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Melatonin alleviates ovarian function damage and oxidative stress induced by dexamethasone in the laying hens through FOXO1 signaling pathway

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ABSTRACT Oxidative stress can trigger follicular atresia, and decrease follicles quantity in each development stage, thereby alleviating reproductive activity. The induction of oxidative stress in chickens through intraperitoneal injection of dexamethasone is a reliable and stable method. Melatonin has been shown to mitigate oxidative stress in this model, but the underlying mechanism remains unclear. Therefore, this study aimed to investigate whether melatonin can recover aberrant antioxidant status induced by dexamethasone and the specific mechanism behind melatonin-dependent protection. A total of 150 healthy 40-wk-old Dawu Jinfeng laying hens with similar body weights and laying rates were randomly divided into three groups, with five replicates per group and 10 hens per replicate. The hens in the control group (**NS**) received intraperitoneal injections of normal saline for 30 d, the dexamethasone group (**Dex+NS**) received 20 mg/kg dose of dexamethasone for the first 15 d, followed by the 15 d of normal saline treatment. While in the melatonin group (**Dex+Mel**), dexamethasone (20 mg/kg dose) was injected intraperitoneally in the first 15 d, and melatonin (20 mg/kg/d) was injected in the last 15 d. The results showed that dexamethasone treatment significantly enhanced oxidative stress ($P < 0.05$), while melatonin not only inhibited the oxidative stress but also notably enhanced the antioxidant enzymes superoxide dismutase (**SOD**), catalase activity (**CAT**), glutathione peroxidase (**GSH-Px**), and antioxidant genes CAT, superoxide dismutase 1 (**SOD1**), glutathione peroxidase 3 (**GPX3**), and recombinant peroxiredoxin 3 (**PRDX3**) expression ($P < 0.05$). Melatonin treatment also markedly reduced 8-hydroxy deoxyguanosine (**8-OHdG**), malondialdehyde (**MDA**), and reactive oxygen species (**ROS**) levels ($P < 0.05$) and apoptotic genes Caspase-3, Bim, and Bax in the follicle. In the Dex+Mel group, the Bcl-2 and SOD1 protein levels were also increased ($P < 0.05$). Melatonin inhibited the forkhead Box Protein O1 (**FOXO1**) gene and its protein expression ($P < 0.05$). In general, this investigation revealed that melatonin might decrease oxidative stress and ROS by enhancing antioxidant enzymes and genes, activating the antiapoptotic genes, and inhibiting the FOXO1 pathway in laying hens.

Key words: melatonin, oxidative stress, dexamethasone, FOXO1, laying hen

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INTRODUCTION

With the advancements in the livestock and poultry industry in China, the differences between the breeding environment and the biological characteristics of animals have increased. Various environmental stresses often trigger the generation of ROS in the body and tissues, producing oxidative stress, which ultimately declines the reproductive performance of poultry (Liu and Meng, 2019). Multiple studies have indicated that ROS has a critical function in intracellular signal transduction, follicular growth, and ovulation (Tamura et al., 2020). As a signal molecule, ROS is responsible for activating and regulating the expression of genes with different physiological activities, such as growth, proliferation, differentiation, and apoptosis (Surai et al., 2019). Follicular development and atresia are the physiological basis of animal reproduction. In poultry follicles, follicular granulosa cells have the largest proportion and

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function. Their proliferation, differentiation, and apoptosis regulate follicular development and atresia (Lillpers and Wilhelmson, 1993). Increasing evidence indicates that excess ROS can trigger granulosa cell apoptosis, leading to sinus follicular atresia (Liu et al., 2018a). Therefore, how to improve ovarian oxidative stress, reduce the rate of follicular atresia after the egg production peak, extend the duration between egg laying, and achieve "500 eggs at 100 wk of age" have become important topics of research by domestic and foreign breeding companies.

Melatonin (N-acetyl-5-methoxytryptamine; MW = 232) is an indoleamine, released from the pineal gland, which critically controls the circadian rhythm, immune and inflammatory responses, and scavenges ROS (Reiter et al., 2016). Melatonin can directly eliminate ROS, involving superoxide anion, hydroxyl radical, singlet oxygen, and hydrogen peroxide ($\mathbf{H_2O_2}$) (Mortezaee and Khanlarkhani, 2018). It also protects biofilm and DNA molecules and improves the body's antioxidant level by activating antioxidant enzymes such as GSH-Px, SOD, and CAT (Paradies et al., 2017; Yang et al., 2019). Additionally, it regulates follicular growth, oocyte maturation, ovulation, and luteal function. Melatonin not only improves the quality of mouse oocytes but also enhances the ovulatory process in older mice (Tamura et al., 2017). Research also suggests that melatonin can potentially relieve oxidative stress-induced apoptosis (Zhou et al., 2012). It is widely distributed in ovarian tissue (including follicular fluid) and its receptors can be detected in the entire ovary (Tian et al., 2017), however, the melatonin binding sites are aggregated more in the granular layer of sinus follicles (Yie et al., 1995). On the contrary, blocking the production of melatonin through pinealectomy accelerates the process of ovarian atresia in mammals (Soares et al., 2003). Therefore, it can be hypothesized that melatonin also regulates follicular development and atresia in poultry. At present, there is no systematic report on melatonin's effects and mechanisms on follicular propagation and atresia in poultry. Its protective mechanism against oxidative stress-induced apoptosis of granulosa cells remains to be further explored.

Forkhead O (**FOXO**) signaling pathway is a subfamily of transcription factors, which includes FOXO1, FOXO3, FOXO4, and FOXO6, and is associated with many cellular mechanisms such as cell death, repair of DNA damage, cell cycle arrest, oxidative stress, etc. (Xing et al., 2018). FOXO1 is the most critical member of the FOXO family. In female mammals, it regulates the growth, maturation, and atresia of follicles through multiple pathways (Zhang et al., 2016). The function of FOXO1 depends on the regulation of downstream targets (Matsuzaki et al., 2016; Murtaza et al., 2017; Xing et al., 2018). Under oxidative stress, FOXO1 mRNA and protein expression increased significantly, and excessive FOXO1 protein enters the nucleus, thereby increasing downstream apoptotic gene expression. Further research found that FOXO1 induces granulosa cell apoptosis by activating the downstream gene PUMA (Liu et al., 2015). In conclusion, oxidative stress induces antral follicle atresia in animals, and FOXO1 is a key regulatory factor of oxidative stress that triggers follicular granulosa cell apoptosis.

Oxidative stress has been known to induce follicular atresia and decrease the number of follicles in each developmental stage, thus inhibiting reproductive activity. The FOXO1 signaling pathway is a major regulatory pathway associated with oxidative stress-induced follicular atresia. Melatonin has been suggested as an antioxidant that may protect against oxidative stress induced by dexamethasone. However, the specific mechanism underlying this protection remains unclear. Thus, this study aims to evaluate whether melatonin-dependent protection can restore the abnormal antioxidant status induced by dexamethasone and elucidate the underlying mechanism. As a cell survival regulator and its potential role in cell cycle control, studying the expression pattern of FOXO1 and the resistance mechanism of melatonin in ovaries of laying hens under oxidative stress can put forth a theoretical basis for the identification of mechanisms associated with follicular growth and for prolonging the utilization cycle of laying hens.

MATERIALS AND METHODS

Animals

For this research, 150 hens (initially weighing about 1.4 ± 0.17 kg), 40 wk of age were acquired from Breeding Poultry of Hebei Dawu Group (Baoding, China). These hens were categorized randomly into 1) the control group (**NS**), 2) the dexamethasone (**Dex+NS**) group, and 3) the melatonin (**Dex+Mel**) group. They were kept in a standard environment, in individual cages, and under 23°C ± 2°C temperature with relative humidity (65 ± 10%). This investigation was a 30-days experiment and hens were housed in a 16:8 h light and dark cycle. The feed and water were available ad libitum. Before any experimental procedure was performed, for 7 d the birds were housed in cages so to allow habituation. The composition and chemical composition of the basal diet during the experiment are shown in Table 1. All the experiments were carried out by following the standard animal handling recommendations provided by the relevant national and local animal welfare bodies. This investigation was authorized by the Animal Care and Use Committee of the College of Animal Science and Technology, Hebei Agriculture University, PR China.

Dexamethasone Treatment to Construct an In Vivo Oxidative Stress Model

Dexamethasone (Sigma, St. Louis, MO) was prepared by dissolving it in absolute ethanol (10% in the final solution) and then diluting it to the desired volume with normal saline. The test period was 30 d. The hens in the control group (NS) were intraperitoneally injected with normal saline for 30 d, the dexamethasone group (Dex +NS) was administered with dexamethasone (20 mg/kg dose) for the first 15 d, and then normal saline for the

Table 1. Composition and nutrient levels of the basal diet (air-dry basis).

Ingredients	Content (%)	Nutrient levels[b]	Content (%)
Corn	62.00	ME/(MJ/kg)	11.44
Soybean meal	24.00	CP	17.00
Soybean oil	1.00	Ca	3.57
Fish meal	2.00	TP	0.54
$CaHPO_4$	1.00	AP	0.34
Stone powder	9.00	Lys	0.90
NaCl	0.32	Met	0.41
Methionine	0.12		
Threonine	0.10		
Choline chloride	0.10		
Premix[a]	0.30		
Phytase	0.02		
Lysine	0.04		
Total	100.00		

[a]The premix provides: V_A 10,000 IU, V_{D3} 000 IU, V_E 29 mg, V_{B1} 3 mg, V_{B2} 8 mg, V_{B6} 6 mg, V_{B12} 30 ug, niacin 50 mg, folate 1.5 mg, Cu 12.5 mg, Fe 90 mg, Zn 82.8 mg, Mn 95.4 mg, I 0.19 mg/kg of feed. The nutritional level is the calculated value.

[b]Metabolic energy is the calculated value, while the rest are the measured values.

later 15 d, While in the melatonin group (Dex+Mel), dexamethasone (20 mg/kg dose) was injected intraperitoneally for the first 15 d, and melatonin (20 mg/kg/d) was injected in the last 15 d. All the treatments were given every day at the same time (16:00th h).

Determination of 8-OHdG, MT, SOD, MDA, GSH-Px, CAT, and ROS Concentrations in Plasma

On the 15th and 30th d, 5 mL blood from each hen was acquired from the vein present under the wing. The collected blood was centrifuged for 15 min at 3,000 rpm. The separated plasma was stored at −20° C for subsequent studies. Levels of 8-OHdG, SOD, MDA, GSH-Px, CAT, MT as well as ROS were evaluated by ELISA with the help of a commercial kit (Shanghai Jianglai Biotechnology Co., Ltd., Shanghai, China), by following the protocol provided by the manufacturer (Sihong et al., 2019; Hao et al., 2020b; Cheng et al., 2021).

Ovarian Follicle Counting

On the last day of the experiment, 10 random chickens from each group were euthanized by cervical dislocation. Their ovaries were dissected and their follicular diameter and quantity were determined. The follicular diameter was evaluated by the following categories using the vernier caliper (Mitutoyo, 410300, Osaka, Japan): 1) Preovulatory follicle (**PF**) = >10 mm; 2) small yellow follicle (**SYF**) = 8 to 10 mm; 3) large white follicle (**LWF**) = 6 to 8 mm; 4) medium white follicle (**MWF**) = 4 to 6 mm; 5) small white follicle (**SWF**) = 2 to 4 mm; 6) Primary follicle (**PE**) = <2 mm (Johnson, 2015).

RNA Extraction and Real-Time PCR Analysis

On the last day of the experiment, 2 chickens from each treatment were dissected and their granular cell layer and ovaries were sampled and stored at -80°C. The expressions of SOD1, CAT, GPX3, PRDX3, BIM, BCL-2, CASPASE-3, BAX, and FOXO1 genes in these samples were determined via RT-PCR. Total RNA was eluted from these samples by following the protocol of the kit used, that is, total RNA extraction kit (Invitrogen, Carlsbad, CA, 12183-555). The quality and extracted RNA concentration were evaluated via agarose gel electrophoresis and the nucleic acid was quantified in an acid quantification analyzer (SmartSpec Plus BIO-RAD), respectively. SuperScript III First-Strand Synthesis SuperMix (Invitrogen, 11752-050) kit was utilized to synthesize total cDNA according to the manufacturer's instructions. Successful cDNA synthesis was confirmed by amplifying the β-actin amplicon with PCR using a 20 μL PCR reaction comprising 10 μL Power SYBR Green PCR Master Mix (Roche, 4913914001, CH, GER), 1 μL cDNA, 0.5 μL forward and 0.5 μL reverse Primer (Huada Biological Engineering Technology & Service, Beijing, China), and 8 μL ddH_2O. Products after PCR were confirmed by agarose gel (1%) electrophoresis, followed by sequencing. Pooled cDNA was utilized to generate standard curves. The qPCR primer sequences are enlisted in Table 2. Each gene's relative expression was elucidated in triplicate/sample via the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Western Blot Analysis

Using ovarian lysate, total protein was acquired by the T-PER tissue protein extraction reagent (Thermo Pierce, Waltham, MA, 78510) augmented with a protease inhibitor cocktail (Thermo Pierce, Waltham, MA, 78440). BCA Protein Assay (Angle Gene Technologies, VB0002, CHN) was performed for protein quantification. For protein separation, 20 μg lysate per lane was loaded in 7.5% Tris−HCl gels and separation was performed via SDS−PAGE electrophoresis. Then proteins were transferred from the gel onto PVDF membranes (Merck Millipore, Rockville, MD, IPVH00010). Tris-buffered saline (**TBS**) with 5% skim milk solution was prepared and used at room temperature for blocking the membranes for 1 h, followed by membrane overnight incubation with primary antibodies at 4°C. After incubation, membranes were rinsed in Western washing buffer (TBS-T) for 15 min (Cell Signaling Technology, Danvers, MA) and then incubated with Goat Anti-Rabbit IgG H&L (HRP) (Abcam, ab6721, Cambridge, MA, 1:5,000 dilution), consecutively rinsed again with TBS-T 3 time for 20 min. Chemiluminescence ECL detecting reagents (GE, Pittsburgh, PA, RPN810) and exposure of the Super Signal West Dura Extended Duration Substrate (Thermo Pierce, Waltham, MA, 34075) were employed for detecting the signals. This protocol was carried out for the analysis of BCL-2 (Proteintech, 26593-1-AP, 1:500 dilution), SOD1 (Proteintech, 10269-1-AP, 1:500 dilution), FOXO1 (Bioss, bs-7068R, 1:500 dilution), and Actin

Table 2. Primers list.

Gene		Primer sequences (5′−3′)	Size (bp)
Bim	NC_060926.1	TTCTTGGATAACCGTGCTGGAA GATGAGGCGGATGATGTAATGC	72
Prdx3	XM_426543.5	AATACCTCGTGCTCTTCTTCTACC CACCTCGCAGTTCACATCGT	110
Gpx3	NC_052544.1	CCAAGGAGCAGAAGGTCTACAG GTTCTTGACAGTGGCGATGTTG	200
Sod1	NM_205064.1	TTGTCTGATGGAGATCATGGCTTC TGCTTGCCTTCAGGATTAAAGTGAG	98
Sod2	XM_015285700.2	CTGTATCAGTTGGTGTTCAAGGAT GCAATGGAATGAGACCTGTTGT	129
Cat	NM_001031215.1	GAGGTAGAACAGATGGCGTATGA CGATGTCTATGCGTGTCAGGATA	113
Bax	NC_006094	TTCGGCTGTTTCTCAC ATCCTTATCTCCGCTCT	243
Caspase3	NM_204725.1	GGCCTGTAGAGGAACAGAATTAG AGTAGCCTGGAGCAGTAGAA	125
Bcl2	NM_205339.2	TTGTACGGCAACAGTATGAGG TCCAAGATAAGCGCCAAGAG	111
Foxo1	NC_006088.5	GTTGGGTGTCAGGCTAGGAT CTGCCAACTCTGACGAAAGG	199
Actin	NM_205518.1	GAGAAATTGTGCGTGACATCA CCTGAACCTCTCATTGCCA	152

(Abcam, ab6276, 1:1,000) which was set as the endogenous control.

Data Analysis

Experimental data in tables and figures are presented as mean ± SD. Statistical analysis of differences between groups (NS, Dex+NS, and Dex+Mel) was performed using 1-way ANOVA followed by Duncan's multiple comparisons tests using IBM SPSS 21.0 (Chicago, IL). Independent sample T tests were used to compare blood indicators at different times on d 15 and d 30. a/b, *, $P < 0.05$ indicates significance. A/B, **, $P < 0.01$ indicates extreme significance. Graphs were generated by GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA).

RESULTS

Effect of Melatonin on the Production Performance of Laying Hens Under Oxidative Stress Induced by Dexamethasone

The effect of melatonin on laying hens' production performance under oxidative stress induced by dexamethasone is displayed in Table 3. On d 15th, the egg-laying rate was significantly reduced after dexamethasone injection ($P < 0.05$), however, with melatonin treatment, the rate in the Dex+Mel group enhanced notably than the Dex+NS group ($P < 0.05$). The average daily feed intake (**ADFI**) of the Dex+Mel group markedly increased compared with NS and Dex+NS groups ($P < 0.05$). On d 30th, the egg-laying rate of the Dex+Mel group reversed significantly and was markedly higher than NS and Dex+NS groups ($P < 0.05$).

Effects of Melatonin on Egg Quality of Laying Hens Under Oxidative Stress Induced by Dexamethasone

The effects of melatonin on the egg quality of laying hens under oxidative stress induced by dexamethasone are shown in Table 4. The Dex+NS group and the Dex +Mel group did not affect the egg quality of hens ($P > 0.05$).

Effects of Melatonin on Oxidative Stress-Related Indexes of Laying Hens Under Oxidative Stress Induced by Dexamethasone

The effects of melatonin on oxidative stress-related indexes of laying hens under oxidative stress induced by dexamethasone are shown in Figures 1 and 2. Figure 1 reveals that on d 15th, the SOD, GSH-Px, and CAT levels in Dex+NS and Dex+Mel groups were extremely reduced than the NS group ($P < 0.01$), while on the 30th d, after supplemental injection of melatonin, the SOD, GSH-Px, and CAT levels in Dex+Mel group enhanced markedly than the Dex+NS group without melatonin supplementation ($P < 0.01$). The d 30th levels of SOD, GSH-Px, and CAT were more than that observed on d 15th ($P < 0.01$). The MDA level in Dex+NS and Dex +Mel groups was notably higher in comparison with the NS group on the 15th d ($P < 0.01$), while on the 30th d, its level was higher in the Dex+NS group compared with the NS and Dex+Mel groups ($P < 0.01$).

Table 3. Effect of melatonin on the production performance of laying hens under oxidative stress induced by dexamethasone.

Period	Items	NS	Dex+NS	Dex+Mel	P value
D 15	ADFI (g)	108.54±5.67[b]	105.21±3.28[b]	118.68±4.58[a]	0.016
	AEW (g)	58.27±4.37	59.24±6.81	60.35±3.14	0.547
	LR (%)	83.23±6.69[ab]	80.73±4.16[b]	84.96±3.58[a]	0.041
	FER	2.40±0.21	2.58±0.12	2.32±0.15	0.067
D 30	ADFI (g)	117.11±7.57	124.81±2.82	122.47±5.37	0.156
	AEW (g)	60.69±3.48	62.42±2.25	61.18±5.84	0.244
	LR (%)	84.33±5.47[b]	80.14±3.53[b]	88.37±2.09[a]	0.018
	FER	2.28±0.18[b]	2.40±0.22[a]	2.26±0.15[b]	0.034

Abbreviations: ADFI, average daily feed intake; AEW, average egg weight; FER, feed-egg ratio; LR, laying rate.
Laying rate = egg number/the number of hen-days × 100%.
Average egg weight = total egg weight/total egg number.
Daily intake = total feed intake/feeding days.
Feed-egg ratio = daily feed consumption/average egg weight.
Values are means ± SD.
[a,b]Means within each row possessing different superscripts and differ significantly ($P < 0.05$, $n = 50$). If there is no marked letter, the difference is not significant.

Table 4. Effects of melatonin on egg quality of laying hens under oxidative stress induced by dexamethasone.

Period	Items	NS	Dex+NS	Dex+Mel	*P* value
D 15	ESI	1.32±0.05	1.33±0.04	1.31±0.02	0.314
	EST (mm)	0.36±0.05	0.36±0.03	0.37±0.02	0.508
	EW (g)	58.29±6.5	60.34±3.2	57.45±5.81	0.175
	AH (mm)	6.56±0.76	5.38±2.34	6.23±0.86	0.138
	HU	77.70±8.60	74.46±10.28	78.50±8.85	0.076
	ESS (N)	44.26±5.26	42.21±3.85	42.67±4.69	0.645
D 30	ESI	1.33±0.04	1.32±0.06	1.32±0.02	0.248
	EST (mm)	0.37±0.02	0.36±0.04	0.38±0.03	0.776
	EW (g)	60.98±5.73	62.53±3.18	62.33±4.52	0.222
	AH (mm)	6.08±0.91	6.15±1.33	5.85±1.87	0.088
	HU	76.08±9.61	77.79±8.17	78.48±9.37	0.578
	ESS (N)	40.44±7.24	44.04±6.46	43.02±5.68	0.344

Abbreviations: AH, albumen height; ESI, egg-shape index; ESRW, eggshell relative weight; ESS, eggshell strength; EST, eggshell thickness; EW, egg weight; EYRW, egg yolk relative weight; HU, Haugh unit.

Values are means ± SD.

Figure 2 indicates that on the 15th d, the melatonin level in Dex+NS and Dex+Mel groups was extremely reduced as compared to the NS group ($P < 0.01$), while on the 30th d, its level in the Dex+NS group was markedly reduced than the other two groups ($P < 0.01$). The ROS and 8-OHdG levels in Dex+NS and Dex+Mel groups were extremely increased compared with the NS group on the 15th d ($P < 0.01$), however, their levels on the 30th d in the Dex+NS group were notably enhanced than that of the NS and the Dex+Mel groups ($P < 0.01$).

Effect of Melatonin on the Ovarian Follicular Quantity of Laying Hens Under Oxidative Stress Induced by Dexamethasone

The effects of melatonin on the ovarian follicular quantity of laying hens under oxidative stress induced by dexamethasone are shown in Figure 3. Figure 3 reveals that on d 15th, the follicular numbers in Dex+NS and Dex+Mel groups were extremely less than the NS group, while on d 30th, after supplemental injection of melatonin, the follicular numbers of the Dex+Mel group increased notably as compared to the Dex+NS group without melatonin supplementation ($P > 0.05$).

Effect of Melatonin on Apoptosis-Related Genes of Laying Hens Under Oxidative Stress Induced by Dexamethasone

The effects of melatonin on apoptosis-related genes of laying hens under oxidative stress induced by dexamethasone are shown in Figure 4. Figure 4 shows the effects of melatonin on Bcl-2, Caspase-3, Bim, and Bax apoptotic genes of laying hens under dexamethasone oxidative stress. It reveals that Bcl-2 levels in the Dex+Mel group were more than in the NS group in PE, SWF, MWF, SYF, and ovary ($P < 0.01$), and LWF and PF ($P < 0.05$). While in the DEX+NS group, the Bcl-2 expression in the SWF, MWF, SYF, and ovary was notably reduced than in the Dex+Mel group ($P < 0.01$), LWF, and PF ($P < 0.05$). In the PE, SWF, SYF, and ovary, the expression levels of caspase-3 in Dex+NS and Dex

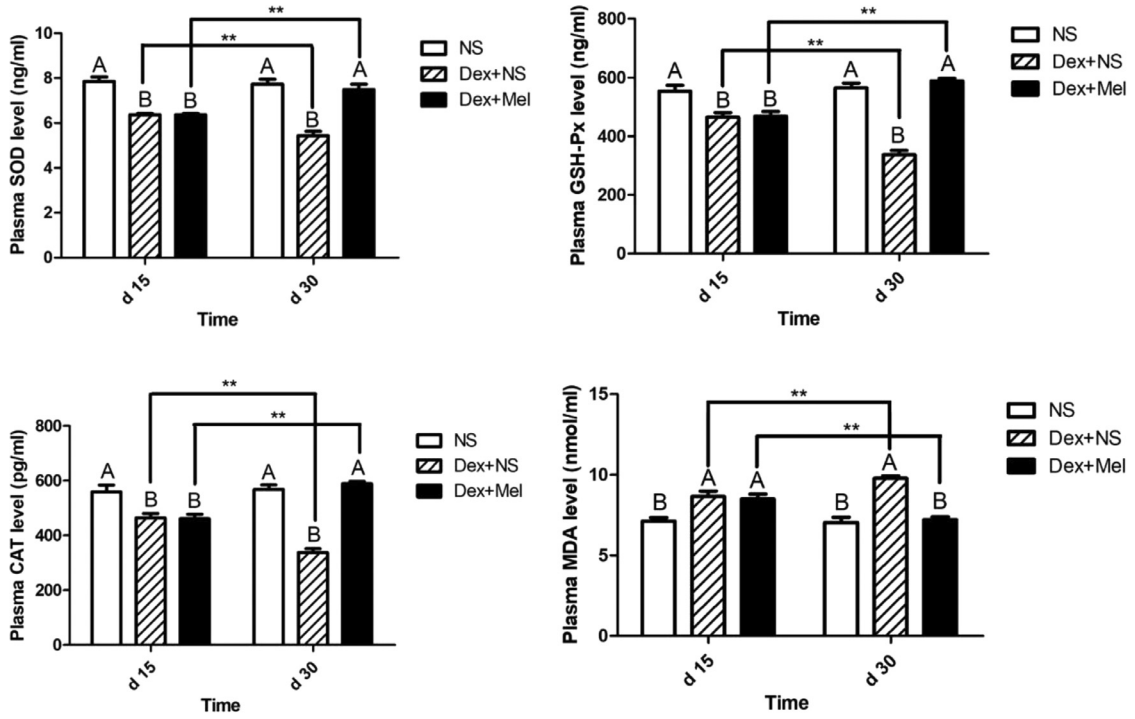

Figure 1. Effects of melatonin on oxidative stress-related indexes of laying hens in dexamethasone oxidative stress model. Diagram displaying SOD, GSH-Px, CAT, and MDA levels of animal groups. Comparison of the levels of SOD, GSH-Px, CAT, and MDA between the control (NS), dexamethasone (Dex+NS), and melatonin (Dex+Mel) groups. Data are shown as means ± SD; $N = 20$ chicken/group. *, $P < 0.05$ indicates significance compared with control values, **, $P < 0.01$ indicates extremely significance from respective control values. A and B indicate extreme significance compared to control values.

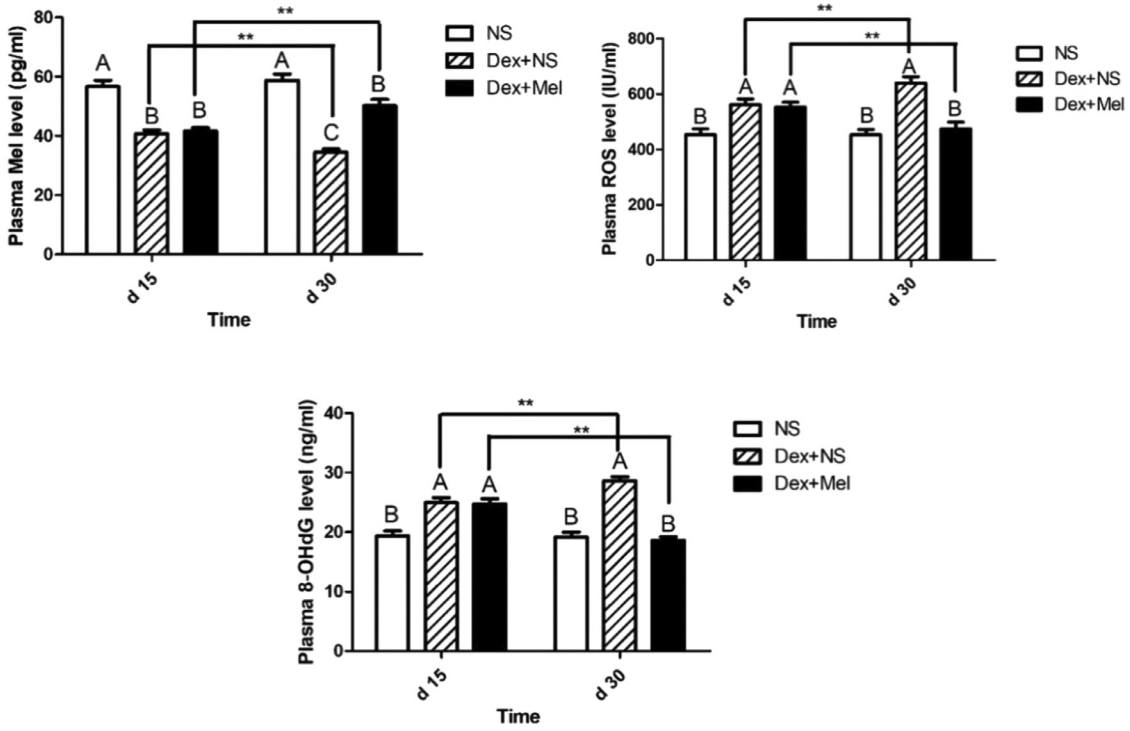

Figure 2. Effects of melatonin on Melatonin, ROS, and 8-OHdG levels of laying hens in the dexamethasone oxidative stress model. Diagram displaying melatonin, ROS, and 8-OHdG levels of animal groups. Comparison of the levels of melatonin, ROS, and 8-OHdG between the control (NS), dexamethasone (Dex+NS), and melatonin (Dex+Mel) groups. Data are shown as means ± SD; $N = 20$ chicken/group. *, $P < 0.05$ indicates extreme significance compared to control values, **, $P < 0.01$ indicates extreme significance from respective control values. A and B indicate extreme significance compared to control values.

+Mel groups were notably enhanced than in the NS group ($P < 0.01$). While in SWF, MWF, LWF, SYF, and ovary the expression levels of caspase-3 in the Dex +Mel group were extremely reduced than that in the Dex+NS group ($P < 0.01$). In the PE, SWF, LWF, PF, and ovary, the expression level of Bim in the Dex+NS group was higher than that in the NS group ($P < 0.01$), whereas, in group Dex+Mel it was markedly decreased compared with the NS group ($P < 0.01$). Compared with Dex+NS and Mel+NS groups, the gene expression of Bim increased significantly ($P < 0.01$). Bax expression in SWF, SYF, PF, and ovary of the Dex+Mel group was notably reduced than that in the NS group ($P < 0.01$), while in the Dex+NS group, it was significantly enhanced than the NS group ($P < 0.01$). Compared with Dex+NS and Mel+NS groups, the Bax gene expression decreased significantly in PE, SWF, MWF, LWF, and ovary ($P < 0.01$). Protein analysis revealed that protein expression of the Bcl-2 ratio declined with mRNA expression (Figure 7). These results demonstrate that melatonin can inhibit apoptosis caused by dexamethasone.

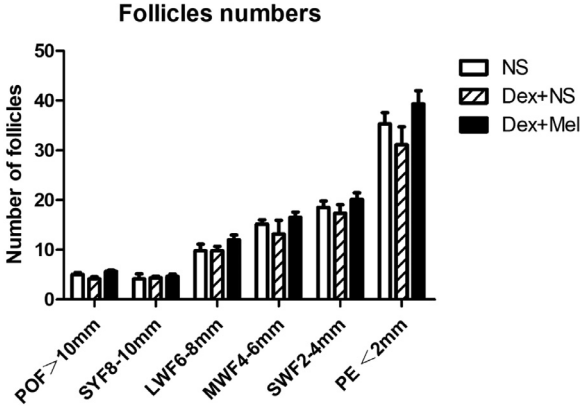

Figure 3. Effect of melatonin on ovarian follicular numbers of laying hens in the dexamethasone oxidative stress model. Comparison of the number of follicles between the control (NS), dexamethasone (Dex +NS), and melatonin (Dex+Mel) groups. Data are shown as means ± SD; $N = 6$ chicken/group. *, $P < 0.05$ indicates extreme significance compared to control values, **, $P < 0.01$ indicates extreme significance compared to control value.

Effect of Melatonin on Antioxidative Stress-Related Genes of Laying Hens Under Oxidative Stress Induced by Dexamethasone

The effects of melatonin on antioxidative stress-related genes of laying hens under oxidative stress induced by dexamethasone are shown in Figure 5. Figure 5 shows melatonin's effects on antioxidative stress-associated genes of CAT, SOD1, GPX3, and PRDX3 in the dexamethasone oxidative stress model in laying hens, suggesting that dexamethasone treatment inhibits the expression of these genes ($P < 0.05$), while melatonin treatment enhances their expression to varying degrees ($P < 0.05$). Protein analysis indicated that the protein expression of the SOD1 ratio declined with mRNA expression (Figure 7). Suggesting that melatonin could restore oxidative stress caused by dexamethasone.

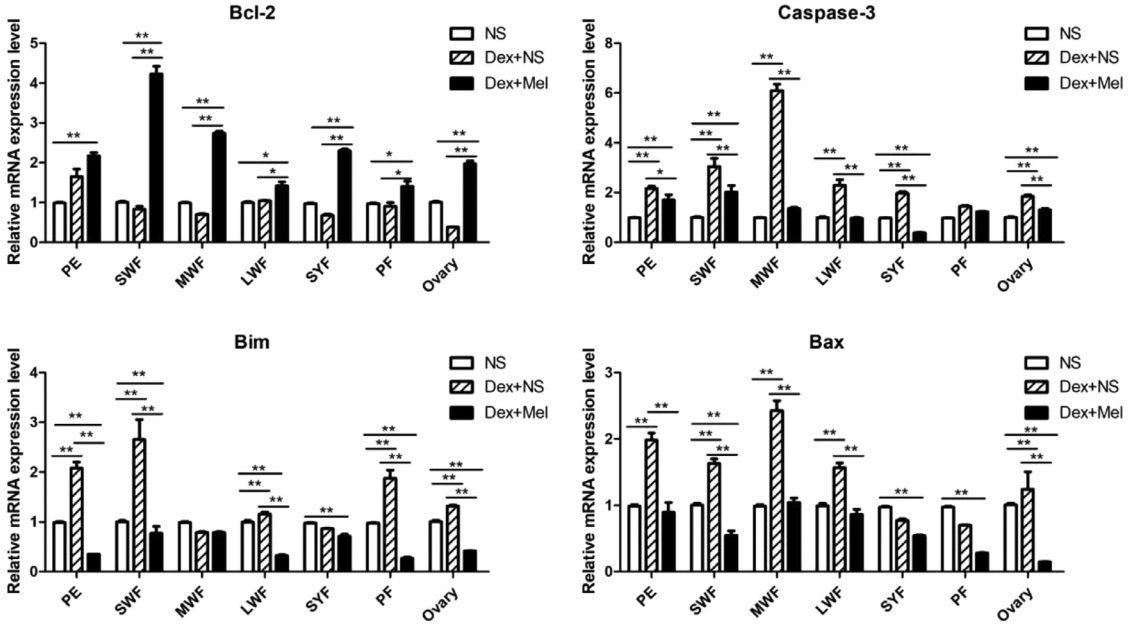

Figure 4. Effect of melatonin on apoptosis-related genes of Bcl-2, Caspase-3, Bim, and Bax of laying hens in the dexamethasone oxidative stress model. Comparison of the gene expression of Bcl-2, Caspase-3, Bim, and Bax between the control (NS), dexamethasone (Dex+NS), and melatonin (Dex+Mel) groups. Data are shown as means ± SD; $N = 3$ chicken/group. *, $P < 0.05$ indicates significance with respect to control values, **, $P < 0.01$ indicates extremely significance with respect to control values.

Effect of Melatonin on FOXO1 Expression of Laying Hens Under Oxidative Stress Induced by Dexamethasone

Figure 6 shows the effect of melatonin on the FOXO1 expression of laying hens under oxidative stress induced by dexamethasone. It indicates that in SWF, MWF, LWF, SYF, and ovary, dexamethasone treatment increases the expression of the FOXO1 gene ($P < 0.01$), while melatonin treatment inhibits its expression as compared with the NS group ($P < 0.01$). Protein analysis suggested that the FOXO1 ratio expression downregulated with mRNA expression (Figure 7), thereby resisting dexamethasone-induced oxidative stress via melatonin treatment.

DISCUSSION

The present study demonstrated that melatonin significantly reduced the generation of reactive oxygen

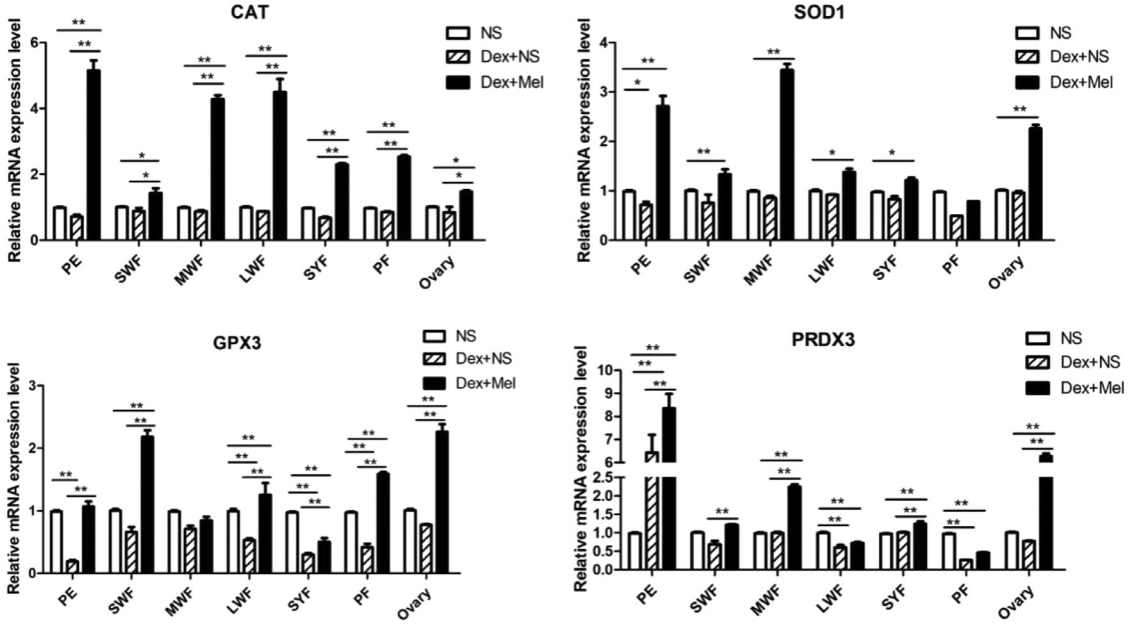

Figure 5. Effect of melatonin on antioxidative stress-related genes of CAT, SOD1, GPX3, and PRDX3 of laying hens in the dexamethasone oxidative stress model. Comparison of the gene expression of CAT, SOD1, GPX3, and PRDX3 between the control (NS), dexamethasone (Dex+NS), and melatonin (Dex+Mel) groups. Data are shown as means ± SD; $N = 3$ chicken/group. *, $P < 0.05$ indicates significance with respect to control values, **, $P < 0.01$ indicates extremely significance with respect to control values.

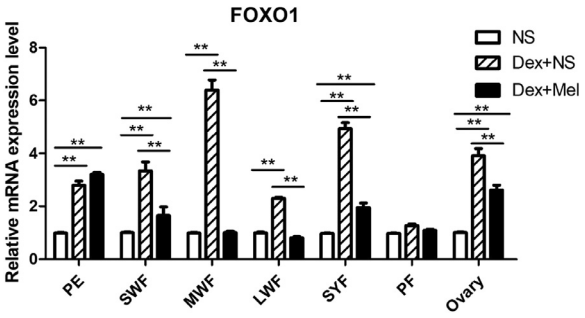

Figure 6. Effect of melatonin on FOXO1 of laying hens in the dexamethasone oxidative stress model. Comparison of the gene expression of FOXO1 between the control (NS), dexamethasone (Dex+NS), and melatonin (Dex+Mel) groups. Data are shown as means ± SD; $N = 3$ chicken/group. *, $P < 0.05$ indicates significance compared with control values, **, $P < 0.01$ indicates extremely significance compared with control values.

species in laying hens, which may be directly linked to the inhibition of oxidative stress by melatonin. The results also revealed that melatonin improved the antioxidant ability and decreased oxidative stress by downregulating the expression of FOXO1 in laying hens under oxidative stress induced by dexamethasone.

Oxidative stress produces multiple effects on different reproduction mechanisms such as oocyte maturation, ovarian follicular development, fertilization, conception, and embryo formation (Agarwal et al., 2005). Treatment with antioxidants during in vitro and in vivo settings has shown to counteract oxidative stress and increase female fertility (Agarwal et al., 2005; Ruder et al., 2008). Maintaining a suitable level of melatonin has been found to have potential benefits in relieving oxidative stress, regulating metabolism, and delaying the aging process in mammals, including humans (Maryam et al., 2018) and chickens (Jia et al., 2016). Our results showed that the laying rate was significantly reduced after Dexamethasone injection at 15 d, but with the addition of melatonin in the later 15 d, the laying rate of the Dex+Mel group was notably higher than the Dex +NS group. The egg-laying rate of the Dex+Mel group was reversed significantly. Ovarian senescence is manifested with gradual ovarian follicle quantity and quality reduction (Zakaria et al., 1983; Lillpers and Wilhelmson, 1993; Faddy, 2000). The mechanism involved in the accelerated rate of ovarian senescence is still not fully understood during the later laying period. Previous research found that the antioxidant capacity decreased in the late egg-laying stage and the body's ROS levels increased. The oxidative stress caused by ROS is thought to be the main factor linked with aggravating

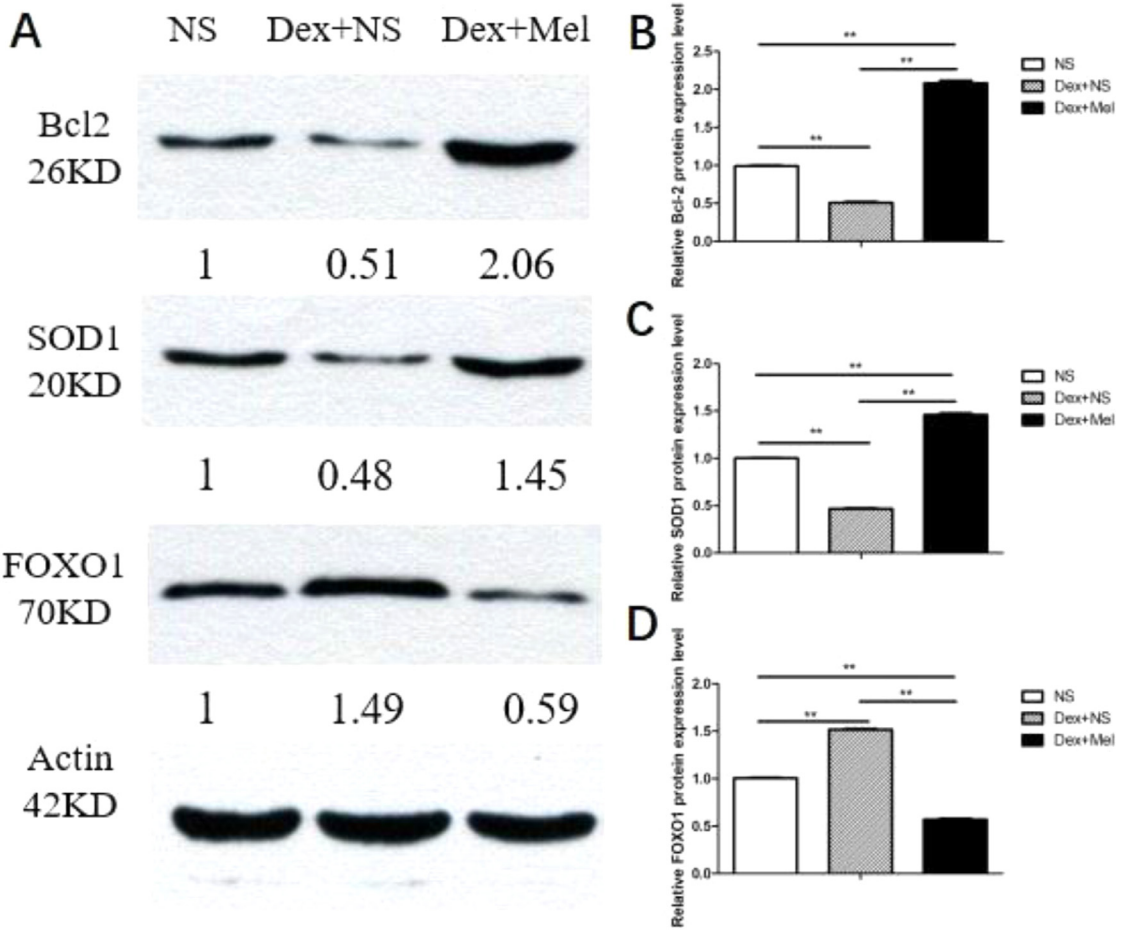

Figure 7. Effect of melatonin on protein expression of Bcl-2, SOD1, and FOXO1 of laying hens in the dexamethasone oxidative stress model in the ovary. Comparison of the gene expression of FOXO1 between the control (NS), dexamethasone (Dex+NS), and melatonin (Dex+Mel) groups. (A) shows the protein bands in the gel. (B–D) show the quantitative expression of BCL-2, SOD1, and FOXO1 proteins, respectively. Data are shown as means ± SD; $N = 3$ chicken/group. *, $P < 0.05$ indicates significance compared with control values, **, $P < 0.01$ indicates extremely significance compared with control values.

ovarian senescence (Toshio et al., 2004; Grondahl et al., 2010). Therefore, it was hypothesized that reduced melatonin levels as age increases promote oxidative stress, causing ovarian senescence (Hardeland, 2012). The CAT, SOD1, GPX3, and PRDX levels in the Dex+NS group markedly decreased on the 15th d, while on d 30th, after melatonin treatment, their levels in the Dex +Mel group were notably higher than Dex+NS group without melatonin supplementation. The levels of ROS and 8-OHdG in the Dex+NS and Dex+Mel groups were extremely higher than those in the NS group on d 15th, whereas, on d 30th the levels were increased in the Dex +NS group than NS and Dex+Mel groups. SOD is a critical human antioxidant enzyme, that effectively eliminates oxygen free radicals from the body, reduces oxidation level, and directly reflects the body's antioxidant capacity, thereby acting as an antioxidant capacity bio-indicator (Ragel et al., 2006). Dodi et al. observed a significant increase in antioxidant power in the swine corpus luteum when evaluating the effect of melatonin (Dodi et al., 2021). Our research found the same results. 8-OHdG is an oxidative additive produced after reactive oxygen free radicals attack DNA, which can be used as an indicator to directly reflect the degree of oxidative damage in the body (Liu et al., 2018b). The data acquired from this study were consistent with the results published by Behram et al. (2017), who showed that in rats, melatonin administration increases SOD activity (Behram et al., 2017). In a previous study on aging laying hens, Hao et al. (2020a) reported that melatonin treatment resulted in a significant increase in the levels of antioxidant enzymes such as SOD and T-AOC, as well as reproductive hormones like E2 and LH (Hao et al., 2020a). Similarly, in our study on chickens, we observed a similar trend, where melatonin treatment reduced the concentration of 8-OHdG, a marker of oxidative stress-induced DNA damage. Tamura found that intrafollicular 8-OHdG concentrations in women with increased oocyte degeneration were markedly higher than in those with lower oocyte degeneration rates. There was a negative relation between the follicular 8-OHdG concentrations and melatonin (Tamura et al., 2008), which is also apparent from our data. Here, melatonin showed a high capability to enhance the CAT, SOD1, GPX3, and PRDX levels and detoxify ROS and 8-OHdG, to directly inhibit the oxidative effect by stimulating endogenous antioxidant production.

The number of follicles is closely related to the egg-laying rate, while the number and the health of follicles are associated with follicular atresia. Most follicles (>99%) have follicular atresia. Apoptosis of oocytes, granulosa cells, and membrane cells is the main feature of follicular atresia (Inoue et al., 2011). How to convert more follicles into dominant form, promote their development and maturation, and reduce follicular atresia has recently become a main focus of research. Follicular atresia is highly regulated by antiapoptotic/proapoptotic factors. In animals, the Bcl-2 family is critically involved in follicular atresia and granulosa cell apoptosis (Matsuda et al., 2012). Bim is a key regulatory protein of apoptosis-promoting factors Bax and Bak during follicular atresia. In the process of porcine folliculogenesis, the addition of melatonin can inhibit the ubiquitination of Bim, thereby reducing the expression of apoptosis-promoting proteins and inhibiting follicular atresia (Wang and Zeng, 2018). Ricassio et al. performed an in vivo study, where mice were given 20 mg/kg melatonin and it was revealed that melatonin can effectively reduce ovarian oxidative damage induced by cisplatin oxide and increase the expression of Metallothionein-1 (**MT1**), and Bcl-2 (Barberino et al., 2017). Talpur et al. showed that inhibition of MT1 would increase Bax protein and decrease Bcl-2 in granulosa cells of mice, and promote apoptosis of granulosa cells. On the contrary, the follicular stimulating hormone could counteract the above changes and promote the proliferation of granulosa cells (Talpur et al., 2017). The caspase family is also seen to effects follicular development and atresia. Usually, the antiapoptosis research is carried out simultaneously with the Caspase and Bcl-2 families. For example, Ricassio et al. measured Caspase-3 in mice and showed that feeding 20 mg/kg melatonin reduces the activation and expression of Caspase-3 and inhibits follicular atresia (Barberino et al., 2017). Riaz et al. also showed that melatonin could increase the Bcl-2 protein expression and inhibit Caspase-3 activation and expression in bovine oocytes in vitro (Riaz et al., 2019). Melatonin nuclear receptors RORα/RORγ mediate G→B-inhibited bursal B lymphocyte apoptosis via reducing caspase-3 and Bax expression in the bursa of chickens (Li et al., 2022). Melatonin treatment enhanced proliferation and suppressed apoptosis in chicken granular cells at 20 mmol/L and 200 mmol/L by upregulating cyclin D1 and Bcl-2 and downregulating P21, caspase-3, Beclin1, and LC3-II (Hao et al., 2020a). This research revealed that in PE, SWF, MWF, LWF, and ovary, the caspase-3, Bim, and Bax expressions in the Dex+NS group were extremely increased than the NS group, while melatonin treatment markedly reduced their expression, which is consistent with the aforementioned studies.

FOXO is a transcription factors subfamily and includes FoxO1, FoxO3, FoxO4, and FoxO6. These are associated with many cellular mechanisms such as differentiation, growth, metabolism, survival, and lysis (Sengupta et al., 2009). FOXO1 (key member) modulates normal follicular development (Liu et al., 2009; Shen et al., 2012). When growing follicles become atretic and degenerate, most granulosa cells undergo apoptosis, and FOXO1 is said to take part in this process (Park et al., 2005; Liu et al., 2009). Recent research has revealed that FOXO1 critically regulates oxidative stress-induced cellular death. It triggers apoptosis under oxidative stress and increases the expression of apoptosis-promoting genes (Zhang et al., 2019). Li et al. found that melatonin inhibits TM3 cells' autophagy caused by H_2O_2 via the AKT/FOXO1 pathway (Li et al., 2022). In this investigation, it was found that in SWF, MWF, LWF, SYF, and ovary, dexamethasone treatment enhances FOXO1 gene expression, while melatonin treatment inhibits it in comparison with the NS group.

CONCLUSIONS

In summary, under dexamethasone-induced oxidative stress, the expression of FOXO1 and oxidative stress indicators increased. However, melatonin administration partially reversed these alterations, increased FOXO1 levels, and improved oxidative stress by inhibiting apoptotic genes of Bcl-2, Caspase-3, Bim, and Bax and enhancing antioxidant genes of CAT, SOD1, GPX3, and PRDX3 in the plasma, follicle, and ovary. Investigating the mechanism of melatonin resistance in the ovaries of laying hens under oxidative stress can provide a theoretical basis for identifying mechanisms related to follicular growth and extending the utilization cycle of laying hens.

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Institutional Review Board Statement: The study was conducted following the guidelines of the Experimental Animals of the Ministry of Science and Technology pertaining to the care and use of animals for experimental and scientific purposes. Hebei Agricultural University's Research Ethics Committee of Animal Care and Use approved the study.

Informed Consent Statement: Not applicable.

Author Contributions: B. K. and H. E. conceived and designed the experiment and wrote the manuscript. C. H. and S. L. revised the manuscript and provided guidance and advice. C. Y. and W. D. provided experimental operation. C. H. and H. R. provided technical and material support. All authors have read and agreed to the published version of the manuscript.

DISCLOSURES

The authors declare no conflicts of interest.

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