Influences of lead exposure on its accumulation in organs, meat, eggs and bone during laying period of hens

Shujie Wang,* Qiao Li,* Yanan Gao,† Zhenlei Zhou,*,[1] and Zhen Li†

*College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China; and †College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China

ABSTRACT Elevating levels of environmental lead (Pb) results in serious hazards to health of animals and human beings. In this study, daily diet with three different levels of Pb (Pb nitrate at doses of 1, 10, and 100 mg/kg body weight) were fed to ISA Brown layers. It showed that the kidney and liver have the relatively high Pb concentration (2.34 and 0.51 ppm) after culture, while the meat has the Pb concentration as low as 0.07 ppm (lower than the standard of Codex Alimentarius). It was also confirmed that egg laying worked as a potential pathway for hens to excrete Pb as Pb concentrations in eggshell and yolk increased from 0.10 to 3.11 ppm. However, the Pb concentration in egg white remains at a safe level (<0.10 ppm). Furthermore, even the intake of low dose Pb can cause a decline of bone mineral density and bone strength. Raman spot and mapping analysis indicated that carotenoids content in humerus from the hens of high dose group increased significantly, which hence can be applied as an indicator for resist stress. The degradation of bone quality will further damage the health of laying hens. Therefore, Pb exposure not only toxifies organs and reduces physiological features (e.g., body weight and laying rate) instantly, but also hurts poultry via degrading bone quality in long term. Additionally, the probability of excessive Pb in poultry meat is less than those of viscera and eggs, indicating its low risk to food safety.

Key words: laying hen, lead concentration, organ, egg, bone carotenoid

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INTRODUCTION

Lead (Pb) is currently one of the major heavy metal pollutants. With the rapid developments in painting, mining, and petroleum industry, anthropogenic Pb has been distributed all over the Earth, even in Antarctic snow and oceanic basins (Angelidis et al., 2011; Rosman et al., 1994). Due to the nonbiodegradable nature and continuous application of Pb-related materials, its levels rise in almost every country, posing serious threats to food security (Hu et al., 2017). The poultry sector is one of the largest sectors in agriculture, and more than 50 billion chickens are raised as food source yearly (Okoye et al., 2015). Therefore, exposure of poultry industry to Pb should be addressed.

Poultry products are enriched in nutritional value. Their eggs are important sources of high-quality natural proteins, as well as essential amino acids, fatty acids, carotenoids, vitamins, and essential minerals (Applegate, 2000; Kiliç et al., 2002; Surai and Sparks, 2001). Trace Pb concertation, as low as 1.0 mg/kg in feed can significantly affect the growth of broilers (Bakalli et al., 1995). In recent decades, chickens are constantly exposed to heavy metal pollution through poultry feed and water, which has increased at an unprecedented rate (Bortey-Sam et al., 2015; Temiraev et al., 2017). For example, in California, the daily Pb intake may exceed the recommended Pb limits for children when a child consumed one average-sized egg (Bautista et al., 2014; Sobhakumari et al., 2019). Moreover, Pb was detected between 10 and 167 μg/kg in 48% of New York City eggs (Spliethoff et al., 2014), over a 100 μg/kg guidance value.

The absorption and retention of soluble Pb is less than 10% of the total intake under normal dietary conditions (Van Barneveld and Van den Hamer, 1985). However, the absorbed Pb is distributed in organs through circulation and is then slowly excreted from them through urine or feces (Agrawal, 2012). For mammals and birds, Pb can be substantially accumulated in bones (Berglund et al., 2000; Scheuhammer, 1987). Pb deposition in bone is highly persistent due to the formation of stable Pb-phosphate complexes (Agrawal, 2012). Moreover, laying

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[1]Corresponding authors: zhouzl@njau.edu.cn

females accumulated much higher leaves of Pb in bone than that of non-layers (Finley and Dieter, 1978). The greater deposition of Pb may be related to the elevated Ca mobilization of bones during laying period (Wang et al., 2019b). Studies of birds have shown a remarkable decrease in the degree of mineralization as Pb concentration enhancing in bone. This may induce bone osteoporosis and bone weakness (Álvarez-Lloret et al., 2014; Fleming et al., 2000; Gangoso et al., 2009). The accumulation of Pb will lead to oxidative stress. Carotenoids are common pigments in birds, and are also powerful antioxidants because of their ability to quench singlet oxygen and to scavenge free radicals. It is proposed that maternally transferred carotenoids play an important role in maintaining redox homeostasis during embryonic development and the beginning after hatching (McGraw et al., 2005). Therefore, the antioxidant potential of carotenoids may protect bones from Pb toxicity.

Kidney and liver usually have the highest Pb content in organs (Custer et al., 1984). Recent literature suggested that the Pb concentration of chicken meat is much lower than that of kidney and liver, which can hence be considered as a safe source of human nutrition (Korish and Attia, 2020; Ogbomida et al., 2018). However, meat products still undergo a high risk of excessive heavy metals (Korish and Attia, 2020).

Poultry eggs might contain rising levels of environmental Pb due to the intake of Pb by hens (Demirulus, 2013; Kabeer et al., 2020). The nutritional components of eggs such as ovalbumin, and phosvitin can bind a variety of metals (Castellani et al., 2004; Dobrzański et al., 2007), which greatly increases the risk of heavy metal accumulation in eggs. The transport rate of Pb from female birds to eggs was considered to be significantly related to the concentrations in livers (Dauwe et al., 2005). Previous study illustrated that Pb may interact with Ca metabolism, making it easier for Pb incorporated into eggshells (Scheuhammer, 1987). In addition to the excretion of Pb through urine or feces, egg laying may hence be a pathway for female birds to excrete Pb (Dauwe et al., 2005). However, Pb accumulation in egg, shell, and protein has not been fully differentiated.

ISA Brown is a popular brown layer globally. It is estimated that one out of 10 farms cultivates ISA brown chickens. Extensive testing shows that the ISA Brown has exceptional feed conversion, capable of laying 500 high-quality eggs. Therefore, the ISA Brown layer was selected in this study to investigate the Pb concentration in egg white, yolk, shell, meat, and different organs of Pb-exposed hens. Additionally, Raman spectroscopy was used to detect bone composition changes.

MATERIALS AND METHODS

Animal Treatment and Sample Collection

Eighty ISA Brown layers (Qinglong Mount Hen Company, Nanjing, China), with an average initial weight of ~1.75 kg and age of 20 wk old. They were randomly divided into 4 groups, that is, control, low dose, medium dose, and high dose after a week of pre-feeding, with 5 replicates (4 layers/replicate) for each treatment. All birds were raised in a room with environmental control (temperature: 20 ± 5°C, relative humidity: 50 ± 10%, frequent ventilation, 16 h light and 8 h dark cycle). The hens had free access to water and were fed the diet according to the nutrition requirement of the flock. The diet contains 58 wt.% corn, 17 wt.% soybean meal, 19 wt.% wheat bran, 1 wt.% stone powder, and 5 wt.% of pre-mix. All the studied laying hens were healthy and no hormone was used. Low dose group, medium dose group, and high dose group were given Pb nitrate solution by intragastric administration each day, and the dosages were 1 mg/kg, 10 mg/kg, and 100 mg/kg respectively. The control group was given the same volume of ultrapure water. The eggs of each group were collected to calculate the laying rate, egg weight, and egg shape index. Hens were killed after 20 d of administration (stop feeding and Pb intragastric administration for 24 h before killed). All animal work was performed according to the Guidelines for Experimental Animals of the Ministry of Science and Technology, and protocols were approved by the Experimental Animal Welfare and Ethics Committee of Nanjing Agricultural University (#NJAU-Poult-2017003).

Heart, liver, spleen, lung, kidney, and pectoralis from each hen were collected, and then were rinsed with saline (0.9%). The samples were preserved at −20°C refrigerator before analysis.

Tibia, femur, and humerus were also collected from each hen and cleaned of all adherent tissue. The eggs of the twentieth day were cleaned with ultrapure water for later determination of Pb residues.

Pb Concentration

Samples (0.5 g) of heart, liver, spleen, lung, kidney, pectoralis, eggshell, egg yolk and egg white were soaked in 5 ml 4:1 HNO_3/HClO acid. The samples were heated by using a defined schedule of temperature. Then, samples were diluted with dilute nitric acid to adjust the final volume to 10 mL. The Pb concentration was analyzed by ICP-MS (iCAP Q ICP-MS, Thermo Fisher Scientific, Inc., Waltham, MA, USA) after filtration through 0.22 μm membrane. The concentrations of standard solution were 10, 20, 40, 60, 80, 100, and 120 ppb.

Bone Evaluation

The bone mineral density (**BMD**) of femur, tibia, and humerus were measured by a dual-energy X-ray absorption measuring instrument (InAlyzer, Medikors, Inc., Gyeonggi-do, Korea). The detection mode was set to fast scan with a high energy parameter of 80 kVp/1.0 mA and a low energy parameter of 55 kVp/1.25 mA. The acquired images were analyzed by using the InAlyzer 1.0 image processing system.

The bone strength of femur, tibia, and humerus were analyzed by the universal materials testing machine (LR10K PLUS, Lloyd Instruments, Ltd., Hampshire,

Table 1. Body weight and average daily feed intake (ADFI) of laying hens (n = 5).

		Control	Low dose	Medium dose	High dose
Weight (kg)	initial	1.74 ± 0.07	1.75 ± 0.02	1.77 ± 0.04	1.76 ± 0.05
	20d	1.79 ± 0.03[a]	1.66 ± 0.02[b]	1.72 ± 0.02[ab]	1.62 ± 0.05[b]
ADFI (g)		110.15 ± 1.19[a]	103.58 ± 0.82[b]	104.16 ± 1.20[b]	101.84 ± 1.02[b]

[a,b]No common superscripts within the row of each classification are significantly ($P < 0.05$) different.

UK). The bone samples were loaded to failure in three-point bending fracture mode. The testing bone was positioned on the support to match the mid-diaphysis, with the maximum stability under the loader. A vertical load at the rate of 10 mm/min was applied and remained constant until bone fracture.

Mid-diaphyseal segments (~1 cm long) were collected from humerus, and the marrow within the bones was washed away by ultrapure water. The humerus shafts (cortical bone) were carefully cleaned by ultrapure water and then air-dried. No additional chemicals were used in bone preparation. The changes in the chemical composition of bone were measured by Raman imaging microscope (DXR2xi, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cortical bone of humerus was detected. The spectral region of 100 to 3400 cm^{-1} was recorded using 532 nm laser. The laser power was set to 10.0 mw, and each point performed 200 × 0.01 s scans. The point spacing of Raman mapping measurements was 5 μm.

Statistical Analyses

All the data were analyzed using IBM SPSS Statistics 21 (IBM Corp., Armonk, NY). The differences among the groups were determined by one-way analysis of variance (ANOVA, Turkey and Dunnet's T3). The data are presented as the mean ± standard error. Significant differences were accepted if $P < 0.05$.

RESULTS

Weight and Egg-Laying Performance

The initial weight of the hens in control group, low dose group, medium dose group, and high dose group are 1.74, 1.75, 1.77, and 1.76 kg, respectively (Table 1). The weight value in the control group increased to 1.79 kg at 20 d, while the weight value in the Pb treated groups decreased. In addition, the weights of hens in low dose group ($P = 0.004$) and high dose group ($P = 0.001$) are significantly lower than that in the control group at 20 d.

The laying rate decreased after 10 consecutive days in the hens from the medium and low Pb treatment. However, the high dose Pb treatment significantly reduced egg production throughout the experimental period (Figure 1). The egg mass from the control group, low dose group, medium dose group, and high dose group after 20 d are 23.45, 22.76, 22.08, and 11.94 kg, respectively. All eggs have been tested for weight and egg shape index. Pb treatment had no significant effect on the index of egg shape (Figure 2). The egg weight value

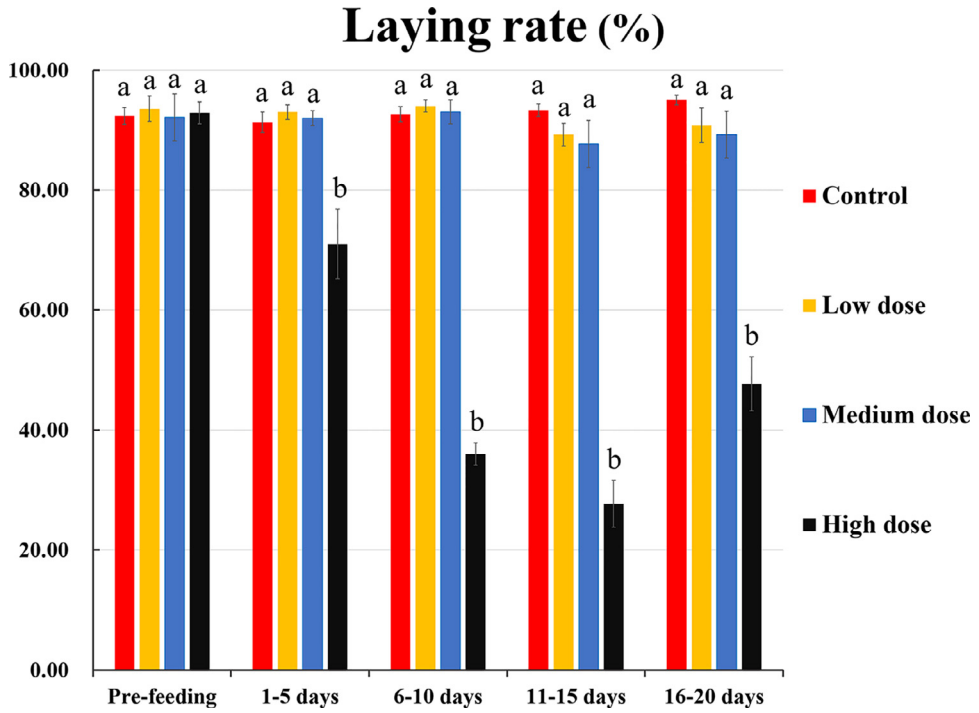

Figure 1. Laying rate of hens in different periods during 20 d administration of lead. Error bar represents the standard error. Bars with different letters between each group indicated the significant difference at $P < 0.05$ level.

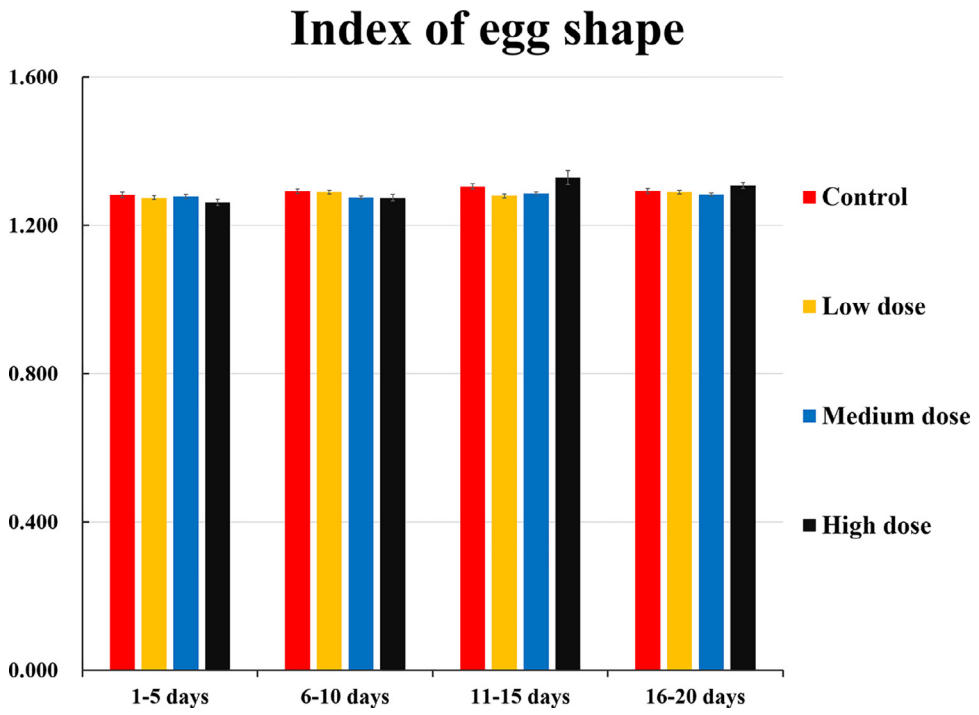

Figure 2. The index of egg shape in different periods during 20 d administration of lead. Error bar represents the standard error. There is no significant difference between each group.

in the medium dose and low dose groups slightly decreased, while the egg weight value in the high dose group increased significantly (Figure 3).

Pb Concentrations of Organs, Meat, and Eggs

There were no visible lesions in all organs. The Pb concentrations of all organs and meat increased with the Pb intake. The Pb concentrations varied greatly (see Figure 4). The kidney had the highest Pb content. It reached >10 mg/kg in high dose group, followed by liver, lungs, spleen, heart, and pectoralis in descending order.

The Pb concentrations of eggshell and egg yolk were also positively correlated with Pb intake (Figure 5). However, the Pb concentrations of egg white in the high dose, medium dose and low dose groups maintained at a very low level, similar to the control group (0.09 ppm).

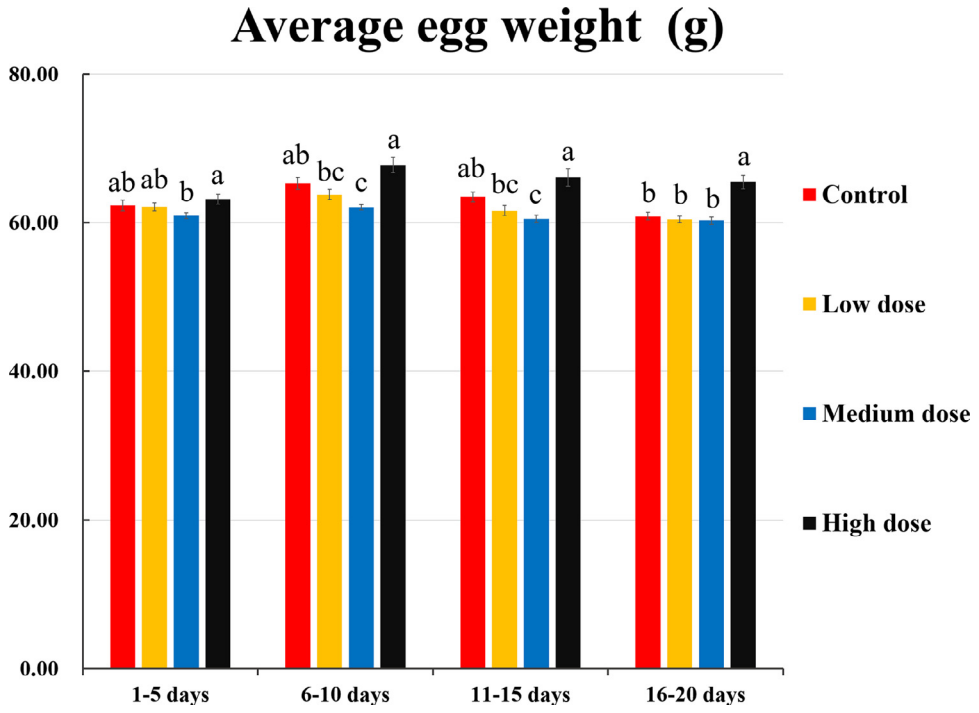

Figure 3. The average egg weight in different periods during 20 d administration of lead. Error bar represents the standard error. Bars with different letters between each group indicated the significant difference at $P < 0.05$ level.

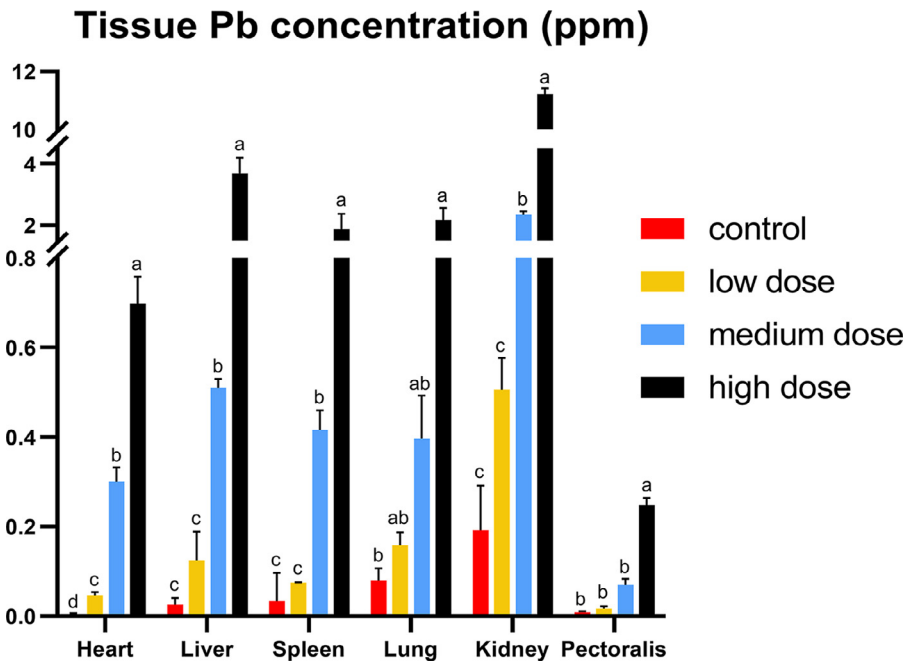

Figure 4. Pb concentrations in different organs and meat (n = 5). Error bar represents the standard error. Bars with different letters between each group indicated the significant difference at $P < 0.05$ level.

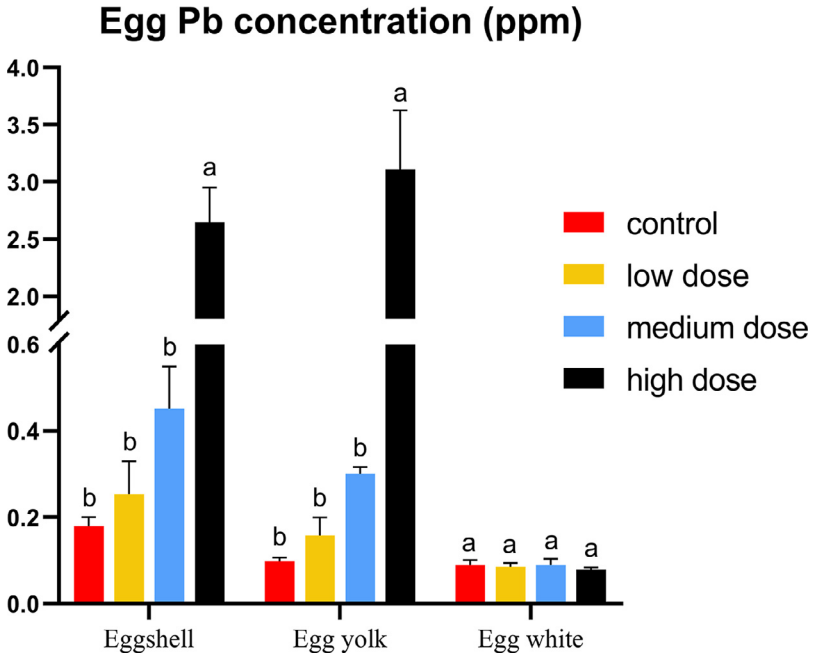

Figure 5. Pb concentrations in eggshell, egg yolk, and egg white (n = 5). Error bar represents the standard error. Bars with different letters between each group indicated the significant difference at $P < 0.05$ level.

Moreover, the Pb content of eggshell (0.18 ppm) was much higher than that of egg white (0.09 ppm) and egg yolk (0.10 ppm) in the control group.

BMD and Bone Strength

The BMD and bone strength of humerus were much lower than those of tibia and femur (Table 2). In addition, the BMD of tibia, femur and humerus in the low dose and medium dose groups was lower than that in the control group. This showed that relatively low dose exposure to Pb reduced BMD. However, the BMD of the hens in high dose group was higher than those in other groups. The trend of bone strength change was consistent with that of BMD. Meanwhile, bone strength value in the high dose group was significantly higher than that in the low dose group.

Raman Analysis on Humerus

In Figure 6, all the spectra were normalized to intensity of the 960 cm^{-1} peak, and the intensity can be applied to estimate the relative contents of the corresponding compounds (Li and Pasteris, 2014). The

Table 2. Bone mineral density and bone strength of laying hens (n = 5).

Parameters	Bone	Control	Low dose	Medium dose	High dose
Bone mineral density (g/cm^3)	Tibia	0.46 ± 0.01	0.44 ± 0.02	0.41 ± 0.01	0.48 ± 0.01
	Femur	0.43 ± 0.02	0.41 ± 0.03	0.40 ± 0.02	0.47 ± 0.02
	Humerus	0.24 ± 0.01	0.22 ± 0.01	0.24 ± 0.02	0.26 ± 0.02
Bone strength (N)	Tibia	128.82 ± 8.03	121.10 ± 3.25	124.24 ± 7.34	149.52 ± 9.97
	Femur	144.04 ± 11.83[ab]	121.42 ± 11.94[b]	130.70 ± 7.94[ab]	170.35 ± 14.92[a]
	Humerus	92.65 ± 3.34	81.57 ± 4.96	91.04 ± 7.07	97.92 ± 6.72

[a,b]No common superscripts within the row of each classification are significantly ($P < 0.05$) different.

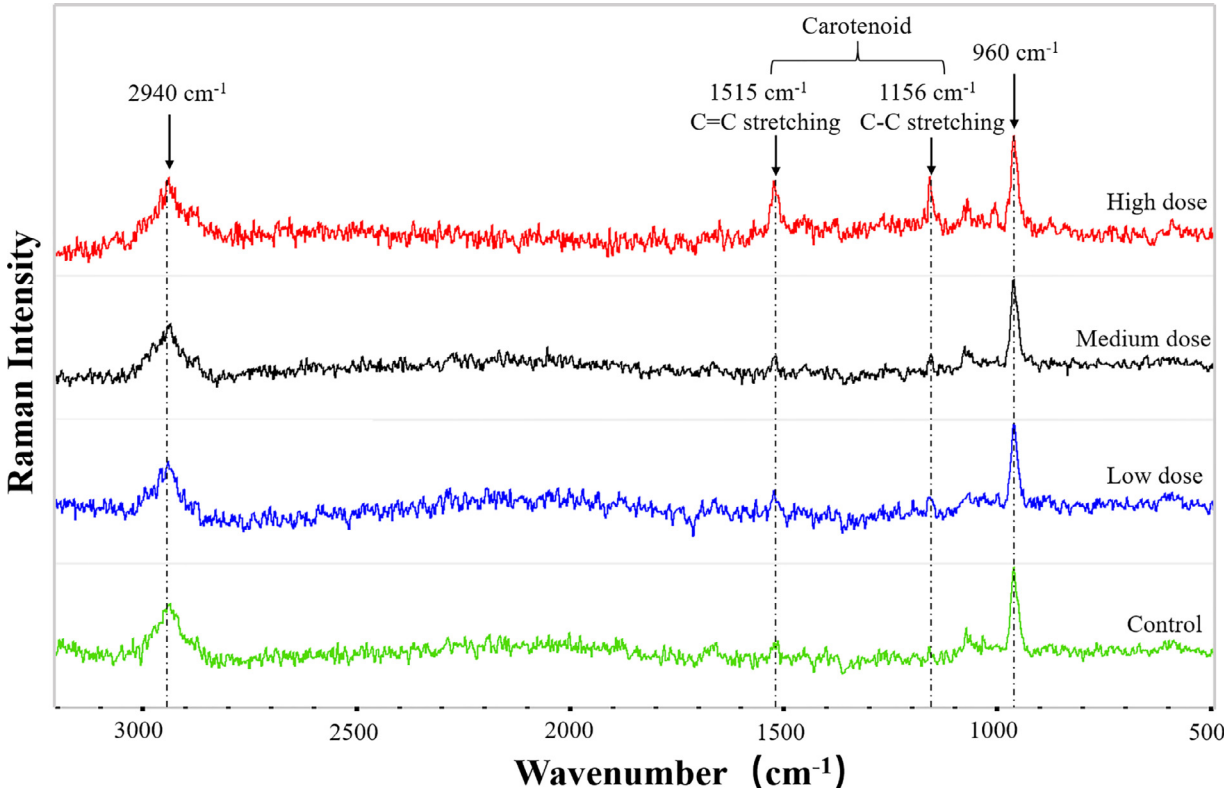

Figure 6. Raman spectra of humerus from the hens in three lead-treated groups and control group (n = 5). The intensity of the 1515/cm and 1156/cm peaks in high dose group was significantly higher than the other groups (All the spectra were normalized to the intensity of the 960/cm peak).

most intense peak in cortical bone was the ν1 P-O symmetric stretch located at ~ 960 cm^{-1}, which confirmed the phosphate phase in chicken bone (Li et al., 2013; Wang et al., 2017).

An envelope of peaks centered at ~2940 cm^{-1} was assigned to C-H stretching vibrations in multiple organic matters (Penel et al., 2005). Two strong peaks observed at 1,156 cm^{-1} and 1515 cm^{-1} can be attributed to the C−C and C=C stretching respectively, both of which are characteristic peaks of carotenoids (Wang et al., 2019a). The intensity of the characteristic peaks of carotenoids in high-dose group was significantly greater than that of other groups (Figure 6), indicating the raised content of bone carotenoids. However, the changes in peak intensity were not evident among the control, low dose group and medium dose groups. Raman mapping confirmed the elevated carotenoids on humerus surface (Figures 7 and 8). In particular, it showed that carotenoids were widely distributed in the cortical bone of high dose group.

DISCUSSION

The affinity of organs for metals is highly specific, varying with the chemical properties of metals and physiological properties of organs (Bowen, 1979; Storelli et al., 2006). Kidney has the highest level of Pb as it concentrates Pb through glomerular filtration (Goyer, 1971; Hall, 2016). Previous studies have identified several high-affinity lead-binding proteins in the kidney, which contribute to the excretion of Pb into urine (Fowler and DuVal, 1991; Gonick, 2011). The Pb concentration of liver was second to kidney. The liver is responsible for detoxifying various metabolites, which has been considered as the largest Pb repository of soft tissues in animals (Patra et al., 2001; Patrick, 2006). The liver contains a large amount of glutathione, which binds to the Pb and helps it to discharge through urine (McGowan and Donaldson, 1986). Meanwhile, the higher volume of blood in liver may also be the cause of its high Pb content (Morgan et al., 1977). However, the Pb

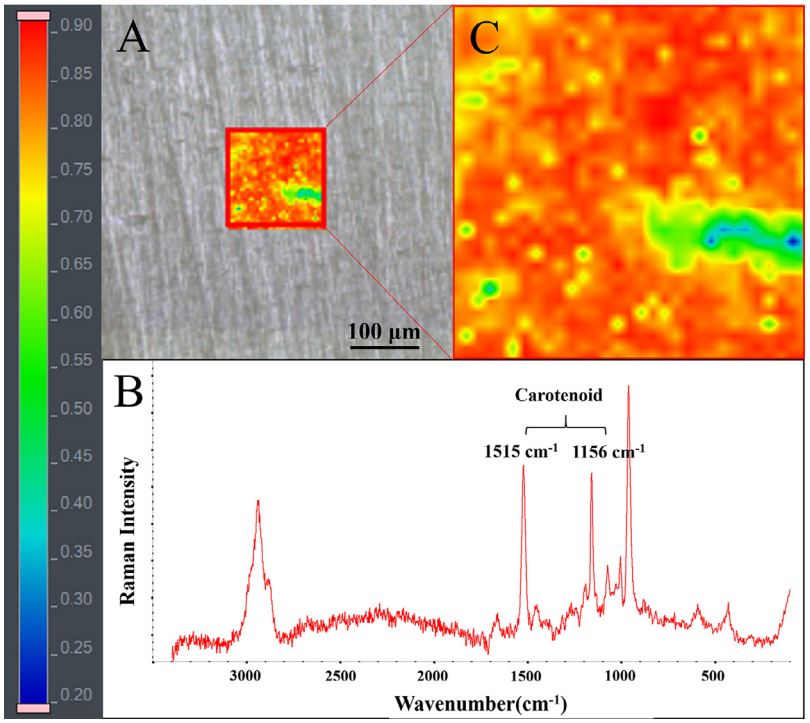

Figure 7. Raman mapping and spot analysis on the humerus collected from the hens in high dose group (n = 5). (A) The detection area of Raman mapping. (B) Representative Raman spectra of bone sample. (C) Distribution maps, the color of the scanning area represents the correlation with the representative Raman spectra.

content in muscle of laying hens exposed to Pb was much lower than that in viscera. Muscle cells are filled with protein filaments of actin and myosin, which have a relatively low affinity to Pb (Goering, 1993; Saladin and Porth, 2010; Tarrago and Brown, 2017). Lead intoxication in broilers indicated that muscles had the lowest deposition of Pb (Bakalli et al., 1995). The Codex Alimentarius Commission limits the Pb concentration in poultry meat and viscera to less than 0.1 mg/kg (0.1 ppm) and 0.5 mg/kg (0.5 ppm) respectively. Based on

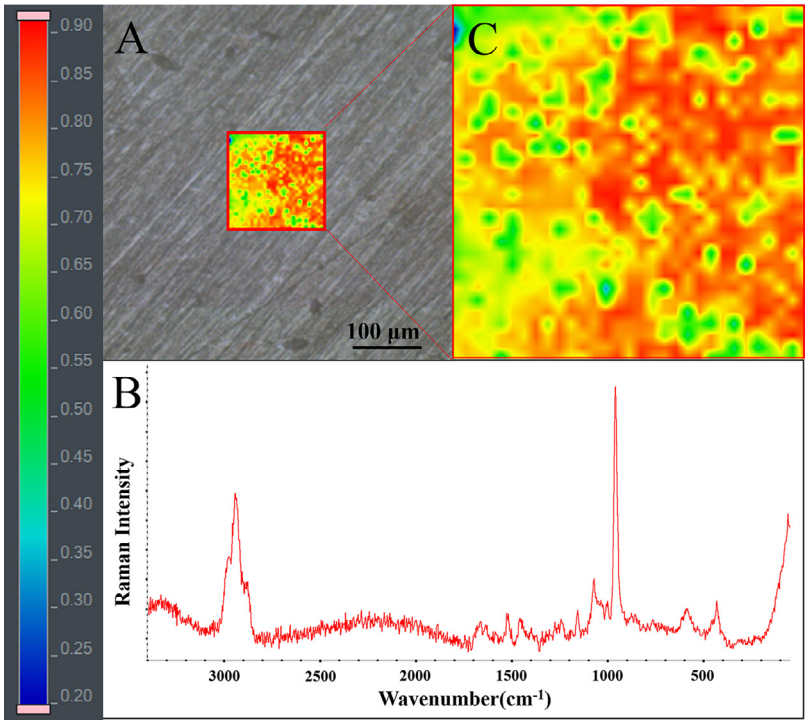

Figure 8. Raman mapping and spot analysis on the humerus collected from the hens in control group (n = 5). (A) The detection area of Raman mapping. (B) Representative Raman spectra of bone sample. (C) Distribution maps, the color of the scanning area represents the correlation with the representative Raman spectra.

our results, daily intake of 10 mg/kg Pb for laying hens will not cause excessive Pb content in poultry products, except liver and kidney.

Egg laying is an effective pathway for hens to excrete Pb, especially for eggshell (Burger, 1994). Pb cannot be metabolized in the body, and it is primarily excreted through urine (Hossain et al., 2014). In the control group, the Pb concentration of eggshell was raised to as high as that of kidney. The eggshell formation is the most rapid mineralization process in vivo, which occurs in uterine fluid as fast as 20 h (Nys et al., 1999; Sun et al., 2013). However, the uterus cannot store Ca^{2+} and Mg^{2+}. These cations are continuously transferred from the blood plasma via the uterine glandular cells (Jonchère et al., 2012; Solomon, 1971). The ion radius of Pb^{2+} and Ca^{2+} are similar. Then, Pb^{2+} may enter the uterine fluid from the blood through passive diffusion. Moreover, the solubility product of $CaCO_3$ is higher than that of $PbCO_3$ (Hu et al., 2019), suggesting that Pb ions in the uterine fluid may be rapidly mineralized in preference to Ca ions. Then, Pb in blood can be continuously incorporated into the eggshell along the concentration gradient. Without egg laying, blood Pb levels in roosters were hence significantly higher than that in hens exposed to the same Pb dose (Mazliah et al., 1989).

There is a significant difference regarding Pb concentration between egg white and egg yolk. The accumulation of most trace minerals in egg white is usually limited (Richards, 1997; Skřivan et al., 2005). Even if the laying hens take high dose of Pb, the Pb in egg white remains at an extremely low level (less than 0.09 ppm). This is probably due to the low ability of egg white to bind metal cations. To the contrast, egg yolk possesses a relatively high affinity towards Pb. The phosvitin of egg yolk is a heavily phosphorylated protein. Its unique primary structure makes this protein one of the strongest metal-chelating agents (Castellani et al., 2004). Furthermore, the precursor of phosvitin is synthesized in the liver, which accumulates abundant Pb. Overall, the Pb-enrichments in the eggshell and egg yolk indicate egg laying being as an important pathway for hens to excrete Pb. Therefore, Pb exposure primarily increases the risk of egg yolk contaminated by Pb, whereas egg white is relatively safe.

For birds and mammals, the highest concentration of Pb typically occurs in bone (Bakalli et al., 1995; Berglund et al., 2000; Hossain et al., 2014; Trampel et al., 2003). It was suggested that Pb can affect osteoblast and osteoclast function, and may increase the risk of fractures or osteoporosis (Berglund et al., 2000; Pounds et al., 1991). Therefore, although bone is not the edible part of chicken, the changes in bone can be used as important indicators for Pb exposure and poultry health (Álvarez-Lloret et al., 2014). Moreover, it is worth noting that Raman analysis demonstrated the sharply elevated carotenoids in the bone of high dose group. It was reported that exposure to heavy metals can cause oxidative stress (Sánchez-Virosta et al., 2015). Then, the antioxidant potential of carotenoids may protect bones from Pb toxicity (Fiedor and Burda, 2014; Flora et al., 2013; Vallverdú-Coll et al., 2015). Furthermore, carotenoids have stimulatory effects on osteoblastic bone formation and may increase BMD (Wattanapenpaiboon et al., 2003; Yamaguchi, 2012; Zhang et al., 2016). In this study, we firstly identified evident enhancement of carotenoids in chicken bone by using Raman spectroscopy, which allows rapid and accurate estimation of carotenoids in fresh bones. Therefore, carotenoids have the potential to be applied as a new reference indicator for Pb contamination in laying hens.

The intake of Pb nitrate as low as 1 mg/kg can cause a decrease in BMD, which can be identified in vivo by X-ray instrument and computed tomography. The decreased bone strength will increase the risk of fracture. BMD and bone strength both increased in high dose group. This phenomenon might be attributed to the following two reasons. Firstly, the egg laying rate of the high dose group dropped significantly, resulting in a decrease in calcium loss due to egg production. Secondly, the elevated carotenoid content in bone reduced the toxic effects of Pb and may also promote the bone formation.

In summary, viscera and egg yolks are prone to Pb deposition, especially for liver and kidney. A Pb concentration as low as 1 mg/kg intake for laying hens may cause excessive Pb content. Children living in a lead contaminated area should avoid eating locally produced egg yolk products and animal offal. In addition, the eggshell and animal bones in polluted areas cannot be used as calcium supplements for animal feed. The yolk can be selected for detection, or the mixture of egg yolk and egg white (must be mixed evenly). Moreover, continuous Pb exposure impairs bone quality, including a decrease of bone density and bone strength. Raman analysis indicated that bone carotenoids content of hens in high dose group sharply increased, which has great potential to be a new indicator for heavy metal stress.

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DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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