Aquatic Toxicology 124–125 (2012) 133–138

Contents lists available at SciVerse ScienceDirect

Aquatic Toxicology

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

Sea urchin coelomocytes are resistant to a variety of DNA damaging agents

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ARTICLE INFO

Article history:
Received 3 July 2012
Received in revised form 7 August 2012
Accepted 7 August 2012

Keywords:
Abasic sites
DNA damage
Sea urchins
Coelomocytes

ABSTRACT

Increasing anthropogenic activities are creating environmental pressures that threaten marine ecosystems. Effective environmental health assessment requires the development of rapid, sensitive, and cost-effective tools to predict negative impacts at the individual and ecosystem levels. To this end, a number of biological assays using a variety of cells and organisms measuring different end points have been developed for biomonitoring programs. The sea urchin fertilization/development test has been useful for evaluating environmental toxicology and it has been proposed that sea urchin coelomocytes represent a novel cellular biosensor of environmental stress. In this study we investigated the sensitivity of coelomocytes from the sea urchin *Lytechinus variegatus* to a variety of DNA-damaging agents including ultraviolet (UV) radiation, hydrogen peroxide (H_2O_2), methylmethane sulfonate (MMS) and benzo[a]pyrene (BaP). LD_{50} values determined for coelomocytes after 24 h of exposure to these DNA damaging agents indicated a high level of resistance to all treatments. Significant increases in the formation of apurinic/apyrimidinic (AP or abasic) sites in DNA were only detected using high doses of H_2O_2, MMS and UV radiation. Comparison of sea urchin coelomocytes with hemocytes from the gastropod mollusk *Aplysia dactylomela* and the decapod crustacean *Panulirus argus* indicated that sensitivity to different DNA damaging agents varies between species. The high level of resistance to genotoxic agents suggests that DNA damage may not be an informative end point for environmental health assessment using sea urchin coelomocytes however, natural resistance to DNA damaging agents may have implications for the occurrence of neoplastic disease in these animals.

1. Introduction

Marine environmental health assessment is used to predict negative impacts of natural and anthropogenic stress on individual species and ecosystems. Monitoring changes in the biology of sentinel organisms provides a useful approach to assessing aquatic ecosystem health. Biological changes at a cellular or molecular level that can ultimately affect organismal processes such as development, growth, reproductive output and survival are particularly useful biomarkers of environmental stress. Measuring cellular damage or changes in expression of stress-response genes or proteins provides a rapid and sensitive assessment of stress often before organismal level changes are apparent (Jha, 2008). In the marine environment, sessile species are particularly well suited to monitoring studies and bivalve mollusks such as mussels (e.g. *Mytilus edulis* and *Mytilus galloprovincialis*) have been widely used for monitoring aquatic ecosystem health (Dixon et al., 2002; O'Connor, 2002). However inter-species differences in vulnerability to toxicity has led to the use of additional sentinel species such as polychaete worms (e.g. *Arenicola marina*, *Nereis diversicolor*, *Nereis virens*) and teleost fish (e.g. *Lipophrys pholis*) (Lewis and Galloway, 2008; Lima et al., 2008). Ultimately, an integrated approach involving multiple biomarkers and a variety of species or cell types may be required to understand ecological consequences of environmental stress (Jha, 2008).

Sea urchins represent an important sentinel animal but its use has been mainly limited to evaluating toxicity in early embryogenesis and larval development. The sea urchin embryo test is a sensitive and cost-effective biological tool for marine monitoring however it lacks an automatically readable endpoint limiting its widespread routine use (Saco-Álvarez et al., 2010). It has been suggested that sea urchin coelomocytes represent a novel cellular biosensor of environmental stress (Pinsino et al., 2008). In support of this idea, an increase in the number of red amoebocytes and an increased expression of heat shock protein-70 were detected in coelomocytes at a site with a persistent contaminating metal source (Pinsino et al., 2008). To further explore the possibility of using sea urchin coelomocytes as cellular biosensors, we investigated the sensitivity of coelomocytes from the sea urchin

Abbreviations: AP sites, apurinic or apyrimidinic sites; BaP, benzo[a]pyrene; BER, base excision repair; CMFSW-E, calcium and magnesium-free sea water with EDTA; H_2O_2, hydrogen peroxide; MMS, methyl methanesulfonate; UV, ultraviolet.

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http://dx.doi.org/10.1016/j.aquatox.2012.08.008

Lytechinus variegatus to DNA-damaging agents. The results reveal that coelomocytes are highly resistant to a variety of treatments suggesting that DNA damage may not be an informative end point for environmental health assessment using sea urchin coelomocytes. However, natural resistance to DNA damaging agents may have an implication for the occurrence of neoplastic disease in these animals.

2. Materials and methods

2.1. Collection of animals and treatment of coelomocytes or hemocytes

L. variegatus were collected from Richardson's Bay or Mangrove Bay, Bermuda in 1–2 m of water and left to equilibrate in flow-through aquaria for 5–7 days to recover from any stress induced by the collection process. Coelomic fluid was collected following removal of Aristotle's lantern and aliquoted into 3 ml volumes for various treatments. Hydrogen peroxide (H_2O_2) (Sigma, 349887) and MMS (Sigma, M4016) were added at the indicated concentrations. BaP (Sigma B1760) was dissolved in DMSO and added at indicated concentrations with a final concentration of DMSO of 0.1%. For all treatments the control samples were given an equivalent volume of vehicle without the genotoxic agent. For ultraviolet radiation exposure, 3 ml aliquots of coelomic fluid were placed into 6 cm glass petri dishes and exposed (uncovered) to 254 nM in a UV Stratalinker 1800 (Stratagene) to deliver total doses of 300, 1000, 3000 or 9999 J/m^2. The cells were maintained at room temperature (23 °C) throughout all treatments and coelomic fluid was not pooled from different individuals for any treatments. At indicated times following exposure (1 h, 6 h or 24 h) the cells were diluted with an equal volume of CMFSW-E media (460 mM NaCl, 10 mM KCl, 7 mM Na_2SO_4, 2.4 mM $NaHCO_3$, 30 mM EDTA, pH 7.4) and centrifuged at 6000 × *g* for 5 min. The cells were resuspended in 1 ml of CMFSW-E media and an aliquot was removed for counting using the trypan blue exclusion method. For this, the cells were diluted with an equal volume of 0.8% trypan blue in CMFSW-E and loaded into a hemacytometer where bright cells were counted as live, while blue cells were considered dead under light microscopy. Counting indicated no increase in cell number during the 24 h exposure period. At the 24 h time point, the percent of dead cells was plotted versus dose of DNA damaging agent and the LD_{50} value (the dose where 50% of the cells were dead) was calculated using regression analysis. The remaining cells were washed twice with CMFSW-E media and cell pellets were frozen at −80 °C prior to DNA extractions. In indicated experiments, the coelomocytes were washed and resuspended in CMFSW-E media prior to treatments.

Aplysia dactylomela (spotted sea hares), collected outside the entrance to Coot Pond on the Northeast side of Bermuda, were allowed to acclimate in flow-through aquaria for 2–3 days prior to extracting hemolymph. Hemolymph was collected from the haemocoel along the side of the head using a syringe fitted with an 18 gauge needle. Hemolymph was divided into 1 ml aliquots for treatments as described above for sea urchins. At the same time 0.1 ml aliquots were mixed with an equal volume of CMFSW-E (to prevent cell aggregation) prior to treatments. Twenty-four hours following treatment, the 1 ml aliquots were centrifuged, washed and pellets frozen prior to DNA preparations and the 0.1 ml aliquots were used for cell counting using trypan blue exclusion as described above.

Panulirus argus (spiny lobsters) were collected in the Eastern Inshore Area near North Rock off Bermuda and acclimated in holding tanks for 7 days prior to drawing hemolymph. Hemolymph was collected with a syringe fitted with an 18 gauge needle inserted into the anterior-most ventral soft segment. Hemolymph was immediately mixed with equal volume of CMFSW-E to prevent clotting and 1 ml aliquots were used for treatments as described above for sea urchins. After 24 h of treatment, 20 μl was removed for cell counting as described above and the remaining cells centrifuged, washed and pellets frozen for DNA preparations.

2.2. DNA extractions and AP sites assay

DNA was prepared from the coelomocytes and hemocytes using a modification of the CTAB procedure of Doyle and Doyle (1987). Briefly, cell pellets were resuspended in CTAB buffer (100 mM Tris–HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% Cetyl Trimethyl Ammonium Bromide, 0.1% beta-mercaptoethanol) and incubated with 0.5 mg/ml proteinase K for 1 h at room temperature. Following an additional incubation with 20 μg/ml RNase at room temperature for 15 min, the samples were extracted twice with chloroform and precipitated with ethanol. To assess heat-labile DNA damage, the proteinase K portion of the extraction procedure was conducted at 65 °C. DNA was visualized on agarose gels using the KODAK Image Station 4000R (Carestream Health Inc.) AP sites were measured in DNA from *L. variegatus* using the Oxiselect Oxidative Damage (AP sites) kit (Cell BioLabs). The plates were read on a VERSA Max microplate reader (Molecular Devices).

2.3. Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics – v19.0 (IBM SPSS Inc., Chicago, IL) and Minitab – v15 (Minitab, State College, PA) statistical software. Two-way repeated measures ANOVAs were used to assess the independent and interactive effects of treatment dose and treatment duration with both dose and treatment duration as within-subjects factors. Degrees of freedom were Greenhouse-Geisser adjusted to account for the violation of sphericity assumption when necessary. Post hoc tests were performed using paired-*T* tests with Holm's sequential Bonferroni correction.

3. Results

3.1. LD_{50} values for sea urchin coelomocytes exposed to DNA damaging agents

Table 1 shows the LD_{50} values determined for *L. variegatus* coelomocytes exposed to H_2O_2, MMS and UV radiation. To rule out the possibility that the coelomocytes are protected from damage by components of the coelomic fluid, the exposures to H_2O_2 and UV were repeated using cells that were washed and resuspended in CMFSW-E prior to exposure. Washing had no effect on the LD_{50} values (data not shown). The LD_{50} value was not determined for BaP as it was above the highest concentration tested (10 μg/ml) which is beyond the solubility of BaP in sea water. The percent of dead cells following 24 h exposure to the highest dose (10 μg/ml) of BaP was 8.3 ± 1.4% (n = 6) for *L. variegatus* coelomocytes.

Table 1
LD_{50} values for cells exposed to UV radiation, H_2O_2 and MMS.

Cell type	UV LD_{50} (J/m^2)	H_2O_2 LD_{50} (mM)	MMS LD_{50} (mM)
L. variegatus (coelomocytes)	6101 ± 1019 (n = 4)	120.1 ± 20.6 (n = 6)	15.4 ± 0.6 (n = 6)
A. dactylomela (hemocytes)	322 ± 20 (n = 4)	2.3 ± 0.4 (n = 4)	3.3 ± 0.9 (n = 4)
P. argus (hemocytes)	>9999 (n = 4)	2.2 ± 0.3 (n = 4)	8.1 ± 2.0 (n = 4)

Values shown are mean ± standard error of the mean.

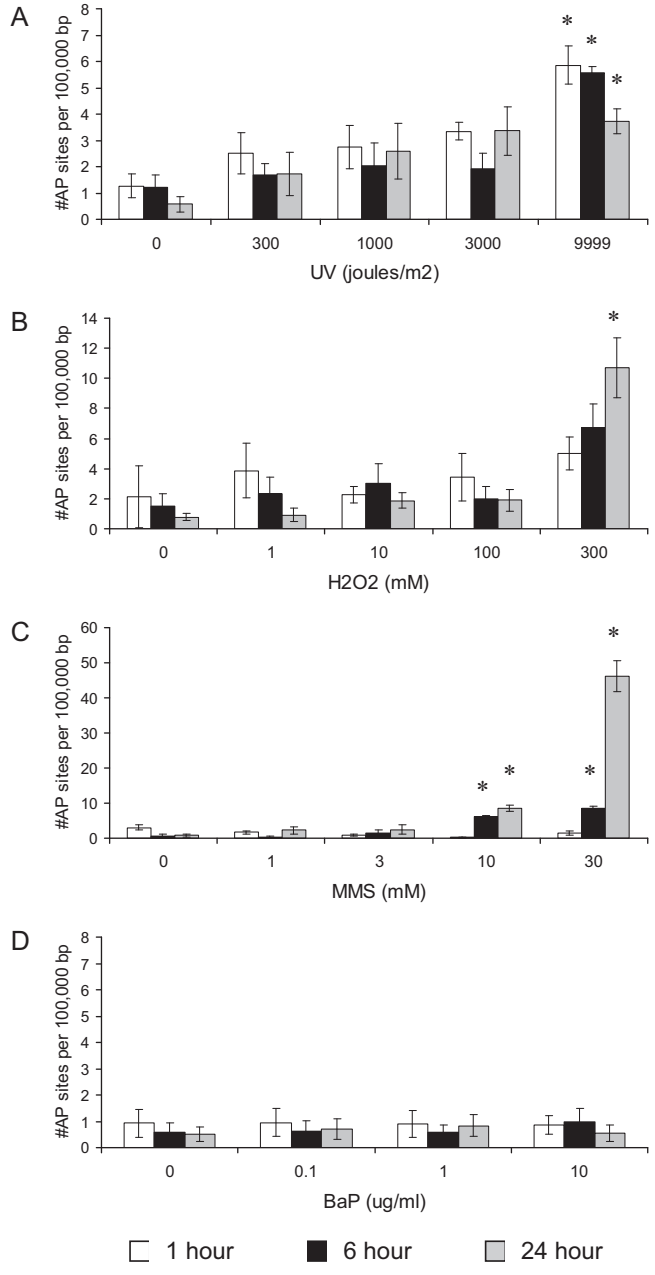

Fig. 1. AP sites in sea urchin coelomocytes treated with DNA damaging agents. The number of AP sites (per 100,000 bp) was measured in DNA isolated from coelomocytes treated with indicated concentrations of UV radiation (panel A), H_2O_2 (panel B), MMS (panel C) and BaP (panel D) for 1 h (white bars), 6 h (black bars) and 24 h (gray bars). The bars represent mean ± standard error; $n = 6$ for H_2O_2 and $n = 4$ for UV radiation, MMS and BaP. Asterisks indicate a significant increase ($p < 0.05$) from values obtained for untreated cells.

3.2. AP sites in DNA isolated from coelomocytes exposed to DNA damaging agents

The level of apurinic/apyrimidinic (AP or abasic) sites was assessed at different times throughout 24 h of exposure to the DNA damaging agents (1 h, 6 h and 24 h) (Fig. 1). Abasic or AP sites are one of the most common forms of DNA damage which are formed either directly as a result of oxidative attack on the deoxyribose moiety or during DNA repair processes (Atamna et al., 2000). There was no increase in AP sites detected in coelomocyte DNA following BaP exposures (Fig. 1, Panel D). Two-way repeated measures ANOVAs show a significant effect of dose for UV radiation

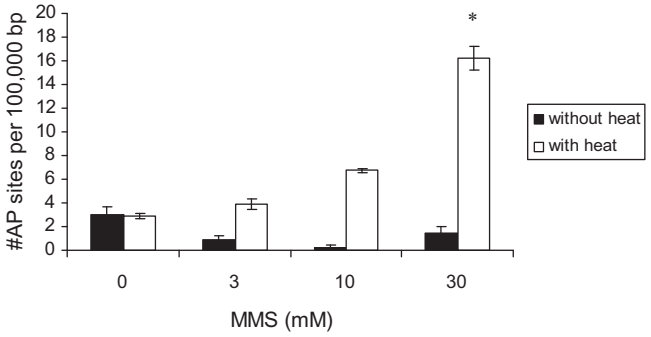

Fig. 2. Heat-labile AP sites in sea urchin coelomocytes treated with MMS for 1 h. The number of AP sites (per 100,000 bp) was measured in DNA isolated with heat (white bars) and without heat (black bars) from coelomocytes treated with MMS. The bars represent mean ± standard error; $n = 4$. The asterisk indicates a significant increase ($p < 0.05$) from values obtained for untreated cells.

($F_{1.697,5.091} = 11.780$, $p < 0.05$) with the number of AP sites significantly above background following exposure to the highest dose, 9999 J/m^2 (Fig. 1, Panel A). Two-way repeated measures ANOVAs also show a significant effect of dose for H_2O_2 ($F_{1.475,7.376} = 8.931$, $p < 0.014$) with the level of AP sites significantly above background following exposure to 300 mM for 24 h (Fig. 1, Panel B). We found no significant increase in AP sites following exposure to low concentrations of H_2O_2 (1 μM, 10 μM, 100 μM) (data not shown) in contrast to a report that submillimolar levels of H_2O_2 were more effective at inducing aldehydic DNA lesions than millimolar levels in some cell types (Nakamara et al., 2003). Washing the coelomocytes and resuspending them in CMFSW-E prior to exposure to H_2O_2 and UV radiation had no effect on the level of AP sites (data not shown). Two-way repeated measures ANOVAs revealed that MMS concentration and treatment duration showed independent and interactive effects (dose: $F_{1.334,4.003} = 86.566$, $p < 0.05$; treatment duration: $F_{2,6} = 48.596$, $p < 0.005$; interaction: $F_{1.459,4.377} = 58.839$, $p < 0.05$) (Fig. 1, Panel C). Post hoc analysis showed a significant increase in AP sites at 6 and 24 h after treatment with 10 mM and 30 mM MMS. It has been reported that alkylated DNA is heat-labile (Lundin et al., 2005) and therefore the DNA extractions for the cells treated with MMS for 1 h were repeated using heat (65 °C) during the proteinase K digestion. Two-way repeated measures ANOVA shows a significant effect of both MMS concentration and heat on the number of detectable AP sites (heat: $F_{1,3} = 123.099$, $p < 0.005$; concentration: $F_{3,9} = 83.432$, $p < 0.001$; interaction: $F_{3,9} = 113.217$, $p < 0.001$) (Fig. 2). Although there was an increase in AP sites with increasing concentration of MMS, the post hoc analysis revealed that this was only significant at the highest concentration. Heat-treatment during the DNA extractions for cells treated for 1 h with UV, H_2O_2 or BaP resulted in no increase in the amount of AP sites detected (data not shown).

Agarose gel electrophoresis of DNA isolated from coelomocytes treated with increasing concentrations of DNA damaging agents showed little gross structural damage to the DNA except at the highest concentrations of MMS, H_2O_2 and UV after 6 or 24 h (Fig. 3).

3.3. Comparison of sea urchin coelomocytes with hemocytes of other marine animals

To determine if the resistance to DNA damaging agents extends to cells of other marine invertebrates, we repeated the treatments using hemocytes from the gastropod mollusk *A. dactylomela* and the decapod crustacean *P. argus*. The LD_{50} values calculated at 24 h for hemocytes exposed to H_2O_2, MMS and UV indicate that sensitivity to different DNA damaging agents varies between species (Table 1). For H_2O_2 and MMS the hemocytes from *A. dactylomela*

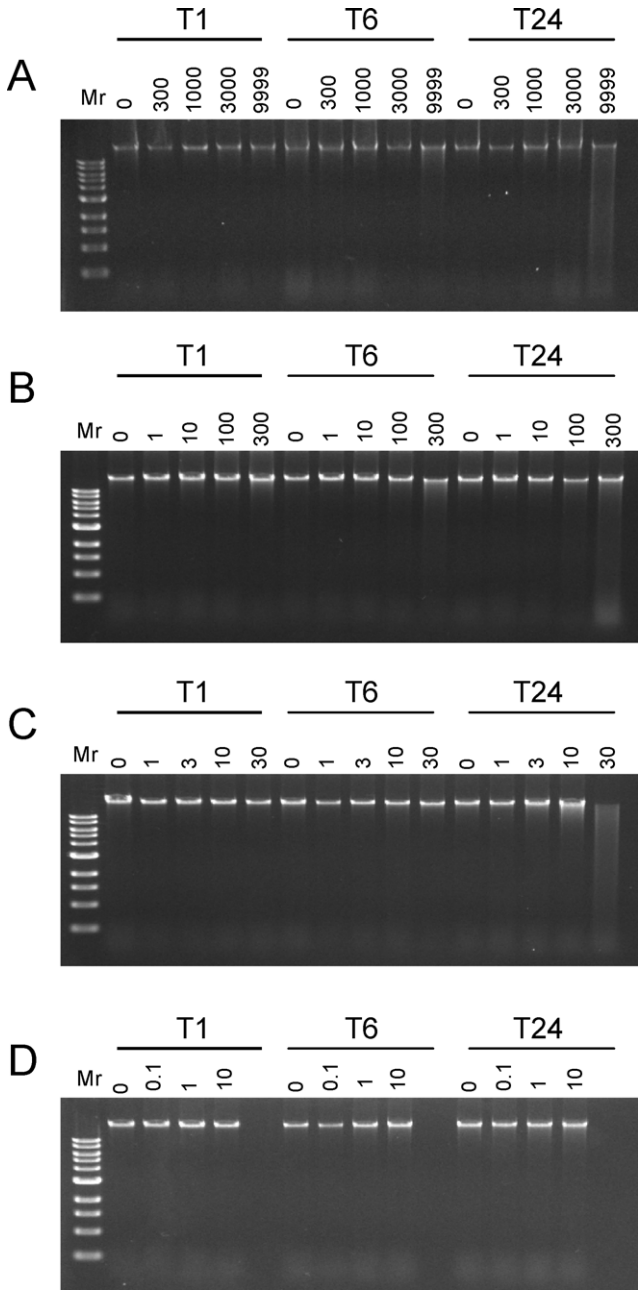

Fig. 3. Agarose gel electrophoresis of DNA isolated from sea urchin coelomocytes treated with UV radiation in J/m^2 (panel A), H_2O_2 in mM (panel B), MMS in mM (panel C) and BaP in μg/ml (panel D) for 1 h (T1), 6 h (T6) or 24 h (T24). Molecular weight markers (Mr) are the 1 kb DNA ladder from New England Biolabs with size range from 0.5 to 10 kb.

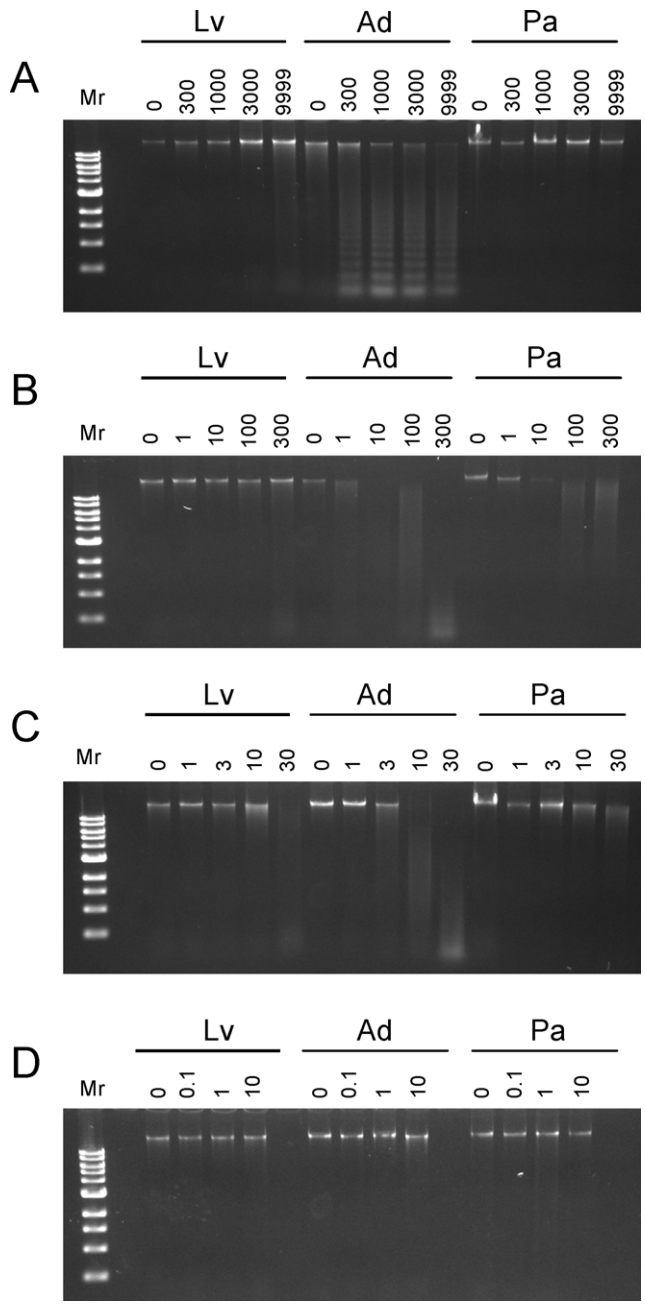

Fig. 4. Agarose gel electrophoresis of DNA isolated from coelomocytes of *L. variegatus* (Lv) and hemocytes of *A. dactylomela* (Ad) and *P. argus* (Pa) treated with UV radiation in J/m^2 (panel A), H_2O_2 in mM (panel B), MMS in mM (panel C) and BaP in μg/ml (panel D). Molecular weight markers (Mr) are the 1 kb DNA ladder from New England Biolabs with size range from 0.5 to 10 kb.

and *P. argus* were more sensitive than *L. variegatus*, however *P. argus* proved to be very resistant to UV radiation with only 33.2 ± 2.0% cell death at 24 h following treatment at the highest dose (9999 J/m^2). For BaP treatments, the percent dead cells at 24 h using the highest dose (10 μg/ml) was 8.3 ± 1.4%, 17.2 ± 2.9% and 34.2 ± 6.9% for *L. variegatus*, *P. argus* and *A. dactylomela*, respectively. Agarose gel electrophoresis of DNA isolated from hemocytes treated with increasing concentrations of DNA damaging agents was consistent with the relative sensitivities and also revealed that UV radiation induced a DNA banding pattern typical of apoptosis in the hemocytes of *A. dactylomela* (Fig. 4). AP sites were not measured in DNA from *P. argus* and *A. dactylomela.*

4. Discussion

Sea urchin coelomocytes provide a convenient primary cell model for examining cellular response to stress for ecotoxicology studies. However, our results show that coelomocytes from *L. variegatus* are resistant to a variety of DNA damaging agents with well characterized yet distinct modes of action: benzo[a]pyrene (BaP), methyl methanesulfonate (MMS), ultraviolet radiation (UV) and hydrogen peroxide (H_2O_2). These agents can generate abasic or AP sites either as a result of oxidative attack on the deoxyribose moiety or indirectly through the DNA base excision repair (BER) pathway (Atamna et al., 2000; Nakamara et al., 2003; Xue and Warshawsky,

2005). However, in our study, AP sites were only detected in coelomocytes treated with high doses of UV, MMS and H_2O_2. Washing the cells prior to UV and H_2O_2 treatments show that the resistance is not the result of protection or defense by components in the coelomic fluid such as UV-absorbing mycosporine-like amino acids or catalase activity. The high level of resistance to UV radiation in *L. variegatus* is consistent with the report of low levels of apoptosis in coelomocytes of *Paracentrotus lividus* exposed to up to 2000 J/m^2 of UVB (312 nm) (Matranga et al., 2006). The fact that the coelomocytes show high resistance to the alkylating agent MMS suggests that the resistance involves more than just antioxidant defenses. The presence of heat-labile AP sites in cells treated for 1 h with MMS indicated that the DNA was being effectively alkylated, however the reason we did not detect AP sites in the non heat-treated samples as a result of the BER process remains to be determined. Consistent with our findings in coelomocytes, sea urchin embryos are quite resistant to MMS as high doses (>10 mM) were required to invoke cell cycle arrest and evidence of cell death (Le Bouffant et al., 2007).

An LD_{50} value could not be determined for coelomocytes treated with BaP and no AP sites were detected in DNA for up to 24 h following treatment. This is in contrast to the observation that low levels of BaP (0.5 ng/ml) induce cytogenetic and developmental abnormalities in *Strongylocentrotus purpuratus* embryos (Hose et al., 1983). The genome of *S. purpuratus* contains genes encoding 120 cytochrome P450 (CYP) enzymes including eleven *CYP1* genes (Goldstone et al., 2006). The CYP1 enzymes catalyze the activation of polycyclic aromatic hydrocarbons prior to reductive or conjugative modification and elimination from cells. Differences in expression of these genes or activity of the gene products between embryos and adult cells or between the two different species (*L. variegatus* and *S. purpuratus*) could explain these inconsistent results. The sensitivity of embryos may reflect differences in biotransformation enzyme activity or may be the result of other differences in cell physiology such as high rate of cell division in embryos. It has been shown that embryos of teleost fish are several orders of magnitude more sensitive than adults to certain chemical carcinogens (Spitsbergen et al., 2000).

The cells in the sea urchin coelom are comprised of a mixture of at least four morphologically distinct cell types; macrophage-like cells, granular cells including white and red spherule cells and vibratile cells (Hibino et al., 2006; Matranga et al., 2006). Macrophage-like cells play a role in phagocytosis, encapsulation and clotting functions while granular cells have been shown to have antibacterial properties (Hibino et al., 2006; Matranga et al., 2006). It is possible that these populations differ in their ability to sense and repair DNA damage or their susceptibility to apoptosis. It would be of interest to investigate the response of the different cell types in future studies. Cell-specific differences in sensitivities to DNA damaging agents have been reported for coelomocytes of polychaetes worms (Lewis and Galloway, 2008) and hemocytes of mussels (Venier et al., 1997). In addition, future studies should be extended to include other cells and tissues of sea urchins to determine whether the high resistance is limited to coelomocytes or is a more general property of sea urchin cells.

The sea urchin genome contains homologues for the major genes that function in cell division and its checkpoints in addition to a complex repertoire of apoptosis genes (Fernandez-Guerra et al., 2006; Robertson et al., 2006). In particular, homologues of the key players that respond to DNA damage such as ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia and Rad3 related), the checkpoint kinases, Chk1 and Chk2 and the p53 tumor suppressor are found in the *S. purpuratus* genome (Fernandez-Guerra et al., 2006). Activation of DNA damage checkpoints, DNA repair and apoptosis in sea urchin embryos has been demonstrated in response to genotoxic agents such as MMS, bleomycin or exposure to ultraviolet radiation (Lamare et al., 2006; Le Bouffant et al., 2007; Lesser et al., 2003). Investigating DNA damage checkpoints, DNA repair and apoptotic pathways in coelomocytes will be important to understand the resistance to DNA damaging agents observed in this study.

Comparison of *L. variegatus* coelomocytes with hemocytes from the gastropod mollusk *A. dactylomela* and the decapod crustacean *P. argus* indicate that sensitivity to different DNA damaging agents varies greatly between species. The variation in sensitivity among cells from these three species indicate that selection of animals is crucial for environmental health assessment where the response of a selection of sentinel animals is used to predict consequences at the population or community level. This is supported by other studies in which species-specific and cell-specific differences in levels of sensitivity to MMS and BaP have been reported (Lewis and Galloway, 2008). Differences between species and the response of isolated cells versus whole organisms could explain the results of studies in which some echinoderms have been shown to be sensitive to some DNA damaging agents (Canty et al., 2009; Taban et al., 2004).

Varying natural resistance to DNA damaging agents may contribute to the variation in incidence of neoplastic disease reported for some animal groups. It is often generalized that cancer is more frequent in vertebrates compared to invertebrates and among the invertebrates, occurrence in mollusks is more frequent than in crustaceans and echinoderms (Robert, 2010; Vogt, 2008). The results for *L. variegatus* coelomocytes and hemocytes from the gastropod mollusk *A. dactylomela* and the decapod crustacean *P. argus* are consistent with this assertion. It is also noteworthy that cells from these marine animals tolerated higher doses of the genotoxic agents than those reported for mammalian cells treated under similar experimental conditions (Long et al., 2004; Murakami et al., 2003; Salmon et al., 2008). However more study is required to determine if the high resistance to DNA damaging agents in sea urchin cells contributes to the low incidence of neoplastic disease and to understand the cellular mechanisms protecting DNA which may have wider implications for the prevention of cancer in higher animals.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

We would like to thank Stan Harris for the collection of lobsters and Chloé Newcomb Hodgetts for assistance collecting and bleeding sea hares and lobsters. We thank Jesse Farruggella for his assistance with the UV treatments of coelomocytes. The authors are grateful for the financial support from the Eranda Foundation, the Ray Moore Endowment and Hannover Life Re (Bermuda) Ltd. that made this work possible.

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