The Journal of Animal & Plant Sciences, 26(3): 2016, Page: 674-679
ISSN: 1018-7081

EFFECTS OF 30 DAY SUB-LETHAL EXPOSURE OF CADMIUM AND LEAD MIXTURE ON DNA DAMAGE IN FISH

F. Ambreen[1] and M. Javed[2]

Department of Zoology, Wildlife & Fisheries, University of Agriculture, Faisalabad-38040, Pakistan
Corresponding Author e-mail: faiza_zool@yahoo.com

ABSTRACT

The present study was undertaken to examine the DNA damage induced by binary metal mixture (cadmium-lead) in peripheral blood erythrocytes of freshwater carp, *Cyprinus carpio* by using Comet bioassay. The 96-hr LC_{50} value of mixture was estimated for 180-old fingerlings of *Cyprinus carpio* in a static system and then four sub-lethal concentrations viz. SL-I (10.42mgL^{-1}), SL-II (7.81mgL^{-1}), SL-III (6.25mgL^{-1}) and SL-IV (5.21mgL^{-1}) were calculated and fish were exposed to these concentrations, separately in glass aquaria for 30 days along with negative and positive control at constant water temperature (30ºC), pH (7.75) and total hardness (225mgL^{-1}). Peripheral erythrocytes were sampled after 30 day exposure for DNA damage assessment. Statistically significant effects were observed at sub-lethal concentrations in-terms of percentage of DNA damage, cumulative tail lengths (µm) and genetic damage index. Concentration dependent response was observed in fish erythrocytes with induction of maximum DNA damage, due to positive control, followed by at highest concentration (SL-I) of mixture. This study also concluded that comet bioassay can be used for in-vivo experiments, using fish as a model for the screening of genotoxic and mutagenic pollutants in aquatic environment.

Key words: Carp, Comet bioassay, DNA damage, Metal Mixture, Sub-lethal.

INTRODUCTION

Pollution of aquatic environment due to heavy metals not only endangers the physiology and survival of the organisms but can also induce genetic alterations which lead to mutation and cancer (Russo *et al*., 2004). Naturally, heavy metals present in water due to various processes such as weathering and erosion (Damodara *et al*., 2008) but anthropogenic activities are the main cause of heavy metal pollution in the aquatic environments. Some metals are essential for living organisms in only trace amounts (e.g. cobalt, copper and zinc, iron) while others like cadmium, lead, arsenic, mercury and chromium have no significant biological roles (Javed, 2012; Ambreen *et al*., 2015). Cadmium is a non-essential heavy metal with carcinogenic, teratogenic and mutagenic effect on aquatic organisms. Integrity of freshwater ecosystems is heavily threatened by cadmium due to greater sensitivity of aquatic organisms than that of mammals (Burger, 2008; Jia *et al*., 2011). Among different heavy metals lead is most common in earth crust, used for thousands of years. Inorganic and organic lead enters in the environment through several different ways and poses diverse effect on organisms. Lead can mimick the essential elements like magnesium, iron, calcium and zinc, increased the incorporation of erroneous nucleotides in which it implicated as a co-carcinogen and effect on DNA repairing mechanisms (Godwin, 2001; Frascasso *et al*., 2002).

Although each metal have unique mechanism of toxicity but there are some common mechanism which include mimicry, adduct formation with DNA or protein and oxidative damage. Generation of reactive oxygen species is induced by heavy metals in their ionic forms resulting in oxidative modifications of DNA, inducing aberrant gene expression and carcinogenesis (Ballatori, 2002; Baselt, 2004). Cocktails of compounds create huge problem as toxicity of a mixture is not easily linked to individual toxicities of components in the mixture (Fernandez-Alba *et al*, 2001). Much work on acute toxicity of individual metals for fish was done by many scientists (Abdullah *et al*., 2007; Azmat *et al*., 2012; Javed, 2012) however, the genotoxic effects of metals in the form of mixture are scant in the literature. Therefore, present study was aimed to evaluate the DNA damage in freshwater carp, *Cyprinus carpio* after 30-day exposure to sub-lethal concentrations of cadmium and lead mixture.

MATERIALS AND METHODS

Experimental Fish and Chemicals: The fingerlings of freshwater common carp, *Cyprinus carpio* were purchased from the local outlets and transported to Fisheries Research Farms, University of Agriculture, Faisalabad, Pakistan. Fish fingerlings of 180-day old (similar weight) were acclimatized under laboratory conditions in cemented tanks for two weeks prior to start the experiment and fed with pelleted diet twice a day (34% Digestible Protein and 3 Kcal/g Digestible Energy).

Appropriate quantity of technical grade $CdCl_2$ and $PbCl_2$ were separately dissolved in deionized water for stock solutions preparation while binary mixture of both metals was prepared by its further dilution on ions equivalence basis (1:1 ratio).

Comet Bioassay: Healthy fingerlings of *Cyprinus carpio* were exposed to four test concentrations (10.42, 7.81, 6.25 and 5.21mgL^{-1}) separately, in glass aquaria having seventy litter water capacity. These exposure concentrations were selected on the basis of 96-hr LC_{50} value (31.25mgL^{-1}) of cadmium-lead mixture on test animal. Based on this 96-hr LC_{50} value four sub-lethal concentrations viz. SL-I (1/3rd of LC_{50}), SL-II (1/4th of LC_{50}), SL-III (1/5th of LC_{50}) and SL-IV (1/6th of LC_{50}) were calculated and used for in vivo genotoxicity experiments. Simultaneously, one group of fish was maintained in metal mixture free water considered as "Negative Control" while cyclophosphamide was used as "Positive Control". The fish were fed daily with small quantity of nutritious food (34% Digestible Protein and 3 Kcal/g Digestible Energy). Water temperature (30 ºC), pH (7.75) and total hardness (225mgL^{-1}) were kept constant throughout the experiment. The peripheral blood erythrocytes were sampled after 30 day exposure to four different test concentrations and controls, and subjected to Comet bioassay. Whole experiment was conducted with three replications for each sub-lethal concentration. Peripheral blood erythrocytes were sampled from caudal vein of fish, immediately transferred in eppendorf and initially treated with anticoagulants (Kousar and Javed, 2015). Comet bioassay was performed as three layer procedure, followed by lysis, electrophoresis and staining by using methods of Singh *et al*. (1988). Two slides per specimen were prepared, scored randomly and analyzed by using an image analysis system attached to Epi-Fluorescence microscope (N-400M, American Scope; USA) equipped with light source of mercury short arc reflector lamp filters for ethidium bromide at 400X magnification and low lux camera (MD-800, USA).

The length of DNA migration in the comet tail is an estimate of DNA damage. The DNA damage was quantified by visual classification of cells into the five categories "comets" corresponding to the tail lengths as undamaged (Type 0); low level damage (Type I); medium level damage (Type II); high level damage: (Type III) and complete damage (Type IV). The cells with no DNA damage possess intact nuclei without a tail, while cells having damaged DNA showed comet like appearance. The extent of DNA damage was examined as the mean percentage of cells with medium, high and complete damaged DNA, which was calculated as the sum of cells with Types II+ III + IV. TriTek CometScore ™ software was used to measure the comet tail length of damaged cells (Jose *et al*., 2011) and cumulative tail length (µm) was obtained by adding the tail length of all examined cells (n = 50/replicate). From the arbitrary values assigned to the different categories (from Type=0 to Type IV) a genetic damage index (GDI) was calculated by using the following formula:

$$GDI = \frac{(\text{Type I}) + 2(\text{Type II}) + 3(\text{Type III}) + 4(\text{Type IV})}{\text{Type 0} + \text{Type I} + \text{Type II} + \text{Type III} + \text{Type IV}}$$

Statistical Analyses of Data: Means were compared for statistical differences through Tukey's Student Newnan-Keul test by using the MSTATC computer software (Steel *et al*., 1996). A p-value less than 0.05 were considered as statistically significant.

RESULTS

Table 1 shows the variable frequency of undamaged nuclei (Type 0), damaged nuclei (Type I to IV), percentage of damaged cells (Type II+III+IV), cumulative tail lengths and genetic damage indices at four sub-lethal concentrations of metal mixture induced in the peripheral erythrocytes of *Cyprinus cario* along with negative and positive control after 30-day exposure period. Among all test treatments, proportions of Type 0 cells were observed higher in negative control (92.67±3.06%) while same was lower due to SL-II concentration. Percentage of Type I damaged cells were observed maximum due to SL-III concentration exposure as evident from their mean value of 52.67±1.15%. Percentage of Type II and Type III damaged cells were observed maximum at SL-II and positive control as compared to negative control. Regarding different treatments, Type IV damaged nuclei in peripheral blood erythrocytes of *Cyprinus carpio* followed the sequence: positive control > SL-I > SL-II > SL-III ≥ SL-IV > negative control. However, Type IV damaged cells at SL-III and SL-IV did not vary significantly at $p<0.05$.

The extent of DNA damage was examined as the mean percentage of cells with medium, high and complete damaged DNA, calculated as sum of Type II, III and IV. Statistically significant ($p<0.05$) DNA damage was observed during whole exposure period due to different test concentrations. Regarding different treatments (negative control, positive control, SL-I, SL-II, SL-III and SL-IV) the DNA damage frequency was observed significantly higher ($p<0.05$) due to positive control, followed by that of SL-I, SL-II, SL-III, SL-IV and negative control indicating dose/concentration dependent DNA damage. However, among four test concentrations, SL-III and SL-IV showed non-significant differences for percentage of damaged cells induction in peripheral erythrocytes of *Cyprinus carpio*. *Cyprinus carpio* also respond differently towards DNA damage induction determined in terms of cumulative tail length of comets (µm). Cumulative tail length of comets, induced due to different concentrations of metal mixture, negative and positive controls, ranged between the mean values of

Table 1. DNA damage in peripheral erythrocytes of *Cyprinus carpio* exposed for 30-days to cadmium-lead mixture.

Treatments	Exposure Concentrations (mgL^{-1})	Undamaged Nuclei (%)	Damaged Nuclei (%)				*Damaged Cells (%)	**CTL (µm)	***GDI
		Type-0	Type-I	Type-II	Type-III	Type-IV			
Negative Control	0.00	92.67±3.06^{a}	7.33±3.06^{f}	0.00±0.00^{e}	0.00±0.00^{e}	0.00±0.00^{e}	0.00±0.00^{e}	3.44±0.06^{f}	0.07±0.03^{f}
Positive Control	CP ($20\mu gg^{-1}$)	36.00±4.00^{bc}	11.33±3.06^{ef}	17.33±3.06^{bc}	13.33±1.15^{a}	22.00±2.00^{a}	52.67±1.15^{a}	132.12±0.11^{a}	1.74±0.04^{a}
SL-I	10.42	28.00±4.00^{d}	33.33±3.06^{d}	16.67±3.06^{c}	9.33±1.15^{b}	12.67±1.15^{b}	38.67±4.16^{bc}	120.65±0.11^{b}	1.45±0.10^{bc}
SL-II	7.81	18.00±2.00^{e}	45.33±4.16^{c}	20.67±1.15^{ab}	8.67±1.15^{b}	7.33±1.15^{cd}	36.67±2.31^{c}	120.36±0.05^{c}	1.42±0.04^{c}
SL-III	6.25	30.67±3.06^{cd}	52.67±1.15^{ab}	6.67±3.06^{d}	5.33±1.15^{cd}	4.67±2.31^{d}	16.67±4.16^{d}	107.54±0.08^{d}	1.01±0.11^{de}
SL-IV	5.21	35.33±5.03^{bc}	48.00±2.00^{bc}	8.67±3.06^{d}	3.33±1.15^{d}	4.67±1.15^{d}	16.67±3.06^{d}	100.81±0.14^{e}	0.94±0.09^{e}

* Damaged Cells (%) = Type II+Type+III+TypeIV;

** CTL = Cumulative Tail Length (µm);

*** GDI (Genetic Damage Index) = {(Type I) +2 (Type-II) +3 (Type-III) +4 (Type-IV) / Type-0+Type-I+Type-II+Type-III+Type-IV};

The means with similar letters in a single column for each variable are statistically non-significant at $p<0.05$.

3.44 to 132.12µm with statistically significant differences at $p<0.05$. Among different treatments, positive control caused significantly maximum genetic damage index of 1.74±0.04, followed by that of SL-I, SL-II, SL-III and SL-IV. However, least GDI value was observed in specimens of negative control group.

DISCUSSION

During present experiment, peripheral blood erythrocytes of carp (*Cyprinus carpio*) showed concomitant increase in DNA damage with increase in metallic ion concentrations. Similarly, Cok *et al*. (2011) observed significant DNA damage in common carp, *Cyprinus carpio*, sampled from natural environment by using comet bioassay in terms of tail lengths (µm), tail intensity (%) and tail moment. Low concentration of cadmium can induce oxidative stress in *Cyprinus carpio*. DNA damage percentage, tail length and tail moment were significantly increased even at $0.41mgL^{-1}$ of cadmium exposure in common carp as observed by Jia *et al*. (2011). Concentration dependent increase in DNA damage in the blood cells of *Oreochromis mossambicus* was also observed upon metal exposure (Ahmed *et al*., 2011a) as compared to control. Kousar and Javed (2015) also reported DNA damage in peripheral blood erythrocytes of four fish species, under 30-day exposure to metals. Statistically significant extent of DNA damage in terms of percentage of damage cells, cumulative tail lengths of comets and genetic damage index due to different sub-lethal concentrations of heavy metals were observed in their study. Significantly larger tail lengths of comets in peripheral erythrocytes of *Cobitis elongate* due to toxicity of industrial effluents were also observed by Kopjar *et al*. (2008). Similarly, Pereira *et al*. (2013) observed genotoxic potential of cadmium and aluminum for zebra fish, which exhibited significantly ($p<0.05$) higher DNA damage due to aluminum as compared to cadmium. Present results are in accordance with Ahmed *et al*. (2010) who also observed concentration dependent DNA damage at $2mgL^{-1}$, followed by that of 1.0 and $0.1mgL^{-1}$ of cadmium exposure in freshwater climbing perch, *Anabas testudineus*. Similarly, Ahmed *et al*. (2011b) observed genetic damage due to exposure of three sub-lethal concentrations of lead (0.1, 1.0 and $2.0mgL^{-1}$). They also observed concentration dependent DNA damage in freshwater climbing perch in terms of different comet parameters viz. %age of DNA in comet tail and %age of DNA in comet heads. Cestari *et al*. (2004) and Ferraro *et al*. (2004) reported that breakage of single strand is actually the main reason of lead induced DNA damage. Significantly higher DNA damage in blood cells observed during present study can be comparable with results of Kousar and Javed (2014) that also worked with blood cells of carps and reported significant ($p<0.05$) DNA damage due to heavy metals. Direct relationships of heavy metals viz. cadmium, lead, arsenic and copper toxicities with DNA strand breakage were also observed by Costa *et al*. (2008) in *Solea senegalensis*. In study performed by Matsumoto *et al*. (2006) a significant increase in the level of DNA strand breaks was observed in the peripheral blood erythrocytes of *Oreochromis niloticus* exposed to chromium. Similarly, concentration/dose dependent increase in DNA damage in *Misgurnus anguillicaudatus* (loach) after exposure to Pb+Cd+Zn mixture was also observed by Zhang *et al*. (2008). Concentration dependent DNA damage as observed during present study is also supported by Chandra and Khuda-Bukhsh (2004) who found dose dependent DNA aberrations in *Oreochromis mossambicus*. Genotoxicity of heavy metals is mainly related to accumulation of free radicals, clastogenic process or simultaneously to clastogenic and aneugenic action in fish (Nepomuceno *et al*., 1997).

DNA damage induced by mixture of cadmium and lead suggested a serious concern towards their potential danger to the survival of carp in natural environment.

Acknowledgement: The author is grateful to the Higher Education Commission Pakistan for providing funds under the Indigenous Ph.D. Fellowship Program to complete this work as a part of Ph.D. Research.

REFERENCES

Abdullah, S., M. Javed, and A. Javid (2007). Studies on acute toxicity of metals to the fish (*Labeo rohita*). Int. J. Agric. Biol. 9 (2): 233-237.

Ahmed, M. K., M. Habibullah-Al-Mamun, M.A. Hossain, M. Arif, E. Parvin, M.S. Akter, M.S. Khan, and M.M. Islam (2011a). Assessing the genotoxic potentials of arsenic in tilapia (*Oreochromis mossambicus*) using alkaline comet assay and micronucleus test. Chemosphere 84: 143-149.

Ahmed, M.K., E. Parvin, M. Arif, M.M. Islam, and M.S. Akter (2011b). Genetic damage induced by lead chloride in different tissues of freshwater climbing perch *Anabas testudineus* (Bloch). Environ. Monit. Assess. 182: 197-204.

Ahmed, M. K., E. Parvin, M. Arif, M.S. Akter, M.S. Khan, and M.M. Islam (2010). Measurements of genotoxic potential of cadmium in different tissues of fresh water climbing perch *Anabas testudineus* (Bloch), using comet assay. Environmental Toxicology and Pharmacology 30: 80-84.

Ambreen, F., M. Javed, and U. Batool (2015). Tissue specific heavy metals uptake in economically important fish, *Cyprinus carpio* at acute

exposure of metals mixtures. Pakistan J. Zool. 47 (2): 399-407.

Azmat, H., M. Javed, and G. Jabeen (2012). Acute toxicity of aluminum to the fish (*Catla catla*, *Labeo rohita* and *Cirrhina mrigala*). Pakistan Vet. J. 32 (1): 85-87.

Ballatori, N. (2002). Transport of toxic metals by molecular mimicry. Environ. Hlth. Perspect. 110: 689-694.

Baselt, R.C. (2004). Deposition of toxic drugs and chemicals in man. 7th Ed. Foster City, CA: Biomedical Publications.

Burger, J. (2008). Assessment and management of risk to wildlife from cadmium. Sci. Total Environ. Res. 56: 471-502.

Cestari, M.M., P.M.M. Lemos, C.A.D. Ribeiro, J. Costa, E. Pelletier, and M.V.M. Ferraro (2004). Genetic damage induced by trophic doses of lead in the neotropical fish *Hoplias malabaricus* (Characiformes, Erythrinidae) as revealed by comet assay and chromosomal aberrations. Genetics and Molecular Biology 27: 270-274.

Chandra, P. and A.R. Khuda-Bukhsh (2004). Genotoxic effects of cadmium chloride and azadirachtin treated singly and in combination in fish. Ecotoxicol. Environ. Saf. 58: 194-201.

Cok, I., O.K. Ulutas, O. Okusluk, E. Durmaz, and N. Demir (2011). Evaluation of DNA damage in common Carp (*Cyprinus carpio* L.) by Comet assay for determination of possible pollution in Lake Mogan (Ankara). The Scientific World J. 11:1455-1461.

Costa, P.M., J. Lobo, S. Caeiro, M. Martins, A.M. Ferreira, M. Caetano, C. Vale, T.A. DelValls, and M.H. Costa (2008). Genotoxic damage in *Solea senegalensis* exposed to sediments from the Sado Estuary (Portugal): effects of metallic and organic contaminants. Mutat. Res. 654: 29-37.

Damodara, G.K.M., V.M. Nair, and N.K. Subbalakshmi (2008). Effect of 15 days exposure to sublethal concentrations of cadmium toxicity on the level of metabolic fuels in Indian Major carps, *Labeo rohita*. The J. Physiological Sciences 21 (1): 18-25.

Fernandez-Alba, A.R., L.H. Guil, G.D. Lopez, and Y. Chisti (2001). Toxicity of pesticides in wastewater: a comparative assessment of rapid bioassays. Analytica. Chimica. Acta. 426: 289-301.

Ferraro, M.V.M., A.S. Fenocchio, M.S. Mantovani, C.D. Ribeiro, and M.M. Cestari (2004). Mutagenic effects of tributyltin and inorganic lead (Pb II) on the fish *H. malabaricus* as evaluated using the comet assay and the piscine micronucleus and chromosome aberration tests. Genetics and Molecular Biology 27 (1): 103-107.

Frascasso, M.R., L. Perbellini, S. Solda, G. Talamini, and P. franceschetti (2002). Lead induced DNA strand breaks in lymphocytes of exposed workers: Role of reactive oxygen species and protein kinase. C. Mutation Res. 515: 159-169.

Godwin, A.H (2001). The biological chemistry of lead. Current Opinion in Chemical Biology 5: 223-227.

Javed, M. (2012). Effects of metals mixture on the growth and their bioaccumulation in juvenile major carps. Int. J. Agric. Biol. 14 (3): 477-480.

Jia, X., H. Zhang, and X. Liu (2011). Low levels of cadmium exposure induced DNA damage and oxidative stress in the liver of Oujiang colored common carp *Cyprinus carpio* var. *color*. Fish Physiol. Biochem. 37: 97-103.

Jose, S., P. Jayesh, A. Mohandas, R. Philip, and I.S.B. Singh (2011). Application of primary haemocyte culture of *Penaeus monodon* in the assessment of cytotoxicity and genotoxicity of heavy metals and pesticides. Marine Environ. Res. 71: 169-177.

Kopjar, N., P. Mustafic, D. Zanella, I. Buj, M. Caleta, Z. Marcic, M. Milic, Z. Dolenec, and M. Mrakovcic (2008). Assessment of DNA integrity in erythrocytes of *Cobitis elongate* affected by water pollution: The alkaline comet assay study. Folia. Zool. 57 (1-2): 120-130.

Kousar, S. and M. Javed (2014). Assessment of DNA damage in peripheral blood erythrocytes of fish exposed to arsenic under laboratory conditions. In. J. Curr. Microbiol. App. Sci. 3 (11): 877-888.

Kousar, S. and M. Javed (2015). Diagnosis of metals induced DNA damage in fish using comet assay. Pakistan Vet. J. 35 (2): 168-175.

Matsumoto, S.T., M.S. Mantovani, M.I.A. Malaguttii, A.U. Dias, I.C. Fonseca, and M.A. Morales (2006). Genotooxicity and mutagenicity of water contaminated with tannery effluents, as evaluated by the micronucleus test and comet assay using the fish *Oreochromis niloticus* and chromosome aberrations in onion root-tips. Genet. Mol. Biol. 29: 148-158.

Nepomuceno, J.C., I. Ferrari, M.A. Spano, and A.C. Centeno (1997). Detection of micronuclei in peripheral erythrocytes of *Cyprinus carpio* exposed to metallic mercury. Environ. Mol. Mutagen. 30: 293-297.

Pereira, S., I. Cavalie, V. Camilleri, R. Gilbin, and C. Adam-Guillermin (2013). Comparative genotoxicity of aluminum and cadmium in embryonic zebrafish cells. Mutat. Res. 750: 19-26.

Russo, C., L. Roco, M.A. Morescalchi, and V. Stingo (2004). Assessment of environmental stress by the micronucleus test and comet assay on the genome of teleost populations from two natural environments. Ecotoxicology and Environmental Safety 57 (2): 168-174.

Singh, N.P., M.T. McCoy, R.R. Tice, and E.L. Schneider (1988). A simple technique for quantization of low levels of DNA damage in individual cells. Exp. Cell Res. 175: 184-191.

Steel R.G.D., J.H Torrie, and D.A Dickey (1996). Principles and procedures of statistics (3rd Ed.). McGraw Hill Book Co., Singapore.

Zhang, Y., Y. Wang, R. Yu, S. Zhang, and Z. Wu (2008). Effects of heavy metals Cd^{2+}, Pb^{2+} and Zn^{2+} on DNA damage of loach *Misgurnus anguillicaudatus*. Front. Biol. China 3: 50-54.