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Induction of testicular oxidative stress by nonylphenol in cichlid fish, *Etroplus maculatus* (Bloch, 1795)

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Abstract
The present study was undertaken in order to evaluate the induction of oxidative stress by the exposure to nonylphenol in testis of cichlid fish, *Etroplus maculatus*. Nonylphenol at one-fifth (178 µg/ L) and one-tenth (89 µg/ L) of LC_{50} concentrations were exposed to fishes for 24, 72 and 96 h. Analysis of various antioxidant enzymes as superoxide dismutase, catalase and glutathione reductase were measured in the crude homogenate of testis. The levels of hydrogen peroxide generation and lipid peroxidation were also tested at both concentrations in all treatment groups. The results pointed out that nonylphenol treatment caused significant (P<0.05) decrease in the activities of antioxidant enzymes with concomitant significant (P<0.05) increase in the levels of hydrogen peroxide generation and lipid peroxidation in concentration-dependant and time-dependant manner. Therefore, the present study reveals that nonylphenol exposure increased reactive oxygen species production in testicular cells that could potentially resulted in enhanced oxidative stress.

Keywords: Nonylphenol, *Etroplus maculatus*, antioxidant enzymes, lipid peroxidation, oxidative stress, testis

Introduction
During the past few decades there has been an increasing concern about the potential harmful effects of the surfactants containing nonylphenol ethoxylates. Nonylphenol (NP) is an organic environmental contaminant widely used in the production of non-ionic surfactant, lubricant additives, plastics such as polystyrene and polyvinyl chloride, food contact plastics, polymer stabilizers, paints, wetting agents, herbicides, pesticides, cosmetics and other formulated products [1]. NP is one of the environmental contaminants possessing estrogenic properties that are active contributor to uphold environmental health risk to exposed organisms. Due to the increase in the commercial use of NP in variety of products it contributes to the large scale discharge into the water bodies and poses toxicity to both marine and freshwater animals. Its ability to bioaccumulate in the organs of aquatic species is considered as potential health hazard that could ultimately affect the human health.
Most of the research data examined on the estrogenic effects of NP in fish by demonstrating female specific protein vitellogenin [2, 3] and on the expression of vitellogenin mRNA in male fish [4, 5]. Due to the estrogenic activities, NP can stimulate or inhibit the action of endogenous estrogens and disrupts estrogenic nuclear hormone receptor action [6]. NP has been shown to change the sex ratio and the occurrence of testis-ova was also reported in medaka [7]. Therefore, it is well understood that NP exposure lead to detrimental effects in the reproductive system of fish. More recently, researchers has shown special attention on the contribution of environmental contaminants on the induction of oxidative stress in reproductive and non-reproductive cells/ tissues. Reactive oxygen species (ROS) such as superoxide anion radical, hydrogen peroxide and hydroxyl radical are highly toxic to cells. All biological systems have well developed enzymatic and non-enzymatic antioxidant mechanisms that normally protect the cell from the oxidative damage due to the toxic effects of free radicals. Since oxidative stress levels may vary between organs/ tissues, organisms are able to adapt to such stress by their well established antioxidant defense system. When the balance between the ROS production and the antioxidant defense is lost would result in oxidative stress [8].

There are several data reporting the adverse effects of NP by the induction of oxidative stress in epididymal sperm of rats [9], gill and liver of freshwater fish, *Oreochromis mossambicus*[10, 11]. However, the effect of nonylphenol in free radical generation in testis of fish remains unclear. Taken together, the objective of the present study was aimed to determine the effects of nonylphenol on the induction of oxidative stress in testis of cichlid fish, *Etroplus maculatus*.

2. Materials and Methods

2.1 Animal

E. maculatus, cichlid fish (7 ± 0.5 g; 7 ± 1.5 cm) were collected from local fish farm near Parappanangadi, Malappuram district, Kerala, India. Fishes were acclimatized to the laboratory conditions prior to experiments by exposing to constant supply of dechlorinated water and good lighting system and were maintained in well-aerated aquarium tanks (40 L capacity). The experiment was conducted during the pre-monsoon period ie., January to February month as catches are comparatively low during the period.

2.2 Preliminary tests

The physico-chemical features of the tap water were estimated as per APHA [12]. Standard water temperature (28 ± 2°C), oxygen saturation of water (70 and 100%), pH (6.5 to 7.5) was maintained throughout the experiment in both control and treated groups.

2.3 Chemicals

Technical grade Nonylphenol, 4-(2, 4-dimethylheptan-3-yl) phenol of 97% purity was purchased from SISCO Research Laboratories Pvt. Ltd., Mumbai, India. Malondialdehyde, NADPH, glutathione oxidized, thiobarbituric acid and pyrogallol were obtained from Himedia Laboratories, Mumbai, India. All other chemicals were of analytical grade and obtained from local commercial sources.

2.4 Treatment

After acclimatization, fishes were housed in different tanks for the experiment maintaining ten animals per group. Nonylphenol was dissolved in 1% DMSO and therefore used as a solvent (vehicle) control in the experiment. In the treatment groups, two sub-lethal concentrations such as one-fifth (178 µg/ L) and one-tenth (89 µg/ L) of LC_{50}-96 h concentrations were used. The exposure period was set up for 24, 72 and 96 h maintaining negative and positive controls. The experiment was designed as follows:

Control Groups: Group 1 – Solvent-free; Group 2 – With solvent (1% DMSO)

Treatment Groups

One-fifth of LC_{50}-96 h of nonylphenol (178 µg/ L)

Group 3 – maintained for 24 h
Group 4 – maintained for 72 h
Group 5 – maintained for 96 h

One-tenth of LC_{50}-96 h of nonylphenol (89 µg/ L)

Group 6 – maintained for 24 h
Group 7 – maintained for 72 h
Group 8 – maintained for 96 h

At the end of every experiment, fishes were caught very gently using a small dip net, one at a time with least disturbance and were decapitated. Testis were dissected and stored at 4ºC until the biochemical analyses were performed. A 1% (w/ v) homogenate of testis was prepared in ice-cold normal saline with the help of a motor-driven glass Teflon homogenizer on crushed ice for a minute. The homogenate was centrifuged at 8000 *g* for 15 min at 4ºC to obtain the supernatant, which was then used for the biochemical analyses. Protein was estimated by the method of Lowry *et al.*[13] with BSA as the standard. Activity of superoxide dismutase[14], catalase[15], glutathione reductase [16], level of hydrogen peroxide generation[17], level of lipid peroxidation [18] were measured in crude homogenate.

2.5 Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test using statistical package SPSS 19.0. Differences were considered to be significant at $P<0.05$ against control groups. Data are presented as mean ± SD for ten animals per group. All biochemical estimations were carried out in duplicate.

3. Results and Discussion

Nonylphenol, an environmental toxicant that functions as xenoestrogen has been shown to cause adverse effects in male reproduction of mammals [9,19]. NP at low micromolar concentration has been shown to induce testicular oxidative stress and cytotoxicity *in vitro* [20]. Previous studies from our laboratory determined the median lethal concentration (LC_{50}-96 h) of nonylphenol in *E. maculatus* by using probit analysis, which is 890 µg/ L [21]. The most detected concentration range of nonylphenol in freshwater ranged from 0.00001 to >0.1mg/ ml [22]. The present study was performed to analyse the effects of nonylphenol on the induction of oxidative stress in testicular cells of fish, *E. maculatus*.

The outcome of the present study showed that nonylphenol exposure caused significant ($P<0.05$) decrease in the activities of antioxidant enzymes such as superoxide dismutase (Fig.1), catalase (Fig. 2) and glutathione reductase (Fig. 3). However, there was a significant ($P<0.05$) increase in the levels of hydrogen peroxide generation (Fig. 4) and lipid peroxidation (Fig. 5) in concentration-dependant and time-dependant manner when compared to the control groups.

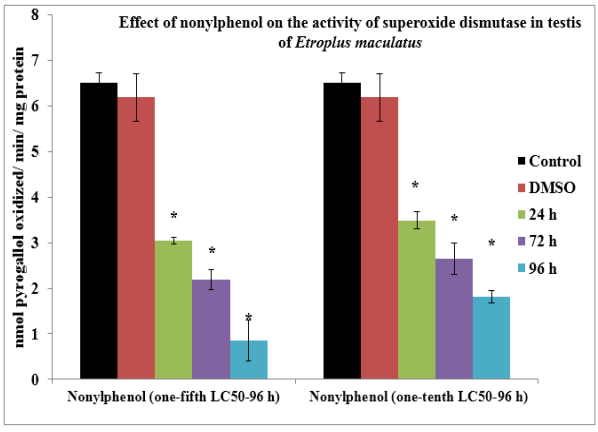

Fig 1

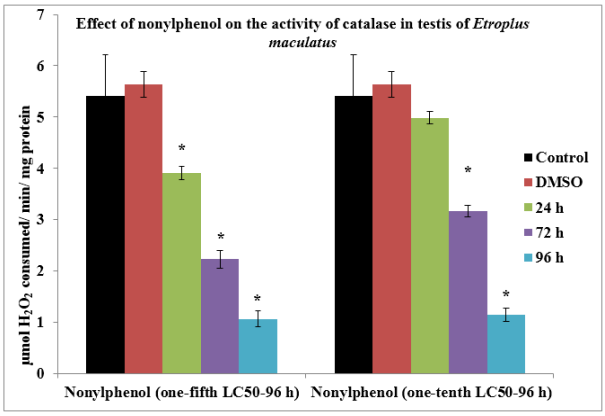

Fig 2

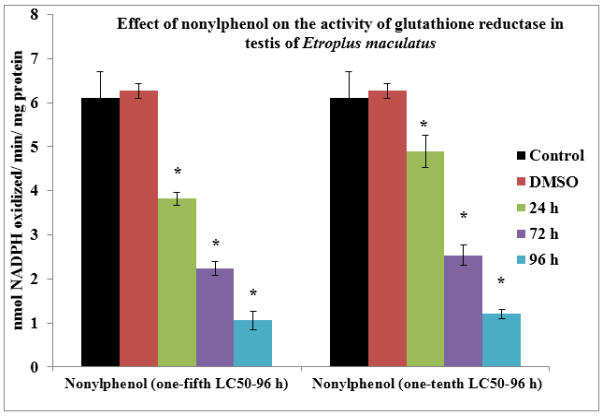

Fig 3

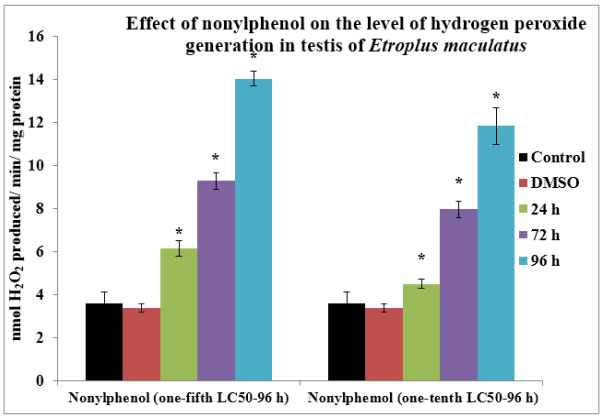

Fig 4

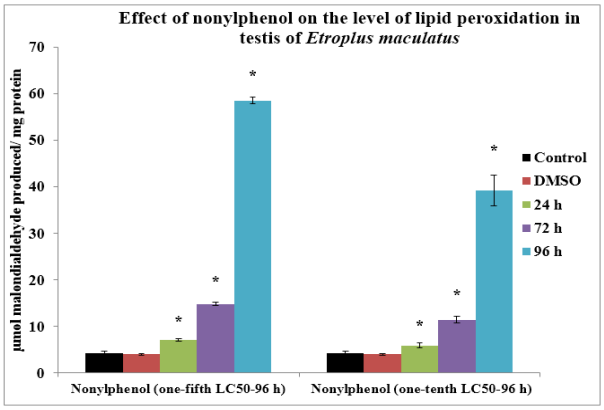

Fig 5

Free radicals and other reactive oxygen species (ROS) are produced in the metabolic pathways of aerobic cells and have been shown to affect a number of biological processes. At a molecular level, free radicals have been found to modify proteins and inactivate enzymes, cause oxidative DNA damage, alter cellular transcriptional machinery and initiate the chain reactions that peroxidize lipids [23]. Superoxide dismutase is the prime antioxidant enzymes involved in the conversion of superoxide radical into molecular oxygen or hydrogen peroxide. The decrease in the activity of superoxide dismutase after exposure to nonylphenol at both sublethal concentrations reveals the failure of the enzyme to eliminate the free radicals. As a result, hydrogen peroxide gets accumulated in the testicular tissue, which is a recognized toxicant that could cause damage to testis through the production of hydroxyl radical.

Catalase is ubiquitous enzyme that can eliminate hydrogen peroxides formed by decomposing into water and oxygen thereby mitigates the toxic effects of hydrogen peroxide. Nonylphenol exposure for 96 h decreased the activities of catalase and glutathione reductase and this clearly indicates the imbalance in the status of antioxidant enzymes in testis of fish. Hydrogen peroxide is considered as a more stable reactive oxygen species responsible for oxidative damage to cells as it can easily pass through the plasma membrane [24]. Several literatures have reported the toxicant-related effects on the level of lipid peroxidation. In the present study nonylphenol treatment significantly elevated the level of hydrogen peroxide along with the increase in the level of lipid peroxidation. Therefore, measurement of lipid peroxidation is essential to address the action of free radicals on lipids that contain polyunsaturated fatty acids (PUFA). Testis is more susceptible to oxidative stress as its membrane is rich in PUFA and the elevated level of lipid peroxidation due to nonylphenol exposure clearly indicate the oxidative damage in testis of fish.

4. Conclusion

Antioxidant defense system is the sensitive system in fish that results in an imbalance due to the exposure to environmental contaminants. The present study addressed the generation of free radicals and the induction of oxidative stress in testis due to the exposure of nonylphenol. Under the stressful environmental condition of fish when exposed to the toxicant, nonylphenol the induction of oxidative stress and/ or oxidative imbalance occurs in testis of fish.

5. Acknowledgement

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