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Hematological changes following copper and zinc manipulations in the common carp (*Cyprinus carpio*) diet

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Abstract: Measurement of hematological parameters of fish exposed to toxicant are used to predict the toxic effects of toxicant. Experimental carps weighing 55.49±6.09g divided into 9 different groups contains 12 fish, were fed daily at 3% of their body weight for 8 continuous weeks. The diets contained copper as sulfate salt (25.18 or 38.15mg Cu/kg diet) and zinc as chloride salt (96.09 or 120.28mg Zn/kg diet) and control group was fed with diet containing no additional copper or zinc. A full factorial combination of above treatments (3×3) was performed. At the end of experiment, five fish were randomly withdrawn from each tank and blood samples were taken from the caudal peduncle vein. The blood was used for determination of Hb (Hemoglobin), Hct (Hematocrit) and RBC (Red Blood Cell) count. Erythrocyte indices, including MCV, MCH and MCHC were also calculated. Our results showed that manipulation of copper and zinc contents in *Cyprinus carpio* diet induce changes in some hematological parameters. The RBC, Hb and MCHC showed a significant decrease in single zinc-treatments ($P<0.05$). There was a significant difference among RBC count and MCHC of single copper-treatments and control group ($P<0.05$). There was significant differences between RBC, MCV, MCH and MCHC indices of combined treatments and single ($P<0.05$). No changes in Hct levels was observed ($P>0.05$). Changes in the amounts of copper and zinc in diet resulted in altering hematological parameters in carp. Although copper and zinc are essential trace elements for fish, it needs to be carefully used as a supplement, because its high concentration in diet may reveal some adverse effects.

Keywords: *Cyprinus carpio*, Hematocrit, Hemoglobin, Trace elements.

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Introduction

Many essential trace elements such as zinc (Zn^{2+}) and copper (Cu^{2+}) are required for fish. Cu^{2+} has a variety of biological functions, including brain neuro-transmitters and collagen synthesis (Davis & Mertz 1987; Lall 2002). Furthermore, Cu^{2+} is a vital component of several enzymes (e.g., cytochrome oxidase and superoxide dismutase) that are involved in oxidation–reduction reactions. Zn^{2+} serves essential structural, catalytic and regulatory functions in many biological systems (Eide 2006; Maret & KrEl 2007). Zn^{2+} is also, an integral part of metaloenzymes, such as carbonic anhydrase and alkaline phosphatase, which undertakes its role in regulating many processes of carbohydrate, lipid and protein metabolisms (Watanabe et al. 1997).

Reduction in Cu^{2+} concentrations in blood and liver following dietary Zn^{2+} (Fischer et al. 1981) led to suppress Hb and Hct levels (Hamilton et al. 1979) and reduce the activities of Cu^{2+}-requiring metalloenzymes (L'Abbe & Fischer 1984). Similar changes were observed in the case of Hb (Knox et al. 1982) and Hct (Knox et al. 1982, 1984) in rainbow trout (*Oncorhynchus mykiss*) fed high levels of Zn^{2+}.

In fish, exposure to heavy metals can induce either increase or decrease in the levels of blood parameters. Hematological indices like Hb content, Hct, RBC, white blood cell (WBC) count, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular volume (MCV) may change in fish after exposure to heavy metals (Javed & Usmani 2014). Blood parameters are considered as a significant tool in evaluating fish health (Pimpao et al. 2007). Therefore, the contents of Cu^{2+} and Zn^{2+} (as trace elements playing important roles in hematopoiesis) in diet of common carp (*Cyprinus carpio*) were manipulated in order to investigate their effects on some hematological parameters.

Material and Methods

Experimental design: A feeding trial of 8 weeks was conducted during February to March in the laboratory of Isfahan University of Technology, Isfahan, Iran. Juvenile common carps were obtained from a local commercial hatchery and were kept in a 300L well-aerated tank. The fish were acclimated for two weeks prior to experiment. A total of 108 fish with 55.49±6.09g for body weight and 15.53±1.9cm for total length were randomly divided into 9 circular tanks (300L), including 12 fish in each tank. The basic diet contained a proximate composition of 35% protein, 10% moisture, 8.2% lipid, 9.1% ash. Nine experimental diets containing 25.18 or 38.15mg Cu/kg and 96.09 or 120.28mg Zn/kg in a completely factorial design were prepared by adding appropriate amounts of Cu^{2+} ($CuSO_4.5H_2O$- Scharlau Barcelona, Spain) and Zn^{2+} ($ZnCl_2$, Anhydrous- Scharlau Barcelona, Spain). Possible interactions between dietary Cu^{2+} and Zn^{2+} in *C. carpio* were investigated with a 3×3 factorial design (Table 1). Fish were fed on a dry matter basis at a rate of 3% body weight daily.

Table 1. Experimental diets of common carp (Mean±SD).

Treatments	Zn^{2+} (mg/kg DW)	Cu^{2+} (mg/kg DW)
Control	68.89±0.005	18.23±0.004
Zn1	96.09±0.005	19.72±0.001
Zn2	120.28±0.006	17.91±0.003
Cu1	69.36±0.009	25.18±0.007
Cu2	73.03±0.015	38.15±0.006
Zn1+Cu1	93.59±0.005	23.23±0.002
Zn1+Cu2	93.56±0.02	40.16±0.002
Zn2+Cu1	116.87±0.006	24.88±0.003
Zn2+Cu2	115.29±0.016	35.36±0.003

Water quality parameters: Water quality parameters were monitored during the trial period (APHA 1998; Table 2).

Statistical analysis: Analyze of Variance (two-way ANOVA) was applied to determined simple and interaction of each variable. Complimentary Duncan's multiple range tests was used to indicate any significant differences if *P*-value was lower than 5%. All analyses were performed by using SPSS 18 software. Data are presented as mean±standard deviation (SD).

Sample collection: At the end of experiment, five fish were withdrawn from each tank randomly (totally 45 fish) and 2ml of blood was collected from the caudal peduncle region with heparinized syringes (sodium citrate, 10%) caudal vein. The blood was used for the estimation of Hb, Hct, RBC count and Erythrocyte indices.

Hematological parameters: RBC was counted by Thoma counting chamber. Hb concentrations were determined by using Cyanmethaemoglobin method (Drabkin 1964) and Hct was determined by the microhematocrit set (Unipan set, type 346, Poland). Erythrocyte indices, including MCV, MCH and MCHC were also calculated according to Saravanan et al. (2011) as follow:

$$MCV\ (fl) = \frac{Hct}{RBC} \times 10$$

$$MCH\ (pg) = \frac{Hb}{RBC} \times 10$$

Table 2. Water quality parameters.

Water temperature (˚C)	pH	Dissolved oxygen (mg/L)	Total hardness (mg/L $CaCO_3$)	Ammonia (mg/L)	Nitrite/Nitrate (mg/L)
18–22	7.8–8.4	6–7.3	480-520	228–245	0.3–1.9, 7.4–9.6

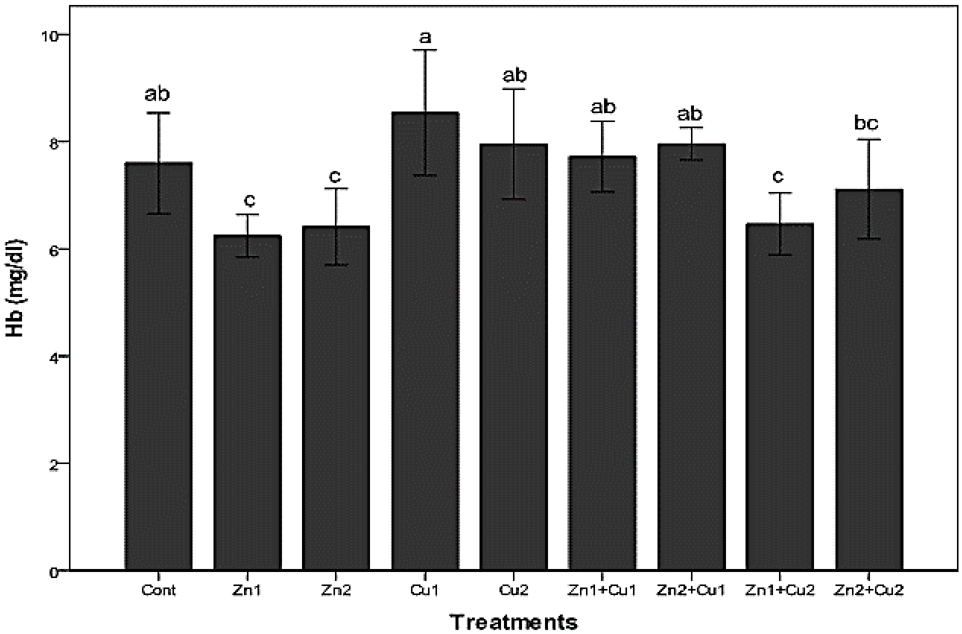

Fig.1. Hemoglobin (Hb) in the common carp after 56 days feeding with diets containing varying concentrations of Cu^{2+} and Zn^{2+}. Values are mean± SD (n=5) and values within the same column with different letters are significantly different (P<0.05).

$$MCHC\ (mg.dl^{-1}) = \frac{Hb}{Hct} \times 100$$

Results

Addition of Zn^{2+} in the diet (96.09 and 120.28mg Zn/kg diet) led to a significant reduction in Hb content by 17.79 and 15.55% for Zn1 and Zn2, respectively, in comparison with the control group (P<0.05). The Zn1+Cu2 treatment led to a decrease in Hb content of the fish by 18.63% in comparison with Cu2 group (P<0.05, Fig.1). The data presented in Figure 2 showed that changes in Cu^{2+} and Zn^{2+} contents of diet for 8 weeks did not led to a significant change in Hct percentage of the fish in all treatments.

RBC content showed a significant decrease in single Zn^{2+}-treatments (Zn1 and Zn2) in comparison with control group (1.27 and 1.77 vs. 2.43, P<0.05). Cu^{2+}-treated fish (Cu1 and Cu2 containing 69.36 and 73.03mg Cu/kg diet respectively) led to a significant decrease (P<0.05) in RBC content in comparison with untreated control group. RBC content in the combination treatments, showed an elevation when compared with Cu1 group (37.33 and 39.75% for Zn1+Cu1 and Zn2+Cu1 respectively, P<0.05). Also, there were significant elevations in Zn1+Cu2 and Zn2+Cu2 treatments when compared with Cu2 group (P<0.05, Fig.3).

In hematological indices, MCV content was obviously higher in fish were fed with both single Zn^2+ diets than control group (88.47 and 36.86% in Zn1 and Zn2 respectively, P<0.05). MCV content showed a significant reduction in Cu1-treated fish in comparison with control group (154.98fl, P<0.05). Zn1+Cu1 and Zn2+Cu1 treatments lead to the significant reduction of MCV content in comparison with Cu1 group (by 32.50 and 28.57%, respectively, P<0.05). Moreover, MCV content in Zn1+Cu2 treatment was lower than Cu2 group (129.18 vs. 163fl, Fig. 4). MCH content showed a significant increase in Zn^{2+} and Cu^{2+} single-treatments in

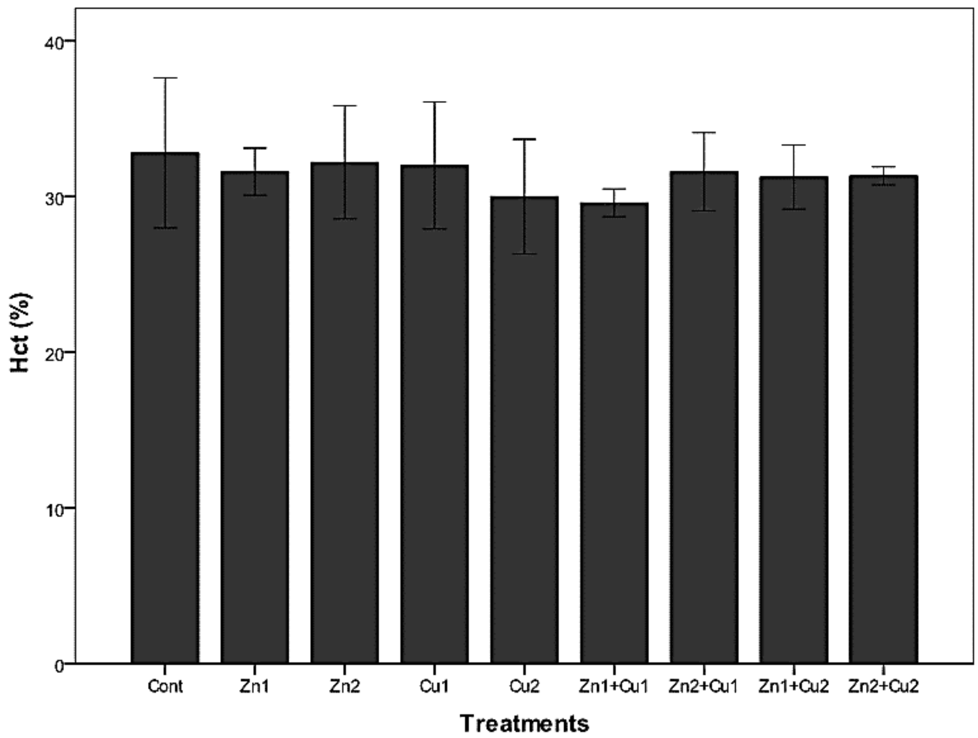

Fig.2. Hematocrit (Hct) in the common carp after 56 days feeding with diets containing varying concentrations of Cu^{2+} and Zn^{2+}. All means and SDs was calculated from 5 random samples.

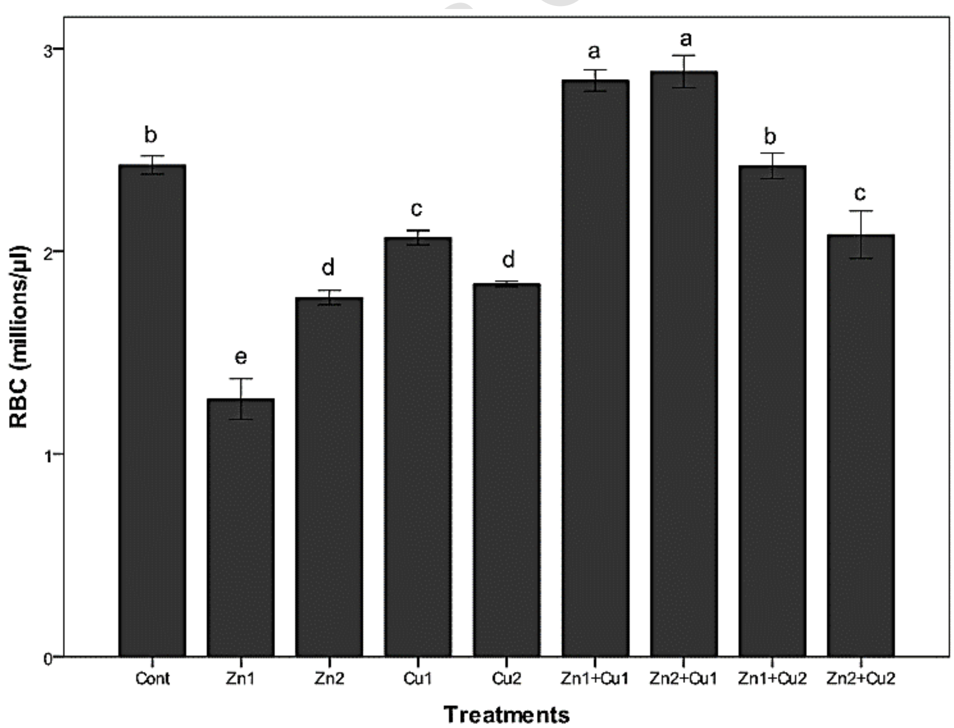

Fig.3. Red Blood Cell (RBC) in the common carp after 8 weeks feeding with diets containing varying concentrations of Cu^{2+} and Zn^{2+} has been shown. Values are mean±SD (n=5) and values within the same column with different letters are significantly different (P<0.05).

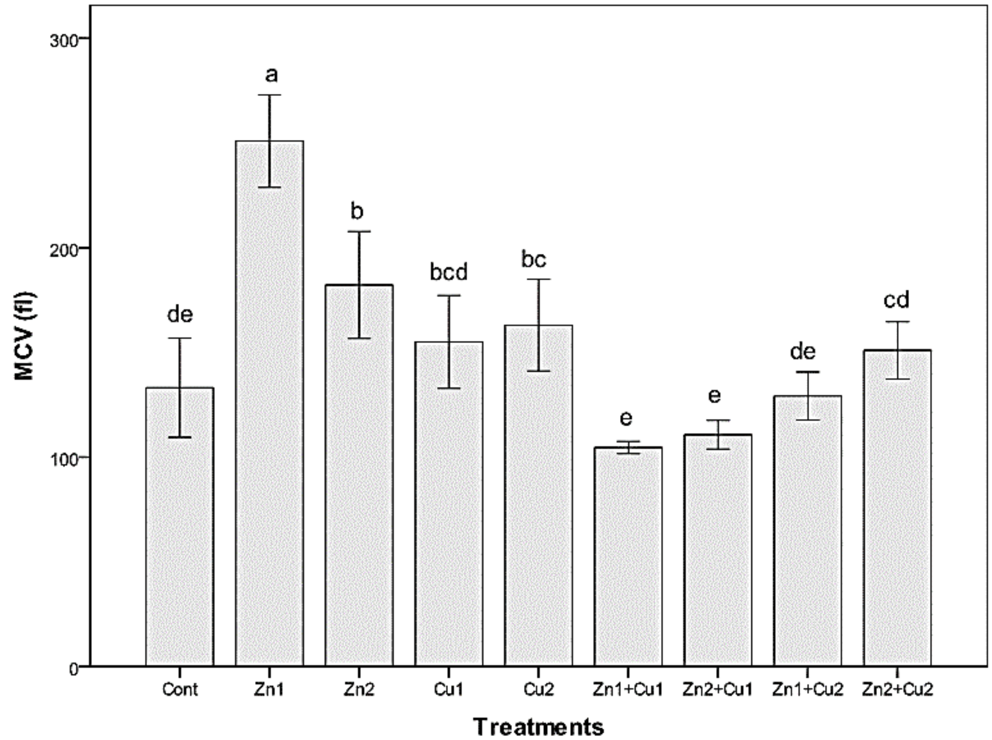

Fig.4. Mean corpuscular volume (MCV) in the common carp after 56 days feeding with diets containing varying concentrations of Cu^{2+} and Zn^{2+}. All means and SDs was calculated from 5 random samples.

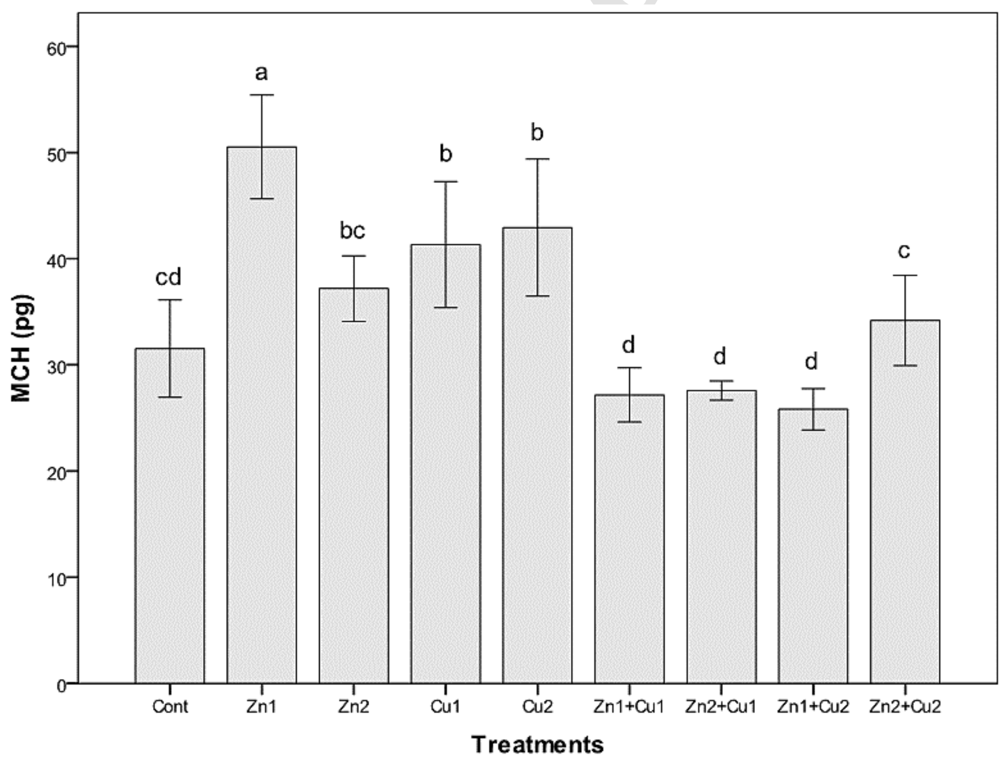

Fig.5. Mean corpuscular hemoglobin (MCH) in the common carp after 56 days feeding with diets containing varying concentrations of Cu^{2+} and Zn^{2+} has been shown. Values are mean±SD (n=5) and values within the same column with different letters are significantly different (P<0.05).

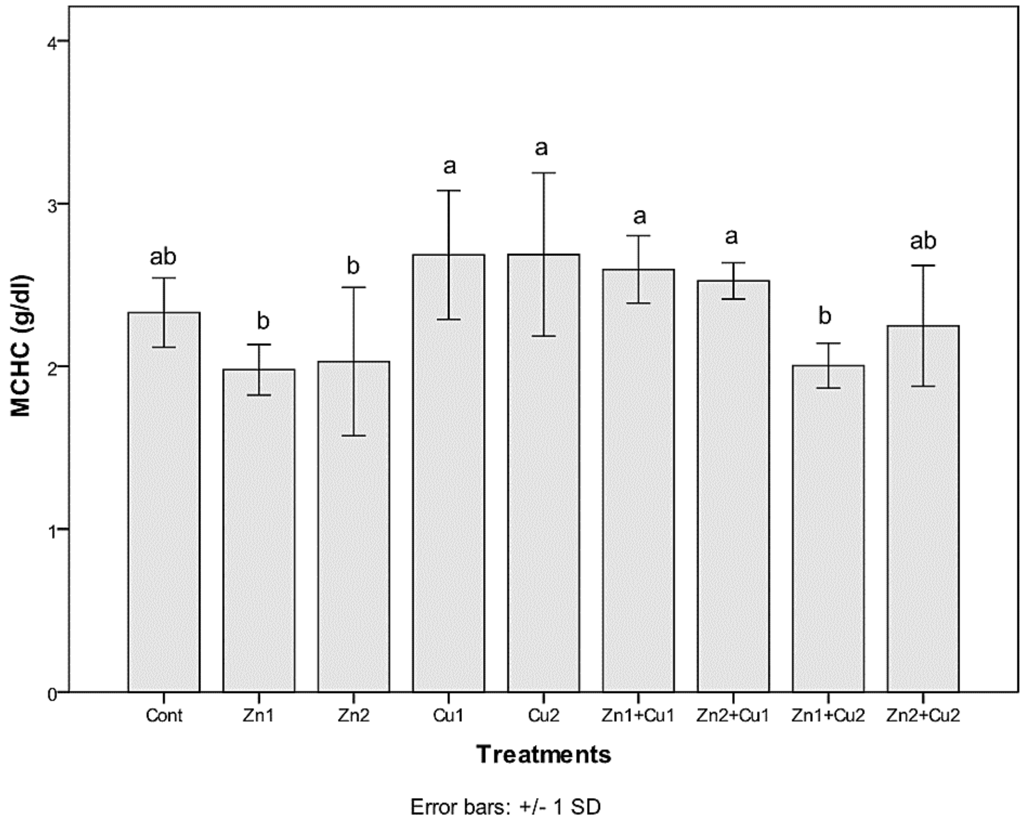

Fig.6. Mean corpuscular hemoglobin concentration (MCHC) in the common carp after feeding with diets containing varying concentrations of Cu^{2+} and Zn^{2+} has been shown. All means and SDs was calculated from 5 random samples.

comparison with control group (50.52, 37.18, 41.32 and 42.92pg, respectively in Zn^{2+} and Cu^{2+} treatments). MCH content in control group was similar to combination-treatments but was significantly (*P*<0.05) lower than the single treatments (Fig. 5). The MCHC content in Zn1+Cu1 was significantly higher than Zn1-treatment (2.59 vs. 1.98g/dl, *P*<0.05). MCHC content decreased significantly in Zn1+Cu2 treatments fish in comparison with Cu2 group (2 vs. 2.69, *P*<0.05, Fig. 6).

Discussion

Our results showed that manipulation of Cu^{2+} and Zn^{2+} contents in carp diet affects some of hematological parameters. Significant decreases in RBC, Hb and MCHC were observed following single Zn^{2+}-treatments (*P*<0.05). There were significant differences among RBC count and MCHC of single Cu2+-treatments with control group (*P*<0.05). There were significant differences between RBC, MCV, MCH and MCHC indices of combined treatments and single Cu^{2+} treatments (*P*<0.05). No changes in Hct percentage was observed (P>0.05).

The exposure to Cu^{2+} induces deteriorations in blood parameters in aquatic species, including *Colisa fasciatus* (Mishra & Srivastava 1980), *Hetero-pneustes fossilis* (Singh & Reddy 1990), *O. mykiss* (Vosyliene 1996), *C. carpio* and *Salvelinus fontinalis* (Witeska 2005) and *Prochilodus lineatus* (Caravalho & Fernandes 2006), characterizing by an increase on the Hb concentration as well as increase in RBC count. Moreover, according to other authors, in *C. carpio* (Svobodova et al. 1994), *Clarias lazera* and *Tilapia zilli* (Hilmy et al. 1987) and *Oreochromis mossambicus* (Celik et al. 2013) exposure to Zn^{2+} show that significant increases in RBC content, Hct and Hb. Also, increase in RBC number and Hct levels were reported in *Mystus vittatus* exposed to sub-lethal and lethal concentrations of Cu^{2+} and Zn^{2+}

(Singh & Singh 1982). This fact may be attributed to a compensatory effect in reaction to oxygen transport capacity (Mazon et al. 2002).

Azarin et al. (2012) demonstrated the negative effect of Cu^{2+} on hematology (Hb, RBC and MCH) of *Rutilus frisii kutum* (*P*<0.05). Also, Thangam et al. (2014) reported decrease in the RBC, WBC and Hb in carp when exposed to Cu^{2+}. Exposures to contaminants may reduce the Hb concentration in fish, could be occurred due to the inhibitory effect of such toxic substances on the function of enzyme, responsible for biosynthesis of Hb (Pamila et al. 1991; Vutukuru 2005).

Likewise, the number of RBC in *Colossoma macropomum* and *Channa punctatus* was reduced following Cu^{2+} treatment (Griffin et al. 1999). Increase in RBC size maybe associated with several parameters (anesthesia, hypoxia and etc.) and it is generally considered as a response against stress. Since metals cause changes on blood gases, the swelling of erythrocyte could also be involved in the reaction of fish against heavy metals. These alterations may be attributed to the damage that Cu^{2+} causes in gills and hematopoietic organs (Mazon et al. 2002).

Heavy metal caused significant decrease in the RBC, Hb concentration of fish due to impaired intestinal absorption of iron (Singh et al. 2008). Anemia, under Cu^{2+} induced stress, probably due to blood cell damage and disrupted Hb synthesis. The anemia in fish may be induced by significant decrease in number of erythrocytes (Joshi et al. 2002).

Blood cell indices like mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration seem to be changes that are more sensitive and can cause reversible changes in the homeostatic system of fish. Fluctuations in these indices correspond with values of RBC count, hemoglobin concentration (Vasantharaja et al. 2012). Earlier works also reported a fall in RBC count and hemoglobin percent and decrease in MCH, MCHC and MCV in freshwater fishes exposed to Zn^{2+} (Sen et al. 1992). Singh et al. (2008) evaluated the hematologic data after exposure to Cu^{2+} in *C. punctatus* and reported a significant increase in the MCHC, MCH and MCV indices (*P*<0.05).

Celik et al. (2013) demonstrated the negative effect of Zn^{2+} on hematology of *O. mossambicus*. In all groups, an increase in MCV, MCH and MCHC values observed in medium and high concentrations (*P*<0.05). The significant change in the MCH, MCV and MCHC may be due to the reduction in cellular blood iron, resulting in reduced oxygen carrying capacity of blood and eventually stimulating erythropoiesis.

Although, reports on Cu^{2+} and Zn^{2+} toxicities have shown significant varying effects on almost physiological systems of different fish species with different degree. The $CuSO_4$ and $ZnCl_2$ toxicity may also vary significantly between fish species due to other parameters such as fish size, exposure dose and time, species unique mechanisms for the metabolism of Cu^{2+} and Zn^{2+} (De Boeck et al. 2004), physiological situations of the individuals, water quality parameters and absorption of minerals from diets or water. Although Cu^{2+} and Zn^{2+} are essential elements to fish, our findings indicated that they need to be carefully used as additives to fish diet. When the Cu^{2+} and Zn^{2+} concentrations were out-of-range the tolerating level, fish may be affected acutely or chronically. Most changes in blood cells would cause interference in the fluid volume and ionic status (Tavares-Dias et al. 2011).

Blood parameters are therefore considered as patho-physiological indicators of the whole body and therefore, are useful to recognize the structural and functional status of fish exposed to toxicants (Adhikari et al. 2004). Thus, it is concluded that the hematological parameters are the most sensitive measurements in monitoring the toxicity of metal (Pimpao et al. 2007) and may be changes in hematological variables in response to metal exposure indicate ion regulatory or respiratory disturbances that imply an increase in energy consumption to restore homeostasis instead of other physiological functions and growth.

The hematopoietic system of fish is basically located in the head kidney and spleen. Therefore, a fall in the hematological factors may be attributed to the disfigurement of the hematopoietic system caused by morphological changes in those organs structure. Moreover, the changes in hematological parameters can be implied as a compensatory response that improves the oxygen carrying capacity to maintain the gas transfer and a change in water blood barrier for gas exchange in gill lamellae (Thangam et al. 2014). The study suggested that the presence of trace elements in diet has strong influence on the hematological parameters in the fresh water fish, i.e. common carp.

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تغییرات خون‌شناسی متعاقب دستکاری مس و روی جیره غذایی ماهی کپور معمولی (*Cyprinus carpio*)

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چکیده: اندازه‌گیری پارامترهای خون‌شناسی ماهی در معرض سموم برای پیش‌بینی اثرات این سموم استفاده می‌شود. ماهیان کپور معمولی با میانگین وزنی ۶/۰۹±۵۵/۴۹ گرم در ۹ گروه ۱۲ تایی تقسیم شدند. در طی ۸ هفته، غذادهی روزانه به میزان ۳ درصد وزن بدن در دو وعده انجام شد. از جیره‌های غذایی حاوی مس (یون مس با استفاده از نمک سولفات مس در دو سطح ۲۵/۱۸ یا ۳۸/۱۵ میلی‌گرم بر کیلوگرم)، روی (یون روی با استفاده از نمک کلرید روی در دو سطح ۹۶/۰۹ یا ۱۲۰/۲۸ میلی‌گرم بر کیلوگرم) و یک گروه به عنوان شاهد (بدون افزودن مس و روی) استفاده شد. تیمارهای ترکیبی مس و روی نیز به صورت فاکتوریل کامل ۳×۳ در نظر گرفته شد. در پایان آزمایش، تعداد پنج قطعه ماهی به‌طور تصادفی از هر مخزن صید شد و از محل ساقه دمی خونگیری به‌عمل آمد. خون به دست آمده برا ی تعیین میزان هموگلوبین، هماتوکریت و شمارش گلبول قرمز استفاده شد. شاخص‌های گلبولی (MCV, MCH و MCHC) با روش‌های متداول آزمایشگاهی مورد اندازه‌گیری قرار گرفت. نتایج نشان داد که دستکاری محتوای مس و روی جیره غذایی ماهی کپورمعمولی سبب تغییر در برخی پارامترهای خون‌شناسی می‌شود. کاهش معنی‌داری در میزان گلبول قرمز، هموگلوبین و MCHC تیمارهای مستقل روی مشاهده شد (P>۰/۰۵). میزان گلبول قرمز و MCHC تیمارهای مس مستقل با تیمار شاهد تفاوت معنی‌داری نشان داد (P>۰/۰۵). میزان گلبول قرمز، MCV, MCH وMCHC تیمارهای ترکیبی و مستقل تفاوت معنی‌داری داشت (P>۰/۰۵). هیچ‌گونه تغییری در میزان هماتوکریت مشاهده نشد (P>۰/۰۵). براساس نتایج حاصل از این مطالعه، تغییر در مقادیر مس و روی جیره غذایی سبب تغییر پارامترهای خون‌شناسی ماهی کپور می‌شود. بنابراین، اگرچه مس و روی عناصر کمیاب ضروری برای ماهی هستند، بایستی دقت کافی در استفاده از این عناصر به عنوان مکمل لحاظ گردد، زیرا غلظت بالای این عناصر می‌تواند اثرات مضر داشته باشد.

کلمات‌کلیدی: کپور معمولی، هماتوکریت، هموگلوبین، عناصر کمیاب.