Dietary hawthorn-leaves flavonoids improves ovarian function and liver lipid metabolism in aged breeder hens

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ABSTRACT Hawthorn-leaves flavonoids (**HF**), extracted from hawthorn leaves, were reported to exert antioxidant, anti-inflammatory and hypolipidemic properties. The aim of our study was to investigate the effects of dietary HF on the reproduction performance and liver lipid metabolism of aged breeder hens. A total of 270 aged Qiling breeder hens (60-wk-old) were randomly divided into 3 treatments: 1) basic corn-soybean diet (**CON**); 2) basic corn-soybean diet supplemented with 30 mg/kg HF (**LHF**); 3) basic corn-soybean diet supplemented with 60 mg/kg HF (**HHF**). The results showed that supplemented HF significantly improved the egg-laying rate and hatching rate of aged breeder hens ($P < 0.05$). HF treatment reduced the serum TG, T-CHO and L-LDL levels ($P < 0.05$), and upregulated the mRNA expressions of *ESR1, ESR2, VTGII, ApoB,* and *ApoVI* in the liver ($P < 0.05$). Serum estrogen levels in HF treated groups were elevated compared with the CON group ($P < 0.05$). In the HHF group, the number of the primordial follicles was higher in comparison with the CON group ($P < 0.05$). Furthermore, dietary supplementation with HF improved the activity of antioxidant enzymes (T-AOC, GSH-Px) ($P < 0.05$), following with the reversed ovarian apoptosis and morphological damage. In addition, 60 mg/kg dietary HF upregulated the protein expression of PCNA and Nrf2 in the ovary ($P < 0.05$). In summary, dietary supplementation with HF could improve the reproduction performance through regulating liver lipid metabolism and improving ovarian function in aged breeder hens.

Key words: hawthorn leaves flavonoids, reproduction performance, lipid metabolism, antioxidant

INTRODUCTION

In the poultry industry, breeder hens in the late egg-laying period have poor reproduction performance, manifesting as the degraded egg laying rate and hatching performance. The decrease of fertility in aged breeder hens is closely related to the decline of liver-blood-ovary axis functions (Peebles et al., 2001; Saleh et al., 2019). Fatty liver syndrome, the most common metabolic disease in aged laying hens, can decrease egg production, fertilization rate, and hatching rate (Zhang et al., 2018). The main induces of fatty liver can be attributed to high-energy diet and lack of exercise in practice (Trott et al., 2014; Rozenboim et al., 2016). In addition, aging of the breeder hens is certainly accompanied with the decreased estrogen production (Miao et al., 2019).

This will inevitably influence the synthesis and transport of estrogen-regulated yolk precursor of the liver, thereby affecting the reproduction performance of aged breeder hens (Liu et al., 2018b). Moreover, decreased productivity in aged breeder hens is widely believed to be ascribed to ovarian senescence (Devine et al., 2012; Luderer, 2014). Oxidative stress caused by reactive oxygen species (**ROS**) is reported to be involved in ovarian aging (Liu et al., 2018a; Peters et al., 2020). Therefore, it is of great significance to find a solution to improve the function of the liver-blood-ovary axis in aged breeder hens from the perspective of nutritional regulation.

Hawthorn tree (*Crataegus pinnatifida Bge*) is a perennial woody plant widely distributed among Asia, Europe and America. Hawthorn leaves have been used to treat cardiovascular and fatty liver diseases (Yin et al., 2014). Hawthorn leaves flavonoids (**HF**), extracted from hawthorn leaves, have been proved to exert antihypertensive action, anti-inflammatory, free radical scavenging, and antimicrobial activities (Fu et al., 2013; Shi et al., 2019). A previous study has suggested that HF can alleviate nonalcoholic fatty liver disease by enhancing the adiponectin/AMPK pathway in NAFLD rats (Li et al., 2015).

In diabetic rats, HF regulates glucose and lipid metabolism through inactivating PKC signaling pathway (Wang et al., 2011a; Min et al., 2017). In addition, non-alcoholic steatohepatitis (**NASH**) rats are intragastrically administered hawthorn leaves flavonoids improves lipid metabolism and antioxidant capacity by regulating the Nrf2/HO-1 signaling pathway (Wang et al., 2018a). The antioxidant and anti-inflammatory effects of HF have also been shown to treat cataracts and polycystic ovaries in rats (Wang et al., 2011b; Shi, Kong, Yin, Zhang and Wang, 2019). However, the effect of HF on the reproduction performance and lipid metabolism of aged breeder hens remains unclear. Therefore, the objective of this study was to determine the effect of dietary HF on the lipid metabolism and reproductive performance of aged breeder hens, and elucidate its possible molecular mechanism.

MATERIALS AND METHODS

Ethics Statement

All experimental protocols performed during this research were carried out in complete accordance to the regulatory guidelines of Animal Ethical and Welfare Committee (**AEWC**) of Nanjing Agricultural University, Nanjing, China (PZ2019105).

Animals and Experimental Groups and Diet Regimes

In this study, a total of 270 Qiling breeder hens (60-wk-old), with similar reproductive performance, were randomly divided into 3 treatments: 1) breeder hens fed with a basic corn-soybean diet (**CON**); 2) breeder hens fed with a basic corn-soybean diet supplemented with 30 mg/kg HF (**LHF**); 3) breeder hens fed with a basic corn-soybean diet supplemented with 60 mg/kg HF (**HHF**). Each treatment contained 6 replicates of 15 hens per replicate in each group (5 cages for each replicate with 3 birds per cage). Qiling breeder hen, used in the present study, is an important local Chinese native breed. The basic diet used in this study was according to National Research Council (1994) (Table 1). The purity of HF (Jingwei Inc, Nantong, Jiangsu, China) used in this study is more than 80% (vitexin-2″-O-rhamnoside, 48%; vitexin-4″-O-glucoside, 12%; vitexin, 8%; hyperoside, 6%). All birds were raised in 2-level cages in the Tushan Breeding Farm (Changzhou, China) under the standard conditions. The house temperature was controlled between 18°C and 25°C, with a photoperiod of 16 h of daily light. After 2 wk of pre-experiment recording, we confirmed that the egg-laying rates were almost the same among the groups. The formal experiment lasted 8 wk. All raised hens have free access to water. In an 8-wk experiment, each hen distributed an average of 113 g of feed at 6 am every day.

Table 1. Ingredient compositions and nutrient levels of the basic corn-soybean diet.

Ingredient	Content (%)	Nutrient level	Content (%)
Corn	54.99	Metabolizable energy (MC/Kg)	2.83
Soybean meal	34.15	Crude protein	16.10
Limestone	7.23	Available phosphorus	0.47
Soybean oil	0.50	Methionine	0.34
Dicalcium phosphate	2.09	Lysine	0.81
NaCl	0.35	Methionine	0.63
Trace mineral premix	0.30	Threonine	0.60
Choline chloride (50%)	0.12	Tryptophan	0.18
Methionine	0.17	Calcium	3.66
Vitamin premix	0.10		
Total	100.00		

Supplied the following per kg complete diet: Cu 8 mg; Zn 75 mg; Fe 80 mg; Mn 100 mg; Se 0.15 mg; I 0.35 mg; vitamin K3 (menadione) 2.65 mg; thiamine 2 mg; riboflavin 6 mg; vitamin B12 (cobalamin) 0.025 mg; biotin 0.0325 mg; folic acid 1.25 mg; pantothenic acid 12 mg; niacin 50 mg; vitamin A(retinol) 12500 IU; vitamin D3 (cholecalciferol) 2500 IU; vitamin E (α- tocopherol) 30 IU.

Laying Performance and Reproduction Performance

The number of eggs and average egg weight were recorded every day to assess the laying performance. All the hens were subjected to artificial insemination daily by experienced breeders. To evaluation of fertility and hatchability, 90 hatching eggs (15 eggs per replicate) from each group were collected at the last week of experiment. Hatching was taken place in a separate incubator in Tushan Farm; recording the number of fertilized eggs in each replicate on the 19th d of hatching to calculate the fertilization rate. Dead embryos and chicks were made a memo on the 21st d of hatching to evaluate the embryo survival rate and hatchability in vitro.

Fertilization rate (%)

$$= \frac{Number\ of\ fertilized\ eggs}{Number\ of\ incubation\ eggs} \times 100\%$$

Embryo survival rate (%)

$$= \frac{Number\ of\ surival\ embryos}{Number\ of\ fertilized\ eggs} \times 100\%$$

Hatchability in vitro (%)

$$= \frac{Number\ of\ hatching}{Number\ of\ fertilized\ eggs} \times 100\%$$

Sample Collection

According to the period of egg-laying, the sample collection was scheduled for 9:00 am at the age of 68 wk. Each replicate was randomly selected one bird. Blood was collected from the wing vein. The serum was then centrifuged from the blood at 3,000 g for 15 min at 4°C and stored at -20°C for analysis of biochemical

parameters. After euthanasia by CO_2 asphyxiation, the liver and ovary were collected immediately, a portion was frozen in liquid nitrogen for measurements of gene expression and biochemical analysis, another portion of the liver and ovary were fixed in 4% paraformaldehyde for histological analyses. The live body weight, liver, and ovary were weighed before sampling.

Serum Biochemical Parameters, Progesterone, and Estradiol Levels

Commercial kits (A111-1, A113-1-1, A112-1-1, A110-1-1, A028-2-1, C009-2-1, Jiancheng Biotechnology, Nanjing, China) and a microplate reader (Spark 20M, TECAN, Männedorf, Switzerland) were used to measure cholesterol (**CHO**), low-density lipoprotein cholesterol (**LDL-C**), high-density lipoprotein cholesterol (**HDL-C**), triglyceride (**TG**), albumin (**ALB**), and alanine aminotransferase (**ALT**).

The serum progesterone and estradiol levels were detected using commercial RIA kits (Beijing North Institute of Biological Technology, Beijing, China) by the company of Shanghai Xinfan Biotechnology. The sensitivity for progesterone determinations was 0.02 ng/mL, and the sensitivity for estradiol determinations was 2.7 pg/mL. The intra-assay coefficient of variation was <10%, and the interassay coefficient of variation was <15%.

Morphology Analysis of Liver and Ovary

After fixation in 4% paraformaldehyde for 24 h, the liver and ovary were soaked through a graded series of ethanol and xylene, embedded in paraffin, and sectioned at 4 μm with a Lecia RM2235 microtome (Leica Biosystems, Buffalo Grove, IL). The sections were deparaffinized with xylene and rehydrated through a graded dilutions of ethanol, and stained with hematoxylin and eosin (**HE**). The images of the liver and ovary were acquired using an Olympus simon-01 microscope (Olympus Optical, Beijing, China).

Evaluation of the Oxidative Status in the Ovary

The ovary was precisely weighed 0.2 g and homogenized in 2 mL of ice-cold PBS. After being centrifuged at 12,000 g for 10 min at 4°C, the supernatants were collected to measure the oxidative status. The protein content of the supernatants was measured with a BCA Protein Assay Kit (P0010, Beyotime Biotechnology, Shanghai, China). We assessed catalase (**CAT**) activity, glutathione peroxidase (**GSH-Px**) activity, total antioxidant capacity (**T-AOC**), and malondialdehyde (**MDA**) content in the ovary using commercial reagent kits (S0051, S0056, S0121 and S0131, Beyotime Biotechnology, Shanghai, China). All experimental procedures were performed according to the manufacturer's instructions. All results were normalized to protein concentration in each sample.

TUNEL Assay

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) was used to assay the apoptosis in the ovary. The whole experiment was performed using a commercial TUNEL BrightRed Apoptosis Detection Kit (A113, Vazyme Biotech, Nanjing, China) according to the manufacturer's instructions. First, we deparaffinized, rehydrated the sections of ovary. The sections were then incubated with Proteinase K (20 μg/mL) at room temperature for 20 min. Second, the sections were incubated with the terminal deoxynucleotidyl transferase (**TdT**) enzyme with BrightRed Labeling Mix at 37°C for 60 min in the dark. Finally, the sections were stained with 4′,6-diamidino-2-phenylindole staining solution (C1005, Beyotime Biotechnology, Shanghai, China) for 5 min in the dark. To ensure there was no nonspecific reaction, we performed the negative control without incubation of the TdT enzyme. The fluorescent images were acquired using a LSM 700 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany). The bright of apoptotic cells (red color) was measured using the Image-J (Media Cybernetics, Bethesda, MD).

Western Blotting

The ovary was homogenized in Radio Immunoprecipitation Assay (RIPA) buffer containing phenylmethylsulfonyl fluoride (PMSF) to extract the proteins. Equal amounts of proteins (60 μg) were electrophoresed in 10% (w/v) SDS-PAGE, and then transferred on to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The membranes were blocked with TBST buffer containing 5% bovine serum albumin (**BSA**) for 2 h at room temperature and were incubated overnight at 4°C with primary antibodies. The primary antibodies were PCNA (1:1000, ab29, Abcam, Cambridge, UK), NRF2 (1:1500, 16396, Proteintech Group, Inc., Chicago, IL) and β-actin (1:5000, 66009, Proteintech Group, Inc., Chicago, IL). After washing the membranes with TBST buffer for 4 times, the membranes were incubated with secondary antibody (1:5000, AS003, ABclonal Biotechnology Co., Ltd., Wuhan, China) for 60 min at room temperature. Finally, the blots were developed using an enhanced chemiluminescence (ECL) kit (Thermo Scientific, Wilmington, DE), and were visualized using a Luminescent Image Analyzer LAS-4000 (Fuji Film, Tokyo, Japan). The blots were normalized to β-actin. The intensities of the immunoreactive bands were quantified by Image J to estimate the protein expressions.

RNA Extraction and Detection, cDNA Synthesis, and Real-Time PCR Analysis

TRIzol (TaKaRa, Dalian, Liaoning, China) was used to isolate total RNA in the tissue according to the instructions. The integrity, concentration and purity of total RNA were assessed using the Nanodrop. cDNA

Table 2. Primers used for quantitative real-time PCR analysis.

Gene	Primer sequence (5'→3')	Amplicon size (bp)	GeneBank accession number
ESR1	F: TGTGCTGTGTGCAACGACTA	167	NM_205183.2
	R: CAGGCCTGGCAACTCTTTCT		
ESR2	F: CGGGCGTGGTGACATTAAAC	126	NM_204794.2
	R: CCAGGATGAAGGGTGTGCAA		
VTGII	F:TTGCAAGCTGATGAACACACAC	192	NM_001031276.1
	R: GATTGCTTCATCTGCCAGGTC		
APOB	F:GCCGTTTGACTGGGAGTACA	126	NM_001044633.1
	R: TCTTCCCATTTCCTGGTGCC		
APOV1	F:CAAATGGGGAAACAAAGCAGGA	220	XM_015295934.1
	R: CTTCAGGGACAGTGGTGCTA		
β-actin	F: TGTTACCAACACCCACACCC	110	NM_205518.1
	R: TCCTGAGTCAAGCGCCAAAA		

Abbreviations: F, represents forward; R, represents reward.

synthesis was performed using HiScript II 1st Strand cDNA synthesis kit (+gDNA wiper) (Vazyme, Nanjing, China) according to the manufacturer's instructions (2 μL 500 μg/μL RNA, 4 μL 4*g DNA wiper mix, 1μL Olido [dT]23VN[10 μM], 9μL RNase-free ddH$_2$O, 4μL 5*Select No RT Control Mix). The onestep real-time RT-PCR was performed using ChamQTM SYBR qPCR Master Mix (Vazyme, Nanjing, China) in a real-time PCR machine (QuantStudio 7 Flex, Thermo, Walltham, MA) following the manufacturer's guidelines (Denaturing 95°C 30 s, number of cycles 40, melting curves: 95°C 15 s, 60°C 60s, 95°C 15 s). The primer pairs used are shown in Table 2. β-actin was used as the housekeeping gene. Relative mRNA expression levels of each target gene were normalized to the control using the $2^{-\Delta\Delta CT}$ method. The ratio of the target gene to β-actin represents the amount of gene expression. The average level of 2 repeats was used for statistical analysis.

Statistical Analysis

Data were analyzed by one-way ANOVA using SPSS 24.0. Results were described as the mean ± SEM. $P <$ 0.05 was considered to be statistically significant.

RESULTS
Laying Performance, Reproduction Performance, and Organ Weight

The effects of HF on laying performance are presented in Table 3. The egg-laying rates were observed no significant differences between LHF and HHF group ($P >$ 0.05). Compared with the CON group, HF treatment groups increased the egg-laying rate at the end of the experiment ($P < 0.05$). During the whole experiment period, the egg-laying rate of HF-treated groups was increased in comparison with the CON group ($P <$ 0.05), the average egg weight among the 3 groups showed no differences ($P > 0.05$). Furthermore, we observed that the relative weight of the ovary was increased in group LHF and HHF ($P < 0.05$), but the relative weight of liver was no difference among the 3 groups ($P > 0.05$). In addition, as shown in Table 4,

compared with the CON group, the hatchability in vitro was elevated in group LHF and HHF ($P < 0.05$). No differences were caught sight of fertilization rate and embryo survival rate among 3 groups ($P > 0.05$).

Table 3. Effects of dietary supplementation with HF on the egg-laying rate and egg weight.

Items	Week	CON	LHF	HHF	P-value
		Groups			
Egg-laying rate (%)	1	44.8±2.7	47.8±2.1	46.2±1.7	0.643
	2	46.1±1.2	47.9±2.1	45.1±1.1	0.436
	3	44.9±1.7	45.1±1.5	49.3±3.4	0.355
	4	42.0±1.1	44.8±1.3	42.2±2.5	0.484
	5	37.1±0.8	43.3±0.7	41.4±3.4	0.122
	6	33.8±1.9	39.5±2.3	41.5±4.1	0.188
	7	31.3±0.9[b]	39.4±2.1[a]	40.3±3.4[a]	0.031
	8	30.5±1.2[b]	37.6±1.9[a]	37.1±2.6[a]	0.038
	1-8	38.8±1.1[b]	43.2±0.8[a]	42.9±1.1[a]	0.002
Average egg weight (g)	1	58.8±0.7	57.8±0.8	60.0±0.2	0.387
	2	58.7±0.7	58.9±0.8	59.3±0.3	0.783
	3	59.1±0.9	59.4±0.7	60.1±0.6	0.656
	4	58.9±0.4	59.4±0.8	59.9±0.3	0.438
	5	59.7±0.7	59.6±0.7	59.6±0.3	0.972
	6	59.4±0.6	59.8±0.8	59.3±0.7	0.857
	7	59.5±0.5	59.1±0.5	59.9±0.3	0.458
	8	57.6±0.9	60.0±0.7	59.6±0.8	0.105
	1-8	58.9±0.2	59.2±0.3	59.6±0.2	0.170

Abbreviations: CON, breeder hens fed with a basic diet; HHF, breeder hens fed with a basic diet supplemented with 60 mg/kg HF; LHF, breeder hens fed with a basic diet supplemented with 30 mg/kg HF.

Data are presented as mean value ± SEM (n = 6).

Without the same mark (a, b) represent statistically significant differences ($P < 0.05$).

Table 4. Effects of dietary supplementation with HF on organ weight and incubation performance.

Items	CON	LHF	HHF	P-value
	Groups			
Ovary relative weight (g/kg)	15.8±0.9[b]	19.4±1.1[a]	18.3±0.5[a]	0.024
Liver relative weight (g/kg)	10.7±0.7	10.6±0.5	10.9±0.7	0.950
Fertilization rate (%)	92.1±3.2	91.1±2.8	96.7±1.5	0.306
Embryo survival rate (%)	97.6±2.4	98.9±1.1	97.8±1.4	0.852
Hatchability in vitro (%)	91.3±1.2[b]	96.2±1.6[a]	97.6±1.5[a]	0.031

Abbreviations: CON, breeder hens fed with a basic diet; HHF, breeder hens fed with a basic diet supplemented with 60 mg/kg HF; LHF, breeder hens fed with a basic diet supplemented with 30 mg/kg HF.

Data are presented as mean value ± SEM (n = 6).

Without the same mark (a, b) represent statistically significant differences ($P < 0.05$).

Table 5. Effects of dietary supplementation with HF on the serum biochemical and hormone of aged breeder hens.

Items	Groups			P-value
	CON	LHF	HHF	
T-CHO (mmol/L)	7.9±0.6[a]	6.5±0.6[ab]	5.8±0.6[b]	0.046
TG (mmol/L)	36±4[a]	26 ± 3[b]	24±4[b]	0.045
LDL-C (mmol/L)	1.77±0.27[a]	0.88 ± 0.15[b]	0.85±0.09[b]	0.030
HDL-C (mmol/L)	89±3	94±3	94±2	0.192
ALB (g/L)	10.7±0.9	11.5±1.0	12.0±1.1	0.688
AST (U/L)	13.6±2.2	14.2±2.5	14.2±0.8	0.970
ALT (U/L)	3.5±0.3	3.7±0.7	3.8±0.4	0.924
Estradiol (pg/mL)	196±25[b]	306±41[a]	310±31[a]	0.006
Progesterone (ng/mL)	0.13±0.08	0.11±0.06	0.16±0.08	0.404

Abbreviations: CON, breeder hens fed with a basic diet; HHF, breeder hens fed with a basic diet supplemented with 60 mg/kg HF; LHF, breeder hens fed with a basic diet supplemented with 30 mg/kg HF.

Data are presented as mean value ± SEM (n = 6).

Without the same mark (a, b) represent statistically significant differences ($P < 0.05$).

Serum Biochemical Parameters, Progesterone, and Estradiol levels

Table 5 showed the effects of dietary supplementation with HF on the serum biochemical and hormones of aged breeder hens. Serum concentrations of TG and LDL-C were decreased in LHF and HHF groups ($P < 0.05$). In addition, HHF group had lower T-CHO compared with CON group ($P < 0.05$). Moreover, the serum estradiol levels were increased in LHF and HHF groups ($P < 0.05$), while, no differences on the serum progesterone levels among the 3 groups ($P > 0.05$).

Hepatic Histomorphology and Genes Expressions

We performed HE staining to observe the liver morphology (Figure 1). Lipid and inflammatory cell infiltration were obviously observed in the liver tissue of CON group and the symptoms in group LHF were relieved. Moreover, HHF group showed less inflammatory cell. The mRNA expressions in the liver were represented in Figure 2. We observed that the expressions of *ESR1, ESR2, VTGⅡ, ApoB,* and *ApoV1* in LHF and HHF groups were remarkably increased ($P < 0.05$).

TUNEL Assay and Morphology of Ovary

To estimate the effect of HF supplementation on apoptotic index of ovary, we performed TUNEL assay (Figure 3). The results showed that group LHF and HHF obviously alleviated the apoptosis of ovary ($P < 0.05$).

Additionally, we performed HE staining to observe the ovarian morphology (Figure 4A). We observed that the medulla in the group LHF and HHF was densely structured and blood vessels were distributed compared to the CON group. Besides, 3∼5 preovulation dominant follicles (F1∼F6 grade follicles, 15∼34 mm in diameter) can perceive in group LHF and HHF. We also recorded the number of the primordial follicles in ovaries (Figure 4B). The results showed that hens in group HHF exhibited greater values ($P < 0.05$) in the number of primordial follicles in ovary.

Oxidative Status of Ovary

Figure 5 showed the effects of dietary supplementation with hawthorn leaves flavonoids on the oxidative status of ovary. LHF and HHF group significantly increased the activity of T-AOC and GSH-Pχ in the ovary ($P < 0.05$). Besides, the MDA content in the ovary was obviously reduced in LHF and HHF groups ($P <
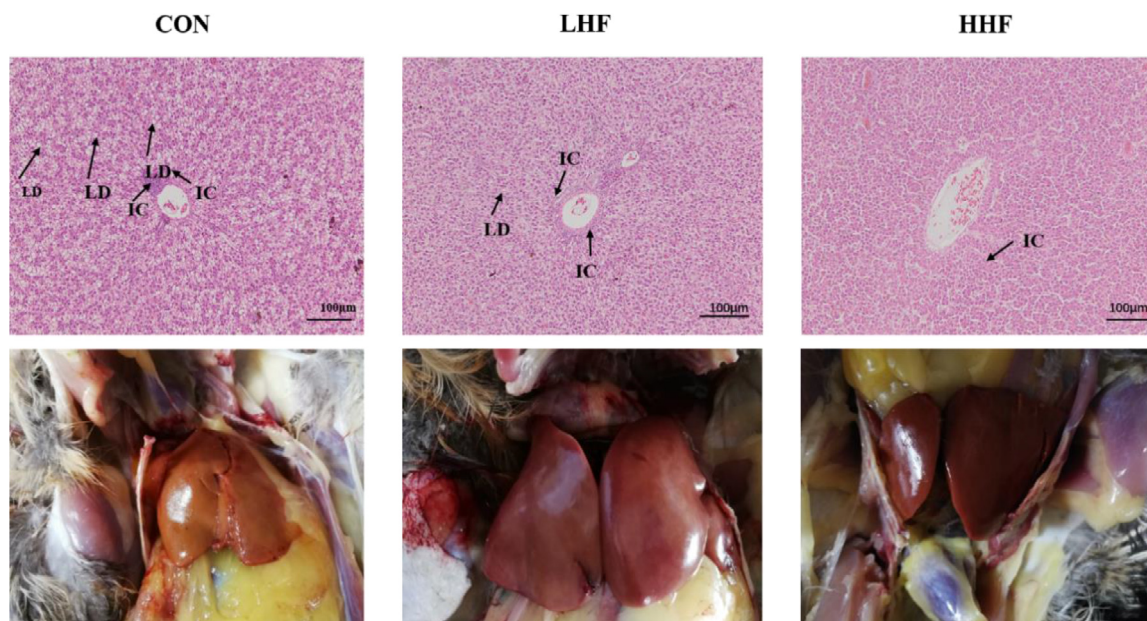

Figure 1. Representative liver histopathological sections. Scale bar = 100 μm. Abbreviations: CON, breeder hens fed with a basic diet; HHF, breeder hens fed with a basic diet supplemented with 60 mg/kg HF; IC, inflammatory cell; LD, lipid droplet; LHF, breeder hens fed with a basic diet supplemented with 30 mg/kg HF.
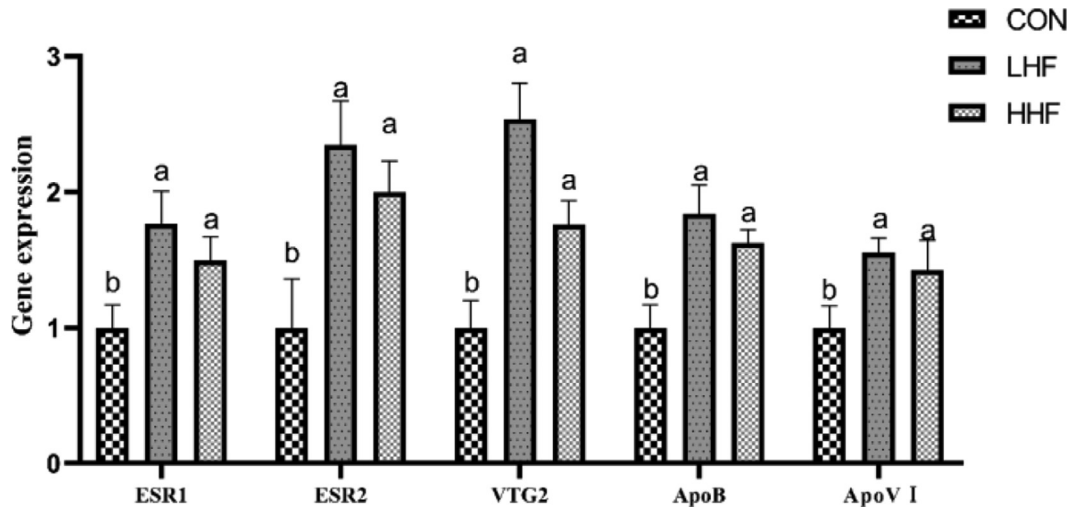
Figure 2. Effects of dietary supplementation with HF on the gene expression in the liver of aged breeder hens. Data are presented as mean value ± SEM (n = 6). Without the same mark (a, b) represents statistically significant differences ($P < 0.05$). Abbreviations: *ApoB*, apolipoprotein B; *ApoV1*, apolipoprotein VLDL I; CON, breeder hens fed with a basic diet; *ESR1*, estrogen receptor 1; *ESR2*, estrogen receptor 2; HHF, breeder hens fed with a basic diet supplemented with 60 mg/kg HF; LHF, breeder hens fed with a basic diet supplemented with 30 mg/kg HF; *VTG2*, vitellogenin 2.
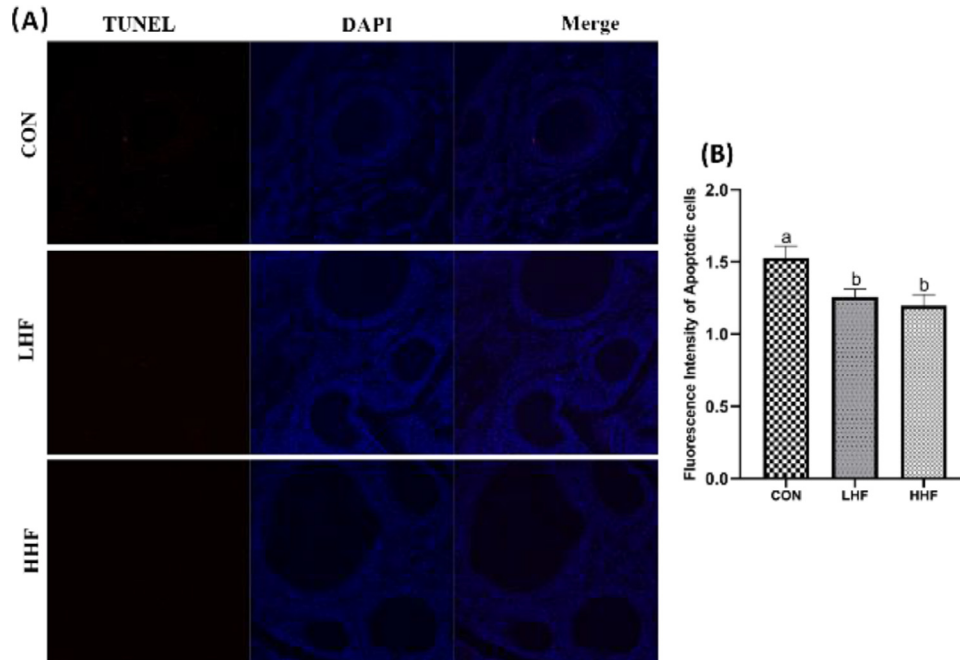
Figure 3. TUNEL assay of ovary in aged breeder hens. The blue color represents the total cells, and red color represents the apoptosis cells in ovary. (A) TUNEL assay of ovary. (B) Statistical analysis of apoptotic index in ovary. Data are presented as mean value ± SEM (n = 6). Without the same mark (a, b) represents statistically significant differences ($P < 0.05$). Abbreviations: CON, breeder hens fed with a basic diet; HHF, breeder hens fed with a basic diet supplemented with 60 mg/kg HF; LHF, breeder hens fed with a basic diet supplemented with 30 mg/kg HF.

0.05). No differences were caught sight of the activity of CAT ($P>0.05$).

Protein Expression of PCNA and Nrf2

LHF and HHF groups exhibited extremely higher expression in ovary PCNA ($P < 0.05$) (Figure 6C). Moreover, HHF group significantly increased the protein expression of PCNA than LHF ($P < 0.05$). Additionally, HHF administration elevated the protein expression of

Nrf2 in the ovary ($P < 0.05$), whereas LHF group had no significantly effect on it ($P>0.05$) (Figure 6D).

DISCUSSION

During the late egg-laying period, the reproduction performance of aged breeder hens decreased rapidly (Nobakht et al., 2006; Sirri et al., 2018; Zhang et al., 2019). It is reported that dietary flavonoid improves the laying performance of aged Hy-Line Brown Hens
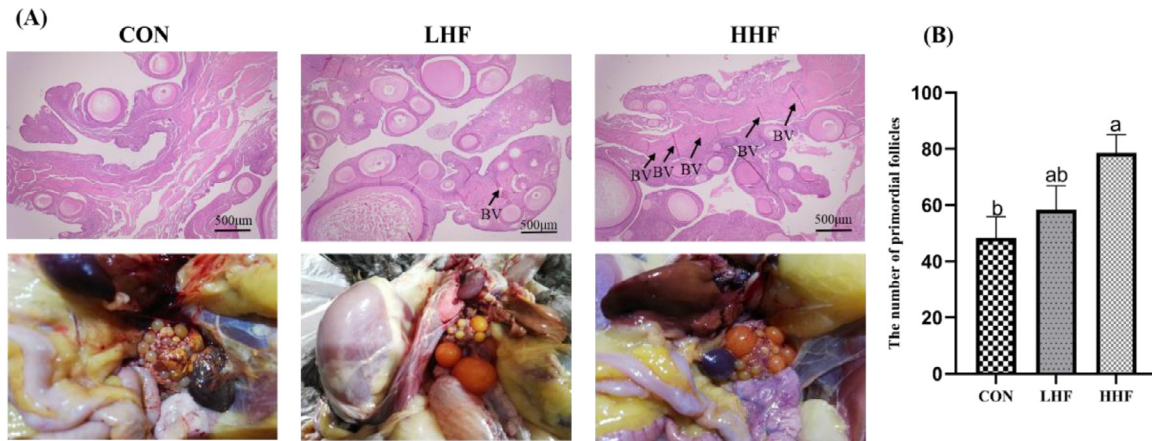
Figure 4. (A) Representative image of ovary of aged breeder hens. (B) Statistical analysis of the number of primordial follicles in ovary. Data are presented as mean value $\pm$ SEM (n =6). Without the same mark (a, b) represent statistically significant differences ($P < 0.05$). Scale bar = 500 μm. Abbreviations: BV, blood vessel; CON, breeder hens fed with a basic diet; HHF, breeder hens fed with a basic diet supplemented with 60 mg/kg HF; LHF, breeder hens fed with a basic diet supplemented with 30 mg/kg HF.

(Wang et al., 2018b). In the current study, dietary supplementation with HF improved the laying performance of aged breeder hens during the late period of the experiment. Several studies have indicated that egg-laying rates have a positive correlation with blood estrogen levels (Long et al., 2017; Dai et al., 2020). Herein, we found that dietary HF increased the serum estrogen levels. Thus, HF could improve the egg-laying performance of aged breeder hens, which was closely related to the changes of serum estrogen.

ROS progressively increased with aging, which contributes to poor production performance in aged female (Wan et al., 2018; Seo et al., 2019). As we all know, the egg-laying performance relates to the process of follicle development and ovulation, which is under the influence of reproductive hormone (Lv et al., 2019). Growing evidence has proved that ROS is associated with ovary dysfunction, which leads to the disorder of steroidogenesis (Sobinoff et al., 2013; Lu et al., 2018). Furthermore, apoptosis induced by senescence and oxidative stress in the ovary is bound to cause lower estrogen levels, thereby reducing the egg-laying performance of aged hens (Manolagas, 2010). It is reported that flavonoids can increase the serum level of estrogen of old laying hen by alleviating the ovary apoptosis (Anwar et al., 2018; Yang et al., 2018; Maues et al., 2019). The current study indicated that adding HF into the diet of aged breeder hens increased the serum levels of estrogen and alleviated the ovary apoptosis. In addition, previous findings have illuminated that decreased apoptosis could be attributed to alleviation of oxidative stress (Sack et al., 2017; Zhai et al., 2017). MDA is the product of oxidative degradation, and it reflects the level of lipid peroxidation. T-AOC, CAT and GSH-Pχ constitute the antioxidant defense system. T-AOC is one of the most important indices reflecting the total antioxidant capacity. CAT and GSH- Pχ act as free radical scavengers to decompose H_2O_2. Hawthorn leaves flavonoids have been reported to increase antioxidant enzyme activity and reduce MDA level in selenite-induced cataract (Qin et al., 2019). In our study, decreased level of MDA

and increased activities of T-AOC and GSH-Pχ demonstrated that the ability of eliminating free radicals was improved in the ovary. Moreover, Nrf2 is a redox-sensitive transcription factor, which increases the activity of antioxidant enzymes and is protective against oxidative stress (Liu et al., 2018a). Our data revealed that dietary HF supplementation could increase the protein expression of Nrf2 in ovary. Similarly, a previous study has indicated that natural flavonoids can reduce oxidative damage of human lung epithelial cells by activating Nrf2 (Li et al., 2018). The expression of protein PCNA (an indicator for cell proliferation) was increased with the alleviation of ovary apoptosis and the enhancement of antioxidant capacity. On the other hand, as female aged, the number of developing oocytes in the ovary is gradually exhausted, which leads to a decline in fertility (Sutherland et al., 2018). Primordial follicles are the reservoir for all developing oocytes, and maintaining the number of primordial follicles in the ovaries, at any point, will determine a female's reproductive lifespan (Fuller et al., 2017; Sutherland et al., 2018). A previous study has indicated that flavonoids can inhibit follicular activation and reduce the incidence of atresia, and thus protect the primordial follicles (Chen et al., 2010; Naseer et al., 2017). Herein, our data revealed that dietary HF supplementation at the level of 60 mg/kg could effectively maintain the number of primordial follicles in aged breeder hen's ovary. Therefore, our results suggest that HF could improve the reproduction performance of the aged breeder hen by relieving ovarian oxidative stress and maintaining the number of primordial follicles.

Fatty liver syndrome, a frequent metabolic disease in aged hens, often exhibits lower egg production, fertilization rate, and hatching rate (Trott et al., 2014; Hamid et al., 2019). The primary cause of fatty liver in caged hens could be imputed to high-energy diets and restricted movement (Gao et al., 2019; Shini et al., 2019). Inflammatory cell infiltration is one of the characteristics of fatty liver. Previous investigations have proved that HF exerts anti-inflammatory properties in
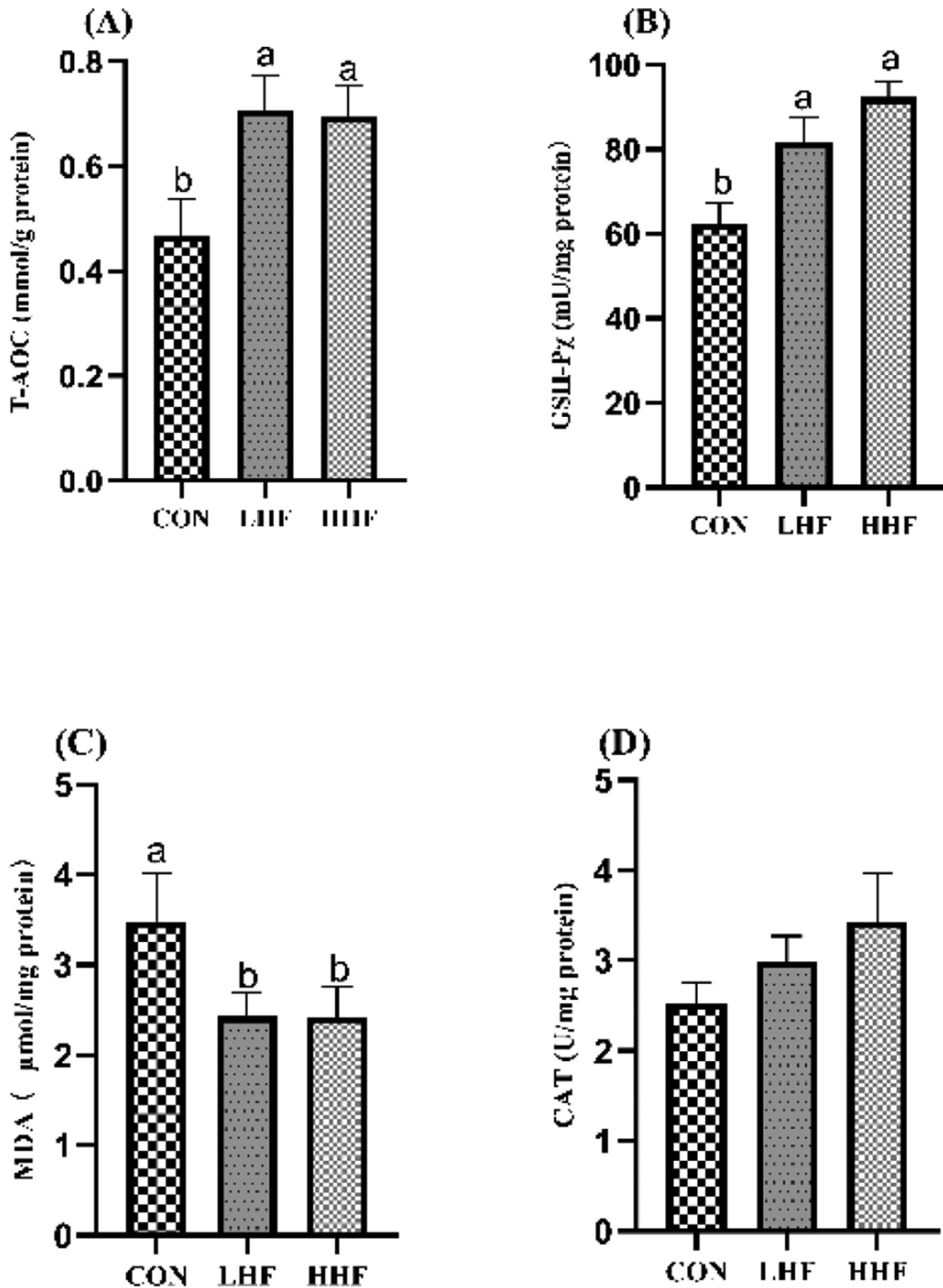
Figure 5. Effects of dietary supplementation with HF on the oxidative status of ovary in aged breeder hens. (A) Activity of T-AOC. (B) Activity of GSH-Px. (C) Activity of MDA. (D) Activity of CAT. Data are presented as mean value ± SEM (n = 6). Without the same mark (a, b) represent statistically significant differences ($P < 0.05$). Abbreviations: CAT, catalase; CON, breeder hens fed with a basic diet; GSH-Pχ, glutathione peroxidase; HHF, breeder hens fed with a basic diet supplemented with 60 mg/kg HF; LHF, breeder hens fed with a basic diet supplemented with 30 mg/kg HF; MDA, malondialdehyde; T-AOC, total antioxidant capacity.

NAFLD rodent (Li et al., 2015; Shi et al., 2019). In current study, HF treated groups reduced inflammatory cell infiltration in the liver of aged breeder hens. HF also has been shown to inhibit the accumulation of TG and free fatty acid in nonalcoholic steatohepatitis mammals (Wang et al., 2011a). In our current study, we also found

that HF treatment showed the effect of antihyperlipidemic in aged breeder hens by decreasing the serum level of TG, T-CHO, and LDL-C. Furthermore, the main form of lipoprotein particles synthesized in the liver is low-density lipoprotein (LDL) and high-density lipoprotein (HDL). ApoB and ApoV1 are the major
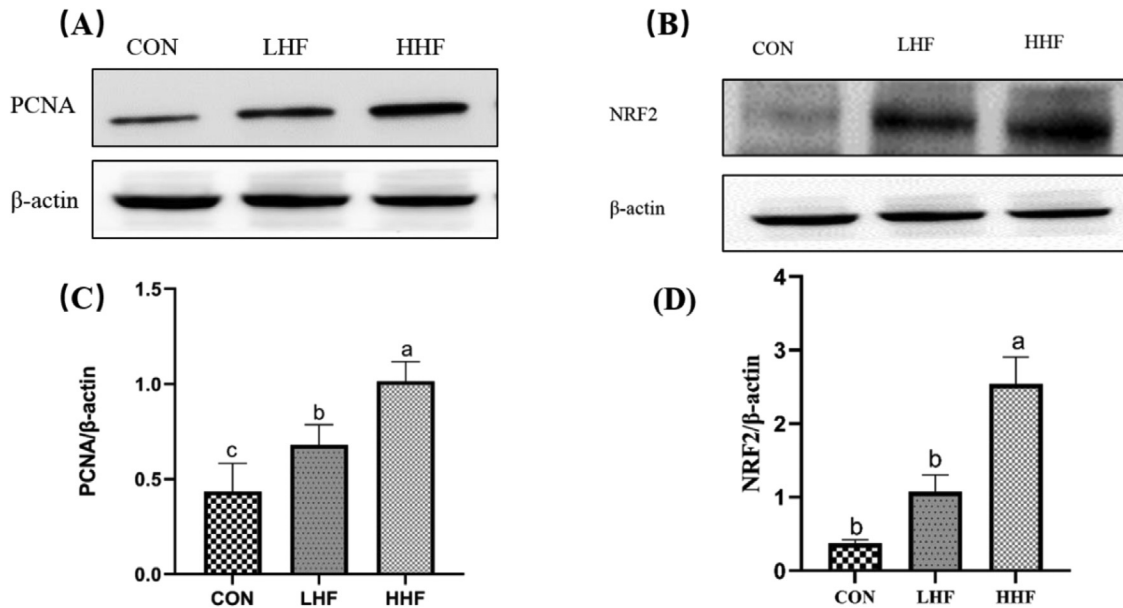
Figure 6. Effects of HF supplementation on the protein expression of PCNA and Nrf2. (A) Western blot analysis of PCNA in the ovary. (B) Western blot analysis of Nrf2 in the ovary. (C) Statistical analysis of PCNA in the ovary. (D) Statistical analysis of Nrf2 in the ovary. Data are presented as mean value $\pm$ SEM (n = 6). Without the same mark (a, b) represent statistically significant differences ($P < 0.05$). Abbreviations: CON, breeder hens fed with a basic diet; HHF, breeder hens fed with a basic diet supplemented with 60 mg/kg HF; LHF, breeder hens fed with a basic diet supplemented with 30 mg/kg HF.

apolipoproteins of chicken VLDL and HDL, which play a role in sustain hepatic lipid homeostasis (Song et al., 2017; Yu et al., 2018). In the present study, dietary HF notably increased the expression of apolipoprotein genes (*ApoB* and *ApoV1*) of aged breeder hens. Additionally, an unfavorable maternal environment may affect the rate of fertilization and hatchability (Wen et al., 2020), and also raise the risk profile for disease in offspring (Wesolowski et al., 2018). Maternal fatty liver may reduce the immune and antioxidant capacity of the fetus, which leads to increased mortality in the offspring (Grossmann et al., 2019). Our data showed that dietary HF supplementation can relieve the symptoms of fatty liver and increase the hatching rate of the fertilized eggs. As we all know, VTG, a female-specific plasma lipid protein, synthesized by the female liver of oviparous. The secretion of VTG is under the stimulation of estrogen by the estrogen receptor (**ER**) pathway (Whitlock et al., 2019). In addition, VTG is the essential precursor of the oocyte yolk, which is fundamental for embryo growth (Nicholls et al., 2002). Here, we found that dietary HF could promote the secretion of estradiol and up-regulate the mRNA expression of *VTGII* and *ER* genes in aged breeder hens. This may be another reason for the increased hatching rate. Based on these results, we found that HF could alleviate the fatty liver by regulating lipid metabolism, and then improve the reproduction performance of aged breeder hen.

CONCLUSIONS

In conclusion, dietary supplementation with HF could improve antioxidant capacity of ovaries and lipid metabolism of liver, thereby enhancing the laying performance of aged breeder hens. Therefore, HF can be used as feed additives to maintain reproductive health of aged breeder hens and the optimal additive dose is 30 mg/kg.

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DISCLOSURES

The authors declare no conflict of interest.

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