Journal of Animal and Veterinary Advances 9 (3): 529-534, 2010
ISSN: 1680-5593

Hypoxic Adaptation and Myoglobin Expression in Heart Tissue of Tibet Chicken Embryo

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Abstract: Myoglobin (Mb) is a classical member of the globin family and plays an important role in the oxygen transportation or storage. As a unique native chicken breed in high altitude, Tibet chicken has the good adaptation to hypoxia. Here we present the first detailed analysis of Mb expression on hypoxic adaptability in chicken heart. In the present study, fertile eggs of Tibet chicken, Shouguang chicken and Silky chicken were exposed to sustained hypoxia (13% O_2) and normoxia (21% O_2). Chicken embryo hearts were collected on days 16 and 20 of incubation to examine the effect of hypoxia on Mb. The results showed that there is no significant difference in content or expression levels of Mb among the three chicken breeds in the normoxic environment. Tibet chicken embryos had the heaviest heart weight among these chicken breeds and the Mb content of all chicken breeds had increased in hypoxia comparing with that in normoxia. Under the hypoxic environment, Mb mRNA levels of Tibet chicken were lower than that of Shouguang and Silky chicken, indicating that the hypoxic degree between Tibet chicken and other lowland chicken was different. The smaller changes of Mb content and expression levels in hypoxia in Tibet chicken embryo suggests Tibet chicken had the better hypoxic adaptation ability in utilizing oxygen in heart tissue. The results showed that Mb is up-regulated by hypoxia and may play an important role in mediating heart hypoxic adaptation.

Key words: Myoglobin, Tibet chicken embryo, hypoxic adaptation, Mb content, eggs

INTRODUCTION

Myoglobin, a protein with a rich history is a cytoplasmic hemoprotein, developed in red muscle in response to mitochondrial demand for oxygen (Wittenberg, 1970) and transports oxygen from the sarcolemma to the mitochondria of vertebrate heart and red muscle cells (Wittenberg and Wittenberg, 1989; Takahashi and Doi, 1998; Wittenberg and Wittenberg, 2003), which reversibly binds O_2 by its heme residue (Ordway and Garry, 2004). In the recent decade, gene disruption technologies have been utilized to study the functions of Mb under the hypoxic stress (Garry *et al*., 1998; Meeson *et al*., 2001; Schlieper *et al*., 2004).

Hypoxia is the main physiological challenge which threatens the survival of organisms in high-altitude areas. Many researchers found that Mb content and expression levels in skeletal muscle of humans have increased at high altitudes (Terrados, 1992; Gelfi *et al*., 2004) and Fraser *et al*. (2006) improved hypoxia can also inducible Mb expression in nonmuscle tissues. In the Tibetan plateau, Tibet chicken has developed subtle mechanism of surviving in a hypoxic environment in the long-term adaptation, demonstrated that the adaptability of Tibet chicken embryos to hypoxia is significantly higher than other lowland chicken breeds (Gou *et al*., 2005; Wang *et al*., 2007; Bao *et al*., 2007; Zhang *et al*., 2008; Li and Zhao, 2009). Until now, our group had confirmed that the embryo death was mainly observed between Tibet chicken and the other lowland chicken when the egg was incubated at days 18-21, showing that the embryos at late phase of incubation were most sensitive to hypoxia (Bao, 2007; Gou *et al*., 2007; Zhang *et al*., 2007). Therefore, we supposed that the different levels of Mb content and mRNA expression at days 16 and 20 in the heart under the hypoxic condition may be helpful for Tibet chicken to adapt the environment. After examining the role of Mb related with the hypoxic adaptation between the highland and lowland chicken breed, we therefore conducted a detailed study on Mb regulation by hypoxia.

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Table 1: Primer sequence and size of Real-time PCR analysis

Target gene	GenBank No.	Sense/Antisense	Size (bp)
Mb	XM_416292	F: 5' GAAAAGTGGAGGCCGACAT 3'	141
		R: 5' TCAGATCTTCAGAGCCCTTCA 3'	
28s rRNA	M59792	F: 5'-GGAGCCCCGGGGAGAGTTC-3'	140
		R: 5'-GGATTTTCACGGGCCAGCGAGAG-3'	
Hprt	AJ132697	F: 5'- CAACCTTGACTGGAAAGAATGT-3'	171
		R: 5'-CAACAAAGTCTGGCCGATAT-3'	

MATERIALS AND METHODS

Animals: Hens of Tibet chicken, Shouguang chicken and Silky chicken at the same age were raised in the Experiment Chicken Farm of the China Agricultural University (CAU, Beijing, 100 m altitude) with the same management procedure. At the end of week 40, they were fertilized by artificial insemination. Tibet chicken (n = 260), Shouguang chicken (n = 300) and Silky chicken fertile eggs (n = 300) were incubated in a hypoxic simulation hatching machine, using a gas mixture containing 13% oxygen and 87% nitrogen simulating an altitude of 4,000 m. At the same time, fertile eggs of Tibet chicken (n = 100), Shouguang chicken (n = 60) and Silky chicken (n = 60) incubated for 21 days in a normoxic hatching machine as controls.

Sampling procedure: On days 16 and 20 of incubation, eggshells were opened at the air-cell and living embryos were pulled out with a nipper and hearts were collected and immediately put into 2 mL microcentrifuge tubes in an ice bath.

Myoglobin content: All hearts were weighed and homogenized (10% w/v homogenate) in ice-cold buffer (2 mM $MgCl_2$, 1 mM EDTA, 75 mM Tris, pH7.2, as described by Bailey and Driedzic (1992) and Reed *et al.* (1994)). The samples were centrifuged at 4°C and 12,000 g for 15 min. The supernatant was collected and Mb content was assayed with an ultraviolet spectrophotometery. A wavelength from 500-610 nm was carried out comparing the supernatant to buffer. The peak absorbance above background light scatter at 581 nm was determined. Mb concentration was calculated using 12.8 as the millimolar extinction coefficient (Hardman *et al.*, 1966; Egginton, 1986) and is expressed as $nmol g^{-1}$ wet mass.

RNA isolation and RT-PCR: Heart tissues from 10 live embryos of Tibet chicken, Shouguang chicken and Silky chicken were sampled respectively in hypoxia and normoxia. RNA was extracted using TRNzol and RNA samples were treated with RNaseout and DNase following the manufacturer's instructions (Qiagen, Germany). Aliquots of total RNA were reverse transcribed using random primers and M-MLV Reverse transcriptase (Promega, US).

Real-time PCR: cDNA PCR was performed using the SYBR Green master mix (ABI Applied Biosystems, US) with the primers shown in Table 1. Standard curves for the target genes of interest (Mb) and the housekeeping gene (Hprt, hypoxanthine phospho-ribosyl transferase) was drawn for each assay. Reactions were run on an ABI PRISM 7900 Sequence Dectecor (ABI Applied Biosystems, US). The cycling conditions comprised 2 min at 50°C, 10 min at 95°C and 40 cycles at 95°C for 25 sec, 57°C for 30 sec and 72°C for 25 sec. Each real-time PCR, including the non-template control was performed in triplicate. A melting curve was obtained after completions of the cycles to verify the present of a single amplicon. The real-time PCR products were sequenced to certain the correct target sequences. Based on the standard curves for each gene, cDNA copy number of the samples was calculated according to the samples Qty mean. Finally, each of the calculated copy numbers for Mb was normalized against the corresponding hprt copy numbers. Standard curves and RNA expression were analyzed using SDS2.2 software (ABI Applied Biosystems, US).

Statistical analysis: Data of embryo heart weights, Mb contents, Mb mRNA expressions were subjected to GLM analysis using SAS software (Version 8.02, SAS Inc, US) respectively. Values of $p<0.05$ were considered statistically significant and $p<0.01$ or $p<0.001$ were considered highly statistically significant.

RESULTS

Heart weight of chicken embryo: The heart weights, including Tibet chicken and the other chicken breeds, were indicated in Fig. 1. It should be noted that the heart weight of two lowland chicken breeds on day 16 in hypoxia were significantly lower than in normoxia ($p<0.05$ in Silky chicken and $p<0.01$ in Shouguang chicken), while three chicken breeds on day 20 in hypoxia had lower heart weight than that of in normoxia ($p<0.001$ in all chicken breeds).

Under the hypoxic condition, the heart weight of Tibet chicken were higher than that of lowland chicken breeds ($p<0.001$ in Shouguang chicken and $p<0.01$ in Silky chicken on day 16 and $p<0.05$ in Shouguang and Silky chicken on day 20).

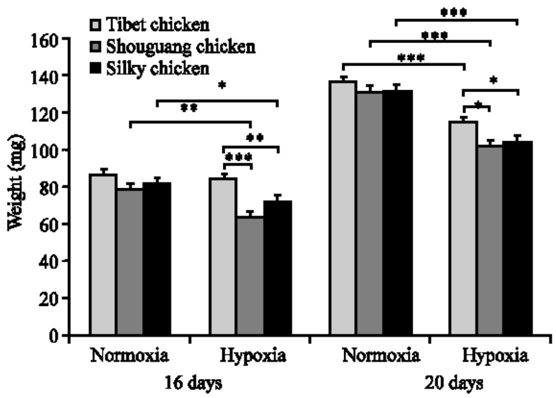

Fig. 1: Weight of hearts in different chicken breeds under hypoxic and normoxic conditions. Each bar represents the mean±SE for each group and Tibet chicken contains 16 samples and Shouguang chicken and Silky chicken contain 10 samples, respectively. *Significant difference at 5% level, **Significant difference at 1% level, ***Significant difference at 0.1% level

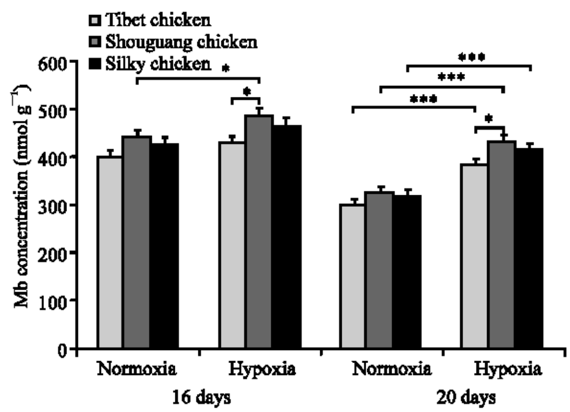

Fig. 2: Mb concentration in different chicken breeds under hypoxic and normoxic conditions. Each bar represents the mean±SE for each group and Tibet chicken contains 16 samples and Shouguang chicken and Silky chicken contain 10 samples, respectively

Myoglobin contents of chicken embryo heart tissues: Figure 2 shows the results of Mb content of the three chicken breeds. The contents in Silky chicken and Tibet chicken on day 16 in normoxia were lower than in hypoxia, though the difference is not statistically significant. It should be also noted that Shouguang chicken show a considerably higher content of Mb than that of Tibet chicken in hypoxia ($p<0.05$ on day 16 and 20). All chicken breeds on day 20 also occurred higher Mb contents in hypoxia than in normoxia ($p<0.001$ in three chicken breeds).

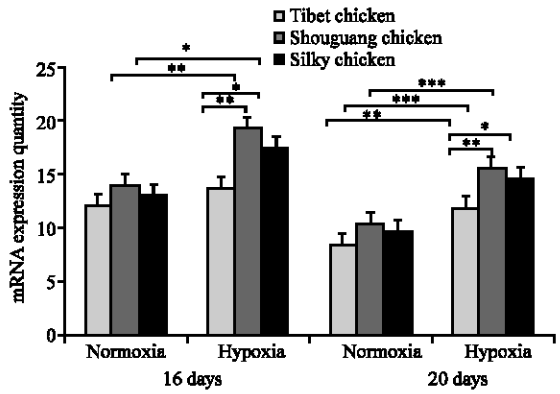

Fig. 3: Mb mRNA expression in different chicken breeds under hypoxic and normoxic conditions. Each bar represents the mean±SE for each group and each group contains 10 samples for mRNA expression level

Mb mRNA expression levels: As shown in Fig. 3, no significant difference on the interactions of breed x oxygen concentration was discovered. The expressions of Mb mRNA in Tibet chicken on two stages were lower than other chicken breeds in hypoxia ($p<0.05$ in Silky chicken and $p<0.01$ in Shouguang chicken). Results showed that Mb mRNA expressions were higher in hypoxic condition than that of normoxic environment in the three chicken breeds ($p<0.01$ in Shouguang chicken and $p<0.05$ in Silky chicken on day 16, $p<0.001$ in Shouguang and Silky chicken and $p<0.01$ in Tibet chicken on day 20).

DISCUSSION

Although, Mb is certainly among the best studied proteins in terms of function, structure, genetics and evolution, relatively little is known about its actual role in highland bird metabolism. As a model animal, chicken embryo is an ideal material to examine hypoxic adaptation in high attitude.

Heart weight and myoglobin levels in different chicken breeds: As shown in Fig. 1, the decrease of the heart weight in three chicken breeds suggested that hypoxia might retard their embryo development. The data of heart weight of Tibet chicken on day 16 showed the development of heart in Tibet chicken is normal. The significance decreased of heart weight on day 20 in all chicken breeds indicated heart growth was affected by hypoxia. Wei (2005) also indicated that the heart growth of Tibet chicken embryo also was influenced by the hypoxic environment but the adverse effect on Tibet chicken lower than that of Dwarf chicken embryos. The results is similar with the report in the hypoxic study of mice, in which the house mice from Morococha had also much larger heart weights than those from sea level (Reynafarje and Morrison, 1962).

Comparing with sea-level dwellers, Mb concentration of permanent high-altitude residents was elevated in biopsies of sartorius muscle but it did not differ in heart Mb content (Reynafarje, 1962). The similar results were observed by Wei (2001). In the present study, there was also no difference in heart Mb concentration between Tibet chicken and the other two chicken breeds in the normoxia. The results indicated that Mb level rise in the heart tissues under hypoxic condition at two stages. A high myoglobin concentration could facilitate oxygen supply under hypoxic conditions when training or competing at altitude (Hoppeler and Vogt, 2001). The possible mechanism to increase oxygen flow into the cardiac muscle is therefore to raise Mb concentrations (Roesner *et al*., 2006) and these demands associated with higher mitochondrial capacities which may drive the increase in cardiac Mb concentrations (Lurman *et al*., 2007). The data showed that Shouguang and Silky chicken breeds had always higher Mb content than that of Tibet chicken in hypoxia, suggesting the two lowland chicken breeds may increase the Mb levels to keep the metabolic demands of survival.

Hypoxia induces myoglobin mRNA expression in chicken embryo heart: No significant differences (Fig. 3) were discovered in mRNA expression of Mb among the three chicken breeds in the normoxic environment showing that the changes of Mb expression in the three chicken breeds under the hypoxia were caused by the oxygen effects.

In human and rat, the regulation of Mb expression in hypoxia had been considered an adaptive response to hypoxic stress (Kanatous *et al*., 2009). During high altitude training, Mb mRNA expression levels in muscle tissues is up-regulated in response to increased demand for oxygen or during hypobaric hypoxia (Hoppeler and Vogt, 2001) and increased mRNA contents of Mb in skeletal muscle were evoked (Vogt *et al*., 2001). In zebrafish heart, the Mb expression of mRNA levels had also increased in severe hypoxia (Roesner *et al*., 2006). These studies showed Mb expression is up-regulated by hypoxia but not all agree (Levine and Stray-Gundersen, 2001). Until now, few research work of Mb gene in heart tissue was reported, although the study of Mb in skeletal muscle had become a hotspot project in human at high altitude hypoxia (Gelfi *et al*., 2004; Robach *et al*., 2007, 2009). In chicken embryo heart, we observed that hypoxia induces Mb mRNA expressions. This change is in line with a major oxygen supply role of Mb for the heart.

In the present study, the results firstly showed the differential response of the three chicken breeds to hypoxia, which is that Silky and Shouguang chicken embryos were more sensitive to hypoxia than Tibet chicken embryos when they were incubated in the same simulated hypoxic environment. The decreased mRNA of Mb in Silky chicken was less than that of Shouguang chicken in hypoxia. The reason may be that Silky chicken come from the mountanious region (750 m altitude, Taihe country in Jiangxi province, China), while Shouguang chicken was from plain region (25 m altitude, Shouguang country in Shandong province, China), therefore Silky chicken had a little stronger adaptability to the hypoxic environment. Durand (1982) thought adaptation to high altitude is characterized by different changes to maintain homeostasis with minimum expenditure of energy. These phenomena may be attributed to the lower oxygen consumption of Tibet chicken embryos in the hypoxic environment, which low oxygen levels can cause a decrease of activity, enhanced ventilation and reduction of the metabolic rate (Smith *et al*., 1996; Ton *et al*., 2003; Van Der Meer *et al*., 2005).

CONCLUSION

In summary, the results showed that the expression of Mb in the three chicken breeds increased in hypoxia on two stages and it could be concluded that chicken Mb is up-regulated by hypoxia. The Mb content or mRNA expression levels of Tibet chicken embryos in hypoxia had less change than that of the other chicken breeds, suggesting that Tibet chicken embryos had the better hypoxic endurance. Until now, the mechanism that contributes to the oxygen transfer of embryos of mountain species is not completely understood yet and further studies are needed to investigate the regulating mechanism of Mb in Tibet chicken embryo in the adaptability to hypoxia.

ACKNOWLEDGEMENTS

The research was supported by the National Basic Research Program (973) of China (No. 2006CB102101) and the Project of National Fundamental Platform for Scientific Work (No. 2005DKA21100-02).

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