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Efficacy of Calcium Reducing Lead Toxicity in Hematology of *Oreochromis niloticus*

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ABSTRACT

This study was investigated the effects of waterborne lead (Pb) and calcium (Ca) dietary on Nile tilapia (*Oreochromis niloticus*) using hematology, micronucleus (MN) and nuclear abnormality (NA) analysis. The values of 96 h LC_{50} of Pb to tilapia in our laboratory was 182.38 mg L^{-1}. Therefore, lead concentration tested in this sub-lethal experiment was 45 mg L^{-1} (correspond to 25% of the 96 h LC_{50}). The dietary supplement with 0, 20 and 60 mg Ca^{2+} g^{-1} fish food were feeding for 30 days. Sixty fish were equally divided into six groups (G1: normal diet and water; G2: normal diet and waterborne lead; G3: low Ca^{2+} diet and water; G4: high Ca^{2+} diet and water; G5: low Ca^{2+} diet and waterborne lead; and G6: high Ca^{2+} diet and waterborne lead). After 30 days treatment, blood collection for hematology, MN and NA analysis. It was observed that, fish showed significant sensitivity to the different treatments. The results of hematological analysis including, hematocrit, hemoglobin, erythrocytes and leukocytes counts, were increased in Pb exposure when compare those of the control group, but no statistically significant. The percentages of immature erythrocyte count were increased from 1.1 to 3.8% through Pb exposure. NA shapes in erythrocytes were scored into blebbed nuclei (BL), lobed nuclei (LB), notched nuclei (NT) and binuclei (BN). In general, the highest value of both MN and NA cells were significantly increased in the Pb treated group followed by the combination of Pb and Ca^{2+} treated group. The frequencies of each NA shape in erythrocytes of all treatments were observed in the following NT>LB>BN>BL. Our results demonstrated the efficacy of calcium in reducing toxicity in fish induced by waterborne lead.

Key words: *Oreochromis niloticus*, nile tilapia, lead nitrate, calcium, hematology

INTRODUCTION

The application of environmental toxicology studies on non-mammalian vertebrates is rapidly expanding and for aquatic systems, fish have become indicators for the evaluation of the effects of noxious compounds. Heavy metal contamination has been reported in aquatic organisms. These metal build up in food chain and are responsible for chronic illness and death in aquatic organisms, which probably move up to the top of food chain (Jiraungkoorskul *et al*., 2002). Lead (Pb) is a soft and corrosion resistant material, which is also a common contaminant that enters the water column mainly through industrial practices. It is useful for industrial used including battery production

and some in paints, such as pigments and colored inks (WHO, 1995). In addition, Pb in a non-nutrient metal found in the earth's crust, which can enter the aquatic environment easily through natural processes, such as geological weathering, volcanic emissions as well as through anthropogenic practices, for instant mining, refining and smelting of Pb (Alves *et al*., 2006). As a result, Pb becomes one of the most everywhere environmental poisons encountered in everyday life.

Waterborne Pb causes the disruption of Na^+, Cl^- and Ca^{2+} regulation during acute exposure, the induction of spinal deformities and black tails during chronic exposure and disruption in hemoglobin synthesis during both types of exposure (Rogers and Wood, 2004). Generally, divalent metals such as lead, cadmium and zinc are considered Ca^{2+} antagonists. The previous study in our laboratory, Nile tilapia (*Oreochromis niloticus*) were fed with 20 and 60 mg Ca^{2+} g^{-1} fish food and then exposed to 45 mg L^{-1} of waterborne lead for 30 days, had reduced Pb tissue burdens (Lamchumchang *et al*., 2007). Similarly, several more recent studies (Baldisserotto *et al*., 2005; Franklin *et al*., 2005) have shown that dietary Ca^{2+} is protective against the uptake of both waterborne and dietary cadmium, as well as against the uptake of waterborne zinc (Niyogi and Wood, 2006).

Erythropoietic activity, as reflected by changes in hematological parameters such as hematocrit, hemoglobin concentration and erythrocyte counts are modulated in fish by several factors like hypoxia (Valenzuela *et al*., 2002), stress (Pages *et al*., 1995). Greater numbers of mature than immature erythrocytes are found in peripheral blood. Immature erythrocytes or polychromatophils are commonly found in low percentages range from 0 to 10%, values that may increase up to 20% through stress, also reflected in the heightened hematocrit and in the increase of total erythrocyte numbers (Leonardi *et al*., 2003). A variety of biomarkers and bioassays in the laboratory and field studies used in determining the effects of genotoxic pollution including the presence of DNA adducts chromosomal aberrations, DNA strand breaks and measurement of micronuclei frequencies. Among the currently available test systems, the micronucleus assay is the most widely applied method due to its simplicity, reliability, sensitivity and proven suitability for fish species. This test is a measure of subcellular process such as induced chromosomal breaks (clastogenesis) or cell spindle malfunction (aneugenesis) (Heddle *et al*., 1991).

Nile tilapia is a common freshwater species that can be found in most tropical waters. It belongs to the Cichlids Family and the Oreochromis Genus. In Thailand, Thai tilapia is one of the most popular because of its economic values (Manosroi *et al*., 2003). Whereas comprehensive information is available on the effects of heavy metals exposure on fish, there are few reports on the efficiency of fish dietary supplementation on reducing their toxicity. In view of this background, the present study was designed to examine the efficiency of the dietary Ca^{2+} intake reduces the toxicity of lead in Nile tilapia (*O. niloticus*) via hematology, micronucleus (MN) and nuclear abnormality (NA) analysis.

MATERIALS AND METHODS

Experimental fish: This study was performed at the Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok, Thailand in 2010. Nile tilapia, *O. niloticus*, 14-18 g in body weight and 8-10 cm in total length, were purchased from a commercial hatchery in Thailand. Tap water was filtered to eliminate chemical contamination. The physicochemical characteristics of water were measured daily, according to the experimental procedures described in Standard Methods for the Examination of Water and Wastewater (APHA, 2005). Acclimatization to

laboratory conditions for 30 days was done using dechlorinated tap water. Fish were fed twice a day with commercial fish food. The quantity of food was 2% of the initial body weight per day. A 16 h light and 8 h dark photo-period was maintained. Lead (II) nitrate ($Pb(NO_3)_2$ Merck, Germany) was directly diluted in water to obtain the desired exposure concentrations.

Diet preparation: All diets were prepared with commercially fish food that was used to feed the fish during acclimation. The manufacturer's specifications for the fish food were of 37% crude protein; 14% crude fat; 3% crude fiber; 12% ash and 1% sodium (Charoen Pokphand Group, Bangkok, Thailand). This fish food was ground in a blender, followed by hydration with approximately 0.7 mL g^{-1} distilled water. To prepare the calcium-supplemented diet, the control diet was supplemented with $CaCO_3$ (Merck, Germany) to yield an experimental diet with 20 and 60 mg $Ca^{2+}g^{-1}$ dry wt. of food. Calcium carbonate was dissolved in the distilled water and added to the food paste. The resulting paste was mixed and extruded through a minced-meat processing machine. Later, the mixture was broken into small pellets by hands and air dried at 60°C in hot air oven for 48 h.

Sublethal toxicity test: The 96 h LC_{50} value of Nile tilapia exposed to Pb was determined in our laboratory as 182.38 mg L^{-1} (Lamchumchang *et al*., 2007). In this study, fish were exposed to 45 mg L^{-1}, which correspond to 25% of the 96 h LC_{50}. Fish (n = 60) were randomly divided into six groups. Each fish was transferred to each aquarium as follows: group 1: normal diet and water (control); group 2: normal diet and waterborne lead; group 3: low Ca^{2+} diet and water group 4: high Ca^{2+} diet and water; group 5: low Ca^{2+} diet and waterborne lead and group 6: high Ca^{2+} diet and waterborne lead. All glass flow-through aquaria (50 x 50 x 120 cm^3) with continuous aeration were filled with 200 L of dechlorinated tap water, whose physicochemical characteristics were the same as those described previously. After 30 days treatment, fish from each group were anesthetized with 0.2 g L^{-1} MS-222 (tricaine methan sulphonate, Sigma, Germany), weighed and measured. Peripheral blood samples were drawn from the caudal vessel and placed in tube containing EDTA for hematological analysis.

Hematological analysis: Blood from the EDTA tubes was drawn into microhematocrit tubes and the hematocrit (Ht) was determined after centrifugation for 5 minutes at 10,000 x g. Numbers of erythrocytes and leukocytes were determined by the Neubauer hemocytometer method using a Natt and Herrick solution (Natt and Herrick, 1952). The percentage of immature erythrocytes was determined relative to 1,000 erythrocytes counted in Wright's staining slides. Absolute values were calculated according to the above-obtained counts. Hemoglobin concentration (Hb) was determined measurement by the cyanometahemoglobin method. The blood indexes, Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC) were then calculated from Hct (Hb) and RBC according to Lee *et al*. (1998). This method of manually determining total WBC and differential counts has been recommended for avian (Zinkl, 1986) and fish (Stoskopf, 1993) blood because nucleated RBC prevent accurate enumeration using automated analysis.

Qualitative analysis of erythrocytes: Qualitative descriptions of erythrocytes followed the characteristics proposed by Fijan (2002): (a) *Shape*: the more immature the erythrocytes, the rounder the cells, (b) Size and degree of nuclear condensation: the more immature erythrocytes,

greater the nucleus area covered within the cells and less chromatin condensation, (c) Cytoplasmic color: the More immature cells, more basophilic their cytoplasm. In accordance to these criteria, ellipsoid cells with no basophilia were classified as mature erythrocytes (Er), ellipsoid cells with slight basophilia as Type 2 polychromatophils (P2, young erythrocytes) and round cells with strong basophilia as Type 1 polychromatophils (P1, polychromatic erythrocytes). Polychromatophils with intermediate characteristics (between Type 1 and 2) were identified and named intermediate polychromatophils (Pi).

Micronuclei and nuclear abnormality analysis: Blood was smeared on clean grease free microscope slides, air dried for 12 h and then fixed in absolute ethanol for 20 min. Each slide was stained with Wright's staining. From each slide 1000 cells were scored under 1000× magnifications using a Nikon E600 light microscope and photographed using a Nikon DXM 1200 digital camera (Tokyo, Japan). A single observer using blind review scored slides. Frequencies of MN and NA were expressed per 1000 cells (%).

Micronuclei and nuclear abnormality cells scoring: Only the cells clearly isolated from the surrounding cells were scored. The criteria for the identification of MN were earlier described: (a) MN must be smaller than one-third of the main nuclei (b) MN must be clearly separated from the main nuclei (c) MN must be on the same plane of focus and have the same color (Fenech *et al*., 2003). Nuclear abnormality shapes were scored into one of the following categories: blebbed nuclei (BL), lobed nuclei (LB), notched nuclei (NT) and binuclei (BN) (Carrasco *et al*., 1990). The result was expressed as the mean value (%) of the sum for all the individual abnormality observed.

Statistical analysis: All data were expressed as means + SD. A two-way analysis of variance was performed separately tested in each group. The Least-Significant Difference (LSD) was used for determination of significant differences at $p<0.05$.

RESULTS

General growth parameters: Mortality did not occur during the experiments and the control fish did not show any gross or behavioral changes. Otherwise, the treated fish were less active than the controls and a few of the treated fish rested on the bottom of the aquaria. The swimming became slower and there was reduction in their rate of feeding. Overall, the exposure to lead caused only a minor reduction in mean growth rate. These differences were not statistically significant.

Hematological analysis: The results including Hct (Hb), erythrocytes and leukocytes counts, were increased in Pb exposure, when compare those of the control group, but no statistically significant (Table 1). Qualitative description of tilapia erythrocytes in this experiment is shown in Fig. 1a-c. The percentages of immature erythrocyte count were increased from 1.1 to 3.8% through Pb exposure.

Micronuclei and nuclear abnormality analysis: Normal erythrocyte, approximately diameter 7 μm, was contained mainly elliptical nuclei (Fig. 2). The small non-refractile circular or ovoid particle lying in the cytoplasm and resembling a nucleus with respect to staining properties was considered as micronuclei. The size of the micronuclei varied to some extent (between 1/25th and 1/5th that of nuclear size) but the number was always one. The position of the micronuclei in the

Table 1: Hematological parameters of *O. niloticus* exposed to different treatments

Hematological parameters	G1	G2	G3	G4	G5	G6
Hct (%)	27	33	18	24	33	30
Hb (g dL^{-1})	9.3	11.1	6.8	8.3	11.8	10.2
Leukocyte count (10^3 μL^{-1})	28.6	31.6	17.6	23.6	28.6	30.0
Erythrocyte count (10^6 μL^{-1})	1.26	1.67	1.48	1.22	1.73	1.40
Immature erythrocyte count (10^6 μL^{-1})	0.014	0.063	0.024	0.016	0.042	0.039
MCV (fL)	214.3	197.6	121.6	196.7	190.8	214.3
MCH (pg)	73.8	66.5	45.9	68.0	68.2	72.9
MCHC (g dL^{-1})	34.4	33.6	37.8	34.6	35.8	34.0

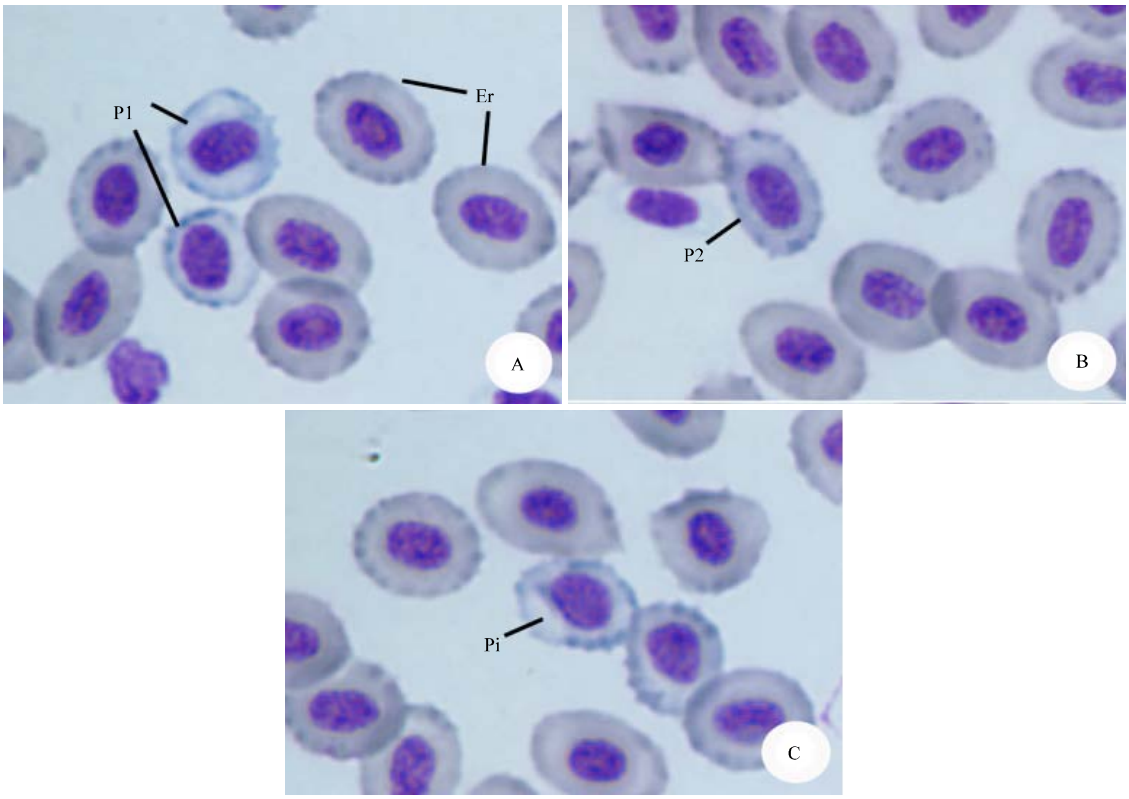

Fig. 1: Qualitative changes in *O. niloticus* erythrocyte(A) P1 = Type 1 Polychromatophils (late basophilic-polychromatic erythrocytes). (B) P2 = Type 2 Polychromatophils (young erythrocytes). (C) Pi = Intermediate Polychromatophils Characteristics.
Note: Er=Mature Erythrocyte

cytoplasm also varied, some located very near to the nucleus or some located vary far even at the periphery of the cell. Some of the nuclei clearly deviated from their normal shape and were either blebbed (BL), lobed (LB), notched (NT) and binucleated cells (BN). All abnormalities of nuclei were scored. In briefly, cells with two nuclei were considered as binucleates. The two nuclei should be approximately equal size, staining pattern and staining intensity, within the same cytoplasmic boundary. Blebbed nuclei presented a relatively small evagination of the nuclear membrane, which

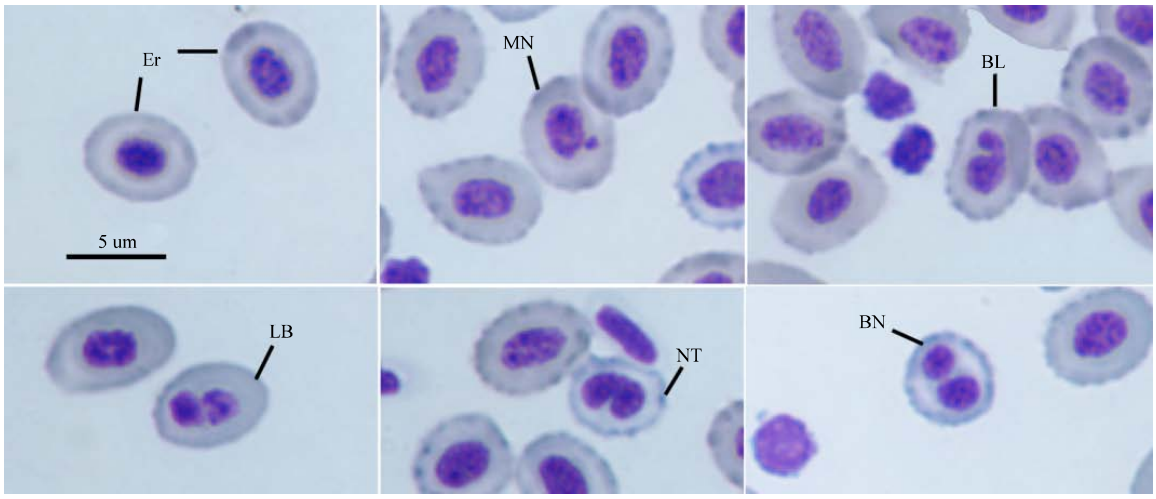

Fig. 2: Photomicrographs of erythrocytes with normal nucleus (Er); with micronuclei (MN) and with nuclear abnormalities: blebbed nuclei (BL); lobed nuclei (LB); notched nuclei (NT); binuclei (BN) of *O. niloticus*

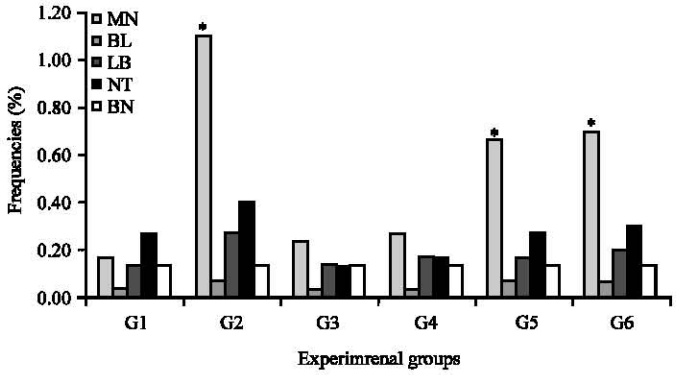

Fig. 3: Frequencies (%) of micronuclei and nuclear abnormalities in erythrocytes of *O. niloticus* exposed to different treatments. *Mean difference was significant when compared the control group ($p<0.05$)

contained euchromatin. Evaginations larger than the blebbed nuclei, which could have several lobes were classified as lobed nuclei. Nuclei with depth into a nucleus were recorded as notched nuclei.

The frequencies of MN and NA observed in various treated and control fish have been showed in Fig. 3. It is evident that the differences in MN were statistically significant in treated group when compared with those of the control group. The frequencies of MN were significant highest number in fish treated with Pb alone. However, when both Pb and calcium were conjointly administered, the MN appeared to be reduced to some extent. Similarly to MN, the frequencies of NA were also significant greater number in fish treated with Pb. When NA in erythrocytes were analyzed separately, it was observed that the frequency of each abnormality shapes were found in the following order: NT>LB>BN>BL.

DISCUSSION

The results of this study similar to our previous report in *Puntius altus* exposed to cadmium (Jiraungkoorskul *et al*., 2007), *O. niloticus* exposed to copper (Kosai *et al*., 2009). Hematological parameters include; pack cell volume, hemoblogbin, total erythrocytes and leucocytes count were used more often when fish was applied to determine the effect of pollutants (Al-Attar, 2005; Mohammed and Sambo, 2008). Fish exposed to Pb showed a statistically significant ($p<0.05$) increase in the hematological parameters. MN is cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosome fragments or intact whole chromosomes lagging behind in the anaphase stage of cell division. Their presence in cells is a refection of structural and/or numerical chromosomal aberrations arising during mitosis (Heddle *et al*., 1991). MN and NA tests in fish are generally performed in enucleated peripheral blood erythrocytes mainly due to its technical feasibility. It is well known that heavy metals interferes the regular chromosome segregation during cell division mainly by inhibition of polymerization of actin tubules, an essential structure of the mitotic spindle (Miura and Imura, 1987). It was suggested that the mechanism of Pb genotoxicity is mainly conditioned by single strand breaks in DNA through the direct lead-DNA interactions as well as by the action of incision nucleases and/or DNA-glycosylase during DNA repair like those of cadmium (Privezentsev *et al*., 1996). Correspondingly, most of the toxic chemicals that produce genotoxic effects have been known to form reactive oxygen species as well as electrophilic free-radical metabolites that interact with DNA to cause disruptive changes. It has been suggested that during the heavy metal exposure, electrophilic ions and radicals are produced, interacting with nucleophilic sites in DNA and leading to breaks and other related damage in the latter.

Arkhipchuk and Garanko (2005) reported the highest increases of cells having micronuclei were observed in blood after fish exposure to cadmium. In the present study, there was a general tendency of occurrence of notched and lobed types in higher frequencies. An analysis revealed spontaneous frequencies of nuclear abnormalities in erythrocytes were found in the following order: NT>LB>BN>BL. Thus, our results seem to be in agreement with previous studies (Cavas and Ergene-Gozukara, 2003; Mallick and Khuda-Bukhsa, 2003).

Previous studies have found significant reductions in whole body cadium uptake from the water and diet when rainbow trout were fed a diet supplemented with 60 mg $CaCO_3$ g^{-1} (Baldisserotto *et al*., 2004a, 2005; Franklin *et al*., 2005), or 60 mg $CaCl_2$ (Zohouri *et al*., 2001; Baldisserotto *et al*., 2004b). Freshwater fish have two primary uptake pathways for essential ions i.e., Ca^{2+}, Na^{+}, the gills as waterborne ions and the gastrointestinal tract as dietary ions. They can regulate the total uptake by changing the proportion of each kind of uptake depending on the environmental situations. For example, tilapia (*O. mossambicus*) up-regulate intestinal Ca^{2+} uptake when living in water with low Ca^{2+} concentration (Flik *et al*., 1995). Therefore, if the fish can acquire more ions via the gastrointestinal route, they may decrease branchial ion uptake rates and thereby subsequently reduce the uptake of metals sharing the common branchial pathway. For example, Cd^{2+}, Zn^{2+} shares the same transport pathway with Ca^{2+} (Verbost *et al*., 1989; Niyogi and Wood, 2004) and previous studies have shown that dietary Ca^{2+} supplementation decreased waterborne Ca^{2+} uptake and subsequently waterborne Cd^{2+} uptake (Baldisserotto, *et al*., 2004a, b, 2005). There is no available data of Ca^{2+} concentrations in the natural diet of feral fish, but Ca^{2+} is available in abundance in crustacean exoskeleton and mollusk shells. Interestingly though, Sherwood et al. (2000) reported that wild yellow perch (*Perca flavescens*) in heavy metal impacted lakes tend to eat relatively more invertebrates than fish.

From our results, it is possible to conclude that the dietary calcium supplementation can reduce the effect of waterborne lead uptake in fish.

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