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Effect of montmorillonite on arsenic accumulation in common carp

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The effect of montmorillonite (MMT) on dietary arsenic (As(III)) accumulation in tissues of common carp was investigated. Growth rates and survival do not appear to be sensitive indicators of dietary As(III) toxicity under lower exposure concentration. However, the toxicity increases as As(III) exposure concentration increase, and this can be alleviated with added MMT concentration. Exposure of common carp to dietary As(III) resulted in a significant As(III) accumulation in the tissues of common carp in the order: intestine>bone>gill>liver>muscle>brain ($P<0.05$), indicating accumulation of these tissues. When fed with MMT, there was a reduction in As(III) concentration of these tissues compared with the control group. The ratio of oxidized glutathione to reduced glutathione (GSSG/GSH) revealed a significant effect with MMT concentration (1.0%) ($p<0.05$) and post-hoc analysis also revealed that the group treated with MMT exhibited a decrease in the ratio of GSSG/GSH when compared to the fish not treated with MMT. Moreover, the addition of MMT to the diet with As(III) decreased metallothionein concentration. The added dietary MMT was therefore clearly protective against the bioaccumulation of As(III).

Key words: Montmorillonite, arsenic, common carp.

INTRODUCTION

Arsenic (As) is widely distributed in the environment and it is known to be highly toxic to humans. It is now well recognized that consumption of arsenic even at low levels may lead to carcinogenesis (Zhu et al., 2008). Owing to its toxic potential to humans, it is a high priority hazardous substance all over the world. Moreover, arsenic is found to be accumulative in freshwater fish. Thus, understanding the accumulation of arsenic in fish is important because of the concerns regarding human and wildlife exposure via consumption (Lewis et al., 2002).

Montmorillonite (MMT) is composed of units made up

of two silica tetrahedral sheets (T) with a central alumina octahedral sheet (O) of alumina, oxygen and hydroxyl, these units. It has a large specific surface area, high and replenished with H₂O and changeable irons between cation exchange capacity (Gao et al., 2006; Bhattacharyya and Gupta, 2008) and a high affinity to various heavy metal ions (Bhattacharyya and Gupta, 2008). Based on these characteristics, MMT has been widely used in biomedicine and clinical therapies (Fan et al., 2007; Guo et al., 2007). For example, MMT has been used to reduce toxin bioavailability in the intestinal tract (Abdel-Wahhab et al., 2005; Abbes et al., 2006b). MMT is also used as a food additive to enhance growth in livestock.

Our major objectives in this study were to evaluate the effect of the MMT on arsenic behaviors in common carp and to test the possible protective effect of MMT against

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As(III)-induced toxicity in the common carp.

MATERIALS AND METHODS

Common carp sampling

The omnivorous common carp is the most important fish species for fishery in China. Common carp for tests reported here were obtained from Jiaozhou Aquafarm of Qingdao. These were collected from a non-contaminated area. A group of fish ranging in mass from 20 to 40 g were kept in tanks with freshwater, which was exchanged partially every day, and the water temperature was maintained at 27 °C. They were fed commercial dry pellets (As, MMT-contaminated diet) for common carp at 1.5 to 2.5% of body weight two times a day during the experimental period. The parameters of the test water were as follows: pH 7.0 to 7.5, dissolved oxygen 5.5 to 7.0 mg/L.

After acclimatization, a total of 180 common carps were randomly allocated to four dietary treatments, each of which has three replicates of 15 fishes per tank. Common carp were treated with As(III) (0.1 and 2.0 mg/L), MMT (1.0%) and As(III) (2.0 mg/L) + MMT (1.0%) in 30 days, respectively. The fish were anesthetized with MS222 containing 100 mg/L in a plastic barrel for 1 to 2 min for sacrificing. Specimens were weighed, measured, and their intestine, bone, gills, liver, muscle and brain tissues were dissected as soon as possible in a sterile condition and the tissue samples were stored in liquid nitrogen after labeling. Tissues were transported to the laboratory in a Dewar flask. Samples were stored at -80 °C until assay preparations at the laboratory and designed control group in the experiments.

Growth performance and specific growth rates

Growth performance was analyzed in terms of daily mass gain (DWG) given as $DWG (g) = (\text{final body mass} - \text{Initial body mass}) / \text{rearing period (day)}$. Specific growth rates (SGR) expressed as a percent (%) per day were determined using linear regression of the natural logarithm of mean bulk weight versus time.

GSH and GSSG assay

The contents of reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined by the method of Hissin and Hilf (1976). About 0.2 g frozen liver was homogenized after addition of 2.0 ml of 1.0 mmol/L ethylenediaminetetraacetic acid (EDTA) and 20 ml perchloric acid (HClO_4), then the extractions were centrifuged in a refrigerated centrifuge at 15,804 g at 4 °C for 10 min for the measurement of reduced GSH levels. For the determination of GSSG levels, 0.5 ml original tissue supernatant was incubated at room temperature with 200 ml of 0.04 M N-ethylmaleimide for 30 min to interact with GSH present in the tissue. To this mixture, 4.3 ml of 0.1 M NaOH was added. A 100 ml mixture was used to assay GSSG, using the procedure described above for GSH measurement, except that 0.1 M NaOH was used as diluent rather than phosphate-EDTA buffer.

Metallothionein measurement

Aliquots of liver, intestine and muscle samples were homogenized in 3 volumes of Tris-HCl buffer (10 mM Tris-HCl, 86 mM NaCl, pH 7.4) at 4 °C with an Ultra-turrax T3 homogenizer. Tissue homogenates were centrifuged at 16,000 g for 20 min to isolate the cytosolic fraction (supernatant). Total metallothionein concentrations

in the cytosolic fraction were determined using the As(III)-chelex assay (Bartsch and Klein, 1989).

As (III), Cu and Zn analysis

Tissue samples (0.5 to 1.0 g) were digested with 2 ml 68% nitric acid, 1 ml 30% hydrogen peroxide and 2 ml ultrapure water in a CEM Mars 5 microwave accelerated reaction system, and then the resulting solution was made up to 25 ml with ultrapure water. Flow injection-hydride generation-atomic absorption spectrometry (FI-HG-AAS) was performed using a Model Analyst-700 spectrometer equipped with a Hewlett-Packard (Houston, TX) Vectra computer with GEM software, PerkinElmer EDL System-2, arsenic lamp (lamp current 380 mA), Cu and Zn concentration were determined by atomic absorption spectrophotometry with flame technique.

Statistical data

Tissue levels of As(III) were compared between groups by a one-way ANOVA. The post-ANOVA test of Neumann-Keuls was used to determine specific changes in tissue As(III) levels among groups of common carp. A two-way-factor ANOVA was used for metallothionein studies. All data are expressed as means \pm SEM. Differences were regarded as significant at $P < 0.05$.

RESULTS AND DISCUSSION

Effect of dietary MMT on As(III) in growth and survival of common carp

MMT has been used as food additive to improve feed manufacture and enhance the nutritive value of food (Hu et al., 2007). MMT (10 to 30 $\mu\text{g}/\text{kg}$) added in diet could promote the growth of chickens and swine (Venglovsky et al., 1999; Tauqir and Nawaz, 2001). Dietary As(III) concentration added in our study was selected to mimic environmentally relevant concentrations (2.0 to 23.26 mg/L) in terms of those found in vertebrates at both contaminated and uncontaminated sites in the environment. There was no significant difference ($P < 0.05$) in growth and survival between MMT and the control group in the present study as shown in Table 1. The reason might be attributed to low As(III) (0.1 mg/L) and MMT concentration (1.0%) and short exposure time (30 days). Xu et al. (2004) also found that growing pigs fed with 0.5% MMT-added diet for 83 days exhibited no growth promoting activity in growth. Based on the above results, common carp fed with 0.1 mg/L and MMT mixture showed similar growth performance to fish fed with As(III) only ($P > 0.05$). In conclusion, there were no effects on growth performance for common carp fed with the MMT-only, As(III)-only and As(III) and MMT diets when compared to the control group under lower concentration.

Dietary exposure at 2.0 mg As(III)/L resulted in significant effects on the growth and survival of common carp over 30 days. Treatment with 2.0 mg As(III)/L resulted in 6% mortality of the fish and significantly decreased body weight gain and survival, whereas these

Table 1. Mean weight, specific growth rates (SGR) and survival rate of common carp fed different As (β) and/or MMT diets for 30 days.

Treatment	Mean weight (g)	SGR (%/day)	Survival rate (%)
0 mg As (III)/L	35 \pm 3 ^a	1.53 \pm 0.11 ^b	100
0.1 mg As (III)/L	34 \pm 3 ^a	1.41 \pm 0.13 ^b	100
MMT (1.0%)	36 \pm 3 ^a	1.68 \pm 0.15 ^b	100
0 mg As (III)/L+ MMT (1.0%)	37 \pm 3 ^a	1.66 \pm 0.12 ^b	100
2.0 mg As (III)/L	33 \pm 2 ^a	1.32 \pm 0.08 ^b	94
2.0 mg As (III)/L+ MMT (10%)	32 \pm 3 ^a	1.35 \pm 0.14 ^b	95
2.0 mg As (III)/L+ MMT (15%)	32 \pm 2 ^a	1.34 \pm 0.09 ^b	96
2.0 mg As (III)/L+ MMT (20%)	31 \pm 3 ^a	1.31 \pm 0.11 ^b	97

a Values are in mean \pm S.E.M.; b SGR was calculated using the linear regression of the natural logarithm of mean bulk weight vs. time. Mean \pm S.E.M. is based on the regression, not on the tank divisions.

parameters were comparable with those of controls in the fish treated with MMT alone. Enhanced-MMT diets (10%) had effects on the growth and survival despite a tendency for specific growth rates (SGR) to be lower in fish fed these diets. There were significant effects on fish weight and survival along with the increased MMT diets (15 and 20%) in this experiment. These results are consistent with Russell and David, (2011) who investigated juvenile rainbow trout exposed for 28 days to a range of arsenic concentrations in water and in a live oligochaete diet, separately and in combination. In clean water, fish fed worms previously exposed to arsenate at 4 or 8 mg As/L showed pronounced reductions in growth, but fish exposed to these same water concentrations and a clean diet experienced less or no effect. Moreover, increasing waterborne arsenate to 16 or 32 mg As/L had substantial effects on both growth and survival. Hence, growth reduction was strongly correlated to total arsenic accumulation in the fish tissue.

Overall, growth rates and survival do not appear to be sensitive indicators of dietary As(III) toxicity under lower exposure concentration, and the toxicity increase with As(III) exposure concentration, but can be alleviated with added MMT concentration as it protected the fish from As(III) exposure. The possible mechanism may be that MMT can absorb metal such as Cd and As(III) via ion exchange reactions (Barbier et al., 2000; Kim and Du, 2009).

Effect of dietary MMT on arsenic (III) in tissues of common carp

We selected As(III) (2.0 mg/L) and MMT concentration (1.0%) as the standard exposure concentration for the experiments according to the primary experiment. Exposure of common carp to dietary As(III) resulted in a significant As(III) accumulation in the tissues of common carp in the following order: intestine>bone>gill>liver>muscle>brain ($P<0.05$), indicating accumulation of these tissues (Figure 1). In common carp fed with MMT,

there was a reduction in As(III) concentration of these tissues compared with the control group. The reason may be that some arsenic accumulation in tissues of common carp fed with the basal diet was bioavailable. Addition of MMT to the diet with As(III) resulted in decrease by 19.2% ($P<0.05$) in the intestine, 34.7% ($P<0.05$) in the bone, 29.4% ($P<0.05$) in the gill, 38.4% ($P<0.05$) in the liver, 37.5% ($P<0.05$) in muscle and 50% ($P<0.05$) in brain. Thus, MMT efficiently reduced As(III) accumulation in tissues of common carp. These results are consistent with Dai and Du, (2010) who found that exposure of tilapia to dietary Pb resulted in a significant Pb accumulation in the tissues of tilapia, and there was a significant decrease in Pb burden in tissues of tilapia fed with MMT and Pb compared to fish fed with Pb only. In addition, Kim and Du, (2009) studied the influence of montmorillonite on cadmium accumulation in carp (*Cyprinus carpio*) and found that the exposure of *C. carpio* to dietary Cd resulted in a significant Cd accumulation in tissues, but MMT inhibited cadmium accumulation in all tissues. It may be suggested that the MMT binds metal such as As(III), Pb, Cd, etc, thus reducing heavy metal bio-availability and uptake at the gastrointestinal tract (He et al., 1999; Barbier et al., 2000; Xu et al., 2004; Kim and Du, 2009).

Effect of dietary MMT on arsenic (III) on GSH and GSSG/GSH ratio

Intracellular specific activities of the enzymes involved in the GSH metabolism were assayed, for which GSH and GSSG levels were measured. The effects of exposure of As(III) on GSH and GSSG contents were determined after exposure for different time (Table 2). As shown in Figure 2, the reduced GSH concentrations were inhibited on the whole during all the exposure time and reached the lowest value at the 30th day, and the content of GSH was about 2.55 $\mu\text{mol/g}$ tissue. GSSG levels increased with the exposure time in initial phase and reached the highest with an average value of 0.58 $\mu\text{mol/g}$ tissue after

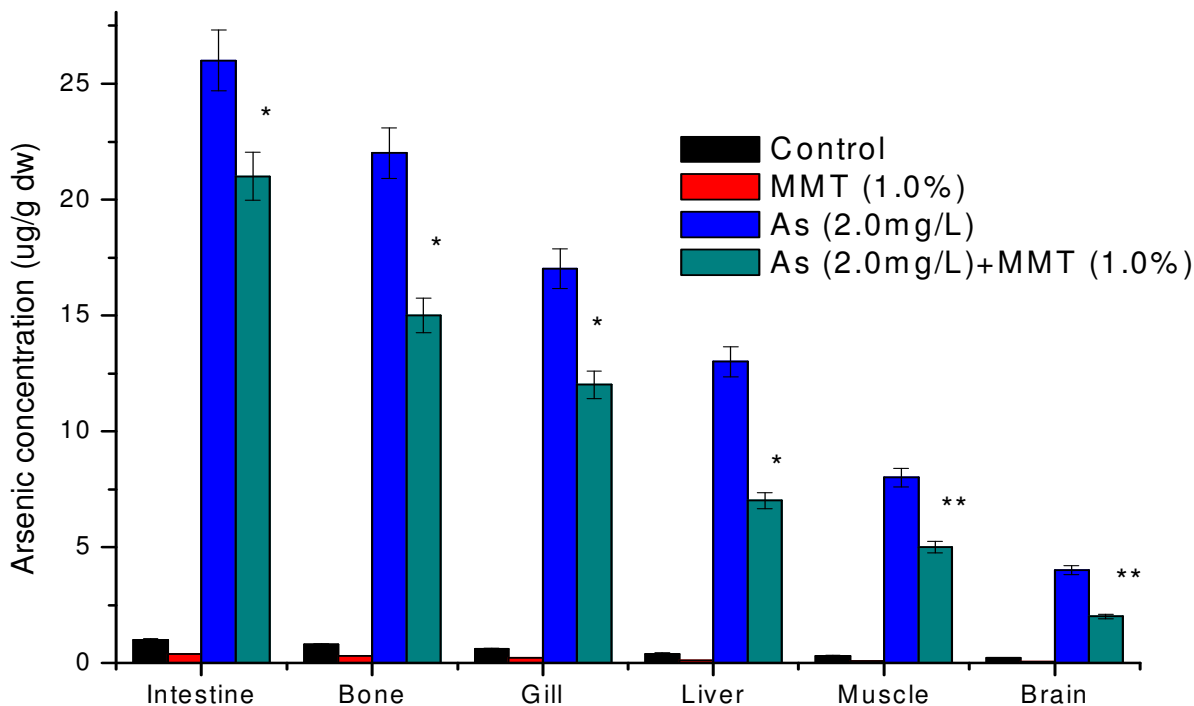


Figure 1. As(III) concentration (mean \pm S.E.) in intestine, bone, gills, liver, muscle and brain of common carp exposed for 30 days. The statistically different means from control are noted by either a single asterisk (* p <0.05) or a double asterisk (** p <0.01).

Table 2. Variations in the levels of GSH and GSSG in the liver of common carp after exposure to As (β) and/or MMT diets for 30 days (All data are means \pm S.E.M; unit: μ mol/g tissue).

Exposure day		0	5	10	20	30
Control (GSH)		3.62 \pm 0.13	3.63 \pm 0.11	3.64 \pm 0.12	3.63 \pm 0.14	3.65 \pm 0.11
2.0 mg As (III)/L	GSH	3.64 \pm 0.11	3.52 \pm 0.12	3.41 \pm 0.10	3.11 \pm 0.12	2.55 \pm 0.14
MMT (1.0%)		3.62 \pm 0.12	3.61 \pm 0.13	3.62 \pm 0.11	3.61 \pm 0.13	3.62 \pm 0.12
2.0 mg As (III)/L + MMT (1.0%)		3.64 \pm 0.12	3.65 \pm 0.11	3.68 \pm 0.12	3.72 \pm 0.09	3.73 \pm 0.07
Control (GSSG)			0.55 \pm 0.02	0.56 \pm 0.01	0.55 \pm 0.03	0.54 \pm 0.02
2.0 mg As (III)/L	GSSG	0.53 \pm 0.02	0.56 \pm 0.01	0.58 \pm 0.02	0.55 \pm 0.01	0.52 \pm 0.02
MMT (1.0%)		0.55 \pm 0.03	0.55 \pm 0.02	0.55 \pm 0.03	0.53 \pm 0.02	0.54 \pm 0.01
2.0 mg As (III)/L + MMT (1.0%)		0.53 \pm 0.02	0.51 \pm 0.02	0.47 \pm 0.03	0.45 \pm 0.02	0.42 \pm 0.01

10 days of exposure, but decreased later until the end of the exposure days, although still higher than the controls. Meanwhile, the GSSG/GSH ratio increased continuously and reached the maximal value 0.2 at the end of the exposure As(III) phase. However, the ratio of GSSG/GSH revealed a significant effect with MMT concentration (1.0%) (p <0.05); post-hoc analysis revealed that treated group with MMT exhibited a decrease the ratio of GSSG/GSH when compared to the fish not treated with MMT (1.0%). If the production of GSSG is higher than the reduction back to GSH by glutathione reductase (GR), then GSSG accumulates and is translocated outside the cell by specific transporters to avoid NADPH exhaustion

(Kaplowitz et al., 1996; Keppler et al., 1997), which is followed by a depletion of the GSH pool (Kretzschmar, 1996). It is obvious therefore that the ratio of GSSG/GSH has been proposed to be a sensitive index of oxidative stress. Indeed, GSSG is formed in antioxidant reactions that involve GSH and can accumulate with increase oxidative processing in the cell (Toborek and Henning, 1994; Bains and Shaw, 1997).

The GSSG/GSH ratio, a potential indicator for oxidative stress (Lange et al., 2002) increased with the exposure time in our study which demonstrated that the exposure to 20 mg/L As(III) did induce oxidative stress. This result is also in agreement with earlier report on Cd-induced

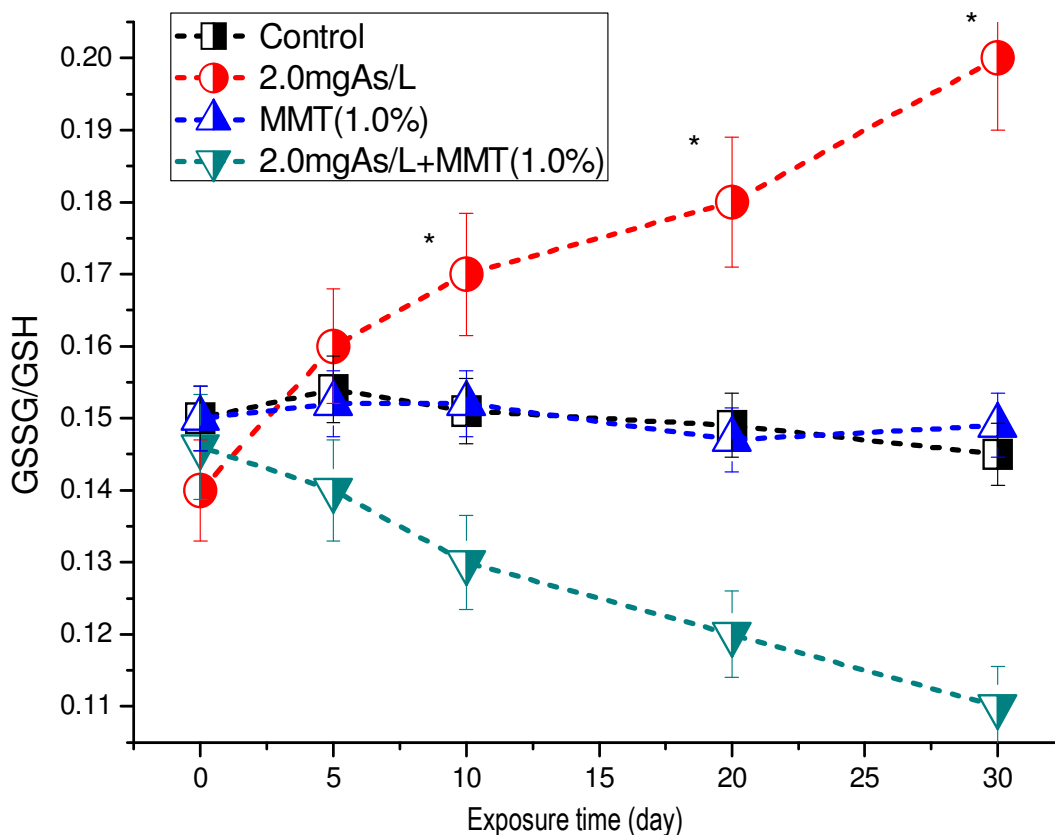


Figure 2. Changes in GSSG/GSH ratio in the liver of common carp after exposed to As (□) and/or MMT for 30 days. The statistically different from control means are noted by either a single asterisk (* $p < 0.05$).

oxidative damage in liver (Bagchi, et al., 1996). GSH is regarded as the first line of defense against oxidative stress (Potter and Tran, 1993). The results in the present study showed that the exposure of common carp to As(III) led to significant changes in GSH metabolism due to the oxidative stress, and the changes were associated with the exposure period. The intracellular glutathione concentration is the final result of a balance between GSH production and the combined rate of GSH consumption by reactive oxygen species (ROS) and removal of the resulting GSSG; inactivation of ROS involves oxidation of GSH into glutathione disulfide (GSSG) (Griffith, 1999). It could be concluded that GSH turnover was strengthened against oxidative stress induced by a short-term As(III) exposure. MMT can protect against toxicity of As(III). Clay minerals have been shown so far to protect against toxicity of various natural or synthetic chemical products, including heavy metal ions (Abdel-Wahhab et al., 1998; Gupta and Gardner, 2005; Abbès et al., 2006a). The mechanism of protection by clay minerals has been primarily attributed to the adsorption and cation exchange capacity to reduce the bioavailability of the toxins or the heavy metal ions in the gastrointestinal tract when added to contaminated diet (Abdel-Wahhab et al., 2005; Abbès et al., 2006b).

Effect of dietary MMT on arsenic (III) on metallothionein in common carp

Metallothionein (MT), a tissue protein with high affinity for heavy metals (such as As, Pb, Cd and Hg), forms chelates with heavy metals *in vivo*. This complex formation represents an important mechanism for detoxification or transport of As. Metallothionein has been applied in both laboratory and field studies (De Smet et al., 2001; Hansen et al., 2006) as a biomarker of metal exposure. In the present study, metallothionein was measured in liver, gill and intestine tissues of common carp to provide evidence for MMT effect on the As(III) level. There was no obvious change of the metallothionein level in common carp supplied with MMT in liver, gill and intestine tissues, respectively. As seen in Figure 3, metallothionein levels were significantly higher in common carp fed with As(III) compared to the control in the three tissues.

The results obtained from this study demonstrate that *de novo* MT synthesis is induced in common carp after exposure to As(III), which leads to higher intracellular As(III)-MT concentrations. However, large differences in the rate of *de novo* synthesis of MTs and the increase of As(III)-MT concentrations were found between liver, gill

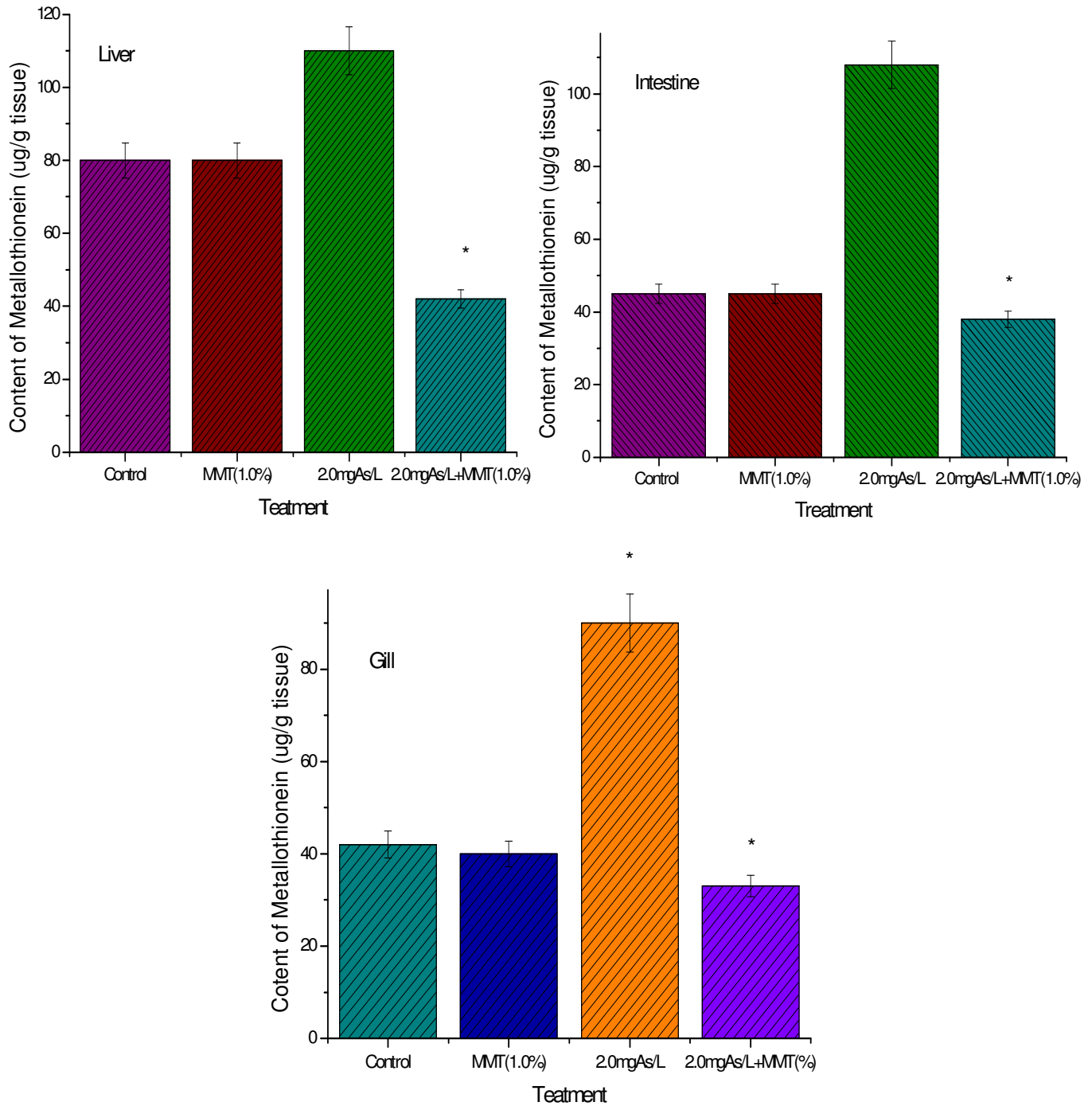


Figure 3. Metallothionein content in liver, intestine and gill of common carp after exposed to As (III) and/or MMT for 30 days. The statistically different from control means are noted by either a single asterisk (* $p < 0.05$).

and intestine tissues. Liver As(III)-MT levels in the control common carp were approximately three times higher compared with those in gills and intestine. Of the three studied tissues, the gills were least capable of sequestering As(III) by As(III)-MT (Figure 3). On the basis of these results, it can be concluded that carp liver is more capable of sequestering the incoming As(III) to As(III)-MT,

compared to gills and intestine. Therefore, potential toxic effects of As(III) caused by the binding to other molecules than MT will be the lowest in this organ. Moreover, the addition of MMT to the diet with As(III) decreased metallothionein concentration. In liver, gill and intestine tissues, contents of As(III)-MT decreased from 135, 103 and 89 $\mu\text{g/g}$ to 52, 39 and 29 $\mu\text{g/g}$, respectively.

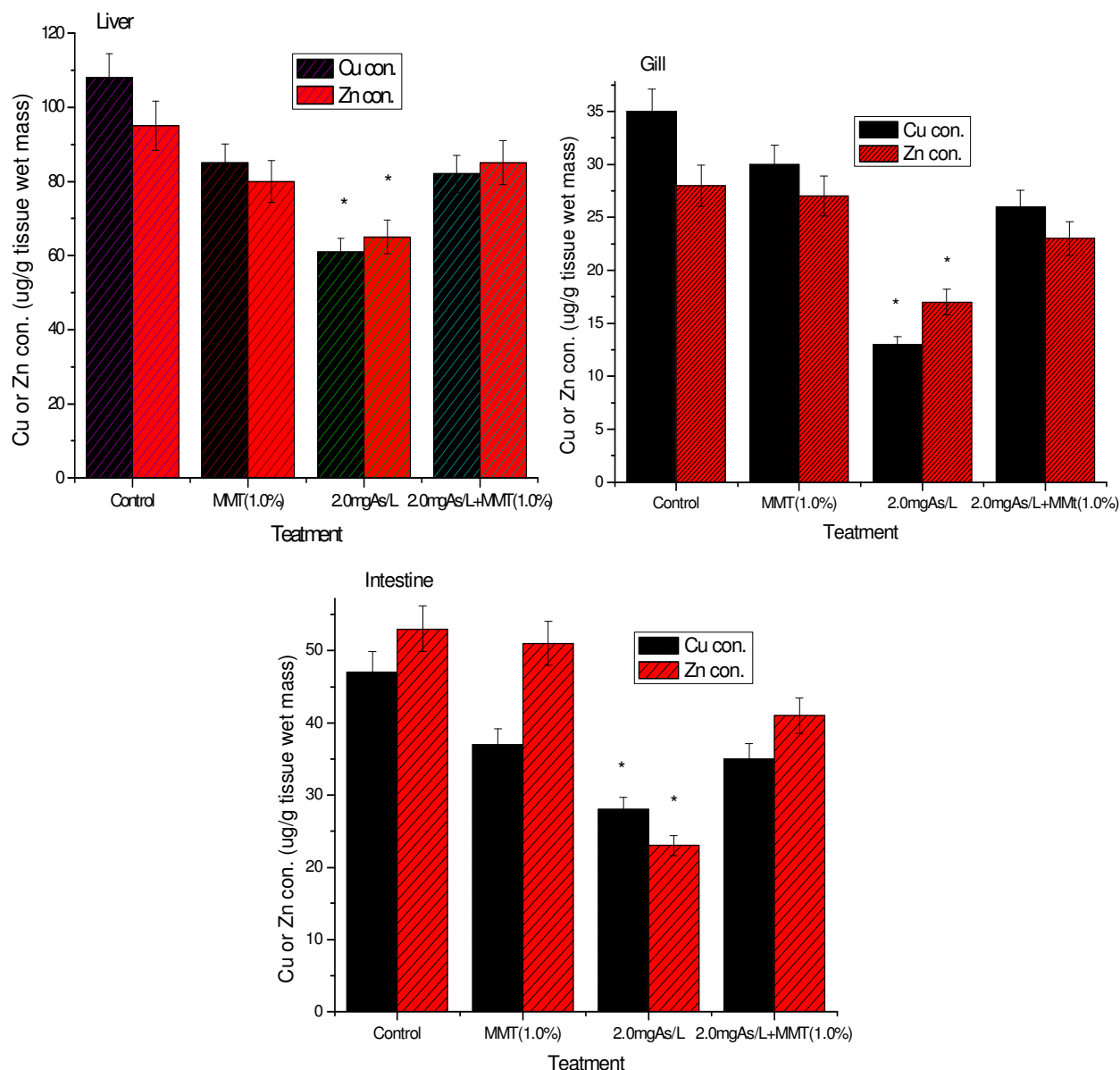


Figure 4. Trace elements (Cu or Zn) concentration in sampled tissues of common carp after exposure to As(III) and/or MMT for 30 days. The statistically different means from control are noted by either a single asterisk (* $p < 0.05$).

One possible explanation is that the metallothionein contents were sufficient high to detoxify As(III). Two fundamental properties of metallothionein are their high kinetic reactivity and their high affinity to bind metal ions (Stillman, 1995), therefore complexing As(III) and rendering it non-toxic. This result also indicated that MMT reduced the content of As(III) in tissues of common carp.

Effect of dietary MMT on arsenic (III) on trace Cu and Zn in common carp

Trace elements such as manganese, cobalt, iron, nickel, vanadium, copper, zinc and selenium, are considered

essential (FAO, 2004) for fish development. Non-essential elements in fish are unregulated and they perform no biological roles (Kojadinovic et al., 2007). The United States Agency for Toxic Substances and Disease Registry classified mercury, lead, cadmium, and arsenic as potentially toxic to human health due to their known or suspected toxicity (ATSDR, 2006). Co-exposure to essential and non-essential elements produced antagonistic and synergistic effects at both relatively high and low dose levels in a biomarker-specific manner (Wang et al., 2008). In the present study, Cu and Zn concentration decreased in sampled tissues of common carp exposed to dietary As(III) compared to the tissues of common carp in the control group (Figure 4). Similar results were

observed in other studies (Blanusa et al., 1989; Vivante et al., 2008). Heavy metal such as As and Pb could compete with trace elements such as Cu, Fe, Zn and P in absorption in the intestine (Flora et al., 1989; Sangita et al., 2011). Cu and Zn concentrations in tissues of common carp exposed to MMT and As(III) increased compared to common carp exposed to As(III) only ($P < 0.05$). This suggests that MMT in the diet most likely binds the dietary As(III) in non-bioavailable form, which in turn reduces As(III) ability to interact or interfere with the transport and/or absorption of Cu and Zn at the gastrointestinal tract. Added dietary MMT was clearly protective against the bioaccumulation of As(III) and exerted its greatest effect in the liver, and the least effect at the gill. The protective effect of added dietary MMT against As(III) bioaccumulation may result in part from direct Cu and Zn versus As(III) competition for uptake mechanisms at the gastrointestinal tract.

Conclusion

There are significant effects of MMT on As(III) behaviors in common carp. To our knowledge, the present study is the first to show that MMT when co-administered with As(III), growth rates and survival do not appear to be sensitive indicators of dietary As(III) toxicity under lower exposure concentration, and the toxicity increase as As(III) exposure concentration. Increased MMT diets could reduce As(III) accumulation in the tissues of common carp, and the ratio of GSSG/GSH presented a significant effect with MMT concentration. The addition of MMT to the diet with As(III) decreased metallothionein concentration. Trace elements (Cu and Zn) concentrations in tissues of common carp exposed to MMT and As(III) increased compared to common carp exposed to As(III) only. It can be seen that added dietary MMT was clearly protective against the bioaccumulation of As(III) in common carp.

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