In-Vivo Cytogenetic Damage in Freshwater Cyprinid Crucian Carp (*Carassius carassius* L.) upon Endosulfan Exposure

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Abstract

The *in vivo* cytogenetic damage in crucian carp, experimentally exposed to sub-lethal concentrations of endosulfan, was evaluated. The $LC_{50\text{-}96h}$ (95% confidence limits) value of endosulfan was 0.070 (0.046-0.093) ppm; on its basis three test concentrations (sub-lethal-SL-I: 0.052, II: 0.035 and III: 0.017 ppm) were selected for *in vivo* exposure. Autopsy was done on 24, 48, 72 and 96 h post exposure for assessment of chromosomal aberrations (CA) and micronuclei formation (MN). Peripheral blood samples, withdrawn by caudal puncture, were used for micro-nuclei assay and chromosome preparations were made from highly mitotic anterior kidney cells. All the tested concentrations of endosulfan showed increased frequencies of CA and MN in a concentration-dependent manner, with induction of maximum genotoxic effects at highest concentration (SL-I; $p < 0.05$). The group exposed to positive genotoxin, cyclophosphamide also showed significant induction ($p < 0.05$) of CA and MN. The results of the present investigation indicated that endosulfan could potentially induce genotoxic effects in fish, even at sub-lethal concentrations and among the numerous bioindicators used in the context of water monitoring, genotoxicity assessment is one of the most important tools. The potential role of these parameters as bioindicators of aquatic pollutants is discussed.

Keywords: Endosulfan, fish, micronucleus, chromosomal aberration, environmental monitoring.

Introduction

Although modern agriculture seeks to achieve a sustainable use of agro chemicals, the amount of pesticides applied in pest control still represents one of the major burdens to the environment. Environmental pollution caused by pesticides, especially in aquatic ecosystems, poses a potential threat for aquatic organisms, and ultimately the entire food chain (Velisek *et al*., 2010; Ondarza *et al*., 2014). Contamination of aquatic bodies has been well-documented worldwide and constitutes a major issue at local, regional, national, and global levels (Angélique *et al*., 2013).

Some Persistent Organic Pollutants (POPs) are found in the environment and can bioaccumulate in an organism along the food chain. Endosulfan, a cyclodiene insecticide, is one such organochlorine compound that has been classified as highly toxic by the majority of environmental protection agencies (Dar *et al*., 2015). In India, endosulfan is classified as an ''extremely hazardous'' pesticide (Ganeshwade *et al*., 2012). Although its use is restricted to certain crops, residues of this compound have been detected in aquatic environment, and it

consequently affects the fish that play a highly important role at the tropic level. Therefore, there is an urgent demand for studies that can correlate the effects of this pesticide in fish (Hoang *et al*., 2011).

It has been suggested that the progress of environmental toxicology requires the development of a battery of bioindicators to evaluate chemical hazards (Brain and Cedergreen, 2008). In the biomarker selection sensitivity is the key factor because greater the biomarker sensitivity to the xenobiotic exposure the earlier will be its response, avoiding occurrence of deleterious effects on the organism or the population (Den Besten and Munawar, 2005).

Toxic effects of pesticides have been studied in several fish species (Monteiro *et al*., 2006; Toni *et al*., 2010). The use of fish as a bio-indicator of pollutant effects is being more and more used since fish are very sensitive to changes in their environment and play significant roles in assessing potential risks associated with contamination of new chemicals in aquatic environment (Lakra and Nagpure, 2009). Ecotoxicological characteristics of freshwater fish, *Carassius carassius* L., such as its wide distribution, availability throughout the year, easy maintenance and commercial importance makes it an excellent model for toxicity studies. Since there is growing concern over the presence of pesticide residues in the aquatic environment, it is important to develop or standardize existing methods for assessing the deleterious effects of xenobiotics in aquatic organisms (Dar *et al*., 2014).

Negative effects of endosulfan on fish have been well documented including histological (Ballesteros *et al*., 2007), physiological (Dorval *et al*., 2003), hematological (Rehman, 2006), neurological (Dutta and Arends, 2003), behavioural (Rehman, 2006), immunological (Ganeshwade *et al*., 2012) and endocrine-disrupting potential (Bisson and Hontela, 2002), but there is a dearth of data on the cytogenetic damage induced by endosulfan at environmentally realistic concentrations. This is the aim of the present study.

Materials and Methods

Experimental fish and chemicals

Healthy fish specimen of *C. carassius* L. (Family: Cyprinidae and Order: Cypriniformes) of a length of 12.5 ± 1.6 cm and weight of 33 ± 5 g were procured with the help of a local fisherman from the Dal Lake (34°07′N 74°52′E) in the vicinity of the University of Kashmir, Srinagar, India, transported live to the laboratory, and subjected to a prophylactic treatment by bathing in a 0.05 % aqueous solution of potassium permanganate for 2 min to avoid dermal infection. The fish stock was then acclimatized for at least 3 weeks to a 1:1 diurnal photoperiod in well aerated 60 L glass aquaria with 24 h aged dechlorinated tap water (pH 7.6 – 8.4) and fed *ad libitum* daily with commercially available fish food (Feed Royal®, Maa Agro Foods, Visakhapatnam, Andhra Pradesh, India). Waste products were siphoned off daily to prevent 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. Endosulfan and cyclophosphamide were purchased from the Sigma Aldrich, Bengaluru, India.

Determination of acute toxicity

Determination of the $LC_{50\text{-}96h}$ of endosulfan to *C. carassius* was conducted in a semi-static system with 60 L glass aquaria, changing the endosulfan solution (99.5 % pure) every alternate day to maintain its similar concentration. Briefly, triplicate sets of 10 fish each were exposed to endosulfan at concentrations of 0.01, 0.03, 0.05, 0.07, 0.09, 0.1, 0.5 and 1 ppm derived from a range finding test. Fish were not fed throughout the experiment and lethality was the toxicity end-point. Fish were visually examined daily and considered dead when no respiratory movements or no sudden swimming in response to gentle touching were observed. The $LC_{50\text{-}96h}$ of endosulfan was determined by probit analysis (Finney and Stevens, 1948).

In- vivo exposure

The experiments consisted of five treatments each with 4 replicates, in total 20 aquaria, containing 60 L dechlorinated and well-aerated tap water with 10 fish in each aquarium. Fish maintained in dechlorinated tap water

served as negative (treatment 1) and those exposed to cyclophosphamide at a concentration of 4 ppm (Özkan *et al*., 2011) as positive control (treatment 2). In treatments 3 – 5, the fish were kept in water containing endosulfan at concentrations of 0.052 (SL-I; 3/4 of $LC_{50\text{-}96h}$), 0.035 (SL-II; 1/2 of $LC_{50\text{-}96h}$) and 0.017 ppm (SL-III; $1/4^{th}$ of $LC_{50\text{-}96h}$) and autopsy was done at 24, 48, 72 and 96 h. On each sampling interval, ten fish were sacrificed; five fish were processed for the chromosomal aberration test (0.05% colchicine treatment was given prior to 3 h of autopsy) and the micronucleus assay was carried out from the blood of the rest five fish as per standard protocols (Dar *et al*., 2014, 2015). Some important physico-chemical properties of test water like temperature 18.2–23.3°C, pH 7.5–8.4, dissolved oxygen 7.9–8.4 mgL^{-1}, total alkalinity 69 –73 mgL^{-1} and ammonical nitrogen 25–29 μgL^{-1} were analyzed throughout the study by standard methods (APHA, AWWA and WPCF, 2005).

Micronucleus test

Blood samples were withdrawn by caudal puncture and peripheral blood smears were immediately made by applying two micro drops of blood on precleaned slides using the standard method of Al-Sabti and Metcalfe (1995). For every sampling time, replicate slides per specimen were prepared and a minimum of 10,000 erythrocytes scored in each treatment group, were examined for the presence of MN. The frequency of MN was calculated per 1000 cells (Raisuddin and Jha, 2004), and was evaluated by scoring the slides under oil immersion at 1000x magnification using Olympus BX 50 microscope (Tokyo, Japan). Coded and randomized slides were scored using blind review by a single observer to avoid any technical variation. The criteria for the identification of micronuclei were according to standard procedures (Fenech *et al*., 2003).

Chromosomal aberration test

Chromosome preparations were made from the highly mitotic head kidney cells, following the techniques of Cucchi and Baruffaldi (1990), and observed with light microscope (100x) for chromosomal aberrations. Replicate slides were selected per fish and a minimum of 25 metaphases were scored from each slide in each group including control (since n = 5 per group/exposure time, minimum 250 metaphases). Notwithstanding the conventional method of scoring, the CA was recorded under two broad categories i.e. classical aberration and non classical aberration. In the classical aberrations, both chromosome and chromatid type breaks, including acentric fragments, sister chromatid union and multiple aberrations (polyploidy, aneuploidy, rings etc) were counted and non-classical aberration comprised of stickiness, pulverization and c-metaphases.

Statistical analysis

Probit analysis was performed with the SPSS (version 16.0) computer program (SPSS Inc. Chicago, IL, USA), with the consultation of Finney's table (Finney and Stevens, 1948). Data was compared for statistically significant difference between control and treatment groups using one-way analysis of variance (ANOVA). Significant difference in ANOVA were further analyzed by post-hoc Bonferroni's, Newman-Keuls and Dunnett's multiple comparison test using Graph Pad Prism 5 software (Graph Pad Software, Inc. San Diego, CA). The p-values less than 0.05 were considered statistically significant.

Results

Acute toxicity

In acute toxicity bioassay, the LC_{50} values (with 95% confidence limits) of different concentration of endosulfan in *C. carassius* (Fig. 1) were found to be 0.215 (0.158-0.272), 0.15 (0.112-0.191), 0.095 (0.075-0.114) and 0.070 (0.046-0.093) ppm for 24, 48, 72 and 96 h, respectively. A dose dependent increase and time dependent decrease were observed in mortality rate such that as the exposure time increases from 24 to 96 h, the median concentration

required to kill thefish was reduced. Based on the $LC_{50\text{-}96h}$ value, the SL-I, II and III were determined as 0.052, 0.035 and 0.017 ppm, which were further used for *in vivo* exposure.

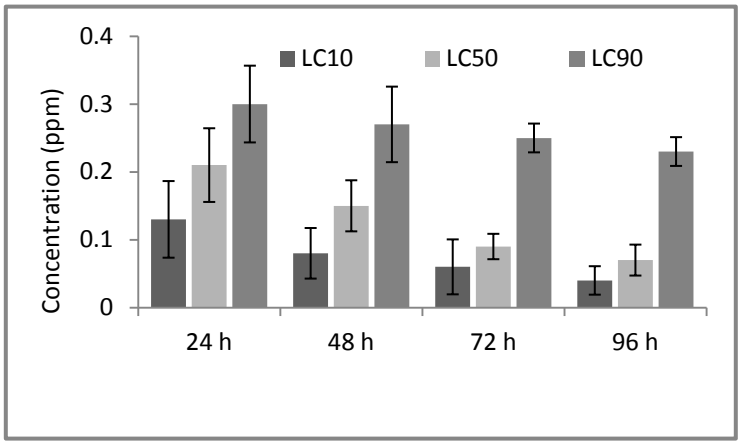

Fig. 1. Lethal concentration (LC) of endosulfan depending on exposure time for *C. carassius*.

Micronuclei Induction

The results of MN induction in peripheral blood erythrocytes of *C. carassius* after exposure to different concentrations of endosulfan are presented in Table 1. It caused one, two and three micronucleated cells but single MN was predominant in the erythrocytes analyzed (Fig. 2). The induction was signficantly ($P \leq 0.05$) hi gher in all the treatment groups compared to the control at all the exposure durations. The maximum MN frequency was observed on day 4 (6.071%; $p < 0.001$) at the highest concentration (SL-I), followed by SL-II and SL-III concentrations. Treatment with genotoxic agent (cyclophosphamide; positive control) also resulted in an extremely significant increase ($p < 0.001$) in the MN frequencies at all the sampling intervals. A concentration-dependent response in MN induction was observed.

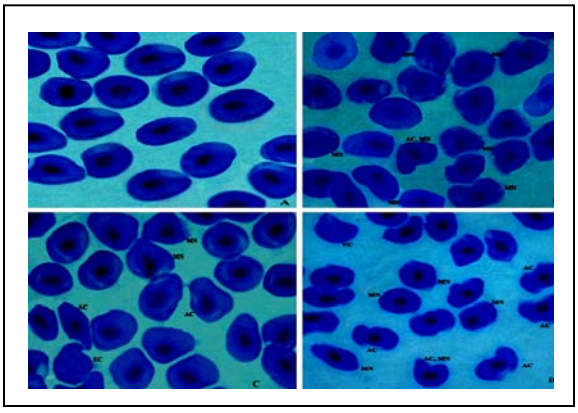

Fig. 2. Some features of erythrocyte alterations and micronuclei induction by endosulfan in *C. carassius* (96 h). A: normal control, B: SL-I (0.017 ppm), C: SL-II (0.035ppm), D: SL-III (0.052 ppm), single micronucleus (MN), two micronuclei (TM), altered cell (AC) and enucleated (EN) condition is unique to Endosulfan (magnification 1000×).

Table 1. Mean (S.E.) percentage micronuclei frequency in peripheral erythrocytes of *C. carassius* exposed to sub-lethal concentrations of endosulfan (n =~ 10000 cells/concentration/exposure time).

Chemical	Conc. (ppm)	Exposure time (days)			
		1	2	3	4
NC	----	0.181 (0.007)	0.230 (0.023)	0.191 (0.016)	0.300 0.015)^{3}
PC	4.00	3.483 (0.126)^{C}	4.100 (0.060)^{C3}	4.386 (0.045)^{C3}	7.526 (0.113)^{C3}
Endosulfan					
SL I	0.052	2.590 (0.080)^{C}	3.171 (0.065)^{C3}	3.575 (0.028)^{C2}	6.071(0.058)^{C3}
SL II	0.035	1.270 (0.021)^{C}	1.801 (0.022)^{C3}	2.168 (0.046)^{C2}	2.550(0.033)^{C2}
SL III	0.017	0.671 (0.017)^{A}	0.911 (0.016)^{A3}	1.010 (0.028)^{A2}	1.406 (0.025)^{A3}

NC: Negative control (tap water), PC: Positive control (cyclophosphamide), SL I: Sub lethal I (¾of LC_{50}), SL II: Sub lethal II (½ of LC_{50}), SL III: Sub lethal (¼ of LC_{50}). Values with different letter superscripts ($^{A}p<0.05$: significant. $^{B}p<0.01$: highly significant. $^{C}p<0.001$: extremely significant) differ significantly from the negative control, whereas values with different numeric superscripts ($^{1}p<0.05$: significant. $^{2}p<0.01$: highly significant. $^{3}p<0.001$: extremely significant) differ significantly between exposure times within concentration.

Chromosomal aberrations

The typical diploid metaphase complements of fish, *C. carassius*, were found to consist of 100 chromosomes of four types such as submetacentric, metacentric, subtelocentric and acrocentric. Various forms of chromosome damage recorded were chromosome and chromatid breaks, fragments, sister chromatid union, dicentric, multiple aberrations, stickiness, pulverization and c-metaphases; whereas gaps were excluded. The frequency of CA observed in *C. carassius* after exposure to different concentrations of endosulfan and cyclophosphamide were significantly ($P \leq 0.05$) higher when compared to the control 1 (Table 2, Fig. 3), at all the exposure durations, and the chromatid and chromosome breaks were more frequent than the other types of aberrations. The maximum CA, like MN frequency, was observed on day 4 (12.14%; $p < 0.01$) at the highest concentration (SL-I). In general, a concentration-dependent response was also observed in case of CA.

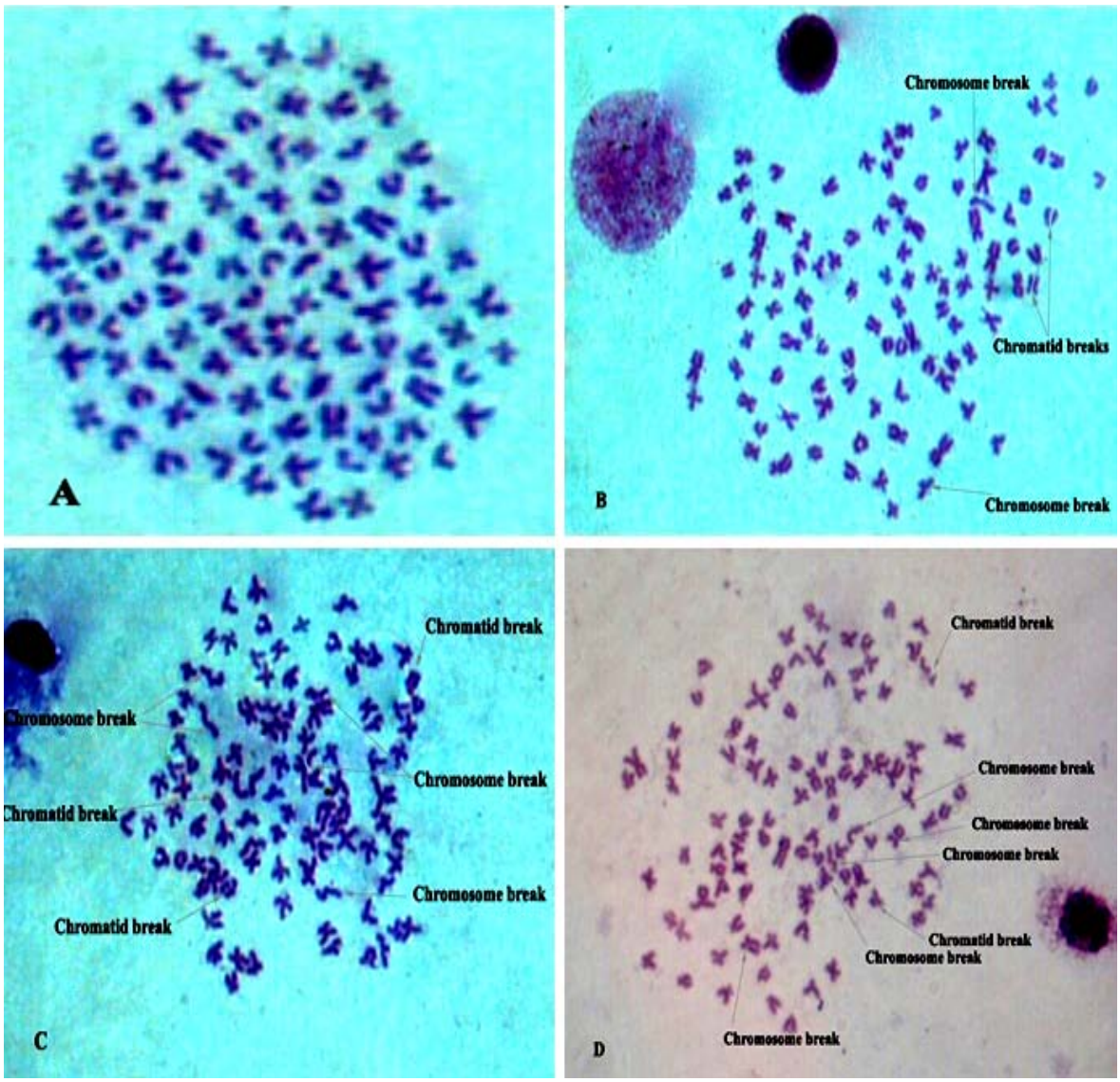

Fig. 3: Metaphase plates prepared from kidney cell of *Carassius carassius* showing (A) normal chromosomes (2n = 100), (B, C and D) chromosomal aberrations from endosulfan exposed fish (SL I-0.052, SL II-0.035, SL III-0.017 ppm), respectively for 96 h.

Table 2. Chromosomal aberration frequencies induced by endosulfan in *Carassius carassius* kidney cells

Exp. days	Treatment	TMS	Classical aberrations						Non-classical aberr.		TA mean (%) ± S.D.
			Csb	Ctb	Frg	Scu	Dic	Mla	Stp	Cmt	
1	NC	103	1	–	–	–	–	–	1	–	1.94 ± 0.13^{2}
	PC	105	3	1	2	–	–	–	2	1	8.57 ± 0.41^{B}
	SL I	113	2	1	1	–	–	–	1	2	6.19 ± 0.30^{B2}
	SL II	101	2	1	1	–	–	–	1	–	4.95 ± 0.25^{B2}
	SL III	107	1	2	–	–	–	–	–	1	3.73 ± 0.23^{A2}
2	NC	105	2	1	–	–	–	–	–	–	2.85 ± 0.21^{2}
	PC	109	3	2	3	–	–	–	1	1	9.17 ± 0.46^{B}
	SL I	117	2	2	1	1	1	–	2	–	7.69 ± 0.37^{B1}
	SL II	108	1	2	1	–	–	–	1	1	5.55 ± 0.26^{B2}
	SL III	113	1	2	–	–	–	–	1	1	4.42 ± 0.20^{A2}
3	NC	104	1	1	–	–	–	–	1	–	2.88 ± 0.16^{2}
	PC	115	3	2	2	1	1	1	2	–	10.43 ± 0.44^{B}
	SL I	110	2	2	1	1	1	–	2	1	9.09 ± 0.37^{B}
	SL II	106	3	1	1	–	–	1	1	–	6.60 ± 0.34^{B2}
	SL III	109	2	2	1	–	–	1	–	–	5.50 ± 0.29^{B2}
4	NC	119	2	1	–	–	–	–	1	–	3.36 ± 0.22^{2}
	PC	116	3	3	2	2	2	2	2	2	15.51 ± 0.58^{B}
	SL I	107	2	2	1	2	2	2	1	1	12.14 ± 0.47^{B2}
	SL II	102	2	1	1	–	1	1	1	1	7.84 ± 0.30^{B2}
	SL III	112	2	1	1	1	1	–	1	–	6.25 ± 0.27^{B2}

Exp: Exposure time in days. **TMS:** Total metaphasic plates studied. **NC:** Negative control (tap water). **PC**: Positive control (cyclophosphamide: 4 ppm). **SL I:** Sub lethal I (1/25 of LC_{50}: 0.052 ppm). **SL II**: Sub lethal II (1/50 of LC_{50}: 0.035 ppm). **SL III:** Sublethal III (1/75 of LC_{50}: 0.017 ppm). **Csb:** chromosome break**. Ctb:** Chromatid break. **Frg:** fragment. **Scu:** sister chromatid union. **Dic:** dicentric. **Mla:** multiple aberrations. **Stp:** stickiness and pulverization. **Cmt:** c-metaphase. Values with different letter superscripts differ significantly from the negative control (Newman-Keuls and Dunnett's multiple comparison tests), whereas values with different numeric superscripts differ significantly from the positive control (Dunnett's multiple comparison test).

Discussion

At present, more than 1000 chemicals have been classified as pesticides and studies using different models have indicated that some of them have genotoxic properties (Zeljezic and Garaj-Vrhovac, 2002). Fish are often used as sentinel organism for ecotoxicological studies because they play a number of roles in the tropic web, accumulate toxic substances and respond to low concentration of mutagens in a similar way to higher vertebrates (Osman *et al*., 2007).

The $LC_{50\text{-}96h}$ value of the endosulfan in the present study was 0.070 ppm which indicated that it is very toxic to fish. Our estimate is higher than the $LC_{50\text{-}96h}$ value of 0.0035 ppm for *Channa striatus* (Ganeshwade *et al*., 2012). The variation may be due to the difference and hardiness of the test species and water quality parameters. Micronucleus test, as a genotoxic endpoint, for clastogenic effects of pollutants has been extensively used in fish such as prussian carp (*Carassius auratus*), rainbow trout (*Oncorhynchus mykiss*), tilapias (*Oreochromis mossambicus*) and salmoniform fish (*Umbra pygmea*) (Vernier *et al*., 1997). In the present study, all concentrations of endosulfan induced significantly higher number of MN in erythrocytes and CA in head kidney cells compared to the control and their frequency increased in concentrations and time dependent manner. These results are more environmentally relevant than previous studies, which have typically used injection as the route of exposure, because waterborne exposure is more realistic of what occurs in nature. Presumably, endosulfan has affected the genetic material by absorption through the gill epithelium. Earlier, it has been emphasized that exposure of fish to genotoxic chemicals, for various interval of time, by the respiratory route following the absorption of chemicals through gill epithelium could be occurred (Rishi and Grewal, 1995; Farah *et al*., 2006). Our results are in agreement with some earlier studies (Bahari *et al*., 1994; Ali *et al*., 2009; Dar *et al*., 2015), which have reported the induction of MN from exposure to various xenobiotics present in the aquatic environment. An advantage of chromosomal studies is that they reveal a measure of sub-lethal effects of xenobiotics *in vivo*. The CA were more at higher as compared to lower concentrations tested, throughout the post exposure, except at the termination of the experiment where the CA showed the constancy effect at all the tested concentrations, as reported in case of dichlorvos impacts on *Channa punctatus* (Rishi and Grewal, 1995). The chromatid and chromosome breaks were more frequent than the other types of aberrations at all the exposure durations in our study. An increase in chromatid break and chromosomal exchange due to water pollution has also been reported (Chaurasia *et al*., 2007) Similar results have also been reported (Yadav and Trivedi, 2009) in *Orechromis mosambicus* on exposure to various xenobiotics. The current study, thus, emphasized that the CA and MN assays are sensitive biological makers for evaluating the genotoxic effects of various clastogenic xenobiotics, especially in the aquatic environment.

Conclusions

Considering the mutagenic and genotoxic effects of endosulfan on *C. carassius* obtained in this study by MN and CA assays, there is serious apprehension about the potential danger of this pesticide to aquatic organisms, especially to fish, and indirectly to human beings. Moreover, in the absence of other convenient or practical methods, the MN and CA will continue to play an important role in assessing the genotoxicity induced by pesticides. Information obtained through these integrated studies in fish model may be used as bioindicators for monitoring the genomic damage from environmentally hazardous contaminants in the aquatic environment.

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