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Excessive copper in feed not merely undermines animal health but affects food safety

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ABSTRACT

Background: Blackened intestines in slaughtered pigs have been commonly observed in China in recent years. However, no cause has been reported.
Objectives: We attempted to determine whether the blackening of the pig intestine was related to an excess of copper (Cu) in their feed.
Methods: In this study, we observed and collected porcine intestines in small- and large-scale pig slaughterhouses in Shandong province from May to October 2018. Twelve types of metal ions were detected in the black intestinal samples.
Results: The Cu level in the intestine samples was mostly higher than the Chinese national limit for food. Further study showed that Cu supplementation in most commercial porcine feed also exceeded the national standard. An animal model (mouse) that could mimic the intestinal blackening in pigs was established. Compared to control mice, Cu accumulated in the liver and intestines of mice fed an excessive Cu level, confirming the excessive Cu in the feed may be considered the major cause of blackened porcine intestines. Microscopic examination revealed that black intestines had many particles containing Cu in the lamina propria of the intestinal mucosa, and the intestinal mucosal epithelial cells showed degeneration and necrosis.
Conclusions: In conclusion, overuse of Cu in animal feed can lead to animal poisoning and Cu accumulation in animal products. Such overuse not only harms the health of livestock but can also affect public health.

Keywords: Pork products; Cu; black intestine; copper poisoning; feed

INTRODUCTION

Pork is one of the most popular meats in China, and there is also a notably large consumption of pork around the world. Pork meat quality and related food safety issues always attract societal attention, even leading to food safety crises. In recent years, it was not uncommon to observe blackened intestines in slaughter pigs in China. The blackened appearance of

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Liu M; Data curation: Ma Z, Li Y, Han Z, Liu Z, Wang H, Meng F; Formal analysis: Liu S, Chen D, Liu M; Funding acquisition: Liu S; Investigation: Ma Z, Li Y, Han Z, Liu Z, Wang H, Meng F, Liu M; Methodology: Ma Z, Chen D, Liu M; Software: Ma Z, Han Z, Liu M; Supervision: Liu S, Liu M; Validation: Ma Z, Li Y; Writing - original draft: Ma Z; Writing - review & editing: Liu S, Chen D, Liu M.

affected intestines could not be removed, and no common pathogens were detected. The inferior-appearing pork product bothered slaughterhouse owners because it was associated with considerable economic loss and potential food safety risks [1]. However, there were no conclusive reports on the cause of the blackening.

A potential factor we first considered was copper (Cu) poisoning. Cu is a commonplace animal feed additive due to its vital biological significance for animal growth and development. It is an important component of many enzymes and is involved in many important metabolic pathways, including regulating cell respiration, and it is involved, neurotransmitter transmission, anti-stress, anti-oxidation, assimilation of relevant metal ions, and other cellular functions [2-6]. According to the "Safety Standards for Feed Additives" (No. 2625) issued by the Ministry of Agriculture and Rural Affairs of the People's Republic of China, the maximum concentration of Cu in feed is 125 mg/kg for piglets less than 25 kg, while the Cu content in feed for fattening pigs should be in the range of 3 to 6 mg/kg and cannot exceed 25 mg/kg.

Nevertheless, for the purposes of growth promotion and hair brightening, excessive Cu may be intentionally added to animal feed by some feed manufacturers [7]. It has been reported that excessive Cu in feed may stimulate the gastrointestinal mucosa of pigs and lead to porcine gastroenteritis, swollen liver, and kidney degeneration [8]. It also affects the absorption of iron and zinc ions in the gastrointestinal tract, resulting in anemia, hemolysis, and jaundice, and even threatens animal and human health [9,10].

Previous research into Cu poisoning has mainly focused on the damage done to the animals' intestines. No study has reported that Cu poisoning can cause intestines to blacken. Based on the above background information, we speculated that the blackening of pig intestines was caused by Cu in feed. In order to explore the reason of the black intestines, we conducted a systematic investigation that included detection of metal ions in blackened intestines and animal feed and an animal model feeding experiment.

MATERIALS AND METHODS

Ethics approval

All protocols and procedures were performed according to the Chinese Regulations of Laboratory Animals (State Council of the People's Republic of China, available from http://www.gov.cn/gongbao/content/2017/content_5219148.htm). The study was approved by the Committee on the Animal Ethics of Shandong Agricultural University. Experiments were carried out in accordance with approved guidelines (No. SDAUA-2016-004).

Experimental materials

BALB/c mice, mouse feed, and $CuSO_4 \cdot 5H_2O$ were purchased from the SPF Biotechnology, China. Russian Blue Iron Stain and Cu salt dye were purchased from Beijing Leagene Biotech, China, and the In Situ Cell Apoptosis Detection Kit I (POD) was purchased from Boster Biological Technology, China. Mouse interleukin 2 (IL-2) enzyme-linked immunosorbent assay (ELISA) kit and mouse ovalbumin specific immunoglobulin G (OVA sIgG) ELISA kits were purchased from Cusabio Technology, USA. Ovalbumin was purchased from Thermo Fisher (China), China. Inductively coupled plasma-mass spectrometry (ICP-MS) was performed by Thermo Fisher (China), China. A microwave digestion instrument, CEM

MARS6, was purchased from Pynn Corp., USA, and an enzyme-labeled instrument was purchased from Tecan, Australia.

Black pig intestines were randomly collected in slaughterhouses, and pig feed was randomly purchased from animal feed markets in Shandong province, China. The RAL K7 colorimetric card was purchased from RAL, Germany. Macroscopic pictures were taken with a Sony Camera a6000, and microscopic pictures were obtained using an Olympus CX41 microscope. The photographs did not undergo post-processing.

Samples collection

We observed and collected porcine intestines in large-scale pig slaughterhouses in Shandong, China. Forty intestines were randomly selected every Monday from May to October 2018. Their origin was recorded, and their color was compared with those on the RAL K7 colorimetric card. If the intestine color was darker than RAL-7021 (black-grey), the sample was considered a black intestine. Twenty black intestinal tissue samples and five normal ones were collected for further examination. Twenty-two brands of commercial pig-fattening feed were randomly bought from several markets in Shandong province.

Sample handling and metal ion detection

Screening for the presence of 12 ion types, Cu, Fe, Zn, Mn, Al, Cr, Co, Ni, As, Se, Cd, and Pb, was performed for the collected pig intestinal tissue samples. A tissue sample (0.5g) was mixed with 5 mL 65% nitric acid and 1 mL of 30% hydrogen peroxide and then digested by using the CEM MARS6 microwave digestion instrument. When digested, the tissue sample was supplemented with sterilized distilled water to obtain a 25 mL sample. The ion content of the sample was quantitatively determined by ICP-MS [11]. In addition, intestinal tissue samples were fixed in 10% formalin solution and embedded in paraffin. Sections of embedded samples underwent hematoxylin and eosin (H&E), Prussian blue, or Cu salt staining. The Cu, Fe, Zn, As, Cd, Pb, and Cr content in the pig feed samples were determined by ICP-MS.

Grouping and feeding of mice experiment

A mouse model was used to simulate the process of Cu poisoning in pigs. Eighty, 21-day-old weaned BALB/c mice (40 male and 40 female) were equally assigned into one of four groups (A, B, C, and D). In each group, five mice of the same gender were raised in an individually ventilated cage with sufficient space for activity. Their feed and clean water were checked and supplemented every day. The animal housing area provided day-night alternation to ensure 12 h light and 12 h darkness. Ambient temperature was maintained at 26°C and the relative humidity at 50%. The cage pallets were cleaned twice a week.

Each group of animals was raised under comparable experimental conditions except for type of feed. According to the People's Republic of China experimental animal compound feed nutrient composition standard (GB14924.3-2010), the Cu ion content of experimental mouse feed should be not less than 10 mg/kg and not over 20 mg/kg. The concentration of Cu ions in the mouse feed was dependent on the level of Cu in the sampled pig feed. The Cu content in the mouse feed was adjusted as the lifespan of mice was shorter than that of pigs.

The amount of Cu added to the feed of each group differed. Group A was the control group and the Cu level in the feed was within the limits of the GB14924.3-2010 standard. Group B: Cu was added to the feed to achieve a level of 1,000 mg/kg (50 times the upper standard limit). Group C: Cu was added to the feed to obtain a 2,000 mg/kg level (100 times the upper

standard limit). Group D: Cu content in feed was adjusted to obtain a level of 4,000 mg/kg (200 times the upper standard limit). The Cu ion content in the feed was determined based on the relative molecular mass of $CuSO_4 \cdot 5H_2O$.

Mouse indicators

Body weights of all mice were measured each week. The average initial body weight and the average weight gain of each group were calculated. The feed and water intakes of each of the groups were recorded.

Mouse immune factor detection

Once the mice were weaned, they were injected with ovalbumin at a dose of 100 μg each. One week later, they were injected with a second dose, and the feeding experiment started. Three mice from each group were randomly selected for tail blood sampling each week, and one of them was euthanized for necropsy. The collected blood serum was examined to assess immunoglobulin G (IgG) and IL-2 immune factor levels by following the ELISA kit manufacturers instructions.

Animal welfare experiment

At the beginning of the animal experiment, all mice were healthy, lively, and their fur was assessed as bright. Their initial scores were all set as 10, and each mouse's score was reduced by one when they had one of the following conditions: messy fur, signs of mental depression, aggressive behavior, irritability, marasmus. For welfare assessment, mice were observed for one hour under light conditions every day. The average scores of each group were calculated weekly. Observations continued for six weeks.

Mouse organ assessment

Mouse colon, liver, and muscle samples were collected from each necropsy. A portion of the colon tissue was fixed in phosphate-buffered formalin (4%) for 24 h before being processed for H&E staining, Cu salt staining, or cell apoptosis assay. The liver and muscle tissues were examined for Cu content using the same detecting method as that intestine tissues.

Statistical analysis

Statistical analyses were conducted using Statistix version 9.0 (Analytical Software, USA). The Mann-Whitney *U* test was used to assess differences in the IgG levels of the different groups. Differences in IgG level, IL-2 level, body weight, and feed intake between the different groups were determined by applying two-sample *t*-tests or Wilcoxon rank-sum tests. Statistical significance was designated as $p < 0.05$.

RESULTS

Detection of black pig intestines

We examined 960 porcine intestines from slaughtered pigs in slaughterhouses in Shandong province. Field observations revealed that blackened portions the porcine intestines were mainly in the rectum and posterior colon areas (**Fig. 1**). The overall black intestine detection rate was 22% (211/960). The black intestine detection rate was 4% (28/686) in samples collected from large-scale farms (> 300 pigs/farm) and was 59% (162/274) in samples collected from small-scale farms (< 300 pigs/farm). The black intestine detection rate of the slaughtered pigs was significantly related to the farm size ($p < 0.05$; χ^2 test).

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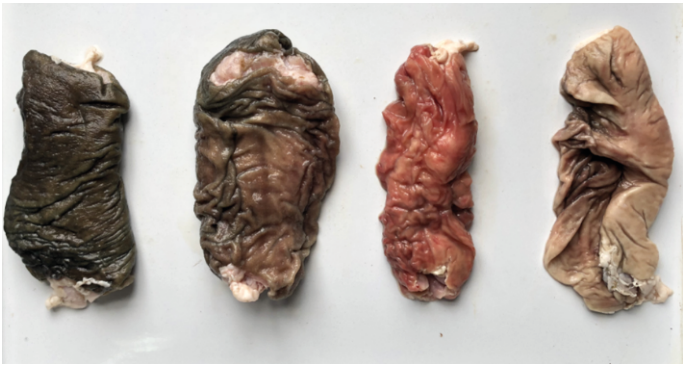

Fig. 1. Blackened porcine large intestines.

Contents of metal ions in porcine intestinal tissues

Twenty black porcine intestines and five normal ones were collected (**Supplementary Table 1**). Compared to the average Cu content in the five normal porcine intestinal tissues (0.156 mg/kg), the 20 black intestinal tissues' average Cu content was significantly higher (73.423 mg/kg). The black intestine Cu level was remarkably higher than the 10 mg/kg Cu limit in feed specified by standard GB15199-1994. The content of Cu in the black intestine tissues was 471 times that of the control tissues. The black intestines' Fe content was also higher than that of the control group, but no significant differences were detected among the other ions assessed in the two groups.

Metal ion content in feed

Seven metal ions were detected in the pig feed (**Supplementary Table 2**). According to the feed additive requirements issued by the Ministry of Agriculture and Rural Affairs of the People's Republic of China, the Cu content in finishing feed should be within the 3–6 mg/kg range, and the maximum should not exceed 25 mg/kg; similarly, the acceptable content values for Fe and Zn are 40–100 mg/kg and 80 mg/kg, respectively. The test results showed that only five brands of feed obtained from feed markets did not exceed the Cu limit, but all far exceeded the national standards of Fe and Zn.

Pathologic changes in black porcine intestines

Histopathological observation was performed by using H&E, Cu salt, and Prussian blue staining. **Fig. 2A, C, and E** presents results from black porcine intestines, while **Fig. 2B, D, and F** present control intestine results. Microscopic examination using H&E stain (**Fig. 2A**) revealed an increased number of brown macrophages in the lamina propria of the intestinal mucosa of black intestines, and scattered tan granules were observed on the outside of the cells. The intestinal mucosal epithelial cells showed mild degeneration and necrosis. Cu salt staining (**Fig. 2C**) resulted in Cu ions being stained dark green while macrophages and some granules were stained brown in the intestinal mucosa's lamina propria. Prussian blue staining (**Fig. 2E**) resulted in Fe ions appearing blue. Microscopic examination revealed only a few blue particles in the cytoplasm of macrophages in the intestinal mucosa's lamina propria. A small number of blue particles were scattered around the macrophages. The results showed that macrophages in the lamina propria of the intestinal mucosa phagocytized a large amount of Cu, and additional Cu particles were scattered around the macrophages.

Mouse experiment data

The mice in groups A, B, and C survived for more than 6 weeks, but all mice in group D died within 1 week. The average body weights of each group are summarized in **Table 1**.

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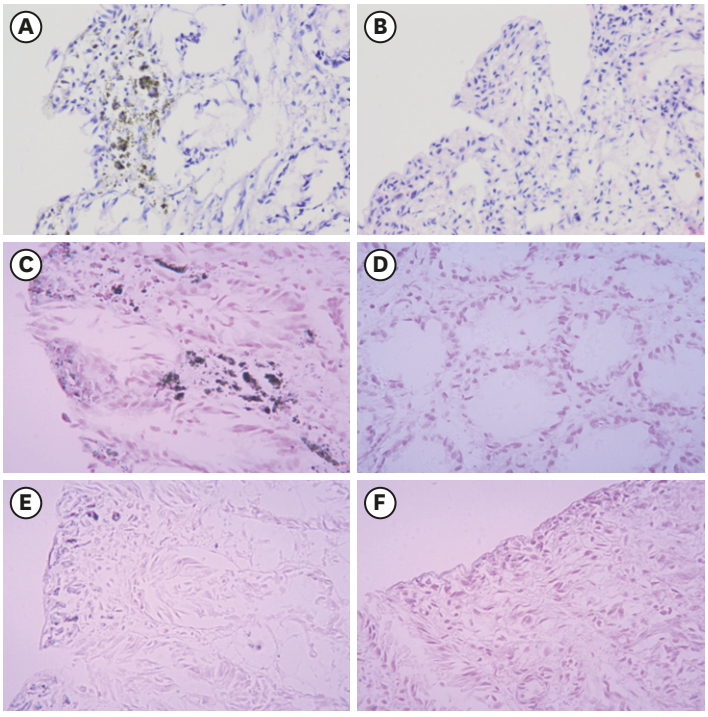

Fig. 2. Microscopic examination of pigs' large intestines subjected to 3 different staining methods.
(A) There are many brown granules in the lamina propria of the intestinal mucosa, and the macrophages are brown. (B) No brown granules shown. (C) The lamina propria of the blackened intestinal mucosa is dark green, and a small amount of dark green particles are also visible around the mucosa. (D) No dark green particles present. (E) The blackened intestinal mucosa has a small number of blue particles in the macrophages' cytoplasm. (F) No blue particles are present. (A, B) (hematoxylin and eosin stain; ×400), (C, D) (copper salt stain; ×400), (E, F) (Prussian blue stain; ×400).

Considering the differences in initial body weights, we compared the weight gain of groups A, B, and C every week. Group C's weight gain was significantly lower than that of the other groups (**Fig. 3A**). Over the duration of the experiment, average water intake increased slowly, with no significant differences among the groups (**Fig. 3B**). Group B had a significantly higher average feed intake than that of group C from the fifth week onward (**Fig. 3C**). The levels of serum IL-2 and IgG are summarized in **Fig. 3D and E**. The results show that as the intake of Cu increased, the IL-2 and IgG levels gradually decreased. Over the duration of the experiment, the ceca of the mice in groups B and C became black, and their upper small intestine appeared to deepen in color, while mice in group A had no apparent lesions. The animal health scores are presented in **Fig. 3F**.

Table 1. Average body weight of mice in each study group (unit: g)

Group	First immunization	Second immunization	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
A								
Male	10.1	15.6	19.8	20.8	22.6	23.2	24.4	25.3
Female	9.3	12.8	16.5	18.1	18.9	19.5	20.2	21.0
B								
Male	9.8	15.4	18.8	19.9	22.4	25.7	27.5	28.8
Female	9.1	12.4	15.7	16.9	18.1	19.4	20.4	21.6
C								
Male	10.1	15.8	14.6	17.7	19.2	19.9	20.4	20.9
Female	9.0	12.5	11.6	13.1	15.4	16.1	17.3	18.1

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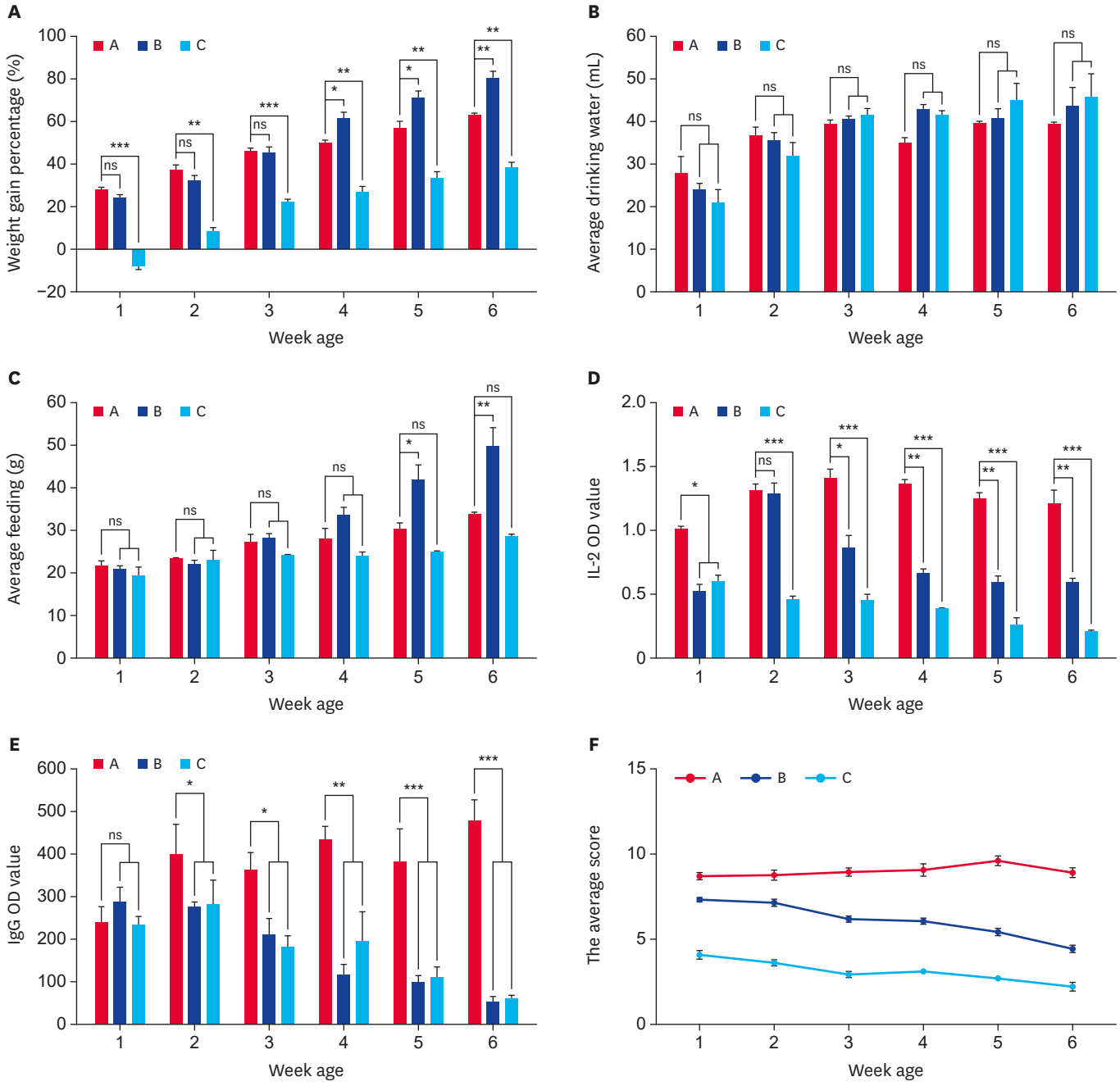

Fig. 3. Data records for animals in the mouse-model experiment. (A) Weight gain of mice in different weeks and different experimental groups. (B) Average water intake of mice in different weeks and experimental groups. (C) Average food intake of mice in different weeks and experimental groups. (D) Serum IL-2 antibody content in different groups of mice. (E) Serum IgG antibody content in different groups of mice. (F) Animal health scores.
NS, not significant; IL-2, interleukin 2; OD, optical density; IgG, immunoglobulin G.
$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Mouse necropsy

Mouse autopsies were conducted in the first and sixth week (**Fig. 4**). The intestines from group A had no visible lesions during the experiment. The ceca and feces from mice in groups B and C became black in the first week. Moreover, the upper small intestines of the mice in group C also began to darken in the first week.

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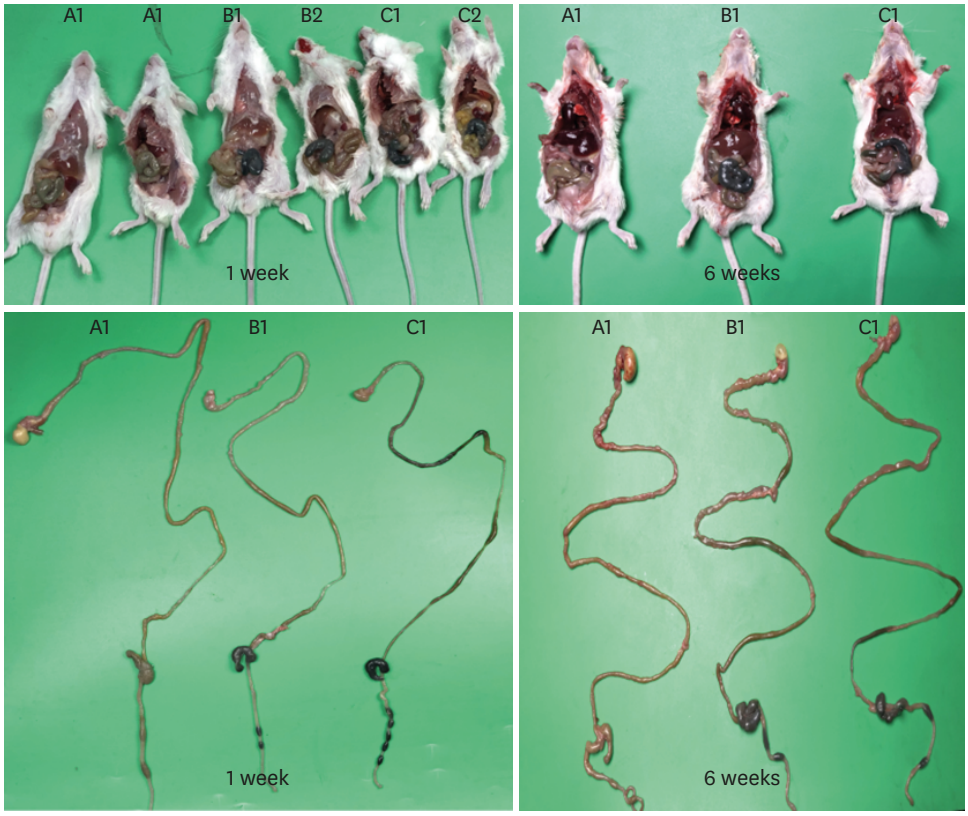

Fig. 4. Mouse necropsy results in different weeks. Two mice of groups A, B, and C were necropsied in the first week, and additional mice were necropsied in the sixth week. The intestines from group A remained normal, while the ceca and feces from mice in groups B and C began to darken in the first week.

Detection of Cu content in mouse organs

The Cu levels within the sampled mouse organs are shown in **Table 2**. Compared with the conventional feed group (group A), the levels of Cu in the intestines and livers of the high-Cu diet group (groups B and C) were extraordinarily high and gradually increased with time. In the sixth week, the intestinal Cu level of mice in group B was almost 100 times than that of mice in group A. In group C, the Cu level was 407.1 mg/kg in the first week, similar to that detected in group B in the sixth week of sampling. The results indicate that mice fed a high Cu content feed can experience a significant accumulation of Cu in intestines and livers.

Pathologic changes in black mouse intestines

The results of the histopathological examinations of large intestine tissue samples are shown in **Fig. 5**. The samples collected from the first week of the mouse experiment are shown in **Fig. 5A and B** shows results from the sixth week of the experiment. **Fig. 5A** shows results for

Table 2. Detection results of copper in organs of mice (unit: mg/kg)

Weeks	Intestine			Liver			Muscle		
	A	B	C	A	B	C	A	B	C
1	5.3	145.0	407.1	39.8	99.9	152.5	1.11	1.31	1.52
2	5.1	161.2	711.6	40.3	104.4	176.3	1.03	1.29	1.73
3	5.8	198.9	1,293.8	42.1	139.1	191.9	1.09	1.37	1.60
4	5.0	231.0	1,322.9	40.9	167.8	203.3	1.25	1.61	1.81
5	5.1	371.5	1,374.2	37.3	193.4	229.0	1.20	1.59	1.71
6	5.3	483.6	1,455.6	41.4	222.9	287.3	1.16	1.58	1.93

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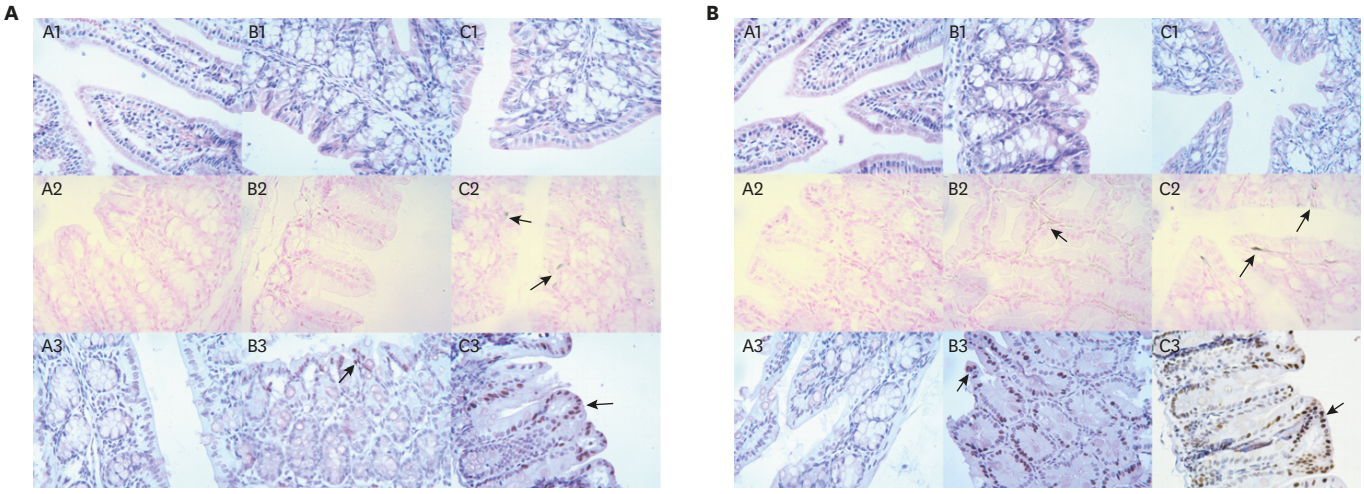

Fig. 5. Pathological sections of mice organs. (A) First week. Group A: no pathological changes detected. Group B: apoptosis occurred in a few cells. Group C: few dark green Cu ions present and cell apoptosis had occurred. (B) Sixth week. Group A: no pathological changes observed. Group B: H&E staining showed a slight thickening of intestinal mucosa and slight edema of intestinal cells; Cu ions were observed in the intestinal mucosa's lamina propria (Cu salt staining result); mucosal surface appeared to undergo necrocytosis (cell apoptosis assay result). Group C: H&E staining showed severe thickening of intestinal mucosa; Cu ions detected in the lamina propria of the intestinal mucosa, which were stained dark green by Cu salt staining; apoptotic staining showed cells were severely necrotic and there was cell shedding throughout the intestine. A1, B1, C1 (H&E stain; ×400), A2, B2, C2 (Cu salt stain; ×400), A3, B3, C3 (cell apoptosis assay results; ×400). H&E, hematoxylin and eosin; Cu, copper.

group A (control) mice, while the other parts of **Fig. 5** show results from mice in groups B and C. The results revealed that Cu ions were scattered in the intestinal mucosal epithelial cells of groups B and C, the high-Cu diet groups. The mucosa lamina propria in those groups showed Cu in macrophages; in addition, apoptotic intestinal mucosal epithelial cells were present.

DISCUSSION

The first factor that had been associated with observations of blackened pig intestines was climate. According to the reports of several slaughterhouses in China, black porcine intestines were commonly observed during the summer period (unpublished data). An exact effect of seasonal variation was not determined due to sampling period and sampling amount limitations. However, a significant relationship was observed between the black intestine detection rate and slaughtered pigs' source. During summers, pigs can be under heat stress conditions, which may lead to damage of the epithelial cells of the intestinal mucosa. During such conditions, harmful substances, like Cu, might successfully invade epithelial cells and macrophages and accumulate in the host's lamina propria. Blackened intestines were more commonly observe in pigs from small-scale farms without systematic feed management procedures. Lack of proper farm management and use of cheap metal-contaminated feed are possible reasons for the results from small-scale farms.

The assessment of 12 kinds of metal ions in black intestine tissue samples revealed that Cu was the most prominent metal contaminant, and the level of Cu exceeded by 6-fold the national standard. Seven types of metal ions were detected in the randomly purchased feed samples. The levels of Cu, Fe, and Zn exceeded the national standards, and notably, high Cu content was observed in black intestinal tissues. Microscopic examination also revealed that Cu was phagocytized and aggregated by macrophages in the lamina propria, which may explain why the pig large intestines, especially in the posterior colon and rectum regions, became blackened.

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To determine whether the excess level of Cu in feed could cause the animal's large intestine to turn black, we conducted animal experiments using mice as a model. The concentration of Cu in the feed for group B was over 50 times that of the control group. We observed behavioral changes in the Cu-treated mice, and the hair of the Cu-treated mice appeared brighter than that of the control mice. Moreover, feed intake and growth rate appeared to be greater in the Cu-treated mice. Those increases may be related to the excess Cu added to feed during the production process. In the first week of the experiment, weight loss was observed in group C mice fed feed that contained 100 times the standard Cu level. Both weight gain and feed consumption of mice in group C were lower than those in groups A and B. Mice in group D, which were fed feed with the highest Cu level, were dead within one week. Necropsy revealed that the intestines of mice in group B and C appeared black. Pathological observations showed that excessive Cu content in feed could induce the intestinal mucosa to darken and cause apoptosis of intestinal mucosal epithelial cells. High Cu content in fee and a longer feeding period can lead to Cu accumulation in the hosts, subsequently causing serious tissue damage. The IgG and IL-2 levels of mice fed a high-Cu diet were significantly lower than those of the control group. It is believed that Cu poisoning can seriously affect an animal's immune function. In this study, behavioral observations suggested that mice in the high Cu feeding group were in a poor welfare state compared to that in the control group.

Cu has an important role in the body's normal metabolism [4], and addition of an appropriate amount of Cu to feed can promote animal growth and improve hair color [12]. Many feed manufacturers apparently intend to add an excessive amount of Cu to feed, some exceeding the national feed standard by dozens of times. Such additions can result in an overaccumulation of Cu in intestines, liver, and other organs, endangering animal health [13]. Excessive Cu in animal feed can also affect public health, Chinese people commonly eat liver, intestines, and other internal organs of pigs; therefore, if the pigs have been fed an excess of Cu, consumers may unknowingly intake an excess of Cu ions. Various symptoms in humans, such as toxic jaundice and a hemoglobin decrease after consumption of excessive Cu ions, have been reported [14]. As early as 1,785, there was a report of Cu poisoning—the patient, a 17-year-old female, developed skin rashes, diarrhea, abdominal pain, vomiting, and other symptoms due to inadvertent consumption of excessive Cu-containing foods [15,16]; unfortunately, the patient died within a few days. When the body's intake of food Cu ions exceeds accepted standards by 100-fold, the body may develop serious diseases such as hemolytic anemia and necrotizing hepatitis [17]. Zhou et al. [14] applied pig manure fertilizer to tea gardens and vegetable plots for 11 years. Upon analysis, the level of Cu ions in the soil was 2,022.22% higher than that in normal soil. It is conceivable that high Cu levels in feces may contaminate the environment [18].

In conclusion, there is an urgent need to develop policies and regulations to control the excessive addition of Cu ions to pig-fattening feed. Cu-contaminated feed can cause porcine intestines to darken and Cu to accumulate in porcine organs. Moreover, it may reduce immunity, as Cu was observed to have been phagocytosed by macrophages and to accumulate in the lamina propria of intestines. This phenomenon might be exacerbated when pigs are under heat stress. Cu poisoning can damage an animal's nervous system, potentially resulting in death. Cu ions that accumulate in animal organs or discharged to the environment can also threaten human health through the food chain. In this investigation, Cu-affected porcine intestines had been discarded, but Cu-contaminated livers had been sold, and their final location could not be determined. As a result, it appears that a high Cu concentration in porcine feed not only harms the pigs' health status but also undermines public health.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1

Detection of ions content in pigs' large intestines (mg/kg)

Click here to view

Supplementary Table 2

Detection of ions content in pig fatting feeds (mg/kg)

Click here to view

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