Rearing system influences the testicular development, semen quality and spermatogenic cell apoptosis of layer roosters

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ABSTRACT The objective of this study was to compare the testicular development, semen quality, and spermatogenic cell apoptosis of roosters reared in colony, single, and large cages. Rohman parental layers (n = 540) were randomly allocated into cages of rearing system groups (135 males and 405 females). The experimental period was 70 to 210 d of age. We compared testicular development and plasma main reproductive hormones (Follicle-stimulating hormone; Luteinizing hormone; Testosterone; Estrogen2;) from d 70 to 210 of roosters among the three systems. In addition, routine semen quality indexes, apoptosis of testicular spermatogenic cells and sperm apoptosis of breeding roosters under three rearing systems on d 175 and d 210 were evaluated. Roosters during the growing period (from d 70 to 140) have rapid testis growth and increasing main reproductive hormones in plasma. At the peak of sexual maturity (d 210), in colony cage, the females have a positive effect and promote the testis development of males. However, the stocking density in colony cage has no effect on testicular development; compared with the single and large cage. Roosters reared in the natural mating system had better semen quality, particularly in semen volume, density, and viability; the hatching % of fertilized eggs and healthy chicks were higher for the colony than single and large cages. Furthermore, the sperm density was higher for colony than single and large cages, which was related to the apoptosis of spermatogonia and spermatocyte, not the apoptosis of mature sperm. This study provided the basic data for the reproductive performance research of chicken reared in the colony cages.

Key words: male breeders, rearing system, reproductive hormones, semen quality, spermatogenic cell apoptosis

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INTRODUCTION

The single cage system requires artificial insemination, involving semen collection, insemination, and other operations, which may cause stress response of breeding chickens and lack of animal welfare (Janczak and Riber, 2015) However, the colony cage system allows for natural-mating which is labor-saving and reduce the possibility of cross-infection of disease (Brillard, 2004). Moreover, the colony system improves the mechanization level of feeding. Under the background of large-scale poultry breeding and animal welfare, the colony cage system may become a development trend of breeding chickens (Yilmaz et al., 2016).Male turkeys housed on the floor produce less semen volume than those kept in cages or cubicles (Woodard and Ablanalp, 1975) Rota et al. (2018) evaluated the effect of housing system, with the animals grouped together in a paddock or kept in individual boxes, on sexual behavior, cortisol and testosterone concentrations and semen characteristics of adult donkeys. They found that sperm motility of donkeys in the paddock were significantly higher than those in boxes. Salivary cortisol was influenced by housing system, both before and 60 min after ejaculation, being higher when donkeys were housed in paddocks. On the contrary, overall and basal testosterone concentrations were significantly higher when animals were kept in boxes. Therefore, the rearing system has a certain influence on the quantity and quality of semen. Furthermore, most of the studies on rearing systems mainly focus on the breeding performance of laying hens and the development of cage-rearing equipment and technical parameters (Blatchford et al., 2016; Al-Ajeeli et al., 2018;

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Bari et al., 2020). Despite the attention mainly paid to the reproduction of laying hens, reseach conducted to determine the potential relationship between rearing system and the male fertility in poultry is space. Moreover, little is known about the interaction of all these traits when males are housed in cages with or without females. The purpose of the present study was to determine the effects of three rearing systems (colony, single, and large cage) on the testicular development, semen quality and spermatogenic cell apoptosis.

MATERIALS AND METHODS

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University (No. DKY-S20123129). All research work was conducted in strict accordance with the Sichuan Agricultural University (SAU) Laboratory Animal Welfare and Ethics guidelines.

Animals and Management

Five hundred and forty 70-day-old Rohman breeders (135 males and 405 females) were selected with similar body condition and purchased from Sichuan Holy Land Ecological Food Co. Ltd., Mianyang, Sichuan, China. Birds were raised at the poultry breeding farm of Sichuan Agricultural University and managed with restricted feeding. The feed was purchased from Chengdu Quanwei Feed Co. Ltd., Chengdu, Sichuan, China. The diet (d 70 to 210) (Table 1) was formulated to meet National Research Council (NRC, 1994)-recommended requirements for all nutrients, and our feeding protocols ensured that the nutrients and amounts consumed by females and males in each group were the same The lighting procedure and the nutritional levels are summarized in Tables 2 and 3, respectively. There were five repeated units in colony and large cage group, respectively, each containing nine roosters; 81 hens were placed in each repeated unit of the colony cage, and no hens were placed in the large cage. There were 9 replicates in the single cage group, 1 male in each replicate. The cage size and the ratio of male to female are shown in Table 4.

Sample Collection

Body weights of the roosters under 3 rearing systems were obtained. Nine roosters were randomly selected from each group and the blood samples were collected from the wing vein at 9:00 am on d 70, 105, 140, 175, and 210, respectively and the both sides testicles from each bird were collected and weighed (Sarabia Fragoso et al., 2013) The left testis was cleaned with PBS and put into 4% paraformaldehyde for fixation for subsequent sectioning, while 100 mg of right testis was collected, put into 1.5 mL epoxy tube and preserved in liquid nitrogen for subsequent RNA extraction.

Table 1. The nutritional level for males and females from d 70 to 210.

Conponents	Growing period (d 70−126)	Pre-laying period (d 127−139)	Laying period (d 140−210)
Calcium (%)	1	2.00	3.5
Phosphorus (%)	0.42	0.45	0.4
Lysine (%)	0.75	0.85	0.8
Methionine (%)	0.3	0.36	0.38
Crude protein (%)	15	17.50	16
Energy (Kcal)	2.75	2.72	2.7

Table 2. The photoperiods for birds from d 1 to 210.

Age (d)	Light: dark (h)	Light intensity (Lux)
1−3	24:0	60
4−7	16:8	60
8−14	14:10	60
15−21	12:12	30
22−69	10:14	30
70−119	9:15	10
120−126	10: 14	25
127−133	11: 13	25
134−140	12: 12	25
141−146	13: 11	25
147−154	14: 10	25
161−210	14: 10	25

Table 3. The daily feed volume per chicken from d 70 to 210.

Age (d)	Feed volume (g)	Feed type
70	57	Growing
77	60	Growing
84	63	Growing
91	66	Growing
98	69	Growing
105	72	Growing
112	75	Growing
119	75	Growing
126	81	Growing
133	84	Pre-laying
140−210	87	Laying

Testicular Seminiferous Tubule Area

The fixed testicular tissue samples were collected from the paraformaldehyde, washed with distilled water for 4 hours, and the normal paraffin section of the testis samples were made according to the method reported by Goncalves (Goncalves et al., 2018). The hematoxylin-eosin stained sections of testis were observed under the microscope and photographed (400 ×). Each of 3 visual fields were taken and saved. The area of testicular seminiferous tubules for each group was analyzed by Image-Pro Plus software (de Melo et al., 2013).

ELISA Assay

Hormone concentrations of nine blood samples from each group were determined by ELISA assay. One milliliter of blood was placed in the heparin anticoagulant tube and centrifuged at 4,500 r/min at 4°C and the plasma stored at −80°C. The concentrations of hormones Follicle-stimulating hormone (**FSH**), Luteinizing

Table 4. The cage size and ratio of male to female.

Housing system cage	Size (Length × Width × Height) cm	Ratio of male to female	Unit replicates
Colony	200 × 100 × 80	♂:♀= 9:81	5
Large	200 × 100 × 80	♂:♀= 9:0	5
Single cage	50 × 40 × 45	♂:♀= 9:0	5

hormone (**LH**), Testosterone (**T**), and Estrogen2 (**E2**) in plasma of each group were determined according to the protocols of the FSH (Andygene AD0476Ch), LH (Andygene AD0447Ch), T (Andygene AD0067Un), and E2 (Andygene AD0010Un) Elisa Hormone Assay Kit (Andygene, Beijing, China).

Semen Quality Evaluation

On d 175 and 210, semens of 9 males from each group were collected with the dorsoabdominal massage method (Burrows and Quinn, 1937). Semen qualities were determined according to the method of human semen quality provided by the World Health Organization (WHO) (Menkveld, 2010).

Fertilization Rate and Hatchability

On d 210, the semen of the single cage group and the large cage group (9 roosters for each) were collected, and the color and density of the semen was visually evaluated. Then 2 groups of hens (27 hens with the same age and similar body weight as those in colony cage for each) were artificially inseminated with pooled semen from the single and large cage group every three days, respectively. The fertilized eggs were collected for 2 weeks (total 300 eggs were collected for each group per week and then hatched once a week). The eggs collected every day and stored in an air-conditioned equipped environment. The percentage of fertilization, hatching, and healthy chicks for each group were recorded. The fertilization (%) = number of fertilized eggs / number of hatching eggs × 100%, The hatching of fertilized eggs (%) = number of chicks / number of fertilized eggs × 100%. The healthy chicks (%) = number of healthy chicks / number of chicks × 100%.

Terminal Deoxynucleotidyltransferase Mediated Dutp-Biotin Nick End Labeling Assay

The paraffin section of the testis was made according to the method of Goncalves (2018) and the samples dyed according to the instructions of the Terminal Deoxynucleotidyltransferase Mediated Dutp-Biotin Nick End Labeling **(TUNEL)** Apoptosis Assay Kit (Solarbio Life Science, Beijing, China). In brief, testis on the slides were incubated for 20 min with 4% formaldehyde fixative buffer, and then permeated with 0.2% Triton X-100 in PBS for 10 min at room temperature. After washed with PBS, cells were incubated with TUNEL reaction solution at 37°C for 1 h, followed by staining with 4′, 6-diamidino-2-phenylindole, and the antifluorescence quenching sealing solution was added into the section, The number of TUNEL-positive nuclei was counted in 5 random fields under × 400 magnification using a fluorescence microscope (Ecliope E400, Nickon, Tokyo, Japan).

Real Time Quantitative PCR

The total RNA of testicular tissues and spermatozoa were extracted using Trizol (Molecular Research Center, Cincinnati, OH) following the manufacturer's protocol, and the concentration and purity of RNA were determined using UV spectrophotometer (IMPLEN, Germany). The RNA integrity was detected by Bioanalyzer 2100 system (Agilent Technologies, China). Total RNA was reverse transcribed into cDNA using a high capacity cDNA Reverse Transcription kit (TaKaRa Bio Inc., Dalian, China). A CFX 96 Touch Real-Time PCR Detection systems (BIO-RAD) was used with an aliquot of cDNA (1.5 μL) in 15 μL qPCR reaction containing SYBR Green PCR Master Mix (TaKaRa Bio Inc., China) for measurement of target genes such as chicken *Caspase-8, Caspase-3, P53, Bcl-xl*, and *Bcl-2,* and *β-actin* was used as the housekeeping gene of the internal control group. Detailed information for primers is summarized in Table 5. The PCR reaction program was as follows: predenaturation at 95°C for 2 min, denaturation at 95°C for 3 s, annealing at X°C for 15 s (X is related to the target gene and the related annealing temperature is shown in Table 5), extension at 72°C for 10 s, and 40 cycles.

Immunohistochemistry (IHC) Assay

The IHC of testis was carried out according to the instructions of the Caspase-3 apoptosis antibody and Bcl-2 antiapoptosis antibody (Youningwei Biotechnology Co., Ltd., China). The expression and localization of Caspase-3 apoptosis protein and Bcl-2 antiapoptosis protein in spermatogenic cells were carried out according to the methods of Song (2015).

Flow Cytometry Assay

The semen was collected as mentioned above, quickly transferred to the laboratory with warm container and incubated at 38°C. Then following washing and centrifugation, sperm were resuspended in 100 μL binding buffer with 5 μL Annexin V-FITC and 5 μL PI (556420; Becton, Dickinson and Company, USA) for 15 min in the dark. Subsequently, cell apoptosis was detected using a Flow Cytometer (Beckman Coulter, Fullerton, CA)

Table 5. Primers used for real-time PCR.

Genes	Sequence	Product Length (bp)	Annealing temperature (°C)	Reference serial number
β-actin	F: GAGAAATTGTGCGTGACATCA R: CCTGAACCTCTCATTGCCA	152	60	NM_001031257
Bcl-2	F: ATCGTCGCCTTCTTCGAGTT R: ATCCCATCCTCCGTTGTCCT	150	58	NM_204305
Bcl-xl	F: AAGCGATGCGCGAAAGGTC R: CACCTGAGACGCCCTCAATG	121	61	NM_204686.2
Caspase8	F: CTGGGGAAACAGCAACAGCAG R: TTATTTTGGTTCTGGGGATGA	103	60	XM_015294370.1
Caspase3	F: TCTCTTCAAACAGCGACAC R: TGAGGAAAGGGTTAGCAC	192	60	NM_205079.1
P53	F:ATGAAATGACCGGCTTTGAC R:GCAAAGGAGAGGTTGCACAT	241	60	NM_204936.1

Table 6. The reaction system for real-time PCR.

Reagent	Reagent dosage (μL)
SYBR® Premix Ex Taq™ (2 ×)	6.25
Forward Primer	0.3
Reverse Primer	0.3
cDNA	1.5
Rnase Free H_2O	6.7
Total	15.0

within 1 h. The sperm apoptosis rate was identified with a flow cytometer (Beckman Coulter, Fullerton, CA).

Statistical Analysis

Gene expression data were calculated with $2^{-\Delta\Delta Ct}$ method (Arocho et al., 2006). All data were analyzed by ANOVA, using Standard least square method of JMP 10 (SAS Institute Inc., NC). The model for trait was as follow:

$$Y_{ij} = \mu + A_i + R_j + (AR)_{ij} + e_{ij},$$

Where Y_{ij} = the trait of bird at i days- old raised at j system, μ= the general mean, A_i = the effect of age i (i = 175 and 210, or i = 70, 105, 140, 175, and 210), R_j = the effect of rearing system (j = colony, single, and large cage), $(AR)_{ij}$ = the interaction effect of age I × rearing system j, e_{ij} = the random residual effect. When main effects of age and rearing system and their interactions were significant, means were tested by Tukey-Kramer multiple comparisons. Significance was assigned at $P < 0.05$. The results were shown as mean ± SE.

RESULTS

Body Weight and Testicular Development

The bodyweight and testicular development for the three housing systems from the sexual development period to sexual maturation were summarized in Figure 1. The bodyweight of rooster increased from 1 kilogram to 2 kilograms within 140 d. On d 105, 140, and 175, the bodyweight of the colony cage group was significantly greater than that of the single cage group ($P < 0.05$), whereas on d 70 and 210, there was no difference for the bodyweight among three groups ($P > 0.05$, Figure 1A). Relative testes weight of the three groups increased rapidly from d 70 to 140 and then slowly from d 140 to 210. The exception was the colony cage group, where the relative testes weight increased from 0.07 on d 175 to 0.09 on d 210 and that was dramatically higher than those of the other two groups on d 210 ($P < 0.05$, Figure 1B). However, on d 70, 105, 140, and 175, there was no significant difference for relative testicle weight among the three groups.

Testis Histology

The seminiferous tubules of the three groups were similar on d 70, and lumen appeared from d 105; the

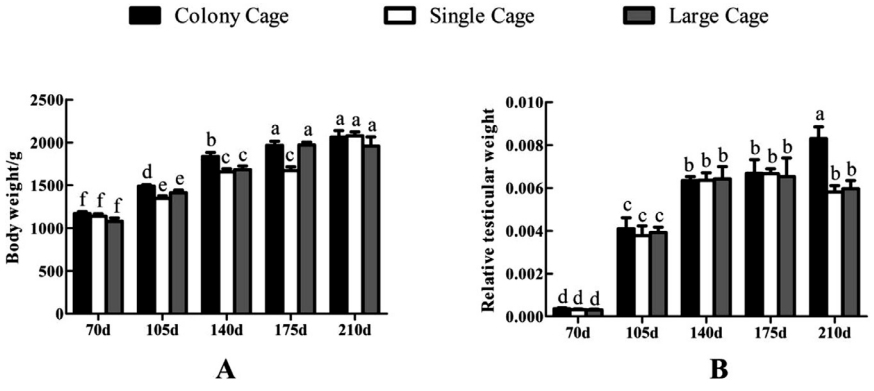

Figure 1. Body weights and relative testicular weights on d 70, 105, 140, 175, and 210. (A) Body weights. (B) Relative testicular weights. The height of a column is the mean of experimental values (n = 9) and the error bar shows the SE. Groups without the same letter differed ($P < 0.05$).

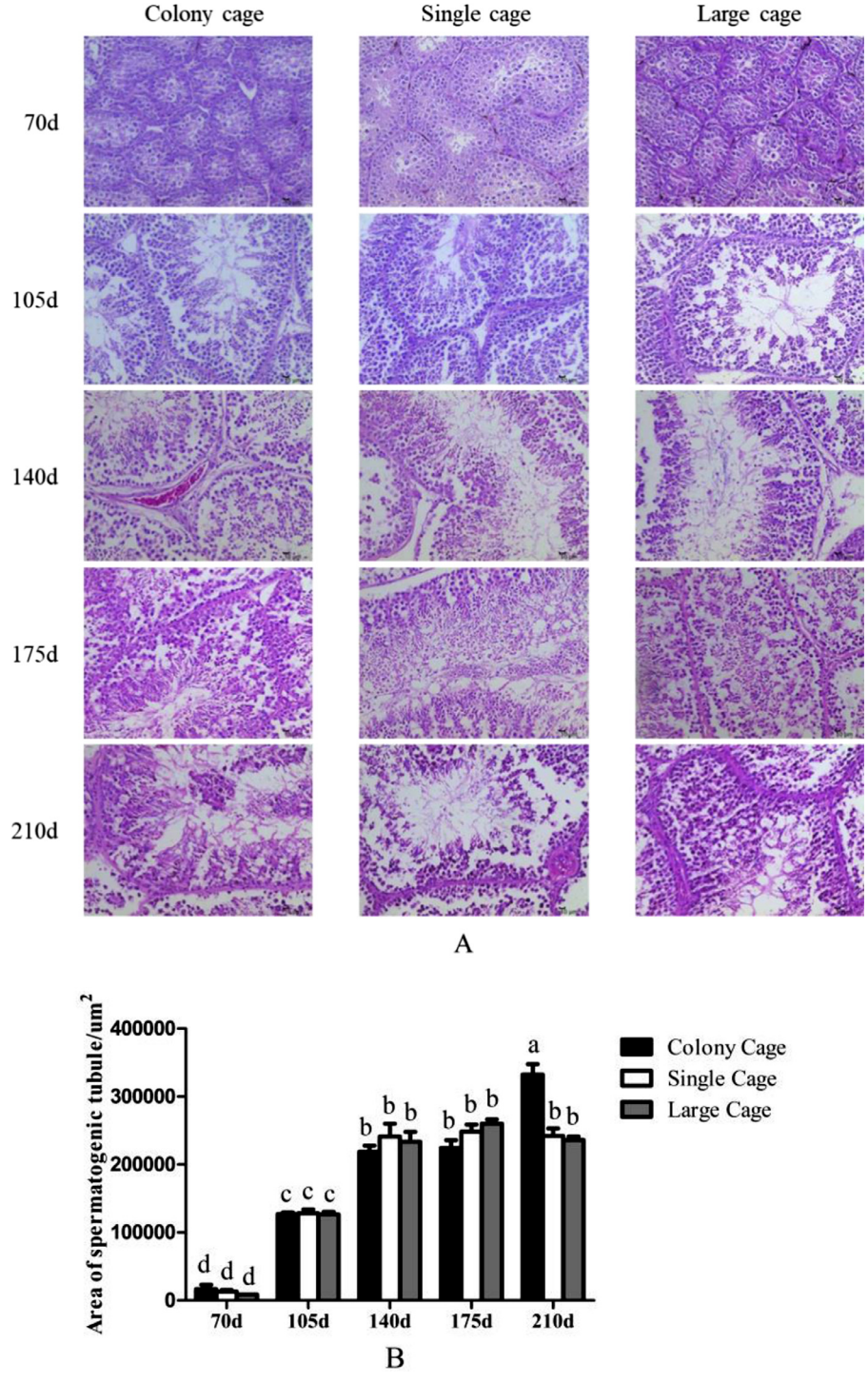

Figure 2. The microstructure of seminiferous tubules on d 70, 105, 140, 175, and 210 (400 ×). (A) HE staining of testicular tissue (400 ×). (B) Spermatogenic tubule area (100 ×). The height of a column is the mean of experimental values (n = 9) and the error bar shows the standard error. Groups without the same letter differed ($P < 0.05$).

seminiferous cells appeared in the seminiferous tubules of the three groups on d 140, and spermatogenesis started (Figure 2A). The area of seminiferous tubules for three groups increased significantly from d 70 to 140 and then slowed down from d 140 to 210 (Figure 2B). There were no significant differences in the area of seminiferous tubules among the three groups on d 70, 105, 140, and 175 ($P > 0.05$). The area of seminiferous tubules in the colony cage group on d 210 was larger ($P < 0.05$), but similar between single and large cage groups ($P > 0.05$).

Plasma Reproductive Hormone in Roosters

The contents of plasma FSH, LH and T for 3 groups increased with age, while the content of E2 decreased. There were no differences among groups at any age for plasma FSH, LH, T, and E2 ($P > 0.05$, Figure 3). The concentrations of plasma FSH, LH, and T in colony cage group were the highest on d 210, compared with those of the single and large cage groups ($P < 0.05$). Meanwhile, the content of E2 in colony cage group was significantly lower than those of single and large cage groups on d 210 ($P < 0.05$).

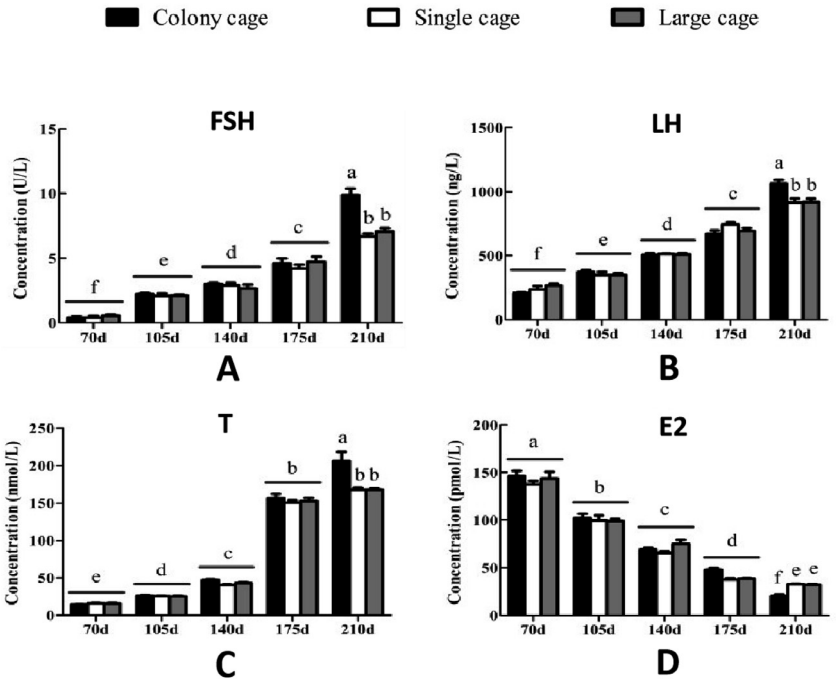

Figure 3. The reproductive hormone contents in plasma on d 70, 105, 140, 175, and 210. Levels of FSH (A), LH (B), T (C), E2 (D) in the blood serum were detected using Elisa kits from d 70 to d 210. The height of a column is the mean of experimental values (n = 9) and the error bar shows the SE. Groups without the same letter differed ($P < 0.05$).

Semen Quality

The results of semen quality characteristics are mentioned in Table 7, and none of the semen quality characteristics was influenced by the rearing system on d 175. However, sperm density and sperm activity rate of colony cage group were higher than those of the single and large cage group on d 210 ($P < 0.05$), respectively. Meanwhile, the pH and sperm viability among 3 groups did not differ ($P > 0.05$). Compared to d 175, roosters have better semen quality on d 210, particularly in sperm concentration and motility ($P < 0.05$).

Fertility and Hatchability

The results of fertility and hatchability are summarized in Table 8. Fertility in the colony cage group was lower than that of the single and large cage groups on d 210 ($P < 0.05$). However, the hatching of fertilized eggs (%) and healthy chicks (%) in the colony cage group was higher than that in the single cage group ($P < 0.05$). There was no difference between the colony and large cage groups ($P > 0.05$).

Cell Apoptosis in the Testis

TUNEL detections of apoptosis in the testis of 3 groups are shown in Figures 4A and 5A. The positive control group showed strong apoptosis fluorescence (green); the negative control group showed no apoptosis fluorescence. Testis tissues of three groups on d 175 were detected apoptosis fluorescence, with the intensity of

Table 7. Semen quality on d 175 and 210.

Trait	Age (d)	Colony cage	Single cage	Large cage	*P* value
Ejaculate volume (mL)	175	0.39 ± 0.04	0.38 ± 0.04	0.41 ± 0.04	ns
		*	ns	ns	
	210	0.67 ± 0.04^{a}	0.48 ± 0.04^{b}	0.45 ± 0.04^{b}	*
pH	175	7.28 ± 0.07	7.16 ± 0.07	7.13 ± 0.07	ns
		ns	ns	ns	
	210	7.33 ± 0.06	7.20 ± 0.06	7.17 ± 0.06	ns
Sperm concentration × 10^9	175	25.30 ± 1.60	21.45 ± 1.60	23.52 ± 1.60	ns
		*	*	*	
	210	48.37 ± 1.67^{a}	34.35 ± 1.67^{b}	37.05 ± 1.67^{b}	*
Sperm motility (%)	175	0.67 ± 0.04	0.63 ± 0.04	0.68 ± 0.04	ns
		ns	ns	ns	
	210	0.87 ± 0.03^{a}	0.82 ± 0.03^{a}	0.83 ± 0.03^{a}	ns
Sperm viability (%)	175	59.67 ± 2.19	52.33 ± 2.19	59.17 ± 2.19	ns
		*	*	ns	
	210	77.00 ± 2.16^{a}	62.07 ± 2.16^{b}	64.00 ± 2.16^{b}	*

Note: Sample replicate number for each group was 9. Values are shown as mean ± SE. Values in a line without the same uppercase differed significantly ($P < 0.05$).

Table 8. The percentage of fertilization and hatching of eggs by cage system.

Traits	Colony cage	Single cage	Large cage
Number of hatching eggs	300	300	300
Fertilization (%)	88 ± 0.8^{b}	93 ± 0.8^{a}	90 ± 0.8^{a}
Hatching (%)	88 ± 0.6^{a}	83 ± 0.6^{b}	88 ± 0.6^{a}
Healthy chick (%)	88 ± 0.7^{a}	82 ± 0.7^{b}	87 ± 0.7^{a}

Note: The values were shown as mean ± SE. Values in a row without the same uppercase differed significantly ($P < 0.05$).

apoptosis was similar among three groups ($P > 0.05$, Figure 4B). Whereas the total light density value of apoptosis fluorescence in testis tissue of colony cage group on d 210 was significantly lower than those of single and large cage groups ($P < 0.05$, Figure 5A).

The Expression of Apoptotic Genes in the Testis

The expression abundances of genes *Caspase-8, Caspase-3, P53, Bcl-2,* and *Bcl-xl* in testis tissue on d

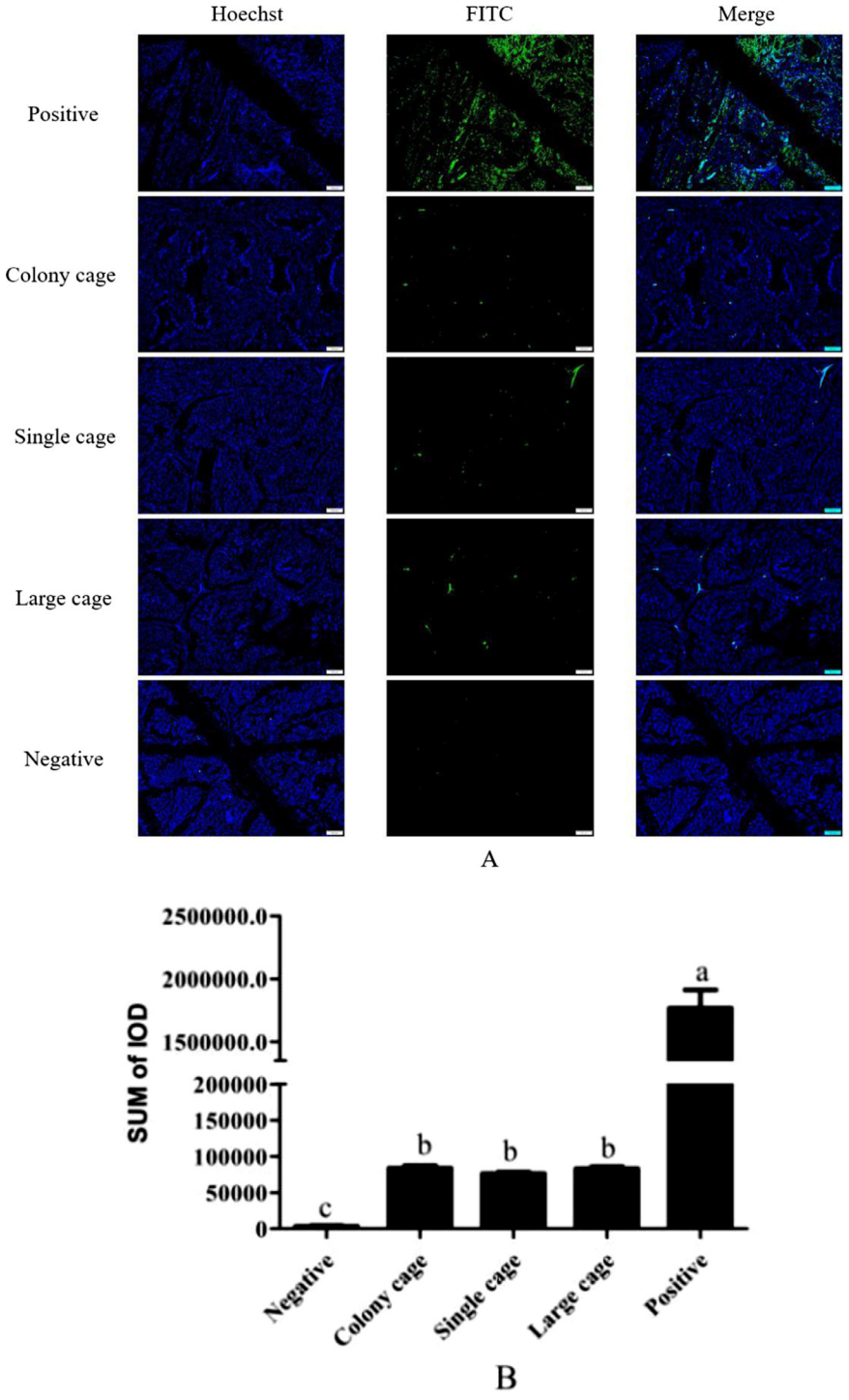

Figure 4. The detection of apoptotic cells in testicular tissue by TUNEL on d 175 (100 ×). (A) The testicular tissue stained with TUNEL (100 ×). (B) The statistical results of apoptotic green fluorescence intensity.The height of a column is the mean of experimental values (n = 9) and the error bar shows the SE. Groups without the same letter differed ($P < 0.05$).

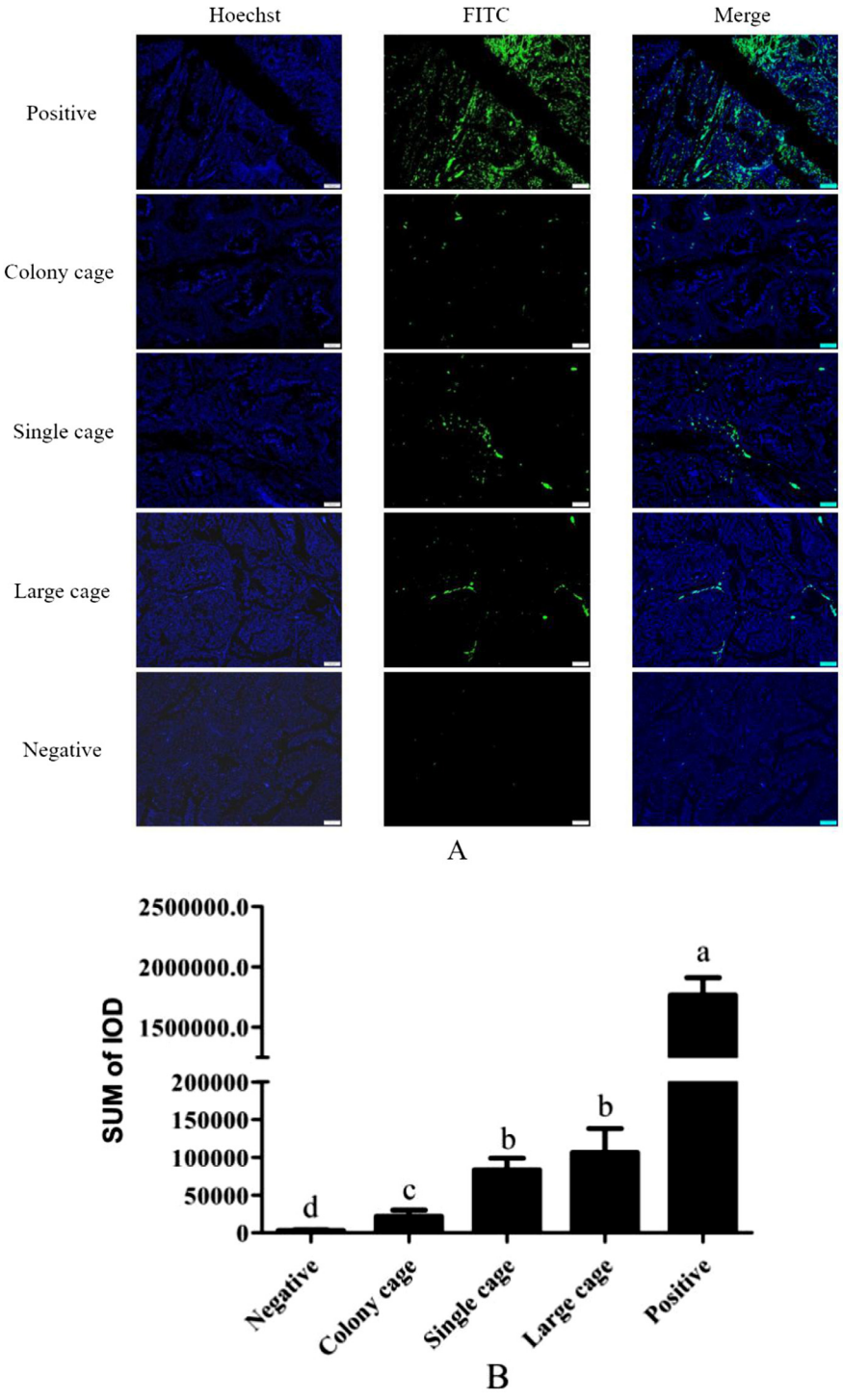

Figure 5. The detection of apoptotic cells in testicular tissue by TUNEL on d 210 (100 ×). (A) The testicular tissue stained with TUNEL (100 ×). (B) The statistical results of apoptotic green fluorescence intensity. The height of a column is the mean of experimental values (n = 9) and the error bar shows the SE. Groups without the same letter differed ($P < 0.05$).

175 and 210 are shown in Figure 6. Expressions were similar among the three groups on d 175 ($P > 0.05$). Expression of *Caspase-8, Caspase-3*, and *P53* in testis of colony cage group were lower than those for the single and large cage groups on d 210 ($P < 0.05$). Expressions of antiapoptosis genes *Bcl-2* and *Bcl-xl* in the testis of the colony cage group were higher than those of the single and large cage groups ($P < 0.05$). The gene expression abundance was not different between the other two groups on d 175 and 210 ($P > 0.05$), respectively.

Expression and Location of the Apoptotic Proteins in Spermatogenic Cells

The expression and location of Caspase-3 and Bcl-2 antiapoptotic protein in the testis of each group on d

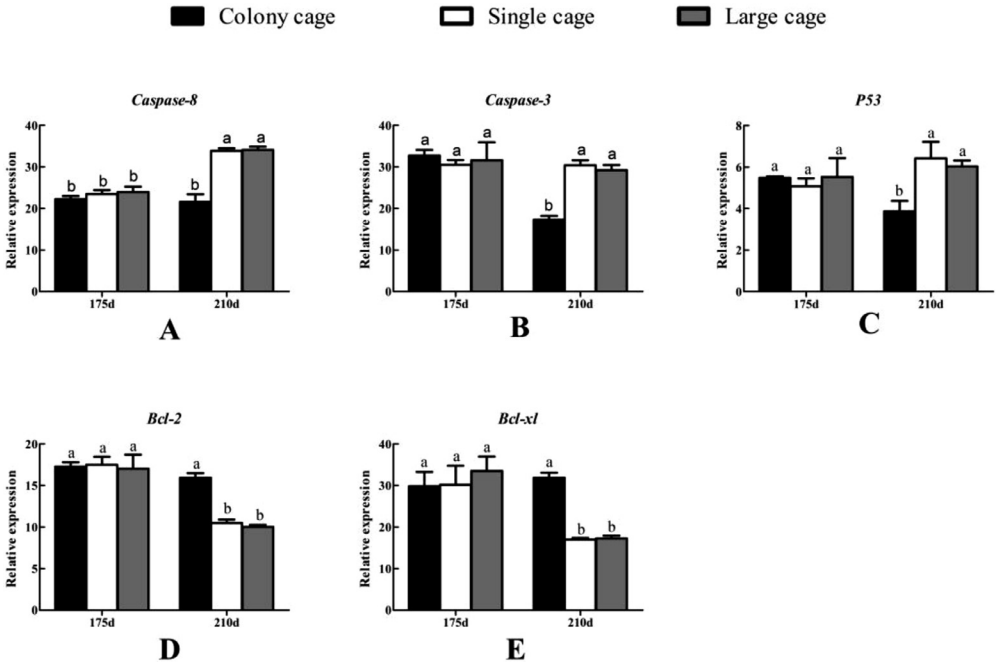

Figure 6. The expression abundances of apoptotic genes in testicular tissue on d 175 and 210. (A−E) The were the results of relative expression abundances of apoptotic genes *Caspase-8, Caspase-3, P53*, and anti-apoptotic genes *Bcl-2* and *Bcl-xl* in testicular tissue, respectively. The height of a column is the mean of experimental values (n = 9) and the error bar shows the SE. Groups without the same letter differed ($P < 0.05$).

175 and 210 are shown in Figures 7 and 8, respectively. Caspase-3 was expressed in Sertoli cells, spermatogonia, primary spermatocytes, and secondary spermatocytes, and was highly expressed in spermatocytes of three groups. There were no expression in the negative control group at either time point (Figure 7A). The total optical density of Caspase-3 apoptotic protein expression was lower on d 175 but higher on d 210. While Caspase-3 in the colony cage group was lower expressed than that in the other two groups on d 210 ($P < 0.05$, Figure 7B). Additionally, Bcl-2 antiapoptotic protein was expressed in spermatogonia, primary spermatocytes, secondary spermatocytes and spermatid of the three groups; on d 175 and 210, it was highly expressed in spermatid and spermatogonia, respectively (Figure 8A). The total optical density of Bcl-2 antiapoptotic protein was lower on d 175 but higher on d 210; Expression of Bcl-2 in the colony cage group was higher than that in the other two groups on d 210 ($P < 0.05$, Figure 8B).

Effect of Rearing Systems on Sperm Apoptosis

The results of sperm cell apoptosis rate and apoptotic genes expression on d 175 and 210 are shown in Figures 9 and 10, respectively. Total apoptosis among the three groups of was not influenced by the rearing system either on d 175 or 210 ($P > 0.05$, Figure 9). Moreover, the expression of *Caspase-8, Caspase-3, P53, Bcl-2,* and *Bcl-xl* genes in the mature sperm of the three groups were not significantly different ($P > 0.05$, Figure 10).

DISCUSSION

The development of testes is very important to the level and maintenance of fertility of the flock. The growth and development of testes are mainly in the state of cell division between 2 and 15 wk, and the increase of testis weight in this stage is relatively limited (Sarabia, 2013) However the proliferation and differentiation of spermatogonia in this stage are important to the fertility in the future. Prior to 10 wk of age, although the weight of testis is not increased much, the number of spermatogonia can increase to more than 1 million (van Bragt et al., 2008). The spermatogonia not only provide nutrition for sperm growth and development but also has a considerable impact on the ability of testis to produce sperm (Okwun et al., 1996). After 15 wk of age, the weight of the testes increases significantly, and testicular weight and semen volume generally peak at 28 to 30 wk of age. At this time, the vas deferens are well developed, and the testicles have proper vascular distribution and a healthy color (Jia et al., 2010). After 35 wk of age, testis weight naturally atrophied, semen production decreased, and egg fertilization decreased (van Haaster and De Rooij, 1993) In addition, the physical (visual and auditory) and chemical (sex hormone) stimulation by hens can promote testicular development and hormone FSH and LH secretion in roosters (Yilmaz et al., 2016). The results showed that the relative testicular weight and the area of seminiferous tubules increased rapidly from d 70 to 140, stabilized between d 140 and 175 and reached the peak on d 210. Rohman males

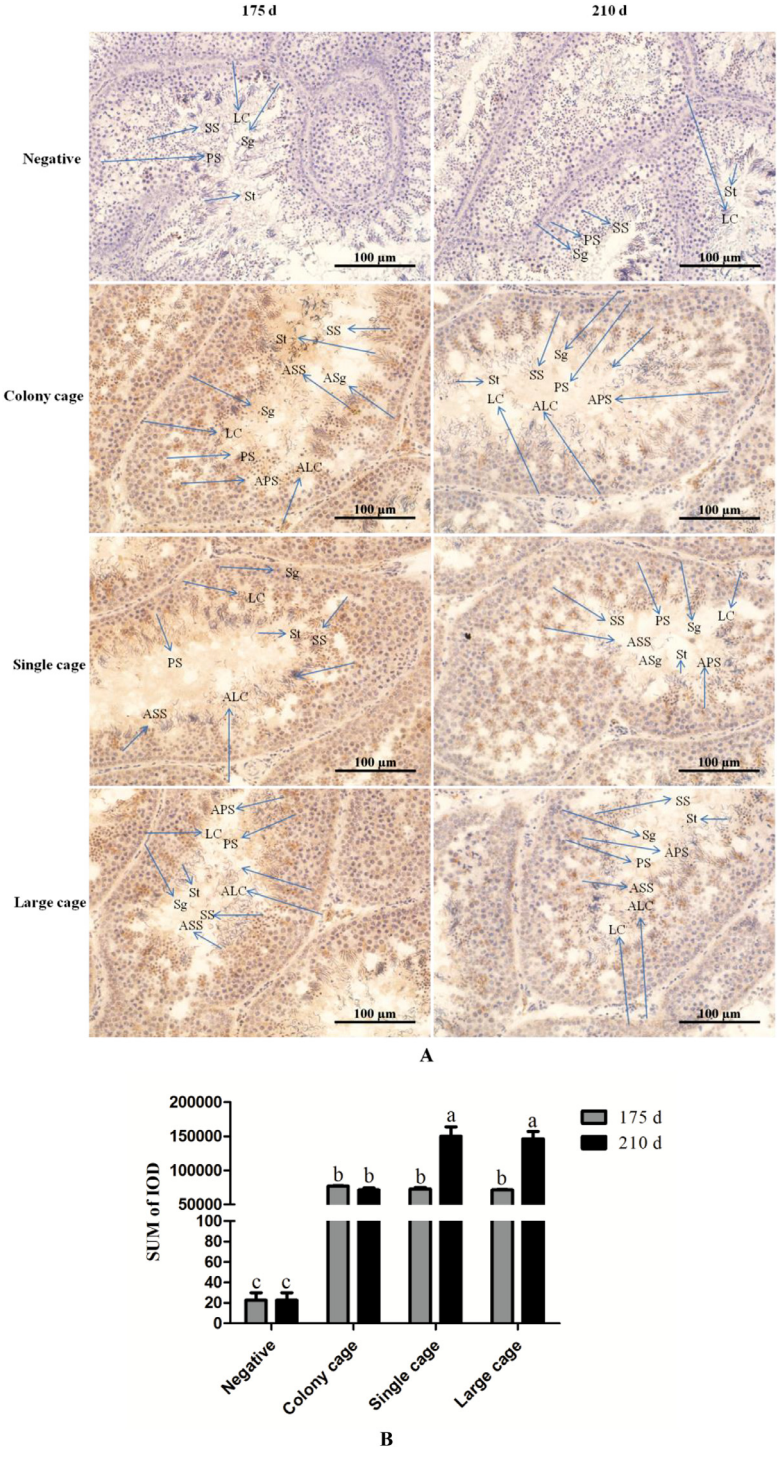

Figure 7. The expression and localization of Caspase-3 protein in testicular tissues in different groups on d 175 and 210 (200 ×). (A) The immuno histochemical result of Caspase-3 protein. (B) The statistical result of total optical density of brown cells. Testicular sections from negative control group were randomly selected from colony cage group. The height of a column is the mean of experimental values (n = 5) and the error bar shows the SE. Groups without the same letter differed ($P < 0.05$). Abbreviations: ALC, apoptotic Leydig cells; ASg, apoptotic spermatogonia; APS, apoptotic primary spermatocyte; ASS, apoptotic secondary spermatocyte; ASt, apoptotic spermatid; LC, Leydig cells; PS, primary spermatocyte; Sg, spermatogonia; SS, secondary spermatocyte; St, spermatid.

used in this study attained sexual maturity on d 105 when fully formed spermatozoa were present both in the tubular lumen and the lumen of excurrent duct system. This was earlier than the d 140 reported by Brillard (1986) in Guinea fowls. The differences in the time of sexual maturity between the 2 flocks may be attributed by possible differences in breed and management, including diet (Abbaspour et al., 2019) and photoperiod (Siegel et al., 1969), which can dramatically alter the onset of meiosis and sustained spermatogenesis. In addition, we found that the relative testicle weight of the colony cage group was significantly higher than that of the single and large cage groups. Largest area of seminiferous

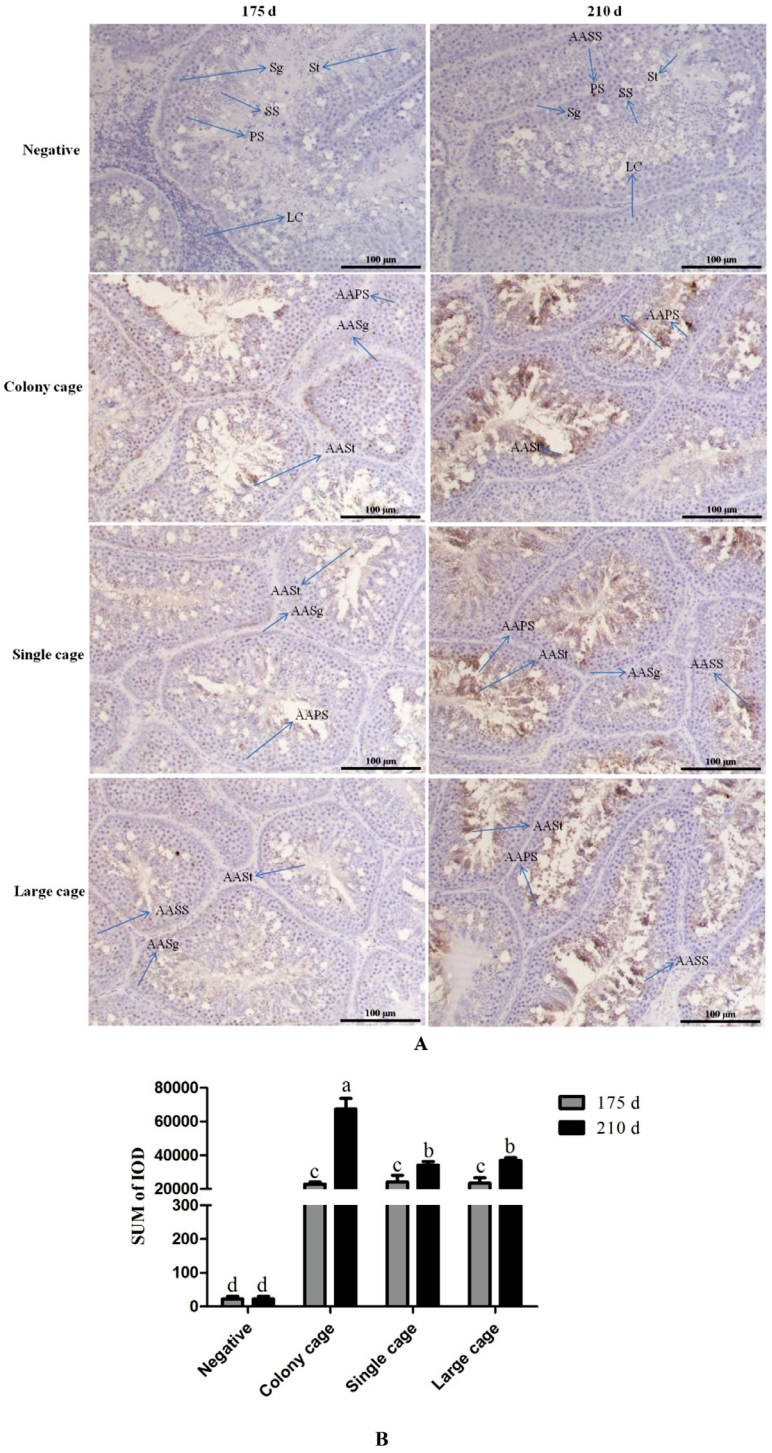

Figure 8. The expression and localization of Bcl-2 protein in testicular tissues in different groups on d 175 and 210 (200 ×). (A) The immunohistochemical result of Bcl-2 protein. B. The statistical result of total optical density of brown cells. Testicular sections from negative control group were randomly selected from colony cage group. The height of a column is the mean of experimental values (n = 5) and the error bar shows the SE. Groups without the same letter differed ($P < 0.05$). Abbreviations: AALC, antiapoptotic Leydig cells; AASg, antiapoptotic spermatogonia; AAPS, antiapoptotic primary spermatocyte; AASS, antiapoptotic secondary spermatocyte; AASt, antiapoptotic spermatid.

tubules in the colony group may contribute to the result.

The previous studies have shown that the testis is weakly affected by relevant hormones from the embryonic stage to prepuberty. However, the influence of some androgens, particularly T levels, on testis weight and secondary sexual characteristics at sex maturity period has been reported by Vizcarra et al., 2010 FSH and LH also played important roles in regulating spermatogenesis via influencing the proliferation and differentiation of Sertoli cells and Leydig cells activation (Huhtaniemi et al., 1999; Layman, 2000). Dramatical inceasing area of seminiferous tubules in 3 rearing system, especially in conoly cage from d 70 to d 210 in the present study may be due to the increase of FSH and LH levels at this period. LH and FSH were secreted by pituitary gland,

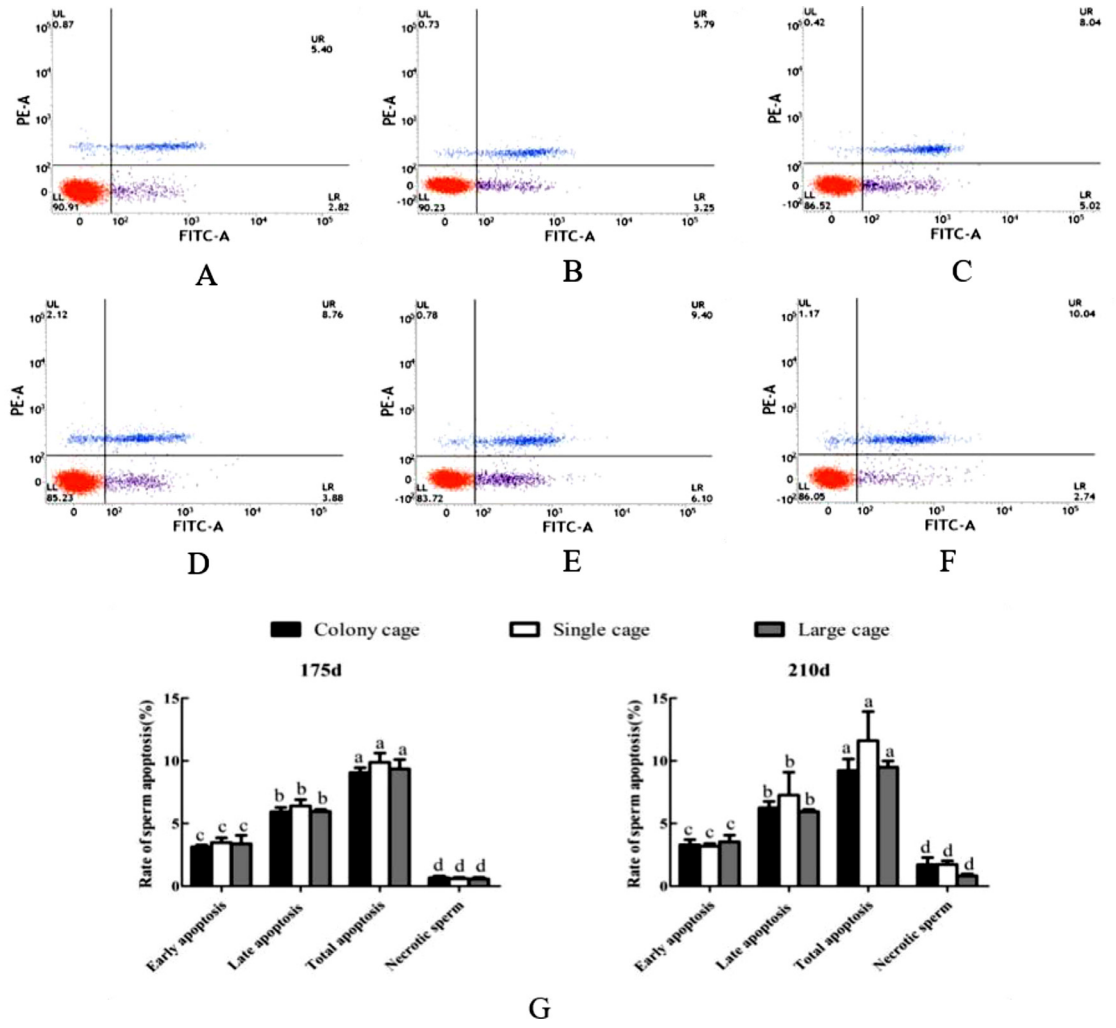

Figure 9. The flow cytometry results for sperm apoptosis on d 175 and 210. (A−C) The flow cytometry results for sperm apoptosis on d 175 for group colony cage, single cage, and large cage, respectively. (D−F) The flow cytometry results for sperm apoptosis on d 210 for group colony cage, single cage, and large cage, respectively. (G) The statistical results of sperm apoptosis on d 175 and 210. The height of a column is the mean of experimental values (n = 5) and the error bar shows the SE. Groups without the same letter differed ($P < 0.05$).

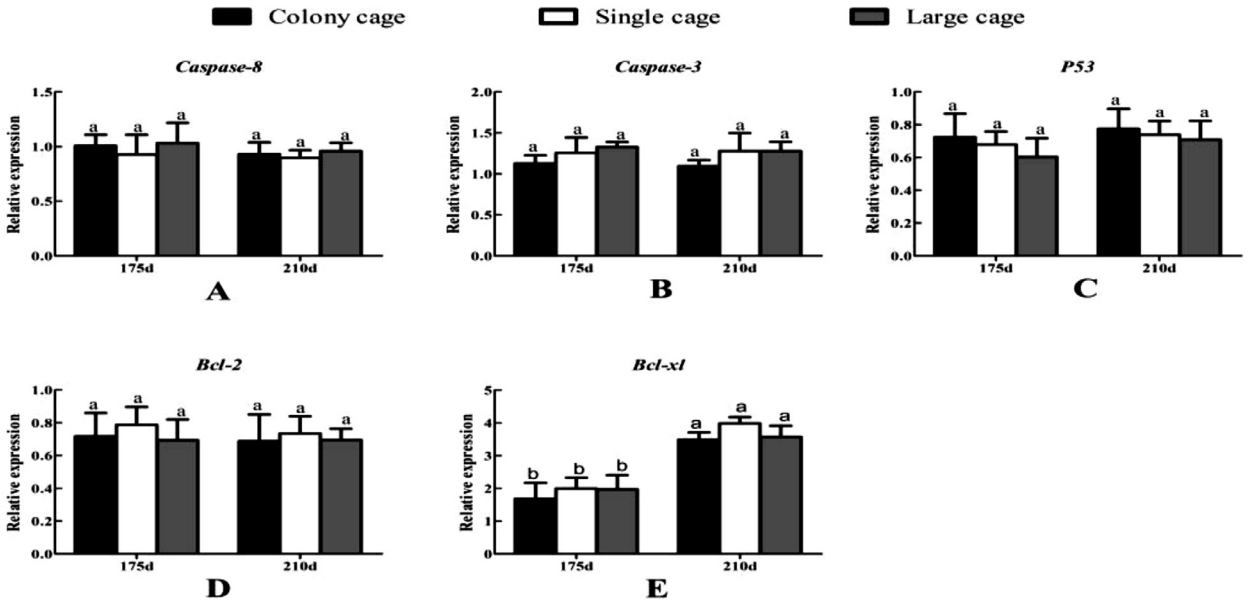

Figure 10. The expression abundances of apoptotic gene in sperm in different groups on d 175 and 210. (A−E) The results of relative expression abundances of apoptotic genes *Caspase-8, Caspase-3, P53, Bcl-2*, and *Bcl-xl* in sperm, respectively. The height of a column is the mean of experimental values (n = 5) and the error bar shows the SE. Groups without the same letter differed ($P < 0.05$).

and T was secreted by Leydig cells of the testes (Dupont et al., 2000). Also GnRH in hypothalamus could remotely promote the expression of 3 β-hydroxysteroid dehydrogenase and increase the content of T (Lin et al., 2008).

On d 210, the concentrations of FSH, LH, and T in the colony cage group were higher than those in single and large cage groups in current study. Li et al. (2017) studied the effects of these hormones on secondary sexual characteristics in males chickens. Results indicated that under the action of these hormones, spermatogenic cells developed, the number of spermatogenic cells increased, and the average area of seminiferous tubules increases rapidly. Both studies illustrate that these hormone lead to the raise of testis volume and weight. The area of seminiferous tubules reached its maximum at 3-mo after birth in chickens, and the testicular weight increased sharply (Rotter et al., 1993). We found that the relative testis weight and the area of seminiferous tubules for the colony cage group were significantly larger than single and large cage groups on d 210. This difference may be due to the inceased reproductive hormone content of the roosters induced by hens in colony cage.

The semen quality of animals is a heritable trait, which will be affected by different genetic background lines in chickens (Flowers, 2015). Additionally, seasons, environment, nutrition, body index, and number of times of semen collection may also influence the semen quality (Sonseeda et al., 2013). The semen quality of breeders will affect flock productivity. The weight of a rooster is an important indicator of his reproductive capacity, the fatty or skinny one had poor semen quality (Li et al., 2013). Compared the body weights between the roosters raised in single and large cages, the results showed that those raised in large cage were lighter than those raised in single cages, suggesting that the rearing system may indirectly influence the semen quality. Also, we found that the semen quality of roosters raised in colony cages was superior to those raised in single cage on d 210. Additionaly, although fertility of the eggs from colony cage was lower than that in single cage, the hatching and healthy chicks of fertilized eggs from colony cages were higher than those in single cage, which was related to the mate preference of roosters in colony cage. Males choose females with potential good genes to increase their reproductive success, which optimizes the quality of the descendents in terms of sexual attractiveness (Kotiaho and Puurtinen, 2007).

Previous studies have shown that both spontaneous and induced apoptosis of spermatogenic cells occurs during spermatogenesis, and excessive apoptosis of spermatogenic cells directly affects the number of spermatozoa finally produced by males (Flowers et al., 2015) Our results showed that the difference of sperm concentration among three rearing systems at d 210 was related to the apoptosis of spermatogenic cells in testis tissue, and the females may reduce the apoptosis of spermatogenic cells in colony cages. Although at different stocking densities, large and single cages had no difference on sperm concentration Yang et al. (2002) reported the relationship between sexual hormones in semen and germ cell apoptosis in male population, the sperm apoptosis rate in the low sperm concentration group was significantly higher than that in the normal group The levels of FSH, LH, T were negatively correlated with sperm apoptosis rates. It was indicated that sperm apoptosis and sexual hormone would directly influence the sperm concentration. In our results indicated that the difference in sperm concentration among three rearing systems was not due to the apoptosis of mature sperm, but due to the difference of sexual hormone levels.

To further explore the apoptotic types of spermatogenic cells in three rearing systems, we detected the expression and localization of Caspase-3 apoptotic protein and Bcl-2 antiapoptotic protein in the testis of roosters. It was reported that apoptosis occurs in spermatogenic cells at all levels of mammalian spermatogenesis (Tapanainen et al.,1993; Yadav et al., 2018). In the process of mouse spermatogenesis, there was a large number of spontaneous apoptosis in spermatocytes, but the rate of spermatogonia apoptosis is relatively low, and almost no apoptosis occurs in spermatid (Xu et al., 2018). In the spermatogenesis of rats and hamsters, spontaneous apoptosis mainly occurs in spermatogonia and spermatocytes (Oliveira et al., 2017). The spermatogonia, spermatocytes, and spermatids all had apoptosis during the development of chicken spermatogenic cells (Song et al., 2015). Our results showed that Caspase-3 and Bcl-2 proteins were expresssed in spermatogonia, spermatocytes, and spermatid, which was consistent with previous studies.

CONCLUSION

In general, roosters during the growing period had rapid testis growth and increasing main reproductive hormones in plasma. At the peak of sexual maturity, the females in colony cage had a positive effect and promoted the testis development of males. Roosters reared in the natural mating system had better semen quality, particularly in semen volume, density, and viability; the hatching % of fertilized eggs and healthy chicks were higher for the colony than single and large cages. Moreover, the sperm concentration was higher for colony than single and large cages, which was related to the apoptosis of spermatogonia and spermatocyte.

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DISCLOSURES

The authors declare that they have no conflict of interest.

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