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RESEARCH ARTICLE

Effect of Cadmium on the Concentration of Ceruloplasmin and its mRNA Expression in Goats under Molybdenum Stress

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The present study aim at evaluating the effects of cadmium on the concentration of ceruloplasmin (CP) and its mRNA expression in goats under molybdenum stress. $CdCl_2$ and $[(NH_4)_6Mo_7O_{24}\cdot 4H_2O]$ were selected as the source of cadmium and molybdenum, respectively. Thirty-six healthy goats were divided into four groups randomly, including control group (0mg/kg Mo+0mg/kg Cd), Mo exposure group (30mg/kg Mo), co-exposure group A (30mg/kg Mo+0.5mg/kg Cd) and co-exposure group B (30 mg/kg Mo+1 mg/kg Cd). Fifty days after exposure, blood and liver samples were collected. The results showed that all animals in experimental groups had shown some visible clinical signs of copper deficiency. The body weight and activity of CP protein in those animals decreased significantly as well. Meanwhile, Mo, Cd and Cu were significantly increased in the liver in all treatments, and contents of Mo, Cd and Cu in serum and liver of animals in co-exposure (Mo+Cd) groups were significantly higher than those in Mo exposure group. In addition, the accumulated level of cadmium positively correlated with time and dose. However, the mRNA expression of ceruloplasmin in Mo exposure group increased significantly ($P<0.05$), while in co-exposure groups, the level of expression increased before day 25 and then decreased rapidly. This study suggests that Cd has a promotional effect on molybdenosis which involves in aggravating the deposition of heavy metals (Mo, Cd and Cu).

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INTRODUCTION

Molybdenum (Mo) is an essential trace element for humans beings and animalssince 1953, but its requirement in animals is extremely low (Barceloux, 1999). High concentrations of Mo could poison animals by its interruptive interaction with other trace elements. Previous studies revealed that ruminants were often harmed by high concentrations of Mo, especially in areas of mining and other industrial businesses (Gardner *et al*., 2003; Suttle, 2012). Low doses of 3 and 50mg/kg of Mo could induce clinical signs of molybdenosis in cattle and sheep respectively (Zhuang *et al.*, 2015). Similarly, cadmium (Cd) is a nonessential trace element and widespread metal contaminant. It has been classified as the sixth toxic substance that endangered human health by the Agency for Toxic Substances and Disease Registry (ATSDR), due to its protracted biological high-life, low rate of excretion, predominant storage in soft tissues, and diverse toxic effects (Marettova*et al*., 2010).

One of the largest rare earths mines around the world located in Jiangxi province, China contributes to more than 90% of the world rare earth production (Wei *et al.*, 2001). Nevertheless, exploitation of rare earth mine resources could cause environmental problems such as heavy metal dissemination (Rauf*et al.,* 2012). Recent epidemiological studies showed that many heavy metals such as Mo and Cd had severely contaminated the soil and pasture in the area. Additionally, Fan firstly reported Mo toxicity in ruminant species-cattle. Significant clinical signs were found, of which they called "red skin combined with white fleece syndrome (RSCWFS)" (Gu*et al.,* 2015). Their subsequent research revealed that the

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syndrome (RSCWFS) was induced by a combination of Mo and Cd intake (Gu*et al.*, 2015). Recently, researches still mainly focused on the toxicity of Mo or Cd alone, whereas very few concentrated on their combined effects. However, heavy metal diseases are always caused by two or more elements. Therefore, we tested the combined effects of Mo and Cd on goats in order to explore the mechanism underlay interaction between these two elements. Moreover, the concentration of cuprum enzyme (ceruloplasmin) and its mRNA expression was also investigated.

MATERIALS AND METHODS

Experimental animals and treatments: Thirty-six healthy purebred bore goats weighing approximately 20kg each were procured from a commercial rearing farm. Animal care was approved by the Department of Animal Science of Jiangxi Agriculture University. The basal diet was formulated to meet or exceed all nutrient requirements for goats (NRC, 1997) (Table 1). The goats were randomly divided into 4 groups with 9 goats in each group. Subjects in the control group was orally administered corresponding quantitative deionized water, meanwhile the animals in all treatment groups were orally administered 30 mg $Mo \cdot kg^{-1} \cdot BW$, and those in co-exposure group A and B were orally administered 0.5 mg $Cd \cdot kg^{-1} \cdot BW$ and 1 mg $Cd \cdot kg^{-1} \cdot BW$ per day, respectively. $CdCl_2$ and $[(NH_4)_6Mo_7O_{24} \cdot 4H_2O]$ were selected as the source of cadmium and molybdenum. The experimental period lasted for 50 days. Goats were fed at 07:00 and 18:00 in equal allotments per day, but were allowed to drink water (with less than 0.01mg Cu/kg, 0.01mg Mo/kg, 0.0001 Cd/kg after analysis) ad libitum.

Recording keeping: The body weights of individual animals were recorded at the beginning of the experiment and thereafter in the morning of every tenth day before feeding. The animal's clinical signs were also recorded.

Collection and preservation of samples: Jugular vein blood samples of each goat were collected into vacutainer tubes with non-heparinized on day 0, 10, 20, 30, 40 and 50 for detecting serum ceruloplasmin (CP) concentration. Liver samples were collected on day 0, 25 and 50 by slaughtering three goats randomly chosen from each group. The serum and liver samples were frozen in liquid nitrogen and stored at -20 and -80ºCin individual freezing tube, respectively.

Biochemical Assays

Determination of CP concentration: According to the manufacturer's instructions, the activity of CP in serum was detected using the commercial ceruloplasmin kit (Nanjing Jiancheng Bioengineering Institute, China).

Determination of molybdenum, cadmium and copper concentration in liver and serum: Small pieces were removed from the frozen liver with a scalpel and were put in a baking oven for 12 h with 110°C. After which 0.5 g samples were transferred into beakers. For digestion, 25 mL of concentrated HNO_3/HCl (4:1) was added into each beaker and warmed on a low temperature electric hot plate until solution became transparent. The samples were adjusted to 10 mL using a volumetric flask by 0.5% HNO_3 (Liu *et al*., 2012), then measured contents of Mo, Cd and Cu in samples and then analyzed by graphite furnace atomic absorption spectrometry (Model 5100, HGA-600 Graphite Furnace; Perkin-Elmer, USA), using an established assay universally used for the determination of trace-element concentrations (Sahin *et al*., 2010; Lasagna-Reeves *et al*., 2010).

Expression of CP mRNA in the liver: Hepatic total RNA was isolated by using the RNesay mini kit (QIAGEN) from 20-50 mg tissue and then was quantified by spectrophotometry. Reverse transcription was performed using a commercial kit (Takara, Japan). Primers and probes were shown in table 2. The amplification cycle for gene were analysed using iQ SYBR Green Supermix (Bio-Rad) on a Real-time PCR thermocycler (Bio-Rad, CA, USA).

Statistical analysis: Statistical analysis was performed using SPSS 16.0 (Chicago, IL), and all parameters were presented as mean±SD. $P<0.05$was shown as significantly change by one-way ANOVA. Differences between means were assessed using Tukey's honestly significant difference test for post hoc multiple comparisons.

RESULTS

Clinical signs: Goats of the control group were under a healthy condition through clinical observation. However, shuffling gait, watery diarrhea, achromotrichia, emaciation and anorexia were observed in the Mo exposure and co-exposure (A and B) groups. Meanwhile, onset time of abnormal clinical signs in co-exposure (A and B) groups was earlier than those in Mo exposure group. Abnormal clinical signs in co-exposure group B (30mg/kg Mo+1mg/kg Cd), co-exposure group A (30mg/kg Mo+0.5 mg/kg Cd) and Mo exposure group were firstly observed on day 36, 43, 47, respectively. Clinical signs of watery diarrhea, emaciation, anorexia and achromotrichia in co-exposure group B (30mg/kg Mo+1mg/kg Cd) were more serious than those in co-exposure group A (30mg/kg Mo+0.5 mg/kg Cd). Screams and red skin were observed at mid-night since day 41 in both co-exposure groups A and B. Red skin was observed in both co-exposure groups. None was observed in the Mo exposure group.

Body weight change: Records of body weight was shown in Table 3. Body weight of the goats in the control group had a significant increase ($P<0.05$) during the experimental period. However, body weight in the Mo exposure and co-exposure (A and B) groups decreased significantly ($P<0.05$) compared with control group. Body weight was significantly lower ($P<0.05$) in the Mo exposure and co-exposure (A and B) groups than that in the control group. Body weight in the co-exposure group B (30mg/kg Mo+1mg/kg Cd) decreased significantly ($P<0.05$) compared with the Mo exposure or co-exposure group A (30mg/kg Mo+0.5 mg/kg Cd) on day 40 and 50.

Table 1: Composition and nutrient levels in the basal diet (DM basis, %)

Items	Content (%)
Ingredients [a]	
Corn	52.5
Deoiled rice	19.00
Soybean meal	10.0
Rapeseed meal	7.0
Cottonseed meal	7.0
CaHPO4	1.0
Limestone	1.5
Salt	1.0
Additives[b]	1.0
Total	100
Nutrient levels	
DM(%)	87.84
ME/(MJ/kg)	8.89
CP (%)	11.90
EE(%)	2.71
Calcium	0.90
AP (%)	0.78

[a]As fed basis. [b]Additives provided as follows per kilogram of additives: nicotinic acid, 2000mg; vitamin A, 1000000 IU; vitamin D_3, 2500000 IU: vitamin E, 24000 mg; iron($FeSO_4 \cdot H_2O$), 2000 mg; zinc($ZnSO_4 \cdot H_2O$), 140000 mg; manganese($MnSO_4 \cdot H_2O$); 3000 mg; iodine(KI,3%),180 mg; selenium($NaSe_3O_4 \cdot H_2O$),100 mg;

Analyses of the Concentrations of Mo, Cd and Cu in serum: Concentrations of Mo, Cd and Cu in serum were presented in Fig. 1. No significant differences (P>0.05) were observed on concentrations of Mo, Cd and Cu in the control group during the experimental time. Concentration of Mo in co-exposure (A and B) groups was significantly higher than the Mo exposure group (Fig. 1A). However, concentration of Cd in co-exposure (A and B) groups significantly increased (P<0.05) while in the Mo group decreased significantly (P<0.05) (Fig. 1B). On day 50, concentrations of Mo and Cd in co-exposure group B (30mg/kg Mo+1mg/kg Cd) were significantly (P<0.05) higher than those in co-exposure group A (30mg/kg Mo+0.5 mg/kg Cd). Concentration of Cu in the Mo group and co-exposure (A and B) groups increased compared with the control group. However, concentration of Cu in Mo group increased significantly compared with the co-exposure (A and B) groups on day 50 (Fig. 1C).

Analyses of the Contents of Mo, Cd and Cu in liver: Contents of Mo, Cd and Cu in liver were presented in Fig. 2. No significant differences (P>0.05) were observed on contents of Cu, Cd and Cu in the control group during experimental time. However, contents of Mo and Cu in the Mo exposure and co-exposure (A and B) groups increased significantly (P<0.05) compared with control group during experimental time. And contents of Cd in the co-exposure groups increased significantly (P<0.05) while the contents of the Mo exposure group decreased. Both metallic elements (Cd and Cu) in the co-exposure (A and B) groups increased compared with the Mo exposure group.

Analyses of the activity of CP in serum and expression of CP mRNA in liver: Activity of CP in serum was presented in Table 4. No significant differences (P>0.05) were observed on the activity of CP in the control group during the experimental period. Activity of CP in the Mo exposure and co-exposure (A and B) groups decreased significantly (P<0.05) compared with control group.

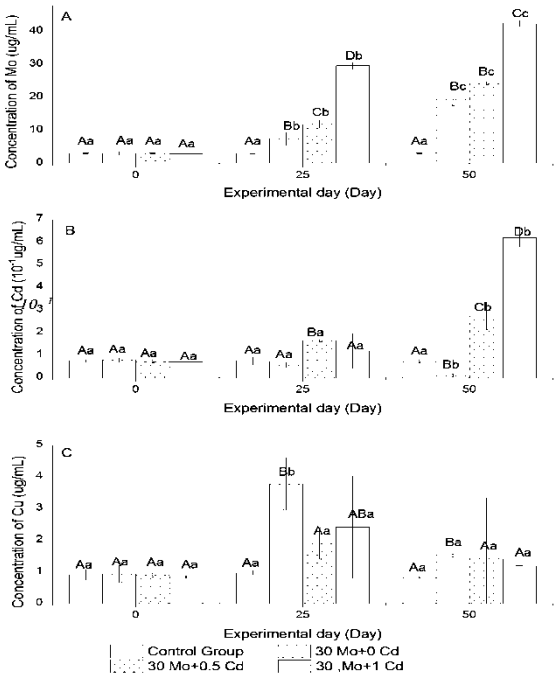

Fig.1: Concentration of Mo, Cd and Cu in serum.

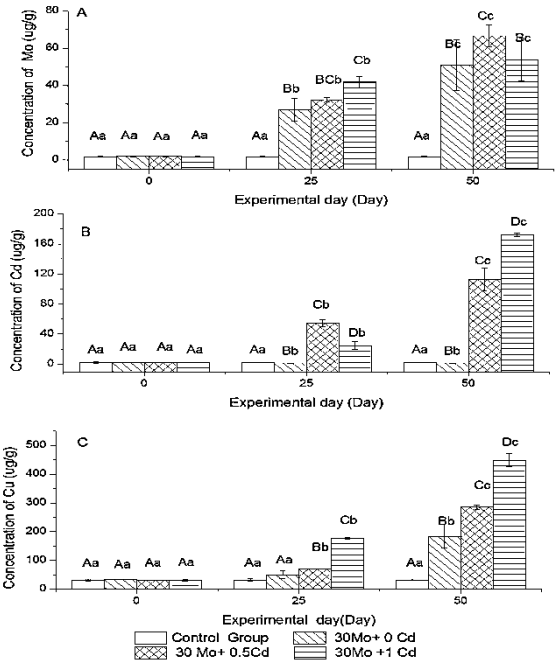

Fig.2: Concentration of Mo, Cd and Cu in liver.

Expression of CP mRNA was presented in Fig. 3. No significant differences (P>0.05) were observed of the expression of CP mRNA in the control group during the experimental period. On day 50, expression of CP mRNA in the Mo exposure group and co-exposure (A and B) groups increased significantly (P<0.05) compared with the control group. However, on day 25, expression of CP mRNA in the co-exposure (Mo+Cd) groups increased significantly compared with the Mo exposure group. And on day 50, expression of CP mRNA in the Mo exposure

group increased markedly while it had a slight decline in the co-exposure (A and B) groups.

Table 2: Primer and Probe Sequences

Gene name	Gene serial number	Primer and Probe Sequence(5'to3')	Amplified fragment length
CP	*NM_001009733.1*	Upstream：TGCTATTAATGGAAGGATGTTTGG Downstream：ACCGAGTGCAAGTCTACTTCATTG Probe：AACCTGCAAGGCCTCAC	78bp
β-Actin	*U39357.1.1*	Upstream TCACGGAGCGTGGCTACAG	63bp

Table 3: Effect of cadmium on average body weight of goats under molybdeum stress (kg/Goat)

Dose (mg·kg^{-1}·BW)		Experimental period (Day)					
Cd	Mo	0	10	20	30	40	50
0	0	21.38±1.10^{Aa}	22.27±0.41^{Aa}	22.60±0.29^{Aab}	23.19±0.08^{Aab}	23.83±0.27^{Ab}	24.38±0.14^{Ab}
0	30	21.35±1.29^{Aac}	21.72±1.06^{Ac}	19.97±0.52^{Bab}	19.02±0.28^{Bbd}	18.62±0.13^{Bbd}	16.92±0.39^{Bd}
0.5	30	21.88±0.51^{Aa}	21.94±0.93^{Aa}	20.69±0.76^{Bab}	18.97±1.03^{Bbc}	17.75±0.51^{Bcd}	16.25±0.36^{Bd}
1	30	21.43±1.12^{Aa}	21.23±0.54^{Aa}	20.74±0.49^{Ba}	18.59±1.34^{Bb}	16.77±0.91^{Bbc}	14.77±0.62^{Bc}

Mean±SD with different lower case letters within a line are statistically different (P<0.05), and mean with different capital case within a column are statistically different (P<0.05).

Table 4: Effect of cadmium on the activity of CP in serum under molybdenum stress (μmol/L)

Dose (mg·kg^{-1}·BW)		Experimental period (Day)					
Cd	Mo	0	10	20	30	40	50
0	0	42.24±1.93^{Aa}	42.45±1.34^{Aa}	40.73±1.49^{Aa}	39.33±0.46^{Aa}	41.91±1.37^{Aa}	39.66±0.23^{Aa}
0	30	44.71±1.59^{Aa}	39.69±0.97^{Ab}	38.61±1.94^{ABb}	25.31±7.98^{Bc}	20.96±6.84^{Bd}	19.18±1.14^{Bd}
0.5	30	39.82±0.23^{Aa}	40.84±2.78^{Aa}	36.11±1.37^{Ba}	24.50±0.46^{Bb}	22.73±0.23^{Bb}	21.28±2.28^{Bb}
1	30	40.09±0.37^{Aa}	38.21±0.68^{Aab}	36.43±1.12^{Bab}	33.53±12.31^{Bb}	24.18±2.74^{Bc}	18.38±2.74^{Bd}

Mean±SD with different lower case letters within a line are statistically different (P<0.05), and mean with different capital case within a column are statistically different (P<0.05).

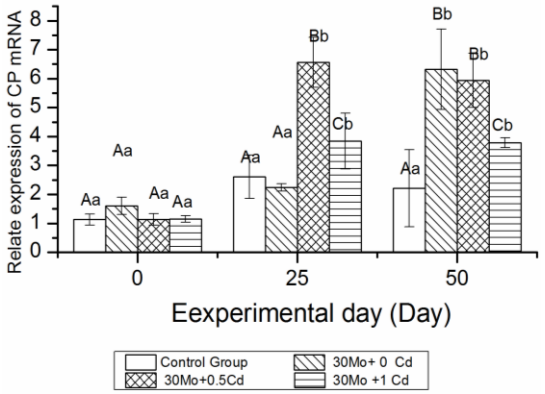

Fig.3 Relative of expression CP mRNA in liver. Data are mean±SD. Different letters indicate statistical significance at P<0.05 among a group (a, b, c) or inter-groups (A, B, C).

Expression of CP mRNA was presented in Fig. 3. No significant differences (P>0.05) were observed of the expression of CP mRNA in the control group during the experimental period. On day 50, expression of CP mRNA in the Mo exposure group and co-exposure (A and B) groups increased significantly (P<0.05) compared with the control group. However, on day 25, expression of CP mRNA in the co-exposure (Mo+Cd) groups increased significantly compared with the Mo exposure group. And on day 50, expression of CP mRNA in the Mo exposure group increased markedly while it had a slight decline in the co-exposure (A and B) groups.

DISCUSSION

The adverse effects and toxicityof Mo and Cd in animals have been widely acknowledged in the past fifty years. Even in practical feeding conditions, some ruminants might be exposed to heavy metal in the environment and develop relative diseases. Previous studies revealed that high levels of Mo intake could induce secondary copper deficiency in many species (Raisbeck*et al*., 2006; Zhang *et al*., 2011), and the toxic effects of Mo can be reflected by testing the enzyme activity and gene expression of ceruloplasmin (CP), which is a copper containing metalloenzyme found in blood, and mainly synthesized in liver and carries approximately 95% of the total plasma copper (Rombach*et al*., 2003). However, it was reported that the element Cd had some effects on copper deficiency. Therefore, analyzing the enzyme activity and gene expression of CP allows us to explore the combined effect of Mo and Cd by adding a set level of Mo with differentiated levels of Cd.

In this study, the combined effect of Mo and Cd was investigated and the results of clinical signs of watery diarrhea, shuffling gait, achromotrichia and anorexia were observed in all treatment groups and the onset time among these groups were distinct, especially in body weight of co-exposure group B, which significantly decreased compared with the Mo group. The result of this study, which was consistent with previous studies about molybdenosis or copper deficiency (Yang *et al*., 2011; Kessler *et al*., 2012), suggested that the level of Cd had aggravated molybdenosis. Through observation of the levels of Mo, Cd and Cu in liver, the increase of supplemental cadmium induced the deposition of Mo and Cu in the co-exposure groups compared with the Mo exposure group, and this interaction was shown an time and dose-dependent On one hand, cadmium might have accelerated the absorption of Mo according to the analysis of serum elements. However, numerous studies revealed that high level of Mo can reduce the bioavailability of Cu by forming insoluble complexes of Cu-Mo in ruminants. On the other hand, Cd have a stronger connection with MT proteins. Metallothionein (MT) is a cysteine-rich, low

molecular weight metal sequestering protein that has been shown to be involved in essential metal homeostasis and in the detoxification of heavy metals (Cobbett and Goldsbrough, 2002). Cd and Cu have a high affinity for MT (Sabolic*et al*., 2010). Cd can lead to increased MT expression, which traps Cu in the liver, consequently disturbs the metabolism of copper. In addition, cadmium exposure was associated with alteration in oxidative stress (Klaassen*et al.*, 1999; Moulis, 2010), inhibited CP (Samsam*et al.*, 2009), and was closely associated with microflora species (Fazeli*et al*., 2011). The microflora species in lumen play an essential role in the development and health of the host by improving the intestinal tract microbial balance as well as detoxification and elimination of harmful compounds from the livestocks (Ravikumar*et al*., 2007). Nevertheless, concentration of Cu in serum increased before day 25 and then reduced, combined with the results of contents of Cu and copper deficiency, this phenomenon suggested that high level of Mo supplementation induced systemic copper deficiency. Copper reserves were mobilized initially, and then induced the animal body copper re-distribution. Nevertheless, the serum in Cu decreased in the later period as the result of depletion of available Cu. In addition, as a result of copper deficiency, the expression of CP in the treatment groups was significantly higher in compensation than that in the control group. Preceruloplasmin in liver has no sufficient copper to form mature ceruloplasmin. Preceruloplasmin released into the blood then degraded rapidly (Braga *et al*., 2013). Nevertheless, expression of CP mRNA in liver decreased on day 50 in co-exposure groups. High heavy metal (Mo, Cd and Cu) deposition in liver caused oxidative stress, ultimately led to disturbance of the liver's function.

Conclusions: In summary, Cd has a promotional effect on molybdenosis, and it can aggravate the deposition of heavy metals (Mo, Cd and Cu) in liver.

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Author's contribution: HBC and GLH conceived and designed the experiment. YZ and PL executed the experiment. YZ and CYZ analyzed the experimental samples. All authors interpreted the date, critical revised the manuscript for important intellectual contents and approved the final version.

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