

DNA damage in male gonad cells of Green mussel (*Perna viridis*) upon exposure to tobacco products

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Abstract. DNA damage (determined by the Comet Assay) and the occurrence of deformed nuclei were measured as endpoints of genotoxicity in male gonad cells of the marine mussel (*Perna viridis*). Upon exposure of the organism to varying concentrations of extracts of smoked and non-smoked cigar tobacco over a period of 16 days, DNA damage was found to be highest in marine mussels exposed to extracts of smoked cigar tobacco. Conversely, more deformed nuclei were detected in marine mussels exposed to extracts of non-smoked cigar tobacco. The level of DNA damage and the number of deformed nuclei reach a maximum at day 12 of exposure to both extracts but decrease thereafter. This phenomenon is attributed to the organism's capacity to maintain the integrity of its genetic material upon exposure to potential genotoxicants present in the tobacco extracts. A dose response in DNA damage and deformed nuclei was also detected in isolated gonad cells upon in vitro exposure to hydrogen peroxide a known DNA strand breaking agent. The results of this study indicate that the DNA in male gonad cells of the marine mussel is damaged upon exposure to genotoxicants, and suggests the suitability of the organism for future investigations into the effect of such agents on its reproductive capacities.

Keywords: *Genotoxicity, DNA damage, Comet assay, Cigar tobacco extracts, H₂O₂*

Introduction

Marine pollution is a global problem and can arise from natural occurrences as well as human activities, resource uses and interventions such as infrastructure development and construction, agricultural activities, industrial developments, urbanization, tourism, etc. Chemical contaminants of major concern in a polluted environment include persistent organic pesticides, radionuclides, oils, nutrients, heavy metals, etc. Some of these chemicals are known genotoxicants and can cause damage to the genetic material of the exposed organism (Shugart, 1990). Damaged DNA may potentiate subsequent deleterious cellular events such as disease (e.g., cancer) (Phillips, 1983) and reduced reproductive competence (Zenzes, 2000). In marine organisms such effects may lead to the disturbance of population and community dynamics thus affecting the marine trophic balance (Bayne, 1976).

DNA damage in somatic cell in the form of adducts, DNA strand breakage, chromosomal aberrations, sister chromatid exchange and micronuclei formation can occur upon exposure to genotoxicants (Ostling and Johanson,1984; Shugart,1990). However, little information is available on the occurrence of these endpoints of DNA damage in the reproductive cells of a marine invertebrate.

In the work reported here, gonad cells of *Perna viridis* (Indian green mussel) were examined for DNA damage upon exposure to extracts of cigar tobacco. Cigar tobacco is reported to contain various mutagenic and carcinogenic compounds, like polycyclic aromatic hydrocarbons (PAHs), carbon monoxide, tar, nicotine (Shihadeh and Saleh, 2005), nitroso-compounds, heterocyclic amines (HCA) (Hoffmann et al.,1976), that are also ubiquitous genotoxicants of anthropogenic origin. The Indian green mussel was selected as the experimental organism because it is a marine invertebrate found in coastal waters of India. It is known to be the marine pollution indicator organism, and has been using as a bio indicator of pollution. So many workers have been working up on this animal to know effects of genotoxicants (Ching et al., 2001).

To measure DNA damage, single cell gel (SCG) electrophoresis (comet assay, Wilson et al., 1998) was performed on: a) male gonad cells isolated from *P. viridis* exposed *in vivo* to extracts of cigar tobacco, and b) cell suspensions of gonad cells exposed *in vitro* to hydrogen peroxide. Data are expressed as percentage of normal and deformed nuclei and nuclei that exhibited DNA streaming (i.e., the comet effect).

Materials and Methods

Design of experiments

Green Mussels were collected from the lower intertidal zone of the Arabian Sea, Goa, India and brought to the wet lab at the National Institute of Oceanography where they were maintained in glass tanks containing clean, aerated filtered sea water at 2 litres /animal. Live animals were exposed to two types of cigar tobacco extracts viz: ash (burnt) cigar tobacco and non-smoked cigar tobacco. Conversely, cell suspensions of the gonad tissue excised from normal, untreated mussels were exposed to hydrogen peroxide.

Exposure to Cigar Tobacco Experiments

Stock solutions of cigar tobacco were prepared by boiling a known quantity of burnt cigar ash and tobacco from normal cigar separately in water overnight, and filtering through Whatman no 1 filter paper. Separate exposure experiments were conducted at four different doses for each cigar extract at final concentrations of 2 µg, 5 µg, 10 µg and 15 µg of extract per ml of seawater.

Each set of experiment comprised of 4 tanks out of which 3 were given the same treatment, and 4th one was kept as untreated control. Experiment was conducted in triplicates. Each glass tank was of 45 X 23 X 30 cm size. It was housed with 9 adult green mussels of size range from 5-8 cm shell-length. The other general environmental conditions in all tanks were maintained similar (salinity of seawater, 35 ±5 PPT and temperature 28 ±2 °C, expressed in SD and n=3).

The experiments in all tanks for both the treatments were conducted for different durations of time varying from 0-16 days, water changed twice a day and treatment was given accordingly. On each even number day i.e. day 2, 4, 6, 8, 10, 12, 14 and 16, one of the test animal from each treatment was sacrificed and the gonad tissue excised, placed in 3-4 ml of calcium magnesium-free saline (CMFS: 20 mM HEPES, 500 mM NaCl, 12.5 mM KCl, 5 mM EDTA) in a watch glass and chopped finely. About 2 ml of the cell suspension was transferred to a microfuge tube, centrifuged at 2000 rpm for 5 min and the pellet was processed further by the comet assays.

As a positive control, cell suspensions of gonad tissue (750 µl), prepared as stated above were exposed to different doses (final concentration: 0 µM, 100 µM, 250 µM, 500 µM and 1000 µM) of the known DNA strand breaking agent, hydrogen peroxide in a 2 ml microfuge tube. After 1 hour the cells were processed further for the comet assay.

Alkaline single cell gel electrophoresis (Comet assay)

Pellets were suspended in 150 µl of 0.65% Low Melting Agarose (LMA, Genel). About 100 µl aliquots were poured onto a frosted microscopic slides which had previously been coated with 200 µl of 0.65% Normal Melting Agarose (NMA,

Genel) dissolved in 1X TAE buffer. Other protocols were followed after Mckelvey-Martin et al.,1993; Wilson et al.,1998; Dhawan et al., 2001. Slides were stained with 50 µl ethidium bromide (10 µg/ml of distilled water). A coverslip was placed over the slides and examined in an Epifluorescence microscope under 10X-eye lens and 40X objective. The number of normal nuclei, DNA damage interns of Comets and deformed nuclei were counted. Data were converted into percentages and tabulated. Where in deformed nuclei indicate DNA damage interns of irregularity in the shape of nuclei (abnormal nuclei).

Statistical data analysis

Effect of treatments on two different characters viz., percent in comets and percent in deformed nuclei were compared and analyzed to know the individual effects of different duration of days and different concentrations of treatments using 3X3 Analysis of Variance by nesting days in to concentrations.

Results

Statistical analyses of the data show that, both the characters and treatments were highly significant at $P \leq 0.001$. Similarly, different concentrations and days were also highly significant ($P \leq 0.001$). However, nesting of days and concentration together was observed to be insignificant ($P \geq 0.001$).

The results of the Cigar tobacco exposure experiments are depicted in Figures 1, 2 and 3. Consisted of Cigar ash extract treated animals indicated that there was an increase in percent comet nuclei from 5% (2nd day) to 70% (12th day). But after there was a decline to 60% (16th day, Fig,1). With deformed nuclei there was an increase from 3% (2nd day) to 21% (12th day), once again there was a decline to 12% (16th day, Fig,2). In those experiments with non smoked cigar tobacco extract, treated animals showed damage in the form of percent of comet nuclei, varying from 4% (2nd day) to 67% (12th day) and then decline to 59% (16th day, Fig,1). Percent of deformed nuclei also increased from 4% (2nd day) to 23% (12th day) but later there was a decline to 15% (16th day, Fig,2).

The mean percentage in comets in cigar ash extract treated animals was higher (43.8%, Fig,1) than non-smoked cigar tobacco extract treated animals (39.85%, Fig,1) and there was an increase in both, number of percent comets and percent

deformed nuclei from 6th day onwards due to increase in the dose of a treatment of an extract from 2 µg/ml to 5 µg/ml as indicated in Fig, 3. But there was a decline in all types of nuclei damages, after 12th day of the treatment.

The results of the cell suspension experiments performed with a known strand-breaking agent H₂O₂ are given in Fig,4. As depicted therein, there is a direct relationship of dose enhancement on increase in number of comets and number of deformed nuclei, for example percent comets in 100 µM was 20% where as it increased to 36% in 1000 µM.

Discussion

In this study we explore the potential use of Indian green mussel (*Perna viridis*) as an indicator organism to know the extent of DNA damage using comet assay on its male gonad cells, upon exposure to different genotoxicants. Treatments with different known concentrations had showed a direct damaging effect on DNA. Where in the animals treated with cigar ash extract showed higher DNA damage interns of more number of comet nuclei, where as non smoked cigar tobacco extract treated animals showed more of deformed nuclei, which clearly indicates the genotoxic nature of cigar ash and cytotoxic nature of tobacco. But as a total there is a direct relationship between concentrations of a treatment and to a total DNA damage, earliar, Nacci et al.,1996 found a significant increase in DNA strand breaks in oyster (*Crassostrea virginica*) following 53 days of exposure to a carcinogen (PAHs). Xu et al.,1999; and Ching, et al., 2001, described the formation of DNA adducts and DNA strand breaks in green lipped mussels, when exposed to Benzo[a]pyrene is a dose and time dependent.

Even though there is a direct relationship between concentration of the treatment and DNA damage but after 12th day of the treatment, DNA damage started decreasing. This phenomenon is attributed to the organism's capacity to maintain the integrity of its genetic material upon exposure to potential genotoxicants present in the tobacco extracts. Hook and Lee, 2004, showed that oxidative lesions in DNA were repaired more rapidly in stage 7 than in stage 4, in the embryos of grass shrimp (*Palaemonetes pugio*) exposed to various concentrations of benzo[a]pyrene (BP), Cr(VI) and hydrogen peroxide.

In cell suspension experiments carried out with a known strand breaking agent H_2O_2 , showed a direct relationship of a dose with an increase in DNA damage. Mello Filho and Meneghini, 1984, has described strand breaking nature of H_2O_2 and Wilson et al., 1998, recorded DNA damage in *Mytilus edulis* on exposure to hydrogen peroxide is a dose dependent in the form of comets. The present experiments also show the induction of comets, which are indicative of DNA damage in the hydrogen peroxide-exposed gonad cells of the green mussel.

The experiments conducted in this study show that DNA damage, as measured by the Comet Assay, occurs in male gonad cell of the green mussel (*P. viridis*) exposed to extracts of Cigar ash and non-smoked Cigar tobacco. Cigar ash extract appears to be more harmful than the non-smoked cigar tobacco extracts, and there was a direct relationship with the increase in dose and time of exposure to the damage of DNA. The result of the cell suspension experiment shows a similar trend suggesting the use of hydrogen peroxide as a positive control. It also suggests the suitability of the mussel for future investigations into the effect of carcinogens on its reproductive capacities of the organism as well as to understand the role of DNA damage in particular stages of spermatogenesis.

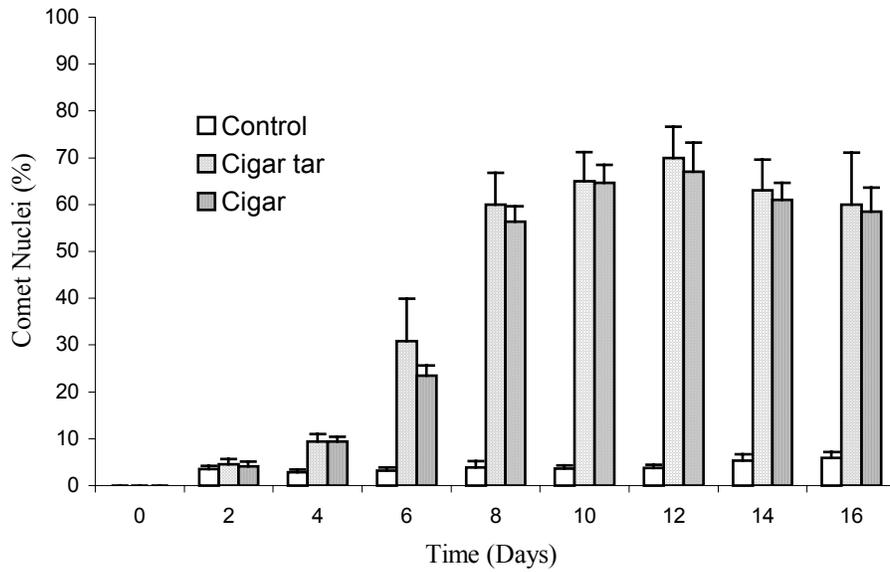
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Fig,1 Level of DNA Damage expressed as comet nuclei in mussel male gonad cells following exposure to various doses of cigar tobacco extracts across different duration of time (days). Error bar indicates standard deviation (SD), where n=3.

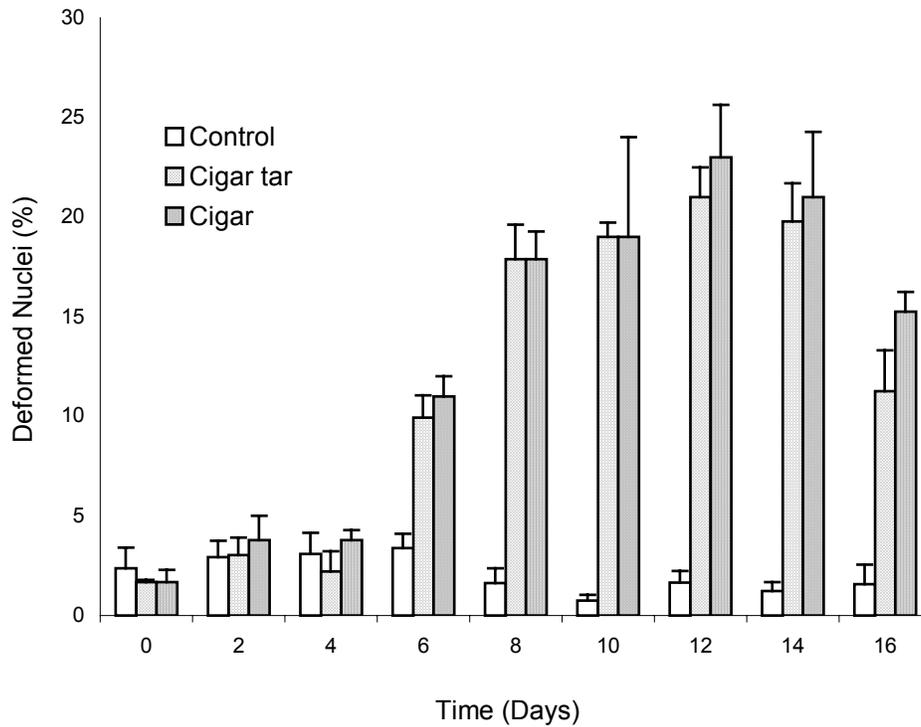


Fig:2 Level of DNA Damage expressed as Percent deformed nuclei in mussel male gonad cells following exposure to various doses of cigar tobacco extracts across different duration of time (days). Error bar indicates standard deviation (SD), where n=3.

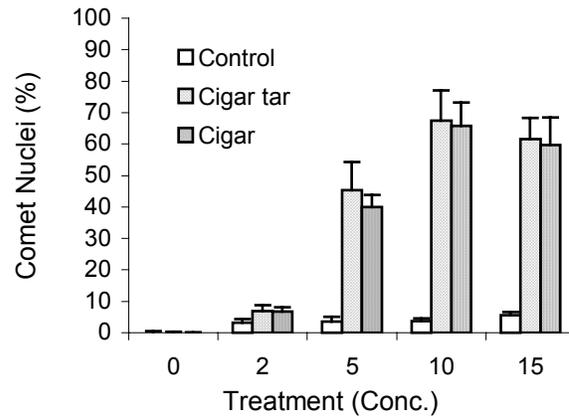


Fig:3 Level of DNA Damage expressed as Percent Comets in mussel male gonad cells exposure to various Concentration (μg), of cigar tobacco Extracts (treatment). Error bar indicates standard deviation (SD), where $n=3$.

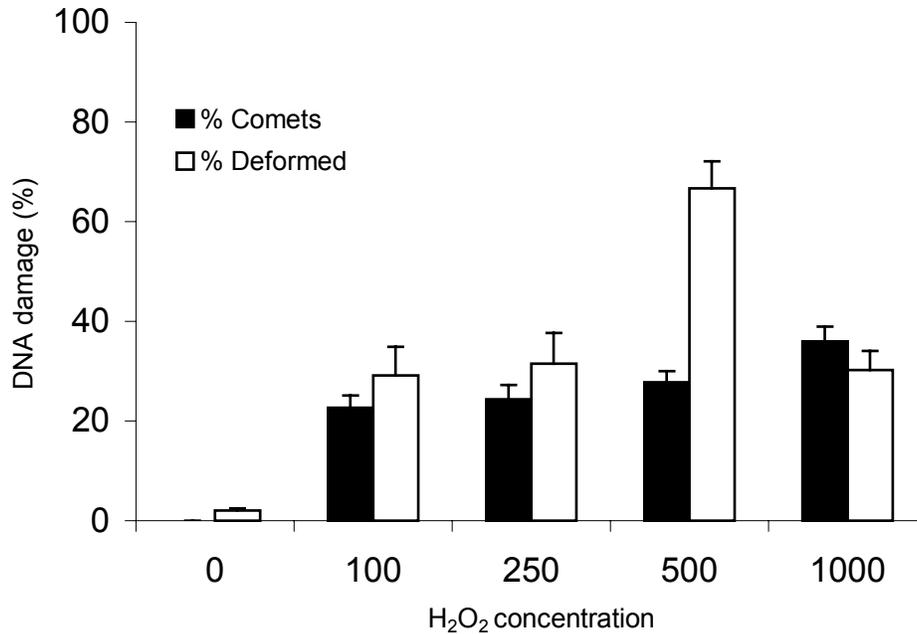


Fig:4 Level of DNA Damage in mussel gonad cells following cell suspension exposure to H₂O₂ Concentration (μM). Error bar indicates standard deviation (SD), where $n=3$.