20 °C for 20 min, followed by freezing in 'control-rate freezer at −80 °C' or 'dry shipper–vapour phase of liquid nitrogen (LN) for 3 h'.

Protocol 4: Followed protocol 3, except that haemocytes were exposed to treatment 2 before cryopreservation and subsequent screening with the comet assay.

2.5. Thawing of haemocytes

Prior to the comet assay, cryopreserved haemocytes were thawed from liquid nitrogen by immersion in a water bath at 37 °C for 30s, followed immediately by mixing in LMP agarose as described in the comet-assay section below.

2.6. Comet assay

Methods were adapted from the protocol of the comet-slide manufacturer (Trevigen Gaithersburg, MD). Briefly, 10 μL of each haemocyte sample was added in an eppendorf tube containing 160 μL of 0.5% LMP agarose solution (0.25 g LMP agarose dissolved in 50 mL mussel physiological saline at 37 °C). Haemocytes in LMP agarose solution were placed on a circle of the Trevigen slide. Slides were kept in the dark at 4 °C for 10 min to ensure agarose was completely set. Slides were then immersed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma base, 1% *N*-lauroyl-sarcosine, 1% Triton X-100, 10% DMSO, pH 10.0) for 30 min at 4 °C in dark. After lysis, slides were washed with distilled water, then incubated in an opaque electrophoresis tank in alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13.0) for 30 min at 20 °C to allow DNA unwinding. Electrophoresis for DNA migration was performed in the same buffer at 0.7 V cm^{-1} (18 V, 300 mA) for 30 min at 20 °C. Slides were taken out and washed three times in distilled water, then with 100 μL of neutralising buffer (0.4 M Trizma base, pH 7.5). Slides were dehydrated in methanol for storage before examination.

For examination, each test sample on the slide was stained with 10 μL ethidium bromide solution (10.0 μg/mL) and sealed with a glass cover slip. All slides were examined with a Nikon Optiphot 2 microscope with a 100-W Short-Arc Mercury lamp (HBO). Fifty cells were randomly scored (at 200× magnification) from each replicate. DNA damage was expressed as the % tail intensity. The images were measured with Comet Assay IV (Perceptive Instruments), and data were compiled with Comet Assay Spreadsheet Generator Version 1-3-1 (Perceptive Instruments).

2.7. Data analysis

All the data of DNA damage from each group in tests were presented as mean and corresponding standard error of the mean (SEM). Data normality was analysed with the Shapiro-Wilk test and analysis of variance (ANOVA) was performed to evaluate the significance of differences across all test groups including positive and negative controls (SigmaPlot Version 12, Systat Software Inc). Data with $p < 0.05$ were classified as significant.