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RESEARCH ARTICLE

Expression of Pyruvate Dehydrogenase Kinase (PDK) in Lungs during Progression of Pulmonary Hypertension Syndrome in Broilers

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A B S T R A C T

Pulmonary hypertension syndrome (PHS) has always been of significant importance related to cardiovascular diseases. The PHS is one of the major reasons for the high morbidity and mortality in broilers negatively impacting the broiler farming industry. In this study samples from lungs and blood of broilers with enlarged and normal abdomen were collected and fluorescence quantification, immunohistochemistry and histopathology of the selected organs were done to diagnose this disease in broilers. Clinically, it was observed that morbid broilers were depressed, dyspneic, right heart hypertrophied, right ventricle to total ventricle volume (RV/TV) was greater than 0.25 and abdomen was enlarged having yellowish fluid. The diagnosis of PHS was confirmed by the RV/TV and clinical symptoms whereas, the histopathological observations revealed that with age the pulmonary vascular density decreased, lumen of the pulmonary vessels was narrowed and the middle layer of pulmonary vessels was thickened. Hematoxylin and Eosin (HE) staining of the selected samples was done. It was found that age increased in greater interstitial spaces in the lungs of PHS broilers along with inflammatory cells infiltrating the lungs. Results of fluorescence quantification and immunohistochemistry revealed that all types of PDK (PDK1, PDK2, PDK3, PDK4) exhibited significant upregulation in the lungs of broilers with PHS onset and PDK4 was significantly expressed on pulmonary vessels. Therefore, it was concluded that PHS leads to remodeling of the pulmonary vasculature leading to ascites and PDK plays an important role in the progression of PHS.

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INTRODUCTION

The occurrence of ascites in fast-growing commercial broilers has been observed at high altitudes compared to plain areas (Wang *et al.*, 2012). Broiler ascites syndrome is also known as pulmonary hypertension syndrome (PHS) which is a chronic progressive disease characterized by pathological alterations in the pulmonary vascular (Wideman *et al.*, 2013). Lower oxygen levels and cold environments at high altitudes stimulate PHS in broilers (Yu *et al.*, 2022). Chronic hypoxia leads to respiratory depression, swift constriction of small pulmonary arteries and capillaries, narrowing of vascular lumen and increased incidence of pulmonary vascular remodeling (Khajali and Wideman, 2016). Reduced pulmonary vascular volume increases pulmonary vascular resistance, forcing right ventricular dilatation and increased pulmonary artery pressure. Existing at a low-pressure hypoxic environment for a longer time leads to continuous constriction of small pulmonary arteries, increased resistance of pulmonary circulation and impaired blood circulation which leads to right heart failure and PHS.

Pyruvate dehydrogenase kinase (PDK) is a mitochondrial substrate enzyme that regulates glucose and lipid metabolism (Song *et al.*, 2021) and is divided into four isoforms PDK1, PDK2, PDK3 and PDK4 which exhibit 70% homozygosity (Stacpoole, 2017). It contributes to the loss of PDH activity by phosphorylating any of the three serine residues (Ser293, Ser300, Ser232) on the E1α subunit of pyruvate dehydrogenase (PDH) (Ma *et al.*, 2020). Normally pyruvate complex is oxidatively decarboxylated by PDH for producing acetyl coenzyme A

and NADH, which are involved in mitochondrial tricarboxylic acid cycle, oxidative phosphorylation, lipid metabolism and are converted to CO_2, adenosine triphosphate (ATP) and citric acid that provide energy for normal metabolic activities of cells (Wang *et al.*, 2021). However, PDK can inactivate PDH by phosphorylation preventing the aerobic oxidative breakdown of the pyruvate complex to produce energy and allowing the cell to produce bioenergy and lactate through glycolysis (Liu *et al.*, 2017). The PDK has the ability to reduce the tricarboxylic acid cycle and oxidative phosphorylation, resulting in the inhibition of mitochondrial function, reduced glucose oxidation and increased uncoupled glycolysis which affects cellular lipid metabolism and glucose metabolism (Yang and Guo, 2018; Padilla and Lee, 2021).

MATERIALS AND METHODS

Animal treatment: Six healthy and 6 PHS broilers of 14 and 28 days of age were selected from the same broiler farms in Qingyang, Gansu Shengyue Agricultural and Animal Husbandry Development Co. The selection criteria for PHS broilers were enlarged abdomen, yellow transparent fluid or peptone-like exudate in the abdominal cavity and right ventricle as a percentage of the total ventricle (RV:TV) greater than 0.25 (Rahimi *et al.*, 2023).

Heart index evaluations: All broilers were weighed before killing, after which their chest cavity was quickly opened and heart was removed by wiping the blood with a qualitative filter paper. Afterwards, the heart was dissected sagittally using a scalpel to isolate the atria and ventricles. The residual blood clot within the ventricles was wiped away using qualitative filter paper. The total broiler ventricle was weighed using an electronic balance and then the right ventricular wall attached to the interventricular septum was separated using ophthalmic forceps with ophthalmic scissors and the right ventricular wall was weighed using an electronic balance. Finally, the broiler cardiac index was calculated using the following formula = heart weight (g)/broiler body weight (g) × 10 × 100 %.

Broiler RV/TV ratio: RV/TV ratio = right ventricular wall weight (g)/total ventricular weight (g) × 100%.

Serum biochemical tests: Blood was collected from broiler samples and anticoagulated with lithium heparin. The blood was centrifuged at 3000 rpm and 4°C for 5 min to separate the serum (Zhu *et al.*, 2022). Serum triglycerides (TG), serum urea (UREA), albumin (ALB-II), alkaline phosphatase (ALP), alanine aminotransferase (ALT), calcium (Ca), creatine kinase (CK), creatinine (CREA), globulin (GLB), direct bilirubin (I-Bil), lactic dehydrogenase (LDH), phosphorus (P), total bilirubin (T-Bil), total serum protein (TP), urea nitrogen (UREA), γ-glutamyl transpeptidase (γ-GT), serum glucose (GLU) and aspartate aminotransferase (AST) concentrations were determined by using the Myeri bs-380 automated biochemical analyzer.

Histopathological observations: Histopathology was performed as described by Yi *et al.* (2022). Briefly, fresh lung tissues were collected, rinsed well with PBS and fixed in 4% paraformaldehyde solution for 48 hours. Subsequently, lung tissues were subjected to paraffin embedding, sectioning, hematoxylin eosin (H&E) staining and Masson staining.

H&E staining: After paraffin sections were dewaxed and rehydrated, hematoxylin staining was performed for 5 minutes, 1% hydrochloric acid alcohol staining was performed for 10 seconds, eosin staining was performed for 5 minutes, graded ethanol dehydration was performed, xylene clearing was performed, and the slices were blocked with a neutral gel.

Masson staining: Paraffin sections were rehydrated after dewaxing, stained with weight iron hematoxylin for 10 minutes, stained with weak acid for 10 seconds, washed with phosphomolybdic acid for 2 minutes, stained with aniline blue for 2 minutes, stained with weak acid for 1 minute, Dehydrated and transparent, then sealed with a neutral adhesive.

Immunohistochemical assay: Immunohistochemical study methods were performed as described by Liu *et al.* (2022). Briefly, Paraffin sections of lung tissue were dewaxed, rehydrated with stepped alcohol, antigenically repaired, then treated in 3% hydrogen peroxide for 15 minutes and closed for 1 hour. Primary antibody incubation at 4°C was done for 16 hours followed by secondary antibody incubation at room temperature for 40 minutes. The DAB was developed which followed hematoxylin nuclear staining for 5 minutes and neutral adhesive sealing. Primary antibody of PDK4 (1:200, ABClonal, China) was used for this experiment.

RT-qPCR analysis: Follow the methodological steps of the previous study (Ouyang *et al.*, 2021). The primer sequences used for RT-PCR are shown in Table 1.

Statistical analysis: Data collected is expressed as mean ± standard error and analyzed by one-way analysis of variance (ANOVA). All results were analyzed using GraphPad Prism 9.5.0 (GraphPad Inc., USA). The differences were considered significant at $P<0.05$.

RESULTS

Clinical diagnosis of PHS broilers: The results of clinical symptoms are shown in Fig. 1A. Compared to healthy broilers PHS broilers were depressed, dyspneic, having enlarged and fluctuating abdomens. Gross anatomical characteristics of healthy broilers and PHS broilers are shown in Fig. 1B. Compared to healthy broilers, PHS broilers had a large amount of yellowish fluid in their abdominal cavity and yellowish staining of abdominal mucosa. The fluid had no specific odor and formed jelly-like clots when exposed to air for longer duration. Moreover, the body weight of PHS broilers at 14 and 28 days of age was significantly lower than that of healthy broilers (**$P<0.01$, ***$P<0.001$) with the highest significant difference in body weight observed at 28 days of age (Fig. 1C). The cardiac index of PHS broilers was also significantly higher (*$P<0.05$,***$P<0.001$) and the upward adjustment of the cardiac index of PHS broilers at

Table 1: qRT-PCR primers

Genes name	Forward sequence (5′→3′)	Reverse sequence (5′→3′)
β-actin	CCGTGCTGTGTTCCCATCTA	TCTGGGCTTCATCACCAACG
PDK1	TGAGTGATCGTGGCGGAGGTG	CGTACAGGCGTGATATGGGCAAG
PDK2	ACGTGGTGAGAGATGCCTAC	AAAGCCGGCCTTGAAGAG
PDK3	CATCCCCAAGCAGATCGAGTA	CACGACCGAAGTCGAGGAAC
PDK4	TGCAATCACCAAAGGTCACCA	TGCAGTAGCTGAAGCTGTGTT
Cyclin D1	GACTTTTGTGGCTCTGTGCG	TGTTCTTGGCAGGCTCGTA
p21	TCCCTGCCCTGTACTGTCTAA	GCGTGGGCTCTTCCTATACAT
p53	GAGATGCTGAAGGAGATCAATGAG	GTGGTCAGTCCGAGCCTTTT

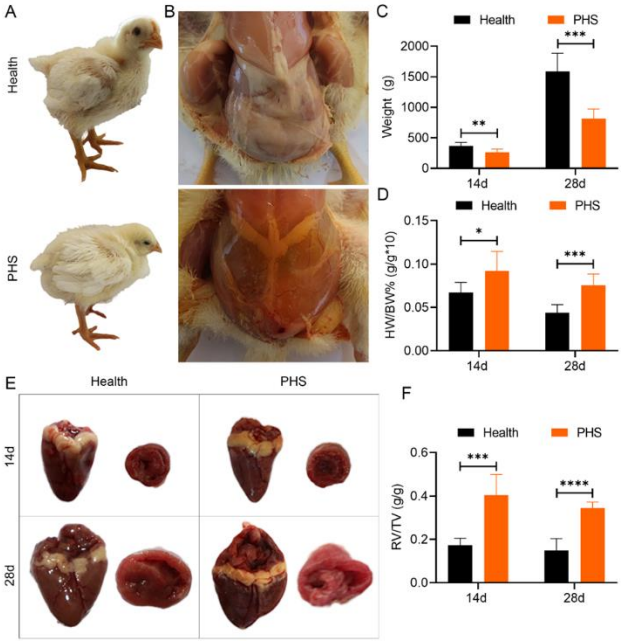

Fig. 1: Gross anatomy of 14-, 28-day-old healthy and PHS broilers. (A) Appearance of healthy broilers and PHS broilers. (B) Abdomen of healthy and PHS broilers. (C) Body weight ratio of healthy and PHS broilers. (D) Heart index. (E) Heart anatomy of healthy and PHS broilers. (F) RV/TV ratio of healthy and PHS broilers.

28 days of age was highly significant (Fig. 1D). The apparent comparison of the ventricular cavities between healthy and PHS broilers is shown in Fig. 1E. It was observed that healthy broilers did not show any dilatation of the RV as its wall was tightly adhered to the septum which could not be detached easily. In PHS broilers, the RV cavities were dilated and the RV wall was flaccid. The results of comparing RV/TV ratios of broiler hearts are shown in Fig. 1F. The RV/TV ratios of PHS broilers at 14 and 28 days of age were significantly higher than those of healthy broilers (***P<0.001, ****P<0.0001).

PHS broiler serum biochemical assay: The serum biochemical results are shown in Fig. 2. 14-day-old PHS broilers exhibited significant down-regulation of LDH (*P<0.05), P (*P<0.05), TG (***P<0.001), TP (*P<0.05), γ-GT (**P<0.01), ALT (***P<0.001), AST (**P<0.01) and up-regulation of URER (**P<0.01), ALP (**P<0.01), AST/ALT (**P<0.01), Ca (*P 0.05). The 28-day-old PHS broilers showed significant down-regulation of γ-GT (*P<0.05), ALT (*P<0.05), CK (**P<0.01) and up-regulation of URER (**P<0.01), ALB (*P<0.05).

Histopathological observations on the lungs of PHS broilers: The results of HE staining displayed that the interstitial space of lung tissue of PHS at 14 and 28 days of age was increased accompanied by inflammatory cell infiltration (Fig. 3A). The HE staining and Masson staining revealed that the pulmonary vessels of PHS were significantly thickened compared with those of healthy broilers and the intima of the vessels were bulging due to hyperplasia. The tunica intima-media layer of the vessels was significantly thickened and lumen of the vessels was narrowed (Fig. 3B - C).

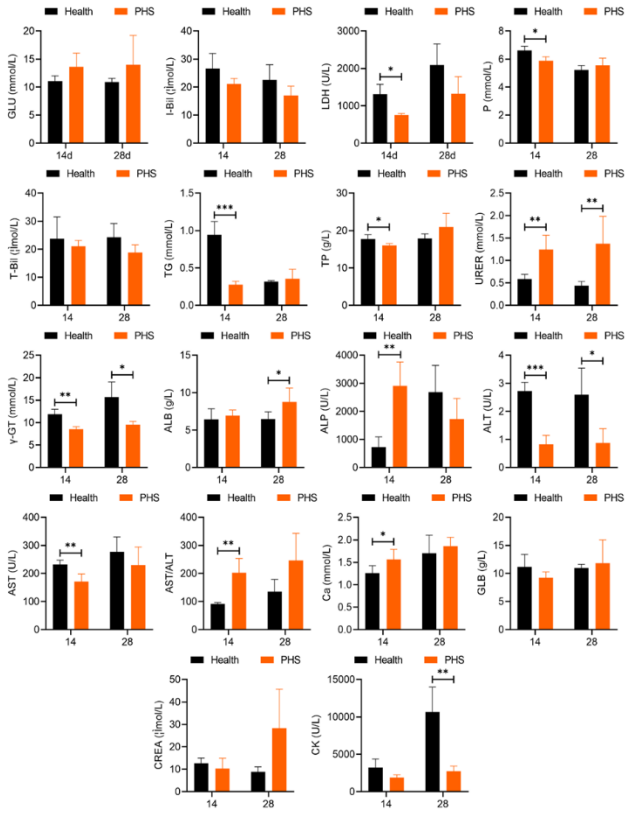

Fig. 2: Results of serum biochemical tests in 14- and 28-day-old healthy broilers and PHS broilers.

Expression of genes related to pulmonary vascular remodeling: The results of lung tissues of 14- and 28-day-old healthy broilers and PHS broilers detected by fluorescence quantification are shown in Fig. 4A-C, PHS broiler lung tissues showed up-regulation of cyclin D1 (**P<0.01), p21 (*P<0.05), and p53 (*P<0.05, ***P<0.001).

Expression of PDK1, PDK2, PDK3, PDK4 in lung tissue of PHS broilers: The results of lung tissues of 14- and 28-day-old healthy broilers and PHS broilers detected by fluorescence quantification are shown in Fig. 5A-D, PHS broiler lung tissues showed up-regulation of PDK1 (*P<0.05), PDK2, PDK3, and PDK4 (*P< 0.05, **P<0.01), especially PDK4 was significantly upregulated. The results by immunohistochemistry were

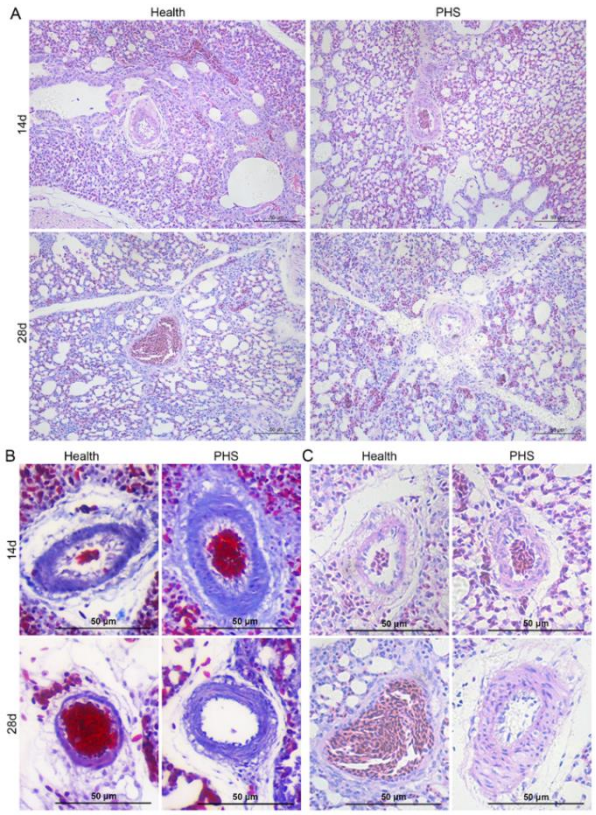

Fig. 3: Lung histopathology in 14- and 28-day-old healthy and PHS broilers. (A) HE staining of lung tissue from healthy and PHS broilers. (B) Masson staining of pulmonary vessels in healthy and PHS broilers. (C) HE staining of pulmonary vessels in healthy and PHS broilers.

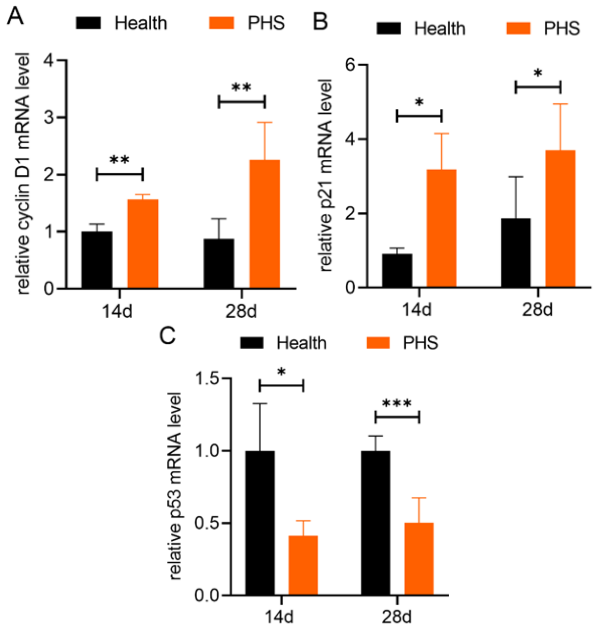

Fig. 4: Fluorescence quantitative detection of cyclin D1, p21 and p53 results. (A) Detection of cyclin D1 expression in lung tissues by fluorescence quantification. (B) Detection of p21 expression in lung tissues by fluorescence quantification. (C) Detection of p53 expression in lung tissues by fluorescence quantification.

shown in Fig. 5E-F, PDK4 was expressed on the lung vessels, especially on the lung vessels of PHS broilers significantly, and the expression of PDK4 (**P<0.01, **** P<0.0001) on the pulmonary vasculature increased with increasing day oldness.

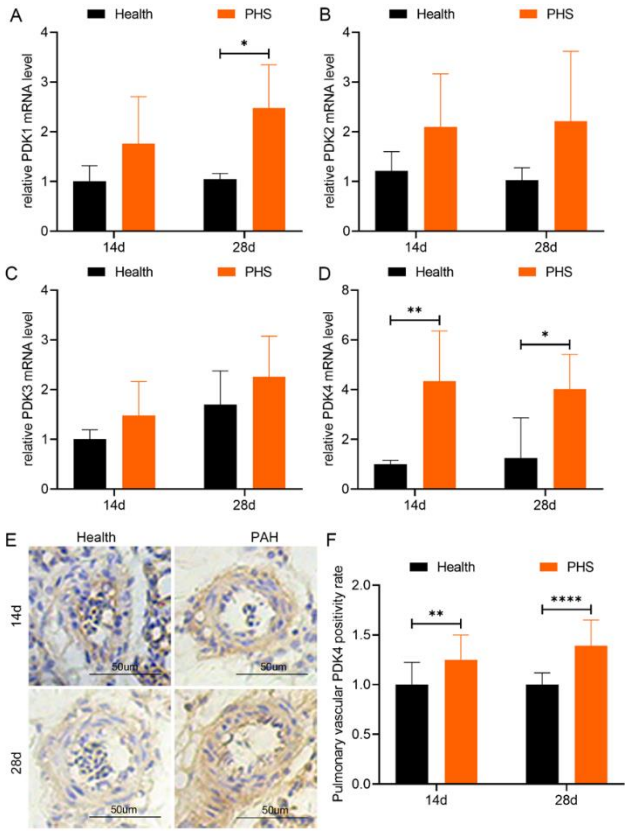

Fig. 5: Expression of PDK1, PDK2, PDK3, PDK4 in lung tissues of 14- and 28-day-old healthy and PHS broilers. (A) Detection of PDK1 expression in lung tissues by fluorescence quantification. (B) Detection of PDK2 expression in lung tissues by fluorescence quantification. (C) Detection of PDK3 expression in lung tissues by fluorescence quantification. (D) Detection of PDK4 expression in lung tissues by fluorescence quantification. (E) Detection of PDK4 expression on pulmonary vessels by immunohistochemistry. (F) Immunohistochemical detection of PDK4 positivity in the pulmonary vasculature.

DISCUSSION

Pulmonary hypertension is a common cardiovascular disease whose prevalence increases with age (Virsinskaite *et al.*, 2023). Physiologically, a pulmonary artery pressure of ≥25 mmHg is usually defined as pulmonary hypertension (Pascall and Tulloh, 2018). Moreover, PHS can lead to emphysema and pulmonary fibrosis (Olsson *et al.*, 2023). While observing the clinical symptoms of PHS broilers we found that PHS broilers exhibited dyspnea, sluggishness, weight loss, abdominal enlargement and a fluctuating sensation when palpated. By comparing the weights of healthy broilers and PHS broilers at 14 and 28 days of age, it was found that the weight loss of PHS broilers was more significant as the age of the bird increased. Moreover, PHS broilers had a large amount of slightly yellowish liquid and jelly-like coagulated lumps from the abdomen. It was also observed that PHS broilers had pericardial effusion and the hearts of PHS broilers were significantly larger than healthy broilers, especially the right ventricle was significantly dilated. The cardiac index was also significantly higher, especially in 28-day-old PHS broilers with significantly up-regulated cardiac index. The presence or absence of RV dilatation and the RV/TV ratio are often used to diagnose PHS in broiler chickens (Yang *et al.*, 2007). The RV/TV ratios greater than 0.25 are considered to be indicative of right ventricular hypertrophy, whereas RV/TV ratios

greater than 0.29 are considered as indicators of right heart failure in chickens (Bahadoran *et al.*, 2023). We found that the RV/TV of PHS broilers was significantly higher than that of healthy broilers and the ratio was greater than 0.25. All these symptoms indicated that the broilers were suffering from PHS.

Serum biochemical tests in PHS broilers revealed significant up-regulation of URER, ALP, AST/ALT, Ca, ALB and down-regulation of LDH, P, TG, TP, γ-GT, ALT, AST and CK. Elevated urea nitrogen during PHS often indicates a poor prognosis (Kang *et al.*, 2019). Hyperuricemia promotes development of PHS by activating endothelial dysfunction, oxidative stress, inflammation and the renin-angiotensin system (Savale *et al.*, 2021). Elevated serum calcium concentration activates calcium channels, triggering vasoconstriction and [Ca^{2+}] regulates pulmonary vascular tone (Minareci and Sadan, 2014). Abnormalities of ALP, ALT and AST indicate that PHS damages the liver and affects its functions (Yang *et al.*, 2021a). Moreover, the increase of inflammatory proteins in serum also promoted the development of PHS and aggravated liver injury in broilers.

The main pathological feature related to PHS is the remodeling of pulmonary vasculature (Sluiter *et al.*, 2012). The pulmonary vasculature is divided into endothelial, mesothelial and exothelia layers. Among them, the intimal layer is mainly composed of endothelial cells, the middle intimal layer is composed of smooth muscle cells and the outer intimal layer is mainly composed of myofibroblasts and fibroblasts (Huang *et al.*, 2022). Vascular remodeling refers to the dysregulation of migration, proliferation and apoptosis of cells resulting in the thickening or thinning of the vessel wall (Ye *et al.*, 2022). Vascular smooth muscle cells play an important role in vascular remodeling. By the changes observed in the vasculature of PHS broilers it can be established that compared to healthy broilers the pulmonary vascular mesothelial and intima-media layers of PHS broilers exhibited significant thickening. Furthermore, abnormal proliferation of vascular cells during pulmonary vascular remodeling is closely related to cell cycle dysregulation (Yang *et al.*, 2021b). cyclinD1 promotes transcription of E2F target genes by phosphorylating retinoblastoma proteins (Weiss *et al.*, 2019). Transcription factor E2F accelerates cell entry into G1-S phase (Li *et al.*, 2017). p53 is a transcription factor that regulates the expression of many genes and is involved in the regulation of the cell cycle and the promotion of apoptosis (Wu *et al.*, 2022). Up-regulation of p21 is associated with DNA damage, which promotes cells to enter senescence. DNA damage is also one of the hallmarks of pulmonary vascular remodeling (Born *et al.*, 2023). Our findings revealed significant upregulation of cyclinD1, p21 and significant downregulation of p53 in lung tissues, suggesting that PHS broilers exhibit abnormal cell proliferation as well as pulmonary vascular remodeling.

The PDK4 expression was significantly increased by pre-transcriptome sequencing study. Therefore, we found that PDK1, PDK2, PDK3, PDK4 showed a trend of up-regulation in lung tissues of PHS broilers by fluorescence quantitative detection, especially PDK4 was significantly up-regulated. It was found that PDK4 was expressed in the pulmonary vasculature by immunohistochemical results, and the expression of PDK4 in the pulmonary vasculature increased significantly during PHS. From this it is confirmed that PDK4 plays a role in the progression of PHS. In PHS, pulmonary vascular cells develop an antiapoptotic phenotype and undergo metabolic remodeling which is characterized by increased glycolysis, decreased glucose oxidation and reduced mitochondrial respiration. These metabolic changes play a key role in vascular remodeling, a process that has also been termed the Warburg effect (Wakasugi *et al.*, 2019; Willson *et al.*, 2019). One of the central mechanisms of the Warburg effect is the inhibition of PDH (Archer, 2017). Whereas, PDK4 plays a crucial role in catalyzing the mitochondrial production of acetyl coenzyme A from pyruvate (the end product of glycolysis) through inhibition of PDH, leading to glycolytic degradation and reduced mitochondrial pyruvate utilization (Michelakis *et al.*, 2017).

Conclusion: It can be concluded that commercial broilers at higher altitudes are susceptible to PHS caused by low oxygen. For adapting to excessive growth rates of broilers and less oxygen supplying capacity of lungs, the blood flow rate of broilers is enhanced, which promotes a series of alterations in its normal physiology such as pulmonary vascular remodeling, increased resistance to blood flow, right heart hypertrophy and abdominal effusion. All types of PDK (PDK1, PDK2, PDK3, PDK4) were up-regulated during the course of PHS and PDK was involved in PHS.

Data availability statement: All data generated or analyzed during this study are included in this published article.

Authors contribution: Xiaojuan Huang: Conceptualization, Formal analysis, Validation, Investigation, Methodology, Writing - original draft. Xiaoqin Liu, Wanli Xu, Yuanliang Li, Ping Wu, Xuqing He, Dongyang Zhong and Farid S. Ataya: Formal analysis, Validation, Investigation, Writing - review & editing. Ying Li: Project administration, Funding acquisition, Writing - review & editing.

Declaration of competing interest: There is no conflict of interest.

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