Assessment of di-n-butyl phthalate (DBP) induced genotoxicity in freshwater cyprinid fish crucian carp (*Carassius carassius* L.) using comet assay

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Abstract: Di-n-butyl phthalate (DBP) is a manufactured chemical, commonly used as a plasticizer. It is a ubiquitous environmental contaminant. It was listed as priority pollutant by US Environmental Protection Agency. The aim of the present study was to evaluate the genotoxic potential of DBP on *C.Carassius* using comet assay. The fishes were exposed to sublethal ($1/2$ LC_{50} -3.88ppm) for 96 hr. After the completion of exposure period, fishes were anesthetized and cardiac puncture was done. The comet assay was performed and the statistical analysis revealed significant ($p<0.05$) DNA damage in DBP treated group. The study demonstrated the utility of comet assay for in vivo laboratory studies using fish for screening the genotoxic potential of DBP.

Keywords- di-n-butyl phthalate, genotoxicity, *C.carassius*, Comet Assay.

1. Introduction

Fishes have significant economic importance and are quite sensitive to the wide array of pollutants discharged in the aquatic ecosystems. Fishes are widely used to evaluate water standard of aquatic environment because they serve as pollution bioindicators and play notable roles in assessing potential risk associated with contamination of new chemicals in aquatic ecosystem (Lakra and Nagpure, 2009). In Kashmir Cyprinids are the most notable family of fish, and its members are distributed globally. These family members are distributed broadly in fresh water sources (Demirsoy, 1988, Geldiay and Balik, 1998). Freshwater Cyprinid fish dominates global aquaculture production. Some characteristics of *C. carassius* L. (Cyprinidae) such as its wide distribution and availability throughout the year, cost-effectiveness, easy handling and acclimatization in the laboratory make it an excellent ecotoxicological model. Toxicity tests have been performed on fishes to estimate the effect of toxins on various aquatic organisms under laboratory conditions.

Due to swift advancement in technology and the geometric progress of the global population growth, plastic materials have found versatile applications in every aspect of modern human life. Different substances are mingled in plastics at various proportions to improve their performance and reduce cost (Tokiwa et al, 2009). Phthalates, which are esters of phthalic acid, are primarily used as plasticizers to enhance plasticity of industrial polymers. They are also used in number of consumer end products such as paints, toys, adhesives, lubricants, packaging and building materials, and personal care items and are unavoidable part of modern life (Shea, 2003, Horn et al. 2004). Presently the overall yearly global use of phthalates is estimated at over 30 million tons with the yearly consumption of Europe alone is about 1million ton (Net et al, 2015). While these plasticizing agents induce advantageous properties to plastics, they are not attached to the polymer by a covalent linkage which makes them vulnerable to leaching from the matrix and entering environment via volatilization, abrasion, leaching and direct diffusion to exposure matrices i,e dust (Rudel et al. 2003, Fromme et al. 2012). Once entering the environment, they pose remarkable toxicological threats to the myriad of non target organisms, discover its way to the food chain, and threaten ecological balance

and biodiversity of nature. The effluents generated from waste water treatment plants have been considered as main source of plasticizers in aquatic environment (Kolpin et al. 2002, Loraine and Pettigrove, 2006).

Di-n-butyl phthalate(DBP) is one of the commonly used phthalate essentially as plasticizer to ameliorate the flexibility and workability of the products, such as polyvinyl chloride, plastic packaging films, adhesives, lubricants, cellulose materials, cosmetics and insecticides (Gao and Wen, 2016). DBP is not chemically attached to the polymer matrix like other phthalates, directing to its ubiquitous existence in the diverse environmental matrices (Net et al, 2015). DBP has been directly assessed for reproductive and developmental toxicity in addition to the monitoring of testicular germ cell toxicity and testicular atrophy in standard estimation (Cater et al. 1977, Oishi and Hiraga, 1980, Gray et al. 1982, Barber et al. 1987, Srivastava et al.1990). DBP is considered very dangerous substances in the EU REACH regulation and is classified as category 1B in the Commission Directive 2007/19/EC (cannot be used to make toys, childcare articles, and cosmetics) and risk reduction measures are required for its safe use. Canada and the United States have also taken regulatory actions restricting their use (Ventrice et al, 2013). Furthermore, they pose a particular risk to aquaculture.

The importance of evaluation of genotoxicity of various pollutants including Phthalates in fish lies in the fact that higher vertebrates, including humans feeding on fish, are conveniently exposed to the genotoxic agents that are trapped in fish body. Since there is increasing concern over the existence of genotoxins in the aquatic environment. It is significant to develop or systematize the already existing methods for determination of genotoxic chemicals in aquatic organisms (Pandey et al, 2006). Fish species can be used to calculate the potential impacts of toxicants to develop carcinogenic and teratogenic effects in human (Harshbarger and Clark, 1990). Singh et al. (1988) established most economical and sensitive technique under alkaline state for the identification of genetic damage at cellular level, The Comet assay having sensitivity for identifying minimum intensity of DNA fragmentation and necessitate a small number of blood cells per fish specimen (Tice et el, 2000). The principal advantage of Comet assay are the small sample size requirement, its swiftness and the probability to distinguish between cell types concerning the degree of DNA damage or DNA repair level.

2. Materials and method

This chapter deals with the details of chemicals, reagents, fish and experimental design used to evaluate the DBP induced genotoxicity.

2.1 Chemicals and reagents

The chemicals used in the current study were of high clarity. Di-n-butyl phthalate ($C_{16}H_{22}O_4$, DBP, CAS No. 84-74-2, 99% purity) was procured from Sigma- Aldrich; Bengaluru, India is a colorless to faint yellow viscous liquid. Acetone, $(CH_3)_2C0$, CAS No.67-64-5, 99% purity was purchased from Hi- Media Labs, Mumbai, India. Agarose-normal melting (molecular biology grade-MB), Agarose-low melting (MB), sodium chloride (analytical reagent grade-AR), potassium chloride (AR), disodium hydrogen phosphate (AR), potassium dihydrogen phosphate (AR), di sodium EDTA(AR), Tris base (AR), sodium hydroxide (AR), sodium lauryl sarcosinate (AR), triton-100 (MB), Hydrochloric acid (AR), Ethanol, Ethidium bromide(MB) and benzocaine (AR) etc were purchased from himedia and are 99.95% of purity.

2.2 Test organism

C. carassius L. (Family: Cyprinidae and Order: Cypriniformes was selected as the experimental model. Locally known as "Gang Gad", it is a freshwater fish occurring in the standing and slow flowing waters, especially the flat land lakes of the Kashmir Valley. Live juvenile fish were procured with the help of a local fisherman, using hand nets, from the Dal Lake (34°07′N 74°52′E), in the vicinity of the University of Kashmir, Srinagar, India. They were transported live in plastic jars to the Cytogenetics and Molecular Biology Laboratory, Centre of Research for Development (CORD), University of Kashmir and subjected to a prophylactic treatment by bathing in a 0.05 % aqueous solution of potassium permanganate for 2 m to avoid dermal infection. Their average length and wet weight (± SD) were recorded as 12.5 ± 1.64 cm and 33 ± 4.94 g, respectively.

2.3 Acclimatization

The fish stock was acclimatized before the commencement of the experiment for at least 3 weeks to a 1:1 diurnal photoperiod in well aerated 60 L glass aquaria at 19.7 ± 2.6°C with 24 h aged dechlorinated tap water (pH 7.6 – 8.4) and fed daily with commercially available fish food (Feed Royal®, Maa Agro Foods, Visakhapatnam, Andhra Pradesh, India). Only active specimen with no sign of injury and distress were used in the study. Waste products were siphoned off every day to check increase of ammonia in the water. Every effort as suggested by Bennett and Dooley (1982) was taken to maintain optimal conditions during acclimatization: no fish died during this period. The acclimatized fish were used for the experiments. Studies involving experimental animals were conducted in accordance with the guidelines described for maintenance, care and conducting toxicity tests of fish in Standard Methods for the Examination of Water and Wastewater, American Public Health Association (APHA, AWWA and WPCF, 2005)

2.4 Genotoxicity testing using Comet assay

2.4.1 Exposure conditions

Acclimatized fish specimen were subjected to sublethal concentration (1/2 LC_{50}) of DBP for the period of 4 days. Following standardized OECD testing guidelines 40 fishes were dived into 4 groups comprising of 10 fish each. Group I (negative control) fish were given no treatment, Group II was solvent group (Acetone), Group III fish were exposed to sub lethal DBP concentration (3.88ppm) and group IV fish were (positive control) exposed to 5ppm of ethyl methanesulphonate (EMS). Aquaria were continually aerated during the experiment and medium were renewed on daily basis and fresh solutions were spiked to maintain water quality with DBP level. During exposure period, fishes were examined for abnormal behaviour and external appearance.

2.4.2 Comet assay/Single cell gel electrophoresis (SCGE)

The comet assay was undertaken according to Singh et al. (1988). The single cell gel electrophoresis (SCGE)/ comet assay, evolved by N.P. Singh, merges the simpleness of biochemical procedures for investigating DNA single strand breaks incomplete excision repair sites, frank strand breaks , alkali-labile sites and cross linking with the single cell perspective representative of cytogenetic assays.

Prior to blood collection, fish were anaesthetized with 0.12 mg/L benzocaine (Marques de Miranda Cabral Gontijo et al, 2003). Cardiac puncture was done and 0.3mL of blood was drawn . 5µL of blood sample was diluted in 1000µL of PBS. Cell viability was assessed by Trypan Blue Exclusion technique (Anderson et al, 1988) and the blood samples showing 84% cell viability were processed for the Comet assay.10µL of cell suspension mixed with 120µL of 0.5% low melting point Agarose (LMPA) at 37%, were layered on previously 1% normal melting pointing Agarose (NMPA) coated slides. The slides were carefully cover-slipped and placed in refrigerator at 4˚C for 10-15 min, for solidification of the gel. Once the blood-LMPA layer was solidified, cover slip was carefully removed avoiding avulsion of the underlying layer. 75µL of LMPA was added onto the Agarose gel mixture layer and a new cover slip was placed carefully over the gel mixture layer, gel was allowed to solidify at 4˚C in a refrigerator for 10-15 min. After solidification of gel, cover slips were carefully removed and slides were immersed into freshly prepared cold lysing solution and refrigerated for atleast 1hr at 4˚C. After lysing, the slides were benignly removed from lysis solution and placed exactly perpendicular to both the electrodes with the Agarose-coated side facing upwards in horizontal submarine gel electrophoresis system. The electrophoresis tank was filled with cold fresh electrophoresis buffer till the buffer completely covered the slides without formation of air bubbles over the Agarose gel. The slides were kept in alkaline buffer for 30 min to allow the unwinding of DNA strands and expose the alkali labile sites (alkali unwinding). Power supply was turned on with 0.74V/cm (between electrodes) and current was adjusted to 300mA by raising and lowering the buffer level. Electrophoresis was carried out for 30min. After 30 min of electrophoresis, power supply was turned off and slides were gently lifted from electrophoresis buffer and were placed on a drain tray. Drop wise slides were coated with neutralization buffer for 5min and the buffer was drained; the process was repeated two more times followed by numerous was with distilled water. After draining, slides were immersed in 100% ethanol for for dehydration. Slides were air dried and placed in an oven for 50min .The slides were then stored at dry area. For staining, slides were rehydrated with chilled distilled water for 30 min and were stained with 50µL of Ethidium bromide and cover slipped. Before viewing excess stain was blotted away from back and edges. For visualization of Ethidium bromide–stained slides, fluorescent microscope equipped with an excitation filter of 515-560nm with barrier filter of 590nm and a magnification of 200X was used.

2.4.3 Visualization and comet scoring

For the assessment of DNA damage, images of 300 randomly selected cells (100 per slide) were analyzed for each sample. The cells were scored visually according to tail length into five categories, from undamaged class (0) to complete damaged class (type IV) (Anderson et al, 1994). The data are presented as the frequency of cells with and without DNA damage, score and distribution of classes. The calculation of score was done by multiplying the number of nuclei found in a class times the class number. Statistical analysis was performed with student's t- test. Analysis was carried out using SPSS (version 24.0) for windows considering a significance level of $p<0.05$.

3. Results and Discussion

Analysis of number of cells with comets in the blood samples demonstrated that there was a significant DNA damage in DBP treated group (1/2 LC_{50} -3.88ppm) compare to all 3 control groups; positive control (5ppm EMS), negative control group (untreated) and solvent control group (acetone). There was approximately 10 times the number of cells with DNA damage in treated group comparing to the negative and solvent control groups. However, comparing positive control group, the treated group showed borderline significance in DNA damage level. Figure 1 shows the different classes of comets formed in DBP treated group.

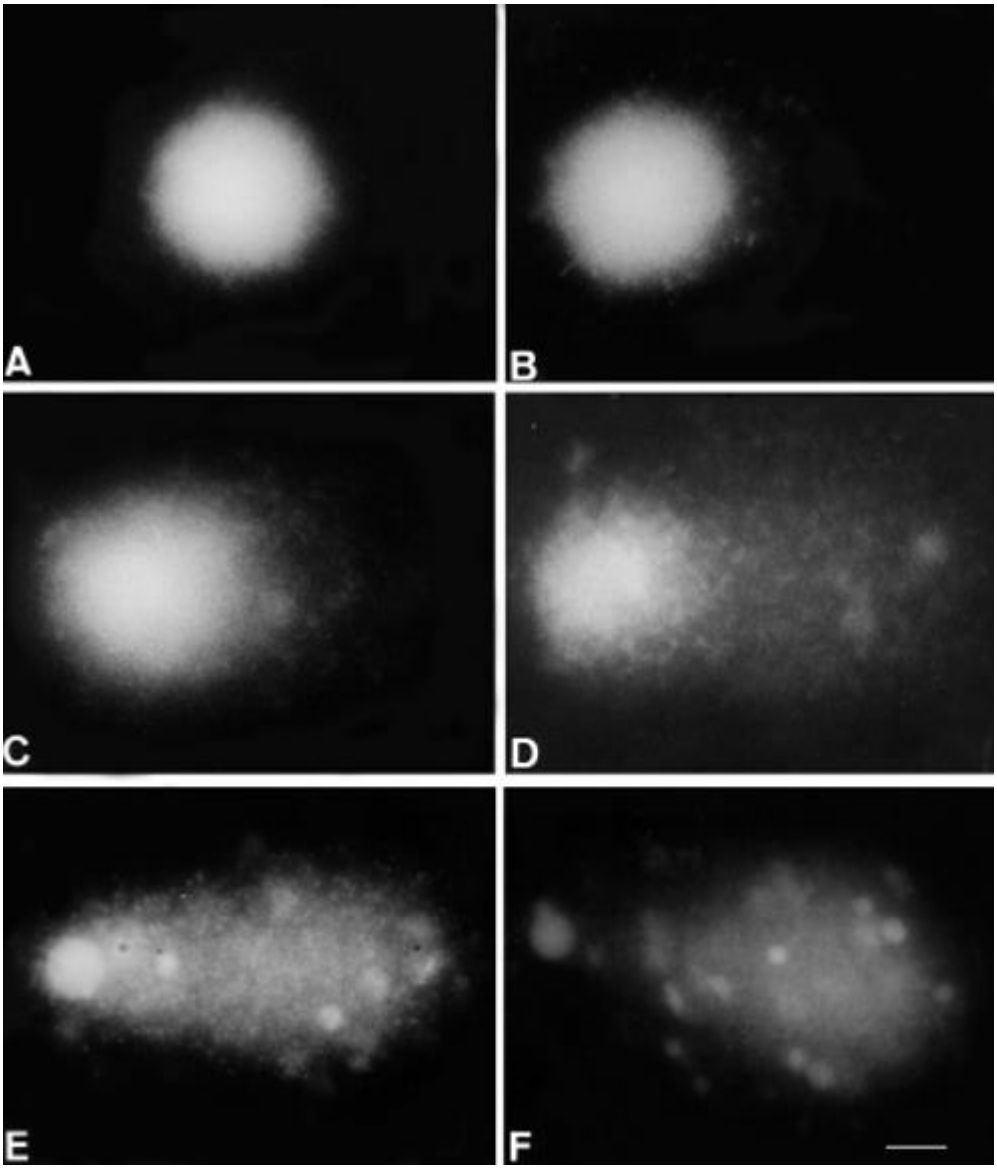

Figure 1: Photomicrographs showing the classification of blood cells ,treated with sublethal DBP (3.88ppm), in the comet assay. A, Class 0, undamaged; B, class 1; C, class 2; D, class 3; E, class 4, maximum damage; F, apoptotic cell.

The mean score, which indicates the degree of damage in the analysis of the blood cells, found in the treated group(μ= 282) was little higher than that of positive control (μ=247.3). However the mean score in treated

group was approximately about 10 times the value determined in the negative control (μ=25) and solvent control(μ=24.6). Statistical analysis demonstrated that the mean score of treated group was significantly greater ($p<0.05$) when comparing to all three control groups as shown in table 1, 2 and 3.

Table I: showing mean score and standard deviation of DBP and EMS. Since the p value is < 0.05 depicting significantly greater DNA damage in DBP treated group compare to EMS (positive control) treated group.

DBP *vs* EMS (positive control)					
	Factor	N	Mean	Std. Deviation	P value
Level	DBP	3	282.0000	8.00000	.049
	EMS	3	247.3333	20.03331	

Table 2: showing mean score and standard deviation of DBP and water (negative control). Since the p value is < 0.05 depicting significantly greater DNA damage in DBP treated group .

DBP *vs* water (negative control)					
	Factor	N	Mean	Std. Deviation	P value
Level	DBP	3	282.0000	8.00000	.000
	Water	3	25.0000	3.60555	

Table 3: showing mean score and standard deviation of DBP and Acetone (solvent group). Since the p value is < 0.05 depicting significantly greater DNA damage in DBP treated group compare to acetone (solvent control) group.

DBP *vs* Acetone (solvent control)					
	Factor	N	Mean	Std. Deviation	P value
Level	DBP	3	282.0000	8.00000	.000
	Acetone	3	24.6667	2.08167	

During the exposure period no clinical signs were noticed in control groups. However, within 8h of exposure to DBP, Carps in DBP treated group showed different intoxications symptoms. Abnormal behaviour was noted immediately such as loss of equilibrium, erratic swimming movements, lethargy and motionlessness followed by convulsions. The fish under experimental study exhibited difficulty in breathing represented by speedy breathing coexisting with rapid movement of operculum and failure to respond to escape reflex. Furthermore dark discoloration of skin with thick layer of mucous was also noted. Postmortem studies revealed congestion of internal organs and excessive slime deposition on gills

At present, numerous chemicals have been classified as plasticizers and studies using different models have indicated that some of them have toxic properties. Pollution of aquatic environment due to plastic residues is well documented and fish are often used as sentinel organisms for eco-toxicological studies as they are able to accumulate genotoxic substances and respond to low concentration of mutagens in a manner similar to higher vertebrates (Spitsbergen and Kent, 2003, Cavas et al. 2005). Therefore, the use of fish biomarkers as indices of the effects of pollution, are of great importance, and help in early detection of aquatic environmental problems (Van Der Oost et al, 2003).

In the present study, pre-treatment of (0.05 %) solution of potassium permanganate was given to the fish for 2 min to avoid any dermal infection and after that the specimen were acclimatized for at least 3 weeks under laboratory conditions to remove the residual effects of other chemicals prior to start of the experiment. Several investigators (Pandey et al. 2006, Sharma et al. 2007, Ali et al. 2009) have used potassium permanganate solution for prophylactic treatment before starting their experiments, and like our study, they did not report any adverse effects in the test organism due to prophylactic treatment.

The genotoxic impact of environmental contaminants can be tracked using wide variety of both Invitro and invivo biomarker assays but the comet assay is getting more popularity over the other assays due to its advantages like sensitivity in detecting low levels of DNA damage (0.1 DNA break per 10^9 DA) (Gedik et al, 1992), completion of assay in short time, low number of cells required, cost effectiveness, precision, ease of application, The cells used in this assay do not need to be mitotically active, requires neither metaphases nor knowledge of chromosome number (Bücker et al. 2006, Belpaeme et al.1998, Buschini et al. 2003, Collins, 2004, Tice et al. 2000). Accordingly, it has been broadly used in the fields of genotoxicology and environmental biomonitoring (Tice, 1995a, Tice et al. 2000) inclusive of aquatic organisms (Lee and Steinert, 2003) as potent tool to measure the relationship between DNA damage and the exposure of aquatic organisms to genotoxic contaminants. The alkaline comet assay (Singh et al, 1988) is able to identify DNA damage, i.e. single strand breaks or other lesions, such as alkali labile sites, DNA cross-links (Tice, 1995b) and incomplete excision repair events (Gedik et al, 1992). Strand breakage level in DNA has been suggested as a sensitive indicator of genotoxicity and as effectual biomarker in biomonitoring of the environment (Belpaeme et al, 1996).

In our study, within the same group blood cells were distributed in different degrees of DNA damage to indicate that comet assay was capable to detect intercellular differences in DNA damage of heterogeneous mixture of cells. The distribution of the DNA damage grades in all the DBP treated groups were significant then the control ($p<0.05$). Though there is sparse data on the genotoxic potential of DBP on fishes, our study shows relevance with the studies conducted by other authors. Khalil et al. (2016) did a study to find the genotoxic impact of di-n-butyl phthalate on juvenile Nile tilapia (*oreachromis niloticus*) by using Alkaline comet assay. The studies confirmed that there was significant DNA damage in fish exposed to DBP. Zeid et al. (2014) demonstrated the DNA damaging potency of chronic exposure of DBP on Nile tilapia fingerlings using comet assay. The result showed significant increase in DNA damage. The study concluded comet assay as sensitive tool detecting lower levels of DNA damage.

The pervasiveness of genotoxic contaminants in the aquatic ecosystems is one of the crucial concern in the field of environmental science and this has demanded the need to evolve sensitive techniques to monitor the genotoxic potency of these chemicals in aquatic organisms (Hayashi et al,1998). Fishes are astonishing model organisms for Genotoxicological studies and furnish prior warnings for toxicants induced environmental degradations and alterations (Pawar, 2012) including evaluation of contaminants in aquatic ecosystems (Akiyma et al, 2001) . The SCGE /Comet assay is being broadly used as a biomarker for the identification of genotoxic impacts of chemicals in aquatic organisms (Ateeq et al. 2005, Jha, 2004, Pandey et al. 2006, Campos de ventura et al. 2008, Yin et al. 2008) including detection of DNA damages propitiously in fish (Cavalcante et al.2008, Caliani et al. 2009).

4.Conclusion

The current study offers considerable advantage over the other cytogenetic assays to detect DNA damage like chromosome aberrations, sister chromatid exchange and the micronucleus test, because to carryout comet assay , the cells don't need to be mitotically active , On the basis of experimental evidence obtained, we suggest that DNA damage in blood cells of freshwater cyprinid, *C.carassius* may potentially be used as bioindicators for detecting the genotoxic nature of DBP, one of the commonly used phthalate as plasticizer.

5.Acknowledgement

Authors are highly thankful to the Centre of Research for Development (CORD), University of Kashmir for providing laboratory facilities to carryout present research work.

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