Changes in Trace Element Contents and Morphology in Bones of Duck Exposed to Molybdenum or/and Cadmium

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Abstract Cadmium (Cd) and high molybdenum (Mo) can lead to adverse reactions on animals, but the coinduced toxicity of Mo and Cd to bone in ducks was not well understood. The objective of this study was to investigate the changes in trace elements' contents and morphology in bones of duck exposed to Mo or/and Cd. One hundred twenty healthy 11-day-old male ducks were randomly divided into six groups and treated with commercial diet containing Cd or/and Mo. On the 60th and 120th days, the blood, excretion, and metatarsals were collected to determine alkaline phosphatase (ALP) activity and the contents of Mo, Cd, calcium (Ca), phosphorus (P), copper (Cu), iron (Fe), zine (Zn), and selenium (Se). In addition, metatarsals were subjected to histopathological analysis with the optical microscope and radiography. The results indicated that Mo and Cd contents significantly increased while Ca, P, Cu, and Se contents remarkably decreased in metatarsals in coexposure groups ($P < 0.01$). Contents of Fe and Zn in metatarsals had no significant difference among groups ($P > 0.05$). Ca content in serum had no significant difference among experimental groups ($P > 0.05$), but P content was significantly decreased in HMo and HMo + Cd groups ($P < 0.05$). Contents of Ca and P in excretion and ALP activity were significantly increased in coinduced groups ($P < 0.05$). Furthermore, osteoporotic lesions, less and thinner trabecular bone were observed in combination groups. The findings suggested that dietary of Cd or/and Mo could lead to bone damages in ducks via disturbing the balance of Ca and P in body and homeostasis of Cu, Fe, Zn, and Se in bones; moreover, the two elements showed a possible synergistic relationship.

Key Words Molybdenum · Cadmium · Bone · Trace element · Duck

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All authors have read the manuscript and agreed to submit it in its current form for consideration for publication in the Journal.

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Introduction

Molybdenum (Mo) is widely found in the Earth's crust at 1–1.5 mg/kg [1], and it is a necessary trace element for almost all animals and plants, which forms the catalytic center of a large variety of enzymes such as nitrate reductases (NAR), nitrogenase, sulphite oxidase, and xanthine oxidoreductases (XODs) [2]. Animal experiments have showed that Mo deficiency inhibited growth, peculiarly in early stages of development [3]. However, high dose of Mo could cause inhibition of fetal development, growth retardation and skeletal deformities, listless, diarrhea and hair discoloration or fall off [4, 5], liver and kidney damages [6, 7]. It has been reported that the higher dose of Mo caused bone cortex thinning in male sheep, reducing the number of trabecular bone and mineral density [8, 9]. Mo is widely used in industry, and many studies reported that animal Mo poisoning is usually caused by the Mo-contaminated feed and water [10, 11]. Bone lesions such as joint deformity, fractures, and osteoporosis were commonly found in chronic Mo poisoning [12].

Cadmium (Cd) is an extremely toxic heavy metal which is obtained from the environment [13]. Cd has a long biological half-life of 10–30 years in body, and sources of Cd exposure in usual population are polluted food and air [14]. It accumulates in a variety of organs, especially in the bone, liver, kidney, and reproductive organs [15, 16]. Study showed that bone was one of the important target organs for Cd toxicity [17]. Cd exposure was associated with loss of bone mineral content and disturbance of activation of vitamin D in the kidney and increased the risk of osteoporosis [18], which also caused hypercalciuria, increased fecal excretion of calcium (Ca) and bone damage, and disrupted Ca metabolism, leading to Ca deficiency [19, 20].

More and more researchers have investigated the effects of metals combination toxicity in recent decades. In China, Southern Jiangxi Province is rich in mineral reserves such as tungsten ore. However, improper mining and indiscriminately dumping wastewater aggravated environmental pollution [21]. Our previous studies showed that coexposure of Mo and Cd caused damages to kidney, spleen, and testes in ducks, and the damages in Mo combined with Cd groups were more severe than those in separate Mo or Cd group [7, 22, 23]. Although several researches showed that Cd and Mo were noxious chemicals which caused bone damages, there were few studies focused on the bone resulted from subchronic toxicity of Mo combined with Cd, especially on waterfowl. This study was carried out to investigate the changes in trace elements' contents and morphology in bones of duck exposed to Mo or/and Cd; Mo, Cd, Ca, phosphorus (P), copper (Cu), iron (Fe), zinc (Zn), and selenium (Se) concentrations and alkaline phosphatase (ALP) activity were determined. Bone damages were assessed by X-ray and pathological evaluation. In addition, the Ca and P concentrations were also examined in excretion.

Materials and Methods

Animals and Treatments

All animal care and experimental procedures were approved by the institutional ethics committee of Jiangxi Agricultural University. One hundred twenty healthy 11-day-old male ducks were randomly divided into six groups with 20 ducks every group. Duck model of excessive exposure of Mo or/and Cd was developed as described in our previous publication [22]. Ducks in each group were fed with basal diet with different levels of Mo or/and Cd: control group (0 mg/kg Cd, 0 mg/kg Mo), low Mo group (15 mg/kg Mo), high Mo group (100 mg/kg Mo), Cd group (4 mg/kg Cd), LMo + Cd group (4 mg/kg Cd, 15 mg/kg Mo), and HMo + Cd group (4 mg/kg Cd, 100 mg/kg Mo). The basal diet was formulated according to the National Research Council (NRC) (1994).

Ammonium heptamolybdate ($[(NH_4)_6Mo_7O_{24}\cdot4H_2O]$) and cadmium sulfate ($3CdSO_4\cdot8H_2O$) were selected as the sources of Mo and Cd, respectively. Ducklings were fed with duckling basal diet and duck basal diet before and after 21 days, respectively. All ducks were raised in separated cages at a constant temperature with good ventilation and light and were guaranteed free access to water and diet. The experiment lasted for 120 days. The contents of Mo, Cd, Cu, Zn, Fe, and Se in the basal diet and water are shown in Table 1. Ducks in this study were handled and treated in accordance with the strict guiding principles of the National Institution of Health for experimental care and use of animals.

Sample Collection

After being treated for 60 and 120 days, the blood collected via wing vein was allowed to clot, incubated at 37 °C for 2 h, and the serum was obtained by centrifugation at 1000g for 10 min at 4 °C. The serum was stored at −20 °C. Ten ducks from each group were sacrificed with an overdose intravenous injection of sodium pentobarbital (Nembutal, Abbot Labs, IL, USA, 50 mg/kg). Metatarsals were isolated from soft tissues, the left metatarsals for the elements' contents were determined after radiography, and the right metatarsals were fixed in 10 % neutral buffered formalin for bone histopathology. In addition, excretion samples were collected for detection of Ca and P concentrations.

Determination of Elements

The collected serum and water samples were wet-digested with 10 % HNO_3. Metatarsals, feed, and excretion were dried, weighed, ashed, and solubilized in 15 % HNO_3. The elements including Mo, Cd, Cu, Fe, Zn, Se, and Ca in metatarsal, serum, excretion, feed, and water were measured using Agilent 240AA atomic absorption spectrophotometer (Agilent, America). P content was determined by UV spectrophotometer (Perkin Elmer, America). All analyses were carried out according to the manufacturer's instruction.

Measurement of ALP Activity

Based on the International Society for Animal Clinical Biochemistry, the activity of ALP was measured by liquid reagent photometry using detection kits (Nanjing Jiancheng Bioengineering Institute, China).

Radiography and Pathological Examination of Metatarsal

All left metatarsals were radiographed using the same equipment and the same program, with an exposure of 68 kV and 30 mAS, bone samples were laid directly on the film together. Hematoxylin and eosin (HE) staining was used in the

Table 1 The contents of Mo, Cd, Cu, Zn, Fe, and Se in the basal diet and water

Trace elements	Duckling feed (µg/g)	Duck feed (µg/g)	Water (µg/mL)
Mo	4.151	4.729	0.010
Cd	0.247	0.476	0.008
Cu	191.351	109.330	0.021
Fe	747.835	709.081	0.188
Zn	210.741	189.874	0.152
Se	1.048	1.052	0.015

histology analysis. All collected right metatarsal were fixed in 10 % neutral buffered formalin, followed with decalcification in 14 % hydrochloric acid. Then, metatarsal bones were dehydrated through a series of ascending ethanol solutions (70 to 100 %), cleared with dimethylbenzene, enclosed in paraffin, and sliced with 8-µm thickness with a rotary microtome (Leica, Germany) for HE stain. Finally, photos were taken with a microscope for analysis (Nikon, Japan).

Statistical Analysis

Statistical analyses of all data were performed using the SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.01 (GraphPad Inc., La Jolla, CA, USA). Differences between means were assessed using one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. All data showed a normal distribution and passed equal variance testing. Data were expressed as mean ± SEM.

Results

The Contents of Ca and P in Serum, Metatarsal, and Excretion

The contents of Ca and P in serum are shown in Fig. 1a, b. Concentration of Ca had a downtrend in HMo group and HMo + Cd group on days of 60 and 120 ($P > 0.05$). There was no significant difference in total experiment period among other experimental groups ($P > 0.05$) (Fig. 1a). On the 60th day, compared with control group, concentration of P was significantly decreased in HMo, Cd, LMo + Cd, and HMo + Cd groups ($P < 0.05$ or $P < 0.01$), but there was no significant difference between control group and LMo group, concentration of P was significantly higher ($P < 0.05$) in HMo group than HMo + Cd group. On the 120th day, compared with control group, concentration of P was observed no significant difference in LMo, Cd, and LMo + Cd groups ($P > 0.05$), but it had a significant downtrend in HMo group and HMo + Cd group ($P < 0.05$) (Fig. 1b).

Figure 1c, d illustrates the contents of Ca and P in metatarsal. The contents of Ca and P showed a similar trend on the 60th day. Contents of Ca and P had no significant difference between control group and experimental groups ($P > 0.05$). On day 120, contents of Ca and P in all treated groups significantly decreased compared with the control group ($P < 0.05$ or $P < 0.01$).

The contents of Ca and P in excretion are shown in Fig. 1e, f, and the contents of Ca and P had an uptrend on days of 60 and 120. On day 60, there were no significant changes among all groups ($P > 0.05$). On day 120, concentrations of Ca and P had a significant increase in HMo + Cd group compared with control group ($P < 0.05$).

The Contents of Mo, Cd, Cu, Fe, Ze, and Se in Metatarsal

As shown in Fig. 2, on day 60, compared to control group, content of Mo had no significant change in LMo + Cd group and Cd group ($P > 0.05$), and it was significantly increased in LMo, HMo, and HMo + Cd groups ($P < 0.05$ or $P < 0.01$); Mo content was significantly increased in HMo group compared to HMo + Cd group ($P < 0.05$). On day 120, compared with control group, Mo content was significantly increased in LMo, HMo, LMo + Cd, and HMo + Cd groups ($P < 0.05$ or $P < 0.01$), but it was significantly decreased in Cd group ($P < 0.05$) (Fig. 2a). Cd concentration highly significantly increased in Cd, LMo + Cd, and HMo + Cd groups compared with control group ($P < 0.01$) while it was no significant change in LMo and HMo groups ($P > 0.05$) (Fig. 2b). Cu concentration was decreased in LMo, HMo, Cd, LMo + Cd, and HMo + Cd groups ($P < 0.05$ or $P < 0.01$) compared with control group on days 60 and 120 (Fig. 2c). Fe and Zn concentrations had no significant change in experimental groups compared with control group, while they presented a declining trend in combined groups ($P > 0.05$) (Fig. 2d, e). Se concentration had no significant change in experimental groups on day 60, while it was highly significantly decreased in experimental groups except LMo group compared with control group at day 120 ($P < 0.01$). Meanwhile, Se concentration had no significant change in HMo + Cd group compared with Cd group and HMo group ($P > 0.05$) (Fig. 2f).
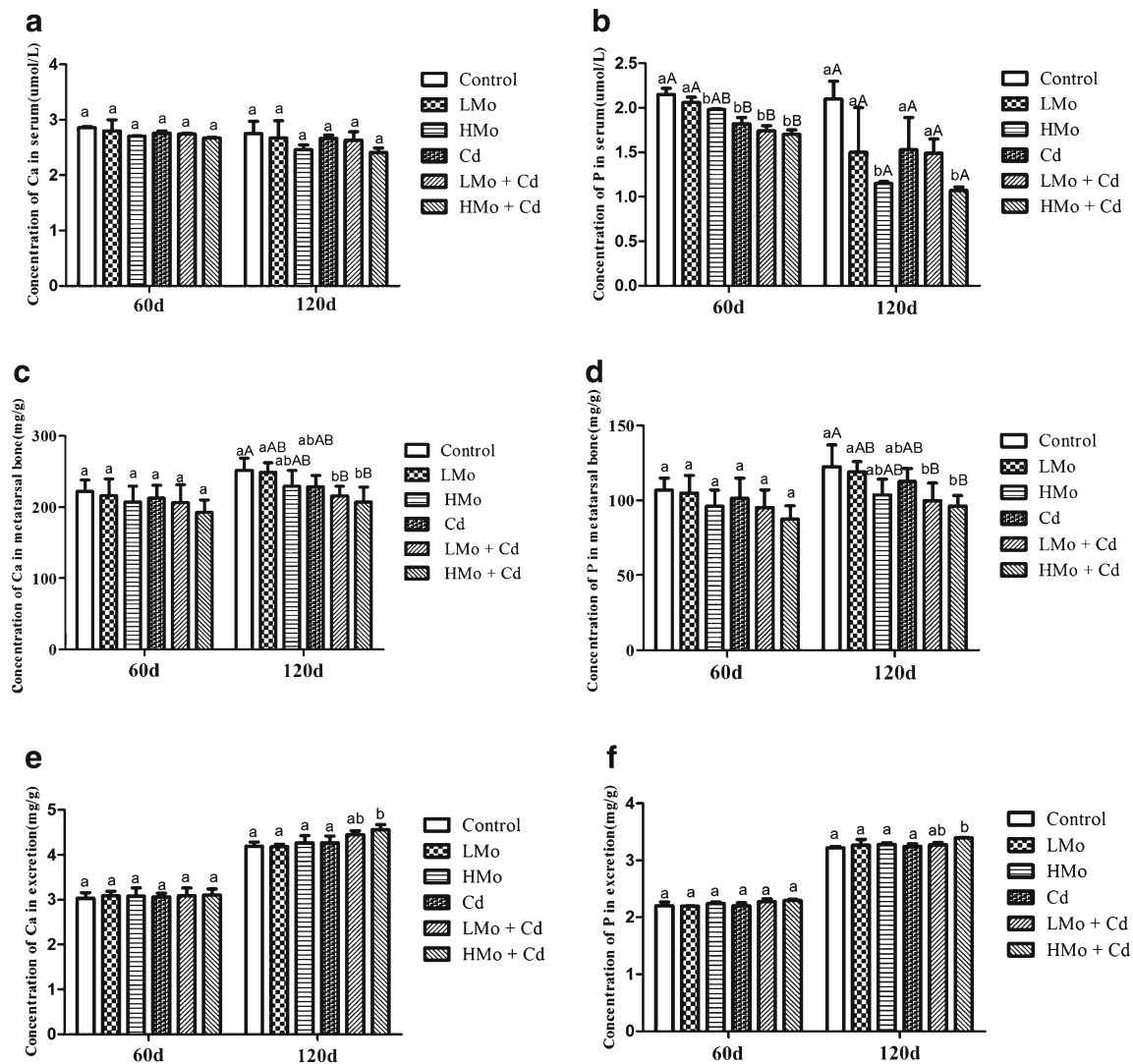
Fig. 1 The contents of Ca and P in serum, metatarsal, and excretion at 60 and 120 days. **a–f** The contents of Ca and P in serum, metatarsal, and excretion, respectively. Statistically significant differences: means with *different lowercase letters* are significantly different between groups ($P < 0.05$), means with *different uppercase letters* are highly significantly different between groups ($P < 0.01$), and means with *common lowercase or uppercase letters* are not significantly different between groups ($P > 0.05$). Each value represented the mean ± SEM. Below is the same

ALP Activity in Serum

As shown in Fig. 3, compared with control group, ALP activity had an increasing trend in HMo, LMo + Cd, and HMo + Cd groups at day 60, but there was no significant difference among these groups ($P > 0.05$); ALP activity was significantly increased in HMo group ($P < 0.05$) and highly significantly increased in Cd, LMo + Cd, and HMo + Cd groups ($P < 0.01$) at day 120.

Radiography of Metatarsal

Radiography of the metatarsal is shown in Fig. 4. The metatarsals in the control group showed no osteopenia during the experiment (Fig. 4a, g) on day 60, and radiography of metatarsals in LMo, HMo, Cd, and LMo + Cd groups had no osteoporotic lesions (Fig. 4b–e), but metatarsals in HMo + Cd group had obvious osteopenia (Fig. 4f); on day 120, significant osteoporotic lesions were not observed in LMo group and Cd group (Fig. 4h, j), X-ray penetrable increased in HMo and coinduced groups (Fig. 4i, k, l), and the bone mineral content deficiency was severe in HMo + Cd group (Fig. 4l).

Pathological Observation of Metatarsal

As shown in Fig. 5, under optical microscopy, the metatarsal of control group had many connected trabecular bones, showing the normal structure of trabecular bones during the experiment (Fig. 5a, g). At days 60 and 120, trabecular bone volume showed slight decrease in LMo,
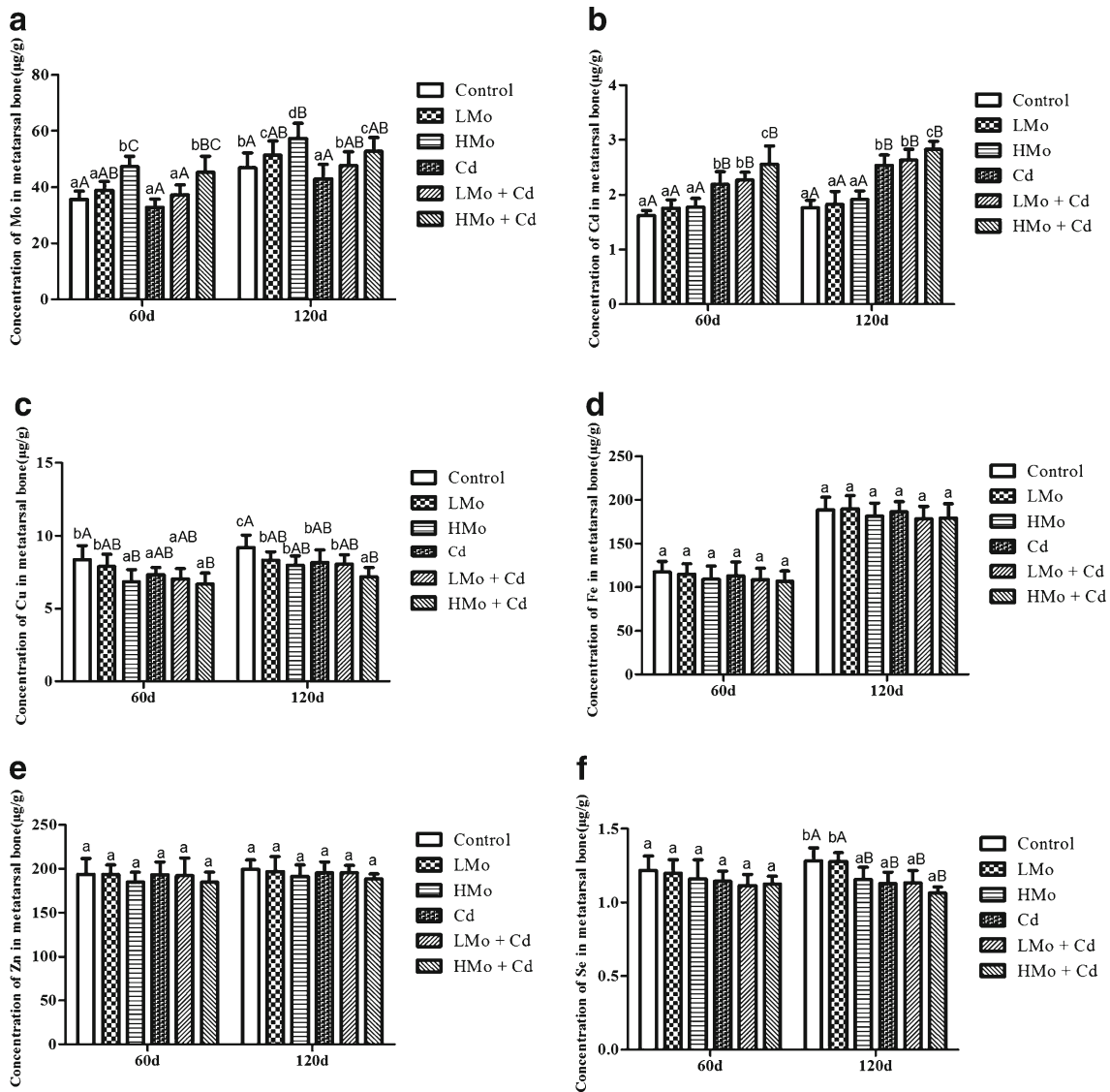
Fig. 2 The contents of Mo, Cd, Cu, Fe, Zn, and Se in metatarsal at 60 and 120 days. a–f The contents of Mo, Cd, Cu, Fe, Zn, and Se in metatarsal, respectively

HMo, and Cd groups (Fig. 5b–d, h–j), and LMo + Cd group and HMo + Cd group showed significantly less and thinner trabecular bones than control group (Fig. 5e, f, k, l). Moreover, trabecular bones arranged disorderly and deformity were observed in HMo + Cd group (Fig. 5f, l).
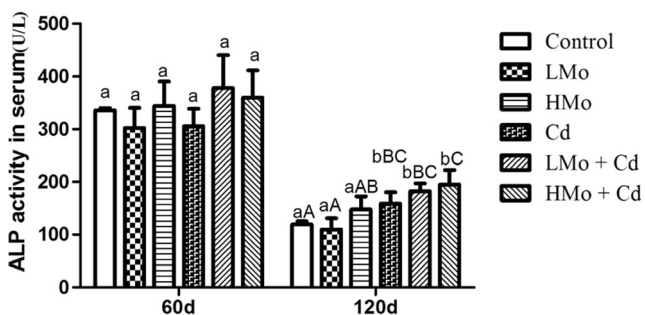

Fig. 3 ALP activity in serum at 60 and 120 days

Discussion

It is widely recognized that Mo and Cd can deposit in the bone and affect the bone tissue [19, 24], but nearly few study observed the combined effects of Mo and Cd on bone in ducks. This study was attempted to report the combined effects of Mo and Cd on bone in ducks based on a subchronic toxicity experimental animal model. The results of the study showed the contents of Mo and Cd increased significantly in co-treated duck bones. Mo could promote the secretion of serum parathyroid hormone (PTH) [25], and high level PTH could reduce bone mineral density because bone calcification got abnormal [26]. Cd inhibits the hydroxyapatite formation, resulting in bone mineral dissolution [27], and it not only accelerates osteoclast differentiation and activation but also inhibits osteoblast activity and promotes apoptosis, leading to the

Fig. 4 Radiologic observation results of metatarsal in ducks at 60 and 120 days. **a**, **g** Control group; **b**, **h** LMo group; **c**, **i** HMo group; **d**, **j** Cd group; **e**, **k** LMo + Cd group; **f**, **l** HMo + Cd group. *Lowercase letters* represent results at day 60; *uppercase letters* represent results at day 120
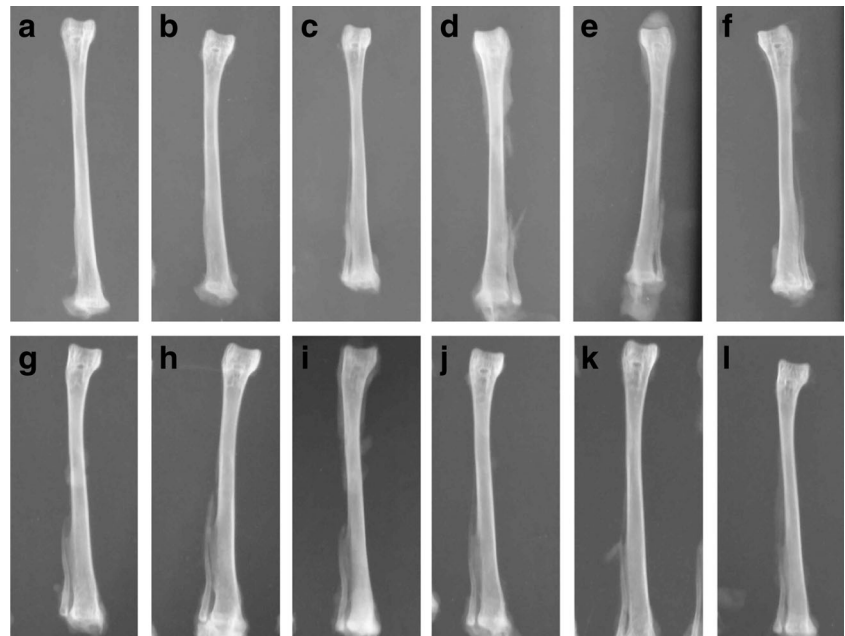

increased bone resorption [28]. Cd also disturbed the activation of vitamin D in the kidney and Ca and P metabolism [29].

Ca and P play critical structural roles in bone. Most of Ca and P in the body are in the form of hydroxyapatite which are mineral compositions of bone and tooth [30, 31]. Previous study also had shown that metabolism of Ca and P was intimately linked and should not be considered separately [32]. Ca or P deficiency could lead to lower bone mineral content and bone mineral density, osteomalacia, and osteoporosis [33, 34]. High dietary intake of Mo could decrease contents of P in serum and increase P loss in excretion [35]. And, cadmium can disturb Ca metabolism during osteogenesis and bone homeostasis, increasing calciuria and decreasing intestinal

Ca absorption [36, 37]. It has been suggested that Cd can inhibit hydroxyapatite formation and the Cd^{2+} ions competition with Ca^{2+} ions for incorporation into bone, resulting in bone mineral dissolution [38]. This study observed that exposure to Mo or/and Cd could disorder Ca and P homeostasis and change their mineral status; moreover, the concentrations of Ca and P in serum and bone decreased while this two elements contents were increased in excretion as a result of exposure to Mo and Cd. It suggested that combined action of Mo and Cd could disturb the metabolism of Ca and P.

ALP can effectively monitor the rate of bone mineralization [39]. Many studies reported that when bones were in growth and development stage, ALP activity was significantly

Fig. 5 Histological observation results of metatarsal in ducks at 60 and 120 days (HE staining, ×40). **a**, **g** Control group; **b**, **h** LMo group; **c**, **i** HMo group; **d**, **j** Cd group; **e**, **k** LMo + Cd group; **f**, **l** HMo + Cd group. *Lowercase letters* represent results at day 60; *uppercase letters* represent results at day 120
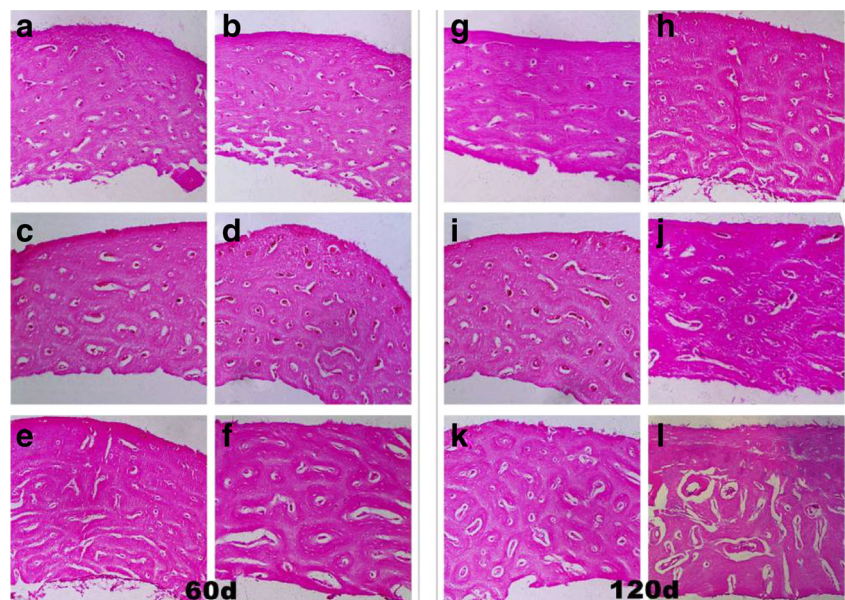
higher [40]. High dietary of Mo considerably increased the activity of ALP [41, 42], and different levels of Cd could disturb the metabolism of ALP [43], which affected the function of osteoblast and inhibited the degree of bone mineralization [44]. In this study, ALP activity was increased in ducks when coexposure to Mo and Cd, which suggested Mo combined with Cd could disrupt the bone salt deposition. What is more, ALP activity was higher in all groups on day 60 than that in day 120; the results were consistent with previous studies [40, 45].

The homeostasis of trace elements is necessary for the body to maintain protein structures and catalyze enzymatic reactions [46, 47]. The present study revealed that subchronic Mo or/and Cd exposure could lead their accumulation in duck bones, which disturbed the balance of trace elements such as Fe, Se, Cu, and Zn. Fe is an essential trace element for bone, and Fe deficiency could decrease bone mineral density and mass and alter microarchitecture [48, 49]. When molybdenosis transport and utilization of Fe became disordered, it caused Fe deficiency in the body [50]. Subchronic exposure to high Mo could decrease the Fe content in duck testicles [22], and Cd could decrease the level of Fe in mice pancreas [51]. In this study, Fe content in bones had a slight downtrend in treated groups compared with control group, but it had no significant difference, which indicated that a longer period of time or higher dosage of Mo or/and Cd exposure might reduce Fe concentration in duck bones. It has been reported that the relationship between Se and Mo was competitive for a regular intestinal transport mechanism in chick small intestine [52]. Se and Cd concentrations were also inversed in blood [53]. Animal experiments showed Se deficiency caused bone and joint abnormalities, including growth retardation and osteopenia [54]. Se deposition in bones revealed a decreasing tendency in HMo, Cd, LMo + Cd, and HMo + Cd groups on day 120 in this study, which suggested that exposure to Cd and high dose of Mo could cause Se decrease in duck bones. Cu is an important element for bone, and Cu deficiency contributed to the development of general osteoporosis and overgrowth of the epiphyseal cartilage [55]. Animals due to Cu deficiency have presented with inhibition of osteoblasts and crosslinking disorder between collagen and elastin, reducing strength of the bone matrix and impairment of bone formation [56]. It has been reported that high level of Mo in diet could cause cattle secondary Cu deficiency and intensify the disturbance of Cu absorption and excretion of Cu [57]. In this study, the concentration of Cu in metatarsals was decreased when Mo or/and Cd were added, which might indicate that Mo or/and Cd accumulation could cause Cu disorder. Zn is related to more than 200 enzyme activities and impaired bone growth and ossification would happen if Zn deficiency [58]. It has been

confirmed that the transporters of Zn were involved in Cd elevation in mammalian cells, and Mo could reduce the concentration of Zn in both liver and muscle [41, 59]. However, no obvious changes were observed in the deposition of Zn in bone in the study. The dose of Mo and Cd implemented or experiment time was possibly not enough to affect the metabolism of Zn in bones.

Bone damage is mainly manifested in the reduction of bone mineral content [60], and trabecular bones decrease and increase the risk of fractures [61, 62]. In the present study, X-ray transmittance increased in combination of Mo and Cd groups, which showed reduction of bone mineral content. In the pathological section of metatarsal, trabecular bone volume decreased and disordered trabecular bones were observed in HMo + Cd group. The results revealed that the combination of Mo and Cd could cause more severe bone damages.

Conclusion

In summary, dietary of Cd or/and Mo could lead to bone damages in duck via disturbing the balance of Ca and P in body and homeostasis of Cu and Se in bones; moreover, the two elements showed a possible synergistic relationship.

Compliance with Ethical Standards All animal care and experimental procedures were approved by the institutional ethics committee of Jiangxi Agricultural University.

Conflict of Interest Statement The authors declare that there are no conflicts of interest.

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