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EVALUATION OF ACUTE TOXICITY OF COPPER SULPHATE IN DIFFERENT TISSUES OF EUPHLYCTIS CYANOPHLYCTIS

Preetpal Kour

Department of Zoology, University of Jammu, Jammu, Jammu & Kashmir, India

N.K. Tripathi

Department of Zoology, University of Jammu, Jammu, Jammu & Kashmir, India

Poonam

Department of Zoology, University of Jammu, Jammu, Jammu & Kashmir, India

ABSTRACT

Copper sulphate, $CuSO_4.5H_2O$ (Fine Chemical, India), Purity (99%) and CAS No. 7758-98-7, a broad-spectrum inorganic pesticide, has many-fold uses as fungicide, molluscicide and weedicide. The adult Indian skittering frog, Euphlyctis cyanophlyctis was treated In vivo with different sublethal concentrations of $CuSO_4$ (3.5, 5.0, 6.5 and 8.0mg/kg) after calculating the LC_{50} value which was found to be 10mg/kg. Each dose was injected intraperitoneally once in the treatment period for 24, 48, 72 and 96hrs to study its potential toxic effect using micronucleus test. It was observed that copper sulphate induced a significant increase in frequency of micronuclei at different concentrations in frog for 24, 48, 72 and 96hrs when compared with the control. The results lead us to the conclusion that there is a dose-effect relationship in the induction in the frequencies of MN in different somatic tissues of the frog due to its genotoxic and cytotoxic properties.

Keywords: Genotoxicity, Copper sulphate, *Euphlyctis cyanophlyctis*, LC_{50} value, Micronucleus test.

1. INTRODUCTION

Chemical stressors such as acid deposition, industrial chemicals, pesticides, heavy metals, salts and nitrogen fertilizers are possible causes for the decline of some amphibian populations. The global phenomena of localized amphibian population declines, a high prevalence of amphibian malformations and the increasing presence of amphibians on threaten or endangered species lists have currently stimulated research investigating amphibian response to both natural and anthropogenic stressors [1]. Heavy metals are usually detected in measurable levels in industrial effluents because metallic compounds are common constituents of several raw materials, which

serve as feedstocks, catalysts, lubricants and clean up chemicals employed in industrial production processes. The main route by which heavy metals enter the aquatic environment is through the discharge of metal-laden municipal and industrial effluents directly into water bodies or indirectly through drainage and canals [2]. Copper, in trace amounts, is essential for life while in excess is toxic. Its importance in health and disease is well documented [3-9]. It has also been established in the literature that lead and copper reach drinking water through the dissolution of plumbing materials [10]. A number of metals present in the occupational environment have been reported to be human carcinogens [11]. However, there is no direct positive correlation between Cu exposure and cancer [6, 12].

In spite of the indiscriminate use of synthetic organic pesticides, a number of inorganic pesticides are still widely used in present day agriculture. Copper sulphate, a broad-spectrum inorganic pesticide, has many-fold uses as fungicide, molluscicide and weedicide. It causes no DNA damage in prokaryotes [13] and enhances viral transformations [14]. De Flora, et al. [15] reported the non-mutagenic property of copper sulphate in several strains of Salmonella tiphimurium and the negative result in DNA repair tests with several repair-deficient strains of Escherichia coli. The genotoxicity of Cu compounds has been reported in animal cell cultures [16, 17]. Its clastogenic effects have been reported in mice in vivo by Bhunya and Pati [18] and Agarwal, et al. [19] while Tinwell and Ashby [20] reported its negative effect in the mouse bone marrow micronucleus assay. The strong clastogenic potential of copper has been reported in plants [21]. There is limited literature on the clastogenic effect of copper sulphate in frogs in vivo test system. This gives us further impetus to evaluate the clastogenic and genotoxic potential of copper sulphate in the Indian Skipper Frog or Skittering Frog (*Euphlyctis cyanophlyctis*) which 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 potential of copper sulphate by using the micronucleus test.

2. MATERIALS AND METHODS

2.1. Test Chemical

Copper sulphate, $CuSO_4.5H_2O$ (Fine Chemical, India), Purity (99%) and CAS No. 7758-98-7 was used as test chemical.

2.2. Animals and Treatment

Experiments were performed on Indian skittering frog *Euphlyctis cyanophlyctis*. Adult live and apparently healthy specimens of, *E. cyanophlyctis* weighing 30-40gms caught from local unpolluted lentic habitats were acclimatized to laboratory conditions in large well aerated plastic containers for at least 5 days prior to use. Frogs were divided into 1 control and 4 treatment groups. Four frogs were kept in each group. LC_{50} value was estimated using standard method of Finney [22] and was found to be 10mg/kg. Based on LC_{50} value of copper sulphate, four sublethal concentrations were arbitrarily chosen and the frogs were treated with these doses (3.5mg/kg, 5.0mg/kg, 6.5mg/kg and 8.0mg/kg) through an intraperitoneal injection by using 1ml syringe only

once in the treatment period. Frogs of all groups were subjected for 24, 48, 72 and 96hrs of exposure periods.

2.3. Micronucleus Test (MNT) Procedure

Fresh blood samples from each frog, experimental as well control, were taken after each duration exposure and smeared onto the clean slides, air-dried for 1-2hrs and then fixed in absolute methanol for 10 min. The slides were stained in Giemsa stain (2%) for 30-35 minutes. Then the animals were anaesthetized and dissected to take out the intestine and kidney. The tissues were hypotonised with 0.50M NaCl solution for 1hour at room temperature. Fixing of the tissues was done in Conroy's fixative for 45min changing the solution every 15 minutes. The material was then dabbed on clean slides, air-dried and stained with 2% Giemsa stain for 30-35 minutes.

2.4. Examining of Slides

The frequency of MN in erythrocytes as well in intestinal and kidney tissues was established by calculating 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 cells were photomicrographed at 1000X magnification under a binocular research microscope (Olympus CH20iBIMF) using Sony SSC-DC378P camera. Only cells with intact cellular and nuclear membrane were scored.

2.5. 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 $p<0.05$.

3. RESULTS

3.1. Effect of Copper Sulphate Treatment in Intestine

Frequencies of micronuclei determined in the different treatments are summarized in Table 1 and Figure 1 & 4. Frogs exposed different concentrations and durations of copper 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 48, 72 and 96hrs, $p<0.01$). In frog treated in vivo with 3.5mg/kg of copper, frequency of MN was recorded to be 0.97±0.865(at24hrs) with a slight decrease to 0.95±0.404 (at 48hrs), followed by an increase in value at 72hrs (1.12±0.670) and at 96hrs (1.13±0.942). An elevated response was observed in the frequency of MN (exposed to 5.0mg/kg) from 24 to 96hrs of exposure (1.14±0.834 after 24hrs, 1.97±0.531 after 48hrs, 2.15±0.3 after 72hrs and 3.47±1.114 after 96hrs). Specimens exposed to 6.5mg/kg showed significantly elevated incidence of MN at 24hrs (1.47±0.35), at48hrs (2.12±0.618), at 72hrs (3.97±1.102) and at 96hrs (3.97±1.717). Values of MN frequency were same at 72 and 96hrs of exposure. Treatment with 8.0mg/kg showed a highly significant increase of micronuclei in relation to the respective

controls at all the exposure periods. The values recorded were 3.32±0.942 (at 24hrs), 5.47±1.117 (at 48hrs), 8.12±0.618 (at 72hrs) and 11.62±0.865 (at 96hrs).

Figure-1. Shows the micronuclei frequency in Intestinal cell.

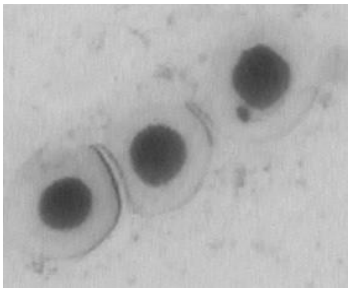


Figure-2. Shows the micronuclei frequency in RBCs.

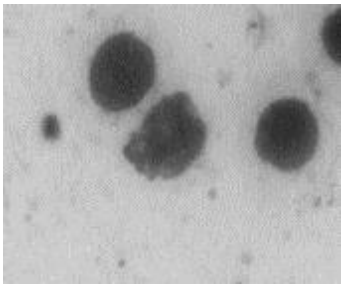
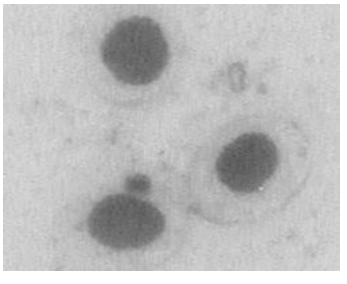


Figure-3. Shows the micronuclei frequency in Kidney cells.



3.2. Effect of Copper Sulphate Treatment in RBCs

Values of MN in the peripheral erythrocytes after different treatments and exposure periods have been shown in Table 1 and Figure 2 & 5. Frog treated *In vivo* with 3.5mg/kg of copper revealed minimum value (0.8±0.627) of MN frequency at 24hrs and maximum value (1.47±0.35) of MN frequency at 72hrs. 0.62±0.531 and 1.12±0.670 are the values of MN recorded at 48hrs and 96hrs respectively. An elevated response was observed during treatment with 5.0mg/kg wherein the values obtained were 1.32±0.942, 1.62 ±0.865, 1.8±1.0 and 3.3 ±1.205 after 24, 48, 72 and 96hrs of exposure respectively. Exposure to 6.5mg/kg induced 0.97±0.865, 1.97±0.531, 3.47±0.35and 4.8±1.232, MN frequency after 24, 48, 72 and 96hrs respectively. Frogs exposed to sublethal concentration of 8.0mg/kg revealed significant induction of MN frequency after all the exposure

periods in comparison to control. Minimum frequency (2.97±0.865) and maximum frequency (3.32±0.942) and maximum frequency (11.8 ±0.848) was recorded after 24 and 96hrs of exposure through 5.62 ±0.865 and 7.47±2.232 at 48 and 72hrs of exposure respectively. Data was found to be statistically significant in all treatment doses versus respective controls (at 24hrs, $p<0.05$; at 48, 72 and at 96hrs, $p<0.01$) as shown in Table 1.

3.3. Effect of Copper Sulphate Treatment in Kidney Cells

Frequencies of MN recorded in the kidney tissue after different treatments and exposure periods have been shown in Table 1 and Figure 3 & 6. MN frequencies observed in frog treated in vivo with 3.5mg/kg increased from 0.62±0.531 at 24hrs to 1.47±0.67 at 96hrs through 0.95±0.404 at 48hrs and 1.47±0.35 at 72hrs, the value being same at 72 and 96hrs of exposure. Increased levels of MN were observed after all the exposure periods in experimental frog treated with 5.0mg/kg.

Table-1. Frequencies of micronuclei (%) in different tissues of *Euphlyctis cyanophlyctis* exposed to Copper Sulphate.

Conc. (mg/kg)	Duration (Hrs)	No. of specimens	Total interphase cells studied	Frequency of micronuclei (Mean±SD)		
				Intestine	RBCs	Kidney
Contr.	24	4	6000	0.3±0.346	0.3±0.346	0.45±0.3
	48	4	6000	0.45±0.3	0.45±0.3	0.45±0.3
	72	4	6000	0.15±0.3	0.3±0.346	0.3±0.346
	96	4	6000	0.47±0.618	0.15±0.3	0.15±0.3
3.5	24	4	6000	0.97±0.865[a]	0.8±0.627[a]	0.62±0.531[a]
	48	4	6000	0.95±0,404[b]	0.62±0.531[b]	0.95±0.404[b]
	72	4	6000	1.12±0.670[b]	1.47±0.35[b]	1.47±0.35[b]
	96	4	6000	1.13±0.942[b]	1.12±0.670[b]	1.47±0.67[c]
5.0	24	4	6000	1.14±0.834[a]	1.32±0.942[a]	1.62±0.865[a]
	48	4	6000	1.97±0.531[b]	1.62±0.865[b]	2.47±0.618[b]
	72	4	6000	2.15±0.3[b]	1.8±1.0[b]	2.97±1.164[b]
	96	4	6000	3.47±1.117[b]	3.3±1.205[b]	3.45±1.350[c]
6.5	24	4	6000	1.47±0.35[a]	0.97±0.865[a]	0.95±0.404[a]
	48	4	6000	2.12±0.618[b]	1.97±0.531[b]	1.97±0.531[b]
	72	4	6000	3.97±1.102[b]	3.47±0.35[b]	4.15±0.834[b]
	96	4	6000	3.97±1.717[b]	4.8±1.232[b]	4.65±1.223[c]
8.0	24	4	6000	3.32±0.942[a]	2.97±0.865[a]	2.15±0.834[a]
	48	4	6000	5.47±1.117[b]	5.62±0.865[b]	4.3±1.288[b]
	72	4	6000	8.12±0.618[b]	7.47±2.232[b]	5.95±2.087[b]
	96	4	6000	11.62±0.865[b]	11.8±0.848[b]	9.8±1.840[c]

Maximum frequency of MN was obtained after exposure period of 96hrs (3.45±1.350) and minimum frequency was obtained after exposure of 24hrs (1.62±0.865). Significant difference (at 24hrs, $p<0.05$, at 48 and 72hrs, $p<0.01$ and at 96hrs, $p<0.001$) in the incidence of MN between experimental and control values were also observed after all the exposure periods in treatment with 6.5mg/kg and 8.0mg/kg. Maximum value recorded was 4.65±1.223 and 9.8±1.840 after 96hrs of

exposure in treatment with the respective concentrations. Similarly, minimum values (0.95±0.404 and 2.15±0.834 for 6.5 and 8.0mg/kg treatment respectively) were recorded after 24hrs of exposure.

Figure-4. Frequencies of micronuclei in Intestine after treatment with Copper.

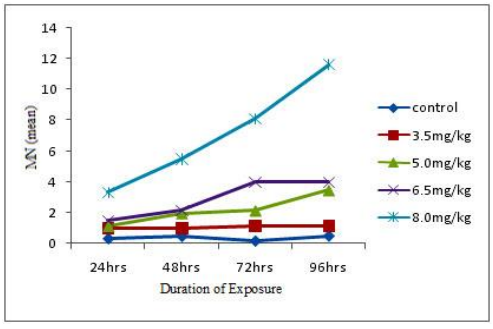


Figure-5. Frequencies of micronuclei in RBCs after treatment with Copper.

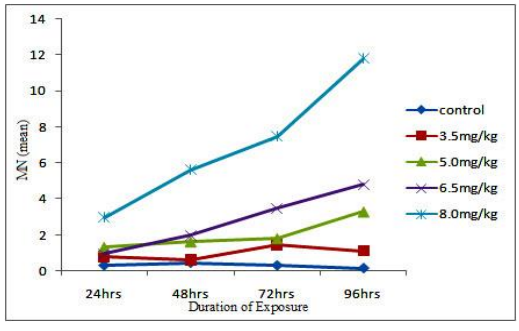
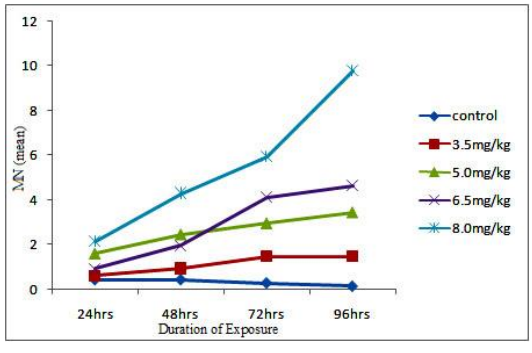


Figure- 6. Frequencies of micronuclei in Kidney after treatment with Copper



4. DISCUSSION

As part of the global loss of species, amphibian populations are declining throughout world [23-27]. Concern about amphibians is due in part to their value as indicators of environmental stress [28]. Many are in close contact with water as larvae, and most have some contact with land as adults. Therefore they experience both aquatic and terrestrial stressors. Since 1989 amphibians

have been widely advocated as excellent biological indicators or sensitive indicators of environmental health [23, 29-31].Amphibians have moist, permeable skin and unshelled eggs that are directly exposed to soil, water and sunlight, and that can readily absorb toxic substances such as heavy metals and pesticides. Although environmental pollution may interfere with amphibian growth and development, the induction of genetic damage after chronic exposure to low doses of chemicals especially heavy metals is perhaps the most important biological effect. Pesticides also effect frogs and other amphibians in different ways since they destroy their natural biotic balance in fields, induce the growth of extra legs and eyes [32]. Some pesticides are cholinesterase inhibitors. This is indication that field application of these pesticides and chemicals may be deleterious to amphibians [33-36]. Moreover, amphibians are important components of many ecosystems, acting as prey, predators or herbivores. Because of their contribution to trophic dynamics, loss of amphibians will probably affect other organisms [26, 37]. Khan, et al. [38], [39] and Khan and Yasmeen [40] studied the induced effects of various chemicals on *Rana cyanophlyctis* and *R. tigrina* and reported that amphibians in general are sensitive but *R. cyanophlyctis* is more sensitive to the chemicals used by them than *R. tigrina*. Jaylet, et al. [41] first adapted the MN test to amphibians. Many MN tests on amphibians have been proven to be suitable for evaluating mutagens and genotoxic agents [42-44]. It has been used as a measure of genotoxicity in amphibians [45, 46] and has shown potential for *In situ* monitoring of water quality [47]. 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) [48, 49]; Heddle, et al. [50].

In the present study of the genotoxic effect of coppersulphate on different tissues of frog, *Euphlyctis cyanophlyctis*, the data revealed a time dependent increase in micronucleus frequencies with the increase in doses of copper in intestine, RBCs and kidney. With respect to the dose, maximum effect was found to be induced at the highest dose of exposure i.e. 8.0mg/kg while prominent effect with respect to the duration of exposure was induced after the maximum period of exposure i.e. 96hrs. Comparison between the micronucleus frequencies induced in the various tissues revealed highest MN frequencies in RBCs, followed by a narrow margin in intestine and least in kidney. In intestine, the value of MN frequency recorded ranged from 0.97±0.865 (at 24hrs with a dose of 3.5mg/kg) to 11.62±0.865 (at 96hrs with a dose of 8.0mg/kg) showing a significant increase in value of micronuclei with the increase in dose and duration of exposure excepting for 48hrs (at 3.5mg/kg) treatment dose, where a fall in value of MN frequency was observed whereas at treatment dose of 6.5mg/kg, the MN frequency was almost same at 72 and 96hrs of duration. The incidence of MN frequencies observed in kidney tissue ranged from 0.62±0.531 (at 24hrs with a dose of 3.5mg/kg) to9.8±1.840 (at 96hrs with a dose of 8.0mg/kg). However, the value of MN frequencies recorded at 72 and 96hrs of duration (with a dose of 3.5mg/kg) were almost the same.

In general, while comparing the effect of different concentrations on the intestine, RBCs and kidney, at concentration of 3.5mg/kg, minimum MN frequency was observed in kidney and RBCs (at 24 and 48hrs respectively) and maximum was observed in the kidney only at 96hrs of duration. With treatment dose of 5.0mg/kg, minimum and maximum MN frequency value was observed in

the intestine only (at 24hrs and 96hrs of duration respectively). At treatment dose of 6.5mg/kg, minimum value of MN frequency was observed in kidney and maximum in the RBCs while at treatment dose of 8.0mg/kg, minimum value was recorded in kidney (at 24hrs) and maximum value recorded in RBCs (at 96hrs). 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 (3.5mg/kg, 5.0mg/kg, 6.5mg/kg, 8.0mg/kg) at 24hrs of duration showed significant results ($p<0.05$) in all the studied tissues but with increase in duration of the doses, the result obtained were highly significant, ($p<0.01$) in intestine and RBCs and ($p<0.001$) in kidney.

5. CONCLUSION

The present work investigated the cytotoxicity and genotoxicity of copper sulphate ($CuSO_4$) on erythrocytes and other tissues (intestine and kidney) of the Indian skittering frog, *Euphlyctis cyanophlyctis* using micronucleus test in controlled laboratory conditions with different sub-lethal doses to a maximum of 96hrs. The results and the data evaluated indicates that $CuSO_4$, which is used as a fungicide, molluscicide and weedicide can be genotoxic at higher concentrations, therefore if inefficiently used in aquatic and agricultural systems, might reach levels that pose genotoxicity to tadpoles and frogs making it a potential threat to water ecosystems and to human health.

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