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Cytotoxic and genotoxic effects of di-isononyl phthalate and di-(2-ethylhexyl) phthalate: An *in vitro* and *in vivo* approach on toxicological assessment

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ABSTRACT

The aim of the present study was to analyze the cytotoxic and genotoxic effects of two selected phthalates, di-isononyl phthalate (DINP) and di-(2-ethylhexyl) phthalate (DEHP) *in vivo* and *in vitro* using fish model, *Oreochromis mossambicus* and cell lines as Chinese Hamster Ovary-K1 (CHO-K1) cells, TA97A, TA98 and TA100 strains of *Salmonella typhimurium* and human peripheral erythrocytes. *In vivo* toxicological assessment showed genotoxicity of DINP and DEHP at 300 ppm and 60 ppm concentrations exposed for 60 days, which was evident by the formation of micronuclei and other nuclear abnormalities in erythrocytes of the fish. However, comet analysis revealed no genotoxic effects of the phthalates in which tail length and percent tail DNA remained unchanged. *In vitro* analysis showed mutagenicity of DINP and DEHP in *Salmonella typhimurium* tester strains. Study on human peripheral blood lymphocytes showed the formation of micronuclei, nucleoplasmic bridges and nuclear bud. Exposure of phthalates in CHO-K1 cells showed significant ($P<0.05$) increase in the tail length and tail DNA percent in DEHP while DINP showed negative results. Cell viability test using MTT assay in CHO-K1 cells showed significant ($P<0.05$) decrease in cell viability while lactate dehydrogenase release test showed cytotoxicity of exposed phthalates. The study illustrated that DINP and DEHP exerted genotoxic effects both *in vivo* and *in vitro* could be relatively unsafe for the organisms. Further, the study can be used as a biomonitoring tool to alarm the risk of plasticizers that enter into the aquatic or nearby environment from the production or release sites.

Key words: DINP, DEHP, cytotoxicity, genotoxicity, *in vivo, in vitro*

Introduction

Recently, many new compounds have been synthesized for industrial and commercial purposes, and its widespread use and high demand in the market concerns for the potential toxic effects on humans and other living organisms. Phthalates are a group of newly emerging chemicals often called plasticizers, which are widely used to extend the ductility and durability of plastic products such as medical supplies, plastic bags, toys and so on (Heudorfa et al., 2007). Humans are extensively exposed to phthalates by consuming foods and drinks that have been in contact with containers and products containing phthalates. Besides the dietary sources, dermal absorption and inhalation are the major routes of human exposure. Most of the phthalates are excreted in the form of conjugated monoesters in urine, while others undergo secondary metabolism like oxidative biotransformation (Silva et al., 2003). The occurrence of phthalates and their metabolites have been detected in breast milk, serum, semen and urine with the concentrations ranging from nanogram to milligram per millilitre (Hogberg et al., 2008). In the past two decades, there are several researches that linked phthalates to negative health impacts on humans such as asthma, obesity, diabetes, breast cancer, attention deficit hyperactive disorder, autism, neurodisorder, behavioural alteration, reproductive abnormalities and infertility (Stojanoska et al., 2017; Wang et al., 2019). There are more than 25 phthalates widely used in commercial products, among which di-isononyl phthalate (DINP) and di-(2-ethylhexyl) phthalate (DEHP) are the most popular around the world. Since the annual global production of phthalates exceeds 8 million tonnes in 2015, it is necessary to evaluate the potential role of phthalates in adverse health effects of human and other animals.

The European Union in 1999 banned the use of DINP and DEHP in children's toys however; there was no such regulation in other parts of the world, including India and China. Owing to the widespread use and ubiquitous in the environment there is a great risk of phthalate exposure in aquatic environments. Fish is the predominant species found in the aquatic ecosystem, and also serves as the direct protein food to humans. Thus humans are either exposed directly or indirectly to several phthalates, which concerns the ecotoxicologists in recent years. Previous studies from our laboratory have documented that DINP and DEHP induced oxidative stress, imbalanced endocrine system and impaired reproductive functions in the freshwater fish, *Oreochromis mossambicus* (Revathy & Chitra, 2018a; 2018b; 2018c; 2019). There are extensive data suggesting the carcinogenic effects of DINP and DEHP in several animal models mediated through peroxisome proliferator-activated receptors (PPAR-α) (Takashima et al., 2008). Phthalates are well-known peroxisome proliferators and endocrine disruptors, which enable to generate reactive oxygen species (ROS), and may result in genetic aberrations and several other diseases in organisms (Yang et al., 2012). Thus, the analysis of phthalate genotoxicity is a challenging task owing to the discrepancies reported in several *in vivo* and *in vitro* studies. One of the researches has reported that DINP as non-mutagenic when tested in *Salmonella* and mouse micronucleus assay (McKee et al., 2000). However, another study has reported that DINP was mutagenic in the Ames assay, and also induced significant levels of unscheduled DNA synthesis and DNA strand breakage (Quinn-Hosey et al., 2012). Similarly, there is a lack of agreement in the carcinogenic and mutagenic effects of DEHP and its metabolites. The hydrolysis product of DEHP namely mono-(2-ethylhexyl)phthalate (MEHP) has been reported as mutagenic while DEHP has been considered as a non-genotoxic carcinogen when evaluated using bacterial reverse mutation assay (Kanode et al., 2017). On the contrary, the induction of DNA damage by DEHP has been reported in human blood cells using comet assay (Anderson et al., 1999).

The present study, therefore, aimed to evaluate the genotoxic potential of two phthalates, DINP and DEHP, using both *in vivo* and *in vitro* models. The common, rapid and reliable *in vitro* testing models include bacterial reverse-mutation or Ames test using different tester strains of *Salmonella typhimurium* and Chinese Hamster ovary cell lines. Phthalates induced DNA damages were examined using micronucleus test, comet assay, cell viability test and cytotoxicity analysis by lactate dehydrogenase (LDH) release, which was considered as the most reliable standard battery of genotoxicity tests. *In vivo* toxicity assessment was also adopted in this study using peripheral erythrocytes of the freshwater fish, *Oreochromis mossambicus*.

Materials and Methods

In vivo genotoxicity tests

Chemicals

Diisononyl phthalate (DINP; CAS No. 28553120 - 99% purity) and di(2-ethylhexyl)phthalate (DEHP; CAS No. 117817 – 99.7% purity) was obtained from Sigma Aldrich chemical Co., USA. Foetal bovine serum, Giemsa, Triton X-100, ethidium bromide, agarose, cytochalasin B, NADH, sodium azide, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide was obtained from Himedia Research Laboratories, Mumbai, India. All other chemicals were of analytical grade and obtained from local commercial sources.

Test animal

Freshwater fish, *Oreochromis mossambicus* (5.5±1.5 cm; 3.5±0.75 g) collected from Safa aquarium, Kozhikkode, Kerala, India were brought to the laboratory without stress to the animal. Fish were acclimatized in dechlorinated and well-aerated water (40 L capacity) for two weeks prior to the experiments. The physicochemical characteristics of water such as pH (6.5 to 7.5), temperature (28±2ºC), oxygen saturation (70-100%) were maintained throughout the study as per APHA guidelines (1998).

Preparation and exposure of test chemicals

Stock solutions of DINP and DEHP were prepared just before exposure by sonication at 50 Hz for 5 and 15 min, respectively with 30-sec pulse interval using propylene glycol as solvent. The concentrations of DINP at 300 ppm and DEHP at 60 ppm were selected based on the maximum solubility as described earlier (Revathy & Chitra, 2015). Fish were then exposed to DINP at 300 ppm and DEHP at 60 ppm concentrations for 4, 7, 14, 30 and 60 days durations maintaining propylene glycol as the vehicle control group, and solvent or toxicant-free as the negative control group. Ten healthy fish were maintained in each experimental group, which were maintained in properly aerated tubs covered with monofilament nets, to avoid the fish jumping out from the test solutions.

Collection of blood

At the end of every experimental period, fish was caught very gently using a small dip net, one at a time with least disturbance to avoid stress to the animal. Blood from the control and treatment groups were collected by cardiac puncture and used immediately for genotoxicity tests.

Micronucleus test

One drop of blood collected from control and experimental fish were placed on the slide and mixed with foetal calf serum. A thin blood smear was made; air dried and

fixed in absolute methanol. Staining was done with 10% Giemsa and air dried for 10 min (Heddle, 1973; Schmid, 1975). A total of 1000 erythrocytes from both control and treatment groups were scored for nuclear and cytoplasmic anomalies (Carrasco et al., 1990) which was then observed under a microscope at 100x magnification. Nuclear anomalies such as micronucleus, blebbed, notched and lobed nucleus were calculated as follows:

$$\text{Nuclear anomalies (\%)} = \frac{\text{Number of abnormal cells}}{\text{Total number of cells counted}} \times 100$$

Comet assay

Alkaline comet assay or single cell gel electrophoresis (SCGE) was performed according to the method of Singh et al. (1988) with slight modifications. Slides were prepared by pre-coating 1% normal melting point agarose (NMA) in double distilled water and stored at 4ºC. The second supportive layer, 0.5% low melting point agarose (LMA) was spread uniformly over the first layer of agarose using a coverslip. The slide was further kept at 4ºC for 5 min to allow complete polymerization of the agarose. After the coverslips were removed, 30 µl of the blood sample was gently mixed with 50 µl of 1% LMA agarose, pipetted on the supportive layer of 1% NMA agarose and again covered with a coverslip. After keeping coverslips for 5 min on ice, they were removed and the slides were placed in freshly made cold lysis buffer (2.5 M NaCl; 100 mM Na_2EDTA; 10 mM Tris; 1.5% Triton X-100; 1% SDS at pH10 along with 10% dimethyl sulfoxide) for 2 h. Slides were then placed in electrophoresis chamber containing cold alkaline electrophoresis buffer (300 mM NaOH; 1 mM Na_2EDTA at pH13) for 20 min to unwind DNA. Electrophoresis was performed by applying electric current at 0.8 V/cm at 4ºC for 20 min. After electrophoresis, slides were washed thrice in freshly prepared neutralization buffer (0.4 M Tris, pH 7.5) for 15 min. Ethidium bromide was used for staining and the stained slides were observed using fluorescence inverted microscope (Olympus CKX41), using Green filter (Excitation filter BP480-550C) at 40× magnifications. Images were captured using C-mount camera (Optika pro5 CCD camera) and comet images were scored using the software Comet Imager V 2.2.1 (MetaSystems, GmbH, Germany). Images of 50 cells were analysed randomly from each group and the tail length and percent tail DNA are the parameters chosen to evaluate DNA damage.

***In vitro* genotoxicity tests**

Cell culture

Different tester strains of *Salmonella typhimurium* such as TA97A, TA98, TA100, and Chinese Hamster Ovary (CHO-K1) cell line purchased from National Centre for Cell Sciences (NCCS), Pune, India was used for Ames test and comet assay, respectively. Peripheral blood obtained from healthy non-smoker volunteer unexposed to ionising radiations, mutagenic agents, and drug or chemical therapy, and without a history of chromosomal fragility or viral infections were cultured for CBMN assay. CHO-K1 cells were also used for cell viability and cytotoxicity assays such as MTT and LDH release tests, respectively.

Bacterial reverse mutation assay or Ames test

Bacterial reverse mutation assay or Ames test was performed in three histidine-required tester strains of *Salmonella typhimurium* namely TA97A, TA98 and TA100 according to the OECD Guideline 471. Three concentrations of DINP and DEHP such as 1, 2 and 5 µg/ml were tested on different tester strains of *Salmonella typhimurium*. Briefly, sterile glucose minimal agar plates were prepared and 25 ml of the prepared solution was poured into Petri-plates to solidify at room temperature. Top agar was prepared and 2 ml of the top agar with sodium phosphate buffer, 50 µl test sample and 100 µl bacterial culture was taken in a sterile glass tube, mixed well at 43ºC in water bath. It is then added to the prepared glucose minimal agar plate and set to solidify. The cultures were then incubated at 37ºC for 72 h in inverted position and colonies were counted, and compared with sodium azide as positive control along with negative and solvent (vehicle) control groups.

Cytokinesis-block micronucleus (CBMN) assay

CBMN assay was performed according to the method as described by Fenech (2007). Briefly, peripheral blood from a donor was drawn to make triplicates of each treatment group. Blood cell cultures were set up by mixing 0.5 ml of whole blood with 4.5 ml of Hikaryo XL (HiMedia) ready mix media. Then two different concentrations of DINP and DEHP at 0.5 and 1 µg/ml were treated with the peripheral blood lymphocyte culture at 44 h and cytochalasin B was added at 48 h to explore the frequency of genotoxic events in the treated and untreated cultures. Harvesting was done at the end of 72 h where the cells were treated hypotonically with cold 0.075 M KCl for 8 min, followed by fixation with methanol-acetic acid (3:1). Subsequently, slides were prepared and stained with Giemsa solution (4%). The cells were scored for anomalies such as micronucleus, nucleoplasmic bridges and nuclear buds (Fenech et al., 2003).

Comet assay

Comet assay was performed as per the method of Singh et al. (1988). CHO-K1 cells were cultured in 6 well plates and treated with 13.3 and 29.1 µg/ml concentrations of DINP and DEHP, respectively, which was then incubated overnight. The cells were trypsinized, washed with fresh media, and the fully frosted microscope slides were pre-coated with 1 ml of

1% normal melting point agarose (NMA Invitrogen, USA) and stored at 4ºC. This layer was removed before use and 120µl of 1% NMA was pipetted on to the slides, which were then covered with coverslips. Treated and untreated cell suspensions were mixed with 50 µl of low melting point agarose (Invitrogen, USA) and pipetted over the first layer of agarose, and NMA (80 µl) was used as a final protective layer. After each step, the slides were incubated at 4ºC for 10 min to set agarose. Slides were placed in a cold lysing solution containing 2.5 M NaCl; 100 mM Na_2EDTA; 10mM Tris base at pH 10 and 1% SDS to which 10% DMSO and 1% Triton X 100 were added immediately prior to use for 1 h. After lysis, slides were placed in electrophoresis buffer (300 mM NaOH; Na_2EDTA at pH13) for 20 min to allow unwinding of DNA. Electrophoresis was conducted in the same buffer by applying an electric current of 0.8V/cm (300 mA) for 20 min. Finally, slides were washed in neutralization buffer (0.4 µl Tris at pH7.5) three times for 5 min each, dried and stained with 50 µl ethidium bromide (EtBr) (20 µg/ml) for 15 min followed by washing with PBS to removed excess EtBr. The slides were photographed using an inverted epifluroscent microscope (Olympus CKX41) attached with Opitka Pro5 CCD camera. Comets were scored using Comet Imager V 2.2.1 software (MetaSystems, GmbH, Germany).

MTT Assay

CHO-K1 cells were treated with 2.5, 5, 7.5 and 10 µg/ml concentrations of DINP and DEHP for 24 h. After the treatment period, the medium was changed and cells were incubated with 30 µl of MTT (Sigma-Aldrich, St. Louis, MO, USA) under normal culture conditions for 4 h. Cell viability was evaluated by the conversion of the tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) to dark-blue coloured formazan by mitochondrial dehydrogenases (Mosmann, 1983). Colour development was measured spectrophotometrically in a microplate reader at 570 nm after cell lyses in DMSO (100 µl/well) against the blank.

Lactate dehydrogenase (LDH) leakage assay

LDH assay was carried out according to Decker and Lohmann-Matthes (1988). Briefly, the control and treated cell culture supernatants were added to phosphate buffer (100 mM; pH 7.4) sodium pyruvate (30 mM) and NADH (6.6 mM). Then the absorbance was read in microplate reader immediately and after 5 min at 490 nm to determine LDH activity. The amount of LDH released is proportional to the number of cells damaged or lysed.

Statistical analysis

All experiments were performed in triplicates for the accuracy of the results. Statistical analysis of the data was performed using the statistical package SPSS 17.0. Students t-test was used to determine the statistical significance where $P<0.05$ was set significant against the control samples.

Results

In vivo genotoxicity tests

Effect of DINP and DEHP, phthalate plasticizers, on micronuclei formation and other nuclear abnormalities in erythrocytes of the fish, Oreochromis mossambicus

Fish when exposed to the selected phthalate plasticizers, DINP and DEHP for 60 days showed induction in the micronuclei formation, and other nuclear abnormalities such as blebbed, notched and lobed nucleus in a time-dependent manner (Table 1; Figure 1). DINP exposure showed 69.2% nuclear abnormalities while DEHP treatment showed 75.7% abnormalities in the erythrocytes of the fish when compared to the control groups (Table 1).

Table 1. *Effect of DINP and DEHP, phthalate plasticizers, on micronuclei formation and other nuclear abnormalities in erythrocytes of the fish, Oreochromis mossambicus (Mean ± SD; n = 10; * P<0.05 against the control groups).*

	Treatment groups	Micronucleus	Blebbed nucleus	Notched nucleus	Lobed nucleus	Abnormalities (%)
	Control	4.3 ± 0.6	3.5 ± 0.5	1.3 ± 0.5	1.5 ± 0.7	10.60
	Vehicle	4.5 ± 0.7	3.0 ± 1.4	1.5 ± 0.7	1.0 ± 0.0	10.00
DINP (300 ppm)	4 days	5.5 ± 0.6	4.3 ± 0.7	1.6 ± 0.5	2.0 ± 0.4	13.40
	7 days	7.4 ± 1.5*	6.1 ± 0.9*	2.3 ± 0.7	2.7 ± 0.5	18.50
	14 days	8.7 ± 0.6*	6.0 ± 0.1*	7.6 ± 0.5*	8.0 ± 0.2*	30.30
	30 days	12.0 ± 0.1*	9.6 ± 0.7*	11.3 ± 1.2*	11.3 ± 0.5*	44.20
	60 days	21.0 ± 1.0*	15.6 ± 0.6*	14.6 ± 1.1*	18.0 ± 0.4*	69.20
DEHP (60 ppm)	4 days	4.3 ± 0.3	3.3 ± 0.5	1.7 ± 0.6	2.0 ± 0.3	11.30
	7 days	5.7 ± 0.5	4.3 ± 0.5	2.7 ± 0.4	3.0 ± 0.1	15.70
	14 days	9.3 ± 0.6*	6.7 ± 0.5*	7.7 ± 0.5*	8.6 ± 1.2*	32.30
	30 days	13.6 ± 0.5*	10.0 ± 0.2*	12.0 ± 1.0*	13.3 ± 0.6*	48.90
	60 days	24.6 ± 1.2*	16.6 ± 1.1*	15.3 ± 1.4*	19.2 ± 1.7*	75.70

Effect of DINP and DEHP on DNA damage in erythrocytes of the fish, Oreochromis mossambicus using comet assay

There was no significant variation in the tail length and percent tail DNA in the erythrocytes of *Oreochromis mossambicus* when exposed to DINP and DEHP for 60 days (Table 2). Comet morphology of the erythrocytes showed no DNA damage after DINP and DEHP exposure for 60 days (Table 2; Figure 2).

Table 2. *Variation in tail length and tail DNA (%) after phthalates exposure in the fish, Oreochromis mossambicus (Mean ± SD)*

	Treatment groups	Tail length	Tail DNA (%)
	Control	8.88 ± 2.84	0.014 ± 0.002
	Vehicle	8.78 ± 2.76	0.011 ± 0.009
DINP (300 ppm)	4 days	8.85 ± 2.87	0.011 ± 0.005
	7 days	8.84 ± 2.75	0.012 ± 0.008
	14 days	8.83 ± 2.69	0.011 ± 0.006
	30 days	8.81 ± 1.92	0.012 ± 0.006
	60 days	8.92 ± 2.76	0.010 ± 0.004
DEHP (60 ppm)	4 days	8.87 ± 2.67	0.010 ± 0.008
	7 days	8.79 ± 2.79	0.012 ± 0.005
	14 days	8.84 ± 2.83	0.012 ± 0.007
	30 days	8.88 ± 2.72	0.011 ± 0.006
	60 days	9.03 ± 2.85	0.012 ± 0.001

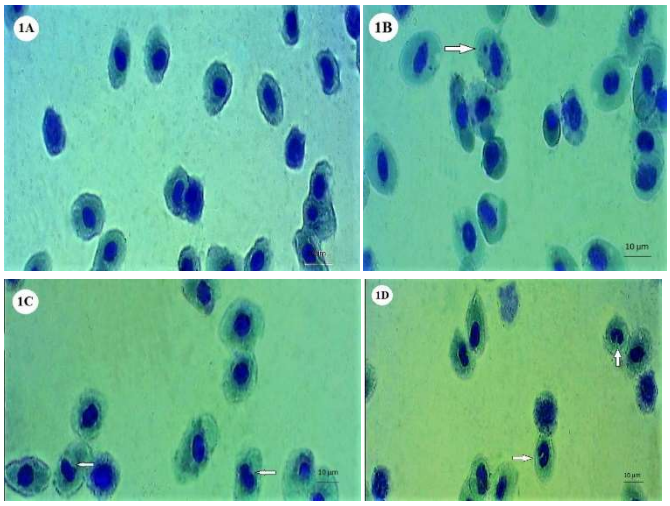

Figure 1. *Phthalates exposed fish erythrocytes showing A - Normal erythrocytes (control); B – erythrocytes with micronucleus (→); C – notched nucleus (←); and D - lobed nucleus (→, ↑)*

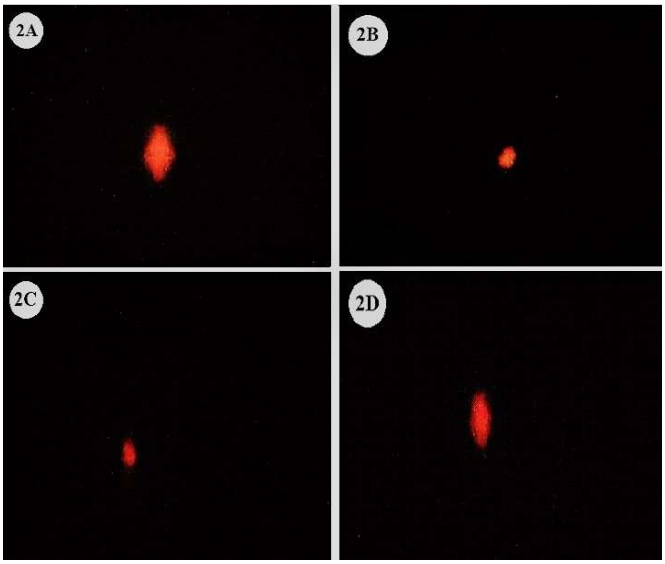

Figure 2. *Comet morphology showing no DNA damage after phthalates exposure in erythrocytes of Oreochromis mossambicus. A – Control; B – Vehicle; C – DINP (300 ppm for 60 days); D – DEHP (60 ppm for 60 days).*

***In vitro* genotoxicity tests**

Mutagenicity of DINP and DEHP in Salmonella typhimurium tester strains

DINP and DEHP exposure at 1, 2 and 5 µg/ ml concentrations induced reverse mutation in TA97A, TA98 and TA100 tester strains of *S. typhimurium* in a concentration-dependent manner (Table 3). The mutagenicity induced by the selected phthalate plasticizers are significantly ($P<0.05$) increased in a concentration-dependent manner in each tester strain (Table 3; Figures 3 and 4).

Effect of DINP and DEHP on cytokinesis-block micronucleus (CBMN) assay in human peripheral blood lymphocytes

Human peripheral blood lymphocytes treated with DINP and DEHP each at 0.5 and 1 µg/ ml concentrations showed significant ($P<0.05$) increase in nuclear anomalies such as the formation of micronuclei, nucleoplasmic bridge and nuclear buds in a concentration-dependent manner when compared to the control groups (Figures 5 and 6).

Comet morphology after DINP and DEHP exposure to CHO cell lines

CHO-K1 cell lines treated with DINP at 13.3 µg/ ml concentration did not cause DNA damage when compared to the control groups (Table 4; Figure 7). However, a significant ($P<0.05$) increase in the tail length and tail DNA percentage was observed after DEHP exposure at 29.1 µg/ ml concentration (Table 4; Figure 7).

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Table 3. *Mutagenicity of DINP and DEHP, phthalate plasticizers, in Salmonella typhimurium tester strains.*

		Number of colonies per plate (Mean ± SD; n = 3)		
		TA 97A	**TA 98**	**TA 100**
	Control	1 ± 0	1.66 ± 0.57	1.33 ± 0.57
	Vehicle	0.66 ± 0.57	1.33 ± 0.57	1.66 ± 1.15
	Positive control (Sodium azide)	134.66 ± 12.09*	3042.66 ± 176.3*	2384 ± 4*
DINP	1 µg/ ml	9.67 ± 0.57*	228.65 ± 17.47*	586.33 ± 34.67*
DINP	2 µg/ ml	12.30 ± 2.51*	518.33 ± 57.4*	828.66 ± 52.9*
DINP	5 µg/ ml	24.33 ± 5.50*	703 ± 65.3*	1305.65 ± 63.6*
DEHP	1 µg/ ml	15.33 ± 1.15*	263.67 ± 7.37*	978.61 ± 21*
DEHP	2 µg/ ml	17.67 ± 2.51*	376.60 ± 52*	1017.30 ± 5.03*
DEHP	5 µg/ ml	48.30 ± 3.51*	751 ± 18.7*	1123.66 ± 13.05*

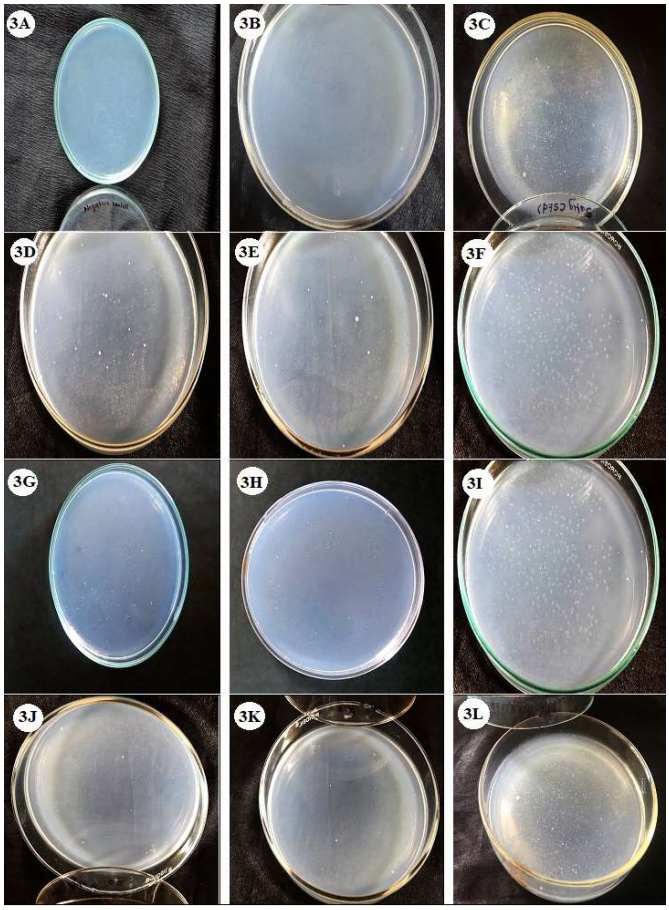

Figure 3. *Colonies of Salmonella typhimurium strains A – Control; B – Vehicle; C – Sodium azide (10 µg/ ml); D – F: TA97A strain exposed to DINP at 1, 2 and 5 µg/ml, respectively; G – I: TA98 strain exposed to DINP at 1, 2 and 5 µg/ ml, respectively; J-L: TA100 strain exposed to DINP at 1, 2 and 5 µg/ ml, respectively.*

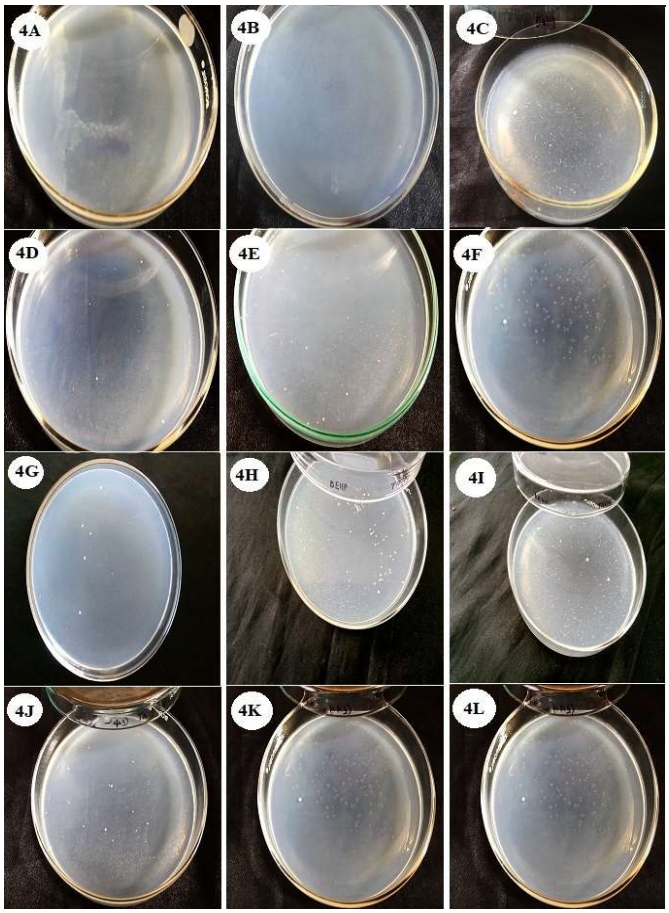

Figure 4. *Colonies of Salmonella typhimurium strains A – Control; B – Vehicle; C – Sodium azide (10 µg/ml); D – F: TA97A strain exposed to DEHP at 1, 2 and 5 µg/ml, respectively; G – I: TA98 strain exposed to DEHP at 1, 2 and 5 µg/ml, respectively; J – L: TA100 strain exposed to DEHP at 1, 2 and 5 µg/ml, respectively.*

Effect of DINP and DEHP on cell viability by MTT assay in CHO cell lines

The percentage of cell viability assessed using methyl tetrazolium (MTT) assay showed significant ($P<0.05$) decrease in a concentration-dependent manner after DINP and DEHP exposure when compared to the respective control groups (Figure 8-10).

Cytotoxicity of DINP and DEHP in CHO cell lines by LDH release assay

Assessment of cytotoxicity by lactate dehydrogenase (LDH) release assay revealed significant ($P<0.05$) increase in LDH release after 5, 7.5 and 10 µg/ ml concentrations of DINP and DEHP exposure, which was concentration-dependent (Figure 11).

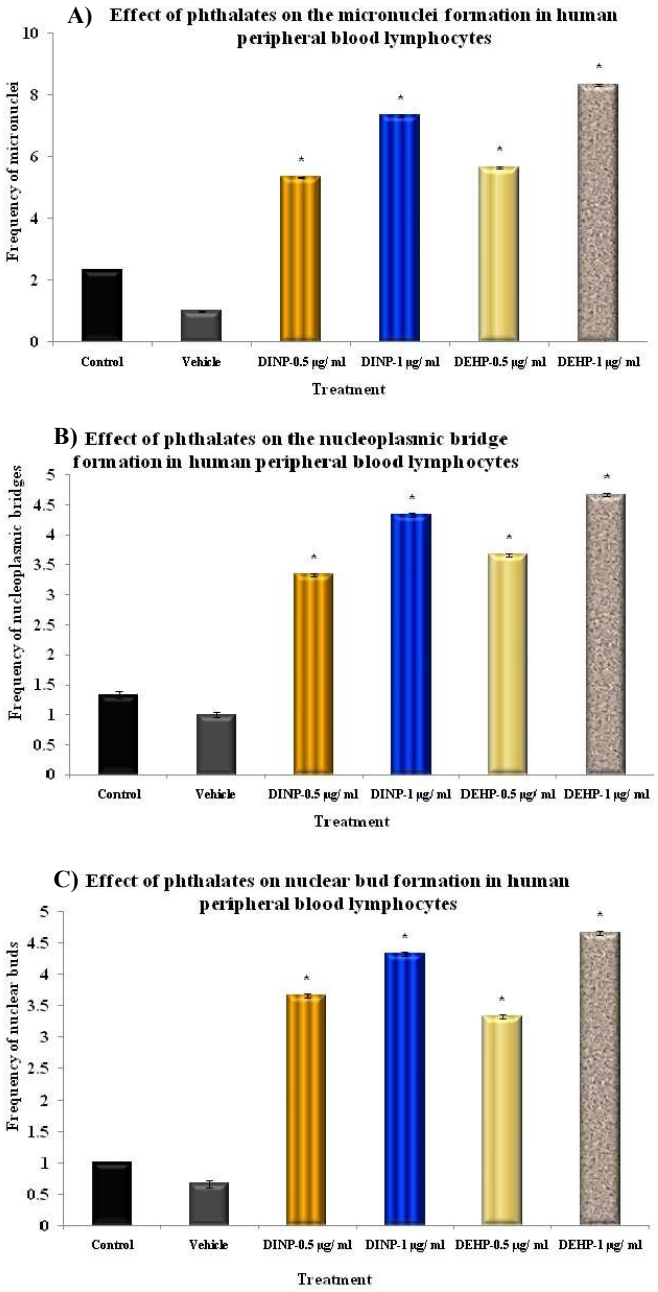

Figure 5. *Effect of phthalates on the micronuclei (A), nucleoplasmic bridge (B) and nuclei bud (C) formation in human peripheral blood lymphocytes.*

Discussion

The cytotoxicity and genotoxicity assessment of the selected phthalate plasticizers, DINP and DEHP were performed on the freshwater fish, *Oreochromis mossambicus* and on human lymphocyte cells, Chinese Hamster Ovary cell lines and in *Salmonella typhimurium*. Genotoxicity studies of chemical substances are widely used to identify the adverse effects of chemicals with respect to DNA damages in the form of gene mutation, recombination, numerical changes and structural chromosomal aberration. Most of the genetic changes are responsible for the heritable effects of the chemicals on germ cells that ultimately risks the future generations (Wassom, 1992). Among the available tests for the genotoxic assessment, micronucleus test and comet assay have attracted much attention. Micronucleus test detects the exposed toxicants as chromosomal damaging agents that act either as chromosome breakage agents or clastogens and/ or spindle dysfunction agents or aneugens (Maistro, 2014). The alkaline comet assay or single-cell gel electrophoresis (SCGE) assay, primarily used to measures DNA strand breakage in single cells identify substances that cause single- and double-strand DNA breaks, as a result of its direct or other interactions with DNA molecule (Tice et al., 2000). In the present study *in vivo* genotoxicity assessment was performed using micronucleus and comet assays in the erythrocytes of the freshwater fish, *Oreochromis mossambicus*.

DINP at 300 ppm concentration and DEHP at 60 ppm concentration exposed to fish for 60 days showed induction in the formation of micronuclei along with other nuclear abnormalities such as blebbed, notched and lobed nucleus in a time-dependent manner. Micronucleus test is one of the technically easier, rapid and simplest techniques used to identify genomic alterations in animals. The assay targets interphase cells of any proliferating cell population regardless of its karyotype, and are used as a biomarker in environmental biomonitoring programmes (Heddle et al., 1983). The formation of micronucleus occurs during the process of cell division but the expression takes place at different times after the DNA damage event, depending on the cell cycle kinetics and the mechanism of induction (Bolognesi and Hayashi, 2011). DINP and DEHP induced micronuclei formation and other nuclear abnormalities revealed the failure of metabolic capacity, DNA repair efficiency and deprived defensive mechanism of the fish, *Oreochromis mossambicus* against the exposed toxicants. The percentage of nuclear abnormalities was more prominent in DEHP treated fish showing 75.7% deformity thereby stating high genotoxic potency of the compound than DINP, the high molecular weight phthalate. *In vivo* DNA damage further assessed by comet assay showed no significant variation in the tail length and percent tail DNA in the erythrocytes of *Oreochromis mossambicus* when exposed to DINP and DEHP for 60 days. The absence of genotoxic activity for DINP and DEHP in comet assay was contradictory to the positive results obtained in the micronucleus test of fish erythrocytes.

It was difficult to generalize the genotoxicity of phthalates *in vivo* thus it required a battery of *in vitro* genetic tests. Several methods including Ames test, cytokinesis-block micronucleus (CBMN) assay, comet assay, cytotoxicity test using MTT assay and LDH release have been used to determine genotoxic properties of phthalates in this study. The mutagenicity induced by the selected phthalate plasticizers, DINP and DEHP, increased in a concentration-dependent manner in TA97A, TA98 and TA100 tester strains of *Salmonella typhimurium*. Tester strains TA97A and TA98 are used to determine frameshift mutation and TA100 strain has been widely used to assess point mutations (Mortelmans & Zeiger, 2000). Ames test is the widely used bacterial assay to identify mutagenic substances that produce genetic damage leading to gene mutations. The tester strains of *Salmonella* are with pre-existing mutations, which makes the bacteria unable to synthesize histidine amino acid, and therefore unable to grow and form colonies. However, new mutations induced by any mutagens, such as phthalates in this study, at the site or nearby the pre-existing mutations, restore the functions of a gene, which allow the bacterial cells to synthesize histidine. Thus the newly mutated cells can grow in the absence of histidine and form colonies, which are referred to as reverants (Mortelmans & Zeiger, 2000). The present results suggested that the phthalates functions as mutagens and penetrated into the cell wall of bacterial cells as evident by the increase in the number of revertant colonies thereby induced mutagenicity. Similar results have been observed when butyl cyclohexyl phthalate induced mutagenic activity in the presence of S9 mix with the *Salmonella* TA100 strain in the Ames test (Koksal et al., 2016).

The cytokinesis-block micronucleus (CBMN) assay widely used for measuring micronucleus in cultured human and/or mammalian cells scores specifically restricting once-divided binucleated cells, which are the cells that can express micronucleus (Fenech, 2000). CBMN assay quantify genomic instability such as chromosome breakage, DNA misrepair, loss or non-disjunction of chromosome, necrosis, apoptosis and cytostasis (Fenech et al., 1999). This method also determines nucleoplasmic bridges, a biomarker of dicentric chromosomes resulting from telomere end-fusions or DNA misrepair (Thomas et al., 2003), and nuclear buds, a biomarker of gene amplification (Shimizu et al., 1998).

Table 4. *Variation in tail length and tail DNA (%) after phthalates exposure in Chinese Hamster Ovary (CHO-K1) cells (Mean ± SD; * significance at P<0.05 against the control groups).*

Treatment groups	Tail length	Tail DNA (%)
Control	11.55 ± 0.62	1.89 ± 0.67
Vehicle	14.18 ± 0.66	0.74 ± 0.03
DINP (13.3 µg/ ml)	12.62 ± 0.34	0.98 ± 0.04
DEHP (29.1 µg/ ml)	23.00 ± 0.67*	83.32 ± 1.74*

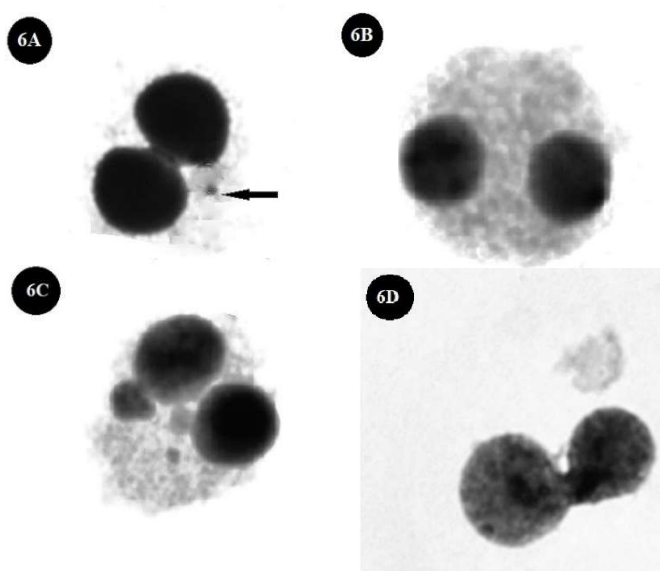

Figure 6. *Formation of A – micronuclei (←); B – Binucleated cells; C – Nuclear bud; D – Nucleoplasmic bridges after phthalate exposure in human peripheral blood lymphocytes.*

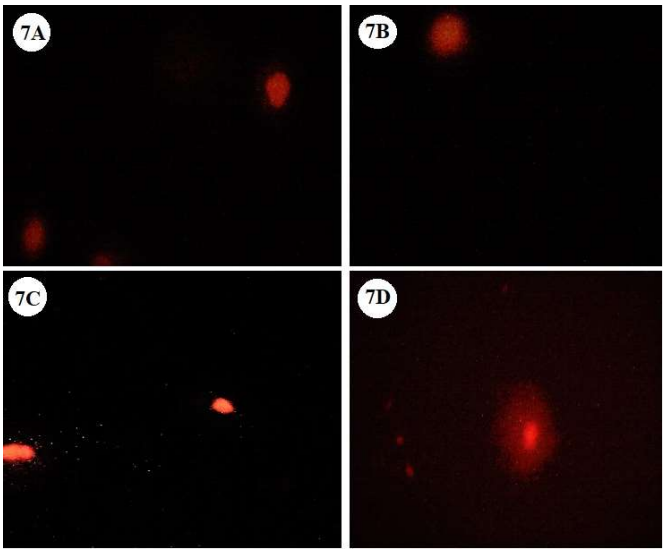

Figure 7. *Comet morphology showing the intensity of DNA damage after phthalates exposure in Chinese Hamster Ovary (CHO-K1) cells. A – Control; B – Vehicle; C – DINP (13.3 µg/ml); D – DEHP (29.1 µg/ml).*

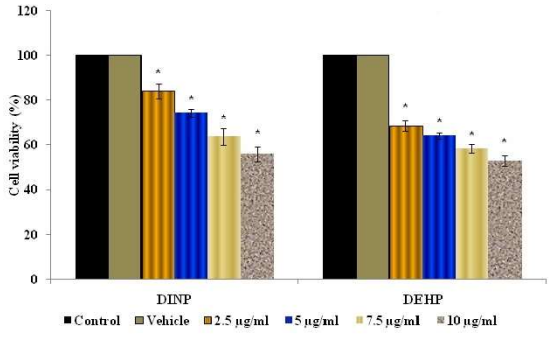

Figure 8. *Effect on phthalate plasticers on cell viability by MTT assay in Chinese Hamster Ovary (CHO-K1) cells.*

Nucleoplasmic bridges have been known to occur when centromeres of dicentric chromosomes are pulled to opposite poles of the cell at anaphase (Hoffelder et al., 2004). The process of nuclear budding occurs during S phase which is characterized by having the same morphology as micronucleus, but an exception that they are linked to the nucleus by a narrow or wide stalk of nucleoplasmic material depending on the stage of the budding process (Serrano-Garcia & Montero-Montaya, 2001). Formation of micronucleus has also been used as a surrogate marker of DNA hypomethylation where increased genome damage may be caused by hypermethylation of CpG islands within or adjacent to the promoter regions of housekeeping genes involved in cell-cycle check points and DNA repair (Norppa & Falck, 2003). Human peripheral blood lymphocytes treated with DINP and DEHP each at 0.5 and 1 µg/ ml concentrations showed an increase in nuclear anomalies such as the formation of micronuclei, nucleoplasmic bridge and nuclear buds in a concentration-dependent manner when compared to the control groups. The present observations indicated that exposure to phthalate induced chromosome damage thereby leading to genomic instability in human peripheral blood lymphocytes.

The comet assay or single cell gel electrophoresis used to assess DNA damage is based on the relaxation of supercoiled DNA in agarose-embedded nucleoids that allows the DNA to drawn out towards the anode under electrophoresis and the comet-like images are formed, which are observed under a fluorescence microscope (Azqueta & Collins, 2013). DNA damages such as single- and double-strand breaks together with alkali-labile sites and crosslinking are evaluated using comet assay. In the present study, comet assay was performed in CHO-K1 cell lines treated where DINP treatment did not cause DNA damage, however, DEHP treated at 29.1 µg/ ml concentration showed increase in the tail length and tail DNA percent thereby suggested DNA damage induced by DEHP and not DINP, thus correlating more genotoxic potential of DEHP. The indication of chromosome damage by an increase in micronucleus formation and tail DNA percent has been reported after exposure to different phthalate esters in TK6 human lymphoblast cell lines (Al-Saleh et al., 2017).

Cell viability and cytotoxicity tested using MTT method is based on the reduction of yellow tetrazolium MTT [3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] into insoluble (E,Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan or formazan, which depends upon cellular metabolic activities due to NAD(P)H-dependent cellular oxidoreductase enzymes (Berridge et al., 2005). Similarly,

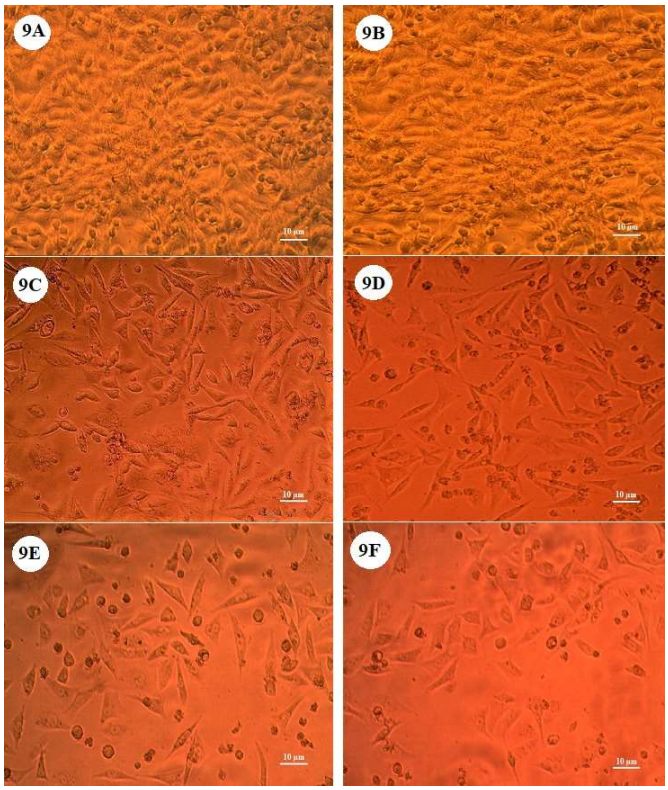

Figure 9. *Effect of DINP on cell viability by MTT assay in Chinese Hamster Ovary (CHO-K1) cells. A-Control; B-Vehicle; C-DINP (2.5 µg/ ml); D-DINP (5 µg/ ml); E-DINP (7.5 µg/ ml); and F-DINP (10 µg/ ml).*

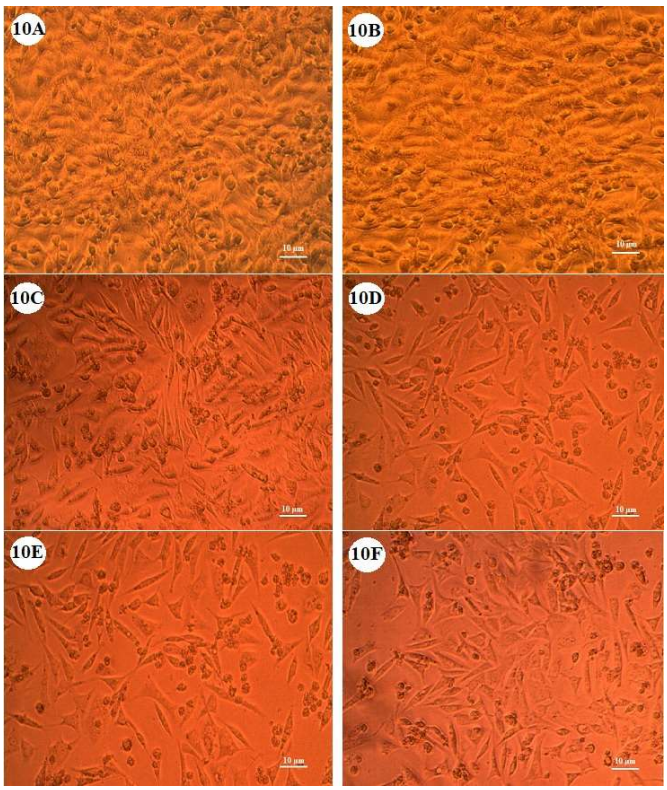

Figure 10. *Effect of DEHP on cell viability by MTT assay in Chinese Hamster Ovary (CHO-K1) cells. A-Control; B-Vehicle; C-DEHP (2.5 µg/ ml); D-DEHP (5 µg/ ml); E-DEHP (7.5 µg/ ml); and F-DEHP (10 µg/ ml).*

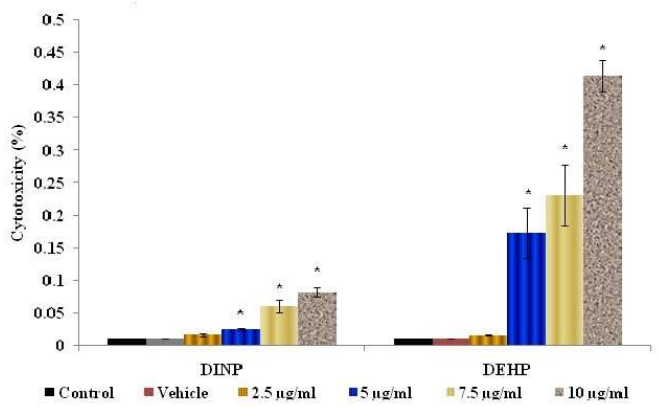

Figure 11. *Effect of phthalate plasticisers on LDH release in Chinese Hamster Ovary (CHO-K1) cells.*

another study suggested that diethylhexyl phthalate, di-n-butyl phthalate exposed to human sperm showed a concentration-and time-dependent decrease in cell viability, which has been found associated to increase in reactive oxygen species (ROS) generation and lipid peroxidation production (Pereira et al., 2007; Pant et al., 2011).Our previous studies have reported that DINP and DEHP induced ROS generation in reproductive and non-reproductive tissues of the freshwater fish, *Oreochromis mossambicus* (Revathy & Chitra, 2018a; 2018b; 2018c; 2019). There are several *in vivo* and *in vitro* studies correlating toxicant-induced oxidative stress and DNA damage. The role of ROS in the production of DNA single strand breaks has been demonstrated as one of the probable mechanisms of toxicity (Azqueta et al., 2009; Collins, 2009).

Cell viability test in CHO-K1 cell lines treated with DINP and DEHP showed a decrease in purple colour intensity in a concentration-dependent manner. While untreated cells was healthy and rapidly growing cells that exhibited high rates of MTT reduction to formazan with high purple colour intensity. In the treated cells, the intensity of purple colour decreased thereby signifies the reduction in cell number and cytotoxicity of the exposed phthalates. Further, the effect of DINP and DEHP on the inhibition of cell growth was analysed using lactate dehydrogenase (LDH) release test. LDH, a cytosolic enzyme present in most eukaryotic cells, are released into culture medium upon cell death due to damage of the plasma membrane. The culture supernatant of CHO-K1 cells treated with DINP and DEHP showed a concentration-dependent increase in LDH activity, which indicated a proportional increase in the number of lysed cells. Besides phthalates, our laboratory studies have reported that several other nanoparticles also induced cytotoxicity in the CHO-K1 cells due to their rapid internalisation and unique physico-chemical properties (Vidya & Chitra, 2018).

Conclusion

The present *in vivo* and *in vitro* observations suggested that the selected phthalate esters, di-isononyl phthalate and di-(2-ethylhexyl) phthalate are the potent cytotoxic and genotoxic agents, and thus the concentrations found similar in some clinical or personal care products may affect the human health status. Therefore, further studies are necessary to shed light on the possible mechanism of genotoxicity focusing on human subjects.

References

Al-Saleh, Al-Rajudi T, Al-Qudaihi G, Manogaran P. 2017. Evaluating the potential genotoxicity of phthalates esters (PAEs) in perfumes using *in vitro* assays. Environ. Sci. Pollut. Res. Int., 24(30): 23903-23914.

Anderson D, Yu TW, Hıncal F. 1999. Effect of some phthalate esters in human cells in the comet assay. Teratog. Carcinog. Mutagen., 19: 275–280.

APHA. 1998. Standard methods for the examination of water and waste water, 20th Edition, Washington.

Azqueta A, Collins AR. 2013. The essential comet assay: a comprehensive guide to measuring DNA damage and repair. Arch. Toxicol., 87(6): 949-968.

Azqueta A, Lorenzo Y, Collins AR. 2009. *In vitro* comet assay for DNA repair: a warning concerning application to cultured cells. Mutagenesis, 24:379-381.

Berridge MV, Herst PM, Tan AS. 2005. Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. Biotechnol Annu Rev., 11: 127-152.

Bolognesi C, Hayashi M. 2011. Micronucleus assay in aquatic animals. Mutagenesis, 26(1): 205-213.

Carrasco KR, Tilbury KL, Myers MS. 1990. Assessment of the piscine micronucleus test as *in situ* biological indicator of chemical contaminant effects. Canadian J. Fish. Aquat Sci., 47: 2123- 2136.

Collins AR. 2009. Investigating oxidative DNA damage and its repair using the comet assay. Mutat. Res., 681(1): 24-32.

Decker T, Lohmann-Matthes ML. 1988. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor TNF activity. J. Immunol. Methods, 115(1): 61-69.

Fenech M, Chang WP, Kirsch-Volders M, Holland N, Bonassi S, Zeiger E. 2003. HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutat. Res., 534(1-2): 65-75.

Fenech M, Crott J, Turner J, and Brown S. 1999. Necrosis, apoptosis, cytostasis and DNA damage in human lymphocytes measured simultaneously within the cytokinesis-block micronucleus assay: description of the method and results for hydrogen peroxide. Mutagenesis, 14(6): 605-612.

Fenech M. 2000. The *in vitro* micronucleus technique. Mutat. Res., 455(1-2): 81-95.

Fenech M. 2007. Cytokinesis-block micronucleus cytome assay. Nat Protoc. 2(5): 1084-1104.

Heddle JA, Hite M, Kirkhart B, Mavoumin K, Mac Gregor JT, Newell GT, Salamone MF. 1983. The induction of micronuclei as a measure of genotoxicity. A report of the U.S. environmental protection agency gene-tox program. Mutat. Res., 123(1): 61-118.

Heddle JA. 1973. A rapid *in vivo* test for chromosomal damage. Mutat. Res., 18(2): 187-192.
Heudorfa U, Sundermann V, Angerer J. 2007. Phthalates: toxicology and exposure. Int. J. Hyg. Environ. Health., 210(5): 623-634.
Hoffelder DR, Luol L, Burke NA, Wattkins SC, Gollins SM, Saunders WS. 2004. Resolution of anaphase bridges in cancer cells. Chromosoma., 112(8): 389-397.
Hogberg J, Hanberg A, Berglund M, Skerfving S, Remberger M, Calafat AM, Filipsson AF, Jansson B, Johansson N, Appelgren M. 2008. Phthalate diesters and their metabolites in human breast milk, blood or serum, and urine as biomarkers of exposure in vulnerable populations. Environ. Health Perspect., 116(3): 334-339.
Kanode R, Chandra S, Sharma S. 2017. Application of bacterial reverse mutation assay for detection of non-genotoxic carcinogens. Toxicol. Mech. Methods., 27(5): 376-381.
Koksal C, Nalbantsoy A, Karabay-Yavasoglu NU. 2016. Cytotoxicity and genotoxicity of butyl cyclohexyl phthalate. Cytotechnology., 68(2): 213-222.
Maistro EL. 2014. The *in vivo* rodent micronucleus test. In: Genotoxicity and DNA repair: A practical approach (Sierra LM and Gaivão I, eds.). Humana Press, New York.
McKee RH, Przygoda RT, Chirdon MA, Engelhardt G, Stanley M. 2000. Di(isononyl) phthalate (DINP) and di(isodecyl) phthalate (DIDP) are not mutagenic. J. Appl. Toxicol., 20: 491-497.
Mortelmans K, Zeiger E. 2000. The Ames Salmonella/ microsome mutagenicity assay. Mutat. Res., 455(1-2): 29-60.
Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods, 65(1-2): 55-63.
Norppa H, Falck GC. 2003. What do human micronuclei contain? Mutagenesis, 18(3): 221-233.
OECD 471. 1997. Bacterial reverse mutation test. Organization for Economic Cooperation and Development, Paris.
Pant N, Pant A, Shukla M, Mathur N, Gupta Y, Saxena D. 2011. Environmental and experimental exposure of phthalate esters: the toxicological consequence on human sperm. Hum. Exp. Toxicol., 30(6): 507-514.
Pereira C, Mapuskar K, Rao CV. 2007. Effect of diethyl phthalate on rat testicular antioxidant system: a dose-dependent toxicity study. Pestic. Biochem. Physiol., 90: 52-57.
Quinn-Hosey KM, Roche JJ, Fogarty AM, Brougham CA. 2012. A toxicological assessment of endocrine disrupting chemicals found in the BMW (Border, Midland and Western) region of Ireland. J. Environ. Prot., 3(4): 304-315.
Revathy V, Chitra KC. 2015. Acute exposure to diisononyl phathalate (DINP) influenced histopathological and behavioural modification on the freshwater fish, *Oreochromis mossambicus* (Peters, 1872). Int. J. Res., 2(4): 465-469.
Revathy V, Chitra KC. 2018a. Effects of diisononyl phthalate on the antioxidant status in gill, liver and muscle tissues of the fish, *Oreochromis mossambicus*. Asian J. Adv. Basic Sci., 6(1): 37-48.
Revathy V, Chitra KC. 2018b. Di(2-ethylhexyl)phthalate-induced lipid peroxidation and associated oxidative stress in gill, liver and muscle tissues of the fish, *Oreochromis mossambicus* (Peters, 1852). Int. J. Theor. Appl. Sci., 10(2): 1-9.
Revathy V, Chitra KC. 2018c. Di-isononyl phthalate (DINP) impairs reproduction in the freshwater fish, *Oreochromis mossambicus* (Peters, 1852). Asian Fish. Sci., 31(4): 284-296.
Revathy V, Chitra KC. 2019. Role of di-(2-ethylhexyl)-phthalate on the antioxidant status in testes and ovary of the fish, *Oreochromis mossambicus* (Peters, 1852). Int. J. Pharm. Biol. Sci. 9(1): 1186-1194.
Schmid W. 1975. The micronucleus test. Mutat. Res., 31(1): 9-15.
Serrano-Garcia L, Montero-Montaya R. 2001. Micronuclei and chromatid buds are the result of related genotoxic events. Environ. Mol. Mutagen., 38(1): 38-45.
Shimizu N, Itoh N, Utiyama H, Wahl GM. 1998. Selective entrapment of extra chromosomally amplified DNA by nuclear budding and micronucleation during S phase. J. Cell Biol., 140(6): 1307-1320.
Silva MJ, Barr DB, Reidy JA, Kato K, Malek NA, Hodge CC, Hurtz D, Calafat AM, Needham LL, Brock JW. 2003. Glucuronidation patterns of common urinary and serum monoester phthalate metabolites. Arch. Toxicol., 77(10): 561-567.
Singh NP, McCoy MT, Tice RR, Schneider E L. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res., 175(1): 184-191.
Stojanoska MM, Milosevic N, Milic N, Abenavoli L. 2017. The influence of phthalates and bisphenol A on the obesity development and glucose metabolism disorders. Endocrine, 55(3): 666-681.
Takashima K, Ito Y, Gonzales FJ, Nakajima T. 2008. Different mechanisms of DEHP-induced hepatocellular adenoma tumorigenesis in wild-type and Ppar alpha-null mice. J. Occup. Health, 50(2): 169-180.
Thomas P, Umegaki K, Fenech M. 2003. Nucleoplasmic bridges are a sensitive measure of chromosome rearrangement in the cytokinesis-block micronucleus assay. Mutagenesis, 18(2): 187-194.
Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryo SC, Sasaki YF. 2000. Single cell/gel comet assay: guideline for *in vitro* and *in vivo* genetic testing. Environ. Mol. Mutagen., 35(3): 206-221.
Vidya PV, Chitra KC. 2018. Evaluation of genetic damage in *Oreochromis mossambicus* exposed to selected nanoparticles by using micronucleus and comet bioassays. Croat. J. Fisheries, 76: 140-158.
Wang Y, Zhu H, Kannan K. 2019. A review of biomonitoring of phthalate exposures. Toxics., 7(2): pii: E21.
Wassom J. 1992. Origins of genetic toxicology and the environ mutagen society. Environ. Mol. Mut., 14(S16): 1-6.
Yang G, Zhou X, Wang J, Zhang W, Zheng H, Lu W and Yuan J. 2012. MEHP-induced oxidative DNA damage and apoptosis in HepG2 cells correlates with p53-mediated mitochondria-dependant signalling pathway. Food Chem. Toxicol., 50(7): 2424-2431.