MOLECULAR AND CELLULAR BIOLOGY

Manipulation of broiler chickens sex differentiation by *in ovo* injection of aromatase inhibitors, and garlic and tomato extracts

Nahid Fazli,[*] Ahmad Hassanabadi,[†,1] Majid Mottaghitalab,[‡] and Hosna Hajati[†]

[*]*Department of Animal Science, University of Zanjan, Zanjan, Iran;* [†]*Department of Animal Science, Ferdowsi University of Mashhad, PO Box 91775–1163, Mashhad, Iran; and* [‡]*Department of Animal Science, Faculty of Agricultural Science, University of Guilan, PO Box 41635-1314, Rasht, Iran*

ABSTRACT The influence of *in ovo* administration of aromatase inhibitors, clomiphen citrate, tomoxifen, and garlic and tomato extracts on sex differentiation in broiler chickens were investigated in 2 experiments. Five hundred, and 1,000 fertile eggs from Ross 308 strain were used in experiments 1 and 2, respectively. In both experiments, eggs were divided into 5 groups: control group (DW, 0.1 mL/egg), tomoxifen (0.05 mg/egg), clomiphene citrate (0.05 mg/egg), garlic and tomato extracts (0.1 mL/egg). Eggs were sanitized and prepared for incubation in a regular automatic hatchery. Experimental preparations were injected into eggs at day 5 of the incubation period. Injection sites on the eggs were cleaned with 70% ethylic alcohol, bored by a needle, and aromatase inhibitors were injected into the white from the thin end of the eggs by insulin syringe and then sealed by melted paraffin. In experiment 1, hatched one-day-old chicks (mixed-sex) were raised till 42 days of age in 25 floor pens with a completely randomized design. Experiment 2 was designed to investigate the effects of sex and treatments on the feed-to-gain ratio of broiler chicks. In experiment 2, hatched one-day-old chicks were feather sexed and raised till 42 days of age in 50 floor pens. A completely randomized design with a 2 × 5 factorial arrangement of treatments (sex×treatment) was used. Gonads of the chicks were checked to determine their sex on day 42 by optic microscope to make sure feather sexing was correct. At the end of both experiments, on day 42, one bird from each pen was slaughtered for carcass analysis. In experiment 1, hatchability and the one-day-old weight of chicks showed no significant differences among treatments ($P > 0.05$). However, *in ovo* administration of garlic and tomato extracts caused the highest percentage of male chicks ($P < 0.05$). Also, the percentage of thighs and wings of the males were significantly higher than those of females ($P < 0.05$). In experiment 2, feed-to-gain ratio of male and female broiler chicks showed no significant differences among treatments ($P > 0.05$).

Key words: *in ovo* injection, aromatase inhibitors, broiler chickens

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INTRODUCTION

In avian species, genetically females are heterogametic (sex chromosome ZW), whereas the homogametic sex (sex chromosome ZZ) are genetic males (Matsushita et al., 2006). The W chromosome positively controls early aromatase synthesis and, consequently, estrogen production (Shimada, 1998; 2002). Estrogens and their receptors are essential for female sexual differentiation. Chicken embryonic gonads are bipotential at an early stage. During development of the female, the left gonad differentiates to a single ovary or oviduct, and the right gonad regresses, developing a permanent female phenotype. This sexual differentiation occurs as a result of aromatase expression in the left gonad at d 6.5 and the production of estrogen from testosterone (Yoshida et al., 1996; Shimada, 1998). In the male genotype, both gonads develop into 2 testes (Shimada, 1998). The time- and sex-dependent expression of enzymes involved in steroid production, which determine the ratio of androgens:estrogens produced by the gonads, has been extensively investigated in recent years (Clinton, 1998; Nishikimi et al., 2000). Although details of the mechanism of sexual differentiation in birds remains unclear, downstream gene expression has been elucidated (Shimada, 2002; Shimada et al., 2007; Smith et al., 2007). Namely, in females, P450 aromatase may be involved because of its exclusive mRNA expression in the female gonad in association with the beginning of ovary formation at around 6 to 7 days of incubation, but no expression is observed in the male

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[1]Corresponding author: hassanabadi@um.ac.ir

gonad of the chickens (Nomura et al., 1999; Nishikimi et al., 2000; Vaillant et al., 2001; Akazome et al., 2002; Yamamoto et al., 2003). In contrast, in males anti-mullerian hormone may be involved because of its higher mRNA expression in the male gonad in association with the beginning of testis formation, but has lower expression in female gonad occurs (Carre-Eusebe et al., 1996; Oreal et al., 2002; Nishikimi et al., 2000).

Experimental sex reversal has been performed using antiestrogens, androgens, aromatase inhibitors, and synthetic steroids (Elbrecht and Smith, 1992; Abinawanto et al., 1998; Shimada, 1998). Differences between male and female gonadal differentiation and development depend on the absence of aromatase and estrogen, whereas an estrogen receptor is present in the gonads of males before sexual differentiation (Smith et al., 1997).

Recently, studies have been conducted on various antiestrogen compounds used for the control of physiological processes. Clomiphen and tamoxifen selective estrogens receptor modulators have been used most frequently in poultry. Reports have been published on the effects of these preparations on birds at various ages, for example during the first days of embryo development (Rozenboim et al., 1990) or even at 42 to 90 days of age (Rosenstrauch et al., 1986). The application of clomiphene and tamoxifen at various frequencies have been examined, e.g., from every second or third day (Zeman et al., 1989) until eight weeks (Rosenstrauch et al., 1986); various administration techniques were also studied: *in ovo* (Rozenboim et al., 1990), *per os* in gelatine capsules (Bednarczyk and Dobalova, 1990), intra muscular injection (Leitner et al., 1996) as well as the use of different preparation doses, e.g., 0.5; 1.0; 5.0; 10.0 or even 25 mg/kg body weight.

Lycopene, an acyclic unsaturated carotenoid, has received much attention in recent years because of its beneficial effects and its important antioxidant properties to suppress free radicals. Lycopene can be extracted from the sources it occurs naturally. Besides lycopene and other carotenoids, the presence of certain fatty acids in tomato, e.g., palmitic (16:0), stearic (18:0), and oleic (18:1n-9) acids, have also been described previously (Takasova et al., 1995).

The present study was conducted to investigate the effect of clomiphene citrate, tamoxifen, and garlic and tomato extracts on sex reversal and their effects on gonad growth, differentiation and performance characteristics of the broilers during the period of 1 to 42 days of age.

MATERIALS AND METHODS

Animals

Experiments were conducted according to the protocol approved by University of Zanjan Animal Care and Use Committee, Iran. Fertile eggs from Ross 308 strain broiler breeder hens were obtained from a commercial farm.

Table 1. Ingredients and nutrient composition of experimental diets.

Ingredient (%)	1–21 d	22–42 d
Corn, ground	57.25	60
Soybean meal	33	30.4
Fish meal, Anchovy	6	4
Soybean oil	–	2
Dicalcium phosphate	1.2	1.1
Oyster shell	1.4	1.4
Common salt	0.3	0.3
Minerals mix[1]	0.3	0.3
Vitamins mix[2]	0.3	0.3
DL-Methionine	0.1	0.1
L-Lysine hydrochloride	0.15	0.1
Calculated composition		
ME (kcalkg)	2800	2944
CP (%)	20.9	18.99
Ca (%)	1.25	1.09
NPP (%)	0.54	0.45
Lysine (%)	1.47	1.26
Methionine	0.51	0.46
Methionine+Cystine	0.85	0.76
Arginine	1.55	1.4

[1]Mineral mix supplied the following per kg of diet: Cu, 20 mg; Fe, 100 mg; Mn, 100 mg; Se, 0.4; Zn, 169.4 mg.

[2]Vitamins mix supplied the following per kg of diet: vitamin A, 18,000 IU; vitamin D3, 4,000 IU; vitamin E, 36 mg; vitamin K_3, 4 mg; vitamin B_{12}, 0.03 mg; thiamine, 1.8 mg; riboflavin, 13.2 mg; pyridoxine, 6 mg; niacin, 60 mg; calcium pantothenate, 20 mg; folic acid, 2 mg; biotin, 0.2 mg; choline chloride, 500 mg.

Experimental Diets

The experimental diets were formulated according to NRC (1994) recommendations. The ingredient content and composition of the diets is shown in Table 1.

Garlic Material

Steam distillation method was used to obtain garlic extract. Briefly, mature garlic bulbs (*ALLIUM SATIVUM* L.) were purchased from a local farmer. These were then cleared of any adhering dried material. The garlic bulbs were peeled, washed, dried over paper towels, and finely chopped by a grinder. Then, 200 g of garlic sample was mixed with 200 mL double distilled water and placed in a 1,000 mL distillation flask connected to the steam distillation apparatus. The steam distillation was continued for 3 h at 100°C (Sun-Neo et al., 2003). Finally, the collected aqueous garlic extract was injected into the eggs at the level of 0.1 ml per egg.

Tomato Material

Suprex (Pittsburgh, PA) MPS/225 integrated SFE-SFC system was used for tomato extraction. The 25 g sample of crushed fresh tomato was placed into the extraction vessel and a stream of carbon dioxide was passed through while the pressure and the temperature were maintained at 32 MPa and 50°C, respectively; as

described previously (Gomez-Prieto et al., 2002). The decrease in solvating power resulting from lowering the pressure (to 15 MPa) and increasing the temperature up to 80°C in a separation vessel (and consequently, from lowering the carbon dioxide density) allowed the most insoluble and less volatile compounds to be collected as a deep red solid. The extract was recovered by washing the vessel with 5 mL dichloromethane. Extraction was achieved 10 times, total extracted material was diluted with 10 mL distilled water and the extract was injected into the dedicated eggs at the level of 0.1 mL per egg.

In Ovo Injection

Five hundred eggs were divided into 5 groups: DW (0.1 mL/egg), clomiphene citrate (0.05 mg/egg), tamoxifen (0.05 mg/egg), and garlic and tomato extracts (0.1 mL/egg,). The materials were prepared and injected into the eggs white from the thin end of the eggs on d 5 using a 1 mL syringe with a 23-gauge needle. Injection sites on the eggs were cleaned with 70% ethylic alcohol, and then sealed by melted Merck paraffin. In the control group, 0.1 mL/egg of distilled water was injected into the eggs in a similar manner.

The eggs were incubated at 37.5°C in a RH of 56% in a commercial hatchery. The eggs were automatically turned once per hour. At day 10, the eggs were checked by candling, and the eggs without embryo development were discarded. In experiment 1, hatched one-day-old chicks were raised till 42 days of age (mixed-sex) in 25 floor pens in a completely randomized design. There were 5 treatments, 5 replicates, and 12 chicks per replicate. Experiment 2 was designed to investigate the effects of sex and treatments on the feed-to-gain ratio of broiler chicks. In this experiment, hatched one-day-old chicks were feather sexed and raised till 42 days of age in 50 floor pens in a 2 × 5 factorial arrangement (sex×treatment) with a completely randomized design. There were 10 treatments, 5 replicates, and 10 chicks per replicate. Treatments in both experiments, included *in ovo* injection of distilled water (0.1 mL/egg), tomoxifen, clomiphene citrate (0.05 mg/egg), and garlic and tomato extracts (0.1 mL/egg). Gonads of chicks were checked to determine their sex on day 42 by optic microscope to make sure feather sexing was correct. At the end of both experiments, on day 42, one bird from each pen was slaughtered for carcass analysis.

Statistical Analysis

All percentage data were subjected to arc sine transformation prior to analysis. While conclusions were drawn from the transformed data, only untransformed data are presented for relevance. Statistical analysis was conducted using the GLM procedure of SAS software. Data of the experiment 1 were statistically analyzed using a completely randomized design (SAS Institute, 2002). In experiment 2, data were analyzed in a completely randomized design with a 2 × 5 factorial arrangement (SAS Institute, 2002). This model included 2 sexes (male, female) and 5 experimental treatments. Means were compared using Duncan's new multiple range test (Duncan, 1955). The level of significance was reported at $P < 0.05$.

Table 2. Effect of garlic and tomato extracts, tomoxifen, and clomiphen citrate on hatchability, male ratio, and one-day-old weight of broiler chickens.

Treatment	Hatchability (%)	Male (%)	1-old weight (g)
DW(control)	77	53.98[b]	35.8
GAR	68	80.43[a]	37
TOM	72	70.97[a]	35.6
TMX	77	61.34[b]	36.1
CLC	67	57.62[b]	34.3
Pr>F	0.1201	0.0145	0.1076

Means within columns with different superscripts differ significantly ($P < 0.05$).

DW, distilled water; GAR, garlic extract; TOM, tomato extract; TMX, tomoxifen; CLC, clomiphene citrate.

RESULTS

Experiment 1

Effects of In Ovo Exposure to Aromatase Inhibitors, Garlic and Tomato Extracts on Hatchability, Male: Female Ratio and Day-Old Weight of Broiler Chickens. *In ovo* injection of the experimental preparations did not affect the hatchability of the eggs ($P > 0.05$). Garlic and tomato extract caused a higher percentage of male chicks ($P < 0.05$). There were no significant differences in the one-day-old weight of the chicks (Table 2).

Effects of In Ovo Exposure to Aromatase Inhibitors, Garlic and Tomato Extracts on Performance and Carcass Characteristics of Broiler Chickens. There were no significant differences among the bird's body weight, weight gain, feed intake, and feed-to-gain ratio (Table 3). Also, there were no significant differences among the relative weight of the carcass organs of the birds. However, thigh and wing weight

Table 3. Effect of garlic and tomato extracts, tomoxifen, and clomiphen citrate on performance of chickens from 1to 42 days of age.

Treatment	BW, 42 d (g)	ADWG (g)	FI (gbd)	FCR (gg)
DW(control)	2346	55	110	2.01
GAR	2263	53	107	2.02
TOM	2388	56	111	1.99
TMX	2220	52	104	2.01
CLC	2344	55	110	2
Pr>F	0.609	0.347	0.216	0.0703

BW, body weight; ADWG, average daily weight gain; FI, feed intake; FCR, feed to gain ratio.

DW, distilled water; GAR, garlic extract; TOM, tomato extract; TMX, tomoxifen; CLC, clomiphene citrate.

Table 4. Effects of garlic and tomato extracts, tomoxifen, and clomiphen citrate on relative carcass characteristics (%) of broiler chickens at 42 days of age.

Carcass characteristics	DW	CLC	TMX	GAR	TOM	Pr>F	male	female	Pr>F
Thighs	0.33	0.32	0.36	0.33	0.35	0.1138	0.36	0.32	0.0018
Breast	0.31	0.3	0.33	0.3	0.33	0.3254	0.32	0.31	0.5493
Wings	0.12	0.1	0.11	0.1	0.11	0.3519	0.11	0.1	0.0101
Back & neck	0.26	0.26	0.28	0.24	0.27	0.0663	0.27	0.26	0.1837
Abdominal fad pad	0.03	0.03	0.03	0.03	0.04	0.4209	0.03	0.03	0.1596
Liver	0.04	0.04	0.04	0.04	0.04	0.961	0.04	0.04	0.149
Gizzard & proventriculus	0.06	0.06	0.07	0.06	0.07	0.1328	0.06	0.06	0.2337

DW, distilled water; CLC, clomiphene citrate; TMX, tomoxifen; GAR, garlic extract; TOM, tomato extract.

Table 5. Effects of garlic extract and tomato extracts, tomoxifen, and clomiphen citrate on feed to gain ratio of males and females during 2 rearing periods.

Period of rearing	DW	CLC	TMX	GAR	TOM	Pr>F	male	female	Pr>F
Starter (1–21 d)	1.857	1.923	1.803	1.791	1.874	0.857	1.848	1.842	0.9592
Grower (22–42 d)	2.103	2.075	2.074	1.975	1.941	0.263	1.964	2.115	0.189

DW, distilled water; CLC, clomiphene citrate; TMX, tomoxifen; GAR, garlic extract; TOM, tomato extract.

percentages of the males were significantly higher than those of the females (Table 4).

Experiment 2

Effects of In Ovo Exposure to Aromatase Inhibitors, Garlic and Tomato Extracts on Feed to Gain Ratio between Male and Female Birds of Broiler Chickens. Results of current experiment showed that the feed-to-gain ratio of male and female birds were not affected by treatments (Table 5).

DISCUSSION

It is known that the physiological functions of the reproductive organs of male chickens (testes activity and semen production) are affected by many factors: age, photoperiod, season, nutrition, diurnal rhythm, management system (cage vs. floor), inheritance, health status (diseases), individual variability, and other factors (Akang et al., 2010; Edens, 2011; Lisowski and Bednarczyk, 2005). Gonadal steroid hormones are critical for sexual differentiation of endocrine and behavioral components of reproduction. In birds, estradiol appears to be central in the sexual differentiation of females in a number of species (Ottinger and Abdelnabi, 1997). Steroid treatment of quail during the embryonic period differentiates the left gonad of the females to a single ovary or oviduct and the right gonad moves backward. This sexual differentiation occurs as a result of aromatase expression and production of estrogen from testosterone in the left gonad at d 6.5 (Yoshida et al., 1996; Shimada, 1998). However, in both female and male right gonads, the cortex is not noticeable by d 6 of incubation. The cortex in the right gonad is not developed because of the intrinsic absence of estrogen receptor gene expression. In contrast, the gene transcript for aromatase is present in the regressing right gonad (Nakabayashi et al., 1998). Thus, aromatase is an important factor for regression of the medulla of the right gonad in birds. Aromatase is the enzyme responsible for the conversion of testosterone to estradiol. Embryonic exposure to aromatase inhibitors, such as tamoxifen and fadrozole, has been shown to defeminize the ovary and accessory structures (Ottinger and vom Saal, 2002). In chickens, females who developed bilateral testes were capable of complete spermatogenesis (Elbrecht and Smith, 1992). These females not only had the physical appearance of males, but exhibited the behavior of males as well. In this experiment, growth and breast muscle development of chickens were not affected by *in ovo* injection of the preparations, which is in agreement with the results of Burke and Henry, 1999; Rickes et al., 1992. Under the conditions of this study, it was concluded that *in ovo* injection of aromatase inhibitors is a way of manipulating chicken sex differentiation.

In the present study, *in ovo* injection of the experimental materials did not affect the hatchability of the eggs ($P > 0.05$). However, garlic extract, tomato extract, tomoxifen, and clomiphene citrate lowered the hatchability numerically compared to the control group. *In ovo* injection of anti-aromatase materials may thus reduce the hatchability of the eggs. Garlic and tomato extracts caused a higher percent of male chicks in comparison to other groups ($P < 0.05$). Thus, garlic and tomato extracts may have positive effects on the hatchability of the eggs. However, further investigation is needed to explore whether these findings apply to poultry hatcheries. There were no significant differences in the one-day-old weight of the chicks. This is in agreement with earlier studies (Burke and Henry, 1999). *In ovo* injection of an aromatase inhibitor, Fadrazole®, did not affect the birds' body weight (Burke and Henry, 1999).

In the present study, there were no significant differences among bird's body weight, weight gain, feed intake, and feed-to-gain ratio. Also, there were no significant differences among carcass relative parts and organs of the broiler chickens. However, thigh and wing weight percentages of the males were significantly higher than those of the females.

Growth and pectoral muscle development of chickens was not affected by *in ovo* injection of an aromatase inhibitor, Fadrazole®, in agreement with previous studies (Rickes et al., 1992; Burke and Henry, 1999). In contrast to our results, Dewil et al. (1998) reported that *in ovo* injection of Vorazole, another azole type aromatase inhibitor, affected body weight in the early post hatching period, but not at 5 wk of age when the experiment was terminated. Percentage abdominal fat pad was reduced by the highest level of the drug in that study. The present study showed that feed-to-gain ratio of male and female birds were not affected by treatments. These contradictory results may be explained by a variety of factors such as drug dosage, time of injection, strain of chickens, or differences in activity of the drugs used.

In conclusion, garlic and tomato extracts showed aromatase inhibitor effect in broiler embryos and they may be introduced to hatcheries as natural materials with anti-aromatase effects to increase the male-to-female ratio.

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