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Effect of Dietary Daidzein on Egg Production, Shell Quality, and Gene Expression of ER-α, GH-R, and IGF-IR in Shell Glands of Laying Hens

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Our previous studies demonstrated that dietary daidzein improves egg production in ducks during the late period of the laying cycle. The present study was aimed to investigate the effect of daidzein in laying hens, with more focus on eggshell quality. The expression of ER-α, GH-R, and IGF-IR mRNA in shell glands was determined to identify the target genes of daidzein action and to reveal the relationship between shell quality and profiles of gene expression in shell glands of laying hens. 1000 ISA hens, at 445 days of age, were allotted at random to two groups and given the basal diet with or without 10 mg of daidzein per kg diet for 9 weeks. Daidzein supplement significantly increased the egg laying rate and the feed conversion ratio. The eggshell thickness increased, while the percentage of cracked eggs decreased in daidzein-treated hens. Serum E_2 and phosphate concentrations were not altered, but the level of serum Ca^{2+} and the tibia bone mineral density were significantly increased in the daidzein-treated group compared with their control counterparts. In parallel with the significant increase of oviduct weight, significant down-regulation of GH-R and IGF-IR mRNA and a trend of decrease in ERα mRNA expression in shell glands were observed in daidzein-treated hens. The results indicate that dietary daidzein improves egg laying performance and eggshell quality during the late (postpeak) laying stage of hens, which is associated with modulations in gene expression in the shell gland.

KEYWORDS: Daidzein; egg laying performance; shell gland; gene expression; ISA hens

INTRODUCTION

Egg quality, especially the eggshell strength, has received great attention in recent years due to the introduction of the free-range system for laying hens (*1*). Eggshell is deposited in the shell gland of the avian oviduct, which is equivalent to the uterus of mammals. It has been well documented that the chicken oviduct demonstrates a massive amount of cell proliferation in response to estrogen. However, as most of the research on estrogen in the oviduct was done in the 1970s and 1980s, the molecular mechanisms and the target genes underlying this proliferative response remain largely undefined (*2*, *3*).

It is well documented that the GH/IGF-I system is involved in the regulation of oviduct growth (*3*). The increased shell thickness of eggs laid by GH-treated hens suggests that the avian oviduct, particularly the shell gland, is a site of GH action (*4*). This possibility is supported by the presence of GH-Rs and GH-R mRNA in these tissues (*5*). Apart from its direct action via its own receptor, GH may act indirectly through the mediation of hepatic or local IGF production, as well as the IGF receptors in the reproductive tract (*3*, *6*). Futhermore, the IGF-I receptor signaling cascade is found to be activated by estrogen exclusively via estrogen receptor α (ERα), suggesting a possible interaction between estrogen and the GH/IGF-1 system in the regulation of oviduct function (*7*). However, no data is available regarding the changes of gene expression for ERα and GH/IGF-I receptors in the oviduct of birds, especially in shell glands, induced by an estrogenic substance.

It is generally conceived that the eggshell thinning among wild birds is originated from exposure of the laying adult female to estrogenic environmental contaminants (*8*, *9*). A variety of phytoestrogens have been shown to exert biological functions by binding to estrogen receptors (ERα and ER*β*) (*10*), and a recent publication suggested that phytoestrogens (genistein, daidzein, coumestrol) are able to reduce the adverse effect of polychlorinated biphenyls (PCBs) on both ovarian and uterine functions (*11*).

Daidzein is one of the phytoestrogens existing widely in natural plants, particularly in soybeans and other legumes (*12*).

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Table 1. Nucleotide Sequences of Specific Primers and PCR Conditions

target genes[a]	reference/GenBank accession no.	PCR products	primer sequences	PCR conditions
cER-α	X03805	418 (1339–1756)	F: 5′-GATATTGATGATCGGCTTAG-3′ R: 5′-GTGCTCCATTCCTTTGTT-3′	94 °C for 30 s, 50 °C for 30 s, 72 °C for 60 s 32 cycles
cGHR	AB075215	346 bp (283–628)	F: 5′-TTACTTCAACACATCCTACACC-3′ R: 5′-TCATAATCTCTTCCCATCTTCA-3′	94 °C for 30 s, 50 °C for 30 s, 72 °C for 60 s 32 cycles
cIGF-IR	BQ037565	397 bp (3085–3481)	F: 5′-GTACTTCAGTGCTTCGGATGTG-3′ R: 5′-CTTCTTCAGAGTTGGAGGTGCT-3′	94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s 31 cycles
cβ-actin	L08165	282 (721–1002)	F: 5′-ACGTCGCACTGGATTTCGAG-3′ R: 5′-TGTCAGCAATGCCAGGGTAC-3′	

[a] c = chicken.

The molecular structure of daidzein is similar to endogenous estrogen, and the bioactivity is about 10^{-3}−10^{-5} times as much as that of 17β-estradiol (*9*). Some observations indicate that phytoestrogens including daidzein may act as estrogen agonists or antagonists depending on dose, duration of use, individual metabolism, and intrinsic estrogenic state (*13*, *14*).

Dietary supplement of daidzein significantly improved the laying performances of Shaoxing duck breeders during the postpeak but not the prepeak laying stage (*15*). Nevertheless, the effect of dietary daidzein on the laying performance of chickens has not been elucidated and the mechanism is still unclear. Therefore, the present study was aimed to investigate the effect of dietary daidzein on egg production in laying hens, with more focus on eggshell quality. The expression of ER-α, GH-R, and IGF-IR mRNA in shell glands was determined to identify the target genes of daidzein action and to reveal the relationship between egg shell quality and profiles of gene expression in shell glands of laying hens.

MATERIALS AND METHODS

Animals. 1000 ISA laying hens during the postpeak period of egg laying (445 days of age, 60%−70% egg laying rate) were selected and randomly allocated to control and daidzein-treated groups, fed with basal diet and the daidzein supplemented diet at the level of 10 mg per kg diet, respectively, for a period of 9 weeks. Hens were kept in a commercial layer farm following the feeding and housing standards, being provided 120 g of feed per day and allowed free access to water. Daily egg laying rate, cracked egg rate, mean egg weight, and feed consumption were recorded. 100 eggs from each group were randomly selected before the termination of the experiment for determining the eggshell quality. At the end of the experiment, 20 hens from each group were sacrificed for blood and tissue sampling. The sera were stored at −20 °C until hormone assay. Tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until RNA isolation.

Diet Composition. The basal diet was formulated according to the nutritional requirements of the flock and contains 11.55 MJ/kg metabolic energy, 16.5% crude protein, 3.63% calcium, 0.40% phosphorus, 0.35% methionine, and 0.95% lysine.

Materials. Daidzein is a synthetic product of Zhang Jia Kou Xuanhua Chemical Plant with a purity of 99.9% and was added on top of the basal diet. The avian myeloblastosis virus (AMV) reverse transcriptase, Taq DNA polymerase, and other related reagents were products of Promega, USA. Agarose was a product of Roche Applied Science, Switzerland (catalog no. 1441).

Cracked Eggs, Shell Thickness, and Bone Density Analysis. Cracked eggs, including soft shell and cracked shell eggs, were recorded weekly. Shell thickness was the mean number of the thickness of the sharp end, the middle part, and the blunt end measured with a Vernier Caliper. Tibia bone mineral density was detected with the BMD equipment (BMD400, Beijing, China).

Radioimmunoassay for Serum Hormone Levels. The serum E_2 was measured with radioimmunoassay (RIA) using commercial kits purchased from Shanghai Institute of Biological Products. The detection range of the assay was between 5 and 4000 pg/mL. The inter- and intra-assay coefficients of variation were 10% and 15%, respectively.

RNA Extraction. Total RNA was extracted from the tissue samples by the acid guanidinium thiocyanate−phenol−chloroform method (*16*), and the RNA concentration was then quantified by measuring the absorbance at 260 nm in a photometer (Eppendorf Biophotometer). Ratios of absorption (260/280 nm) of all preparations were between 1.8 and 2.0. Aliquots of RNA samples were subjected to electrophoresis with 1.4% agarose−formaldehyde gels stained with ethidium bromide to verify their integrity.

Reverse Transcription (RT) and Polymerase Chain Reaction (PCR). The reverse transcription reaction for first-strand cDNA synthesis included 1×RT buffer, 0.5 mmol/L dNTPs, 20U RNase inhibitor, 10U AMV reverse transcriptase, 2.5 μmol/L oligo (dT_{15}) primer, and 2 μg of total RNA per 20 μL of reaction volume. RNA samples were denatured at 80 °C for 5 min and placed on ice for 5 min together with oligo (dT_{15}) primer and dNTP before reverse transcription (RT). Tubes were incubated for 1 h at 42 °C and at 5 min at 95 °C and then chilled to 4 °C. 2 μL of the RT reaction mix was used for PCR in a final volume of 50 μL containing 1 U Taq DNA polymerase, 5 mmol/L Tris-HCl (pH 9.0), 10 mmol/L NaCl, 0.1 mmol/L DDT, 0.01 mmol/L EDTA, 5% (w/v) glycerol, 0.1% (w/v) Triton X-100, 0.2 mmol/L each dNTP, 1.0−2.0 mmol/L $MgCl_2$, and 0.5 μmol/L specific primers for respective target genes. The PCR primers for ERα, GH-R, IGF-IR, and β-actin were designed using Primer Premier 5.0 and were synthesized by Takara Biocompany (Haojia, China). The nucleotide sequences of these primers and the PCR conditions set for respective genes are shown in **Table 1**. β-Actin was coamplified with each target gene in the same reaction as an internal control. The pooled samples made by mixing equal quantities of total RNA from all samples were used for optimizing the PCR condition and normalizing the intra-assay variations. The linear amplifications were ensured by optimizing the cycles for each gene. Different controls were set to monitor the possible contaminations of genomic and environment DNA both at the stage of RT and PCR. All samples were included in the same run of RT-PCR and repeated at least 3 times. Both RT and PCR were performed in a Gene Amp PCR System 9600 (Perkin-Elmer, U.S.A.).

Quantitation of PCR Products and Statistical Analysis. An aliquot (10−20 μL) of PCR products was analyzed by electrophoresis on 2% agarose gels. The gel was stained with ethidium bromide and photographed with a digital camera. The net intensities of individual bands were measured using Kodak Digital Science 1D software (Eastman Kodak Company, Rochester, NY).

The ratios of net intensity of target genes to β-actin were used to represent the relative level of target gene expression. The average level of three repeats was used for statistical analysis. The results were expressed as mean ± SD or mean ± SEM (for gene expressions), and differences were considered significant when $p < 0.05$, tested by ANOVA with SPSS 11.0 for windows.

RESULTS

Egg Laying Performance. As shown in **Table 2**, dietary supplementation of daidzein significantly increased the egg laying rate and decreased the percentage of cracked eggs over

Table 2. Effect of Daidzein on Egg-Laying Rate, Average Egg Weight, and Percentage of Cracked Eggs during the 9-Week Supplementation in ISA Laying Hens[a]

	weeks								
groups	1	2	3	4	5	6	7	8	9
					Egg-Laying Rate (%)				
control	65.84 ± 1.01	68.81 ± 2.14	68.35 ± 1.54	67.02 ± 2.48	62.13 ± 2.35 a	61.23 ± 1.38 a	61.55 ± 1.83	59.31 ± 1.66	54.90 ± 2.65 a
daidzein	67.86 ± 2.20	67.93 ± 1.82	68.94 ± 2.28	69.35 ± 1.29	67.12 ± 2.20 b	65.53 ± 1.27 b	64.57 ± 2.82	63.46 ± 3.98	61.11 ± 3.18 b
					Average Egg Weight (g)				
control	64.24 ± 1.23	65.43 ± 0.65	65.57 ± 1.04	66.10 ± 2.89	66.55 ± 4.51	65.85 ± 2.16	67.69 ± 2.61	69.17 ± 2.03	69.06 ± 2.12
daidzein	64.52 ± 0.35	64.33 ± 0.89	65.04 ± 1.13	65.75 ± 0.57	65.39 ± 2.61	66.66 ± 1.07	67.41 ± 0.69	68.93 ± 2.47	67.77 ± 0.49
					Percentage of Cracked Eggs (%)				
control	0.74 ± 0.30 a	1.00 ± 0.27 a	0.88 ± 0.32	0.77 ± 0.44 a	0.60 ± 0.39	0.94 ± 0.38 a	1.51 ± 0.47 a	1.13 ± 0.65	1.47 ± 0.54 a
daidzein	0.42 ± 0.16 b	0.47 ± 0.24 b	0.67 ± 0.33	0.46 ± 0.33 b	0.56 ± 0.41	0.58 ± 0.33 b	0.77 ± 0.33 b	0.95 ± 0.39	0.68 ± 0.34 b

[a] Values are means ± SD. Mean values without a common identifier (a, b) differ significantly between two groups ($p < 0.05$, $n = 100$).

Table 3. Effect of Daidzein on the Quality of Eggshells and the Level of Serum E_2 of ISA Laying Hens at the Ninth Week of Daidzein Supplement[a]

exptl group	oviduct/body weight (g/kg)	shell thickness (mm)	shell parameter (width/length)
control	32.21 ± 1.74 a	0.44 ± 0.04 a	0.77 ± 0.03
DA (10 mg/kg)	39.87 ± 1.05 b	0.48 ± 0.05 b	0.78 ± 0.03

[a] Values are means ± SD. Mean values without a common identifier (a, b) differ significantly between two groups ($p < 0.05$, $n = 100$).

Table 4. Effect of Daidzein on Serum E_2, Calcium (Ca^{2+}), and Phosphate (P) Concentrations and Tibia Bone Mineral Density (BMD) of ISA Laying Hens at the Ninth Week of Daidzein Supplement[a]

exptl group	serum E_2 (pg/mL)	serum Ca^{2+} (mmol/L)	serum phosphorus (mmol/L)	BMD (g/cm^2)
control	175.21 ± 69.25	2.82 ± 0.78 a	5.31 ± 2.20	0.199 ± 0.024 a
DA (10 mg/kg)	160.43 ± 43.15	3.46 ± 0.51 b	4.69 ± 1.18	0.236 ± 0.035 b

[a] Values are means ± SD. Mean values without a common identifier (a, b) differ significantly between two groups ($p < 0.05$, $n = 20$).

the whole experimental period of 9 weeks ($p < 0.05$), without affecting the average egg weight.

Eggshell Quality and Oviduct Weight. Daidzein supplementation significantly increased the shell thickness and the ratio of oviduct weight relative to body weight ($p < 0.05$). No significant alteration in the egg shape parameter (width/length) was observed in the daidzein-treated group before the termination of the 9 week dietary daidzein treatment (**Table 3**).

Serum Concentrations of E_2, Calcium, and Phosphorus and Tibia Bone Mineral Density. As shown in **Table 4**, serum concentrations of E_2 and phosphorus were not altered in hens after 9 weeks of daidzein supplement compared with their control counterparts; however, serum calcium and tibia bone mineral density were found to be significantly increased ($p < 0.05$).

Shell Gland Expression of mRNAs for ERα, GH-R, and IGF-IR. As shown in **Figures 1**−**3**, the abundance of ERα mRNA in shell glands was not altered by daidzein supplement; however, significant down-regulations were observed in the mRNA abundances of both the GH receptor and the type 1 IGF receptor (GH-R and IGF-IR) in the shell glands of daidzein-treated hens ($p < 0.05$).

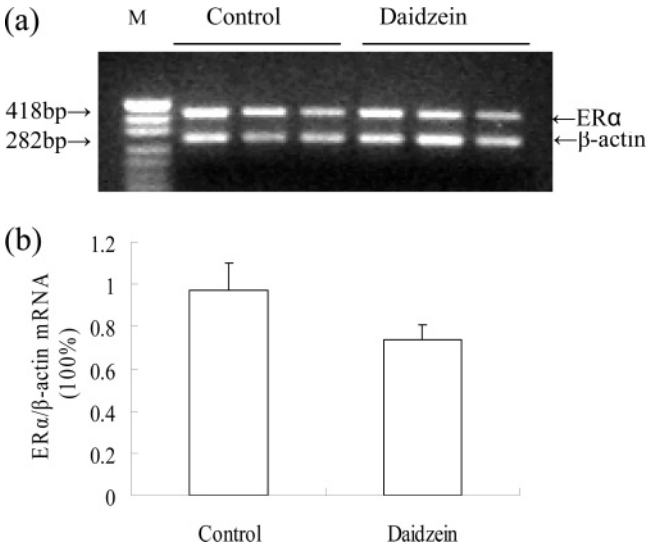

Figure 1. Effect of daidzein supplement on ER-α mRNA expression in shell glands of ISA hens during the postpeak period of egg laying. (**a**) Representative electrophoresis photo of RT-PCR products for ER-α and β-actin mRNA. M: DNA molecular weight marker PUC19. (**b**) Results of statistical analysis for ER-α mRNA levels expressed as arbitrary units relative to β-actin mRNA. The results were expressed as mean ± SEM, and differences were considered significant when $p < 0.05$, tested by ANOVA with SPSS 11.0 for Windows. Mean values with (a, b) differ significantly between control and daidzein treatment hens ($p < 0.05$, $n = 20$).

DISCUSSION

The present study provided evidence that dietary daidzein significantly increased the egg production and the feed efficiency during the postpeak period of egg laying in ISA hens. The oviduct weight and eggshell thickness increased, while the percentage of cracked eggs decreased in daidzein-treated hens, which was accompanied with decreased expression of GH-R and IGF-IR in shell glands.

Early studies suggested that phytoestrogens cause infertility in animals (*2*) and apparently inhibit reproduction and prevent the production of young in wild quail (*17*). However, such adverse effects on reproductive functions were not always observed in our previous studies. We found that daidzein exerted a negative influence on reproduction before the onset of lay and during the early (prepeak) stage of laying cycle but improved the laying performance during the late (postpeak) stage of the laying cycle in a dose-dependent manner in ducks (*15*). In the present study, the positive effect of daidzein on laying

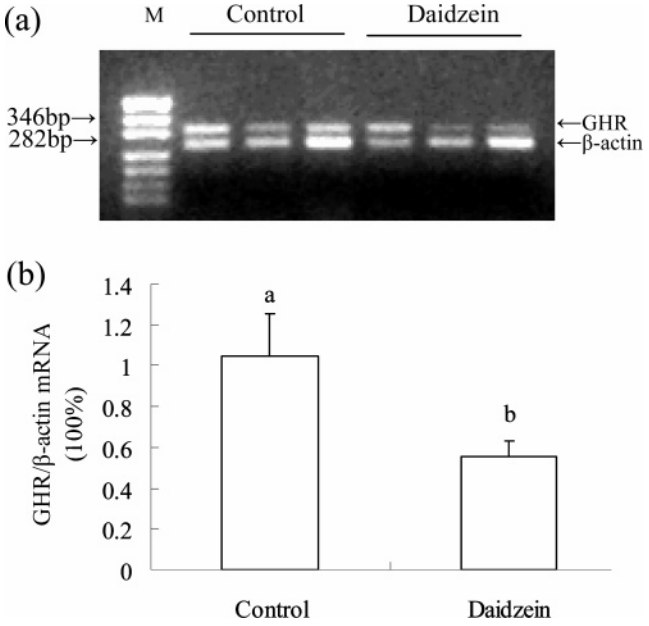

Figure 2. Effect of dietary daidzein on the relative abundance of GH-R mRNA in shell glands of ISA hens during the postpeak period of egg laying. (**a**) Representative electrophoresis photo of RT-PCR products for GH-R mRNA and β-actin mRNA. M: DNA molecular weight marker PUC19. (**b**) Results of statistical analysis for GH-R mRNA levels expressed as arbitrary units relative to β-actin mRNA. The results were expressed as mean ± SEM, and differences were considered significant when $p < 0.05$, tested by ANOVA with SPSS 11.0 for Windows. Mean values with (a, b) differ significantly between control and daidzein treatment hens ($p < 0.05$, $n = 20$).

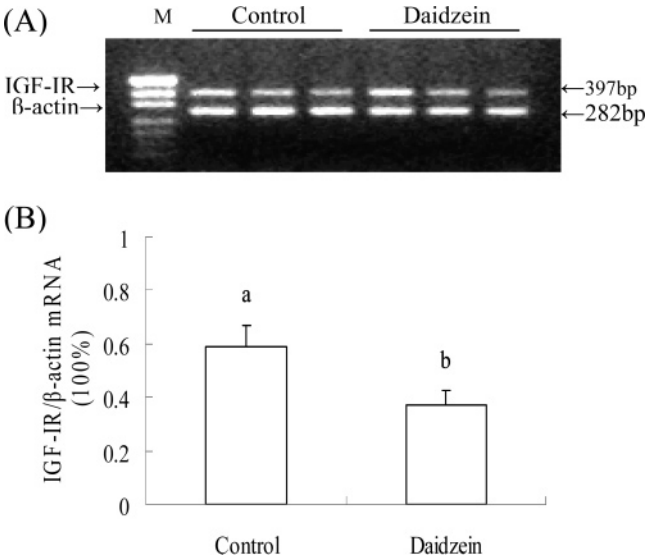

Figure 3. Effect of dietary daidzein on the relative abundance of IGF-I receptor mRNA in shell glands of ISA hens during the postpeak period of egg laying. (**A**) Representative electrophoresis photo of RT-PCR products for IGF-IR mRNA and β-actin mRNA. M: DNA molecular weight marker PUC19. (**B**) Results of statistical analysis for IGF-IR mRNA levels expressed as arbitrary units relative to β-actin mRNA. The results were expressed as mean ± SEM, and differences were considered significant when $p < 0.05$, tested by ANOVA with SPSS 11.0 for Windows. Mean values with (a, b) differ significantly between control and daidzein treatment hens ($p < 0.05$, $n = 20$).

performance during the postpeak period of the egg laying cycle has been confirmed again in ISA hens.

It has been well documented that phytoestrogens including daidzein exert both agonistic and antagonistic effects depending on the intrinsic estrogen levels (*13*). When endogenous E_2 concentrations are low, daidzein may serve as an agonist to occupy vacant estrogen-binding sites, resulting in a total increase of the systemic estrogenic effect; otherwise, daidzein may act as an antagonist by competing with estrogen for estrogen receptors and produce a much weaker estrogenic response (*14*). Daidzein was shown to increase the postpeak egg-laying performance, including egg production and feed efficiency, indicating the agonist effects of estrogen as observed in our previous studies on ducks (*15*, *18*).

Our results demonstrated that daidzein supplement significantly increased the oviduct weight, which is in agreement with the studies on ovariectomized rats that reported that daidzein prevented uterine atrophy in a dose-dependent manner (*19*). The phytoestrogen genistein at a pharmacological dose increased uterus weight as well as serum estradiol level in prepubertal rats (*20*). Additionally, a dose−response relationship between plasma E_2 and oviduct growth and function has been well documented in broiler breeder chicks (*21*, *22*). Higher serum E_2 concentration was observed in our previous study on ducks during the postpeak period of egg laying by daidzein treatment, while in this study serum E_2 was not altered over 9 weeks of daidzein supplement in hens. Apart from the differences in inclusion levels of daidzein (5.0 mg/kg vs 10.0 mg/kg) and species (duck vs chicken) employed in the experiments, the ovulatory status at the time of sampling might be an important factor that accounts for the divergent results of serum E_2, especially in laying hens, since it was reported that the level of E_2 varies dramatically during the ovulatory cycle in hens (*23*).

In the present study, the egg shell thickness was significantly increased and the percentage of cracked eggs decreased by daidzein supplement, which was accompanied by increased serum Ca^{2+} concentration. The higher concentration of Ca^{2+} in serum may be caused by higher absorption of Ca^{2+} from the gastrointestinal tract or higher Ca^{2+} released due to the increased bones resorption. However, the latter could be ruled out by the higher density of shank bones observed in daidzein-treated hens in the present study. The positive effect of estrogenic substance on intestinal calcium absorption has been implicated previously in humans (*24*).

A variety of phytoestrogens have been shown to bind to both isoforms of the estrogen receptor ERα and ERβ both *in vivo* and *in vitro*. However, the binding preference of receptor subtypes is largely tissue-specific. In tissues expressing relatively more abundant ERα, such as oviduct, phytoestrogen might prefer to bind to the ERα subtype (*25*−*28*). Therefore, ERα was proposed to mainly mediate the bioactivity of daidzein in chicken oviduct. Indeed, ERβ mRNA was hardly detectable in shell glands of laying hens with RT-PCR methods (data not shown). A trend of decrease in mRNA expression of ERα in shell glands was shown in the daidzein-supplemented group. No conclusion can be drawn whether daidzein affects the ERα expression in oviduct or whether the action of daidzein is mediated by ERα. It is possible that the effect of daidzein would be shown more clearly at the post-transcriptional level of ERα, as it was reported that the level of endogenous E_2 significantly affected the amount of ERα protein in the shell glands of hens (*29*).

GH and IGF-1 are both found to be produced locally in the chicken reproductive tract (*6*), implicating the autocrine or paracrine action of the GH/IGF-1 system, in addition to their endocrine function (*5*, *30*). In this study, the abundances of

GH-R and IGF-IR mRNA decreased significantly with daidzein treatment, which was contrary to our previous expectation that GH-R and IGF-IR mRNA expressions in shell glands would be up-regulated significantly by daidzein supplement. However, the significant decrease of the steady-state mRNA would also indicate faster turnover of mRNA; therefore, GH-R and IGF-IR would be up-regulated at protein level in hens with daidzein supplement. The detailed pathway or mechanism underlying the daidzein action on oviduct growth and function is worthy of further investigation.

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