Acid-base balance, osmoregulation and hematological changes in tilapia (*Oreochromis niloticus*) after sublethal cadmium exposure

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Abstract

Tilapia exposed to two different Cd concentrations in freshwater had varying levels of Cd in their gills, with fish exposed to 2 mg/L of Cd having a higher Cd level than fish exposed to 1 mg/L of Cd. The higher Cd level exposure caused considerable metabolic acidosis (lower pH and higher pCO_2) and inhibited gill carbonic anhydrase. Fish exposed to higher Cd levels had lower osmolality, levels of plasma Cl^- and K^+ but constant levels of Na^+. All hematological parameters tested in this study, including red blood cells, hemoglobin, hematocrit, and mean cellular hemoglobin concentration hematocrit, decreased as a result of increased Cd exposure. This is consistent with the pO_2 measurement, which indicates that fish exposed to greater levels of Cd had a considerable reduction in pO_2.

Introduction

Cadmium (Cd) is known as the non-element since it lacks essential nutritional properties such as Cu, Zn, Co, and Mn (McGeer et al. 2012). Cd is naturally found in water bodies in trace concentrations of around 0.003 mg/L; however, background levels are increasing, particularly in areas polluted by major industrial, agricultural, and mining activities, and have reached 2 mg/L. (Cao et al. 2012; Adesiyan et al. 2018). Cd rapidly bioaccumulates and bioconcentrates in all tissues and organs of aquatic species, with relatively high levels in the liver, kidney, and gills and substantially lower levels in muscle tissue (Heydarnejad et al. 2013; Soegianto et al. 2022a).

Fish exposed to waterborne Cd may experience a variety of deleterious effects, including changes in respiration (Shaffı et al. 2001), decreased growth (Okorie et al. 2014), alteration in plasma ion regulation (McGeer et al. 2000), hematological abnormalities (Adhim et al. 2017; Handayani et al. 2020), and enzyme activity (Nursanti et al. 2017; Ma'rifah et al. 2019). There has been limited research on the effect of Cd on acid-base balance in fish. Previous studies reported that some heavy metals such as Zn, Cu and Pb can affect blood acid-base balance of rainbow trout (*Salmo gairdneri*) (Spry and Wood, 1985), cod (*Gadus morhua*) (Larsen et al. 1997), and groovy mullet (*Liza dumerili*) (Mzimela et al. 2002). Other forms of inorganic pollution, such as nitrite, increased blood nitrite, methaemoglobin, and oxygen partial pressure (pO_2) in European eels (*Anguilla anguilla*), although blood pH, pCO_2, and HCO_3^- were negatively correlated to nitrite concentrations in media (Huang and Chen 2002). Following nitrite exposure, giant river prawn *Macrobrachium rosenbergii* showed an increase in haemolymph pO_2 and ammonia excretion as well as a reduction in haemolymph pH (Chen and Lee 1997). Alteration in acid-base balances and other hematological parameters induced by heavy metal and other pollution exposure occurred from a quick series of events in which hypoxemia, most likely caused by gill destruction, led in tissue hypoxia and a combined acidosis, both were lethal (Brauner and Rummer 2011).

All animal generates approximately the same quantity of CO_2 that O_2 consumes during metabolism. The blood transports O_2 from the environment to the tissues, while the tissues release CO_2 and the blood transports it back to the environment. The red blood cell (RBC) contains hemoglobin (Hb), which is

required for O_2 and CO_2 transport in the blood of all vertebrates (Brauner and Rummer 2011). Since acid–base compensation relies mostly on the direct movement of H^+ and HCO_3^- through the gill in exchange for Na^+ and Cl^-, acid–base regulation is also associated to ionic regulation in fish. In consequence, ensuring ionic and osmotic balance in fish requires regulation of NaCl transport through the gill. CO_2 excretion, ionic regulation, and acid–base balance is all regulated by carbonic anhydrase (CA) (Gilmour and Perry 2009). Many in vitro studies have indicated that heavy metals suppress CA activity in fish (Soyut and Beydemir 2012; Kaya et al. 2013; Caglayan et al. 2020; Kurici et al. 2021), but there has been very few in vivo research on the influence of heavy metals on fish CA.

The tilapia, *Oreochromis niloticus*, was selected as the experimental test species in this research because it is among the most commonly consumed freshwater fish in Indonesia and the world's second most popular fish after carps, according to the Food and Agriculture Organization. It can endure a variety of environmental conditions and is widespread in many areas of the country (Pabru et al. 2019; Handayani et al. 2020). Nonetheless, because tilapia farms utilize waters from rivers that are regularly contaminated by heavy metals from anthropogenic sources, the consequences of Cd in tilapias are a major issue. In order to investigate the physiological and hematological effects of sub-lethal Cd on *O. niloticus* reared in freshwater, we evaluated acid-base regulation, CA level, plasma osmolality, ions level, and blood parameters over a four-day exposure period.

Materials And Methods

Sampling protocol and laboratory acclimation

Tilapia *O. niloticus* (10.1 ± 0.7 cm total length) were purchased from a local farm located in Pasuruan, East Java, and brought to the laboratory in oxygenated waters. The fish were placed in 250 L holding tanks with dechlorinated tap water (Putranto et al. 2014) in the laboratory and maintained at 28–29°C for about 7 days with photoperiods of 12 h light and 12 h dark (Handayani et al. 2020). Water quality was maintained by constant circulation via a biological filter consisting gravel, sand, and sponge filters. Fish were fed commercially available fish feed on a daily basis during this time period. To maintain the water quality suitable for fish, feces, residual food not eaten by the fish, and other debris were siphoned out daily. Temperature, pH, and dissolved oxygen (DO) were measured daily with a glass thermometer, pH meter (Hanna HI 98150, Beijing, China), and DO meter (Lutron DO 5510, Taiwan). Temperature, pH, and DO concentrations during acclimation and experimentation were 28°C–29°C, 7.7–8.1, and 6.9–7.6 mg/L, respectively.

Cd stock solution

A Cd (1000 mg/L) stock solution was made by dissolving 2.744 g $Cd(NO_3)2.4H_2O$ (Merck, Darmstadt, Germany) in 1 L deionized water and placing it in borosilicate glass bottles. Based on the acute toxicity test LC50 value (7.5 mg/L) (Soegianto et al. 2022a), a control (no Cd in test media), 1 mg/L, and 2 mg/L

of Cd were chosen for the experiment These levels are likely to be experienced by fish in their natural environment (Cao et al. 2012).

Exposure experiment

Following acclimation, 30 healthy fish were chosen at random from the storage tank and divided into six tanks (n = 5 fish per tank). Each tank had 40 L of testing media, which included 1 mg/L of Cd (lower sublethal level), 2 mg/L of Cd (higher sublethal level), and the control (no Cd) at freshwater media. Each concentration had two tanks. The exposure lasted 96 hours. Due to the limited volume of blood, five fish from each treatment were chosen at random for acid-base and hematological parameters, and five other fish for plasma osmolality and ions level. Following the experiment, the Cd-containing waste water was disposed of in a metals waste tank. Experiments were carried out in accordance with the University's Institutional Animal Care standards and procedures.

Measurement of physiological and hematological parameters

The fish were sedated with a clove solution (200 mg/L) before blood collection since it had no detrimental impact on blood parameters (Mohseni et al. 2008). Each fish's blood sample was quickly taken from the caudal aorta using a 1 mm heparinized plastic syringe. An automated hematology analyzer (SFRI Blood Cell Counter 33, Jean d'Illac, France) was used to determine RBC count, hematocrit (Ht) level, hemoglobin (Hb) level, and mean cellular Hb concentration in blood samples (MCHC). The chemicals needed to run the hematology analyzer were provided by SFRI Corporation.

To evaluate blood pH, pCO_2, and pO_2, 50 µL blood sample was quickly injected into a sensor card (Techno Medica 091, Japan), then introduced into an automated blood gas analyzer (GASTAT-Navi, Japan), and pCO_2 and pO_2 were expressed in mmHg.

To measure the plasma osmolality and Na^+, Cl^-, and K^+ levels, blood samples were centrifuged at 5,000 rpm for 10 minutes at 4°C to obtain the plasma, then a 20 µL the plasma sample was determined using a Fiske® 210 Micro-Sample Osmometer (Norwood, MA, USA) and expressed in mOsm/kg. To assess the levels of Na^+, Cl^-, and K^+, 22 µL of plasma sample was analyzed using an electrolyte analyzer (SpotChem EL SE-1520, Kyoto, Japan) and expressed in mmol/L.

To eliminate any remaining blood, the dissected gills were properly washed in PBS (pH 7.4) and weighed. Gills were then homogenized in PBS on ice using a glass homogenizer and centrifuged at 2000–3000 rpm for about 20 minutes at 4^oC. CA was measured using a sandwich-ELISA according to the Bioassay Technology Laboratory Biotech Co. Ltd, Shanghai, China guidelines. The microtiter plates were all pre-coated with an anti-CA antibody. Each well received a 50 µL standard sample, a blank, and a 40 µL sample to determine the CA level. Immediately, 10 µL anti-CA antibody and 50 µL streptavidin-horseradish peroxidase were put to each well except the blank control, mixed thoroughly, covered with a plate sealer, and incubated at 37°C for 60 minutes. The plate was automatically aspired and washed 5 times with

wash buffer after the sealer was removed. Paper towels were used to clean the plate. The plates were then sealed with sealer and incubated at 37°C in the dark for about 10 minutes with 50 µL of substrate solution A and 50 µL of substrate solution B in each well. Each well received 50 µL of stop solution to cease the enzyme action. After the blue color quickly became yellow, the sample was measured with an automatic microplate reader (Bio-Rad, model iMark, Japan) at 450 nm within 10 minutes after applying the stop solution. The CA level was provided in ng/ml.

Measurement of Cd concentration in gills

The Cd level in the gills was determined using the Soegianto et al. (2022b) method. Each gill was crushed and dried in an oven at 65°C for 48 hours to get a constant weight.

0.5 g crushed tissue was decomposed for 4 hours at 90°C in 3 mL H_2NO_3 (Merck, Darmstadt, Germany). After cooling, the sample was filtered through Whatman filter paper (0.45 µm) with deionized water until it reached 50 mL. Cd concentrations were determined using an atomic absorption spectrophotometer (Shimadzu AA-7000, Tokyo, Japan), and the results were represented mg/kg dry weight. Analytical blanks were processed similarly to the samples, and concentrations were measured using a standard produced from the same acid solution. Cd measurement accuracy was validated using dogfish muscle reference material (DORM-4) provided by the National Research Council of Canada, with a Cd recovery of 106 percent and a detection limit of 0.008 mg/kg. All of the reagents used in this research were of high analytical quality.

Statistical analyses

The mean and standard deviation are used to express all data. Prior to statistical analysis, their normality was tested using the Kolmogorov–Smirnov test. IBM® SPSS® Statistics version 25 was used for all statistical analyses. To evaluate the effect of Cd on plasma osmolality, ion levels, blood and acid-base parameters, CA level, and Cd level in gills, data were statistically analyzed using one-way ANOVA followed by Tukey's HSD post hoc comparison test. When the p-value was less than 0.05, the difference was judged statistically significant.

Results

During the experiment, no fish died. Cd accumulation in the gills of fish exposed to sublethal concentrations of Cd (1 and 2 mg/L) for up to 4 days increases with increasing Cd in the medium (Fig. 1). The highest values were found in fish that had been exposed to 2 mg/L of Cd.

Blood pH and pO_2 levels in fish exposed to 1 mg/L Cd did not change substantially from controls. While blood pH and pO_2 were lower in fish exposed to 2 mg/L than in controls. In contrast, fish exposed to 2 mg/L Cd had the highest blood pCO_2 level. Gill CA levels were significantly lower after 4 days of Cd exposure compared to the control (Fig. 2).

Plasma osmolality in fish exposed to Cd at 1 mg/L did not differ significantly from the control. The level of plasma osmolality in fish exposed to 2 mg/L was lower than in controls. The concentration of Cl^- in the plasma of Cd-exposed fish was lower than in the control, but it did not differ significantly between fish exposed to 1 mg/L and 2 mg/L of Cd. Na^+ concentration in the plasma of Cd-exposed fish was not significantly different from that of the controls. However, K^+ concentrations in plasma significantly decreased in fish exposed to 1 and 2 mg/L of Cd (Fig. 3).

The RBC content, Ht and MCHC of fish exposed to 2 mg/L of Cd was lower than the control, while there was no difference between the RBC content, Ht and MCHC of fish administered to 1 mg/L of Cd and the control. Fish hemoglobin levels were lower in fish exposed to 1 and 2 mg/L of Cd than in controls, with the fish exposed to 2 mg/L having the lowest level. (Fig. 4).

Discussion

In this study, tilapia exposed to two different Cd concentrations in freshwater exhibited different levels of Cd in their gills, with fish exposed to 2 mg/L of Cd having a greater Cd level than fish exposed to 1 mg/L of Cd. The higher Cd level exposure resulted in a significant metabolic acidosis (decrease pH and increase pCO_2). Elevated pCO_2 in Cd-exposed fish might be due to increased barrier to gas diffusion by the injured epithelium of the gills, which is most likely the primary cause of decreased pO_2 (Spry and Wood 1984). Another cause might be that Cd inhibits gill CA, leading in a restriction in the ability of cells to convert CO_2 to HCO_3^- (Larsen et al. 1997). This is consistent with our findings, which indicated that CA levels in Cd-exposed fish were lower.

In general, heavy metals have a strong influence on the regulation of monovalent ions, causing net ion drop in hyperosmoregulating freshwater fish (Wilson and Taylor 1993; Adhim et al. 2017; Handayani et al. 2020) and net ion uptake in hypoosmoregulating marine fish (Stagg and Shuttleworth 1982). Fish exposed to Cd had a drop in plasma Cl^- while maintaining a consistent level of blood Na^+. It is probable that gill Cl^-/HCO_3^- exchange regulation is crucial in acid-base control during hypercapnia, whereas Na^+/H^+ exchange is minimal. Both freshwater and marine fishes seem to be relied on chloride-mediated processes rather than sodium-mediated mechanisms during hypercapnia (Larsen and Jensen 1997). Measuring Na^+ and Cl^- fluxes between animals and their surroundings has revealed that Na^+ fluxes are often small and quick, but Cl^- fluxes are higher and last longer (Goss et al. 1992). The impairment of the function of the branchial $Cl^-/HCO3^-$ exchanger could be due to heavy metal suppression of Na^+/K^+-ATPase in chloride cells (Larsen et al. 1997). Copper exposure resulted in a constant decrease in haemolymph osmotic and ionic concentrations of *Carcinus maenas*, possibly due to increased activity of Na^+/K^+ ATPase, stressing the enzyme's involvement in osmoregulation (Boitel and Truchot, 1990). The reduction in K^+ in Cd-exposed fish could be attributed to injured branchial epithelium, which affects gill ion permeability and consequently passive fluxes. Because fish gills are permeable to K^+, efflux outweighs influx. Lower K^+ uptake, rather than increasing K^+ loss, is a more relevant consequence,

according to Patridge and Lymbery (2008). Meanwhile, Nussey et al. (1995) proposed that the drop in plasma K^+ level was due to osmotic adaptation.

Other blood parameter changes were typically consistent with acidosis. The drop in plasma Cl^- might be attributed to a shift of Cl^- into RBCs as a result of the influence of lower plasma pH on the Donnan distribution for Cl^- and/or a penetration of Cl^- into the intracellular compartment to balance lactate outflow (Kurbel 2011). Turner et al. (1983) hypothesized such an exchange when they discovered that a rise in blood lactate concentration corresponded with a nearly comparable decline in plasma Cl^- in intensely exercised trout. Furthermore, the decrease in MCHC was associated with erythrocyte shrinkage. Large red cell shrinkage in higher Cd-exposed fish may have resulted in a significant decline in blood O_2-affinity, as seen by smaller carp red cells (Jensen, 1990). There is a possibility that blood O_2 transport will be disrupted.

All hematological parameters examined in this study, such as RBC, HB, Ht, and MCHC reduced as a result of higher Cd exposure. This indicates that the erythrocytes are disintegrating or that the haemopoietic system is deteriorating (Svobodova et al., 1994). A decrease in RBC counts, Hb and Ht has been also demonstrated in *Oreochromis aureus* (Allen, 1993), *Clarias gariepinus* (Olanike et al., 2008), and *O. niloticus* (Adhim et al., 2017) after exposed to different level of Pb, and in *O. niloticus* after Hg exposure (Handayani et al., 2020). A considerable reduction in the number of RBC revealed that Cd may damage RBC throughout the circulating erythrocytes process (Heath, 1995). According to Al-Rudainy (2015), the decrease in Hb level in fish could possibly be due to heavy metals inhibiting the enzyme system responsible for Hb synthesis. The decrease in RBC, together with the decreases in Hb and Ht, indicates that *O. niloticus* was subjected to anaemic conditions or haemodilution. This is consistent with the pO_2 measurement, which shows a significant decrease in pO_2 in fish exposed to higher levels of Cd. The capacity of fish to provide adequate oxygen to tissues is considerably diminished under this state, resulting in decreased physical activity (Wepener et al. 1992; Nussey et al. 1992).

Conclusion

Cd accumulation in the gills of fish exposed to sublethal Cd concentrations increases as the Cd content in the medium increases. This accumulation has a significant impact on acid-base balance parameters, osmotic and ionic regulation, and blood parameters, particularly in fish exposed to higher levels of Cd. Cd-exposed fish may experience a significant decrease in blood O_2-affinity, disruption of blood O_2 transport, and consequently a significant loss in the fish's ability to provide sufficient oxygen to tissues, as well as a decrease in physical activity.

Declarations

Competing interests

The authors stated that they do not have any conflicting interests.

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Figures

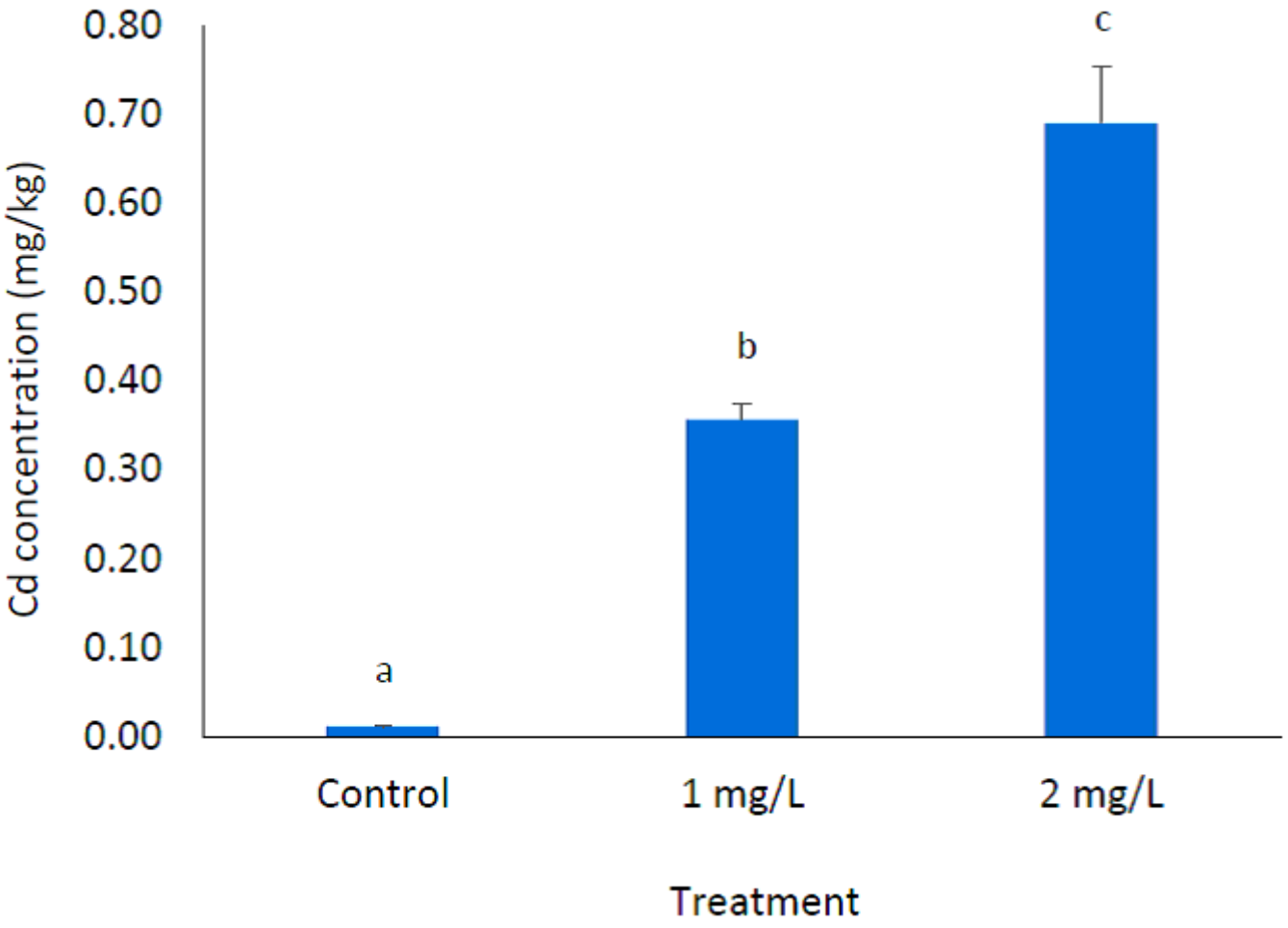

Figure 1

Cd concentration in fish gills exposed to various Cd concentrations in medium.

Different letters represent a statistically significant difference ($p < 0.05$).

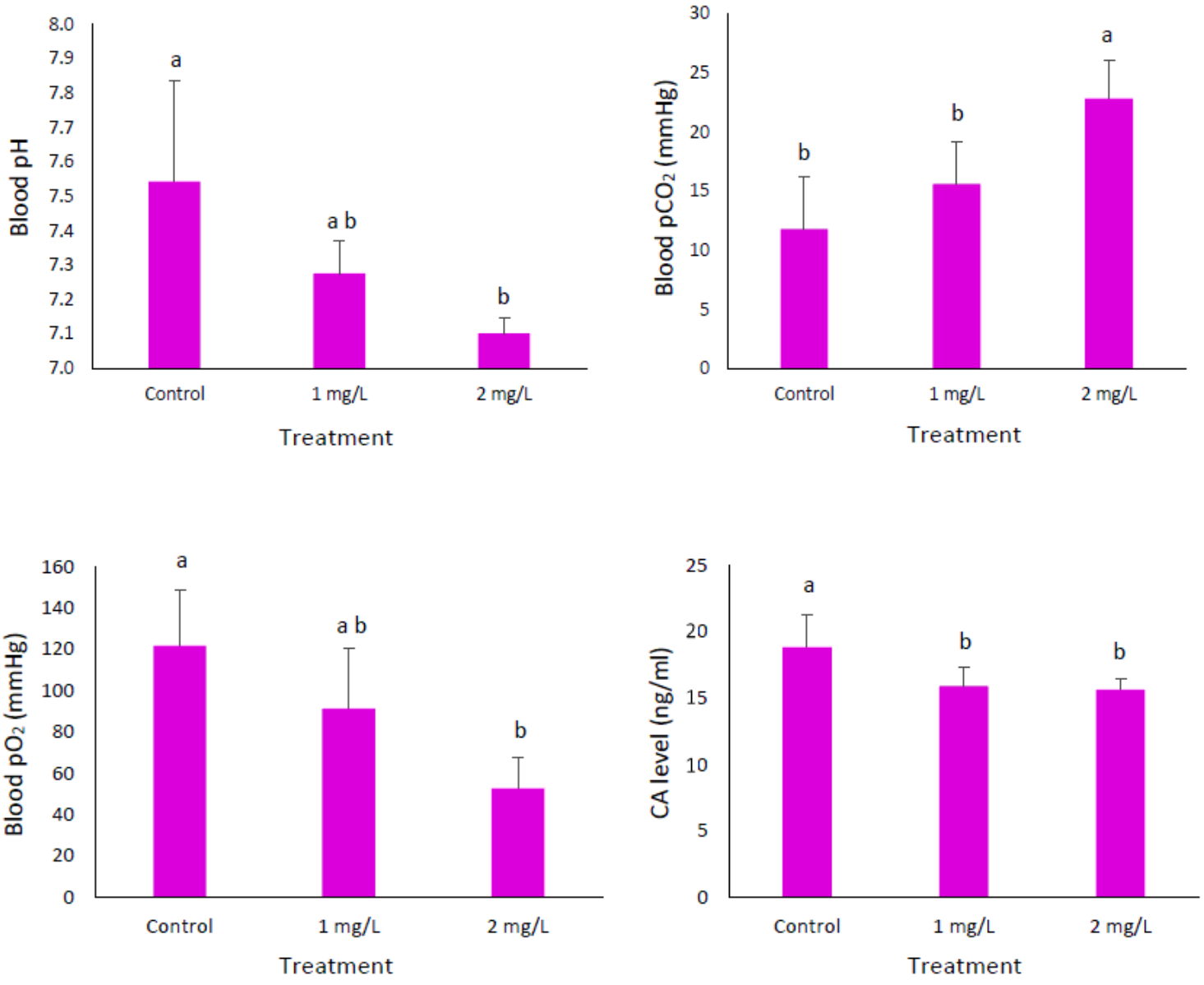

Figure 2

Blood pH, pCO_2, pO_2, and CA levels in fish exposed to varied concentrations of Cd in the medium. A statistically significant difference ($p < 0.05$) is indicated by a different letter.

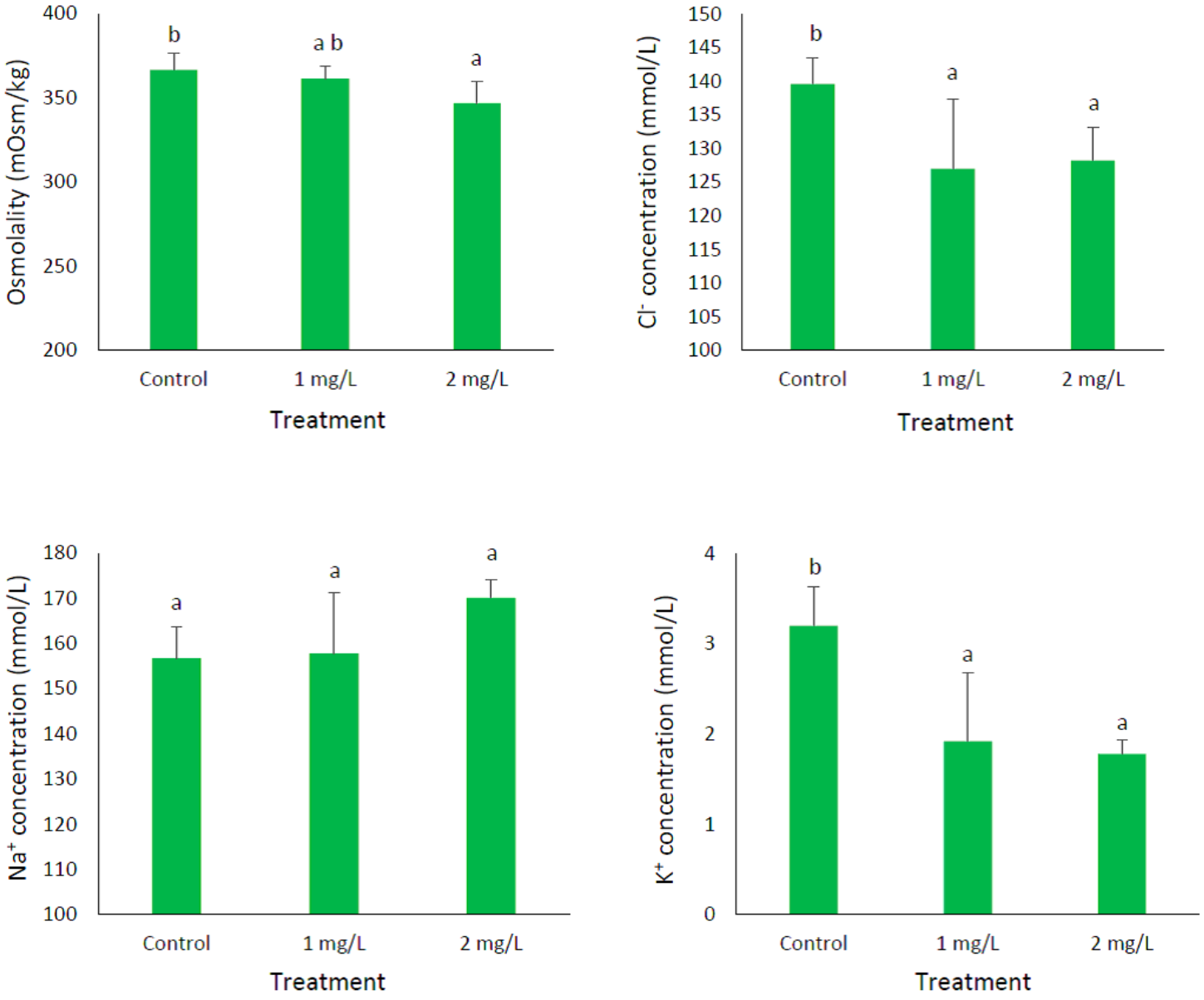

Figure 3

Osmolality and ion (Cl^-, Na^+, and K^+) levels in fish exposed to different levels of Cd in the medium. A statistically significant difference ($p < 0.05$) is represented by different letters.

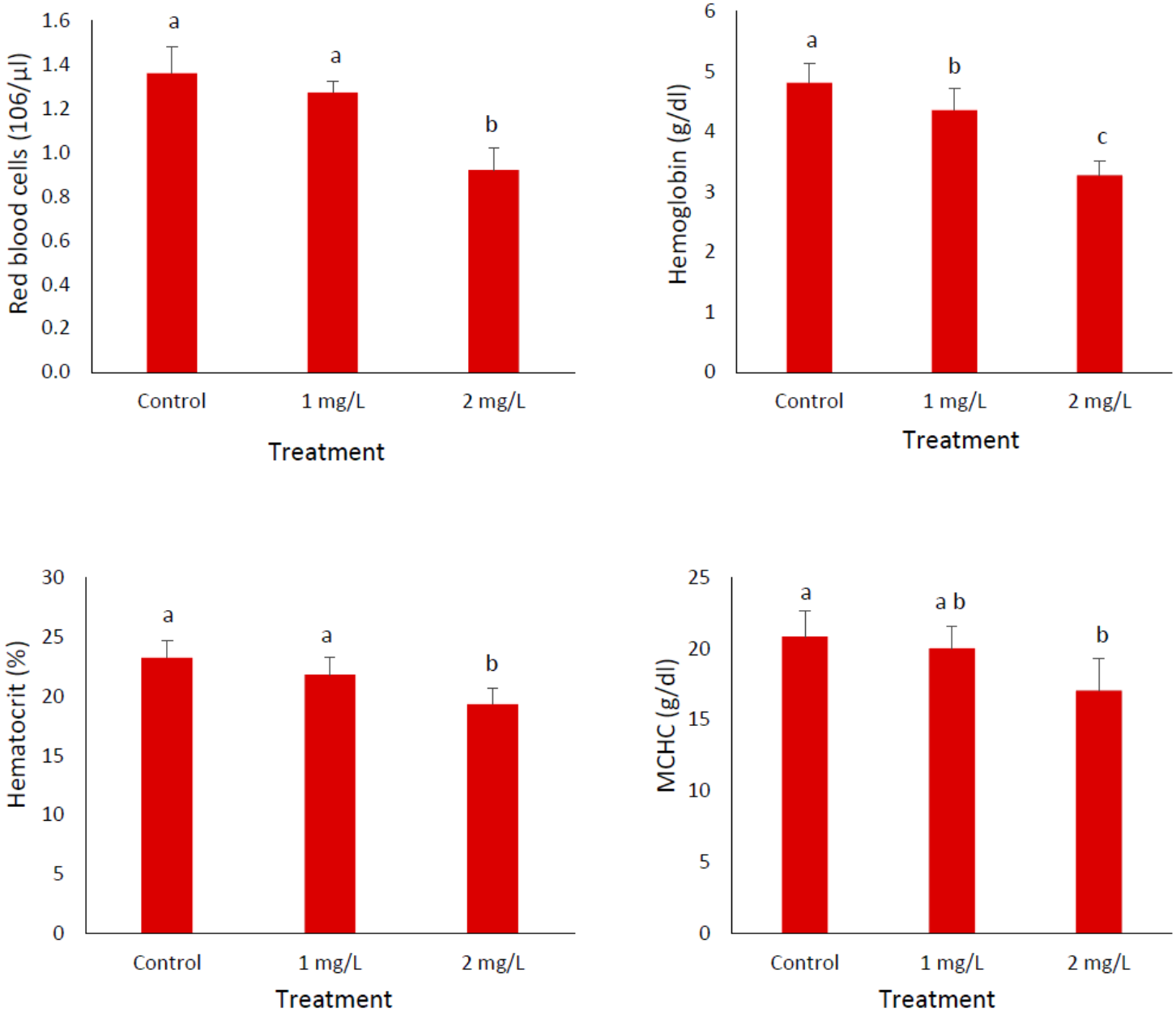

Figure 4

Red blood cells, hemoglobin, hematocrit and MCHC in fish exposed to varying levels of Cd in the medium. A statistically significant difference ($p < 0.05$) is marked with a different letter.