Genotoxicity assessment of textile effluent in Eudrilus eugeniae earthworms coelomocytes by comet assay and micronucleus test

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Abstract:

Textile effluents and its present different chemical compounds are extensively used worldwide and considered as possible environmental pollutant. Earthworms are useful to model organism in monitoring soil pollution. Earthworms are indicators of soil quality and are widely used as model organisms in terrestrial ecotoxicology. In this study, the earthworm Eudrilus eugeniae (10 for each group) were introduced to 48 hrs for textile effluents in five different concentrations like 10 ml, 20ml, 30ml, 40 ml, and 50 ml to find LD50. The LD50 was found at 40 ml. So, the aim of the study was find out the LD50/2, LD50, 2XLD50 for 48 h was used. Evaluate the genotoxicity in Eudrilus eugeniae earthworms. After that we collect earthworm's coelomocytes by using parameters like micronucleus (MN) test and comet assay (CA). MN and CA demonstrated the increase both genotoxicity and cytotoxicity. The results showed that high concentration of textile effluent increase DNA damage and chromosomal aberrations in Eudrilus eugenie earthworms.

Keywords: Textile effluent, Earthworm, Coelomocytes collection, LD50, Genotoxicity, Micronuclues, comet assay,

1. Introduction

Earthworms are chief decomposers of soil organic matter and, thus aid in improving soil quality and fertility. So far, there are about 3920 named species of earthworm reported worldwide. Moreover, in India, 509 species referable to 67 genera and 10 families have been stated (Kale, 1991). Earthworms are widely used as model organisms in terrestrial ecotoxicology and they serve as a good indicator of heavy metal contamination due to their innate sensitivity to pollutants. The assessment of genotoxic effects caused by environmental pollutants is of great concern because of their relevance in carcinogenesis (Susanna Sforzini, et al., 2012). Coelomocytes are immunocompetent cells which are affected by the chemicals and are measured as a very sensitive biomarker of environment health (Homa et al., 2005; Zhan, 2012). Amidst the numerous industrial sectors, the textile and paper industries are exclusively challenging since they

generate substantial quantities of wastewater that may have detrimental impacts when released into the environment without any treatment. The environmental problems associated with textile activities are caused mainly by the widespread use of dyes, heavy metals which pose a threat to public health because of its persistence, biomagnifications and accumulation in the food chain (Issazadeh et al. 2014; Peralta-Zamora et al., 2003). With the number of industries being increased day by day in the modern world, the concentration of heavy metals such as cadmium, chromium, mercury, lead, nickel, cobalt, and copper is also being increased (Smrithi and Usha 2012). A number of studies have been conducted to intricate the effects of these heavy metals on living organisms which provide damages by affecting the cell membranes, by altering the specificity of the enzymes, by the cellular functions and by damaging the structure of the DNA (Chisti 2004; Ozer and Pirincci 2006). The alkaline comet assay (CA) is a well-established indicator to quantify genetic toxicology (Cotelle and Ferard, 1999; Singh et al., 1988). Micronucleus test (MN), which is a cytogenetic technique to identify chromosomal aberrations and nuclear abnormalities has been used as a profound method to find micronuclei (MN) and binuclei (BN) (Cavas et al., 2005; Sanchez-Galan et al., 2001). Thus, the present study was designed to assess the cytotoxicity and genotoxicity of textile effluent in *Eudrilus eugeniae* by using the CA and MN tests.

2. Materials and Methods

2.1. Earthworm and effluent collection

The earthworms were collected from the pallipalayam natural and clean surrounding area. Species identification was done by the specified key of taxonomic classification. The textile effluent was collected from Erode.

2.2. The experimental method

The worms were cultured in molecular biology and genetics laboratory, using a stock soil in the darkroom at 25 ± 2 ^{o}C. Water was sprinkled thrice a week to keep the soil wet. All adult earthworms were weighing between 500 and 600 mg with a well-developed clitellum. E.eugeniae was introduced for 48 h to textile effluents. The earthworms (10 for each group) were introduced to five Different series of concentrations of textile effluent (10ml, 20ml, 30ml, 40 ml, 50 ml) was used. Textile effluents were used to find LD50. In each group of concentration, equal numbers of earthworms were used and these were representative of the pool sample. The effect of different textile effluent concentrations (LD50/2, LD50, and LD50X2) was measured on DNA and chromosomal damage by using the CA and MN tests, respectively for 48 h. All experiments were carried out in the dark at 25 ± 2^{o}C for 48 h. Probit program was used to determine the 48 h LD50. Distilled water was used in the control group and the same experimental procedure was also applied for the control group.

2.3. Alkaline comet assay:

The coelomic fluid was collected from the coelomic cavity using the extrusion buffer (NaCl 4.161 g, EGTA 1.091 g and 9.09 g Guaiacol glycerol 2 ether were made up to 950 ml with distilled water with the extra addition of 50 ml absolute ethanol to make it to 1 L and pH

was adjusted to 7.5). Coelomocytes were collected into a 1-ml centrifuge tube. CA was carried out, as described by Reinecke and Reinecke (2004), with slight modifications. All protocol was conducted in very faint light at 4 _C to avoid any additional DNA damage. Initially, coelomocytes were centrifuged at 2000 rpm for 5 min. Supernatant discarded and 10 ml mixed with 100 ml of LMP agarose in a 1 ml centrifuge tube. The cell suspension was overlaid on the microscope slide, precoated with normal LMP agarose, and covered immediately with a cover glass. Slides were kept on ice packs for 2 min to solidify the gel. Then, slides were immersed in salt lysis solution for 1 h after gently removing the coverslips. Later to the lysing procedure, the slides were kept in a gel electrophoresis chamber containing the electrophoretic buffer in a horizontal position for 20 min to uncoil the DNA. The gel electrophoresis chamber was placed at the cool place (4 _C) and then electrophoresis was carried out at 25 V (1 V cm_1) up to 20 min. Subsequent electrophoresis, cold distilled water was flowed over the slides smoothly to neutralize it. Each slide was then stained by 70 mL ethidium bromide (20 mg/ml) and finally, coverslip was placed on it. Two slides from each earthworm of each concentration were examined. The extent of DNA damage was evaluated by the visualizing 100 comets per slide and scored 0 to 4.

2.4. Micronucleus test:

The remaining coelomic fluid was used for the MN test. MN was performed, as defined by Muangphra and Gooneratne (2011), with slight modifications. Potassium chloride (1 ml) added in coelomic fluid, waited for 5 min and centrifuged at 1200 rpm for 5 min. Then, coelomic fluid was centrifuged (1200 rpm for 10 min) with 1 ml fixative I (50 ml fixative II, 50 ml 0.09%Nacl) and 1 ml fixative II (400 ml glacial acetic acid, 200 ml methanol), respectively. An aliquot of coelomic fluid cell suspension was smeared on a clean wet microscope. The slides were air-dried for 24, stained with Giemsa for 15 min and permanently fixed by with entellan solution after 1 day. A total of 3000 coelomocytes from 3 slides per concentration (including the negative control) were scored using a compound microscope at 400 _ magnification and the frequencies of coelomocyte micronuclei (MNi) and binuclei (BN) cells were used to assess chromosomal aberrations and inhibition of cytokinesis.

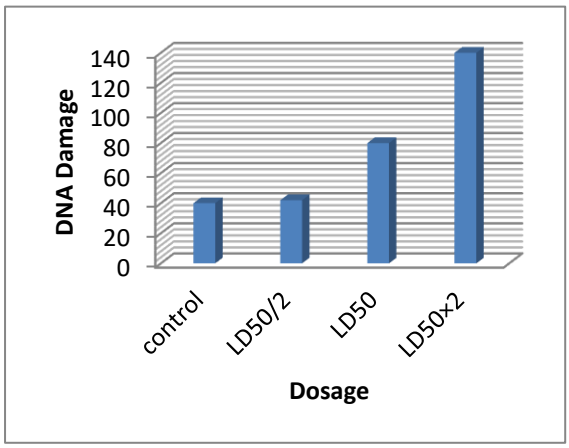
3. Statistical analysis

Results were analyzed by analysis of variance (ANOVA) by using SPSS version 15.0 for Windows software. Mann-Whitney U-test was used for comparing the different concentration of groups with control and $P < 0.05$ kept as statistical significance cut-off value.

4. Results

Effects of textile effluent on mean coelomocyte DNA damage score evaluated by the alkaline CA in coelomocytes of E.eugeniae are shown in Fig. 1. A clear dose-dependent response was observed. The LD50 for textile effluent was found at 28 ml. Significant difference ($P < 0.05$).

Fig. 1. Mean coelomocyte DNA damage score in earthworms (n ¼ 10) exposed to different concentrations of Textile effluent error bars are



standard deviations. *Significantly different from the control ($P < 0.05$).

was found between the mean DNA damage for LD50 and 2XLD50 as compared to the control group. The mean coelomocyte DNA damage score increased significantly on exposure to 2XLD50. So, numbers of comets were increased as the concentration of textile effluent increased. The effects of textile effluent on chromosomal damage in the coelomocytes were evaluated by the MN test, and shown in Fig. 2. A total of 1000 coelomocyte (MNi and BN) cells were counted from each earthworm. The sums of MNi and BN were higher in each concentration and were statistically significant ($P < 0.05$) from LD50/2 onwards when compared with the control. The highest number of Mni and BN were observed at 2XLD50. There was a clear statistically significant ($P < 0.05$) response of MNi and BN between the different concentrations and control group. Overall, the frequency of MNi was greater than for binuclei. The highest toxic dose was found at 2XLD50 for both DNA damage and chromosomal aberration.

5. Discussion

Nowadays, The CA and MN tests, are considered as key genotoxic tests to assess the genotoxicity of chemicals (Benedicte et al., 2009; Ci_gerci et al., 2013). Heavy metals induce genotoxicity in earthworms (Muangphra and Gooneratne, 2011). The current study showed clear effects of Textile effluent on DNA damage of earthworms. The dose-dependent relationship showed that a higher concentration of Textile effluent causes more DNA damage. The CA allows for the detection of primary DNA injuries due to the balance of DNA damage induction and repair mechanisms by different toxic agents (Rojas et al., 1999). MN test results also showed a significant increase in MNi and BN in coelomocytes exposed to higher concentrations in their aqueous solutions. This detection of MNi and BN, which are usually formed in the bi- or multinucleated interphase cells, is indicative of chromosomal damage caused by exposure to Textile effluent Heavy metals being greater as the toxicant concentration increased. MN test discloses exposed genotoxic damage accumulated during the lifetime of the cells (Bolognesi and Hayashi, 2011).

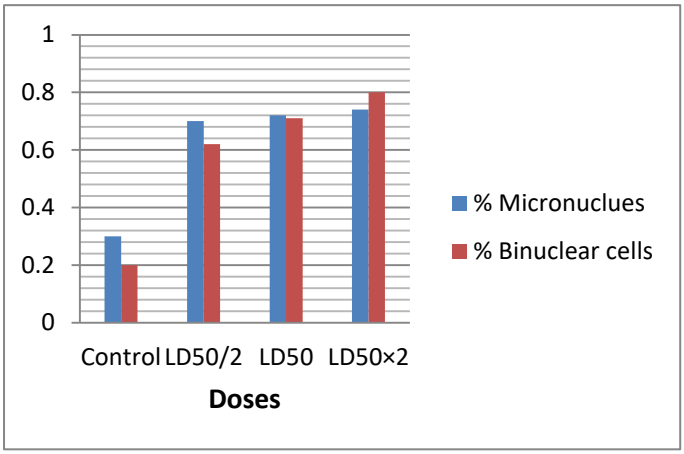


Fig. 2. Total micronuclei (MNi) and binuclei (BN) in coelomocytes exposed to different exposure of Textile effluent concentrations (n ¼ 10), error bars are Standard deviations. Asterisks indicate significant differences ($P < 0.05$) from control.

Moreover, DNA strand breaks produced in the coelomocytes may not the cause of MNi generation. Generally, MN demonstrates the chromosomal damage in the form of bi or multi-nuclei which is formed by the toxin exposure during metaphase and anaphase of mitosis. In the present study, coelomic fluid was used to apply the genotoxic parameters, as it is considered suitable target for evaluating genotoxic damage since it is the non-invasive method of extraction, short slide-preparation time and ease in sample manipulation (Bonnard et al., 2009; Eyambe et al., 1991). Previously, the sensitivity of these cells in revealing DNA variations by genotoxic compounds induction is well established (Bigorgne et al., 2010; Eyambe et al., 1991). Surprisingly, the levels of DNA damage and chromosomal aberration in the control animals were also somewhat higher. However, the basal level of DNA damage in earthworms is still unknown. Therefore, the little bit ‘‘higher’’ levels of DNA damage in the control group may be due to the natural levels of DNA damage, or it could even indicate the presence of a mixed coelomocytes culture (Fourie et al., 2007).

S.No	The concentration of Textile effluent	DNA Damage in Coelomocytes %	Micronucleus %	Binuclear cells %
1	Control	10	0.1	0.1
2	LD50/2	42	0.7	0.62
3	LD50	80	0.72	0.71
4	LD50×2	140	0.74	0.8

Table.1. DNA damage, Total micronuclei (MNi) and binuclei (BN) in coelomocytes exposed to different concentrations of Textile effluent.

Moreover, The CA and MN have been revealed to be effective in assessing the levels of DNA and chromosomal damage in the earthworm E. *eugeniae* exposed to Textile effluent. Thus, these tests could be used as sensitive biomarkers of genotoxicity for terrestrial species to find environmental pollutant.

6. Conclusions

Textile effluent heavy metals caused DNA damage, cytokinesis failure and chromosomal aberrations in E. *Eugenia* earthworms. Overall, it is revealed that even the lowest concentrations of Textile effluent, induced both DNA and chromosomal damage in earthworm coelomocytes. Thus, the combined application of CA and the MN signifies the demonstration of induced genotoxic effects in these invertebrates by environmental pollutants in terrestrial ecosystems.

Acknowledgments:

We are sincerely thankful to Human Molecular Genetics Lab, Department of Zoology, Sri Vasavi College, Erode- 638316, Tamilnadu,

India for her valuable suggestions on the present paper.

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