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Juvenile Tilapia (*Oreochromis mossambicus*) Strive to Maintain Physiological Functions After Waterborne Copper Exposure

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Abstract Juvenile tilapia were acutely exposed to 0.2 and 2 mg/L Cu^{2+} for up to 144 h. The Na^+-K^+-ATPase (NKA)-specific activity in the gills of tilapia exposed to 0.2 mg/L Cu^{2+} significantly decreased over 48–72 h and was restored to the control level after 96 h, but was again depressed during 120–144 h. The whole-body Cl^- levels significantly decreased after 48 h, but recovered shortly afterwards and continued to do so until 144 h with 0.2 mg/L Cu exposure. During 48–72 h, the numbers of the wavy-convex type of mitochondria-rich (MR) cells appeared to significantly increase and the cortisol content also significantly increased. Changes in MR cell morphology might be necessary in order to enhance Cl^- uptake, and this might be related to changes in cortisol levels. Whole-body Na^+ concentrations had significantly decreased by 72 h, but recovered during 96–144 h. Whole-body Cu^{2+} concentrations also significantly increased compared to the initial concentration during 72–144 h of Cu exposure. All measured parameters (NKA activity, Na^+ concentration, and MR cell numbers) significantly decreased in fish exposed to 2 mg/L Cu, and no recovery was observed. These data demonstrate that juvenile tilapia strived to maintain physiological functions after exposure to sub-lethal concentrations of Cu.

Keywords Copper · Teleost · Sodium pump · Compensation

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Introduction

The toxicity of copper to adult fish, its accumulation in tissues, and its impact on physiological mechanisms have been well studied (Dethloff et al. 1999). Copper directly causes necrosis of mitochondria-rich (MR) cells (Bury et al. 1998), and much evidence shows that Cu toxicity also disrupts Na^+ and K^+ homeostasis and growth in larval (Wu et al. 2003), juvenile, and adult fish (Taylor et al. 1996, Li et al. 1998, Perschbacher and Wurts 1999, McGeer et al. 2000). One reason for these changes is that NKA-specific activity in fish is very sensitive to waterborne Cu exposure (Li et al. 1998, De Boeck et al. 2001). Free Cu^{2+} has been shown to covalently bind to SH-groups of NKA, thus causing conformational changes in the protein (Kone et al. 1990), even in fish exposed to sub-lethal concentrations of Cu for a short time. For example, NKA-specific activation in gills was found to have significantly decreased following four hours of 0.1 mg/L Cu exposure, and by 96 h, NKA-specific activation in gills had decreased to 65% of its initial level in carp (*Cyprinus carpio*) (De Boeck et al. 2001). Rainbow trout (*Oncorhynchus mykiss*) exhibited hypoxia in gill epithelium after four hours of 105 μg/L Cu exposure (Heerden et al. 2004). Gills are the first organ to be targeted by heavy metal exposure (Taylor et al. 1996), and fish apparently induce some mechanism to adapt to Cu-polluted environments. It is well known that the process of Cu accumulation in gills upon exposure to sub-lethal concentrations of Cu is characterized as a damage-repair model (McDonald and Wood 1993). Alternatively, acute Cu inhibition of branchial Na^+ and Cl^- uptake can initiate a cascade of effects that leads to mortality (Grosell et al. 2002). Fathead minnows (*Pimephales promelas*) exposed to either lethal or sub-lethal concentrations of

Cu exhibited a strong relationship between larval survival and whole-body Na^+, once acclimation had developed (Sellin et al. 2005). Javanese carp (*Puntius gonionotus*) have been exposed to lethal concentrations of Cu in order to determine the $LC_{50,}$ and challenged with sub-lethal Cu concentrations to monitor the immune response (Shariff et al. 2001). However, few integrated studies have compared the physiological responses to, and connections between, both sub-lethal and lethal Cu exposure in fish.

Past studies reported that morphological alterations of MR cells occur when ion disturbances are induced in freshwater teleosts (Perry and Laurent 1993, Perry and Goss 1994). These phenotypic changes were suggested to be a crucial mechanism for ion regulation (Hirose et al. 2003). In addition, morphological characteristics of gill MR cells are affected by hormones, especially cortisol which stimulates the proliferation and differentiation of ion-transporting cells (MR cells) of the gills, as well as NKA expression by those cells (Bindon et al. 1994, Seidelin et al. 1999). Cortisol also reduces MR cell necrosis with lower-concentration Cu treatments, in an in vitro study (Bury et al. 1998). It is well known that sub-lethal Cu disrupts ion homeostasis in fish (McGeer et al. 2000, Wu et al. 2003). Therefore, we hypothesized that fish are able to modify the morphology of MR cells in order to enhance cation or Cl^- uptake upon sub-lethal Cu exposure, and MR cells either may be adversely affected by longer exposures or by higher doses of Cu, which may be related to changes in cortisol levels.

In tilapia (*Oreochromis mossambicus*), copper accumulates in MR cells (Dang et al. 1999), has significant effects on the respiratory and osmoregulatory functions of gills (Nussey et al. 1996), and inhibits NKA activity (Li et al. 1998). The reasons for these actions might be related to the amount of NKA, which is estimated to be 10^8 molecules in a single MR cell (Karnaky 1986). These MR cells decrease in a time-dependent manner through degeneration by apoptosis and necrosis in Cu-exposed fish (Bury et al. 1998, Li et al. 1998), but fish respond with compensatory mechanisms including stimulation of NKA synthesis and enhanced MR cell turnover (Dang et al. 2000a). Therefore, the NKA activity should be restored following multiplication of MR cells.

In this study, fish were challenged with a sub-lethal concentration (0.2 mg/L) and a lethal concentration (2 mg/L) of copper and exposed for 0–144 h to compare the temporal changes in NKA activity, Na^+ concentration, cortisol level, and number of histologically determined MR cells. However, NKA immunoreactivity (NKAI) reactions, Cl^- levels, and Cu accumulation were only measured after 0.2 mg/L Cu exposure.

Materials and Methods

Fish and Experimental Media

Mature adult tilapia (O. mossambicus) were collected from the Mariculture Research Center of the Taiwan Fisheries Research Institute, Tainan, Taiwan. Fish were reared in 182-L glass aquariums with plastic chips for gravel. Each tank was supplied with circulated, aerated, and de-chlorinated local tap water (FW) at 26–28°C under a photoperiod of 12–14 h, and fish were fed commercial fish food pellets. Because our previous studies had reported that the physiological responses of fish should show a higher variance if samples are collected from different broods and the number of hatched larvae in a single brood is limited (Hwang and Wu 1993, Wu et al. 2003), different broods were used in separate experiments for examining the effects on various parameters. Larvae were incubated under the same conditions as above until they reached the juvenile stage, and juvenile tilapia at 2–4 cm total length and 0.3–0.5 g body weight were used in the present study. Duplicate experiments were conducted using different broods.

Completely dehydrated $CuSO_4$ (Merck, Darmstadt, Germany) was dissolved in double-deionized water (ddH_2O, DI-S4, Millipore, USA) to prepare the 1000 mg/L Cu^{2+} stock solution. This stock solution was diluted to the desired concentrations with local tap water, as described by Wu et al. (2003). In preliminary experiments the medium with 2 mg/L Cu caused 40–60% mortality during six days of exposure, but no mortality occurred with exposure to 0.2 mg/L Cu during 0–144 h of treatment. Therefore, the Cu test media were prepared at concentrations of 0, as a control, 0.2 mg/L as the sub-lethal concentration, and 2 mg/L Cu as the lethal concentration. The medium in the test containers was changed daily, and the variance in Cu concentrations was less than 5% within 24 h. The Cu concentration in local tap water was less than 0.01 mg/L (according to data provided in a routine report by the Taiwan Water Supply Corporation, Chiayi, Taiwan). The exposure media (i.e., local tap water) also contained a hardness of 1.46 ± 0.06 mM $CaCO_3$, 1.55 ± 0.01 mM Na^+, 0.08 ± 0.003 mM K^+, 0.75 ± 0.06 mM Ca^{2+}, 0.83 ± 0.01 mM Mg^{2+}, and a pH of 8.45 ± 0.25 (n = 6).

Specific Activity of NKA

Aliquots of gill filament homogenates were used for determining protein and NKA activities. NKA activity was assayed by adding the supernatant to a reaction mixture [100 mM imidazole-HCl buffer (pH 7.6), 125 mM NaCl, 75 mM KCl, 7.5 mM $MgCl_2$, and 5 mM

Na_2ATP]. The reaction media were incubated at 37°C for 30 minutes, and then the reaction was stopped by the addition of 200 µl of ice-cold 30% trichloroacetic acid. The inorganic phosphate concentration was measured according to Peterson's method (1978). The enzyme activity of NKA was defined as the difference between the inorganic phosphate liberated in the presence and absence of 3.75 mM ouabain in the reaction mixture. Six replicates were run for each sample.

Scanning Electron Microscopy (SEM) and Abundances of MR Cells

For SEM, the gill filament treatment was as described by Lin et al. (2004). Briefly, tissues were fixed at 4°C in phosphate-buffered 4% paraformaldehyde plus 5% glutaraldehyde (at pH 7.2) for 24 h, and then in 1% osmium tetroxide (at pH 7.2) for 1 h at 4°C. Tissues were dehydrated in ascending concentrations of ethanol from 50% to absolute and then in 100% acetone, and dried using a Hitachi HCP-2 critical-point drier. After sputter-coating with a gold–palladium complex for 90 s, specimens were examined using a Hitachi S-3500N scanning electron microscope. MR cell densities were counted using SEM as described by Lee et al. (2000). The abundances of MR cells were determined by counting them using SEM. Areas on the filament afferent side near the lamellae (not including the interlamellar regions) were chosen at random for counting at 1000× magnification. Three areas (30×30 µm, or 900 μm^2, each) were counted on each of six gill filaments from each fish per group (with 0–144 h of exposure). Numbers of wavy-convex MR cells and pavement cells were counted.

Whole-Body Na^+, Cl^-, and Cu^{2+} Measurement

Tilapia were weighed, and then digested with 13.1 $NHNO_3$ at 230°C for 45 min in a microwave sample preparation system (Antion Paar, Multiwave, Austria). Digestion solutions were diluted with ddH_2O for subsequent analysis. Digested samples, test media, and appropriate blanks, including deionized water, were analyzed by atomic absorption spectrophotometry (Z-5000, Hitachi, Japan), with an air/acetylene flame for the Na^+ analyses and a graphite furnace for the Cu^{2+} analysis. Three standard ion solutions from Merck were used to create the standard curves for the measurements. The addition of the standard solution to the test samples was used for background correction to estimate the matrix effect (following the manufacturer's instructions for the Hitachi spectrophotometer). Six individuals were analyzed for Cl^- concentration for each sampling time. The method of Cl^- quantification was modified from the ferricyanide method (Wu et al. 2003), and concentrations were measured using a spectrophotometer (Z-2000, Hitachi) at 450 nm.

NKA Immunoreactivity

To determine NKA immunoreactivity, gills were immersed in Bouin's fixative solution for 24 h. After dehydration and embedding in paraffin, 5-µm-thick tissue sections were cut, mounted on poly-L-lysine-coated slides (Sigma, St Louis, MO, USA), and processed according to the manual of the PicTure kit (Zymed, San Francisco, CA, USA), to visualize the MR cells. In brief, after dewaxing and blocking the endogenous peroxidase with 3% H_2O_2 for 10 min, slides were washed in 0.1 M phosphate-buffered saline (PBS). Slides were incubated overnight in a humid chamber at 4°C with a mouse monoclonal antibody to NKA (IgG a5; Developmental Studies Hybridoma Bank, USA) at a working dilution of 1:200, followed by incubation with a horseradish peroxidase (HRP) polymer conjugate at room temperature for 30 min. After washing, the 3-amino 9-ethylcarbazole (AEC) chromogen was added and incubated for 15 min, and the peroxidase catalyzed the substrate (hydrogen peroxide) and converted the chromogen to a red deposit, which allowed visualization of the location of the NKA.

Cortisol Extraction and Measurement with Enzyme-Linked Immunosorbent Assay

The method for cortisol extraction followed Hwang and Wu (1993). The cortisol content was determined by an enzyme-linked immunosorbent assay (ELISA), following the method of our previous paper (Wu et al. 2005). Briefly, diluted antiserum of cortisol was coated in a 96-well microtiter plate for 24 h at 4°C. Blocking buffer was reacted for 24 h at 4°C, followed by three washes with washing buffer. Tissue extract, plasma, and the standard solution were mixed with cortisol-conjugated HRP. O-phenylenediamine (OPD; Sigma) was added for color development, and the results were measured at 490 nm with an automatic microtiter plate ELISA reader (Dynes MRX, Chantilly, VA, USA). The displacement curve for the serial dilutions of sample extracts was found to be parallel to that of the cortisol standard. The linear regression coefficient was determined using Microsoft Excel 97 SR-1 (1997). The logarithm of the cortisol standard concentrations was 0.99, and the slope was –0.18. The coefficients of the intra- and interassay variations were 1.5–3.7% and 5.4–6.3%, respectively.

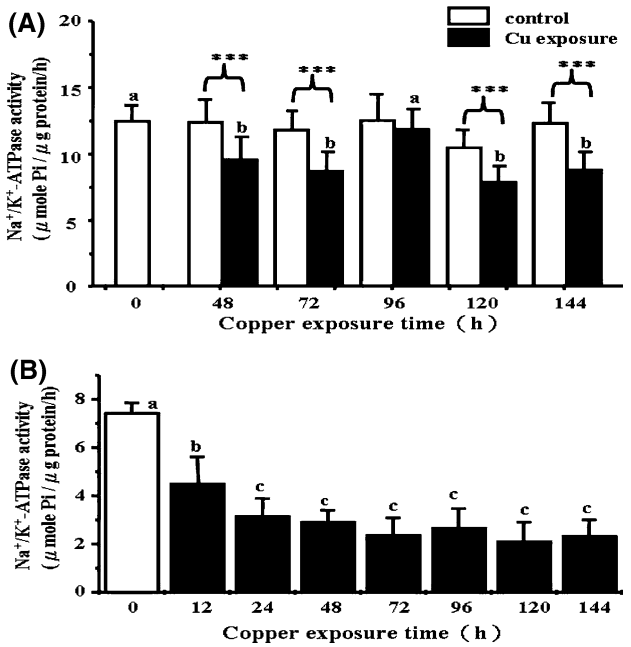

Fig. 1 Comparisons of changes in Na^+-K^+-ATPase activity in juvenile tilapia with **(A)** 0.2 mg/L Cu or **(B)** 2 mg/L Cu for 0–144 h. Values are mean ±SD ($n = 6$). Different superscript letters indicate a significant difference ($p < 0.05$) among treatment durations (ANOVA analysis with Tukey's comparison). *** ($p < 0.001$) indicates a significant difference between the control and treatment groups at the same time point

Exposure to Cu

Experiment 1

(1) NKA activity measurement: six to eight tilapia juveniles were collected from each of the six test tanks and sampled at 0, 48, 72, 96, 120, and 144 h, after 0 or 0.2 mg/L Cu exposure. Fish were anesthetized with MS222 (Sigma) immediately after removal from the experimental tanks during sampling. Gill filaments were excised and homogenized after weighing them, and then NKA activity was measured as described above.

(2) SEM: fish were treated under the same conditions as above, and then excised gills were placed in 0.5 mM phosphate buffer (pH, 7.2). Gills were dehydrated after being fixed, and then MR cells were observed with SEM.

(3) Measurement of Na^+ and Cu^{2+} accumulation, Cl^- and cortisol concentrations: every parameter measured was independently sampled, with one fish as one sample, to detect the whole-body Na^+, Cu^{2+}, Cl^-, and cortisol concentrations. For cortisol- and Cu-content measurements, no sample was treated with the control at the different times because our past study had found that the handling stress caused cortisol to rise after 12 h, and it should recover after 24 h (Wu et al. 2006). Furthermore, Cu content wasn't affected by the handling stress (Wu et al. 2003). All parameters in this study were measured at least three times.

Experiment 2

In the second experiment, sampling times (0, 12, 24, 48, 72, 96, 120, and 144 h) and the Cu^{2+} exposure dose (2 mg/L) differed slightly. However, all other parameters were consistent with those in experiment one, except for Cl^-, Cu^{2+}, and NKAI. These parameters were not measured because the wavy-convex type of MR cells only appeared during 48–72 h after 0.2 mg/L Cu exposure, and we supposed that the morphology of these apical surfaces changed in order to increase Cl^- uptake (Lin and Hwang 2004). Therefore, the whole-body Cl^- concentration of juvenile fish was measured after sub-lethal Cu exposure. In addition, because NKA activity was restored to the control level at 96 h of Cu exposure but the density of MR cells did not show a similar profile, active or inactive MR cells were detected with NKAI after sub-lethal Cu exposure. The whole-body Na^+ concentration was measured because it had been restored to the control level after 96 h of sub-lethal Cu^{2+} exposure and compared with Cu^{2+} accumulation to determine their relationship.

Statistical Analysis

Data are presented as the mean ±standard deviation (±SD), and were analyzed by one-way analysis of variance (ANOVA) with Tukey's multiple-comparison analysis or Student's *t*-test. Statistical significance was accepted for *p*-values less than 0.05.

Results

NKA activity in fish exposed to 0.2 mg/L Cu significantly decreased, compared to control fish, from 48 h to the end of the experiment, except at 96 h (Fig. 1A). The activity again significantly decreased, by 35%, during the period of 120–144 h of Cu exposure, compared to the control (Fig. 1A). However, NKA activity significantly decreased after exposure to 2 mg/L Cu for 12 h, and continued to decrease by 57%, 60%, and 70% after 24, 48, and 72 h of exposure to 2 mg/L Cu, respectively (Fig. 1B). More of the deep-hole type of MR cells were present in the gill filaments in the control group (0 h of Figs. 2 and 3). Compared to the deep-hole and shallow-basin types of MR cells, densities of

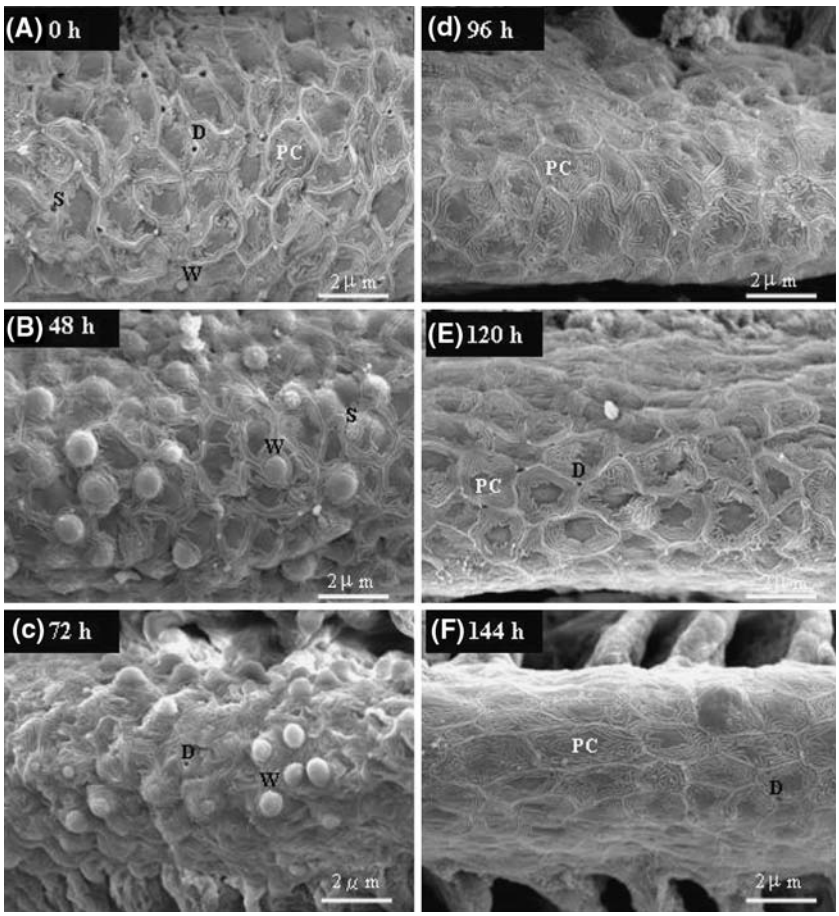

Fig. 2 Scanning electron microscopic images of branchial mitochondria-rich (MR) cells in juvenile tilapia exposed to 0.2 mg/L Cu for 0, 48, 72, 96, 120, and 144 h. D, deep-hole MR cells; W, wavy-convex MR cells; S, shallow-basin MR cells; PC, pavement cells. Magnification, ×1000

the wavy-convex type of MR cells were significantly greater between 48 and 72 h of exposure to 0.2 mg/L Cu, but they were lower during the period of 96–144 h (Table 1; Fig. 2). A few of the deep-hole type of MR cells remained, but both shallow-basin and wavy-convex types of MR cells had disappeared by 120 h of 0.2 mg/L Cu (Fig. 2) and 96 h of 2 mg/L Cu exposure (Fig. 3). Compared with the control group at each exposure time, differences in the Cl^- concentration were not significant, except for fish exposed to 0.2 mg/L Cu for at least 48 h (Fig. 4). In control fish, NKAI cells (MR cells) were exclusively found in the filament epithelium, with occasional, small NKAI cells at the base of the lamellae (Fig. 5a). The abundance of MR cells in the filament epithelium of fish exposed to 0.2 mg/L Cu for 96 h was similar to that of control fish (Fig. 5d); however, the densities were significantly lower than the control group after 120–144 h of Cu exposure (Fig. 5e, f).

There was a peak in the cortisol content after 48 h of exposure to 0.2 mg/L Cu, and then it decreased after 72–96 h of exposure. It obviously differed upon acute Cu exposure. There were no significant differences in the cortisol content between treatment with 2 mg/L Cu and the control, except after 96 h of Cu exposure (Table 2).Within 48–72 h from the start of the experiment, Na^+ concentrations significantly decreased, by 50%, in fish exposed to 0.2 mg/L Cu, however, levels increased to control levels within 96–144 h (Fig. 6A). Following 2 mg/L Cu exposure, Na^+ concentrations had significantly decreased by 48 h and remained constant until 96 h (Fig. 6B). Compared with the control, at each of the exposure times, Cu concentrations significantly increased after 72 h, though Cu accumulation remained constant after 96 h of 0.2 mg/L Cu exposure (Fig. 7). Neither whole-body Na^+ nor NKA activity showed a significantly difference among various treatment times of the control group in experiment 1. Therefore, there were no control data to compare with the treatment group at the same time in experiment 2 (Figs. 1b and 6b).

Discussion

The results of this study are similar to those of previous papers that reported that NKA-specific activity in fish is very sensitive to waterborne Cu exposure (Li et al. 1998, De Boeck et al. 2001). However, a lethal Cu^{2+} concentration only disrupted Na^+ homeostasis; the whole-body Na^+ concentration was reduced by about 50% at 72 h in fish

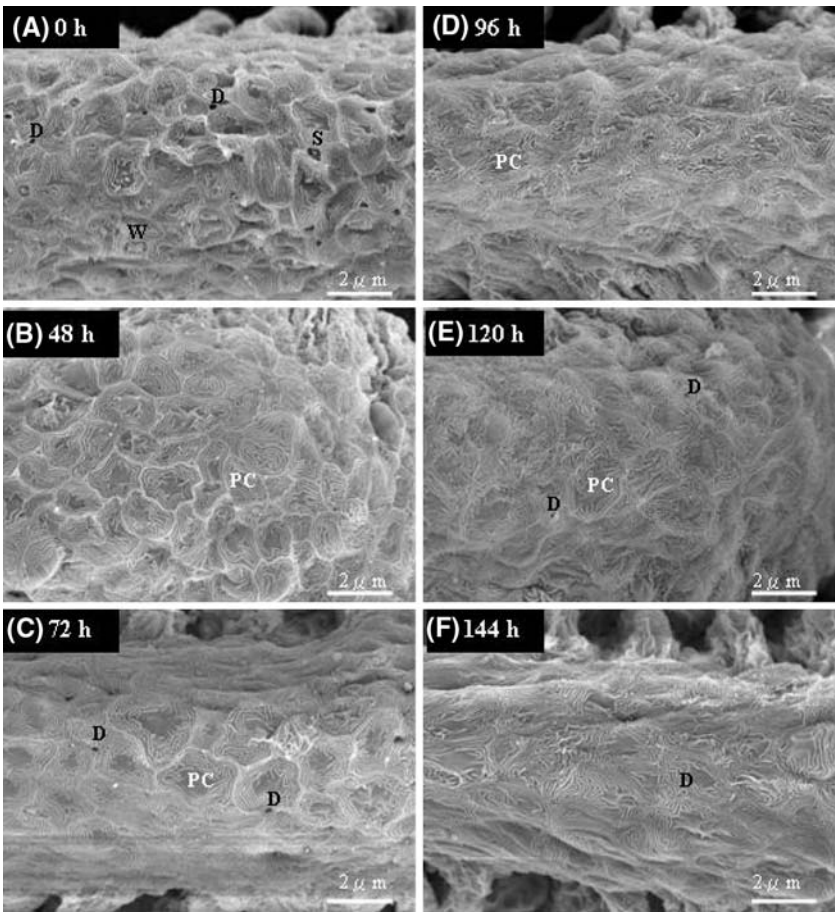

Fig. 3 Scanning electron microscopic images of branchial mitochondria-rich (MR) cells in juvenile tilapia exposed to 2 mg/L Cu for 0, 48, 72, 96, 120, and 144 h. D, deep-hole MR cells; W, wavy-convex MR cells; S, shallow-basin MR cells; PC, pavement cells. Magnification, ×1000

Table 1 Changes in the density (cell number/900 μm^2) of three types of mitochondria-rich (MR) cells (W, wavy-convex; S, shallow-basin; D, deep-hole) and pavement (PV) cells in gills of juvenile tilapia, following either 0.2 or 2 mg/L Cu exposure for 0, 48, 72, 96, 120, and 144 h

Cu^{2+} exposure dose	Cell type	Duration of Cu exposure (h)					
		0	48	72	96	120	144
0.2 mg/L	PV	50.4 ±17.1^{a}	49.7 ±13.4^{a}	46.4 ±10.7^{ab}	35.9 ±7.1^{b}	38.9 ±9.7^{b}	38.1 ±9.3^{b}
	W-MR	5.2 ±3.4^{a}	18.6 ±6.3^{b}	18.0 ±5.6^{b}	0.4 ±0.9^{c}	1.3 ±2.1^{c}	1.1 ±2.3^{c}
	S-MR	5.1 ±2.3^{a}	6.1 ±3.6^{a}	5.4 ±3.6^{a}	0.6 ±0.9^{b}	1.0 ±1.3^{b}	0.8 ±0.8^{b}
	D-MR	10.6 ±4.0^{a}	2.4 ±1.8^{b}	3.7 ±1.9^{b}	1.3 ±1.3^{b}	2.4 ±1.4^{b}	1.7 ±1.2^{b}
2 mg/L	PV	51.2 ±11.8^{a}	49.4 ±12.8^{a}	50.2 ±13.2^{a}	45.7 ±9.9^{a}	43.5 ±9.4^{a}	43.2 ±9.4^{a}
	W-MR	2.8 ±2.4^{a}	0.4 ±0.6^{b}	0.4 ±0.7^{b}	0.4 ±0.7^{b}	0.6 ±0.8^{b}	0.7 ±0.7^{b}
	S-MR	5.1 ±2.2^{a}	0.8 ±0.9^{b}	1.1 ±0.9^{b}	0.5 ±0.8^{b}	0.8 ±0.9^{b}	0.7 ±0.7^{b}
	D-MR	9.1 ±3.5^{a}	1.5 ±1.2^{b}	1.9 ±1.9^{b}	1.3 ±1.2^{b}	1.6 ±1.6^{b}	1.4 ±1.2^{b}

Values are the mean ±SD (n = 18). Different superscript letters indicate a significant difference ($p < 0.05$) among various times of Cu exposure by one-way ANOVA analysis with Tukey's comparison

exposed to 0.2 mg/L Cu^{2+}, but it recovered to the control level and remained constant until 144 h, while Cl^{-} was not significantly affected in juvenile tilapia with sub-lethal Cu^{2+} treatment. Furthermore, we observed that juvenile tilapia attempted to acclimate by changing the morphological type of MR cells, and stimulating cortisol release when they were exposed to an ambient sub-lethal concentration of Cu^{2+}. Several studies support the idea that waterborne Cu exposure leads to decreased branchial NKA activity and increased cortisol levels in fish (McDonald and Wood 1993, Wendelaar Bonga 1997). Cortisol plays many important roles in regulating acclimation of fish to environments polluted with heavy metals; for instance, metallothionein expression is induced (Wu et al. 2006),

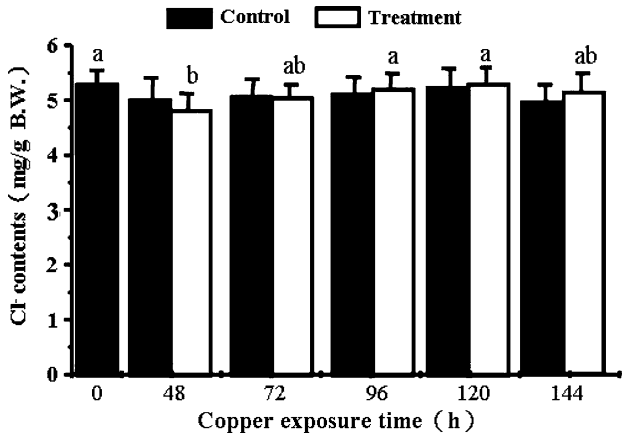

Fig. 4 Cl^- concentrations of juvenile tilapia treated with 0 and 0.2 mg/L Cu for 0, 48, 72, 96, 120 and 144 h, respectively. Values are the mean ±SD ($n = 6$). Different superscript letters indicate a significant difference ($p < 0.05$) among times of treatment (ANOVA analysis with Tukey's comparison)

which protects against Cu-induced necrosis of MR cells (Bury et al. 1998) and is a stress indicator of heavy metal toxicity (Wu et al. 2006). In addition, cortisol stimulates the proliferation and differentiation of ion-transporting (MR) cells of the gills, as well as stimulating NKA expression within those cells (Seidelin et al. 1999). In this study, we found that cortisol increased, and the MR cell morphology changed, after 48–72 h of sub-lethal Cu^{2+} exposure. However, the density of MR cells significantly decreased and cortisol levels significantly increased after 96 h of 2 mg/L Cu^{2+} exposure. The present data suggest that the initial cortisol induction might be related to MR cell transformation in order to enhance ion uptake and protect against Cu-induced necrosis of MR cells. However, a further increase in the cortisol concentration at 96 h after 2 mg/L Cu^{2+} exposure may have been induced by Cu toxicity.

Morphological changes and the increase in density of MR cells induced by Cu in fish gills illustrate two types of response: defense and compensation (via cell proliferation or mucus secretion) (Cerqueira and Fernandes 2002). Both responses help to reduce the entry of toxicants and prevent damage caused by the direct effects of Cu. However, gills additionally function in gas exchange, ionic and osmotic regulation, and acid–base equilibrium; the histopathological

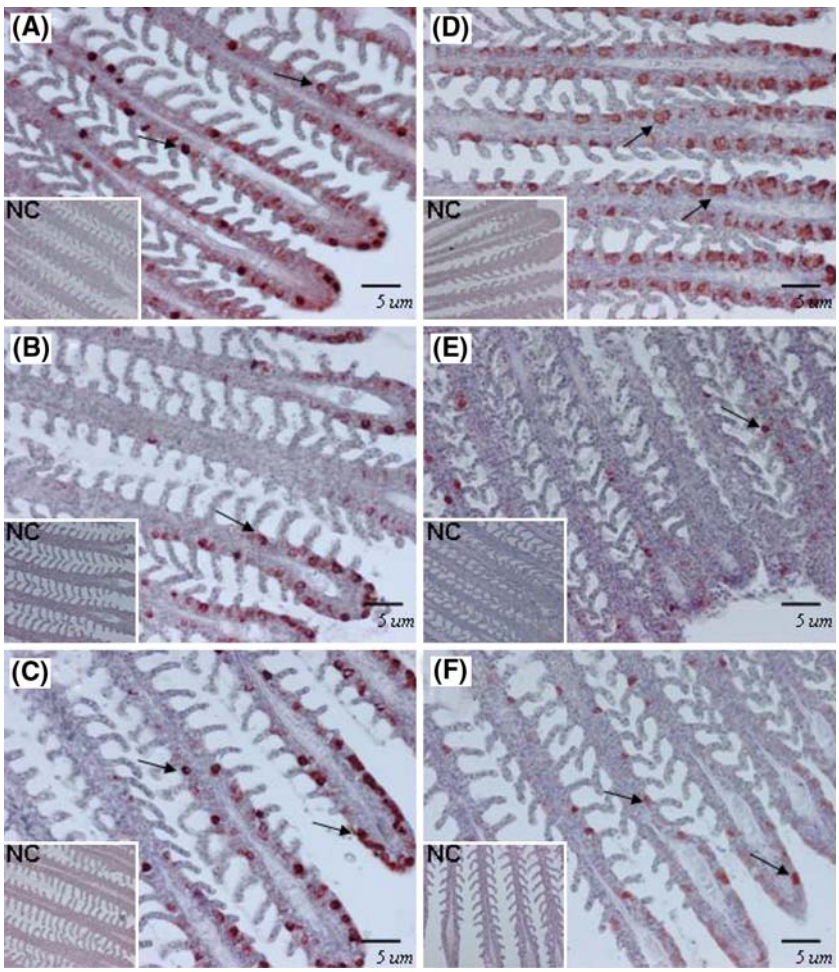

Fig. 5 Na^+-K^+-ATPase immunoreactive (NKAI) cells of gills in **(A)** control tilapia, and tilapia exposed to 0.2 mg/L Cu for **(B)** 48, **(C)** 72, **(D)** 96, **(E)** 120, and **(F)** 144 h. The negative control (NC) is presented in the lower left corner of each picture (insert). Magnification, ×200

Table 2 Changes in the whole-body cortisol concentration (ng/g body weight) following either 0.2 or 2 mg/L Cu exposure

Cu exposure dose	Duration of Cu exposure (h)				
	0	24	48	72	96
0.2 mg/L	10.3 ±2.9^{a}	14.4 ±6.0^{a}	40.3 ±9.3^{c}	28.4 ±5.0^{b}	18.1 ±4.4^{a}
2 mg/L	5.2 ±1.3^{a}	9.7 ±1.8^{ab}	10.2 ±4.9^{a}	10.1 ±4.9^{ab}	13.5 ±6.0^{b}

Values are the mean ±SD ($n = 10$). Different superscript letters indicate a significant difference ($p < 0.05$) among various times of either 0.2 or 2 mg/L Cu treatment by one-way ANOVA analysis with Tukey's comparison

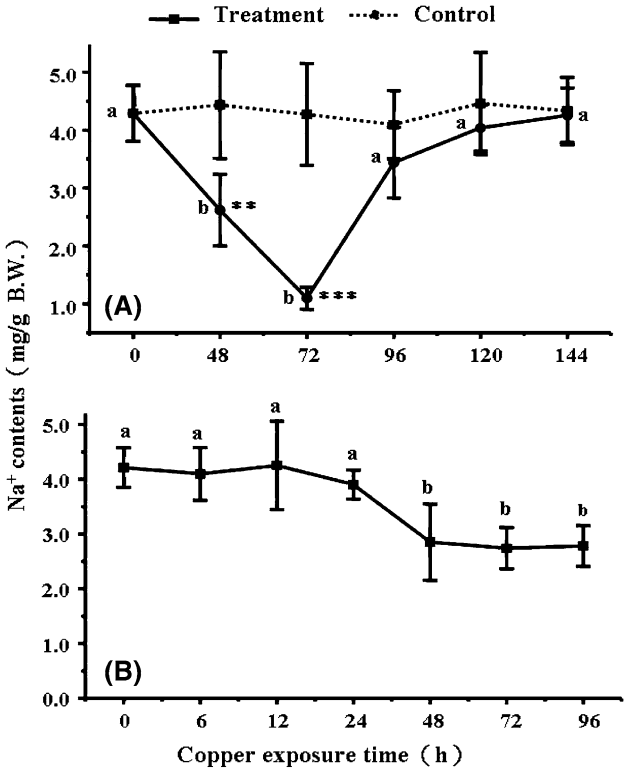

Fig. 6 Na^{+} concentration in tilapia juveniles treated with waterborne Cu (**A**) 0 and 0.2 mg/L for 0–144 h; (**B**) 0 and 2 mg/L for 0–96 h). Values are the mean ±SD ($n = 6$). Different superscript letters indicate a significant difference ($p < 0.05$) among times of treatment (ANOVA analysis with Tukey's comparison). ** ($p < 0.01$) and *** ($p < 0.001$) indicate a significant difference between the control and treatment groups at the same time point

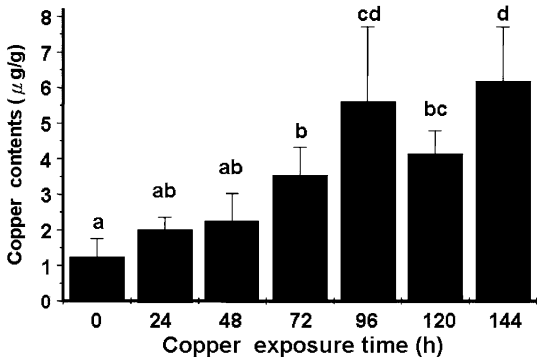

Fig. 7 Comparisons of Cu accumulation in juvenile tilapia treated with waterborne Cu concentrations (0 and 0.2 mg/L) for 0–144 h. Values are the mean ±SD ($n = 6$). Different superscript letters indicate a significant difference ($p < 0.05$) among times of treatment (ANOVA analysis with Tukey's comparison)

responses to Cu result in respiratory disturbances and electrolytic imbalances. MR cells malfunction due to direct Cu-mediated inhibition of NKA (Li et al. 1998) and Cu-induced necrosis and apoptosis of mature MR cells (Dang et al. 2000b). Although MR cell hyperplasia and hypertrophy following metal exposure are considered compensatory responses and help maintain ion balances, the increased MR cell turnover may result in increased subpopulations of newly emerged, necrotic and apoptotic MR cells, exhibiting low NKA density and activity per cell (Dang et al. 2000b), which could produce ion imbalances. NKA activity continued to decrease during a period of 48–72 h after sub-lethal Cu exposure, returned to control levels after 96 h of Cu exposure, and again decreased during 120–144 h of Cu exposure. It was noted that the density of MR cells was lower after 96 h of Cu exposure than the control (Table 1).

Previous studies reported significantly higher apoptotic MR cells at 96 h after 0.2 mg/L Cu^{2+} exposure in tilapia compared to the control, and a positive relationship appeared between the number of MR cells and NKA levels (Li et al. 1998). However, the present data showed an inverse relationship between MR cell density and NKA activity with 0.2 mg/L Cu^{2+} exposure at 96 h. We speculated that the experimental media contributed to the different responses between the studies. Local tap water used in the present study contained higher Na^{+} (1.55 mM) and Ca^{2+} (0.75 mM), which can help enhance the Cu resistance of fish (Wu et al. 2007). Lin and Hwang (2004) have reported that unexposed MR cells are functionally inactive. The present data showed that both the NKAI and NKA activities had recovered by 96 h, but they were reduced again during 120–144 h of Cu exposure. However, MR cells had almost completely disappeared by that time. Summarizing these results, we suggest that MR cells might contact the apical surface at 96 h after Cu exposure, and that MR cells might gradually undergo apoptosis during a period of 120–144 h of Cu exposure.

The three types of MR cells in freshwater tilapia are wavy convex, shallow basin, and deep hole (Lee et al. 1996). Their relative abundances were found to differentially vary when tilapia were acclimated to media of different Cl^- and Ca^{2+} compositions, leading to the suggestion that wavy-convex and shallow-basin MR cells are mainly responsible for the uptake of Na^+/Cl^- and Ca^{2+}, respectively (Lin and Hwang 2001). Furthermore, the dominant MR cell type in tilapia gills changes from deep-hole to wavy-convex within 6 h of acclimation to a low-Cl^- medium (Chang et al. 2003). It is thus evident that the MR cell type is very sensitive to ambient ions. In studies of tilapia (Pelgrom et al. 1995), flounder (Stagg and Shuttleworth 1982), and rainbow trout (Wilson and Taylor 1993), Na^+ and Cl^- concentrations were similarly affected by Cu exposure. In the present study, Na^+ and Cl^- concentrations recovered to control levels during 96–144 h of Cu exposure, and MR cells changed to a wavy-convex morphology after a period of 48–72 h of Cu exposure. This might have been an effort to manage Cl^- uptake enhancement, via morphological changes in MR cells. Additionally, the NKA activity appeared to have compensated at 96 h after Cu^{2+} exposure. The cortisol levels also significantly increased during 48–72 h. According to these results, cortisol might also be related to changes in MR cell morphology and protection of MR cells against necrosis during 48–72 h of Cu^{2+} exposure. Even though cortisol also increased at 96 h after 2 mg/L Cu^{2+} exposure, NKA activity still decreased and the MR cell morphology showed no change under that treatment condition. We suggest that the cortisol level rose at 96 h due to a stress response caused by Cu toxicity.

In most cases, Na^+ absorption across epithelia involves the diffusive entry of Na^+ into cells from the external medium through ion channels or protein carriers (facilitated diffusion) (Handy et al. 2002). In addition, branchial Na^+ uptake is the result of NKA-dependent Na^+ influx, and Na^+ efflux occurs via passive diffusional losses (Wood 1992) or via a Ca^{2+}/Na^+ exchanger on the basolateral membrane (Verbost et al. 1994). NKA extrudes intracellular Na^+ from the branchial epithelium into the blood, while H^+-ATPase in the apical membrane pumps protons out of cells, which increases Na^+ uptake. Both of these actions affect the electrochemical gradient between the external and internal environments (Pyle et al. 2003). To sum up those reports, whole-body Na^+ contents were affected by a primarily diffusive Ca^{2+}/Na^+ exchanger (Verbost et al. 1994), by H^+-adenosine triphosphatase (H^+-ATPase) activity in the apical membrane, by the electrochemical gradient, and most importantly, by gill permeability (Pyle et al. 2003). In the present study, the whole-body Na^+ contents were restored after 96 h, even though gill NKA concentrations still decreased during the period of 120–144 h of Cu exposure. Therefore, the data seems to demonstrate that Na^+ contents were restored by means other than NKA-dependent Na^+ influx.

There is some evidence to support the uptake of Cu^{2+} through Cu-specific channels or through Na^+ channels, because of its smaller ionic radius (Handy et al. 2002). Cu ions may inhibit basolateral NKA activity and Na^+ influx, resulting in a net loss of Na^+ (Handy et al. 2002). Grosell and Wood (2002) presented evidence of two high-affinity mechanisms for branchial Cu^{2+} uptake in gills of rainbow trout: one that directly competes for external Na^+ and another that is independent of external Na^+. Our previous studies have shown that the whole-body Na^+ concentration significantly increases between 24 and 72 h, with 0.05 and 0.03 mg/L Cu^{2+} exposures in tilapia larvae, but Cu^{2+} accumulation showed a steady state between 72 and 96 h of 0.1 mg/L Cu exposure (Wu et al. 2003). In tilapia larvae exposed to ambient hyper Na^+ with 0.075 mg/L Cu^{2+} after 48 h, both whole-body Na^+ and Cu^{2+} accumulations significantly increased compared with 0.075 mg/L Cu^{2+} exposure (Wu et al. 2007). In this study, the increase in the Cu^{2+} concentration appeared to be time dependent from 24 to 96 h of 0.2 mg/L Cu^{2+} exposure, then remained constant for the period of 96–144 h; Na^+ content was evidently inhibited by Cu^{2+} during 48–72 h, but was restored during 96–144 h of Cu^{2+} exposure. To sum up these experiments, Na^+ uptake was affected by an interaction of the Cu^{2+} treatment dose with the time course. It is likely that tilapia adapt to certain doses of Cu^{2+}, and compensate for Na^+ uptake in order to maintain normal physiological functions. Many studies support Cu accumulation being time and dose dependent, but it can reach a maximal capacity (Marr et al. 1996, Berntssen et al. 1999, Wu et al. 2003). The present study showed that Cu^{2+} accumulation did not increase during 96–144 h of Cu^{2+} exposure. It seems that juvenile tilapia achieve a capacity for Cu^{2+} accumulation during this time.

In conclusion, our data indicate that MR cells might be produced through membrane turnover, in order to increase Cl^- uptake, upon sub-lethal Cu^{2+} exposure. Also, cortisol may have several important functions in modulating the apical surface structure, as reflected in the density of MR cells and NKA activity compensation. Lastly, contrasting profiles were found for Cu^{2+} accumulation and Na^+ concentration during 24–72 h, while similar profiles appeared during 96–144 h after sub-lethal Cu^{2+} exposure. We suggest that Na^+ uptake competed with Cu^{2+} at the beginning of Cu^{2+} exposure, and later, juvenile tilapia achieved a capacity for Cu^{2+} accumulation and compensated for Na^+ uptake under these experimental conditions.

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