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Effects of Caponization on Expression of Gonadotropin-Releasing Hormone-I and Gonadotropin Subunits Genes in Roosters

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We evaluated the effects of caponization on mRNA levels of gonadotropin-releasing hormone-I (*GnRH-I*), gonadotropin subunit and other hypothalamic and hypophyseal peptide genes in male chicken. Thirty roosters (25 d) with similar weight were equally divided into the experimental (capons) and control (sham-operated males) groups randomly. Caponization was performed at 28 days of age and birds were slaughtered at 140 days of age. Caponization resulted in increasing levels of luteinizing hormone β (*LHβ*) and follicle-stimulating hormone β (*FSHβ*) mRNA in the pituitary gland and levels of LH and FSH in serum ($P<0.05$ or $P<0.01$). There were no significant differences in levels of *GnRH-I*, Gonadotropin releasing hormone receptor (*GnRHR*), neuropeptide Y (*NPY*) and Proopiomelanocortin (*POMC*) mRNA between the two groups. Capons exhibited lower levels of follistatin (*FS*), estrogen receptor α (*ERα*) and higher levels of androgen receptor (*AR*) mRNA in the pituitary gland compared with sham-operated males ($P<0.05$). These results suggest that increased LH and FSH concentrations in serum and *LHβ* and *FSHβ* mRNA levels in pituitary after castration are not depended on GnRH synthesis. And changed expression of *ERα, AR* and *FS* genes in the pituitary gland may be important components of regulating gonadotropin in capons.

Key words: Caponization, *FSHβ, GnRH-I*, *LHβ*, rooster

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Introduction

In avian species, reproduction is controlled by the hypothalamo-pituitary-gonadal (HPG) axis. Hypothalamic gonadotropin-releasing hormone (GnRH) is released into portal blood in a pulsatile fashion and transported to receptors on gonadotrope cells in the pituitary gland and differently regulated the biosynthesis and secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Sharp *et al*., 1990). Two forms of GnRHs (cGnRH-I and cGnRH-II) have been identified in avian species (Miyamoto *et al*., 1984). cGnRH-I fibers project to the median eminence (Van Gils *et al*., 1993), and immunization against cGnRH-I results in decreased plasma LH (Sharp *et al*., 1990), suggest that cGnRH-I could regulate the function of gonadotrope in chicken (Maney *et al*., 1997). However, there is a controversy about whether cGnRH-II can regulate gonadotrope (Sharp *et al*., 1990; Guémené *et al*., 1992; Millam *et al*., 1998; Proudman *et al*., 2006).

LH and FSH are consists of a common α-subunit, while have distinct β-subunits that confer their biological activity (Terada *et al*., 1997). Chicken pituitary have a separate population of LH and FSH cells and a different distribution pattern of hormone producing cells than those of mammals (Proudman *et al*., 1999). Differential synthesis and secretion of LH and FSH while in part dependent on the GnRH signal pattern, also appears to result from the actions of local and gonadal peptide hormones. (Burger *et al*., 2004). Sun *et al*. (2012) reported that estrogen receptor α (ERα) and androgen receptor (AR) are different distributions in gonadotrope cells suggest that the regulating mechanisms of estrogen and androgen on the pituitary hormones secretion are different. Local production of activin and follistatin (FS) appear to stimulate and inhibit secretion of FSH, respectively (Winters *et al*., 2001).

In chicken, studies have consistently reported that gonadectomy results in an increase in serum concentrations of LH (Wilson and Sharp, 1976; Zadworny and Etches, 1987; Bruggeman *et al*., 1998) and LHβ mRNA levels in pituitary gland (Petrowskl *et al*., 1993; Terada *et al*., 1997). However, literature about the effects of caponization on expression of *GnRH* and gonadotropin subunit genes is still scarce.

In present study, we wished to investigate the effects of caponization on mRNA levels of *GnRH-I*, gonadotropin subunit, *AR* and *ERα* genes to gain insight into the HPG axis

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in chicken. This study also provided the opportunity to investigate the effect of caponization on the expression of *NPY* and *POMC* genes which have been proposed to modulate the excitability of GnRH neurons (Contijoch *et al*., 1993; Millam *et al*., 2002).

Materials and Methods

Ethical Approval of the Study Protocol

The study protocol was approved by the Animal Care and Use Committee of Anhui Agricultural University (Hefei, China).

Management of Experimental Birds

Thirty roosters (Guang-xi Yellow chickens an indigenous breed in China age, 25 days) with similar body weight were divided randomly into two groups of 15: experimental (capons) and control (sham-operated males). Caponization was performed at 28 days of age. Preoperatively, roosters were not given food or water for 12 h. A 1.0-cm incision was made between the two last ribs, and the bilateral testes were removed. All procedures were undertaken under local anesthesia and iodine-alcohol applied to the incision site. All birds were reared in the same house and raised up to 20 wk of age. Food and water were provided *ad libitum*. At the end of experiment (140 days), blood samples (5 mL) were collected *via* the wing vein. After collection of blood samples, animals were anesthetized. Samples were taken from the hypothalamus and pituitary gland and frozen in liquid nitrogen.

Serum Hormone Determination

Concentrations of LH, FSH, Testosterone (T) and estrogen (E_2) were measured by ELISA with commercial kits provided by Nanjing Jiancheng Bioengineering Co. Ltd. (Nanjing China). As mentioned in manufacturer instructions the quantitative sandwich enzyme immunoassay technique was employed to detecting hormone concentrations. Purified anti-chicken LH, FSH, E_2 and T polyclonal antibody have been pre-coated onto microtiter plate. Standards or samples solution were added to the appropriate microtiter plate wells and incubated at 37℃ for 1 h then washed five times with phosphate-buffered saline containing 0.05% Tween 20 (PBST). After washing, the biotinylated chicken anti-LH, FSH, E_2 and T polyclonal antibodies were added to wells and incubated at 37℃ for 1 h. After washing five times with PBST, the microplate was incubated at 37℃ for 1 h with Avidin-Horseradish Peroxidase (HRP) conjugate. Colour developed after the addition of the substrate 3, 3′, 5, 5′-tetramethylbenzidine (TMB) and the reaction was stopped after 15 mins by adding a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of LH, FSH, E_2 and T in the samples is then determined by comparing the optical density (OD) of the samples to the standard.

Quantitative Real-time Polymerase Chain Reaction (qPCR)

Total RNA of samples from the hypothalamus and pituitary gland was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The purity and concentration of total RNA was determined with an Ultraviolet Spectrophotometer (Beckman Coulter Inc., Brea, CA, USA). Electrophoresis on 1.0% agarose gels was used to verify the integrity of RNA. Then, 0.5 μg total RNA from hypothalamus samples and 0.8 μg of total RNA from pituitary-gland samples in a final volume of 20 μL was used to generate cDNA using an iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) in accordance with manufacturer instructions. The gene-specific primers that we employed are listed in Table 1. qPCR was undertaken with iTaq Universal SYBR Green Supermix (Bio-Rad) using 25-μL reaction mixtures with 0.3 μM of each oligonucleotide primer on a Rotor-Gene 6000 Real-time Cycler (Corbett Research, Cambridge, UK). The PCR program comprised a 10-min activation step at 95℃ followed by 40 cycles of 10 s at 95℃, 15 s at 60℃ and 20 s at 72℃. After cycling, products were melted by increasing the temperature from 72℃ to 95℃. Each sample was run in triplicate, negative (without template) and positive control reactions were carried out for each assay. Relative expression levels of candidate genes were analyzed according to the $2^{-\Delta\Delta Ct}$ method using the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene for normalization (Livak and Schmittgen, 2001).

Statistical Analysis

Data are the mean±SEM. Significance was determined using the Student's *t*-test. $P<0.05$ was considered significant. Analyses were undertaken using SAS v9.1 (SAS Institute, Cary, NC, USA).

Results

The effects of caponization on mRNA levels of *GnRH-I*, *POMC* and *NPY* in the hypothalamus are detailed in Table 2. There was no significant difference ($P>0.05$) in the mRNA levels of *GnRH-I*, *POMC* and *NPY* genes in the hypothalamus between the two groups. Levels of *LHβ*, *AR* and *FSHβ* mRNA were 1.95-fold, 1.29-fold and 1.89-fold higher ($P<0.05$) in capons than in sham-operated males. However, capons displayed significantly lower levels of *FS* and *ERα* mRNA in the pituitary gland ($P<0.05$). There was no significant difference ($P>0.05$) in the mRNA levels of *GnRHR* between the two groups (Table 3). After castration, serum concentrations of LH, FSH and E_2 were increased by approximately 100%, 92.6% and 84.4% ($P<0.05$ or $P<0.01$) and serum concentrations of T were decreased by approximately 120.7% (Table 4).

Discussion

In avian species, the hypothalamic NPY and POMC neuronal elements appeared to contact GnRH perikarya and axons, suggesting that they can modulate the excitability of GnRH neurons (Contijoch *et al*., 1993; Millam *et al*., 2002). Stansfieid and Cunningham. (1987) reported that the endogenous opioid peptides inhibited LHRH secretion in cockerel. NPY have been shown to stimulate GnRH secretion (Contijoch *et al*., 1993). We found *POMC* mRNA content did not affected by castration. We also found no significant differences in *NPY* mRNA levels between the two groups. This result was supported by Kameda *et al.* (2001) who

Table 1. ***qRT-PCR* primers for candidate genes**

Gene	Primer sequence	Product size (bp)	Reference
GnRH-I	F-GCTTGGCTCAACACTGGTCT R-CTGGCTTCTCCTTCGATCAG	202	NM_001080877.1
NPY	F-CCTCATCACCAGGCAGAGAT R-GCTGAAAATCCCATCACCAC	137	NM_205473.1
POMC	F-GAGGAAGATGGAGAAGGGTTG R-TACACCTTGATGGGTCTCCTC	110	NM_001031098.1
FSHβ	F-ACTCACCAGTCTCATCTGTTCA R-CATTGATTGCTTCCATTGTGAC	220	NM_204257.1
LHβ	F-TAACGGTGGCGGTGGAGAA R-CCCAAAGGGCTGCGATACA	109	HQ872606.1
GnRHR	F-GGTCATCGTCTCCTCCTTCA R-GGTGAAGAGCCCATAGGTGA	175	NM_001012609.1
AR	F-ATTTGGTCTTCAACGAGT R-GCCACTGGAATAATACTGA	157	NM_001040090.1
ERα	F-CTGGGCAAAGAGAGTTCCAG R-GATTTCCACCATGCCCTCTA	196	NM_205183.2
FS	F-GACTGTGGACCTGGGAAGAA R-GCTTTGAGAAGGGCACACTC	149	NM_205200.1
GAPDH	F-CATCACACGGACACTTCAGG R-ACAAACATGGGGGCATCAG	244	NM_204305.1

Table 2. **Relative expression of *GnRH-I*, *POMC* and *NPY* genes in the hypothalamus of capons and sham-operated males**

Gene	Capons	Sham-operated males
GnRH-I	0.96±0.17	0.82±0.13
POMC	0.91±0.19	1.19±0.20
NPY	0.97±0.18	1.17±0.14

GnRH-I, gonadotropin-releasing hormone-I; *POMC*, proopiomelanocortin; *NPY*, neuropeptide Y.
Values are the mean±SEM.

Table 3. **Relative expression of mRNA in the pituitary gland of capons and sham-operated males**

Gene	Capons	Sham-operated males
FSHβ	1.04±0.15*	0.55±0.07
LHβ	3.28±0.48*	1.68±0.34
AR	1.42±0.09*	1.10±0.10
GnRHR	1.01±0.11	1.00±0.12
ERα	0.33±0.04	0.75±0.08*
FS	1.59±0.34	3.62±0.68*

FSHβ, follicle-stimulating hormone beta; *LHβ*, luteinizing hormone beta; *ERα*, estradiol receptor alpha; FS, follistatin. *AR*, androgen receptor; *GnRHR*, Gonadotropin releasing hormone receptor
Values are the mean±SEM; * $P \leq 0.05$.

Table 4. **Serum concentrations of LH, FSH, T and E_2 in capons and sham-operated males**

Item	Capons	Sham-operated males
LH (mIU/mL)	4.11±0.53**	2.05±0.27
FSH (mIU/mL)	4.45±0.69*	2.31±0.50
T (pg/ mL)	189.0±23.3	417.3±57.4***
E_2 (pg/ mL)	19.1±2.1**	10.4±1.3

LH, luteinizing hormone; FSH, follicle-stimulating hormone; E_2, estradiol. T, Testosterone.
Values are the mean±SEM; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

demonstrated that the concentrations of *NPY* mRNA in chicken hypothalamus remain unaffected after castration. These findings suggest that testicular testosterone is not involved in POMC and NPY regulating GnRH release in roosters.

Gonadal hormones regulate expression of gonadotropin subunit gene by acting at the hypothalamus to alter GnRH pulsatility. Testosterone reduces gonadotrophin subunit mRNAs by inhibiting GnRH secretion (Dalkin *et al*., 1992); Oestradiol replacement at the time of gonadectomy prevents the rise in LH-*β* mRNA concentrations by altering GnRH secretion (Dalkin *et al*., 1990). The present study shows that the serum levels of LH in capons increased twofold com-

pared with sham birds 112 days after castration, parallelly changed levels of LHβ mRNA in the pituitary. These results are in agreement with Terada *et al*. (1997), who reported that ovariectomy increased plasma concentrations of LH as well as *LHβ* mRNA levels in the pituitary of chicken. Similarly, caponization resulted in increasing serum FSH concentrations and *FSHβ* mRNA levels which is in agreement with Bruggeman *et al*. (1998) who reported that FSH concentrations significantly increased after ovariectomy in Hybro G broiler breeder (Euribrid, Boxmeer). Additionally, serum concentrations of E_2 and T were increased and decreased after castration, respectively. However, the content of *GnRH-I* mRNA was unaltered in castration roosters, which corroborated the results of Wiemann *et al*. (1990). These results suggest that increased in serum LH and FSH concentrations accompany with up-regulating levels of *LHβ* and *FSHβ* mRNA after castration is not depended on GnRH synthesis in the hypothalamus. There are other factors can explain increased LH and FSH secretion after castration. Katt *et al*. (1985) reported that castration increased pituitary sensitivity to GnRH by increasing GnRH receptor number. We further examined *GnRHR* mRNA in pituitary among the two groups and found that *GnRHR* mRNA did not differ between the two groups. Thus, increased pituitary sensitivity to GnRH after castration cannot account for the rise in gonadotropins. However, the present data do not exclude potential roles in conversion of precursor pro-GnRH to the mature bioactive GnRH decapeptide or in rate of degradation of pro-GnRH.

Gonadal hormones also can regulate gonadotropin subunit gene expression by acting directly on the pituitary gonadotropes. It is well-known that estrogens and androgens influence pituitary functions by binding to the corresponding receptors in pituitary. Sun *et al*. (2012) reported that ERα and AR was expressed in LH cells about 68% and 37%, respectively, in pituitary of adult cockerels. They also deduced that ER and AR-positive cells (approximately 64%) are FSH-secreting gonadotrope. The present study showed that levels of *ERα* and *AR* mRNA were decreased and increased, respectively, in capons. We speculated that the increased serum LH and FSH levels parallelly change levels of *LHβ* and *FSHβ* mRNA in pituitary after castration, which could be associated with decreasing *ERα* and increasing *AR* mRNA levels in pituitary gland but the related mechanisms need to be investigated in future study. Additionally, the expression of *FSHβ* gene and secretion of FSH also appeared to be modulated by local production (Activin and FS) (Winters *et al*., 2001). Activin is produced by the gonads and gonadotropes. Activin works through specific activin receptors to stimulate FSH synthesis (Carroll *et al*., 1989; Huang *et al*., 2001) FS is produced by the gonads, gonadotropes, and folliculo-stellate cells. FS inhibits expression of *FSHβ* mRNA by binding to and neutralizing activin (Shimonaka *et al*., 1991). We found that levels of *FS* mRNA decreased in castrated chickens. However, Popovics *et al*. (2011) reported that *FS* mRNA was increased by 57% compared with sham-operated rats and Winters *et al*. (2001) found that *FS* mRNA remain unaffected in castrated adult male rhesus monkeys. The reasons for these discrepancies could be attributed to difference in species. These findings suggest that increased the *FSHβ* mRNA and serum FSH are also associated with the decreasing of *FS* mRNA in pituitary glands of castrated roosters.

Conclusion

The present study suggests that increased LH and FSH in serum and mRNA levels in pituitary after castration are not depended on GnRH synthesis. Our data also suggest that changed expression of *ERα, AR* and *FS* genes in the pituitary gland may be important components of regulating gonadotropin in capons

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