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# Effects of hot melt extrusion processed nano-iron on growth performance, blood composition, and iron bioavailability in weanling pigs

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## Abstract

This study was conducted to investigate the effects of hot melt extrusion (HME) nano-iron as an alternative for the common ferrous sulfate on iron (Fe) bioavailability, growth performance, nutrient digestibility, intestinal morphology, and intestinal microbiota of weanling pigs. A total of 200 piglets (Landrace × Yorkshire × Duroc) were randomly allotted to seven treatments on the basis of initial body weight (BW) and sex. Treatments were the INO100 (100 ppm Fe as $FeSO_4$), HME-Fe levels (50, 75, and 100 ppm nano-Fe as $FeSO_4$). ORG100 (100 ppm Fe as iron methionine). In phase 1, the HME50 pigs showed the lowest Fe content in feed and feces. Plasma Fe concentration was increased in HME100 and ORG100 pigs. In phase 2, there were significantly lower concentration of Fe in feed and feces of HME50 pigs ($p < 0.01$). A lower Fe concentration in the plasma and liver were observed in HME50 pigs compared with HME100 pigs. Concentration of red blood cell (RBC) was the lowest ($p < 0.01$) for HME50 pigs. During phase 2, the HME100, HME75, and ORG100 pigs showed a higher RBC and hemoglobin values compared with HME50 pigs. Digestibility of gross energy (GE) and crude protein (CP) were significantly higher in HME100 pigs compared with HME50 pigs. There was an increased ($p < 0.01$) villus height in the duodenum and jejunum of HME100 pigs compared with HME50 pigs. It is concluded that dietary Fe does not improve growth performance of weanling pigs; however, increasing the dietary iron concentration in weanling piglets increased the RBC and hemoglobin. In addition, the potential ability of HME to be used at a lower level (HME75) was observed.

**Keywords:** Weanling pigs, Nanoparticle, Iron, Hemoglobin, Villus height, Growth performance

## Background

Iron (Fe), an essential trace element for pigs, is needed for proper blood hemostasis and count of hemoglobin [1,2]. It is also a component of some crucial antioxidant enzymes such as superoxide dismutase that decrease the peroxides damage during stressful condition [3]. Anemia is a common problem in suckling piglets due to the deficient transfer of Fe from the placenta to the fetus, as well as the limited content of Fe in sow milk [4]. Therefore, weanling piglets eventually suffer from the carry over effects of iron deficiency from the suckling period. The high growth rate in modern swine farms increases the susceptibility of heavy piglets to iron deficiency than small and medium-sized piglets due to the higher blood volume and hemoglobin utilization capacity [2]. The demand for dietary iron is high at weaning, however, the unstable intestinal microbiota due to change in feed form from milk to solid

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may decrease the absorption efficiency during the weaning period. Inorganic form of Fe as ferrous sulfate had been used widely in swine diets. However, inorganic type had been known to have a lower absorption or having a high fluctuation in bioavailability ratio based on the source of diet, and the dietary content of ascorbic acid, pectin, phytate, protein, amino acid, and other mineral source [3,5]. To compensate the low absorption rate, the organic form of mineral was introduced with higher bioavailability due to a lower binding capacity with chelates [3,6]. Apart from the interactions between minerals and chelates in intestine, the size of particles is the key factor responsible for nutrient absorption through gastrointestinal mucosa [7]. Desai et al. [8] suggested that nutrients with 100 nm or lower particle size can directly increase the absorption rate for at least 10 times compared with big particles. The smaller particle size increases the availability and accessibility of particles in the intestinal mucosa [9]. Hot melt extrusion (HME) technique had been introduced as an effective nano-process in the pharmacology field [10]. In this method, inorganic Fe (ferrous sulfate, $FeSO_4$) was diffused and mixed with insolvent to prepare the nano particles in this study. In HME processing, target molecules overpassed the compounding and extruding step and achieved a homogenous dispersion. We hypothesized that Fe nanoparticles prepared through HME process will improve absorption and utilization of Fe in weanling pigs and thus may be more efficient in comparison to common inorganic Fe. The aim of this study was to investigate the effects of supplementation of HME processed Fe on growth performance, nutrient digestibility, and blood metabolites of Fe in weanling pigs.

## Materials and Methods

### Preparation of HME Fe

$FeSO_4$, Span 80, Tween 80 and PEG 6000 were mixed at 20:12:4:64 ratio prior to the feeding process. That processed mixture was moved to a feed hopper at a 45 g/min speed. Twin-screw system worked with hot-melt extruder (STS-25HS, Hankook E.M. Ltd., Pyeongtaek, Korea) connected with a round-shaped die (1 mm diameter) was used for the preparation of extruded materials [10]. The temperatures of barrel and die section were maintained at 45℃ and 40℃, respectively. The speed of screw was 0.025 × g during HME process. By passing through conveying and kneading sections in the barrel, samples were extruded from the die section. Extruded substances were solidified and they were milled by the HBL-3500S grinder (Samyang Electronics Co., Gunpo, Korea). Particle properties of $FeSO_4$ nanoparticles dispersed in distilled water were elucidated. The hydrodynamic size, polydispersity index, and zeta potential of $FeSO_4$ nanoparticles were measured by dynamic light scattering and laser Doppler methods (ELS-Z1000; Otsuka Electronics, Tokyo, Japan). Powder of $FeSO_4$ extrudate was dispersed in DW at 20 mg/mL and their particle properties were tested. The particle shape of $FeSO_4$ extrudate was observed by transmission electron microscopy (TEM). The dispersion of $FeSO_4$ nanoparticles was loaded onto the copper grid with film and dried for 10 min. That sample was then observed by TEM (JEM 1010; JEOL, Tokyo, Japan).

### Animals and experimental design

A total of 200 weanling pigs (Yorkshire × Landrace × Duroc, 21 ± 1 day old) were randomly allotted with an average initial body weight (BW) of 6.76 ± 1.14 kg on the basis of initial BW according to a randomized complete block (RCB) design in the Research Center of Animal Life Sciences at Kangwon National University. There were 4 replicates in five treatments and each treatment with 10 pigs/replicate. The treatment included: Inorganic Fe (INO100, 100 ppm Fe as $FeSO_4$), HME Fe (HME50, 50 ppm Fe; HME75, 75 ppm Fe; HME100, 100 ppm Fe as $FeSO_4$), Organic Fe (ORG100, 100 ppm Fe as $FeSO_4$). The organic Fe was purchased from TMC Company (Gimhae, Korea). The experimental diets were fed for 28 days in 2 phases (phase 1, d 0 to 14; and phase 2, d 15 to 28). The diets were formulated to provide all of the nutrients to meet or exceed the nutrient requirements (Table 1) listed in swine nutrient specification [11] with the exception of Fe.

### Experimental procedure and sampling

#### *Concentration of Iron*

To measure the level of iron concentration from experimental diet, feces and serum, inductively coupled plasma emission spectroscopy (ICP) was used with the method of AOAC [12]. Diet and fecal samples were collected by 1 g and dried in a muffle furnace for 1 hour (600℃; 24h for diet and 48h for feces). Then, those samples were dissolute with 10 mL of HCl 50% (v/v) for 24 hrs. After this step, dried samples were filtered via Whatman filter paper in 100 mL flask and diluted with distilled water and measured a concentration through ICP. The 1 mL of serum samples was moved into crucible and burned in a muffle furnace (600℃; 1 h). The liver samples were collected after slaughtering at the end of phase 2 to investigate the iron concentration. Then, those samples were dried (105℃; 24h) and ground by the mixer. A 1 g of liver sample also burned in a muffle furnace (600℃; 1 h). The serum and liver samples were dissolute with 10 mL of HCl 50% (v/v) for 24 hrs and filtered via Whatman filter paper in 100 mL flask and measured an iron concentration by ICP.

#### *Blood sampling*

To measure a white blood cell (WBC), red blood cell (RBC), hemoglobin and hematocrit, blood samples were collected via the

**Table 1. Formula and chemical composition of experimental basal diets (as-fed basis)**

| Item | Phase I (0-14 d) | Phase II (15-28 d) |
|---|---|---|
| Ingredient (g/kg) | 1,000 | 1,000 |
| Corn | 380.7 | 465.7 |
| Whey powder | 170 | 153.8 |
| Fish meal (60%) | 50 | 30 |
| Soybean meal dehulled | 244.9 | 294.9 |
| Soy protein concentrate | 50 | - |
| Soy oil | 33.8 | 26 |
| Mono calcium phosphate | 4 | 3.6 |
| Limestone | 7.7 | 8.3 |
| Salt | 3 | 3 |
| DL-Methionine (98%) | 1.4 | 0.8 |
| L-Lysine (98%) | 3.3 | 2.8 |
| L-Threonine (98.5%) | 1.4 | 1.3 |
| L-Tryptophan (10%) | 2.2 | 1.7 |
| Vitamin premix[1)] | 2.5 | 2.5 |
| Mineral premix[2)] | 2.5 | 2.5 |
| Choline-chloride (50%) | 0.5 | 0.5 |
| Phytase | 0.1 | 0.1 |
| Chromic oxide | 2.5 | 2.5 |
| Lactose | 39.5 | - |
| Calculated composition (%) | | |
| ME (MJ/kg) | 14.226 | 14.226 |
| CP | 23 | 21 |
| Ca | 0.8 | 0.7 |
| Av.P | 0.4 | 0.33 |
| SID. Lys | 1.35 | 1.23 |
| SID. Met | 0.41 | 0.36 |
| SID. Met + Cys | 0.74 | 0.68 |
| SID. Thr | 0.79 | 0.73 |
| SID. Trp | 0.22 | 0.2 |
| Lactose | 15 | 10 |

[1)]Supplied per kilogram of diet: vitamin A, 20,000 IU; vitamin $D_3$, 4,200 IU; vitamin E, 10 IU; vitamin $K_3$, 5.6 mg; vitamin $B_1$, 2.8 mg; vitamin $B_2$, 5.5 mg; vitamin $B_6$, 4.2 mg; vitamin $B_{12}$, 0.042 mg; pantothenic acid, 14 mg; niacin, 42 mg; biotin, 0.105 mg; folic acid, 1.05 mg.

[2)]Supplied per kilogram of diet: 0.3 mg Se; 0.20 mg Co; 30 mg Cu; 30 mg Mn; 20 mg Zn; 0.35 mg I; Fe based on the treatments.

jugular vein from 6 pigs per treatment, which randomly selected on the last day of each phase. To analyze WBC and RBC, Natt-Herrick solution was used and microhaematocrit method was used to hematocrit analysis. For hemoglobin analysis, cyanmethaemoglobin method was used to measure hemoglobin concentration [13]. Blood samples were centrifuged at 25,000 × g at 4℃ for 15 min to obtain the serum. The haemolysis-free serum was transferred immediately and frozen at −80℃ until further biochemical analyses.

### Growth performance

The BW was recorded in first day of this experiment and end of each phase. In feed intake, after the end of each phase, remained feed weight from each feeder was used to calculate for average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR).

### Nutrient digestibility

The nutrient balance examine was conducted after each phase 1 and phase 2 of feeding trial to investigate total digestibility, dry matter (DM), crude protein (CP) and gross energy (GE). Prior to the end of each phase, day 7 (phase 1) and day 28 d (phase 2), 2 pigs from each replicate were allotted in individual cages (one pig / cage) to collect excreta samples. For analysis, fed a 2.5 g $kg^{-1}$ chromium oxide in the diet as an indigestible marker during experiment. Excreta samples were collected during 12–14 d (phase 1) and 26–28 d (phase 2). These collected samples were dried in a forced air-drying oven (60℃, 72 h) and minced with a Wiley laboratory mill (Thomas Model 4 Wiley®Mill, Thomas schientific, Swedesboro, NJ, USA) by 1 mm screen.

The proximate analysis for experimental diet and feces was followed by AOAC [12] method and DM and CP were analyzed. For GE analysis, bomb calorimeter (Model 1261, Parr Instrument Co., Moline, IL., USA) was used and spectrophotometer (Jasco V-650, Jasco Crop., Tokyo, Japan) was used for analysis of $Cr_2O_3$ concentration.

The total nutrient utilization used this formula as:

$$\text{Total nutrient utilization (\%)} = 100 - \left[100 - \left(\frac{\text{Cr in feed (\%)}}{\text{Cr in excreta (\%)}} \times \frac{\text{Nutrient in excreta (\%)}}{\text{Nutrient in feed (\%)}}\right)\right]$$

### Microbial analyses

To determine microbiology, fecal samples were carried out at the end of each phase and sealed. The analysis was conducted on the same day of fecal collection. In those samples, total anaerobic bacteria (TAB, plate count agar, Difco laboratories, Detriot, MI, USA), *Lactobacillus* spp. (MRS agar + 0.200 g/L $NaN_3$ + 0.500 g/L $_L$-cystine hydrochloride monohtdrate) and *Clostridium* spp. (Tryptose sulphite cycloserine agar, Oxoid, Hampshire, UK) were analyzed for this experiment. For microbial groups, anaerobic conditions were generated and gaspak anaerobic system (BBL, No. 260678, Difco, MI, USA) was used. The microbial populations were calculated by colony-forming unit (CFU) and transformed into $\log_{10}$ before statistical analysis.



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### *Small intestinal morphology*

A total of 15 pigs was chosen to male pigs with respect to mean of BW in the group. Carcasses were dissected to get samples. After carcasses were dissected, the intestines were removed and laid out on a plastic surface. Three 5 cm segments were taken from the sections at 28%, 50%, and 75% of the total intestinal length, each of which was referred to proximal duodenal, jejunal, and ileal segment. The small intestine samples were stored at 10% neutral buffered formalin solution (pH 7.2–7.4) for 24 h. Three cross-sections of each intestinal sample were prepared after staining with azure A and eosin using standard paraffin embedding procedures [14]. Well-oriented crypt-villus groups (total 10 intact) were chosen in triplicates for analyzing each intestinal cross-section. Crypt depth was characterized as the depth of the invagination between the following villi and height of villus was determined from the villus crypt junction to the edge of the villi. By using an image which is processing and analyzing system all of the morphological characteristics were measured (crypt depth or villus height) in 10-μm increments (Media Cyber genetics, Optimus software version 6.5, North Reading, MA, USA).

### Statistical analysis

Statistical analysis of the current experimental data was completed by using the GLM procedure of SAS (SAS Inst. Inc., Cary NC) in a completely randomized design. Pen replicate was the experimental unit for all measurements. Treatment means were separated by Tukey multiple range tests at $p < 0.05$ statistical level.

## Results

### Iron concentration

The effects of dietary Fe concentration and source on feed, feces and plasma presented in Table 2. During phase 1, there were significant differences observed in the concentration of feed, feces, and plasma ($p < 0.01$), and the HME50 pigs showed the lowest Fe content in feed and feces. Plasma Fe concentration was higher in HME100 and ORG100 pigs. In phase 2, there were significantly lower concentration of Fe in feed and feces of HME50 pigs ($p < 0.01$). A lower Fe concentration of the plasma and liver were observed in HME50 pigs compared with HME100 pigs.

### Serum profiles

The effects of dietary Fe concentration and source on serum profiles are presented in Table 3. In phase 1, there were no significant differences in WBC and hematocrit. However, RBC concentration was significantly lower ($p < 0.01$) in HME50 pigs. There was a significant ($p < 0.05$) difference in blood hemoglobin between pigs fed the HME100 and HME50 diets. During phase 2, the WBC and hematocrit did not show any significant differences among treatments. The HME100, HME75, and ORG100 pigs showed a higher ($p < 0.01$) counts of RBC and concentration of hemoglobin compared with HME50 pigs.

### Growth performance

The effects of dietary Fe concentration and source on growth performance presented in Table 4. There was no difference in ADG, ADFI, and FCR in phase 1 and phase 2. The overall experimental period also showed no significant difference in ADG, ADFI, and FCR.

### Nutrient digestibility

The effects of dietary Fe concentration and source on apparent total tract digestibility of nutrients presented in Table 5. No difference was observed in digestibility of DM, GE, and CP in phase 1. In phase 2, there was no significant difference in DM digestibility. However, digestibility of GE and CP were significantly higher in

**Table 2. Effect of dietary Fe concentration and source on Fe concentration of feed, feces and plasma in weanling pigs**

| Item | Inorganic (ppm) | HME (ppm) | | | Organic (ppm) | SEM | *p*-value |
|---|---|---|---|---|---|---|---|
| | 100 | 50 | 75 | 100 | 100 | | |
| Phase I (d 14) | | | | | | | |
| Feed (mg/kg) | 173$^{a}$ | 130$^{c}$ | 151$^{b}$ | 177$^{a}$ | 171$^{a}$ | 4.32 | 0.001 |
| Feces (mg/kg) | 268$^{a}$ | 221$^{c}$ | 245$^{b}$ | 267$^{a}$ | 266$^{a}$ | 4.67 | 0.001 |
| Plasma (ug/dL) | 103$^{ab}$ | 92$^{b}$ | 102$^{ab}$ | 108$^{a}$ | 106$^{a}$ | 1.79 | 0.027 |
| Phase II (d 28) | | | | | | | |
| Feed (mg/kg) | 176$^{a}$ | 131$^{c}$ | 154$^{b}$ | 174$^{a}$ | 172$^{a}$ | 4.10 | <0.001 |
| Feces (mg/kg) | 260$^{a}$ | 217$^{d}$ | 232$^{c}$ | 245$^{b}$ | 254$^{ab}$ | 3.77 | <0.001 |
| Plasma (ug/dL) | 114$^{ab}$ | 96$^{b}$ | 108$^{ab}$ | 116$^{a}$ | 114$^{ab}$ | 2.22 | <0.001 |
| Liver | 238$^{a}$ | 217$^{b}$ | 245$^{a}$ | 252$^{a}$ | 242$^{a}$ | 6.01 | 0.004 |

$^{ab}$Values with different superscripts of the row significantly differ ($p < 0.05$).

HME, hot melt extrusion; SEM, standard error of means.

**Table 3.** Effect of dietary Fe concentration and source on complete blood count in weanling pigs

| Item | Inorganic (ppm) | HME (ppm) | | | Organic (ppm) | SEM | *p*-value |
|---|---|---|---|---|---|---|---|
| | 100 | 50 | 75 | 100 | 100 | | |
| Phase I (d 14) | | | | | | | |
| WBC ($10^3$/μL) | 21.21 | 19.22 | 21.44 | 21.90 | 21.45 | 0.98 | 0.076 |
| RBC ($10^6$/μL) | 6.42$^{a}$ | 6.22$^{b}$ | 6.40$^{a}$ | 6.53$^{a}$ | 6.42$^{a}$ | 0.04 | <0.001 |
| Hemoglobin (g/dL) | 11.52$^{ab}$ | 11.42$^{b}$ | 11.54$^{ab}$ | 11.86$^{a}$ | 11.78$^{ab}$ | 0.13 | 0.028 |
| Hematocrit (%) | 43.82 | 43.08 | 42.84 | 46.82 | 44.73 | 1.52 | 0.093 |
| Phase II (d 28) | | | | | | | |
| WBC ($10^3$/μL) | 22.59 | 21.33 | 22.21 | 23.54 | 22.62 | 0.74 | 0.54 |
| RBC ($10^6$/μL) | 6.46$^{bc}$ | 6.26$^{c}$ | 6.50$^{b}$ | 6.73$^{a}$ | 6.50$^{b}$ | 0.07 | <0.001 |
| Hemoglobin (g/dL) | 11.50$^{bc}$ | 11.28$^{c}$ | 11.58$^{ab}$ | 11.75$^{a}$ | 11.60$^{a}$ | 0.09 | <0.001 |
| Hematocrit (%) | 48.43 | 48.00 | 47.38 | 50.27 | 47.90 | 1.22 | 0.117 |

$^{a-c}$Values with different superscripts of the row significantly differ ($p < 0.05$).

HME, hot melt extrusion; SEM, standard error of means; RBC, red blood cell; WBC, white blood cell.

**Table 4.** Effect of dietary Fe concentration and source on growth performance in weanling pigs

| Item | Inorganic (ppm) | HME (ppm) | | | Organic (ppm) | SEM | *p*-value |
|---|---|---|---|---|---|---|---|
| | 100 | 50 | 75 | 100 | 100 | | |
| Phase I (0-14 d) | | | | | | | |
| ADG (g) | 281 | 277 | 283 | 282 | 279 | 6.13 | 0.999 |
| ADFI (g) | 394 | 395 | 389 | 411 | 413 | 6.33 | 0.723 |
| FCR | 1.40 | 1.45 | 1.37 | 1.46 | 1.49 | 0.03 | 0.684 |
| Phase II (15-28 d) | | | | | | | |
| ADG (g) | 420 | 406 | 419 | 431 | 433 | 6.17 | 0.708 |
| ADFI (g) | 704 | 680 | 692 | 724 | 722 | 6.03 | 0.068 |
| FCR | 1.68 | 1.68 | 1.66 | 1.69 | 1.68 | 0.03 | 0.999 |
| Overall (0-28 d) | | | | | | | |
| ADG (g) | 350 | 342 | 351 | 356 | 356 | 4.88 | 0.901 |
| ADFI (g) | 549 | 537 | 541 | 567 | 568 | 4.75 | 0.098 |
| FCR | 1.57 | 1.58 | 1.54 | 1.59 | 1.60 | 0.02 | 0.893 |

HME, hot melt extrusion; SEM, standard error of means; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio.

**Table 5.** Effect of dietary Fe concentration and source on apparent total tract digestibility (%) of nutrients in weanling pigs

| Item | Inorganic (ppm) | HME (ppm) | | | Organic (ppm) | SEM | *p*-value |
|---|---|---|---|---|---|---|---|
| | 100 | 50 | 75 | 100 | 100 | | |
| Phase I (d 14) | | | | | | | |
| DM | 80.67 | 81.09 | 82.02 | 82.41 | 81.75 | 0.80 | 0.177 |
| GE | 80.20 | 76.15 | 77.28 | 78.71 | 77.43 | 0.50 | 0.087 |
| CP | 77.64 | 79.85 | 80.81 | 81.31 | 80.73 | 1.18 | 0.172 |
| Phase II (d 28) | | | | | | | |
| DM | 81.04 | 80.36 | 81.26 | 81.86 | 81.18 | 0.68 | 0.185 |
| GE | 75.50$^{ab}$ | 74.82$^{b}$ | 75.63$^{ab}$ | 77.06$^{a}$ | 75.51$^{ab}$ | 0.49 | 0.005 |
| CP | 81.17$^{a}$ | 79.16$^{b}$ | 81.32$^{a}$ | 81.99$^{a}$ | 81.11$^{a}$ | 0.40 | 0.004 |

$^{ab}$Values with different superscripts of the row significantly differ ($p < 0.01$).

HME, hot melt extrusion; SEM, standard error of means; DM, dry matter; GE, gross energy; CP, crude protein.


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HME100 pigs compared with HME50 pigs.

### Intestinal morphology

The effects of dietary Fe concentration and sources on small intestinal morphology presented in Table 6. In the duodenum and jejunum, there were no significant differences in crypt depth and VH/CD ratio among the treatments. However, there was an increased ($p < 0.01$) villus height in the duodenum and jejunum of HME100 pigs compared with HME50 pigs. There were no differences in villus height, crypt depth and VH/CD ratio in the ileum.

### Fecal microflora

The effects of dietary Fe concentration and source on fecal microflora presented in Table 7. During phase 1, there was no difference in the population of TAB and *Lactobacillus* spp. However, the population of coliforms was increased in ORG100 pigs compared with HME100 pigs ($p < 0.05$). In phase 2, there was no significant difference observed in TAB. HME100 and ORG100 pigs showed an increased *Lactobacillus* spp. population compared with pigs fed HME50 diet ($p < 0.01$). The population of Coliforms analysis showed that pigs fed HME50 diet had a higher colonization of coliforms compared with HME100 and ORG100 pigs ($p < 0.01$).

## Discussion

It was reported that supplementation of Fe is required for optimal performance [15]. In the present study, no difference was observed in ADG between the treatments. The former reference suggested that a dietary level of 90 ppm Fe can sufficiently maximize growth performance and feed intake [16]. Feng et al. [17] reported that the ADG of weanling piglets responded with a linear increase of dietary organic Fe to 120 ppm. Lewis et al. [18] observed that increased dietary Fe supplementation up to 200 ppm resulted in higher ADFI, ADG, and gain to feed ratio in weanling pigs. However, in agreement with the results of this study, several studies showed that growth performance is not a sensitive criterion for Fe-related biological response [5,15]. Interestingly, no relationship was shown between the ADG and digestibility of nutrients in spite of the significant contribution of Fe to increased nutrient digestibility. Higher concentrations of dietary Fe increased the dry matter and CP digestibility in broiler chickens were under the iron depleted conditions [6].

Crypt hyperplasia and villus atrophy occur during weaning [19]. The present study indicated that villus height in the duodenum was increased in HME100 and ORG100 piglets. Furthermore, villus height in the jejunum was increased only by HME100 or INO100 supplementation. Recent literature has also indicated improved villus height in the jejunum and duodenum of weanling pigs fed Fe supplemented diets [20]. Intestinal morphology status indicates the capability of nutrient digestion [19,21]. An increase in villus height may lead to improved nutrient digestion capacity in weanling pigs. Higher digestibility of GE and CP in HME100 pigs during the phase 2 may be associated with the improved villus height.

One of the aims of the present study was to evaluate the bioavailability of different Fe sources. A higher dietary dose of Fe can be reflected in Fe concentration of plasma [2,5]. Fe is absorbed and subsequently transported to the bloodstream, as plasma Se was higher in HME100 or ORG100 pigs than HME50. Ferrous methionine as an organic Fe absorbs via methionine transporters and

**Table 6. Effect of dietary Fe concentration and source on small intestinal morphology of weanling pigs (d 28)**

| Item | Inorganic (ppm) | HME (ppm) | | | Organic (ppm) | SEM | *p*-value |
|---|---|---|---|---|---|---|---|
| | 100 | 50 | 75 | 100 | 100 | | |
| Duodenum | | | | | | | |
| Villus height (VH; μm) | 449$^{ab}$ | 430$^{b}$ | 436$^{ab}$ | 459$^{a}$ | 444$^{ab}$ | 7.30 | 0.025 |
| Crypt depth (GD; μm) | 237 | 233 | 231 | 223 | 229 | 3.75 | 0.856 |
| VH/CD | 1.87 | 1.86 | 1.86 | 2.06 | 1.95 | 0.03 | 0.481 |
| Jejunum | | | | | | | |
| Villus height (μm) | 357$^{ab}$ | 344$^{b}$ | 359$^{ab}$ | 371$^{a}$ | 364$^{ab}$ | 7.11 | 0.045 |
| Crypt depth (μm) | 237 | 233 | 223 | 226 | 221 | 4.65 | 0.515 |
| VH/CD | 1.51 | 1.49 | 1.61 | 1.64 | 1.73 | 0.03 | 0.282 |
| Ileum | | | | | | | |
| Villus height (μm) | 239 | 229 | 247 | 253 | 238 | 3.82 | 0.347 |
| Crypt depth (μm) | 142 | 140 | 146 | 141 | 142 | 1.66 | 0.455 |
| VH/CD | 1.64 | 1.65 | 1.69 | 1.79 | 1.75 | 0.02 | 0.335 |

$^{ab}$Values with different superscripts of the row significantly differ ($p < 0.05$).

HME, hot melt extrusion; SEM, standard error of means..

**Table 7. Effect of dietary Fe concentration and source on fecal microflora in weanling pigs**

| Item | Inorganic (ppm) | HME (ppm) | | | Organic (ppm) | SEM | *p*-value |
|---|---|---|---|---|---|---|---|
| | 100 | 50 | 75 | 100 | 100 | | |
| Phase I (d 14) | | | | | | | |
| Total anaerobic bacteria | 9.37 | 9.53 | 9.42 | 9.39 | 9.35 | 0.08 | 0.190 |
| *Lactobacillus* spp. | 8.19 | 8.16 | 8.15 | 8.18 | 8.13 | 0.02 | 0.096 |
| *Coliforms* spp. | 6.59$^{ab}$ | 6.61$^{ab}$ | 6.69$^{ab}$ | 6.56$^{b}$ | 6.74$^{a}$ | 0.04 | 0.013 |
| Phase II (d 28) | | | | | | | |
| Total anaerobic bacteria | 9.67 | 9.70 | 9.65 | 9.49 | 9.55 | 0.24 | 0.275 |
| *Lactobacillus* spp. | 8.44$^{ab}$ | 8.36$^{b}$ | 8.50$^{ab}$ | 8.58$^{a}$ | 8.54$^{a}$ | 0.05 | 0.006 |
| *Coliforms* spp. | 5.96$^{ab}$ | 6.06$^{a}$ | 5.97$^{ab}$ | 5.87$^{b}$ | 5.91$^{b}$ | 0.04 | 0.002 |

$^{ab}$Values with different superscripts of the row significantly differ ($p < 0.05$).

HME, hot melt extrusion; SEM, standard error of means.

convert to ferrous in the liver to provide Fe for heme synthesis [6]. The previous studies reported the use of organic sources, such as ferrous glycine is superior to inorganic sources [3]. In contrast, this study does not show the superior absorption of Fe in ORG pigs and no difference was observed between HME75 and ORG100 in plasma Fe concentration. A Fe atom normally spends no longer than two hours in plasma [4]. The higher plasma Fe due to increasing levels of dietary Fe in this study is similar to results reported by Perri et al. [2]. Yu et al. [5] also reported a linear plasma Fe when dietary Fe concentration increased. In case of fecal Fe content, HME and ORG Fe sources were more efficient than the INO at 100 ppm/kg of diet. A different absorption pathway of organic mineral, and a smaller particle size of HME may contribute to the higher intestinal absorption. Particle size is a key parameter to improve absorption efficiency. The smaller size of Fe nanoparticle (around 200 nm) allows Fe to be absorbed through the intestinal mucus barrier [22]. In addition, the lower dietary Fe or decreased plasma Fe may explain the reduced Fe concentration in liver of HME50 pigs. Underwood and Suttle [23] indicated that the liver Fe depletion occurs during Fe deprivation. Therefore, the concentration of Fe in the liver can be decreased in weanling pigs fed lower Fe. For this experiment, mean liver Fe concentration (239 mg/kg) was within the normal range for the weanling pig reported by Rincker et al. [4]. Feng et al. [3] also reported that the hepatic Fe concentration of pigs was linearly increased when supplemental Fe was increased from 0 to 120 mg/kg. However, comparing the influence of the three different Fe sources, no difference was detected. Regarding the role of liver as a major storage site for nutrients, the liver Fe content may be mobilized when the demand for Fe is high.

Fe status is commonly evaluated by several indicators such as hemoglobin content in blood. Hemoglobin is responsible for the diverse biological pathways and also transports oxygen and carbon dioxide from the organs to the lung [24]. We studied the effect of different Fe sources on blood profile of weanling pigs and found that the plasma hemoglobin and RBC in the HME100 pigs was higher than that of the HME50. A whole blood hemoglobin concentration of 10.0 g/dL is considered sufficient, whereas 8.0 g/dL suggests to be a borderline anemia [25]. The hemoglobin of piglets in this study fall in adequate range. The differences in blood hemoglobin between weanling pigs fed low and high Fe is an expected result due to the positive connection between dietary Fe level and hemoglobin production. Considering the significant differences in the phase 2 and insignificant result in phase 1, this study shows that in a long term, high blood Fe may reflect in higher plasma hemoglobin and RBC. The Fe availability influences the blood hemoglobin in weanling pigs [2,18]. The increase in blood hemoglobin in HME100 and ORG100 pigs is in line with previous literature, in which the hemoglobin in blood from either inorganic Fe [4] or the organic source [5]. Hemoglobin is commonly used as an indicator of iron status, because hemoglobin includes 80% to 90% of the total iron present in the piglet [26]. A linear increase in hemoglobin, hematocrit, and plasma transferrin of suckling pigs was reported by Rincker et al. [4] when dietary Fe increased to 150 ppm. Feng et al. [17] also found the hemoglobin, and plasma Fe concentrations increased linearly as the supplemental feeding level of Fe increased to 120 ppm.

Along with hemoglobin, other indicators such as blood RBC and hematocrit are also determinant factors for assessing iron status in piglets. Iron deficient weanling pigs have fewer RBC and containing less hemoglobin, compared with normal piglets [2]. Hematocrit is proportional to the volume of RBCs in blood. Initial mean hematocrit for weanling pigs in this study were in the same range reported by Rincker et al. [4]. The hydration status affects hematocrit of the animal, with a falsely hematocrit by high dehydration [4]. The hydration state of pigs can be affected by stress at weaning. The result of this study showed only a tendency for higher hematocrit when dietary Fe levels increased in phase 1. These


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results are in agreement with those of Dove and Haydon [27] and Rincker et al. [4], who reported higher percentages of hematocrit after 28 days as the dietary concentration of Fe increased. Yu et al. [5] also showed a linear increase in hematocrit as the dietary level of Fe enhanced up to 120 mg Fe/kg. A limitation to this study is the use of a single Fe dose of organic and inorganic sources, and according to these results the best dose of Fe as HME cannot be recommended for weanling pigs in different phases. In addition, a confirmed optimal dose for nano-Fe has not been determined and the recommended dietary level for Fe is still under debate. The high cost of nanoparticle and organic sources has restricted their consumption compared to the common inorganic source. However, apart from the cost, the environmental issues have to be taken into consideration. Therefore, it is crucial for sustainable swine industry to use highly absorbable Fe to decrease environmental pollution.

In conclusion, the weanling pigs fed the HME75 diet exhibited a decreased fecal Fe content compared with INO100, HME100, and ORG100 without any adverse effects in blood hemoglobin. According to the nutrient digestibility and blood parameters results, a higher dose of Fe is recommended during the weaning period.

### Competing interests

No potential conflict of interest relevant to this article was reported.

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### Availability of data and material

Upon reasonable request, the datasets of this study can be available from the corresponding author.

### Authors' contributions

Conceptualization: Cho HJ, Chae BJ.
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### Ethics approval and consent to participate

The project underwent proper ethical standards and the experiments (KW-170519-1) were approved by the Institutional Animal Care and Use Committee of Kangwon National University, Chuncheon, Korea.

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