

# Naringin prevents follicular atresia by inhibiting oxidative stress in the aging chicken

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**ABSTRACT** Oxidative stress is an essential inducement in follicle atresia and ovarian aging, resulting in decline in female fecundity. As a natural and effective antioxidant, naringin was investigated to relieve chicken follicle atresia and ovarian aging. First, the cultured small white follicles (SWFs) from D280 hens were pre-treated with 0.5 mM naringin for 24 h and then treated with H<sub>2</sub>O<sub>2</sub> for 72 h to establish the oxidative stress model to evaluate the putative attenuating effects of naringin on follicle atresia. Meanwhile, SWFs of D580 hens were treated with naringin for 72 h to examine the attenuating effect on the physiological aging of SWFs. Finally, each hen was fed with naringin at a dose of 50 mg/kg every day to explore the effect of naringin on follicular development and laying performance in D580

hens. Results showed that naringin could rescue the antioxidant capacity decline by increasing the antioxidant-related indexes and expression of antioxidation-associated genes. It could also maintain the homeostasis of SWFs in both the H<sub>2</sub>O<sub>2</sub>-induced group and natural physiological aging group. In addition, naringin increased estrogen levels, capacity of antioxidants, and the laying performance in aged laying chickens. The thickness and strength of the eggshell were increased in the naringin-treated group as well. In conclusion, this study showed that naringin is capable of relieving SWFs atresia that was induced by oxidative stress and maintaining the laying performance of aging low-yielding hens by reducing oxidative stress.

**Key words:** naringin, chicken, aging ovary, follicular atresia, oxidative stress

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## INTRODUCTION

In the production of poultry eggs, age-related oxidative stress is accompanied by a deterioration of antioxidant capacity, which aggravates the decline of ovarian function, and ultimately leads to atresia of follicles and reduces egg production (Liu et al., 2018a). At around 80 wk of age, ovarian function significantly declines, and the rate of egg production is also reduced (Molnár et al., 2016). In addition, starting from the 60th wk of age, the thickness of the eggshell decreased by 0.23  $\mu$ m per week. The quality of eggshells gradually declines with age, which inevitably leads to a significant increase in the incidence of cracked eggs (Joyner et al., 1987; Molnár et al., 2016).

In laying hens, a previous study demonstrated that the appearance of long-term age-related reduction in

antioxidant status is caused by a decrease of antioxidant enzymes activity in tissues and antioxidant function (Liu et al., 2018a). Many studies in rodents and humans also showed that excessive levels of reactive oxygen species (ROS) in ovarian tissue leading to granulosa cell apoptosis, antral follicle atresia, and reduced reproductive ability (Agarwal et al., 2012; Cabry et al., 2014). At the same time, the level of 17 $\beta$ -estradiol (E<sub>2</sub>) and the function of oxidative stress relieving decreased with aging (Mohamad et al., 2020). In the mouse, oxidative stress displayed an inhibitory effect on the growth of follicles and the inductive effect on atresia of the antral follicles (Gupta et al., 2006). Granulosa cells play an important role in regulating the growth and atresia of follicles during follicular development. And oxidative stress-induced granulosa cells apoptosis is a significant cause of follicular atresia (Matsuda et al., 2012; Zhang et al., 2021).

Currently, effective measures for alleviating ovarian recession have been explored in poultry production based on oxidative stress attenuation. Therefore, key measures to maintain sustained high egg production performance in aging poultry were focused on increasing the

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level of antioxidants, improving the activities of antioxidants, reducing the oxidative stress in the ovary, and alleviating follicular atresia by supplementation of natural phytoantioxidants such as lycopene and grapeseed proanthocyanidins (Liu et al., 2018b; Zhou et al., 2022).

Phytoestrogens have the common features of one phenolic ring and 2 hydroxyl groups, which can combine with estrogen receptors to exert antioxidant effects. As one of phytoestrogens, naringin has the potential to act on estrogen receptors in the treatment of osteoporosis in related studies, and in terms of antioxidants, it is found that naringin pretreatment can regulate related pathways and increase the expression and activity of antioxidant enzymes to protect cells from oxidative damage (Torrens-Mas and Roca, 2020; Yu et al., 2020). Naringin has important medicinal values due to its various effects such as antioxidation, anti-inflammation, and anti-apoptosis, and has significant therapeutic potential in many diseases. The number of studies on naringin's application in livestock and poultry production is relatively minor and single. Most studies have focused on naringin's effects on fat metabolism, meat quality, and livestock and poultry body immunity (Simitzis et al., 2019; Hager-Theodorides et al., 2021). Therefore, the purpose of this study was to explore the protective effect of naringin on the follicular development of laying hens during late laying period. The results of the study may provide a foundation for prolonging the laying period of aging laying hens by alleviating the decline of ovarian function.

## MATERIALS AND METHODS

### *Animals and Tissue Collection*

Hyline white hens (D280 for 280-day-old hens and D580 for 580-day-old hens) were raised in a local commercial farm and subjected to conventional breeding management conditions. All procedures in this study were performed in accordance with the *Guiding Principles for the Care and Use of Laboratory Animals of Zhejiang University (ZJU20220085)*. And the experimental protocol of this study was approved by the Committee on the Ethics of Animal Experiments of Zhejiang University (No. ZJU2015-156-12). Hens were sacrificed after anesthesia, and the ovaries were separated for collection of small white follicle (SWFs) (2–4 mm). Composition and nutrient levels of basal diet is the same as that in a published article (Ma et al., 2020).

### *Culture of SWFs and Treatment of Chemicals*

Single SWF from D280 and D580 hens was cultured in the complete Dulbecco's Modified Eagle's Medium (Hyclone, Tauranga, New Zealand) with 10  $\mu\text{g}/\text{mL}$  insulin, 5  $\mu\text{g}/\text{mL}$  transferrin, 30 nM selenite (ITS, Sigma-Aldrich, St. Louis, MO), 100 IU/mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin, and 5% fetal calf serum (FCS, Hyclone, UT) at 38.5°C and 5% CO<sub>2</sub> for 96 h (D280) or 72 h (D580). The media were renewed every

24 h. For the 5-bromo-20-deoxyuridine (BrdU) incorporation test, the follicles were incubated with complete medium supplemented with 10  $\mu\text{g}/\text{mL}$  BrdU (Sigma-Aldrich) at the last 24 h. 1) For the culture of D280 SWFs in vitro, firstly, to screen the optimal concentration of naringin (Shanghai Yuanye Bio-Technology Co., Ltd.), different concentrations of naringin (0, 0.05, 0.5, and 5 mM) were used to pre-treat for 24 h. Then, 1 mM H<sub>2</sub>O<sub>2</sub> (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) was added and incubated for 72 h to establish a model of atretic small white follicles (ASWFs) (Yao et al., 2020). 2) Afterwards, the optimal concentration of naringin treatment was selected based on histomorphology, cell proliferation, and cell apoptosis. Next, SWFs were randomly divided into 4 groups as control, 1 mM H<sub>2</sub>O<sub>2</sub>, 0.5 mM Naringin and H<sub>2</sub>O<sub>2</sub> + Naringin. After 96 h of treatment, the SWFs were collected and fixed in 4% paraformaldehyde for morphological observation and fluorescence immunohistochemistry. Some of the SWFs were used for biochemical analysis and qRT-PCR. 3) In addition, SWFs from D580 hens were cultured and treated with 0.5 mM Naringin for 72 h.

For the experiment in vivo, thirty D580 hens (~2 kg BW) were randomly and evenly divided into 2 groups (experimental group/control group) with 3 replicates in each group and 5 hens in each replicate in flat cages. Each hen in the experimental group was fed with naringin at a dose of 50 mg/kg with 2.5 mL pure water every day for 15 consecutive days. The control group was assigned an equal volume of pure water. At the end of the experiment, 3 hens were randomly selected from each replicate group for sampling (SWFs and blood). During the experiment, the egg production rate of each group was recorded every day, and on the 13th d, eggs from each treatment group were collected to assess egg quality parameters. Blood samples were collected from the wing vein on the 16th d for the preparation of plasma before the hens were sacrificed. Furthermore, the number of follicles and atretic follicles before containing SWFs were recorded to calculate the follicle atresia rate in the in vivo experiments.

### *Morphological Observation*

The SWFs from D280 and D580 hens were fixed in a 4% paraformaldehyde solution at 4°C for at least 24 h, and then treated with a grade ethanol and xylene, and embedded in paraffin, subsequently sectioned for subsequent experiments. Hematoxylin and Eosin (H&E) staining were performed according to a standard histological procedure. An Eclipse 80i microscope (Nikon, Tokyo, Japan) was used to capture the H&E staining results.

### *Biochemical Analysis*

**Measurements of Oxidative Parameters** The plasma samples and the supernatant obtained from SWFs homogenized and centrifuged in phosphate-buffered

saline (**PBS**) were used for the determination of all oxidative parameters including the activity of catalase (**CAT**), superoxide dismutase (**SOD**), glutathione peroxidase (**GSH-Px**), glutathione S-transferase (**GSH-ST**), and total antioxidant capacity (**T-AOC**), and the concentration of malonaldehyde (**MDA**) according to the manufacturer's kit instructions (Nanjing Jiancheng Institute of Bioengineering, Nanjing, China).

**Determination of Plasma E<sub>2</sub>** Plasma E<sub>2</sub> was determined using an E<sub>2</sub> ELISA test kit (ERK R7005, Endocrine Technologies, Inc., San Francisco, CA) according to the manufacturer's instruction.

**Eggshell Quality** The eggshell thickness and eggshell strength were measured using a digital egg tester (DET6000, NABEL Co., Ltd, Kyoto, Japan).

**RNA Extraction and qRT-PCR** Trizol reagent (Invitrogen, Carlsbad, CA) was used for the extraction of total RNA from SWFs. RNA concentration was measured with a NanoDrop 2000c (Thermo Scientific, Waltham, MA). According to the manufacturer's protocol, the cDNA was generated from 2  $\mu$ g total RNA using a HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China) and qRT-PCR was performed using SYBR Premix Ex Taq kit (Vazyme) on the ABI 7500HT real-time PCR machine (Applied Biosystems, Foster City, CA) under the following conditions: 95°C 10 min, then 95°C 30 40 cycles of seconds, 64°C 34 s and 72°C 30 s. The relative mRNA expression levels were determined by the  $2^{-\Delta\Delta C_t}$  formula method standardized to  $\beta$ -actin. The primers for PCR were listed in Table 1.

**Immunofluorescence Staining** As previously reported, the immunofluorescence (**IF**) experiment procedure was carried out (Liu et al., 2018a). The primary antibodies used in the IF experiment were as follows: mouse

anti-BrdU monoclonal antibody (1:200, G3G4, DSHB, IA), rabbit anti-PCNA antibody (1:100, bs-0754R, Bioss, Beijing, China). The secondary antibodies used in the IF experiment were as follows: goat anti-mouse IgG (H + L)-TRITC (1:100, BS11502, Bioworld Technology, Inc., Minneapolis, MO), goat anti-rabbit antibody (1:50, HA1016, HuaBio, Hangzhou, China). After that, the IX70 fluorescence microscope (Olympus, Tokyo, Japan) was used to observe the fluorescence image.

**TUNEL Assay** TUNEL assay was performed using the TUNEL assay kit (Vazyme) according to the manufacturer's protocol. TUNEL-positive cells were marked in green and visualized using an Olympus microscope (IX70).

## Statistical Analysis

All experimental data were expressed as the means  $\pm$  standard error of the means (**SEM**), and analyzed by one-way analysis of variance (**ANOVA**) with Tukey's multiple comparisons test or independent samples *t* test using GraphPad Prism 5 software. In addition, all experiments in this study were repeated at least 3 times, and *P* < 0.05 was considered a statistically significant difference.

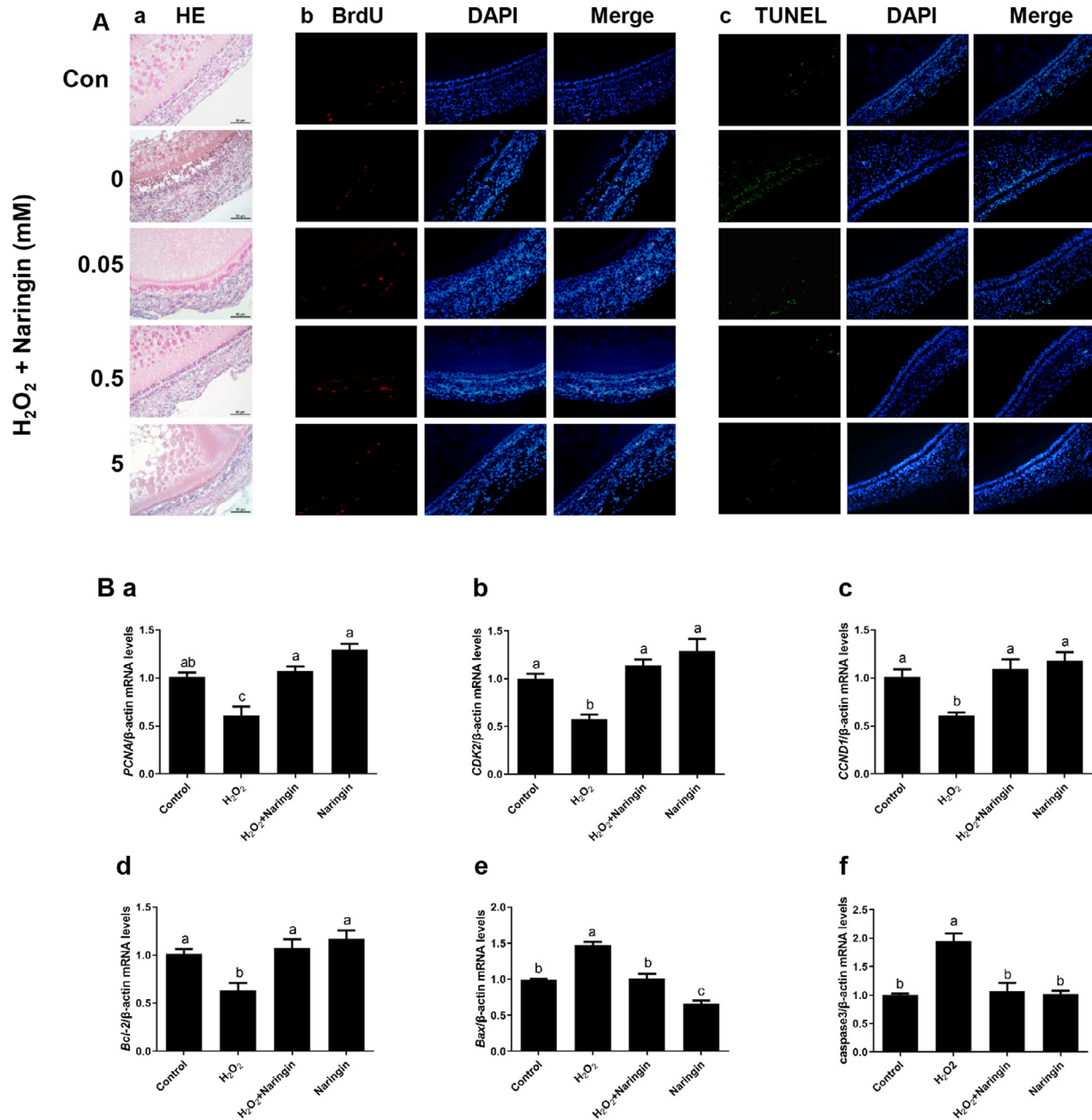
## RESULTS

### Effects of Naringin on the Cell Proliferation and Cell Apoptosis in the H<sub>2</sub>O<sub>2</sub>-Induced ASWFs

According to the results of H&E staining, after 72 h treatment of SWFs with 1 mM H<sub>2</sub>O<sub>2</sub>, granulosa cells

**Table 1.** Sequences of the primers for PCR.

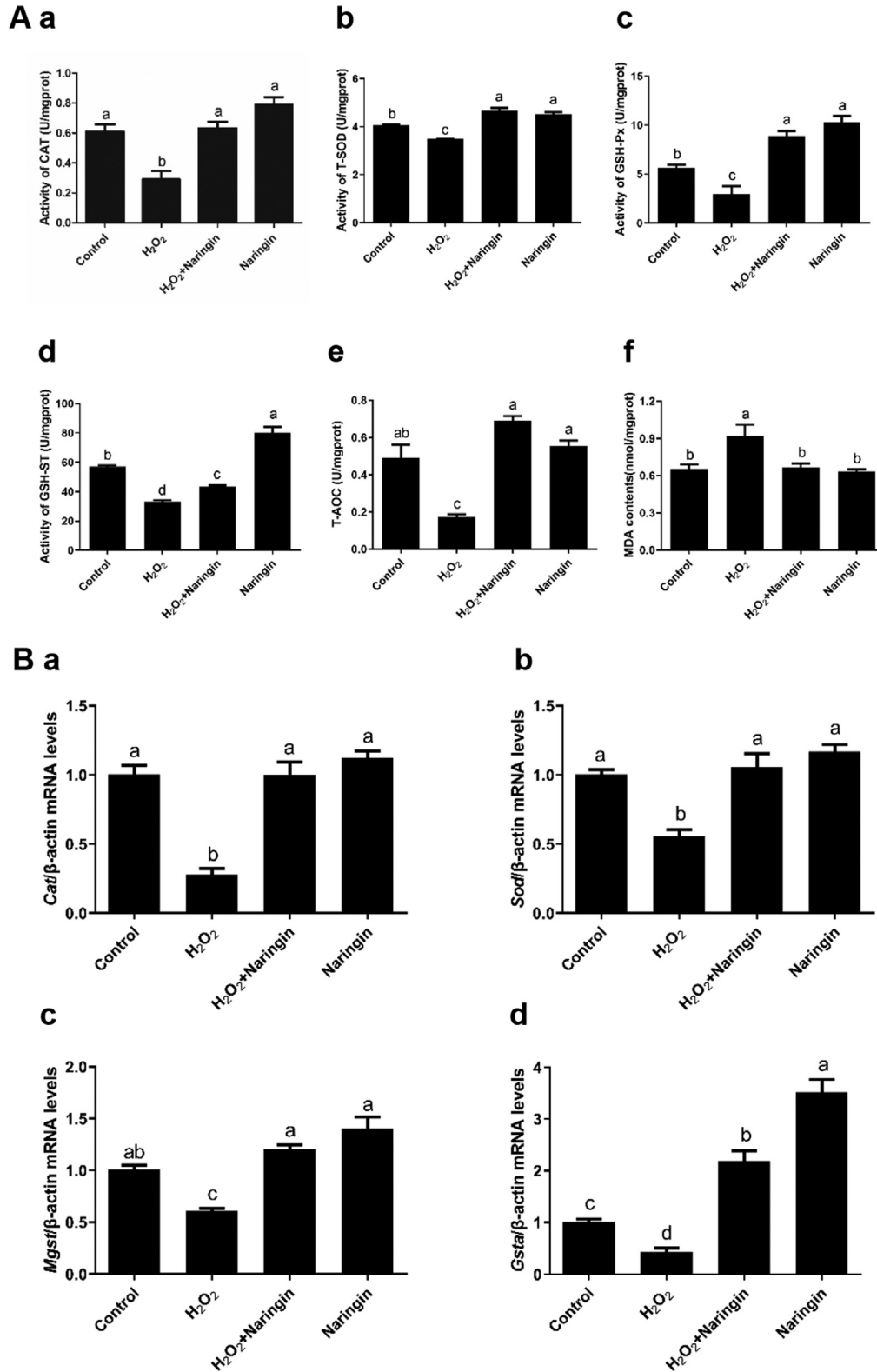
Gene name	Accession no.	Primer sequence (5'-3')	Product size (bp)
<i>PCNA</i>	NM_204170.2	F: GGGCGTCAACCTAAACAGCA R: AGCCAACGTATCCCGATTGT	97
<i>CCND1</i>	NM_205381.1	F: CCTCAAGAAAAGCCGGTTGC R: CTGCGGTTCAGAGGAATCGTT	86
<i>CDK2</i>	NM_001199857.1	F: TCCGTATCTTCCGCACGTTG R: GCTTGTGGGATCGTAGTGC	183
<i>Bcl-2</i>	NM_205339.2	F: ATCGTCGCCCTTCTTCGAGTT R: ATCCCATCCTCCGTTGTCTT	150
<i>caspase3</i>	NM_204725.1	F: CAGCTGAAGGCTCCTGGTTT R: GCCACTCTGCGATTTACACG	98
<i>Bax</i>	XM_015290060.2	F: GGATGACAGGAAAGTACGGCA R: TCACCAGGAAGACAGCGTAT	173
<i>CYP17A1</i>	NM_001001901.2	F: GGAGCTGACAGATGACCACC R: TCTTCTGGACCTCGGGGRAG	125
<i>CYP19A1</i>	NM_001001761	F: CCTCTGCTGGAGATGGTTT R: GCTGATCCACTTTAGTCACTCTGA	68
<i>Cat</i>	NM_001031215.2	F: TCAGGAGATGTGCAGCGTTT R: TCTTACACAGCCTTTGGCGT	109
<i>Sod</i>	NM_205064.1	F: GGCAATGTGACTGCAAAGGG R: CCCCTTACCAGGTCATCA	133
<i>Mgst</i>	NM_001135550.1	F: GGCATTTGCCAACCCAGAAG R: CAAGGTCATTGAGGTGGCCT	116
<i>Gsta</i>	NM_204818.2	F: GCAGAGCCATCCTCAGCTAC R: CCTTTGCCCTCAGGTGGAGAG	150
$\beta$ -actin	NM_205518	F: ACACCCACACCCTGTGATGAA R: TGCTGCTGACACCTTACCATTTC	136



**Figure 1.** Effect of naringin on cell proliferation and apoptosis of H<sub>2</sub>O<sub>2</sub>-induced ASWFs. (A) a, H&E, b, BrdU, and c, TUNEL assay in ASWFs after treatment with different concentrations of naringin. Scale bar: 50  $\mu$ m. (B) Effect of different concentrations of naringin on the expression of proliferation and apoptosis-related genes in ASWFs (a–f, *PCNA*, *CDK2*, *CCND1*, *Bax*, *Bcl-2*, and caspase3). Data were expressed as means  $\pm$  SEM. Different lowercase letters indicate significant difference ( $P < 0.05$ ).

were damaged, and the arrangement of granulosa cells became loose and irregular (Figure 1A, a). From the results of BrdU fluorescence staining, H<sub>2</sub>O<sub>2</sub> treatment alone decreased the BrdU labeling rates in the SWFs compared with the control group (Figure 1A, b). The results of TUNEL assay showed that after H<sub>2</sub>O<sub>2</sub> treatment, the percentage of TUNEL-positive cells in SWFs was significantly higher than that in the control group (Figure 1A, c). The pretreatment measures with different concentrations of naringin alleviated these adverse changes in growing follicles and granulosa cells in a dose-dependent manner. Combined with H&E, BrdU, and TUNEL staining, 0.5 mM naringin was the best concentration to alleviate the damage of

H<sub>2</sub>O<sub>2</sub> to SWFs. In addition, H<sub>2</sub>O<sub>2</sub> significantly down-regulated the expression of *PCNA*, *CDK2*, and *CCND1* mRNAs (Figure 1B, a–c). The results of qRT-PCR showed that in H<sub>2</sub>O<sub>2</sub>-induced SWFs, the *Bcl-2* expression level was reduced, while the expression of *Bax* was increased significantly, as well as the expression of caspase3 was increased compared with the control group (Figure 1B, d–f). However, compared with the H<sub>2</sub>O<sub>2</sub> treatment group, the expression of *Bax* and caspase3 mRNAs in the H<sub>2</sub>O<sub>2</sub>+Naringin group were decreased, and naringin increased and recovered the decrease in *Bcl-2* expression induced by H<sub>2</sub>O<sub>2</sub>. Meanwhile, naringin itself had no apparent adverse effect on follicles and granulosa cells



**Figure 2.** Effect of naringin on the decreased antioxidant status and downregulation of the antioxidant gene expression in H<sub>2</sub>O<sub>2</sub> -induced ASWFs. (A) Antioxidase levels (a–e, CAT, T-SOD, GSH-Px, GSH-ST, T-AOC) and oxidative products (f, MDA) in the SWFs after 24 h of naringin pretreatment. B: The levels of *Cat*, *Sod*, *Mgst*, and *Gsta* mRNAs in SWFs from the control group, H<sub>2</sub>O<sub>2</sub> group, H<sub>2</sub>O<sub>2</sub>+Naringin group, and Naringin group. Data were expressed as means  $\pm$  SEM. Different lowercase letters indicate significant difference ( $P < 0.05$ ).

(Figure 1). These data indicated that naringin reversed the H<sub>2</sub>O<sub>2</sub>-induced damage to the structure of growing follicles and inhibited the decline in proliferation and increase in apoptosis of H<sub>2</sub>O<sub>2</sub>-induced ASWFs.

### **Effect of Naringin on the Antioxidant Capacity in ASWFs Induced by H<sub>2</sub>O<sub>2</sub>**

From the results of the antioxidant capacity of SWFs, compared with the control group, T-AOC in the SWF

was significantly reduced after  $H_2O_2$  treatment (Figure 2A, e). At the same time, the activities of CAT, T-SOD, GSH-Px, and GSH-ST were markedly decreased after 72 h of  $H_2O_2$  treatment (Figure 2A, a-d). The application of naringin prevented these downward trends. After pretreatment with naringin alone, the activities of T-SOD, GSH-Px, and GSH-ST were increased compared with the control group (Figure 2A, b-d). The content of MDA in the  $H_2O_2$  group was significantly higher than the other three groups (Figure 2A, f). The qRT-PCR results showed that  $H_2O_2$  significantly reduced the expression of *Cat*, *Sod*, *Mgst*, and *Gsta* mRNAs, and these changes were standardized by naringin pretreatment (Figure 2B). These results indicated that naringin restored the decline of antioxidant capacity and partially rescued the changes in the expression of antioxidant genes in ASWFs induced by  $H_2O_2$ .

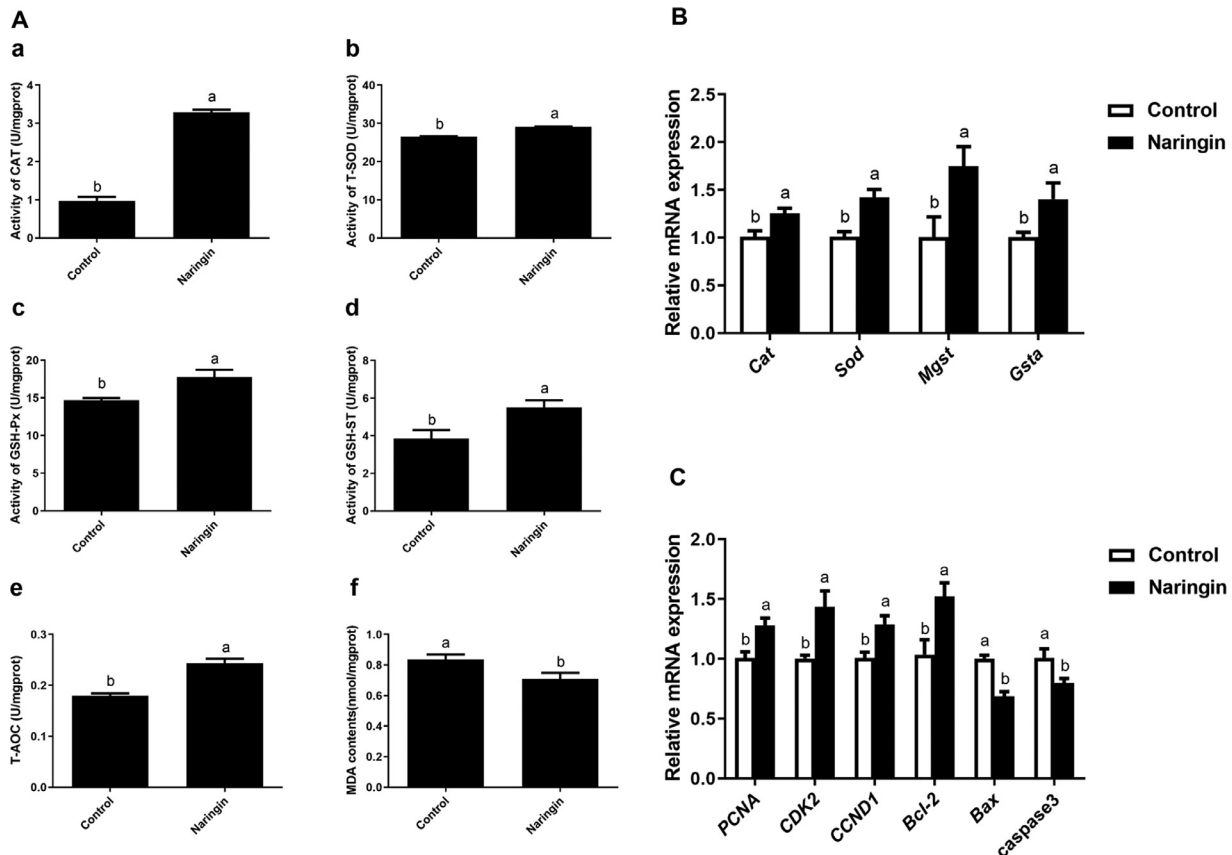
### Naringin Protected Natural Aging SWFs From Oxidative Stress In Vitro

To examine whether naringin treatment could protect the SWFs during natural aging from oxidative stress, SWFs from D580 hens were cultured with/without naringin treatment for 72 h. Then the antioxidant capacity, cell proliferation and apoptosis of the SWFs in each group were measured. After 72 h of naringin treatment,

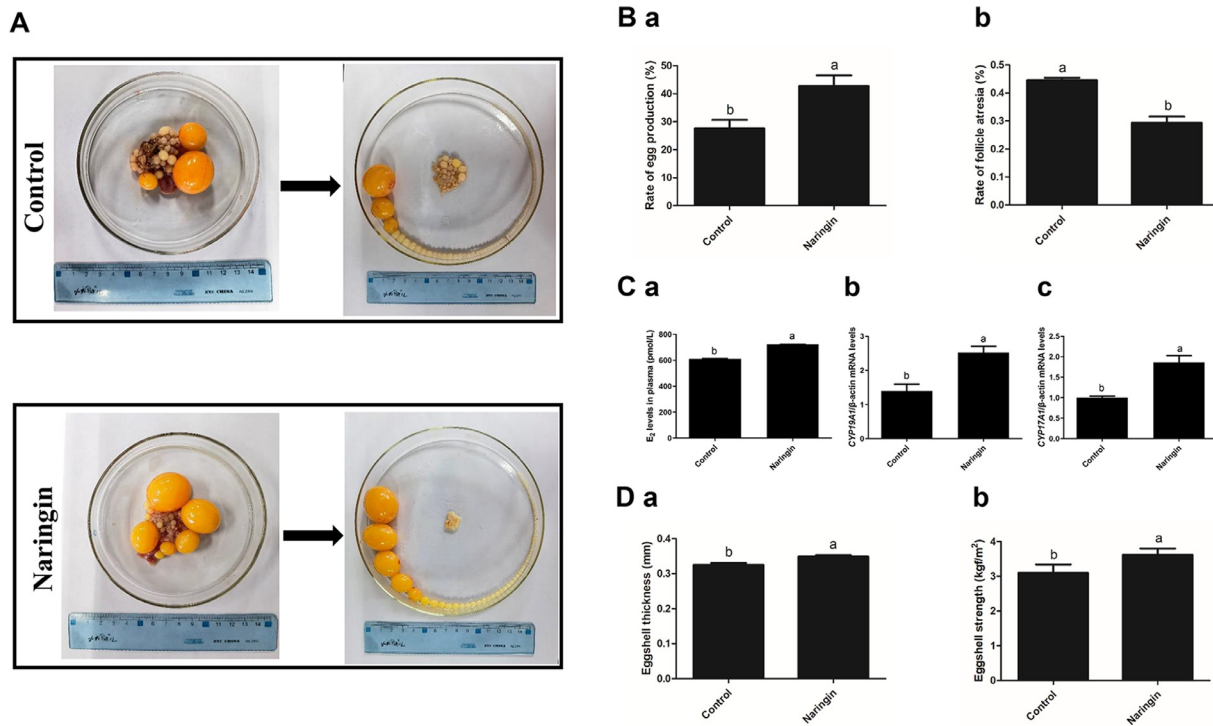
the T-SOD activity and T-AOC content in D580 SWFs were significantly increased (Figure 3A, b and e). Naringin treatment increased CAT and activated GSH-Px and GSH-ST in SWFs at D580 (Figure 3A, a, c and d). Meanwhile, naringin treatment reduced the content of the MDA in cultured SWFs (Figure 3A, f). qRT-PCR results showed that naringin treatment significantly increased the expression of *Cat*, *Sod*, *Mgst*, and *Gsta* mRNAs (Figure 3B) and increased the expression of *PCNA*, *CCND1*, *CDK2*, and *Bcl-2* in D580 SWFs (Figure 3C). In addition, after 72 h of naringin treatment, the expression of *Bax* and caspase3 in D580 SWFs were decreased significantly (Figure 3C).

### Effects of Naringin on Ovarian Development and Levels of Biochemical Parameters in D580 Hens

In order to investigate the effect of naringin on low-production hens in vivo, each hen was given naringin at a dose of 50 mg/kg for 15 consecutive days. During the experiment, the egg production rate of each group was recorded every day. The results showed that compared with the control group, the naringin group increased the egg production rate of the late laying hens during this period and reduced the follicular atretic rate (Figures 4A and 4B). Meanwhile, the naringin group



**Figure 3.** Effect of naringin on the decreased antioxidant status and downregulation of the antioxidant genes expression in D580 SWFs in vitro. (A) The antioxidant enzyme levels of CAT, T-SOD, GSH-Px, GSH-ST, and T-AOC, and the oxidation product level of MDA. (B) The levels of *Cat*, *Sod*, *Mgst*, and *Gsta* mRNAs in SWFs from the control group, and Naringin group in SWFs in vitro experiments. C: Effect of naringin on the expression of *PCNA*, *CDK2*, *CCND1*, *Bcl-2*, *Bax*, and caspase3 mRNAs. Data were expressed as means  $\pm$  SEM. Different lowercase letters indicate significant difference ( $P < 0.05$ ).



**Figure 4.** Effect of naringin on ovarian development and laying of D580 hens. (A) Effect of grade follicles development and the number of atresia of follicles in the control group and the naringin-fed group. (B) Effect of feeding naringin on egg production and follicular atresia ratio of old hens. a-b, Egg production rate, follicle atresia ratio. (C) Effect of naringin on estrogen levels in plasma and the expression of *CYP17A1* and *CYP19A1* mRNAs in D580 SWFs. (D) Effect of naringin on eggshell thickness and eggshell hardness in D580 hens. Data were expressed as means  $\pm$  SEM. Different lowercase letters indicate significant difference ( $P < 0.05$ ).

increased the thickness of the eggshell by 6.7% and the eggshell strength by 13.6% compared with the control group (Figure 4D). At the same time, E<sub>2</sub> in plasma was increased, and the expression of *CYP17A1* and *CYP19A1* mRNAs were up-regulated in SWFs in the naringin group (Figure 4C).

### Effect of Naringin on Proliferation and Apoptosis of SWFs in D580 Hens

PCNA fluorescent staining, TUNEL and qRT-PCR were used to evaluate the effect of naringin on the development of SWFs (Figure 5A). The results showed that naringin increased PCNA-labeled cells while reduced TUNEL-labeled cells (Figure 5A). qRT-PCR results showed that compared with the control, naringin treatment increased the expression of *PCNA*, *CCND1*, *CDK2*, and *Bcl-2* mRNAs in SWFs and decreased the expression of *Bax* and caspase3 mRNAs, which was consistent with the results of fluorescent staining (Figure 5B).

### Effect of Naringin on the Antioxidant Capacity and Antioxidant Genes in D580 Hens

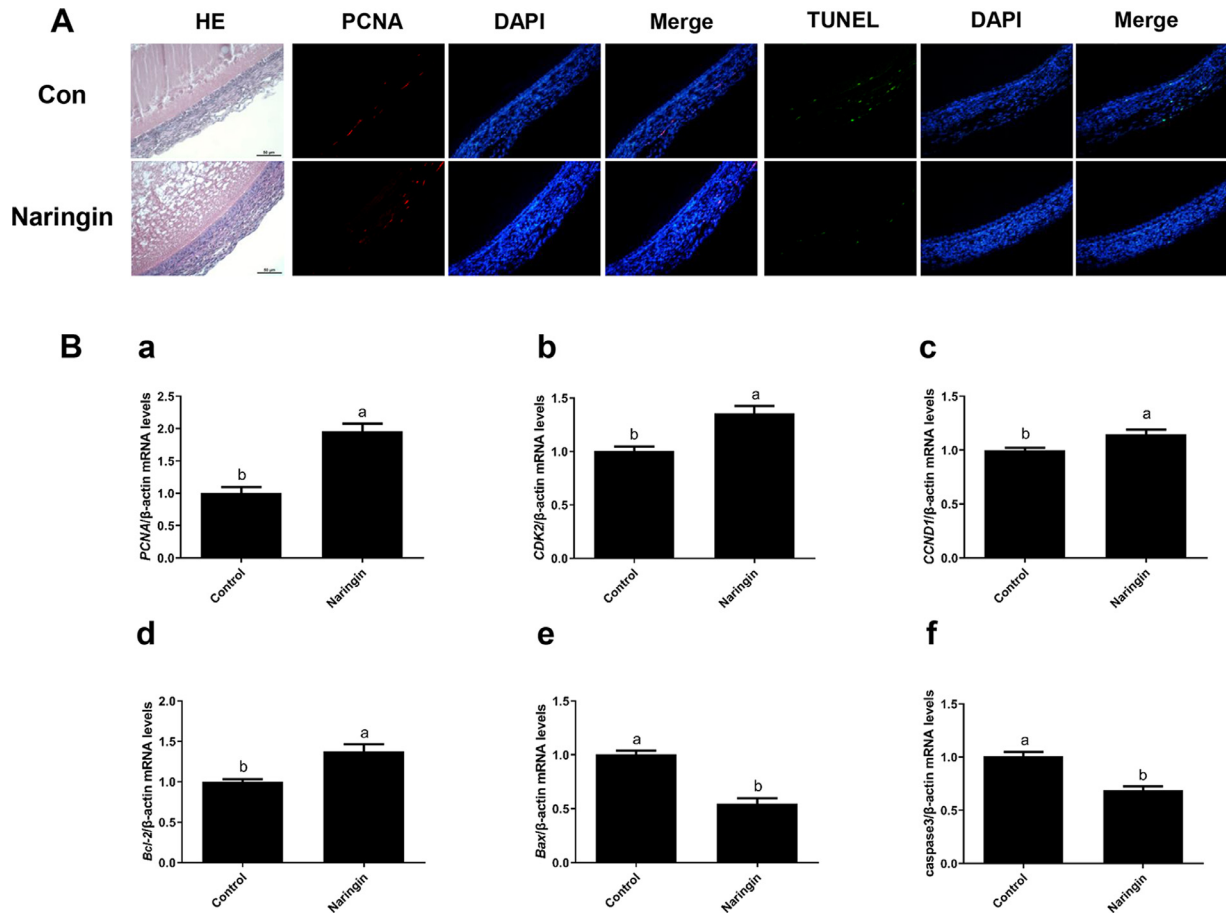
In order to evaluate whether naringin treatment could protect the naturally aged SWFs from oxidative stress, the levels of antioxidant indicators both in plasma and SWFs and the expression of

antioxidant genes in SWFs were also tested. The results showed that whether in plasma or SWFs, the naringin group reduced the content of the MDA (Figure 6A, f and Figure 6B, f). At the same time, the T-AOC content and T-SOD activity in the naringin group were significantly increased, as well as the CAT and the GSH-ST and GSH-Px in SWFs also were increased (Figures 6A and 6B). Furthermore, the results of qRT-PCR showed that naringin treatment significantly increased the expression of *Cat*, *Sod*, *Mgst*, and *Gsta* mRNAs in D580 SWFs (Figure 6C).

## DISCUSSION

Decreased antioxidant capacity and increased oxidative stress in age-related decline ovaries lead to imbalances in cell proliferation and apoptosis, which further leads to an increased in atretic follicles (Lim and Luderer, 2011; Liu et al., 2018a). Ovarian recession in aging laying hens is manifested as a decline in egg production and egg quality. In this study, we used the H<sub>2</sub>O<sub>2</sub>-induced ASWFs model established by the previous laboratory and the SWFs of naturally aged hens to study the effect of naringin on reducing oxidative stress in aged laying hens (Yao et al., 2020).

As an important ROS, H<sub>2</sub>O<sub>2</sub> has been widely used as a model of exogenous oxidative stress in apoptosis studies (Shi et al., 2021). To a certain extent, apoptosis is required for average cell renewal and maintaining tissue



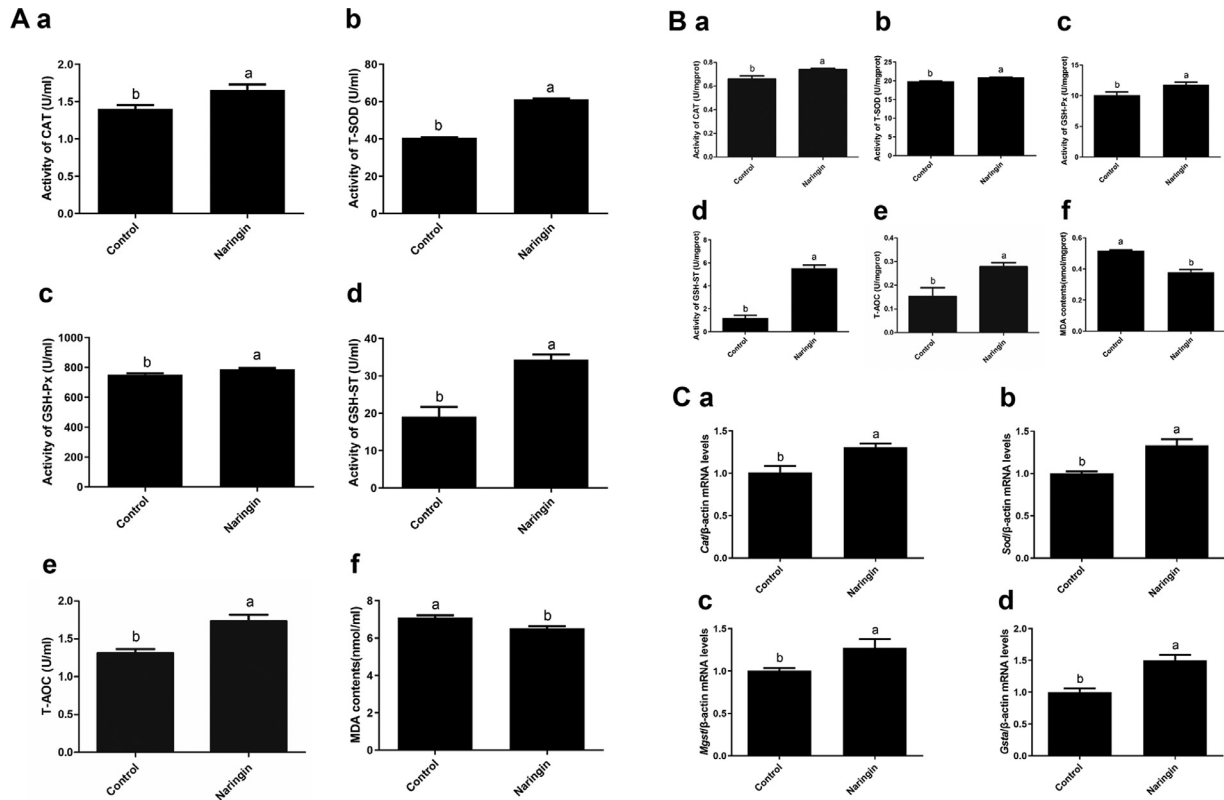
**Figure 5.** Effect of naringin on the proliferation and apoptosis of SWFs in D580 hens. (A) Naringin increased the number of PCNA-positive cells (red) in the SWFs of D580 hens and decreased the number of TUNEL-positive cells (green). Scale bar: 50  $\mu$ m. (B) Effect of naringin on the expression of *PCNA*, *CDK2*, *CCND1*, *Bcl-2*, *Bax*, and caspase3 mRNAs. Data were expressed as means  $\pm$  SEM. Different lowercase letters indicate significant difference ( $P < 0.05$ ).

homeostasis (Tower, 2015). However, excessive ROS in the cell will induce cell apoptosis, which further may lead to organ dysfunction (Narezkina et al., 2021). Expression of apoptosis-signaling genes was increased in atretic follicles compared with normal follicles (Yao et al., 2020). As a pro-apoptotic factor, *Bax* plays an important role in initiating the apoptosis program, which leads to a series of downstream caspase cascades and also induces the apoptosis of granulosa cells in the early stage of follicular atresia (Sai et al., 2011). Moreover, *Bcl-2* prevents apoptosis by binding to *Bax* (Qiu et al., 2019). In this study,  $H_2O_2$  treatment decreased the expression of *Bcl-2*. At the same time,  $H_2O_2$  treatment increased the expression of *Bax* and caspase3 in the results of qRT-PCR, which was consistent with the finding that the labeling rate of TUNEL-positive cells was markedly increased in the  $H_2O_2$  group. Similar to the reaction caused by hydrogen peroxide in the previous study (Jiang et al., 2020). BrdU incorporation and the results of qRT-PCR showed that  $H_2O_2$  treatment significantly inhibited the cell proliferation of SWFs. Phytoestrogens can reduce oxidative stress-induced apoptotic cells and improve proliferation rates (Petrine and Del Bianco-Borges, 2021). Naringin pretreatment reversed these changes caused by  $H_2O_2$ , which indicated that naringin had a regulatory effect on the

homeostasis of SWFS cells. This effect of naringin was also observed in D580-day-old laying hens.

High-concentration  $H_2O_2$  treatment could cause a decrease in the expression of antioxidant genes (Alhasani et al., 2018). In this study, after treatment with  $H_2O_2$  for 72 h, the content of the T-AOC and the activities of T-SOD, CAT, GSH-ST, and GSH-Px were decreased significantly, accompanied by the increased MDA level in the SWFs. And we found that naringin alleviated the decrease in antioxidant capacity caused by  $H_2O_2$  by increasing the activity of these antioxidant enzymes in SWFs that could effectively scavenge free radicals, inhibit the body's oxidation and maintain the body's homeostasis. In addition, the results of qRT-PCR showed that naringin could increase the level of the transcription of antioxidant genes respond to oxidative stress. Naringin also ameliorated the decline in antioxidant status in D580 naturally aged laying hens in the same way. The antioxidant capacity of naringin has been verified in another study (Gerçek et al., 2021). High concentrations of  $H_2O_2$  can induce excessive production of ROS and reduce the expression level of Nrf2 mRNA, which has a negative impact on the antioxidant status in chickens (Chen et al., 2021). The nuclear transcription factor Nrf2 is an important factor involved in activating the oxidative stress defense system to regulate the





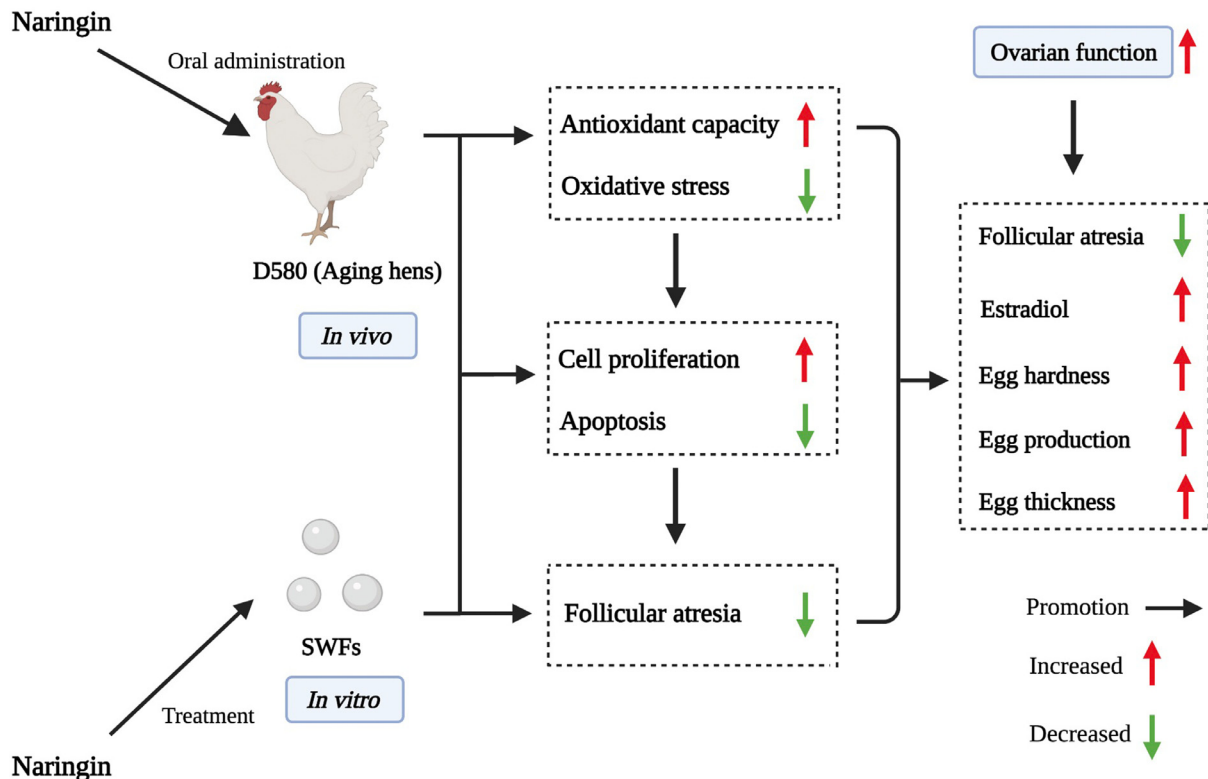
**Figure 6.** Naringin promoted antioxidant levels in D580 hens. (A) Effect of naringin on the antioxidant capacity of D580 plasma in vivo. Levels of CAT, T-SOD, GSH-Px, GSH-ST, T-AOC, and MDA were determined in plasma. (B) Effect of naringin on the antioxidant capacity of D580 SWFs in vivo. Levels of CAT, T-SOD, GSH-Px, GSH-ST, T-AOC, and MDA were determined in SWFs. (C) Effect of naringin on the downregulation of antioxidant genes expression in D580 SWFs in vivo. Comparison of the levels of antioxidant genes *Cat*, *Sod*, *Mgst*, and *Gsta* mRNAs between the control and naringin groups. Data were expressed as means  $\pm$  SEM. Different lowercase letters indicate significant difference ( $P < 0.05$ ).

transcription of antioxidant genes. In related experiments in mice, naringin can alleviate acetaminophen-induced acute liver injury by activating the Nrf2 pathway to exert antioxidant and antiapoptotic (Zhai et al., 2022). In addition, naringin can protect hADMSCs from oxidative stress-induced inhibition of osteogenic differentiation by activating the Wnt signaling pathway (Wang et al., 2015). Naringin also ameliorated arsenite-induced cardiotoxicity in rats by modulating TGF- $\beta$ /Smad-3, Nrf-2/HO-1 pathways and reducing cardiomyocyte apoptosis (Adil et al., 2016). In this experiment, the mechanism by which naringin exerts its antioxidant activity remains to be further studied.

Estrogen, playing a key role in defending against oxidative stress and stimulating cell proliferation and promoting follicle growth and inhibiting atresia, is a steroid hormone present in the blood and ovaries of poultry and derived from the follicular inner membrane cells of the ovary, mainly estradiol, which can be converted from androgens catalyzed by aromatase (Quirk et al., 2004; La Colla et al., 2017; Rani et al., 2021). Whether in mammals or poultry, female aging is accompanied by a decline in  $E_2$  levels. And phytoestrogens have their beneficial effects in the presence of estrogen deficiency, and a diet derived from phytoestrogens has a positive effect on the urogenital system of postmenopausal women (Petrine and Del Bianco-Borges, 2021). At low concentrations or in the absence of endogenous estrogen,

naringin exhibits estrogen agonist activity, and induces proliferation of estrogen-sensitive MCF-7 cells (Guo et al., 2011). This study found that naringin improved the antioxidant capacity and decreased apoptosis of D580 SWFs in vivo and in vitro experiments and played an important role in regulating estrogen. After 15 d of continuous oral administration of naringin, the level of  $E_2$  in the plasma of aged laying hens was increased significantly accompanied by elevated expression of *CYP17A1* and *CYP19A1*.

Eggshell is a biomineralization process in which calcium is obtained from intestinal absorption and completed in the uterus as the predominant form of calcium carbonate. It has been reported that age-related oxidative stress inhibits intestinal calcium absorption, resulting in reduced eggshell calcium deposition and the decline of eggshell quality in aged laying hens (al-Batshan et al., 1994; Areco et al., 2020). Estrogen has been shown to promote intestinal calcium absorption and increase eggshell strength at the end of the laying period (Wistedt et al., 2014; Nie et al., 2020). Meanwhile, naringin has been reported to protect intestinal calcium absorption by enhancing its antioxidant properties, especially GSH levels that is essential for intestinal calcium absorption (Rodríguez et al., 2017). The improvement in eggshell quality (eggshell thickness and hardness) in our study may be due to increased intestinal calcium absorption regulated by



**Figure 7.** Preventing effect of naringin on follicular atresia by inhibiting oxidative stress in the aging chickens.

estrogen as well as by elevated levels of antioxidants in the body. Its specific mechanism needs further study. Based on these arguments, naringin played an influential role in improving the quality of eggshells and inhibiting follicular atresia by regulating the follicles' antioxidant capacity and hormone levels.

## CONCLUSIONS

As shown in Figure 7, this study demonstrated that naringin improved the antioxidant capacity, alleviated oxidative stress inhibition on the proliferation, and reduced the increased apoptosis of  $H_2O_2$ -induced and naturally aging SWFs cells in vitro, thereby preventing atresia of follicles. Naringin also promoted the increase of  $E_2$  levels in aging laying hens and improved the decline of egg production. At the same time, the thickness and hardness of the eggshell were enhanced to reduce the loss rate in the egg during transportation to a certain extent. In summary, this study may prove the potential utilization of naringin in protecting laying hens from oxidative stress in decline in aging ovarian function by regulating antioxidant capacity, reducing follicular atresia, and improving eggshell quality.

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## DISCLOSURES

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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