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RESEARCH ARTICLE

Mitochondrial Dynamics Disturbances in the Liver of Broilers with Pulmonary Hypertension Syndrome

Wenting Zhang[1], Yuxuan Li[1], Xuan Ye[1], Zhijian Ma[1], Xiuhua Wei[1], Jiaqiang Pan[1*], Zhaoxin Tang[1], Lianmei Hu[1], Ying Li[1], Dalia Fouad[2] and Farid Shokry Ataya[3]

[1]College of Veterinary Medicine, South China Agricultural University, Guangzhou 510642, China
[2]Department of Zoology, College of Science, King Saud University, PO Box 22452, Riyadh 11495, Saudi Arabia
[3]Department of Biochemistry, College of Science, King Saud University, PO Box 2455, Riyadh 11451, Saudi Arabia
*Corresponding author: panjq@scau.edu.cn

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A B S T R A C T

Pulmonary hypertension syndrome (PHS) is a poultry disease that causes significant economic losses in broilers. Portal hypertension and liver cirrhosis can cause ascites formation. Disturbance of mitochondrial dynamics causes several diseases; however, its mechanism of action in PHS is not yet fully understood. In this research, 210 broilers were split randomly into two groups: control and PHS. Broilers in the control group were injected with normal saline and those in the PHS group were injected with CM-32 cellulose particles at 20 days of age. Transmission electron microscopy (TEM) was used to assess mitochondrial ultrastructure. Changes in mitochondrial dynamics were measured by immunohistochemical staining, RT-PCR and western blotting. On days 28 and 42, the liver and heart were collected from each group, and the ascites heart index (AHI) and hepatic coefficient were calculated. The results showed that PHS induced hepatocyte damage, reduced mitochondrial size, and increased mitochondrial number. Furthermore, RT-PCR demonstrated the downregulation of Mfn1, Mfn2, and Opa1 mRNA, and the upregulation of Drp1 and Mff mRNA. Protein expression levels detected by immunohistochemistry and western blotting were similar to those obtained by RT-PCR. This study showed that mitochondrial fusion in the liver was diminished and mitochondrial fission in the liver was enhanced in PHS broilers.

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INTRODUCTION

Pulmonary hypertension syndrome (PHS) is a common disease in broilers, characterized by increased pulmonary pressure, progressive right-sided heart failure, and death. After seven days of age, the growth rate of bones, muscles and feathers in broilers is significantly accelerated, leading to a state of high load on the cardiopulmonary function. It becomes difficult to accommodate the rapid increase in cardiac output in the reserve capacity of the pulmonary vasculature, which eventually leads to right-sided congestive heart failure, persistent hypoxemia, venous congestion, and elevated portal blood pressure (Wideman *et al.*, 2013). Metabolically active liver cells cannot obtain enough oxygen to support the mitochondria to produce energy, resulting in liver cell necrosis and cirrhosis. Cirrhosis and portal hypertension are the direct causes of ascites. After intravenous injection of cellulose particles, the particles in blood vessels are transported back to the lungs through venous blood (Wideman *et al.*, 2002). The anterior capillary artery was blocked by cellulose particles, resulting in increased pulmonary vascular resistance and successful induction of PHS (Wideman *et al.*, 2002). Therefore, we used a CM-32 cellulose suspension to simulate PHS and explore equilibrium changes in mitochondrial dynamics.

Mitochondria take an important part in maintaining an active metabolism in liver cells. Mitochondria control oxidative phosphorylation to generate energy, coordinate various metabolic pathways, regulate apoptosis, and maintain calcium homeostasis (Faas and de Vos, 2020; Wu *et al.*, 2022). Mitochondria adjust their morphology and numbers through constant fusion and fission to maintain their integrity and homeostasis (Adebayo *et al.*, 2021). These changes are defined as mitochondrial dynamics. Mitochondrial fission is mainly mediated by two dynamin-related proteins: dynamin-related protein 1 (Drp1) and

mitochondrial fission factor (Mff). Whereas, mitochondrial fusion is regulated by superfamily: optic atrophy 1 protein (Opa1), mitofusin 1 (Mfn1), and mitofusin 2 (Mfn2). Mfn1 and Mfn2 anchored in the mitochondrial outer membrane are required to promote mitochondrial docking and outer membrane fusion. Opa1 plays a regulatory role in mitochondrial inner membrane fusion. Mitochondrial dynamics are linked to variety of cellular processes, including the production of reactive oxygen species (ROS), mitosis, apoptosis, and the cell cycle (Horbay and Bilyy, 2016).

There is growing evidence that shows that disturbances in mitochondrial dynamics are integral to the pathogenesis of several cardiovascular diseases. Enhanced Drp1-mediated mitochondrial division has been described in patients with pulmonary hypertension (PH) (Chen *et al.*, 2018). Hypoxia-induced human pulmonary artery smooth muscle cells (PASMCs) show decreased Mfn2 and upregulation of Drp1 protein expression (Parra *et al.*, 2017). Previous research has shown that mitochondrial dynamics disturbances induce cellular dysfunction through different pathways. Under hypoxia conditions, mitochondrial hyper-fission leads to mediated endoplasmic reticulum stress that causes pulmonary artery smooth muscle dysfunction (Zhuan *et al.*, 2020). Previous studies have observed that mitochondrial respiration was impaired in PHS broilers livers. (Iqbal *et al.*, 2001). This suggests that mitochondrial function was impaired during PHS in broilers. However, the changes in mitochondrial dynamics in PHS-affected broilers remain unknown. And the mechanisms involved are not fully clarified how mitochondrial dynamics in the pathological processes of PHS, which needs to further explore. The aim of this study was to provide new ideas for the prevention and cure of PHS by targeting mitochondrial dynamics.

MATERIALS AND METHODS

Research animals: After fourteen days of feeding, 210 one-day-old healthy white-feathered broilers were routinely immunized and split at random into control and PHS groups (n= 105 for both groups). On day 20, broilers in the PHS group were injected with 0.35 mL of CM-32 carboxymethylcellulose suspension (mean particle size of 24–63 μm) through the wing vein, and broilers in control group were injected with an equal amount of saline similarly. The cellulose suspension consisted of 0.02 g/L, and 150 U/mL heparin sodium saline, which was mixed continuously by stirring during use. Twenty broilers were randomly selected from control and PHS groups and were euthanized on days 28 and 42. Heart and liver tissues were subsequently collected. Liver tissue was fixed in a paraformaldehyde (4%) solution, and others were stored at -80℃.

Ascites heart index: After collecting the heart, the atria were removed, and the total ventricular (TV) was weighed. The right ventricular weight (RV) was measured by cutting along the margin of the ventricular septum. The total (TV) and right ventricular weight (RV) of each sample were recorded.

$$\mathrm{AHI} = \mathrm{RV}/\mathrm{TV}$$

Hepatic coefficient evaluations: The body weight and liver weight of each sample were recorded.

$$\text{Hepatic coefficient} = (\text{Liver weight (g)} * 100\%)/\text{body weight (g)}.$$

Histopathological examination: The samples were fixed and embedded in paraffin. Tissue sections (4μm thickness) were stained (hematoxylin and eosin, H&E, respectively). Subsequently, the pathological sections were examined using a Leica DM1000 optical microscope.

Mitochondria ultrastructure observation by electron microscopy: The tissues were treated as follows: alcohol, acetone, and resin were used to dehydrate and permeabilize, drying box treatment was provided at three conditions (37℃ for 12 h, 45℃ for 12 h, and 60℃ for 48 h). Finally, the sections were examined using transmission electron microscope (Hitachi, Japan).

Immunohistochemistry analysis: For immunohisto-chemical analysis, antigen repair of the sections (4μm) was performed for 10 min using a sodium citrate solution, followed by treatment with 3% H_2O_2 solution for 15 min. Subsequently, incubation with primary antibodies against Mfn1, Opa1, and Drp1 for 14 h at 4°C. After incubation with secondary antibodies, the color reaction was developed using DAB (Beyotime, China). Sections were observed under a microscope (Leica, Germany).

RT-PCR analysis: Gene sequences used for detecting mitochondrial dynamics were obtained from NCBI GenBank. β-actin was used as a reference gene. RNA was extracted from the livers of the broilers by using the TRIzol reagent (TaKaRa, Ltd, Japan), and the HiScript 1st Strand cDNA Synthesis Kit (Vazyme, Ltd, Nanjing, China) was used to reverse-transcribe the extracted RNA into cDNA. The SYBR Premix Ex Taq II Kit (Vazyme, Ltd., Nanjing) detected samples, followed by cDNA amplification using a LightCycler 480 Real-Time System (Roche, Germany). The primer sequences for all genes and the reaction system are shown in Table 1 and 2, respectively.

Western blot analysis: The proteins extracted from broilers liver were added to the pores of the colloidal medium. Each membrane was incubated overnight with diluted primary antibodies against Mff (Affinity Biosciences., LTD., China) and Opa1, Mfn1, Mfn2, Drp1, β-actin (Proteintech Group, INC, China). After incubation with secondary antibodies, chromogenic agents were added dropwise and covered with a PVDF film. Subsequently, ImageJ software was used to analyze the gray value of the blot to calculate the protein expression levels.

Statistical analysis: Paired samples one-way ANOVA and student's t-test were performed to assess the significance of differences between two groups. In this research, all data were statistically analyzed using GraphPad Prism 7.0 (GraphPad Inc., La Jolla, CA, USA). Significance was defined as *P<0.05, **P<0.01, and ***P<0.001.

RESULTS

Clinical observation of PHS: The appearance of the broiler heart and liver in control and PHS groups is shown in Fig. 1A and B. There were no obvious abnormal changes in the heart of broilers in control group. In PHS group, the heart of broiler was obviously hypertrophy. Compared with the PHS group on day 28, the PHS group broiler hearts on

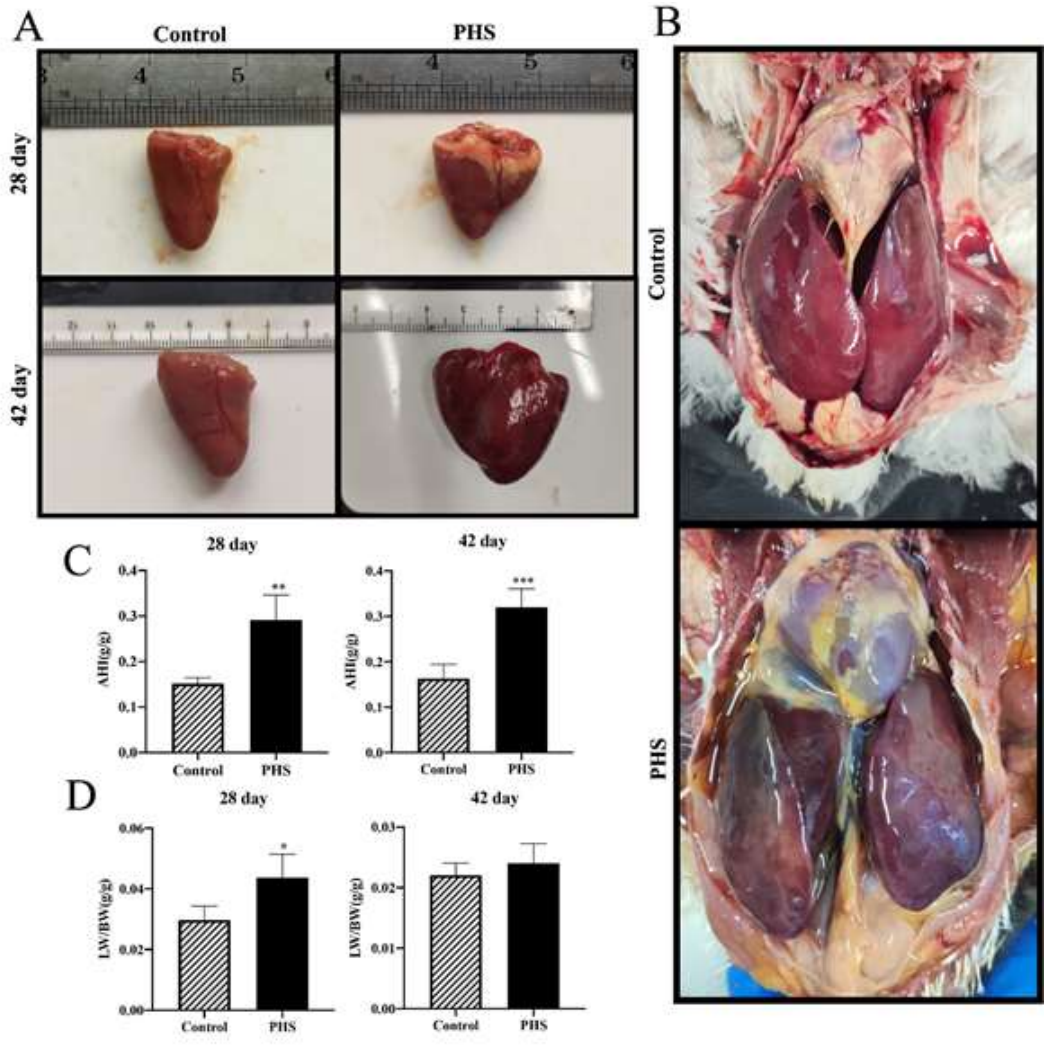

Fig. 1: Effect of PHS on appearance of the heart and liver, ascites heart index (AHI), and hepatic coefficient of both groups. A) Appearance of broilers heart on days 28 and 42. B) Appearance of broilers liver on day 42. C) AHI of the control group and PHS group on days 28 and 42. D) Hepatic coefficient of the control group and PHS group on days 28 and 42. (*P<0.05, ** P<0.01, *** P<0.001).

Table 1: Primers for RT-PCR

Genes	Forward sequence (5′→3′)	Reverse sequence (5′→3′)	GenBank ID
Opa1	TCCCCTACGCAAAACAAAAC	CACCTACAGCAGACCCCAAT	NM_001039309.2
Mfn1	ATGGAGCGCTGTCTGACTTT	CCTTCTGTCTCCTGGCACTC	NM_001396185.1
Mfn2	AACACCAGCAAATCCCAGTC	GTGACCATCGACACCATGAG	XM_040689233.2
Drp1	GGCAGTCACAGCAGCTAACA	GCATCCATGAGATCCAGCTT	XM_046907548.1
Mff	ACTCCTCCCCGTGTTATT	TTCTCTCTTCAGCCTTCC	XM_040679333.2
β-actin	GTTGGTATGGGCCAGAAAGA	CCGTGTTCAATGGGGTACTT	NM_205518.2

Table 2: RT-PCR reaction mixture

Name	Volume (μL)
2×ChamQ SYBR qPCR Master Mix	5
Primer1 (10 μM)	0.5
Primer2 (10 μM)	0.5
Template cDNA	2
ddH_2O	2

day 42 had more serious hyperemia and congestion. The livers of broilers in the control group have no significant change. Compared with controls, the PHS group showed ecchymosis and hepatotropic on day 42. In the PHS group, yellowish brown ascites was also observed in the abdominal cavity.

Ascites heart index and hepatic coefficient: The AHI changes are shown in Fig. 1C. The AHI values in the PHS groups were greater than 0.25 on days 28 and 42. The AHI value in the PHS group, compared with the control group, was significantly increased on days 28 and 42 (P<0.01, P<0.001, respectively). As shown in Fig. 1D, the hepatic coefficient in the PHS group was significantly higher than that in the control group on day 28 (P<0.05). Compared whit the control group, the hepatic coefficient in the PHS group showed an upward trend on day 42(P>0.05).

Histopathological changes: As shown in Fig. 2, the PHS group showed some liver damage. The plates of the liver tissue in the control group were clearly and closely arranged. In the PHS group, hyperplastic connective tissue and the hepatic sinusoidal space was enlarged. Furthermore, the intrinsic hepatic structure was disrupted; necrotic hepatocytes; the nuclei were dissolved and the cytoplasm was coagulated and stained in the PHS group.

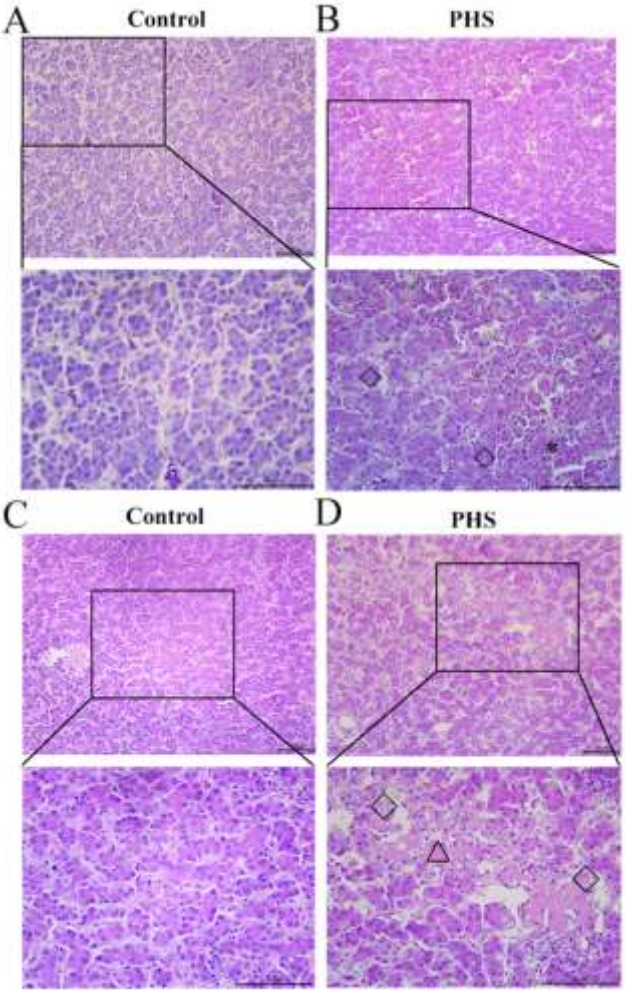

Fig. 2: Histopathological changes of both groups. A&B) Histopathological changes in the liver in control and PHS groups on day 28. C&D) Histopathological changes in the liver in control and PHS groups on day 42. All images were taken with ×20 and ×40 objective lenses. * Hyperplastic connective tissue. ◇ Nuclei dissolved. △ Cytosolic coagulation.

Mitochondria ultrastructure observation: The results of the mitochondrial structure observations are indicated in Fig. 3A and B. The mitochondrial morphology was clear, the arrangement of the cristae was regular, and the shape of the mitochondria was regular in the control group. Comparatively, Fig. 3B showed decreased electron density, partial cristae dissolution, and an unstable shape in the PHS group. Additionally, the mitochondria became smaller, and their numbers significantly increased in the PHS group.

Effect of PHS on mitochondrial fusion: To identify the effects of PHS on fusion, expression levels of fusion-related genes and proteins were examined. As shown in Fig. 5A and B, Mfn1, Mfn2, and Opa1 mRNA expression in the livers of the PHS group were lower than those in the control group. On day 28, the expression levels of Mfn1 and Mfn2 in the PHS group significantly decreased ($P<0.01$). The mRNA expression of Opa1 was also decreased but not significantly. On day 42, Mfn1, Mfn2, and Opa1 expression levels in the PHS group were significantly lower than those in the control group ($P<0.05$, $P<0.01$, or $P<0.001$, respectively). Additionally, immunohisto-chemical analysis using ImageJ (Fig. 4A and 4C) showed that the expression of Mfn1 and Opal

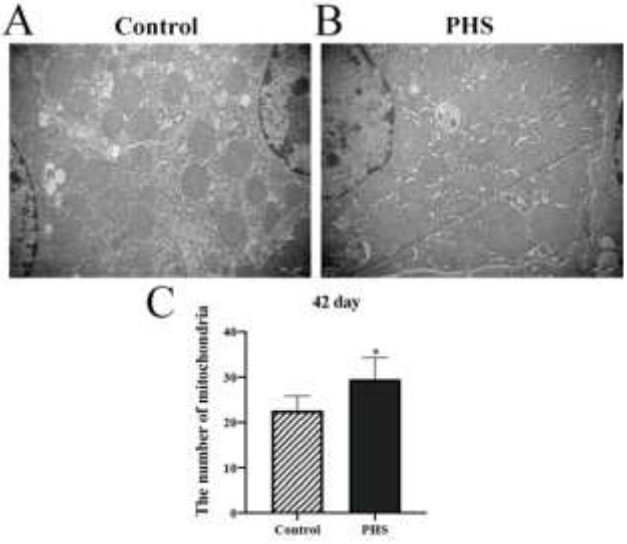

Fig. 3: Micro-photographs of the liver mitochondria from the control group and PHS group on day 42. A&B) Mitochondrial from the control and PHS groups. ×60,000. C) Number of mitochondria in liver of 42-day broilers. (*$P<0.05$)

decreased in the livers of the PHS group compared to that in the control group on days 28 and 42. Among these, Mfn1 expression was significantly decreased on days 28 and 42 ($P<0.05$). Fig. 5C and 5D illustrate the expression levels of mitochondrial fusion proteins, and Fig. 5E and 5F demonstrate western blot results. Protein expression levels showed a trend similar to that of mRNA expression. The results indicate that PHS causes liver damage by downregulating Mfn1, Mfn2 and Opa1 expression.

Effect of PHS on mitochondrial fission: As shown in Fig. 6A and 6B, compared with that in the control group, the mRNA expression of Drp1 and Mff were significantly upregulated in the PHS group on days 28 ($P<0.01$ or $P<0.001$, respectively) and 42 ($P<0.05$). The protein expression levels were consistent with their respective mRNA expression trends (Fig. 6C, 6D, 6E, and 6F). On day 28, the Mff protein level in the PHS group was significantly increased ($P<0.01$). On day 42, Drp1 protein levels were significantly upregulated in the PHS group ($P<0.05$). Drp1 expression in the liver was detected by immuno-histochemistry (Fig. 4). The expression of Drp1 was upregulated in the PHS group compared with that in the control group, and the expression of Drp1 on day 28 was significantly higher ($P<0.05$). Based on these results, PHS causes liver damage by upregulating mitochondrial fission-related genes and proteins.

DISCUSSION

During the occurrence of PHS, metabolically active hepatocytes cannot obtain enough oxygen due to hypoxia and portal vein congestion, causing hepatocyte necrosis and cirrhosis and inducing plasma leakage from the liver surface to the abdominal cavity (ascites). These alterations are closely associated with profound and persistent mitochondrial dysfunction (Wideman *et al*., 2013). Studies have shown that mitochondrial dysfunction is observed in human PH and leads to various complications, including decreased mitochondrial membrane potential, altered ROS,

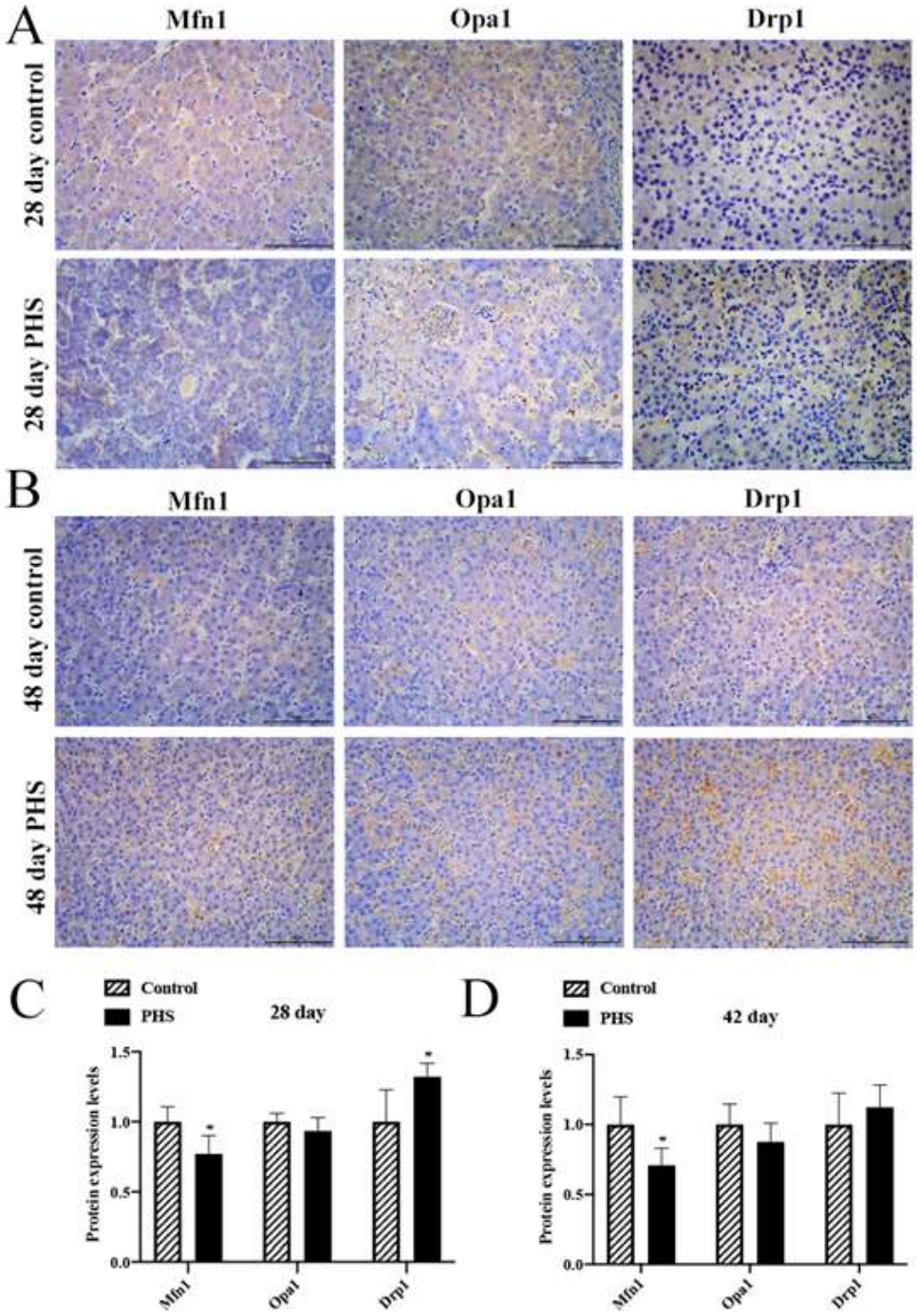

Fig. 4: Immunohistochemistry staining analysis of the liver tissues. A&B) Mfn1, Opa1, and Drp1 protein expression on days 28 and 42. C&D) Comparison of Mfn1, Opa1, and Drp1 protein expression levels between the PHS and control groups on days 28 and 42. All images were taken with ×40 objective lenses. (*P<0.05).

and increased fragmentation of mitochondrial (Culley and Chan, 2018). Changes in mitochondrial dynamics in PHS broilers livers remain unknown. According to our findings, PHS causes mitochondrial fission to increase and mitochondrial fusion to decrease.

In this study, broilers developed right heart hypertrophy and PH with increasing AHI values (AHI >0.25) in the PHS group, which is consistent with the results of previous studies (Li *et al.*, 2013). Liver damage was indicated by an increased hepatic coefficient and histopathological changes. In the PHS group, the natural structure partially disappeared, connective tissue was hyperplastic, and the divided liver plate formed a pseudolobule. Simultaneously, the hepatocytes appeared necrotic. These changes are consistent with the pathological characteristics of PHS, which include cirrhosis. The ultrastructure of mitochondria in the liver showed an increased number and smaller volume of mitochondria in the PHS group, which were significantly different from mitochondrial traits observed in the control group, which is possibly associated with excessive mitochondrial fission (Duranova *et al.*, 2020). Furthermore, some studies suggest that cardiac mitochondrial fragmentation and lack of crista in rat models of PH (Shults *et al.*, 2019). Additionally, in the PHS group, mitochondrial electron density was lower with

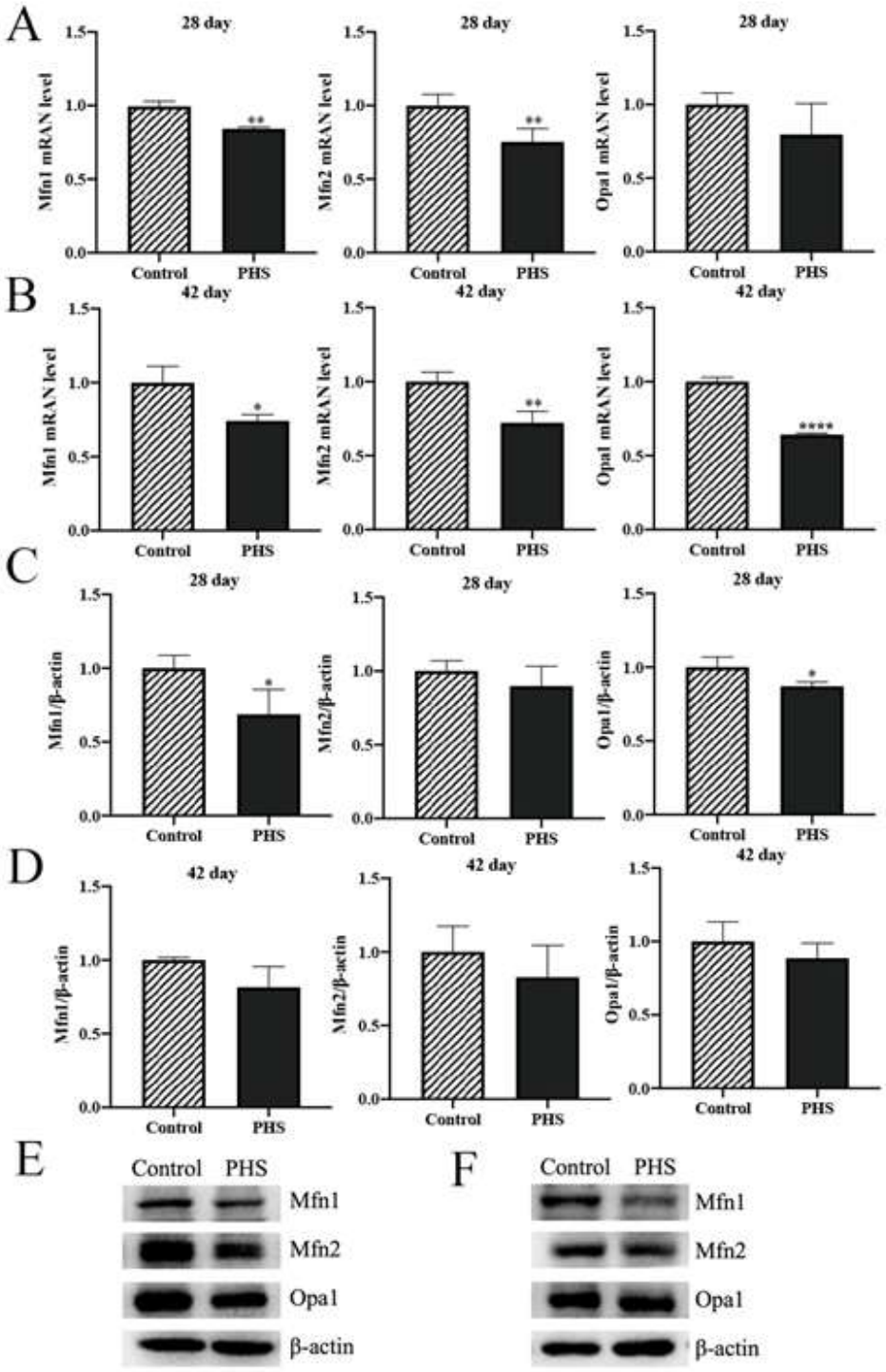

Fig. 5: Effect of PHS on the expression of genes and proteins related to mitochondrial fusion. A) mRNA levels of mitochondrial fusion-related genes on day 28. B) mRNA levels of mitochondrial fusion-related genes on day 42. C) Quantitative analysis of protein expressions of mitochondrial fusion-related protein on day 28. D) Quantitative analysis of protein expressions of mitochondrial fusion-related protein on day 42. E) Western blot shows expressions of mitochondrial fusion-related protein on day 28. F) Western blot shows expressions of mitochondrial fusion-related protein on day 42. (*P<0.05, ** P<0.01, *** P<0.001).

compared to the control group. A decrease in electron density is related to the complexity of the internal mitochondrial structure. The high intensity of fission leads to damage to the mitochondrial internal structure and dissolution of the cristae. Opa1 acts as an important part of the remodeling and maintaining mitochondrial cristae structure (Baker *et al.*, 2019). Within the mitochondria, there are two forms of Opa1: membrane-resident and activated soluble forms, which combine into an oligomer that maintains the bottleneck structure of the cristae and prevents their widening (Ulivieri, 2010). In this study, the decrease in Opa1 content in the PHS group was consistent with the damage to the cristae structure mediated by Opa1 deficiency.

The dynamic changes in mitochondria not only meet the daily basic metabolic requirements of the cell but can also regulate the internal or external pressure of the cell. It has been previously demonstrated that Drp1 expression in PASMCs is increased at the onset of human PH and is

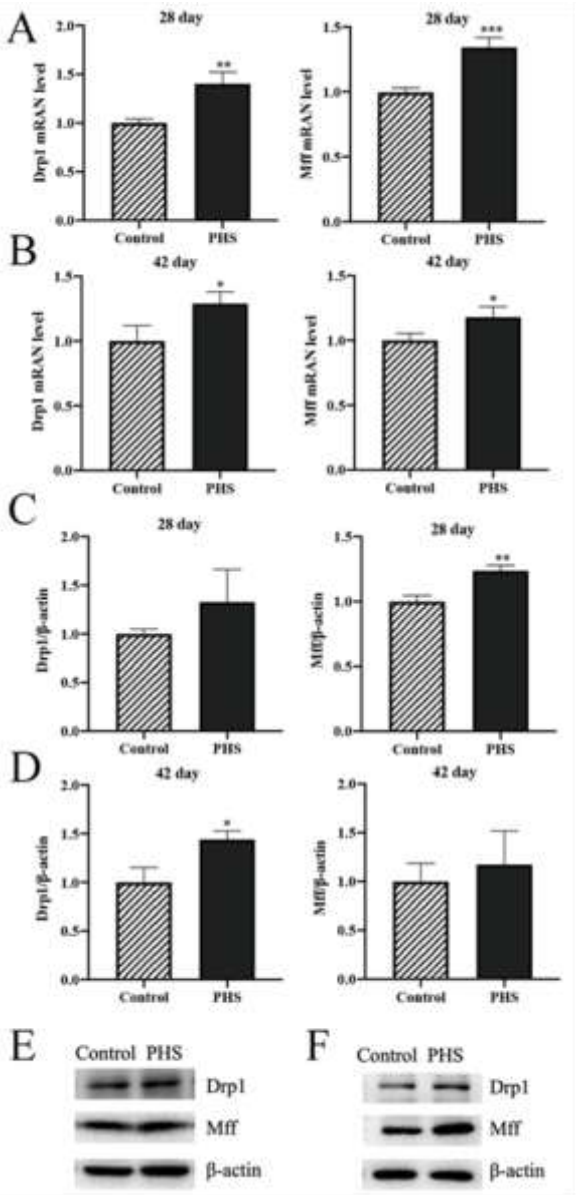

Fig. 6: Effects of PHS on the expression of genes and proteins related to mitochondrial fission. A) mRNA levels of mitochondrial fission-related genes on day 28. B) mRNA levels of mitochondrial fission-related genes on day 42. C) Quantitative analysis of protein expressions of mitochondrial fission-related protein on day 28. D) Quantitative analysis of protein expressions of mitochondrial fission-related protein on day 42. E) Western blot shows expressions of mitochondrial fission-related protein on day 28. F) Western blot shows expressions of mitochondrial fission-related protein on day 42. (* P<0.05, ** P<0.01, *** P<0.001).

positively correlated with two Drp1 receptors: MiD49 and MiD51 (Chen *et al*., 2018). In PH, mitochondrial damage activates Hypoxia-Inducible Factor-1α (HIF-1α) release. It is consistent with current reports that the HIF-1α upregulation decreased peroxisome proliferator-activated receptor α coactivator 1α (PGC1α) production, reduced the Mfn2 downregulated and mitochondrial fragmentation (Ryan and Archer, 2015). Previously, miR-140 was upregulated and Mfn1, its target protein, was downregulated in rats with hypoxia-induced PHS (Joshi *et al.*, 2016). Drp1-mediated enhancement of mitochondrial fission also plays an important part in PH pathogenesis. Drp1 regulates mitochondrial fission in a variety of ways during mitochondrial dysfunction, resulting in the removal of damaged or stressed mitochondria (Xiao *et al.*, 2022). In a rat model of monocrotaline-induced PHS, extracellular signal-regulated kinase 1/2 (ERK1/2) signaling activated Drp1 phosphorylation and increase mitochondrial fragmentation (Feng *et al.*, 2021). Hypoxia-induced murine model of PH was used to demonstrate that M6A regulates Drp1 promoter methylation during PHS progression by triggering decay of pyroptosis (Wang *et al.*, 2022). In this study, the expression levels of mitochondrial dynamic-related genes to explore changes in broilers with PHS. The results showed that downregulation expression of Mfn1, Mfn2, and Opa1 mRNAs and Drp1 and Mff mRNAs were upregulated in PHS broilers. The protein expression levels of Drp1, Mfff, Opa1, Mfn1, and Mfn2 measured by immunohistochemistry and western blotting were similar to the gene expression trends obtained by RT-PCR. These results suggest that broilers with PHS appear to have mitochondrial dynamic disorders due to increased expression of fission-related genes and decreased expression of fusion-related genes. Changes in mitochondrial dynamics in the liver of broiler chickens were similar to those observed in human PASMCs under similar conditions.

An imbalance in mitochondrial dynamics is a cellular pathological process (Yapa *et al.*, 2021). Mfn2 is dominant in fusion and is associated with apoptosis (Guo *et al.*, 2007). By constructing an overexpression Mfn2 vector, proliferation was reduced and apoptosis was restored to normal levels in PHS PASMC (Ryan *et al.*, 2015). Based on these properties, Mfn2 is considered a potential target for PHS therapy and could treat PHS by promoting mitochondrial fusion, whose potential has been demonstrated in rodent models (Ryan *et al.*, 2013). Overexpression of Drp1 decreases mitochondrial function, increases mitochondrial fission, and disruption of cellular metabolism (Dai *et al.*, 2020). The selection of suitable inhibitors to affect Drp1 depending on its different stages in mitochondrial fission also provides a new avenue for the treatment of PHS (Marsboom *et al.*, 2012). Inhibitors can be divided into three categories: inhibitors of Drp1 activation (Wong *et al.*, 2019), inhibitors of Drp1 GTPase activity (Wu *et al.*, 2020) and inhibitors of Drp1 ligands (Mo *et al.*, 2019). Our results are consistent with the mitochondrial dynamic disorder of human PH, providing a new idea for the prevention and treatment of PHS.

As a result of this study, we found that changes in mitochondrial dynamics caused by liver injury due to PHS are mediated by enhanced mitochondrial fission and attenuated mitochondrial fusion in broilers. A broiler model with PHS showed augmented Drp1 and Mff protein expression and downregulated Mfn1, Mfn2, and Opa1 protein levels. Consistent with our expectations, the changes in mitochondrial dynamics were similar to those observed in the pathogenesis of human PASMCs with PHS. This is similar to alterations in mitochondrial dynamics and increased mitochondrial respiration in an endothelial cell model of PH (Tura-Ceide *et al.*, 2021). Fission and fusion imbalance are key pathways in PHS-induced liver damage, and the suppression of mitochondrial fission by fission inhibitors or fusion agonists may antagonize the damaging effects by regulating mitochondrial quality control. Correcting mitochondrial fusion/fission disturbances using mitochondrial fission inhibitors or fusion agonists may be a viable treatment for reducing liver damage during PHS. It is also feasible to prevent PHS in fast-growing broilers through mitochondrial quality control methods, such as the

prevention of mitochondrial disorders and regulation of mitochondrial dynamics.

Conclusions: In summary, our study demonstrated hepatocyte damage in broilers with PHS. Mitochondrial ultrastructure analysis showed that although mitochondria increased in number, their sizes were reduced during the onset of PHS. Mitochondrial dynamics were disturbed in the livers of PHS broilers, as indicated by the decreased expression of Opa1, Mfn1, and Mfn2, and the increased expression of Mff and Drp1, resulting in reduced mitochondrial fusion and increased fission.

Authors contribution: WZ, YL, XY and ZM carried out the conceptual and experimental work. XW, JP, LH and YL wrote the first draft of the manuscript. WZ, YL, XY, DF and FSA contributed to the writing and review of the manuscript. JP and ZT supervised the study. All authors have approved the manuscript for publication.

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Conflict of interest: None of the authors has any conflict of interest.

Ethical Approval: All the experiments were performed after the approval of the Institutional Animal Welfare and Research Ethics Committee of South China Agricultural University, Guangzhou, China.

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