Genotoxic Effect of Cadmium on NileTilapia (*Oreochromis niloticus*)

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Abstract-

Micronucleus test has been an excellent tool for assessing genotoxicity of waterborne substances in Nile Tilapia. Also, comet assay is a rapid, simple and sensitive procedure for quantifying DNA in individual cells. The current study was conducted to evaluate toxic effects of cadmium on Nile tilapia using cytogenetic and molecular methods. Fish were exposed to several doses of cadmium at different time intervals. The results revealed that micronucleus formation was proven to be less with low concentration of cadmium and short time interval than that obtained with high concentration and long exposure time interval giving evidence that treatment duration has affected the genomic system. Comet assay in erythrocytes of Nile Tilapia revealed that fish treated with high dose of cadmium and long duration of exposure showed a significant increase both in the number of damaged nucleus and in the comet scores compared with negative control of fish. The results suggested that formation of the micronuclei exhibited significant variations which might be related to the inhibition of the cadmium on the antioxidant enzymes. Moreover, the significant increase in DNA damage estimated by comet assay could be probably due to the elevation of cellular reactive oxygen species (ROS).

Index terms: Nile Tilapia; Heavy metals; micronucleus test; comet assay, genotoxicity.

1 INTRODUCTION

Aquatic animals have often been used in bioassays to monitor water quality. The development of biological monitoring techniques based on fish offers the possibility of checking water pollution with fast responses on low concentrations of direct acting toxicants (1; 2). Increasing contamination of the environment by toxic chemicals has resulted in the need for sensitive assays to be used in risk assessment of polluted sites. Cytogenetic procedures were developed, including chromosomal aberrations and micronucleus test, to assess the mutagenic damage of petrochemicals and low-level radioactivity on indigenous terrestrial and aquatic wildlife populations. These procedures are sensitive to the perturbation of DNA that results from exposure to mutagenic contaminants in both field and laboratory studies. The use of natural populations of animals in biomonitoring, combined with traditional chemical assays, will ultimately provide sufficient information to estimate the risk to human health and environmental quality from anthropogenic pollution (3). Evaluation of the monitoring

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systems which use aquatic organisms to assess the genotoxicity of polluted water in the field and in the laboratory is considered as important need.

In a field study, micronucleus assay was shown to be applicable to freshwater and marine fishes. Additionally, gill tissues have been found to be more sensitive than hematopoietic cells to micronucleus inducing agents (4). Lyne et al. (1992)(5) reported that micronucleus test considered as sensitive tool for determination of pollution with heavy metals such as cadmium induced genotoxicty in freshwater fish species. Furthermore, Porto et al. (2005)(6) indicated that use of micronucleus test in fish tissues exposed to water pollution has been found to be an excellent tool for assessing genotoxicty of waterborne substances. Moreover, alkaline single-cell gel electrophoresis (SCGE), namely comet assay, is a rapid, simple and sensitive procedure for quantifying DNA damage in individual cells. It is used for environmental monitoring and for detecting DNA damage in aquatic animals such as fish, clams, shellfish and mussels (7;).

The Nile tilapia (*Oreochromis niloticus*) is a common fish species in Egypt. Recently, Nile tilapia has been used as experimental model for toxicological studied, since it is highly susceptible to nutritional deficit and is extremely vulnerable to toxic insult from diverse chemicals (8; 9). Therefore, the aim of this study is to assess genotoxicity of cadmium in Nile tilapia using micronucleus test and comet assay.

2 MATERIALS AND METHODS

2.1 Nile Tilapia (*Oreochromis niloticus*)

Two-month old of Nile tilapia (Oreochromis niloticus) fish weighing 100 ±10 g were purchased and transported in large plastic water containers supplemented with O2 source. Fish were maintained on glass fish aquariums. After an acclimation period of two weeks, fishes were divided into 3 experimental groups (10 fishes/ group) and each group was individually placed into a fish aquarium containing 110 ± 2 L of dechlorinated tap water. The fish were fed by standard fish diet contained 25% protein content (10).

2.2 Experimental design

2.2.1. Determination of LC50:-

Fish groups were treated with four doses (16, 18, 20 and 22 mg/L) of cadmium. LC50 was calculated according to Behreues and Karbeur 1953 (10) using the following formula:

$$LC50 = \text{biggest dose} - \frac{\sum (A \sum B)}{N}$$

Where N is the present population size (measured as numbers of individuals), A and B are the number of individuals which are die and emigrate from the population.

2.3 Fish treatment*:*

Two concentrations of cadmium chloride were used for exposure of fish with Cd namely Cd (a) and Cd (b). The Cd concentrations were 1/10 and 1/8 of LC50 in which 1/10 of LC50 was = 1.95 mg/L and 1/8 of LC50 was = 2.44 mg/L. The total treatment duration was 28 days for fish used in the study. Samples collection was taken at zero exposure time, 24 h, 7 days, 14 days, 21 days and 28 days of the treatment. Water quality was monitored for assessment of changes in several parameters such as temperature, pH,

dissolved oxygen, total dissolved solids, and conductivity.

2.4 Micronucleus test

The peripheral blood smears were obtained from blood cells of gills tissues using fetal calf serum. For each individual fish 3000 erythrocytes were examined to detect micronuclei formation. The slides were examined using inverted microscopy with 1000 X oil- immersion lens.

2.5 Preparation of blood samples for comet assay

Few drops of blood samples were collected from individual fish and mixed with fetal calf serum. The samples were stored in Eppendorf tubes on ice and protected from light until processed (11; 12). Before running the comet assay, cell viability for erythrocytes and gill cells was determined using the trypan blue exclusion method.

2.6 Comet assay

Comet assay was used according to (13, 14), as well as (15) with minor modifications. Samples were spread on cleaned slides and covered with agarose layers. Low melting agarose was used in the second and third layers. Roughened microscope slides were dipped briefly into 1.0% hot normal melting agarose (NMA) prepared in phosphate-buffered saline (PBS) as soon as the agarose had boiled. The slides were dried overnight at room temperature and then stored until used in clean area. Subsequently, blood (7-10 µl) samples mixed with 95 ml of 0.75% low melting point agarose (LMA) (Gibco BRL) at 37^{o}C was spread on the slide using a cover slip and then allowed to solidify at 4^{o}C in a moist box. After removal of the cover slip, the slides were immersed in freshly prepared cold (4oC) lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris; pH 10-10.5; 1% Triton X-100 and 10% DMSO added just before use) for at least 1 h and for up to four weeks.

All procedures up to the lysis were done in the lab. The alkaline unwinding, electrophoresis and neutralization steps were performed as described by (14). The slides were removed from the lysis solution and placed in the electrophoresis chamber, which was then filled with freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 12.6). The cells were exposed to alkali for 30 min to allow for DNA unwinding and the expression of alkali-labile sites. Subsequently, the DNA was run electrophoresis for 30 min at 300 mA and 24 V in an ice bath. The extended comet tails would be clearly distinguishable from the heads, and thus be easier to evaluate (16). After electrophoresis, the slides were placed in a horizontal position and washed three times (5 min each) with 0.4 M Tris buffer, pH 7.5, to neutralize the excess alkali. Finally, 70 µl of ethidium bromide (2 mg/ml) was added to each slide, which was then covered with a cover slip, stored in a humidified box at 4^{o}C and analyzed using a fluorescence microscope with a calibrated eyepiece. The slides maintained a good fluorescent image for at least four days. Images of 50 randomly selected cells (25 cells from each of two replicate slides) were analyzed from each

animal. Comet tail lengths (nuclear region + tail) were measured in arbitrary units. One unit was approximately 5 mm at 40X magnification. The fluorescence microscope was equipped with a BP546/12-nm excitation filter and a 590-nm barrier filter. Cells were also scored visually into four classes, according to tail size (from undamaged-0, to maximally damaged-d) and a value was assigned to each comet according to its class. The final overall rating for the slide, DNA damage score, between 0 (completely undamaged) and 200 (maximum damage), was obtained by summation (17).

2.7 Statistical analysis

Data of water parameters were analyzed according to the standard methods (18) which maintained that the D.O. saturation level was 8 ± 0.5 using aeration sources. In addition, data of micronucleus formation and DNA damage were analyzed statistically using one-way analysis of variance (ANOVA) and by using (SPSS) regression correlations, 10. Data were expressed as Mean ± S. E for all experiments and the levels of significance were expressed in which $P < 0.05$, (12).

3 Results and Discussion

3.1 Micronucleus test

The results revealed that percentages of micronucleus formation in gills cells was proven to be less with low concentration of cadmium and short time interval than that obtained with high concentration and long exposure time interval giving evidence that treatment duration has affected the genomic system.

The results indicated that erythrocytes in gills tissues were affected by cadmium dependent on dose and time intervals. Figures 1, 2 and 3 as well as Table 1 represent micronuclei formation and deformed nucleus with different doses of Cd throughout duration of treatment (28 days). Cytogenetic examination after treatment with different doses of cadmium revealed that deformed nuclei in addition to the main type of aberration (micronucleus) were observed. Statistical analysis using two way ANOVA ($P<0.05$, 12) method observed significant differences between cadmium doses and with different time intervals.

Table 1. Mean values of micronucleated erythrocytes examined in gills of Nile tilapia treated with different doses of cadmium for 28 days.

Cd Doses (mg/L)	Exposure time	Averages M.N. Cells
Before treatment	Zero Time	12
Control (no treatment)	24 h	15
1.95 (low dose)	24 h	42
2.44 (high dose)	24 h	51
Control (no treatment)	7 days	18
1.95 (low dose)	7 days	48
2.44 (high dose)	7 days	54
Control (no treatment)	14 days	21
1.95 (low dose)	14 days	54
2.44 (high dose)	14 days	60
Control (no treatment)	21 days	24
1.95 (low dose)	21 days	63
2.44 (high dose)	21 days	72
Control (no treatment)	28 days	27
1.95 (low dose)	28 days	69
2.44 (high dose)	28 days	81

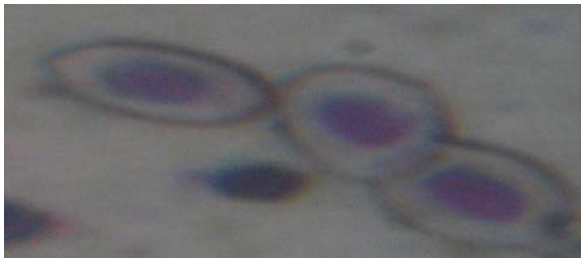

Figure (1): Normal cells stained with Gimsa for micronucleus test.

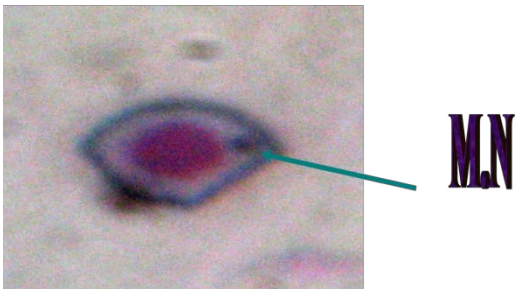

Figure (2): Photomicrograph showing small micronucleus in blood cells from gills of Nile tilapia.

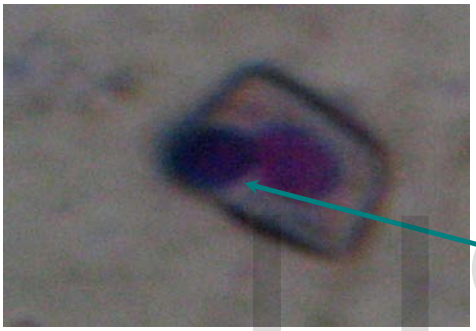

Figure (3): Photomicrograph showing large micronucleus in blood cells from gills of Nile tilapia.

The current study revealed that erythrocyte micronucleus test has been used with different cadmium doses and different time intervals to monitor aquatic pollutants displaying mutagenic features. The obtained results supported the fact demonstrated by Pantaleao *et al. (*2006)(19), who demonstrated that fish inhabiting polluted waters have greater influences on frequencies of micronuclei. The micronuclei frequencies may vary according to the season, kind of pollution involved, and treatment intervals. In fish, it is known that gills are responsible for erythropoiesis and filtration. Rabello-Gay (1991)(20) found that upon fish exposure to toxins defective erythrocytes undergo passage from the gills into the peripheral blood. The current study showed that fish supplemented with high level of cadmium and long exposure time interval showed higher frequencies of micronuclei than those exposed to low dose and short time interval in which they showed lower frequencies of micronuclei. This could be attributed to the repair system in gills which avoided the negative effect of low dose of cadmium and short time interval compared with the higher dose and longer time interval. According to the results demonstrate that Nile tilapia fish can respond in completely different ways in different doses of treatment over different time intervals to given genotoxic agents. Kligerman., 1982(21) indicated that depending on the toxic treatment and the duration of exposure, the behavior of micronuclei rates may exhibit significant variations, probably related to the inhibition of the cadmium on antioxidant enzymes. Therefore, Al-Sabti *et al*., 1995(22) and Grisolia and Cardeiro., 2000(23) indicated that micronuclei formation in fish erythrocytes collected from polluted waters increased significantly with greater. Rabello-Gay., 1991(20) and Lyne *et al*., 1992(5) found that the micronuclei frequencies may vary according to the season, the dose of pollution involved, and the exposure duration. It is concluded from this study that gills erythrocytes of Nile tilapia (*Oreochromis niloticus*) can be used for estimating the genotoxic effects of waterborne pollutants.

3.2 Comet assay

The results obtained in the current study using the comet assay revealed that that fish treated with high dose of cadmium and long time interval showed a significant increase both in the number of damaged nucleotides and in the comet scores compared with control fish (Table 2 and Fig. 4). The results revealed that blood cells collected from fish treated with cadmium for only 24 h showed low incidence of damaged cells and in the comet score. However, after 28 days treatment interval of cadmium a significant increase was observed in the number of damaged nuclei which remained similar to the results obtained from MN. In contrary, other time intervals of exposure with cadmium did not show significant increase of damage DNA (Table 2). Furthermore, the results of

the number of comet cells scored into 4 classes (Fig 4a, b, c, and d) revealed that the cell viability was higher (above 94% of viable blood cells) in control fish compared with cadmium treated fish.

Jha, 2004 (24) found substantial progress has been made in the last decades to evaluate the impact of physical and chemical genotoxins in aquatic organisms. Shugart., 2000 (25) and Van Der *et al*. 2003(26) thought that the development of new methods and the application of assays those are more sensitive in the detection of genotoxicity for various chemicals in aquatic biota have been the main determinants for attaining these advances. Therefore, the genotxic effect of the cadmium in the present work was evaluated based on the comet assay applied to the analysis of peripheral blood erythrocytes of Nile Tilapia (*Oreochromis niloticus*). Although the comet assay is suitable for genotoxicity studies in any nucleated eukaryotic cells as that was done by Sharma *et al*. 2007(27), there may be various practical limitations to the application of this assay including the first stage of cell isolation that were put by Mitchelmore *et al*., 1998(28). Blood cells were chosen for the determination of the DNA damage by the comet assay in which it was conducted by Lee and Steinert, 2003(29). However, the results obtained in genotoxicity tests must be first checked in relation to the sensitivity of the viability and the overall credibility of the test system. In this context, the utilization of negative control group is part of the recommended guidelines used by Matsumoto and Colus ,2000(30).

Table 2: Frequency of nucleoids observed in each comet class (0, 1, 2 and 3) and the number of damaged nucleoids (mean±S.E.) in blood cells treated with different doses of cadmium for 28 days.

Cd Doses (mg/L)	No. of Sample	Comet Cells	Types of Comet cells			
			A	B	C	D
Before treatment	Zero Time	1	199	1	0	0
Control (no treatment)	24h	1	199	1	0	0
1.95 (low dose)	24h	4	196	3	1	0
2.44 (high dose)	24h	5	195	3	1	1
Control (no treatment)	7 days	2	198	1	1	0
1.95 (low dose)	7 days	5	195	4	1	0
2.44 (high dose)	7 days	15	185	9	6	0
Control (no treatment)	14 days	2	198	2	0	0
1.95 (low dose)	14 days	20	180	10	2	8
2.44 (high dose)	14 days	25	175	15	9	1
Control (no treatment)	21 days	0	200	0	0	0
1.95 (low dose)	21 days	28	172	18	7	3
2.44 (high dose)	21 days Cd (b)	42	158	26	13	3
Control (no treatment)	28 days	2	198	2	0	0
1.95 (low dose)	28 days Cd (a)	38	162	9	14	15
2.44 (high dose)	28 days Cd (b)	52	148	8	22	24

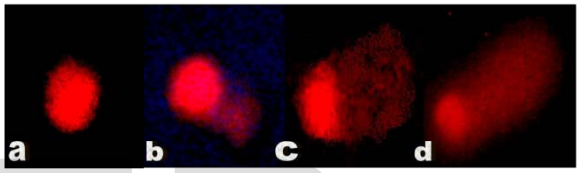

Figure 4: Types of comet cells [a] Normal cell without comet tail (tail/cell length = 0). [b] Narrow comet cells (0.55 > tail/cell length > 0.0). [c] Moderate comet cells (0.75 > tail/cell length > 0.65). [d] Large comet cells (tail/cell length< 0.75).

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