USE OF THE COMET ASSAY FOR DETECTION OF DNA DAMAGE IN OYSTER HEMOCYTES FROM CRASSOSTREA GIGASEXPOSED IN VIVOTO STRESSORS

ABSTRACT
The Comet assay or single-cell gel electrophoresis assay is a simple, non-specific method of detecting DNA damage in individual cells. Ideally, the Comet assay can be performed using any eukaryotic cell type. In this study, oyster hemocytes were analyzed for DNA damage using this assay which was first standardized. Oysters and other shellfish are excellent environmental indicator organisms due to their sessile and filter-feeding nature. Oysters were subjected to in vivo stresses, and hemocytes tested for DNA damage. Stresses investigated were high organic pollution levels, and incubation with poliovirus type 1. Results showed a slight increase in percent DNA damage (23.42%) after one week of pollution exposure when compared to the controls. The study was continued for nine weeks of exposure, after which no significant increase was observed. Incubation with two poliovirus concentrations (4.1 x 10^5 and 4.1 x 10^7 pfu) for 1, 3, and 6 hours showed large increases in percent of DNA damage (from 72.50% to 86.83%). These results suggest that this assay can be used as an indicator of viral contamination in oysters, and also indicate that oysters can repair their nucleic acids and continue their growth even in very polluted environment.
INTRODUCTION

The Comet assay, also known as the single cell gel electrophoresis assay (SCGE), was first introduced in 1984 by Ostling and Johanson (1), as a nonspecific technique for detection of DNA damage in individual cells. Since then, this assay has been modified and is very simple, sensitive, rapid and inexpensive, and has been adopted for a wide range of applications including investigation of chemical genotoxicity, responses to clinical treatments and environmental bio-monitoring (2, 3, 4, 5). The Comet assay enables the visual detection of single-strand breaks and alkali-labile sites (6, 7). Sample preparation requires a cell suspension embedded in low melting-point agarose on a pre-coated microscope slide. Cells are then lysed by detergents and high salt concentrations to liberate the DNA, followed by alkaline unwinding. After this, the slide-gels are subjected to electrophoresis, neutralized and stained with a fluorescent DNA binding dye. When viewed under epifluorescence microscopy, cells with increased DNA damage show increased migration of DNA from the nucleus towards the anode giving the appearance of a comet. The degree of damage in each cell can be empirically classified based on differing tail length, DNA content of the nucleus, and DNA content of the tail, all based on fluorescence measurements. Commonly, cells are scored on a point scale from 0 to 4 with 0 having no damage, and 4 having extensive damage (8).

Theoretically, the Comet assay can be performed using any eukaryotic cell type. Mammalian cells have been studied extensively, and recently cells from a marine invertebrate (the mussel *Mytilus edulis*) have been investigated (9). Bivalve mollusks are widely distributed sessile filter-feeders, and are therefore specially vulnerable to marine contaminants. For this reason, these organisms are suitable bioindicators of marine pollution (10). Many pollutants have genotoxic effects (2, 3), and consequently the presence of such agents in contaminated seawater may have an effect on the integrity of DNA in marine species. Specifically, Steinert’s group (11) studied the effects of contaminant exposure on the DNA of mussel hemocytes from *Mytilus edulis*. The hemocytes contained in the hemolymph are involved in digestion, excretion, and internal defenses against pathogens (12). The antimicrobial response of hemocytes includes the production of reactive oxygen species such as hydrogen peroxide (13). Due to this DNA-damaging oxidative stressors, along with the presence of genotoxic chemicals, hemocytes exposed to high levels of environmental pollution exhibit increased levels of DNA damage.

The present study evaluates the Comet assay as a method of detecting DNA damage in oyster hemocytes subjected to in vivo stresses. The bivalve mollusks examined were Pacific oyster species *Crassostrea gigas*. The specific stresses investigated were high levels of pollution and incubation with the enterovirus poliovirus type 1, which is an attenuated vaccine strain. Possible applications of this study include a non-specific detection of marine contaminants, quality monitoring of commercial oysters, and optimization of cultivation conditions based on DNA damage levels.

MATERIAL AND METHODS

Standardization of the Comet assay

The protocol used was similar to that outlined by Steinert and his group (11) for the analysis of mussel hemocytes. Oysters were opened to a width of approximately 1cm and hemolymph removed from the posterior adductor muscle using a 5ml syringe and a 26 gauge needle. Positive controls were run by addition of H2O2 to 200, 500, and 1000 mM. One hundred µl aliquots of hemolymph were placed in 1.5ml Eppendorf tubes and they were incubated for one hour at room temperature. The hemolymph was centrifuged for 2 min at 2000Xg and supernatant was then discarded, and the pellets were resuspended in 150µl KLMA (0.5% Gibco low melting DNA grade agarose in Kenny’s Salt Solution consisting of 0.4M NaCl, 9mM KCl, 0.7mM K2HPO4, and 2mM NaHCO3, pH 7.5) at 37°C. Fifty microliters of the cell suspension were then transferred onto frosted slides previously coated with one layer of 1.5% Gibco Normal Melting Agarose (NMA) in PBS free of Ca2+ and Mg2+, gelled, and a second layer of 0.75% NMA in PBS free of Ca2+ and Mg2+.

A coverslip was placed over and left to gel at 4°C for 5 min. Coverslips were then removed and slides placed in glass screw-top Coplin jars containing lysis solution (2.5M NaCl, 10mM Tris, 0.1M EDTA, 1% sarcosyl, 1% Triton X-100, and 10% DMSO, pH 10.0) and incubated at 4°C for at least 2h. Slides were then placed to in a submarine gel electrophoresis chamber filled with 300mM NaOH and 1mM EDTA, and the DNA was
allowed to alkaline unwinding for 15 min at 4°C. Following unwinding, the samples were subjected to electrophoresis at 300mA, 25V, for 10 min at 4°C. The slides were then neutralized with three 5-min rinses in 0.4M Tris, removed, excess solution blotted away and DNA stained with 40ml of a 20mg/ml solution of ethidium bromide in distilled water, and a coverslip applied. Slides were analyzed under epifluorescence microscope (excitation filter 510-560nm green light, barrier filter 590nm) at 200X magnification. For each slide, 100 cells were graded for level of DNA damage with a score of 0 indicating no damage to 4 indicating extensive damage. A percentage of DNA damage was then calculated for each slide.

Pollution study
Twenty live oysters cultivated in a farm at Sambaqui Beach on the Island of Santa Catarina, Santa Catarina State, Brazil were relocated to a highly polluted site near the Hercilio Luz Bridge (downtown) in Florianópolis City, in Santa Catarina state. At 0, 1, 2, 4, 6, and 9 weeks after relocation, three oysters were randomly chosen for analysis. Oysters were transported on ice to the laboratory, the shells were scrubbed through running tap water, and hemolymph withdrawn and pooled. The Comet assay, as described above, was performed in triplicate. Hemolymph from fresh Sambaqui Beach oysters were used as negative controls for each experiment. Hemolymph samples treated with 200, 500 and 1000μM H₂O₂ were used as positive controls.

Enterovirus Study
Poliovirus titration by pfu assay was done as previously described (14). Poliovirus type 1 (attenuated vaccine strain) was propagated in VERO cells cultivated in Sigma Medium 199, supplemented with 5% fetal bovine serum (Gibco). Confluent monolayers of VERO cells in 75 cm² cell culture flasks were infected with this virus. Following one hour of adsorption, excess viruses were removed in the supernatant and fresh medium added. After 24h of infection (3 virus replication cycles), flasks were frozen at -80°C. Virus was prepared by three freeze-thaw lysis cycles and clarified by centrifugation at 20000Xg for 30 min. Virus titer was quantified using a plaque assay, performed using a 12-well plate containing a confluent layer of VERO cells. The maintenance media was removed from each well and 500μl of virus diluted in Medium 199 were added. Virus dilutions analyzed ranged from 10⁻³ to 10⁻⁵. After one hour of incubation with rocking agitation, unadsorbed viruses were removed, and 1ml of 0.75% carboxymethyl cellulose was added to each well. After 24h, the overlay was removed, and 500μl of a 1% solution of naphthalene black in distilled water were added. After 5min, the dye was removed, and lysis plaques were counted.

Oysters were incubated for 0, 1, 3, and 6h with zero, 4.1 x 10⁶ and 4.1 x 10⁷ pfu of poliovirus type 1 added to the containers. Each container was filled with 1.2 L filtered seawater from Barra da Lagoa Aquaculture Laboratory, and three live oysters from Sambaqui Beach. Viruses were added with approximately 2g Emerald Entree Fresh and Marine Omnivore Diet. The experiments were carried out at room temperature. Analysis was performed by pooling the hemolymph of three oysters and using the Comet assay, as described above, in triplicate.

RESULTS AND DISCUSSION
The Comet assay has been used to analyze DNA damage of oyster hemocytes under conditions of a highly polluted environment, or exposed to viral contamination. A large number of variables involved in the Comet assay procedure using oyster hemocytes may be due to the in vivo nature of the studies. The amount of hemocytes in a given amount of hemolymph fluctuated greatly between samples. Therefore, to obtain a consistent amount of cells for analysis was a very difficult task. Crowding of cells can complicate classification of comets, producing less accurate results. Along with variations in cell number, there are also considerable variations in cell types. Oysters possess two types of hemocytes: granulocytes have small and round nuclei whereas the nuclei of hyalinocytes are large and irregular in shape (12). For consistency, in this study only hyalinocytes were analyzed in all experiments.

Pollution study.
After one week of exposure in the polluted site, percentage of DNA damage, as determined by the Comet assay, increased to a maximum of 24.8%. The samples collected after 14, 28, 42, and 63 days of exposition had lower levels of
DNA damaged than those at one week. Negative controls showed DNA damage ranging from 4.3% to 9.8% (Figure 1).

These results indicated an increase in hemocyte DNA damage after one week of relocation to the polluted site, followed by a decrease in damage. A slight increase was observed between two and nine weeks of incubation, but this difference was not significant due to high data variability. The negative samples, which were from oysters obtained from an uncontaminated site, were observed by the Comet assay to have less than 10% DNA damage. The low levels of DNA damage in the negative controls may have been caused by several factors. It is possible that during hemolymph extraction and hemocytes isolation some DNA damage was introduced. Although the procedures were performed under artificial light, some ultraviolet exposure could have occurred and may have caused the observed DNA damage. Additionally, mechanical agitation of cells during withdrawal and centrifugation of hemocytes could lead to DNA damage. It is also possible that a small percentage of DNA damage naturally occurs at the hemocytes even under unpolluted conditions. The immediate increase in DNA damage, followed by a rapid recovery is contrary to the time-dependant increase we expected. One possible explanation for this observation is an adaptive response by the oysters. Oysters may initially be susceptible to the genotoxic agents in the water, but later develop resistance, possibly through a selective filtration mechanism. It is also possible that after one week period oyster tissue was saturated with contaminants, at which time no more genotoxic substances could be absorbed. Then, upon the regeneration of hemocytes, a decrease in DNA damage was observed. Similar results were observed in a study realized by Wilson and his group (9) who analyzed the gill cells of the marine mussel Mytilus edulis by using the Comet assay. Their results for untreated control animals were extremely variable over time, mussels showed a sharp increase in DNA damage levels after just 24h of relocation to clean, aerated seawater. This increase was followed by a two day recovery period, where levels of DNA damage decreased. These results suggest that the stress of relocation itself can produce a significant increase in DNA damage levels. An important consideration with in vivo studies is the possibility for DNA repair during the transport and preparation of samples (15). In order to decrease the activity of repair enzymes, oysters in this study were stored on
ice during transport from the collection site to the laboratory. Additionally, samples were always processed immediately after arrival in the laboratory, (approximately 30 min after collection). However, even this short period could allow for some DNA repair, resulting in underestimation of DNA damage.

**Enterovirus study**

Hemocyte samples from the oysters incubated with both amounts of poliovirus (4.1 x 10^5 and 4.1 x 10^7 pfu), for all times analyzed (1, 3, and 6 hours), showed large percentages of DNA damage, as determined by the Comet assay. Percentages of DNA damage in samples exposed to poliovirus ranged from 72.5% to 86.8%. In contrast, samples not exposed to poliovirus showed DNA damage ranging from 3.8% to 15.6% (Figure 2).

This study showed high levels of DNA damage by using the Comet assay. The values were in the range of 80% after as little as one hour of incubation with the attenuated poliovirus vaccine strain. Similar results were observed with both virus concentrations analyzed (4.1 x 10^5 and 4.1 x 10^7 pfu) for all incubation times. Samples from untreated controls showed DNA damage ranging from 3.8% to 15.6%. The rapid response to the presence of poliovirus may be partially attributed to the high filtration rate of oysters. It is known that these mollusks can filter over 100L of water in a single hour. With only 1.2L of seawater in the experimental containers used, it is conceivable that after only one hour of incubation, all the poliovirus had been removed from the water and concentrated in oyster tissues. In fact, other researchers (16) found that viruses could be detected in mussel tissues after just one hour of contact and maximum levels were observed after six hours. The possibility of DNA repair during the period between virus exposure and analysis is less of a concern in this study than in the pollution exposure experiment. Incubation was performed within the laboratory, eliminating the need for transport of samples. Hemolymph was immediately removed following virus exposure to prevent the repair of induced damage. Negative controls showed higher levels.
of damage than those used for the pollution study, as discussed above. This could be due to incubation at room temperature, which was ca. 25°C as opposed to the in situ environmental exposure negative samples that were maintained at ca. 15 to 18°C during the period of this study (October/November 1999). As seen in Figure 2, higher levels of damage in untreated oysters were observed after 3 and 6h of incubation. The optimal temperature for living oysters is approximately 15-18°C (9). This elevated temperature could cause thermal stress even in the absence of poliovirus. However, it is evident that the presence of poliovirus at the concentrations used for exposure had a significant effect on the level of DNA damage in oyster hemocytes.

Further work should be done to investigate the genotoxic effects of lower concentrations of poliovirus in a similar study. This would allow for the determination of the lower limits of detection of DNA damage induced by incubation with poliovirus. The effects of incubation with other enteroviruses should also be investigated, and compared to those here using the attenuated vaccine strain of poliovirus.

REFERENCES


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