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GENOTOXIC POTENTIAL OF NONYLPHENOL IN FRESHWATER FISH, *OREOCHROMIS MOSSAMBICUS*

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ABSTRACT

Nonylphenol is the breakdown product of alkylphenol ethoxylates (APEs), which are an important class of non-ionic surfactants used for a variety of industrial, household and commercial applications including plastics, cosmetic products, inks, paints and textiles. Genotoxic potential of nonylphenol is evaluated by micronucleus and Ames test in *Oreochromis mossambicus.* Median lethal concentration of nonylphenol (1.5 mg/ L) was computed on the basis of probit analysis. Fishes were treated for 24 h, 96 h and 7 days with one-tenth of the concentration to represent the sublethal dose. Nuclear abnormalities such as micronuclei, and other nuclear malformations as fragmented apoptotic cells, binucleated cells and sticky adherent cells are noticed after nonylphenol exposure. In the present study Ames test reports more colonies in one-tenth of the test dose compared with the controls. Thus the present findings reports that nonylphenol is mutagenic and causes genotoxicity in *O. mossambicus*.

KEYWORDS: Ames Test, Genotoxicity, Micronucleus, Nonylphenol, *Oreochromis*, *Salmonella*

INTRODUCTION

Aquatic toxicity is the lethal consequence of any compounds with suspected detrimental effects and that are usually determined on any organisms with several endpoints. Any man-made or the natural environmental chemicals present in the aquatic environment are able to interfere with the various systems in the aquatic organisms and has been shown to cause adverse effects on growth, behavior, reproduction, immune system or may cause genotoxicity. Some of such environmental toxicants listed as genotoxicants have been known to be released into the environment that is produced during post-emission chemical or biological transformation. Consequently, it is not surprising that during the past decade there has been increasing concern regarding the genotoxic hazards associated with the release of complex industrial wastes and effluents.

According to research, approximately 300,000 tones of alkylphenols are produced per year and 60% are known to be released into the environment. This is then converted into the biodegradation products as nonylphenol and octylphenol, which are more toxic than alklyphenol itself (Schrenk-Bergt and Steinberg, 1998). Nonylphenol is more persistent in the environment than the parent nonylphenol ethoxylate. Nonylphenol bioaccumulates in aquatic organisms, especially on fish and the breakdown products of these compounds are persistent in the environment. The bio concentration factor (BCF) of nonylphenol in fish varies from 3 to 1300 (Ahel et al., 1993) and relatively low concentrations of nonylphenol and/ or nonylphenol ethoxylates has been shown to cause death in fishes (Cox, 1996).

One of the studies has reported that nonylphenol shows positive genotoxic effects with increased nuclear abnormalities on *Oreochromis spirulus* fish (Al-Sharif, 2012). On contrary, another literature reported that nonylphenol did

not cause genotoxicity, however it accelerated the maturation of oocytes and spermatocytes in *Oreochromis niloticus* (Rivero et al., 2008). Therefore the present study was designed to evaluate the genotoxic potential of nonylphenol in another species of Oreochromis (*O. mossambicus*) and our present findings reveal that nonylphenol is genotoxic as evidenced by micronucleus and Ames test.

MATERIAL AND METHODS

Collection and Maintenance of Animal

Oreochromis mossambicus weighing 9 ± 1.5 g and length 7.5 ± 1cm were collected from a fish farm, Kaloos Aquarium, Kottakal, Malappuram District, Kerala. Fishes were acclimatized to the laboratory conditions prior to experiments and were exposed with constant supply of water and good lighting system. They were maintained in well-aerated tubs (40 L capacity), which was dechlorinated and sustained with fresh water flow and waste water discharge.

Preliminary Tests

The physico-chemical features of the tap water were estimated as per APHA (1998). Water temperature in the test ranged from 28 ± 2°C during the experiment, oxygen saturation of water ranged between 70 and 100 %, pH is 6.5 to 7.5 which were monitored using a standardized procedures. The LC_{50} values in the respective time intervals were determined by probit analysis, with a confident limit of 5 % level (Finney, 1971). Preliminary tests were conducted to provide guidance on range of concentration of toxicant to use in the bioassay. The specimens were not fed a day prior to and during toxicity tests to reduce faecal and excess food contaminating the test solution. Five specimens were placed in each tub of replicates so that ten fishes were maintained in each test and aerated using tubed motorized pumps. Monofilament netting was used to cover the tanks to prevent the specimens from jumping out of test solutions.

Evaluation of Median Lethal Concentration (LC_{50})

The concentration of the toxicant at which 50 percentage of the test animals dies during a specific period or the concentration lethal to one half of the test population is referred to as median lethal concentration (LC_{50}) or median tolerance limit. For determining LC_{50} concentration separate circular plastic tubs of 40 L of water capacity were taken and different concentrations of nonylphenol were added. Then, 10 fish were introduced into each tub. A control tub with 40 L of water and 10 fishes were also maintained (no toxicant). The lethal concentration for 50 % killing (LC_{50}) values was computed on the basis of probit analysis (Finney, 1971) for 96 h, which was 1.5 mg/ L. One-tenth of the dosage (0.15 mg/ L) of nonylphenol was chosen in the present study.

Chemicals

Technical grade Nonylphenol, 4-(2,4-dimethylheptan-3-yl)phenol of 97% purity was obtained from SISCO Research Laboratories Pvt. Ltd., Mumbai, India. Giemsa stain, Histidin and Biotin were obtained from Sigma Aldrich Laboratories, USA. *Salmomella typhimurium* was purchased from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. All other chemicals were of analytical grade and obtained from local commercial sources.

Treatments

There were four groups, three tanks with toxicant doses (nonylphenol dissolved in DMSO) and a control tank. Single dose with three durations were used in the present study. Ten fish specimens were used for every test and also in control. The first group of fish was maintained in toxicant free water and used as control and the second group was treated

with nonylphenol at 0.15 mg/ L for 24 h; the third group was treated with nonylphenol at 0.15 mg/ L for 96 h and the fourth group was also treated with nonylphenol at 0.15 mg/ L for 7 days.

Killing of Animals

The fish was caught very gently using a small dip net, one at a time with least disturbance. At the end of each exposure time, a drop of blood was taken by cardiac puncture and used for micronucleus test. Mutageni city test was performed *in vitro* using *Salmonella typhimurium* (TA 100) culture.

GENOTOXICITY ANALYSIS

In Vivo Micronucleus Test

The micronucleus test was performed according to Heddle, 1973; and Schmid, 1975, and nuclear abnormalities were evaluated according to Carrasco et al., 1990 with slight modification. A drop of blood from fish was taken by cardiac puncture and mixed well with a drop of fetal calf serum in a clean glass slide and smeared. It was then air dried followed by fixing in absolute methyl alcohol for ten minutes. The slide was then stained with 5% Giemsa for 10 minutes and 1000 cells under 40 x magnifications were scored for nuclear abnormalities.

Bacterial Reverse Mutation Assay

The mutagenicity of nonylphenol was done by the method as described by Ames et al., 1975. Sterile glucose minimal agar plates were prepared and 25ml of the prepared solution was poured into petri plates for solidifying at room temperature. Top agar was then prepared and 2ml of the top agar with 0.5 ml sodium phosphate buffer, 50 µl test sample and 100 µl bacterial culture (*Salmonella typhimurium*) was taken in a sterile glass tube, mixed well at 43˚C in water bath. It is then added to the prepared glucose minimal agar plate and set to solidify. The cultures were then incubated at 37˚C for 48 h in inverted position and colonies were counted.

Statistical Analyses

Statistical analyses of micronucleus test were performed using statistical package SPSS 19.0. Differences were considered to be significant at $p<0.05$ against control group. Data are presented as mean ± SD for ten animals per group.

RESULTS

Nonylphenol treatment showed several nuclear abnormalities as micronucleus, binucleated cells, sticky cells, fragmented-apoptotic cells and lobed cells at varying percentage in time-dependent manner as shown in Table 1.

Table 1: Effect of Nonylphenol on Micronuclei and other Nuclear Abnormalities in Blood Cells of *Oreochromus Mossambicus*

Treatment Groups	MN	BN	SC	FA	L	Total	%	Mean ± SD
Group1 (Control)		0	0	4	2	0	60	66±0.33
Group2 (24 h)		4	8	8	6	2	280	2828±1.26
Group 3 (96 h)		8	10	18	8	6	500	5050± 0.62*
Group 4 (7 days)	12	16	24	10	8	700	70	70±0.52*

MN = Micronuclei; BN = Bi-nucleated; SC = Sticky cells; FA = Fragmented-apoptotic cells; L = Lobed.
No. of fishes = 10; No. cells examined = 1000/ fish
* represents statistically significant at $P< 0.05$ from the control group

The morphological features are shown in Figure 1 and 2. Ames test reports more colonies of *Salmonella typhimurium* in treated dose as compared without and with DMSO shown in Figures 3 and 4.

DISCUSSIONS

Nonylphenol, one of the estrogenic environmental contaminants, get released into the environment from the waste water run-off and get bioaccumulated in the aquatic organisms as fish. It is considered as directly toxic to fish and even low concentrations of nonylphenol lead to estrogenic effects that might have long-term consequences, such as disrupting population dynamics or interactions within the aquatic communities. In the present study freshwater fish, *Oreochromis mossambicus* was used as an experimental model and nonylphenol at sub lethal concentration, 0.15 mg/ L, was exposed in acute as well as sub-chronic duration for 24 h, 96 h (acute) and 7 days (sub-chronic) to study its genotoxic potential in blood cells of *Oreochromis*.

In fish, micronucleus test is an important parameter that helps to identify the status of water quality, the health of species and its potential risk (Al-Sabti and Metcalfe, 1995). Fish erythrocytes are especially preferred for micronucleus test and the abnormal nuclear morphology has been measured as an indicator of genotoxic damage in fish (Talapatra and Banerjee, 2007). Alternatively, various abnormal morphological forms of erythrocytes are also proved as effective indicators of cytotoxicity as apoptotic cells are formed by actively dying cells that include cell shrinkage, membrane blebbing and chromatin condensation (Talapatra and Banerjee, 2007).

The freshwater teleostian fish tilapia, *Oreochromis mossambicus* was first introduced into Indian pond ecosystem in 1952 and soon it was stocked in the reservoirs of south India. By the end of 1960s, most of the reservoirs in Palakkad and Trissur districts of Kerala, India were regularly stocked with tilapia. Performance of tilapia in ponds of south India has been encouraged mainly due to its early maturity, continuous breeding and over-population.

It has been reported to mature at 6 cm length at an age of 75 days and to breed at an interval of one month under the tropical conditions. In the present study *Oreochromis mossambicus* was chosen because of its widespread usage in south India. Apart from this tilapia is considered as one of the highly sensitive and bio-indicator fish when exposed to environmental chemicals, and are widely used as an ideal model organisms for the evaluation of toxic effects on aquatic ecosystem. Thus it is significant to use this species as a potential bio-indicator for freshwater environmental contaminants including nonylphenol.

In the present study the results of micronucleus showed positive genotoxic effects as measured by increase in nuclear abnormalities as micronucleus, bi-nucleated cells, sticky cells, fragmented-apoptotic cells and lobed cells when exposed to sub lethal concentration of nonylphenol in all treatment groups. This reveals that nonylphenol bioaccumulate in *Oreochromis mossambicus* in a time-dependent manner.

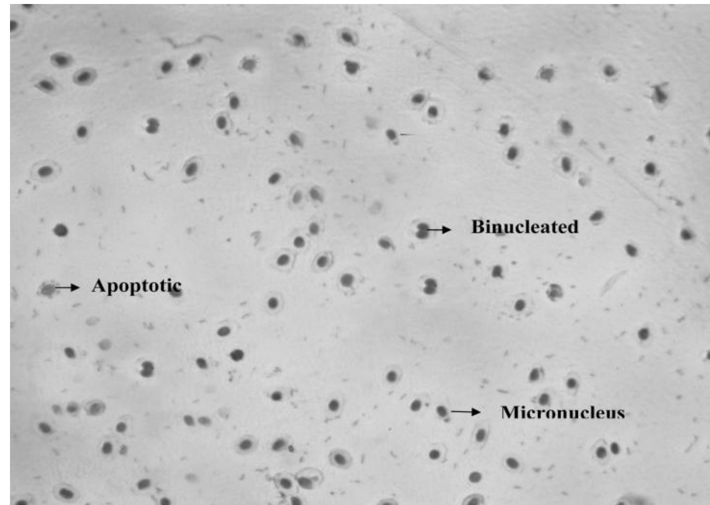

Figure 1

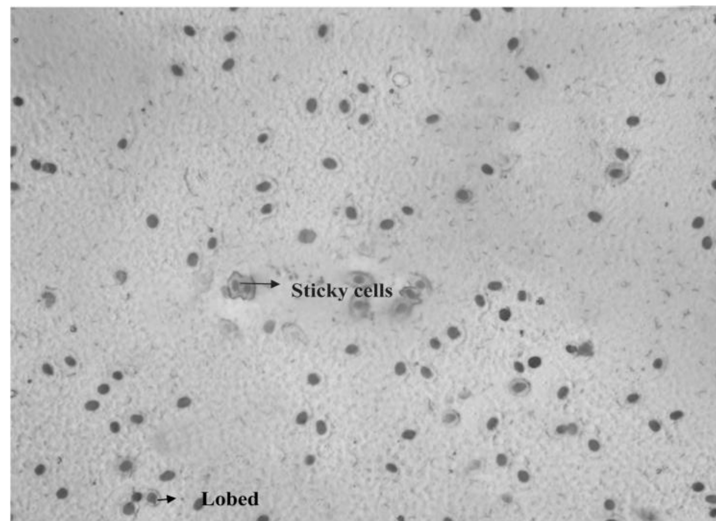

Figure 2

The Salmonella mutagenicity test is the most validated short-term assay for mutagenicity assessment of environmental toxicants. It is relatively straightforward, exceptionally reliable, requires little specialized equipment, and is readily amenable to bioassay directed fractionation. The most commonly used Salmonella tester strains include TA98, TA100, TA97, and TA102. However, in the present study TA100 strain is used for detection of mutagenic activity of nonylphenol. TA100, the base-pair strain, contains a single base substitution mutation in a leucine codon (Mortelmans and Zeiger, 2000). In all the plates few small non-revertant colonies are also noticed and it is referred to as "pinpoint colonies", which consist of histidine-dependent bacteria that survived high chemical toxicity. These colonies are readily visible by the naked eye and may be mistaken for revertant colonies.

Microscopic inspection of the plates revealed that there was a total absence of background lawn. The pinpoint colonies occur as a result of more availability of histidine to the surviving His^{-} bacteria on a per cell basis. Therefore, these bacteria can undergo additional cell divisions until the depletion of the histidine. The histidine dependency was checked by streaking a few pinpoint colonies on agar plates supplemented with biotin but without histidine in the absence of the test chemical. After the plates are removed from the incubator, the colonies are counted and the results are expressed as revertant colonies per plate. Hand-counting of the colonies, including positive and negative control plates was done as

there was poor contrast between the colony and the agar and this could be that the test chemical discolors the agar which prevents sufficient light from passing through the agar. In the present study nonylphenol at one-tenth of the concentration showed double the number of colonies when compared with those of controls.

The sample was considered to be mutagenic when the number of revertant colonies in the test plates was doubled than the number of revertants in solvent control (Kutlu et al., 2004). Thus nonylphenol was considered as mutagenic.

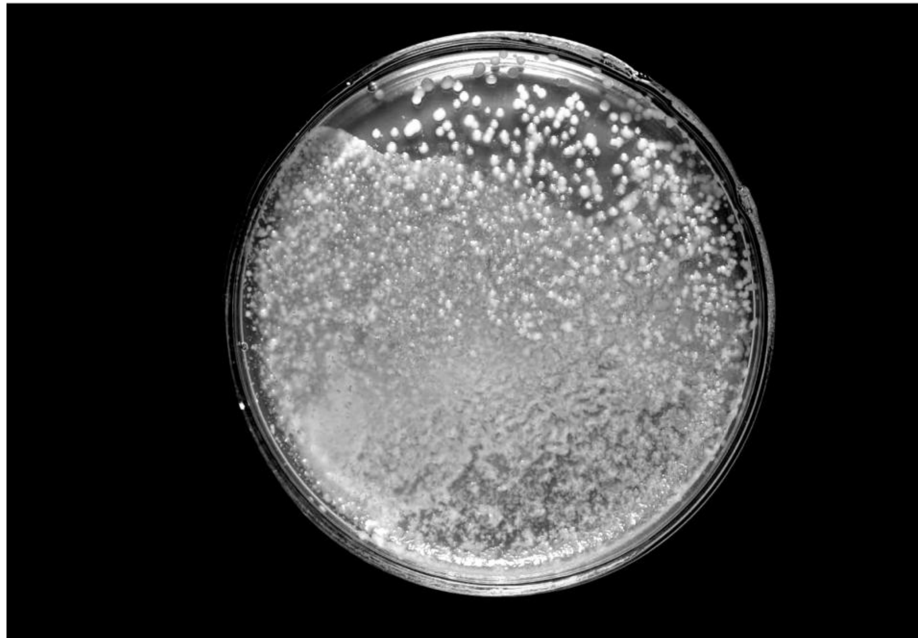

Figure 3a: CONTROL

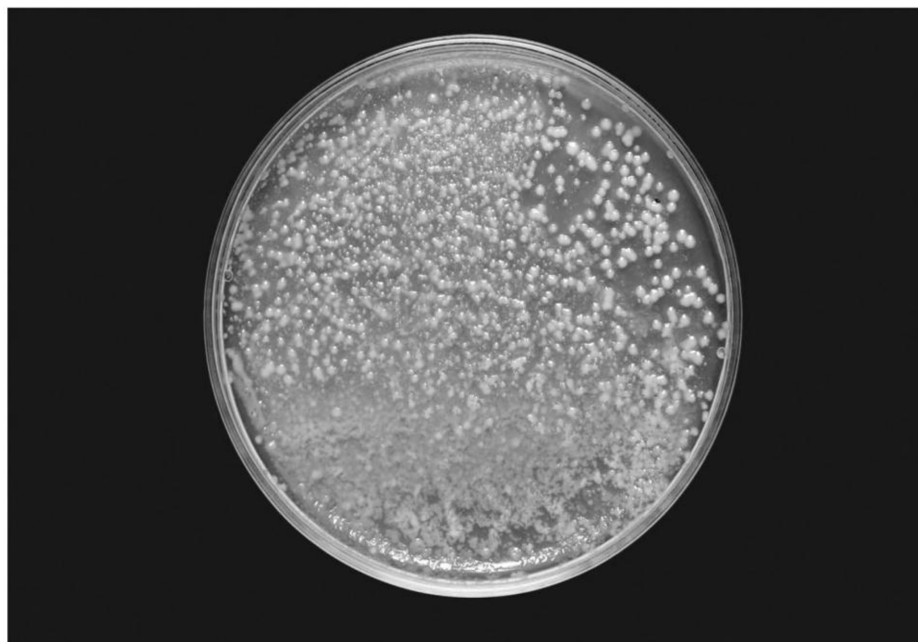

Figure 3b: DMSO

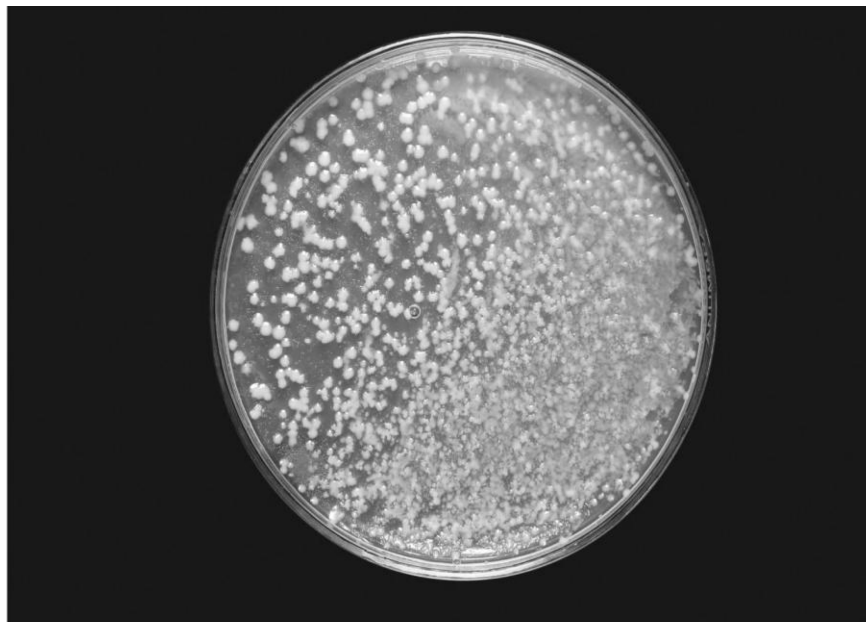

Figure 4: NONYL PHENOL

CONCLUSIONS

In conclusion, micronucleus test and Salmonella mutagenicity test was used as an investigation tools for the evaluation of the genetic damage of the *Oreochromis mossambicus* induced by the exposure to nonylphenol. Thus the present findings reports that nonylphenol is mutagenic and causes genotoxicity in *O. mossambicus*.

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