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Effects of massive ovulation on oxidation state and function of the ovaries in laying hens

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Abstract: Modern laying hens show fast reduction in egg production rate after 60 weeks of age, which is associated with the decline in ovarian function. The aim of this study was to examine the influence of massive ovulation on the oxidation state and ovarian function in hens. In total, 48 Jing Hong hens aged 17 weeks were randomly divided into high ovulation (HO) and low ovulation (LO) groups. The LO hens were exposed to 6 h of light per day (6 L:18 D) up to 32 weeks to delay the initiation of egg production and then were switched to 16 L:8 D. The HO hens were reared at 16 L:8 D from 19 weeks. The treatment effects were analyzed by the independent samples t-test using SAS 9.0 software (SAS Institute, Cary, NC, USA). At 36 weeks, there were no significant differences in body weight ($P > 0.05$) between the two groups and the egg production rate of both groups reached 98%; however, the cumulative egg number in the HO group was much higher than that in the LO group (71.3 versus 18.5, respectively; $P < 0.001$). The following parameters decreased in the HO hens compared with the LO hens: the number of small yellow follicles (SYFs, $P < 0.001$) and large white follicles (LWFs, $P < 0.01$), mRNA expression of luteinizing hormone receptor ($P < 0.001$), and follicle-stimulating hormone receptor ($P < 0.001$), while the atretic rates of SYFs and LWFs increased ($P < 0.001$ and $P < 0.01$, respectively). In addition, compared with the LO group, the HO hens had reduced activity of superoxide dismutase (plasma: $P < 0.01$; liver: $P < 0.001$; ovary: $P < 0.001$) and glutathione peroxidase (plasma: $P < 0.01$; liver: $P < 0.01$; ovary: $P < 0.01$), while the levels of methane dicarboxylic aldehyde (plasma: $P < 0.01$; liver: $P < 0.001$; ovary: $P < 0.001$) and mRNA expression of mitochondrial transcription factor A ($P < 0.001$) in granulosa cells were higher. These results indicated that massive ovulation aggravated oxidative stress and had adverse effects on ovarian function in laying hens.

Key words: Laying hens, massive ovulation, ovarian function, oxidative stress

1. Introduction

With the development of breeding technology and nutrition regulation, the egg production rate of modern hens can reach more than 95% at 24 or 25 weeks of age. However, after 60 weeks, hens show a fast reduction in the egg production rate (1), which is linked to the decline of ovarian function (2–4), slower follicular growth (5,6), accelerated follicular atresia (5), and decrease in follicular numbers at different developmental stages of the ovaries (7,8). However, compared with domestic hens, wild seabirds exhibit little or no reproductive decline even at the age of 10 or more years (9). Liu et al. (10) delayed the onset of egg production of turkey hens to 64 weeks by light restriction and found that egg production and the number of hierarchical follicles (HFs) in older turkeys (60 weeks old) were similar to those of younger birds (30 weeks old) reared under normal light conditions. These results indicate that aging may not be the only reason for the rapid decline of ovarian function in modern laying hens. Miyamoto et al. (11) induced repeated ovulation in mice by injecting pregnant mare serum gonadotropin and human chorionic gonadotropin, and they found that repeated ovulation reduced oocyte numbers and increased follicular atresia while inhibiting the activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in the ovaries and increasing the level of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a biomarker of oxidative stress, in the oocytes. Therefore, we hypothesized that massive ovulation in modern laying hens may promote oxidative stress and rapid decline of ovarian function. In the current study, we tested this hypothesis by comparing the oxidation state and ovarian function of laying hens in light-restricting and normal conditions.

2. Materials and methods

2.1. Animals and treatments

The animals were reared in compliance with the guidelines for experimental animals established by the Ministry of Science and Technology (Beijing, China). Forty-eight Jing Hong hens aged 17 weeks were randomly divided into the high ovulation (HO) and low ovulation (LO) groups consisting of 12 replicates (2 birds per replicate).

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The LO group was exposed to 6 h of light per day (6 L:18 D photoperiod) up to 32 weeks, which delayed the onset of egg production in the LO hens, and then quickly switched to a 16 L:8 D photoperiod, whereby the rate of egg production reached 98% at 36 weeks. The HO group was reared in normal conditions (16 L:8 D photoperiod) from 19 weeks and also kept 98% egg production rate at 36 weeks. Diet composition and nutrient content are shown in Table 1. A certain amount of food was provided for both groups during the experiment.

2.2. Sample collection and preparation

At the age of 36 weeks, the oviposition time of every hen was recorded. Five hours after oviposition, blood samples were collected via the wing vein by one person, and plasma was separated and stored at –20 °C. The hens were then sacrificed, and the livers were collected and stored at –20 °C until analysis of oxidative stress parameters. Granulosa cells were directly isolated from F1 and F5 follicles as previously described (12), rapidly frozen in liquid nitrogen, and stored at –80 °C for mRNA expression analysis. The ovaries without HFs, small yellow follicles (SYFs), and large white follicles (LWFs) were weighed and stored at –20 °C for the analysis of oxidative stress parameters. The number of normal and atretic HFs, SYFs, and LWFs was counted as described by Gilbert et al. (13).

Table 1. Composition and nutrient content of experimental diet for laying hens (dry matter basis).

Ingredients	%
Corn	63.58
Soybean meal	24.70
Soybean oil	0.50
Limestone	8.30
Dicalcium phosphate	1.50
Sodium chloride	0.30
DL- Methionine	0.12
Premix[1]	1.00
Energy and nutrients	
Metabolic energy (MJ/kg)	11.30
Crude protein	16.50
Calcium	3.50
Available phosphate	0.38
Lysine	0.80
Methionine	0.38
Methionine+cysteine	0.65

[1]Premix provided the following nutrients per kilogram of diet: vitamin A, 8000 IU; vitamin D3, 3000 IU; vitamin E, 10 IU; vitamin K, 2 mg; vitamin B12, 0.04 mg; vitamin B1, 4.5 mg; vitamin B2, 2.5 mg; vitamin B6, 3 mg; pantothenic acid, 5 mg; nicotinic acid, 20 mg; folic acid, 0.3 mg; biotin, 0.1 mg; choline, 500 mg; $MnSO_4 \cdot 5H_2O$, 80 mg; $ZnSO_4 \cdot 7\ H_2O$, 65 mg; $FeSO_4 \cdot 7\ H_2O$, 80 mg; $CuSO_4 \cdot 5\ H_2O$, 8 mg; KI, 0.5 mg; Na_2SeO_3, 0.3 mg.

2.3. Oxidative stress parameters

The activity of SOD and GSH-Px and the level of malondialdehyde (MDA) in the liver, ovary, and plasma were measured using a commercially available kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and were determined by spectrophotometer. Total protein content in the liver and ovary was measured by the Bradford protein assay (Beyotime Institute of Biotechnology, Shanghai, China); bovine serum albumin was used as a standard (14).

2.4. mRNA expression analysis

Total mRNA was extracted from granulosa cells of F1 and F5 follicles using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol and quantified by spectrophotometry. Total RNA was reverse-transcribed to cDNA for 15 min at 37 °C and 5 s at 85 °C using the MMLV-RT kit (Takara Biotechnology Co. Ltd., Dalian, China). mRNA expression of cholesterol side-chain cleavage enzyme P450 (*P450scc*), luteinizing hormone receptor (*LHR*), follicle-stimulating hormone receptor (*FSHR*), and mitochondrial transcription factor A (*TFAM*) were analyzed by PCR in a total volume of 50 µL containing 0.5 µM specific primers and 0.5 U of Taq DNA polymerase (Takara Biotechnology Co. Ltd.) using the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Specific primers were designed using the Primer Express program (Applied Biosystems) and are shown in Table 2. Relative mRNA expression of the target genes was quantified by the $2^{-\Delta\Delta CT}$ method and normalized to the levels of *β-actin* used as a housekeeping gene.

2.5. Statistical analysis

The treatment effects were analyzed by the independent samples t-test using SAS 9.0 software (SAS Institute, Cary, NC, USA). All data were expressed as the means ± SD, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of light restriction on body weight and cumulative egg numbers in laying hens

At 36 weeks of age, laying hens of the two groups were all at peak production and had similar body weight (BW) ($P > 0.05$; Figure 1A). However, the cumulative egg number of the HO hens was much higher than that of the LO hens (71.3 versus 18.5, respectively; $P < 0.01$, Figure 1B).

Table 2. Primer sequences for the *P450scc*, *LHR*, *FSHR*, *TFAM*, and *β-actin* genes.

Gene[1]	Primer sequence	Product size, bp
P450scc	F: CTGCTCCGCCACCTCAAC R: CCCGGTAAACGTTTGGATACA	100
LHR	F: ACTCCTGCGCAAACCCATT R: GCTCGGCTCTTACAGCAACCT	100
FSHR	F: CATGTCTCCGGCAAAGCAA R: AAAACGCGTGCCATAATGG	100
TFAM	F: GCTTCCTGAGGGACAACC R: CAGCCAACTGCTCTTCGTATT	171
β-actin	F: AACACCCACACCCCTGTGAT R: TGAGTCAAGCGCCAAAAGAA	100

[1]P450scc = cholesterol side-chain cleavage enzyme P450; LHR = luteinizing hormone receptor; FSHR = follicle-stimulating hormone receptor; TFAM = mitochondrial transcription factor A.

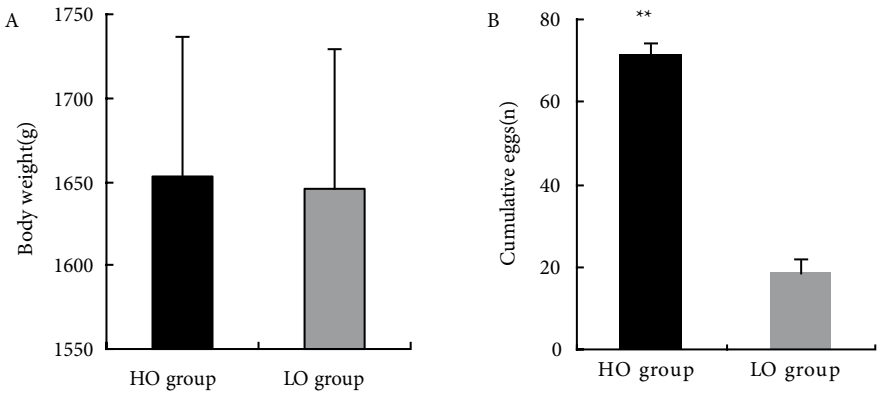

Figure 1. Effects of light restriction on body weight (A) and cumulative egg numbers (B) in laying hens; **P < 0.01. HO = high ovulation; LO = low ovulation.

3.2. Effect of massive ovulation on the number of normal and atretic follicles in laying hens

The numbers of SYFs and LWFs in the ovaries of the HO hens were lower than in the LO group (P < 0.01 for both parameters). The rates of atretic SYFs and LWFs in the ovaries of the HO group were higher than the LO group (P < 0.001 and P < 0.01, respectively). There was no significant difference in the number of HFs between the two groups (P > 0.05; Table 3).

3.3. Effect of massive ovulation on the expression of *P450scc, LHR, FSHR,* and *TFAM* mRNA in granulosa cells

No difference was found in *P450scc* mRNA expression between the two groups (P > 0.05; Figure 2). Compared with HO hens, mRNA expression of *LHR* in F1 follicles and *FSHR* in F5 follicles increased (both P < 0.001), while that of *TFAM* in F1 follicles decreased (P < 0.001) in the LO hens.

3.4. Effect of massive ovulation on oxidative stress parameters in the liver, ovary, and plasma

The activities of SOD (plasma: P < 0.001; liver: P < 0.001; ovary: P < 0.001) and GSH-Px (plasma: P < 0.001; liver: P < 0.001; ovary: P < 0.01) in the HO hens were lower than in the LO hens, while the levels of MDA were higher (plasma: P < 0.05, liver: P < 0.001, ovary: P < 0.001) (Table 4).

4. Discussion

In this study, the onset of egg production in the LO hens was delayed to 32 weeks by light restriction (6 L:18 D). In previous studies, the onset of egg production was delayed to 44 weeks in broiler breeder hens (15) and to 63 weeks in turkey hens by the same light-restricting regime (10).

Table 3. Effect of massive ovulation on the number of normal and atretic follicles in laying hens.

Treatment[1]	HFs (n)2	SYFs (n)[3]	LWFs (n)[4]	Atretic rate of SYFs (%)	Atretic rate of LWFs (%)
HO group	5.33 ± 0.65	17.25 ± 0.78[a]	11.38 ± 2.38[a]	64.12 ± 11.82[a]	17.08 ± 3.76[a]
LO group	5.00 ± 0.83	19.63 ± 1.15[b]	15.13 ± 2.59[b]	38.42 ± 5.79[b]	12.66 ± 3.22[b]
P-value	>0.05	< 0.001	<0.01	< 0.001	<0.01

[a,b]Values marked with different letters in the same column are significantly different.
[1]HO = high ovulation; LO = low ovulation.
[2]HFs = hierarchical follicles.
[3]SYFs = small yellow follicles.
[4]LWFs = large white follicles.

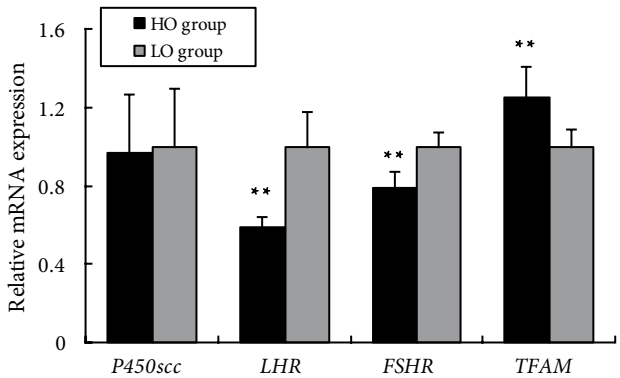

Figure 2. Effect of massive ovulation on the expression of *P450scc*, *LHR*, *FSHR*, and *TFAM* mRNA in granulosa cells of laying hens; **P < 0.01. For F1 follicle, *P450scc*, *LHR*, and *TFAM* mRNA were analyzed, while for F5 follicle, *FSHR* mRNA was analyzed. P450scc = cholesterol side-chain cleavage enzyme P450; LHR = luteinizing hormone receptor; FSHR = follicle-stimulating hormone receptor; TFAM = mitochondrial transcription factor A; HO = high ovulation; LO = low ovulation.

Table 4. Effect of massive ovulation on the activity of SOD and GSH-Px, and the levels of MDA in the plasma, liver, and ovaries of laying hens.

Parameter[1]	HO group[2]	LO group[3]	P-value
SOD activity			
Plasma (U mL^{-1})	309.03 ± 19.52[a]	346.73 ± 21.39[b]	<0.001
Liver (U mg^{-1} prot)	135.09 ± 4.21[a]	162.85 ± 15.72[b]	<0.001
Ovary (U mg^{-1} prot)	705.95 ± 17.99[a]	822.59 ± 24.16[b] 24.16[b]	<0.001
GSH-Px activity			
Plasma (U mL^{-1})	3862.94 ± 364.89[a]	4460.55 ± 261.48[b]	<0.001
Liver (U mg^{-1} prot)	11.32 ± 1.07[a]	12.89 ± 0.62[b]	<0.001
Ovary (U mg^{-1} prot)	358.73 ± 43.41[a]	407.75 ± 34.59[b]	<0.01
MDA level			
Plasma (nmol mL^{-1})	9.65 ± 1.49[a]	8.48 ± 0.38[b]	<0.05
Liver (nmol mg^{-1} prot)	0.147 ± 0.016[a]	0.070 ± 0.008[b]	<0.001
Ovary (nmol mg^{-1} prot)	4.79 ± 0.89[a]	3.42 ± 0.34[b]	<0.001

[a,b]Values marked with different letters in the same row are significantly different.
[1]SOD = superoxide dismutase; GSH-Px = glutathione peroxidase; MDA = malondialdehyde.
[2]HO = high ovulation; LO = low ovulation.

The light regime was quickly switched to normal (16 L:8 D) after 32 weeks, and the egg production rate of the LO hens reached its maximum (98%) at 36 weeks. The HO group hens also kept a 98% egg production rate at this time. All birds in the two groups reached the peak of egg production and had similar BW at the same age; however, the cumulative egg number of the HO hens was significantly higher than that of the LO hens.

Hens in the HO group had lower numbers of SYFs and LWFs, higher rates of follicle atresia, and reduced mRNA expression of *LHR* and *FSHR* in granulosa cells compared with the LO group. The binding of LH and FSH to respective receptors on granulosa cells plays an important regulatory role in follicular development (16,17). Other studies revealed that the reduction in *LHR* expression affects the sensitivity of granulosa cells to LH and may induce a decline in ovarian function in aging hens (18,19). In the present study, massive ovulation inhibited mRNA expression of *LHR* and *FSHR*, which may be associated with the decline of ovarian function in modern laying hens.

In the HO hens, the activity of SOD and GSH-Px in different tissues was significantly decreased, while MDA level and *TFAM* expression in F1 follicles were significantly increased compared with the LO hens. TFAM is an mtDNA-binding protein essential for the maintenance, replication, and transcription of mtDNA (20–22). When the level of intracellular reactive oxygen species (ROS) increases, *TFAM* expression is induced to prevent further ROS generation and subsequent oxidative stress (23–25). The present results indicate that massive ovulation may aggravate oxidative stress in laying hens. In vitro studies have shown that excessive ROS generation inhibits follicular development, induces granular cell apoptosis, and causes follicular atresia (26–30). The decline of ovarian function in modern laying hens may be associated with oxidative stress induced by massive ovulation.

In conclusion, massive ovulation aggravated oxidative stress and had adverse effects on ovarian function in laying hens. Oxidative stress induced by massive ovulation may be a reason for the fast reduction in the egg production rate of modern laying hens after 60 weeks of age.

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