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Iron Supplementation Attenuates the Inflammatory Status of Anemic Piglets by Regulating Hepcidin

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Abstract Iron deficiency is common throughout the world and has been linked to immunity impairments. Using piglets to model human infants, we assessed the impact of systemic iron homeostasis on proinflammatory status. Artificially reared piglets were parenterally supplied with iron dextran by intramuscular administration at the age of 3 days. Relative to no iron supplementation (control), iron dextran-treated (FeDex) piglets increased hematological parameters as well as iron levels in serum and tissues from days 21 to 49. High expression of hepcidin was observed in FeDex-treated piglets, which correlated with suppressed expression of ferroportin in duodenum. Lower levels of proinflammatory cytokine (IL-6, TNF-α, IFN-γ, and IL-1β) transcripts were detected in ileum of FeDex-treated piglets, which indicated that iron supplementation could attenuate the increase of inflammatory cytokines caused by iron deficiency. Histopathological analysis of liver and duodenum proved the less inflammatory responses after iron supplementation. Hepcidin was highly stimulated by FeDex supplementation and attenuated the inflammation of anemia, which implied that hepcidin might had antiinflammatory function and is a candidate regulator of the cross-talk between iron regulation and inflammation.

Keywords Hepcidin · Iron dextran · Inflammatory cytokines · FeDex · Piglets

Introduction

Iron deficiency is considered to be the most common mammalian nutritional deficiency and is the primary cause of anemia in humans especially in the neonatal period [1]. Newborn piglets are a suitable model to explore the multifaceted etiology of iron deficiency, because they are born with limited iron stores and can quickly become deficient without an adequate source [2, 3]. As sow's milk is not sufficient to meet iron requirement, the use of parenteral injection of exogenous iron to prevent deepening iron deficiency in suckling piglets has been well-documented and is obligatory in pig breeding [4–6].

Hepcidin is a peptide hormone that regulates iron homeostasis and acts as an antimicrobial peptide. It is expressed and secreted mainly by hepatocytes in response to iron loading and inflammation. Hepcidin mediates iron homeostasis by binding to the iron exporter ferroportin (FPN), inducing its internalization and degradation [7]. Hepcidin expression in mammals is regulated transcriptionally, increasing in response to iron supplementation and decreasing in response to iron need [8]. Hepcidin transcription is also regulated in response to infection, as hepcidin transcription is increased in response to activation of toll-like receptors (TLRs) as well as to inflammatory cytokines such as interleukin-6 (IL-6) [9, 10]. However, little is known whether hepcidin itself could regulate inflammatory response [11]. Therefore, the aim of this study was to determine the ability of iron supplementation to improve the hematological status, regulate hepcidin expressions, and affect inflammatory responses in the piglets. We hypothesized that iron dextran-treated piglets would have corresponding increases in hepcidin expression, which would account for the changes of proinflammatory status associated with iron deficiency.

Materials and Methods

Piglets and Experimental Diets

All of the experimental piglets were provided by Zhejiang Academy of Agricultural Sciences (Hangzhou, China).

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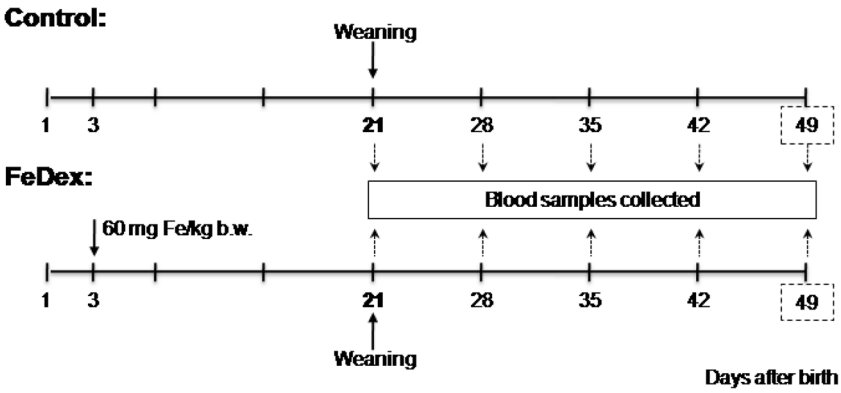

Fig. 1 Experimental design scheme. *Numbers* indicate the age of piglets. Piglets were weaned at 21 days after birth. Blood samples were collected on days 21, 28, 35, 42, and 49 after birth. *Boxed numbers* indicate the day on which piglets were sacrificed, and biological samples were collected

Animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University. A total of 12 large white Duroc piglets housed in standard conditions (approximately 70 % humidity and a temperature of 22±2 °C in standard cages) were used in the experiments. Piglets were taken from two litters delivered by two multipara sows. They were randomly alloted to two treatments (six piglets per treatment): intramuscular injection with PBS (control) or iron dextran (60 mg/kg Fe b.w.) at third day of age (FeDex). The creep feed (iron content 185 mg/kg Fe) was offered to piglets from day 7 to day 49. They were weaned at the age of 21 days (Fig. 1). The basal diet was formulated based on NRC recommendations to meet or exceed the pig's requirement for all nutrients except Fe and was not pelleted. The supplemental Fe was provided as $FeSO_4$.

Blood and Tissue Sample Collection

Blood was collected from vena cava cranialis of the piglets on days 21, 28, 35, 42, and 49. Ethylenediaminetetraacetic acid (EDTA) was used as anticoagulant for hematological examination. Piglets were sacrificed by narcotization and exsanguination at the age of 49 days. Immediately after death, the selected tissue fragments including liver, spleen, heart, kidney, lung, duodenum, and ileum were excised, frozen in liquid nitrogen, and held at −80 °C until used.

Hematological Analysis and Iron Parameters

Red blood cells (RBC) count, hemoglobin level (HB), hematocrit (HCT), and mean cell volume (MCV) were determined using an automated hematology analyzer (Sysmex K-1000D, Sysmex Inc., Kobe, Japan). Concentrations of serum iron, total iron-binding capacity (TIBC), and ceruloplasmin (CP) were measured using the method of fielding [12]. Quantification of the nonheme iron content of tissue fragments was performed using BIOCHEMTEST as described previously [13].

Real-Time PCR Analysis

Specific primers were designed based on the published gene sequences of hepcidin, ferroportin 1 (FPN), 18 s

Table 1 Oligonucleotide primers used for real-time PCR analysis

mRNA	Primer sequence (5′-3′)	Size (bp)	Accession number
Hepcidin	F:GAGCCACCGCTGGTTTGAC R: ACATCCCACAGATTGCTTTGC	108	AF516143.1
FPN	F:GAATAATGGGAACTGTGG R: AAGTGGCTCTGTCTGAAT	139	XM003359590
IL-6	F:TGGCTACTGCCTTCCCTACC R: CAGAGATTTTGCCGAGGATG	132	AF518322.1
TNF-α	F: CCAATGGCAGAGTGGGTATG R: TGAAGAGGACCTGGGAGTAG	116	NM_214022.1
IFN-γ	F:CAAAGCCATCAGTGAACTCATCA R: TCTCTGGCCTTGGAACATAGTCT	100	NM_213948.1
IL-1β	F: ACAAAAGCCCGTCTTCCTG R: ATGTGGACCTCTGGGTATGG	105	NM_214055.1
18 s	F: CCCACGGAATCGAGAAAGAG R: TTGACGGAAGGGCACCA	122	AY265350.1

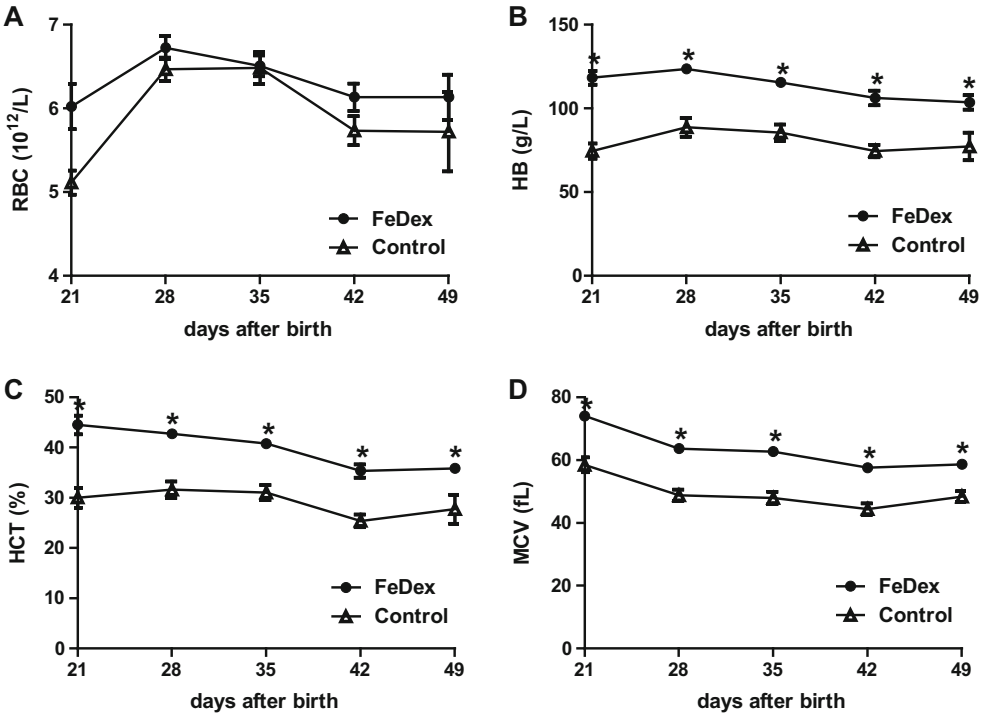

Fig. 2 Hematological parameters of control and FeDex-supplemented piglets. Blood cell indices were determined for seven piglets from each group at each time point. **a** Red blood cell (RBC) count, **b** hemoglobin (HB) concentration, **c** hematocrit (HCT) value, **d** mean cell volume (MCV). Values are expressed as the mean±SEM. *Asterisks* indicate significant differences ($p \leq 0.05$) in comparison with control values at the given day after birth

rRNA, and some cytokines including IL-6, tumor necrosis factor (TNF-α), interferon γ (IFN-γ), and interleukin-1β (IL-1β) in pigs using Primer 5.0 and GenBank (Table 1). Total RNA was extracted by Trizol Reagent kits (Sigma). Real-time PCR was conducted using the iQTW5 real-time multiplexing system (Bio-Rad, USA). M-MuLV Reverse Transcriptase (Thermo Fisher Scientific) and oligo primer transcriptase were used to reverse RNA to cDNA. The fluorescence quantitative PCR mixtures contained 1.0 μl cDNA, 5.0 μl SYBR green PCR

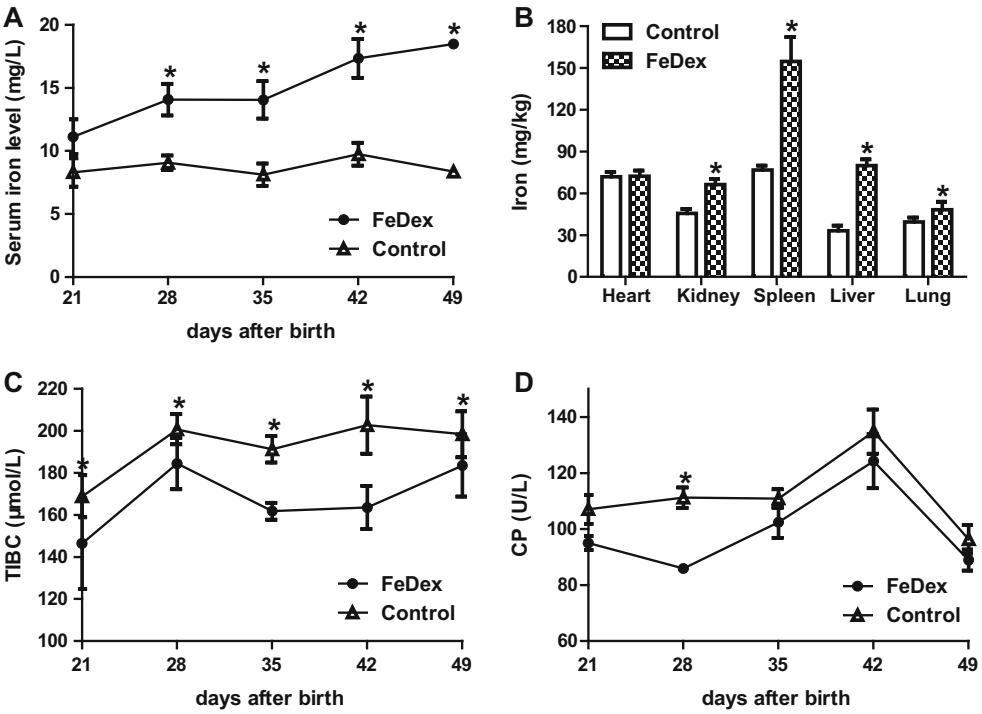

Fig. 3 Iron parameters of piglets supplemented with FeDex. **a** Serum iron level, **b** tissue iron level, **c** total iron-binding capacity (TIBC), **d** Ceruloplasmin (CP). Values are expressed as the mean ±SEM. *Asterisks* indicate significant differences ($p \leq 0.05$) in comparison with control values at the given day after birth

master mix (Roche, USA), 3.4 μl RNase-free deionized water, 0.3-μl upstream primers, and 0.3-μl downstream primers. The standard cycling conditions consisted of 1 cycle at 95 °C for 1 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Results were normalized to 18 s rRNA. The relative quantification was performed using Relative Expression Software Tool-384 (Bio-Rad, USA), and the data were calculated based on $2^{\Delta\Delta Ct}$ value [14].

Histopathology and Immunohistochemistry

Hematoxylin and eosin (HE) staining for liver and duodenum was performed to determine the extent of tissue damage. Liver and duodenum were removed and placed overnight in fixative containing 10 % formalin. Samples were then paraffin-embedded and cut at 5 μm in the longitudinal plane. Sections were examined under the microscope for the extent of and compared with the control group. Immunohistochemistry was performed to determine the expression of hepcidin protein in liver and FPN protein in duodenum, respectively. Tissues were fixed in 10 % formalin sectioned in paraffin and then subjected to microwave antigen retrieval and immunohistochemistry (AP-blue kit, USA) with primary antibodies against hepcidin (1:200; Uscn Life Science Inc. USA) and FPN (1:200; Accurate Chemical, Bioss, CHN). The stains were developed using diaminobenzidine as a chromogen. The sections were then counterstained with hematoxylin and examined by conventional light microscopy (Olympus BX51, Olympus Optical, Tokyo, Japan). The value of mean optical density (OD) was calculated by IPP 6.0.

Statistical Analysis

Statistical analyses were performed using SPSS 16.0. *t* Test was used to compare the differences between two breeds of pigs. Data were presented as mean±SEM. The results were considered significantly different at $p \leq 0.05$.

Results

Iron Supplementation Prevents the Anemic State of Piglets

Low value for RBC count, hematocrit percentage, hemoglobin concentration, and mean erythrocyte volume of piglets in

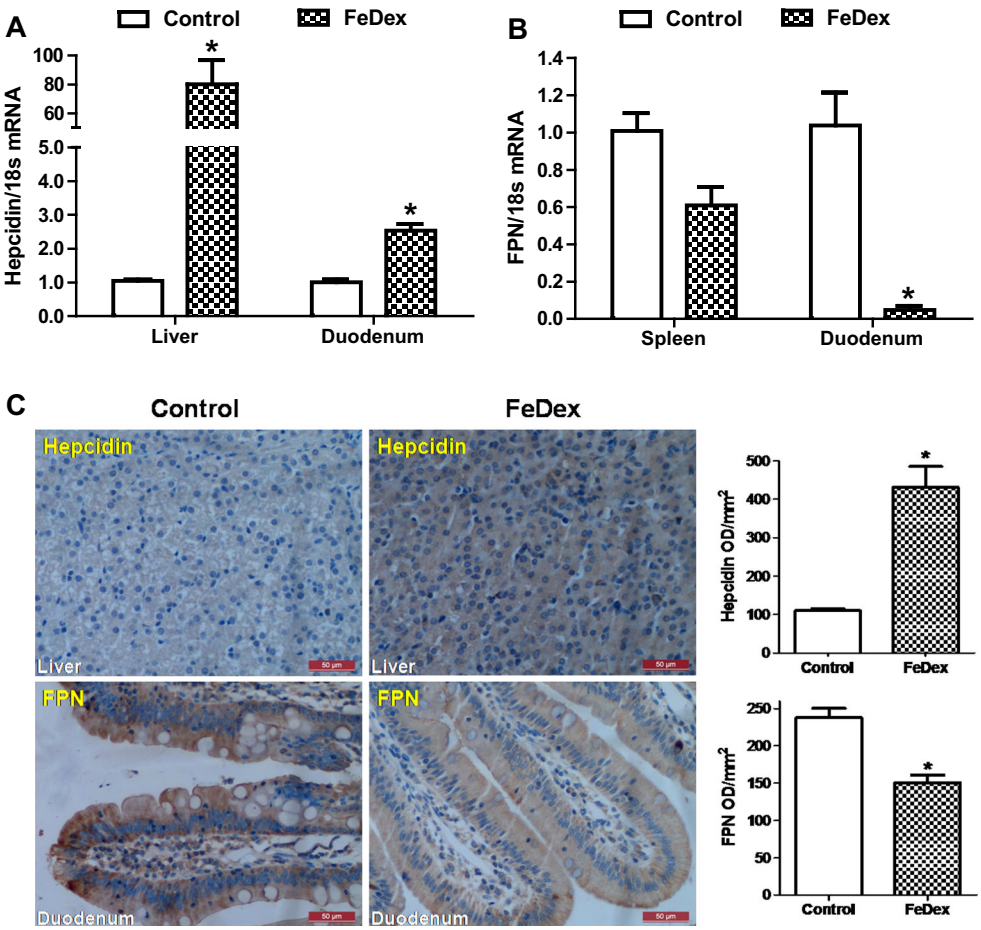

Fig. 4 Expression of hepcidin and FPN genes in tissues. Quantitative RT-PCR analysis of hepcidin (**a**) and FPN (**b**) transcripts in the liver, spleen, and duodenum homogenates. The expression levels of hepcidin are given as the amount relative to the expression of the housekeeping gene 18 s rRNA in each sample. *Bars* indicate means (n=6), and *error bars* indicate mean±SEM. *Asterisks* denote statistically significant differences between two different treated groups ($p \leq 0.05$). Immunohistochemical staining of hepcidin in the liver and FPN in the duodenum (**c**, *left*). The mean optical density value of hepcidin and FPN (**c**, *right*)

control group indicated the occurrence of severe iron deficiency anemia (Fig. 2a–d). However, the piglets in FeDex group showed significantly higher hematocrit percentage, hemoglobin concentration, and mean erythrocyte volume. Hemoglobin values in FeDex groups increased significantly, and no piglets were below anemic limit of 80 g/L [15] in any period of the trial (Fig. 2b).

Iron Supplementation Improves the Iron Status of Piglets

Iron is an important part of the body especially in growing animals, which is mostly located as functional iron in hemoglobin, and the rest forms myoglobin and different enzymes [2]. Serum iron level, tissue iron level, TIBC, and CP were examined to evaluate the iron status. Compared with the control piglets, serum iron levels were higher in FeDex supplied piglets, while TIBC and CP were lower (Fig. 3a–d). Iron concentration in most tissues of piglets in FeDex group had been significantly improved, of which spleen and liver were the sharpest improvement ($p \leq 0.05$) (Fig. 3b).

Relationship Between Hepcidin and FPN Levels

Iron was the first biological factor shown to induce hepcidin expression. As expected, FeDex-treated piglets showed the relatively high hepcidin transcript abundance both in liver and duodenum (Fig. 4a). FPN levels in duodenum and spleen were measured to determine their relationship with hepcidin expression and molecular potential for iron absorption. The observed decrease in FPN transcrpits in duodenum and spleen of FeDex group piglets was associated with an opposite increase in hepcidin levels (Fig. 4a and b). Immunohistochemical staining

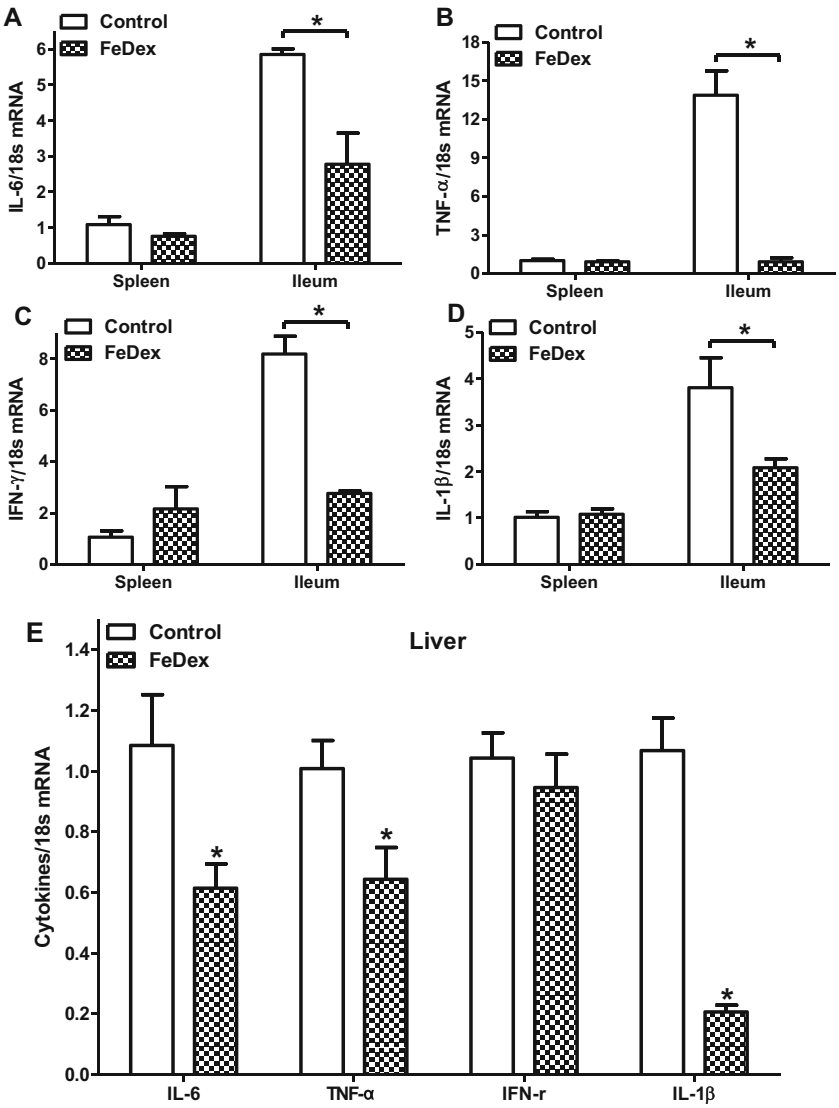

Fig. 5 Analysis of cytokine mRNA expression in the spleen and ileum. The PCR products were normalized to 18 s rRNA as the reference genes, and data shown are cytokine mRNA expression as a ratio of control in spleen (**a**–**d**) or in liver (**e**). Data plotted represent mean±SEM (n=5). *Asterisks* indicate significant differences ($p \leq 0.05$)

was used to assess the hepcidin and FPN protein levels. Hepcidin was significantly higher in FeDex-treated piglets and distributed evenly in liver cells, while FPN was localized to the basolateral membrane of enterocytes and was significantly lower (Fig. 4c).

Proinflammatory Cytokine mRNA Expressions Are Decreased in Ileum of FeDex-Supplemented Piglets

To determine whether the upregulation of hepcidin mRNA expression correlated with inflammatory status, the transcripts of cytokines were measured. Preliminary experiment showed that ileum was the most abundant tissue to express cytokines genes (data not shown). This analysis showed that cytokines were highly deficient ($p \leq 0.05$) in ileum of FeDex-supplemented piglets including IL-6, TNF-α, IFN-γ, and IL-1β (Fig. 5a–d). The expression of TNF-α and IFN-γ mRNA in FeDex piglets was ~15.2-fold and 3.0-fold lower ($p \leq 0.05$), respectively, than those in control piglets. In the liver, IL-6and IL-1β transcripts were also decreased in iron-supplemeted piglets (Fig. 5e).

Histopathological Analysis of Liver and Duodenum

HE staining was used to determine the extent of tissue damage. Swollen cytoplasm, vacuolar generation, and pyknotic hepatocyte nuclei were found in the live cells of control piglets (Fig. 6). Goblet cells were increased, and inflammatory lymphocytes infiltrated in duodenal lamina propria of iron deficiency piglets. However, FeDex supplementation significantly attenuated the pathological status both in the liver and duodenum.

Discussions

Iron and its homeostasis are intimately tied to the inflammatory response. The adaptation to iron deficiency, which confers resistance to infection and improves the inflammatory condition, underlies what is probably the most obvious link: the anemia of inflammation or chronic disease [16]. Iron deficiency anemia is probably the most prevalent micronutrient deficiency disorder in pigs and the most frequent form of anemia in mammals. The goal of the present study was to investigate the molecular regulation of iron metablism in piglets and to evaluate the function of hepcidin to regulate its inflammatory status during iron supplementation.

It is well-established that iron dextran (100–200 mg) injected intramuscularly into piglets within several days of birth will prevent iron deficiency anemia [17]. Once injected into the piglets, iron released from the iron dextran complex is presumed to enter the reticuloendothelial system through lymphatic circulation, store in ferritin and deliver to the bone marrow or other tissues by transferring slowly [18]. It is apparent from the hematologic data in this study that iron is adequately utilized. Iron supplementation (60 mg/kg Fe b.w.) on 3-day-old piglets was sufficient to keep HCT and HB within normal range for young pigs [2] and to have liver nonheme iron higher than in animals not injected with iron. However, TIBC in these piglets is very unsaturated and may be indicative of a rapidly growing animal in which iron supply is barely adequate. It suggested that iron supplementation effiently prevented the deterioration of the hematological status of piglets and contributed to the recovery of animals from the anemic state.

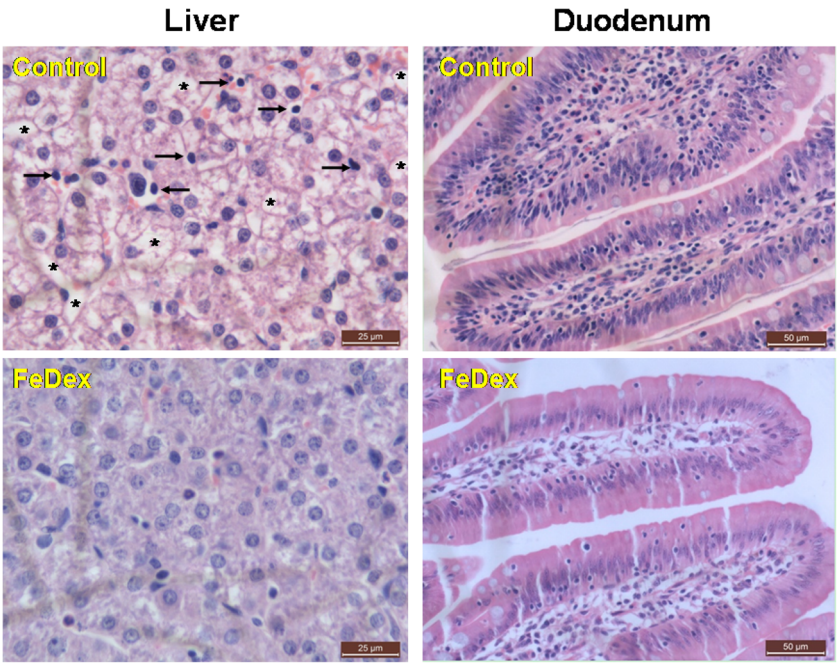

Fig. 6 Histopathological analysis of liver and duodenum sections stained with hematoxylin and eosin (HE). The *asterisks* show swollen cytoplasm and vacuolar generations. The *arrows* show pyknotic hepatocyte nuclei

The role of hepcidin and FPN in regulating systemic iron homeostasis is well-recognized, as mutations in either result in iron overload disease [8]. In this study, hepcidin was suppressed in iron deficiency. However, iron supplementation stimulated hepcidin production, including hepcidin transcripts and proteins. Hepcidin was induced highly in liver, which is in line with other reports that hepatocytes are the main cellular source of hepcidin [19]. FPN mRNA abundance and protein expression were both decreased significantly in duodenum. It showed that FPN mRNA levels are also regulated by iron, suggesting that the direct interaction of hepcidin with FPN protein might not be the only pathway. It is reported that FPN also functions in the uptake of iron at the apical membrane of enterocyte, possibly by modulating the activity of divalent metal transporter 1 (DMT1) [20]. It suggested that increased concentrations of hepcidin in turn inhibit FPN expression and block dietary iron absorption, thus preventing further iron loading.

Hepcidin is not only an iron regulatory hormone but also an important linker between host defense and iron metabolism. During infection and inflammation, hepcidin synthesis is markly increased by a mechanism that is independent of iron status or erythropoietic activity [21]. Increased hepcidin levels in response to inflammation result in decreased iron export and macrophage iron retention. As infectious agents require iron, decreased iron export is thought to be antimicrobial [16]. In present study, we demonstrated that iron supplementation inhibited the proinflammatory response with consequent decrease of inflammatory cytokines (IL-6, TNF-α, IFN-γ, and IL-1β), along with high expression of hepcidin. However, in theory, both iron deficiency and lack of hepcidin might be responsible for this effect. It is well known that IL-6 mediates hypoferremia of inflammation by inducing the synthesis of hepcidin [21]. Surprisingly, in contrast to anemic piglets, FeDex-supplemented piglets showed less inflammation response but high hepcidin expression. A possible explanation for this phenomenon may be that when both factors existed; hepcidin expression and secretion are principally regulated by iron loading, while inflammation is secondary. Another possible reason might be that increased hepcidin could affect inflammatory responses directly. Consistent with our results, pretreatment with hepcidin rescued the inflammatory phenotype of spleen macrophages in iron deficiency mice [22].

In summary, our results open an interesting field for speculations regarding the relationship between iron supplementation, regulation of iron metabolism, and inflammatory status. In conditions of iron deficiency, the production of hepcidin is suppressed. Besides controlling iron availability to microbes, hepcidin is a candidate regulator of the cross-talk between iron metablism and inflammation. FeDex supplementation attenuated the inflammatory status of piglets and induced high expression of hepcidin, which implied the proposed antiinflammatory function of hepcidin.

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Conflict of Interest No conflict of interest exists in the submission of this manuscript, and manuscript is approved by all authors for publication. The work described was original research that has not been published previously and not under consideration for publication elsewhere.

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