

Full Length Research Paper

Expression of androgen and estrogen receptors in the testicular tissue of chickens, quails and chicken-quail hybrids

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36 New Roman cocks, 30 Korean male quails and 30 chicken-quail hybrids of different day-age were selected and their body weight and testes weights were measured and as well, their testes were collected. Real-time polymerase chain reaction (RT-PCR) was performed to evaluate the messenger ribonucleic acid (mRNA) expression patterns of androgen receptors (AR) and estrogen receptors (ER) genes in testicular tissue of chickens, quails and chicken-quail hybrids at different growth stages. The results show that the testes of chickens and quails grew and developed normally with body weight gain, but the testes of chicken-quail hybrids had a slower growth rate and stunted growth. Real-time PCR showed AR and ER mRNA expression patterns in testes of chickens and quails at different growth stages were similar. AR mRNA expression in chickens and quails reached a significant peak at 80 and 30 days of age, respectively and their ER gene expression showed fluctuation slightly. The AR and ER expression of chicken-quail hybrids were different from the above expression patterns; the hybrids AR gene expression showed a gradual decline and ER gene expression gradually increased. The chicken-quail hybrids AR and ER gene expression was abnormal and we speculate this is an important molecular factor for the testicular dysplasia of chicken-quail hybrids. Our results show that AR gene expression was upregulated by ER gene and we suggest that the synergetic effect of AR and ER gene regulated the normal testis growth and development of chicken and quail.

Key words: Chicken, quail, chicken-quail hybrid, testis, androgen receptors (AR), estrogen receptors (ER) expression.

INTRODUCTION

Both chicken and quail are from the same pheasant family Phasianidae of the class Aves, order Galliformes, but they belong to *Gallus gallus* and *Coturnix coturnix*, respectively (Khosravinia et al., 2005). The crossing between chickens and quails is a typical distant hybridization. Studies show that the chicken-quail hybrids have

a significant heterosis for growth rate, body size, meat quality and other production performance (Liao et al., 2008) and even have a very strong resistance to birds ribonucleic acid (RNA) tumor virus (Greenfield et al., 1986). On the other hand, there are some problems or challenges in the distant hybridization, such as the incompatibility of heterogenous germ cells, low rate of fertilization (11.5%), hybrid reproductive organs dysplasia and hybrid sterility (Wilcox and Clark, 1961; Takashima and Mizuma, 1981).

It is also interesting and confusing that the chicken-quail hybrids embryos had males and females before 120 h incubation and only males were found by cytological inspection after they had been hatched (Yoshihiro, 1982).

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Hence, the chicken-quail hybrids can be used as a good material for animal genetic studies.

Androgen is one kind of steroid hormones in male animal body and exists mainly in the form of testosterone and dihydrotestosterone (DHT), which plays an important role in maintenance of the male signs as well as in the growth and development (Wang et al., 2009). The development and function of male sexual organs are regulated mainly by androgen and estrogen is also essential for the development and function of male reproductive organs (Ferlin et al., 2010). The physiological functions of androgen and estrogen can be achieved only through their receptors (Walters et al., 2010; Bi et al., 2005). Androgen receptor gene (AR) and estrogen receptor gene (ER) are members of nuclear hormone receptor family (Heinlein and Chang, 2002; Luconi et al., 2002). AR gene mutation or deletion can cause developmental abnormalities in male reproductive organs or infertility (Brinkmann et al., 1995). The expression of ER mRNA in the gonad of the male and female chicken embryos at 26 weeks (4.5 days incubation) was found by whole-mount *in situ* hybridization (Elbrecht and Smith, 1992). The estrogen receptors are expressed earlier than gonadal differentiation in chick embryo development and estrogen plays a permanent role in sex differentiation in birds (Andrews et al., 1997). Transgenic experiments showed that the testes of ER α knockout male mice were significantly smaller than those of wild-type mice, and the seminiferous epithelium was thinning, sperm count and animals mating were reduced, and fertility was decreased (Eddy et al., 1996). However, the effects of AR and ER genes in the growth and development of chicken-quail hybrid testes have not been reported.

Since the success of hybridization between chickens and quails, some studies of chicken-quail hybrids were carried out, such as effects of lactate dehydrogenase and alcohol dehydrogenase on the development of hybrids embryos and individuals, molecular mapping of neuropeptide and peptide, heredity of mitochondrial mRNA and coat color (Boswell et al., 1998; Meyerhof and Haley, 1975; Le Vine and Haley, 1975; Minvielle et al., 2002; Watanabe et al., 2005). The aim of this study was to define AR and ER expression patterns in testis in different growth stages of chickens, quails and chicken-quail hybrids using real time quantitative PCR, to further our understanding of the role of androgen and estrogen in chickens and quails genital development and to clarify the molecular factors for testicular dysplasia of chicken-quail hybrids.

MATERIALS AND METHODS

Experimental animals

New Roman chickens, Korean egg-type quails and chicken-quail hybrids were raised under the same conditions at Shihezi University Experiment Station. Chicken-quail hybrids were produced through the mating of New Roman cocks and Korean quail hens by artificial insemination. Six cocks were selected at the age of 20, 40, 60, 80,

100 and 120 days respectively. Six male quails and six chicken-quail hybrids were selected at the age of 10, 20, 30d, 40 and 50 days respectively. A total of 36 cocks, 30 male quails and 30 chicken-quail hybrids were chosen. The selected animals were weighed and then sacrificed. The testes were rapidly removed and weighed. They were then frozen in liquid nitrogen immediately and then stored at -80°C. Before storage, the testes from a 120-day-old cock, a 50-day-old male quail and a 50-day-old chicken-quails hybrid were photographed with a digital camera (CANON-IXUS 1000 HS, Japan) and slices with a thickness of 0.5 cm were harvested from the left and right testes at the center line. The slices were then fixed in 10% formalin solution for 24 h. After washing, dehydrating and paraffin-embedding, they were sliced to 4 μ m sections using paraffin slicing machine (Leica, Germany), which were then stained with hematoxylin-eosin and photographed under optical microscope (Olympus, Japan) with 40-fold magnification.

Total ribonucleic acid (RNA) extraction and reverse transcription

Total RNA was extracted using TRIZOL reagent according to the manufacture's protocol. The integrity of total RNA was detected on 1.2% agarose denaturing formaldehyde gel. RNA concentration was measured at 260 nm using a Smart SpecTM Plus UV spectrophotometer (BIO-RAD, U.S.A). Total RNA was reverse transcribed into first strand complementary deoxyribonucleic acid (cDNA) using SYBR[®] PrimeScriptTM RT-PCR Kit (TaKaRa, Dalian, China).

Isolation of AR and estrogen receptors ER cDNA fragment

Based on the chicken AR and ER mRNA sequence (GenBank accession Nos. NM_001040090 and AF442965, respectively), primers were designed using Primer 5.0 to amplify AR and ER cDNA fragments on quails (Table 1). The PCR products were ligated into the pGEM-T easy vector system (TaKaRa, Japan) and then transformed into competent Top10 cell. Plasmid DNA was purified and sequenced in Sangon company (Shanghai, China) using an automated ABI3730 analyzer (Applied Biosystems, CA, USA).

SYBR green real-time polymerase chain reaction (PCR) analysis of expression pattern

The expression of AR and ER genes were detected by iQTM5 Multicolor real-time PCR detection system (USA). The gene expression level was quantified relative to the expression of β -actin gene (GenBank accession No. AY550069). The forward and reverse primers for AR and ER genes are listed in Table 1. Real-time PCR was performed in triplicate in 25 μ l mixture containing 12.5 μ l SYBR Premix Ex TaqTM (2 \times), 0.5 μ l of forward and reverse primers, 2 μ l of template cDNA, and 9.5 μ l of ddH₂O. The cycling condition consisted an initial cycle of 15 s at 95°C followed by 45 cycles of 10 s at 95°C (for denaturation), 10 s at 60°C (for annealing) and 20 s at 72°C (for polymerization). The expression level was calculated using double-standard curve method. The differences in means of expression level was examined using one-way analysis of variance (ANOVA) and Duncan's multiple comparison test ($p < 0.05$).

RESULTS

Growth and development of testes of chickens, quails and chicken-quail hybrids

Body weights and testes (pair) weight of chickens, quails

Table 1. Conditions of PCR and parameters of oligonucleotide primer pair.

Target gene	GenBank number	PCR product	Sequence of primer(5'→3')	Tm
AR	NM_001040090	280 bp	F: CCAGATTTGGTCTTCAACG R: GCTGGTAAAACCGCCTA	56 °C
ER	AF442965	253 bp	F: CCCTTCATCCATCACCACA R: ACAAGACCAGACCCATAAT	56 °C
β-actin	L08165	152 bp	F: CCTGAACCTCTCATTGCCA R: AGAAATTGTGCGTGACATCA	56 °C

Table 2. Living weight and testis weight of cock, quail and chicken-quail hybrid (g).

Breed	Trait	Day								
		10	20	30	40	50	60	80	100	120
Cock	Living weight	/	84.37	/	327.62	/	680.33	1048.29	1263.87	1527.36
	Testis weight	/	0.00552	/	0.2871	/	1.2357	6.4362	12.7252	18.6401
Quail	Living weight	22.41	46.46	8.83	142.79	182.48	/	/	/	/
	Testis weight	0.0082	0.0437	1.7835	3.3572	6.6735	/	/	/	/
Chicken- Quail hybrid	Living weight	29.33	66.73	128.65	286.62	352.56	/	/	/	/
	Testis weight	0.0065	0.0131	0.0581	0.2153	0.3626	/	/	/	/

and chicken-quail hybrids of different day-ages are listed in Table 2. Body weight and testes weight of chickens increased by 18 times and 337 times from 84.37 and 0.0552 g at 20-day-age to 1527.36 and 18.6401 g at 120-day-age, respectively. Body weight and testes weight of quails increased by 8 times and 814 times from 22.41 and 0.0082 g at 10-day-age to 182.48 and 6.6735 g at 50-day-age, respectively. Body weight and testes weight of chicken-quail hybrids increased by 12 times and 56 times from 29.33 and 0.00065 g at 10-day-age to 352.56 and 0.3626 g at 50-day-age, respectively. It was indicated that testicular growth and development of chicken-quail hybrids was very slow. From the cumulative growth curves of the body weight and testis weight of chicken, quail and chicken-quail hybrids (Figure 1), it can be seen that the three cumulative growth and developmental curves of body weight of chicken, quail and chicken-quail hybrids were consistent, but their growth and developmental curves of testes were very similar only in chicken and quail. However, hybrids did not have rapid growth and developmental period. Through the comparative study of the sections of testicular tissue of three adults (Figure 2), it was discovered that the hybrid's testicular structure was significantly abnormal, the number of sertoli cells was small, seminiferous tubules diameter was also small, and secondary spermatocyte, spermatid and sperm could not be found within the lumen, which showed significant dysplasia. The typical male testis organ was also

discovered in the external morphology of the hatched hybrid (Figure 3). The adult testis of hybrid individual was small and there was no surrounding vascular distribution. Through the above comparative study, normal testicular growth and development was found in both chicken and quails, while the testes of hybrids showed developmental deficiency and typical male sterility.

The expression of AR mRNA in the testicular tissue in cock, quail and hybrid

AR mRNA expression analysis is shown in Figure 4. Figure 4 shows that the relative expression of AR mRNA was highest at the age of 80 days, and lowest at the age of 120 days; the difference was significant ($P < 0.01$). It was highest at the age of 30 days in quails and the lowest at the age of 10 days; the difference was significant ($P < 0.01$). It was highest at the age of 10 days in chicken-quail hybrids and the lowest at the age of 50 days; the difference was significant ($P < 0.01$). From the expression curve figure of the testicular tissue AR gene in chicken, quails and their hybrids (Figure 4), it could be seen that the relative expression curves of the testicular tissue AR mRNA in chicken and quails were similar, which reached the peak in the adolescent (chicken at 80 day-old, quail at 30 day-old) and showed increase first followed by a decline after the peak; the expression of hybrid testis AR mRNA declined linearly from 10-day-old

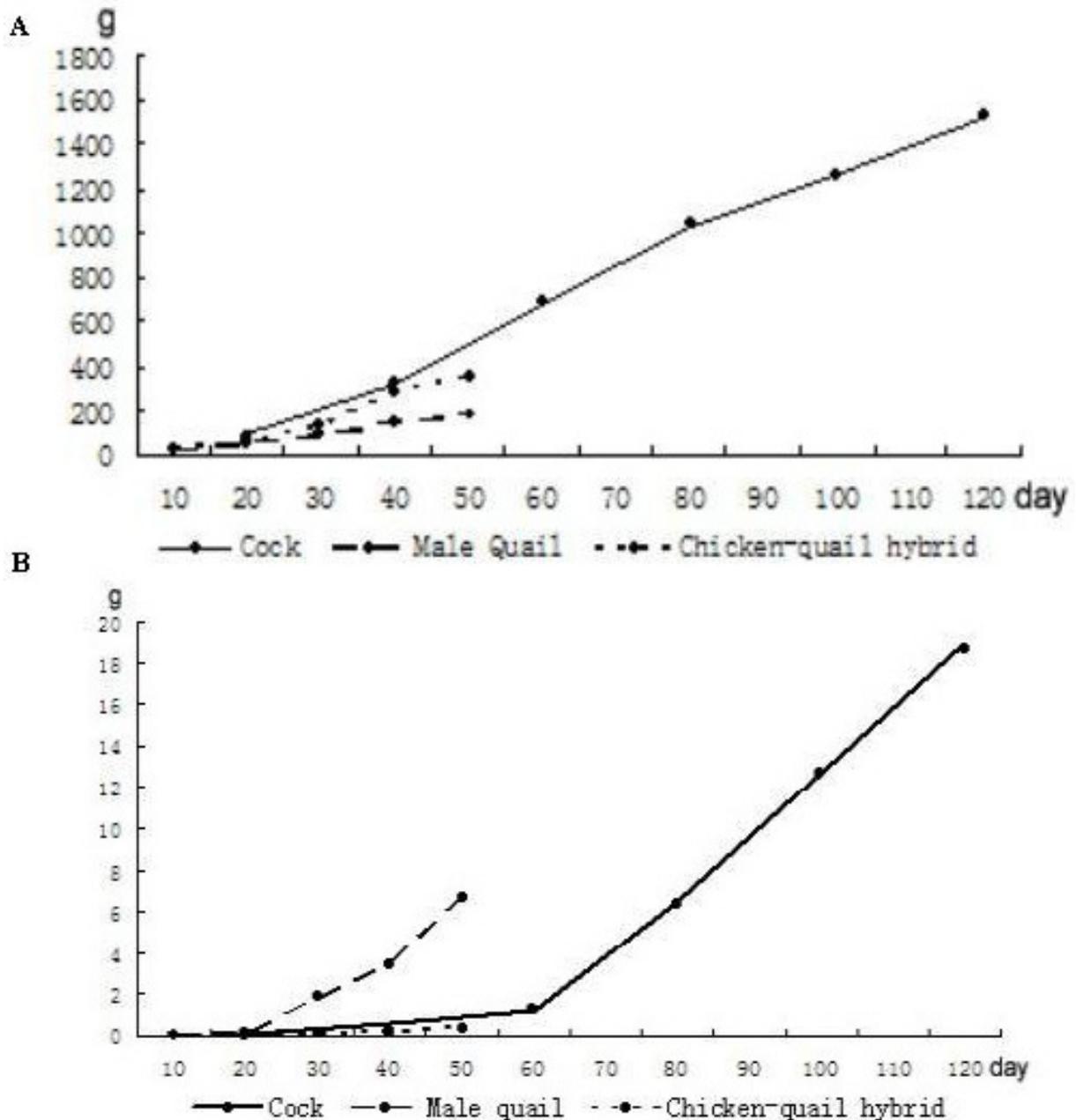


Figure 1. The growth curve of cock, quail, and hybrid cumulative body and testis weights. A, Cumulative growth curves of body weights of cock, quail and chicken-quail hybrid; B, cumulative growth curves of testis weights of cock, quail and chicken-quail hybrid.

to 50-day-old.

The expression of ER mRNA in testicular tissue in cock, quail and hybrid

The results of ER mRNA expression analysis are shown in Figure 5. Figure 5 shows that the mRNA expression of chicken ER was highest at 60 days and lowest at 80 days and the difference was significant ($P < 0.01$); it was the

highest at the age of 50 days in quails, and lowest at the age of 40 days; the difference was significant between the two ($0.01 < P < 0.05$), it was highest at the age of 50 days and lowest at the age of 10 days in chicken-quail hybrids, the difference was significant ($P < 0.01$). The expression curves of testicular tissue ER genes in chicken, quail and their hybrids (Figure 6) showed that at the different developmental stages of chicken and quails, ER mRNA expression in testicular tissue appeared to have fluctuating sequential changes; it was the highest at

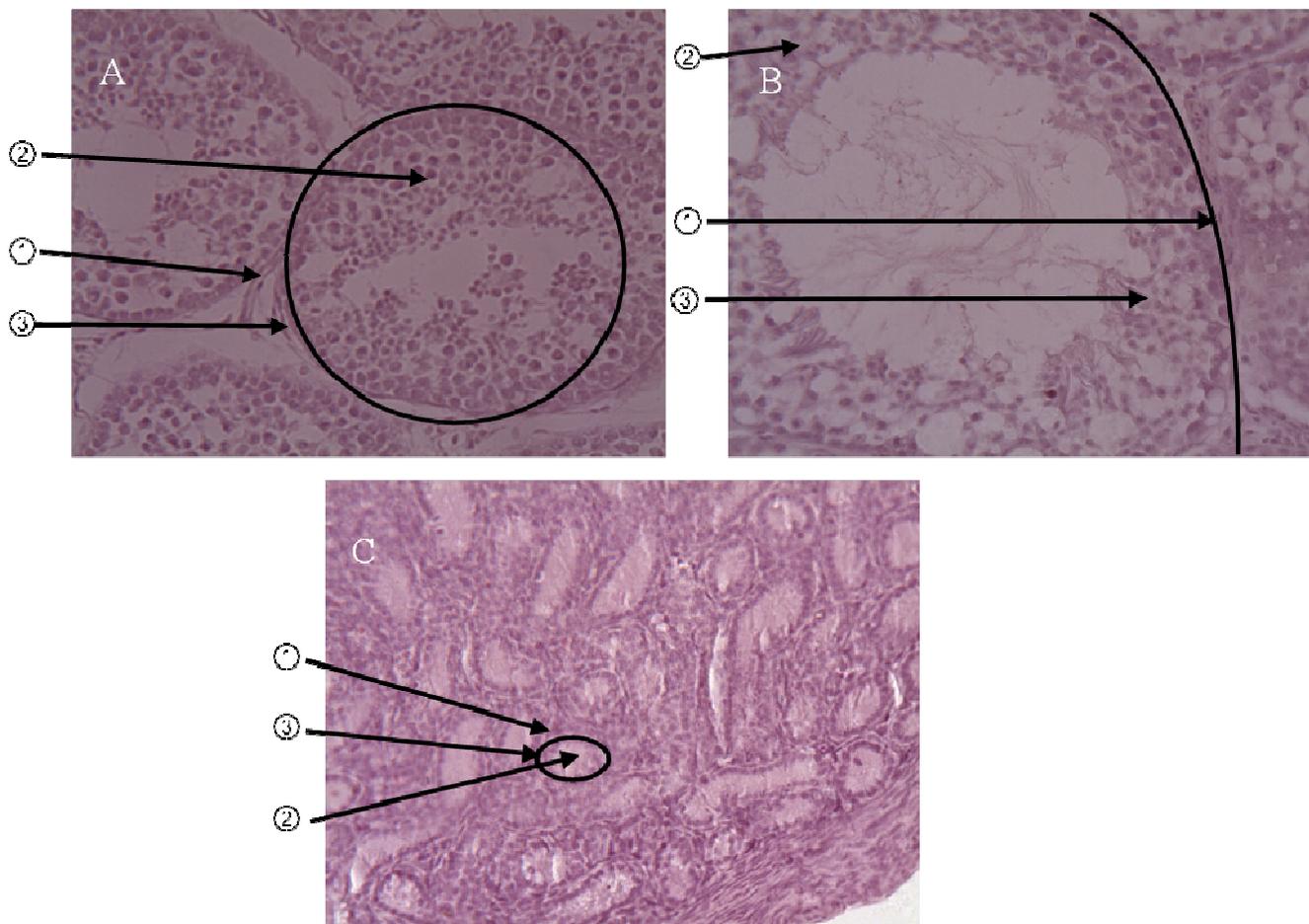


Figure 2. Schematic diagram of testicular biopsy (40 times). A, Cock; B, male quail; C, chicken-quail hybrid; ① mesenchymal cells; ② sertoli cells; ③ seminiferous tube cavity; (The contorted seminiferous tubules caliber (μm) of cock, male quail and chicken-quail hybrid were 275.09 ± 46.07 , 328.28 ± 30.15 , 20.98 ± 10.43 , respectively. The seminiferous epithelium depth (μm) of cock, male quail and chicken-quail hybrid were 97.01 ± 8.12 , 100.89 ± 11.02 , 4.223 ± 2.507 , respectively).

the age of 20 days in chicken, and the lowest at the age of 40 days, it gradually increased from 40 to 80 days and later decreased gradually; it also decreased gradually in the age of 10 to 40 days in quail, with the lowest point at the age of 40 days and the highest point at the age of 50 days. The hybrid ER mRNA expression in testis continued to rise linearly from 10 to 50 days, and with the increase in age, its ER mRNA expression was much more than those in chicken and quail.

DISCUSSION

AR gene expression in testis tissue of chicken, quail and chicken-quail hybrid

Testicular development and function are dependent on many factors, the most important of which is androgen, which is a class of hormones that promote the development of male genitals and secondary sex

characteristics and stimulate the differentiation of spermatogenic cells and the sperm generation. The biological activity of androgen is mediated by the AR. Therefore, AR is essential for the male gonadal development (Jarow and Zirkin, 2005). It was showed that AR gene mutation or deletion may cause developmental abnormalities in male reproductive organs or infertility (Carreau et al., 2003). Within puberty in rats, a higher AR levels in leydig cells can promote testosterone secretion and with luteinizing hormone (LH) together, the synthesis ability of androgen in interstitial cells of adolescent rat testis increase many times and further promote the testis development (Hardy et al., 1990). It was also found by *in situ* hybridization that AR mRNA expression in rat leydig cells rose shortly in adolescence and reached the highest on the 35th day after birth. It was indicated that AR gene plays an important role on the differentiation of mesenchymal cells (O'Shaughnessy et al., 2002). O'Shaughnessy et al. (2002) found AR expression disorder with age and the mesenchymal cells

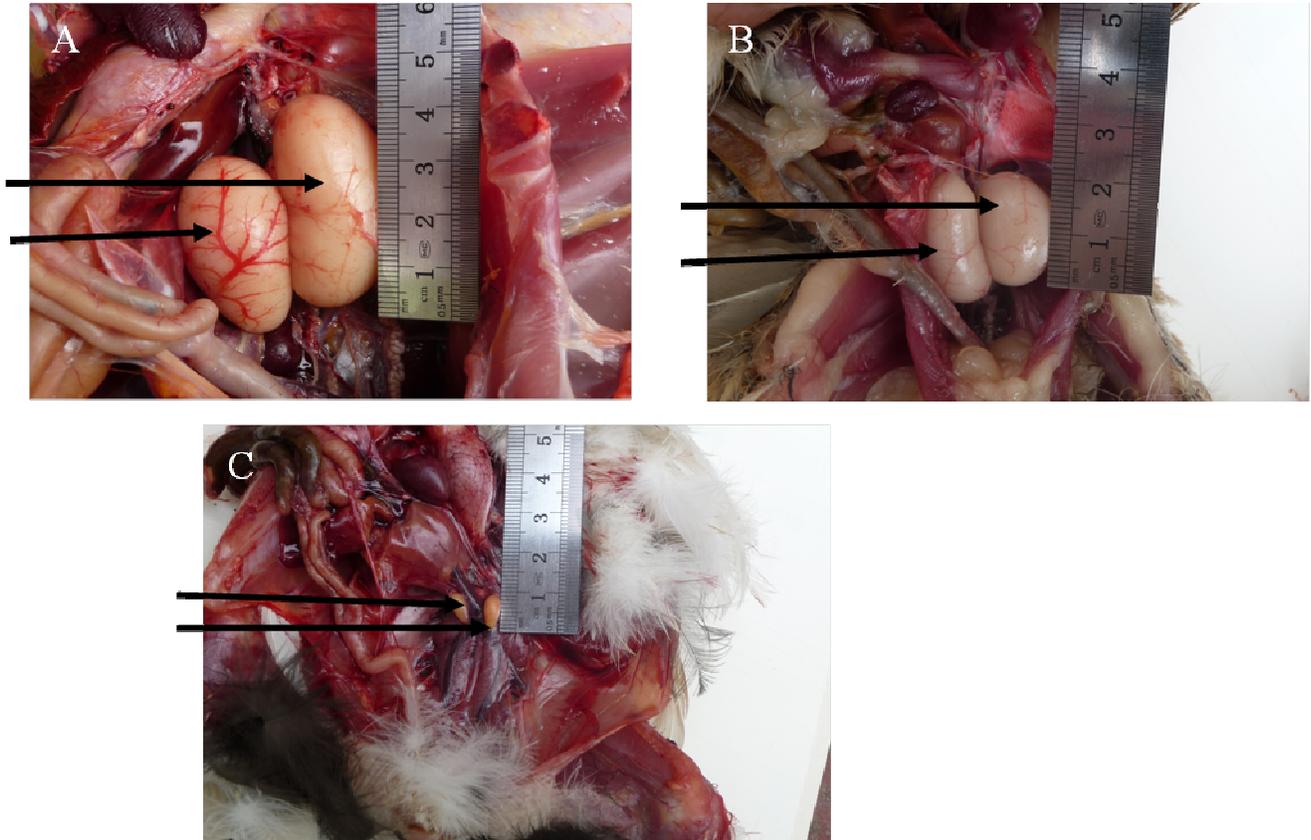


Figure 3. Testicular morphology. A, Cock; B, male quail; C, chicken-quail hybrid.

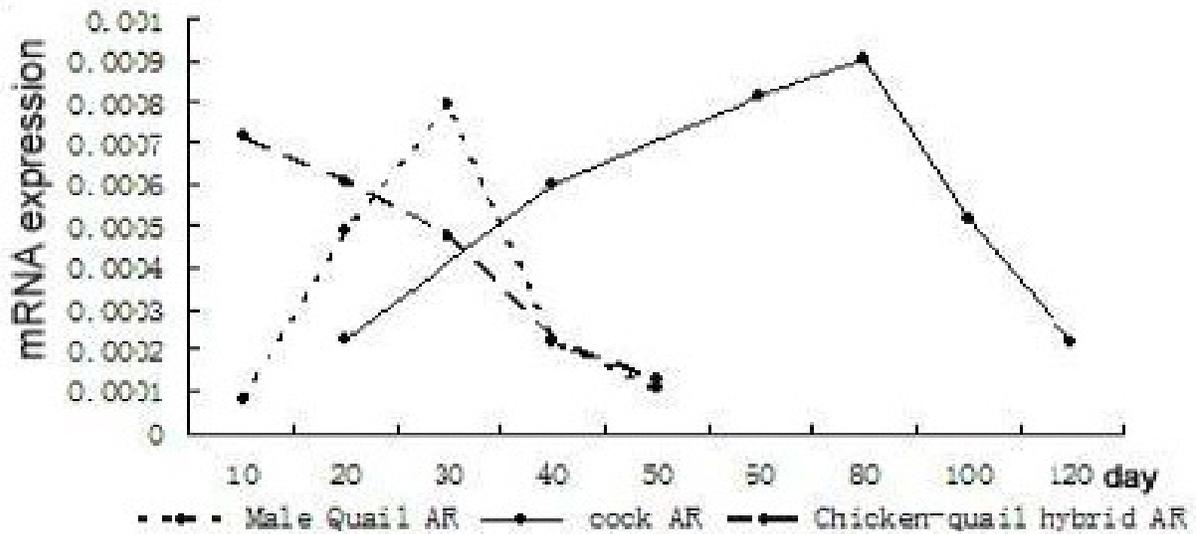


Figure 4. The expression of AR mRNA in the testicular tissue in cock, quail and chicken-quail hybrid.

development is also abnormal, only with partial function of interstitial cells, although embryonic mesenchymal cells develop normally in mice with lack of AR function. It was indicated that AR plays an important role in

promoting the normal development of stromal cells. AR expression in testicular tissue of rat, goat and human had similar time series. AR expression measured by *in situ* hybridization on 21, 35 and 90 days of rat increases firstly

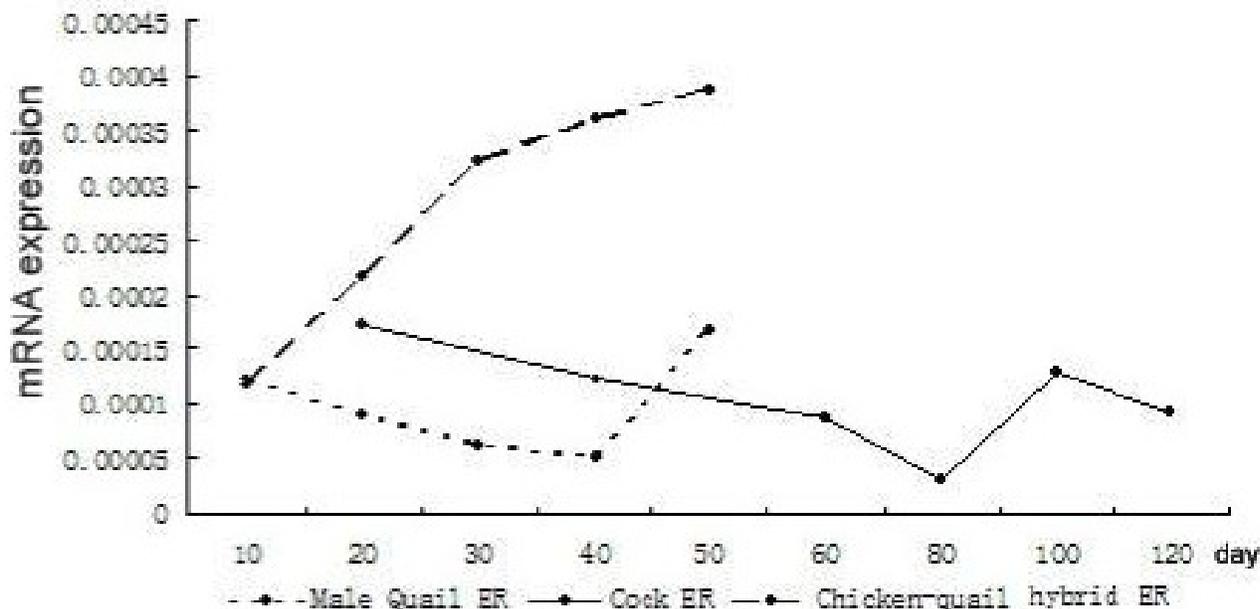


Figure 5. The expression of ER mRNA in testicular tissue in cock, quail, and chicken-quail hybrid.

and decrease after reaching its peak (Shan et al., 1995). The intensity of AR immunostaining of rat and goat testicular sertoli cells also increase with increasing age and reach the strongest at sexual maturity (Goyal et al., 1997). It was also found that AR immunostaining in the human adult testis cell has cyclical changes, that is, the intensity of AR immunostaining is the strongest in spermatogenesis on the 3rd stage and decrease within the 4 to 5 and 1 to 6 stage (Suárez-Quian et al., 1999). In our study, AR expression in testicular tissue of chickens and quails (Figure 4B) also showed similar time series, but the AR expression pattern of chicken-quail hybrids was different. Our results suggest that AR expression with time series regulates the normal growth and development of the testis supporting cell of cock and male quail; by contrast, the AR expression of chicken-quail hybrids, which showed a linear decline and was different with that of cock and male quail, is abnormal. The abnormal AR expression in the hybrid testis reduced the function of androgen regulating sertoli cells and the growth of testicular supporting cells of hybrids was blocked, leading to testicular growth and slow development of hybrid; there was no strong period, the testes of 50-day hybrids were undersized and showed a significant dysplasia.

ER gene expression in testis tissue of chicken, quail and chicken-quail hybrid

Estrogen not only indirectly affects germ cell development in the testis seminiferous tubules, but also directly

affects spermatogenesis and inhibits the apoptosis of germ cells (Pentikainen et al., 2000; Shetty et al., 1997). Small doses of estrogen can promote spermatogenesis in minor voles. On the contrary, large doses of estrogen or the estrogen receptor antagonists is bad to testis and will lead to testicular atrophy (Gancarczyk et al., 2004). ER α expression is carried through mainly in stromal cells in the testis development of rats, mice, pigs, dogs, marmosets and humans from juvenile to adult (Zhou et al., 2002; Rago et al., 2004; Nie et al., 2002; Pelletier and El-Alfy, 2000; Sar and Welsch, 2000; Fisher et al., 1997). It was found by *in situ* hybridization that ER mRNA is expressed in the gonad both in the male and female chicken 26-stage embryos (4.5-day incubation). ER β expression was found in germ cells and sertoli cells, except in leydig cells at different developmental stages of male (Carreau et al., 2003). Hence, ER β gene directly affects germ cells in the process of testis development and spermatogenesis. Transgenic experiments showed that the testis ER α -knockouted male mouse with thinning seminiferous epithelium, reducing sperm count, reducing mating number and decreased fertility, was significantly smaller than wild-type mice (Gancarczyk et al., 2004). Our results show that the model of ER mRNA expression in testis of chicken-quail hybrids is different from that of chickens and quails. The ER mRNA expressions of hybrids increased linearly continuously and gradually out distant that of chickens and quails with days (Figure 5B). Therefore, we speculate that this model of the sustained incremental expression and higher levels of expression of ER gene is the important factor that causes azoospermia in the testicular seminiferous tubules of hybrids.

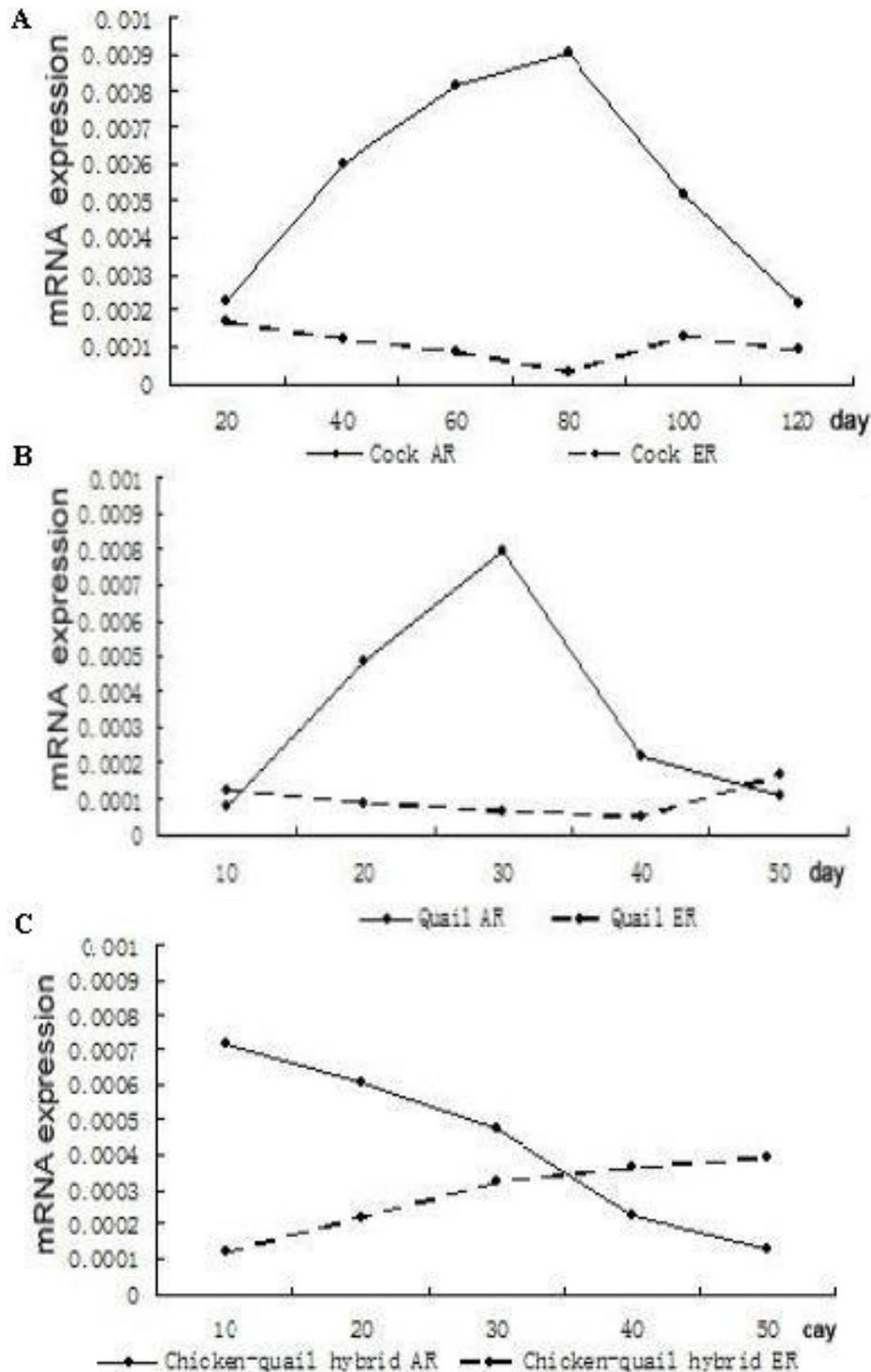


Figure 6. The expression curves of AR and ER gene mRNA in the chicken, quail, and chicken-quail hybrid testis.

AR and ER gene in chickens, quails and chicken-quail hybrids testis tissue

Female and male hormones promote male reproductive system with a synergistic effect through their receptors

(Oliveira et al., 2004; Carreau and Hess, 2010). The lack of estrogen in young male can directly affect the reproductive capacity in adult. Figure 6 shows that, the length of growth and developmental periods of chickens and quail were different, but the AR and ER expression

patterns in their testes were similar and showed a time series. In all ages, AR expression was higher than the corresponding ER, and three had a common characteristic which was when AR expression was at the highest, ER was at the lowest point. On the contrary, ER expression was at the peak while AR tended to be at the lowest point. It demonstrated that AR and ER genes synergistically regulated the growth and development of testicular tissue. We speculate ER gene has the function of up-regulating AR gene or AR gene has the function of down-regulating ER gene. The reason could be due to the amount of androgen secretion which increased AR, whereas competition reduced estrogen secretion which led to the decrease in ER. Therefore, it is speculated that the opposite and synergistic expression pattern is the molecular regulation of normal growth patterns in chicken and quail testis. In this regulatory pattern, the testes of chicken and quails grew and developed normally, while the expression of testis AR mRNA continued to decline in the hybrids with the continuous increase in ER which is completely different from the normal regulatory patterns of cocks and male quails, showing abnormal expression, and resulting in abnormal testicular growth and development in the hybrids from chicken and quail hybridization.

Conclusion

AR and ER genes both participate in the regulation of the testicular tissue growth in chicken, quails and their hybrids. Its regulation has certain time sequence. Since there are changes in the mRNA regulatory patterns of AR and ER genes in hybrid testicular tissue, an extreme type of uncoordinated abnormal expression was shown resulting in slow growth and development of hybrid testis, testicular tissue structural abnormalities and the absence of normal reproductive functions.

ACKNOWLEDGMENT

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ABBREVIATIONS

AR, Androgen receptors; **ER**, estrogen receptors; **RT-PCR**, real-time polymerase chain reaction; **mRNA**, messenger ribonucleic acid; **LH**, luteinizing hormone.

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