Punjab University Journal of Zoology

OPEN ACCESS

36(2): 153-158 (2021)
https://dx.doi.org/10.17582/journal.pujz/2021.36.2.153.158

Research Article

# Micronucleus Assay as a Biomarker to Diagnose Lead, Chromium and Cadmium Induced Genotoxicity in Erythrocytes of Carnivorous Fish, *Wallago attu*

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**Article History**
Received: February 22, 2021
Revised: July 19, 2021
Accepted: August 13, 2021
Published: October 08, 2021

**Authors' Contributions**
SA supervised the study and guided the author in planning the research work. HN helped in laboratory work. ZA, TA and UN executed the research work. MB helped in statistical analysis. AAQ assisted in manuscript writing.

**Keywords**
Blood, Nuclear abnormalities, Metal toxicity, Carnivorous fish

**Abstract** | Heavy metals discharged from agriculture and industries into water bodies are a significant cause of water contamination. This increase in heavy metals cause genetic damage in exposed species like fish. Therefore, this study focused on the genotoxic effects of lead (Pb), chromium (Cr) and cadmium (Cd) on nuclear abnormalities in peripheral erythrocytes of *Wallago attu* were assessed by micronucleus assay. Fish were exposed to different concentrations ($1/3^{rd}$, $1/4^{th}$, $1/5^{th}$and $1/7^{th}$ of $LC_{50}$) of Pb, Cr and Cd, separately, for three weeks. Blood sample of fish was collected to see the micronuclei (MN) and deshape nuclei (DN) in peripheral erythrocytes of fish after one-week interval. Fish *W. attu* showed significant induction of MN and DN in blood due to metals (Pb, Cr and Cd) exposure. It was noted that Pb and Cd exposure caused significant formation of MN and DN in RBCs of fish throughout the experimental period. However, Cr treated fish showed higher formation of MN and DN during initial two weeks of exposure after that both were decreased. The toxic potential of different concentration of Pb, Cr and Cd induced genotoxicity followed order: $1/3^{rd}$>$1/4^{th}$>$1/5^{th}$>$1/7^{th}$ of $LC_{50}$. The metal specific response of fish followed the trend: Pb>Cr>Cd. Statistical analysis revealed that different concentrations and duration of Pb, Cr and Cd have significant effect on the frequency of micro-nucleated erythrocytes. The inferences of this study will be helpful in monitoring aquatic ecosystems using fish biomarkers.

**Novelty Statement** | The study is novel for detecting the genetic damage at cellular level of fish (*Wallago attu*) caused by heavy metals and industrial toxicants in water.

**To cite this article:** Abdullah, S., Naz, H., Abbas, Z., Nazir, U., Basharat, M., Ahmed, T. and Qazi, A.A., 2021. Micronucleus assay as a biomarker to diagnose lead, chromium and cadmium induced genotoxicity in erythrocytes of carnivorous fish, *Wallago attu*. *Punjab Univ. J. Zool.*, **36**(2): 153-158. https://dx.doi.org/10.17582/journal.pujz/2021.36.2.153.158

## Introduction

Metal pollution of freshwater bodies such as streams, rivers, and lakes has been recorded around the world due to its harmful effects not only on humans but also on other living species. Heavy metal pollution severely deteriorates the quality of environment by interfering with ecological equilibrium of an aquatic ecosystem (Farombi *et al*., 2007). In aquatic bodies, fish are the animal which cannot escaped from harmful impact of these heavy metals and that's the reason fish is commonly used as bio-indi-

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cator of metals toxicity in water (Agah *et al.*, 2009). The direct contact of fish with environment makes them more vulnerable to all chemical and physical modifications occurred due to pollution in water (Ip *et al.*, 2005). Fish shows behavioral, physiological and molecular alteration upon exposure to contaminants (Navarro and Martinez, 2014; Prado *et al.*, 2014).

Chromium (Cr) is known to be a toxic metal for health and environment due to its high mobility and solubility (Xu and Wang, 2012). Due to its non-biodegradable and bioaccumulation property, it is toxic to living organisms. The indiscriminate discharge of Cr from industries to aquatic bodies severely affects the growth and survival of aquatic life particularly fish (Mishra and Mohanty, 2008). Lead (Pb) is a common, growing and subtle environmental waste material that provokes a wide range of behavioral, biochemical and physiological impairments (Patra *et al.*, 2001; El-Magd *et al.*, 2016). Lead toxicity is associated with the formation of reactive oxygen species (ROS) that cause reduction in antioxidant defense systems of cell and damage to DNA (El-Ashmawy *et al.*, 2006). According to Hong *et al.* (2007) Pb+2 can directly bind with DNA by a covalent bond. Some non-essential heavy metals like cadmium (Cd) can induce harmful effects in aquatic animals even at very low dose (Bertin and Averbeck, 2006; Cambier *et al.*, 2010). Cd have ability to bio-accumulate and induce toxicity in fish by changing their physiology, histology, osmoregulation, reproduction, immune and enzyme response (Dang and Wang, 2009; Garcia-Santos *et al.*, 2011; Guardiola *et al.*, 2013; Li *et al.*, 2014).

The genotoxic (DNA damage) affects of metals on aquatic animals can effectively be examined by the application of simple and reliable techniques such as Micronucleus (Frenzilli *et al.*, 2009; Bolognesi and Hayashi, 2011) and nuclear abnormalities (notched nuclei, blebbed, lobbed, budding, fragmenting nuclei and bi-nucleated cells) assays both are most commonly used tests due to their verified suitability for aquatic species (Kirschbaum *et al.*, 2009). The presence of both MN and NAs in RBCs has been widely used as a biomarker of environmental genotoxicity in fish exposed to complex mixtures and single substances (Carrola *et al.*, 2014; Seriani *et al.*, 2015).

*Wallago attu* is a valuable fish species for studying the toxicological effects due to its higher adaptation and easy reproduction. It is native species of Pakistan and can be used as sentinel organisms to evaluate the geno-toxic effects of metals even at a very low dose. Information regarding metal induced genotoxicity in carnivorous fish is very limited. Therefore, the main objective of current research was to assess the metal-, concentration-, and time-specific response of nuclear anomalies in *Wallago attu* erythrocytes exposed to chronic metals.

## Materials and Methods

*Fish sampling and experimental layout*

Carnivore fish, *Wallago attu* were acquired from natural habitat, and were shifted to the Fisheries Research Farm, University of Agriculture Faisalabad. Fish were acclimatized to laboratory conditions for 14-day. A group of fish (n=10) were kept in each glass aquarium having 100-liter water capacity. Total five aquaria were used for each metal. Fish were exposed to different sub-lethal concentrations viz. 1/7$^{th}$, 1/5$^{th}$, 1/4$^{th}$ and 1/3$^{rd}$ of $LC_{50}$ (96-h) of lead (Pb), chromium (Cr) and cadmium (Cd), separately, for three weeks. The $LC_{50}$ (96-h) values of Pb, Cr and Cd for *W. attu* were calculated as 25.08, 61.38 and 32.94 $mgL^{-1}$, respectively (Batool *et al.*, 2014, 2018). The pure chloride compound of cadmium and lead, and nitrate compound of chromium were used to make metals solutions. The cyclophosphamide was injected into the positive control (PC) fish however, the negative control (NC) fish were kept in water without any treatment. Some water quality parameters including water temperature (30°C), hardness (250mg/L) and pH (7.5) were kept stable during the experimental trail. Fish caudal vein was ruptured to collect the blood sample after 7 days of exposure.

*Micronucleus assay*

Fish caudal vein was ruptured and blood was instantly smeared on slide and dried in air for few minutes. Methanol was applied to fix the smear and allowed to dry for 10 minutes. The blood smear was stained with wright-giemsa (Sigma Aldrich) stain for 8 minute (Barsiene *et al.*, 2004). The erythrocytes with micronuclei and de-shaped nuclei were counted (per 1,000 cell) by using binocular microscope (1000 X magnification) for PC, NC and metals (Pb, Cr and Cd) stressed fish. Blind counting of abnormal nuclei was done by adopted the criteria of Fenech *et al.* (2003).

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

*Data analysis*

Data obtained from this experiment was subject to statistical analyses. Analysis of variance was done to determine the significance of the effect of concentration and period of treatment on NAs formation. All statistical analyses were performed by using statistix 8.0 version software.

## Results and Discussion

According to Souza and Fontanetti (2006) any biological, chemical and physical agent can stimulate the formation of micronuclei by interacting with non-genomics constituents (mitotic apparatus) of cell and, resulting in

failure of segregation of chromosome. The micronuclei assay is used to visualise and quantify all the abnormalities induced by these agents. At specific concentration, metals ions are also noted as geno-toxicants because they can bind with thiol groups and cause disturbance if the formation of spindle in cells (Patra *et al*., 2004).

Fish *W. attu* showed significant induction of MN and DN in blood due to metals (Pb, Cr and Cd) exposure. It was noted that Pb and Cd exposure caused significant formation of MN and DN in RBCs of fish throughout the experimental period while in case of Cr higher formation were noted during initial two weeks of treatment after that same were decreased in third week. The concentration specific response of MN and DN in RBCs of fish exposed to metals followed the order: PC>1/3$^{rd}$>1/4$^{th}$>1/5$^{th}$>1/7$^{th}$>NC (Tables 1, 2 and 3). The metal specific response of fish followed the trend: Pb>Cr>Cd. The variation in genotoxic response to different chemicals is primarily related to metabolism and other pharmacokinetic factors in animals (Al-Sabti and Metcalfe, 1995). While each metal has its own toxicity mechanism but some are common including mimicry, DNA or protein adduct formation, and oxidative stress. Heavy metals forms produce reactive oxygen species (ROS), which cause oxidative changes in DNA, resulting in abnormal gene expression and carcinogenesis (Ballatori, 2002). Razzaq *et al*. (2021) reported the concentration and duration dependent induction of MN and DN in RBCs of *L. rohita* due to cobalt+chromium mixture exposure.

**Table 1: Nuclear abnormalities in erythrocytes of *Wallago attu* expose to cadmium.**

| Parameters | Sampling time | NC | PC | Sub-lethal concentrations | | | |
|---|---|---|---|---|---|---|---|
| | | | | 1/3$^{rd}$ | 1/4$^{th}$ | 1/5$^{th}$ | 1/7$^{th}$ |
| MN | 1$^{st}$ Week | 0.0 | 15 | 13 | 10 | 6 | 5 |
| | 2$^{nd}$ Week | 0.0 | 18 | 15 | 11 | 7 | 7 |
| | 3$^{rd}$ Week | 0.0 | 20 | 18 | 14 | 10 | 8 |
| Frequency (%) | 1$^{st}$ Week | 0.00±0.00C$^{f}$ | 0.75±0.05C$^{a}$ | 0.65±0.04C$^{b}$ | 0.50±0.01C$^{c}$ | 0.30±0.02C$^{d}$ | 0.25±0.01C$^{e}$ |
| | 2$^{nd}$ Week | 0.00±0.00B$^{f}$ | 0.90±0.07B$^{a}$ | 0.75±0.01B$^{b}$ | 0.55±0.06B$^{c}$ | 0.35±0.01B$^{d}$ | 0.35±0.02B$^{e}$ |
| | 3$^{rd}$ Week | 0.00±0.00A$^{f}$ | 1.00±0.01A$^{a}$ | 0.90±0.05A$^{b}$ | 0.70±0.01A$^{c}$ | 0.50±0.06A$^{d}$ | 0.40±0.03A$^{e}$ |
| DN | 1$^{st}$ Week | 0.0 | 19 | 17 | 10 | 6 | 5 |
| | 2$^{nd}$ Week | 0.0 | 26 | 25 | 18 | 12 | 9 |
| | 3$^{rd}$ Week | 0.0 | 28 | 28 | 22 | 16 | 11 |
| Frequency (%) | 1$^{st}$ Week | 0.00±0.00C$^{f}$ | 0.95±0.06C$^{a}$ | 0.85±0.08C$^{b}$ | 0.50±0.05C$^{c}$ | 0.30±0.03C$^{d}$ | 0.25±0.01C$^{e}$ |
| | 2$^{nd}$ Week | 0.00±0.00B$^{f}$ | 1.30±0.08B$^{a}$ | 1.25±0.07B$^{b}$ | 0.90±0.01B$^{c}$ | 0.90±0.07B$^{d}$ | 0.45±0.06B$^{e}$ |
| | 3$^{rd}$ Week | 0.00±0.00A$^{f}$ | 1.40±0.08A$^{a}$ | 1.40±0.08A$^{b}$ | 1.10±0.07A$^{c}$ | 0.80±0.06A$^{d}$ | 0.55±0.04A$^{e}$ |

Small alphabet superscripts show the difference between treatments within the same row While capital alphabet shows significant (P < 0.05) among different durations of exposure within the same column.

**Table 2: Nuclear abnormalities in erythrocytes of *W. attu* exposed to lead.**

| Parameters | Sampling time | NC | PC | Sub-lethal concentrations | | | |
|---|---|---|---|---|---|---|---|
| | | | | 1/3$^{rd}$ | 1/4$^{th}$ | 1/5$^{th}$ | 1/7$^{th}$ |
| MN | 1$^{st}$ Week | 0.00 | 20 | 22 | 18 | 13 | 11 |
| | 2$^{nd}$ Week | 0.00 | 23 | 27 | 19 | 15 | 9.0 |
| | 3$^{rd}$ Week | 0.00 | 28 | 33 | 25 | 20 | 17 |
| Frequency (%) | 1$^{st}$ Week | 0.00±0.01A$^{f}$ | 2.0±0.1C$^{b}$ | 2.2±0.1C$^{a}$ | 1.8±0.1C$^{c}$ | 1.3±0.01C$^{d}$ | 1.1±0.01B$^{e}$ |
| | 2$^{nd}$ Week | 0.00±0.1A$^{f}$ | 2.3±0.1B$^{b}$ | 2.7±0.01B$^{a}$ | 1.9±0.1B$^{c}$ | 1.5±0.1B$^{d}$ | 0.9±0.01C$^{e}$ |
| | 3$^{rd}$ Week | 0.00±0.01A$^{f}$ | 2.8±0.01A$^{b}$ | 3.3±0.01A$^{a}$ | 2.5±0.1A$^{c}$ | 2.0±0.01A$^{d}$ | 1.7±0.1A$^{e}$ |
| DN | 1$^{st}$ Week | 0.00 | 25 | 35 | 26 | 18 | 14 |
| | 2$^{nd}$ Week | 0.00 | 29 | 28 | 26 | 19 | 10 |
| | 3$^{rd}$ Week | 0.00 | 33 | 40 | 31 | 25 | 16 |
| Frequency (%) | 1$^{st}$ Week | 0.0±0.01A$^{f}$ | 2.5±0.01C$^{c}$ | 3.5±0.01B$^{a}$ | 2.6±0.01B$^{b}$ | 1.8±0.10C$^{d}$ | 1.4±0.01B$^{e}$ |
| | 2$^{nd}$ Week | 0.0±0.10A$^{f}$ | 2.9±0.01B$^{a}$ | 2.8±0.10C$^{b}$ | 2.6±0.01B$^{c}$ | 1.9±0.01B$^{d}$ | 1.0±0.10C$^{e}$ |
| | 3$^{rd}$ Week | 0.0±0.01A$^{f}$ | 3.3±0.10A$^{b}$ | 4.0±0.01A$^{a}$ | 3.1±0.01A$^{c}$ | 2.5±0.10A$^{d}$ | 1.6±0.10A$^{e}$ |

Small alphabet superscripts show the difference between treatments within the same row While capital alphabet shows significant (P < 0.05) among different durations of exposure within the same column.

**Table 3: Nuclear abnormalities in erythrocytes of *Wallago attu* exposed to chromium.**

| Parameters | Sampling time | NC | PC | Sub-lethal concentrations | | | |
|---|---|---|---|---|---|---|---|
| | | | | 1/3rd | 1/4th | 1/5th | 1/7th |
| MN | 1st Week | 0.0 | 10 | 8 | 6 | 6 | 4 |
| | 2nd Week | 0.0 | 15 | 14 | 7 | 8 | 5 |
| | 3rd Week | 0.0 | 11 | 10 | 8 | 6 | 5 |
| Frequency (%) | 1st Week | $0.00\pm0.01C^{f}$ | $0.50\pm0.04C^{a}$ | $0.40\pm0.04C^{b}$ | $0.30\pm0.03C^{c}$ | $0.30\pm0.02C^{d}$ | $0.20\pm0.01C^{e}$ |
| | 2nd Week | $0.00\pm0.03A^{f}$ | $0.75\pm0.01A^{a}$ | $0.70\pm0.01A^{b}$ | $0.35\pm0.04A^{c}$ | $0.40\pm0.05A^{d}$ | $0.25\pm0.05A^{e}$ |
| | 3rd Week | $0.00\pm0.02B^{f}$ | $0.55\pm0.01B^{a}$ | $0.50\pm0.07B^{b}$ | $0.40\pm0.06B^{c}$ | $0.30\pm0.05B^{d}$ | $0.25\pm0.04B^{e}$ |
| DN | 1st Week | 0.00 | 12 | 11 | 9 | 7 | 6 |
| | 2nd Week | 0.00 | 20 | 13 | 12 | 10 | 8 |
| | 3rd Week | 0.00 | 14 | 18 | 15 | 13 | 10 |
| Frequency (%) | 1st Week | $0.0\pm0.01A^{f}$ | $0.60\pm0.03B^{a}$ | $0.55\pm0.01C^{b}$ | $0.45\pm0.02C^{c}$ | $0.35\pm0.01C^{d}$ | $0.30\pm0.01C^{e}$ |
| | 2nd Week | $0.0\pm0.10A^{f}$ | $2.00\pm0.01A^{a}$ | $1.30\pm0.01B^{b}$ | $0.60\pm0.03B^{c}$ | $0.50\pm0.02B^{d}$ | $0.40\pm0.02B^{e}$ |
| | 3rd Week | $0.0\pm0.01A^{f}$ | $0.70\pm0.02B^{b}$ | $1.80\pm0.03A^{a}$ | $0.75\pm0.03A^{c}$ | $1.30\pm0.01A^{d}$ | $0.50\pm0.01A^{e}$ |

Small alphabet superscripts show the difference between treatments within the same row While capital alphabet shows significant ($P < 0.05$) among different durations of exposure within the same column.

Similar results were observed by Kousar and Javed (2015) who noted the copper induced concentration specific formation of MN and NAs frequency in RBCs of three fish species exposed for 30-days. Similarly, *Oreochromis niloticus* showed higher frequency of MN and NAs in RBCs during initial hours of Cd and Zn exposure after that both were decreased in duration dependent manner (Abu-Bakar *et al*., 2014). Rasal *et al*. (2011) also reported the Cr induced MN in blood of *Labeo rohita*. Kousar and Javed (2016) also documented the dose specific induction of MN and NAs in four species of fish exposed to arsenic for a period of 30 days. Al-Tamimi *et al*. (2015) noted the progressive increase in MN formation in erythrocytes of *Cyprinus carpio* as the concentration of copper exposure increased. Alimba *et al*. (2015) also noted the time dependent formation of MN and NAs frequency in blood of *Clarias gariepinus* exposed to effluent from Bodija, Nigeria for 7-, 14- and 28-day. Ashmawy *et al*. (2015) also documented the formation of MN in RBCs of *Oreochromis niloticus* exposed to different concentrations of cadmium. Hussain *et al*. (2018) observed the 96% reduction in *Labeo rohita* population due to MN and NA induction captured from polluted River Chenab, Bhawana, Faisalabad. Mahboob *et al*. (2014) also confirmed the concentration dependent increase in MN frequency of *Clarias gariepinus* exposed to mercuric chloride for 7-day.

The different concentrations of lead nitrate have ability to induce of MN in *Channa punctatus* erythrocytes (Choudhary *et al*., 2012). According to Jindal and Verma (2015) RBCs of *Labeo rohita* showed significant formation of MN and NAs exposed to Cd for different time. Ahmed *et al*. (2013) noticed the dose reliant increase in MN frequency in RBCs of *Heteropneustes fossilis* during initial hours of Cr exposure after that MN formation was decreased. Monteiro *et al*. (2011) also reported the lead induced NAs in blood of *Prochilodus lineatu*.

## Conclusions and Recommendations

It was concluded that metals have ability to induce geno-toxicity in erythrocytes of *W. attu*. It was also concluded that the measurement of genotoxic damage by micronucleus assay is the most promising method for evaluating the pollution load in aquatic ecosystem which effects the population of fish species like *W. attu*. More research is needed, with a focus on priority pollutants and other ecological receptors, especially those used as human food.

*Conflict of interest*

The authors have declared no conflict of interest.

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