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Research Article

CYTOTOXIC AND GENOTOXIC EFFECT OF OXYTETRACYCLINE ON FISH *CYPRINUS CARPIO* AFTER ACUTE EXPOSURE

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ABSTRACT

Objective: Cytotoxicity in blood cells and genotoxic alteration in gill, liver, and kidney of *Cyprinus carpio* acutely (96 h) exposed to 80 mg/L oxytetracycline (OTC) including a control (non-exposed group) were evaluated in the present study. Genotoxic endpoints reflecting different types of genetic damage in cells of the liver, gill, and kidney were also determined by analysis of nuclear and cytoplasmic abnormalities.

Methods: *C. carpio* was divided into two groups, one control and others treated with 80 mg/L OTC. After 96 h, sampling was done and slides were prepared for different tissues as well as for measuring polychromatic erythrocyte (PCE) frequency in blood. Slides were scored for micronucleus, nuclear abnormalities, swollen cells, and vacuolated cytoplasm.

Results: The hereby data obtained showed a higher and significant increase in the genotoxic effect in all the tissues tested. Furthermore, gill cells showed the highest genotoxic effect followed by liver and kidney, while PCE frequency increases up to 72 h of exposure, on the other hand, a significant decrease in the value was observed at 96 h of exposure.

Conclusion: The present study revealed that OTC has cytotoxic and genotoxic effect on different organs and blood cells of *C. carpio* at this concentration and suggests gill as sensitive tissue for genotoxic assessment.

Keywords: Oxytetracycline, Genotoxicity, Polychromatic erythrocyte frequency, Micronucleus assay, Normochromatic erythrocytes.

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INTRODUCTION

Pharmaceuticals are synthetic or natural chemicals, manufactured for use as therapeutic medicines and veterinary drugs. The major route for pharmaceuticals to enter in aquatic environments is hospital disposal, agricultural runoff, and aquaculture waste [1-3]. Throughout the world, more than 100 pharmaceuticals and their metabolites have been found in water bodies at concentrations ranging from ng to mg/L [4]. Recently, Khanm *et al*. [5] have suggested that there are irrational use and inappropriate prescription pattern of antibiotics in the rural area of developing countries. Studies carried out during the past few years in Europe, Brazil, Spain, Germany, and USA have shown that chemical substances in the form of secondary metabolite extracts from pharmaceutical products are found as toxin in aquatic environments is more numerous every year [6]. The concern further increases for the release of active pharmaceutical ingredient into the environment because they are designed for biological activity [7]. Hence, the European Union water framework directive has included pharmaceuticals on a dynamic watch list based on their potential toxic effect on aquatic organisms [8].

Oxytetracycline (OTC) is the broadspectrum tetracycline group of antibiotics and an antibacterial agent which is used for the treatment of bacterial diseases, mainly caused by Gram-negative bacterial infectious agent [9,10]. Maximum of OTC is given to fishes through the feed, but fish have very less absorption for OTC, so 70–80% of OTC is excreted as waste material into the water. OTC can be retained in the sludge and bottom deposits of fish farms due to their lipophilic and non-degradable nature [11,12]. The excessive use of OTC in common carp aquaculture may also increase the possibility of transfer of antibiotic resistance to human pathogens; as a result, it is dangerous to humans [12,13]. In Asian countries, it was noticed that liquid waste and sewage discharged into the river at concentrations up to mg/L [14,15]. It has been found that 59 pharmaceuticals were present in effluent samples collected from Patancheru common effluent treatment plant (Patancheru Enviro Tech Limited) near Hyderabad, India [14].

Under severe stress condition, tissue alterations can be seen in fish organs. OTC is found in blood, kidney, liver, and muscle tissues of *Lateolabras janopicus* and black sea bream *Sparus macrocephalus* [16]. Gill, kidney, and liver are sensitive organs used for assessing the health status of fish. The liver is the organ involved in accumulation and metabolizing the xenobiotics. The liver is the main organ for OTC deposition, while gill is the organ which comes in direct contact to pollutant and the kidney is the main organ of excretion and osmoregulation [17].

Micronucleus (MN) assay is used to investigate the clastogenicity, genotoxicity, and aneugenicity and it is one of the most delicate markers for noticing DNA damage [18]. In polychromatic erythrocyte (PCE) the presence of a MN is used as an index for genotoxic potential, and for an index of the cytotoxicity, PCE ratio is used [19]. The decrease in the ratio of (P/N) and PCE to normochromatic erythrocytes (NCE) is an indicator of toxicity [20].

Fish is an important bioindicator due to their role in aquatic chain and due to their sensitivity to low concentrations of toxic substance [21]. *Cyprinus carpio* is species of greater commercial importance and is highly cultured species. *C. carpio* is selected due to its greater commercial value, easy adaptability, and high growth rate and tolerate a wide range of temperature. To check the toxicity of different pollutant,

C. carpio is widely used in various studies [22,23]. Studies related to cytogenotoxicity of OTC on fish are very scarce, so the present study was aimed to evaluate the putative toxic effect elicited by OTC in fish *C. carpio*.

METHODS

Common carps with almost the same size and weight were collected from the Department of Fisheries Farm at CSK Himachal Pradesh Agricultural University, Palampur (India), and they were safely transported to the laboratory in large bucket with proper covering. For removing the dermal infection fish were treated with 0.2% $KMnO_4$ for 2 min. Fish were stocked in a large aquarium containing dechlorinated water for 10 days and were fed on the farm made pelleted feed once in a day. The debris was removed from the aquaria after each feeding. To remove metabolic waste, water medium was changed regularly.

Acute exposure

Fish *C. carpio* (total length 23.5±17 cm and total weight of 160±60 g) were exposed in 50 L aquaria for 96 h with an OTC concentration of 80 mg/L. This concentration was sublethal and selected on the basis the work done by Ambili *et al*. [12]. In this experiment, an additional control group (without chemical) was also included in the study. The treatment was given to the fish for 96 h and after every 24 h exposure medium was 100% renewed. During this exposure fish were not fed.

For cells from gill, liver, and kidney tissue

After dissection, gill arches, liver, and kidney tissue were removed and transferred to Carnoy's fixative. Before slide making gills were placed in 20% acetic acid, liver, and kidney were placed in 45% acetic acid for 30 min for tissue maceration. After this chemical maceration, epithelial cells were then scraped off from the gills and placed slides were made by removing tissue clumps. Tissues were gently minced and filtered. Then, the cell suspensions were smeared on dry slide. Slides were air dried and stained with 10% Giemsa solution for 25–30 min.

Photomicrography and scoring of slides

Scoring of micronucleus cells (MNC) and binucleated cells (BNC) was done according to Sharma and Chadha [17]. Photomicrography of normal cells and cells with binucleus and MN, and other cellular abnormalities were taken using the Olympus digital camera (Olympus, ×3) with a trinocular compound light microscope (Olympus C ×41). The scoring was done under magnification (×100) using Magnus immersion oil. One thousand cells were scored from each tissue at the treatment 96 h as well as from the control group. The mounted slides were examined by three experienced observers.

PCE frequency

For determining PCE frequency, blood samples were collected from each treated fish and control fish at 24, 48, 72, and 96 h and the blood smears were prepared. A blood smear was dried at room temperature and fixed in absolute ethanol and dried again at room temperature. Then, the fixed slides were stained with 10% Giemsa for 30–35 min. Slides were scored according to Pacheco and Santos [24] for NCE and PCE.

PCE frequency was calculated as:

$$\text{PCE frequency} = \frac{\text{PCE} \times 100}{\text{PCE} + \text{NCE}}$$

Statistical analysis

One way analysis of variance was applied to study the effect of duration on parameters studied. Tukey's test was applied to see the significant difference between different time intervals. All the statistical analysis was done using SPSS.

RESULTS

Table 1 shows the value of different parameter micronucleated cells, swollen cells (SC), nuclear abnormality (NA), lysed cytoplasm, and vacuolated cells in kidney, liver, and gill tissue in control and post 96 h treatment with OTC. The values significantly increase in post-treated group when compared with control group (Tukey's test) of all the parameters in all the three tissues tested. The highest micronucleated cells were found in gill tissue followed by liver and least were found in the kidney. A time-dependent increase in the values of all the parameters has been found. A similar trend was found for NAs, while SC and vacuolated cytoplasm were found higher in liver cells. The result showed that gill is the most affected organ followed by liver and kidney.

PCEs frequency

PCE frequency in fish after treatment with 80 mg/l concentration of OTC is shown in Fig. 1. An increase in PCE frequency was observed at 24 h, but the change was not significant (Tukey's test). A significant increase was observed in PCE frequency from at 48 and 72 h of exposure, but after 72 h the PCE frequency significantly decreases at 96 h of exposure. The highest PCE frequency was observed at 72 h of exposure, while the lowest percent frequency of PCE was observed at 24 h of exposure. The highest PCE frequency was 3.39 times more than the lowest PCE frequency.

DISCUSSION

Along with blood cells, tissue-specific genotoxic response has also been studied in the present study. Sharma and Chadha [25] suggested that the use of different cell type for genotoxicity assessment can be used to see the overall condition of the organism. Three mitotically active tissues, i.e., kidney, gill, and liver were used and tested for genotoxicity assessment. In the present study, it is observed that the liver and gill cells showed more damage than kidney cells when treated with OTC. The gill cell shows more damage as they are constantly vulnerable to an assortment of traumatic, toxicological, and infectious insults [26]. Liver cells also show high damage as the liver is the site for metabolism of xenobiotics, processing, and storage of nutrients and enzyme synthesis. Furthermore, Andrade and Tulkens [27] found that OTC shows nephrotoxicity and hepatotoxicity, which is closely associated with oxidative stress. The same results have been observed by Talapatra and Banerjee [28] in *Labeo bata* from sewage fed fish farm. Fagr *et al*. [29] suggested that gills showed MN induction as they are more sensitive. Sharma and Chadha [25] also found the MN frequency in gill cells

Table 1: Percent frequency of MN, SC, NA, LC, and VC in different parameters in kidney, liver, and gill tissue after treatment with 80 mg/L concentration of oxytetracycline at different times of exposure

Parameters	Kidney tissue		Liver tissue		Gill tissue	
	Control	96 h	Control	96 h	Control	96 h
MN	0.09±0.037[a]	0.46±0.021[b]	0.15±0.254[a]	1.46±0.021[b]	0.03±0.076[a]	1.96±0.105[b]
SC	5.816±0.075[a]	24.66±0.211[b]	5.617±0.183[a]	40.33±.211[b]	0.833±0.076[a]	10.33±0.211[b]
NA	14.73±0.1.9[a]	41.00±0.258[b]	10.500±0.126[a]	32.500±0.224[b]	9.583±1.64[a]	43.167±0.105[b]
LC	3.707±0.067[a]	38.667±0.211[b]	5.400±0.073[a]	13.75±0.112[b]	5.583±0.083[a]	20.33±0.167[b]
VC	2.65±0.085[a]	10.500±0.224[b]	0.817±0.17[a]	82.667±0.105[b]	0.633±0.131[a]	2.41±0.154[b]

The values are given as mean ±standard error. Different letters ([a,b]) are significantly different (Tukey's test, p≤0.01) and signify the effect of duration of exposure at concentration 80 mg/L. MN: Micronucleus, SC: Swollen cell, NA: Nuclear abnormalities, LC: Lysed cytoplasm, VC: Vacuolated cytoplasm

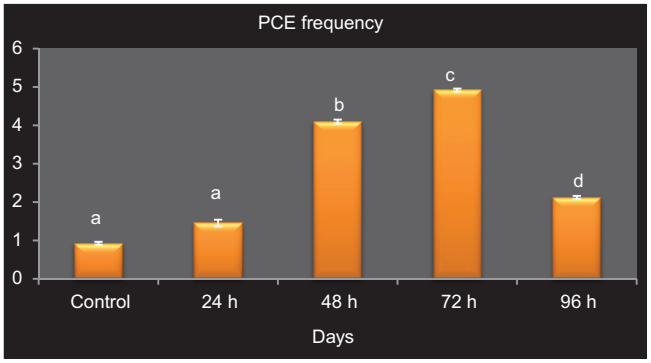

Fig. 1: Mean frequency of polychromatic erythrocyte in blood of *Cyprinus carpio* after treatment with 80 mg/L concentrations of oxytetracycline for different time intervals

was higher as compared to liver and kidney cells. Ahmad *et al.* [30] revealed that fish gills are more susceptible to oxidative damage than other organs and respond earlier for any pollutants. The vacuolated cell frequency is increased in liver cells of treated fish as compared to the control. Nunes *et al.* and Rodrigues *et al.* [31,32] also observed vacuolization in cells of fish exposed to tetracyclines. Ahmad *et al.* [30] used pentachlorophenol (0.1–0.4 ppm) in *Heteropneustes fossilis* and reported the induction of MNC in treated groups. Jiraungkoorskul *et al.* [33] observed the increase in MN and NAs in gill, liver, and fin cells of fish *Puntius altus* when exposed to chromium. Sharma and Chadha [17] studied percent frequency of micronucleated and BNC in different tissues (liver, gill, and kidney) of fish *Channa punctatus* to examine the genotoxic effect. Different tissues have varied alkali labile sites, different metabolic activity, repair activity, antioxidant concentration, and other factors, which can explain the variable DNA damage pattern in different tissues [34].

Different parameter in tissues (kidney, liver, and gill) of 96 h exposed fish shows a significant change, so they are good indicators of genotoxicity. Several researchers have also identified nuclear bud, fragmented nucleus, vacuolated nucleus, and NAs as an indicator of genotoxicity [35-37]. The possible toxicological mechanism for OTC toxicity may be generation of free radicals along with toxic metabolites which are formed during its metabolism, which may interrupt the enzymatic activity, and cause oxidative stress [38]. Further alterations and cell damage are caused by generated free radicals in fish tissues [31,39]. Second, OTC makes bond with divalent ions such as Ca^{2+} and Mg^{2+} which may not be bioavailable and leads to a toxic effect on *C. carpio* further may disrupt many biological activities. Furthermore, antibiotics inhibit DNA synthesis, which may be due to the disarrangement of molecular organization leading to degeneration [40]. NAs caused by OTC may be due to increased oxidative stress results in to break down of microtubules and F actin cytoskeleton [32]. The enlarged liver cells were also observed with or without concomitant increase in nuclear size. This may be due to glycogen or lipid vacuolization, hydropic degeneration, and organelle proliferation [41]. Mild to severe histopathological alterations were observed in gill and liver of *Sparus aurata* after acute and subchronic exposure of OTC by Rodrigues *et al.* [32].

The present study indicates that initially there is an increase in the PCE/NCE frequency at 24–72 h of exposure. However, a significant decrease in the value was observed at 96 h of exposure. The initial increase may be due to a higher demand for blood cells due to respiratory stress in fish. Murad *et al.* [42] suggested that due to respiratory stresses such as an increase in oxygen demand, reduction in blood oxygen-carrying capacity, transient hypoxia, and immature cell division may increase the PCE frequency, whereas the decrease in PCE frequency at 96 h of exposure may be due to excessive damage to genetic material, chromosomal breakage, and cell death by the OTC supported by Cavaş and Könen [43] in rodents. Balansky *et al.* [44] exposed *Carassius auratus* with mercury chloride and lead acetate and observed a significant reduction in PCE/NCE ratio in the peripheral blood. Pacheco and Santos [24] indicated that the PCE frequency gets decreased in European eel after exposure to benzopyrene and dehydroabietic acid. Fagr *et al.* [29] observed that the PCE/NCE ratio is not linear when *C. punctatus* was exposed to 4-nonylphenol. Initially, the PCE/NCE value decreases, but at 96h its value increases.

CONCLUSION

Therefore, the above study pointed that the OTC is able to generate the genocytotoxic effect in the blood, gill, liver, and kidney cell of fish *C. carpio*. The rational management and disposal of pharmaceutical waste, hence warrant a combined effort of government entities with healthcare industries and professionals so as to minimize the toxic effect on flora and fauna.

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AUTHORS' CONTRIBUTION

1. Dr. Madhu Sharma: Design the study and draft the manuscript
2. Jyoti Thakur: Performed the experiment and analyzed the data
3. Dr. Subhash Verma: Drafting of the manuscript
4. Dr. Pardeep Sharma: Drafting of the manuscript.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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