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RESEARCH ARTICLE

INDUCTION OF MICRONUCLEI DUE TO CYTOTOXIC AND GENOTOXIC EFFECT OF LEAD ACETATE METAL SALT IN *EUPHLYCTIS CYANOPHLYCTIS* (AMPHIBIA: ANURA)

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ABSTRACT

The aim of the present study was to investigate the genotoxic effects of lead acetate in different somatic tissues (Intestine and RBCs) of skittering frog (*Euphlyctis cyanophlyctis*) using micronucleus test. LC_{50} value of lead acetate was calculated to be 42mg/kg. The four different sublethal concentrations of lead acetate (4.5, 9.0, 18.0 and 36.0mg/kg) were injected intraperitoneally once in the treatment period for 24, 48, 72 and 96hrs. It was observed that this heavy metal induced a significant increase in frequency of micronuclei at different concentrations in frog for 24, 46, 72 and 96hrs when compared with the control. These results lead us to the conclusion that lead may have genotoxic and cytotoxic properties due to the induction in the frequencies of MN in different tissues of the frog.

INTRODUCTION

Micronuclei are cytoplasmic chromatin-containing bodies that appears in the cell like a small satellite nucleus around the cell nucleus, due to chromosome fragments or entire chromosomes that are not incorporated in the main nucleus after cell division. The presence of micronuclei (MN) in cells is considered as a biomarker of damage to the DNA. The micronucleus test is an *in vivo* and *in vitro* short-time screening cytogenetic test, introduced by Heddle (1973) and Schmid (1975) is a widely used method for assessing genotoxicity of chemicals in organisms (Meier *et al.,* 1999). The micronucleus test has been used because it is technically easy to master, reliable, least expensive and extremely rapid screening system. The micronucleus test for mutagenicity screening has been well established in several systems i.e. ovary, bone marrow, epithelial tissue, peripheral blood, liver, exfoliated buccal cells and fetus cells of several laboratory animals or human (Heddle, 1990; Saleh and Zeytinoglu, 2001). Micronuclei formation occurred in any dividing tissue of any species (Heddle *et al.,* 1983; Zuniga *et al.,* 1996). Micronucleus test was used to investigate environmental pollution in plants (Ma *et al.,* 2005), fish (Cavas and Ergene Gozukara, 2005), birds (Bhunya and Jena, 1996) and frogs (Mouchet *et al.,* 2005 and 2006). Furthermore, (Hayashi *et al.,* 1998) evaluated the monitoring systems that use aquatic organisms to assess the genotoxicity of water in the field and in the laboratory. In a field study, micronucleus assay was shown to be applicable to micronucleus inducing agents in frogs (Saleh and Zeytinoglu, 2001). Aquatic animals have often been used in bioassays to monitor water quality of effluent and surface water (Fernandez and L Haridon, 1992). The development of biological monitoring techniques regarding frogs and fishes offer the possibility of checking water pollution with fast responses on low concentrations of direct acting toxicants (Al Sabti, 1986; Al Sabti and Metcalfe, 1995; Andrade *et al.,* 2004). Amphibians have also proved to be valuable biological models for the study of micronucleus induction in erythrocytes, leading to the normalized micronucleus test MNT (Jaylet *et al.,* 1986; Krauter *et al.,* 1987; Gauthier, 1996; Ferrier *et al.,* 1998; Jaylet and Zoll-Morreux, 1990; Zoll-Morreux *et al.,* 1990; Djomo *et al.,* 2000; Bekaert *et al.,* 2002). Determining the direct and indirect effects of agro and industrial chemicals to amphibian species continues to be identified, as a research need. The conclusiveness of published information on the ecotoxicology of metal contaminants to amphibians is limited in many cases (Sparling *et al.,* 2000).

As one of these metal contaminants, lead is extensively used as industrial materials such as in the manufacture of batteries, metal product, paints and ceramic glazes. The largest source of lead in the atmosphere has been from lead gasoline combustion. Lead enters the organisms through food chain and absorbed mainly by ingestion and inhalation. Since it cannot be discharged, the lead accumulates in tissues and in over doses the lead has toxic effects (Uysal, 1997). Lead acetate is a chemical compound, which is also known as mutagen and carcinogen.

Amphibians are considered as excellent "bio indicators" of environmental health (Schuytema and Nebeker, 1999; Tejedo, 2003; Garcia-Munoz *et al.,* 2010. The Indian Skipper Frog or Skittering Frog (*Euphlyctis cyanophlyctis*) is a common frog found in South Asia and is the native frog of Kashmir. They are often seen at the edge of bodies of water with their eyes above the water. *E. cyanophlyctis* was chosen in the present study to evaluate the genotoxic potential of lead acetate by using the micronucleus test.

MATERIALS AND METHODS

Sample Collection

Healthy and actively living adult frog specimens of *Euphlyctis cyanophlyctis* were collected from unpolluted ponds and ditches in the vicinity of Jammu region. They were collected using hand nets to prevent injury to animals during capture since they are

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active animals. The weight of frogs ranged from 35-40g. These frogs were kept under laboratory conditions for about 5 days, at ambient temperature and natural photoperiod, according to the time of the year, in which each test was performed, for acclimatization before starting the assay. They were kept in 15L plastic containers. The water was changed daily to avoid accumulation of toxic substances.

Heavy metal treatment

After adaptation, the frogs were separated in 5 experimental groups, 1 group as control and 4 treated groups in separate plastic containers of 10L capacity. Four frog specimens were kept for treatment with each heavy metal salt concentration (sublethal conc.) for 24, 48, 72, and 98 hrs. Separate batch of same number of frogs were kept as control for each duration of exposure i.e. 24, 48, 72, and 98hrs. LC_{50} value at 96hrs was calculated using standard method of Finney (1980) and was found to 42mg/kg. Based on the LC_{50} value of lead acetate, the four sublethal concentrations were arbitrarily chosen and the frogs were treated with these doses (i.e. 4.5mg/kg, 9.0mg/kg, 18.0mg/kg and 36.0mg/kg) through an intraperitoneal injection by using 1ml syringe, only once in the treatment period.

Slides preparation and staining

For each frog, experimental as well control, fresh blood samples were taken after each duration of exposure and smeared onto the clean slides. The slides were air dried for 1-2hrs and then fixed in absolute methanol for 10min. After fixing, the same slides were stained in Giemsa (2%) for about 30min.

Examining and scoring of MN slides

Red blood cells in lower vertebrates such as amphibians are nucleated and undergo cell division in the circulation. These cells are therefore suitable for micronuclei detection which can be readily counted in blood smears. The frequency of MN in erythrocytes was established by estimating the number of MN in at least 1500 interphase cells/ specimen (total of 6000 interphase cells from four specimens used for conc. and duration). The micronucleated interphase erythrocytes were photomicrographed at 1000X magnification under a binocular microscope (Olympus). Only cells with intact cellular and nuclear membrane were scored. The following criteria were used as described by previous studies:

a) MN should be one-tenth and one-third diameter of main nucleus.
b) They should be on the same place of focus.
c) They should have the same colour, texture and refraction as the main nucleus.
d) They should be clearly separated from the main nucleus.

Statistical analysis

The frequency of micronuclei obtained from the experimental as well as controlled group were expressed as mean ±SD. Statistical analysis of the data was carried out using the non-parametric Kruskal-Wallis test and the computer software called 'PRIMERS-4.0' was used for it. Difference between means are regarded as significant if $p<0.05$.

RESULTS

Effect of Lead acetate treatment on Intestine

Frequency of MN determined in different treatment is summarized in Table 1 and Fig 1 and 3. Frog exposed to different concentration and durations of Lead acetate showed a dose and time dependent increase in the incidence of micronuclei which were significantly higher than that of controls (at 24hrs, $p<0.05$; at 48hrs, 72hrs and 92hrs, $p<0.010$ verses respective controls in all treatment groups). In frog treated in vivo with 4.5mg/kg lead acetate, the frequency of MN was recorded to be 1.15±0.83 (24hrs), 1.45±0.83 (48hrs), 1.65±0.40 (72hrs) and 2.13±1.45 (96hrs) wherein the incidence of micronuclei increased along with the duration of the same dose.

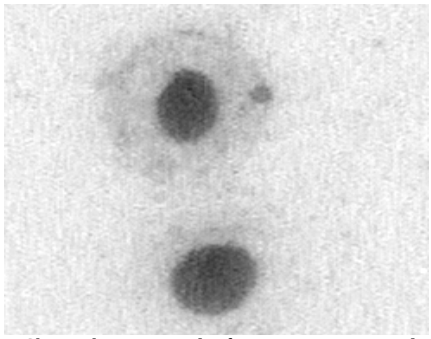

Fig 1 Shows the micronuclei frequency in Intestinal cell

An elevated response was observed up to 96hrs of exposure in the frequency of MN (2.30±0.85 at 24hrs, 3.30±0.57 at 48hrs, 3.65±1.38 at72hrs and 4.30±0.35 at 96hrs) after treatment with 9.0mg/kg. Specimens exposed to 18.0mg/kg showed significantly decrease in MN at 24hrs (1.65±0.40) but an elevated increase in frequency of MN were observed at 48hrs (3.63±0.87), 72hrs (4.98±1.62) and 96hrs (5.80±0.63). Treatment with 36.0mg/kg showed a highly significant increase of micronuclei in relation to respective controls at all the exposure periods. The values recorded were 2.30±0.35 (24hrs), 4.13±1.49 (48hrs), 6.40±0.62 (72hrs) and 8.63±2.12 (96hrs).

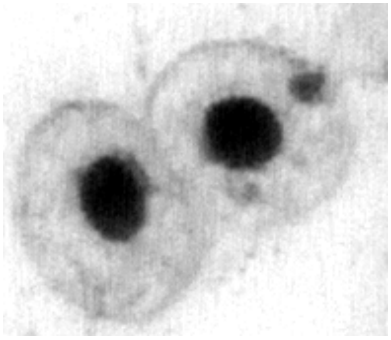

Fig 2 Shows the micronuclei frequency in RBC

Effect of Lead acetate treatment on RBCs

Values of MN in the peripheral erythrocytes after different treatment and exposure periods have been shown in Table 1 and Fig 2 and 4. Frog treated *In vivo* with 4.5mg/kg showed a highly significant increase (at 24, 48, 72 and 96hrs, $p<0.010$ versus control values) in the incidence of MN versus respective controls at all the exposure periods. The values recorded were 0.60±0.00, 1.12±0.67, 1.12±0.67, 1.82±0.35 after 24hrs, 48hrs,72hrs and 96hrs respectively, wherein the frequency of MN were same at 48hrs and 72hrs of treatment. Frogs exposed to sub-lethal concentration of 9.0mg/kg, 18.0mg/kg and 36mg/kg also revealed highly significant increase ($p<0.010$ at all the exposure periods versus respective control values) in the incidence of MN. At 9.0mg/kg of treatment, the values recorded were 0.77±0.35, 4.12±0.62, 3.65±0.40 and 3.95±1.10 after 24hrs, 48hrs, 72hrs and 96hrs respectively, where maximum frequency of MN were recorded at 48hrs of treatment.

Table 1 Frequencies of micronuclei (%) in different tissues of *Euphlyctis cyanophlyctis* exposed to Lead acetate.

Conc. (mg/kg)	Duration (hrs)	No. of Specimens	Total interphase cells studied	Frequency of micronuclei (Mean±SD)	
				Intestine	RBC
Contr.	24	4	6000	0.60±0.00	0.30±0.34
	48	4	6000	0.60±0.00	0.15±0.30
	72	4	6000	0.30±0.36	0.45±0.30
	96	4	6000	0.30±0.36	0.45±0.30
4.5	24	4	6000	1.15±0.83^{a}	0.60±0.00^{b}
	48	4	6000	1.45±0.83^{b}	1.12±0.67^{b}
	72	4	6000	1.65±0.40^{b}	1.12±0.67^{b}
	96	4	6000	2.13±1.45^{c}	1.82±0.35^{b}
9.0	24	4	6000	2.30±0.85^{a}	0.77±0.35^{b}
	48	4	6000	3.30±0.57^{b}	4.12±0.62^{b}
	72	4	6000	3.65±1.38^{b}	3.65±0.40^{b}
	96	4	6000	4.30±0.35^{c}	3.97±1.10^{b}
18.0	24	4	6000	1.65±0.40^{a}	1.65±0.40^{b}
	48	4	6000	3.63±0.87^{b}	3.30±0.57^{b}
	72	4	6000	4.98±1.62^{b}	4.95±0.40^{b}
	96	4	6000	5.80±0.63^{c}	4.45±2.15^{b}
36.0	24	4	6000	2.30±0.35^{a}	2.47±1.15^{b}
	48	4	6000	4.13±1.49^{b}	4.30±1.61^{b}
	72	4	6000	6.40±0.62^{b}	5.45±1.83b
	96	4	6000	8.63±2.12^{c}	9.48±1.48^{b}

a=p<0.05,b=p<0.01,c=p<0.001,ns= non-significant(represent values significantly differently from the respective controls; Kruskal-Wallis test; df=4), Contr.= Control

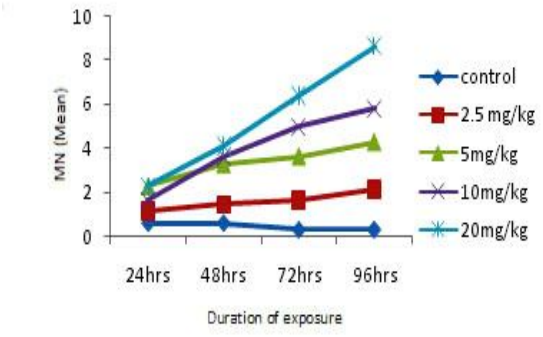

Fig 3 Frequencies of micronuclei in Intestine after treatment with Lead acetate

Exposure to 18.0mg/kg induced 1.65±0.40, 3.30±0.57, 4.95±0.40 and 4.45±2.15 MN frequency after 24hrs, 48hrs, 72hrs and 96hrs, here the maximum frequency were observed at 72hrs treatment period. An elevated response was observed during treatment with 36.0mg/kg wherein the values recorded were 2.47±1.15, 4.30±1.61, 5.45±1.83 and 9.48±1.48 after 24, 48, 72 and 96hrs of exposure respectively.

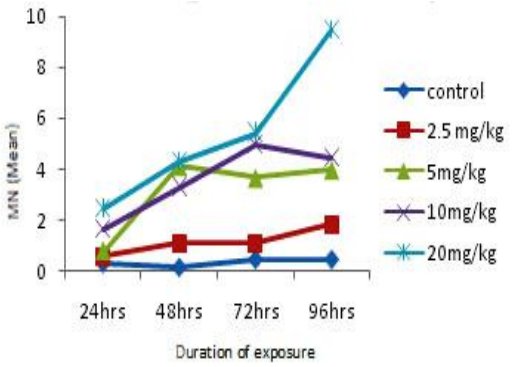

Fig 4 Frequencies of micronuclei in RBCs after treatment with Lead acetate

DISCUSSION

Over the past decades, the decline of the amphibian populations have been extensively reported (Houlahan *et al.*, 2000; Stuart *et al.*, 2004). Amphibian are reliable indicators of environmental pollution due to their biphasic life (aquatic and terrestrial) and semi-permeable skin (Lee and Stuebing, 1990). In some cases this phenomenon is associated with pollution by pesticides and heavy metals. Although environmental pollution may interfere with amphibian growth and development, the induction of genetic damage after chronic exposure to low doses of chemicals is perhaps the most important biological effect. The blood of amphibians is very plastic tissue. In fact, variation of several hematological parameters in response to natural changes in the environment have widely described by researchers (Krauter, 1993; Stansley and Roscoe, 1996). Jayet *et al.*, (1986) first adapted the MN test to amphibians. Many MN tests on amphibians have used genotoxic agents (Van Hummelen *et al.*, 1989; Chen and Xia, 1993; Zoll Morreux and Ferrier, 1999). It has been used as a measure of genotoxicity in amphibians (Ferrier *et al.*, 1998; Compana *et al.*, 2003) and has shown potential for *in situ* monitoring of water quality (Gauthier, 1996). MN derivative from chromosomal fragments or whole chromosomes which are not incorporated into main nucleus during cell division as a consequence of DNA fragmentation (clastogenic origin) or of alteration of the mitotic apparatus (an eugenic origin) (Schmid, 1975; Heddle *et al.*, 1991; Norppa and Flack, 2003).

In the present study of genotoxic effect of Lead acetate using micronucleus test revealed that there is a significant induction of micronuclei in all the tissues (intestine and RBCs) of *E. cyanophlyctis*, studied as compared to positive control groups. The frequencies of micronuclei in different tissues was evaluated (Table 1) and expressed as Mean±SD. A comparison between micronucleus frequencies in both the tissues revealed highest micronuclei frequencies at 96hrs duration in RBCs (Fig 4) followed by Intestine (Fig 3). In intestine, the value of MN recorded ranged from 1.15±0.83 (at 24hrs with a dose of 4.5mg/kg) to 8.63±2.12 (at 96hrs with a dose of 36.0mg/kg) showing a significant increase in the value of micronuclei with the increase in dose and duration of exposure excepting for 24hrs and

48hrs, each for 18mg/kg and 36.0mg/kg treatment dose, where a slight fall in value of the MN frequency was observed. In RBCs the MN frequency ranged from 0.60±0.00 (at 24hrs with a dose of 4.5mg/kg) to 9.48±1.48 (at 96hrs with a dose of 36.0mg/kg). There was significant increase in the MN frequency for all the exposure periods (24, 48, 72 and 96 hrs) at 4.5mg/kg but with the increase in dose (9.0 and 18.0 mg/kg) fluctuation in the MN value was observed with a maximum value (4.12±0.62) at 48hrs of duration with 9.0mg/kg treatment of the test chemical and with 18.0 mg/kg treatment, maximum value (4.95±0.40) was recorded at 72hrs exposure period. However, with the increase in dose of the test chemical the value of MN frequency showed a statistical increase. Thus, a positive relationship between metal concentrations and micronuclei frequencies were recorded for all the treatment groups with respect to their dose and the exposure periods compared to the control groups. The frog treated with different doses of the lead acetate (4.5mg/l, 9.0mg/l, 18.0mg/l,36.0mg/l) at 24hrs of duration showed significant results ($p<0.05$) but with increase in duration of the doses, the result obtained were highly significant ($p<0.01$).

CONCLUSION

Present results, thus suggested that sublethal doses of lead acetate caused detectable genome damage in various tissues of frog, *Euphlyctis cyanophlyctis*. Further studies should include more of the different lead doses and different stages of development of the frog to gain a better insight in the significance of present findings and to elucidate the mechanism of lead genotoxicity and also different genotoxic tests can be taken into account.

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