Chironomus ramosus larvae exhibit DNA damage control in response to gamma radiation

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Abstract

Purpose: Chironomus ramosus is one of the recently reported radiotolerant insects. Salivary gland cells of fourth instar larvae respond to ionizing radiations with increases in the levels of antioxidant enzymes and chaperone proteins. Here we made an attempt to study the state of nuclear DNA after exposure of larvae to a lethal dose for 20% of the population (LD20) of gamma radiation (2200 Gy, at a dose rate 5.5 Gy/min).

Materials and methods: Genomic DNA preparations were subjected to competitive ELISA (Enzyme linked immunosorbent assay) for detection of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and dynamic light scattering (DLS) to monitor any radiation-induced damage. Single salivary gland cells were subjected to alkaline single cell gel electrophoresis (ASCGE), comet assay and pulsed field gel electrophoresis (PFGE) to check for DNA double-strand breaks.

Results: Results from all four experimental procedures confirmed damage of nucleobases and fragmentation of nuclear DNA immediately after radiation. Some 48 h after radiation exposure, modified 8-oxodG residues returned to basal level, homodisperity of genomic DNA reappeared, the length of comet tail regressed significantly (ASCGE) and PFGE pattern matched with that of high molecular weight unirradiated DNA.

Conclusion: Chironomus ramosus larvae showed control of DNA damage as observed over 48 h in post irradiation recovery which could be attributed to their ability to tolerate gamma radiation stress.

Keywords: Chironomus ramosus, alkaline comet assay, gamma radiation stress, DNA damage response, Pulsed field gel electrophoresis, dynamic light scattering

Introduction

Tolerance to high doses of ionizing radiation makes non-biting midges (Diptera: Chironomidae) an extraordinary group of stress-tolerant insects (Watanabe et al. 2006, Datkhile et al. 2009a, Jorgelina and Karsten 2010). Members of this primitive group thrive under diverse ecological and climatic conditions (Ferrington 2008), sustain a wide variety of biotic and abiotic stresses (Choi 2004, Warrin et al. 2012) including accumulation of uranium in simulated laboratory conditions (Muscabella and Liber 2009). So far, tolerance to very high doses of ionizing radiation has been reported in rare life forms like the radioresistant tardigrades (Harikawa and Hitashi 2004), bdelloid rotifers (Gladyshevet and Meselson 2008), or radioresistant bacteria Thermococcus gammatolerans (Jolivet et al. 2003) and Deinococcus radiodurans (Slade and Radman 2011). Among the dipteran insects, Polypedilum vanderplanki, is radiotolerant. Resistance to radiation here is suggested to be a consequence of their evolutionary adaptation to survive desiccation as both radiation and desiccation are DNA damage stressors (Gusev et al. 2010). Gamma radiation doses affecting the developmental stages of Indian tropical midge Chironomus ramosus are remarkably high compared to other known insects (Datkhile et al. 2009a). One of the hallmarks of ionizing radiation on biological systems is damage caused to cellular DNA. Damage can be direct through the generation of single- or double-strand breaks in DNA or indirectly by the generation of reactive oxygen species (ROS), which gives rise to an additional stress leading to oxidatively damaged biomolecules. The most commonly formed adduct in nuclear DNA, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) residues has been a ubiquitous biomarker for measurement of DNA oxidation (Cooke et al. 2003, Cadet et al. 2012). The presence and repair of modified base 8-oxodG has emerged as a quantifiable biomarker for the detection of oxidatively damaged and repaired DNA (Rosen et al. 1996, Kasai 1997, Elliott et al. 2000, Shen et al. 2007). The extent and nature of radiation-induced damage however, depends on the type of radiation, rate of exposure, effective cumulative dose to specific cellular and sub-cellular compartments and lastly, upon tolerance threshold of each organism.

In midges, salivary gland cells (SGC) are the most metabolically active larval organs containing giant polytene chro-
mosomes. High transcriptional activity, content of abundant proteins and genomic DNA make it the most widely used tissue for cytotoxicity, genotoxicity and molecular studies (Nath et. al. 2005, Thorat and Nath 2010). Our earlier study on radiation tolerance of Indian tropical midge, C. ramosus showed that the larvae contained relatively high antioxidant enzymatic activities concomitant with an elevated level of 70 kDa Heat Shock Protein (HSP 70) expression in the irradiated SGC during recovery from radiation stress (Datkhile et al. 2009b, 2011). Increased DNA repair capabilities of tissue cultured cells have been reported from other insects in the past (Koval 1994, Chandana et al. 2004). In this study, biochemical and biophysical methods were implemented to observe effects of gamma radiation on SGC nuclear DNA of C. ramosus larvae.

Material and methods

Rearing of Chironomus and gamma radiation exposure

The inbred line of laboratory culture of C. ramosus was originated from a single egg mass (isofemale line) from a water body of Pune city, India (Lat. 18°33’ 17.73” N, Long. 73°51’ 48.06” E) inhabited by the natural population of C. ramosus. Culture was maintained by mass rearing technique (Nath and Godbole 1998) at 25 ± 2°C temperature, relative humidity of 50 ± 10%, and a photoperiod of 14 h light and 10 h darkness in the insectary of Bhabha Atomic Research Centre (BARC), Mumbai. Thirty-day-old fourth instar larvae were put in a glass beaker containing tap water and exposed to a dose of 2200 Gy a lethal dose for 20% of the midge population (LD20) determined by dosimetric studies for the determination of percent mortality of irradiated larvae followed by Probit analysis (Datkhile et al. 2009a) from a cobalt 60Co source (dose rate 5.5 Gy/min, Gamma Cell 220, Atomic Energy of Canada Ltd, Ottawa, Canada). Another 60Co source (dose rate 55.6 Gy/min, Gamma Cell 5000 BRIT, Mumbai, India) was used to check the effect of shorter exposure time for the same dose of radiation. Equal numbers of fourth instar larvae from the same generation were kept outside the gamma chamber for the same duration of time in a beaker containing tap water. All subsequent experiments were done with larvae that survived radiation exposure. Fifty larvae each from control and test groups were dissected to obtain 100 larvae that survived radiation exposure. Fifty larvae each with peroxidase (Sigma) at 37°C for 90 min at 37°C for 30 min. After five washes of PBS-T the plates were incubated with 100 μl (1:10000) avidin conjugated with peroxidase (Sigma) at 37°C for 30 min. Finally, after three washes of PBS-T followed by two washes with phosphate buffer saline (PBS-T) for 10 min incubation using Spectra max 250 ELISA reader (Erba Microscan, Verkaufsburo, Germany). For Anti 8-oxodG antibody (synthesized and characterized in the laboratory, Modak et al. 2009), titer of the antibody used was 1:1000.

Alkaline single cell gel electrophoresis (ASCGE)

Single cell suspensions of salivary glands were mixed with 1.5 ml of 0.8% agarose solution (Sigma, St. Louis, MO, USA) at 42°C and carefully poured over pre-chilled frosted slides (Fisher Scientific, Carlsbad, CA, USA) to make a uniform layer. In-gel lysis of cells was carried out for 1 h at 4°C by immersing slides in lysis solution containing 2.5 M NaCl (HiMedia, Mumbai, India), 100 mM Na2EDTA (ethylenediaminetetra-
cetic acid) (HiMedia), 1% Triton X-100 (HiMedia) and 10% DMSO (Dimethyl sulfoxide) (HiMedia). Slides were washed three times in chilled alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na2EDTA, pH 13.0) and kept in the same buffer for 20 min to allow unwinding of polytene chromosomal DNA. Electrophoresis (Amersham Biosciences, Piscataway, NJ, USA) was carried out in cold for 20 min at 25 V, and 300 mA in horizontal tanks (Singh et al. 1988). Slides were washed by flooding with neutralizing solution 0.4M Tris, pH 7.5 (HiMedia) avoiding physical damage to DNA from handling. SYBR Green-I dye (Molecular Probes, Eugene, OR, USA) stained cell nuclei were scored under Fluorescence microscope (CarlZeiss/AxioplanequippedwithKY-F55BE3CCD, JVC Yokohama, Japan). Digital images of comets were analyzed using SCGE-Pro software (BARC, Mumbai, India).

Competitive enzyme linked immunosorbent assay (ELISA) for detection of oxidatively damaged DNA

Levels of 8-oxodG were estimated by competitive enzyme linked immune-sorbent assay (Modak et al. 2009). Genomic DNA was extracted from 100 SGC per sample (i.e., control larvae and larvae at different recovery phases post exposure to radiation) following standard organic phase separation method (Sambrook et al. 1989).

ELISA assay

To each well of polystyrene flat bottom microtiter plate (Sigma) pre-coated with 0.03% protamine sulphate (Sigma) 100 ng of 8-oxodG was added for competitive ELISA assay. Single stranded DNA (100 ng) samples were incubated with 100 μl of monoclonal antibody against 8-oxodG (1 μg/ml) for 90 min at 37°C. After five washes with 0.05% Tween 20 in Phosphate Buffer Saline (PBS-T) (Sigma) 100 μl (1:2000) of goat anti mouse Immunoglobulin G (IgG)-conjugated with biotin (F(ab’)) fragment (Sigma) in PBS (Sigma) was added to each well and incubated at 37°C for 30 min. After five washes of PBS-T the plates were incubated with 100 μl (1:10000) avidin conjugated with peroxidase (Sigma) at 37°C for 30 min. Finally, after three washes of PBS-T followed by two washes with phosphate buffer saline (pH 5.0), 100 μl of substrate solution containing 10% of 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) (Sigma) and 0.06% H2O2 in phosphate citrate (Bangalore GeNei, Bangalore, India) was added to each well. Absorbance was measured at 410 nm after 10 min incubation using Spectra max 250 ELISA reader (Erba Microscan, Verkaufsburo, Germany). For Anti 8-oxodG antibody (synthesized and characterized in the laboratory, Modak et al. 2009), titer of the antibody used was 1:1000.

Pulsed field gel electrophoresis (PFGE)

DNA agarose plugs containing either salivary gland cells or cell nuclei were prepared under sterile conditions and were stored at 4°C after complete removal of lysis buffer containing additional 1 mg/ml Pronase-A (MBI Fermentas, Vilnius, Lithuania) (Cantor et al. 1988).

Preparation of nuclei

Salivary gland tissues were homogenized in 400 μl of nuclei isolation buffer (NIB) containing 80 mM KCl (HiMedia),
10 mM EDTA (HiMedia), 1 mM spermidine trihydrochloride (Sigma), 1 mM spermine tetrahydrochloride (Sigma), 10 mM Trizma (HiMedia), 0.5% Triton X-100 (HiMedia), 0.5 M sucrose (Bangalore GeNi) and 0.15% 2-mercaptoethanol (Bangalore GeNi). Homogenates were filtered through micro cloth to remove the cell debris and centrifuged at 10,000 g for 10 min (4°C) to obtain nuclear pellet. Pellets were washed two times with ice-cold NIB, resuspended in 50 μl of NIB (Zhang et al. 2012).

Restriction endonuclease digestion of DNA in agarose plugs

Agarose plugs were transferred to microfuge tubes (Tarson, Kolkata, India) containing 10 volumes of 1× restriction enzyme buffer-O (MBI Fermentas) for 30 min at room temperature. This was replaced with 3 volumes of 1× buffer-O containing 20 units of Nru-I restriction endonuclease (MBI Fermentas) and incubated at 37°C for 12 h to obtain complete restriction of embedded genomic DNA in plugs.

Figure 1. Alkaline comet assay of single salivary gland cells. Fourth instar larvae of C. ramosus were exposed to 2200 Gy gamma radiation and allowed to recover until 48 h. (A) Distribution of SYBR Green-I stained DNA in head and tail of comets from control larvae (left side panel) and irradiated larvae (right side panel) under 40× magnification (bracket indicates under measurement intensity). (B) Histogram showing percent DNA in tail. Data points represent (mean ± SE) of the tail lengths of comets from three independent experiments with 50 larvae per experimental group. Significance of difference p < 0.05. This Figure is reproduced in color in the online version of International Journal of Radiation Biology.
Electrophoresis parameters
Broma Chiller system 2015 Pulsaphor electrophoresis unit (LKB), with 2301 macrodrive-1 power supply and 2015 pulsaphor control unit, (Amersham Biosciences), was used for PFGE of genomic DNA in 0.8% agarose gels prepared in 0.5× (Tris Borate EDTA) TBE buffer (45 mM Tris, 90 mM boric acid, and 1 mM EDTA pH 8.0) and subjected to 150 volts (10 V/cm) for 48 h with pulse control switch times of 90 s. Thereafter, gels were stained with 1 mg/ml ethidium bromide (Bangalore GeNei) solution for 1 h and de-stained for 30 min. Gels were visualized and photographed under Ultra-Violet (UV) Trans-illuminator (Syngene, Bangalore, India).

Dynamic light scattering (DLS)
Ten whole larvae each of irradiated and non-irradiated groups from 0, 24 and at 48 h PIR were homogenized in 500 μl of DNAzol solution (Molecular Research Centre, Cincinnati, OH, USA), centrifuged at 15,000 g for 30 min at 4°C and genomic DNA was precipitated from the supernatant. After RNase (Sigma) treatment, 1 μg/ml of DNA in Milli Q water (Millipore, Darmstadt, Germany) was subjected to DLS (Malvern ZP, Worcestershire, UK) at 20°C fixed scattering angle. Size by volume variations of DNA in solution was measured.

Statistical analysis
Alkaline comet assay and DLS results were analyzed using MicroCal, Origin Version 6, (Northampton, MA, USA). Mean, standard error (± SE) for each group is depicted in figures. Statistical analysis was carried out using one-way analysis of variance (ANOVA) with p < 0.05 for alkaline comet assay. For DLS data, Student’s t-test was used for analysis with p < 0.05.

Results
Measurement of comets
Percent DNA in comet tail (% T-DNA), tail moment (TM) and tail length (TL) of comets were evaluated in radiation exposed SGC at 0, 24 and 48 h PIR to study the fate of DNA in individual cells (Figure 1A). Total fluorescence intensity of DNA in head and tail of comets showed wide variations among the control and irradiated groups immediately after exposure and up to 24 h PIR. This difference was not significant among samples taken at 48 h PIR. Comparative values of percent DNA estimated from the tail moment and tail length clearly indicated that maximum damage occurred immediately after exposure to radiation stress and during PIR (Figure 1B) these values came down to normal levels.

Estimation of 8-OxodG
A two-fold increase in the level of 8-oxodG 34,600 ± 2200 nmoles to 73,800 ± 1730 nmoles per μg of DNA was observed immediately after exposure to radiation stress. The amount of 8-oxodG reduced significantly (25,800 ± 1220 nmoles) in DNA extracts prepared from SGC of larvae after 48 h post exposure to radiation stress indicating efficient removal of modified bases from the system by this time period (Figure 2).

Migration of DNA from restricted and non-restricted DNA-agarose plugs
High molecular weight DNA fragments resolvable in agarose gel showed differences between the migration patterns of Nru I restricted DNA in control and irradiated larvae. Low molecular weight DNA fragments observed in SGC of irradiated larvae at 0 h shifted towards high molecular weight DNA on the gel after 24 h PIR and this was more pronounced by 48 h PIR (Figure 3A). Shift of fragment sizes were compared with molecular weight markers (Figure 3B). Salivary gland cell nuclei plugs prepared from larvae exposed to higher dose rate radiation showed similar effects of radiation on high molecular weight DNA even without restriction digestion (Figure 3C). Immediately after removal from radiation environment DNA damage was maximal.

Figure 2. Competitive ELISA for measurement of 8-oxodG in salivary gland cells of C. ramosus. Level of oxidized products of 8-oxodG residue measured using anti 8-oxodG antibody to check for gamma radiation-induced DNA base damage. Histogram showing levels of 8-oxodG in control and irradiated larvae at 0, 24 and 48 h PIR. Each bar represents (mean ± SE) from three independent experiments (##p < 0.05, #p > 0.05).

Figure 3. Representative image from three independent pulsed field gel electrophoresis of salivary gland cell DNA and nuclei of C. ramosus. Pattern of migration of ethidium bromide stained genomic DNA on 0.8% agarose gel during recovery from gamma radiation stress. (A) Restriction enzyme (NruI) digested genomic DNA: lane-1. Control; lane-2. LD20 0 h; lane-3. LD20 24 h; lane-4. LD20 48 h. (B) High molecular weight DNA marker. (C) SG nuclei after high dose rate radiation and without restriction digestion: lane-1. Control; lane-2. LD20 0 h; lane-3. LD20 24 h; lane-4. LD20 48 h.
Estimation of DNA fragmentation by dynamic light scattering (DLS)

The size by volume distribution of total DNA molecules in solution showed additional peaks from fragmented DNA in irradiated samples. Hydrodynamic diameter of control DNA sample, i.e., 122.42 d nm (diameter in nanometers) with 19.16% volume distribution (Figure 4Aa) changed to two peaks of 78.8 d nm with 12.7% volume distribution and 615.1 d nm with 8.7% volume distribution after gamma radiation (Figure 4Ab). After 24 h PIR, hydrodynamic diameter of DNA was 396.0 d nm with 15.0% volume distribution (Figure 4Ac). By 48 h post radiation, average size of larval DNA was 220.2 d nm with 13.9% volume distribution showing a reverse trend as it was found in the non-stressed condition (Figure 4Ad). A histogram plotted from three independent observations showed maximum polydispersity of DNA (i.e., larger hydrodynamic diameter) immediately after removal from stress conditions (Figure 4B). Over a period of 24 and 48 h, the hydrodynamic diameter reduced gradually towards that found in control larvae. Importantly this is a trend where we did not find a complete reversal to baseline levels unlike what was observed in the experimental results with 8-oxodG and the alkaline comet assay.

Discussion

DNA damage is the critical target of any ionizing radiation-induced biological endpoint. Extent of damage and inherent capacity to repair determines fate of survival of an organism under radiation stress. Some prokaryotes have evolved extremely efficient mechanisms of DNA repair; allowing restoration of DNA structures even after very high doses of gamma radiation (Battista 1998, Billi et al. 2000). DNA repair mechanisms are well documented in radiotolerant eukaryotes

![Figure 4. Dynamic light scattering of larval genomic DNA from *C. ramosus*. Effect of gamma radiation on changes in hydrodynamic diameter of DNA (Zeta analyzer, Malvern). One microgram per ml concentrations of larval genomic DNA (control and 2200 Gy gamma radiation exposed) was used (A) to measure size by volume and percent intensity distribution of molecules in solution (a) Control sample, (b) Sample taken immediately after radiation exposure, i.e., 0 h PIR, (c) 24 h PIR, and (d) 48 h PIR. All samples were analyzed at 20°C with fixed scattering angle. (B) Histogram showing hydrodynamic diameter of DNA in control and irradiated samples at different time-points in PIR (Student’s t-test, *p* < 0.05 and **p** > 0.1).]
(Koval 1994, Elliott et al. 2000), but it is not considered as the primary mechanism for radiation tolerance in multicellular animals (Clegg 2001, Chandna et al. 2004). Radiation tolerance in C. ramosus larvae was demonstrated by us in SGC in earlier studies (Datkhile et al. 2011), hence the current investigation was focused on the same tissue. Also, DNA content is higher in SGC compared to other diploid tissues of the midge larvae (Daneholt and Edström 1967, Macgregor and Varley 1988, Nath and Godbole 1997). Detailed dosimetry studies of C. ramosus had established that 2200 Gy dose was well tolerated by 80% larvae over a period of 24 h observation, hence this LD20 dose was chosen for studying effect on DNA. Comet formation and PFGE have been chosen as preferred experimental methods to demonstrate DNA damage (Metzger and Iliakis 1991, Chaubey et al. 2001, Speit and Hartmann 2006, Herschleb et al. 2007). Differences in the tolerance dose could be noticed in the literature with reference to DNA damage experiments. Chandna et al. (2004), compared lepidopteran SF-9 cell line with the threshold dose used for human BMG-1 glioma cell line. In our case, where whole organism is exposed to radiation, tolerance data from the cell lines would not be appropriate for comparison. Inclusion of other insect model systems for the comparison of the parameters between radioresistant and radiosensitive individuals at the whole organismal level would be useful in future. Here, we introduced DLS, a biophysical technique for measuring the size of molecules in suspension in order to assess radiation stress induced changes in DNA (Banerjee et al. 2014). The method is presently gaining momentum for evaluation of DNA and chromatin damage (Jain et al. 2014). To our knowledge this is the first report of this method using an insect model organism, as well as optimization of PFGE for separation of large polytene DNA of Chironomus midge salivary gland cells. Resistance to migration in agarose gel was observed over time of PIR suggesting rejoining of damaged SGC DNA to higher molecular weight. Experimental conditions for PFGE studies (different dose rates of gamma source) were designed to achieve the same dose of 2200 Gy in a shorter exposure time in order to confirm that DNA fragmentation were indeed an effect of radiation and not any other physiological parameters that may prevail during the longer exposure time for the same dose. Indirect evidence for existence of oxidative damage control system was also shown for the first time in C. ramosus by ELISA. Lower than basal levels of modified 8-oxoG bases present in irradiated larvae (after 48 h in PIR) could be transient due to differences in the metabolic state of the two groups. Nevertheless, further studies are warranted to elucidate the mechanism of this remarkable system(s) of control of DNA damage caused by gamma radiation.

Conclusion
Evidence obtained in the present study in conjunction with our earlier findings encourage us to hypothesize that in C. ramosus, tolerance to gamma radiation could be an integrative cellular defense mechanism comprising of control of DNA damage, role of antioxidant enzymes and radiation responsive stress proteins. Evolutionarily, the ancient history of existence of insects may be an appropriate justification for such adaptability seen in Chironomus species.

Acknowledgements
Authors thank Dr R.C. Chaubey and Dr H. N. Bhiwade for their help with microscopy and SCGE data analysis software. Dr R. Shashidhar for his help in PFGE experiments and Dr Lata Panicker for DLS analysis. Authors acknowledge use of all the facilities provided in Molecular Biology Division of the Bhabha Atomic Research Centre, Mumbai and Department of Zoology, University of Pune, for experimental work. This work was supported by University Grant Commission-Centre for Advance Studies (UGC-CAS), Department of Science and technology-Promotion of University Research and Scientific Excellence (DST-PURSE) and Department Research and Development Programme (DRDP). Support from Department of Atomic Energy (DAE), India, for fellowship to KDD and PG under BARC-UoP (Bhabha Atomic Research Centre-University of Pune) collaborative PhD programme (BBN and RM) is duly accredited.

Declaration of interest: The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online

Supplementary Tables and Figures available online at http://informahealthcare.com/abs/doi/10.3109/09553002.2015.1021962