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RESEARCH ARTICLE

Basic Analysis of Glycolysis in Cardiac Tissue in Broiler Chickens Presenting with Ascites Syndrome

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ABSTRACT

Ascites syndrome (AS) is one of the most noticeable metabolic disorders in broiler chickens. In the present study, histopathology, immunohistochemistry, quantitative real-time PCR and Western blot were performed to investigate the influence of metabolic functions on the development of AS. Pathological examination of cardiac tissues of AS broilers revealed loosely arranged myocardial fibers, blurred plasma membrane boundaries, interstitial edema, and congestion. Levels of glycolysis were analyzed in cardiac tissue samples based on the expression of major rate-limiting enzymes in the related pathway. The results showed that the mRNA levels of PFK and PKM were significantly increased 2.19-fold and 3.82-fold vs. the control group in AS group ($P<0.05$), respectively. IHC results showed increased protein levels of PFK1, p-PFK2 and PKM2, which were in accordance with the changes in the levels of mRNA. Then, the Western blot analysis demonstrated that the expression percentage of PFK1, p-PFK2 and p-PKM2 were increased by 48.71, 45.41 and 67.14% than healthy broilers ($P<0.01$). The results indicated that the acidic intermediate products induced by the activation of glycolytic pathway might trigger fluid accumulation in the abdominal cavity of broilers with AS.

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INTRODUCTION

Broiler chickens are carefully selected for their productive traits (Baghbanzadeh and Decuypere, 2008), as a nearly seven fold increase in body mass within 50 days cannot be sustained without equally dramatic increases in the functional capacities of the heart and lungs. Broilers require rigorous conditions with strict requirements for nutrition, temperature, air quality, and raising density, among others. When these requirements are not met, inefficient production and emergence of various metabolic diseases may occur. Ascites is a multifactorial syndrome caused by interactions among environmental, physiological and genetic factors. In broiler chicken, the symptoms of ascites syndrome (AS, also known as pulmonary hypertension) include generalized edema, hydro-pericardium (pericardial effusion) in the abdominal cavity (Balog *et al*., 2003), epicardial fibrosis, lung edema, enlarged flaccid heart, hypertrophy, dilation of the heart (particularly the right ventricle) (Decuypere *et al*., 2000), hypoxaemia, comb paleness, and high blood hematocrit (Luger *et al*., 2003). Although many studies have been published on the effects of pulmonary hypertension (Sutendra and Michelakis, 2014; Gurtu and Michelakis, 2015), the pathogenesis of AS remains unclear.

AS is a metabolic disorder in broilers (Pakdel *et al*., 2005), and the fast growth is the main factor contributing to the susceptibility of broilers to ascites. The left ventricle of the heart pumps oxygenated blood to support basal metabolism, activity, and growth (Wideman *et al*., 2013). Broilers with high metabolic-rate exhibit sustained elevation of pulmonary arterial pressure and right ventricular hypertrophy, which ultimately result in ascites and then heart failure (Hassanzadeh *et al*., 2005).

In meat-type poultry, rapid growth and heavy body weight increased the oxygen requirement of the chicken.

Therefore, hypoxia is regarded as the driving force behind the development of AS (Wang *et al*., 2008). Glycolysis preferentially occurs in the body under hypoxic conditions to maintain the energy supply of broilers. However, the molecular mechanism underlying this process remains unclear. The glycolytic pathway includes three key enzymes, namely, hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK) (Weljie and Jirik, 2011). HK, the first rate-limiting enzyme in the Embden-Meyerhof-Parnas pathway, can rapidly phosphorylate glucose and promote the conversion of glucose into glucose-6-phosphate. HK has high specificity, and its enzymatic activity is detected only in the lungs. PFK functions as the gatekeeper of glycolysis and is therefore highly regulated (Mullarky and Cantley, 2015). Mg^{2+} can catalyze PFK-dependent ATPs to produce fructose-1,6-diphosphate in the second phosphorylation step, and is critical in determining the flux rate of glycolysis (Hasawi *et al*., 2014). PK catalyzes the last step of glycolysis, during which phosphoenolpyruvate is phosphorylated to produce pyruvate and ATP. PKM2 is considered a rate-limiting enzyme because its activity is sensitive to allosteric regulation (Christofk *et al*., 2008) and it catalyzes a thermodynamically favorable reaction. AS is primarily characterized by hypertrophy of the right ventricle and lung-specific expression of HK. This study, we assessed the expression of PFK and PKM in the right ventricle of broilers with AS.

MATERIALS AND METHODS

Samples collection: Broilers were provided by Da Xiang farm in Taigu, Shanxi Province. The clinical features of AS include weakness of breath, distended abdomen, fluid retention, dullness, and depression; broilers with AS also manifest enlarged right ventricles and reduced elasticity. Normal broilers were selected from the same flocks as control animals. There were 5 replicates per treatment. After weighing, the broilers were treated with urethane (24 mg/gram of body weight), by intraperitoneal injection, and then euthanized by vein bloodletting following anesthesia. The heart was harvested from the thorax and divided into two portions: one portion was fixed in Bouin solution and the other was stored at -80°C. All the procedures involving the broilers were approved by and performed in accordance with the guidelines of the Animal Ethics Committee of the Henan Institute of Science and Technology.

Histopathological examination: Cardiac tissues were fixed in Bouin solution for 24 h, washed in a vessel with running water for 10 h, and then dehydrated in an increasing alcohol gradient. Thereafter, the blocks were washed thoroughly with pure xylene to remove residual alcohol. The cardiac tissues were subsequently embedded in paraffin, serially sectioned into 5 μm slices, and then stained with hematoxylin and eosin for histopathological observation through a light microscope.

Immunohistochemical analysis: After dewaxing and rehydrating, tissues were incubated in 10% H_2O_2 to block endogenous peroxidase activity. Antigen retrieval was performed using 0.1 M trypsin for 20 min, followed by blocking with 5% BSA to reduce nonspecific binding by the primary antibody for 45 min. Sections were incubated at 4°C overnight with antibodies against PFK_1 (1:200), p-PFK_2 (Ser466, 1:150), and PKM_2 (1:200), washed thoroughly with phosphate-buffered saline with Tween (PBST), and then incubated with horseradish-peroxidase-conjugated antibodies at room temperature for 40 min. Afterward, the sections were incubated with streptavidin-biotin complex (SABC) for 25 min to enhance the sensitivity for detecting positive signals. Peroxidase activity signals were developed using 3,3-diaminobenzidine (DAB) as the substrate. Finally, the sections were lightly counterstained with hematoxylin, dehydrated, cleared and saved for analysis.

Determination of mRNA expression levels through quantitative RT-PCR: Total RNA was isolated from the cardiac tissues by using TRIzol reagent in accordance with the manufacturer's recommendation (Invitrogen, USA). Subsequently, 1 μg of the total RNA was converted into first-strand cDNA by using a cDNA synthesis kit (Promega, USA). The primers of the target transcripts were designed using the complete cDNA sequences deposited in GenBank. The primers used as follows: PFK (F-GCTCTCAACACCATCAC, R-ATGC TGAAGTGCTCCT,); PKM (F-CGAACTGGACTCATC AAG, R-GCTGCCCACATCTATAAC); and β-actin (F-GCCAACAGAGAGAAGATG, R-CAGAGTCCATCAA TACC). Briefly, qPCRs were performed with a 10 μL reaction volume consisting of 2 μL of the first-strand cDNA (dilution 10:1), 5 μL of 2 × SYBR Green Master Mix, 0.5 μL (20 mM) each of the forward and reverse primers, and 2 μL of RNase-free H_2O. The qPCR conditions were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 20 s. PFK and PKM transcript levels were estimated by $2^{-\delta\delta}$CT method, where δδCT represents the difference in CT values between a target gene and GAPDH.

Western blot analysis: Cardiac tissue samples were homogenized in a glass homogenizer in the presence of protease inhibitors. The homogenate was incubated on ice for 30 min and then centrifuged at 12,000 × g for 10 min at 4°C, and the protein concentrations of the resulting supernatants were determined using a BCA protein assay kit (P0009, Beyotime Biotechnology). Protein samples were mixed with SDS loading buffer and denatured for 10 min in boiling water. Proteins were separated using 10% SDS-PAGE and then transferred onto a nitrocellulose filter membrane. The membranes were blocked with 5% skim milk for 2 h at room temperature, incubated overnight in diluted primary antibodies, including PFK_1 (1:150), p-PFK_2 (Ser466, 1:300), PFK_2 (1:300), PKM_2 (1:1500), p-PKM_2 (Tyr105, 1:1000), and GAPDH (1:3000), treated with the corresponding secondary antibodies, and then incubated for another 2 h. Protein bands were visualized by adding an enhanced chemiluminescent substrate, and the target protein bands were analysed with Image J (National Institutes of Health, USA).

Statistical analysis: Data are presented as mean ± SEM. Statistical analyses were performed using one-way ANOVA and paired-sample *t*-tests using SPSS version 19.0

software (IBM Corp, Armonk, NY, USA). P<0.05 was considered to indicate significant differences and P<0.01 was considered to indicate highly significant differences.

RESULTS

Cardiac tissues revealed damage in AS broilers: Histologically normal cardiac tissues are characterized by a regular arrangement of myocardial fibers (Fig. 1A). Compared with the control group, AS broilers displayed loosely arranged myocardial fibers. Edema was observed in the myocardial interstitium (Fig. 1B) and plasma membranes boundaries appeared blurred and exhibited different degrees of congestion. Further, damage of myocardial parenchyma was found in AS broilers.

Immunohistochemical staining of PFK and PKM increased in AS broilers: Immunohistochemical (IHC) analysis was performed to detect protein expression levels of PFK_1, p-PFK_2, and PKM_2 in cardiac tissues. As shown in Fig. 2, positive immunoreactivity was observed for PFK_1 (Fig. 2B), p-PFK_2 (Fig. 2D), and PKM_2 (Fig. 2F) in the cytoplasm and plasma membranes of myocardiocytes. Signal intensities revealed an increasing trend from the negative group to the normal group to the AS.

The mRNA expression levels of PFK and PKM increased in AS broilers: To investigate the metabolic phenotypes of AS broilers, real-time qPCR was performed to detect the gene expression of the rate-limiting enzymes PFK and PKM. Compared with the corresponding levels found in the cardiac tissues of the control group, the mRNA expression of PFK increased (Fig. 3, 2.19-fold, P<0.05) and that of PKM significantly (3.82-fold, P<0.01) in the cardiac tissues of AS broilers.

Protein expression of PFK and PKM increased in AS broilers: Western blot analysis was used to evaluate the expressions of PFK and PKM in cardiac tissues of AS broilers. Compared with the control group, the expression of PFK_1 were increased by 48.71% (Fig. 4, P<0.05), as well as p-PFK_2 and p-PKM_2 were significantly elevated in the AS cardiac tissues (45.41 and 67.14%, respectively, P<0.01).

DISCUSSION

The main function of the heart is to enable the continuous circulation of blood throughout the body to deliver nutrients to organs and remove metabolic products from the body. The poultry industry plague with circulation system disease frequently, especially in fast-growing broilers, which consume large amounts of feed and oxygen to satisfy their high energy and metabolic requirements (Xu *et al*., 2009). The alarming increase in metabolic diseases, such as heart failure syndrome, ascites, and edema in the lungs and heart, can be directly related to an insufficient oxygen supply.

AS as causes of mortality in broiler chickens are increasing steadily. The two most distinctive features of AS are accumulation of yellow liquid in the abdominal cavity and the hypertrophy of right ventricle of the heart (Shi *et al*., 2014; Naeije *et al*., 2014; Ahmadpanah *et al*., 2017). Hypertrophy of the heart is a compensatory mechanism that can become a deleterious pathologic feature over time, and ultimately lead to the structural and functional failure of the heart (Shi *et al*., 2014). Marina reported that the edema was the most prominent histopathological change confirmed in the heart of ascites syndrome chicken, while the myocardiofibriole disruptions were predominant lesions (Tišljar *et al*., 2011). In this study, we selected the right ventricle to study the pathological change in heart of AS chicken. The results of HE staining confirmed that edema and congestion change

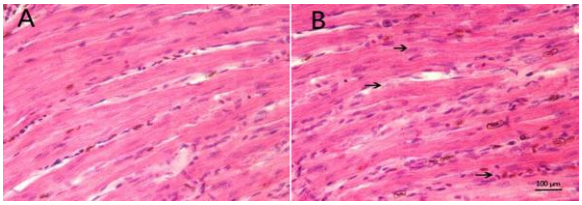

Fig. 1: Hematoxylin and Eosin staining of broiler hearts. A: Control group; B: Ascites syndrome group; (400×).

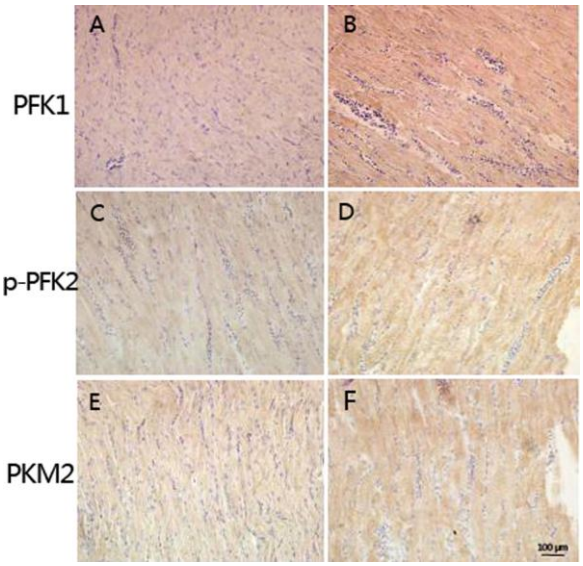

Fig. 2: Immunohistochemistry of phosphofructokinase and pyruvate kinase M_2. Phosphofructokinase (PFK) and pyruvate kinase M (PKM) are rate-limiting enzymes of the glycolytic pathway. A: Control group of PFK_1, B: AS group of PFK_1; C: Control group of p-PFK_2, D: AS group of p-PFK_2 expression; E: Control group of PKM_2, F: AS group of PKM_2.

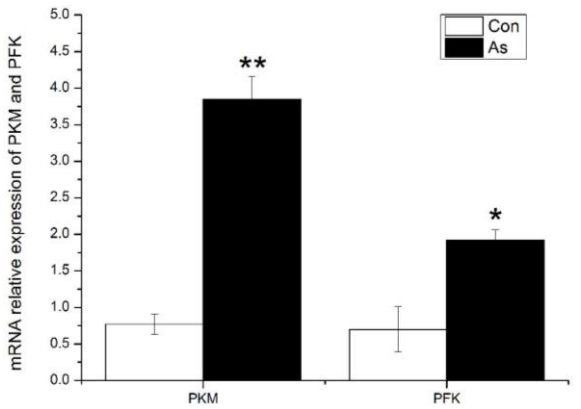

Fig. 3: mRNA expression of PFK and PKM. Bars indicate the mean ± SEM of three independent experiments. *P<0.05, **P<0.01, as compared with the control group (LSD multiple comparison test after one-way ANOVA).

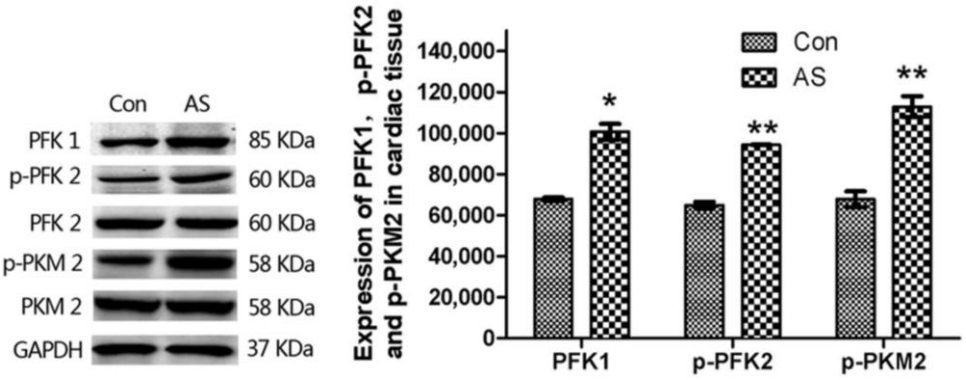

Fig. 4: Protein expression of PFK_1, p-PFK_2 and p-PKM_2. In this study, phosphorylation (Ser466) of PFK_2. Phosphorylation (Tyr105) of PKM_2. *P<0.05, **P<0.01, as compared with the control group.

significantly in the myocardial interstitium of AS group. In addition, compared with healthy chicken, cardiac tissues of AS broilers revealed loosely arranged myocardial fibers, blurred plasma membrane boundaries. This result in accordance with most research opinion. We thus speculate that heart dysfunction is an important aspect of the pathogenesis in AS.

To meet the daily need of people, fast growth chicken has been active selection of parents to produce birds. An increased growth rate and a faster synthesis of tissues demanding a high metabolic rate means that more oxygen is required to produce increased amounts of free energy. Further investigation is necessary to verify the specific mechanism of changes in cardiac tissue during the development of AS. There's plenty of research showed that hypoxia is one of the main reasons causes AS in birds. Hypoxia causes an increase in pulmonary arterial pressure in humans and animals, resulting in oedema in different parts of the body (lung oedema, hydro-pericardium, and ascites). (Scheele, 1997) Glycolysis provides energy for the anoxic myocardium. However, accumulation of acidic intermediate products occurs during glycolysis. Acidic physiological conditions in broilers exert deleterious effects on the structure and function of their cardiac tissues, resulting in disruption of pulmonary circulation, eventually, disrupt all organ systems of the organism.

Phosphorylation is an essential post-translational modification in glycolysis (Tripodi *et al*., 2015). Phosphorylation activates a number of metabolic enzymes that interact with carbohydrate substrates (Donthi *et al*., 2004), such as PKs and the two subunits of phosphofructokinase (PFK_1 and PFK_2). They are key factors for glucose metabolism. Nodoubt, glycolytic shift is implicated in the pathogenesis of ascites syndrome (Bonnet and Paulin. 2019). Many studies showed that the bifunctional enzymes 6-phosphofructo-2- kinase/fructose-2,6-bisphosphatases (PFKFB3) is upregulated in hypoxia (Minchenko *et al.,* 2003), which can synthesize and degrade PFK1. In this study, we investigated the expression of rate-limiting enzymes of glycolysis in the cardiac tissues of AS broilers. The mRNA expression level of PFK and PK were significantly higher in the hearts of AS broilers than in the tissues of the normal group (P<0.05). In addition, Western blot results showed that PFK_1, p-PFK_2, and p-PKM_2 are significantly increased in the cardiac tissues of AS broilers (P<0.01). The result of immunohistochemical analysis in accordance with this experiment. Pyruvate kinase M2 is a critical enzyme that regulates cell metabolism and growth under different physiological conditions, which exists mainly in the cytosol as a glycolytic enzyme. PKM2 is considered as a significant regulator of the Warburg effect in many cells (Luo and Semenza, 2012), and its upregulation is mostly correlated with increased glucose utilization in diseases. The studies indicated that insulin stimulation increased the expression of PKM2 cancer cells, while the knockdown of PKM2 partially inhibited glucose uptake and lactate production (Iqbal *et al.,* 2013). Other researcher reported that increased PKM2 expression was also shown to be associated with obesity and steatohepatitis (Luca *et al.,* 2018). After hypoxia, PKM2 catalyzes the last glycolytic step to generate more ATP, which is likely necessary to meet the heart energy demands. On the other hand, increased PKM2 expression promotes cell proliferation of myocardial cell, which caused the hypertrophy of cardiac tissues. These data demonstrate that glycolysis is closely related to pathogenesis of AS.

In conclusion, our results showed that pathological changes occur in AS broilers, especially in the heart. This finding reveals that the heart is one of the primary organs involved in the development of the syndrome. Our results revealed that PFK_1, PFK_2, and PKM_2 expression is significantly increased in the myocardium of AS broilers, and that the glycolytic pathway is promoted in the cardiac tissues of AS broilers under hypoxic conditions. Acidic intermediate products accumulate in the blood, ultimately causing fluid buildup in the pericardium and abdominal cavity.

Authors contribution: YG and HN conceived and designed the study. YL, LC and HL executed the experiment. HH and NH collected tissue samples. WW and XW analyzed the data. All authors interpreted the data and approved the final version. The authors declare no competing financial interest.

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