Article
Industrial Health

Toxicology and Industrial Health
1–8

DOI: 10.1177/0748233717733599
journals.sagepub.com/home/tih
SAGE

Evaluation of gamma radiation-induced DNA damage in *Aedes aegypti* using the comet assay

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Abstract

The study was undertaken to evaluate gamma radiation-induced DNA damage in *Aedes aegypti*. The comet assay was employed to demonstrate the extent of DNA damage produced in adult male *A. aegypti* exposed to seven different doses of gamma radiation, ranging from 1 Gy to 50 Gy. DNA damage was measured as the percentage of comet tail DNA. A significant linear increase in DNA damage was observed in all samples; the extent of damage being proportional to the dose of gamma radiation the organism received, except in those treated with 1 Gy. The highest amount of DNA damage was noticed at 1 h postirradiation, which decreased gradually with time, that is, at 3, 6 and 12 h postirradiation. This may indicate repair of the damaged DNA and/or loss of heavily damaged cells as the postirradiation time increased. The comet assay serves as a sensitive and rapid technique to detect gamma radiation-induced DNA damage in *A. aegypti*. This could be used as a potential biomarker for environmental risk assessment.

Keywords

Gamma radiation, DNA damage, comet assay, genotoxicity

Received 9 April 2017; Revised 19 July 2017; Accepted 24 August 2017

Introduction

Living organisms are constantly exposed to ionizing radiations from radionuclides that exist in various natural and artificial sources. In past decades, scientific and regulatory activities related to radiation protection focused on the radiation exposure of humans. The prevailing view has been that if humans were adequately protected, then other living things are also likely to be sufficiently protected (EMRAS, 2007; Moller and Mousseau, 2013). Over time, the general validity of this view has been questioned on occasion, and therefore, consideration has been given to the potential effects of exposure to ionizing radiation on non-human biota (UNSCEAR, 2011). The study of radiological effects on various plants and animals is currently a subject of widespread scientific interest (Singhal et al., 2009).

Aedes aegypti is the primary vector for dengue fever/dengue haemorrhagic fever (DF/DHF) and major public health problems in many subtropical and tropical countries (Ahmad et al., 2007). It is also the best known insect species from the standpoint of both basic and applied science. Because of its ready adaptability to laboratory culture and short lifespan with high reproductive potential, this species has been used as a test animal for many physiological, developmental and genotoxicity studies (Craig and Hickey, 1967;

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Clemons et al., 2010). It has been extensively reported that gamma radiations can break covalent bonds and can directly affect DNA structure by inducing DNA breaks, particularly single-strand breaks and double-strand breaks (DSBs) in living cells (Azzam et al., 2012; Borrego-Soto et al., 2015; Lee and Steinert, 2003).

Numerous methods have been developed for detecting damage to DNA strands (Tice et al., 2000). Single cell gel electrophoresis (SCGE), also known as the comet assay, is an extremely promising genotoxicity test developed in recent years to measure and analyse DNA damage in single cells. SCGE is less resource intensive than conventional genotoxic techniques and permits both qualitative and quantitative assessment of DNA damage in any eukaryotic cell population. The simplicity and sensitivity of the comet assay has resulted in a rapid and widespread application of this technique in many areas, including environmental monitoring (Cavallo et al., 2002; Rajaguru et al., 2002), *in vivo* and *in vitro* genotoxicity testing (Anderson et al., 1996; 2001; Dhawan et al., 2002) and epidemiological and biomonitoring studies in human populations exposed to radiation occupationally, environmentally or clinically (Bajpayee et al., 2002; Marczynski et al., 2002; Mohankumar et al., 2002). The assay detects DNA strand breakage and alkali-labile sites by measuring the migration of DNA from immobilized individual cell nuclei.

Invertebrates are an interesting subject of ecotoxicological research because of their significance in ecosystems. In this context, the comet assay has been successfully used for genotoxicity assessment in marine and freshwater invertebrates (Cotelle and Ferard, 1999; Lee and Steinert, 2003; Mitchelmore and Chipman, 1998). More specifically, it can be applied to explore pesticide resistance or the selection of environmental pollutant immunity, as well as to better understand the ageing of insects. To date, terrestrial species of several orders, including insects that are significant to the human economy, have been examined using this assay. This includes organisms such as *Liriomyza trifolii* of order Diptera (Koo et al., 2012); *Tenebrio molitor* (Wright et al., 2004), *Curculio sikkimensis* (Todoriki et al., 2006), *Sitophilus zeamais* (Hasan et al., 2008), and *Lasioderma serricorne* (Kameya et al., 2012) of order Coleoptera; *Plodia interpunctella* (Imamura et al., 2004), *Plutella xylostella* (Koo et al., 2011) of order Lepidoptera; and aquatic species, including *Chironomus riparius* (Martinez-Paz et al., 2013; Morales et al., 2013) and *Chironomus kiiensis* (Al-Shami et al., 2012). Undoubtedly, evaluations of damage to genetic material will be important in insects that are of substantial importance to humans, such as crop pests, disease vectors and social insects. In view of this, the present study was undertaken to assess the different doses of gamma radiation-induced genotoxicity in *A. aegypti* at different time intervals using the alkaline comet assay.

Materials and methods

Mosquito rearing

A. aegypti larvae collected from the J. P. Nagar area of Bengaluru, India were reared at 25 $\pm$ 1°C and 75 $\pm$ 5% relative humidity under a 14-h photoperiod in the insectary of the Centre for Applied Genetics, Bangalore University following standard protocol (Shetty, 1983).

Gamma irradiation

Experiments were performed in triplicate, along with a control. Overall, a total of 1680 adult males, 2–3 days of age, were irradiated with different doses of gamma radiation from a ^{60}Co (Theratron 780-C Telecobalt Unit, AECL, Ontario, Canada) source with a dose rate of 253.56 cGy/min at the Kidwai Memorial Institute of Oncology, Bengaluru. The mosquitoes were placed in plastic boxes (5 $\times$ 4 $\times$ 2.5 cm) covered with fine net cap during irradiation. Doses of 1, 5, 10, 20, 30, 40 and 50 Gy were chosen for the study. Dosimetry was employed to quantify the dose received by the irradiated insects and confirm that all the doses delivered lay within a 5% error range. Each batch consisted of 240 adult mosquitoes receiving a specific dose of the radiation. The irradiated mosquitoes maintained in the insectary for further analysis.

Genotoxicity study using comet assay

The DNA damage studies were carried out using SCGE. The protocol followed is described by Singh et al. (1988) with minor modifications as described below.

Slide preparation

Whole body homogenates were prepared by pooling 20 irradiated males, each at four different time intervals, that is, 1, 3, 6 and 12-h postirradiation. A control set was prepared in a similar manner. Twenty

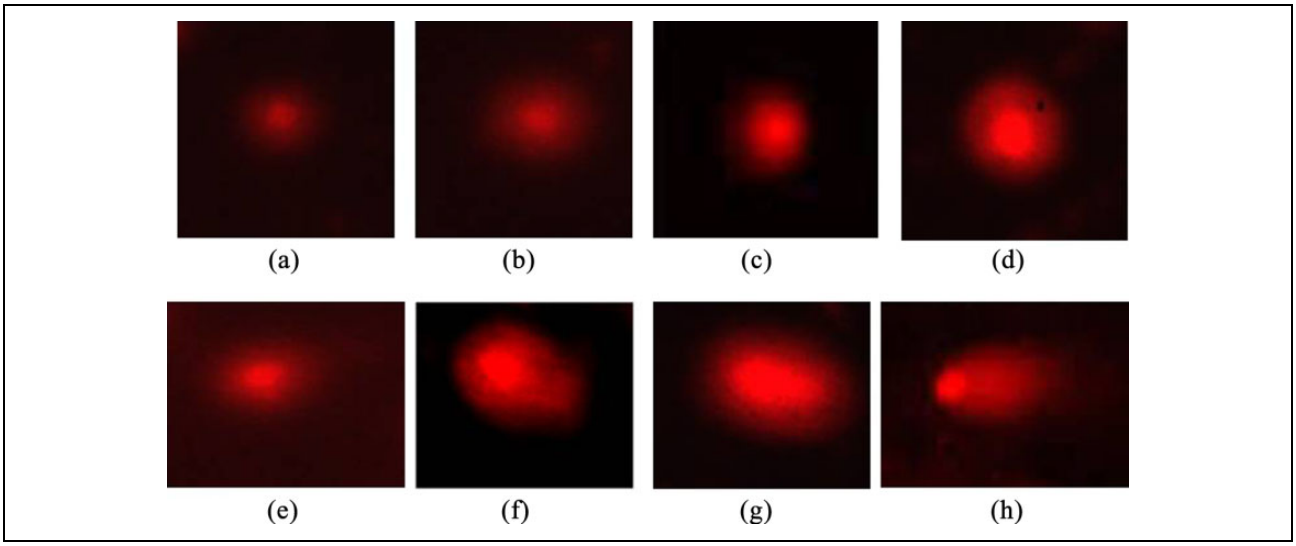

Figure 1. Representative figures of the comet assay at 1 h postirradiation. (a) Control, (b) 1 Gy, (c) 5 Gy, (d) 10 Gy, (e) 20 Gy, (f) 30 Gy, (g) 40 Gy and (h) 50 Gy.

mosquitoes were homogenized in 10% (w/v) homogenizing buffer (0.075 M NaCl and 0.024 M EDTA). The homogenate mixture was centrifuged at 1000 r/min for 10 min using a cold centrifuge at 4°C. The pellets were gently resuspended in 1 ml of chilled homogenizing buffer for nuclei preparation. Roughened frozen microscopic slides were marked, placed horizontally and then a thin, homogenous layer of 1% normal melting agarose was cast onto the slide. The slides were dried at room temperature and then placed at 4°C until used. Subsequently, each precoated slide was cast with 100 µl of isolated nuclei and 1% low melting agarose (1:4) mixture using a cover slip and allowed to solidify at 4°C for 20 min. After the removal of the cover slip, the slides were immersed into freshly prepared chilled lysis buffer (2.5 M NaCl, 100 mM EDTA pH 10, 5% DMSO and 1% Triton X-100) for 1 h in the dark, at 4°C. Following this, the slides were incubated in alkaline electrophoresis buffer (1 mM EDTA and 300 mM NaOH, pH > 13) in an ice-cold electrophoresis chamber for 20 min to facilitate unwinding of DNA strands; the process was subsequently conducted for 20 min at 25 volts/300 mA. The slides were washed thrice by incubating the slides for 5 min each in neutralizing buffer (0.4 M Tris, pH 7.5). Just before visualization, the slides were stained with ethidium bromide (20 µg/ml, 40 µl/slide) for 10 min in the dark or without direct exposure to light. The slides were then washed once in chilled distilled water by dipping to remove excess ethidium bromide and subsequently were covered with a cover slip. The slides were stored in a dark, humidified chamber and analysed within 3 ± 4 h.

Comet capture

A total of 50 cells from each slide were analysed at 40 × magnification, using a Zeiss Axioskop 2 fluorescence microscope (ZEISS, Germany) with an extinction filter of 515–560 nm and a barrier filter of 590 nm. AxioVision Rel. 4.8 software was used for photography. Comet tail length and the percentage of DNA damage in the tail were measured with CASP comet software (CaspLab 1.2.3beta2 version).

Statistical analysis

Analysis of variance (ANOVA)-General Linear Model (GLM) followed by Tukey's post hoc test was performed to analyse the significant difference in the percentage of tail DNA in-between test samples. In addition, trend analysis in the form of linear regression was performed using SPSS, and a significant dose–rate response relationship was indicated by a slope significantly different ($p \leq 0.05$) from zero.

Results

This study detects, at the microscopic level, the extent of DNA damage on a temporal scale ranging from 1 to 12 h, following gamma irradiation with doses ranging from 1 to 50 Gy. The postirradiation DNA damage as observed in individual cells of adult *A. aegypti* is illustrated in Figure 1. This representation figure

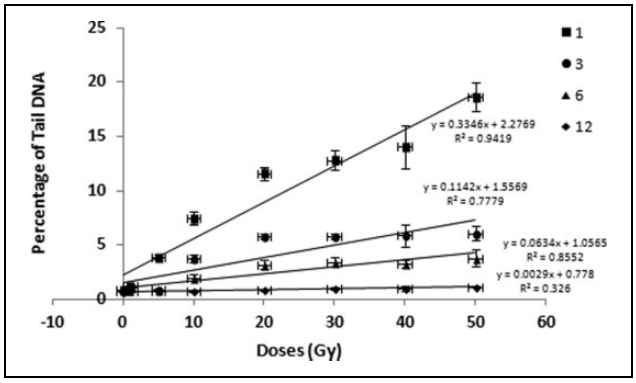

Figure 2. Dose–response relationship for DNA damage at 1 h, 3 h, 6 h and 12 h after gamma irradiation on adult male *Aedes aegypti*.

shows the comet assay images of adult *A. aegypti* taken 1 h after irradiation to different doses of gamma rays. Gamma radiation breaks DNA strands, increasing the migration of short-chain fragments leading to the formation of a comet-like shape with a long tail, following electrophoresis. The dose- and time-dependent increase of DNA damage, induced by gamma radiation, and their statistical significance are represented in Figure 2.

A significant increase in DNA damage was observed in all the samples of *A. aegypti* irradiated with various doses of gamma radiation except those treated with 1 Gy (1 h postirradiation), 1 and 5 Gy (3 h postirradiation), 1, 5 and 10 Gy (6 h postirradiation), and 12 h postirradiation for all doses, which did not show a significant increase ($p \geq 0.05$) in the percentage tail DNA with reference to the control.

The dose–response study showed that there was a dose-dependent increase in the intensity of radiation and DNA damage with a minimum percentage of tail DNA (1.22 ± 0.24) at the lowest dose of 1 Gy and the maximum percentage of tail DNA (18.64 ± 1.36) at the highest dose of 50 Gy at 1 h postirradiation. It was also observed that the dose–response effect was linear for different time points (i.e. 1 h, 3 h and 6 h) except 12 h postirradiation. When significant dose–rate response trends were found using linear regression, the goodness of fit (R^2 adjusted) was high for the samples exposed to different doses of gamma radiation and at the time intervals, that is, 1 h, 3 h and 6 h after radiation except in the samples 12 h postirradiation (Figure 2; 1 h, slope $= 0.334$, $R^2 = 94\%$, $p \leq 0.05$; 3 h, slope $= 0.114$, $R^2 = 77\%$, $p \leq 0.05$; 6 h, slope $= 0.063$, $R^2 = 85\%$, $p \leq 0.05$; 12 h, slope $= 0.002$, $R^2 = 32\%$, $p \geq 0.05$). There were few comets of the apoptotic types found in each dose especially at 1 h postirradiation. Since these comets showed a very high percentage of tail DNA (ranging from 50% to 80%), they were not considered for the count as comets of the apoptotic types give high variation in the mean percentage of tail DNA. A one-way ANOVA of the data on the dose-dependent DNA damage observed in the control and treated samples showed significance at $p \leq 0.05$ ($F = 97.44$) at the first three time points studied (i.e. 1 h, 3 h and 6 h, respectively).

A time-response study indicated significant DNA damage at initial three time points, that is, at 1 h, 3 h and 6 h for the 10, 20, 30, 40 and 50 Gy doses of gamma radiation that were studied. Samples of adult *A. aegypti* for all the above said doses, post 12 h of irradiation on the other hand, did not show any significant difference ($p \geq 0.05$) in the percentage of tail DNA, when compared to the control. The highest DNA damage (18.64 ± 1.36) was observed in the 1 h post-treatment 50 Gy exposed samples, and it decreased at the later time points reaching a minimum (3.81 ± 0.75) at 6 h and normal (1.16 ± 0.18) at 12 h postirradiation (1 h, $p \leq 0.05$; 3 h, $p \leq 0.05$; 6 h, $p \leq 0.05$; 12 h, $p \geq 0.05$). A similar trend was also observed for the other doses 5, 10, 20, 30 and 40 Gy.

Discussion

Gamma rays are known to induce various types of cellular and subcellular damage in living organisms (Garrison and Uyeki, 1988). Radiation, which acts on the cellular components, breaks chemical bonds (and also DNA DSBs) and provokes the production of free radicals, which results in oxidation and subsequent damage. The comet assay has previously confirmed that irradiation (both electron beam and gamma ray induced) can cause severe DNA damage in a dose-dependent manner in the investigated species (Augustyniak et al., 2016). Several studies have been conducted to evaluate radiation-induced DNA damage in agricultural pests, such as the Indian meal moth *P. interpunctella* (Imamura et al., 2004), the maize weevil *S. zeamais* (Hasan et al., 2008), the diamond-back moth *P. xylostella* (Koo et al., 2011) and the Oriental leafworm moth *Spodoptera litura* (Yun et al., 2014). However, this is the first time a similar study has been conducted on the mosquito vector species, *A. aegypti*.

We show a significant increase in gamma radiation-induced DNA damage by an increase in the mean percentage of tail DNA, using the comet assay at doses ranging from 5 Gy to 50 Gy of gamma

radiation, when compared to the control. From the study, the data indicated that gamma radiation is a potential genotoxic inducer in *A. aegypti,* especially at 1 h postirradiation in all the doses ranging from 5 Gy to 50 Gy. Exposure to 1 Gy, however, showed no significant increase in the percentage of tail DNA at any time point, when compared to the control. In a similar radiation study on *S. zeamais,* exposure to doses 0.5 kGy and 1 kGy was analysed using the comet assay, which recorded an increase in DNA damage with an increase in radiation dose for all the developmental stages, clearly indicating that radiation-induced DNA damage in all the stages was dose dependent (Hasan et al., 2008). Todoriki et al. (2006) employed the comet assay to evaluate electron beam radiation sensitivities in mature larvae of chestnut weevil, *C. sikkimensis,* and showed that DNA damage increases significantly as dose increases.

In the current study, we employed a temporal comet assay to understand DNA damage following gamma radiation. The study showed that significant DNA damage occured at 3 and 6 h postirradiation, showing a minimum amount of increase in the percentage of tail DNA, and 12 h postirradiation showed no significant increase in the percentage of tail DNA for any of the doses exposed. The results, thus, suggest that the genotoxic effect of gamma radiation does not last for a long period in *A. aegypti*. In a similar manner, DNA damage in *L. serricorne* exposed to gamma radiation of 1 kGy was evaluated using the comet assay under alkaline conditions, wherein broken DNA strands appeared to be repaired as the postirradiation period lengthened (Kameya et al., 2012). The decrease in genetic damage at later times may indicate either repair of damaged DNA or loss of heavily damaged cells or both (Revankar and Shyama, 2009; Saleha Banu et al., 2001).

It was recorded that high-dose irradiation (30 Gy) lengthened the prepupal period in wild-type (Canton S and Oregon R) and mutant strains such as DNA damage sensing (mei-41), DNA repair (mus209, mus210, mus309, rad54) and free radical detoxification (sod) strains of *Drosophila melanogaster.* The obtained results suggest the important role of free radical detoxification, DNA damage sensing and DNA repair mechanisms in the whole organism radiation-induced effects (Shaposhnikov et al., 2009). Exposure of gamma radiation doses (1–50 Gy) to *A. aegypti* showed a 10–12 days' increase in longevity at 4 Gy; however, the lifespan decreased following exposure to higher doses ranging from 30 Gy to 50 Gy (Shetty et al., 2016). Such hormetic dose responses to gamma radiation have been recorded in many insect species (Seong et al., 2011; Vaiserman et al., 2003). Similar results were recorded in a study on the adult/pupal stages of *Anopheles arabiensis*, where it was shown that an overall similar or higher survival is observed in the irradiated samples when compared to the control (Helinski et al., 2006). For *Anopheles pharoensis*, a slight increase in longevity of males irradiated with doses ranging from 5 Gy to 70 Gy was reported (Abdel-Malek et al., 1966). Irradiation-induced reduction in longevity has been recorded in several anopheline species such as *Anopheles stephensi, An. pharoensis* and *Anopheles gambiae* s.s. as the dose increases beyond 80 Gy (Abdel-Malek et al., 1967; Curtis, 1976; Sharma et al., 1978). In *D. melanogaster,* it was shown that DNA damage and the following overexpression of different DNA repair genes led to both positive and negative effects on lifespan and stress resistance (Shaposhnikov et al., 2015).

The possible mechanism of radiation-induced DNA damage in *A. aegypti* may be the generation of reactive oxygen species (ROS) by gamma radiation, therefore causing DNA damage. ROS at high and/or sustained levels can cause severe damage to DNA, protein and lipids (Lau et al., 2008). Various stressors present in the environment, including pesticides and radiation, are capable of reacting with DNA and causing DNA damage. Stressors also have the capability to generate ROS, one of the possible mechanisms for the induction of DNA damage may be through the generation of ROS (Joseph et al., 2014; Rastogi et al., 2014; Wilson et al., 2003). After irradiation, the ability of an insect to survive and reproduce is closely related to the level of DNA damage (Augustyniak et al., 2016; Yun et al., 2014). The effects that are detected by SCGE, such as chromosomal aberrations due to DSBs and less extensive damage, especially in germ cells, can affect the fitness of the entire population if it reaches a critical level (Augustyniak et al., 2016). *A. aegypti* is also a well-known mosquito vector for dengue, chikungunya and DHF (Gubler, 1998). Numerous vector control measures have been initiated to curb its proliferation. One such measure includes the radiation-induced sterile insect technique (SIT) which involves the release of sterile males into the environment in an attempt to control its population. Several irradiation studies have been conducted and isolated radiation-induced chromosomal translocations in mosquito species such as

Culex quinquefasciatus (Shetty, 1993), *Culex pipiens* complex (Bhalla et al., 1974), *Anopheles fluviatilis* (Shetty, 1983), *An. stephensi* (Gayathri and Shetty, 1992) and *A. aegypti* (Rai et al., 1970), and it was shown that it could be effectively employed for genetic control programmes. It was also noted that in combination with selecting appropriate translocations, the doses of ionizing radiation which induced potentially deleterious effects while extending lifespan, probably offer an effective method of genetic control of mosquitoes using SIT (Shetty et al., 2016).

From this study, it appears that the alkaline comet assay is a promising technique to assess the genotoxic potential of gamma radiation in *A. aegypti*. A dose-dependent increase and a time-related decrease of genotoxicity of gamma radiation were observed in *A. aegypti*. This could be used as a sensitive biomarker for environmental risk assessment. Further, this assay may be used to explain the connection between the stress that is induced by radiation and DNA damage, as well as the repair efficiency under limited energy conditions.

Acknowledgements

We are grateful to the Department of Radiation Physics, Kidwai Memorial Institute of Oncology, Bengaluru, for providing radiation facility.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

This work was supported by the grants of the Board of Research in Nuclear Sciences (BRNS), Department of Atomic Energy (DAE), Bhabha Atomic Research Centre (BARC), Mumbai (No.2009/36/80-BRNS/2394 Dated 9/12/2009).

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