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Postnatal Growth Changes of Myoglobin and its Gene Expression Level in M. longissimus Dorsi Muscle of Jinhua and Landrace Barrows

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Abstract: The study investigated the developmental changes of Myoglobin (MB) in muscles of Jinhua pig (China's fatty breed) and the breed differences between Jinhua and Landrace (lean) pigs. About 30 Jinhua and Landrace barrows (three of each breed, at birth, age 60, 90, 120 and 150 days) were used to detect the postnatal growth changes of MB, peroxisome proliferator-activated receptor gamma, co-activator 1 alpha (PPARGC1A) and Peroxisome Proliferator-Activated Receptor Delta (PPARD) mRNA expression and soluble MB concentration (SM) in M. Longissimus Dorsi muscle (LD). Results showed that MB and PPARD mRNA expression in LD were significantly affected by ages of pig and PPARGC1A mRNA expression in LD was lower at birth and significantly higher from age 60-150 days ($p<0.05$). The differences of MB, PPARGC1A and PPARD mRNA expression and SM in LD between two pig breeds were also investigated. The data showed that MB and PPARD mRNA expression and SM in LD of Jinhua barrows were significantly higher ($p<0.05$) than that of Landrace barrows from 60-150 days but no significant difference at birth and PPARGC1A mRNA expression were similar but the age was only at 120 days ($p<0.01$). At last, MB, PPARGC1A and PPARD mRNA expression and the correlation among gene, protein, CIE (a uniform colorimetric system defined by the Commission Internationale de l'Eclairage) and pH were evaluated. The data showed that the correlation between MB, SM, PPARGC1A and PPARD mRNA expression in LD were high and the correlation coefficients between MB and SM, PPARGC1A, PPARD, CIE L* and CIE a*-values were 0.81, 0.44, 0.81, -0.26 and 0.69, respectively. Correspondingly, the correlations between SM and PPARGC1A, PPARD, CIE L*, CIE a* were 0.44, 0.74, -0.26 and 0.73, respectively. In summary, the study is the first one which was conducted to explain the postnatal growth changes of MB mRNA expression in skeletal muscles of Jinhua pigs and provides information for development of nutritional methods to regulate MB in regulating and improving meat quality.

Key words: Jinhua barrows, landrace barrows, MB, PPARGC1A, PPARD, gene expression, SM, LD

INTRODUCTION

Meat quality has gained more and more attention with the increasing of meat production. Eating quality and sensory experience including flavor, tenderness, juiciness and color have been regarded as the most critical characteristics. Providing consumers with both satisfactory eating quality and sensory experience is the aim for increasing and maintaining repeated consumer purchases. China's characteristic pigs have more advantages than foreign pigs breed in meat quality and Jinhua pigs are excellent in some aspects like high Intamuscularfat (IMF), redder and tender meat and good taste flavor. In China, there are some studies on the meat quality of Jinhua pigs found that Jinhua pigs have more total pigment content and redder color in meat than Landrace pigs. The physiological basis of meat color is MB and meat color largely depends on MB concentration, its chemical state and the overall physical state of meat (pH, myofibril proteins state and denaturation degree, etc.) (Ledward, 1992). Lin *et al.* (2002) found that as PPARGC1A is expressed preferentially in muscle enriched in type I fibres so, when PPARGC1A is expressed at physiological levels in transgenic mice driven by a

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Muscle Creatine Kinase (MCK) promoter, a fibre type conversion is observed: muscles normally rich in type II fibres are redder and activate genes of mitochondrial oxidative metabolism. Notably, putative type II muscles from PPARGC1A transgenic mice also express proteins characteristic of type I fibres such as troponin I (slow) and MB. Wang *et al.* (2004) also found that in transgenic mice activation of PPARD the level of MB increased. Interestingly, both of them found that muscles in the transgenic mice appeared redder this result suggests that PPARGC1A or PPARD may be an upstream transcription factor of MB. There are few publications concerning *MB* gene expression in pigs and no information has yet been published on the differences of *MB* gene expression between Jinhua and Landrace pigs. The main objective of this study is to compare the postnatal growth changes of MB mRNA expression in the two breeds of pig from birth to 150 days of age.

MATERIALS AND METHODS

Animals: All handling of the animals was done in accordance with Chinese law for the protection of animals and the guidelines for animal care of Canadian Council on Animal Care.

A total of 30 pigs were fed with the same commercial diet to study the postnatal changes of *MB* gene expression and its relationship with meat color measurement. Three barrows of five age-levels were used: birth and 60, 90, 120 and 150 days.

Sample collection: The experimental pigs of different age levels were exsanguinated using the commercial slaughter method. Samples of LD at the 10th rib were collected and immediately frozen in liquid nitrogen. LD samples were divided into two halves, one half were stored at 4°C which were intended for the detection of MB concentration and objective measures. Another half were stored at -80°C for determination the mRNA expression levels of *MB* gene. The remaining samples were stored at -80°C.

Meat color and pH: All sample muscles were immediately obtained after slaughter the experimental pigs. The a* (redness), b* (yellowness) and L* (lightness) values of meat color were spectrophotometrically detected at the 45 min postmortem using ColorFlex (The Self-Contained Color Measurement Spectrophotometer). The pH was measured on the 10th rib face of the LD using a Mettler-Toledo pH meter (SevenMulti, Greifensee, Switzerland) equipped with an electrode (InLab®427, Mettler Toledo). Three measurements were taken from each sample and averaged.

Soluble myoglobin content: A 4.45 cm diameter core was removed from each sample and the top 1/3 of each core was removed and chopped finely. Of the finely chopped muscle, 5 g were homogenized with 25 mL of 2 mM phosphate buffer (pH 7.0) for 45 sec in a Waring blender. The homogenate was centrifuged at 35000 g for 30 min at 4°C and the supernatant filtered through Whatman#541 filter paper. The supernatant was oxidized with 1-2 crystals of $K_3Fe(CN)_6$, poured into 10000 molecular weight cut off dialysis tubing (Spectra/Por, Spectrum Industries inc., Rancho Dominguez, CA) and dialyzed at 4°C against 2 mM phosphate buffer (pH 7.0) with two phosphate buffer change-overs.

After dialysis, approximately 3 mL of the muscle extract was placed in a standard cuvette and absorbance read at 572, 565, 545 and 525 nm using an Ultrospec 2000 UV/visible spectrophotometer (Pharmacia Biotech). SM was calculated using the equation described by Krzywicki (1982).

Total RNA extraction: Total RNA was extracted from collected tissues by Trizol reagent (Invitrogen) according to the manufacture's protocol. RNA quality was assessed by OD_{260nm} calculation method and all the ratios of OD_{260nm}/OD_{280nm} were in the range 1.8 and 2.0. Approximately 4 μg of total RNA was used for reverse transcription using ThermoScript Real-time PCR system (Invitrogen) and random primers. The specific procedure was carried out according to methods of Shan *et al.* (2008, 2009). The cDNA products were initially confined by 18S ribosome RNA (18S rRNA) amplification using semi-quantitative PCR before Real-Time PCR detection.

Reverse transcription: A mixture of 4 μg of total RNA and 2 μL of random primers were added up to 11 μL using nuclease free water before denaturation at 70°C for 5 min. The following components were then added in the following order: 4 μL of 5×reaction buffer, 2 μL dNTP mix (10 mM each of dATP, dCTP, dGTP and dTTP), 1 μL M-MLV reverse transcriptase (200 U μL^{-1}, Promega) with nuclease free water added to a final volume of 20 μL. After gently mixed the reaction mixture, the mixture was incubated at 37°C for 60 min.

Quantitative RT-PCR for MB in RNA expression: Real-time PCR was performed using SYBR green I nucleic acid dye on StepOnePlus Real-Time PCR System (Applied Biosystems). Primer pairs designed by Primer Premier 5.0 software (Premier Biosoft International, USA) for *MB*, *PPARGC1A*, *PPARD*, *18S rRNA* genes were

Table 1: Primer pairs designed for the determination of the porcine MB and other correlative gene mRNA levels using Real-time PCR. The target gene mRNA levels were normalized against 18S mRNA levels

Genes name	Direction	Sequence	Length (m)	GenBank accession no.	Product length (bp)
MB	-F	5'-ccgcacttgctctgtttctct-3'	21	NM214236f	102
	-R	5'-gacatcagcctccaccttcc-3'	20		
PPARGC1A	-F	5'- gtgtcgccttcttgttcttctttt-3'	24	AY346131	92
	-R	5'-cgcatcctttggggtcttt -3'	19		
PPARD	-F	5'-agtcagcgtcgtgtggtgtt -3'	20	AY188501	106
	-R	5'-ggcagttcctgtcaacctcttt -3'	22		
18S	-F	5'-cccacggaatcgagaaagag-3'	20	AY265350	122
	-R	5'-ttgacggaagggcacca-3'	17		

shown in Table 1. As 18S rRNA had been found to be stable between the samples containing equal amounts of analyzed cDNA (Wimmers *et al*., 2008) so, the researchers used it as an internal control in all real-time PCR detections, according to following thermal profile: 95°C for 10 sec, followed by 40 cycles of 95°C for 10 sec, 60°C for 34 sec, last by 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec. To calculate the expression of mRNA of the *MB*, *PPARGC1A* and *PPARD* gene, the Ct values were used for the comparison of the two genes and the internal control, using the $2^{-\Delta\Delta Ct}$ method (Shan *et al*., 2008, 2009). Briefly, 1 μL of cDNA template was added to each well in a 96 well reaction plate and the transcripts of each gene were amplified in triplicate. Average CT valus were computed for each gene. Subsequently, ΔΔCT was computed for each gene by subtracting the average ΔCT for the control group. The final fold differences were computed as $2^{-\Delta\Delta Ct}$ for each gene. These measurements were repeated 3 times.

Date analysis: Statistical analysis was carried out with SPPS 15.0 software using one-way ANOVA. The MB mRNA level was determined by RT-PCR and presented as a ratio to 18S rRNA levels for each sample. The correlations was analyzed use Pearson's bivariate correlation analysis of SPSS 15.0 to examine the relationships between SM, MB, PPARGC1A, PPARD, CIE L*, CIE a*, CIE b* and pH_{45} values. All experimental data was presented here as means±SE. Statistical significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Meat color and pH: The value of CIE L* and CIE b* generally decreased with age from birth to 150 days while that of a*-value increased as a whole and there was no significant difference with pH_{45} values in the experiment (Table 2).

Soluble myoglobin content: SM was apparently affected by the age of Jinhua barrows (Table 2) and significantly higher from the age of 60-150 days ($p<0.001$) compared to at birth.

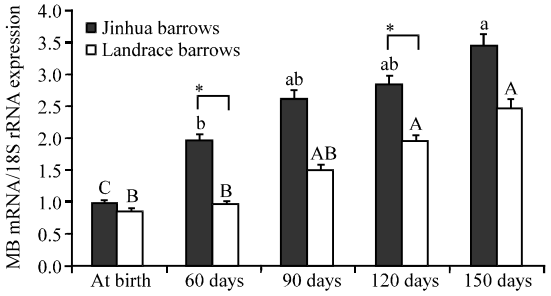

Fig. 1: Effect of age on MB mRNA expression levels in LD of Jinhua and Landrace barrows. Myoglobin mRNA expression was detected by Real-time PCR and presented as normalized to 18S rRNA expression and expressed relative to MB mRNA levels at birth of Jinhua pigs. Each column represents the mean of three experimental pigs. $^{a, b, A-C}$Mean without a common letter (the lower case for Jinhua pig and the capital for Landrace) differs significantly between ages ($p<0.05$). *$p<0.05$, significantly different between the two breeds at the same age

Postanal growth changes of *MB* gene expression in LD of Jinhua and landrace barrows: MB mRNA expression was generally increased from birth to 150 days as a whole and significantly affected by age of pigs (Fig. 1) and the MB mRNA levels in Jinhua barrows were significantly higher from ages of 60-150 days ($p<0.05$) compared with Landrace barrows.

Other correlative gene expression in Jinhua and Landrace barrows: To obtain further proof for a functional role of *MB* gene expression in muscle of pig, the PPARGC1A and PPARD mRNA levels in LD were evaluated (Fig. 2 and 3). The expression of both PPARGC1A and PPARD mRNA showed a similar pattern to that of MB mRNA specifically, PPARD was significantly higher from ages of 60-150 days ($p<0.01$) while the PPARGC1A was significantly higher only at 120 days ($p<0.01$) in Jinhua barrows compared with Landrace barrows.

Table 2: Postanal growth changes of live weight, instrumental color, pH and SM in LD of Jinhua and Landrace barrows

	Age					
Traits[†]	Breed[‡]	At birth±SE	60 days±SE	90 days±SE	120 days±SE	150 days±SE
Live weight (kg)	J	1.100±0.120^{e}	10.330±0.070^{d}	23.030±0.280^{c}	36.670±0.330^{b}	46.000±2.310^{a}
	L	1.180±0.060^{E}	15.150±0.400^{D}***	38.330±1.860^{C}**	62.670±2.910^{B}**	78.330±1.200^{A}***
L*	J	49.290±3.040^{a}	44.080±0.240^{b}	45.940±0.410^{ab}	43.120±1.170^{b}	41.230±0.460^{b}
	L	50.750±0.640^{A}	44.540±0.470^{B}	44.300±0.490^{B}	39.590±1.070^{C}	41.290±1.000^{C}
a*	J	10.380±0.090^{d}	11.320±0.390^{c}*	11.800±0.130^{c}*	13.430±0.120^{b}***	14.640±0.300^{a}***
	L	8.940±0.560^{B}	9.760±0.350^{AB}	9.490±0.710^{AB}	10.220±0.090^{AB}	10.570±0.150^{A}
b*	J	12.740±0.080^{a}	10.290±0.310^{b}	12.070±0.330^{a}	11.630±0.660^{ab}*	10.520±0.660^{b}*
	L	12.170±0.210^{A}	10.740±0.120^{B}	10.950±0.520^{B}	8.190±0.410^{C}	8.740±0.140^{C}
pH_{45}	J	6.430±0.430^{a}	6.410±0.210^{a}	5.970±0.140^{a}	6.150±0.010^{a}	6.370±0.090^{a}
	L	6.430±0.450^{A}	6.560±0.160^{A}	6.410±0.110^{A}	6.500±0.140^{A}	6.320±0.360^{A}
SM (mg g^{-1})	J	0.067±0.008^{d}*	0.419±0.099^{c}	0.629±0.026^{b}**	0.848±0.044^{a}*	0.931±0.015^{a}***
	L	0.031±0.002^{D}	0.248±0.038^{C}	0.369±0.023^{AB}	0.431±0.134^{AB}	0.536±0.021

[A†]CIE L* = black (0) to white (100) scale; CIE a* = red (+) to green (-) color scale; CIE b* = yellow (+) to blue (-) color scale; pH_{45} = pH of LD 45 min postmortem; SM: Soluble Myoglobin content, mg g^{-1} (as is/wet basis) ‡J = Jinhua pig, L = Landrace SE = Stand Error of the mean. [a-e, A-E]Mean without a common letter (the lower case for Jinhua pig and the capital for Landrace) differs significantly between ages ($p<0.05$).*$p<0.05$, **$p<0.01$,***$p<0.001$, significantly different between the two breeds at the same age

Table 3: Correlation of biochemical, gene, objective color measurements and physical for LD

Traits[a]	SM	MB	PPARGC1A	PPARD	CIE L*	CIE a*	CIE b*	pH_{45}
SM	1	0.81**	0.44**	0.74**	-0.26	0.73**	-0.14	-0.13
MB	-	1	0.44**	0.81**	-0.26	0.69**	-0.21	-0.27
PPARGC1A	-	-	1	0.52**	-0.28	0.45**	-0.19	-0.34*
PPARD	-	-	-	1	-0.21	0.88**	-0.08	-0.38*
CIE L*	-	-	-	-	1	-0.19	0.74**	-0.11
CIE a*	-	-	-	-	-	1	-0.01	-0.18
CIE b*	-	-	-	-	-	-	1	-0.12
H_{45}	-	-	-	-	-	-	-	1

[a]SM = Soluble Myoglobin content (mg g^{-1}) (as is/wet basis); CIE L* = black (0) to white (100) scale; CIE a* = red (+) to green (-) color scale; CIE b* = yellow (+) to blue (-) color scale; pH_{45} = pH of LD 45 min postmortem; *$p<0.05$; **$p<0.01$

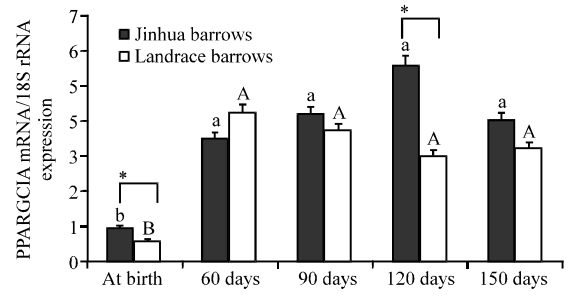

Fig. 2: Effect of age on PPARGC1A mRNA expression levels in LD of Jinhua and Landrace barrows. This mRNA expression was detected by Real-time PCR and presented as normalized to 18S rRNA expression and expressed relative to the PPARGC1A mRNA levels at birth of Jinhua pigs. Each column represents the mean of three experimental pigs. [a-c, A,B]Mean without a common letter (the lower case for Jinhua pig and the capital for Landrace) differs significantly between ages ($p<0.05$). *$p<0.05$, significantly different between the two breeds at the same age

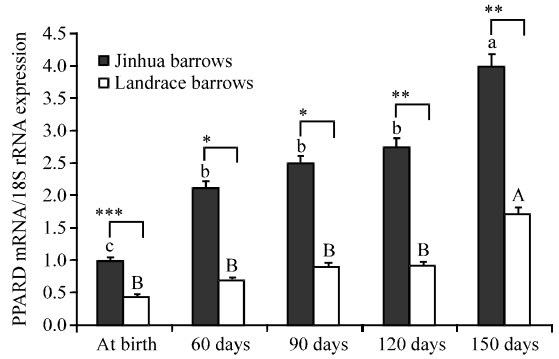

Fig. 3: Effect of age on PPARD mRNA expression levels in LD of Jinhua and Landrace barrows. This mRNA expression was detected by Real-time PCR and presented as normalized to 18S rRNA expression and expressed relative to the PPARD mRNA levels at birth of Jinhua pigs. Each column represents the mean of three experimental pigs. [a-c, A-C]Mean without a common letter (the lower case for Jinhua pig and the capital for Landrace) differs significantly between ages ($p<0.05$). *$p<0.05$, **$p<0.01$, ***$p<0.001$, significantly different between the two breeds at the same age

Correlations between meat color, MB content and *MB* gene expression in Jinhua and Landrace pigs: The correlation coefficients between the investigated meat quality traits and MB from the investigated groups of pigs were shown in Table 3. The absolute value of the correlation between *MB* gene expression and other meat quality indicator traits were 0.81 or less. The correlation coefficients between MB and SM, PPARGC1A, PPARD, CIE L*, CIE a* were 0.81, 0.44, 0.81, -0.26 and 0.69,

respectively. Correspondingly, the correlation coefficients between SM and PPARGC1A, PPARD, CIE L*, CIE a* were 0.44, 0.74, -0.26 ansd 0.73, respectively.

Meat color and pH: As shown in Table 2, a*-value was affected significantly by age and breeds as a whole but not L*-value, b*-value and pH_{45} in two breeds which meant meat from Jinhua borrows were redder than Landrace borrows. These results were different from previous results reported by Miao *et al.* (2009). Miao found that developmental changes of the L*- and b*-value followed same descending trends in the two breeds except for a*-value and L*-values were higher in Landrace than in Jinhua pigs. May be it was because the test pigs selected by Miao were sex balance while barrows by us.

Soluble myoglobin content: Apparently, SM were significantly higher in LD of Jinhua barrows than Landrace barrows at ages of birth, 90, 120 and 150 days ($p<0.05$) and more than that the correlation between SM and a*-value is very close and their correlation coefficient is 0.73 which indicates that myoglobin is the important factor that affect a*-value and Jinhua pigs could be selected a superior breed as breeding animals because they contain more myoglobin in muscle.

Postanal growth changes of MB and other correlative gene expression in LD of barrows: This research demonstrated for the first time the postanal growth changes of the *MB* gene and other correlative gene expression in LD of Jinhua and Landrace barrows. Both of them were at lower level at birth and became higher and stable after birth. Tong *et al.* (2004) found that MB mRNA expression was low in Erhualian and Large White boars at 3 days and divergent trends were followed by different breed of pigs thereafter. No significant changes in MB mRNA expression were observed in Large White boars during the period of study although, a higher level was seen at 120 days. In Erhualian boars however, the level of MB mRNA rose significantly ($p<0.01$) from 3-20 days and stayed high consistently afterwards. The results were similar but not the same which may means that *MB* gene expression pattern has something in common but with differentiated rules in different breed of pigs.

CONCLUSION

The present study investigated for the first time that the MB mRNA expression pattern in Jinhua pigs and its correlation with other meat quality traits. First, the researchers found that *MB* gene expression and SM in LD were significantly affected by pig's age and there was a high correlation coefficient between them of 0.81 ($p<0.01$) in accordance with Weller *et al.* (1986). This indicates that the regulation of *MB* gene expression is at translational level. Second, the *MB* gene expression and SM were both highly correlated with a*-value, consistent with previous study that SM in porcine skeletal muscles was an indicator of the CIE a*-values (redness) (Leseigneur-Meynier and Gandemer, 1991). All of these data was consistent with previous reports that meat color in pigs was significantly affected by age (Lindahl *et al.*, 2006). The researchers found that *PPARGC1A* and *PPARD* gene expression in LD were also significantly affected by the age and the correlation between MB and PPARD was higher than for MB and PPARGC1A. Lin *et al.* (2002) and Wang *et al.* (2004) found that transgenic PPARGC1A and PPARD expression, respectively could robustly induced the expression of genes specifically enriched in type I fibres such as MB. Thus, the results suggest that PPARD may play a more important role than PPARGC1A in regulating *MB* gene expression. Nevertheless, more studies are necessary to gain wider knowledge about the molecular regulation mechanism of MB mRNA and how to improve MB content in skeletal muscles of pigs to develop higher meat quality in the future.

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