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Investigation on exogenous testosterone and growth hormone injection on liver and ovary IGF-I gene expression and thyroid hormones of the native breeder hens

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ABSTRACT

The effect of growth hormone (GH) and testosterone injections on ovarian and liver insulin-like growth factor system was studied in Iranian native chickens. Two hundred hens were randomly assigned into four treatments. Each treatment consisted of five replicates. The number of hens in each replicate was 10. The daily ration contained 20% CP and 2800 cal ME/kg body weight (BW). Each hen received a daily allowance of 100 g of the ration and water was offered ad libitum. Treatments were: (1) injection (subcutaneous) of hGH (100 µg/kg BW), (2) injection of testosterone (500 µg/kg BW), (3) injection of hGH (100 µg/kg BW) + testosterone (500 µg/kg BW) and (4) injection of 100 µl distilled water (control group). Injections were made before puberty at 21st week of age before 5% oviposition of flock (flock puberty). The liver and ovary were removed 8 hours after each injection and immediately transferred stored at -80°C until used for determination of the IGF-I relative gene expression (Ovary extract was used for the determination of gene expression). Before each injection and again at 8 hours, blood samples were taken from the wing vein into EDTA-tubes from 15 hens in each experiment. The plasma concentration of estradiol, T3 and T4 were measured with ELISA. The data were analyzed using SAS 9.13 and the Real-time PCR softwares. The level of significance was set at $P < 0.05$. Estradiol concentration significantly decreased in testosterone and GH+testosterone groups. Plasma levels of T3 and T4 significantly increased in GH+testosterone hens. All hormonal injections resulted in a decreased expression of IGF-I gene in the liver. Hormonal treatments resulted in decreased expression of IGF-I in the ovarian extract as compared with the control hens. The expression of IGF-I gene in the liver significantly increased in testosterone-treated birds, compared with the control. The results indicated that injections of growth hormone and testosterone may influence the egg production by modifying the follicular growth and oviposition and so it can mediated via IGF-I gene.

Key words: Growth hormone; Testosterone; Gene expression; IGF-I.

INTRODUCTION

Avian GH is protein with a weight of 22 KD and 191 amino acids. In birds a complex pattern of structural variants in GH has been found. Due to the chicken pituitary GH is mainly Mmonomeric, and Ddimeric and polymeric forms also by gel poly acrylic amayd have been identified [1]. In birds, GH secretion is pulsed, pulsed release, which is a1.5-2 hour. Intra-muscular injection of 100 μg / Kg GH to chicken, blood GH increased after 40 minutes and then decreased exponentially

Hormonal status of hen have important role on egg production and quality. Testosterone and growth hormone (via IGFs system) can be effective in egg production. The ovary of the hen secretes estrogen (estradiol and estrone), progestin (progesterone), and androgen [testosterone (T) and dihydrotestosterone (DHT)] [2].

GH usually, but not always, required for the timing of sexual maturation, since delayed or absent puberty is often associated with GH-deficient or GH-resistant states and GH administration accelerates puberty [3]. GH may play a particularly important role in early, follicle-stimulating hormone (FSH)-independent, since GH-binding activity peaks during early folliculogenesis in porcine follicles [4] and fish ovarian homogenates [5]. Indeed, in vivo and in vitro studies suggest that GH stimulates growth and prevents atresia in small follicles. For instance, GH administration in vivo increases the number of small follicles in cattle [6,7] and horses [8].

In the domestic hen, an increase in plasma progesterone, originating from the mature and maturing preovulatory ovarian follicles induces a preovulatory release of luteinizing hormone (LH) [9], by stimulating the release of gonadotrophin-releasing hormone[10]. The increase in plasma progesterone and LH 3–6 h before ovulation is preceded by increased plasma testosterone and plasma estradiol [11]. Estradiol does not participate directly in the positive feedback control of LH release, but it is necessary to prime the hypothalamus to allow the positive feedback action of progesterone. A role for the preovulatory release of testosterone in the ovulatory process is suggested by the finding that injection of testosterone in laying hens with mature preovulatory follicles induces ovulation [12] and a preovulatory-like release of LH, while blockage of testosterone action by passive immunization or active immunization against testosterone [13] blocks ovulation. Further, active immunization against testosterone induces atresia of preovulatory yellow yolky follicles, but does not prevent their [13], while chronic treatment with the steroidal androgen receptor antagonist, cyproterone acetate, blocks ovulation and induces ovarian regression[14]. While injection of hens with mature preovulatory follicles with progesterone induced ovulation within 8 h, injection with testosterone induced ovulation after more than 9 h, and suggested that testosterone must first be converted to an “active substance” before ovulation could be induced. Croze and Etches (1980) found that ovulation could only be induced using doses of testosterone which produced unphysiologically high plasma concentrations, and suggested that the preovulatory release of testosterone has “a preparatory or priming action on the hypothalamo–pituitary–ovarian system which facilitates the preovulatory release of LH”. Blocking the action of the preovulatory surge of testosterone, with its specific antagonist flutamide (a non-steroidal androgen receptor antagonist; [15]; will halt the predicted oviposition and the preovulatory surges of plasma testosterone, progesterone, estradiol and LH in the laying hen. Rangel, et al. (2006) demonstrated that in the domestic hen acute blockage of testosterone action during the ovulatory cycle, by the inhibition of its specific receptor with flutamide, blocks egg laying and the associated preovulatory surges of progesterone, estradiol and LH. Earlier studies suggested that testosterone must first be converted to an “active substance” before it can induce ovulation [17] or act to prime the

hypothalamo–pituitary–ovarian system to facilitate the preovulatory release of LH [12]. The possibility that testosterone must be first converted to an "active substance" to exert a direct stimulatory effect on LH release is unlikely since all evidence points to progesterone being the principal steroid directly inducing the preovulatory release of LH [18] and progesterone is not a metabolite of testosterone [19]. The possibility that testosterone primes the hypothalamo–pituitary–ovarian system to facilitate the preovulatory release of LH therefore merits closer analysis. A combination of estrogen and progesterone treatment primes the hypothalamo–pituitary system of the ovariectomised hen to make it responsive to the stimulatory action of progesterone on LH release [20]. It has not been established whether testosterone might mimic the priming effect of estrogen. However, it seems unlikely that the preovulatory increase in plasma testosterone is solely responsible for priming the hypothalamo–pituitary system for the stimulatory action of progesterone on LH release since the base-line plasma concentrations of estrogen in the flutamide-treated hens were not depressed and should have been adequate to exert a priming effect on the hypothalamo–pituitary system [16]. It is therefore possible that the preovulatory peak of testosterone may act to prime the ovary to facilitate the preovulatory release of progesterone. The principal ovarian source of progesterone for the preovulatory surge is the granulosa cell layer of the mature preovulatory follicle, with subsidiary contributions from the granulosa layer of the next most mature preovulatory follicle [21]. These granulose cells are targets for testosterone since they contain nuclear androgen receptors [22]. Ovulations and ovipositions ceased in the arrested laying hens, but the entrance of follicles into the follicular hierarchy and hierarchical growth continued, leading to an accumulation of numerous mature follicles in the ovary.

The present study was performed to obtain evidence for the direct action of one injection of testosterone and growth hormone, before puberty, on the egg production and it's characteristics in native breeder hens.

It Found that injections of growth hormone corticosterone synthesis are increased (a direct effect on adrenal) and May increase the concentration of thyroxin. Injection of growth hormone causes receptor down-regulation of hormone. Probably testosterone serge before ovulation the starter for progesterone releasing from ovary. The major source of progesterone for Serge before ovulation is the layer of granulosa cells in mature follicles before ovulation However, with the follicular granulosa layer that will be mature later [16].

In birds, testosterone increases use of peripheral thyroid hormones. Also, Plasma testosterone concentration in brooding hens rises. Decrease in thyroid hormones can reduce egg production, egg weight, shell thickness and ovarian weight. On the other hand, for pituitary somatotroph cell differentiation, TRH released from the hypothalamus, which increase thyroid hormone levels, so, according to that the study of thyroid hormone in reproductive studies is important [23].

MATERIALS AND METHODS

To determine the effects of exogenous injection of testosterone and growth hormone on livere and ovary IGF-I gene expression and plasma concentration of thyroid hormones two hundred native pullets were used. The pullets were randomly assigned into four treatments. Each treatment consisted of five replicates with 10 hens in each replicate. The pullets were maintained under day-light (about 12h), and had free access to water. All hens used in this study were from the same hatch. All birds were fed on same and common diet (contained 20% CP and 2800 cal ME/kg body weight (BW)) and hormones were injected subcutaneously based on body weight (BW). Each hen received a daily allowance of 100 g of the ration and water was offered ad

libitum. Treatments were: (1) subcutaneous injection of hGH (100 µg/kg BW), (2) subcutaneous injection of testosterone (500 µg/kg BW), (3) subcutaneous injection of hGH (100 µg/kg BW) + testosterone (500 µg/kg BW) and (4) subcutaneous injection of 100 µl distilled water (control group). Injections were made before puberty at 21st week of age before 5% oviposition of flock (flock puberty). Before each injection and again at 8 hours, blood samples were taken from the wing vein into EDTA-tubes from 15 hens in each experiment. The plasma concentration of estradiol (Adaltis kit), T3 and T4 **[**24**]** were measured with ELISA. The liver and ovary were removed 8 hours after each injection and immediately transferred stored at -80°C until used for determination of the IGF-I relative gene expression (Ovary extract and liver was dissected for the determination of gene expression). RNA isolation was By RNA purification kits (Qiagen) that 80 to 100 mg of tissue was used. For cDNA synthesis 1µl oligo-dt and 10µl RNA were poured in sterile tube, then incubated it in PCR for 5 min at 70°C. After this, tubes were put on ices and the contents of the tubes were transferred to kit (AccuPower RT PreMix) and then 12µl RNase free water was added. The reaction mixture was incubated at 42 °C for 60 min, followed by heat inactivation of the enzyme (Reverse transcriptase) at 95 °C for 5 min. then, the cDNA was stored at -20 °C to be used for Real-time PCR. Real-time has done by Corbett machine. For each sample 7.5 µl Qiagen SYBR Green Master with 5 µl RNase Free Water poured and 1 µl forwarded primer and 1 µl backward primer were mixed and then cDNA was added. IGF-I sense primer; **5′**-TGGCCTGTGTTTGCTTACCTT-3′ and antisense primer were **5′**-TTCCTTTTGTGCTTTTGGCAT-3′ (M32791, 301bp), respectively. The β-actin sense and antisense primers were; **5′**-GAGAAATTGTGCGTGACATCA-3′ and 5′-CCTGAACCTCTCATTGCCA-**3′** (L08165**,** 152 bp), respectively. The Real-time PCR raw data were analyzed using the comparative threshold cycle method (CT) [25]. The Kolmogorov-Smirnov test was used to determine the normality of distribution, and variables were found to be normally distributed. The data were analyzed using SAS 9.1 [26] and the Real-time PCR softwares. The level of significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Results of this research indicated that injection of both hormone (GH and Testosterone) significantly affected the liver and ovary IGF-I gene expression, plasma concentration of Estradiol, triiodothyronine and thyroxin.the results showed in Table 1 and Figures 1 and 2.

Table 1. Effect of GH and testosterone injection on plasma concentration of Estradiol, triiodothyronine and thyroxin in hen

Parameter	Treatment*				
	Control	GH	Testosterone	GH + Testosterone	SEM**
Estradiol (pg/ml)	600.76^{a}	517.74^{a}	239.62^{b}	344.66^{b}	21.71
Tri-iodothyronine (ng/dl)	3.55^{bc}	3.41^{c}	4.593^{ab}	4.720^{a}	0.19
Thyroxin (ng/dl)	33.80^{b}	28.30^{b}	30.200^{b}	52.663^{a}	1.05
Tri-iodothyronine/thyroxin	0.084	0.093	0.092	0.083	0.005

**100 µg/kg BW, hGH, 500 µg/kg BW, testosterone and100 µl distilled water (Control) was used.*
***Standard error of mean*

General effects of steroid hormones on the growth and the structure of oviduct have been well studied. Large increase in oviduct weight after using of gonadal steroid hormones, it has been proven. Also, Synergistic effects of testosterone and estrogen on oviduct growth has also been reported. Estrogen increases the synthesis of proteins, but the excretory potential of oviduct requires the cooperation of progesterone, testosterone together and with estrogen.

Estradiol concentrations in testosterone and growth hormone+testosterone treatments were significantly decreased ($p<0.05$). Probably puberty age and this age not appropriate for injection these hormones. And injection of testosterone and growth hormone in puberty will reduce the sensitivity of receptors of these hormones; Testosterone during puberty can also have negative effects on estradiol production.

Figure 1. Exogenous testosterone and growth hormone injection on liver IGF-I gene expression

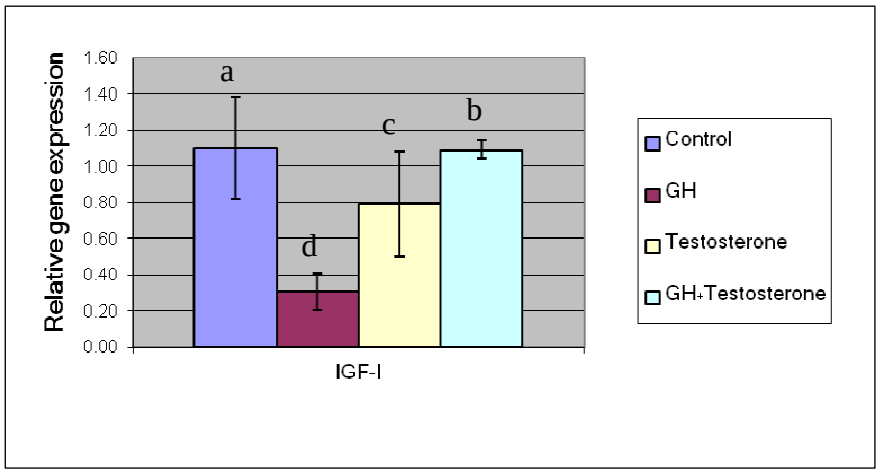
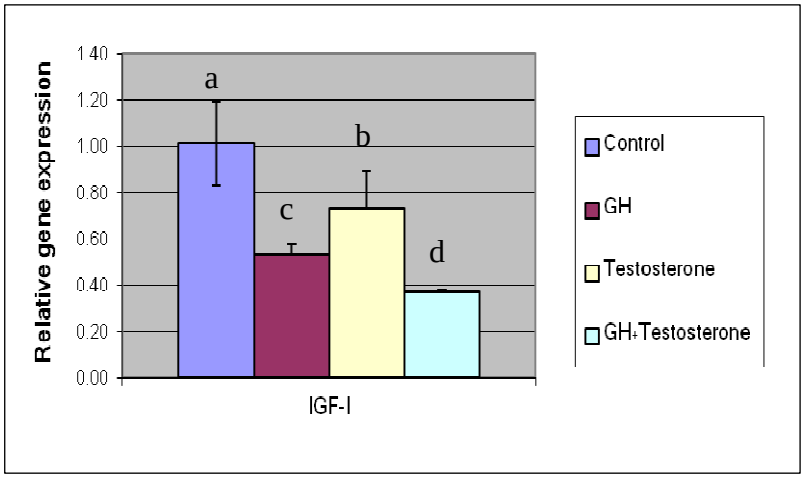


Figure 2. Exogenous testosterone and growth hormone injection on ovary IGF-I gene expression



T3 and T4 concentrations in the treatment of growth hormone + testosterone was significantly increased ($p<0.05$). But no change in the ratio of T4 to T3 was found ($p>0.05$).

Since TRH causes differentiation of pituitary somatotroph cells and growth hormone secretion and growth hormone increases the secretion of TRH [23]. So may the injection of growth hormone during laying, increases the concentration of thyroid hormones. High concentrations of the thyroxin in all treatments may be due to hen blood collection at night at this time the concentration can reaches its maximum value [27]. GH can reduce the activity of the deiodinase 3 in the liver, Hence conversion of T4 to T3 reduced and increasing the T4 Concentration in the blood. On the other hand, GH can increase the activity of the deiodinase 1 activity that increase T4 to T3 conversion and increasing the T3 Concentration in the blood [28, 29, 30, 31]. Treatment that used in this research may be had the same effect on thyroid hormone conversion cycle and

the pattern of changes in a manner identical and same for T4 and T3 and T3 to T4 ratio is not changed.

IGF-I gene expression in the liver in all treatments showed a decrease compared to controls, and because the effects of GH in young chickens are very different and varies. And it is possible that it's effect at the puberty age (and in the young chicken) as well as older hens not same, Thus, the reduced expression of genes can be high concentrations of IGFs. In fact GH injections can be reduced in the IGF. In ovary, IGF-I gene expression was reduced in all treatments compared to controls. growth hormone injections can reduce IGF-I concentration and consistency of its impact on IGF by reducing of gene expression as seen in this study . IGF-I gene expression in the ovary of birds by Roberts and colleagues (1994) have been reported [32]. Using RT-PCR, it was shown that the IGF-I mRNA is expressed in chicken granulosa cells.

Armstrong and Hogg (1996) genes expression of IGF-I and IGF-II in theca and granulosa cells also proved [33]. It has been reported that IGF-1 and IGF-II production in middle size follicle more than large follicles (F1 and F2) and at all IGF-II production in growing yellow follicle more than IGF-1 [34, 35, 36].

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