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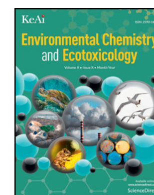
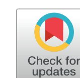


Evaluation of genotoxicity, enzymatic alterations and cadmium accumulation in Mozambique tilapia *Oreochromis mossambicus* exposed to sub lethal concentrations of cadmium chloride



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ABSTRACT

Cadmium (Cd), a heavy metal, is widely used in modern industries including in metal and mining industries as well as in the manufacture of Ni—Cd batteries. Its bioaccumulation is reported to induce hazardous effects in aquatic organisms including fish. Hence, the present study was undertaken to evaluate the toxic potential of cadmium in Tilapia (*Oreochromis mossambicus* Peters). Fish were exposed to 3 sub-lethal concentrations of $CdCl_2$: 7.4 μg/L (high), 3.7 μg/L (medium) and 1.85 μg/L (low) for a period of 21 days. Ten fish were used for each group and each exposure was done in triplicates. A group of 10 fish without exposure to $CdCl_2$ was used as control. Genotoxic effects of Cd were assessed employing micronucleus assay and the comet assay. Along with these tests, alterations in the amount of the enzymes acetylcholine esterase (AChE) and catalase (CAT), as well as, the quantum of Cd accumulated in different tissues of the organism were also studied. A significant increase in DNA damage as % tail DNA and micronuclei were observed in the fish exposed to $CdCl_2$. $CdCl_2$ also induced a significant increase in the activity of CAT whereas a decrease in the activity of AChE was observed. A significant association was also observed between DNA damage parameters and catalase activity as well as Cd concentration in the gills. Cd may thus induce genotoxicity in *O. mossambicus* via oxidative stress and tissue accumulation. The combined use of these biomarkers in *O. mossambicus* could be used to monitor Cd contamination in the aquatic environment.

1. Introduction

Metal pollution in aquatic ecosystems is a matter of serious concern since the last few decades. Natural aquatic systems are prone to contamination by metals released from domestic, industrial and other man-made activities. This results in devastating effects on the ecological balance of the aquatic environment and the diversity of resident aquatic organisms [1–3]. Aquatic species such as fishes, molluscs and crustaceans that are exposed to these pollutants exhibit a plethora of complications with regard to their physiology, biochemistry, genetics, behaviour as well as population [4,5].

Cadmium (Cd) is one of the twenty three heavy metal toxicants widely used in modern industry having various applications such as anticorrosive agents, stabilizers in PVC products, in pigments, manufacture of nickel–cadmium batteries and also in phosphate fertilizers [6]. As a consequence, Cd can potentially enter the aquatic environment via rainwater runoffs from metal mining sites, agricultural farms treated with phosphate fertilizers, mine drainage water, sewage treatment plants, landfills and hazardous waste sites [7,8]. These excess amounts in addition to naturally occurring levels, gradually build up to toxic levels in the aquatic ecosystem thereby causing damage to the biota. In fish, acute and sub-chronic exposure to Cd leads to alterations in gill epithelium, liver and kidneys [9,10]. The major effect of Cd on DNA may be indirect, via the action of reactive oxygen species and thus leading to oxidative DNA damage [11]. Cd exposure can induce carcinogenesis in aquatic organisms either through oxidative stress or inhibition of DNA repair processes [12]. Cadmium accumulation in fish has also been linked to the damage to organ structure, changes in the levels of glucose, osmotic regulation and alteration in enzyme activities [13].

Fishes play a very important role as consumers in an aquatic ecosystem. Further, they may accumulate a number of pollutants in the aquatic environment, thereby contributing to their bioaccumulation / biomagnification through the food chain. A number of fish have been used as bioindicators

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of aquatic pollution. One such fish, the Tilapia (*Oreochromis mossambicus*) has been used extensively in field studies to assess the pollution status of water bodies as well as toxicity assessments in the lab [14,15,16,17]. Additionally, it has significant economic importance and is relatively easy to handle in laboratory conditions. Therefore in the present study, Tilapia (*Oreochromis mossambicus*) was used as a model organism to know the extent of accumulation of Cd in its tissues and also to evaluate the potential of $CdCl_2$ to induce genotoxicity, neurotoxicity and oxidative stress in them. This study also aims to provide an understanding on the mechanism of Cd toxicity in tilapia and could thus be used to monitor Cd contamination in the aquatic environment.

2. Materials and methods

2.1. Quality assurance and quality control

The appropriate quality assurance methods of sample preparation, handling and preservation were carried out in accordance with APHA, AWWA, WEF [18] procedures. All chemicals used were of analytical grade from Himedia (Himedia, India) unless specified otherwise.

2.2. Experimental setup

In the present study Tilapia (*Oreochromis mossambicus*) were handled and maintained based on the protection of animals used for scientific purposes by the European Union directive [19]. Tilapia were procured from Directorate of Fisheries, Old Goa, Tiswadi, Goa which were cultured and maintained in fish ponds within the facility. The water parameters in these fish ponds are regularly monitored so as to ensure minimal metal contamination as well as the health of the fish. Seven month old male fish (15 ± 2.3 cm) were collected by net and were transported in oxygenated polythene bags to the laboratory. They were maintained in aquaria (25 L capacity) filed with dechlorinated and Millipore filtered tap water. The aquaria were fitted with aerators and temperature regulators and water conditions were maintained as follows: temperature 28 °C, 12 h light/dark cycle, pH 7.0 ± 0.15, DO 8.5 ± 1.0 mg/L, TDS 212.45 ± 7.83 mg/L, conductivity 278.56 ± 9.59 μS. Fishes were fed with high quality commercial aquarium fish feed twice a day and fecal matter was siphoned off once a day. Water was changed after every alternate day. In this condition, they were allowed to acclimatize to laboratory conditions for 30 days.

2.3. Treatment schedule

The fishes were divided into groups, each containing 10 individuals. Exposure concenrations were selected based on LC_{50} values of cadmium chloride in *Oreochromis mossambicus* [20]. Accordingly, tilapia (12.0 ± 1.03 cm) were exposed in triplicates to three concentrations of cadmium chloride viz. 7.4 mg/L (high), 3.7 mg/L (medium) and 1.85 mg/L (low). During the exposure period, the water was changed once daily and the respective concentration of $CdCl_2$ was added to the aquarium. Fishes were then sacrificed after 24 h, 7 days, 14 days and 21 days of exposure (time response). A group without cadmium chloride exposure was maintained in parallel and served as the negative control.

2.4. Genotoxicity analysis

2.4.1. Single cell gel electrophoresis (comet assay)

The comet assay was performed according to the protocol of Ferraro et al. [21] with some modifications. All the steps were carried out in dim light and at 4 °C to prevent photo-oxidation of DNA. Peripheral blood was withdrawn from the caudal vein and suspended in phosphate buffered saline (Ca + Mg + free, pH 7.2). 20 μL of the blood suspension was mixed with low-melting agarose (LMA) and smeared on a base layer of normal-melting agarose on frosted microscopic slides. A third layer of LMA was smeared over the second layer and allowed to solidify. These slides were then placed in a cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 Mm Tris, 10% DMSO And 1% Triton-X pH 10, 4 °C) for 1 h. The slides were then placed in unwinding buffer (pH > 10) for 20 min to allow the DNA to unwind. Electrophoresis was then performed under alkaline conditions (pH 10) for 20 min at 300 mA, 25 V. Following electrophoresis, the slides were placed in neutralization buffer (400 mM Tris base, pH 7.5) for 5 min. To visualize the cells, 25 μL of ethidium bromide stain was applied in small, equally sized droplets over the gel, covered with a coverslip and examined using a fluorescence microscope (Olympus BX53) with a green filter at 200× magnification. Two slides per fish were prepared and 100 "comets" were screened per slide. Images of the comets of non-overlapping cells were captured using an attached camera and analyzed with the help of computer software, CASP [22]. The % tail DNA was recorded which is used as a reliable measure of DNA damage [23].

2.4.2. Micronucleus test

Micronucleus test was done following the procedure described by Baršiene et al. [24]. Blood was withdrawn by caudal puncture and was smeared on pre-cleaned slides and was air-dried at room temperature. Smears were then fixed by dipping these slides in absolute methanol for 10 min and were stained in 5% Giemsa in phosphate buffer for 30 min. The percentage of micronuclei (% MN) was evaluated by screening 5000 cells per fish at 1000× magnification (Olympus BX53). MN were identified as structures with the following morphological features: (1) spherical or ovoid-shaped extra nuclear bodies in the cytoplasm (2) a diameter of 1/3–1/20 of the main nucleus (3) non-refractory bodies (4) colour texture resembling that of the nucleus, and (5) the bodies completely separated from the main nucleus [25].

2.5. Catalase activity

Catalase activity (CAT) was estimated as per Aebi [26] based on the decrease in absorbance of the test sample by the decomposition of H_2O_2. Muscle tissue was excised from the fish and was homogenized in Tris buffer (50 mM, pH 7.4) containing EDTA (1 mM) and sucrose (0.3 M). This homogenate was then centrifuged at 10,000 ×*g* for 20 min at 4 °C and the supernatant was carefully collected. The reaction mixture consisted of 13.2 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0) and 0.1 mL of the supernatant. The reduction in absorbance was measured at 240 nm using a multi-well plate reader (Analytical Technologies Ltd.) at 25 °C over 3 min. Total protein concentration was measured by Bradford's method [27]. The activity of catalase was expressed as μmol H_2O_2 min^{-1} mg^{-1} protein.

2.6. Acetylcholinesterase activity

The acetylcholinesterase (AChE) activity from the muscle was determined using the Ellman et al. [28] with modifications as described by Galloway et al. [29] and Rao et al. [30]. Briefly, 50 μL of supernatant (as previously mentioned in the protocol for CAT) was incubated in microtitre plates with 150 μL DTNB (270 μM in 50 mM sodium phosphate pH 7.4) at 25 °C for 5 min. The enzyme activity was initiated by the addition of 3 mM acetylthiocholine iodide and the absorbance was measured at 412 nm. The activity of AChE was expressed as nmol $thiocholine^{-1} min^{-1}$mg protein.

2.7. Estimation of cadmium in tissues

The concentration of Cd in the tissues was determined according to Begum et al. [31]. Fish from each group were collected and their gill and muscle tissues were dissected out. Tissue samples (dry weight 2 g) were then heat-digested with nitric acid and perchloric acid at 150 °C for 2 h in Teflon tubes. The solutions were evaporated to 5 mL and diluted upto 50 mL using deionized water. Cadmium concentrations were then assayed using Flame Atomic Absorption Spectroscopy (GBC 932 AA). The calibration curve was prepared using a certified reference Cadmium standard

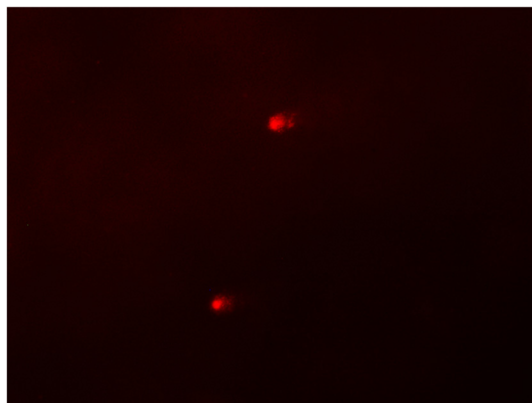

Fig. 1. Blood cells of *Oreochromis mossambicus* with DNA damage (% Tail DNA) as observed by the comet assay (Magnification 200×).

(Sigma TraceCERT certified for AAS). Results were expressed as μg/g of wet tissue.

2.8. Statistical analysis

The data was recorded as mean with standard deviation. The statistical analysis was carried out using IBM SPSS 23 statistical software package. All the data was expressed as mean ± SD. Data of MN test, CAT, AChE and Cd concentration in tissues were log transformed and that of % tail DNA, were arc sine transformed to improve linearity and were tested for normality and homogeneity using Shapiro-Wilk's test and Levene's test respectively prior to subsequent analyses. The significance between controls and treated were analyzed by using student's *t*-test. Analysis of variation (ANOVA) was done to analyze the variation between treatment groups and time intervals. The significance of the data of all the analyses between the treatment groups was analyzed employing a post hoc Dunnet's test. A step-wise multiple regression model with a combination of forward selection and backward elimination was used to evaluate the factors affecting the DNA damage parameters. The data were regarded as statistically significant at $p < .05$, $p < .01$ and $p < .001$.

3. Results

Significant DNA damage as % tail DNA was observed in the erythrocytes of *Oreochromis mossambicus* exposed to all the concentrations of cadmium chloride at all the time intervals of study (except for the low concentration at 1 day of exposure) compared to their respective control (Fig. 1). There was a significant increase in the DNA damage with increasing

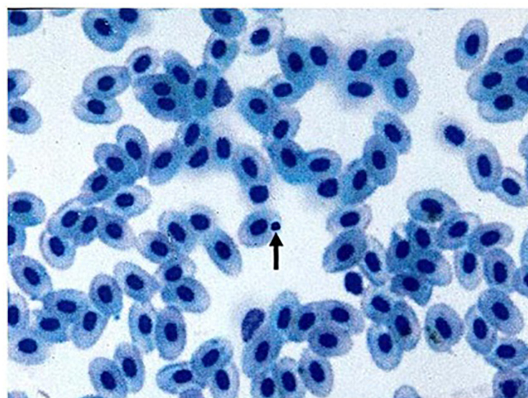

Fig. 2. DNA damage (%) in erythrocytes of Tilapia exposed to various concentrations of $CdCl_2$ at different time intervals (* $p < .05$, ** $p < .01$, *** $p < .001$).

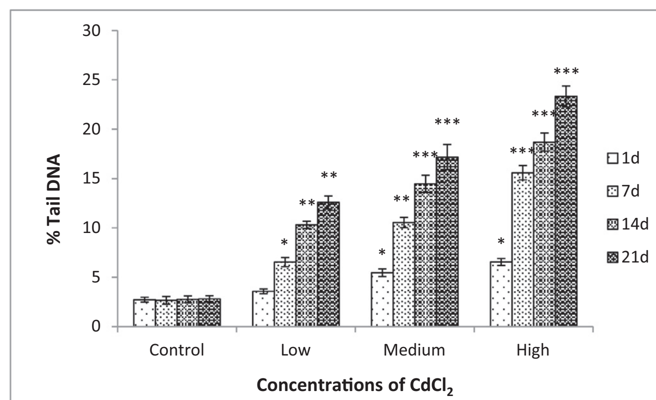

Fig. 3. Micronucleus in a blood cell of *Oreochromis mossambicus* (Magnification 1000×).

concentration of cadmium (F = 705.71, $p < .001$) as well as with advancing time (F = 359.21, $p < .001$). The highest DNA damage was observed in tilapia at the highest concentration of $CdCl_2$ exposed for 21 days ($p < .001$) (Fig. 2).

All the concentrations of $CdCl_2$ also induced significant micronuclei (MN) in the erythrocytes of tilapia (Fig. 3) at all the time intervals studied except at 1 day of exposure. The occurrence of MN was found to increase in a concentration-dependent (F = 1152.21, $p < .001$) as well as time-dependent manner (F = 1108.55, $p < .001$) except after 1 day of exposure, with the highest % MN being induced in the highest concentration of $CdCl_2$ at 21 days of exposure (Fig. 4).

The data on the activity of catalase in the muscle tissue of tilapia exposed to $CdCl_2$ are given in Fig. 5. CAT activity in was found to increase significantly with increasing concentration of $CdCl_2$ (F = 1016.97, $p < .001$) as well as with progressing time (F = 602.71, $p < .001$) except at 1 day of exposure.

Acetylcholinesterase activity in the muscle tissue of tilapia was found to decrease significantly in a concentration-dependent manner (F = 119.87, $p < .001$) as well as time-dependent manner (F = 33.25, $p < .001$) except for the low and medium concentration groups at 1 day of $CdCl_2$ exposure (Fig. 6). AChE activity was observed to be as low as 21.57 ± 1.11 in the tilapia exposed to the highest concentration of $CdCl_2$ after 21 days of exposure.

The concentration of Cd was found to be elevated in the gill and muscle tissues of tilapia exposed to all the concentrations of $CdCl_2$ with increasing dose [F = 2712.57, $p < .001$ (gill); F = 982.99, p < .001 (muscle)] as well as with advancing time [F = 843.28, p < .001 (gill); F = 701.46, p < .001 (muscle)] (Fig. 7). The concentration of Cd was found to be the highest in the gill tissues exposed to the highest concentration of $CdCl_2$ on 21st day

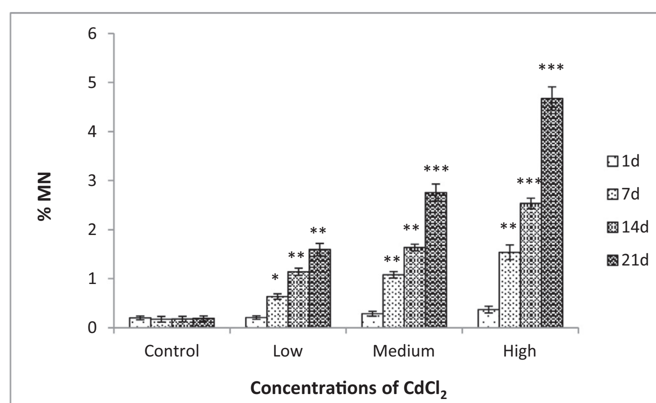

Fig. 4. Micronuclei (%) in erythrocytes of Tilapia exposed to various concentrations of $CdCl_2$ for different time intervals (* $p < .05$, ** $p < .01$, *** $p < .001$).

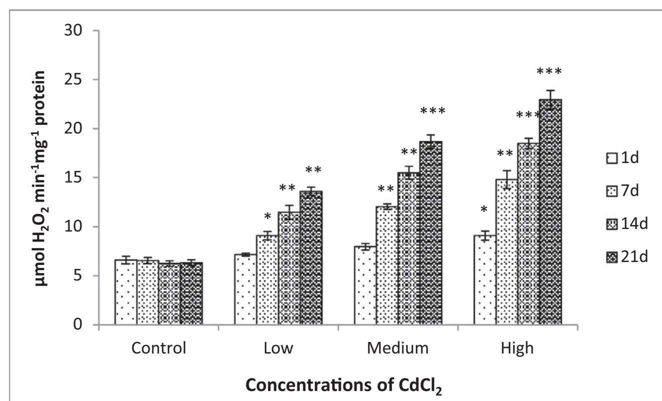

Fig. 5. Catalase activity in Tilapia exposed to various concentrations of $CdCl_2$ for different time intervals (* $p < .05$, ** $p < .01$, *** $p < .001$).

(0.801 ± 0.04 μg/g). Further comparison between the two tissues for a particular concentration revealed that the gill tissue was found to contain a higher concentration of Cd than muscle tissue.

A two-way ANOVA was carried out using % tail DNA, % MN, CAT, AChE, Cd concentration in muscles or Cd concentration in gills as the dependent variable with concentration and time as the independent variables (Table 1). The variance between all the dependent variables with concentration as well as with time was found to be highly significant. A similar trend was also observed with time as the independent variable. The variance between all the dependent variables with the interaction of concentration and time was also found to be significant. High F values in the concentration or time groups indicate that within-groups variance is large compared to between-groups variance. This is especially the case in parameters such as % MN, CAT and Cd in gills in which a large variability was observed for between-group treatments compared to within-group treatments.

Based on the multiple regression model (Table 2), the induction of % tail DNA was largely affected by % MN, CAT and Cd concentration in the gills ($R^2 = 0.972$). The AChE and concentration of Cd in muscle did not contribute significantly to this model. Similarly, the incidence of % MN was predicted by a model which included % Tail DNA, AChE, CAT and Cd concentration in gills ($R^2 = 0.957$). The concentration of Cd in muscles also did not contribute to the model significantly indicating that it may not be a predictor of genotoxicity.

4. Discussion

The present study revealed the toxic effect of Cadmium in Tilapia (*Oreochromis mossambicus*).

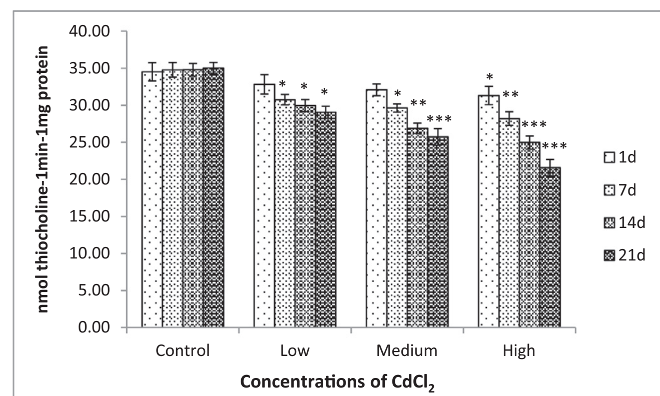

Fig. 6. Acetylcholinesterase activity in Tilapia exposed to various concentrations of $CdCl_2$ for different time intervals (* $p < .05$, ** $p < .01$, *** $p < .001$).

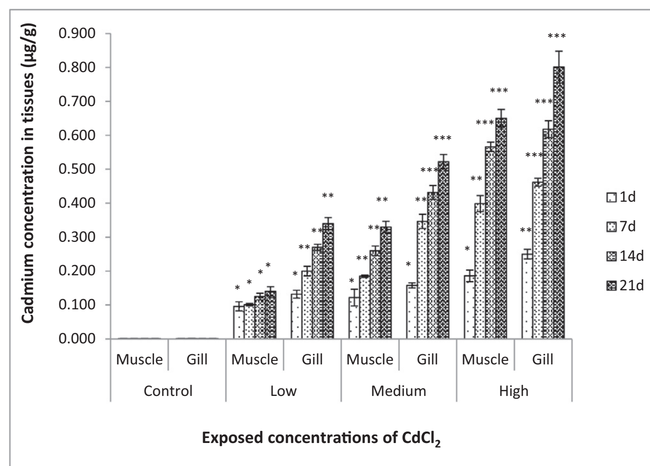

Fig. 7. Quantum of Cadmium in tissues (Muscle and Gills) of *Oreochromis mossambicus* exposed to various concentrations of Cadmium for different time intervals (* $p < .05$, ** $p < .01$, *** $p < .001$).

The results of the comet assay indicate that cadmium chloride was able to cause significant concentration-dependent as well as time-dependent increase of % tail DNA. This indicates that $CdCl_2$ is capable of inducing DNA damage in the form of single-stranded breaks in *O. mossambicus*. These results are in agreement with that of Ahmed et al. [15] wherein they observed a significant increase in % tail DNA in different tissues of *Anabas testudineus* exposed to various concentrations (0.5–2.0 mg/L) of $CdCl_2$. Our results also find similarities with that of Jindal and Verma [32] in which they reported significant concentration-dependent increase of % tail DNA in the peripheral erythrocytes of *Labeo rohita* exposed to $CdCl_2$ for a period of 100 days.

The micronuclei were also found to increase significantly in a concentration- as well as time-dependent manner. This is on par with the studies of Özkan et al. [33] in which they reported significant induction of micronuclei in the peripheral blood of *Oreochromis niloticus* exposed to sub-lethal concentrations of $CdCl_2$ for 10 days. They further reported that MN induction occurred in a concentration- and time-dependent manner. Our results are also in agreement with that of Abu Bakar et al. [34] in which they observed a significant time-dependent increase of MN in the erythrocytes of *O. niloticus* exposed to a single concentration of $CdCl_2$ and observed for 24, 48, 72 and 96 h.

The CAT activity which is a measure of oxidative stress was found to increase significantly in Cd exposed fish with increasing concentration and time. This is on par with the studies of Basha and Rani [35] in which they reported a significant increase in CAT activity in *O. mossambicus* exposed to 5 mg/L of $CdCl_2$ for a period of 30 days. Our results are also in agreement with the findings of Almeida et al. [36] in which CAT activity was significantly increased in *O. niloticus* exposed to 0.75 mg/L of $CdCl_2$ for 15 days. Increase in oxidative stress could therefore also lead to the formation of

Table 1
Two-way ANOVA testing the influence of $CdCl_2$ concentrations and exposure time as well as the interaction between them (concentration × time) on the parameters in *O. mossambicus.*

Dependent variable	Independent variables					
	Factors				Interaction	
	Concentration		Time		Concentration × Time	
	F value	p	F value	p	F value	p
% Tail DNA	705.71	< 0.001	359.21	< 0.001	49.96	< 0.001
% MN	1152.21	< 0.001	1108.55	< 0.01	219.37	< 0.001
CAT	1016.97	< 0.001	602.97	< 0.001	95.42	< 0.001
AChE	119.87	< 0.001	33.25	< 0.001	10.42	< 0.001
Cd in gills	2712.57	< 0.001	843.28	< 0.001	181.94	< 0.001
Cd in muscle	982.99	< 0.001	701.46	< 0.001	126.84	< 0.001

Table 2
Multiple regression model using % tail DNA or % MN as dependent variables (or independent variables) and CAT, AChE, Cd concentration in gills and muscles as independent variables in different combinations (* $p < .05$, ** $p < .01$, *** $p < .001$).

Dependent variable	Independent variable	Beta Coefficients	SE	t value	Significance	R square
% Tail DNA	% MN	0.322	0.172	3.948	***	0.972***
	CAT	0.578	0.058	6.048	***	
	Cd in gills	0.115	0.004	3.299	**	
	Excluded variables: AChE and Cd in muscle					
% MN	% Tail DNA	0.531	0.061	4.091	***	0.957***
	AChE	−0.136	0.050	−2.392	*	
	CAT	0.481	0.040	3.468	***	
	Cd in gill	−0.197	0.002	−4.863	***	
	Excluded variable: Cd in muscle					

reactive oxygen species which affect DNA and thereby induce damage indirectly [37]. Some of the mechanisms by which cadmium causes damage to DNA are inhibition of DNA repair mechanisms as well as apoptosis [38,39]. As a result, the increase in ROS coupled with faulty DNA repair and apoptosis mechanisms could potentially lead to mutagenicity in Cd-exposed organisms [40].

The AChE activity was found to decrease significantly with increasing concentration of $CdCl_2$ and advancing time compared to the control. This observation can be compared with that of Silva and Pathiratne [41] wherein *O. niloticus* fingerlings exposed to comparatively low concentrations of Cd for 28 days exhibited a significant depression of tissue cholinesterase activities. Our results also find similarities with that of Jebali et al. [10] in which they reported decreased AChE activity in *Seriola dumerilli* exposed to 100 and 250 μg/kg of Cd for 2 days. This decrease in AChE activity could also attributed to ROS induced by Cd exposure. Another possible mechanism is the interaction of Cd with active sites of synapses which in turn affects the hydrolysis of acetylcholine thereby resulting in an inhibition of AChE [42]. Decrease in AChE activity due to $CdCl_2$ exposure may also lead to behavioural abnormalities such as decreased motor coordination and swimming behaviour [43].

In the present study, the concentration of Cd was found to be significantly high in the gills and muscles of *O. mossambicus*. These findings are on par with that of Al-Asgah et al. [44] in which they observed a similar accumulation of Cd in the gills of *O. niloticus* exposed to various concentrations of $CdCl_2$. Cadmium absorption is predominantly due to free Cd^{2+} and is considered to be the most bioavailable form and can readily be taken up by the gills [45]. With regard to this, we observed that the accumulation of Cd was higher in the gills than in the muscle tissues for all the concentrations studied.

According to the step-wise multiple regression model, the induction of DNA damage was dependent on CAT as well as the concentration of Cd in the gills. Since the gills accumulate Cd from the environment, this Cd could enter into the body through the blood stream and can either affect DNA directly or indirectly via the formation of reactive oxygen species (ROS) and inhibition of DNA repair mechanisms and apoptosis as mentioned earlier. The results of the two-way ANOVA indicate that concentration and time as well as the interaction of concentration x time had a significant effect on all the parameters studied. Thus, the longer the exposure to the various concentrations of Cd, the more significant will be DNA damage and oxidative stress. Thus Cd contamination for prolonged periods in the environment could induce severe impairment of alterations in DNA integrity and biochemical parameters in exposed fauna such as fish.

5. Conclusion

The present study suggests that cadmium chloride is able to induce oxidative stress, genotoxicity and neurotoxicity in *O. mossambicus* and also gets accumulated in its gills and muscles. Discharges which contain cadmium will ultimately enter the aquatic ecosystem and have toxic effects on the fish such as tilapia and other associated species. Hence, the monitoring of contaminants such as Cd in freshwater bodies is of utmost importance as it could lead to significant declines in the natural populations of fish. Further, the integrated use of multiple biomarkers using *O. mossambicus* will give us an understanding of the extent of Cd contamination in the aquatic environment and could potentially be applied in field studies.

Declaration of Competing Interest

None.

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