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Toxic effects of antibiotic oxytetracycline in the fish, *Cyprinus carpio* using a multi-biomarker approach

J. Thakur[2], M. Sharma[1]*, P. Sharma[1] and T. Shah[3]

[1]*Department of Fisheries, COVAS, Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishwavidyalaya, Palampur-176 062, India*
[2]*Department of Zoology, Central University of Himachal Pradesh, Dharamshala-176 215, India*
[3]*Department of Veterinary Medicine, COVAS, Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishwavidyalaya, Palampur-176 062, India*

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***Corresponding Author Email :** madhu.srma@gmail.com ***ORCiD:** https://orcid.org/0000-0002-1528-0549

Abstract

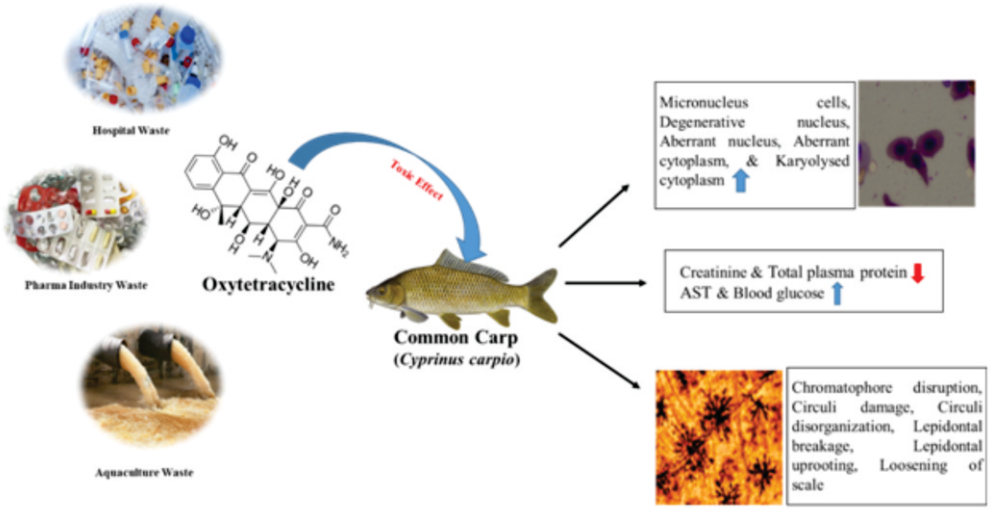
Aim: The present study was designed to assess the toxic effect of oxytetracycline in the fish *Cyprinus carpio* using multi-biomarker approach following acute exposure.

Methodology: The fish were exposed to sub-lethal concentration (80 mg l^{-1}) of oxytetracycline for 96 hrs. Blood samples were collected 24, 48, 72 and 96 hrs after treatment. Biomarkers such as genotoxicity, biochemical parameters and alteration of the morphology of scales were used to assess the health status of fish after exposure to oxytetracycline. Genotoxicity was assessed using blood cell micronucleus assay.

Results: Significant alterations in the nucleus and cytoplasm were noticed such as the aberrant nucleus and vacuolated cytoplasm which were time dependent in the treatment group compared to the control. Biochemical parameters such as blood glucose, total plasma protein, aspartate transaminase and creatinine levels were significantly altered in the oxytetracycline exposed groups. The examination of body scales of treated fish revealed significant alterations such as uprooted and damaged lepidonts and dispersed chromatophores.

Interpretation: The data of the present study indicates that oxytetracycline is toxic to fish at sub-lethal concentration.

Key words: Antibiotics, Biomarkers, *Cyprinus carpio*, Genotoxicity, Oxytetracycline, Scale



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Introduction

India is now among the world's top five pharmaceutical markets (Chander *et al.,* 2016). The Central and State Governments of India has implemented an excise duty-free zone for the pharmaceutical manufacturing sector, which has resulted in large-scale production pharmaceutical. Pharmaceutical waste management has become a herculean task due to a lack of strict and targeted regulations, which exacerbates the issue of ever-increasing environmental pollution (Chander *et al.,* 2016). Antibiotics mainly enter the aquatic environment through manufacturing industries, livestock, inefficient sewage treatment plants, municipal wastewater, aquaculture, and agricultural practices (Burkina *et al.,* 2015; Johnson *et al.,* 2015). Furthermore, since antibiotics are not metabolized in the body and up to 30 to 70 percent are released in the environment (Carvalho and Santos 2016; Perez *et al.,* 2018; Palacio *et al.,* 2023).

Pharmaceuticals are bioactive, and their continuous release in the aquatic environment may impair a variety of metabolic pathways, including oxidative defense, neurotoxicity, and immunotoxicity of aquatic fauna (Grill and Maganti, 2011). This is in contrast to conventional pollutants, which include pesticides, surfactants, metals and detergents (Guardiola *et al.,* 2012; Santos *et al.,* 2010). These antibiotics are categorized (Sanderson *et al.,* 2004) as a high priority contaminant and a great matter of concern in coming time. Oxytetracycline is a widely used broad-spectrum antibiotic which belong to class tetracycline (Li *et al.,* 2018). It is most commonly used antibiotic for bacterial treatment in fish farming due to low cost and easy availability over the counter. Therefore, large amount of antibiotic wastewater is generated and is of more concern in countries like India. Oxytetracycline is poorly metabolized and excreted via urine and feces (Slana and Dolenc 2013).

Oxytetracycline has high hydrophilic characteristics, low volatization and significantly persist in water (Daghrir and Drogvi, 2013). Pereiro *et al*. (2015) detected oxytetracycline concentration between 9.1 and 33.3 ng l^{-1} in river water located downstream aquaculture system. Dietze *et al.* (2005) reported oxytetracycline concentration in intensive fish hatcheries @ 10 µg l^{-1}. In Asian countries, it was noticed that liquid waste and sewage discharged into river contained pharmaceutical products up to 1 mg l^{-1} (Larsson *et al.,* 2007). It has been discovered that wastewater samples taken from the Patacheru Common Effluent Treatment Plant (PETL) close to Hyderabad, India, contained 59 phameceutical products (Larsson *et al.,* 2007). Additionally, the researchers found that 21 of the 59 phameceuticals were present in quantities greater than 1 g l^{-1}. Antibiotics were detected by Diwan *et al.* (2010) in hospital wastewater in Ujjain, India, in a range of 1.4-6.6 μg^{-1}. Balakrishna *et al.,* 2017 also reviewed that in India the concentration of different pharmaceuticals was 40 times more is sewage treatment water as compared to countries in Europe, Australia, Asia and North America.

A variety of biomarkers have been found to be beneficial in obtaining integrated health responses from organism (Sharma *et al.,* 2016). It is widely acknowledged that relying on a single biomarker may not accurately reflect the complexity of health issues. Genotoxicity testing has gained widespread acceptance due to its applicability across a diverse range of species (Sharma and Chadha, 2019). DNA integrity and stability are essential for the survival and health of an organism, as a single change in DNA may disrupt the normal cell process, ultimately leading to cell death (Guilherm, 2012). Therefore, it is strongly advised to use multiple- biomarkers for toxicity related studies (Sharma and Chadha 2017; Sharma *et al.,* 2020).

Biochemical parameters are widely used to monitor the health condition and biological function of an organism (Vutukuru 2003). Scales are exoskeleton structures that from first line of defense. As a fish grow, their scales also increase in size. There is continuous formation of circuli with the deposition of calcium in the scale. The anterior region of the scale is buried in the dermis, circuli have tooth like structures called lepidonts that firmly attach the scale to the skin. The posterior end of the scale is the exposed part having several rows of pigment granules called trabecule. The colour imparting chromatopohores are present in tubercles. Hence, a study of scale deformations is crucial in assessing the toxic impacts on fish.

Fish can serve as early warning systems for minor environmental disturbances as they are sensitive to trace amount of pollutants (Sharma *et al.,* 2019). Their position in the food web, ability to accumulate toxins, sensitivity to low pollutant concentrations, and nutritional value to humans make them excellent indicator for toxicity studies. The biological sensitivity of aquatic organisms gets elevated due to direct contact with the aquatic environment. The present study will provide the first data of the effects of oxytetracycline using different parameters *i.e*., micronucleus assay, biochemical analysis and effect on scale structure of Common carp.

Materials and Methods

Common carp *(Cyprinus carpio)* taken from the Fish farm of Department of Fisheries, Dr. GC Negi College of Veterinary and Animal Sciences, Chaudhary Sarwan Kumar Himachal Pradesh Krishi Visawavidyalaya, Palampur (India) were used for the experiment. Fish of average length of 19±0.5 cm and weight 103.9±6.5 g were subjected to disinfectant by dip treatment in 0.05% $KMnO_4$ for 2 min. Fish were acclimatized under laboratory conditions for 2 weeks and fed with pelleted feed during this period.

The antibiotic oxytetracycline immersion ZYDUS AH manufacturer (Steclin injection 100%) was purchased from the local medical store. The treatment method was semi-static bioassay and every 24 hours the test medium was changed along with drug oxytetracycline to replenish the initial concentration (Sharma and Chadha, 2016). The experimental glass aquaria used were of 100 l capacity. Control fish were subjected to the same water change schedule without the addition of

oxytetracycline. Fish were exposed for 96 hours, with a sub-lethal concentration level of oxytetracycline set at 80 mg l^{-1} of water, based on the survival to mortality ratio value provided by Ambili *et al.* (2013). Blood samples were collected from the caudal vein using a heparinized syringe at 24, 48, 72, and 96 hrs from both treated and control groups, with approximately 1ml of blood taken from each fish. The experiment was carried out in triplicate.

Micronucleus assay: Blood smears were prepared on clean slide immediately after each sampling and dried at room temperature for micronucleus assay (Sharma and Chadha 2016). Slides were fixed in absolute alcohol for 10 min and stained with 10% Giemsa (Himedia). Slides were finally scored for the presence of micronucleus and other nuclear and cytoplasmic aberrations (Tsarpali *et al.,* 2020) under microscope (Olympus compound light microscope CX41) using 100x oil immersion lens. In each fish, three slides were scored and from each slide 1000 blood cells were scored.

Biochemical parameters: Blood samples were collected from fish after different duration of exposure. Blood was collected from caudal vein in anticoagulant free centrifuge tubes. Serum was obtained by centrifugation of blood at 3000 rpm for 10 min. Serum samples were stored at -80°C until further analysis. From the plasma samples, blood glucose, total protein, aminotransferase and creatinine kinase were analyzed using a biochemical analyzer.Various plasma biochemical parameters were also estimated as per standard protocol designed by Agappe Diagnostics Ltd. 'Agappe Hills', Dist. Ernakulam, Kerala- 683 562. Biochemical analysis was done using Microlab 300 Clinical Chemistry Analyzer (Merck Limited, Mumbai).

Scale structure: Scales were taken from the second row to eight dorsal fin rays with the help of forceps (Kaur and Dua, 2012). They were washed with distilled water and studied under different magnification to observe the lepidontal damage and structural changes in the circuli and chromatophores.

Statistical analyses: The results were expressed as mean±S.E. One-way ANOVA followed by Tukey-HSD test was applied the significance between treated and control groups at 99% confidence level. The statistical analyses was conducted using SPSS-20 softwere.

Results and Discussion

Various characteristics such as micronucleated cells, degenerative nuclei, aberrant nuclei, aberrant cytoplasm and karyolyzed cytoplasm were identified during the micronucleus assay. With treatment duration, there was an observed increase in the values of all these parameters (Table 1). The number of micronucleated cells, degenerative nucleus and cells with karyolysed cytoplasm increased with duration, showing its maximum value at 72 and 96 hrs post treatment. A significant rise ($p \leq 0.001$) in the value was detected at all durations. The maximum increase was observed in the cells with aberrant cytoplasm. Similarly, studies conducted on fish exposed to high concentrations oxytetracycline reported the genetic material (DNA) damage (Qu *et al.,* 2004; Li *et al.,* 2006; Zhang *et al.,* 2015). Rodrigues *et al.* (2017a) and Khan *et al.* (2003) investigated the genotoxicity oxytetracycline induced in *O. mykiss,* and concluded that oxytetracycline has high affinity to bind with DNA and form tetracycline – DNA (TC–DNA) binary complexes, as it changes the secondary structure of DNA-duplex. Rodrigues *et al.* (2017) reported that oxytetracycline can directly act on the nuclear enzyme proteins leading to protein dysfunction. The formation of reactive oxygen species (ROS) occurs as a result of oxytetracycline metabolism.

Oxytetracycline metabolism results in the formation of ROS which have already been reported to cause damage to DNA (Khan *et al.,* 2003; Rodrigue *et al.,* 2017; Botelho *et al.,* 2015). The other possible reason for DNA damage in the present study might be the adduct formation in which OTC can directly bind to DNA, causing irrepairable damage (Rodrigues *et al.,* 2017). Under stressful conditions, antioxidant defense is overwhelmed by ROS production and they attack cell components like nucleic acid, protein, lipid and membrane. Nakano *et al.* (2018) found a reduction in HSP70 (stress protein) when fish Coho salmon was exposed to oxytetracycline. Furthermore, Banni *et al.* (2015) found that oxytetracycline causes a drastic down-regulation of mRNA encoding hsp 27 protein. Similar studies by Franzellitti and Fabbri (2013) suggested that the impairment of regulatory pathways triggered by low concentrations of pharmaceuticals may affect the ability of animals to elaborate defense strategies or adapt to stress factors. Oxytetracycline establishes a strong complex with Ca^{2+}

Table 1: Effect of acute exposure of oxytetracycline on nuclear and cytoplasmic alteration in blood cells of fish *C. carpio*

Parameters	Control	24 hr	48 hr	72 hr	96 hr
Micronucleus cells	0.15±0.02^{a}	0.96±0.02^{a}	1.46±0.02^{b}	1.73±0.02^{c}	2.01±0.01^{d}
Degenerative nucleus	0.11±0.01^{a}	3.95±0.02^{b}	13.76±0.03^{c}	22.5±0.14^{d}	6.30±0.09^{e}
Aberrant nucleus	6.18±0.07^{a}	22.30±0.06^{b}	41.65±0.07^{c}	55.25±.09^{d}	2.23±1.34^{e}
Aberrant cytoplasm	4.1±0.02^{a}	30.15±0.06^{b}	53.10±0.03^{c}	70.30±0.09^{d}	80.30±0.09^{e}
Karyolysed cytoplasm	0.13±0.02^{a}	7.05±0.02^{b}	10.58±0.04^{c}	12.53±0.04^{d}	13.90±0.03^{e}

Values are mean ± S.E. Different letters (a, b,c,d,e) are significantly different (Tukey's test, $p \leq 0.01$) and signify the effect of duration of exposure at 80 mg l^{-1} concentration of oxytetracycline.

Table 2: Effect of acute exposure of oxytetracycline on the biochemical parameters of *C. carpio*

Parameters	Control	24 hr	48 hr	72 hr	96 hr
Creatinine (mg dl^{-1})	0.42±0.02^{a}	0.35±0.03^{a}	0.15±0.01^{b}	0.41±0.03^{a}	0.36±0.03^{a}
Aminotransferase (IU l^{-1})	23.52±0.39^{a}	43.87±2.09^{b}	66.73±2.17^{c}	101.23±3.79^{d}	244.27±7.8^{e}
Total plasma protein (g dl^{-1})	2.82±0.03^{a}	1.64±0.08^{b}	1.22±0.01^{c}	1.02±0.01^{d}	0.92±0.02^{e}
Blood glucose (mg dl^{-1})	98.83±1.23^{a}	122.27±3.22^{b}	150.97±2.06^{c}	196.63±5.72^{d}	177.83±1.22^{d}

Values are mean ± S.E. Different letters (a, b, c, d, e) are significantly different (Tukey's test, p≤0.01) and signify the effect of duration of exposure at 80 mg l^{-1} concentration of oxytetracycline.

Table 3: Effect of acute exposure of oxytetracycline on morphological alterations observed in the structure of scale of *C. carpio*

Parameters	Control	24 hr	48 hr	72 hr	96 hr
Chromatophore disruption	-	+	++	+++	++++
Circuli damage	-	-	+	++	+++
Circuli disorganization	-	+	+	++	+++
Lepidontal breakage	-	+	+	++	+++
Lepidontal uprooting	-	-	+	++	+++
Loosening of scale	-	-	-	+	++

(-) None, (+) Mild, (++) Moderate, (+++) Strong (++++) Severe

and Mg^{2+}, which may also have implications on its biological activity. Oxytetracycline acts as an inhibitor of protein synthesis (Leal *et al.,* 2018). A single alteration in the DNA molecule of an organism may lead to serious biological consequences, disrupting the normal cell process and leading to cell death. In the present study, under nuclear abnormalities, the outflow of genetic material from the blood cell was observed. This may be due to the alteration in the nuclear wall because oxytetracycline can directly react with membrane components. Klajn (2001) observed that tetracyclines can cause the outflow of genetic material and other complexes out of the cells by altering the bacterial membrane. Yonar (2012); Ren *et al.* (2017) suggested that oxytetracycline residues have the ability to directly react with lipids in the membrane resulting in destabilized and damaged membrane. Similar DNA damage was observed by Sharma *et al.* (2019) and Zahra *et al.* (2023) in fish exposed to oxytetracycline.

Many nuclear bridges were observed during this study, which showed that under stressful condition fish requires more blood cells to compensate the damaged cells. Therefore, regular and improper mitotic division occures, which results in nuclear bridge formation. The above statements also confirmed that oxytetracycline can bind with the protein (which help in breakage of nuclear bridges) and block the function of that protein. Different type of cytoplasmic alterations (aberrant and karyolysed cytoplasm) were observed in the blood cells in the present study. Similar results were observed by Chai *et al.* (2014) in human RBC's treated with oxytetracycline in which deformed cells along with more cytoplasmic projections were reported. Furthermore, Botelho *et al.* (2015) and Rodrigues *et al.* (2019) observed different nuclear abnormalities in the blood cells of fish exposure to oxytetracycline.

Biochemical parameters such as aspartate transaminase, creatinine, glucose and total protein, were found to be significantly altered in serum samples of oxytetracycline treated fish when compared with the control group (Table 2). Aminotransferase, total plasma protein and blood glucose were found to be significantly different at all the exposure durations when compared with control, while creatinine was found significantly altered at 48 hours of exposure (Tukey's test). Increased concentration of the enzyme may be due to efflux from the damaged organ into circulation. Stress may affect the enzyme activity in different ways, either by direct enzyme inhibition or elevated serum enzyme via tissue damage or sometimes by alteration in the enzyme activity. A significant increase in the AST level was found in a time-dependent manner after oxytetracycline treatment. AST enzyme usually reflects liver health, an elevated AST activity indicate heaptotoxicity. Antibiotics can affect the energy metabolism pathways as reported in the literature (Limbu *et al.,* 2018). Similar results have been reported by Limbu (2024) on Nile tilapia, Nakano *et al.* (2018) on salmon, Rodrigues *et al.* (2019) in fish liver and Hoseini and Yousefi (2018) in rainbow trout. The magnitude and severity of cell damage determines the degree of enhanced activity of cellular enzymes. Increased blood level of AST is a result of liver cell membrane damage and subsequent leakage of intracellular enzyme into circulation. Change in the creatinine is found to increase with each hour of exposure, but a significant change was seen at only 48 hrs (p≤0.001)). Creatinine level in the blood plasma reflects kidney function (Hernandez and Coulson, 1967).

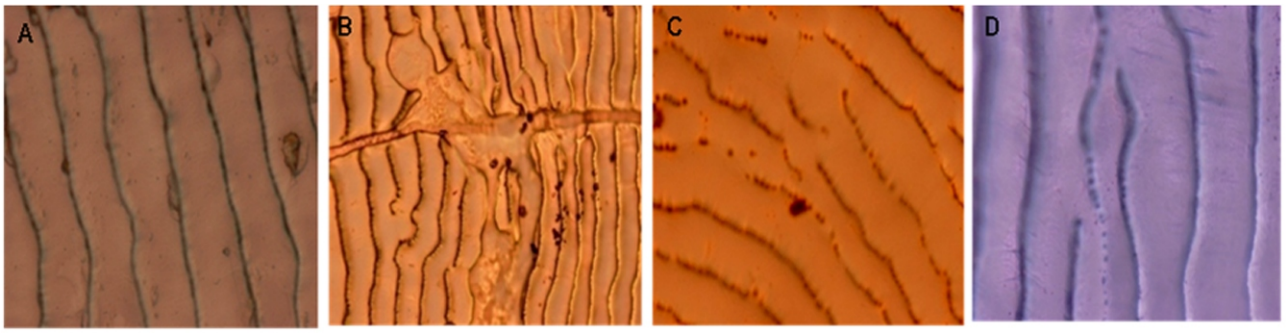

Fig. 1: Photomicrograph of scale of *C. carpio*; (A) Circuli region in control and (B,C,D) Changes observed in treated group (results in gap in circuli, breaking of whole row and uprooted lepidonts.

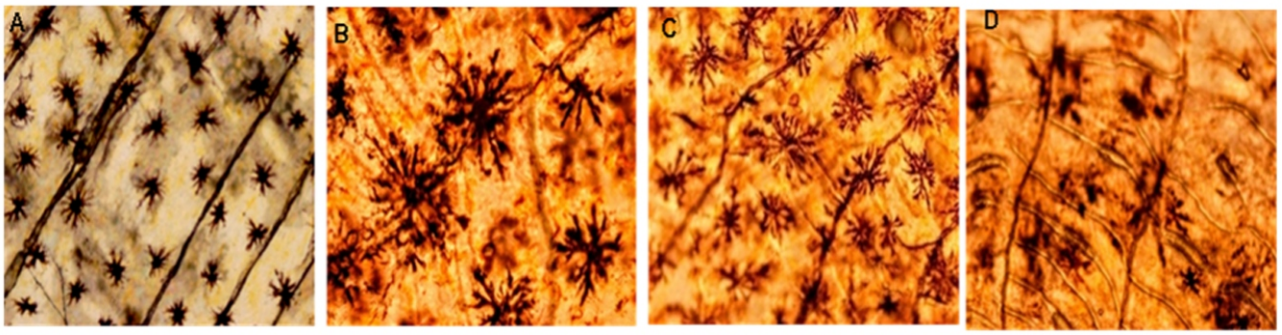

Fig. 2: Photomicrogaph of posterior part of the scale of *C. carpio*: (A) Reticulated and punctuated chromatophores in control fish; (B) dispersed chromatophores; (C) Super dispersed chromatophores and (D) Broken fragments of chromatophores are visible.

Creatinine level increases as kidney function decreases. In this study, a mild change in creatinine level was noted, however, was not significant. This shows that oxytetracycline does not affect kidney functioning in fish. Although a decrease creatinine level was observed in *Oreochromis nilotics* after exposure to oxytetracycline (Reda *et al.,* 2013). Time dependent significant decrease ($p \leq 0.001$) in total plasma protein content was observed and the lowest plasma protein was observed at 96 hr post exposure, which indicates the inhibition of protein synthesis. Moreover, the induction of heat stress protein can result in a decrease in protein level. Fish under stressed condition may mobilize protein to meet the energy demand (Ramesh *et al.,* 2014). The other probable reason may be the formation of lipoproteins which are utilized to repair the damaged cell. Oxytetracycline decreased the lysosomal membrane stability in mussels (Banni *et al.,* 2015) and enhance protein catabolism (Viarengo *et al.,* 2007).

Increase in glucose concentration is considered as a stress indicator in fish. In this study, the glucose level was increased after exposure to oxytetracycline which may be due to increased metabolic demand caused by OTC. Change in blood glucose level indicates improper carbohydrate metabolism. Alteration in glucose levels may be observed in fish under high energy requirements which may be the result of internal and external stress (Poopal *et al.,* 2017). Under stressful conditions, certain hormones are released to compensate energy demand and affect hypothalamus-pituitary internal axis leading to hyperglycemic condition (Ambili *et al.,* 2013).

Cycloid type of scale has focus and circuli and grooves are present in the region, which remain embedded in the body, while the exposed part of scale has two types of pigmented structures, darkly coloured chromatophores and yellow coloured xanthophores. The circuli were normal in control fish with a proper tooth like structure embedded in sockets called lepidonts and the shape of tuberacles was oval or semi-oval, however, after exposure to oxytetracycline different structural deformities were observed in both parts of the scale (Table 3). Uprooting of lepidonts, lepidontal breakage and damaged circuli were observed. With an increase in duration of exposure individual lepidontal breakage to the uprooting of whole lepidontal row and sloughing of lepidont from their original position were observed (Fig. 1). At 96 hrs, severe lepidontal breakage, disorganized calcareous architect of the circuli and extreme and pronounced damage were observed. Additionally, a loss of architectural

pattern was observed in the circuli resulting in a loss of scale.

As far as the effect on colored pigment is concerned, the dispersed chromatophores were observed in scale after treatment with oxytetracycline. With an increase in the duration of exposure the dispersion of chromatophores also increased. Initially, the dark pigmented area was seen in the center and dispersion of the chromatophore with increased size was seen, but with an increase in duration, the central part started fading and further dispersion of the chromatophore was observed. At the highest duration of exposure, the structure of chromatophore was seen completely distorted and lost their cellular entity along with lost dendritic processes (Fig. 2).

Oxytetracycline acts as corrosive and cause time-dependent increase in breakage and branching of circuli, sloughing of lepidonts and rupturing of tubercle. Damage to the morphological structure of the scale has been reported (Johal and Dua 1994; Rishi and Jain 1998; Dua and Gupta 2005; Kaur *et al.,* 2019). Previous studies have reported that oxytetracycline binds to Ca^{2+} ions and inhibit osteoblast activity and destroys the bony matrix. Merino *et al.* (2012) suggested that under stressful condition, Ca^{2+} is absorbed by fish. Reason behind the decline and disappearance of circuli and lepidont may be inhibition of desorption of bone. Brraich and Jangu (2016) collected *L. rohita* from Harike wetland and reported uprooting of lepidonts, sloughing off of lepidonts and structural damage in the form of lesions in the scale of *L. rohita.* Khanna *et al.* (2007) observed severe, irreversible damages to the circuli and lepidonts resulting in loosening of scales in fish due to anthropogenic activities. Dua and Gupta (2005) exposed *C. punctatus* to a low concentration of Hg and reported damage in the circuli, redii (anterior) part of the scale.

Tubercles have chromatophores and impart colour to the fish. oxytetracycline has been reported to cause membrane disruption which disrupts in the tubercles. Rupturing of tubercles may be due to lipid peoxidation and membrane disruption activity of oxytetracycline. Ahmad *et al.* (2018) reported chromatophore change from reticulate to punctuate-stellate in fish *H. fossilis* after exposure to cadmium chloride. Saikia *et al.* (2017) observed the effect on the scale of *C. punctatus* due to the pathogenic effect of *Pseudomonas aereginosa* and found chromatophore disruption, circuli damage, circuli disorganization, lepidontal breakage, lepidontal uprooting in pathogen load dependent manner. Kaur and Dua (2012) found significant alterations in lepidants and chromatophore in *C. punctatus* exposed to waste water. Sugimoto (2002) and Kaur and Dua (2015) observed black pigmented body due to the fragmentation of melanophores. Similarly, Barraich and Jangu (2016) observed uprooted and damaged lepidonts and altered elemental composition in *C. carpio* with effect to heavy metals.

The findings of this study, underscore the severe impact of oxytetracycline on *C. carpio,* highlighting the environmental hazards associated with oxytetracycline. It is imperative to conduct further research to determine the safe threshold for this pharmaceutical and to identify the minimum concentration that could be detrimental to biological systems. This issue serves as an urgent call for both state and central governments concerning the use of oxytetracycline in aquaculture sector. If not addressed promptly and effectively, it could lead to enduring health hazards, thereafter comprehensive approach is required to tackle these issues.

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