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DNA damage in spleen as a indicator of genotoxicity in *Channa punctatus* exposed to 4-nonylphenol

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Abstract

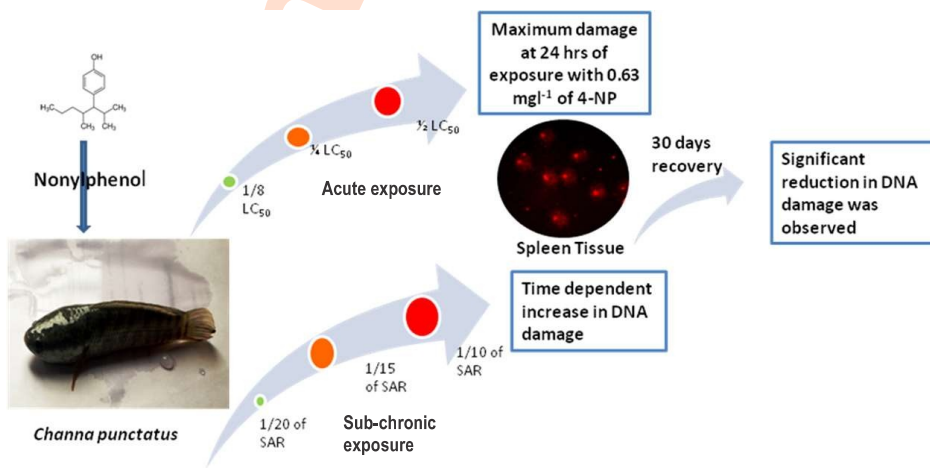
Aim : The present study was performed to evaluate the genotoxic effect of 4-nonylphenol after acute and subchronic exposure in spleen tissue of *Channa punctatus*, recovery in DNA damage was also ascertained after 30 days of cessation of exposure.

Methodology : Tail length (TL), tail intensity (TI), tail moment (TM), Olive tail moment (OTM) was used as biological indicators of DNA damage. The fish were exposed to different sublethal concentrations of 4-NP for 96 hrs (acute exposure) and for 90 days (sub chronic exposure).

Results : Exposed groups showed significantly higher DNA damage in both acute and sub chronic exposure as compared to control groups. In the case of acute exposure, the highest damage was observed at 24 hr of exposure followed by a decline in the value of all the parameters, while in the later hours of exposure these value further increased. On the other hand, in the case of sub-chronic exposure, the highest damage was observed after treatment with 0.10 mg l⁻¹ concentration of 4-NP at 90 days of exposure. Recovery experiment showed a decrease in the values of all the parameter's studied, however, a significant decrease was observed only at the highest concentration.

Interpretation : The results conclude the DNA damaging potential of 4-nonylphenol and highlighted the usage of spleen tissue for genotoxicity testing.

Key words: 4-Nonylphenol, *Channa punctatus*, Comet assay, Genotoxicity, Spleen



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Introduction

Worldwide the concern is growing for the release of xenobiotics in the environment, especially surface active agents. Among these emerging contaminants alkylphenols (nonylphenol, octylphenol, etc.) also known as endocrine disruptors (EDCs) are of more concern today as they are persistent in nature (Lee *et al.*, 2018), and they are also not even regulated in developing countries (Barrios-Estrada *et al.*, 2018). Nonylphenol is a ubiquitous contaminant primarily released from agricultural, household and industrial waste waters (Gavrilescu *et al.*, 2015; Kourouma *et al.*, 2015). Due to its low solubility and higher hydrophobicity, nonylphenol is considered as one of the persistent organic pollutants (POPs). Due to its bioaccumulation nature, it induces a toxic effects on aquatic fauna. Nonylphenol have low solubility, hence they have a high tendency to accumulate in soil and sediments (Careghini *et al.*, 2015). Their residues have been detected frequently in soil, sediment, water and air (Sharma and Chadha 2018a; Cao *et al.*, 2019). High concentrations of 4-NP have been reported in the rivers of developing countries as compared to developed countries. Gautam *et al.* (2015) reported nonylphenol concentration ranging between 12.40 $\mu\text{g l}^{-1}$ to 16.29 $\mu\text{g l}^{-1}$ in the river Ganga and Varuna in India, while Jie *et al.* (2018) reported the concentration of 4-NP in river Xiangjiang in China, ranging between 0.174 to 3.411 $\mu\text{g l}^{-1}$, respectively.

Nonylphenol has attracted the attention of scientists and environmentalists as they interfere with the endocrine system and bioaccumulate in aquatic organisms (Vilela *et al.*, 2018). It has been associated with reproductive impairment (Wang *et al.* 2019), nerve impairment (Guo *et al.*, 2019) and immune system weakening (Sharma *et al.*, 2018; Sayed *et al.*, 2019). The effect of 4-NP as a toxicant have been studied using different animal models (Ayanda *et al.*, 2018; Sharma and Chadha, 2016a) as well as different tissues (Sharma and Chadha, 2017; Tasmeen and Yasmeen 2018), showing that the effect are different from one animal model to another as well as from one tissue to another depending upon their sensitivity. Till date toxicological research on endocrine disrupting compounds (EDCs) using fish is mainly focused on their effect on reproduction whereas little attention has been paid to possible immune-endocrine interaction (Sayed *et al.*, 2018).

Spleen, an organ of the immune system act as a storage site for lymphocytes and provides immunity and defense against infection and filtering blood in fish (Ajdari *et al.*, 2014; Zhang *et al.*, 2010). Thereby providing protective immunity (Li *et al.*, 2019). Being an organ that is interposed in the blood stream, it also stands as the body's largest blood filter that furthermore contribute in detecting senescent, mechanically damaged and aberrant cells. Effect on spleen will lead to immune suppressive response. Severe or chronic stress is often associated with poor performance and has long been associated with immune-suppression in fish (Sayad *et al.*, 2019; Sharma and Chadha, 2018b). Comet assay is used to assess the genetic status of an

organism. Comet assay is a flexible, rapid, simple, inexpensive and sensitive assay as it quantifies DNA damage in individual cell and detects DNA damage as strand break. Comet assay is well suited to be applied to spleen tissue as it does not rely on proliferating cells as well as it allows a broad range of DNA damage.

Fish is a marvelous animal model for testing genotoxicity as they provide early warning signs for environmental changes and degradation. They also play an important role in maintaining balance of the aquatic system. Several investigations have emphasized the use of fish as a sentinel species. In the present study, *Channa punctatus* was selected as test model, as it has a wide distribution in India due to its hardy nature and is being consumed in several parts of the country. This fish has high growth rate, easy adaptability to laboratory conditions, and can be fed on artificial feed. A number of authors have reported that *C. punctatus* has been reported as a good bio model for genotoxicity evaluation (Sharma and Chadha 2017; Sharma and Chadha 2019). Keeping in view the above, the present study aimed to verify the acute and subchronic genotoxic effects of 4-NP on spleen cells of *C. punctatus* along with the recovery tendency of spleen cells of fish *C. punctatus* after cessation of exposure.

Materials and Methods

Experimental design: 4-nonylphenol used in the present study was obtained from Himedia (India). A stock solution was prepared in ethanol. In order to eliminate the leaching potential of 4-nonylphenol, plastic material was avoided and 200 l glass aquaria were used for the experiment. The 96 hr LC₅₀ value of 4-NP was determined as 1.27 mg l⁻¹ for *C. punctatus*. Three sub lethal concentrations for acute exposure was determined as 1/2 of LC₅₀ (0.63 mg l⁻¹), 1/4 of LC₅₀ (0.31 mg l⁻¹) and 1/8 of LC₅₀ (0.15 mg l⁻¹). For sub chronic exposure, safe application rate was determined by the method of Basak and Konar (1977). 1/10th (0.15 mg l⁻¹), 1/15th (0.10 mg l⁻¹) and 1/20th (0.07 mg l⁻¹) of SAR were decided as sublethal concentrations for sub-chronic exposure. Exposure was given for 96 hr in acute treatment, while for 90 days in case of sub-chronic experiment. Sampling was done at 24, 48, 72 and 96 hr after acute exposure while at 30, 60 and 90 d (720, 1440, 2160 hr) for sub-chronic exposure. The specimens maintained in tap water served as negative control while in ethanol as positive control.

Quantification of DNA damage: Comet assay was performed by the method of Singh *et al.* (1988). A 100 μl blood was diluted in 1000 μl phosphate saline buffer (PSB) followed by three agarose layer on slide one with the diluted blood. The slides were then subjected to lyse in refrigerator followed by incubated for 20 min in 300 mM + 1 mM EDTA buffer (pH>13). Electrophoresis at 25 v and 300mA for 20 min was carried out. Slides were neutralized with 0.4 M Tris for 15 min and were stained with ethidium bromide. Two slides were prepared for each group and analyzed further under fluorescent microscope. The captured digital images were scored through CASP Lab. Hundred randomly selected cells were

scored through CASP Lab. DNA damage was quantified by studying different parameters including Tail length (TL), Tail moment (TM), percent tail DNA (% DNA) and Olive tail moment (OTM).

Statistical analysis: The data, expressed as mean \pm SE, was analyzed through one-way analysis of variances (ANOVA), followed by Tukey's test by software ASSISTAT to see the significant difference.

Results and Discussion

In the present investigation, the DNA damaging effect of different sublethal concentrations of 4-NP after acute and subchronic exposure on spleen cells of *C. punctatus* was assessed. Also the recovery of damage induced after subchronic exposure of 90 days was assessed. Perusal of Table 1 reveals the genotoxic effect of different concentrations of 4-NP on spleen cells of fish after acute exposure to 24, 48, 72 and 96 hrs. DNA damage was analyzed by comet assay using four parameters like tail length (TL), tail intensity (TI), tail moment (TM) and olive tail moment (OTM). Different researchers have used different comet parameters for analyzing genotoxicity. Some suggested tail length as good indicator of genetic damage (Rajaguru et al., 2003; Nwani et al., 2010; Pereira et al., 2012). Some emphasized the evaluation of TL and TM (Duez et al., 2003). Further, Sunjog et al. (2013) suggested that OTM and percent tail intensity could be used for scientific purposes. Kopjar et al. (2008) used comet tail length, tail intensity and tail moment to study genotoxicity of

polluted water in *Cobitis elongate*. While Cavas and Konen (2008) used the percent damaged cell and genetic damage index as parameters for the comet assay for testing domoic acid toxicity on fish *O. niloticus*. Tail moment was used as parameter to detect DNA damage by Adeyemi et al. (2015) in fish *Danio rerio* co-exposed to arsenic and atrazine.

The values of all the parameters were found to be significantly higher in all the treatment groups as compared to positive and negative control. Effect of duration was also found to be significantly higher (One way ANOVA $p \leq 0.05$). These results are in line with the previous studies on genotoxicity as higher values for different comet parameters were found in *C. punctatus* after exposure to monocrotophos and profenfos (Ali and Kumar, 2008; Pandey et al., 2011). Increased DNA damage was also studied in other species of fish after treatment with glyphosate based herbicide (Guilherme et al., 2012). Recently, Hussain et al., (2018) assessed the DNA damage in *Labio rohita* collected from the polluted area of river Chenab.

During acute exposure, the maximum effect was seen after 24 hrs of exposure followed by a decrease in the value of all the parameters. TL was found to increase 63.88 μ m as compared to the control group. Similarly, TI was found 41.2 % higher than the control group after treatment with 4-NP. Furthermore, TM increased 29.98 % and OTM increased by 19.16 % when compared with negative control. At lowest concentration, the value of TL decreased upto 72 hr, while a slight increase in the value was seen at 96 hr. On the other hand, the value of all other

Table 1: Values of different comet parameters (TL, TI, TM, OTM) in spleen cells after acute exposure to different concentrations of 4-NP for 24, 48, 72 and 96 hrs

Doses		TL (μ m)	TI (%)	TM	OTM
0.15 mg l ⁻¹	Control	3.65 \pm 0.02 ^a	0.34 \pm 0.07 ^a	0.06 \pm 0.06 ^a	0.08 \pm 0.01 ^a
	Ethanol	5.24 \pm 0.02 ^a	0.92 \pm 0.08 ^a	0.18 \pm 0.005 ^a	0.16 \pm 0.01 ^a
	24 hr	22.3 \pm 0.98 ^b	12.91 \pm 1.9 ^b	3.59 \pm 0.28 ^b	4.39 \pm 0.36 ^b
	48 hr	24.77 \pm 3.44 ^b	14.21 \pm 2.79 ^b	6.68 \pm 0.68 ^b	5.01 \pm 0.02 ^b
	72 hr	8.28 \pm 1.10 ^a	4.2 \pm 0.65 ^a	0.49 \pm 0.13 ^a	1.19 \pm 0.28 ^{ab}
	96 hr	10.11 \pm 0.51 ^a	1.53 \pm 0.17 ^a	0.35 \pm 0.12 ^a	0.54 \pm 0.008 ^a
0.31mg l ⁻¹	Control	3.60 \pm 0.002 ^a	0.54 \pm 0.43 ^a	0.14 \pm 0.06 ^a	0.11 \pm 0.007 ^a
	Ethanol	5.73 \pm 0.25 ^a	1.73 \pm 0.0001 ^a	1.03 \pm 0.03 ^a	0.50 \pm 0.17 ^{ab}
	24 hr	51.85 \pm 7.46 ^b	31.33 \pm 6.22 ^c	17.53 \pm 9.42 ^a	13.96 \pm 3.6 ^c
	48 hr	38.95 \pm 6.17 ^b	17.81 \pm 0.45 ^b	9.17 \pm 0.66 ^a	7.44 \pm 0.70 ^{bc}
	72 hr	58.19 \pm 0.40 ^c	38.37 \pm 0.47 ^c	26.25 \pm 1.3 ^c	17.27 \pm 1.22 ^c
	96 hr	54.98 \pm 1.17 ^b	25.61 \pm 2.26 ^{bc}	11.82 \pm 0.309 ^a	11.58 \pm 0.785 ^c
0.63 mg l ⁻¹	Control	4.00 \pm 0.28 ^a	0.52 \pm 0.86 ^a	0.04 \pm 0.01 ^a	0.11 \pm 0.01 ^a
	Ethanol	5.28 \pm 0.003 ^a	1.93 \pm 0.04 ^a	0.99 \pm 0.008 ^a	0.19 \pm 0.005 ^a
	24 hr	67.88 \pm 3.3 ^c	41.72 \pm 2.77 ^c	30.02 \pm 2.84 ^c	19.27 \pm 1.42 ^d
	48 hr	36.68 \pm 2.88 ^b	18.93 \pm 1.56 ^b	10.18 \pm 0.77 ^b	6.72 \pm 0.45 ^c
	72 hr	46.28 \pm 0.95 ^b	18.28 \pm 0.90 ^b	15.35 \pm 0.77 ^b	11.46 \pm 1.07 ^b
	96 hr	39.36 \pm 3.41 ^b	25.21 \pm 1.6 ^b	11.84 \pm 1.64 ^b	10.08 \pm 1.29 ^{bc}

Values are mean \pm standard error. Different letters (a, b, c, d) with in columns are significantly different (Tukey's test) and signify the effect of duration of exposure after treatment with each concentration. TL-Tail length; TI: Tail intensity; TM-Tail moment and OTM-Olive tail moment

Table 2: Values of different comet parameters in spleen cells after sub-chronic exposure to different concentrations of 4-NP for 30, 60 and 90 days

Doses		TL (μm)	TI (%)	TM	OTM
0.07 mg l ⁻¹	Control	3.63±0.013 ^a	0.40±0.03 ^a	0.05±0.008 ^a	0.09±0.007 ^a
	Ethanol	5.22±0.013 ^a	0.84±0.04 ^a	0.184±0.002 ^a	0.15±0.005 ^a
	30 days	15.78±0.75 ^b	12.31±1.33 ^b	2.47±0.27 ^b	3.85±0.42 ^c
	60 days	14.70±0.45 ^b	17.89±0.23 ^c	3.59±0.41 ^c	2.76±0.28 ^b
	90 days	15.38±0.28 ^b	18.51±0.61 ^c	4.02±0.137 ^c	3.53±0.08 ^{ab}
0.10 mg l ⁻¹	Control	3.6±0.001 ^a	0.58±0.02 ^a	0.2±0.03 ^a	0.12±0.003 ^a
	Ethanol	5.5519±0.12 ^{ab}	1.73±0.001 ^a	1.0075±0.01 ^a	0.65±0.88 ^a
	30 days	10.708±0.05 ^b	8.05±0.49 ^b	1.455±0.11 ^a	2.04±0.126 ^b
	60 days	24.81±0.11 ^c	14.45±0.04 ^c	4.72±0.03 ^b	3.84±0.016 ^c
	90 days	48.24±2.75 ^d	25.29±0.57 ^d	13.14±0.80 ^c	9.003±0.44 ^d
0.15 mg l ⁻¹	Control	4.25±0.14 ^a	0.44±0.04 ^a	0.58±0.006 ^a	0.107±0.005 ^a
	Ethanol	5.28±0.001 ^b	1.89±0.02 ^a	0.98±0.004 ^b	0.18±0.002 ^a
	30 days	8.83±0.01 ^c	6.8±1.05 ^b	0.81±0.16 ^b	1.07±0.06 ^b
	60 days	11.45±0.08 ^d	6.36±0.10 ^b	0.89±0.003 ^b	1.13±0.03 ^b
	90 days	12.46±0.003 ^e	11.32±0.36 ^c	1.73±0.01 ^c	2.21±0.05 ^c

Values are mean ± SE. Different letters (a, b, c, d, e) within columns are significantly different (Tukey's test) and signify the effect of duration of exposure after treatment with each concentration

Table 3: Values of different parameters of the comet assay in spleen cells of fish *C. punctatus* after exposure to different concentrations of 4-NP for 90 days and after 30 days of recovery

Doses		TL (μm)	TI (%)	TM	OTM
0.07 mg l ⁻¹	Exposed gp	15.78±0.75 ^a	12.31±1.33 ^a	2.47±0.27 ^a	3.85±0.42 ^a
	Recovery gp	11.31±1.66 ^a	7.40±1.45 ^b	1.25±0.42 ^a	1.97±0.63 ^a
0.10 mg l ⁻¹	Exposed gp	10.70±0.05 ^a	8.05±0.49 ^a	1.45±0.11 ^a	2.04±0.126 ^a
	Recovery gp	8.31±1.61 ^a	5.88±2.06 ^a	1.14±0.25 ^a	1.17±0.60 ^a
0.15 mg l ⁻¹	Exposed gp	8.83±0.01 ^a	6.8±1.05 ^a	0.80±0.16 ^a	1.07±0.06 ^a
	Recovery gp	6.97±0.23 ^b	2.56±0.41 ^b	0.31±0.081 ^a	0.49±0.05 ^b

Values are mean ± SE. Different letters (a, b) are significantly different (Tukey's test, $p \leq 0.01$) and signify difference between the exposed and recovery group.

three parameters TI, TM and OTM decreased upto 96 hr of exposure, but a different pattern was observed at the highest concentration value of TL decreased upto 96 hr while the value of all other parameters increased at the highest exposure period. One explanation may be due to repair of damaged DNA or replacement of highly damaged cells or both (Banu *et al.*, 2001). More comets in the early hour of exposure may be due to apoptosis of cells in the later hour of exposure. Another possibility is gene activation like cytochrome p450 which activate the metabolizing enzymes which provide a defensive mechanism against genotoxicants (Wong *et al.*, 2001).

After sub-chronic exposure also significantly higher values of all parameters were observed in the treatment groups as compared to control (Table 2). The highest DNA damage was observed after treatment with 0.07 mg l⁻¹ concentration at 90 days of exposure (Table 2). Where TL was 44.04 μm higher and TI was

24.75% higher than the negative control group. Similarly, value of TM increased by 12.94 % and the increase of 8.88 % in OTM were seen. The reasons behind this might be a threshold repair theory. According to it DNA repair enzymes get activated only when tissue accumulates the toxicant above a threshold level below which repair mechanism operate only at basal level (Kienzler *et al.*, 2013). This must be the reason of the observed low value of comet parameters after 0.10 mg l⁻¹ and 0.15 mg l⁻¹ compared to 0.07 mg l⁻¹ treated group.

4-NP is reported to induce microtubular disruptive activity, mutation and genomic rearrangements which may lead to DNA adduct formation (Sharma and Chadha, 2017). Another possible reason for DNA damage may be formation of reactive intermediates which may cause DNA damage. DNA damage leads ultimately to apoptosis. Miura *et al.* (2005) observed apoptosis in sertoli cells after exposure to 4-NP. Similarly

Makkawy et al. (2011) found an increase percentage of apoptotic cells in fish *C. galpinus* after exposure to 4-NP. Sayed et al. (2018) reported the increase production of ROS and depletion of enzymatic and non-enzymatic antioxidant and upregulation of oxidative stress related genes under NP induced stress.

Recovery study showed a decrease in the values of all the parameters (Table 3). In spleen cells of recovery group, a significant recovery was observed only in the group treated with 0.15 mg l⁻¹ concentration of 4-NP, where as decrease in TL by 1.86 µm, TI by 4.24 % and OTM up to almost 50% were found. The probable reason for this may be the genotoxic response is a cell-specific process, with toxicant exposure required to reach a threshold level before DNA repair systems are initiated (Langan et al., 2018). The results of both acute and subchronic exposures show the genotoxic potential of 4-NP is genotoxic in the spleen cells of *C. punctatus*. The usage of spleen cells for genotoxicity assessment has been scarcely explored. Spleen tissue has recently used in toxicity and immunomodulatory studies (David and Kartheek, 2015; Wang et al., 2019). Spleen act as filters for blood and after metabolizing xenobiotics and may cause damage to the body, specifically the immune system which may ultimately make them prone to a number of diseases and death of fish. In the present study, we found that the spleen is a sensitive tissue for genotoxicity testing and suggest that the spleen is a good indicator of genotoxicity and can be used to assess the genotoxic effect to see the overall effect of pollutant on health, specifically on the immune system of fish.

In conclusion, the present study sheds light on the genotoxic effect of 4-NP on the spleen tissue of *C. punctatus* and confirming its utility as a sensitive organ for toxicological biomonitoring. Both acute and sub chronic exposure leads to DNA damage. Recovery tendency was found to be low in spleen cells as significant recovery was shown by only one group. Therefore, attention of environmental agencies should be aroused regarding its concentration in water bodies especially in the developing countries like India.

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