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Evaluation of Genotoxicity induced by Cobalt to Freshwater Fish, *Cirrhina mrigala* using Micronuclei Assay

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ABSTRACT

Due to industrial advancement, a variety of toxic chemicals including metals are released into the aquatic environment which not only disturbs the physico-chemical properties of the water bodies but also influences the aquatic food chain to cause physiological and cytogenetic alternations in the aquatic animals. Metals have the ability to produce reactive oxygen species (ROS) that would cause the oxidative of nucleic acid. Micronucleus test has been commonly used for the estimation of biological impacts of water pollutants on genotoxic damage in fish. Therefore, the present research work was designed to check the genotoxic potential of cobalt for fish *Cirrhinus mrigala* by using a micronuclei assay. Fish were exposed to the various sub-lethal concentrations of cobalt metal such as 2/3rd, 1/3rd, 1/4th, and 1/5th of LC_{50} concentration for one month and sampling was done after 10 days intervals. Blood sample from the caudal vein of fish was collected to see the micronuclei and binucleated nuclei. Results showed that all test concentrations induced micronuclei and binucleated nuclei in peripheral erythrocytes of *C. mrigala*. Maximum nuclear abnormalities in peripheral erythrocytes of *C. mrigala* were observed in 2/3rd concentration followed by the orders: 1/3rd >1/4th >1/5th.

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INTRODUCTION

Aquatic bodies were polluted with a variety of toxicants has become a matter of great concern from last few years (Vutukuru, 2005). Aquatic ecosystems are at risk to various pollutant that are mainly released from sewage treatment plants, effluents discharged, industries, agricultural, urban zones and anthropogenic activities (Lopez *et al.,* 2002; Karbassi *et al.,* 2006).

Among these pollutants, metals constitute a significant part of inorganic pollutants (Ghosh and Singh, 2005; Gad and Saad, 2008; Jadhav *et al.,* 2010) and cause severe damage to aquatic animals due to their chemical stability and non-biodegradable as compared to other aquatic organic pollutants (Wepener *et al.,* 2001; Begam, 2004; Ambreen *et al*., 2015). Heavy metals like zinc, cobalt, lead, arsenic, cadmium, manganese and chromium and present at very high concentrations affecting aquatic life, especially fish (Patil and Shrivastava, 2003).

Contamination of heavy metals not only disturbed the physiology and survival of the individual but also cause genetic disorders (Russo *et al*., 2004). Among aquatic animals fish are very good indicator of metallic ion pollution in water and also reflect the biological effects of environmental pollution. In recent years, contamination of aquatic ecosystem was attracted the attention of researchers around all over the world (Dutta and Dalal,

2008; Agah *et al*., 2009).

Cobalt is the 33rd most abundant (ATSDR, 2004) oligo-element which is crucial for the formation of vitamin B12 and other cobalamines (Garoui *et al.,* 2011). It is naturally present in water as low concentrations. Cobalt is a genotoxicant that can cause chromosomal fragmentation and DNA damage (Figgitt *et al.,* 2010).

Metals can cause gene amplification (Rossman and Wolosin, 1992), cellular transformation (Huang *et al.,* 1995), DNA-protein cross-links and DNA strand breakage (Gebel *et al.,* 1998). Genotoxicity of heavy metals can be effectively evaluated in aquatic environment by using Micronucleus test, is a reliable technique for assessing DNA damage, on exposed sentinel species (Frenzilli *et al*., 2009; Bolognesi and Hayashi, 2011). The micronucleus test also detects both aneugenic and clastogenic effects (Heddle *et al.,* 1991). Application of the erythrocytes (fish) micronucleus assay in the measurement of genotoxic compounds, acts as a valuable biomarker in field surveys, in monitoring studies and in comparing different levels of pollutants (Omar *et al.,* 2012) and acts as a bioindicator for environmental mutagenicity studies over other cytogenetic techniques (Anbumani and Mohankumar, 2011). Genotoxic compounds represent major ecological challenge because they may lead to unusual disorders that could be transmitted to the next generations (Haldsrud and Krokje, 2009). In current study, formation of micronucleus and binucleated nuclei in peripheral erythrocytes of *Cirrhinus mrigala* exposed to various sub-lethal concentrations of cobalt for different time intervals was evaluated.

MATERIALS AND METHODS

Experimental Layout

Cirrhinus mrigala was chosen as an experimental fish. The fingerlings of freshwater fish *Cirrhinus mrigala,* were purchased from Fish Seed Hatchery, Faisalabad. This research work was conducted in the wet laboratory at Fisheries Research Farm, Department of Zoology, Wildlife and Fisheries, University of Agriculture Faisalabad. Before the start experiment, fish were acclimatized to laboratory condition for two weeks. After acclimation, fish were transferred to 100-liter glass aquarium. Ten fishes were kept in each aquarium. The 96-hr LC_{50} of cobalt for *C. mrigala* was determined as 117.39 mgL^{-1} (Batool and Javed, 2015). Fish were exposed to the various sub-lethal concentration of cobalt metal such as, $2/3^{rd}$ (78.26 mgL^{-1}), $1/3^{rd}$ (39.13 mgL^{-1}), $1/4^{th}$ (29.35 mgL^{-1}) and $1/5^{th}$ (23.48 mgL^{-1}) for one month. The sampling was done after 10 days interval. A group (n=10) of fish were also kept in metals free water known as negative control (NC) and cyclophosphamide was used as positive control (PC).

Physico-chemical Parameters

The water pH (7.5), temperature (30°C) and total hardness (250 mgL^{-1}) of test media were kept constant during whole the experiment. The other physico-chemical parameters of water such as total ammonia, hardness, calcium, magnesium, sodium, potassium and CO_2 were also maintained (A. P. H. A., 1998).

Micronucleus Test

The sample of blood was collected from caudal vein of fish. A drop of blood was directly smeared on slide and air dried. After that smear was fixed in methol for 10 minute and stained with wright-giemsa stain for 8 minute (Barsiene *et al*., 2004). The frequency of micronuclei and bi-nucleated erythrocytes were evaluated (per 1,000 cell) by scoring at a 1000 X magnification by using a binocular microscope (LABOMED CX_{R3}) under oil emersion (100 X) lens. A total of 2,000 erythrocyte with intact cellular and nuclear membranes were examined for *C. mrigala*. Blind scoring of micronuclei and binucleated nuclei were performed on coded slides. Ovoid or round shaped non-refractory particles with the color and structure similar to chromatin, with a diameter equaling $1/3^{rd}$ or less of the main nucleus and clearly detached from it were interpreted as micronuclei. In general, the color intensity of MN was the same or lower than of the main nuclei using criteria described by Fenech *et al.* (2009).

$$MN\% = \frac{\text{Number of cells containing micronucleus}}{\text{Total number of cells counted}} x100$$

Statistical Analysis

Data obtained from control and exposed fish groups were statistically compared by using the non-parametric Mann-Whitney U-test was performed. Regression analysis was also applied to find out the relationships among various parameters under study.

RESULTS

The exposure of different sub-lethal concentrations of cobalt induced significant ($p<0.01$) formation of micronuclei and binucleated nuclei in peripheral erythrocytes of fish as compared to control. Results showed a duration and concentration dependent effect of cobalt on peripheral erythrocytes. Comparison among concentrations showed that significantly maximum frequency of micronuclei and binucleated nuclei were observed at $2/3^{rd}$ concentration of cobalt. In present study cyclophosphamide (CP) was used as positive control. It was also concluded that CP had more potential to induce nuclear abnormalities in peripheral erythrocytes of fish as compared to cobalt. Table 1-2 shows the data regarding to nuclear abnormalities in peripheral erythrocytes of *C. mrigala* exposed to sub-lethal concentration of cobalt.

Table 3 shows the micronuclei and binucleated nuclei frequency in peripheral blood erythrocytes of *C. mrigala* had significantly higher dependence on cobalt concentration. The partial regression coefficients for micronuclei and binucleated nuclei frequency were positive and significant at $P<0.05$. Moreover, the high value of R2 for micronuclei and binucleated nuclei frequency predicts significantly high reliability of these regression models.

Table 1. Micronuclei frequency (Mean±SD) in peripheral erythrocytes of *Cirrhinus mrigala* expose to various sub-lethal concentrations of cobalt.

Duration of Exposure	Negative Control	Positive Control	Sub-lethal Concentrations of Cobalt				*Overall Means
			$2/3^{rd}$ of LC_{50}	$1/3^{rd}$ of LC_{50}	$1/4^{th}$ of LC_{50}	$1/5^{th}$ of LC_{50}	
10 days	0.10±0.01f	1.50±0.05a	1.25±0.05b	0.95±0.03c	0.65±0.02d	0.50±0.01e	0.83±0.54C
20 days	0.15±0.02f	2.10±0.06a	1.80±0.03b	1.35±0.03c	1.00±0.01d	0.80±0.05e	1.20±0.76B
30 days	0.15±0.02f	2.55±0.05a	2.25±0.03b	1.75±0.02c	1.45±0.04d	1.20±0.02e	1.58±0.94A
Overall Means	0.13±0.03F	2.05±0.53A	1.77±0.50B	1.35±0.48C	1.03±0.40D	0.83±0.35E	

Means with similar letters in a single row or *column is statistically similar at $p<0.05$. ANOVA followed by HSD Tukey test.

Table 2. Binucleated nuclei (Mean±SD) in peripheral erythrocytes of *Cirrhinus mrigala* expose to various sub-lethal concentrations of cobalt.

Duration of Exposure	Negative Control	Positive Control	Sub-lethal Concentrations of Cobalt				*Overall Means
			$2/3^{rd}$ of LC_{50}	$1/3^{rd}$ of LC_{50}	$1/4^{th}$ of LC_{50}	$1/5^{th}$ of LC_{50}	
10 days	0.05±0.01f	0.76±0.04a	0.55±0.01b	0.35±0.02c	0.21±0.02d	0.15±0.03e	0.34±0.28C
20 days	0.10±0.01f	1.20±0.04a	0.90±0.03b	0.65±0.02c	0.50±0.01d	0.40±0.06e	0.63±0.41B
30 days	0.10±0.01f	1.55±0.03a	1.15±0.04b	0.95±0.03c	0.70±0.03d	0.55±0.04e	0.83±0.54A
Overall Means	0.08±0.03F	1.17±0.40A	0.87±0.30B	0.65±0.30C	0.47±0.25D	0.37±0.20E	

Means with similar letters in a single row or *column is statistically similar at $p<0.05$. ANOVA followed by HSD Tukey test.

Table 3. Relationship between concentrations of cobalt metal and frequency of nuclear abnormalities induced in peripheral erythrocytes of *Cirrhina mrigala.*

	Regression	SE	R	R2
Micronuclei Frequency (%)	0.565 + 0.0160 *(conc.)	0.003	0.959	0.920
Binucleated Nuclei (%)	= 0.227 + 0.00853 *(conc.)	0.001	0.958	0.918

Conc.=Concentration; SE: Standard Error; r: Multiple Regression Coefficient; R2: Coefficient of Determination;
* Significant at $P<0.05$

DISCUSSION

Environmental pollution and its effects are very hot issue of societies and living organisms. Therefore, identification of these contaminants and prevention of their environmental dispersion are one of the necessities in this field. Increased concern for environmental health problems resulted in considerable interest in monitoring the contamination level of water bodies. The toxicants that exist in aquatic eco-systems have potential to induce genotoxicity and mutagenesis in organisms (Ohe et al., 2004). DNA integrity is very essential for the maintenance of any cell or individual and passed genetic information to the offspring, the measurement of DNA damage is a key parameter for evaluating the stress induced by pollutants in living creatures (Depledge, 1998). Micronucleus formation has been extensively used to evaluate genotoxic potential of toxicants in many freshwater organisms (Bolognesi and Hayashi, 2011). Micronuclei are very small masses of chromatin material which are formed from broken segment of chromosome or from the chromosomes which could not be transformed into daughter nuclei (Fagr *et al*., 2008).

In present work, genotoxic potential of cobalt metal was observed in peripheral erythrocytes of C. mrigala. Results showed that exposure of cobalt at sub-lethal concentrations significantly induced the micronuclei and binucleated in peripheral erythrocytes of fish. Several authors reported the formation of micronuclei (Olivero et al., 1995; Van-Goethem et al., 1997; De Boeck et al., 2003a; Miller et al., 2001;) by ultrafine metal cobalt and soluble cobalt salts. Many metallic ions act as important genotoxins at particular concentrations due to their ability to bind with thiol groups and induce instability in the spindle formation in the cells (Patra *et al*., 2004). Metals have ability to produce reactive oxygen species (ROS) that would cause oxidation of nucleic acid. Thus, production of ROS and inhibition of DNA repair would lead to oxidative stress, and genomic instability, cause DNA strand breakage in fish (Rossman, 2003; Ventura-Lima *et al.,* 2009).

During present investigation the ability of cobalt to induce micronuclei and other nuclear abnormalities in their peripheral erythrocytes of fish increased with increasing concentration and duration of exposure. Hooftman and Raat (1982) reported the duration-dependent increase in micronuclei induction in peripheral erythrocytes of *Umbra pygmaea* exposed to Ethyl Methane sulfonate. A dose dependent increase in the formation of micronuclei in peripheral erythrocytes of *Heteropneustus fossilis* exposed to mitomycin and paper mill effluents was reported by Das and Nanda (1986). Hoshina *et al.* (2008) reported that frequencies of micronuclei and other nuclear abnormalities induced in peripheral erythrocytes of tilapia were significantly higher due to exposure of petroleum refinery effluents containing barium, chromium, cadmium and copper iron, cobalt, nickel, zinc and lead as compared to the control fish. Bolognesi *et al.* (1999) reported the mutagenic potential of heavy metals (cadmium, copper and mercury). Ahmed *et al*. (2011) observed concentration depended on increase in micronuclei frequency in the erythrocytes of *Oreochromis mossambicus*. In *Channa punctatus*, the frequency of micronuclei was found significantly higher due to heavy metals exposure as compared to control fish (Patowary *et al.,* 2012). Summak *et al.* (2010) reported significantly positive correlation (r=0.980) between metal concentrations and frequency of nuclear abnormalities in *Oreochromis niloticus* also. There is little information about cobalt induced genotoxicity in fish however, some work was done on other vertebrates. Hussien and Mohamed (2018) reported the increased number of micronucleated in vertebrates due to Co3O4NPs administration. Hassan et al. (2010) investigated the clear interaction of cobalt and zinc exposure with number of abnormal micronucleus in Oreochromis niloticus captured along the river. Razzaq *et al*. (2021) also confirmed the duration and dose specific induction of MN and deshaped nuclei in *Labeo rohita* exposed to Co+Cr Mixture.

CONCLUSION

In current study it was concluded that the cobalt had ability to induced nuclear abnormalities in fish erythrocytes. It was also demonstrated that micronucleus assay is a useful technique for detecting the genotoxic pollutants in aquatic environment.

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