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Iron Glycine Chelate on Meat Color, Iron Status and Myoglobin Gene Regulation of *M. longissimus dorsi* in Weaning Pigs

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Abstract

Effects of iron glycine chelate (Fe-Gly) on meat color, iron status and gene expression of myoglobin was studied in weaning pigs. A total of 180 pigs, weight about7.81 kg were selected and allotted to six dietary treatments. The treatments consisted of 0, 30, 60, 90, 120 mg Fe/kg diets from Fe-Glys, and 120 mg Fe/kg from $FeSO_4$, respectively. Feeding trial lasted for 40 days, the results showed that a significant increase (P<0.05) in a* values for *M. longissimus dorsi* was observed when pigs were fed with Fe-Gly (60, 90 or 120 mg Fe/kg). A linear response on myoglobin in *M. longissimus dorsi* was observed with increasing Fe-Gly levels. In addition, the increasing Fe-Gly levels enhanced the content of total iron and heme iron in *M. longissimus dorsi*, but had no significant effects in nonheme iron contents between the treatments. Supplementation with 60 or 90 mg/kg Fe-Gly greatly increased the relative abundance of myoglobin mRNA (P<0.05 or P<0.01). To conclude, adding Fe-Gly to diets for pigs improved the meat color, increased muscle total iron and heme iron content, and enhanced gene expression of myoglobin in *M. longissimus dorsi* pork. © 2013 Friends Science Publishers

Keywords: Iron glycine chelate; Meat color; Myoblogin; Weanling pigs

Introduction

Meat color is an important factor of meat products that influences consumer purchase decision and affects their perception of freshness of the product (Wilborn *et al.*, 2004; Adzitey and Huda, 2012). Iron concentration has a direct effect on meat color (Lawrence *et al.*, 2004), and red meat muscle contains higher heme iron and nonheme iron than light muscle (Kongkachuichai *et al.*, 2002). Dietary Fe addition may increase concentration of muscle total iron and heme iron in pork (Yu *et al.*, 2000). The primary pigment of myoglobin, a heme protein, is mainly responsible for meat color in animals (Mancini and Hunt, 2005). Previous studies showed that diets with addition of iron significantly increased the myoglobin content of the *longissimus dorsi* in calives (Bray *et al.*, 1959) or in pork (Lin *et al.*, 2002).

In studies with rats and humans, it was demonstrated that iron glycine chelate maintains high bioavailability. Our previous study also found that compared with ferrous sulfate, appropriate dietary Fe-Gly could improve growth performance, haematological characteristics in weanling pigs (Feng *et al.*, 2007, 2009). Whereas data about the relationship between organic iron and meat color indexes in animal are limited. The objectives of our present research were to evaluate the effects of iron glycine chelate on meat color, iron status and myoglobin gene regulation of *M. longissimus dorsi* in weaning pigs.

Materials and Methods

Animals and Experimental Design

One hundred and eighty pigs (Duroc×Landrace×Yorkshire), weighing about 7.81 kg were selected and allotted to six dietary treatments. Each treatment includes three replications with ten pigs per replicate.

The treatments consisted of 0 (control), 30, 60, 90, 120 mg Fe/kg diets from Fe-Gly, and 120 mg Fe/kg from $FeSO_4$, respectively. The basal diet was formulated based on National Research Council (NRC, 1998) (Table 1). All pigs were given *ad libitum* access to feed and water in the 40 days feeding trial.

Sample Collection

On day 40, four pigs of each treatment were randomly selected and slaughtered. Chops of *M. longissimus dorsi* were removed for sensory, iron status and myoglobin analysis. For color measuring chops were stored at 4°C, other chops stored at –70°C until analysis.

Color Measurement

At 60 min after slaughtering, L^* (indicates lightness), a^* (indicates redness), and b^* (indicates yellowness) values were determined with a Minolta colorimeter (CR-310; Minolta, Tokokawa, Japan).

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Measurement of Myoglobin

Measurement was performed as described by Henry and Bratzler (1960). Duplicate 25 g aliquots of the samples were minced in a blender for two minutes with 100 mL of cold distilled water. After blending, the solutions were centrifuged for 20 min at 2500 rpm. All centrifugation was carried out at temperatures of 4°C. Solid mono and dibasic potassium phosphate was added to precipitate the hemoglobin. To alleviate the difficulty encountered in dissolving the monobasic potassium phosphate it was first ground and sifted through a 250-micron sieve. In calculating the concentration of myoglobin per gram of fresh tissue, the extinction coefficient of 11.5, equivalent to that of cyanomethemoglobin and a molecular weight of 16,500 for myoglobin was assumed.

Total Iron, Heme and Nonheme Iron Analysis

Total iron concents were determined on wet ashed samples according to Helrich (1990) method. An atomic absorption spectrometer (AA6501, Shimadzu Corp., Kyoto, Japan) was used to sample absorbance at a wavelength of 248.3 nm. Comparisons were made to a standard curve using 0, 1, 2, 5, and 10 mg/kg of iron. A method of Schricker *et al.* (1982) was carried out to determine nonheme iron concentrations of *M. longissimus dorsi.*

Total RNA Extraction and Reverse-Transcription-PCR Assay (RT-PCR)

Total RNA of *M. longissimus dorsi* was extracted using Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA). Primer sequences of myoglobin and 18S rRNA housekeeping gene for RT-PCR were designed as shown in Table 2. Components of PCR assay mixture (50 μL) contained: 1 μL cDNA product, 5 μL 10×PCR reaction buffer, 1 μL sense primer (20 mM), 1 μL antisense primer (20 mM) and 0.5 μL Taq DNA polymerase, 3 μL $MgCl_2$ (25 mM), 1 μL dNTPs mix.

PCR for myoglobin and 18S rRNA was done under the following thermal cycles: myoglobin at 94°C for 2 min, 29 cycles (94°C for 45 sec, 58°C for 45 sec, 72°C for 1 min), 72°C for 10 min; 18S rRNA at 94°C for 2 min, 29 cycles (94°C for 45 sec, 53°C for 45 sec, 72°C for 1 min), 72°C for 10 min.

Expression of Myoglobin mRNA in *M. longissimus dorsi*

Quantitative real-time PCR (Q-PCR) reactions were performed on the real-time PCR detection system (Bio-Rad, Hercules, CA, USA). SYBR Green I was used as the fluorescence reporter. Q-PCR was performed in duplicate in 25 μL reaction mixtures under the following protocol: 95°C for 30 sec, 40 cycles (95°C for 30 sec, 58°C for 30 sec) 95°C for 1 min, 55°C for 1 min, followed by a final stage of 95°C for 15 sec, 60°C for 15 sec, and 95°C for 15 sec. The linearity of the dissociation curve was analyzed using the iCycler iQ software 3.0, and the threshold cycle (Ct) was determined. Each sample was analyzed in duplicate and normalized to18S rRNA as the following equation: $\Delta Ct_{GENE} = Ct_{GENE} - Ct_{18S\ rRNA}$. The fold change can be estimated by the formula: $2^{(-\Delta\Delta CtGENE)}$, where $\Delta\Delta CtGENE = \Delta Ct_{GENE}$ of the control $- \Delta Ct_{GENE}$ of each pig (Liao *et al.*, 2007).

Table 1: Ingredient and chemical composition of the basal diet on an as-fed basis

Ingredient	g/kg	Nutrient[a]	
Corn	543.5	DE (MJ/kg)	14.38
Soybean meal	170	Crude protein (g/kg)	207.2
Extruded-soybean	100	Calcium (g/kg)	10.5
Whey	80	Phosphorus (g/kg)	7.6
Fish meal	60	Lysine (g/kg)	13.5
Wheat middling	10	Fe (g/kg)	78.55
Calcium hydrogen phosphate	10	Cu (g/kg)	58.13
Limestone	8	Zn (g/kg)	146.2
Soybean oil	5		
Vitamin mineral premix[b]	10		
Salt	2		
Lysine	1.5		

[a]DE based on calculated values, others were analyzed value
[b]Supplied the following per kilogram of diet: vitamin A 15,000 IU; vitamin D2 3000 IU; vitamin E 30 IU; vitamin B2 5 IU; vitamin B1 3.0 mg; vitamin B12 0.025 mg; biotin 0.06 mg; pantothenic acid 20 mg; nicotinic acid 15 mg; Cu 50 mg; Zn 120 mg; Mn 60 mg; Se 0.67 mg; Co 1 mg

Table 2: Specific primers for the myoglobin and 18S rRNA genes

Gene	Accession number	Primer source	Oligonucleotide sequence	Length (bp)
Myoglobin	M14433	Pig	5'-GGGGAAGGTGGAGGCTGATGTC-3' (sense primer) 5'-GGAACCTGGATGATGGCTTCTGAGT-3' (antisense primer)	335
18S rRNA	AY265350	Pig	5' - CTCCACCAACTAAGAACGG-3' (sense primer) 5' - AAGACGGACCAGAGCGAAA-3' (antisense primer)	375

Data Analysis

General Line Model procedures of SAS were used for data analysis. The planned single-d.f. tests included the linear and quadratic effects of Fe-Gly, the control versus $FeSO_4$ (120 mg Fe/kg), $FeSO_4$ versus Fe-Gly treatments (120 mg Fe/kg). The relative abundance of mRNA for myoglobin after different treatments was compared on the basis of the myoglobin to 18S rRNA ratios. $P<0.05$ was set as differences significant.

Results and Discussion

Meat Color

Compared with the control, Fe-Gly supplementation in diets (60, 90, or 120 mg Fe/kg) increased *a** values for *M. longissimus dorsi* ($P<0.05$), and 120 mg Fe/kg as ferrous sulfate also improved *a** value ($P<0.05$) (Table 3).

It was reported that *a** values in veal enhanced when calves were fed increasing levels of Fe (Gariépy *et al.*, 1998). The red sensory of meat was positively correlated to *a** values ($P<0.01$). Ludeen *et al.* (2004) found that increasing level of dietary organic iron improved the meat color of *M. longissimus dorsi*.

Myoblobin Concentrations

A linear response on myoglobin in *M. longissimus dorsi* was observed with increasing Fe-Gly levels (Fig. 1). There was no difference on myoblobin concentrations when diets were supplemented with Fe-Gly or FeSO4. Meat color is affected by the concentration and properties of the meat pigments' myoglobin (Olsson and Pickova, 2005). The results from the current experiment were consistent with the studies of Bray *et al.* (1959) and Lin *et al.* (2002), supplementation of diet with iron had a significant effect upon muscle myoglobin concentration in the *longissimus dorsi* muscle. Furthermore, the increased *a** value or meat color may be a result of enhanced myoglobin contents in response to dietary Fe level (Yu *et al.*, 2000; Ludeen, 2004).

Iron Status

Iron concentration has a direct effect on meat color (Lawrence *et al.*, 2004) and the red meat muscle contains higher heme and non-heme iron than light muscle (Kongkachuichai *et al.*, 2002). Present study found that the total iron and heme iron contents increased when pigs were fed increasing levels of Fe-Gly (Table 4). Yu *et al.* (2000) reported that increasing iron concentration in the muscle agreed with the trend toward redness of skin color, and both total-iron and heme-iron concentrations increased significantly along with the level of Availa-Fe (0-120 mg/kg) supplement. However, it should be noted that supplementing swine diets with 50-150 mg/kg of Fe (Availa-Fe, produced by Zinpro Corp.) did not alter concentrations of LM total, heme and non-heme iron. This point needs further study.

Development of Myoglobin Expression (RT-PCR and Q-PCR Assay)

Compared with the control, supplementation Fe-Gly (60, 90, 120 mg Fe/kg) enhanced the relative abundance of myoglobin ($P<0.05$; Fig. 2). When the piglets were fed with 60, 90, or 120 mg/kg Fe-Gly, myoglobin mRNA expression were higher than the piglets of the control group in Q-PCR assay (Fig. 3). In addtion, myoglobin mRNA expression of *M. longissimus dorsi* peaked when pig fed with 90 mg/kg Fe-Gly among all the treatments. The 18S rRNA mRNA levels did not differ among treatments.

Myoglobin, an iron-binding protein, is the main pigment of red muscles, and its concentration in muscle is the most important factor responsible for meat color. Bray *et al.* (1959) found that the myoglobin concentration of the *longissimus dorsi* was nearly twice as high in calves fed iron

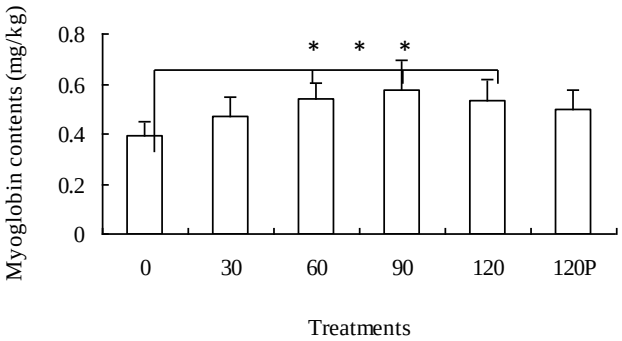

Fig. 1: Effects of Fe-Gly on content of myoglobin in *M. longissimus dorsi* from weaning pigs. Treatments consisted of: 0-120 mg Fe/kg diet from Fe-Gly groups and positive control (120P, 120 mg Fe/kg diet from $FeSO_4$). Values are means ± SD, "*"indicates significant difference compared with the control

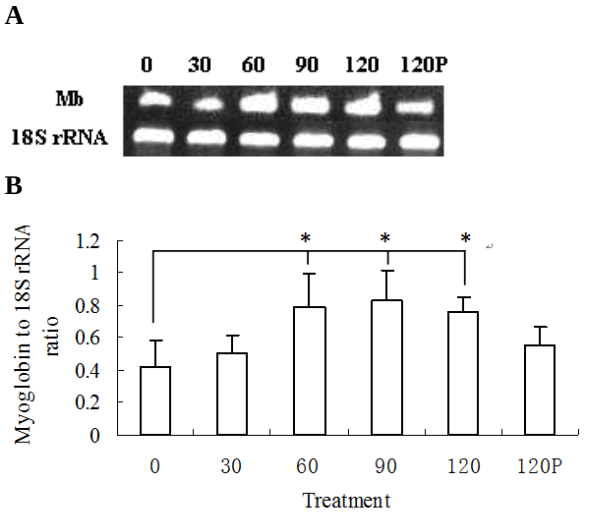

Fig. 2: Effect of Fe-Gly on the expression of myoglobin in the *M. longissimus dorsi* of weanling pigs

(A) Electrophoresis results of reverse-transcription-PCR for myoglobin and 18S rRNA in *M. longissimus dorsi*. Lane 1: control group; lane 6: 120 mg/kg FeSO4 group; lane 2-5: 30, 60, 90, 120 mg/kg Fe-Gly group

(B) The integrated optical density (IOD) ratio of each band of myoglobin and 18S rRNA for the relative group. Myoglobin gene expression was shown as myoglobin to 18S rRNA ratios

supplemented diets as in control animals. Lin *et al.* (2002) showed that the concentration of myoglobin in muscle was greatly increased with dietary Fe level (0-400 mg/kg). Therefore, it would be expected that myoglobin expression is increasing as iron levels. Lacking information on myoglobin expression and iron levels, Stephen and Robert (1983) reported that treatment with a variety of Fe(Ⅲ) chelates had no effect on myoglobin expression but enhanced both iron accumulation and ferritin synthesis in L6 cells. The early observation found that inorganic iron promotes protein synthesis in reticulocytes by stimulation of heme synthesis (Grayzel *et al.*, 1966), and has no

Table 3: Effects of different levels of iron glycine on meat color of *M. longissimus dorsi* in weanling pigs [a]

Item	Fe-Gly[b]					$FeSO_4$[b]	S.E.M.[c]	P-value[d]			
	0[e]	30[e]	60[e]	90[e]	120[e]	120[e]		Control *vs.* $FeSO_4$	$FeSO_4$ *vs.* Fe-Gly	Fe-Gly	
										Linear	Quadratic
a*	6.75	7.04	10.18	10.88	8.94	7.26	0.421	0.767	0.301	0.038	0.121
b*	11.6	12.85	13.57	12.56	13.70	12.81	0.275	0.215	0.667	0.713	0.304
L*	38.76	41.58	38.91	38.62	39.55	40.75	0.597	0.353	0.423	0.789	0.819

Table 4: Total iron, heme iron and non-heme iron in meat from weanling pigs [a] (mg/kg)

Item	Fe-Gly[b]					$FeSO_4$[b]	S.E.M.[C]	P-value[d]			
	0[e]	30[e]	60[e]	90[e]	120[e]	120[e]		Control *vs.* $FeSO_4$	$FeSO_4$ *vs.* Fe-Gly	Fe-Gly	
										Linear	Quadratic
Total Fe	7.57	8.61	10.25	10.42	9.16	9.00	0.001	0.098	0.789	0.001	0.000
Heme Fe	3.91	5.20	6.79	7.25	6.03	5.99	0.001	0.005	0.837	0.000	0.000
NonhemeFe	3.65	3.41	3.46	3.17	3.13	3.01	0.236	0.108	0.123	0.070	0.931

[a]Values are presented as means: n=4 for per treatment and data reported on a fresh basis
[b]Fe source
[c]Standard error of the mean
[d]Non-orthogonal comparisons between the control *vs.* $FeSO_4$ (120 mg Fe of per kg diet), and the $FeSO_4$ (120 mg/kg) *vs.* Fe-Gly treatments (30~120 mg Fe of per kg diet). Linear and quadratic effects of increasing dietary Fe supplementation (0~120 mg/kg) as Fe-Gly
[e]Fe addition (mg/kg)

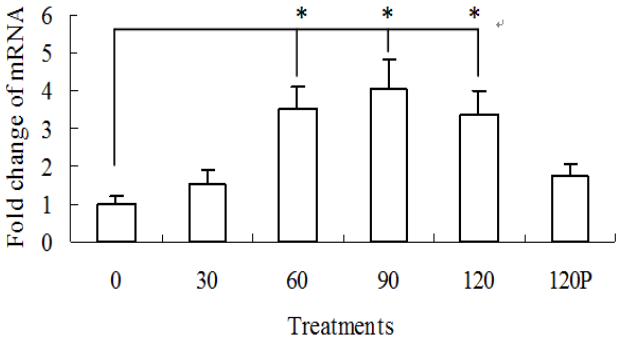

Fig. 3: Myoglobin mRNA expression in *M. longissimus dorsi* of weanling pigs

The extracted mRNA was analyzed by Q-PCR. PCR products were quantified for relative levels of mRNA using image by comparing Mb with 18S rRNA. Treatments consisted of: 0-120 mg Fe/kg diet from Fe-Gly groups and positive control (120P, 120 mg Fe/kg diet from FeSO4 group). Values are means±SD, "*"indicates significant difference compared with the control

effect on either myoglobin or total protein accumulation (Bailey *et al.*, 1990). The results of the present study indicated that Fe-Gly could promote myoglobin protein synthesis. This point warrants further research.

Conclusion

Adding Fe-Gly to weaning pigs diets could improve meat color, increase muscle total iron and heme iron content, and enhance gene expression of myoglobin in *M. longissimus* dorsi of pork.

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