104 (2025) 105330

Contents lists available at ScienceDirect

Poultry Science

journal homepage: www.elsevier.com/locate/psj

Dietary resveratrol alleviates liver and **intestinal** injury in ducks under cage rearing system

Yong Tian [a,b], Xiangshan Liu [a], Li Chen [a,b], Tao Zeng [a,b], Tiantian Gu [a,b], Wenwu Xu [a,b], Jindong Ren [a,b], Lizhi Lu [a,b,*]

[a] State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-products, Key Laboratory of Livestock and Poultry Resources (Poultry) Evaluation and Utilization, Ministry of Agriculture and Rural Affairs, Institute of Animal Science & Veterinary, Zhejiang Academy of Agricultural Sciences, Hangzhou, 310021, China

[b] Zhejiang Provincial Key Laboratory of Livestock and Poultry Biotech Breeding, Hangzhou, 310021, China

ARTICLE INFO

Key words:
Resveratrol
Cage rearing
Duck
Oxidative stress
Inflammation

ABSTRACT

Cage rearing is a promising farming method. However, our previous studies have demonstrated that changes in farming practices induce oxidative stress and inflammation in the liver and duodenum of ducks. Resveratrol (RES), a natural plant polyphenol, possesses antioxidant, anti-inflammatory, and cytoprotective properties. This study evaluated the alleviating effects of RES against cage-rearing-induced duck health problems, emphasizing the involvement of redox imbalance, inflammatory response, endoplasmic reticulum (ER) stress, apoptosis, and PI3K/AKT and MAPK/ERK pathways. A total of 120 healthy 12-week-old female ducks were transferred to a cage system and randomly assigned to two dietary RES groups with 6 replicates each (10 ducks per replicate), including basal diet + 0 mg/kg RES (control group, CON), and basal diet + 500 mg/kg RES (RES-treated group, RES). During the early stages (within 10 days) of cage rearing, blood, liver, and duodenal samples were collected for analysis. The results demonstrated that RES reduced histopathological damage in the liver and duodenum of cage-reared ducks. It also reduced serum albumin levels, increased serum aspartate aminotransferase and alanine aminotransferase levels, and enhanced antioxidant (increased CAT, GSH-Px, SOD, and T-AOC activities in the serum, liver, and duodenum, and reduced the increase in MDA) and anti-inflammatory properties (reduced pro-inflammatory cytokines interleukin (IL)-1β and IL-6 secretion and increased anti-inflammatory cytokine IL-4 levels). Additionally, quantitative real-time polymerase chain reaction revealed that RES intervention reversed the abnormal mRNA abundance of biomarkers associated with inflammatory injury (iNOS and COX2) in the liver, and ER stress (GRP78) and apoptosis (Bax and Bcl2) in the liver and duodenum of cage-reared ducks. Further analysis of key proteins in the PI3K/AKT and ERK MAPK signaling pathways revealed that RES promoted AKT phosphorylation in the liver and duodenum of cage-reared ducks and reduced cleaved caspase-3 protein content. Overall, RES prevents cage-rearing stimuli-induced liver and intestinal injury in ducks by enhancing liver function, improving antioxidant properties, inhibiting inflammation, ER stress, and apoptosis, and activating the PI3K/AKT signaling pathway.

Introduction

The rearing population of laying ducks in China is large, accounting for approximately 90% of the world's total, and the number of laying ducks raised in 2023 was approximately 150 million (China Waterfowl Industry Technology Research System). Traditional duck rearing mode is based on water surface grazing, which has many disadvantages, such as environmental pollution, low economic benefits, high disease incidence, and poor product quality (Liang, 2016; Zhao et al., 2016). As environmental pressure continues to increase, the impact of environmental constraints on duck farming will become more obvious. From the perspective of development, the cage-rearing mode will become dominant, which can promote the rapid development of large-scale duck farming. It was found that laying ducks are usually 70-90 days of age; therefore, changing from flat farming to cage rearing can improve egg production and reduce the feed-to-egg ratio and eggshell breakage rate

Resveratrol alleviates damage in caged ducks

* Corresponding author.
E-mail address: lulizhibox@163.com (L. Lu).

https://doi.org/10.1016/j.psj.2025.105330
Received 4 March 2025; Accepted 21 May 2025
Available online 22 May 2025

(Zhao et al., 2016). However, laying ducks are timid by nature, and any changes in feeding and management factors can cause a stress response in the body, resulting in slow growth, decreased production performance, immunity, and product quality, and even death. Our previous studies have confirmed that ducks are highly susceptible to strong stress reactions in the early stages of cage rearing, leading to tissue damage in the liver and the duodenum (Zhang et al., 2019; Gu et al., 2019; Gu et al., 2020).

Inflammation is the body's defense response to adverse stress-induced tissue damage, helping to eliminate harmful substances and repair damaged tissue; however, excessive or prolonged inflammation may cause damage to the body (Medzhitov, 2021; Figus et al., 2021). We have reported that cage stress induces increased expression of inflammatory cytokines in ducks, impairs the body's antioxidant capacity, and causes tissue damage (Zhang et al., 2019; Gu et al., 2019). Moreover, the endoplasmic reticulum (ER) stress marker GRP78 significantly increased following tissue injury (Zeng et al., 2023; Ma et al., 2024). Our previous study also confirmed that caged rearing increased the ER stress genes GRP78 expression levels in the duck liver (Zhang et al., 2019). It has been suggested that ER stress is closely related to apoptosis. Early malnutrition-induced ovarian follicle reduction in adult rats may be mediated by increased ovarian ER stress in a manner that increases follicular apoptosis (Chan et al., 2015). Chronic heat stress enhanced ER stress that induced hepatocyte apoptosis, and caused liver injury (Ma et al., 2022). In addition, hypoxic stress impairs the antioxidative capacity of the liver and induces oxidative injury and apoptosis through XIAP-p53-bax-bcl2 and caspase-dependent pathways, but enhances host immunity and maintains homeostasis in the black rockfish *in vivo* by regulating nonspecific immune indicators and related gene expression (Jia et al., 2023). Thus, manipulating antioxidant activity, inflammation, ER stress, and apoptosis may effectively alleviate oxidative damage in laying ducks caused by adverse stimuli, including changes in rearing practices.

Plant polyphenols are a class of secondary metabolites with a polyphenolic structure, which are widely found in plants and have potential health benefits (Behl et al., 2020; Del Bo' et al., 2021; Rinott et al., 2022). Resveratrol (3,4′,5-trihydroxystilbene, RES) is a non-flavonoid polyphenolic compound that is a phytoalexin produced by various plants and is abundant in grapes, wines, peanuts, soybeans, berries, and *Polygonum cuspidatum*. RES has been reported to have several biological effects, including anti-inflammatory activity (Omraninava et al., 2021) and antioxidant capacity (Blanton et al., 2023), as well as the ability to modulate signaling pathways (Cui et al., 2022; Zhu et al., 2023) and control cellular metabolism (Timmers et al., 2011), autophagy (Chang et al., 2017), proliferation, and apoptosis (Chen et al., 2020). It has been well established that PI3K/AKT and MAPK/ERK are important signal transduction pathways mediating apoptosis, growth and cell survival. RES can inhibit cardiomyocyte apoptosis, thus attenuating coronary microembolization-induced myocardial injury by activating the PI3K/Akt pathway (Li et al., 2021). Findings *in vitro* and *in vivo* have suggested that RES treatment potentiated human umbilical cord-derived mesenchymal stem cells repair for cisplatin-induced acute kidney injury, which was achieved by triggering the ERK signaling pathway and inhibiting renal tubular cell apoptosis (Zhang et al., 2018).

RES, as a natural antioxidant, has been widely used in animal production due to its multiple targets and few side effects (Meng et al., 2023). However, the role of RES in the liver and intestinal damage in caged ducks remains unclear. In this study, we investigated the protective effects of RES against cage stress-induced liver and intestinal injury and explored its mechanisms by conducting *in vivo* studies focusing on the duck liver and duodenum. These results may provide a theoretical basis for developing RES as a target drug for treating tissue damage induced by cage rearing in ducks.

Materials and methods

Ethical statement

All animal experimental procedures in this study were carried out in accordance with the national standard of Laboratory animal−Guideline for ethical review of animal welfare (GB/T 35892-2018, General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China, Beijing, China) and approved by the Institute of Animal Husbandry & Veterinary Science, Zhejiang Academy of Agricultural Sciences (Hangzhou, China).

Animals, treatments, and management

The experimental animals were female Shaoxing ducks (*Anas platyrhynchos*) provided by Hubei Shendan Health Food Co., Ltd. (Xiaogan, China). The ducks were kept in a floor-rearing system before the experiment. At 12 weeks of age, all birds were transferred to a cage feeding system to begin the formal trial. A total of 120 healthy ducks were randomly allocated to one of the two dietary treatment groups, with six replicates per treatment and 10 birds per replicate. Dietary treatments included a control basal diet without additives (CON) and the basal diet supplemented with 500 mg/kg of resveratrol (RES). The basal diet was formulated following the national standard GB/T 41189-2021 (State Administration for Market Regulation and Standardization Administration of China, Beijing, China). RES additive was provided by Hubei Nongteng Feed Co., Ltd. (Xiaogan, China). During the early stage of cage rearing (experimental period: 0–10 days), ducks were observed daily for mental status, activity, fecal pattern and color, food intake, and water intake.

The ducks were housed in three-tier battery cages (length × width × height, 28 × 40 × 40 cm), with two birds per cage, and each cage was equipped with a feed trough and nipple drinker. Diets and water were available *ad libitum*. The room temperature was maintained at 21 ± 2 °C, and the relative humidity was approximately 55%. Throughout the trial, all ducks were subjected to a daily lighting regimen of 12 h light and 12 h dark (12L: 12D), and the ducks did not receive any vaccinations or antibiotic treatments.

Sample collection and processing

On days 2, 4, 7, and 10 of cage rearing, six ducks from each group (one per replicate) were selected, and blood was collected aseptically from the wing vein. Blood samples were centrifuged, and serum was separated and stored at –20 °C for subsequent analysis. The ducks selected at the last four sampling time points (2, 4, 7, and 10 days) were then sacrificed. The ducks were euthanized by injection of sodium pentobarbital (150 mg/kg). The abdominal cavity of each duck was opened immediately, and samples of the liver and duodenum (2 cm posterior to the gizzard) were collected. All liver and duodenum samples were divided into two parts; one part was fixed in 4% paraformaldehyde solution for histological analysis, and the other was immediately transferred to liquid nitrogen and then stored at −80 °C until analysis.

Histopathologic analysis

For histopathological analysis, the liver and duodenal tissues fixed in 4% paraformaldehyde solution were embedded in paraffin, and serial sections of 4–5 μm thickness were prepared and stained with hematoxylin and eosin (HE) according to standard protocols. The sections were analyzed under an Olympus light microscope (Tokyo, Japan) to detect any evidence of tissue damage. According to the degree of the lesion from mild to severe, no lesion or very small amount was marked as 0; mild or small amount was marked as 1; moderate or medium amount was marked as 2; severe or multiple amount was marked as 3; and very severe or large amount was marked as 4.

Liver function test

Liver function indicators were determined in serum using commercial kits (Biosino Biotechnology and Science Inc., China) on a Mindray BS420 Fully-Auto Biochemistry Analyzer (Shenzhen Mindray Bio-Medical Electronics Co., Ltd., China) using the colorimetric method. The indices measured included total protein (TP), albumin (ALB), globulin (GLB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (TBIL), as well as the ALB-to-GLB and AST-to-ALT ratios.

Determination of serum inflammatory cytokine levels

Three inflammatory cytokines, interleukin (IL)-1β, IL-6, and IL-4, were analyzed in the serum using commercial kits (Beijing Sino-UK Institute of Biological Technology, China), following the manufacturer's instructions. The levels of these cytokines were measured using a colorimetric assay with an A-6 Semi-automatic biochemical analyzer (Beijing Shining Sun Technology Co., Ltd., China).

Antioxidant capacity measurement

Antioxidant indices were assayed simultaneously in serum, liver, and intestinal samples using an A-6 Semi-automatic biochemical analyzer (Beijing Shining Sun Technology Co., Ltd., China). Antioxidant capacity, including catalase (CAT), glutathione peroxidase (GSH-Px), total antioxidant capacity (T-AOC), superoxide dismutase (SOD), and malondialdehyde (MDA) levels, were measured using commercial kits following the manufacturer's protocols (Beijing Sino-UK Institute of Biological Technology, China).

RNA extraction and real-time qPCR

Total RNA was isolated from the liver and intestinal samples using TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. Total RNA was reverse-transcribed into cDNA for qPCR using SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen, USA) following the manufacturer's protocol.

Real-time PCR assays were performed to determine mRNA expression using Power SYBR® Green PCR Master Mix (Applied Biosystems, USA) on a CFX384 Touch^{TM} Real-Time PCR Detection System (Bio-Rad, USA). The primer pairs used in this experiment are listed in Table 1. *GAPDH* served as an internal reference gene for normalization. The qPCR was performed in a 20 μL reaction mixture containing 10 μL of Power SYBR® Green Master Mix (Applied Biosystems, USA), 1.0 μL of template cDNA, 0.5 μL of each primer (10 μM), and 8 μL of ddH_2O. The reaction mixtures were incubated in a 96-well plate at 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s and 63 °C for 25 s. At the end of the amplification cycle, a melting curve analysis was performed to confirm the specificity of the amplification. All reactions were conducted in triplicate. The relative expression levels of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot assay

Proteins from the liver and intestinal samples were obtained using a tissue protein extraction reagent (Thermo Fisher Scientific, USA), and protein concentrations were determined using a BCA Protein Assay Kit (Beyotime, China) following the manufacturer's instructions. Protein samples were separated by SDS-PAGE, transferred to PVDF membranes (Millipore, USA), blocked in TBST containing 5% nonfat milk, and incubated with anti-p-AKT (1:1,000; Sigma, USA), -AKT (1:1,000; CST, USA), -p-ERK1/2 (1:1,000; Sigma, USA), -ERK1/2 (1:1,000; Sigma, USA), -cleaved caspase-3 (1:1,000; Abcam, England), and -GAPDH (1:10,000; Abcam, England) antibodies overnight at 4 °C. GAPDH was served as the loading control. Membranes were then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:5000; Thermo Fisher Scientific, USA), HRP-conjugated anti-rabbit IgG (1:5000; Thermo Fisher Scientific, USA), and HRP-conjugated anti-goat (1:5000; Thermo Fisher Scientific, USA) at room temperature for 1 h. The ECL working solution prepared with SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific, USA) was used for visualization. Protein levels were analyzed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA) and normalized to the relative density of the control group. The ratio of phosphorylated protein to total protein was used to evaluate the phosphorylation levels.

Statistical analysis

Statistical Package for Social Sciences software (SPSS Inc., Chicago, IL, USA; version 26.0) was used for all data processing and analyses. Data are presented as the mean ± standard deviation (SD). An independent sample *t*-test was used for comparisons between two groups, and a one-way analysis of variance was used for multiple comparisons. A $P < 0.05$ was considered statistically significant. All graphs with standard deviation bars were generated using GraphPad Prism software (GraphPad Software, San Diego, CA, USA; version 9.0).

Results

Hepatic and intestinal morphology

A histological analysis was performed to determine the effects in the tissues of caged ducks fed diets supplemented with RES, and the morphological changes of liver and duodenum are presented in Figs. 1A and 1B, respectively. The results indicated that duck liver tissue exhibited a certain degree of damage at the initial stage of cage rearing, and the damage was aggravated with the extension of time, particularly on the 7th day; inflammatory cell infiltration was obvious and displayed hepatocyte degeneration and cell vacuolization. And the liver tissue damage was markedly ameliorated in ducks fed RES-containing diets compared to those fed basal diets (Fig. 1A). The microstructure of

Table 1
Primer sequences and product lengths of target gene fragments.

Genes	Accession number	Primer sequence (5′-3′)		Product length (bp)
iNOS	FJ966247.1	F:	GCTCCTTGAGGTGGGAGGTCT	92
		R:	CATCACAGAAGTCTCGCACTCCTAT	
COX2	XM_005015351.3	F:	GTGTTTGGTTTGGTCCCAGGTT	100
		R:	CCCACTCTGGATGCTCCTGTTT	
GRP78	XM_005024444.2	F:	CGATGCCAAGCGGCTCATA	128
		R:	GTCCACCGCCAACATCAACT	
Bax	KY788660.1	F:	GGGACGAGCTGGACAGCAA	185
		R:	CTGCGAGAACAGAGCCTTGATG	
Bcl2	XM_027451677.1	F:	CTGGATGACCGAGTACCTGAAC	159
		R:	GCTCCCACCAGAACCAAACTC	
GAPDH	XM_005016745.2	F:	GGAGCTGCCCAGAACATTATC	141
		R:	GCAGGTCAGGTCCACGACA	

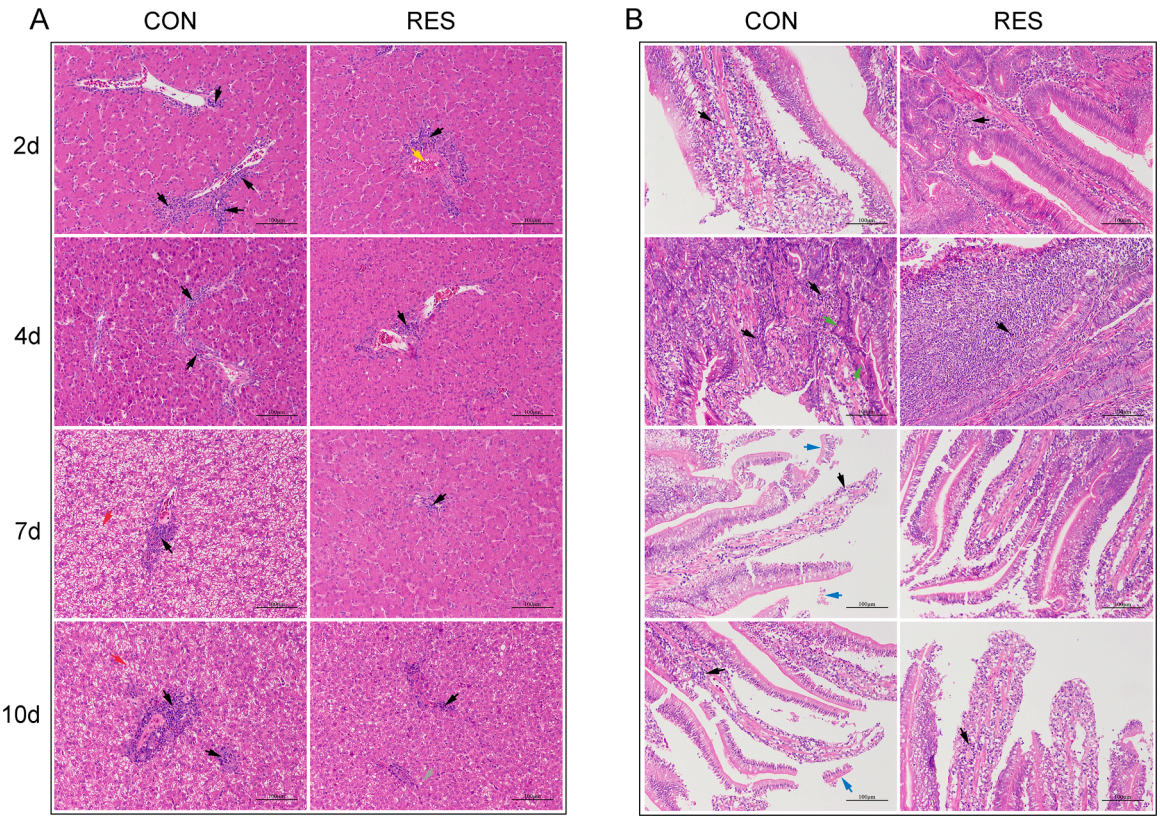

Fig. 1. Representative photomicrographs of duck liver (A) and intestine (B) tissue sections (HE staining; magnification, 200 ×; scale bars, 100 μm). Inflammatory cell infiltration (black arrow); congestion (yellow arrow); hepatocyte degeneration with swollen cytosol, and loose and lightly stained cytoplasm (red arrow); cytoplasmic vacuolization (gray arrow); necrosis (green arrow); epithelial cell detachment (blue arrow).

intestine illustrated that duodenal damage was also present in the control group of caged ducks fed with a basal diet, as evidenced mainly by mucosal detachment and inflammatory cell infiltration, which were particularly severe on the 7th and 10th days after the implementation of the cage rearing system, while the intestinal damage was markedly improved in caged ducks fed the RES-containing diet (Fig. 1B).

Biochemical changes in liver function

To monitor changes in liver function-related serum biochemical indices of ducks fed dietary RES during the early stage of cage rearing, blood samples were collected at 2, 4, 7, and 10 days after the formal feeding experiment. The effects of a feeding diet supplemented with RES on the liver function of ducks reared in a cage system are presented in Fig. 2. Certain serum biochemical parameters, such as TP, ALB, GLB, ratio of ALB to GLB, AST, ALT, and ratio of AST to ALT, varied between

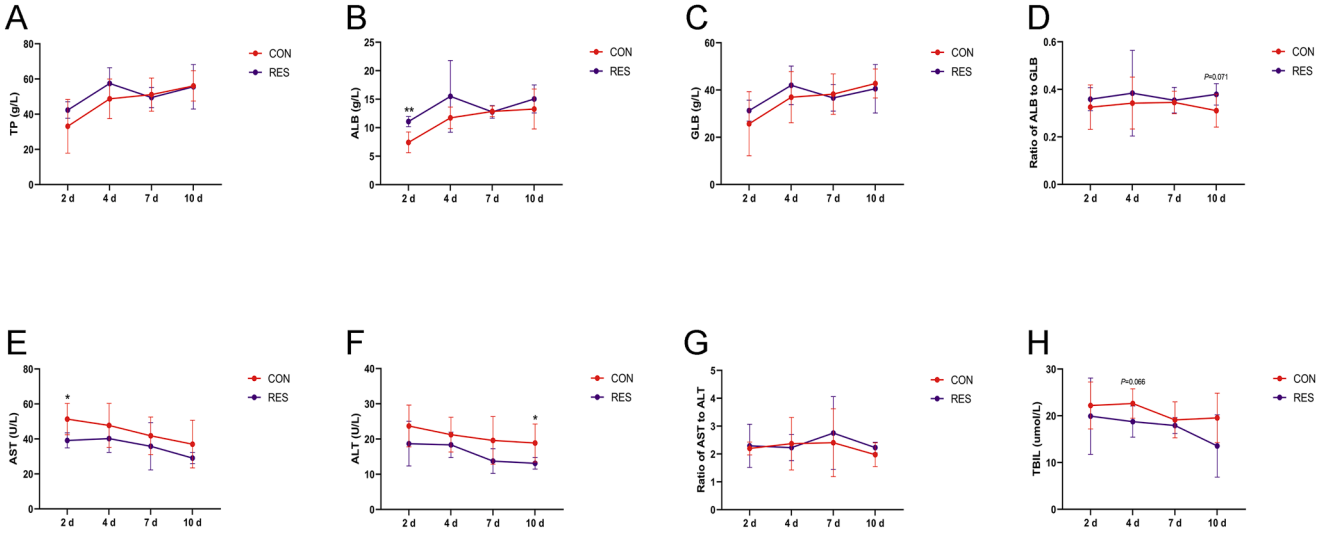

Fig. 2. Effects of dietary RES supplementation on liver function in ducks reared in the cage system. Difference analysis was performed using an independent sample *t*-test between CON and RES groups for the same time of cage rearing. $^{*}P < 0.05$ and $^{**}P < 0.01$. Values are expressed as the mean ± SD (n = 6).

the CON and RES groups. RES significantly increased ALB levels in ducks on day 2 after cage rearing ($P < 0.01$), and also tended to increase the ratio of ALB to GLB in ducks on day 10, yet the difference was not statistically significant ($P = 0.071$). The serum levels of AST on day 2 and ALT on day 10 were significantly lower in the RES group than in the CON group ($P < 0.05$). RES also decreased serum TBIL levels on day 4, but this result was non-statistically significant ($P = 0.066$).

Antioxidant status

To characterize the effects of RES on the antioxidant capacity of caged ducks, serum CAT, GSH-Px, SOD and T-AOC activity, and MDA levels were determined. As presented in Figs. 3A-3E, RES improved antioxidant indices in serum sample from cage-reared ducks. Compared to the basal diet group, RES supplementation significantly increased CAT levels on days 4, 7, and 10 of cage rearing ($P < 0.01$), and also increased CAT level on day 2, but there was a non-significant difference ($P = 0.077$). GSH-Px levels were significantly higher in the RES group than in the control group on days 7 and 10 of cage rearing ($P < 0.01$), and a similar trend was observed on day 4, yet this difference was not significant ($P = 0.064$). Compared with the control group, the SOD level in the RES group was significantly increased on days 2, 4, and 10 ($P < 0.01$). Dietary RES supplementation significantly increased T-AOC levels in ducks on days 2 ($P < 0.01$) and 10 ($P < 0.05$) of cage rearing compared to ducks fed a basal diet. The MDA level in the RES group was significantly lower than in the control group ($P < 0.01$). Further analysis revealed that with the extension of cage rearing, the change trends of antioxidant indexes in both groups were consistent; specifically, CAT, GSH-Px, and SOD levels depicted an increasing trend. Besides, T-AOC first increased slowly and then decreased to the initial level, while MDA levels exhibited a significant decreasing trend.

The effects of dietary RES supplementation on the antioxidant capacity of duck hepatic tissues are demonstrated in Figs. 3F-3J. Compared with the control group, CAT activity in the liver tissues of the RES group was significantly elevated on days 2 ($P < 0.01$), 4 ($P < 0.05$), 7 ($P < 0.01$), and 10 ($P < 0.01$) of cage rearing, and similar results were found in the comparisons of GSH-Px activity between the two groups. SOD activity in liver tissues of ducks fed the RES-supplemented diet on days 2 ($P < 0.01$), 4 ($P < 0.01$), and 10 ($P < 0.05$), and T-AOC activity on days 4 ($P < 0.01$) and 7 ($P < 0.05$) were significantly higher than those of the control ducks. RES also reduced the levels of oxidative damage products (MDA) in the liver and reached a significant level on day 4 ($P < 0.05$).

The activity of antioxidative enzymes was detected in the intestinal tissue of ducks after feeding with RES (Figs. 3K-3O). The intestinal CAT, GSH-Px, and SOD activities were significantly elevated on days 2, 4, 7, and 10 of cage rearing in RES-fed ducks compared to the basal diet group ($P < 0.01$). RES significantly increased the T-AOC activity of intestine tissues in caged ducks on days 4, 7, and 10 ($P < 0.01$), and partially increased its activity on day 2, though the result was not statistically significant ($P = 0.059$). The intestinal MDA levels in ducks fed RES were significantly lower than in the control group ($P < 0.01$).

Serum inflammatory cytokines

The results of the assay for RES-induced cytokine changes in caged ducks are demonstrated in Fig. 4. RES significantly reduced IL-1β levels on days 2 ($P < 0.01$) and 7 ($P < 0.05$) of cage rearing, and reduced its level on day 10, although the difference was not significant ($P = 0.074$). RES significantly decreased IL-6 levels in caged ducks on days 2 ($P < 0.01$), 4 ($P < 0.05$), and 7 ($P < 0.05$); and increased IL-4 levels on days 2 ($P < 0.01$), 7 ($P < 0.01$), and 10 ($P < 0.05$), as well as increasing IL-4 level on day 4, but this result was not statistically significant ($P = 0.064$).

mRNA expressions of inflammatory factors and ER stress-related genes

To evaluate the effects of dietary RES on markers associated with inflammatory mediators (iNOS and COX2) and ER stress (GRP78), the mRNA expression levels of these three genes in the liver and intestine of cage-reared ducks were examined using qRT-PCR. Our results demonstrated that the mRNA expression levels of liver iNOS and COX2 gene on

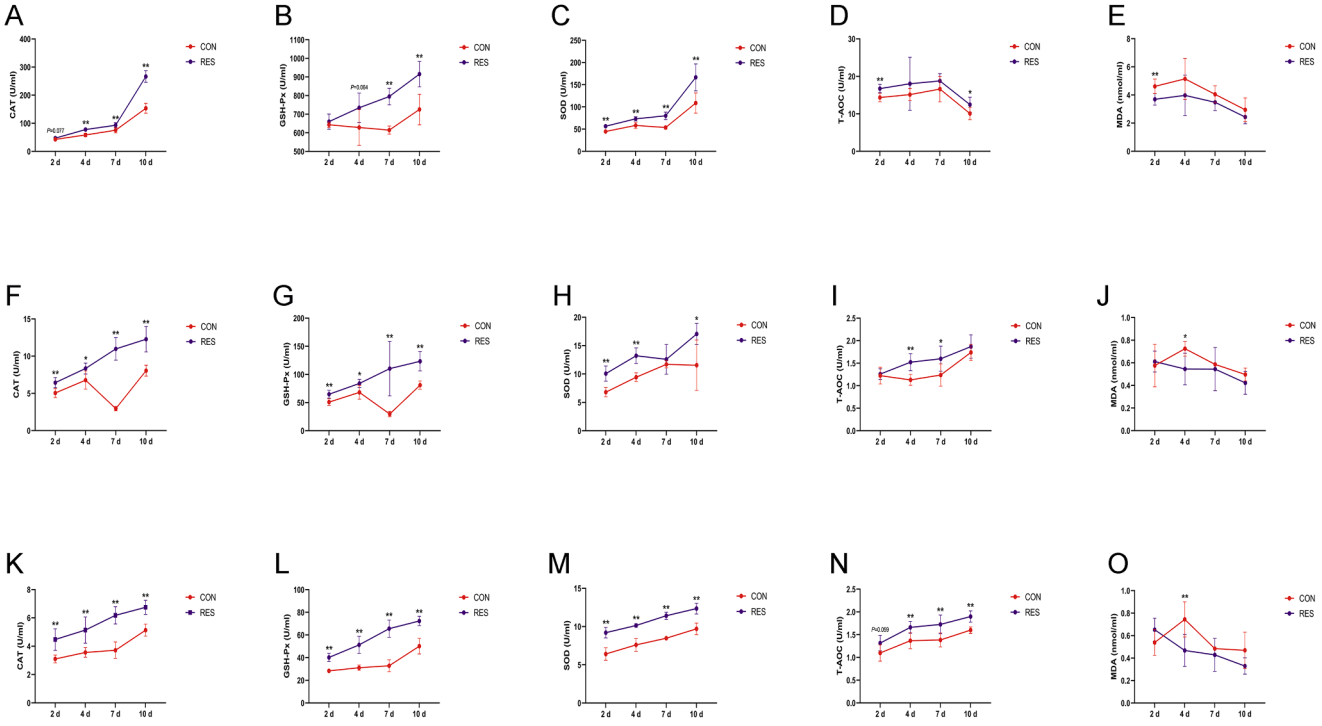

Fig. 3. Effects of RES on antioxidant levels in cage-reared ducks. (A–E) Serum antioxidant indices, (F–J) liver antioxidant indices, and (K–O) intestinal tissue antioxidant indices. Difference analysis was performed using an independent sample *t*-test between CON and RES groups for the same time of cage rearing. *$P < 0.05$ and **$P < 0.01$. Values are expressed as the mean ± SD (n = 6).

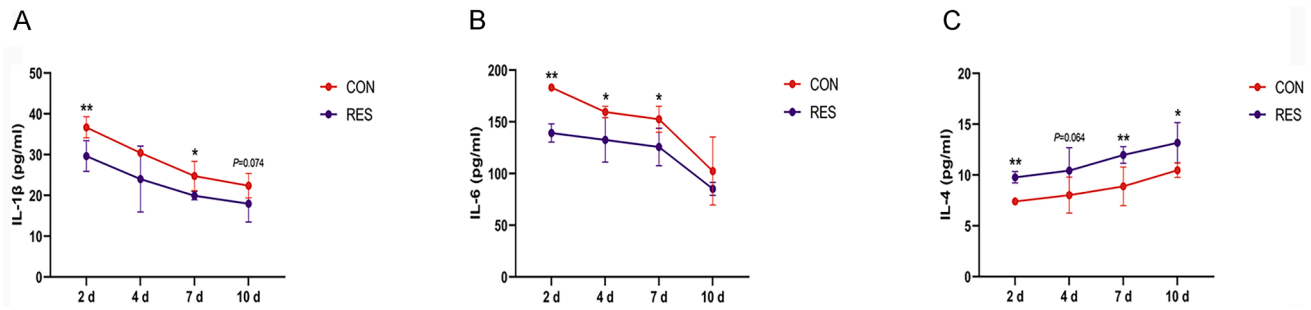

Fig. 4. Serum cytokine level of cage-reared ducks with RES supplementation. Difference analysis was performed using an independent sample *t*-test between CON and RES groups for the same time of cage rearing. $^{*}P < 0.05$ and $^{**}P < 0.01$. Values are expressed as the mean ± SD (n = 6).

day 2 ($P < 0.01$) and liver GRP78 gene on days 2 ($P < 0.01$) and 4 ($P < 0.05$) in the RES group were significantly lower than those in the control group (Figs. 5A-5C). For intestinal tissues, on the 7th day of cage rearing, the mRNA expression level of the GRP78 gene in ducks fed diets supplemented with RES was significantly lower than that in ducks fed basic diets ($P < 0.01$). In contrast, there was a statistically non-significant difference in the relative mRNA expression levels of iNOS and COX2 genes between the RES and CON groups (Figs. 5D-5F).

mRNA expression levels of apoptosis-related genes

To determine the effects of RES on apoptosis-related factors, Bax and Bcl2 gene expression was measured by qRT-PCR in the liver on day 2 and the intestine on day 7 after cage rearing of ducks. As illustrated in Figs. 6A-6B, we found that the levels of Bax mRNA in the liver of ducks in the RES group were lower than those in the control group, although this result was not significant ($P = 0.07$), while the difference in Bcl2 mRNA expression between these two groups was non-statistically significant. Similarily, compared with the control ducks, the expression of Bax mRNA was significantly decreased in the intestine of ducks fed diets supplemented with RES ($P < 0.05$), while the mRNA levels of duck intestinal Bcl2 were non-significantly changed in the RES group when compared with those in the control group (Figs. 6C-6D).

Effects of dietary RES on AKT and ERK1/2 pathways in caged ducks

To explore the involvement of AKT and ERK signaling molecules in RES amelioration of tissue injury in caged ducks, a western blot assay was performed to detect the phosphorylation status of AKT and ERK1/2 and the expression of activated caspase-3. The results demonstrated that RES supplementation increased p-AKT and p-AKT/AKT in the liver and p-AKT and AKT in the intestine, compared with the basal diet. Furthermore, RES supplementation decreased the level of cleaved caspase-3 in the liver, and the same trend was observed in the intestine. However, no changes in the protein phosphorylation levels of ERK were observed (Fig. 7). These data indicate that RES may promote cell survival by activating the AKT pathway, thereby ameliorating liver and intestinal injury in cage-reared ducks.

Discussion

Inflammation underlies a wide range of physiological and

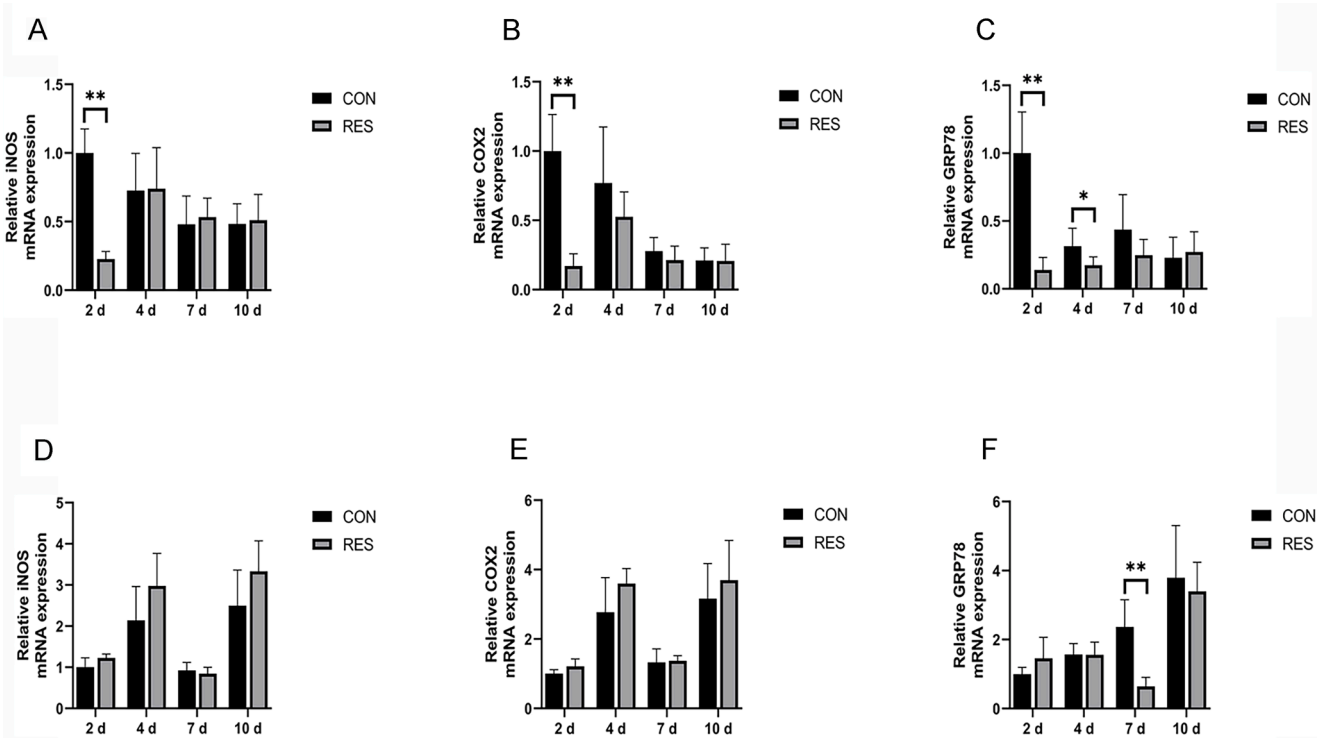

Fig. 5. Effects of dietary RES supplementation on mRNA expression levels of iNOS, COX2, and GRP78 genes in cage-reared ducks. (A–C) liver. (D–F) duodenum. Difference analysis was performed using an independent sample *t*-test. $^{*}P < 0.05$ and $^{**}P < 0.01$. Values are expressed as the mean ± SD (n = 6).

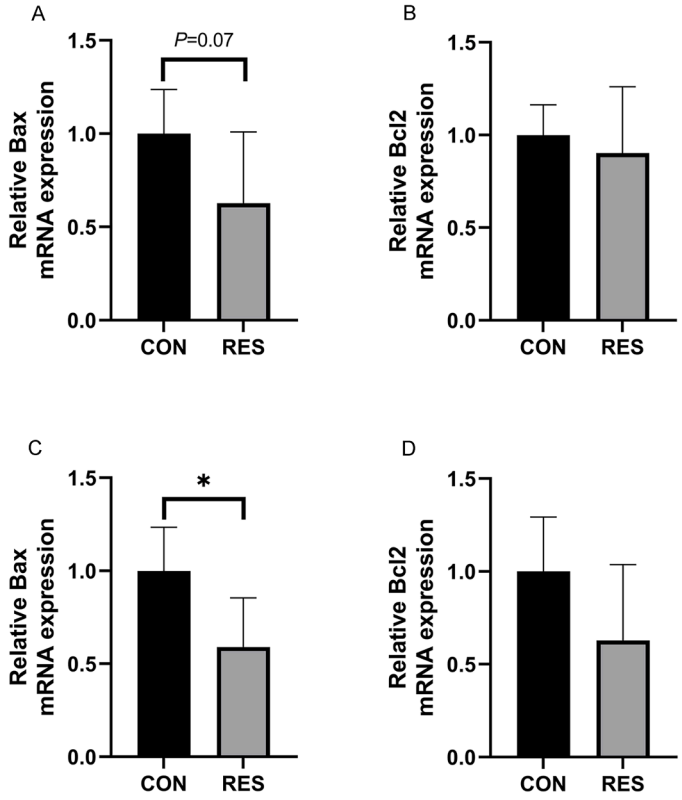

Fig. 6. Effects of dietary RES supplementation on mRNA expression levels of Bax and Bcl2 genes in cage-reared ducks. (A-B) liver. (C-D) duodenum. Difference analysis was performed using an independent sample *t*-test. $^{*}P < 0.05$ and $^{**}P < 0.01$. Values are expressed as the mean ± SD (n = 6).

pathological processes typically triggered by infection and/or tissue damage, and various harmful stimuli can cause oxidative damage to the body. Accordingly, injury to an organism is usually accompanied by an inflammatory response, and RES is proven to improve liver damage caused by a variety of factors. Dietary RES supplementation has been reported to reduce inflammatory cell infiltration and attenuate liver oxidative damage induced by chronic heat stress in broilers (Ding et al., 2023). Che et al. (2020) found that RES prevented liver damage in methionine-choline deficiency-induced steatohepatitis mice by promoting the gene transcription that negatively regulates inflammatory cytokine expression. Notably, RES has a potential role in enhancing lipid metabolism and protecting against liver damage. Milton-Laskibar et al. (2022) investigated the effects of RES on preventing fattening diet-induced liver injury and found that RES ameliorated dietary hepatic steatosis and hepatic inflammation by histopathological analysis. Researchers fed RES-added high-fat diet to male rats, which were a model of metabolic dysfunction-associated steatotic liver disease (MASLD) prepared using a high-fat diet, and found that the compound reduced the development of histological steatosis in the liver (Heebøll et al., 2016). Consistent with these findings, HE staining of duck liver tissue from the normal control group exhibited aggravated inflammatory cell infiltration and hepatocellular degeneration with the extension of cage rearing time in the present experiment, whereas liver morphology of ducks in the RES group was significantly improved.

With reference to human serological characteristics and liver function tests (Gong et al., 2023), eight indicators, namely AST, ALT, AST/ALT, TBIL, TP, ALB, GLB, and ALB/GLB, were filtered as evaluation indices for the effects of RES on liver function in ducks reared with the cage system. AST is widely expressed in the mitochondria of hepatocytes, whereas ALT is mainly found in the cytoplasm of liver cells, and AST and ALT are released into the blood if hepatocytes are damaged. TBIL is the sum of direct and indirect bilirubin levels, which is an important indicator of the severity of jaundice. ALB synthesis is an essential function of the liver, and its ability to synthesize proteins declines with hepatocellular injury, resulting in decreased serum TP and

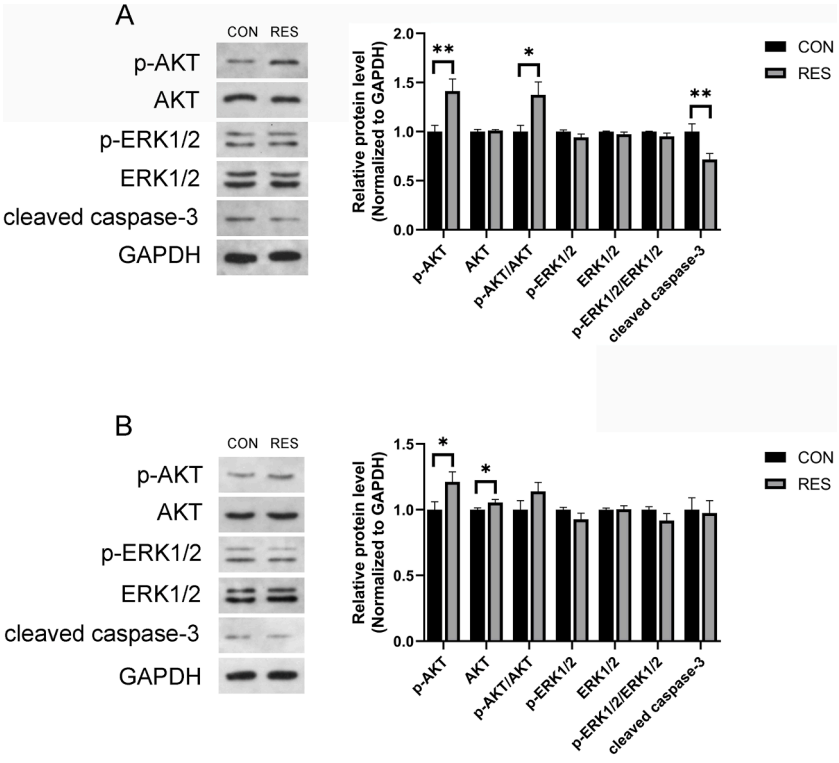

Fig. 7. RES induces AKT phosphorylation and suppresses specific cleavage of caspase-3 in the liver (A) and intestine (B) in cage-reared ducks. Difference analysis was performed using an independent sample *t*-test. $^{*}P < 0.05$ and $^{**}P < 0.01$. Values are expressed as the mean ± SD (n = 3).

ALB levels (Zhu and Han, 2015). As expected, we found an overall decreasing trend in serum ALT, AST, and TBIL, along with increases in TP, ALB, and GLB, in ducks fed RES-supplemented diets compared to those fed a normal ration under cage system farming conditions, suggesting that RES could reduce cage rearing-induced hepatic injury in ducks.

The intestine is the largest immune organ in animals and is also the main digestive, absorption, and metabolism site of the body. Maintaining intestinal homeostasis is essential for maintaining animal health. The gut is among the earliest involved and most sensitive organs at the onset of the stress response and is also the most critical peripheral target organ (Liu et al., 2021). Adverse stimuli, also known as stressors, can impair the integrity and function of the intestinal mucosal barrier, leading to increased permeability, which seriously affects intestinal health and is an important causative factor in various diseases (Aleman et al., 2023). Therefore, identification of appropriate natural antioxidants that can effectively ameliorate intestinal damage caused by cage rearing in ducks is essential for managing oxidative stress-induced diseases. The alleviating effect of RES on intestinal injury caused by different factors has been confirmed in many cases. RES treatment has been reported to attenuate methotrexate or radiation-induced intestinal injury at a histopathological level (Yulug et al., 2015; Zhang et al., 2017; Radwan and Karam, 2020). Wang et al. (2023) found that RES alleviated intestinal ischemia-reperfusion injury via *in vivo* and *in vitro* experiments. Zhuang et al. (2019) demonstrated that RES could protect IPEC-J2 cells from oxidative stress, suggesting that RES may be an effective feed additive against intestinal damage in livestock production. These reports strongly supported the effective protective role of RES against intestinal injury caused by various unfavorable factors. In this study, our histopathological assessment of the duodenum of ducks reared in cages revealed that the duodenum exhibited some tissue damage as the stress time increased, as evidenced by the shedding of intestinal epithelial cells, accompanied by more inflammatory cellular symptoms, and that the inflammatory response and tissue damage in the duodenal tissues of ducks reared in cages were significantly mitigated by RES supplementation in the diets.

Oxidative stress is closely related to inflammation and has long been recognized as a key contributor to various tissue injuries (Rocca et al., 2020; Zou et al., 2022; Li et al., 2023; Lu et al., 2023). Our previous work has also found that tissue damage caused by cage rearing in laying ducks is related to the reduced antioxidant capacity, as well as inflammation (Zhang et al., 2019; Gu et al., 2019; Gu et al., 2020). CAT, SOD and GSH-Px are important antioxidant enzymes, and T-AOC is an important indicator for assessing the overall function of the antioxidant defense system (Jomova et al., 2024). Concurrently, MDA is a signature product of lipid peroxidation, which is an important indicator for evaluating the level of lipid peroxidation and the extent of oxidative damage in tissues. In the current work, RES decreased the serum, liver, and intestinal oxidative stress-related indices CAT, GSH-px, SOD, and T-AOC levels and increased MDA content in cage-reared ducks, indicating that cage-rearing-induced tissue oxidative damage was significantly reversed by RES treatment. This finding is consistent with previous reports that RES has strong antioxidant properties (Zhang et al., 2015; He et al., 2022; Wang et al., 2024). Additionally, RES has been demonstrated to be a promising therapeutic agent for various inflammatory diseases. As the cytotoxic effects of adverse stimuli are believed to be a consequence of cytokine cascade responses, RES with anti-inflammatory properties may have a potential role in attenuating stress-mediated injury. It has been reported that RES administration could remarkably inhibit inflammatory responses in the liver and intestinal injuries caused by multiple factors (Wang et al., 2014; Radwan and Karam, 2020; Kasprzak-Drozd et al., 2024). The analysis of serum inflammatory cytokines in this experiment exhibited that the levels of pro-inflammatory cytokines IL-1β and IL-6 were remarkably depressed in the RES group, accompanying an increasing level of anti-inflammatory cytokine IL-4 than the normal group. These data suggest that the protective effects of RES against liver and intestinal injuries in cage-reared ducks may be partially due to its potent antioxidant and anti-inflammatory activities.

The overproduction of nitric oxide (NO) and prostaglandin E2 (PGE2) is associated with inflammatory diseases, and iNOS and COX2, the upstream enzymes of NO and PGE2 synthesis, respectively, are key enzymes mediating the inflammatory process. We have previously found that cage-rearing induced inflammatory injury in duck liver and elevated iNOS and COX2 mRNA expression (Zhang et al., 2019), suggesting that the upregulated expression of these two genes may serve as markers of cage-rearing-induced hepatic injury. This study demonstrated that the expression levels of hepatic inflammation-related genes iNOS and COX2 were significantly reduced in the RES-added group than in the basal diet group. Consistent with our findings, previous studies have reported that RES exerts its anti-inflammatory and protective effects by downregulating COX2 and iNOS expression (Wang et al., 2016; Lin et al., 2022). Moreover, it has been extensively demonstrated that ER stress is involved in the pathophysiological processes of inflammatory diseases (Keestra-Gounder et al., 2016; Stengel et al., 2020), and ER and OS are intimately linked within cells (Liu et al., 2019; Camargo et al., 2023). ER stress is mediated by activation of the unfolded protein response (UPR), a complex network initiated by three ER transmembrane receptors. During ER homeostasis, GRP78 binds to these transmembrane proteins to maintain a stable nonreactive state. When ER homeostasis is imbalanced, GRP78 dissociates from them and activates the UPR (Walter and Ron, 2011). Consequently, GRP78 is an essential marker of ER stress. A large amount of evidence indicates that unfavorable stimuli can induce ER stress with an increase in GRP78 expression, and we also reported activation of the ER stress response in cage-reared ducks (Zhang et al., 2019). In this study, we evaluated the effects of RES on ER stress in the liver and duodenum of cage-reared ducks and found that it significantly reduced the expression of GRP78 mRNA in these tissues, suggesting that RES could alleviate ER stress induced by cage-rearing.

RES is a promising natural compound, and there is growing evidence in the literature that it can prevent and treat various human diseases, such as tumors and cardiovascular disorders, by regulating apoptosis and survival pathways. Bcl-2/Bax forms ion channels in the mitochondrial outer membrane and regulates the outer membrane permeability by controlling the opening of ion channels. Bcl2 and Bax have been suggested to play key roles in apoptosis (Ke et al., 2017). The Bcl2 family regulates the upregulation and downregulation of anti-apoptotic proteins (Bcl-xL and Bcl2) and pro-apoptotic proteins (Bax) in the mitochondria to determine whether cells undergo apoptosis or survive (Lin et al., 2015). In a recent report on the improvement of ovarian status by RES, Liang et al. (2023) found that Bcl2 expression was significantly elevated and Bax levels were significantly reduced in RES-treated human ovarian granulosa cells than the H_2O_2-induced oxidative stress model group. Hou et al. (2018) investigated the neuroprotective effects of RES on cerebral ischemia/reperfusion injury and found that it could inhibit apoptosis-related pathways by downregulating Bax and activating Bcl2. However, in tumor cells, RES plays a role in promoting apoptosis (Sajadimajd et al., 2020; Najafiyan et al., 2024). Indeed, the two diametrically opposed modes of apoptosis regulation, in which RES inhibits or promotes apoptosis in different cells, are not contradictory, as its ultimate effect is to promote survival. In this study, Bcl2 mRNA levels tended to increase, and Bax mRNA expression decreased in the liver and duodenum of cage-reared ducks after RES treatment, suggesting that RES exerts its protective effect by inhibiting apoptosis in cage-induced duck injury.

PI3K/AKT and MAPK signaling pathways have been extensively studied for their cytoprotective effects against tissue damage. AKT is an essential serine/threonine kinase that helps cell proliferation, cell death, and cell differentiation. MAPKs are serine/threonine kinases that play crucial roles in cell survival, proliferation, the cell cycle, and apoptosis signal transduction pathways. Studies have suggested that activation of AKT and/or ERK1/2 is essential in cellular survival and death

(Konieczny et al., 2022; Zhang et al., 2022; Bellizzi et al., 2022; Burguete et al., 2023). Caspases are an evolutionarily conserved cysteine protease family vital for cell death and inflammatory responses. Activation of caspases occurs only when they are cleaved, and activating effector caspases (for example, caspase-3) to induce apoptosis requires initiator caspase activation (Van Opdenbosch and Lamkanfi, 2019). Research has indicated that apoptosis regulation of RES occurs through the PI3K/AKT and ERK1/2 pathways, which were found to act upstream of caspase-3 (Mondal and Bennett, 2016; Liu et al., 2022). Western blot results demonstrated that RES elevated p-AKT protein levels and decreased cleaved caspase-3 protein in this experiment, while there was a non-significant effect on ERK1/2 and p-ERK1/2 protein levels. Combined with the results of apoptosis and tissue damage assessments, this suggests that PI3K/AKT/caspase-3 may be involved in the protective effect of RES. Additionally, many studies have demonstrated that activation of the PI3K/AKT pathway protects against tissue damage via synergistic upregulation of antioxidant and anti-inflammatory activities, as well as inhibition of ER stress and apoptosis, and that these processes are interconnected (Yu et al., 2019; Patel et al., 2020; Yuan et al., 2023; Deng and Zhou, 2023). Our findings indicate that RES-mediated antioxidant, anti-inflammatory, and anti-apoptotic properties protect against injury in cage-reared ducks, and these properties may be mediated by activating the AKT pathway, which requires further experimental verification.

Conclusions

Our study demonstrated that RES supplementation improved histopathology and liver function, elevated antioxidant and anti-inflammatory properties, attenuated ER stress, inhibited apoptosis, and activated the AKT pathway in caged ducks. These results suggest that RES has a protective effect against liver and small intestinal injury in cage-reared ducks, which is partly mediated by the activation of the PI3K/AKT pathway. The present findings regarding RES-mediated molecular mechanisms and signaling pathways provide new insights and therapeutic targets for alleviating injury in cage-reared ducks. Future studies should focus on whether RES-mediated antioxidant, anti-inflammatory, and anti-apoptotic effects in cage-reared ducks are mediated through the PI3K/AKT pathway and the specific mechanisms. Also, the data in the current study provide further evidence that RES mediates multiple biological processes and thus exerts cytoprotective effects in different species.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 32172688), Zhejiang Science and Technology Major Program on Agricultural New Variety Breeding (No. 2021C02068-10), and China Agriculture Research System of MOF and MARA (No. CARS-42-6).

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