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Physiological Adaptability of Tibet Chicken Embryo Liver to Hypoxia and its Protein Profile Analysis

[1]M. Song, [1]W. Han, [1]H. Bao, [2]C. Liu, [1]C. Wu and [1]C. Zhao
[1]College of Animal Science and Technology,
[2]State Key Laboratory of Agrobiotechnology,
China Agricultural University, Beijing 100193, China

Abstract: In the present study, embryo liver tissues collected from hypoxic incubation of Tibet chicken and Shouguang chicken were analyzed on their histological structures and antioxidant capacity as well as differential proteomics to study the mechanism of Tibet chicken's adaptability to hypoxia. The results of histological study conducted with paraffin and ultrathin sections showed that Shouguang chicken had a weaker liver structure with less cell layers and the development of its liver was more impaired compared with Tibet chicken and Tibet chicken maintained relatively better mitochondria than Shouguang chicken. The content of Maleic dialdehyde in Tibet chicken was lower than Shougguang chicken, but the total antioxidant capacity and the content of Superoxide Dismutase in Tibet chicken were higher than Shouguang chicken. Using two-dimensional electrophoresis and mass spectrometry, we identified 8 differential expression liver proteins between Tibet chicken and Shouguang chicken, in which 6 proteins (enolase I, pyruvate dehydrogenase, ATP synthase, ubiquinol-cytochrome C reductase, superoxide dismutase and apolipoprotein A-I) showed higher expression level in Tibet chicken than that in Shouguang chicken while the expressions of the other 2 proteins (Triose phosphate isomerase, adenosine 5-diphosphosugar pyrophosphatase) were less in livers of Tibet chicken. These results indicated that Tibet chicken had higher glucose oxidation level, electron transfer efficiency and antioxidant ability than Shouguang chicken under the hypoxic condition and these differences made Tibet chicken have better adaptability to hypoxia than Lowland chicken. The study made a good basis for the further study of the genetic mechanism of adaptation to hypoxia.

Key words: Hypoxic incubation, chicken embryo, liver protein, antioxidant ability, 2D electrophoresis

INTRODUCTION

In altiplano, hypoxia, as a major stimulating factor, influences the subsistence and development of animals, plants and human being seriously. Tibet chicken, a unique native chicken breed in altiplano, shows better genetic adaptability than the breeds at the low altitude. Previous hatching experiment in our lab showed that hatchability of Tibet chicken were 87.4, 79.51 and 30.70%, respectively at altitude of 100, 2900 and 3650 m (Zhang *et al*., 2008), while those of Shouguang chicken, an indigenous Chinese chicken breed, which

Corresponding Author: C. Zhao, College of Animal Science and Technology, China Agricultural University, Beijing 100193, China Tel: +86-10-62894888

inhabits in Shouguang county of Shandong province with an altitude less than 100 m, were correspondingly 90.1, 32.21 and 3.81%. The results indicated that Tibet chicken showed similar hatchability with Shouguang chicken at the low altitude, but the former exhibited higher hatchability than the latter when it comes to the high altitude. Moreover, it was shown that hypoxia influences the hatchability mainly by increasing the mortality in the later period (Atherton and Timiras, 1970). Thereby, higher hatchability in the hypoxic environment is an important aspect of hypoxic adaptation.

As the center of metabolism of animals, liver could be injured sensitively by hypoxia (Hiratsuka *et al*., 2000; MacDonald *et al*., 2001) and many changes occurred with liver injured, such as systemic hypoxemia, sinusoidal capillarization and formation of portal-systemic collateral vessels and intrahepatic shunts (Moon *et al*., 2009), could boost further hepatocellular hypoxia (Ji *et al*., 1982; Rosmorduc *et al*., 1999; Corpechot *et al*., 2002), resulted from disrupted hepatic blood flow and sinusoidal fibrin deposition. A number of transcription factors, including Hypoxia-Inducible factors (HIFs), were activated to regulate gene expression to maintain the oxygen homoeostasis in hypoxia (Gaber *et al*., 2005).

In the present study, using Shouguang chicken as control, we explored the potential mechanism of hypoxic adaptation of Tibet chicken embryo based on the histological study, antioxidant assays of liver and analysis of comparative proteomics.

MATERIALS AND METHODS

Animals and Sampling Procedure

The fertile eggs of Tibet chicken and Shouguang chicken from the Experiment chicken Farm of the China Agricultural University (CAU, Beijing, 100 m altitude, June 2007) were incubated in a stable hypoxic environment using an incubator with a gas mixture containing 13% oxygen and 87% nitrogen (Zhang *et al*., 2010).

On the 16th day of incubation, the eggshells were broken at the air-chamber and the embryos were pulled out, from which the livers were collected after being washed by phosphate-buffered solution. Forty five samples were collected for each breed, of which 5 samples were used for paraffin section with hematoxylin and eosin (HE) stain according to Method of Erinn B. Rankin *et al*. (2009) and other 5 samples were made for ultrathin section and observed using Transmission Electron Microscopy (TEM) at the TEM lab of China Agricultural University.

Determination of Antioxidant Ability

Liver was ground into homogenate with 9 times of saline solution and centrifuged at 1000~1500 rpm for 10 min. Then the supernatant of 3 ground livers was mixed and 10 mixed samples were made for each breed. Then the protein was quantified using Bradford method. Total Antioxidant Capacity (TAC) and the content of maleic dialedhyde (MDA), superoxide dismutase (SOD) and Glutathione peroxidease (GSH-PX) were determined according to the manufacturer's protocols (Jiancheng Co., Nanjing, China).

Statistical Analysis

Data of this study were subjected to variance analysis using SAS software (Version 8.02, SAS Inc., US) and the significance level was fixed to $p<0.05$ and extreme significance $p<0.01$.

2D Electrophoresis and Mass Spectral Analysis

Liver sample was ground into fine powders by pestles in mortars filled with liquid nitrogen and added with Lysis Solution containing 7 M urea, 2 M thiourea, 65 mM DTT, 4%

w/v CHAPS and 0.1% v/v IPG Buffer and placed in ice overnight. Then the mixture was centrifuged at 14000 rpm at 4°C for 30 min. Five available samples were mixed into a pool and each pool was measured twice after the protein concentrations were measured by spectrophotometry using the Bradford method (Thongboonkerd *et al*., 2006). Three replications of such pools were conducted for analysis of 2D eletrophoresis for each chicken breed.

Immobilized pH gradient (IPG) strips (nonlinear pH 5-8, 17 cm) were rehydrated overnight with appropriate protein derived from the pools mentioned above and premixed with rehydration buffer containing 8 M urea, 4% w/v CHAPS, 20 mM DTT, 0.2% v/v IPG buffer and trace bromphenol blue. The first dimensional electrophoresis, isoelectric focusing (IEF), was performed in PROTEAN IEF Cell (Bio-Rad) at 20°C, using the mode 250 V, 30 min, then 1000 V, 1 h, 10000 V, 5 h, 10000 to 60000 V•h and 500 V at last, according to the method of Gorg *et al*. (1999) and the manufacturer's protocols (Bio-Rad, USA). After the IEF, the strips were equilibrated with a buffer containing 50 mM Tris-HCl (pH 8.8), 30% v/v glycerol, 2% w/v SDS and 0.1 g DTT for 15 min and then with another buffer containing 50 mM Tris-HCl (pH 8.8), 30% v/v glycerol, 2% w/v SDS and 0.125 g IAA for 15 min. The equilibrated strips were moved onto 12% polyacrylamide slab gels. After covered with 0.5% agarose, the second dimensional separation was performed at 14°C, using the mode 80 V, 30 min, 250 V until the bromophenol blue was moved out of the gels. The resolved protein spots were then stained using silver nitrate (Shevchenko *et al*., 1996).

The images were obtained by Image Scanner (Bio-Rad) at 256 gray scale with 300 dpi and analyzed by PDQuest 2D software (Bio-Rad).

Protein spots which were differentially expressed with two-time difference in intensity were excised from the 2-D polyacrylamide gels and washed with equal volume of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate until the color was removed thoroughly, then washed with water to terminate the reaction. The gel pieces were dehydrated with 100% acetonitrile (ACN) after washed by 25 mM NH_4HCO_3/50% ACN and dried in vacuum for 20 min, then swollen with 5~10 μL of 10 ng μL^{-1} trypsin in 25 mM NH_4HCO_3 at 37°C for 12~16 h. The peptide fragments were subsequently extracted twice with 0.1% trifluoroacetic acid (TFA) and 0.1% TFA/70% ACN, respectively (Thongboonkerd *et al*., 2006; Laville *et al*., 2009). The protein pellets were then dried at ultralow temperature for 2 h.

The sample spots were analyzed by AUTOFLEX II TOF-TOF. The data of PMFs input into Matrix Science (http://www.matrixscience.com) were matched with the NCBI database.

RESULTS AND DISCUSSION

Paraffin Section with HE Stain

Morphologic characters of livers of Tibet chicken and Shouguang chicken embryos hatched in hypoxia were shown, respectively in the Fig. 1a and b. It can be seen from the two figures that the liver tissue of Shouguang chicken embryo was more porous and showed worse development with fewer cellular layers compared with Tibet chicken.

Ultrathin Sections Observed with Electron Microscopy

The result of TEM ultrathin sections of livers of the Tibet chicken and Shouguang chicken embryos hatched in hypoxia were showed in the Fig. 2a and b. Obviously, the mitochondria in the livers of both Tibet chicken and Shouguang chicken exhibited swelling and abnormal. And the membrane and cristae in both breeds were impaired or even removed, whereas, the damage suffered by Tibet chicken was less than that of Shouguang chicken.

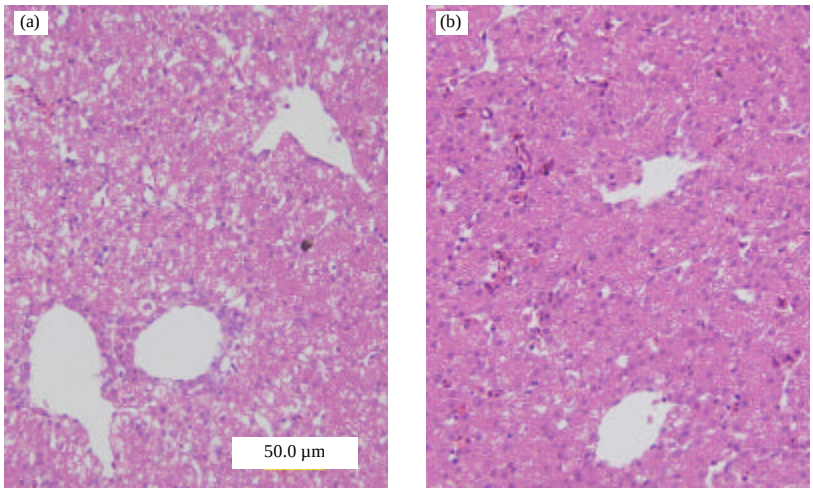

Fig. 1: The liver slices of chicken embryos under hypoxic incubation (stained by HE). (a) The liver slice of Shouguang chicken and (b) the liver slice of Tibet chicken

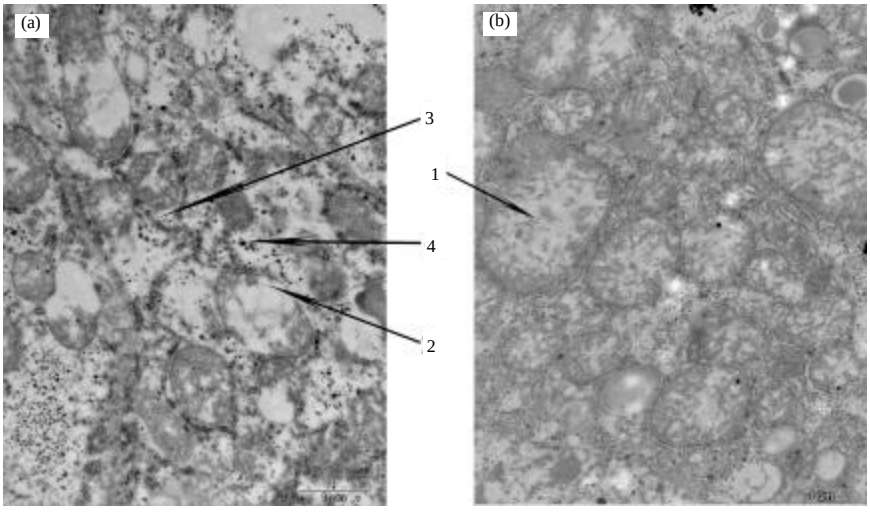

Fig. 2: The liver ultrastructure of chicken Embryos under hypoxic incubation condition. (a) The liver ultrastructure of Shouguang chicken embryo; (b) The liver ultrastructure of Tibet chicken embryo; 1: Swelling mitochondria, 2: Impaired cristae, 3: Fractured Endoplasmic Reticulum, 4: Shed ribosomes

Determination of Antioxidant Capacity

Table 1 showed that the livers of Tibet chicken contain more TAC, SOD and less MDA than that of Shouguang chicken, indicating that Tibet chicken liver suffered lower degree of lipid peroxidation than Shouguang chicken in hypoxic conditions and thus had stronger antioxidant capacity. These features may make the Tibet chicken better adapt to hypoxic environment.

Table 1: Antioxidant ability of embryo livers of Tibet chicken and Shouguang chicken in hypoxia

Determination of indicators	Shouguang chicken	Tibet chicken
MDA (nmol mg^{-1})	1.4879±0.0531[a]	1.4049±0.0862[b]
TAC (U mg^{-1})	4.6460±0.7324[a]	5.3320±0.6305[b]
SOD (U mg^{-1})	65.5190±7.6163[a]	72.5060±6.1444[b]
GSH-PX (U mg^{-1})	95.5150±13.325	98.5170±14.965

Values in the same row with different letters means that the difference between them is significant difference ($p<0.05$)

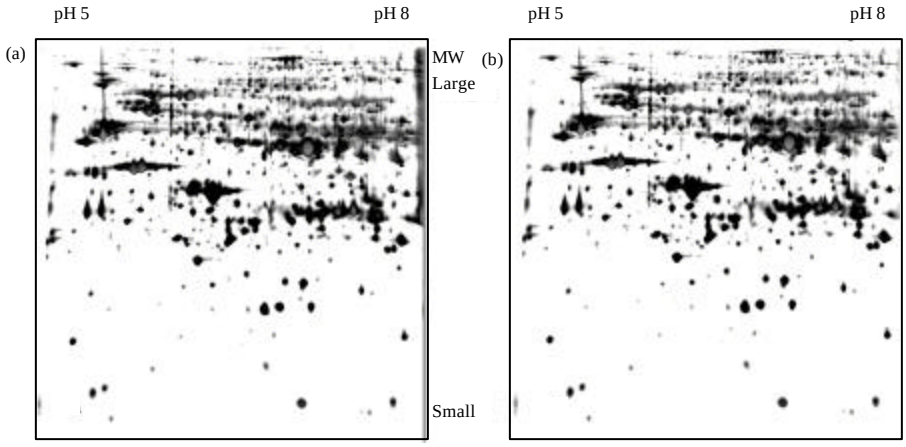

Fig. 3: 2-DE profile of fetal liver in hypoxic incubation condition (stained by silver nitrate). (a) The profile of Shouguang chicken. (b) The profile of Tibet chicken

Protein Profile Analysis

Totally, 6 subjects were included for proteome analysis. On the basis of chicken breeds, the subjects were divided equally into two groups, 3 replications for each. The results of the liver protein profile analysis were showed in Fig. 3a and b. The MW and pH of proteins separated by 2D-electrophoresis range, respectively from 10~110 kDa and pH 5~8 and the matching rate between different replications was up to 95%.

In hypoxic condition, there were 13 differentially expressed proteins between Tibet chicken and Shouguang chicken embryos on the 16th day of incubation and eight of them were successfully identified by mass spectral analysis and searching database and named T16-24, T16-33, T16-92, T16-59, T16-63, T16-80, T16-38 and T16-73, respectively. Six of the eight proteins, including enolase 1, ATP synthase, pyruvate dehydrogenase, coenzyme Q cytochrome C reductase, superoxide dismutase 2 and apolipoprotein AI were more highly expressed in Tibet chicken than in Shouguang chicken, while the rest proteins-triosephosphate isomerase and adenosyl acid pyrophosphorylase had lower expression level in Tibet chicken than in Shouguang chicken. The identified proteins were classified into four functional groups based on their major roles in biological processes, including glucose metabolic enzymes (triose-phosphate isomerase, enolase 1 and pyruvate dehydrogenase), electron transfer of mitochondria (ATP synthase and coenzyme Q cytochrome C reductase), antioxidants (superoxide dismutase 2) and lipid metabolic protein (apolipoprotein AI) (Table 2).

Tibet chicken, as a native breed in altiplano, showed better adaptation in hypoxia, especially the higher hatching rate (79%) than the breeds at low altitudes (31%). The long-term adaptation in hypoxia has resulted in genetic difference of Tibet chicken comparing

Table 2: Proteins with differential expression in embryo livers of Tibet chicken and shouguang chicken under hypoxic incubation condition

No.	Protein	Biological function	Expression comparison
T16-24	Triosephosphate isomerase	Glycolysis	Tibet<Shouguang
T16-59	Adenosyl acid pyrophosphorylase	Catalysis	Tibet<Shouguang
T16-33	Enolase 1	Glycolysis	Tibet>Shouguang
T16-38	Superoxide dismutase 2	Antioxidant	Tibet>Shouguang
T16-92	ATP synthase	Energy generation	Tibet>Shouguang
T16-63	pyruvate dehydrogenase	Aerobic oxidation of glucose	Tibet> Shouguang
T16-73	Apolipoprotein AI	Lipid metabolism	Tibet>Shouguang
T16-80	Coenzyme Q cytochrome C reductase	Electron transfer of mitochondria	Tibet>Shouguang

with the breeds at low altitudes (Gou *et al*., 2005; Wang *et al*., 2007; Bao *et al*., 2008). In the present study, Shouguang chicken, a typically native breed in low altitude was set as a control. Observation of ultrathin sections with electron microscopy, antioxidant capacity assays and comparing proteome assays were performed and some important results were obtained.

Influence of Hypoxia on the Morphology of Liver

Earlier study suggested that the breeds at low altitudes would demonstrate many physiologic changes when exposed continuously to hypoxia, such as the increase of Mb (Garcia *et al*., 2000), ventricular hyperplasia, the retard of growth of embryo (Atherton and Timiras, 1970) and so on. The dwarf chickens had been hatched in altiplano to study the liver growth of embryo (Zhang *et al*., 2007) and it had been found that the growth of embryos and livers was significantly retarded. In this study, the same results were obtained about the influence of hypoxia on fetal liver. Moreover, fetal liver of Tibet chicken showed a better development than Shouguang chicken in the same hypoxic hatching condition, which is one of physiologic indicators of hypoxic adaptation of Tibet chicken embryo.

The results of histological study showed the mitochondria of Tibet chicken were less impaired by hypoxia than that of Shouguang chicken. As the centre of energy supply, mitochondria needed oxygen to perform respiration. Exposure of the embryos to continuous hypoxia or low oxygen pressure beyond the tolerance of the liver cells may interrupt the respiration chain (Korge *et al*., 2008), harm the liver and lead to morphologically granular degeneration which was observed in the present study. Long-term exposure to low oxygen pressure can even injure the subcellular ultrastructures (Mantena *et al*., 2009) of the organism and exacerbate the granular degeneration further into vacuolar degeneration, then ruin and disintegrate endoplasmic reticulum. The less damage of mitochondria and endoplasmic reticulum suffered by Tibet chicken than that of Shouguang chicken suggested that the former has better hypoxic adaptability than the latter on the subcellular level.

Antioxidant Capacity of Liver

Lipid peroxidation, which mainly does harm to the membrane and subcellular, is a series of free radical reactions of polyunsaturated fatty acids. MDA, the final product of lipid peroxidation, can reflect the rate and intensity of lipid peroxidation directly (Xu *et al*., 2009). SOD and GSH-PX, the major indicators of antioxidant capacity of organisms, can eliminate the oxyradical efficiently to reduce the harm of peroxidation. Many studies have demonstrated that intermittent exposure to hypoxia could promote adaptability mechanism of organisms (Nagasaka and Satake, 1969). But it has not yet been conclusively known which factors are responsible for the adaptation.

The antioxidant capacity of fetal liver of Tibet chicken and Shouguang chicken hatched in hypoxia was firstly determined and the effects of hypoxia on the antioxidant capacity of

chick embryo liver were assessed in this study. The result indicated that the level of lipid peroxidation in Tibet chicken was lower than that in Shouguang chicken in hypoxia due to reduction of MDA and increase of SOD and TAC, indicating that the antioxidant enzyme capacity of organism was activated by hypoxia. The increase of the expressions of GSH-PX and SOD in liver cells can accelerate the disintegration of lipid peroxide (LPO) (Savransky *et al*., 2009) to prevent branched-chain reactions of lipid peroxidation induced by LPO, which is a significant protective mechanism that makes organisms free from the harm of LPO when they were exposed in hypoxia. These phenomena may be adaptive compensation of organisms, in which organisms modulate expression of the interrelated gene spontaneously in hypoxia to enhance their antioxidant capacity.

Interrelated Proteins of Liver Involving in the Hypoxic Adaptation of Tibet Chicken Embryo

All physiologic and biochemical functions of organism are undertaken by proteins, which are considered as the ultimate executor of metabolism. Proteomics is a rapidly advancing field of study relevant to protein expression on the overall level of cell (Wilkins *et al*., 1996; Pandey and Mann, 2000; Haynes and Yates, 2000) and can provide insight for uncovering the mechanism of hypoxic adaptation on the level of proteome. In this experiment, studies of fetal liver of Tibet chicken and Shouguang chicken were performed using comparing proteomics and some differentially expressed proteins were identified and their biofunctions are described as follows:

Triose Phosphate Isomerase (TPI) plays an important role in glycolysis, synthesis of fatty acid, gluconeogenesis and pentose phosphate pathway, by transforming the dihydroxyacetone phosphate generated in glycolysis into Fischer-Bear ester and meets energy need of organism. Absence of TPI can lead to many diseases of innate immune system disorders (Eber *et al*., 1991). In this study, the expression of TPI in Shouguang chicken livers was higher than that in Tibet chicken livers in hypoxia, suggesting that Shouguang chicken may adapt to hypoxia by switching on the compensatory mechanism that regulates the expressions of enzymes in glycolysis.

Enolase I, an enzyme involving in glycolysis, plays an important role in energy metabolism by catalyzing dehydration of 2-phosphoglycerate into Phosphoenolpyruvate Pyruvate. Increase of expression of this enzyme was observed in the proteomic research of breast and intrahepatic bile duct cancer (Verrills *et al*., 2003; Srisomsap *et al*., 2004). This study showed higher expression of enolase I in Tibet chicken livers than that in Shouguang chicken in hypoxia, indicating the mechanism of hypoxic adaptation of Tibet chicken may be in related to the expression of enzymes regulating intracellular energy metabolism.

Pyruvate dehydrogenase (PDH), the major component of pyruvate dehydrogenase complex, plays an important role in TCA cycle as a rate limiting enzyme, which catalyzed the pyruvate into acetyl coenzyme A (Wagner *et al*., 2005). The content of PDH in Tibet chicken is higher than that in Shouguang chicken, suggesting that Tibet chicken could make use of the limited oxygen much more effectively to yield more energy for organism in hypoxia.

Coenzyme Q-cytochrome reductase and ATP synthase, both are interrelated to the synthesis of ATP. As a key enzyme in process of electron transfer in respiration, the functional activity of Coenzyme Q-cytochrome reductase influences the synthesis of ATP directly. The activity of enzymes in mitochondrial respiratory chain complexes was regulated by hypoxia to reduce the synthesis of ATP (Michiels, 2004). ATP synthase also participates in the oxidative phosphorylation by catalyzing hydrolysis of ATP to ADP and phosphate anion to generate energy. The content of coenzyme Q-cytochrome reductase and ATP synthase was higher in Tibet chicken than in Shouguang chicken in hypoxia, suggesting that Tibet chicken can synthesize more ATP to meet the needs of organic metabolism.

Superoxide Dismutase (SOD), shown in our study, was richer in livers of Tibet chicken than that of Shouguang chicken in hypoxia. Hypoxic stimulation can induce much unstable oxyradical which can in turn induce the lipid peroxide of polyunsaturated fatty acid in biomembrane to generate MDA that can ultimately damage the membranous structures of cell and subcell (Iimuro *et al*., 2000). As one of the important antioxidant enzymes in the cells of organisms, SOD exists broadly in aerobic, oxytolerant and anaerobic organisms, playing vital roles in regulating the balance between anti-and oxidant of organism. Higher expression of SOD in Tibet chicken was observed in the study, suggesting that better antioxidant system in Tibet chicken could help the organism reduce the damage of oxyradical, which may also contribute to the hypoxic adaptation of Tibet chicken.

Apolipoprotein A-I, as a kind of special proteins that can bind and transfer lipid, synthesized mainly in liver and bowel, is the major component of high density lipoprotein (HDL). It can remove the cholesterol by activating the lecithin-cholesterol acyltransferase to prevent the accumulation of lipid in peripheral tissues by eliminating the cholesterol. Besides, it takes part in the transport process of cholesterol from peripheral tissues into liver. Absence of apolipoprotein A-I but normality of high density lipoprotein cholesterol (HDL-C) could lead to atherosclerosis and coronary heart (Saitta *et al*., 1999; Cohen *et al*., 2004). The results of the present study showed that there was higher amount of apolipoprotein A-I in Tibet chicken than in Shouguang chicken, indicating the better metabolism of HDL and function of liver in Tibet chicken.

In summary, there are significant differences between Tibet chicken and lowland chicken breed-Shouguang chicken in terms of morphology, antioxidant capacity and expression of proteome in livers of embryos, which may be the causative factors for the hypoxic adaptation of Tibet chicken.

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