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 hypophysal peptide genes in male chicken. Thirty roosters (25 d) with similar weight were equally divided into the experimental (capon) and control (sham-operated males) groups randomly. 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

Introduction

In avian species, reproduction is controlled by the hypothalamic-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 (Petrowski 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...
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 (capon) 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*. 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 (E₂) 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, E₂ 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°C 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, E₂ and T polyclonal antibodies were added to wells and incubated at 37°C for 1 h. After washing five times with PBST, the microplate was incubated at 37°C for 1 h with Avidin-Horseradish Peroxidase (HRP) conjugate. Colour developed after the addition of the substrate 3,3′,5,5′-tetramethyl benzidine (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, E₂ 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°C followed by 40 cycles of 10 s at 95°C, 15 s at 60°C and 20 s at 72°C. After cycling, products were melted by increasing the temperature from 72°C to 95°C. 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^ΔΔCt 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 E₂ 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). Stansfield 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
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, parallely changed levels of LHβmRNA 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 LHβmRNA levels in the pituitary of chicken. Similarly, caponization resulted in increasing serum FSH concentrations and FSHβmRNA 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 E2 and T were increased and decreased after castration, respectively. However, the content of GnRH-I mRNA was unaltered in caponization 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 possible 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 parallely change levels of LHβ and FSHβmRNA 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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