

RESEARCH ARTICLE

STUDY ON THE COMET ASSAY AND MICRONUCLEUS TEST IN *EUDRILUS EUGENIAE* EARTHWORMS' COELOMOCYTES EXPOSED WITH TANNERY INDUSTRIAL EFFLUENTS**Mathivanan, R. and Chandirasekar, R. ***

Department of Zoology, Sri Vasavi College, Erode, Tamil Nadu, India.

ABSTRACT

Tannery effluents having the different kind's chemical compounds are extensively used to the production of leather industries and it's considered as rich potential of environmental pollutant. Earthworms are easily affecting the toxic chemical in environmentally and in this organism is good experimental animal for monitoring the soil pollution and terrestrial ecosystem. In this study, we taken from the industrial raw tannery effluents and the experimental animal in earthworm species of *Eudrilus eugeniae* (10 for each group) were introduced to 48 hrs for tannery effluent in five different concentrations like 10 ml, 20ml, 30ml, 40 ml, and 50 ml and to find out LC50 level. The LC50 was found at 35 ml concentration. The study was used to the LC50/2, LC50, 2XLC50 for 48 hrs. Then the genotoxicity level was evaluated in *Eudrilus eugeniae* species. After that we collect earthworm's coelomocytes by using the micronucleus (MN) test and comet assay (CA) test. Result of the study, MN and CA level was significantly increased in both genotoxicity and cytotoxicity assays and the high concentration of effluent to promote the increased level of DNA damage and micronucleus in *Eudrilus eugenie* species.

Keywords: Tannery effluent, Earthworm, Coelomocytes collection, LC50, Genotoxicity, Micronuclues, comet assay.

1. INTRODUCTION

Earthworms are chief decomposer of soil organic matter and thus aid in improving soil quality and fertility. About 3920 earthworm species are reported worldwide. Moreover, in India, 509 species, 67 genera and 10 families have been reported (1). Earthworms are widely used as model organisms in terrestrial ecosystem and they serve as a good indicator of heavy metal contamination due to their innate sensitivity to pollutants. Coelomocytes are immunocompetent cells which are affected by the toxic chemicals and are measured as a sensitive biomarker of environment health (2). The tannery and paper industries are exclusively challenging since they generate substantial quantities of wastewater that may have detrimental impacts when released into the environment with or without any treatment. The tannery effluents are ranked as the highest pollutants (3). Tanneries are one of the most prominent sources of chromium pollution to the aquatic environment. If not adequately treated, wastewater from tanneries contaminates surface water and sediments to an unacceptable level of chemicals (4-7). 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 (8). 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 (9,10) (Chisti 2004; Ozer and Pirincci 2006).

The alkaline comet assay (CA) is a well-established indicator to quantify genetic toxicology (11,12). Cytogenetic techniques are used to identify the nuclear abnormalities like micronuclei (MN) and binuclei (BN) cells (13,14). Thus, the present study was designed to assess the cytotoxicity and genotoxicity of tannery effluent in *Eudrilus eugeniae* by using the CA and MN assays.

2. MATERIALS AND METHODS*2.1. Earthworm and effluent collection*

The earthworms were collected from the Western Ghats of Erode district natural and clean surrounding area. Species identification was done, using the specified keys of taxonomic classification. The tannery effluent was collected from tannery industrial area of 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 °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 hrs to tannery effluents. The earthworms (10 Numbers for each group) were introduced to five Different series of concentrations of tannery effluents (10ml, 20ml, 30ml, 40ml, 50ml) was used. LC50 was findouted. In each group of concentration, equal numbers of earthworms were used and these were representative of the pool sample. The effect of different tannery effluent concentrations (LC50/2, LC50, and LC50X2) was measured on DNA and nuclear abnormalities by using the CA and MN tests, respectively for 48 hrs. All the experiments were carried out in the dark at $25 \pm 2^\circ\text{C}$ for 48 hrs. Profit program was used to determine the 48 hrs LC50. Normal water was used to the control group and the same experimental procedure was followed.

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 (15), 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 (16), 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.

2.5. 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.

3. RESULTS

Effects of tannery effluent on mean coelomocyte DNA damage score evaluated by the alkaline CA in coelomocytes of *E. eugeniae* are shown in Fig. 1 and Table 1. A clear concentration-dependent response was observed.

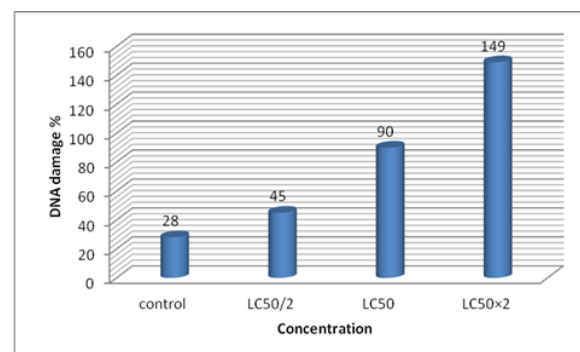


Fig. 1. Mean coelomocyte DNA damage score in earthworms (n = 10) exposed to different concentrations of Tannery effluent error bars are standard deviations. *Significantly different from the control ($p < 0.05$).

The LC50 for tannery effluent was found at 35 ml. significant difference ($p < 0.05$) was found between the mean DNA damage for LC50 and 2XLC50 as compared to the control group. The mean coelomocyte DNA damage score increased significantly on exposure to 2XLC50. So, the numbers of comets were increased as the positively correlated with the increased concentration of tannery effluent. The effects of tannery effluent on nuclear abnormalities in the coelomocytes were evaluated by the MNI test, and

shown in Fig. 2 and Table 2. A total of 1000 coelomocyte (MNI and BN) cells were counted from each earthworm. The sums of MN and BN were higher in each concentration and were statistically significant ($p < 0.05$) from LC50/2 onwards when compared with the control. The highest number of MNI and BN were observed at 2XLC50. 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 binuclear. The highest toxic concentration was found at 2XLC50 for both DNA damage and chromosomal aberration.

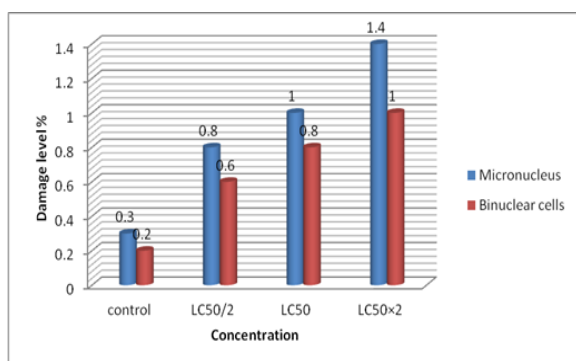


Fig. 2. Total micronuclei (MNI) and binuclei (BN) in coelomocytes exposed to different exposure of Tannery effluent concentrations (n ¼ 10), error bars are Standard deviations. Asterisks indicate significant differences (P < 0.05) from control.

4. DISCUSSION

The CA and MN test are considered as key genotoxic tests to assess the genotoxicity of chemicals (17,18). Heavy metals induce genotoxicity in earthworms (16). The current study showed clear effects of Tannery effluent on DNA damage of earthworms. The concentration-dependent relationship showed that a higher concentration of Tannery 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 (19). 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 Tannery effluent Heavy metals being greater as the toxicant concentration increased. MN test discloses exposed genotoxic damage accumulated during the lifetime of the cells (20).

Moreover, DNA strand breaks produced in the coelomocytes may not be 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 (21,22). Previously, the sensitivity of these cells in revealing DNA variations by genotoxic compounds induction is well established (22,23). 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 (24).

Table 1. Comparison of the parameters of the collected Tannery wastewater sample with the permissible limit stipulated by WHO

S.No.	Parameters	Sample	Permissible Limit
1	DO (mg/L)	2.72	4.5
2	TDS (mg/L)	21300	2100
3	TSS (mg/L)	1250	600
4	EC (μ S/cm) 1200	42500	1200
5	BOD (mg/L)	4464	30
6	COD (mg/L)	12840	250
7	pH 5.5-9	8.3	5.5-9
8	PO43- (mg/L) 17.1	17.1	5
9	Cl- (mg/L)	13.8	1000
10	Pb (mg/L)	0.1818	0.1
11	Cu (mg/L)	0.4112	0.1
12	Cr (mg/L)	11.34	2
13	Zn (mg/L)	1.8241	1
14	Fe (mg/L)	16.675	10

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 Tannery effluent. Thus, these tests could be used as sensitive biomarkers of genotoxicity for terrestrial species to find environmental pollutant.

Table 2. DNA damage, Total micronuclei (MNi) and binuclei (BN) in coelomocytes exposed to different concentrations of Tannery effluent.

S.No	The concentration of Tannery effluent	DNA Damage in Coelomocytes %	Micro-nucleus %	Binuclear cells %
1	Control	28	0.3	0.2
2	LC50/2	45	0.8	0.6
3	LC50	90	1.0	0.8
4	LC50×2	149	1.4	1.0

4. CONCLUSION

Tannery 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 Tannery 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.

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REFERENCES

- Kale, V.S. and Phansalkar, V. G. (1991). Purana basins of peninsular India: a review. *Basin Res.* 3(1): 1-36.
- Homa, J., Olchawa, E., Sturzenbaum, S.R., Morgan, A.J. and Plytycz, B. (2005). Early-phase immunodetection of metallothionein and heat shock proteins in extruded earthworm coelomocytes after dermal exposure to metal ions. *Environ. Pollut.* 135: 275-280.
- Shen T.T. (1999). Industrial pollution prevention (2nd Ed.). Springer, pp: 40.
- Wardas M., Budek, L. and Rybicka, E.H. (1996). Variability of heavy metals content in bottom sediments of the Wilga River (Kraków area, Poland). *J. Appl. Geochem.* 11: 197.
- Walsh, A.R. and O'halloran, J. (1996). Chromium speciation in tannery effluent – ii

- . Speciation in the effluent and in a receiving estuary. *Water Res.* 30: 2401.
- Khawaja, A.R., Singh, R. and Tandon, S. (2001). Monitoring of ganga water and sediments vis-à-vis tannery pollution at kanpur (india): a case study. *Environ. Monit. Assess.* 68: 19.
- Molik, A., Siepak, J., Swietlik, R. and Dojlido, J.R. (2004). Identification of Chromium Species in Tanning Solutions. *Polish J. Environ. Stud.* 13(3): 311.
- Smrithi, A. and Usha, K. (2012) Isolation and characterization of chromium removing bacteria from tannery effluent disposal site. *Int. J. Adv. Biotechnol. Res.* 3: 644-652.
- Chisti, Y. (2004) Environmental impact of toxic pollutants. *Biotechnol. Adv.* 6: 431-432.
- Ozer, A. and Pirincci, H.B. (2006) The adsorption of Cd (II) ions on sulphuric acid treated wheat bran. *J. Hazard Mater.* 13: 849-855.
- Cotelle, S. and Ferard, J.F. (1999). Comet assay in genetic ecotoxicology: a review. *Environ. Mol. Mutagen* 34: 246-255.
- Singh, N.P., McCoy, M.T., Tice, R.R. and Schneider, E.L. (1988). *Exp. Cell Res.* 175:1 84-191.
- Cavas, T., Garanko, N.N. and Arkhipchuk, V.V. (2005). Induction of micronuclei and binuclei in blood, gill and liver cells of fishes subchronically exposed to cadmium chloride and copper sulphate. *Food. Chem. Toxicol.* 43: 569-574.
- Sanchez-Galan, S., Linde, A.R., Ayllon, F. and Garcia-Vazquez, E. (2001). Induction of micronuclei in eel (*Anguilla anguilla* L.) by heavy metals. *Ecotoxicol. Environ. Saf.* 49, 139e143.
- Reinecke, S.A. and Reinecke, A.J. (2004). The comet assay as a biomarker of heavy metal genotoxicity in earthworms. *Arch. Environ. Contam. Toxicol.* 46: 208-215.
- Muangphra, P. and Gooneratne, R. (2011). Comparative genotoxicity of cadmium and lead in earthworm coelomocytes. *Appl. Environ. Soil Sci.* 1-7.
- Benedicte, R.A., Johanna, W. and Kathryn, M.R. (2009). Large scale geographic clines of parasite damage to *Populus tremula* L. *Ecography* 33, 483e493.
- Cigerci, I.H., Liman, R., Ozgül, E. and Konuk, M. (2013). Genotoxicity of indium tin oxide by allium and comet tests. *Cyto* 67: 157-163.
- Rojas, E., Lopez, M.C. and Valverde, M. (1999). Single cell gel electrophoresis assay: methodology and applications. *J. Chromatogr.* 722, 225-254.
- Bolognesi, C. and Hayashi, M. (2011). Micronucleus assay in aquatic animals. *Mutagenesis* 26: 205-213.

21. Bonnard, M., Eom, I.C., Morel, J.L. and Vasseur, P. (2009). Genotoxic and reproductive effects of industrially contaminated soil on the earthworm *Eisenia fetida*. *Environ. Mol. Mutagen* 50: 60e67.
22. Eyambe, G.S., Goven, A.J., Fitzpatrick, L.C., Venables, B.J. and Cooper, E.L. (1991). A noninvasive technique for sequential collection of earthworm (*Lumbricus terrestris*) leukocytes during subchronic immunotoxicity studies. *Lab. Anim.* 25: 61e67.
23. Bigorgne, E., Cossu-Leguille, C., Bonnard, M. and Nahmani, J. (2010). Genotoxic effects of nickel, trivalent and hexavalent chromium on the *Eisenia fetida* earthworm. *Chemosphere* 80: 1109-1112.
24. Fourie, F., Reinecke, S.A. and Reinecke, A.J. (2007). The determination of earthworm species sensitivity differences to cadmium genotoxicity using the comet assay. *Ecotoxicol. Environ. Saf.* 67: 361e368.

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