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Study on DNA damaging effects of 4-nonylphenol using erythrocytes from peripheral circulation, gill and kidney of fish *Channa punctatus*

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Abstract

The present study aimed to evaluate the genotoxic effect of 4-nonylphenol (NP) on blood cells of fish *Channa punctatus*. Fish were exposed to three sublethal concentrations (0.15 mg l^{-1}; 0.31 mg l^{-1} and 0.63 mg l^{-1}) of 4-NP for 24, 48, 72 and 96 hrs. Blood cells from kidney, gills and peripheral circulation were analyzed for the presence of micronuclei and other changes in the erythrocytes. Significant changes were observed in all the experimental groups tested when compared with control. Highest genotoxicity was observed in blood cells obtained from gills (MN-2.92%, aberrant cell- 70.64%), followed by kidney (MN-1.34%, aberrant cells-64.94%), were least effect was observed in blood cells obtained from peripheral circulation (MN-0.88%, aberrant cells-46.27%).Therefore, micronucleus test performed on blood cells obtained from different sources showed that gills were more sensitive as compared to peripheral blood and kidney revealing genotoxic effect of 4-NP on fish *C. punctatus*.

Key words

Blood cells, *Channa*, Genotoxicity, Micronucelus, 4-nonylphenol

Introduction

Surfactants are known worldwide for their solubility and cleansing properties. Alkyl phenol ethoxylates, together with another class of surfactants called alcohol ethoxylates, are the two largest classes of nonionic surfactants in current use (Ivankovic, 2010). About 80% of alkyl phenol ethoxylates (APES) are nonyl phenol ethoxylates. Consequently, they are included in detergents and cleaning products. Surfactant gets dispersed into different environmental compartment (soil, water and sediments) after release from different sources. Approximately, 60% is estimated to end up in water bodies around the world (Bhattacharya *et al*., 2008). Once cast out into the environment, nonyl phenol ethoxylates break down by microbial transformation into metabolic intermediates that are nonyl phenol, nonyl phenol monoethoxylate, nonyl phenol diethoxylate and other related compounds.

4-nonyl phenol been has identified as most critical metabolite of alkyl phenol ethoxylates as it is persistent and virtually ubiquitous contaminant in an environment that is toxic to aquatic life. Due to persistence in the environment, it is bioconcentrated in organisms and is extremely toxic to aquatic life (Lozano *et al*., 2012). So in recent time concern has increased about their usage as it mimics natural hormones so affect the immune system and reproductive system (Ying *et al*., 2002; Masuno *et al.,* 2003). So far most studies of nonyl phenol have focused on its estrogenic effects like morphological changes and developmental abnormalities (Ali and Legler, 2011) or changes of sex ratio, but little is known about their genotoxicity. Screening for genotoxicity of endocrine disrupting compounds is a reliable tool for evaluation of genetic hazard and to obtain information regarding possible carcinogenic potential.

Micronucleus test is one of the simplest, short term test for biomonitoring of aquatic systems. Micronucleus (MN) refers to fragment of damaged chromosomes or whole chromosomes, which fail to find their room onto the spindle

during cell division.As compared to other cytogenetic assays, it is one of the easier and the fastest method. Besides micronuclei, other nuclear and cytoplasmic abnormalities (Nuclear buds, blebbed, notched, fragmented, deformed nuclei, nuclear bridge, vacuolated nucleus, vaculated cytoplasm and caryolysis) can also be taken as an index of cytogenetic damage (Barsiene*et al*., 2006a, b, c; Pacheco *et al.,* 2005; Talapatra and Banerjee, 2007).

Erythrocytes of several fish species have been used for genotoxic assessment as these are easily acquired and no cellular dissociation is required (Ayllon and Garcia-Vazquez, 2000, 2001; Gravato and Santos, 2002; Pacheco *et al.,* 2005). Gill and kidney erythrocytes were chosen along with peripheral erythrocytes as both these organs are metabolically active. Gills are directly exposed to environmental pollutants and involved in respiration, osmoregulation, while kidneys erythropoises and filtration. As erythrocytes mature in kidneys so toxins directly affects kidney erythrocytes at an early stage than peripheral erythrocytes. Several ecotoxicological characteristics of *C. punctatus* such as wide distribution in freshwater environment, availability throughout the year, easy acclimatization to laboratory conditions and commercial importance make this species an excellent test specimen for toxicity studies (Pandey *et al.,* 2005). So in the present study, micronucleated and altered erythrocytes in peripheral circulation, blood from kidney and gills of *C. punctatus* were assessed.

Materials and Methods

Chemical : 4-nonylphenol used in the present study was procured from Himedia (India). A stock solution was prepared in ethanol.

Experimental fish specimens and chemicals : Freshwater fish *C. punctatus* of average weight 16.50 g and length 11.40 cm, respectively were procured from local fish market. The specimens were acclimatized for two weeks under laboratory conditions after treatment with 0.02% $KMnO_4$ for disinfection in static and renewable system. They were fed with boiled eggs and other waste materials were siphoned off daily to reduce ammonia content in water. In order to eliminate the leaching potential of 4-nonylphenol, plastic material was avoided and glass aquaria of 200 l capacity were used for the experiment.

Determination of sub-lethal concentrations : 96 hours LC_{50} value of 4-nonylphenol was determined as 1.27 mgl^{-1} for *C. punctatus* (Sharma *et al.,* 2014), following probit analysis method as described by Finney (1971). Based on 96 hours LC_{50} value, three test concentrations of nonyl phenol *viz*; sub-lethal concentration I (SL-I; $1/8^{th}$ of LC_{50} =0.158mgl^{-1}), concentration II (SL-II; 1/4th of LC_{50} =0.317mgl^{-1}) and concentration III (SL-III; 1/2nd of LC_{50} =0.635mgl^{-1}) were estimated and used for *in vivo* experiment.

***In vivo* exposure experiment :** Blood samples were taken at interval of 24, 48, 72 and 96 hr at the rate of five fish per interval. The specimens maintained in tap water were considered as negative control, while in ethanol as positive control. On each sampling day, blood was collected and immediately processed for MN assay. Physico-chemical properties of test water, namely temperature (23.9±0.19), pH (7.4±0.20), dissolved oxygen (3.2±0.30mgl^{-1}), total alkalinity (16.65±0.30), free CO_2 (8±0.23mgl^{-1}), TDS (0.4±0.01gl^{-1}), TSS (0.5±0.01), TS (0.9±0.02gl^{-1}) were

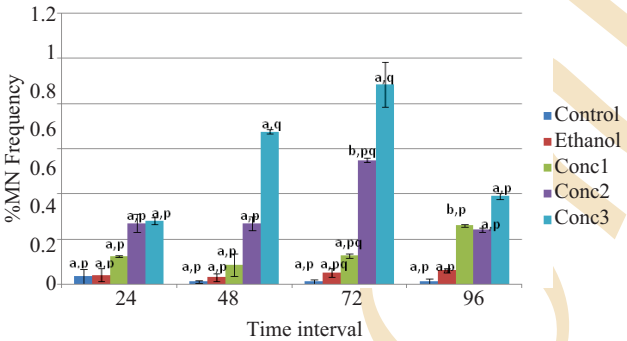

Fig. 1 : Mean frequency of micronucleated cells in peripheral erythrocytes of *C. punctatus* after treatment with different concentrations of 4-nonylphenol at different time intervals. Error bars represent standard errors (SE). Different letters (a, b, c, d) show the significant different (Tukey's test, $p \leq 0.01$) and signify the effect of 4-nonylphenol at different time intervals. Similarly, different letters (p, q, r, s) show significant different (Tukey's test, $p \leq 0.01$) and signify the effect of different concentrations of 4-nonylphenol at the same time intervals

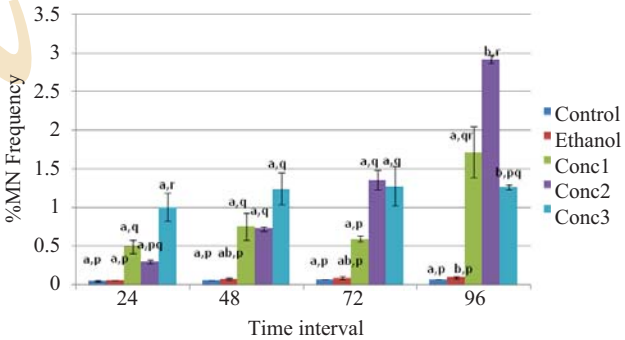

Fig. 2 : Mean frequency of micronucleated cells in gill erythrocytes of *C. punctatus* after treatment with different concentrations of 4-nonylphenol at different time intervals. Error bars represent standard errors (SE). Different letters (a, b, c, d) show the significant different (Tukey's test, $p \leq 0.01$) and signify the effect of 4-nonylphenol at different time intervals. Similarly, different letters (p, q, r, s) show significant different (Tukey's test, $p \leq 0.01$) and signify the effect of different concentrations of 4-nonylphenol at the same time intervals

analyzed by standard methods (APHA, AWWA and WPCF, 2005).

Micronucleus assay : For each experimental group, as well as, control groups drops of blood taken from heart (as peripheral blood), gills and kidneys are smeared on clean slides. Slides were then fixed in absolute ethanol for 10 min and stained with Giemsa 10% (Palhares and Grisolia, 2002). In each group, 1000 erythrocytes were counted under a binocular microscope (Olympus) using 100x oil immersion lens.

Statistical analysis : The results were expressed as mean ±S.E., and to study the significant of difference in frequency of micronucelated and altered erythrocytes among treated and control group ANOVA followed by Tukey-HSD test was applied using SPSS program.

Results and Discussion

Chemical stability of DNA is one of the fundamentals of life. Alteration in chemical structure of DNA occurs frequently, which results in cell death if allowed to accumulate. Contaminants released in aquatic environment have potential to damage DNA of exposed organisms, resulting in genetic disorders. In fish species, micronucleus assay is usually performed to test genotoxicity of contaminants using erythrocytes since, these cells contain nucleus (Bolognesi and Hayashi, 2011). Nonyl phenol is an aquatic contaminant which is bio-accumulative and

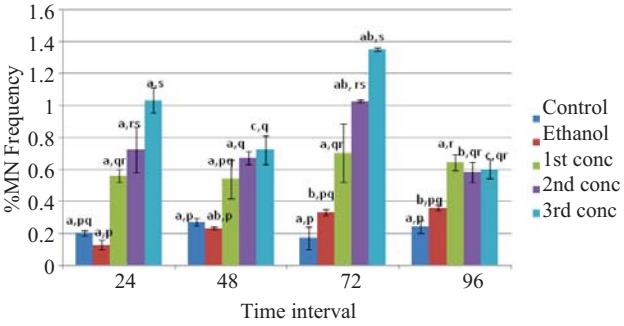

Fig. 3 : Mean frequency of micronucleated cells in peripheral erythrocytes of *C. punctatus* after treatment with different concentrations of 4-nonylphenol at different time intervals. Error bars represent standard errors (SE). Different letters (a, b, c, d) show the significant different (Tukey's test, $p \leq 0.01$) and signify the effect of 4-nonylphenol at different time intervals. Similarly, different letters (p, q, r, s) show significant different (Tukey's test, $p \leq 0.01$) and signify the effect of different concentrations of 4-nonylphenol at the same time intervals.

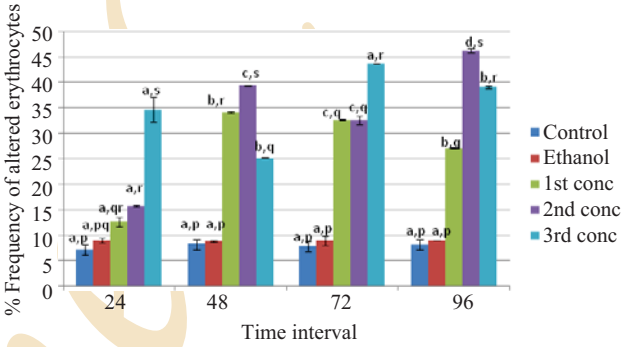

Fig. 4 : Mean frequency of altered erythrocytes in peripheral erythrocytes of *C. punctatus* after treatment with different concentrations of 4-nonylphenol at different time intervals. Error bars represent standard errors (SE). Different letters (a, b, c, d) show the significant different (Tukey's test, $p \leq 0.01$) and signify the effect of 4-nonylphenol at different time intervals. Similarly, different letters (p, q, r, s) show significant different (Tukey's test, $p \leq 0.01$) and signify the effect of different concentrations of 4-nonylphenol at the same time intervals

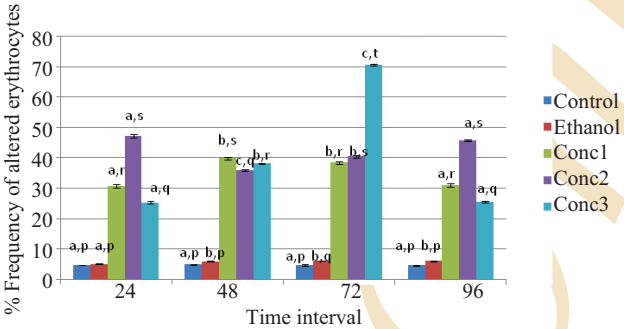

Fig. 5 : Mean frequency of altered erythrocytes in gill erythrocytes of *C. punctatus* after treatment with different concentrations of 4-nonylphenol at different time intervals.Error bars represent standard errors (SE). Different letters (a, b, c, d) show the significant different (Tukey's test, $p \leq 0.01$) and signify the effect of 4-nonylphenol at different time intervals. Similarly, different letters (p, q, r, s) show significant different (Tukey's test, $p \leq 0.01$) and signify the effect of different concentrations of 4-nonylphenol at the same time intervals

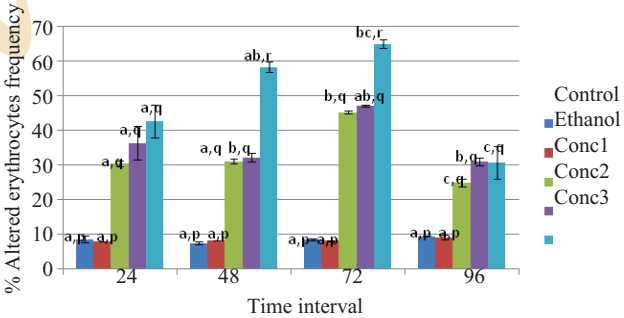

Fig. 6 : Mean frequency of altered erythrocytes in kidney erythrocytes of *C. punctatus* after treatment with different concentrations of 4-nonylphenol at different time intervals.Error bars represent standard errors (SE). Different letters (a, b, c, d) show the significant different (Tukey's test, $p \leq 0.01$) and signify the effect of 4-nonylphenol at different time intervals. Similarly, different letters (p, q, r, s) show significant different (Tukey's test, $p \leq 0.01$) and signify the effect of different concentrations of 4-nonylphenol at the same time intervals

persistent in nature. It is a hydrophobic chemical with endocrine disrupting and acute narcotic effects on aquatic biota. Because of their extensive use, there is concern about the ultimate fate of nonyl phenol in aquatic ecosystem and, about its bioaccumulation.

In the present study, genotoxic effect of 4-nonyl phenol in peripheral erythrocytes, blood cells from gill and blood cells from kidney of *C. punctatus* was analyzed after 24, 48, 72 and 96 hrs exposure to three sub lethal concentrations of 4-nonyl phenol (Fig. 1-6). Time and dose related increase in frequency of micronucleated cells and altered erythrocytes was detected in all the experimental tissues, even after 24 hrs exposure as compared to control. MN frequency and altered erythrocyte frequency increased progressively with increasing duration of exposure, as well as, with an increase in concentration. Fig. 1 is revealing MN frequency in peripheral blood cells and highest effect was seen at 0.635 mgl^{-1} concentration after 72 hrs of exposure duration. A slight decrease was observed at 96 hrs.

Fig. 2 shows MN frequency in blood cell of gills. Lowest frequency of MN was found in negative control followed by positive control group. Highest MN frequency (2.92%) in erythrocytes was noted after 96 hours of exposure at 0.317 mgl^{-1} concentration. In kidney erythrocytes of control group, lowest frequency of MN was observed (Fig. 3). Highest induction of 1.34% was noted at 72 hrs and 0.635 mgl^{-1} concentration. In gill and kidney erythrocytes; a significant time and dose related increase was found ($p \leq 0.0001$). In case of MN in the peripheral blood significant increase was found after 24 hrs of exposure ($p \leq 0.01$).

Fig. 4-6 shows altered erythrocyte frequency in case of peripheral blood, gills and kidneys respectively. Altered erythrocyte frequency increased with time duration and increase in concentration in all the tissues studied. In case of peripheral blood increase was up to 96 hrs and 6.8 times as compared to control. While in case of gill and kidney erythrocytes, increase was concentration and duration dependent but highest effect was seen at 72 hrs of exposure. In gills highest frequency 70.64% of altered erythrocytes was seen at 0.635 mgl^{-1} concentration after 72 hrs. In kidney, highest value 64.99% at 0.635 mgl^{-1} concentration after 72 hrs. While in blood, highest frequency (46.27%) 0.317 $mg\,l^{-1}$ concentration after 96 hrs of exposure respectively.

In the present study, increase in MN frequency and altered erythrocytes was observed in blood cells from all the tissues considered as compared to controls. The effect of dose and time of exposure was also considered. A similar dose response relationship of MN frequency was studied in *C. punctatus* after treatment with cadmium chloride (Parveen and Shadab, 2012). Induction of MN was also studied in other fish like *Heteropneustes fossilis* after exposure of pentachlorophenol (Ahmed *et al*., 2002). A large number of clastogenic and aneugenic compounds have been reported to cause nuclear lesions in kidney erythrocytes of fresh water fish (Ayllon and Garcia-Vazquez, 2000, 2001).Palhares and Grisolia (2002), found that experimental treatment of turbot with 30 ppb of nonylphenol significantly induced formation of MN in peripheral blood and cephalic kidney erythrocytes, and also induced formation of BN and fragmented apoptosis. Mekkawy *et al*. (2011) found that nonyl phenol increase apoptosis cells, micronucleated cells and altered erythrocytes in blood of *C. galiepinus* with increase in concentration of nonyl phenol. Al-Sharif (2012) observed a significant induction of nuclear buds and fragmented apoptotic cells in blood of Tilapia after exposure to 30 $\mu g\,l^{-1}$ of nonyl phenol.

Estimation of altered erythrocytes seems to be quite helpful as it presents a strong positive correlation with enumeration of MN. Finally, the fact that there are more pollutants presenting statistical differences when total abnormalities are examined supports the hypothesis that all the nuclear lesions should be taken into account to improve detection of chromosomal and cytoplasmic damage, as well as, to improve interpretation of results (Serrano-Garcia and Montero-Montoya, 2001; Muranli and Guner, 2011). These nuclear protrusions and deformations could be attributed to the detrimental effects caused by clastogenic pollutants, which cause problems in chromosomal attachments or gene amplification. These abnormalities cause deformed nuclei as indicated by the presence of lobed, blebbed, binucleated, notched, budded and other deformed nuclei during elimination of amplified DNA from nucleus (Bolongesi *et al*., 2006; Ergene *et al.,* 2007)

In the present study, gill cells showed significant increase in frequency of micronucleated cells and other aberrant erythrocytes, which increased with concentration. Higher damage in gill cells might be due to constant exposure to DNA damaging chemicals dissolved in water. Early investigations in various fish species indicated higher sensitivity of gill cells to DNA damage than kidney and erythrocytes (Ali *et al*., 2009). Fish gills are particularly sensitive to the presence of surfactants, and several studies have documented destruction and marked deterioration of gills exposed to nonyl phenol ethoxylates (Cox, 1996). The results of the present study showed that there was inter tissue differences in MN frequency. Similar inter-tissue differences in frequency of MN in cod and turbot (Cristaldi *et al.,* 2004) was observed for which might be due to elimination of damaged erythrocytes from peripheral blood system. Data showed spleen activity in selective removing of micronucleated erythrocytes from peripheral blood system. In fish blood, low level of fragmented-apoptotic cells were observed. Such type of cells are recognized as one of the main

ways for which might be due to elimination of micronucleated cells (Micic *et al.,* 2002).

These genotoxicants have been reported to cause mutations because they form strong covalent bonds with DNA, resulting in formation of DNA adducts preventing accurate replication (Hartwell *et al.,* 2000; Luch, 2005). Genotoxins affecting germ cells (sperm and egg cells) can pass genetic changes down to descendants (Hartwell *et al.,* 2000). In the present study, nonylphenol was found to exert DNA damaging effect which might be due to its micro tubular disrupting activity (Vazquez- Duhalt *et al*., 2005) or due to its biotransformation into reactive intermediates, which might cause changes at DNA level. The present study emphasize that nonyl phenol is genotoxic to fish *C. punctatus* and gill as most sensitive tissue. The present study is a preliminary study showing genotoxicity of 4-nonyl phenol to fish.

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