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Effects of the *in ovo* injection of luteoloside on growth performance, serum biochemical indices, and gene expression in broiler chickens

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Abstract

This study investigated the effects of the *in ovo* injection of luteoloside on broiler chickens. A total of 160 eggs were divided into four groups and injected on day 14 of incubation with 0, 10, 25, or 100 nmol of luteoloside, with these treatments designated as the C, L, M, and H groups, respectively. The results showed that the luteoloside injection did not significantly affect hatchability, live weight, carcass percentage, or organ indices at 1 or 7 days post-hatch. However, the chemical analysis of the serum showed that the malondialdehyde level in the H group was significantly lower than that in the C, L, and M groups at day 7. In contrast, the total superoxide dismutase activity in the M and H groups was significantly higher than that in the C and L groups. Gene expression analysis indicated that on day 1, the M and H groups showed significant upregulation of peroxisome proliferator-activated receptor gamma and downregulation of fatty acid synthase. The H group also had higher lipoprotein lipase and leptin expression. On day 7, the H group exhibited the highest lipoprotein lipase expression, while insulin-like growth factor 2 expression was significantly higher in all luteoloside-injected groups compared to the control. In conclusion, *in ovo* injection of luteoloside had no significant impact on growth performance but effectively alleviated oxidative stress, reduced oxidative damage, and markedly influenced the expression of key genes in the liver.

Keywords: chicken embryo, flavonoid, gene expression, *in ovo* injection, luteoloside

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Introduction

Luteoloside, also known as luteolin-7-O-glucoside or cynaroside, is a polyphenol flavonoid present in medicinal plants of the Caprifoliaceae family (Zhu *et al*., 2020; Han *et al*., 2023), particularly in honeysuckle (*Lonicera japonica*). Studies have shown that this compound possesses a broad spectrum of pharmacological activities, including antioxidant (Zang *et al*., 2016), anti-inflammatory (Feng *et al*., 2021b; Pei *et al*., 2021), anticancer (Ji *et al*., 2021), antitumour (Baskar *et al*., 2010), antiviral (Zhu *et al*., 2020), and lipid-lowering effects (Sun *et al*., 2021). Recent studies have elucidated several molecular mechanisms through which luteoloside mediates its biological effects. In the context of cardiovascular protection, luteoloside has been shown to mitigate hypoxia/reoxygenation injury in cardiomyocytes by modulating the 14-3-3η signalling pathway (Liu *et al*., 2018). In cancer research,

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luteoloside suppresses the activation of the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome, thereby inhibiting proliferation, migration, and other malignant behaviour in hepatocellular carcinoma (Sarkar *et al*., 2014). Additional evidence indicates that luteoloside protects H9c2 cardiomyocytes from H_2O_2-induced apoptosis by reducing intracellular reactive oxygen species levels, maintaining mitochondrial integrity, and influencing apoptotic signalling through the c-Jun N-terminal kinase (JNK) and p53 pathways (Sun *et al*., 2011). Moreover, in glucose metabolism, luteoloside facilitates Akt kinase phosphorylation and promotes glycogen synthesis through cooperative actions with other metabolic modulators (Defferrari *et al*., 2018).

In ovo injection is a method for delivering exogenous nutrients directly into developing avian eggs, and has been shown to effectively support embryos during late developmental stages and enhance growth performance after hatching (Uni & Ferket, 2019; Han *et al*., 2020; Duan *et al*., 2021; Geng *et al*., 2022). Studies confirm that the procedure does not adversely affect hatchability or later development (Ohta *et al*., 1999), and can play a role in promoting healthier conditions and optimising growth throughout the incubation period (Jha *et al*., 2019; Bednarczyk *et al*., 2021). Previous studies have mainly explored the use of conventional exogenous nutrients in *in ovo* injections, including carbohydrates (Zhai *et al*., 2011), vitamins (Tufarelli *et al*., 2021), and amino acids (Toghyani *et al*., 2018). However, the application of luteoloside via *in ovo* injection in broiler embryos remains unreported, and, to date, the biological functions of luteoloside have been primarily investigated in mammalian models. Nonetheless, luteoloside has been shown to exert cardioprotective effects against hypoxia/reoxygenation injury (Liu *et al.*, 2018), to attenuate inflammation through nuclear factor-κB (NF-κB) inhibition (Cao *et al.*, 2022), and to reduce oxidative stress via activation of the nuclear factor erythroid 2-related factor 2 (Nrf2)/haem oxygenase-1 (HO-1) pathway (Xiang *et al.*, 2022).

In poultry, however, the bioactivity of luteoloside and its potential effects on embryonic development and post-hatch growth remain largely unknown. Thus, while the multifaceted bioactivities of luteoloside are recognised, its impact on post-hatch organ development, oxidative status, and related gene expression in broilers after administration via *in ovo* injection remains unexplored. This study aimed to determine whether the *in ovo* injection of luteoloside promotes growth and development in chickens, improves antioxidant indicators, and modulates the expression of hepatic genes. The results are expected to provide a foundation for future research on nutritional regulation with luteoloside in Arbor Acres broiler chickens.

Materials and methods

The animal experiment protocol was approved by the Experimental Animal Ethics Committee of Linyi University, Shandong Province (approval number: LYU20250111).

Egg selection and incubation

A total of 160 Arbor Acres broiler breeder eggs (mean egg weight: 60.2 ± 0.5 g) were obtained from the same parent flock at Tangyin Quanda Poultry Breeding Co. Ltd. and were selected based on comparable egg weight (P >0.05). Upon arrival, the eggs were wiped with 0.1% benzalkonium bromide solution, allowed to rest for 12 hours at room temperature, and then immediately transferred to the incubator (i.e. no prolonged storage was applied). Throughout the incubation period, a constant temperature of 38 °C was maintained, while relative humidity was controlled between 55% and 66%. The eggs were automatically turned for 3 minutes every 2 hours. This study was designed as a preliminary experiment with a relatively small sample size (40 eggs per group). The findings provide a basis for future large-scale investigations.

Experimental design and diets

A single-factor completely randomised design was implemented in this study. On the 14th day of incubation (a stage when the yolk sac is well-developed and vascularised for efficient compound absorption), the eggs were randomly assigned to four experimental groups. Each group contained four replicates, with 10 eggs included in every replicate. Luteoloside (Shanghai Macklin Biochemical Technology Co. Ltd., Shanghai, China, CAS: 5373-11-5) was dissolved in phosphate-buffered saline with 1% dimethyl sulfoxide (PBS-DMSO) (Shi *et al.*, 2020) to prepare solutions at the required concentrations, specifically 0 nmol/egg (C group), 10 nmol/egg (L group), 25 nmol/egg (M group), or 100 nmol/egg (H group) of luteoloside. The doses (10, 25, and 100 nmol/egg) were selected based on

effective dose ranges of structurally similar flavonoids in chicken embryos, as this study was designed as a preliminary experiment to explore dose effects. On day 14 of incubation, the eggs were candled, and a small hole was drilled in the shell above the yolk sac after disinfection with 75% ethanol. Each embryo received a 0.2 mL injection of the corresponding luteoloside solution into the yolk sac using a 1 mL sterile syringe with a 27-gauge needle. The hole was sealed with paraffin film, and the eggs were returned to the incubator and maintained at 38 °C with 60% relative humidity until hatching. The control group (C) received an equal volume (0.2 mL) of the sterile PBS-DMSO vehicle under the same conditions to control for the effects of the injection procedure and the solvent. Day 14 was selected for *in ovo* injection because it provides sufficient duration (7–8 days) for luteoloside to exert its effects on hatchability and post-hatch development, while avoiding the peak period of early embryonic mortality (days 3–7 of incubation).

For the 7-day post-hatch trial, 48 chicks (12 per group, three per replicate × four replicates) were housed in stainless steel battery cages with *ad libitum* access to feed and water. The 7-day trial period employed a basal diet formulated to meet the nutritional requirements of broilers (in accordance with the Chinese Chicken Feeding Standard, NY/T 33-2004, China). Its composition and nutrient levels are presented in Table 1.

Table 1 Composition and nutrient content of the basal diet (on an as-fed basis)

	Feeding period (days 1–7)
Ingredients (%):	
Maize	54.50
Soybean	32.71
Fish meal	3.00
Shell powder	1.22
$CaHPO_4 \cdot 2H_2O$	1.70
Soybean oil	3.00
Wheat bran	2.50
Salt	0.20
DL-methionine (98%)	0.17
Premix[1]	1.00
Total	100.00
Calculated nutrient composition	
Metabolisable energy (MJ/kg)	12.52
Crude protein (%)	21.52
Calcium (%)	0.97
Available phosphorus (%)	0.45
Lysine (%)	1.21
Methionine (%)	0.53
Methionine + cysteine (%)	0.91

[1] Premix provided per kilogram of diet: 55 mg manganese sulphate, 55 mg zinc sulphate, 44 mg ferrous sulphate, 5.5 mg copper sulphate, 0.44 mg potassium iodide, 0.099 mg selenium selenite, 770 IU vitamin A, 1.2 mg vitamin B_1, 4.8 mg vitamin B_2, 1.6 mg vitamin B_6, 0.011 mg vitamin B_{12}, 255 IU vitamin D_3, 15 mg vitamin E, 2.2 mg vitamin K, 11 mg pantothenic acid, 35.5 mg niacin, 0.66 mg folic acid, 0.11 mg biotin, 500 mg choline, 33.1 mg xanthine, 120 mg antioxidant.

The metabolisable energy and available phosphorus contents of the diet were calculated based on Chinese Feed Composition and Nutritive Values (31st edition, 2020). Crude protein, calcium, and amino acid concentrations were determined using the Kjeldahl method, EDTA titration, and acid hydrolysis, respectively, following the Chinese national standards (GB/T 6432-2018, GB/T 6436-2018, and GB/T 18246-2019, respectively). The lighting schedule during the rearing period was set at 24 hours

of light per day, with the room temperature maintained at 33–35 °C and the relative humidity controlled at between 50%–70%. Feed and water were provided twice daily (08:00 and 18:00), with feeders kept continuously supplied between refills to maintain *ad libitum* access. All birds were vaccinated following the standard immunisation protocol.

Sample collection

The total number of hatched chicks was recorded to calculate the hatchability rate for each group. The hatch window (time from the first to the last chick hatching in each replicate) was not recorded in this study. On day 1 post-hatch, chicks were fasted for 12 hours immediately after hatching (feed withheld, but with free access to water) to allow for yolk sac absorption and to standardise their nutritional status. After this 12-hour fasting period, three chicks per replicate (four replicates per group, yielding 12 chicks per treatment group) were randomly selected and weighed. The chicks were then euthanised by cervical dislocation, and approximately 1 mL of blood was immediately obtained from the heart of each chick and placed in a sterile 1.5 mL centrifuge tube. After collection, the samples were centrifuged at 3000 rpm for 5 minutes to isolate serum. The resulting serum was aliquoted and preserved at −80 °C for further analysis. For 1-day-old chicks, only organ weights (heart, liver, and gizzard) were recorded to calculate organ indices; carcass traits were not measured at this age. A portion of the liver was immediately sampled, with one sample being placed into a 1.5 mL RNase-free centrifuge tube, flash-frozen in liquid nitrogen, and stored at −80 °C for further use, and another sample being transferred to a 10 mL centrifuge tube containing fixative solution for subsequent haematoxylin and eosin (H&E) staining and histological analysis.

Similarly, on day 7 post-hatch, after a 12-hour fasting period with free access to water, three chickens per replicate (four replicates per group, yielding 12 chicks per treatment group) were weighed. The chickens were then euthanised by cervical dislocation, and blood was collected from the heart. For the 7-day-old chicks, the heart, liver, gizzard, proventriculus, lungs, carcass weight, and eviscerated carcass weight were measured. Carcass weight was recorded after bleeding, feather removal, and excision of the feet and head. Eviscerated carcass weight was obtained after removing the internal organs (including the gastrointestinal tract, heart, liver, spleen, and lungs). The organ index, carcass percentage, and eviscerated carcass percentage were calculated. All organ weights were measured using an electronic balance with a precision of 0.0001 g. For clarity and conciseness, the data are presented in the tables rounded to two decimal places; however, all statistical analyses were performed using the original unrounded data

Measurement of parameters

Organ index, carcass percentage, and eviscerated carcass percentage were calculated as the ratio of the respective organ or carcass weight (g) to the pre-slaughter live weight (g), expressed as a percentage.

Serum levels of triglycerides (TG), total cholesterol (T-CHO), malondialdehyde (MDA), and total superoxide dismutase (T-SOD) were measured using a Synergy H1 microplate reader (BioTek Instruments, Inc.). All assay kits were purchased from the Nanjing Jiancheng Bioengineering Institute, China. Serum TG and T-CHO concentrations are expressed as mmol/L, while MDA and T-SOD levels are expressed as nmol/mL and U/mL, respectively.

Total RNA for hepatic gene expression analysis was extracted from liver tissue using an Animal Tissue/Cell RNA Extraction Kit (Beijing Coolaber Co., Ltd, China) according to the manufacturer's instructions. Briefly, approximately 40 mg of tissue was homogenised in 1 mL of lysis buffer. The lysate was then processed through a gDNA-filter column to remove genomic DNA, followed by washing steps. Total RNA was eluted in 50 µL of RNase-free water (Doleshal *et al*., 2008). Complementary DNA was synthesised by reverse transcription of mRNA using the HyperScript™ RT SuperMix for qPCR kit (APE×BIO Company), following the kit instructions. The reverse transcription reaction was carried out using a BIO RAD T100 thermal cycler according to the manufacturer's recommended thermal profile: 25 °C for 2 minutes, 45 °C for 15 minutes, 95 °C for 1 minute, and a 4 °C hold. Primers for target genes and the reference gene *β-actin* were designed based on chicken mRNA sequences using Primer Premier (v6.25). The primer sequences are listed in Table 2. The reverse transcription quantitative polymerase chain reaction (RT-qPCR) was conducted using the HotStart™ 2× Green qPCR Master Mix (APE×BIO Company) on a Roche LightCycler 480 under the following conditions: initial denaturation at 95 °C for 30 seconds, followed by 40 cycles of denaturation at 95 °C for 30 seconds, annealing at the

temperature specified in Table 2 for 30 seconds, and extension at 72 °C for 1 second. Each sample was run in triplicate, and the relative expression of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method with *β-actin* as the internal reference gene.

Table 2 Primer sequences for target genes and the reference gene *β-actin*

Primer name	Primer sequence (5′–3′)	Annealing temperature (°C)	Product size (bp)	Accession number
PPARγ	F: TCTCCTGGCTTCTCTCAT R: TGGGCTCCATAAAGTCAC	57	116	KT899869
LPL	F: TCATTGTTGTGGACTGGC R: TGGACATTGTTGAGAGGGT	58	139	NM_205282
LEPT	F: GACTTCATTCCTGGGCTT R: AGGTCATTGGCTATCTGC	58	122	AF012727
IGF2	F: CTATGCGTTGGATTCAGC R: CCTGTTATTTCGTCCCAC	58	133	NM_001030342
FAS	F: TGAAGGAGGAAGTCAACG R: GATGGTGAGGAGTCGGAT	55	196	J04485
β-actin	F: GTGACATCAAGGAGAAGC R: CATCAGGGAGTTCATAGC	55	105	L08165

PPARγ: peroxisome proliferator-activated receptor gamma; *LPL*: lipoprotein lipase; *LEPT*: leptin; *IGF2*: insulin-like growth factor 2; *FAS*: fatty acid synthase.

Liver histological sectioning

Liver tissue morphology was examined using H&E staining. Fixed liver samples were embedded in paraffin, sectioned, stained with H&E reagents, and mounted with neutral gum. The sections were observed under a microscope for image acquisition and analysis.

Statistical analysis

Data were analysed by one-way analysis of variance using SPSS 27.0, as the experimental data met the assumptions of normality and homogeneity of variances. Significant differences among treatment groups' means were further evaluated with Duncan's multiple comparison test. Results are expressed as the mean ± the standard error (SE). Differences were considered statistically significant at $P \leq 0.05$, and non-significant at $P > 0.05$.

Results and discussion

Hatchability and growth performance

The effects of *in ovo* luteoloside injection on hatchability, growth performance, and organ indices are shown in Table 3. *In ovo* injection of luteoloside did not significantly affect hatchability, growth performance, organ weights, or organ indices in either 1- or 7-day-old chicks. Embryonic mortality rates, calculated as 100% minus hatchability, were 17.5%, 15.0%, 17.5%, and 12.5% for the C, L, M, and H groups, respectively. These findings indicate that luteoloside administration via *in ovo* injection at the tested doses is safe for embryonic development and early post-hatch growth. The lack of significant effects on growth performance may be explained by the fact that growth is a complex, tightly regulated trait that is resistant to short-term nutritional or pharmacological modulation. Alternatively, the dosage used in this experiment may have been insufficient to produce a measurable growth response, and the 7-day trial period may have been too short to detect differences in body weight gain at this early stage of development.

Table 3 Effects of *in ovo* injection of luteoloside on the hatchability of chicken embryos and the growth performance and organ indexes of chicks

	Treatment groups				*P*-value
	C group	L group	M group	H group	
Hatchability (%)	82.5	85.0	82.5	87.5	0.495
Day 1					
Live weight pre-slaughter (g)	44.67 ± 0.71	46.39 ± 0.59	44.68 ± 1.00	45.34 ± 0.78	0.373
Heart index (%)	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.166
Liver index (%)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.986
Gizzard index (%)	0.05 ± 0.00	0.04 ± 0.00	0.05 ± 0.00	0.04 ± 0.00	0.615
Day 7					
Live weight pre-slaughter (g)	212.64 ± 5.94	218.90 ± 8.35	202.89 ± 12.62	202.81 ± 9.20	0.510
Carcass (%)	0.95 ± 0.00	0.96 ± 0.00	0.95 ± 0.00	0.95 ± 0.00	0.333
Eviscerated carcass (%)	0.68 ± 0.00	0.67 ± 0.01	0.66 ± 0.01	0.66 ± 0.01	0.061
Heart index (%)	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.546
Liver index (%)	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.124
Gizzard index (%)	0.04 ± 0.00	0.04 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.176
Proventriculus index (%)	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.200
Lung index (%)	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.638

Treatment groups: luteoloside *in ovo* injected at 0 nmol/egg (C group), 10 nmol/egg (L group), 25 nmol/egg (M group), and 100 nmol/egg (H group).

Serum biochemical parameters

The effects of *in ovo* luteoloside injection on serum biochemical parameters at 1 and 7 days post-hatch are presented in Table 4. At 1 day post-hatch, no significant differences were observed in serum levels of TG, T-CHO, MDA, or T-SOD among the groups. Similarly, at 7 days post-hatch, *in ovo* luteoloside injection did not significantly affect serum TG or T-CHO levels. However, the MDA concentration in the H group was substantially lower than that in the C, L, and M groups (P <0.01) at 7 days post-hatch, with no significant differences among the C, L, and M groups. Total superoxide dismutase activity in the M and H groups was higher than that in the C and L groups (P <0.01), whereas no significant differences were detected between the C and L groups or between the M and L groups.

The observed changes in MDA and T-SOD levels indicate that luteoloside exerts significant antioxidant effects in chickens. Superoxide dismutase is a fundamental antioxidant enzyme that protects against oxidative stress by neutralising free radicals and breaking chain reactions of oxidation; higher SOD activity therefore indicates an enhanced capacity for eliminating reactive oxygen species (Xie *et al.*, 2021). Conversely, MDA is a direct measure of oxidative injury to lipids and indirectly reflects the degree of cell damage (Li *et al.*, 2019). The reduction in MDA and elevation in T-SOD activity observed in the present study are consistent with previous reports demonstrating that luteoloside possesses significant antioxidant properties through its ability to inhibit lipid peroxidation (Süzgeç *et al.*, 2005) and activate the Nrf2 signalling pathway, leading to the upregulation of key antioxidant enzymes such as SOD (Son *et al.*, 2020). Furthermore, luteoloside has been shown to inhibit the formation of free radicals within reaction systems containing 13-hydroperoxyoctadecadienoic acid and iron ions (Iwahashi *et al.*, 2009). Taken together, these findings suggest that luteoloside may alleviate oxidative stress in poultry through a dual mechanism: direct free radical scavenging and enhancement of endogenous antioxidant defences.

Table 4 Effects of *in ovo* injections of luteoloside on serum biochemical profiles in broiler chicks

	Treatment groups				*P*-value
	C group	L group	M group	H group	
Day 1					
TG (mmol/L)	1.13 ± 0.14	1.26 ± 0.15	1.28 ± 0.14	1.38 ± 0.14	0.692
T-CHO (mmol/L)	6.34 ± 0.34	5.48 ± 0.37	5.62 ± 0.63	5.52 ± 0.40	0.507
MDA (nmol/mL)	16.18 ± 1.26	16.18 ± 0.31	16.32 ± 1.31	13.68 ± 0.85	0.227
T-SOD (U/mL)	0.70 ± 0.07	0.67 ± 0.04	0.56 ± 0.07	0.53 ± 0.06	0.163
Day 7					
TG (mmol/L)	0.76 ± 0.05	0.75 ± 0.05	0.75 ± 0.06	0.78 ± 0.08	0.990
T-CHO (mmol/L)	8.15 ± 0.48	8.22 ± 0.69	9.09 ± 0.29	8.12 ± 0.28	0.485
MDA (nmol/mL)	28.74^{A} ± 1.33	31.01^{A} ± 1.18	26.85^{A} ± 0.68	21.77^{B} ± 0.72	<0.001
T-SOD (U/mL)	0.99^{B} ± 0.02	0.96^{B} ± 0.03	1.26^{A} ± 0.02	1.36^{A} ± 0.03	<0.001

Treatment groups: luteoloside *in ovo* injected at 0 nmol/egg (C group), 10 nmol/egg (L group), 25 nmol/egg (M group), and 100 nmol/egg (H group). TG: triglycerides, T-CHO: total cholesterol, MDA: malondialdehyde, T-SOD: total superoxide dismutase. ^{AB} Values within a row sharing the same uppercase superscript letters indicate no significant difference (P >0.05), while different uppercase superscript letters indicate significant differences (P <0.01).

Liver morphology

The histology of liver sections stained with H&E is shown in Figure 1. At both 1 and 7 days post-hatch, liver tissue from all groups exhibited intact architecture with no significant necrosis or congestion. Hepatocytes were arranged normally, without observable pathological abnormalities. These results indicate that the *in ovo* injection of luteoloside at various concentrations did not induce adverse effects on liver morphology, which is consistent with the organ index results observed in this experiment. The lack of histological changes suggests that luteoloside's mechanism of action operates primarily at the molecular and cellular levels through pathway regulation, without exerting negative impacts on organ structure (Cao *et al.*, 2022).

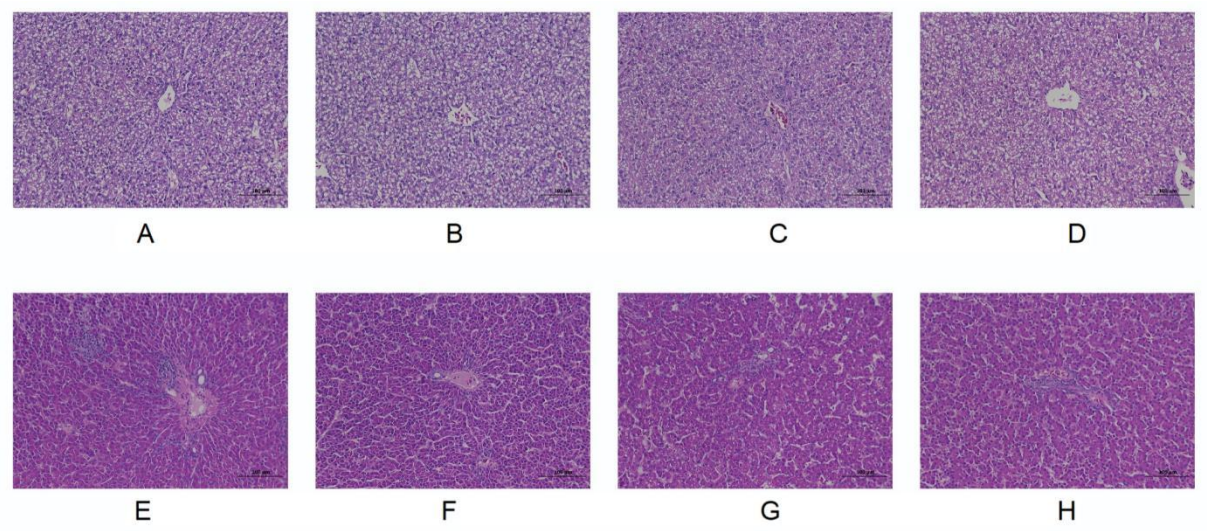

Figure 1 Microstructure of liver tissue sections from broiler chicks *in ovo* injected with luteoloside (haematoxylin and eosin staining, 200× magnification, scale bar represents 100 μm). Treatment groups: luteoloside *in ovo* injected at 0 nmol/egg (C group, A = day 1, E = day 7), 10 nmol/egg (L group, B = day 1, F = day 7), 25 nmol/egg (M group, C = day 1, G = day 7), and 100 nmol/egg (H group, D = day 1, H = day 7).

Hepatic gene expression

The expression levels of liver-related genes were detected by RT-qPCR using complementary DNA templates from liver tissues collected at 1 and 7 days post-hatch, with *β-actin* as the internal reference gene. The results are presented in Figures 2 and 3.

At 1 day post-hatch (Figure 2), the expression of peroxisome proliferator-activated receptor gamma (*PPARγ*) in the M and H groups was higher than that in the C and L groups (P <0.01), with no significant differences observed between the C and L groups or between the M and H groups. Lipoprotein lipase (*LPL*) expression in the H group was higher than that in the L and M groups (P <0.05), but did not differ significantly from the C group. No significant differences were detected among the C, L, and M groups. Leptin (*LEPT*) expression in the H group was higher than that in the M group (P <0.05), whereas no significant differences were found among the C, L, and M groups. The expression of fatty acid synthase (*FAS*) in the M and H groups was lower than that in the C group (P <0.05), with no significant differences observed between the C and L groups or among the L, M, and H groups. The expression level of insulin-like growth factor 2 (*IGF2*) did not differ significantly among the groups.

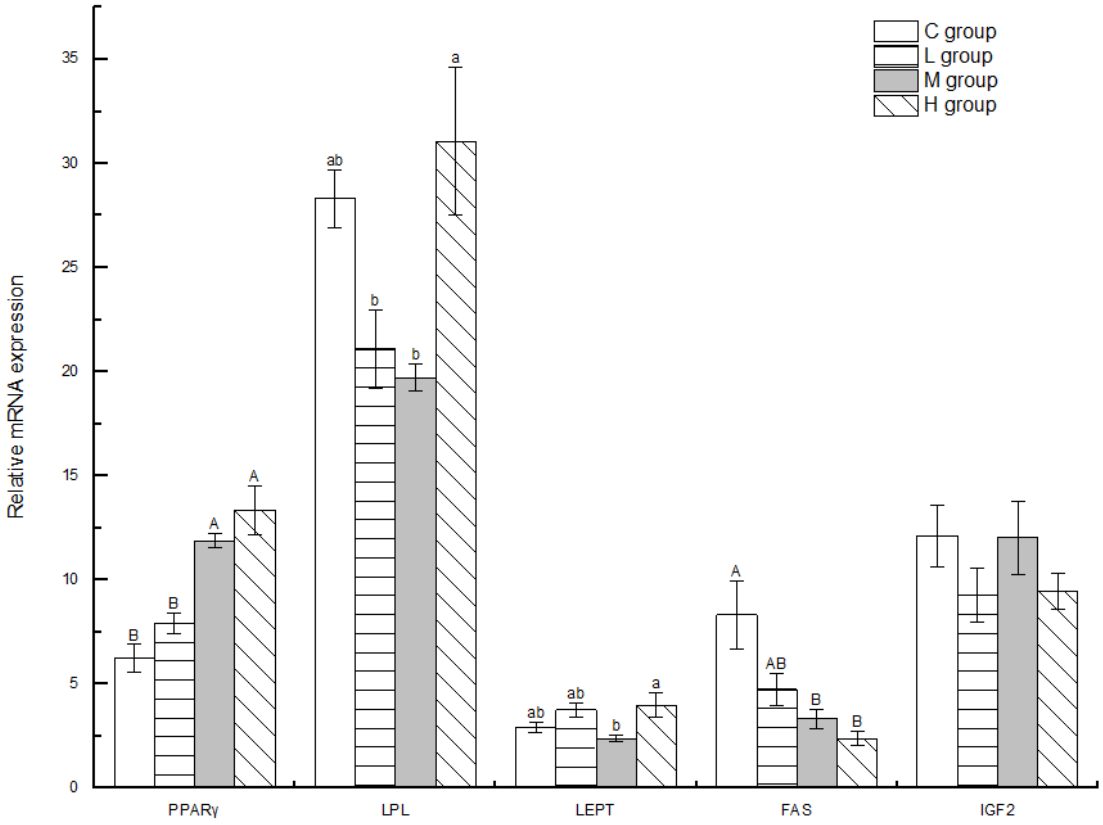

Figure 2 Effects of the *in ovo* injection of luteoloside on gene expression in the livers of 1-day-old chicks (luteoloside *in ovo* injected at 0 nmol/egg (C group), 10 nmol/egg (L group), 25 nmol/egg (M group), and 100 nmol/egg (H group)). Within the same dataset, an absence of superscript letters or shared letters indicates no significant difference (P >0.05). Different lowercase letters (ab) indicate significant differences (P <0.05), while different uppercase letters (AB) indicate highly significant differences (P <0.01). Values are expressed as relative mRNA expression normalised to *β-actin* ($2^{-\Delta\Delta Ct}$ method) and are unitless.

At 7 days post-hatch (Figure 3), no significant differences were observed in the expression of *PPARγ*, *LEPT*, or *FAS* among the groups. The *LPL* expression in the M group was lower than that in the other three groups (P <0.01), while the H group exhibited substantially higher *LPL* expression compared to the other groups (P <0.01). The expression of *IGF2* in the L, M, and H groups was higher than that in the C group (P <0.01), with the M group also showing higher expression than the L group (P <0.01).

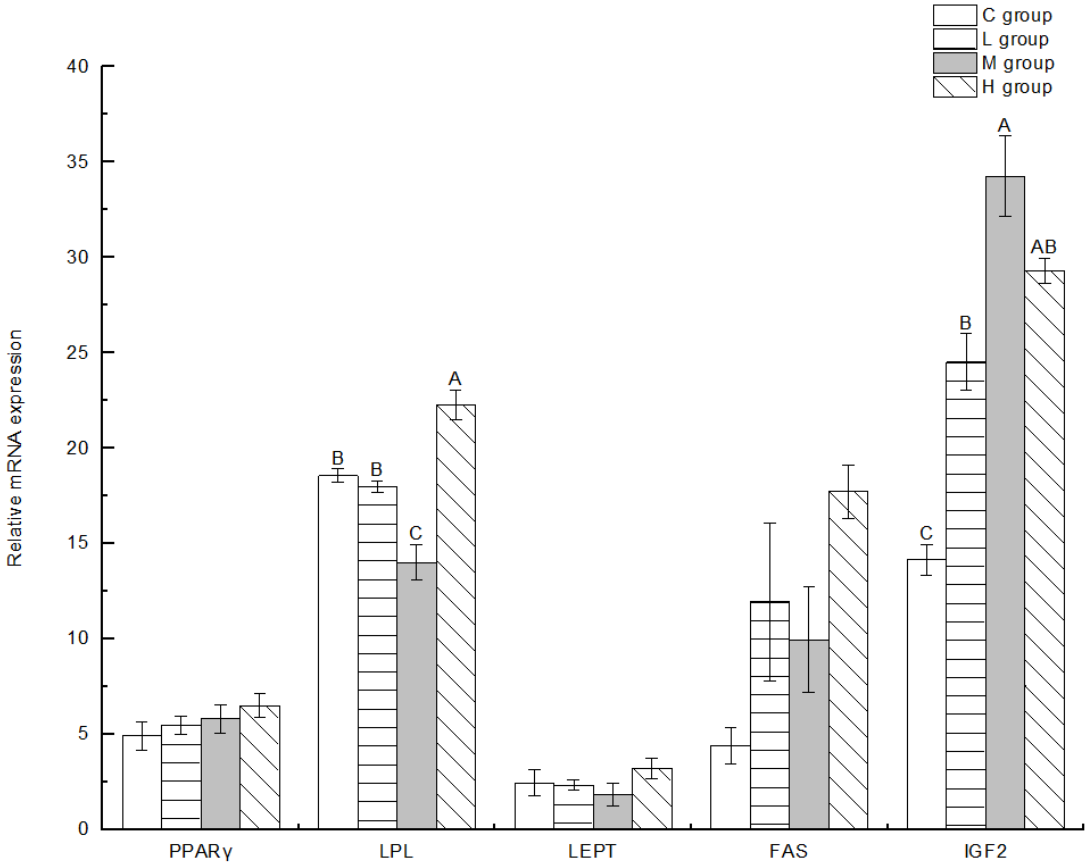

Figure 3 Effects of the *in ovo* injection of luteoloside on gene expression in the livers of 7-day-old chicks (luteoloside *in ovo* injected at 0 nmol/egg (C group), 10 nmol/egg (L group), 25 nmol/egg (M group), and 100 nmol/egg (H group)). Within the same dataset, an absence of superscript letters or shared letters indicates no significant difference (P >0.05). Different uppercase letters (AB) indicate highly significant differences (P <0.01). Values are expressed as relative mRNA expression normalised to β-*actin* ($2^{-\Delta\Delta Ct}$ method) and are unitless.

To our knowledge, this is the first report on the effects of luteoloside on hepatic gene expression in broilers. Previous studies on luteoloside's bioactivity have been primarily conducted in mammalian models (Liu *et al.*, 2018; Cao *et al.*, 2022), and no direct comparison baseline is available for Arbor Acres broilers at day 1 post-hatch in a commercial setting.

The distinct temporal patterns observed between 1 and 7 days suggest that luteoloside exerts stage-dependent effects on hepatic gene expression. At 1 day post-hatch, luteoloside upregulated *PPARγ* but downregulated *FAS*, a pattern that contrasts with the typical co-downregulation observed under lipid-lowering conditions (Vig *et al.*, 2015). This divergence likely reflects the integrated nature of gene regulation, which involves multiple signals, including nutrition, hormones, metabolism, and inflammation, rather than a single pathway (Zhang *et al.*, 2022). Fatty acid synthase is a multi-enzyme complex that catalyses a key step in *de novo* fatty acid synthesis and is tightly regulated by the transcription factor sterol regulatory element-binding protein (SREBP-1c) (Vig *et al.*, 2015). Luteoloside is known to reduce blood lipid levels (Sun *et al.*, 2021), and the activation of the AMP-activated protein kinase (AMPK) pathway can inhibit the expression of both *SREBP-1c* and *FAS*, thereby decreasing hepatic lipid synthesis (Liu *et al.*, 2022). The observed dose-dependent decrease in *FAS* expression at 1 day post-hatch suggests that this effect may be mediated by luteoloside-induced AMPK activation. The PPARγ/liver X receptor alpha (LXRα) signalling pathway has been reported to inhibit pro-inflammatory genes, thereby modulating lipid metabolism and suppressing inflammatory responses (Zhang *et al.*, 2022). We therefore speculate that luteoloside intervention may upregulate *PPARγ*

expression at 1 day post-hatch through the activation of this pathway. The decrease in *PPARγ* and *LEPT* expression by 7 days post-hatch, with significant intergroup differences only at 1 day post-hatch, suggests that the transcriptional effects of luteoloside may diminish over time, possibly due to metabolic adaptation or the clearance of the compound (Wang *et al.*, 2021). Conversely, the increase in *IGF2* expression across all groups by 7 days post-hatch, with significant differences among groups not present at 1 day post-hatch, indicates that luteoloside may exert delayed effects on the growth hormone/IGF axis, which warrants further investigation.

The current understanding of luteoloside's biological activity is mainly derived from mammalian models, while its specific molecular mechanisms in poultry remain insufficiently studied. Emerging evidence indicates that luteoloside can modulate the toll-like receptor 4 (TLR4)/NF-κB pathway to exert antitumour, anti-inflammatory, and antioxidant effects in birds (Cao *et al.*, 2022; Xiang *et al.*, 2022). In a mechanistic study, Wang *et al.* (2021) demonstrated in a septic mouse model that luteoloside significantly protected against sepsis, an effect closely associated with the suppression of the high-mobility group box 1 (HMGB1)/TLR4/NF-κB/mitogen-activated protein kinases (MAPKs) pathway. According to Feng *et al.* (2021a), luteoloside not only blocks the nuclear translocation of NF-κB but also enhances autophagic activity. Although studies on luteoloside's role in poultry are limited, its proven anti-inflammatory and antioxidant effects in mammals – particularly through regulating the TLR4/NF-κB pathway – suggest that it may similarly reduce intestinal inflammation and oxidative stress in birds (Cao *et al.*, 2022). This perspective provides a plausible theoretical basis for future poultry research.

This study found that the *in ovo* administration of luteoloside at suitable concentrations is safe for hatchability and growth performance in broilers. Based on the results, luteoloside exhibits significant antioxidative effects in chickens, as evidenced by reduced MDA and elevated T-SOD activity, which aligns with the cited literature on its antioxidant properties. However, the molecular network through which luteoloside exerts its effects in poultry is not yet fully understood. Future research should explore a broader range of luteoloside doses, the effects of injection at different embryonic stages, and its efficacy across various poultry breeds. More definitive techniques, such as western blot, chromatin immunoprecipitation, or metabolomic analysis, are recommended to clarify its molecular mechanisms. Such comparative studies will help determine the optimal timing and dosage for improving embryonic development, post-hatch growth, and carcass traits in broilers.

Conclusions

In conclusion, the *in ovo* injection of luteoloside at suitable concentrations was safe for embryonic development and effectively mitigated oxidative stress in chickens. This was demonstrated by a significant decrease in MDA in the H group and an increase in T-SOD activity in both the M and H groups at 7 days post-hatch. Additionally, luteoloside significantly influenced the expression of liver-related genes, and at 1 day post-hatch, *PPARγ* expression was upregulated while *FAS* expression was downregulated. Under the experimental conditions of this study, 100 nmol luteoloside/egg yielded optimal experimental results.

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Authors' contributions

Conceptualisation: Xing, J.Y. and Li, X.L. Methodology: Xing, J.Y. Software: Pang, Y.T. Validation: Pang, Y.T. and Zhang, Y.J. Formal analysis: Pang, Y.T. Investigation: Pang, Y.T. and Duan, S.Y. Resources: Xing, J.Y. and Li, X.L. Data curation: Xing, J.Y. and Pang, Y.T. Writing (original draft preparation): Pang, Y.T. Writing (review and editing): Xing, J.Y. and Zhang, Y.J. Visualisation: Pang, Y.T. and Liu, H.T. Supervision: Xing, J.Y. and Li, X.L. Project administration: Xing, J.Y. and Li, X.L. Funding acquisition: Xing, J.Y. and Liu, H.T. All authors have read and agreed to the published version of the manuscript.

Conflict of interest declaration

The authors have no conflicts of interest to declare.

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