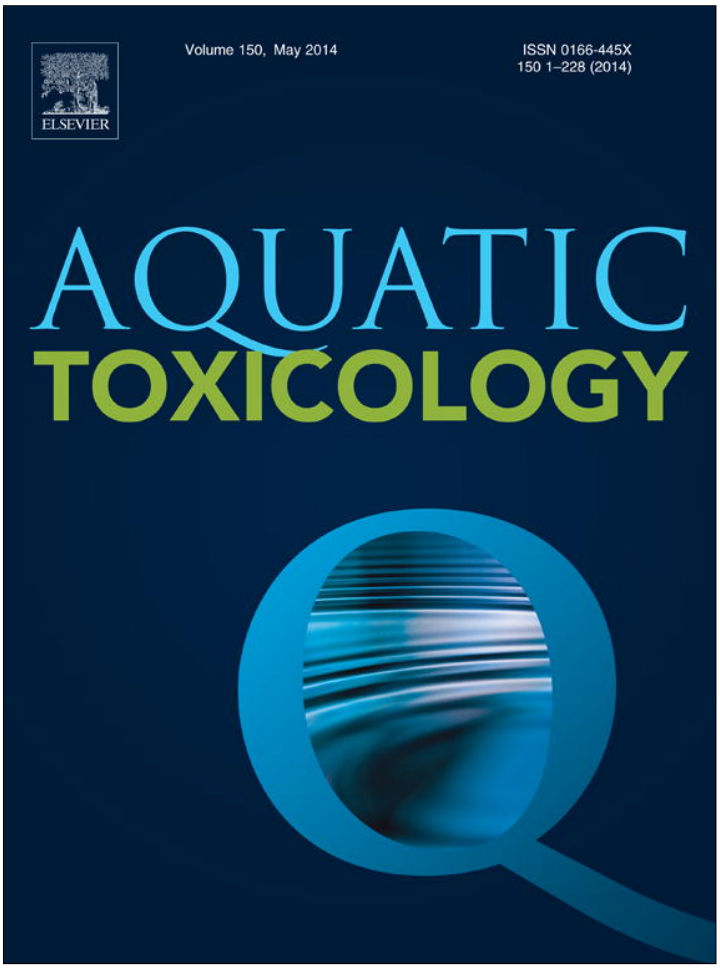

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Evaluation of γ-radiation-induced DNA damage in two species of bivalves and their relative sensitivity using comet assay

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ABSTRACT

Ionizing radiation is known to induce genetic damage in diverse groups of organisms. Under accidental situations, large quantities of radioactive elements get released into the environment and radiation emitted from these radionuclides may adversely affect both the man and the non-human biota. The present study is aimed (a) to know the genotoxic effect of gamma radiation on aquatic fauna employing two species of selected bivalves, (b) to evaluate the possible use of 'Comet assay' for detecting genetic damage in haemocytes of bivalves as a biomarker for environmental biomonitoring and also (c) to compare the relative sensitivity of two species of bivalves *viz. Paphia malabarica* and *Meretrix casta* to gamma radiation. The comet assays was optimized and validated using different concentrations (18, 32 and 56 mg/L) of ethyl methanesulfonate (EMS), a direct-acting reference genotoxic agent, to which the bivalves were exposed for various times (24, 48 and 72 h). Bivalves were irradiated (single acute exposure) with 5 different doses (*viz.* 2, 4, 6, 8 and 10 Gy) of gamma radiation and their genotoxic effects on the haemocytes were studied using the comet assay. Haemolymph was collected from the adductor muscle at 24, 48 and 72 h of both EMS-exposed and irradiated bivalves and comet assay was carried out using standard protocol. A significant increase in DNA damage was observed as indicated by an increase in % tail DNA damage at different concentrations of EMS and all the doses of gamma radiation as compared to controls in both bivalve species. This showed a dose-dependent increase of genetic damage induced in bivalves by EMS as well as gamma radiation. Further, the highest DNA damage was observed at 24 h. The damage gradually decreased with time, i.e. was smaller at 48 and 72 h than at 24 h post irradiation in both species of bivalves. This may indicate repair of the damaged DNA and/or loss of heavily damaged cells as the post irradiation time advanced. The present study reveals that gamma radiation induces single strand breaks in DNA as measured by alkaline comet assay in bivalves and comet assay serves as a sensitive and rapid method to detect genotoxicity of gamma radiation. This study further indicates that both *M. casta* and *P. malabarica* exhibit almost identical sensitivity to gamma radiation as measured by DNA damage.

Introduction

Applications of nuclear technologies have made a very significant contribution to modern civilization. Nuclear technology is useful to humans in several ways, including as a source of power, as a tool for medical diagnostics and also as an industrial tool. Exploding human population and the changing needs/lifestyle have resulted in a drastic increase in the production, consumption and disposal of chemical contaminants into our environment. The aquatic environment is often the ultimate recipient of a wide range of contaminants including chemical and radioactive wastes, a large proportion of which could be potentially genotoxic and carcinogenic (Moore et al., 2004; Jha et al., 2000a). Radioactive wastes emit radiation in different forms, e.g. α-, β-particles and gamma rays, which could pose a potential risk to human health and also to our environment (Dallas et al., 2012). Environment and man may be exposed to a moderate dose of 110 Gy of gamma radiation as a result of radiation accidents or nuclear/radiological terrorism alone or in conjunction with bioterrorism (Coleman et al., 2003). During nuclear accidents, radionuclides are released into the

Abbreviations: Gy, gray; h, hour; γ, gamma; EMS, ethyl methanesulfonate.

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environment, either in modest amounts or on a larger scale, such as that caused by the Chernobyl NPP (Ukraine, formerly USSR) in 1986 or the earthquake-tsunami at Japan's Fukushima Daiichi NPP in 2011. The risk to non-human biota due to ionizing radiation exposure is of considerable current interest to both the International Commission on Radiological Protection (ICRP) and the International Atomic Energy Agency (IAEA) and they recommend the impact assessment of radiation on some natural organisms (IAEA, 1992; ICRP, 1991, 2007). Very little information is available on the potential detrimental effects of ionizing radiation on aquatic invertebrates (Dallas et al., 2012). They are very important human food source (Barnes and Rawlinson, 2009; Ren et al., 2010) and are also a source of food for various commercially important fish species (Pedersen et al., 2008). Further, they are frequently used as model organisms for toxicological tests (Depledge, 1998).

Haemocytes, the cells of the open vascular system of mussels, have been extensively used for genotoxicological studies, including for monitoring cytogenetic damage (Mersch et al., 1996; Pavlica et al., 2001). The usage of haemocytes provides a relatively non-invasive source of material for biomonitoring (Fossi et al., 1994; Mitchelmore and Chipman, 1998; Taddei et al., 2001). Further, these cell types are suitable for the comet assay and the MN assay because they can be rapidly and easily sampled without any need for cell dissociation (Belpaeme et al., 1998; Jha, 2008; Canty et al., 2009). These advantages have the benefit of shortening the time required for slide preparation and facilitating the sample process. The comet or single-cell gel electrophoresis assay is used as a very quick, sensitive method for measuring DNA damage in eukaryotic cells for the study of genetic damage associated with exposures to potentially genotoxic agents (Lovell and Omori, 2008). It is also used in regulatory and biomonitoring studies in a range of mammalian and non-mammalian both under in vitro and in vivo systems (Chaubey et al., 2001; Collins, 2004; Brendler-Schwaab et al., 2005; Malladi et al., 2007). It offers considerable advantages over several other cytogenetic methods used in DNA damage detection, such as sister chromatid exchange studies, micronucleus test and the chromosomal aberrations. Further, there is no need for cells to be in a dividing state. Other advantages include its rapidity, i.e. results can be obtained in a single working day and wide applicability to virtually any nucleated cell type (Jha, 2008; Canty et al., 2009).

In addition to the detection and quantification of contaminants in the environment in order to reveal their environmental impact, it is very essential to identify their toxic effects on living systems, which are the ultimate recipients of toxicant-induced mutation (Claxton et al., 1998). Mussels are globally used as bioindicators for pollution of coastal and estuarine environments by metals and radionuclides (Lonsdale et al., 2009). Hagger et al. (2005) have reported the β-radiation induced genotoxic, cytotoxic, developmental and survival effects of tritiated water in the early life stages of the marine mollusc, *Mytilus edulis*. Further, the genotoxic effects of tritium (^{3}H) in the adult life stage of *Mytilus edulis* have been evaluated by employing micronucleus (MN) test and comet assay (indicating DNA single strand breaks/alkali labile sites) in the haemocytes of exposed individuals (Jha et al., 2005, 2006). Tritiated water (HTO) and tritiated glycine (T-Gly) induced a significant number of micronuclei in the haemocytes of *Mytilus edulis* (Jaeschke et al., 2010). External and internal dose rates of ionizing radiation altered the DNA strand breakage and *RAD51* mRNA expression in marine mussel *Mytilus edulis* which was observed using gene expression study and comet assay (Alamri et al., 2012). However, studies on the genotoxic potential of radiation in the estuarine bivalves are lacking. Hence, the present study is aimed (a) to know of genotoxic effect of gamma radiation on estuarine bivalves (b) to evaluate the use of 'Comet assay' for detecting genetic damage in haemocytes of bivalves as a biomarker for biomonitoring and also (c) to compare the relative sensitivity of two species of bivalves *Paphia malabarica* and *Meretrix casta* to gamma radiation.

Materials and methods

Experimental bivalve specimens

Two species of estuarine bivalves, *P. malabarica* and *M. casta* which are abundant in Goa and used as a common sea food by locals were selected as the experimental animals. Healthy specimens were procured from an unpolluted site at Siolim, Goa and brought to the laboratory. Animals measuring an average length of 3.0 ± 0.4 cm were selected for the current study. They were maintained in aerated glass aquaria with sand, pebbles and estuarine water, procured from the same above cited unpolluted site. Water in the aquaria was replaced once in every day. These bivalves were acclimatized under the laboratory conditions for 2 weeks in semi static systems before exposure to reference agent as well as to different doses of gamma radiation.

Validation of comet assay

Prior to the evaluation of genotoxic effects of gamma radiation the comet assay was fully optimized and validated against a direct-acting reference genotoxin, ethyl methanesulfonate (EMS; Sigma, UK). The range of EMS concentrations used in the study was based on earlier studies (Jha et al., 2005). Both species of bivalves (10 animals per group) were exposed to different concentrations of EMS (18, 32 and 56 mg/L) dissolved in estuarine water.

Sample collection from bivalves

Four hundred (400) microliter of haemolymph was collected from the sinus region (located near the posterior adductor muscle) of each of the control and EMS-exposed bivalves using a hypodermic syringe under dim light at exposure times of 24, 48 and 72 h. Each sample was transferred to a microcentrifuge tube placed on ice in an ice box to prevent endogenous DNA damage occurring during sample preparation and also to inhibit DNA repair in the unfixed cells (Siu et al., 2004).

Cell viability assay

Prior to the comet assay, the cell count and the cell viability were checked to ensure that there were an optimum number of living cells to perform the assay. The cell count and viability assessment were conducted with a haemocytometer and trypan blue dye exclusion test. Haemolymph samples showing more than 90% viability and a cell count of 10^6 cells/mL were used for the comet assay.

Comet assay

The technique of lysis, unwinding and electrophoresis were standardized for haemocytes by adopting the protocol of Singh et al. (1988) and Tice (1995) with slight modifications, prior performing the comet assay. The comet assay was carried out in a dark room with dim red light. Haemolymph (15 μL) was mixed with 75 μL of 0.5% low melting point (LMP) agarose at 37 °C and rapidly spread on a frosted microscope slide (Fisherfinest premium) precoated with 1% normal melting point (NMP) agarose. A cover slip was applied on the smear and the slide was allowed to solidify for 5 min in a freezer at 0 °C. The cover slip was then gently removed and the slide was immersed in a fresh cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 10% DMSO 1% and Triton X-100) placed in a Petri dish for 2 h at 4 °C. Comet slides were later immersed in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) in a horizontal gel box for 30 min to allow the DNA to unwind. These slides were subjected to electrophoresis by applying 20 V and 275 mA for 20 min and later on the unwound DNA

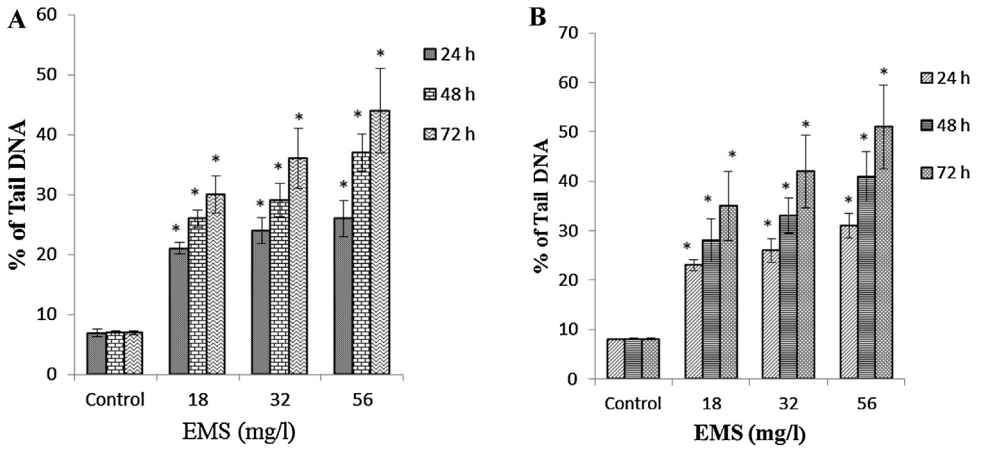

Fig. 1. (A and B) Percentage of DNA damage in haemocytes of *Paphia malabarica* (A) and *Meretrix casta* (B) exposed to 18, 32 and 56 mg/L of EMS at various time intervals. Data are mean ± SD (*$P < 0.001$ denotes statistically significant difference from the control, Student's *t*-test significance).

in the slides were neutralized in cold neutralization buffer (0.4 M Tris–HCl, pH 7.5) for 10 min in a Petri dish, rinsed in distilled water and neutralized again. The DNA was stained with 15 μg/mL ethidium bromide and examined under a BX53 Olympus fluorescence microscope (Japan) at 200× magnification. Two slides per specimen and a total of 500 comets per group (25 cells per slide) were selected for analysis. The images of these comets were captured using ProgRes® Capture Pro 2.7. The captured images were analyzed using CASP (Konca et al., 2003) image analysis software and the percentage of tail DNA content (% tail DNA) was measured as an indicator of DNA damage (single strand breaks) (Kumaravel and Jha, 2006).

Selection of the radiation doses

A dose range of 2–10 Gy of gamma radiation was selected for the present study as this quantum of radiation is suspected to be released into our environment during any nuclear accident.

Irradiation of bivalves

Bivalves, *P. malabarica* and *M. casta* (10 animals per group) were exposed to a whole body irradiation of a single dose of 2, 4, 6, 8 and 10 Gy allowing 0.5 cm deviations at the center of the dose rate from a gamma radiation source of a Cobalt Teletherapy Unit at Goa Medical College, Goa. Unirradiated bivalves were employed as control animals. After each time (24, 48 and 72 h) post irradiation of different doses of gamma radiation and the control group without irradiation, 400 μL samples of haemolymph were taken from each bivalve, the cell count and cell viability were checked as described above, and the rest of the sample was used for the comet assays.

Statistical analysis

Statistical analysis was performed by using PRISM and STATISTICA 6.0 packages. Data was analyzed with student's *t*-test and ANOVA. Within the single experiment, the significance of the data for each dose against that of the respective control was evaluated by the Student's *t*-test. Dose respondents as well as time response of gamma irradiation on bivalves were determined by one-way ANOVA. The difference of DNA damage between the *P. malabarica* and *M. casta* was analyzed by student's *t*-test. A level of probability of $P < 0.05$ was considered as statistically significant data.

Results

Validation of comet assay

The DNA damage (% tail DNA) induced by different concentration of EMS (18, 32 and 56 mg/L) in the haemocytes of *P. malabarica* and *M. casta* at various time intervals (24, 48 and 72 h) are shown in Fig. 1(A and B). EMS induced a concentration-dependent increase of DNA damage in both the species of bivalves. The highest amount of DNA damage (% of tail DNA) was observed in both the species of bivalves exposed to the highest concentration of EMS (56 mg/L). Further, a time-dependent increase of DNA damage was observed for all the concentrations of EMS. All EMS concentrations induced a significant increase (*t*-test) of DNA damage in comparison with the controls in both bivalve species.

Irradiation of bivalves

DNA damage in P. malabarica

The dose and the time dependent increase of DNA damage induced by gamma radiation and their statistical significance are represented in Fig. 2A and Fig. 3A, , respectively.

Dose–response assay. Significant DNA damage ($P < 0.001$) was seen in all individuals of *P. malabarica* irradiated by various doses of gamma radiation in comparison to their respective controls as per the *t*-test (Fig. 2A). Further, a dose-dependent increase of the mean % tail DNA was observed, with a minimum (60.57 ± 8.30) at the lowest dose (2 Gy) and the maximum (75.21 ± 9.05) at the highest dose (10 Gy) at 24 h of post irradiation. One way ANOVA of the data on the dose-dependent DNA damage observed in the control and treated animals showed significance at $P < 0.0001$ ($F = 54.8$, 59.8 and 27.3) at all the time intervals studied (24, 48 and 72 h, respectively).

Time-response assay. Time-response study indicated significant DNA damage at all the time intervals for all the doses of gamma radiation studied as per the *t*-test (Fig. 3A). The highest DNA damage (60.57 ± 8.30) was observed at 24 h post treatment. The damage decreased considerably in the later time points and reached the minimum (29.4 ± 8.0) at 72 h (2 Gy). A similar trend was also observed for the other doses (4, 6, 8 and 10 Gy). One way ANOVA of the DNA damage observed at different time points showed significant decrease in the DNA damage with the $P < 0.0001$ ($F = 34.10$,

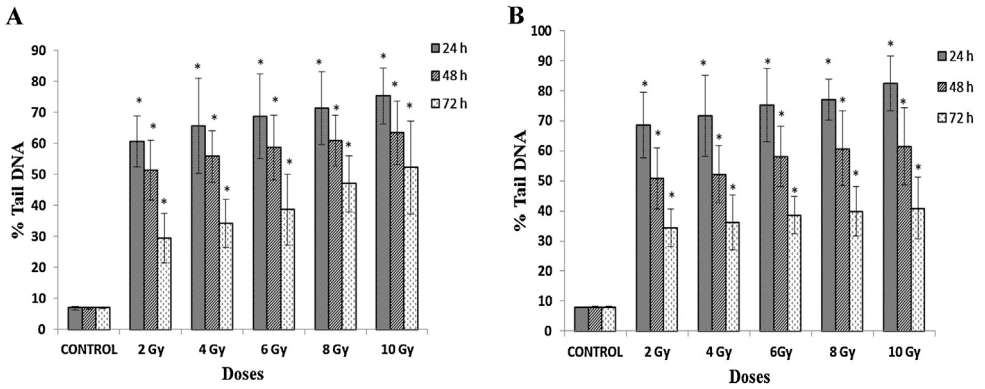

Fig. 2. (A and B) Percentage of DNA damage in haemocytes of *Paphia malabarica* (A) and *Meretrix casta* (B) exposed to 2, 4, 6, 8 and 10 Gy of gamma radiation at various time intervals. Data are mean ± SD (*$P < 0.001$ denotes statistically significant difference from the control, Student's *t*-test significance).

21.20, 16.59, 16.08 and 9.96) for all the doses studied (2, 4, 6, 8 and 10 Gy, respectively).

DNA damage in M. casta

The dose–response and the time-dependent DNA damage induced by gamma radiation and their statistical significance are represented in Fig. 2B and Fig. 3B, respectively.

Dose–response assay. Significant DNA damage ($P < 0.001$) was induced by all the doses of gamma radiation in the haemocytes of bivalves, in comparison to their respective controls (*t*-test) at all the time points studied (Fig. 2B). Results also indicated a dose-dependent increase of % tail DNA in these bivalves. The minimum % tail DNA (68.52 ± 10.25) was observed at the lowest dose (2 Gy) and the maximum (82.43 ± 9.11) was observed at the highest dose (10 Gy) at 24 h of post irradiation. One way ANOVA of the DNA damage observed in the control and treated animals showed significance at $P < 0.0001$ ($F = 80.0$, 39.7 and 26.5) at all the time intervals studied (24, 48 and 72 h, respectively).

Time-response assay. The significant DNA damage was observed at all the time intervals for all the doses of gamma radiation studied (*t*-test; Fig. 3B). Maximum % tail DNA (68.52 ± 10.25) was observed at 24 h which gradually declined (34.4 ± 6.24) until 72 h (2 Gy) in a time-dependent manner. A similar trend was observed for the other doses, too. One way ANOVA of the DNA damage observed at different time intervals showed significance at $P < 0.0001$ ($F = 33.6$, 27.5, 33.3, 47.7 and 37.5) for all the doses studied (2, 4, 6, 8 and 10 Gy, respectively).

Comparison between P. malabarica and M. casta

Dose–response assay

The DNA damage induced by various doses (*viz.* 2, 4, 6, 8 and 10 Gy) of gamma radiation in the haemocytes of *P. malabarica* and *M. casta* at 24, 48 and 72 h time intervals are compared each other and represented graphically in Fig. 4(a–c).

24 h post-treatment study. Data on DNA damage at 24 h post treatment with gamma radiation is represented graphically in Fig. 4a. A linear increase in DNA damage in the form of % tail DNA was observed with the increasing doses of gamma radiation in both the species of bivalves. The *M. casta* showed more DNA damage when compared with *P. malabarica* in all the doses of gamma radiation at 24 h post treatment, although the difference was not statistically significant.

48 h post-treatment study. The DNA damage at 48 h post treatment with gamma radiation is represented in Fig. 4b. Interestingly, *P. malabarica* showed almost the same DNA damage as *M. casta* in all the doses of the gamma radiation.

72 h post-treatment study. Gamma radiation induced DNA damage at 72 h post treatment is represented graphically in Fig. 4c. At 72 h of post irradiation *M. casta* showed a gradual, linear increase of DNA damage with increasing doses, whereas *P. malabarica* showed comparatively less DNA damage at lower doses (2–6 Gy) but at higher doses (8–10 Gy) it showed a drastic increase of DNA damage as compared to *M. casta*. However, the differences observed

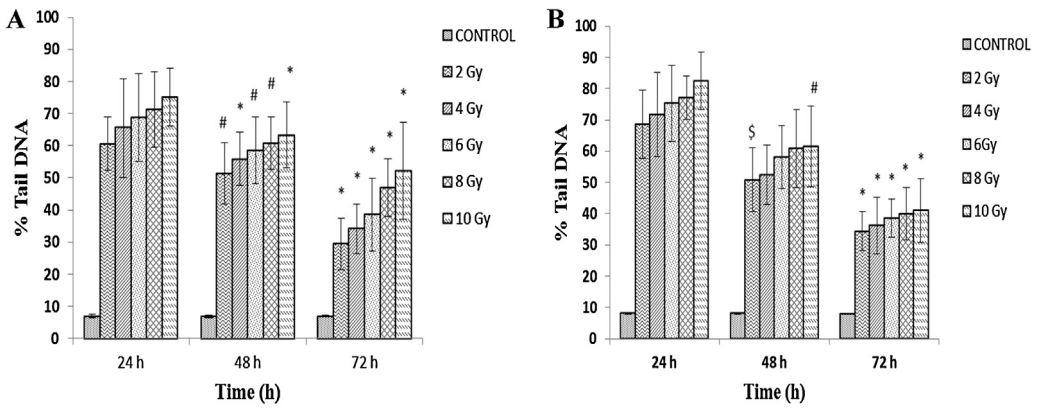

Fig. 3. (A and B) Percentage of DNA damage in haemocytes of *Paphia malabarica* (A) and *Meretrix casta* (B) exposed to various doses of gamma radiation at 24, 48 and 72 h time intervals. Data are mean ± SD ($^{\$}P < 0.05$, $^{\#}P < 0.01$, $^{*}P < 0.001$ denotes statistically significant difference from the damage at 24 h, Student's *t*-test significance).

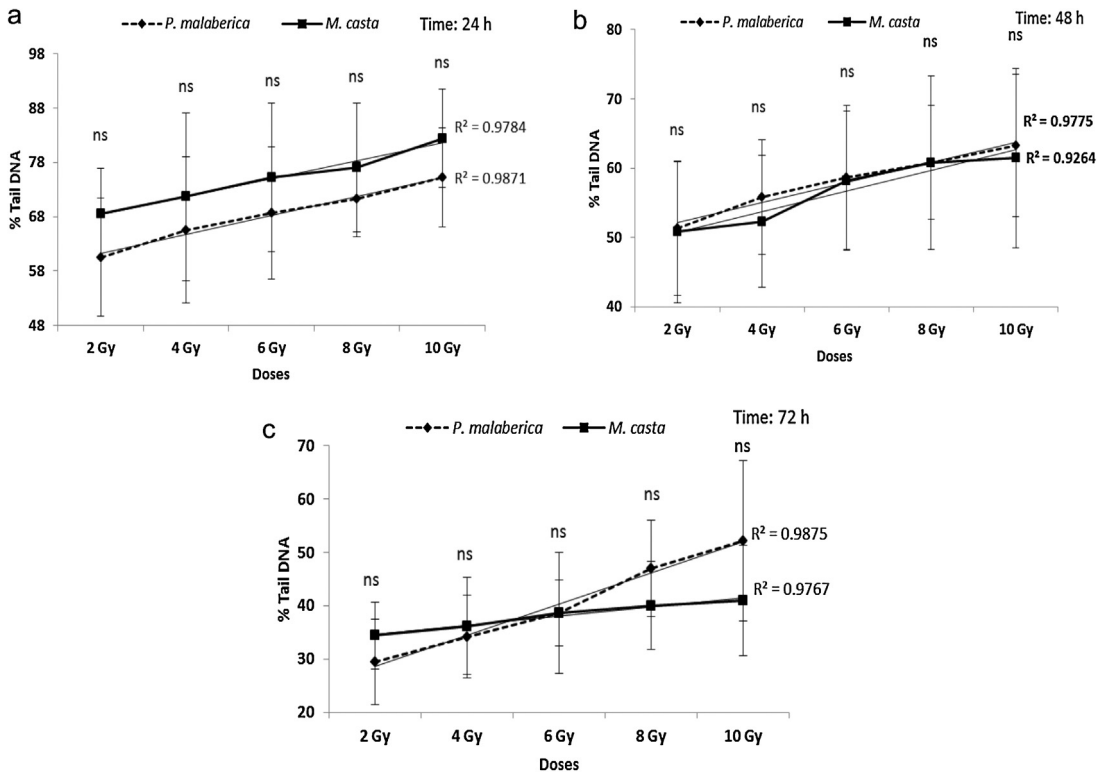

Fig. 4. (a–c) Comparison of the DNA damage induced by various doses (*viz.* 2, 4, 6, 8 & 10 Gy) of gamma radiation in the haemocytes of *Paphia malabarica* and *Meretrix casta* at 24, 48 and 72 h time intervals using *t*-tests. Each point indicates the mean ± SD of P*aphia malabarica* and *Meretrix casta*. *Note*: ns indicates a non-significant difference.

were not statistically significant, neither at lower nor at higher doses.

Time-response assay

Comparison between the DNA damage induced by various doses (*viz.* 2, 4, 6, 8 and 10 Gy) of gamma radiation at 24, 48 and 72 h after the radiation in the haemocytes of *P. malabarica* and *M. casta* are compared to each other and represented graphically in Fig. 5(a–e). Data on DNA damage at 24 h post treatment with various doses of gamma radiations in *M. casta* showed slightly higher quantum of genetic damage when compared with *P. malabarica*, although the difference is not statistically significant. The overall comparison between *P. malabarica* and *M. casta* has shown that there is considerable difference in DNA damage induced by gamma radiation in these bivalves, although it is not statistically significant.

Discussion

Validation studies of comet assays

The increased DNA damage observed in the present study in the haemocytes of EMS-exposed bivalves (both species) indicates their sensitivity to genotoxic agents as well as their possible use for genotoxicity studies using the comet assay. Many of earlier studies have shown that the comet assay is a sensitive and reproducible method to detect genotoxic effects in the haemocytes of bivalves following exposure to a variety of directly and indirectly acting genotoxins (Dixon et al., 2002; Jha et al., 2005, 2006). We could make similar observations in EMS-exposed bivalves (both species) in the present study. Further, similar to other reports, our study also suggests that haemocytes of bivalves are a sensitive and reliable cell type for evaluating the genotoxic effects of reference and environmental agents.

Gamma radiation-induced DNA damage

Significant increase of mean % tail DNA damage observed by comet assay in the present study at all the doses of gamma radiation when compared to controls in both bivalve species indicate the genotoxic potential of gamma radiation in these bivalves, viz. *P. malabarica* and *M. casta*. Bivalves, as members of the phylum Mollusca, which is the second largest group of invertebrates, have been exploited worldwide for food, ornamentation, pearls, etc. and also used for biomonitoring of pollutants. Most of the earlier studies have reported radiation-induced developmental toxicity or survival of the early life stages of aquatic invertebrates (Hingston et al., 2003; Dallas et al., 2012). However, reports on the use of the comet assay in the cells of a bivalve to study the effect of gamma radiation are very scanty (Jha et al., 2005; Jaeschke et al., 2010; Alamri et al., 2012).

Dose–response assay

The dose-dependent increase of DNA single-strand breaks, in the form of comet induction (% tail DNA) induced by irradiation in bivalves in the present study is in line with the observations of Jha et al. (2005, 2006); they exposed *Mytilus edulis* to a series of tritium activities and consequently observed a dose-dependent increase of MN and DNA damage in haemocytes. Larger radioactive particles caused increased comet % of tail DNA and frequency of MN in the haemolymph of *Mytilus edulis* (Jaeschke, 2012). Radiation-induced DNA damage has recently been reported in the haemocytes of *Mytilus edulis* with the help of the comet assay and MN test by Jaeschke et al. (2010) and Alamri et al. (2012).

Time-response assay

The significant genetic damage induced by gamma radiation at 24 h time intervals which decreased by the 48 h and further declined by 72 h in both the species of bivalves in the present

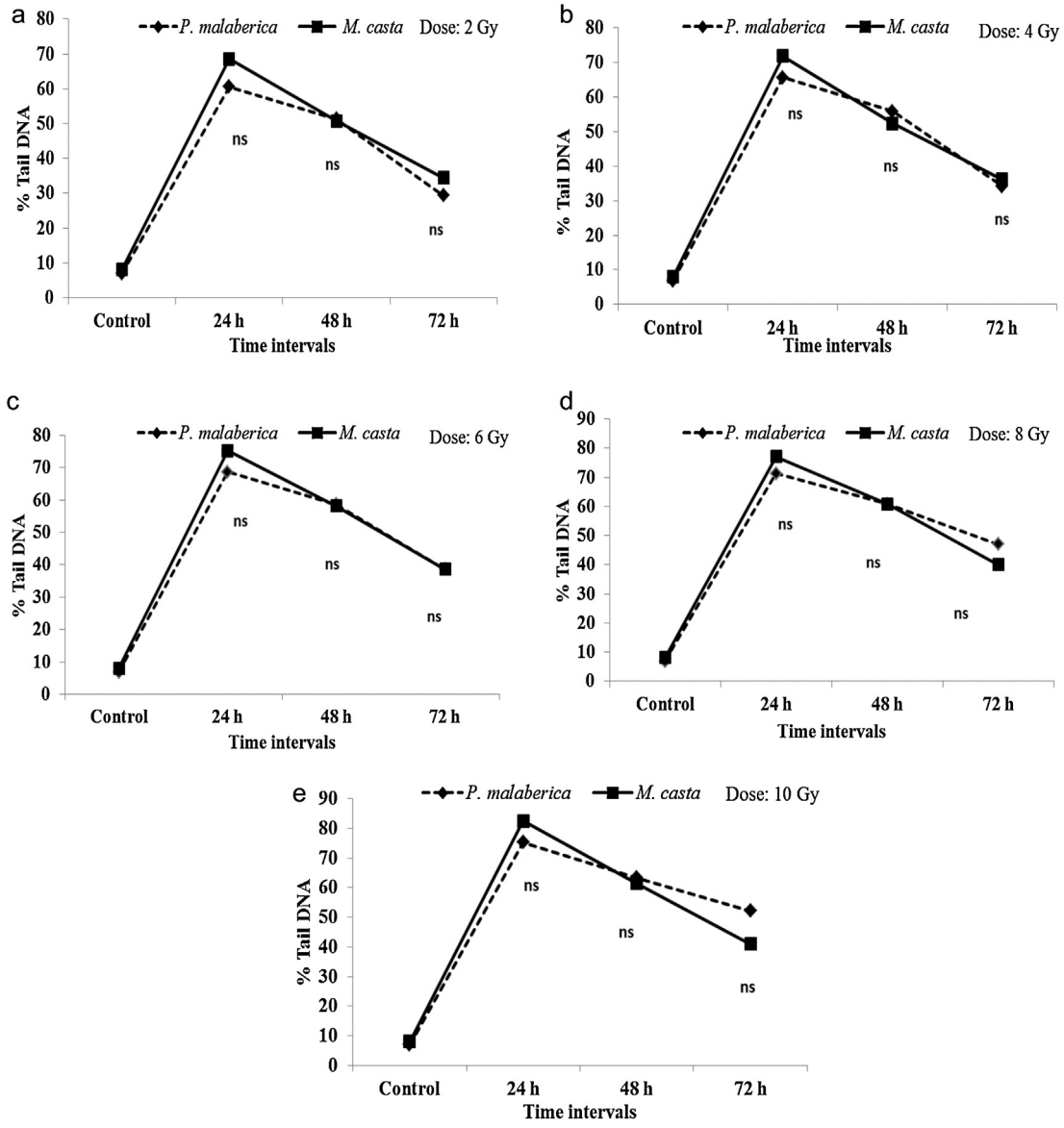

Fig. 5. (a–e) Comparison of the DNA damage induced at various time intervals of gamma radiation in the haemocytes of *Paphia malabarica* and *Meretrix casta* by various doses (*viz.* 2, 4, 6, 8 & 10 Gy). Each point indicates the mean ± SD of P*aphia malabarica* and *Meretrix casta*. *Note*: ns indicates a non-significant difference.

time-dependent study suggests that the genotoxic effect of gamma radiation does not last for a long period. This is in agreement with the observation of Jaeschke et al. (2010) who observed that the frequency of MN in mussels exposed to HTO became insignificantly different from the control value after 21 days of depuration. It is also in line with similar reports by several scientists using chemical toxicants (Miyamae et al., 1997; Wong et al., 2001; Rank and Jensen, 2003; Sharma et al., 2007). Decrease in genetic damage at later times may indicate either repair of damaged DNA or loss of heavily damaged cells or both (Banu et al., 2001; Preeti and Shyama, 2009).

Comparison between P. malabarica and M. casta

Almost similar DNA damage induced by various doses of gamma radiation in the haemocytes of *P. malabarica* and of *Meretrix cast* in the present study may indicate that the DNA of both of these bivalves is similarly sensitive to gamma radiation. Varying levels of relative sensitivity are reported in ecologically relevant invertebrates exposed to various contaminants under laboratory conditions (Jha et al., 2000b; Cheung et al., 2006; Canty et al., 2009) and in situ (Dallas et al., 2013). In the present study, we observed an interesting dose–response at 72 h in *P. malabarica*. It showed comparatively less DNA damage at lower doses (2–6 Gy) which increased drastically later on at higher doses (8–10 Gy) as compared to *M. casta*. *M. casta* did not show a drastic increase of DNA damage with the increased doses of irradiation. However, this interesting trend doesn't show significant difference between the two species. Present observation on the relative sensitivity of two bivalves suggests that the same doses of gamma radiation produce similar amount of genotoxic response in closely related species. This may be due to their close phylogenetic relationship, whereby also their antioxidant defense mechanisms may be similar.

Conclusion

The result of the comet assay-based study presented here has indicated the positive mutagenic effect of gamma radiation on bivalves. Further, a dose-related increase and a time-dependent decrease of genotoxicity of gamma radiation were also observed in

the haemocytes of both bivalve species. These responses indicate that haemocytes can be used as sensitive indicators (biomarkers) of a genotoxicant within an environmentally realistic range. Overall, the assay provides a convenient, highly sensitive and non-invasive monitoring tool of environmental exposure to genotoxicants. Further, the alkaline comet assay appears to be a promising technique to assess the genotoxic potential of gamma radiation in estuarine bivalves. This study also showed that *P. malabarica* and *M. casta* show similar sensitivity to gamma radiation.

Conflict of interest

None.

Acknowledgments

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