Effect of fly ash on biochemical responses and DNA damage in earthworm, *Dichogaster curgensis*

Vijaykumar L. Markad, Kisan M. Kodam*, Vikram S. Ghole

Division of Biochemistry, Department of Chemistry, University of Pune, Pune 411007, India

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ABSTRACT

Fly ash is receiving alarming attention due to its hazardous nature, widespread usage, and the manner of disposal; leading to environmental deterioration. We carried out bio-monitoring and risk assessment of fly ash in earthworms as a model system. *Dichogaster curgensis* were allowed to grow in presence or absence of fly ash (0–40%, w/w) for 1, 7, and 14 d. The biochemical markers viz. catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione S-transferase (GST), and malondialdehyde (MDA) level were measured. The comet and neutral red retention assays were performed on earthworm coelomocytes to assess genetic damages and lysosomal membrane stability. The results revealed increased activities of SOD, GPx, GST, and MDA level in a dose–response manner while GR activity was decreased with increasing concentrations of fly ash. No obvious trend was observed in the CAT activity and fly ash concentration. Lysosomal membrane destabilization was noted in the earthworms exposed to 5% and more fly ash concentration in a dose and time dependent manner. The comet assay demonstrated that the fly ash induced DNA damage and DNA–protein crosslinks in earthworm coelomocytes.

1. Introduction

About 75% of the electricity in India is generated from coal-based thermal power plants. However, these power plants produce vast amounts (over 100 million tons/year) of fly ash [1]. Disposal and management of this fly ash is a major environmental concern. Although, 38% of the total fly ash is utilized in agriculture as a soil amendment or in manufacturing of cement and concrete bricks, most of it is disposed in ash ponds near the power plants [2–4]. Heavy metals (Cu, Zn, Cd, Pb, Ni, Cr, etc.) and polyhalogenated compounds [5–7] in fly ash have adverse effects on terrestrial and aquatic ecosystems [8–10]. Repetitive application of fly ash as soil amendment, may lead to soil contamination. Therefore, bio-monitoring and risk assessment is necessary before utilization of fly ash as soil amendment. Earthworms have been extensively used to evaluate biological responses of pesticides, polychlorinated biphenyls, polycyclic hydrocarbons, and heavy metals [11–14]. Few studies have been carried out on the ecotoxicology of fly ash [15,16]; yet, there is paucity of data regarding the antioxidant and genotoxic responses of fly ash on earthworms.

Antioxidant enzymes protect the cells from various reactive oxygen species (ROS) and hence considered as biomarkers for assessing the environmental impact of contaminants. Enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST), and lipid peroxidation (LPO) [14,17–20] have been studied as biomarkers of environmental pollution. The lysosomal membrane stability is a sensitive cellular biomarker widely used in ecotoxicology, provides useful information on cellular damage, and was evaluated using the neutral red retention assay. It has been used to assess the effects of Cu, Cd, Ni, and Zn on earthworm coelomocytes [21–25].

The comet assay or single cell gel electrophoresis is yet another effective tool to measure the DNA damage in individual cells, widely used in the area of ecotoxicology [1]. It measures single and double strand breaks, alkali labile sites, oxidative DNA damages, DNA–DNA/DNA–protein/DNA–drug cross-linking, and DNA repair. Some modifications in the standard protocol of comet assay have been proposed for sensitive detection of DNA-crosslinks [26–29]. The comet assay has been demonstrated to be effective in measuring the DNA damage by various genotoxins in earthworm coelomocytes [1,30–32].

Therefore, the objective of the present study was to better understand the biological effects of fly ash on the earthworm, *Dichogaster curgensis* and underlying mechanisms in order to provide additional information on their toxicological effects. Various

* Corresponding author. Tel.: +91 20 25691728; fax: +91 20 25691728.
E-mail addresses: kodam@chem.unipune.ac.in, kisankodam@gmail.com
(K.M. Kodam).

biomarkers viz. biochemical responses, lysosomal membrane stability, and DNA damage in the fly ash exposed earthworms were evaluated.

2. Materials and methods

2.1. Chemicals and fly ash

Glutathione reductase, thiobarbituric acid, low melting agarose, and epinephrine were purchased from Sigma (St. Louis, Mo, USA). Aroclor 1260 (Cat No. 4-8704) and PAHs mix (Cat No. 48905-U) were purchased from Supelco, USA. All other chemicals used were of analytical grade, purchased from Sisco Research Laboratories (India), Himedia (India), and Merck (India). The fly ash sample was collected from the thermal power station, Nashik district (19°30′–20°45′N and 73°15′–74°45′E), Maharashtra, India.

2.2. Animals

A stock culture of earthworm, *D. curgensis* was maintained in laboratory on hand collected, dried, homogenized cattle manure with 12/12 h dark–light cycle, 40–50% humidity, and temperature at 22 ± 2 °C. The adult healthy worms (average weight ~250–300 mg) with well-developed clitellum were used.

2.3. Preparation of experimental sets and exposure of worms

The experiments were carried out under laboratory conditions in polythene culture pots (20 cm × 10 cm × 8 cm). Two types of experimental beds were prepared as follows: (i) control set (cattle manure only); (ii) test set (cattle manure + fly ash). The test set was amended with 2.5, 5, 10, 20, and 40% (w/w) fly ash [3]; moisture content was maintained at 40% and temperature at 22 ± 2 °C. The sets were left for 2 d undisturbed prior to experimentation, for stabilization. The earthworms ($n = 15$) were exposed to different doses of fly ash for 1, 7, and 14 d. All the experiments were carried out in triplicate.

The pH and electrical conductivity of the samples were measured (in 0.01 M CaCl$_2$) as described earlier [33]. The organic matter was determined by combusting the samples in a furnace by heating for 1 h at 200 °C, 1 h at 400 °C, and 6 h at 500 °C. The organic matter was calculated as the loss on ignition [33].

2.4. Biochemical assays

The earthworms ($n = 3$ for each group) were randomly selected at an interval of 1, 7, and 14 d of exposure to fly ash, were rinsed with distilled water and kept for 48 h on moist filter paper in Petri dishes to depurate their gut contents. The earthworms were homogenized in Tris–HCl buffer (100 mM, pH 7.5) for 1 min at 4 °C using Potter-Elvehjem homogenizer and centrifuged at $12,000 \times g$ for 20 min. The supernatant was used as an enzyme source and aliquots were stored at −80 °C until further use. The enzyme assays were performed using temperature-controlled dual beam UV–Vis spectrophotometer (Jasco V-630). All the assays were carried out in triplicate.

The CAT activity was determined using procedure described by Saint-Denis et al. [19] and the decomposition of hydrogen peroxide was measured spectrophotometrically at 240 nm. The reaction mixture contained 0.1 M Tris–HCl buffer (pH 7.5), 15 mM H$_2$O$_2$, and the sample (30 μl) and a molar extinction coefficient of 40 M^{-1} cm^{-1} was used for activity expression. The SOD activity was determined as described by Misra and Fridovich [34] in a reaction mixture containing 0.1 M carbonate buffer (pH 10.2), 0.2 mM EDTA, 0.34 mM adrenaline, and the sample (30 μl). The rate of adrenaline autooxidation was monitored at 480 nm and

degree of inhibition was assessed. Glutathione reductase activity was determined according to Racker [35] in a reaction mixture containing 0.1 M Tris–HCl buffer (pH 7.5), 2 mM oxidized glutathione, 0.1 mM NADPH, and the sample (100 μl). The GPx activity was measured as described by Paglia and Valentine [36] against H$_2$O$_2$. The reaction mixture contained 0.1 M Tris–HCl buffer (pH 7.5), 1 mM sodium azide, 1 mM reduced glutathione, 1 U of glutathione reductase, 0.1 mM NADPH, 0.1 mM H$_2$O$_2$, and the sample (100 μl). The oxidation of NADPH was monitored at 340 nm and a millimolar extinction coefficient (6.22 mM^{-1} cm^{-1}) was used for calculation of both GR and GPx activities.

The GST activity was measured using the method of Habig et al. [37] in a reaction mixture containing 0.1 M Tris–HCl buffer (pH 7.0), 1 mM 1-chloro 2,4-dinitrobenzene, 2 mM reduced glutathione, and the sample (30 μl) and reaction was followed in terms of absorbance at 340 nm. A millimolar extinction coefficient of 9.6 mM^{-1} cm^{-1} was used for activity determination. All enzyme activities were assayed at 25 °C keeping appropriate blanks for non-enzymatic reaction. Lipid peroxidation was estimated spectrophotometrically as described by Livingstone et al. [17]. The reaction mixture contained sample (200 μl), 20% trichloroacetic acid (800 μl), and 0.67% thiobarbituric acid (2 ml). The reaction mixture was incubated at 100 °C for 15 min and the formation of thiobarbituric acid reactive substances was quantified in terms of malondialdehyde (MDA) equivalents at 532 nm. The MDA concentration was presented as μmol of MDA produced per mg protein using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. Protein concentration was estimated by the Lowry method [38] using bovine serum albumin as a standard.

2.5. Determination of metals and organic matter

The total metal content of fly ash, cattle manure, and fly ash amended test beds were performed as described earlier [39]. The samples were digested with concentrated nitric and perchloric acid and the digests were diluted to 25 ml with Milli-Q water. Similarly, a pool of 4 earthworms (depurated for 48 h, freeze killed) was acid digested and diluted to 25 ml with Milli-Q water. The metal contents were estimated using Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES, ARCOS, Spectro, Germany).

The PAH and PCB concentrations were determined as described earlier [12]. The samples (fly ash and cattle manure) were Soxhlet extracted with hexane–acetone (2:1) mixture, while freeze-dried earthworms were extracted with hexane–dichloromethane (2:1) mixture. The extracts were pre-cleaned with anhydrous Na$_2$SO$_4$, evaporated to 1 ml, and further purified by solid phase extraction for separation of PAHs and PCBs as described earlier [12]. The PAHs and PCBs were estimated by gas chromatography mass spectroscopy (GCMS-QP 5050, Shimadzu).

2.6. Cytotoxic and genotoxic studies

2.6.1. Coelomocytes harvesting

The earthworm coelomocytes were obtained by simple, non-invasive technique described by Eyambe et al. [40]. Three earthworms were randomly selected from each test group on 1, 7, and 14 d of exposures. An individual adult earthworm was washed with distilled water and placed in a glass vial containing chilled extrusion medium (NaCl, 71.2 mM; ethylene glycol tetra acetic acid, 5 mM; and guaicol glycerol ether, 50.4 mM; pH 7.5). The extruded coelomocytes were washed thrice with phosphate buffered saline (PBS) (100 mM, pH 7.3) to remove mucous. The cell viability was checked using trypan blue exclusion method and final cell density was adjusted to 1×10^6 cells ml^{-1} with PBS.

2.6.2. Neutral red retention assay

The neutral red retention assay was performed on coelomocytes of the control and fly ash exposed earthworms using method of Weeks and Svendsen [21]. In brief, a working solution of neutral red ($80\,\mu g\,ml^{-1}$) in Hanks balanced salt solution (NaCl, 0.137 mM; KCl, 5.4 mM; Na_2HPO_4, 81 mM; KH_2PO_4, 14.70 mM; $CaCl_2$, 1.3 mM; $MgSO_4$, 1.0 mM; and $NaHCO_3$, 4.2 mM; pH 7.3) was prepared. $30\,\mu l$ of neutral red solution was added to $10\,\mu l$ of cell suspension and examined for stained or unstained cytosol for 1 min under the light microscope. The slides were then transferred to the light proof humidity chamber containing moist filter paper to prevent drying out of slides. The cells were counted repeatedly at an interval of 5 min until ratio of cells with fully stained to unstained cytosol was 1:1. The time required to attain this ratio was taken as neutral red retention (NRR) time.

2.6.3. Single cell gel electrophoresis/comet assay

The comet assay was carried out according to the method of Singh et al. [41] with slight modifications. Microscopic slides were prepared as described previously [1]. Lysis step was performed by dipping the slides in freshly prepared lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.0 and 1% Triton X-100) for 1 h at $4\,^{\circ}$C. Slides were then placed in alkali buffer (1 mM EDTA and 0.3 M NaOH, pH > 13.0) for 20 min to allow unwinding of DNA and electrophoresis was carried out at $0.7\,V\,cm^{-1}$ and 300 mA for 20 min. After electrophoresis, excess alkali was neutralized repeatedly (3–4 times) with neutralizing buffer (0.4 M Tris–HCl buffer, pH 7.5). Each slide was then stained with $75\,\mu l$ ethidium bromide ($20\,\mu g\,ml^{-1}$) for 5 min and excess stain was removed by dipping in distilled water. The slides were observed under fluorescence microscope (Olympus CX41, Japan) and images were analyzed by a computer-assisted image analysis system (Comet Score, Tritek, USA). The Olive tail moment (OTM, arbitrary units, defined by percentage of DNA in the tail multiplied by the distance between the intensity centroids of the head and tail region of the comet) was evaluated for each cell as a parameter of DNA damage.

The DNA–protein crosslink (DPC) levels within the earthworm coelomocytes were determined using modified comet assay as described by Merk and Speit [29]. After alkali lysis step, slides were washed thrice with TE-buffer (10 mM Tris, 1 mM EDTA, pH 10.0), and were treated with proteinase K ($1\,mg\,ml^{-1}$ TE-buffer) for 2 h at $37\,^{\circ}$C in the moist chamber. The slides were then subjected to comet assay as described above.

2.7. Statistical analysis

Data were expressed as the mean $\pm$ S.E.M. Significant differences between the results of the different treatment groups were determined using one-way ANOVA and Tukey–Kramer multiple comparison post hoc test. Means were compared by two-way ANOVA with dose and duration treatments as independent variables. The level of significance was considered $p < 0.05$. All statistical analyses were carried out using GraphPad Prism software (Version 5.0, USA).

3. Results

3.1. Metal concentration and organic matter

Physico-chemical analysis and metal contents of fly ash, cattle manure, and fly ash amended beds are summarized in Table 1. The initial pH of the cattle manure (6.97 ± 0.05) was marginally increased in the fly ash amended beds. The decrease in the organic matter (from 48.88 to 26.15%) was observed with increase in fly ash concentrations. The metal contents of the fly ash amended beds were found to be increased with increase in fly ash concentration (Table 1). No significant changes were observed in the metal body burdens of *D. curgensis* on 1 and 7 d of exposure (data not shown), while bioaccumulation of Cd, Pb, As, and Cr was noted after 14 d of exposure to fly ash when compared to control earthworms (Table 2). The survival and the body weights of the earthworms were not affected throughout the experimental period. The concentrations of PAHs and PCBs in fly ash were 1.4 and $1.9\,\mu g\,kg^{-1}$ respectively, while it was undetectable in cattle manure and earthworms.

3.2. Biochemical assays

The effect of fly ash on the biochemical responses of *D. curgensis* is listed in Table 3. The CAT activity was increased after 7 d exposure to fly ash (40%), while, no significant differences were observed after 1 d exposure. After 14 d, the decline in CAT activity was observed in earthworms exposed to 20 and 40% fly ash, whereas, it was increased in earthworms exposed to 10% fly ash. However, no

Table 1
Physico-chemical properties and metal contents of fly ash, and fly ash amended beds.

Parameters	Fly ash	Dose of fly ash (%, w/w)					
		Control (0)	2.5	5	10	20	40
pH	8.02 ± 0.1	6.97 ± 0.05	6.97 ± 0.08	7.00 ± 0.08	7.04 ± 0.06	7.1 ± 0.05	7.21 ± 0.07
OM (%)	0.1 ± 0.01	48.9 ± 0.5	48.85 ± 0.35	46.6 ± 0.71	45 ± 1.41	$36 \pm 0.71^{*}$	$26.15 \pm 0.21^{**}$
Si ($g\,kg^{-1}$)	87.7 ± 11.15	42.5 ± 5.4	45.88 ± 2.54	50.94 ± 3.92	52.61 ± 3.78	57.61 ± 6.21	$71.81 \pm 5.18^{**}$
Ca ($g\,kg^{-1}$)	27.2 ± 24.11	25.5 ± 11.6	24.36 ± 5.08	26.52 ± 16.6	27.21 ± 12.61	27.56 ± 11.79	26.55 ± 12.38
P ($g\,kg^{-1}$)	10.5 ± 1.8	12.5 ± 1.5	13.21 ± 1.33	13.93 ± 5.69	12.82 ± 1.02	12.79 ± 2.08	11.54 ± 3.41
Na ($g\,kg^{-1}$)	1.9 ± 0.282	3.3 ± 0.3	4.49 ± 2.91	3.36 ± 0.56	4.16 ± 0.69	2.96 ± 0.18	2.38 ± 0.45
Mg ($g\,kg^{-1}$)	80.1 ± 9.60	7.1 ± 0.9	7.85 ± 0.26	12.55 ± 1.64	$15.03 \pm 1.03^{*}$	$19.70 \pm 2.19^{*}$	$21.12 \pm 5.79^{**}$
Fe ($g\,kg^{-1}$)	62.5 ± 40.23	35.4 ± 3.9	33.29 ± 3.05	36.67 ± 1.16	40.18 ± 5.62	$49.28 \pm 2.61^{*}$	$60.24 \pm 3.95^{*}$
Al ($g\,kg^{-1}$)	58.8 ± 36.55	32.8 ± 4.6	28.21 ± 1.82	35.64 ± 1.13	37.41 ± 1.08	$43.38 \pm 2.07^{*}$	$49.51 \pm 5.37^{**}$
Mn ($mg\,kg^{-1}$)	795 ± 21.21	171 ± 59.4	285.4 ± 37.6	$305.2 \pm 41.0^{*}$	$334.6 \pm 22.1^{*}$	$359.7 \pm 5.3^{*}$	$445.8 \pm 29.8^{**}$
Pb ($mg\,kg^{-1}$)	10 ± 0.2	<0.01	<0.01	1.0 ± 1.06	$1.25 \pm 0.71^{*}$	$1.87 \pm 0.53^{*}$	$2.62 \pm 0.17^{**}$
As ($mg\,kg^{-1}$)	10.0 ± 0.1	<0.01	0.12 ± 0.17	0.5 ± 0.35	$2.62 \pm 0.88^{*}$	$4.25 \pm 0.35^{*}$	$5.37 \pm 0.88^{**}$
Zn ($mg\,kg^{-1}$)	155 ± 7.1	50.0 ± 2.8	54.0 ± 2.12	57.12 ± 5.83	$69.75 \pm 2.12^{*}$	$89.0 \pm 3.18^{*}$	$111.2 \pm 8.8^{**}$
Ni ($mg\,kg^{-1}$)	40.0 ± 14.1	5.0 ± 7.1	12.12 ± 1.59	15.5 ± 2.12	$18.62 \pm 0.53^{*}$	$22.87 \pm 1.24^{*}$	$29.5 \pm 3.18^{*}$
Cd ($mg\,kg^{-1}$)	20.0 ± 0.8	<0.01	<0.01	1.25 ± 1.77	$2.63 \pm 0.17^{*}$	$4.0 \pm 0.35^{*}$	$6.0 \pm 1.41^{**}$
Cr ($mg\,kg^{-1}$)	57.5 ± 3.53	27.5 ± 6.4	26.5 ± 0.35	30.75 ± 1.76	34.12 ± 1.53	$39.5 \pm 2.12^{*}$	$48.87 \pm 4.53^{*}$
Cu ($mg\,kg^{-1}$)	70.0 ± 4.2	35.0 ± 4.2	36.12 ± 1.59	39.5 ± 1.41	41.75 ± 0.70	$46.63 \pm 1.24^{*}$	$54.12 \pm 1.88^{*}$

Values are mean $\pm$ S.E.M. OM – organic matter. Means were compared by ANOVA and Tukey's multiple comparison tests.

* Statistical significance from the control groups are indicated as: $p < 0.05$.
** Statistical significance from the control groups are indicated as: $p < 0.01$.

Table 2
Metal contents in *D. curgensis* before and after exposure to fly ash.

Metal ($\mu g\,g^{-1}$)	Before exposure	After exposure to different doses of fly ash (%, w/w)					
		Control (0)	2.5	5	10	20	40
Fe	390.65 ± 23.9	429.56 ± 21.7	441.1 ± 26.2	390.71 ± 26.8	406.12 ± 19.9	373.38 ± 22.6	454 ± 31.2
Al	305.32 ± 22.8	314.68 ± 18.8	270.4 ± 33.15	267.89 ± 36.25	273.06 ± 29.68	295.16 ± 31.58	325.44 ± 12.31
Mn	9.86 ± 1.22	10.22 ± 0.82	13 ± 0.79	9.29 ± 3.21	12.45 ± 1.32	9.68 ± 2.54	9.78 ± 1.95
Pb	0.28 ± 0.08	0.27 ± 0.1	0.19 ± 0.08	0.49 ± 0.09	1.22 ± 0.11*	1.11 ± 0.15*	1.44 ± 0.09**
As	0.31 ± 0.07	0.27 ± 0.08	0.6 ± 0.1	0.41 ± 0.09	0.51 ± 0.08	0.50 ± 0.12	0.76 ± 0.16*
Zn	425.14 ± 22.08	433.30 ± 11.27	440.4 ± 15.48	448.02 ± 12.20	430.10 ± 21.25	445.36 ± 13.32	431.78 ± 15.26
Ni	0.58 ± 0.18	0.64 ± 0.16	0.6 ± 0.09	0.52 ± 0.11	0.51 ± 0.12	0.60 ± 0.08	0.78 ± 0.07
Cd	0.15 ± 0.09	0.20 ± 0.09	0.19 ± 0.11	0.31 ± 0.10	0.47 ± 0.07	0.54 ± 0.09	3 ± 1.3*
Cr	0.82 ± 0.18	0.79 ± 0.14	1.04 ± 0.17	0.94 ± 0.09	1.13 ± 0.21	0.95 ± 0.18	0.91 ± 0.13
Cu	42.21 ± 6.90	43.88 ± 12.21	40.70 ± 7.089	41.20 ± 9.937	39.80 ± 7.326	38.41 ± 14.21	42.55 ± 8.96

Values are mean ± S.E.M. Means were compared by ANOVA and Tukey's multiple comparison tests.

* Statistical significance from the control groups are indicated as: $p < 0.05$.
** Statistical significance from the control groups are indicated as: $p < 0.01$.

significant differences in the CAT activity were noted in 2.5 and 5% fly ash exposed earthworms when compared to control. The CAT activity decreased after 14 d of exposure when compared to 7 d in all doses of fly ash.

The SOD activity increased at all doses and time points. The SOD level was decreased after 14 d when compared to 7 d of exposure. The significant decrease ($p < 0.05$) in the GR activity was noted in the treatment groups that exposed to 20 and 40% of fly ash at all durations (1, 7, and 14 d). However, GR activity significantly decreased in 10% fly ash exposed earthworms on 1 and 7 d of exposure and recovered on 14 d. While no significant differences were noted in the earthworms exposed to 2.5 and 5% fly ash. The significant increase ($p < 0.001$) in GPx activity was observed in 10, 20 and 40% fly ash exposed animals after 14 d.

A significant increase ($p < 0.01$) in GST activity was observed in earthworms exposed to 10 and 40% fly ash after 1 d, while no change was noted after 7 d exposure. The GST activity slightly decreased after 7 d of exposure when compared to 1 d. However, after 14 d of exposure, GST activity was significantly increased ($p < 0.001$) with dose in all the treatment groups. A strong positive correlation was observed between MDA levels and fly ash concentrations. Significant increase ($p < 0.05$) in MDA level was observed after 7 d exposure in 5–40% fly ash exposed earthworms. After 14 d, MDA

levels were significantly increased ($p < 0.001$) with dose when compared with control. The multivariate analysis revealed significant influence of dose and duration of exposure on all studied biochemical responses. The CAT, GR, GST activities, and MDA level were significantly affected by both dose and duration of exposure, while SOD and GPx activities were not influenced by this interaction (Table 4).

3.3. Lysosomal membrane stability

The viability of coelomocytes derived from control and exposed earthworms, was always found to be >90%. A significant reduction ($p < 0.05$, $p < 0.001$) in the NRR times was observed in *D. curgensis* exposed to fly ash ($\geq 5\%$), when compared to control earthworms. The reduction in NRR time was continued till end of the experiment. The NRR time of the earthworms exposed to 2.5% fly ash was similar to that of control (Fig. 1).

3.4. Comet assay

Fig. 2 represents the effect of fly ash on DNA damage (OTM) in coelomocytes of *D. curgensis*. A significant increase ($p < 0.05$) in OTM values was observed in earthworms exposed to all doses of fly

Table 3
Biochemical responses of *D. curgensis* exposed to fly ash.

Biochemical measurements	Duration of exposure (d)	Dose of fly ash (%, w/w)					
		Control (0)	2.5	5	10	20	40
CAT activity ($\mu mol\,mg^{-1}$ protein min^{-1})	1	11.50 ± 0.55	11.38 ± 0.37	10.04 ± 0.31	10.94 ± 0.57	11.87 ± 0.38	12.36 ± 0.56
	7	13.18 ± 0.56	13.77 ± 0.50	12.43 ± 0.66	14.28 ± 0.35	13 ± 0.50	14.89 ± 0.76
	14	10.99 ± 0.56	11.07 ± 0.66	10.78 ± 0.71	12.67 ± 0.64	10.07 ± 0.40	9.89 ± 0.32
SOD activity ($U\,mg^{-1}$ protein min^{-1})	1	9.86 ± 0.46	11.4 ± 0.68	10.12 ± 0.53	11.56 ± 0.29	11.38 ± 0.35	11.79 ± 0.36
	7	13.17 ± 0.19	14.24 ± 0.56	14.47 ± 0.93	16.9 ± 1.29	16.65 ± 1.97	15.5 ± 1.23
	14	12.4 ± 0.41	13.99 ± 0.87	12.56 ± 0.49	14.02 ± 0.47	15.48 ± 0.74*	16.87 ± 0.44***
GR activity ($\mu mol\,mg^{-1}$ protein min^{-1})	1	6.56 ± 0.27	5.98 ± 0.15	7.11 ± 0.38	5.38 ± 0.29*	5.03 ± 0.27**	5.36 ± 0.13*
	7	6.58 ± 0.14	6.38 ± 0.42	6.03 ± 0.08	5.20 ± 0.08**	5.57 ± 0.22*	4.43 ± 0.16***
	14	8.19 ± 0.49	8.81 ± 0.43	7.85 ± 0.21	7.47 ± 0.13	5.90 ± 0.11***	4.38 ± 0.28***
GPx activity ($\mu mol\,mg^{-1}$ protein min^{-1})	1	3.85 ± 0.40	4.12 ± 0.41	3.94 ± 0.42	3.70 ± 0.34	4.11 ± 0.44	4.47 ± 0.40
	7	5.76 ± 0.07	6.04 ± 0.28	6.19 ± 0.13	6.43 ± 0.17	6.46 ± 0.18	6.29 ± 0.15
	14	5.49 ± 0.11	5.91 ± 0.15	5.65 ± 0.19	6.79 ± 0.11***	6.47 ± 0.19***	6.84 ± 0.12***
GST activity ($\mu mol\,mg^{-1}$ protein min^{-1})	1	20.31 ± 1.26	22.46 ± 0.71	22.37 ± 0.44	25.51 ± 0.97**	22.8 ± 0.82	24.43 ± 0.96*
	7	18.18 ± 0.91	20.64 ± 1.48	20.35 ± 0.59	20.43 ± 0.58	18.26 ± 1.15	21.41 ± 0.74
	14	22.79 ± 0.78	28.4 ± 0.89***	28.94 ± 0.58***	28.89 ± 0.44***	33.27 ± 0.90***	36.48 ± 1.13***
MDA content ($\mu mol\,mg^{-1}$ protein)	1	0.15 ± 0.02	0.17 ± 0.04	0.15 ± 0.02	0.17 ± 0.01	0.18 ± 0.02	0.18 ± 0.03
	7	0.19 ± 0.03	0.22 ± 0.06***	0.26 ± 0.02***	0.27 ± 0.01***	0.26 ± 0.01***	0.28 ± 0.02***
	14	0.28 ± 0.01	0.32 ± 0.02**	0.31 ± 0.02	0.39 ± 0.02***	0.44 ± 0.02***	0.50 ± 0.02***

Results are expressed as mean ± S.E.M. ($n \geq 6$). Means were compared by ANOVA and Tukey's multiple comparison tests. CAT, catalase; SOD, superoxide dismutase; GR, glutathione reductase; GPx, glutathione peroxidase; GST, glutathione-S-transferase; MDA, malondialdehyde.

* Statistical significance from the control groups are indicated as: $p < 0.05$.
** Statistical significance from the control groups are indicated as: $p < 0.01$.
*** Statistical significance from the control groups are indicated as: $p < 0.001$.

Table 4
Results of ANOVA tests on the biochemical responses of *D. curgensis* exposed to fly ash.

Biochemical measurements	Dose			Duration			Dose × Duration		
	df	F	P	df	F	P	df	F	P
CAT activity	5	3.14	0.0328*	2	43.00	<0.0001*	10	2.64	0.0350*
SOD activity	5	6.57	0.0012*	2	43.48	<0.0001*	10	1.14	0.3880
GR activity	5	41.52	<0.0001*	2	48.62	<0.0001*	10	7.66	0.0001*
GPx activity	5	4.00	0.0129*	2	130.00	<0.0001*	10	1.38	0.2634
GST activity	5	19.37	<0.0001*	2	192.90	<0.0001*	10	7.13	0.0002*
MDA content	5	8.29	0.0003*	2	102.90	<0.0001*	10	2.68	0.0332*

CAT, catalase; SOD, superoxide dismutase; GR, glutathione reductase; GPx, glutathione peroxidase; GST, glutathione-S-transferase; MDA, malondialdehyde; df, degree of freedom.

* Dose or the duration of exposure had a significant effect ($p < 0.05$).

ash and durations when compared to control earthworms. A dose dependent increase in DNA damage was observed in the earthworms exposed to 2.5 and 5% of fly ash. While, an unexpected decrease in the DNA migration was observed in the earthworms exposed to 10, 20, and 40% fly ash, although the OTM values were statistically significant ($p < 0.05$) when compared to control. This decrease in the DNA migration was continued further after 7 and 14 d exposure to fly ash. In order to find out the involvement of DNA–protein crosslinks in the comets, slides were treated with proteinase K. A significant increase in DNA migration was noted after proteinase K treatment at all doses of fly ash and durations. The OTM data were further analyzed in terms of percentage distribution of cells (OTM, <2 to >10). The percentage of damaged cells after proteinase K treatment was found to be significantly increased when compared to before treatment, confirming the formation of DNA–protein crosslinks (Supplementary Fig. S1–S3).

4. Discussion

Biological molecular markers are regarded as fast, diagnostic, and prognostic early warning system to detect and assess the environmental impact of wide range of contaminants, which cannot be achieved from mere chemical analysis of environmental samples [14,32,42]. The present study investigated in details, the toxicological effects of fly ash on *D. curgensis* using biochemical response, lysosomal membrane stability and DNA damage as endpoints.

Fly ash used in the present investigation showed the predominance of various heavy metals (Zn, Cd, Cr, Cu, Ni, Pb, As, and Mn). Earthworm survival and biomass were not affected by fly ash concentration. The bioavailability of metal varied with the pH and organic matter [32,39]. In the present study, the bioaccumulation of non-essential elements viz. Cd, As, and Pb was observed while other

metals were at par with control earthworms. This might be due to presence of high organic matter in cattle manure. However, the observed metal accumulation was less than earlier reports [14,32]. The amount of PAHs and PCBs in used fly ash was far less when compared to earlier studies [43].

The enzyme activities and cytotoxic effects varied with doses of fly ash and duration of exposure. Superoxide dismutase catalyzes dismutation of superoxide anion into oxygen and hydrogen peroxide, while catalase protects the cells by eliminating hydrogen peroxide [19]. The SOD level was induced with increase in dose and duration of exposure of fly ash, consistent with the earlier report of earthworms exposed to heavy metal contaminated soils [14]. However, the SOD level was reduced on the 14 d when compared to 7 d of exposure, this may be due to the elimination of the highly reactive superoxide or inactivation of SOD by singlet oxygen, peroxyl radicals, and hydrogen peroxide [44–47] or adaptation of the organism to its micro environment. The variation in CAT activity with fly ash concentration supports earlier findings by Saint-Denis et al. [12,13], where, CAT was of minor importance than glutathione and related enzymes in the protection against ROS. Another reason for inhibition of CAT activity could be high cellular stress, especially due to maximal concentrations of fly ash or presence of high levels of ROS. It was reported that, superoxide anion exceeds the dismutation capacity of SOD, they become CAT inhibitors [48,49]. The results of the present study suggested that, fly ash is likely to inhibit CAT activity.

Earthworms are particularly susceptible to peroxidation of lipids due to high content of polyunsaturated fatty acids; various contaminants like metals are known to induce lipid peroxidation through the formation of ROS [12,13]. The MDA is an oxidized product of cellular lipid membranes and could be used as a sensitive biomarker of cell injury [13,17]. Interestingly, MDA levels were found to be increased in *D. curgensis* after 7 d of fly ash exposure and further increased with increase in fly ash concentration, suggesting the formation of ROS. Similar observations were reported in *Eisenia fetida andrei* and polychaeta *Laenereis acuta* exposed to Pb and Cu [13,20].

Glutathione S-transferase is one of the most important phase II enzymes involved in glutathione conjugation to xenobiotics, fatty acid hydroperoxides, and aldehydic products of lipid peroxidation such as MDA [50,51]. The decline in GST activity after 7 d of fly ash exposure was similar to earlier reports by Saint-Denis et al. [13] and Schereck et al. [49] in earthworms, exposed to lead and pesticides. The increase in GST activity after 14 d of fly ash exposure could be correlated with an increased MDA level, which is directly proportional to lipid peroxidation in earthworm tissues [52]. A similar response in GST activity was also observed in the earthworms exposed to Pb, Cu, and Zn [13,53].

Glutathione peroxidase eliminates H_2O_2 by using reduced glutathione as a hydrogen donor, while glutathione reductase reduces oxidized glutathione to maintain the cellular antioxidant status. The GR activity decreased with an increase in the concentrations
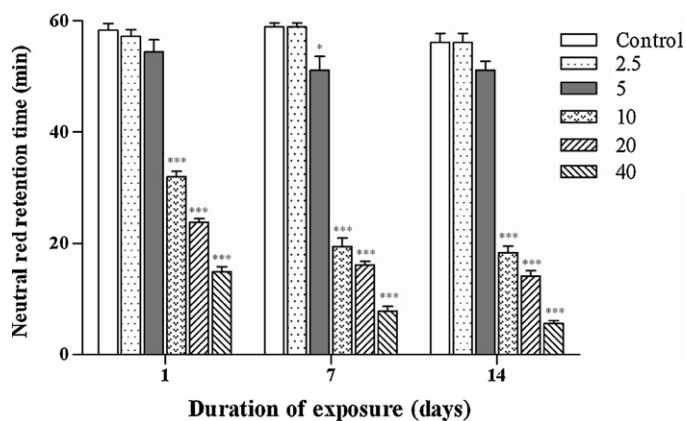

Fig. 1. The neutral red retention time (min) of coelomocytes derived from *D. curgensis* exposed to fly ash, assessed after 1, 7, and 14 d. Data are expressed as mean ± S.E.M. *($p > 0.05$), ***($p > 0.001$), compared with control.
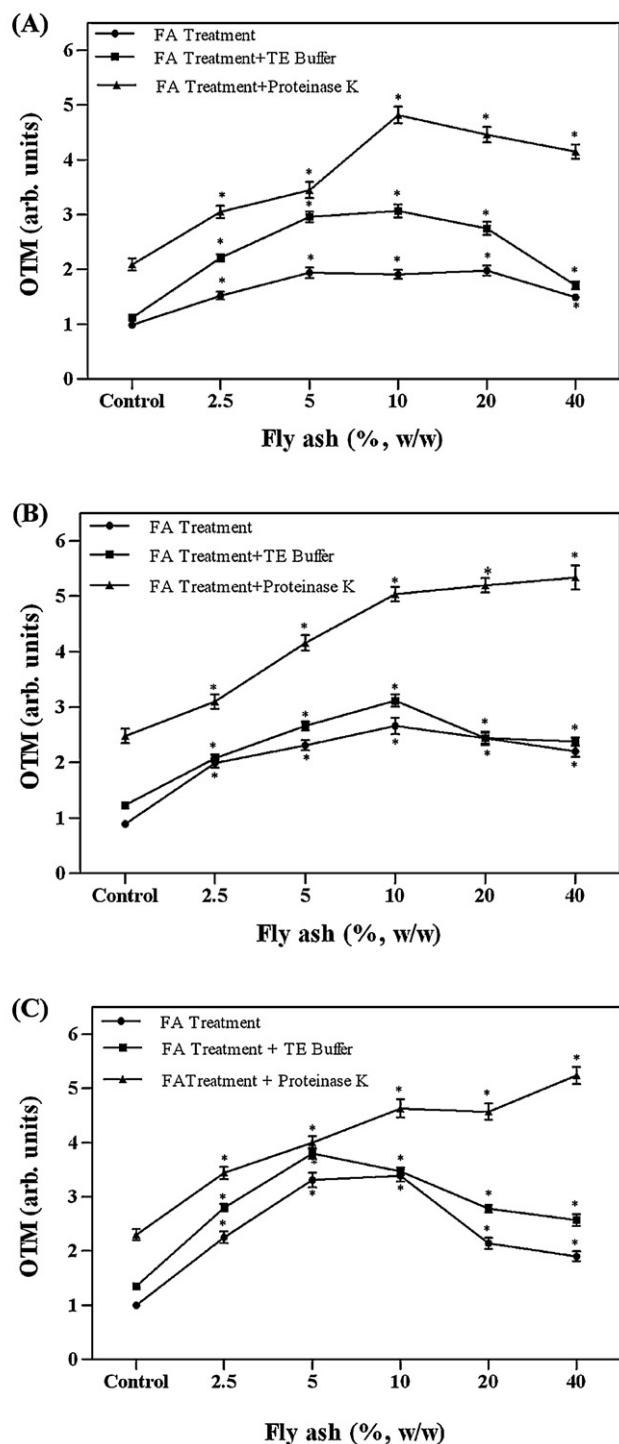
Fig. 2. DNA damage (Olive tail moment, OTM) in coelomocytes of *D. curgensis* exposed to fly ash. (A)–(C) represents OTM values for the earthworms after 1, 7, and 14 d of fly ash exposure, respectively. OTM values were measured after the exposure period, with and without proteinase K treatment to reveal DNA–protein crosslinks. Data are expressed as mean ± S.E.M. *Significantly different when compared with control ($p > 0.05$).

corroborates with earlier findings in the earthworms exposed to heavy metals [13,14].

Lysosomal membrane stability is a potential biomarker of various environmental contaminants, including heavy metals such as Cu, Ni, Zn, and Cd [21–25,52]. Our results are in good agreement with previous reports, where it has been shown that lysosomal membrane stability was decreased with increase in concentration of metals [21–25,54]. The mechanism causing alterations in membrane stability of lysosomes is not well understood. The evidences suggest that ROS produced in the cellular system plays a major role in metal-induced cellular responses and affects various cellular organelles and their repair systems [55,56]. Our results with respect to various antioxidant enzymes clearly suggested ROS production in the earthworms exposed to fly ash. Lysosomes may aid in ROS formation due to its iron content, which undergoes redox cycling to form ROS that may lead to oxidative stress in the cellular systems [57].

The genotoxicity of fly ash in *D. curgensis* was evaluated using comet assay. The fly ash induced DNA damage in the coelomocytes after 1 d of exposure, whereas, a decrease in DNA migration was observed at higher concentrations of fly ash. This reduction in DNA migration can be attributed to the DNA crosslinks (DNA–protein, DNA–DNA) [58]. These crosslinks were confirmed using a modified comet assay and it was observed that DNA migrates in a dose–response manner. The mechanism causing the formation of DPC in earthworm coelomocytes was unclear but it may be due to the complex mixture of toxic metals present in the fly ash. The fly ash used in the present study contained substantial amounts of Cr, Ni, Pb, and As, which might be sufficient to induce DPCs in the earthworms. It must be pointed out that various chemical and physical agents such as Cr, Cu, Pb, Ni, Pt compounds, various aldehydes, ionizing radiation, and UV light are known to produce DPCs [59–62]. The results obtained in the present study supports the earlier findings by Grumiaux et al. [63,64], where it was hypothesized that chronic toxicity of fly ashes could be attributed to its mutagenic or genotoxic effects; leading to several detrimental effects in *Eisenia andrei*.

Toxicological studies of fly ash and their leachates were reported in various animal models [1,10,16,63–65]. We have reported earlier that, the fly ash leachate induces DNA damage in vitro in the coelomocytes of *D. curgensis* [1]. The metal accumulation reported in this study may not be significantly high but toxicity determined in terms of biochemical, cytotoxic, and genotoxic responses was clearly visible. This may be due to synergistic effect of toxic components present in the fly ash mixture rather than individual component existing in lower concentration.

5. Conclusions

Toxicological effects of fly ash on earthworm *D. curgensis* were evaluated using multiple biomarkers as endpoints. The risk assessment and management of heavy metals present in fly ash was overviewed and found that fly ash induces harmful effects including oxidative stress, destabilization of lysosomal membranes and DNA damage. The formation of DNA–protein crosslink in the earthworm coelomocytes was also confirmed. Therefore, indiscriminate use of fly ash as soil amendment may lead to potential environmental hazards. To the best of our knowledge, this is the first report on fly ash toxicity in earthworms, using multiple biomarkers.

of fly ash and duration of exposure. Contrary to GR, GPx activity significantly increased after 14 d of exposure. This may be due to a compensatory mechanism between GPx and GR [14] or NADPH availability and/or inactivation of GR by binding of metals to biomolecules [13]. The decreased GR and increased GPx activity

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Appendix A. Supplementary data

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