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Genotoxicity of 4-Nonylphenol (4NP) on *Oreochromus spilurs* Fish

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Abstract: 4-Nonylphenol Compound is widely used as an element of detergents, paints, insecticides and many others products. It is known that the existence of this compound may lead to the emission of estrogenic responses in mammals, birds and fish. It is described as pollutant since it cause disorder of endocrine glands. In previous studies, it was proven that this compound exists in water and in the materials precipitated in Red Sea coast in Jeddah near the drains of processed drainage water and near the drainage site of the residuals of paper factories. Therefore, this study aimed to evaluate the cytogenetic aberrations caused by 4-nonylphenol through exposing Talapia Fishes to aquatic solution of the compound with 0, 15, 30 microgram/Liter for one month. Samples of gills and liver were collected for micro nucleuses, nuclear abnormalities and measuring DNA and RNA amount in the treated fish. The results pointed out that there is a significant increase in the numbers of micro nucleuses in the fish exposed to the former concentrations as compared to the control group. Exposing fishes to 4-nonylphenol resulted in an increased amount of both DNA and RNA, compared to the control group. There is a positive correlation between the amount of the compound (i.e. dosage dependent effect) and the inspiring for cytogenetic effect on Talapia fishes in Jeddah. Therefore, micronucleus test, DNA and RNA contents can be considered as an index of cumulative exposure, which appear to be a sensitive model to evaluate genotoxic effects of 4-Nonylphenol compound on fish.

Key words: Genotoxic · 4-nonylphenol · Micronuclei · Fish · DNA · RNA

INTRODUCTION

Nonylphenol ethoxylate (NPE) has been found in aquatic environments, particularly in sediments, sewage and river water [1, 2]. This compound is widely used in the manufacture of non-ionic surfactants, lubricants, stabilizer polymers, antioxidants, alkylphenol chemicals, detergents, paints and various pesticide formulations, such as insecticides and herbicides. In the aquatic environment NPE breakdown to 4-nonylphenol,which is more stable and persistent [3- 6].

Research has been conducted to determine the effects at the DNA level. This is surprising considering that estrogens are known to induce DNA damage including for example single strand breaks, chromosomal damage and diverse types of DNA Roy *et al*., [7]. A genotoxicity study on MCF-7 human breast tumor cells revealed genomic instability by telomeric associations and chromosomal aberrations caused by nonylphenol [8].

DNA and chromosomal damage are the most important critical events following the exposure to carcinogenic and/or genotoxic agents. Chromosomal damage, as a result of inefficient or incorrect DNA repair, is expressed during the cell division and represents an index of accumulated genotoxic effects. Chromosomal effects could be measured as macrolesions in cells exposed to genotoxic agents. Analysis of chromosomal aberrations is difficult in fish because fish chromosomes are generally small in size and high in number. The micronucleus test is the most applied technique to evaluate chromosomal damage in different organisms [9-13]. Micronuclei (MN) arise from chromosomal fragments or whole chromosomes that are not incorporated into daughter nuclei at mitosis. MN are small fragments of chromatin separated from the main cell nucleus which are index of chromosomal breaking or mitotic spindle dysfunctions [14]. This test has the advantage that it can be applied in interphase to any

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proliferating cell population regardless of the karyotype. MN can be analysed in different fish cell types such as peripheral erythrocytes, gill, kidney, hepatic and fin cells [13, 15].

Fish have shown susceptibile to pesticides and other industrial water contaminants after being exposed through gills, skin and food [16]. Oreochromis niloticus have been used in many studies of genetic monitoring of toxic chemicals, such as textile mill effluent, refinery effluent and treated sewage effluent [17-19].

The aim of this study was to investigate the genotoxicity of 4-Nonylphenol and its effect on the liver and erythrocytes of Tilapia, Oreochromus spilurs fish.

MATERIALS AND METHODS

Chemical: 4-Nonylphenol **(**4-NP) was obtained from Fluka, U.K. the mixture, which was composed of several isomers, had a purity of greater than 98% but contained trace concentrations of cumyl and dodecalphenol. A stock solution of 1 gm NP/L was prepared by dissolving NP in ethanol as a carrier solvent, to give the desired concentrations in water.

Fish: " *Oreochromus spilurs* " the "tilapia " fish has been selected, as the most appropriate species farmed in sea environment, as it is farmed in the Kingdom of Saudi Arabia, namely in Jeddah. 15-17g fish is characterized by the ability to grow in a wide range of salinity and great potentiality of production and resistance to diseases and parasites, regarding their weights, the fish was divided into three categories as seen in the in experimental design below.

Experimental Design

The First Part of the Experiment (Group1): Tilapia fish (weight: 16.25+0.43g. and length 8.5+0.48 cm.) were obtained from fish laboratory farms in special basins from egg laying, hatching and larvae were collected and divided into three sub-groups. Each group contained 30 fish: The first sub-group was reared without treatment until maturity. The second sub-group was exposed to (15 µg/L) 4-nonylphenol. The third sub-group was exposed to (30 µg/L) 4-nonylphenol.

The Second Part of the Experiment (Group2): The experiment were to be continued, where the treated fish mother with 4-NP15µg/L concentration were bred until eggs were laid and hatched and larvae reared until maturity and then were divided into three sub-groups (each group containing 30 fish): The first sub-group was reared without treatment until maturity. The second group was exposed to (15 µg/L) 4-nonylphenol. The third sub-group was exposed to (30 µg/L) 4-nonylphenol.

Specimens were obtained during the experiment, which extended from July until December 2008, fish were maintained under suitable light, temperature and feeding conditions. Waste pipelines and ventilation lines were routinely cleaned to remove dirt and to keep pollution as low as possible.

Analysis of Micronucleus (MN) and Other Nuclear Abnormalities: A drop of blood from the gills was mixed with a drop of fetal calf serum on a glass slide and then air- dried. The specimen was then fixed in absolute methyl alcohol for five minutes and the slide was stained with 10% Giemsa stain for 10 minutes [20]. The frequency of MN, bi-nucleated, fragmented-apoptotic cells and nuclear buds were scored. Ten slides per treatment were prepared. For each animal, 1000 cells were scored under 100 x magnification to determine the frequencies of micronucleated cells. Coded and slides taken at random were scored using a blind review by a single observer. Nuclear buds, bi-nucleated and fragmented-apoptotic cells were identified earlier using criteria described by M. Fenech with co-authors [21]. Their morphological features are shown in Fig. 1.

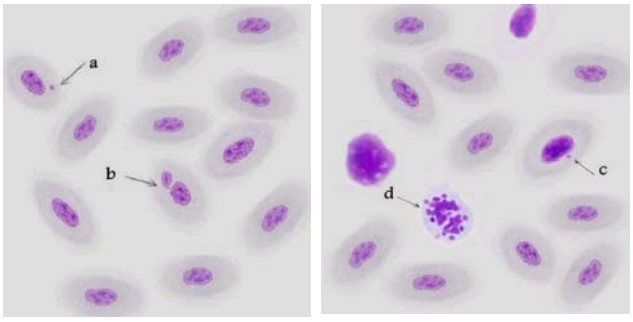

Fig. 1: Photomicrography showing different nuclear abnormalities (a) erythrocytes with micronuclei, (b) bi-nucleated cells (c), nuclear bud and (d) fragmented-apoptotic

DNA and RNA Contents: DNA and RNA from liver were isolated by modified method based on that Shibko *et al* [22]. RNA was estimated using visible spectrophotometers by orcinol reaction as described by Ashweel [23], while DNA was estimated by diphenylamine (DPA) colour reaction described by Burton [24].

Statistical Analysis: All the obtained results from chemical analysis are presented as means ±SE. Data obtained were subjected to T-test using the Statistical Package for the Social Sciences (SPSS). The Significancy differences between the hematological and blood biochemistry values of the control and treated groups were estimated.

RESULTS

Induction of MN in Peripheral Blood: Table 1 shows very highly significant (P<0.001) increase in micronucleus occurrence after treatment with 15 µg/L 4NP concentration for the first generation. Moreover, the concentration 30 µg/L showed a very highly significant increase in the number of micronuclei, compared to the control sample. Also, Table 1 shows the results of processing with 4NP, with the aforementioned two concentrations for the second generation which exhibited very highly significant (P<0.001) increase in the number of micronuclei, compared to the control sample. These results were also confirmed using the t-test and multiple comparisons.

Induction of Other Nuclear Abnormalities: The frequency of nuclear buds, bi-nucleated and fragmented apoptotic cells was determined in Talapia mature (peripheral blood) erythrocytes as a number of the abnormalities/1000 cells (‰).

In the control fish group, the means of frequency of nuclear buds, bi-nucleated and fragmented-apoptotic cells were very similar (values ranged from 0.36 to 0. 42 ‰). In blood of exposed Tilapia groups, the significant induction of nuclear buds and fragmented-apoptotic cells were detected in sub-g3 (Table 1). Elevated levels of bi-nucleated cells were not observed in all treatment groups. The frequency of nuclear buds were increased and the levels of fragmented-apoptotic cells were elevated after exposure to 30 µg/L of 4NP for the second generation (P < 0.001). Statistically very highly significant in induction of fragmented-apoptotic cells after exposure in, sub-g6 was found (Table 1).

The Effect of 4-Nonylphenol on DNA Content: Table 2 shows, that the average DNA content of the control sample during non exposure to the 4NP for the first generation was 113.55 µg/ml. Treatment with 4NP with concentration of 15µg/L led to significant increase in DNA content (138.63 µg / ml), compared to the control. When the fishes were exposed to the 30 µg/L concentration, the average DNA content showed highly significant increase (207.41 µg / ml). Also, Table 2 shows a very highly significant (P<0.001) increase when the second generation of fishes were exposed to both concentrations (15 and 30mg./L, respectively).

Table 1: Statistical analysis of Micronuclei and other nuclear abnormalities in blood cells of *Oreochromus S.* Fishes treated with different concentrations of 4 - Ninolphenol

		Nuclear abnormalities / exposure							
		Micronuclei		Nuclear buds		Bi-nucleated cells		Fragmented-apoptotic cells	
Type of Treatment		Total No.	% Mean±SE	Total No.	% Mean±SE	Total No.	% Mean±SE	Total No.	% Mean±SE
Group1	Sub group1 (Control)	50	0.5 5.00± 0.42	36	0.36 3.60±0. 37	42	0.42 4.20± 0.59	40	0.40 4.00±0.33
	Sub group 2 (15mg/L)	231	2.31 23.10±1.89 ***	39	0.39 3.90 ±.0.31	44	0.44 4.40± 0.26	40	0.40 3.90± 0.31
	Sub group 3 (30mg/L)	397	3.97 39.70± 2.51 ***	49	0.49 4.90±0.48 *	62	0.62 6.20±0.86	150	1.50 15.00±1.92***
Group2	Subgroup 4 (Control)	52	0.52 5.20± 0.32	44	0.44 4.40±0.37	55	0.55 5.50±0.52	38	0.38 3.80±0.35
	Subgroup 5 (15mg/L)	389	3.89 38.90± 3.00 ***	40	0.40 4.0±0.55	53	0.53 5.30±3.71	45	0.45 4.50±0.34
	Subgroup 6 (30mg/L)	768	7.68 76.80±18.546***	103	1.03 10.30± 1.39**	59	0.59 5.90±2.234	246	2.46 24.60±2.64***
Signiifcancy	Groups	P < 0.05	P< 0.001	NS	NS				
level	Dose	P< 0.001	P< 0.001	NS	P< 0.01				
	Groups * Dose	NS	P< 0.01	NS	P<0.05				

MN = Micronuclei. No. individuals Examined = 50 fish No. cells examined = 1000 / fish Statistically. NS: non Significant * Significant (P≤0.05), **highly significant (P<0.01), *** very highly significant (P<0.001) from the control

Table 2: Statistical analysis of DNA and RNA level in liver content treated with different concentrations 4 - Ninol phenol (4NP) for Tilapia *Oreochromus spilurs* Fishes

		Mean±SE	
Type of Treatment		DNA (µg / ml)	RNA (µg / ml)
Group1	Sub group1 (Control)	113.55±1.00	135.66± 0.87
	Sub group 2 (15mg/L)	138.63± 0.46 ***	140.79±1.64***
	Sub group 3 (30mg/L)	207.41±0.27***	143.43±0.87***
	Subgroup 1 (Control)	113.55± 1.00	135.66±0.87
Group2	Subgroup 2 (15mg/L)	148.63±0.46 ***	167.16 ±1.25 ***
	Subgroup 3 (30mg/L)	248.69±0.45***	246.88± 2.83 ***
Statistical Significancy level	Groups	P<0.001	P<0.001
	Dose	P<0.001	P<0.001
	Groups * Dose	P<0.001	P<0.001

No. individuals Examined = 50 fish No Statistically * Significant ($P \leq 0.05$), **highly significant (P<0.01), *** very highlysignificant (P<0.001) from the control.

The Effect of 4-Nonylphenol on RNA Content: Table 2 shows that the average RNA content of the control for the first generation was 135.66 µg / ml. Treatment with 4NP with concentration of 15 µg/L led to significant increase in average RNA content (140.79 µg /ml), as compared to the control. When the fishes were exposed to the 30 µg/L concentration, the average RNA content showed highly significant increase (143.43 µg /ml). Also, Table 2 shows a very highly significant (P<0.001) increase in average RNA content (167.16 and 246.88 µg/ml) when the second generation fishes were exposed to concentrations 15 µg/L and 30 µg/L respectively. Levels of statistical significancy for testing differences among treatment are listed in Table 2.

DISCUSSION

Alkyl phenol-ethoxylates (APEOs) are common surfactants used in cleaning products and involved in many industrial processes requiring wetting agents and emulsifiers. APEOs and their biodegradable products have xenoestrogenic characteristics. Acting as estrogen agonists, NPEOs or 4-nonylphenol ethoxylate caused distinct changes and growth in germ cells of feathed minnows and extensive cell proliferation in the mammary glands of noble rats[25]. In addition, some biodegradable products of estrogen-like APEOs, especially 4-nonylphenol ethoxylate, also demonstrate cytotoxic effects in aquatic organisms, which has led to increasing concern about the environmental hazards of these substances [26].

Previous studies determined the lethal concentration (LC 50% = 0.032 ml l-1) of NP against *Oreochromus spilurs* niloticus by exposure for 72 h suggesting the sensitivity of this fish to NP toxicity. Also some authors reported that NPEO induced cytotoxicity in many aquatic organisms at concentrations ranging from 0.069 to 5.0 mg/mL [26, 27]. Most detected concentrations of 4-nonylphenol ethoxylates in river water ranged from 0.00001 to >0.1 mg/mL, lower than these cytotoxic levels [28].

The micronucleus test in peripheral erythrocytes provides a feasible approach to monitor the effects of environmental genotoxic agents in fish. A large variability in spontaneous MN frequency in fish was evident from the analysis of the scientific literature [29]. Tilapian fish have been widely used as a biological indicator and in the monitoring of toxic substances in an aquatic environment [13, 18, 19, 30, 31].

The present results showed positive genotoxic effects as measured by increase in MN frequency in erythrocytes from fish exposed to 4NP. A number of papers recently appeared in the scientific literature reported an association between the frequency of nuclear anomalies and the exposure to genotoxic agents [32, 33]. A comprehensive application has been proposed for the MN assay in human lymphocytes, including a set of parameters of genotoxicity and citotoxicity: MN as markers of chromosomal breakage and/or loss, nucleoplasmic bridges as markers of chromosomal rearrangements, nuclear evaginations or buds as markers of gene amplification, cellular necrosis and apoptosis [34].

Higher cytotoxicity of 4-NP to peripheral erythrocytes of *Oreochromus S* was observed in the treatments at concentration of 30 µg/ L, which showing an increased frequency in nuclear abnormalities. [5] Showed that the bioaccumulation of NP in the fish species Onchirynchus mykiss occurs in a time dependent manner, as observed through histological changes in the liver.

The current study showed that exposure of fish to either dose 15mg/l or 30mg/l of 4NP significantly increased the levels of DNA in liver of 4-NP intoxicated fish in both mother and its first, second generations offspring compared with normal control ones. The first generation was dramatically affected by the toxic effect of 4-NP indicated by an increased DNA level compared with the mother fish. Also, the results revealed marked increment in RNA concentration in livers of intoxicated first generation fish compared to the normal ones, however, significant increase in RNA level in liver of mothers exposed to 4-NP in relation to normal fish. The present results may indicate the genotoxic effect of this compounds on liver tissue. Similar genotoxic effect of nonylphenol on human lymphocytes and sperm cells was reported [35]. A genotoxicity study on MCF-7 human breast tumor cells revealed genomic instability by telomeric associations and chromosomal aberrations caused by nonylphenol [36]. In a previous study on blood samples of 2 donors, nonylphenol caused DNA damage using concentrations from 1 to 500 mM.

Previous investigation on human lymphocytes showed a dose-dependent increase in DNA damage at concentrations starting with 5 mg/mL of 4-nonylphenol ethoxylate, whereas lower concentrations (0.15-1.5 mol/ml) showed no significant differences in genotoxicity compared to the negative control in test system [37]. Concentrations higher than 30 mg/ml with increasing genotoxic effects were accompanied by excessive cytotoxicity (viability <70%). Viabilities below 70% are considered to be incompatible with reliable genotoxicity measurements[38].Concentration levels causing dose-dependent genotoxicity with viabilities higher than 70% in comparison to the concentrations resulting in cytotoxicity in aquatic organisms [39].

Concerning the toxic effect of 4NP on Mn of blood cells, the results revealed Higher cytotoxicity of NP on blood cells of *Oreochromus spilurs*. niloticus treated with 15mg/l or 30mg/L as indicated by pronounced increase in frequencies of abnormal Mn of treated fish when compared with normal untreated ones. These results may confirm the genotoxicity of the 4-NP to *Oreochromus spilurs*. niloticus. The current results are in agreement coped with Kaptaner [40]. Uguz *et al*. [5] showed that the bioaccumulation of NP in the fish species Onchirynchus mykiss occurs in a time dependent manner, as documented through histological changes in the liver.

Kaptaner and Unal [40] reported that NP dependent nepatotoxicity was causally related to the increase in apoptosis where NP(200 µg L^{-1}) caused significant increases in the extent of apoptosis in liver.

In conclusion the results of this study indicated a positive correlation between the amount of the compound (i.e. dosage dependent effect) and the inspiring for cytogenetic effect on Talapia fishes in Jeddah.

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