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Tea tree oil inhibits hydrogen sulfide-induced oxidative damage in chicken lungs through CYP450s/ROS pathway

Yilei Liang,[*,†,1] Li Jiang,[†] Mao Hu,[†] Xuegang Luo,[*,†] Tingting Cheng,[*,†] and Yachao Wang[*,†,2]

[*]*Biomass Center, School of Life Science and Engineering, Southwest University of Science and Technology, Mianyang, Sichuan, China, 621000; and* [†]*School of Life Science and Engineering, Southwest University of Science and Technology, Mianyang, Sichuan, China, 621000*

ABSTRACT A large amount of hydrogen sulfide (**H_2S**) is produced in the process of chicken breeding, which can cause serious inflammation and oxidative damage to the respiratory system of chickens. Tea tree oil (**TTO**) has antioxidant and anti-inflammatory properties. No studies have been reported on the use of TTO in H_2S-induced lung injury in chickens. Therefore, in this study, 240 one-day-old Roman pink laying hens were randomly and equally divided into 3 groups: control group (**CON**), H_2S exposure group (**AVG**, containing H_2S), and TTO treatment group (**TTG**, containing H_2S and 0.02 mL/L TTO) to establish an experimental model of TTO treatment with H_2S exposure for a period of 42 d. Hematoxylin and eosin (**H&E**) staining was used to detect lung histopathology. Gene expression profiles were analyzed using transcriptomics. The underlying mechanism of the amelioration of lung injury by TTO was further revealed by antioxidant enzyme assays and qRT-PCR. The results showed that H_2S exposure induced significant gene expression of CYP450s (CYP1B1 and CYP1C1) ($P < 0.05$), and caused intense oxidative stress, apoptosis and inflammation compared with CON. TTO could reduce ROS production and enhance antioxidant capacity (SOD, CAT, T-AOC, and GSH-PX) by regulating the CYP450s/ROS pathway ($P < 0.05$). Compared with the control group, the treatment group showed significantly decreased expression of apoptotic (Caspase-8, Caspase-3, Bid and Fas) ($P < 0.05$) and inflammatory (IL-4, IL-16, NF-κB, TNF-α and IFN-γ) ($P < 0.05$) factors in the lung. This study revealed that TTO regulated CYP450s/ROS pathway to alleviate H_2S-induced lung injury in chickens. These results enrich the theory of the action mechanism of TTO on H_2S-exposed chicken lungs and are of great value for the treatment of H_2S-exposed animals.

Key words: CYP450s, H_2S, oxidative stress, tea tree oil

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INTRODUCTION

With the rapid development of intensive farming, high-density feeding environments have become the norm in the industry. However, under such environmental conditions, the decomposition of feed residues and manure of chickens can produce large amounts of hydrogen sulfide (**H_2S**), which poses a serious threat to livestock and poultry health (Song, 2024). Although the traditional window ventilation and exhaust method can help to reduce the concentration of H_2S to a certain extent, it is often difficult to completely remove H_2S from livestock and poultry breeding houses (Hansen et al., 2018; Guo et al., 2022) . Therefore, the long-term exposure of livestock and poultry to H_2S seriously affects the growth performance and health of livestock and poultry (Gyte and Kelsey, 2024). Hydrogen sulfide exposure can damage the respiratory system (Li et al., 2024) and even cause tissue damage and pulmonary edema (Xu et al., 2017). Studies have shown that H_2S can induce the expression of a variety of inflammatory factors, such as tumor necrosis factor alpha-like (**TNF-α**), which initiated inflammatory injury in the lung (Li et al., 2024). In addition, H_2S could change the gene expression of Interleukin-1β (**IL-1β**), Interleukin-4 (**IL-4**), Interleukin-6 (**IL-6**), TNF-α and interferon gamma (**IFN-γ**) gene, resulting in immune and pathological damage (Hu et al., 2018). More seriously, H_2S can also induce the expression of apoptosis-related genes, such as caspase 3, 8, 9, thereby promoting cell apoptosis (Hu et al., 2018; Liu et al., 2022c). At the same time, H_2S exposure can also induce mitochondria to produce a large number of reactive oxygen species (**ROS**), leading to severe oxidative stress, which may

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[1]The first author was Yilei Liang.
[2]Corresponding author: wangyachao@swust.edu.cn

not only reduce the content of superoxide dismutase (**SOD**), catalase (**CAT**) and glutathione peroxidase (**GSH-Px**), but also increases the expression of cytochrome P450s (CYP450s) family genes (Zheng et al., 2019). Previous studies have shown that CYP450s might produce a large amount of ROS in the process of metabolizing H_2S, which would aggravate oxidative stress and destroy the oxidative balance of the body (Zheng et al., 2019; Olubadewa et al., 2020).

Tea tree oil (**TTO**), a plant essential oil derived from tea leaves (Reuveni et al., 2023) has antioxidant and anti-inflammatory effects (Yang et al., 2022) and improved growth Performance (Liu et al., 2023b) . It is reported that TTO can increase the average daily gain and average daily feed intake of pigs and reduce the diarrhea rate to gain weight (Wang et al., 2021). In sheep, the gene expression of IL-1β, IL-6, TNF-α and nuclear factor of kappa (**NF-κB**) was significantly decreased after feeding with TTO (Hu et al., 2021). In addition, TTO has been shown to inhibit the production of inflammatory mediators by interfering with NF-κB and mitogen-activated protein kinase pathways (Nogueira et al., 2014). Meanwhile, TTO termite-4-alcohol and α-pineol inhibited the production of superoxide, and then increased the levels of total antioxidant capacity (T-AOC), SOD, CAT and GSH-Px in the body, and reduced the content of malondialdehyde (**MDA**) (Wang et al., 2021; Yang et al., 2022). TTO also regulated CYP1A1(cytochrome P450 family 1 subfamily A member 1), and GPX4 (glutathione peroxidase 4) gene expression (Liu et al., 2022a). In addition, further studies found that TTO treatment regulated mitochondrial apoptosis through p53-dependent pathway by regulating the expression of Bax (bcl2-associated X), Bcl-2 (B-cell lymphoma) and caspase-3 (Ramadan et al., 2019).

Therefore, this study focused on the effects of TTO on the respiratory system and lung of chickens exposed to H_2S. Transcriptome analysis and qRT-PCR were used to verify the mRNA expression levels of related genes, and whether TTO has effects on CYP450, oxidative stress, apoptosis, and inflammatory immunity. This study is expected to provide a new solution to mitigate the adverse effects of H_2S on chickens and provide strong support for the healthy development of livestock and poultry farming.

MATERIALS AND METHODS

Experimental Materials

TTO was purchased from Chongqing Huaruilong Biotechnology Co., LTD. (Chongqing, China), in which the principal components are terpine-4-alcohol and γ-terpinene, with a content of 40.44% and 20.04%, respectively (Annex 1). Roman pink egg roosters were purchased from the Guangzhou Xiying Rare Poultry Breeding Co., LTD. (Guangzhou, China). Total protein (**TP**), ROS, T-AOC, CAT, MDA, SOD, hydrogen peroxide (**H_2O_2**) and GSH-Px the kit purchased from Nanjing Jian Cheng Bioengineering Institute (Nanjing, China).RNA extraction kit and reverse transcription kit were purchased from Baori Doctor Material Technology Co., LTD. (Beijing, China). Paraformaldehyde 4% was purchased from Chengdu Cologne Chemicals Co., LTD. (Chengdu, China).

Experimental Design

The experiment was approved by the Animal Care Committee of Southwest University of Science and Technology. A total of 240 healthy 1-day-old chickens were divided into 3 groups: control group (**CON**), H_2S-exposed group (**AVG**), and TTO treatment group (**TTG**). Four replicates were set up for each treatment group with 20 chickens per replicate. It was optimized according to the protocol of Song et al. (2021) and housed in 3 separate standard animal rooms. An Innova-1412 Luma Sense photoacoustic field gas monitor was used to measure the concentration of H_2S. For the first 21 d, the control group did not add TTO and the H_2S content was within the safe range (less than 0.5 mg/m^3), while the H_2S exposure group kept the H_2S concentration at 3.5 to 4.5 mg/m^3. TTO treatment groups were exposed to 3.5 to 4.5 mg/m^3 of H_2S and treated with 0.02 mL/L TTO in drinking water (Runmin et al., 2018). The concentration of H_2S was adjusted to 19.5 to 20.5mg/m^3 in the H_2S exposure group and the TTO treatment group 21 d later, and other conditions were unchanged. Roman pink laying hens had free access to water and basal diet during the experiment, and other conditions were kept the same. On the 42nd d of the whole experiment cycle, the chickens were pacified with carbon dioxide followed by cervical dislocation. The upper lobe of the left lung was taken, and the size was appropriate and uniform ($2.0 \times 2.0 \times 0.3$ cm^3). The samples were fixed in 4% paraformaldehyde, and a part of the tissue samples were stored at -80℃ for transcriptome sampling and qRT-PCR detection.

Weight Measurement

Feed was forbidden for 12h before the end of the experiment, and then the body weight of the chickens in each group was determined after 42 d (Liu et al., 2023b).

Lung Histopathology

Tissue samples (upper lobe of left lung) fixed with 4% paraformaldehyde were dehydrated in gradient alcohol (50, 60, 75, 85, 95, 100, and 100%), cleared (absolute alcohol: xylene = 2:3), immersed in wax, and encapsulated. The slices were then cut into 5 μm thick sections at room temperature and finally stained with hematoxylin and eosin staining (**HE**). The gel-sealed sections were observed intact under an electron microscope (OLMPUS, Tokyo, Japan). Normal tissues and obvious lesions were photographed and recorded, and the damage of tissue samples was described (Wang et al., 2017).

Oxidation Index Determination

Samples from the lung (upper lobe of the left lung) were weighed and 1 mL saline was added, while steel balls were added to ground the tissue blocks. The grinding tissue blocks were further added with normal saline according to the ratio of tissue weight (g): volume (mL) = 1:9. The homogenate was mechanically homogenized under the condition of ice water bath. The homogenate was centrifuged at 3,000 r/min for 10 min, and the supernatant was stored in a refrigerator at -20℃ until measured (Chi et al., 2020). The concentrations of TP, ROS, T-AOC, CAT, MDA, SOD, H2O2 and GSH-Px were determined according to the instructions of the kit manufacturer.

Transcript Sequencing Analysis

Total RNA was extracted using Trizol method (Wei et al., 2024). Extracted RNA samples were collected on an Agilent 2,100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) on the test quality (Ranjani et al., 2020). Transcriptome sequencing and analysis were performed by OE Biotechnology Co., LTD (Shanghai, China). Libraries were sequenced on an Illumina HiSeq X Ten platform. Raw reads in fastq format were processed by fastp software, and low-quality reads were removed to obtain clean reads for subsequent data analysis (Chen et al., 2018). The clean reads were aligned to the chicken reference genome using HISAT2 (Daehwan et al., 2015). Differential expression analysis was performed using the DESeq2 (Mills et al., 2016). Screening of differentially expressed genes (**DEGs**) conditions for | Fold Change (FC) |≥2 and $P < 0.05$).

Functional Enrichment Analysis

Gene ontology (**GO**) enrichment analysis was performed on all DEGs in this experimental design. The GO analysis results were visualized using the bioinformatics website (www.cloud.oebiotech.cn) (Huang et al., 2008).

Real-Time Fluorescent Quantitative PCR

Primer sequences were designed using primer 5 software, and primer specificity was verified using the Primer-BLAST online tool. The primer sequences were shown in Annex 1. Primers were synthesized by Sangon Biotechnology (Shanghai) Co., LTD. The genes involved in this study included glutathione peroxidase 2 (**GPX2**), glutathione S-transferase alpha 2 (**GSTA2**), nitric oxide synthase 2 (**NOS2**), bcl-2 interacting domain death agonist (**Bid**), Caspase-8, Caspase-3, fas cell surface death receptor (**Fas**), fas associated via death domain (**FADD**), interleukin 16 (**IL-16**), IL-4, INF-γ, TNF-α, NF-κB, vascular endothelial growth factor C (**VEGFC**), cytochrome P450 family 1 subfamily B member 1 (**CYP1B1**) and cytochrome P450, family 1, subfamily C, polypeptide 1 (**CYP1C1**). About 0.1g of lung tissue samples were weighed into ribonase-free tubes, and 1 mL of Trizol was added and ground with a microtissue homogenizer. Total RNA was extracted from lung tissue using an RNA extraction kit (TaKaRa, Japan). RNA integrity was detected by electrophoresis of 1.0% of the extracted total RNA on agarose gel, and RNA purity and concentration were measured by micro-UV spectrophotometer. The ratio of OD260 / OD280 between 1.9 and 2.1 was valid. cDNA was synthesized using a reverse transcription kit (TaKaRa, Japan). Primers were synthesized by Sangon Biotechnology (Shanghai) Co., LTD. (Annex 2). qRT-PCR reactions were performed using SYBR Green I chimeric fluorescence assay. Relative gene expression levels were calculated using the classical $2^{-\Delta\Delta CT}$ method using β-ation as the reference gene (Arocho et al., 2006).

Data Analysis

SPSS 18.0 statistical software was used for the one-way analysis of variance. The experiment was repeated 3 times, and the data are expressed as mean ± standard deviation (M±SD). The processed data were plotted using GraphPad Prism 5 (GraphPad Software, CA). Three samples from each group of histopathological sections were compared and analyzed. Different letters between groups indicated statistically significant differences ($P < 0.05$). In contrast, no mark or identical letter marks indicated that the difference between groups was not significant ($P > 0.05$).

RESULTS

Weight Change

As shown in Figure 1A, compared with the CON, the body weight of the AVG and TTG decreased significantly ($P < 0.05$). Compared with AVG, the weight of TTG increased, but there was no significant difference ($P > 0.05$).

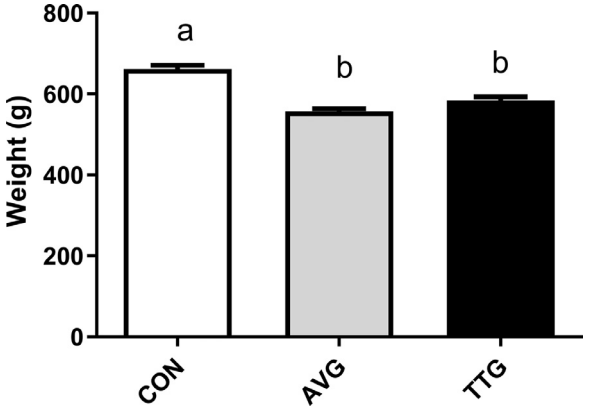

Figure 1. Changes in body weight of chickens after 42 d of the experiment. CON: control group. AVG: H_2S exposed group. TTG: TTO treatment group. Different letters between groups indicated significant differences ($P < 0.05$), and data were expressed as mean ± standard deviation (M±SD).

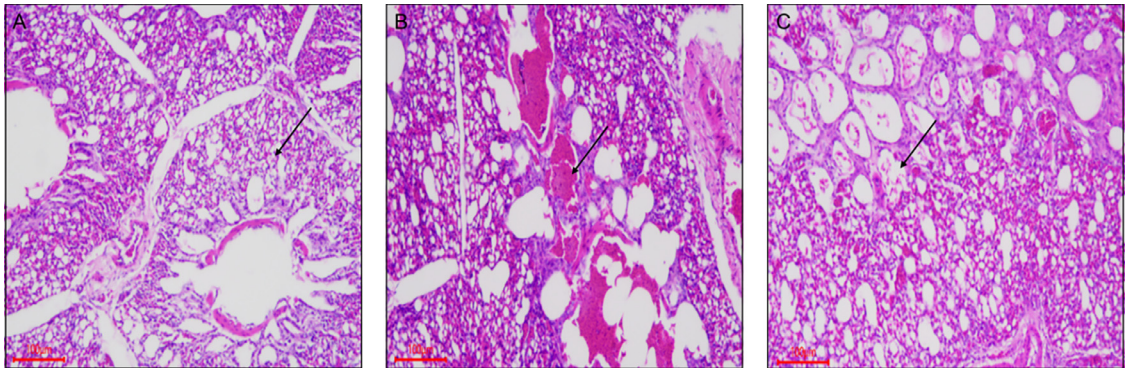

Figure 2. Lung tissue sections. (A) Control group (CON) lung tissue. (B) Lung tissue from H_2S-exposed group (AVG). (C) TTO treatment group (TTG) lung tissue. The arrow points to the alveolus, H&E, Bar = 100μm.

Pathological Section Observation of Lung Tissue

The results showed that the lung tissue structure in CON was normal, without obvious histopathological damage. In the lungs of AVG, hemorrhage was seen in the secondary bronchi and atria of the lungs, and respiratory capillaries were congested. In TTG, there was slight hemorrhage in the lung and pulmonary secondary bronchi, and no hyperemia was observed in the pulmonary atria or respiratory capillaries (Figure 2).

Effects of Tea Tree Oil Treatment and Hydrogen Sulfide Exposure on Transcriptome

As shown in Figure 3A, there were significant differences ($P < 0.05$) among treatments. As shown in Figures 3B and 3C, 1,940 DEGs were found in AVG, of which 1,092 were upregulated and 848 were downregulated. In total, 1,175 DEGs were identified in the tree oil treatment group, of which 639 were upregulated and 536 were downregulated (FC > 2 and $P < 0.05$).

GO enrichment analysis was performed for all DEGs to explore the key functions affected by the DEGs. The results showed that most of the genes were mainly enriched in biological processes. In AVG, DEGs were mainly enriched in cellular processes, biological regulation, biological process regulation, etc. Among the cellular components, DEGs were mainly enriched in the cells and cellular fractions. In terms of molecular function, the DEGs were mainly enriched in binding and catalytic activity (Figure 3D). In TTG, the DEGs were mainly enriched in cellular processes, biological regulation, and biological process regulation in the biological process. Among the cellular components, the DEGs were mainly

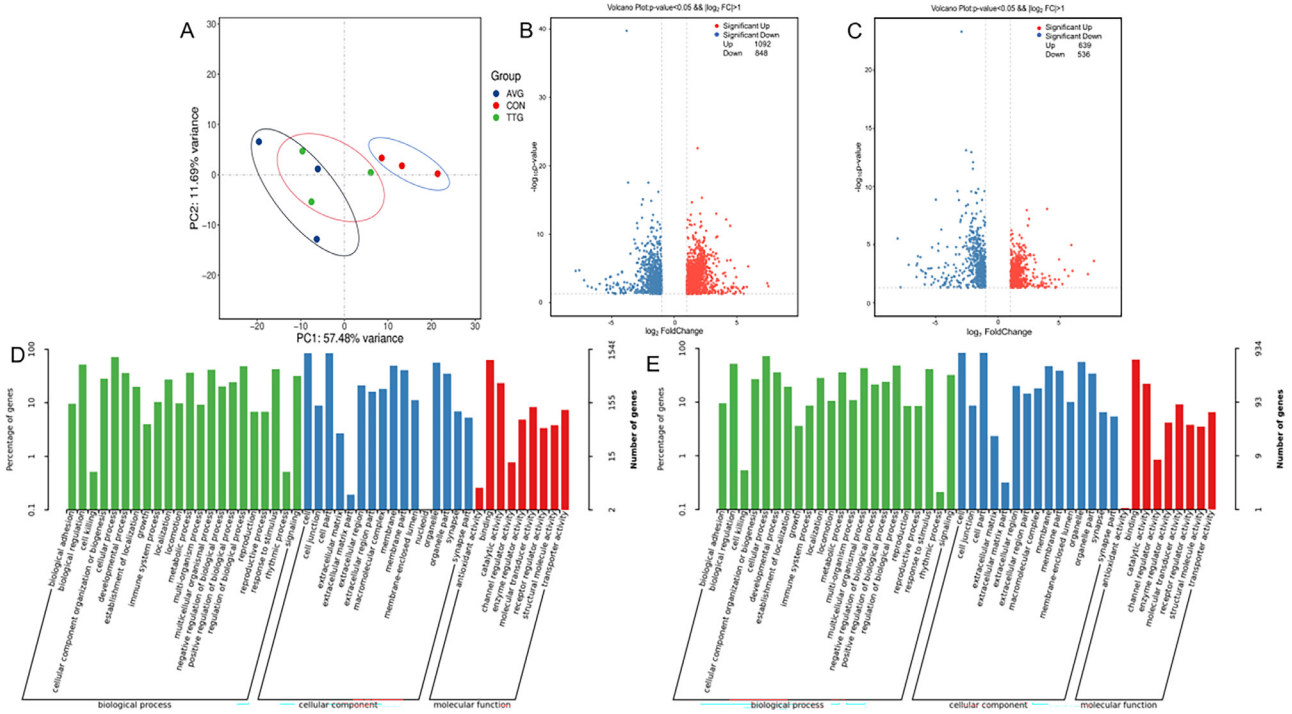

Figure 3. Transcriptome sequencing analysis. (A) PCA analysis, Points of the same color indicate the same group. (B) Volcano plot of H_2S exposure group, red indicates up-regulated genes and blue indicates down-regulated genes. (C) Volcano plot of TTO treatment group. (D) GO was enriched in the H_2S-exposed group. (E) GO enrichment in the TTO treatment group. CON: control group. AVG: H_2S exposed group. TTG: TTO treatment group.

enriched in cells, cell fractions, and organelles. In terms of molecular function, the DEGs were mainly enriched in binding, catalytic activity, and molecular transduction activity (Figure 3E).

Effect of TTO on CYP Expression in Chicken Lungs

As shown in Figure 4A, 16 differentially expressed CYP450 genes were detected in the lungs of AVG, while 6 differentially expressed CYP450 genes were detected in TTG. To further confirm that TTO altered the transcription of CYP450s in chicken lung tissue, 2 CYP450 genes with large expression differences CYP1C1 and CYP1B1 were selected and subjected to qRT-PCR (Figures 4D and 4E). The results showed that TTO treatment reduced the expression levels of CYP1C1 and CYP1B1 genes in AVG ($P < 0.05$), returning them to normal levels. This was consistent with the trend of log2FC obtained by transcription (Figure 4C).

Effect of TTO on Oxidative Stress in H2S Chicken Lungs

Figure 5A showed that 29 oxidative stress-related differential genes were detected in chicken lungs, modulated by H_2S exposure. By contrast, only 13 DEGs related to oxidative stress were detected in TTG (Figure 5B). Compared with the H_2S-exposed group, TTO treatment significantly increased the contents of antioxidant enzymes GSH-Px, CAT, SOD, and T-AOC, significantly reduced the contents of ROS, MDA, and H_2O_2 and returned them to normal levels (Figure 5C) ($P > 0.05$).To further confirm the changes in oxidative stress genes in chicken lungs between TTG and AVG, GSTA2, GPX2, and NOS2 genes in the transcriptome were selected to verify whether the log2FC trend obtained by transcription was consistent with the qRT-PCR results (Figure 5D). The qRT-PCR results showed that compared with CON, the expression levels of GSTA2, GPX2, and NOS2 genes in AVG and TTG were significantly increased ($P < 0.05$). This was consistent with the trend of log2FoldChange obtained from the transcription studies (Figure 5D).

TTO Modulates the Inflammatory Effects of H2S in Chicken Lungs

The effect of TTO on cytokines in H_2S-exposed lungs was shown in Figure 6. The heat map in Figure 6A showed that 28 inflammatory and immune-related differential cytokines were found in the lungs of chickens in the H_2S-exposed group. Among these 28 DEGs, 11 DEGs were downregulated, and 17 DEGs were upregulated (Figure 5B). The heat map in Figure 6C showed that 17 inflammatory and immune-related differential cytokines were found in the lungs of chickens in TTG. Among these 17 DEGs, 7 DEGs were downregulated, and 10 DEGs were upregulated (Figure 6D). To further confirm the effect of TTO on cytokines in H_2S-exposed lungs, we performed qRT-PCR (Figure 6B) to measure the mRNA levels of IL-4, VEGFC, IL-16, NF-κB, TNF-α, and IFN-γ in chicken lungs. The results showed that compared with the H_2S-exposed group, the mRNA levels of IL-4, IL-16, NF-κB, TNF-α, and IFN-γ in the lungs of TTG were significantly downregulated ($P < 0.05$), and returned to normal levels. However, the level of VEGFC mRNA was significantly increased compared with the H_2S-exposed group ($P < 0.05$) (Figure 6E).

TTO Regulates Apoptotic Gene Expression

To investigate the effect of TTO on apoptosis in lungs exposed to H_2S. Eleven DEGs related to apoptosis in AVG were detected by transcriptional sequencing (Figure 7A). Among them, 3 DEGs were upregulated, and 8 DEGs were downregulated (Figure 7B). However, no DEGs related to apoptosis were detected in TTG. To

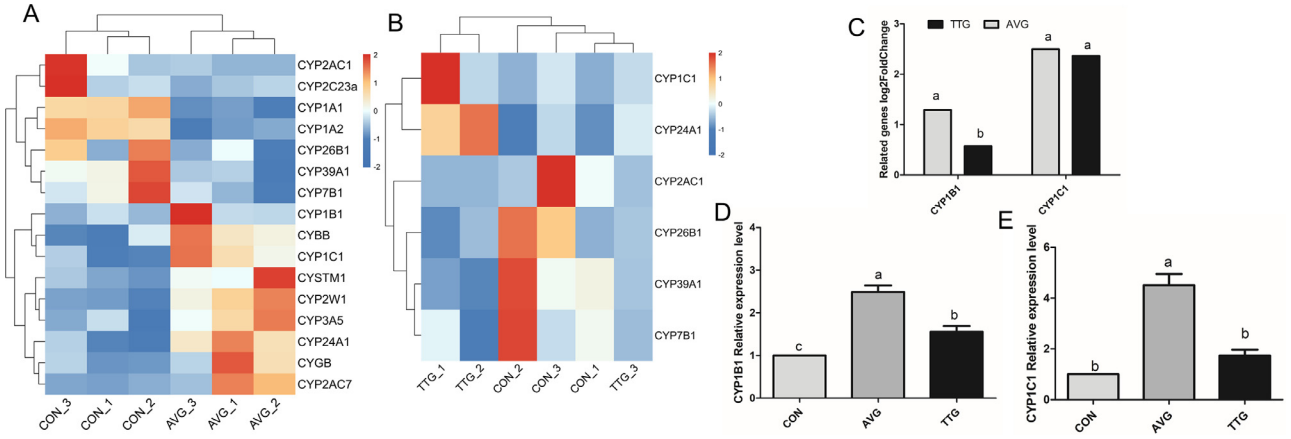

Figure 4. Effect of TTO on CYP450s genes in the lungs of H_2S-exposed chickens. (A) Heat map of log2FC of AVG CYP450s genes, with up-regulated genes in red and down-regulated genes in blue. (B) log2FC heatmap of TTG CYP450s genes, with up-regulated genes in red and down-regulated genes in blue. (C) log2FC trend of CYP1C1 and CYP1B1. (D) CYP1B1 gene expression. (E) CYP1C1 gene expression. Control group (**CON**), H_2S exposure group (AVG), TTO treatment group (**TTG**). Different letters between groups indicated significant differences ($P < 0.05$), and data were expressed as mean ± standard deviation (M±SD).

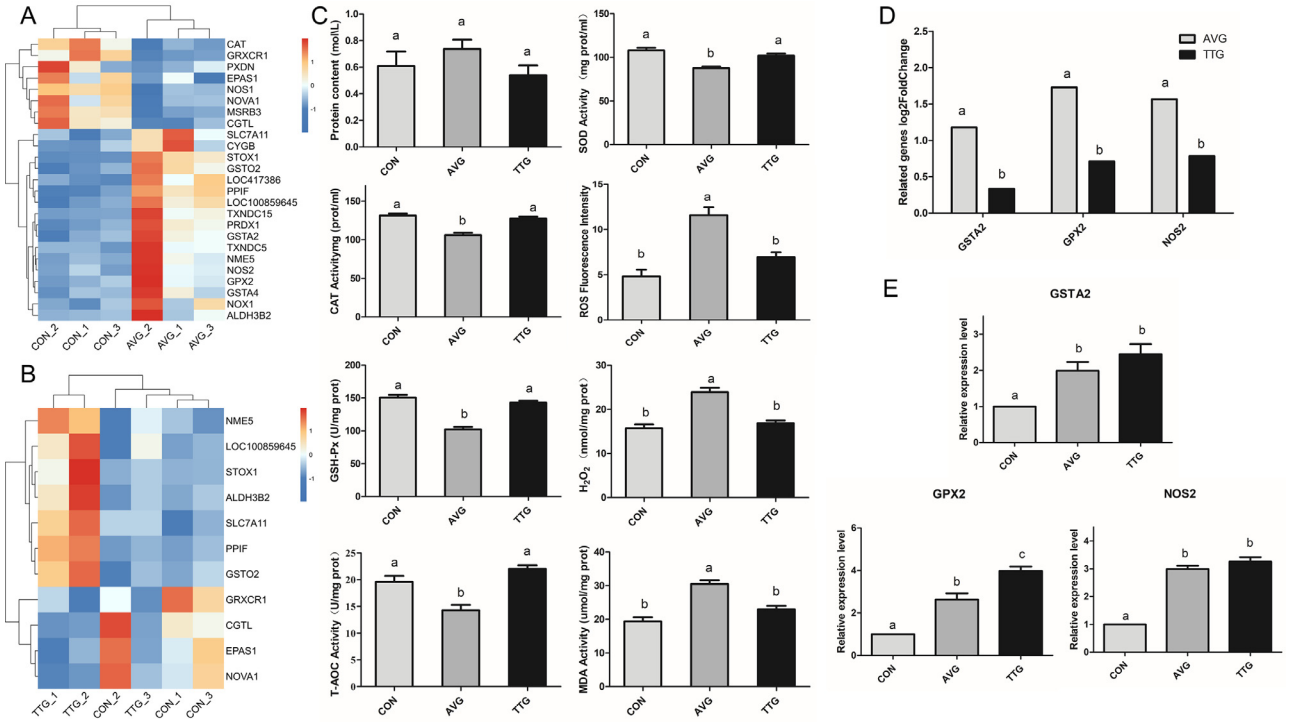

Figure 5. The effect of TTO on the antioxidant system. (A) Heat map of Log2FC of AVG oxidative stress genes, Up-regulated genes are shown in red and down-regulated genes are shown in blue. (B) TTG oxidative stress gene Log2FC heatmap, Up-regulated genes are shown in red and down-regulated genes are shown in blue. (C) Oxidative stress indicators. (D) Log2FC of GSTA2, GPX2, NOS2 genes. (E) GSTA2, GPX2, NOS2 gene expression. CON: control group. AVG: H_2S exposed group. TTG: TTO treatment group. Different letters between groups indicated significant differences ($P < 0.05$), and data were expressed as mean ± standard deviation (M±SD).

verify the reliability of the transcriptome data, B-cell lymphoma 2-related protein A1 (**BCL2A1**) was selected for validation, and the results were consistent with the log2FC trend obtained from the transcription studies (Figure 7C), indicating that TTO has a significant effect on maintaining the anti-apoptotic activity of BCL2A1. In this experiment, the expression levels of FADD, Caspase-8, Caspase-3, Bid, and Fas genes were

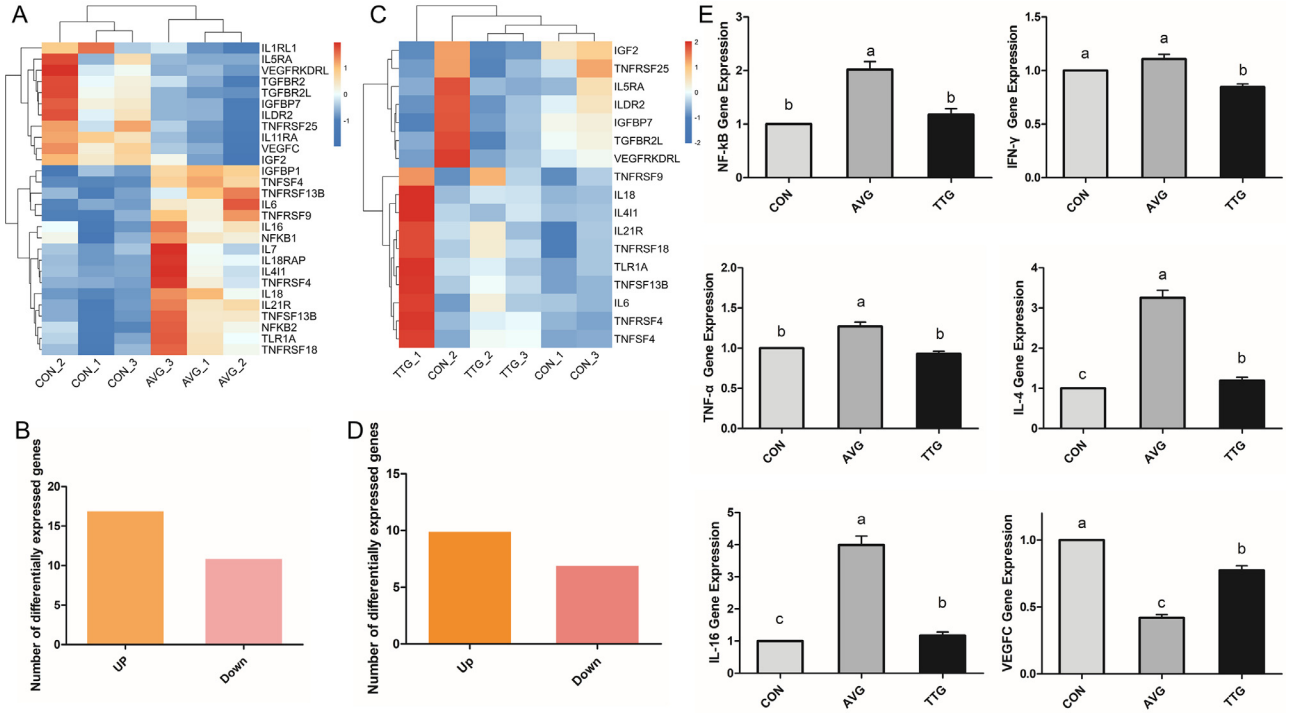

Figure 6. The effect of TTO on inflammation. (A) AVG cytokine Log2FC heatmap, Up-regulated genes are shown in red and down-regulated genes are shown in blue. (B) Number of down-regulated genes on AVG. (C) TTG cytokine Log2FC heatmap,Up-regulated genes are shown in red and down-regulated genes are shown in blue. (D) number of down-regulated genes on TTG. (E) Gene expression of IL-4, VEGFC, IL-16, NF-κB, TNF-a, and INF-γ. CON: control group. AVG: H_2S exposed group. TTG: TTO treatment group. Different letters between groups indicated significant differences ($P < 0.05$), and data were expressed as mean ± standard deviation (M±SD).

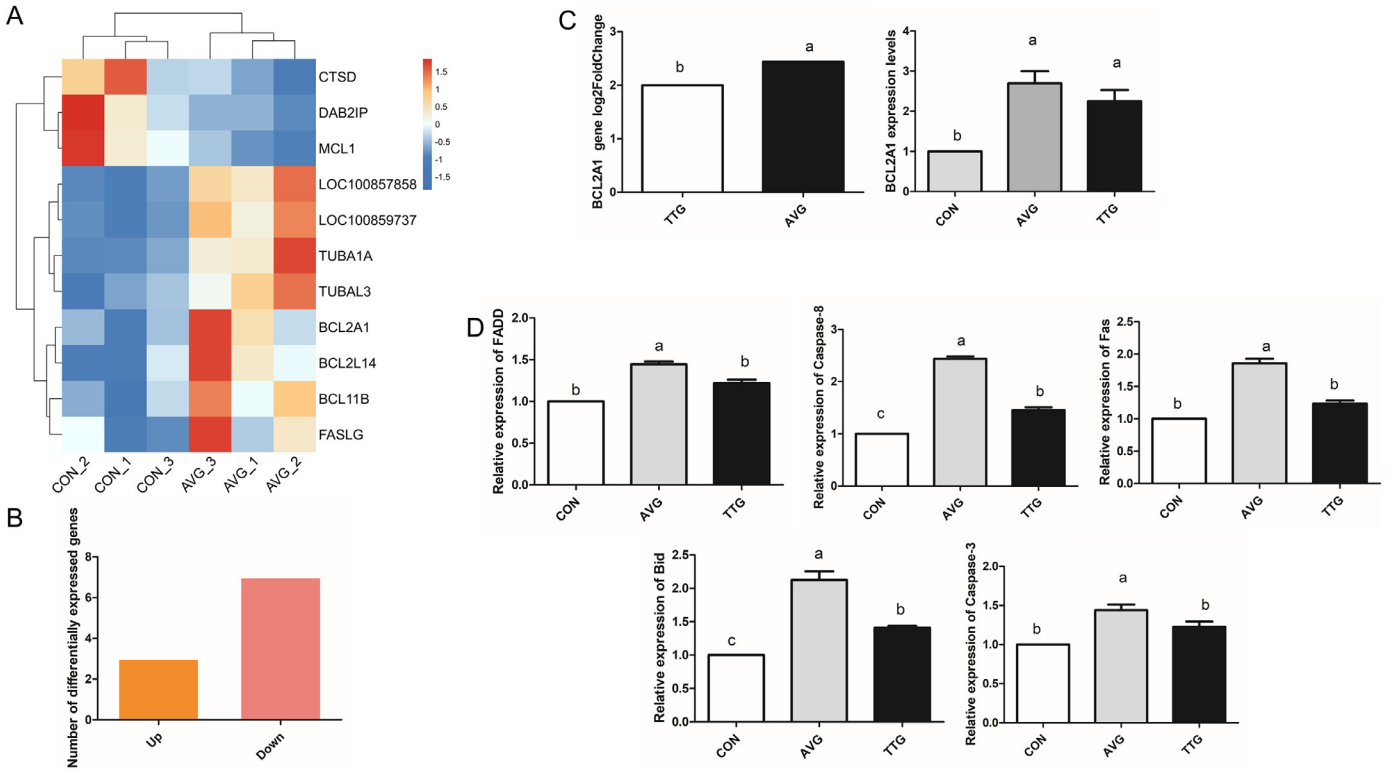

Figure 7. Effect of TTO on apoptotic genes. (A) AVG apoptotic genes cytokine Log2FC heatmap, Up-regulated genes are shown in red and down-regulated genes are shown in blue. B: number of up-regulated and down-regulated genes in AVG exposed group; C Log2FC of BCL2A1 gene. D qRT-PCR was used to detect FADD, Caspase-8, Caspase-3, Bid, and Fas genes. TTG: TTO treatment group. Different letters between groups indicated significant differences ($P < 0.05$), and data were expressed as mean ± standard deviation (M±SD).

also detected by qRT-PCR, and the results showed that TTO could significantly reduce the expression of apoptotic genes induced by H_2S (Figure 7D).

DISCUSSION

H_2S is a highly toxic pollutant in chicken houses and is widely present in breeding houses, posing a serious threat to the health of livestock and poultry animals. TTO, as an additive that can enhance immunity, inhibit inflammation, improve antioxidant levels, and promote growth, is used in the farming industry. Therefore, in the present study, we analyzed the morphological damage and transcriptome data of chicken lungs induced by the treatment of H_2S with TTO added to drinking water and verified the key genes by qRT-PCR. This is the first study to demonstrate that TTO can reduce CYP450 and H_2S metabolism that causes oxidative stress, leading to inflammation, immune dysfunction, and apoptosis in chicken lungs.

TTO is widely used as an additive in the aquaculture industry with growth and digestion-promoting functions (Liu et al., 2023b). In this trial, it was found that the addition of TTO to drinking water increased the body weight of chickens. This may be due to the fact that TTO can effectively ameliorate the effect of vulcanization on chicken gut microbial balance. In previous studies, H_2S was found to disrupt the gut microbiota balance, thereby affecting feeding and growth in suckling pigs (Guo et al., 2022). However, the addition of TTO to the diet can increase the villus height of the ileum, improve the feed conversion rate, nutrient digestion, and absorption rate, and increase the body weight of broilers (Liu et al., 2023a).

Transcriptome analysis provides important information for studying the mechanism of the environmental stress response in organisms (Saul and Kosinsky, 2023). All DEGs in the AVG and TTG in this study were annotated using GO analysis. All DEGs between AVG and TTG were annotated using GO analysis. In AVG, biological processes, cellular processes, and molecular processes were mainly represented as cellular processes, cellular, and catalytic activity. These were mainly related to the injury of lung cells by H_2S. However, the most significantly enriched pathways for biological, cellular, and molecular processes were biological regulation, organelle, and molecular transduction activity, respectively, in the chicken lungs supplemented with TTO. These pathways were mainly related to the regulation of the cellular metabolism of H_2S by TTO.

CYP450 is present in the tissues of most organisms and participates in the metabolism of H_2S in vivo (Tang et al., 2018). Previous studies have shown that acute H_2S poisoning inhibited the activities of CYP2B6 (cytochrome P450 family 2 subfamily B member 6), CYP2D6 (cytochrome P450 family 2 subfamily D member 6), CYP1A2, and CYP2C9 (cytochrome P450 family 2 subfamily C member 9) enzymes in rats (Wang et al., 2014). Consistent with the results of previous studies, H_2S exposure in this study activated 18 differentially expressed CYP450 genes. However, only 6 CYP450

genes were differentially expressed in lung tissues after TTO treatment. In the gene validation experiment, the mRNA levels of CYP1B1 and CYP1C1 in TTG were significantly lower than those in AVG, which also supported the idea that TTO could reduce lung damage caused by H_2S metabolism by regulating the gene expression of CYP450s. CYP450, as a terminal oxidase in a mixed-function oxidase system, metabolizes endogenous substrates and xenobiotics, and is closely related to the oxidative stress response of organisms (Havrylyuk et al., 2024). The upregulation of CYP450s (CYP1A1, CYP1B1, and CYP1C1), found in zebrafish, increases the expression of oxidative stress response genes GPx1 (glutathione peroxidase 1), and GCLC (glutamate-cysteine ligase catalytic subunit) (Dong et al., 2009).

In the present experiment, H_2S exposure resulted in significantly higher mRNA levels of CYP1C1 and CYP1B1 in chicken lungs. However, the mRNA levels of CYP1C1 and CYP1B1 were significantly decreased in the lungs of the TTO-treated chickens. In the liver tissue of tree mice, it was found that the increased content of CYP450s caused a significant decrease in the activities of CAT, GSH-Px, and SOD (Xing, 2013). The above studies suggested that CYP450 plays a role in regulating antioxidant defense. In this study, it was found that with the alteration of CYP450 expression, the transcriptome profile of the antioxidant system in chicken lungs also changed significantly. In qRT-PCR validation, GPX2 mRNA levels were significantly higher in TTO than that in AVG, while NOS2 and GSTA2 mRNA levels were slightly but not significantly increased. GSTA2 is an oxidative stress factor involved in the generation of GPX2 (Choi et al., 2023). The NOS2 gene is related to the production of NO, and the change in NO levels may affect the oxidative stress and immunity of the body (Klusek et al., 2024). The abnormal expression of GPX2 and GSTA2 genes may be due to free radicals leading to the synthesis of enzyme activities related to transcriptional reactions and altering the oxidative stress balance in the lungs. H_2S exposure significantly increased the levels of MDA, ROS, and H_2O_2, while the activities of CAT, SOD, GSH-Px, and T-AOC were significantly lower than those of the control group. However, lung antioxidant enzyme activities were significantly increased, and oxidative stress products were significantly reduced in chickens treated with TTO. This is mainly due to the metabolism of H_2S by CYP450 to produce ROS, and a large amount of ROS accumulates in the cell and cannot be removed, leading to the destruction of the antioxidant defense system, with the decrease in antioxidant enzyme activity and increase in oxidative stress products (Song et al., 2024). However, TTO treatment increased the activity of antioxidant enzymes and enhanced the scavenging capacity of the antioxidant system for ROS. Related studies have confirmed that terpinen-4-ol, the active ingredient in TTO, can activate the antioxidant system and clear ROS (Liu et al., 2021). TTO also enhanced antioxidant capacity by activating the Keap1-Nrf2 and Relish-Imd signaling pathways (Liu et al., 2022b). These results indicated that TTO can effectively treat the oxidative stress induced by H_2S metabolism by CYP450s.

Oxidative stress is a basic response characteristic of organisms to external stimuli (Noctor et al., 2016; Che et al., 2024). When the homeostasis imbalance of redox reaction is broken, it can cause a variety of adverse reactions in the body, including cell apoptosis, morphological damage and inflammatory response. Oxidative stress can induce the production of inflammatory cytokines, such as TNF-α, TGF-β (transforming growth factor beta), and PTGES (prostaglandin E synthase). The transcriptome profiles of inflammation-related genes were dramatically altered in AVG and TTG. In the present study, it was found that H_2S exposure activated the NF-κB pathway, resulting in an increase in the mRNA levels of NF-κB, TNF-α, IFN-γ, IL-4, and IL-16. However, the mRNA levels of TNF-α, NF-κB, IL-4, INF-γ, and IL-16 in lung tissues were significantly downregulated after TTO treatment. These data suggested that TTO alleviated H_2S-induced redox homeostasis disorder in the chicken lungs, which in turn reduced inflammation. In addition to inflammation, oxidative stress leads to immune dysfunction. IL-4 is a key factor involved in immune activity. Its main role is to stimulate the differentiation of B lymphocytes, and then induce the production of immunoglobulin E (**IgE**). IFN-γ acts as a major cytokine of Th1 cells. Studies have found that in birds, oxidative damage increased the mRNA levels of IL-4 and TNF-α, and decreased the mRNA levels of IFN-γ, leading to immunotoxicity (Yang et al., 2022). In chickens, it was found that H_2S exposure induced oxidative stress through the CYP450s/ROS pathway, which caused an increase in the mRNA levels of NF-κB, TNF-α, IL-10, VEGFC, and PTGES, and then damaged the immune system (Zheng et al., 2019). In mice, oxidative stress-induced immune dysfunction was mediated by the activation of NF-κB signaling pathway, which upregulated the expression of TNF-α, IFN-γ, and IL-4 (Tewari et al., 2015). As a chemotactic cytokine, IL-16 binds to CD4+ cells and affects the activation of T cells when the body is injured, thereby participating in the inflammatory response (Dorota Purzycka et al., 2016). The IL-16 cytokine can be used as a potential marker of disease activity and plays an important role in regulating cellular activity and immunity (Dorota Purzycka et al., 2016). The inflammatory response induced by H_2S is due to the activation of the NF-κB pathway (Sun et al., 2019), which leads to an increase in the IL-16 mRNA level. Previous studies have shown that TTO can regulate oxidative stress through the NF-κB signaling pathway (Liu et al., 2022b) and reduce the expression level of IL-16 (Zhan et al., 2020), which in turn suppresses inflammation. TTO has a significant effect on improving immunity. The levels of TNF-α and IL-12 in the guts of goats fed with TTO were significantly downregulated, and the expression level of IL-2 was significantly increased, leading to immunity (Lv et al., 2022). However, TTO was found to significantly reduce the content of NF-κB in the rumen of sheep, thereby inhibiting the expression of inflammatory cytokines (Hu et al., 2021).

This is mainly due to the fact that TTO reduces the proliferation of inflammatory cells without affecting their ability to secrete anti-inflammatory cytokines. Data from this study showed that TTO has a significant effect on treating inflammation and immune disorders caused by H_2S exposure.

Many environmental toxins are known to trigger apoptosis, and oxidative stress is one of them (Wang et al., 2024). Caspases are key genes or proteins required for apoptosis. FADD can act on Caspase-8, which activates Caspase-3, which in turn clears borane domain proteins, such as Bid, to amplify apoptotic signals and induce apoptosis (Jiang et al., 2022). When the redox balance is disrupted, it leads to enhanced activities of Bid, Caspase-3, and Caspase-8, which leads to the occurrence of apoptosis (Jiang et al., 2022). Oxidative stress activates Caspase-8/3 and FADD expression to induce apoptosis (Kang et al., 2022). This is consistent with the results of this study. However, the mRNA levels of FADD, Caspase-8, Caspase-3, Bid, and Fas in TTG were significantly lower than those in the AVG. These results indicated that TTO had a certain effect on inhibiting H_2S-induced apoptosis, corroborating previous findings that TTO can regulate cell apoptosis (Assmann et al., 2018). This also fully proves that the regulation of apoptosis by TTO via CYP450/ROS pathway is feasible.

In summary, the results of this study provide evidence that TTO treatment improves the changes in the transcriptomic profile of chicken lungs induced by H_2S. TTO can reduce the redox homeostasis disorder induced by H_2S metabolism by regulating the expression of CYP450 genes, thereby reducing morphological damage, inflammatory response, cell apoptosis, and immune dysfunction in chicken lungs.

CONCLUSIONS

Adding 0.02 mL/L TTO to chicken drinking water can alleviate inflammation, apoptosis, and oxidative stress disorder in chicken lungs caused by H_2S exposure through CYP450s/ROS pathway. These results demonstrate the feasibility of TTO in treating H_2S-induced lung injury through CYP450s/ROS pathway, and provide a new strategy for solving H_2S-induced respiratory diseases.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Yilei Liang: Conceptualization, Methodology, Data curation, Writing – review & editing. **Li Jiang:** Formal analysis, Data curation, Investigation. **Mao Hu:** Methodology, Investigation. **Xuegang Luo:** Supervision, Funding acquisition. **Tingting Cheng:** Methodology. **Yachao Wang:** Investigation, Writing – review & editing.

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DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

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