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Genotoxic Effect of Zinc and Cadmium Following Single and Binary Mixture Exposures in Tilapia (*Oreochromis niloticus*) Using Micronucleus Test

(Kesan Genotoksik Zink dan Cadmium Secara Tunggal dan Campuran Binari dalam Ikan Tilapia (*Oreochromis niloticus)* dengan Menggunakan Ujian Mikronukleus)

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ABSTRACT

The aim of this study was to investigate the genotoxicity effect of Cd and Zn and their binary mixtures in tilapia fish Oreochromis niloticus *using the micronucleus test. Two cytogenetic end points were considered; the frequencies of micronucleated cells and nuclear abnormalities. Fishes were exposed to 4.63 mg/L Cd, 7.50 mg/L Zn and 4.63 mg/L Cd + 7.50 mg/L Zn mixture for the period of 24, 48, 72 and 96 h. The results showed that the frequencies of micronuclei and nuclear abnormalities in the erythrocyte were significantly increased in all groups of treatments when compared with the control group (0 exposures). In addition, the highest frequencies of micronucleated and nuclear abnormalities were obtained after 48 h exposure in almost all cases (except in the mixture of Cd+Zn) and decreased after 72 and 96 h exposure. Frequencies of micronuclei and erythrocytes with nuclear abnormalities exposed to a mixture of Cd+Zn in* O. niloticus *were always lower at all-time points (after 24, 48, 72 and 96 h) than that of a single Cd and Zn exposure. Therefore, the study demonstrated that the genotoxic potential of these metal compounds and the simultaneous treatment of Cd and Zn suggest the presence of antagonistic interactions.*

Keywords: *Binary mixtures; genotoxicity; heavy metals; micronucleus test;* Oreochromis niloticus

ABSTRAK

Kajian ini dilakukan untuk menilai kesan genotoksid Cd dan Zn serta campuran binari dua logam berat tersebut ke atas ikan tilapia, Oreochromis niloticus. *Dengan menggunakan ujian mikronukleus, dua jenis kesan sitogenetik ke atas sel darah merah atau eritrosit seperti sel yang mempunyai mikronuklei dan sel yang mempunyai abnormal nuclear telah direkodkan. Ikan telah didedahkan kepada 4.63 mg/L Cd, 7.50 mg/L Zn dan 4.63 mg/L Cd + 7.50 mg/L Zn campuran bagi tempoh 24, 48, 72 dan 96 jam. Keputusan menunjukkan kekerapan mikronuklei dan sel eritrosit yang mempunyai abnormal nuklear telah meningkat dengan ketara dalam kesemua kumpulan rawatan dengan logam berat berbanding kumpulan ikan kawalan. Tambahan lagi, didapati kekerapan sel yang mempunyai mikronuklei yang mempunyai abnormal nuklear menunjukkan jumlah tertinggi selepas 48 jam pendedahan kepada semua kumpulan logam berat (kecuali di dalam kumpulan ikan yang didedahkan kepada campuran Cd+Zn) dan semakin berkurang selepas 72 dan 96 jam. Kehadiran mikronuklei dan sel yang mempunyai abnormal nuklear di dalam kumpulan ikan yang didedahkan kepada campuran Cd+Zn pada setiap masa ujian dilakukan (selepas 24, 48, 72 dan 96 jam) menunjukkan kekerapan yang lebih rendah berbanding dengan kumpulan yang didedahkan kepada Cd atau Zn sahaja. Oleh itu, penemuan ini menunjukkan bahawa logam berat seperti Cd dan Zn berpotensi untuk menghasilkan kesan genotoksid serta pendedahan secara serentak Cd+Zn mencadangkan berlakunya interaksi antagonistik.*

Kata kunci: Campuran binari; genotoksid; logam berat; Oreochromis niloticus*; ujian mikronukleus*

Introduction

Heavy metals released into the rivers constitute one of the major sources of water pollution. In Malaysia, the rivers of Juru and Bukit Tambun of the Penang state have been found to contain copper (0.05 mg/L), lead (0.25 mg/L), zinc (0.05 mg/L), cadmium (0.14 mg/L), chromium (0.20 mg/L), arsenic (3.44 mg/L) and mercury (0.01 mg/L) (Abbas et al. 2007). The steady increase in emission of heavy metals in natural waters due to various industrial and agricultural activities have led to the damage in the genetic materials of exposed organisms which include fish and therefore causing further genotoxic effects (Francoise et al. 2011). This study focused on the metals cadmium (Cd) and zinc (Zn). Cd and Zn are related to heavy metals, almost always occurring together in pollution incidents, which have been used widely in industry and are released into the environment as a by-product of ore smelting (Morley et al. 2001). Cd is a non-essential or toxic metal even in low concentration, capable of inducing DNA damage and is classified as a human carcinogen by the IARC (Aitio et al. 1993). Meanwhile, Zn is an essential metal that plays important role in the biological function of several proteins and enzymes. However, like Cd, Zn may pose the same toxicity in excessive concentration.

Simultaneous exposure of the aquatic environment to both the essential and non-essential metal mixtures warrants careful consideration. Fundamentally, the joint effect of multiple toxicants on an organism could be: the additive toxic effect of one chemical to the other; the toxic effects caused by the mixture being significantly less than the sum of the toxic effects of the individual constituents (antagonism) and the toxic effects caused by the mixture may significantly exceed the sum of effects of the individual constituents (synergism) (Otitoloju 2002). Mixture toxicity has commonly predicted additive effects if the proportional independent contributions of each toxicant was simply added. However, previous studies on aquatic organisms showed that mixtures of heavy metals showed different type of interactions occurring between the heavy metals (Cooper et al. 2009; Lange et al. 2002; Obiakor et al. 2010). As essential heavy metals, the protective effect of Zn against Cd toxicities has been known for many years (Leber & Miya 1976; Parizek 1957; Webb 1972).

Most studies have been carried out on the single effect of the Cd or Zn alone, however few studies have been done on the combined contamination of Zn and Cd in *Oreochromis niloticus*. Özkan et al. (2011) demonstrated the genotoxic effects of sub-lethal doses of cadmium in peripheral erythrocytes of *Oreochromis niloticus*. The authors found that the exposure to 0.5 and 1.0 mg/L Cd produced significantly higher frequency of micronucleated and nuclear abnormalities ($p<0.05$). Similarly, Jiraungkoorskul et al. (2007) reported significant levels of micronucleated induced in erythrocytes in three freshwater fishes (Butterfish, Red-tailed tinfoil barb and Nile tilapia) exposed to 25% of 96h LC_{50} value of three single heavy metals (lead, copper and cadmium). Following that, a study on the genotoxicity of copper and zinc mixture in erythrocyte of *Synodontis clarias* and *Tilapia nilotica* has been demonstrated by (Obiakor et al. 2010). Increased formation of the micronuclei were observed in all the three concentrations studied ($0.25LC_{50}$, $0.125LC_{50}$ and $0.0625LC_{50}$) and the frequency of micronuclei was observed to increase significantly ($p<0.05$) when the fish species were exposed to binary mixture compared with single exposure.

O. niloticus is a commercially important species for local consumption and some have even been exported to other countries. It is widely cultured in ponds, cages and tanks, pen culture and as well as in large water bodies such as in Kenyir Lake and Temenggor Lake, Peninsular Malaysia. In addition, *O. niloticus* is one of the most sensitive fish species for genotoxicity study as reported by (Jiraungkoorskul et al. 2007; Obiakor et al. 2010). In this study, we aimed to investigate the genotoxic effects of Cd and Zn and their binary mixtures (Cd+Zn) on the erythrocytes of Tilapia fish (*O. niloticus*) using the micronucleus test.

Materials and Methods

Fish and Chemical Reagent

Mature *O. niloticus* (with the length range 13-16 cm; weight range 80-100 g), obtained from Bukit Tinggi Aquaculture Centre, Pahang, Malaysia were acclimated in 100 L glass aquaria under controlled conditions for one week prior to the start of the experimentation. The aquaria were supplied with dechlorinated local tap water and oxygen had been kept saturated by aeration. The physico-chemical characteristics of the aquarium water were as follow: pH 6.9±0.3; dissolved oxygen 4.8±0.2 mg/L; hardness 86.55 4- 9.83 mg $CaCO_3$ 1-1; alkalinity 244.83 4- 12.69 mg $CaCO_3$ l-l; temperature 23.59±0.24°C and under a photoperiod of 12-14 h. Fish were fed twice a day with commercial fish food pellet and the aquarium water was replaced every 24 h to minimize contamination from metabolic waste.

Cadmium chloride ($CdCl_2$) and zinc sulphate ($ZnSO_4$) were purchased from Sigma. A stock solution of 1000 mg/L for each heavy metal was prepared by having it dissolved in distilled water and was added to the aquaria to achieve the desired final concentrations.

Heavy Metals Exposure

After the acclimatization period, fish were divided into four groups. One group served as control and the other three were exposed to 4.63 mg/L Cd; 7.50 mg/L Zn and 4.63 mg/L Cd + 7.50 mg/L Zn mixture for the period of 24, 48, 72 and 96 h. The concentration of the two metals used were $1/4^{th}$ of 96 h LC_{50} value from preliminary single exposure static renewal experiments (Almeida et al. 2002). Each concentration was tested in triplicate with three fish per test group and a total of 48 aquaria were used to stock one fish per aquarium (20 L). Fish were not fed 24 h before starting and during the 96 h of experiment. The experiment was conducted in static renewal systems. Water in all aquaria was changed daily and calculated additions of metals were added up to reach the desired nominal concentrations.

Micronucleus Test

At different times (24, 48, 72 and 96 h), three fishes from each group were anesthetized with 0.2 g/l 2-phenoxyethanol and weighed. Blood samples of *O. niloticus* were taken from the caudal vein by using heparinised syringes. Blood smear was prepared on slides and left to air-dry at room temperature. The slides were fixed with absolute methanol for 20 min and dried for 24 h and finally stained with 5% Giemsa for 20 min. A total of 2000 erythrocytes were examined for each fish under the light microscope, with a total of four slides used for each fish. For the scoring of micronuclei, the diameter of the micronucleus should be separated, that is less than 1/5th of the main nucleus and should have similar staining properties with the main nucleus. Erythrocytes with nuclear abnormalities other

than micronuclei in erythrocytes were classified into five groups which are binucleated cells, cells with 'blebbed' nuclei, cells with 'lobed' nuclei, cells with 'notched' nuclei and cells with nuclei bearing 'broken-eggs' (Nehls & Segner 2004). For the statistical analysis, the data from micronuclei and nuclear abnormalities analyses were analysed using R software. All data were expressed as mean±SD. The one-way ANOVA, followed by Tukey Multiple Comparisons test was employed to compare mean differences in frequency between the control and different exposure groups and time.

Results and Discussion

The incidences of micronuclei and erythrocytes with nuclear abnormalities observed in erythrocytes of *O. niloticus* after 24, 48, 72 and 96 h in their exposure to 1/4th of the LC_{50} value from the acute toxicity test of single and binary mixtures of Cd and Zn are summarized in Tables 1 and 2. As shown in Table 1, there were significant increases in the frequencies of micronuclei in all treated groups when compared with the control group. The highest frequencies of micronucleated erythrocytes were induced in fish treated with Cd at almost all time points (with the exception of the highest micronuclei which were found in Zn-treated groups at 96 h). Although the frequencies of micronucleated erythrocytes were always higher in Cd than Zn and their binary mixtures treatment, the apparent difference was not statistically significant in all cases (Table 1, Tukey Multiple Comparisons test, p=0.23–0.78). Conversely, frequencies of micronuclei in fish exposed to a mixture of Cd+Zn in *O. niloticus* were always lower at all time points (significantly different after 48 and 96 h but no significant difference at 24 and 72 h) than that of a single Cd and Zn exposure. In addition, the highest frequencies of micronuclei were obtained after 48 h exposure in all cases and decreased after 72 and 96 h exposure.

Different kinds of nuclear abnormalities such as binucleated cells, cells with 'blebbed' nuclei, cells with 'lobed' nuclei, cells with 'notched' nuclei and cells with nuclei bearing 'broken-eggs' were induced in the erythrocytes of the fish exposed to Cd, Zn and their binary mixture at all exposure times (Table 2 and Figure 1), although the observed differences of each kind was not statistically significant in every group (Tukey Multiple Comparisons test, p=0.19-0.79). Similarly to micronuclei, the frequencies of erythrocytes with nuclear abnormalities were also significantly greater at all time points (24, 48, 72 and 96 h) in fish treated with Cd followed by Zn and mixtures of Cd+Zn. The frequencies of erythrocytes with nuclear abnormalities in single exposure of Cd and Zn increased at 24 h, peaked at 48 h and decreased after 72 h and 96 h exposure. In comparison, the highest frequencies of nuclear abnormalities in the binary mixture Cd+Zn were obtained after 72 h and decreased after 96 h. It was observed that the mean frequencies of each type of nuclear abnormality in all treatments were found as followed: 'notched'>binuclear>'broken-eggs'>'lobed'> 'blebbed'.

Micronuclei are formed during cellular division and they reflect cytogenetic effects that is chromosomal rearrangement and change in chromosome number; loss of chromosomal fragment or whole chromosomes that are not included in the parent nucleus during anaphase (Muranli & Guner 2011). The incidence of micronuclei has served as an index of chromosomal breaks and mitotic spindle apparatus dysfunction for over 40 years since the first study on mouse developed by Boiler and Schmid (1970). This test is then widely employed and was subsequently modified by Hooftman and de Raat (1982) for the application of genotoxicity biomarkers in the laboratory for the fish. Today, micronucleus assays are widely validated and have been extensively used in the detection of genotoxicity in fish erythrocyte as it is simple, reliable and sensitive. Previous studies have identified nuclear abnormalities including 'blebbed', 'lobed' and 'notched' nuclei and 'binucleated cells' as possible indicators of genotoxicity (Muranli & Guner 2011; Nehls & Segner 2004) and therefore may complement the scoring of micronuclei in routine genotoxicity surveys. The formation of binucleated cells may have surfaced due to the difficulty in the formation of mitotic fuse caused by the aneugenic action of the heavy metals (Fernandes et al. 2007) and may be associated with failure of tubulin polymerisation (Ventura et al. 2008). The cells with 'blebbed' nuclei, 'lobed' nuclei and nuclei bearing 'broken-eggs' probably occurred from irregularities in the cell division, while 'notched' nuclei

TABLE 1. Micronuclei frequencies in erythrocytes of *Oreochromis niloticus* exposed to different treatments of cadmium, zinc and their binary mixtures

Time (h)	Total of erythrocytes analyzed	Frequencies of micronuclei (mean±SD)			
		Control	Cd	Zn	Cd+Zn
24	2000	0.100±0.100	0.900±0.100[a,c]	0.783±0.257[a,c]	0.567±0.076[a,c]
48	2000	0.033±0.057	2.700±0.436[a]	2.133±0.126[a]	1.500±0.100[a,b]
72	2000	0.133±0.057	2.000±0.458[a]	1.967±0.058[a]	1.217±0.126[a]
96	2000	0.167±0.104	1.600±0.229[a]	1.700±0.229[a]	1.000±0.050[a,b]

[a] p<0.05; represents values significantly different from the controls
[b] p<0.05; represents values significantly different for each time (h) when compared with other groups in the same row
[c] p<0.05; represents values significantly different for each group when compared with other times (h) in the same column

TABLE 2. Different kinds of nuclei abnormalities frequencies in erythrocytes of *Oreochromis niloticus* exposed to different treatments of cadmium, zinc and their binary mixtures

Nuclear abnormalities	Time (h)	Control	Cd	Zn	Cd+Zn
binucleated cells	24	0.017±0.029	0.567±0.076[a,c]	0.700±0.278[a]	0.483±0.189[a,c]
	48	0.050±0.050	1.067±0.104[a]	0.967±0.161[a]	0.767±0.208[a,b]
	72	0.067±0.058	0.967±0.077[a]	0.817±0.225[a]	0.917±0.126[a]
	96	0.050±0.087	0.983±0.104[a,b]	0.633±0.115[a,b]	0.983±0.076[a,b]
'blebbed' nuclei	24	0.033±0.029	0.417±0.076[a,c]	0.367±0.153[a]	0.633±0.161[a,b]
	48	0.067±0.029	1.150±0.100[a,b]	0.317±0.161[a,b]	0.750±0.150[a,b]
	72	0.017±0.029	0.800±0.100[a,b]	0.383±0.076[a,b]	0.583±0.104[a,b]
	96	0.067±0.029	1.100±0.180[a,b]	0.333±0.153[a]	0.53±0.189[a]
'lobed' nuclei	24	0.017±0.029	0.483±0.176[a,c]	0.400±0.100[a]	0.483±0.176[a]
	48	0.033±0.058	1.050±0.150[a,b]	0.317±0.104[a,b]	0.633±0.153[a,b]
	72	0.067±0.029	0.983±0.126[a,b]	0.417±0.176[a,b]	0.733±0.076[a,b]
	96	0.050±0.087	1.283±0.202[a,b]	0.450±0.229[a]	0.567±0.076[a]
'notched' nuclei	24	0.067±0.029	1.033±0.252[a,b]	0.533±0.293[a.]	0.567±0.246[a]
	48	0.050±0.050	1.283±0.275[a]	0.667±0.076[a,b]	1.067±0.076[a,c]
	72	0.017±0.029	1.066±0.126[a,b]	0.617±0.161[a,b]	0.883±0.126[a,b]
	96	0.017±0.029	1.050±0.100[a,b]	0.350±0.132[a,b]	0.750±0.132[a,b]
nuclear bearing 'broken eggs'	24	0.033±0.028	0.467±0.209[a,c]	0.333±0.153[a]	0.717±0.351[a]
	48	0.017±0.029	0.900±0.100[a,b]	0.417±0.104[a]	0.583±0.161[a]
	72	0.017±0.029	1.000±0.229[a,b]	0.467±0.257[a,b]	0.800±0.100[a,b]
	96	0.050±0.087	0.933±0.252[a]	0.617±0.126[a,b]	0.583±0.225[a,b]
Total frequencies	24	0.167±0.764	2.583±0.247[a,b,c]	1.617±0.126[a,b,c]	1.500±0.100[a,c]
	48	0.233±0.764	4.950±-.507[a,b]	4.150±0.304[a,b]	2.517±0.076[a,b]
	72	0.183±0.764	4.583±0.407[a,b]	4.000±0.436[a,b]	2.550±0.180[a,b]
	96	0.250±0.087	4.333±0.611[a,b]	3.850±0.218[a]	2.317±0.076[a,b]

[a] $p<0.05$; represents values significantly different from the controls.
[b] $p<0.05$; represents values significantly different for each time (h) when compared with other groups in the same row.
[c] $p<0.05$; represents values significantly different for each group when compared with other times (h) in the same column

are induced by the disintegration of the nuclear membrane. The appearances of 'vacuolated' nuclei may be associated with the presence of morphological alterations, resulting from necrotic processes. In addition, the formation of these abnormalities may represent a way to eliminate any amplified genetic material from the cell nucleus (Shimizu et al. 1998).

The peak values for micronuclei and nuclear abnormalities in erythrocyte had been obtained mostly at 48 h. Similar time-dependent effects have been previously reported in other fresh water fish erythrocytes exposed to lead, copper, cadmium (Jiraungkoorskul et al. 2007) and cadmium chloride (Ayllon & Gracia-Vazquez 2000). The absorption and accumulation of metal in fish after exposure was initially obtained at a high rate and eventually level stabilized when equilibrium is achieved between the rate of metal uptake and excretion rate. This explains the observed decrease in frequencies of both micronuclei and nuclear abnormalities in erythrocytes after 72 h and 96 h. Besides that, Udroiu (2006) suggested that among teleosts, a peak in micronucleated erythrocytes appears to occur between 1 and 5 days after exposure, mostly after 2 or 3 days. However, the time required to reach the peak of micronucleus induction in peripheral blood varies greatly among the species.

The exposure of *O. niloticus* to the Zn alone resulted in a significantly increased inductions of micronuclei and erythrocytes with nuclear abnormalities compared with the control groups (Tables 1 and 2), consistent with Obiakor et al. (2010), who demonstrated significant levels of micronucleated erythrocytes in freshwater fishes, *Synodontis clarias* and *Tilapia nilotica* exposed to 0.238 mg/L and 0.948 mg/L Zn. Although the concentrations used were considered very low compared to concentration used in this study, the micronucleus values obtained (Table 1) were similar with Obiakor et al. (2010) using similar micronucleus test and thus did not indicate concentration-dependent relationship. This accumulation patterns suggest that the uptake of Zn take place concurrently with excretion. While new Zn is entering the body in significant amount, an equivalent amount of Zn is excreted to match the rate of Zn uptake to keep the tissue concentrations within the levels required for metabolism (Daka et al. 2006). On the other hand, several studies have reported that high Zn concentrations can interfere with ROS detoxification processes and thus contributes to ROS accumulation (Nzenguea et al. 2011). In addition, an *in vitro* study showed that excess Zn can induce chromosomal instability and DNA double stranded breaks in human lung cell (Xie et al. 2009), but the underlying molecular mechanism of Zn toxicity remains unknown.

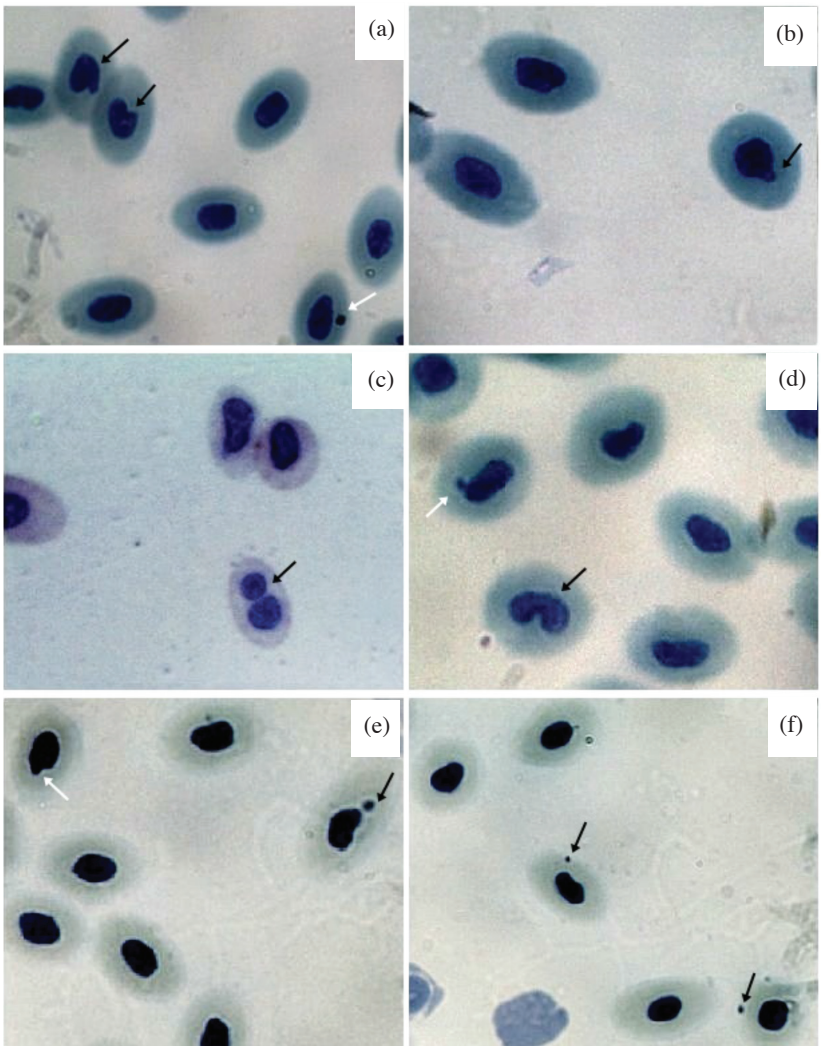

FIGURE 1. Erythrocyte cells with the presence of MN and NA (a) micronucleus, (b) binuclear, (c) notched nuclei, (d) lobed nuclei, (e) broken-egg and (f) blebbed nuclei

The observation of increased frequency of micronucleated in fish exposed to Cd have already been demonstrated in other species such as in the erythrocytes of *Anguilla anguilla* (Sanchez-Galan et al. 2001) and in *Cyprinus carpio*, *Carassius gibelio* and *Corydoras paleatus* (Cavas et al. 2005). Cd is a non-essential toxic metal that sometimes imitates the action of an essential component in the body and hence, interferes with the metabolic process to cause illness. The basis of Cd toxicity is its ability to interrupt the enzymatic systems of cells by substitution of essential metals ions (i.e. Zn^{2+}, Cu^{2+} and Ca^{2+}) in metallothionein and because of its very strong affinity to biological structures containing - SH groups, such as proteins, enzymes and nucleic acid (Jacobson & Turner 1980; Stohs & Bagchi 1995). In addition, Lutzen et al. (2004) suggested that the mechanism of Cd genotoxicity is mainly conditioned by single stranded breaks in DNA through direct Cd-DNA interactions as well as by the action of incision nucleases and/or DNA-glycosylase during DNA repair. Besides that, it has been reported that in the presence of Cd, reactive oxygen species (ROS) are formed and could be responsible for the alterations in protein, DNA and membrane structures (Isani et al. 2009).

Our results showed that the frequency of micronucleated erythrocytes and erythrocytes with nuclear abnormalities exposed in the Cd+Zn combinations at all times point were lower compared to single Cd and Zn exposure. The predicted antagonistic interactions of binary mixture of Cd+Zn presume that Zn may reduce the toxic effects of Cd on *O. niloticus*. These observations agree with those derived by Thorp and Lake (1974) who demonstrate antagonistic interactions between Zn and Cd when tested jointly against the freshwater shrimp *Paratya tasmaniensis*. Furthermore, Otitoloju (2002) reports that in most of the test combinations of the mixture, Zn compound is found to consistently reduce the toxic effect of Cu and Cd compound when tested jointly against *Tympanotonus fuscatus var radula* (L.).

The antagonistic response of Zn on Cd toxicity might have been due to many similarities in their chemical affinities between Cd and Zn and thus they may share

similar uptake pathways into organism (Rainbow 1997). In biological systems Cd and Zn are linked to macromolecules, primarily through sulphur (S), oxygen (O) and nitrogen (N) and interact readily with S-, O- and N donors. They bind preferentially to the same proteins like albumin in the bloodstream, metallothionein and other proteins in tissues. The interactions between these metals occur through the competition with Zn in metallothioneins in the cytosol or by acting on the nucleic acid structure or on membranes (Wang 1992), resulting in a decrease in the binding of Cd with macromolecules, thereby contributing to the protective nature of Zn. Although concentrations of Zn (7.50 mg/L) were higher than Cd (4.63 mg/L) in this study, they both results in the same level of toxicity ($1/4^{th}$ of 96 h LC_{50}). Zhang and Xiao (1998) suggested that when the concentration of Zn is higher than Cd, the replacement of Zn by Cd was inhibited and thus maintains the activity of zinc-containing enzymes.

Conclusion

This study has demonstrated the induction of genotoxic effects of the single and binary mixture of Cd and Zn in erythrocytes of *O. niloticus* under a short-term exposure. A significant increase in micronuclei and nuclear abnormalities are found in the treatment with single metals and binary mixtures, compared with the micronuclei and nuclear abnormalities in the control group. This study showed the antagonistic interaction between Cd and Zn.

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