Effects of High Dietary Iron on Iron Transporter Gene Expression in Duodenal Mucosa and Tissue Trace Element Content in Broiler Chickens (Postprint)

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Date: 2017-10-23T00:00:00+00:00

Abstract

This experiment aimed to investigate the effects of dietary iron content on the concentrations of essential trace elements iron, manganese, copper, and zinc in tissues of broiler chickens and on the gene expression of major iron transporters in duodenal mucosa, and to explore the influence of iron on trace element absorption and metabolism in broiler chickens and its underlying mechanisms. A total of 336 one-day-old commercial Ross 308 male broiler chicks were randomly divided into 4 groups based on body weight, with 6 replicates per group and 14 chickens per replicate. The control group was fed a basal diet without additional iron supplementation (actual iron content of 77.7 mg/kg), while the iron-supplemented groups were fed experimental diets supplemented with 100, 250, or 500 mg/kg iron in the form of ferrous sulfate heptahydrate (FeSO4·7H2O) (actual iron contents of 166, 308, and 579 mg/kg, respectively). The experimental period lasted 21 days. Chickens from each group were slaughtered at 7, 14, and 21 days of age to analyze iron, manganese, copper, and zinc contents in the liver, heart, pancreas, duodenal mucosa, and tibia, as well as mRNA expression levels of divalent metal transporter 1 (DMT1) and ferroportin 1 (FPN1) in duodenal mucosa. The results showed: 1) The 500 mg/kg iron supplementation group had significantly lower average daily gain during 1-7 days and 8-14 days of age compared to the other three groups ($P<0.10$), and the 250 and 500 mg/kg iron supplementation groups had significantly lower average daily feed intake during 1-7 days of age compared to the other two groups ($P<0.10$). 2) Dietary iron content had no significant effect on plasma total iron binding capacity, whole blood hemoglobin concentration (except at 7 days of age), or hematocrit in broiler chickens at 7, 14, and 21 days of age ($P>0.10$), but significantly affected plasma iron content and iron saturation at 7, 14, and

21 days of age (P<0.10), both of which increased with increasing dietary iron content. 3) Iron contents in the heart at 7 and 14 days of age and in the liver, duodenal mucosa, pancreas, and tibia ash at 7, 14, and 21 days of age increased with increasing dietary iron content; manganese contents in duodenal mucosa, pancreas, and tibia ash at 7, 14, and 21 days of age decreased with increasing dietary iron content; dietary iron supplementation significantly decreased pancreatic zinc content at 7 days of age (P<0.10), but had no significant effect on pancreatic zinc content at other ages, zinc content in other measured tissues at all ages, or copper content in all measured tissues at all ages (P>0.10). 4) Dietary iron content significantly affected DMT1 and FPN1 mRNA expression levels in duodenal mucosa at 7, 14, and 21 days of age (P<0.10), with expression levels of both DMT1 and FPN1 decreasing with increasing dietary iron content at all ages. These results suggest that high-iron diets may reduce manganese and zinc absorption in the intestine by regulating the expression of DMT1 and FPN1 genes in duodenal mucosa, thereby decreasing manganese and zinc deposition in tissues.

Full Text

Effects of High Dietary Iron on Iron Transporter Gene Expression in Duodenal Mucosa and Tissue Trace Element Contents in Broiler Chickens During 1–21 Days of Age

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Abstract: This study investigated the effects of dietary iron (Fe) content on the concentrations of essential trace elements (Fe, manganese [Mn], copper [Cu], and zinc [Zn]) in tissues and on the expression of key iron transporter genes in the duodenal mucosa of broiler chickens, aiming to elucidate the mechanisms through which Fe influences trace element absorption and metabolism. A total of 336 one-day-old commercial Ross 308 male chicks were randomly allocated to four groups based on body weight, with six replicates per group and 14 chicks per replicate. The control group received a basal diet without supplemental Fe (analyzed Fe content: 77.7 mg/kg), while the Fe-supplemented groups received experimental diets containing 100, 250, or 500 mg/kg Fe added as ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), yielding analyzed Fe concentrations of 166, 308, and 579 mg/kg, respectively. The 21-day feeding trial involved slaughtering birds at 7, 14, and 21 days of age to analyze Fe, Mn, Cu, and Zn contents in the liver, heart, pancreas, duodenal mucosa, and tibial ash, as well as mRNA expression levels of divalent metal transporter 1 (DMT1) and ferroportin 1 (FPN1) in the duodenal mucosa. The results showed: (1) The 500 mg/kg Fe group exhibited significantly lower average daily gain (ADG) during days 1–7 and 8–

14 compared to the other three groups ($P < 0.10$), while the 250 and 500 mg/kg Fe groups showed significantly reduced average daily feed intake (ADFI) during days 1–7 ($P < 0.10$). (2) Dietary Fe content did not significantly affect plasma total iron-binding capacity, whole-blood hemoglobin concentration (except at 7 days), or hematocrit at 7, 14, or 21 days of age ($P > 0.10$), but significantly influenced plasma Fe concentration and transferrin saturation at all three time points ($P < 0.10$), both of which increased with dietary Fe levels. (3) Tissue Fe concentrations in the heart (at 7 and 14 days), liver, duodenal mucosa, pancreas, and tibial ash (at all ages) increased linearly with dietary Fe content. Conversely, Mn concentrations in the duodenal mucosa, pancreas, and tibial ash decreased as dietary Fe increased. Dietary Fe supplementation significantly reduced pancreatic Zn content at 7 days of age ($P < 0.10$) but had no significant effect on Zn in other tissues or on Cu concentrations in any tissue at any age ($P > 0.10$). (4) Dietary Fe content significantly affected DMT1 and FPN1 mRNA expression in the duodenal mucosa at all ages ($P < 0.10$), with expression levels decreasing as dietary Fe increased. These findings suggest that high dietary Fe may reduce intestinal absorption of Mn and Zn by downregulating DMT1 and FPN1 gene expression in the duodenal mucosa, thereby decreasing their deposition in tissues.

Keywords: iron; iron transporter gene expression; tissue trace element content; broiler chickens

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Trace elements are essential nutrients in animals that serve as critical components of enzymes and specific proteins, playing vital roles in antioxidant defense, bone development, energy metabolism, oxygen transport and storage, DNA synthesis, and protein metabolism. However, synergistic or antagonistic interactions during intestinal absorption and metabolic processes often cause excess of one element to impair absorption and metabolism of others. Previous studies have identified divalent metal transporter 1 (DMT1) in the duodenal mucosa as a protein capable of transporting not only Fe but also other divalent metals such as Mn, Cu, and Zn [**?**]. Ferroportin 1 (FPN1), located on the basolateral membrane, transports both Fe and Mn into circulation [**?**] and may also participate in the transport of Zn, Cu, cobalt, and cadmium [**?**]. In weaned piglets, high dietary Fe reduced DMT1 mRNA levels in the duodenal mucosa while linearly increasing Fe and decreasing Mn concentrations in the liver and duodenal mucosa [**?**]. Similarly, in weaned calves, high dietary Fe decreased duodenal FPN1 mRNA expression and tended to reduce DMT1 mRNA, increased liver Fe content, and significantly lowered duodenal Mn content [**?**]. During commercial broiler feed processing, substantial Fe contamination often results in dietary Fe levels far exceeding requirements, yet no studies have examined the effects of high dietary Fe on intestinal iron transporter gene expression or tissue trace element concentrations in broilers. Therefore, this experiment investigated how dietary Fe content affects tissue Mn, Cu, and Zn concentrations and duodenal DMT1 and FPN1 mRNA expression in broilers to explore the mechanisms

of Fe' s influence on trace element absorption and metabolism, providing an experimental basis for improving trace element utilization efficiency.

A corn-soybean meal basal diet was formulated according to the Chinese Feeding Standard for Chickens [**?**] for broilers aged 1–21 days (Table 1 ; analyzed Fe content: 78 mg/kg). Three experimental diets were prepared by substituting equivalent amounts of corn starch in the basal diet with ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) to provide supplemental Fe at 100, 250, and 500 mg/kg (analyzed Fe contents: 166, 308, and 579 mg/kg, respectively).

Table 1 Composition and nutrient levels of the basal diet for broilers during 1 to 21 days of age (as-fed basis), %

Note: Reagent grade. Feed grade. Provided per kilogram of diet: VA 12,500 IU, VD_3 3,750 IU, VK_3 2.5 mg, VE 20 IU, VB_1 2.5 mg, VB_2 8 mg, VB_{12} 0.015 mg, VB_6 2.5 mg, nicotinic acid 32.5 mg, D-pantothenic acid 12.5 mg, biotin 0.125 mg, folic acid 1.25 mg, choline 700 mg, chlortetracycline 50 mg, Cu (as copper sulfate) 8 mg, Zn (as zinc sulfate) 60 mg, Mn (as manganese sulfate) 100 mg, Se (as sodium selenite) 0.15 mg, I (as potassium iodide) 0.35 mg. CP, Ca, and Fe were measured values; others were calculated values.

Three hundred thirty-six one-day-old commercial Ross 308 male chicks were randomly assigned to four groups based on body weight, with six replicates per group and 14 chicks per replicate. The control group received the basal diet without Fe supplementation, while the three treatment groups received the experimental diets. Birds were housed in stainless-steel cages by replicate, with ad libitum access to feed and tap water (no detectable Fe). Management followed the Ross Broiler Management Manual. Health status and mortality were recorded daily. Body weight and feed consumption were measured per replicate at weekly intervals to calculate average daily feed intake (ADFI), average daily gain (ADG), feed-to-gain ratio (F/G), and mortality rate. The trial lasted 21 days.

Sample Collection and Preparation

After an 8-hour fast, four chicks per replicate were selected at 8 and 15 days of age, and two chicks per replicate at 21 days of age, all with body weights close to the replicate mean. Blood was collected via cardiac puncture into EDTA-coated tubes for hemoglobin and hematocrit analysis (stored at 4°C) and into heparinized tubes for plasma preparation (centrifuged at 3,000 rpm for 10 minutes, plasma stored at -20°C for Fe and total iron-binding capacity [TIBC] analysis). Following blood collection, chicks were slaughtered. The duodenum was rinsed twice with saline, opened 1 cm from the pylorus, and the upper 4–5 cm of mucosa was scraped with a glass slide, snap-frozen in liquid nitrogen, and stored at -80°C for mRNA analysis. The lower ~10 cm of mucosa was scraped for mineral analysis. The left lobe of the liver, heart, pancreas, and left tibiotarsus were collected and stored at -20°C for Fe, Mn, Cu, and Zn determination. Samples from each replicate were pooled in equal amounts for

analysis [**?**].

Analytical Methods

Mineral Analysis: Fe, Mn, Cu, and Zn concentrations in tap water, diets, duodenal mucosa, liver, heart, pancreas, and tibial ash were determined by wet digestion with mixed acid (HNO_3:$HClO_4$ = 20:1) followed by inductively coupled plasma optical emission spectrometry (Model IRIS Intrepid II, Thermal Jarrell Ash, Waltham, MA) [**?**].

Blood Parameters: Hematocrit and hemoglobin concentration were measured using an automatic hematology analyzer (HC-3000). Plasma Fe and TIBC were determined colorimetrically using commercial kits (Nos. A039 and A040, Nanjing Jiancheng Bioengineering Institute) according to the method of Ma [**?**]. Transferrin saturation (TS) was calculated as (plasma Fe/TIBC) × 100%.

DMT1 and FPN1 mRNA Expression: Real-time quantitative PCR was performed according to Bai [**?**]. Total RNA was extracted using Trizol reagent (No. 15596-026, Invitrogen), and RNA concentration and purity (OD_{260}/OD_{280} ratio) were assessed using a P330-31 nucleic acid analyzer (Implen). RNA integrity was verified by 1% formaldehyde denaturing agarose gel electrophoresis. cDNA was synthesized using the SuperScript™ III First-Strand Synthesis System for RT-PCR kit (No. 205311, Qiagen). DMT1 and FPN1 mRNA levels were quantified by SYBR Green real-time PCR (No. 4367659, ABI) on an ABI 7500 system, with β-actin as the internal reference gene. Primer sequences are listed in Table 2 and were synthesized by Invitrogen (Shanghai).

Table 2 Primer sequences for real-time quantitative PCR

Gene	Primer sequences (5' -3')	Product length (bp)	Accession No.
DMT1	F: AGCCGTTCACCACT-TATTTCGR: GGTCCAAATAGGC-GATGCTC	206	GI 206597489
FPN1	F: GAGACTGGGTGGA-CAAGAACTCR: ATGCATTCTGAACAAC-CAAGGA	597	NM_{205518}
β-actin	F: ACCTGAGCGCAAG-TACTCTGTCTR: CATCGTACTCCT-GCTTGCTGAT	610	GI 61098365

Statistical Analysis: All data were analyzed using the General Linear Model (GLM) procedure in SAS 8.0, with replicate as the experimental unit. When

significant differences were detected, means were compared using the Least Significant Difference (LSD) test. Significance was declared at $P < 0.10$, following common practice in international literature [**?**].

Results and Analysis

The effects of dietary Fe content on growth performance are presented in Table 3 . Only one bird in the 250 mg/kg Fe group died during days 14–21, with no direct correlation between dietary Fe and mortality, so mortality data were omitted from Table 3. Dietary Fe content significantly affected ADG during days 1–7 and 8–14 and ADFI during days 1–7 ($P < 0.10$), but had no significant effect on other performance metrics ($P > 0.10$). The 500 mg/kg Fe group showed significantly lower ADG than the other three groups during both days 1–7 and 8–14 ($P < 0.10$), while the 250 and 500 mg/kg Fe groups had significantly lower ADFI during days 1–7 compared to the control and 100 mg/kg Fe groups ($P < 0.03$).

Table 3 Effects of dietary Fe content on growth performance of broilers during 1–21 days of age ($n = 6$)

Note: Values with different letter superscripts within a column differ significantly ($P < 0.10$). The same applies to Tables 4–8.

Dietary Fe content effects on blood parameters are shown in Table 4 . Plasma TIBC, whole-blood hemoglobin concentration (except at 7 days), and hematocrit were not significantly affected by dietary Fe at any age ($P > 0.10$). However, plasma Fe concentration and transferrin saturation were significantly influenced ($P < 0.01$), increasing linearly with dietary Fe levels. Hemoglobin concentration at 7 days was significantly affected by dietary Fe ($P < 0.01$), but not at 14 or 21 days.

Table 4 Effects of dietary Fe content on blood parameters of broilers during 1–21 days of age

Effects of dietary Fe on tissue Fe, Mn, Cu, and Zn concentrations are presented in Tables 5–8. Dietary Fe significantly affected Fe content in the heart at 7 and 14 days and in the liver, duodenal mucosa, pancreas, and tibial ash at all ages ($P < 0.10$), with concentrations increasing linearly with dietary Fe ($P < 0.001$). Dietary Fe had no significant effect on Mn content in the liver or heart ($P > 0.10$), but significantly reduced Mn in the duodenal mucosa, pancreas, and tibial ash at all ages ($P < 0.10$). Pancreatic Zn content at 7 days was significantly decreased by Fe supplementation ($P < 0.10$), but Zn in other tissues and Cu in all tissues were unaffected ($P > 0.10$).

Table 5 Effects of dietary Fe content on Fe content in tissues of broilers during 1–21 days of age (fresh weight basis)

Table 6 Effects of dietary Fe content on Mn content in tissues of broilers during 1–21 days of age (fresh weight basis)

Table 7 Effects of dietary Fe content on Zn content in tissues of broilers during 1–21 days of age (fresh weight basis)

Table 8 Effects of dietary Fe content on Cu content in tissues of broilers during 1–21 days of age (fresh weight basis)

Dietary Fe effects on duodenal mucosal DMT1 and FPN1 mRNA expression are shown in Table 9 . Expression levels of both genes were significantly affected by dietary Fe at all ages ($P < 0.10$), decreasing as dietary Fe content increased.

Table 9 Effects of dietary Fe content on mRNA expression levels of DMT1 and FPN1 in duodenal mucosa of broilers during 1–21 days of age

Note: Values with different letter superscripts within a row differ significantly ($P < 0.10$).

Discussion

Effects of Dietary Fe Content on Growth Performance

Cao et al. [**?**] reported that adding 400, 600, or 800 mg/kg Fe to a corn-soybean meal diet (containing 188 mg/kg Fe) reduced ADFI and ADG in broilers during days 1–7, 8–14, and 15–21, though differences among Fe-supplemented groups were not significant. Ma [**?**] found that adding 100 mg/kg Fe to a corn-soybean meal diet (containing 67 mg/kg Fe) did not reduce ADFI or ADG during days 1–21. In the current study, the 100 mg/kg Fe group showed no difference in ADG or ADFI compared to the control, whereas the 250 and 500 mg/kg Fe groups had significantly reduced ADFI during days 1–7, and the 500 mg/kg Fe group showed significantly lower ADG during days 1–7 and 8–14. This indicates that Fe excess exerted negative effects at these levels, with the trend toward reduced ADFI and ADG in the high-Fe groups at later ages possibly reflecting adaptation to high dietary Fe.

Approximately 60–70% of body Fe is used for hemoglobin synthesis, making hemoglobin concentration a common indicator of Fe status [**?**, **?**]. While Fe deficiency reduces hemoglobin concentration [**?**], adding 100–500 mg/kg Fe to non-deficient diets does not increase it [**?**, **?**, **?**]. In this study, the control group had significantly lower hemoglobin only at 7 days compared to the 100 and 500 mg/kg Fe groups, likely because the basal diet (78 mg/kg Fe) was only marginally deficient. The lack of significant increase in hemoglobin or hematocrit in Fe-supplemented groups indicates that Fe requirements were met, which is supported by transferrin saturation data. Transferrin saturation below 16% indicates deficiency, while values above 60% indicate excess [**?**]. The 250 mg/kg Fe group had transferrin saturation around 60% at all ages, suggesting borderline excess, whereas the 500 mg/kg Fe group exceeded 60%, confirming Fe overload.

Effects of Dietary Fe Content on Tissue Fe, Mn, Cu, and Zn Concentrations

Liver, kidney, duodenal mucosa, and tibial ash Fe concentrations increase linearly with dietary Fe [**?**, **?**, **?**, **?**, **?**], confirming these tissues as Fe storage sites. Manganese is primarily stored in mitochondria-rich tissues, with pancreatic, renal, hepatic, cardiac, and skeletal Mn deposition increasing linearly with dietary Mn [**?**]. In weaned piglets fed diets with 20 mg/kg Fe plus 100 or 500 mg/kg supplemental Fe, liver and duodenal Fe increased linearly while Mn decreased [**?**]. In weaned calves fed 750 mg/kg Fe, liver Fe increased significantly, duodenal and cardiac Fe tended to increase, and only duodenal Mn decreased significantly [**?**]. Few studies have examined Fe' s effects on Cu, Zn, and Mn metabolism in broilers. Jiang et al. [**?**] reported that Mn apparent retention decreased as dietary Fe increased from 231–296 mg/kg with 30 or 60 mg/kg added Fe.

In this study, Fe concentrations in the liver, pancreas, duodenal mucosa, and tibial ash increased linearly with dietary Fe, while Mn concentrations in the pancreas, duodenal mucosa, and tibial ash decreased linearly, suggesting that high dietary Fe reduces Mn absorption by downregulating DMT1 expression. The observed decrease in duodenal DMT1 and FPN1 mRNA expression with increasing dietary Fe supports this hypothesis.

Regarding Fe' s effects on Cu and Zn absorption, some studies report reduced Zn [**?**] and Cu [**?**] absorption with Fe supplementation, while others show no effect on Cu absorption in broilers [**?**], rats [**?**], or piglets [**?**]. In this study, pancreatic Zn at 7 days was lower in all Fe-supplemented groups, suggesting reduced Zn absorption, whereas Cu concentrations were unaffected, indicating no significant impact on Cu absorption under these experimental conditions. Discrepancies among studies may reflect differences in diet composition and Fe supplementation levels.

Dupic et al. [**?**] reported that rats fed Fe-deficient or high-Fe diets (2% ferric carboxymaltose) for 2 weeks showed increased duodenal mRNA expression of iron absorption-related genes (Dcytb, DMT1, FPN1, TfR1) in the Fe-deficient group and decreased expression in the high-Fe group. Hansen et al. [**?**] observed reduced duodenal FPN1 mRNA in calves fed 750 mg/kg Fe, with a trend for lower DMT1. In 21-day-old piglets fed 100 or 500 mg/kg Fe, duodenal DMT1 mRNA decreased significantly [**?**]. Similarly, broilers fed a diet with 141 mg/kg Fe (as ferric citrate) for 6 weeks showed significantly lower duodenal DMT1, FPN1, and Dcytb mRNA compared to an unsupplemented control (51 mg/kg Fe) [**?**]. Consistent with these reports, our study demonstrated sustained reductions in duodenal DMT1 and FPN1 mRNA expression after just one week of Fe supplementation, indicating that broilers regulate intestinal Fe transporters to limit excess Fe absorption, which consequently reduces absorption of divalent metals like Mn and Zn, as evidenced by decreased tissue concentrations.

Conclusions

1. Adding 250 or 500 mg/kg Fe to a corn-soybean meal basal diet containing 78 mg/kg Fe induced borderline and overt Fe excess, respectively, in broilers during days 1–21.
2. High dietary Fe reduced Mn concentrations in the pancreas, duodenal mucosa, and tibial ash, and decreased pancreatic Zn concentration at 7 days of age.
3. High dietary Fe downregulated DMT1 and FPN1 mRNA expression in the duodenal mucosa of broilers during days 1–21.

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Note: Figure translations are in progress. See original paper for figures.