Flammulina velutipes stem regulates oxidative damage and synthesis of yolk precursors in aging laying hens by regulating the liver−blood−ovary axis

Haoyuan Wu,* Jing Yuan,* Haixu Yin,* Bo Jing,* Chang Sun,* Ivan Stève Nguepi Tsopmejio,* Zhouyu Jin,* and Hui Song*,†,‡,1

*School of Life Science, Jilin Agricultural University, Changchun 130118, P. R. China; †Engineering Research Center of the Chinenese Ministry of Education for Bioreactor and Pharmaceutical Development, Changchun, Jilin, 130118, P. R. China; and ‡Engineering Research Center of Chinese Ministry of Education for Edible and Medicinal Fungi, Changchun, Jilin, 130118, P. R. China

ABSTRACT Egg production levels in late laying hens are negatively correlated with increasing age. Decreased liver and ovarian function in aging laying hens is accompanied by decreased antioxidant capacity, reproductive hormone levels, and follicular development, resulting in decreased synthesis of yolk precursors. The golden needle mushroom (*Flammulina velutipes*) has been reported to exhibit anti-inflammatory, antioxidant, and hypolipidemic properties. We aimed to reveal the therapeutic effects of *F. velutipes* stem (**FVS**) on liver−blood−ovary axis and investigate the underlying mechanisms. A total of 360 sixty-seven-wk-old laying hens were randomized into 4 treatment groups: 1) basal maize−soybean meal diet (**CON**); 2) basal maize + 20 g/kg FVS (2% FVS); 3) basal maize + 40 g/kg FVS (4% FVS); and 4) basal maize + 60 g/kg FVS (6% FVS). FVS groups demonstrated significantly increased egg production and ovarian development compared with the CON group. The addition of FVS increased the levels of antioxidant enzymes (GSH-Px, T-SOD, and T-AOC) in the liver, serum, and ovaries and decreased malondialdehyde levels by regulating the expression of proteins related to the Keap1-Nrf2/ARE signaling pathway. Additionally, FVS significantly decreased ovarian apoptosis by regulating *Bax, Bcl-2*, and *caspase3* mRNA and protein expression levels. FVS significantly increased the expression levels of estradiol, progesterone, luteinizing hormone, and follicle stimulating hormone and their respective receptors. With increased levels of estradiol transported to the liver through the bloodstream, targeted binding to estrogen receptor (**ER**)-α and ER-β led to significant increases in *ApoVLDL II, ApoB*, and *VTG II* mRNA expression associated with yolk precursor synthesis. FVS decreased the levels of triglyceride and total cholesterol and significantly increased the expression of lipid metabolism, and transport-related mRNAs (*FAS, PPAR-a/γ*, and *MTTP*) in the liver. Therefore, the dietary supplementation of FVS can maintain the productive performance of aging laying hens by alleviating the degree of oxidative stress and regulating the transport of functional substances along the liver−blood−ovary axis, thereby improving the synthesis of yolk precursors.

Key words: *Flammulina velutipes* stem, liver−blood−ovary signal axis, oxidative stress, reproductive hormone, yolk precursor synthesis

2023 Poultry Science 102:102261
https://doi.org/10.1016/j.psj.2022.102261

INTRODUCTION

Female fertility declines with age, mainly because of a decline in oocyte quality and a decrease in the number of follicles (May-Panloup et al., 2016). In female animals, aging begins with the decline of the reproductive system and is manifested by abnormal secretion of steroid hormones and disorders of lipid metabolism, and the gradual loss of reproductive capacity while greatly increasing the risk of disease (Ko and Kim, 2020). Reactive oxygen species (**ROS**) damage is one of the many causes of age-related changes in ovarian function (Wang et al., 2021b). Excess ROS leads to oxidative damage to oocytes and granulosa cells in laying hens follicles, resulting in decreased steroid hormone production and increased follicular atresia (Yoshimura and Tamura, 1988; Zhu et al., 2021). In addition, several studies have found that poultry exhibits a correlation between reproductive function and age as well as the

© 2022 The Authors. Published by Elsevier Inc. on behalf of Poultry Science Association Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Received August 9, 2022.
Accepted October 12, 2022.

[1]Corresponding author: songhuisk@jlau.edu.cn

intrusion of ROS into their reproductive system (Liu et al., 2018b).

It has been well documented that decreased egg production in laying hens is directly related to decreased levels of gonadotropins and sex hormones and decreased production of yolk precursors in the liver (Ciccone et al., 2005; Meng et al., 2013). The balance between ROS production and elimination is disrupted, resulting in oxidative stress, decreased reproductive hormone levels and primordial follicular reserve in the ovary, decreased lipid metabolism and transport in the liver, and decreased production of yolk precursors and their deposition in the oocyte, which in turn leads to a significant decrease in egg production and faces the problem of being culled (Liu et al., 2018b; Gu et al., 2021).

Yolk precursors include very low-density lipoprotein Y (**VLDLy**) and yolk proteinogen vitellogenin (**VTG**), which are the basis for yolk formation (Schneider, 1992). Apolipoprotein B (**ApoB**) and very low-density apolipoprotein II (**ApoVLDLII**) play an important role in the synthesis and processing of VLDLy assembly and modification (Walzem et al., 1999). The primary role of the abovementioned yolk precursors is to transport energy sources, such as triglycerides (**TG**) and total cholesterol (**T-CHO**), synthesized by the liver to the ovary to facilitate the development of follicles in the oocyte and provide essential energy sources for yolk synthesis (Barber et al., 1991). The number of yolk precursors produced is controlled by estradiol (**E_2**), which is transported from the ovary to the liver, where it regulates the synthesis of vitellogenin II (**VTGII**), ApoB, and ApoVLDLII by targeting estrogen receptor (**ER**)-α and ER-β, thereby promoting the production of yolk precursors (Li et al., 2014). VLDLy and VTG reach the ovary from the bloodstream and enter the oocyte via very low-density lipoprotein receptor (**VLDLR**)-mediated cytokinesis, facilitating its development into follicles and ultimately yolk formation (Bujo et al., 1994).

Considering the abovedescribed egg production mechanism of the liver−blood−ovary signaling axis, delaying the aging of laying hens and preventing the deterioration of their liver and ovary functions can not only improve egg production but also prolong their life span, save feed costs, and increase economic benefits. In recent years, many researchers have found that the addition of natural antioxidants or plant extracts, such as resveratrol, lycopene, proanthocyanidins, betaine, and quercetin, to the diet can maintain normal egg production by alleviating oxidative stress during liver or ovarian decline, thereby increasing the production of yolk precursors (Liu et al., 2018a; Omer et al., 2018; Yang et al., 2018; Xing et al., 2020).

The golden needle mushroom (*Flammulina velutipes*) exhibits hypolipidemic, anti-inflammatory, antioxidant, and immunomodulatory activities as a food and medicinal fungus (Yeh et al., 2014). It is rich in polysaccharides, polyphenols, flavonoids, vitamins, and other active components, among which polysaccharides, polyphenols, and flavonoids have exhibited strong antioxidant properties in vitro and in vivo (Rahman et al., 2015; Hu et al., 2016, 2019). Other studies have shown that because some flavonoids can exert estrogen-like effects in animals as phytoestrogens, the golden needle mushroom may act as an agonist to maintain reproductive hormone homeostasis in the body during low estrogenic states (Sirtori et al., 2005). The *F. velutipes* stem (**FVS**), which is the base of the stalk, is often a waste product in production of the mushroom. Using it as a feed additive not only regulates the immune function, intestinal flora, and lipid metabolism of laying hens and broilers but also avoids environmental pollution due to its incineration or use in landfills (Mahfuz et al., 2019a; Liu et al., 2020b). A study by Nguepi Tsopmejio et al. (2021) in our laboratory found that golden needle mushroom improved intestinal inflammation and antioxidant factors in mice through the NF-κB and Nrf2/Keap1 signaling pathways. Based on the active components contained in FVS, this study investigated the effects of FVS on the regulation of antioxidant capacity via the liver−blood−ovary axis in 67-wk-old laying hens, which in turn regulates ovarian apoptosis and liver lipid metabolism and transport, and verified the beneficial effects of reproductive hormones on follicle development and yolk precursor synthesis.

MATERIALS AND METHODS

Experimental Animals and Diet

All experimental birds related to this study were approved by the Animal Care and Use Committee, Jilin Agricultural University, China. One-day-old Hyline-Brown laying hens were purchased from Yinong Poultry Co., Ltd. (Harbin, China), and the rearing site was provided by Huiling Egg Breeding Professional Cooperative (Liaoyuan, China). A total of 360 Hyline-Brown laying hens (1-day-old) were randomly divided into four treatment groups, with 6 replicates of 15 hens per replicate. The experimental treatments were as follows: laying hens fed with a basic corn-soybean diet (**CON**); laying hens fed with a basic corn-soybean diet supplemented with 20 g/kg FVS (2% FVS); laying hens fed with a basic corn-soybean diet supplemented with 40 g/kg FVS (4% FVS); laying hens fed with a basic corn-soybean diet supplemented with 60 g/kg FVS (6% FVS). The analyzed nutritional composition of the experimental diets is presented in Table 1, and the chemical compositions of FVS were summarized in a previous study by our group (Mahfuz et al., 2019a). All laying hens were raised in stepped 3-tier cages equipped with feeders and drinkers, with three hens in each cage. The house temperature was controlled between 19°C and 24°C, with a photoperiod of 16 h of daily light. The *F. velutipes* mushroom was supplied by Xueguogaorong Biotechnology Co., Ltd. (Changchun, China), the mushroom stem was dried under sun and transferred to Hanghong Animal Husbandry Co. Ltd. (Changchun, China) for further uses.

Growth Parameters, Laying Performance, and Egg Quality

At the entire experimental period, feed intake, egg production, and egg weight of each group of hens were

Table 1. Ingredients and nutrient composition of the basal diet and experimental diets (%, as fed-basis).

Items	Groups			
	CON	2%FVS	4%FVS	6%FVS
Ingredient (%)				
Maize corn	62.65	60.8	59.00	57.20
Soyabean meal	25.35	25.10	24.80	24.52
Soyabean oil	0.75	0.85	0.95	1.03
FVS	-	2.00	4.00	6.00
Lysine	0.10	0.10	0.10	0.10
Methionine	0.12	0.12	0.12	0.12
Steamed bone meal	0.70	0.70	0.70	0.70
Limestone	8.93	8.93	8.93	8.93
Common salt	0.30	0.30	0.30	0.30
Vit-mineral premix[1]	0.10	0.10	0.10	0.10
Total	100	100	100	1000
Nutrient level (%)				
ME (MJ/kg)	11.30	11.30	11.30	11.30
CP	17.39	17.39	17.39	17.39
Ca	3.36	3.36	3.36	3.36
P	0.36	0.36	0.36	0.36
EE	3.45	3.45	3.45	3.45
CF	2.55	2.55	2.55	2.55
Lysine	0.95	0.95	0.95	0.95
Methionine	0.40	0.40	0.40	0.40
Cystine	0.31	0.31	0.31	0.31

Abbreviations: CON, laying hens fed with a basic diet; 2%FVS, laying hens fed with a basic diet supplemented with 20 g/kg FVS; 4%FVS, laying hens fed with a basic diet supplemented with 40 g/kg FVS; 6%FVS, laying hens fed with a basic diet supplemented with 60 g/kg FVS. ME, metabolisable energy; CP, crude protein; Ca, calcium; P, phosphorus; EE, crude fat; CF, crude fiber.

[1]The premix provided the following per kg of diets: vitamin A, 4,500 IU; vitamin D3, 1,200 IU; vitamin E, 2,500 IU; vitamin B1, 5,000 mg; vitamin B2, 20,000 mg; vitamin B3, 45,000 mg; vitamin K1, 10,000 mg; pantothenic acid, 35,000 mg; biotin, 1,500 mg; folic acid, 3,000 mg; vitamin B12, 40 mg; Zn, 45 mg; Mn 50 mg; Fe, 30 mg; Cu, 4 mg; Co 120 μg; I, 1 mg; Se, 120 μg.

recorded daily, the feed conversion ratio (**FCR**), egg-laying rate, and average egg weight were calculated. Thirty randomly selected eggs from each group collected were subjected to egg quality measurements by ORKA EA-01 egg quality analyser and EFR-01 eggshell strength tester (ORKA Food Techonology Ltd., Herzeliya, IL) to obtain egg quality data including Haugh unit and shell strength. Determination of eggshell thickness without inner membrane was used by MXRL-25 high-precision digital display micrometer (Starrett, Athol, MA).

The liver, ovary, and abdominal fat were collected and weighed to calculate the liver index, ovary index and abdominal fat index, respectively. Afterward, pre-ovulatory follicles (F1∼F6, 10−40 mm), big yellow follicles (8−10 mm), small yellow follicles (6−8 mm), big white follicles (4−6 mm), small white follicles (2−4 mm) and primary follicles (0.4−2 mm) in the ovaries were separated and the pre-ovulatory follicles index was calculated (Figure S1). The remaining ovarian tissues were then digested using trypsin (Solarbio Science & Technology Co., Ltd., Beijing, China), and the primordial follicles were counted with the aid of a DM4B microscope (Leica Biosystems, Buffalo Grove, IL) and a finer needle.

$$\text{Liver index}(\%) = \text{Liver weight/live body weight} \times 100\%$$

$$\text{Ovary index}(\%) = \text{Ovary weight/live body weight} \times 100\%$$

$$\text{Pre} - \text{ovulatory follicles index } (\%) = \text{Pre} - \text{ovulatory follicles weight/live bodyweight} \times 100\%$$

$$\text{Abdominal fat index } (\%) = \text{Abdominal fat weight/live body weight} \times 100\%$$

Serum and Tissue Sample Collection

Samples were collected at 67 wk of age, a period reflecting the rapid decline in production performance of hens in the late egg-laying period, after being fed the Table 1 diet from 47 wk of age for 20 wk.

One hen was randomly selected from each replicate. Blood was collected from the wing vein, centrifuged at 3,000 *g* for 10 minutes at 4°C to obtain the serum and stored at −20°C. The hens were euthanised and the livers and ovaries were immediately collected for weighing and morphological photography. A small piece of tissue (approximately 0.3 g) from each group was cut and stored in an embedding box and then immersed in 4% paraformaldehyde for histological analyses. The remaining liver and ovary tissues were stored in self-sealing bags and immediately frozen in liquid nitrogen and stored at −80°C.

Oxidative Stress Parameters Assay in the Serum, Liver, and Ovary Tissue

The livers and ovaries were precisely weighed 0.1 g and homogenised in 1 mL of PBS. After centrifugation at 12,000 *g* for 10 min at 4°C, the supernatants were collected to determine the degree of oxidative stress. Total superoxide dismutase (**T-SOD**) and malondialdehyde (**MDA**) were determined by using a chemical colorimetric method according to the manufacturer's instructions of the biochemical kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Their absorbance was then measured respectively at 550 nm and 532 nm. Total antioxidant capacity (**T-AOC**) was calculated by a ABTS method and the absorbance was read at 405 nm, while glutathione peroxidase (**GSH-Px**) was measured at 412 nm by using the enzymatic reaction rate determination method.

Histological Staining

After being fixed in 4% paraformaldehyde solution for 24 h, liver and ovary tissues were soaked in a series of different concentrations of ethanol and xylene, embedded with paraffin. Tissue sections were made using an RM2235 microtome (Leica Biosystems), followed by

hematoxylin-eosin (**HE**) staining and histological observation under a DM4B microscope (Leica Biosystems).

Serum Levels of Reproductive Hormones

According to the instructions of enzyme-linked immunosorbent assay (**ELISA**) kits (Jingmei Biological Technology Co., Ltd, Yancheng, China), 10 μL of serum and 40 μL of sample dilution were added to the antibody wells in triplicate accurately and incubated at 37°C for 30 min. After washing with concentrated washing solution, the enzyme standard reagent was added and incubated at 37°C for 30 min. After washing again, the chromogen solution and stop solution were added to each well and the levels of E2, progesterone (**P_4**), follicle-stimulating hormone (**FSH**), and luteinizing hormone (**LH**) were measured at 450 nm using a spectrophotometer (Thermo Fisher Scientific Co., Shanghai, China).

Yolk Precursors and Their Receptor Synthesis Capacity

The levels of TG, T-CHO, aspartate transaminase (**AST**), and alanine transaminase (**ALT**) were measured in livers and serum using biochemistry kits (Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China) according to the manufacturer's instructions. The levels of VTG and VLDLy were also measured from the serum using enzyme-linked immunosorbent assay (ELISA) kits (Jingmei Biological Technology Co., Ltd) with guidance from the manufacturer.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted with Trizol reagent (GenStar, Beijing, China) according to the manufacturer's protocol. Their concentrations and purities were determined using a Infinite M200 Pro NanoQuant (Tecan Trading AG, Switzerland), using samples with A260/280 values between 1.8 and 2.0. Furthermore, single-stranded cDNA was synthesized using the SPARKscriptⅡ RT Plus Kit (Sparkjade Biotechnology Co., Shandong, China) according to the manufacturer's instructions. The cDNA (2 μL), 2 × SYBR qPCR Mix (10 μL), forward and reverse primers (0.4 μL) (Table 2), ROX Reference DyeII (0.4 μL) and RNase Free dH2O (6.8 μL) were mixed to form a 20 μL system. The quantitative real-time PCR (**qRT-PCR**) was performed using a Real-Time PCR machine (Agilent Mx3000P, Santa Clara, CA) with the following reaction conditions: pre-denaturation at 95°C for 30 s followed by 40 cycles of 95°C for 5 s, 60°C for 30 s and finally 72°C for 30 s. In this experiment, all the samples were replicated 3 times and β-actin was used to normalize the relative abundance of each transcript. The relative mRNA expression of the target gene was calculated using the 2-ΔΔCT method.

Western Blot Analysis

Liver and ovarian samples were homogenised in Radio Immunoprecipitation Assay (**RIPA**) buffer (Solarbio Science & Technology Co.,Ltd.) supplemented with phenylmethylsulfonyl fluoride (**PMSF**) (Solarbio Science & Technology Co.,Ltd.) and denatured at 100°C for 10 min. Protein concentrations were determined using a BCA analysis kit (Biotechnology Co., Beijing, China), equal amounts of proteins were electrophoresed in 10% SDS-PAGE and transferred onto polyvinylidene difluoride (**PVDF**) membranes blocked with skimmed milk diluted in TBS-0.1% tween 20 for 3 h and incubated overnight at 4°C with primary antibodies. The primary antibodies were nuclear factor E2-related factor 2 (**Nrf2**) (Wanlei Biotechnology Co., Shenyang, China), kelch-like ECH-associated protein 1 (**Keap1**) (Cell Signaling Technology, Inc., Beverly, MA), heme oxygenase 1 (**HO-1**) (Wanlei Biotechnology Co., Shenyang, China), Bcl-2 associated X protein (**Bax**) (Proteintech Group, Inc., Wuhan, China), B cell lymphoma 2 (**Bcl-2**) (Proteintech Group, Inc., Wuhan, China), caspase3 (Wanlei Biotechnology Co., Shenyang, China), and β-actin (Cell Signaling Technology, Inc.). Then, the membranes were washed and incubated with secondary antibody (Bioss Biotechnology Ltd., Beijing, China) for 90 min at room temperature. The positive bands were developed by enhanced chemiluminescence (**ECL**) kits (Sparkjade Biotechnology Co., Ltd.), and were visualized using a Fusion FX6. EDGE Systems (VILBER BIO IMAGING Co., Paris, France). The blots were normalized to β-actin and quantified via Fusion.

Statistical Analysis

Significances of means between different groups were tested by one-way analysis of variance (**ANOVA**) followed by Ducan test for multiple comparisons by SPSS version 26.0 (SPSS Inc., Chicago, IL). All statistics are analysed using Graphpad Prism (GraphPad Software Inc., San Diego, CA). All experimental data were presented as mean ± standard error (**SE**). Compared with the CON group, * means $P < 0.05$, ** means $P < 0.01$.

RESULTS

Growth Parameters, Laying Performance, and Egg Quality

The results presented in Table 3 showed that there was no difference in the liver index between the CON group and all FVS groups ($P > 0.05$). Furthermore, we observed that the ovary and pre-ovulatory follicles index were increased in both the 4%FVS and 6%FVS groups. For abdominal index, we observed significant decreases in the 4%FVS and 6%FVS groups as compared to the CON group. For laying performance and egg quality, compared with the CON group, the 2%FVS group increased the egg-laying rate remarkably, but no differences were caught sight of avergage egg weight and feed

Table 2. Gene-specific primers sequences for quantitative real-time PCR.

Gene	Primers (from 5′ to 3′)	Length (bp)	GeneBank accession number
MTTP	F: TTCAGGCATTCCGTGACCAAGTATG R: TCCAACATTTCTGCTTTCCCTCTCC	83	NM_001109784.2
FAS	F: TCTCTGCCATCTCCCGAACTTCC R: TCTCAATTAGCCACTGTGCCAACTC	102	NM_205155.1
PPAR-α	F: CCTTTCACCAGCATCCAGTCCTTC R: TGTACTCCGTAATGGTAGCCTGAGG	138	NM_001001464.1
PPAR-γ	F: GAATGCCACAAGCGGAGAAGGAG R: TTTGGTCAGCGGGAAGGACTTTATG	140	NM_001001460.1
ER-α	F: TGTGCTGTGTGCAACGACTA R: CAGGCCTGGCAACTCTTTCT	167	NM_205183.2
ER-β	F: CCCTCCCAGCAGCAAACAACTC R: ACATCTCCAGCAGCAAGTCATACAC	145	NM_204794.2
APOVLDL-II	F: AGCAGGACAGCAGGTCTCTTGG R: TCAGGGACAGTGGTGCTAAGGA	114	NM_205483.2
APOB	F: GCCGTTTGACTGGGAGTACA R: TCTTCCCATTTCCTGGTGCC	126	NM_001044633.1
VTGII	F: TTGCAAGCTGATGAACACACAC R: GATTGCTTCATCTGCCAGGTC	192	NM_001031276.1
FSHR	F: ACCTGCCTGGATGAGCTAAA R: ATCCATGACTTGGCAGGAAG	136	NM_205079.1
LHR	F: CGTCCTCATAACCAGCCACT R: AGCATCCACCGAAGCAAT	115	NM_204936.1
BAX	F: GTGATGGCATGGGACATAGCTC R: TGGCGTAGACCTTGCGGATAA	90	XM_422067.4
BCL-2	F: ATCGTCGCCTTCTTCGAGTT R: ATCCCATCCTCCGTTGTCCT	150	Z11961.1
CASPASE3	F: ACTCTGGAATTCTGCCTGATGACA R: CATCTGCATCCGTGCCTGA	129	NM_204725.1
NRF2	F: GGGACGGTGACACAGGAACAAC R: GCTCTCCACAGCGGGAAATCAG	93	NM_205117.1
KEAP1	F: CATCGGCATCGCCAACTT R: TGAAGAACTCCTCCTGCTTGGA	113	XM_025145847.1
HO-1	F: GCTGGGAAGGAGAGTGAGAGGAC R: GCGACTGTGGTGGCGATGAAG	107	NM_205344.1
NQO1	F:TCGCCGAGCAGAAGAAGATTGAAG R: CGGTGGTGAGTGACAGCATGG	191	NM_001277619.1
MAF	F: CCTGGCCATGGAATATGTTAATGA R: TGATAATGCGATCGGTCTCCAC	83	NC_000074.7
GPX-1	F: GCTGTTCGCCTTCCTGAGAG R: GTTCCAGGAGACGTCGTTGC	118	NM_001277853.2
SOD-1	F: TCTTACCGGACCACACTGCATC R: ACGAGGTCCAGCATTTCCAGTTA	115	NM_205064.1
β-actin	F: AGCCAACAGAGAGAAGATGACAC R: CATCACCAGAGTCCATCACAATA	134	NM_205518.1

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

conversion ratio (FCR) among 4 groups ($P > 0.05$). Furthermore, the Eggshell thickness was remarkably elevated in all FVS groups as compared to the control group. However, there was no difference in the haugh unit and eggshell strength between the CON group and all FVS groups.

Antioxidant Capacity and MDA Levels of the Serum, Liver, and Ovary Tissue

As shown in Table 4, the activities of T-SOD, GSH-Px, and T-AOC and the amount of MDA in the livers of 6%FVS group were remarkably changed compared to those of the CON group. Moreover, T-AOC activity was dramatically enhanced, and the MDA level was greatly inhibited in the 4%FVS group. And the T-AOC activity of the 2%FVS group had a significant downward trend ($P < 0.01$). The levels of T-SOD and GSH-Px in the serum of all FVS groups were higher compared to the CON group ($P < 0.01$), and the MDA levels were significantly decreased ($P < 0.01$). Besides, the serum T-AOC level was significantly elevated in the 2%FVS group. Compared with the CON group, the levels of T-SOD, GSH-Px and T-AOC in the ovaries of the 2% and 4%FVS groups were remarkably enhanced, and the MDA levels were significantly decreased. Interestingly, the antioxidant enzyme activities and MDA level in the 4%FVS group did not show any changes ($P > 0.05$).

Genes and Proteins Significantly Differentially Expressed in the Nrf2-Keap1/ARE Signaling Pathway of the Liver and Ovary

As demonstrated in Figure 1A, the mRNA levels of *Nrf2* in liver and ovary were significantly increased in the 2%FVS and 4%FVS groups ($P < 0.01$), but the Nrf2 level in liver of 6%FVS group had no difference with the CON group. By contrast, the mRNA levels of *Keap1* in the liver tissues of all FVS groups were greatly inhibited

Table 3. Effects of dietary supplementation with FVS on Growth parameters and laying performance and egg quality.

Items[1]	Groups			
	CON[2]	2%FVS	4%FVS	6%FVS
Organ parameters (%)				
Liver index	1.48 ± 0.15	1.52 ± 0.11	1.57 ± 0.18	1.51 ± 0.23
Ovary index	1.96 ± 0.08[b]	2.16 ± 0.11[ab]	2.42 ± 0.22[a]	2.40 ± 0.13[a]
Pre-ovulatory follicles index	1.49 ± 0.10[b]	1.67 ± 0.09[ab]	1.82 ± 0.10[a]	1.89 ± 0.12[a]
Abdominal fat index	3.07 ± 0.38[a]	2.22 ± 0.36[ab]	1.21 ± 0.34[b]	1.41 ± 0.30[b]
Laying performance				
Avergage egg weight (g)	58.14 ± 3.23	61.81 ± 5.61	60.33 ± 5.24	56.98 ± 5.34
Egg laying rate (%)	0.75 ± 0.04[bc]	0.86 ± 0.02[a]	0.73 ± 0.04[c]	0.83 ± 0.03[ab]
FCR (g of feed/g of egg)	3.09 ± 0.19	2.68 ± 0.20	3.31 ± 0.21	2.91 ± 0.27
Egg quality				
Haugh unit	67.66 ± 4.54	71.43 ± 3.27	66.05 ± 4.07	68.66 ± 3.54
ES (kgf/m^2)	34.05 ± 2.48	34.49 ± 2.50	33.72 ± 1.65	34.25 ± 2.06
ET (mm)	0.30 ± 0.01[b]	0.34 ± 0.01[a]	0.34 ± 0.01[a]	0.33 ± 0.01[a]

Without the same mark (a, b) represent statistically significant differences ($P < 0.05$).
The values are represented as mean ± SEM, n = 6.
[1]Abbreviations: CR, feed conversion ratio; ET, eggshell thickness; FES, eggshell strength; Kgf/m^2, kilogram-force/m^2.
[2]CON, laying hens fed with a basic diet; 2%FVS, laying hens fed with a basic diet supplemented with 20 g/kg FVS; 4%FVS, laying hens fed with a basic diet supplemented with 40 g/kg FVS; 6%FVS, laying hens fed with a basic diet supplemented with 60 g/kg FVS.

Table 4. Effects of FVS on the antioxidant capacity and MDA levels of the liver, serum, and ovary of hens.

Items[1]	Groups[2]				SEM	*P* value
	CON	2%FVS	4%FVS	6%FVS		
Liver						
T-SOD (U/mg prot)	14.29[bc]	13.89[c]	14.43[b]	15.36[a]	0.31	0.25
GSH-Px (U/mg prot)	144.25[b]	153.24[b]	147.36[b]	168.82[a]	3.74	0.05
T-AOC (mmol/g prot)	0.62[c]	0.78[a]	0.69[b]	0.78[a]	0.04	<0.01
MDA (nmol/mg prot)	0.44[a]	0.38[ab]	0.31[bc]	0.28[c]	0.04	0.21
Serum						
T-SOD (U/mL)	98.34[b]	111.86[a]	112.24[a]	111.76[a]	8.46	0.14
GSH-Px (U/mL)	234.28[c]	287.44[b]	299.18[b]	355.23[a]	5.75	<0.01
T-AOC (mmol/L)	0.44[bc]	0.46[a]	0.43[c]	0.45[ab]	0.01	0.02
MDA (nmol/mL)	18.60[a]	6.75[b]	8.26[b]	7.03[b]	1.21	<0.01
Ovary						
T-SOD (U/mg prot)	20.01[bc]	22.36[a]	18.30[c]	21.25[ab]	0.90	0.02
GSH-Px (U/mg prot)	297.66[b]	269.16[b]	204.81[b]	424.94[a]	52.82	0.61
T-AOC (mmol/g prot)	0.52[b]	0.84[a]	0.55[b]	0.79[a]	0.12	0.02
MDA (nmol/mg prot)	4.27[a]	3.04[b]	4.28[a]	2.98[b]	0.60	0.07

Without the same mark (a, b, c) represent statistically significant differences ($P < 0.05$).
The values are represented as mean ± SEM, n = 6.
[1]Abbreviations: GSH-Px, glutathione peroxidase; MDA, malondialdehyde; T-SOD, total superoxide dismutase; T-AOC, total antioxidant capacity.
[2]CON, laying hens fed with a basic diet; 2%FVS, laying hens fed with a basic diet supplemented with 20 g/kg FVS; 4%FVS, laying hens fed with a basic diet supplemented with 40 g/kg FVS; 6%FVS, laying hens fed with a basic diet supplemented with 60 g/kg FVS.

as compared to the CON group ($P < 0.01$); and the mRNA levels of *Keap1* in the ovary of the 2%FVS and 4%FVS groups were significantly decreased as compared to the CON group. Futhermore, the mRNA expression of *small Maf proteins* (***sMaf***) in the liver of the 2%FVS and 6%FVS groups was significantly elevated compared with the CON group; and the mRNA abundance of *sMaf* in the ovary of the 4%FVS and 6%FVS groups was extremely higher as compared to CON group. For mRNA expression of *HO-1* in liver tissues, the values of the 2%FVS, 4%FVS, and 6%FVS groups were significantly higher than the CON group ($P < 0.01$); and the mRNA expression level of *HO-1* in the ovary was significantly increased in the 2%FVS and 6%FVS groups as compared to the CON group ($P < 0.01$). In addition, the mRNA expression of *NAD(P)H quinone oxidoreductase 1* (***NQO1***) in the liver and ovary tissues was remarkably enhanced in all FVS groups as compared to the CON group ($P < 0.01$). Moreover, we observed that the mRNA expression of *glutathione peroxidase 1* (***GPX1***) in the 4%FVS and 6%FVS groups was significantly higher as compared to the CON group ($P < 0.01$); the mRNA expression of *GPX1* in the ovary was significantly elevated in the 2%FVS and 6%FVS groups ($P < 0.01$). Furthermore, compared with the CON group, the mRNA expression of *superoxide dismutase 1* (***SOD1***) in the liver and ovary tissues was remarkably increased in the 2%FVS and 4%FVS groups. As shown in Figures 1B and 1C, 2%FVS and 4%FVS could remarkably increase the expression of Nrf2 and HO-1 proteins in liver tissues ($P < 0.01$); however, for ovary, the protein levels of Nrf2 and HO-1 were significantly enhanced in the 2%FVS, 4%FVS, and 6%FVS groups ($P < 0.01$). By contrast, FVS remarkably reduced Keap1 expression in a

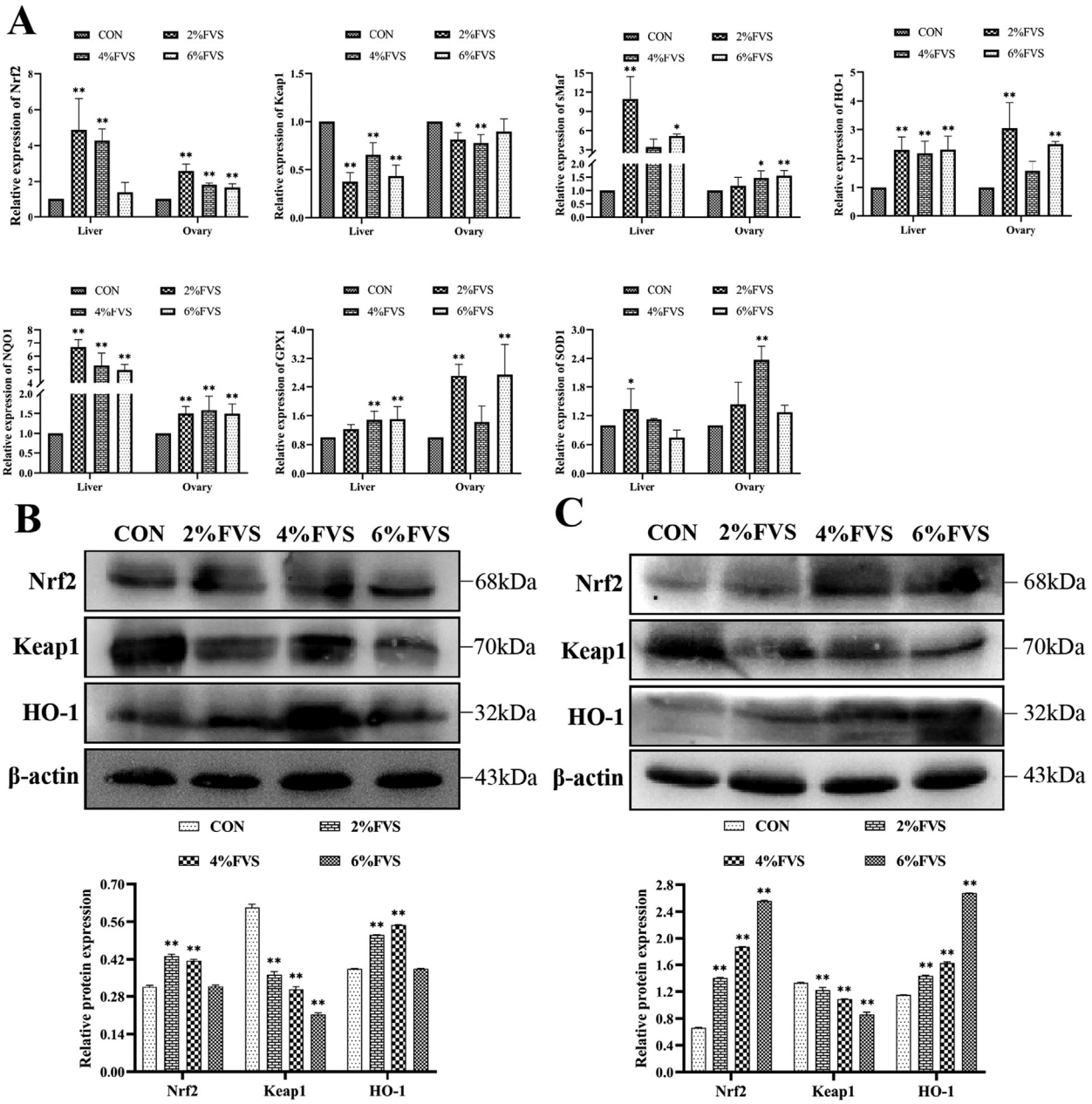

Figure 1. FVS affected the Nrf2-Keap1/ARE pathway in the liver and ovary. (A) The mRNA abundance of Nrf2, Keap1, and downstream genes in the liver and ovary. (B) Effects of FVS on the protein levels of Nrf2, Keap1 and HO-1 in the liver. (C) Effects of FVS on the protein levels of Nrf2, Keap1 and HO-1 in the ovary. Abbreviations: Nrf2, nuclear factor E2-related factor 2; Keap1, kelch-like ECH-associated protein 1; sMaf, small Maf protein; HO-1, heme oxygenase 1; NQO1, NAD(P)H quinone oxidoreductase 1; GPX1, glutathione peroxidase 1; SOD1, superoxide dismutase 1; CON, laying hens fed with a basic diet; 2%FVS, laying hens fed with a basic diet supplemented with 20 g/kg FVS; 4%FVS, laying hens fed with a basic diet supplemented with 40 g/kg FVS; 6%FVS, laying hens fed with a basic diet supplemented with 60 g/kg FVS. The results are expressed as the mean ± SEM (n = 4); $^{*}P < 0.05$ and $^{**}P < 0.01$.

concentration-dependent trend compared with the CON group ($P < 0.01$). Simultaneously, the gene expressions of Nrf2, Keap1, and HO-1 were consistent with the results of the protein expression.

Ovarian Histomorphology, and the Expression Levels of Genes and Proteins Associated With Apoptosis

As shown in Figure 2A, the morphology and structure of the ovaries were normal in all groups. Different numbers of primordial and pre-ovulatory follicles (F1−F6, 15−34 mm in diameter) could be found. We observed that the medulla in all FVS groups were densely structured compared to the CON group, and the number of atretic follicles was decreased. Furthermore, by observing the denseness of the membrane layers in each group of mature follicles, we hypothesized that FVS might have facilitating effects on granulosa cells development. Moreover, we recorded the number of the primordial follicles and pre-ovulatory follicles indexes (Figures 2B and 2C). These results indicated that the values of 4%FVS and 6%FVS groups were dramatically enhanced as compared to the CON group.

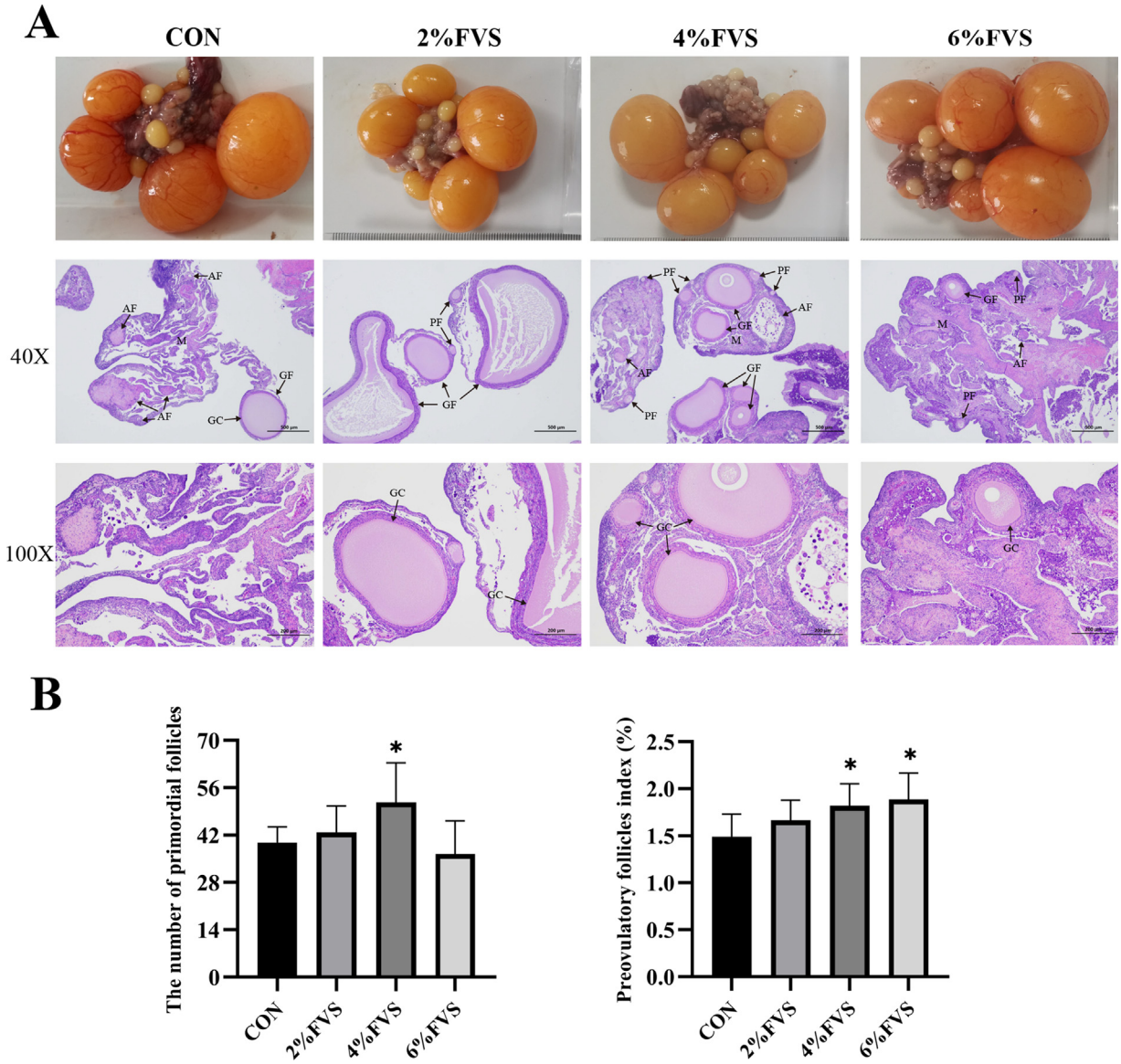

Figure 2. Influence of FVS on the follicular parameters and histology of the ovary in hens. (A) Hematoxylin-eosin (HE) staining of the hen's ovary tissue. (B) Statistical analysis of the number of primordial follicles and pre-ovulatory follicles index in ovary. Scale bar = 200 and 500 μm. Abbreviations: AF, atresia follicle; CON, laying hens fed with a basic diet; GF, growing follicles; GC, granulosa cells; M, medulla; PF, primary follicles; 2%FVS, laying hens fed with a basic diet supplemented with 20 g/kg FVS; 4%FVS, laying hens fed with a basic diet supplemented with 40 g/kg FVS; 6%FVS, laying hens fed with a basic diet supplemented with 60 g/kg FVS. The results are expressed as the mean ± SEM (n = 6); $^{*}P < 0.05$ and $^{**}P < 0.01$.

Figure 3A shows the transcription level of Bax was reduced in the 4%FVS and 6%FVS groups as compared to the CON group. Bcl-2 was significantly increased in the 2%FVS group ($P < 0.01$), but there was no difference observed among the 4%FVS, 6%FVS, and the CON groups. Moreover, the mRNA expression of *caspase3* was greatly inhibited in all FVS groups ($P < 0.01$). To further validate the molecular mechanism, we evaluated the protein levels of Bax, Bcl-2 and caspase3 (Figures 3B and 3C). The protein level of Bax was significantly decreased in all FVS groups ($P < 0.01$). In addition, FVS remarkably enhanced Bcl-2 expression and decreased Bax/Bcl-2 level in a dose-dependent manner compared with the CON group ($P < 0.01$). By contrast, the caspase3 levels of the 2%FVS and 6%FVS groups were significantly higher than that in the CON group ($P < 0.01$). Interestingly, the expression of the caspase3 protein in 4%FVS group did not show any changes.

The Levels of Reproductive Hormone and Their Receptors

The results presented in Figure 4A indicated that the levels of E_2 of 2%FVS and 4%FVS groups were extremely higher as compared to the CON group. Besides, the levels of P_4 and LH of the 2%FVS group were remarkably higher than those of the CON group ($P < 0.01$). In addition, we observed that the levels of FSH of 4% and 6%FVS groups were significantly higher compared to the CON group. In Figure 4B, our results indicated that the mRNA expression of *ER-α* was

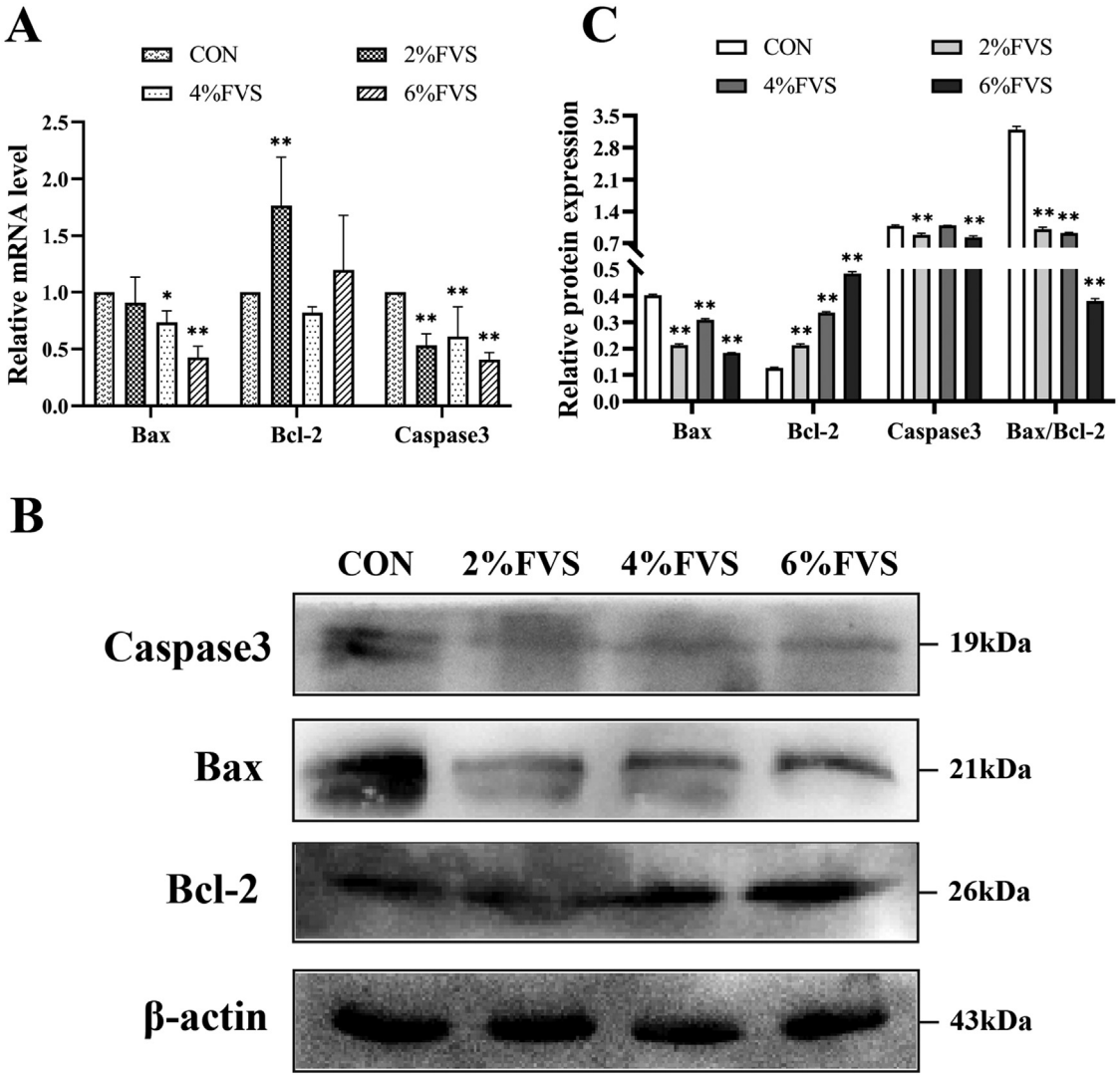

Figure 3. FVS alleviated ovarian apoptosis via the Bax/Bcl-2 and caspase3 pathway. (A) Effects of FVS on the relative expression of apoptosis-related genes in the ovary tissues. (B and C) Effects of FVS on the protein levels of Bax, Bcl-2 and caspase3 in the ovary. Abbreviations: CON, laying hens fed with a basic diet; 2%FVS, laying hens fed with a basic diet supplemented with 20 g/kg FVS; 4%FVS, laying hens fed with a basic diet supplemented with 40 g/kg FVS; 6%FVS, laying hens fed with a basic diet supplemented with 60 g/kg FVS. The results are expressed as the mean ± SEM (n = 4); $^{*}P < 0.05$ and $^{**}P < 0.01$.

remarkably increased in 2%FVS group ($P < 0.01$). The mRNA expression of *ER-β* was enhanced in all FVS groups as compared to the CON group ($P < 0.01$). For the mRNA abundance of *FSHR* and *LHR*, the 4%FVS and 6%FVS groups had more elevated levels as compared to CON groups ($P < 0.01$), but no difference was observed between 2%FVS and CON groups ($P > 0.05$).

Determination of Yolk Precursors and Their Receptor Synthesis Levels

Liver histomorphology was observed by HE staining (Figure 5A). The results showed that the liver tissue was significantly infiltrated with lipid and inflammatory cells in the CON group, and the FVS group exhibited decreased lipid droplets and less inflammation. In addition, liver AST and ALT levels were significantly decreased in the 6% FVS group compared with the CON group (Figure 5B), verifying the ameliorative effect of FVS on liver damage and dysfunction in hens.

The 6% FVS group exhibited significantly decreased serum T-CHO levels compared with the CON group, with no significant change in TG levels (Figure 6A). In addition, TG levels were significantly decreased in the liver in the 2, 4, and 6% FVS groups ($P < 0.01$), and T-CHO levels were significantly decreased in the liver in the 4% FVS group. In addition, VTG and VLDLy levels were significantly higher in the 2% FVS group than in the CON group, and VLDLR levels were significantly higher in the ovaries of the 2% FVS group. Figure 6B shows the transcript levels of genes related to liver yolk precursor synthesis and lipid metabolism in laying hens. The expression levels of *ApoB* and *VTG II* mRNA were significantly increased in the 2, 4, and 6% FVS groups compared with the CON group; interestingly, the addition of FVS had no significant effect on *ApoVLDL II* mRNA abundance. The mRNA expression levels of *fatty acid synthase* (***FAS***) were significantly increased in the

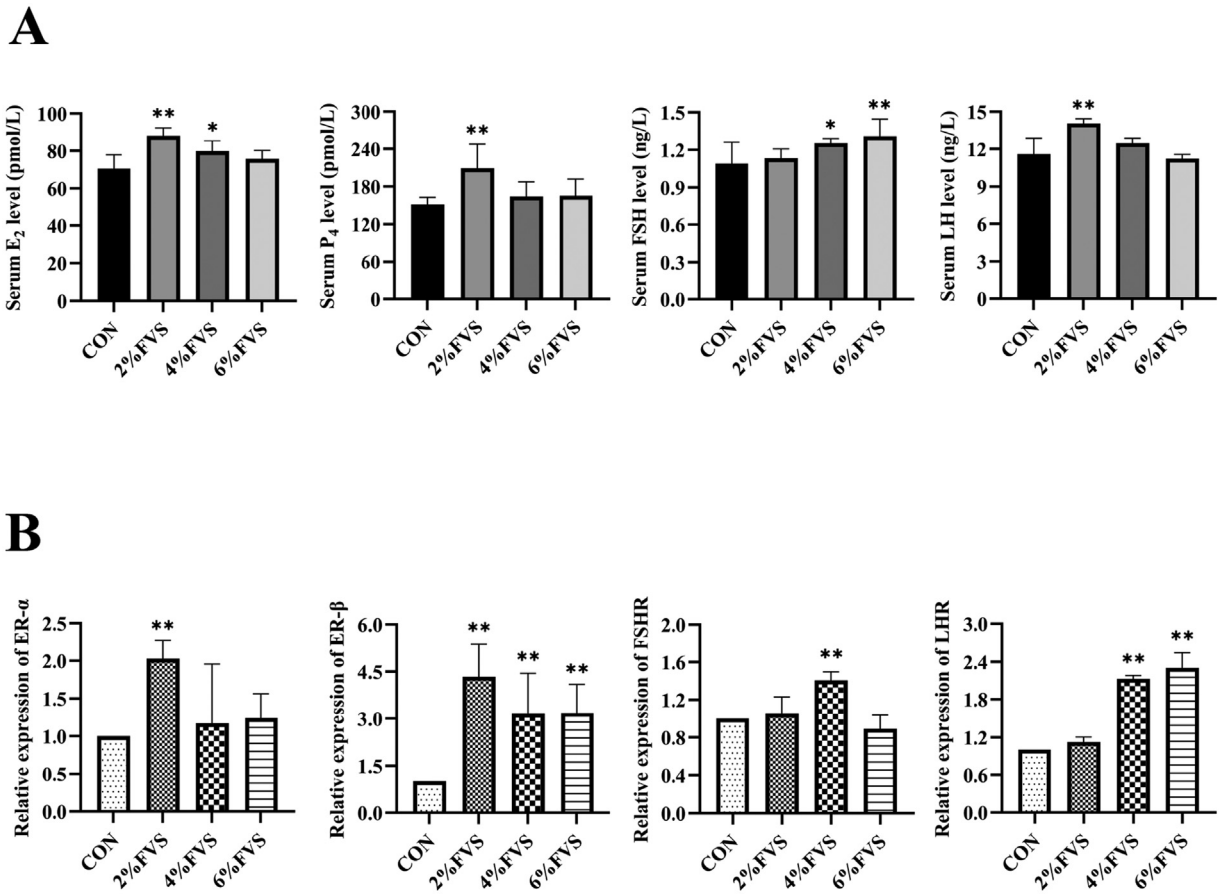

Figure 4. Effects of FVS on the levels of serum reproductive hormone and their receptors. (A) The levels of serum E_2, FSH, LH and P_4. (B) The levels of mRNA expression of hormone receptors in the ovary of hens. Abbreviations: CON, laying hens fed with a basic diet; E_2, estradiol; ER-β, estrogen receptor-β; ER-α, estrogen receptor-α; FSH, follicle-stimulating hormone; FSHR, follicle-stimulating hormone receptor; LH, luteinizing hormone; LHR, luteinizing hormone receptor; P_4, Progesterone; 2%FVS, laying hens fed with a basic diet supplemented with 20 g/kg FVS; 4%FVS, laying hens fed with a basic diet supplemented with 40 g/kg FVS; 6%FVS, laying hens fed with a basic diet supplemented with 60 g/kg FVS. The results are expressed as the mean ± SEM (n = 6); $^*P < 0.05$ and $^{**}P < 0.01$.

livers of hens in the 2 and 4% FVS groups compared with hens in the CON group. The mRNA expression levels of *peroxisome proliferator activated α* (***PPAR-α***) were significantly increased in the 6% FVS group, whereas those of peroxisome *proliferator activated γ* (***PPAR-γ***) did not change significantly. The mRNA expression levels of *microsomal triglyceride transport protein* (***MTTP***) in the livers of hens in the 2, 4, and 6% FVS groups were all significantly increased compared with hens in the CON group.

DISCUSSION

Increasing evidence suggests that female fertility is age dependent and that ovarian longevity is a major determinant of reproductive function (Wang et al., 2021b). In ovoviviparous hens, the liver plays an essential role in ovulation and production, as does the ovary (Cui et al., 2020). During egg production, the liver produces large amounts of apolipoproteins, yolk proteins, and lipids, which are processed into yolk precursors that are transported through the blood to the ovary to promote oocyte maturation; therefore, their levels can affect egg production (Nimpf and Schneider, 1991). In addition, the synthesis of yolk precursors is regulated by the production of estrogen in the ovary (Tramunt et al., 2021). It is therefore essential to investigate the mechanism of action of the liver−blood−ovary axis to prolong the reproductive cycle in laying hens.

One of the main causes of aging is oxidative stress, and the substantial accumulation of ROS with age causes oxidative damage to the liver and ovaries, resulting in decreased reproductive hormone levels and yolk precursor synthesis, which is the most direct cause of decreased egg production (Luderer, 2014; Liu et al., 2018b).

It has been reported that the addition of dietary antioxidants can effectively alleviate oxidative damage in the body (Nateghi et al., 2019). As a natural antioxidant, the golden needle mushroom exhibits antioxidant, antiaging, anti-inflammatory, and immunomodulatory effects and can protect internal organs, regulate lipid metabolism, and inhibit lipid peroxidation (Yeh et al., 2014; Chen et al., 2015). FVS can be used as an antibiotic substitute to maintain egg production, decrease blood lipid levels, and improve immunity in laying hens (Mahfuz et al., 2019a). In this study, using FVS as a feed substitute improved reproductive organ characteristics, where the ovarian and pre-ovulatory follicular indices and egg production rates were subsequently

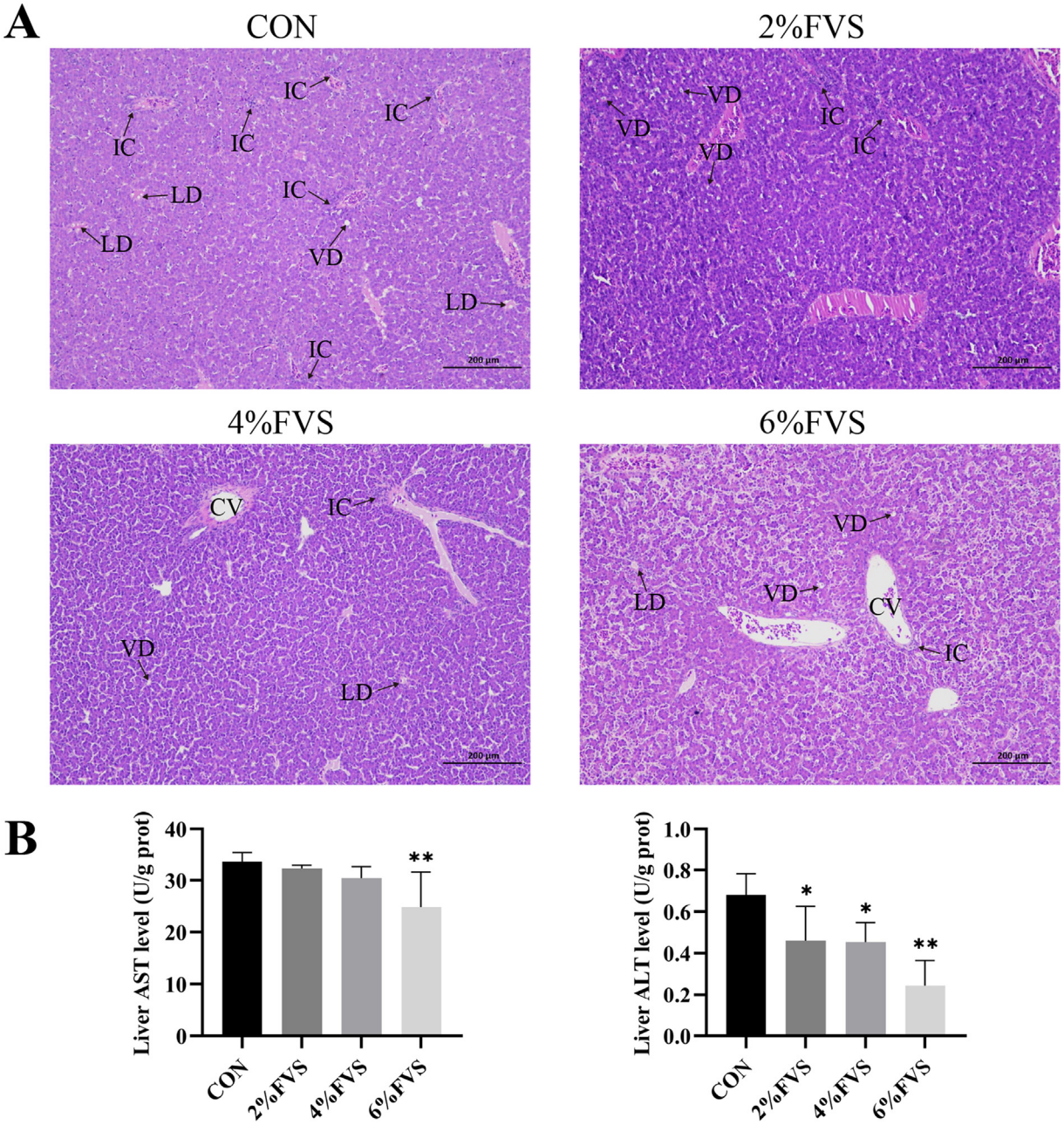

Figure 5. Effect of FVS on liver histomorphology and heptic function damage. (A) Hematoxylin-eosin (HE) staining of the hen's liver tissue. (B) Effect of FVS on hepatic AST and ALT levels. Scale bar = 500 μm. Abbreviations: AST, aspartate transaminase; ALT, alanine transaminase; CON, laying hens fed with a basic diet; CV, central vein; IC, inflammatory cells; LD, lipid droplet; VD, vacuolar degeneration; 2%FVS, laying hens fed with a basic diet supplemented with 20 g/kg FVS; 4%FVS, laying hens fed with a basic diet supplemented with 40 g/kg FVS; 6%FVS, laying hens fed with a basic diet supplemented with 60 g/kg FVS. The results are expressed as the mean ± SEM (n = 4); $^*P < 0.05$ and $^{**}P < 0.01$.

increased. However, FVS did not significantly increase the FCR in hens, which is consistent with the findings of a previous study (Mahfuz et al., 2019b). In the present study, FVS did not significantly affect egg weight, haugh units, or shell strength but it did affect shell thickness, and a previous study reported that FVS can improve calcium utilization and deposition in eggshells, possibly because of this reason (Mahfuz et al., 2018). In summary, the increase in egg production verified that FVS alleviates the decline in reproductive function caused by aging of the organism to some extent, and that the reproductive capacity of aging hens is developing for the better.

The imbalance between oxidants and antioxidants (in favor of oxidants) in the organism causes oxidative stress (Sies, 1997). To control oxidation levels, the body forms a complex antioxidant defense system, in which the enzymatic defense system includes T-SOD, GSH-Px, and T-AOC, whereas MDA, a marker product of lipid peroxidation, is often seen as an unfavorable factor for redox (Gawel et al., 2004; He et al., 2017). Numerous studies have reported that the addition of natural antioxidants, such as lycopene, quercetin, and grape seed proanthocyanidins, significantly increases the levels of antioxidant enzymes and reduces MDA levels in laying hens (Liu et al., 2018a, 2018c; Yang et al., 2018). It has

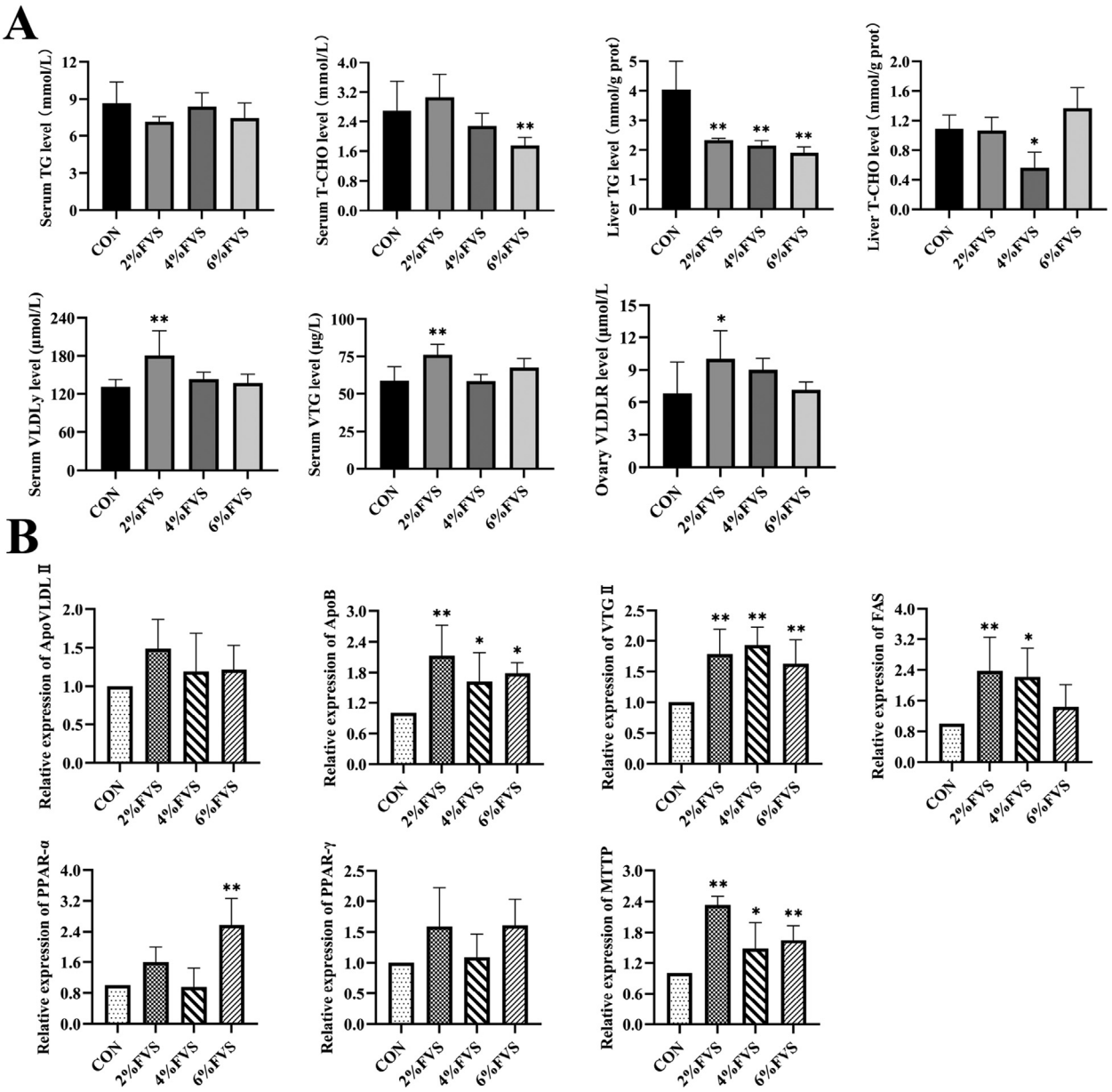

Figure 6. Effect of FVS on the synthesis capacity of yolk precursors and their receptors. (A) Effect of FVS on yolk precursors, TG, T-CHO and VLDLR levels. (B) Effect of FVS on mRNA expression levels of genes related to yolk precursor synthesis and lipid metabolism. Abbreviations: ApoB, apolipoprotein B; ApoVLDLⅡ, apolipoprotein VLDL Ⅱ; TG, triglycerides; T-CHO, total cholesterol; VLDLy, very low-density lipoprotein Y; VTG, vitellogenin; VLDLR, very low-density lipoprotein receptor; VTGⅡ, vitellogenin Ⅱ; CON, laying hens fed with a basic diet; 2%FVS, laying hens fed with a basic diet supplemented with 20 g/kg FVS; 4%FVS, laying hens fed with a basic diet supplemented with 40 g/kg FVS; 6%FVS, laying hens fed with a basic diet supplemented with 60 g/kg FVS. The results are expressed as the mean ± SEM (n = 6); $^{*}P < 0.05$ and $^{**}P < 0.01$.

been shown that active components, such as polysaccharides and polyphenols of *F. velutipes*, result in enhanced antioxidant capacity of the body and can inhibit lipid peroxidation (Rahman et al., 2015; Ma et al., 2021b; Liang et al., 2022). In the present study, FVS significantly increased the levels of the antioxidant enzymes T-SOD, GSH-Px, and T-AOC and decreased MDA levels in the liver, serum, and ovary compared with CON. These results are consistent with the findings of Chen et al. (2019) and Liu et al. (2020a), which showed that feeding FVS increased T-SOD, GSH-Px, and T-AOC levels and decreased MDA levels in the serum of laying hens and piglets. One study found that nuclear factor E2-related factor 2 (Nrf2), a redox transcription factor, exerts protective effects against oxidative stress and can regulate the expression of antioxidant genes such as *SOD, GSH, HO-1*, and *NQO1* (Loboda et al., 2016). Nrf2 activates the antioxidant response element (**ARE**) transcription system after binding to sMaf, and HO-1 and NQO1 exert antioxidant effects as downstream proteins of this pathway (Yang et al., 2012). Keap1, a negative regulator of Nrf2 and a sensor of oxidative stress, plays a decisive role in the expression of Nrf2 (Motohashi and Yamamoto, 2004). It has been found that medicinal fungi, such as *Ganoderma lucidum*, Shiitake mushroom, and Monkey head mushroom, can positively affect oxidative stress in the body through the Nrf2/HO-1 signaling pathway (Lee et al., 2016; Gao et al., 2019; Kushairi et al., 2019). In the present study, we found

that FVS effectively alleviated oxidative stress in aging laying hens by activating the Nrf2/Keap1-ARE signaling pathway in the liver and ovaries, thereby activating the expression of downstream antioxidant genes or proteins.

It is well known that egg production rate is related to follicle development and ovulation processes (Li et al., 2021). There is an increasing evidence that follicular atresia due to oxidative stress may be the underlying cause of reduced fertility in older laying hens (Wang et al., 2021a). Moreover, ovarian senescence and oxidative stress-induced apoptosis inevitably lead to reduced estrogen levels, which in turn inhibit follicular development and egg production (Manolagas, 2010). In this study, the mRNA and protein expression levels of the proapoptotic genes *Bax* and *caspase-3* were significantly reduced, whereas those of the antiapoptotic gene *Bcl-2* were significantly increased and the Bax/Bcl-2 protein ratio was significantly decreased in the ovaries of hens fed the FVS diet. These results are in concordance with previous studies showing that the decrease in the expression of genes involved in the apoptosis may be closely related to the decrease of those involved in oxidative stress. (Sack et al., 2017; Li et al., 2019). It is known that the number of primordial follicles in the ovary determines a woman's reproductive lifespan, and the depletion of the limited reservoir of primordial follicles implies a gradual reduction in the number of grade follicles selected to enter the pre-ovulatory hierarchy (Sobinoff et al., 2013). Furthermore, the decline in the quality of pre-ovulatory follicles is associated with decreased levels of estrogen and gonadotropins (FSH and LH) and their respective receptors during oocyte development, leading to an impairment of granulosa cell proliferation and consequently follicular maturation (Zhang et al., 1997). In this study, we demonstrated that FVS maintained the denseness of the granulosa cell membrane layer, the number of primordial follicles, and the level of the pre-ovulatory follicle index in aging laying hens by reducing oxidative stress and apoptosis in the ovary, thereby improving egg production.

Granulosa cells are important mediators of follicle production and estradiol secretion, and oxidative stress-induced granulosa cell death is a common cause of follicular atresia, whereas FSH has been shown to prevent oxidative damage to granulosa cells (Caicedo et al., 2016; Shen et al., 2017). It is reported that serum E_2 and FSH levels can be increased by alleviating oxidative stress and apoptosis in the ovaries of aging laying hens (Yang et al., 2019). In this study, the FVS groups exhibited increased serum levels of E_2, P_4, FSH, and LH and their respective receptors ER-α/β, follicle-stimulating hormone receptor (**FSHR**) and luteinizing hormone receptor (**LHR**) compared with the CON group and had a mitigating effect on hormone levels in aging hens, which correlated with the results that the anterior segment FVS inhibits ovarian apoptosis and promotes follicular development. Furthermore, these results are consistent with those reported by Ma et al. (2021a) demonstrating that epigallocatechin-3-gallate as an antioxidant was able to increase the antioxidant effects in the liver and ovaries of laying hens via the Nrf2-Keap1-sMaf signaling pathway, which in turn increased the levels of E_2, P_4, FSH, and LH in laying hens. In addition, it has been found that phytoestrogens are biologically active phenolic compounds of plant origin with structure and activity similar to those of ovarian estrogens (Sirtori et al., 2005). The mechanism of action is through binding to ER-α or ER-β, which exert estrogenic effects in organs such as the regulated ovary and the hypothalamus−pituitary. Phytoestrogens may act as estrogen antagonists in a high-estrogen environment and as estrogen agonists in a low-estrogen environment (Hwang et al., 2006). Several studies have identified flavonoids such as quercetin, gallic acid, ferulic acid, rutinoside, arbutin, epicatechin, allantoin, apigenin, kaempferol, and mangostemonin in golden needle mushrooms, verifying their strong antioxidant and organ-protective effects (Rahman et al., 2015; Hu et al., 2016; Ma et al., 2021b). It has also been reported that quercetin can act as both an antioxidant and a phytoestrogen to increase the levels of sex hormones and their receptors in laying hens, thereby regulating egg production (Amevor et al., 2021). This study demonstrates that FVS alleviates the decline in levels of reproductive hormones and their receptors in laying hens due to aging by increasing the antioxidant and antiapoptotic levels in ovaries, and that the flavonoids contained in FVS may act as phytoestrogens, exerting a positive regulatory effect at low estrogen levels.

The results of this study clearly indicate that with increased levels of E_2 and ER-α/β expression, the expression levels of *ApoVLDLII, ApoB*, and *VTGII* mRNAs and genes related to yolk precursor production increased in the liver of laying hens, and serum levels of VLDLy and VTG increased significantly. In addition, caged hens may be obese due to an ad libitum diet, restricted exercise, or reduced levels of lipid metabolism, which may be a natural cause of metabolic diseases such as fatty liver syndrome in older laying hens (Rozenboim et al., 2016; Dai et al., 2021). The present study verified that FVS significantly reduced the abdominal lipid rate, attenuated the number of lipid droplets and the amount of inflammatory infiltration in the liver, resulted in significantly lower AST and ALT levels, and reduced the accumulation of TG and T-CHO in the liver and serum, with significantly higher gene expression levels associated with lipogenesis (FAS), lipid transport (MTTP), and lipolysis (PPAR-α/β). The results of this study are consistent with those of Li et al. (2014) showing that *ApoVLDLII, VTGII, FAS*, and *MTTP* mRNA expression levels in the liver of laying hens exhibited upregulation with increasing serum E_2 levels, indicating a correlation among the liver, blood, and ovary during yolk synthesis. Furthermore, Amevor et al. (2021) found that the combination of the phytoestrogen quercetin and the antioxidant vitamin E increased the levels of antioxidants, estrogens, and their receptors, modulated lipid metabolism and transport in hens, and thus enhanced the production of yolk

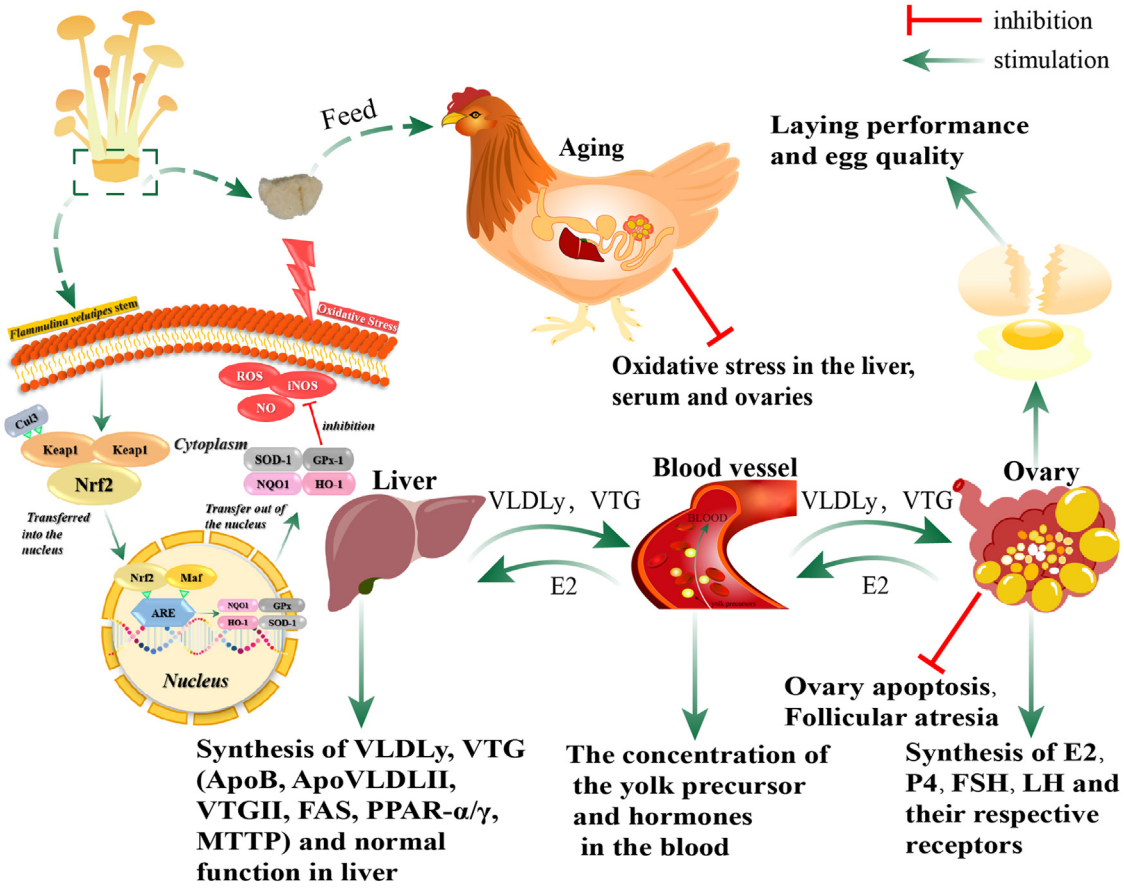

Figure 7. Schematic diagram outlining the regulation of reproductive function in aging laying hens by Flammulina velutipes mushroom stem through the liver-blood-ovary axis.

precursors, which is consistent with the results of this trial. In conclusion, FVS promotes yolk precursor production by increasing serum E_2 and liver ER-α/β levels. On the other hand, FVS can reduce reproductive stress caused by fat accumulation and improve the synthesis of yolk precursors by regulating liver lipid metabolism, thus improving production performance.

CONCLUSIONS

In this study, we used the natural antioxidant FVS as a feed substitute and verified that it could enhance antioxidant capacity in the liver, serum, and ovaries by regulating the Nrf2-Keap1/ARE signaling pathway, delaying the decline in hepatic and ovarian functions, and decreasing ovarian apoptosis, thereby maintaining lipid metabolism homeostasis in the liver, enhancing reproductive hormone secretion and follicle development, and increasing estrogen and its receptor levels. In addition, the mRNA expression levels of proteins associated with the synthesis of yolk precursors increased significantly, which ultimately delayed the decline in egg production in aging hens. These results demonstrate that FVS, as a functional feed substitute, exhibits potential hepatic and ovarian protective effects, facilitating the transport and exchange of various substances via the liver−blood−ovary axis and, to a certain extent, maintaining reproductive homeostasis in aging laying hens (Figure 7).

ACKNOWLEDGMENTS

This work was supported by the Key R&D Project of Jilin Provincial Department of Science and Technology "R&D of new safe and efficient feed products" (20180201019NY).

Haoyuan Wu: drafting the manuscript, conception and design; Jing Yuan and Haixu Yin: performed the experiments; Bo Jing and Zhouyu Jin: analyzed the data; Ivan Stève Nguepi Tsopmejio: revising the manuscript. Chang Sun: Given final approval of the version to be published. Hui Song: Agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

DISCLOSURES

The authors have declared that no competing interest exists.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102261.

REFERENCES

Amevor, F. K., Z. Cui, X. Du, Z. Ning, G. Shu, N. Jin, X. Deng, Y. Tian, Z. Zhang, X. Kang, D. Xu, G. You, Y. Zhang, D. Li, Y. Wang, Q. Zhu, and X. Zhao. 2021. Combination of quercetin and vitamin e supplementation promotes yolk precursor synthesis and follicle development in aging breeder hens via liver-blood-ovary signal axis. Animals (Basel) 11:1915.

Barber, D. L., E. J. Sanders, R. Aebersold, and W. J. Schneider. 1991. The receptor for yolk lipoprotein deposition in the chicken oocyte. J. Biol. Chem. 266:18761–18770.

Bujo, H., M. Hermann, M. O. Kaderli, L. Jacobsen, S. Sugawara, J. Nimpf, T. Yamamoto, and W. J. Schneider. 1994. Chicken oocyte growth is mediated by an eight ligand binding repeat member of the LDL receptor family. EMBO J. 13:5165–5175.

Caicedo, R. R., M. P. Nieto, and M. Kamiyoshi. 2016. Effects of steroid hormone in avian follicles. Asian-Australas. J. Anim. Sci. 29:487–499.

Chen, M., S. Mahfuz, Y. Cui, L. Jia, Z. Liu, and H. Song. 2019. The antioxidant status of serum and egg yolk in layer fed with mushroom stembase (Flammulina velutipes). Pak. J. Zool. 52:389–392.

Chen, P., Y. Yong, Y. Gu, Z. Wang, S. Zhang, and L. Lu. 2015. Comparison of antioxidant and antiproliferation activities of polysaccharides from eight species of medicinal mushrooms. Int. J. Med. Mushrooms. 17:287–295.

Ciccone, N. A., P. J. Sharp, P. W. Wilson, and I. C. Dunn. 2005. Changes in reproductive neuroendocrine mRNAs with decreasing ovarian function in ageing hens. Gen. Comp. Endocrinol. 144:20–27.

Cui, Z., F. K. Amevor, Q. Feng, X. Kang, W. Song, Q. Zhu, Y. Wang, D. Li, and X. Zhao. 2020. Sexual maturity promotes yolk precursor synthesis and follicle development in hens via liver-blood-ovary signal axis. Animals (Basel) 10:2348.

Dai, H., Z. Lv, Z. Huang, N. Ye, S. Li, J. Jiang, Y. Cheng, and F. Shi. 2021. Dietary hawthorn-leaves flavonoids improves ovarian function and liver lipid metabolism in aged breeder hens. Poult. Sci. 100:101499.

Gao, Y., Y. Wang, Y. Wang, Y. Wu, H. Chen, R. Yang, and D. Bao. 2019. Protective function of novel fungal immunomodulatory proteins Fip-lti1 and Fip-lti2 from Lentinus tigrinus in concanavalin a-induced liver oxidative injury. Oxid. Med. Cell. Longev. 2019:3139689.

Gawel, S., M. Wardas, E. Niedworok, and P. Wardas. 2004. [Malondialdehyde (MDA) as a lipid peroxidation marker]. Wiad. Lek. 57:453–455.

Gu, Y. F., Y. P. Chen, R. Jin, C. Wang, C. Wen, and Y. M. Zhou. 2021. Age-related changes in liver metabolism and antioxidant capacity of laying hens. Poult. Sci. 100:101478.

He, L., T. He, S. Farrar, L. Ji, T. Liu, and X. Ma. 2017. Antioxidants maintain cellular redox homeostasis by elimination of reactive oxygen species. Cell. Physiol. Biochem. 44:532–553.

Hu, Q., J. Yu, W. Yang, B. M. Kimatu, Y. Fang, N. Ma, and F. Pei. 2016. Identification of flavonoids from Flammulina velutipes and its neuroprotective effect on pheochromocytoma-12 cells. Food Chem. 204:274–282.

Hu, Y. N., T. J. Sung, C. H. Chou, K. L. Liu, L. P. Hsieh, and C. W. Hsieh. 2019. Characterization and antioxidant activities of yellow strain flammulina velutipes (jinhua mushroom) polysaccharides and their effects on ROS content in L929 cell. Antioxidants (Basel) 8:298.

Hwang, C. S., H. S. Kwak, H. J. Lim, S. H. Lee, Y. S. Kang, T. B. Choe, H. G. Hur, and K. O. Han. 2006. Isoflavone metabolites and their in vitro dual functions: They can act as an estrogenic agonist or antagonist depending on the estrogen concentration. J. Steroid Biochem. Mol. Biol. 101:246–253.

Ko, S. H., and H. S. Kim. 2020. Menopause-associated lipid metabolic disorders and foods beneficial for postmenopausal women. Nutrients 12:202.

Kushairi, N., C. W. Phan, V. Sabaratnam, P. David, and M. Naidu. 2019. Lion's mane mushroom, hericium erinaceus (Bull.: Fr.) Pers. Suppresses H2O2-Induced oxidative damage and LPS-Induced inflammation in HT22 hippocampal neurons and BV2 microglia. Antioxidants (Basel) 8:261.

Lee, Y. H., J. H. Kim, C. H. Song, K. J. Jang, C. H. Kim, J. S. Kang, Y. H. Choi, and H. M. Yoon. 2016. Ethanol extract of ganoderma lucidum augments cellular anti-oxidant defense through activation of Nrf2/HO-1. J. Pharmacopuncture 19:59–69.

Li, J., Q. Qin, Y. X. Li, X. F. Leng, and Y. J. Wu. 2021. Tri-ortho-cresyl phosphate exposure leads to low egg production and poor eggshell quality via disrupting follicular development and shell gland function in laying hens. Ecotoxicol. Environ. Saf. 225:112771.

Li, J., Z. Qiao, W. Hu, W. Zhang, S. Shah, and M. Ishfaq. 2019. Baicalin mitigated Mycoplasma gallisepticum-induced structural damage and attenuated oxidative stress and apoptosis in chicken thymus through the Nrf2/HO-1 defence pathway. Vet. Res. 50:83.

Li, J., I. H. Leghari, B. He, W. Zeng, Y. Mi, and C. Zhang. 2014. Estrogen stimulates expression of chicken hepatic vitellogenin II and very low-density apolipoprotein II through ER-alpha. Theriogenology 82:517–524.

Liang, Q., Q. Zhao, X. Hao, J. Wang, C. Ma, X. Xi, and W. Kang. 2022. The effect of flammulina velutipes polysaccharide on immunization analyzed by intestinal flora and proteomics. Front. Nutr. 9:841230.

Liu, X., B. Zhang, H. Liu, G. Zhang, J. Zhao, L. Liu, X. Piao, H. Song, S. Zhang, and Y. Li. 2020a. Determination of the available energy values and amino acid digestibility of Flammulina velutipes stem waste and its effects on carcass trait and meat quality fed to growing-finishing pigs. J. Anim. Sci. Biotechnol. 11:41.

Liu, X., J. Zhao, G. Zhang, J. Hu, L. Liu, X. Piao, S. Zhang, and Y. Li. 2020b. Dietary supplementation with flammulina velutipes stem waste on growth performance, Fecal short chain fatty acids and serum profile in weaned piglets. Animals (Basel) 10:82.

Liu, X., X. Lin, S. Zhang, C. Guo, J. Li, Y. Mi, and C. Zhang. 2018a. Lycopene ameliorates oxidative stress in the aging chicken ovary via activation of Nrf2/HO-1 pathway. Aging (Albany NY) 10:2016–2036.

Liu, X., X. Lin, Y. L. Mi, W. D. Zeng, and C. Q. Zhang. 2018b. Age-related changes of yolk precursor formation in the liver of laying hens. J. Zhejiang Univ. Sci. B 19:390–399.

Liu, X., X. Lin, Y. Mi, J. Li, and C. Zhang. 2018c. Grape seed proanthocyanidin extract prevents ovarian aging by inhibiting oxidative stress in the hens. Oxid. Med. Cell. Longev. 2018:9390810.

Loboda, A., M. Damulewicz, E. Pyza, A. Jozkowicz, and J. Dulak. 2016. Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism. Cell Mol. Life Sci. 73:3221–3247.

Luderer, U. 2014. Ovarian toxicity from reactive oxygen species. Vitam. Horm. 94:99–127.

Ma, Y., Y. Shi, Q. Wu, and W. Ma. 2021a. Epigallocatechin-3-gallate alleviates vanadium-induced reduction of antioxidant capacity via Keap1-Nrf2-sMaf pathway in the liver, kidney, and ovary of laying hens. Biol. Trace. Elem. Res. 199:2707–2716.

Ma, S., H. Zhang, and J. Xu. 2021b. Characterization, antioxidant and anti-inflammation capacities of fermented flammulina velutipes polyphenols. Molecules 26:6205.

Mahfuz, S., T. He, S. Liu, D. Wu, S. Long, and X. Piao. 2019a. Dietary inclusion of mushroom (flammulina velutipes) stem waste on growth performance, antibody response, immune status, and serum cholesterol in broiler chickens. Animals (Basel) 9:692.

Mahfuz, S., H. Song, Y. Miao, and Z. Liu. 2019b. Dietary inclusion of mushroom (Flammulina velutipes) stem waste on growth performance and immune responses in growing layer hens. J. Sci. Food Agric. 99:703–710.

Mahfuz, S., H. Song, Z. Liu, X. Liu, Z. Diao, G. Ren, Z. Guo, and Y. Cui. 2018. Effect of golden needle mushroom (Flammulina velutipes) stem waste on laying performance, calcium utilization, immune response and serum immunity at early phase of production. Asian-Australas. J. Anim. Sci. 31:705–711.

Manolagas, S. C. 2010. From estrogen-centric to aging and oxidative stress: a revised perspective of the pathogenesis of osteoporosis. Endocr. Rev. 31:266–300.

May-Panloup, P., L. Boucret, D. L. B. J. Chao, V. Desquiret-Dumas, V. Ferre-L'Hotellier, C. Moriniere, P. Descamps, V. Procaccio, and

P. Reynier. 2016. Ovarian ageing: the role of mitochondria in oocytes and follicles. Hum. Reprod. Update 22:725–743.

Meng, X. T., N. N. Hou, X. J. Wang, H. C. Jiao, J. P. Zhao, Z. G. Song, and H. Lin. 2013. Increased hepatic yolk precursor synthesis, secretion and facilitated uptake by follicles are involved in the rejuvenation of reproductive performance of molted hens (Gallus gallus domesticus). Gen. Comp. Endocrinol. 194:198–207.

Motohashi, H., and M. Yamamoto. 2004. Nrf2-Keap1 defines a physiologically important stress response mechanism. Trends Mol. Med. 10:549–557.

Nateghi, R., M. M. A. Alizadeh, A. Y. Jafari, R. Fathi, and A. Akhlaghi. 2019. Dietary fish oil and vitamin e reduce cryoinjuries and apoptosis in vitrified laying hens' ovarian tissue. Biopreserv. Biobank. 17:148–156.

Nguepi Tsopmejio, I. S., M. Ding, J. L. Wei, C. Zhao, Y. Jiang, Y. T. Li, and H. Song. 2021. Auricularia polytricha and Flammulina velutipes ameliorate inflammation and modulate the gut microbiota via regulation of NF-κB and Keap1/Nrf2 signaling pathways on DSS-induced inflammatory bowel disease. Food Biosci. 47:101426.

Nimpf, J., and W. J. Schneider. 1991. Receptor-mediated lipoprotein transport in laying hens. J. Nutr. 121:1471–1474.

Omer, N. A., Y. Hu, Y. Hu, A. A. Idriss, H. Abobaker, Z. Hou, H. Dong, and R. Zhao. 2018. Dietary betaine activates hepatic VTGII expression in laying hens associated with hypomethylation of GR gene promoter and enhanced GR expression. J. Anim. Sci. Biotechnol. 9:2.

Rahman, M. A., N. Abdullah, and N. Aminudin. 2015. Antioxidative effects and inhibition of human low density lipoprotein oxidation in vitro of polyphenolic compounds in flammulina velutipes (golden needle mushroom). Oxid. Med. Cell. Longev. 2015:403023.

Rozenboim, I., J. Mahato, N. A. Cohen, and O. Tirosh. 2016. Low protein and high-energy diet: a possible natural cause of fatty liver hemorrhagic syndrome in caged white leghorn laying hens. Poult. Sci. 95:612–621.

Sack, M. N., F. Y. Fyhrquist, O. J. Saijonmaa, V. Fuster, and J. C. Kovacic. 2017. Basic biology of oxidative stress and the cardiovascular system: part 1 of a 3-part series. J. Am. Coll. Cardiol. 70:196–211.

Schneider, W. J. 1992. Lipoprotein receptors in oocyte growth. Clin. Investig. 70:385–390.

Shen, M., Y. Jiang, Z. Guan, Y. Cao, L. Li, H. Liu, and S. C. Sun. 2017. Protective mechanism of FSH against oxidative damage in mouse ovarian granulosa cells by repressing autophagy. Autophagy 13:1364–1385.

Sies, H. 1997. Oxidative stress: oxidants and antioxidants. Exp. Physiol. 82:291–295.

Sirtori, C. R., A. Arnoldi, and S. K. Johnson. 2005. Phytoestrogens: end of a tale? Ann. Med. 37:423–438.

Sobinoff, A. P., E. L. Beckett, A. G. Jarnicki, J. M. Sutherland, A. McCluskey, P. M. Hansbro, and E. A. McLaughlin. 2013. Scrambled and fried: cigarette smoke exposure causes antral follicle destruction and oocyte dysfunction through oxidative stress. Toxicol. Appl. Pharmacol. 271:156–167.

Tramunt, B., A. Montagner, N. S. Tan, P. Gourdy, H. Remignon, and W. Wahli. 2021. Roles of estrogens in the healthy and diseased oviparous vertebrate liver. Metabolites 11:502.

Walzem, R. L., R. J. Hansen, D. L. Williams, and R. L. Hamilton. 1999. Estrogen induction of VLDLy assembly in egg-laying hens. J. Nutr. 129(2S Suppl):467S–472S.

Wang, J., R. Jia, H. Gong, P. Celi, Y. Zhuo, X. Ding, S. Bai, Q. Zeng, H. Yin, S. Xu, J. Liu, X. Mao, and K. Zhang. 2021a. The effect of oxidative stress on the chicken ovary: involvement of microbiota and melatonin interventions. Antioxidants (Basel) 10:1422.

Wang, L., J. Tang, L. Wang, F. Tan, H. Song, J. Zhou, and F. Li. 2021b. Oxidative stress in oocyte aging and female reproduction. J. Cell. Physiol. 236:7966–7983.

Xing, C., Y. Wang, X. Dai, F. Yang, J. Luo, P. Liu, C. Zhang, H. Cao, and G. Hu. 2020. The protective effects of resveratrol on antioxidant function and the mRNA expression of inflammatory cytokines in the ovaries of hens with fatty liver hemorrhagic syndrome. Poult. Sci. 99:1019–1027.

Yang, X., W. Wang, Y. Zhang, J. Wang, and F. Huang. 2019. Moxibustion improves ovary function by suppressing apoptosis events and upregulating antioxidant defenses in natural aging ovary. Life Sci 229:166–172.

Yang, J. X., M. T. Chaudhry, J. Y. Yao, S. N. Wang, B. Zhou, M. Wang, C. Y. Han, Y. You, and Y. Li. 2018. Effects of phytoestrogen quercetin on productive performance, hormones, reproductive organs and apoptotic genes in laying hens. J. Anim. Physiol. Anim. Nutr. (Berl) 102:505–513.

Yang, C. M., S. M. Huang, C. L. Liu, and M. L. Hu. 2012. Apo-8′-lycopenal induces expression of HO-1 and NQO-1 via the ERK/p38-Nrf2-ARE pathway in human HepG2 cells. J. Agric. Food Chem. 60:1576–1585.

Yeh, M. Y., W. C. Ko, and L. Y. Lin. 2014. Hypolipidemic and antioxidant activity of enoki mushrooms (Flammulina velutipes). Biomed. Res. Int. 2014:352385.

Yoshimura, Y., and T. Tamura. 1988. Effects of gonadotrophins, steroid hormones, and epidermal growth factor on the in vitro proliferation of chicken granulosa cells. Poult. Sci. 67:814–818.

Zhang, C., K. Shimada, N. Saito, and N. Kansaku. 1997. Expression of messenger ribonucleic acids of luteinizing hormone and follicle-stimulating hormone receptors in granulosa and theca layers of chicken preovulatory follicles. Gen. Comp. Endocrinol. 105:402–409.

Zhu, M., S. Miao, W. Zhou, S. S. Elnesr, X. Dong, and X. Zou. 2021. MAPK, AKT/FoxO3a and mTOR pathways are involved in cadmium regulating the cell cycle, proliferation and apoptosis of chicken follicular granulosa cells. Ecotoxicol. Environ. Saf. 214:112091.