DNA damage in *Mytella falcata* (Mytiloida, Mytilidae) cells: a new tool for biomonitoring studies in tropical estuarine ecosystems

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Abstract

Bivalve filter feeders are sessile animals frequently used as sentinels in aquatic environments. For this reason it is important to identify native key species in tropical environments, since the majority of studies were conducted in temperate regions. This study was developed to evaluate if *Mytella falcata*, a tropical estuarine bivalve, could be used to detect genotoxic damages by means of the comet assay. Individuals of this species were exposed *in vivo* to methyl methanesulphonate concentrations; a control group, not exposed, was conducted in the same way. Haemolymph and gill cells were submitted to the comet assay, to evaluate the sensitivity of these tissues. DNA damage increase was detected in both tissues, however statistical difference was observed only in haemolymph cells, maybe because of the damages generated during the dissociation process needed in gill cells manipulation. It was concluded that *Mytella falcata* had the expected response to this direct acting mutagenic substance and that haemolymph cells are better material for genotoxicity studies, because they can be easily obtained.

Key words: genotoxicity, gill cells, haemolymph cells, mussel, tropical environments

Introduction

Water pollution directly influences aquatic life. Changes in cell genome, caused by genotoxic agents, can lead to mutations and possibly formation of tumors. Thus, DNA damaging agents must be detected and continuously monitored (Villela et al., 2006). For this reason, assays that can measure the effects of environment pollutants must be developed and applied in a wide range of organisms. Ostling and Johanson (1984) were the first researchers to develop a microgel electrophoresis technique for detecting DNA damages at the level of single cell. Singh et al. (1988) introduced a microgel technique involving electrophoresis under alkaline (pH >13) conditions for detecting DNA damage in single cells, named as comet assay.

The comet assay presents some advantages in comparison with other genotoxicity assays such as: sensitivity for detecting low levels of DNA damage and requirement of low number of cells per sample (Tice et al., 2000). However, studies with mussels as bioindicators of genotoxic damages in aquatic habitats were conducted almost exclusively in temperate regions with *Mytilus* species; these

species are not present in tropical areas, where pollution monitoring strategies must be developed (Tavares et al., 1988; Gregory et al., 2002; Nicholson and Lam, 2005). *Mytella falcata* (Mytiloida, Mytilidae) is a bivalve species found in the Atlantic Coast, from Venezuela to Argentina and also in the Pacific Coast and the Galapagos Islands (Narchi and Galvão-Bueno, 1983). It is considered as an important food item, and it is highly consumed in northeastern Brazil. In many cases it is the only source of protein for the inhabitants of some poor villages (Boffi, 1979).

The aim of this study was to adapt the comet assay to *Mytella falcata* cells and analyze if the comet assay can be used in this species as a reliable indicator of DNA damages. During the experiment, sensitivity of two tissues (haemolymph and gills), exposed *in vivo* to the direct acting genotoxic substance methyl methanesulphonate, was compared.

MATERIAL AND METHODS

Mytella falcata individuals were collected in August 2006 at a low to moderated impacted area of Branco River, Santos/São Vicente estuary (Brazil) (23º55.0,52´S, 46º26.975´W). This site is a tributary of the estuarine channel that shows low to moderated influence of industrial activity (CESTESB, 2001). Samples were immediately transported to the laboratory, where they were kept for 5 days in 5L aquarium with water from the collection site, the water was maintained under constant aeration and 1L of water was changed every day. This time was considered as an adaptation time to the laboratory conditions.

Seven bivalves were placed in Methyl methanesulphonate (MMS) solutions for 40 minutes which were prepared with the water from the collection site at concentrations of 0.6, 1.2 and 2.4 x 10-4 M., this proceedings were taken according to the experiment of Rigonato et al. (2005). As a control group seven bivalves were conducted in the same way but were not exposed to MMS. Haemolymph was collected from the posterior adductor muscle using a syringe and 10 µL aliquots were used in the comet assay. Small gill fragments were removed and gently macerated in phosphate buffered saline (PBS) to obtain a cell suspension out of which 10 µL aliquots were submitted to the comet assay.

Comet assay was conducted according to Singh et al. (1988) with some modifications described below. Microscope slides pre-coated with normal melting point agarose 1.5%, were covered with 10 µL aliquots from the different cell suspensions mixed with 90 µL of low melting point agarose 0.5%. The slides were covered with coverslips and put in refrigerator for 10 minutes. The coverslips were removed and cells were submitted to the lysis solution (2.5 M NaCl, 100mM EDTA and 10mM Tris, pH 10.0-10.5) containing 1% of Triton X-100 and 20% of dimethyl sulfoxide (DMSO) for at least 1 hour and at most 3 hours at 4ºC. Slides were incubated in freshly prepared alkaline buffer solution (300mM NaOH and 1mM EDTA, pH=12.1) for 20 minutes and then submitted to electrophoresis for 15 minutes under 21V and 270mA (0.8V.cm-1). All these steps were conducted under indirect light. Slides were neutralized, washed three times in distilled water and left to dry

overnight at room temperature. Slides were fixed for 10 minutes in fixative solution (trichloroacetic acid 15%, zinc sulphate 5%, glycerol 5%), washed three times in distilled water and dried for 2 hours at 37ºC. The dry slides were re-hydrated for 5 minutes in distilled water and then stained (sodium carbonate 5%, ammonium nitrate 0.1%, silver nitrate 0.1%, tungstosilicic acid 0.25%, formaldehyde 0.15%, freshly prepared in the dark) under constant shaken for 7 minutes at 37 ºC. After staining, the slides were washed two times in distilled water and submitted to stop solution (acetic acid 1%), washed again and dried at room temperature.

The slides were analyzed under the light microscope at a 400x magnification and 100 cells were scored per individual. The comets were visually classified in migration categories according to the size of the comet tail (Figure 1): class 0, no tail; class 1, a small tail less than the head (nucleus) diameter; class 2, tail length equal to or up to twice the head diameter; and class 3, tail more than twice the head diameter (Rigonato et al., 2005). The damage score was calculated as the sum of the number of cells in each class and the total of each class multiplied by the value of this class (0-3); therefore, scores can vary from 0 (all cells without damage – 0x100) to 300 (all cells with the maximum damage - 3x100). International recommendations and guidelines for the comet assay consider the comet visual analysis as a well established evaluation method that can be used with high confidence (Tice et al., 2000).

The damage frequency was calculated as the percentage of cells with a tail. Damage score and damage frequency from the control and exposed animals were statistically analyzed using individual mussels as unit of analysis. The data obtained was 3 normally distributed and variance was homogeneous so the analysis of variance (ANOVA) and Dunnett's multiple comparisons of means were applied at a 0.01 level of significance.

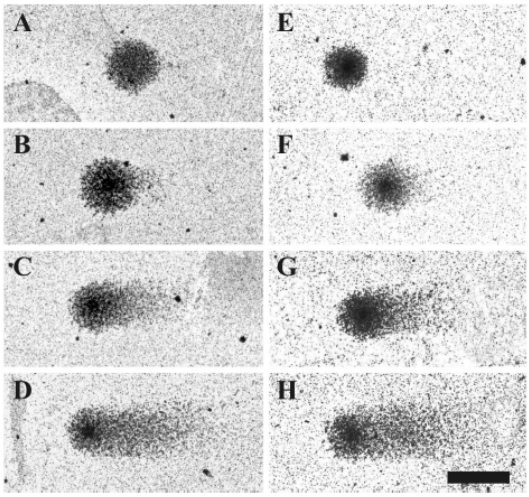

Figure 1. Cell damage classes as detected by the comet assay in haemolymph (**A**,**B**,**C**,**D**) and gill cells (**E**,**F**,**G**,**H**) of *Mytella falcata* exposed to methyl methanesulphonate (MMS). **A,E.** class 0, no tail; **B,F.** class 1, a small tail less than the head (nucleus) diameter; **C,G.** class 2, tail length equal to or up to twice the head diameter; **D,H.** class 3, tail more than twice the head diameter. Bar = 50µm

RESULTS

The dimethyl sulfoxide (DMSO) concentration (20%) in the lysis solution was increased since previous experiments demonstrated that the use of the usual concentration (10%) (Singh et al., 1988; Tice et al., 2000) resulted in high DNA damage in all *Mytella falcata* cells, as well as the pH>13 in the alkaline buffer solution. Those DNA damages made the results difficult to analyze and required very low voltage and time during the electrophoresis to turn the control group reliable.

Haemolymph cells and gill cells showed an increase in the damage score according to the increase of the MMS concentrations (Table 1). When comparing the damage score obtained for the haemolymph control group and the gill cells control group, it was observed that haemolymph cells showed lower damage levels when compared to gill cells (Table 1). A significant difference was observed between the damage score of haemolymph cells control group and all concentrations analyzed (Table 1). On the other hand, the damage score observed for the exposed gill cells did not present significant difference in relation to the data found for the control group of this tissue (Table 1).

The damage frequency had a significant increase according to the increase in MMS concentrations for the haemolymph cells, whereas gill cells groups presented almost the same damage frequency independent of the MMS concentration (Table 1).

When the comet distribution between migration classes was observed it was clear that for haemolymph cells the number of undamaged cells decreased according to the MMS concentrations increase, while the other classes had an increase (Table 1). Gill cells did not show such response and the distribution of comets between migration classes was almost the same in all MMS concentrations. It was also observed that the number of class 3 comets were higher than class 2 comets for all concentration including the control group (Table 1).

DISCUSSION

Mussels exhibit desirable characteristics to serve as sentinel organisms. They are long lived and easily sampled and their sedentary nature means that their geographical relationship to a pollution source can be easily ascertained. Most of the studies that used mussels as sentinels have been concentrated in Europe and North America and have principally centered about their local indigenous species of the *Mytilus* genus. However, interest has been increasing in areas such as the Asia-Pacific region, Australia and South America (Gregory et al., 2002). In Brazil some studies were developed with the freshwater bivalves *Corbicula fluminea* (Rigonato et al., 2005) and *Limnoperna fortunei* (Villela et al., 2006), but studies with species that inhabits estuarine systems were not developed yet. The establishment of a methodology as the comet assay in *Mytella falcata* cells proposed in this study creates an important tool to identify pollutants in tropical estuarine systems.

Table 1. Detection of DNA damage by the comet assay in haemolymph and gill cells of *Mytella falcata* exposed to methyl methanesulphonate (MMS).

	Gill cells						Haemolymph cells					
	Migration categories						Migration categories					
	0	1	2	3	DS [a]	DF [b]	0	1	2	3	DS [a]	DF [b]
	37	15	8	40	151	63	88	5	4	3	22	12
	44	25	7	24	111	56	52	22	10	16	90	48
	20	34	24	22	148	80	62	19	9	10	67	38
Control	53	35	6	6	65	47	70	25	5	0	35	30
	54	19	8	19	92	46	66	28	4	2	45	35
	64	10	9	17	79	36	56	33	7	4	59	44
	48	21	17	14	97	52	62	19	9	10	67	38
M	**45.7**	**22.7**	**11.2**	**20.2**	**106.1**	**54.2**	**65.1**	**21.5**	**6.8**	**6.4**	**55**	**35**
SD±	**14.1**	**9.3**	**6.6**	**10.4**	**32.9**	**14.1**	**11.7**	**8.8**	**2.5**	**5.7**	**22.7**	**11.7**
	43	12	16	29	131	57	39	34	16	11	99	61
	35	20	8	37	147	65	44	41	7	8	79	56
	74	11	7	8	49	26	54	22	13	11	81	46
$0.6x10^{-4}$ M	37	20	20	23	129	63	42	44	10	4	76	58
	50	19	14	17	98	50	29	31	23	17	128	71
	48	18	16	18	104	52	14	63	17	6	115	86
	49	15	17	19	106	51	16	53	12	19	134	84
M	**48**	**16.4**	**14**	**21.5**	**109.1**	**52**	**34**	**41.1**	**14**	**10.8**	**101.7***	**66***
SD±	**12.8**	**3.7**	**4.7**	**9.3**	**31.8**	**12.8**	**14.9**	**13.8**	**5.2**	**5.5**	**24.2**	**14.9**
	29	19	11	41	164	71	1	84	10	5	119	99
	47	24	7	22	104	53	18	39	26	17	142	82
	25	24	20	31	157	75	12	57	15	16	135	88
$1.2x10^{-4}$ M	47	24	16	13	95	53	31	47	15	7	98	69
	58	19	9	14	79	42	21	51	18	10	117	79
	44	26	18	12	98	56	19	31	20	30	161	81
	42	42	8	8	82	58	15	70	10	5	105	85
M	**41.7**	**25.4**	**12.7**	**20.1**	**111.2**	**58.2**	**16.7**	**54.1**	**16.2**	**12.8**	**125.2***	**83.2***
SD±	**11.3**	**7.7**	**5.2**	**11.9**	**34.7**	**11.3**	**9.1**	**18.1**	**5.6**	**9**	**22**	**9.1**
	20	20	23	37	177	80	9	37	27	27	172	91
	24	19	15	42	175	76	17	46	16	21	141	83
	55	18	13	14	86	45	17	48	19	16	134	83
$2.4x10^{-4}$M	36	25	21	18	121	64	30	51	12	7	96	70
	52	32	10	6	70	48	33	52	8	7	89	67
	39	20	24	17	119	61	8	80	7	5	109	92
	37	22	17	24	128	63	9	44	33	14	152	91
M	**37.5**	**22.2**	**17.5**	**22.5**	**125.1**	**62.4**	**17.5**	**51.1**	**17.4**	**13.8**	**127.5***	**82.4***
SD±	**12.9**	**4.8**	**5.2**	**12.8**	**40.4**	**12.9**	**10.2**	**13.6**	**9.7**	**8.1**	**30.6**	**10.2**

[a] damage score: 0 = no damage; 300 = maximum damage

[b] damage frequency: 0% = no damage; 100% = maximum damage

* $p<0.01$; in all cases significance was tested with respect to control using ANOVA and Dunnett's test

Since the comet assay technique was published, it has already been used in a number of studies in a great variety of organisms and tissues, and some alterations have been necessary for its execution. The major alterations made in this work were the increase in the DMSO concentration and the utilization of pH 12.1. DMSO is widely used as a solvent and is known to be a radical scavenger with high affinity for hydroxyl radicals (Repine et al., 2001) and since cell disruption is needed for the comet assay and this process can extravasate harmful substances to the DNA, the increase in DMSO concentration prevents the induction of DNA damage after the experimental development.

The pH variation during electrophoresis is related to the kind of DNA breaks expressed (Lee and Steinert, 2003). The pH>13 is widely used and highly recommended mainly for vertebrate cells due to the fact that this pH detects a greater variety of DNA damages including breaks in single and double strands and alkali-labile sites (Tice et al., 2000). On the other hand, single strand breaks do not have a drastic effect once they are rapidly repaired. Double strand breaks are more severe lesions in effect; besides this they have a much more complex repair. Lesions in the double strand, in this way, can be considered as of greater importance and can be visualized under neutral pH (Dixon et al., 2002). In marine bivalves and polychaetes cases, that have a naturally high incidence of alkali-labile sites, neutral pH can be considered more informative than pH>13 (Dixon et al., 2002). The pH 12.1, used in this study, was capable to demonstrate damages without activating the alkali-labile sites and for this reason is recommended for the comet assay when applied to *Mytella falcate* cells.

Experiments with genotoxic substances, that present known effects on the DNA molecule, are realized to validate the comet assay in organisms that were still not used as bioindicators (Rigonato et al., 2005; Villela et al., 2006). Rank and Jensen (2003) applied the comet assay in *Mytilus edulis* gill and haemolymph cells exposed to MMS, hydrogen peroxide and UV radiation and concluded that all tissues are equally sensitive to these agents, but due to the facility in obtaining haemolymph cells, this cellular type would be the most indicated in genotoxicity studies. Siu et al. (2004) stated that the use of haemolymph cells in genotoxicity studies is interesting because of their role in the immune defense, phagocytosis, transport, excretion and detoxification of xenobiotics. Besides this, it was suggested that the multifunctional characteristics of these cells provide a higher sensitivity to external factors such as genotoxic xenobiotics.

The results obtained in this study were very similar to those found by Rigonato et al. (2005) in *Corbicula fluminea*; applying the comet assay in the digestive gland, haemolymph and gill cells of this bivalve exposed to MMS, the authors concluded that gill cells seem to be less appropriated because they present high levels of basal damage, what can masquerade the results.

Another fact to be considered is that gill filaments presents many cell types. Mitchelmore and Chipman (1998) suggested that a high control response in the comet assay in some cell types may be a feature related to DNA packaging and background alkali-labile sites rather than endogenous strand breaks. When the comet assay is conducted in different tissues the normal DNA damage can be highly variable. Different cell types may have very different background levels of DNA breaks due to variation in excision repair activity, metabolic activity, anti-oxidant concentrations or other factors (Lee and Steinert, 2003). In the present study, there were no differences between gill cells from exposed groups and the control group; this fact can be a result of the variety of cellular types in gills which can present different answers to the mutagenic agent; or can be the result of the tissue manipulation necessary to obtain the cellular suspension.

In conclusion, the increase in DMSO concentration, the pH adaptations and the electrophoretic conditions performed here allowed a better visualization of DNA damages, minimizing the scores of the control group and facilitating the results analysis. These results demonstrated a close relation between DNA damage and the direct acting genotoxic substance concentrations in haemolymph cells. Gill cells presented high damage levels that can lead to misinterpretations of genotoxic actions in the DNA molecule and need a more detailed analysis. The comet assay can be employed pricisely in *Mytella falcata* haemolymph cells to detect DNA damage in biomonitoring studies of tropical estuarine regions.

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